SYSTEMIC REGULATION OF ORGAN HOMEOSTASIS AND IMPLICATIONS OF HORMONES AND IMMUNITY

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SYSTEMIC REGULATION OF ORGAN HOMEOSTASIS AND IMPLICATIONS OF HORMONES AND IMMUNITY

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EDITORIAL
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Editorial: Systemic Regulation of Organ Homeostasis and Implications of Hormones and Immunity

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Keywords: neuroimmune interaction, microglia, neurotrophic factor, Leydig cells, Sertoli cells, circadian rhythm

Editorial on the Research Topic

Systemic Regulation of Organ Homeostasis and Implications of Hormones and Immunity

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Propagating life to the next generation is a tightly controlled, interdependent process whereby tissues and organ systems work together to promote an organism's relative fitness and survival. Groups of organs and tissues performing related functions are organized into systems that interact and work cohesively to achieve normal physiological functions of the organism. Though in-depth research is available on each organ/tissue independently (human and other model organisms), the complex interactions that take place between cells, tissues, organs, and the organism are sometimes overlooked. Tissue-specific changes in the micromilieus have a fundamental influence on cellular/organ functions, and alterations to endocrine/ligand signaling can impact local and systemic tissue function, all of which may contribute to disease pathogenesis.

With this background in mind, the current topic explores the potential interactions between endocrine and immune systems that are important for understanding disease biology and investigating associated treatment strategies. Another major mandate of this Research Topic was to explore optimal treatment and management strategies for various disorders, such as cancer. The research and review articles in this topic address interactional and non-classical functions of hormones and immunity.

Circadian rhythms regulate the physiological processes of an organism, including its immune system. Understanding the molecular mechanisms underlying diurnal variation in hosts due to infection-mediated immune responses is warranted. In this context, Jacquelot et al. describe how neuro-immune interactions create rhythmic activity in innate lymphoid cells, and how minor disruptions lead to the development of chronic inflammation. The immune system and the neuroendocrine system communicate extensively through overlapping receptors and networks that control mechanisms of immunity in addition to regulating development and metabolism. Klein describes the immune-endocrine interactions with an emphasis on the hormones of the hypothalamus-pituitary-thyroid axis. In addition, the processes by which immune system–derived thyroid stimulating hormone (TSH) controls thyroid hormone synthesis and bone metamorphosis are also explained in the context of a novel splice variant of TSHβ, a contributing factor in the development of autoimmune thyroid disease. Likewise, Zhan et al. review the immune checkpoint inhibitors (ICI; group of drugs used for treating various types of

malignant tumors) in relation to immune system reactivation, which results in the death of normal tissues and cells, eventually leading to immune-related adverse events. Zhan et al. also discuss the clinical manifestations, possible pathogenesis, and management of ICI-related thyroid dysfunction.

Marinkovic et al. delineate the influence of constant light (LL) on the maturity of the Leydig cells and their various endocrine roles. The effects of the LL are prominent in puberty with increased *Bmal1*, *Per1/2*, and *Reverba* and decreased pituitary genes encoding gonadotropic hormones (*Cga, Lhb, Fshb*). Further, serum androgens and markers of Leydig cell maturity/differentiation (*Insl3, Lhcgr*) and steroidogenesis (*Scarb1, Star, Cyp11a1, Cyp17a1*) were decreased, with increases in serum corticosterone. The authors conclude that LL slows the maturation of Leydig cells with their endocrine function, leading to the delay of reproductive development.

Khan et al. discuss how endocrine and paracrine pathways are regulated by sex hormones and growth factors that have direct control over Sertoli cell proliferation, differentiation, and maturation, and that can directly impact reproduction.

Benitez et al. discuss a neurotrophic factor and its role in activating noradrenergic and cholinergic systems in the rat ovary. Using estradiol valerate (a polycystic ovary phenotype model), their study provides evidence that the primary signal, nerve growth factor (NGF), activates both noradrenergic and cholinergic systems *in vivo*. This in turn increases both norepinephrine (through an NGF-dependent mechanism) and acetylcholine levels, the former of which through an NGF-dependent mechanism. This implies that NGF is the main regulator of dual autonomic control.

Ghafouri-Fard et al. discuss an interactional aspect of insulin-like growth factor signaling in the pathogenesis of neoplasia in relation to non-coding RNA. In addition, novel therapeutic strategies are suggested based on the modification of IGF signaling and identification of the impact of non-coding RNAs in this pathway.

Fujita and Yamashita focus on microglia, resident immune cells of the central nervous system (CNS), and their role in neural development in both normal physiological and pathological conditions. Further, they discuss the heterogeneity of microglia (functional, morphological, and regional heterogeneity across different CNS regions) and associated epigenetic changes coordinating gene expression. Mechanisms underlying spatiotemporal and functional diversity of microglia during developmental stages and other altered or diseased conditions are also discussed.

Liu and Wu assess the impact of intramuscular and vaginal regimens of progesterone on neonatal outcomes in HRT-frozen-thawed embryo transfer cycles. The study concludes that higher serum progesterone induced by intramuscular regimens did not change the live birth rate or neonatal outcomes compared to vaginal regimens.

CONCLUSION AND FUTURE PERSPECTIVE

The collection of articles presented in this topic introduces important work and extends our knowledge on the overlapping and interlinking pathways across organ systems with specific reference to hormones and immunity. With this work, we hope our community is encouraged to tackle the remaining, unresolved issues to advance the body of scientific research into the interactions between hormones and immunity. Identifying comparable and cohesive interactions locally and systemically will pave the way for better understanding and, ultimately, improve disease management and treatments.

AUTHOR CONTRIBUTIONS

All authors have contributed to the editing and writing equally. All authors contributed to the article and approved the submitted version.

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ORIGINAL RESEARCH
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Progesterone Intramuscularly or Vaginally Administration May Not Change Live Birth Rate or Neonatal Outcomes in Artificial Frozen-Thawed Embryo Transfer Cycles

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Backgrounds: Previous studies suggested that singletons from frozen-thawed embryo transfer (FET) were associated with higher risk of large, post-date babies and adverse obstetrical outcomes compared to fresh transfer and natural pregnancy. No data available revealed whether the adverse perinatal outcomes were associated with aberrantly high progesterone level from different endometrium preparations in HRT-FET cycle. This study aimed to compare the impact of progesterone intramuscularly and vaginally regimens on neonatal outcomes in HRT-FET cycles.

Methods: A total of 856 HRT-FET cycles from a fertility center from 2015 to 2018 were retrospectively analyzed. All patients had their first FET with two cleavage-staged embryos transferred. Endometrial preparation was performed with sequential administration of estrogen followed by progesterone intramuscularly 60 mg per day or vaginal gel Crinone 90 mg per day. Pregnancy outcomes including live birth rate, singleton birthweight, large for gestational age (LGA) rate, small for gestational age (SGA) rate, and preterm delivery rate were analyzed. Student's t test, Mann-Whitney U-test, Chi square analysis, and multivariable logistic regression were used where appropriate. Differences were considered significant if $p < 0.05$.

Results: No significant difference of live birth rate was found between different progesterone regimens (Adjusted OR 1.128, 95% CI 0.842, 1.511, p = 0.420). Neonatal outcomes like singleton birthweight (p = 0.744), preterm delivery rate (Adjusted OR 1.920, 95% CI 0.603, 6.11, p = 0.269), SGA (Adjusted OR 0.227, 95% CI 0.027, 1.934, p = 0.175), and LGA rate (Adjusted OR 0.862, 95% CI 0.425, 1.749, p=0.681) were not different between two progesterone regimens. Serum P level >41.82 pmol/L at 14 day post-FET was associated with higher live birth rate than serum P level ≤41.82 pmol/L in HRT-FET cycles when progesterone was intramuscularly delivered (Adjusted OR 1.690, 95% CI 1.002, 2.849, p = 0.049). But singleton birthweight, preterm delivery rate, SGA and LGA rate were not different between these two groups.

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Conclusions: Relatively higher serum progesterone level induced by intramuscular regimen did not change live birth rate or neonatal outcomes compared to vaginal regimen. Monitoring serum progesterone level and optimizing progesterone dose of intramuscular progesterone as needed in HRT-FET cycles has a role in improving live birth rate without impact on neonatal outcomes.

Keywords: frozen-thawed embryo transfer, hormone replacement therapy, progesterone, live birth, birthweight

INTRODUCTION

As cryopreservation has been an efficient and reliable laboratory procedure, freeze-all policy and elective embryo cryopreservation have been increasingly prevalent with a variety of clinic indications like preventing OHSS, implantation of PGT-A, fertility preservation, etc (1). There is a growing number of FET cycles applied due to the endometrial synchrony and relative steady endocrine environment compared to supraphysiologic estrogen milieu generated by controlled ovarian stimulation (COS) in fresh IVF cycles. However, we must recognize the adverse perinatal outcomes of FET, like higher risk of macrosomia, perinatal mortality, and pregnancy complications (2–5). Reports suggested that singletons from FET were associated with higher risk of large and post-date babies, placenta accrete, pre-eclampsia compared to fresh transfer and natural pregnancy (6–9). The reason for that varied. Some reports suggested embryo cryopreservation altered epigenetics regulation and induced abnormal placentation and fetal growth (7). Some studies advised that excessive estrogen priming set off the obstetrics complications (10, 11). Nevertheless, scarce literature has been focused on the supraphysiologic progesterone exposure to the deep placentation.

Progestin directly advanced vascular proliferation during placentation (12). A supraphysiologic progestin exposure in HRT could initiate excessively deep placentation which would make a difference in infant birthweight and obstetrical consequences (13). Previous studies explored the perinatal outcomes in endometrium preparation and indicated an inferior live birth rate with more obstetrics complications like hypertensive disorder, placenta accrete, post-term birth, and macrosomia in artificial cycle FET than nature cycle FET. They assumed a link between adverse obstetric outcomes and the lack of secretion of endogenous progesterone by functional corpus luteum (14–16). But there is no study investigating whether the adverse obstetrics outcome was related to different methods of endometrium preparation in HRT-FET cycles, especially aberrantly high circulating progesterone value induced by suboptimal dose and route of progesterone. The regimen and amount of progesterone applied in HRT-FET cycles varied a lot from different IVF centers, and the exogenous progesterone administered in HRT usually exceeds the endogenous progesterone of menstrual cycles by folds. There is little agreement on the ideal route and dose of administration. Optimizing it could be crucial to maximize both the clinical and neonatal successful rate. Thus our study aimed to explore whether circulating progesterone values induced by different progesterone regimens impact the live birth rate and neonatal outcomes.

Abbreviations: HRT, hormone replacement therapy; FET, frozen-thawed embryo transfer; COS, controlled ovarian stimulation; IVF, *in vitro* fertilization; ICSI, intracytoplasmic sperm injection; LGA, large for gestational age; SGA, small for gestational age; EVT, extravillous trophoblast; 95% CI, 95% confidence intervals; OR, odds ratios; BMI, body mass index.

METHODS

Participants

The retrospective study was undertaken at the assisted reproduction medicine department in Shanghai General Hospital affiliated to Shanghai Jiao Tong University School of Medicine, including 856 women who had undergone their first FET from January 2015 to December 2018. Inclusion criteria were maternal age <48, undergoing two Day 2 or Day 3 cleavage-stage embryos transfer following HRT endometrium preparation. The patients with cryopreserved oocytes or donor oocytes and with prior attempts at conception *via* IVF and FET were excluded from the study. The final database included 856 women and 240 live birth singletons in the criteria. We kept patients re-examined in the first trimester and followed up by phone calls after the first trimester.

IVF and Laboratory Protocols

Ovarian stimulation, oocyte retrieval, and IVF/ICSI procedures have been previously described (17). For IVF, oocytes were inseminated with human tubal fluid supplemented with 10% serum substitute supplement and with around 300,000 progressively motile spermatozoa. For ICSI, oocytes were placed in the fertilization medium immediately after microinjection. Fertilization was evaluated 18 h after insemination. Embryos were cultured in early cleavage medium before Day3 and in multiblast medium afterwards. All embryos were cultured in incubator at 37° C, 5% O_2 and 6% CO_2 concentration. Embryo development was evaluated on Days 2, 3, 5 and 6. Day 2 or Day 3 cleavage-stage embryos with at least two or six blastomeres respectively and with fragmentation <20% according to guidelines (18) were eligible for cryopreservation. The criteria for good-quality embryo were: four to six cells with less than 10% fragmentation for Day 2 embryos, seven to nine cells with less than 10% fragmentation for Day 3 embryos.

Frozen Embryo Transfer Protocol

In a FET cycle, patients were administered estrogen and progesterone sequentially for endometrial preparation before FET. Patients started with estrogen administered orally (Estradiol Valerate or Estradiol Femoston) 6 mg per day with

or without adding estrogen vaginally (Estradiol Femoston) 2 mg per day. Transvaginal ultrasonography, serum E2, LH, and progesterone level were measured at each visit weekly. Once the time of FET was determined, progesterone intramuscularly (60 mg per day) or vaginal gel Crinone (90 mg per day) was initiated daily. They both combined with dydrogesterone orally 30 mg per day and estradiol orally 6 mg per day. Intramuscular Progesterone or Vaginal Crinone was chosen according to patient preference after fully informing them with the advantages and side-effects of different routes such as vaginal itch and discharge or the subcutaneous swell experienced with intramuscular injection. Patients who were undertaken Day 2 or Day 3 cleavage-staged embryo transferred started the progesterone 2 or 3 days before FET, respectively. The vitrification and thawing procedure were previously presented (17). Embryo transfer was performed *via* the same flexible catheter under transabdominal ultrasound guidance. After FET, daily estrogen and progesterone administration continued until a negative pregnancy test was obtained at the 14th day after embryo transfer. If pregnancy was achieved, hormone administration continued until 12 weeks' gestation.

Outcome Measures and Definitions

In order to evaluate the impact of progesterone delivered regimens on clinical outcome, the primary outcome measure was live birth rate. Secondary outcome measures included clinical pregnancy rate, newborn birthweight, large for gestational age (LGA), small for gestational age (SGA), preterm delivery rate. Live birth was defined as a delivery of a viable infant after the 28th gestational weeks. Clinical pregnancy is a pregnancy confirmed by the confirmation of gestational sac or heartbeat. Gestational age was calculated from 14 days before the embryo transfer. Preterm birth was defined as delivery between 28 to 37 gestational weeks. SGA and LGA were defined as birthweight <10th and >90th percentile, respectively. Z score was administered to calculate birthweight adjusted for gestational age and newborn gender using the formula: Z score = $(\chi-\mu) / \sigma$, where χ is the birthweight of the infant, μ is the mean birthweight for the same sex and same gestational age in the reference group and σ is the standard deviation of the reference group. The reference is the Chinese singletons newborns (19).

Statistical Analysis

Patients and singletons live birth demographic baseline, cycle characteristics, clinical and neonatal outcomes were compared using Student's t-test, Mann-Whitney U test, chi-square, and Fisher's exact tests, as appropriate. Whether binary live birth and clinical pregnancy were modified by the regimens of progesterone was assessed by multivariable logistic regression adjusting for major covariates as maternal age, BMI, the route of estrogen administered, whether duration of estradiol treatment >21 days, whether at least one good quality embryo was transferred. Multivariable logistic regression was applied to evaluate the regimens of progesterone impact on neonatal outcomes adjusting for the major covariates mentioned above plus newborn gender. Adjusted odds ratios (OR) and 95% confidence intervals (95% CI) were reported. All analyses were conducted with SPSS statistics. P value < 0.05 was considered statistically significant.

Ethical Approval

Institutional review board and ethics committee of Shanghai General Hospital approval was obtained.

RESULTS

Clinical Outcomes

This analysis included 856 women and 240 live birth singletons with the following outcomes: 44.04% clinical pregnancy rate, 35.63% live birth rate. There were 333 patients who were progesterone administered intramuscularly and 523 patients who were progesterone administered vaginally. Baseline demographics and characteristics were compared between patients with different progesterone regimens (**Table 1**). Among the 856 women, it did not reveal any significant differences for maternal age, BMI, whether there was at least one good quality embryo transferred, endometrium thickness at progesterone starting day, days of estrogen duration, E2, P, LH level at progesterone starting day between two groups. The proportion of patients with estradiol vaginally and orally delivered together in progesterone vaginally group was significantly higher than in progesterone intramuscularly group. Serum progesterone level at 14th day after embryo transfer was significantly higher in progesterone intramuscularly group than in progesterone vaginally group (40.5 pmol/L *versus* 14.95 pmol/L). No significant difference of live birth rate (Crude OR 1.181, 95% CI 0.895, 1.557, p = 0.282) and clinical pregnancy rate (Crude OR 1.170, 95% CI 0.879, 1.557, p = 0.239) was found between different progesterone regimens (**Table 2**). Controlling for maternal age, BMI, the route of estrogen administration, whether estradiol duration was longer than 21 days, whether there was at least one good quality embryo transferred, progesterone administered regimen did not modify the odds of achieving live birth (Adjusted OR 1.128, 95% CI 0.842, 1.511, p = 0.420) or clinical pregnancy (Adjusted OR 1.144, 95% CI 0.863, 1.518, p = 0.349) (**Table 2**). Maternal age and at least one good quality embryo transferred were the only independent factors that increased the live birth rate and clinical pregnancy rate.

Neonatal Outcomes

To further explore the progesterone regimen impact on singleton birthweight and gestational age, a cohort of 240 live birth singletons from 856 patients was further investigated. Neonatal outcomes stratified by the regimens of progesterone administered were presented in **Table 3**. Newborn gender, gestational age, mean birthweight, Z-scores, preterm delivery rate, SGA and LGA rate were not different across two groups (**Table 3**). In multivariate analyses (**Table 3**), the risk of preterm delivery (Adjusted OR 1.920, 95% CI 0.603, 6.11, p = 0.269), the risk of LGA (Adjusted OR 0.862, 95% CI 0.425, 1.749, p = 0.681), and SGA (Adjusted OR 0.227, 95% CI 0.027, 1.934, p = 0.175) were not significantly different between two groups after adjusting for maternal age,

TABLE 1 | Baseline demographics and cycle characteristics according to different progesterone routes.

	Progesterone intramuscularly (N = 333)	Crinone vaginally (N = 523)	P
Maternal age (y)	30.46 ± 4.5	31.0 ± 4.5	0.051
BMI	21.47 ± 3.1	21.22 ± 3.0	0.26
At least one good quality embryo	294	451	0.383
Endometrium thickness at P starting day (mm)	9.0 (8.38, 9.63) (N = 320)	8.9 (8.4, 9.4) (N = 508)	0.355
Days of estradiol administration			
>21 days	24	36	0.856
≤21 days	209	487	
Estrogen route			
Orally and vaginally	126	229	0.009
Orally only	107	294	
E2 level at P starting day (pmol/L)	1374 (789.3, 3843.3) (N = 312)	1342.5 (822, 5291) (N = 479)	0.340
P level at P starting day (pmol/L)	1.1 (0.68, 1.7) (N = 311)	1.2 (0.65, 1.85) (N = 477)	0.663
LH level at P starting day (pmol/L)	8.8 (4.78, 14.11) (N = 311)	8.6 (4.44, 14.72) (N = 477)	0.561
P level at 14th day after embryo transfer (pmol/L)	40.5 (31.61, 57.57) (N = 262)	14.95 (8.29, 25.55) (N = 410)	<0.001

Data are presented as mean ± SD for continuous variables in formal distribution, median (first quartile, third quartile) for continuous variables in informal distribution. P values were assessed with the use of t tests or Wilcoxon rank sum tests or chi-square.

TABLE 2 | Clinical outcomes according to different progesterone routes.

	Progesterone intramuscularly (N = 333)	Crinone vaginally (N = 523)	Crude OR(95% CI)	P	Adjusted OR(95% CI)	P
Clinical pregnancy	155 (46.55)	222 (42.45)	1.181 (0.895, 1.557)	0.239	1.144 (0.863, 1.518)[a]	0.349
Live birth	126 (37.84)	179 (34.23)	1.170 (0.879, 1.557)	0.282	1.128 (0.842, 1.511)[a]	0.420

[a]*adjusted for maternal age, BMI, transfer with at least one good quality embryo, estrogen regimen, whether estrogen administration days >21. CI, confidence interval; OR, odds ratio.*

TABLE 3 | Perinatal outcomes of live birth singletons according to different progesterone routes.

All singletons	Progesterone intramuscularly (N = 95)			Crinone vaginally (N = 145)		P
Newborn gender						
Female	45			71		0.809
Male	50			74		
Gestational age						
32-36	7			7		0.411
≥37	88			138		
Birthweight	3349.19 ± 487.2			3365.58 ± 469.5		0.744
Z score	0.357 ± 1.047			0.345 ± 1.023		0.928
	Progesterone intramuscularly (N = 95)	**Crinone vaginally (N = 145)**	**Crude OR (95% CI)**	**P**	**Adjusted OR (95% CI)**	**P**
Preterm delivery	7	7	1.568 (0.532, 4.623)	0.411	1.920 (0.603, 6.110)[a]	0.269
SGA	1	6	0.246 (0.029, 2.080)	0.319	0.227 (0.027, 1.934)[a]	0.175
LGA	14	27	0.819 (0.410, 1.637)	0.572	0.862 (0.425, 1.749)[a]	0.681

Data are presented as mean ± SD for continuous variables in formal distribution. P values were assessed with the use of t tests or Mann-Whitney U tests or chi-square (Fisher's exact tests as appropriate).

[a]*adjusted for maternal age, BMI, transfer with at least one good quality embryo, estrogen regimen, whether estrogen administration days >21, newborn gender. SGA, small for gestational age; LGA, large for gestational age.*

BMI, estradiol route, whether estrogen administration lasted more than 21 days, whether at least one good quality embryo was transferred, and newborn gender.

Subgroup Analysis

In order to investigate the circulating serum progesterone impact on singleton gestational weeks and birthweight, a

cohort of 262 patients with progesterone intramuscularly administered and 77 live birth singletons was further investigated. We did not analysis vaginal progesterone cohort because systemic serum progesterone value was not the reflection of progesterone Crinone vaginally absorbed (20). From the cohort, clinical and neonatal outcomes of patients with serum progesterone level >41.82 pmol/L and ≤41.82 pmol/L at the 14th day after embryo transfer were compared in **Table 4**. The median value of serum progesterone level was 41.82 pmol/L at the 14th day after embryo transfer for patients with progesterone intramuscularly administered. Patients with serum P level >41.82 pmol/L demonstrated higher clinical pregnancy rate (Adjusted OR 1.670, 95% CI 1.005, 2.774, p = 0.048) and higher live birth rate (Adjusted OR 1.690, 95% CI 1.002, 2.849, p = 0.049) than patients with serum P level ≤41.82pmolL both in univariate analysis and multivariate analysis adjusting for maternal age, BMI, whether at least one good quality embryo was transferred, estrogen regimen, whether estrogen administration days >21. While birthweight, Z-score, preterm delivery rate, LGA and SGA rate were not different between these two groups. Multivariate analysis was not performed for preterm delivery rate, LGA and SGA rate because the sample size in this category was too small.

DISCUSSION

Main Findings

From our study, we found no difference of live birth rate, clinical pregnancy rate, singleton birthweight, preterm delivery rate, LGA and SGA rate between progesterone vaginally and intramuscularly administrations in HRT-FET cycles. Relatively higher serum progesterone level induced by intramuscular regimen did not increase newborn birthweight or prolong gestational weeks compared to vaginal regimen. As for intramuscular progesterone supplementation, serum progesterone concentration higher than 41.82 pmol/L at day 14 post-FET was associated with improved live birth rate and comparable neonatal outcomes compared to P level ≤41.82 pmol/L. Monitoring serum progesterone level and optimizing progesterone dose as needed in intramuscular progesterone HRT-FET cycles has a role in improving clinical outcomes without impact on perinatal outcomes.

Interpretation of Data

Previous studies indicated FET resulted in increased risk of pregnancy-induced hypertension, LGA and post-date newborns compared to fresh IVF-ET cycles (2, 6–8). The difference of gestational weeks and birthweight between FET singletons and fresh IVF cycles singletons led people to speculate the association of the quality of placentation and hormone characteristics from different cycles. People presumed the reasons behind this phenomenon involved the supraphysiologic estrogen milieu in ovarian stimulated cycles (10, 11), embryo cryopreservation technique *per se* induced epigenetics alteration (7), and the lack of functional corpus luteum in HRT-FET cycles (14–16). However no data available explored whether newborn gestational age, birthweight, and the placental-related obstetric complications were associated with the specific protocols of endometrium preparation in FET cycles, especially progesterone replacement regimens. In HRT-FET cycles, once the adequate proliferation of the endometrium is achieved, progesterone daily starts before scheduled embryo transfer, which elicits decidualization of estrogen-primed endometrial stromal cells and develops endometrial receptivity. Progestin assists with extravillous trophoblast (EVT) invasion and endometrium vascular remodeling, which are important for pregnancy, because defects in extravillous trophoblast invasion could generate first trimester decidual hemorrhage and induce later adverse outcome like pre-eclampsia and post-date newborn (21). Progesterone acts on endometrium inhibiting uterus contractility and creates uterine quiescence (22). There is no functioning corpora luteal and endogenous progesterone production in HRT. The progesterone replacement applied in FET cycles usually created a relatively high P milieu. Several studies reported the negative impact of very high progesterone level on endometrium maturation and implantation in non-human studies (23, 24). Aberrantly higher levels of progesterone in early pregnancy can result in over-invasion of the extravillous trophoblast invasion by affecting the functions of syncytial trophoblast and decidual cells, in this way potentiating the later superficial placentation which influence newborn birthweight and gestational weeks (12, 21, 25).

TABLE 4 | Clinical and perinatal outcomes between P level >41.82 pmol/L and P level ≤41.82 pmol/L groups when progesterone intramuscularly was used in HRT-FET cycles.

	P level >41.82 pmol/L (N = 131)	P level ≤41.82 pmol/L (N = 131)	Crude OR(95% CI)	P	Adjusted OR(95% CI)	P
Clinical Pregnancy	72 (54.9)	55 (41.98)	1.686 (1.034, 2.940)	0.036	1.670 (1.005, 2.774)[a]	0.048
Live Birth	59 (45.04)	43 (32.82)	1.677 (1.016, 2.769)	0.043	1.690 (1.002, 2.849)[a]	0.049
Singletons Neonatal outcome	**P level >41.82 pmol/L (N = 42)**	**P level ≤41.82 pmol/L (N = 35)**	**Crude OR (95% CI)**		**P**	
Birthweight	3331.79 ± 421.19	3354.03 ± 643.51			0.861	
Z score	0.2778 ± 0.9658	0.6248 ± 1.1147			0.153	
Preterm delivery	2	5	0.391 (0.074, 2.051)		0.444	
LGA	5	8	0.610 (0.194, 1.917)		0.393	
SGA	1	0	/		/	

[a]*adjusted for maternal age, BMI, transfer with at least one good quality embryo, estrogen regimen, whether estrogen administration days >21. SGA, small for gestational age; LGA, large for gestational age.*

The ideal administration route, duration, and the dosage of progesterone in HRT-FET cycles have not been well defined. Vaginal Crinone and intramuscular progesterone are two preferred luteal support regimens. Vaginally delivered progesterone reached the uterus directly and induced higher progesterone concentration in the uterine endometrium with relatively lower circulating P level compared to intramuscular regimen (26). Report has suggested micronized vaginal P supplement helped decrease uterine contraction frequency and lowered the risk of displacement of embryo in fresh IVF-ET cycles (27). While vaginal progesterone administration for luteal support has provided strong evidence of similar pregnancy and birth outcomes compared to intramuscular P in fresh IVF cycles (28–30). However in FET cycles, the successful rate is in favor of progesterone intramuscular administration compared to vaginal and oral administration (1, 31, 32). It is noteworthy that comparison between different routes of progesterone administered is beneficial in clarifying not only the most effective but also the relative safer one. Here our research focused on whether large-spanned circulation progesterone values elicited by different luteal support regimens made a difference in both successful clinical outcome and neonatal outcomes including newborn gestational weeks and birthweight.

From our study, we showed that progesterone vaginally administered resulted in comparable live birth rate with intramuscular regimen as luteal support in HRT-FET cycles, in line with similar results found in fresh IVF cycles (33–35). We further analyzed neonatal outcomes stratified by different progesterone regimens and found no significant difference of infant birthweight, preterm delivery rate, SGA and LGA rate between two groups. Although the higher risk of LGA and post-date newborns was found in FET singletons compared to fresh ET cycles (2, 6–8), and study suggested the aberrantly higher levels of progesterone resulted in over-invasion of the EVT which intervened the perinatal outcome (12), relatively higher peripheral serum progesterone level by intramuscularly delivered progesterone did not make higher birthweight and longer gestational weeks compared to vaginal regimen in our cohort. The reason may be speculated that the placenta formation and angiogenesis could be influenced by not only circulating progesterone level, but also uterine local progesterone level. Vaginally delivered progesterone gel might create the high concentration of progesterone at the maternal-fetal interface but it is hard to detect by blood drawing. In our cohort patients received dydrogesterone orally at the same time in case of vaginal malabsorption, though dydrogesterone orally taken did not contribute to the serum progesterone value either. And it shouldn't be neglected that vaginal progesterone uptake distribution and metabolism vary tremendously between patients. Additionally, it could be the lack of corpus luteum and the absence of vasoactive hormones like Relaxin released from corpus luteum in HRT-FET cycles that play the dominant role in the adverse perinatal outcomes (16, 36). So the different extent of high progesterone level induced by different progestin replacement did not change the newborn birthweight and gestational weeks in our analysis. Our study compared specific protocols of endometrial preparation used for artificial FET cycles and added some evidence supporting that large-spanned circulating progesterone levels induced by different progesterone routes didn't change the neonatal outcomes like singleton birthweight and gestational age.

Some studies showed low serum progesterone on the day of embryo transfer is associated with inferior clinical pregnancy both in artificial FET and fresh IVF-ET cycles (37–39). In our study we analyzed the circulating progesterone concentration of 14th day after embryo transfer in HRT-FET cycles, which is also hCG test day. At this time almost no endogenous progesterone from placenta is present. Only little progesterone from trophoblasts contributes to serum progesterone concentration in the peri-implantation period. Our results showed when intramuscularly delivered progesterone was applied as luteal support, higher serum progesterone level at 14th day after embryo transfer got higher successful rate than the lower P level counterparts in HRT-FET cycles. Thus clinicians could optimize intramuscular progesterone supplementation according to the P monitoring. Our findings confirmed previous data that luteal progesterone level outside the range limits reduced clinical pregnancy rate (40–42). But we further explored newborn birthweight and gestational age in this intramuscular progesterone cohort and revealed that systemic P level higher than 41.82 pmol/L did not increase birthweight or prolong gestational weeks, but it increased the live birth rate. Thus it is advised to monitor systemic P concentration and provide higher progesterone dose as needed in intramuscular progesterone patients to optimize the live birth rate without impact on neonatal outcomes. Increasing the dose of intramuscular progesterone when circulating P value is lower than 41.82 pmol/L was presumed to be a superiority. We did not analyze the circulating progesterone value impact in the vaginal progesterone cohort because circulating progesterone concentration does not serve as a surrogate marker for the amount of vaginal progesterone absorbed (20).

Strengths and Limitations

The present study has following strengths. The analysis was performed in one single IVF center which guaranteed the same laboratory procedures and sonographers. The analysis only included patients with their first FET cycles to assure the relative good quality embryo transferred and exclude the recurrent embryo failure cases. We excluded blastocyst transfer to alleviate prolonged *in vitro* embryo culture impact on neonatal outcomes (43, 44). To control for infant gender and gestational age bias, z score was calculated across two groups. There were limitations in this study. It is a retrospective analysis and selection bias was possible. Although we accounted for some associated factors in multivariate analysis, unknown factors might have affected the results. Large randomized prospective study is needed. We couldn't follow up the patient about obstetrics details, which impeded us to assess perinatal outcomes like gestational hypertension, pre-eclampsia, placenta accrete and previa. The obstetrics details would allow us a better understanding of high serum progesterone level's impact on placental formation in pregnancy. Additionally, in order to analyze whether obstetrics inferiority in HRT-FET was associated with a lack of secretions of

endogenous progesterone by functional corpus luteum or a suboptimal dose or route of exogenous progesterone administration, it is better to address this issue by comparing perinatal outcomes between different progesterone regimen protocols in artificial cycles as well as natural cycles.

CONCLUSION

In conclusion, this is the first study to demonstrate the association of progesterone regimen protocols and neonatal outcomes. Relatively higher serum progesterone level induced by intramuscular regimen did not change live birth rate, or increase newborn birthweight or prolong gestational weeks compared to vaginal regimen. During intramuscular progesterone supplementation of HRT-FET cycles, circulating progesterone concentration higher than 41.82 pmol/L at day 14 post-FET was associated with improved live birth rate. Monitoring serum progesterone level and optimizing progesterone dose as needed in intramuscular progesterone HRT-FET cycles has a role in improving clinical outcomes without impact on neonatal outcomes.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the institutional review board and ethics committee of Shanghai General Hospital. The ethics committee waived the requirement of written informed consent for participation.

AUTHOR CONTRIBUTIONS

YL and YW were involved in study concept and design. YL collected and analyzed the data. YL drafted the article. YW revised it for important intellectual content. All authors contributed to the article and approved the submitted version.

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REVIEW
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Alterations in Chromatin Structure and Function in the Microglia

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Microglia are resident immune cells in the central nervous system (CNS). Microglia exhibit diversity in their morphology, density, electrophysiological properties, and gene expression profiles, and play various roles in neural development and adulthood in both physiological and pathological conditions. Recent transcriptomic analysis using bulk and single-cell RNA-seq has revealed that microglia can shift their gene expression profiles in various contexts, such as developmental stages, aging, and disease progression in the CNS, suggesting that the heterogeneity of microglia may be associated with their distinct functions. Epigenetic changes, including histone modifications and DNA methylation, coordinate gene expression, thereby contributing to the regulation of cellular state. In this review, we summarize the current knowledge regarding the epigenetic mechanisms underlying spatiotemporal and functional diversity of microglia that are altered in response to developmental stages and disease conditions. We also discuss how this knowledge may lead to advances in therapeutic approaches for diseases.

