ENDOCRINE AND PARACRINE REGULATION OF SPERMATOGENESIS - A COLLECTION OF UP TO DATE RESEARCH CONTRIBUTIONS ON TESTIS FORMATION AND FUNCTION

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PUBLISHED IN: Frontiers in Endocrinology

frontiers Research Topics

ISSN 1664-8714
ISBN 978-2-88976-859-2
DOI 10.3389/978-2-88976-859-2

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Citation: Goldberg, E., Zirkin, B., Papadopoulos, V., Lishko, P. V., eds. (2022). Endocrine and Paracrine Regulation of Spermatogenesis - A Collection of Up to Date Research Contributions on Testis Formation and Function. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88976-859-2

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frontiers | Frontiers in Endocrinology

TYPE Editorial
PUBLISHED 26 July 2022
DOI 10.3389/fendo.2022.984409

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology

RECEIVED 02 July 2022
ACCEPTED 07 July 2022
PUBLISHED 26 July 2022

CITATION
Goldberg E, Lishko PV, Papadopoulos V and Zirkin B (2022) Editorial: Endocrine and paracrine regulation of spermatogenesis. *Front. Endocrinol.* 13:984409. doi: 10.3389/fendo.2022.984409

Editorial: Endocrine and paracrine regulation of spermatogenesis

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KEYWORDS

endocrine and paracrine regulation of spermatogenesis, endocrine regulation, paracrine regulation, spermatogenesis, testes

Editorial on the Research Topic
Endocrine and paracrine regulation of spermatogenesis - A collection of up to date research contributions on testis formation and function

Introduction

Continuous generation of male gametes occurs through the tightly controlled multistep process of spermatogenesis. This process includes the formation and differentiation of spermatogonia, their entry into meiosis, recombination of the paternal genome during meiosis, and the differentiation of the spermatids that result from meiosis into advanced spermatids/spermatozoa. Somatic cell-cell and somatic-germ cell interactions within the testis define the local milieu and endocrine environment driving germ cell development and spermatogenesis. The spermatozoa formed in the testis gain the ability for forward motility and fertility during their passage though the epididymis. The process of spermatogenesis ensures the propagation of the species while also providing biological diversity and adaptation.

This volume, edited by Erwin Goldberg (Northwestern University), Polina Lishko (University of California, Berkeley), Vassilios Papadopoulos (University of Southern California), and Barry Zirkin (Johns Hopkins University), is dedicated to how the process of spermatogenesis is regulated by endocrine and paracrine mechanisms. It encompasses peer-reviewed perspectives, reviews, opinion papers, and original research reports by scientists at the cutting edges of their fields. The authors were invited by the guest editors to submit articles in the broad area of endocrine and paracrine regulation of spermatogenesis. The topics covered in the volume include the regulation of spermatogonial development from stem cells, spermatogonial division to form cells that enter meiosis, the process of meiosis, spermatogenesis and its regulation, the effects of aging

and of chemical exposure on sperm formation and function, new approaches to male contraception, and the application of new molecular technologies. The editors have organized the papers into four major areas: Spermatogonia: Development and Regulation; Sperm Formation: Molecular and Hormonal Regulation; Effects of Age and Exogenous Influences on Sperm Formation and Function; and Clinical Applications: Testosterone, Spermatogenesis and Contraception. The following descriptions summarize the contents of the papers contained in each of these four major areas:

Spermatogonia: Development and regulation

Analyses of altered activin A in murine models by Moody et al. implicate activin A as a key determinant of early germline formation and highlight the potential for altered activin A levels *in utero* to increase the risk of testicular pathologies that arise from impaired germline maturation. Manku et al., building on their previously identified Ubiquitin-Proteasome System (UPS) enzymes that are dynamically altered during gonocyte differentiation, focus on the role of the RING finger protein 149 (RNF149), an E3 ligase expressed in gonocytes and downregulated in spermatogonia. The new data that are presented support a role for RNF149 in gonocyte proliferation.

Wright reviews the *in vivo* regulation of spermatogonial stem cells (SSCs) in adult testes by Sertoli cell-produced glial cell line-derived neurotrophic factor (GDNF) through the use of a novel chemical-genetic approach to diminish replication and increase the differentiation of SSCs. The data that are reviewed suggest that GDNF may prove to be an effective therapy for men whose testes contain only Sertoli cells (SCO syndrome).

Diao et al. summarize research progress on the regulation of spermatogonial stem cells (SSCs), and the potential application of SSCs for fertility restoration. The authors suggest that *in vitro* spermatogenesis from SSCs produced from induced pluripotent stem cells (iPSCs) might be of use in improvement of spermatogenesis.

Sperm formation: Molecular and hormonal regulation

Leydig cells are the main site of production of the male sex hormone testosterone, a steroid that is critical for the development of male sexual characteristics and spermatogenesis. The commitment, differentiation, and function of Leydig cells require the coordinated action of several transcription factors acting in a time-specific manner. de Mattos et al. review current knowledge on the expression, function, and mechanism of action of various transcription factors that regulate fetal and adult endocrine Leydig cell development and function. The ability of Leydig cells to form androgen is influenced by other cells present in the interstitium, e.g., immune cells such as macrophages. Gu et al. review the literature showing that interstitial testicular macrophages are intimately associated with Leydig cells, controlling Leydig cell development and function, and that certain types of lymphocytes produce and metabolize steroids that might affect the steroidogenic output of the testis. Ruthig and Lamb review recent advances in understanding the interplay of Sertoli cell endocrine and paracrine signals that regulate germ cell state and thus, spermatogenesis. Recent studies of Sertoli cell ablation and transplantation that provide better clarity of the role of the Sertoli cell niche in germ cell development are discussed.

Sertoli cells are a major component of the spermatogonial stem cell niche and provide essential growth factors and chemokines to developing germ cells. Hoffman and McBeath review the activation of master regulators of the niche in Sertoli cells and their targets, and the molecular mechanisms underlying the regulation of growth and differentiation factors such as GDNF and retinoic acid by NOTCH signaling and other pathways.

Male fertility is reliant upon continuous production of sperm. Spermatogenesis involves the coordinated transitions of mitosis, meiosis, and spermiogenesis. Moritz and Hammoud review current understanding of chromatin dynamics during spermiogenesis, and the molecular basis of the histone-to-protamine exchange in idiopathic male infertility. The transition of Type A spermatogonia to differentiated spermatogonia requires the action of retinoic acid (RA). The synthesis of retinoic acid from retinal in the seminiferous epithelium is a result of the action of aldehyde dehydrogenases. Topping and Griswold report that of the three known retinal dehydrogenases involved in RA synthesis, two are required in Sertoli cells for normal spermatogenesis, and that the global deletion of the genes for these two enzymes blocks spermatogenesis, thus offering a potential target for contraception in the male.

Meyer-Ficca et al. address the question of whether age-related NAD+ decline is functionally linked to decreased male fertility. Using a transgenic mouse model, the authors report that decreasing testicular NAD+ levels in young adult mice, to levels that match or exceed the NAD+ decline observed in old mice, results in the disruption of spermatogenesis, and that providing vitamin B3 (niacin) to NAD+-depleted transgenic mice rescues spermatogenesis. The results suggest that NAD+ provided by vitamin B3 is important for complete spermatogenesis and male fertility.

Mundt et al. review publications on extracellular adenosine triphosphate (ATP) as a paracrine mediator of male fertility and sperm production, acting by targeting membrane-bound purinergic receptors and ion channels, and triggering changes in the cell's membrane potential, calcium homeostasis, and cAMP levels. The summarized results demonstrate the importance of purinergic signaling in the control of male reproduction.

Kiyozumi and Ikawa describe biological processes regulated by proteases and protease inhibitors based on the use of gene-

modified organisms. A focus is on the generation and activation of gametes during spermatogenesis. Discussed are proteolysis-related factors and biological processes regulated by proteolysis for successful reproduction, including cleavage of peptide bonds to activate and inactivate enzymes, transcription factors, and receptors.

Effects of age and exogenous influences on sperm formation and function

Paternal age at conception has been steadily increasing globally. Chan and Robaire review results from mammalian animal models showing that increasing paternal age affects progeny outcome. Clinical studies reveal effects on offspring with respect to perinatal health, cancer risk, genetic diseases, and neurodevelopmental deficits. An overview of the potential mechanisms involved in altering germ cells in advanced age is presented. This is followed by an analysis of the current state of management of reproductive risks associated with advanced paternal age, and strategies for mitigating its impact.

Sakib et al. make the case that an *in vitro* system to study testicular maturation would serve as a platform for high-throughput drug and toxicity screening in a tissue-specific context. The authors report conditions that result in the successful generation and maintenance of rat testicular organoids in culture and the use of this system to study testicular cell maturation and the effects of exposure to toxicants.

Infection and inflammation can lead to infertility. The review by Hasan et al. describes evidence for the activation of inflammatory pathways as causative in various forms of male testicular disorders. The focus is on how imbalance of local testicular factors contributes to disturbances of spermatogenesis and steroidogenesis.

Wang et al. discuss the association of perinatal exposure to smoking and childhood asthma. They point out that although the molecular mechanisms underlying childhood asthma induced by perinatal exposure to smoking or nicotine remain elusive, an epigenetic mechanism might be involved. The new data presented in this paper show that perinatal exposure to nicotine leads to alterations in the profiles of sperm RNAs, including mRNAs and small RNAs, and that rosiglitazone, a PPARγ agonist, can reverse the negative effects on RNA.

The study by Starovlah et al. addresses the possible mechanism(s) by which acute psychological stress might affect male fertility in rat models. Included in the study are analyses of numbers of spermatozoa, markers of mitochondrial dynamics, and expression of signaling molecules.

Wang et al. discuss the X-linked miR-465 cluster. The study that the authors present focuses on the role of the miR-465 cluster in murine development. It is reported that ablation of the miR-465 miRNA cluster using CRISPR-Cas9 did not cause infertility, but rather a sex ratio biased toward males in the knockout mice. The data suggest that the miR-465 cluster is required for normal female placental development, and that ablation of the miR-465 cluster leads to a skewed sex ratio with more males (~60%) due to selective degeneration and resorption of the female conceptuses.

Clinical applications: Testosterone, spermatogenesis and contraception

Hypogonadism and priapism have been shown to be common in men with sickle cell disease (SCD). Musicki and Burnett review the use of a mouse model for understanding the relationship of primary hypogonadism to SCD and to priapism. They also discuss the mechanisms involved in reduced cholesterol transport to and into the mitochondria in relationship to reduced testosterone, and how endogenous testosterone production might be restored specifically and safely in men with SCD, thereby reducing episodes of priapism.

Makela and Toppari review data showing that the retinoblastoma protein (RB) binds to E2F transcription factors in the testis, and that their interaction is a key mechanism involved in the establishment and maintenance of male fertility. In particular, evidence from gene knock-out studies is discussed that demonstrates that RB-E2F interaction in Sertoli cells is essential for fertility and is important for germline maintenance and lifelong sperm production.

Schlegel points out that much of what is understood about human spermatogenesis has come from the study of rodent models, but that this approach might not be ideal. This paper focuses on clinical observations of human spermatogenesis, focused mainly on genetic abnormalities in human sperm that are based on analyses conduct with patients presenting with symptoms of severe infertility.

Johnston and Lindsey discuss innovative approaches focused on expanding the contraceptive options available to men and women. They also consider new challenges to clinical development and regulatory approval, and how these challenges can be met so that new discoveries will move “from bench to bedside.”

In the last but not the least of these contributions, Page et al. emphasize the importance of effective contraceptive options for men and women and make the case for expanded male contraceptive methods. The authors discuss the use of exogenous progestins and androgen that suppress the hypothalamic-pituitary-gonadal axis as effective and reversible, and present new data on the use of novel steroids and varied routes of hormone delivery.

Concluding remarks

The editors are sincerely grateful to all authors for their invaluable contributions highlighting current knowledge on spermatogenesis and its regulation by endocrine and paracrine

factors. Moreover, we are grateful to the reviewers for their insightful and constructive reviews. Our hope is that these results are discussed, and the new techniques that now are available to investigators, will inspire further in-depth work in this important field of human biology. We also express our appreciation for the editorial assistance by the Frontiers staff and in particular that from Samuel Manning Journal Specialist, Frontiers in Endocrinology for his timely and helpful responses to our many questions during the entire project period.

Author contributions

EG, PL, VP and BZ contacted potential authors, assigned manuscripts to reviewers, and ultimately made decisions as to the publication status of the manuscripts.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

frontiers | Frontiers in Endocrinology

MINI REVIEW
published: 05 April 2022
doi: 10.3389/fendo.2022.867011

Purinergic Signaling in Spermatogenesis

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Adenosine triphosphate (ATP) serves as the essential source of cellular energy. Over the last two decades, however, ATP has also attracted increasing interest as an extracellular signal that activates purinergic plasma membrane receptors of the P2 family. P2 receptors are divided into two types: ATP-gated nonselective cation channels (P2X) and G protein-coupled receptors (P2Y), the latter being activated by a broad range of purine and pyrimidine nucleotides (ATP, ADP, UTP, and UDP, among others). Purinergic signaling mechanisms are involved in numerous physiological events and pathophysiological conditions. Here, we address the growing body of evidence implicating purinergic signaling in male reproductive system functions. The life-long generation of fertile male germ cells is a highly complex, yet mechanistically poorly understood process. Given the relatively sparse innervation of the testis, spermatogenesis relies on both endocrine control and multi-directional paracrine communication. Therefore, a detailed understanding of such paracrine messengers, including ATP, is crucial to gain mechanistic insight into male reproduction.

Keywords: spermatogenesis, ATP - adenosine triphosphate, purinoceptor, calcium signaling, P2X, P2Y

OPEN ACCESS

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Specialty section:
This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology

Received: *31 January 2022*
Accepted: *08 March 2022*
Published: *05 April 2022*

Citation:
Mundt N, Kenzler L and Spehr M (2022) Purinergic Signaling in Spermatogenesis. Front. Endocrinol. 13:867011. doi: 10.3389/fendo.2022.867011

SPERMATOGENESIS

The generation of fertile spermatozoa is one of the most complex, yet least understood developmental processes in postnatal life. Spermatogenesis describes the differentiation and maturation of diploid spermatogonial stem cells into haploid spermatozoa (1). Spermatogenesis occurs in the seminiferous tubules within the mammalian testis (2) (**Figure 1**). These hollow tubules are coiled loops that converge in the *rete testis*, which feeds into the epididymis (2, 4). Seminiferous tubules comprise a specialized tissue subdivided into three compartments: the lumen, the germinal epithelium, and the tubular wall. The latter is composed of extracellular matrix proteins and flat smooth-muscle-like testicular peritubular cells (TPCs). The germinal epithelium comprises two cell types: somatic Sertoli cells and developing germ cells.

Sertoli cells fulfill essential structural, regulatory, and nourishing functions for the surrounding germ cells. They span from the basal lamina to the lumen and are associated with up to 50 germ cells (5). During the course of differentiation, Sertoli and germ cells remain connected, enabling continuous bidirectional communication. In the basal seminiferous epithelium, Sertoli cells form necklace-like tight junction threads between adjacent Sertoli cells, creating a tight barrier between the basal and adluminal compartments (6). This blood-testis barrier prevents passage of many

molecules and migrating immune cells into the inner, adluminal compartment and, thus, creates a protective, immune-privileged environment for postmeiotic germ cell development (7).

During maturation, germ cells migrate in a complex process from the basal compartment towards the lumen. The first wave of spermatogenesis is initiated upon puberty and divided into four phases (8, 9):

1. Mitotic proliferation of diploid spermatogonia (spermatogoniogenesis)
2. Meiotic division of tetraploid spermatocytes into haploid spermatids
3. Morphological differentiation of spermatids into spermatozoa (spermiogenesis)
4. Sperm release into the tubular lumen (spermiation)

The first mitotic division is asymmetrical as one daughter cell remains in the stem cell pool, while the other spermatogonium is irreversibly determined to differentiate. In subsequent mitotic divisions into various spermatogonial subtypes, the cells lose contact with the basal lamina (10). Due to incomplete cytokinesis, premeiotic germ cells stay connected *via* cytoplasmic bridges allowing small molecule exchange and, hence, synchronized development (11, 12). Spermatogonia then differentiate into primary spermatocytes, which progress through meiosis and cross the blood-testis barrier. Haploid spermatids undergo drastic morphological changes (spermiogenesis), yielding elongated and flagellated spermatozoa that are located close to the tubular lumen. Finally, in a process called "spermiation", spermatozoa are released into the lumen, which marks the endpoint of spermatogenesis (2, 8). Upon release, spermatozoa remain immotile and, thus, need to be actively transported towards *rete testis* and epididymis, where they gain the capacity for motility but remain quiescent (13–15). Sperm transport is mediated by coordinated smooth muscle contractions of TPCs that surround individual tubules (3, 16).

The bewildering complexity of cell types that coexist in the seminiferous epithelium as well as the numerous proliferation and differentiation steps that must be precisely orchestrated pose an obvious question: Which multi-directional cellular communication mechanisms control spermatogenesis?

Given the lack of pronounced seminiferous tubule innervation testicular sympathetic innervation appears restricted to blood vessels and the tunica albuginea (17), spermatogenesis relies on endo-, auto-, and paracrine communication pathways. Therefore, a detailed understanding of the relevant paracrine messengers, including ATP, promises to provide much needed mechanistic insight into male reproduction.

PURINERGIC SIGNALING

One of the paracrine messengers that has attracted increasing scientific interest in a multitude of general physiological

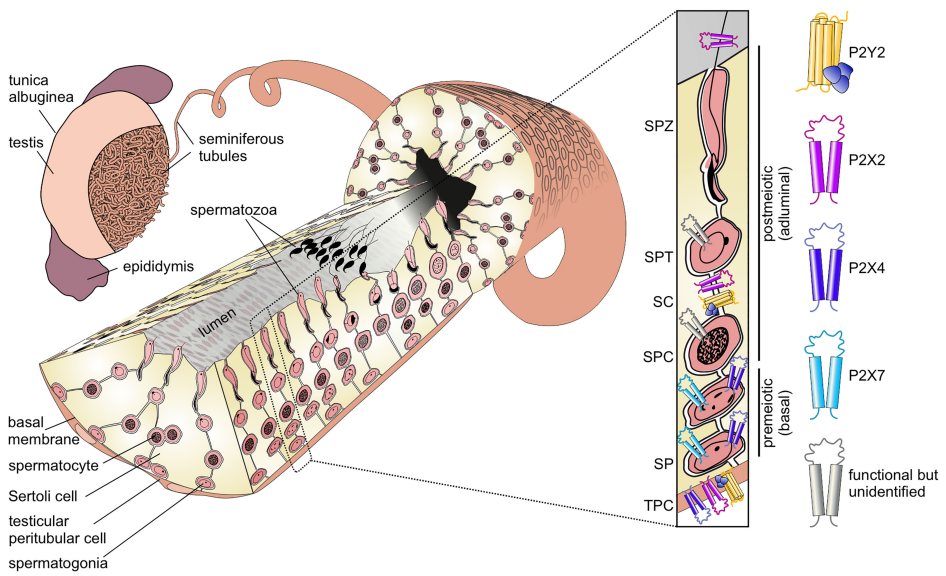

FIGURE 1 | Functional P2 receptor isoform distribution among individual cell types of the seminiferous tubule. Left: Schematic illustration of the mammalian testis and cellular architecture of a seminiferous tubule. A single layer of contractile testicular peritubular cells (TPC) lines the tubular wall. Developing germ cells are distributed between nourishing Sertoli cells (SCs). Undifferentiated spermatogonia (SP) are located near the basal membrane. Spermatocytes (SPC) migrate to the adluminal compartment, where they complete meiosis. The resulting haploid round spermatids (SPT) differentiate into elongated spermatids and, eventually, into highly condensed and compartmentalized spermatozoa (SPZ). These mature, yet immotile spermatozoa are then released into the lumen (spermiation) and undergo further maturation steps once transported to the epididymis. Adapted from: Fleck, Kenzler et al. (3). Right: Distribution of P2 isoforms in various cell types of the seminiferous tubule. Schematic shows the P2 receptor distribution as supported by direct functional (i.e., physiological) evidence.

events is extracellular adenosine triphosphate (ATP) (18–21). Through an evolutionarily conserved route for cell-to-cell communication, extracellular ATP activates members of the membrane-bound P2 purinoceptor family (18). ATP-gated P2 receptors are divided into two classes, namely ionotropic P2X receptors (22, 23), and metabotropic P2Y receptors, which are members of the G protein-coupled receptor (GPCR) superfamily (24). The majority of the eight P2Y receptor isoforms (P2Y1, 2, 4, 6, 11) couple to $G_{\alpha q}$, thus signaling *via* phosphoinositide turnover. $G_{\alpha q}$ activates phospholipase C, which in turn hydrolyzes phosphatidylinositol-4,5-bisphosphate to inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol. Cytosolic increase in IP_3 level triggers Ca^{2+} release from internal Ca^{2+} storage organelles (i.e., the endo/sarcoplasmic reticulum) *via* IP_3 receptors. The main effector of P2Y12, P2Y13, and P2Y14 is $G_{\alpha i/o}$ followed by an activation or inactivation of adenylate cyclase and altered cytosolic cyclic adenosine monophosphate (cAMP) levels (25).

P2X receptors, by contrast, are homo- or heterotrimeric ligand-gated nonselective cation channels. They share a common transmembrane topology – intracellular termini and two transmembrane domains separated by a large extracellular loop (26) – with DEG/ENaC/ASIC channels. Upon ATP binding, conformational changes lead to the opening of a cation-permeable channel pore (27). Among the P2X family, seven homotrimeric (P2X1–7) and several heterotrimeric isoforms have been described, all of which share substantial Ca^{2+} permeability, but are readily distinguished by ligand affinities, activation and desensitization kinetics, as well as distinct pharmacological fingerprints (28). The complexity of both receptor families, which cover a vast dose-response range of effective ATP concentrations, and the broad spatiotemporal response scales of P2 receptors confer both functional specificity and physiological flexibility to a ubiquitous signaling pathway. Accordingly, a given cell's P2 receptor expression profile underlies its unique response phenotype upon ATP exposure. Notably, as both metabotropic and ionotropic ATP response pathways represent substantial cellular Ca^{2+} gates, purinoceptors mediate numerous Ca^{2+}-dependent downstream effects, including control of gene transcription, protein phosphorylation, ion channel function, muscle contraction, and more (29). While the general picture is still incomplete, we here seek to summarize evidence from a growing number of reports about purinergic signaling routes within the seminiferous tubule and their potential implications in spermatogenesis and male (in)fertility.

Purinoceptor Signaling in Germ Cells

Given the broad physiological response scale of purinoceptors, purinergic signaling has been proposed to play a role in controlling germ cell maturation at different developmental stages. In mice, twelve such stages are sequentially transitioned to complete one seminiferous epithelial cycle. Accordingly, immunohistochemical investigation of cell type- and stage-dependent protein expression has been notoriously difficult. Early work described immunoreactivity for several P2X receptor subtypes in the rat testis (30). Various germ cell types throughout different stages of the seminiferous epithelial cycle were found immunopositive for P2X2, P2X3, and P2X5 receptors. By contrast, P2X4 and P2X6 receptors appeared absent from rat testis samples – a finding that was later contradicted by Ko and coworkers (31). P2X1 receptors were exclusively detected in blood vessels and P2X7 antibody staining was restricted to Sertoli cells (30). Notably, P2X2 and P2X3 isoforms, which frequently form functional heteromers in the nervous system (32), were usually observed in the same cell types and stages (30).

Recently, we combined gene expression analysis, immuno- and bioanalytical chemistry, protein knockdown, and single-cell electrophysiology to gather functional evidence for purinergic signaling in male germ cells (33). We identified a multidimensional ATP response pathway consisting of both P2X4 and P2X7 receptors and downstream Ca^{2+}-activated large conductance (BK) K^+ channels in prepubescent mouse spermatogonia (**Figure 2A$_{III}$**). P2X4 and P2X7 receptors display distinct ATP affinities, and their activation triggers transmembrane currents with characteristic kinetics that enable unequivocal electrophysiological isoform identification. Cooperatively activated by concurrent membrane depolarization and increased cytoplasmic Ca^{2+}, hyperpolarizing BK channels provide a negative feedback mechanism that counteracts the effects of P2X receptor activation and ensures swift repolarization of the spermatogonial membrane potential (33).

While some of the apparent discrepancies between the above studies (30, 31, 33) likely result from species [mouse (33) *versus* rat (30, 31)] and/or age [juvenile (33) *versus* adult (30, 31)] differences, they also highlight the limitations of unidirectional (i.e., immunochemistry-only) protein expression analysis. Electrophysiological recordings from postmeiotic germ cells in acute seminiferous tubule slices of adult mice are technically challenging. Our own unpublished data nonetheless indicate functional expression of a fast activating and slowly desensitizing ATP-activated channel in postmeiotic spermatocytes and/or round spermatids (**Figure 2A$_{IV}$**). The molecular identity of this putative P2X receptor remains to be identified.

Given the emerging role of extracellular ATP in numerous physiological signaling processes, it is tempting to speculate that spermatozoa might be exposed to varying concentrations of extracellular ATP in the testis, epididymis, and/or female reproductive tract. ATP might, therefore, play a role in modulating sperm fertilizing capacity. In humans, extracellular ATP has been reported to increase the fertilizing potential of sperm and, accordingly, sperm exposure to ATP during IVF treatment has been suggested (36). Early studies report that extracellular ATP triggers acrosome exocytosis in human sperm *via* P2X-dependent Na^+ influx (37, 38). In rat spermatozoa, P2X7 has been proposed to mediate the ATP-triggered acrosome reaction (39). While the acrosomal membrane is as yet inaccessible to electrophysiological recordings, acrosomal P2X receptor currents remain to be verified. A different mechanism was found in bovine spermatozoa, where extracellular ATP appears to activate P2Y

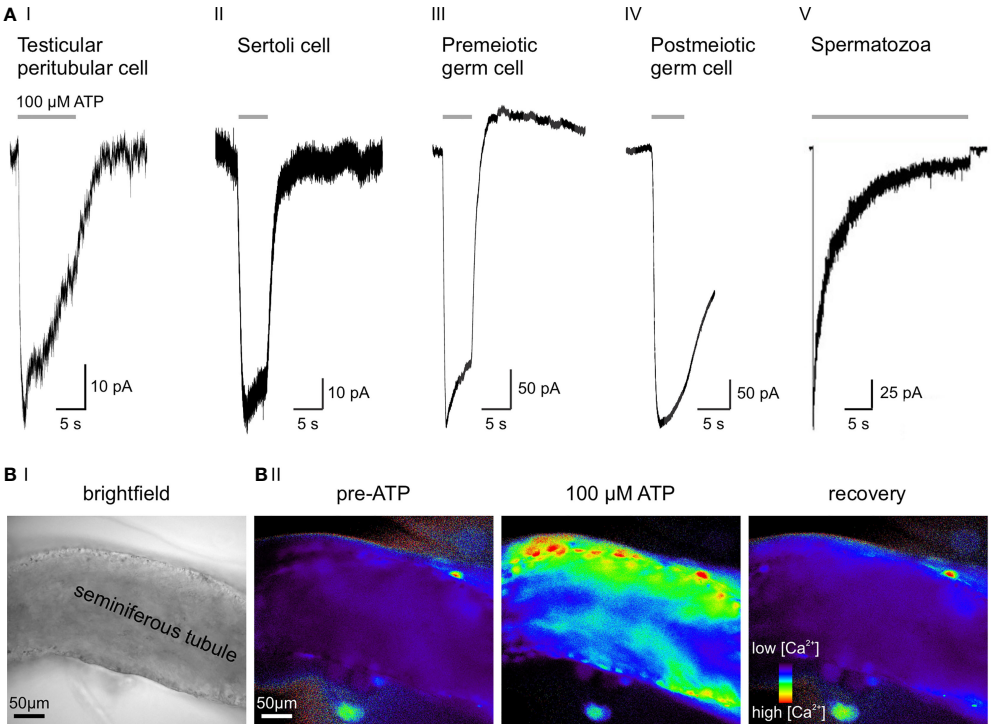

FIGURE 2 | ATP sensitivity across cell types of the seminiferous tubule. **(A)** Representative whole-cell voltage-clamp recordings from various testicular cell types, transiently exposed to extracellular ATP (100 µM). Negative current indicates cation influx through P2X receptors. **(A_I)** Slowly desensitizing P2X2 and/or P2X4 current in a mouse TPC (3). **(A_{II})** ATP activates P2X2 in murine Sertoli cells (33). **(A_{III})** 100 µM ATP selectively activates P2X4, but not P2X7 in premeiotic spermatogonia. Note the delayed BK-mediated outward current (33). **(A_{IV})** Postmeiotic germ cells exhibit an ATP-induced inward current, but the underlying P2X isoform is yet to be identified (unpublished data; recording in an acute seminiferous tubule section from an adult mouse according to (33), extracellular solution containing (mM) 145 NaCl, 5 KCl, 1 $CaCl^2$, 0.5 $MgCl^2$, and 10 HEPES; pH = 7.3, intracellular solution containing (mM) 143 KCl, 2 KOH, 1 EGTA, 0.3 $CaCl^2$, 10 HEPES, and 1 Na-GTP ($[Ca^{2+}]_{free}$ = 110 nM); pH = 7.1, stimulation with 100 µM ATP for 5 s). **(A_V)** Epididymal mouse spermatozoa with characteristic fast-activating and slowly desensitizing P2X2 current evoked by extracellular ATP. Electrophysiological recording was performed on a head plus midpiece fragment by Navarro et al. (2011) (34). **(B)** Combined ionotropic and metabotropic ATP responses of various cells in an acute seminiferous tubule section visualized as Ca^{2+}-dependent changes in fluorescence. Imaging was performed according to published experimental protocols (35). **(B_I)** Brightfield micrograph of the seminiferous tubule section under investigation. **(B_{II})** Fluorescence images of the same seminiferous tubule bulk-loaded with fura-2/AM (30 µM, 30 min at room temperature). Pseudocolor images (rainbow 256 color map) illustrate relative cytosolic Ca^{2+} concentration before, during, and after ATP stimulation (unpublished data).

receptors. The resulting elevation in cytoplasmic Ca^{2+} activates PKCα, which triggers acrosomal exocytosis (40). In 2007, Edwards et al. quantified the effects of extracellular ATP on acrosomal exocytosis, protein tyrosine phosphorylation, and sperm motility parameters in human sperm (41). In healthy and asthenozoospermic donors, ATP had no impact on acrosome exocytosis or tyrosine phosphorylation. However, it significantly altered sperm motility, increasing curvilinear velocity and percentage of hyperactivation. This observation might explain the previously described benefits of ATP supplement during IVF treatment.

Navarro et al. reported a nonselective cation current in the midpiece of mouse spermatozoa that is activated by external ATP exposure (**Figure 2A_V**) (34). This current matches the kinetics and pharmacological profile reported for recombinant P2X2 and, importantly, is absent in $P2X2^{-/-}$ mice. Despite the loss of this ATP-gated current, $P2X2^{-/-}$ spermatozoa show unaltered motility and acrosome reaction. However, $P2X2^{-/-}$ males are subfertile when given the chance to mate at high frequencies, indicating that P2X2 adds a selective advantage under frequent mating conditions. The authors hypothesize that increased intracellular Ca^{2+} through P2X2 energizes sperm mitochondria in the midpiece, presumably as a consequence of Ca^{2+}-dependent potentiation of enzymes in the Kreb's cycle (42).

Purinoceptor Signaling in Sertoli Cells

Work from multiple laboratories suggests that extracellular ATP triggers a rapid and transient increase in the cytosolic Ca^{2+} concentration of Sertoli cells, albeit with partly conflicting propositions for the underlying purinoceptor isoforms (31, 33, 43–47).

Endocrine control of spermatogenesis along the hypothalamic–pituitary–testicular axis converges on Sertoli cells (48). Sertoli cell function is centrally regulated by gonadotropins, either directly by follicle stimulating hormone (FSH) or indirectly by luteinizing hormone-dependent generation of dihydrotestosterone. FSH surges trigger cAMP production and mobilization of cytosolic Ca^{2+} in Sertoli cells (48). Interestingly, both ATP and its uridine

derivative UTP inhibit FSH-dependent cAMP accumulation by 70% in rat Sertoli cells, suggesting that P2Y2 or P2Y4 receptors are involved (43). Moreover, rapid IP_3 accumulation was observed upon ATP exposure in primary cultures of rat and mouse Sertoli cells, in line with P2Y2 or P2Y4 receptor activation (49). In rat Sertoli cells, extracellular ATP evoked 17β-estradiol production/secretion. This effect depended on both membrane depolarization *via* Na^+ influx (implicating P2X receptors) and Ca^{2+} release from internal stores (suggesting a concurrent role of P2Y receptors) (47).

Both receptor identification and direct functional characterization of purinergic signaling in mouse Sertoli cells were performed by Veitinger et al. in 2011 as well as Fleck et al. in 2016 (33, 45). P2X2 and P2Y2 are the prevailing purinoceptors (**Figure 2A$_{II}$**) with confirmatory results obtained from both Sertoli cell–germ cell co-cultures (45) and acute seminiferous tubule sections (33). These (electro-)physiological observations are in accordance with early findings by Foresta et al. in rat Sertoli cells. Here, the authors claimed that ATP exposure generates both an increase in cytosolic Ca^{2+} by release from intracellular stores (P2Y receptors) and a depolarizing Na^+ influx consistent with P2X receptor activation (43). Notably, Veitinger and coworkers establish that mitochondria serve as essential regulatory components of Sertoli cell purinergic Ca^{2+} signaling (45).

Purinoceptor Signaling in Testicular Peritubular Cells

Spermatogenesis completes with the release of still immotile spermatozoa from the seminiferous epithelium into the lumen of the seminiferous tubule. After detachment from Sertoli cells, sperm must therefore be transported towards the *rete testis* and epididymis for further maturation. Accordingly, precisely regulated tubular transport mechanisms are imperative for reproduction.

Early on, observations of minute motions of seminiferous tubule segments (50, 51) have sparked speculation about a critical role for smooth muscle-like TPCs (52, 53) in male (in) fertility through mediating contractile tubule movements (54, 55). However, direct experimental *in vivo* evidence for paracrine control of TPC contractions has been lacking (56) and quantitative live-cell measurements of seminiferous tubule contractions are rare and controversial (57–60). Somewhat surprisingly, early work explicitly excluded extracellular ATP as an activator of TPCs (61). By contrast, we recently reported both ATP-dependent Ca^{2+} signals and adenosine-dependent proinflammatory actions in human TPCs *in vitro* (62, 63). Notably, we also identified purinergic signaling pathways as physiological triggers of tubular contractions both *in vitro* and *in vivo*. By acting on ionotropic (P2X2 and/or P2X4) and metabotropic (P2Y2) purinoceptors (**Figure 2**), extracellular ATP elevates cytosolic Ca^{2+} (**Figure 2B**), activates TPC contractions, and triggers stage-dependent directional sperm movement within the mouse seminiferous tubules (3). Combining recordings from primary mouse and human TPC cultures as well as acute mouse seminiferous tubule slices with intravital multiphoton imaging of intact tubules, we provide direct and quantitative evidence for purinergic TPC signaling that triggers robust peristaltic movement of luminal sperm (3). Electrophysiological and Ca^{2+} imaging data suggest that, while metabotropic P2Y signaling is sufficient to induce ATP-dependent contractions, influx of extracellular Ca^{2+} through ionotropic P2X receptors enhances TPC contractions. While the full picture is admittedly still incomplete, current data support a concept of Ca^{2+}-induced Ca^{2+} release mechanisms that amplify ATP-dependent excitation-contraction coupling.

Being under androgen control, expression of TPC contractility proteins initiates with puberty and, notably, TPC-selective androgen receptor knock-out renders mice infertile (64). Both findings underscore a potential role of TPC contractions in male (in)fertility. Accordingly, pharmacological targeting of purinergic signaling pathways to (re)gain control of TPC contractility represents an attractive approach for male infertility treatment or contraceptive development (3). Still, translation of TPC contractions and their putative role(s) from mice to humans awaits further physiological investigation.

CONCLUDING REMARKS

With recent technical advances in male reproductive physiology, we and others identified functional P2X and/or P2Y receptors in essentially all cell types of the seminiferous tubule, constituting a purinergic signaling network (**Figure 2**). Local ATP elevations will affect the surrounding cells within a limited paracrine radius both electrophysiologically and biochemically by triggering membrane depolarization as well as substantial Ca^{2+} influx and cAMP signaling. Distinct type- and stage-specific purinoceptor repertoires will determine unique response profiles of individual target cells. Moreover, ectonucleotidases provide pathways of local ATP degradation/metabolization, restricting the effective range of paracrine ATP signaling (65). Both Sertoli and germ cells have been proposed as putative ATP release sites (66), but a conclusive picture of extracellular ATP release in the testis requires future investigation.

AUTHOR CONTRIBUTIONS

NM and LK acquired the data that is indicated as unpublished. MS contributed to the conceptualization thereof. NM wrote the first draft of the manuscript and designed the figures. LK and MS wrote sections of the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work has been funded by the Deutsche Forschungsgemeinschaft (DFG), grant reference number 368482240/GRK2416 (NM and MS).

ACKNOWLEDGMENTS

We thank all members of the Spehr laboratory for discussions.

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MINI REVIEW
published: 07 April 2022
doi: 10.3389/fendo.2022.881309

Transcription Factors in the Regulation of Leydig Cell Gene Expression and Function

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Cell differentiation and acquisition of specialized functions are inherent steps in events that lead to normal tissue development and function. These processes require accurate temporal, tissue, and cell-specific activation or repression of gene transcription. This is achieved by complex interactions between transcription factors that form a unique combinatorial code in each specialized cell type and in response to different physiological signals. Transcription factors typically act by binding to short, nucleotide-specific DNA sequences located in the promoter region of target genes. In males, Leydig cells play a crucial role in sex differentiation, health, and reproductive function from embryonic life to adulthood. To better understand the molecular mechanisms regulating Leydig cell differentiation and function, several transcription factors important to Leydig cells have been identified, including some previously unknown to this specialized cell type. This mini review summarizes the current knowledge on transcription factors in fetal and adult Leydig cells, describing their roles and mechanisms of action.

Keywords: transcription factors, gene expression, regulatory element, DNA binding motif, steroidogenesis, Leydig cells

OPEN ACCESS

Edited by:
Vassilios Papadopoulos, University of Southern California, United States

Reviewed by:
Diane Rebourcet, The University of Newcastle, Australia
Peter O'Shaughnessy, University of Glasgow, United Kingdom

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Specialty section:
This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology

Received: *22 February 2022*
Accepted: *15 March 2022*
Published: *07 April 2022*

Citation:
de Mattos K, Viger RS and Tremblay JJ (2022) Transcription Factors in the Regulation of Leydig Cell Gene Expression and Function. Front. Endocrinol. 13:881309. doi: 10.3389/fendo.2022.881309

1 INTRODUCTION

Localized in the testicular interstitium, Leydig cells are the principal source of testosterone and insulin-like 3 (INSL3), two hormones that regulate male reproductive development and function. In mammals, there are at least two distinct populations of Leydig cells, fetal Leydig cells (FLC) and adult Leydig cells (ALC), which are responsible for the synthesis of steroid hormones in the prenatal and postnatal testes, respectively [reviewed in (1, 2)]. Steroidogenesis is a multi-step process requiring various transporters and enzymes to convert cholesterol into a steroid hormone [reviewed in (3)]. The expression of the genes coding for these steroidogenic proteins is finely regulated to avoid steroid hormone insufficiency or excess across the lifespan.

Transcription factors (TFs) are fundamental to the regulation of gene expression. They are specialized proteins that recognize and bind to regulatory DNA sequences, modulating the rate of gene transcription [reviewed in (4)]. TFs typically recruit or interact with other TFs forming a unique molecular code that is key for specifying temporal- and tissue-specific gene expression as well as hormone responsiveness in hormone-sensitive target tissues. Moreover, TFs exhibit a dynamic behaviour that is characterized by their ability to interact with various partner proteins and

to regulate different target genes according to many determinants such as cell type, development stage, and signal stimulus, among others.

In recent years, the development of novel and powerful methodological approaches in molecular genetics has led to the emergence of new information regarding the role of TFs in the regulation of Leydig cell differentiation and function, and by extension, in male fertility and reproductive health. In this mini review, we provide a brief overview of the roles and mechanisms of action of some of the most characterized TFs in Leydig cells. We have adopted the most recent classification of TFs, which is based both on amino acid sequence homology and the tertiary structure of their DNA-binding domains (5). Using this classification, TFs that have been identified in Leydig cells are presented in **Table 1**; **Table 2** lists the target genes for these TFs in Leydig cells.

2 SUPERCLASS OF BASIC DOMAINS

2.1 Class of Basic Leucine Zipper Factors (BZIP)

2.1.1 AP-1 Factors

The activator protein 1 (AP-1) is a dimeric complex that includes members of the JUN, FOS, activating transcription factor (ATF), and musculoaponeurotic fibrosarcoma (MAF) families of TFs (54). Among the AP-1 members, JUN and FOS are the best characterized. The JUN subfamily comprises three members (cJUN, JUNB, and JUND) while four members compose the FOS subfamily [cFOS, FOSB, Fos-related antigens 1 (FRA-1, FOSL1), and Fos-related antigens 2 (FRA-2, FOSL2)]. Members of the JUN family can homodimerize or heterodimerize, whereas FOS family members only form heterodimers. The DNA sequence recognized by AP-1 members differs according to the dimer involved. JUN : JUN and FOS : JUN dimers recognize the TPA-response element (TRE; TGA(C/G)TCA) and the cAMP-responsive element (CRE; TGACGTCA), whereas ATF dimers preferentially recognize the CRE motif, and MAF dimers bind to MAF recognition elements (MAREs), a long palindromic sequence that contains TRE or CRE motifs (55) [reviewed in (56)].

AP-1 members were first described in Leydig cells in the late 1990s (57). AP-1 factors regulate several genes in Leydig cells such as the steroidogenic acute regulatory protein (*Star*) gene, which is activated by cJUN (6, 7, 12). In addition, cJUN cooperates with other TFs, including GATA4, STAT5B, and NUR77 leading to a stronger activation of the *Star* promoter (7–9). Both cJUN and cFOS regulate *Star* promoter activity by recruiting CREB and CBP (10). Transcription of the gap junction protein alpha1 [*Gja1*, also known as connexin43 (*Cx43*)] gene, involved in the initiation and maintenance of sperm production, is also controlled by cJUN, JUNB, and FOSL2, and by a cJUN/cFOS cooperation (11, 58). Furthermore, the ferredoxin 1 (*Fdx1*) promoter is activated by a cJUN/SF1 cooperation (12). *Fdx1* is a partner of *Cyp11a1*, participating in the conversion of cholesterol into pregnenolone, the first and rate-limiting step in steroidogenesis. It is important to note that the nature of the cJUN dimerization partner influences its role in gene regulation. For example, the combination of either FOSL2 or cFOS with cJUN inhibits the stimulatory effect of cJUN on the *Star* promoter (6, 10, 59). AP-1 factors in Leydig cells have been reviewed elsewhere (56).

2.1.2 CREB-Related Factors

CREB-related factors include three members: CRE-binding protein (CREB), cAMP response element modulator (CREM), and CRE-activating transcription factor (ATF-1). CREB factors homodimerize and heterodimerize with other CREB members and with other bZIP TFs, such as AP-1 members (60). CREB factors regulate transcription by binding to a CRE motif (TGACGTCA) similar to that recognized by AP-1 members, leading to overlap and redundancy in their activities (61). Although CREM is the most abundant member in MA-10 Leydig cells, all CREB members activate *Star* transcription through CRE elements located in the proximal promoter region (13, 14). Moreover, CREB factors cooperate with SF1 (NR5A1, Ad4BP) to enhance *Star* transcription (15). CREB also stimulates *CKLFSF2B* promoter activity in response to LH/cAMP (16). *Cklfsf2b* codes for a protein that inhibits steroidogenesis in Leydig cells (16). Therefore, CREB is involved in both activation and repression of steroidogenesis in Leydig cells depending on its target genes.

2.1.3 C/EBP-Related Factors

Members of the CCAAT/enhancer binding protein (C/EBP) subfamily contain a bZIP DNA-binding domain and regulate gene expression by binding to the sequence (A/G)TTGCG(C/T)AA(C/T) as homo- or heterodimers (62). C/EBPβ is the predominant member in Leydig cells (17, 63) where it activates *Star* transcription alone and in cooperation with SF1 and GATA4 (17–19). C/EBPβ also cooperates with NF-κβ p50 to stimulate *Nur77* promoter activity in Leydig cells (20). The *Nur77* gene encodes the orphan nuclear receptor NUR77, which regulates several genes involved in steroidogenesis in Leydig cells (see Section 3.1.2, *NGFI-B/NR4A Receptors*, below).

3 SUPERCLASS OF ZINC-COORDINATING DNA-BINDING DOMAINS

3.1 Class of Nuclear Receptors With C4 Zinc Fingers

TFs belonging to the nuclear receptor class respond to extracellular and intracellular signals to regulate gene expression. They also regulate cellular functions within the cytoplasm (64). In this section we present the nuclear receptors for which the roles and mechanisms of action are, or have begun to be, characterized in Leydig cells. Detailed information can be found in a review article dedicated to nuclear receptors in Leydig cells (65).

3.1.1 COUP-Like/NR2F Receptors

The nuclear receptor subclass 2, group F (NR2F) subfamily consists of three members: chicken ovalbumin upstream promoter transcription factor I (COUP-TFI, NR2F1, EAR3),

TABLE 1 | Classification of transcription factors identified in Leydig cells.

Superclass	Class	Family	Subfamily	Transcription factor
Basic Domains	Basic leucine zipper factors (bZIP)	Jun-related	Jun	cJUN
				JUNB
			NF-E2-like factors	NFE2L2 (NRF2)
		Fos-related	Fos	cFOS
				FRA-2 (FOSL2)
		CREB-related	CREB-like	CREB
				CREM
		C/EBP-related	C/EBP	C/EBPβ
	Basic helix-loop-helix factors (bHLH)	PAS domain	Arnt-like factors	ARNTL (BMAL1)
		bHLH-ZIP	SREBP factors	SREBP
			USF	USF1
				USF2
			n.a.	SPZ1
	Basic helix-span-helix factors (bHSH)	AP-2	n.a.	AP-2
Zinc-Coordinating DNA-Binding Domains	Nuclear receptors with C4 zinc fingers	Steroid Hormone Receptors (NR3)	GR-like receptors (NR3C)	NR3C1 (GR)
				NR3C2 (MR)
				NR3C3 (PR)
				NR3C4 (AR)
			ER-like (NR3A)	Erα; Erβ
		Thyroid hormone receptor-related (NR1)	Retinoic acid receptors (RAR - NR1B)	RARα, RARβ, RARγ
			Thyroid hormone receptors (THR - NR1A)	TRα, TRβ
			PPAR (NR1C)	PPARα, PPARβ/δ, PPARγ
			LXR (NR1H)	LXRα, FXR
		RXR-related receptors (NR2)	Retinoid X receptors (NR2B)	RXRα, RXRβ, RXRβ
			Testicular receptors (NR2C)	TR2 (NR2C1)
			COUP-like receptors (NR2F)	COUP-TFII (NR2F2)
		NGFI-B-related receptors (NR4A)	n.a.	NR4A1 (NUR77, NGFI-B)
				NR4A2 (NURR1)
		FTZ-F1-related receptors (NR5A)	n.a.	NR5A1 (SF-1, FTZ-F1)
				NR5A2 (LRH1)
		DAX-related receptors (NR0B)	n.a.	NR0B1 (DAX1)
				NR0B2 (SHP)
	Others C4 zinc finger-type factors	GATA-type zinc fingers	Two zinc-finger GATA factors	GATA4
	C2H2 zinc finger factors	Three-zinc finger Krüppel-related	Sp1-like	SP1
				SP3
			Kr-like	KLF6
			EGR	EGR1 (NGFI-A)
		More than 3 adjacent zinc fingers	ZNF44-2-like	ZNF44 (GIOT2)
			(unclassified)	ZNF461 (GIOT1)
Helix-Turn-Helix domains	Homeodomain factors	Paired-related HD	ARX	ARX
			RHOX	RHOX4
				PBX1
		HD-LIM	LHX2-like	LHX9
	Fork head/winged helix factors	Forkhead box (FOX)	FOXA	FOXA3 (HNF-3γ)
Alpha-helices exposed by beta-structures	MADS box factors	Regulators of differentiation	MEF2	MEF2A
				MEF2C
				MEF2D
Immunoglobulin fold	Rel homology region (RHR) factors	NF-kappaβ-related	NF-kappaβ p50 subunit-like	NF-κβ p50
			NF-kappaβ p65 subunit-like	NF-κβ p65 (RelA)
	STAT domain factors	STAT	n.a.	STAT5B

n.a., not applicable.

COUP-TFII (NR2F2, ARP1) and COUP-TFIII (NR2F6, EAR2). NR2Fs have been implicated in various physiological and developmental processes by regulating the expression of numerous genes [reviewed in (66, 67)]. *Via* their double zinc finger DNA-binding domain, NR2F factors bind as monomers to the nuclear receptor element AGGTCA and its variants. They also bind as dimers to direct (DR), inverted (IR), and everted (ER) repeats separated by 1-12 nucleotides (68).

TABLE 2 | Transcription factors and their target genes in Leydig cells.

Transcription Factor	Target Gene*	Select References
AP-1 (cJUN/cFOS)	*h, mStar*	(6–10)
	mGja1	(11)
	mFdx1	(12)
CREB/CREM	*mStar*	(10, 13–15)
	hCKLFSF2B	(16)
C/EBPβ	*mStar*	(17–19)
	rNr4a1 (Nur77)	(20)
BMAL1	*mStar*	(21)
AP-2	*m, rLhr*	(22, 23)
NR2F2 (COUP-TFII)	*mStar*	(24)
	mInsl3	(25)
	mAmhr2	(26)
	mAkr1c14	(27)
	mGsta3	(28)
	mInha	(28)
NR4A1 (NUR77, NGFI-B)	*mStar*	(9, 29, 30)
	m, hHsd3b	(31, 32)
	h, mInsl3	(33, 34)
	rCyp17a1	(35, 36)
NR5A1 (SF1, FTZ-F1)	*m, hStar*	(9, 15, 17–19, 37)
	rCyp19a1	(38)
	hHSD3B2	(32)
	hCyp11a1	(37)
	rCyp17a1	(39, 40)
	rPrlr	(41)
	rAmhr2	(42)
	mVanin-1	(43)
	m, hInsl3	(33, 34)
	mFdx1	(12)
NR5A2 (LRH1)	*mStar*	(9)
	rCyp19a1	(44)
	m, hInsl3	(33)
NR0B1 (DAX1)	*mStar*	(45)
GATA4	*h, mStar*	(7, 18, 19, 46, 47)
	hHSD3B2	(32)
	mAmhr2	(46)
	rSrd5a1	(46)
SP1	*rSrbi*	(48)
	mLhr	(22)
SP1/SP3	*mVegf*	(49)
	mPbr	(50)
KLF6	*hINSL3*	(34)
FOXA3 (HNF-3γ)	*rPdgfra*	(51)
MEF2	*mStar*	(47)
	rNr4a1 (Nur77)	(52)
	mGsta1-4	(53)
	mAkr1c14	(27)
NF-κβ p50	*rNr4a1 (Nur77)*	(20)
NF-κβ p65 (RelA)	*rCyp17a1*	(31)
STAT5B	*mStar*	(8)
	rNr4a1 (Nur77)	(8)

**The letter preceding the name of the gene refers to the species: m, mouse; r, rat; h, human.*

Of the NR2F subfamily members, COUP-TFII is by far the most abundant in Leydig cells. Although COUP-TFII is present in mice interstitial cells from early fetal life throughout adulthood, it is only associated with steroidogenically active ALC in postnatal life (24). COUP-TFII is a marker of stem cells giving rise to the ALC population (24, 69). *In vivo* studies using mouse models have shown that COUP-TFII is crucial for Leydig cell development and male reproductive function (70, 71). In Leydig cells, COUP-TFII regulates the expression of several genes involved in lipid metabolism, male gonad development, and steroidogenesis (28). COUP-TFII activates *Star*, *Insl3*, and *Amhr2* expression by binding to their respective promoter sequences (24–26). It cooperates with SF1 on the *Star* and *Insl3* promoters (24, 25) and with SP1 on the *Amhr2* promoter (26). The *Akr1c14* gene, which codes for the 3α-HSD enzyme that catalyzes the interconversion of dihydrotestosterone (DHT) into 5α-androstane-3α,17β-diol (3α-diol), is activated by COUP-TFII in cooperation with MEF2 (27). COUP-TFII also

activates the expression of *Gsta3* and *Inha*, genes involved in the inactivation of reactive oxygen species and in the homeostasis of the hypothalamic-pituitary-gonadal axis, respectively (28). Expression of several other Leydig cell genes including *Cyp17a1*, *Hsd3b1* and *Cyp11a1* is reduced in *Coup-tfii* null mice (71) and in COUP-TFII-depleted MA-10 Leydig cells (28), implying a role for COUP-TFII in their expression.

3.1.2 NGFI-B/NR4A Receptors

The NR4A family consists of three orphan nuclear receptors: neuron-derived clone 77 (NR4A1, NUR77, NGFI-B, TR3), nuclear receptor related 1 (NR4A2, NURR1) and neuron-derived orphan receptor 1 (NR4A3, NOR1). NR4A members can bind to DNA either as monomers, homodimers, or heterodimers. NUR77 and NURR1 also heterodimerize with RXR. As monomers, they bind to a NGFI-B-response element (NBRE; AAAGGTCA), as homodimers and heterodimers to a Nur-response element (NurRE; TGATATTTN$_6$AAATGCCA), and as heterodimers with RXR to a DR5 sequence [reviewed in (72, 73)]. NR4A factors are immediate early response genes involved in the regulation of several physiological and pathological processes, including steroidogenesis (74) [reviewed in (75)].

Leydig cells contain mainly NUR77, followed by NURR1 where both are important regulators of basal and hormone-induced gene transcription (76). *Nur77* expression is strongly increased by LH (76) *via* the CAMKI pathway (29, 77) consistent with its role as a key regulator of several genes in Leydig cells including *Cyp17a1* (31, 35), *Hsd3b* (31), *HSD3B2* (32), *Insl3* (33, 34), and *Star* (29, 30). NUR77 regulates the expression of these genes by cooperating with CAMKI (29), cJUN (9), KLF6 (34), and SF1 (34). In Leydig cells, *Nur77* expression is controlled by distinct regulatory elements for both basal and hormone-induced expression (77), through mechanisms involving MEF2 (52), STAT5B (8), CREB (77), cJUN (9), C/EBPβ (20), and NF-κβ p50 (20).

3.1.3 FTZ-F1-Related/NR5A Receptors

The nuclear receptor 5A (NR5A) family comprises two members: steroidogenic factor 1 (NR5A1, Ad4BP, SF1) and liver receptor homolog 1 (NR5A2, LRH1, FTF). Both factors share high sequence similarity, bind to the same DNA motif, regulate common target steroidogenic genes, and exhibit overlapping expression in several tissues [reviewed in (78, 79)]. Despite this, they have nonredundant roles and cannot fully compensate for each other [reviewed in (78, 79)]. NR5A members regulate gene expression by binding as monomers to the sequence (T/C)CAAGGTCA located in the promoter region of target genes.

SF1 was initially identified as a tissue-specific activator of several cytochrome P450 steroid hydroxylase genes (38, 80). SF1 is essential for steroidogenesis, reproduction, and male sex differentiation, as revealed by mutations in the *SF1* gene in humans and in mouse models where adrenal and gonadal development and function are impaired (37, 81–84) [reviewed in (85, 86)]. Interestingly, *Sf1* knockdown in MLTC-1 Leydig cells leads to downregulation of *Star* and *Cyp11a1* and accumulation of neutral lipids and cholesterol (37). Moreover, SF1 is one of only a handful of TFs that can convert fibroblasts into functional Leydig-like cells, revealing the pivotal role of this nuclear receptor in Leydig cells (87, 88).

In vitro analysis of regulatory elements has shown that the expression of several Leydig cell genes is regulated by SF1. These include *Star* (9, 17, 37), *Cyp19a1* (38), *HSD3B2* (32), *Cyp17a1* (39, 40), *Cyp11a1* (37), *Prlr* (41), *Amhr2* (42), *Vanin-1* (43), *Insl3* (33), and *Fdx1* (12). SF1 activity relies on interactions with a long list of protein partners, such as C/EBPβ (17), cJUN (9, 12), DAX1 (45), GATA4 (89), and KLF6 (34).

Like SF1, LRH1 influences steroidogenesis and fertility. To date, only a few genes are known to be regulated by LRH1 in Leydig cells, including *Star* (in cooperation with cJUN) (9), *Cyp19a1* (44), and *Insl3* (33).

3.1.4 DAX-Related/NR0B Receptors

The DAX-related receptor (NR0B) family comprises two members: critical region on the X chromosome gene 1 (NR0B1, DAX1) and small heterodimer partner (NR0B2, SHP). They lack the typical zinc finger DNA-binding domain and therefore act mainly as transcriptional repressors by inhibiting the activity of other TFs (90, 91). Both members are present in Leydig cells and act as homodimers or heterodimers (92).

In *Dax1*-deficient mice, testis cord organization is compromised and FLC development is arrested (93). *In vitro* studies in Leydig cell lines revealed that DAX1 represses steroidogenesis by inhibiting *Star* expression, while silencing *Dax1* expression increases *Star* transcription leading to enhanced steroidogenesis (45). DAX1 interacts with and represses the activity of NUR77 and SF1, inhibiting *Star* expression (36, 45). Interestingly, *Dax1* knockdown in MA-10 Leydig cells decreases *Cyp11a1* and *Star* expression suggesting that DAX1 could also act as a coactivator in addition to its repressor role (94).

SHP is a repressor of steroidogenesis. In mouse Leydig cells, *Shp* expression is reduced by hCG treatment (95). In *Shp*-deficient mice, testosterone levels as well as *Star*, *Cyp11a1*, and *Hsd3b1* mRNA levels are increased leading to premature sexual maturation (96). SHP inhibits steroidogenesis by interacting and repressing the activity of LHR1 (96). *Shp* mRNA levels are significantly reduced in COUP-TFII- and MEF2-depleted Leydig cells, indicating that *Shp* expression requires these two TFs (28, 97).

3.2 Class of Other C4 Zinc Finger-Type Factors

3.2.1 Two Zinc-Finger GATA Factors

The six GATA members (GATA1 to 6) are crucial for the development and function of several tissues, including the male gonad [reviewed in (98, 99)]. GATA factors regulate gene expression by binding *via* their two zinc fingers to the DNA sequence (A/T)GATA(A/G) in the promoter region of target genes. Of the six GATA factors, GATA4 is the most abundant in Leydig cells *in vivo* (100–102). Its expression is also the broadest being present from the onset of testis morphogenesis and into adult life (103). Considered one of the first gonadal markers in both sexes, GATA4 is required for urogenital ridge development in mice and later for mammalian gonadal differentiation (103, 104).

A *Sf1*-Cre mouse line, which expresses the Cre recombinase in several tissues including Leydig, Sertoli and adrenal cells, was used to conditionally inactivate *Gata4*. The resulting males were undervirilized and had small testes lacking mature sperm (105), thereby supporting a role for this factor in male reproductive function. Transcriptomic analysis of GATA4-depleted MA-10 Leydig cells revealed several deregulated pathways, including cholesterol metabolism and steroidogenesis (46). Consistent with this, GATA4 stimulates the transcription of several genes expressed in Leydig cells such as *HSD3B2* (32), *Cyp19a1* (106), *Star* (46, 106), *Inha* (106), *Sf1* (106), *Amhr2* (46), and *Srd5a1* (46). GATA4 also cooperates with cJUN, C/EBPβ, and MEF2 to upregulate *Star* expression (7, 18, 47). These results emphasize the indispensable role of GATA4 in the differentiation and function of FLC and ALC (46, 107). The critical nature of GATA4 in the Leydig cell differentiation is further supported by the demonstration that GATA4, along with SF1 and DMRT1 or NUR77, are sufficient to reprogram fibroblasts toward the Leydig-like cell fate (87, 88).

4 SUPERCLASS OF HELIX-TURN-HELIX DOMAINS

4.1 Class of Forkhead/Winged Helix Factors

4.1.1 Forkhead Box (FOX) Factors

The forkhead box A3 (FOXA3) is the only member of the FOXA subfamily present in the testes, mainly in ALC (51, 108, 109). So far, the only direct target identified for FOXA3 in Leydig cells is the gene coding for the platelet-derived growth factor receptor alpha (*Pdgfra*) (51), that in response to PDGF signaling, acts in Leydig cell differentiation and testis organogenesis (110). In cAMP-induced steroidogenesis, FOXA3 is proposed to repress *Nur77* expression, which in turn reduces steroidogenic gene expression and testosterone production (111). These findings indicate that FOXA3 participates actively in the control of Leydig cell function and male fertility.

5 SUPERCLASS OF α-HELICES EXPOSED BY β-STRUCTURES

5.1 Class of MADS Box Factors

5.1.1 MEF2 Subfamily

The Myocyte Enhancer Factor 2 (MEF2) factor subfamily comprises four members (MEF2A-2D) that share two highly conserved domains, a MADS box and a MEF2 domain, involved in dimerization and DNA binding [reviewed in (112)]. MEF2 factors form homo- and heterodimers that bind the sequence YTAWWWWTAR (Y=C/T, W=A/T, R=G/A) in the promoter region of their target genes. Because of their conserved DNA-binding domain, MEF2 members share common targets and can compensate for each other. MEF2 members also display unique spatiotemporal patterns in different tissues. Due to their divergent transactivation domain, MEF2 factors respond to different signals and interact with different partners, leading to specific gene expression [reviewed in (112)].

Although widely studied in other organs, the presence of MEF2 in the testes, more specifically in Sertoli and Leydig cells, was only reported in 2014 (52). In Leydig cells, MEF2A and MEF2D and to a lesser extent MEF2C, are expressed from early gonadal development into adulthood (52). MEF2A/2D-depleted MA-10 Leydig cells produce less steroid hormone demonstrating that MEF2 factors have a role in male reproductive function (47). Consistent with this, microarray analysis of MEF2A/2D-depleted MA-10 Leydig cells identified several differently regulated genes known to be involved in fertility, gonad morphology, and steroidogenesis (97). To date, direct gene targets for MEF2 factors in Leydig cells include *Nur77* (52), *Gsta1-4* (53), *Star* (involving a MEF2/GATA4 cooperation) (47), and *Akr1c14* (through a cooperation with COUP-TFII) (27). The complete network of genes regulated by MEF2 factors in Leydig cells as well as MEF2 interacting partners remain to be fully elucidated.

6 SUPERCLASS OF IMMUNOGLOBULIN FOLD

6.1 Class of STAT Domain Factors

6.1.1 STAT Factors

The signal transducer and activator of transcription (STAT) family consists of seven proteins [reviewed in (113)]. Cytokines and growth factors activate STAT members through the Janus kinase (JAK) signaling pathway. In the nucleus, STAT factors regulate gene transcription by binding as homo- or heterodimers to the γ-interferon-activated sequence (GAS; $TTCN_3GAA$) in the promoter region of target genes. So far, STAT5B is the only STAT factor identified in Leydig cells (114). In these cells, STAT5B is activated by growth hormone, an important regulator of steroidogenesis (8). STAT5B activates *Star* expression directly by binding to a GAS element and in cooperation with cJUN (8). STAT5B also activates the *Nur77* promoter (8).

7 OTHER TRANSCRIPTION FACTORS PRESENT IN LEYDIG CELLS

Other TFs have been described in Leydig cells, but their mechanisms of action remain poorly characterized. This includes the nuclear factor E2-related factor-2 (NRF2, NFE2l2), which is an important modulator of reactive oxygen species levels, especially in aging Leydig cells (115–117). Furthermore, the brain and muscle arnt-like protein-1 (BMAL1), a component of the circadian clock system, is also directly involved in the control of Leydig cell function in different species, by regulating the expression of *Star*, *Hsb3b*, and *Cyp11a1* (21, 118, 119). Finally, members of the nuclear factor kappa-beta (NF-κβ) family, involved in immune and inflammatory responses, also contribute to the regulation of steroidogenesis in Leydig cells (20, 31, 120).

8 CONCLUDING REMARKS

As described in this mini review, several TFs belonging to different classes and families are pivotal to ensure proper Leydig cell differentiation and function. This underscores the complex regulatory mechanisms involved. Most of the knowledge acquired so far has relied on *in vitro* analyses of regulatory elements of genes expressed in Leydig cells. Although we are far from fully understanding all the signals, pathways, and TFs involved, technological advances and novel mouse models will certainly lead to significant discoveries in the coming years.

AUTHOR CONTRIBUTIONS

KM wrote the first draft of the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

Supported by a grant from the Canadian Institutes of Health Research (funding reference number MOP-81387) to JT. KM is the recipient of a studentship from the Fonds de recherche du Québec-Santé.

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frontiers | Frontiers in Endocrinology

MINI REVIEW
published: 28 April 2022
doi: 10.3389/fendo.2022.894437

Immune Cells as Critical Regulators of Steroidogenesis in the Testis and Beyond

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Steroidogenesis is an essential biological process for embryonic development, reproduction, and adult health. While specific glandular cells, such as Leydig cells in the testis, are traditionally known to be the principal players in steroid hormone production, there are other cell types that contribute to the process of steroidogenesis. In particular, immune cells are often an important component of the cellular niche that is required for the production of steroid hormones. For several decades, studies have reported that testicular macrophages and Leydig cells are intimately associated and exhibit a dependency on the other cell type for their proper development; however, the mechanisms that underlie the functional relationship between macrophages and Leydig cells are unclear. Beyond the testis, in certain instances immune cells themselves, such as certain types of lymphocytes, are capable of steroid hormone production, thus highlighting the complexity and diversity that underlie steroidogenesis. In this review we will describe how immune cells are critical regulators of steroidogenesis in the testis and in extra-glandular locations, as well as discuss how this area of research offers opportunities to uncover new insights into steroid hormone production.

Keywords: Leydig cell, macrophage, steroidogenesis, testosterone, testis, immune cell, reproduction

OPEN ACCESS

Edited by:
Barry Zirkin,
Johns Hopkins University,
United States

Reviewed by:
Kate Lakoski Loveland,
Monash University, Australia

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Specialty section:
This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology

Received: *11 March 2022*
Accepted: *31 March 2022*
Published: *28 April 2022*

Citation:
Gu X, Li S-Y, Matsuyama S and DeFalco T (2022) Immune Cells as Critical Regulators of Steroidogenesis in the Testis and Beyond. Front. Endocrinol. 13:894437. doi: 10.3389/fendo.2022.894437

INTRODUCTION

Steroid hormones are mainly produced in the adrenal glands, gonads, and placenta, where they play endocrine roles in regulating target tissue or cell function depending on circulating steroid concentrations (1, 2). While specific hormone-producing cells in these tissues have received the major share of focus in the field, previous studies have shown that many peripheral tissues and cell types within the brain, kidney, lung, skeletal muscle, intestine, keratinocytes, adipocytes, astrocytes, and placental trophoblasts have the capacity of *de novo* steroidogenesis or steroid conversion (3–11). This diversity of tissues with steroidogenic capacity indicates that there are multiple cell types that can undertake or mediate steroid hormone production. One cell lineage that has been linked to steroidogenesis is the immune cell lineage, as local sex steroid production has been identified within immune cell populations such as macrophages and T lymphocytes (12–14). Within the testis, macrophages have been implicated in steroid production by Leydig cells (15, 16), although the mechanisms by which macrophages developmentally or functionally regulate Leydig cells are poorly understood. The unexpected and poorly understood steroidogenic capacity of immune cells and

their roles in modulating glandular steroidogenesis is becoming an emerging area of research that is critical for a deeper understanding of the complex immunoregulatory roles of steroid hormones in normal and disease contexts. In this review we will discuss the various roles proposed for testicular macrophages in Leydig cell biology and we will highlight future areas of research that should be pursued to elucidate the mechanisms underlying regulatory functions of immune cells and their potential *de novo* steroidogenesis in the testis and, potentially, beyond.

BIOSYNTHETIC PATHWAY AND SITE OF PRODUCTION OF STEROID HORMONES

Steroidogenesis is a process in which cholesterol is converted into steroid hormones by a series of steps mediated by steroidogenic enzymes. In this process, there are two key rate-limiting steps, which are 1) the transport of cholesterol from the cytoplasm into mitochondria and 2) the conversion of cholesterol into pregnenolone. Free cholesterol is derived from intracellular cholesterol that is synthesized either from acetate, from cholesterol ester stored in lipid droplets, or from uptake of cholesterol-containing low-density lipoproteins (LDLs). Plasma LDLs are the most important source of cholesterol when steroidogenic cells are chronically stimulated. Then steroidogenic acute regulatory protein (StAR) promotes the rapid flux of cholesterol into the mitochondria, where cholesterol is catalyzed to yield pregnenolone by side-chain cleavage enzyme cytochrome P450scc (also known as CYP11A1, encoded by the *CYP11A1* gene) within the mitochondrial inner membrane. Pregnenolone, as an immediate precursor, requires further catalysis by two major families of enzymes, which are cytochrome P450 (CYP) and hydroxysteroid dehydrogenase (HSD) located in both mitochondria and the endoplasmic reticulum, to facilitate the biosynthesis of steroid hormones (2, 17, 18).

In many contexts, steroid hormones are classified based on the organs that produce them and the receptors to which they bind. The adrenal steroids, which consist of glucocorticoids and mineralocorticoids, are secreted by the adrenal cortex. Glucocorticoids such as cortisol in humans and corticosterone in rodents control many cell metabolic processes, including maintaining blood pressure and regulating immune cell function. Aldosterone is the most well-known mineralocorticoid, which maintains the body's water and salt balance by acting primarily on the kidneys. Sex steroid hormones, which are composed of androgens (e.g., testosterone), estrogens (e.g., estradiol), and progestogens (e.g., progesterone), are produced by the gonads and placenta. These sex hormones are responsible for regulating sexual development and promoting fertility. Additionally, the adrenal cortex secretes sex hormones to a lesser extent than the gonads, and the gonads may produce adrenal steroids (1, 19). Aside from dedicated steroidogenic cells like Leydig cells, theca cells, or adrenocortical cells, future research should address the extent to which alternative glandular or extra-glandular cell types in the gonads and adrenal are involved in *de novo* steroidogenesis.

DEVELOPMENTAL LINKS BETWEEN TESTICULAR MACROPHAGES AND LEYDIG CELLS

Early analyses of the immune cells in the testis revealed that macrophages are a large component of the testicular interstitial compartment, comprising approximately 20% of interstitial cells (20). Macrophages and Leydig cells, therefore, occupy the same compartment of the testis and are in intimate contact throughout development (21). Histological and ultrastructural studies of the postnatal and adult rat testis demonstrated that macrophages and Leydig cells form intercellular cytoplasmic digitations (21, 22), which only are observed between these 2 cell types and only upon puberty (22), indicating an intimate relationship linked to testicular maturation. Furthermore, macrophage-deficient osteopetrotic mice mutant for *colony stimulating factor 1* ($Csf1^{op/op}$) are infertile as a result of low testosterone, oligozoospermia, and decreased libido (15, 23, 24). Analyses of normal and cryptorchid testes revealed that there is a robust correlation between the volume density of Leydig cells and macrophages, as well as total mass of Leydig cells and macrophages per testis (25), leading to early ideas of functional coupling between the two cell types. These findings strongly suggest that testicular macrophages have trophic functions in Leydig cell differentiation and promote steroidogenesis, but the developmental and functional links between macrophages and Leydig cells are still open areas of investigation.

Multiple studies by Gaytan et al. in the 1990s revealed that there is an interdependent relationship between macrophages and Leydig cells in both developmental and regenerative contexts (26–28). Using dichloromethylene diphosphonate-containing liposome (Cl_2MDP-lp) injection to deplete testicular macrophages in prepubertal rats, they found that macrophages are required for the development of Leydig cells during postnatal testicular maturation (26). The authors concluded that, in the absence of macrophages, Leydig cell proliferation did not occur, nor were mesenchymal progenitor cells able to undergo differentiation into Leydig cells (26). They further speculated that macrophages were required for Leydig cell responsiveness to lutenizing hormone (LH) and human chorionic gonadotropin (hCG) (29, 30), as hCG-treated Leydig cells in Cl_2MDP-lp-injected testes did not increase in number as in contralateral intact testes. Regeneration of Leydig cells in testes that had selective Leydig cell depletion induced by ethylene dimethanesulfonate (EDS) treatment, which requires LH (31), was also hindered in the absence of macrophages (27, 28) (see next paragraph). These findings suggest that as-of-yet undefined macrophage factors are essential for Leydig cell responsiveness to LH/hCG.

Gaytan et al. demonstrated, again using a Cl_2MDP-lp-mediated ablation method (27, 28), that testicular macrophages are required for adult Leydig cell regeneration after specific

depletion of Leydig cells *via* EDS treatment. In contrast, when macrophages were ablated in intact adult testes, there was no effect on Leydig cell numbers (28), indicating that macrophages are not as essential for steady-state maintenance of adult Leydig cell numbers; a more recent finding showed a similar result, in which a diphtheria-toxin-mediated ablation of adult macrophages did not result in a change in Leydig cell number (although there was a significant drop in testicular testosterone levels) (32).

FUNCTIONAL RELATIONSHIP BETWEEN TESTICULAR MACROPHAGES AND LEYDIG CELLS

Given the tight physical association between testicular macrophages and Leydig cells in the interstitial compartment, in the past 40 years most investigations into testicular macrophage functions focused on Leydig cell steroidogenesis (16, 33). Yee and Hutson in 1985 showed that testicular macrophage-conditioned medium (TMCM) in a dose-dependent manner increases testosterone production of Leydig cells (34). Consistent with this finding, bank vole Leydig cells from a long photoperiod in co-cultures with testicular macrophages or treated with TMCM produced more testosterone (35). However, some subsequent studies demonstrated that non-stimulated testicular macrophages have an inhibitory effect on the production of testosterone by Leydig cells (36–38), whereas TMCM obtained from lipopolysaccharide (LPS)-stimulated macrophages or macrophages isolated from autoimmune orchitis could promote testosterone production (36, 39). Therefore, the role of testicular macrophages in Leydig cell steroidogenesis under physiological conditions has been controversial. Furthermore, testicular macrophages isolated using different methods may have different phenotypes and metabolic properties *in vitro* due to the loss of their complex *in vivo* microenvironment. This could be one of the reasons why testicular macrophages need to be additionally activated in some circumstances in order to function properly. Our recent study found that the depletion of adult testicular macrophages *in vivo* decreases testicular testosterone levels (32), suggesting the beneficial effect of testicular macrophages on Leydig cell steroidogenesis.

Role of Testicular Macrophage-Derived Cytokines in Leydig Cell Steroidogenesis

A number of studies have shown that testicular macrophages from rats and goldfish can secrete pro-inflammatory cytokines, such as interleukin 1 (IL1) and tumor necrosis factor (TNF), which were dramatically increased after stimulation by LPS (40–42). Therefore, these cytokines from testicular macrophages may be key regulators of testosterone production, either enhancing or inhibiting it under physiological and inflammatory conditions. Previous research on the roles of IL1 on Leydig cell steroidogenesis *in vitro* yielded contradictory results. Many studies have shown that IL1B decreases testosterone synthesis of Leydig cells (43–45), whereas some studies reported that IL1B had no effects on testosterone synthesis of Leydig cells (37, 46), or even increased testosterone synthesis (47). Different testicular IL1 isoforms, including 17K IL1A and IL1B, 32K proIL1A, and a 24K splice variant, stimulated testosterone production by Leydig cells from 40- but not 80-day-old rats, and the potency of IL1A was 50-fold more than IL-1B (48). Intratesticular administration of IL1B resulted in a significant increase in basal testosterone secretion *in vitro* and serum testosterone concentration one day after treatment in 21-day-old rats, but it inhibited this process 6 days after treatment (49). A recent study showed that IL1B deficiency induced by treatment with diacerein, an anti-inflammatory agent, impairs Leydig cell function, suggesting a positive effect of IL1B in steroidogenesis under normal conditions (50). These findings suggest that the paracrine roles of IL1 in regulating Leydig cell steroidogenesis may be related to animal age, treatment time, and IL1 isoforms. Generally, numerous studies documented that TNF reduces testosterone production of Leydig cell function *in vitro* and *in vivo*. TNF treatment inhibited steroidogenic enzyme activity or their mRNA expression, such as StAR, CYP17A1, and HSD3B1, in a dose-dependent manner (51–55). Additionally, under LPS stimulation, testicular macrophages also could produce reactive oxygen species (ROS) and nitric oxide (NO) (33). Leydig cell steroidogenesis was inhibited by both hydrogen peroxide (a potent oxidant) (56, 57) and NO (58, 59). These results suggest that under inflammatory conditions, activated testicular macrophages secrete several factors that limit Leydig cell steroidogenesis and even impair testicular function.

Several groups' studies have clearly demonstrated that there are two distinct macrophage populations in adult testis: 1) interstitial macrophages located in the testicular interstitium and in close contact with Leydig cells; and 2) peritubular macrophages located in the myoid layer around seminiferous tubules (32, 60–65). Interstitial macrophages express higher levels of the immunosuppressive M2-type gene *Il10*, while peritubular macrophages highly express the M1-associated inflammatory gene *Il1b* (62). However, whether IL10 and IL1B can be secreted into the testicular interstitial compartment by the two macrophage populations and whether the two populations have unique or overlapping roles in regulating Leydig cell steroidogenesis have been not investigated.

Role of Testicular Macrophage-Derived Lipophilic Factors in Adult Leydig Cell Steroidogenesis

Aside from cytokines, a testicular macrophage-derived factor implicated in steroidogenesis was a lipophilic factor later identified as 25-hydroxycholesterol (25-HC) after it was purified using organic extraction and high-performance liquid chromatography (66, 67). Furthermore, human macrophages have been shown to produce 25-HC, indicating that this phenomenon is not specific to rodents (68). 25-HC is an oxysterol that is synthesized from cholesterol by the addition of a hydroxyl group to the position 25 carbon, and this reaction is catalyzed by cholesterol 25-hydroxylase (CH25H) (69). CH25H is found in the endoplasmic reticulum and is widely expressed in many cell types, particularly macrophages (70). The intracellular

level of 25-HC is primarily determined by the activity of CH25H, which is upregulated *via* TLR4/IRF3/IFN-β/STAT1 signaling pathways in LPS-stimulated macrophages (71).

Recent studies have found that macrophages have the potential to provide an alternative pathway for steroidogenesis by providing 25-HC as a direct substrate for side chain cleavage (16, 72). 25-HC has been shown to increase StAR protein levels in Leydig cells and adrenocortical cells *in vitro* (73). Kazeto et al. transfected non-steroidogenic cells with a complex of eel *P450scc* cDNA (encoding *Cyp11a1*) and discovered that the recombinant CYP11A1 produced in these cells efficiently catalyzed the conversion of 25-HC into pregnenolone (74). A recent study revealed that Leydig cells utilize 25-HC as a substrate for testosterone biosynthesis (72), in which it was proposed that cholesterol is converted into 25-HC by CH25H in macrophages, and the 25-HC is subsequently secreted into neighboring Leydig cells. In Leydig cells, StAR transports 25-HC to mitochondria where is converted into pregnenolone by the CYP11A1 enzyme. 25-HC produced in macrophages promotes testosterone synthesis in Leydig cells, while testosterone produced in Leydig cells inhibits 25-HC production in macrophages (75), which suggests a paracrine negative feedback loop between the two cell types. Therefore, 25-HC could be a paracrine factor that mediates interactions between macrophages and neighboring Leydig cells.

STEROID PRODUCTION BY IMMUNE CELLS

Tissue immune cells, particularly macrophages and T lymphocytes, may be an important source of local steroid production by steroid conversion or *de novo* steroidogenesis. Intracrine and paracrine roles of immune-cell-derived steroids may be essential for cellular functions within various tissues. Therefore, immune cell-derived steroids and steroid metabolites potentially have biological effects within the tissue microenvironment, although their quantities in tissue fluids or blood are likely modest.

Steroid Conversion Capacity of Immune Cells

Immune cells are not only passive targets of steroid hormones due to their expression of hormone receptors, but also have the capacity for steroid hormone conversion and metabolism (14). Human alveolar macrophages can convert androstenedione to testosterone and other steroids through the catalytic activity of 3β-HSD, 3α-HSD, 17β-HSD, and 5α-reductase enzymes (76). These steroidogenic enzymes also are present in the alveolar macrophages of pigs (77), indicating an evolutionary conservation of these steroidogenic functions. In turn, testosterone is converted to androstenedione and dihydrotestosterone (DHT) in primary cultured human synovial macrophages (78, 79). In addition, human monocyte-derived macrophages, rather than monocytes, preferentially convert dehydroepiandrosterone (DHEA) to a physiologically relevant amount of downstream steroid hormones including testosterone, androstenedione, estrone, and estradiol, in the presence of LPS (80). When human peripheral monocyte-derived THP-1 cells and primary monocytes are differentiated to macrophages, they exhibit upregulation of both *CYP19A1* mRNA levels and aromatase activity, which catalyzes the conversion of androgens to estrogens, in response to dexamethasone (a synthetic glucocorticoid) (81). These studies suggest that the conversion of steroid hormones in macrophages may be related to their phenotypic heterogeneity and microenvironmental contexts.

Steroidogenic enzymes are also expressed by T lymphocytes. Splenic T lymphocytes in trauma-hemorrhagic male and proestrus female mice exhibited enzyme activities of 3β-HSD, 17β-HSD, 5α-reductase, and aromatase (CYP19A1). Although most of these steroidogenic enzymes were also found in B lymphocytes, they had lower activity and no 17β-HSD activity. Increased 5α-reductase activity in male T cells is immunosuppressive due to enhanced 5α-dihydrotestosterone synthesis, whereas increased aromatase activity, which triggered 17β-estradiol synthesis, has an immune-protective function in female T cells (82). Furthermore, *CYP19A1* expression and aromatase activity has been reported in tumor-infiltrating lymphocytes (83, 84). However, whether other lymphocytes and/or myeloid cell types in normal tissues have steroidogenic activities that can induce the conversion of steroid hormones to fulfill their immunoregulatory functions is likely a fruitful area for future research.

De Novo Steroidogenesis of Immune Cells

Beyond immune cells' capability of local steroid conversion, recent reports indicate that immune cells have the ability to undertake *de novo* steroidogenesis starting from the initial processing of cholesterol. Type 2 immune cells, including mast cells, basophils, and particularly T helper 2 cells, can *de novo* synthesize pregnenolone during helminth infection and in tumor environments to regulate immune homeostasis and tumor immunosuppression, respectively. T-helper-2-cell-mediated steroidogenesis is likely due to the high expression of CYP11A1 in these immune cells (12, 13). CYP11A1 expression is increased in CD4+ or CD8+ T cells in peanut-induced intestinal anaphylaxis and allergic lung disease (85, 86). Additionally, in peanut-allergic children, CYP11A1 is involved in the regulation of CD4+ T cells in the proallergic immune response (87). These findings may suggest the importance of steroids derived from immune-cell-mediated *de novo* steroidogenesis in healthy and pathological microenvironments with adaptive immunomodulation. In addition, infiltrating myeloid cells in dystrophic skeletal muscles can produce aldosterone, as all genes encoding steroidogenic enzymes in the aldosterone synthesis pathway are expressed by muscle-derived myeloid cells (88). However, whether tissue-resident or inflammation-induced macrophages are capable of *de novo* steroidogenesis has yet to be determined. StAR has been detected in macrophages (89, 90), indicating that macrophages contain at least the ability to produce steroidogenic substrates. Interestingly, primary testicular macrophages produce significant amounts of corticosterone *in vitro* (91), but whether this corticosterone is derived from the conversion of other steroids or from *de novo* steroidogenesis was not investigated in that study. A recent study reported that testicular

macrophages could also produce progesterone, and this steroid production by macrophages may contribute to a local feedback loop between Leydig cells and macrophages that regulates testosterone production (92). Therefore, it is necessary to explore in greater detail whether and how testicular macrophages have the ability to undertake *de novo* steroidogenesis and, if so, to what extent testicular function is dependent on this source of steroidogenesis.

DISCUSSION

The presence of testicular macrophages and their potential roles in Leydig cell steroidogenesis have been investigated for several decades, but the mechanisms underlying their functional relationship is still unclear. One particular area that needs to be rigorously addressed is whether testicular macrophages merely promote steroidogenesis by Leydig cells or if they undergo *de novo* steroidogenesis in a meaningful way to promote spermatogenesis and fertility. Macrophages could impact Leydig cells through a number of mechanisms, such as regulating the cytokine environment, providing steroidogenic substrates, or through modulating Leydig cell ultrastructure *via* unique cell-cell junctions (**Figure 1**). Given recent findings of *de novo* steroidogenesis by T cells in various contexts, the contributions of immune-cell-derived steroids should be addressed in the context of testicular function. Furthermore, as many studies have linked inflammation to infertility, it is also critical to study how macrophage polarization and the subsequent changes in their cellular activities cause or exacerbate testicular pathology. Reports in several fields indicate that immune cell steroid production is a broadly observed and evolutionarily conserved phenomenon; therefore, understanding the roles of immune cells in testicular steroidogenesis and Leydig cell function will likely provide new insights into endocrinology that will extend beyond the boundaries of the testis.

AUTHOR CONTRIBUTIONS

XG, S-YL, and SM performed literature searches and drafted the manuscript. TD conceptualized, drafted, and supervised the manuscript. All authors contributed to manuscript revision and approved the submitted version.

FUNDING

Work from the DeFalco laboratory is supported by National Institutes of Health (grants R35GM119458 and R01HD094698 to TD).

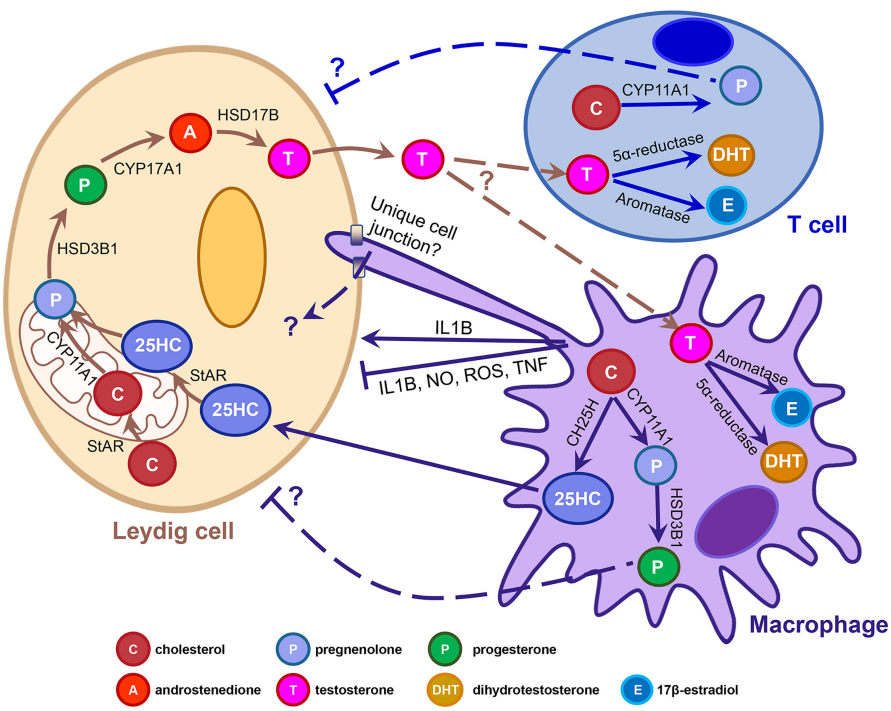

FIGURE 1 | Potential mechanisms underlying macrophage-Leydig cell interactions and immune cell steroidogenesis. Cartoon depicts the adult rodent testicular interstitium, containing a Leydig cell, macrophage, and T cell. Arrows denote the different molecular and cellular pathways that have been implicated in macrophage-Leydig interactions and *de novo* steroidogenesis by immune cells. T-shaped lines indicate an inhibitory interaction. Dashed arrows and lines flanked by question marks indicate that interactions have been proposed but have not been demonstrated experimentally, nor have mechanisms or factors involved been identified definitively. 25HC, 25-hydroxycholesterol; CH25H, cholesterol 25-hydroxylase; IL1B, interleukin 1 beta; NO, nitric oxide; ROS, reactive oxygen species; StAR, steroidogenic acute regulatory protein; TNF, tumor necrosis factor.

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MINI REVIEW
published: 28 April 2022
doi: 10.3389/fendo.2022.897029

Mechanism of Inflammatory Associated Impairment of Sperm Function, Spermatogenesis and Steroidogenesis

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OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology

Received: *15 March 2022*
Accepted: *28 March 2022*
Published: *28 April 2022*

Citation:
Hasan H, Bhushan S, Fijak M and Meinhardt A (2022) Mechanism of Inflammatory Associated Impairment of Sperm Function, Spermatogenesis and Steroidogenesis. Front. Endocrinol. 13:897029. doi: 10.3389/fendo.2022.897029

Infection and inflammation are relevant entities of male reproductive disorders that can lead to sub-/infertility. Associated damage of the testis of affected men and in rodent models include leukocytic infiltration, edema formation, fibrosis, germ cell loss and reduced androgen levels. Negative effects on spermatogenesis are thought to be elicited by oxidative stress sustained mostly by increased levels of ROS and pro-inflammatory cytokines. Under normal conditions these cytokines have physiological functions. However, increased levels as seen in inflammation and infection, but also in obesity and cancer are harmful for germ cells and impair steroidogenesis. As a summary, there is mounting evidence that the activation of inflammatory pathways is a rather common feature in various forms of male testicular disorders that extends beyond established infectious/inflammatory cues. This mini review will focus on relevant entities and the mechanisms of how a dysbalance of local testicular factors contributes to disturbances of spermatogenesis and steroidogenesis.

Keywords: testicular infection, testicular inflammation, autoimmunity, paracrine regulation, oxidative stress, ROS, cytokines, chemokines

CONDITIONS LEADING TO TESTICULAR AND EPIDIDYMAL INFLAMMATION AND THEIR INFLUENCE ON HORMONE LEVELS, STEROIDOGENESIS, SPERMATOGENESIS AND SEMEN QUALITY

The testis is an immune privileged organ that tolerates the introduction of sperm autoantigens at the onset of puberty without eliciting an inflammatory immune response (1). The testis in mammals evolved multiple strategies to preserve this immunocompromised status, namely, the formation of the blood-testis-barrier (BTB) between adjacent Sertoli cells that secludes most of the developing germ cells from the interstitial compartment where leukocytes reside (1, 2). Besides the BTB the Sertoli cells display important immunoprotective functions that may also contribute to immune privilege. This has been shown when Sertoli cells were co-transplanted with allo- or xenografts thereby prolonging the survival of pancreatic islets (3), hepatocytes (4) and neurons (5) as well as other types of cells (6). Moreover, through Sertoli cells, antigens protected from transcellular leakage

by the BTB can egress *via* transcytosis into the interstitial space where antigen-presenting cells (dendritic cells, macrophages) help to maintain Treg tolerance to meiotic antigens. Depletion of the Treg leads to autoimmune orchitis emphasizing the importance of the Sertoli cell-macrophage-Treg axis in maintaining immune privilege (7).

Evidence suggests that immunological and infectious etiologies contribute substantially to male infertility [accounting for 13–15% of cases (2)], a medical and social problem which in total is increasing worldwide (8). The contribution of inflammatory infertility may be underestimated as immune cell infiltration is observed in 20% of testicular biopsies of azoospermic infertile patients (9). Moreover, increased infiltration of immune cells into the testes with concomitant impairment in testicular functions is associated with certain chronic diseases, namely atherosclerosis and cancer (10–12). Given that infection and inflammation are critical drivers of male infertility, we will highlight how these entities can impair the archetypical functions of the male gonad, i.e. spermatogenesis and steroidogenesis.

Local inflammatory conditions of the testis, because of acute infection or inflammatory testicular reactions of unknown origin as well as systemic inflammatory conditions, all can negatively impact spermatogenesis and steroidogenesis. They can do so at the following levels: (a) direct impairment of spermatogenesis, sperm quality and function, e.g. by germ cell death, oxidative stress and impaired mitochondrial activity, (b) disruption of steroidogenesis due to perturbation of the hypothalamic-pituitary-testicular axis, (c) obstruction of the male genital tract or (d) dysfunction of accessory glands (13–15). The following sections will elaborate in more detail on relevant factors and mechanism of disease.

Bacterial infections

In the clinic, Escherichia coli (E. coli), Proteus mirabilis, Staphylococcus aureus, Streptococcus veridans, Ureaplasma urealyticum, Mycoplasma hominis and Chlamydia trachomatis are commonly isolated pathogens in liquid biopsies of men with genitourinary tract infection (16, 17). Among these bacteria, E. coli and Chlamydia trachomatis are the most clinically relevant pathogens and thus are frequently used in animal studies to mimic the human condition (16). Currently, rodent models propose two routes of infection for these microbes with uropathogenic E. coli (UPEC) reaching the epididymis and testis via ascending canicular infection after injection into the vas deferens. Alternatively, for Chlamydia muridarum, a murine-specific pathovar, macrophages were suggested as a vector as luminal spread from the infection site at the urethral orifice was excluded by vasectomy (18–20). Although not seen as vectors for UPEC, infiltrating monocyte-derived macrophages also appear to be crucial in the immunopathology associated with acute epididymo-orchitis which was convincingly shown in $Ccr2^{-/-}$ mice, which lack blood monocytes due to defective egress from the bone marrow (21).

In clinical practice, epididymitis is almost exclusively of infectious origin. Leukocytospermia is seen often in the acute phase of disease; however, approximately 40-50% of epididymitis patients show persistent impaired semen parameters affecting sperm concentrations, motility and morphology (22). In up to 60% of all cases, the testis is also affected in a combined epididymo-orchitis as follow-up biopsies revealed severe hypospermatogenesis indicated by loss of germ cells in the adluminal compartment of the seminiferous epithelium, massive infiltration of the interstitial and even tubular compartment by immune cells, a thickened lamina propria and interstitial fibrosis. These alterations were accompanied by increased FSH levels (23, 24). Of note, persistent azoospermia in 10% and oligozoospermia in 30% of men suffering from acute epididymitis is detected (15, 22). Interestingly, sperm proteome analysis in patients after recovery from epididymitis (3 months) demonstrated long-term alterations in protein composition (25). Besides changes in the proteome, the glycome of sperm was altered in men with a history of epididymitis as seen by a substantial reduction of sialic acid residues on the surface of spermatozoa (26).

Viral infections

Several viruses, namely human immunodeficiency virus (HIV-1), Zika virus (ZIKV), Ebola and Marburg viruses as well as the mumps orthorubulavirus (MuV) can infect not only the testes but also the entire male reproductive tract of human and non-human primates through the hematogenous route (14). These viruses silently propagate inside the organ for an extended time. Recent studies suggest that the testicular macrophages are the reservoir for a few viruses and are critical for initiating infection and later dissemination into other testicular cells. For example, the ZIKV colonized the interstitial $CD206^+$ testicular macrophages and then spread infection into the seminiferous tubules (27). Similarly, another study demonstrated that the $S100A4^+$ macrophages were susceptible to ZIKV infection that facilitated ZIKV dissemination and persistence in the seminiferous tubules (28). After internalizing ZIKV, testicular macrophages skewed towards a pro-inflammatory phenotype and secreted pro-inflammatory cytokines. These disturb the BTB in a paracrine fashion by down-regulating claudin-1 expression and facilitating $S100A4^+$ macrophage entry into the seminiferous tubules (28). In contrast to ZIKV, Marburg virus mainly colonized Sertoli cells leading to a disruption of the BTB. In addition, infection with Marburg virus results in increased infiltration of immune cells in the testis, namely $CD68^+$ macrophages/monocytes, $CD3^+$ T cells and B cells in both the interstitial space and seminiferous tubules leading to spermatogenic cell loss and severe testicular damage (29).

Viral infection alters endocrine, sperm and semen parameters by targeting the male reproductive tract directly and indirectly (systemic). In relation to systemic infections (e.g. influenza), fever could result in increased testicular temperature and subsequent disturbances in spermatogenesis and steroidogenesis by perturbation of the hypothalamo-pituitary-gonadal axis (30, 31). In the context of viral infections, alterations in spermatozoal (count, motility, morphology) and semen parameters (e.g. volume of seminal plasma, viscosity, pH, enzyme concentrations) were reported, in some cases accompanied by orchitis (32–37). Impairment of

spermatogenesis could be related to different mechanisms including inflammatory reactions in the reproductive organ, disruption of the testicular cytokine milieu, decreased testosterone production by Leydig cells, disturbances in the paracrine control by somatic cells, change in testicular temperature due to fever and viral replication within cells of the male genital tract. Of note, macrophages, Sertoli cells and germ cells may serve as viral reservoirs [reviewed in (14)]. In chronic viral orchitis, histology of affected seminiferous tubules reveal degeneration of the germinal epithelium accompanied by thickening of the lamina propria, which ultimately may result in complete hyalinization and fibrosis of the tubules leading to the formation of so called "tubular shadows" (38). In Leydig cells, viral replication can lead to decreased testosterone production (39–41) an observation that was reported to be accompanied by changes in LH, FSH or inhibin B levels (32, 33, 36, 37, 41).

Autoimmunity

Autoimmune orchitis is an inflammation of the testis, where autoimmune reactions against spermatic antigens cause damage to germ cells, and also to testicular somatic cells. It is a rare disease in men with the potential to impede the normal function of the testis. Mutation in the autoimmune regulator (*Aire*) gene results in human autoimmune polyendocrine syndrome APS-type 1 (APS-1), which is characterized by autoimmune reactions in several organs, including the testes (42). This observation is corroborated in *Aire*-deficient mice that reproduced many clinical signs of APS-1 in human (43).

In men, histopathological analysis of testicular biopsies with inflammatory lesions of idiopathic origin show that lymphocytic infiltrates correlate with tubular damage, visible as partial or complete loss of the germinal epithelium, thickening of the lamina propria and tubular fibrosis. These changes are associated with reduced testicular volume and score counts for spermatogenesis, while FSH levels are not increased in these patients (2, 38). Similar histopathological changes are also seen in a mouse model of autoimmune-based epididymo-orchitis (EAEO) elicited by injection of testicular homogenate. Here, the disease can develop progressively up to the formation of granulomas. In rodent EAEO, FSH levels are concomitantly increased, while testosterone levels are reduced. This possibly points to a negative local paracrine influence on Leydig cell steroidogenesis. This assumption is supported by the observation that basal and hCG stimulated production of testosterone is elevated in isolated primary Leydig cells from EAEO rats compared to control. TNF-α abolishes this increase in testosterone [reviewed in (2, 44)].

In addition, systemic low grade inflammatory conditions associated with obesity including complications leading to cardiovascular disease, type 2 diabetes mellitus, malignancy and accelerated aging are connected with alterations in the hypothalamic-pituitary-gonadal axis, poor semen quality and disruption of testicular steroidogenesis. Obesity impacts negatively semen parameters (sperm concentration, motility, viability, morphology) and sperm function (chromatin condensation, DNA fragmentation, apoptosis and epigenetic signatures [reviewed in (45, 46)].

INFLAMMATORY DISORDER RELATED MECHANISMS AND PATHWAYS

Influence of Oxidative Stress on Spermatogenesis and Steroidogenesis

Reactive oxygen species (ROS) play an important role both in the maintenance of fertility in men, but also in pathological alterations of sperm parameters such as viability, motility, maturation, capacitation, hyperactivation and acrosome reaction (47). While ROS is required to combat pathogens and thus account for an effective anti-microbial immune response (48), supraphysiological levels of ROS, particularly for extended periods of time, can induce intense oxidative stress with toxic consequences for cells in general. In this regard, spermatozoa are particularly vulnerable due to their unique cytoarchitecture and biochemical characteristics (49–51). Spermatozoa possess a plasma membrane that is highly enriched in polyunsaturated fatty acids, particularly docosahexaenoic and arachidonic acids making them extremely susceptible to ROS-induced damage (52). Increased ROS production coupled with poor antioxidant capabilities in sperm can result in sperm DNA fragmentation (SDF) (**Figure 1**) (53). Elevated SDF alters the ultrastructure of sperm by leading to vacuolization in the nucleus along with other severe sperm morphological abnormalities that altogether can hinder fertilization by adversely affecting hyperactivation, capacitation and acrosome reaction (54). In this light, it is not surprising that SDF was reported in couples with unexplained recurrent pregnancy loss (55). Moreover, an initiation in the lipid peroxidation cascade can ultimately reduce sperm motility and viability owing to the fact that ROS-induced lipid peroxidation decreases mitochondrial membrane potential with concomitant structural damage in the adjacent axoneme (56, 57). The generation of lipid peroxidation products, particularly lipid aldehydes such as 4-hydroxynonenal (4-HNE), can negatively influence sperm motility as 4-HNE can bind to the dynein heavy chain in the sperm tail and to protein kinase anchoring protein 4 (AKAP4) in the sperm fibrous sheath (51) (**Figure 1**). In developing germ cells, oxidative stress can mediate cell death *via* several apoptotic pathways including activation of death receptors (Fas and TNFR1) and mitochondrial pathways (caspase 9) (58–60). The increased co-expression of Fas and FasL in germ cells implies that cell death *via* the Fas/FasL-mediated apoptotic signal transduction pathway could occur *via* autocrine and/or paracrine mechanisms (59). The susceptibility of germ cells to apoptosis *via* Fas/FasL could be regulated by Sertoli cells when the intracellular death domain of Fas reacts with FasL receptors on Sertoli cells (61, 62). Activated macrophages also play a role in the apoptosis of germ cells by releasing the stress response protein HMGB1 in response to inflammation-induced oxidative stress (**Figure 1**). In turn, HMGB1 causes germ cell death by inducing a decrease in anti-apoptotic Bcl-2 levels and a concomitant increase in pro-apoptotic Bax protein levels, cytochrome c and caspase 3 activity (63).

Alongside apoptosis, autophagy was reported as a pathway involved in disruption of spermatogenesis. In this context,

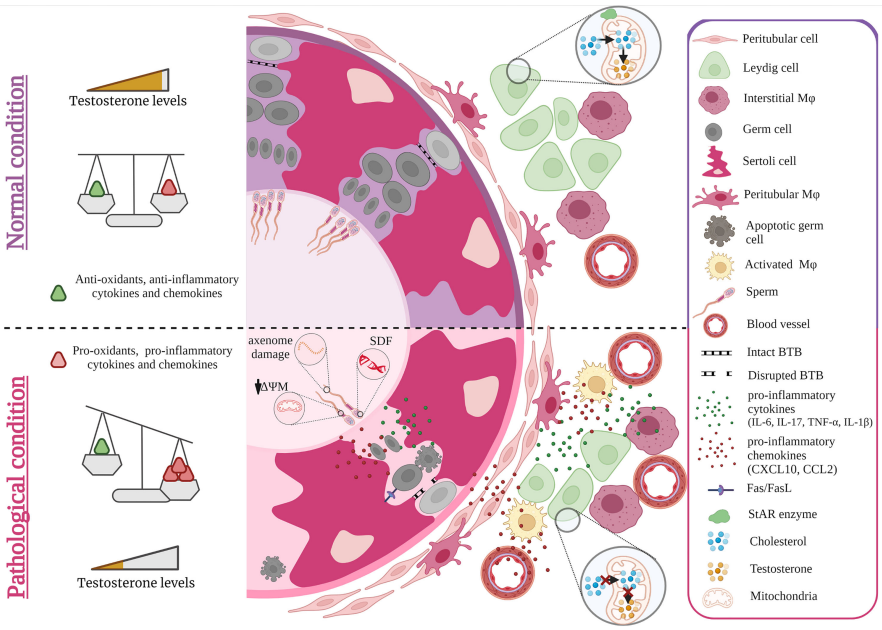

FIGURE 1 | Effect of cytokines and oxidative stress on spermatogenesis and steroidogenesis. Under normal conditions, levels of anti- and pro-inflammatory cytokines, chemokines as well as anti-and pro-oxidants are balanced maintaining steroidogenesis and spermatogenesis. Sterile inflammation and microbial infection both cause an invasion of monocyte derived macrophages that together with increased production of pro-inflammatory cytokines such as IL-6, TNF-α, IL-17, IL-1β and chemokines such as CCL2, CXCL10 by resident testicular cells as well as recruited immune cells result in a shift towards higher levels of pro-inflammatory factors that negatively impact spermatogenesis and steroidogenesis. As a consequence, germ cell death and lower levels of androgens are observed e.g. by ROS diminishing the expression of StAR. ROS induced damage of spermatozoa also occurs during epididymal transit (axoneme damage, decreased mitochondrial potential = ΔΨM, sperm DNA fragmentation =SDF). Figure created with BioRender.com.

increased expression of autophagy-related gene 7 (Atg7) was observed in spermatocytes after heat treatment of mice (64). The knockdown of Atg7, a factor required for formation of autophagosomes (65), *via* siRNA injected into the seminiferous tubules of these mice led to significant protection against heat-induced autophagy that was accompanied with decreased rates of germ cell apoptosis (64).

Intense oxidative stress can also affect Leydig cell steroidogenesis eventually leading to infertility. ROS can disturb Leydig cell mitochondria in diminishing the expression of steroidogenic acute regulatory protein (StAR) which in turn can decrease mitochondrial transport of cholesterol and consequently reduces synthesis of androgens (66, 67). This negative influence on steroidogenesis was reported to be a result of oxidative stress-induced activation of the p38 MAPK protein (68). C-Jun, a further stress responsive MAPK subfamily member, was also shown to be involved in suppressing the expression of steroidogenic enzymes as ROS mediated signaling upregulation of c-Jun inhibits Nur77 transactivation (69). Orphan nuclear receptors like Nur77 are known to be key transcriptional factors regulating the gene expression of steroidogenic enzymes (70, 71). Moreover, steroidogenesis can be downregulated in a paracrine fashion. This is elicited by TNF-α released by activated macrophages which addresses the TNF-α receptor TNFR1 expressed on neighboring Leydig cells. This leads to Leydig cell apoptosis and to activation of p38 MAPK signaling pathway resulting in decreased serum testosterone levels (72).

Paracrine Influence of Cytokines, Chemokines and Growth Factors on Spermatogenesis and Steroidogenesis

Signaling molecules especially cytokines and growth factors and their receptors are widely produced by testicular cells. These signaling molecules play crucial roles in normal testis development and function when expressed at physiological levels, whereas increased levels can lead to disturbed organ function (73, 74). As an example, the activation of toll-like receptors (TLR) following binding of microbial pathogen-associated molecular patterns (PAMPs) and endogenous ligands such as alarmins (which are released during tissue damage) can initiate a cascade of signal transduction pathways which ultimately can culminate in the secretion of a range of signaling molecules including pro-inflammatory cytokines TNF-α, interleukin (IL)-1β and IL-6 in addition to chemokines (CXCL8 and CXCL10) (75) that all act in a paracrine fashion. Pathological consequences are indicated by neutralization of TNF-α in conditioned media of testicular macrophages, which results in decreased apoptosis of germ cells (74). Furthermore, murine *Tnf-α*$^{-/-}$ Sertoli cells were protected from MuV-induced down-regulation of occludin and zonula occludin-1 thus safeguarding the integrity of the BTB. Inhibition of TNF-α

production by the immunomodulatory drug pomalidomide in MuV infected Sertoli cells also prevented the disruption of the tight junction integrity of the BTB. Similar observations were made *in vivo* where TNF-α deficiency prevented the MuV induced disruption in the BTB and loss in spermatids (76).

TNF-α can also induce the production of CXCL10 in Sertoli cells in an autocrine manner, which can in turn induce apoptosis of germ cells *via* caspase-3 activation after binding to CXCR3 on these cells. As a control, the experimental deletion of the genes for CXCL10 or TNF-α in a co-culture of germ cells and Sertoli cells inhibits MuV-induced germ cell apoptosis (77). To add, CXCL10 and another chemokine ligand, CCL2, which is produced by Sertoli cells, Leydig cells and testicular macrophages in response to inflammation could recruit leukocytes resulting in a negative impact on spermatogenesis (**Figure 1**) (78). The role of a dysregulated CCL2/CCR2 axis on spermatogenesis was clearly shown in *Ccr2*$^{-/-}$ mice that were protected from germ cell loss otherwise seen in acute bacterial epididymo-orchitis (21) TNF-α can also lead to elevated expression of activin A - a member of the transforming growth factor-β (TGFβ) family of cytokines - in Sertoli cells (**Figure 1**). Inhibiting activin A *in vivo* by elevating circulating levels of its antagonist follistatin reduced the overall severity of EAEO, associated germ cell loss and fibrotic damage (79). Further credence of a negative role of upregulated pro-inflammatory cytokines on spermatogenesis is derived from *in vivo* and *in vitro* experiments (74, 80–82). Testicular injection of IL-6 or IL-17A induced germ cell sloughing and disruption of the integrity of the BTB, a finding corroborated *in vitro* when murine Sertoli cells cultured with excess IL-6 or IL-17A exhibited a disrupted BTB integrity and permeability concomitant with a decrease in transepithelial electrical resistance that was associated with changes in the distribution of tight junction protein expression (occludin, claudin 11) (81, 83) (**Figure 1**). IL-6 can also directly induce apoptosis of germ cells *in vitro* (74, 84). Infection with Sars-Cov-2 was shown to increase the levels of pro-inflammatory cytokines mainly IL-6, TNF-α, IL-1β and this was accompanied with disruption in the expression of junctional proteins (occludin, claudin-11, connexin-43) along with decreased numbers of Sertoli cells and decreased sperm counts (85–87).

Increase in the aforementioned pro-inflammatory cytokines and chemokines can also negatively influence the ability of Leydig cells to synthesize testosterone mainly by acting as repressors of steroidogenic enzyme gene expression (88–90). TNF-α and TGF- β were found to be implicated in disrupting steroidogenesis directly *via* the competitive inhibitory action of NF-κB subunits on the transactivation of Nur77 and other orphan nuclear receptors (88, 91–93). Activated macrophages, which are physically interacting with Leydig cells, were shown to produce pro-inflammatory cytokines such as IL-1 and TNF-α that can inhibit Leydig cell steroidogenesis (66). In this co-culture setting of Leydig cells with activated testicular macrophages (via lipopolysaccharide stimulation), mRNA expression of steroidogenesis related genes (SF1, StAR and 3β-HSD) was inhibited (94). Moreover, IL-1β added to murine Leydig cells can induce the expression of CCL2, which in turn can decrease steroidogenic enzymes such as CYP17A1 and induce apoptosis as evidenced by cleaved caspase-3. This effect was also documented in human Leydig cells (95). Overexpression of another chemokine -CXCL10- in murine tumor Leydig cells also inhibit StAR expression and decrease cAMP-induced progesterone synthesis in a paracrine fashion (77).

SIGNIFICANCE AND CONCLUSION

Cytokines and chemokines play an important role in the regulation of normal testicular function. They display direct paracrine effects on spermatogenic and Leydig cells that in the case of an upregulation during inflammatory episodes can impose harmful consequences. However, a degree of caution is necessary as a considerable amount of data relies on *in vitro* studies using isolated cells. Moreover, definitive functions of pro-inflammatory factors are difficult to determine as their action is context dependent and influenced by other mediators acting at the same target cell. Research harvesting breakthrough technologies like scRNA-seq and spatial transcriptomic is just about to unravel the overlap of the immune and testicular system and how they are linked in normal and pathological condition.

AUTHOR CONTRIBUTIONS

AM designed the outline of the manuscript. Writing and final editing was performed by all authors. Figure design was done by HH. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by a GRK 1871/2 International Research Training Group Giessen-Monash grant on 'Molecular pathogenesis of male reproductive disorders' (HH, SB, MF, AM) and a project grant BH 93/1-4 (awarded to SB) both from the Deutsche Forschungsgemeinschaft.

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frontiers | Frontiers in Endocrinology

ORIGINAL RESEARCH
published: 28 April 2022
doi: 10.3389/fendo.2022.871225

Global Deletion of ALDH1A1 and ALDH1A2 Genes Does Not Affect Viability but Blocks Spermatogenesis

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The transition of undifferentiated A spermatogonia to differentiated spermatogonia requires the action of retinoic acid (RA). The synthesis of retinoic acid from retinal in the seminiferous epithelium is a result of the action of aldehyde dehydrogenases termed ALDH1A1, ALDH1A2, and ALDH1A3. We used a mouse with a global deletion of the *Aldh1a1* gene that is phenotypically normal and the *CRE*-loxP approach to eliminate *Aldh1a2* genes globally and from Sertoli cells and germ cells. The results show that global elimination of *Aldh1a1* and *Aldh1a2* genes blocks spermatogenesis but does not appear to affect viability. The cell specific elimination of *Aldh1a2* gene showed that retinoic acid synthesis by Sertoli cells is required for the initial round of spermatogonial differentiation but that there is no requirement for retinoic acid synthesis by germ cells. In both the global gene deletion and the cell specific gene deletions the maintenance of *Aldh1a3* activity could not compensate.

Keywords: ALDH1A1, ALDH1A2, retinoic, spermatogenesis, viability

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology

Received: *07 February 2022*
Accepted: *15 March 2022*
Published: *28 April 2022*

Citation:
Topping T and Griswold MD (2022) Global Deletion of ALDH1A1 and ALDH1A2 Genes Does Not Affect Viability but Blocks Spermatogenesis. Front. Endocrinol. 13:871225. doi: 10.3389/fendo.2022.871225

INTRODUCTION

The active form of vitamin A is retinoic acid that is synthesized in precise cellular locations by a two-step mechanism. First, retinol which is the circulating form of the vitamin is oxidized in a reversible reaction to retinal by retinol dehydrogenase (RDH10). Retinal is then oxidized to retinoic acid in a nonreversible reaction by one of 3 retinal dehydrogenases known as ALDH1A1, ALDH1A2, and ALDH1A3 (1).

ALDH1A2 and ALDH1A3 are required during fetal development. ALDH1A2$^{-/-}$ mice die during embryonic development and ALDH1A3$^{-/-}$ mice die shortly after birth (2, 3). However, ALDH1A1$^{-/-}$ mice develop normally (4). In humans, ALDH1A1 mRNA is found in the liver, kidney, testis, brain, lung, red blood cells, and lens of the eye while ALDH1A2 mRNA is found in the testis, uterus, and skeletal muscle, and ALDH1A3 mRNA is localized in the prostate, trachea, intestine, and testis (5). Clearly all three ALDH1A enzymes contribute to RA synthesis during postnatal life. All together these studies underscore the tissue-specific central roles that ALDH1A enzymes play in animal physiology, and the vital significance of obtaining information concerning the expression and essential nature of the activity of these enzymes in human tissues.

In the mouse testis, retinoic acid is essential for the progression of undifferentiated spermatogonia A to become differentiating spermatogonia A1 and enter into spermatogenesis (6). In the absence of retinoic acid, undifferentiated spermatogonia never begin this progression (7). We have previously shown that deletion of the RDH10 gene in Sertoli cells alone will inhibit the

progression of undifferentiated spermatogonia and also the deletion in both germ cells and Sertoli cells blocks this progression (8). Enzyme inhibitors have been used to eliminate the activity of all 3 aldehyde dehydrogenases and using the *CRE*lox P approach all 3 Aldh1a genes have been deleted only in germ cells, only in Sertoli cells and in both cell types (9, 10). Both of these approaches have shown that the retinal dehydrogenases are essential for spermatogenesis and both enzymes are present in germ cells and Sertoli cells. Aldh1a1 is most highly expressed in the Sertoli cells and Aldh1a2 and Aldh1a3 are expressed primarily in the germ cells but all 3 enzymes appear to be expressed at some level in both cell types (11).

It has been known that global deletion of the *Aldh1a1* gene in mice has little effect and does not significantly alter fertility (4). Recent studies have shown that the knockout of Aldh1a2 alone or the simultaneous knockout of Aldh1a1-3 in germ cells has little effect on successful spermatogenesis and fertility (9, 12). However, the simultaneous knockout of Aldh1a1-3 in Sertoli cells does not allow the undifferentiated A spermatogonia to progress to differentiating A1 spermatogonia (9). In this study we have broadened these previous observations by examining the effect on spermatogenesis of leaving the *Aldh1a3* gene intact. We started with a mouse mutant with a global deletion in *Aldh1a1*. From that genotype we used the *CRE*-loxP system to remove the *Aldh1a2* gene in germ cells and/or Sertoli cells and globally in all cells. In addition, and we have included sperm counts and fertility studies.

MATERIALS AND METHODS

Animal Care, Breeding and Genotyping

All procedures involving mice were approved by the Washington State University Committee on the Use and Care of Animals. The mouse colonies were maintained in a temperature-controlled environment with access to food and water *ad libitum*. Mice were euthanized by CO_2 asphyxiation followed by cervical dislocation. Four mouse lines were generated for this study, each expressing Cre recombinases to inactivate the *Aldh1a2* gene in Sertoli cells, germ cells, both Sertoli and germ cells or globally. The *Aldh1a1*$^{-/-}$, *Aldh1a2*$^{fl/fl}$, *ERT*-Cre line was created by breeding *Aldh1a2*$^{fl/fl}$, *ERT*-Cre (12) with *Aldh1a1*$^{-/-}$ (a gift from John Amory and Jisun Paik at the University of Washington with permission from Jackson labs, JAX stock #012247). The offspring who were heterozygous for all 3 alleles were bred and those animals in the next generation who were homozygous for the 3 alleles were experimental animals. In every case the excision of the gene, *Aldh1a2*$^{\Delta}$, was confirmed by genotyping (12).

The *Aldh1a1*$^{-/-}$, *Aldh1a2*$^{fl/fl}$, *Amh*-Cre^{+} line was created by initially breeding *Aldh1a1*$^{+/-}$ with *Aldh1a2*$^{fl/fl}$, to create mice that were *Aldh1a1*$^{+/-}$, *Aldh1a2*$^{fl/-}$. These were bred with mice carrying the *Amh*-Cre transgene (a gift from Marie-Claude Hofman, UT MD Anderson Cancer Center) and those progeny who were *Aldh1a1*$^{+/-}$, *Aldh1a2*$^{fl/-}$ and carrying the transgene were then bred together to accumulate the experimental and control mice *Aldh1a1*$^{-/-}$, *Aldh1a2*$^{fl/fl}$, *Amh*-Cre^{+} and *Aldh1a1*$^{-/-}$, *Aldh1a2*$^{fl/-}$, *Amh*-Cre^{+}, respectively.

The experimental and control mice for the *Aldh1a1*$^{-/-}$, *Aldh1a2*$^{fl/fl}$, *Stra8*-Cre+ and *Aldh1a1*$^{-/-}$, *Aldh1a2*$^{fl/fl}$, *Stra8*-Cre+, *Amh*-Cre^{+} lines were generated from the same breeding scheme. Males carrying the *Stra8*-Cre transgene (13) were bred with *Aldh1a1*$^{+/-}$ females. Male offspring who were *Aldh1a1*$^{+/-}$ and carried the *Stra8*-Cre transgene were paired with *Aldh1a1*$^{-/-}$ females. Males from this breeding who were *Aldh1a1*$^{-/-}$, *Stra8*-Cre^{+} were bred with females, generated above, who were *Aldh1a1*$^{-/-}$, *Aldh1a2*$^{fl/fl}$, *Amh*-Cre^{+}. Male offspring from this pairing who were *Aldh1a1*$^{-/-}$, *Aldh1a2*$^{fl/-}$, *Stra8*-Cre+, *Amh*-Cre^{+} were paired with females, *Aldh1a1*$^{-/-}$, *Aldh1a2*$^{fl/fl}$, *Amh*-Cre^{+}, to generate experimental and control mice for both lines.

To determine the genotypes of the mice, PCR reactions were performed on template generated from a tail clip from each mouse. The primer sets for *Amh*-Cre and *ALDH1A1* are as follows: *Amh-Cre* forward primer GCGGTCTGGCAGTAAAAACTATC and reverse primer GTGAAACAGCATTGCTGTCACTT; *ALDH1A1* forward primer CAACCCTGAGCAAATCCTCCAC, reverse primer for the knockout TGGATGTGGAATGTGTGCG AG and reverse primer for wild-type GACAGATTGAGAGCAG TGTTTACCC. All others have been reported elsewhere (12).

Fertility and Sperm Counts

Males with confirmed KO in germ cells or Sertoli cells or both germ and Sertoli cells or ERT-Cre, tamoxifen treated males and controls were aged to 7 weeks and then were paired with a female of known fertility for 2 months to assess fertility. At the end of the 2 months the males were euthanized for study and the females left for 3 more weeks to continue to monitor for litters. The number of offspring and number of litters for each male was recorded. Following this timeline, each male in this study was euthanized at approximately 4 months. The body was weighed immediately after euthanasia. One testis was placed in Bouin's fixative for immunohistochemistry and one was detunicated, snap frozen and weighed. Both cauda epididymides were placed in DMEM at room temperature and processed for counting sperm. The cauda epididymides were cut into approximately 1mm^3 pieces and incubated at 37°C for 15 minutes. Three µl of the sperm suspension was applied to a Cell Vision disposable counting slide (CV 1020-4CV) and analyzed using a SCACASA system (Fertility Technology Resources, Inc) following the manufacturer's instructions. When the sperm numbers were over 80 million, the sperm suspension was diluted 4 fold with DMEM before counting the sperm.

Histology

Bouin's fixed testes were embedded in paraffin, cut into 4 µm sections and either stained with hematoxylin and eosin or immunohistochemistry was performed using primary antibodies to Stra8 (14).