Keywords: brain, neuron, microglia, genome, development, chromatin 3D architecture

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INTRODUCTION

Microglia are immune cells that have been studied extensively for their roles in pathological conditions. Microglia share many features with other substates of tissue-resident macrophages. Microglia respond rapidly to pathological stimuli via changes in morphology and function, such as releasing inflammatory cytokines, increased proliferation, and exhibiting active phagocytic properties (Ransohoff and Perry, 2009; Kettenmann et al., 2011; Shemer et al., 2015; Ransohoff, 2016). Advanced technologies, such as imaging and omics data analysis have identified roles for microglia that extend beyond their function as immune cells in physiological conditions. For instance, microglia communicate with neurons and survey the brain microenvironment, thus contributing to neuronal survival and maintenance of brain homeostasis (Tremblay et al., 2011; Wake et al., 2013). During brain development, microglia modulate diverse steps in the establishment of neural circuity, such as neuronal survival (Ueno et al., 2013; Fujita et al., 2020), axon outgrowth (Pont-Lezica et al., 2014; Squarzoni et al., 2014), and synaptic elimination (Hanisch and Kettenmann, 2007; Tremblay et al., 2011; Wake et al., 2013; Ueno and Yamashita, 2014; Mosser et al., 2017). These varied functions highlight the heterogeneity of microglia and their diverse responses and activities in both health and disease.

Histologically, microglia demonstrate regional heterogeneity (Tan et al., 2020). Microglia exhibit distinct morphologies and densities across different CNS regions in the healthy brain, which undergo alterations in disease or different stages of life. Microglia originate from yolk-sac macrophages (YSM) and enter the brain on embryonic day (E) 9.5 in mice (the timepoint

at which neurons are first generated) (Casano and Peri, 2015; Ginhoux and Prinz, 2015; Prinz et al., 2017). Upon entering the brain, microglia expand and accumulate around white matter in the early postnatal brain, forming the "fountain of microglia" (Milligan et al., 1991; Monier et al., 2006; Hristova et al., 2010; Verney et al., 2010). These early microglia exhibit an amoeboid morphology, which differs from their ramified morphology in the adult brain (Milligan et al., 1991; Ling et al., 2001; Streit, 2001; Hristova et al., 2010; Ueno et al., 2013). In the adult brain, although most microglia possess ramified morphology with extended branches in physiological conditions, they exhibit unique morphology in certain brain regions. Compared to cortical microglia, microglia in the adult mouse cerebellum have less arbors and smaller somata (Verdonk et al., 2016; Stowell et al., 2018). With regards to density, the total number of microglia in the adult mouse brain is estimated to be $\sim 3.5 \times 10^6$, which is comparable to percentages of ∼5% in the cortex and corpus callosum, and 12% in the substantia nigra of adult CNS cells (Lawson et al., 1990).

Microglia alter their gene expression profiles and characteristics in response to different conditions, highlighting their capacity for plasticity. Recent advances in transcriptomic analysis using bulk and single cell (sc)-RNA-seq have identified specialized substates of microglia across different CNS regions and contexts throughout developmental stages and various disease conditions (Hammond et al., 2019; Masuda et al., 2019; Sankowski et al., 2019). These coordinated gene expression profiles are underpinned by epigenetic modifications, including histone modifications such as acetylation, methylation, and phosphorylation; and DNA methylation. Epigenetic regulation is indispensable for normal brain development, and dysregulation of epigenetic states underscores disease pathology (Holtman et al., 2017; Cheray and Joseph, 2018). Alteration of epigenetic states often occurs in a context-dependent manner. For example, epigenetic mechanisms are involved in microglial function during development, disease, and reprogramming (Datta et al., 2018; Matsuda et al., 2019). In addition to epigenetic modifications in the linear genome, recent advanced technologies such as genome-wide chromatin analysis have revealed the importance of spatial chromatin architecture, such as chromatin loops that permit the association of gene promoters and other regulatory elements such as enhancers over short- and long-range linear genomic distances in transcriptional regulation (Dekker and Mirny, 2016; Szabo et al., 2019; Misteli, 2020).

This review focuses on the role of epigenetic mechanisms in the regulation of microglial heterogeneity and plasticity in physiological and pathological states. We discuss the potential involvement of microglial phenotypes and functions regulated by epigenetic modulators in neurodevelopmental pathologies and neurodegenerative diseases.

MULTILAYERED ORGANIZATION OF THE GENOME

The epigenomic state of cells regulates gene expression, differentiation, and cellular identity (Crotti and Ransohoff, 2016; Holtman et al., 2017; Yeh and Ikezu, 2019). Recent technological advancements and genome wide analysis have identified the spatial structure of chromatin, including chromosome territories, A/B compartments, topologically associating domains, and chromatin loops, which are hierarchically organized in the three-dimensional nuclear space (**Figure 1**) (Phillips-Cremins, 2014; Bonev and Cavalli, 2016; Dekker and Mirny, 2016; Misteli, 2020).

A series of genome-wide analyses have enabled the elucidation of higher-order chromatin architecture and histone modifications. The assay for transposase accessible chromatin (ATAC-seq) is a technique for identifying genome-wide accessible regions of chromatin based on transposase activity that inserts sequencing adapters into open regions of the chromatin (Buenrostro et al., 2013). Chromatin immunoprecipitation coupled with DNA sequencing (ChIP-seq) is a technique to identify protein-chromatin interactions by combining immunoprecipitation and high throughput DNA sequencing. ChIP-seq is widely used for the identification of cis-regulatory DNA elements, such as promoters, enhancers, and silencers, by targeting known histone modifications, transcription factors of interest, or proteins expected to be involved in enhancer activities, such as P300/CBP histone acetyltransferase (Simon and Kingston, 2009; Shlyueva et al., 2014; Andersson and Sandelin, 2020). Promoters and enhancers are typically marked by H3K4me3 and H3K4me1, respectively; both are additionally marked by H3K27ac upon activation. In contrast, silent or repressed promoters and enhancers are often marked by H3K27me3, which has been linked to Polycomb repression. Further, H3K9me3 typically labels transcriptionally silent heterochromatic regions (**Figure 2**).

A series of molecular techniques based on Chromosome-Conformation-Capture (3C), including 4C, 5C, and Hi-C, have been used to analyze the spatial organization of chromatin (Dekker et al., 2002; de Wit and de Laat, 2012). These methods enable the quantification of chromatin-chromatin interactions at different scales: 3C quantifies the interactions between two specific DNA fragments (one vs. one interactions) using PCR, whereas Hi-C quantifies the interactions between all possible pairs of fragments (all vs. all interactions) using paired end sequencing. These techniques have revealed that hierarchically organized spatial chromatin architecture is crucial for the regulation of gene transcription, which in turn is essential for the development and maintenance of various biological processes and epigenetic profiles of the linear genome such as histone modifications and DNA methylation (Phillips-Cremins, 2014). In the following sections, we summarize the current knowledge of the epigenetic profiles of microglia from these aspects and discuss their regulation and alterations in various contexts, including development, homeostasis, and disease.

GENOME STRUCTURE AND FUNCTION OF MICROGLIA IN HOMEOSTASIS

Even in normal conditions, microglia exhibit different transcriptional and epigenetic profiles. Macrophages populate all organs, and each population of tissue-specific macrophages,

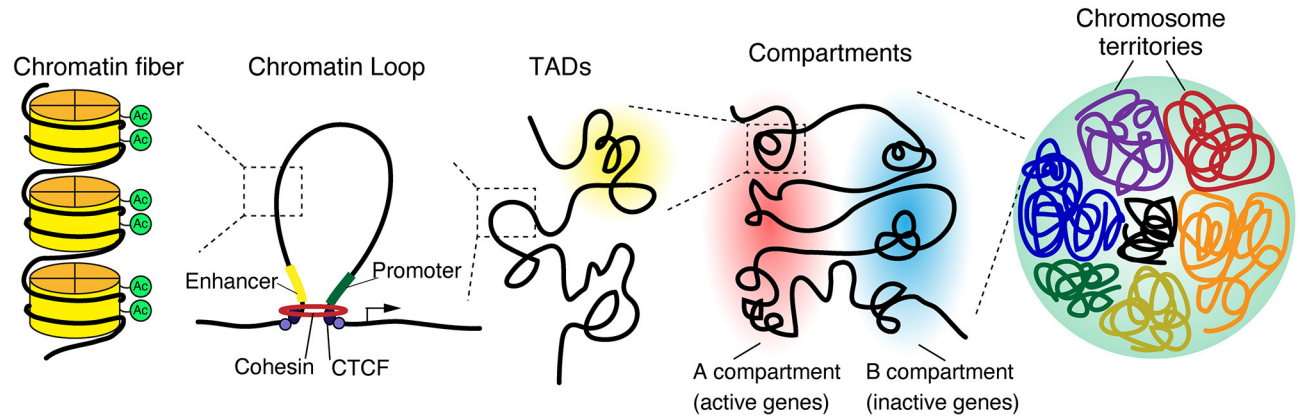

FIGURE 1 | 3D genome organization. The genome is organized in a hierarchical manner, starting at a nuclear level containing all chromosomes down to individual chromatin fibers. Chromosomes occupy distinct regions in the nucleus called chromosome territories and generally avoid overlap. Each chromosome is separated into A and B compartments that include the transcriptionally active or inactive genes, respectively. Both A and B compartments involve topologically associated domains (TADs), whereby the genomic associations strongly occur within the domain. TADs are generally bordered by CCCTC-binding factor (CTCF), which connects to linearly distant DNA sequences and brings them in close proximity, leading to the formation of a three-dimensional chromatin loop.

including microglia (brain-resident macrophages), is considered to adapt to its surrounding environment. Two groups have reported that cell type-specific enhancer and promoter landscapes regulate the identities of tissue-resident macrophages (Gosselin et al., 2014; Lavin et al., 2014). Enhancers are *cis* regulatory regions of DNA that allow the binding of multiple transcription factors to influence gene expression over variable distances, sometimes up to several hundred kilobases (kb) away. Chromatin loop formation enables the association with such distal enhancers to gene promoters (Sanyal et al., 2012; Shlyueva et al., 2014; Schoenfelder and Fraser, 2019). During development, the binding of lineage-specific transcription factors (TFs) to distinct enhancers is thought to be critical for the establishment of cell type-specific transcription by allowing local remodeling of chromatin and permanent accessibility to selective stretches of DNA (Heinz et al., 2015).

Tissue-resident macrophage populations have both common identities among general tissue macrophages and distinct enhancer profiles associated with the tissue specificity of macrophage subsets. Combination analysis of the chromatin landscape, including promoters (H3K4me3), poised enhancers (H3K4me1), and active enhancers (H3K27ac) of seven different tissue-resident macrophages, monocytes, and neutrophils with gene expression profiles and open chromatin regions revealed candidate tissue-specific transcriptional factors that contribute to shaping the chromatin specifications for tissue-resident macrophages (Lavin et al., 2014). In addition, transplant experiments revealed that the environment is partially capable of establishing the chromatin landscape of transplanted bone marrow precursors. Differentiated macrophages can be reprogrammed when transferred into a new microenvironment. Comparison of chromatin openness and transcriptomes between yolk sac-derived microglia and bone marrow graft-derived parenchymal brain macrophages revealed that graft-derived macrophages acquire microglial characteristics such as longevity, ramified morphology, and gene expression features but remain significantly distinct with respect to transcriptomes and chromatin accessibility landscapes (Shemer et al., 2018).

Furthermore, the brain environment also specifies gene expression in human microglia. Comparative studies of human and mouse microglial transcriptomes, including studies at the single cell level have revealed that human microglial gene expression correlates well with murine microglial gene expression, but numerous species-specific differences have been identified that include genes linked to neurological diseases in humans (Gosselin et al., 2017; Masuda et al., 2019). Transitions of human and mouse microglia from *ex vivo* brain tissue to an *in vitro* tissue culture environment resulted in remodeling of their respective enhancer landscapes alongside rapid and extensive down-regulation of genes that are induced in primitive mouse macrophages following migration into the fetal brain (Gosselin et al., 2017). Such environment-sensitive programs of gene expression and corresponding regulatory elements highlight the existence of dynamic transcription factor networks that underscore the identity and function of microglia. Collectively, these findings suggest that both specific factors associated with ontogeny and dynamic environmental factors cooperate to shape tissue-specific chromatin landscapes and gene expression profiles of macrophages.

Microglia also exhibit diversity in brain region-specific expression profiles and functions (Grabert et al., 2016; De Biase et al., 2017). Furthermore, environment-dependent epigenetic landscapes specify the gene expression profiles in both mouse and human microglia (Gosselin et al., 2017; Ayata et al., 2018). Cerebellar, but not striatal or cortical, microglia exhibit a high level of basal clearance activity associated with an elevated degree of cerebellar neuronal death in mice (Ayata et al., 2018). The microglia-specific translating ribosome affinity purification (TRAP) approach (Doyle et al., 2008; Heiman et al., 2008) permits region-specific analysis of microglia-enriched mRNA expression

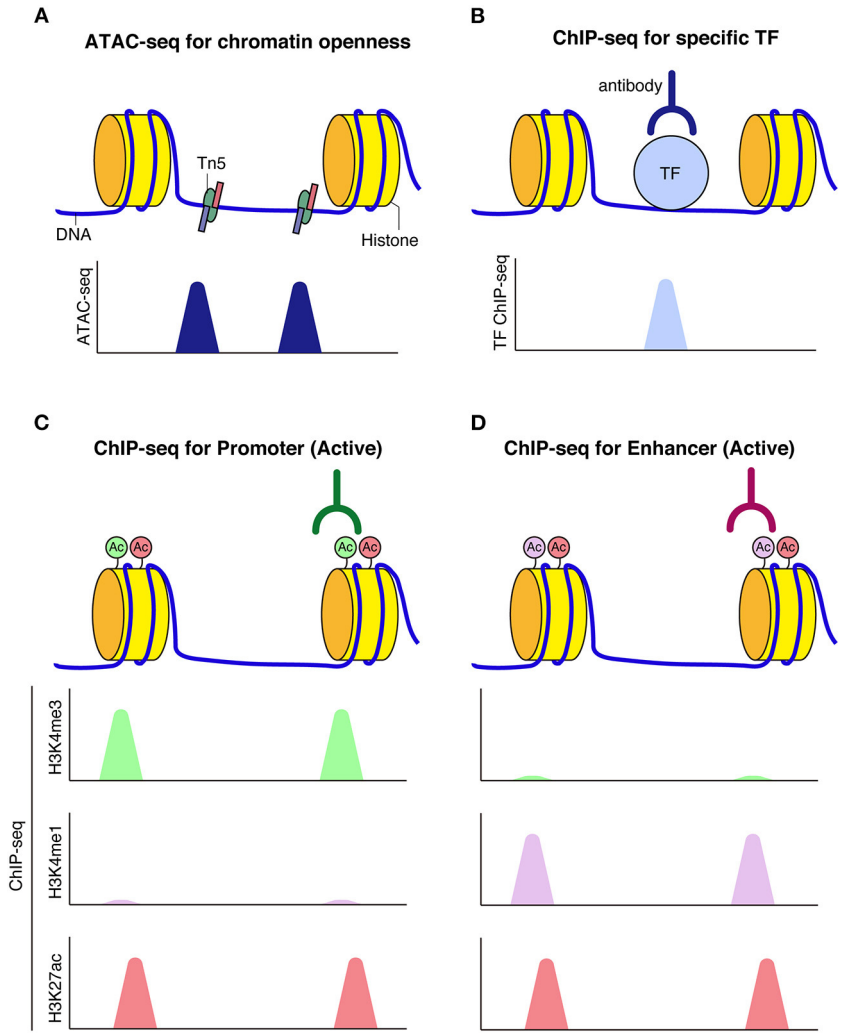

FIGURE 2 | Schematic model of chromatin accessibility and histone marks at regulatory elements. **(A)** Assay for transposase-accessible chromatin using sequencing (ATAC-seq) can be used to capture chromatin openness, where Tn5 transposase simultaneously fragments and tags accessible DNA prior to sequencing. **(B)** When transcription factors bind to DNA, reads corresponding to TF bound fragments are obtained by sequencing. **(C,D)** Histone modifications mark functional genomic elements. Active enhancers are often marked by H3K27ac and H3K4me1 **(C)**. Active promoters are often marked by H3K27ac and H3K4me3 modifications **(D)**.

and precludes nonspecific microglia activation and concurrent upregulation of immediate early and inflammatory genes that occur during commonly used microglia isolation approaches. TRAP studies have revealed that cerebellar microglia exhibit cell-clearance phenotypes associated with exposure to dying cells. PRC2, which catalyzes the repressive chromatin modification histone H3 lysine 27 trimethylation (H3K27me3) (Margueron and Reinberg, 2011), epigenetically restricts the gene expression program that supports clearance activity in striatal and cortical microglia. H3K27me3 is absent from clearance-related gene loci in cerebellar microglia but not in striatal microglia. Loss of PRC2 leads to aberrant activation of clearance-specific genes in striatal microglia, which triggers changes in neuronal morphology and behavior, including decreased spine numbers in striatal medium spiny neurons (MSN) and MSN-mediated locomotor sensitization. These observations suggest that disturbances in epigenetic mechanisms are linked to aberrant activation of microglial clearance of neuronal damage and complex behavioral alterations associated with neurodegenerative and psychiatric diseases.

In normal conditions, microglia exhibit considerable heterogeneity across different CNS region (Tan et al., 2020). The epigenetic landscape of microglia varies among brain regions and may be associated with the maintenance of regional microglia specifications in the adult brain associated with their morphological and functional heterogeneity (Yeh and Ikezu, 2019). Microglia respond to environmental challenges, and their transcriptional epigenetic landscape can be dynamically altered in response to extrinsic stimulation. Therefore, the epigenetic landscape is specialized according to brain region, while retaining the capacity for plasticity and reprogramming (Holtman et al., 2017). A recent study demonstrated that the

epigenetic regulation of microglia plays an important role in the reprogramming of microglia. Direct reprogramming of microglia into neurons has been achieved by expression of a single transcriptional factor, NeuroD1 (Matsuda et al., 2019), which has previously been used to convert astrocytes into neurons (Guo et al., 2014). The expression of NeuroD1 allows remodeling of the chromatin landscape from closed chromatin, associated with bivalent modifications (H3K4me3 and H3K27me3) to the monovalent mark (H3K4me3), associated with the establishment of neuronal identity at later stages of reprogramming. Concordant with pathological states, microglia accumulate and proliferate at injured sites and become the predominant cell type within the glial scar (Annunziato et al., 2013; Cregg et al., 2014). Therefore, modulating epigenetic and transcriptional profiles of existing microglia toward a neuronal phenotype may be a possible therapeutic approach to replenish lost neurons in CNS injury and disease.

GENOME STRUCTURE AND FUNCTION OF MICROGLIA DURING DEVELOPMENT AND CELLULAR DIFFERENTIATION

Microglia possess various epigenomic and associated transcriptomic signatures throughout life, including microglial development and aging. Studies with scRNA-seq have identified distinct spatiotemporal subpopulations of microglia with single cell resolution (Masuda et al., 2020b). Microglia demonstrate greater diversity during development, disease, and in the aging brain than in the normal, healthy adult brain (Hammond et al., 2019; Masuda et al., 2019; Sankowski et al., 2019). Genome-wide analysis of chromatin and expression profiles indicates that microglia undergo three distinct developmental stages, including early, pre-, and adult stages, with characteristic gene expression and functional states. Perturbations of this developmental process, such as knockout of the adult microglial transcription factor MafB, lead to disrupted brain homeostasis via the dysregulation of adult microglial genes and immune response pathways (Matcovitch-Natan et al., 2016).

A study of single-nucleus (sn) ATAC-seq in the mouse forebrain at seven developmental stages (E11.5, 12.5, 113.5, 14.5, 15.5, 16.5, P0) revealed chromatin accessibility profiles in microglia during development (Preissl et al., 2018). This study identified 12 distinct subpopulations of brain cells that exhibited abundant changes through development. Based on this classification, the chromatin accessibility profiles at gene loci of known marker genes have been addressed. The myeloid lineage cluster is restricted to E11.5 and disappears in later developmental stages. snATAC-seq data in the adult (P56) mouse forebrain identified one microglia cluster with accessibility at genes encoding complement factors, including the gene *C1qb*, leading to the inference that the adult forebrain comprises 6% microglia.

Dynamic developmental transitions of transcriptional and epigenetic profiles of human microglia throughout brain development have been analyzed (Schmunk et al., 2020). An integrative analysis of microglia, including transcriptomes, chromatin accessibility data generated using single cell ATAC-seq, and putative enhancer elements among open chromatin regions throughout human brain development has revealed the molecular signatures of stepwise maturation. Notably, human-specific cytokine-associated substates of microglia expressing increased levels of *C-C motif chemokine* (*CCL)2*, *CCL4*, and *interleukin* (*IL) 1B* are present in early brain development around the onset of neurogenesis. These findings demonstrate the dynamic transitions in transcriptional and epigenetic profiles in both mouse and human microglia. However, the molecular mechanisms underscoring the regulation of the epigenetic landscape are not fully understood.

Sexual Dimorphism in Epigenetic Modulation of Microglia

Rodent microglia exhibit sexually dimorphic properties in pain perception, contribute to brain masculinization, and exhibit differences in brain colonization in males and females (Schwarz et al., 2012; Lenz and McCarthy, 2015; Mapplebeck et al., 2016). Furthermore, it was recently reported that microglia demonstrate transcriptomic differences in females and males throughout postnatal development (Hanamsagar et al., 2017). RNA-seq and ATAC-seq have revealed that microglia progressively gain sex-associated transcriptomic signatures and chromatin accessibility landscapes, which diverge in adult males and females (Thion et al., 2018). Microglia purified from female and male mouse brains at E18.5, shortly after the initiation of sex hormone production (Nelson and Lenz, 2017), exhibit low numbers of differentially expressed genes mostly present on the X and Y chromosomes, which may limit embryonic transcriptomic sexual dimorphism in adult females and males, consistent with other studies (Hanamsagar and Bilbo, 2017). In addition, female microglia display higher expression of genes associated with inflammatory responses, apoptotic processes, and responses to lipopolysaccharide (LPS). The absence of the microbiome in germ-free mice are more profoundly perturbed in the microglia of male embryos and female adults, highlighting the prenatal and postnatal impact of temporal and sexually dimorphic factors. ATAC-Seq has also revealed temporal changes in chromatin accessibility in the absence of the microbiome (Thion et al., 2018).

Several disorders exhibit sexual dimorphism. For instance, autism spectrum disorder (ASD) are more prevalent in males, whereas auto-immune diseases are more prevalent in females (McCarthy and Wright, 2017; Nelson and Lenz, 2017). These findings underscore the need to identify how transcriptomic and epigenetic sexual dimorphism in microglia is associated with their differentiation or functional differences linked to CNS diseases. Further work using animal models of these diseases should address the effects of temporal and sexually dimorphic factors in modulating the epigenetic and transcriptomic landscape of microglia in disease onset and progression.

Genome Dynamics During Cellular Differentiation

Cell fates are specified by lineage-determining transcription factors. Epigenetic mechanisms regulate lineage-determining transcription factors which bind to genomic regions in a

cell-specific manner. Macrophages and B cells play essential and complementary roles in the innate and adaptive arms of the immune system. Within the mammalian hematopoietic system, these cell types are derived from a lymphoid-primed multipotential progenitor (LMPP) that subsequently gives rise to common lymphoid progenitors (CLPs). CLPs differentiate into B cells or granulocyte-macrophage progenitors (GMPs) cells that can differentiate into macrophages (Adolfsson et al., 2005). ChIP-seq revealed distinct PU.1 binding patterns within the vicinity of motifs bound by lineage-restricted transcription factors in macrophages and B cells, respectively (Heinz et al., 2010). PU.1 binding induces nucleosome remodeling followed by H3K4 monomethylation (H3K4me1) that may signify accessible chromatin and/or enhancer-like elements (Heintzman and Ren, 2009) at large numbers of genomic regions associated with both broadly and specifically expressed genes. PU.1-bound sites in macrophages also play a role in shaping the transcriptional response to inflammatory stimuli such as LPS, likely by generating cell type-specific regions of open chromatin that allows the recruitment of transcriptional coactivators (Ghisletti et al., 2010). These findings provide insight into the extensive genome-wide and cell type-specific colocalization of transcriptional factors.

A study using the Hi-C approach, which is a genome-wide approach for the detection of interactions between all mappable regions of entire chromatin, has revealed the three-dimensional chromatin arrangement and transcription during cellular differentiation from human monocytes and differentiated macrophages (Phanstiel et al., 2017). This study employed a model using the monocytic leukemia cell line THP-1 treated with phorbol myristate acetate (PMA), which is widely used for studying the differentiation from monocytes to macrophages and provides an ideal system for studying the regulatory dynamics of long-range interactions (Daigneault et al., 2010). A modified Hi-C method, *in situ* Hi-C, permits higher resolution and unbiased genome-wide detection of DNA loops. Compared to static (pre-formed) loops, acquired loops during macrophage differentiation are enriched for H3K27ac, consistent with enhancer activity and gene promoters. Enhancer-bound loop formation and enhancer activation of preformed loops form multiloop activation hubs at key macrophage genes during macrophage development. Each multiloop activation hub in differentiated macrophages involves the interaction of on average 3.4 enhancers to a promoter and exhibits a strong enrichment of the binding sites for activator protein 1 (AP-1), a key transcriptional regulator for the differentiation of monocytic precursors into mature macrophages. These findings suggest that the distal regulation of gene transcription mediated by DNA loops, which bring enhancers in close proximity to their target genes, represents a major mechanism for controlling the developmentally regulated expression of distinct genes.

A recent study showed that the spatial architecture of chromatin is important for inflammatory response rather than the differentiation of immune cells (Stik et al., 2020). CCCTC-binding factor, CTCF binds to DNA and are involved in the formation of TADs and long-range chromatin loop (Phillips and Corces, 2009). CTCF depletion disrupts TAD organization but did not affect the differentiation of human leukemic B cells into macrophages (Stik et al., 2020). In contrast, CTCF depletion in induced macrophages impairs the upregulation of inflammatory genes upon lipopolysaccharide (LPS) stimulation and decreased the frequency of the enhance-promoter interaction at the *IL6* locus (**Figure 3A**).

Although the dynamic alterations of spatial chromatin organization have been addressed, the causes or consequences of 3D genome dynamics still remain unclear. In addition, analyses of microglia from this aspect are lacking, which should be addressed by future studies.

GENOME STRUCTURE AND FUNCTION OF MICROGLIA IN PATHOLOGICAL CONDITIONS

Microglia contribute to various processes including brain development and homeostasis throughout the lifespan. Microglia regulate early wiring, synaptic pruning and formation, and cell death and survival, which are indispensable for establishing and maintaining neural circuits (Ransohoff and El Khoury, 2015; Schafer and Stevens, 2015; Hong et al., 2016; Tay et al., 2017; Thion and Garel, 2017; Wolf et al., 2017). Consistent with their diverse roles, microglia have been linked to the initiation or progression of several developmental and neurodegenerative diseases, including ASD, schizophrenia, Alzheimer's disease (AD), Parkinson's disease (PD), and multiple sclerosis (MS) (Shemer and Jung, 2015; Colonna and Butovsky, 2017). As microglia possess different epigenomes and associated transcriptomes throughout the life course, perturbations of epigenetic regulation may result in diverse effects that may underscore disease onset and progress.

Alzheimer's Disease

Reactive microglia are associated with almost all neurodegenerative diseases (Streit et al., 1999). Reactivity of microglia and elevated cytokine levels are observed in the brains of AD patients. Nevertheless, the mechanisms underlying microglial activation and their contribution to disease progression remain poorly understood. Both transcriptomic and proteomic analysis at the single cell level have revealed the entire immune landscape and different expression profiles in CNS pathology (Keren-Shaul et al., 2017; Mathys et al., 2018; Mrdjen et al., 2018). Depletion of both histone deacetylases Hdac1 and Hdac2 in microglia result in different effects in the developing, homeostatic, and diseased brain (Datta et al., 2018). Hdac1 and Hdac2 are essential for microglial survival during brain development in mice but not during homeostasis in adulthood. In 5xfamilial AD (5xFAD) transgenic mice (an AD mouse model), deletion of microglial Hdac1 and Hdac2 enhances microglial phagocytosis of amyloid plaques and improves cognitive function.

In addition, ten-eleven translocation 2 (TET2) methylcytosine dioxygenase is expressed by amyloid beta (Aβ) plaque-associated microglia in brain tissue in both 5xFAD mice and individuals with AD (Carrillo-Jimenez et al., 2019).

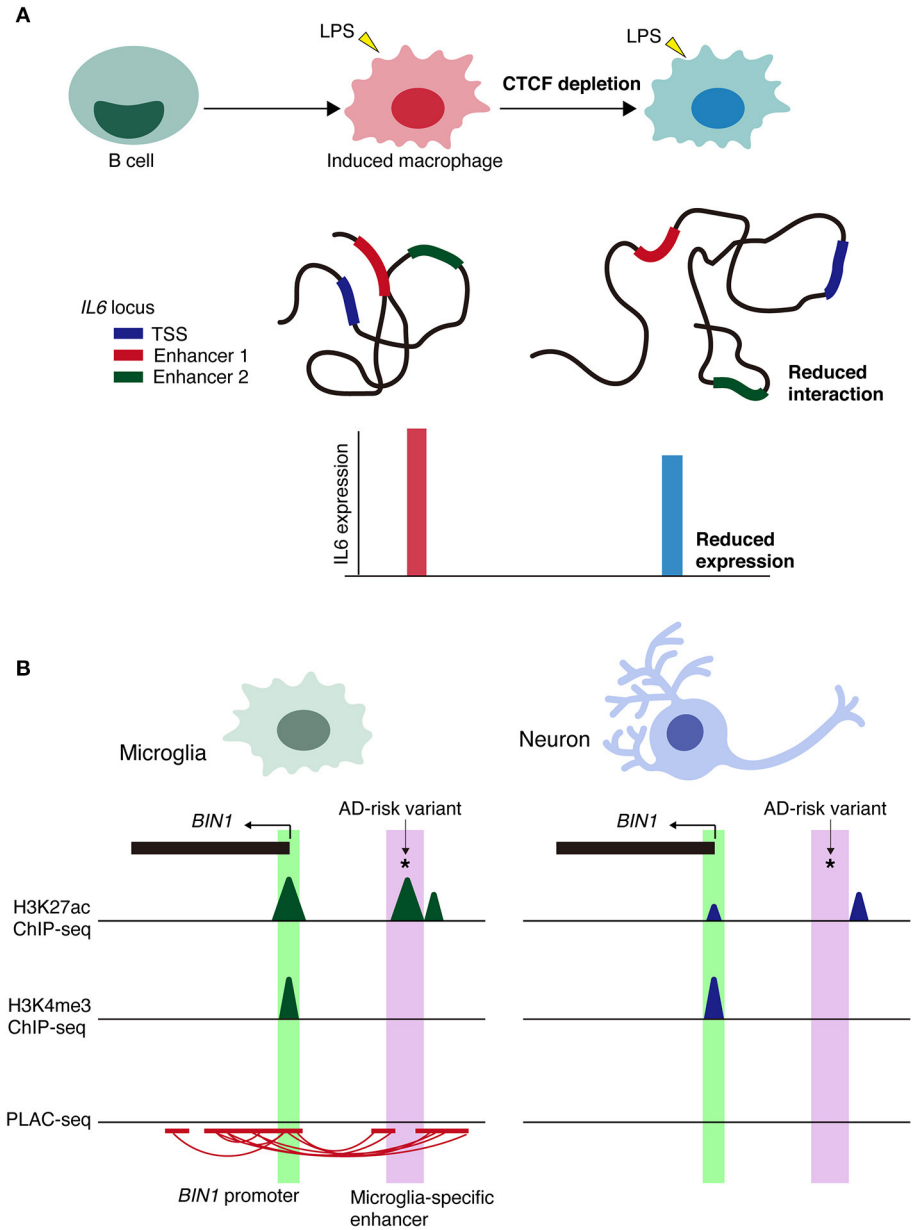

FIGURE 3 | Context-dependent alterations of chromatin interactions. **(A)** Schematic model of the alterations in chromatin architecture and the impairment of inflammatory responses of CTCF depletion in immune cells via lipopolysaccharide (LPS) stimulation. Loss of CTCF reduces the frequency of chromatin interactions, such as enhancer-promoter interactions at *IL6* locus. TSS, transcription start site. **(B)** Schematic representation of microglia-specific enhancer region (highlighted in purple) harboring AD-risk variants at *BIN1* loci. Active promoter regions (highlighted in green) are shared between cell types. PLAC-seq demonstrates that microglia-specific enhancers are linked to the *BIN1* promoter. *Indicates AD-risk variant.

TET2 is involved in early gene transcriptional changes, leading to early metabolic alterations, and later inflammatory responses independently of its enzymatic activity. TET2 is upregulated in microglia upon exposure to inflammatory stimuli via an NF-kB-dependent signaling pathway, which involves epigenetic mechanisms. Following inflammatory stimulation, the level of H3K27ac marking increases at the Tet2 promoter and upstream regions concomitant with the recruitment of p65 to both the promoter and upstream regions.

Injection of interleukin (IL)-33 in APP/PS1 mice (an amyloid-deposition mouse model) ameliorates Aβ pathology by reprogramming microglial epigenetic and transcriptomic profiles to induce a microglial subpopulation with enhanced phagocytic activity (Lau et al., 2020). IL-33 enhances microglial Aβ clearance by inducing a subpopulation of

major histocompatibility complex class II (MHC-II)-positive phagocytic microglia, which in turn are regulated by PU.1-dependent transcriptome reprogramming. ATAC-seq and ChIP-seq analysis revealed that IL-33-induced remodeling of chromatin accessibility and transcription factor PU.1 binding at the signature genes of IL-33-responsive microglia regulate their transcriptome reprogramming. Thus, IL-33-induced epigenetic and transcriptional regulation of microglial state transitions contributes to the alleviation of AD pathology. Although effective therapies for AD are currently lacking, these findings provide novel insight into the therapeutic potential of reprogramming the epigenetic and transcriptome profiles of microglia to treat AD.

Context-specific microglial phenotypes have been reported, including disease-associated microglia (DAM) (Keren-Shaul et al., 2017) and the microglial neurodegenerative phenotype (MGnD) (Krasemann et al., 2017). Comparison of DAM enhancers in wildtype (WT) and 5xFAD mice using a high sensitivity method for ChIP-seq analysis (iChIP) (Lara-Astiaso et al., 2014) revealed a similar level of H3K4me2, which marks promoter and enhancer regions (Keren-Shaul et al., 2017). These findings suggest that the disease-associated regions primed in DAM are already primed in homeostatic microglia.

Innate immune memory is a key mechanism underlying myeloid cell plasticity that occurs in response to environmental stimuli (Netea et al., 2015, 2016). This mechanism can be classified into immune training, which enhances immune responses to subsequent immune insults, and immune tolerance, which suppresses inflammatory responses to subsequent stimuli (Biswas and Lopez-Collazo, 2009; Cheng et al., 2014; Saeed et al., 2014). In a mouse model of AD, cerebral β-amyloidosis is exacerbated by immune training and alleviated by immune tolerance via epigenetic modifications (Wendeln et al., 2018). ChIP-seq analysis revealed that increased H3K4me1 levels in microglia from 1xLPS (immune training) vs. 4xLPS (immune tolerance) WT animals exhibited enrichment for the thyroid hormone signaling pathway, including a putative enhancer for hypoxia inducible factor-1a (HIF-1a). Similar results were observed in AD model mice (APP23) injected with 1xLPS vs. 4xLPS (Wendeln et al., 2018). In addition, microglia from 4xLPS-treated AD model mice demonstrated increased H3K4me1 levels in putative enhancers related to phagocytic function. These observations highlight the differential effects of immune training vs. tolerance due to multiple environmental stimuli, which is reflected in the epigenetic landscape of DAM and/or MGnD in AD mouse models. Further studies should elucidate how environmental stimulation modulates the epigenetic landscape for context-specific microglial functions and their contribution to the progression of neurodegenerative disorders.

Comprehensive studies of the transcriptional and epigenetic landscapes of isolated microglia from human and mouse brain tissue samples using RNA-seq, ChIP-seq, and ATAC-seq have revealed the involvement of microglia in disease mechanisms (Gosselin et al., 2017; Tansey et al., 2018; Nott et al., 2019). The transcriptional profiles of cortical microglia defined 881 transcripts as the unique microglial gene signature (Gosselin et al., 2017). This core transcript set was compared with 46 publicly available microarray or RNA-seq datasets of genes that are differentially regulated in neurodegenerative and behavioral disorders. Of these, 28 exhibited enrichment or depletion of the microglial signature. More than half of the genes associated with noncoding genome-wide association study (GWAS) risk alleles for AD and MS are preferentially expressed in microglia. In contrast, fewer genes associated with PD and schizophrenia risk alleles exhibited preferential expression in microglia. These findings underscore the diverse roles of microglia in the context of different diseases.

Alongside transcriptional alterations, perturbations in gene expression regulation are inferred to be key mechanisms since the majority of disease-associated genetic variation resides in non-coding regions of the genome (Maurano et al., 2012; Khurana et al., 2016). To better understand genetic variation associated with brain diseases, isolated nuclei from different brain cell types, including neurons, astrocytes, microglia, and oligodendrocytes from cortical brain tissue of human individuals were subjected to ATAC-seq to assess open chromatin regions and ChIP-seq for H3K27ac and H3K4me3 to address active enhancers and promoters, respectively, in each brain cell type (Nott et al., 2019). Whereas, active promoters were mostly common across cell types, the fraction of active enhancers that overlapped between different cell types was small, suggesting that cell type specificity is modulated predominantly by the enhancer repertoire.

Linkage disequilibrium score (LDSC) regression analysis can be utilized on GWAS summary statistics to determine SNP-based genetic heritability for a trait or disease. Psychiatric disorders or behavioral traits are primarily associated with variants in transcriptional enhancers and promoters in neurons. In contrast, sporadic AD risk variants are largely confined to microglial enhancers. To detect long-range chromatin interactions at the promoter region, H3K4me3 proximity ligation-assisted ChIP-seq (PLAC-seq), in which proximity ligation is conducted in nuclei prior to chromatin shearing and immunoprecipitation (Fang et al., 2016), has been performed. This method revealed 219,509 significant interactions across cell types (Nott et al., 2019). Interactome maps from PLAC-seq identified several parameters, including: (1) AD-risk variants that were linked to more distal active promoters and not the closest promoter; (2) enhancers harboring AD-risk variants that were PLAC-linked to active promoters of both GWAS-assigned genes and an extended subset of genes not assigned to GWAS loci; and (3) cell type-specific enhancers harboring AD risk variants linked to genes expressed in multiple cell types, suggesting cell type-specific disease susceptibility.

Regarding cell type-specific enhancers, the *BIN1* microglia-specific enhancer is PLAC-linked to the *BIN1* promoter and harbors the AD-risk variant rs6733839, which has the second highest AD-risk score (**Figure 3B**). Deletion of a microglia-specific enhancer harboring AD-risk variants ablated *BIN1* expression in microglia but not in neurons or astrocytes. Collectively, these findings demonstrate the value of chromatin interactome maps to the functional interpretation of GWAS risk alleles associated with neurological and psychiatric diseases. Alterations in cell type-specific enhancer-promoter interactions may be a prominent mechanism underlying genetic

variants in non-coding regions associated with disease onset and/or progression.

Huntington's Disease (HD)

HD is a neurodegenerative disorder caused by specific expansion of a CAG repeat in the coding region of the *HTT* gene (The Huntington's Disease Collaborative Research Group, 1993). Similar to neurodegenerative diseases such as AD and PD, reactive microglia and elevated cytokine levels are observed in the brains of both mice and humans with HD (Sapp et al., 2001; Tai et al., 2007). Genome-wide approaches including RNA-seq and ChIP-seq have revealed that the expression of mutant Huntingtin (mHTT) in microglia but not in bone marrow-derived macrophages causes cell autonomous pro-inflammatory transcriptional activation through increased expression and transcriptional activity of myeloid lineage-determining factors PU.1 and C/EBPs (Crotti et al., 2014). ChIP-seq analysis for PU.1 and H3K4me2, a histone modification associated with enhancers and promoters (Regha et al., 2007; Brykczynska et al., 2010; Chepelev et al., 2012), demonstrated that genomic loci encoding mRNAs that are upregulated in BV2 microglia expressing mHTT generally exhibit higher enrichment of PU.1 binding to promoters/enhancers, exemplified by the Tnf locus. Similar results were observed in ChIP-seq analysis for C/EBPα and C/EBPβ. The binding sites for PU.1 and C/EBPs are highly enriched in enhancers and promoters associated with genes exhibiting constitutive upregulation in mHTT-expressing microglia. Collectively, these observations indicate that disruption of epigenetic and transcriptomic regulation in microglia affects neuronal function. Deeper understanding of the effects of microglial identity on interactions with neurons will provide further insight into the contribution of microglial activation to the pathophysiology of neurodegenerative diseases such as AD and HD.

Rett Syndrome

Aberrant epigenetic regulation in microglia is also implicated in neurodevelopmental and psychiatric disorders. Growing evidence suggests that alterations in spatial chromatin structure is associated with neurodevelopmental and neuropsychiatric disorders. Rett syndrome is an ASD caused primarily by mutations in methyl-CpG binding protein 2 (MeCP2) (Amir et al., 1999) and is characterized by prominent neurologic dysfunction. Accordingly, efforts to understand the function of MeCP2 have largely focused on its role in neurons (Chahrour and Zoghbi, 2007). More recently, the expression and roles of MeCP2 in astrocytes (Ballas et al., 2009; Lioy et al., 2011; Yasui et al., 2013), oligodendrocytes (Nguyen et al., 2013), and microglia (Maezawa and Jin, 2010; Derecki et al., 2012) have been reported. Microglia of MeCP2-null mice, a mouse model of Rett syndrome, exhibit reduced phagocytic activity. Transplantation of MeCP2-null mice with WT microglia ameliorates disease progression, suggesting that the phagocytic properties of microglia are indispensable for normal brain development and function, and deficits in microglial phagocytosis may be associated with disease onset and/or progression. In addition, ChIP-seq analysis revealed that MeCP2 deletion increased histone H4 acetylation at enhancer regions of *Fkbp5* (a canonical glucocorticoid target gene) and recruitment of nuclear receptor corepressor 2 and HDAC3 complex (Cronk et al., 2015). Thus, MeCP2 deletion resulted in the upregulation of *Fkbp5* gene expression thorough epigenetic mechanisms, suggesting that MeCP2 deletion underpins microglial dysfunction in Rett syndrome. In contrast, another study reported that wild-type microglia or specific Mecp2 expression in microglia did not rescue the pathology in Mecp2 null mice (Wang et al., 2015). The contribution of the microglia in Rett syndrome and the therapeutic potential of targeting the microglia in this disease are still being debated.

Pain

Neuropathic pain is a chronic and devastating condition that occurs following nerve damage or in various diseases (Basbaum et al., 2009). Animal studies have demonstrated that characteristic changes in both neurons, glial cells, and neuro-glial interactions, play a key role in the establishment and maintenance of persistent pain (Tsuda et al., 2003; Calvo and Bennett, 2012; Denk et al., 2016). Genome-wide transcriptional profiles of isolated spinal cord microglia following partial sciatic nerve ligation, which is a widely used model of neuropathic pain, have been identified by RNA-seq. H3K4me1 ChIP-seq analysis revealed injury-induced alterations in microglial enhancer profiles, possibly associated with transient transcriptional upregulation (Denk et al., 2016). Although the time-course analysis for expression changes revealed that transcriptional upregulation reverts to baseline by 28 days following pain induction, ChIP-qPCR identified several putative latent enhancer regions with increased H3K4me1 binding levels up to a month following pain induction. These findings reveal persistent injury-specific alterations of the microglial enhancer landscape.

DISCUSSION

Based on cell type-specific isolation of microglia and/or techniques using deep sequencing, epigenetic and transcriptomic profiles of microglia have been identified. However, much remains unknown of their causal or consequential effects, such as the regulation of functional, morphological, and regional heterogeneity of microglia in a context-dependent manner. Animal models with inducible drivers of Cre-recombinase such as Cx3cr1-CreER (Yona et al., 2013) allow the use of microglia-targeted tracing and microglia-specific knockout or overexpression of genes of interest. One study reported that several substates of microglia downregulate CX3CR1, limiting this model's utility in the study of microglia (Stratoulias et al., 2019). Recent studies have highlighted that the CX3CR1 line targets the microglia and CNS border-associated macrophages (Goldmann et al., 2016; Chappell-Maor et al., 2020). Newly developed mouse lines that express inducible Cre or fluorescence reporter genes specifically in the microglia, such as the Trem119-CreERT2 (Kaiser and Feng, 2019), Hexb-CreERT2 (Masuda et al., 2020a) and P2ry12-CreER (McKinsey et al., 2020) transgenic mice would be powerful tool in further studies. These approaches will help address the functional roles of epigenetic changes in

microglial regulation and the manner in which they affect the nervous system *in vivo*.

A further step forward is to decipher how epigenetic profiles, especially spatial structures of chromatin, respond to various environmental cues. Microglia are exposed to diverse cues depending on developmental and pathological context. Even under normal conditions, microglia exhibit altered epigenetic marks. However, the influence of environmental cues on microglial physiological identity and disease-specific responses remains elusive. In addition, not all microglia respond to certain cues, which implies different substate-dependent susceptibility. There exists a unique microglial phenotype, known as the dark microglia, which is identified by the alteration in nuclear chromatin at the ultrastructural level (Bisht et al., 2016). This substate is rarely found in the physiological state in some areas of the brain, including the hippocampus, cerebral cortex, amygdala, and hypothalamus. However, they proliferate in conditions such as chronic stress, aging, fractalkine signaling deficiency, and Alzheimer's disease pathology. These observations suggest the association between the chromatin structure of microglia and their roles in the pathological remodeling of neuronal circuits. Advancements in technologies to elucidate transcriptomes, chromatin accessibility, and the interactions between DNA at a single cell level, including single cell RNA-seq, single cell ATAC-seq, and single cell Hi-C, will help to overcome the aforementioned technical limitations.

Recent studies have identified disturbances in enhancer-promoter interactions in the diseased brain (Rajarajan et al., 2018; Nott et al., 2019). In addition, deletion of regulatory proteins for chromatin loop formation is associated with deficits in synapse formation during brain development and behavioral deficits (Hirayama et al., 2012; Fujita et al., 2017). In this regard, it is plausible that aberrant changes in 3D chromatin structure contribute to neurodevelopmental diseases. Although the mechanisms by which epigenetic marks and changes in spatial chromatin structure regulate microglial function are largely unknown, elucidating these mechanisms will provide a step forward in understanding the role of microglia in neurodevelopmental and neuropsychiatric disorders. Studies in animal models and humans, including tissues and cells derived from induced pluripotent stem cells of patients with relevant diseases will provide novel insight into the role of microglia in disease pathogenesis.

AUTHOR CONTRIBUTIONS

YF wrote the manuscript, and TY revised it. All authors contributed to the article and approved the submitted version.

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Nerve Growth Factor: A Dual Activator of Noradrenergic and Cholinergic Systems of the Rat Ovary

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The functioning of the ovary is influenced by the autonomic system (sympathetic and cholinergic intraovarian system) which contributes to the regulation of steroid secretion, follicular development, and ovulation. There is no information on the primary signal that activates both systems. The nerve growth factor (NGF) was the first neurotrophic factor found to regulate ovarian noradrenergic neurons and the cholinergic neurons in the central nervous system. The aim of this study was to determine whether NGF is one of the participating neurotrophic factors in the activation of the sympathetic and cholinergic system of the ovary *in vivo* and its role in follicular development during normal or pathological states. The administration of estradiol valerate (a polycystic ovary [PCO] phenotype model) increased norepinephrine (NE) (through an NGF-dependent mechanism) and acetylcholine (ACh) levels. Intraovarian exposure of rats for 28 days to NGF (by means of an osmotic minipump) increased the expression of tyrosine hydroxylase and acetylcholinesterase (AChE, the enzyme that degrades ACh) without affecting enzyme activity but reduced ovarian ACh levels. *In vitro* exposure of the ovary to NGF (100 ng/ml for 3 h) increased both choline acetyl transferase and vesicular ACh transporter expression in the ovary, with no effect in ACh level. *In vivo* NGF led to an anovulatory condition with the appearance of follicular cysts and decreased number of corpora lutea (corresponding to noradrenergic activation). To determine whether the predominance of a NE-induced polycystic condition after NGF is responsible for the PCO phenotype, rats were exposed to an intraovarian administration of carbachol (100 μM), a muscarinic cholinergic agonist not degraded by AChE. Decreased the number of follicular cysts and increased the number of corpora lutea, reinforcing that cholinergic activity of the ovary participates in controlling its functions. Although NGF increased the biosynthetic capacity for ACh, it was not available to act in the ovary. Hence, NGF also regulates the ovarian cholinergic system, implying that NGF is the main regulator of the dual autonomic control. These findings highlight the need for research in the treatment of PCO syndrome by modification of locally produced ACh as an *in vivo* regulator of follicular development.

Keywords: nerve growth factor, acetylcholine, norepinephrine, polycystic ovary syndrome, follicular development

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INTRODUCTION

Many studies have shown that ovary function is controlled by the sympathetic nervous system regulating steroid secretion, follicular development, and ovulation (1). Sympathetic nerves communicate with the ovary in two ways: the superior ovarian nerve with fibers localized around the follicles regulates steroid secretion and follicular development, and the ovarian plexus nerve mainly supplies innervation to blood vessels (2, 3). In addition, recent evidence supports the presence of an intraovarian cholinergic system located in granulosa cells (GCs) and involved in the development of ovarian follicles and ovulation (4–6). We have previously shown that the intraovarian cholinergic system mainly participates in the control of follicular development ovulation and atresia of antral follicles (6). Apparently, both noradrenergic and cholinergic systems regulate ovarian functions, probably working together or participating in a balanced way to regulate ovary function, similar to the autonomous regulation of many internal organs of the body (6). Their function is likely linked to maintaining the homeostatic condition of the organ, especially when the other part of the neuroendocrine axis (mainly gonadotropin-dependent control of the ovary) is being modified. In this sense, polycystic ovary syndrome (PCOS), the most frequent ovarian pathology causing infertility in women, is characterized by profound changes in follicular development, resulting in ovarian steroid secretion. In this condition, both neuroendocrine and nervous dysfunction have been observed in many other changes related to the metabolic and cardiovascular events associated with the syndrome (7). Due to the multitude of effects associated with PCOS, studies using animal models of the PCO phenotype are important. Recent studies have found that sympathetic stress, such as chronic exposure to cold (4°C for 3 h each day for 4 weeks), activates not only the sympathetic nerve fibers of the ovary but also the intraovarian cholinergic system (6). However, it is not known which primary signal activates both systems. The nerve growth factor (NGF) was the first neurotrophic factor found to regulate ovarian noradrenergic neurons (8). NGF is one of the most important factors in the regulation of cholinergic neurons in the central nervous system (9, 10), but there is limited information on its action in the ovary. If this is correct, we either can suggest that NGF acting on sympathetic nerves increases NE in the ovary by a direct effect of NGF or induced by stress (11, 12) and can participates in the development of the PCO phenotype in rat. It probably acts increasing the ACh concentration whose participation in ovary physiology is just recently been considered (4, 13); much less is know in pathological conditions such as the PCO phenotype in rat.

Recent evidence supports an additional role of NGF, not as a neurotrophic factor, but rather as a factor that regulates follicular development, affecting the survival or death of the follicular population (14). However, it is not known whether the primary actions are mediated by the NGF acting on nerve activity or NGF directed to the GCs to regulate follicular growth or death during development. Interestingly, NGF and TNF-alpha are part of a feedback loop similar to that associated with the inflammatory response, a common mechanism associated with ovary function (15).

Previous studies on the actions of NGF and norepinephrine on the ovary have shown that NGF's action on follicular development is not only related to nerve activity but also involved in the control of ovarian follicular cells alongside the cholinergic system. Thus, the main aim of this study was to determine whether NGF is one of the neurotrophic factors involved in the activation of the cholinergic system of the ovary *in vivo*. In this work, we present data on a common neurotrophic mechanism acting on noradrenergic neurons and on ACh-producing cells to balance of the autonomic tone of the organ.

METHODS

Animals and Experimental Design

We studied the effect of NGF on noradrenergic and cholinergic system in the rat ovary. Thus, we divided the experiments in two: 1. Studies *in vivo* in which we induced increase in noradrenergic transmission in the ovary by an NGF-mediated EV effect on the activity of the neurons in the ovary (16, 17). 2. the other experiment was chronic *in vivo* exposure by intrabursal administration of NGF to the ovary (11, 16). Once we determined the *in vivo* effect, we studied the effect of NGF *in vitro* to verify for a local effect of NGF on noradrenergic and cholinergic markers. After we defined the role of NGF in the activity of the noradrenergic or cholinergic biochemical markers, we analyzed the reproductive function and follicular development in the NGF treated rats. To differentiate from the cholinergic effect we also analyzed the role of a cholinergic muscarinic agonist Carbachol chronically administrated to the ovary on the follicular dynamic.

A total of 25 female Sprague–Dawley rats were used in this study: Six prepuberal (80–90 g) and 19 adult (250–300 g) animals (**Table 1**). All animals were housed in a maintenance room at a temperature of 20°C with light–dark cycles (12:12 h). The animals were provided food and water *ad libitum*. The estrous cycle of the adult rats was monitored *via* daily vaginal smears observed under a light microscope, as previously described (6, 19). The number of cycles was estimated as the regular passage from proestrus (P) to estrus (E), followed by diestrus (D). Control (sham) rats had regular 4-day estrous activity (Hubscher et al., 2005; Paccola et al., 2013). Ovaries from rats treated with estradiol valerate (EV) (intramuscular [i.m.] single dose, 10 mg/kg) were used. Bioethical regulation suggest to use tissue from other experiments previously published, we used one ovary of a previous study (18), that were stored at −80°C for ACh determination. At the end of the experiments, the rats were euthanized by decapitation, and the ovaries and plasma were collected. Decapitation was performed according to the AVMA Guidelines for the Euthanasia of Animals (2020 Edition) (20) by a specialized personnel. The study was also approved by the Bioethics Committee of the Faculty of Chemistry and Pharmaceutical Sciences at the University of Chile (Protocol number: CBE2017-14 to AB and CBE2017-05 to HL) and complied with the National guidelines (CONICYT Guide for the Care and Use of Laboratory Animals).

TABLE 1 | Experimental groups used for the *in vivo* and *in vitro* studies.

***IN VIVO* STUDIES**							
1.-EV adm.	**N**	**Age (days)**	**Days treatment**	**Biochemical studies ovary**	**Reprod function**	**Ovarian morphology**	**Origin of tissue**
10mg/kg i.m.	5	24 days old	30 days	ACh	———	————	Del Campo et al. (1)
Sham (sesame oil)	5	24 days old	30 days	ACh	———	————	Del Campo et al. (1)
2.- NGF adm. 100uM minipump	5	3-3.5 month old	30 days	TH WB ACh concentration AChase WB	Estrous cycle	Morphometry	Present work
Sham (saline)	5	3 - 3.5 month old	30 days	TH WB ACh concentration AChase WB	Estrous cycle	Morphometry	Present work
3.- Carbachol adm. 100uM minipump	4	3 - 3.5 month old	30 days	———————	Estrous cycle	Morphometry	Present work
Sham (saline)	5	3 - 3.5 month old	30 days	———————	Estrous cycle	Morphometry	Present work
***IN VITRO* STUDIES**							
Control rats	6	Prepuberal (two half)	Control medium	Half ovary Half ovary	ACh, mRNA		Present work
Ovaries cut in half and used		Prepuberal (two half)	NGF(100 ng/ml)	Half ovary Half ovary	ACh mRNA		Present work

(1) Ovaries were stored frozen at -80C from an experimental serie done for the paper of (18).

In Vivo NGF and Varbachol Administration Studies

Nineteen adult female rats were randomly assigned to either the sham group (control group) (n = 5) or NGF group (n = 5) for NGF studies, and sham group (control group) (n = 5) or carbachol group (n = 4) for carbachol studies (**Table 1**). The animals were anesthetized with an intramuscular dose of ketamine 60 mg/kg and xylazine in 10 mg/kg solution under aseptic conditions. To eliminate the possible contribution of the contralateral ovary to steroidogenesis, all sham, NGF- and carbachol-treated animals were subjected to unilateral ovariectomized (ULO) at the moment of the minipump implant (19), performed as previously reported (16).

To eliminate the possibility of a confounding effect of ovary hypertrophy derived from the ULO, all sham and experimental rats, were subjected to hemiovariectomy. Briefly, a transverse midlumbar incision, 1.5 cm long, was made in the flank area on one side of the animal to obtain access to the ovarian bursa. The ALZET osmotic minipump ([0.25 µl/h] Model 2004; Alza Corp. Palo Alto, CA, USA) was connected to the underlying bursa of the left ovary with SILASTIC 0.64 mm ID × 1.19 mm OD CAT 508-003 (Dow Corning Corp, Midland, MI, USA) tubing for 28 days. The treatment was performed as follows:

a) Animals in the NGF group were implanted with osmotic minipumps for intraovarian NGF delivery at a concentration of 100 ng/ml in saline (catalog number N-100; Alomone Labs, Jerusalem, Israel).
b) Animals in the carbachol group were implanted with osmotic minipumps for intraovarian delivery at a concentration of 100 µM in saline (catalog number 212385-M; Calbiochem, Sigma Chemicals, St Louis, MO, USA). Carbachol, is a well-known non-specific muscarinic cholinergic agonist that is not degraded by AChE
c) For the sham group, animals were subjected to surgery and implanted with the cannula filled with saline, the solvent for both drugs, but not the osmotic minipump.

After 28 days, the rats were euthanized and the ovary and trunk blood were collected for analysis. The position of the minipump and the cannula was inspected to verify that they were in place after the two or one month procedure. A picture of both is shown in supplementary data. The ovaries were cut in half, and one half was fixed with Bouin's fluid for morphometric analysis. The other half was cut again in half, and each half was stored at −80°C for ACh determination or western blot analysis for NGF studies.

In Vitro NGF Studies

To study the effects of NGF on ACh production in the ovaries, we used six prepuberal rats (**Table 1**). We used prepuberal rat ovaries because, at this age, there is no ovulation and no corpora lutea; thus, we can study the effect of NGF mainly in GCs (21, 22). The rats were euthanized, and both ovaries were removed through an anterior incision in the midline of the abdomen. The ovaries were halved (2 ovaries = 4 halves per animal), and each half was incubated for 3 h at 37°C in 1.0 ml of Krebs-bicarbonate-albumin buffer (NaCl, 118.6 mM; KCl, 4.7 mM; KH_2PO_4, 1.2 mM; ascorbic acid, 100 µg/ml; $NaHCO_3$, 0.15 M; $CaCl_2$, 25 mM; albumin, 0.1 mg/ml; glucose, 11.2 µg/ml), under 95% oxygen and 5% CO_2. For each condition, the animals were randomly divided into groups of six. One half was incubated only in Krebs-bicarbonate buffer (the control group), and the other half was incubated with NGF at 100 ng/ml (catalog

number N-100, Alomone Labs, Jerusalem, Israel). The concentration of NGF used has been previously demonstrated to be sufficient to increase choline acetyl transferase (ChAT) protein levels in human GCs and ACh levels in bovine luteal cells (9, 10). After incubation, the ovaries were stored at −80°C for mRNA extraction or ACh determination at a later date.

Quantification of Intraovarian Levels of ACh and AChE Activity

The ovary was homogenized in 10 volumes of PBS in ice. ACh and AChE activity was determined in the homogenate using the Amplex® ACh/AChE assay kit (Invitrogen, Carlsbad, CA, USA) according to the instructions recommended by the provider as previously described (4, 6). The results represent the total amount of ACh in µmol per ovary and AChE activity in U per ovary (where one U is defined as the amount of enzyme that hydrolyzes 1.0 µmole of ACh to choline and acetate per minute at pH 8.0 at 37°C, as indicated by the manufacturer). The minimal detectable value for AChE was 0.002 UI/ml and for ACh was 0.3 µM (range, 0.3 µM to 100 µM).

Western Blot Analysis

For western blotting, the ovary was homogenized in 10 volumes of RIPA buffer (1% NP40, 0.5% sodium deoxycholate, and 0.1% SDS in PBS; just before use, 10 µl of the following mixture [10 mg/ml stock solution of PMSF; aprotinin and sodium orthovanadate] was added) in the presence of Complete Mini EDTA-Free Protease Inhibitor Cocktail (Roche, Basel, Switzerland). Proteins were quantified by the Bradford method, and 50 µg was run on a 10% polyacrylamide gel. Proteins were transferred to nitrocellulose, blocked with 5% milk for 1 h, and incubated with an antibody that recognizes all the isoforms of AChE (A-11; Santa Cruz Biotechnology, Dallas, TX, USA) at a dilution of 1:3,000 overnight or with tyrosine hydroxylase (MAB5280; Merck Millipore, Burlington, MA, USA) at a dilution of 1:1,000 overnight. As an internal control, we used GAPDH (G9545; Sigma Chemicals, St Louis, MO, USA) at a dilution of 1:40,000 for 1 h. The secondary antibodies used were goat anti-mouse IgG Fc (HRP) (catalog number 31430; Waltham, Massachusetts, USA) at 1:10,000 dilution for 1 h, and goat anti-rabbit IgG Fc (HRP) (ab97200; Abcam, Cambridge, United Kingdom) at 1:10,000 dilution for 1 h. The antibody complexes were detected by chemiluminescence using an EZ-ECL Enhanced Chemiluminescence Detection Kit (Biological Industries, KBH, Israel). Chemiluminescence was captured using a G-Box Syngene system (Syngene Headquarters, MD, USA).

Morphometric Analysis

The halved ovaries were fixed in Bouin's fluid, embedded in paraffin, cut into 6-µm sections, and stained with hematoxylin and eosin. Morphometric analyses of whole ovaries were performed according to the method of (23) with modifications described previously (16), using n = 5 ovaries for the sham group, n = 5 ovaries for the NGF group, and n = 4 ovaries for the carbachol group. We used the following classification: primordial follicles had one oocyte surrounded by flattened GCs. Primary follicles had one layer of cubical GCs, and secondary follicles had no antral cavity but two or more layers of GCs. Antral follicles were those with more than three healthy GC layers, the antrum, and a clearly visible nucleus of the oocyte. Atretic follicles had more than 5% of the cells with pyknotic nuclei in the largest cross-section and exhibited shrinkage and occasional breakdown of the germinal vesicle. Precystic follicles were large follicles with or without oocyte, containing four or five plicated layers of small, densely packed GCs surrounding a very large antrum with an apparently normal thecal compartment. Cystic follicles were devoid of oocytes and displayed a large antral cavity, a well-defined thecal cell layer, and a thin (mostly monolayer) GC compartment containing apparently healthy cells. All abnormal follicular structures were grouped as cystic structures.

Plasma Levels of Steroid Hormones

Plasma levels of steroid hormones progesterone (P4), androstenedione (Δ4), testosterone, and estradiol (E2) were measured. Serum levels of P4, Δ4, testosterone, and E2 were determined by enzyme immunoassay (EIA), following the manufacturer's instructions (Alpco Diagnostic, Windham, NH, USA). Intra and interassay variations were less than 5% for P4, less than 10% for Δ4, less than 6% for testosterone, and less than 5% for E2, and the minimal detectable values were 0.1 ng/ml, 0.04 ng/ml, 0.02 ng/ml, and 10 pg/ml, respectively.

Real-Time Polymerase Chain Reaction (qPCR)

Total RNA was extracted as described previously (24) from the halved ovary incubated *ex vivo*. The primers used are listed in **Table 2**. A BLAST search was performed to determine the specificity of the sequences. The PCR reaction mix contained 10 µl of Brilliant II SYBR Green QPCR Master Mix (Agilent Technologies, Inc., California, USA), 0.01 µM of each GAPDH primer, 0.1 µM of each ChAT primer or 0.1 µM of each vesicular ACh transporter (VAChT) primer, 2 µg of cDNA, and sterile water for a final volume of 20 µl. PCR reactions were performed using the IQ5 real-time thermocycler (Bio-Rad) under the

TABLE 2 | Primers used for polymerase chain reaction (PCR) amplification.

Gen		Sequences	Access number	Sequence reference
ChAT	*forward*	5′- CTGGATTTCATTGTTTATAAGTTTGACAAC-3′	XM_00106152	(25)
	reverse	5′- CTGGAGGGCCACCTGGAT-3		
VAChT	*forward*	5′- GCCACATCGTTCACTCTCTTG-3′	X80395	(26)
	reverse	5′- CGGTTCATCAAGCAACACATC-3′		
GAPDH	*forward*	5′- GATGCCCCCATGTTTGTGAT -3′	NM_017008.4	(27)
	reverse	5′- GGTCATGAGCCCTTCCACAAT-3′		

following conditions: 95°C for 20 s, 60°C for 20 s, 72°C for 20 s, and a final extension at 72°C for 10 min. All samples for RT-qPCR analysis were run in triplicate (with no reverse transcriptase control as a negative control), and the mean values were used to determine the mRNA levels. Relative quantifications of ChAT and VAChT mRNA were performed using GAPDH mRNA as a housekeeping gene.

Statistical Analysis

The data are expressed as the mean ± SEM. Statistical analyses were performed using Prism GraphPad 6 (GraphPad Software, San Diego, California, USA). To examine statistical differences between the two groups, we used Student's *t* and the Mann-Whitney tests, as described below. To analyze differences between proportions, we used the chi-square test. To determinate normal distribution of our data, we used the Shapiro-Wilk normality test.

The number of animals for all experiments was calculated as the minimum number of animals according to the variability of the experimental procedures and the intrinsic variation between them. The minimum number of animals was calculated according to the following equation (28):

$$n = \frac{2(Z\alpha + Z\beta)^2 \times S^2}{d}$$

where n is the number of animals for each condition, S = standard deviation, d = difference needed to obtain statistical significance, Zα = the probability of type I error (significance), and Zβ = the probability of type II error (power). In the experiments to determine ACh, and AChE activities and levels of plasma hormones, we proposed $\alpha = 0.05$, the probability of finding a statistically significant difference was 0.05; $\beta = 0.3$, the probability of having a difference between the populations; the intrapopulation variation, was 0.2; and d, the smallest difference in the population, was 0.11. Thus, we obtained n = 4.5. Therefore, to obtain a statistically significant difference of $p<0.05$, we needed to use four or five animals per study group.