Tamoxifen Preparation and Administration

Tamoxifen (Sigma T5648) was dissolved in 10% ethanol and 90% sesame oil at a concentration of 10 or 20 mg/ml, and the solution

was wrapped in aluminum foil to protect from light. Mice were injected intraperitoneally with 40 mg/kg tamoxifen once per day from postnatal day 8 to 10 and/or with 80 mg/kg tamoxifen for 5 days starting at day 21 postpartum. Alternatively, at day of birth and postnatal day 1, mice were injected intraperitoneally with 100 mg/kg tamoxifen dissolved in sesame oil only at a concentration of 5 mg/ml. Tamoxifen was stored for a maximum of one week at 4°C and warmed to room temperature before injections. To confirm that the action of tamoxifen on the *ERT*-Cre resulted in excision of the ALDH1A2 gene, *Aldh1a2*$^{\Delta}$ genotyping was performed on tail clips collected after euthanasia.

Retinoic Acid Injections

Retinoic acid (Sigma R2625) was made fresh each day in DMSO. For the mice expressing the *Stra8*-cre and/or *AMH*-cre, 10 µl of 20 mg/ml was intraperitoneally injected once at day 21. For the males expressing the *ERT*-cre, RA at a concentration of 10 mg/ml was injected intraperitoneally at a dose of 12.5 µg/g body weight once at day 21. Males were euthanized after one round of spermatogenesis, 42 days later. As a control the same volume of DMSO was injected at day 21.

RESULTS

Using the *Aldh1a1*$^{-/-}$, *Aldh1a2*$^{fl/fl}$ mice as our starting point we first wanted to see whether the presence of *Aldh1a3* altered the results from the previous studies of Teletin et al. (9). Their data showed that the deletion of all 3 Aldh1a genes in Sertoli cells blocked spermatogenesis at the conversion of A spermatogonia to A1 spermatogonia in mice. However, if these mice were injected with retinoic acid once, the block was removed, and spermatogenesis proceeded normally and continuously. They also showed that spermatogenesis was normal with the deletion of all 3 *Aldh1a* genes in germ cells alone. They concluded that RA from Sertoli cells was necessary for the initial A to A1 conversion of spermatogonia but that RA from germ cells could maintain the process. If RA synthesis was normal in Sertoli cells the presence of RA synthesis in germ cells was not necessary. We created *Aldh1a1*$^{-/-}$, *Aldh1a2*$^{fl/fl}$ mice under control of *AMH Cre* or *Stra8 Cre* to produce deletions of only Aldh1a1 and Aldh1a2 in Sertoli cells or germ cells, respectively. *Aldh1a1*$^{-/-}$ mice have essentially normal spermatogenesis and we previously showed that *Aldh1a2*$^{-/-}$ mice also have normal spermatogenesis (12). However, the deletion of both of these two genes in Sertoli cells or germ cells recapitulated the results from deletion of all 3 genes reported by Teletin et al. (9). We also found that deletion of *Aldh1a1* and *Aldh1a2* genes in Sertoli cells completely blocked spermatogenesis at the conversion of A spermatogonia to A1 and that this block could be overcome by a single injection of RA (**Figure 1**). The knockout of genes coding for both enzymes in germ cells had no effect on sperm production. The results based on histology (**Figure 1**) were reflected in testis weights and number of sperm detected in the cauda epididymis. In breeding studies all of the crosses that had detectable sperm in the cauda produced normal litter numbers and sizes. So, the production of RA by Sertoli cells from either or both *Aldh1a1* or *Aldh1a2* enzymes is required for the initiation of spermatogenesis and the presence of *Aldh1a3* enzymes cannot compensate.

In order to examine the effect of globally deleting *Aldh1a1* and *Aldh1a2* we created *Aldh1a1*$^{-/-}$, *Aldh1a2*$^{fl/fl}$ mice with an inducible ERT-Cre, and then utilized the injection of tamoxifen to activate the CRE activity. We used several protocols for the injection of tamoxifen. We injected once per day for 3 consecutive days starting at 8 days of age or once per day for 5 days at 21 days of age. In both protocols we found that spermatogenesis appeared to be little affected since the sperm counts per cauda epididymis were near normal and most of the mice fathered litters of near normal size. However, if we combined the protocols and injected tamoxifen for 3 consecutive days at 8 days of age and then repeated the injections for 5 days at 21 days of age, we found that by 4 to 5 months of age when the mice were analyzed the sperm counts went to zero and no litters were produced in breeding trials. Apparently, neither of the 2 individual tamoxifen injection regimes were sufficient to eliminate all ALDH1A2 activity. An alternative protocol where on the day of birth and on 1 day of age the males were injected with tamoxifen produced more robust results. Under this protocol none of the males produced any sperm throughout their lifetime. Some of these mice treated with tamoxifen on day of birth and day 1 after birth were raised to 23 days of age and injected with a bolus of RA. The histology of the testes of these animals were examined 42 days after the injection of RA but the block at the conversion of A spermatogonia to A1 spermatogonia was still in place and no sperm were produced (**Figure 1**). This was expected since there is no source of RA from the germ cells or Sertoli cells to support spermatogenesis as was also seen for *Aldh1a1*$^{-/-}$, *Aldh1a2*$^{fl/fl}$, *Stra8-Cre*+, *Amh*-Cre+ (**Table 1** line 6).

The mice with globally deleted *Aldh1a1* and *Aldh1a2* from either tamoxifen injection protocol that resulted in aspermatogenesis were viable and appeared normal in all respects with the exception of the testis. (**Table 2**) A detailed pathological examination was not done but the mice were routinely aged to 4 months and some were left for over 6 months and showed normal body weight and no obvious pathologies.

DISCUSSION

The action of retinoic acid (RA) is required for normal spermatogenesis in rodents and possibly all mammals (15). We have previously shown that RA is synthesized locally in pulses along the seminiferous tubules (16). These pulses are required for the transition of undifferentiated A spermatogonia into A1 spermatogonia and into the differentiation pathway (7). The location of these pulses corresponds to the onset of spermatogenesis and the initiation of the cycle of the seminiferous epithelium. In the absence of RA there is no cycle, and no germ cells advance beyond undifferentiated spermatogonia. It has been

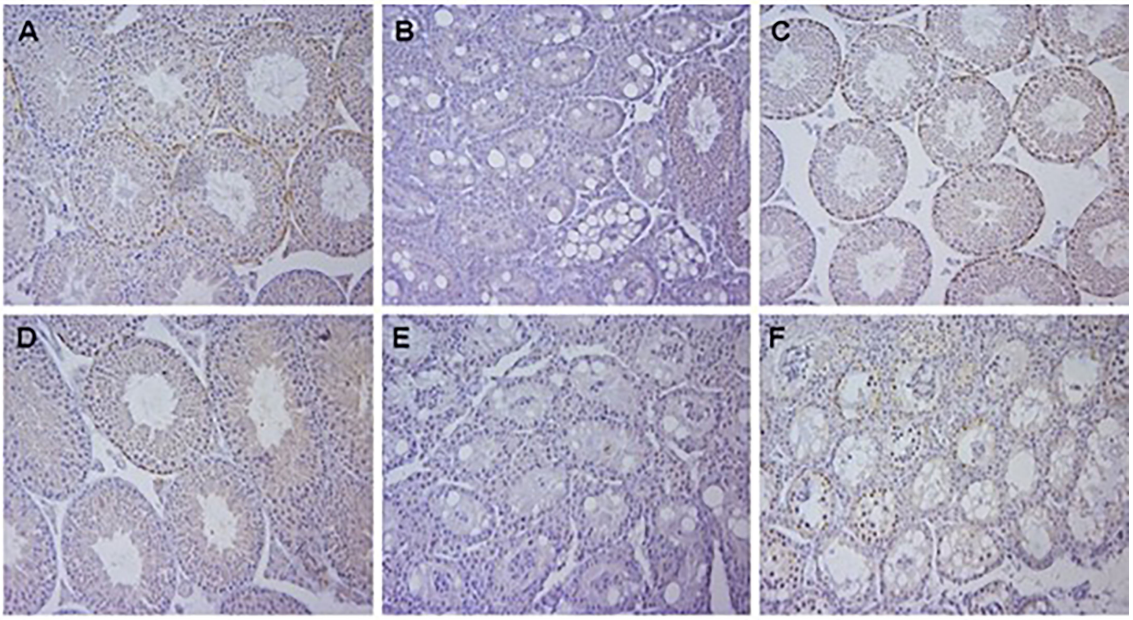

FIGURE 1 | Histology of testis of mice with gene deletions in *Aldh1a1* and *Aldh1a2*. All samples were Bouin's fixed, embedded in paraffin, cut into 4 µm sections and either stained with hematoxylin and eosin and immunohistochemistry was performed using primary antibodies to Stra8. **(A)** *Aldh1a1*$^{-/-}$, *Aldh1a2*$^{-/+}$ 4 week old mice showing normal histology with sperm and Stra8 positive spermatogonia and preleptotene spermatocytes. **(B)** 4 week old mice with genotype of *Aldh1a1*$^{-/-}$, *Aldh1a2*$^{-/-}$ after crossing mice in **(A)** with AMH-Cre mice resulting in deletion in Sertoli cells and a complete block at the progression of undifferentiated spermatogonia. **(C)** Mice with deletion in Sertoli cells as shown in **(B)** 42 days after a single injection of RA. Note recovery of normal spermatogenesis and partial synchrony of Stra8 expression. **(D)** 4 week old mice with genotype of *Aldh1a1*$^{-/-}$, *Aldh1a2*$^{-/-}$ after crossing mice in **(A)** with Stra8-Cre mice resulting in deletion in germ cells. Note normal histology. **(E)** 4 week old mice with genotype of *Aldh1a1*$^{-/-}$, *Aldh1a2*$^{-/-}$ after crossing mice in **(A)** with both AMH-Cre mice and Stra8-Cre mice resulting in deletion in both Sertoli cells and germ cells. **(F)** 4 week old mice with genotype of *Aldh1a1*$^{-/-}$, *Aldh1a2*$^{-/-}$ after crossing mice with ERT-Cre mice and treatment with tamoxifen starting at postnatal day 8 and again at postnatal day 21 as described in the methods. Results illustrate the early block between A and A1 spermatogonia when RA is not available.

established that the pulse of retinoic acid is a result of the localized synthesis of retinal by retinol dehydrogenase 10 (RDH10) and the conversion of retinal to retinoic acid by 3 aldehyde dehydrogenases designated ALDH1A1, ALDH1A2 and ALDH1A3 (8, 9, 17, 18). Both the Sertoli cells and the germ cells have the capacity to synthesize RA (9).

Deletion of either the *Aldh1a1* gene or *Aldh1a2* gene alone has no major consequences to spermatogenesis or the mice. Teletin et al. (9) used a Cre-Lox P approach to eliminate all 3 *Aldh1a* genes from Sertoli cells or from germ cells or from both cell types. From these experiments they determined that RA from Sertoli cells was essential to begin the first wave of germ cell development. Deletion of all 3 genes from germ cells had no effect on spermatogenesis. However, in the Sertoli cell specific triple gene deletion, if RA was present during the first wave in the form of a single injection, spermatogenesis proceeded normally and was continuous suggesting that the germ cell RA was sufficient to maintain spermatogenesis once it had been initiated. We addressed these studies using a different genetic approach where we left the *Aldh1a3* gene intact. While there are only low levels of ALDH1A3 in the testis we wanted to determine if it was sufficient to maintain spermatogenesis.

Our cell specific deletions of only *Aldh1a1* and *Aldh1a2* recapitulated the results from Teletin et al. (9) who deleted all

TABLE 1 | The Aldh1a1$^{-/-}$, Aldh1a2$^{f/f}$ mice were crossed with the designated Cre to delete gene in Sertoli cells or germ cells or both.

Experiment	N	Testis wt.	Sperm/cauda
control	10	0.129+/-.013	92+/-27
Stra8 *Cre*	7	0.118+/-.013	79.6+/-37
AMH *Cre*	6	0.018+/-.002	zero
AMH *Cre* +RA	9	0.077+/-0.018	84+/-21
Stra8 *CRE* and AMH *CRE*	8	0.025+/-.005	zero
Stra8 *CRE* and AMH *CRE* plus RA	7	0.029+/-.005	zero

In some experiments (4 and 6) mice were treated with RA and analyzed 4 weeks later to determine if spermatogenesis could recover. N is number of individual mice. Values plus standard deviation are shown.

TABLE 2 | The Aldh1a1$^{-/-}$, Aldh1a2$^{f/f}$ mice were crossed with the ERT Cre.

Experiment Aldh1a1$^{-/-}$, Aldh1a2$^{f/f}$	N	Testis wt. (mg)	Sperm/2 cauda (millions)
1. *ERT* CRE 8-10 postnatal	10	86+/-17	72.2+/-25.1
2. *ERT* CRE 21-25 postnatal	10	107+/-7	96+/-9.9
3. *ERT* CRE 8-10 postnatal and 21-25 postnatal	8	17+/-7	zero
4. *ERT* CRE 0-1 postnatal	7	11+/-3	zero
5. *ERT* CRE 0-1 d postnatal plus RA	7	29+/-5	zero

Tamoxifen injections were given on the designated days after birth to delete Aldh1a2 gene globally. In experiment 5 mice were treated with RA and analyzed 4 weeks later to determine if spermatogenesis could recover. N is number of individual mice. Values plus standard deviation are shown.

3 *Aldh1a* genes. Deletion of these 2 genes and maintenance of the *Aldh1a3* gene in Sertoli cells completely blocked spermatogenesis unless an injection of RA was made. Thus, the presence of ALDH1A3 alone is not sufficient to maintain spermatogenesis. In addition, deletion of these two genes in germ cells had no effect on sperm production. RA synthesized in Sertoli cells is sufficient to initiate and maintain spermatogenesis while RA from germ cells can only maintain spermatogenesis after it has been initiated.

Because of the absolute requirement of RA for spermatogenesis, it has been proposed that inhibition of the synthesis or the action of RA could be a possible approach for contraceptive development (10, 11, 19). Blocking RA synthesis with an aldehyde dehydrogenase inhibitor or use of RA analogs that inhibit the action of retinoic acid receptors (RAR) have been shown to block spermatogenesis (10, 20–22). However, given the prevalence of the RA signaling system in biology and its absolute requirement in embryogenesis there was serious concern about developing a contraceptive approach for the testis that could have serious consequences for other organ systems. Previously it has been shown that global deletions of the genes for Cyp26A1 and Cyp26b1, the enzymes involved in RA homeostasis, lead to increased concentrations of RA in several organs, reduced lifespan, failure to gain weight, and fat atrophy (23). So, increased RA concentrations in adult mice led to severe physiological consequences. Therefore, one of the goals of this study was to examine the viability of mice after global deletion of 2 of the 3 *Aldh1a* genes and a decreased ability to synthesize RA. We found that the global deletion of *Aldh1a1* and *Aldh1a2* had no apparent effect on the gross viability of the mice. Teletin et al. (9), only reported data on the testis cell specific deletion of all 3 genes coding for ALDH1A enzymes so in our studies it is possible that ALDH1A3 was able to compensate in some tissues other than the testis. Nonetheless, inhibitors targeting ALDH1A1 and ALDH1a2 would certainly act as effective contraceptive compounds while not affecting gross viability. While we did not examine the physiopathology of potentially affected systems such as the immune system these results are significant in attesting to the feasibility of a RA focused contraceptive approach.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by WSU IACUC.

AUTHOR CONTRIBUTIONS

Experimental protocols were done by TT and the experiments were planned by TT and MG.

FUNDING

Supported by HD 10808 from NIH.

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The reviewer KR declared a shared affiliation with the authors to the handling editor at the time of review.

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MINI REVIEW
published: 03 May 2022
doi: 10.3389/fendo.2022.892184

Testosterone Deficiency in Sickle Cell Disease: Recognition and Remediation

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Hypogonadism is common in men with sickle cell disease (SCD) with prevalence rates as high as 25%. Testicular failure (primary hypogonadism) is established as the principal cause for this hormonal abnormality, although secondary hypogonadism and compensated hypogonadism have also been observed. The underlying mechanism for primary hypogonadism was elucidated in a mouse model of SCD, and involves increased NADPH oxidase-derived oxidative stress in the testis, which reduces protein expression of a steroidogenic acute regulatory protein and cholesterol transport to the mitochondria in Leydig cells. In all men including those with SCD, hypogonadism affects physical growth and development, cognition and mental health, sexual function, as well as fertility. However, it is not understood whether declines in physical, psychological, and social domains of health in SCD patients are related to low testosterone, or are consequences of other abnormalities of SCD. Priapism is one of only a few complications of SCD that has been studied in the context of hypogonadism. In this pathologic condition of prolonged penile erection in the absence of sexual excitement or stimulation, hypogonadism exacerbates already impaired endothelial nitric oxide synthase/cGMP/phosphodiesterase-5 molecular signaling in the penis. While exogenous testosterone alleviates priapism, it disadvantageously decreases intratesticular testosterone production. In contrast to treatment with exogenous testosterone, a novel approach is to target the mechanisms of testosterone deficiency in the SCD testis to drive endogenous testosterone production, which potentially decreases further oxidative stress and damage in the testis, and preserves sperm quality. Stimulation of translocator protein within the transduceosome of the testis of SCD mice reverses both hypogonadism and priapism, without affecting intratesticular testosterone production and consequently fertility. Ongoing research is needed to define and develop therapies that restore endogenous testosterone production in a physiologic, mechanism-specific fashion without affecting fertility in SCD men.

Keywords: nitric oxide, testosterone replacement, TSPO, PDE5, erectile dysfunction, infertility, oxidative stress

OPEN ACCESS

Edited by:
Vassilios Papadopoulos, University of Southern California, United States

Reviewed by:
Carol Podlasek, University of Illinois at Chicago, United States
Peter Liu, The Lundquist Institute, United States

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Specialty section:
This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology

Received: *08 March 2022*
Accepted: *31 March 2022*
Published: *03 May 2022*

Citation:
Musicki B and Burnett AL (2022) Testosterone Deficiency in Sickle Cell Disease: Recognition and Remediation. Front. Endocrinol. 13:892184. doi: 10.3389/fendo.2022.892184

1 INTRODUCTION

Sickle cell disease (SCD) is the most common hereditary hematologic disorder in the United States, which affects an estimated 100,000 Americans, mostly African-Americans, and millions of people globally (1). Patients with SCD experience acute complications, such as painful vaso-occlusive episodes, and chronic multi-organ damage, which heighten their risks for morbidity and mortality (2). SCD was long considered to be a disease of children and young adults because of its devastating natural progression. Due mostly to universal newborn screening and early therapeutic intervention, life expectancy in patients with SCD has steadily improved over the last 30 years, and recent studies have estimated the median survival for patients with SCD at 60 years (3). Extended survival outcomes have, however, led to an increase in long-term complications of this disease.

SCD is associated with hypogonadism (total testosterone levels below 300 ng/dl), which develops in up to 25% of men with this disease (4). This rate contrasts with the 6-12% prevalence rate of symptomatic hypogonadism in otherwise healthy middle aged and older men, who manifest an age-related decline in testosterone production (5). The impact of testosterone deficiency in the SCD male population is evident, based on its symptomatic effects, e.g., impaired physical and sexual maturation, reduced libido, erectile dysfunction, decreased physical strength, fatiguability, mood changes, and infertility (6, 7). Attempts to address this problem are, however, hampered by limited understanding of the mechanism of hypogonadism in SCD.

This review focuses on the mechanism of testosterone deficiency in SCD, the impact of hypogonadism on health- and reproduction-related issues in SCD males, and novel strategies to drive endogenous testosterone biosynthesis. These strategies may translate into clinical therapeutic opportunities for preserving sexual function and fertility, and possibly other conditions, adversely affected by hypogonadism in SCD.

2 SICKLE CELL DISEASE

SCD is caused by a single point mutation in the β-globin gene of hemoglobin, leading to the expression of abnormal sickle hemoglobin (HbS). Traditionally, the pathophysiology of SCD was thought to result exclusively from the polymerization of HbS under hypoxic conditions, causing erythrocytes to become deformed, sludge, and occlude blood vessels, along with oxidative stress, inflammation, and hemolytic anemia (8). More recent studies show that SCD is also characterized by a chronic deficiency of the endogenous vasodilator nitric oxide (NO) and vascular dysfunction (8, 9). As a consequence, SCD leads to progressive multi-organ failure resulting in pulmonary hypertension, leg ulcers, renal failure, stroke, infarct, retinopathy, neurocognitive impairment, bone loss, and priapism (2, 9, 10).

2.1 Hypogonadism in Sickle Cell Disease

Clinical research has documented a high frequency of testosterone deficiency in SCD, with prevalence rates as high as 25% (4). In a small number of clinical studies investigating hypogonadism in SCD, findings regarding its etiology and clinical implications have varied. Studies have reported elevated luteinizing hormone (LH) and follicle-stimulating hormone (FSH) levels in patients with SCD (primary hypogonadism; 6, 11–14). Repeated testicular infarction is observed in some men with SCD, attributed to erythrocyte sickling, obstructed blood flow, and hypoxia (15), and this course has been proposed to be a contributing factor for testicular failure (16–19). In contrast, studies report decreased LH and FSH in patients with SCD (secondary hypogonadism; 4, 20, 21). Furthermore, compensated hypogonadism (characterized by increased gonadotropins and normal testosterone levels) has also been identified in men with SCD (22). Smaller testis size in SCD men (6, 23) and reduced testis weight in SCD mice (24) is further evidence of hypogonadism related to this disease.

In recent years, progress has been made toward understanding the mechanism of testosterone deficiency in SCD, and primary hypogonadism has now been established as the principal cause for this hormonal abnormality. Oxidative/nitrosative stress is implicated in defective testosterone production by affecting the expression or enzymatic activation of several steroidogenic enzymes, or by depletion of antioxidants (25–27). In the vasculature of humans and experimental animals with SCD, reactive oxygen species (ROS)-generated enzymes NADPH oxidase (NOX) and xanthine oxidase, endothelial NO synthase (eNOS) uncoupling, autooxidation of HbS, heme iron release, and increased asymmetric dimethylarginine have been described (28, 29). Diverse stimuli associated with these redox sources include hypoxia, angiotensin II, proinflammatory cytokines, vasoconstrictors, growth factors, metabolic factors, and superoxide itself (30).

The testis of the SCD mouse exhibits upregulation of 4-hydroxy-2-nonenal (4-HNE), a major end product of lipid peroxidation, upregulation of NOX gp91phox subunit, and uncompensated expression of the antioxidant enzyme glutathione peroxidase-1, all consistent with a heightened and uncontrolled redox environment in the SCD mouse Leydig cell (31). Increased NOX-derived oxidative stress reduces protein expression of steroidogenic acute regulatory protein (StAR) (but not cholesterol side-chain cleavage enzyme) in Leydig cells of the SCD mouse testis, which initiates cholesterol transfer into mitochondria. Reduced transport of cholesterol to mitochondria of Leydig cells in the SCD testis accounts for primary hypogonadism (31).

Secondary hypogonadism appears to represent patients having more severe or progressive forms of SCD, who exhibit more frequent abnormalities of LH and FSH in comparison with patients having mild disease (20). While not completely understood, secondary hypogonadism may be the result of vasoocclusion of hypothalamic-pituitary small blood vessels, or pituitary infarction (11).

2.1.1 Hypogonadism, Reproductive Issues, and Health-Related Quality of Life in SCD

Testosterone plays a critical role in muscle physiology, body development, bone density, sexual function, fertility, as well as social, emotional, and neurocognitive functioning in males (32). Patients with SCD exhibit reduced height and weight, decreased

physical strength, and delayed sexual maturation (23). Low levels of testosterone have been associated with very low bone mass density in SCD patients compared with those having normal bone mass density (33). Psychological distress, such as mood changes, increased anxiety, extreme fatigue, social withdrawal, and depression, and neurocognitive impairment, such as impaired executive function, attention, and processing speed, are well recognized complications of SCD (34–36). However, it is not understood whether declines in physical, psychological, and social domains of health in SCD patients are related to low testosterone levels or are consequences of other abnormalities of SCD. Future studies are warranted to evaluate this possible consequence of hypogonadism in SCD.

Although poorly studied in SCD, male infertility is recognized to be a common complication of this disease (23, 37–39). Impaired male fertility in SCD is due to multiple causes, including hypogonadism, gonadal failure and sperm abnormalities (such as oligospermia, reduced sperm motility and density, and abnormal sperm morphology), decreased ejaculate volume, and delayed or impaired sexual development. Prevalence rate of at least one abnormal sperm parameter in male patients with SCD is 91% (40). Erectile dysfunction, largely as a result of penile damage from recurrent or prolonged priapism, further contributes to reduced fertility in SCD men (23).

2.1.2 Hypogonadism and SCD-Related Priapism

Priapism is a pathologic condition of prolonged penile erection in the absence of sexual excitement or stimulation (41). Ischemic priapism, which features little or absent intracorporal blood flow resulting in painful erections, is prevalent in men with SCD, occurring in as many as 48% of men, with a mean age of onset of 15 years (42, 43). Repeated episodes of priapism may lead to irreversible damage to erectile tissue and permanent erectile dysfunction (42, 44, 45) and cause psychological distress, impaired sexual relationships, and reduced quality and function of life (46). The prevalence rate of erectile dysfunction associated with recurrent ischemic priapism in SCD patients is as high as 47.5% (47).

The historical premise is that androgens are causative in the pathophysiology of priapism. However, this notion is now challenged. Reports of no increase in priapism in testosterone deficient men administered testosterone gel at eugonadal levels (48), as well as reduced priapism occurrences in testosterone deficient men with SCD receiving long-acting testosterone undecanoate injections (49) oppose earlier conceptions that testosterone therapies cause priapism. It is now established that physiologic testosterone administration does not cause priapism and, in contrast, this intervention promotes molecular mechanisms that favor normal erection responses. In fact, priapism in SCD is associated with decreased testosterone levels. A potential role for testosterone in correcting priapism acknowledges that androgens contribute to physiologic erectile tissue responses. Testosterone and dihydrotestosterone promote physiologic relaxation of penile arteries and cavernous tissue, and androgen deficiency decreases the expression and enzymatic activities of eNOS, neuronal NOS, and phosphodiesterase type 5 (PDE5) in the penis, the main players in penile erection (50).

The mechanisms by which testosterone deficiency contributes to priapism has recently been elucidated. In a mouse model of SCD, characterized by both primary hypogonadism and priapism (51), testosterone replacement at eugonadal levels corrects priapism. At the molecular level, normalized testosterone levels reverse downregulated eNOS activity *via* a nongenomic mechanism by normalizing downregulated P-Akt (Ser-473) and P-eNOS (Ser-1177) protein expressions in the penis (51). Increased NO reverses downregulated protein expression and activity of PDE5, the enzyme which degrades cGMP in the penis (52–56). Testosterone's effect on PDE5 protein expression is believed to be mediated by increased NO-induced accumulation of cGMP, which binds to cGMP response sequences in the PDE5 promoter (57). Testosterone's effect on PDE5 catalytic activity is due to phosphorylation of PDE5 on Ser-92 by cGMP-mediated activation of protein kinase G, which stimulates binding of cGMP to the regulatory domain of PDE5 (58). Upregulated PDE5 protein expression and activity in the penis restores the mechanism for cGMP degradation, thereby preventing excessive accumulation of this nucleotide upon neurostimulation. By controlling the amount of cGMP, which causes relaxation of smooth muscles in the penis and penile erection, priapic activity is lessened (51). This proof-of-principle study supports testosterone deficiency as a cause for SCD-associated priapism by exacerbating already impaired NO molecular signaling in the penis.

In contrast to its physiologic doses, testosterone at supraphysiologic doses decreases NO production from eNOS and increases oxidative stress in endothelial cells (59–61). This may partially explain findings described in several case reports in men that, at excessive dosing, testosterone may trigger priapism rather than reduce it (62–64).

Priapism is one of very few complications of SCD that has been studied in the context of hypogonadism. It is interesting to observe that low testosterone exhibits opposing erection phenomena in the general population of men vs men with SCD: while low testosterone may contribute to decreased erection in the general population having cardiovascular or metabolic factors affecting erectile tissue function, it results in uncontrolled erection in the SCD population, which has a severely disturbed PDE5 regulatory pathway in the penis. However, it is noted that achieving physiologic "eugonadal" effects in the penis is healthful in both populations.

3 TESTOSTERONE REPLACEMENT STRATEGIES

Traditional approaches for managing testosterone deficiency in general have largely centered on exogenous administration of testosterone. Testosterone therapies and their relative usages are: transdermal testosterone gel therapy (70%), testosterone injections (17%), transdermal testosterone patches (10%), and other forms of testosterone therapy, such as an oral formulation (3%) (65, 66). However, limitations exist with these current therapies. Adverse side effects are commonly described in

association with exogenous testosterone administration, including supraphysiologic levels of testosterone, local irritation with applications, gynecomastia, erythrocystosis, hepatotoxicity, and sleep apnea (67). Adverse prostate health risks of benign prostate enlargement and prostate cancer as well as cardiovascular risks (i.e., edema, heart attack, stroke) have also been contended to be potential risks of testosterone therapy (68). Impaired sperm production and infertility are also documented risks of exogenous testosterone therapies, by virtue of feedback inhibition of central gonadotropin release. Such therapies suppress LH, which in turn suppress Leydig cell-stimulated testosterone production, resulting in reduced intratesticular testosterone concentrations needed for spermatogenesis (67, 69). Because of the contraceptive effect exerted by exogenous testosterone preparations, many young men with hypogonadism desiring to retain reproductive function are precluded from pursuing exogenous testosterone therapies as a therapeutic option.

Alternatives to exogenous testosterone treatment have been explored, with the main objective to drive endogenous testosterone production and in turn preserve fertility. Current options include selective estrogen receptor modulators (SERMs), aromatase inhibitors, and human chorionic gonadotropin (hCG) (70). Both SERMs (e.g., clomiphene citrate and tamoxifen citrate), which serve as estrogen receptor antagonists, and aromatase inhibitors (e.g., letrozole, anastrozole, and testolactone), which block the conversion of testosterone to estradiol, result in decreased estrogen feedback to the hypothalamus thereby effecting a natural increase in gonadotropin release (70). Their efficacy in increasing testosterone production is limited in men with normal or elevated LH levels who manifest a testosterone production defect at the testicular level. hCG, operating as an LH analogue, serves to stimulate Leydig cell production of testosterone. Its efficacy is limited in men whose Leydig cells are not functionally responsive to LH because of decreased receptor function or capacity for testosterone production (65, 71).

These reports indicate that currently available testosterone therapeutic options aiming to enhance endogenous testosterone production fall short in addressing testosterone deficiency associated with testicular failure. This shortcoming is relevant generally and for hypogonadal males with SCD. Specifically in males with SCD, exogenous testosterone would further affect fertility by decreasing intratesticular testosterone production needed for spermatogenesis.

4 ENDOGENOUS MECHANISM-SPECIFIC MOLECULAR TARGETS FOR TESTOSTERONE PRODUCTION

Targeting mechanism-specific endogenous sources of testosterone production in the SCD testis to produce eugonadal levels of the hormone directly addresses primary hypogonadism. As transfer of cholesterol from the outer to the inner mitochondrial membrane of Leydig cells in the testis is the principal site of regulation of steroid hormone biosynthesis, and is impaired in SCD, targets for stimulating testosterone production may involve transduceosome protein components. The transduceosome is an ensemble of mitochondrial and cytosolic proteins responsible for cholesterol translocation from intracellular stores to the inner mitochondrial membrane (72). Translocator protein (TSPO) is a high-affinity drug- and cholesterol-binding mitochondrial protein, and its protein expression is decreased in the testis of SCD mice (73, 74). The TSPO-dependent import of StAR into mitochondria and the association of TSPO with the outer/inner mitochondrial membrane contact sites drives intramitochondrial cholesterol transfer and subsequent steroid formation (73). Previous studies have shown that TSPO drug ligands activate steroid production by MA-10 mouse Leydig tumor cells and by mitochondria isolated from other steroidogenic cells (75–77). Furthermore, pharmacologic stimulation of TSPO stimulates testosterone production, both *in vitro* by Leydig cells isolated from aged rats and *in vivo* in aged rats, without reducing intratesticular testosterone concentrations or sperm number (78, 79). These studies oppose several previous reports which questioned the role and extent of involvement of TSPO in mitochondrial cholesterol import and steroidogenesis (80, 81).

A recent study in a SCD mouse model demonstrated that pharmacologic stimulation of TSPO corrects priapism. Treatment of SCD mice with TSPO-selective drug ligand N,N-dihexyl-2-(4-fluorophenyl) indole-3-acetamide (FGIN-1-27) produces eugonadal levels of testosterone. Normalized testosterone levels corrects priapism without decreasing intratesticular testosterone production (74). At the molecular level, TSPO ligand, by normalizing testosterone levels, restores PDE5 activity and decreases NOX-mediated increase in oxidative stress in the penis. Conceivably, this effect of testosterone pertains to recovered control of NO/cGMP responsiveness associated with restored PDE5 function. The mechanism underlying testosterone's inhibitory effect on NOX expression and activity is not known, but may be indirect through the improvement of endothelial function. In human endothelial cells and mouse aorta, NO S-nitrosylates and inhibits p47phox subunit of NOX, inhibits protein expression of gp91phox and p47phox subunits of NOX, and inhibits superoxide production (82–84). These findings suggest that targeting endogenous testosterone production in the SCD testis by pharmacologic activation of protein components involved in cholesterol transport could be a novel, targetable pathway to correct primary hypogonadism and ameliorate testosterone deficiency-associated health conditions without affecting fertility.

While not examined, it is plausible that, in addition to TSPO, other cytosolic or outer mitochondrial membrane protein components involved in cholesterol transport from intracellular stores to the inner mitochondrial membrane (such as voltage dependent anion channel 1, negative protein adaptor 14-3-3ϵ, or AAA domain-containing protein 3A) (72), may be targeted in the SCD testis to increase endogenous testosterone production. Because pharmacologic activation of TSPO is

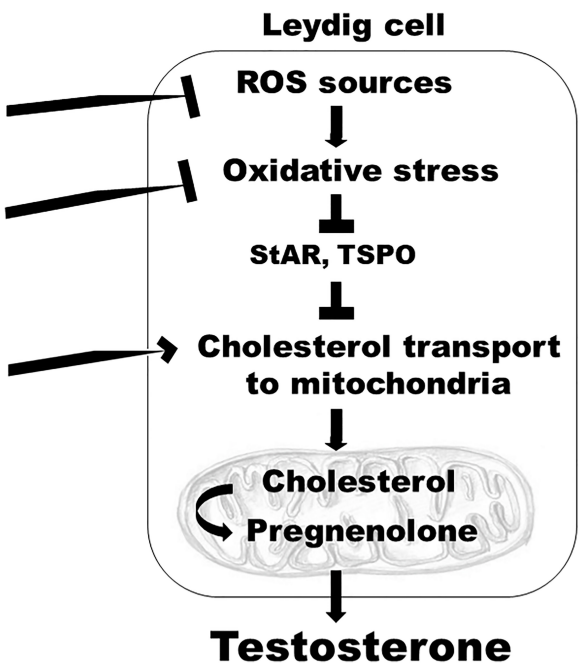

FIGURE 1 | A model depicting mechanism-specific endogenous targets within the Leydig cells of the testis which can be modulated to reverse hypogonadism in SCD. Targets include: inhibition of enzymatic sources of oxidative stress, such as NOX; inhibition of increased oxidative stress, which decreases protein synthesis of enzymes involved in cholesterol transport to the mitochondria, such as StAR and TSPO; stimulation of cytosolic or outer mitochondrial membrane protein components involved in cholesterol transport from intracellular stores to the inner mitochondrial membrane; ROS, reactive oxygen species; StAR, steroidogenic acute regulatory protein; TSPO, translocator protein.

independent of LH, it is conceivable that this approach may treat secondary hypogonadism, or mixed primary and secondary hypogonadism, as well. Other possible mechanism-based targets in the SCD testis include increased oxidative stress, or enzymatic sources of oxidative stress (such as NOX), which are enhanced in SCD-associated primary hypogonadism (**Figure 1**).

Of note, L-glutamine, one of the 3 recently FDA-approved treatments for SCD (L-glutamine, crizanlizumab, and voxelotor), increases glutathione-dependent anti-oxidation in the testis and testosterone levels, at least in sleep-deprived rats (85), while alleviating primary hypogonadism and protecting erythrocytes against oxidative damage.

5 DISCUSSION

SCD affects millions of people throughout the world, mostly of African ancestry, and is recognized by the World Health Organization and United Nations as a global health issue. In the United States, health outcomes for people with SCD have improved in the past few decades. Despite medical advances, life expectancy for individuals with SCD in the United States remains 20 to 30 years lower than that of the average American. It has been recognized that research and treatment efforts for SCD lag behind that of other chronic genetic illnesses, such as hemophilia and cystic fibrosis, requiring legislative attention (86, 87). In correlation, less FDA-approved therapies are currently available for SCD. The Sickle Cell Disease Comprehensive Care Act, signed into law in December 2018, represents a commitment by the government to continue research towards increasing the understanding of prevalence, distribution, outcomes, and therapies associated with SCD.

Amidst health care disparities among ethnic populations in the United States, limited knowledge and action surround hypogonadism in SCD, in spite of its long-term and costly health problems. While many studies have evaluated the mechanism and health-related issues of hypogonadism in the general adolescent population, very few studies have focused on hypogonadism in the SCD population. For example, although an estimated 1 in 4 SCD patients exhibits low testosterone levels, no studies have assessed the testosterone-dependent health-related quality of life profiles of SCD patients.

Despite inequity in federal and foundation research funding, basic scientific advances and potential new directions to target testosterone deficiency in SCD are being made in recent years. The objective of finding and targeting mechanism-specific endogenous sources of testosterone production appears necessary for preserving sexual function and fertility in the SCD young adult population, particularly in light of the harms of exogenous testosterone therapies.

AUTHOR CONTRIBUTIONS

The authors confirm contribution to the manuscript as follows: BM and AB critically reviewed the literature. BM drafted the article. BM and AB reviewed and revised the manuscript. BM and AB contributed to the article and approved the submitted version.

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ORIGINAL RESEARCH
published: 04 May 2022
doi: 10.3389/fendo.2022.893863

Perinatal Exposure to Nicotine Alters Sperm RNA Profiles in Rats

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Perinatal exposure to smoking has been associated with childhood asthma, one of the most common pediatric conditions affecting millions of children globally. Of great interest, this disease phenotype appears heritable as it can persist across multiple generations even in the absence of persistent exposure to smoking in subsequent generations. Although the molecular mechanisms underlying childhood asthma induced by perinatal exposure to smoking or nicotine remain elusive, an epigenetic mechanism has been proposed, which is supported by the data from our earlier analyses on germline DNA methylation (5mC) and histone marks (H3 and H4 acetylation). To further investigate the potential epigenetic inheritance of childhood asthma induced by perinatal nicotine exposure, we profiled both large and small RNAs in the sperm of F1 male rats. Our data revealed that perinatal exposure to nicotine leads to alterations in the profiles of sperm-borne RNAs, including mRNAs and small RNAs, and that rosiglitazone, a PPARγ agonist, can attenuate the effect of nicotine and reverse the sperm-borne RNA profiles of F1 male rats to close to placebo control levels.

Keywords: asthma, nicotine, smoking, lung, epigenetic inheritance, small RNA, large noncoding RNA

OPEN ACCESS

Edited by:
Barry Zirkin,
Johns Hopkins University,
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Reviewed by:
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Specialty section:
This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology

Received: *10 March 2022*
Accepted: *07 April 2022*
Published: *04 May 2022*

Citation:
Wang H, Liu J, Gao J, Yan W and Rehan VK (2022) Perinatal Exposure to Nicotine Alters Sperm RNA Profiles in Rats. Front. Endocrinol. 13:893863. doi: 10.3389/fendo.2022.893863

INTRODUCTION

Asthma is one of the most common childhood diseases with an increasing prevalence over the past decades (1, 2). Among a multitude of potential causes, perinatal exposure to smoking has been associated with childhood asthma and a lifelong decrease in pulmonary functions in both humans and animal models (3, 4). In general, exposure to smoke constituents *in utero* and/or during early postnatal development has been regarded as the primary cause as it is well-established that the chemicals released from smoking, especially nicotine, adversely affect the developing lung, rendering increased susceptibility to childhood asthma (5–9). Interestingly, we and others have shown that childhood asthma induced by perinatal exposure to nicotine can be transmitted across multiple generations even in the absence of the same exposure (10–14). This finding is of great interest and significance because it suggests that perinatal exposure to smoking/nicotine not only causes asthma in the immediate offspring but also results in changes in their germline, leading to the transgenerational inheritance of childhood asthma.

Given that the childhood asthma induced by perinatal exposure to nicotine arises in one generation and the distribution of this disease phenotype in subsequent generations never follows Mendel's Law (11–14), it is highly unlikely that the asthma phenotype results from genetic

mutations caused by nicotine exposure. Instead, transgenerational inheritance of the phenotype is most probably mediated by an epigenetic mechanism. Both inter- and trans-generational epigenetic inheritance of disease phenotypes induced by exposure to environmental chemicals, over-or under-nutrition (e.g., high-fat diet (HFD) or starvation), or traumatic stress has been convincingly demonstrated at least in animal models (15–17). However, the underlying molecular mechanisms remain elusive. Since mammals reproduce sexually, the epigenetic codes that induce the acquired traits must lie in the gametes, sperm, and eggs. Indeed, epimutations, including changes in sperm DNA methylome (e.g., 5mC), histone modifications, and small RNA profiles, have been associated with various acquired traits in both human and animal models (18, 19). However, the causative relationship between specific epimutations (e.g., altered DNA methylation or histone marks) and specific phenotypes has not been established. Interestingly, several studies have shown that sperm total or small RNAs from male mice with an epigenetic phenotype (e.g., metabolic disorders induced by HFD and the whitetail tips caused by *Kit* paramutation), seem capable of inducing a similar phenotype in offspring derived from zygotes injected with either total or small RNAs isolated from the sperm, suggesting that sperm RNAs may function as the epigenetic codes responsible for the paternal transmission of certain acquired traits (19–21). Our previous studies have shown that the sperm 5mC profiles and histone marks are altered in the male rats with perinatal exposure to nicotine (11, 22). Given that both DNA methylation and histone marks in sperm are largely established during testicular development and spermatogenesis (23, 24), it is plausible to hypothesize that both large and small sperm-borne RNAs may also be altered in the male rats of our perinatal nicotine rat models.

Here, we report that indeed, both mRNA and small RNA transcriptomes were altered in the sperm of F1 male offspring of F0 dams with the perinatal treatment of nicotine. Consistent with our earlier reports (25, 26), we also found that a PPARγ agonist could attenuate the effects of perinatal exposure to nicotine on sperm RNA profiles in the F1 male offspring.

MATERIALS AND METHODS

The Perinatal Nicotine Exposure Rat Model

The perinatal nicotine exposure rat model used in this study was established as described previously (12, 22, 27, 28). Briefly, time of mating-matched, first-time pregnant, pair-fed adult (2 months of age) Sprague Dawley rat dams (F0) with bodyweight between 200-250 g received either placebo (saline, n = 3), nicotine (1 mg/kg, subcutaneously, n = 3), or nicotine (1 mg/kg, subcutaneously) plus rosiglitazone (RGZ) (3 mg/kg, intraperitoneally, n = 3) in 100 μL volumes once daily from embryonic day 6 (E6) of gestation to postnatal day 21 (PND21). The dose of nicotine used (1 mg/kg/day) is within the range of nicotine exposure in moderately heavy smokers (29–31). At this dose, the pulmonary structural, molecular, and functional changes that we observed in the rat model used are similar to those demonstrated in numerous other perinatal nicotine and smoke exposure models (12, 27, 32–35). Animals were maintained in a 12h-light and 12h-dark cycle, pair-fed according to the previous day's food consumption by the nicotine-treated group and were allowed free access to water. Following spontaneous delivery at term, the F1 pups were allowed to breastfeed ad libitum. At PND21, pups were weaned and maintained in separate cages. At PND60, males [n = 3 (from 3 separate litters) for each group] were euthanized by pentobarbital overdose injected intraperitoneally, followed by epididymis collections as quickly as possible. The epididymides were kept in ice-cold F12 culture medium until sperm isolation within 1-2 hours of the collection, as outlined below. All animal procedures were performed following the National Institutes of Health guidelines for the care and use of laboratory animals and approved by the Institutional Animal Care and Use Committee at The Lundquist Institute for Biomedical Innovation at Harbor-UCLA Medical Center.

Collection and Purification of Sperm Cells

At culling, each epididymis was isolated by cutting the vas deferens and muscle connections with the testis. After trimming the surrounding connective tissue, the two epididymides from each animal were placed in a tissue culture plate containing 3 mL of HTF culture medium (Sigma, EmbryoMax® Human Tubal Fluid (HTF) (1X), Cat No. MR-070-D) on ice. The spermatozoa were released into the culture media by making 6-8 small cuts to each epididymis with a sharp blade, and the plates were placed in a culture incubator at 37°C for 30 minutes. Following incubation, the medium containing spermatozoa was filtered through a cell strainer (Genesee Scientific, 70 μm Advanced Cell Strainers, Cat No. 25-376) to a 50 mL conical tube, and the filtrate was divided into four 1.5 mL micro-centrifuge tubes. The tubes were centrifuged at 1000×g for 5 minutes, supernatants discarded, and 1 mL lysis buffer (0.05% SDS and 0.005% Triton X-100 in distilled water) added to each tube to gently suspend the pellet. The tubes were kept on ice for 5 minutes to lyse and remove the somatic cell contamination. After confirming the purity of isolated sperms microscopically, the samples were centrifuged at 3000×g for 5 minutes. The supernatants were discarded, and each pellet gently suspended in 1 mL ice-cold PBS. The suspensions from two tubes were pooled and centrifuged at 3000×g for 5 minutes. The supernatants were discarded, and pellets stored at -80°C until RNA isolation and establishment of cDNA library.

Total RNA Extraction

Sperm samples were pooled (n = 3 mice/biological replicate) and subjected to RNA extraction for RNA-seq, as described below. RNA was extracted from cells according to the manufacturer's instructions using the mirVana miRNA Isolation Kit (ThermoFisher, Cat No. AM1560). The Qubit RNA High Sensitivity Assay Kit (Invitrogen, Cat No. Q32855) was used to quantify the extracted RNA and measured on the Qubit 2.0 Fluorometer (Invitrogen).

Large RNA Libraries Construction

Large RNA libraries were constructed using KAPA Stranded RNA-seq Kit with RiboErase (Roche, Cat No. KK8484) according to the manufacturer's instructions, as described previously (36), and sequenced using HiSeq 2500 system for paired-end 50 bp sequencing.

Large RNA-Seq Data Analysis

The following workflow was used in bioinformatic analyses of the RNA-seq data: QC check (fastQC) ➔ alignment (Hisat2) ➔ featureCounts (subread) ➔ Differential gene expression analysis (DESeq2) ➔ Pathway Enrichment, GO analysis (Bioconductor clusterProfiler). To ensure the quality of RNA-seq data, fastq files were subjected to fastQC (37) to check their quality and changes after adaptor and quality trim. MultiQC (38) was then utilized to analyze and integrate the QC reports (**Figure S1**). HISAT2 was used to perform alignment (39). Each sample yielded a bam file after being aligned to the genome. Feature counts from each bam file that map to the genomic features in the provided annotation file was realized by subread function (40). DESeq2 was used to analyze the gene differential expression (41). Markers/genes with the sum of reading count across all cases and controls at 10 or greater were kept for further analyses. To interpret the expression data, a universal enrichment tool named "clusterProfiler" was applied to infer gene set enrichment (42).

Annotation of lncRNAs From Large RNA-Seq Data

LncRNA information was first obtained with gene symbols by merging two Ensembl releases (release-105 Rattus_norvegicus.mRatBN7.2.ncRNA.fa.gz and release-104 Rattus_norvegicus.Rnor_6.0.104.gtf.gz). The gene symbols were then used in differential expression analyses as keywords to search in Ensemble to obtain the lncRNA information that can be annotated. Finally, those two are combined to obtain all the lncRNA gene symbols. The extracted padj of these lncRNAs were much larger than 0.05.

Small RNA Libraries Construction

Small RNA libraries were constructed using NEBNext® Small RNA Library Prep Set for Illumina® (Multiplex Compatible) (NEB, Cat No. E7330L) according to the manufacturer's instructions, as described previously (43), and sequenced using HiSeq 2500 system for single-end 50 bp sequencing.

Small RNA-Seq Data Analysis

Cutadapt (44) was used to remove adaptors and trim low quality reads (q > 20). The fastq files after QC filter were used to run the AASRA pipeline using default parameters (45). Eight small species, incuding miRNA, tRNA, piRNA, rRNA, snRNA, snoRNA, Mt_rRNA and Mt_tRNA, were annotated. The subsequent analyses using Featurecounts and DESeq2 were performed the same as large RNA-Seq. TargetScan was used to identify potential miRNA targets, the candidate target genes were used for gene ontology (GO) enrichment analyses using "clusterProfiler".

qPCR Analysis

cDNAs for large and small RNA were prepared as previously described (43). Briefly, large RNAs were reverse transcribed to cDNAs using SuperScriptTM II Reverse Transcriptase (Thermo Fisher Scientific, Cat No. 18064014). Then qPCR analyses for large RNA were conducted using Fast SYBR® Green Master Mix (Thermo Fisher Scientific, Cat No. 4385616). Gapdh was used for large RNA expression normalization. Small RNAs were poly(A) tailed using E. coli Poly(A) Polymerase (NEB, Cat No. M0276L) followed by reverse transcription with LD_CDS primer using SuperScript™ II Reverse Transcriptase. qPCR analyses for small RNA were then performed using TaqMan™ Gene Expression Master Mix (Thermo Fisher Scientific, Cat No. 4369016) with Illu lib quant probe. U6 was used for small RNA expression normalization. The primer sequences used in this study for qPCR are listed in Datasets S1.

Statistical Analysis

All data were subjected to statistical analysis using the SPSS program (IBM, SPSS, New York, NY, USA) and shown as mean ± standard error of the mean (SEM). And statistical differences between two groups were assessed by two samples *t*-test. Symbols *, ** and *** represent $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.

Availability of Data and Materials

The RNA-seq data have been deposited into the National Center for Biotechnology Information Sequence Read Achieve database (accession no. PRJNA813596).

RESULTS

mRNA Profiles in Sperm From the Male Rats Born to Control, Nicotine-Treated, and Nicotine Plus RGZ-Treated Dams

Adult female rats (F0 dams) received placebo (saline subcutaneously as controls), nicotine (1 mg/kg, B.W. subcutaneously), or nicotine (1 mg/kg, B.W. subcutaneously) plus RGZ (3 mg/kg, B.W., intraperitoneally) between E6 and PND21 (**Figure 1**). Cauda epididymal spermatozoa of F1 male offspring (n = 3, from 3 separate litters in each group) were collected at PND60 and used for large RNA deep sequencing (RNA-seq), followed by bioinformatics analyses using the pipeline as illustrated (**Figure 2A**).

The 3D principal component analyses (3D-PCAs) verified that the differential transcriptomes of placebo control, nicotine-treated (NIC) and nicotine plus RGZ-treated (NR) sperm samples were indeed clustered separately (**Figure 2B**). A total of 29 differentially expressed mRNAs (21 upregulated and 8 downregulated mRNAs) satisfied the criteria of padj (adjusted p-value) less than 0.05, and fold change greater than 0.2 and less than −0.2 (logFC ± 0.2) in NIC sperm samples compared to placebo controls (Datasets S2). The MA plots (**Figure 2C**, left panel) illustrate the differentially expressed genes (DEGs). In contrast, no significantly dysregulated mRNAs (padj <= 0.05 and

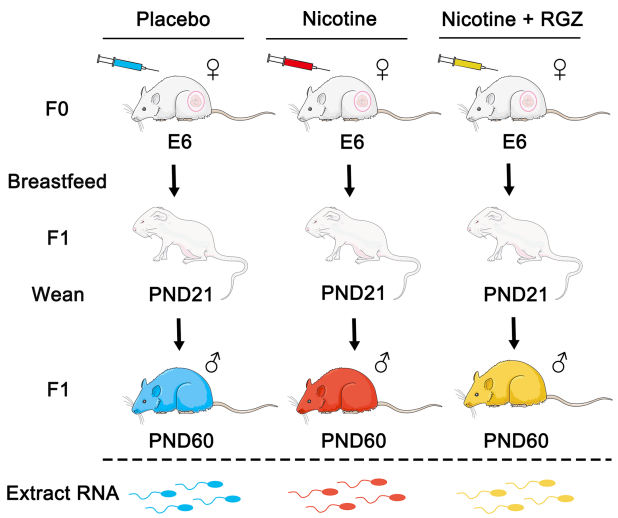

FIGURE 1 | The perinatal nicotine exposure rat model used in the present study. Dams (F0) received either placebo (saline), nicotine (1 mg/kg, subcutaneously), or nicotine (1 mg/kg, subcutaneously) plus rosiglitazone (RGZ) (3 mg/kg, intraperitoneally) once daily from embryonic day 6 (E6) of gestation to postnatal day 21 (PND21). The F1 pups were allowed to breastfeed ad libitum. At PND21, the F1s were weaned and maintained in separate cages. Pure sperm cells of F1 male rats were collected at PND60 and sperm RNA was extracted and used for RNA-seq analyses.

|log2FC| ≥ 2) were detected in sperm of the control and NR groups (**Figure 2C**, right panel and Datasets S3).

Many of the upregulated genes in nicotine-treated sperm are known to be involved in asthma pathogenesis, including *L-Histidine decarboxylase (Hdc)*, *Fc receptor-like 3 (Fcrl3)*, *Endothelin receptor type B (Ednrb)*, and *Complement C4A (C4a)*. *Hdc* encodes a unique enzyme in mammals which catalyzes histamine formation from L-histidine and histamine plays a critical role in the pathogenesis of bronchial asthma. In particular, the level of *Hdc* mRNA is elevated in asthmatic patients (46). Furthermore, *Hdc* allele Glu644 in homozygotes increases the risk of rhinitis in the study population, supporting a prominent role for genetic variants associated with histamine homeostasis in developing allergic disease risk (47). In studying a single nucleotide polymorphism (SNP) in *Fcrl3* in asthma and/or AR patients and healthy controls in a Chinese Han population, novel SNP rs7528684 appears to be associated with asthma with comorbid AR, and *Fcrl3_3* (rs7528684) and *Fcrl3_6* (rs3761959) SNPs are protective against asthma in Mexican male patients (48, 49). As the receptor for asthma related gene *EDN1*, the 30G>A SNP in *Ednrb* is strongly associated with the degree of airway obstruction, especially in patients with factors that induce airway remodeling, such as asthma or smoking (50). And in the murine model of asthma, *Ednrb* receptor antagonists is found to effectively inhibit allergic reactions (51). When compared with the children without asthma, an increasing serum level of *C4* component of the complement system is observed in the majority of the patients with intermittent atopic asthma, representing a biomarker for diagnosis of intermittent atopic asthma (52). In addition, the level of *C4a* increases in the plasma of patients with aspirin-induced asthma, and significantly correlated with FEV1 (53).

However, several genes are newly implicated in asthma, including *Rho GTPase activating protein 15 (Arhgap15)*, *Pleckstrin (Plek)*, and *Transcription factor EC (Tfec)*. *Arhgap15* has been called a "master negative regulator of neutrophil functions", and validated as a differentially expressed novel transcript in patients with asthma (54, 55). *PLEK* is a major substrate for protein kinase C signaling, a pathway strongly implicated in asthma pathogenesis was upregulated in severe asthmatics and exhibited a moderate ability to distinguish between severe and mild-moderate asthmatics (56). Furthermore, earlier studies have revealed an *IL-4/STAT-6/Tfec/IL-4Rα* positive feedback regulatory loop, in which *Tfec* transcribes *IL-4Rα* expression to promote M2 programming in macrophages, which was implicated in asthma pathogenesis (57). In addition, several genes were involved in lung cancer and other lung diseases, such as *Ceacam1* (58, 59), *Ereg* (60, 61), *Selp* (62, 63), and *Pik3r5* (64, 65). The most conspicuous genes among downregulated ones are members of the keratins (KRTs) and keratin-associated proteins (KRTAPs), including *Krt34*, *Krtap3-1*, and *Krtap7-1*, which are important for epidermal development and hair follicle morphogenesis (66, 67), respectively. qPCR analyses of seven dysregulated genes, including *C4a*, *C4b*, *Sult1c2*, *Arhgap15*, *Ednrb*, *Ceacam1*, and *Pik3r5*, further validated the RNA-seq data (**Figure 2D**). In addition, bioinformatic analyses showed no long non-coding RNAs (lncRNAs) were significantly dysregulated (padj <= 0.05) in sperm samples from the three groups (**Figure S2**, Datasets S4 and S5). To further understand the functions of these DEGs, gene ontology (GO) term enrichment analyses were performed, and the dysregulated genes appeared to be involved in integrin activation and platelet activation (**Figure 2E**, Datasets S6).

Taken together, perinatal exposure of nicotine appears to induce altered profiles of sperm mRNAs, but not those of lncRNAs; however, administration of RGZ appeared to attenuate the nicotine effects on the sperm mRNA profiles.

Profiles of Small Non-Coding RNAs (sncRNAs) in Sperm From the Male Rats Born to Control, Nicotine-Treated, and Nicotine Plus RGZ-Treated Dams

To determine the effects of perinatal exposure to nicotine and nicotine plus RGZ on the sperm sncRNAs profiles of F1 males, sperm small RNAs were isolated and sncRNAs-seq was performed followed by bioinformatics analyses using AASRA (68) (**Figure 3A**). A total of eight sncRNA species, including miRNAs, mitochondrial DNA-encoded rRNA (Mt_rRNA) and tRNA (Mt_tRNA) fragments, piRNAs, rRNA fragments, tRNA fragments, snoRNAs, and snRNAs, were annotated and their normalized total counts were compared among the three groups (**Figure S3**).

Principal component analyses verified that the differential transcriptomes of the three groups were clustered separately (**Figure 3B**). A total of 139 sncRNAs were identified to be

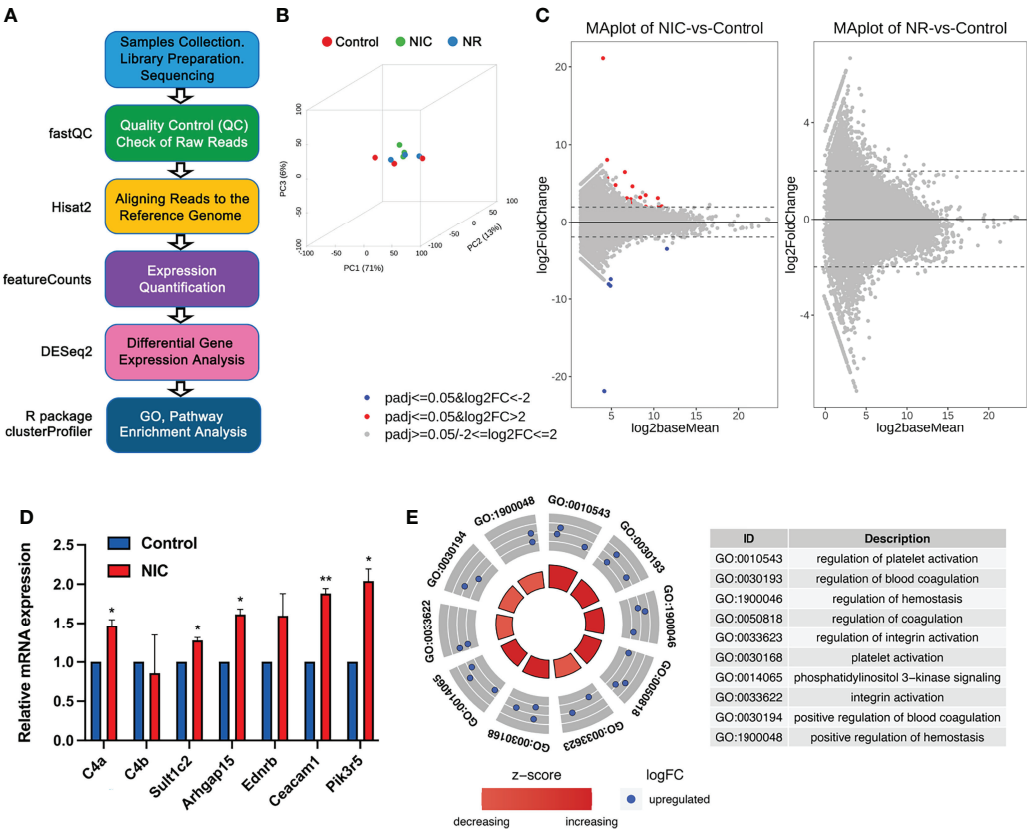

FIGURE 2 | RNA-seq analyses of sperm mRNA profiles in placebo, nicotine-treated and nicotine plus RGZ-treated F1 male rats. **(A)** The workflow for large RNA-seq data analyses, showing the major steps and bioinformatic tools used in the study. **(B)** Three-dimensional principal component analyses of the large RNA-seq data from nicotine-treated, nicotine plus RGZ-treated, and placebo-treated sperm samples. **(C)** MA plots showing differentially expressed genes (upDEGs and downDEGs) detected between nicotine-treated and placebo-treated sperm samples (left panel) and between nicotine plus RGZ-treated and placebo-treated sperm samples (right panel). The Log2baseMean represent the Log2 mean value of DESeq2 normalized counts between nicotine-treated and placebo-treated sperm, or between nicotine plus RGZ-treated and placebo-treated sperm. Log2 fold change (Log2FC) was calculated by the Log2 mRNA counts of nicotine-treated sperm/placebo-treated sperm, or nicotine plus RGZ-treated sperm/placebo-treated sperm. Genes that pass a threshold of padj <= 0.05, log2FC > 2 and padj <= 0.05, log2FC < -2 in differential expression analysis were designated by red (up-regulated) and blue (down-regulated) in nicotine-treated or nicotine plus RGZ-treated sperm relative to placebo-treated control sperm cells. **(D)** qPCR validation of mRNA expression levels in placebo and nicotine-treated sperm. *Gapdh* was used as an internal control. Data are presented as mean ± SEM (n = 3). **P < 0.01; *P < 0.05. **(E)** Circle plots showing the top 10 Gene Ontology (GO) terms of biological process analyzed from 16 significantly dysregulated genes in sperm samples from rats injected with nicotine compared to those from rats injected with placebo.

significantly dysregulated (padj <= 0.05 and |log2FC| ≥ 0 between sperm from NIC and control groups (**Figure 3C**, left panel and Datasets S7). These dysregulated sncRNAs included 47 miRNAs, 83 piRNAs, 1 tRNA, and 8 other sncRNAs. In contrast, no significantly dysregulated sncRNAs (padj <= 0.05) were detected between sperm from NR and placebo control groups (**Figure 3C**, right panel and Datasets S8). All of the dysregulated miRNAs and the vast majority (79 out of 83) of the dysregulated piRNAs were upregulated between NIC and control sperm (**Figure 3D**). Interestingly, while miRNA and piRNA levels were upregulated, other sncRNAs were mostly downregulated in nicotine-treated sperm. To validate the sncRNAs-seq data, we performed qPCR analyses on eight miRNAs (let-7a-1-3p, miR-101b-3p, 293-5p, 148-3p, 192-5p, 340-5p, 1b, and 598-3p) and five piRNAs (piR-rno-62944, rno-62902, rna-62978, rno-62740, rno-62736) in nicotine-treated and placebo control sperm. The results showed that levels of miRNAs and piRNAs were much higher in nicotine-treated sperm compared to controls (**Figure 3E**). Together, perinatal exposure to nicotine appears to alter the sncRNAs profiles, and this effect can be abolished by RGZ.

Given that miRNAs are known to function as a post-transcriptional regulator by targeting the 3'UTRs of mRNAs, we further determined the potential targets of the 47 significantly dysregulated miRNAs (Datasets S9 and S10) using TargetScan (69). Those target genes included those previously implicated in asthma pathogenesis, such as *ADAM metallopeptidase domain 33 (Adam33)*, *PHD finger protein 11 (Phf11)*, *Dipeptidyl peptidase like 10 (Dpp10)*, *Interleukin 4 (Il4)*, *Brain-derived neurotrophic factor (Bdnf)*, *Serine peptidase inhibitor, Kazal type 5 (Spink5)*, *Cd69 molecule (Cd69)*, etc. Following linkage studies, *Adam33* (70), *Phf11* (71) and *Dpp10* (72) have been identified to be associated with asthma and asthma-related phenotypes. Studies

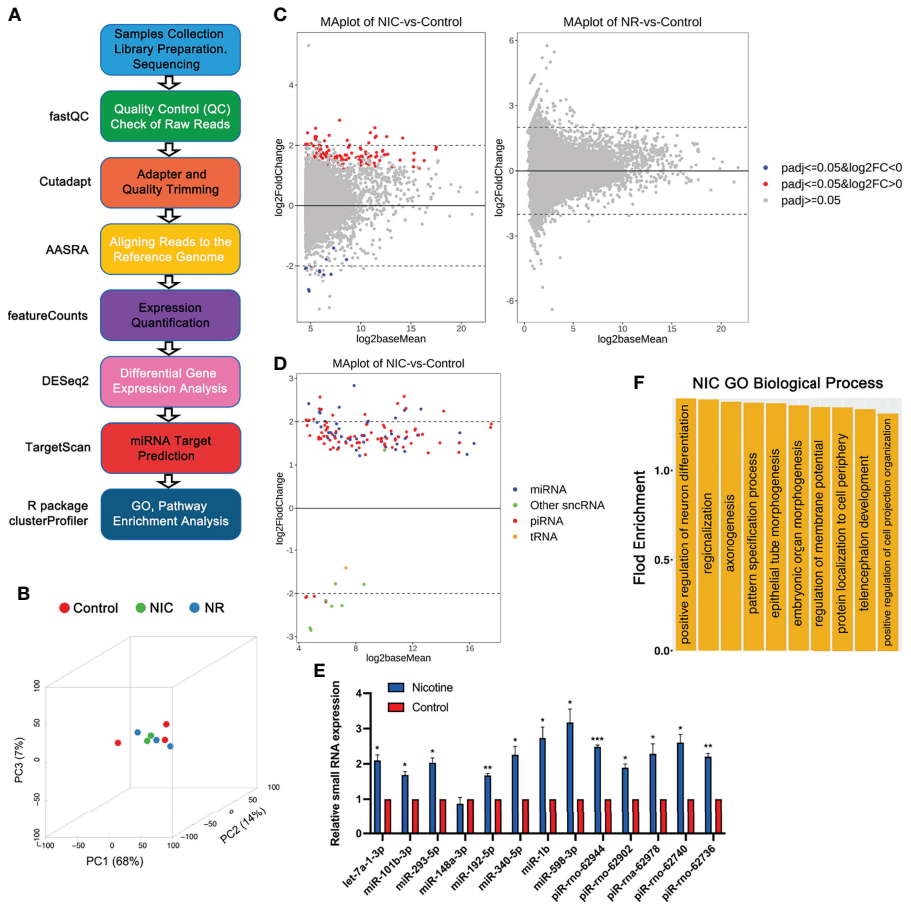

FIGURE 3 | RNA-seq analyses of small non-coding RNAs (sncRNAs) in placebo, nicotine-treated and nicotine plus RGZ-treated sperm samples. **(A)** The workflow for small RNA-seq data analysis, including the major steps and bioinformatics tools used in the study. **(B)** Three-dimensional principal component analyses of the small RNA-seq data from nicotine-treated, nicotine plus RGZ-treated, and placebo-treated sperm samples. **(C)** MA plots showing the differentially expressed sncRNAs detected between nicotine-treated and placebo-treated sperm samples (left panel) and between nicotine plus RGZ-treated and placebo-treated sperm samples (right panel). The Log2baseMean represent the Log2 mean value of DESeq2 normalized counts between nicotine-treated and placebo-treated sperm. Log2 fold change (Log2FC) was calculated by the Log2 sncRNA counts of nicotine-treated sperm/placebo-treated sperm. SncRNAs that pass a threshold of padj <= 0.05, log2FC > 0 and padj <= 0.05, log2FC < 0 in differential expression analysis were designated by red (up-regulated) and blue (down-regulated) in nicotine-treated or nicotine plus RGZ-treated sperm relative to placebo-treated control sperm cells. **(D)** MA plots showing the number of significantly differentially expressed sncRNAs (padj <= 0.05) between nicotine-treated and placebo-treated sperm samples. **(E)** qPCR validation of sncRNA expression levels in placebo and nicotine-treated sperm. U6 was used as an internal control. Data are presented as mean ± SEM (n = 3). ***P < 0.001; **P < 0.01; *P < 0.05. **(F)** GO term enrichment analyses of potential target genes of significantly dysregulated miRNAs in nicotine-treated sperm. Outputs (biological processes) are sorted and plotted against fold enrichment.

showed that *IL4*, a key effector Th2 cytokine in allergic asthma, was essential for B cells autophagy induction *in vivo* and *in vitro*, thereby further sustaining B cell survival and enhanced B cell antigen presentation (73). *BDNF* may contribute to normal lung function and immune response and may serve as a potential peripheral biomarker for asthma, especially that is aspirin-sensitive (74). Studies have shown that *SPINK5* has biological actions other than protease inhibition, which may be related to the pathogenesis of asthma (75). *CD69* was known as an early activation marker antigen of lymphocytes, had a crucial role in the pathogenesis of arthritis and allergic airway inflammation and could be a possible therapeutic target for arthritis and asthma in human patients (76). Furthermore, many of the target genes were known to be involved in other lung diseases, including *High mobility group AT-hook 2 (Hmga2)* (77), *Ubiquitin-conjugating enzyme E2C (Ube2c)* (78), *Adrenoceptor beta 3 (Adrb3)* (79), *Coronin 1C (Coro1c)* (80), *Sp1 transcription factor (Sp1)* (81), *Ras homolog family member B (Rhob)* (82), *Serum/glucocorticoid regulated kinase 1 (Sgk1)* (83), *BTG anti-proliferation factor 2 (Btg2)* (84), *Homeobox D8 (Hoxd8)* (85), *Bone morphogenetic protein 4 (Bmp4)* (86), *Protein regulator of cytokinesis 1(Prc1)* (87), etc.

GO term enrichment analyses identified that the affected target genes were mostly involved in the biological processes including embryonic organ morphogenesis, regionalization, epithelial tube morphogenesis, positive regulation of neuron

differentiation, telencephalon development, protein localization to the cell periphery, pattern specification process, axonogenesis, regulation of membrane potential, and positive regulation of cell projection organization (**Figure 3F**, Datasets S11).

DISCUSSION

Epidemiological studies have shown that grandma's smoking when pregnant with the mother increases the risk of asthma in the grandchild independent of the mother's smoking status, suggesting a potential transgenerational effect of perinatal smoking on the incidence of childhood asthma (88, 89). However, considering many confounding factors, this notion remains highly correlative. Given that it would take decades to follow up on multiple generations on any transgenerational effect, we and others have developed animal models to demonstrate that childhood asthma induced by perinatal exposure to nicotine in F0 dams can persist for at least three generations in the absence of continuous perinatal exposure to nicotine in F1-F3 (11, 12). Such intergenerational and transgenerational transmission of the induced disease phenotype must be mediated by the gametes (sperm and eggs) given sexual reproduction. Indeed, our earlier data have shown that both histone marks and DNA methylation (5mC) patterns are altered in F1 sperm (10). Since the sperm DNA methylation patterns are largely established during fetal testicular development and further modified during spermatogenesis, the DMRs in F1 male rat sperm must have arisen in pro-spermatogonia and/or the subsequent spermatogenic cells including spermatogonia, spermatocytes, or spermatids. Since DNA methylation changes affect gene expression, it is possible that the mRNAs that are produced in spermatogenic cells and packed into the sperm nuclei might be altered as well. In contrast, the vast majority of nuclear histones are replaced by transition proteins and ultimately by protamine during the elongation of round spermatids (20, 90). Consequently, only trace amounts of histones (<1% in rodents and <5% in humans) are retained in sperm (91). Therefore, altered histone levels and chemical modifications must have occurred during late spermiogenesis. Since both large and small RNAs are believed to be packed into the condensing nuclei of spermatids upon elongation during spermiogenesis, the altered histone profiles may also indicate altered mRNA and small RNAs that are packed into the nuclei of sperm. Indeed, our data clearly show that the mRNA and small RNA profiles are indeed altered in the sperm of F1 male rats born to dams with perinatal exposure to nicotine.

Sperm-borne mRNAs are delivered to the oocytes during fertilization (92). It remains unclear whether these mRNAs are functional and thus necessary for fertilization and early embryonic development. Small RNAs have been detected in sperm nuclei, and miRNAs and endo-siRNAs have been shown to be essential for fertilization and early embryonic development, most likely through functional as post-transcriptional regulators (93, 94). Increasing lines of evidence also suggest that both sperm-borne large and small RNAs may have a role in mediating epigenetic inheritance of acquired traits (19). This notion is largely based on the observations that injection of either total RNA or small RNAs isolated from male mice with the specific acquired traits (e.g., metabolic disorders induced by HFD, stress response conditioned to specific odor, wound healing response conditioned to repeated liver injury, etc.) into wildtype oocytes can produce offspring displaying a similar phenotype. However, the exact molecular actions of these sperm-borne RNAs remain elusive. In the present study, we identified 29 differentially expressed mRNAs in nicotine exposed F1 male rats compared to placebo control male rats. These DEGs may represent the consequences of the dysregulated epigenome, as reflected by numerous DMRs and aberrant histone marks detected (11, 22), in spermatogenic cells during spermatogenesis. An alternative function would be that these sperm-borne mRNAs, once delivered into the cytoplasm of the oocytes, can produce proteins that participate in early embryonic development. Given that these F1 male rats all have normal fertility, the changed levels of the proteins encoded by these DEGs must be compatible with successful fertilization and embryonic development. However, it remains unknown whether these proteins can be involved in epigenetic regulations that can lead to childhood asthma. Among the differentially expressed small RNAs, miRNAs and piRNAs appear to be the dominant small RNA species in the nicotine exposed F1 male rats. miRNAs are known to function as a post-transcriptional regulator by binding the 3'UTR of mRNAs to control mRNA stability and translational efficiency (95). Sperm-borne piRNAs are largely produced in spermatocytes and spermatids, and these piRNAs are believed to control the timely degradation of mRNAs during late spermiogenesis (96, 97). It remains unclear how miRNAs and piRNAs function as carriers of epigenetic information in sperm although both have been shown to be involved in the transmission of acquired traits inter-or trans-generationally. Several studies have shown that microinjection of sperm total or small RNAs (total, miRNAs, tsRNAs) isolated from the male mice with acquired traits can induce similar phenotypes in offspring although the phenotypic penetrance varies (98–102). It would be of great interest to see whether injection of the dysregulated small RNAs in male F1 rats with perinatal exposure to nicotine also transmits the asthma phenotype to the subsequent progeny. Moreover, examination of the epigenome of the F1 lung tissue in both nicotine-exposed and placebo control males during fetal and postnatal development may shed light on the effects of the dysregulated sperm small RNAs in the future.

Rosiglitazone is a PPARγ agonist that has shown a beneficial effect in both mice and humans with asthma (103, 104). In asthmatic mice and patients, PPARγ activation appears to inhibit airway inflammation and remodeling by downregulating proinflammatory gene expression and inflammatory cell functions (105). In our rat model of childhood asthma, induced by perinatal nicotine exposure, RGZ administered in conjunction with nicotine attenuates the development of asthma (25, 26). More interestingly, the altered levels of 5mC and several

histone modifications including H3 acetylation and H4 acetylation also get reversed in the lung and gonad of F1 rats (11, 22). These data suggest that RGZ has an epigenetic effect on both the target tissue (i.e., lung) and germ cells, which can largely restore the gene networks required for normal airway functions. Consistent with these previous data, our RNA-seq analyses of total RNA expression profiles in the sperm of F1 male rats also show that RGZ can attenuate the adverse effects of perinatal exposure to nicotine on the sperm RNA profiles. The effect may directly affect the expression of certain mRNAs and small RNAs. Alternatively, the altered transcriptomes may result from RGZ's effect on DNA methylation and/or histone modifications. Nevertheless, the fact that a PPARγ agonist attenuates the effect of nicotine on sperm large and small RNA transcriptome further supports the notion that PPARγ agonists is a promising class of drugs for treating childhood asthma.

In summary, we report here that perinatal exposure to nicotine leads to alterations in the profiles of sperm-borne RNAs, including mRNAs and small RNAs, and that rosiglitazone can attenuate the effect of nicotine and reverse the sperm-borne RNA profiles of F1 male rats to close to placebo control levels.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee at the Lundquist Institute for Biomedical Innovation at Harbor-UCLA Medical Center.

AUTHOR CONTRIBUTIONS

VR designed the research. HW and JL performed the experiments. JG performed bioinformatic analyses. HW and WY analyzed the data and wrote the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by grants from the NIH (HD098593, HD0085506, HD099924 to WY; and HL151769, H127137, HD071731, and HL152915 to VR); the Templeton Foundation (PID: 61174 to WY); and the TRDRP (23RT-0018, 27IP-0050, and T29IR0737 to VR).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.893863/full#supplementary-material

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frontiers | Frontiers in Endocrinology

MINI REVIEW
published: 04 May 2022
doi: 10.3389/fendo.2022.897196

Updates in Sertoli Cell-Mediated Signaling During Spermatogenesis and Advances in Restoring Sertoli Cell Function

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Since their initial description by Enrico Sertoli in 1865, Sertoli cells have continued to enchant testis biologists. Testis size and germ cell carrying capacity are intimately tied to Sertoli cell number and function. One critical Sertoli cell function is signaling from Sertoli cells to germ cells as part of regulation of the spermatogenic cycle. Sertoli cell signals can be endocrine or paracrine in nature. Here we review recent advances in understanding the interplay of Sertoli cell endocrine and paracrine signals that regulate germ cell state. Although these findings have long-term implications for treating male infertility, recent breakthroughs in Sertoli cell transplantation have more immediate implications. We summarize the surge of advances in Sertoli cell ablation and transplantation, both of which are wedded to a growing understanding of the unique Sertoli cell niche in the transitional zone of the testis.

Keywords: sertoli cell (SC) niche, transitional zone (TZ), Sertoli cell ablation, Sertoli cell transplantation, Spermatogenesis, FSH signaling, AR signaling, Exosome extracellular vesicle (EV)

OPEN ACCESS

Edited by:
Barry Zirkin, Johns Hopkins University, United States

Reviewed by:
Michael Griswold, Washington State University, United States

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Specialty section:
This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology

Received: *15 March 2022*
Accepted: *31 March 2022*
Published: *04 May 2022*

Citation:
Ruthig VA and Lamb DJ (2022) Updates in Sertoli Cell-Mediated Signaling During Spermatogenesis and Advances in Restoring Sertoli Cell Function. Front. Endocrinol. 13:897196. doi: 10.3389/fendo.2022.897196

INTRODUCTION

Although germ cells are the stars of spermatogenesis, Sertoli cells are the sustaining lead, without which, spermatogenesis would cease to occur. Sertoli cells provide the supportive framework within which germ cells will safely undergo rounds of mitosis and meiosis (**Figure 1**). This structure which includes tight junctions between adjacent Sertoli cells, divides the seminiferous epithelium into the basal and adluminal compartments, serving a protective role as the testicular region within the seminiferous tubules that is immuno-privileged (1–5). Sertoli cells act as the mediator between germ cells and endocrine signaling, from controlling spermatogenesis by hormones (follicle stimulating hormone [FSH] and testosterone [T]), originating from outside of the seminiferous tubule (6–8). Sertoli cells also have direct impacts on germ cell development through paracrine signaling (9–11). These roles are all key elements required to orchestrate the symphonic cyclicity of steady-state spermatogenesis within the adult testis. When aberrations in Sertoli cell function occur, this intricate exchange breaks down and spermatogenic failure may occur, ultimately challenging the fertility of the male. Recent research into the niche population of Sertoli cells at the transition zone between the rete testis and seminiferous tubules, as well as studies of Sertoli cell transplantation, are

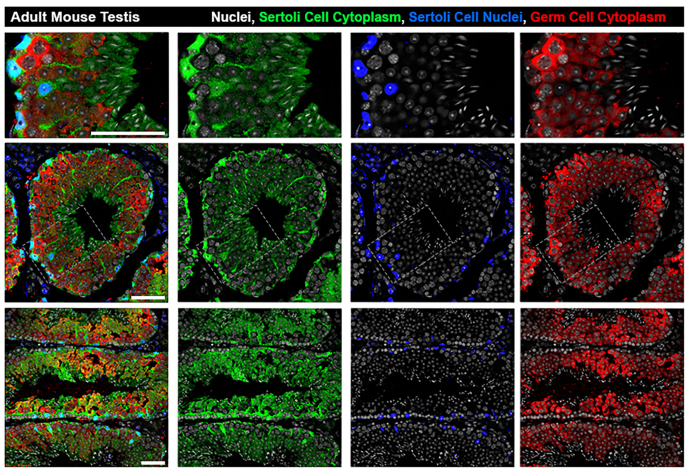

FIGURE 1 | Architecture of Sertoli cells in the adult mouse seminiferous tubule. The bodies of Sertoli cell cytoplasm (green) can be seen engulfing germ cells (red) from basal lamina to lumen while Sertoli cell nuclei (blue) are located basally. Top row: zoomed inset from grey boxed region in Middle Row: seminiferous tubule cross section at stage V-VI. Bottom Row: Longitudinal sections showing multiple stages. All scale bars are 50μm.

bringing new insights to the field. Both branches of investigation offer the promise of a deeper understanding into how Sertoli cells come to reside properly in the testis, and methods for getting functional Sertoli cells in to replace Sertoli cells that are deficient.

Aside from the germ cell based histological staging of spermatogenesis defined by consistent cell associations present in cross-sections of the seminiferous tubule (**Figure 2**), generally the stages of the cycle can also be defined by unique metabolic and molecular Sertoli cell identities (22–24). Specifically in regards to the androgen signaling pathway, Sertoli cells display stage specific temporal peaks of AR expression in rodents (stages VI-VIII) (25–27) (**Figure 2A**), and humans (stage III) (28) (**Figure 2B**). For germ cells, as one progresses concentrically towards the seminiferous tubule lumen, this AR peak period coincides with: undifferentiated type A spermatogonia meiotic entry, elongating spermatid adhesion, and spermiation (29–32).

ENDOCRINE AND PARACRINE SIGNALS

Larose et al. 2020 (33) took a more granular look at the direct impact of AR presence in Sertoli cells on germ cell meiotic progression. Using SCARKO mutant mice (Sertoli cell androgen receptor knockout) they defined a Sertoli cell-AR androgen independent period of germ cell development from meiotic initiation to early prophase. Germ cells in these mice that did not undergo apoptosis (and many germ cells did) progressed up to what, histologically, appeared to be relatively normal pachytene spermatocytes. But upon deeper investigation using scRNA-seq, the most advanced germ cells were transcriptionally defined and resembled leptotene or zygotene spermatocytes (33). This discrepancy between transcriptomic and histological cell-identity was also reported in *Pdrm9* mutant germ cells (34). This finding calls into question the many definitive studies using models of androgen deficiency or receptor deletion causing a defined maturation arrest that predates the use of scRNA-seq technology and relied solely on classical histological assessment. Revisiting these classic maturation arrest studies with modern bioinformatics tools has the potential to elucidate other molecular details similar to those reported by 33.

Transcriptomic analysis on SCARKO mutant mice also identified a set of genes (including: *Fabp9*, *Gstm5*, *Ybx3*, *Meig1*, *Spink2*, *Rsph1*, *Aldh1a1*, *Igfbps*, *Piwil1*, *Mael*) regulated by AR signaling in Sertoli cells. Collectively this gene set seems to license spermatocytes for the first meiotic division, as well as for spermiogenic competency (33). Another gene, *Rhox5*, initially transcribed in Sertoli cells, is an androgen-inducible transcription factor (35–39). RHOX5 regulates Sertoli cell gene expression controlling cell surface and protein secretion in relation to germ cells (7, 40–43). *Rhox5* has two promoters, distal and proximal. Previously, these promoters were understood to drive different tissue-specific expression, with the exception that both promoters are active in adult Sertoli cells Bhardwaj et al. 2022 defined a postnatal temporality to *Rhox5* promoter activity (44). The proximal promoter is activated shortly after birth, while the distal promoter is dormant until late in the postnatal period also identified novel androgen-responsiveness for the *Rhox5* distal promoter. The group then established that the proximal promoter can act as an enhancer for the distal promoter and further, that RHOX5 up-regulates its own transcription *via* the distal promoter (44).

Rhox5 expression in Sertoli cells is dependent on FSH signaling (36). Unlike *Ar*, in adult mouse Sertoli cells *Fshr* has a consistent expression level throughout the stages of spermatogenesis (23) and knockout experiments have shown there is a degree of added redundancy in the FSH pathway when working synergistically with the AR pathway (45, 46). Reported activity of both proximal and distal *Rhox5* promoters into adulthood specifically in Sertoli cells at Stages II-V (outside AR peak) and VI-VIII (within AR peak) (44). Potentially, *Rhox5* is

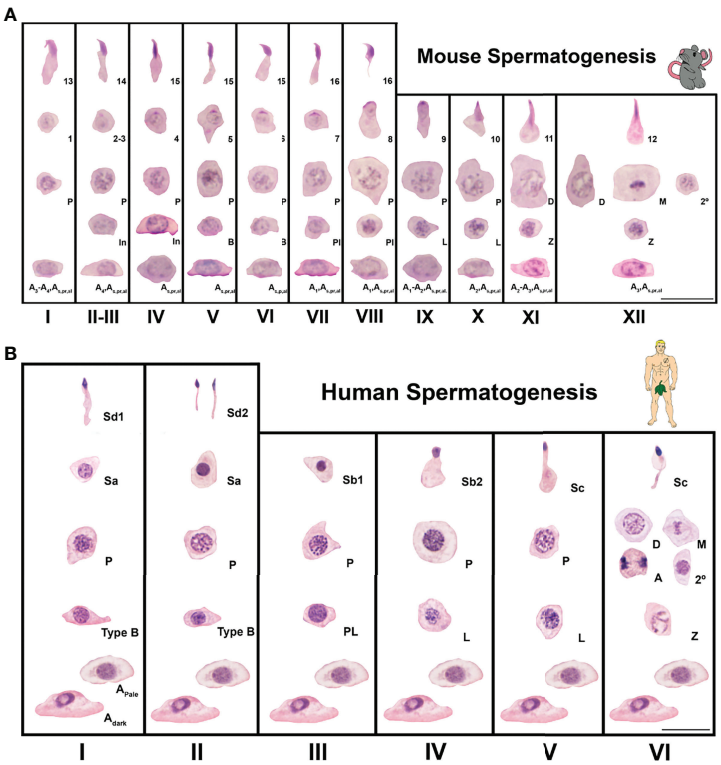

FIGURE 2 | Seminiferous epithelial stages of mouse and human spermatogenesis as classic spermatogenesis cycle staging charts using germ cell associations and morphology. Spermatogenesis is the process of sperm development and involves phases of mitosis, meiosis, and spermiogenesis (morphological cell changes). **(A)** Spermatogenesis in mice is a cycle that takes ~8.6 days (12–14). The time necessary for a germ cell to go from type A spermatogonia to spermatozoa (the complete process or duration of spermatogenesis) is about 35 days (12, 13, 15). In mice, spermatogenesis is divided into 12 stages (I-XII) and 16 spermatid developmental steps. A, In, and B are type A, intermediate, and type B spermatogonia, respectively. Pl, L, Z, P, D, M, and 2º are preleptotene, leptotene, zygotene, pachytene, diplotene, meiotic, and secondary spermatocytes, respectively. Steps of spermatid development are numbered 1-16. Sections were stained with Periodic Acid Schiff's regent-hematoxylin (PAS-H), which is a conventional staining for staging of mouse testis sections. Scale is 20µm. **(B)**. Spermatogenesis in men is a 16 day cycle with a complete duration that was classically determined to be 64 days but modern methods show to be closer to 74 days (16–21). In humans, spermatogenesis is divided into 6 stages (I-VI) and 6 spermatid developmental steps. Adark, Apale and B are type A dark, type A pale and type B spermatogonia, respectively. Pl, L, Z, P, D, M, A and 2º are preleptotene, leptotene, zygotene, pachytene, diplotene, meiotic metaphase, meiotic anaphase and secondary spermatocytes, respectively. Steps of spermatid development are labeled Sa, Sb1, Sb2, Sc, Sd1 and Sd2. Sections were stained with Periodic Acid Schiff's regent-hematoxylin (PAS-H), which is a conventional staining for human testis histology assessment. Scale is 20µm.

yet another recipient of synergistic T and FSH action. This would add another layer to the evolutionary pressure postulated by 44. According to the authors, this pressure drove retention of the *Rhox5* distal and proximal promoters. This evolutionary pressure was probably directed at the initial temporally-staggered promoter expression of *Rhox5* postnatally. During the first wave of spermatogenesis, *Ar* and *Fshr* are known to have dynamic expression patterns in mouse Sertoli cells (24, 44).

T and FSH synergism is not limited to Sertoli cell transcription factors. A newer player in the realm of intercellular signaling is the extracellular vesicle, which can hold and transport an array of different molecules including: growth factors, cytokines, mRNAs, bioactive lipids, and microRNAs (47–49). A recent report by Mancuso et al 2015 utilized a porcine Sertoli cell culture system to define the extracellular vesicle components with FSH-alone and synergistic T+FSH stimulation (50). Proteomic analysis showed FSH-alone increased proteins generally linked to modulating the hypothalamic-pituitary axis regulating testosterone biosynthesis, the blood-testis-barrier, and spermiation (INHA, INHB, PLKA, HPT, SERA and AT1A1). While stimulation (50) with T+FSH increased proteins generally linked to blood-testis-barrier adherens junctions, and gating endocrine and paracrine regulation of spermatogenesis (INHA, INHB, TPA, EGFL8, EF1G and SERA). These extracellular vesicles also contained transcripts (*Amh*, *Inhb*, *Abp*, *Fshr*), which the authors postulate could function in loading germ cells, and other testicular cells, with mRNA that will later be translated (50).

Extracellular vesicles are generally accepted to belong to 3 categories: exosomes, microvesicles, and apoptotic bodies (51, 52). Exosomes, were recently the focus of exciting findings in the field. Aside from transporting mRNA, extracellular vesicles, specifically exosomes, can also transport microRNA (53).

Paracrine signaling from Sertoli to germ cells by exosomes containing microRNA would putatively be to silence genes. Indeed, a recent report by Li et al. 2021, revealed that Sertoli exosomes contain the microRNA miR-486-5p (54). The authors used a culture system of adult Sertoli cells and P6 germ cells enriched for spermatogonial stem cells. Using this system demonstrated that Sertoli cell exosomes with miR-486-5p down-regulated spermatogonial stem cell expression of *Pten* by targeting of the Pten-3'UTR by miR-486-5p. The authors further identified that both *Stra8*. and *Sycp3* were indirectly up-regulated in spermatogonial stem cells by the decrease in repressive PTEN. Ultimately this exosome exchange would seem to be part of the differentiation signal from Sertoli cells to spermatogonia (54).

The observations of Li et al. 2021 about Sertoli cell miR-486-5p containing exosomes adds to the evolving school of thought on how undifferentiated spermatogonia enter meiosis (54). Spermatogonial differentiation and meiotic entry is established to be highly dependent on retinoic acid (RA) signaling (55, 56). The commonly proposed paracrine source of germ cell stimulating RA is Sertoli cells and spermatocytes (32, 57–60). Much like AR, RA levels in the seminiferous epithelium are also cyclic and peak at stage VIII, the same stage at which undifferentiated spermatogonia commit to meiosis (61). Timing for meiotic entry is critically important, and inherent in understanding the control of this timing is the need to define how spermatogonia control RA-responsiveness. In the fetal testis CYP26B1, which catabolizes RA, is a key regulator in blocking fetal male germ cell meiotic entry (62–65). Using the first wave of spermatogenesis as a synchronized model of spermatogenesis, Velte et al. 2019 (66) showed that CYP26 also blocks meiotic entry at postnatal day 6 (P6) in undifferentiated spermatogonia that are poised to respond to RA. Spermatogonial poising for RA responsiveness is generally thought to be accomplished through RARG (RA receptor gamma) expression (66). Indeed, this model was eloquently validated by in Suzuki et al. (67), who defined two sub-populations of undifferentiated spermatogonia in the adult mouse testis. Early-undifferentiated spermatogonia did not express RARG, while late-undifferentiated spermatogonia did express RARG (67). However deeper analysis in a follow-up study further sub-divided late-undifferentiated spermatogonia into a group expressing *Dppa3* (*Dppa3*+) and RARG that quickly transition to a differentiating spermatogonia (KIT+) state upon RA stimulation. While the other group of late-undifferentiated spermatogonia express RARG but not *Dppa3* (*Dppa3*-) and have delayed differentiation (68). Whether or not *Dppa3* transcript presence is the product of exosome-mediated microRNA silencing is still an open question.

SERTOLI CELL TRANSPLANTATION AND TRANSITIONAL ZONE SERTOLI CELL NICHE

Clinically, men can suffer from an array of Sertoli cell-origin infertility. In some cases the ligand is the issue: gonadotropin-deficient men, mutations (69) and androgen dysregulation (70). In other cases the receptor is the issue, such as complete or partial androgen insensitivity syndromes resulting from polymorphisms or deletions of the androgen receptor (71, 72). Extracellular vesicles may offer the possibility of a cell-free treatment for some forms of infertility due to specific types of Sertoli cell deficiencies. Theoretically extracellular vesicles could be injected clinically through the rete testis using the ultrasound-guided injection technique (73–76). Although these types of therapeutics are still years away, extracellular vesicles could become clinically relevant sooner due to their diagnostic potential. Two recent studies demonstrated the value of seminal exosome analysis as markers of Sertoli cell damage by varicocele (77), and predictive of testicular sperm presence in NOA men (78).

Another exciting technology that has seen a surge of progress lately is Sertoli cell transplantation. Ralph Brinster pioneered germ cell transplantation over a quarter century ago, his technique was later applied to transplant the somatic cells of the seminiferous epithelium, Sertoli cells (79). Some of the earliest reporting of Sertoli cell transplantation as a method for repairing the spermatogonial stem cell niche goes back to the early 2000's (80, 81). A challenge to restoring Sertoli cell function through transplantation of functional Sertoli cells is what to do about clearing out the dysfunctional Sertoli cells from the seminiferous epithelium to make space. Previously transgenic lines and cadmium has been used for Sertoli cell ablation (81–84). Although effective, from a clinical perspective these methods are not feasible and pose adverse risks, respectively.

Yokonishi et al. 2020 (85) recently identified a safe alternative to cadmium, benzalkonium chloride (BC), which is an FDA-approved non-toxic agent present in over-the-counter eye drops and hair conditioner (86). The authors show that admission of 0.02% benzalkonium chloride through the mouse rete testis is sufficient to ablate Sertoli cells. Further this group defines the temporal windows for host Sertoli cell ablation, donor Sertoli cell transplantation, and donor germ cell transplantation. The window for host germ cell survival is also detailed, the method is tested with cryopreserved testicular cells, and a culture version of the method demonstrates benzalkonium chloride utility in large mammals (dog) (85). In a follow-up study the same group shower that fetal mouse gonadal cells transplanted into an ablated adult mouse testis are competent to colonize, mature, and support host germ cell spermatogenesis (87). An added level of temporality in transplanted donor Sertoli cell colonization after ablation, was recently defined in another robust ablation study. Using a transgenic system of Sertoli cell ablation, Imura-Kishi et al. 2021 showed that donor Sertoli cells first colonize the transitional zone where they resume repression of spermatogenesis. After reaching an equilibrium in the transitional zone Sertoli cells then proliferate further, repopulating the host seminiferous epithelium where the donor Sertoli cells will support host spermatogenesis (88).

The transitional zone of the testis goes by many names (Sertoli valve, transitional region, tubulis rectus, intermediate region, terminal segment) expertly reviewed in (89). Foundation

papers first describing this area between the rete testis and spermatogenic seminiferous epithelium date back to the 60's (90–97). Sertoli cells in the transitional zone are morphologically distinct having long string-like cell bodies that extend distally into the rete testis, structurally giving the zone a valve appearance histologically (98). At least a sub-population of these transitional zone Sertoli cells has been documented by multiple labs to be proliferatively competent (99–103). Specifically, because some transitional zone Sertoli cells do not express the maturation markers p27, GATA4 and AR (101). AR is not just a marker for Sertoli cell maturation and proliferative cessation (104, 105). Loss of AR has been shown to inhibit Sertoli cell maturation (106). In men and rodents, germ cells that reside in this region are exclusively spermatogonia that seem to be predominantly undifferentiating spermatogonia (88, 92, 99, 107). Collectively the transitional zone represents a unique Sertoli-germ cell niche within the testis.

During their ablation experiments, Imura-Kishi et al. 2021, identified transitional zone Sertoli cell *Cyp26a1* expression that is at least partially responsible for blocking RA signaling to the spermatogonia in the transitional zone. Due to the proximity to the rete testis, the authors also showed retrograde rete derived FGF signaling may also competitively inhibit RA signal in the transitional zone (88). A separate recent report defined two sub-populations of transitional zone Sertoli cells that were KRT8+, DMRT1- or KRT8+,DMRT1+ (108). DMRT1 is essential in differentiation of Sertoli cells into a non-proliferative state (109). These studies elucidated the molecular uniqueness of the transitional zone niche, but there is still much we do not understand about cell identity and function in the transitional zone. Given the recent reports on exosomes, one cannot help but wonder if there is also a unique population of transitional zone Sertoli cell extracellular vesicles that are part of maintaining this niche.

DISCUSSION

Ablation and transplantation are done *via* injection through the rete testis (110). Even when done by the most skilled pair of hands, this represents a traumatic event to the surrounding tissue. The plasticity of the Sertoli cell population in the transitional zone and the robustness of this epithelium is a fortunate coincidence for this method, but also represents an intriguing source for discoveries in reversing Sertoli cell dysfunction and repopulating a Sertoli cell deficient testis. Sertoli cells in human testes partially resume proliferation after gonadotropin suppression with coincident reduction of AR (111). Continued research into maintenance and control of proliferative transitional zone Sertoli cells in conjunction with Sertoli cell transplantation has the potential to unlock new therapeutics for treatment of Sertoli cell based male infertility, and reversing the reproductive harm done by gonadotoxic cancer treatment.

AUTHOR CONTRIBUTIONS

VAR generated the direction for the manuscript, and produced the figures. DJL supervised the creative process providing expert feedback and insight. VAR and DJL wrote the manuscript and reviewed the manuscript. All authors contributed to the article and approved the submitted version.

ACKNOWLEDGMENTS

Figure 1 is from unpublished immunofluorescent imaging done by VAR while training under Dr. Blanche Capel, Duke University. **Figure 2** is from unpublished light microscopy imaging done by VAR while training under Dr. Monika A Ward, University of Hawai'i at Mānoa. All imaging processing and final layouts were done by VAR in FIJI (version 2.3.0/1.53f) (112) and Adobe Photoshop (version 23.1.0). Support provided by the National Institute of General Medical Sciences (F32GM129956 to VAR); the Frederick J. and Theresa Dow Wallace Fund of the New York Community Trust (DJL); the National Institute of Diabete and Digestive and Kidney Diseases (1R01DK078121 to DJL); the Small Business Innovation Research fund (1R43HD108826-01 to Inherent Bio and DJL); the Eunice Kennedy Shriver National Institute of Child Health and Human Development (1P50HD106793-01 to The Population Council Inc and DJL); and grants 1P50HD100549-01 (L. Levin), and 5P01HD087157 (M.M. Matzuk) from the Eunice Kennedy Shriver National Institute of Child Health and Human Development of the National Institutes of Health (DJL).

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Conflict of Interest: DJL serves on the Ro advisory board, and as a consultant, and has equity; and for Fellow has equity; and serves as Secretary-Treasurer for the American Board of Bioanalysts with honorarium.

The remaining author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers | Frontiers in Endocrinology

REVIEW
published: 04 May 2022
doi: 10.3389/fendo.2022.876370

Proteolysis in Reproduction: Lessons From Gene-Modified Organism Studies

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The physiological roles of proteolysis are not limited to degrading unnecessary proteins. Proteolysis plays pivotal roles in various biological processes through cleaving peptide bonds to activate and inactivate proteins including enzymes, transcription factors, and receptors. As a wide range of cellular processes is regulated by proteolysis, abnormalities or dysregulation of such proteolytic processes therefore often cause diseases. Recent genetic studies have clarified the inclusion of proteases and protease inhibitors in various reproductive processes such as development of gonads, generation and activation of gametes, and physical interaction between gametes in various species including yeast, animals, and plants. Such studies not only clarify proteolysis-related factors but the biological processes regulated by proteolysis for successful reproduction. Here the physiological roles of proteases and proteolysis in reproduction will be reviewed based on findings using gene-modified organisms.

Keywords: protease, fertilization, proteolysis, protease inhibitor, pseudoprotease, gene-modified animal models, ubiquitin-proteasome system, sperm maturation

OPEN ACCESS

Edited by:
Erwin Goldberg, Northwestern University, United States

Reviewed by:
Toshinobu Tokumoto, Shizuoka University, Japan
Martine Culty, University of Southern California, United States

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Specialty section:
This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology

Received: *15 February 2022*
Accepted: *28 March 2022*
Published: *04 May 2022*

Citation:
Kiyozumi D and Ikawa M (2022) Proteolysis in Reproduction: Lessons From Gene-Modified Organism Studies. Front. Endocrinol. 13:876370. doi: 10.3389/fendo.2022.876370

INTRODUCTION

Although a simple peptide bond between two amino acids in water at room temperature has a half-life of several years (1), the hydrolysis of a peptide bond is significantly accelerated under the presence of proteases. As well as mediating non-specific protein hydrolysis, proteases also act as processing enzymes that perform highly selective, limited, and efficient cleavage of specific substrates. As many biological processes are influenced by this irreversible post-translational protein modification, dysregulation of the expression and/or function of proteases underlie many human pathological processes and have therefore been an intensely studied class of targets for drug discovery.

Abbreviations: ACE, angiotensin converting enzyme; ADAM, a disintegrin-like and metalloproteinase domain; ADAMTS, a disintegrin-like and metalloproteinase domain with thrombospondin type 1 motif; CSN, constitutive photomorphogenic-9 signalosome; EMS, ethylmethane-sulfonate; GGT, glutamyltranspeptidase; IαI, inter-α-trypsin inhibitor; KI, knock-in; KO, knockout; OVCH2, ovochymase 2; S-Lap, sperm-Leucylaminopeptidase; SUMO, small ubiquitin-related modifier; TASP1, threonine aspartase 1; TMP,trimethylpsoralen; TNFα, tumor necrosis factor-α; UPS, ubiquitin-proteasome system; USP, ubiquitin-specific protease.

By searching *Saccharomyces cerevisiae*, *Drosophila melanogaster*, and *Caenorhabditis elegans* genome databases with a gene ontology term "peptidase activity" (GO:0008233), 51, 506, and 448 genes encoding proteases, respectively, can be identified (2–4). In the mouse and human genome, 628 and 553 protease genes exist, respectively (5). In *Arabidopsis thaliana*, 723 protease genes were reported (6). Based on catalytic mechanisms, proteases can be divided into five classes: cysteine proteases, serine proteases, metalloproteases, threonine proteases, and aspartic proteases. After activation of the amide, cysteine, serine, and threonine proteases utilize the namesake residue to attack the amide carbonyl group, whereas metalloproteases and aspartic proteases use an activated water molecule as a nucleophile. As proteases bind their substrates between the substrate side chains and well-defined substrate-binding pockets within the active site, they have their own preference for substrate amino acid sequence proximal to the cleavage site (7). There are some enzymatically inactive pseudoproteases encoded in the mammalian genome in which the amino acid residues indispensable for catalytic activity are substituted. As proteases are potentially toxic, their activities are strictly regulated as such by pH, specific ion concentrations, posttranslational modifications, and spatiotemporal expression of protease inhibitors.

The contribution of proteases depends on their intracellular or extracellular localization where they act on substrate proteins. The ubiquitin-proteasome system (UPS) is a complex but sophisticated intracellular proteolytic system in eukaryotes; this complex system degrades unneeded or damaged proteins by proteolysis. When target proteins are post-translationally labeled with ubiquitin, a protein of 76-amino acid residues exhibiting high sequence conservation among eukaryotes, they will be recognized and degraded by the proteasome.

Proteolytic processing events are fundamental in reproductive processes including gametogenesis, fertilization, and embryonic development. Recent advances in generating gene-modified animals have identified many proteases and their regulators associated with reproduction in various species including yeast, invertebrates, vertebrates, and plants. In the following sections the physiological importance of proteolysis in reproduction will be overviewed based on findings obtained by gene-modified organism studies. Proteolysis-related genes essential in reproduction identified by gene-modified animal studies are listed in **Table 1**. Few proteins are known to be proteolytically processed under certain reproductive situations. They are, however, not included in this review as the physiological roles of such processing in reproduction are not fully clarified at present.

UNICELLULAR ORGANISMS

Saccharomyces cerevisiae

S. cerevisiae, Baker's yeast, is a model diploid unicellular organism. *S. cerevisiae* can stably exist as either a diploid or a haploid. When stressed, *S. cerevisiae* can undergo meiosis to produce four haploid spores. Haploid cells are capable of fusing with other haploid cells of the opposite mating type (an 'a' cell can only mate with an 'α' cell, and vice versa) to produce a stable diploid cell. a and α cells produce mating peptide pheromones a-factor and α-factor, respectively. Ste24p and Axl1p encoded by *ste24* and *alx1*, respectively, are metalloendopeptidases that process precursor peptide to produce mature mating a-factor pheromone (8, 9).

MULTICELLULAR ORGANISMS I: INVERTEBRATES

The body of multicellular organisms consists of two types of cells with different lineages, i.e., germ cells and somatic cells. Germ cells produce gametes for fertilization, whereas somatic cells develop reproductive organs to support gametogenesis and fertilization by germ cells. Therefore, dysfunction of proteolysis in either cell lineage can result in fertility defects.

Nematodes

Caenorhabditis elegans is androdioecious; i.e., it has two sexes, hermaphrodite and male, whereas *Ascaris suum* is dioecious, being either male or female. They develop two U-shaped gonads in which gametes are generated and fertilization occurs. Several proteases and inhibitors have been identified to regulate nematode reproductive processes.

Oogenesis and fertilization are affected when *cpi-2a*, encoding a cystatin-like cysteine protease inhibitor, is mutated (10). Nullification of *dss-1* encoding a 26S proteasome subunit provokes sterility because of deficient oogenesis (14). Knockdown of puromycin-sensitive aminopeptidase encoded by *pam-1* causes delayed oocyte maturation and subfertility (17). Deletion of *dpf-3* encoding a serine protease causes sterility because of impaired spermatogenesis (15). *gon-1* encoding a disintegrin-like and metalloproteinase domain with thrombospondin type 1 motif (ADAMTS) is necessary for morphogenesis of U-shaped gonads (11, 12). A mutant worm lacking *timp-1* encoding a tissue inhibitor of metalloproteinase also shows deficient gonadal development (13). A double mutant in which *sup-17* and *adm-4*, encoding nematode orthologs of mammalian membrane metalloproteases ADAM10 and ADAM17, respectively, are sterile because of aberrant spermathecal function (16).

Unlike mammalian flagellated sperm, nematode sperm are amoeboid cells. For successful fertilization, sperm must be activated prior to contacting an oocyte in both *C. elegans* and *A. suum*. This sperm activation is called spermiogenesis through which round immobile spermatids transform into motile, fertilization-competent spermatozoa. Mechanistically, spermiogenesis occurs by sensing extracellular signals and can be reproduced *in vitro* by exposing spermatids to proteases such as Pronase and proteinase K. A trypsin-like secreted protease encoded by *try-5* is expressed in the vas deferens and triggers activation of spermatids (18). *swm-1* encodes a secreted protein with a trypsin inhibitor-like domain, and *swm-1* mutant males

TABLE 1 | Proteolysis-related genes associated with reproduction.

Gene	Protein feature	Protein localization	Gene-modified organism	Fertility	Phenotype	Refs.
S. cerevisiae						
ste24	Prenyl protein-specific endoprotease	Intracellular membrane	Ethylmethane-sulfonate (EMS) mutagenesis	Sterile	MAT a-specific sterility.	(8)
axl1	Metalloprotease	Intracellular	UV exposure	Sterile	Defect in a-factor pheromone secretion.	(9)
C. elegans						
cpi-2a	Cystatin-like cysteine protease inhibitor	Extracellular	Deletion mutant	Sterile	Oocyte-specific sterility.	(10)
gon-1	Metalloprotease	Extracellular	EMS mutagenesis	Sterile	Gonadal developmental defect.	(11, 12)
timp-1	Metalloprotease inhibitor	Extracellular	Trimethylpsoralen (TMP)–UV-mutagenesis	Sterile	Gonadal growth defect.	(13)
dss-1	26S proteasome subunit	Intracellular	Deletion mutant	Sterile	Defects in oogenesis.	(14)
dpf-3	Serine protease	Intracellular	Deletion mutant	Sterile	Impaired spermatogenesis.	(15)
sup-17; *adm-4*	ADAM metalloproteases	Cell membrane	EMS mutagenesis; TMP)–UV-mutagenesis	Sterile	Aberrant spermathecal function.	(16)
pam-1	Metalloprotease	Intracellular	RNAi knockdown	Subfertility	Decreased brood size. Expanded pachytene.	(17)
try-5	Serine protease	Extracellular	Deletion mutant	Fertile	*try-5* functions in parallel to *spe-8* for male fertility.	(18)
swm-1	Trypsin inhibitor-like	Extracellular	EMS mutagenesis	Reduced male fertility	Ectopic sperm activation within the male reproductive tract. Failure of sperm transfer to hermaphrodite.	(19)
gcna-1	Metalloprotease	Nucleus	Deletion by CRISPR/Cas9	Fertility defects	Decrease of fertility in later generations because of genomic instability	(20)
T12E12.6	Metalloprotease	Intracellular	RNAi knockdown	Subfertility	Decreased brood size.	(17)
zmp-2	Metalloprotease	Extracellular	RNAi knockdown	Subfertility	Reduced offspring production.	(21)
D. melanogaster						
CG9000; *CG9001*; *CG9002*	Yeast ste24p ortholog proteases	Intracellular membrane	Ends-out gene targeting	Male fertility defects	Abnormal spermatid maturation.	(22)
Prosalpha6T	Proteasome subunit	Intracellular	KO	Male infertility	Spermatogenic defects in sperm individualization and nuclear maturation.	(23)
Duba	Deubiquitylating enzyme	Intracellular	Imprecise P-element excision	Male infertility	Defects in spermatid individualization.	(24)
Dronc	Cysteine protease	Intracellular	Transgenic expression of dominant-negative DRONC	Uncertain	Defects in spermatid individualization.	(25)
Dredd	Cysteine protease	Intracellular	EMS mutagenesis	Fertile	Defects in spermatid individualization.	(25)
Dark	Caspase activator	Intracellular	Enhancer trap	Male infertility	Defects in spermatid individualization.	(25)
Htra2	Serine protease	Mitochondria	P element mobilization	Male infertility	Sperm were completely immotile	(26)
			EMS mutagenesis	Male infertility	Defective spermatogenesis.	(27)
S-Lap1-8	Leucylamino-peptidase	Intracellular	Classical mutant, CRISPR/Cas9	Male infertility or subfertility	Deficient accumulation of paracrystalline material in mitochondria.	(28)
Sems	Trypsin-like protease	Extracellular	Knockdown	Male subfertility	Females laid fewer number of eggs when mated to *Sems* knockdown males. Sperm remained in storage in the seminal receptacle.	(29)
Nep4	Metalloprotease *Mmel1* ortholog	Cell membrane	KO	Male infertility	Mutant sperm are quickly discarded by females.	(30)
Dcp-1	Cysteine protease	Intracellular	Female carrying germline KO clone	Female infertility	Defective oogenesis.	(31)

(Continued)

TABLE 1 | Continued

Gene	Protein feature	Protein localization	Gene-modified organism	Fertility	Phenotype	Refs.
mh	Metalloprotease	Nucleus	EMS mutagenesis, P element mobilization	Female infertility	The integration of paternal chromosomes in the zygote was specifically affected.	(32, 33)
Ance	Angiotensin-converting enzyme	Extracellular	EMS mutagenesis	Male infertility	Compound heterozygotes for two different lethal alleles are male sterile.	(34)
Slfc	Serine protease	Extracellular	RNAi knockdown	Male infertility	Details are unknown. Females also show slightly decreased fertility.	(35)
ome	Serine protease	Cell membrane	EMS mutagenesis	Male subfertility	Details are uncertain.	(36)
Mmp2	Metalloprotease	Extracellular	RNAi	Female subfertility	Ovulation was blocked.	(37)
A. socius						
ejac-sp	Serine protease	Extracellular	RNAi knockdown	Male subfertility	Reduced ability to induce a female to lay eggs.	(38)
Bombyx mori						
Osp	Serine protease	Cell membrane	KO	Female infertility	Mutant females laid fewer eggs than wild-type females and eggs did not hatch	(39)
Ser2	Serine protease	Extracellular	KO	Male infertility	Wild-type females mated with mutant males laid eggs normally but the eggs did not hatch.	(40)
Spodoptera litura						
Osp	Serine protease	Cell membrane	KO	Female infertility	Mutant females laid fewer eggs than wild-type females and eggs did not hatch.	(39)
Plutella xylostella						
Ser2	Serine protease	Extracellular	KO	Male infertility	Mutant sperm morphology is normal but they do not enter eggs.	(40)
Hyphantria cunea						
Hcser2	Serine protease	Extracellular	RNAi knockdown, KO	Male infertility	The growth, development, mating behavior, or egg laying was not affected.	(41)
Bactrocera dorsalis						
Bdcp-1	Cysteine protease	Intracellular	RNAi knockdown	Female infertility	Impaired ovary development.	(42)
M. musculus						
Psma8	Proteasome component	Nucleus	KO	Male infertility	Arrested spermatogenesis at spermatocyte stage.	(43)
Psme3	Proteasome activator	Intracellular	KO	Male subfertility	Decreased sperm number and motility.	(44)
Psme4	Proteasome activator	Nucleus	KO	Severe male subfertility	Defective spermatogenesis.	(45)
Psme3; *Psme4*	Proteasome activator	Intracellular	Double KO	Male infertility	Morphologically normal sperm with motility defect.	(46)
Cops5	Metalloprotease	Intracellular	KO	Male infertility	Male infertility. Germ cells undergo significant apoptosis at a premeiotic stage.	(47)
Usp2	Ubiquitin-specific protease	Nucleus	KO	Male subfertility	Defects in sperm motility.	(48)
Usp9x	Ubiquitin-specific protease	cytoplasm	*Vasa-cre*; $Usp9x^{fl/Y}$	Male infertility	Apoptosis of spermatocytes.	(49)
Usp26	Ubiquitin-specific protease	Intracellular	KO	Severe male subfertility	Unsynapsed chromosomes in pachynema and defective chiasma formation in diplonema, apoptosis of metaphase spermatocytes and decrease of spermatids.	(50, 51)
Usp1	Ubiquitin-specific protease	Nucleus	KO	Male infertility	Impaired spermatogenesis.	(52)
Apaf1	Caspase activator	Intracellular	KO	Male infertility	Degeneration of spermatogonia resulting in the absence of sperm.	(53)
Agbl5	Metalloprotease	Intracellular	KO	Male infertility	Defective spermatogenesis	(54, 55)
Gcna	Metalloprotease	Nucleus	KO	Male infertility	Nearly devoid of sperm.	(56)
Tasp1	Endopeptidase	Nucleus	KO	Male infertility	Release immature germ cells.	(57)
Tysnd1	Serine protease	Peroxisome	KO	Male infertility	Globozoospermia, no acrosomal cap.	(58)
Spink2	Serine protease inhibitor	Extracellular	KO	Male infertility	Oligoasthenoteratozoospermia in heterozygotes, azoospermia in homozygotes.	(59)

(Continued)

TABLE 1 | Continued

Gene	Protein feature	Protein localization	Gene-modified organism	Fertility	Phenotype	Refs.
Serpina5	Serine protease inhibitor	Extracellular	KO	Male infertility	Abnormal spermatogenesis due to destruction of the Sertoli cell barrier.	(60)
Adamts2	Metalloproteinase	Extracellular	KO	Male infertility	Marked decrease in testicular sperm.	(61)
Acr	Serine protease	Acrosome	KO	Male subfertility	Delayed fertilization.	(62, 63)
Pcsk4	Serine protease	Acrosomal membrane	KO	Male subfertility	Putatively due to impaired fertilization.	(64, 65)
Tmprss12	Serine protease	Plasma membrane	KO	Male infertility	Deficient sperm migration into oviduct.	(66)
Prss55	Serine protease	Plasma membrane	KO	Male infertility	Deficient sperm migration into oviduct.	(67, 68)
Tryx5	Serine protease	Plasma membrane	KO	Male infertility	Deficient sperm migration into oviduct.	(69)
Prss37	Pseudoprotease	Plasma membrane	KO	Male infertility	Deficient sperm migration into oviduct.	(70)
Ace	Metallo-carboxypeptidase	Plasma membrane	KO	Male subfertility	Deficient sperm migration into oviduct.	(71)
Adam1a	Pseudoprotease	Plasma membrane	KO	Male infertility	Deficient sperm migration into oviduct.	(72)
Adam2	Pseudoprotease	Plasma membrane	KO	Male subfertility	Deficient sperm migration into oviduct	(73)
Adam3	Pseudoprotease	Plasma membrane	KO	Male infertility	Deficient sperm migration into oviduct.	(74, 75)
Adam6	Pseudoprotease	Plasma membrane	KO	Male infertility	Deficient sperm migration into oviduct.	(76)
Cst8; *Cst9*; *Cst11*; *Cst12*; *Cst13*; *Cstdc1*; *Cstdc2*; *Cstl1*	Cystatin-like inhibitor	Extracellular	Multiple KO	Male infertility	Deficient sperm migration into oviduct.	(77)
Ovch2	Serine protease	Extracellular	KO	Male infertility	Deficient sperm migration into oviduct.	(78)
Mmel1	Metalloprotease	Extracellular	KO	Male infertility	Normal spermatogenesis but reduced egg fertilization.	(79)
Prss21	Serine protease	Plasma membrane	KO	Male subfertility, decreased *in vitro* fertility	Mutant spermatozoa possessed decreased motility, angulated and curled tails, and fragile necks. Decreased *in vitro* zona pellucida binding and acrosome reaction.	(80, 81)
Cpe	Metalloprotease	Extracellular	Spontaneous mutation	Male subfertility	Abnormal sexual behavior. Abnormal testis morphology in older mutant males.	(82)
Adam24	Pseudoprotease	Plasma membrane	KO	Male subfertility	Polyspermic fertilization.	(83)
Adam7	Pseudoprotease	Plasma membrane	KO	Male subfertility	Decreased cell height in caput epididymis, spermatic granuloma, kinked sperm flagellum and reduced sperm motility.	(84)
Cst3	Cysteine protease inhibitor	Extracellular	KI (Leu68Gln)	Male subfertility	Reduced viability of spermatozoa and large agglutinated clumps.	(85)
Serpine2	Serine protease inhibitor	Extracellular	KO	Male subfertility	Inadequate semen coagulation and deficient vaginal plug formation upon copulation	(86)
Tmprss6	Serine protease	Plasma membrane	KO	Female infertility	Marked retardation in ovarian maturation.	(87)
Ambp	Serine protease inhibitor	Extracellular	KO	Female subfertility	Defective cumulus matrix expansion.	(88, 89)
Psen1	Aspartic protease	Endoplasmic reticulum, Golgi, endosome, plasma membrane	KI (Leu166Pro)	Female infertility	Primordial follicles near the ovarian cortex and consisting largely of ovarian stromal elements.	(90)
Adamts1	Metalloprotease	Extracellular	KO	Female subfertility	Fewer numbers of mature follicles in ovary, thick and convoluted uterus.	(91, 92)
Lonp	Serine protease	Mitochondria	*Gdf9-cre* or *Zp3-cre*; *Lonp1*$^{fl/fl}$	Female infertility	Impaired follicular development, progressive oocyte death, ovarian reserve loss.	(93)
Furin	Serine protease	Golgi, endosome, plasma membrane, extracellular	*Gdf9-cre* or *Zp3-cre*; *Furin*$^{fl/fl}$	Female infertility	Arrest of early secondary follicles.	(94)
Pappa	Metalloprotease	Extracellular	KO	Female subfertility	Reduced litter size and reduced ovulatory capacity, probably because of decreased bioavailability of ovarian insulin-like growth factor.	(95)
Astl	Metalloprotease	Extracellular	KO	Female subfertility	No ZP2 cleavage after fertilization.	(96)

(Continued)

TABLE 1 | Continued

Gene	Protein feature	Protein localization	Gene-modified organism	Fertility	Phenotype	Refs.
Fetub	Metalloprotease inhibitor	Extracellular	KO	Female infertility	Premature zona pellucida hardening.	(97)
Serpinc1	Serine protease inhibitor	Extracellular	KI (Arg48Cys)	Female subfertility	Thrombosis in placenta and penile vessels.	(98)
Adam10	Metalloprotease	Cell membrane	*Tie2-cre*; *Adam10*$^{fl/fl}$	Female subfertility	Impaired decidualization.	(99)
Adamts18	Metalloprotease	Extracellular	KO	Female infertility or subfertility	Fifty percent of mutant females are infertile because of vaginal obstruction due to either a dorsoventral vaginal septum or imperforate vagina.	(100)
Plg	Serine protease	Extracellular	KO	Female subfertility	Compromised female fertility.	(101, 102)
Timp1	Metalloprotease inhibitor	Extracellular	KO	Female subfertility	Reduction in reproductive lifespan.	(103)
Pcsk2	Serine protease	Extracellular	KO	Female subfertility	Details are uncertain.	(104)
Espl1	Cysteine protease	Nucleus	KI *Meox2*^{cre}; *Espl1*$^{+/S1121A}$	Male infertility	Spermatogonia cell depletion.	(105)
			Zp3-cre; *Espl1*$^{fl/fl}$	Female infertility	Prevention of chiasmata resolution. Failure to extrude polar bodies in Meiosis I.	(106)
			Meox2^{cre}; *Espl1*$^{+/S1121A}$	Female infertility	Primordial germ cell depletion by apoptosis during embryonic oogenesis.	(105, 107)
			Zp3-cre; KI (Ser1121Ala)	Female infertility	Failure in preimplantation development.	(108)
Agtpbp1	Metalloprotease	Intracellular	Spontaneous mutation, insertional mutation	Male infertility	Defective spermatogenesis.	(109–112)
				Female subfertility	Poor development of secondary follicles into antral follicles.	(113)
Clpp	Serine protease	Mitochondria	KO	Male infertility	Disrupted spermatogenesis at the spermatid stage.	(114)
				Female infertility	Ovarian follicular differentiation failure, premature reproductive aging.	(114)
Npepps	Metallo-aminopeptidase	Nucleus, cytosol	Gene trap	Male infertility	Lack of copulatory behavior, impaired spermatogenesis.	(115)
				Female infertility	Impaired formation of corpus luteum in pregnancy.	(116)
Ggt1	Protease	Plasma membrane	KO	Male infertility	Reduced testis and seminal vesicle size, reduced seminiferous tubule diameter.	(117)
				Female infertility	Hypogonadal, absence of antral follicles and corpora lutea and follicular degeneration.	(117)
Immp2l	Serine protease	Mitochondria	KO	Severe male subfertility	Erectile dysfunction.	(118)
				Female infertility	Defective folliculogenesis and ovulation.	(118)
Adam17	Metalloprotease	Extracellular	*Sox9-cre*; *Adam17*$^{fl/fl}$	Male subfertility	Details are uncertain.	(119)
				Female infertility	Details are uncertain.	(119)
Mesocricetus auratus						
Acr	Serine protease	Acrosome	KO	Male infertility	Sperm failure in zona pellucida penetration.	(120)
R. norvegicus						
Adamts16	Metalloprotease	Extracellular	KO	Male infertility	Cryptorchidism.	(121, 122)
D. rerio						
adamts9	Metalloprotease	Extracellular	KO	Female infertility	Ovary malformation.	(123)
H. sapiens						
SPINK2	Serine protease inhibitor	Extracellular	Spontaneous mutation	Male infertility	Azoospermia.	(59)

(Continued)

TABLE 1 | Continued

Gene	Protein feature	Protein localization	Gene-modified organism	Fertility	Phenotype	Refs.
GCNA	Metalloprotease	Nucleus	Spontaneous mutation	Male infertility	Non-obstructive azoospermia and cryptoospermia.	(124, 125)
A. thaliana						
A36	Aspartic protease	Plasma membrane	T-DNA insertion	Decreased male transmission	Reduced pollen germination.	(126)
A36; *A39*	Aspartic protease	Plasma membrane	Double KO by T-DNA insertion	Severely compromised male transmission	Programmed cell death of microspores. Compromised micropylar guidance of pollen tubes.	(126)
PCS1	Aspartic protease	Endoplasmic reticulum	T-DNA insertion	Reduced male and female transmission	Degeneration of both male and female gametophytes.	(127)
UND	Aspartic protease	Mitochondria	siRNA and artificial microRNA	Partial male sterility	Apoptosis-like programmed cell death in tapetum and pollen.	(128)
CEP1	Cysteine protease	Vacuole, endoplasmic reticulum	T-DNA insertion	Male subfertility	Mutants exhibited aborted tapetal PCD and decreased pollen fertility with abnormal pollen exine.	(129)
SPF1; *SPF2*	SUMO-specific cysteine protease		Double KO by T-DNA insertion	Male and female sterility	Severe abnormalities in microgametogenesis, megagametogenesis, and embryo development.	(130)
O. sativa						
OsAP65	Aspartic protease	Vacuole	T-DNA insertion	Male sterility	No germination or elongation of mutant pollen.	(131)

are infertile because of ectopic premature activation of sperm (19). Like in *C. elegans*, activation of spermatozoa by exposure to extrinsic protease *in vitro* can also be seen in several insect species (132, 133). *spe-4* encoding a presenilin, an aspartyl protease with intramembrane proteolytic activity prevents spermatid activation because *spe-4* mutant males progress directly to functional spermatozoa without the need for an activation signal (134).

gcna-1 encodes nuclear metalloprotease. *gcna-1* deletion causes genomic instability decreasing fertility in later generations (20). *T12E12.6* encodes intracellular metalloprotease whereas *zmp-2* encodes secreted metalloproteases. Knockdown of either of them results in reduced offspring production (17, 21).