RESULTS

Estradiol Valerate (EV) Increased Ovarian ACh Levels

A previous report showed that after 30 days of estradiol valerate (EV) exposure, there was an increase in NGF level in the ovary; 60 days after EV, there was also an increase in norepinephrine level (17). We found that ovarian ACh levels had also increased ([mean ± SEM] 5.0 ± 0.6 μmol/mg ovary for sham vs. 12.3 ± 3.1 μmol/ovary for EV-treated rats, $p < 0.05$, unpaired Mann-Whitney test, n = 5).

Effect of *In Vivo* Intraovarian Exposure to NGF on Autonomic Neurotransmitter

The concentration of NGF used would be sufficient to promote biological actions on the sympathetic neurons fibers that innervate the ovary (29–31). To ensure sympathetic activation, we determined tyrosine hydroxylase (TH) levels (MW: 56 kDa) by western blot analysis (**Figure 1A**). **Figure 1B** shows the quantification of protein levels relative to the mean of the sham group level. *In vivo* NGF treatment produced a 10-fold increase in TH.

We found a decrease in ovarian ACh levels per ovary after 28 days of stimulation with 100 ng/ml NGF (**Figure 1C**). No differences were found in ovarian weight (data not shown). When we compared the ACh levels of the ovaries between before and after NGF treatment (where the ovaries before treatment corresponded to the contralateral ovary removed before starting the osmotic minipump implantation or sham surgery) for each condition, we found a significant decrease in ACh in the NGF-treated ovaries (**Figure 1D**).

Effect of *In Vivo* Intraovarian Exposure to NGF on Ovarian AChE Isoform and Enzyme Activity

ACh levels are mainly regulated by cholinesterases. In the ovary, the main cholinesterase is AChE (32). Ovarian rat cells express two isoforms: AChE subtypes –R and –S (4). Therefore, to determine if the decrease in ovarian ACh levels is mediated by an increase in AChE, we analyzed its levels by western blot. In this sense, while AChE-R has a size of approximately 55 kDa (33–35), AChE-S has several posttranslational modifications, and its size could be 70, 60, and even 55 kDa (33–36). **Figure 2** shows the western blot analysis results for AChE, and we found two main bands at 70 kDa and 55 kD (**Figure 2A**). The total group data are presented in **Figures 2B, C**. *In vivo* NGF treatment produced a two-fold increase only in the AChE 55 kDa-isoform (**Figure 2C**). We used a monoclonal antibody that is targeted to the *N*-terminal (A-11, Santa Cruz Biotechnology), common to both isoforms, so we could not discriminate between AChE subtypes.

To determine the amounts of enzymatic proteins, especially the AChE 55kDa-isoform, we determined the ACh hydrolysis capacity of the ovary samples because this isoform is mainly present in the ovary and not butyryl cholinesterase (32). We did not find changes in enzyme activity between the NGF-treated and sham groups (**Figure 2D**). Therefore, the increase in AChE 55 kDa-isoform levels was not related to an increase in its catalytic activity.

In Vitro NGF Incubation Increased ChAT and VAChT mRNA Levels But Not ACh Levels

In vivo NGF treatment affects not only the ovarian cholinergic system but also extrinsic sympathetic fibers that innervate the organ as well as other factors that are present in a rat. To rule out all these extrinsic factors, **Figure 3** shows the effect of *in vitro* ovary culture treated with 100 ng/ml of NGF on ChAT and VAChT mRNA expression and ACh levels in rat ovary. Reverse transcription qPCR (RT-qPCR) studies showed that *in vitro* NGF incubation for 3 h produced a four-fold increase in ChAT and VAChT mRNA levels compared to the control condition (**Figures 3A, B**). AChE mRNA levels showed no changes (data not shown). A slight but not significant increase in ovarian ACh levels was observed in two-thirds of the samples

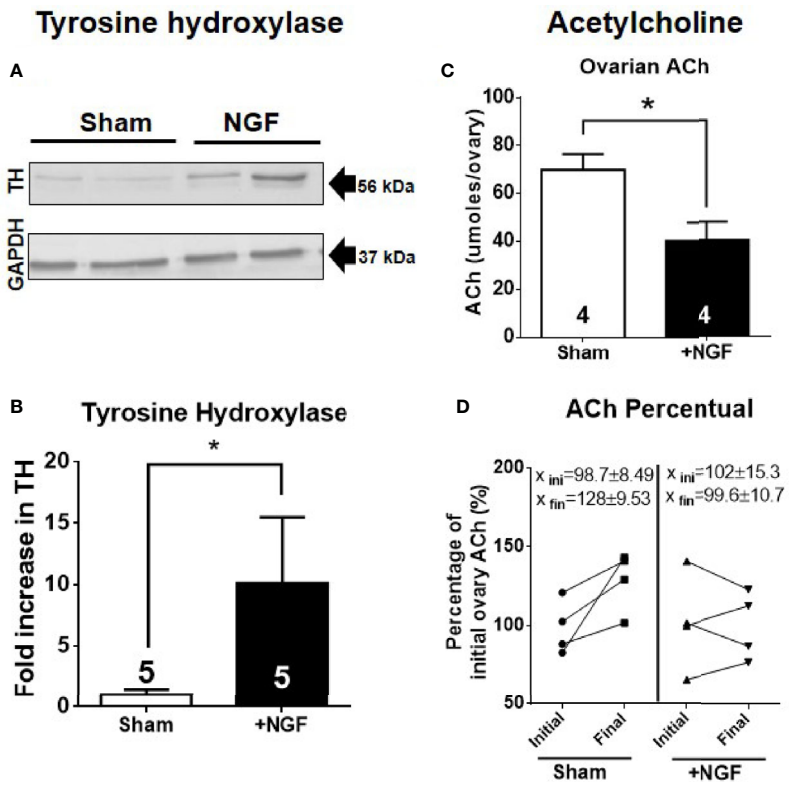

FIGURE 1 | *In vivo* intraovarian nerve growth factor (NGF) administration increases tyrosine hydoxylase (TH) levels and decreases acetylcholine. **(A)** Western blot analysis of tyrosine hydroxylase (~56 kDa) and GAPDH in the ovary. Representative membranes of each protein are shown. **(B)** Bar charts show quantification of protein levels of TH compared to GAPDH control in each condition. Pixels were counted using ImageJ software (U.S. National Institutes of Health, Bethesda, Maryland, USA). **(C)** Decreased levels of ovarian ACh level. Unpaired Student's t test. No change was found in ovary weight after 28 days of treatment between the sham and NGF groups (sham = 74.61 ± 8.189 mg; NGF = 52.78 ± 10.44 mg). **(D)** A decrease in percent ACh levels after NGF treatment relative to initial level – Initial ovary corresponds to contralateral ovary that was removed on day 0: Sham = 128 ± 9.53% vs. NGF = 99.6 ± 10.7%. *$P < 0.05$, Chi-square test. All values correspond to the mean ± SEM. *$P < 0.05$.

after 3 h of stimulation (**Figure 3C**). Interestingly, ACh was reduced but only in incubation media.

Effect of *In Vivo* NGF Exposure Administration on Estrus Cycle

The exposure of the ovary to NGF during the 28-d disrupted the estrous cycle (**Figure 4A**), as previously described (11). Compared to the sham group, the treated group showed a significant decrease in the percentage of time of proestrus (NGF = 15.4 ± 2.2% vs. sham = 21.3 ± 1.3%; *$P < 0.05$) and an increase during estrus (NGF = 33.7 ± 3.1% vs. sham = 26.0 ± 1.7%; *$P < 0.05$). There was a significant decrease in the number of ovulatory estrous cycles (NGF = 25.0 ± 6.9% vs. sham = 64.1 ± 7.9%; *$P \leq 0.05$) (**Figure 4B**). All of these changes were previously described (11)

Ovarian Follicular Dynamic Was Altered by *In Vivo* NGF Administration

Morphometric analysis results of the ovary exposed to excess NGF for 28 days are shown in **Figure 5**. As previously described (11), important alterations in normal follicular development were found, with a reduction in the number of secondary follicles (**Figure 5A**) and healthy antral follicles (**Figure 5B**). Moreover, there was an increase in atretic antral follicles (**Figure 5C**), and a decrease in the number of corpora lutea (**Figure 5D**). NGF exposure for 28 days resulted in the appearance of cystic structures (**Figure 5E**). A decrease in the number of healthy antral follicles and an increase in atretic antral follicles, along with the appearance of cystic structures, have also been observed in mice and rats treated with an excess NGF (11).

Plasma Concentration of Progesterone Reduced After Chronic NGF Administration

Table 3 shows the plasma levels of ovarian steroids, progesterone, androstenedione, testosterone, and estradiol at the end of the experimental protocol. NGF treatment for 28 days led to a reduction in the progesterone levels. No changes were found in androstenedione, testosterone, and estradiol levels.

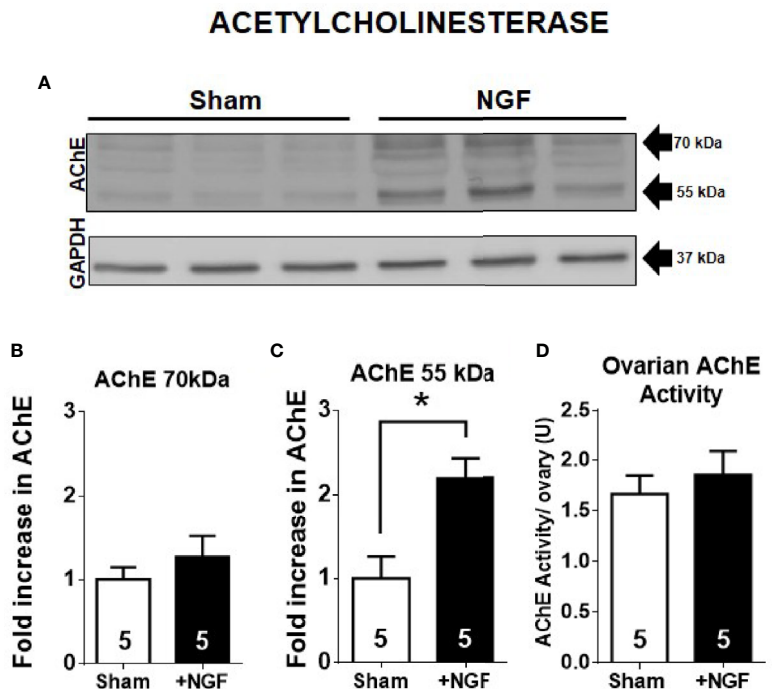

FIGURE 2 | 55 kDa-isoform AChE is increased by *in vivo* nerve growth factor (NGF) treatment. **(A)** Western blot analysis of AChE and GAPDH in the ovary. Representative membranes of each protein are shown, and two main bands at ~70 kDa and ~55 kDa were identified. GAPDH was used a loading control. **(B)** Bar chart shows quantification of protein levels of AChE 70 kDa-isoform. No significant change was found. Pixels were counted using ImageJ software. **(C)** Bar charts shows the quantification of protein levels of AChE 55 kDa-isoform. All values correspond to the mean ± SEM. **(D)** Ovarian AChE activity was not affected by *in vivo* administration of NGF. All values correspond to the mean ± SEM of n = 5 experiments, $^*P < 0.05$, unpaired Mann-Withney test.

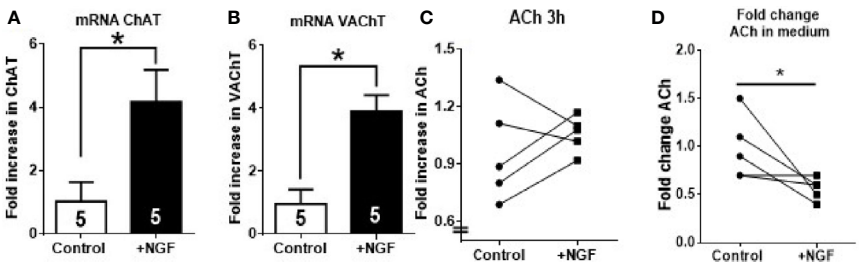

FIGURE 3 | *In vitro* nerve growth factor (NGF) incubation increases ChAT and VAChT mRNA but decrease ACh in media. Half ovaries were incubated in Krebs buffer for 3 h: Control, incubated only in media; NGF, incubated with NGF at 100 ng/ml. **(A, B)** Fold increase in the mRNA expression of ChAT and VAChT after incubation with NGF. GAPDH mRNA was used as housekeeping gene. **(C)** A slight but not significant increase was found in ACh levels in 60% of animals. **(D)** ACh fold change in incubation media. All values are relative to control mean and, for each group, correspond to the mean ± SEM of n = 5 experiments. *P < 0.05 unpaired Mann-Withney test.

In Vivo Carbachol Administration Promoted Follicular Development

We found that *in vivo* NGF treatment increases intraovarian TH levels, reduces intraovarian Ach levels, and causes aberrant follicular development. To determine if the decrease in ACh mediates this enhanced response of NGF to the sympathetic nerves, we used chronic administration of carbachol, a muscarinic agonist that is not degraded by AChE. No changes in the estrous cycling activity was found (not shown). Morphometric analysis of the ovaries after carbachol exposure showed no changes in secondary and antral follicles, but increased numbers in the corpus luteum and a decrease in cystic structures (**Figure 6**), suggesting cholinergic promotion of the healthy pathway.

DISCUSSION

Recent evidence strongly suggests that when the balance between the sympathetic and cholinergic pathways is altered, this may

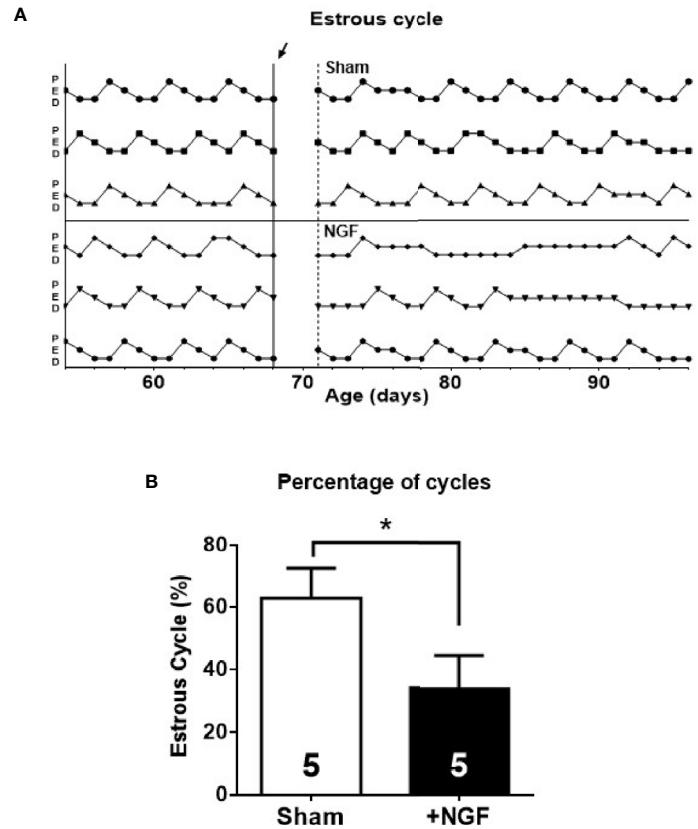

FIGURE 4 | Estrous cyclicity was affected by *in vivo* nerve growth factor (NGF) administration. Sham, animals exposed to sham surgery; and NGF, animals exposed to 100 ng/ml of NGF locally delivered to the ovary by the means of an osmotic minipump. Animal weights after 28 days of treatment were: Sham = 311.3 ± 8.147 g vs. NGF = 298.3 ± 12.91 g (n.s). **(A)** Three representatives estrous cycle profiles before (14 days) and during treatment (28 days) for each condition are shown (*arrow* indicates osmotic minipump implantation or sham surgery). Vertical axis depicts different stages of the estrous cycle. **(B)** Each bar represents the number of estrus cycles estimated as the regular passage from proestrus (P) to estrus (E) followed by diestrus (D) over the observation days. All values correspond to the mean ± SEM of n = 5 animals. *$P < 0.05$, unpaired Student's t test.

lead to pathologic conditions, such as that induced by estradiol valerate treatment, a model that resembles PCOS (16, 37, 38). Sympathetic fibers innervate the ovary in the establishment of PCOS conditions. ACh is involved in several processes related to ovarian function, but how its ovarian production is regulated is unclear. Recently, we found that chronic sympathetic stimulation by stress stimulated the intraovarian cholinergic system (6). Here, we found that estradiol valerate produces an increase in ACh levels, similar to that observed after chronic cold stress. Our most important observation was the stimulatory effect of NGF *in vitro* on the increase in the metabolizing enzymes of ACh, and the effect was repeated after long-term *in vivo* treatment. The fact that it was not translated into changes in ACh led to an unbalanced sympathetic/cholinergic system, resulting in aberrant follicular development with a concomitant decrease in progesterone plasma levels. Since NGF regulator of the dual autonomic control, which is essential for maintaining the homeostasis of ovary function.

In sympathetic neurons, estradiol valerate treatment leads to an increase in ovarian noradrenaline levels and an ovarian phenotype similar to that in PCO (17, 39). After 30 days, estradiol valerate has been found to produce an increment in NGF and NGFR levels in rat ovary which is also associated with PCO phenotype in rats (16). The implant in the ovary of cells overexpressing NGF are involved in the development of PCO in mouse (14, 40). It is not surprising to found estrogen dependent changes in neurotrophin in sympathetic nerves and in cholinergic neurons because it has been amply demonstrated that estrogens converge with neurotrophin signaling pathways (41, 42). The increase in the biosynthetic enzymes for ACh indicate that NGF regulates ovarian ACh production. Previous reports also suggest that NGF stimulates ACh production (6, 9, 10). This increase could protect follicular development from the actions of an over-activated sympathetic pathway and chronic increase in ovarian noradrenaline, caused by the hyperinnervation of the organ (39, 40). To explore the effects of NGF on the activities of the enzymes involved in the biosynthesis and degradation of intraovarian ACh in the rat ovary, we utilized *in vivo* and *in vitro* approaches.

In Vivo NGF Treatment Reduced ACh Levels and Enhanced Production of 55kDa-Isoform AChE

In vivo treatment with intrabursal NGF disrupted the estrous cycle, as demonstrated in previous studies that either grafted the

TABLE 3 | Plasma concentration of progesterone, androstenedione, testosterone, and estradiol after NGF treatment.

	Sham	NGF-treated
Progesterone (ng/ml)	10.8 ± 1.9	4.5 ± 1.8 (*)
Androstenedione (ng/ml)	0.33 ± 0.2	0,29 ± 0.1
Testosterone (ng/ml)	0.32 ± 0.1	0.24 ± 0.1
Estradiol (pg/ml)	22.3 ± 2.6	26.3 ± 5.0

*Results correspond to five animals in each condition and are expressed as mean value ± SEM. *P < 0.05, unpaired Student's t test.*

ovary with cells overexpressing NGF (11) or used transformed cells producing NGF (11, 14). Locally delivered NGF was effective in activating sympathetic neurons, as evidenced by the increased expression of TH, the rate-limiting enzyme in the biosynthesis of NA. The ovaries of animals treated with NGF were hyperinnervated by catecholaminergic fibers, giving an enhanced sympathetic tone to the gland (16). Although NGF increased the activities of the enzymes involved in the biosynthesis of ACh, NGF unexpectedly decreased ACh levels, even with no changes in AChase activity.

When we compared the ovaries at the end of the treatment with NGF with the contralateral ovary collected at before the minipump installation, we found that the NGF-treated ovaries had significantly lower ACh levels per ovary than the non-treated ovaries. We do not have information about the release mechanism inside the GCs and whether it is regulated by other factors (43). However, in primary cultures of cholinergic neurons, NGF has been found to promote ACh release (vesicular as well as spontaneous) faster than enhancing ChAT activity, in a concentration- and time-dependent manner up to 10 days (43, 44). (6), proposed a model in which ACh was stored in vesicles inside the GC and would not be exposed to degradation by AChE. However, ACh, once released, does not act on muscarinic receptors to exert trophic action. Further research is needed to evaluate ACh release mechanisms in GC.

ACh levels are also regulated by cholinesterases. It has been reported that ovarian cholinesterase activity is mainly mediated by the ovarian AChE (32). AChE isoforms –S and –R have been recently identified in rat ovaries (4). It is known that they have different characteristics and distributions: isoform S is able to

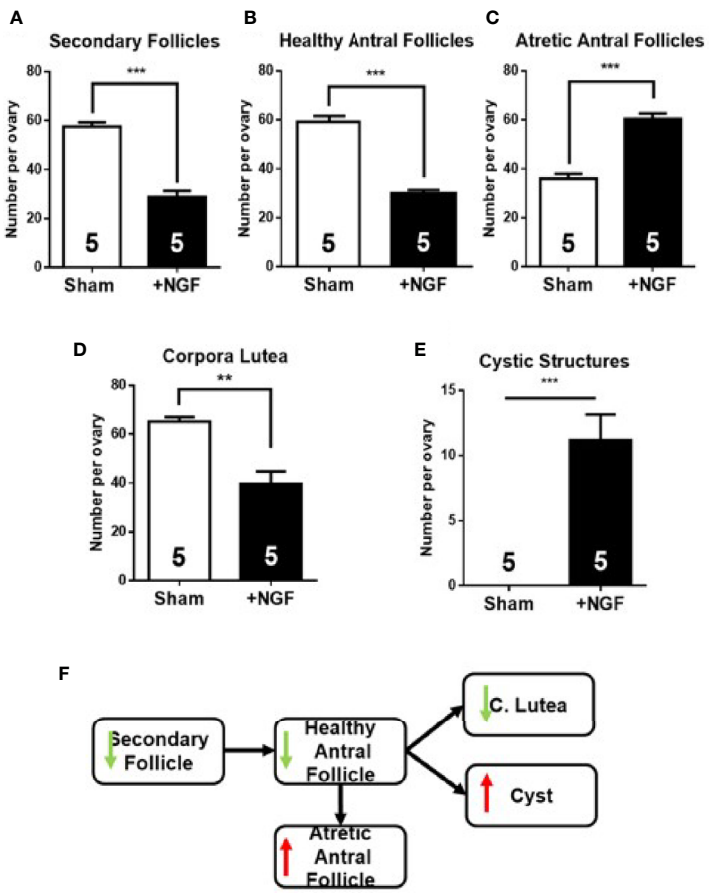

FIGURE 5 | Morphometric analysis of ovaries after *in vivo* intrabursal exposure during 28-day with 100 ng/ml nerve growth factor (NGF). Altered follicular dynamic was found in NGF-treated animals: decrease in number of secondary **(A)** and healthy antral **(B)** follicles, an increase in number of atretic antral follicles **(C)**, minor increase in corpora lutea, **(D)** and appearance of cystic structures **(E)** is visible. **(F)** Summary of changes found in follicular development. All values correspond to the mean ± SEM (n = 5 for each group). **(A–C, E)** ***P* < 0.001, **(D)** **P* < 0.01, unpaired Student's t test.

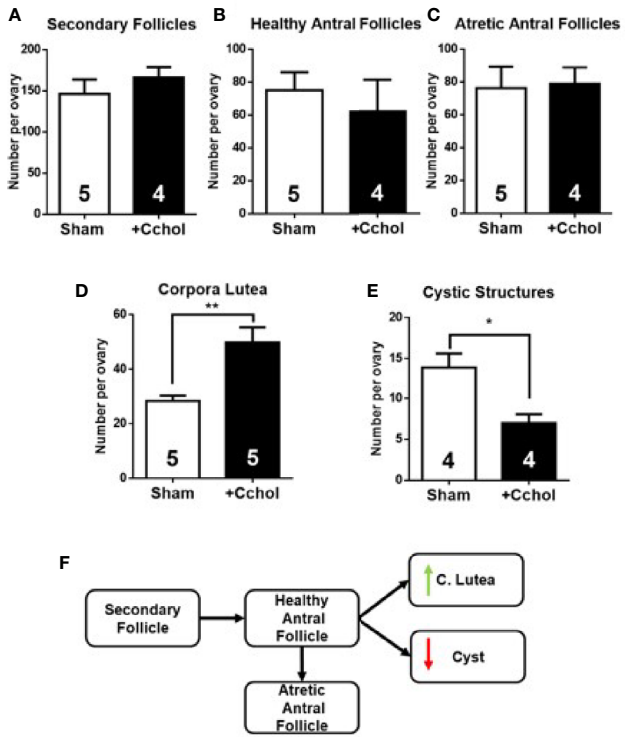

FIGURE 6 | Morphometric analysis of ovaries after *in vivo* intrabursal exposure during 28-day with 100 uM Carbachol (Cchol). No significant changes in weight gain were observed between experimental groups: Sham, animals exposed to sham surgery; and carbachol (Cchol), animals exposed to 100 uM of Cchol locally delivered to the ovary by means of an osmotic minipump. Follicular development was enhanced: No changes in number of secondary **(A)**, healthy **(B)**, and atretic antral **(C)** follicles, but an increase in number of corpora lutea **(D)**, and a decrease in number of cystic structures **(E)** are visible. **(F)** Summary of changes found in follicular development. All values correspond to the mean ± SEM of n = 4 or 5 animals as shown in the figure. **(D)** $^{**}P < 0.01$, **(E)** $^{*}P < 0.05$, unpaired Student's t test.

form multimers that join to anchoring proteins and bind them to the synaptic membrane, while AChE-R cannot form it and once secreted, it is soluble. In studies involving mice and humans, AChE-S has shown different molecular weights (55, 60, and 70 kDa) depending on posttranslational modifications (33–36). The molecular size of AChE-R was found to be 55 kDa in studies involving mice and humans (33–35). When we analyzed AChE by western blotting after NGF stimulation and found a 2-fold increase only in the 55 kDa-isoform. The monoclonal antibody (A11, Santa Cruz Biotechnology) that we used did not discriminate between both isoforms. Besides, it is known that AChE levels increase to compensate for the excess in ACh in the brain, such as the hippocampus and caudate nucleus (45, 46). However, in our study, there was no increase in ACh or AChE activity. Therefore, we thought that this increase in the mass of the protein might be related to AChE-R. In this sense, the action of stress or AChE inhibitor leads to alternative splicing that produces a large amount of AChE-R mRNA (46). However, AChE-R mRNA is less stable than AChE-S mRNA (47), and its expression is limited to the duration of its stimulus. Further research is needed to explore this hypothesis.

NGF Increased ChAT and VAChT mRNA But Not ACh Production

Three hours of incubation of the ovary in the presence of NGF increased the level of ChAT mRNA. This change is in agreement with results in previous reports where 100 ng/ml of NGF stimulated ChAT production in bovine luteal cells and human GCs (9, 10). Although it has not been established that ACh is stored in vesicles, VAChT has been found to be expressed in GCs (21). Both proteins are expressed together in many neuronal models because they share the same transcriptional direction, since VAChT genes are located in the first intron of ChAT genes (48). We also found an increase in VAChT mRNA after NGF treatment; therefore, the effects related to the expression of proteins probably stimulated ACh production and storage. However, despite these promising results, when we analyzed ACh levels in our *in vitro* model, we found a slight but not significant increase in ACh levels in 66% of samples after 3 and 24 h of NGF treatment. Several factors were considered in our experimental protocol. First, we used prepubertal rat ovaries to rule out the interference of structures that do not produce ACh in rats, such as the corpus luteum (21, 49). Second, our model has been demonstrated to be useful for evaluating the ovarian effects of different neuropeptides, including NGF (19, 50, 51). Isolated ovaries eliminate the exogenous contributions from cholinergic and sympathetic neuronal fibers that innervate the organ. In the incubation medium, we found a decrease in ACh levels, but this may be related release or increase in AChE activity. In septohippocampal co-cultures and hippocampal slices, 3–4 weeks of NGF stimulation is needed to observe changes in the neurotransmitter and in ChAT activity (52, 53). However, 4 weeks of *in vivo* treatment was insufficient to produce an increase in ACh. Thus, although we demonstrated increased levels of the metabolic enzymes by NGF, we did not find increased levels of ACh, suggesting another function of the cholinergic system not related to its action as a neurotransmitter and probably with trophic actions affecting follicular development.

Unbalanced Intraovarian Cholinergic System and Over-Activated Sympathetic Pathway Due to Intraovarian NGF Excess Produced Altered Follicular Dynamics

Ovarian function was altered in rat ovaries exposed to NGF. The initial recruitment was altered because the number of secondary follicles was reduced, despite the fact that NGF promotes their growth and differentiation (54–56). Dissen et al. did not find changes in preantral follicles after grafting NGF-producing cells in rat ovaries. However, they were treated for 60 days with NGF, and compensatory mechanisms in initial recruitment could be

responsible for their results (57). Regarding cycle recruitment, we found a decrease in the number of healthy antral follicles and an increase in atretic antral follicles. Since the GCs of healthy antral follicles express ChAT (21, 22), the reduction in their number could explain the reduction in acetylcholine levels after NGF treatment. The increase in atretic antral follicles could be an NGF-dependent atretic process mediated by its low-affinity NGFR (also termed as p75NTR) (58). However, NGFR is expressed at very low levels or is undetectable in the GCs of rat ovaries (59); therefore, the increment in atretic follicles may be promoted by other causes, such as necroptosis. Recently, Du et al. reported that the use of necrostatin, an inhibitor of a kinase of the necroptosis pathway, promotes the increase in the size of oocytes and follicles cultured *in vitro* (60). In this sense, the ARP fragment of AChE-R induces cell death activating kinases of the necroptosis pathway, and, as described above, induced by AChE-R, which thought that is the isoform incremented by our *in vivo* protocol. ARP induces necroptosis in human GCs (32). Hence, there is a relationship between the promotion of atresia by NGF and the increment in AChE-R and hence a decrease in ACh. Further research is needed to determine the mechanism involved in this process.

Cruz (5) suggested that ACh regulates ovulation through muscarinic receptors. We reported a decrease in the number of corpora lutea, specifically of newly formed (large) corpora lutea, and accumulation of small corpora lutea (data not shown). The increase in small corpora lutea is associated with a decrease in the number of ovulations. Moreover, NGF stimulates the formation of cystic structures. It is well known that cysts produce impairments in ovarian function, such as hyperandrogenism, anovulation, and infertility (12, 17, 51, 61). Overall, all these changes show that chronic NGF treatment produced a decrease in ovarian function, but not by a selective increase in noradrenergic tone but rather in both neurotransmitters, being a noradrenergic predominance because of the increase in the AChE degrading enzyme.

However, the present findings are not explained only by the hyperactivation of the sympathetic pathway in the ovary due to the increase in noradrenaline levels. Indeed, 4 weeks of cold stress treatment (chronic noradrenergic activation) increases ovarian noradrenaline and aberrant follicular development, but only it induces a decrease in number of secondary follicles, corpora lutea and appearance of precystic structures (6, 12, 62). Recently, it was described that CUMS chronic stress decreases NGF in the ovary (63). Probably is related to a non-specific increase in corticoids as it was demonstrated to occur in the ovary after a restraint/cold stress (61). The most important observation was that *in vitro* incubation with NGF reverse the changes induced by CUMS suggesting changes in the balance of the induced neurotransmitters (probably ACh) or in the inhibition of corticoids synthesis in the rats. Imbalance in the intraovarian cholinergic system may be the factor affecting normal follicular development, increasing ACh levels by inhibiting AChE-enhanced follicular development and reducing the number of cystic structures (4). Herein, the imbalance in ACh production/degradation favored its degradation. In addition, the increase in the AChE 55 kDa-isoform (probably AChE-R), which could produce inflammation directly or indirectly through ARP (which itself promotes necroptosis), could explain the more profound effects of NGF on aberrant follicular development (including the appearance of pre-cyst and cyst structures). These abnormalities with the over-activated sympathetic pathway may favor the promotion of the atretic pathway and cystic structures over healthy follicles.

In fact, if a decreased ovarian ACh concentration is the cause of the predominance of the noradrenergic tone responsible for the PCO phenotype, we obtained strong evidence of a regulating effect of ACh in the ovarian follicular development by the use of carbachol, a non-specific muscarinic agonist, which is not degraded by AChE. This drug produced clear changes in ovarian morphology, showing the results of a pure cholinergic effect; thus, the cholinergic stimulation led to an increase in the number of corpora lutea (i.e., ovulation). A decrease in cystic follicles indicates that cholinergic activation is in balance with sympathetic activation. These results are also supported by the experiments of Urra et al (4). in which they demonstrated that the increase in ACh in a normal ovary induced an increase in ovulation and fertility. In addition this is a characteristic of ACh because it is able to reverse the PCO phenotype induced by cold stress in rats (13).

Our results indicate that ACh is a key factor in follicular development, promoting the healthy pathway over the atretic or cystic pathway. The most important finding of this work is that NGF stimulates ovarian ChAT and VAChT mRNA and, probably, ACh production. *In vivo*, we found that NGF mainly induced the activation of the sympathetic pathway and increase in AChE 55 kDa-isoform, resulting in an imbalance in the ovarian cholinergic system and aberrant follicular development. Overall, it seems that NGF is a key factor in maintaining homeostasis in the dual autonomic control system, balancing the output of the sympathetic and cholinergic systems to regulate ovarian function. These results open the possibility to pharmacologically control the sympathetic and cholinergic activity by direct delivery of drug affecting the ovarian follicular development in the rat. These results needs to be validate in human ovary as it has accumulated for the noradrenergic control (7).

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 the Bioethics Committee of the Faculty of Chemistry and Pharmaceutical

Sciences at the University of Chile (Protocol number: CBE2017-14 to AB and CBE2017-05 to HL). Sergio Livingstone 1007.

AUTHOR CONTRIBUTIONS

AB performed most of the experimental work with rats, biochemical analysis, data collection, and manuscript preparation. MdC performed estradiol studies. RR and CA performed the morphometric analysis in NGF and carbachol studies, respectively. HL conceived the idea, participated in the study design, and directed the work and manuscript preparation. All authors contributed to the article and approved the submitted version.