Insects

The reproductive system of *Drosophila melanogaster* is more complex compared with nematodes; it is composed of gonads, genital ducts, and accessory structures. Several proteases have been implicated in *D. melanogaster* spermatogenesis. In the *D. melanogaster* genome, there are five genes paralogous to *S. cerevisiae ste24* encoding a type I prenyl protease. Deletion of three tandemly arrayed *ste24* paralogs results in male fertility defects manifesting late in spermatogenesis (22).

All *Drosophila* spermatid nuclei descended from a primary spermatocyte remain connected to each other *via* an extensive network of cytoplasmic bridges. Spermatids should therefore be physically dissociated from each other by a process referred as individualization and a ubiquitin-proteasome system regulates this process. Males in which *Prosalpha6T* encoding a testis-specific proteasome core particle subunit was ablated are sterile because of defects in sperm individualization and nuclear maturation (23). *Duba* encodes a deubiquitylating enzyme and *Duba* null mutants are male sterile and display defects in spermatid individualization (24). The non-apoptotic function of caspases also contributes to individualization. DARK is a *Drosophila* homolog of mammalian caspase activator Apaf-1, whereas DRONC and DREDD are *Drosophila* apical caspases. Flies deficient in DARK or expressing a dominant-negative version of DRONC failed individualization (25, 135). *Dredd*-null flies also often show individualization defects (25).

In *D. melanogaster* sperm, mitochondrial derivatives run along the entire flagellum to provide structural rigidity for flagellar movement. Two mitochondrial derivatives (i.e., major and minor) differentiate and major one accumulates paracrystalline material by the end of spermatogenesis. S-Lap1-8, Sperm-Leucylaminopeptidase (S-Lap) family members are constituents of paracrystalline material. S-Lap mutants possess defects in paracrystalline material accumulation and abnormal structure of the elongated major mitochondrial derivatives and male sterility (28). *Htra2* encodes a mitochondrial serine protease. In one *Htra2*-null mutant line males are infertile because sperm are completely immotile (26), whereas spermatogenesis is defective in another *Htra2* mutant line (27).

Seminal fluid produced in the accessory gland includes proteases and protease inhibitors and is thought to contribute to fertilization in a post-mating manner. Seminase is a trypsin-like protease encoded by *Sems* and included in seminal fluid. When females mated with *Sems* knockdown males, they laid significantly fewer eggs (29). In cricket, *Allonemobius socius*, an ejaculate serine protease encoded by *ejac-sp* is expressed in male reproductive accessory glands. RNAi knockdown of *ejac-sp* resulted in a significant reduction of the male's ability to induce a female to lay eggs (38). *Nep4*, a drosophila ortholog of mammalian *Mmel1*, encodes a metalloprotease expressed in male gonads (136). *Nep4* mutant males are infertile; mutant sperm are quickly discarded by females (30). When *Dcp-1*

encoding a cysteine protease was ablated in their germline, the resulting females were infertile because of defective oogenesis (31).

Several proteases also of concern in *Drosophila* reproduction include *maternal haploid* or *mh* encodes the *Drosophila* homolog of SPRTN, a conserved metalloprotease essential for resolving DNA–protein cross-linked products. Paternal chromatids of *mh* mutants are unable to separate in the anaphase of the first embryonic mitosis and form a chromatin bridge. As a consequence, haploid nuclei of maternal origin rapidly separate from the damaged paternal chromosomes and haploid embryos develop but become lethal in a maternal effect manner (32, 33, 137). *Ance* encodes a putative homologue of mammalian angiotensin-converting enzyme (ACE). Compound heterozygote for two different *Ance* lethal alleles exhibit male sterility (34), but the molecular details are unknown. RNAi knockdown of *Slfc* encoding a secreted serine protease causes male infertility (35). When a membrane serine protease encoded by *ome* was mutated, males became subfertile (36). RNAi knockdown of a secreted metalloprotease encoded by *Mmp2* caused female subfertility because ovulation was blocked (37).

Several pest control attempts target reproduction-associated proteases. In pests *Spodoptera litura* and *Plutella xylostella*, targeted inactivation of serine protease genes *Osp* and *Ser2*, respectively, resulted in female and male infertility as also observed in silkworm moth *Bombyx mori* (39, 40). In other pests *Hyphantria cunea*, and *Bactrocera dorsalis*, RNAi knockdown of *Hcser2*, and *Bdcp-1* encoding serine protease and cysteine protease, respectively, also resulted in infertility (41, 42). Thus, proteases are potential targets for pest population control.

MULTICELLULAR ORGANISMS II: VERTEBRATES

Findings in vertebrates were obtained by genetic studies in rodents, fish, and human patients. Genes disrupted in these species include those encoding proteases, protease inhibitors, and non-catalytically active pseudo-proteases. Proteolysis-related factors are included in various aspects of male and female reproductive processes such as gamete production, gamete maturation, fertilization, post-fertilization events, and mating behavior.

UPS in Gamete Production

For the fine-tuning of cellular processes, intracellular proteins are timely degraded by UPS. The proteasome localizes in the nucleus and cytoplasm where it degrades ubiquitylated proteins. Spermatoproteasome, a testis-specific proteasome, is one of the three tissue-specific proteasomes identified together with the immunoproteasome and the thymoproteasome in mammals (138). Deletion of *Psma8*, which encodes a testis-specific 20S proteasome component, leads to spermatogenesis arrest at the spermatocyte stage (43). *Psme3* encodes REGγ, a proteasome activator. *Psme3*-null males are subfertile with decreased sperm number and motility (44). This is probable because REGγ regulates p53-mediated transcription of *Plzf*, a transcription factor necessary for spermatogonial stem cell self-renewal and proliferation (139). *Psme4* encodes PA200 proteasome activator. *Psme4*-null males have reduced fertility due to defects in meiotic spermatocytes and post-meiotic spermatids (45). *Psme3*;*Psme4* double KO males were infertile; mutant sperm appeared morphologically normal but exhibited remarkable defects in motility and decreased proteasome activity (46).

Proteasome target proteins are ubiquitylated by E3 ubiquitin ligases which transfer the ubiquityl group from E2 ligase to the target protein. There are ∼600 E3 ligases encoded in the mammalian genome (140). The ubiquitin ligases, which are not proteases but included in ubiquitin-proteasome system-mediated protein degradation, indispensable for mammalian reproduction are listed in **Table 2**. Here only Huwe1 is mentioned as how E3 ligases function in reproductive processes. Huwe1 ubiquitylates histone H2AX, which is phosphorylated in response to DNA damage and is essential to the efficient recognition and repair of DNA double-strand breaks. Germline-specific *Huwe1* ablation increased histone H2AX level, elevated DNA damage response, and caused Sertoli cell only phenotype. Thus Huwe1 likely regulates the response to spontaneous DNA damage by UPS-mediated H2AX degradation to maintain cell survival (156).

Cullin-RING E3 ubiquitin ligases are known to be reversibly neddylated, i.e., conjugated with NEDD8, a ubiquitin-like protein. By conjugation with NEDD8, cullin-RING E3 ligases increase their stability and ligase activity. The constitutive photomorphogenic-9 signalosome (CSN) deneddylates cullin-RING E3 ligases by cleaving the isopeptide bond of neddylated lysine to regulate the cellular ubiquitylation status. COPS5 is the fifth component of the CSN and abundant in mouse testis (185). *Cops5*-null males were infertile because of significant reduction of sperm number caused by premeiotic apoptosis of germ cells (47).

Ubiquitylated proteins can be deubiquitylated by deubiquitylating enzymes such as ubiquitin-specific proteases (USPs), cysteine endopeptidases encoded by *Usp* genes, thereby expression levels and activity of target proteins are regulated. USP1 deubiquitylates FANCD2 which is included in the repair of DNA crosslinks. *Usp*1 null males were infertile and the seminiferous tubules were markedly atrophic and mostly devoid of spermatogenic cells in the mutant testis. *Usp2*-null males possessed severely reduced fertility and the mutant sperm were defective in sperm motility and egg fertilizing ability *in vitro* (48). Germ cell-specific ablation of *Usp9x* using *Vasa-cre* possessed spermatogenic cell apoptosis at the early spermatocyte stage and resulted in complete infertility (49). *Usp26* is an X-linked gene exclusively expressed in testis (186). *Usp26* -null males are subfertile because of reduced number of haploid cells in testis (50, 51). *Usp*1-null female mice showed reduced fertility probably because of a reduced number of oocytes in ovaries (52). Thus, UPS is critically important for germ cell production in both sexes.

TABLE 2 | The ubiquitin ligases indispensable for mammalian reproduction.

Gene	Type	Gene-modified organism	Phenotype	Refs.
D. melanogaster				
rae1	E3 ligase component	*ms (2)Z5584* mutation	Male infertile, striking defects in primary spermatocyte nuclear integrity, meiotic chromosome condensation, segregation, and spindle morphology.	(141)
parkin	E3 ligase	P element insertion	Female infertility.	(142)
cul3	E3 ligase	EMS mutagenesis	Male infertility	(143)
C. elegans				
mel-26	E3 ligase	EMS mutagenesis	Germ cell depletion and sterility.	(144)
skr-1, *skr-2*	E3 ligase component	RNAi knockdown	Hermaphrodites are sterile. Arrested germline development in pachytene stage, expanded transition zone, and the presence of gaps in the gonad arm.	(145)
vhl-1	E3 ligase	RNAi knockdown	Reduced fertility.	(146)
M. musculus				
Chfr	E3 ligase	KO	30% of KO male were infertile.	(147)
Cul4a	E3 ligase component	KO	Male infertility phenotype resulted from a combination of decreased spermatozoa number, reduced sperm motility and defective acrosome formation.	(148, 149)
Cul4b	E3 ligase	*Vasa-cre*; *Cul4b*$^{fl/Y}$	Male infertility.	(150)
		Cul4b$^{-/Y}$	Male infertility.	(151)
Dcaf17	E3 ligase	KO	Male infertility due to abnormal sperm development.	(152)
Dcaf8	E3 ligase	KO	Pronounced sperm morphological abnormalities with typical bent head malformation.	(153)
Dcun1d1	E3 ligase component for neddylation	KO	Malformed spermatozoa with supernumerary and malpositioned centrioles.	(154)
Fbxw7	E3 ligase component	*Amh-cre*; *Fbxw7*$^{fl/fl}$	Impaired testis development, which is characterized by age-dependent tubular atrophy, excessive germ cell loss, and spermatogenic arrest, and the mutant males were infertile at 7 months old	(155)
Huwe1	E3 ligase	*Ddx4-cre*; *Huwe1*$^{fl/Y}$	Male infertile, Sertoli cell only phenotype. Increased level of histone H2AX and an elevated DNA damage response.	(156)
	E3 ligase	*Stra8-cre*; *Huwe1*$^{fl/Y}$	Male infertile, spermatogenesis arrest. Accumulation of DNA damage response protein γH2AX.	(157)
	E3 ligase	*Zp3-cre*; *Huwe1*$^{fl/fl}$	Oocyte death and female infertility.	(158)
Mdm2	E3 ligase	*Pgr-cre*; *Mdm2*$^{fl/fl}$	Female infertility. Impaired oocyte maturation, ovulation, and fertilization.	(159)
		Gdf9-cre; *Mdm2*$^{fl/fl}$	Female infertility. Complete lack of follicular structures resembling human premature ovarian failure.	(160)
		Zp3-cre; *Mdm2*$^{fl/fl}$	Female infertility.	(160)
		Amh-cre; *Mdm2*$^{fl/fl}$	Male infertile. degenerated testes with no organized seminiferous tubules and a complete loss of differentiated germ cells.	(161)
Mgrn1	E3 ligase	Spontaneous	Male infertility.	(162)
Phf7	E3 ligase	KO	Male infertility due to impaired protamine replacement in elongated spermatids.	(163)
Rnf20	E3 ligase	*Stra8-cre*; *Rnf20*$^{fl/fl}$	Male infertility because of arrested spermatogenesis at the pachytene stage.	(164)
Rnf216	E3 ligase	KO	Disrupted spermatogenesis and male infertility.	(165)
Rnf8	E3 ligase	KO	Male infertility.	(166)
		Gene trap	Male infertility.	(167)
Siah1a	E3 ligase	KO	Female subfertility and male infertility. Interrupted spermatogenesis because of impaired progression past meiotic metaphase I.	(168)
Spop	E3 ligase	*Pgr-cre*; *Spop*$^{fl/fl}$	Female infertility because of impaired uterine decidualization.	(169)
Syvn1 (Hrd1)	E3 ligase	*Alb-cre*; *Hrd1*$^{fl/fl}$	Female infertility.	(170)
Trim37	E3 ligase	KO	Male and female infertility.	(171)
Trim71	E3 ligase	*Nanos3-cre*; *Trim71*$^{fl/-}$	Male infertility because of Sertoli cell-only phenotype.	(172)
Ubr2	E3 ligase	KO	Male infertility caused by arrested spermatogenesis at meiotic prophase I.	(173)
Uhrf1	E3 ligase	*Stra8-cre*; *Uhrf1*$^{fl/fl}$	Failure of meiosis and male infertility.	(174)
		Zp3-cre; *Uhrf1*$^{fl/fl}$	Female infertility.	(175)
Rad6b	E2 ligase	KO	Male infertility because of the loss of spermatogenesis	(166)
Ube2i	E2 ligase	*Gdf9-icre*; *Ube2i*$^{fl/fl}$	Female infertility with major defects in stability of the primordial follicle pool, ovarian folliculogenesis, ovulation and meiosis.	(176)

(Continued)

TABLE 2 | Continued

Gene	Type	Gene-modified organism	Phenotype	Refs.
Ube2j1	E2 ligase	KO	Male infertility because of deficient spermatogenesis.	(177)
Ube2q1	E2 ligase	KO	Reduced female fertility. Altered estrus cycle, abnormal sexual behavior and reduced offspring care, and significantly increased embryonic lethality in the uterus of mutant females.	(178)
H. sapiens				
RNF220	E3 ligase	Spontaneous mutation	Small-headed sperm.	(179)
A. thaliana				
PUB4	E3 ligase	T-DNA insertion	Male sterility.	(180)
SAP	E3 ligase component	Two-element Enhancer-Inhibitor transposon system	Male and female sterility. Severe aberrations in inflorescence and flower and ovule development. Carpelloid sepals, short and narrow or absent petals, and degenerated anthers.	(181)
SIZ1	SUMO E3 ligase	T-DNA insertion	Arrest of funicular and micropylar pollen tube guidance.	(182)
MMS21	SUMO E3 ligase	T-DNA insertion	Severely reduced fertility, deficient gametogenesis.	(183)
O. sativa				
SIZ1	SUMO E3 ligase	T-DNA insertion	Spikelet sterility caused by defective anther dehiscence.	(184)

Non-Proteasomal Intracellular and Extracellular Proteolysis Factors in Sperm Production

Intracellular and extracellular proteolysis factors critically function in spermatogenesis. Cleavage of specific peptide bonds also contributes to spermatogenesis. *Apaf1* encodes a caspase activator, and *Apaf1*-null males are infertile because of degeneration of spermatogonia, which results in the absence of sperm (53). *Agbl5* encodes an intracellular metalloprotease. *Agbl5*-null males are infertile because of defective spermatogenesis (54, 55). A cytosolic carboxypeptidase 1, another metalloprotease encoded by *Agtpbp1* deglutamylates polyglutamylated proteins. *Agtpbp1* mutant mice known as *Purkinje cell degeneration* (*pcd*) possess male infertility (109–112) because of defective spermatogenesis (110). A germ cell nuclear antigen encoded by *Gcna* contains a metalloprotease domain. *Gcna*-null males are nearly devoid of sperm and infertile (56). In human, *GCNA* spontaneous mutations were identified in spermatogenic failure patients (124, 125).

Separin, a caspase-like cysteine protease encoded by *Espl1*, plays a central role in chromosome segregation by cleaving the SCC1/RAD21 subunit of the cohesin complex (187–189). A point mutation in *Espl1* which substitutes inhibitory phosphorylation site Ser^{1121} to Ala depletes spermatogonia because of chromosome misalignment during proliferation of the postmigratory primordial germ cells and following mitotic arrest, aneuploidy, and cell death (105). Threonine aspartase 1 (TASP1) is an intracellular endopeptidase that cleaves after distinct aspartate residues of the conserved IXQL(V)D/G motif (190). TASP1 cleaves general transcription factor TFIIAα–β to enable testis-specific transcription; *Tasp1*-null male mice were unable to activate spermatogenic gene activation, which lead to the release of immature germ cells and infertility (57). A serine protease ClpP is located in the mitochondrial matrix and participates in mitochondrial protein quality control by degrading misfolded or damaged proteins. In *Clpp*-null mutants spermatogenesis was disrupted by the spermatid stage (114). *Tysnd1* encodes a serine protease that processes peroxisomal leader peptides. *Tysnd1*-null mutant males possess globozoospermia and their spermatozoa lack the acrosomal cap (58). *Spink2* encodes a Kazal-type serine protease inhibitor abundantly expressed in testis and epididymis (191). *Spink2*-null males had azoospermia, and a homozygous splice mutation of *SPINK2* was found in infertile men (59). Ablation of *Serpina5* encoding another serine protease inhibitor also results in an abnormality in sperm production in the testis (60).

Puromycin-sensitive aminopeptidase encoded by *Npepps* is also an intracellular protease. It appears to contribute indirectly to spermatogenesis. *Npepps*-null testes and seminal vesicles were significantly reduced in weight, spermatogenesis was impaired, and copulatory behavior was lacking. It is suggested that the defects in the testes likely arises from dysfunction of Sertoli cells, whereas the lack of copulatory behavior results from defects in the brain (115).

A null mutation of *Adamts2* encoding secreted metalloproteinase caused male infertility (61). Decreased spermatogenesis was observed but copulatory behavior and/or copulatory plug formation may also be impaired because a copulatory plug was never observed (61).

Proteolysis Factors Associated With Sperm Function

Acrosomal Function

The acrosome is a Golgi-derived sperm head organelle in which many digestive enzymes such as proteases and hyaluronidases are included to penetrate egg surroundings. Acrosin is a serine protease and a major component of the acrosome. Although acrosin-deficient male mice are fertile (62, 63), disruption of hamster acrosin resulted in complete male infertility (120). *In vitro*, mutant hamster spermatozoa attached to the zona

pellucida, but failed to penetrate it (120), suggesting that acrosomal function can be attributed to specific factors in a species-specific manner.

Proprotein convertases convert inactive precursor proteins into their mature and active forms. PCSK4 is a member of proprotein convertases expressed on the sperm surface overlying the acrosome (64). *Pcsk4*-null males showed impaired fertility (64, 65) and mutant sperm exhibited accelerated capacitation, precocious acrosome reaction, reduced binding to egg zona pellucida (64). Acrosome formation during spermatogenesis was also abnormal (192).

Sperm Maturation

A group of genes encoding proteases, enzymatically inactive pseudoproteases, and protease inhibitors is apparently associated with the same physiological function, i.e., maturation of sperm conferring abilities to migrate into female oviduct and bind with zona pellucida. Ablation of *Tmprss12* (66), *Prss55* (67, 68), *Tryx5* (69), *Prss37* (70), *Ace* (71), *Adam1a* (72), *Adam2* (73), *Adam3* (74, 75), and *Adam6* (76) results in deficient sperm migration into the oviduct and binding to the zona pellucida of eggs. Among them, *Adam1a*, *Adam2*, *Adam3*, *Adam6*, and *Prss37* encode catalytically inactive pseudoproteases. A disintegrin and metallopeptidase domain (ADAM) 3, a catalytically inactive transmembrane pseudoprotease appears to be central to a molecular mechanism that governs sperm migratory and adhesion abilities, because ADAM3 expression is a prerequisite for sperm to acquire these abilities (193).

ADAM3 is expressed as a precursor and the processed into mature form as spermatozoa mature in epididymis (194). Similarly, enzymatically inactive pseudoproteases ADAM2 and ADAM6 are processed during sperm maturation in epididymis (195, 196). Therefore, they are rather substrates for other proteases. Ablation of ADAM2 or ADAM6 also results in significant decrease or loss of ADAM3 from epididymal sperm (74, 76) indicating the involvement of both ADAM2 and ADAM6 in ADAM3 expression. PRSS37 supports ADAM3 precursor translocation to the sperm cell surface by collaborating with PDILT, a testis-specific protein disulfide isomerase indispensable for ADAM3 surface expression (197, 198). TMPRSS12, PRSS55, and TRYX5, all of which are serine proteases and retain catalytic triad residues, are necessary for the production or stable localization of processed ADAM3 on the cell surface of epididymal spermatozoa (66–69), although it remains uncertain whether these proteases directly cleave ADAM3.

Cystatins are secreted cysteine proteinase inhibitors. Cystatin genes *Cst8*, *9*, *11*, *12*, *13*, *dc1*, *dc2*, and *l1* are clustered on mouse chromosome 2 and expressed in both testis and epididymis. Their simultaneous ablation resulted in the loss of ADAM3 from epididymal sperm and deficient sperm migration into the oviduct (77), implying the importance of regulated proteolysis in sperm maturation. Ovochymase 2 (OVCH2) is a chymotrypsin-like serine protease. OVCH2 is specifically expressed in the caput epididymis under the regulation of lumicrine signaling, in which testis-derived secreted protein NELL2 transiting through the luminal space acts on the epididymal epithelium by binding to its receptor ROS1 tyrosine kinase to differentiate (78). Ablation of *Ovch2* results in abnormal sperm ADAM3 processing and deficient sperm migration into the oviduct (78). Thus, regulated proteolysis on or outside spermatozoa apparently modulates sperm maturation.

NL1 encoded by *Mmel1* is a zinc metallopeptidase expressed in testis. NL1 is expressed as a type II transmembrane protein but released as a soluble form. *Mmel1*-null mice show normal spermatogenesis but reduced egg fertilization, suggesting the role of NL1 in sperm maturation (79). It remains, however, uncertain whether NL1 is included in ADAM3-mediated sperm maturation. Testisin encoded by *Prss21* is a GPI-anchored serine protease. *Prss21* KO males are subfertile because mutant spermatozoa possessed decreased motility, angulated and curled tails, and fragile necks (80). In another *Prss21* mutant line *in vitro* sperm binding to egg zona pellucida, acrosome reaction, and fertility were decreased (81).

Other Proteolytic Factors Associated With Male Reproduction

Several cell surface and extracellular proteases and inhibitors seem to regulate male fertility in more indirect manners. *Adamts16* homozygous mutant rat males resulted in cryptorchidism and male sterility (121). The mutant testis undescended during development because of the failure of gubernacular migration (122). γ-glutamyltranspeptidase 1 (GGT1) is a type II transmembrane protein which cleaves γ-glutamyl bond of extracellular glutathione (γ-Glu-Cys-Gly), glutathione conjugates, and other γ-glutamyl compounds. The resulting cysteinyl-glycine is further cleaved by dipeptidase into free amino acids. *Ggt1*-null males are infertile because of decreased epididymal sperm number and failure in copulatory plug formation (117). Although *Ggt1*-null testis was small, spermatogenesis inside seminiferous tubules appeared normal and seminal vesicles were hypoplastic. As *N*-acetylcysteine-fed mutant mice were fertile, the observed infertility is a consequence of cysteine deficiency (117),. Carboxypeptidase E (CPE) is a metallo-carboxypeptidase and functions as a prohormone processing exopeptidase. $Cpe^{fat/fat}$ males are infertile and deficient in Pro-gonadotropin-releasing hormone processing in the hypothalamus (82). ADAM24 is a metalloproteinase localized on the mature sperm surface. *Adam24*-null males are subfertile and polyspermic fertilization increased *in vitro* and *in vivo*, suggesting a physiological role of ADAM24 for prevention of polyspermy (83). ADAM7 is a membrane-anchored protein with a catalytically-inactive metalloproteinase domain abundantly expressed in the epididymis (199). *Adam7* ablation resulted in a modest reduction of male fertility; impaired epididymal morphology and integrity may affect sperm maturation (84).

Cystatin C encoded by *Cst3* is a cysteine protease inhibitor abundantly expressed in testis and epididymis. Substitution of Leu^{68} to Gln is an amyloid-forming mutation found in a hereditary form of cystatin C amyloid angiopathy. Heterozygous male mice were infertile and increased levels of

amyloid was observed in the epididymal fluid (85). Nonpathological function of amyloid during epididymal sperm maturation is also suggested (200).

Immp2l encodes an inner mitochondrial membrane peptidase 2-like. *Immp2l*-null homozygous males were severely subfertile because of erectile dysfunction (118). Tumor necrosis factor-α (TNFα) converting enzyme encoded by *Adam17* is involved in the proteolytic release of the ectodomain of diverse cell surface proteins. Conditional ablation of *Adam17* with *Sox9-cre* severely impaired male fertility but the details are uncertain (119).

Serpine2 encodes protease nexin-1, a serine protease inhibitor expressed in seminal fluid. *Serpine2*-null males possessed reduced fertility because of impaired semen coagulation and copulatory plug formation (86).

Proteolytic Factors in Ovary and Follicle Development

Both intracellular and extracellular proteolytic factors are included in ovary and follicle development. Conditional ablation of separase under the control of *Zp3-cre* hindered extrusion of the first polar body and caused female sterility (106). Introduction of a Ser^{1121} to Ala deregulatory mutation into separase led to primordial germ cell apoptosis during embryonic oogenesis (107). Ablation of cytosolic carboxypeptidase 1 encoded by *Agtpbp1* results in female subfertility because secondary follicles poorly develop into antral follicles (113). Oocyte-specific ablation of nuclear cysteine protease separase causes female infertility because mutant oocytes are able neither to extrude polar bodies in meiosis I nor to resolve chiasmata (106).

A deregulatory mutation into separin encoded by *Espl1* at early embryonic period caused primordial germ cell depletion by apoptosis during embryonic oogenesis, which led to female infertility (105, 107). The introduction of the same mutation at later oocyte development by using *Zp3-cre* also resulted in female infertility but because of failure in preimplantation development (108).

Matriptase encoded by *Tmprss6* is a type II transmembrane serine protease which functions in iron homeostasis by cleaving cell surface proteins associated with iron absorption. *Tmprss6*-null females possessed marked retardation in ovarian maturation (87), probably because of severe decrease in plasma iron levels. The defective ovarian follicle development and female infertility can be mimicked by a low iron diet (201).

The inter-α-trypsin inhibitor (IαI) family are abundantly found in body fluids including blood plasma and urine and possess inhibitory activity for serine proteases. They are composed of bikunin, a proteoglycan with a single chondroitin sulfate chain, and heavy chains covalently bound to chondroitin sulfate chain of bikunin. IαI family members are able to transfer their heavy chains from IαI to hyaluronan in the presence of tumor necrosis factor-stimulated gene-6. This reaction results in the modified hyaluronan covalently linked heavy chain and is necessary for hyaluronan-rich cumulus matrix expansion. When the bikunin-coding region was deleted from *Ambp* gene, the resulting homozygous females ovulate oocytes deficient in hyaluronan-rich cumulus matrix expansion, leading to female infertility (88, 89).

γ-secretase is an endoprotease complex that catalyzes the intramembrane cleavage of integral membrane proteins. *Psen1* encodes presenillin-1, a catalytic subunit of γ-secretase. Female mice homozygous with a Leu^{166} to Pro mutation, an aggressive mutation found in familial Alzheimer's disease patients, are infertile and their ovaries consisted largely of stromal elements with primordial follicles near the cortex (90).

ADAMTS1 is a secreted metalloproteinase expressed in the granulosa cell layer of mature follicles in the ovary (91). *Adamts1*-null females possessed lower numbers of mature follicles in the ovary and a thick and convoluted uterus (92). In another mutant mouse line, ovulation in null females was impaired because mature oocytes remained trapped in ovarian follicles (91). In zebrafish, *adamts9*-null females possess ovarian malformation and are unable to ovulate (123).

Lonp encodes a mitochondrial serine protease. Oocyte-specific *Lonp* ablation by *Gdf9-cre* or *Zp3-cre*; $Lonp1^{fl/fl}$ results in female infertility because of impaired follicular development, progressive oocyte death, ovarian reserve loss (93). *Furin* encodes a transmembrane serine protease localized in Golgi appratus, endosome, plasma membrane; it is necessary for mature protein release by cleaving at RX(K/R)R consensus motif. Conditional ablation of *Furin* by *Gdf9-cre* or *Zp3-cre*; $Furin^{fl/fl}$ result in female infertility because of the arrested oogenesis at early secondary follicles (94). *Pappa* encodes an extracellular metalloprotease. *Pappa* KO females decreased their litter size and ovulatory capacity, probably because of decreased bioavailability of ovarian insulin-like growth factor (95).

Loss of GGT1 causes infertility in not only males but females. In the *Ggt1*-null females, antral follicles and corpora lutea were absent and follicles degenerated due to the reduced intracellular cysteine levels (117).

Mitochondrial proteases also affect ovarian follicle development. Ablation of *Clpp* encoding mitochondrial matrix ClpP protease caused relatively small ovaries in which follicular differentiation was impaired probably because of the reduction of the granulosa cell layers (114). When the inner mitochondrial membrane peptidase 2-like encoded by *Immp2l* was ablated, the resulting mutant females were deficient in folliculogenesis and ovulation and infertile, probably because of low availability of nitric oxide caused by mitochondrial dysfunction (118).

Proteolytic Factors in Post-Fertilization Events of Female Reproduction

Several proteolysis-associated secreted proteins contribute to post-fertilization events including the hardening of the egg-surrounding zona pellucida. Ovastacin encoded by *Astl* is a secreted metalloendopeptidase deposited in cortical granules of oocytes. Ovastatin is secreted into the extracellular space in response to egg activation triggered by fertilization. In *Astl*-null eggs, ZP2 cleavage necessary for zona pellucida hardening and the postfertilization block to polyspermy did not occur after fertilization (96). Fetuin is a cystatin family protease inhibitor abundantly expressed in blood plasma. Fetuin-B prevents

premature ZP hardening probably by inhibiting ovastacin derived from spontaneous cortical granule release, as fetuin-B inhibited ovastacin protease activity *in vitro* and *Fetub*-deficient oocytes undergo premature zona pellucida hardening (97).

Antithrombin encoded by *Serpinc1* inhibits thrombin and some other coagulation factors by binding heparin and heparan sulfate. When an Arg^{48} to Cys mutation, which corresponds to human thrombosis mutation, was introduced into mice, the resulting homozygous females had decreased their litter size, probably because thrombosis occurred in placenta (98).

Adam10 encodes a membrane metalloprotease. Conditional ablation of vascular *Adam10* by *Tie2-Cre*; $Adam10^{fl/fl}$ causes impaired decidualization and female subfertility (99). *Adamts18* encodes a member of secreted metalloprotease ADAMTS. *Adamts18*-null females suffer from vaginal obstruction, due to either a dorsoventral vaginal septum or imperforate vagina and infertility or subfertility (100).

Other Proteolytic Factors in Female Reproduction

Several proteolysis-associated factors regulate female reproduction in a more indirect manner. *Npepps*-null females lacking a puromycin-sensitive aminopeptidase impairs corpus luteum formation and are infertile, probably because of disruption of the hypothalamic-pituitary axis (116). Plasmin is a secreted serine protease generated from plasminogen through activation by tissue-type or urokinase-type plasminogen activators. The fertility of plasmin-deficient *Plg*-null female mice appeared to be compromised (101, 102). It seems not to be the consequence of the impaired proteolytic process essential for ovulation, as plasminogen-deficient mice had normal ovulation efficiency (202). *Timp1* encodes a tissue inhibitor of metalloproteinases 1, an inhibitor for matrix metalloproteinases. *Timp1* mutation reduced the reproductive lifespan of female but not male mice (103). When *Pcsk2* encoding neuroendocrine convertase 2 was ablated, the number of consecutive litters from mutant female mice was small and *Pcsk2*-null female mice sometimes gave birth to dead pups (104) for uncertain reason. Conditional ablation of TNFα converting enzyme by *Sox9-cre*; $Adam17^{fl/fl}$ resulted in female infertility but details are uncertain (119).

FERTILITY-ASSOCIATED PROTEASES IN PLANTS

Several aspartic proteases are associated with pollen development and function. In *Arabidopsis thaliana*, A36 and A39 are GPI-anchored putative aspartic proteases predominantly expressed in pollen and the pollen tube. In *a36*; *a39* double mutant, pollen grains underwent apoptosis-like programmed cell death and the pollen tube compromised micropylar guidance (126). *UND* encodes a secreted aspartic protease UNDEAD, and its silencing using small interfering RNA caused premature tapetal and pollen programmed cell death (128).

In *Oryza sativa*, *OsAP65* encodes an aspartic protease localized in the pre-vacuolar compartment. T-DNA-inserted *OsAP65* mutant alleles could not be transmitted through the male gamete; the mutant pollen matured normally, but did not germinate or elongate, indicating its essentiality in pollen germination and tube growth (131). *PCS1* encodes an aspartic protease and its loss-of-function mutation caused degenerated male and female gametophytes (127).

A cysteine protease also contributes to pollen development; when a papain-like vacuolar cysteine protease encoded by *CEP1* was ablated, the resulting mutants are male subfertile because of aborted tapetal programmed cell death and decreased pollen fertility with abnormal pollen exine (129).

Some aspect of *A. thaliana* reproduction includes Small Ubiquitin-related Modifier (SUMO). SPF1 and SPF2 are cysteine proteases and function in desumoylation of sumoylated proteins. *spf1*; *spf2* double mutants exhibit severe abnormalities in microgametogenesis, megagametogenesis, and embryo development (130). There are SUMO-E3 ligases involved in gametophyte development (182, 183) in *A. thaliana* and in anther dehiscence in *O. sativa* (184).

CONCLUSION AND PERSPECTIVE

By a comprehensive survey, it has been demonstrated that proteolysis regulates reproduction in various species including yeast, insects, nematodes, vertebrates, and plants. Regulation of reproduction by proteolysis already exist in unicellular yeast. In multicellular organisms, proteolysis regulates the formation and function of gametes derived from germ cells as well as the development and function of reproductive organs by somatic cells, thereby securing successful reproduction. In these cell lineages, both limited proteolysis and degrative proteolysis by ubiquitin-proteasome system play critical roles.

One of intriguing paradigms emerging in this review is that many sperm surface and extracellular proteases, pseudoproteases, and inhibitors are included in the acquisition of mammalian sperm conferring abilities to migrate into the oviduct and to bind to the zona pellucida of eggs. As spermatozoa are transcriptionally and translationally silent, post-translational modification mechanisms such as proteolysis may largely contribute to sperm maturation.

Many compounds have been designed to inhibit the enzymatic activity of proteases. Clinically, there have been numerous successes including angiotensin-converting enzyme inhibitors for cardiovascular disorders (203), thrombin inhibitors for thromboembolism and bleeding disorders (204, 205), and HIV protease inhibitors in the treatment of HIV and AIDS (206), among others (207, 208). In addition, enzymatically active proteases could also be good druggable targets for contraceptives.

Genome editing techniques developed in recent years will identify fertility-associated proteolytic factors further. In addition to identifying novel factors, more intense studies on the molecular basis of proteolysis including the identification of

substrates will clarify how proteolytic events govern reproduction. It will also clarify the physiological significance of molecular events governed by proteolysis in reproduction.

AUTHOR CONTRIBUTIONS

DK and MI wrote the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported in part by Ministry of Education, Culture, Sports, Science and Technology (MEXT)/Japan Society for the Promotion of Science (JSPS) KAKENHI grants (JP21H00231 to D.K. and JP21H05033 to MI), Japan Science and Technology Agency (21460710 to D.K. and 21467777 to MI), National Institutes of Health (R01HD088412 and P01HD087157 to MI), and the Bill & Melinda Gates Foundation (Grant INV-001902 to MI). Under the grant conditions of the Foundation, a Creative Commons Attribution 4.0 Generic License has already been assigned to the Author Accepted Manuscript version that might arise from this submission.

ACKNOWLEDGMENTS

We thank Dr Julio Castaneda for critical reading of this manuscript.

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ORIGINAL RESEARCH
published: 06 May 2022
doi: 10.3389/fendo.2022.896356

Low NAD$^+$ Levels Are Associated With a Decline of Spermatogenesis in Transgenic ANDY and Aging Mice

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Advanced paternal age has increasingly been recognized as a risk factor for male fertility and progeny health. While underlying causes are not well understood, aging is associated with a continuous decline of blood and tissue NAD$^+$ levels, as well as a decline of testicular functions. The important basic question to what extent ageing-related NAD$^+$ decline is functionally linked to decreased male fertility has been difficult to address due to the pleiotropic effects of aging, and the lack of a suitable animal model in which NAD$^+$ levels can be lowered experimentally in chronologically young adult males. We therefore developed a transgenic mouse model of acquired niacin dependency (ANDY), in which NAD$^+$ levels can be experimentally lowered using a niacin-deficient, chemically defined diet. Using ANDY mice, this report demonstrates for the first time that decreasing body-wide NAD$^+$ levels in young adult mice, including in the testes, to levels that match or exceed the natural NAD$^+$ decline observed in old mice, results in the disruption of spermatogenesis with small testis sizes and reduced sperm counts. ANDY mice are dependent on dietary vitamin B3 (niacin) for NAD$^+$ synthesis, similar to humans. NAD$^+$-deficiency the animals develop on a niacin-free diet is reversed by niacin supplementation. Providing niacin to NAD$^+$-depleted ANDY mice fully rescued spermatogenesis and restored normal testis weight in the animals. The results suggest that NAD$^+$ is important for proper spermatogenesis and that its declining levels during aging are functionally linked to declining spermatogenesis and male fertility. Functions of NAD$^+$ in retinoic acid synthesis, which is an essential testicular signaling pathway regulating spermatogonial proliferation and differentiation, may offer a plausible mechanism for the hypospermatogenesis observed in NAD$^+$-deficient mice.

Keywords: vitamin B3, niacin, nicotinamide, testis, aging, retinoic acid, spermatogonia, male fertility

OPEN ACCESS

Edited by:
Vassilios Papadopoulos, University of Southern California, United States

Reviewed by:
Michael A. Palladino, Bloomfield College, United States
Silvana A. Andric, University of Novi Sad, Serbia

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Specialty section:
This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology

Received: *15 March 2022*
Accepted: *30 March 2022*
Published: *06 May 2022*

Citation:
Meyer-Ficca ML, Zwerdling AE, Swanson CA, Tucker AG, Lopez SA, Wandersee MK, Warner GM, Thompson KL, Chini CCS, Chen H, Chini EN and Meyer RG (2022) Low NAD$^+$ Levels Are Associated With a Decline of Spermatogenesis in Transgenic ANDY and Aging Mice. Front. Endocrinol. 13:896356. doi: 10.3389/fendo.2022.896356

INTRODUCTION

Associated with socioeconomic considerations, for example increased time needed for education and professional development, mean paternal age has increased over the past 44 years from 27.4 to 30.9 years (1). This is concerning because paternal age has been shown to negatively affect fertility, pregnancy rates and children's health (2, 3). How exactly the aging process exerts its negative effects on male fertility is not clear, because of its pleiotropic effects on the body, including the testis (4, 5). Although underlying mechanisms are not yet well understood, one of the hallmarks of aging is a steady decline of cellular, tissue and plasma NAD^+ concentrations, observed during chronological aging in humans, worms, flies, and mice (6–10). NAD^+ and $NADP^+$, and their reduced forms NADH and NADPH, are important coenzymes for most cellular redox reactions, and as such essential for maintaining cellular metabolism and respiration. In addition to its function as a redox cofactor, NAD^+ is also consumed by enzymes involved in chromatin modification, gene regulation, and DNA repair, including poly(ADP-ribose) polymerases (PARP family of enzymes), as well as NAD-dependent protein deacetylases (sirtuins) and CD38 (11–13).

Unfortunately, the links between aging, low NAD^+ levels and declining fertility are not well understood because systematic investigations have been hampered by basic metabolic differences present between laboratory rodents and humans in their ability to generate NAD^+ from their diet.

In certain mammals, including humans, nicotinic acid (NA), nicotinamide (Nam) and Nam riboside (NamR), collectively referred to as niacin or vitamin B3, are the main nutritional precursors of NAD^+ and its phosphorylated form, $NADP^+$. Humans depend on dietary niacin as their main source of NAD^+ and $NADP^+$ precursors and can become niacin-deficient when their food lacks sufficient amounts of vitamin B3. Niacin deficiency is characterized by very low levels of NAD^+ and in its most extreme form, pellagra, can be debilitating and even deadly, which is now rare in western countries. However, milder forms of clinical niacin deficiency are commonly seen with increasing age, and in cancer patients, alcoholics and people without access to quality food (14, 15). While this may be clinically relevant on its own, it is unlikely that a lack of dietary vitamin B3 intake is at the root of age-related NAD^+ decline. Instead, age-related increases in the activity of NAD^+-consuming enzymes such as PARP1 and CD38, or potential mitochondrial dysfunction, or both, provide a more plausible explanation [(12, 16, 17), reviewed in (18)].

Physiological effects of low NAD^+ status and their potential impact on male fertility have been difficult to study because of a lack of suitable animal models. Wild-type laboratory rodents are able to completely satisfy their NAD^+ needs by metabolizing tryptophan (Trp) to NAD^+ *via* the kynurenine (*de novo* synthesis) pathway and, unlike humans, do not depend on intake of dietary niacin. In order to address this problem and to investigate the impact of low NAD^+ levels as a potential factor contributing to the decline of fertility in aging males, we therefore generated mice with tetracycline-inducible overexpression of a transgene encoding the enzyme human aminocarboxymuconate semialdehyde decarboxylase (hACMSD) to create a mouse model of human-like NAD^+ metabolism (ANDY, acquired niacin dependency) (19). In this mouse, hACMSD overexpression diverts the central kynurenine pathway in the liver and kidney to produce acetyl-CoA instead of NAD^+ which makes the animals dependent on dietary niacin intake as the main source of NAD+ synthesis, similar to humans (19) (**Figure 1A**). ANDY mice with hACMSD overexpression reproducibly become NAD^+-deficient in various tissues over the course of 6 weeks on a defined diet that is devoid of niacin (ND diet), but not on a control diet that is chemically identical to ND but supplemented with 30 mg/kg nicotinic acid (CD diet). Previous data showed that ANDY mice had significantly lower NAD^+ and $NADP^+$ levels in blood, liver, and other tissues when they received a niacin-free ND diet and doxycycline (Dox, a water-soluble tetracycline) in their drinking water (19). If maintained at very low NAD^+ levels, male ANDY mice sired smaller litters than control males (data not shown).

The goal of the current study has therefore been to investigate the impact of NAD^+ deficiency on spermatogenesis in young adult ANDY mice to test the hypothesis that low NAD^+ levels have a negative impact on male fertility, independent of chronological age.

MATERIALS AND METHODS

Animal Model and Induction of NAD Deficiency

Details of the generation of the transgenic animal model C57BL/6J-Gt(Rosa)26Sor$^{tm1(rTTa*M2)JaeCol1a1^{tm6(tetO-hACMSD)MMF}}$ and the biochemical basis of NAD-dependency in these mice has been described previously (19). Briefly, administration of doxycycline, a water-soluble tetracycline, in the drinking water induces overexpression of the human aminocarboxymuconate semialdehyde decarboxylase (hACMSD) gene. Increased ACMSD activity renders these transgenic mice dependent on dietary niacin uptake in a manner similar to humans. In the absence of dietary niacin, these ANDY mice become measurably NAD^+ deficient in blood and body tissues (19). Mice were bred and housed under standard conditions. Transgene expression was only induced in adult mice during the feeding trials. Breeding, postnatal and pubertal development occurred in the absence of doxycycline-induced transgene overexpression and on normal, niacin-containing chow diet. Animal studies and experimental procedures were approved by the Institutional Animal Care and Use Committees (IACUC protocol number 10056) of Utah State University and of Mayo Clinic, Rochester, Minnesota.

Defined Feeds and Feeding Trials

Standard chow diet was Teklad Rodent diet 8604 (24% crude protein, 63 mg/kg niacin, Envigo, Madison, WI, USA). Niacin-deficient diet (ND, TD.140376) and control diet (CD, TD.140375) were defined, purified diets compounded by Teklad laboratory animal diets (Teklad Custom Diets, Envigo) as modifications of AIN-93G standard chow (19). Both, ND and CD contained 10% alcohol-washed casein as a vitamin-free

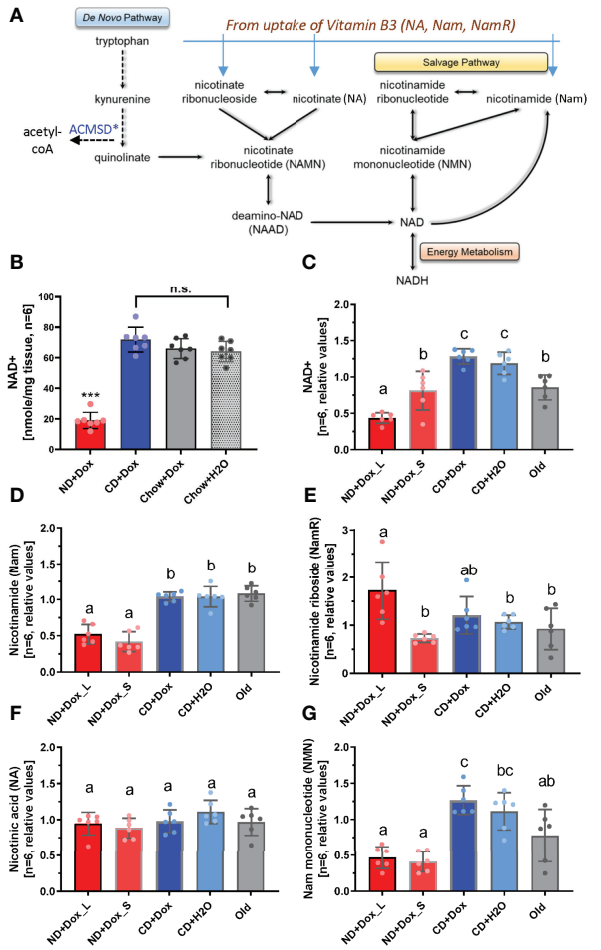

FIGURE 1 | Dietary niacin deficiency altered NAD metabolite profiles in ANDY mice. ANDY mice were kept on niacin-deficient (ND) for up to 12 weeks (ND +Dox_S) or longer (ND+Dox_L) or on control diet with Dox for ACMSD transgene induction (CD+Dox) or without Dox (CD+H2O). Old mice were 31 months old. **(A)** The NAD *de novo* synthesis pathway from tryptophan can provide all of the NAD in wild-type rodents in absence of all other dietary NAD precursors such as nicotinic acid (NA, nicotinate), nicotinamide (Nam), or nicotinamide ribonucleotide (nicotinamide riboside, NamR). Dox-mediated induction of a human ACMSD transgene overexpression diverts the central kynurenine pathway in the liver and kidney from NAD^+ production towards acetyl-CoA formation, and ultimately makes these mice dependent on dietary niacin to maintain tissue NAD^+ levels similar to humans **(B)** Testicular NAD^+ levels decline in ANDY mice on vitamin B3 (niacin) – free diet (ND). Data were generated using enzymatic cycling assays. **(C)** Metabolomic analyses confirms data in B, and indicates that a short-term period on ND diet (ND +Dox_S) results in a milder decline of NAD^+ that is comparable to old mice. **(D)** Nam levels declined already after short-term dietary niacin-deficiency, while NamR **(E)** values appeared to increase again in mice with long-term niacin deficiency. Hypothetically, the latter may result from loss of spermatogonia, spermatocytes and spermatids that occurs at later stages of NAD^+ decline. **(F)** Whole NA (nicotinate/nicotinic acid) did not change on the ND diet, while NMN **(G)** was significantly lowered in both short-and long-term ND fed mice compared to controls, but not old mice. Identical letters indicate group categories that are not significantly different from each other; different letters indicate statistically significant differences (One-way ANOVA with Tukey's multiple comparison analysis, $p<0.05$ considered a significant difference; ***$p > 0.001$).

protein source, either without niacin (ND) or with 30 mg/kg niacin (CD).

Age- and weight-matched animals were randomly assigned for the experiments when they were sexually mature, young adult mice between 7-14 weeks of age. During each feeding trial, mice were either fed ND or CD. Doxycycline (Sigma Aldrich, D9891; Alfa Aesar J6057922) was added to the drinking water [2 mg/ml] to induce ACMSD expression. Drinking water was changed twice per week. Durations of feeding trials are indicated in the results and figure legends. In recovery studies, animals were first kept on ND +Dox for the indicated time interval to induce NAD+-deficiency, then switched back to CD for the indicated recovery time.

At termination of each study, animals were euthanized, heparinized blood samples and tissues were collected rapidly, tissues weighed, snap frozen in liquid nitrogen and stored at -80°C until further analyses. Samples for histology were fixed immediately. Sperm numbers were determined in epididymal sperm isolated from the cauda epididymides and the vas deferens using a Neubauer hemocytometer.

Histology and Evaluation

Tissues for histological analyses were fixed in Bouin's solution (Sigma Aldrich, HT10132) or 10% neutral buffered formalin (Sigma Aldrich, HT501128-4L) immediately after tissue collection. Paraffin embedding, sectioning and hematoxylin eosin staining was performed by the Utah Veterinary Diagnostic Laboratory's histology core facility according to standard histological procedures.

Testicular tubules were analyzed for abnormalities in a blinded manner by an individual that had been trained in identifying spermatogenic stages in the mouse. Hematoxylin/eosin stained paraffin sections of testes were analyzed using bright field microscopy (Axio Scope A.1, Zeiss, Jena, equipped with AxioVision software). Tubules were classified as abnormal if they were missing a complete layer of cells that are normally present in a given tubular stage and that are used for classification of tubules, e.g. the absence of spermatocytes or round spermatids in stages I-VIII, or absence of spermatocytes and/or condensing spermatids in stages IX-XII, and/or if absence of several cell layers prevented stage identification. One hundred tubules were evaluated per animal and testis section, and statistical analysis was performed using 1-way ANOVA with Tukey's multiple comparison test.

NAD Measurements

Testicular tissue NAD^+ was quantified using an enzymatic cycling assay method described previously (19–21). Briefly, frozen testis tissue was lysed in NaOH, and neutralized with H_3PO_4. Protein was removed by $HClO_4$ precipitation, and supernatant was treated with KOH. NAD^+ was quantified in the supernatant in a 96-well microplate format on a SpectraMax Plus 384 plate reader (Molecular Devices, Sunnyvale, CA). All chemicals were from Sigma, Aldrich (St Louis, MO).

Testosterone Measurements

Testicular testosterone was quantified by radio immuno-assay as described previously (22, 23). In brief, snap-frozen pieces of testicular tissue were extracted in 2 ml of assay buffer and

testosterone was measured using standard RIA procedure with a testosterone specific antibody (ICN Biomedicals, Costa Mesa, CA) and ^{3}H-T (NEN Life Science Products, Boston, MA).

Quantification of Metabolites in Testicular Tissue

Testicular testosterone, nicotinamide adenine dinucleotide (NAD), nicotinamide (Nam), nicotinamide riboside (NamR), nicotinic acid (NA) and Nam mononucleotide (NMN) were quantified with Ultrahigh Performance Liquid Chromatography-Tandem Mass Spectroscopy (UPLC-MS/MS) on the Metabolon Platform (Metabolon, Norrisville, NC). Frozen testis samples were prepared using the automated MicroLab STAR® system (Hamilton), proteins were precipitated with methanol followed by centrifugation. The resulting extract was analyzed by two separate reverse phase (RP)/UPLC-MS/MS methods with positive ion mode electrospray ionization (ESI), by RP/UPLC-MS/MS and by HILIC/UPLC-MS/MS, both with negative ion mode ESI. UPLC-MS/MS was performed on a Waters ACQUITY UPLC and a Thermo Scientific Q-Exactive high resolution/accurate mass spectrometer interfaced with a heated electrospray ionization (HESI-II) source and Orbitrap mass analyzer operated at 35,000 mass resolution. The sample extract was dried, then reconstituted in solvents compatible to each of the four spectroscopy methods. Each reconstitution solvent contained a series of standards at fixed concentrations to ensure injection and chromatographic consistency. The MS analysis alternated between MS and data-dependent MSn scans using dynamic exclusion. The scan range varied slighted between methods but covered 70-1000 m/z. Raw data was extracted, peak-identified and QC processed using Metabolon's hardware and software. Compounds were identified by comparison to library entries of purified authenticated standards, and peaks were quantified using area-under-the-curve.

Graphing and Statistical Analyses

GraphPad Prism software versions 7.04 & 9.2.0 (GraphPad Software, San Diego, CA) were used for graphing and statistical analyses (One-way ANOVA, Tukey's multiple comparison, Welch's t-test, Pearson Correlation analysis; $p<0.05$ was considered significant).

RESULTS

ANDY Mice on Niacin Free Diet Have Significantly Reduced Testicular NAD+

In the absence of dietary niacin, blood NAD of ANDY mice declined steadily over time a time span of 6 weeks, and then remained at significantly lower levels compared to those in control animals (**Supplementary Figure 1**). Similar to blood, testes of ANDY mice became niacin-deficient, i.e. had significantly decreased NAD^+ tissue concentrations. NAD^+ levels in ANDY mice that were maintained on ND+Dox for 24 weeks dropped to about 1/3 of the NAD^+ content measured in ANDY mice fed niacin-containing CD diet or chow (**Figure 1B**), as measured using a sensitive enzymatic cycling assay (**Supplementary Table 1**). Similar changes were observed using comparative metabolomics analyses of testes from animals fed ND for 24 weeks (long-term, ND+Dox_L) or 12 weeks (short-term, ND+Dox_S). The metabolomic LC-MS/MS quantification confirmed the significant lowering of testicular NAD^+ levels in the ND+Dox_L group (long-term on ND diet, i.e. >12 weeks, one-way ANOVA, p-values from <0.0001 to 0.0052 with Tukey's multiple comparison test, **Figure 1C**). Compared to this group, NAD^+ levels were higher in ANDY mice kept on ND for 12 weeks (ND+Dox_S, p=0.0052), but still significantly lower than the control groups (p-values from 0.0004 to 0.0055). NAD^+ contents in controls CD+Dox and CD+H2O were not significantly different from each other, indicating that ACMSD overexpression and doxycycline administration on their own did not have any measurable effect on NAD^+ levels in the testis. Interestingly, NAD^+ content in the testes of the ND+Dox_S group was not significantly different from that of old mice at 31 months of age. Nam levels were low in both short- and long-term ND groups (**Figure 1D**). Unexpectedly, NamR levels were significantly higher in the ND+Dox_L group than ND+Dox_S (p=0.0011), but not significantly different from the CD+Dox control and the old mice (**Figure 1E**). NA values did not vary between the different treatment groups (**Figure 1F**). Similar to NAD^+ and NamR, NMN was not significantly different between mice in the ND+Dox_S group and old mice (**Figure 1G**).

Taken together, ACMSD overexpression in combination with niacin-free feed significantly lowered testicular NAD^+ levels of ANDY mice, which is also reflected in an altered NAD^+ metabolite profile. Moreover, the NAD^+ levels created in ANDY mice of the ND+Dox_S group were similar to those in old mice at 31 months of age.

Declining Testicular Weight and Sperm Counts in NAD+-Deficient ANDY mice

Sperm counts of mice that were kept on ND diet for ten weeks decreased significantly compared to control animals, and were similar to sperm numbers in old mice (**Figure 2A)**. After two additional weeks on deficient diet, sperm numbers declined abruptly (**Figure 2A**). Along with falling sperm counts, testes of mice in the ND+Dox group became significantly smaller than testes of any other treatment group as soon as 10 weeks on this diet, and continued to shrink until week 24 (**Figure 2B**). When recovered on the CD diet for 9 weeks, testis weights returned to normal values (**Figures 2B, C**). These results demonstrate that declining NAD^+ levels resulted in testicular shrinkage that was reversed by niacin supplementation which restored NAD^+ levels.

NAD+-Deficiency Causes a Reversible Cessation of Spermatogenesis

Histological evaluation of the testicular shrinking process (**Figure 3**) in testis from animals on the niacin-free ND diet revealed progressive seminiferous epithelial defects compared to control animals on CD +Dox (**Figures 3A–D**). Seminiferous tubules showed a lack of ongoing spermatogenesis with severely decreased numbers of spermatogonia and spermatocytes, as well as an abnormal spatio-temporal organization (**Figures 3C, D**). Seminiferous epithelia of

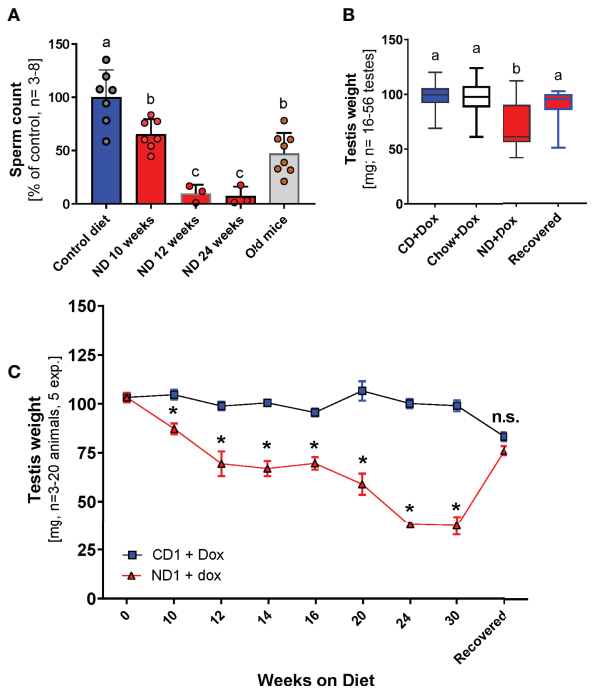

FIGURE 2 | Testicular decline when NAD+ levels are low. **(A)** Sperm counts of old mice and ANDY mice on ND+Dox diet (10 weeks) were significantly lower than controls, but not statistically different from each other. Sperm counts of ND+Dox fed mice were dramatically lower after 12 weeks and remained low after that (one-way ANOVA with Dunett's multiple comparison analysis comparing % of sperm count at indicated time points with sperm counts at start of study, only sperm from ND diets were significantly different; P-values range was 0.0001 to 0.01). **(B)** Testes of ANDY mice on ND diet were significantly smaller on average (1-Way ANOVA, p= 0.0001 Tukey's multiple comparisons: ND group is significantly different from recovered ND (9 weeks recovery on CD diet): p<0.0001; Chow:: p<0.0001 CD1: p<0.0001. ND recovered and CD- or chow-fed group chow were not significantly different from each other. **(C)** Testis weights significantly declined in ANDY mice on niacin-deficient (ND) diet over the course of 24 weeks compared to ANDY mice on control diet (CD, 30 mg/kg niacin). After changing ANDY mice that had initially been on ND diet to CD diet for 9 weeks, testis weights recovered in these animals and were no longer significantly different from the CD-fed group. Multiple t tests of row stats, significant difference in weeks 10-30, p<0.0039 and smaller; * indicates significant difference to the control value, values at time points 0 and after recovery not significantly different. Groups marked with the identical letter (a, b or c) were not significantly different from each other; differing letters indicate a significant difference; n.s. indicates no significant difference.

animals kept on ND+Dox for 24 weeks, followed by recovery on niacin-containing CD+Dox for 9 weeks, were restored to full cell complements, consistent with the observed reversal of testicular shrinkage (**Figures 2B, D**). Seminiferous tubules of older mice at 20 month-old appeared mostly normal, except for the appearance of sporadic abnormal seminiferous tubules (asterisk in **Figure 3F**), while seminiferous tubules in testes of 31 month-old mice displayed marked and frequent disorganization of the seminiferous tubules (**Figure 3G**). After 24 weeks on ND+Dox diet, seminiferous tubules were lined mostly by Sertoli cells and some spermatogonia, and contained cells that appeared to be mostly residual round and some elongated spermatids (**Figure 3H**). Quantification of abnormal tubules in testis sections after 16 weeks on the indicated diets showed that NAD+-deficient testes contained significantly more tubules with abnormal composition of the seminiferous epithelium than controls or mice that were first kept on ND+Dox diets for 24 weeks and then recovered on CD for 9 weeks (**Figure 3I**). Taken together, the histological results suggested that a lack of spermatogonial proliferation led to a paucity of promeiotic and meiotic germ cells, which together make up more than half of the testicular weight and size in a normal animal. The remarkable recovery of spermatogenesis and subsequent doubling of testicular volume to a normal state in animals recovered on CD diet further indicates that spermatogonial stem cells remained intact and capable of restoring full spermatogenesis once NAD+ levels returned to normal levels (**Figures 2B, C** and **3E**).

NAD+-Deficiency Did Not Affect Testicular Testosterone Content, but Was Associated With Increased Testicular Retinol Concentrations

We initially hypothesized that the pronounced, but reversible, detrimental effect of low NAD+ levels on testicular function could result from impaired testosterone synthesis and metabolism. Unexpectedly, however, significant differences in testosterone levels due to individual NAD+ status could be detected neither by radioimmuno-assays (**Figure 4A**) nor by LC-MS/MS analyses (**Figure 4B**). Each diet group contained animals with markedly higher testosterone levels than those in their group mates, indicating that the ability to synthesize testosterone was not principally suppressed in any of the diet groups. Because retinoic acid (RA) signaling is essential for spermatogonial proliferation and differentiation, we used LC-MS/MS to analyze vitamin A metabolites in ANDY mice on different diets. Retinal and RA were not detectable using this method, but levels of the precursor molecule retinol were determined to be significantly elevated in ND +Dox_L, ND+Dox-_S, and old mice compared to control animals, with concentrations being the highest in the ND+Dox_L group (**Figure 4C**), suggesting a possible negative correlation of testicular retinol- and NAD+-levels (**Figure 1C**). Testicular retinol and testicular NAD content were highly significantly inversely correlated (**Figure 4E**). The accumulation of testicular retinol may be interpreted as resulting from a reduction or block of the rate-limiting step in the RA synthesis pathway that oxidizes retinol to retinal (**Figure 4D**), which is mediated by the NAD+-dependent enzyme retinol dehydrogenase (RDH10). Retinal is further oxidized to RA by aldehyde dehydrogenases ALDHA1/2/3, which are NAD (P)+ dependent enzymes as well. Therefore, NAD+ deficiency could lead to an inhibition of RA in the testis, potentially contributing to the observed spermatogonial proliferation and differentiation defect observed in ND+Dox mice.

DISCUSSION

The main results of this study are (i) that NAD+ deficiency can be produced in ANDY mouse testes on a niacin-free diet, and that

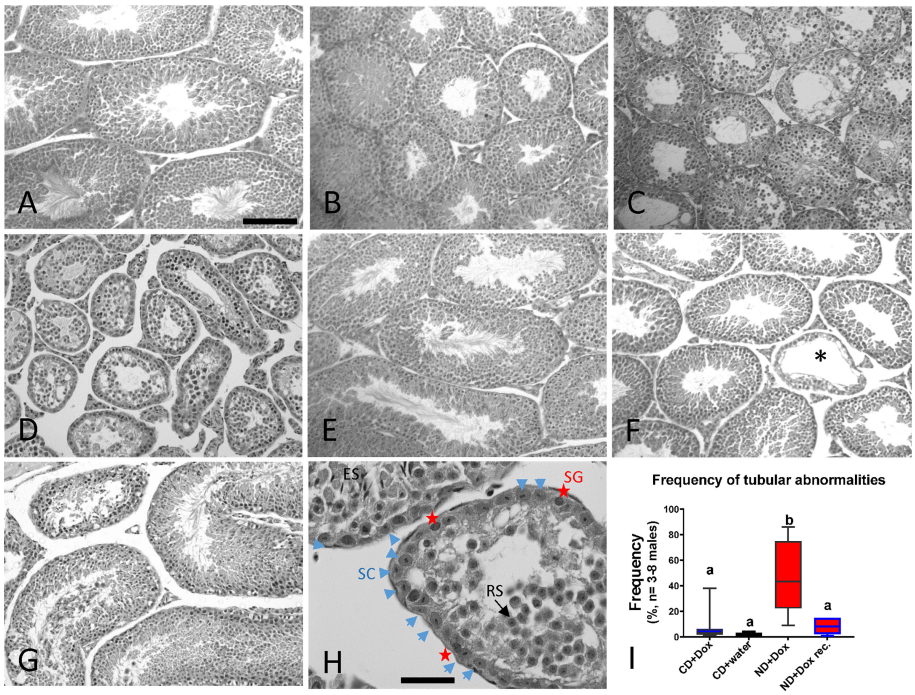

FIGURE 3 | Impact of testicular NAD+ levels and male age on the seminiferous epithelium. Hematoxylin/eosin-stained testicular sections of testes from young adult male fed **(A)** CD+Dox control diet, **(B)** ND+Dox diet for 8 weeks, **(C)** ND+Dox for 14 weeks; damaged tubules **(D)** ND+Dox for 24 weeks; normal tubules +/- absent **(E)** ND for 24 weeks followed by 9 weeks of recovery on niacin-containing CD diet; tubules mostly restored. **(F)** Control testis at 20 months of age. Asterisk marks a tubule with abnormal seminiferous epithelium. **(G)** Control testis of 31 month-old mouse. **(H)** After 24 weeks on ND diet, seminiferous tubules are lined mostly by Sertoli cells (SC, blue arrow heads) interspersed with spermatogonia (SG, red stars), as identified by histological morphology of the cells. Tubular lumen contain mostly cells resembling round spermatids (RS, black arrow) and occasionally elongated spermatids (ES)). **(I)** NAD+-deficient testis contain significantly more tubules with abnormal composition of the seminiferous epithelium in testis of mice that were on indicated diets for 16 weeks (CD+Dox, CD+water, ND+Dox) or ND+Dox that were subsequently recovered on niacin containing diet for 9 weeks. One hundred tubules were evaluated per testis section, ANOVA with Tukey's multiple comparison, b is significantly different from a, p=0.0003 to 0.001. Scale bar: 250 mm in a.-f., 40 mm in (g) & h.

the degree of this deficiency increases over time (**Figure 1**). To our knowledge, this is the first time this has been accomplished in a laboratory research animal. (ii) The degree of NAD+ decline that was achieved by keeping ANDY mice on niacin-free diet for 10-12 weeks was typical of an aging mouse (**Figure 1**). (iii) Low testicular NAD+ levels resulted in the attenuation of spermatogenesis and testicular atrophy due to impaired spermatogonial proliferation and differentiation (**Figure 3**). (iv) Recovery of mice on a niacin-containing control diet fully reversed testicular shrinkage and fully restored spermatogenesis (**Figure 3E**). Because NAD+ decline resulted in attenuation of spermatogenesis in ANDY mice, it may represent a link between low NAD metabolism as a hallmark of aging, and the decline of male fertility as males age.

Based on our data, low testosterone levels were not the determining factor for the observed hypo- and aspermatogenesis (**Figure 4**). However, the loss of mature germ cell stages in severely NAD+ depleted testes and overall seminiferous tubule histology was reminiscent of vitamin A-deficient males, where tubules appear to have only Sertoli cells and early stages of spermatogonia left in the tubular lumen (24). Vitamin A1 (retinol) is essential for spermatogenesis because it is the dietary precursor for RA synthesis (25) (**Figure 4i**). RA signaling is indispensable for spermatogonial proliferation and differentiation. If blocked by the inhibitor WIN 18,446 in adult rodents or humans, spermatogonial differentiation is disrupted and a vitamin A deficiency phenotype is created in the testis (24, 26–28). The rate-limiting step of RA synthesis is the oxidation of retinol to retinal by retinol dehydrogenase (RDH10), which is entirely dependent on the availability of NAD+ as a cofactor. In addition, the next step in RA synthesis is the conversion of retinal to retinoic acid, which is dependent on NADP+, whose levels are linked to cellular NAD+ stores (**Figure 4D**). This step, which is mediated by the aldehyde dehydrogenase (ALDHA) family of enzymes is also essential for testicular RA synthesis, and thus for the execution of spermatogenesis (27). Our finding that retinol appeared to accumulate to significantly higher levels in both NAD+-deficient and aging mice in a manner significantly inversely correlated with NAD+ levels (**Figure 4E**) therefore provides an intriguing clue that low NAD+ levels may block RA synthesis and thus cause the observed spermatogenic failure. However, additional investigations will be necessary to provide further confirmation of this hypothesis. Mechanisms underlying the aging process are still poorly understood, in part because effects of chronological aging are numerous and difficult to separate from environmental and intrinsic factors affecting a given individual over time. The NAD+ decline

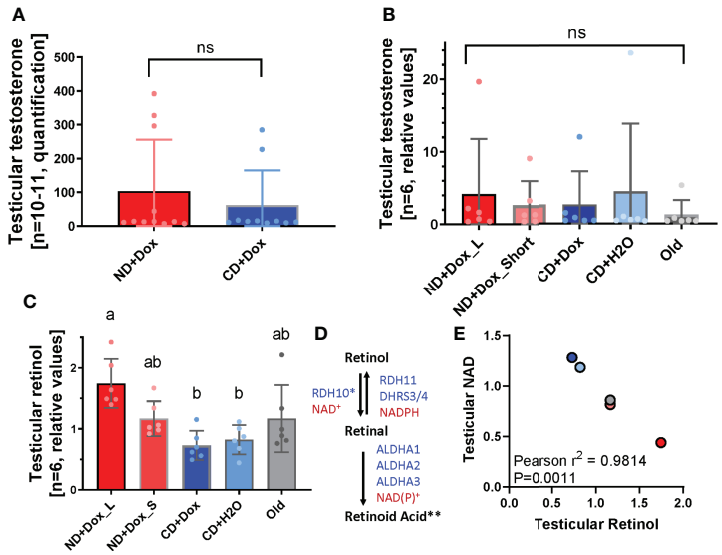

FIGURE 4 | Unchanged testicular testosterone, elevated retinol levels in niacin-deficient ANDY mice with NAD+ decline. **(A)** Testicular testosterone levels were not significantly different between ND+Dox and CD+Dox groups (one-tailed unpaired students t-test, p=0.238). **(B)** Metabolomic analysis confirmed that result. **(C)** Mice in the ND+Dox groups had significantly elevated testicular retinol (vitamin A) concentrations (1-Way ANOVA, p= 0.0005; Tukey's multiple comparisons: ND+Dox_L is significantly different from CD+Dox: p=0.0005; CD+H2O: p=0.0015, but not different from ND+Dox_S and old mice. Differences between CD+Dox, CD+H2O, ND +Dox_S and old mice did not reach statistical significance. Groups marked with the identical letter (a, b) were not significantly different from each other; differing letters indicate a significant difference; ns indicates no significant difference. **(D)** Retinol metabolism to form retinoic acid, which is an essential signal molecule in spermatogonial differentiation and proliferation and regulator of meiosis. Retinol (vitamin A) is transported into the testicular germ and Sertoli cells, and then converted into retinal by NAD^+-dependent oxidation mediated by retinol dehydrogenase 10 (RDH10), which is the rate-limiting step (*) in the conversion to retinal. Alternatively, retinol can be converted to retinal in an NADPH-dependent fashion by RDH11, or by DHRS3 and DHRS4. Retinal is then further oxidized by aldehyde dehydrogenases ALDHA1/2/3 in an $NAD(P)^+$-dependent way to form the active compound retinoic acid [**, after Gewiss, Topping and Griswold, 2019 (26)]. **(E)** Pearson's correlation analysis reveals highly significant inverse correlation between testicular NAD (Y-axis) and testicular retinol (X-axis), with p=0.0011.

observed in aging animals and humans appears to be a consequence of the aging process, for example by means of failing mitochondrial activity, or through elevated consumption of NAD^+ by PARP enzymes or elevated tissue activity of the NAD glycohydrolase CD38 (7, 16–18, 29–32). However, to what extent NAD^+ decline itself may also be a driver of the aging process has remained an open question. The current study takes full advantage of the novel ANDY mouse model that allows for the first time that NAD+ levels in rodents can be lowered significantly, independent of the chronological age of the animal. The results of this initial investigation suggest that low and very low levels of NAD+ result in testicular decline in mice, similar to that observed in aging males. This finding suggests that NAD^+ decline itself may promote aspects of the pathophysiology of aging.

NAD and NADP serve not only as an essential cofactor for enzymatic reaction in energy metabolism; they are also essential cofactors for several cellular mechanisms that protect the genome against DNA damaging insults, e.g. from reactive oxygen species (ROS). There is an age-related increases in ROS, the so-called "free radical theory of aging", that is also evident in context of spermatogenesis and sperm quality (33–35). In fact, aging has been associated with reduce genetic quality in spermatogenic cells and sperm (36–38). Because NAD and NADP are required for both, maintaining a sufficient pool of the active antioxidant glutathione GSH, and for the enzymatic activity of PARP1, an important DNA repair factor, lower testicular NAD could potentially contribute to the aging-related accumulation of ROS and decline in sperm quality. S Investigations are currently underway to address this important question.

A potential limitation of the present study is that the degree of testicular NAD^+-decline produced in ANDY mice that were on ND for a long period of time (exceeding 12 weeks) may arguably be more severe than the NAD^+ deficiency measured in the 31 month-old mice. On the other hand, while the spermatogenic defects observed in these old mice may be less severe, they were clearly detectable and may at least in part be caused by NAD^+ deficiency. Furthermore, the dynamics of human testicular NAD^+ decline with age may be different from mice, along with its importance for human male fertility, which will require further research. Additional investigations are currently underway to determine the role of NAD^+-decline in the aging process in ANDY mice. In summary, this study is the first one to show that experimentally induced low testicular NAD^+ levels result in reversible disruption of spermatogenesis, adding vitamin B3 to the list of vitamins that are essential for proper spermatogenesis in humans. The study also provides clues to the role of NAD^+ decline in the age-related decline of testicular function and male fertility.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committees (IACUC) of Utah State University and of Mayo Clinic, Rochester, Minnesota.

AUTHOR CONTRIBUTIONS

MM-F and RM contributed conception and design of the study, experimentation, data analysis and preparation of the manuscript. AZ, CS, AT, SL, MW and HC performed experiments, collected and analysed data. GW, KT, CC, and EC contributed materials. All authors contributed to manuscript revision, read, and approved the submitted version.

FUNDING

Research reported in this publication was supported by the Eunice Kennedy Shriver National Institute Of Child Health & Human Development of the National Institutes of Health under Award Number HD100970 (to RM) and HD103027 (to MM-F) and by the National Institute On Aging of the National Institutes of Health under Award Number AG069745 (to MM-F and EC), and by a Utah Agricultural Experiment Station grant, Utah State University (UTA01403, to RM).

CS was supported by a Veterinary Student Summer Research Fellowship of the School of Veterinary Medicine, Utah State University.

ACKNOWLEDGMENTS

The authors gratefully acknowledge assistance with testosterone measurements by Barry R. Zirkin.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.896356/full#supplementary-material

Supplementary Figure 1 | Time course of blood NAD levels in ANDY mice kept on indicated diets. Adult, sexually mature ANDY males ranging in age from 7-14 weeks were placed on indicated diets. Blood samples were taken prior to initiation of diet (pre-diet), after 2, 4, 6 and 11 weeks on diet. NAD content was determined using enzymatic cycling assays. Blood NAD levels decline between 2-6 weeks on diet. No further drop in blood NAD levels were observed between 6 and 11 weeks on diet. Significance of differences in A. was determined using 2-way ANOVA with Tukey's multiple comparison, in B. 1-way ANOVA. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.Letters a, b, c in figure B indicate significant changes with p-values in the *** to **** range. Identical letters indicate that there was no statistically significant difference.

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MINI REVIEW
published: 12 May 2022
doi: 10.3389/fendo.2022.895528

Roles of Spermatogonial Stem Cells in Spermatogenesis and Fertility Restoration

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OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology

Received: *14 March 2022*
Accepted: *31 March 2022*
Published: *12 May 2022*

Citation:
Diao L, Turek PJ, John CM, Fang F and Reijo Pera RA (2022) Roles of Spermatogonial Stem Cells in Spermatogenesis and Fertility Restoration. Front. Endocrinol. 13:895528. doi: 10.3389/fendo.2022.895528

Spermatogonial stem cells (SSCs) are a group of adult stem cells in the testis that serve as the foundation of continuous spermatogenesis and male fertility. SSCs are capable of self-renewal to maintain the stability of the stem cell pool and differentiation to produce mature spermatozoa. Dysfunction of SSCs leads to male infertility. Therefore, dissection of the regulatory network of SSCs is of great significance in understanding the fundamental molecular mechanisms of spermatogonial stem cell function in spermatogenesis and the pathogenesis of male infertility. Furthermore, a better understanding of SSC biology will allow us to culture and differentiate SSCs *in vitro*, which may provide novel stem cell-based therapy for assisted reproduction. This review summarizes the latest research progress on the regulation of SSCs, and the potential application of SSCs for fertility restoration through *in vivo* and *in vitro* spermatogenesis. We anticipate that the knowledge gained will advance the application of SSCs to improve male fertility. Furthermore, *in vitro* spermatogenesis from SSCs sets the stage for the production of SSCs from induced pluripotent stem cells (iPSCs) and subsequent spermatogenesis.

Keywords: spermatogonia, spermatogenesis, *in vivo*, *in vitro*, stem cell, 3D culture, male infertility

INTRODUCTION

Early in human development, a small group of cells is set aside or allocated to become the germ cells that give rise to the sperm and oocytes that will transmit genetic and epigenetic information to subsequent generations (1). In males, the process of spermatogenesis maintains the production of spermatozoa, the final cell carrier of inheritable material, throughout the lifetime of male mammals (2). Continuous spermatogenesis depends on the appropriate self-renewal and differentiation of spermatogonial stem cells (SSCs) throughout the life of the male (3). The SSCs are the resident stem cell population that resides at the basal membrane of seminiferous tubules of the testis (4, 5). The SSCs can undergo mitotic divisions for self renewal to maintain a steady stem cell pool or they can differentiate through sequential and extensive processes into spermatozoa (6). The balance of self-renewal and differentiation of SSCs is critical, not only for maintaining normal spermatogenesis but

also for sustaining lifelong fertility (7). A tilt to self-renewal is a risk factor for germ cell tumors, while a tilt towards differentiation results in exhaustion of germ cell pools, leading to male infertility (8). Numerous studies have demonstrated that the balance between self-renewal and differentiation is precisely controlled by a combination of intrinsic genetic and epigenetic factors within SSCs as well as the extrinsic signals that eminate from the somatic niche (9, 10).

Significantly, SSCs have extraordinary therapeutic potential in assisted reproduction for male infertility (11, 12). Transplantation of SSCs can restore spermatogenesis in patients who suffer from impaired spermatogenesis (13). One application example is fertility preservation of prepubertal boys with cancer and undergoing chemotherapy (14). SSCs can be isolated from testicular biopsy and cryopreserved before chemotherapy, followed by stem cell transplantation into the seminiferous tubules to restore fertility (15, 16). In addition, germline gene therapy using SSCs has been proposed, albeit with obvious concerns regarding legitimate ethical issues, as a promising and feasible approach to treat endocrine disease and metabolic disorders with germline gene mutations (17). Currently, the major hurdle to the use of SSCs in assisted reproductive technology is the difficulty of identificating and isolating endogenous SSCs and directing their differentiation to haploid cells *in vitro*.

This review provides a brief overview summary of some of the existing knowledge and research progress regarding use of SSCs for inducing spermatogenesis *in vivo* and *in vitro* for fertililty restoration. We hope that this summary review may spur further inquiries into details and ongoing studies of practical applications of SSCs in human reproduction and regenerative medicine.

REGULATION OF SSCS

Human germ cell development begins with the specification of a small group of cells to form the primordial germ cells (PGCs) (18), which are thought to arise from the dorsal amnion at the onset of gastrulation (19). Following their specification, PGCs actively proliferate and migrate to the developing gonad (20–22) where they will occupy the genital ridge and undergo sex-determination by entering either male or female sex-specific developmental pathways (23). External signals from the somatic environment determine the sex of PGCs (24). For male germ cell development, once PGCs occupy the seminiferous tubules of the male gonad, they are termed gonocytes (25), which later interact with the niche cells to become spermatogonia (26). Note that nomenclature is not universal or all inclusive as subtypes exist (example: type A, type b, light and dark spermatogonia), different stages of development are sometimes indicated (examples: early or late spermagonia or undifferentiated and differentiating), or reference to marker content (example: c-kit+ spermatogonia).

The Niche

The architecture of the testes is characterized by two structurally distinct compartments (**Figure 1**), the seminiferous tubule and the interstitial tissue (27). Within the seminiferous tubule, Sertoli cells form a tight blood-testis barrier to divide the seminiferous epithelium into basal and luminal compartments (28). Developing spermatogonia reside on the basal membrane and are further defined by three types of cells: undifferentiated spermatogonia (quiescent SSCs), differentiating spermatogonia (SSCs that undergo active mitosis), and differentiated spermatogonia (29, 30). The Sertoli cells are the supporting cells for the germ cell population in the testes and are essential for maintaining normal spermatogenesis by providing the cellular matrix and by secreting specific growth factors (31). The surrounding interstitial space consists of various cell types that include the Leydig cells, mesenchymal cells, and immune cells, in addition to lymph vessels, nerve fibers, and connective tissues (27). Leydig cells produce the hormone testosterone and cytokines that may function both directly and indirectly to regulate self-renewal of SSCs (32).

External and Intrinsic Factors

The fine-tuned balance between self-renewal and differentiation of SSCs is regulated by the interplay of extrinsic and intrinsic factors. GDNF, a growth factor produced by the somatic niche cells, is critical for the maintenance of SSCs both *in vivo* and *in vitro* (33). It regulates several essential downstream genes, including the germ cell specific and ubiquitously-expressed genes *Nanos2*, *Etv5*, *Lhx1*, *T*, *Bcl6b*, *Id1*, and *Cxcr4*, to promote SSC self-renewal and inhibit differentiation (34–39). CXCL12/CXCR4 (39), FGFs (33, 40), and VEGF-A (41) act in synergy with GDNF to maintain SSC stem cell status. In contrast, retinoic acid (RA), a hormone secreted primarily by Sertoli cells, plays an indispensable role in inducing differentiation of SSCs by downregulation of GDNF expression and activation of differentiation-promoting factors, such as BMP and SCF (42–45). Genetic ablation studies in mice indicate that several transcription factors are involved in regulating SSC maintenance and recruitment to spermatogenesis. The PLZF transcription factor is expressed by SSCs and interacts with GDNF signaling as one of the master regulators to promote the self-renewal of SSCs (46, 47). Loss of PLZF results in progressive germ cell loss, testicular hypoplasia, and infertility (46–48). One of the downstream targets of PLZF is the SALL4 protein, which is required for the self-renewal of SSCs and maintenance of ability to enter spermatogenic differentiation (49). A potential upstream regulator of PLZF is PRMT5. Disruption of the *PRMT5* gene results in a dramatic reduction of *PLZF* gene expression, and subsequent progressive loss of SSCs leading to male infertility (50). Another transcription factor important for maintenance of SSC self-renewal is FOXO1, which regulates a number of genes that are preferably expressed in SSCs (51). Deletion of the *FOXO1* gene results in defects in SSC maintenance and ultimately spermatogenic failure. In addition, recent research has identified numerous microRNAs as critical regulators in spermatogenesis. Some microRNAs regulate the self-renewal of SSCs. For example, miR-202 plays a crucial role in the maintenance of SSC stemness or self-renewal of the stem cell population (52). Other microRNAs, such as miR-1908-3p (53), miRNA-122-5p (54), and miRNA-31-5p (55), enhance the

proliferation and inhibit the early apoptosis of human SSCs *via* targeting key downstream pathways. Conversely, several microRNAs facilitate differentiation *via* regulation of the expression of genes associated with SSC differentiation. MiR-34c promotes SSC differentiation by inhibiting the function of the *NANOS2* gene, leading to the up-regulation of meiotic-related proteins, STRA8, in mice (56). Similarly, miR-486-5p secreted by Sertoli cells stimulates differentiation of SSCs in mice by up-regulating the expression of STRA8 and SYCP3 (57). Further, impaired spermatogenesis is observed in mice carrying a deficiency in miR-17-92 or a gene deletion of miR-17-92 (58, 59). miR-202 similarly regulates spermatogenesis *via* orchestration meiotic initiation by preventing precocious differentiation of mouse SSCs (52). Taken together, numerous genes act to balance self-renewal and differentiation of SSCs.

FERTILITY RESTORATION THROUGH *IN VIVO* SPERMATOGENESIS

SSCs within the testicular tissues have the potential to complete the entire process of spermatogenesis *in vivo* and produce functional spermatozoa for fertility restoration (**Figure 2**). Thus, cryopreservation of testicular tissue prior to gonadotoxic treatment for prepubertal boys is proposed as a helpful strategy for fertility preservation (60). To restore fertility through *in vivo* spermatogenesis, testicular tissues could be either autotransplanted to the same individual or the tissues might be dissociated to obtain SSCs for autotransplantation. Xenotransplantation would carry the obvious complication of mixing of sperm from different individuals.

Transplantation of Testicular Tissues

Autotransplantation of testicular tissues has achieved success in multiple animal models, which results in live offspring (61–65). However, the approach has the risk of re-introducing malignancy is a concern (66). Studies of xenotransplantation, which transplants immature testicular tissue under the back skin of immune-deficient animals, have been used to examine potential complications including malignancy. In 2002, Nagano and colleagues, for example, transplanted human SSCs into immunodeficient mice for the first time (67). Human SSCs survived in mouse testes for at least six months and proliferated during the first month after transplantation.

Transplantation of SSCs

To avoid potential complications of malignancy, isolation of SSCs from cryopreserved testicular tissues followed by transplantation has been proposed as the leading alternative stratgey. To separate SSCs from somatic cells, antibodies that recognize human SSC-specific proteins are used for FACS (fluorescent-activated cell sorting) or MACS (magnetic-activated cell sorting) for sorting SSCs from other cell types. Antibodies that have been shown to be useful for sorting SSCs include GFRα (68), GPR125, ID4 (69), ITGA6 (70), SSEA4 (71), PLPPR3 (72), and OCT4 (73). An alternative to cell sorting is to take advantage of different physical properties between SSCs and somatic cells such as velocity sedimentation and differential affinity to extracellular matrices on the culture plate (74–78). Once isolated, SSCs are cultured with growth factors shown to be optimal or essential for SSC maintenance [GDNF, BFGF, EGF, and LIF (79–81)].

A major limitation of SSC transplantation *in vivo*, for fertility restoration in clinical practice, is the scarcity of SSCs within the testicular tissue. This has necessitated exploration of alternatives including the establishment of a robust *in vitro* culture system to maintain and expand human SSCs. Extensive effort has been focused on optimization of culture conditions for long-term maintenance and propagation of human SSCs. Multiple culture substrates, including hydrogel, matrigel, and laminin, have been shown to promote the propagation of human SSCs under feeder-free conditions (82). Currently, several markers are used for the verification of human SSCs. However, many of these markers are also expressed in testicular somatic cells. For example, UCHL1, which was used to identify SSCs from humans, is also expressed in Leydig cells and nerve fibers (83). The most stringent assay to assess the function of SSCs is to generate offspring after homologous transplantation. However, despite success in animal models, including non-human primates, no studies are reporting the generation of human functional spermatozoa following autotransplantation or xenotransplantation of testicular tissue or isolated human SSCs for fertility restoration.

FERTILITY RESTORATION THROUGH *IN VITRO* SPERMATOGENESIS

The establishment of a system to recapitulate spermatogenesis and generate spermatozoa *in vitro* can not only be directly applied in assisted reproduction, such as *in vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI), but also provide a convenient system to study the molecular mechanisms and genetic causes for male infertility. Building a functional somatic microenvironment is critical for *in vitro* spermatogenesis. Several strategies, including exploitation of intrinsic somatic microenvironment by organotypic culture, two-dimensional culture, and three-dimensional culture of testis cell suspensions.

Organotypic Culture

Since 1959, a gas-liquid interface was used to culture testicular fragments of the adult rats (84). In this culture system, the differentiation of SSCs was limited up to pachytene spermatocytes (85). In 2003, round spermatids were observed after two weeks of culture in a gas-liquid interface culture system (86). Several other organotypic culture systems have been developed to recapitulate the entire process of spermatogenesis *in vitro*. One of the breakthroughs in the research was reported in 2011 with the demonstration of live offspring that were generated from *in vitro*-produced haploid germ cells (87). In this study,

testicular tissue fragments from neonatal mice were cultured on an agarose gel-based organ culture system. Subsequently, microfluidic technology was adopted for organ culture, with the goal of providing a better culture environment for SSCs by facilitating the exchange of gases, nutrients, and waste products (88). Recently, successful recapitulation of human testicular organogenesis from fetal gonads was achieved, and *in vitro*-derived haploid spermatids were shown to undergo meiotic recombination (89).

Two-Dimensional Culture

2D culture systems with testis cell suspensions have been widely used for SSC proliferation and differentiation with two primary types of 2D culture systems most common: (1) SSCs cultured on mitotically-inactivated feeder cells, (2) SSCs co-cultured with somatic cells (90). Using the support of 2D culture sytems, numerous studies have reported that haploid male germ cells could be induced (91–95), and offspring can be produced from these *in vitro* derived haploid male germ cells in rodent (96). However, the 2D culture system has not been optimized for human germ cells. This may be due to the lack of spatial structure of seminiferous tubules and proper interactions between germ cells and somatic cells.

Three-Dimensional Culture

To better mimic the testicular niche, various 3D culture systems have been developed. In 2006, testicular cells isolated from rats were cultured on collagen gels to mimic the composition of the basal membrane of seminiferous tubules (97). Later, the soft-agar culture system (SACS) was developed (98), and mice haploid germ cells from undifferentiated germ cells were generated in this system in 2012 (99). The SACS system also supports the differentiiation of SSCs of non-human primates. The most commonly used alternate material in 3D culture system is methylcellulose. The methylcellulose culture system (MCS) also supports the differentiation of immature germ cells.

In order to artificially reproduce the *in vivo* form and function of the seminiferous epithelium, a 3D engineered blood-testis barrier (eBTB) system was designed in 2010 (100). Testicular peritubular myoid cells were first cultured on the underside of culture inserts, and then germ cells and Sertoli cells were added on top of the inserts. The testicular cells from neonatal mice form the aggregate by culturing on a V-shaped plate. The aggregate plated on the top of agarose gel blocks, and the haploid male germ cells were obtained after 30-51 days of incubation (101).

The 3D decellularized testicular scaffold with hyaluronic acid and chitosan provides the condition for the differentiation and proliferation of mice SSCs (102). The proliferation and self-renewal of mice SSCs was stimulated by culturing on the 3D scaffold consisting of alginate hydrogel with Sertoli cells (103). The mice germ cells were cultured in 3D printed one-layer scaffolds at the air-medium interface simulating the tubule-like structure. This culture system provided the condition for long-term survival and differentiation (104).

Soft agar and agarose gel are the most common material used to establish the 3D culture system for human SSCs. A soft agar culture system has been shown to support the proliferation and differentiation of human SSCs (105). Another material that has been used in 3D culture systems for human SSCs is a polycaprolactone (PCL) nanofiber matrix (106). This material may mimic the physical form of collagen fibers in the natural extracellular matrix (107).

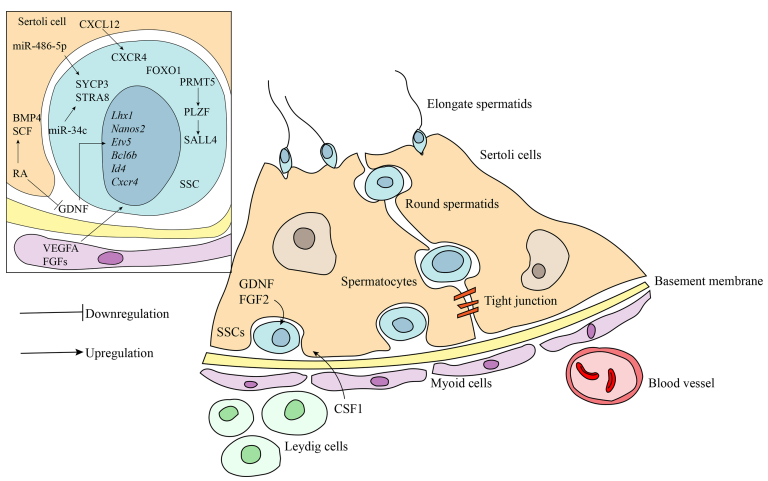

FIGURE 1 | Schematic diagram of the niche of SSCs and the regulatory factors involved in maintaining the stemness and self-renewal of SSCs. Undifferentiated SSCs are localized at the basement membrane. Germ cells maintain the close contact with the Sertoli cells inside the seminiferous epithelium. Peritubular myoid cells surround the seminiferous tubules to form testicular cords. The interstitial compartment consists of many somatic cell types including Leydig cells, mesenchymal cells and immune cells. Bioactive factors in the niche play crucial role in self-renewal and differentiation of SSCs. CXCL12/CXCR4, FGFs, and VEGFA act in synergy with GDNF to maintain SSCs. Retinoic acid (RA) induces the differentiation of SSCs by downregulation, at least in part, of GDNF expression and activation of SCF and BMP4. Transcription factors, PLZF and FOXO1, are involved in regulating SSCs maintenance and spermatogenesis by acting on a subset of downstream target gene. MicroRNAs, including miR-1908-3p, miR-112-5p and miR-31-5p, also act as critical regulators in spermatogenesis.

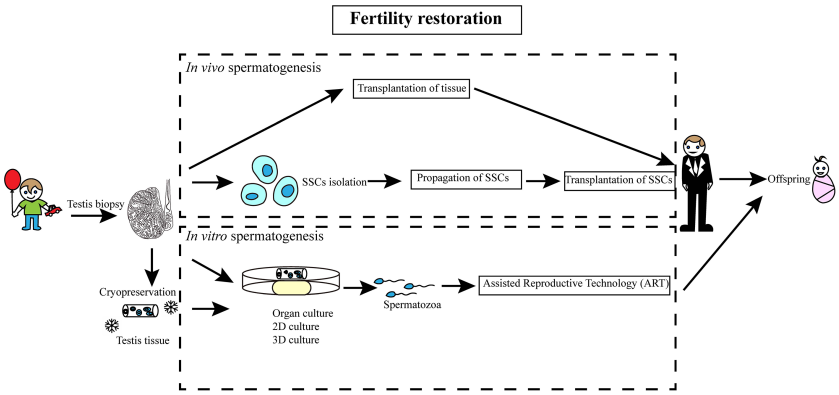

FIGURE 2 | Schematic diagram of SSC-based fertility restoration in humans. A sample of testicular tissue of prepubertal boys, who receive gonadotoxic treatment, is retrieved and cryopreserved. Spermatogenesis may be induced after treatment either *in vivo* or *in vitro*.

CONCLUSION AND PERSPECTIVES

With the development of technologies, including -omics at the single-cell level, lineage-tracing, spermatogonial transplantation, and *in vitro* culturing and differentiation, we start decoding the secrets of SSCs. However, the application of SSCs to treat male infertility necessitates extensive studies to ensure safety and efficacy. An efficient culture condition for human SSCs to ensure their propagation, as well as proper animal models for xenotransplantation, will assist in assessing safety and efficacy as indicated by recent studies (108). Furthermore, establishing a robust system for *in vitro* spermatogenesis is also helpful for pharmaceutical or toxicological studies for new drugs. Finally, *in vitro* spermatogenesis from SSCs sets the stage for the production of SSCs from induced pluripotent stem cells (iPSCs) and subsequent spermatogenesis. For example, studies are underway to integrate data and practices from divergent fields to promote spermatogenesis from iPSCs *via* co-culture with Sertoli cells in a 2D-, 3D- or a modified environment, similar to those used in other physiological systems, that might more faithfully mimic spermatogenic dynamics including circulation (109, 110).

AUTHOR CONTRIBUTIONS

All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by an NIH grant to RRP #HD096026 and National Natural Science Foundation of China #32070830 to FF.

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Conflict of Interest: Authors PJT and CMJ are founders of the company MandelMed. No funding from MandelMed is associated with this study.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
published: 13 May 2022
doi: 10.3389/fendo.2022.896507

Role of the Ubiquitin Ligase RNF149 in the Development of Rat Neonatal Gonocytes

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OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to
Reproduction,
a section of the journal
Frontiers in Endocrinology

Received: 15 March 2022
Accepted: 11 April 2022
Published: 13 May 2022

Citation:
Manku G, Kong C-C and Culty M
(2022) Role of the Ubiquitin Ligase
RNF149 in the Development
of Rat Neonatal Gonocytes.
Front. Endocrinol. 13:896507.
doi: 10.3389/fendo.2022.896507

Male reproductive function depends on the formation of spermatogonial stem cells from their neonatal precursors, the gonocytes. Previously, we identified several UPS enzymes dynamically altered during gonocyte differentiation. The present work focuses on understanding the role of the RING finger protein 149 (RNF149), an E3 ligase that we found to be strongly expressed in gonocytes and downregulated in spermatogonia. The quantification of RNF149 mRNA from postnatal day (PND) 2 to 35 (puberty) in rat testis, brain, liver, kidney, and heart indicated that its highest levels are found in the testis. RNF149 knock-down in PND3 rat gonocytes was performed to better understand its role in gonocyte development. While a proliferative cocktail of PDGF-BB and 17β-estradiol (P+E) increased both the expression levels of the cell proliferation marker PCNA and RNF149 in mock cells, the effects of P+E on both genes were reduced in cells treated with RNF149 siRNA, suggesting that RNF149 expression is regulated during gonocyte proliferation and that there might be a functional link between RNF149 and PCNA. To examine RNF149 subcellular localization, EGFP-tagged RNF149 vectors were constructed, after determining the rat testis RNF149 mRNA sequence. Surprisingly, two variant transcripts were expressed in rat tissues, predicting truncated proteins, one containing the PA and the other the RING functional domains. Transfection in mouse F9 embryonal carcinoma cells and C18-4 spermatogonial cell lines showed differential subcellular profiles of the two truncated proteins. Overall, the results of this study support a role for RNF149 in gonocyte proliferation and suggest its transcription to variant mRNAs resulting in two proteins with different functional domains. Future studies will examine the respective roles of these variant proteins in the cell lines and isolated gonocytes.

Keywords: germ cells, gonocytes, ubiquitin proteasome system, ubiquitin ligase, proliferation

INTRODUCTION

Spermatogenesis is a process that encompasses numerous steps including phases of quiescence, proliferation, differentiation, migration, and apoptosis to ensure the production of sperm throughout the lifetime of a male (1). At the origin of sperm formation is the existence of a pool of germline stem cells, the spermatogonial stem cells (SSCs), originating from neonatal precursors, the gonocytes, also known as pre- or pro-spermatogonia (1, 2). We have previously shown that rat neonatal gonocytes undergo proliferation in response to platelet-derived growth factor (PDGF)-BB (P) and 17β-estradiol (E) while activating both the PDGF receptor (PDGFR) and Estrogen Receptor (ER) signaling pathways (3, 4). Furthermore, we have shown that gonocyte differentiation is induced by retinoic acid (RA) with activation of the PDGFR, JAK2, STAT5, and SRC signaling pathways (5, 6). While studying genes involved in gonocyte apoptosis, we also found that pro-apoptotic genes Cycs and Gadd45α were significantly upregulated in differentiating gonocytes, indicating their possible role in gonocyte apoptosis, a necessary step for eliminating gonocytes that failed to differentiate to spermatogonia (7). Studies have suggested that improper development of gonocytes can lead to the formation of testicular germ cell tumors (TGCTs), the most common type of cancer in young men (8, 9). Testicular cancer rates have been steadily increasing for the past few decades for reasons that are not completely known (10) and as a result, a better understanding of gonocyte development can help provide a more thorough insight into how testicular tumors form and why the incidence has been increasing.

We have previously shown that the ubiquitin proteasome system (UPS) is involved in gonocyte differentiation and that proteasome inhibition significantly reduced RA-induced gonocyte differentiation (11). UPS is the main pathway by which proteins are degraded in eukaryotes, involving a succession of enzymatic reactions involved in the attachment of an ubiquitin chain to a substrate protein targeted for degradation, including an E1 activating enzyme, an E2 conjugating enzyme, and an E3 ligase (12, 13). UPS activity regulates a variety of developmental and biological functions, such as myogenesis, bone formation and immune function (14–16). During the ubiquitination process, the E1 activating enzyme activates the ubiquitin molecule which is then transferred to the E2 conjugating enzyme and finally to an E3 ligase. The E3 ligase attaches the ubiquitin tag to the substrate to be targeted, which is then recognized by the 26S proteasome and degraded. Deubiquitinating enzymes modulate the pathway by removing ubiquitin molecules as needed (17, 18). Besides its major role in protein degradation, the UPS has been shown to be involved in other processes including signal transduction, kinase activation, cell cycle progression, cell proliferation, and protein interaction regulation (12, 13, 19, 20), while also playing an important role in the later stages of spermatogenesis (21). Given that spermatogenesis occurs *via* multiple tightly timed biological events (1), it is not surprising that the UPS is involved in this process, as spermatogenesis requires a large amount of protein turnover and degradation. Our study of UPS gene and protein expression in rat PND3 gonocytes and PND8 spermatogonia showed that the E3 ubiquitin ligase RNF149 (also known as DNA polymerase-transactivated protein 2; DNAPTP2) was downregulated in spermatogonia compared to neonatal gonocytes, as well as in gonocytes that had undergone RA-induced differentiation compared to undifferentiated germ cells of the same age (11). This suggested a likely role of RNF149 in neonatal gonocyte development. The present study further examined the role of E3 ubiquitin ligase RNF149 in gonocyte development, revealing its involvement in cell proliferation, and the existence of two variant forms of the protein with distinct functional moieties observed in different subcellular compartments.

MATERIALS AND METHODS

Animals and Tissue Collection

Newborn male Sprague Dawley rats obtained from Charles Rivers Laboratories (Saint-Constant, QC, CA). Rats aged from PND2 to PND35 were euthanized and handled according to protocols approved by the McGill University Health Centre Animal Care Committee and the Canadian Council on Animal Care. Several organs, including brain, heart, liver, kidneys and testes, were collected and either frozen for gene expression analysis by quantitative Real Time PCR analysis (qPCR), or fixed in 4% paraformaldehyde for immunohistochemical analysis.

Gonocyte Isolation

Gonocytes were isolated from PND3 rat testes following a well-established protocol as previously described (3, 4, 6). In short, testes from 40 rats were isolated and decapsulated. Gonocytes were isolated by sequential tissue enzymatic digestion and differential plating overnight in RPMI 1640 medium (Invitrogen, Thermo Fisher Scientific, ON, CA) with 5% fetal bovine serum (FBS) (Invitrogen), 2% penicillin/streptomycin (CellGro, Manassas, VA, USA), and 1% amphotericin B (CellGro). During overnight plating, somatic cells adhered to the culture plates while germ cells remained non-adherent. The next day, non-adherent germ cells were further separated using a 2-4% bovine serum albumin (BSA) (Roche Diagnostics, Indianapolis, IN, USA) gradient. Gonocytes were judged by morphology and larger size compared to Sertoli/myoid cells by phase contrast microscopy. Fractions containing the most gonocytes were pooled, centrifuged, and collected for treatments or RNA analysis with a purity of at least 85%. Cell viability was assessed by trypan blue exclusion assay, together with live gonocyte quantification on hemacytometer. The enrichment efficacy, viability, and identity of the gonocytes were validated using a variety of approaches in previous studies (3, 7, 11, 22). Samples with lower purity were used for immunocytochemical analysis. All experiments were performed using a minimum of three independent gonocyte preparations.

RNF149 Silencing and Gonocyte Treatment

For RNF149 silencing, after gonocyte collection from BSA gradient, cells were plated at a density of 10000 cells/well in a 24-well plate using RPMI 1640 media free of antibiotics, 2.5% FBS, and amphotericin B, as they are not recommended while using siRNA protocols. Gonocytes were then treated with Mock, Scrambled (10nM), or RNF149 silencing duplexes (at various concentrations) (siRNA TriFECTa Kit, IDT Inc., San Jose, CA, USA) using Lipofectamine RNAiMAX (Invitrogen) and Opti-MEM transfection medium (Invitrogen). The three silencing duplexes used were: (1) Sense Strand: 5'-GGAAUUGUGAA AUGUAGUUCCUUAT-3', Antisense Strand: 5'-AUAAGGAA CUACAUUUCACAAUUCCAC-3', (2) Sense Strand: 5'-ACCUGUAAAGUGAGAAAUCUUGCCA-3', Antisense Strand: 5'-UGGCAAGAUUUCUCACUUUACAGGUUC-3', (3) Sense Strand: 5'-GGAAACUAAGAAGGUUAUUGG CCAG-3', Antisense Strand: 5'- CUGGCCAAUAACCUUCUU AGUUUCCUU-3'. A red fluorescent dye was transfected at 10nM and served as a positive control. Cells were transfected for 48 hours and were then treated with or without PDGF-BB (Sigma Aldrich, Oakville, ON, CA) and 17β-estradiol (Sigma Aldrich) for an additional 24 hours. This additional 24 hour treatment contained 2.5% FBS and antibiotics. Cells were then collected for RNA analysis and immunocytochemical analysis on microscopic slides.

F9 Mouse Embryonal Teratocarcinoma Cell Culture

As previously described, F9 cells were maintained in DMEM medium (Invitrogen) containing 10% fetal bovine serum (FBS) (Invitrogen) at 37°C and 5.0% CO_2 (5, 6). Cells were plated on gelatin-coated culture dishes on day 1 and treated on day 2. Cells were plated at a density of 30000 cells/well in a 6-well plate using DMEM media free of antibiotics, FBS, and amphotericin B, as they are not recommended while using siRNA protocols. Cells were then treated with Mock, Scrambled (10nM), or RNF149 silencing duplexes (20nM/duplex) (siRNA TriFECTa Kit, IDT Inc., San Jose, CA, USA) using Lipofectamine RNAiMAX (Invitrogen) and Opti-MEM transfection medium (Invitrogen). The three silencing duplexes used were: (1) Sense Strand: 5'-GGCAUACAGUAAUGUCUUUAAAUGA-3', Antisense Strand: 5'- UCAUUUAAAGACAUUACUGUAUGCCUA-3', (2) Sense Strand: 5'- AGCGGAGACUGUAGAACUUGG AAAT-3', Antisense Strand: 5'- AUUUCCAAGUUCUACA GUCUCCGCUCA-3', (3) Sense Strand: 5'- CGCGGGAACA GGAAACAUAGUCGTC-3', Antisense Strand: 5'- GACGAC UAUGUUUCCUGUUCCCGCGUG-3'. A red fluorescent dye was transfected at 10nM and served as a positive control. Cells were transfected for 48 hours and were then treated with or without Glial Cell-Derived Neurotrophic Factor (GDNF, 100ng/ml, Millipore, Etobicoke, ON, CA), Fibroblast Growth Factor (FGF2, 10ng/ml, Millipore) and GDNF Family Receptor 1A (GFRα1, 300ng/ml, R&D Systems, Minneapolis, MN, USA) to promote proliferation or retinoic acid (RA, 10^{-7}M, Sigma Aldrich) to promote differentiation for an additional 24 hours. This additional 24 hour treatment contained 10% FBS and antibiotics. Cells were then collected for RNA analysis.

C18-4 Mouse Spermatogonia Cell Culture

C18-4 cells (a gift from MC Hofmann, Houston TX, USA) were maintained in DMEM medium (Invitrogen) containing 10% fetal bovine serum (FBS) (Invitrogen) at 34°C and 5.0% CO_2. Cells were plated on day 1 and treated on day 2 similarly to how F9 cells were treated above.

RNA Extraction and cDNA Synthesis

Total RNA was extracted from cell pellets using the PicoPure RNA isolation kit (Arcturus, Mountain View, CA, USA) and digested with DNase I (Qiagen, Valencia, CA, USA) as previously described (11). Tissue total RNA of testis and other organs in PND2 to PND35 rat pups were extracted using QIAGEN RNAeasy Mini kit (Qiagen, Santa Clarita, CA) as previously described (19). For quantitative PCR (qPCR) analysis, cDNA was synthesized from the extracted RNA by using the single strand cDNA transcriptor synthesis kit (Roche Diagnostics) following the manufacturer's instructions.

Reverse Transcriptase (RT)-PCR Analysis

RNF149 gene expression in various tissue samples was examined by PCR and gel electrophoresis. Two primer sets were used: (1) RNF149 cloning primers (Reverse, 5'- CGAGCGGTCTC ACTCTTCC-3'; Forward: 3'-TGAGGCTGTCAATGAAGACG-5'), and variant (VA) form testing primers (Reverse: 5'-AAGGAATTCCAGTAAAAATGAGG; Forward: 3'-TTAAAGTTTTCAATACACACTGC-5'). PCR reactions were carried out using GoTaq® DNA polymerase (Promega, Madison, WI, USA) and amplified using the iCycler thermal cycler (Bio-Rad, Hercules, CA, USA). PCR cycle conditions: 95°C for 3 min; 45 cycles of 95°C for 60 sec, 55°C for 60 sec, and 72°C for 2 min; followed by a 10 minute extension at 72°C and a cool down to 4°C. PCR products were then run alongside molecular weight standards (New England BioLabs, Whitby, ON, CA) on a 1.5% agarose gel. Gel densitometry analysis was performed using Multi-Gauge software (FujiFilm, Mississauga, ON, CA).

Quantitative Real Time PCR (qPCR)

QPCR was performed using a LightCycler 480 with a SYBR Green PCR Master Mix kit (Roche Diagnostics) as previously described (6, 22). The primer sets used were designed using the Roche primer design software (Roche Diagnostics) and are listed in **Table 1**. QPCR cycling conditions: initial step at 95°C followed by 45 cycles at 95°C for 10 sec, 61°C for 10 sec, and 72°C for 10 sec. The comparative threshold cycle (C_t) method was used to analyze the data and 18S rRNA was used for data normalization. We initially determined the Ct values of three potential housekeeping genes, GAPDH, Tubulin, and 18S rRNA in cDNA samples from isolated gonocytes cultured for 1 day after siRNA interference, and 18S rRNA showed that it presented minimal changes in Ct values between samples. Assays were performed in triplicate. All experiments were performed using a minimum of three independent sample preparations and the mean ± SEM are plotted.

TABLE 1 | Quantitative real time PCR Primers.

Species	Gene	Forward Primer	Reverse Primer
Rat	Rnf149	TGCACCTTCAAGGACAAGGT	GCGCTCCTGGTTGTAGACC
Rat	Pcna	CGTAGTATCACCAGATGGCATCTTTA	GGACTTAGACGTTGAGCAACTTGG
Rat	Ccnb2	AAAACCTCACCAAGTTCATCG	GAGGGATCGTGCTGATCTTC
Rat	18S	cgggTGCTCTTAGCTGAGTGTCCcG	CTCGGGCCTGCTTTGAACAC
Mouse	Rnf149	CGGTCAGTCTGTGGTGTTTG	CCTTCTTAGTCTCCTTCCTATGATTC
Mouse	Stra8	CTCTCCCACTCCTCCTCCACTC	CGGTATTGCTTGTAAAAGTTGAGATA
Mouse	18S	CGGAATCTTAATCATGGCCTCAGTTC	ACCGCAGCTAGGAATAATGGAAT

Immunohistochemistry

RNF149 protein expression was determined in paraformaldehyde fixed, paraffin-embedded sections of PND2-35 testes and PND3 and PND10 brain, heart, liver, and kidney sections. Slides were stained using previously described methods (22). In brief, slides were first dewaxed and rehydrated using Citrosolv (Fisher Scientific, Toronto, ON, CA) and Trilogy solution (Cell Marque IVD, Rocklin, CA, USA). Following treatment with Dako Target Retrieval solution (DAKO, Burlington, ON, CA), the sections were incubated with PBS (Invitrogen) containing 10% goat serum (Vector Laboratories, Burlington, ON, CA), 1% BSA (Roche Diagnostics) and 0.02% Triton X100 (Promega) for one hour to block non-specific protein interactions. Slides were subjected to a 30% hydrogen peroxide/methanol solution incubation. The sections were then treated with the RNF149 antibody (Santa Cruz, Dallas, TX, USA) diluted in PBS (Invitrogen) containing 1% BSA (Roche Diagnostics) and 0.02% Triton X100 (Promega) overnight at 4°C. Once the overnight incubation was complete, sections were incubated with biotin-conjugated goat anti-rabbit secondary antibody (BD Pharmingen, San Jose, CA, USA) diluted in PBS (Invitrogen) containing 1% BSA (Roche Diagnostics) for 60 minutes at room temperature. Immunoreactivity was detected using streptavidin-peroxidase (Invitrogen) and AEC single use solution (Invitrogen). The sections were then counter-stained with hematoxylin (Sigma Aldrich), coated with Crystal Mount (Electron Microscopy Sciences, Hatfield, PA, USA) and dried, and then cover-slipped using Permount (Fisher, Thermo Scientific) and glass coverslips (Fisher Scientific). Slides were then examined under bright-field microscopy with a BX40 Olympus microscope (Olympus, Center Valley, PA, USA) coupled to a DP70 Olympus digital camera (Olympus). Negative controls were performed by incubating sections with pre-immune Rabbit IgG (Invitrogen).

Immunocytochemistry

Microscopic slides were prepared on a cytospin centrifuge using Mock, Scrambled, and siRNA treated gonocytes (with or without PDGF-BB and 17β-estradiol) for protein analysis. C18-4 cells (detailed above) were also cultured in 8-well chamber slides (BD Falcon, Oakville, ON, CA) and analyzed using immunocyto chemistry. The protocol used for immunocytochemistry, as previously described, was as follows (11). In brief, slides were treated with Dako Target Retrieval solution (DAKO) and then blocked with PBS (Invitrogen) containing 10% goat serum (Vector Laboratories), 1% BSA (Roche Diagnostics) and 0.02% Triton X100 (Promega) for one hour to block non-specific protein interactions. Slides were then incubated with the phospho-ERK antibody (Cell Signaling, Danvers, MA, USA) for gonocyte analysis and the RNF149 and PCNA antibodies (Santa Cruz) for C18-4 cell analysis. Antibodies were diluted in PBS (Invitrogen) containing 1% BSA (Roche Diagnostics) and 0.02% Triton X100 (Promega) overnight at 4°C. Once the overnight incubation was complete, the slides were incubated with a biotin-conjugated goat anti-rabbit or anti-mouse secondary antibody (BD Pharmingen) diluted in PBS (Invitrogen) containing 1% BSA (Roche Diagnostics) for one hour at room temperature. Immunoreactivity was then detected using a combination of streptavidin-peroxidase (Invitrogen) and AEC single use solution (Invitrogen). Slides were counter-stained with hematoxylin (Sigma Aldrich), coated with Crystal Mount (Electron Microscopy Sciences) and dried, and then cover-slipped using Permount (Thermo Scientific) and glass coverslips (Fisher Scientific). The slides were then viewed using a BX40 Olympus microscope (Olympus, Center Valley, PA, USA) coupled to a DP70 Olympus digital camera (Olympus). For RNF149 silencing analysis, phospho-ERK positive gonocytes were easily distinguished from the remaining somatic cells by their much larger size on the cytospin slides. They were counted and their number was normalized to the total gonocyte number for each treatment condition, and the data means plotted as percent of the total gonocyte numbers.

Recombinant DNA Constructs and Amplification

Template RNF149 cDNA was cloned from PND3 testis total cDNA with RNF149 cloning primers by GoTaq® DNA polymerase (Promega) to create poly-A tailing. PCR products were then separated and extracted from 1.5% agarose gels. Purified segments were then ligated to pGEM®-T Easy Vector System I (Promega) and transformed into DH5-α competent cells (Invitrogen) overnight at 37°C. Single colonies were collected and cultured in LB (Invitrogen) for 8 hours. Plasmids were purified by QIAprep Spin Miniprep kit (Qiagen) and sent for sequencing (Genome Quebec, Montreal QC, CA). After sequencing, two confirmed variant forms of RNF149 were then further amplified and purified with HiSpeed Plasmid Maxi Kit (Qiagen). These two variant forms were ligated into pEGFP-N1 and pEGFP-C2 (Clontech, Mountain View, CA, USA). Based on gene maps constructed using SnapGene® software (Version 2.8, GSL Biotech, Chicago, IL, USA), restriction sites were selected at HindIII and KpnI with the 2.1 buffer (New England BioLabs). The following constructs were used: N-terminal EGFP-tagged VA1 (pPA-EGFP) and VA2 (pRING-EGFP), C-terminal EGFP-tagged VA1 (pEGFP-PA) and VA2 (pEGFP-RING). Gene maps

of RNF149-EGFP plasmids are shown as **Supplementary Figures 1**, **2**.

Transfections and Live Cell Imaging

Both cell lines were grown on 35mm fluoro-dish cell culture dishes (World Precision Instruments, Sarasota, FL, USA) at a cell density of 25 million cells/dish before transfection. Cells were transfected with the plasmids mentioned above with either Set 1: BFP-KDEL (Blue ER tubule marker, Addgene, Cambridge, MA, USA) and DsRed-Mito (Red mitochondria marker, Clontech) or Set 2: pDsRed2-ER (Red ER marker, Clontech), one day before confocal microscopy observation, using Lipofectamine™ 3000 (Invitrogen) according to the manufacturer's protocol. LysoTracker Blue DND-22 (60nM, Life Technologies) is added to Set 2 cells before observation for 30 minutes. Before observation under confocal microscope, cells are gently washed with culture medium, and then 1ml Opti-MEM medium (Life Technologies) is added to replace culture medium. Cell samples were analysed by Zeiss LSM880 Laser Scanning Confocal and Super-Res SIM/PALM/dSTORM system (Zeiss) at the McGill University Health Centre Research Institute Molecular Imaging Core Facility. Images were collected over a 60 minute time period.