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REVIEW
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The Interplay Between Non-coding RNAs and Insulin-Like Growth Factor Signaling in the Pathogenesis of Neoplasia

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The insulin-like growth factors (IGFs) are polypeptides with similar sequences with insulin. These factors regulate cell growth, development, maturation, and aging via different processes including the interplay with MAPK, Akt, and PI3K. IGF signaling participates in the pathogenesis of neoplasia, insulin resistance, diabetes mellitus, polycystic ovarian syndrome, cerebral ischemic injury, fatty liver disease, and several other conditions. Recent investigations have demonstrated the interplay between non-coding RNAs and IGF signaling. This interplay has fundamental roles in the development of the mentioned disorders. We designed the current study to search the available data about the role of IGF-associated non-coding RNAs in the evolution of neoplasia and other conditions. As novel therapeutic strategies have been designed for modification of IGF signaling, identification of the impact of non-coding RNAs in this pathway is necessary for the prediction of response to these modalities.

Keywords: IGF, miRNA, lncRNA, expression, disorders

Abbreviations: IGFs, insulin-like growth factors; MAPK, mitogen-activated protein kinase; IGFBP, insulin-like growth factor binding proteins; lncRNAs, long non-coding RNAs; miRNAs, microRNAs; IGFBP-3, IGF binding protein-3; EMT, epithelial-mesenchymal transition; NNTs, nearby normal tissues; GBM, Glioblastoma; OC, ovarian cancer; OS, osteosarcoma; HCC, hepatocellular carcinoma; BCa, breast cancer; NPC, nasopharyngeal carcinoma; GC, gastric cancer; NSCLC, non-small cell lung carcinoma; LGG, low-grade gliomas; WT, wilms tumor; RB, retinoblastoma; OSCC, oral squamous cell carcinoma; CRC, colorectal cancer; RCC, renal cell carcinoma; ULM, uterine leiomyoma; PTC, papillary thyroid carcinoma; EC, endometrial carcinoma; M, melanoma; SCCHN, squamous cell carcinoma of head & neck; HUVECs, human umbilical vascular endothelial cells; CHD, coronary heart disease; DR, diabetic retinopathy; PE, preeclampsia; PCOS, polycystic ovary syndrome; PNI, peripheral nerve injury; RA, rheumatoid arthritis; IPF, idiopathic pulmonary fibrosis; NAFLD, non-alcoholic fatty liver disease; SCI, spinal cord injury; AMI, acute myocardial infarction; LDD, lumbar disc degeneration; DMR, differentially methylated region; PaC, pancreatic cancer; TSCC, tongue squamous cell carcinoma; VSMC, vascular smooth muscle cell; TNBC, triple-negative breast cancer; PaC, prostate adenocarcinoma; ACC, adrenocortical carcinoma; GIST, gastrointestinal stromal tumor; HDACi, histone deacetylase inhibitors; NICTH, non-islet cell tumor hypoglycemia; HB, hepatoblastoma; LA, lung adenocarcinoma; MM, multiple myeloma; EOC, epithelial ovarian cancer; DLBCL, diffuse large b-cell lymphoma; EWS, ewing sarcoma; liver CSCs, liver cancer stem cells.

BACKGROUND

The insulin-like growth factors (IGFs) are involved in growth and developmental processes and are evolutionarily conserved among several species (Rosenzweig, 2020). The functions of IGFs are mediated through two receptor tyrosine kinases and receptors for IGF1 and insulin. Besides, several IGF binding proteins selectively inhibit IGF1 or IGF2. IGF1 receptors have been shown to be up-regulated in tumors, thus participating in the tumorigenesis, resistance to therapies, and facilitation of metastasis in various cancer kinds (Rosenzweig, 2020). IGF1 receptors are known inducers of the Akt and mitogen-activated protein kinase (MAPK) (Pollak, 2008). Besides, IGF signaling is involved in the pathogenesis of insulin resistance and other disorders (Rosenzweig, 2020). The contribution of IGF in the pathogenesis of a wide assortment of human disorders including neoplasia and other disorders is explained by its influence on energy metabolism and cell growth (Pollak, 2008). IGF1 acts downstream of the growth hormone and through activation of MAPK and PI3K pathways and anabolism, it promotes growth and maturation of almost all tissues. Therefore, it is also involved in the aging process (Wrigley et al., 2017). **Figure 1** depicts an overview of Insulin-like growth factor (IGF) signal transduction and two downstream signaling pathways: PI3K/AKT and MAPK/ERK. The IGF signaling network is composed of three receptor tyrosine kinases (IGF1R, IGF2R, and INSR), three ligands (insulin, IGF1, and IGF2), and six serum insulin-like growth factor binding proteins (IGFBP). **Figure 1** shows the IGF signal transduction and its downstream effectors.

Recent investigations have verified the influence of regulatory non-coding RNAs on IGF signaling (Chen B. et al., 2019). Most investigations in this regard have focused on long non-coding RNAs (lncRNAs) and microRNAs (miRNAs) (Chen B. et al., 2019). LncRNAs are transcripts with sizes of more than 200 nucleotides which are principally produced by RNA polymerase II. These transcripts have various functions in the modulation of genomic structure, chromatin configuration, mRNA stability, alternative splicing, and enhancement or inhibition of transcription. The other types of regulatory non-coding RNAs, i.e., miRNAs mainly influence gene expression at the post-transcriptional phase via binding with the 3′ UTR of their specific targets. Both classes of non-coding RNAs participate in the pathogenesis of human diseases. We designed the current study to search the available data about the role of IGF-associated non-coding RNAs in the evolution of neoplasia and other conditions.

IGF-ASSOCIATED miRNAs IN HUMAN DISORDERS

Several IGF-associated miRNAs have been dysregulated in neoplastic conditions. For instance, experiments in ovarian cancer cells have shown that miR−19a−3p suppresses the levels of IGF binding protein−3 (IGFBP−3), thus promoting the growth and migration of these cells. Notably, the expression of this miRNA can be modulated by NF−κB (Bai et al., 2019). Shastri et al. have demonstrated the inhibitory effects of the miR-29 family on IGF-1. Members of the miR-30 family can inhibit both IGF-1 and IGF-1R. Notably, calorie restriction has resulted in the over-expression of miR-29 and miR-30 in the normal liver and the liver being metastasized by breast cancer cells, indicating a possible role for dietary modifications in the management of liver metastases (Shastri et al., 2020). In nasopharyngeal squamous cell carcinoma cells, miR-30a inhibitor could reverse IGF-I-associated epithelial-mesenchymal transition (EMT). The IGF-1R/Src/miR-30a/E-cadherin axis has been identified as an important pathway in the regulation of EMT in these cells (Wang et al., 2016). miR-99a is another miRNA that can inhibit proliferation, migration, and invasion of breast carcinoma via suppression of IGF-1R (Xia et al., 2016). Being up-regulated in hepatocellular carcinoma cells, miR-155 can increase expression of IGF-II and IGF-1R, while decreasing IGFBP-3 expression. Through these pathways, miR-155 can increase proliferation, migration, and clonogenicity of hepatocellular carcinoma cells (El Tayebi et al., 2015). In the same type of cancer, miR-342-3p can inhibit cell proliferation through the suppression of the IGF-1R-associated Warburg effect (Liu et al., 2018d). In colorectal cancer cells, the oncogenic protein IGF2BP2 has a functional interaction with miR-195 through which it regulates RAF1 expression and participates in the carcinogenic process (Ye S. et al., 2016). Meanwhile, miR-197 can inhibit the expression of IGFBP3 through binding with its 3′-UTR, hence enhancing cell migratory potential and invasion of colorectal cancer cells (Zhou et al., 2018). **Supplementary Table 1** reviews the results of studies that displayed the role of IGF-associated miRNAs in the neoplastic conditions.

The interaction between IGF-related proteins and miRNAs has been also assessed in non-neoplastic conditions. For instance, IGF-1 is targeted by miR-17. This miRNA has been over-expressed in ox-LDL treated human umbilical vascular endothelial cells (HUVECs) in association with down-regulation of IGF-1. Up-regulation of miR-17 has enhanced cell viability and suppressed the apoptosis of ox-LDL exposed cells. Such effects have been accompanied by down-regulation of Bax and Caspase3 expressions, while up-regulation of Bcl-2, suggesting a role for miR-17 as a biomarker for coronary heart disease (Chen Z. et al., 2019). Expression of miR-30a-3p has been elevated in the placenta samples of women with preeclampsia. This miRNA has been shown to regulate the expression of IGF-1, therefore influencing the invasive capacity and apoptosis of trophoblasts (Niu et al., 2018). Over-expression of miR-129 has suppressed proliferation and migration of Schwan cells and axonal outgrowth of dorsal root ganglion neurons through modulation of several targets including IGF-1 (Zhu H. et al., 2018). Yang et al. have reported up-regulation of miR-143-3p in synovial tissues of patients with rheumatoid arthritis compared with those affected with osteoarthritis. Down-regulation of miR-143-3p has inhibited cell proliferation, enhanced apoptosis, and reduced production of inflammatory cytokines. miR-143-3p has been shown to target IGF1R and IGFBP5 and regulate the Ras/p38 MAPK axis (Liu et al., 2018c). In colonic smooth muscle cells, miR-155 has been shown to down-regulate IGF-1

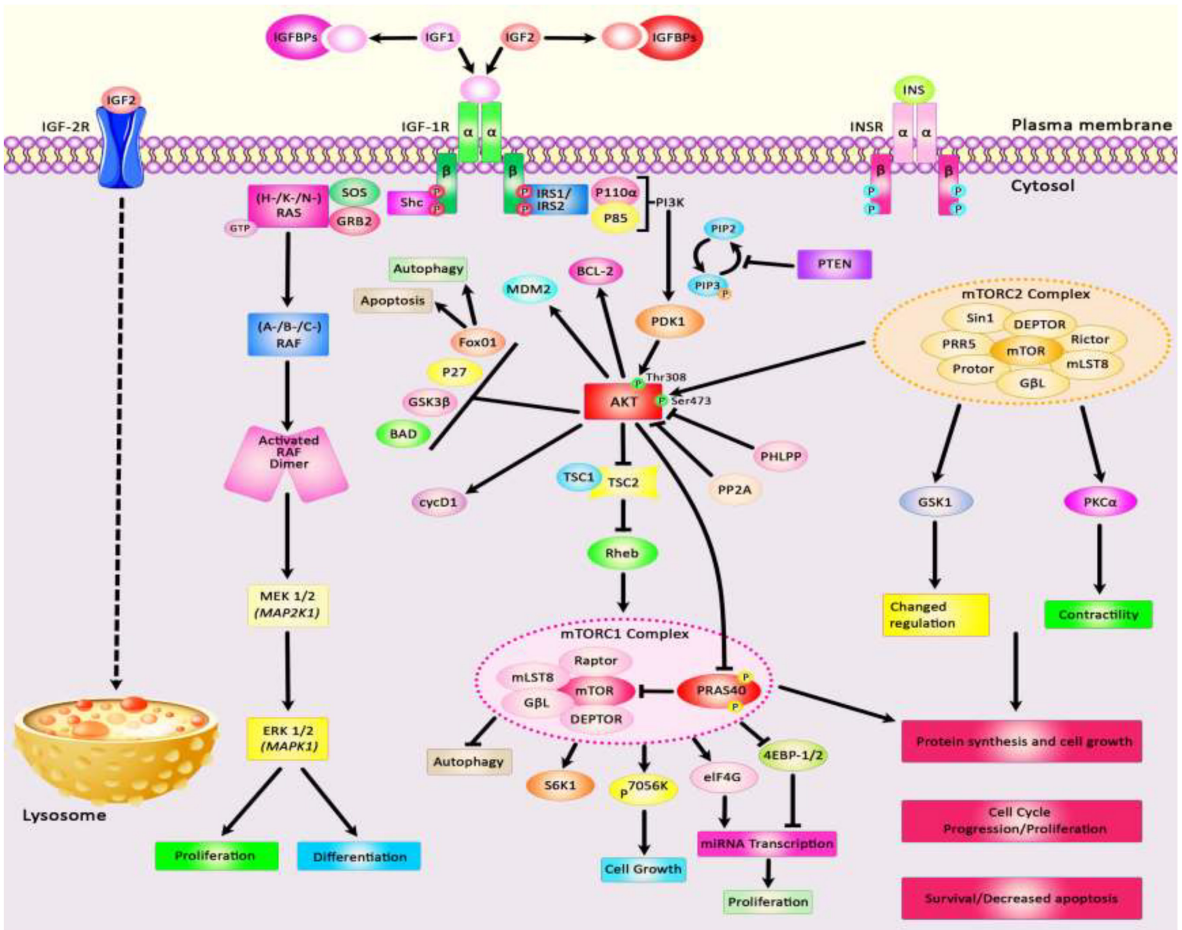

FIGURE 1 | A schematic diagram of the insulin-like growth factor (IGF) signal transduction and main downstream effects. Both IGF-1 and IGF-2 could bind with plasma membrane IGF-1R and IGF-1/Insulin hybrid receptor leading to autophosphorylation of this target receptor in the intracellular β-subunits and thus triggering the catalytic function of the IGF-1R, while insulin binds only to the Insulin-R. IGFBPs could regulate the bioavailability of both IGF-1 and IGF-2 signaling cascades. The bioavailability of IGF-2 could also be regulated via binding to the IGF-2R which results in receptor-triggered internalization and endosomal degradation of IGF-2 in the lysosomes. Phosphorylated β-subunits could in turn create docking sites for the adaptor proteins IRS-1/2, and Shc that modulate the activation of two signaling pathways: PI3K/AKT and MAPK/ERK. Activation of the PI3K and AKT pathway leads to modulation of a variety of cell signaling cascades, such as regulation of TSC1/2 to suppress mTORC1 complex and modulation of 4EB-P1 and S6K1/2 phosphorylation, promoting cell survival via activation or suppression of major effectors like the Foxo transcription factors, BCL-2, BAD, and P27, upregulation of transformation of glucose to glycogen through suppression of GSK-3β, increasing protein synthesis, as well as suppressing apoptosis and autophagy. Activation of AKT family of kinases via PDK1 and mTORC2 leads to the phosphorylation at Thr308 and Ser473, respectively. Besides, docking of Grb2 to the phosphorylated IGF-1R β subunits could trigger the Ras/Raf/MEK/ERK axis. Shc binding to activated IGF-1R leads to stimulation of the MAPK/ERK cascade which regulates a kinase signaling pathway and eventually results in promoting cellular proliferation via enhancing transcription factors activities including ELK1.

levels. Up-regulation of miR-155 has increased apoptosis of these cells and reduced the thickness of the related tissue in the diabetic mice, suggesting the role of this miRNA in the aggravation of colonic dysmotility (Shen et al., 2020). **Figure 2** illustrates the IGF signaling cascade modulating by dysregulated miRNAs in various human diseases as well as cancers. **Table 1** reviews the role of IGF-associated miRNAs in non-neoplastic conditions.

Overexpression of IGF-associated miRNAs namely miR-30a-3p, miR-155, miR-199a-3p, and miR-486-5p has important roles in different conditions such as preeclampsia, hepatocellular carcinoma, estrogen-mediated autophagy, and congenital heart disease (El Tayebi et al., 2015; Fu et al., 2018; Niu et al., 2018; Fan et al., 2019). Besides, dysregulation of miR-210-3p, miR-491-5p, and miR-615-3p contributes to the pathogenesis of atherosclerosis, colorectal carcinoma, and non-small lung cancer through modulation of IGF2 expression level (Liu j. et al., 2019; Lu et al., 2019; Qiao et al., 2020). Besides, aberrant expressions of miR-204-5p, miR-197, and miR-155-5p participate in the pathogenesis of papillary thyroid carcinoma, colorectal cancer, and non-small lung cancer through affecting expressions of IGFBP5, IGFBP3, and IGFBP1, respectively (Ling et al., 2015; Liu L. et al., 2015; Zheng et al., 2018). miR-99a, miR-503, and miR-1275 contribute to the pathogenesis of polycystic ovary syndrome, coronary heart disease, and hepatocellular carcinoma by affecting IGF-1R levels (Fawzy et al., 2015;

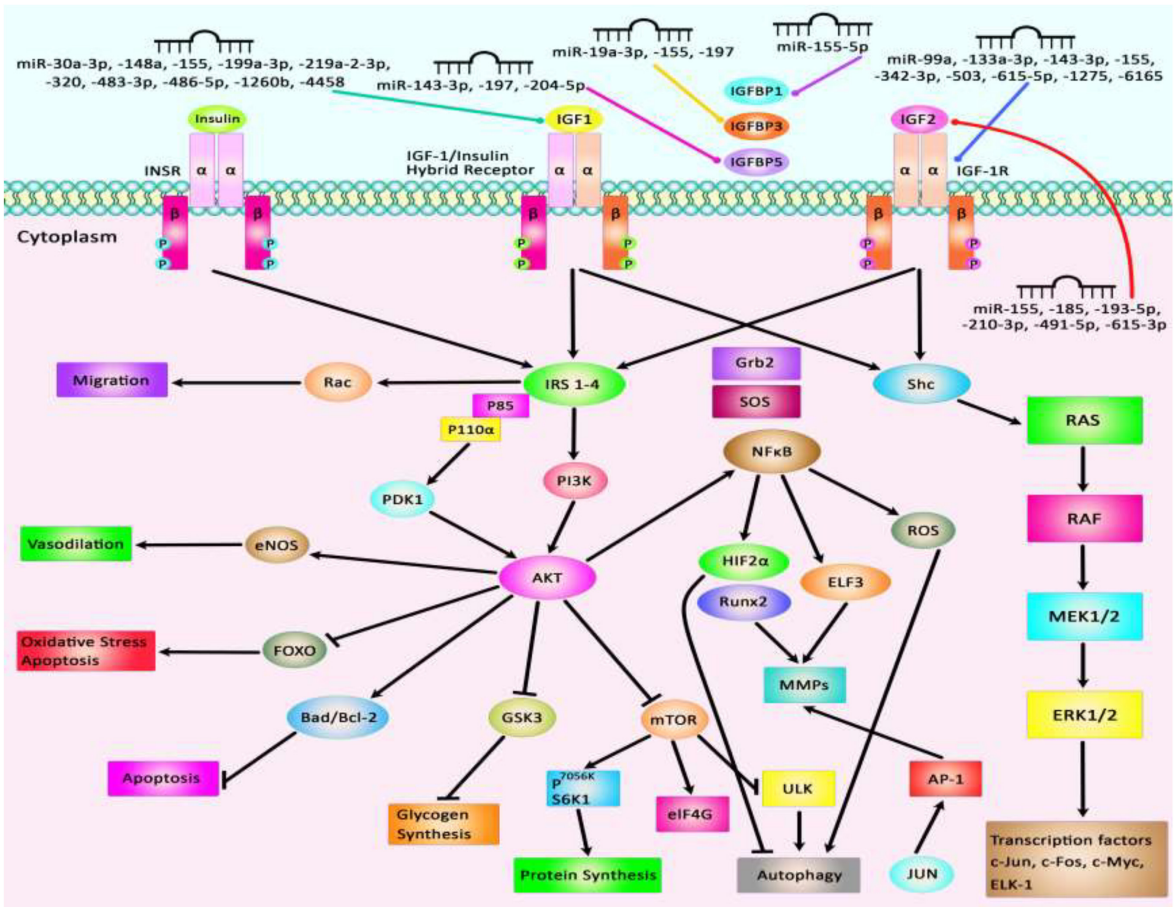

FIGURE 2 | Several proteins in the IGF signaling pathway such as IGF1, IGF2, IGF-1R, and IGFBPs are regulated by miRNAs. Through modulation of these proteins, miRNAs can affect several cellular processes such as apoptosis, autophagy, protein synthesis, response to oxidative stress, and cell migration.

Zhu W. et al., 2018; Geng et al., 2019). **Figure 2** summarizes the role of a number of IGF-associated miRNAs in human disorders including cancers.

IGF-ASSOCIATED lncRNAs IN HUMAN DISORDERS

Several lncRNAs have functional links with IGF-related proteins. Wang et al. have demonstrated over-expression of circ_0014130 in Non-Small Cell Lung Carcinoma tissues and cells. Down-regulation of this cirCRNA has suppressed cell proliferation and enhanced cell apoptosis in these cells. Circ_0014130 has functional interactions with miR-142-5p and IGF-1. Small interfering RNA-mediated circ_0014130 silencing has enhanced IGF-1 levels through up-regulation of miR-142-5p (Wang M. et al., 2020). Another study in this kind of cancer has shown up-regulation of HOXA-AS2 in the tumor samples. HOXA-AS2 silencing has decreased the expression of IGF2. Therefore, HOXA-AS2 promotes the migratory and invasive capacities of lung cancer cells by enhancing IGF2 expression (Zheng et al., 2019). In cervical cancer cells, the expression of linc00319 has been increased. Linc00319 silencing has suppressed cell proliferation, invasion, and migration of cervical cancer cells. This lncRNA interacts with miR-147a to modulate the expression of IGF1R (Ma et al., 2020). DBH-AS1 is another oncogenic lncRNA in hepatocellular carcinoma. Up-regulation of this lncRNA has been associated with the down-regulation of miR-138. DBH-AS1 knockdown and miR-138 up-regulation have decreased cell viability, repressed colony formation, and increased cell apoptosis. DBH-AS1 enhanced tumor growth and activated FAK/Src/ERK axis by modulating the expression of miR-138 (Bao et al., 2018). H19 is another up-regulated lncRNA in melanoma. H19 silencing has increased the sensitivity of melanoma cells to cisplatin, suppressed colony formation, and enhanced apoptosis of cisplatin-resistant melanoma cells. This lncRNA regulates IGF-1 expression through modulation of miR-18b expression (An et al., 2020). Honda et al. have assessed the methylation pattern of the H19 differentially methylated region (DMR), loss of heterozygosity, and allelic expression of IGF2 in hepatoblastoma. They reported associations between biallelic IGF2 expression and hypermethylation of H19 DMR. On the other hand, the monoallelic expression of IGF2 has been correlated with normal methylation of this region. They

TABLE 1 | IGF-associated miRNAs in non-malignant disorders.

Type of disease	microRNA	*P*-value	Animal	Clinical samples (human)	Cell lines	Target	Pathway	Function	References
Coronary Heart Disease (CHD)	miR-17	<0.01	–	–	HUVECs	IGF-1, Caspase-3, Bax, Bcl-2	–	Overexpression of miR-17 via targeting IGF-1 could promote the proliferation and inhibit apoptosis of HUVECs.	Chen Z. et al., 2019
Diabetic Retinopathy (DR)	miR-18b	<0.01	–	–	HRECs	IGF-1	AKT, MEK, ERK	Downregulation of miR-18b by targeting IGF-1 could increase the proliferation of HRECs exposed to VEGF secretion and normal glucose.	Wu et al., 2016
Polycystic Ovary	miR-19b	<0.01	–	PCOS (*n* = 18), normally menstruating women (*n* = 10)	KGN	IGF-1, CDK1, Cyclin-D1	–	Downregulation of miR-19b by targeting IGF-1 could enhance ovarian GCs proliferation in PCOS.	Zhong et al., 2018
Preeclampsia (PE)	miR-30a-3p	<0.05	–	PE (*n* = 25), normal pregnant women (*n* = 20)	HTR-8/SVneo, JEG-3	IGF-1	–	Overexpression of miR-30a-3p via targeting IGF-1 could induce the apoptosis of trophoblast HTR-8/SVneo cells.	Niu et al., 2018
Polycystic Ovary Syndrome (PCOS)	miR-99a	<0.05	–	15 pairs of married women with PCOS and a control group of women without PCOS	COV434	IGF-1R	–	Overexpression of miR-99a by targeting IGF-1R could reduce the proliferation and promote apoptosis of human granulosa cells (GCs).	Geng et al., 2019
Peripheral Nerve Injury (PNI)	miR-129	<0.01	Male SD rats	–	SCs, 293T	IGF-1	–	Overexpression of miR-129 by targeting IGF-1 could suppress the proliferation and migration of SCs, and axonal outgrowth of DRG neurons in PNI.	Zhu H. et al., 2018
Rheumatoid Arthritis (RA)	miR-129-5p	<0.05	–	RA (*n* = 15), healthy controls (*n* = 12)	FLSs	IGF-1R, Caspase-3/8	Src/ERK/Egr-1	Overexpression of miR-129-5p by targeting IGF-1R and activating Src/ERK/Egr-1 signaling could inhibit cell proliferation and induce apoptosis of RA cells.	Zhang Y. et al., 2019
Idiopathic Pulmonary Fibrosis (IPF)	miR-130b-3p	<0.05	–	4 IPF patients and 3 normal lung tissues	A549, ATII, MRC5	IGF-1	–	Downregulation of miR-130b-3p by enhancing IGF-1 production from the epithelium of the lung could activate fibroblasts to increase the proliferation, migration ability, and expression of collagen I of fibroblasts in co-culture systems.	Li S. et al., 2016
Diabetic Retinopathy (DR)	miR-142-5p	<0.001	Male SD rats	–	HRECs, 293T	IGF-1, VEGF	PI3K, ERK, AKT, VEGF	Inhibition of miR-142-5p via blocking the IGF-1/p-IGF-1R pathway could promote HREC proliferation in response to DR conditions.	Qiao et al., 2020
Rheumatoid Arthritis (RA)	miR-143-3p	<0.01	–	5 pairs of RA and normal control	MH7A	IGF-1R, IGFBP-5, TNF-α, Bax, Bcl-2, Caspase-3	Ras/p38 MAPK	Downregulation of miR-142-3p could reduce cell proliferation and promotes cell apoptosis by targeting IGF-1R and IGFBP-5.	Liu et al., 2018c
Fracture Healing	miR-148a	<0.0 1	Male Wistar rats	–	293T, rBMSCs	IGF-1, Runx2, OCN, OPN	–	Downregulation of miR−148a by targeting IGF-1 could promote the expression of osteogenesis−related proteins and regulate bone BMSCs−mediated fracture healing.	Liu et al., 2018a
Diabetes Mellitus	miR-155	<0.05	–	–	SMCs	IGF-1	–	Overexpression of miR-155 by regulating the IGF-1 could decrease the thickness of colonic smooth muscle tissues in diabetic mice and also could increase apoptosis of colonic SMCs.	Shen et al., 2020
Non-alcoholic Fatty Liver Disease (NAFLD)	miR-190b	<0.05	Male C57Bl/6 mice	15 pairs of NAFLD and NNTs	L02	IGF-1, ADAMTS9	IRS2/AKT	Downregulation of miR-190b by directly targeting IGF-1 and ADAMTS9 could regulate lipid metabolism and insulin signaling pathway *in vitro* and could reduce the hepatic steatosis and insulin resistance *in vivo*.	Xu et al., 2018
Diabetic Cardiomyopathy	miR-193-5p	<0.05	Wistar rat	–	MMEC	IGF-2	–	Downregulation of miR-193a-5p by inhibiting IGF-2 could reduce cell migration and proliferation in type 2 diabetic cardiomyopathy.	Yi et al., 2017

(Continued)

TABLE 1 | Continued

Type of disease	microRNA	*P*-value	Animal	Clinical samples (human)	Cell lines	Target	Pathway	Function	References
Estrogen-Mediated Autophagy	miR-199a-3p	<0.05	–	–	MLO-Y4, MC3T3-E1	IGF-1	mTOR	Overexpression of miR-199a-3p by targeting IGF-1 and inhibiting the mTOR signaling pathway could induce autophagy in osteocyte-like MLO-Y4 cells.	Fu et al., 2018
Atherosclerosis	miR-210-3p	<0.01	Male C57BL/6J mice	–	THP-1	IGF-2, TNF-α, MCP-1, IL-6, iNOS	NF-kB	Overexpression of miR-210-3p by inhibiting the IGF-2/IGF-2R axis could inhibit the expression of CD36 and NF-κB, then led to a reduction in the inflammatory response of macrophages and lipid accumulation in atherosclerosis.	Qiao et al., 2020
Spinal Cord Injury (SCI)	miR-219a-2-3p	<0.01	Female SD rats	–	NSCs, PC12	IFG-1, BAX, Bcl-2, Beclin-1, Caspase-3	NF-kB	Exposure of exosomes derived from NSCs to IGF-1 via the miR-219a-2-3p-dependent pathway could suppress the nerve inflammation, inhibit apoptosis, and promote nerve regeneration after the SCI.	Moharamoghli et al., 2019
Acute Myocardial Infarction (AMI)	miR-320	<0.05	Female Wistar rats	–	–	IGF-1, ASK1, Bcl-2, Bax, Caspase-3	p38, JNK	Downregulation of miR-320 via increasing IGF-1 could suppress cardiomyocyte apoptosis.	Song et al., 2016
Cerebral I/R Injury	miR-320	<0.01	Male C57BL mice	–	PC12	IGF-1	–	overexpression of miR-320 by targeting IGF-1 could enhance brain infarction volume and edema volume in MCAO/R mice.	Liang et al., 2018
Polycystic Ovary Syndrome (PCOS)	miR-323-3p	<0.05	–	20 pairs of PCOS lesion and normal ovarian cortex tissue samples	KGN, CCs	IGF-1, AR, AMHR-II, CYP19A, EGFR, GATA-4	–	Downregulation of miR-323-3p by targeting IGF-1 could enhance apoptosis and increase the steroidogenesis in KGN cells.	Wang et al., 2019b
PCOS	miR-483	<0.001	–	20 pairs of PCOS lesion and normal ovarian cortex tissue samples	KGN	IGF-1, CCNB1, CCND1, CDK2	–	Overexpression of miR-483 possibly by targeting IGF-1 could inhibit KGN cell viability and proliferation and induces cell cycle arrest.	Xiang et al., 2016
Rheumatoid Arthritis (RA)	miR-483-3p	<0.001	–	Synovial tissues from RA patients (n = 10), healthy controls (n = 6)	HFLS, HFLS-RA	IGF-1	–	Overexpression of miR-483-3p via targeting IGF-1 could promote cell proliferation, the G0/G1-to-S phase transition, and suppress apoptosis in RA FLSs.	Wang Y. et al., 2020
Acute Myocardial Infarction (AMI)	miR-483-3p	<0.05	–	6 pairs of AMI patients and normal volunteers	H9c2	IGF-1, Bax, Bcl-2, Caspase-3, Caspase-9	–	Overexpression of miR-483-3p by targeting IGF-1 could promote apoptosis in the AMI model.	Sun et al., 2018
Congenital Heart Disease	miR-486-5p	<0.01	–	–	H9C2	IGF-1, Bcl-2, Bax, Caspase-3, Caspase-9,	–	Downregulation of miR-486-5p by targeting IGF-1 could increase cardiomyocyte growth in hypoxic conditions.	Fan et al., 2019
Coronary Heart Disease (CHD)	miR-503	<0.05	–	–	H9c2	IGF-1R, Cyto-C, c-PARP, Caspase3	PI3K/AKT	Overexpression of miR-503 by inhibiting the PI3K/AKT pathway via targeting IGF-1R could accelerate hypoxia-induced injury.	Zhu W. et al., 2018
Lumbar Disc Degeneration (LDD)	miR-4458	<0.05	–	24 LDD samples and 22 normal controls	SV40	IGF-1	PI3K/AKT	Overexpression of miR-4458 by decreasing both total IGF-1R and phosphorylated IGF-1R could lead to a decrease of phosphorylated AKT. Also, miR-4458 by suppressing the PI3K/AKT pathway via inhibiting IGF-1R could promote the development of LDD.	Liu Z. Q. et al., 2016

also reported over-expression of IGF2 and predominance of the embryonic P3 transcript in most hepatoblastoma with retention of imprinting (Honda et al., 2008). **Table 2** summarizes the role of IGF-associated lncRNAs in cancers.

Via regulation of the IGF−1 signaling pathway, H19 can modulate proliferation and apoptosis of male germline stem cells. H19 silencing has reduced the cell quantities in the seminiferous tubule (Lei et al., 2019). Expression of the lncRNA 150Rik has been enhanced in renal tissue of animal models of diabetic nephropathy and in mesangial cells cultured in hyperglycemic media. This lncRNA regulates mesangial cell proliferation through interacting with miR-451, thus regulating the IGF1R/p38MAPK axis (Zhang et al., 2018). LncIRS1 has been shown to act as a molecular sponge for miR−15a, miR−15b−5p, and miR−15c−5p to modulate the expression of IRS1 a downstream target of the IGF1-R. Up-regulation of lncIRS1 has enhanced IRS1 expression and increased phosphorylation of AKT as an important element in the IGF-1 pathway. LncIRS1 can also regulate the expression of atrophy−associated genes and affect muscle atrophy (Li Z. et al., 2019). TUG1 is an up-regulated lncRNA in ox-LDL-exposed vascular smooth muscle cell (VSMC) and HUVEC. Its silencing has suppressed proliferation and enhanced apoptosis in ox-LDL-exposed VSMC but has exerted opposite effects in HUVEC. miR-148b has been identified as a target of TUG1 in these cells. In turn, miR-148b has been shown to target IGF2. Therefore, TUG1 enhances IGF2 levels by sequestering miR-148b (Wu X. et al., 2020). HCP5 is a lncRNA that is involved in the pathogenesis of polycystic ovarian syndrome (PCOS). Down-regulation of this lncRNA inhibits cell proliferation via inducing cell cycle arrest at the G1 phase and stimulating the mitochondrial apoptotic route. miR-27a-3p has been recognized as a direct target of HCP5. This miRNA can bind with IGF-1. Therefore, HCP5 can be involved in the development of PCOS via modulating the miR-27a-3p/IGF-1 axis (Luo L.-H. et al., 2020). **Figure 3** represents the dysregulation of various types of lncRNAs which have a remarkable role in negatively modulating IGF1, IGF2, IGFBP2, and IGF-1R through the IGF signaling pathway in different human cancers. **Table 3** summarizes the information about the role of IGF-associated lncRNAs in non-neoplastic conditions.