Statistical Analysis

Statistical analysis was performed using an unpaired two-tail Student's t-test using statistical analysis functions in the GraphPad Prism 5.0 program (GraphPad Inc., San Diego, CA, USA). All experiments were performed where N equals a minimum of three independent experiments. A P-value less than 0.05 was considered statistically significant.

RESULTS

RNF149 Expression Profile in Neonatal to Pubertal Rat Organs

As previously mentioned, we have shown that RA-induced gonocyte differentiation requires an active ubiquitin proteasome system (UPS), and identified a number of UPS genes and proteins differentially expressed between PND3 gonocytes and PND8 spermatogonia (11). Amongst those identified, RNF149 was found to be more abundant in gonocytes than spermatogonia, suggesting that this UPS gene is decreased during the process of differentiation and remains low thereafter. In order to confirm this hypothesis and to understand the role of RNF149 in rat development, tissues such as testis, kidney, liver, heart, and brain were collected from rat pups aged from PND2 to PND35 for gene expression and immunohistochemistry studies.

Using qPCR analysis, we found that RNF149 mRNA expression was highest in the testis at all ages analyzed when compared to RNF149 mRNA expression levels in the brain, heart, liver, and kidney at the same ages (**Figure 1A**). In neonatal testes, RNF149 was mainly expressed in gonocytes and was found in Sertoli cells only at older ages (**Figure 1B**). Interestingly, RNF149 was found highly expressed in the nucleus of PND2 and PND3 gonocytes, with a weaker staining in gonocyte cytoplasm. RNF149 also appeared to translocate to the cytoplasm in spermatogonia (**Figure 1B**). Although gene expression levels of RNF149 in other organs were lower than in the testis, we also observed weak RNF149 staining in the brain, liver, and heart at PND3, a more robust staining in the PND10 liver and a strong signal in cells of kidney tubules at both ages (**Figure 1C**). This suggests that RNF149 might play an important role in regulating the development of different tissues, mainly testis and kidney.

RNF149 Silencing Leads to Reduced Cell Proliferation in Neonatal Gonocytes

To understand the possible mechanism of RNF149 in regulating gonocyte development, we started by testing the function of RNF149 in gonocyte proliferation. RNF149 mRNA was efficiently knocked down after 48 hours of treatment with a triad of siRNA duplexes using Lipofectamine transfection (**Figure 2A**). Compared to mock treatment conditions and those of scrambled, there was a significant decrease in *Rnf149* expression in gonocytes treated with siRNA, indicating an efficient knockdown (**Figure 2A**). We have previously shown that a combination of PDGF-BB (P) and 17β-estradiol (E) induces gonocyte proliferation. Here, we found that when gonocytes were treated with P+E, there was a significant increase in *Rnf149* mRNA expression in the mock treated cells, indicating a possible role of RNF149 in gonocyte proliferation. When analyzing Proliferating Cell Nuclear Antigen (*Pcna*) mRNA levels as a marker for gonocyte proliferation, we found that silencing RNF149 led to a significant decrease in proliferation (**Figure 2B**). Furthermore, as expected, the addition of P+E to gonocytes in the mock or scrambled conditions significantly upregulated *Pcna* expression. We also found that when P+E was added to the treated cells, siRNA-treated gonocytes had significantly lower *Pcna* expression compared to Mock cells treated with P+E, indicating that regardless of P+E stimulation, silencing RNF149 had a negative effect on proliferation. To confirm these findings, we also analyzed cyclin B2 (*Ccnb2*) mRNA expression (**Figure 2C**), which is another marker used to assess cell proliferation as it is an essential part of the cell cycle regulatory machinery involved in controlling the G2/M transition (23). However, unlike PCNA, we found that *Ccnb2* mRNA levels were significantly upregulated upon RNF149 silencing, although *Ccnb2* induction by P+E was reduced upon RNF149 silencing, suggesting that RNF149 disrupts *Ccnb2* expression in basal and proliferative conditions. Taken together, the analysis done on PCNA and CCNB2 gene expression indicates a complex and dynamic role of RNF149 in gonocyte proliferation.

To further explore the role of RNF149 in gonocyte proliferation we analyzed the activation of ERK, since we had previously shown that the MEK/ERK signalling pathway is involved in gonocyte proliferation (4). Thus, we examined whether there were any changes in levels of ERK phosphorylation upon P+E treatments in control cells and cells in which RNF149 was knocked down with

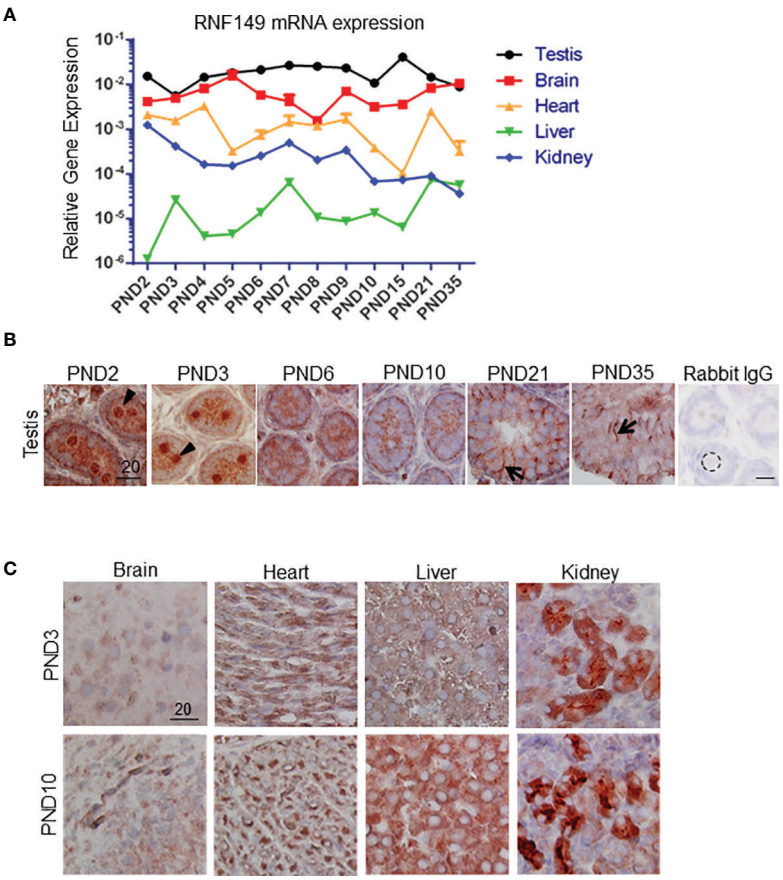

FIGURE 1 | RNF149 expression profile in various organs in rats at various ages. **(A)** RNF149 mRNA levels in various tissues from PND2 to PND35. **(B)** RNF149 protein expression in testes from PND2 to PND35. Arrowhead: germ cell; arrow: Sertoli cell. **(C)** RNF149 expression in brain, heart, liver, and kidney at PND3 and PND10. 20μm scale shown. Representative images shown.

siRNA. Gonocytes were immunostained to determine the levels of phosphorylated ERK, and the number of gonocytes positively stained for phospho-ERK in each condition was determined. As expected, there was a significant increase in phospho-ERK-positive gonocytes treated with P+E compared to Mock gonocytes alone (**Figure 2D**). Furthermore, there was a decrease in ERK activation when RNF149 was silenced in gonocytes with or without P+E treatment, similarly to *Pcna* mRNA expression. Taken together, these data suggest that when RNF149 is knocked down, there is a significant decrease in the expression of markers for gonocyte proliferation, further supporting its role in gonocyte proliferation.

RNF149 Knockdown in C18-4 Mouse Spermatogonia Cells Does Not Affect Proliferation or Differentiation

Although there is no cell line model available for gonocytes, the mouse-derived C18-4 spermatogonia cell line is commonly used to study type A- spermatogonial development (24). Here, we used C18-4 cells to determine whether RNF149 knockdown had a similar effect on spermatogonial development. After confirming efficient RNF149 mRNA knockdown by qPCR (**Figure 3A**), we found that upon RNF149 mRNA silencing, there was a significant increase in Stra8 mRNA levels (**Figure 3B**). Stra8 mRNA level increases were also seen when Mock C18-4 cells were treated with RA (**Figure 3C**), confirming its use as a marker for spermatogonial differentiation. Unlike gonocytes, there was no significant change in the mRNA levels of PCNA upon RNF149 silencing (data not shown). Furthermore, when treating C18-4 cells with a cocktail of glial-cell derived neurotrophic factor (GDNF), fibroblast growth factor (FGF2), and GDNF family receptor alpha 1 (GFRα1), known to promote proliferation (25), there was no significant change seen in PCNA mRNA expression (data not shown). Taken together, this data indicates that unlike in gonocytes, RNF149 is likely negatively involved in spermatogonial differentiation, and not proliferation.

RNF149 Silencing in F9 Mouse Embryonal Teratocarcinoma Cells Does Not Affect Their Proliferation or Differentiation

Our lab previously showed that F9 cells, considered as surrogate for embryonic stem cells, also share similar traits with gonocytes, especially in their ability to express the spermatogonial marker

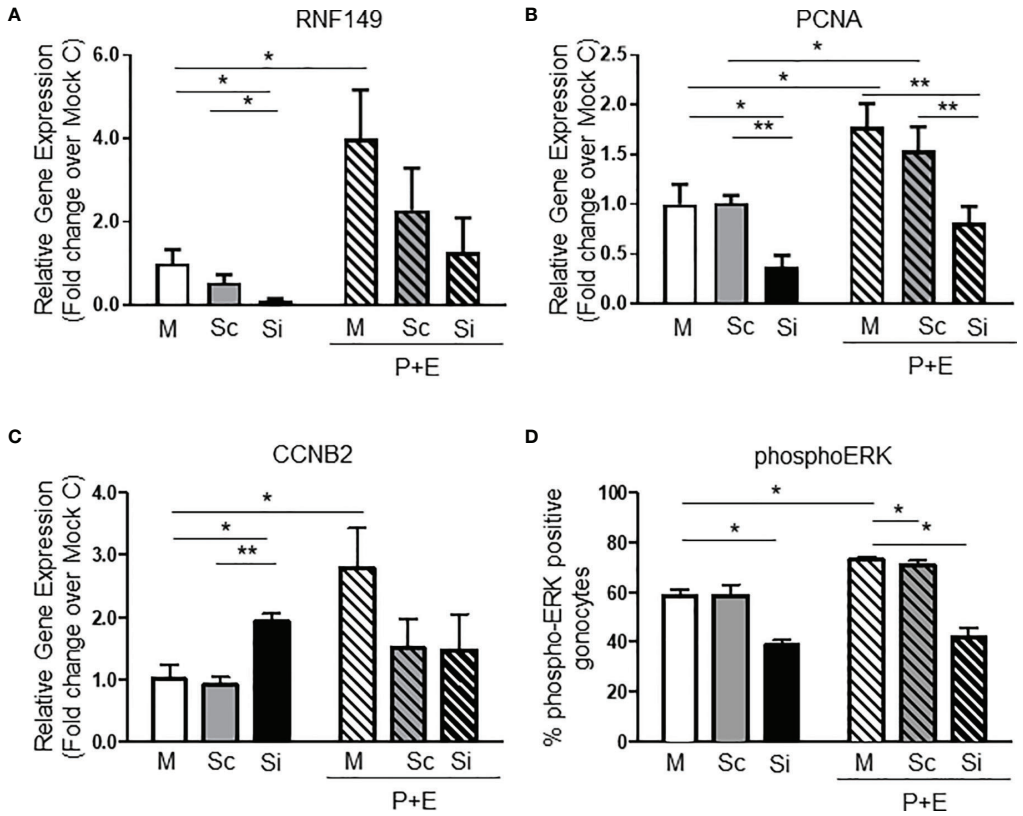

FIGURE 2 | RNF149 knockdown in PND3 gonocytes. **(A)** RNF149 mRNA expression in gonocytes first treated with mock (M), scrambled siRNA (Sc), or RNF149 siRNA (Si) for 48 hours and then treated with or without PDGF-BB (10^{-9}M) and 17β-estradiol (10^{-6}M) [with 2.5% fetal bovine serum (FBS)] for an additional 24 hours. Results shown are from N=3-5 independent germ cell preparations (each done in duplicate) and are plotted mean ± SEM. *p-value<0.05. **(B)** PCNA mRNA expression and **(C)** CCNB2 mRNA expression in similarly treated cells. **p-value<0.01. **(D)** Treated gonocytes were immunostained using phospho-ERK antibody. Total number of gonocytes and positively stained cells were counted. Percentage of phospho-ERK-positive cells was graphed. Results shown are from N=3 independent germ cell preparations. *p-value<0.05.

STRA8 in responses to RA treatment, and in the existence of crosstalk between RA and PDGFR signaling pathways (6). Moreover, F9 cells proliferate in response to PDGF-AA, similarly to gonocytes that proliferate in response to PDGF-BB. Due to their similarities, F9 cells can be used as a model for the study of gonocyte differentiation. Here, we used F9 cells to determine whether RNF149 knockdown had a similar effect on these cells as it did on gonocyte proliferation. Upon confirmation of efficient RNF149 mRNA knockdown (**Figure 4A**), we found that independent of F9 cells being treated with siRNA or not, there was a significant increase in Stra8 mRNA levels upon RA treatment, as expected (**Figures 4B, C**). Silencing RNF149 mRNA had no significant effect on F9 cell proliferation also (data not shown). Furthermore, there was no significant change seen in PCNA mRNA expression in cells where RNF149 was silenced (data not shown). Taken together, these data suggest that although there are many similarities between the development of F9 cells and gonocytes, the involvement of RNF149 in their proliferation is not a common characteristic between these two cell types.

Two Variant Forms of RNF149 Transcripts Are Expressed in Rat Tissues

Given that there is limited information about RNF149 and its possible role in germ cell development, we proposed to build EGFP-tagged RNF149 vectors to better understand its cell localization and mechanism of action (**Figure 5A**). Interestingly, with primers designed based on a predicted sequence that would generate a single product approximately 1185bp in size, three major variant forms were found in all tissue sample examined, showing stronger expression in the testis and kidney than in the liver (**Figure 5B**). This finding was in agreement with both the qPCR and tissue staining results presented above. Furthermore, the three major variant forms were also present in the rat testes at ages PND2 to PND35 at varying intensities (**Figure 5C**).

Two PCR products that were approximately 1000bp in size were then cloned into pGEM®-T Easy Vectors for sequencing. The largest band obtained was 1400bp but because it was much larger than the expected size of full length RNF149 (1185bp), this band was not further used. However, one cannot exclude that it may correspond to a true RNF149 variant mRNA with intron

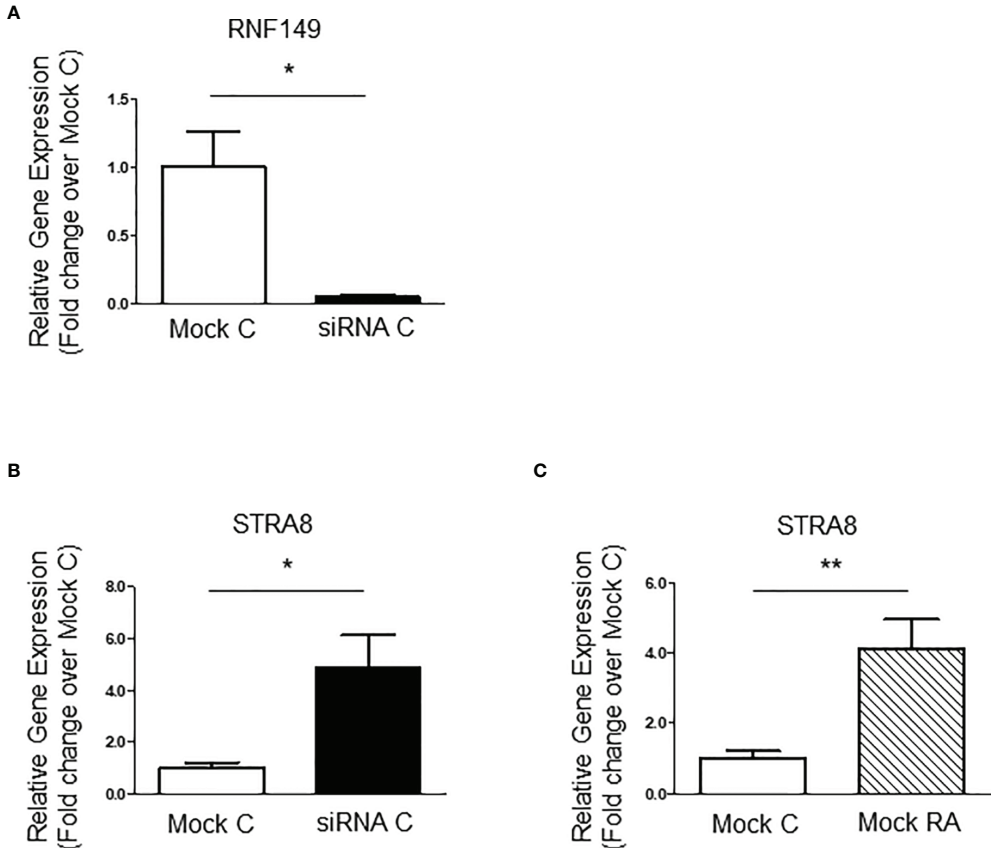

FIGURE 3 | RNF149 knockdown in C18-4 cells. **(A)** RNF149 mRNA expression in C18-4 cells treated with mock and siRNA for 48 hours. Results shown are from N=3 independent cell passages and are plotted mean ± SEM. *p-value<0.05. **(B)** Stra8 mRNA expression in C18-4 cells treated with mock and siRNA for 48 hours. *p-value<0.05. **(C)** Stra8 mRNA expression in mock cells treated with or without RA (10^{-7}M) for an additional 24 hours. **p-value<0.01.

retention, as found for a number of germ cell and cancer variant transcripts.

Sequencing results indicated that there are two variant forms in rat tissues: VA1 (1066bp) that expresses the PA domain and VA2 (862bp) that expresses the TM-RING domain (**Figure 6A**). Although a great number of mutated RNF protein expressions have been found in cancer patients, these might be the first naturally expressed variant forms of RNF proteins in rat. Based on sequencing results, a primer set was designed to verify the expression of these two variant forms in other organs, which for VA1 would produce a 182bp product, and for VA2, a 301bp product. The results showed that almost all organs tested had three bands, two of which matched our predictions (**Figure 6B**). Taken together, this data suggests the presence of variant RNF149 forms present in the rat testis. The exact functions of these variants remain to be elucidated.

Subcellular Localization of EGFP-Tagged RNF149 Isoforms in C18-4 and F9 Cell Lines

C18-4 cells are immortalized spermatogonial cells that exhibit the general properties of type-A spermatogonia and we found these cells to express both RNF149 and PCNA (**Figure 7A**). RNF149 protein expression was found mainly to be cytosolic, in agreement with the cytosolic expression observed in PND8 spermatogonia (data not shown). Given that C18-4 cells express RNF149, this cell line can be used as a potential model to study its role in various germ cell related mechanisms. Four vectors that express either C-termini or N-termini EGFP-tagged VA1 and VA2 forms of RNF149 were transfected into both F9 and C18-4 cell lines independently, with or without the co-transfection of either blue or red ER marking plasmid and lysosome tracker. In C18-4 cells, the location of EGFP on either C-termini or N-termini changed the distribution of RNF149 (**Figure 7B**). Therefore, C-terminal EGFP plasmids were used for subsequent studies. To test where RNF149 isoforms localized in C18-4 cells, two C-terminal EGFP plasmids were co-transfected with markers for the ER and mitochondria. The RING domain-containing RNF149 isoform was widely expressed in C18-4 cell cytoplasm and nucleus (**Figure 7C**), whereas the PA domain-containing isoform co-localized with ER, but not with the mitochondria or lysosome (**Figures 7D, E**). The differential localization of the two variant forms suggests differential functions. Moreover, the PA domain might be required for RNF149 to reside in the ER membrane.

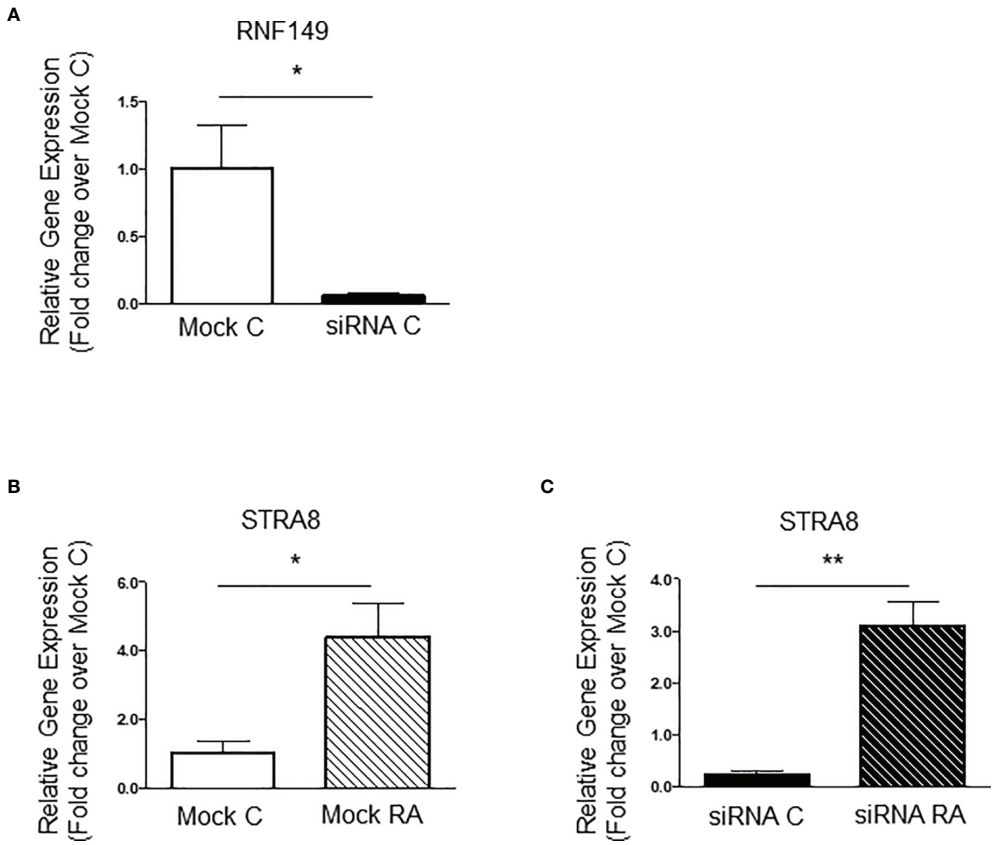

FIGURE 4 | RNF149 knockdown in F9 cells. **(A)** RNF149 mRNA expression in F9 cells treated with mock and siRNA for 48 hours. Results shown are from N=3 independent cell passages and are plotted mean ± SEM. *p-value<0.05. **(B)** Stra8 mRNA expression in F9 cells in mock cells treated with or without RA (10^{-7}M) for an additional 24 hours. *p-value<0.05. **(C)** Stra8 mRNA expression in siRNA treated cells also treated with or without RA (10^{-7}M) for an additional 24 hours. **p-value<0.01.

Interestingly, similar expression patterns were observed in F9 cells transfected with PA-EGFP plasmid with markers for ER and mitochondria, where PA-EGFP RNF149 co-localized with the ER rather than mitochondria (**Figure 8A**). When co-transfected with an ER marker and stained with lysosome tracker, PA-EGFP RNF149 did not co-localize with lysosomes in F9 cells, which corresponds to the expression pattern seen in C18-4 cells (**Figure 8B**). However, RING-EGFP RNF149 did not co-localize with ER, but was highly aggregated in lysosomes (**Figure 8C**). These results suggest that in both C18-4 and F9 cells, the PA-domain of RNF149 is a key factor for RNF149 localization in the ER, and that the PA variant protein might have a role in ER, while the RING-domain variant protein might be an essential element for RNF149 localization in the lysosome, related to the protein degradation pathway.

DISCUSSION

The ubiquitin proteasome system has been widely studied due to its multiple functions in regulating protein degradation, kinase activation, DNA repair, trafficking, translation, and signal pathway activation (12–17). In addition, among the three key enzymes of the UPS, E3 ligases play the most important role as they provide specificity to the entire process (14). RING-type E3 ligases have been reported to be important regulators in many diseases, such as Mdm2, that can ubiquitinate P53 (26), and Skp2, that can degrade c-Myc (27), thus their involvement in various disease states. Some studies have been conducted on transmembrane RING-type E3s such as RNF128 (also called GRAIL) and RNF5, which were reported to participate in cell proliferation and differentiation processes (28). While a functional UPS is required for the regulation of cell proliferation and differentiation in physiological processes such as the formation of ocular lenses, its dysregulation leading to improper proliferation or differentiation is associated with diseases such as cancer and osteoporosis (15, 29, 30). Interestingly, RNF128 was proposed to be linked to cancer and sepsis *via* its role in immunologic tolerance (31).

In contrast, little is known about RNF149, our protein of interest. A study carried in human colon cancer and embryonic kidney cell lines reported that RNF149 indirectly regulated cell differentiation by reducing BRAF, a kinase known for its pro-proliferation function (32). On the other end, a recent study

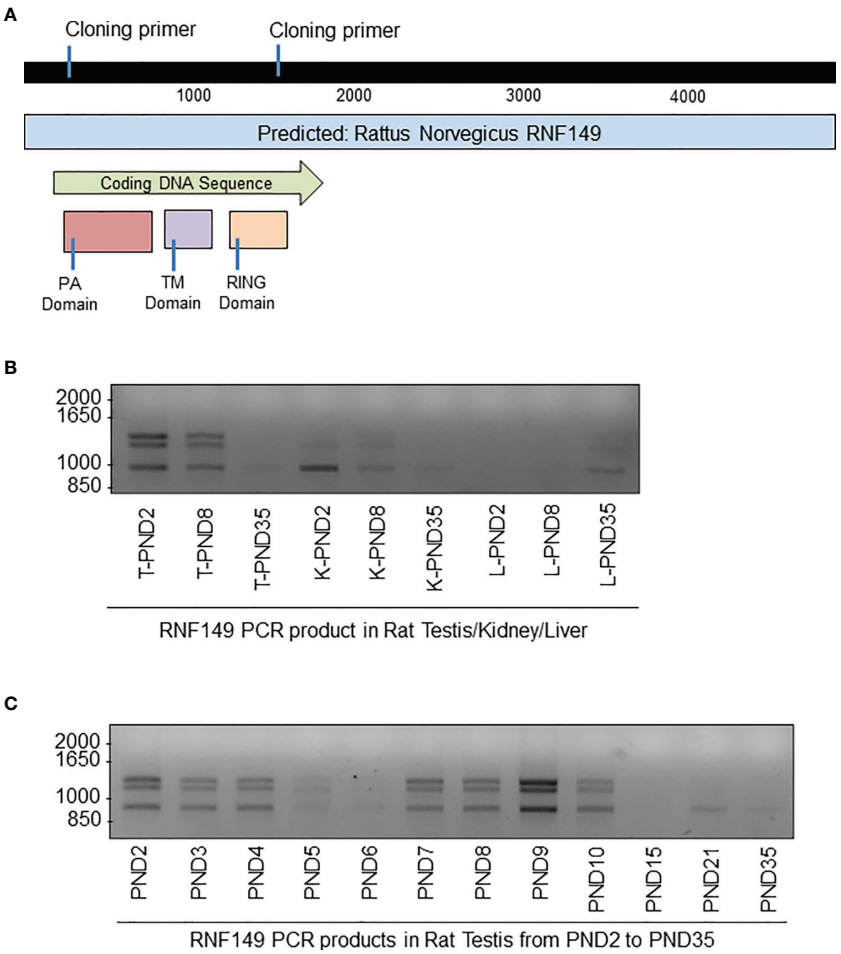

FIGURE 5 | PCR analysis of RNF149 sequences cloned from tissues of rat pups at different ages. **(A)** Gene map of RNF149. Blue bar represents total predicted gene, green bar represents the RNF149 coding sequence that includes the PA domain (red), transmembrane TM domain (purple), and RING domain (orange). **(B)** PCR products of RNF149 in rat testes at various ages. **(C)** PCR products of RNF149 in various rat organs and various ages. T, testis; K, kidney; L,liver.

searching germline modifier genes possibly associated with aggressive prostate cancer, by GWAS and human prostate tumor expression data set analyses, identified RNF149 among four genes that could contribute to disease aggressiveness in PC patients. Ectopic overexpression of these genes in *in vitro* cell growth and *in vivo* tumor growth assays found that the overexpression of only one gene, CCDC115, decreased tumor growth, while the other genes had not significant effects in these assays (33). This finding agrees with their observation that RNF149 expression was associated with an increase in disease burden and tumor stage in the patients, whereas CCDC115 high expression was associated with decreased tumor burden (33).

Our previous study showing that RNF149 is strongly expressed in gonocytes and is downregulated during their differentiation to spermatogonia (11), suggests that RNF149 may need to be removed before gonocytes can undergo differentiation to form spermatogonia. This trend is also observed in testes sections, where RNF149 has the highest protein expression in PND2 and PND3 gonocytes, especially in the nucleus, in contrast to its expression in Sertoli cell cytosol in older, pubertal testis. While the full-length rat RNF149 does not have an obvious nuclear signal sequence according to the Nuclear Localization Signal Data Base website (https://rostlab.org/services/nlsdb/), it is possible that RNF149 translocates to the nucleus as part of a protein complex. In addition, RNF149 is found highly expressed in certain cell types in kidney and other organs from PND2 to 35 (puberty), the strongest being in cells from kidney tubules. It is interesting to note that RNF149 profiles reported in the Human Protein Atlas public website (data not shown) were similar to our findings at younger ages in the rat. Indeed, RNF149 was strongly expressed at the surface of spermatocytes and in Sertoli cell cytoplasm in adult human testes, in kidney tubule cells, and in bile duct cells. We also found strong RNF149 expression in adult PND120 Sertoli cell cytoplasm as well as in the cytoplasm of elongated spermatids (data not shown). Thus, our results indicate that RNF149 might

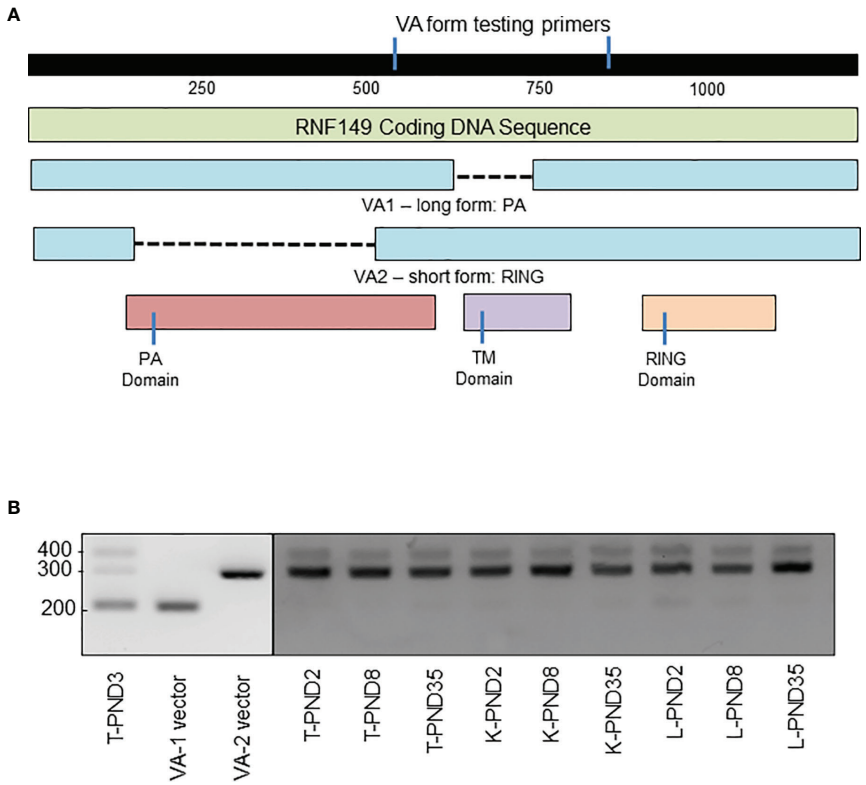

FIGURE 6 | PCR analysis of RNF149 variant forms in rat testes, kidney, and liver at various ages. **(A)** Gene map of RNF149 coding sequence. Green bar represents total RNF149 coding sequence that includes the PA domain (red), transmembrane TM domain (purple), and RING domain (orange). Blue bars represent two variant forms of RNF149 found in the rat. **(B)** PCR products of RNF149 in PND3 rat testes, and VA1 and VA2 plasmids compared to kidney and liver at various ages. T, testis; K, kidney; L, liver.

be an essential regulator for the postnatal development of different organs. Moreover, its expression varies in testicular germ cells from a nuclear expression in neonatal gonocytes to a cytoplasmic localization in spermatogonia and elongated spermatids, suggesting specific roles in restricted phases of germ cell development. Also of interest is its presence in the cytoplasm of pubertal to adult Sertoli cells, suggesting a potential role in differentiated, but not immature, Sertoli cells.

The analysis of neonatal gonocyte proliferation in response to a mixture of PDGF-BB and 17β-estradiol (P+E), previously found to induce gonocyte proliferation (3, 4), in cells expressing normal levels of RNF149 showed that the expression levels of RNF149 were increased in mock cells simultaneously to PCNA by the proliferative agents, whereas blocking RNF149 expression with siRNA reduced its P+E driven induction by nearly 70% and decreased PCNA induction by more than 50% of the levels found in P+E-treated mock cells. Together with the fact that phospho-ERK, a downstream effector of gonocyte proliferation, was similarly affected by knocking down RNF149, these results suggest that RNF149 may be a positive regulator of gonocyte proliferation. Moreover, the role of RNF149 in neonatal gonocytes appeared specific to this stage of germ cell development, since RNF149 silencing did not affect spermatogonial proliferation. Similarly, the lack of effect of RNF149 silencing on the proliferation of F9 cells suggested that it may not be involved in stem cell proliferation. However, one cannot exclude the possibility that the data obtained with the C18-4 immortalized spermatogonial and the F9 teratoma cell lines might not reflect the function of RNF149 in primary spermatogonia or embryonic stem cells, since these cell lines have deficient cell cycle regulation that could mask a potential role of RNF149 in their proliferation.

A study published in a Chinese journal proposed that RNF149 might be directly involved in cell proliferation *via* degrading CD9 (34). Other studies have linked CD9 to the maintenance of stemness in spermatogonia and its presence in human male germ cells related to their ability to repopulate rodent testes after transplantation (35). It is possible that the target(s) of RNF149 in gonocytes is different from CD9 or that it requires the recruitment of other proteins to affect CD9. Another ubiquitin ligase, RNF144A, was reported to exert a positive effect on cell proliferation in EGF-dependent human cancer and immortalized embryonic cell line models, by maintaining EGFR expression (36). In this study, RNF144A was shown to prolong EGF effects by promoting EGFR ubiquitination, and that RNF144A depletion using CRISPR/Cas9 system decreased EGF-dependent cell proliferation. Subcellular localization studies led to the hypothesis that RNF144A may regulate EGFR transport

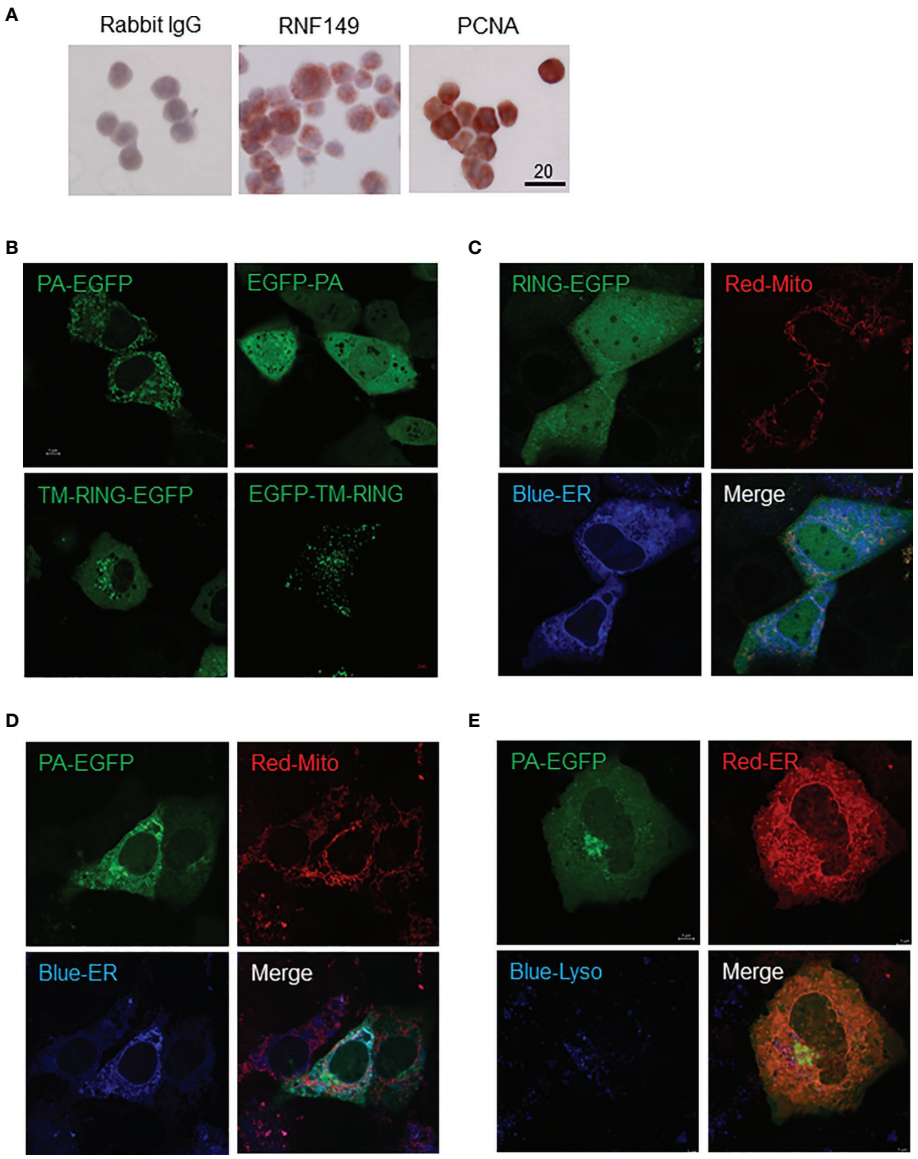

FIGURE 7 | C18-4 cells as a potential model for studying RNF149 function. **(A)** Colorimetric analysis of C18-4 cells positively expressing RNF149 and PCNA. 20μm scale shown. Representative cells shown. **(B)** Single transfection of EGFP-tagged RNF149 isoforms. **(C)** RNF149 is co-localized with ER by PA domain. RING-EGFP vector co-transfected with mitochondria marker (red) and ER marker (blue). **(D)** PA-EGFP vector co-transfected with mitochondria marker (red) and ER marker (blue). Both co-transfections were performed using Lipofectamine™ 3000 (Invitrogen) 24 hours before microscopic observation (according to the manufacturer's protocol). **(E)** RNF149 is co-localized with ER by PA domain but not the lysosome. PA-type RNF149 isoform is co-transfected with ER marker (red) and lysosome marker [blue; LysoTracker Blue DND-22 (Life Technologies)]. Representative images shown.

to intracellular vesicles in EGF-treated cells (36). These findings extend the possibility of RNF proteins regulating cell proliferation not only by regulating the ubiquitination of their target proteins, but also their subcellular localization.

Although very little is known about RNF149, it has been reported to be a transmembrane protein mostly expressed on ER membranes and lysosomes. Besides its potential role in cell proliferation, RNF149 might also participate in the regulation of recycling endosome trafficking. Goliath and Godzilla, two Drosophila members of the PA-TM-RING RNF protein family and their human homologue RNF167, were reported to regulate recycling endosome trafficking *via* ubiquitylation of the VAMP3 (vesicle-associated membrane protein 3) SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) protein and induce enlargement of EEA1 (early endosome antigen 1)/Rab5-positive early endosomes both *in vitro* and *in vivo* (37). Moreover, a study conducted on LGR5+ stem cells demonstrated that two other PA-TM-RING family proteins

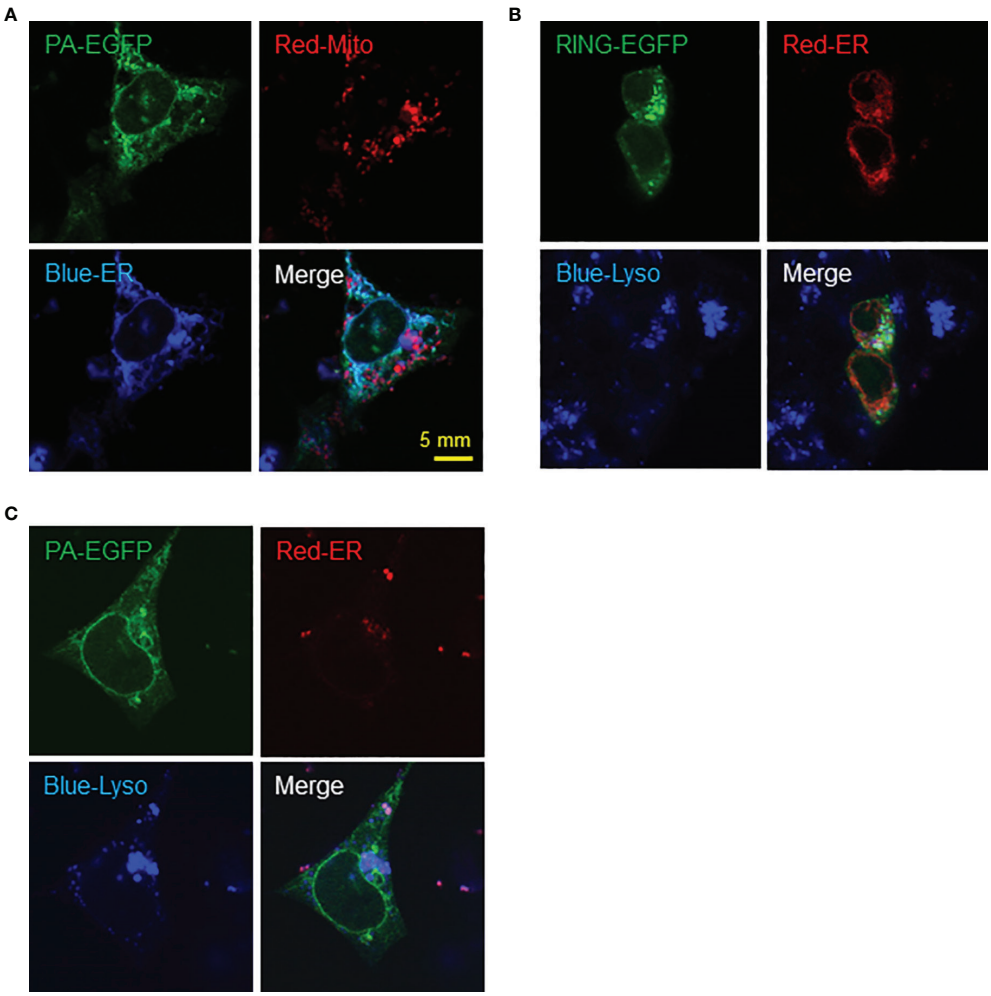

FIGURE 8 | F9 cells as a potential model for studying RNF149 function. **(A)** RNF149 is co-localized with ER by PA domain but not the mitochondria. PA-type RNF149 isoform is co-transfected with ER marker (blue) and mitochondria marker (red). **(B)** PA-domain potentially co-localizes with ER while RING-domain is co-localized with lysosomes. RING-EGFP vector is co-transfected with ER marker (red) and lysosome marker (blue). **(C)** PA-EGFP vector is co-transfected with ER marker (red) and lysosome marker (blue). Representative images shown.

RNF43 and ZNRF3, were able to reduce Wnt signals by enhancing endocytosis of Frizzled receptors *via* its ubiquitylation, hence cell growth arrest (38). As a matter of fact, massive activation of Wnt signaling is found in either mice lacking these genes or cancer cells harboring loss-of-function mutations of RNF43 (39). These results implicate a shared regulatory function for PA-TM-RING ubiquitin ligases in intracellular trafficking/sorting and suggest that abrogation of their function may lead to cellular signaling disorder, which can eventually cause cancer.

Our next goal was to generate overexpression vectors coding for rat RNF149 mRNA and to express the recombinant protein in cell lines to determine its subcellular localization. Using publicly available rat RNF149 mRNA sequence, we cloned the gene from rat testes and other tissues. Interestingly, two variant mRNA forms of RNF149, VA1 and VA2, were found in rat testes, liver, and kidney cDNA libraries. Sequence analysis and the positions of start and stop codons showed that VA1 included the sequence of the PA domain but lacked the RING domain. On the other hand, the start codon and stop codon of VA2 defined a sequence including TM and RING domains. Thus, VA2 was referred to as the RING form. These two constructs were then ligated to N1/C2 EGFP vector for mammalian expression of RNF149, to examine their subcellular localization in F9 and C18-4 cells used as models.

Weak RNF149 protein expression was observed in primary rat spermatogonia cytoplasm in PND6-8, in agreement with its expression in mouse C18-4 cells, an immortalized cell line considered as type-A spermatogonia, including spermatogonial stem cells (24). Interestingly, RNF149 expression appeared to be stronger in some but not all C18-4 cells than *in vivo* PND8 spermatogonia, suggesting two subpopulations in growing C18-4 cells, in support of this cell line containing type A spermatogonia at different phase of differentiation, as observed with isolated

spermatogonia from juvenile mice (40–42). Alternatively, these different patterns in RRNF149 expression levels could be related to the cells being at different phases of the cell cycle. As a type I transmembrane protein, RNF149 shares common features, such as N-terminal signal peptides (NS) and transmembrane domains, with other members, suggesting that a C-terminal fusion protein of EGFP and RNF149 should not disrupt the N-terminal signal peptides, allowing the fusion protein to remain in the cytoplasm, whereas the fusion of EGFP at the N-terminal might affect its PA-domain function. In RNF protein-related studies, due to the existence of predicted N-terminal signal peptides, EGFP is mostly conjugated to the C-termini. Here, EGFP ligated at the N-termini changed the localization of VA1 and VA2 RNF149, making them either widely spread in the nucleus and cytoplasm, likely due to EGFP hindering the PA domain, resulting in the loss of ability to reside in the ER and other potential sites in the cell, or leading to condensation into smaller spots as seen with VA2. This further suggests that the RING domain participates in intracellular trafficking/sorting. Therefore, to reduce the interference effect caused by EGFP, C-terminal EGFP tagged VA1 and VA2 RNF plasmids will be used in further studies.

In C18-4 and F9 cell lines co-transfected with either the ER, mitochondria, or lysosome marker, VA1 RNF149 was localized in the ER, suggesting a potential function of the PA domain, in agreement with other studies. In contrast, VA2 is localized mainly to lysosomes in F9 cells, which was not observed in C18-4 cells. These results suggest that in both C18-4 and F9 cells, the PA-domain of RNF149 potentially exhibits its function in the ER, while in F9 but not C18-4 cells, the RING-domain may be an essential element for RNF149 translocation to the lysosome, in relation to the protein degradation pathway.

The apparent difference in RING domain localization between F9 cells and C18-4 cells is interesting, since F9 cells correspond to pluripotent embryonic stem cells with both somatic and germ line potentials, whereas C18-4 cells represent more advanced undifferentiated spermatogonia. To date, only BRAF and CD9 are known targets for RNF149, and how they take part and react with both the PA and RING domains of RNF149 remains unclear. Therefore, further studies focused on finding other potential substrates of RNF149 and its actual mechanism of action in these cell lines and gonocytes are required.

In summary, this study demonstrated the potential function of RNF149 in gonocyte development, highlighting the correlation between RNF149 expression and proliferation marker PCNA during PDGF-BB+17β-estradiol co-treatment, the variant forms of RNF149 found in rat tissues, and the potential roles of PA and RING domain-containing variant proteins. Although these studies were not able to fully identify the role of RNF149 and the identity of its substrates in response to proliferation or differential stimulation in gonocytes, C18-4 cells, and F9 cells, they revealed the possibility of RNF149s involvement in gonocyte proliferation and tested the potential use of F9 and C18-4 cell lines as models to study the function of RNF149. The importance of the UPS system in preventing the accumulation of misfolded proteins was recently highlighted, in parallel to the role of autophagy in maintaining cell integrity and functionality (43). Our previous finding that inhibiting proteasome activity impaired gonocyte differentiation (11) and the present study emphasizing RNF149 role in gonocyte proliferation, suggest that multiple UPS enzymes exert different effects in the regulation of these cells and that RNF149 is only a piece of the puzzle. Taken together, the findings that RNF149 expression is induced by proliferating agents, that its silencing decreases proliferation and increases differentiation genes, and that it is downregulated during differentiation, supports the hypothesis that RNF149 plays a role in gonocyte proliferation, while its downregulation may be part of the differentiation process. Moreover, the perturbation of RNF149 function might lead to disorder in membrane protein trafficking and degradation.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by McGill University Health Centre Animal Care Committee and the Canadian Council on Animal Care.

AUTHOR CONTRIBUTIONS

GM contribution includes participate to the study design, performing experiments, data analysis, writing the manuscript, and preparing the table and figures. C-CK was involved in the study design and performing experiments. MC contributed in developing the concept and design of the project, analysing data, preparing and editing the manuscript and figures. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by a grant from the Natural Sciences and Engineering Research Council of Canada (NSERC) (Discovery Grant # 386038-2013), Canada and by funds from the USC School of Pharmacy to MC. GM was supported by funds from the Division of Endocrinology, Department of Medicine, McGill University, Canada.

ACKNOWLEDGMENTS

The authors thank Dr. Marie-Claude Hoffman, Houston TX, USA for the gift of the C18-4 spermatogonial cell line, and they are thankful to Dr. Jinjiang Fan for his technical assistance with the GFP-tagged cell-line studies.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.896507/full#supplementary-material

Supplementary Figure 1 | Gene map of RNF149-EGFP plasmid. **(A)** RNF149 VA1-EGFP gene map. **(B)** RNF149 VA2-EGFP gene map.

Supplementary Figure 2 | Gene map of EGFP-RNF149 plasmid. **(A)** RNF149 EGFP-VA1 gene map. **(B)** RNF149 EGFP-VA2 gene map.

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PERSPECTIVE
published: 18 May 2022
doi: 10.3389/fendo.2022.889959

Human Spermatogenesis: Insights From the Clinical Care of Men With Infertility

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Human spermatogenesis is a qualitatively and quantitatively different process than that observed for most other mammals. In contrast with most other mammals, human spermatogenesis is characterized by reduced quantitative production and more abnormal sperm morphology. Until recently, direct evaluation and observations of human sperm production has been limited and the majority of scientific knowledge regarding spermatogenesis was derived from rodent models of study. Unique opportunities to observe human spermatogenesis have occurred as a consequence of the treatment of severe male infertility. These patients have sperm production so limited that no sperm reach the ejaculate so their fertility treatment involves surgical sperm retrieval from the testis, coupled with use of those sperm with advanced assisted reproductive techniques. Treatment of men with severe male infertility has enhanced identification of new genetic abnormalities that may cause this condition, since they now seek medical care. Three key novel concepts have resulted: (a) spermatogenesis is spatially heterogeneous in the human male, especially when sperm production is compromised, (b) genetic abnormalities are common in men with severe male infertility, particularly in men with diffuse maturation arrest and (c) rodent studies may not be an ideal model for understanding human male infertility. Scientific understanding of human spermatogenesis has been enhanced by these clinical observations.

Keywords: human, spermatogenesis, male infertility, genetics, treatment

OPEN ACCESS

Edited by:
Barry Zirkin, Johns Hopkins University, United States

Reviewed by:
John Amory, University of Washington, United States

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Specialty section:
This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology

Received: *04 March 2022*
Accepted: *15 March 2022*
Published: *18 May 2022*

Citation:
Schlegel PN (2022) Human Spermatogenesis: Insights From the Clinical Care of Men With Infertility. Front. Endocrinol. 13:889959. doi: 10.3389/fendo.2022.889959

INTRODUCTION

With the advent of assisted reproductive technologies, the ability to treat severe forms of male infertility has been significantly enhanced. Spermatozoa that would have had limited chances of oocyte fertilization can now routinely be used for fertilization and subsequent pregnancy. In addition, the ability to make direct observations from use of single male gametes in assisted reproduction further increases our ability to understand the role of a male factor in human fertility.

The treatment of severe male factor infertility, where sperm production is so limited that no viable sperm are present in the ejaculate requires surgical sperm retrieval. This surgical intervention has also given us opportunities to directly investigate the internal function of the testis through clinical observation. The most effective form of sperm retrieval for non-obstructive azoospermia is microdissection testicular sperm extraction (microTESE) (1, 2) where direct evaluation of the seminiferous tubules within the testis is done to identify the sites of sperm production. In many cases, dissection is used to examine hundreds of the seminiferous tubules inside of the testis; a unique opportunity to evaluate and characterize spermatogenesis for these men. These observations provide the basis for some of the novel concepts described in this perspective manuscript.

Evaluation of men with severe male infertility now occurs routinely as part of their treatment process. The routine genetic testing for potential abnormalities such as Y chromosome microdeletions (3) prior to treatment has provided the opportunity to examine their genome on a broader scale than may otherwise have occurred. Our experience with treatment of men has resulted in a large number of men being referred for treatment, and, fortunately, most of them are willing to allow additional genetic testing under IRB oversight for novel conditions that may cause infertility, as well as the known causes of severe male infertility including karyotypic abnormalities and Y chromosome microdeletions (4).

HETEROGENEITY OF HUMAN SPERMATOGENESIS

Spermatogenesis in the rodent has been carefully characterized since the 1800s (5) and qualitatively documented since Clermont's work in 1952 to have specific carefully coordinated and timed facets for the spermatogenic process (6). The documentation of 12 specific stages in the mouse reflects a uniquely organized process for spermatogenic development (7). Central to the observations of rodent spermatogenesis in nearly every publication is that the germ cell developmental process is uniform throughout the testis, with variations from tubule to tubule only based on tightly organized specific spermatogenic stages.

In distinction, human spermatogenesis has been typically characterized as being chaotic, with no reliable staging within the seminiferous tubule. Although attempts to define spermatogenic cycles in the human have been proposed by Nikkanen (8), and a complex interlocking spiral process of spermatogenic cell development was described by Schulze (9), human spermatogenesis is widely observed to be more chaotic than organized.

The treatment of men with non-obstructive azoospermia is complex since, by definition, these men have spermatogenesis so impaired that no sperm are observed in the ejaculate. As a result, surgery coupled with assisted reproduction is needed for fertility. Testicular histology is uniformly abnormal in these men. Interestingly, even men with predominant Sertoli cell-only pattern will have focal areas of sperm production identifiable with mTESE (10). Since sperm production is grossly abnormal in these testes, heterogeneity of sperm production is required for sperm production to be present in the testes; only men with at least focal spermatogenesis (different from their baseline or overall pattern of spermatogenesis) will have sperm retrieved. The microsurgical exploration and dissection of testicular tissue is needed to find these isolated areas of sperm production, reflecting why microTESE is the preferred method of sperm retrieval. Men with diffuse, uniform-appearing maturation arrest can be very challenging to treat. However, these men with diffuse maturation arrest will often have rare sperm in the ejaculate, even if wide dissection of their testicular tissue shows no obvious evidence of focally normal sperm production. So, even they have focal differences in sperm production despite a nearly uniform histologic appearance in the testes.

Large studies examining men with a normal karyotype have shown that only 52% of men will have normal spermatogenesis on testis biopsy, with the remainder showing globally decreased sperm production or late defects in spermatogenic development (11). Taken together, especially for men with impaired spermatogenesis, it is clear that human spermatogenesis is typically a heterogeneous process. This is markedly different from the process of spermatogenesis in other mammals, especially rodents.

GENETIC ABNORMALITIES IN NON-OBSTRUCTIVE AZOOSPERMIA

Defined genetic abnormalities causative of non-obstructive azoospermia are detected in approximately 20% of men with severely impaired sperm production, and include microdeletions of the AZFa, AZFb or AZFc regions of the Y chromosome as well as karyotypic abnormalities. Although Klinefelter syndrome is the more common karyotypic abnormality associated with non-obstructive azoospermia, autosomal translocations can also be found in men with this condition. Another 5% of men have a history of chemotherapy or radiation associated with their azoospermia, and about 10% of men have a history of cryptorchidism and prior orchiopexy. That leaves another 65% of men for whom we can either characterize their etiology based on histologic appearance (Sertoli cell-only and/or maturation arrest) or simply refer to them as being idiopathic. Since histologic characterization does not reflect an etiology, the majority of men with non-obstructive azoospermia could have a genetic cause for their severely impaired sperm production.

The recognition that infertility could be hereditary is not a new concept. In 1981, well before advanced genetic techniques identified specific genetic abnormalities, Cantu et al. recognized the presence of maturation arrest in 3 of 13 brothers from a

consanguineous marriage, suggesting the possibility of an autosomal recessive genetic defect that could be causal for disordered spermatogenesis (12). In a limited population study, Fakhro et al. examined a cohort of 8 families using whole-exome sequencing to identify genes associating with non-obstructive azoospermia. They found that 10 of 16 men with infertility had novel genes with homozygous mutations segregating with the men who had infertility (but not present in their siblings). Of note, the majority of these five novel genes were associated with maturation arrest, with one associated with a Sertoli cell-only pattern. Gene expression was noted to be remarkably testis-specific, with evidence in experimental animals for their potential role in spermatogenesis for 4 of 5 genes. Among an additional 75 unrelated men, they found a 13% frequency of additional recessive variants, with no variants in fertile controls (13).

More recent evaluation of a population of 96 men from Northern Africa who were negative for karyotypic or Y microdeletion defects found 23% of these men had highly deleterious variants identified using a panel of only 151 genes. Six of the 16 variants identified in these 22 men had novel genes associated with their infertility (14). As discussed below, seven of the men had variants in piwi or DNA repair pathways with 12 having meiotic process gene defects identified. Of note, the men with defects in meiotic pathways did not have sperm retrieved, suggesting a potential prognostic role of such genetic testing.

Despite having a uniform, identifiable genetic abnormality, men may still result in a variable spermatogenic pattern within the testis. Deletions of AZFc are uniformly associated with impaired spermatogenesis; about 40% of these men are azoospermic and the remainder have severe oligospermia or even cryptozoospermia. However, within the testis, we commonly observe heterogeneity between different seminiferous tubules. So, although the AZFc deletion is the same in every cell of the body, individual tubules may have Sertoli cell-only, maturation arrest or hypospermatogenesis. Typically, each tubule will have the same pattern of spermatogenesis within the tubule, but an adjacent seminiferous tubule will often have a different histologic pattern. The explanation for such variation between tubules remains elusive, even when the genetic defect is uniform within the testis.

Unfortunately, genetic variants that cause spermatogenic failure may have unique or varied roles when evaluated in different ethnic groups or countries. For example, when Iberian investigators looked for 6 variants found in an Asian population, they observed that 3 variants were associated with spermatogenic failure in both additive and dominant models, with an associated negative predictive value for sperm retrieval for one of the variants. Of note, some of the variants are associated with lincRNAs, non-coding RNAs longer than 200 bp that are transcribed autonomously and do not overlap coding genes. It is widely accept that these lincRNAs control the expression of nearly genes in a tissue-specific manner. Of note, the testis represents the most enriched tissue in lincRNAs in humans (15).

The search for specific causal variants in populations with male infertility has been slow and tedious with only rare mutations identified using large study groups of well-characterized patients with severe male infertility. The genetic variants have also been identified as having a broad series of potential roles in spermatogenesis, including roles in genome integrity (16) as well as piRNA processing (16, 17). Definition of the specific cause of male infertility is particularly important, as it is now recognized that severe male infertility is a risk factor for future cancer development (4, 18). Testing that would allow clearer identification of the patient's risk would be much more useful than simple counseling about "increased risk". Patients are obviously confused and frustrated when increased risk exists but clinicians are unable to provide focused recommendations on how prior infertility patients should be screened for cancers. Specific identification of the causal etiology for infertility, whether a DNA repair defect or otherwise, would be critical for clinical recommendations in long-term follow up for cancer risk. Although accumulating evidence suggests that a genetic cause is common for severe male infertility, a clinically informative gene testing panel to aid in diagnosis is not currently widely available.

TRANSLATION BETWEEN RODENT AND HUMAN MODELS

Rodents have remarkably high spermatogenic efficiency, and uniform patterns of histology, both of which are very different from human spermatogenesis. It is likely that a toxic effect on human fertility can occur without detection during screening in a rodent model. One example where rodent models have not been helpful is in the detection of the adverse effects of selective serotonin antagonists on male fertility potential. Whereas testosterone levels drop by 200 ng/dL and 50% of a cohort of normal men will have abnormal sperm DNA integrity produced within weeks of taking the SSRI (19), paroxetine, this defect was not detected in rodent models at high dose. This may be related to the fact that SSRIs act on sperm transport rather than sperm production, it is possible that the qualitatively and quantitatively limited human sperm production can be adversely affected by a drug, such as finasteride, without observing such an effect in a rodent model.

Another area where rodent models were limited in their ability to identify and/or quantify the role of specific genetic defects in spermatogenesis was for genes on the Y chromosome. In part, this relates to the observation that mammalian Y chromosomes can be highly divergent, but also that Y-gene targeting is made more difficult by the highly repetitive nature of the Y, also limiting genetic sequencing difficult with classical approaches (20). From a clinical standpoint, we know that several regions on Yq are critical for human spermatogenesis,

including genes on AZFa as well as AZFb and SRY. However, it has been proposed by some that only two Y genes are essential for murine male fertility (21).

Certainly, there continue to be roles for murine models of spermatogenesis, to detect or confirm a putative genetic cause of male infertility. At a minimum, such data help to support statistical evaluations of an association between genetic variants and impaired spermatogenesis. However, there are substantial limitations to using a rodent model to predict human spermatogenesis.

SUMMARY

Human spermatogenesis is unique in mammalian models of testicular function. Not only should we avoid assuming that an observation in a rodent model will predict human testicular function, but continued work to evaluate human spermatogenesis directly will be required to understand male fertility.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

This work is based on the sole authors observations, writing, editing and review of final submission.

FUNDING

The article was funded in part by NICHD P50HD096723, as well as The Reeve Foundation fund of Weill Cornell Medicine.

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Conflict of Interest: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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REVIEW
published: 19 May 2022
doi: 10.3389/fendo.2022.903684

Retinoblastoma-E2F Transcription Factor Interplay Is Essential for Testicular Development and Male Fertility

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The retinoblastoma (RB) protein family members (pRB, p107 and p130) are key regulators of cell cycle progression, but also play crucial roles in apoptosis, and stem cell self-renewal and differentiation. RB proteins exert their effects through binding to E2F transcription factors, which are essential developmental and physiological regulators of tissue and organ homeostasis. According to the canonical view, phosphorylation of RB results in release of E2Fs and induction of genes needed for progress of the cell cycle. However, there are eight members in the E2F transcription factor family with both activator (E2F1-3a) and repressor (E2F3b–E2F8) roles, highlighting the functional diversity of RB-E2F pathway. In this review article we summarize the data showing that RB-E2F interaction is a key cell-autonomous mechanism responsible for establishment and maintenance of lifelong male fertility. We also review the expression pattern of RB proteins and E2F transcription factors in the testis and male germ cells. The available evidence supports that RB and E2F family members are widely and dynamically expressed in the testis, and they are known to have versatile roles during spermatogenesis. Knowledge of the function and significance of RB-E2F interplay for testicular development and spermatogenesis comes primarily from gene knock-out (KO) studies. Several studies conducted in Sertoli cell-specific *pRB*-KO mice have demonstrated that pRB-mediated inhibition of E2F3 is essential for Sertoli cell functional maturation and cell cycle exit, highlighting that RB-E2F interaction in Sertoli cells is paramount to male fertility. Similarly, ablation of either pRB or E2F1 in the germline results in progressive testicular atrophy due to germline stem cell (GSC) depletion, emphasizing the importance of proper RB-E2F interplay for germline maintenance and lifelong sperm production. In summary, while balanced RB-E2F interplay is essential for cell-autonomous maintenance of GSCs and, the pRB-E2F3 system in Sertoli cells is critical for providing GSC niche thus laying the basis for spermatogenesis.

Keywords: retinoblastoma protein, spermatogenesis, testis, E2F transcription factor, germ cell, Sertoli cell

OPEN ACCESS

Edited by:
Barry Zirkin, Johns Hopkins University, United States

Reviewed by:
Kate Lakoski Loveland, Monash University, Australia
Wei Yan, Lundquist Institute for Biomedical Innovation, United States

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Specialty section:
This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology

Received: *24 March 2022*
Accepted: *11 April 2022*
Published: *19 May 2022*

Citation:
Mäkelä J-A and Toppari J (2022) Retinoblastoma-E2F Transcription Factor Interplay Is Essential for Testicular Development and Male Fertility. Front. Endocrinol. 13:903684. doi: 10.3389/fendo.2022.903684

RB-E2F PATHWAY

Strict regulation of the cell cycle is critical during testicular development and steady-state spermatogenesis. The mechanisms that define whether a cell stays in the G_1 state or transits to S phase or G_0 are ultimately responsible for normal development of any tissue and its function under homeostasis. The G_1/S transition is controlled by the interaction between retinoblastoma (RB) tumor suppressor proteins and E2F transcription factors. In G_1 RB family proteins form complexes with E2Fs and various chromatin modifiers to repress E2F activity on the promoters of the genes that are needed to enter the S phase. The function of RB is thus growth-inhibitory and misregulation of RB-E2F interplay is one of the hallmarks of cancer (1). The RB-dependent repression on E2F-driven transcription is relieved upon phosphorylation of RB by cyclin-dependent kinases (CDKs) resulting in cellular growth, DNA synthesis and advancement of the cell cycle (**Figure 1**). It is considered that many different stimuli that affect cell fate decisions are channeled through CDKs to control the phosphorylation status of RB to ultimately control the progress or arrest of the cell cycle at G_1 (2).

Efficient entry in the S phase depends on numerous phosphorylation events on RB resulting in the dissociation of the RB-E2F complex and activation of E2F transcription factors. While numerous different kinases have a capacity to phosphorylate RB (3), the role of CDK4/6 and CDK2 in G1/S transition is probably the most critical (4). The transcriptional targets of E2F include *cyclin E*, *Cdk1, DNA polymerase-alpha* and *E2f*s themselves (5). While the molecular control of CDK-RB-E2F pathway has been much studied and relatively well understood, the versatility in RB and E2F protein families brings a layer of complexity to the big picture.

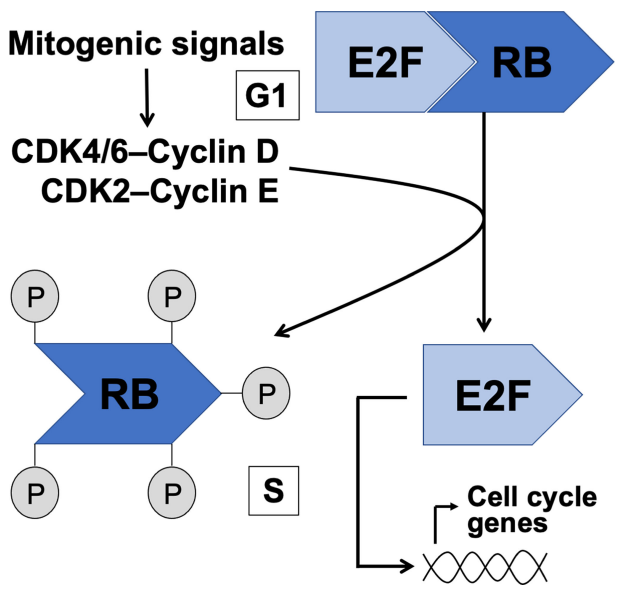

FIGURE 1 | The canonical RB-E2F pathway in control of G1 to S phase transition. Intra- and extracellular mitogenic signals are considered to converge on CDK/cyclin activation resulting in phosphorylation of RB, release of E2F and transcription of S phase genes.

The retinoblastoma protein family consists of three proteins (pRB, p107 and p130; also known as RB1, Retinoblastoma-like 1 and Retinoblastoma-like 2 (RBL1-2), respectively) that share significant homology, yet only *pRb* is found frequently mutated in cancer (6). While some degree of functional redundancy in the RB protein family is apparent - especially between p107 and p130, as observed in mouse knock-out studies - the data support an indispensable role for pRB in development and oncogenesis [reviewed in (7)]. Yet, in a physiological context evaluation of such aspects is often irrelevant due to cell-type dependent expression patterns, as we will later see also in the testis. Thus far eight different *E2f* genes (*E2f1-8*) are known, giving rise to nine distinct E2F proteins. E2F1, E2F2 and E2F3A are generally considered as transcriptional activators, whereas E2F3B and E2F4-8 have been assigned transcriptional repressor functions. However, at least in some developmental contexts E2Fs display functional plasticity and may interconvert between repressor and activator functions (8–12). While activator E2Fs are generally considered to function in cell proliferation, repressor E2Fs are involved in cell cycle exit and differentiation (13). However, such clear-cut functional dichotomy in a physiological context barely exists, and the downstream effects of E2F activation are context-dependent. A notable feature in the E2F protein family is functional redundancy, and in many cases two or more *E2f* genes need to be ablated in order to achieve a phenotypic change (14).

RB family proteins are so called pocket proteins that interact widely with different proteins (>300 proteins have been identified as possible binding partners), not just with E2Fs (15, 16). Within the E2F family they show preferences in terms of interaction partners, and while E2F1-3 predominantly associate with pRB, E2F4-5 preferentially interact with p107 or p130 (7). Conversely, E2F6 is an RB-independent transcriptional repressor and instead forms a complex with Polycomb group proteins (17), and E2F7/8-driven transcriptional repression is also independent from CDK-mediated phosphorylation of RBs (18). Notably, the function of RBs goes beyond gatekeeping G_1/S transition and they play critical roles in quiescent, senescent and differentiating cells by maintaining G_0/G_1. Among E2Fs the interaction of E2F1 with pRB is considered unique and even hyperphosphorylation of pRB does not fully preclude this interaction (19, 20). Conspicuously, E2F1 is also the most extensively studied of the E2F family members and future investigations will be required to elucidate the nuances in tissue-, cell- and context-dependent action of different E2Fs.

In addition to its canonical role in control of the cell cycle and proliferation, the RB-E2F pathway has also been implicated in regulation of heterochromatin and chromosome stability, and apoptosis (2), all of which are very relevant to spermatogenesis. A number of studies have shown that increased E2F activity due to RB loss-of-function has an adverse effect on chromosome stability and causes aneuploidy (2, 21). One of the underlying mechanism may be deregulation of the pericentric heterochromatin, an

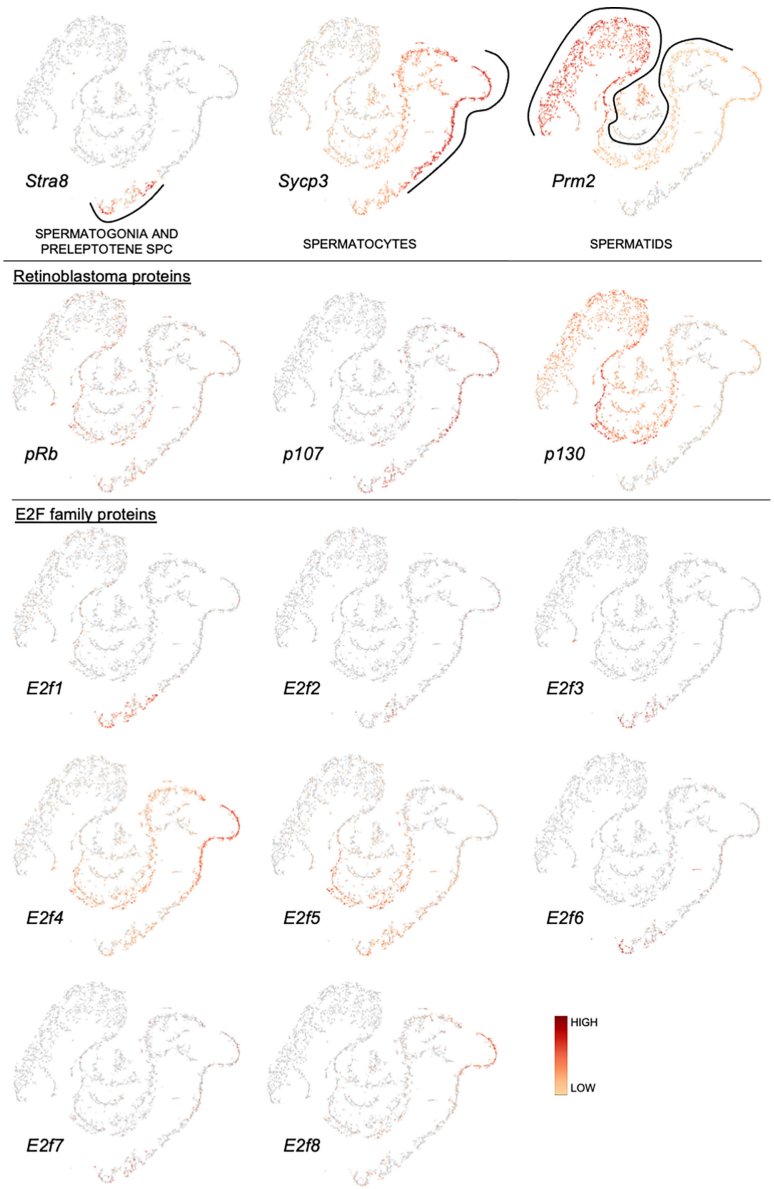

FIGURE 2 | Expression of retinoblastoma protein family and E2F transcription factor family mRNAs along a pseudotime trajectory of adult mouse spermatogenic cells. The data is extracted from Hermann et al. 2018 (26) using Loupe Cell Browser v6.0.0 from 10x Genomics. *Stra8*, *Sycp3* and *Prm2* are included to denote spermatogonia/preleptotene spermatocytes, spermatocytes and spermatids, respectively.

essential chromosomal region for proper segregation during mitosis and meiosis (22, 23). Considering the role of activator E2Fs in cell cycle progression, it is somewhat counterintuitive that increased E2F activity is also able to induce apoptosis *via* DNA damage signaling pathways. According to a model proposed by Dick & Rubin (2013) this involves extensive post-transcriptional modifications in both RB and E2F1 allowing expression of pro-apoptotic genes, but repression of E2F-dependent cell cycle genes (2). All these different functional aspects of RB-E2F pathway make it a key regulator of testicular development and physiology, as will be highlighted in this article.

DYNAMIC EXPRESSION OF RBS AND E2FS IN RODENT TESTIS

mRNA Expression in Mouse Spermatogenic Cells

Application of single-cell RNA-sequencing technology (scRNA-seq) for the analysis of gene expression during spermatogenesis has provided a powerful tool to better understand its molecular regulation and identify the genes/pathways involved in it in various organisms (24–32). We took advantage of a previously published adult mouse testis scRNA-seq dataset (26) and Loupe

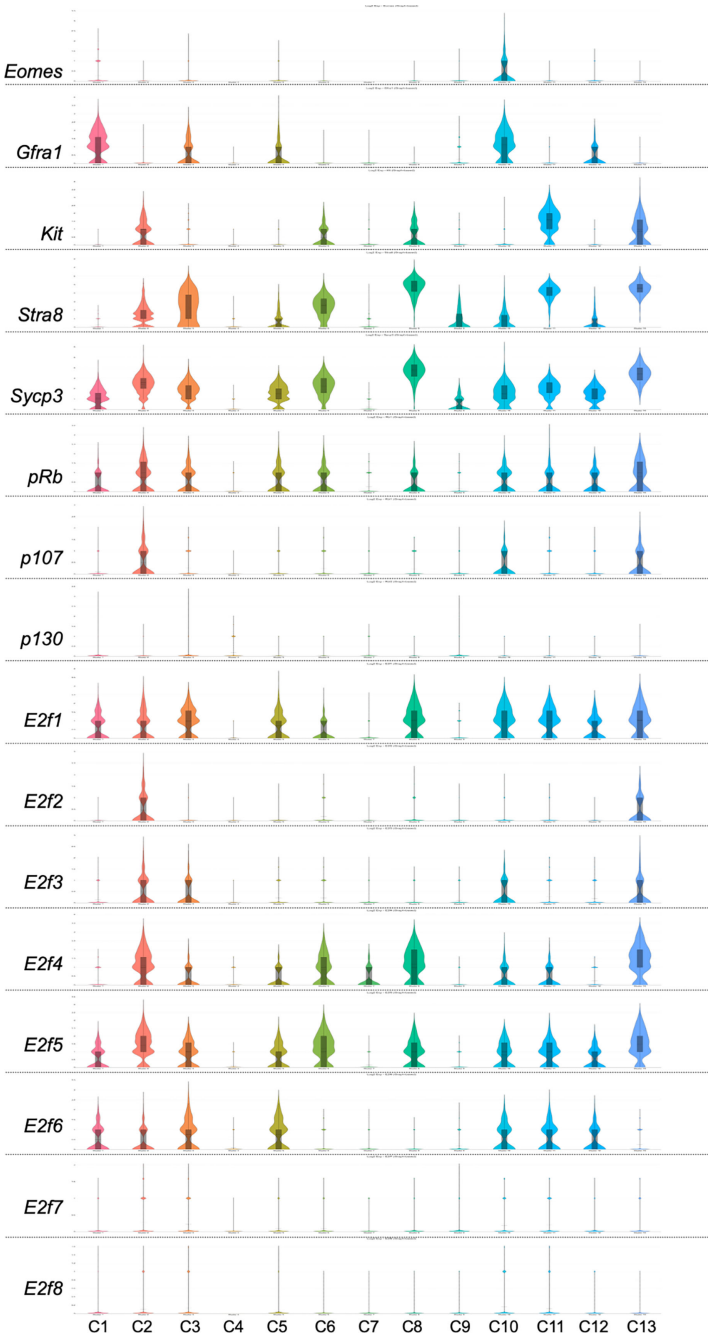

FIGURE 3 | Violin plots displaying the expression of *Eomes, Gfra1, Kit, Stra8, Sycp3, pRb, p107, p130* and *E2f1-E2f8* in spermatogonial cells of an adult mouse. The data is extracted from Hermann et al. 2018 (26) using Loupe Cell Browser v6.0.0 from 10x Genomics. Spermatogonia are divided into clusters 1-13 (C1-13).

Cell Browser v6.0.0 from 10x Genomics to visualize the distribution of RB and E2F family mRNAs along the entire spermatogenic trajectory (**Figure 2**), or specifically in spermatogonia (**Figure 3**) and round spermatids (**Figure 4**). We chose the dataset of Hermann et al. (2018) because it is the first comprehensive scRNA-seq analysis of adult mouse spermatogenic cells distinguishing 11 cell types (26) and enabling feasible analysis of their gene expression signature. For this analysis spermatogonia were further divided into clusters (1-13; **Figure 3**), with clusters 1, 3, 5 and 10 representing undifferentiated spermatogonia as defined by expression of *Gfra1* (33, 34) and *Eomes* (35, 36), and clusters 2, 3, 6, 8, 11 and 13 representing differentiating spermatogonia with characteristic expression for *Kit* (37, 38), *Stra8* (39, 40) and *Sycp3* (41, 42). Round spermatids were divided into early, mid and late

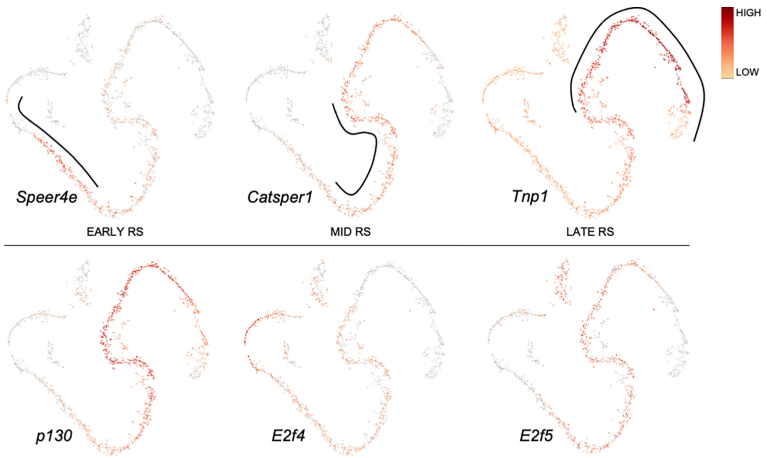

FIGURE 4 | Expression of *p130, E2f4* and *E2f5* along a pseudotime trajectory of sorted mouse round spermatids. The data is extracted from Hermann et al. 2018 (26) using Loupe Cell Browser v6.0.0 from 10x Genomics. Expression for *Speer4e, Catsper1* and *Tnp1* are included to demarcate early, mid and late round spermatids, respectively.

steps (**Figure 4**) based on expression of *Speer4e*, *Catsper1* and *Tnp1* (26). Besides the entire spermatogenic trajectory (**Figure 2**), we decided to focus on GSCs/spermatogonia (**Figure 3**) because they are the foundation of spermatogenesis, and round spermatids (**Figure 4**) because *p130*, *E2f4* and *E2f5* display intriguing expression patterns in these cells. It should be noted that the data presented in **Figures 2–4** are from a previously published dataset (26) and their reliability have not been validated with independent approaches.

pRB (Rb1)

pRB is expressed rather uniformly throughout spermatogenic differentiation (**Figure 2**), and also in most cell clusters within the spermatogonial compartment (**Figure 3**).

p107 (Rbl1)

p107 is expressed in spermatocytes, and to a lesser extent in early spermatids (**Figure 2**) and spermatogonia (**Figure 3**).

p130 (Rbl2)

p130 is expressed in spermatids (**Figure 2**) with the highest levels in mid-to-late round spermatids (**Figure 4**).

E2f1

E2f1 is expressed in spermatogonia and early spermatocytes (**Figure 2**), which is also supported by RNA *in situ* results (43). *E2f1* expression is rather uniform in all spermatogonial clusters (**Figure 3**) from GSCs (*Eomes/Gfra1*+; clusters 1 and 10) to differentiating spermatogonia (*Kit/Sycp3/Stra8*+; clusters 2, 3, 6, 8, 11 and 13).

E2f2

There are very few cells expressing *E2f2* along the spermatogenic trajectory (**Figure 2**).

E2f3

E2f3 is expressed in a limited fashion in spermatogonia and preleptotene spermatocytes (**Figure 2**).

E2f4

E2f4 is expressed in spermatogonia, spermatocytes and round spermatids (**Figure 2**). In spermatogonia (**Figure 3**) the highest *E2f4* levels are seen in clusters 2, 6, 8 and 13 (*Kit/Sycp3/Stra8*+) corresponding to differentiating spermatogonia. In round spermatids *E2f4* levels are highest in early and mid steps (**Figure 4**).

E2f5

E2f5 closely follows the expression pattern of *E2f4* but is clearly less expressed in spermatocytes and early round spermatids (**Figure 2**). Within the round spermatid population the highest levels are seen in mid to late steps (**Figure 4**). Expression in spermatogonial clusters is rather uniform but with highest levels in differentiating spermatogonia (**Figure 3**).

E2f6

E2f6 expression is restricted to spermatogonia and only sporadically detected elsewhere (**Figure 2**). Within the spermatogonial compartment *E2f6* is enriched in clusters that are also positive for *Gfra1* (1, 3, 5 and 10) and thus potential GSCs (**Figure 3**).

E2f7-8

Spermatogenic expression for *E2f7* and *E2f8* is low. Late spermatocytes and/or early round spermatids show limited *E2f8* expression (**Figure 2**).

Protein Expression in Rodent Testis

The protein expression of all RB and most E2F family members have been studied in a handful of articles. The expression pattern for each protein is summarized in **Figure 5**.

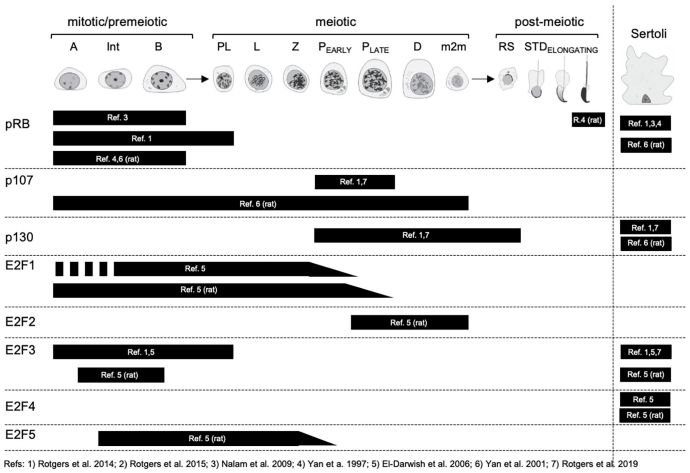

FIGURE 5 | Expression of RB and E2F family proteins in spermatogenic cells and Sertoli cells. Mitotic/premeiotic germ cells are divided into type A (A), type Intermediate (Int) and type B (B) spermatogonia. Meiotic germ cells are presented as preleptotene (Pl), leptotene (L), zygotene (Z), early and late pachytene (P) and diplotene (D) spermatocytes, and secondary spermatocytes, m2m. Post-meiotic germ cells are round spermatids (RS) and elongating spermatids (STD).

pRB (RB1)

In the mouse testis Nalam et al. (2009) reported the staining of pRB in Sertoli cells and spermatogonia (44), while Rotgers et al. (2014) observed the same but extended the staining to preleptotene spermatocytes (45). In the rat testis Yan et al. (2001) confirmed pRB staining in Sertoli cells and spermatogonia but also showed that the highest levels of Sertoli cell pRB in seminiferous epithelial stages VII-VIII (46). The protein expression of pRB in spermatogenic cells is to some extent in conflict with mRNA level findings (**Figure 2**), and suggests that *pRB* mRNA is not translated to protein in post-mitotic male germ cells.

p107 (RBL1)

Rotgers et al. (2014 & 2019) reported p107 staining solely in pachytene spermatocytes in adult WT mouse (45), which is in agreement with single-cell RNA-sequencing data (**Figure 2**). In the rat testis p107 was localized to spermatogonia and spermatocytes (46).

p130 (RBL2)

In the mouse testis Sertoli cells were found positive for p130 (45, 47). Interestingly, the authors did not mention a weak staining for p130 in spermatocytes and round spermatids that would also be supported by enrichment of *p130* mRNA in these germ cells (**Figure 2**). In the rat testis p130 protein expression is seen in Sertoli cells, Leydig cells and peritubular myoid cells, and excluded from differentiating germ cells (46).

E2F1

The expression of E2F1 in the mouse testis has been reported only in one study. El-Darwish et al. (2006) showed the expression in Intermediate to type B spermatogonia and in leptotene to early pachytene spermatocytes (48). The highest levels of E2F1 were observed in stages IX-XI, *i.e* leptotene and zygotene spermatocytes. These findings align with mRNA expression analysis, although leave the protein expression in type A spermatogonia elusive. Based on functional analyses (discussed later in the text) it is probable that E2F1 is expressed at the protein level already in type A spermatogonia. In the rat testis the E2F1 staining differs slightly with type A spermatogonia already expressing it, and the expression extending to mid-to-late pachytene spermatocytes (48).

E2F2

El-Darwish et al. (2006) also studied the localization of E2F2 in rodent testis. In the rat testis E2F2 is most highly expressed in pachytene and diplotene spermatocytes of stages VII-XIII, but also in secondary spermatocytes (48). In the mouse the results show abundant E2F2 staining in nearly all mouse testicular cell types, excluding condensing spermatids. Compared to the very restricted pattern of expression in the rat and the scanty mRNA expression in mouse (**Figure 2**) the results concerning the staining in the mouse would require further investigation and validation.

E2F3

The data concerning E2F3 expression in the mouse testis is contradictory. While the expression in Sertoli cells is supported by all available data (45, 47, 48), germ cell expression remains somewhat elusive. While El-Darwish et al. (2006) and Rotgers et al. (2014) reported E2F3 staining in spermatogonia and preleptotene spermatocytes (45, 48), which is compatible with mRNA expression (**Figure 2**), Rotgers et al. (2019) did not confirm spermatogonial staining with E2F3a-specific or pan-E2F3 antibodies in the adult mouse testis (47). Isoform unspecific E2F3 antibody, however, did stain spermatogonia in the juvenile testis. In the adult rat testis E2F3 expression was seen in Sertoli cells and spermatogonia (48).

E2F4

Strong E2F4 staining is seen in the nuclei of testicular somatic cells: Sertoli cells, Leydig cells and peritubular myoid cells both in

mouse and rat (48). The authors speculate though, that only the Sertoli cell staining is specific because parallel staining of *E2f4* KO mouse testis sections show that only Sertoli staining was absent. Interestingly, there is a granular staining for E2F4 from spermatocytes to elongated spermatids both in mouse and rat.

E2F5

E2F5 is expressed from type Intermediate spermatogonia to mid-Pachytene spermatocytes in the rat testis (48). To our knowledge E2F5 localization in the mouse testis has not been studied.

As far as we know there are no studies where the localization of E2F6, E2F7 or E2F8 in rodent testis would have been studied.

FUNCTIONAL CONSEQUENCES OF RB-E2F PATHWAY LOSS-OF-FUNCTION IN THE TESTIS

pRB Is Essential for Establishment and Maintenance of GSCs

The fact that *pRB*-null mice are embryonically lethal (49–51) has complicated the study of pRB's tissue-specific functions. In the testicular context, however, this limitation has been circumvented by creation of cell type-specific conditional KO mice (44, 45, 52–54) and *ex vivo* culture (55). Of note, *p107*- and *p130*-deficient mice are viable, healthy and fertile (56, 57). We will first focus on the effects of germ cell-specific ablation of pRB and then summarize the role of pRB in Sertoli cells. The available literature indicates that pRB is indispensable for formation and maintenance of germline stem cells (GSCs). Lifelong ability of sperm production depends on GSCs, whose cell fate decisions need to be tightly balanced in order to maintain high and continuous production of sperm throughout the reproductive life span. In theory, GSCs must undergo both self-renewal divisions to sustain the GSC pool and differentiation divisions to give rise to transit-amplifying progenitor spermatogonia that are destined to complete spermatogenesis. Mechanistically it is not entirely clear to what extent these cell fate decisions are cell-autonomous and how/when the molecular cues in the GSC niche microenvironment are incorporated into the regulation of these events. The stem cell capacity in the mouse testis is thought to reside in a subset of undifferentiated spermatogonia (A_{undiff}), although in regenerative conditions A_{undiff} display functional plasticity suggesting that the number of potential stem cells markedly exceeds that of steady-state stem cells (58, 59).

Given its role as the gatekeeper of G1/S transition, or quiescence vs. proliferation, pRB is understandably a critical regulator of GSCs. The evidence for this comes from various mouse models where *pRB* has been conditionally deleted from germ cells at different ages: prenatally from primordial germ cells (*pRB*-KO^{Blimp1}) (53) or ED15.5-17 gonocytes (*pRB*-KO^{Ddx4}) (52, 54), and postnatally from progenitor-A_{undiff} (*pRB*-KO^{Ngn3}) (54) and progenitor/differentiating spermatogonia (*pRB*-KO^{Stra8}) (54). Contrary to its role in somatic stem cells where inactivation of RB family proteins often results in stem cell expansion, increased apoptosis, altered cell fate/differentiation defects, and initiation of cancer (60), pRB-deficient male GSCs have been reported to lose their capacity to self-renew, possibly explaining why no testicular tumors were observed (52, 54). Notably, the differentiation capacity of *pRB*-null germ cells was not affected but young adult males (until 2-3 months of age) were able to sire offspring. However, due to gradual depletion of GSCs they became infertile with age (52, 54).

Despite the fact that pRB is expressed very early in the germ lineage, its absence does not seem to have any effects on fetal germ cells, termed gonocytes (or prospermatogonia), before ED14.5 (53, 55) which is the time when WT gonocytes start to enter mitotic quiescence (61). Recently, Du et al. (2021) discovered that at ED16.5, when practically all control gonocytes had stopped proliferating, the majority of pRB-KO^{Blimp1} were still engaged in the cell cycle resulting in two-fold higher number of germ cells at the same time point. Subsequently, there is a massive wave of apoptosis in pRB-KO^{Blimp1} testes that ablates the germline by the time of onset of the first round of spermatogenesis (PND3.5-6.5) (53). Interestingly, in this time window the classical effects of pRB-deficiency are also recapitulated in the germline: mitotic overexpansion followed by increased apoptosis. It is not exactly clear what induces programmed cell death in practically all pRB-KO^{Blimp1} germ cells perinatally, but there are a number of factors that might contribute to it, including a failure of pRB-KO^{Blimp1} gonocytes to inhibit the onset of meiosis, a demarcating feature of all fetal male germ cells (53). It has been well documented that the gonocyte population experiences a wave of apoptosis between ED13.5 and ED17.5 (62, 63), which is considered to eradicate genetically or epigenetically defective male germ cells that are developmentally incompetent (64). Considering that the cellular functions of pRB are not limited to cell cycle regulation, it is likely that the reason for germline ablation in pRB-KO^{Blimp1} mice is due to failure of pRB-deficient gonocytes to pass this developmental quality control check-point.

Perinatal testicular development in *pRB*-KO^{Ddx4} mice (*pRB* deleted in ED15.5-17 gonocytes) is apparently normal although the number of spermatogonia in the pre/peripubertal testis is higher than in control mice (52). Despite a previously established role for pRB in control of gonocyte cell cycle exit before birth (55), this is not due to extended proliferation of gonocytes and *pRB*-KO^{Ddx4} mice are born with a normal number of germ cells (52), suggesting that conditional deletion of *pRB* at the time of gonocyte quiescence, does not disrupt this state. Notably, and in contrast to pRB-KO^{Blimp1} mice, the GSC pool is formed in pRB-KO^{Ddx4} mice because GFRa1-positive (GDNF family receptor alpha 1) cells are observed at the basement membrane of the seminiferous epithelium (52). These GSCs, however, show very limited, if any, self-renewal capacity (52, 54). Although it has not been studied whether the GSC niche forms properly in pRB-KO^{Ddx4} mice, it is conceivable that inability to self-renew is likely GSC-intrinsic rather than due to extrinsic factors, such as lack of GDNF (glial cell line-derived neurotrophic factor) (36, 65). Hu et al. (2013) also report that pRB-KO^{Ddx4} GSC exit from self-renewal is followed by expansion of progenitor-A_{undiff} population (52). This is supported by increased density of

spermatogonia in peripubertal mice but suffers some limitations because the authors use PLZF (promyelocytic leukemia zinc finger) as a marker of A_{undiff}, while PLZF is also expressed in differentiating spermatogonia (66, 67). Nonetheless, these data support that pRB regulates the timely and stage-dependent cell cycle entry and exit in spermatogonia.

If pRB is ablated in progenitor-A_{undiff} or differentiating spermatogonia, as in *pRB*-KO^{Ngn3} and *pRB*-KO^{Stra8} mice, there are no effects on male fertility, but the testes of respective mice are often cystic and have areas that are devoid of spermatogenic cells, and sometimes, as Yang et al. (2013) report, house cells with neoplastic features (54). These results suggest that the consequences of pRB-deficiency in differentiation-committed and differentiating germ cells and all subsequent spermatogenic cell types are significant but possibly compensated by other RB family members, as also suggested by **Figure 2**, given the continuation of qualitatively normal spermatogenesis in adult *pRB*-KO^{Ngn3} and *pRB*-KO^{Stra8} mice.

To summarize, the spermatogenic phenotype that germ cell-specific *pRB* deletion inflicts highly depends on the developmental stage where the deletion has been induced. While *pRB*-KO^{Blimp1} mice are sterile, pRB-KO^{Ddx4} mice undergo 1-2 rounds of spermatogenesis, and both *pRB*-KO^{Ngn3} and *pRB*-KO^{Stra8} mice display qualitatively normal spermatogenesis and are fertile (52–54). These data demonstrate that pRB is a critical regulator of formation and maintenance of GSCs but dispensable for spermatogenic differentiation of their progeny.

RB-E2F3 in Sertoli Cell Maturation

In addition to germ cells, the developmental and functional consequences of pRB ablation have also been studied in Sertoli cells (44, 45). Sertoli cells are the only somatic cell type inside the seminiferous tubules and they are paramount to testicular development, function and spermatogenesis (68). It is considered that the number of Sertoli cells ultimately defines sperm production capacity because each Sertoli cell is able to support a finite number of spermatogenic cells (69). Therefore, any factors that impinge on Sertoli cell proliferation or apoptosis are a potential threat to male fertility. Sertoli cells are specified in the mouse XY gonad following the expression *Sry* (sex-determining region Y) and its most significant downstream target gene *Sox9* (SRY-box 9) at ED10.5-11 (61). Sertoli cells then coordinate the differentiation of all other testicular cell types, including the germ cells. Sertoli cells undergo maturation in pubertal testis involving polarization, formation of the blood-testis barrier, a profound change in the transcriptome/proteome and exit from the cell cycle. Notably, Sertoli cells of an adult mouse do not proliferate but are in a seemingly terminally-differentiated state. However, under specific *in vitro* circumstances they are able to resume the cell cycle (70, 71). As discussed in detail below, similar to many other somatic cells, but not germ cells, Sertoli cells fail to enter a functionally mature mitotically-quiescent state if lacking pRB (44, 45, 52, 60).

The consequences of Sertoli cell-specific ablation of pRB have been explored in three studies, all of which relied on Cre expression under *Anti-Müllerian hormone* promoter (*pRB*-KO^{Amh}) resulting in pRB loss of function from ED14.5 (44, 45, 47). The studies show congruent results: pRB-deficiency leads to rapid testicular atrophy and male infertility. However, while the gene expression is misregulated at least as early as in PND10 testis, testicular development and onset of spermatogenesis in juvenile mice appear unaffected in *pRB*-KO^{Amh} mice, and until 6-weeks of age there are no obvious changes in testicular histology (44, 45). Subsequently, the changes in the phenotype are fast and the male mice become infertile by early adulthood. The testicular phenotype of *pRB*-KO^{Amh} mice also includes Sertoli cell vacuolization, sloughing of Sertoli cells and immature germ cells from the seminiferous epithelium, increased germ cell apoptosis and Leydig cell hyperplasia (44, 45, 47). Interestingly, this might present an evolutionarily conserved function of RB proteins given a comparable phenotype in *Drosophila* following somatic deletion of *Rbf* (a pRB homolog) (72, 73).

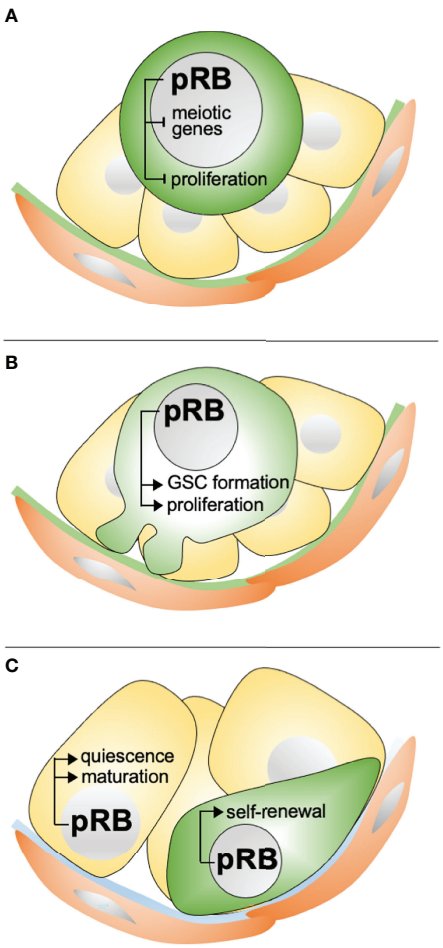

FIGURE 6 | Known developmental roles of pRB in the testis. **(A)** In ED14.5-16.5 gonocytes, pRB is needed for cessation of proliferation and entry into a mitotically quiescent state. pRB likely also inhibits the expression of meiotic genes. **(B)** In the perinatal testis, pRB is needed for cell cycle re-entry and formation of the GSC pool. **(C)** In pubertal and adult testis, pRB is required for functional maturation of Sertoli cells, including their exit from the cell cycle, and self-renewal of GSCs.

There are a few remarkable changes in pRB-ablated Sertoli cell physiology that have a common nominator: inability to mature functionally. First of all, while WT Sertoli cells exit the cell cycle at around PND15, *pRB*-KO^{Amh} Sertoli cells continue to proliferate at a constant rate at least until PND30 (45). This is followed by increased apoptosis probably due to activation of p53 pathway, thus precluding tumor formation (44). While the likely reason for spermatogenic failure in these mice is complex and multifactorial [loss of BTB (blood-testis barrier) integrity, apoptosis of pRB-deficient Sertoli cells to nurture and maintain spermatogenic cells etc.] it has not been carefully studied at which step spermatogenesis fails and whether functional GSCs were sustained in adult *pRB*-KO^{Amh} mice. Considering the critical cell-autonomous role of pRB in GSCs, as described above, this is an interesting question and would deserve further investigation. Given the timeline of male infertility in these mice [2-3 months (44) and 3-6 months (45)] it is conceivable that GSCs are able to undergo at least some rounds of self-renewal in these conditions. This is also supported by qPCR data showing reduced but measurable levels of GSC-associated transcripts: *Gfra1* and *Lin28* (47). However, *Pou5f1* (*Oct4*) expression is lost in *pRB*-KO^{Amh} testis suggesting a loss of progenitor-A$_{undiff}$, where *Pou5f1* expression is enriched (36).

Interestingly, according to Rotgers et al. (2014) the BTB first organizes normally in 6-week old *pRB*-KO^{Amh} mice but then disintegrates (45). However, the data from Nalam et al. (2009) indicate that the BTB is not functional even at this early time point and allows the leakage of a tracer from the interstitium to the adluminal compartment of seminiferous tubules, an immune-privileged site in normal testis (44). These findings suggest that pRB is not developmentally needed for formation of the BTB but is indispensable for its integrity and maintenance, which is another sign of Sertoli cell dedifferentiation and immaturity in *pRB*-KO^{Amh} mice. Interestingly, Sertoli cells deficient for pRB display seminiferous epithelial cyclic activity, as judged by fluctuation of androgen receptor (AR) staining intensity between seminiferous tubule cross-sections (47). AR is one of the proteins that are known to be expressed in Sertoli cells in a seminiferous epithelial stage-dependent manner with the highest levels in early-to-mid stages and lowest levels in late stages (74, 75).

In WT testis pRB interacts with E2F3, the only activator E2F that is expressed in Sertoli cells (45). *E2f3* gene encodes two isoforms, *E2f3a* and *E2f3b*, both of which are expressed in the mouse testis. Interestingly, while Sertoli cells also express p130, Rotgers et al. (2014) do not confirm an interaction between E2F3 and p130 in WT testis. In *pRB*-KO^{Amh} testis, however, E2F3 is found to form a complex with both p130 and p107, whose expression is induced in pRB-deficient Sertoli cells (45). This is in agreement with previous findings showing that when pRB is absent p107 and p130 also bind to activator E2Fs (E2F1-3), not just the repressors (E2F4-5) (76). While these findings imply that there might be a compensatory mechanism, the severity of the phenotype indicates that such a mechanism does not have any functional relevance. Already in their original paper Rotgers et al. (2014) provide data showing that impairment of spermatogenesis due to pRB-deficiency is attenuated by simultaneous silencing of *E2f3 in vivo* (45). These results were corroborated by a follow-up study where *pRB-E2f3* compound-KO^{Amh} mice were created (47). These mice display full spermatogenesis but interestingly the litter size is markedly reduced when compared to control. This might be due to disturbance of non-canonical, *i.e.* non-E2F-mediated, pRB functions in compound-KO^{Amh} mice, including chromatin organization and nuclear architecture which are pronouncedly relevant in the spermatogenic context (77, 78). While spermatogenesis is qualitatively normal in *pRB*-KO^{Amh}/*E2f3*-haploinsufficient mice, they display age-dependent testicular atrophy, and continuously cycling Sertoli cells, suggesting that *E2f3* gene dosage is critical for spermatogenesis. Notably, adult Sertoli cells of *pRB-E2f3* compound-KO^{Amh} mice are non-proliferative and seem to be functionally comparable to their WT counterparts (47). In summary, these data show that spermatogenic failure, prolonged proliferation, increased apoptosis and dedifferentiation of Sertoli cells are mediated by adverse and non-restricted action of E2F3 in Sertoli cells of *pRB*-KO^{Amh} mice, and highlights the role of pRB as a key regulator in life-long maintenance of the non-renewable Sertoli cell population.

Considering the spermatogenic rescue experiments by simultaneous deletion (47) or shRNA-mediated *in vivo* knock-down of *E2f3* (45) on a *pRB*-KO^{Amh} background, it is surprising that *E2f3*-KO^{Amh} mice, where *E2f3* has been deleted from fetal Sertoli cells, are fertile and show no signs of impaired spermatogenesis (47). The authors hypothesize that E2F4 might fully compensate for lack of E2F3 in these mice. While this might be possible there are, however, at least two obvious pitfalls in this interpretation. Firstly, E2F4 is considered a transcriptional repressor, whereas E2F3a isoform is classically considered a transcriptional activator. Secondly, E2F4 primarily associates with p107 and p130, not with pRB. Thus, further investigations are warranted to address this question. Results from these studies further support the concept that Sertoli cells are not terminally-differentiated but in a state of continuous cell cycle repression (79), and show that pRB is responsible for maintaining this non-proliferative state. These conclusions, however, suffer some limitations because pRB is ablated already in fetal Sertoli cells. An inducible *pRB*-deletion in adult Sertoli cells would be required to further elucidate this question. The developmental roles of pRB in the testis are summarized in **Figure 6**.

pRB Is Not Involved in Testicular Tumorigenesis

To our knowledge, there are only very limited data available supporting a relationship between pRB-deficiency and increased tumorigenesis in the testis. Targeted deletion of *pRB* from prenatal germ cells results in germline ablation and infertility (44, 53, 54), and focal GCNIS-like (germ cell neoplasia in situ) like cells are only observed when *pRB* is deleted from progenitors and/or differentiating spermatogonia, arguably due to inability to commit to the spermatogenic lineage (54), an interpretation that might have some merit given that progenitor-A$_{undiff}$ are enriched

for pluripotency-associated transcripts (36). Ablation of pRB in Sertoli cells leads to continued engagement in the cell cycle but also increased apoptosis thus precluding tumor formation (44). Interestingly, Nalam et al. (2010) also studied the compound effects of *pRB* ablation on an inhibin-α-knockout (*Inha* KO) background (80). *Inha* KO mice are known to develop gonadotropin-dependent gonadal stromal tumors, which originate from Sertoli cells (81). Unexpectedly, the double-KO mice did not display exacerbation of the tumorigenic phenotype but Sertoli cell dysfunction took place earlier in these mice when compared to *pRB*-KO^{Amh} mice (80). In summary, there are no strong indications that pRB would have tumor suppressor properties in the mouse testis.

E2F1 Has a Multifaceted Role in the Testis

Similar to other tissues and cellular contexts, E2F1's role in the testis has deserved most attention within the E2F family. E2F1 transcription factor is a critical regulator of the cell cycle, and a direct target of pRB. However, like all activator E2Fs, E2F1 in particular displays intriguing functional dichotomy and is able to induce both proliferation and apoptosis (13). This is apparent also in the testis. E2F1 has been associated with progressive degeneration of the seminiferous epithelium (43, 82–84), GSC maintenance (43, 84), apoptosis of germ cells (43, 85) and Sertoli cells (44), germ cell neoplasia *in situ (*86), testicular tumors (82), testicular descent in human (87, 88) and mouse (84), and human male infertility (87, 88).

Our understanding of E2F1's testicular function comes primarily from mouse work and use of *E2f1*^{tm1Meg} mouse strain (89) (referred to *E2f1*-null hereafter). *E2f1*-null mice display characteristics of progressive testicular atrophy that is manifested in a background strain-dependent manner implying that genetic background of the mice has an impact on the time course and severity of the phenotype (43, 82–84, 89). Interestingly, on C57Bl/6 background the first signs of spermatogenic impairment can be observed as early as 20 days of age when the number of meiotic cells is reduced in *E2f1*-null mice when compared to control (43). E2F1 is an early regulator of the spermatogonial compartment and likely promotes spermatogonial apoptosis during the first wave of spermatogenesis (43). Somewhat counterintuitively, loss of E2F1 does not result in accumulation of spermatogonia during steady-state spermatogenesis but their gradual depletion and occurrence of tubules with Sertoli-cell-only phenotype (43, 82, 84). While the underlying mechanisms are not fully understood it is likely that GSCs in *E2f1*-null mice are lost *via* loss of self-renewal capacity and escape to differentiation. The phenotype thus shares remarkable but limited resemblance to pRB-KO^{Ddx4} mice (52). While *E2f1*-null males remain fertile for at least 5-6 months (43, 82, 84), pRB-KO^{Ddx4} mice become infertile by 2-3 months (52, 54). This is a significant difference because it shows that GSCs are able to self-renew in *E2f1*-null but not in pRB-KO^{Ddx4} mice.

While the exact explanation for the discrepancy in time course is yet to be discovered, there are a couple of likely explanations: compensation by other E2F activators and non-E2F-mediated effects of pRB (discussed above). Notably, apart from *E2f1* no other *E2f*s were differentially expressed in *E2f1*-null testis casting doubts for compensation at the transcriptional level (43), yet leaving the door open for post-transcriptional mechanisms. Interestingly, when human E2F1 is overexpressed in the mouse testis, increased germ cell apoptosis is observed, accompanied with accumulation of undifferentiated spermatogonia (85), thus parallelling GDNF or NANOS2 overexpression phenotype (65, 90). Together these data demonstrate that E2F1 is a critical regulator of GSC maintenance and germ cell apoptosis. Although the definitive data are missing, as how E2F1 might be involved in GSC fate decisions, the literature provides some clues about the potential mechanisms. Transcriptomic changes in *E2f1*-null testis have been studied at 20 days of age by Rotgers et al. (2015) (43), and 3 and 7 months of age by Jorgez et al. (2021) (84). However, choice of the time point and method of study (microarray vs. qPCR) makes the results of these studies hard to compare. While the histological differences in a 20-day-old *E2f1*-null and WT mouse testis are relatively small making comparison between the genotypes feasible at this time point, the transcriptome at this age is different from adult testis with full spermatogenesis which complicates the comparison between the time points. Then again, the cellularity already in a 3-month-old and particularly in a 7-month-old *E2f1*-null testis is dramatically different from WT testis thereby complicating the analysis. For these reasons we will look at the findings separately.

Rotgers et al. (2015) identify a couple of E2F1 candidate target genes in a 20-day old mouse testis that, based on available studies, might be relevant for the development of the phenotype: *Cnot1 (CCR4-NOT Transcription Complex Subunit 1)* and *Chd1* (Chromodomain Helicase DNA Binding Protein 1) both of which are downregulated in *E2f1*-null testes (43). CNOT1 is particularly interesting because it directly interacts with NANOS2, a key intrinsic regulator of the male germline (90, 91). NANOS2 is involved in both degradation and sequestration of specific mRNA molecules in ribonucleoprotein complexes, and its loss results in GSC depletion and rapid germline ablation (59, 90–93). CNOT1 mediates NANOS2 interaction with CCR4-NOT, a major cytoplasmic deadenylase that primes mRNAs for degradation (91, 93). Importantly, CCR4-NOT–CNOT1–NANOS2 interaction is critical for NANOS2 function and maintenance of the male germline (91), a plausible mechanism also explaining GSC depletion in *E2f1*-null mice. Another interesting E2F1-candidate gene discovered by the microarray analysis is *Chd1*. While its role in spermatogenesis has not been studied, two of its family members CHD4 and CHD5 are critical for sperm production, albeit at the opposite ends of this complex process. CHD4 has been shown to be highly expressed in GSCs where its involved in their maintenance and self-renewal (94), whereas CDH5 is required for spermiogenesis and especially for chromatin condensation in elongating spermatids (95, 96).

Jorgez et al. (2021) studied the expression of select 66 mRNAs by qPCR and subsequent proteomic analyses (84). They found changes in the expression of cyclin genes and other E2F1 cell cycle targets. Interestingly, Wnt signaling pathway genes were differentially expressed between the genotypes, and all studied

Wnt ligands were found generally upregulated, especially WNT4. Wnt signaling has been thought to prime GSCs for differentiation and promote exit from the self-renewing state (97–101). Boyer et al. (2012) reported that treatment of cultured GSCs with WNT4 reduces their stemness both *in vitro* and after transplantation (100). These data would support a model where GSCs in *E2f1*-null testis are subjected to differentiation-priming environment at the expense of self-renewal, thus resulting in their depletion over time. To explore this possibility Jorgez et al. (2021) generated a compound *E2f1*-null/*Wnt4*-null mouse line, where *Wnt4* was conditionally deleted from *Stra8*-expressing germ cells, that is progenitor-A_{undiff}/differentiating spermatogonia. Remarkably, the spermatogenic capacity is qualitatively restored in these mice and they display nearly normal fertility parameters, demonstrating that many of the adverse effects of E2F1-deficiency can be overcome by simultaneous removal of WNT4. While the above findings provide a mechanism for GSC loss in *E2f1*-null mice, further studies are needed to address how the RB-E2F pathway is integrated in the control of GSC fate in WT mice, and how is it mechanistically linked with GDNF signaling (36, 65, 102) and mTOR (mammalian target of rapamycin) pathway (67, 103, 104) to balance self-renewal and differentiation of GSCs.

A typical feature of spermatogenic impairment when E2F1 is either deleted or over-expressed is spermatocyte apoptosis (43, 82, 85). While this leads to subfertility, *E2f1*-null male mice are able to sire viable offspring because some spermatocytes manage to avoid cell death. The reason for *E2f1*-null spermatocyte apoptosis is not known but there is data to support that DNA damage is not the underlying cause (43). Further studies are needed to unveil what induces spermatocyte apoptosis if E2F1 is either absent or overexpressed. Considering the expression pattern of E2F1 (highly expressed in premeiotic cells and early spermatocytes; **Figures 2**, **5**) and its role as a transcriptional activator, it is conceivable that the expression of E2F1-target genes needs to be delicately balanced for successful meiosis. It is likely that spermatocyte apoptosis is induced in a cell-intrinsic manner, also considering the fact that despite being expressed in peritubular cells and in the testicular interstitium, E2F1-deficiency was not found to affect testicular somatic cells or gonadotropin or androgen levels (43, 84). However, albeit not known to express E2F1, the Sertoli cells of *E2f1*-null mice display some transcriptomic changes when compared to controls. Surprisingly, while some of the changes may be explained by a change in cellularity in *E2f1*-null vs. control mice testis (43), misregulation of genes involved in tight and adherens junctions were shown to have functional consequences and the blood-testis barrier of 3-month-old *E2f1*-null mice was found leaky (84).

Table 1 provides a summary of the spermatogenic phenotypes of different KO mouse strains where one or two genes encoding proteins of RB-E2F pathway has been deleted.

Repressor E2Fs E2F4 and E2F5 Are Needed for the Development of Male Reproductive Tract

Compared to activator E2Fs, the functions of repressor E2Fs have been less investigated. While both *E2f4* and *E2f5*-deficient mice are viable, they have a shortened lifespan (105–107). *E2f4*-mutants display developmental defects in multiple tissues and have a high early postnatal lethality due to susceptibility to infections (106, 107). *E2f4*-deficient male mice were found subfertile/infertile although the histology of male reproductive organs was reported normal (106, 107), and the underlying basis for this observation therefore remains elusive. There is a lack of knowledge concerning fertility of *E2f5*-mutant mice. However, *e2f5* mutation in the zebrafish results in male infertility due to a spermatogenic arrest in prophase I, and subsequent apoptosis of spermatocytes (108). Rotgers et al. (2014, 2019) report a clear decrease in *E2f4* and *E2f5* expression in *pRB*-KO^{Amh} mouse testis which is, however, likely due to lack of meiotic and postmeiotic germ cells (cf. **Figure 2**). Interestingly, E2F4 and E2F5 have been shown to display redundant roles in controlling the development of the male reproductive tract. *E2f4*-deficiency within the efferent ducts on a *E2f5* heterozygous background leads to a loss of multiciliated cells from the efferent ducts, dilation of the seminiferous tubules and the rete testis, and infertility.

The other repressor E2Fs have been even less studied in the testis. *E2f6*-deficient mice are born at an expected Mendelian frequency, are viable and fertile, and grow and develop normally (109). Pohlers et al. (2005) have reported that E2F6 is needed to suppress the expression of germline genes in somatic tissues (109).

TABLE 1 | Summary of spermatogenic phenotypes in different mouse strains with a deletion in gene(s) encoding RB-E2F pathway proteins.

Mouse strain	Time of deletion	Lineage where deleted	Normal establisment of the GSC pool	Qualitatively/Quantitatively normal 1st round of spermatogenesis	Qualitatively/Quantitatively normal spermatogenesis in adulthood	Ref
pRB-KO^{Blimp1}	ED6.5	germline	No	No/No	No/No	(53)
pRB-KO^{Ddx4}	ED15.5-17	germline	n.a.	Yes/No	No/No	(52, 54)
pRB-KO^{Ngn3}	postnatal	germline	Yes	Yes/n.a	Yes/No	(54)
pRB-KO^{Stra8}	postnatal	germline	Yes	Yes/n.a	Yes/No	(54)
pRB-KO^{Amh}	ED14.5	Sertoli	Yes	Yes/Yes	No/No	(44, 45)
E2f3-KO^{Amh}	ED14.5	Sertoli	Yes	Yes/Yes	Yes/Yes	(47)
pRB-KO^{Amh}/*E2f3*-KO^{Amh}	ED14.5	Sertoli	Yes	Yes/n.a	Yes/No	(47)
pRB-KO^{Amh}/*E2f3*$^{+/-}$	ED14.5	Sertoli	Yes	Yes/n.a	Yes/No	(47)
E2f1^{tm1Meg}	zygotic	universal	Yes	Yes/No	No/No	(43, 84)
E2f1^{tm1Meg}/*Wnt4*-KO^{Stra8}	zygotic/ postnatal	universal/ germline	Yes	Yes/n.a	Yes/No	(84)

n.a, not analyzed.

However, the significance or functions of E2F6 in the germline cells are not known but mRNA level analysis (**Figure 2**) suggests they might be subtle. No fertility problems have been reported in mice deficient for *E2f7* or *E2f8*, whereas the double-null mice are early embryonically lethal precluding any analysis on germline effects (110).

CONCLUDING REMARKS

Considering its role as the gatekeeper of cell cycle, it is no wonder that the RB-E2F pathway is critically important for development and function of the testis. Both the germline and the somatic Sertoli cells depend on proper regulation of its activity during formation of the testis and under steady-state spermatogenesis. Continuous production of sperm relies on lifelong maintenance of GSCs and functionally mature Sertoli cells, two fundamental outcomes of normal function of RB-E2F pathway, highlighting its importance for male fertility. Despite the fact that the functional consequences of pRB, E2F1 and E2F3-deficiency in the testis have been relatively well characterized, further studies are warranted to elucidate how RB-E2F is integrated into the regulation of the germline at the mechanistic level plus shed light on the diversity of RB-E2F signaling beyond pRB and E2F1 and E2F3. Notably, despite relatively high level of mRNA expression in germ cells, the spermatogenic functions of p107, p130, E2F4 and E2F5 are virtually undefined. Examining their roles would probably require creation of compound inducible KO mouse models to circumvent functional redundancy within both the RB and E2F families, and for targeted analyses of spermatogenic functions. Their temporally restricted patterns of expression along the spermatogenic trajectory are intriguing and worthy of deeper investigation.

AUTHOR CONTRIBUTIONS

J-AM and JT: Conception of the article, writing and editing. All authors contributed to the article and approved the submitted version.

ACKNOWLEDGMENTS

The authors want to acknowledge Dr. Sheyla Cisneros-Montalvo (University of Turku) for drawing the cell types to **Figure 5**. Sigrid Juselius Foundation, Kirsten and Freddy Johansen Fund, and Turku University Hospital Special Governmental Funding are acknowledged for financial support.

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frontiers | Frontiers in Endocrinology

ORIGINAL RESEARCH
published: 23 May 2022
doi: 10.3389/fendo.2022.893854

Ablation of the *miR-465* Cluster Causes a Skewed Sex Ratio in Mice

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The X-linked *miR-465* cluster is highly expressed in the testis, sperm, newborn ovary, and blastocysts as well as in 8-16 cell embryos. However, the physiological role of the *miR-465* cluster is still largely unknown. This study aims to dissect the role of the *miR-465* cluster in murine development. Despite abundant expression in the testis, ablation of the *miR-465* miRNA cluster using CRISPR-Cas9 did not cause infertility. Instead, a skewed sex ratio biased toward males (60% males) was observed among *miR-465* KO mice. Further analyses revealed that the female conceptuses selectively degenerated as early as embryonic day 8.5 (E8.5). Small RNA deep sequencing, qPCR, and *in situ* hybridization analyses revealed that the miRNAs encoded by the *miR-465* cluster were mainly localized to the extraembryonic tissue/developing placenta. RNA-seq analyses identified altered mRNA transcriptome characterized by the dysregulation of numerous critical placental genes, e.g., *Alkbh1*, in the KO conceptuses at E7.5. Taken together, this study showed that the *miR-465* cluster is required for normal female placental development, and ablation of the *miR-465* cluster leads to a skewed sex ratio with more males (~60%) due to selective degeneration and resorption of the female conceptuses.

Keywords: *miR-465*, microRNA, sexual dimorphism, sex ratio, extraembryonic tissues, placenta, CRISPR-Cas9

OPEN ACCESS

Edited by:
Erwin Goldberg, Northwestern University, United States

Reviewed by:
Renee Reijo Pera, McLaughlin Research Institute, United States
Jonathan LaMarre, University of Guelph, Canada

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Specialty section:
This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology

Received: *10 March 2022*
Accepted: *11 April 2022*
Published: *23 May 2022*

Citation:
Wang Z, Meng N, Wang Y, Zhou T, Li M, Wang S, Chen S, Zheng H, Kong S, Wang H and Yan W (2022) Ablation of the miR-465 Cluster Causes a Skewed Sex Ratio in Mice. Front. Endocrinol. 13:893854. doi: 10.3389/fendo.2022.893854

INTRODUCTION

Sexual dimorphism refers to different characteristics beyond the sex organs between the two sexes within the same species, e.g., appearance, structure, behavior, etc. (1). Data from a recent study of 14,250 wild-type (WT) and 40,192 mutant mice suggest that 9.9% of qualitative and 56.6% of quantitative traits display sexual dimorphism (2). Sexual dimorphism commences as early as embryonic development, e.g., X chromosome inactivation in the female embryo. Sexual dimorphism is also reflected by differential gene expression profiles in placental, fetal, and adult tissues (3–5). To date, a role of miRNAs in sexual dimorphism has not been reported although miRNAs are well known to be critical for early development (6–9). miRNAs are ~22 nucleotide small non-coding RNAs that regulate gene expression at post-transcriptional levels (10). Inactivation of either DICER or DROSHA, the two enzymes required for miRNA biogenesis, leads to early embryonic lethality in mice, indicating an essential role of miRNAs in early

development (6–9, 11–13). Our previous studies have shown that the X-linked *miR-465* cluster, which encodes 6 pre-miRNAs and 12 mature miRNAs, belongs to a large X-linked *miR-506* family (14). Their high abundance in the testis, sperm, newborn ovary, blastocysts, and 8-16-cell embryos (14–17) suggests a potential role in gametogenesis and early embryonic development in mice. However, their physiological role has not been investigated *in vivo*. Here, we report that the *miR-465* cluster miRNAs are also abundantly expressed in the developing placenta, and ablation of the *miR-465* cluster does not affect fertility but causes a skewed sex ratio favoring males due to selective degradation of the female placenta during early embryonic development.

MATERIALS AND METHODS

Generation of *miR-465* KO Mice

The animal use protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Nevada, Reno (Protocol# 00494). Generation of global *miR-465* KO mice and mouse genotyping were performed as described (14, 18, 19). gRNA and genotyping primers are listed in **Table S1**.

DNA and RNA Isolation, Library Construction, and qPCR Analyses

DNA and RNA were extracted from WT and KO embryos using the mirVana™ miRNA Isolation Kit as previously described (19). The sexes of the conceptuses were determined based on PCR amplification of *DYzEms3* (a Y chromosome-specific repetitive sequence) and *Rn18s* (a housekeeping transcript as the internal control). Males display two bands (*DYzEms3* and *Rn18s*), while females show only one band (*Rn18s*). Large RNA libraries were constructed using KAPA Stranded RNA-Seq Kits with RiboErase (Cat. # 07962282001, Roche) according to the manufacturer's instructions. Small RNA libraries were constructed using NEBNext® Small RNA Library Prep Set for Illumina® (Cat. # E7330L, NEB) according to the manufacturer's instructions. miRNA qPCR was performed as described (14). All oligos for sex determination and qPCR are listed in **Table S1**.

In Situ Hybridization

Cryosections (10 μm) were adhered to poly-L-lysine-coated slides and fixed in 4% paraformaldehyde (Cat. # P6148, Sigma-Aldrich) solution in PBS for 1 h at room temperature. The sections were then washed 3 times in PBS for 5 min each, acetylated for 10 minutes (0.25% acetic anhydride), washed 2 times in PBS for 5 min each, and hybridized with DIG-labeled probes overnight at 50°C. Hybridization buffer contained 1X salts (200 mM NaCl, 13 mM Tris, 5 mM sodium phosphate monobasic, 5mM sodium phosphate dibasic, 5 mM EDTA), 50% formamide, 10% (w/v) dextran sulfate, 1 mg/ml yeast tRNA (Cat. # 10109509001, Roche), 1×Denhardt's [1% (w/v) bovine serum albumin, 1% (w/v) Ficoll, 1% (w/v) polyvinylpyrrolidone], and RNA probe (final concentration: 1 μM). Post-hybridization washes were followed by an RNase treatment (20 μg/ml RNase A). After blocking in 20% heat-inactivated sheep serum (Cat. # ZLI-9021, Beijing Zhongshan Jinqiao Biotechnology Company) and 2% blocking reagent (Cat. # 12039672910, Roche) for 1 h, sections were incubated overnight in blocking solution containing anti-DIG antibody (1:2500 dilution; Cat. # 11093274910, Roche) at room temperature. After washing, the color was developed using NBT/BCIP according to the manufacturer's instructions (NBT: Cat. # N1332, Gentihold; BCIP: Cat. # B1360, Gentihold). Sections were counterstained in Nuclear Fast Red (Cat. # G1321, Solarbio), dehydrated in gradient alcohol, cleared in xylene, and mounted in neutral resins. All oligos used for RNA ISH were listed in **Table S1**.

RNA-Seq Data Analysis

The Sailfish (20) and SPORTS1.0 (21) pipelines were used to quantify the large RNA expression and small RNA expression, respectively. Transcript per million reads (TPM) was used as the unit of gene expression level. Groupwise differential expression was estimated by the likelihood ratio test and the RNAs with a false discovery rate < 5% were deemed differentially expressed.

Luciferase Assay

Luciferase assays were performed as described (22). *cel-mir-67* was used as a negative control. *Renilla* luciferase signals were normalized to *Firefly* luciferase signals to correct the transfection efficiency. All oligos for constructing 3'UTR luciferase vectors are listed in **Table S4**.

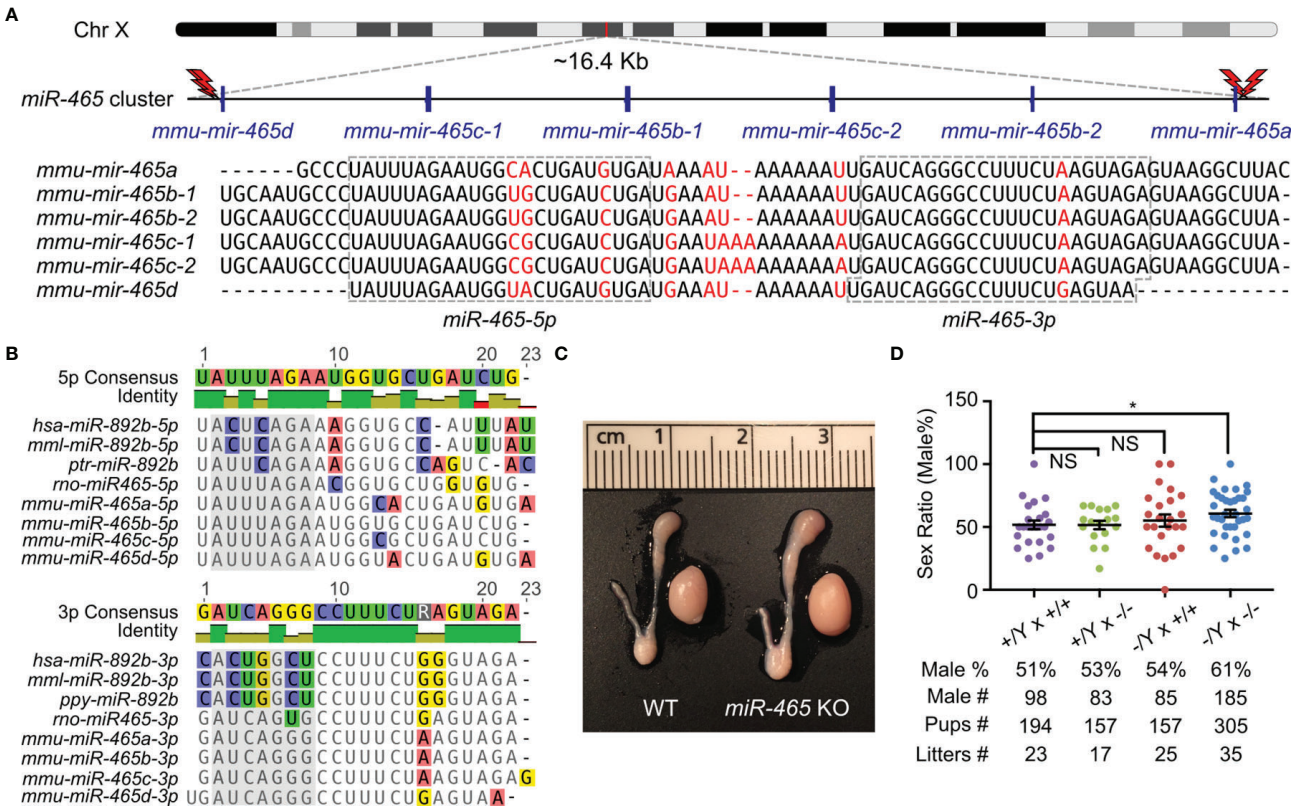
Statistical Analyses

Data are presented as mean ± SEM, and statistical differences between datasets were assessed by two samples t-test unless stated otherwise. $p < 0.05$, 0.01, 0.001, and 0.0001 are considered statistically significant and indicated with *, **, ***, and ****, respectively.

RESULTS

Ablation of the *miR-465* Cluster Leads to a Male-Biased Sex Ratio

The *miR-465* cluster consists of 6 miRNA genes encompassing a ~16.4 kb region on the X chromosome in mice (**Figure 1A**). Although 6 pre-miRNAs and 12 mature miRNAs are produced in mice, only 6 mature miRNAs can be distinguished based on their sequences, including *miR-465a-5p*, *miR-465b-5p*, *miR-465c-5p*, *miR-465d-5p*, *miR-465a/b/c-3p* and *miR-465d-3p* (**Figure 1A**). The *miR-465* cluster has orthologs in humans, monkeys, and chimpanzees, which have been annotated as *miR-892b* in the miRBase and contain some U-to-C or A-to-G substitutions (**Figure 1B**). Like the *miR-465* cluster, *miR-892b* is also flanked by *Slitrk2* and *Fmr1* on the X chromosome (14). To define their physiological roles, we deleted the entire *miR-465* cluster in the mouse genome using CRISPR-Cas9 (**Figures 1A**; **S1A**), as previously described (14, 18, 19). PCR genotyping and Sanger sequencing confirmed that the genomic loci of these miRNAs were successfully deleted (**Figures S1B, C**).



	+/Y x +/+	+/Y x -/-	-/Y x +/+	-/Y x -/-
Male %	51%	53%	54%	61%
Male #	98	83	85	185
Pups #	194	157	157	305
Litters #	23	17	25	35

FIGURE 1 | Generation of *miR-465* KO mice. **(A)** The genomic location and sequences of the *miR-465* cluster on the X chromosome of mice. The red lightning bolts represent the gRNAs used, and their right and left orientations indicate the reverse and forward strands targeted by the gRNAs, respectively. **(B)** The orthologs of the *miR-465* cluster in primates and rodents. Bases highlighted in the grey background are the potential seed regions. **(C)** A representative image of the testis and epididymis of WT and *miR-465* cluster KO mice. One unit on the ruler is 1 mm. **(D)** The sex ratios among pups from different breeding schemes. *, p<0.05; NS, statistically not significant.

The *miR-465* KO mice were fertile with normal testis size (**Figure 1C**). Both the litter size (8.4 ± 0.85, n=35) and litter interval (25.4 ± 0.86, n=34) of the KO mice were comparable to those of WT controls (Litter size: 8.6 ± 1.59, n=23; litter interval: 26.6 ± 1.42, n=22) (**Figure 1D**), suggesting that these miRNAs are dispensable for both spermatogenesis and folliculogenesis. Interestingly, unlike the equal distribution of the two sexes (~50%) among pups from the WT breeding pairs (+/*Y* × +/+), the sex ratio is significantly skewed toward the male (61%, p<0.05) among the *miR-465* KO pups derived from the homozygous breeding pairs (-/*Y* × -/-) (**Figure 1D**). Of interest, ~60% appears to be the most common skewed sex ratio observed in previous reports (**Table S2**) (23–28).

The Skewed Sex Ratio Occurred During Early Embryonic Development

The skewed sex ratio could result from either a distorted X/Y sperm ratio or a loss of female embryos/fetuses during development. If the sex ratio is already skewed in X/Y sperm, the bias should be observed among pups from the breeding pairs of KO males (-/*Y*) and WT females (+/+), but not in those from the breeding pairs of WT males (+/*Y*) and homozygous KO females (-/-). However, the sex ratio among the pups from the -/*Y* × +/+ breeding pairs was slightly, but not significantly, skewed toward males (54%) (**Figure 1D**), suggesting that the significantly skewed sex ratio likely occurs during development. To identify when the skewed sex ratio occurs, we collected early embryos at E3.5, E7.5, and E10.5 in the homozygous breeding pairs (-/*Y* × -/-). Males accounted for ~50% among all of the KO embryos at E3.5 and E7.5, whereas the ratio of the males increased to ~61% at E10.5 (**Figure 2A**), suggesting that some female embryos are lost between E7.5 and E10.5. Indeed, we observed that on average 1-2 conceptuses per uterus were either being resorbed or had already been resorbed between E8.5 and E10.5. More intriguingly, 6 out of 7 conceptuses that appeared to degenerate were all female KOs (**Figures 2B, C**). Together, these data suggest that inactivation of the *miR-465* cluster leads to selective degeneration and absorption of female conceptuses between E7.5 and E10.5.

The *miR-465* miRNAs Are Abundantly Expressed in the Extraembryonic Tissues at E7.5

Although the loss of the *miR-465* cluster leads to female-biased lethality, it remains unknown whether the primary defects lie in

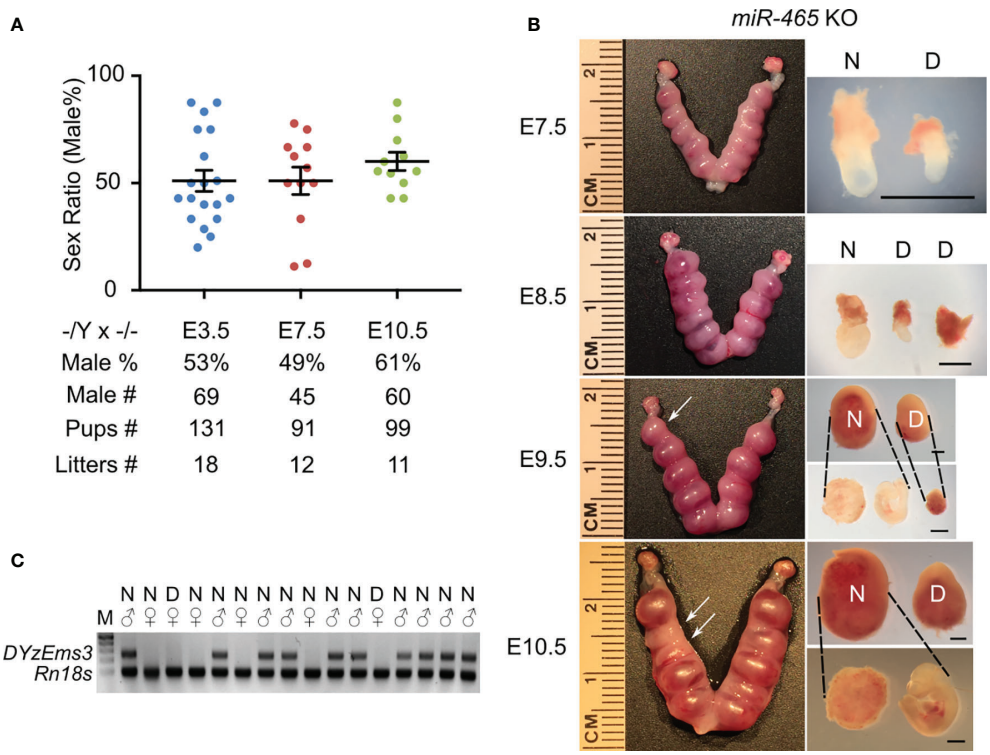


FIGURE 2 | The phenotype of *miR-465* KO mice. **(A)** The sex ratios of pups from homozygous inbreeding (-/Y × -/-) at E3.5, E7.5, and E10.5. **(B)** Representative images of the KO uteri and conceptuses collected between E7.5 and E10.5. Arrows point to the degenerating/degenerated conceptuses (D) among the normal-looking (N) ones. Scale bars = 1mm. **(C)** A representative gel image of genotyping results. *DYzEms3*, a Y-linked genomic fragment, was amplified to identify male conceptuses, and *Rn18s*, which encodes 18s ribosomal RNA, was used as a loading control in the PCR-based genotyping analyses.

the embryos or the extraembryonic/placental tissue. To address this question, we collected both WT and KO embryos and extraembryonic/placental tissues from both sexes at E7.5 and E10.5 and performed small RNA sequencing (sRNA-seq) (**Figures 3A–C**; **S2**). sRNA-seq data confirmed that the *miR-465* cluster miRNAs were indeed absent in the KO embryos and extraembryonic/placental tissues (**Figures 3A**; **S2A–C**). While no significant sex differences in miRNA levels were observed in WT embryos and extraembryonic tissues at E7.5 (**Figures S2D, E**), the *miR-465* cluster miRNAs were predominantly expressed in extraembryonic tissues, as compared to embryos of both sexes at E7.5 (**Figures 3B**; **S2F**), and these miRNAs were significantly downregulated from E7.5 to E10.5 (**Figures 3C**; **S2G–I**). Indeed, the TaqMan real-time PCR analyses further confirmed the sRNA-seq results (**Figure 3D**). We next further performed miRNA *in situ* hybridization (ISH) assays (**Figure 3E**) to corroborate the cellular localization of the *miR-465* cluster. Consistent with the sRNA-seq and qPCR data, miRNA ISH results showed that the *miR-465* cluster miRNAs were predominantly expressed in extraembryonic tissues, especially in the ectoplacental core and chorion (**Figure 3E**). Although the *miR-465* cluster miRNAs were also detected in maternal decidua (**Figure 3E**), potential decidual defects are highly unlikely based on our breeding data showing normal sex ratio among offspring of the +/*Y* × -/- breeding pairs (**Figure 1D**). Given the predominant expression of the *miR-465* cluster in the extraembryonic tissues, it is highly likely that the loss of some female embryos was secondary to placental defects.

Ablation of the *miR-465* Cluster Leads to Dysregulated mRNAs in the Female, but Not the Male, Extraembryonic Tissues

To identify the targets of the *miR-465* cluster miRNAs, we then conducted RNA-seq assays on WT and KO embryos and extraembryonic tissues of both sexes at E7.5. We chose E7.5 because, at this point, despite no obvious degeneration and resorption, the transcriptomic alterations should have accumulated in the implicated female KO conceptuses (**Figure 2B**). Principal component analyses (PCA) identified two major clusters, each containing either embryos or extraembryonic tissues of both WT and most of the KO of both sexes except for two outliers (**Figure 4A**). The two outliers turned out to be one female KO embryo and its extraembryonic tissue, suggesting that this conceptus most likely represents a "to-be-degenerating" KO female. While WT and non-degenerating KO embryos and extraembryonic tissues of both sexes displayed similar mRNA transcriptomes (**Figure 4B**; **Table S3**), numerous differentially expressed genes (DEGs) were identified between

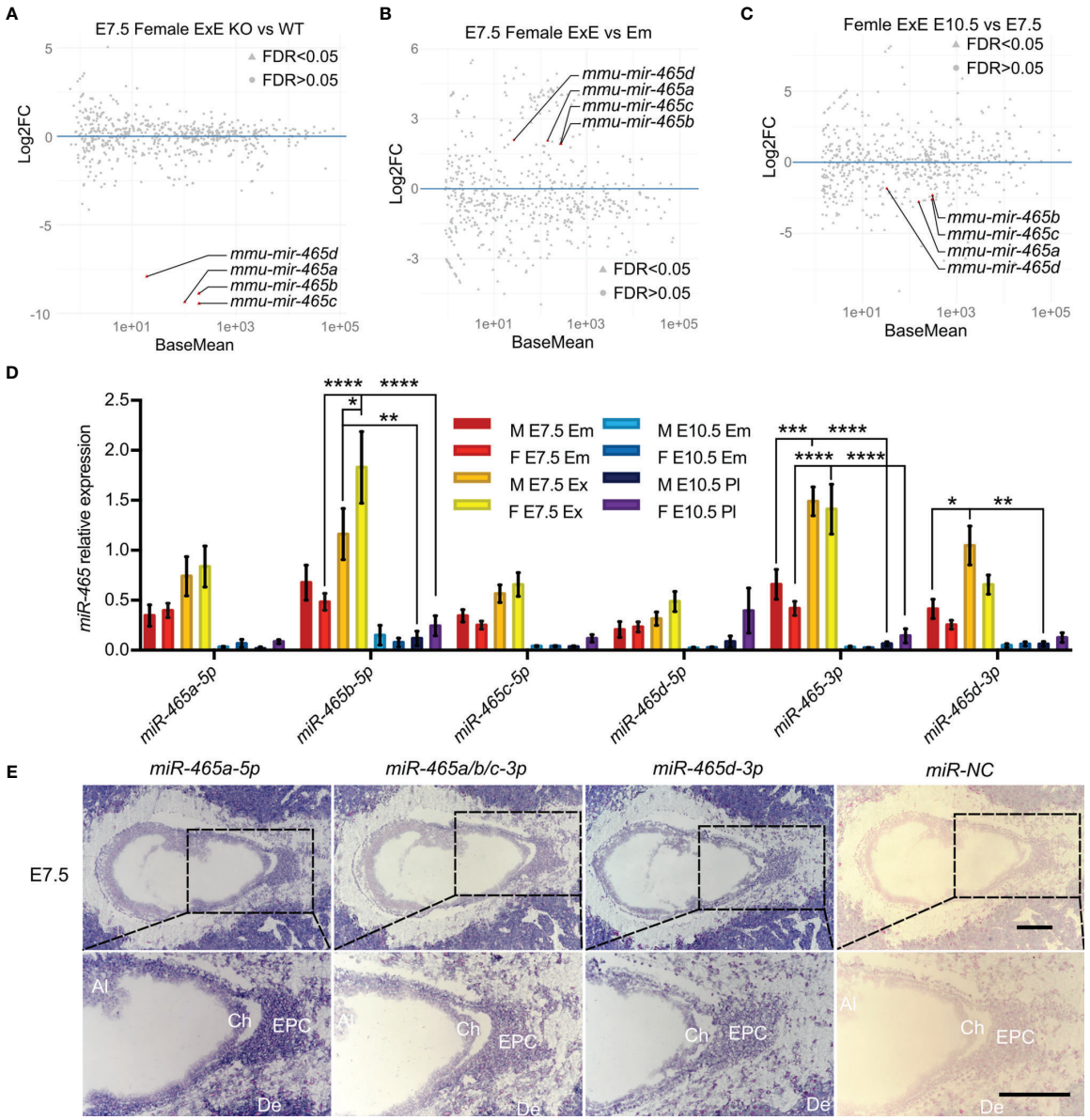


FIGURE 3 | Expression profiles of the *miR-465* cluster. **(A)** Differentially expressed miRNAs between WT and KO female extraembryonic tissues at E7.5. **(B)** Differentially expressed miRNAs between WT female extraembryonic tissues and embryos at E7.5. **(C)** Differentially expressed miRNAs between WT female extraembryonic tissues/placentas at E7.5 and E10.5. Data points representing the *miR-465* cluster miRNAs are marked in red. sRNA-seq analyses were conducted in biological triplicates (n=3). **(D)** TaqMan qPCR analyses of expression levels of the *miR-465* cluster miRNAs in extraembryonic tissues/placenta and embryos at E7.5 and E10.5. M, male; F, female; Em, embryo; ExE, extraembryonic tissue; Pl, placenta. *, p<0.05; **, p<0.01; *** p<0.001, **** p<0.0001. **(E)** Representative miRNA-ISH results showing localization of the *miR-465* cluster miRNAs in female conceptuses at E7.5. Ch, chorion; EPC, ectoplacental core; Al, allantois; De, decidua. Scale bars = 200 μm.

the extraembryonic tissues from the "to-be-degenerating" KO female and those from non-degenerating KO females (**Figure 4B**; **Table S3**). Gene ontology (GO) term analyses identified that the DEGs were primarily involved in extraembryonic/placental development (**Figure 4C**). Among the dysregulated genes responsible for placental development, 8 out of 44 were either imprinted genes or sex-biased genes (**Table S4**). Luciferase assays further confirmed that some of the dysregulated genes were indeed the targets of the *miR-465* cluster miRNAs (**Figure S3**). Given the similar expression levels of the *miR-465* cluster miRNAs in the extraembryonic tissues of both sexes (**Figures S2D, E**), it is likely that the sexually dimorphic role of the *miR-465* cluster is achieved through miRNA-mediated post-transcriptional regulation of the sex-biased target genes. For example, *Alkbh1*, a target of *miR-465* (**Figure S3**), is a tRNA demethylation enzyme (29) highly expressed in chorion and the

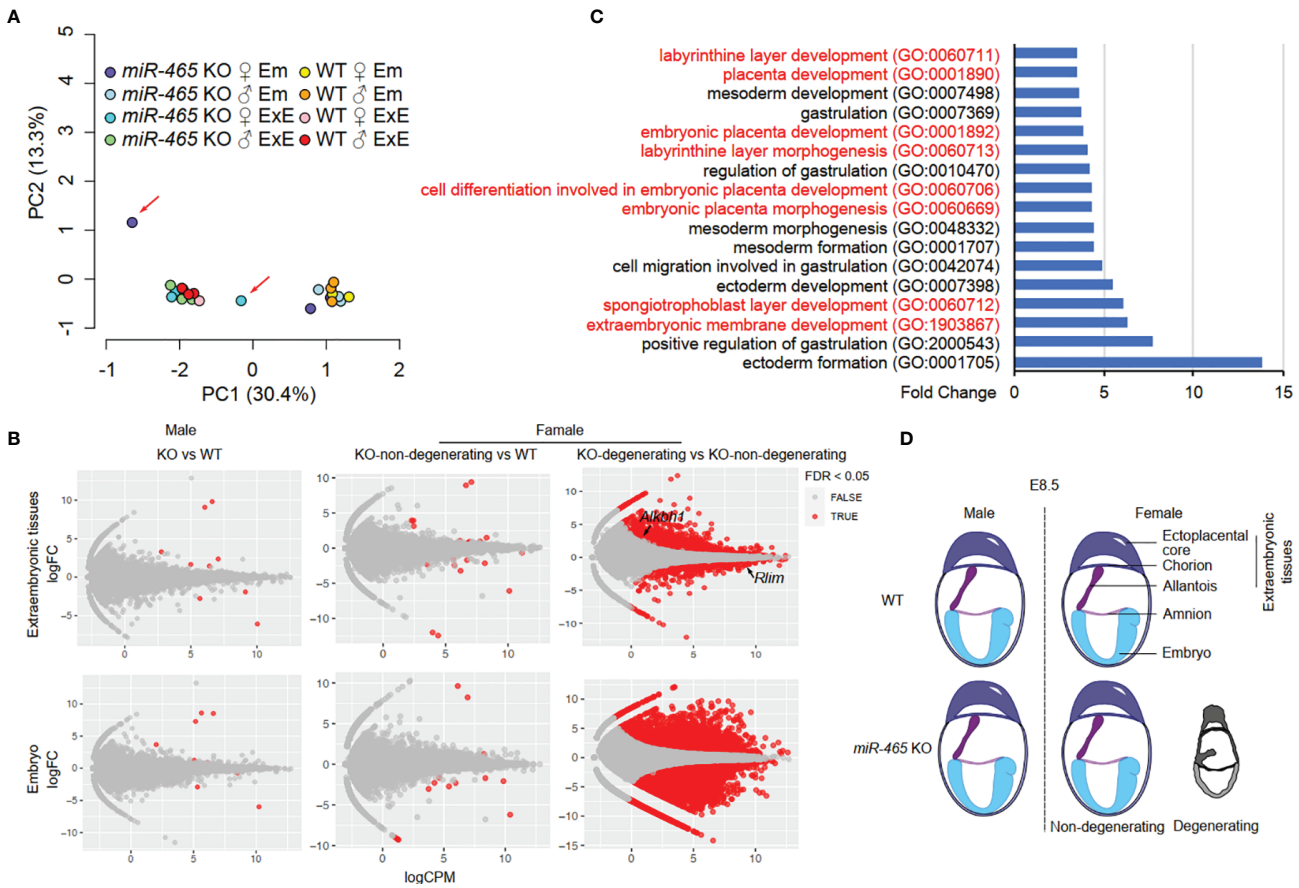


FIGURE 4 | RNA-seq analyses of *miR-465* KO and WT conceptuses. **(A)** Principal component (PC) analyses of RNA-seq data from embryonic (Em) and extraembryonic (ExE) tissues of *miR-465* KO and WT mice. The red arrows indicate the degenerating embryo and extraembryonic tissues from a *miR-465* KO female conceptus. **(B)** Differentially expressed genes (DEGs) identified in the following three comparisons: between the *miR-465* KO and WT males (left), between the *miR-465* KO non-degenerating and WT females (middle), and between *miR-465* KO degenerating and non-degenerating females (right). **(C)** GO term enrichment analyses of DEGs between the degenerating and non-degenerating *miR-465* KO female extraembryonic tissues. GO terms related to extraembryonic development are highlighted in red. **(D)** Schematics showing a critical role of the *miR-465* cluster in supporting the full developmental potential of the female placenta and embryos.

ectoplacental cone at E8.5 (30); its ablation also induces female-biased lethality (27).

DISCUSSION

The X chromosome is known to be enriched in protein-coding genes critical for reproduction and fertility (31, 32). Our earlier work has also shown that several large miRNA clusters, including the *miR-465* cluster, are either exclusively or preferentially expressed in the testis, suggesting a role in controlling spermatogenesis and male fertility (14, 33). Although ablation of the *miR-506* cluster compromises the male fertility (14), inactivation of the *miR-465* miRNA cluster does not affect either gametogenesis or fertility. Surprisingly, a lack of the *miR-465* miRNAs leads to a skewed sex ratio biased toward males due to selective degeneration of the female conceptuses between E7.5 and E10.5. Given its predominant expression in the extraembryonic tissue, the selective degeneration and absorption of female conceptuses in the absence of *miR-465* miRNAs likely reflect the compromised development of the extraembryonic/placental tissue rather than the embryos/fetuses. Therefore, the *miR-465* miRNAs appear to be required for proper development of the female, rather than the male, extraembryonic/placental tissue, supporting a role in sexual dimorphism in placental development. While sexual dimorphism is believed to mainly result from the differential gene expression between the male and female embryos (5), our study provides evidence that the placental development also displays sexual dimorphism, which can lead to a screwed sex ratio in offspring.

The 60% sex ratio seems subtle, but it is quite common in all the previous studies involving biased sex ratios (**Table S2**) (23–28). The X-linked *miR-465* cluster belongs to the SpermiRs/*miR-506* family (14, 34), and these X-linked miRNAs have no

homologs on the Y chromosome. Member miRNAs of the *miR-506* family share numerous targets despite their different seed sequences (14, 34). One previous study has shown that *miR-465a-5p* is upregulated when the *miR-741* is inactivated in the cultured mouse spermatogonial stem cells (SSCs) (34), suggesting genetic compensation between these two miRNAs during spermatogenesis. Indeed, a similar phenomenon was observed in the *miR-465* KO testes. Other *miR-506* family members, including *miR-201*, *miR-463*, *miR-471*, *miR-741*, *miR-871*, *miR-883a*, and *miR-883b*, were upregulated in the *miR-465* KO testes when compared to the WT testes (**Figure S4A**; **Table S5A**). Comparisons between WT male and female extraembryonic tissues at E7.5 yielded no differentially expressed miRNAs. However, comparisons between the KO counterparts, the *miR-10a*, *miR-10b*, and *miR-196b* were upregulated in the KO male extraembryonic tissues (**Figure S4B**; **Table S5B**). Although *miR-10a*, *miR-10b*, and *miR-196b* do not belong to the *miR-506* family, they share a large number of target genes with the *miR-465* cluster (**Figure S4C**), indicating that these miRNAs may compensate for the loss of the *miR-465* cluster in the male extraembryonic tissues, and that the *miR-465* cluster plays a sexual dimorphic role during extraembryonic tissues development. Comparisons between the *miR-465* KO male and WT male or between the KO non-degenerating female and WT female extraembryonic tissues at E7.5 found no upregulated miRNAs, whereas 74 miRNAs were found dysregulated in between the KO degenerating and KO non-degenerating females (**Figures S4D, E**; **Tables S5C–S5E**), suggesting that the degenerating females are more "sensitive" to the *miR-465* KO. Of interest, 26 miRNAs of the 74 dysregulated miRNAs all target *Alkbh1*, one of the validified targets of the *miR-465* cluster that has a sex dimorphic role during extraembryonic development (27). No significant changes in mRNA transcriptome were detected between either WT and KO males, or between WT and the KO non-degenerating females; however, drastic changes were observed between the KO degenerating and KO non-degenerating females. Among these dysregulated genes, some of them are either sex-specific (e.g., *Alkbh1* and *Rlim*) or imprinted genes, further confirming that the *miR-465* cluster influences the extraembryonic development in a sex-specific manner through mediating sex differential genes.

Spontaneous embryonic resorption during early pregnancy is common in most mammalian species, including mice, rats, rabbits, voles, ewes, red pandas, swine, and humans (35–44). Moreover, spontaneous embryonic resorption during early pregnancy does not necessarily lead to reduced litter size (35, 36). Given that the embryonic resorption occurs randomly without obvious sex ratio bias, it is highly likely that both male and female embryos are resorbed at a similar rate to maintain a balanced sex ratio. Some studies have correlated embryonic loss with aberrant placental development (44). A recent in-depth survey of 103 knockout mice lines that display embryonic lethality has revealed that ~68% of these embryonic resorption cases are caused by placental dysfunction (45). Our data that almost all of the resorbed *miR-465* KO embryos are females suggest a sexual dimorphic role of the *miR-465* cluster in extraembryonic/placental development. Like the other X-linked miRNA clusters (14), the *miR-465* has its orthologue in humans, which was named *miR-892b*, suggesting that the findings in mice may apply to humans. Supporting this hypothesis, a recent study in humans showed that the *miR-892b* was downregulated in the plasma collected from preeclampsia pregnancies (46), which is often accompanied by fetal growth restriction and placental abruption (47).

Taken together, our study uncovered an essential role of the *miR-465* cluster in supporting the full developmental potential of the female, but not the male, extraembryonic tissues/placentae (**Figure 4D**). The male-biased sex ratio among *miR-465* KO mice results from selective degeneration of the female placenta and resorption of the female embryos in the absence of the *miR-465* cluster.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found here: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA669325/.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee (IACUC) of the University of Nevada, Reno (Protocol# 00494).

AUTHOR CONTRIBUTIONS

ZW and WY designed the research. Z W, NM, YW, SW, SC, and HZ performed bench experiments. ZW, TZ, and ML performed bioinformatic analyses. SK and HW contributed reagents and protocols. ZW and WY wrote the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by grants from the NIH (HD098593, HD0085506, HD099924 to WY) and the Templeton Foundation (PID: 61174 to WY).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.893854/full#supplementary-material

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ORIGINAL RESEARCH
published: 31 May 2022
doi: 10.3389/fendo.2022.896747

The Impact of Activin A on Fetal Gonocytes: Chronic Versus Acute Exposure Outcomes

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Activin A, a TGFβ superfamily member, is important for normal testis development through its actions on Sertoli cell development. Our analyses of altered activin A mouse models indicated gonocyte abnormalities, implicating activin A as a key determinant of early germline formation. Whether it acts directly or indirectly on germ cells is not understood. In humans, the fetal testis may be exposed to abnormally elevated activin A levels during preeclampsia, maternal infections, or following ingestion of certain medications. We hypothesized that this may impact fetal testis development and ultimately affect adult fertility. Germ cells from two mouse models of altered activin bioactivity were analysed. RNA-Seq of gonocytes purified from E13.5 and E15.5 *Inhba* KO mice (activin A subunit knockout) identified 46 and 44 differentially expressed genes (DEGs) respectively, and 45 in the E13.5 *Inha* KO (inhibin alpha subunit knockout; increased activin A) gonocytes. To discern direct effects of altered activin bioactivity on germline transcripts, isolated E13.5 gonocytes were cultured for 24h with activin A or with the activin/Nodal/TGFβ inhibitor, SB431542. Gonocytes responded directly to altered signalling, with activin A promoting a more differentiated transcript profile (increased differentiation markers *Dnmt3l, Nanos2* and *Piwil4*; decreased early germ cell markers *Kit* and *Tdgf1*), while SB431542 had a reciprocal effect (decreased *Nanos2* and *Piwil4*; increased *Kit*). To delineate direct and indirect effects of activin A exposure on gonocytes, whole testes were cultured 48h with activin A or SB431542 and collected for histological and transcript analyses, or EdU added at the end of culture to measure germ and Sertoli cell proliferation using flow cytometry. Activin increased, and SB431542 decreased, Sertoli cell proliferation. SB431542-exposure resulted in germ cells escaping mitotic arrest. Analysis of FACS-isolated gonocytes following whole testis culture showed SB431542 increased the early germ cell marker *Kit*, however there was a general reduction in the impact of altered activin A bioavailability in the normal somatic cell environment. This multifaceted approach identifies a capacity for activin A to directly influence fetal germ cell development, highlighting the potential for altered activin A levels *in utero* to increase the risk of testicular pathologies that arise from impaired germline maturation.

Keywords: activin A, signalling, fetal germ cell, fetal testis, differentiation

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Edited by:
Barry Zirkin, Johns Hopkins University, United States

Reviewed by:
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Jodi Anne Flaws, University of Illinois at Urbana-Champaign, United States

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Specialty section:
This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology

Received: *15 March 2022*
Accepted: *11 April 2022*
Published: *31 May 2022*

Citation:
Moody SC, Whiley PAF, Western PS and Loveland KL (2022) The Impact of Activin A on Fetal Gonocytes: Chronic Versus Acute Exposure Outcomes. Front. Endocrinol. 13:896747. doi: 10.3389/fendo.2022.896747

INTRODUCTION

The complex processes governing the successful transformation of a primordial germ cell into a spermatogonial cell requires signals from the dynamic somatic milieu of the growing testis. There are gaps in our knowledge of these cues in the fetal testis which are particularly evident in the interval following assignment of a male fate and birth. In mouse, male sex determination initiates around embryonic day (E) 10.5, with *Sry* expression in pre-Sertoli cells which proliferate and surround the proliferating germ cells as testis cords are formed. Germ cells commit to the male fate by about E12.5 in response to signals from somatic cells (1, 2). From E13.5, these male germ cells, called gonocytes or pro-spermatogonia, enter mitotic arrest in an asynchronous manner to become uniformly quiescent by E15.5 (3). During this interval, transcripts that indicate their more differentiated status increase significantly, including *Nanos2*, *Dnmt3l* and *Piwil4*, while markers expressed in their less-differentiated precursors, such as *Kit*, *Nodal* and *Tdgf1*, decrease (4–6). These are hallmark indicators of the male germ cell genome transitioning to an epigenetically more stable state, as the piRNA pathway components, *Piwil4, Dnmt3l, Mov10l1, Tdrd1, Tdrd9*, are upregulated in a sex-specific manner.

Proteins in the transforming growth factor β (TGFβ) superfamily produced by several testis cell types shape the growing fetal and postnatal testes and affect germ cell development. This superfamily contains over 30 different ligands, including transforming growth factor-betas (TGFβs), bone morphogenetic proteins (BMPs), activins, Nodal and growth and differentiation factors (GDFs) (7, 8). They share a conserved dimeric ligand structure, and signal through both shared and distinct signalling moieties, making the potential for signalling crosstalk and synergy of context-dependent importance (**Figure 1A**). For example, both activin A and TGFβ1 are implicated in stimulating germline exit from the cell cycle; genetically modified mice with decreased signalling by either one leads to a modest but significant increase in the proportion of germline cells that continue to proliferate at E15.5 (10, 11). However *in vitro* exposure to an inhibitor that blocks both pathways, SB431542, yielded a more robust outcome when testis fragments were cultured from E12.5 to E15.5 (12), suggesting that these pathways are partially redundant in the context of fetal germline maturation. In the context of human pregnancy, the premature elevation of activin A is an established indicator of pre-eclampsia that has been identified as early as the first trimester (13–15), the period of development in humans during which the germline initiates and progresses through sex-specific development. Understanding how disruptions to activin A signalling affect fetal germline development may provide clues to human reproductive pathologies.

In mouse and human, there are four activin subunits, inhibin βA (encoded by *Inhba)*, inhibin βB, inhibin βC and inhibin βE, which can form either hetero- or homo-dimeric ligands to signal. As with all other TGFβ superfamily members, the mature activin A protein consists of two INHBA subunits joined by a disulphide bond (16). Indicative of its importance, activin A mature protein subunits are 100% identical between these species. A mouse model with global knockout of the gene encoding the mature activin A subunit, *Inhba*, was first reported in 1995; pups with homozygous deletion of *Inhba* die within 24 hours of birth primarily due to their inability to suckle, amongst other defects that illustrate widespread contributions of activin A to fetal organ development (17). Within the mouse testis, *Inhba* transcript levels increase from E11.5 until shortly after birth (10). At postnatal day 0, *Inhba* KO mice have smaller testes, fewer Sertoli cells and higher gonocyte numbers compared with testes of wildtype littermates. This phenotype emerges after E13.5, with a small but significant increase in gonocyte numbers at E15.5 in KO testes (10), highlighting the potential for activin A to directly suppress germ cell proliferation.

Circumventing the neonatal lethality of *Inhba* KO mice, *Amhr2*-cre driven deletion of *Inhba* in Leydig cells resulted in smaller testes at E19.5, reduced coiling of the fetal testis cords and reduced Sertoli cell proliferation (18). This identified fetal Leydig cells as a key source of activin A. An *Sf1*-cre conditional knockout of *Inhba* in Sertoli and other somatic cells further revealed that activin A synthesized by gonocytes or immune cells did not rescue this phenotype (19). Adults with either of these conditional *Inhba* deletions had smaller testes, larger seminiferous tubule diameters, and tubules with abnormal or absent spermatogenesis. Such results demonstrated the potential for long term effects of reduced activin A on adult male fertility, some of which could be attributed to its roles in postnatal Sertoli cell proliferation and immunomodulatory functions (20–23).

More recently, activin A levels were shown to determine both steroidogenesis and lipid metabolism in the fetal testis. Activin A-deficient E13.5 and E15.5 testes in *Inhba* KO mice have drastically reduced levels of the *Hsd17b1* and *Hsd17b3* transcripts which encode the enzymes that convert androstenedione (A4) to testosterone. In the fetal testis, these enzymes are exclusively synthesized in Sertoli cells, and consequently, A4 produced from cholesterol in Leydig cells is not efficiently converted into testosterone (T) in *Inhba* KO testes. At E17.5 these testes exhibit an abnormal accumulation of lipid droplets within the testis cords and an elevated A4/T ratio (9), both indicating a profound impact of activin A signalling pathway on processes central to masculinization in a key developmental window (24).

The present study addresses the poorly understood question of how altered activin A signalling affects germ cell development, focussing on events that occur in the fetal testis after sex determination while testis *Inhba* transcript levels are rising and phenotypic changes in the *Inhba* KO testes are emerging between E13.5 and E15.5. Utilising a multi-pronged approach, we examined the impact of both the chronic (*in vivo*) and transient (*in vitro*) changes in activin A bioactivity on fetal male mouse germ cells. The results presented identify that both direct and indirect affects/mediators are likely to influence germline development depending on local levels of activin A during this key developmental window.

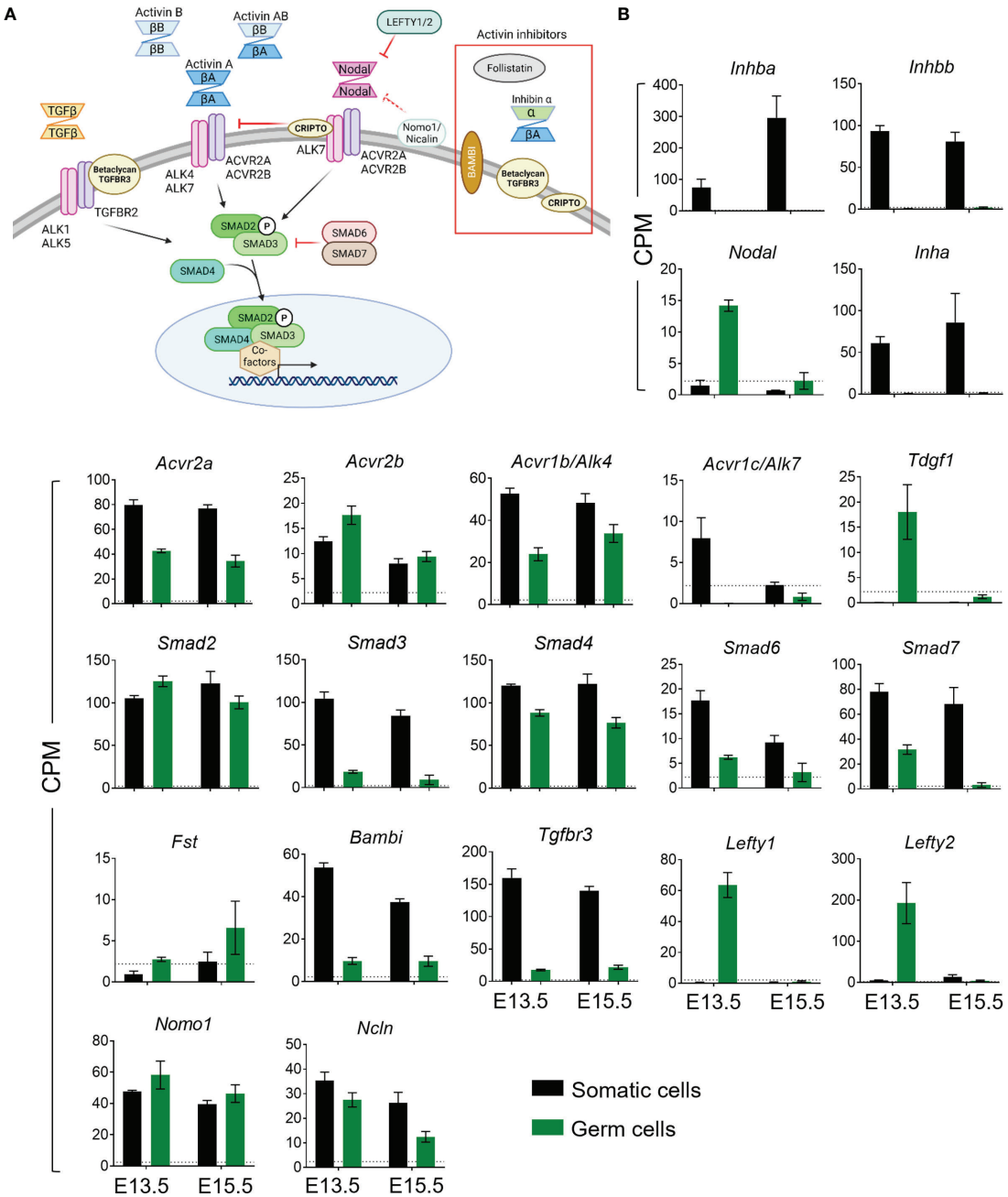


FIGURE 1 | Activin and Nodal signalling component expression profiles in somatic and germ cells from wildtype E13.5 and E15.5 testes. **(A)** Overview of activin/Nodal/TGFβ signalling pathway and modulators. Dimeric ligands bind to two Type 2 receptors with a constitutively active serine-threonine kinase (STK) (purple) which then recruit, phosphorylate and thereby activate Type 1 receptor subunits with STK activity (pink). The complex can phosphorylate SMADs 2 and 3, and two of these complex with SMAD4 for transport into the nucleus, where interactions with nuclear co-factors effect changes in gene transcription. Crosstalk between family members is a feature of this pathway. This is mediated by the shared utilization of receptors (e.g. between activin A and Nodal), SMADs (common to activin/Nodal/TGFβ), and the inhibitory impact of Nodal pathway components (e.g. Cripto and Lefty1/2) on activin A signaling. **(B)** RNA-sequencing was performed on somatic and germ cell populations isolated from E13.5 and E15.5 *Inhba x Oct4-Gfp* mouse testes. The somatic cell data was published previously (9). Transcript levels of activin and Nodal ligands, type 1 and type 2 receptors, intracellular Smads, and activin and Nodal inhibitors in wildtype somatic (black columns) and germ cells (green columns) are shown in counts per million (cpm). Data are presented as mean ± SD. The detection limit for the *Inhba x Oct4-Gfp* RNA-Seq data set was 2.2 cpm (dotted line).

MATERIALS AND METHODS

Animals

All animal procedures were carried out in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes under Monash University Animal Ethics Committee approval. Mice were housed at Monash Medical Centre Animal Facility under a 12-hour dark/light cycle and with food and water available *ad libitum*. For all experiments except RNA-Seq, timed-mates were set up between Swiss females and transgenic *Oct4-eGfp* males (OG2; pure 129T2svJ background) (25, 26). Females were checked daily and the presence of a vaginal plug marked as E0.5. At E13.5, pregnant females were culled by cervical dislocation and the uterine horns removed and placed in phosphate buffered saline (PBS). Embryos were removed and euthanised by decapitation and the developmental stage was determined by the time since mating, and fore- and hind-limb morphology. The gonad/mesonephros complex was dissected out of each embryo and the mesonephros removed. Sex of the embryo was identified by the absence or presence of testis cords in the gonads, visualised using an upright dissecting microscope (MZFLIII, Leica, Wetzlar, Germany).

For RNA-Seq, mice lacking the inhibin βA [encoded by *Inhba*; no activin A (17)] or inhibin alpha subunits [*Inha*; high activin A (27)] on a C57/Bl6 background were crossed with *Oct4-eGfp* mice (9, 25, 26). For each line, heterozygous timed mates were set up and fetal gonads from E13.5 and E15.5 embryos collected as above. Tails were collected from each embryo for commercial genotyping (Transnetyx, USA).

Whole Gonad Culture

E13.5 testes were randomly assigned treatment groups. Testes were cultured on 0.4 μM Millicell cell culture inserts (Merck Millipore, Germany) in 6-well plates with each well containing 1.4 mL media (DMEM/F12, Thermo Fisher Scientific, Waltham, MA, USA; 10% FBS, Bovogen, Keilor East, VIC, Australia; 1% penicillin-streptomycin, Thermo Fisher Scientific) (28). Previous cell culture experiments with mouse postnatal day 6 and 15 Sertoli cells revealed SMADs localise to the nucleus following exposure to 5 ng/mL activin A (29). Further, the human germ cell-like line, TCam-2, is responsive to activin A at 5 ng/mL (30). However, the local concentration of activin A in the fetal testis is unknown, therefore we determined the optimum concentration of activin A by culturing whole testes with 0 (vehicle control; 4 μM HCl), 5, 25, 50 or 100 ng/mL human recombinant activin A (R&D Systems, Minneapolis, MN, USA) and activin-responsive genes measured. Following analysis of changes in activin A-responsive genes, testes were cultured in media containing 50 ng/mL activin A as the optimal dose (described in Results), or 10 μM SB431542 (Sigma-Aldrich, St Louis, MS, USA) and their respective controls (DMSO for SB431542). PBS was placed in the gaps between wells to maintain humidity. E13.5 testes were cultured for 48 hours with a full media change at 24 hours. Following culture, gonads were imaged using bright field and fluorescence using an Olympus IX70 inverted microscope to visualize gross gonad structure and GFP-positive germ cells. Gonads were removed from the membrane, washed in PBS and individually snap-frozen on dry ice for transcript analysis, fixed in 4% paraformaldehyde (PFA) for histological analysis, or dissociated for flow cytometry.

Testis Dissociation and Germ Cell Isolation by Fluorescent Activated Cell Sorting (FACS)

For RNA-Seq experiments, paired testes from one embryo yielded a single biological replicate. From whole gonad cultures (E13.5 + 48h), single testes were a biological replicate. For E13.5 germ cell cultures, 6 - 10 paired testes were pooled. Testis dissociation and isolation of germ and somatic cells were performed as previously described (9). Briefly, testes were dissociated in 0.25% Trypsin-EDTA. Dissociation was halted with media containing 10% FBS. Cells were passed through a 35 μM strainer to obtain a single-cell suspension then centrifuged. Cell pellets were resuspended in 0.4% BSA/PBS and propidium iodide was added for exclusion of non-viable cells. GFP-positive and GFP-negative cells were sorted by Monash FlowCore staff using either an Influx or ARIA Fusion (BD Biosciences) machine. Sorted cells were pelleted, supernatant removed, and stored at -80°C for transcript analyses. Gonocyte cell culture is described below.

EdU Incorporation and Flow Cytometry

This protocol was based on a previously published method using the Click-iT™ Edu Alexa Fluor™ kit (Thermo Fisher Scientific) (31). For EdU incorporation, a final concentration of 20 μM was added to culture media two hours before collection. Then testes were washed in PBS and dissociated in 0.25% Trypsin-EDTA at 37°C for 5 to 10 minutes. Dissociation was halted with DMEM/F12 containing 10% FCS, and the cells were passed through a 35 μm mesh cell strainer to obtain a single cell suspension. Following centrifugation and removal of supernatant, cells were resuspended in 4% PFA and fixed for 15 minutes at room temperature. After 3 washes in permwash (1X saponin-based permeabilisation reagent (Thermo Fisher Scientific) in 1% BSA/PBS) cells were stored in permwash for no more than one week before immunostaining. For all steps, solutions were made up in and washes done with permwash and performed at room temperature.

Cells were centrifuged and resuspended in 5% donkey serum (Sigma) for 15 minutes. Cells were incubated with anti-DDX4 (detection of germ cells; R&D Systems; AF2030; goat polyclonal; 1:100) and anti-DNMT3L (Abcam; ab194095; rabbit polyclonal; 1:200), or anti-SOX9 (Millipore; AB5535; rabbit polyclonal, 1:300) and anti-AMH (Anti-Mullerian Hormone; Santa Cruz; sc6886; 1:300), or anti-DDX4 and anti-SOX9 in combination for 45 minutes. Dissociated mesonephros was used as a negative control for DDX4 staining, and dissociated E13.5 ovaries were used as a negative control for SOX9 staining. Cells were washed twice then incubated 45 minutes with secondary antibodies (Donkey anti-rabbit biotin, Thermo Fisher Scientific; Donkey anti-goat Alexa Fluor 488, Thermo Fisher) diluted 1:300. Cells were washed twice then resuspended and incubated in the Click-iT reaction cocktail containing Alexa Fluor 647 for 30 minutes.

Following two washes, samples were incubated with Streptavidin Pacific Blue (Thermo Fisher Scientific; 1:500) for 30 minutes. Cells were washed twice, then resuspended in 300 µL permwash containing 20 µg/mL propidium iodide (Sigma) to measure cellular DNA content. Samples were run on the same day on the BD Biosciences FACS CANTO-II at Monash FlowCore (Monash Health and Translational Precinct (MHTP) Node) and data analysed using FlowJo X.0.7 software (Ashland, OR, USA). Single, intact cells were analysed following gating using forward and side scatter characteristics, and DNA content.

Double Immunofluorescence Staining of Mouse Testis Sections

Cultured and uncultured testes were fixed in 4% PFA for immunofluorescence (IF) analysis. After standard ethanol processing conditions, they were paraffin embedded and sectioned at 4 µM onto Superfrost Plus slides.

Unless stated, all steps were performed at room temperature. Sections on slides underwent dewaxing in histosol, followed by rehydration in a graded ethanol series (100%, 95% and 70% ethanol). Slides were briefly washed in water and incubated for 30 mins at 98°C in Citrate buffer (pH 6; DAKO) for antigen retrieval. After cooling, slides were rinsed in distilled water, washed once in PBS, then sections permeabilised in 0.1% Triton-X-100 (Merck) in PBS for 30 mins. Slides were washed twice in PBS and a wax circle drawn around sections using a PAP pen (Cederlane Laboratories, Burlington, Canada). Non-specific antibody binding was blocked by incubation with 10% donkey serum (Sigma-Aldrich) in 5% bovine serum albumin (BSA)/PBS for 1 hour. The blocking liquid was tapped off and primary antibodies in dual combination were diluted in 1% BSA/PBS and added to sections, with 1% BSA/PBS serving as a control lacking primary antibody. Primary antibodies against the following proteins were used: DNMT3L (Abcam; ab194094; 1:200), VASA (R&D Systems; AF2030; raised in goat; 1:400), VASA (Cell Signalling Technologies; 8761S; raised in rabbit; 1:400), cKIT (R&D Systems; AF1356; 1:100), Laminin (Sigma; L9393; 1:200) and AMH (Santa Cruz; sc6886; 1:200). Slides were incubated overnight at 4°C in a humid chamber. The next day, primary antibodies were removed, and slides washed 3 x 5 minutes in PBS. Secondary antibodies (Donkey anti-Rabbit Alexa Fluor 594, Invitrogen, A21207; Donkey anti-Goat Alexa Fluor 488, A11055) were diluted 1:300 in 1% BSA/PBS and added to sections for 2 hours. Slides were washed one time in 0.1% Triton-X-100 in PBS, then twice for 5 minutes each in PBS. DAPI (Thermo Fisher Scientific) was applied to sections at 5 µg/mL in PBS for 15 minutes. Following three washes in PBS, slides were mounted under glass coverslips with ProLong Gold Antifade Mountant (Thermo Fisher Scientific) and set overnight. Imaging was performed by Monash Histology Platform (MHTP node) using the VS120 Slide Scanner (Olympus, Tokyo, Japan) and images were processed using OlyVIA software (Olympus).

Gonocyte Cell Culture

Following dissociation of Swiss x *Oct4-Gfp* E13.5 testes and isolation of gonocytes *via* FACS, germ cells were counted using a haemocytometer. In each well (48-well plate), 20,000 germ cells were added in 500 µL media (MEM-α, 10% FBS, 1% penicillin-streptomycin) containing 10 µM SB431542, 5 ng/mL activin A, or relevant vehicle control. A lower concentration of activin A was used compared with the whole testis cultures, as cells grown as a monolayer have been demonstrated to be robustly responsive to 5 ng/mL activin A (29, 30). Cells were cultured for 24 hrs in 5% CO_2/air, after which the cells were collected for transcript analysis. Because the germ cells were lightly adherent, media and one PBS wash were collected to avoid losing cells, and 200 µL of 0.1% Trypsin-versene added per well and incubated for approximately 5 minutes or until all remaining cells were detached. Trypsin was quenched with media containing 10% serum, and all contents were transferred to a 1.5 mL tube containing the original media and PBS wash. Cells were centrifuged at 1020 g, supernatant removed, and cell pellets stored at -80°C.

RNA Extraction, cDNA Synthesis and qRT-PCR

All RNA extractions and on-column DNase treatment were performed using the NucleoSpin RNA XS kit (Machery-Nagel, Germany) according to the manufacturer's protocol. RNA concentration was quantified using a NanoPhotometer (Implen, Munchen, Germany). RNA was subjected to reverse transcription in a reaction containing 200 Units SuperScript III Reverse Transcriptase (Thermo Fisher Scientific), 50 ng random primers and 500 ng oligo dTs (Promega, Madison, USA) per sample. For whole gonads, 100 ng RNA was added to the reaction. For isolated cell cultures and cells isolated following culture, 40 ng and 15 ng respectively, was used in each cDNA reaction and RNaseOUT Recombinant Ribonuclease Inhibitor (Thermo Fisher Scientific) added to each 20 µL reaction as per the manufacturer's protocol.

Real time PCR was conducted on the QuantStudio Fast Real-time PCR System at the MHTP Medical Genomics Facility (Clayton, Australia), and data generated using SDS software (Applied Biosystems). Each reaction contained power SYBR Green Master Mix (Thermo Fisher) and specific primer pairs (**Table 1**; Integrated DNA Technologies, Coralville, IA, USA) facilitating transcript measurements in 384 well plates. Primers pairs were designed to span exon-exon junctions or have pairs separated by an intron where possible. Each cDNA was diluted 1:20 or 1:10 for whole testes and isolated cells respectively. Every sample was measured in triplicate, and amplification of a single product was indicated by detection of a single peak in a melt curve analysis. Data were normalised to the *Canx* housekeeper gene (33) and analysed using the $2^{-\Delta Ct}$ method.

Additionally, transcript levels in isolated E13.5 germ cell cultures were measured using the Fluidigm Biomark™ 96x96 Dynamic Array IFC by the MHTP Medical Genomics Facility. Taqman assays (Thermo Fisher Scientific; **Table 2**) were used for amplification of specific transcripts. The geometric mean of two housekeeper genes, *Canx* and *Mapk* (33), was used for normalisation of data following a Pearson correlation between the two Ct values (R^2>0.92). Data were normalised to the

TABLE 1 | Forward and reverse primers for qRT-PCR (SYBR Green).

Gene	Accession	Forward (5'- 3')	Reverse (5'- 3')
Canx	NM_001110499.1	TTCCAGACCCTGATGCAGA	TCCCATTCTCCGTCCATATC
Piwil2	NM_021308.2	TTGGCCTCAAGCTCCTAGAC	GAACATGGACACCAAACCTACA
Piwil4	NM_001368831.1	GGGGCTCGTTGTCCTTACCA	ACTGCCTTCATCAGGCGGAA
Tdrd1	NM_001002241.2	TCTTCCCACAGCACCATCTGTA	CACTCTTCACTTCAATGGCCT
Tdrd9	NM_029056.1	TGGCGAGTTGACCTTCCTGG	CTGAACGCCTCCACAAGTGC
Dnmt3a	NM_007872.4	GGCCCGTTACTTCTGGGGTA	TGGCTATTCTGCCGTGCTCC
Dnmt3l	NM_001284197.1	ATGATCAAGAGGGAGCGGGC	CGAGCCGTACACCAGGTCAA
Mov10l1	NM_031260.2	AAGAGTACCTGGTCATCGTCATCTC	CAGCAGTGCTTTGGGTCTTG
Mvh	NM_001145885.1	CATCGAATTGGACGCACTG	GGCAATCTCTTCTAGCCATGC
Oct4	NM_013633.3	GTTGGAGAAGGTGGAACCAA	CTCCTTCTGCAGGGCTTTC
Kit	NM_001122733.1	TCATCGAGTGTGATGGGAAA	GGTGACTTGTTTCAGGCACA
Nodal	NM_013611.5	ACATGTTGAGCCTCTACCGAGAC	AACGTGAAAGTCCAGTTCTGTCC
Tdgf1*	NM_011562.2	GGCCATTTCCAGTGCGTTT	GCAAGGTCTCTCCCAGCAAC
Nanos2	NM_194064.2	TCTCCATGGACCATTCACG	CTTCCTCTTATTCCTGATGGACA
Sox9	NM_011448.4	TGAACGCCTTCATGGTGTG	TTCTCGCTCTCGTTCAGCAG
Mmp2	NM_008610.3	TCGCTCAGATCCGTGGTGAG	TCATTCCCTGCGAAGAACACA
Ccl17	NM_011332.3	AATGTAGGCCGAGAGTGCTG	TGGCCTTCTTCACATGTTTG
Cldn11	NM_008770.3	AGTTCTCCCCTGCATCCGAA	TCACAGCACCGATCCAACCT
Gja1	NM_010288.3	AGGAGTTCCACCACTTTGGCG	AAATGAAGAGCACCGACAGCC
Serpina5	NM_172953.3	TCTTCACCACCCATGCTGAC	GAATGTGAAGATGGCTCCTGTG
Hsd17b1	NM_010475.2	CACTACCTGCGTGGTTATGAGC	GAAGCGGTTCGTGGAGAAGTAG

*Souquet et al., (32).

TABLE 2 | Taqman assays for Fluidigm analysis.

Gene	Taqman assay
Mapk	Mm00442479_m1
Canx	Mm00500330_m1
Nodal	Mm00443040_m1
Tdgf1	Mm03024051_g1
Lefty2	Mm00774547_m1
Dnmt3l	Mm00457635_m1
Slc43a3	Mm00469627_m1
Msi1	Mm01203522_m1

housekeepers, stable across samples, and analysed using the $2^{-\Delta Ct}$ method. Multiple experiments were analysed on the same array, accounting for the remaining samples and Taqman assays which make up the 96x96 array.

RNA-Sequencing

RNA-Sequencing was performed on gonocyte and somatic cells isolated from E13.5 and E15.5 *Inhba x Oct4-Gfp*, and germ cells isolated from E13.5 *Inha* x *Oct4-Gfp* wildtype and knockout testes. RNA sample quality was assessed on the Agilent 2100 Bioanalyzer using the Eukaryote total RNA Pico Kit, providing a measure of RNA integrity (RIN). All samples were high quality (RIN 8.4 – 9.9). Double stranded cDNA was prepared from 2-20 ng total RNA using Trio RNA-Seq or RNA-Seq V2 kits and SPIA amplification (Tecan/NuGEN, Leek, The Netherlands). These methods both use full length linear amplification to minimise bias. RNA-Seq libraries were then prepared with unique barcodes to allow multiplexing during sequencing. Illumina single end sequencing was performed on the HiSeq 3000 or NextSeq2000 (Illumina, San Diego, CA, USA). All RNA quality control, library preparation, and sequencing were performed by staff at the MHTP Medical Genomics Facility.

RNA-Sequencing Analysis

Sequencing from E13.5 and E15.5 *Inhba x Oct4-Gfp* returned 35-40 million 80 base pair reads. Sequencing from E13.5 *Inha x Oct4-Gfp* returned 65-85 million 100 base pair reads. The *Inhba* and *Inha* datasets were analysed independently of each other. RNA sequencing data were processed and analysed by Monash University Bioinformatics Platform. Sequencing reads were aligned to the Ensembl mouse reference genome GRCm38 (Ensembl release 84) and analysis was performed using the RNAsik pipeline with STAR aligner (34). Differential gene analysis was performed on Degust V4.1.5 (David R. Powell, Monash Bioinformatics Platform), using *Limma-Voom* (35, 36). Heatmaps were generated using ClustVis (37). RNA-Seq data are available *via* accession number GSE201520.

Inhba x Oct4-Gfp analysis: Following principal component analysis of the samples, two samples were excluded as outliers: one sample from the E13.5 *Inhba* KO somatic cell group, and one sample from the E13.5 *Inhba* WT gonocyte group. Further scrutiny of these samples led us to conclude these may have been contaminated or swapped, and their exclusion was supported following consultation with a bioinformatician (Monash Bioinformatics Platform).

The detection limit was determined by calculating the median of the entire array of counts per million (cpm) values for the datasets. For the entire dataset (germ and somatic cells), the detection limit was determined as 2.2 cpm, while the detection limit for the germ cell only dataset was calculated at 2.391 cpm. Values greater than these were determined as being detectable. Analysis of these data confirmed the purity of the germ and somatic cell populations through absence or presence of *Ddx4* (germ cells), and *Sox9* and *Nr5a1* (somatic cells) (**Supplementary Figure 1A**). Absence of *Inhba* in knockout animals was confirmed in the somatic cell population with a 4-

fold increase measured from E13.5 to E15.5 (**Supplementary Figure 1B**), consistent with previously published data (10, 12).

Differentially expressed genes were identified as having a false discovery rate (FDR) <0.05, a LogFC>0.585 and <-0.585 (i.e. a 1.5-fold change up or down, respectively), and two or more samples across genotypes being greater than the detection limit of 2.391cpm. There were 44 DEGs identified in the E15.5 *Inhba* KO gonocyte dataset. None were identified in the E13.5 *Inhba* KO dataset, therefore further analysis was performed to generate a list of transcripts that are altered in E13.5 gonocytes, described below. The data was processed in Degust using *Limma-Voom*, and the p-value was calculated within the software using the trimmed mean of M-values (TMM) normalised voom-transformed expression levels. Differentially expressed genes at E13.5 were identified using a LogFC> 0.585 and <-0.585, p-value <0.01 and restriction to at least two samples across wildtype and knockout animals being greater than the detection limit of 2.391 cpm. This approach enabled less abundant transcripts to be considered, and it resulted in the identification of 46 DEGs.

Inha x Oct4-Gfp analysis: Two wildtype and knockout littermate pairs were analysed using batch correction. Mitochondrial genes were filtered out as they were highly variable and genes with a minimum of 2 cpm in at least 2 samples included. Differentially expressed genes were identified by FDR<0.05 and LogFC>0.585. This led to the identification of 45 DEGs. Germ cell purity was assessed by the presence of *Ddx4* and absence of *Sox9* and *Nr5a1* (**Supplementary Figure 1C**). *Inha* genotypes were confirmed in the somatic cell population by qRT-PCR (**Supplementary Figure 1D**).

Gene lists obtained after analysis (**Supplementary Tables 1–3**) were submitted to the PANTHER classification system (38, 39) to identify molecular functions, biological processes, and protein classes of the DEGs. A Venn diagram was created following input of DEG lists to JVenn (40).

Statistical Analysis

All statistical analyses were performed using GraphPad Prism Software (San Diego, CA, USA). Normal distribution of control and treatment groups was determined using a Shapiro-Wilk or D'Agostino and Pearson normality test. qRT-PCR and flow cytometric data from whole gonad cultures were analysed using an unpaired Student's t-test for normally distributed data, or a Mann-Whitney test for data that was not normally distributed. For statistical analysis of the isolated cell culture experiments, a paired t-test or a Wilcoxon matched-pairs signed rank test was performed. Data was determined as significantly different when the p-value was less than 0.05.

RESULTS

Germ Cells Express the Signalling Machinery to Respond to Activin A

Levels of transcripts encoding activin and Nodal ligands, signalling machinery and inhibitors were obtained from RNA-Seq analysis of germ and somatic cell populations collected from wildtype *Inhba* E13.5 and E15.5 testes (**Figure 1B**). These data reveal the complexity and dynamic nature of signalling potential of these selected components of the TGFβ superfamily within the testis during this window of development that is crucial to testis and embryo masculinization.

Inhba and *Inhbb*, encoding activin A and B subunits, respectively, were detected in somatic cells at both ages (**Figure 1B**), while *Inhbc* was below the detection limit in all samples (data not shown). *Inhba* increased 4-fold from E13.5 to E15.5 (73.8 ± 27.1 to 295.7 ± 69.7 cpm), and *Inhbb* levels were relatively constant (93.3 ± 6.7 cpm and 81.0 ± 10.9 cpm). At E13.5, *Nodal* was measured in germ cells, but not somatic cells, and it decreased to undetectable levels at E15.5 (**Figure 1B**). The levels of *Inhba*, *Inhbb* and *Nodal* were consistent with previous reports (12, 41). Transcripts encoding the Type 2 receptors for activin A, activin B and Nodal, *Acvr2a* and *Acvr2b*, were present in both somatic and germ cells at both ages highlighting the potential for each of these to respond, however *Acvr2a* was present at higher levels in both ages and cell types (**Figure 1B**). *Acvr1b*, encoding the type 1 receptor for activin A, activin B and Nodal, was present in both cell types at E13.5 and E15.5, while the transcript encoding the Nodal and activin B receptor, *Acvr1c*, was present only at low levels in E13.5 somatic cells (8.0 ± 2.3 cpm), indicating that *Acvr1b*, and not *Acvr1c*, is the predominant receptor for Nodal actions in germ cells at E13.5. Nodal signalling additionally requires the co-receptor, Cripto, encoded by *Tdgf1*, also known to antagonise activin A (42); this transcript was detected in E13.5 germ cells only (18.0 ± 1.2 cpm). These results illustrate the potential for Nodal to specifically impact on the germline cells which are exiting their proliferative state. Transcripts encoding the intracellular signalling components required for activin/Nodal signalling, *Smad2* and *Smad4*, were present at both ages in somatic and germ cells, however Smad3 was predominantly detected in the somatic cell samples (**Figure 1B**).

Activin and Nodal inhibitors are also present during fetal testis development, and these would be expected to fine-tune the responsiveness of cells expressing their receptors (**Figure 1A**). *Inha*, encoding the inhibin α subunit which forms a potent activin A inhibitor when dimerised with an activin β subunit, was detected only in somatic cells at both E13.5 (60.8 ± 8.1 cpm) and E15.5 (85.9 ± 34.7 cpm). Follistatin (*Fst*) was detected at low levels (<7 cpm) in all samples (**Figure 1B**), consistent with previous studies demonstrating that *Fst* is only expressed in the fetal ovary compared with the testis (43, 44). The transcript encoding the decoy receptor Bambi (45) was expressed at both ages in somatic and germ cells, with consistently higher levels in somatic cell samples compared with those in germ cells (53.6 ± 2.3 and 37.3 ± 1.7 cpm in somatic cells, and 9.7 ± 1.6 and 9.6 ± 2.4 cpm in germ cells). Transcripts encoding the inhibitory Smad6 and Smad7 were predominantly expressed in the somatic cells, but were also measured in germ cells at both ages. Betaglycan, encoded by *Tgfbr3*, is a co-receptor for TGFβs which is required for TGFβ2 signalling, and it can inhibit activin A (46). It was highly expressed in somatic cells compared to germ cells at both ages (**Figure 1B**).

There are several Nodal antagonists which could dampen its capacity to compete with activin A. *Lefty1* was identified in germ cells at E13.5 (**Figure 1B**; 63.6 ± 8.1 cpm) and undetectable by E15.5, consistent with previous observations (12). *Lefty2* was expressed at higher levels in E13.5 germ cells (192.8 ± 49.9 cpm) and dropped to 4.3 ± 1.5 cpm by E15.5. *Lefty2* transcripts were also low in the somatic cells at both ages (**Figure 1B**). The *Cerberus* transcript, encoding another Nodal inhibitor, was below the detection limit in all samples (data not shown). The Nomo/Nicalin complex has been identified as a Nodal antagonist in zebrafish (47), however its roles in the mouse are not known. Transcripts for each component were present in the mouse fetal testis (*Nomo1* and *Ncln*) at E13.5 and E15.5 in both somatic and germ cells (**Figure 1B**), indicating these proteins may also reduce Nodal activity in the fetal testis.

Transcriptional Changes in Gonocytes in the Absence of Activin A (*Inhba* Knockout)

In germ cells lacking activin A (*Inhba* KO), there were 46 and 44 differentially expressed genes (DEGs) at E13.5 and E15.5, respectively (**Figures 2A, B**; **Supplementary Tables 1, 2**). At E13.5, there were no DEGs by FDR (<0.05), therefore we utilised p-value (<0.01) and LogFC (>0.585 and <-0.585) to assess any differences between genotypes (**Figure 2A**). There were 21 downregulated, and 25 upregulated DEGs, which were primarily associated with binding and catalytic functions, and cellular

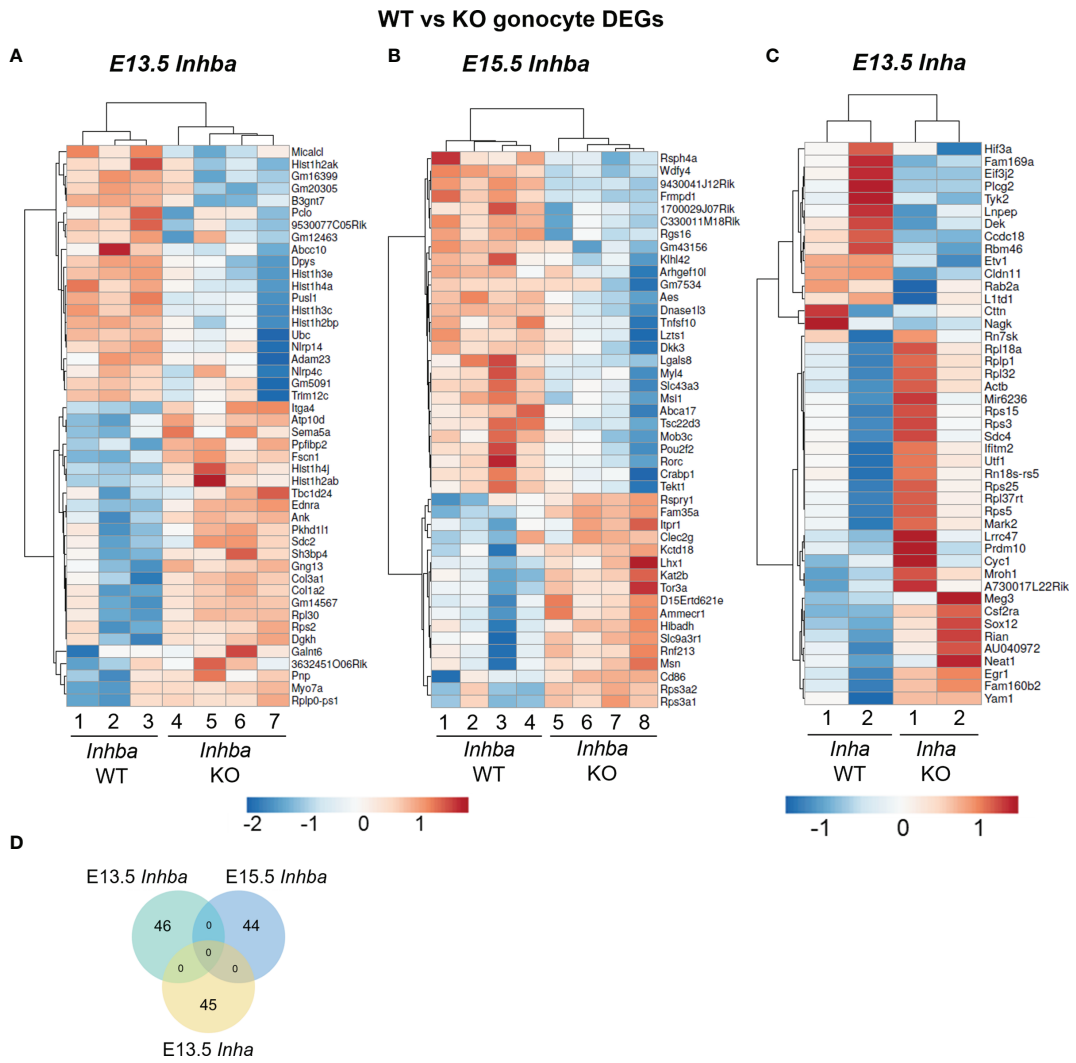


FIGURE 2 | Heatmaps generated from differentially expressed genes (DEGs) following RNA-Seq analysis. **(A)** 15 downregulated and 30 upregulated DEGs in E13.5 *Inha* knockout (KO) gonocytes compared with wildtype (WT) littermates (n=2 per genotype). DEG criteria: FDR<0.05, LogFC>0.585, <-0.585. **(B)** 21 downregulated and 25 upregulated DEGs in E13.5 *Inhba* KO (n=3) compared with WT (n=4) gonocytes. DEG criteria: LogFC>0.585, p-value<0.01. **(C)** 27 downregulated and 17 upregulated DEGs in E15.5 *Inhba* KO compared with WT gonocytes (n=4 per genotype). DEG criteria: FDR<0.05, LogFC>0.585, <-0.585. **(D)** Venn diagram demonstrating lack of overlap of DEGs from the E13.5 and E15.5 *Inhba* KO, and E13.5 *Inha* KO gonocytes.

processes. The top protein class was identified as being metabolite interconversion enzymes, which convert one small molecule to another (PANTHER, **Table 3**), however, the function of these genes in the testis are unknown. Interestingly, an association between *Galnt6* and piRNAs has been identified in an oral squamous cell carcinoma mouse model (48).

At E15.5, there were 27 downregulated, and 17 upregulated DEGs (**Figure 2B**), however there was no overlap in DEGs between E13.5 and E15.5. These transcripts were similarly associated with binding and catalytic activity (PANTHER, **Table 3**). The top biological processes were cellular processes, and biological regulation, with the top protein classes identified as cytoskeletal proteins, gene-specific transcriptional regulators, protein binding activity modulators, and transporters (PANTHER, **Table 3**). Within the DEGs, Musashi-1 (*Msi1*), an RNA-binding protein in the Musashi family of proteins which function in translational regulation, was identified as lower in KO germ cells compared with WT counterparts. Its essential role in governance of postnatal transitions of murine spermatogenesis has been established, and it was previously shown to be expressed in gonocytes (49). There was no overlap in DEGs between E13.5 and E15.5, indicating age-specific responses of germ cells occurred in the absence of activin A.

A recent study identified that activin A promotes a less differentiated transcript profile in the human germ cell-like cell line, TCam-2 (30). To determine if germline differentiation was similarly altered in *Inhba* KO mice, we examined early and differentiation-associated germ cell transcripts in the *Inhba* WT and KO RNA-Seq dataset. Early germ cell transcripts *Nodal*, *Tdgf1*, *Kit*, *Lefty1*, *Lefty2*, and *Nanog* were all downregulated between E13.5 and E15.5 in both WT and KO samples, while differentiation markers *Nanos2* and *Dnmt3l* were upregulated (**Supplementary Figure 2**). We also observed higher expression of piRNA pathway transcripts such as *Piwil1, Piwil2* and *Piwil4, Dnmt3a, Dnmt3l, Tdrd1, Tdrd9, Mael and Mov10l1* at E15.5 relative to E13.5 in WT and KO samples. This is consistent with the activation of the piRNA pathway and *de novo* methylation from around E14.5-E15.5 in quiescent germ cells (50); the higher level of piRNA pathway transcripts encoding components such as in our dataset is consistent with the normal progression of developmental events associated with this phenomenon (**Supplementary Figure 2**). Of these transcripts, *Mov10l1* was decreased ($p<0.05$, Mann-Whitney test) in the KO germ cells at E15.5, however this was not determined to be differentially expressed in the RNA-Seq dataset by FDR and fold change, as presented in **Figure 2B**. Transcripts associated with pluripotency and differentiation showed no differences in isolated germ cells from *Inhba* KO testes compared with WT counterparts at either E13.5 or E15.5. The germ cell-specific transcripts, *Ddx4* and *Pou5f1* (*Oct4*), were both detected at relatively high levels in germ cells, with *Ddx4* increasing 1.6-fold from E13.5 to E15.5 in WT cells.

While *Inhba* KO germ cells appear to differentiate normally based on classical germ cell markers, a subset of genes was altered, indicating that loss of activin A modulates some aspects of early male germline transcription. However, the significance of the outcomes remains to be determined.

The E13.5 Germ Cell Transcriptome Is Altered in the High Activin A Environment of the *Inha* Knockout Testis

The elevation of activin A levels linked with pre-eclampsia in human pregnancy can occur in the second and third trimesters when male germ cells are mainly quiescent. The inhibin α subunit encoded by *Inha*, forms a dimer with an INHBA subunit to form the inhibin A protein, a potent inhibitor of activin A. In *Inha* KO mice, activin A bioactivity is elevated due to the combined absence of inhibitory inhibin proteins, and to the greater availability of INHBA subunits for dimerization to form activin proteins. In wildtype mice, *Inhba* is detectable from E11.5, with its levels increasing until just after birth (10, 51). As the phenotype of the E13.5 testis appears normal but is significantly different by E15.5 (data not shown), we examined the germ cell transcriptome in *Inha* KO compared to WT littermates, prior to gross morphology changes. RNA-Seq analysis of germ cells isolated from two independent wildtype and knockout littermate pairs identified 45 DEGs (**Figure 2C** and **Supplementary Table 3**; FDR<0.05, LogFC>0.585, <-0.585). Thirty upregulated transcripts included ribosome structural components such as *Rps15, Rps25, Rps5, Rplp1* and *Rps3*. These transcripts are also associated with RNA binding. Pathway analysis revealed that the top molecular functions of the 45 DEGs were binding and catalytic activity, with cellular processes the top associated biological process. *Inha* KO DEG were associated with translational proteins (primarily the ribosomal structural component transcripts), and gene-specific transcriptional regulators, which included *Sox12*, *Egr1*, *Etv1* (upregulated), and *Prdm10* (downregulated) (**Table 3**). Interestingly, there were no reciprocal DEGs between *Inha* E13.5 germ cells and the E13.5 or E15.5 *Inhba* germ cells (**Figure 2D**). Collectively, these results demonstrate that gonocytes which develop in an environment of altered activin bioactivity are different from their wildtype counterparts, leading us to investigate whether this effect is direct or indirect.

Germ Cells Can Respond Directly to Activin A

RNA-Seq revealed differences in male germ cell mRNA profiles in mice with altered activin A bioavailability (**Figure 2**). To test whether activin A can directly affect germ cells, gonocytes isolated from E13.5 testes were cultured for 24 hours in 5 ng/mL activin A or 10 μM SB431542, and appropriate vehicle controls. After 24 hours in culture, germ cells retained *Oct4*-eGFP expression, as observed by fluorescence microscopy (**Figure 3A**). Transcripts encoding markers of germ cell differentiation were measured in isolated E13.5 gonocytes and first compared with levels in cells cultured for 24 hours in control conditions (**Supplementary Figure 3**). After 24 hours in culture, the early germ cell marker *Kit* had declined to 85% of E13.5 levels, and *Nodal* was at 10% of E13.5 levels. The differentiation marker *Nanos2* was moderately increased (1.8-fold), while *Dnmt3l, Piwil4, Tdrd1* and *Mov10l1* were all higher after 24 hours in culture compared with E13.5 levels (16-, 11-, 6- and 3-fold, respectively). Interestingly, germ cell

TABLE 3 | PANTHER analysis of RNA-Seq DEGs.

PANTHER analysis	*Inhba* E13.5	*Inhba* E15.5	*Inha* E13.5
Molecular function	**Binding (GO:0005488)**	**Binding (GO:0005488)**	**Binding (GO:0005488)**
	Hist1h2ak	Dkk3	Rplp1
	Gng13	Pou2f2	Dek
	Fscn1	Mob3c	Tyk2
	Ubc	Lhx1	Cttn
	Sema5a	Slc9a3r1	Hif3a
	Tbc1d24	Rorc	Rps5
	Itga4	Msn	Etv1
	Myo7a	Crabp1	Lnpep
	Catalytic activity (GO:0003824)	Itpr1	Sox12
	Atp10d	**Catalytic activity (GO:0003824)**	L1td1
	Pusl1	Hibadh	Egr1
	Pnp	Mob3c	Rbm46
	Tbc1d24	Abca17	**Catalytic activity (GO:0003824)**
	Dgkh	Rspry1	Rplp1
	Dppys	Kat2b	Tyk2
	Myo7a		Plcg2
	Abcc10		Mark2
			Lnpep
			Nagk
Biological processes	**Cellular Process (GO:0009987)**	**Cellular Process (GO:0009987)**	**Cellular Process (GO:0009987)**
	Atp10d	Lzts1	Rplp1
	Sdc2	Dkk3	Dek
	Pusl1	Rsph4a	Tyk2
	Gng13	Arhgef10l	Ifitm2
	Fscn1	Pou2f2	Rps15
	Ubc	Mob3c	Cttn
	Sema5a	Lhx1	Plcg2
	Pclo	Rspry1	Hif3a
	Ppfibp2	Kat2b	Mark2
	Dgkh	Slc93r1	Rps5
	Itga4	Rorc	Etv1
	Dpys	Msn	Sdc4
	Myo7a	Cd86	Lrrc47
	Abcc10	Tekt1	Cldn11
	Rps2	Dnas1l3	Lnpep
		Biological Regulation (GO:0065007)	Sox12
		Lzts1	L1td1
		Dkk3	Egr1
		Arhgef10l	
		Pou2f2	
		Mob3c	
		Lhx1	
		Kat2b	
		Rorc	
		Msn	
		Cd86	
Protein Class	**Metabolite interconversion enzyme**	**Cytoskeletal protein**	**Translational protein**
	Pusl1	Rsph4a	Rplp1
	Pnp	Msn	Eif3j2
	Dgkh	Myl4	Rpl32
	B3gnt7	Tekt1	Rps15
	Dpys	**Gene-specific transcriptional regulator**	Rpl18a
	Galnt6	Pou2f2	Rps5
		Lhx1	Rps3
		Rorc	Lrrc47
		Protein binding activity modulator	Rps25
		Arhgef10l	**Gene-specific transcriptional regulator**
		Mob3c	Hif3a
		Rgs16	Etv1
		Transporter	Sox12
		Abca17	Egr1
		Itpr1	Prdm10
		Slc43a3	

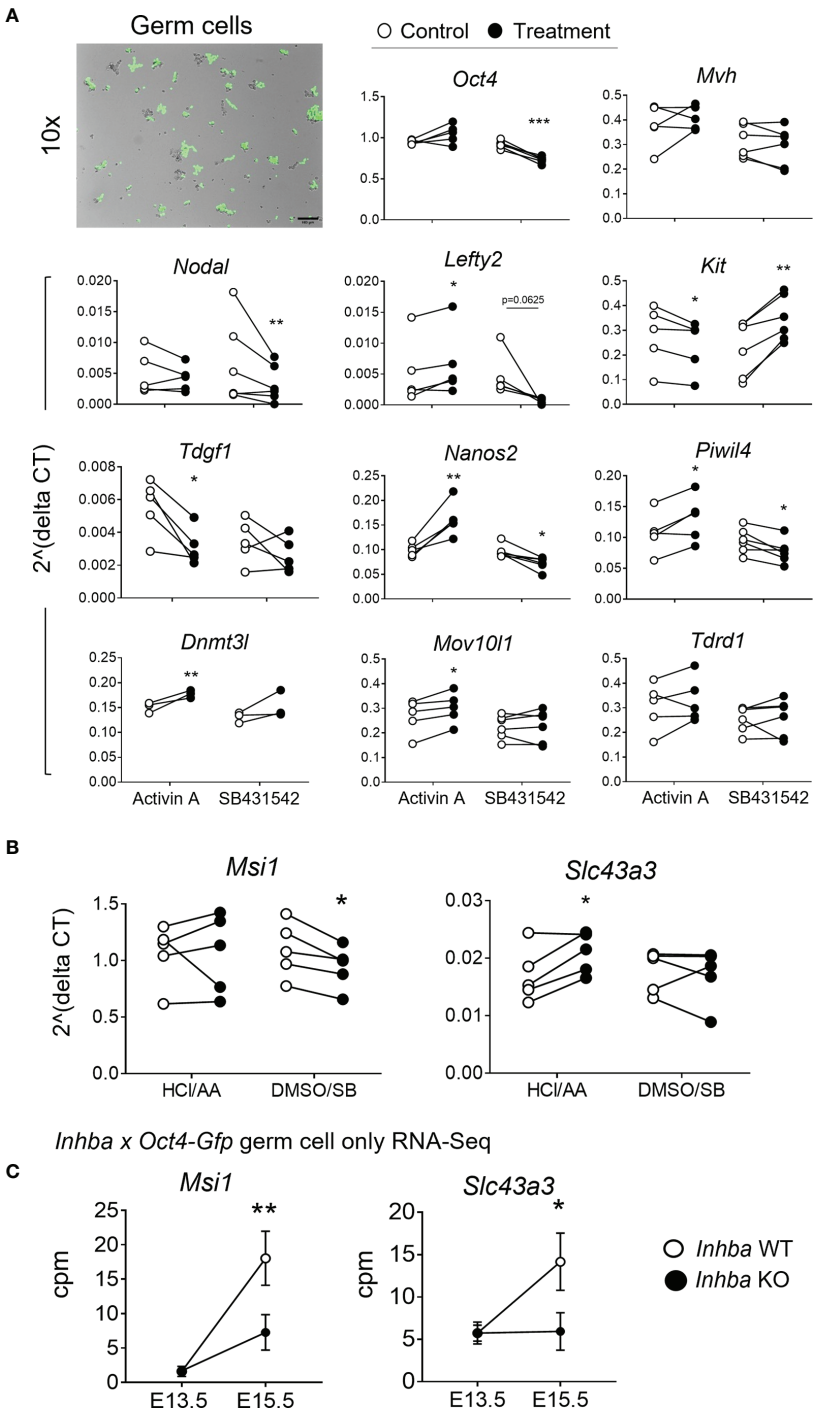


FIGURE 3 | Germ cells can respond directly to activin A modulation. **(A)** E13.5 germ cells isolated *via* FACS were cultured with 5 ng/mL activin A or 10 μM SB431542 for 24 hours. Early germ cell and differentiation-associated transcripts were measured by qRT-PCR and normalised to *Canx* or the mean of *Canx* and *Mapk* housekeepers. Data was analysed using the 2^deltaCT method, and each individual experiment is graphed. Following the Shapiro-Wilk normality test, significance was determined by a paired t-test or Wilcoxon test and indicated by asterisks (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). Top left-hand image represents brightfield and fluorescent overlay of Oct4-positive germ cells after 24 hours in culture. Germ cells were lightly adherent, accounting for the overlap shift. Scale bar represents 100 μm. **(B)** Transcript levels of Musashi-1 (*Msi1*) and *Slc43a3*, as described above. **(C)** Transcript levels of *Msi1* and *Slc43a3* in the *Inhba* x *Oct4-Gfp* E13.5 and E15.5 WT and KO germ cell RNA-Seq dataset (counts per million; cpm), presented as mean ± SD.

markers *Oct4* and *Mvh* increased over time (**Supplementary Figure 3**). The decrease in *Nodal* and increase in differentiation markers suggests that E13.5 germ cells can autonomously differentiate outside of the somatic environment.

Oct4 and *Mvh* transcripts were unaffected by activin A exposure, however SB431542 resulted in a significant decrease in *Oct4* (0.8-fold). *Nodal* and *Lefty2* levels were also unaffected by activin A exposure, however both were lower in SB431542-treated cells, consistent with previous reports (12). *Kit* was significantly lower following activin A exposure (0.85-fold), and significantly higher following SB431542 (1.75-fold), and *Tdgf1*, encoding the Nodal co-receptor, was significantly reduced by activin A (**Figure 3A**). The mRNA encoding the Nodal inhibitor Lefty2 is a known activin A-responsive gene, demonstrated in mouse embryonic stem cells and P19 embryonic carcinoma cells (52, 53), and in human TCam-2 cells (30). This responsiveness was also demonstrated here in isolated gonocytes, with a 1.61-fold increase in *Lefty2* following activin A exposure (**Figure 3A**).

In addition, treatment of E13.5 gonocytes with activin A resulted in a more differentiated transcript profile, with significant elevation of *Nanos2*, *Piwil4*, *Dnmt3l* and *Mov10l1*. Further, SB431542 decreased *Nanos2*, consistent with whole gonad culture, and *Piwil4*, while *Kit* increased These results demonstrate that gonocytes respond directly to activin A and the inhibition of its pathway in culture (**Figure 3A**).

Two transcripts, Musashi-1 (*Msi1*) and Solute carrier family 43 member 3 (*Slc43a3*), identified as DEGs in the RNA-Seq data from E15.5 activin A knockout mouse testes (**Figure 2B**), were investigated in these samples. Following exposure to SB431542, *Msi1* was significantly decreased to 0.86-fold of control levels in E13.5 gonocytes after 24 hours, but it was not affected by activin A (**Figure 3B**). In *Inhba* WT germ cells, *Msi1* normally increases 10-fold between E13.5 and E15.5. This was reduced to a 4-fold increase between E13.5 and E15.5 in *Inhba* KO germ cells, resulting in a significant difference in expression levels between wildtype and knockout germ cells at E15.5 (60% decrease, **Figure 3C**). In the isolated somatic cells of *Inhba WT* and *Inhba* KO testes, examined using RNA-seq, the level of *Msi1* recorded was greater than in germ cells (>20 cpm; data not shown) (9) but was not different between genotypes. Thus, *Msi1* appears to be a germ cell-specific activin A target gene, a conclusion supported by the results in the E13.5 isolated germ cell cultures in which *Msi1* was significantly decreased following activin/Nodal/TGFβ inhibition, and that it was significantly reduced in germ cells of *Inhba* KO animals at E15.5.

Slc43a3, originally identified as an equilibrative nucleobase transporter, has also been identified as influencing fatty acid flux (54, 55) but its function in the testis is unknown. *Slc43a3* was lower in the E15.5 *Inhba* KO germ cells compared to WT (**Figures 2B**, **3C**), and was significantly higher in activin A-treated gonocytes (1.26-fold of controls) (**Figure 3B**) suggesting that it is upregulated by activin A directly in germ cells. While *Slc43a3* was not altered following SB431542 exposure; this may be due to a difference between the chronic absence of activin A in the *Inhba* KO mouse and acute inhibition in these cultures *via* SB431542. It is also important to consider that the germ cells may be developmentally different, or that *Slc43a3* transcript may be relatively stable and therefore not reduced within the 24-hour window examined in the isolated E13.5 germ cells.

Dose-Dependent Response of Activin A Somatic Target Genes in Whole Testis Culture

After determining that gonocytes can directly respond to activin A and SB431542 through altered gene expression, we cultured whole testes to assess the outcome of altered signalling on germ cells within their somatic niche. We first performed a dose-response, to determine the optimal concentration of activin A. E13.5 testes were cultured with 5, 25, 50 or 100 ng/mL activin A for 48 hours and compared with control samples cultured in the vehicle. Levels of known activin A-induced somatic cell transcripts, *Hsd17b1*, *Ccl17* and *Serpina5* (9), were monitored to determine the optimal dose at which responses were evident. *Hsd17b1* was significantly higher in testes exposed to 25, 50 and 100 ng/mL, while *Ccl17* and *Serpina5* were significantly higher in testes exposed to 50 and 100 ng/mL of activin A, when compared with vehicle controls (**Figure 4**). Because all three transcripts were increased following exposure to at least 50 ng/mL activin A, this concentration was chosen for subsequent experiments.

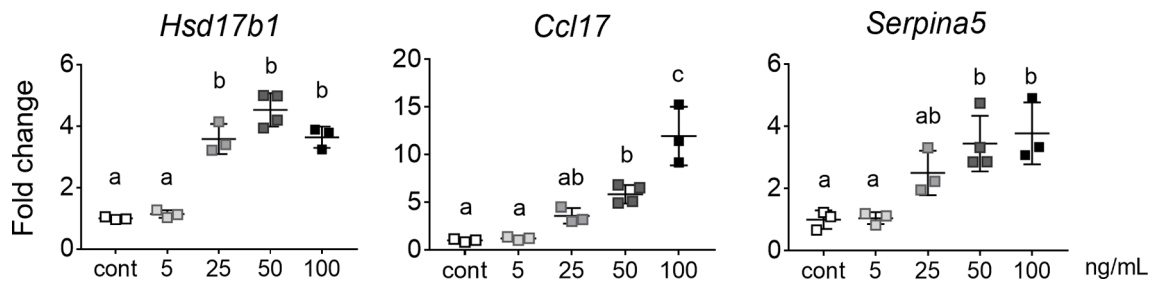


FIGURE 4 | Dose-dependent response of activin A target genes. qRT-PCR analysis of transcripts of E13.5 whole gonads exposed to 5 to 100 ng/mL activin A for 48 hours in culture and compared with control (n=3 or 4 per group). Data were normalised to the *Canx* housekeeper and expressed as fold change compared to control (no activin A). Data are presented as mean ± SD, and significance was determined by one-way ANOVA following the Shapiro-Wilk normality test. Significant differences are indicated by different letters.

Acute Effects of Activin A and SB431542 on Somatic Cells

E13.5 testes were cultured with 50 ng/mL activin A or with the activin/Nodal/TGFβ inhibitor, SB431542, which blocks ligand access to the Type 1 receptors, ALK4, ALK7 and ALK5 (56). Testes were photographed immediately after collection at E13.5 and after 48 hours of culture. Testis cords were easily observed in the E13.5 testes, and after 48 hours of culture in either vehicle, the cords appeared elongated, contained GFP-positive germ cells, and were grossly of the shape normally observed *in vivo* at E15.5 (**Figure 5A**). In contrast, after 48 hours in culture the effectiveness of inhibitor treatment was evident based on the appearance of cords that were fatter and appear stunted, compared with the DMSO controls (**Figure 5A**), previously demonstrated by Miles and colleagues in cultures beginning at E12.5 (12). Cords in testes cultured with activin A were grossly similar to control testes but appeared to be slightly thinner. Activin A target gene transcripts were measured by qRT-PCR. *Ccl17*, *Serpina5*, *Hsd17b1* and *Gja1* (encoding gap-junction protein Connexin 43, expressed in Sertoli cells) were significantly higher than in corresponding control samples following activin A exposure (5.8-, 3.5-, 4.5- and 1.7-fold, respectively; **Figure 5B**), and significantly lower in SB431542-treated testes (0.18-, 0.3-, 0.04- and 0.63-fold of control) (**Figure 5C**), confirming the efficacy of these treatments and demonstrating a dose-dependency of these transcript levels as previously reported *in vivo* (9). *Cldn11*, also encoding a component of Sertoli cell tight junctions, decreased in post-pubertal rat Sertoli cell *in vitro* cultures following activin A exposure (22). The finding that *Cldn11* was significantly lower in activin A-treated fetal testes (0.36-fold), and significantly increased in SB431542-treated testes (3.3-fold) (**Figures 5B, C**) indicates that the responsiveness of these genes to activin bioactivity is likely to be conserved through the Sertoli cell lifespan.

Matrix metalloproteinases are involved in tissue remodelling and have been detected in the fetal testis (57, 58). Exposure of the human gonocyte-like seminoma cell line, TCam-2, to activin A increased both MMP2 transcript and protein levels (59). Therefore, *Mmp2* was also assessed as a potential activin A target in the mouse fetal testis. Activin A exposure did not alter *Mmp2* transcript in fetal mouse testes, however SB431542 significantly decreased 0.43-fold of controls (**Figures 5B, C**). *Mmp2* may not be solely upregulated by activin A, as its decrease following SB431542 exposure could be due to the inhibition of TGFβs or Nodal. Alternatively, *Mmp2* synthesis could have already been at the highest level normally reached by activin A stimulation by the levels present at E13.5. Opposing regulation of *Ccl17, Serpina5*, *Hsd17b3, Gja1* and *Cldn11* by activin A and SB431542 demonstrates the effectiveness of each in culture, while extending our knowledge of how transcripts encoding extracellular matrix components are regulated in the fetal gonad.

To assess Sertoli cells, *Sox9* transcription was measured following whole gonad culture with activin A or SB431542. Interestingly, *Sox9* transcript was significantly lower in activin A-treated gonads (0.76-fold) and significantly higher (1.33-fold) in SB431542-treated gonads (**Figures 5B, C**). This was consistent with our RNA-sequencing analysis of fetal somatic cells from *Inhba* KO mice (data not shown) which collectively suggests that *Sox9* transcription or turnover may be modulated by activin A.

Testes in fetal mice lacking activin A have a reduced proportion of proliferative Sertoli cells (10, 19), and E12.5 testes exposed to SB431542 for 72 hours exhibited a five-fold decrease in Sertoli cell proliferation (12). To assess the effects of activin A and SB431542 on cell proliferation in cultured whole testes, Edu-incorporation followed by flow cytometry was employed. Fetal Sertoli cells, detected by AMH immunostaining, comprised 14% of the total cell population after 48 hours in culture with vehicle controls (HCl, DMSO); testes exposed to activin A had a significantly higher proportion of fetal Sertoli cells, with a 1.5-fold increase to 21% of AMH-positive cells. Conversely, SB431542 exposure significantly reduced the proportion of Sertoli cells to 9% (0.65-fold of DMSO levels) (**Figure 5D**). Consistent with this, we observed a significant increase in the proportion of EdU-positive Sertoli cells following activin A exposure (1.7-fold), demonstrating that activin A increased Sertoli cell proliferation, and a decrease following SB431542 exposure (0.37-fold), demonstrating decreased Sertoli cell proliferation (**Figure 5E**). In addition, the mean fluorescent intensity (MFI) of AMH in AMH-positive cells was also measured as an indication of relative protein levels; Flow cytometric analysis revealed that SB431542 significantly reduced the AMH MFI (**Figure 5F**), and this was confirmed in sections of SB431542-treated testes analysed using immunofluorescence staining (**Figure 5G**).

Testis Culture Supports Germ Cell Development

The *Oct4-Gfp* transgene allowed visualisation of germ cells by fluorescent microscopy after culture. Based on GFP localisation, germ cells appeared restricted to the cords (**Figure 5A**). Levels of germ cell transcripts, assessed by qRT-PCR, were compared between E13.5 whole testes and testes cultured for 48 hours in vehicle. These were also examined against the RNA-seq data of wildtype E13.5 and E15.5 gonocyte populations isolated from *Inhba* x *Oct4-Gfp* mice. Early germ cell transcripts *Kit, Nodal, Nanog* and *Tdgf1* were lower in testes after 48 hours in culture compared with E13.5 testes, and the differentiation markers *Nanos2, Dnmt3a, Dnmt3l, Mov10l1, Piwil2, Piwil4, Tdrd9* and *Tdrd1*, normally upregulated by E15.5, were all increased after 48 hours in culture. These findings were consistent the changes measured by RNA-Seq (**Figure 6A**) and demonstrate the suitability of the culture system for investigating effects on germ cell development.

A Small Proportion of Gonocytes Escape Mitotic Arrest Following SB431542-Exposure

Treatment of E12.5 testes with 10 μM SB431542 for 72 hours previously resulted in an increased proportion of germ cells escaping mitotic arrest, with a 4-fold increase (3% to 14%) in germ cells incorporating EdU (12). This indicates that blocking

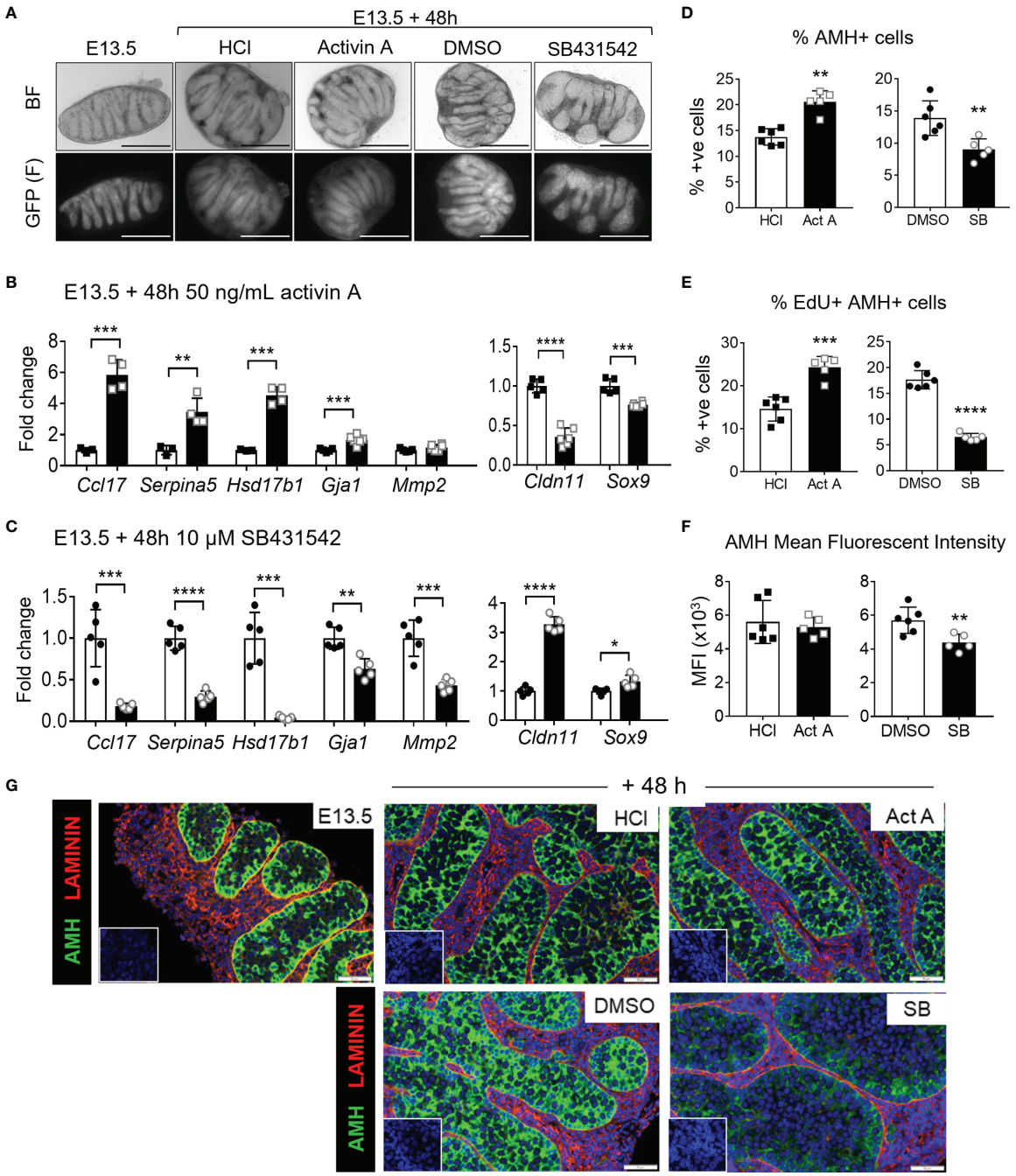
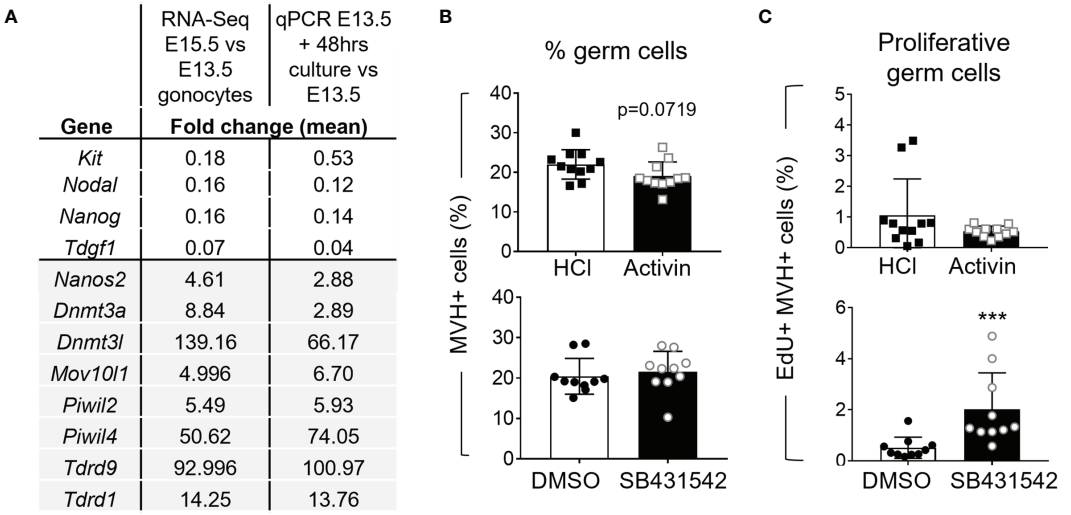
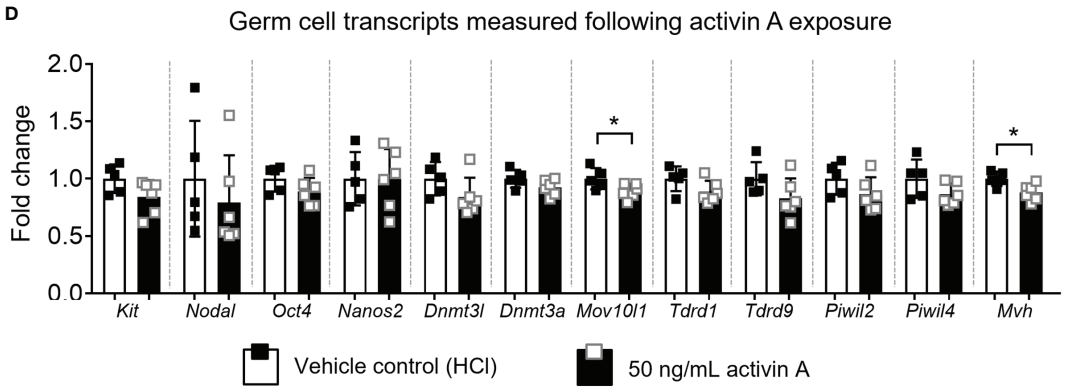


FIGURE 5 | Acute activin A and SB431542 effects on fetal testicular somatic cells. E13.5 testes were cultured for 48 hours on membranes. **(A)** Representative brightfield (top panel) and fluorescent (GFP, bottom panel) images of E13.5 testis and E13.5 testes cultured 48 h with 50 ng/mL activin A, 10 µM SB431542, or vehicle controls (HCl and DMSO respectively). **(B)** Somatic cell transcripts of individual testes (n=3-5 per group) cultured for 48 hrs with 50 ng/mL activin A (black bars) compared with control (white bars). **(C)** Somatic cell transcripts of individual testes (n=3-5 per group) cultured for 48 hrs with 10 µM SB431542 (black bars) compared with control (white bars). All transcripts were measured by qRT-PCR and data normalised to *Canx* housekeeper. **(D–F)** Flow cytometry analysis of dissociated E13.5 testes cultured with 50 ng/mL activin A or 10 µM SB431542 for 48 hrs compared with controls. Proportion of **(D)** AMH-positive (Sertoli) cells, **(E)** EdU-positive AMH-positive (proliferating Sertoli) cells, and **(F)** mean fluorescent intensity (MFI) of AMH-positive population. **(G)** Representative images of AMH (green) and Laminin (red) immunofluorescence staining of 48hr-cultured E13.5 testes. Sections were counterstained with DAPI (blue) for nuclear detection and scale bar is 50 µm. All graphical data are presented as mean ± SD and significant differences determined by a Student's t-test or Mann-Whitney test following the Shapiro-Wilk normality test and indicated by asterisk (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).

A

Gene	RNA-Seq E15.5 vs E13.5 gonocytes	qPCR E13.5 + 48hrs culture vs E13.5
	Fold change (mean)	
Kit	0.18	0.53
Nodal	0.16	0.12
Nanog	0.16	0.14
Tdgf1	0.07	0.04
Nanos2	4.61	2.88
Dnmt3a	8.84	2.89
Dnmt3l	139.16	66.17
Mov10l1	4.996	6.70
Piwil2	5.49	5.93
Piwil4	50.62	74.05
Tdrd9	92.996	100.97
Tdrd1	14.25	13.76





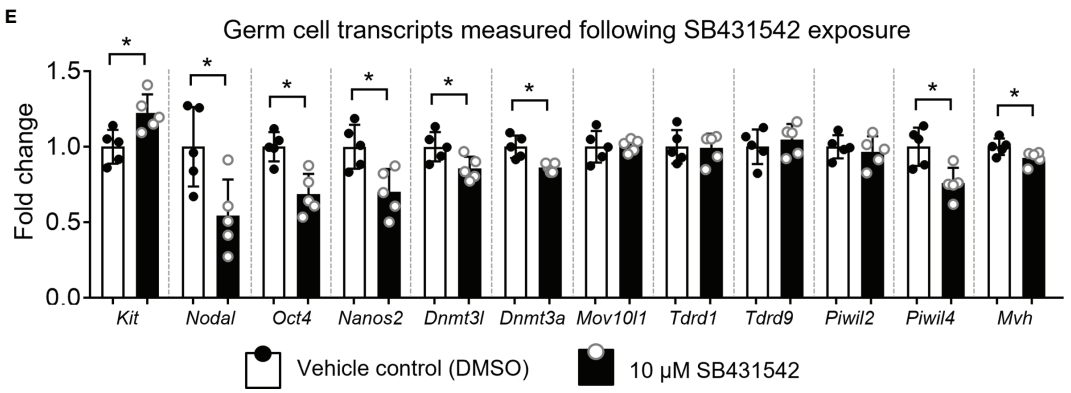


FIGURE 6 | Effect of acute activin A and SB431542 exposure on germ cells. E13.5 testes were cultured for 48 hours on membranes. **(A)** Table presents the fold change of transcript levels. The second column presents fold change of E15.5 compared to E13.5 germ cell transcripts measured by RNA-Sequencing (counts per million (CPM); n=3-4 animals per age). The third column represents the transcript fold-changes of E13.5 testes cultured under normal conditions for 48 hours compared with E13.5 testes measured by qRT-PCR (data was normalised to *Canx*; n=5 per group). **(B, C)** Flow cytometry analysis of E13.5 testes cultured with 50 ng/mL activin A or 10 μM SB431542 for 48 hours compared with respective vehicle controls (HCl and DMSO). **(B)** Proportion of germ cells (MVH+ SOX9-) and **(C)** EdU-positive proliferating germ cells following culture with activin A or SB431542 (black columns) compared with controls (white columns). For activin A and controls, n=5 per group, for SB431542 and controls, n=5 and n=4 respectively. **(D, E)** qRT-PCR analysis of early germ cell and differentiation-associated markers in individual E13.5 testes cultured with **(D)** 50 ng/mL activin A (black bars; n=6) or HCl control (white bars; n=5) or **(E)** 10 μM SB431542 (black bars) or DMSO control (white bars; n=5 per group). Transcripts measured by qRT-PCR were normalised to *Canx* housekeeper and fold change compared to control group shown. All graphical data are presented as mean ± SD and significant differences determined by a Student's t-test or Mann-Whitney test following the Shapiro-Wilk or D'Agostino and Pearson normality tests and indicated by asterisk (*$p < 0.05$, ***$p < 0.001$).

ALK4/5/7 signalling has a robust effect on mitotic arrest. To assess the window of vulnerability of germ cells to this disruption, and to assess whether the proportion of germ cells in this sub-population was sustained, we investigated whether E13.5 testes were similarly susceptible to SB431542 treatment, an age when most germ cells have already entered mitotic arrest. In parallel, we sought to determine whether exposure to exogenous activin A would influence germ cell numbers or proliferation. For these studies, EdU-incorporation and flow cytometry were employed detect proliferating MVH-positive (germ) cells after 48 hours in culture. Germ cells comprised approximately 20% of the total cell population in the cultured testes. There were no significant differences in this value between treatment and control samples after 48 hours in culture, but there was a trend to fewer germ cells following activin A treatment (0.86-fold, p=0.0719) (**Figure 6B**). The proportion of Edu^+ germ cells in the SB431542 treatment group was increased (2% Edu^+, compared with controls, 0.5% Edu^+) (**Figure 6C**). This was statistically significant, and indicates that a small proportion of germ cells in E13.5 testes retain the capacity to escape mitotic arrest. Moreover, together the observations that SB431542 diverts a greater proportion of the germ cell population from mitotic arrest at E12.5 compared to E13.5, indicate that there is a window at around E12.5 during which inhibiting AKL4/5/7 can divert germ cells from their normal entry into mitotic arrest.

Activin/Nodal/TGFβ Inhibition in E13.5 Mouse Testes Promoted a Less-Differentiated Germ Cell Phenotype

To further examine the relevance of this pathway to fetal germ cell differentiation in these whole fetal testis cultures, key markers were measured by qRT-PCR. Early germ cell marker transcripts *Nodal*, *Kit* and *Oct4* were not different following activin A treatment, and amongst key transcripts normally upregulated between E13.5 and E15.5 (*Nanos2*, *Dnmt3l*, *Dnmt3a*, *Mov10l1*, *Tdrd1*, *Tdrd9*, *Piwil2* and *Piwil4*), only *Mov10l1* was affected and was 11% lower than in the control sample. However, the germ cell marker *Mvh* was reduced by 12% following activin A treatment (**Figure 6D**).

E13.5 testes exposed to 10 μM SB431542 exhibited a less-differentiated transcript profile. *Nodal* is highly expressed at E13.5 in germ cells and decreases to <20% by E15.5 (**Figure 1B**). After 48 hours of culture with SB431542, *Nodal* was downregulated to 54% of the control level (**Figure 6E**). Nodal upregulates its own expression (32, 41), and because SB431542 blocks Nodal signalling through ALK4/5/7 inhibition, this downregulation of *Nodal* was expected, and consistent with findings from Miles and colleagues (12). The early germ cell marker *Kit* was significantly higher following SB431542 exposure (1.22-fold, compared with controls; **Figure 6E**). While *Kit* is also expressed in somatic cells, the *Inhba* KO RNA-Seq data shows that at E13.5, *Kit* is predominantly expressed in germ cells (196 ± 5 cpm vs 36 ± 4 cpm in somatic cells; **Figure 7A**) suggesting that the increase in *Kit* is most likely due to an effect of activin/Nodal/TGFβ inhibition on germ cells.

In SB431542-treated testes, the gonocyte differentiation marker *Nanos2* was reduced to 70% of controls, and several PIWI/piRNA pathway components, which normally increase by E15.5 when germ cells are quiescent, were also reduced. The *de novo* DNA methyltransferases *Dnmt3a* and *Dnmt3l* were both reduced by 14% to 86% of control levels in SB431542-treated gonads compared to controls. Similarly, *Piwil4* was reduced to 76% of controls, but there was no change in *Mov10l1*, *Tdrd1*, *Tdrd9* or *Piwil2* levels. The germ cell markers *Oct4* and *Mvh* were reduced to 69% and 93% of control levels following SB431542 exposure, however there was no change in germ cell numbers (**Figure 6E**). Collectively, these changes indicate a modest transcriptional response of these genes to activin/Nodal/TGFβ inhibition.

Because the early germ cell marker *Kit* was upregulated, and the differentiation marker *Dnmt3l* was downregulated following SB431542 exposure, immunofluorescence staining for these two markers was performed on E13.5 testes, and on the activin A and SB431542 treatment samples. KIT was co-localised with MVH in E13.5 germ cells, corresponding with transcript data, but was not detectable in SB431542-treated testes (**Figure 7B**), despite transcript up-regulation. *Dnmt3l* values in germ and somatic cells at E13.5 are below 1 cpm and increase in germ cells to 115 ± 50 cpm at E15.5 (**Figure 7A**). By immunofluorescence, DNMT3L was not detectable in any MVH-positive germ cells at E13.5 but was detected in the nucleus of germ cells after 48 hours of culture in every treatment group, consistent with its normal upregulation by E15.5 (**Figure 7C**). There were no obvious differences between activin A- or SB431542-treated testes compared with their respective controls. DNMT3L appeared to be heterogeneously distributed, with bright and dim staining present in individual germ cell nuclei (**Figure 7C**), however flow cytometry revealed no difference in DNMT3L-positve germ cells (**Figure 7D**) or its MFI between treatment groups (**Figure 7E**). Further scrutiny of the data did not reveal any distinct "bright" or "dim" populations, nor differences in their distribution across treatment groups.