LncRNAs that regulate the expression of IGF1, IGF2, IGF-1R, and IGFBPs can participate in the pathogenesis of human disorders. H19, NR2F1-AS1, and SNHG7 participate in the development of melanoma and breast cancer through modulation of IGF1 (Boone et al., 2019; An et al., 2020; Zhang et al., 2020). NEAT1, THOR, and HOTTIP via targeting IGF2 affect carcinogenic processes in colorectal cancer, tongue squamous cell carcinoma, and renal cell carcinoma (Wang Q. et al., 2018; Yang et al., 2019; Zhuang et al., 2020). Additionally, downregulation of circVANGL1 through suppressing the expression level of IGFBP2 could attenuate breast cancer cell invasion, migration, and proliferation (Yang D. et al., 2020). Also, IRAIN, Linc00319, and DLEU1 through negatively regulating IGF-1R could cause breast cancer, cervical cancer, and hepatocellular carcinoma (Pian et al., 2018; Zhang W. et al., 2019; Ma et al., 2020). **Figure 3** summarizes the role of these IGF-associated lncRNAs in human disorders.

DIAGNOSTIC/PROGNOSTIC VALUES OF IGF-ASSOCIATED miRNAs/lncRNAs IN CANCERS

A number of miRNAs and lncRNAs which are functionally linked with IGF signaling have potential applications as diagnostic/prognostic markers in cancers. Zhuang et al. have demonstrated high accuracy of NEAT1 levels in distinguishing colon cancer tissues from normal ones (area under the receiver operating curve = 0.89) (Zhuang et al., 2020). Expression levels of the IGF-associated miRNAs miR-485-5p and miR-155-5p have been associated with the survival of patients with lung cancer and Wilms tumor, respectively (Huang et al., 2018; Luo X. et al., 2020). Also, Linc00319, H19, AFAP1-AS1, SNHG7, HOTTIP, linc01023, DLEU1, and NEAT1 have been identified as prognostic markers in diverse kinds of cancer (**Table 4**).

IMPORTANCE OF IGF-ASSOCIATED PATHWAYS IN RESPONSE TO CHEMOTHERAPY

IGF-associated molecules have been involved in the resistance of cancer cells to chemotherapeutic agents. In some cases, miRNAs or lncRNAs have been identified as molecules that mediate this phenotype. For instance, H19 silencing has enhanced the sensitivity of cancer cells to cisplatin and increased apoptosis of cisplatin-resistant melanoma cells through modulation of IGF1 expression (An et al., 2020). In a number of ovarian cancer cell lines, IGF-2 expression has been higher in Taxol-resistant cells compared with chemosensitive cell lines. Transient IGF2 silencing has enhanced Taxol sensitivity in these cells. However, IGF1R blocking did not affect the chemosensitivity of these cells. These results have supported the role of IGF-2 as a possible therapeutic target in drug-resistant ovarian cancer (Brouwer-Visser et al., 2014). IGF-1 has been shown to confer resistance to docetaxel in prostate cancer cells. IGF-I treatment has reduced expression of miR-143 expression, while enhanced expression IGF-1R and IRS1, direct targets of this miRNA. Up-regulation of miR-143 has stopped IGF-I-associated resistance to docetaxel, reduced expressions of IGF-I, IRS1, and VEGF in these cells (Niu et al., 2017). **Table 5** reviews the importance of IGF-related pathways in response to chemotherapy.

IGF SIGNALING PATHWAY IN TUMORIGENESIS AND PROGRESSION OF CHEMOTHERAPEUTIC DRUG RESISTANCE PROVIDING THE NEW CONCEPTS IN CANCER THERAPY

One of the major impediments to current cancer remedy endeavors is the induction of drug resistance by tumors. Despite recent improvements in diagnostic methods and surgical interventions, many aggressive tumors have a poor response to adjuvant or neoadjuvant chemotherapy and radiation. The

TABLE 2 | IGF-associated lncRNAs in cancers (NNTs: nearby normal tissues).

Type of Cancer	lncRNA	*P*-value	Animal	Clinical Samples (Human)	Cell Lines	Target	Pathway	Function	References
NSCLC	circ_0014130	<0.01	–	20 pairs of NSCLC and NNTs	H1299, A549, BEAS-2B	miR-142-5p, IGF-1	–	Downregulation of circ_0014130 via upregulating miR-142-5p and downregulating IGF-1 expression could inhibit NSCLC cell proliferation and promotion of cell apoptosis.	Wang M. et al., 2020
NSCLC	HOXA-AS2	<0.01	–	63 pairs of NSCLC and NNTs	SPCA1, A549, PC-9, H1975, 16HBE	IGF-2	–	HOXA-AS2 via upregulating IGF-2 could promote cell migration and invasion in NSCLC.	Zheng et al., 2019
Cervical Cancer (CC)	LINC00319	<0.01	–	60 pairs of CC and NNTs	Ect1/E6E7, HeLa, SiHa, Caski, C33A, Me180	IGF-1R, miR-147a, Vimentin, MMP2, p21, E-cadherin	–	Downregulation of Linc00319 via targeting the miR-147a and IGF-1R could inhibit CC cell proliferation, invasion, and migration.	Ma et al., 2020
Melanoma	DBH-AS1	<0.001	–	62 pairs of M and NNTs	A375, HaCaT, A875	IGF-1R, miR-223-3p, EGFR, GLUT1	AKT	Overexpression of DBH-AS1 via miR-223-3p/EGFR/AKT axis could enhance the glycolytic activity and reduce cancer progression.	Bao et al., 2018
Melanoma	H19	<0.01	–	30 pairs of M and NNTs	HEMa, A375, WM35, M8, SK-MEL-2, A2508	miR-18b, IGF1	–	H19 via acting as a molecular sponge of miR-18b can regulate IGF-1 expression and sensitivity of melanoma cells to DDP.	An et al., 2020
Hepatoblastoma	H19	<0.05	–	HB (n = 54), normal controls (n = 5)	HuH6, HepG2	IGF-2, PLAG1, CTNNB1	–	There was an association between biallelic IGF2 expression and hypermethylation of H19 DMR.	Honda et al., 2008
Follicular Thyroid Cancer (FTC)	H19	<0.05	Male nude mice	45 pairs of FTC and NNTs	FTC-133, FTC-238, Nthy-ori 3-1	IGF2BP1, IGF-1, SOCS3, Pax5, miR-29-3p	JAK/STAT	H19 via the IGF1/JAK/STAT axis could suppress metastasis of FTC.	Xu et al., 2019
Breast Cancer (BCa)	IRAIN	<0.05	–	–	MDA-MB-31	IGF-1R	–	IRAIN via targeting IGF1R could alter the phenotypes of MDA-MB-231 tumor cells.	Pian et al., 2018
Bladder Cancer	circVANGL1	<0.001	BALB/c nude mice	60 pairs of BLC and NNTs	SV−HUC, T24, EJ, J82, RT−4, UM−UC−3, TCC	miR−1184, IGFBP2	–	Downregulation of circVANGL1 via inhibiting IGFBP−2 could inhibit cell invasion, migration, and growth.	Yang D. et al., 2020
Breast cancer (BCa)	NR2F1-AS1	<0.0001	Female NOD/SCID mice	–	MDA-MB-231, MCF-7, HUVECs	IGF-1	ERK	Overexpression of NR2F1-AS1 by increasing miR-338-3p and activating IGF-1and ERK pathway could enhance the HUVEC proliferation, tube formation, and migration ability in BCa cells.	Zhang et al., 2020
BCa	SNHG7	<0.05	–	TCGA database	MCF7, T47D, MDA-MB-231, MCF10A	IGF-1	MAPK	The silencing of SNHG7 could lead to cell cycle arrest in G0/G1. A negative feedback loop between SNHG7 and IGF-1 could regulate transcript levels and proliferation in BCa cells.	Boone et al., 2019
Colorectal Cancer (CRC)	circRUNX1	<0.001	Male BALB/c nude mice	52 pairs of CRC and NNTs	SW480, SW620, HCT116, HT29, LoVo, RKO	miR-145-5p, IGF-1	–	Overexpression of circRUNX1 via miR-145-5p/IGF-1 Signaling could enhance cell proliferation and migration and also inhibit apoptosis and metastasis in CRC cells.	Chen et al., 2020
CRC	LINRIS	<0.01	female BALB/c nude mice	60 pairs of CRC and NNTs	LOVO, CCD841, RKO, CW2, SW1116, SW480, SW620, DLD-1, HCT116, HT29, COLO205	IGF2BP2,	–	LINRIS by stabilizing IGF2BP2 could promote the aerobic glycolysis in CRC cells.	Wang et al., 2019e

(Continued)

TABLE 2 | Continued

Type of Cancer	lncRNA	*P*-value	Animal	Clinical Samples (Human)	Cell Lines	Target	Pathway	Function	References
Colon Cancer	NEAT1	<0.001	–	10 pairs of CRC and NNTs	SW620 HT-29, HCT 116, LoVo, SW480, NCM460	IGF-2, miR-185-5p	–	Overexpressed NEAT1 via the miR-185-5p/IGF-2 axis could promote invasion and migration of colon cancer cells.	Zhuang et al., 2020
Pancreatic Cancer (PaC)	AFAP1-AS1	<0.01		63 pairs of PaC and NNTs	AsPC-1, BxPC-3, PANC-1, PaCa-2, SW1990, HPDE6c7	IGF-1R	–	Downregulation of AFAP1-AS1 by upregulating the IGF1R oncogene via sequestration of miR-133a could suppress the tumor cell growth and invasion in PaC.	Chen B. et al., 2018
PaC	HOTAIR	<0.05	BALB/c nude mice	25 pairs of PaC and NNTs	BXPC3, 293T, CFPAC-1, Panc-1, L3.6pl	IGF-2, miR-663b, Caspase-3, Caspase-9	–	HOTAIR by inhibiting miR-663b via upregulating IFG-2 could promote PaC cell proliferation.	Cai et al., 2016
Renal Cell Carcinoma (RCC)	HOTTIP	<0.01	–	TCGA database, 57 pairs of RCC and NNTs	A-498, 786-O, Caki-1, Caki-2, ACHN, HK-2, 293T	IGF-2, hsa-miR-615-3p	–	HOTTIP by regulating the miR-615/IGF-2 axis could promote RCC progression.	Wang Q. et al., 2018
Glioma	linc01023	<0.001	nude mice	Glioma (n = 169), normal brain tissues (NBTs, n = 30)	U87, U251, NHA	IGF-1R	AKT	Knockdown of linc01023 by regulating the IGF-1R/AKT axis could restrain glioma proliferation, migration, and invasion.	Yu et al., 2019
Hepatocellular carcinoma (HCC)	DLEU1	<0.01	Male BALB/c nude mice	56 pairs of HCC and NNTs	SMMC-7721, Hep3B, HepG2, Huh-7, LO2	IGF-1R, miR-133a, E-cadherin, N-cadherin Vimentin	PI3K/AKT	DLEU1 by sponging miR−133a to regulate IGF−1R expression through the PI3K/AKT pathway could promote HCC progression.	Zhang W. et al., 2019
Tongue Squamous Cell Carcinoma (TSCC)	THOR	<0.05	–	55 TSCC and 31 NNTs	Tca-8113 and Cal-27	IGF2BP1, IGF-2, Cyclin-E1, Cyclin-D1, p21, p27,	MEK-ERK	THOR by stabilizing IGF2BP1 could increase TSCC cell proliferation.	Wang et al., 2019a

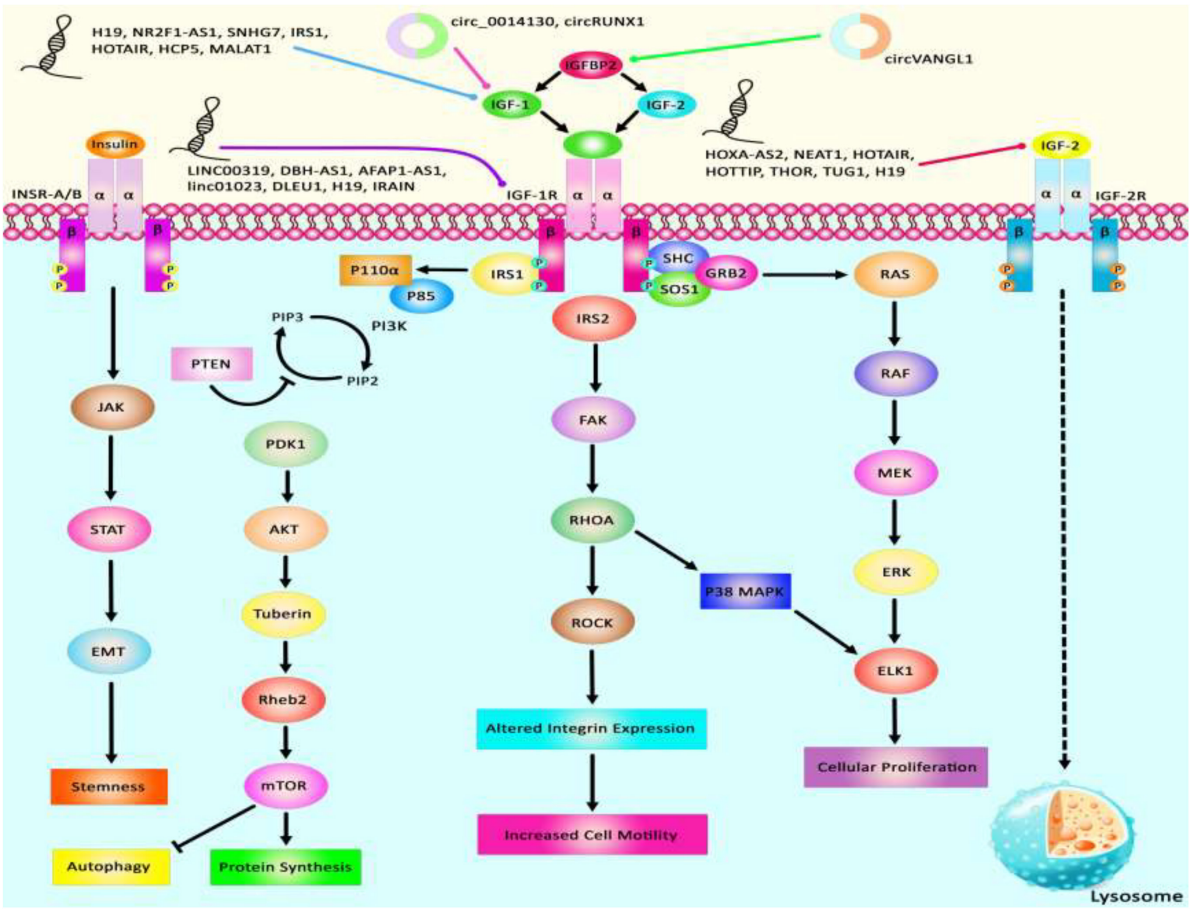

FIGURE 3 | A schematic summary of lncRNAs that target IGF signaling cascade. IGF1, IGF2, IGFBP2, and IGF-1R are among proteins that are regulated by lncRNAs. Abnormal levels of lncRNAs can affect the carcinogenesis process by influencing autophagy, cell proliferation, protein synthesis, and stemness.

IGF signaling axis has been detected to have a pivotal role in the progression and development of a variety of tumors (Denduluri et al., 2015). The IGF-1R is involved in various human cancers, such as ovarian, breast, pancreatic, glioma, hepatocellular, lymphoma, and non-small lung cancers. In some cases, its anti-apoptotic attributes strengthen cancerous cells to resist the cytotoxic characteristics of chemotherapeutic agents or radiotherapy (Beauchamp et al., 2009; Dool et al., 2011; Awasthi et al., 2012; Zhou, 2015). Zhou et al. demonstrated that the IGF-1R kinase inhibitor nVp-ADW742 combined with temozolomide could trigger inhibition of P38, GSK3β, and AKT phosphorylation along with a considerable reduction in the intracellular expression levels of Bcl-2, P38, and GSK3β, thereby resulting in promoting response to chemotherapeutic drug temozolomide in medulloblastoma to a large extent (Zhou et al., 2011). Also, Vewinger et al. have illustrated that the IGF signaling pathway has an important role in HGNET-BCOR brain tumor since IGF-1R could be a significant target to improve the sensitivity of vinca alkaloids, vinblastine, doxorubicin, ceritinib, and actinomycin D as efficient drugs in patients affected with this kind of brain tumor. As a consequence, utilizing the off-target IGF1R suppressor ceritinib may pave the way for the remedy of tumor cells driven by IGF1R and IGF2 (Vewinger et al., 2019). In another study, Valerie et al. have indicated that the activity of histone deacetylase inhibitors (HDACi) has reduced in Ewing sarcoma patients. Drug combinations of temozolomide with the dual ALK and IGF-1R inhibitor, AZD3463 could suppress AKT and STAT3 to promote the cytotoxic impacts of temozolomide, and thereby decreasing cell proliferation and enhancing apoptosis via cleavage of PARP and caspase-3 indicating that AKT and STAT3 activation could be modulated by ALK and IGF-1R signaling pathway (Sampson et al., 2015). Additionally, Refolo et al. have figured out that the combined treatment with regorafenib, vitamin K1, and two IGF-1R tyrosine kinase inhibitors GSK1838705A or OSI-906 could strengthen antitumor effects of the target drug, improving their actions and decreasing their toxicity to a large extent. Therefore, both IGF1-R inhibitors could enhance the pro-apoptotic and antiproliferative impacts of regorafenib and VK1 in hepatocellular carcinoma downregulating both MAPK and PI3K/AKT signaling pathways (Refolo et al., 2017). **Supplementary Table 2** summarizes the results of various studies that indicate utilizing IGF-1R drug inhibitors with the aim of suppressing the anti-apoptotic properties of IGFR

TABLE 3 | IGF-associated lncRNAs in non-malignant disorders.

Type of disorder	lncRNA	*P*-value	Animal	Clinical Samples (Human)	Cell Lines	Target	Pathway	Function	References
–	H19	<0.05	Bovin	–	GC1, mGSCs	IGF-1, IGF-1R, C-MYC, PNCA, p53, Caspase-3,	AKT, ERK	H19 via the IGF−1 pathway could regulate the proliferation of bovine male germline stem cells.	Lei et al., 2019
Diabetic Nephropathy (DN)	150Rik	<0.01	Male db/db mice	–	–	miR-451/IGF1R, Ago2	p38 MAPK	Overexpression of 150Rik via miR-451/IGF-1R/p38 MAPK pathway could promote mesangial cell proliferation in DN.	Zhang et al., 2018
Skeletal Muscle Atrophy	IRS1	<0.05	Hypertrophic (WRR) and leaner broilers (XH)	–	DF-1	IGF1	PI3K/AKT	Overexpression of lncIRS1 via increasing miR-15 to activate IGF1-PI3K/AKT signaling could promote muscle proliferation, differentiation, and muscle mass.	Li Z. et al., 2019
Atherosclerosis	TUG1	<0.001	–	–	HUVEC, VSMC	IGF-2, miR-148b, Bax, Bcl-2, PCNA	–	TUG1 by regulating the miR-148b/IGF2 axis could regulate apoptosis and proliferation in ox-LDL-stimulated HUVEC and VSMC.	Wu X. et al., 2020
Polycystic Ovary Syndrome (PCOS)	HOTAIR	<0.01	Rat	–	Granulosa cells	IGF-1, miR-130a	–	Downregulation of HOTAIR by reducing the expression of IGF-1 via miR-130a could alleviate PCOS in rats.	Jiang et al., 2020
Polycystic Ovarian Syndrome (PCOS)	HCP5	<0.001	–	–	KGN	IGF-1, miR-27a-3p, caspase-9, Bax, Bcl-2	–	Downregulation of HCP5 via the IGF-1/miR-27a-3p axis could induce apoptosis and also could suppress cell proliferation by arresting cell cycle progression at the G1 phase.	Luo L.-H. et al., 2020
Preeclampsia (PE)	MALAT1	<0.01	–	30 pairs of PE and matched normal pregnant women	HTR-8/SVneoc, JEG-3	miR-206, IGF-1	PI3K/AKT	Downregulation of MALAT1 via knockdown of IGF-1 and upregulating miR-206 could suppress the trophoblast cell migration and invasion.	Wu H. Y. et al., 2020
PE	MALAT1	<0.01	–	30 patients with PE and 30 normal samples	HTR-8/SVneoc, JEG-3	IGF-1, miR-206	PI3K/AKT	MALAT1 via regulating the miR-206/IGF-1 axis through the PI3K/AKT pathway could regulate trophoblast cell migration and invasion.	Wu H. Y. et al., 2020
Liver Fibrosis	H19	<0.01	Male Sprague-Dawley rats	–	HSC-T6	IGF1R, MeCP2, a-SMA, Col1A1, miR-200a	–	H19 by targeting the MeCP2/IGF1R axis control hepatic stellate cell proliferation.	Yang et al., 2016
Endometriosis	H19	=0.035	–	–	Human endometrial stromal cells	IGF-1R, let-7	–	Downregulation of H19 via the let-7/IGF-1R axis could reduce the proliferation of endometrial stromal cells.	Ghazal et al., 2015

TABLE 4 | Prognostic values of miRNAs/lncRNAs in cancers (NNTs: nearby normal tissues).

Sample number	Kaplan-Meier analysis	Univariate/multivariate cox regression	References
87 pairs of lung cancer and NNTs	Downregulation of miR-485-5p was correlated with poor prognosis in NSCLC.	–	Huang et al., 2018
87 pairs of WT tissues and paracarcinoma kidney tissues	miR-155-5p and IGF2 level did not correlate with the survival time of WT patients.	–	Luo X. et al., 2020
60 pairs of cervical cancer samples and NNTs	Higher Linc00319 expression level was related to the low survival rate	–	Ma et al., 2020
44 pairs of resected OSCC and NNTs	Downregulation of miR-375 is correlated with tumor progression and poor prognosis of OSCC patients.	–	Zhang et al., 2017
60 pairs of HCC tissues and NNTs	Reduced expression of miR-505 was correlated with the worse prognosis of HCC patients.	Tumor size, Lymph node metastasis, and TNM stage were correlated with prognosis.	Ren et al., 2019
150 pairs of HCC tissues and NNTs	Downregulation of miR-216b was correlated with poor prognosis in HCC.	–	Liu F. Y. et al., 2015
62 paired HCC samples and NNTs	Downregulation of miR-29a-3p was correlated with poor prognosis in HCC.	–	Wang X. et al., 2017
80 pairs of CRC and NNTs	The decreased miR-491-5p expression level was associated with poor overall survival in CRC patients.	Differentiation level and TNM stage were correlated with prognosis.	Lu et al., 2019
30 pairs of malignant melanoma tissues and NNTs	Lower expression of lncRNA H19 was associated with better overall survival.	–	Men et al., 2020
89 pairs of LGG tissues and NNTs	Low expression of miR-138 was associated with poor prognosis in LGG patients.	–	Yang Y. et al., 2020
90 pairs of GC tissues and NNTs	Downregulation of miR-598 was correlated with poor prognosis in GC.	–	Liu et al., 2018c
63 pairs of PC and NNTs	Upregulation of AFAP1-AS1 was correlated with poor overall survival in patients with PC.	–	Chen B. et al., 2018
40 pairs of glioblastoma tissues and non-tumor tissues	Lower expression of miR-15b was associated with a shorter survival rate.	–	Wang J. et al., 2017
TCGA database	Higher expression of SNHG7 was associated with a lower OS rate.	–	Boone et al., 2019
TCGA database, 57 pairs of RCC and NNTs	Higher expression of HOTTIP was associated with lower OS and DFS rates.	Higher expression of HOTTIP was associated with pathological grade, tumor size, and TNM stage.	Wang Q. et al., 2018
Glioma (n = 169), NBTs (n = 30)	Higher expression of linc01023 was associated with a lower OS rate.	–	Yu et al., 2019
56 pairs of HCC and NNTs	Higher expression of DLEU1 was associated with a lower OS rate.	–	Zhang W. et al., 2019
10 pairs of CRC and NNTs	Higher expression of NEAT1 was associated with a lower OS rate.	–	Zhuang et al., 2020

which cause cancerous cells to resist the cytotoxicproperties of chemotherapeutic drugs or radiotherapy.

EPIGENETIC REGULATION OF IGF-I, IGF-II, IGF-1R, AND IGFBPS OF IGF AXIS IN A VARIETY OF HUMAN CANCERS

Accumulating evidence indicates that dysregulation of epigenetic systems has an important role in cancer pathogenesis resulting in overexpression of altered target genes as well as malignant cellular transformation. Since the IGF axis could contribute to cancer progression and invasion, it is now widely accepted that aberrant methylation of IGFBP7, IGFBP-4, IGFBP-3, IGF-1R, IGF-1, and IGF-II promoters could be a potential factor in various common human cancers (Qian et al., 2011; Sato et al., 2011; Bolomsky et al., 2015; Ye P. et al., 2016). Beeghly et.al have demonstrated that differential promoter P2 and P3 methylation patterns of the IGF-II gene could be remarkably related to promoting the risk of disease progression in epithelial ovarian cancer, especially hypermethylation of P2 could be associated with unpleasant symptoms of this serious disease (Beeghly et al., 2007). Additionally, another research indicated that epigenetic alterations in the IGF signaling pathway could play an effective role in the emergence of hepatocellular carcinoma. Therefore, considerable demethylation and upregulation of IGFBP3 via employing 5-Aza-2′- deoxycytidine and trichostatin A therapy results in attenuating cell proliferation and decreasing colony formation in HCC cells (Han et al., 2015). Chang et al. have illustrated that hypermethylation of the IGFBP-3 promoter which dramatically suppressed the expression level of this target gene could be substantially related to poor prognosis among Non-Small Cell Lung Carcinoma patients. Therefore, utilizing demethylation agents to upregulate the expression of IGFBP-3 could pave the way for providing a pivotal remedial procedure for these patients (Chang et al., 2002). Besides, Dar et al. have discovered that epigenetic silencing of IGFBP3 via hypermethylation of its promoter in human melanoma cells. Upregulation of IGFBP3 through applying 5AZA treatment resulting in inhibiting cancer cell survival, triggering tumor

TABLE 5 | Importance of IGF-associated pathways in response to chemotherapy.

Type of cancer or disease	microRNA/ lncRNA	Animal	Clinical Samples (Human)	Cell lines	Target	Pathway	Function	References
Melanoma	H19	–	30 pairs of M and NNTs	HEMa, A375, WM35, M8, SK-MEL-2, A2508	miR-18b, IGF1	–	Downregulation of H19 could increase the sensitivity of melanoma cells to DDP.	An et al., 2020
Ovarian cancer (OC)	–	Female athymic nude mice (Harlan)	489 cases of high grade serous OC	A2780, HEY, NIH: OVCAR-8, HET-T30, A2780-T15, HEY-B20, HEY-Epo8, OVCAR-8-D30	IGF-2	AKT, ERK	Downregulation of IGF-2 could activate taxol sensitivity in drug-resistant OC.	Brouwer-Visser et al., 2014
OC	–	–	–	HEY, OVCAR-8, SKOV-3, BG-1, A2780, HEY-T30	IGF-1R	PI3K, ERK	Overexpression of the IGF-1 could induce cisplatin resistance of OC cells.	Eckstein et al., 2009
OC	–	–	134 pairs of OC and NNTs	A2780, HEY, HEY-T30	IGF2	AKT	Downregulation of IGF-2 could reduce drug-resistant OC cells to taxol.	Huang et al., 2010
OC	–	–	212 pairs of OC and NNTs	–	IGF-II	–	IGF-II and its SNP could be associated with elevated risks of disease progression and death in epithelial OC.	Lu et al., 2013
Breast Cancer (BCa)	–	–	–	Hs578T, Hs578T/PTX	IGFBP-3, Caspase-3	–	Paclitaxel could increase the endogenous IGFBP-3 production, then induce apoptosis of Hs578T human BCa cells.	Fowler et al., 2000
BCa	–	–	–	MCF-7, CAMA-1, MDA-MB-361, HCC1954, BT474, MDA-MB-453, UACC893, HCC70, MDA-MB-435S, ZR75-30, HCC1419, SKBR-3, BT549, T47D, ZR75-1, MDA-MB-231	IGF-1R, IRS-1	PI3K/AKT	NVPAEW541 treatment via Inhibiting IGF-1R and the PI3K/AKT pathway could lead to inhibit cell growth and increase the effect of chemotherapeutic drugs.	Mukohara et al., 2009
BCa	–	–	24 BC patients and 16 healthy women	–	IGF-1	–	Adjuvant cyclophosphamide, methotrexate, and 5-fluorouracil could decrease the plasma concentration of IGF-1 in premenopausal BCa women.	Kajdaniuk et al., 2001
BCa	–	–	73 pairs of BCa and NNTs	Py230, 4T1, MDA-MB231	IGF-1/2	–	Downregulation of IGF1/2 in combination with paclitaxel could reduce tumor cell proliferation and lung metastasis in pre-clinical BCa models.	Ireland et al., 2018
Triple-Negative Breast Cancer (TNBC)	–	Female BALB/c nude	–	MDA-MB-468, HCC1806	GFBP-3, EGFR, S1P, SphK-1, Caspase-3	–	Inhibition of EGFR and SphK could lead to a block of IGFBP-3-dependent signaling and inhibit cell proliferation in TNBC.	Julovi et al., 2018
Pancreatic Cancer	–	Female Fox Chase SCID Beige mice	–	KP-4, BxPC-3, Capan-2, CFPAC-1, HPAF-II, SU8686, SW1990, AsPC1, PANC1	IGF-1R, ErbB3	PI3K/AKT	IGF-1 and HRG by targeting the PI3K/AKT pathway could reduce pancreatic cancer cell sensitivity to gemcitabine or paclitaxel.	Camblin et al., 2018
Prostate Cancer	miR-143	Male BALB/cA-nu nude mice	–	PC-3, DU145, 293T, DU145/DTX, PC-3/DTX	IGF-1/R, VEGF	–	Overexpression of miR-143 could reduce IGF-1-induced chemoresistance to docetaxel treatment and inhibit tumor growth *in vivo*.	Niu et al., 2017

(Continued)

TABLE 5 | Continued

Type of cancer or disease	microRNA/ lncRNA	Animal	Clinical Samples (Human)	Cell lines	Target	Pathway	Function	References
Prostate Adenocarcinoma (PaC)	–	–	–	DU145, PC-3, DU145/DTX, PC-3/DTX	IGF-I, IGFBP-2	PTEN	Downregulation of IGFBP-2 could increase the sensitivity of Cap cells to docetaxel.	Uzoh et al., 2011
CRC	miR-143	–	62 pairs of CRC and NNTs	–	IGF-1R	–	Upregulation of miR-143 by targeting IGF-1R could inhibit cell proliferation, migration, and also increase chemo-sensitivity to oxaliplatin.	Qian et al., 2013
CRC	–	–	–	WiDr, SW620, HMEC-1,	IGF-1, HIF-1α	–	IGF-1 could increase the cell viability of stromal and cancer cells in response to chemotherapy in CRC.	Volkova et al., 2014
CRC	miR-497		131 pairs of CRC and NNTs	HCT116, HCT28, LoVo, Colon205, SW480, SW620, CRL-1831	IGF-1R	PI3 K/AKT	Overexpression of miR-497 via inhibiting IGF1-R activity could increase sensitivity to apoptosis induced by chemotherapeutic drugs in CRC cells.	Guo et al., 2013
Adrenocortical Carcinoma (ACC)	–	–	17 ACCs and 6 normal adrenal tissue samples samples	H295R, HAC15	IGF-2, IRA, IGF-1R, IGF-2R	mTOR	Linsitinib treatment by IGF pathway could inhibit cell growth in the H295R and HAC15 cell lines	De Martino et al., 2019
Gastric Cancer	–	–	3 pairs of GC and NNTs	–	IGFBP-3, ICAM-1, VCAM-1, p65, NF-kB, IkB	–	Overexpression of IGFBP-3 could increase cell growth inhibition via suppressing the NF-kB activity by regulating ICAM-1 and VCAM-1 in GC cells.	Kim and Lee, 2015
Gastrointestinal Stromal Tumor (GIST)	–	–	–	GIST-882, GIST-T1, GIST-882/imatinib, GIST-T1/imatinib	IGF-1R,	–	Knockdown of CCDC26 by regulating IGF-1R could induce imatinib resistance in GIST cells.	Xie et al., 2019
Esophageal Squamous Cell Carcinoma (ESCC)	–	–	–	SLMT-1, SLMT-1/CDDP1R, SLMT-1R-pcMV3, SLMT-1R-IGFBP5	IGFBP5	–	Downregulation of IGFBP5 could induce cisplatin-resistance in ESCC cells.	Chan et al., 2018
Brain Tumor	–	–	–	MCH-BT-31, MCH-BT-39, MCH-BT-30, MCH-BT-52, HTB-14	IGF-1R, IGF-I, IGF-II, P-gp	PKC	Tamoxifen treatment could reduce PKC activity and IGF-II expression in brain tumor cells.	Ramachandran et al., 2004
Glioma	–	–	–	U-87MG, KNS-42	CPP32, Bcl-2	p53	Overexpression of IGF-I by increasing the expression of Bcl-2 and decreasing the activity of CPP32 could decrease apoptosis in glioma cells.	Yin et al., 2005
Non-Small-Cell Lung Carcinoma (NSCLC)	–	Nude mice	NSLC (n = 14), patients without cancer (n = 9)	SCC-25, HeLa, SCC-25/CP, KB-3-1, KB-CP, 2008/CP, IGROV1/CP, A2780/CP, PC-9/CDDP, PC-14/CDDP, SBC-3/CDDP	IGFBP7, MKP3	MAPK Erk1/2, MEK/Erk, Stat3	Downregulation of IGFBP7 could increase cellular resistance to cisplatin.	Okamura et al., 2012
NSCLC	–	–	–	SKMES1, SKMES, SKLu-1, Calu-3, H1299, H460, H157, HCC44, A549, H1975	IGF-1, IGF-1R, VEGF	AKT, MAPK	AVE1642 treatment by targeting IGF-1 could increase the paclitaxel-mediated anti-tumor effect.	Spiliotaki et al., 2011

(Continued)

TABLE 5 | Continued

Type of cancer or disease	microRNA/ lncRNA	Animal	Clinical Samples (Human)	Cell lines	Target	Pathway	Function	References
NSCLC	miR-223	Male nude mice	–	PC-9, PC-9/ER, PC-9/ER-EV	IGF-1R	AKT/S6	Overexpression of miR-223 by relating IGF-1R could inhibit tumor growth in nude mice and also increase the sensitivity to erlotinib.	Zhao et al., 2016
NSCLC	–	–	–	NCI-H460, H1299, A549	IGF-1, Chk2, Chk1	p53	Overexpression of IGF-1 could recover cisplatin-derived inhibition of proliferation and apoptosis in NSCLC cells.	Jeon et al., 2008
NSCLC	–	Female athymic nude mice	–	H460	IGF-1, IGFBP-3	–	Targeting of the IGF-1 receptor using siRNA could result in the sensitization of cisplatin-R-cells to cisplatin and radiation.	Sun et al., 2012
NSCLC	–	–	–	A549, A549/PTX	IGF-1, SphK1, Vimentin, Fibronectin, N-cadherin, E-cadherin	ERK, AKT	IGF-1 treatment via activating SphK1, ERK, and AKT could decrease the sensitivity of A549 cells to paclitaxel.	Wu et al., 2019
NSCLC	LUCAT1	–	–	A549/DDP, A549	IGF-2	–	Overexpression of LUCAT1 by regulating IGF-2 could promote the cisplatin resistance in NSCLC.	Wang et al., 2019c
Cardiac Toxicity	–	–	–	H9c2	IGF-1R, IGFBP-3	p53	Doxorubicin via inhibiting IGF-1R and upregulating IGFBP-3 through p53 could lead to resistance to IGF-1 that may contribute to doxorubicin-initiated apoptosis.	Fabbi et al., 2015

cell death, decrease colony formation and invasion, inducing expression of the pro-apoptotic genes containing PUMA, p21, and BAX as well as caspase 3 cleavage and downregulating phosphorylation of AKT (Dar et al., 2010). Furthermore, Schayek et al. have indicated that hypermethylation of AR promoter in metastatic prostate cancer cells results in downregulation of IGF1R expression levels which indicates the fact that the IGF1R gene has been detected s a downstream target for AR action. Employing 5-Aza treatment could trigger demethylation of AR promoter and as a consequence the expression level of IGF1R could increase significantly which may consider as a promising therapy in human prostate cancer (Schayek et al., 2010). An overview of promoter methylation and epigenetic modulation of various genes relevant to the IGF signaling pathway in different human cancers is represented in **Supplementary Table 3**.