These data suggest that inhibition of activin/Nodal/TGFβ activity in E13.5 testes cultured for 48 hours results in a less-differentiated germ cell transcript profile. Considering that a small subpopulation of germ cells escaped mitotic arrest in SB-treated gonads (**Figure 6C**), it is possible that the changes observed in the transcript profiles may reflect only the small population of germ cells that have not yet entered quiescence.

Delineating Direct and Indirect Effects of Activin A and SB431542 on Gonocytes

After documenting the impact on fetal germ cells of chronic activin A disruptions in transgenic mouse models and demonstrating that isolated germ cells in culture can respond directly to activin A, we wanted to extend our knowledge of how exogenous activin A and SB431542 exposures each affect the germ cells within the intact testis environment. Whole E13.5 testes were dissociated after 48h culture with activin A or SB431542, and the gonocytes isolated by FACS for transcript analysis. Known activin A target genes were analysed in the

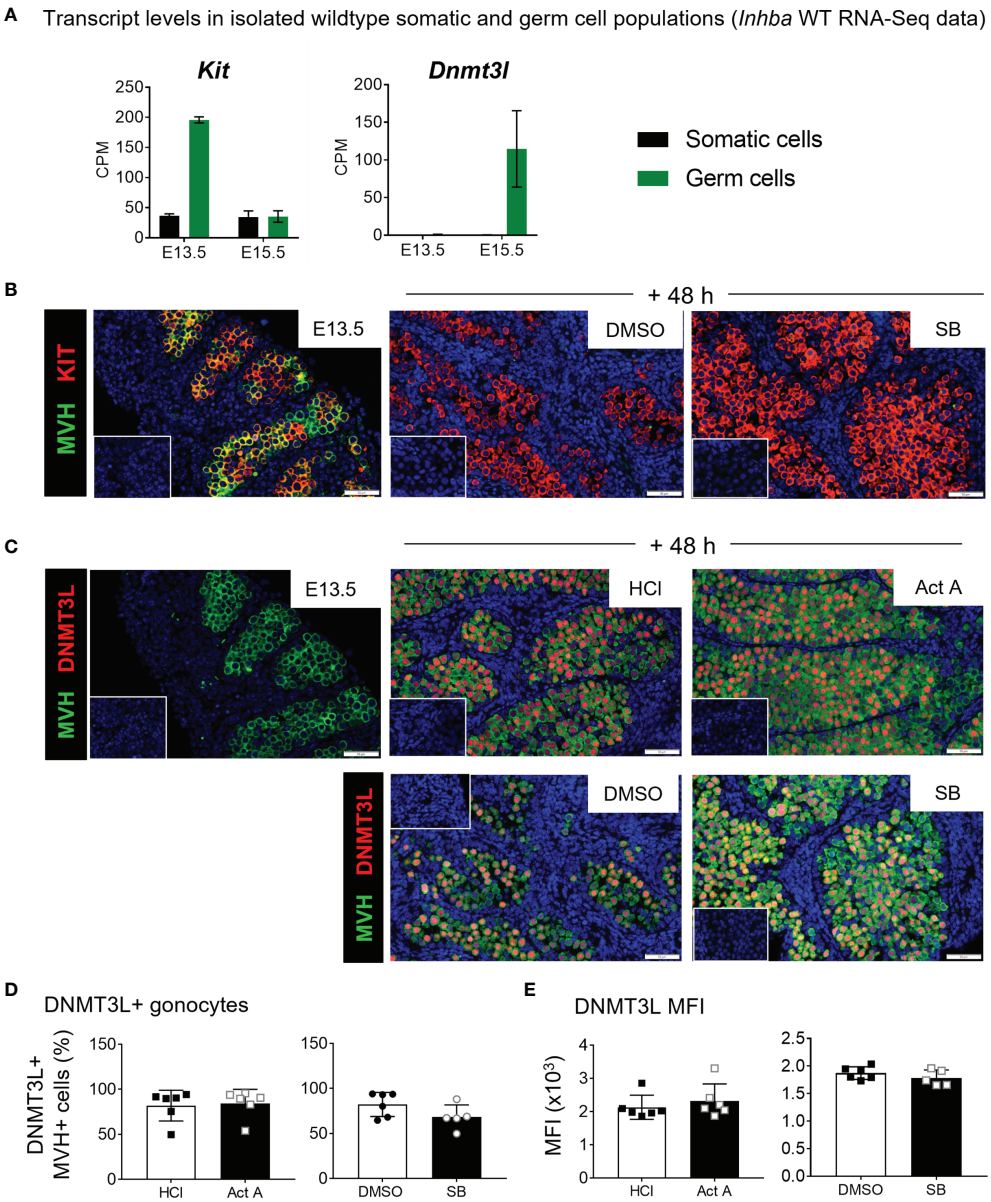


FIGURE 7 | KIT and DNMT3L expression in E13.5 testis cultures. **(A)** E13.5 and E15.5 transcript levels of *Kit* and *Dnmt3l* from FACS-sorted *Inhba* WT somatic and germ cell RNA-Seq data, expressed in counts per million (CPM). **(B, C)** Immunofluorescence staining of E13.5 and cultured testes. **(B)** Detection of KIT (red) and MVH (green) marking germ cell cytoplasm in E13.5 testis and E13.5 testes cultured for 48 hours with 10 μM SB431542 or DMSO control. **(C)** Detection of DNMT3L (red) and MVH (green) in E13.5 testis and those cultured 48 hours with 50 ng/mL activin A or 10 μM SB431542 and respective vehicle controls. DAPI staining in blue marks nuclei. Scale bars are 50 μm, insets represent controls lacking primary antibody. **(D, E)** Flow cytometry measuring **(D)** proportion of DNMT3L-positive MVH-positive germ cells and **(E)** mean fluorescent intensity (MFI) of DNMT3L-positive population following 48 hr culture with activin A or SB431542. All graphical data are presented as mean ± SD and significant differences determined by a Student's t-test or Mann-Whitney test following the Shapiro-Wilk or D'Agostino and Pearson normality tests.

isolated somatic cells to confirm the effectiveness of activin A and SB431542 treatments in the cultures. These results were consistent with the previous whole gonad cultures (**Supplementary Figure 4**). Transcript analysis of isolated gonocytes after whole testis culture with activin A revealed no changes in the early germ cell (*Kit*), or differentiation (*Dnmt3l, Nanos2, Mov10l1, Piwil4* or *Dnmt3a*) markers. SB431542 exposure resulted in significantly increased *Kit* levels (2-fold), consistent with whole testis analysis and isolated germ cell cultures (**Figure 8**). Interestingly, SB431542-exposure did not result in decreased levels of the differentiation marker transcripts *Dnmt3l*, *Nanos2*, *Piwil4* or *Dnmt3a*. Unexpectedly, *Mov10l1* was significantly increased (1.4-fold; **Figure 8**). These data suggest that, while gonocytes can directly respond to perturbed activin A

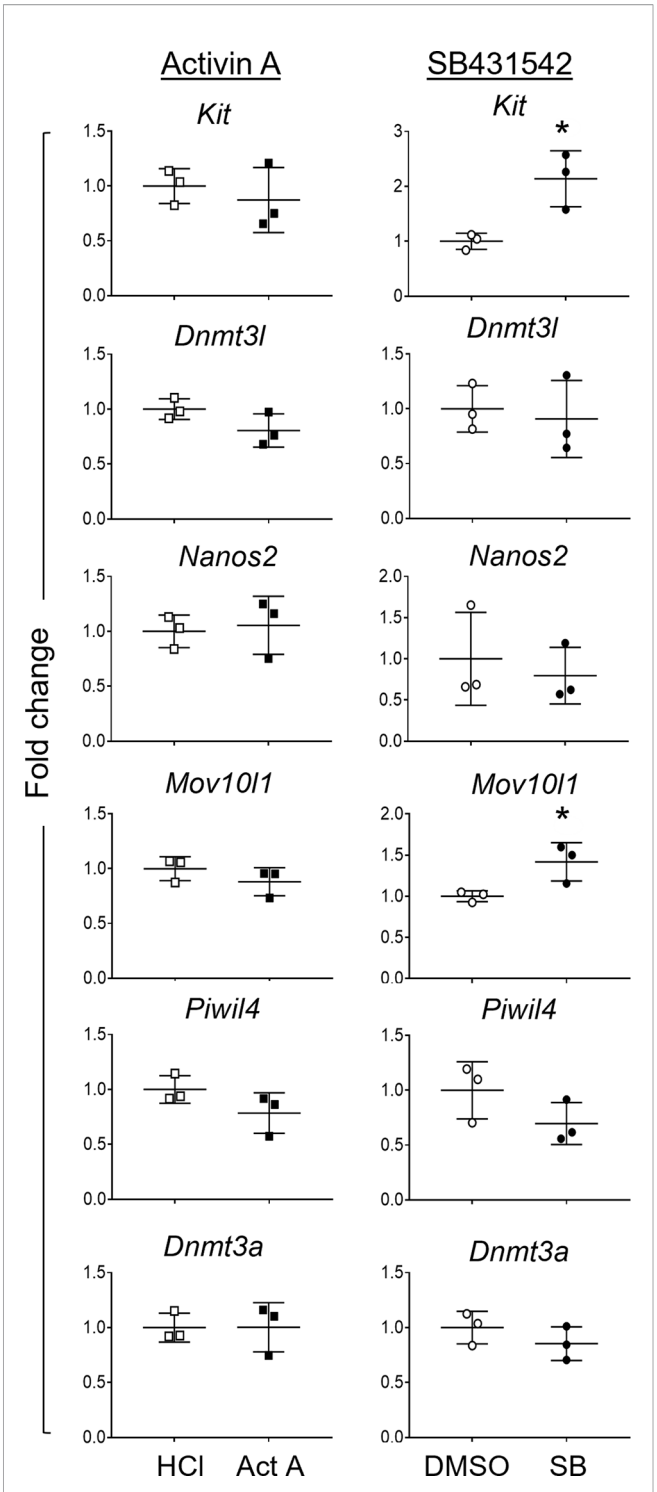


FIGURE 8 | Germ cell transcripts in gonocytes isolated after 48-hour whole testis culture. Germ cells were isolated *via* FACS from E13.5 testes cultured for 48 hours with 50 ng/mL activin A (Act A, black squares) or 10 μM SB431542 (SB, black circles) and their controls (white symbols). Transcripts of germ cell genes were measured by qRT-PCR. Treatment groups are presented as fold change compared to control (n=3 individual testes per group). Data were normalised to the *Canx* housekeeper gene, and presented as mean ± SD. Significance was determined by a Mann-Whitney test or Student's t-test following the Shapiro-Wilk normality test, and indicated by asterisk (*p < 0.05).

and TGFβ signalling, the effects are minimized whilst they reside in an intact somatic environment.

DISCUSSION

The outcomes of this study have demonstrated that germ cells respond directly and indirectly to conditions with which levels of activin A, or its signalling pathway are altered. These findings highlight the value of understanding the contribution of TGFβ superfamily crosstalk to the complex processes required for normal testis development, as the inhibition of receptors shared by activin A, Nodal and TGFβ had a robust impact. The effects of acute pathway inhibition on germ cells was more prominent in intact testes: they displayed a delayed differentiation profile and a smaller proportion of germ cells entered mitotic arrest. Although analyses of intact E13.5 testes cultured for 48 hours with activin A identified minimal effects on germ cells, those germ cells isolated from mice with chronically altered activin A levels have altered transcriptomes at both E13.5 and E15.5. Analysis of cells isolated from *Inhba* mutant mice at two different ages provides evidence of age-specific readouts of activin A signaling, as there was no overlap in DEGs between the ages. A general comparison of what were expected to be equivalent samples (i.e. duplicates at each genotype and age) show variations that would be expected for transcripts that are undergoing dynamic regulation at each of these time points. While culture of isolated germ cells demonstrated their cell-intrinsic capacity to directly respond, with exogenous activin A promoting advanced differentiation transcript profiles, we did not observe reciprocal gene expression changes in the two models. This may reflect signalling interactions between activin A and other pathways.

Murine male germ cells enter mitotic arrest starting from E13.5, and the vast majority are quiescent by E15.5 (3, 26). In the present study, a small but significantly higher population of germ cells (2% of population) were identified as mitotic (in S-phase) in E13.5 whole testes cultured with SB431542 for 48 hours compared with controls. A similar analysis of E12.5 testes cultured with SB431542 for 72 hours reported that approximately 20% of germ cells escaped mitotic arrest, an outcome not observed using the TGFβ-specific inhibitor Alk5i; this result indicated that entry into quiescence was selectively disrupted by activin and/or Nodal signalling in these cultures (12). Thus, the findings in this study are consistent with previous reports, and identify the potential for TGFβ signalling disruptions to alter the maturation pace of fetal male germ cells, including by allowing a small proportion of germ cells to delay mitotic arrest. This may be relevant to human pathologies that arise from disruptions to the differentiation of just a small number of cells. It is well documented that altered activin A signalling disrupts normal testicular somatic cell development, with the Sertoli cells the main target of activin A actions (9, 10, 18, 19). Because spermatogenic development is reliant on the somatic niche, germ cells are susceptible to local environmental changes that could include changes to hormones, growth factors,

and extracellular matrix composition which influence somatic cell functions. In humans, arrested or disrupted differentiation of fetal germ cells is deemed to underpin the emergence of the GCNIS cells which can progress to form testicular germ cell tumours in young men (60). Therefore, minor disruptions to TGFβ signalling could lead to significant consequences in adulthood that may be more impactful in species such as humans which have a long pre-pubertal period.

The combined inhibition of several ligands using SB431542 resulted in a stronger phenotypic change in both culture systems. It is therefore important to consider the combined actions of TGFβ superfamily ligands on testis development and their potential for functional redundancy. TGFβs have a role in regulating germ cell proliferation in the testis. Exogenous TGFβ1 and TGFβ2 decrease the number of gonocytes and increase the number of apoptotic germ cells in fetal rat testis cultures (61). In mouse, 24-hour cultures of E13.5 testes with TGFβ2 decreased gonocyte numbers, and blocking TGFβ signalling in germ cells *in vivo* increased the proportion of proliferative germ cells (11). Treatment of E11.5 and E12.5 XX gonads with a combination of FGF9, TGFβ1, activin A and activin B led to a greater induction of male characteristics than did exposure to a single ligand (62).

Germ cells isolated from E13.5 testes autonomously continue to develop in culture in the absence of a somatic environment. This was previously documented, as E13.5 male germ cells cultured up to 6 days upregulated *de novo* DNA methylation, autonomously establishing genomic imprints (63). The capacity for isolated germ cells to develop in different culture conditions (collagen-coated inserts with 20% serum in the Iwahashi study, vs on plastic with 10% serum, used here) suggest that fetal germ cells harbour a robust cell-autonomous developmental program. In the present study, isolated gonocytes exposed to activin A decreased the early germ cell marker *Kit* and increased differentiation markers such as *Nanos2* and *Piwil4*. In contrast, SB431542 exposure increased *Kit*, and decreased *Nanos2* and *Piwil4*. These data are consistent with, and extend the findings by Wu and colleagues (64).

In both whole testes and isolated germ cells cultured with SB431542, *Kit* levels were increased, consistent with a delayed differentiation profile. In contrast, *Oct4*, a pluripotency marker, was lower in SB431542-treated testes and isolated germ cells. However, Nodal has been shown to promote *Oct4* transcription in a mouse spermatogonial cell line (65), and *Oct4* has been demonstrated to be a direct target of SMAD2 binding in mouse ES cells (52). Therefore, inhibition of activin/Nodal/TGFβ signalling may negatively regulate *Oct4* levels. While isolated germ cells retain their differentiation trajectory, they are sensitive to external signalling cues such as altered TGFβ superfamily signalling.

The importance of identifying targets of activin A signalling relates to the value of understanding how *in utero* environmental exposures may impact on adult fertility. Entry into quiescence signifies a key differentiation step of fetal germ cells and coincides with an increase in the differentiation marker *Nanos2* and of transcripts encoding PIWI/piRNA pathway components such as DNMT3L, DNMT3A and PIWIL4. The decreased levels of these transcripts in E13.5 testes exposed to SB431542 during an interval when they would normally be increasing indicates their differentiation is delayed. The PIWI/piRNA pathway plays an important role in the genomic methylation of retrotransposons during epigenetic reprogramming (5, 50). Mice lacking either PIWIL4 or DNMT3L are sterile, and to various degrees exhibit reduced methylation and increased levels of transposable elements (50, 66, 67), and DNMT3A methylates the maternally imprinted *H19* gene (68). *Mov10l1*, essential for the primary processing of piRNA precursors that have translocated to the cytoplasm (69), is decreased in *Inhba* KO E15.5 gonocytes and increased in activin A-treated gonocytes. Interestingly, *Mov10l1* was increased in germ cells isolated following whole testis culture with SB431542. Loss of primary piRNAs in *Mov10l1* mutant mice completely disrupts the PIWI/piRNA pathway, leading to de-repression of retrotransposons and increased levels of LINE1 and IAPs in postnatal germ cells (70). Similar to other mouse models with genetic modifications of the PIWI/piRNA pathway, the absence of *Mov10l1* causes male-specific sterility (50, 71–73). Because the consequences of PIWI/piRNA pathway disruption often severely affect fertility, it will be useful to determine if the functional consequences of aberrant activin A signalling include altered DNA methylation, increased levels of retrotransposons or reduced levels of piRNAs in germ cells.

Musashi-1 (*Msi1*) encodes an RNA-binding protein, first characterised in *Drosophila* as a regulator of germ cell stemness (74) and shown to impact on germline development in the postnatal testis in mice. MSI1 is present in the cytoplasm of gonocytes and spermatogonia, and in the nucleus of the more differentiated pachytene spermatocytes. Its overexpression impairs spermatogenesis a finding linked to its role in nuclear delivery of an mRNA required for meiotic progression (49, 75). The present study identified *Msi1* reduction in E15.5 germ cells of *Inhba* mutant mice (lacking activin A), and also in E13.5 isolated germ cells exposed to SB431542, providing the first evidence that *Msi1* may be a novel target of activin/TGFβ superfamily signalling.

The somatic cell environment is ultimately essential for fetal germ cell development and therefore crucial to consider when investigating the effect of signalling pathways on testis growth. Anti-Mullerian hormone (AMH), produced by fetal Sertoli cells from E12.5 until puberty (76), is essential for Mullerian duct regression. In the present study, AMH protein levels measured by immunofluorescence on sections, was markedly reduced in SB431542-treated testes. This is in accordance with the report that exposure of human first trimester testes to SB431542 for two weeks in a hanging drop culture system abolishes the AMH signal in cells and reduces its secretion into the media (77). Interestingly, MMP2 is also essential for Mullerian duct regression, and mice lacking AMH have decreased *Mmp2* expression in Mullerian ducts (78). Mouse testes also exhibited reduced *Mmp2* levels following SB431542 treatment, which may be a consequence of reduced AMH levels. This result highlights the challenges inherent in delineating indirect versus direct

signalling pathway outcomes. Gonads of AMH-deficient mice have been examined, but only up to E12.5; testis morphology appeared normal (79), however they did not assess later development *in utero*, when the testis cords are expanding and elongating. AMH is phylogenetically conserved, and the ortholog is present in species that lack Mullerian ducts, such as fish. In medaka fish, AMH is essential for regulating germ cell proliferation; loss-of-function mutations result in excessive proliferation and premature meiosis in male fish (80). It will be of interest to determine the roles of AMH on both somatic and germ cells within the fetal testis.

The integration of cellular development in the fetal testis provides the foundation for ongoing spermatogenesis throughout adulthood. This study has shown that gonocytes can respond directly to activin A and its inhibition. Chronic absence or elevation of activin A can alter the gonocyte transcriptome, and combined activin, Nodal and TGFβ inhibition leads to a less-differentiated phenotype. Importantly, it appears that the somatic cell environment can dominate, and potentially attenuate, gonocyte responsiveness to altered TGFβ superfamily pathway signalling. The use of several complimentary approaches will be required to fully discern how fetal germ cells develop normally in response to somatic cues and to understand the impact of inappropriate cues arising from maternal exposures or genetic factors. Studies such as this one capitalise on the general similarities in the developmental chronology of mouse and human testis growth to learn about germ cell development. The identification of activin A target genes, in addition to others potentially affected by TGFβ superfamily signalling disruptions, provides the opportunity to unearth how germ cells respond to signalling cues and potential outcomes within the complex cellular milieu of the fetal testis. Such information can ultimately identify processes that are of relevance to human pathologies.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI, GSE201520.

ETHICS STATEMENT

The animal study was reviewed and approved by Monash University Animal Ethics Committee (Monash Medical Centre).

AUTHOR CONTRIBUTIONS

SM, KL, and PSW designed the experiments. SM performed experiments. PAFW assisted with RNA-Seq material collection. SM performed primary analysis, with KL and PSW performing critical review of results. SM and KL and PSW wrote the manuscript. All authors read and approved the final version of the manuscript.

FUNDING

This work was supported by the National Health and Medical Research Council (NHMRC) of Australia grants (Ideas grant ID1181516 to KL, and project grant ID1081987 to KL and PSW). SM was supported by an Australian Government Research Training Stipend. This work also received support from the Victorian State Government Operational Infrastructure Scheme.

ACKNOWLEDGMENTS

The authors would like to acknowledge the Monash Medical Centre Animal Facility, Monash Bioinformatics Platform, MHTP Medical Genomics Facility, MHTP Monash FlowCore for sorting of cells using FACS, and the MHTP Monash Histology Platform. Special thanks to our feline friends for emotional support during pandemic lockdowns. **Figure 1A** was created using BioRender.com.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.896747/full#supplementary-material

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frontiers | Frontiers in Endocrinology

PERSPECTIVE
published: 03 June 2022
doi: 10.3389/fendo.2022.891589

Hormonal Male Contraception: Getting to Market

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Rates of unplanned pregnancies are high and stagnant globally, burdening women, families and the environment. Local limitations placed upon contraceptive access and abortion services exacerbate global disparities for women. Despite survey data suggesting men and their partners are eager for expanded male contraceptive options, efforts to develop such agents have been stymied by a paucity of monetary investment. Modern male hormonal contraception, like female hormonal methods, relies upon exogenous progestins to suppress the hypothalamic-pituitary-gonadal axis, in turn suppressing testicular testosterone production and sperm maturation. Addition of an androgen augments gonadotropin suppression, more effectively suppressing spermatogenesis in men, and provides androgenic support for male physiology. Previous contraceptive efficacy studies in couples have shown that hormonal male methods are effective and reversible. Recent efforts have been directed at addressing potential user and regulatory concerns by utilizing novel steroids and varied routes of hormone delivery. Provision of effective contraceptive options for men and women is an urgent public health need. Recognizing and addressing the gaps in our contraceptive options and engaging men in family planning will help reduce rates of unplanned pregnancies in the coming decades.

Keywords: androgen, testosterone, sperm, male contraception, male contraception emerging market, population growth, acceptability

OPEN ACCESS

Edited by:
Vassilios Papadopoulos, University of Southern California, United States

Reviewed by:
Dolores Jean Lamb, Cornell University, United States
Patricia Morris, The Rockefeller University, United States

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Specialty section:
This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology

Received: *07 March 2022*
Accepted: *26 April 2022*
Published: *03 June 2022*

Citation:
Page ST, Blithe D and Wang C (2022) Hormonal Male Contraception: Getting to Market. Front. Endocrinol. 13:891589. doi: 10.3389/fendo.2022.891589

INTRODUCTION

Globally, unplanned pregnancy rates have remained high over the last three decades, a time that has coincided with global warming, population growth and increasing calls for policies that decrease greenhouse gas emissions (1). Limited access, education and engagement in modern, effective contraception remains a global problem that disempowers women, contributes to a cycle of poverty, and impacts the health and welfare of girls and adolescents. However, even with increased access, many women experience side effects from currently available contraceptives or have health conditions that limit contraceptive use. While 16% (6% Africa, 29% North America) of current global contraceptive use is male-directed (condoms, vasectomy and withdrawal) (2), male engagement in contraception is variable around the globe and stymied by limited choices and high rates of method failure (condoms, withdrawal). Vasectomy is effective but requires a highly

skilled provider, is invasive and largely irreversible, limiting the population willing to use this method. We are overdue for new options for male contraception, including both reversible and permanent methods.

Despite the imperatives of climate change and population growth, investment in development of novel, reversible contraceptives for men is minimal, compared with the late 20th century when academia, non-governmental organizations, pharmaceutical entities, and governmental agencies were investors. In the last decade, pharmaceutical companies have largely abandoned their male contraceptive development programs. Can this trend be reversed? We believe we are at a turning point with new male contraceptive methodologies showing promise and strongly positive receptivity from both men and their partners (3–6). It is time for a sea change in investment in male contraception, a potential game-changer for family planning, female agency and reproductive rights.

IS THERE A MARKET FOR NOVEL MALE CONTRACEPTIVES?

Conceptually, male contraceptives, including hormonal male methods, appear to have high acceptability amongst potential users. Multinational survey data from the early 2000s suggest interest and enthusiasm among men from a variety of countries (3–6). Women in committed relationships state they are likely to trust their partners to use these methods (5) and demand for these methods is likely to grow with increasing public awareness. Data obtained from participants in male contraceptive clinical trials represent real user experience, albeit self-selected volunteers with baseline willingness to engage in male contraceptive development (7–10). Across multiple studies employing various modes of administration, participants are overwhelmingly positive regarding the products evaluated, with 50-85% of men reporting willingness to use the product and pay out-of-pocket if commercially available.

Creative methods to demonstrate user desires and preferences are needed to harness the interest of the pharmaceutical industry to support male contraceptive development. Landscape surveys of potential users in least-resourced regions to assess men's willingness to share the burden and costs for contraception are necessary to advance the funding, development, and marketing of new male contraceptive methods. A non-profit (Male Contraceptive Initiative) committed to helping develop male contraceptives has recently conducted consumer market research among men in the United States. Their findings echo earlier enthusiasm; approximately 50% of US men, ages 18-49 who have sex with women and do not wish to father a pregnancy express a high level of interest in novel male contraceptives (11). Data have long suggested that male attitudes toward family planning, including child spacing and family size, have a strong influence on contraceptive use by women and within a couple (12), including in Africa and Southeast Asia where the global burden of maternal death is the highest (WHO trends in maternal mortality). Indeed, pilot projects in sub-Saharan Africa, such as the Malawi Male Motivation project, demonstrate that contraceptive education programs aimed at men improve contraceptive uptake and communication around sexual health within couples, even among couples who have never used contraception (13). As men become more engaged in reproductive health, updated work is needed to better understand the modern contraceptive landscape.

For effective uptake, novel contraceptives must be cost-effective for users and for public health programs aimed to assist family planning. In this context, it is noteworthy that in the United States, long-acting reversible contraceptives (LARCs) female intrauterine devices and implants, are the most cost-effective contraceptive methods, despite high up-front costs (14). Modelling predicts that introduction of novel, reversible male methods could significantly decrease unintended pregnancies as much as 30-40%, particularly in areas where contraceptive uptake is currently low (15). The toll of unplanned pregnancies, as well as medical abortions, is high, affecting mental, physical and economic well-being of women and families. Data on immediate health care costs alone support significant cost-effectiveness for increased contraceptive use among high-risk populations (16). Introduction of novel, cost-effective male contraceptives could have important downstream global health and economic benefits that, over time, could decrease health disparities.

HORMONAL MALE CONTRACEPTION IS EFFECTIVE AND REVERSIBLE

Like hormonal female methods, hormonal male contraceptives utilize exogenous steroids to interrupt physiologic hypothalamic-pituitary-gonadal pathways. Exogenous steroids suppress secretion of gonadotropins, LH and FSH; lack of gonadotropins impairs testosterone production and sperm maturation in the testes, resulting in profound reductions in sperm output 4-12 weeks following initiation of the method. Proof-of-principle studies in the 1980s performed by the World Health Organization (WHO) demonstrated that exogenous high-dose androgens given to healthy men markedly, and reversibly, suppressed spermatogenesis and provided effective contraception for couples (17–19). The use of exogenous progestins more profoundly suppresses gonadotropin secretion in men and allows for physiologic dosing of androgens, largely eliminating hyper-androgenic side effects and minimizing time to suppression to effective contraceptive thresholds (<1 million sperm/ml of ejaculate) (20).

To date, a total of eight hormonal male contraceptive efficacy studies have been conducted, five utilized only testosterone derivatives and three administered a progestin plus testosterone (9, 18, 21–26). Over 2000 couples have been enrolled in these trials, with >1500 completing the efficacy phase (wherein the study drug is relied upon as the sole contraceptive method) after achieving a predetermined sperm threshold of <1-5 million sperm/ml. The Pearl Index, a measure of failure rate, has ranged from 0-2.3 pregnancies/100 person-years in male hormonal

contraceptive clinical trial when a sperm threshold of < 1 million/ml. This compares favorably with female hormonal methods ranging from 0 to 0.3 for intrauterine devices and implants to 1 to 3% for perfect use of the oral contraceptive pill. However, the typical failure rate for female injectable contraceptives is estimated as 6%, for female oral contraceptives is 7.2% and for male condoms is 13%. Whilst regulators have yet to provide firm guidance regarding acceptable failure rates for novel male contraceptives, investigators have advocated for approval of new male methods that fall in the typical use range of condoms. In all studies of these male contraceptive regimens, the methods were fully reversible (27). Thus, although data are limited to the clinical trial context, hormonal male contraceptive methods are highly effective.

WHAT IS IN THE MALE CONTRACEPTIVE CLINICAL PIPELINE?

Hormonal male contraceptive trials over the last five decades have largely centered upon longer-acting hormonal therapies administered by a clinician (i.e. implants, intramuscular injections). With the approval of transdermal formulations of testosterone, research supported by the *Eunice Shriver Kennedy* National Institute of Child Health and Human Development (NICHD) in collaboration with the Population Council, has evaluated transdermal gels delivering a novel progestin, segesterone acetate (also known as Nestorone®) and testosterone to inhibit sperm production. This transdermal NES/T gel has the potential to provide more independence and less discomfort for users than injections and implants and has few side effects whilst delivering physiologic doses of androgens (28–31).

We are conducting a Phase 2b contraceptive efficacy study of NES/T transdermal gel. This multi-national study enrolling 400 couples is the first to evaluate contraceptive efficacy of a daily, self-delivered male contraceptive agent. Importantly, with sites in the United States, Europe, South America and Africa, it will provide information from a diverse group of potential users and is the first male contraceptive efficacy study to include a site in Sub-Saharan Africa. Early clinical studies of NES/T gel demonstrated high effectiveness at suppressing gonadotropins and sperm production (29–31), and very high acceptability amongst users (32) who were eager to know when this product will be commercially available for male contraception. Clinical evaluation of the potential for transfer of the transdermal hormones to a partner was reassuring when the gel was used as instructed (33). Most men found the product easy to use and they adapted the daily gel application to their routine grooming. Results to date indicate that the product is highly effective and acceptable to both partners. Large Phase 3 pivotal studies to further demonstrate safety and contraceptive efficacy are needed for regulatory approval and will require involvement of the pharmaceutical industry.

A notable deficiency in hormonal male contraceptive development and clinical testing has been candidate oral formulations. Many men report they would prefer an oral agent to other modes of contraceptive delivery (3). Until recently, approved oral testosterone formulations have been associated with hepatoxicity (methyltestosterone). A recently approved oral formulation of T undecanoate (34) is safe but the requirement for twice-daily dosing with food is not convenient for a contraceptive regimen. To fill this gap, NICHD is developing several novel androgens as oral formulations in an effort to design the elusive "male pill".

Dimethandrolone undecanoate (7-alpha, 11-beta-dimethyl-19-nortestosterone undecanoate (DMAU)) and 11-beta-methyl-19-Nortestosterone 17-beta-dodecylcarbonate (11β-MNTDC), are synthetic pro-drugs under investigation as both oral and injectable contraceptive agents. DMAU is converted to the active drug, DMA, and 11β-MNTDC to 11β-MNT, *in vivo*, by endogenous esterases. DMA and 11β-MNT activate both androgen and progesterone receptors (35). These progestogenic androgens have potential to be single-agent male hormonal contraceptives. Neither androgen requires 5alpha-reduction (36) to exert maximal androgenic action and neither is aromatized to an aromatic A-ring compound (37). *In vitro*, DMAU is a more potent androgen, while 11β-MNTDC has more balanced androgen and progestogenic activity (35, 38); thus, they exhibit slightly different pharmacodynamics in men.

Preclinical studies in rodents demonstrated that DMAU reversibly suppressed gonadotropins, spermatogenesis and fertility while maintaining non-gonadal androgenic effects (39–41). Both DMAU and 11β-MNTDC support androgenic body composition and bone mineral density in mice (39). Initial studies of single oral doses of DMAU and 11β-MNTDC in men demonstrated that concomitant food ingestion is required for effective oral absorption of these synthetic steroids (42, 43). A subsequent dose-finding study in healthy men,100-400 mg of DMAU taken once-daily for 28 days, provided further evidence that oral DMAU is safe, well-tolerated and markedly suppressed serum gonadotropins and sex-steroid concentrations (44). Remarkably, participants receiving DMAU rapidly developed castrate serum testosterone concentrations (<50 ng/dL), yet had few or no symptoms of hypogonadism, a profound *in vivo* demonstration of the androgenic activity of DMA previously observed *in vitro* (35). A longer study of daily oral DMAU, 100-400 mg, to determine its impact on spermatogenesis is underway. A Phase 1 study of DMAU as an injectable male contraceptive is also underway (NCT02927210). Intramuscular administration of DMAU is unlikely to cause changes in serum lipids by avoiding the well-recognized first-pass effects of oral androgen administration on cholesterol metabolism (45) and may act as a "depot" formulation allowing for injection intervals of 2-6 months.

Compared to other androgens, 11β-MNTDC is the least hepatotoxic when given in repeated oral doses to rabbits (40), making it a promising oral agent. Like DMAU, a 28-day daily dosing study of 200- 400 mg doses of 11βMNTDC demonstrated profound suppression of serum testosterone and gonadotropins, particularly at the higher dose (46). Side effects noted were mild and similar to DMAU. Longer studies of these dual-action androgens are ongoing to determine their relative potency as reversible inhibitors of spermatogenesis; they show considerable promise as a male pill.

Non-hormonal approaches to reversible male contraception aim to reversibly disrupt testes or germ-cell specific targets. These targets include structures and molecules involved in sperm transport, spermiation, and sperm motility among others. A recent review of these approaches has been published (47); major hurdles in advancing development of non-hormonal contraception for men includes ensuring specificity, drugability, safety, and efficacy in animal models. With the exception of trials in India of reversible vaso-occlusion (48), where reversibility remains a major challenge, novel non-hormonal contraceptives for men have not reached clinical trials. It is likely that hormonal male contraceptives will be the first novel, reversible male method to reach the marketplace, hopefully paving the way for additional methods to contribute to the male contraceptive menu going forward.

RISKS AND BENEFITS OF HORMONAL MALE CONTRACEPTIVES

Similar to female hormonal contraceptives, some men who use investigational hormonal male contraceptives may experience unwanted side effects. In general, side effects are seen in a minority of men and mirror those experienced and tolerated by, women who use hormonal methods; namely mild weight gain, mood lability, and impacts on libido. Early hormonal male contraceptive efficacy studies utilized supraphysiologic dosages of intramuscular testosterone. Reported androgenic side effects in some participants, included significant increases in hematocrit, creatinine, and triglycerides across the studies (17–19). Utilizing progestins facilitates physiologic androgen dosing side effects were minimal in recent male contraceptive efficacy studies (22) (21). Pre-efficacy studies in male volunteers demonstrate that the frequency and severity of side effects may be impacted by the progestin used (and its relative androgenicity) and the mode of administration. For example, a series of studies combining oral levonorgestrel with physiologic doses of intramuscular testosterone demonstrated that reductions in levonorgestrel dosing minimized side effects such as weight gain without impacting sperm suppression (49–51). Transdermal delivery of Nestorone®/Testosterone gel is well-tolerated. The most common side effects some men experience are modest weight gain (2-5kg) and acne (30). Fine-tuning the dose of testosterone and the progestin may minimize some side effects that were observed in earlier studies. Nestorone is a potent progestin, that may have advantages over other progestins; its lack of cross reactivity with androgen and estrogen receptors may limit side effects (28).

Validated tools to prospectively identify and quantify potential impacts on mood, libido and sexual function are critical to include in all placebo-controlled Phase 1 and Phase 2 male contraceptive studies, as well as in future efficacy trials, to better understand possible effects of exogenous steroids on these health parameters in men. A placebo-controlled sperm suppression study using a long-acting progestin implant and long-acting TU injections for T replacement, highlighted that male hormonal contraceptives might have mood-related side effects in some men (52). A subsequent efficacy study of separate injections of a long-acting progestin and TU was prematurely terminated due to similar concerns (9). The potential for hormonal imbalance of progestin and testosterone with long-acting formulations may explain the mood effects observed in these trials. Studies of Nestorone and Testosterone combined in a gel and applied once-a-day have been reassuring to date, with no indication of changes in mood. Mild changes in libido without impact on sexual function were observed in a small minority of participants (30). Importantly, most participants report high satisfaction and both partners express a desire to continue to use this method, suggesting that side effects are minimal and acceptable to most users (29, 32). DMAU and 11β-MNTDC, despite leading to marked suppression of endogenous testosterone, were able to maintain sexual function with minor anticipated effects on hematocrit and lipid profiles (53–55). In short-term studies, participants found the once–a-day oral capsules highly acceptable (56, 57).

The risk/benefit ratio for male contraception is complex. Women weigh the side effects of contraceptive methods relative to effects of an unwanted pregnancy; however, the personal risk/benefit health ratio is different for men, raising questions regarding their tolerance for potential side effects. Ideally, male methods that have positive health benefits for the user (such as reductions in long-term disease risk, improvements in well-being, improved metabolic or skeletal risk profiles) will be identified, similar to benefits of some female hormonal methods. While men do not experience medical risks of pregnancy, exploring the mental and economic costs and benefits men *and their partners* incur with unwanted pregnancy will be important to quantify as we assess the potential impact of any novel male contraceptive. Indeed, the concept of "shared risk" is not novel in healthcare, and the importance of applying this principle to male contraceptives that provide substantial benefits to women and men must not be overlooked (58).

IS THERE A PATH TO THE MARKET FOR NOVEL MALE CONTRACEPTIVES?

A major impediment to moving male contraceptive development forward is a lack of regulatory guidance, inhibiting both scientific and financial investment. While work is ongoing to develop effective and well-tolerated products, it is not known what regulatory agencies such as the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA) will find permissible for initial approval of the first hormonal male contraceptive. Consensus recommendations from the research community have been published (59) but whether these will be adopted by regulators is unknown

Along with scientific and clinical investment and innovation, behavioral studies to understand and address the impact of user variables, including product preferences, compliance, barriers to uptake, social biases, and access to contraceptives are critical to advancing the field of male contraception. Male-directed contraception is not new, but the last novel method, the condom, was introduced to the marketplace over 200 years

ago. Novel male contraceptive methodologies demonstrate strongly positive receptivity from both men and their partners. Innovative experimental designs are needed to understand behavioral aspects of modern male contraceptive use. In parallel, engaging reproductive-age male and female advocacy groups will be critical to disseminating accurate information regarding novel male contraceptive methods, helping to reduce misinformation and disparities in access to products. Engaging pharmaceutical companies to co-develop products and initiate new pathways to product development is critical to moving the field forward. Lastly, fair pricing, prescribing practices and health care coverage will be necessary to ensure male contraceptives impact unplanned pregnancies and the global health of future generations of men and women.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

All authors (SP, DB, and CW) contributed to the concept, writing and editing of this manuscript. All authors read and approved the final manuscript.

FUNDING

SP receives funding from the National Institutes of Health through the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development for Clinical Evaluation of Male Contraceptives (Contract number: HHSN275201300025I). SP is also supported by the Robert McMillen Professorship in Lipid Research. CW receives funding form the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development for Clinical Evaluation of Male Contraceptives: Contract number HHSN275220130024I and 75N94020D0007 and P50 HD098593 National Male Reprodutive Epigenomics Center and the National Center for Advancing Translational Sciences UL1TR 001881 UCLA Clinical and Translational Science Institute.

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REVIEW
published: 03 June 2022
doi: 10.3389/fendo.2022.896390

The Regulation of Spermatogonial Stem Cells in an Adult Testis by Glial Cell Line-Derived Neurotrophic Factor

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This review focuses on the *in vivo* regulation of spermatogonial stem cells (SSCs) in adult testes by glial cell line-derived neurotrophic factor (GDNF). To study adult mouse testes, we reversibly inhibited GDNF stimulation of SSCs *via* a chemical-genetic approach. This inhibition diminishes replication and increases differentiation of SSCs, and inhibition for 9 days reduces transplantable SSC numbers by 90%. With more sustained inhibition, all SSCs are lost, and testes eventually resemble human testes with Sertoli cell-only (SCO) syndrome. This resemblance prompted us to ask if GDNF expression is abnormally low in these infertile human testes. It is. Expression of FGF2 and FGF8 is also reduced, but some SCO testes contain SSCs. To evaluate the possible rebuilding of an SSC pool depleted due to inadequate GDNF signaling, we inhibited and then restored signaling to mouse SSCs. Partial rebuilding occurred, suggesting GDNF as therapy for men with SCO syndrome.

Keywords: GDNF (glial cell line-derived neurotrophic factor), spermatogonial stem cells (SSC's), sertoli cell-only syndrome, mature testis, male infertility, sertoli cell

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology

Received: *15 March 2022*
Accepted: *11 April 2022*
Published: *03 June 2022*

Citation:
Wright WW (2022) The Regulation of Spermatogonial Stem Cells in an Adult Testis by Glial Cell Line-Derived Neurotrophic Factor. Front. Endocrinol. 13:896390. doi: 10.3389/fendo.2022.896390

INTRODUCTION

Spermatogonial stem cells (SSCs) are the foundation of male fertility. Preserving this foundation requires that their replication sustains a stem cell pool of normal size and also produces sufficient numbers of differentiating progenitor spermatogonia to ensure continuous production of the large numbers of sperm required for fertility (1). As with all other stem cells, SSCs reside in a special physiological environment or niche that in the testis is created by testicular somatic cells, including Sertoli and peritubular myoid cells (2, 3). These somatic cells secrete numerous growth factors and cytokines that regulate SSC replication and differentiation. Glial cell line derived neurotrophic factor (GDNF) was the first growth factor demonstrated to be essential for the normal function of the SSC niche (4). Prepubertal $GDNF^{+/-}$ mice do not generate the numbers of SSCs necessary to sustain spermatogenesis in the adult. However, until recently, the role of GDNF in a normal adult testis had not been evaluated.

Given this gap in our knowledge and the obvious importance of SSCs to human male fertility, we focused our research on the role of GDNF in the regulation of SSCs within in a mature mouse testis. Results of those studies then prompted us to ask whether a deficit in GDNF expression might contribute to the most severe form of human nonobstructive azoospermia, Sertoli cell-only (SCO)

syndrome. This syndrome is characterized by the apparent absence of spermatogenic cells in histological sections of testes (5). This review summarizes the results of our studies that collectively address three hypotheses:

Hypothesis 1: GDNF is essential for sustaining SSCs in an adult mouse testis. We predict that inhibition of GDNF signaling causes numbers of these stem cells to rapidly decline, due to their differentiation and cessation of self-renewing replication.

Hypothesis 2: Sertoli cells in human SCO testes express abnormally low levels of GDNF.

Hypothesis 3: A mouse SSCs pool that has been partially depleted due to inhibition of GDNF signaling will rebuild if GDNF signaling is restored.

This review also places our results in the context of current literature on the presence of multiple subtypes of SSCs in a mature testis, the transcriptomes of human SSCs and Sertoli cells and analyses by other laboratories of human SCO testes. Building on this summary, we end with a proposal for a potential new therapy for some men with SCO syndrome. As this proposal for human therapy is founded on our analysis of the restoration of a depleted pool of mouse SSCs, it is appropriate to begin with a brief summary of similarities and differences between these stem cells in mice and men (6). Obviously, the first important similarity is that mice and men contain SSCs, as defined by their abilities to survive and replicate when transplanted into a germ cell-deficient mouse testes (7, 8). We acknowledge that transplanted mouse but not human SSCs generate the entire spermatogenic lineage when transplanted into mouse testes (7). Hermann and colleagues attributed the results with human SSCs as being due to the evolutionary distance between mice and men (7). This suggestion is reasonable since testes of another primate species, Rhesus macaques, contains SSCs as defined by their ability to seed the entire spermatogenic lineage when transplanted into germ cell-deficient monkey testes (9). The second similarity is that mouse and human SSCs express many of the same stem cell markers (7). These include: GFRA1, the ligand binding subunit of the GDNF receptor (10), UTF1, a stimulator of self-renewing stem cell replication (11), LIN28 a regulator of stem cell pluripotency and metabolism (12), ZBTB16, a transcription factor necessary for preservation of SSC stemness (13), and ID4, a dominant-negative inhibitor of basic helix-loop-helix transcription factors (14). Importantly, SSCs of neither species express KIT, the receptor for Kit ligand, a stimulator of spermatogonial differentiation (15).

There is, however, a major difference between mouse and human SSCs. As discussed in detail in two excellent reviews by Orwig and co-workers, numbers of SSCs per gram testis are much higher in humans than in mice (6, 7). It has been proposed that the higher number of human SSCs compensates for fact that human spermatogonia replicate fewer times before the start of meiosis (7). Based on the data presented in those 2 reviews we estimate that numbers of SSCs per gram testis are 4-fold higher in men than mice. This estimate is consistent with our analyses; numbers of $GFRA1^+$ spermatogonia per mm^2 tubule surface are 4.2-fold higher in men than mice (5, 16).

HYPOTHESIS 1: GDNF IS ESSENTIAL FOR SUSTAINING SSCs IN AN ADULT MOUSE TESTIS

The Experimental Model

A prerequisite for studying the role of GDNF in a normal mature mouse testis was that experiments start with animals whose testes contained a full complement of SSCs and differentiated spermatogenic cells. Meeting this prerequisite required that GDNF signaling to SSCs be altered only in the adult animal. Furthermore, to test our third hypothesis, this alteration must be reversible. Consequently, we developed a novel chemical-genetic approach that allowed specific and reversible inhibition of stimulation of SSCs by GDNF (**Figures 1A–C**). Our approach had two components: First, we developed a line of mice with a single amino acid mutation (V805A) in Ret, the kinase subunit of the GDNF receptor (19). This mutation enlarged the size of the ATP binding pocket of Ret, without affecting normal RET kinase activity. However, this enlargement enabled Ret(V805A) to bind a bulky, high affinity ATP competitive inhibitor, 1NAPP1-HCl (hereafter called 1NAPP1). Ret(V805A) mice were normal and fertile. However, injection of these mice with 1NAPP1, blocked the ability of GDNF to stimulate its target cells (**Figure 1C**). Surprisingly, while RET is expressed in many adult mouse organs (https://www.gtexportal.org/), we only detected an effect of 1NAPP1 in the Ret (V805A) mouse testis (19). Of equal importance, normal Ret signaling was restored to any remaining SSCs when injection of the inhibitor ceased.

First Test of Hypothesis 1

Our first test of the hypothesis that GDNF is essential for sustaining SSC s in an adult testis took advantage of the fact that the sustained loss of SSCs from a seminiferous tubule is followed by the sequential loss of all remaining spermatogonia, of spermatocytes and then of spermatids. Eventually maturation depletion results in a tubule devoid of all germ cells. It follows that the higher the percentage of germ cell-deficient tubules, the lower the numbers of SSCs at the time testes are collected for analysis. In several experiments, we injected between three and five Ret(V805A) mice with 1NAPP1 once a day for 7 to 30 days and then waited 35 or 60 days for maturation depletion to occur. We then prepared 1-micron cross sections from 4 to 6 different areas of each testis, and determined the percentage of tubule cross sections without germ cells, including spermatogonia. Results demonstrated that this percentage increased as the duration of inhibited GDNF signaling increased (**Table 1**). Importantly, when mice were treated for 11 and 30 days, 97% and 100% of tubules, respectively lacked all spermatogenic cells (**Table 1**). (We examined a total of 1200 (11 days) and 1500 (30 days) tubule cross sections in that experiment.) In contrast, when mice were treated for 7 or 9 days, about 5% and 47% of tubules, respectively, lacked germ cells. As SSCs are the foundational spermatogenic cells, and as after 30 days of treatment, all seminiferous tubules we examined were devoid of spermatogenic cells, we conclude that GDNF is essential for maintenance of SSCs in an adult mouse testis. However, because of the length of time

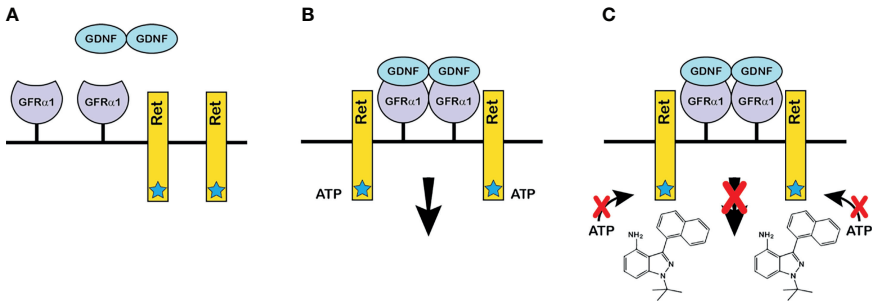

FIGURE 1 | The chemical-genetic approach to reversible inhibition of GDNF signaling *in vivo*. **(A)** GDNF and the components of the GDNF receptor: GDNF is secreted as a disulfide-bonded dimeric protein (17). GFRα1 is the ligand binding subunit of the GDNF receptor, which is linked to the plasma membrane by glycosylphosphatidylinositol (18). Ret is the receptor's protein kinase subunit. The star on Ret denotes the enlarged ATP binding pocket of Ret(V805A). **(B)** The GDNF receptor-ligand complex and the initiation of intracellular signaling: Dimeric GDNF cross-links two GFRα1 receptor subunits, which then recruit two Ret receptor subunits. Formation of the ligand-tetrameric receptor complex stimulates Ret kinase activity, and Ret-bound ATP donates a phosphate to tyrosine residues on the intracellular domain of Ret. Phosphorylation initiates an intracellular signaling cascade. The V805A mutation has no effect on normal Ret kinase activity. **(C)** How the chemical-genetic approach works: The bulky ATP-competitive inhibitor, 1NAPP1 (structure shown on figure) binds with high affinity to the ATP binding pocket of Ret(V805A). Consequently, 1NAPP1 prevents RET phosphorylation. Daily injections of 1NAPP1 are sufficient to inhibit GDNF signaling but signaling is restored when injections cease.

between treatment and analysis, those experiments did not reveal whether inhibition of GDNF signaling caused rapid stem cell loss.

Second Test of Hypothesis 1

To address whether inhibition of GDNF signaling caused rapid loss of SSCs, we injected mice for 9 days and used a functional test for SSCs, their ability to seed spermatogenesis when transplanted into a germ cell-deficient testis. Transplantation of 1NAPP1- and vehicle injected animals occurred 2-4 days after the last injection. Two months later, we enumerated colonies of spermatogenic cells in the testes that received the transplants. As shown in **Figure 2A**, inhibition of GDNF signaling for 9 days reduced numbers of transplantable SSCs to 10% of control. Thus, during a 9-day period, almost all SSCs depend on GDNF to maintain their stemness. These results plus those obtained in the first tests of hypothesis 1 support the conclusion all SSCs are GDNF-dependent at some time during a 30-day period.

We acknowledge that other studies have identified SSCs that do not express GFRA1, the ligand binding subunit of the GDNF receptor. Cells that do not express the GDNF receptor are by definition GDNF-independent. Some of the evidence for GDNF-independent SSCs is as follows: First, some transplantable SSCs in a mature mouse testis do not express GFRA1 (21). Second, 5-10-day old mouse testes contain a subset of SSCs that can be propagated *in vitro* in a GDNF-independent, FGF2-dependent manner. These cells seed spermatogenesis when transplanted into germ cell-deficient testes. Third, there is considerable heterogeneity in expression of markers of SSCs within a population of highly undifferentiated mouse spermatogonia, a subset of which are SSCs (22). Some of these cells do not express GFRA1 but do express other stem cell markers (6).

In comparing results demonstrating that some SSCs do not express the GDNF receptor with our conclusion that all SSCs are at some time GDNF-dependent, it is important to keep in mind that the demonstration of GFRA1$^-$ SSCs represents a "snapshot" of SSCs at one point in time. We examined the consequences of inhibiting GDNF signaling over an extended period. We demonstrated a duration-dependent effect of 1NAPP1-treatment, not only on numbers of GFRA1$^+$ A single (A_s) spermatogonia but also on numbers of A_s cells that expresses a different SSC marker, ZBTB16 (19). These A_s spermatogonia are thought to encompass both SSCs and the most undifferentiated of

TABLE 1 | Effect of the duration of treatment of mice with 1NAPP1-HCl on the percent of Seminiferous Tubules that lack all Spermatogenic Cells 35 or 60 days after treatment.

Treatment Duration (days)	1NAPP1-HCl mg/kg body weight	Days From Treatment to Tissue Collection	Number of Treated Animals in Experiment	Total No. Tubules Examined in Experiment**	% Tubules Lacking All Germ Cells*	Citation
30	62.5	35	5	1500	100	6
11	62.5	35	4	1200	97	6
9	43.7	36	3	900	50	13
9	43.7	60	3	900	45	22
7	43.7	35	3	900	5	13

**1-micron histological sections were prepared from 4-6 different areas of each testis. 300 tubules from each testis were examined.*
***Numbers of animals X 300 tubules analyzed/animal.*

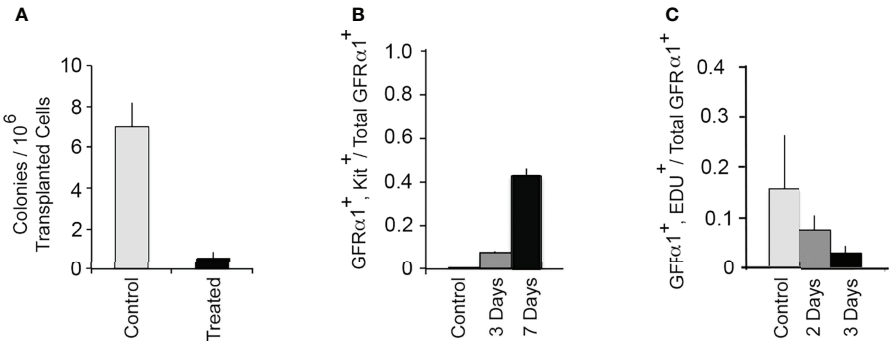

FIGURE 2 | GDNF is essential for sustaining SSCs in an adult mouse testis. **(A)** Effect of inhibition of GDNF signaling for 9 days on numbers of transplantable SSCs. The Ret (V805A) mice used in this study were heterozygous for bacterial β–galactosidase (*Rosa 26*) and for *Id4-GFP*. Adult mice were injected daily for 9 days with 1NAPP1 (treated) or with vehicle (control). Two to four days after the last injections, germ cells were isolated from these mice, transplanted into testes of germ cell-deficient mice, and testes analyzed 2 months after transplantation. Numbers of transplanted SSCs were estimated by enumerating colonies of Rosa 26^+ spermatogenic cells in each testis. Data (mean + SEM) demonstrate that inhibition of GDNF signaling for 9 days results in loss of 90% of transplantable SSCs. Data are from: (16). **(B)** Inhibition of GDNF signaling results in rapid differentiation of SSCs. In this experiment we defined SSCs as GFRα1^+, A single (A_s) spermatogonia, and we used their co-expression of Kit to identify differentiating cells (1). Adult Ret(V805A) mice were treated with vehicle or with 1NAPP1 for 3 or 7 days, seminiferous tubules collected 24 hours after the last injection, and tubules processed for identification of both GFRα1^+ and GFRα1^+, Kit^+ A_s spermatogonia. Data (mean + SEM) are presented as numbers of GFRα1^+, Kit^+ A_s spermatogonia divided by total numbers of total GFRα1^+A_s spermatogonia. Results demonstrate that in control mice, only 0.0008 of all GFRα1^+, A single (A_s) spermatogonia express Kit. However, after 3 and 7 days of treatment with 1NAPP1, 0.08 and 0.42, respectively of all GFRα1^+ A_s spermatogonia, expressed Kit. Data are from: (20). **(C)** Acute inhibition of GDNF signaling causes a rapid decrease in replication of SSCs in normal, mature testes. Ret(V805A) mice were injected with 1NAPP1 for 2 or 3 days, and also with the thymidine analogue, EdU on the last day of treatment. Tubules were collected 24 hrs. later and processed for detection of GFRα1 and EdU incorporation. Data are expressed as fraction of all GFRα1^+, A_s spermatogonia, that had incorporated EdU^+. Results (mean + SEM) demonstrate that inhibition of GDNF signaling results in a rapid decrease in SSC replication. After 3 days, this replication was reduced to 19% of control. Data are from: (20).

progenitor spermatogonia (1). When mice were injected for 11 consecutive days with 1NAPP1, numbers of $ZBTB16^+$ cells decreased to 12% of control. Thus, most $ZBTB^+$ spermatogonia are GDNF-dependent some time during that 11-day period. Furthermore, it was reported that when $GFRA1^-$ cells are transplanted, some transplanted cells began to express GFRA1 (21). It was proposed that niche factors stimulate $GFRA1^-$ SSCs to express GFRA1. This is consistent with the proposals of both Guo et al. (23), and of Sharma et al. (24) that SSCs exist in metastable states that allow their adaption to a dynamic stem cell niche. Thus, we propose than an individual SSC may be GDNF-dependent at one point in time, but not at another. Finally, we acknowledge that the isolation and passaging of FGF2-dependent, GDNF-independent transplantable SSCs is experimentally more than one snapshot in time. However immature testes were the source of these SSCs, and the transcriptomes of these cells differ from the transcriptomes of SSCs in adult mouse testes (25). Thus, our experiments differ in a significant way from those that conclude that some SSCs are GDNF-independent. We have examined the effects of inhibition of GDNF signaling over time, not just at one time. Furthermore, we have studied SSCs in mature testes, not stem cells isolated from immature testes.

Inhibition of GDNF Signaling Causes Differentiation of SSCs and Suppresses Their Replication

There is abundant evidence that *in vitro* GDNF suppresses differentiation and stimulates replication of SSCs, but evidence that this was true *in vivo* was lacking when we began our experiments (3, 8). Thus, we tested whether GDNF suppresses differentiation and stimulates replication of SSCs within a mature mouse testis (20). We defined SSCs morphologically, as $GFRA1^+$ A single (A_s) spermatogonia We used the expression of Kit as a marker of stem cell differentiation, and incorporation of the thymidine analogue, 5-ethynyl-2'-deoxyuridine (EdU), to identify replicating cells. To examine SSC differentiation, we treated Ret(V805A) mice for 3 or 7 days with 1NAPP1 or vehicle and stained cells in whole mounts of seminiferous tubules for both GFRA1 and Kit. Results demonstrated that in controls, fewer then 1% of the $GFRA1^+$ A_s spermatogonia expressed Kit. However, after 3 and 7 days of treatment, 8% and 40% of these cells, respectively, expressed this differentiation marker (**Figure 2B**).

To test if GDNF is an acute regulator of SSC replication, mice were injected with 1NAPP1 for 2 or 3 days and with EdU on the last day of treatment. Tubules were collected and analyzed 24 hours later. Results showed that inhibition of GDNF signaling for 2 or 3 days decreased SSC replication to 44% and 19% of control, respectively. (**Figure 2C**). However, consistent with these cells' long cell cycle times, inhibition of GDNF signaling for 2 or 3 days did not decrease cell numbers (20).

Taken together, our results support the hypothesis that GDNF is essential for sustaining SSCs in a normal adult mouse testis. Moreover, this growth factor suppresses SSC differentiation and acts as an acute regulator of the replication of SSCs in a normal adult testis.

HYPOTHESIS 2: SERTOLI CELLS IN HUMAN SCO TESTES EXPRESS ABNORMALLY LOW LEVELS OF GDNF

The results from our tests of Hypothesis 1 demonstrated that inhibition of GDNF signaling for 11 days caused loss of SSCs and, subsequently, all spermatogenic cells from almost all seminiferous tubules of mature Ret(V805A) mice. Consequently, the testicular histology that the mice developed closely resembled that of human SCO syndrome (**Figure 3**). However, it should be noted that testes of 15%-20% of men with SCO syndrome contain one or more seminiferous tubules with focal areas of active spermatogenesis, allowing sperm retrieval by microdissection testicular sperm extraction (micro-TESE) (26, 27). As loss of mouse germ cells in the Ret(V805) mice resulted from inhibition of GDNF signaling, and as Sertoli cells are the sole and a major source of GDNF in rats and mice, respectively (2, 28), we predicted that Sertoli cells in human SCO testes expressed abnormally low levels of GDNF (5). This prediction was consistent with a preliminary report that cultured Sertoli cells isolated from 2 human SCO testes contained less GDNF mRNA than Sertoli cells obtained from testes of patients with active spermatogenesis. The SCO Sertoli cells also contained significantly lower levels KITL mRNA, a growth factor which stimulates progenitor spermatogonia to differentiate into Type A spermatogonia (29, 30).

First Test of Hypothesis 2

We first tested hypothesis 2 by comparing GDNF mRNA levels in normal and SCO human testes (5). Results showed that GDNF

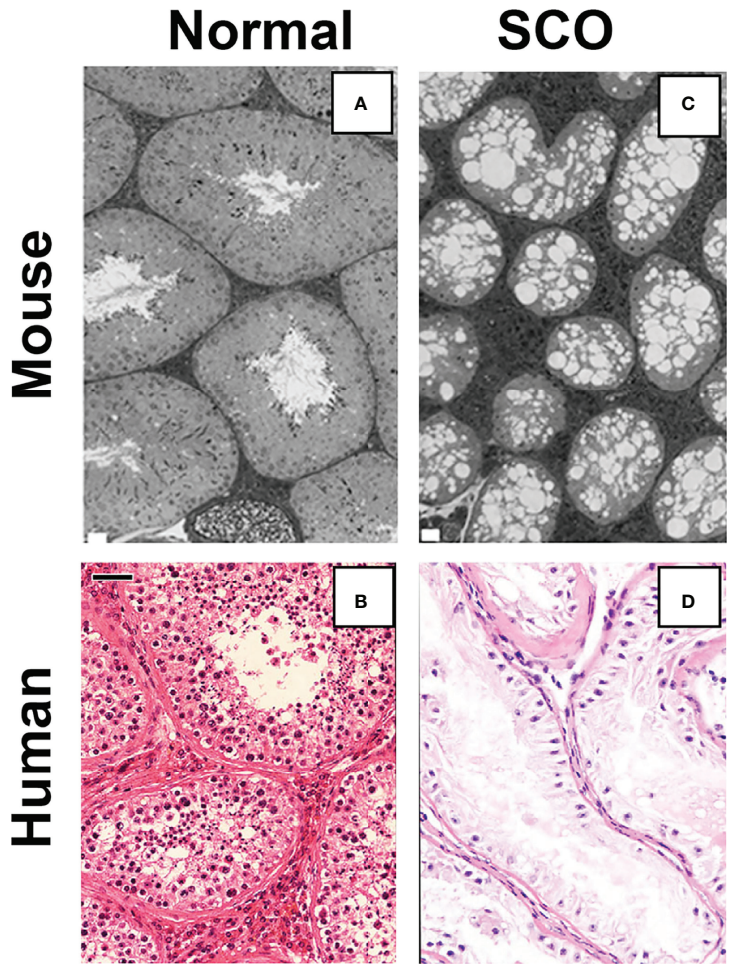

FIGURE 3 | A comparison of the testicular histology of control **(A)** and treated Ret(V805A) mice **(C)** with the histology of normal **(B)** and SCO **(D)** human testes. No spermatogenic cells are evident in the image of testes from treated mouse testis or the image of a human SCO testis. Ret(V805A) mice were injected for 30 days with vehicle **(A)** or with 1NAPP1 **(C)** for 30 days and testes collected 35 days later. Mouse testes were fixed with glutaraldehyde, embedded in epon and 1-micron thick sections stained with Toluidine blue. Sections of human testes were prepared from biopsies collected as part of standard clinical care. Five-micron thick sections were stained with hematoxylin and eosin and all patient identifiers were removed before sections became available for microscopic analysis. The white bars on panels **(A)** and **(C)** are equal to 20 microns on the original section. The black bar on panel **(B)** is equal to 40 microns. Micrographs are from: (5, 19).

mRNA levels were 5.2-fold lower in SCO testes (**Figure 4A**). However, in contrast to the previous report (29), we detected significantly elevated amounts of KITL mRNA in SCO testes (**Figure 4B**). As Sertoli cells are the only source of KITL in a human testis (31), we propose that while we quantified this transcript in testis samples, our results reflect KITL mRNA expression by Sertoli cells. Therefore, we suggest that the previous report of diminished KITL mRNA in SCO Sertoli cells may be due to changes in gene expression caused by the culture conditions.

Second Test of Hypothesis 2

We next used FACS to determine if Sertoli cells in SCO testes contain markedly reduced levels of GDNF (5). Single cell suspensions were prepared from normal and SCO testes, and cells were immunolabeled for GDNF and for SOX9, a specific marker of human Sertoli cells (23, 32) (**Figure 4C**). Results of 5 independent experiments demonstrated that in a normal human testis, GDNF is produced by a single population of Sertoli cells. Sertoli cells were also the only source of GDNF in SCO testes. However, in SCO testes we identified two different Sertoli populations based on GDNF content (**Figure 4D**). The content of the smaller population was like Sertoli cells in normal testes, but the GDNF content of the predominant population was substantially lower. Thus, in SCO testes most but not all Sertoli cells express abnormally low GDNF levels. This deficit in Sertoli cell function results in GDNF concentration of SCO testes to be only 30% of normal (5).

A recent report from Zhao et al. (32) supports our conclusion that SCO testes contain two populations of Sertoli cells. Results from single cell sequencing led the authors to conclude that SCO testes contain one population of Sertoli cells that is similar to healthy immature Sertoli cells, while the other population is similar to Sertoli cells that have begun to mature. The authors also demonstrated that culturing SCO Sertoli cells in the presence of a Wnt signaling inhibitor resulted in transcriptomes of those cells becoming more like those of mature Sertoli cells. Therefore, they concluded that the dysfunction of Sertoli cells in SCO testes is an intrinsic characteristic to those cells and is not due lack of stimulus from spermatogenic cells, with which Sertoli cells normally interact.

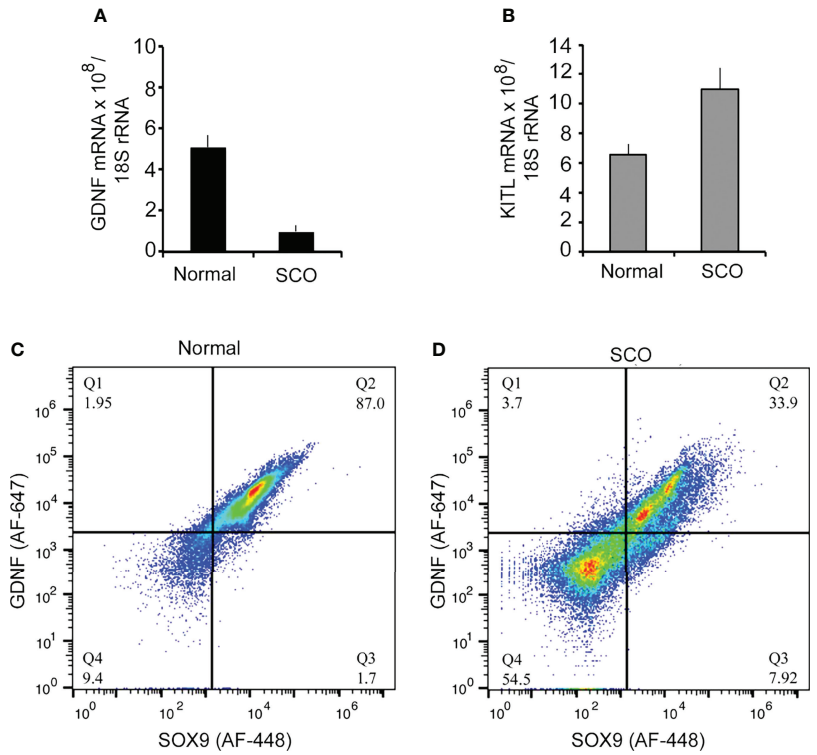

FIGURE 4 | A comparison of the expression of GDNF, and KITL by normal and SCO human testes. RNA was extracted from testis biopsies and expression of GDNF mRNA **(A)**, and KITL mRNA **(B)** assayed by real-time PCR. Data were normalized for βactin mRNA in each sample. GDNF mRNA expression was 5.2-fold higher in normal testes than in SCO testes, while KITL mRNA expression was 1.7-fold higher in SCO testes than in normal testes. **(C, D)** Fluorescence-activated cell sorting was used to determine if human Sertoli cells express GDNF and if this expression is significantly reduced in SCO testes. Single cell suspensions were prepared from biopsies of human normal **(C)** and SCO testes **(D)** and cells incubated with fluorochrome-labeled antibodies for GDNF and the human Sertoli cell specific marker, SOX-9 (23). Data are presented as relative amounts of GDNF and of SOX 9 in each cell. Results, which are representative of 5 independent experiments, demonstrate that normal testes contain a single GDNF-expressing population of Sertoli cells. However, SCO testes contain two populations of Sertoli cells, and the predominant population contains less GDNF than cells in normal testes. Results are from: (5).

While we are fascinated by the data presented by Zhao et al. (32), we consider their conclusion premature, for there is abundant evidence in rodents and in humans of extensive morphological interactions between Sertoli and spermatogenic cells (33). Furthermore, proteins, genes and pathways, that are molecular bases for these interactions have been identified by studying rats and mice. For example, formation of the blood-testis barrier is considered an important milestone in Sertoli cell maturation (34). However, when a mature seminiferous epithelium experiences sequential loss and restoration of spermatogenic cells, its blood-testis barrier is disassembled and later reformed (16). Furthermore, spermatogenic cells have a profound effect on gene expression by mature rodent Sertoli cells. We identified 198 genes whose expression by rat Sertoli cells waxed and waned from 4 to 900-fold as adjacent germ cells progressed through the stages of the cycle of the seminiferous epithelium (35). The rat cathepsin L (CTSL) gene has proven an excellent model with which to understand how germ cells regulate Sertoli cell gene expression. Stage specific CTSL expression is controlled by sequential stimulatory and inhibitory signals from germ cells, which regulate transcription *via* transcriptional activators and repressors within the CTSL gene promoter (36–39). We acknowledge that as of this date, no one has investigated potential interactions between human germ cells and Sertoli cells. However, Sertoli cells and spermatogenic cells of all mammalian species are organized similarly within the seminiferous epithelium, and developing spermatogenic cells in all mammals translocate along the surface of Sertoli cells in a similar manner. Furthermore, as occurs in all other mammals, the human spermatogonia, spermatocytes and spermatids adjacent to the same Sertoli cell mature synchronously and progress through of the stages of the cycle (33, 40). It therefore seems probable that in a fertile human testis, germ cells significantly affect Sertoli cell gene expression. It follows that an absence of germ cells may be one reason that the transcriptomes of Sertoli cells in SCO testes differ from Sertoli cells in normal testes.

Some SCO Testes Contain SSCs

When we measured GDNF and KITL mRNA levels in normal and SCO human testes, we also measured DDX4 mRNA, a specific human germ cell marker (41). Surprisingly, DDX4 mRNA was detectable in all SCO testes, albeit at very low levels, which suggested that some SCO testes contain SSCs (5). To explore this possibility, we used RNAseq to define the transcriptomes of 4 normal and 7 SCO human testes. (RNA was isolated from 5-40 mg testis biopsies. Patients gave informed consent for their collection and analysis.) We then searched those transcriptomes for the presence of 5 transcripts considered to be selectively expressed by SSCs (42). All SCO testes contained these transcripts. Shiraishi et al. (43) expanded our observation when they reported that immunocytochemical analysis of some SCO testes identified cells that express the germ cell-specific marker, DDX4.

Since our analysis of the transcriptomes of SCO testes, several laboratories published the transcriptomes of every cell type in the human testes that were obtained by single cell RNA sequencing. We identified 13 transcripts that 3 different reports identify as selectively expressed by human SSCs (23, 44, 45). We reasoned that identification of these 13 SSC markers in SCO testes would further support the hypothesis that some SCO testes contain SSCs. All 13 markers were detected in the transcriptomes of the 4 normal testes. Nine were present in the transcriptomes of all 7 SCO testes (**Figure 5A**, arrowheads). Three were present 5 of these transcriptomes (**Figure 5A**, arrows). One consensus SSC marker, NANOS 2, was present in the transcriptomes of all 4 normal testes but in none of the SCO transcriptomes. This absence of NANOS2 mRNA in SCO testes might be explained by the facts that GDNF stimulates NANOS2 expression by mouse SSCs (46), and that the GDNF concentration in SCO testes is substantially lower than in normal human testes (5).

To illustrate the differences between SCO and normal testis in the average abundance of each SSC marker, we first normalized data by dividing the abundance of each SSC marker in each transcriptome by abundance of β-actin mRNA. We then calculated the average normalized abundance of each SSC marker in the transcriptomes of normal testes and in SCO testes. Finally, we divided the average normalized abundance in each marker in SCO testes by the average normalized abundance in normal testes (**Figure 5A**). Except for ID4, each SSC marker was substantially less abundant in SCO testes than in normal testes. The median abundance of the 13 SSC markers in SCO testes was 7% of normal.

We previously reported that in SCO testes the expression of 2 putative markers of pachytene spermatocytes were reduced to much greater extent than expression of putative SSC markers. To further support for this observation, we used the same strategy we used to compare abundance of each consensus SSC marker in SCO and normal testes. We first identified 6 consensus pachytene spermatocyte markers. All 6 were identified in two recent reports as selectively expressed by human pachytene spermatocytes, and proven to be essential for the fertility of male mice (23, 45, 47–52). These 6 markers are identified in **Figure 5B**. Four consensus markers were present in all 7 SCO testes (Black arrows, **Figure 5B**). DMC1 and SPO11 were present in the transcriptomes of 5 and 1 of the SCO testes, respectively (**Figure 5B**). To compare the abundance of each of the 6 consensus markers in SCO testes with their abundance in normal testes, we divided the average normalized abundance of each transcript in SCO testes by their average normalized abundance in normal testes (**Figure 5B**). The median abundance of these 6 markers in SCO testes was 0.4% of normal, which is much lower than the median abundance (7%) of SSC markers in the same transcriptomes. Thus, while some SCO testes contain SSCs, most do not give rise to pachytene spermatocytes.

Reduced Expression of FGF2 and FGF8 in SCO Testes

Studies of mice have identified numerous growth factors and chemokines that in addition to GDNF regulate SSC replication, differentiation and/or function. These include FGF8, FGF2, CXCL12 and CSF1. Mouse SSCs express FGFR1, a receptor for both FGF8 and FGF2 and Cre-mediated excision of this receptor

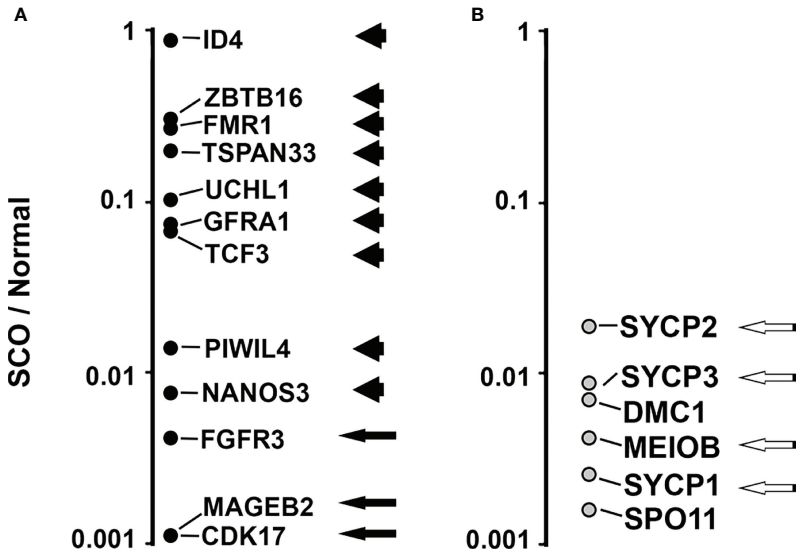

FIGURE 5 | Comparing the abundance of 13 transcripts that are consensus markers of human SSCs **(A)** and 6 transcripts that are consensus markers of human pachytene spermatocytes **(B)** in the transcriptomes of 7 human SCO testes and 4 normal testes. All 19 consensus markers were identified in the transcriptomes of the 4 normal testes. Nine of the 13 SSC markers were identified in the transcriptomes of all 7 SCO testes (Panel **A**, arrowheads). Three SSC markers were present in the transcriptomes of 5 SCO testes (Panel **A**, black arrows). An additional SSC marker, NANOS2, was not present in the transcriptome of any SCO testes. (NANOS2 data are not shown in panel **(A)**. Four markers of pachytene spermatocytes, were present in all 7 SCO transcriptomes (Panel **B**, white on black arrows). DMC1 and SPO11 were present in 5 and 1 of the transcriptomes of SCO testes, respectively. To compare differences between SCO and normal testes in the abundance of each marker, we first normalized data by dividing the abundance of each marker in each transcriptome by the abundance of β actin mRNA in the same transcriptome. We then calculated the average normalized abundance of each marker in the transcriptomes of SCO testes and in the transcriptomes of normal testes. To illustrate the normalized abundance of 18 of the 19 markers in SCO testes (NANOS2 not shown), data for each SSC consensus marker **(A)** and each pachytene spermatocyte consensus marker **(B)** are presented as a ratio (SCO/Normal). Except for ID4, the abundance of each SSC marker is substantially lower in SCO testes than in normal testes. The median ratio (SCO/Normal) for the abundance of the 13 SSC markers was 0.07. The median ratio for abundance of the 6 pachytene spermatocyte markers was 0.004. Thus, some SCO testes contain SSCs, but few support the production of pachytene spermatocytes. The transcriptomes of normal and SCO human testes are described in Paduch et al. (42), and RNAseq data deposited in the NCBI dbGAP database, accession number: phs001777.v1.p1.

from spermatogenic cells, results 24 months later in a significant decrease in numbers of $GFRA1^+$ spermatogonia (53). *In vivo*, testicular overexpression of FGF8 causes numbers of $GFRA1^+$spermatogonia to double within 15 days of virus injection, while injection of FGF2-containing microspheres stimulates formation of large clusters of these cells (53, 54). *In vitro*, CXCL12 stimulates proliferation and suppresses differentiation of SSCs (55). *Furthermore, in vivo*, this chemokine acts as a homing signal for SSCs, and, thus, may play an important role in the migration of SSCs into empty niches (56). *In vitro*, CSF1 stimulates self-renewing SSC replication, and transient depletion of testicular macrophages, a major CSF1, diminishes numbers of $ZBTB16^+$ spermatogonia (55, 57). As human SSCs and/or progenitor spermatogonia express receptors for these growth factors, we examined the transcriptomes of normal and SCO testes to determine whether expression of any of these factors was markedly lower in human SCO testes (**Figure 6A**) (58, 59). As a control, we also examined the abundance of GDNF, to ensure that the RNAseq data replicated our previous results (See: **Figure 4**). It did. Furthermore, the transcriptomes of SCO testes revealed significantly reduced abundance of FGF2 and FGF8 mRNAs, but not of CXCL12 and CSF1 mRNAs. As the abundance of FGF8 mRNA in SCO testes was only 2% of control, we used FACs analysis to evaluate if this reduced expression reflected a specific deficit in Sertoli cell function. Result (**Figure 6B**) show in normal testes, FGF8 is expressed by a single population of Sertoli cells. In contrast, SCO testes contained two populations. One population contained markedly lower amounts of FGF8 than Sertoli cells in a normal testis (**Figure 6C**).

In summary most Sertoli cells in human SCO testes express abnormally low levels of GDNF as well as reduced levels of FGF2 and FGF8. Furthermore, some human SCO testes contain SSCs, though most do sustain production of pachytene spermatocytes. However, since a subset of the Sertoli cells contain normal amounts of GDNF, some of these cells may support the foci of active spermatogenesis present in some SCO testes (26).

Why Do the SSCs in Human SCO Testes Give Rise to So Few Pachytene Spermatocytes?

As already discussed, a comparison of the transcriptomes of normal and SCO human testes suggests that in SCO testes few SSCs give rise to pachytene spermatocytes. The cause of this apparent maturation arrest might be intrinsic to the cells, themselves. Alternatively, it might result from inadequate

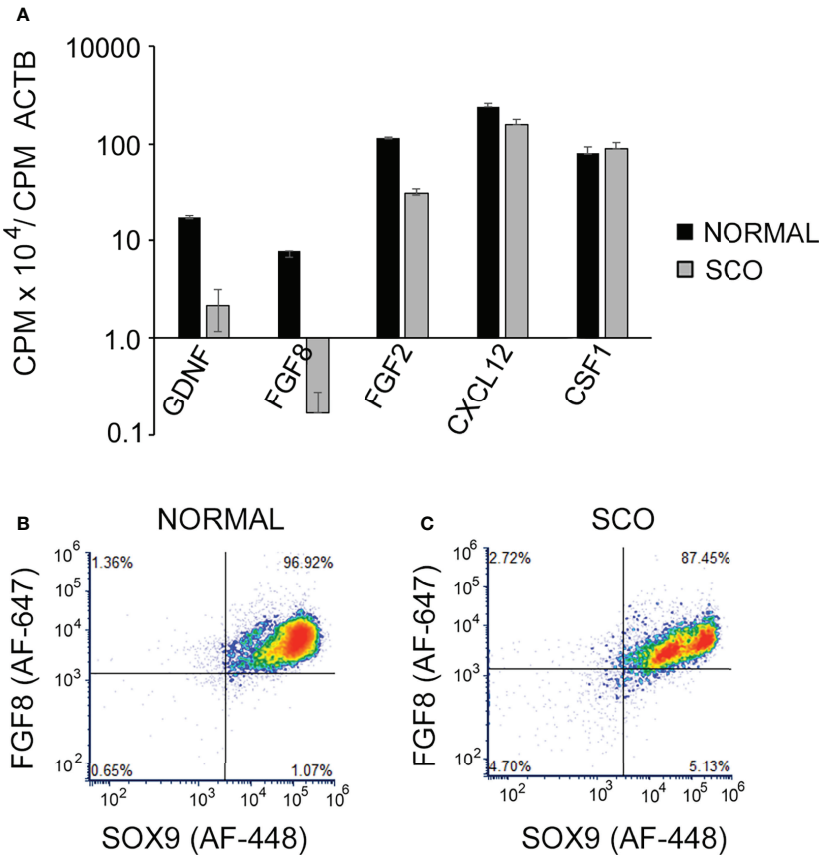

FIGURE 6 | The abundance of GDNF, FGF8, GF2, CXCL12 and CSF1 mRNAs in the transcriptomes of human normal and SCO testes. **(A)** CPMs of each transcript in each of the transcriptomes of 4 normal and 7 SCO testes were normalized for CPM of β actin. Normalized data (mean + SEM) confirm that GDNF mRNA levels are markedly lower in SCO testes and reveal that expression of both FGF8 and FGF2 mRNA are also significantly reduced in SCO testes. **(B, C)** Fluorescence activated cell sorting was used to determine if human Sertoli cells express FGF8 and if this expression is significantly reduced in SCO testes. Single cell suspensions were prepared from normal and SCO testes, cells were immunolabeled for FGF8 and the human Sertoli cell-specific marker SOX9 (23) and FGF8 and SOX9 expression analyzed. Results, which are representative of 3 independent experiments, present FGF8 and Sox 9 expression by individual cells. This analysis demonstrates that normal human testes contain a single population of FGF8-expressing Sertoli cells. SCO testes contain 2 populations, and cells in the larger population contain less FGF8 than Sertoli cells in normal testes. Results are from: (42).

testicular levels of extrinsic stimulators of progenitor spermatogonia differentiation. Kit ligand is one such stimulator (1), but as already discussed, KITL mRNA levels are elevated in SCO testes. However, there may be deficiencies in testis levels of a second stimulator, retinoic acid, for a preliminary study reported that testicular levels of 13-cis retinoic acid are lower in men with an abnormal semen analysis than in men with a normal one (60, 61). While we did not quantify testicular retinoic acid concentrations, we reasoned that if RA levels were normal in SCO testes, their transcriptomes should reveal normal expression of the enzymes that catalyze the 2-step conversion of retinol to retinoic acid (62). In the testis, the first and rate-limiting step, the conversion of retinol to retinal, is catalyzed by retinal dehydrogenase 10 (RDH10), and Cre-mediated excision of this gene in both Sertoli cells and germ cells of prepubertal mice results in maturation arrest of progenitor spermatogonia (63). The second step, the conversion of retinal to retinoic acid can be catalyzed by one of three different retinaldehyde dehydrogenases expressed in testes, ALDH1A, ALDH1A2 and ALDH1A3 (62). Human Sertoli and peritubular myoid cells express ALDH1A1, pachytene spermatocytes and round spermatids express ALDH1A2, and Sertoli cells and pachytene spermatocytes express ALDH1A3 (64).

A comparison of the transcriptomes of normal and SCO testes reveals that expression of RDH10 in SCO testes is only 10% of normal (**Figure 7**). However, expressions of ALDH1A1 and ALDH1A3 are normal, while expression of ALDH1A2 is reduced, as would be expected for a gene expressed by germ cells (**Figure 7**). As retinoic acid stimulates differentiation of progenitor spermatogonia, as RDH10 is the rate limiting step in the conversion of retinol to retinoic acid, and as expression of this enzyme is markedly reduced in SCO testes, a comparison of retinoic acid concentrations in normal and SCO human testis is warranted.

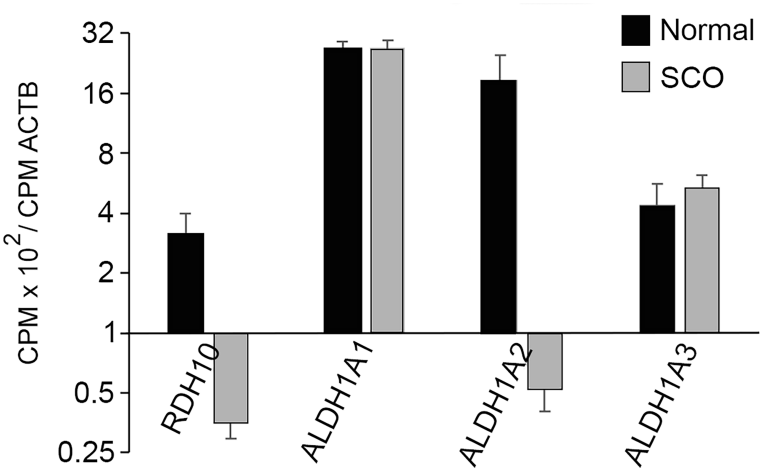

FIGURE 7 | The abundance in the transcriptomes of normal and SCO human testes of transcripts that encode the enzymes catalyzing the two-step conversion of retinol to retinoic acid. CPMs of each transcript in each of the transcriptomes of 4 normal and 7 SCO testes were normalized for CPM of β actin. Normalized data (mean + SEM) demonstrate that expressions of RDH10 and ALDH1A2 are substantially reduced in human SCO testes.

HYPOTHESIS 3: A MOUSE SSCs POOL THAT HAS BEEN PARTIALLY DEPLETED DUE TO INHIBITION OF GDNF SIGNALING WILL REBUILD IF GDNF SIGNALING IS RESTORED

The fact that most Sertoli cells in human SCO testes express low amounts of GDNF raised the question of whether a pool of SSCs that has been depleted due to inadequate GDNF stimulation would rebuild if adequate stimulation to the remaining stem cells was restored. We took the first step to answering this question by use of our mouse model. We injected mice for 9 days with 1NAPP1 and sacrificed mice 2-4 days or 2 months later after injections ceased. Loss and restoration of SSCs were evaluated using two different morphological approaches. The first enumerated cells that co-expressed two different SSC markers, ID4-GFP and GFRA1. The second counted seminiferous tubules that 2 months after treatment were characterized as exhibiting normal spermatogenesis, incomplete spermatogenesis, or SCO syndrome (absence of all germ cells). Tubules exhibiting incomplete spermatogenesis contained 2-4 generations of germ cells, rather the normal 4 to 5 generations (33).

All images of control tubules, all images of tubules collected 2-4 days after treatment and 75% of the images from tubules collected 2 months after treatment, showed ID4-GFP$^+$, GFRA1$^+$ cells to be A_s spermatogonia (**Figure 8A**), another morphological characteristic of SSCs (1). However, 2 months after treatment, 25% of the images revealed clusters or chains of ID4-GFP$^+$, GFRA1$^+$ spermatogonia. We suggest that these clusters or chains exist at the interface between areas of tubules with refilled stem cell niches and areas with empty niches. Morphometric analysis demonstrated that 9 days of treatment reduced numbers of ID4-GFP$^+$, GFRA1$^+$ cells by 84%. Two months later, their numbers were normal (**Figure 8B**).

Histological analysis demonstrated that 2 months after treatment, 71% of tubule cross sections exhibited either normal or incomplete spermatogenesis (**Figure 8C**). The remaining 29% of the tubules were characterized as SCO.

Taken together, those data demonstrate that an SSCs pool that has been substantially depleted due to inadequate GDNF stimulation will substantially rebuild if the remaining SSCs are provided adequate GDNF stimulation.

POTENTIAL THERAPY FOR SOME INFERTILE MEN WITH SCO SYNDROME

The data presented in this review prove that GDNF is essential for maintaining a normal pool of SSCs in an adult mouse testis. We and others have demonstrated that most Sertoli cells in human SCO testes express abnormally low levels of GDNF, and analysis of GDNF$^{+/-}$ mice indicate that such levels are insufficient to sustain a normal stem cell pool (4, 43). Our data also demonstrated that the few SSCs remaining after 9 days of inhibited GDNF signaling will partially rebuild the stem cell pool within 2 months if adequate GDNF stimulation resumes. Taken together, these observations suggest that increasing the concentration of GDNF in a human SCO testis might stimulate the few SSCs they contain to increase in numbers, whether those stem cells are in areas of a tubule without other spermatogenic cells or whether they are present in foci of active spermatogenesis. As GDNF stimulates the migration of SSCs (65), an increase in GDNF testicular concentration, might also stimulate SSCs to migrate to empty niches and potentially seed active spermatogenesis in a previous barren area of tubule. Such an increase in the size or number of spermatogenic foci would increase the probability of successful sperm retrieval by micro-TESE.

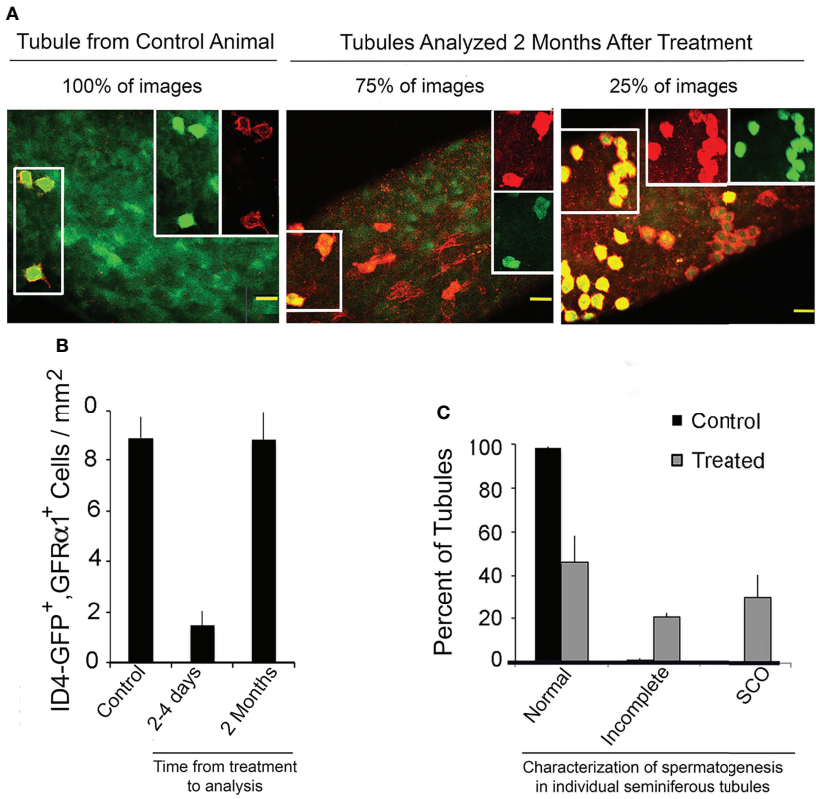

FIGURE 8 | A mouse SSCs pool that has been partially depleted due to inhibition of GDNF signaling will rebuild if GDNF signaling is restored. Mice used for this experiment were from the same liters that provided mice for enumeration of transplantable SSCs (See: **Figure 2A**). Ret(V805), Rosa 26 $^{+/-}$, ID4-GFP $^{+/-}$ mice were injected for 9 days with vehicle (control) or with 1NAPP1, tubules were collected 2 to 4 days or 2 months thereafter and GFRα1-expressing cells detected by immunocytochemistry. Tubules were imaged by confocal microscopy and SSCs were defined as co-expressing GFRα1 (red fluorescence) and ID4-GFP (green fluorescence). Intact testes from additional control and treated mice (n = 3/group) were collect 2 months after the last injections and process for light microscopy as described for **Figure 3**. Spermatogenesis in each tubule cross section was examined. **(A)** Confocal micrographs of tubules of control mice, and of tubules from mice sacrificed 2 months after treatment with 1NAPP1. Cells that expressed both GFRα1 and GFP are outlined by a box on the left had side of each image. Separate red and green channels for the same cells are shown in the boxes on the right side. GFRα1$^+$, ID4-GFP$^+$ cells were identified as A_s spermatogonia in all images of tubules from control mice, in all images of tubules from mice sacrificed 2-4 days after treatment and in 75% of the images of tubules collected 2 months after treatment. In the other 25% of those images, GFRα1$^+$, ID4-GFP$^+$ cells were present as chains or clusters of cells. **(B)** Numbers of GFRα1$^+$, ID4-GFP$^+$ spermatogonia per mm^2 of tubule in control mice, in mice sacrificed 2-4 days after treatment and in mice sacrificed 2 months after treatment. Data are presented as mean + SEM for 5 control animals and for 3 mice at each of the two time points. **(C)** Characterization of spermatogenesis in seminiferous tubules of control mice and of mice sacrificed 2 months after treatment (n = 3/group). Tubules were characterized as having normal spermatogenesis, incomplete spermatogenesis (containing 2-4 generations of germ cells) or lacking all germ cells and thus exhibiting SCO syndrome. Normal or incomplete spermatogenesis was observed in 71% of tubules of treated mice. Taken together, these results demonstrate that when GDNF signaling is inhibited for 9 days and then restored, the depleted SSC pool is partially rebuilt. Results are from: (16).

Given the above considerations, how could GDNF be developed as therapy for SCO syndrome? A potential approach is suggested by methods that are being developed to stimulate repair injured neurons by local administration of GDNF. Three different methods have been described for this local administration: Driving *de novo* expression of GDNF at the site of injury by injection of non-replicating virus that encode GDNF, implantation of GDNF-containing microspheres, and implantation of cells that express recombinant GDNF (66–70). In the last approach, GDNF-secreting cells are encapsulated in matrices that protected those cells from immune attack, while allowing free diffusion of proteins to and from the cells.

It is well established that chronically increasing the concentration of testicular GDNF in rodent testes substantially suppresses SSC differentiation, causing their substantial overaccumulation (4, 69, 71). Eventually the structure of the seminiferous epithelium is disrupted, and spermatogenesis fails. As noted by Sharma et al. (71) the likely reason for this overaccumulation is that normally, the expression of GDNF by rodent Sertoli cells changes more than 10-fold as the adjacent germ cells progress through the stages of the cycle of the seminiferous epithelium (28, 71). We have proposed that this cycle of GDNF expression results in SSC replication at some stages of the cycle and SSC differentiation at others (28). We anticipate that successful use of GDNF as therapy for SCO

syndrome will require that the therapy cycle the testes between periods of elevated GDNF concentration and periods of lower concentration. This goal might he achieved by implanting SCO testes with encapsulated cells that drive GDNF expression *via* a bacterial Tet operon (72). By interspersing days of oral tetracycline administration with days of placebo administration, a cycle of testis GDNF levels might be achieved.

We recognize that development of this proposed therapy will take much effort, time, and resources. Defining the proper number of implanted cells will be essential, as well as their placement within a testis. Developing an efficacious tetracycline dosing schedule will also be critical. Moreover, as FGF2 and FGF8 expression is also abnormally low in SCO testes, implants of cells expressing one or both of growth factors also may be required. However, the successful development of this new therapy may allow some infertile men with SCO syndrome to father their own children.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

FUNDING

Supported by HD 1RO1HD074542.

ACKNOWLEDGMENTS

Many students and colleagues contributed to the success our research. Joseph Savitt generated the Ret(V805A) mouse line, Kevan Shokat generously provided 1NAPP1-HCl for all experiments that employed the chemical-genetic approach. My colleagues at Weill Cornell Medical School, Darius Paduch and Peter Schlegel, recruited and obtained informed consent for patients in our studies, and collected testis biopsies. Dr. Schlegel obtained approval for studies with human patients from the Weill Cornell IRB. Dr. Paduch performed FACs analysis of Sertoli cell content of GDNF and FGF8. Dolly Singh analyzed the transcriptomes of normal and SCO human testes. Nicole Parker performed most of the experiments with mice. I am particularly grateful to Pamela Wright for her expert editing of manuscripts and grants and for artwork, including **Figure 1** of this review.

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frontiers | Frontiers in Endocrinology

ORIGINAL RESEARCH
published: 09 June 2022
doi: 10.3389/fendo.2022.892342

Organotypic Rat Testicular Organoids for the Study of Testicular Maturation and Toxicology

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An *in vitro* system to study testicular maturation in rats, an important model organism for reproductive toxicity, could serve as a platform for high-throughput drug and toxicity screening in a tissue specific context. *In vitro* maturation of somatic cells and spermatogonia in organ culture systems has been reported. However, this has been a challenge for organoids derived from dissociated testicular cells. Here, we report generation and maintenance of rat testicular organoids in microwell culture for 28 days. We find that rat organoids can be maintained *in vitro* only at lower than ambient O_2 tension of 15% and organoids cultured at 34°C have higher somatic cell maturation and spermatogonial differentiation potential compared to cultures in 37°C. Upon exposure to known toxicants, phthalic acid mono-2-ethylhexyl ester and cadmium chloride, the organoids displayed loss of tight-junction protein Claudin 11 and altered transcription levels of somatic cell markers that are consistent with previous reports in animal models. Therefore, the microwell-derived rat testicular organoids described here can serve as a novel platform for the study of testicular cell maturation and reproductive toxicity *in vitro*.

Keywords: testicular organoid, spermatogonia, MEHP, cadmium chloride, Sertoli cell

OPEN ACCESS

Edited by:
Barry Zirkin, Johns Hopkins University, United States

Reviewed by:
Marie-Claude Hofmann, University of Texas MD Anderson Cancer Center, United States
Martine Culty, University of Southern California, Los Angeles, United States

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Specialty section:
This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology

Received: *08 March 2022*
Accepted: *02 May 2022*
Published: *09 June 2022*

Citation:
Sakib S, Lara NLM, Huynh BC and Dobrinski I (2022) Organotypic Rat Testicular Organoids for the Study of Testicular Maturation and Toxicology. Front. Endocrinol. 13:892342. doi: 10.3389/fendo.2022.892342

INTRODUCTION

Male factor infertility is responsible for 40-50% of all cases of infertility worldwide (1) and around 9-16% of all men suffer from infertility (2). The causes of infertility are varied; some are due to pathophysiological conditions, while others may be caused by environmental toxicants such as exposure to phthalates and heavy metals (3–5).

Spermatogenesis is a highly orchestrated process that is dependent on a tightly regulated stem cell niche (6). This spermatogonial stem cell niche is primarily composed of spermatogonia and somatic cells, such as Sertoli cells, peritubular myoid cells and Leydig cells (6). The regulation of spermatogonial cell fate is tightly modulated by these somatic cells (7, 8). *In vitro* culture systems to model this niche have primarily been limited to conventional two-dimensional co-culture systems (9), which fail to mimic the cell-cell signaling seen *in vivo* (10, 11). A three-dimensional organotypic culture system can bridge the gap between cell cultures and whole animal models to better study developmental processes such as spermatogonial differentiation and reproductive toxicology *in vitro*

(12, 13). Several three-dimensional testicular organoid systems have been developed with this goal in mind (11, 14–18).

Here, we describe the adaptation of microwell-derived testicular organoids, previously established in porcine, murine, human and primate models (11), to rat testicular organoids and highlight the species-specific challenges encountered during this endeavor. Rats have been extensively studied as an animal model for testicular maturation and reproductive toxicology (19–22). We report here that organotypic rat testicular organoids can be derived and maintained for 28 days only at lower than ambient (15%) O_2 tension. We also characterize rat organoids cultured at both 37°C and 34°C and show that rat organoids cultured at lower temperature better support the differentiation of spermatogonia. Finally, we present proof of principle with known environmental toxicants to establish the utility of rat testicular organoids for the study of reproductive toxicity.

MATERIALS AND METHODS

Preparation of Rat Starting Testicular Cell Population

SAS Sprague Dawley rats (Strain: code 400, Charles River), aged P4-P5, were euthanized and testes were removed. All animal procedures were performed as approved by the Animal Care Committee, University of Calgary. Using a pair of forceps, the tunica albuginea was removed to release the tubules, which were then washed in Hank's Balanced Salt Solution (HBSS, Gibco, cat# 14025092) containing 1% penicillin-streptomycin (ThermoFisher Scientific, cat# 15070063). The tubules were digested using collagenase type IV (Worthington-Biochem, cat# LS004189) in HBSS (2 mg/mL) for 25 min at 37°C. The tubules were then sedimented by centrifugation at 90x g for 1.5 min and washed with 5 mL HBSS thrice. Finally, the tissue was digested with 0.25% trypsin–EDTA (Sigma-Aldrich, cat# T4049) and DNase I (Sigma-Aldrich, cat# DN25) in HBSS (10 mg/mL) for 5 min to obtain the starting cell population (11). All experiments were replicated using a minimum of three independently prepared cell suspensions.

Generation of Organoids

AggreWell 400 plates (STEMCELL Technologies Inc, Vancouver, Canada, cat# 34450) were treated with Anti-adherence Rinsing Solution (STEMCELL Technologies Inc, Vancouver, Canada, cat# 07010) according to the manufacturer's instructions. Each well was filled with 500 µL of organoid formation medium (OFM) (Dulbecco Modified Eagle Medium F/12 (Gibco, cat# 11330-032) supplemented with insulin 10 µg/mL, transferrin 5.5 µg/mL, selenium 6.7 ng/mL (Gibco, cat#41400-045); 20 ng/mL epidermal growth factor (R & D Systems, cat# 236-EG); 1% Penicillin-Streptomycin) and the plates were then centrifuged at 2000x g for 2 minutes to release any trapped air bubbles (11). Each well was then seeded with 6×10^5 rat testicular cells suspended in 500 µL OFM. Finally, the plates were centrifuged at 500x g for 5 minutes to sediment the cells in the microwells. 500 µL of media were removed from each well and fresh media supplemented with Corning Matrigel Growth Factor Reduced (GFR) Basement Membrane Matrix (1:100 dilution; Life Sciences, cat# 354230) was used to replenish each well. Microwell plates were divided into three groups and placed in 3 incubators setup for three conditions: (i) ambient O_2 tension (18.4% or 6.34 mM O_2, 5% CO_2, 37°C), (ii) 15% O_2 tension at 37°C (15% or 5.18 mM O_2, 5% CO_2) and (iii) 15% O_2 tension at 34°C (15% or 5.22 mM O_2, 5% CO_2). Culture in OFM was carried out for 3-5 days with 50% media changes every second day.

Maturation and Differentiation of Organoids

After culturing the organoids for 3 days, which were designated as day 0 undifferentiated organoids, the OFM media was completely removed, and the culture was continued in organoid differentiation medium (ODM). ODM was composed of Minimum Essential Medium α (ThermoFisher Scientific, cat# 12571063) supplemented with 10% KnockOut Serum Replacement (ThermoFisher Scientific, cat# 10828028), hepatocyte growth factor (5 ng/mL) (R&D Systems, cat# 294-HG), activin A (100 ng/mL) (Sigma-Aldrich, cat# a4941), follicle stimulating hormone (1 ng/mL) (Sigma-Aldrich, cat# F4021), luteinizing hormone (1 ng/mL) (Sigma-Aldrich, cat# L5259), testosterone (1 µM) (Steraloids, cat# A6950-000), recombinant human BMP-4 (20 ng/mL) (R&D Systems, cat# 314-BP), recombinant human BMP-7 (20 ng/mL) (R&D Systems, cat# 354-BP), 3,3',5-triodo-L-thyronine sodium (2 ng/mL) (Sigma-Aldrich, cat# T6397), l-ascorbic acid-2-glucoside (1 mM) (Matrix Scientific, cat# 092375) and 1% Penicillin-Streptomycin (22, 23). Culture was carried out for an additional 28 days, with full media changes every second day. The organoids were sampled every 7 days, including day 0, for analysis.

Immunohistochemistry

Testes tissue from 43-day old rats were fixed in 4% paraformaldehyde, dehydrated with a gradient series of ethanol, and embedded in paraffin wax to prepare sections of 5 µm thickness. The rat starting cell populations and organoids were fixed using 2% paraformaldehyde and spun down on slides using cytospin centrifugation (1000 rpm for cells and 500 rpm for organoids) (Cytospin 4, Thermo Scientific). The samples were then permeabilized using a gradient series of methanol (24) and blocked with 10% donkey serum. The testes tissue was incubated with anti-γ-H2AX (Gamma H2A Histone Family X) (25) and anti-SYCP3 (Synaptonemal Complex Protein 3) (26) (**Supplementary Table 1**). For rat testicular cells, the slides were incubated with anti-GATA4 (GATA Binding Protein 4) (27), anti-VASA (DEAD-Box Helicase 4) (28), anti-α-SMA(alpha-Smooth Muscle Actin) (29), anti-3β-HSD (3 Beta-Hydroxysteroid Dehydrogenase) (28) (**Supplementary Table 1**). In addition to the antibodies mentioned above, rat organoids were also incubated with anti-Collagen IV (30), anti-Laminin (31), anti-Fibronectin (32), anti-Claudin 11 (33), anti-UCHL1 (Ubiquitin *C*-terminal Hydrolase L1), anti-TNP1 (Transition Protein 1) (34), anti-PRM1 (Protamine 1) (35), anti-ACR (Acrosin) (36) and anti-AR (Androgen Receptor) (37) antibodies overnight at 4°C (**Supplementary Table 1**). Fluorescence labelling was done with

secondary antibodies conjugated with Alexa Fluor 488 and 555 (**Supplementary Table 1**). DAPI (4′,6-diamidino-2-phenylindole) (Vector, cat# H1200) was used for labelling the nuclei. The cells were analyzed using Zeiss Imager.M2 fluorescence microscopy and the percentages of testicular cell types were determined by counting the cells with ImageJ software. The organoids were analyzed using a Leica TCS-SP8 confocal laser scanning microscope with the Leica Las X software.

Cell Number Quantification Within Organoids

Immunohistochemistry for VASA was performed on day 7 and 28 organoids, while SYCP3 staining was performed on day 28 organoids. Using confocal microscopy, organoids were selected blindly based on only DAPI and scanned across the z-axis to quantify the number of $VASA^{+ve}$ and $SYCP3^{+ve}$ cells in each organoid. From each of the three independent experiments (n = 3) performed, a total of 30 organoids were analyzed for $VASA^{+ve}$ and 10 organoids were analyzed for $SYCP3^{+ve}$ cell counts.

Reverse Transcription Quantitative Polymerase Chain Reaction

RNA was isolated from 1200 organoids using RNeasy Micro Kit (QIAGEN, cat # 74004) and then reverse transcription was performed using SuperScript™ IV VILO™ Master Mix (Thermo Fisher Scientific, cat# 11756050). RT-qPCR with the primers listed in **Supplementary Table 2** was performed with a 7.500 Fast Real-Time PCR System (Applied Biosystems) using SsoFast Eva Green Supermix with Low ROX (Bio-Rad laboratories, cat# 1725211). The expression levels were presented relative to *Gapdh*. Statistical analysis was performed on the mean of ΔΔCt.

MEHP and $CdCl_2$ Treatment

Day 19 organoids were treated with 1 μM MEHP and 0.25 μM $CdCl_2$. Controls were treated with equivalent volumes of DMSO. After 48 hours of treatment, at day 21, the organoids were harvested and analyzed with immunofluorescence and RT-qPCR.

Dose Determination

Day 5 organoids were treated with 0.5, 1 and 1.5 μM of MEHP (Sigma, Cat# 796832) and 0.01, 0.05, 0.25 and 1.25 μM of $CdCl_2$ (Sigma, Cat# 202908). The control groups for MEHP and $CdCl_2$ were treated with equivalent volumes of DMSO for 1.5 μM of MEHP and 1.25 μM of $CdCl_2$, respectively. Controls of 1.5 μM and 1.25 μM DMSO were treated with equivalent volumes of phosphate buffered saline (Thermo Fisher Scientific, cat# 14190144). At day 7 (48 hours after treatment), the organoids were harvested and approximately 60 organoids suspended in 50 μL ODM were seeded in each well of a 96-well plate as duplicates to perform the MTT assay (Abcam, cat# ab211091) according to manufacturer's instructions. Absorbance at OD 590 nm was measured using SpectraMax i3x plate reader (Molecular Devices). MTT assay was used to measure cellular metabolic activity as an indicator of cell viability.

Statistical Analysis

All the results described here are from at least three independent experiments performed with three separately prepared rat starting cell population (n = 3). Data were analyzed using the GraphPad Prism 8 software. Unpaired two-tailed *t*-tests were performed for single comparisons between two groups. For more than two groups, one-way ANOVA with Tukey's multiple comparison tests were performed. A value of $p < 0.05$ was set as the limit of statistical significance.

RESULTS

Rat Testicular Cells Generate and Maintain Organotypic Testicular Organoids at 15% O_2 Tension

A rat (P4-P5) testicular starting cell population which contained 92.2 ± 1.28% $GATA4^{+ve}$ Sertoli cells (38), 1.7 ± 0.3% $VASA^{+ve}$ germ cells (a marker for spermatogonia, spermatocytes and round spermatids) (39), 2.6 ± 0.7% 3β-HSD^{+ve} Leydig cells (40) and 19.97 ± 2.8% α-SMA^{+ve} peritubular myoid cells (41) (n = 3) was used to generate rat testicular organoids with organoid formation media (OFM). Since lower O_2 tension is known to support higher differentiation potential of rat testicular cells (22), initial cultures were carried out in incubators set up for ambient and 15% O_2 tensions (37°C). Both culture conditions supported the initial generation of testicular organoids (72 hours), with organotypic morphology similar to our previously published porcine and murine model (11). However, unlike the porcine or murine models (11), the rat organoids cultured in ambient O_2 tension underwent a loss of testis-specific tissue architecture at day 6 of culture while day 6 organoids cultured at 15% O_2 tension had distinct internal-interstitial and external-seminiferous epithelial compartments. The two compartments were separated by a collagen IV^{+ve}, $fibronectin^{+ve}$ and $laminin^{+ve}$ basement membrane (**Figure 1A**). The external compartment was composed of $VASA^{+ve}$ germ cells and $GATA4^{+ve}$ Sertoli cells. α-SMA^{+ve} peritubular myoid cells were located lining the basement membrane in the interior compartment, while 3β-HSD^{+ve} Leydig cells were distributed throughout the interior compartment (**Figure 1A**). In contrast, the organoids cultured at ambient O_2 tension showed increased Sertoli cell numbers, generation of large Sertoli cell clusters and complete or partial separation of internal and external compartments (**Figure 1B**). Thus, subsequent experiments were carried out at 15% O_2 tension.

Under Optimized Conditions, Rat Testicular Organoids Undergo Maturation and Support Spermatogonial Differentiation

Matsumura et al. (22) and Sato et al. (23) reported efficient spermatogenic differentiation of rodent testicular organ cultures at 34°C. To evaluate the effect of temperature on somatic cell and spermatogonial maturation, rat organoids were generated with OFM (72 hours: day 0 undifferentiated organoid) and then

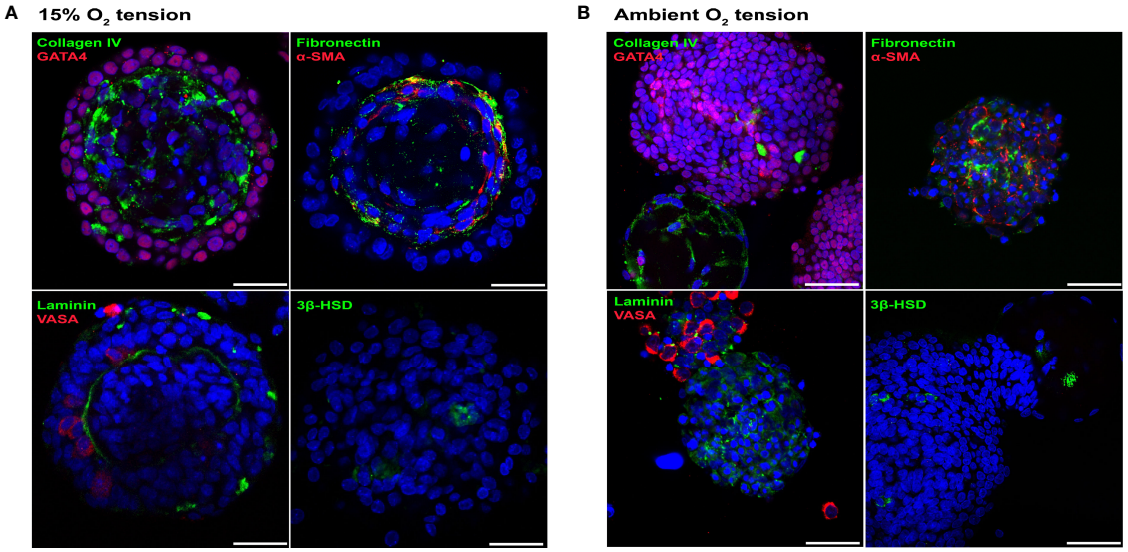

FIGURE 1 | Rat testicular organoids cultured at 15% O_2 tension maintained organotypic morphology. **(A, B)** Immunofluorescence images of day 6 rat testicular organoids at 15% **(A)** and ambient **(B)** O_2 tensions showing the distribution of basement membrane (collagen IV, fibronectin, laminin), Sertoli cells (GATA4), peritubular myoid cells (α-SMA), germ cells (VASA) and Leydig cells (3β-HSD). Scale bars measure 25 μm.

cultured with organoid differentiation medium (ODM) at 37°C and 34°C (22, 42) for up to 28 days. Immunofluorescence analysis revealed no morphological differences between the two groups at day 28 (**Figure 2A**) and both cultures showed expression of Claudin 11, a component of Sertoli cell tight junctions (**Figure 2B**) (43). Except for expression of *Shbg* (sex hormone binding globulin) (Sertoli and Leydig cells), which was increased 5.7-fold ($n = 3$, $p < 0.05$) in 34°C cultures, no significant differences in transcription levels were observed between the two groups for the somatic cell markers *Fshr* (follicle stimulating hormone receptor) (Sertoli cells), *Star* (steroidogenic acute regulatory protein), *Cyp17a1* (cytochrome P450 family 17 subfamily a member 1) (Leydig cells) and *Hsd17b3* (hydroxysteroid 17-beta dehydrogenase 3) (Sertoli and Leydig cells) (**Supplementary Figure 1A**) ($n = 3$, $p > 0.05$) (44–47). At day 7, rat testicular organoids contained $UCHL1^{+ve}$ undifferentiated spermatogonia at both temperatures (**Supplementary Figure 1B**). Both at day 7 and 28, there was no difference in the number of $VASA^{+ve}$ germ cells between 37°C and 34°C cultures ($n = 3$, $p > 0.05$) (**Figures 2C, D**). However, the number of $VASA^{+ve}$ germ cells was lower at day 28 compared to day 7 at both temperature conditions ($n = 3$, $p < 0.05$) (**Figure 2D**). $SYCP3^{+ve}$ spermatogenic cells were observed in both conditions, with a staining pattern similar to spermatocytes in 43-day old rat testes (**Supplementary Figure 1E**), starting from day 21. γ-H2AX, which is induced by the DNA double stranded break in leptotene and early zygotene, was also observed in both culture conditions with a staining pattern similar to 43-day old rat testes (**Supplementary Figures 1D, E**) (25). The number of $SYCP3^{+ve}$ was quantified and their number was found to be higher at day 28 in the organoids cultured at 34°C compared to 37°C ($n = 3$, $p < 0.05$) (**Figures 2E, F**) (48, 49). Therefore, the 37°C cultures were excluded from further analysis.

To characterize maturation during the 28-day long culture, transcription levels of the immature Sertoli cell marker *Amh* (anti-mullerian hormone) and Sertoli and Leydig cell markers *Fshr*, *Shbg*, *Star*, *Hsd17b3* and *Kitlg* were analyzed for day 0, 7, 14, 21, 28 organoids. The transcription levels of *Amh* were undetectable within a week ($n = 3$, $p < 0.05$) while expression of *Cyp17a1* showed no significant changes over the duration of the culture ($n = 3$, $p > 0.05$). *Fshr*, *Shbg* and *Kitlg* were upregulated by 2.7-, 17.9- and 3.3-fold at day 21 ($n = 3$, $p < 0.05$) (**Figure 2G**). Transcription levels of *Star* and *Hsd17b3* increased 26.9- and 10-fold, respectively, by day 28 of culture ($n = 3$, $p < 0.05$) (**Figure 2G**). In addition, an increasing number of Sertoli cells in the organoids started to express AR with subsequent weeks of culture, indicating cell maturation (**Figure 2H**). Along with punctate SYCP3 staining (potentially leptotene spermatocytes) (**Figure 2E**), an elongated pattern of SYCP3 staining (as observed in early zygotene spermatocytes) (50) (**Supplementary Figure 1C**) was also detected at day 28. However, cells found with elongated SYCP3 expression were no longer adhered to the organoids at the time of analysis (**Supplementary Figure 1C**). In contrast, no $PRM1^{+ve}$, $TNP1^{+ve}$ or ACR^{+ve} cells were observed in the cultures.

Rat Testicular Organoids to Model Reproductive Toxicity: Proof of Principle

Initial dose-response experiments on monolayers of rat testicular cells (P4-P5) were used to select the dosages of 0.5, 1 and 1.5 μM for phthalic acid mono-2-ethylhexyl ester (MEHP); and 0.01, 0.05, 0.25 and 1.25 μM for the heavy metal cadmium chloride ($CdCl_2$) to be tested on organoids. Then, relative cell viability assessments were performed on day 7 (treatment began on day 5) organoids treated with the aforementioned dosages with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay, revealing

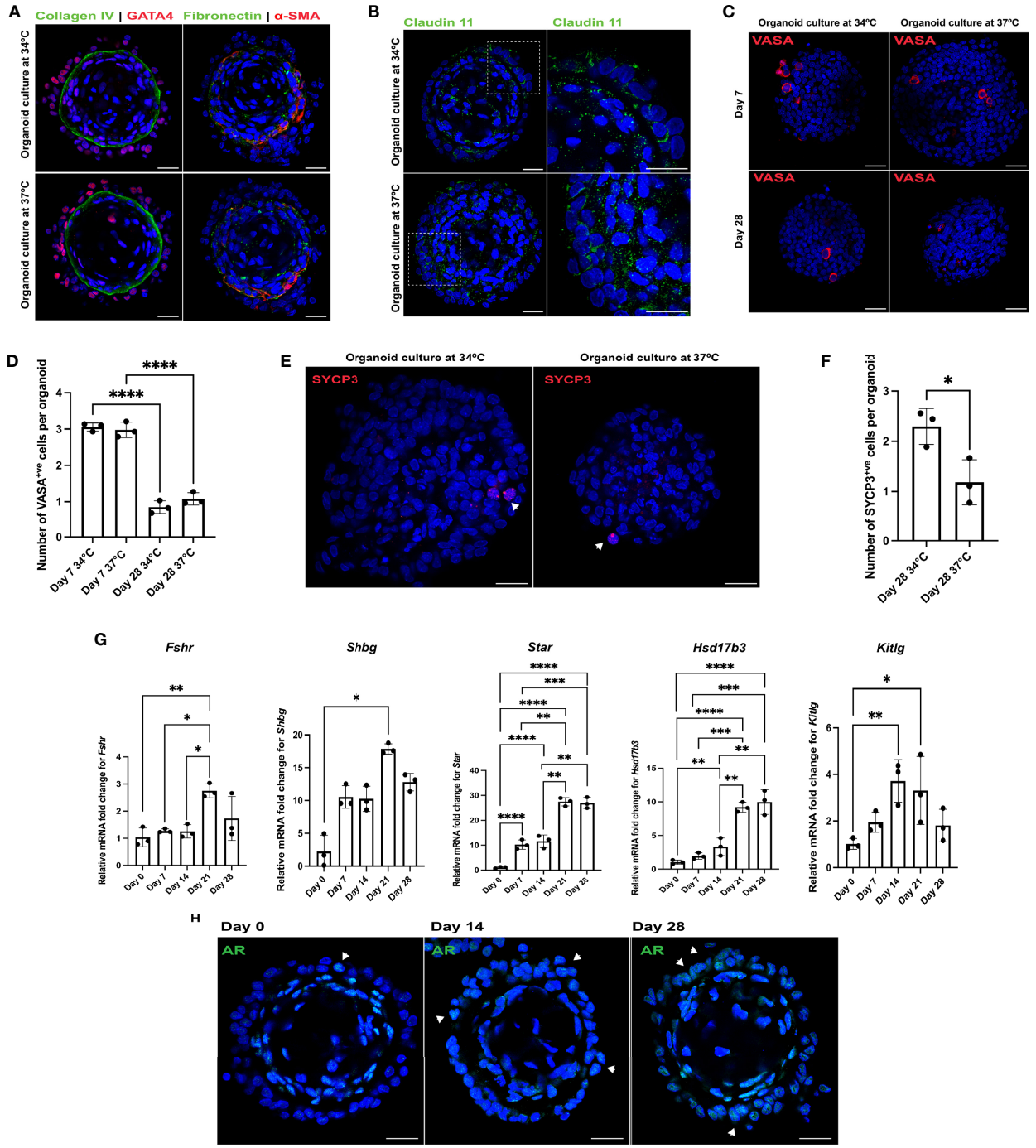

FIGURE 2 | Rat testicular organoids support somatic cell maturation and spermatogonial differentiation at 34°C culture. **(A–C, E)** Immunofluorescent images of day 28 rat testicular organoids cultured at 34°C and 37°C showing **(A)** basement membrane (collagen IV, fibronectin), Sertoli cells (GATA4), peritubular myoid cells (α-SMA), **(B)** tight junction protein (Claudin 11) (inserts showing the magnified area on the right panel), **(C)** germ cells (VASA) and **(E)** meiotic cells (SYCP3) (indicated with white arrows). Scale bars measure 25 μm. **(D)** Number of germ cells adhered to each organoid. Bars indicate mean ± SD, n = 3. Analysis was performed using one-way ANOVA followed by Tukey's multiple comparison test. **(F)** Number of meiotic cells adhered to each organoid. Bars indicate mean ± SD, n = 3. Analysis was performed using unpaired two-tailed *t*-test. **(G)** Relative mRNA fold change of *Fshr*, *Shbg*, *Star*, *Hsd17b3* and *Kitlg* for organoids cultured at 34°C. Bars indicate mean ± SD, n = 3. Analysis for *Fshr*, *Star*, *Hsd17b3* and *Kitlg* was performed using one-way ANOVA followed by Tukey's multiple comparison test and analysis for *Shbg* was performed using Kruskal-Wallis test with Dunn's multiple comparison test. **(H)** Immunofluorescent detection of AR (indicated with white arrows) in the 34°C culture at day 0, day 14 and day 28. Scale bars measure 25 μm. $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***), $p \leq 0.0001$ (****). Only significant differences are indicated with asterisks.

that 1 µM MEHP and 0.25 µM $CdCl_2$ were the highest doses without adverse effects on viability (n = 3, p < 0.05) (**Figures 3A, B**). Thus, all other dosages were excluded from further analyses. Since most of the maturation markers showed robust upregulation at day 21, toxicological effects of MEHP and $CdCl_2$ were evaluated at day 21 by treating the organoids with MEHP and $CdCl_2$ at day 19 and then harvesting and analyzing 48 hours later. MEHP treatment was associated with upregulation of *Fshr* and *Star* and downregulation of the expression of *Cyp17a1* (n = 3, p < 0.05) (**Figure 3D**). Expression of *Fshr*, *Shbg*, *Hsd17b3* and *Cyp17a1* was drastically downregulated upon exposure to $CdCl_2$ (n = 3, p < 0.05) (**Figure 3D**). Transcription of *Star*, in contrast, was upregulated 7-fold compared to DMSO controls (n = 3, p < 0.05) (**Figure 3D**). In contrast to MEHP which led to partial loss of tight-junction protein Claudin11, $CdCl_2$ treatment caused a total loss of Claudin 11 (**Figure 3C**).

DISCUSSION

In the last few years, we and others have reported the generation of testicular organoids from dissociated primary testicular cells (11, 14–18). In the current study, we adapted our previously established approach to generate porcine, murine, human and primate testicular organoids (11) to the formation of organotypic rat testicular organoids. Rats represent an important animal model for studying spermatogenesis and have been the main model for the study of reproductive toxicology (19–22). It was therefore necessary to establish the optimal conditions required for the generation and maturation of microwell-derived rat testicular organoids to support spermatogonial differentiation.

Rat testicular organoids presented some unique challenges that were not observed in our previously reported organoids (11). While porcine and murine organoids can be maintained for up to 45 days at ambient O_2 tension, which translates to 18.4% or 6.34 mM O_2 at 1084 m elevation in Calgary, Alberta, Canada (51, 52), rat organoids collapsed at day 6 by purging the interstitial compartment. This is likely due to the perturbation of the ratio of Sertoli cells to interstitial cells, caused by increased Sertoli cell proliferation. Sertoli cell proliferation levels are higher at high O_2 tension compared to more hypoxic conditions (15%, 10%, 5% O_2) (53). Unlike porcine and murine testicular organoids, where organoid formation leads to a contact inhibition effect on the cells, the Sertoli cells of rat testicular organoids seem to retain their ability to proliferate, which is exacerbated at ambient O_2 tension. This increased number of

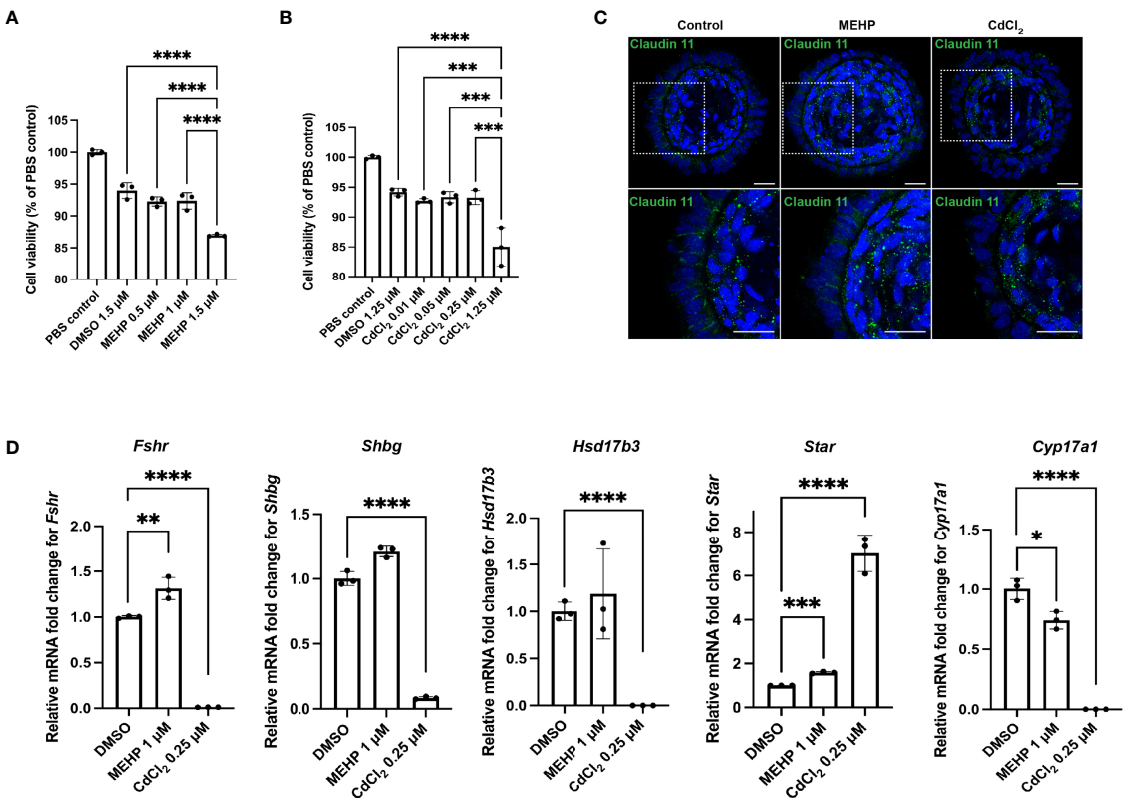

FIGURE 3 | Rat testicular organoids allow for modeling reproductive toxicity. **(A, B)** Relative cell viability of organoids after MEHP and $CdCl_2$ treatments. **(C)** Immunofluorescent images of day 21 rat testicular organoids treated with MEHP and $CdCl_2$ showing tight junction protein (Claudin 11) (inserts on the top panels are showing the magnified area on the bottom panel). Scale bars measure 25 µm. **(D)** Relative mRNA fold change of *Fshr*, *Shbg*, *Star*, *Hsd17b3* and *Cyp17a1* in day 21 organoids after MEHP and $CdCl_2$ treatment. Bars indicate mean ± SD, n = 3. Analysis was performed using one-way ANOVA followed by Tukey's multiple comparison test. $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***), $p \leq 0.0001$ (****). Only significant differences are indicated with asterisks.

Sertoli cells likely leads to a loss of their affinity for the basement membrane of the organoids. As a result, the Sertoli cells migrate to form separate aggregates which leads to complete or partial expulsion of the interstitial compartment.

Culture at 37°C can impair the glucose transport of spermatids and render spermatids and spermatozoa fragile (42, 54). It can also have deleterious effects on testis tissue *in vitro* (55). We observed higher expression of *Shbg*, increased numbers of early meiotic cells and the presence of cells with elongated SYCP3 staining pattern in 34°C culture conditions, indicating a positive effect on Sertoli cell maturation and spermatogonial differentiation, which is consistent with previous work (22, 45, 50, 54). As expected, number of germ cells were similar at both temperatures. This is consistent with previous reports which have found that the proliferation and survival of spermatogonia do not seem to be affected by temperature (54). However, the number of germ cells decreased over the duration of culture and zygotene spermatocytes, identified by the typical elongated staining pattern of SYCP3, were no longer adhered to the organoids. This gradual loss or dislodgement of loosely adhered germ cells is likely due to extensive media changes throughout the 28-day long culture. Such loss of a critical cell type may be mitigated by adapting the microwell system to support a continuous perfusion system (56). This would allow a slow and constant perfusion of media and reduce extensive handling for long-term cultures.

After establishing the optimal conditions for promoting maturation and early spermatogonial differentiation, we performed a proof of principle experiment to evaluate the utility of rat organoids for toxicological evaluation of drugs and environmental toxicants. Exposure to MEHP, a fairly common plasticizer (57), led to increased expression of *Fshr* and *Star* and decreased expression of *Cyp17a1*. This is consistent with previous reports, which have shown that phthalates can modulate basal steroidogenic machinery in both Sertoli and granulosa cells (58–60). Cadmium, a heavy metal that is often used as stabilizer in production of polymers and dyes, can cause endocrine disruption in the testis (61). We observed cadmium mediated disruption of Sertoli and Leydig cell function by downregulation of *Fshr*, *Shbg*, *Hsd17b3* and *Cyp17a1*, and upregulation of *Star*, which has been reported previously (61, 62). Both MEHP and $CdCl_2$ are known to disrupt the blood testes barrier by downregulation of tight-junction proteins such as Claudin 11 (63, 64). We witnessed a similar effect upon exposure of organoids to MEHP and $CdCl_2$. Human exposure to $CdCl_2$ and MEHP can depend on a number of factors such as cumulative effects, metabolism by the liver, accumulation due to continuous environmental and occupational exposure (65, 66). While all of these considerations are beyond the scope of this current study, the dosages used here show definite disruptive effects on the steroidogenic machinery. This proof of principle experiment shows that the rat testicular organoids can serve as viable platforms for modeling male reproductive toxicity.

In conclusion, we report a rat testicular organoid system that reflect testis specific morphology and can support early testicular maturation. In addition, this system supports germ cell development to early meiosis up to the zygotene stage. Further optimization of the differentiation conditions may be warranted to support full *in vitro* spermatogenesis.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Materials**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by University of Calgary Animal Care Committee.

AUTHOR CONTRIBUTIONS

SS and ID conceived and designed the study. SS, NLML, and BH performed the data analysis, data interpretation and statistical analysis. SS prepared the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by NIH/NICHD HD091068-01 to ID; a graduate student scholarship to SS from the Alberta Children's Hospital Research Institute (ACHRI); and a Canadian Institutes of Health Research (CIHR) fellowship (MFE-176542) and an ACHRI postdoctoral fellowship to NLML.

ACKNOWLEDGMENTS

We thank Dr. Mark Ungrin and his laboratory for their guidance and support.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.892342/full#supplementary-material

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REVIEW
published: 09 June 2022
doi: 10.3389/fendo.2022.897101

Advanced Paternal Age and Future Generations

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Paternal age at conception has been increasing. In this review, we first present the results from the major mammalian animal models used to establish that increasing paternal age does affect progeny outcome. These models provide several major advantages including the possibility to assess multi- transgenerational effects of paternal age on progeny in a relatively short time window. We then present the clinical observations relating advanced paternal age to fertility and effects on offspring with respect to perinatal health, cancer risk, genetic diseases, and neurodevelopmental effects. An overview of the potential mechanism operating in altering germ cells in advanced age is presented. This is followed by an analysis of the current state of management of reproductive risks associated with advanced paternal age. The numerous challenges associated with developing effective, practical strategies to mitigate the impact of advanced paternal age are outlined along with an approach on how to move forward with this important clinical quandary.

Keywords: spermatozoa, oxidative stress, animal models, artificial reproduction technologies, progeny outcome

OPEN ACCESS

Edited by:
Barry Zirkin,
Johns Hopkins University,
United States

Reviewed by:
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Specialty section:
This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology

Received: *15 March 2022*
Accepted: *13 April 2022*
Published: *09 June 2022*

Citation:
Chan PTK and Robaire B (2022) Advanced Paternal Age and Future Generations. Front. Endocrinol. 13:897101. doi: 10.3389/fendo.2022.897101

INTRODUCTION

We are witnessing the progressive increase of paternal age at conception. The birth rate among 35 to 49 year old American men in 2015 was 69.1 per thousand compared with 42.8 per thousand in 1980 (1). Other countries have reported a similar trend (2) that appears to be consistent across all races, ethnicities, regions and level of education (3). While controversies exist, a preponderance of evidence from recent scientific literature affirms a negative impact of advanced paternal aging on reproductive health. In this review we will begin by discussing the role of animal models as a valuable research tools to study the effects of paternal aging. After presenting how advanced paternal age impacts the fertility status of men, reproductive outcomes and offspring health, we will provide an opinionated analysis on the challenges faced by healthcare providers and health authorities in the development and implementation of practical strategies designed to reduce or mitigate the negative impact of advanced paternal age from a public health point of view.

ANIMAL MODELS FOR STUDYING PATERNAL AGING

A wide range of animal models ranging from insects to worms, birds, fish and mammals have been used to investigate the effects of paternal aging on male reproduction function. However, rodent models have become the predominant species for determining the cellular and molecular changes with aging that occur in the testis and epididymis (4–6). Outbred rodents are often used in drug testing or environmental exposure studies so to increase the genetic variability in the population. However, inbred rodents are preferred for aging studies that focus specifically on the mechanistic pathways in question. A potential limitation is that several pathologies associated with aging including pituitary, adrenal or testis tumors may complicate result interpretation. An ideal animal model should be long-lived and free from the systemic aging-related diseases, while maintaining other reproductive changes that emulate those in aging men.

Mouse Models

Studies using mouse models that lack any known or induced mutations have demonstrated a quantitative reduction in spermatozoa with increased age. Testicular architecture reveals changes in tubule segments with impaired spermatogenesis, increased number of vacuoles in Sertoli and germ cells, a thinning of the seminiferous epithelia, and a reduction in the number of spermatocytes and spermatids (7, 8). An increase in age-related germ cell mutations has also been reported (9).

Several inbred strains of mice, such as the senescence-accelerated mouse (10, 11), and transgenic mice, such as Klotho mice (12, 13), have been developed to model accelerated aging in humans. These mice exhibit defects in a wide range of organs (e.g., vessels, lungs, kidney, brain, skin and testes), and thus are poor models to study aging of male germ cells as many interfering systems could be operant. However, an advantage of the mouse model is the feasibility for genetic manipulations for both over-expressed and knocked out genes, and consequently allows for studies investigating mechanisms involved in aging. Mice overexpressing catalase have reduced ROS and do not exhibit the age-dependent loss of spermatozoa, do show aging-associated loss in testicular germ and Sertoli cells, and show reduced 8-oxodG lesions in spermatozoa (14). In contrast, null mutations for superoxide dismutase show exacerbated age-induced damage in both the testis histology and spermatozoa quality (15).

Rat Models

With its long lifespan and relatively free of age-related pathologies including tumors and obesity, the Brown Norway (BN) rat is a highly robust model for the study of male reproductive aging (16–19). Striking age-related changes in the seminiferous tubules (16), Leydig cells (5) and epididymides (20) of these animals have been reported. Several genes in the testis (Leydig and germ cells) and in the epididymis have altered expression as a function of aging (21–23). With advancing age, Sertoli cells, the niche-forming "nurse" cells that surround the germ cells and ensure their normal development, display anomalies in the structure of the endoplasmic reticulum and nuclei; large intracellular spaces are observed between Sertoli cells, rather than the normally embedded germ cells (24). Genes and proteins associated with the formation of the blood-testis barrier decline prior to the barrier becoming "leaky" (25). Effects of aging are also seen in the hypothalamic-pituitary function (17, 26). Importantly, the changes seen in testis and hypothalamic-pituitary functions in the BN rat with age reflect those reported in aging men (27, 28).

Mating of male BN rats of increasing age (3–24 months) to young females result in an increase in pre-implantation loss, a decrease in the average fetal weight, and an increase in neonatal deaths (29). Together, these results show that the quality of spermatozoa decreases as BN male rats age. The basis for these age-related declines in reproductive function remains unclear. In isolated populations of testicular germ cells, the expression of a number of genes is affected during ageing (21, 30). The findings of a large increase in sperm with abnormal flagellar midpieces, decrease in the percentage of motile spermatozoa and elevation of immature spermatozoa retaining their cytoplasmic droplets in the cauda epididymides of old rats suggests a defective spermatozoa formation in aging testes (31) and impaired epididymal function in supporting sperm maturation. We reported previously aging related increase in basal sperm chromatin damage with age (32) which suggest an accumulation of DNA damage and/or mutations in the germ line that may contribute to adverse health outcomes of their offspring.

Advantages and Limitations of Animal Models Over Human Studies

Animal models have clear advantages for control over the homogeneity of the genetic pool, for conducting controlled mating studies and for access to all cells of the reproductive system for analyses. Indeed, studies using animal models have unequivocally established that increased paternal age is associated with decreased sperm number and chromatin quality, and adverse progeny outcome. For therapeutic and interventional studies, animal models allow for control of confounders seen often in human studies such as obesity, diet, exposure to toxins and the age of female mates. Finally, it is possible to assess multi- transgenerational effects of paternal age on progeny in a relatively short time window.

Aging studies with animal are not without limitations. The relatively shorter lifespan of rodents limits the wide range of environmental exposures to chemicals that can impinge on sperm function and production. Further, quantitatively and qualitatively, men are far less effective at producing sperm per gram of testis (33), possibly due to postural position and bypass of temperature regulation for optimal spermatogenesis. Finally, although the number of genes in man and rodents are similar, the human genome contains far more non-genomic DNA that likely plays a role in epigenetic regulation of germ cell functions (34). Thus, a comprehensive understanding of how paternal age affects both the genome and epigenome of spermatozoa, and the

consequences of these effects will require complementary animal and human studies.

IMPACT OF ADVANCED PATERNAL AGE IN MEN AND ON THEIR PROGENY

Impact of Advanced Paternal Age on Male Fertility Status

Various studies have indicated an age-related decline of conventional semen parameters including semen volume, total sperm count, motility and morphology (35). Not surprisingly, natural fertility rates decline as men age, as demonstrated by a survey that conception at 1yr is 30% less for men >40yrs versus those <30yrs (36). Similar findings were reported by Hassan and Killick (37). Natural conceptions with men >35yrs were found to be 1.26 times more likely to miscarry than those with men <35yrs (38). In a retrospective cohort study from 1989–2005, pregnancies sired by father >45yrs showed a 48% increased risk of late stillbirth, a 19% increased risk of low birth weight, a 13% increased risk of preterm birth and a 29% increased risk of very preterm birth (39).

Impact of Advanced Paternal Age on Assisted Reproductive Outcomes

Advanced paternal age has been associated with various adverse outcomes with assisted reproductive technologies (ARTs) including poor embryo quality, increased miscarriage rates, reduced fertilization, implantation, pregnancy, and live birth rates (40–48). Inconsistency and conflicting data exist (49–51) likely due to the results of confounders and bias in the design of the studies, small sample size, retrospective nature and heterogeneity of the subjects. One proposed mechanism of the adverse reproductive outcomes in natural and assisted reproduction is impaired sperm chromatin integrity and increased DNA fragmentation rates (52). In a recent systematic review, 17 out of 19 studies demonstrated an association of advanced paternal age with significant increase in DNA fragmentation (53), mostly measured by Sperm Chromatin Structure Assay ® and sperm chromatin dispersion test. The two studies that did not find the effect of advanced paternal age on sperm DNA fragmentation utilized terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labelling (TUNEL) assay. Each sperm chromatin integrity and DNA fragmentation examines different structural aspects of the target molecule with intrinsic advantages and limitations; thus, it is clearly important to use a complementary panel of assays to fully assess sperm quality at the molecular level.

Impact of Advanced Paternal Age on Offspring Perinatal Health

In a population based cohort study, advanced paternal age was found to increase risk of premature birth, gestational diabetes and newborn seizures (54). The odd ratios of birth defects including cleft lip, diaphragmatic hernia, right ventricular outflow tract obstruction, pulmonary stenosis was found to increase significantly, after adjustment for multiple confounders, with each year of increase in paternal age (55).

Impact of Advanced Paternal Age on Risks of Malignancy in Offspring

Results from a prospective cohort study of over 180,000 subjects indicate that men >35yrs had a 63% higher risk of having offspring who develop hematologic cancers compared with those whose fathers were <25yr, with a significant linear dose-response association noted (56). In a nationwide cohort study of close to two million children born in Denmark from 1978–2010, the risk of childhood acute lymphoblastic leukemia increases by 13% for every 5 years increase in paternal age (57). Other offspring malignancies associated with advanced paternal age include central nervous system tumors and breast cancer (58–61). One proposed mechanism for increased cancer risk with advanced paternal age is telomeres lengthening (62, 63). Telomere shortening is associated with various diseases and is thought to be a limitation of longevity. Leukocytes telomeres are lengthened in offspring of older fathers by 0.5 -2 times per year of paternal age (62–64). While this may confer some health and longevity advantage, a higher risk for malignancy has been noted (63, 64).

Impact of Advanced Paternal Age on Risks of Offspring Mental Health

Advanced paternal age is also linked to psychological and neurodevelopmental disorders in offspring (65). The relative risk (RR) of offspring diagnosed with schizophrenia increase progressively with paternal age from 34 years (RR 2.02, 95% CI, 1.17-3.51 for the 45-49 age group; RR 2.96, 95% CI, 1.6-5.47 for the older than 50 group) (66). Other investigators have also reported an increased risk of offspring schizophrenia with advanced paternal age (67–69) unaccounted by other factors such as family history of psychosis, maternal age, parental education and social ability, family social integration, social class, birth order, birth weight or birth complications (70). Additionally, the risk of obsessive-compulsive disorder in offspring was reported to increase with advanced paternal age (71). After adjusting for maternal and family history, the risk of offspring of men >54yrs diagnosed with bipolar disorder was found to be 1.37 times higher than those of men 20–24yrs old (72). Using paternal sibling comparisons, another cohort study reported a 24-fold increase of bipolar disorder in offspring born to fathers 20–24yrs versus those aged 45yrs or older (73). In a population-based cohort study of over 130,000 births, offspring from men aged >40yrs were more than fivefold more likely to develop autism spectrum disorders compared to offspring of men <30yrs (74), consistent with a registry study using paternal sibling comparisons (73).

Impact of Advanced Paternal Age on Risks of Genetic Disorders in Offspring

Several genetic diseases that occur with a low frequency in the general population are associated with advanced paternal age. These include Apert, Crouzon and Pfeiffer syndromes, achondroplasia and other conditions (75). Many of these

disorders follow an autosomal dominant pattern, consistent with the opinion that these are mainly *de novo* mutations in the germline. Although the incidences of these conditions in advanced paternal age are generally lower than 1% (76, 77), they are nonetheless associated with severely debilitating phenotypes. Hence, prospective parents with advanced paternal age concerns should be informed and counselled for such risks.

Approximately 0.33% of infants are born with an altered number of chromosomes. Aneuploidies derive mainly from non-disjunction events during meiotic divisions, represent the most common heritable chromosomal anomaly (78). Though most constitutional aneuploidies originate in the female germline (79), all men produce approximately 3–5% of aneuploidy sperm (80) and non-disjunction events, particularly in sex chromosomes, are more likely to occur with aging (81). Most *de novo* structural chromosomal abnormalities are found to be of paternal origin (82–87). Several studies have shown a significant age related increase in sperm structural chromosomal abnormalities (88–93). Results from studies on the association of advanced paternal age and increased risks of offspring aneuploidies and structural chromosome anomalies are inconsistent (82, 94–101). This is in part related to the fact that the vast majority of chromosome aneuploidies are not compatible with fetal development, leading to implantation failure or early miscarriage. Structural chromosomal rearrangements that are balanced are usually phenotypically normal and are thus undetected during childhood, while the vast majority of those that are unbalanced are not compatible with fetal development.

PROPOSED MECHANISMS ON ADVANCED PATERNAL AGE IMPACT

Studies in animal models suggest that the constitution of the male germline is relatively robust with far fewer spontaneous mutations compared to somatic tissues (102, 103). This high level of genetic fidelity in part explains why even after exposure to chemotoxic agents or radiation in men, no increase in the incidence of birth defects, sperm DNA chromatin abnormalities or *de novo* germline mutations are noted in their offspring (104, 105). In contrast, paternal aging has been shown to be unique for the creation of *de novo* mutations in male germline (106). Several mechanisms of age-induced *de novo* germline mutations have been proposed. Cumulative replication error from repeated cell divisions represents a significant source of germline mutation (107, 108). Based on whole-genome sequencing studies of parent-offspring trios, approximately one to three *de novo* mutations are introduced to the germline mutational load of the offspring for each additional year in the father's age at conception (109, 110). Selfish spermatogonial selection from preferentially amplified mitotic clonal expansion of mutated spermatogonial stem cells (111–113) is another proposed mechanism to explain why several genetic diseases associated with advanced paternal age follow the autosomal dominant pattern. Age-related epigenomic modifications in men, as reported by our group (114) and others (115) are speculated to increase the risk of some rare epigenetic disorders in offspring conceived with ARTs (116). Other proposed mechanisms involve post meiotic damage of sperm DNA secondary to the combined effects of increased oxidative stress (117) and nuclease activities and aberrant or inadequate repair of such damage by oocytes (118).

CURRENT STATE OF MANAGEMENT OF REPRODUCTIVE RISKS ASSOCIATED WITH ADVANCED PATERNAL AGE

Few professional organizations have provided a clear definition of advanced paternal age. The American College of Medical Genetics has defined advanced paternal age as >40yrs at conception (76) for the purpose of risk counselling. While the American Society of Reproductive Medicine states that the sperm donor should be "young enough" (119), the Canadian Fertility and Andrology Society have set an upper age limit for sperm donation at 40yrs (120). However, no organizations have made any clear statements as to whether access to reproductive technologies after this age should be restricted.

The lack of clear, authoritative clinical guidelines not only poses challenges to health providers to decline services, but it also inadvertently allows patients to downplay or ignore the negative impact of paternal aging. Additional factors further aggravate the situation: increased access to contraceptives (121), delayed marriage, high divorce and remarriage rates, increased life-expectancy (122), increased access to erectile dysfunction treatment (123) leading to extension of active sex-life expectancy, continuous spermatogenic activities with aging, social acceptance in delaying fatherhood as modeled by a number of male celebrities having children at advanced age, and widespread usage of social media and dating apps to increase the odds of courtship (124). These factors have provided elements for a perfect storm resulting in a rising number of aging men entering or re-entering fatherhood.

CHALLENGES IN DEVELOPING EFFECTIVE, PRACTICAL STRATEGIES TO MITIGATE THE IMPACT OF ADVANCED PATERNAL AGE

Though experts recognize the importance of disseminating current knowledge on the negative impacts of advanced paternal age to clinicians and prospective parents, in practice, this task is far from simple to execute. For example, when counselling a couple with an aging male partner seeking fertility care, merely informing the couple of the potential adverse outcomes serves little more than risk disclosure. Obviously, the couple could do nothing to change the age factor. Alternative options such as using donor gametes or adoption are unlikely to be accepted when the male partner still has functional sperm. From their perspectives, risk is

not a certainty. Infertile couples who are determined enough to pursue fertility treatment may feel entirely rational to accept such risks (125). Additionally, there is ample evidence suggesting that children born to parents of more advanced age may enjoy further benefits in life chances such as financial security, parental psychological maturity and a wider network of support for upbringing, education and future career development (125, 126). Taken together, the impact of counselling solely for risk disclosure may not be effective in modifying behavior or improving treatment outcomes.

To add yet another layer of complexity, denying this couple further fertility evaluation is not correct since there could be significant medical conditions including varicoceles, obstruction of the excurrent ductal system, genetic and endocrinological disorders that can contribute to impaired semen parameters. Some causes of male infertility may be correctable to improve the fertility status of the male partner and allow for a better chance of conception. Further, detection of impaired semen parameters may lead to early detection of potential chronic diseases such as cardiovascular diseases and diabetes mellitus, and even cancers (127–129). It may be unethical not to diagnose and treat their infertility. Even for these couples with no correctable male infertility factors who choose to use ARTs, denying such care based solely on age may be viewed as age-discrimination. Additionally, there is a substantial number of children born to aging fathers from natural pregnancies, yet healthcare providers generally take no action in prohibiting aging men in the society at large to have children. Is it rational for them not to intervene with all men at advanced age who are attempting to have children?

One may propose that a more sensible strategy is perhaps through general public education for a "preventative" approach. Unfortunately, this will also encounter obstacles at a different level. The message that "delayed parenthood could lead to adverse outcomes" may be misinterpreted as "education and career commitment are less important" (130, 131). which would not echo well with the ambitious-minded youngsters Further, as the negative impact of female aging on reproduction risks is arguably stronger than that in male aging (44), if the message is therefore more strongly emphasized to young females than to young males, one could only imagine the severity of backlash it would spark from the public.

With regards to reproductive technologies, though planned or elective egg freezing for non-medical reasons is an established strategy to reduce the reproductive risks associated with female aging, planned or elective sperm freezing has not been shown to be effective in mitigating reproductive and offspring health risks associated with paternal aging. This is in part related to the fact that the well-documented chromatin cryodamage from sperm cryopreservation (132–136) can potentially offset any potential benefits from sperm banking. Though sperm cryopreservation is non-invasive and widely accessible, the fees associated with semen storage for years can be significant. Of note, ARTs are required when using cryopreserved sperm. Intra-uterine insemination (IUI) can be used but given its lower success rate compared to *in vitro* fertilization (IVF) and intracytoplasic sperm injection (ICSI), multiple semen samples may have to be cryopreserved to allow for repeated trials of IUI to have a reasonable success rate. In practice, advanced assisted reproduction such as ICSI are often required when using cryopreserved sperm. Aging men who previously banked sperm at a younger age may opt to attempt conception through intercourse when they realize the cost, invasiveness and potential risks on the female partners and offspring associated with using ICSI (137–139). Ultimately, large scale studies to unbiasedly compare the reproductive outcomes and long-term offspring health of with natural conception versus long-term cryopreserved sperm with ICSI are required to establish the benefits and cost-effectiveness of planned or elective sperm freezing against male aging.

Accumulating evidence from the past two decades links impaired sperm chromatin integrity and DNA fragmentation to increased risk of pregnancy loss and reduced success rate with assisted reproductive technologies. Growing interest in recent years on various sperm selection strategies has led to studies that provided preliminary evidence of improving reproductive outcomes in selected infertile couples (140, 141). However, the question of whether these sperm selection strategies are effective in cases of advanced paternal age, particularly in lowering the risks of health conditions linked to aberrant chromatin, remains to be answered.

LOOKING FORWARD

In dealing with the risks association with advanced paternal age, too often wrong questions were asked: "how old is too old?", "What is the paternal age cut-off at which we can justify imposing restriction of access to reproductive care?" Although most experts agree that the negative impacts of advanced paternal age can be detected in some men after the age of 40 years, currently there is no consensus on the optimal definition of advanced paternal age as studies have used different age inclusion and different outcomes with different definitions. The progressive nature of the physiological changes associated with male aging is a main reason why it is challenging for investigators to reach agreement on a clear definition for aging.

To begin the mission to reduce risks associated with paternal aging, paradoxically, the focus of discussion must first be shifted away from chronological age to gamete-mediated risk on reproductive outcomes and offspring health. In other words, advanced paternal age should be treated as other male factor infertility causes with a focus on identifying elements that can be ameliorated, assessment of gamete functional status, and selection of the gametes with the best chance for a successful procreation. Health policy makers and healthcare providers may have to accept the fact that the growing number of aging men having children is an inevitable phenomenon in the current direction of societal evolution. It is equally important to recognize that strategies aiming to prohibit or dissuade this behavior through establishing a clear paternal age limit for provision of fertility care or through education and counselling can readily be challenged and therefore deemed ineffective.

An alternative approach is to have policy makers, clinicians and investigators work closely together to synthesize information on the risks that can be disseminated to prospective parents to allow them to engage in a shared decision-making model with their healthcare providers. Risks on adverse reproductive outcomes and offspring health that are gamete mediated should be comprehensively assessed and defined, using established diagnostic tools at the molecular levels. It is important to emphasize that, in addition to aging, gamete mediated risks may well be attributed to other health conditions such as intrinsic genetic disorders, gonadotoxin exposure, history of cytotoxic therapies, metabolic derangements, obesity, smoking, and varicoceles. Thus, communication of gamete mediated reproductive risks should be conducted across the board as a standard of practice to all male partners seeking fertility care and not just to those at an advanced age. Shifting the focus of counselling from chronological age to gamete mediated risks allows clinicians to formulate a treatment plan or decline treatment without being accused of age discrimination. Finally, additional psychosocial concerns beyond gamete quality in the context of advanced paternal age such as life-expectancy of parents, should also be an important consideration in this shared decision-making model.

To minimize or mitigate the negative impact of advanced paternal age, comprehension of the collective body of scientific evidence is only the first step. Continued dialogues must be maintained among stakeholders at all levels, including investigators, healthcare providers, health policy makers and patients, on emerging data and their implications at the personal as well as societal levels. Most importantly, it is imperative for all parties to collaborate rigorously, with the goal of catalyzing a new agenda to reconceptualize the management strategy of advanced paternal age in the context of reproductive care of prospective parents.

AUTHOR CONTRIBUTIONS

PC and BR contributed to the writing and the editing of this manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This research has been funded by the CIHR Institute for Gender and Health Team Grant TE1-138298. BR is a James McGill Professor.

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MINI REVIEW
published: 10 June 2022
doi: 10.3389/fendo.2022.897062

Sertoli Cell-Germ Cell Interactions Within the Niche: Paracrine and Juxtacrine Molecular Communications

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Male germ cell development depends on multiple biological events that combine epigenetic reprogramming, cell cycle regulation, and cell migration in a spatio-temporal manner. Sertoli cells are a crucial component of the spermatogonial stem cell niche and provide essential growth factors and chemokines to developing germ cells. This review focuses mainly on the activation of master regulators of the niche in Sertoli cells and their targets, as well as on novel molecular mechanisms underlying the regulation of growth and differentiation factors such as GDNF and retinoic acid by NOTCH signaling and other pathways.

Keywords: Sertoli cell, germ cell, growth factors, self-renewal, differentiation

OPEN ACCESS

Edited by:
Vassilios Papadopoulos, University of Southern California, United States

Reviewed by:
Martine Culty, University of Southern California, United States
Alan Diekman, University of Arkansas for Medical Sciences, United States

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Specialty section:
This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology

Received: *15 March 2022*
Accepted: *25 April 2022*
Published: *10 June 2022*

Citation:
Hofmann M-C and McBeath E (2022) Sertoli Cell-Germ Cell Interactions Within the Niche: Paracrine and Juxtacrine Molecular Communications. Front. Endocrinol. 13:897062. doi: 10.3389/fendo.2022.897062

INTRODUCTION

The Niche Microenvironment

Maintenance, repair, and regeneration of many mammalian organs depend on adult stem cells. Stem cells proliferate and differentiate to replace mature functional cells within tissues that have either high turnover such as blood, testis, and epithelia (intestine, skin, and respiratory tract), or tissues that have low turnover but a high regenerative potential upon disease or injury such as liver, pancreas, skeletal muscle, and bone (1). Precise regulation of adult stem cell fate is therefore critical for the support of tissue homeostasis, and stem cell maintenance must involve a fine balance between genetic and epigenetic mechanisms, external factors from the microenvironment and systemic support, and multiple signaling pathways elicited by paracrine and juxtacrine factors.

Over the years, evidence has accumulated showing that stem cell self-renewal depends on the constituents of their microenvironment called the niche (2, 3) and that in turn stem cells influence their own environment (4–6). The constituents of the niche can be classified into adjacent supporting cells such as fibroblasts, tissue macrophages, glial cells (brain), osteoblasts (bone marrow), Sertoli cells (testis) and myofibroblasts (gut), together with paracrine and juxtacrine factors secreted by these supporting cells, and the extracellular matrix. Once they leave the niche, stem cells become progenitor cells that are less plastic and differentiate at the expense of their immortality. Over the last 15 years, critical cellular and molecular components of the specialized niche microenvironment have begun to be unveiled in several tissues. Advanced techniques in lineage-tracing, endogenous cell and gene/protein deletions in animal models, and high-resolution

microscopy have significantly improved our understanding of the molecular and cellular intricacies that maintain and integrate the many activities required to sustain tissue homeostasis.

The Spermatogonial Stem Cell Niche

In the mammalian testis, the male germline produces a life-long supply of haploid spermatozoa through the highly regulated and coordinated process of spermatogenesis. This process starts with the self-renewal of a small pool of diploid stem cells called spermatogonial stem cells (SSCs or A_{single} spermatogonia), which can self-renew to maintain the pool or give rise to more mature germ cells called A_{paired} and $A_{aligned}$ spermatogonia. Collectively, A_{single}, A_{paired} and $A_{aligned}$ spermatogonia are called undifferentiated spermatogonia (7). These cells further differentiate into differentiating spermatogonia and spermatocytes that undergo meiosis, producing haploid spermatids that will mature into spermatozoa. The longevity and the high output of sperm cell production relies therefore primarily on the proper maintenance of the pool of SSCs and their proliferation. Like other types of stem cells, SSCs rely on their micro-environment to sustain their growth and to initiate differentiation that signals their release from the basal part of the seminiferous epithelium and exit from the niche.

SSCs reside on the basement membrane that supports the seminiferous epithelium. They are in intimate physical contact with highly specialized somatic niche cells, the Sertoli cells, which directly provide soluble growth factors and membrane-bound signals to the germ cells (8). Other niche cell types have been recently investigated, including peritubular myoid cells, interstitial cells (macrophages and Leydig cells), and endothelial cells from the vascular network, which all produce critical growth factors (**Figure 1**) (9–15). Because of their direct physical association with germ cells, their secretion of growth factors and basement membrane components, and their architectural support of the seminiferous epithelium, Sertoli cells are considered the most important contributor of the testicular niche, and the regulation of their molecular communications with SSCs and more mature premeiotic germ cells will be the subject of this review.

SERTOLI CELLS AS STRUCTURAL NICHE ORGANIZERS

It is now established that the number of Sertoli cells increases during fetal development due to growth stimulation through FSH/FSHR signaling. Sertoli cells proliferate up to day 15 after birth in mice and 17 days after birth in rats, after which the number of Sertoli cells reaches its peak and remain constant throughout life unless altered by insult and aging. Therefore, the number of Sertoli cells is finite and its maintenance is crucial for life-long spermatogenesis. Several years ago, de Franca et al. induced experimental hypothyroidism in the rat with propylthiouracil (PTU) administrated neonatally. The treatment significantly increased the period of Sertoli cell proliferation and therefore increased their number at puberty and beyond. This also increased germ cell number and the size of the testes (16). However, direct evidence that Sertoli cells indeed provide a structural and functional SSC niche support was provided by Oatley and colleagues (17). The authors treated male mouse pups with PTU, which led to

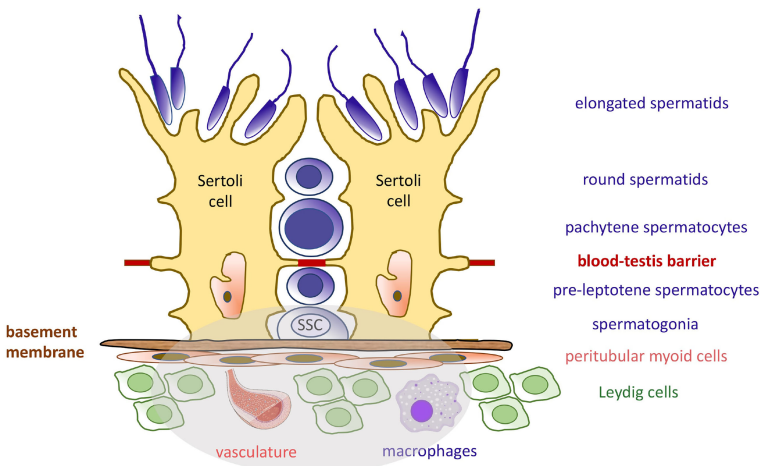

FIGURE 1 | Seminiferous Epithelium Organization and the Spermatogonial Stem Cell Niche. The seminiferous epithelium consists of germ cells (blue) and the somatic Sertoli cells (yellow). Sertoli cells produce many factors needed at various developmental steps during the spermatogenic process. The blood-testis barrier separates diploid germ cells from more mature cells and provide an immuno-privileged microenvironment for the completion of meiosis. Like Sertoli cells, the spermatogonial stem cells (SSCs) are attached to the basement membrane. They rely on specific growth factors for self-renewal and maintenance of the pool. These molecules are produced by Sertoli cells, peritubular myoid cells, Leydig cells, and macrophages, as well as the vasculature. The components of the SSC niche are highlighted in the grey area.

increased Sertoli cell and germ cell numbers in the adult testes. Next, by using these mice as germ cells recipients after busulfan treatment destroyed their endogenous germ cells, they showed a significant increase of colonization by normal donor SSCs after transplantation. This demonstrated an increased presence of functional niches. Because neither the vasculature nor interstitial cell populations were altered in the PTU recipient model, they concluded that Sertoli cells are the most critical somatic cell type in the testis and that they create the SSC niche.

MASTER REGULATORS OF THE NICHE

The germ cell and Sertoli cell behaviors leading to the establishment of the spermatogenic stem cell niche in the early postnatal testis are well known. In addition to Sertoli cell proliferation leading to the expansion of the niche units until puberty, one of the most striking cellular behavior is the movement of pro-spermatogonia, or gonocytes, toward the periphery of the cords at around day 3-4 after birth in rodents, and 8-12 weeks after birth in humans (18, 19). By postnatal day 6 in the mouse, about 90% of pro-spermatogonia have reached the basal lamina, have become SSCs and rapidly differentiate (20), whereas germ cells that failed to migrate have died (21). The past fifteen years have seen a growing interest in understanding how these processes are regulated and the discovery of Sertoli cell-specific genes that are master determinants of the niche has become a priority.

DMRT1 (*Doublesex* and *Mab-3* related transcription factor 1) is a conserved gene that is expressed in the testes of all vertebrates. In the mouse, DMRT1 expression starts at the genital ridge stage and continues throughout adult life. DMRT1 is required for normal sexual development, and defective expression leads to abnormal testicular formation and XY feminization (22). While both germ cells and Sertoli cells express the gene, Sertoli cell-specific knockout of *Dmrt1* led to testicular abnormalities at around day 7 post-partum (22–25). Sertoli cells lacking DMRT1 re-expressed Forkhead box L2 (FOXL2), a female gonad determinant (26). The cells could not polarize, reprogrammed into granulosa cells, and seminiferous tubule lumens did not form (22). Consequently, SSCs and undifferentiated spermatogonia were not maintained at the tubule periphery, the germ cell population remained disorganized, and germ cells died after meiotic arrest. This indicated that DMRT1 antagonizes FOXL2 and functions as a repressor of the female gonad development. Further, DMRT1 is also a known activator of androgen receptor (AR) (27, 28) and is crucial for cellular junction formation and function by driving the expression of *Claudin 11 (Cldn11), Vinculin (Vcl)*, and *gap junction protein alpha 3 (Gja3)* (**Table 1**), therefore controlling the structural niche as well (28, 48, 79, 120).

In 2015, Chen and colleagues demonstrated that targeted loss of *Gata4*, a known Sertoli cell marker also involved in mouse genital ridge initiation, sex determination, and embryonic testis development (72–74), resulted in a loss of the establishment and maintenance of the SSC pool, and led to Sertoli cell-only syndrome (41). Loss of *Gata4* altered the expression of a number of chemokines, including *Cxcl12* (SFD1, binding to the CXCR4 receptor) and *Ccl3* (binding to the CCR1 receptor), which are known to guide pro-spermatogonia toward the basement membrane and the niche provided by Sertoli cells (39, 40). Similarly, another Sertoli cell transcription factor, ETV5, was found to directly bind to the promoter of the chemokine *Ccl9*. CCL9 facilitated chemoattraction of stem/progenitor spermatogonia, which express CCR1, the receptor for CCL9 (42) (**Table 1**). Together, these results revealed a novel role for GATA4 and ETV5 in organizing the SSC niche *via* the transcriptional regulation of chemokine signaling shortly after birth. More recently, Alankarage and colleagues demonstrated that *Etv5* in Sertoli cells is directly under control of SOX9, a transcription factor that specifies the function of Sertoli cells and their differentiation from somatic cell precursors (61).

Migration of pro-spermatogonia to the basement membrane and niches provided by Sertoli cells is also dependent on AIP1, a β-actin-interacting protein that mediates β-actin (ACTB) disassembly (29, 31). Sertoli and germ cell-specific deletion of mouse *Aip1* each led to significant defects in germ cell migration at postnatal day 4, which corresponded to elevated numbers of actin filaments in the affected cells. Increased actin filaments might have caused cytoskeletal changes that impaired E-cadherin (CDH1) regulation in Sertoli cells and germ cells, decreasing germ cell motility. *Aip1* deletion in Sertoli cells did not affect the expression and secretion of growth factors, suggesting that the disruption of SSC migration and function results from architectural changes in the postnatal niche.

Another determinant of the perinatal niche, CDC42, was recently identified by Mori et al. (46). Together with RAC1 and RHOA, CDC42 is a member of the RHO family of small GTP-ases, which are mainly involved in cell polarity and migration (43, 111). Importantly, a possible role of the small GTP-ases CDC42 and RAC1 in the regulation of the blood-testis-barrier (BTB), tight junction components, and Sertoli cell polarity was suggested by several authors (45, 47, 109). While deletion of *Cdc42* expression in Sertoli cells in the Mori study did not lead to major changes in the BTB integrity and cell polarity, it led to the depletion of the growth factor glial cell line-derived neurotrophic factor (GDNF), a major determinant of spermatogonial proliferation, possibly through the downregulation of canonical PAK1 activity downstream of CDC42 (44).

EPIGENETIC REGULATORS OF THE NICHE

One of the first discovered epigenetic regulators of the SSC niche was the Switch-insensitive 3a (SIN3A) co-repressor protein, part of a massive transcriptional complex that interacts with a wide array of epigenetic regulators (114). The SIN3A transcriptional corepressor complex plays a role in diverse cellular processes

TABLE 1 | Names and functions of proteins discussed in this review.

Protein	UniProt ID (mouse, unless specified)	Cell Type	Function in the testis	References
ACTB	P60710	Sertoli cells	**Beta-Actin**. Component of adherens junctions.	29, 30
AIP1 (WDR1)	P60710	Sertoli cells	**Actin-Interacting Protein 1**. Functions as Actin disassembly factor, promotes germ cell movement toward the basement membrane.	31
AIP1 (WDR1)	P60710	Pro-spermatogonia/ Undifferentiated spermatogonia	**Actin-Interacting Protein 1**. Functions as Actin disassembly factor, promotes germ cell movement toward the basement membrane.	31
AMH	P27106	Sertoli cells, immature	**Anti-Mullerian Hormone**. Regression of Müllerian ducts in male fetuses.	32, 33
AR (NR3C4)	P19091	Sertoli cells	**Androgen receptor**. Responsible for binding of Testosterone/Dihydrotestosterone.	27, 28, 34
ARID4A/ ARI4A	F8VPQ2	Sertoli cells	**AT-Rich Interaction Domain 4A.** Maintains the blood-testis barrier. Knock-out induces meiotic arrest.	33, 35
ARID4B/ ARI4B	A2CG63	Sertoli cells	**AT-Rich Interaction Domain 4B**. Supports the SSC niche. Transcriptional coactivator for AR.	33, 34, 36
BCL6B	O88282	Spermatogonial stem cells	**B-Cell CLL/Lymphoma 6, Member B**. Supports self-renewal.	37, 38
BEX1	Q9HBH7 (human)	Human Sertoli cells, Stage b (8-11 year old)	**Brain Expressed X-Linked Protein 1**. Transcription regulator. Plays a role in cell cycle progression in Stage b human Sertoli cells.	30
CCL3	P10855	Sertoli cells, perinatal	**C-C Motif Chemokine Ligand 3**. Guides pro-spermatogonia toward the basement membrane.	39–41
CCL9	P51670	Sertoli cells, perinatal	**C-C Motif Chemokine Ligand 9**. Guides pro-spermatogonia toward the basement membrane. Maintains SSCs within the niche.	42
CCR1	P51675	Pro-spermatogonia, undifferentiated spermatogonia	**C-C Motif Chemokine Receptor 1**. Receptor for CCL3 and CCL9.	39
CDC42	P60766	Sertoli cells	**Cell Division Cycle Protein 42**. Involved in cell polarity and migration. Regulation of the blood-testis barrier and Sertoli cell polarity.	43–47
CDH1	P09803	Sertoli cells	**E-cadherin/cadherin-1**. Calcium-dependent cell adhesion protein.	29
CLDN11/ CLD11	Q60771	Sertoli cells	**Claudin 11**. Tight junction protein at the blood-testis barrier.	28, 48
CSF1	P07141	Leydig cells	**Macrophage Colony Stimulating Factor 1**. Enhances self-renewal of spermatogonial stem cells.	12
CST9L	Q9H4G1 (human)	Human Sertoli cells, Stage c (17 year old to adult)	**Cystatin 9 Like**. Tissue remodeling during early testis development. Also present in adult Sertoli cells.	30, 49
CTNNB1	Q02248	Spermatocytes and spermatids	**Catenin Beta 1**. Maintenance of post-mitotic germ cells.	50–52
CXCL12/ SDF1	P40224	Sertoli cells	**C-X-C Motif Chemokine Ligand 12.** Guides pro-spermatogonia toward the basement membrane. Maintains SSCs within the niche.	41 53
CXCR4	P70658	Pro-spermatogonia, undifferentiated spermatogonia	**C-X-C Motif Chemokine Receptor 4**. Receptor for CXCL12.	40
CYP26B1	Q811W2	Sertoli cells, immature and postnatal	**Cytochrome P450 Family 26 Subfamily B Member 1**. Inactivates retinoic acid through oxidation.	54–56
DEFB119	Q8N690 (human)	Human Sertoli cells, Stage c (17 year old to adult)	**Defensin Beta 119**. Anti-microbial defense in the male reproductive tract.	30, 57
DMRT1	Q9QZ59	Sertoli cells, immature and adult	**Doublesex And Mab-3 Related Transcription Factor 1.** Required for normal testis development and maintenance. Antagonist of FOXL2.	22, 23, 28, 58
DMRT1	Q9QZ59	Germ cells	**Doublesex And Mab-3 Related Transcription Factor 1**. Required for SSC maintenance and germ cell mitosis/meiosis decision.	24, 25
EGF	P01133 (human)	Human Sertoli cells, Stage a (2-5 year old)	**Epidermal Growth Factor.** Produced by Sertoli cells. Germ cell maintenance/proliferation.	30, 59
EGR3	Q06889 (human)	Human Sertoli cells, Stage a (2-5 year old)	**Early Growth Response 3**. Induced by mitogenic stimulation of Sertoli cells.	30
ENO1/ ENOA	P06733 (human)	Human Sertoli cells, Stage b (8-11 year old)	**Enolase 1**. Growth control, cell metabolism.	30
ERK5/ MAPK7	Q13164 (human)	Human Sertoli cells, Stage a (2-5 year old)	**Mitogen-Activated Protein Kinase 7.** Proliferation, differentiation, transcription regulation and development of Sertoli cells.	30
ETV5	Q9CXC9	Sertoli cells	**ETS Variant Transcription Factor 5**. Induces the production of chemokines and maintains SSC homing within the niche	42, 60, 61

(Continued)

TABLE 1 | Continued

Protein	UniProt ID (mouse, unless specified)	Cell Type	Function in the testis	References
ETV5	Q9CXC9	Spermatogonial stem cells	**ETS Variant Transcription Factor 5**. Induces the production of CXCR4 and Brachyury (T) and maintains SSC homing within the niche.	62 63
FGF2	P15655	Sertoli cells	**Fibroblast Growth Factor 2**. SSC self-renewal.	38, 64–68
FOXL2	O88470	Granulosa cells	**Forkhead Box L2**. Ovarian development and function. Repression of somatic testis determination. Antagonist of DMRT1.	22, 26
FSH	Q60687	Anterior pituitary cells	**Follicle Stimulating Hormone Subunit Beta**. Induces Sertoli cell proliferation in early development. Induces Sertoli cells to secrete androgen-binding proteins (ABPs), and stimulates inhibin B secretion.	69, 70
FSHR	P35378	Sertoli cells	**Follicle Stimulating Hormone Receptor**	71
GATA4	Q08369	Sertoli cells	**GATA Binding Protein 4**. Embryonic testis development, Sertoli cell maintenance, production of chemokines, SSC niche maintenance.	41, 58, 72–74
GDNF	P48540	Sertoli cells, postnatal	**Glial Cell Derived Neurotrophic Factor**. SSC self-renewal	66, 75;
GDNF	P48540	Sertoli cells, prenatal	**Glial Cell Derived Neurotrophic Factor.** Pro-spermatogonia maintenance.	76
GFRA1	P97785	Undifferentiated spermatogonia	**GDNF Family Receptor Alpha 1**. Co-receptor of RET	77, 78
GJA3 (CX46)	Q64448	Sertoli cells	**Gap Junction Protein Alpha 3**. Connexin 46. Gap Junction Protein, component of the blood-testis barrier.	28, 79
HES1	P35428	Sertoli cells	**HES Family BHLH Transcription Factor 1.** Target/mediator of NOTCH signaling. Inhibits GDNF and CYP26B1 expression.	56, 80
HEY1	Q9WV93	Sertoli cells	**Hes Related Family BHLH Transcription Factor With YRPW Motif 1.** Target/mediator of NOTCH signaling. Inhibits GDNF and CYP26B1 expression.	56, 80
HOPX	Q9BPY8 (human)	Human Sertoli cells, Stage c (17 year old to adult)	**HOP Homeobox**. Growth suppression and differentiation.	30, 81
IGF1	P05019 (human)	Human Sertoli cells, Stage a (2-5 year old)	**Insulin-Like Growth Factor 1**. Produced by Sertoli cells. Germ cell proliferation.	30, 82
INHBB	Q04999	Sertoli cell	**Inhibin Subunit Beta B**. Testis development. Marker of Sertoli cells function and germ cell numbers. Regulation of FSH secretion by pituitary.	33, 83, 84
JAG1	Q9QXX0	Undifferentiated spermatogonia	**Jagged 1**. Canonical NOTCH ligand.	55, 85
JUN	P05627	Sertoli cell	**Jun Proto-Oncogene**. AP-1 transcription factor complex subunit. Sertoli cell function, maintenance of the blood-testis barrier.	30, 86
KIT	P05532	Differentiating spermatogonia	**KIT Proto-Oncogene, Receptor Tyrosine Kinase.** Proliferation and differentiation.	87–89
KIT	P05532	Primordial germ cells	**KIT Proto-Oncogene, Receptor Tyrosine Kinase.** Proliferation and Survival.	90
KIT	P10721 (human)	Seminoma cells	**KIT Proto-Oncogene, Receptor Tyrosine Kinase.** Mutated and constitutively activated in 25% of seminoma.	91
KITL	P20826	Sertoli cell	**KIT Ligand.** Proliferation and differentiation of germ cells.	89, 92–95
LIF	P42703	Sertoli cell	**Leukemia Inhibitory Factor**. Maintenance of spermatogonial stem cell survival.	10, 66, 96
LIN28	Q8K3Y3	Pro-spermatogonia, undifferentiated spermatogonia	**Lin-28 Homolog A**. Pluripotency and SSC self-renewal.	97, 98
NFKB1	P25799	Sertoli cell	**Nuclear Factor Kappa B1**. Pleiotropic transcription factor.	99
NOTCH1	Q01705	Sertoli cell	**NOTCH Receptor 1**. Intercellular signaling pathway regulating cell fate specification and differentiation	56, 80, 85, 100
NR3C1	P06537	Fetal and perinatal Sertoli cell	**Nuclear Receptor Subfamily 3 Group C Member 1**. Glucocorticoid receptor. Possible link between stress and testicular function.	33, 101, 102;
NR3C1	P06537	Germ cell (spermatogonia)	**Nuclear Receptor Subfamily 3 Group C Member 1**. Glucocorticoid receptor. Possible link between stress and testicular function.	101
NR4A1	P22736 (human)	Human Sertoli cells, Stage a (2-5 year old)	**Nuclear receptor subfamily 4 group A member 1**. Proliferation, chemotaxis.	30
PAK1	O88643	Sertoli cell	**P21 Protein (Cdc42/Rac)-Activated Kinas**e 1. Canonical target of RHO GTPases.	44
PDGFA	P20033	Sertoli cells, perinatal	**Platelet-derived growth factor subunit A.** Germ cell proliferation.	103–106
PDGFB	P31240	Sertoli cells, perinatal	**Platelet-derived growth factor subunit A.** Germ cell proliferation.	103–106
PLZF (ZBTB16)	A3KMN0	Undifferentiated spermatogonia	**Zinc Finger And BTB Domain Containing 16**. Represses KIT in undifferentiated spermatogonia.	107, 108
RAC1	P63001	Sertoli cell	**Ras-related C3 botulinum toxin substrate 1.** Sertoli cell polarity.	109
RARA/G	P18911	Germ cells, undifferentiated	**Retinoic acid receptor alpha/gamma.** Germ cell differentiation.	68
RBPJ	P31266	Sertoli cells	**Immunoglobulin Kappa J Region Recombination Signal Binding Protein 1.** Transcription factor, mediator of all activated NOTCH receptors	80, 100, 100

(Continued)

TABLE 1 | Continued

Protein	UniProt ID (mouse, unless specified)	Cell Type	Function in the testis	References
RET	P35546	Germ cell, undifferentiated	**Ret Proto-Oncogene, Rearranged During Transfection**. SSC self-renewal, undifferentiated spermatogonia proliferation.	77, 78;
RET	P35546	Germ cell, fetal	**Ret Proto-Oncogene, Rearranged During Transfection**. Maintenance of fetal germ cells.	110
RHOA	P61586 (human)	Human Sertoli cells, Stage b (8-11 year old)	**Transforming protein RhoA.** Sertoli cell polarity, junction remodelling	30, 111
RHOX5	P52651	Sertoli cells	**Homeobox protein Rhox5.** Regulation of germ cell apoptosis.	34, 112,
S100A13	Q99584 (human)	Human Sertoli cells, Stage b (8-11 year old)	**S100 Calcium Binding Protein A13**. Cell cycle progression and differentiation.	30
SIN3A	Q60520	Sertoli cell	**Switch-insensitive 3a (SIN3A).** Co-repressor, regulation of chemokines expression.	113, 114
SOHlH1	Q6IUP1	Differentiating spermatogonia	**Spermatogenesis- and oogenesis-specific basic helix-loop-helix-containing protein 1.** Upregulation of KIT receptor expression.	115;
SOHlH2	Q9D489	Differentiating spermatogonia	**Spermatogenesis- and oogenesis-specific basic helix-loop-helix-containing protein 1**. Upregulation of KIT receptor expression.	115;
SOX9	Q04887	Sertoli cells	**SRY-Box Transcription Factor 9**. Sex determination. Maintenance of Sertoli cell functions.	58, 61, 116
VEGFA	Q00731	Sertoli cells, perinatal	**Vascular endothelial growth factor A**. Maintenance of spermatogonial stem cells.	117, 118
VEGFA	Q00731	Germ cells, perinatal	**Vascular endothelial growth factor A**. Maintenance of spermatogonial stem cells.	117
VEGFA	Q00731	Interstitial cells	**Vascular endothelial growth factor A**. Maintenance of spermatogonial stem cells.	117
VEGFA164	Q00731	Sertoli cells	**Vascular endothelial growth factor A, VEGFA164 isoform.** SSC self-renewal.	119
VCL	Q64727	Sertoli cells	**Vinculin**. Actin filament (F-actin)-binding protein. Cell-cell adhesion, adherens junction, ectoplasmic specializtion.	28, 120
WNT5A	P22725	Sertoli cells	**Wingless-Type MMTV Integration Site Family, Member 5A**. SSC maintenance and survival. CTNNB1 independent.	50, 121
WNT3A	P27467	Sertoli cells	**Wingless-Type MMTV Integration Site Family, Member 5A**. Proliferation of progenitor spermatogonia exiting the SSC state. CTNNB1-dependent.	122
WT1	P22561	Sertoli cells, fetal and adult	**Wilms tumor protein homolog 1.** Testis development, lineage maintenance of Sertoli cells.	27, 33
WTAP	Q9ER69	Sertoli cell	**Wilms tumor protein homolog 1-associated protein**. Mediates N6-methyladenosine (m6A) methylation of RNAs.	33, 123

such as proliferation, differentiation, tumorigenesis, apoptosis and cell fate determination (113). The classical mechanism of action of this complex is transcriptional silencing through histone deacetylation mediated by HDAC1/2. In the mouse testis, Sertoli cell specific *Sin3a* deletion resulted in a decrease of undifferentiated spermatogonia after birth. The Sertoli cell markers *Kit Ligand (KITL)* and *Gdnf*, which support germ cell proliferation, were not diminished. However, chemokine signaling molecules such as CXCL12/SDF1 and CXCR4, expressed by Sertoli cells and germ cells, respectively, were not detected. This again demonstrates that regulators of germ cell movement toward the periphery of testicular cords and the basement membrane after birth are critical for the establishment of the initial postnatal niche. However, the relationship between SIN3A and the signaling networks governed by GATA4 and ETV5 in Sertoli cells are not yet known.

In 2013, Wu and colleagues identified ARID4A and ARID4B (AT-rich interactive domain-containing protein 4A/B) as additional master regulators critical for the establishment of the niche, in particular during the pro-spermatogonia to SSC transition phase (35, 36). Interestingly, ARID4B is a subunit of the SIN3A transcriptional repressor complex. Sertoli cell ablation of *Arid4B* expression resulted in Sertoli cell detachment from the basement membrane, which precluded niche formation and the movement of pro-spermatogonia toward the periphery of the testicular cords. Without niche support, the germ cells underwent apoptosis. The authors also showed that ARID4B can function as a transcriptional coactivator for androgen receptor (AR) and identified reproductive homeobox 5 (*Rhox5*) (124) as the target gene critical for spermatogenesis (34).

Another epigenetic regulator of the niche is WTAP, or Wilms Tumor 1-associated protein (33). WTAP regulates transcription and translation of genes by depositing N^6-methyladenosine (m^6A) marks directly on RNA transcripts or indirectly on transcriptional regulators (125). Jia and colleagues demonstrated that conditional deletion of *Wtap* in mouse Sertoli cells modified pre-mRNA splicing, diminished RNA export and translation, and therefore altered the transcription and translation of many Sertoli cell genes normally marked by m^6A modification. Many of these genes were critical for SSC maintenance, spermatogonial differentiation, retinol metabolism, and the cell cycle, including *Inhbb*, *Wt1*, *Arid4a*, *Arid4b*, *Etv5*, *Ar*, *Dmrt1*, and *Sin3a* (**Table 1**) (23, 27, 35, 60, 83, 114, 126, 127). Consequently, progressive loss of undifferentiated spermatogonia was observed in WTAP-deficient testes and mice were sterile. Interestingly, while not normally marked by m^6A modification, *Gdnf*, which is required for SSC maintenance and self-renewal, was also downregulated. The authors surmised that several of the key transcription regulators that have been reported to be important for *Gdnf* transcription contained m^6A sites and were dysregulated by *Wtap* knockout.

SINGLE CELL RNA-SEQ AND SPATIAL TRANSCRIPTIONAL DISSECTION OF PERINATAL AND MATURE SERTOLI CELLS

Single cell characterization of developing and mature Sertoli cells in rodents and humans, as well as their spatial transcriptional dissection, uncovered many genes potentially important for the organization of the niche, and are providing a large resource for functional analysis of possible signaling pathway networks (102, 128–132). All studies demonstrated that mouse Sertoli cells undergo stepwise changes during the perinatal period, which are dependent on the expression of *SOX9, AMH, GATA1-4, DMRT1, NR3C1* and their target genes (**Table 1**) (32, 58, 101, 102, 116). Notably, as predicted, expression of cell cycle genes decreases as Sertoli cells mature after birth. Further, these data demonstrated a postnatal increase in expression of Sertoli-Sertoli cell junctions and germ cell-Sertoli cell junction signaling (102). Zhao and colleagues identified three stages of postnatal Sertoli cells maturation in humans. In stage a (2-5 years old), the top three differentially expressed genes were *EGR3*, *JUN*, and *NR4A1* (**Table 1**) (30, 86). In stage b (8-11 years) *S100A13*, *ENO1*, and *BEX1* were prominently expressed, while in stage c (17 years to adult) *HOPX*, *DEFB119*, and *CST9L* were upregulated (**Table 1**) (49, 57, 81). Gene Ontology and Ingenuity Pathway Analysis (IPA) at each of the three stages indicated that genes ensuring proliferation and maintenance of cell numbers were prominently expressed in stage a (EGF, IGF, and ERK5 signaling), RHOA/ACTB motility and remodeling of Sertoli-Sertoli epithelial junctions were a feature of stage b, and pathways of cholesterol biosynthesis and germ cell-Sertoli cell junction signaling were increased in stage c (59, 82). In addition, protein transmembrane transport, phagosome maturation, and cellular metabolic processes were upregulated in stage c, confirming that the most important functions of mature Sertoli cells are the production of growth factors, phagocytosis of germ cells and metabolites processing. Collectively, these data indicate that single cell RNA-seq and spatial transcriptomic characterization of Sertoli cells generate reliable resources for future mechanistic studies of master regulators of the niche and their targets at different time points.

SERTOLI CELL FACTORS CONTROLLING SSC MAINTENANCE AND SELF-RENEWAL.

In the seminiferous epithelium, Sertoli cells produce a number of soluble factors that are under the control of the above-described master regulators. These growth factors are critical for pro-spermatogonial maintenance in the fetus, maintenance of the SSC pool, self-renewal of SSCs after birth, and the onset of germ cell differentiation. The most critical factors include glial cell line-derived neurotrophic factor (GDNF) (75), colony-stimulating factor 1 (CSF1) (12), fibroblast growth factor 2 (FGF2) (65, 66), leukemia inhibitory factor (LIF) (10) and WNT family proteins (50, 122). They all bind to their cognate receptors at the surface of SSCs or undifferentiated spermatogonia and activate the MAPK or PI3K/AKT pathway to drive the cell cycle. They also promote SSC proliferation *in vitro*, which can be demonstrated by increased testes colonization after transplantation. KITL, the ligand for KIT receptor, and retinoic acid (RA) are considered major determinants of germ cell differentiation after birth, promote the switch between undifferentiated and differentiating spermatogonia and trigger meiotic entry (94, 133, 134).

Glial Cell Line-Derived Neurotrophic Factor

GDNF is a member of the transforming growth factor beta (TGF-b) superfamily that binds to the GFRA1/RET receptor complex at the surface of SSCs, A_{paired} and some $A_{aligned}$ spermatogonia (75, 77). Meng and colleagues were first to demonstrate that GDNF haploinsufficiency in mice induced fertility defects after birth (75). The mice were fertile but exhibited increased numbers of seminiferous tubules lacking spermatogonia as they aged. In addition, transgenic animals overexpressing *Gdnf* accumulated undifferentiated spermatogonia. In 2006, Naughton and colleagues disrupted the expression of *Ret* and *Gfra1* at the surface of SSCs, which resulted in their loss and led to the definitive proof of the critical function of this receptor-ligand interaction (78). Together with FGF2 and LIF, GDNF is critical for the self-renewal of SSCs in short- and long-term cultures (66). Because of its importance for spermatogenesis, efforts were made to understand the temporal regulation of its expression. Low levels of GDNF and RET are already present in the fetal gonad (76, 110). Since pro-spermatogonia do not proliferate until after birth, GDNF is therefore only necessary for their maintenance, highlighting the importance of its dosage (98). GDNF expression then increases until it reaches a peak at days 3-7 after birth (110, 135, 136). One interesting feature of GDNF expression in the adult is its cyclic pattern throughout the stages of the seminiferous epithelium. Cyclical production of soluble factors according to stages was demonstrated earlier by Johnston and colleagues using transillumination-assisted microdissection and microarray analysis (137). In the rat, GDNF expression is highest at stages XIII-I, and lowest at stage VII of the seminiferous epithelium (138), while in the mouse its expression is highest at stages IX-I and lowest at stages V-VIII when most cells are quiescent and the majority of $A_{aligned}$ spermatogonia transition to the differentiating A1-A4 cells (85, 98, 139). When GDNF was ectopically overexpressed by Sertoli cells in Stages V-VIII, the number of GFRA1+/LIN28$^-$ germ cells, a subtype of A_s spermatogonia with enhanced self-renewal capacity, was increased (97, 98).

Several mechanisms regulating GDNF expression have been recently proposed. Garcia and colleagues established Sertoli cell-specific gain-of-function and loss-of-function mouse models of NOTCH receptor signaling (80, 100). Constitutive activation of this pathway in Sertoli cells led to a complete lack of germ cells by P2, and infertility. Expression of GDNF by Sertoli cells was

significantly downregulated in the perinatal and adult testis and was due to upregulation of *Hes/Hey* transcription factors, which are canonical NOTCH targets and transcriptional repressors that bind to the GDNF promoter (80, 85). Further, loss-of-function of *Rbpj*, a mediator of NOTCH, and downregulation of *Hes/Hey*, led to upregulation of *Gdnf* expression (80) (**Table 1**). Importantly, the NOTCH ligand JAG1 was expressed mainly by undifferentiated spermatogonia (85). Consequently, the accumulation of undifferentiated spermatogonia around stage VII might increase NOTCH activity in Sertoli cells through JAG1, triggering the observed increase of Hes/Hey inhibitors at this stage and decrease in GDNF expression, leading to its cyclic expression. Therefore, spermatogonia, when in sufficient numbers, regulate their own homeostasis through downregulation of GDNF (55). These data are consistent with the observation that in wild type mice, the absence of germ cells triggered by busulfan treatment correlated with higher expression of GDNF (85, 135, 140) (**Figure 2A**).

Other interesting mechanisms of GDNF regulation have been recently proposed. Given the fact that retinoic acid (RA) concentration is high when GDNF is low during the cycles of the seminiferous epithelium (141), Saracino and colleagues tested whether RA was a direct inhibitor of GDNF expression (142). Using *ex vivo* cultured immature testes and staged adult seminiferous tubules, they showed that negative regulation of *Gdnf* by RA indeed takes place in these models and demonstrated that *Gdnf* expression is directly regulated by RA through a mechanism involving a RARE-DR5 binding site on the *Gdnf* promoter. Negative regulation requires retinoic acid receptor (RARα) and induces a strong decrease of histone H4 acetylation levels around the transcription start. Further, because of the existence of a NF-kappaB binding site in the GDNF promoter, the same group investigated how TNF-alpha might influence GDNF expression (99). They demonstrated that in primary Sertoli cells, TNF-alpha induces the expression of the transcriptional repressor *Hes1* by a NF-KappaB-dependent mechanism, which in turn downregulates GDNF. Therefore, TNF-alpha and NOTCH signaling may converge to regulate the expression of *Hes1* and its target genes, including GDNF (**Figure 2A**).

Fibroblast Growth Factor *(FGF2)*

While GDNF is a critical component of the niche, many *in vivo* and *in vitro* experiments demonstrated that other factors are needed to support maintenance and self-renewal of SSCs. Earlier examination of perinatal Sertoli cells demonstrated that they expressed FGF2, and that this expression was stimulated by follicle-stimulating hormone *in vitro* (FSH) (64). Together with EGF, LIF, and GDNF, fibroblast growth factor (FGF2) has been used to sustain the long-term proliferation of SSCs in culture (66, 143). Further, Takashima and colleagues demonstrated that FGF2 could induce SSC self-renewal alone in culture through activation of the transcription factors ETV5 and BCL6B (**Table 1**) (37, 38, 60, 62, 63, 67). They also showed that FGF2-depleted mouse testes produced increased levels of GDNF, which correlated with SSCs enrichment. This suggests that a balance or complementation between FGF2 and GDNF exists to maintain the stem cell pool (67). More recently, additional studies comparing the effects of GDNF and FGF2 on the proliferation of undifferentiated spermatogonia demonstrated that while both factors expanded the GFRA1+ population, FGF2 rather expanded a subpopulation of cells expressing RARG, which were therefore more susceptible to differentiate (68). This emphasizes the complex nature of signaling and a growth factor demand that is modulated upon the need to maintain germ cell homeostasis.

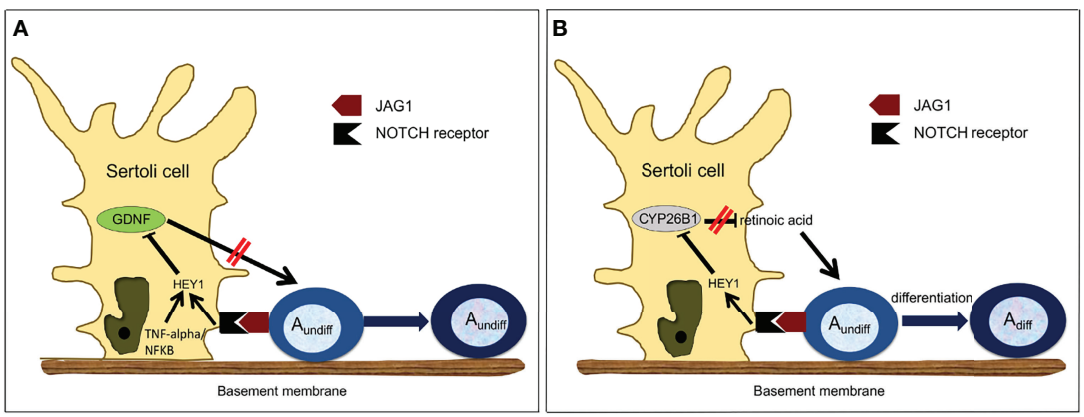

FIGURE 2 | Proposed Model of Regulation of Germ Cell Homeostasis by NOTCH Signaling. **(A)** Regulation of GDNF expression in Sertoli cells. GDNF is produced by Sertoli cells and normally increases Asingle, Apaired and some Aligned spermatogonia proliferation. However, as the number of undifferentiated spermatogonia increases, more JAG1 ligand is available to activate NOTCH signaling in Sertoli cells. Activated NOTCH will down-regulate the expression of GDNF through HES/HEY, which will decrease the number of undifferentiated spermatogonia, re-establishing GDNF production. Inhibition of GDNF by HES/HEY can be potentiated by the TNF-alpha/NF-KappaB pathway. **(B)** Regulation of CYP26B1 expression in Sertoli cells. CYP2681 is produced by Sertoli cells and normally degrades retinoic acid. However, as the number of undifferentiated spermatogonia increases, in particular Aaligned spermatogonia, more JAG1 ligand is available to activate NOTCH signaling in Sertoli cells. Activated NOTCH will down-regulate the expression of CYP26B1, which a llows retinoic acid to trigger the transition from undifferentiated to differentiating spermatogonia.

Other Growth Factors

Platelet-derived growth factor (PDGF) is specifically produced by Sertoli cells. In rodents, PDGF is critical for prospermatogonia proliferation after birth (103, 104) and cooperates with estrogen signaling (106). Exposure to xenoestrogens in the environment might disrupt crosstalk between PDGF and estrogen-driven signaling pathways. This could lead to alteration of prospermatogonia behavior and induce preneoplastic states (105). Vascular endothelial growth factor A (VEGFA) family members and their receptors are all produced by germ cells, Sertoli, cells and interstitial cells (117, 118). However, only the pro-angiogenic isoform VEGFA164 promotes SSC self-renewal, as determined by the SSC transplantation assay (119). WNT signaling plays a role in SSC maintenance (50, 144). WNT5A is produced by Sertoli cells but does not induce self-renewal. It rather promotes SSCs survival through a β-catenin (CTNNB1)-independent mechanism that activates mitogen-activated protein kinase 8 (MAPK8 or JNK) (50). Confirming this data, CTNNB1 ablation in germ cells led to spermatogenesis disruption but not to SSC loss (51, 52). Finally, leukemia inhibitory factor (LIF) has been used for decades to maintain undifferentiated embryonic stem cells *in vitro*, therefore an investigation of its expression in Sertoli cells and its effects on SSCs, at least *in vitro*, was attempted early on (96). LIF production in Sertoli cells was shown to depend on tumor necrosis factor (TNFα) (96) and is still widely used in cultures of primordial germ cells, pro-spermatogonia, and SSCs of many different species. However, LIF does not induce SSC self-renewal, and is rather used to maintain survival and start long-term SSC cultures (10).

SERTOLI CELL FACTORS CONTROLLING SPERMATOGONIAL DIFFERENTIATION

Regulation of KIT/KITL

Activation of the KIT tyrosine kinase receptor by its ligand KITL is required for the survival and proliferation of primordial germ cells (PGCs) (90). KIT is downregulated in pro-spermatogonia, which stop proliferating once they enter the fetal gonads. After birth, KIT is re-expressed in differentiating spermatogonia (87, 88), which proliferate under the influence of KIT ligand (KITL) produced by Sertoli cells. Together with retinoic acid (RA), the KIT/KITL system is important for triggering meiotic entry of type B spermatogonia (92, 93), and KITL has been recently used in culture to differentiate rat spermatogonia without serum or somatic cells (95). Because KIT/KITL signaling is important not only for germ cells, but also for haematopoietic stem cell and melanoblasts, mechanisms controlling KIT transcription have been extensively studied. Further, KIT is mutated in about 25% of seminoma (91), and accounts for secondary mutations that confer resistance to drugs in other cancers. Therefore, regulation of its expression and identification of downstream effectors as druggable targets are of particular interest. Earlier studies have demonstrated that KIT expression in undifferentiated spermatogonia is repressed by PLZF (promyelocytic leukemia zinc finger), which is a transcriptional repressor with local and long-range chromatin remodeling activity (107, 108). Further, Dann and colleagues demonstrated that RA triggered spermatogonial differentiation through downregulation of PLZF (145). Thus, one mechanism by which PLZF maintains the pool of spermatogonial stem cells is through a direct repression of *Kit* transcription. The main mechanism of KIT upregulation involves the helix-loop-helix transcription factors SOHLH1 and SOHLH2 (Spermatogenesis and Oogenesis HLH1/2). Both factors are expressed in differentiating spermatogonia and their deletion leads to the disappearance of KIT-expressing spermatogonia. Further, ChIP-PCR analysis demonstrated that SOHLH1 binds the *Kit* promoter to activate its transcription (115). While investigations have mostly focused on the regulation of KIT, few studies have explored the regulation of KITL expression in the past 10 years. However, one study by Correia and colleagues demonstrated that 100 nM estrogen induced a decrease in *Kit* expression while increasing expression of *Kitl* in adult rat seminiferous tubules cultured ex vivo (89). Altered expression of the KIT/KITL system decreased germ cell proliferation and promoted apoptosis, which is not in accord with the data of previous studies (146).

Regulation of Retinoic Acid Activity

Rats and mice deprived of dietary retinoic acid (RA) can only produce A_{undiff} spermatogonia and are sterile (147, 148). Since these earlier studies, it has been well documented that retinoic acid (RA) activity is a major determinant of the transition between undifferentiated and differentiating germ cells, and that RA also drives the meiotic process and spermatid maturation at stage VIII of the seminiferous epithelium (134, 149). It has been proposed that pulses of RA are triggered around this stage by somatic cells and germ cells to allow proper germ cell differentiation and maturation (150). This implies that RA must be degraded during the other stages. Recently, Parekh and colleagues demonstrated an inverse relationship between the expression of *cytochrome P450 family 26 subfamily B member 1 (Cyp26b1)*, an enzyme that degrades RA (54), and NOTCH activity in Sertoli cells (56). They further provided evidence that in the adult testis activated NOTCH signaling in Sertoli cells down-regulates *Cyp26b1* expression through the HES/HEY transcriptional repressors that bind to the *Cyp26b1* promoter (56). Importantly, expression of these inhibitors is highest at stage VIII of the seminiferous epithelium (85). They also demonstrated that $A_{aligned}$ spermatogonia, through their expression of the NOTCH receptor JAG1, were activating the NOTCH/HES/HEY axis in Sertoli cells and were responsible for *Cyp26b1* down-regulation at stage VIII, allowing RA activity and therefore triggering their own differentiation into A_1 spermatogonia (**Figure 2B**).

CONCLUSION

The Sertoli cell orchestrates spermatogenesis and is a major component of the SSC niche. The past decade has seen an increase in our understanding of these processes at the molecular level. In the perinatal testis, Sertoli cells support multiple aspects of germ cell development through paracrine factors, but the master regulators of the niche and the signaling networks regulating these soluble factors have just begun to be identified. State-of-the-art technologies exist that should help dissect the functions of novel genes and signaling

pathways in Sertoli cells in the future. The efforts that were spent understanding the cyclic regulation of GDNF and Cyp26b1, and by extension RA, should be expanded to other growth and differentiation factors. In particular, surprisingly little is known about the signals that germ cells send to Sertoli cells and their neighboring germ cells. We hope that the use of spatial transcriptomics will help uncover the molecular signals and pathways that germ cells and Sertoli cells use to communicate between each other to direct testis function and maintain homeostasis. We have highlighted JAG1/NOTCH signaling as one possible mechanism that fulfills this role, but other modes of germ cell to Sertoli cell communication exist that still need to be identified.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

FUNDING

This work was funded by NIH R01 HD081244 and NIH R03 HD101650.

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MINI REVIEW
published: 22 June 2022
doi: 10.3389/fendo.2022.895502

The Art of Packaging the Sperm Genome: Molecular and Structural Basis of the Histone-To-Protamine Exchange

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Male fertility throughout life hinges on the successful production of motile sperm, a developmental process that involves three coordinated transitions: mitosis, meiosis, and spermiogenesis. Germ cells undergo both mitosis and meiosis to generate haploid round spermatids, in which histones bound to the male genome are replaced with small nuclear proteins known as protamines. During this transformation, the chromatin undergoes extensive remodeling to become highly compacted in the sperm head. Despite its central role in spermiogenesis and fertility, we lack a comprehensive understanding of the molecular mechanisms underlying the remodeling process, including which remodelers/chaperones are involved, and whether intermediate chromatin proteins function as discrete steps, or unite simultaneously to drive successful exchange. Furthermore, it remains largely unknown whether more nuanced interactions instructed by protamine post-translational modifications affect chromatin dynamics or gene expression in the early embryo. Here, we bring together past and more recent work to explore these topics and suggest future studies that will elevate our understanding of the molecular basis of the histone-to-protamine exchange and the underlying etiology of idiopathic male infertility.

Keywords: sperm chromatin, histone, epigenetics, chromatin remodeling, histone displacement

OPEN ACCESS

Edited by:
Polina V. Lishko, University of California, Berkeley, United States

Reviewed by:
Bernard Robaire, McGill University, Canada

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Specialty section:
This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology

Received: *13 March 2022*
Accepted: *02 May 2022*
Published: *22 June 2022*

Citation:
Moritz L and Hammoud SS (2022) The Art of Packaging the Sperm Genome: Molecular and Structural Basis of the Histone-To-Protamine Exchange. Front. Endocrinol. 13:895502. doi: 10.3389/fendo.2022.895502

INTRODUCTION

Spermatogenesis ensures transmission of genetic information to the next generation by maintaining male fertility throughout life. Three biologically distinct processes safeguard the continuous generation of sperm: mitosis, meiosis, and spermiogenesis – the last of which involves extensive remodeling of both cytoskeleton and chromatin to achieve a significant compaction of the sperm head (1, 2). At both a molecular and structural level, sperm chromatin is highly distinct from the chromatin in oocytes and somatic cells. While nucleosome-based packaging by histone octamers produces a bead-on-a-string structure of chromatin in oocytes and somatic cell nuclei, the sperm genome is packaged by small, arginine-rich basic proteins known as protamines (P1 and P2), which presumably package the DNA into toroidal structures leading to a 10-fold greater chromatin

compaction state than the somatic cell nucleus (2–4). This differential packaging program evolved over 500 million years ago, yet its biological and evolutionary significance remains unknown.

Seminal work used biochemical and genetic approaches to identify intermediate proteins involved in the histone-to-protamine transition; however despite its biological importance, our insight into how chromatin-associated factors/remodelers are involved remains limited (5–9). We lack both genetic and molecular reagents to identify chromatin-associated factors as well as *in vitro* experimental systems to investigate mechanisms. In this review, we summarize the current understanding of chromatin dynamics during spermiogenesis and the advances made to understand sperm chromatin 3D organization. A greater understanding of sperm genome packaging and molecular organization will inform our understanding of how this process is dysregulated in infertility and will aid in the development of clinical assays and therapeutic approaches that may enhance clinical care and reproductive outcomes.

CHROMATIN DYNAMICS DURING SPERMATOGENESIS LEAD TO A UNIQUE PACKAGING MECHANISM OF SPERM CHROMATIN

The histone-to-protamine transition is one of the most poorly understood aspects of spermiogenesis and the sequence of events is also known to vary across species. However, this remodeling process is believed to occur in a stepwise fashion, wherein canonical histones are sequentially replaced by testis-specific histone variants (10–13) followed by transition proteins (TNPs) (14–16) and finally by protamines (17). These sequential events are thought to loosen histone-DNA interactions, thereby facilitating histone removal and permitting protamine incorporation.

Mechanisms Contributing to Nucleosome Destabilization: Histone Variants and Histone Post-Translational Modifications

The hallmark of spermiogenesis is the dramatic reorganization of chromatin in spermatids, in which most histones are sequentially replaced with protamines (**Figure 1**) (5, 6, 18). To achieve this reorganization, the spermatid nucleus undergoes a series of intermediate state transitions, including the incorporation of histone variants (H1t, H2A.X., H2A.Z., H3.3, H3t, TH2A, TH2B) – many of which are testis-specific – during meiosis (19–26) and throughout post-meiotic maturation in round spermatids (H2AL.1/2, HILS) (11, 12, 27, 28). Several *in vitro* studies have demonstrated that the incorporation of histone variants such as H3T, H2AL2, and TH2B induces nucleosome destabilization by altering histone-DNA binding and weakening the associations between H2A/H2B dimers and H3/H4 tetramers, to ultimately promote reorganization of the chromatin (10, 29–31).

Although histone variants are presumed to instruct the chromatin remodeling process, inferring specific roles of these complexes in the exchange process through the analysis of gene loss of function phenotypes is sometimes challenging due to confounding functions outside of the histone-to-protamine exchange. For instance, knockout of H3T results in defective spermatogonial differentiation, ultimately leading to azoospermia (26). On the other hand, H2AL2 knockout males are infertile and exhibit defective genome packaging during spermiogenesis (12). High-resolution electron microscopy (EM) analysis of chromatin compaction in H2AL2 knockout sperm identified more diffuse packaging and many translucent areas, indicative of defective global genome compaction. This defect is due to inefficient assembly of both TNPs and protamines onto chromatin, raising the question of how a histone variant, functioning upstream of both transition proteins and protamines, prevents their proper incorporation onto chromatin (12). However, not all variants incorporated during spermiogenesis are essential for the histone-to-protamine exchange. For example, mice lacking TH2B are fertile because TH2B loss is compensated for by the retention of alternative H2B isoforms and the addition of destabilizing PTMs such as arginine methylation and lysine crotonylation within the histone fold domains of H2A, H2B, H3, and H4, as opposed to the histone tail (25). Similarly, mice lacking the testis-specific linker histone H1t retain alternative H1 isoforms and are fertile (32–34). Therefore, the differences in cellular phenotypes reported for each of the histone variants may be attributed to gene family expansions and the extent to which protein variants have retained ancestral or acquired novel functions. A greater understanding of histone variant evolution and phylogeny may help us predict and/or reconcile reported phenotypes for the different proteins involved in germ cell development and packaging (35–37).

In addition to nucleosome destabilization by the incorporation of histone variants, histone PTMs can alter histone-DNA binding dynamics and aid in promoting chromatin accessibility (**Figure 1**). Preceding the histone-to-protamine exchange, well-documented histone hyperacetylation mechanisms promote chromatin accessibility by inhibiting folding of nucleosomes into chromatin fibers (38–44). Accordingly, genetic knockout of either EPC1 or Tip60, two components of the mammalian NuA4/TIP60 nucleosome acetyltransferase complex, results in a global decrease in H4 hyperacetylation, leading to aberrant spermatid elongation, decreased TNP2 incorporation, and ultimately impaired fertility (45). Similarly, the loss of GCN5, another histone acetyltransferase, in differentiating spermatogonia (using *Stra8-Cre*) leads to aberrant spermatid development and impaired fertility (46). Indicative of defects in remodeling and compaction, conditional GCN5 mutant sperm feature morphological abnormalities such as rounded or blunted triangular-shaped heads. Chromatin characterization further reveals an increase in histone retention and concomitant decrease in sperm protamine levels (46). In related work, loss of the chromatin reader BRDT–which directly binds to acetylated histones and facilitates their removal, thereby initiating repackaging of the genome during spermiogenesis– results in

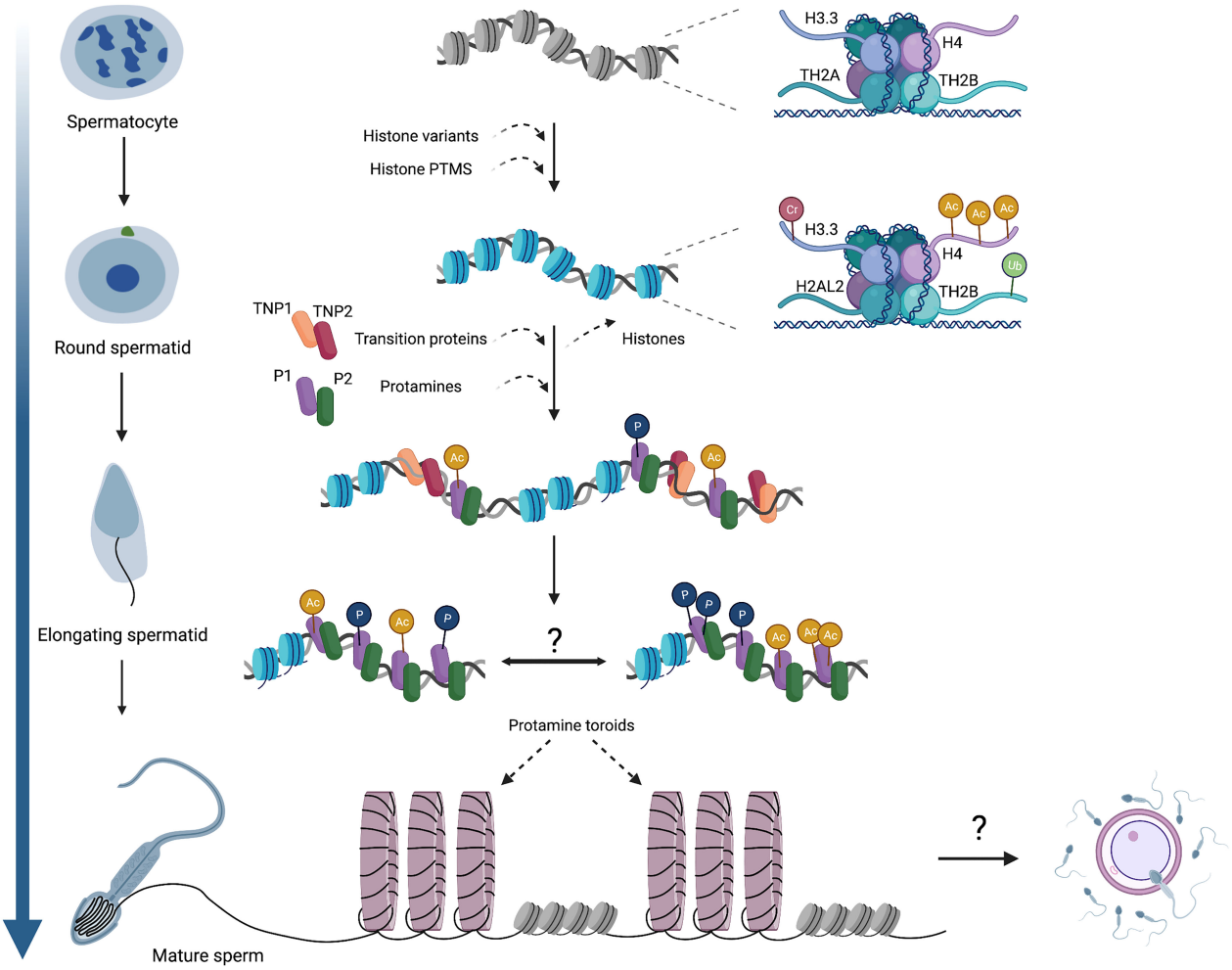

FIGURE 1 | An overview of chromatin dynamics and intermediate stages of the histone-to-protamine exchange. Many histone variants are incorporated in meiotic spermatocytes, including H3.3, TH2A, and TH2B. Histone variant incorporation continues in post-meiotic round spermatids (H2AL2), concomitant with various histone PTMs that induce nucleosome destabilization. As spermatids begin elongation, TNPs and protamines are expressed and incorporated onto chromatin, but whether these act as discrete steps or co-occur remains unknown. It is also established that protamines acquire various PTMs, but the genomic localization of these PTMs (i.e. whether they occur randomly throughout the genome or localize into discrete domains) has not been determined. Ultimately, protamine-DNA binding forms toroidal structures of sperm chromatin, making sperm chromatin distinct from that of both oocytes and somatic cells. The contribution of sperm chromatin structure and the sperm epigenome to embryonic development will also be a fascinating area for future exploration. Cr,crotonylation; Ac, acetylation; Ub, ubiquitination; P, phosphorylation.

decreased chromatin compaction in spermatids, aberrant spermatid elongation, decreased sperm counts, and infertility (47, 48). Together, these studies illustrate that targeted disruption of histone acetylation writers and readers leads to similar phenotypes, underscoring the importance of histone acetylation for histone-to-protamine exchange and sperm function.

Although H4 hyperacetylation is a well-established modification known to precede the histone-to-protamine exchange in multiple species, other modifications, such as di- and trimethylated H3K79, catalyzed by DOT1L, have been reported to temporally overlap with H4 hyperacetylation in both human and mouse spermatids (49, 50). H3K79me3 is enriched at the chromocenter (the constitutive heterochromatin) of round spermatids and at repetitive elements in mESCs, whereas H3K79me2 accumulates at euchromatic regions, often downstream of promoters of actively transcribed genes (51–55). DOT1L loss of function mutants are embryonic lethal (56), therefore preventing the analysis of H3K79 methylation in the histone-to-protamine exchange or spermatid-specific cellular functions. Therefore, a round spermatid-specific conditional knockout of DOT1L or H3K79 point mutant mice will be needed to dissect the impact of the K79 residue or its methylation during spermiogenesis. In related work, histone crotonylation, a newly identified modification, is reportedly enriched in elongating spermatids concomitant with H4

hyperacetylation (49, 57). Histone crotonylation in somatic and germ cells is enriched at TSSs, and largely overlaps with active histone modifications (57). Consistent with a possible role for crotonylation in the histone-to-protamine exchange, CDYL (chromodomain Y-like protein, an eraser of crotonylation) knockout mice exhibit reduced levels of chromatin-bound transition proteins, sperm motility defects, and decreased fertility (58). Given the general enrichment in spermatids for modifications canonically associated with transcriptional activation in somatic cells, together with the well-documented pervasive transcription observed in round spermatids (59–61) and the lack of reported phenotypes for many spermatid-specific expressed genes, this begs the question of whether the physical process of gene transcription may be important in nucleosome destabilization and subsequent exchange–a hypothesis that will need to be evaluated in future studies (62–65).

Transition Proteins

Transition proteins are present in many species including mouse, rat, human, ram, and boar (20, 66, 67). Two major TNPs–TNP1 and TNP2– are prominent in rodent spermatids (68). TNP1 is highly expressed (~2.5x higher in spermatids than TNP2) and conserved in various mammals, while TNP2 sequences are poorly conserved across species and its expression level and protein abundance vary between species (20, 67, 69, 70). Knockout of TNP1 results in male sub-fertility, and sperm exhibit abnormal morphology and decreased progressive motility (71). A detailed analysis of sperm chromatin from $TNP1^{-/-}$ sperm reveals alterations in protein composition–including a compensatory increase in TNP2 in mature sperm as well as an accumulation of unprocessed P2 (71). Interestingly, fertility in $TNP2^{-/-}$ males is unaffected, although progressive sperm motility is decreased, and sperm morphology is slightly abnormal. Like $TNP1^{-/-}$ males, $TNP2^{-/-}$ males also exhibit an increased level of unprocessed P2 in mature sperm. In both $TNP1^{-/-}$ and $TNP2^{-/-}$ males, defects in progressive sperm motility did not impact fertilization rates, as assessed by blastocyst formation resulting from intracytoplasmic sperm injections (ICSI) (72). However, double knockout mice are completely infertile, with a near complete loss of progressive sperm motility and alterations in sperm chromatin composition (72), underscoring the importance of these proteins in finetuning chromatin packaging.

Previous dogma posited that TNPs are incorporated onto chromatin following histone eviction and occupy the majority of the genome in elongating spermatids, thereby acting as intermediates between histones and protamines (73). This initial assumption was based on the knowledge that the two transition proteins- TNP1 and TNP2, are both relatively small and highly basic, with high lysine (~19%) and arginine (~21%) content, that can mediate electrostatic interactions with the phosphate backbone of DNA uniformly along the TNP molecules (74). However, accumulating molecular, genetic, and biochemical data suggest that TNPs may not replace histones completely as initially predicted by the stepwise model.

First, numerous studies observed that transition protein expression does not precede that of protamines, but rather they are co-expressed in spermatids along with other histone variants and can be directly visualized in the spermatid nucleus in specific spermatogenic stages (IX-I, **Figure 1**) (12, 69, 75). This observation suggests the possibility that these proteins act in concert, rather than sequentially, to ensure successful chromatin remodeling. Interestingly, early *in vitro* data shows that TNP1 has a >8-fold affinity for single-stranded DNA (ssDNA) over double-stranded DNA (dsDNA), and in contrast to H1 histone, TNP1 forms less stable structures with DNA even at higher ionic strength (50 mM NaCl), which is still below physiological salt concentrations (76). In contrast, TNP2 has a 40X higher affinity for dsDNA and stabilizes and condenses DNA fibers *in vitro* at a broad range of ionic strengths (77, 78). These observations reveal that DNA binding and stabilizing properties of TNP1 and 2 differ greatly, suggesting that it is unlikely that TNP1 binds dsDNA, but rather it may intercalate between nucleic acid bases resulting in local melting of the DNA duplex, while TNP2 physically replaces histones. However, recent nucleosome invasion assays show that TNP2 does not physically replace canonical nucleosomes or testis-specific variant-containing nucleosomes, but rather TNP2 intercalates the nucleosome, leading to nucleosome destabilization/eviction or TNPs may serve as a scaffold on histones to aid in protamine recruitment/deposition onto chromatin (12). Therefore, various categories of nuclear proteins (histone variants, transition proteins, protamines), act in a concerted manner to mediate a direct transition from histone-to-protamine states, as observed in certain species of birds and fish (79, 80). The differences in the complexity of the remodeling process are intriguing and makes us wonder whether these differences may be due to biochemical and biophysical properties of protamine proteins themselves or whether analogous proteins (variants and TNPs) with similar properties are needed in other species but have not yet been identified.

Protamines

During spermiogenesis, small, sperm-specific, and highly arginine-rich protamines serve to compact paternal DNA, allowing the sperm head to adopt a highly condensed, hydrodynamic shape that protects the paternal DNA during transit to the egg (81, 82). Most mammals, including mice and humans, express two forms of protamine: protamine 1 (P1) and protamine 2 (P2). Rapidly evolving across species (6, 83–86), protamines are subject to strong positive selection that tightly maintains arginine/serine-rich regions, but not strict sequences (85–87). Whether protamines are possibly coevolving with the DNA sequence or if protamines from different species have different binding affinities to certain genomic regions within and across species remains to be determined.

P1 is expressed in its mature form, while P2 is initially expressed as a longer precursor (pro-P2) and undergoes selective proteolytic processing to produce its mature form (P2) once bound to DNA (88, 89). Truncation of the amino terminus of P2, the portion of the protein that is typically cleaved (cP2) in the nucleus, causes infertility due to inefficient import of the protein into the nucleus, resulting in altered protamine ratios and immotile sperm; suggesting that the longer isoform may be required for protamine-chaperone interactions (90).

Across species, the P1:P2 ratio is highly variable but maintenance of a species-specific P1:P2 ratio is critical for normal fertility (91–94). Conversely, alterations in protamine ratios in mice and humans are associated with increased sperm DNA fragmentation, diminished fertilization rates, and defects in sperm morphology and motility (12, 90, 92, 95). Consistent with the importance of P1:P2 ratio correlations, initial haploinsufficiency studies of either P1 or P2 genes resulted in infertility (96). However, subsequent studies using CRISPR-Cas9 engineered P1 or P2-deficient mouse lines found that haploinsufficiency of P1 is sufficient to cause infertility, whereas loss of one P2 allele is tolerated and complete deletion is necessary to cause infertility (97, 98). Together, these results suggest that a defined composition of chromatin is necessary for fertility, and deviations have negative consequences.

Given that protamines were assumed to bind uniformly in the genome and not believed to bear PTMs, their potential role as informational carriers has been largely overlooked. Recent biochemical and mass-spectrometry analysis by us and others led to the discovery that P1 and P2 proteins from mature sperm carry multiple PTMs, including phosphorylation, acetylation, and methylation (95, 99). Dynamic phosphorylation/dephosphorylation of protamines was previously suggested to have a role in modulating protamine-DNA dynamics in a variety of species (100–103). Analysis of radiolabeled proteins from mouse and rat seminiferous tubules by acid urea gel electrophoresis revealed that newly synthesized protamines are phosphorylated and subsequently dephosphorylated shortly after their deposition onto DNA (88), a phenomenon also observed in human sperm (101, 102). More recent studies reported comprehensive catalogs of mouse and human protamine PTMs, with ~53% of P1 peptides in mouse containing PTMs and ~16% of P2 peptides (99, 104). Importantly, the sites of protamine modifications are maintained within a species but not conserved across species, suggesting that these modifications may confer a lineage-specific function (95). The identification of protamine PTMs was surprising since these proteins are placed onto DNA after meiosis and during spermatid maturation–when all transcription in germ cells has halted, suggesting that these modifications have no effect on spermatid gene expression. Rather, these modifications may be required for either 1) mediating protamine protein deposition onto DNA and/or regulating sperm genome packaging, 2) conveying epigenetic information to the zygote, or 3) instructing paternal genome chromatin reorganization.

Indeed, recent studies suggested that protamine phosphorylation during spermiogenesis is important for modulating protamine-DNA dynamics and maximizing chromatin compaction (105, 106). Recently, Gou et al. reported that phosphorylation of serine residues in P1 during early embryogenesis is required to weaken protamine-DNA interactions, thereby permitting male pronuclear remodeling and protamine-to-histone exchange (106). Additionally, we found that loss of acetylation at P1 lysine (K) 49 drastically alters sperm chromatin composition and results in subfertility in the mouse, premature dismissal of P1 from paternal chromatin in the zygote and altered DNA compaction and decompaction rates *in vitro* (95). Together, these studies establish a regulatory role for protamine PTMs in governing sperm chromatin packaging and unpacking in the embryo. Whether PTMs on human protamines similarly influence these processes remains to be determined. Additionally, assessing whether alterations in protamine PTM levels affect embryonic gene expression, as is the case for alterations in histone levels/PTMs, will further provide insight into the function of these modifications *in vivo*.

Although the histone and protamine packaging systems were discovered decades ago, we know relatively little about whether protamine protein placement varies along the sperm genome and how they are placed onto DNA, relative to the wealth of data available for histone proteins. The current models suggest that protamine proteins bind uniformly throughout the genome, but definitive data to support or refute such a model are lacking. The super-condensed protamine-packaged chromatin state does not easily lend itself to mechanistic investigations. Moreover, the scarcity of lysine residues in protamines makes it difficult to crosslink protamine proteins and DNA to prevent protamine on/off dynamics, which can lead to non-biological associations during sample processing.

CHROMATIN REMODELERS INVOLVED IN HISTONE-TO-PROTAMINE EXCHANGE

Studies of chromatin-associated factors/remodelers involved in sperm chromatin remodeling are hampered by the lack of genetic and molecular reagents with which to identify chromatin-associated factors *in vivo* and the lack of experimental systems to model the histone-to-protamine exchange process *in vitro*. However, candidate gene knockout studies have begun to shed insights. For example, in a full body knockout of Chromodomain Helicase DNA Binding Protein 5 (CHD5), with phenotypes ranging from subfertility to infertility, the infertility is not caused by changes in the hypothalamic pituitary axis or somatic cell numbers. Instead, the infertility appears to be germ cell-intrinsic; presenting as defects in spermatid elongation and condensation defects, consistent with CHD5 expression in steps 7-10 of spermatid maturation, immediately preceding and overlapping with the extensive chromatin remodeling (107, 108). Biochemical fractionation of spermatids shows that CHD5 deficiency perturbs histone hyperacetylation and the histone-to-protamine transition, leading to aberrant retention of histones and elevated levels of transition proteins and protamines (107, 108). The overall higher level of protamine mRNA and protein expression in $CHD5^{-/-}$ males, assessed by qPCR and immunoblotting, indicates a possible role for CHD5 in protamine transcriptional and/or translational control (107).

Other studies have explored the roles of ATP-dependent chromatin remodeling complexes SWI/SNF (SWItch/Sucrose Non-Fermentable) and ISWI (Imitation SWItch). A knockout of BRG1 (a SWI/SNF component and transcription activator) in germ cell progenitors resulted in a mid-pachytene arrest,

preventing investigations in post-meiotic round spermatids (109). The zinc finger and bromo-domain protein ACF1/BAZ1A, a component of ISWI, binds to the chromatin remodeler SNF2H and plays an essential role during post-meiotic spermiogenesis, as evidenced by its deletion resulting in infertility with increased DNA damage and spermiation defects (110). At a general level, deletion studies are confounded by upstream functions in spermatogenesis, making it difficult to investigate the specific role of chaperones/remodelers in nucleosome eviction/protamine deposition and to discern whether histone removal and protamine deposition are functionally distinct processes that require unique or shared proteins. As the process of spermiogenesis occurs within the testis, and its byproduct is sperm DNA compaction, monitoring the remodeling process in a living organ is not possible. However, the combination of future targeted proteomic analyses with an *in vitro* chromatin remodeling system holds promise for identifying candidate remodelers and uncovering molecular details of their roles in the histone-to-protamine exchange.

SOMETHING OLD, SOMETHING NEW: EXPERIMENTAL APPROACHES TO UNDERSTAND SPERM STRUCTURE AND 3D ORGANIZATION

Decades of *in vitro* biochemistry and biophysics experiments have provided fundamental insights into protamine-DNA interactions and the structure of sperm chromatin imposed by protamine binding. Early *in vitro* studies primarily relied on measuring the behavior and properties of either polyarginine/polylysine peptides or purified salmon or bull (domestic cattle, *Bos taurus*) sperm protamine (111–116). Raman and nuclear magnetic resonance (NMR) spectroscopy using a polyarginine (R6WGR6) peptide – a representative sequence of the central arginine-rich domain of bull P1 – suggested that protamines bind preferentially to the major groove of DNA, with one protamine molecule bound per turn of the helix (117). Using Particle Induced X-ray emission, *in vivo* measurements of the total amount of nuclear phosphorous and sulfur in sperm from various species estimated that bull P1 binds ~10-11 base pairs of DNA. Assuming that the P1 binding to DNA mode is conserved across species, and given known P1:P2 ratios, calculations of phosphorous:sulfur ratios predict that P2 binds ~15 base pairs, although the exact footprints of P1 and P2 remain to be determined (115).

Several early studies examining the 3-dimensional topology of the sperm genome indicated that sperm DNA, like somatic cell DNA, forms loops, as inferred by the formation of nuclear "halos" when sperm are treated with SDS and stained with ethidium bromide (118–121). The loops formed by hamster sperm were noted to be smaller than those found in somatic cell nuclei by ~60%, and to consist of ~50 kb of DNA on average. Furthermore, these loops are anchored to a structural component of the sperm nucleus – termed the nuclear matrix – at attachment sites known as matrix attachment regions, or MARs (120–126). When isolating DNA loops or nuclear matrices and analyzing the localization of a handful of genes, early data suggested that the 5SRNA gene enriches at the nuclear matrix, while satellite DNA is detected in loops (120, 125, 127, 128), suggesting that DNA organization and sites of DNA attachment to the matrix may not be random, but programmatic. However, future studies are needed to explore such assumptions genome-wide and determine whether MARs are associated with specific DNA sequences or with specific chromatin (histone or protamine-bound) in sperm.

The molecular nature of sperm genome organization was initially difficult to resolve because sperm decondensation by chemical agents was necessary to visualize sperm DNA, which prevented the investigation of the structure of unperturbed sperm chromatin *in vivo*. However, by examining intact native sperm or reconstituted salmon sperm protamine with either lambda phage DNA or linearized plasmid DNA, using a variety of techniques including light scattering (129, 130), electron and atomic force microscopy (116, 131), fluorescence microscopy (132, 133), and DNA elasticity measurements (134), it was discovered that protamine-DNA complexes *both in vivo* and *in vitro* were organized into toroidal structures. The identification of a toroid is intriguing given that other positively charged molecules, including hexamine-cobalt (III), spermine, and spermidine, have also been shown to form DNA toroids (135, 136). While toroids are the identified packaging unit, the exact mechanism of folding and unfolding of the toroid is unknown, but presumed to be mediated by single loops coming together and then separating back out. Recent studies using tethered particle motion assays and AFM found that salmon protamine uses a multi-step process, forming multiple independent loops of a roughly defined diameter that come together before forming a larger toroidal structure (137). Furthermore, the formation of these structures relies on protamine binding-and-bending the DNA, whereby multiple protamine molecules bind locally to a DNA segment to induce bending of the DNA filament to form loops (138). These data are in agreement with previous studies that identified loops formed by sperm DNA *in vivo* (119, 121, 124) as well as our recent EMSA and single molecule DNA curtain assays, which suggest that large-scale genome compaction by protamines is achieved by protamine protein cooperativity (95). Although these experiments provide a basic foundation of knowledge of sperm genome packaging, these data rely on protamines from teleost fish or bull P1 proteins, which are highly divergent from both mouse and human protamines in both sequence and amino acid composition. Therefore, we are currently presuming that protamines from all species display a stereotypic random association with DNA that is sequence-independent. Future studies utilizing mammalian proteins or multiple protamine protein proteoforms (P1, 2, and/or 3) are needed to explore whether packaging is universal regardless of source or combination of proteins used. By learning how protamines guide sophisticated genome self-assembly, one may utilize the inherent rules of cellular machineries to synthesize designer molecular structures *in vitro* which can be used for gene therapy delivery.

Multiple groups have taken advantage of chromatin capture assays to allow high-resolution mapping of the 3D organization of

not only sperm chromatin across a variety of species, but also of pre-implantation embryos, providing foundational insight into sequence-level 3D chromatin organization from gametes to the next generation. Initial Hi-C studies in mouse sperm curiously found that despite sperm being packaged by protamines, sperm 3D organization resembles both fibroblast (139) and mESC (140) genome organization, with the exception that sperm from mouse and macaque possess a significant number of long-range interactions (>2 Mb), with a significant fraction of these interactions being between TADs (141, 142). Likely, these extra-long-range interactions aid in either establishing or maintaining the hypercondensed state of the sperm nucleus. In contrast, zebrafish sperm, which completely lack protamines, lack TADs altogether, and resemble mitotic chromosomes. Contact matrices exhibit "flare-like" structures, indicative of clustering of large extended genomic loops at a set point that is equidistant for all loops (143). Analysis of these flares illustrated that zebrafish sperm do indeed display periodic domains of ~150 kb that repeat every 1-2 mega bases–a chromatin structure resembling the mitotic cell chromatin landscape, and suggesting that the overall 3D chromatin architecture of the zebrafish sperm genome may be distinct from protamine-bound sperm genomes (144). However, since the 3D chromatin structure of a zebrafish sperm, which is fully packaged in histone, is different from somatic cells, this begs the question of whether the published structures of mammalian sperm, which resemble somatic cells and mESCs, are truly representative of the *in vivo* architecture. Given its hyper-condensed state, the protamine-packaged genome is poorly accessible to restriction enzymes. Therefore, applying current Hi-C technology in mammalian sperm is likely to be particularly technically challenging, requiring methodological innovations before Hi-C can be leveraged towards generating a comprehensive view of *the in vivo* sperm genome architecture.

CONCLUSIONS AND FUTURE PERSPECTIVES

Protamine-based compaction of paternal DNA and the unique sperm chromatin state have fascinated scientists for decades. We have gained foundational knowledge about the histone-to-protamine transition, yet, we still lack a comprehensive understanding of the mechanisms governing critical steps of the exchange process. Specifically, it remains unknown which specific factors are required for histone eviction/protamine deposition and importantly, how all basic proteins function together to ensure successful exchange. Future studies examining whether histone variants, transition proteins, and protamines truly function as independent intermediates or act in combined mechanisms will shed light on the regulation of this process and inform development of targeted interventions to treat infertility. The recent discovery of protamine PTMs suggest that nuanced interactions may control aspects of the exchange process and chromatin condensation during spermiogenesis, but whether these modifications constitute a species-specific code analogous to the histone code for instruction of development remains to be determined. Lastly, while both classical and modern approaches have been applied towards understanding the structure of sperm chromatin, structure determination by cryo-EM will undoubtedly provide a more complete picture. These future studies will not only significantly increase our understanding of sperm genome packaging, but may aid in our understanding of idiopathic male infertility or eventually lead to the development of clinical assays that can better predict reproductive success.

AUTHOR CONTRIBUTIONS

LM contributed to manuscript writing and revision. SH contributed to manuscript writing and revision. All authors contributed to the article and approved the submitted version.

FUNDING

Research in the Hammoud lab is supported by National Institute of Health (NIH) grants 1R21HD090371-01A1 (SSH), 1DP2HD091949-01 (SSH), R01HD104680 (SSH), training grants NSF 1256260 DGE (LM), Rackham Predoctoral Fellowship (LM), T32GM007315 (LM), and Open Philanthropy Grant 2019-199327 (5384) (SH).

ACKNOWLEDGMENTS

We thank members of the Hammoud Lab for scientific discussions and manuscript comments. **Figure 1** was created using BioRender.com.

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ORIGINAL RESEARCH
published: 14 July 2022
doi: 10.3389/fendo.2022.896193

Spermatozoa Develop Molecular Machinery to Recover From Acute Stress

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This study was designed to search for the possible mechanism(s) of male (in/sub)fertility by following the molecular response of spermatozoa on acute psychological stress (the most common stress in human society) and on a 20-h time-dependent recovery period. To mimic *in vivo* acute stress, the rats were exposed to immobilization once every 3 h. The recovery periods were as follows: 0 (immediately after stress and 3 h after the light is on—ZT3), 8 (ZT11), 14 (ZT17), and 20 (ZT23) h after stress. Results showed that acute stress provoked effects evident 20 h after the end of the stress period. Numbers of spermatozoa declined at ZT17 and ZT23, while functionality decreased at ZT3 and ZT11, but recovered at ZT17 and ZT23. Transcriptional profiles of 91% (20/22) of tracked mitochondrial dynamics and functionality markers and 91% (20/22) of signaling molecules regulating both mitochondrial dynamics and spermatozoa number/functionality were disturbed after acute stress and during the recovery period. Most of the changes presented as increased transcription or protein expression at ZT23. The results of the principal component analysis (PCA) showed the clear separation of acute stress recovery effects during active/dark and inactive/light phases. The physiological relevance of these results is the recovered positive-acrosome-reaction, suggesting that molecular events are an adaptive mechanism, regulated by acute stress response signaling. The results of the PCA confirmed the separation of the effects of acute stress recovery on gene expression related to mitochondrial dynamics, cAMP, and MAPK signaling. The transcriptional patterns were different during the active and inactive phases. Most of the transcripts were highly expressed during the active phase, which is expected given that stress occurred at the beginning of the inactive phase. To the best of our knowledge, our results provide a completely new view and the first presentation of the markers of mitochondrial dynamics network in spermatozoa and their correlation with signaling molecules regulating both mitochondrial dynamics and spermatozoa number and functionality during recovery from acute stress. Moreover, the interactions between the proteins important for spermatozoa homeostasis and functionality (MFN2 and PRKA catalytic subunit, MFN2 and p38MAPK) are shown for the first time. Since the existing literature suggests the importance of semen quality and male fertility not only as the fundamental marker of reproductive health but also as the fundamental biomarkers of overall health and

OPEN ACCESS

Edited by:
Vassilios Papadopoulos, University of Southern California, United States

Reviewed by:
Pierre Leclerc, Laval University, Canada
Jinyong Chung, Johns Hopkins Medicine, United States

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Specialty section:
This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology

Received: *14 March 2022*
Accepted: *06 June 2022*
Published: *14 July 2022*

Citation:
Starovlah IM, Radovic Pletikosic SMR, Tomanic TM, Medar MLJ, Kostic TS and Andric SA (2022) Spermatozoa Develop Molecular Machinery to Recover From Acute Stress. Front. Endocrinol. 13:896193. doi: 10.3389/fendo.2022.896193

harbingers for the development of comorbidity and mortality, we anticipate our result to be a starting point for more investigations considering the mitochondrial dynamics markers or their transcriptional profiles as possible predictors of (in/sub)fertility.

Keywords: acute psychological stress, stress recovery, mitochondrial dynamics and functionality markers, cAMP signaling markers, MAPK signaling markers, spermatozoa number and functionality

INTRODUCTION

Stress is an important adaptive response of an organism that enables survival and maintains homeostasis (1). However, if it is repeated or persistent/chronic, it can cause diseases (2–6). Many epidemiological studies showed that DNA damage during stress response is regulated through adrenergic signaling (7). It is clear that different types of stress and stressful life events have been linked to reduced adult male reproductive function (8–11). Numerous studies reported connection between male (sub/in) fertility and stressful life (8, 12–14). However, mechanisms causing the (sub/in)fertility are not described yet.

Mitochondria are a very important linking point between stress response and spermatozoa functionality since these organelles are able to produce enormous levels of energy required for both processes (2, 3, 6, 13). Moreover, signaling pathways activated by stress hormone receptors are important for homeostasis of mitochondrial network and spermatozoa functionality (10, 11, 15). The mtDNA is required for male fertility (16) and could be a diagnostic marker for sperm quality in men (17). The disturbed mtDNA was observed in oligo-asthenozoospermic patients (18) and in asthenoteratozoospermia-induced male infertility (19). Since the mitochondrial morphology changes during spermatogenesis (20), the disturbed ultrastructure of mitochondria can explain some of the unexplained cases of asthenozoospermia (21). Moreover, the mitochondrial membrane potential is also important for spermatozoa functionality (22–25). The reduced mtDNA content in human sperm (26) and the expression of TFAM gene correlate with abnormal spermatozoa forms (27, 28). Furthermore, human sperm motility and viability are regulated by mitophagy (29) as well as UCP2 (30) and the MFN2 expression levels (31). Thus, the mitochondria are a crucial organelle for spermatozoa wellbeing and fertility (13).

The homeostasis of the mitochondrial network is regulated by intriguing processes of mitochondrial dynamics including mitochondrial biogenesis, mitofusion, mitofission, and mitophagy (32–35). All processes involve a complex and multistep molecular event required for renewal, adaptation, or expansion of the mitochondrial network (26, 36–38). The main molecular markers of mitochondrial dynamics are not only the main markers of mitochondrial biogenesis (PGC1α, PGC1β, NRF1, NRF2, and TFAM), mitofusion (MFN1, MFN2, and OPA1), mitofission (DRP1 and FIS1), and mitophagy (PINK1 and PARKIN), but also important markers of the respiratory chain function (32–35, 37, 38). In addition, maintaining homeostasis of the mitochondrial network requires intriguing and complex network of signaling pathways (33, 36, 38), which are able to convey a wide variety of different environmental signals: stress (39, 40), temperature (41), energy deprivation (38), availability of nutrients (38), and growth factors (42).

It is important to point out that all signaling pathways regulating mitochondrial dynamics are required for spermatozoa homeostasis (43). Similar signaling pathways are involved in regulation of the function of sperm flagellum (44). Additional complications related to understanding the regulation of spermatozoa functionality are findings that show that murine germ cells highly express genes involved in steroidogenesis and other cell functions, such as genes involved in fatty acid metabolism or synthesis. This supports the possibility of an additional level of regulation of spermatogenesis (45, 46).