APPLYING REMEDIAL CRISPR AND siRNA STATE-OF-THE-ART GENOME EDITING SYSTEMS TO MANIPULATE THE IGF SIGNALING PATHWAY IN VARIOUS HUMAN CANCERS

It is now accepted that gene silencing via CRISPR-Cas9 and small interfering RNA (siRNA) is becoming an inevitable gene-editing tool in biological research, especially to repair genetic defects via editing or knock out various genes related to the IGF signaling pathway. Via applying a CRISPR/Cas9 or siRNA genome editing tool, it could be possible to knock out or edit ectopic expression of various genes related to IGF signaling cascade through which we could be able to improve response to chemotherapeutic agents as well as attenuating tumor cell survival, proliferation, invasion, angiogenesis, and metastasis of different kinds to a large extent (Singh et al., 2008; Brouwer-Visser et al., 2014; Hussmann et al., 2017; Strub et al., 2018). Liu et al. have detected that knockdown of IGF2BP1 expression level through applying a CRISPR/Cas9 genome editing system could play a crucial role in repressing the expression levels of IGF2, Gli1, CD44, and Myc in skin SCC cells through which tumor cell proliferation and survival were suppressed considerably. Likewise, via utilizing siRNA-mediated knockout of IGF2BP1-bound lncRNA THOR, skin SCC cell growth could be suppressed dramatically (Liu et al., 2018e). In addition, another research demonstrated that silencing IGF1R expression through employing a CRISPR/Cas9 genome editing system leads to functional endpoint mechanism for TKI resistance in a targetable direction MET-amplification, and thereby resulting in improving response to treatment via suppressing resistance to Erlotinib in Non-Small Cell Lung Carcinoma cells and inhibiting epithelial-mesenchymal transition in tumor cells (Hussmann et al., 2017). Besides, Strub et al. have demonstrated that via applying a CRISPR–Cas9 screen targeting chromatin regulators the histone deacetylase SIRT6 haploinsufficiency could play an effective role in upregulating IGFBP2 expression level through promoting chromatin availability, H3K56 acetylation at the

TABLE 6 | Pre-clinical studies employing the CRISPR/Cas9 system with the aim of editing or knocking down various target genes related to the IGF signaling pathway in different human cancers.

Type of cancer or disease	Target	*In vitro*	Cell line	Animal	*In vivo*	CRISPR	Vector	Treatment	Pathway	Function	References
Diffuse large B-cell lymphoma (DLBCL)	YAP	+	LY1, LY3	5-week-old female SCID beige mice	+	Knockout	Lentiviral	Doxorubicin	Hippo-YAP	Knockdown of YAP expression level through a CRISPR/Cas9 genome editing system accompanied with suppression of the expression level of IGF-1R leading to the activation of downstream targets CTGF and CYR61, and thereby could remarkably inhibit tumor cell proliferation and cause cell cycle arrest in DLBCL cells.	Zhou et al., 2020
BRAF V600E melanoma	SIRT6	+	SKMel-239	6-week-old female athymic mice (NCrnu/nu)	+	Screening (targeting ∼140 chromatin factors)	Lentiviral	Dabrafenib, Trametinib	IGF-1R/AKT	Employing CRISPRCas9 screen targeting chromatin regulators illuminate that SIRT6 haploinsufficiency could upregulate IGFBP2 expression level as well as attenuate sensitivity to MAPKi, and thereby enhancing BRAFV600E melanoma cell survival via triggering IGF-1R/AKT signaling pathway.	Strub et al., 2018
BRAF V600E melanoma	PTRF	+	MM121224	–	–	Knockout (targeting exon 1)	Lentiviral	Vemurafenib, Encorafenib	TGFβ, MAPK, IGF	Proteomic analysis of CRISPR/Cas derived PTRF knockouts demonstrated that two markers (PTRF and IGFBP7), which are considerably overexpressed, have an effective contribution to MAPKi resistance and EMT as well as promoting cell adhesion and sphere formation in melanoma cells harboring BRAF mutations.	Paulitschke et al., 2019
Skin squamous cell carcinoma (SCC)	IGF2BP1	+	A431	SCID mice	+	Knockout	Lentiviral	–	–	Knockdown of IGF2BP1 expression level via a CRISPR/Cas9 genome editing system could downregulate the expression levels of IGF2, Gli1, CD44, and Myc, and thereby attenuating proliferation and survival of skin SCC cells.	Liu et al., 2018e
Breast cancer (BCa)	IRAIN	+	MDA-MB-231	–	–	Insertion a strong CMV promoter in front of IRAIN to upregulate IRAIN lncRNA via inducing homologous recombination	Lentiviral	–	IGF1R	Via employing CRISPRCas9 gene-editing system IRAIN could compete in cis with the overlapping IGF1R promoter, and thereby suppress the IGF1R signaling cascade that in turn attenuate tumor cell proliferation and metastasis in BCa cells.	Pian et al., 2018
Colorectal cancer (CRC)	CXCR4	+	HT115, COLO201	–	–	Knockout	Plasmid	–	IGF1R	Knockdown of CXCR4 expression level via applying a CRISPR/Cas9 genome editing system in CRC cells could inhibit tumor angiogenesis triggered via IGF1R with the help of SDF-1 in the tumor microenvironment.	Zheng et al., 2017

(Continued)

TABLE 6 | Continued

Type of cancer or disease	Target	*In vitro*	Cell line	Animal	*In vivo*	CRISPR	Vector	Treatment	Pathway	Function	References
Ewing sarcoma (EWS)	PAPPA	+	EW8	6–8-week-old male NSG mice	+	Knockout	Not available	–	IGF-1	Knockdown of PAPPA expression level via applying a CRISPR/Cas9 genome editing system could overwhelmingly attenuate immune evasion in EWS cells triggered by PAPPA via reinforcement of IGF-1 signaling.	Heitzeneder et al., 2019
Glioblastoma (GBM)	ERN1, IGFBP3, IGFBP5	+	U251	–	–	Knockout	Plasmid	12ADT	IGF-1, IRE1α	Inhibition of the expression levels of ERN1, IGFBP3, and IGFBP5 via applying a CRISPR/Cas9 genome editing system could promote sensitivity to 12ADT in GBM cells.	Rodvold et al., 2020
GBM	IGF2BP1	+	A172	–	–	Knockout	Lentiviral	–	–	Knockdown of IGF2BP1 expression level via applying a CRISPR/Cas9 genome editing system leading to upregulation of miR-4500 in GBM cells, and thereby suppressing tumor cell growth and metastasis to a large extent.	Li Z.-W. et al., 2019
Liver cancer stem cells (liver CSCs)	β-Catenin	+	Huh7	–	–	Knockout (targeting exon 1 and 5)	Lentiviral	–	Wnt/β-catenin, IGF/MEK/ERK	Inhibition of the expression level of β-catenin via applying a CRISPR/Cas9 genome editing system demonstrated that IGF/MEK/ERK triggers Tcf7l1 phosphorylation and ubiquitination and controlling its suppression independent of β-catenin in liver CSCs.	Shan et al., 2019
Lung cancer (LC)	Nrf2	+	A549	11–12-week-old female C.B-17 SCID.beige mice	+	Knockout	Lentiviral	–	IGF1R	Suppression of the expression level of Nrf2 via applying a CRISPR/Cas9 genome editing system illustrated that ERBB3 and IGF1R signaling pathway accompanied by thioredoxin and peroxiredoxin proteins play an effective role in KEAP1-mutant cancer cells.	Vartanian et al., 2019
Non-Small Cell Lung Carcinoma (NSCLC)	IGF1R	+	HCC827	–	–	Knockout (targeting exon 2 leading to a deletion of 101 bp)	Plasmid	Erlotinib	IGF1R	Knockdown of IGF1R expression level via applying a CRISPR/Cas9 genome editing system could promote the responsiveness of NSCLC cells to Erlotinib, and thereby suppressing EMT.	Hussmann et al., 2017
Oral squamous cell carcinoma (OSCC)	IGF1R	+	SCC-4	–	–	Knockout	Lentiviral	–	PI3K-AKT, hedgehog	Knockdown of IGF-1 expression level via applying a CRISPR/Cas9 genome editing system could suppress the activation of AKT and Hedgehog signaling pathways, and thereby inhibiting cell proliferation, migration, and tumor aggressiveness in OSCC cells.	Ferreira Mendes et al., 2020

(Continued)

TABLE 6 | Continued

Type of cancer or disease	Target	*In vitro*	Cell line	Animal	*In vivo*	CRISPR	Vector	Treatment	Pathway	Function	References
Osteosarcoma (OS)	IGF1, IGFBP3	+	U2OS	–	–	Knockout	Not available	Graphene Oxide nanoparticles	IGF1, IGFBP3	Knockdown of IGF1 and IGFBP3 expression level via applying a CRISPR/Cas9 genome editing system could promote apoptosis in OS cells which in turn leading to downregulating the expression level of ROS and Nrf-2, and thereby enhancing the sensitivity of Graphene Oxide in tumor cells.	Burnett et al., 2020
Prostate cancer (PCa)	MDA-9/syntenin	+	ARCaPM	Athymic nude mice	+	Knockout	Plasmid	–	STAT3	Knockdown of MDA-9/syntenin expression level via applying a CRISPR/Cas9 genome editing system could downregulate the expression levels of MMP-2 and MMP-9 and inhibit STAT3 activation as well as suppressing pro-angiogenic factors containing IGFBP-2, VEGF-A, IL-8, and IL-6, and thereby attenuating invasion in PCa cells.	Das et al., 2018
Renal cell carcinoma (RCC)	THOR	+	786-O	5–6-week-old female nude mice	+	Knockout	Plasmid	–	–	Knockdown of THOR expression level via applying a CRISPR/Cas9 genome editing system could suppress the expression levels of IGF2BP1-regulated genes, containing IGF2, Myc, and GLI1, and thereby inhibiting proliferation and viability of RCC cells.	Zhu W. et al., 2018

IGFBP2 locus, and overexpression of IGF-1R function as well as downstream AKT signaling cascade. Additionally, elevating the IGFBP2 expression could lead to attenuate sensitivity to MAPK signaling inhibitors, and thereby increasing BRAFV600E melanoma cell survival via triggering IGF-1R/AKT signaling pathway. Thus, incorporating a clinically suitable IGF-1Ri with BRAFi could pave the way for promoting the sensitivity of SIRT6 haploinsufficient melanoma cells (Strub et al., 2018). Besides, another research indicated that POU2F3 can be expressed particularly in variant SCLC cancers that have the insufficient expression of neuroendocrine markers and markers of a chemosensory lineage. They applied domain-focused CRISPR screening as a suitable procedure to identify POU2F3 as a significant transcription factor in a subset of SCLC cells and to display other important associations in POU2F3-expressing SCLC lines, containing the lineage TFs SOX9 and ASCL2 and IGF1R. Besides, this strategy shed light on the fact that upregulation of IGFBP5 through employing lentivirus in POU2F3high SCLC lines could suppress tumor cell growth remarkably (Wu et al., 2018). Baade Rø et al. have illustrated that there are an extreme intricacy and interaction between the chemokine and cytokine network triggering migration. They have detected the positive relevance among the degree of cytokine-induced migration and phosphorylation of PAK. PAK phosphorylation was considerably elevated when tumor cells were triggered by combinations of SDF-1a, IGF-1, and HGF which could play an effective role in promoting myeloma cell migration to the large extent. Therefore, via utilizing small interfering RNA, the expression of PAK was downregulated leading to attenuating cytokine-driven migration (Rø et al., 2013). Another study detected that silencing expression of IGFBP-6 or IGF-I or IGF-II through applying siRNA mechanism as well as knockdown IGF-1R activity on fibroblasts could lead to altering fibroblast mobilization, attenuating tumor invasion and TME remodeling through the IGFs/IGF-1R axis in breast epithelial cells which can be considered as a helpful tool for pivotal therapeutic of breast cancer related to dysregulation of IGF signaling pathway (De Vincenzo et al., 2019). Additionally, Brouwer-Visser et.al indicated that suppressing the expression level of IGF2 in ovarian cancer cells via employing RNA interference technology could elevate paclitaxel sensitivity and could restore sensitivity to both microtubule-stabilizing and destabilizing agents (Brouwer-Visser et al., 2014). A summary of clinical researches with the aim of editing or knocking down aberrant expression of different target genes relevant to IGF signaling pathway in various human cancers via employing CRISPR/Cas9 and siRNA gene-editing tools are demonstrated in **Tables 6**, **7**, respectively.

DISCUSSION

IGFs and the related signal transduction networks partake in the pathogenesis of cancers, diabetes complications, atherosclerosis, PCOS, and other disorders. Meanwhile, these signaling pathways are regulated by hundreds of miRNAs and lncRNAs. Several members of IGF signaling including IGF-I, IGF-II, IGF-1R, and

TABLE 7 | Pre-clinical researches applying the siRNA silencing mechanism to edit or knockdown aberrant expression of target genes relevant to the IGF signaling pathway in various human cancers.

Type of cancer or disease	Target	*In vitro*	Cell line	Animal	*In vivo*	siRNA	Vector	Treatment	Pathway	Function	References
Breast cancer (BCa)	IGF-1R	+	EMT6, C4HD	BALB/c females	+	Inhibition of IGF-1R expression	Cytomegalovirus (CMV)	–	IGF-1R, AKT, ERK	Attenuating tumor cell proliferation, Suppressing phosphorylation of downstream signaling cascades ERK and AKT, Triggering secretion of proinflammatory cytokines IFN-γ and TNF-α, and blocking G0/G1 cell cycle.	Durfort et al., 2012
BCa	IGF-II	+	MCF-7	–	–	Inhibition of IGF-II expression	Not available	Resveratrol (RSV)	PI3K/AKT, MAPK/ERK	Enhancing progression and chemoresistance in BCa cells via negatively regulating Bcl-2 and Bcl-XL.	Singh et al., 2008
BCa	IGF-1R	+	SKBR3	–	–	Inhibition of IGF-1R expression	Plasmid	Docetaxel	IGF-1R	Utilizing the MUC1 Apt-conjugated CH NPs with the aim of co-delivery of Docetaxel and IGF-1R siRNA remarkably inhibiting the expression levels of IGF-1R, MMP9, STAT3, and VEGF.	Jafari et al., 2019
BCa	IGFBP-6, IGF-I, IGF-II, IGF-1R	+	MCF10A-MycER MCF10A-Myc^{ON}, MCF10A-Myc^{OFF}	–	–	Inhibition of IGFBP-6, IGF-1R IGF-I, and IGF-II expression	Not available	–	IGFs/IGF-1R	Downregulation of IGFBP-6 or IGF-I or IGF-II expression levels via siRNAs in breast epithelial cells or knockdown IGF-1R activity on fibroblasts could play an effective role in changing fibroblast mobilization, suppressing TME remodeling and tumor invasion via the IGFs/IGF-1R axis.	De Vincenzo et al., 2019
Triple-negative breast cancer (TNBC)	IGF-1R	+	MDA-MB-231, BT-549	–	–	Inhibition of IGF-1R expression	Not available	–	PI3K-Akt	IGF-1R knockdown via NVP-AEW541, 3-MA, and Atg7 siRNA could induce TNBC cell-protective autophagy and thereby attenuating the efficacy of IGF-1R-modulating therapeutic agents in tumor cells.	Wu W. et al., 2017
Ovarian cancer (OC)	IGF2	+	HEY-T30	–	–	Inhibition of IGF2 expression	Not available	Paclitaxel	IGF	Suppression of the IGF signaling pathway via siRNA could promote sensitivity to paclitaxel in OC cells.	Huang et al., 2010
OC	IGF2	+	HEY-T30	6–8-week-old female athymic nude mice	+	Inhibition of IGF2 expression	Plasmid	Paclitaxel	IGF	IGF2 knockdown via siRNA leading to the suppression of paclitaxel resistance in OC cells.	Brouwer-Visser et al., 2014
Colorectal cancer (CRC)	IGF-1R	+	SW480	–	–	Inhibition of IGF-1R expression	Plasmid	5-Fluorouracil	IGF-1R	Inhibiting CRC cell proliferation and promoting chemosensitization to 5-FU.	Yavari et al., 2010
CRC	IGF-1R	+	SW480	–	–	Inhibition of IGF-1R expression	Not available	–	IGF-1R	Utilizing radioconjugate of IGF-1R siRNA, p-SCN-Bn-DTPA, and 177Lu as radiopharmaceutical to suppress CRC cell proliferation caused by upregulation of IGF-1R via triggering apoptosis.	Fathi et al., 2013
CRC	IGF-1R, IR-A	+	SW480	–	–	Inhibition of IGF-1R and IR-A expression	Plasmid	–	IGF-1R	Inhibiting IR-A expression causing a concomitant promotion of IGF-1R activation through IGF-I and IGF-II, decreasing the formation of IGF-1R: IR-A hybrid receptors, and enhancing IGF-1R homodimer formation in CRC cells.	Brierley et al., 2010

(Continued)

TABLE 7 | Continued

Type of cancer or disease	Target	*In vitro*	Cell line	Animal	*In vivo*	siRNA	Vector	Treatment	Pathway	Function	References
CRC	IGF-1R, PIAS3	+	HT29, HT29-OxR, DLD-1-OxR	–	–	Inhibition of IGF-1R, PIAS3 expression	Not available	Ganitumab, NVP-AEW541, Dasatinib, FOLFOX, CAPOX, FOLFIRI, Oxaliplatin	IGF-1R, AKT, Wnt	Upregulation of PIAS3 could contribute to promoting the expression level of IGF-1R that in turn leading to Wnt pathway activation and thus causing resistance to chemotherapeutic agents. IGF-1R and PIAS3 knockdown via siRNAs leading to the chemotherapy sensitivity in CRC cells.	Codony-Servat et al., 2017
CRC	IGF-1R	+	HCT116	–	–	Inhibition of IGF-1R expression	Plasmid	5-fluorouracil, Cisplatin	IGF-1R, MEK/ERK, PI3K/AKT	IGF-1R knockdown via siRNA could lead to upregulation of miR-497 and activation of PI3K/AKT signaling pathway, which in turn promoting the sensitivity of CRC cells to the chemotherapeutic drugs 5-fluorouracil and cisplatin.	Guo et al., 2013
Gastric carcinoma (GC)	AKT, ERK1, ERK2	+	MGC803, SGC-7901	–	–	Inhibition of AKT, ERK1, and ERK2 expression	Plasmid	–	AKT/ERK	Upregulation of IGF-I could trigger EMT in gastric cancer cells which is accompanied by enhancing ZEB2 expression level. Thus, AKT, ERK1, and ERK2 knockdown via siRNA could reverse IGF-I-induced ZEB2 up-regulation and EMT via promoting the expression of miR-200c.	Li et al., 2014
Pancreatic cancer (PC)	FAK	+	Panc-1, MiaPaca-2	–	–	Inhibition of FAK expression	Adenoviral	–	IGF-1R, FAK	Dual knockdown of FAK and IGF-1R via TAE 226 and siRNA could lead to remarkable suppression of cell viability, reducing ERK and AKT phosphorylation levels, and promoting apoptosis in PC cells which in turn resulting in caspase-3 activation as well as ADP-ribose and PARP cleavage in tumor cells.	Liu et al., 2008
PC	PTEN	+	BxPC-3, SW1990, AsPC-1, MIA PaCa-2, PANC-1	–	–	Inhibition of PTEN expression	Not available	–	IGF-1, PI3K/AKT, NFκB	IGF-1 could trigger tumor cell growth and invasiveness of PC cells leading to promoting activation of PI3K/AKT/NFκB signaling as well as downregulating phosphorylation of PTEN. PTEN knockdown via siRNA could increase PI3K/AKT/NFκB pathway activation and increasing tumor cell proliferation and invasion.	Ma et al., 2010
Prostate adenocarcinoma (PaC)	IGFBP-2	+	DU145, PC3	–	–	Inhibition of IGFBP-2 expression	Not available	Docetaxel	–	Downregulation of IGFBP-2 via siRNA modulating promotion of PTEN activity as well as sensitivity to docetaxel in CaP cells.	Uzoh et al., 2011
Acute myeloid leukemia (AML)	IGF-1R, IR, PI3K	+	U937	–	–	Inhibition of the class Ia PI3K isoforms p110β and p110δ	Plasmid	–	PI3K/AKT, ERK	Targeting isoforms p110β and p110δ via RNAi could reduce AKT activation through IGF-I or insulin, indicating that both PI3K isoforms contributing to the upregulation of IGF-1R or IR in AML cells and improving the sensitivity of tumor cells to chemotherapeutical drugs.	Doepfner et al., 2007

(Continued)

TABLE 7 | Continued

Type of cancer or disease	Target	*In vitro*	Cell line	Animal	*In vivo*	siRNA	Vector	Treatment	Pathway	Function	References
Ewing's sarcoma (ES)	IGF-1R, EWS/FLI-1	+	TC-71	–	–	Inhibition of IGF-1R and EWS/FLI-1 expression	Not available	R1507	IGF, AKT	Transferring EWS/FLI-1 siRNA leading to upregulation of IGF-BP3 levels and downregulation of IGF-1 and IGF-2 levels and following that reducing p-Akt levels, thereby suppressing signaling via p-IGF-1R. As a result, triggering apoptosis and proliferation inhibition in ES cells.	Huang et al., 2011
Lung Cancer (LC)	IGF-1R	+	A549	–	–	Inhibition of IGF-1R expression	Lomustine-loaded chitosan nanoparticles (ChiNPs)	Doxorubicin	IGF-1R, STAT3	The IGF-1R siRNA/DOX co-delivery system loaded chitosan nanoparticles play an effective role in reducing mmp9, STAT3, and VEGF in tumor cells.	Shali et al., 2018
LC	IGF-1R	+	H460	5–6-week-old female athymic nude mice	+	Inhibition of IGF-1R expression	Not available	Cisplatin	IGF-1R	IGF-1R knockdown via siRNA could upregulate the expression level of IGFBP-3 in tumor drug resistance cells and lead to promoting the sensitivity of LC cells to cisplatin and radiation.	Sun et al., 2012
Non-small cell lung carcinoma (NSCLCs)	IGFBP7	+	PC-9, PC-14	–	–	Inhibition of IGFBP7 expression	Not available	Cisplatin	–	IGFBP7 knockdown via siRNA could play an effective role in promoting resistance to cisplatin as well as upregulating the expression level of MKP-3 in NSCLCs.	Okamura et al., 2012
NSCLCs	IRS-1	+	H1299	–	–	Inhibition of IRS-1 expression	Not available	Cisplatin	DSBs repair and checkpoint	IRS-1 knockdown via siRNA indicating that IRS-1 and ATM expression levels are downregulated by IGF-1 that could contribute to promoting cisplatin resistance in NSCLC cells and blocking the activation of DSBs repair and checkpoint pathways as well as cisplatin-induced γH2AX formation.	Jeon et al., 2008
Renal cell carcinoma (RCC)	IGF-2, HOTTIP	+	A-498, 786-O	–	–	Inhibition of IGF-2, HOTTIP expression	Plasmid	–	IGF-2	LncRNA HOTTIP could contribute as a miR-615 sponge which negatively modulates its target IGF-2. There is a positive association between the expression of HOTTIP and IGF-2 in tumor cells. HOTTIP knockdown via siRNA could remarkably suppress cell growth and carcinogenesis, and promote apoptosis in RCC cells.	Wang Q. et al., 2018
Esophageal squamous cell carcinoma (ESCC)	IGFBP5	+	SLMT-1	–	–	Inhibition of IGFBP5 expression	Not available	Cisplatin	IGF	IGFBP5 knockdown via siRNA indicating Cisplatin resistance in ESCC cells, and thereby upregulation of IGFBP5 could play an important role in promoting sensitivity of tumor cells to chemotherapeutic agents.	Chan et al., 2018
Hepatocellular carcinoma (HCC)	IGF-1R	+	Huh7, Hep3B	6-week-old BALB/c nude mice	+	Inhibition of IGF-1R expression	Lentiviral	–	IGF-1R	IGF-1R knockdown via lentivirus-mediated RNAi could remarkably suppress tumor cell growth and apoptosis through attenuating the expression level of midkine in HCC cells.	Bie et al., 2016

IGFBP-3 are targets of regulation by miRNAs and lncRNAs. Therefore, understanding the complex interplay between these factors is a necessary step in the design of appropriate therapeutic options for these conditions. The importance of this task has been further underscored by the availability of several IGF-modifying modalities including receptor-specific antibodies, inhibitors of receptor kinases, and activators of AMP-activated protein kinases (Pollak, 2008). In addition to these types of therapeutics, a number of alternative medicines act by affecting the expression of IGF-related non-coding RNAs. For instance, bufothionine induces gastric cancer cell apoptosis via up-regulating miR-133a-3p which sponges IGF1R and regulates PI3K/Akt associated production of reactive oxygen species (Hu Z. H. et al., 2020).

The data presented above indicate that most of the IGF-associated lncRNAs exert their roles via modulation of miRNAs. Examples of lncRNA/miRNA interactions in the IGF-related pathways are circ_0014130/miR-142-5p, Linc00319/miR-147a, TUG1/miR-148b, H19/miR-18b, HCP5/miR-27a-3p and DBH-AS1/miR-138. The association between lncRNAs/miRNAs and the IGF system has importance in regenerative medicine as well. IGF1R signaling has been shown to partake in the preservation of stem cell features and improvement of efficiency of stem cell therapy, as IGF1R-expressing stem cells exhibit strong pluripotent or multipotent features (Teng et al., 2018). Therefore, the lncRNA/miRNA-mediated regulation of IGF1R signaling might offer putative modalities for maintaining stem cell features and enhancing the effects of these therapeutics in clinical settings.

IGF-related miRNAs and lncRNAs can be used as potential markers for forecasting the prognosis of cancer. Moreover, expression levels of these transcripts can be used as diagnostic markers for neoplastic conditions. The importance of IGF signaling in the modulation of response of melanoma, ovarian cancer, breast cancer, pancreatic cancer, prostate cancer, colorectal cancer, and several other cancers to chemotherapeutic agents has been validated. Some lncRNAs and miRNAs such as H19, LUCAT1, miR-143, miR-497, and miR-223 are involved in this process. However, the role of other transcripts should be assessed in the upcoming researches. Based on the role of IGF-related miRNAs and lncRNAs in the modulation of response o chemotherapeutic agents, these transcripts are putative targets for the improvement of the response of cancer cells to these agents.

Besides, promoter methylation of IGF-1R, IGF-1, IGF-II, and especially IGFBP-3 in various regions could be associated with cancer prognosis (**Supplementary Table 3**). Methylation patterns of these promoters are important for the regulation of their expression and could have pivotal clinical implications in various cancers. Re-expression of IGFBP-3 will be really helpful in curing the majority of aggressive tumors and can solve the problem of intratumoral heterogeneity.

Furthermore, employing CRISPR-Cas9 or siRNAs gene editing tools with the aim of knockdown of ectopic expression of target genes including IGF1R, IGF1, IGF2, IGFBP3, and IGFBP-6 can play an important role in attenuating the tumorigenesis characteristics as well as improving response to treatment in various human cancer cells. Utilizing this effective method will pave the way for future clinical advancement.

CONCLUSION

The advent of novel genome editing modalities and clarification of the role of epigenetic factors including both genomic marks and non-coding RNAs have raised the possibility of management of human cancers particularly neoplastic disorders with novel therapeutics. Meanwhile, concomitant assessment of expression profile and genomic marks of IGF-related genes using high throughput methods would facilitate appropriate stratification of patients with regards to possible response to each therapeutic option. Further investigations are needed to appraise the clinical application of novel therapeutic modalities that target IGF signaling and related lncRNAs.

Availability of Data and Materials

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

AUTHOR CONTRIBUTIONS

MT and SG-F supervised the study, wrote the draft, and edited the submission. HS, AA, and MM performed the data collection, designed the tables and figures. All of the authors are contributed equally and fully aware of submission.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2021.634512/full#supplementary-material

Supplementary Table 1 | IGF-associated miRNAs in cancers (NNTs: nearby normal tissues).

Supplementary Table 2 | Role of different drug inhibitors in suppressing the IGF-1R activity and attenuating tumorigenesis as well as drug resistance in various human cancer cells and promoting response to treatment.

Supplementary Table 3 | Epigenetic regulation of different genes associated with the IGF signaling pathway in human cancers.

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Growing Up Under Constant Light: A Challenge to the Endocrine Function of the Leydig Cells

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The factors influencing Leydig cell maturity and the acquisition of functional capacity are incompletely defined. Here we analyzed the constant light (LL) influence on Leydig cells' endocrine function during reproductive maturation. Rats were exposed to LL from P21 to P90. Data were collected at juvenile (P35), peri/pubertal (P42, P49), and adult (P90) stages of life. The results proved the effect of LL on rats' physiology by changing of bimodal voluntary activity pattern into free-running. Additionally, the peripheral clock in Leydig cells changed in LL condition, indicating disturbed rhythm: the positive element (*Bmal1*) increased in pre-/pubertal but decreased in the adult period, while negative elements (*Per2* and *Reverba*) were increased. The effects of LL were most prominent in puberty: pituitary genes encoding gonadotropic hormones (*Cga, Lhb, Fshb*) decreased; serum corticosterone increased, while serum androgens and mass of testicular and sex accessory organs reduced; markers of Leydig cells maturity/differentiation (*Insl3*, *Lhcgr*) and steroidogenesis-related genes (*Scarb1, Star, Cyp11a1, Cyp17a1*) decreased; the steroidogenic and energetic capacity of the Leydig cell mitochondria decreased; the mtDNA copy number reduced, and mitochondrial dynamics markers changed: fusion decreased (*Opa1* and *Mfn2*), and mitophagy increased (*Pink1*). In adults, the negative effect of LL on mitochondrial function and steroidogenic capacity persists in adult Leydig cells while other parameters reached control values. Altogether, the results indicate that LL slows down Leydig cells' maturation by reducing the endocrine and energy capacity of cells leading to the delay of reproductive development.

Keywords: Leydig cell, puberty, constant light, mitochondria, steroidogenesis, clock

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INTRODUCTION

Most physiological processes in our body oscillate daily and are synchronized with external environmental changes (1). Light is the external environment element that profoundly influences circadian and neuroendocrine control of mammal physiology. According to the photoperiod, almost all hormones essential for life, including reproductive hormones, are secreted in a circadian rhythm. Adequate exposure to environmental cues such as the light/dark cycle is critical to the body physiology's temporal organization. However, the urban lifestyle has reduced many external cues'

daily contrasts, including light/darkness contrasts. Many people are not exposed to a strong light/dark cycle spending most of their time indoors with the low light distinction between day and night (2), which can significantly disrupt rhythms, body physiology, and health. How living and growing up in buffered oscillations of external factors, including the light/dark cycle, affects males' reproductive maturation remains to be clarified.