In search for mechanisms activated by and during stress, we explored molecular events in spermatozoa at four time points in a 20-h time-dependent recovery period after acute stress (once for the duration of 3 h, 7 a.m. to 10 a.m.). Acute stress was chosen since it is the most common stress in human society. Recovery was followed at a different time points during the day (light/inactive and dark/active phase): immediately after acute stress (ZT3) as well as 8 (ZT11), 14 (ZT17), and 20 (ZT23) h after acute stress. Number and functionality of spermatozoa, as well as the transcriptional profiles of 22 mitochondrial dynamics and function markers and 22 related signaling molecules were followed (**Figures 1**–**13**). Two rationales were prevalent in the decision to follow spermatozoal functionality by acrosome reaction. First, acrosome reaction is the event in the timeline that is closer to fertilization than motility or other parameters. Second, working with human samples (640 samples were collected over the last 18 months) from men attending the national IVF program, we came to learn that there are significant numbers of normozoospermic samples with good motility and other parameters of spermiogram, but with negative acrosome reaction, suggesting the possible reason for entering the IVF program.

MATERIALS AND METHODS

All experiments were carried out in the Laboratory for Reproductive Endocrinology and Signaling and Laboratory for Chronobiology and Aging, Faculty of Sciences at University of Novi Sad (wwwold.dbe.pmf.uns.ac.rs/en/nauka-eng/lares). Methods used in this study were carried out following relevant guidelines and regulations and were reported previously [for all references, please see (10, 11, 47)]. Key resource tables and tables containing primers and antibody data are provided in the **Supplementary Material**.

Statement of the Institutional Review Board

The manuscript is approved by the Committee of the Faculty of Sciences, University of Novi Sad, Novi Sad, Serbia.

The authors complied with ARRIVE guidelines and all experiments were in adherence to the ARRIVE guidelines. All experimental protocols were approved (statement no. 01-201/3) by the local Ethical Committee on Animal Care and Use of the University of Novi Sad operating under the rules of the National Council for Animal Welfare and the National Law for Animal Welfare (copyright March 2009), following the NRC publication *Guide for the Care and Use of Laboratory Animals* and the NIH *Guide for the Care and Use of Laboratory Animals*.

Animals and Experimental Model of Acute Stress With a Recovery Period

Adult, 3-month-old, male Wistar rats were used in all experiments. Animals were bred and raised in the accredited Animal Facility of the Faculty of Sciences, University of Novi Sad, Serbia, in controlled environmental conditions [22 ± 2°C; 14-h light and 10-h dark cycle, lights on at 07:00 a.m. (ZT0)] with food and water *ad libitum*. The experimental model of psychophysical stress by immobilization (IMO) was performed by the method previously described (10, 11, 47, 48). To analyze the effects of acute stress with the recovery period (48), animals were subjected to immobilization stress (IMO) for 3 h, once, from ZT0 to ZT3 (1x3hIMO), and allowed to recover (1x3hIMO+R) for 0, 8, 14, and 20 h after the IMO (ZT3, ZT11, ZT17, and ZT23; ZT0 is a time when the light is turned on) (**Figure 1**). The experimental model of psychophysical stress by immobilization was performed by the method previously described (9–11). In short, rats were bound in a supine position to a wooden board by fixing the rats' limbs using thread, while the head motion was not limited. Unstressed, freely moving rats were present as a control group (Control) in each experiment. All activities during the dark phase were performed under the red light. At the end of the experimental period, control and stressed animals were quickly decapitated without anesthesia and trunk blood was collected. In each experiment, both control and stressed animals were randomly divided into four time point groups, with a total of 4 animals in the control group and 6 animals in the 1x3hIMO+R group per time point. The sample size was checked by Power Analysis using the G Power software (http://core.ecu.edu/psyc/wuenschk/Power.htm) according to previously published results. The experiment was repeated two times.

Spermatozoa Isolation and Their Functionality Assessment (Capacitation and Acrosome Reaction)

Isolation of caudal epididymides spermatozoa was carried out following the WHO laboratory manual (https://www.who.int/publications/i/item/9789240030787) with modifications for rat spermatozoa isolation. In short, caudal epididymides were

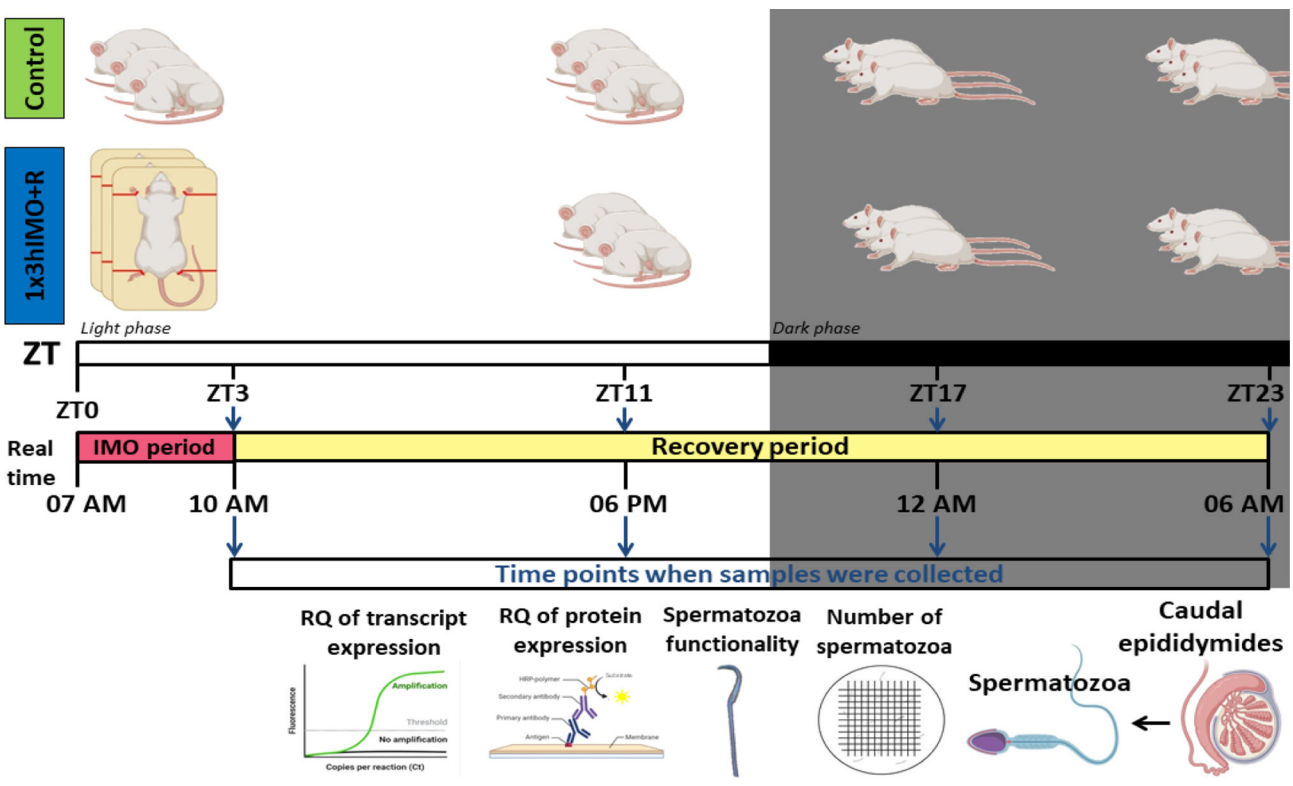

FIGURE 1 | Experimental design of immobilization stress with recovery period used to assess spermatozoa number and functionality (% acrosome reaction) as well as mitochondrial dynamics markers and related signaling molecule expression profiles of transcripts and proteins.

quickly isolated, the surrounding adipose tissue was removed, and epididymides were placed in a petri dish containing medium for isolation and preservation of spermatozoa (1% M199 in HBSS with 20 mM HEPES buffer and 5% BSA) or Whitten's Media (100 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 5.5 mM glucose, 1 mM pyruvic acid, and 4.8 mM lactic acid), depending on the subsequent analysis. Isolated epididymides were finely punctuated with a 25G needle to enable spermatozoa to be released into the medium, and incubated at 37°C for 10 min. Released spermatozoa were collected and centrifuged for 5 min at 700×*g* at room temperature. The supernatant was removed, and the pellet was resuspended in the appropriate medium depending on the subsequent analysis. Concentrations of isolated spermatozoa were calculated using a Makler counting chamber (Sefi-Medical Instruments, Ltd, Israel). Isolated spermatozoa were used for the capacitation and acrosome reaction procedure and the rest of the spermatozoa were stored at −70°C, before RNA isolation and the subsequent gene transcription analysis. To determine the spermatozoa functionality, approximately 1.5×10^5 spermatozoa in 50 µl of Whitten's Media were mixed with 350 µl of WH+ media [Whitten's Media supplemented with the 10 mg/ml BSA (bovine serum albumin) and 20 mM of $NaHCO_3$, to stimulate the capacitation] with a drop of mineral oil, at 37°C (5% CO_2) for 1 h. Fifty microliters of capacitated spermatozoa was transferred into two new tubes, one without the progesterone, present as the control of the acrosome reaction, and one with 15 µM progesterone (PROG) to activate the acrosome reaction, with a drop of mineral oil, and incubated at 37°C (5% CO_2) for 30 min. For the fixation of spermatozoa after the acrosome reaction, 20 µl of the spermatozoa suspension from each tube was mixed with 100 µl of the fixation solution (20 mM Na_2HPO_4, 150 mM NaCl, and 7.5% formaldehyde) and incubated for 20 min at room temperature. Subsequently, fixed spermatozoa were centrifuged for 1 min at 12,000×*g*, and washed with 100 mM ammonium acetate, pH 9. Smears of fixed spermatozoa were prepared on microscopic slides and air-dried. Dried spermatozoa smears were stained using staining solution (0.04% Coomassie Blue–G250, 50% methanol and 10% acetic acid) for 5 min at room temperature. Staining solution was rinsed with distilled water and spermatozoa smears were allowed to air-dry. Stained smears were analyzed using the Leica DMLB 100T microscope with 1,000× magnification, and up to 100 spermatozoa per slide were counted to determine the acrosomal status. Blue staining in the acrosomal region of the head indicated intact acrosome, while spermatozoa without blue staining in the acrosomal region were considered to be acrosome-reacted. Data are presented as the percentage of acrosome-reacted spermatozoa ± SEM.

Isolation of RNA and cDNA Synthesis

Total RNA isolation was performed using the GenElute Mammalian Total RNA Miniprep Kit according to the protocol recommended by the manufacturer, followed by the DNase I (RNase-free) treatment. The first-strand cDNA was synthesized using the High-Capacity Kit for cDNA preparation.

Relative Quantification of Gene Expression

Rat spermatozoa samples isolated from caudal epididymides were stored at −70°C until they were used for the isolation of total RNA. Total RNA isolation was performed using the GenElute Mammalian Total RNA Miniprep Kit according to the protocol recommended by the manufacturer (Sigma Aldrich, Germany, https://www.sigmaaldrich.com). To eliminate DNA from the samples, DNase I (RNase-free) treatment was carried out according to the manufacturer's instructions (New England Biolabs, Massachusetts, United States, https://www.neb.com). The concentration and purity of isolated total RNA were measured using the BioSpec-nano spectrophotometer (Shimadzu, Japan, https://www.shimadzu.com). Furthermore, the first-strand cDNA was synthesized using the High Capacity Kit for cDNA preparation according to the manufacturer's protocol (Thermo Fisher Scientific, Massachusetts, United States, https://www.thermofisher.com). In each set of reactions, negative controls consisting of non-reverse-transcribed samples were included. Quality of RNA and DNA integrity was checked using control primers for *Gapdh*, as described previously by our group [for references, please see (10, 11, 47)]. Relative expression of genes was quantified by real-time PCR (RQ-PCR) using SYBR Green-based chemistry from Applied Biosystems. Each reaction contained 10 ng of cDNA (calculated from starting RNA) in a volume of 2.5 µl and specific primers at a final concentration of 500 nM. Primer sequences used for RQ-PCR analysis, average Ct values, as well as GenBank accession codes for full gene sequences are given in **Supplementary Tables S5–S11**. Relative gene expression quantification of *Gapdh* was measured in each sample and used to correct variations in cDNA content between samples. Relative quantification of each gene was performed in duplicate, three times for each sample of two independent *in vivo* experiments. The real-time PCR reactions were carried out in the Eppendorf Mastercycler ep realplex 4 Real Time PCR and post-run analyses were performed using Mastercycler ep realplex Software. The heat map image was generated with relative fold change values, using the online tool CIMminer (http://discover.nci.nih.gov/cimminer/home.do as of December 13, 2021) to represent the gene expression profile of mitochondrial dynamic and functionality markers and signaling molecules regulating mitochondrial dynamics and functionality in different time points after the acute immobilization stress.

Relative Quantification of Protein Expression and Immunoprecipitation Analysis

Rat spermatozoa samples isolated from caudal epididymides were frozen and stored at −70°C until protein extraction. Cells were lysed and Western blot analysis was performed as described previously (9). Immune-reactive bands were detected using MyECL Imager (Thermo Fisher Scientific Inc.; https://www.thermofisher.com) and analyzed as two-dimensional images using Image J version 1.48 (http://rsbweb.nih.gov/ij/download.html). The optical density of images is expressed as volume adjusted for the background, which gives arbitrary units of adjusted volume. Normalization of the data was done using GAPDH protein expression as the

endogenous control. Immune detection was performed with different antibodies (all details are listed in **Supplementary Table S12**). Antibodies against PGC1, NRF1, NRF2, and GAPDH were purchased from Santa Cruz Biotechnology (https://www.scbt.com).

Spermatozoa samples for immunoprecipitation analysis were lysed in 1 ml of buffer containing 20 mM HEPES, 10 mM EDTA, 2.5 mM $MgCl_2$, 40 mM β-glycerophosphate, 1 mM DTT, 1% NP-40, 0.5 mM 4-(aminoethyl)-benzenesulfonyl fluoride hydrochloride, 1 μM aprotinin, 2 μM leupeptin, and phosphatase inhibitor cocktail tablets [cont. (1R, 2S, 3R, 6S)-1.2-dimethyl-3.6-epoxycyclohexane-1.2-dicarboxylic anhydride]. The concentration of proteins in each sample was estimated by the Bradford method and set at a concentration of 300 μg/ml. An equal amount of protein in each sample (300 μg) was used for the immunoprecipitation. Pre-clearing of the lysate was done using 5 μl of normal goat serum [Santa Cruz Biotechnology, normal goat serum: sc-2043, (https://www.scbt.com)] mixed with 1 ml of lysate and incubated on ice for 1 h. After the incubation, 100 μl of bead slurry was added to each sample and incubated for 30 min at 4°C with gentle agitation. The supernatant for the immunoprecipitation was collected after 10 min and centrifuged at 14,000×*g* at 4°C. After the pre-clearing process, lysates were mixed with MFN2 antibody (Santa Cruz Biotechnology) and incubated at 4°C overnight with constant rotation. During additional overnight incubation at 4°C with constant rotation, immunoprecipitated complexes with MFN2 antibody were recovered by 80 μl of protein G agarose bead slurry. Precipitated proteins were washed two times with 1 ml of lysis buffer and the supernatant was used for further protein analysis (please see **Supplemental Material file**). Final pellets were mixed with protein loading dye, incubated at 100°C for 5 min, and resuspended in the SDS-PAGE 12% gels. Gels were analyzed by one-dimensional SDS-PAGE and proteins were transferred to a polyvinylidene difluoride membrane using a wet transfer. The immunodetection of the MFN2, PRKAc, and p38 MAPK was done with the use of MFN2 antibody (Santa Cruz Biotechnology), PRKAc antibody (BD Transductions Laboratories), and p38 MAPK antibody (Cell Signaling Technology) (all details are listed in **Supplementary Table S12**). Immune-reactive bands were detected using MyECL Imager (Thermo Fisher Scientific Inc.; https://www.thermofisher.com) and analyzed as two-dimensional images using Image J version 1.48 (http://rsbweb.nih.gov/ij/download.html). The optical density of images is expressed as volume adjusted for the background, which gives arbitrary units of adjusted volume. Normalization of the data was done using MFN2 protein expression.

Statistical Analysis

Results of the experiments represent group means ± SEM values of the individual variation from two independent experiments. In each experiment, both control and stressed animals were randomly divided into four time point groups, with a total of 4 animals in the control group and 6 animals in the 1x3hIMO+R group per time point. In each of the two experiments, both control and stress animals were randomly divided into four time-points groups. In the first experiment, spermatozoa samples of each individual animal were used for the RNA extraction, individual cDNA, individual real-time PCR for the analysis of relative expression of transcripts, individual for protein extraction. While in the second experiment spermatozoa sample of each animal were pooled. Results from each experiment were analyzed by Mann–Whitney's unpaired nonparametric two-tailed test (between the 1x3hIMO group and the control group within the same time point), or by one-way ANOVA followed by Dunnett's test, for comparison with the ZT3-Control group. All statistical analyses were performed using GraphPad Prism 5 Software (GraphPad Software 287 Inc., La Jolla, CA, USA). In all cases, p-value <0.05 was considered to be statistically significant.

Principal Component Analysis

Principal component analysis (PCA) was done with the dudi.PCA() function implemented in "ade4" package (49), on scaled and centered data matrix, within the R environment. We decided to retain the first two PCs based on eigenvalues and cumulative variation. In support of such a decision, we performed Horn's parallel analysis for a PCA with the "paran" package, to adjust for finite sample bias in retaining components (50). Biplot visualization were performed with the "factoextra" package (51).

RESULTS

In order to properly understand the connection between acute stress, the most common stress in human society, and male (sub/in)fertility, the immobilization (IMO) stress of 3 h once (1x3hIMO) was applied to the adult male rats (11). The stress period was followed by recovery periods. Immediately after acute stress (ZT3) as well as 8 (ZT11), 14 (ZT17) and 20 (ZT23) h after acute stress, the number and functionality of spermatozoa, as well as the transcriptional profiles of 22 mitochondrial dynamics and function markers and 22 signaling molecules regulating both spermatozoa number/function and mitochondrial dynamics were tracked (**Figures 1**–**13**).

Spermatozoa Number Is Lower 14 and 20 h After Acute Stress, While Functionality Declines Immediately After the Stress and 8 h Later, But Recovers 14 and 20 h After the Stress

The number of spermatozoa (**Figure 2A**) declined in rats having longer recovery periods, i.e., from the ZT17-1x3hIMO +R group (1.5-fold compared to ZT17-Control and 1.6-fold vs. ZT3-Control) and the ZT23-1x3hIMO+R group (2.9-fold compared to ZT23-Control and 2.6-fold vs. ZT3-Control). In contrast, the spermatozoa functionality (positive acrosome reaction) (**Figure 2B**) declined in groups of rats having shorter recovery periods, i.e., from the ZT3-1x3hIMO group (3.6-fold compared to ZT3-Control) and the ZT11-1x3hIMO +R group (1.4-fold compared to ZT11-Control and 1.5-fold vs. ZT3-Control).

In search for the possible mechanism(s) beyond these effects, the transcriptional profiles of mitochondrial dynamics markers and signaling molecules regulating both mitochondrial

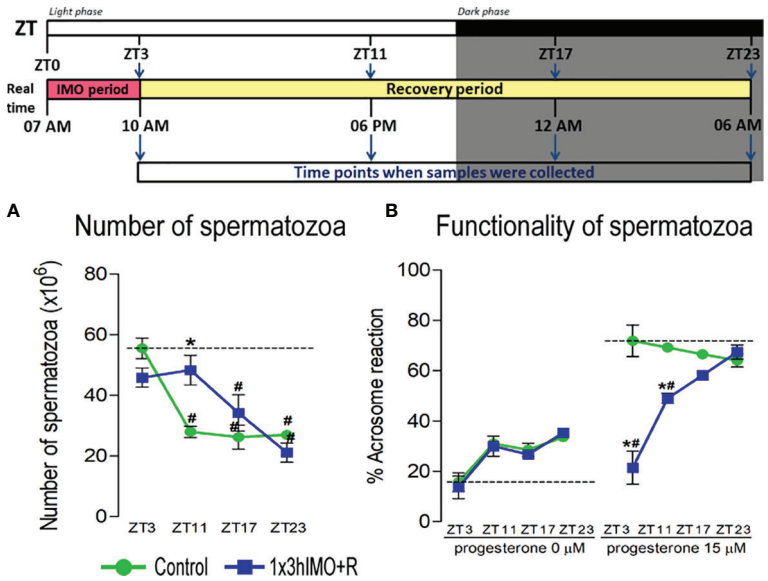

FIGURE 2 | The acute psychophysical stress by immobilization (IMO) decreases functionality and number of spermatozoa in different time points after the IMO stress. Number of spermatozoa **(A)** isolated from caudal epididymides of unstressed rats (control) and rats subjected to acute immobilization stress once for 3 h (1x3hIMO) with recovery periods of 0, 8, 14 and 20 h. **(B)** The functionality of spermatozoa (% of acrosome reacted spermatozoa) isolated from control and acutely (1x3hIMO) stressed rats. Capacitated spermatozoa were stimulated with progesterone (PROG 15 μM) in parallel with spermatozoa not treated with progesterone (PROG 0 μM). Blue staining in the acrosome region of the head indicated intact acrosome, whereas spermatozoa without blue staining in the acrosome region were considered to be acrosome reacted. Data are presented as green dots connected with a green line for the control group, and blue squares connected with a blue line for the 1x3hIMO group, and are mean ± SEM values of two independent *in vivo* experiments. Statistical significance was set at $p < 0.05$: * vs. the control group of the same time point, # vs. the control group of ZT3 time point.

dynamics and spermatozoa number and functionality (important for fertilization) were tracked. Results showed that stress dramatically disturbed expression of transcripts for markers of mitochondrial dynamics and functionality as well as associated signaling pathways in spermatozoa. Expression levels of 40 out of 44 (91%) markers were changed either using ZT3-Control as a calibrator (**Figures 3–9**) or using the corresponding control at a particular ZT time point (**Supplementary Figures S1–S7**).

Significant Changes in Transcriptional Profiles of Mitochondrial Dynamics and Functionality Markers in Spermatozoa From Acutely Stressed Rats Are Evident Up to 20 h After Stress

The transcriptional profiles of molecular markers of mitochondrial dynamics and functionality in spermatozoa are disturbed by acute stress since the transcriptional levels of 20 out of 22 (91%) markers were changed (**Figures 3–7** and **Supplementary Figures S1–S5**).

Mitochondrial biogenesis markers changed 8 out of 8 (100%). The level of transcripts for genes encoding PGC1 (*Ppargc1a* and *Ppargc1b*), very well known as the master regulator involved in the transcriptional control of all the processes related to mitochondrial homeostasis and integrator of environmental signals (32, 33), was disturbed (**Figure 3**). Interestingly, a circadian-like profile was observed in the expression of the *Ppargc1a* transcript since it was differently changed in spermatozoa taken from undisturbed rats at different time points: increased in the ZT11-Control (2.6-fold) and ZT17-Control (2.9-fold) groups compared to ZT3-Control, but decreased in ZT23-Control (2.1-fold). Changes were also detected in spermatozoa obtained from acutely stressed rats with different recovery periods: decrease in ZT11-1x3hIMO+R group (4.8-fold compared to ZT11-Control), but increase in ZT17-1x3hIMO+R (2.4-fold compared to ZT3-Control) and ZT23-1x3hIMO+R (3.6-fold compared to ZT23) groups. Less prominent effects were observed on the transcription of *Ppargc1b*: increased in spermatozoa from the ZT17-Control group (1.7-fold compared to ZT3-Control) and in the ZT23-1x3hIMO+R group (1.4-fold compared to ZT23-Control).

Transcription profiles of PGC1 downstream targets (*Nrf1*, *Nrf2a*, *Tfam*, *mtNd1*, and *Ppard*) that regulate genes for subunits of the oxidative phosphorylation (OXPHOS) also changed.

Tfam transcription was disturbed only at ZT23: decreased in the ZT23-Control group (2.3-fold compared to ZT3-Control), but increased in the ZT23-1x3hIMO+R group (3.7-fold compared to ZT23-Control and 1.6-fold vs. ZT3-Control).

Nrf1 transcript decreased in spermatozoa from ZT23-Control (2.6-fold compared to ZT3-Control), but increased in all stressed groups with recovery period: ZT11-1x3hIMO+R (1.5-fold vs. ZT11-Control, 1.7-fold vs. ZT3-Control group), ZT17-1x3hIMO+R (1.5-fold vs. ZT17-Control), and ZT23-1x3hIMO +R (4.9-fold vs. ZT23-Control, 2.0-fold vs. ZT3-Control).

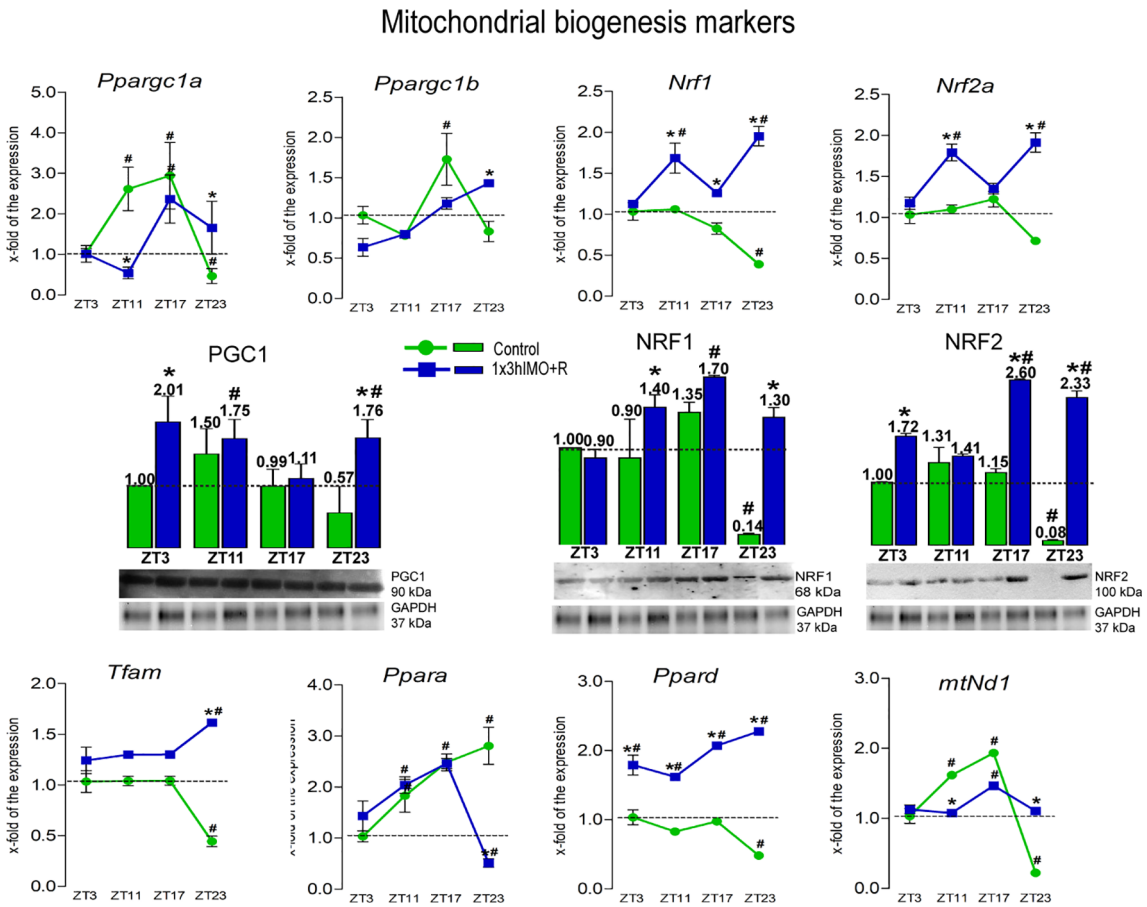

FIGURE 3 | Transcription of mitochondrial biogenesis markers is significantly changed in spermatozoa of acutely stressed adult rats in a time-dependent manner. Isolated RNA and proteins from spermatozoa of undisturbed and stressed rats were used for the analysis of the transcriptional profile and protein expression profile of markers of mitochondrial biogenesis. The representative blots are shown as panels. Data from scanning densitometry were normalized on GAPDH (internal control). Values are shown as bars above the photos of blots. Data are presented as green dots connected with a green line for the control group, and blue squares connected with a blue line for the 1x3hIMO group, and are mean ± SEM values of two independent *in vivo* experiments. Statistical significance was set at $p < 0.05$: * vs. the control group of the same time point, # vs. the control group of ZT3 time point.

Nrf2a transcription was less disturbed than *Nrf1*: increased in ZT11-1x3hIMO+R (1.6-fold compared to ZT11-Control, 1.8-fold vs. the ZT3-Control group) and ZT23-1x3hIMO+R (2.7-fold vs. ZT23-Control, 1.9- fold vs. ZT3-Control) groups.

Ppara transcription profile increased in spermatozoa obtained from rats of all control groups compared to ZT3-Control (1.8-fold in ZT11-Control, 2.5-fold in ZT17-Control, 2.8-fold in ZT23-Control). In spermatozoa from stressed animals, an increased level of *Ppara* transcript was observed in ZT11-1x3hIMO+R (2.0-fold vs. ZT3-Control group) and ZT17-1x3hIMO+R (2.5-fold vs. ZT3-Control) groups, but *Ppara* transcript decreased in the ZT23-1x3hIMO+R group (5.5-fold vs. ZT23-Control, 1.95-fold vs. ZT3-Control).

Ppard transcription was less disturbed than *Ppara* since change/decrease was observed only in the spermatozoa from ZT23-Control (2.1-fold) compared to ZT3-Control. Increased *Ppard* levels were registered in spermatozoa of all stressed groups: ZT3-1x3hIMO+R (1.8-fold vs. ZT3-Control); ZT11-1x3hIMO+R (2.0-fold vs. ZT11, 1.6-fold vs. ZT3-Control), ZT17-1x3hIMO+R (2.1-fold vs. ZT-17, 2.1-fold vs. ZT3-Control), and ZT23-1x3hIMO+R (4.7-fold vs. ZT-23, 2.3-fold vs. ZT3-Control).

mtNd1 transcription profile was similar to *Ppargc1a*. Increased *mtNd1* level was detected in spermatozoa from ZT11-Control (1.6-fold) and ZT17-Control (1.9-fold) groups compared to ZT3-Control, but *mtNd1* decreased in ZT23-Control (4.5-fold). Changes were also detected in spermatozoa obtained from acutely stressed rats with different recovery periods: decreased in the ZT11-1x3hIMO+R group (1.5-fold compared to ZT11-Control), but increased in the ZT17-1x3hIMO+R (1.5-fold compared to ZT3-Control) and ZT23-1x3hIMO+R (5.0-fold compared to ZT23) groups.

Mitochondrial fusion markers changed 3 out of 3 (100%). Changes in transcriptional profiles of all spermatozoal mitofusion as well as mito-architecture markers (*Mfn1*, *Mfn2* and *Opa1*) were observed at the ZT23 time point (**Figure 4A**).

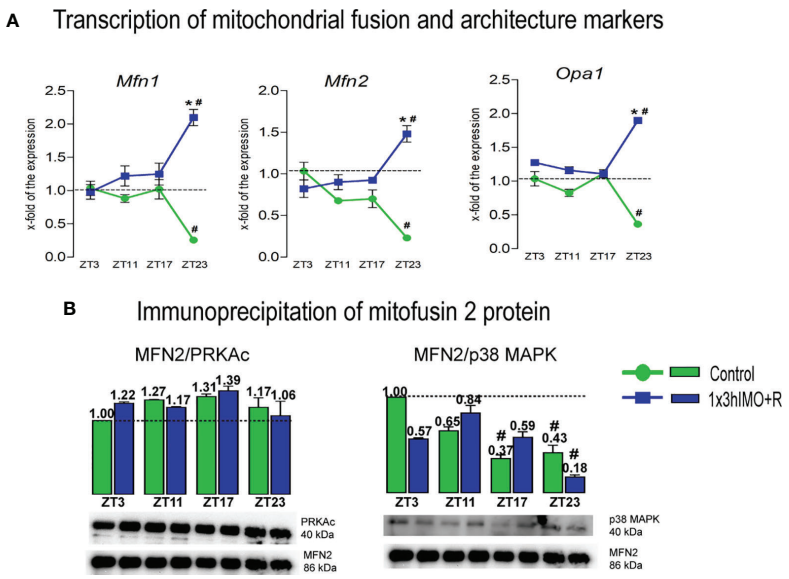

FIGURE 4 | Transcription of mitochondrial fusion and architecture markers, as well as interactions of mitofusin 2 protein and PRKAc and p38 MAPK proteins are significantly changed in spermatozoa of acutely stressed adult rats in a time-dependent manner. Isolated RNA from spermatozoa of undisturbed and stressed rats was used for the analysis of the transcriptional profile of markers of mitochondrial fusion and architecture **(A)**. Isolated proteins from spermatozoa of undisturbed and stressed rats were used for immunoprecipitation analysis with MFN2 antibody, followed by Western blot for PRKAc and p38 MAPK **(B)**. The representative blots are shown as panels. Data from scanning densitometry were normalized on MFN2 (internal control). Values are shown as bars above the photos of blots. Data are presented as green dots connected with a green line or green bars for the control group, and blue squares connected with a blue line or blue bars for the 1x3hIMO group, and are mean ± SEM values of two independent *in vivo* experiments. Statistical significance was set at $p < 0.05$: * vs. the control group of the same time point, # vs. the control group of ZT3 time point.

Mfn1 transcription decreased in spermatozoa from the ZT23-Control group (3.9-fold vs. ZT3-Control), but increased in the ZT23-1x3hIMO+R group (8.1-fold compared to ZT23 control, 2.1-fold vs. ZT3-Control).

Mfn2 transcription profile was similar to *Mfn1*. The level of *Mfn2* transcript decreased in spermatozoa from the ZT23-Control group (4.3-fold vs. ZT3-Control), but increased in the ZT23-1x3hIMO+R group (6.4-fold compared to ZT23 control, 1.5-fold vs. ZT3-Control).

Opa1 transcript profile was similar to *Mfn1* and *Mfn2*. The level of *Opa1* transcript significantly decreased in spermatozoa obtained from the ZT23-Control group (2.7-fold vs. ZT3-Control), but increased in the ZT23-1x3hIMO+R group (5.3-fold compared to ZT23-Control, 1.9-fold vs. ZT3-Control).

Considering the importance of MFN2 expression for spermatozoa motility and viability as well as mitochondrial network homeostasis (31), interactions of MFN2 and proteins regulating both mitochondrial dynamic and spermatozoa number and functionality were followed. Results show that MFN2 protein interacts with the PRKA catalytic subunit in spermatozoa, but there is no significant difference in spermatozoa of the 1x3hIMO+R group compared to the ZT3-Control group. Immunoprecipitation analysis of MFN2 followed by Western blot analysis with p38 MAPK protein shows that the interaction between these proteins exists in spermatozoa, and that there is significant decrease in the control group of ZT17 and ZT23, as well as in the 1x3hIMO+R group of the ZT23 time point, compared to the ZT3-Control group (**Figure 4B**).

Mitochondrial fission markers changed 2 out of 2 (100%). Levels of transcripts for *Drp1* and *Fis1* differently changed at different the ZT time points (**Figure 5**).

Fis1 transcription increased in spermatozoa from ZT17-Control (1.9-fold compared to ZT3-Control). In spermatozoa from stressed rats, a decrease was observed in the ZT17-1x3hIMO+R group (1.5-fold vs. ZT17-Control) and the opposite effect (increase) was detected in the ZT23-1x3hIMO +R group (1.8-fold vs. ZT23-Control, 2.4-fold vs. ZT3-Control).

Drp1 transcript profile was different from *Fis1*, since changes/decreases were evident only in stressed groups: ZT11-1x3hIMO+R (2.8-fold vs. ZT11-Control) and ZT17-1x3hIMO +R groups (1.8-fold vs. ZT17-Control).

Mitochondrial autophagy markers changed 1 out of 3 (33%). Significant changes were evident only on the transcription profile of *Prkn*: decrease in ZT17-Control (3.8-fold compared to ZT3-Control) and ZT17-1x3hIMO+R (5.3-fold vs. ZT3-Control) groups. The transcriptional profile of *Pink1* and *Tfeb* remained unchanged (**Figure 6**).

Mitochondrial functionality markers changed 6 out of 6 (100%). Transcriptional profiles of NRF1/NRF2 downstream targets (CytC, COX4, and UCPs) serving as mitochondrial functional markers as well as the mediators of regulated proton leak and controllers of the production of superoxide and other downstream reactive oxygen species (41) were significantly changed at the ZT23 time point (**Figure 7**).

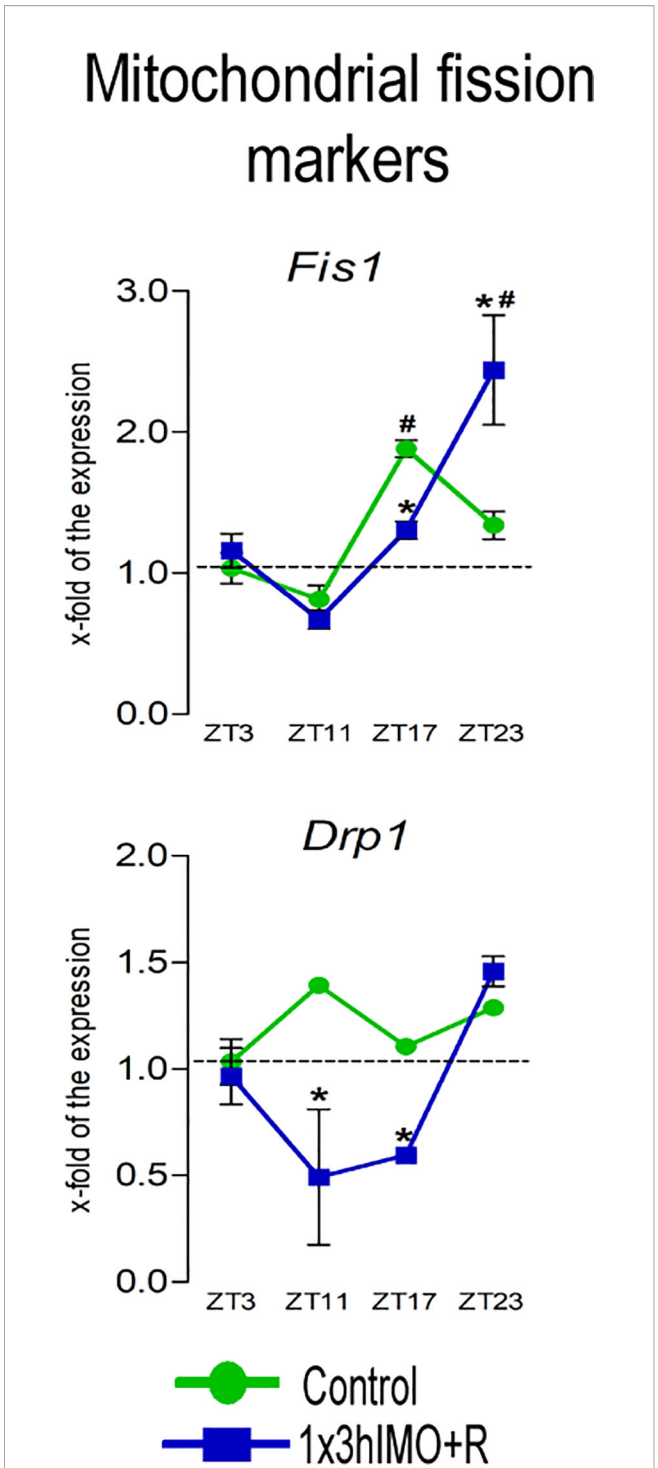

FIGURE 5 | Transcription of mitochondrial fission markers is significantly changed in spermatozoa of acutely stressed adult rats in a time-dependent manner. Isolated RNA from spermatozoa of undisturbed and stressed rats was used for the analysis of the transcriptional profile of markers of mitochondrial fission. Data are presented as green dots connected a with green line for the control group, and blue squares connected with a blue line for the 1x3hIMO group, and are mean ± SEM values of two independent *in vivo* experiments. Statistical significance was set at $p < 0.05$: * vs. the control group of the same time point, # vs. the control group of ZT3 time point.

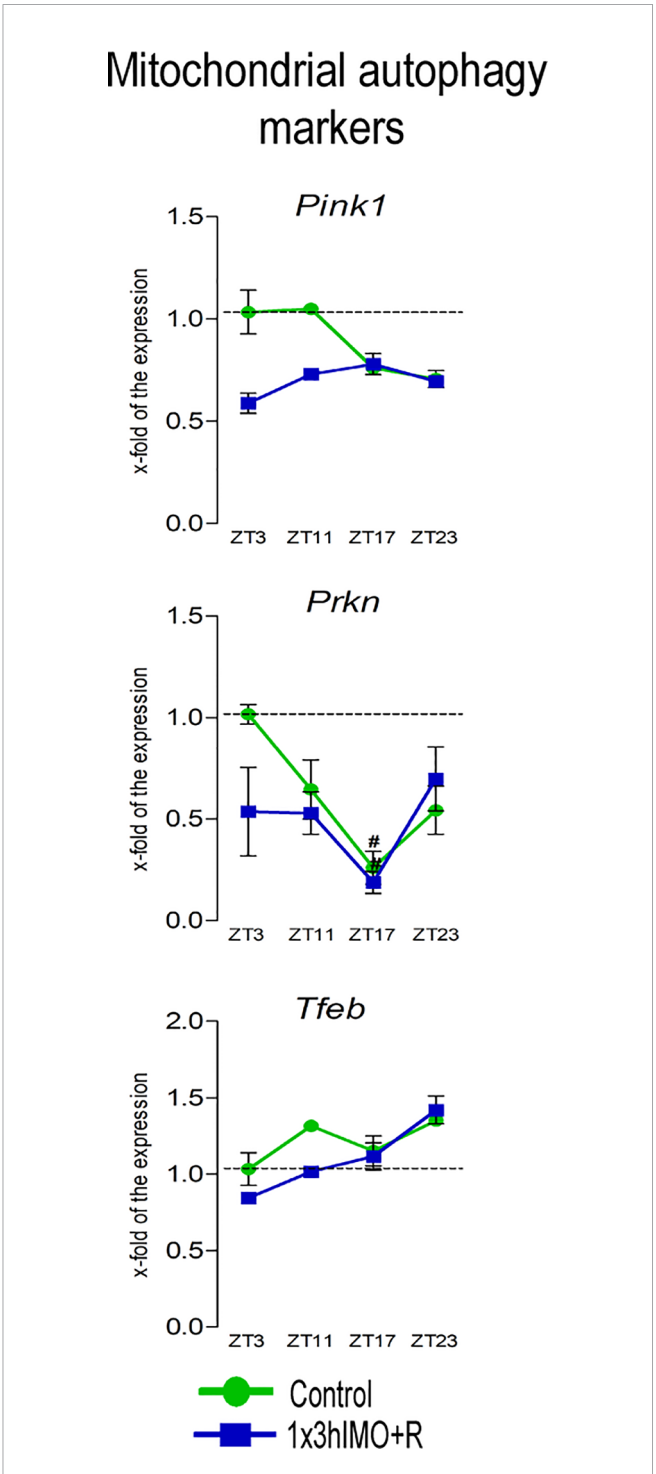

FIGURE 6 | Transcription of mitochondrial autophagy markers is significantly changed in spermatozoa of acutely stressed adult rats in a time-dependent manner. Isolated RNA from spermatozoa of undisturbed and stressed rats was used for the analysis of the transcriptional profile of markers of mitochondrial autophagy. Data are presented as green dots connected with a green line for the control group, and blue squares connected with a blue line for the 1x3hIMO group, and are mean ± SEM values of two independent *in vivo* experiments. Statistical significance was set at $p < 0.05$: * vs. the control group of the same time point, # vs. the control group of ZT3 time point.

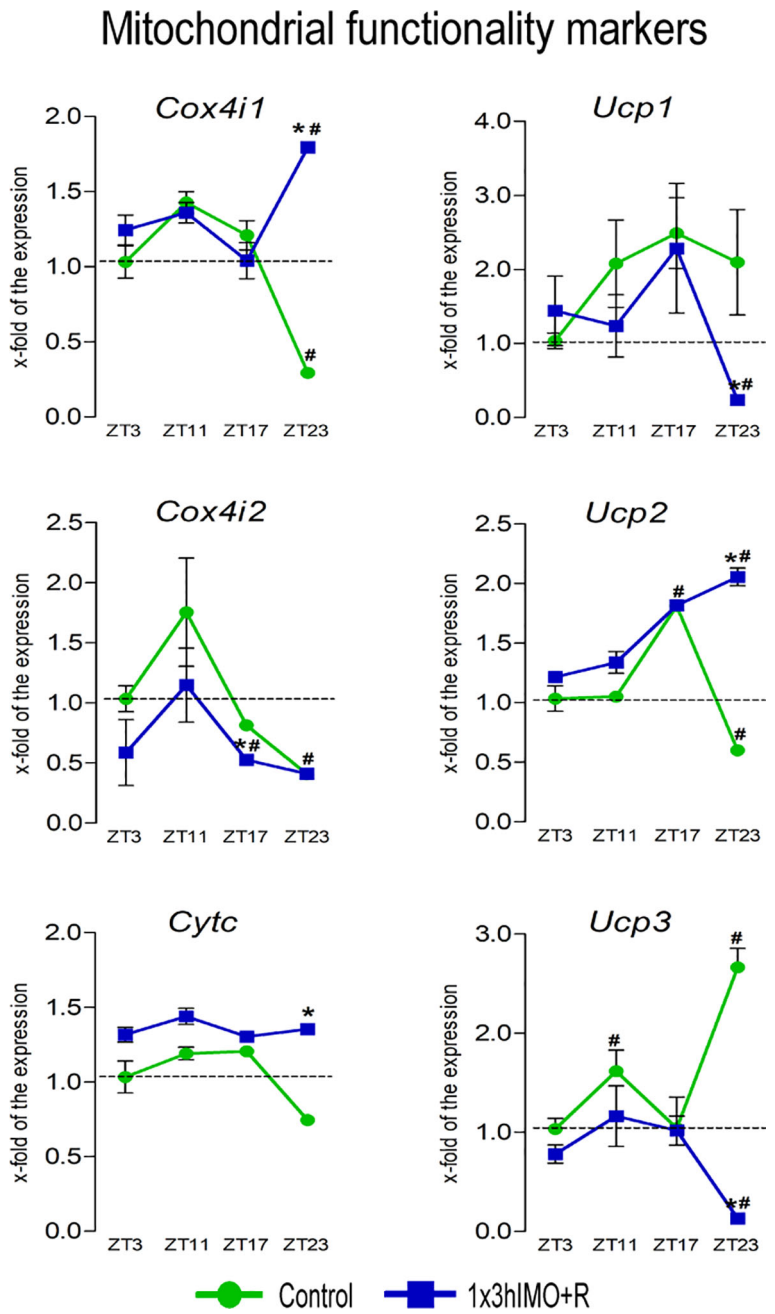

FIGURE 7 | Transcription of mitochondrial functionality markers is significantly changed in spermatozoa of acutely stressed adult rats in a time-dependent manner. Isolated RNA from spermatozoa of undisturbed and stressed rats was used for the analysis of the transcriptional profile of markers of mitochondrial functionality. Data are presented as green dots connected with a green line for the control group, and blue squares connected with a blue line for the 1x3hIMO group, and are mean ± SEM values of two independent *in vivo* experiments. Statistical significance was set at $p < 0.05$: * vs. the control group of the same time point, # vs. the control group of ZT3 time point.

Cox4i1 transcription significantly decreased in spermatozoa from the ZT23-Control group (3.4-fold vs. ZT3-Control) but increased in the ZT23-1x3hIMO+R group (6.1-fold compared to ZT23-Control, 1.8-fold vs. ZT3-Control).

Cox4i2 transcript level significantly decreased in spermatozoa from the ZT23-Control (2.4-fold vs. ZT3-Control) as well as the stressed groups ZT17-1x3hIMO+R (1.6-fold compared to ZT17-Control, 1.9-fold vs. ZT3-Control) and ZT23-1x3hIMO+R (2.4-fold compared to ZT3-Control).

Cytc transcription significantly increased only in spermatozoa obtained from the ZT23-1x3hIMO+R group (1.8-fold compared to ZT23-Control).

Ucp1 transcript level was significantly lower only in spermatozoa obtained from the ZT23-1x3hIMO+R group (8.9-fold compared to ZT23-Control, 4.3-fold vs. ZT3-Control).

Ucp2 transcript in spermatozoa (the most abundantly expressed UCP gene in spermatozoa; *Ucp2*-Ct=22.07>*Ucp3*-Ct=29.81>*Ucp1*-Ct=29.96) was changed at ZT17 and ZT23 time points. *Ucp2* transcript level increased in spermatozoa isolated from ZT17-Control (1.8-fold compared to ZT3-Control), but decreased in spermatozoa from ZT23-Control (1.7-fold vs. ZT3-Control). In spermatozoa from stressed rats, increase was detected in ZT17-1x3hIMO+R (1.8-fold vs. ZT3-Control) and ZT23-1x3hIMO+R (3.4-fold compared to ZT23-Control, 2.0-fold compared to ZT3-Control).

Ucp3 transcription increased in spermatozoa from ZT11-Control (1.6-fold compared to ZT3-Control) as well as ZT23-Control (2.7-fold vs. ZT3-Control), but decreased in ZT23-1x3hIMO+R (20.8-fold compared to ZT23-Control, 7.7-fold compared to ZT3-Control).

The results of the PCA confirmed that separation of the effects of acute stress recovery on mitochondrial dynamics marker elements depends on the day phase. It is clear that the transcriptional patterns were different during the active and inactive phases. Most of the transcripts were highly expressed during the active phase, which is expected given that stress occurred at the beginning of the inactive phase. Expression of the transcripts for proteins involved in mitochondrial dynamics tends to separate across the first two PCs for 74.2% of the total dataset of mitochondria-related gene variability. Also, the results offer different acute stress recovery effects on transcript expression: a pronounced cluster of genes encoding the elements essential for mitochondrial dynamics in the active phase opposes *Ucp3, Ppara*, and *Ucp1* in the inactive phase (**Figure 12A**, variable loadings are shown in **Supplementary Table S1**).

Since the cAMP and MAPK signaling are crucial not only for the regulation of spermatozoa number and functionality (43), but also for the regulation of mitochondrial dynamics and functionality (32, 33, 37), the transcriptional profiles of main signaling molecules were tracked.

Significant Changes in Transcriptional Profiles of Signaling Molecules Regulating the Number and Functionality of Spermatozoa, as Well as the Mitochondrial Dynamics and Functionality in Spermatozoa From Stressed Rats Are Evident Up to 20 h After Stress

Markers of signaling pathways regulating the spermatozoa number/functionality as well as mitochondrial dynamics/functionality, both very important for male fertility, significantly changed during the recovery time course. Transcriptional levels of 20 out of 22 (91%) markers were changed, and most of the changes were increases of the expression (**Figures 8**, **9** and **Supplementary Figures S6** and **S7**).

cAMP signaling markers changed 11 out of 12 (92%). Most of the changes in transcriptional profile of cAMP signaling markers during stress recovery time periods were increased expression of most of the adenylyl cyclases (*Adcy3*, *Adcy5, Adcy6*, and *Adcy7*) except for *Adcy8* (decreased), *Adcy7* (remained unchanged), and *Adcy10* (decreased). In the same spermatozoa samples, the level of the transcripts for all genes encoding the catalytic and the regulatory protein kinase A subunits (*Prkaca, Prkacb, Prkar1a, Prkar2a*, and *Prkar2b*) increased (**Figure 8**).

Adcy3 transcript levels increased in spermatozoa isolated from ZT11-Control (2.4-fold compared to ZT3-Control) and ZT17-Control (2.7-fold vs. ZT3-Control). In spermatozoa from stressed rats, increases were detected in ZT11-1x3hIMO+R (2.8-fold vs. ZT3-Control), ZT17-1x3hIMO+R (1.5-fold vs. ZT3-Control), and ZT23-1x3hIMO+R (4.5-fold compared to ZT23-Control, 4.5-fold compared to ZT3-Control).

Adcy5 transcriptional profile was changed/increased only in spermatozoa from the stressed rats with recovery for 14 or 20 h: ZT17-1x3hIMO+R (1.7-fold vs. ZT17-Control) and ZT23-1x3hIMO+R (1.9-fold compared to ZT23-Control, 1.8-fold compared to ZT3-Control).

Adcy6 transcription increased in spermatozoa isolated from ZT11-Control (1.7-fold compared to ZT3-Control), as well as from ZT23-1x3hIMO+R (2.5-fold compared to ZT23-Control, 1.6-fold compared to ZT3-Control). The opposite changes (decreased expression) were detected in ZT3-1x3hIMO+R (1.9-fold vs. ZT3-Control) and ZT11-1x3hIMO+R (1.4-fold vs. ZT11-Control).

Adcy7 transcript levels decreased in spermatozoa from ZT23-Control (1.6-fold compared to ZT3-Control). In contrast, increased expressions were observed in the stressed group at all recovery time points: ZT11-1x3hIMO+R (2.0-fold vs. ZT11-Control), ZT17-1x3hIMO+R (2.1-fold vs. ZT17-Control), and ZT23-1x3hIMO+R (4.3-fold compared to ZT23-Control, 2.7-fold compared to ZT3-Control).

Adcy8 transcription increased in spermatozoa isolated from ZT23-Control (1.7-fold compared to ZT3-Control). In spermatozoa from the stressed rats, decreases were detected in ZT3-1x3hIMO+R (2.3-fold vs. ZT3-Control), ZT17-1x3hIMO +R (2.4-fold vs. ZT17-Control, 1.7-fold vs. ZT3-Control), and ZT23-1x3hIMO+R (188.9-fold compared to ZT23-Control, 111.1-fold compared to ZT3-Control).

Adcy10 transcriptional profile was changed/decreased only in spermatozoa from the stressed rats at all recovery time points: ZT11-1x3hIMO+R (1.5-fold vs. ZT11-Control), ZT17-1x3hIMO+R (1.4-fold vs. ZT17-Control), and ZT23-1x3hIMO +R (2.5-fold vs. ZT23-Control, 2.5-fold vs. ZT3-Control).

Prkaca transcription was changed only at the ZT23 time point: decreased in spermatozoa from the ZT23-Control group (2.8-fold compared to ZT3-Control), but increased in spermatozoa from the ZT23-1x3hIMO+R group (4.1 fold compared to ZT23-Control, 1.5 fold compared to ZT3-Control).

Prkacb transcription profile was similar to *Prkaca*. The level of *Prkacb* transcripts decreased in spermatozoa from the ZT23-Control group (2.6-fold compared to ZT3-Control), but

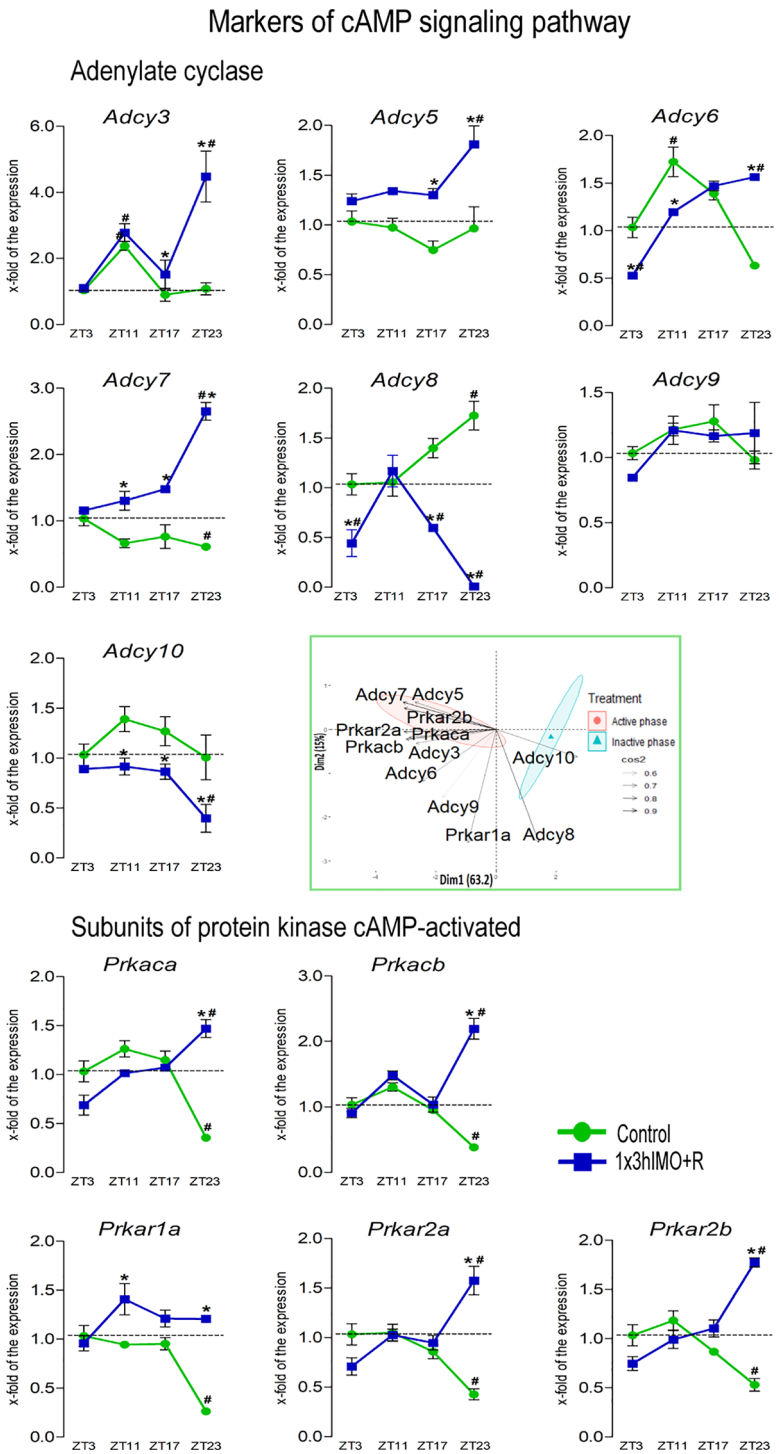

FIGURE 8 | Transcription of markers of cAMP signaling regulating mitochondrial dynamics and functionality as well as spermatozoa number and functionality is changed in spermatozoa of acutely stressed adult rats in a time-dependent manner. Isolated RNA from spermatozoa of undisturbed and stressed rats was used for the analysis of the transcriptional profile of markers of the cAMP signaling pathway. PCA of markers of the cAMP signaling pathway on active/inactive phase; Dim1 and Dim2 represent the first two PCs and % of the retained variation. Cos2 estimates the qualitative representation of variables (**Supplementary Table S2**). Data are presented as green dots connected with a green line for the control group, and blue squares connected with a blue line for the 1x3hIMO group, and are mean ± SEM values of two independent *in vivo* experiments. Statistical significance was set at $p < 0.05$: * vs. the control group of the same time point, # vs. the control group of ZT3 time point.

increased in the ZT23-1x3hIMO+R group (5.8-fold compared to ZT23-Control, 2.2-fold vs. ZT3-Control).

Prkar1a transcription decreased in spermatozoa isolated from ZT23-Control (3.8-fold compared to ZT3-Control). In contrast, transcription increased in the ZT11-1x3hIMO+R (1.5-fold vs. ZT11-Control) and the ZT23-1x3hIMO+R (4.6-fold compared to ZT23-Control) groups.

Prkar2a transcriptional profile was similar to the profiles of transcripts for catalytic subunits of PRKA: decreased in spermatozoa from the ZT23-Control group (2.3-fold compared to ZT3-Control) but increased in the ZT23-1x3hIMO+R group (3.7-fold vs. ZT23-Control, 1.6-fold vs. ZT3-Control).

Prkar2b transcription profile was similar to *Prkar2a*. The level of *Prkar2b* transcripts decreased in spermatozoa from the ZT23-Control group (1.9-fold compared to ZT3-Control) but increased in the ZT23-1x3hIMO+R group (3.3-fold compared to ZT23-Control, 1.8-fold vs. ZT3-Control).

The results of the PCA confirmed the separation of the effects of acute stress recovery on cAMP signaling pathway elements. It is clear that the transcriptional patterns were different during the active and inactive phases and that the transcripts were highly expressed during the active phase. Most of the transcripts were highly expressed during the active phase, which is expected given that stress occurred at the beginning of the inactive phase. Expression of the transcripts for proteins involved in cAMP signaling accounts for 78.2% variability. Also, the results offer different acute stress recovery effects on the transcripts' expression: a pronounced cluster of *Adcy8* and *Adcy10* in the inactive phase opposes gene clusters encoding the other elements of cAMP signaling in the active phase (**Figures 8**, **12B**, variable loadings are shown in **Supplementary Table S2**).

MAPK signaling markers changed 9 out of 10 (90%). The markers of MAPK signaling (*Mapk1, Mapk3, Mapk6, Mapk7, Mapk8, Mapk9, Mapk11, Mapk12, Mapk13*, and *Mapk14*) were affected at all time points and more than the above-mentioned markers in spermatozoa. Transcripts of all markers significantly increase, except for the decreased transcription of *Mapk11*, in spermatozoa isolated from the groups of rats exposed to acute stress for 3 h and recovered for 20 h, while most of the markers increased in spermatozoa from the stressed rats at all recovery time points (**Figure 9**).

Mapk1 transcription decreased in spermatozoa isolated from ZT23-Control (2.7-fold compared to ZT3-Control). Increases were observed in spermatozoa from the stressed rats at all recovery time points: ZT3-1x3hIMO+R (1.9-fold vs. ZT3-Control), ZT11-1x3hIMO+R (2.0-fold vs. ZT11-Control, 2.0-fold vs. ZT3-Control), ZT17-1x3hIMO+R (1.4-fold vs. ZT17-Control, 1.8-fold vs. ZT3-Control), and ZT23-1x3hIMO+R (7.2-fold compared to ZT23-Control, 2.6-fold compared to ZT3-Control).

Mapk3 transcript levels decreased in spermatozoa from the ZT23-Control group (2.3-fold compared to ZT3-Control) but increased in the ZT23-1x3hIMO+R group (4.1-fold compared to ZT23-Control, 1.8-fold vs. ZT3-Control).

Mapk6 transcriptional profile was similar to *Mapk1*. The decline in the *Mapk6* transcription was observed in spermatozoa from ZT23-Control (2.4-fold compared to ZT3-Control). The significant increase was evident in spermatozoa from the stressed rats at all recovery time points: ZT3-1x3hIMO+R (1.6-fold vs. ZT3-Control), ZT11-1x3hIMO+R (2.8-fold compared to ZT11-Control, 1.8-fold vs. ZT3-Control), ZT17-1x3hIMO+R (2.1-fold vs. ZT17-Control; 1.7-fold vs. ZT3-Control), and ZT23-1x3hIMO+R (5.6-fold compared to ZT23-Control, 2.3-fold compared to ZT3-Control).

Mapk8 transcriptional profile was similar to *Mapk1* and *Mapk6*, but the effect was absent at the ZT17 time point. The decreased *Mapk8* transcript level was evident in spermatozoa from ZT23-Control (2.5-fold compared to ZT3-Control). A significant increase was evident in spermatozoa from ZT3-1x3hIMO+R (1.4-fold compared to ZT3-Control), ZT11-1x3hIMO+R (1.5-fold compared to ZT11-Control), and ZT23-1x3hIMO+R (4.9-fold compared to ZT23-Control, 1.9-fold vs. ZT3-Control).

Mapk9 transcription increased only in spermatozoa from the stressed rats recovered for 20 h, i.e., ZT23-1x3hIMO+R (3.0-fold vs. ZT23-Control, 1.9-fold compared to ZT3-Control).

Mapk11 transcriptional profile was changed/increased only in spermatozoa from the stressed rats recovered for 14 and 20 h: ZT17-1x3hIMO+R (1.5-fold compared to ZT3-Control) and ZT23-1x3hIMO+R (2.1-fold compared to ZT23-Control).

Mapk12 transcription significantly decreased in spermatozoa from ZT23-Control (3.3-fold compared to ZT3-Control). In spermatozoa from stressed rats, increases were detected in ZT11-1x3hIMO+R (2.0-fold compared to ZT11-Control, 1.8-fold compared to ZT3-Control) and ZT23-1x3hIMO+R (7.7-fold compared to ZT23-Control, 2.5-fold compared to ZT3-Control).

Mapk13 transcript level significantly decreased in spermatozoa from ZT23-Control (2.7-fold compared to ZT3-Control). Significant increases were evident in spermatozoa from the rats recovered at different time points: ZT11-1x3hIMO+R (1.7-fold vs. ZT11-Control, 1.6-fold vs. ZT3-Control), ZT17-1x3hIMO+R (1.7-fold vs. ZT17-Control), and ZT23-1x3hIMO +R (3.4-fold vs. to ZT23-Control).

Mapk14 transcription was changed only at the ZT23 time point: decreased in spermatozoa from ZT23-Control (2.1-fold compared to ZT3-Control), but increased in spermatozoa from ZT23-1x3hIMO+R (4.3-fold compared to ZT23-Control, 2.1-fold compared to ZT3-Control).

The results of the PCA show significant separation of the effects of acute stress recovery on MAPK signaling pathway elements depending on the day phase. It is clear that the transcriptional patterns were different during the active and inactive phases and that the transcripts were highly expressed during the active phase (**Figures 9**, **12C** as well as **Supplementary Table S3**).

The results of the PCA confirmed the separation of the effects of acute stress recovery on MAPK signaling. The transcriptional patterns were different during the active and inactive phases. Most of the transcripts were highly expressed during the active phase, which is expected given that stress occurred at the beginning of the inactive phase. Expression of the transcript of the proteins involved in MAPK signaling accounts for 82.1% of data variability. The results offered different acute stress recovery effects on the transcript expression: a pronounced *Mapk11* in the inactive phase opposes clusters of other transcripts for elements

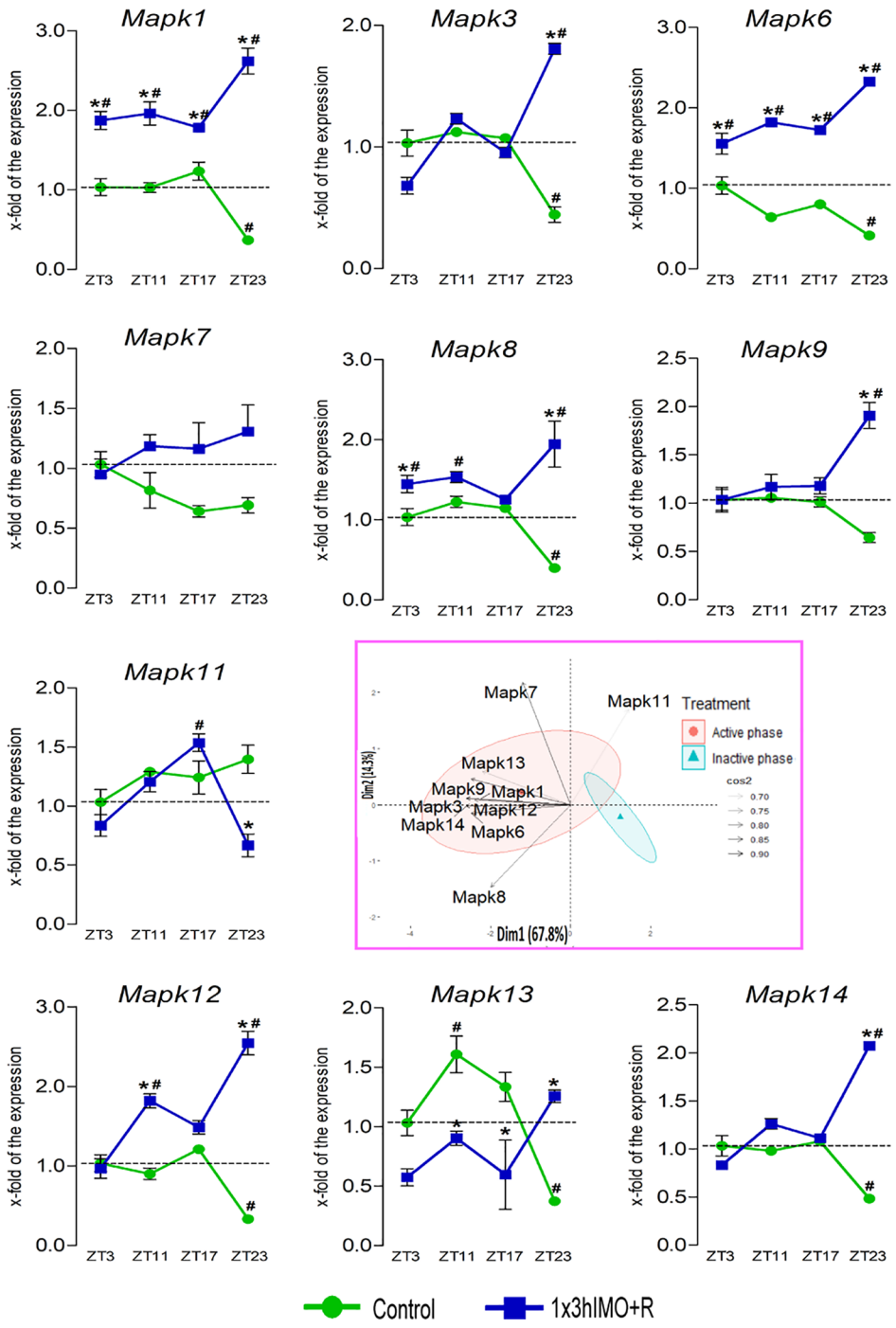

FIGURE 9 | Transcription of markers of MAPK signaling regulating mitochondrial dynamics and functionality as well as spermatozoa number and functionality is changed in spermatozoa of acutely stressed adult rats in a time-dependent manner. Isolated RNA from spermatozoa of undisturbed and stressed rats was used for the analysis of the transcriptional profile of markers of the MAPK pathway. PCA of markers of the MAPK signaling pathway on active/inactive phase; Dim1 and Dim2 represent the first two PCs and % of the retained variation. Cos2 estimates the qualitative representation of variables (**Supplementary Table S3**). Data are presented as green dots connected with a green line for the control group, and blue squares connected with a blue line for the 1x3hIMO group, and are mean ± SEM values of two independent *in vivo* experiments. Statistical significance was set at $p < 0.05$: * vs. the control group of the same time point, # vs. the control group of ZT3 time point.

of MAPK signaling (**Figures 9**, **12C**, variable loadings are shown in **Supplementary Table S3**).

For a better understanding, results showing the transcriptional profiles of mitochondrial dynamics and functionality markers and signaling molecules regulating mitochondrial dynamics and functionality as well as spermatozoa number and functionality in spermatozoa of stressed adult rats with different periods for recovery are summarized in **Table 1**.

DISCUSSION

It is very well known that life starts with fertilization. This process requires highly energizing and perfectly functioning spermatozoa. Unfortunately, many recent publications pointed to increased incidence of unexplained cases of (sub/in)fertility in men as well as a decrease in the fertility rate in men younger than age 30 (8, 12, 13). The semen quality and fertility are important not only as fundamental markers of reproductive health, but also as fundamental biomarkers of overall health (13, 52). Also, the World Health Organization (WHO) stated that the overall burden of infertility in men is high, unknown, and underestimated, and has not shown any decrease over the last 20 years. The WHO called for urgent investigations of the mechanisms of (sub/in)fertility (https://www.who.int/reproductivehealth/topics/infertility/perspective/en/).

In search for possible mechanisms of (sub/in)fertility as well as the connection between stress and male (sub/in)fertility, an *in vivo* model of acute psychological stress, the most common stress in human society, was applied on adult male rats and stress period was tracked with different recovery periods. Four time points were chosen (2 points during 12-h light/inactive phase and 2 points during 12-h dark/active phase): immediately after the 3-h acute stress (ZT3) as well as 8 (ZT11), 14 (ZT17), and 20 (ZT23) h later. The number/functionality (positive acrosome reaction) of spermatozoa and the transcriptional profiles of 22 mitochondrial dynamics/function markers and 22 related signaling molecules were tracked.

Results showed for the first time, to the best of our knowledge, that the acute stress-provoked effects appeared 20 h after the end of the stress, and this is very clearly shown on heat maps (**Figure 10** and **Supplementary Figure S8**). Lower number of spermatozoa was observed at ZT17 and ZT23, while decreased spermatozoa functionality (positive acrosome reaction) was evident at ZT3 and ZT11, but recovered at ZT17 and ZT23. Transcriptional profiles of 91% (20/22) of mitochondrial dynamics and functionality markers and 91% (20/22) of signaling molecules regulating both mitochondrial dynamics and spermatozoa number and functionality were disturbed after acute stress and during the recovery period (**Figures 10**, **11** and **Supplementary Figure S8**). The results of the PCA show the significant separation of effects of acute stress recovery during the active and inactive phase of the day. It is clear that the transcriptional patterns were different during the active and inactive phases and that most of the transcripts were highly expressed during the active/dark phase of the day (**Figure 12**). The physiological relevance is the recovered functionality (positive acrosome reaction), suggesting that molecular events are adaptive mechanism regulated by physiological stress response signaling. With this molecular scenario, the spermatozoa may try to preserve the basic mitochondrial network homeostasis and self-activity.

It is well known that stress signaling is involved in the regulation of spermatogenesis and fertility in a very complex and intriguing manner. Chronic intermittent stress irreversibly decreases sperm number (53–55) as well as sperm motility (56) and spermatozoa quality (57) in male rats. Our recently published articles showed that repeated psychophysical stress also lowered the number of spermatozoa (10, 11). The decline in progressively motile sperm in humans is associated with stress (58) and secondary infertility is significantly higher in patients with post-traumatic stress disorder (59). However, there are no published pieces of evidence related to the effects of stress recovery on spermatozoa number and functionality as well as signaling pathways associated with these processes. Here, we show that the number of epididymal spermatozoa declines 14 and 20 h after stress. It is difficult to give a precise explanation, but one of the reasons could be that mechanisms causing reduction in the number of spermatozoa started at earlier points, maybe as a consequence of stress hormone signaling activation, but they are visible at ZT17 and ZT20. Also, the reason could be, although not significantly, persistently higher levels of cortisol (48).

Since mitochondria are very important for many highly energy-driven processes including spermatozoa functionality and fertilization as well as stress response, it was of interest to follow the transcriptional profile of mitochondrial dynamics/functionality markers as well as signaling molecules regulating mitochondrial homeostasis and spermatozoa functionality. Results of transcriptional analyses clearly showed that effects of acute stress were visible up to 20 h later and most of the effects and prominent effects were observed at ZT23 (**Figures 10**, **11**). All those molecules are very important for spermatozoa functionality. Our results showed a circadian-like type of transcriptional profile of *Ppargc1a*/PGC1 in spermatozoa from both unstressed and stressed rats. It was published that PPARGC1A is changed in spermatozoa from patients suffering from type 2 diabetes mellitus (60) and that increased expression of *Nrf2* diminished testicular inflammation (61). Also, our preliminary results show protein interaction of PGC1 and NRF1 proteins in spermatozoa (data not shown). Moreover, expression of the TFAM gene correlates with sperm DNA fragmentation and mtDNA copy number (27, 28). Heat map analysis of the transcriptional profile of mitochondrial dynamics and functionality markers (**Figure 10A** and **Supplementary Figure 8A**) clearly showed that during recovery from acute stress, spermatozoa most abundantly express the main markers of mitochondrial fusion (*Mfn1, Mfn2*, and *Opa1*). This is very important for keeping the integrity of the mitochondrial network and energetic balance. These results may explain the findings of others showing the relation of the expression level of MFN2 to motility and cryoprotective potentials of human sperm (31, 62). Also, our results show for the first time, to the best of our knowledge, the interaction of MFN2 and the catalytic

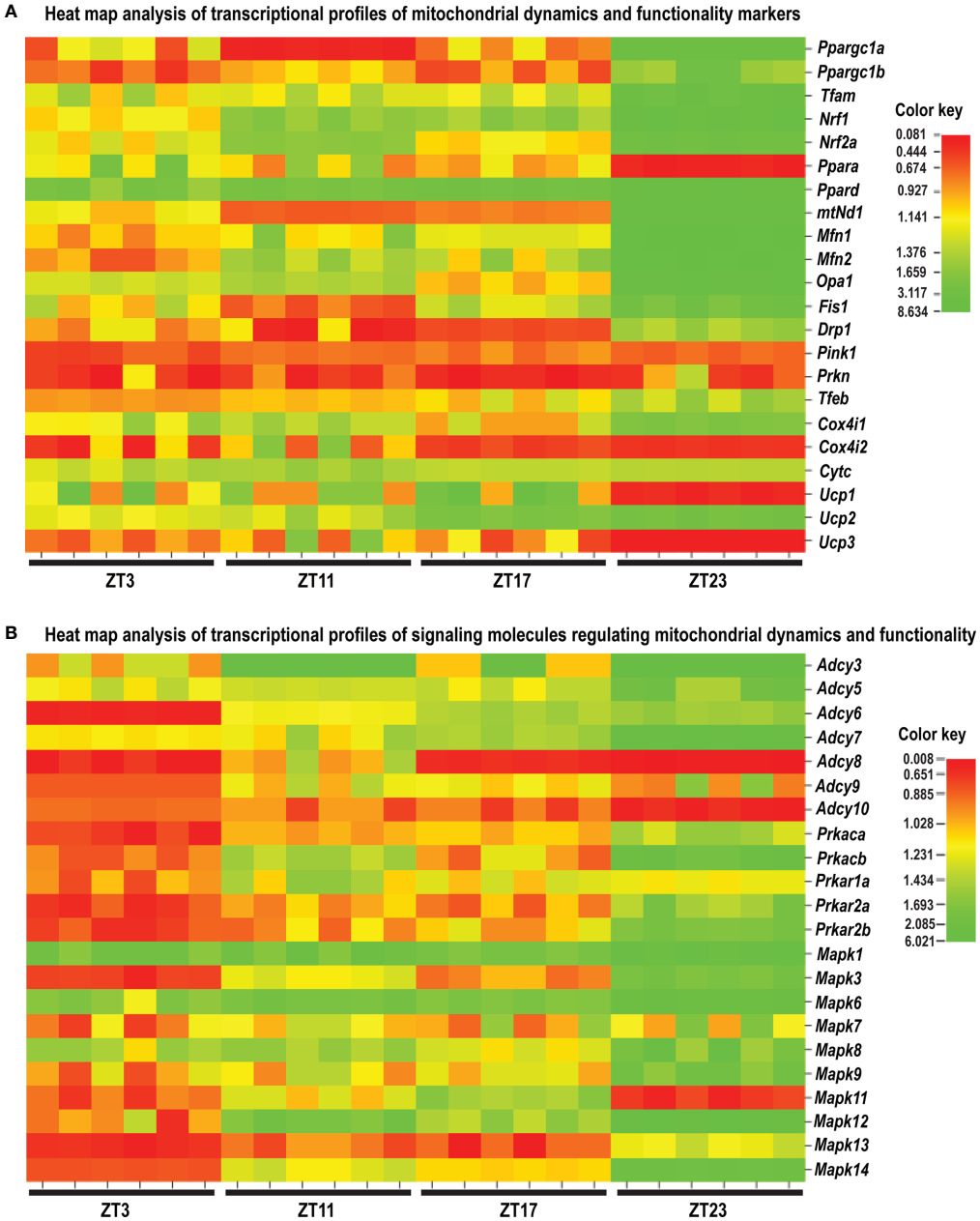

FIGURE 10 | Heat map analysis of the transcriptional profile of the mitochondrial dynamic and functionality markers **(A)** and the signaling molecules regulating mitochondrial dynamics and functionality **(B)** in spermatozoa of acute stressed adult rats. Heat map analysis showing different patterns of transcription at different time points in spermatozoa after the acute immobilization stress. The relative fold change in gene expression for the aforementioned genes was compared in different time points (ZT3, ZT11, ZT17, and ZT23). Color from red to green indicates low to high expression.

subunit of protein kinase A (PRKAc) in spermatozoa. This interaction was already confirmed in other cell types, with PRKA phosphorylation site at Serine 442 (63, 64). Presented results of immunoprecipitation analysis (MFN2/PRKAc) show no difference between the 1x3hIMO+R and the control group of all time points. On the other hand, results of immunoprecipitation analysis show an interaction between MFN2 and p38 MAPK proteins, with a decrease in ZT17-Control, ZT23-Control, and ZT23-1x3hIMO+R groups compared to the ZT3-Control group, suggesting that prolonged acute stress recovery influences the interaction between these two proteins in spermatozoa. Our results show the increased expression of transcript for *Cox4i1* in spermatozoa from stressed rats, the gene that encodes the terminal enzyme in the mitochondrial respiratory chain. It has been shown that this gene is also significantly increased in spermatozoa from obese males (65), and it is important for infertility treatment in men (66). Our results clearly show increased

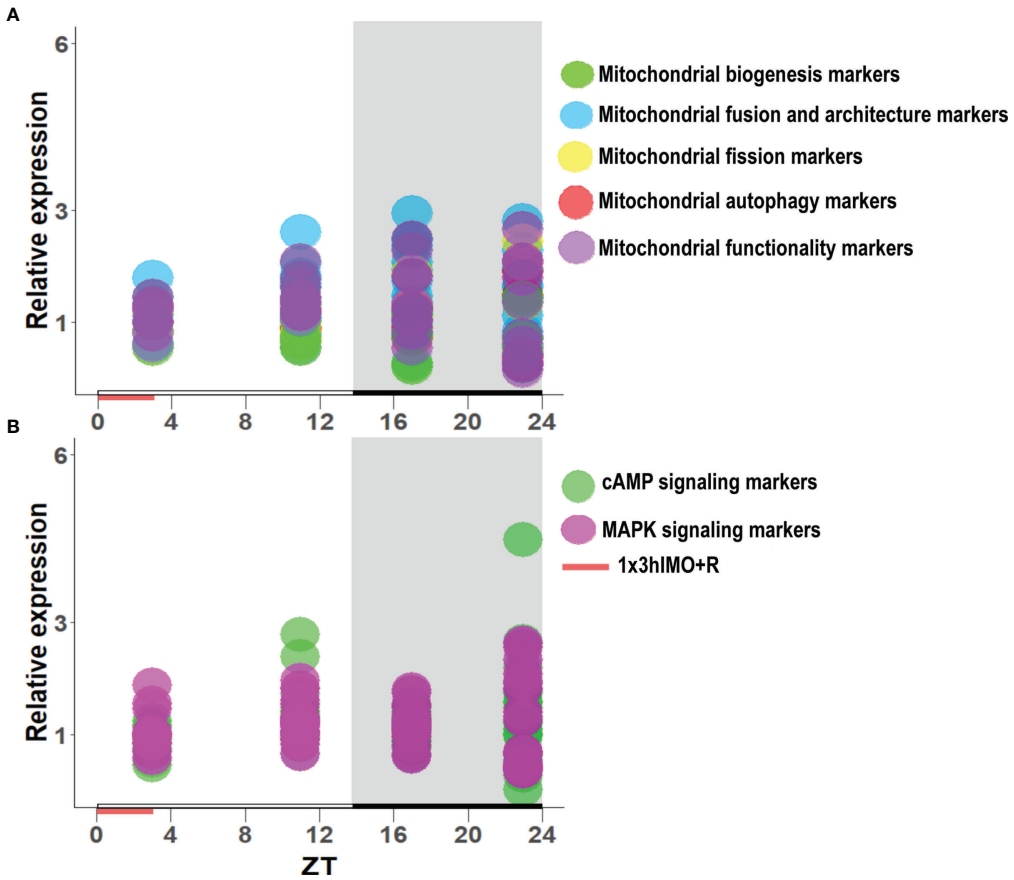

FIGURE 11 | The transcription pattern in spermatozoa of acutely stressed adult rats with different recovery periods (ZT3, ZT11, ZT17, and ZT23). Data shown represent the transcriptional pattern of the genes for mitochondrial dynamics/functionality markers **(A)** as well as cAMP and MAPK signaling pathway-related molecules **(B)**. Points represent a deviation in the transcription of a particular gene at different ZT time points.

expression of transcript for *Ucp2* (most abundantly expressed UCP protein in rat spermatozoa) probably as a consequence of the stress hormone adrenaline (10). Our supplementary results show the trend of the increased mitochondrial membrane potential of spermatozoa treated with adrenaline (**Supplementary Figure S10**). These molecular events can increase spermatozoa motility since it was shown that UCP2 mitigates the loss of human spermatozoa motility (30). The results of the PCA show that most of the transcripts for the

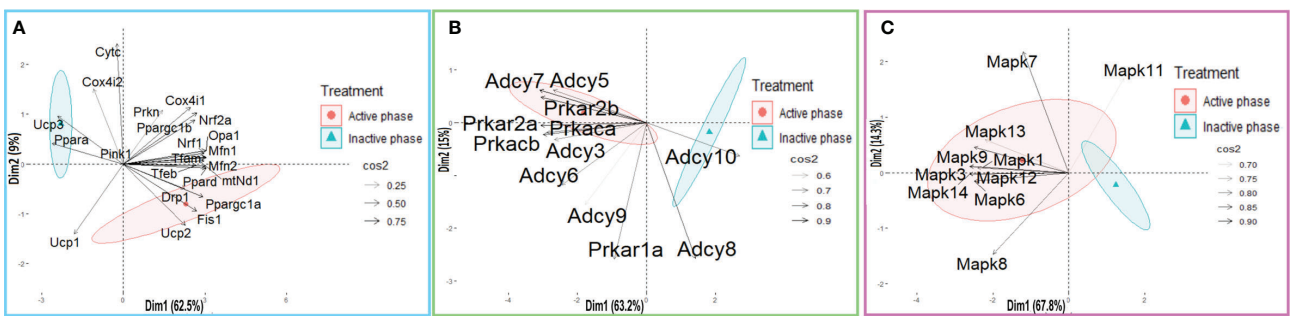

FIGURE 12 | PCA of mitochondrial dynamics **(A)**, cAMP signaling pathway **(B)**, and MAPK signaling pathway **(C)** gene expression on active/inactive phase; Dim1 and Dim2 represent the first two PCs and % of the retained variation. Cos2 estimates the qualitative representation of variables (**Supplementary Tables S1–S3**).

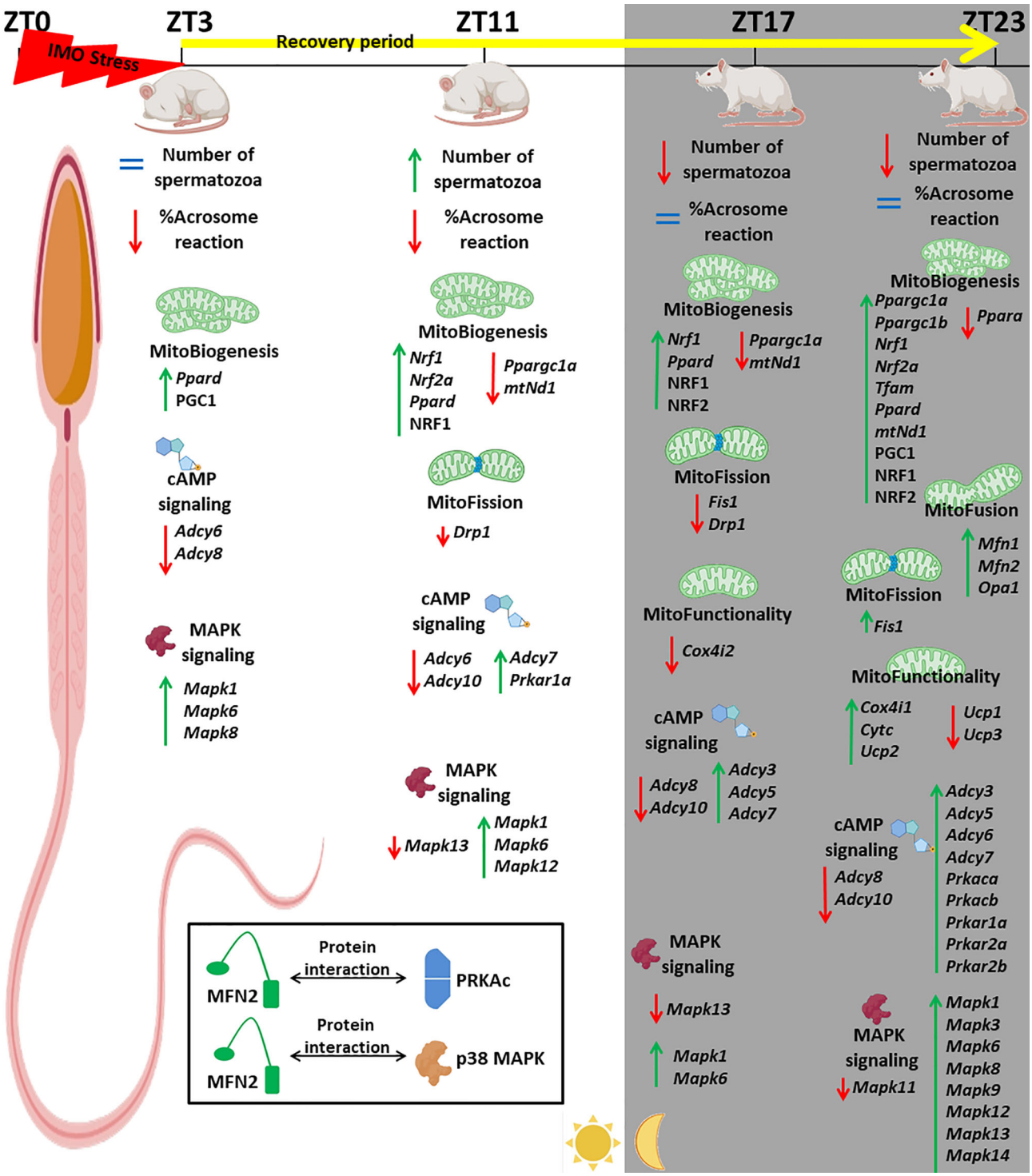

FIGURE 13 | Acute stress, the most common stress in human society, significantly changes 91% of followed mitochondrial dynamics and functionality markers as well as 91% of signaling molecules regulating spermatozoa homeostasis and mitochondrial dynamics/functionality. The most prominent changes were observed 20 h after the end of the stress. The physiological significances are the recovery of spermatozoa number and functionality (positive acrosome reaction). Furthermore, the interactions between the proteins important for spermatozoa homeostasis and functionality (MFN2 and PRKA catalytic subunit, MFN2, and p38MAPK) are shown for the first time.

TABLE 1 | The transcriptional profiles of mitochondrial dynamics and functionality markers and signaling molecules regulating mitochondrial dynamics and functionality as well as spermatozoa number and functionality in spermatozoa of stressed adult rats.

Group Transcript	Time points							
	ZT3		ZT11		ZT17		ZT23	
	Control	1x3hIMO	Control	1x3hIMO	Control	1x3hIMO	Control	1x3hIMO
Ppargc1a	1.0 ± 0.11	1.0 ± 0.20	2.6$^{\#}$ ± 0.54 ↑	0.5* ± 0.14 ↓	2.9$^{\#}$ ± 0.82 ↑	2.4$^{\#}$ ± 0.59 ↑	0.5$^{\#}$ ± 0.18 ↓	1.7* ± 0.65 ↑
Ppargc1b	1.0 ± 0.11	0.6 ± 0.11	0.8 ± 0.01	0.8 ± 0.05	1.7$^{\#}$ ± 0.32 ↑	1.2 ± 0.07	0.8 ± 0.13	1.4* ± 0.03 ↑
Tfam	1.0 ± 0.10	1.2 ± 0.13	1.0 ± 0.05	1.3 ± 0.02	1.0 ± 0.04	1.3 ± 0.01	0.4$^{\#}$ ± 0.05 ↓	1.6*$^{\#}$ ± 0.01 ↑
Nrf1	1.0 ± 0.11	1.1 ± 0.02	1.1 ± 0.04	1.7*$^{\#}$ ± 0.18 ↑	0.8 ± 0.07	1.3* ± 0.03 ↑	0.4$^{\#}$ ± 0.01 ↓	2.0*$^{\#}$ ± 0.12 ↓
Nrf2a	1.0 ± 0.11	1.2 ± 0.07	1.1 ± 0.05	1.8*$^{\#}$ ± 0.10	1.2 ± 0.09	1.4 ± 0.06	0.7 ± 0.03	1.9*$^{\#}$ ± 0.12 ↑
Ppara	1.0 ± 0.11	1.4 ± 0.29	1.8$^{\#}$ ± 0.32 ↑	2.0$^{\#}$ ± 0.16	2.5$^{\#}$ ± 0.17 ↑	2.5$^{\#}$ ± 0.10 ↑	2.8$^{\#}$ ± 0.36 ↑	0.5*$^{\#}$ ± 0.08 ↓
Ppard	1.0 ± 0.12	1.8*$^{\#}$ ± 0.14 ↑	0.8 ± 0.04	1.6*$^{\#}$ ± 0.03 ↑	1.0 ± 0.02	2.1*$^{\#}$ ± 0.00 ↑	0.5$^{\#}$ ± 0.01 ↓	2.3*$^{\#}$ ± 0.04 ↑
mtNd1	1.0 ± 0.12	1.1 ± 0.06	1.6$^{\#}$ ± 0.01	1.1* ± 0.02 ↓	1.9$^{\#}$ ± 0.04 ↑	1.5$^{\#}$ ± 0.04 ↑	0.2$^{\#}$ ± 0.01 ↓	1.1* ± 0.03 ↑
Mfn1	1.0 ± 0.11	1.0 ± 0.11	0.9 ± 0.06	1.2 ± 0.15	1.0 ± 0.15	1.3 ± 0.16	0.3$^{\#}$ ± 0.02 ↓	2.1*$^{\#}$ ± 0.12 ↑
Mfn2	1.0 ± 0.11	0.8 ± 0.11	0.7 ± 0.02	0.9 ± 0.09	0.7 ± 0.11	0.9 ± 0.03	0.2$^{\#}$ ± 0.03 ↓	1.5*$^{\#}$ ± 0.10 ↑
Opa1	1.0 ± 0.11	1.3 ± 0.01	0.8 ± 0.05	1.2 ± 0.05	1.1 ± 0.01	1.1 ± 0.06	0.4$^{\#}$ ± 0.02 ↓	1.9*$^{\#}$ ± 0.01 ↑
Fis1	1.0 ± 0.11	1.2 ± 0.12	0.8 ± 0.10	0.7 ± 0.06	1.9$^{\#}$ ± 0.06 ↑	1.3* ± 0.06 ↑	1.3 ± 0.10	2.4*$^{\#}$ ± 0.39 ↑
Drp1	1.0 ± 0.11	1.0 ± 0.13	1.4 ± 0.04	0.5* ± 0.32 ↓	1.1 ± 0.01	0.6* ± 0.02 ↓	1.3 ± 0.03	1.5 ± 0.07
Prkn	1.0 ± 0.05	0.5 ± 0.22	0.65 ± 0.15	0.6 ± 0.10	0.3$^{\#}$ ± 0.09 ↓	0.2$^{\#}$ ± 0.05 ↓	0.5 ± 0.12	0.7 ± 0.16
Cox4i1	1.0 ± 0.12	1.3 ± 0.10	1.4 ± 0.07	1.4 ± 0.07	1.2 ± 0.10	1.0 ± 0.12	0.3$^{\#}$ ± 0.01 ↓	1.8*$^{\#}$ ± 0.04 ↑
Cox4i2	1.0 ± 0.12	0.6 ± 0.27	1.8 ± 0.45	1.2 ± 0.31	0.8 ± 0.01	0.5*$^{\#}$ ± 0.05 ↓	0.4$^{\#}$ ± 0.03 ↓	0.4$^{\#}$ ± 0.02 ↓
Cytc	1.0 ± 0.12	1.3 ± 0.05	1.2 ± 0.04	1.4 ± 0.05	1.2 ± 0.02	1.3 ± 0.01	0.7 ± 0.01	1.4* ± 0.01 ↑
Ucp1	1.0 ± 0.12	1.4 ± 0.47	2.1 ± 0.59	1.2 ± 0.42	2.5$^{\#}$ ± 0.48 ↑	2.3$^{\#}$ ± 0.88 ↑	2.1$^{\#}$ ± 0.71 ↑	0.2*$^{\#}$ ± 0.04 ↓
Ucp2	1.0 ± 0.11	1.2 ± 0.03	1.1 ± 0.04	1.3 ± 0.09	1.8$^{\#}$ ± 0.02 ↑	1.8$^{\#}$ ± 0.04 ↑	0.6$^{\#}$ ± 0.01 ↓	2.1*$^{\#}$ ± 0.07 ↑
Ucp3	1.0 ± 0.11	0.8 ± 0.09	1.6$^{\#}$ ± 0.21 ↑	1.2 ± 0.31	1.0 ± 0.31	1.0 ± 0.15	2.7$^{\#}$ ± 0.19 ↑	0.1*$^{\#}$ ± 0.02 ↓
Adcy3	1.0 ± 0.11	1.1 ± 0.12	2.4$^{\#}$ ± 0.13 ↑	2.8$^{\#}$ ± 0.27 ↑	0.9 ± 0.20	1.5* ± 0.43 ↑	1.1 ± 0.18	4.5*$^{\#}$ ± 0.77 ↑
Adcy5	1.0 ± 0.11	1.3 ± 0.07	1.0 ± 0.09	1.3 ± 0.01	0.8 ± 0.09	1.3 ± 0.07	1.0 ± 0.22	1.8 ± 0.18
Adcy6	1.0 ± 0.11	0.5 ± 0.01	1.7$^{\#}$ ± 0.16 ↑	1.2 ± 0.01	1.4 ± 0.07	1.5 ± 0.05	0.6 ± 0.01	1.6*$^{\#}$ ± 0.04 ↑
Adcy7	1.0 ± 0.10	1.2 ± 0.01	0.7 ± 0.07 ↑	1.3* ± 0.14 ↑	0.8 ± 0.18	1.5* ± 0.05 ↑	0.6$^{\#}$ ± 0.03 ↓	2.7*$^{\#}$ ± 0.13 ↑
Adcy8	1.0 ± 0.11	0.4* ± 0.13 ↓	1.1 ± 0.14	1.2 ± 0.16	1.4 ± 0.10	0.6*$^{\#}$ ± 0.03 ↓	1.7$^{\#}$ ± 0.14 ↑	0.01*$^{\#}$ ± 0.0 ↓
Adcy10	1.0 ± 0.12	0.9 ± 0.01	1.4 ± 0.13	0.9* ± 0.08 ↓	1.3 ± 0.15	0.9* ± 0.08 ↓	1.0 ± 0.22	0.4*$^{\#}$ ± 0.14 ↓
Prkaca	1.0 ± 0.11	0.7 ± 0.10	1.3 ± 0.08	1.0 ± 0.01	1.2 ± 0.09	1.1 ± 0.03	0.4$^{\#}$ ± 0.03 ↓	1.5*$^{\#}$ ± 0.09 ↑
Prkacb	1.0 ± 0.18	0.9 ± 0.07	1.3 ± 0.06	1.5 ± 0.07	1.0 ± 0.03	1.0 ± 0.12	0.4$^{\#}$ ± 0.01 ↑	2.2*$^{\#}$ ± 0.2 ↓
Prkar1a	1.0 ± 0.10	1.0 ± 0.08	0.9 ± 0.04	1.4* ± 0.16 ↑	1.0 ± 0.06	1.2 ± 0.09	0.3$^{\#}$ ± 0.01 ↓	1.2* ± 0.02 ↑
Prkar2a	1.0 ± 0.13	0.7 ± 0.09	1.1 ± 0.09	1.0 ± 0.06	0.9 ± 0.07	0.9 ± 0.07	0.4$^{\#}$ ± 0.05 ↓	1.6*$^{\#}$ ± 0.14 ↑
Prkar2b	1.0 ± 0.09	0.7 ± 0.07	1.2 ± 0.10	1.0 ± 0.09	0.9 ± 0.01	1.1 ± 0.09	0.5$^{\#}$ ± 0.06 ↓	1.8*$^{\#}$ ± 0.05 ↑
Mapk1	1.0 ± 0.07	1.9*$^{\#}$ ± 0.11 ↑	1.0 ± 0.06	2.0*$^{\#}$ ± 0.15 ↑	1.2 ± 0.11	1.8*$^{\#}$ ± 0.04 ↑	0.4$^{\#}$ ± 0.01 ↓	2.6*$^{\#}$ ± 0.16 ↑
Mapk3	1.0 ± 0.10	0.7 ± 0.07	1.1 ± 0.03	1.2 ± 0.04	1.1 ± 0.04	1.0 ± 0.04	0.4$^{\#}$ ± 0.06 ↓	1.8*$^{\#}$ ± 0.04 ↑
Mapk6	1.0 ± 0.11	1.6*$^{\#}$ ± 0.13 ↑	0.6 ± 0.02	1.8*$^{\#}$ ± 0.04 ↑	0.8 ± 0.05	1.7*$^{\#}$ ± 0.03 ↑	0.4$^{\#}$ ± 0.04 ↓	2.3*$^{\#}$ ± 0.04 ↑
Mapk8	1.0 ± 0.11	1.4*$^{\#}$ ± 0.11 ↑	1.2 ± 0.07	1.5$^{\#}$ ± 0.07	1.1 ± 0.04 ↑	1.3 ± 0.05	0.4$^{\#}$ ± 0.05 ↓	1.9*$^{\#}$ ± 0.29 ↑
Mapk9	1.0 ± 0.17	1.0 ± 0.13	1.1 ± 0.05	1.2 ± 0.13	1.0 ± 0.05	1.2 ± 0.08	0.6 ± 0.05	1.9*$^{\#}$ ± 0.14 ↑
Mapk11	1.0 ± 0.10	0.8 ± 0.09	1.3 ± 0.03	1.2 ± 0.09	1.2 ± 0.14	1.5$^{\#}$ ± 0.07 ↑	1.4 ± 0.12	0.7* ± 0.10 ↓
Mapk12	1.0 ± 0.11	1.0 ± 0.12	0.9 ± 0.07	1.8*$^{\#}$ ± 0.09 ↑	1.2 ± 0.03	1.4 ± 0.09	0.3$^{\#}$ ± 0.04 ↓	2.5*$^{\#}$ ± 0.15 ↑
Mapk13	1.0 ± 0.11	0.6 ± 0.07	1.6$^{\#}$ ± 0.16 ↑	0.9* ± 0.06 ↓	1.3 ± 0.12	0.6* ± 0.29 ↓	0.4$^{\#}$ ± 0.01 ↓	1.3* ± 0.05 ↑
Mapk14	1.0 ± 0.12	0.8 ± 0.01	1.0 ± 0.02	1.3 ± 0.05	1.1 ± 0.01	1.1 ± 0.01	0.5$^{\#}$ ± 0.01 ↓	2.1*$^{\#}$ ± 0.01 ↑

Data are presented as means ± SEM values of two independent experiments. Statistical significance at $p < 0.05$: * *vs. the control group of each time point;* $^{\#}$ *vs. the control group of ZT3 time point. Green arrow indicates the increased level of the transcript, while red arrow indicates decreased level of the transcript.*

main markers of mitochondrial dynamics were highly expressed during the active/dark phase of the day (**Figure 12A**), suggesting the importance of molecular timing in regulation of the above-mentioned markers.

New insights into the understanding of molecular events related to the effects of acute stress on spermatozoa include our finding that shows that 91% of markers of signaling pathways regulating both mitochondrial dynamics and spermatozoa

functionality are changed during the recovery from acute stress. Again, heat map analysis (**Figure 10B**, **Supplementary Figure S8**) clearly showed that changes are most abundant at the ZT23, and they are mostly increased expression. All increased transcripts are, for the signaling molecules, very well known as the essential regulators of spermatozoa number/functionality (43), as well as regulators of PGC1, the biogenesis of OXPHOS, mitofusion, mitofission, and mitophagy (32, 33, 37). Furthermore, all affected molecules are part of the complex signaling network in spermatozoa precisely regulated to provide fertility homeostasis in health and diseases (67). The consequences of the increased expression of transcripts are restored spermatozoa functionality at the ZT17 and ZT23 since it was shown that cAMP signaling improves sperm motility (68, 69) and it is important for the activation of CatSper channels (70). Increased expressions of transcripts for all subunits of PRKA are also a great adaptive and ameliorative mechanism since it was reported that the PRKAR2A reduction in asthenozoospermic patients decreases sperm quality (71), while Prkar2b is sensitive to heat (72). The results of the PCA clearly showed that the transcriptional patterns were different during the active and inactive phases and that most of the transcripts were highly expressed during the active/dark phase of the day. Interestingly, the transcript for the most important spermatozoal ADCY, ADCY10, was highly expressed during the inactive/light phase of the day (**Figure 12B**). Last, but not least, increased transcripts for *Mapk1*, *Mapk3*, and *Mapk14* in spermatozoa from rats recovered for 20 h could be compared with findings that testicular hyperthermia induces both MAPK1/3 and MAPK14 (73) and that MEK1/2 and ERK2 regulate the spermatozoa capacitation (74). The results of the PCA clearly showed that the transcriptional patterns of all analyzed MAPKs, except *Mapk11*, were highly expressed during the active/dark phase of the day (**Figure 12C**). Moreover, *Mapk8* significantly increased at ZT3 and ZT23, and it was shown that phosphorylation of MAPK8 is associated with germ cell apoptosis and redistribution of the Bcl2-modifying factor (75). All molecular events could be possible adaptive responses, and this was proven by recovery of functionality at ZT23.

We believe that results presented here have a significant translational aspect related to the effect of acute stress on male fertility. To prove that our results have translational significance, we started our analysis using spermatozoa obtained from human subjects, and preliminary results showed the correlation of different transcriptional profiles of the mitochondrial dynamics markers and different types of spermiograms. Moreover, according to the questionnaire completed by 115 patients from a governmental ART clinic providing the IVF service for free, 105/115 (91%) reported some degree of stress: 51/115 (44%) reported a low degree of stress, 41/115 (36%) reported frequent stressful situations, and 13/115 (11%) reported a high degree of stress (Tomanic et al., unpublished results).

It is important to point out that our investigation did not consider a possible contribution of epididymal cells to the RNA isolated from spermatozoa. This is important since spermatozoa RNA is subjected to epididymal RNA contamination that is transferred to spermatozoa *via* extracellular vesicles such as epididymosomes. Also, the aim of our study was not to assess motility, but certainly it is well known that mitochondria produce energy for sperm movement. Last, but not least, it is shown that structural abnormalities and decreased spermatozoa motility are associated with decrease in mitochondrial activity and decrease in basal oxygen consumption (76). Since stress stimulates reactive oxygen species and higher concentrations of reactive oxygen species can have detrimental effects on quality of spermatozoa (77), our results showing the increase in the expression of NRF1 and NRF2 transcripts and proteins could be the possible mechanism of adaptation for the restored spermatozoa functionality 20 h after acute stress.

Maybe it is noteworthy that all molecules involved in the regulation of spermatozoa homeostasis and functionality could be possible candidates and eventually responsible for male (in/sub)fertility. Recently, it was described that the sperm-specific form of lactate dehydrogenase is required for fertility and is an attractive target for male contraception (78). Since the existing literature suggests the importance of semen quality and male fertility not only as the fundamental marker of reproductive health but also as fundamental biomarkers of overall health and harbingers for the development of comorbidity and mortality, we anticipate our results to be a starting point for more investigations considering the mitochondrial dynamics markers, or their transcriptional profiles as possible predictors of (in/sub)fertility.

CONCLUSIONS

Acute stress, the most common stress in human society, significantly changes 91% of followed mitochondrial dynamics and functionality markers as well as 91% of signaling molecules regulating spermatozoa homeostasis and mitochondrial dynamics/functionality. This leads to the recovery of spermatozoa number/functionality (positive acrosome reaction), which is important for male (in/sub)fertility. Stress-triggered changes represent adaptive mechanisms to keep spermatozoa functionality, and they are essential for fertility. Besides the effects of stress recovery, our results show the circadian-like nature in the expression of some important regulators of spermatozoa function.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

All experimental protocols were approved (statement no. 01-201/3) by the local Ethical Committee on Animal Care and Use of the University of Novi Sad.

AUTHOR CONTRIBUTIONS

IS—acquisition of the data; analysis and interpretation of the data; drafting of the manuscript; revising manuscript critically for important intellectual content; final approval of the version to be submitted. SR.—acquisition of the data; analysis and interpretation of the data; revising manuscript critically for important intellectual content; final approval of the version to be submitted. TT—acquisition of the data; revising manuscript critically for important intellectual content; final approval of the version to be submitted. MM—acquisition of the data; revising manuscript critically for important intellectual content; final approval of the version to be submitted. TK—acquisition of the data; analysis and interpretation of the data; revising manuscript critically for important intellectual content; final approval of the version to be submitted. SA—the conception and design of the research; acquisition of the data; analysis and interpretation of the data; drafting the manuscript; revising manuscript critically for important intellectual content; final approval of the version to be submitted. All authors—approved the final version of the manuscript; agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved; qualify for authorship, and all those who qualify for authorship are listed.

FUNDING

This research was funded by the Serbian Ministry of Education, Science and Technological Development (CIV-CeRES-2021, 451-03-9/2021-14/200125) and the Autonomous Province of Vojvodina (2708).

ACKNOWLEDGMENTS

This work was supported by grants from the Serbian Ministry of Education, Science and Technological Development (CIV-CeRES-2021; 451-03-9/2021-14/200125) and the Autonomous Province of Vojvodina (2708). The authors are very grateful to Dr. Milan Zupunski for help in PCA as well as to Strahinja Keselj for help with English language editing.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.896193/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

GLOSSARY

1x3hIMO	one time immobilization stress with a duration of 3 h
Adcy3	gene encoding adenylate cyclase 3
Adcy5	gene encoding adenylate cyclase5
Adcy6	gene encoding adenylate cyclase 6
Adcy7	gene encoding adenylatecyclase 7
Adcy8	gene encoding adenylate cyclase 8
Adcy10	gene encodingadenylate cyclase 10
COX4	cytochrome c oxidase subunit 4
Cox4i1	geneencoding cytochrome c oxidase subunit 4i1
Cox4i2	gene encoding cytochromec oxidase subunit 4i2
Cytc	gene encoding cytochrome c
DRP1	dynamin 1-like;
Drp1	gene encoding dynamin 1-like
FIS1	fission mitochondrial 1
Fis1	geneencoding fission mitochondrial 1
IMO	immobilization
MAPK	mitogenactivatedprotein kinase
Mapk1	gene encoding mitogen-activated protein kinase 1
Mapk3	gene encoding mitogen-activated protein kinase 3
Mapk6	gene encoding mitogen-activated protein kinase 6
Mapk8	gene encoding mitogen-activated protein kinase 8
Mapk9	gene encoding mitogen-activated protein kinase 9
Mapk11	gene encoding mitogen-activated protein kinase 11;
Mapk12	gene encoding mitogen-activated protein kinase 12
Mapk13	gene encoding mitogen-activated protein kinase 13
Mapk14	gene encoding mitogenactivated protein kinase 14
MFN1	mitofusin 1
Mfn1	gene encoding mitofusin 1;
MFN2	mitofusin 2
Mfn2	gene encoding mitofusin 2
mtNd1	gene encoding
NADH dehydrogenase 1	mitochondrial
NRF1	nuclear respiratory factor 1
Nrf1	gene encoding nuclear respiratory factor 1
NRF2	nuclear respiratory factor 2;
Nrf2a	gene encoding nuclear respiratory factor 2
OPA1	mitochondrial dynamin like GTPase
Opa1	gene encoding mitochondrial dynamin like GTPase;
OXPHOS	oxidative phosphorylation
PGC1a	peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PGC1b	peroxisome proliferator-activated receptor gamma coactivator 1-beta
PINK1	PTEN induced kinase 1
Ppara	gene encoding peroxisome proliferator-activated receptor alpha
Ppard	gene encoding peroxisome proliferator-activated receptor delta
Ppargc1a	gene encoding peroxisome proliferator-activated receptor gamma coactivator 1-alpha;
Ppargc1b	transcripts for gene encoding peroxisome proliferator-activated receptor gamma coactivator 1-beta
PRKA	protein kinase AMP-activated;
PRKAc	protein kinase AMP-activated catalytic subunit
Prkaca	gene encoding protein kinase cAMP-activated catalytic subunit alpha
Prkacb	gene encoding protein kinase cAMP-activated catalytic subunit beta
Prkar1a	gene encoding protein kinase cAMP-dependent type I regulatory subunit alpha
PRKAR2A	protein kinase cAMP-dependent type II regulatory subunit alpha
Prkar2a	gene encoding protein kinase cAMP-dependent type II regulatory subunit alpha;
Prkar2b	gene encoding protein kinase cAMP-dependent type II regulatory subunit beta
T+DHT	testosterone + dihydrotestosterone
TFAM	transcription factor A mitochondrial
Tfam	gene encoding transcription factor A mitochondrial
UCPs	uncoupling proteins
Ucp1	gene encoding uncoupling protein 1
Ucp2	gene encoding uncoupling protein 2
Ucp3	gene encoding uncoupling protein 3

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OPINION
published: 14 July 2022
doi: 10.3389/fendo.2022.897139

Biomarkers and Diagnostics Will Play Essential Roles in Advancing Innovative Contraception

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Keywords: contraception, biomarker, diagnostic, regulatory, spermatogenesis

OPEN ACCESS

Edited by:
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Reviewed by:
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Daniel G Cyr, Université du Québec, Canada

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Specialty section:
This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology

Received: *15 March 2022*
Accepted: *31 March 2022*
Published: *14 July 2022*

Citation:
Lindsey CC and Johnston DS (2022) Biomarkers and Diagnostics Will Play Essential Roles in Advancing Innovative Contraception. Front. Endocrinol. 13:897139. doi: 10.3389/fendo.2022.897139

INTRODUCTION

The field of contraception development has exciting new momentum. Novel, innovative approaches will expand the contraceptive options available to men and women. However, the associated innovations present fresh challenges to clinical development and regulatory approval. How can stakeholders best ensure that obstacles will be overcome so the new discoveries will move from bench to bedside?

Translational medicine came to prominence over 20 years ago and arose from the need to improve the transfer of biological knowledge into medical utility. Critical components of translational medicine are the formation and utilization of animal models relevant to the human condition, as well as the development of diagnostics and biomarkers which are integral to drug development by informing evidence-based preclinical and clinical decisions. This opinion piece discusses the current need for biomarkers and diagnostics in the development of contraceptives and suggests how the field can work together toward that goal.

Development of Tools

Biomarkers and diagnostics are generally named in connection to their usage. These include, but are not limited to disease biomarkers, patient selection biomarkers, pharmacodynamic (PD) biomarkers, and diagnostics to understand chemical concentration. For example, disease biomarkers indicate potential disease (e.g., prostate specific antigen test for prostate cancer). Patient selection biomarkers are predictive of a given person's response to a drug, guiding clinical use or potentially inclusion in a clinical trial (e.g., HER2 amplification for cancer). Disease and patient selection biomarkers, not typically used directly for contraception, are important for other therapeutic areas and clearly illustrate the application of such tools.

The PD biomarker is relevant to all drug development. It indicates the drug effect on the molecular target in an organism and is typically as proximal to the target modulation site as feasibly possible to minimize any indirect effects. If the target is an enzyme, the PD biomarker is usually the product of the enzyme, and PD activity is read as a change in the concentration of the enzymatic product. If the assay is appropriately sensitive, a small amount of target modulation will result in the significant change in the PD biomarker.

However, other factors also impact the selection of a PD biomarker, such as sample procurement. For example, if the target is a testis-specific enzyme and an invasive testicular

biopsy is required for direct target activity measurement, obtaining that sample for clinical development is unlikely. In such cases, an indirect readout such as ejaculated sperm number, sperm morphology or sperm motility may be employed as an alternative.

Consider the potential use of a PD biomarker for the male contraceptive target Soluble Adenylyl Cyclase (sAC), encoded for by the ADCY10 gene. The enzyme converts ATP to cAMP: a requirement of sperm motility. Targeted deletions of ADCY10 in mice result in male infertility due to impaired sperm motility (1). Two infertile men have been identified with identical frameshift mutations leading to premature translation termination upstream of the nucleotide binding site in sAC (2). Importantly, treatment of sperm lacking sAC activity with a cell-permeable cAMP analogue increases sperm motility significantly. An excellent PD biomarker to evaluate a sAC inhibitor's function is the measurement of cAMP levels in freshly ejaculated sperm prior to and following test subject exposure of a putative sAC inhibitor.

To fully interpret the results of PD biomarker analysis, one must understand the drug pharmacokinetics (PK) at the target site by measuring the drug concentration associated with the target site (e.g., plasma, rete testis fluid, ejaculate, vaginal cavity secretions). The relationship of these two parameters, a "PK/PD relationship," is critical for determining whether the active pharmaceutical ingredient is modulating the target of the drug (indicated by the PD biomarker), and whether the active ingredient concentration required to induce the observed modification of the PD biomarker results in the desired clinical endpoint. Although the science of PK is broader than local drug concentration (it incorporates the absorption, distribution, metabolism, and excretion of drugs), in the context of the PK/PD relationship, 'PK' implies local drug concentration.

Deciding What to Use in the Clinic

For effective use in translational research, the development and validation of biomarkers requires ample time and investment. Novel biomarkers require significant pre-clinical research and development to ascertain their suitability for the clinical environment. Analytical validation, particularly evaluation of the biomarker test's precision and accuracy, is critical. In some cases, biomarkers could be developed for use beyond the clinic and become commercial companion diagnostics (e.g., patient selection biomarkers, disease biomarkers), defined as medical devices by the Food and Drug Administration (FDA).

Similar to constraints that may arise from sample acquisition, the cost/benefit ratio of developing a PD biomarker that is proximal to the target and the significant time and resources needed to develop and validate a clinical biomarker for testing may give reason for more distal PD biomarker strategies to be considered. For example, as discussed above with respect to sAC inhibition, cAMP quantitation in ejaculated sperm is an excellent PD biomarker; however, an indirect method for assessing sAC activity could be evaluating sperm motility. This evaluation may be sufficiently useful without incurring the time and cost of developing a validated clinical cAMP assay for sperm. Andrology laboratories associated with large medical institutions are trained in standardized sperm evaluation methods (e.g., World Health Organization Guidelines) (3). The assumption is that the observed decrease in sperm motility following exposure to a sAC inhibitor is the result of target modulation (inhibition of sAC leading to a decrease in intracellular sperm cAMP concentrations) ultimately leading to decreased sperm motility.

A Changing Paradigm

Attempts to develop a male contraceptive *via* the suppression of intratesticular androgen concentration has a long and well documented history (4–8). Regulatory agencies have been aware of the mechanism of suppressing endogenous androgen production for more than 30 years. The biomarker of azoospermia and its use as a biomarker of clinical utility for contraception is accepted by regulatory agencies.

This is not true for potential male contraceptive products that function *via* non-hormonal methods. The development of male contraceptives directed against highly selective/specific mechanisms of action (e.g., single gene products such as sAC) will require thoughtful interaction with regulatory agencies to efficiently establish a comfort level for each new paradigm (e.g., inhibition of meiosis, perturbation of spermiogenesis, inhibition of capacitation, inhibition of sperm motility).

Discussion

We have attempted to demonstrate that the changing paradigm in the field of contraceptive development will benefit from the development of biomarkers and diagnostics to facilitate the translation from preclinical to clinical research. These tools will also facilitate interactions with regulatory agencies and inform decision making during the clinical trial process. A key question is how to stimulate progress to this end?

The *Eunice Kennedy Shriver* National Institute of Child Health and Human Development (NICHD) has emphasized the importance of biomarker development for contraceptive research programs in recent years. Since 2017, Request for Applications (RFAs) for the Contraception Research Centers have emphasized the need to pursue these studies. Since 2019, the NICHD's Biological Testing Facility (PAR-21-078) and Chemical Screening and Optimization Facility (PAR-19-261) have supported contraceptive development to execute service requests for the generation of data supporting novel contraception development, including biomarker data. Despite these calls and opportunities, applications including biomarker identification and validation have been lacking and biomarker research has not been incorporated into funded contraception development programs.

To stimulate focus more directly in this area, in 2021 the NICHD published RFA-HD-22-018 specifically to support the development of biomarkers and diagnostics for new contraceptive methods by small business. Awards are expected in 2022. This RFA was an important commitment and highlights the priority placed on biomarkers/diagnostics. Hopefully, the resulting research will directly support product development and serve as a 'seed crystal' for programs that have yet to include biomarkers/diagnostics in their product development strategies.

How Do We Best Move Forward in the Field of Contraception?

The field would benefit from the organization and execution of a focused workshop, highlighting late-stage preclinical contraception programs and prioritizing discussions about biomarker implementation. A critical component of such a workshop would be the active participation of key opinion leaders, industry representatives, and regulatory agency personnel (e.g., regulatory agency pharmacology/toxicology and clinical staff). Upon adjournment, timely communication of the workshop outcomes would ideally broadcast the key findings, next steps, and regulatory considerations, and thereby deepen information available to the field and strengthen the prospects of all worthy novel approaches.

Could There be Broader Implications for Reproductive Biology/Health?

We believe so. In recent years industry has shown renewed interest in the reproductive health space. Going forward, if a consortium involving industry as well as innovative product developers from academia and small business were to be established, enormous benefit could be realized. Reaching consensus on diagnostics and biomarkers and their validation would expedite product development. The cumulative impact would benefit not only the field of contraception, but all stakeholders across the spectrum of reproductive biology/health.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

FUNDING

The authors are employees of the National Institutes of Health. *Comments and views of the authors do not necessarily represent the views of the NICHD, NIH, or federal government.*

ACKNOWLEDGMENTS

The authors would like to thank Gregory S. Kopf PhD, Vera Halpern MD, and Neha J. Mehta MPH, RAC for sharing their past regulatory experiences related to the topic of this document.

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