In males, testosterone is a crucial androgenic hormone that regulates fertility, development, and maintenance of the male reproductive system's organs and muscle strength, cognition and sexual function, and overall male phenotype (3). In rat circulation, testosterone exhibits a low-amplitude daily rhythm with a peak at the beginning of the dark phase (4).

Leydig cells are the largest and most important source of androgenic hormones in mammals. In male life, two populations of Leydig cells exist. Fetal Leydig cells produce androgens and INSL3 in embryonic life crucial for the male fetus's masculinization (5, 6). Androgenic activity of the fetal Leydig cells continues for a few weeks after birth, and after that, they disappear, followed by hormonal quiescence until puberty. When the hypothalamic–pituitary–testicular axis is reactivated, the new Leydig cells aroused from the testicular stem cells become able to produce testosterone. In rodents, in postnatal days from P21 to P35, the number of new progenitors Leydig cells increases together with differentiation, causing a rise in serum androgens (7). Those progenitor Leydig cells express genes related to steroidogenesis such are luteinizing hormone receptor (LHCGR), scavenger receptor Class B member 1 (SCARB1), steroidogenic acute regulatory protein (STAR), cytochrome P450 cholesterol side-chain cleavage (CYP11A1), 3β-hydroxysteroid dehydrogenase 1 (HSD3B1), and cytochrome P450 17α-hydroxylase/17,20-lyase (CYP17A1). However, those (progenitors) Leydig cells do not express 17β-hydroxysteroid dehydrogenase 3 (HSD17B3), the enzyme which synthesizes testosterone in the last step of the steroidogenic pathway. The activities of CYP11A1, HSD3B1, and CYP17A1 increase in immature Leydig cells as they mature from progenitor Leydig cells. The immature Leydig cells express HSD17B3, and therefore they can make testosterone from androstenedione. The main secretory product of these cells is not testosterone because they express steroid 5a-reductase 1 (SRD5A1) and 3a-hydroxysteroid hydrogenase (AKR1C14) therefore secrete androstanediol (3, 8, 9). Around P49–P56, the adult Leydig cells are formed in rat testis (7) characterized by higher expression of CYP11A1, HSD3B, CYP17A1, and HSD17B3 and silenced SRD5A1 expression (10, reviewed in Chen et al., 2020), which makes testosterone the primary secretory product of the cells. In general, the transitions along the Leydig cell lineage are associated with the progressive down-regulation of the proliferative activity, and the up-regulation of steroidogenic capacity. This is regulated by many signaling pathways specific for each step, including growth factors, such are Dessert Hedgehog, platelet-derived growth factor-AA, LH, and others (9). Another characteristic of adult Leydig cells is the presence of numerous mitochondria necessary for the initiation of steroidogenesis, namely, the cholesterol is transported into the mitochondria by StAR and other proteins of transduceosome as a consequence of the cAMP-PRKA signaling activation created in a series of events occurring after LH binding to its receptor (LHCGR) (11). In the mitochondria, cholesterol is converted into pregnenolone by CYP11A1, starting series of events culminating with testosterone production (12). However, steroidogenic and bioenergetic cell demands are closely linked to mitochondrial dynamics (13). In cells with stimulated steroidogenesis, a tubular mitochondrial network is dominant (13) enabled by the coordinated action of mitofusion genes and proteins, including *Mtn1/2* and *Opa1*. Also, Leydig cell steroidogenesis is facilitated by the inhibition of mitofission due to PRKA-dependent phosphorylation of DRP1 (14). Moreover, the LH-cAMP signaling is involved in the regulation of new mitochondria generation and mitochondrial fusion/fission coupled with increased steroidogenesis and energetic function (15). Still, studying the events regulating mitochondrial dynamic in developmental Leydig cells critical for establishing normal male fertility at puberty is missing.

During reproductive maturation, the dynamic interaction among genome, epigenome, and stochastic and environmental factors contributes to acquiring the full endocrine capacity of the Leydig cells. In this study, the effect of constant light (LL) on Leydig cells' endocrine ability during the period of reproductive maturation was analyzed. The results indicate that LL slows Leydig cells' maturation by reducing the endocrine and energy capacity of cells and delay in reproductive development.

MATERIAL AND METHODS

Chemicals

Medium 199 containing Earle's salt and L-glutamine (M199), Dulbecco's Modified Eagle Medium (DMEM-F12 medium), Tris-Ethylenediaminetetraacetic (EDTA), Bovine serum albumin (BSA), collagenase Type IA, from *Clostridium histolyticum*, Trypan Blue, tris(hydroxymethyl)aminomethane (Trizma base), and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were obtained from Sigma Chemical Company (St. Louis, MO, USA). TMRE (tetramethylrhodamine ethyl) was purchased from Fluka Company. hCG-Pregnyl 3,000 IU/mg (human chorionic gonadotropin) was from Organon (Roseland, New Jersey, USA). Power SYBR Green PCR Master Mix was purchased from Applied Biosystems (Thermo Fisher Scientific, Waltham, MA, USA), while qPCR primers were from Integrated DNA Technologies (Coralville, Iowa, United States). Anti-testosterone-11-BSA serum No. 250 was kindly supplied by Gordon D. Niswender (Colorado State University, Fort Collins, CO). Testosterone was from New England Nuclear (Brisel, Belgium). Active charcoal—Norit A—was obtained from Serva (Heidelberg, Germany).

Animals

Experiments were carried out on male *Wistar* rats raised and bred in the animal facility of the Faculty of Sciences, University of Novi Sad. Rats were raised at a controlled temperature of 22 ± 2°C with free access to water and commercially balanced food.

When rats were separated from the mother on the 21st day of age, they were divided into two groups. The first group was raised under the controlled light regime of 14 h light–10 h dark (LD, control group), while the second group was exposed to constant light conditions (LL, experimental group) until 35, 42, 49, and 90 postnatal days of life (P35, P42, P49, P90). At a certain age, groups were decapitated in the morning, *i.e.*, 1 h after the light turned on to the control group. All experiments were approved by the Local Ethical Committee on Animal Care and Use of the National Council for animal welfare and the National Law for Animal Welfare (No. 323-07-0-08975/2019-05) and in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications number 80 23, revised 1996, seventh edition). All experiments and laboratory procedures are conducted in accordance to Laboratory biosecurity guidance, WHO, September 2006.

Detection of an Animal's Voluntary Activity

To analyze the voluntary rat's activity, the P60 rats from both groups were placed in individual cages with a running wheel system. The system was set to record turns of the wheel that the animal made every 6 min. The rhythmic activity was monitored from P60 to P90. Based on the collected data, the actograms were formed as one of the standard ways to represent circadian rhythms. The graphical representation of the animal's activity (actograms) was formed using R software (16).

Serum Collection, Body, and Organ Weight Measurement

The animal's body weight was measured. The trunk blood was collected. Individual serum samples were stored at −80°C until usage. The reproductive organs' weights (testes, seminal vesicles, dorsal and ventral prostate) were respectively measured.

Collection and Preparation of Purified Leydig Cells

Leydig cells were isolated according to the same protocol previously described by our research group (17, 18). Briefly, after isolation, testes were decapsulated and the main blood vessel removed. Testicular tissue was placed in 50 ml plastic tubes containing 0.25 mg/ml collagenase; 1.5%-BSA; 20 mM HEPES-M199 (two testes per tube). Cell isolation was continued by placing plastic tubes into a shaking-water bath (15 min/34°C/140 cycles/min). To stop enzymatic reaction, 45 ml of cold medium was added, and seminiferous tubules were separated during filtration through Mesh № 100 (Sigma, St. Louis, Missouri, USA). The remaining interstitial cell suspension was centrifuged (160 × *g* for 5 min) and resuspended in 8 ml/tube DMEM-F12 medium. To separate Leydig cells from the others, the resuspended cell mixture was moved to a Percoll gradient with different densities (1.080, 1.065, and 1.045 g/ml) and centrifuged 1,100 × *g* for 28 min (brake free). When separated, the Leydig cells were collected from specific gradient fragments (1.080/1.065 g/ml and 1.065/1.045 g/ml) washed in M199-0.1% BSA and centrifuged at 200 × *g*/5 min. Cell precipitate was resuspended in 5 ml DMEM/F12 and used for the experiment. According to HSD3B staining (19), the presence of Leydig cells in the culture was more than 90%. As for Trypan blue exclusion test, cell viability was greater than 95%. The controls to validate this purification method's comprehensiveness were challenges of purified cells (although few of them) from the inter-layers with hCG (20). Briefly, the cells from the inter-layers (few of them) were collected following the described procedures and incubated with/without (10 ng/ml), but androgen production was not detected.

Ex Vivo Experiments

Leydig cells' primary culture was obtained by plating 3×10^6 cells in a Petri dish (55 mm) and placed in CO_2 incubator at 34°C to attach and recover for 3 h. After the recovering period, cell media were changed, and cells were treated with/without hCG (50 ng/ml) for 2 h. Cell media were collected and stored for androgen level determination, while cells were stored for further analysis.

Hormone Level Measurement

Androgen concentration was determined by RIA in serum and cell medium samples (17). Antitestosterone serum number 250 used in this study showed 100% cross-reactivity with testosterone and dihydrotestosterone but recognized also other androgens. Samples were measured in duplicate (sensitivity: 6 pg/tube; intraassay coefficient of variation: 5–8%; interassay coefficient of variation: 7.5%). For serum corticosterone levels (21), all samples were measured in duplicate in one assay by the corticosterone EIA Kit (Cayman, Ann Arbor, MI, USA) with 30 pg/ml as the lowest standard significantly different from the blank.

ATP Level and Mitochondrial Membrane Potential ($\Delta\Psi$m) Determination

The ATP level was determined using the ATP Bioluminescence CLS II kit following the manual instruction (Roche Diagnostics, Indianapolis, USA) published previously by our group (15). Leydig cells (1×10^6/tube) were resuspended in boiling water and Tris-EDTA (1:9), incubated in the water bath (100°C/3 min), centrifuged (900 × *g*/1 min), and the supernatant was used for ATP measurement while cell pellet was further used for Bradford method analysis. Sample/standard and Luciferase reagent were mixed 1:1, and luminescence was measured by the Biosystems/luminometer (Fluoroscan, Ascent, FL). For $\Delta\Psi$m detection, as we described before (15, 22), Leydig cells were placed in 96 well-plates (1×10^5 cells/well) and incubated with tetramethylrhodamine (TMRE) staining for 20 min/34°C/5% CO_2. Fluorescence was measured on fluorimeter (Fluoroscan, Ascent, FL) on excitation wavelengths 485 and 550 nm, while emission wavelengths were 510 and 590 nm. Cells were washed with 0.1%BSA–PBS and stored for protein quantification by Bradford method.

Genomic DNA Purification, Total RNA Isolation, and qRT-PCR Analysis

Genomic DNA from Leydig cells was purified by Wizard® Genomic DNA Purification Kit (Promega, Medison, WI, USA),

and total RNA from Leydig cells and pituitary glands was isolated using GenEluteTM Mammalian Total RNA Miniprep (Qiagen, Hilden, Germany) and RNeasy kit reagents (Sigma, St. Louis, Missouri, USA), following a protocol recommended by the manufacturer. RNA quality was measured and validated by BioSpec-nano (Shimadzu Biotech, Kyoto, Japan). Following DNase-I treatment (New England Biolabs, Ipswich, Massachusetts, USA), the first-strand cDNA was synthesized using the High Capacity kit for cDNA preparation and according to the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA, USA). Negative controls consisting of non-reverse transcribed samples as well as positive controls were included in each set of reactions. Quantification of gene expression was performed by real-time PCR reaction with SYBR Green technology on Mastercycler RealPlex gradientS (Eppendorf) device. It was obtained in standard conditions (50°C/2 min, 95°C/10 min; 40 cycles, each 95°C/15 s, and then 60°C/1 min). The reaction was performed in the presence of 5 µl cDNA and specific primers (**Supplemental Table 1**; all primers were designed to flank the intron regions). Each sample was run in duplicate, and *Gapdh* was used as endogen control.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 5. Experimental results T + DHT and TMRE are shown as mean value ± SEM individual variation. qPCR results are shown as mean RQ value ± SEM for each group, where all Ct values are obtained from one measurement. Results from each experiment were analyzed by Mann–Whitney non-parametric test. Parameters of rhythmic activity (p, MESOR, Amplitude, and Acrophase) were obtained by cosinor method using Cosinor software fitted to 24 h period (https://cosinor.online/app/cosinor.php).

RESULTS

In this study, growing up in LL condition and the consequences of male's reproductive maturation were studied. Rats were reared in constant light from the mother's separation (P21) to the adult stage (P90) and analyzed on P35, P42, P49, and P90. The specific ages are chosen because they illustrate Leydig cell maturation: in the testes from juvenile rats, P21–35, dominates proliferative progenitor cells; in the testes from peri/pubertal rats, P42–P49, due to reactivation of reproductive axis a transition from immature to adult Leydig cells occurs; testicles from P90 rats contain the mature adult Leydig cells.

Growing Up Under Constant Light Changed Activity Patterns, Body, and Reproductive Organs' Weight

Living under constant light changed the daily rhythms of the rat locomotor activity. The bimodal activity pattern detected in LD conditions (**Figure 1A**) became free-running so that the entire active period consistently drifted later each day (**Figure 1B**), and activity diminished slightly, leading to delay in acrophase (**Supplemental Table 2**). The period (τ) has been extended to 24.911 ± 0.07 h. Rats that grow up in constant light were 40% less active measured in the period from the sixtieth to the ninetieth postnatal day (**Figure 1C**). The body mass of rats growing up in constant light on days 35, 42, and 49 did not differ significantly from that of the controls. In both groups, the gradual increment of body mass was observed. However, in adulthood (P90), in the LL group an increase in body weight was observed compared to LD rats (**Figure 1D**). The weight of the testicles, seminal vesicles, and the ventral and dorsal prostate, which increased with age, were lower during puberty (42nd and 49th days) in rats that grew up in constant light (**Figure 1E**).

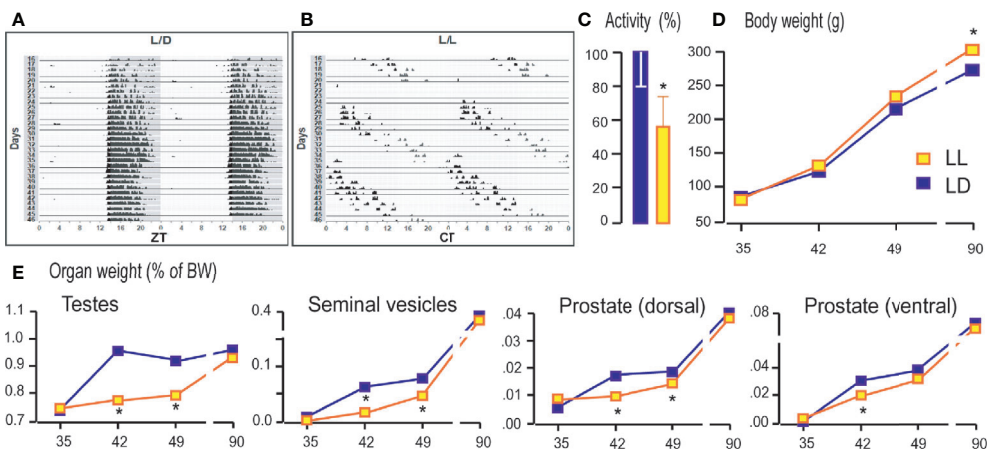

FIGURE 1 | Growing up under constant light changed activity patterns, body and reproductive organs weight. Rats were raised under the controlled light regime of 14 h light–10 h dark (LD) or were exposed to constant light conditions (LL) until P35, P42, P49, and P90. From P60 to P90 the rats' voluntary activity was monitored and actograms formed. The representative actograms from LD **(A)** and LL **(B)** condition as well as average daily activity **(C)** are shown. Rats were monitored for body mass **(D)** and weight of testes and accessory organs **(E)**. Data points/bars represent group means ± SEM values (n = 5–8). *Statistical significance at level $p < 0.05$ compared to the corresponding LD group.

Still, the weight of testes and sex accessory organs reach control values in adults (**Figure 1E**).

Growing Up Under Constant Light Reduced Serum Androgens and Changed the Transcriptional Pattern of Genes Encoding Pituitary Gonadotropic Hormones

Decreased testicular mass and mass of sex accessory organs at puberty suggest that growing up in constant light affects the endocrine testicular function's awakening. In that respect, serum androgens together with a transcription of genes encoding subunits of pituitary gonadotropins and the main marker of Leydig cell differentiation and activity were measured. All variables measured were compared with those observed in P35 rats that lived in LD conditions.

As it is well known, growing is accompanied by increased circulating androgens from the pubertal to the adult period (**Figure 2A**). Increased androgen production was followed by increased expression of *Insl3* (a marker of Leydig cell maturity and functionality; **Figure 2B**) and *Lhcgr* (gene encoding LH receptor, a marker of Leydig cells; **Figure 2C**) indicating the transition from immature to adult Leydig cells.

Rats raised in constant light had lower serum androgens than controls (**Figure 2A**), which may be associated with observed decreased testicular and accessory sex organ mass. Also, in LL conditions, Leydig cells derived from pubertal rats (P42 and P49) transcribed less *Insl3*, but transcript level approached the control values in adult rats (**Figure 2B**). Transcription of *Lhcgr* was decreased during puberty under constant light but came close to the values in Leydig cells from adults (**Figure 2C**). Since *Insl3* is a sensitive marker of Leydig cell maturity/function (23), the decreased androgens in circulation along with reduced *Insl3* and *Lhcgr* could reflect disturbance or delayed puberty due to life in constant light.

Since Leydig cell differentiation and androgenic activity are superiorly governed by hormones operated in the reproductive axis, the transcriptional pattern of genes encoding pituitary gonadotropic hormones (*Cga*, *Lhb*, and *Fshb*) as well as gonadotropin-releasing hormone receptor (*Gnrhr*) was studied. Results showed increased transcription of pituitary *Gnrhr*, *Cga*, and *Lhb* in P42, P49, and P90 compared to P35 (**Figures 2D–F**). Living in constant light reduced gene expression in P35, P42, and P49 but was equated with control values in Leydig cells from adult rats (**Figures 2D–F**). Also, *Fshb* increased with age. The highest expression was detected in pubertal P49 (**Figure 2G**). LL reduced *Fshb* in P49, while in Leydig cells from adults, transcription reached control values (**Figure 2G**).

Growing Up Under Constant Light Changed the Transcriptional Pattern of Steroidogenesis-Related Genes

Further, the mRNA abundance of elements essential for steroidogenesis was analyzed in Leydig cells isolated from P35, P42, P49, and P90 rats that grow up in LD or LL conditions.

The analysis showed an age-associated increment of transcription of some Leydig cell biomarkers, including steroidogenic acute regulatory protein (*Star*), enabled cholesterol transport into the mitochondria and a set of androgen synthases, such as cytochrome P450 cholesterol side-chain cleavage (*Cyp11a1*), 3β-hydroxysteroid dehydrogenase 1/2 (*Hsd3b1/2*), cytochrome P450 17α-hydroxylase/17,20-lyase (*Cyp17a1*), 17β-hydroxysteroid dehydrogenase (*Hsd17b4*) and an androgen metabolizing enzymes, such as aromatase (*Cyp19a1*) (9). Living in constant light decreased transcripts of genes essential for androgen production: scavenger receptor Class B member 1 (*Sarb1*; involved in cholesterol delivery into cells) in Leydig cells from P42, P49, and P90 rats (**Figure 3A**); *Star* in P49 and P90 (**Figure 3B**); *Cyp11a1* in P42 to P90 (**Figure 3C**); *Hsd3b1/2* in P49 (**Figure 3D**); *Cyp17a1* in P42

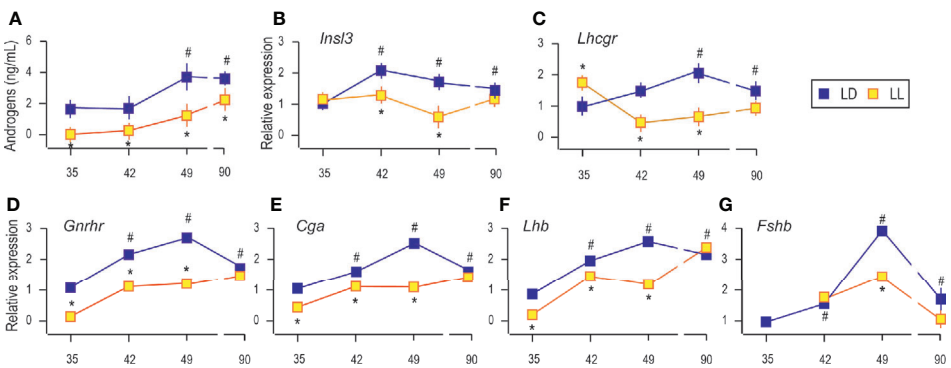

FIGURE 2 | Growing up under constant light reduced serum androgens, changed transcriptional pattern of Leydig cells' maturity markers and genes encoding pituitary gonadotropic hormones. Androgen levels were monitored in serum from rats that lived in LD or LL regime **(A)**. At a certain age Leydig cells were purified, RNA isolated, and qRT-PCR performed in order to measure expression of genes markers for Leydig cells' maturity/activity **(B, C)**. The RNAs were isolated from the pituitary of both groups, and the expression of genes encoding GNRHR **(D)** as well as subunits of gonadotrophic hormones **(E–G)** was measured. Data points represent group means ± SEM values (n = 5–8). *Statistical significance at level $p < 0.05$ compared to the corresponding LD group;#Statistical significance at level $p < 0.05$ compared to the P35 LD group.

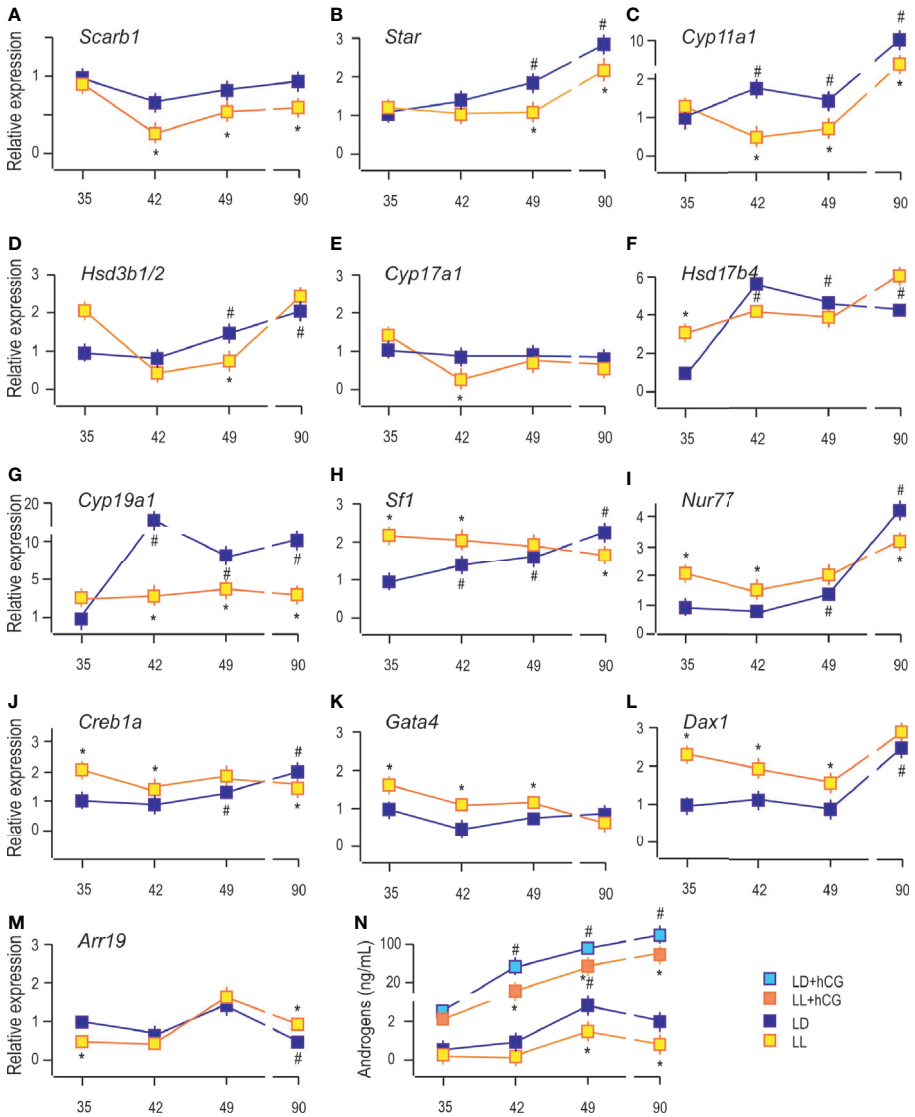

FIGURE 3 | Growing up under constant light changed transcriptional pattern of steroidogenesis-related genes. RNAs were isolated from purified Leydig cells and transcription of steroidogenic genes was monitored **(A–G)** together with transcription of positive **(H–K)** and negative **(L, M)** steroidogenic regulators. Leydig cells obtained from different LD and LL groups were challenged with/wo hCG (bind to LHR; 50 ng/ml) followed by androgen levels determination **(N)**. Data points represent group means ± SEM values (n = 5). *Statistical significance at level $p < 0.05$ compared to the corresponding LD group;#Statistical significance at level $p < 0.05$ compared to the P35 LD group.

(**Figure 3E**), and *Cyp19a1* in Leydig cells from P42 to P90 (**Figure 3G**). The *Hsd17b4* transcription was increased in P35 under the influence of constant light but without changes in other investigated age categories (**Figure 3F**).

Further, the qRT-PCR analysis revealed increased abundance of steroidogenic stimulators, *Sf1, Nur77*, and *Creb1a* (**Figures 3H–J**), as well as a steroidogenic repressor, *Dax1* (**Figure 3L**). In contrast, *Arr19*, a repressor of steroidogenic genes, decreased (**Figure 3M**) in Leydig cells from the adult testis. We could not detect significant changes in the transcriptional pattern of *Gata4* during pubertal maturation (**Figure 3K**). The LL condition stimulated *Sf1, Nur77*, *Creb1a, Gata4,* and *Dax1* in Leydig cells from P35 to P49 rats (**Figures 3H–L**). Still, in P90 Leydig cells, the steroidogenic stimulators (*Sf1, Nur77*, and *Creb1a*) were decreased (**Figures 3H–J**) and repressor, *Arr19*, increased (**Figure 3M**), supporting the observed attenuated transcription of steroidogenic genes. LL did not influence *Gata4* and *Dax1* mRNA levels (**Figures 3K, L**).

Further, to elucidate the resulting differences in maturity observed by the changed expression of *Lhcgr* and steroidogenic genes, Leydig cells obtained from P35 to P90 rats that lived in LD or LL regime were challenged with hCG. Leydig cells derived from rats that lived in the LL regime exhibited a reduced response to hCG stimulation compared to control LD rats

(**Figure 3N**). This was illustrated by the reduced increase of androgen production (**Figure 3N**) and suggested the attenuated steroidogenic machinery level.

Constant Light Changed Leydig Cell's Mitochondrial Function

In Leydig cells, the steroid production begins in the mitochondria enabled by cholesterol availability and mitochondrial steroidogenic enzyme localization. For efficient steroid production, functional mitochondria are necessary (24), so some characteristics which provide insight into mitochondrial functionality were analyzed. Since mitochondrial membrane potential ($\Delta\psi$m) contributes to mitochondrial energetic and steroidogenesis (12), it was essential to analyze possible changes of $\Delta\psi$m in Leydig cells during puberty in LL conditions. Changes in the $\Delta\psi$m was detected by measuring TMRE fluorescence because TMRE fluorescence values are proportional to the magnitude of $\Delta\psi$m. The obtained results showed increased $\Delta\psi$m in Leydig cells during puberty and adulthood (**Figure 4A**), followed by an increase in the number of mtDNA copies estimated through *mtNd1/B2m* ratio (**Figure 4B**). Both data indicate the increased mitochondrial engagement needed for enlarged steroid production. Moreover, a positive correlation was observed between serum androgens and Leydig cells' mtDNA content (R = 0.801). However, life in LL conditions increased $\Delta\psi$m at the beginning of the pubertal period (P35 and

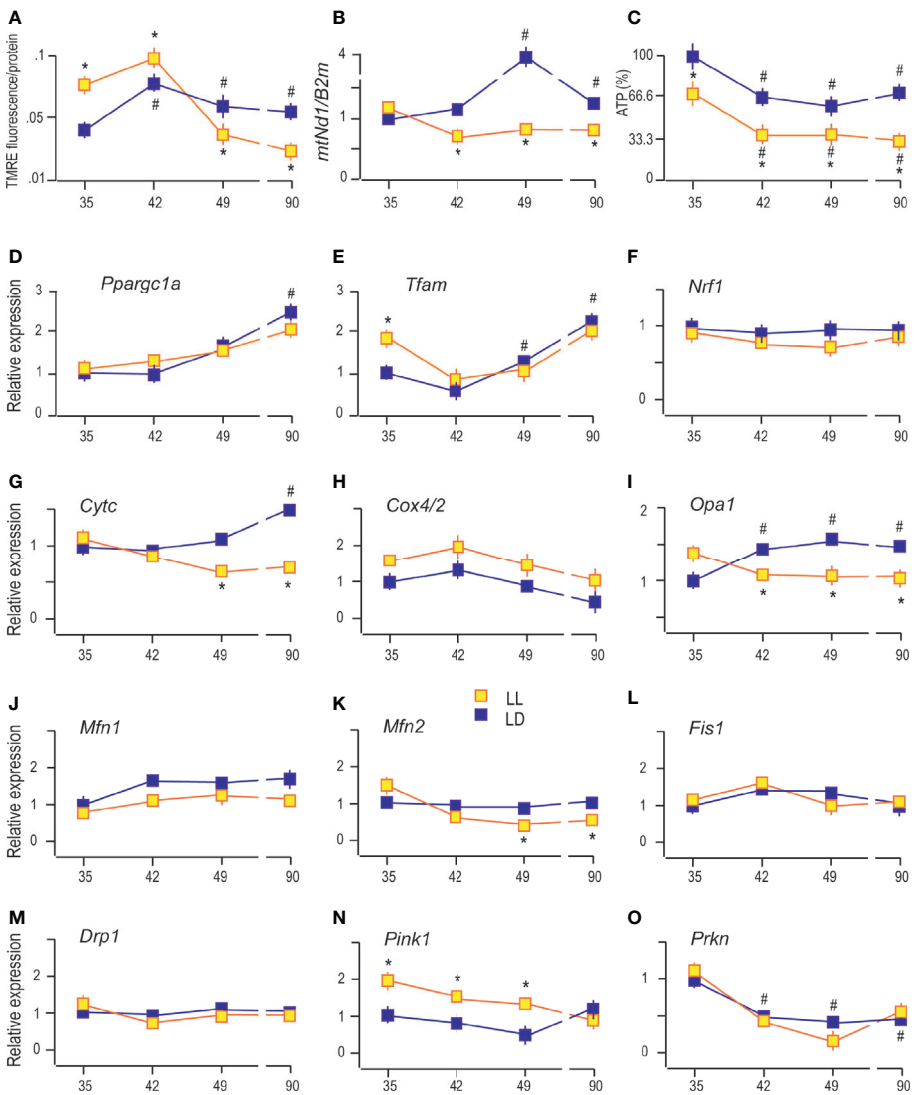

FIGURE 4 | Constant light changed Leydig cell's mitochondrial function. The $\Delta\psi$m was measured by using TMRE fluorescence **(A)**, followed by monitoring of mtDNA content estimated through *mtNd1/B2m* ratio **(B)** and analysis of ATP levels **(C)** in purified Leydig cells isolated from rats of different ages of both (LD and LL) groups. Also, transcriptional patterns of genes important for mitochondrial biogenesis **(D–F)**, genes encoding subunits of respiratory proteins **(G, H)**, mitochondrial fusion **(I–K)**, mitochondrial fission **(L, M)** and mitophagy **(N, O)** were monitored in the same cells. Data points represent group means ± SEM values (n = 5). *Statistical significance at level $p < 0.05$ compared to the corresponding LD group; #Statistical significance at level $p < 0.05$ compared to the P35 LD group.

P42) but was followed by subsequent reduction (P49 and P90) (**Figure 4A**). Additionally, LL caused a decrease in mitochondrial DNA content in the pubertal period and adulthood (**Figure 4B**).

On the other side, ATP production in Leydig cells gradually decreased with rats' ages (**Figure 4C**). Even more, LL reduced the production of ATP in Leydig cells from all four age categories that have been studied (**Figure 4C**). Decreased ATP, mtDNA, and $\Delta\psi m$ together with decreased steroid production support the hypothesis that growing up in LL suppresses mitochondrial function in Leydig cells.

It is well known that mitochondrial function is closely linked with fusion/fission (25), while mitochondrial mass is regulated by mitochondrial biogenesis and mitophagy (26). To see if puberty and growing under LL are accompanied by altered mitochondrial biogenesis or mitophagy and/or by changes in mitochondrial fusion/fission, the expression of genes involved in these processes was monitored.

The obtained results indicate gradually increased expression of *Ppargc1a* (main regulator of mitochondrial biogenesis and function) in Leydig cells from pubertal and adult rats compared with cells from prepubertal rats (**Figure 4D**). The same expression pattern showed its downstream gene *Tfam* (**Figure 4E**) and *Cytc* (**Figure 4G**), indicating increased expression of the markers of mitochondrial biogenesis. However, the expressions of *Nrf1* (activator of genes required for respiration) and *Cox4/2* (encoding subunit of respiratory protein) were not changed during sexual maturation (**Figures 4F, H** respectively). LL did not significantly change the transcriptional pattern of *Ppargc1a, Tfam, Nrf1, and Cox4/2* but decreased *Cytc* in P49 and P90 (**Figures 4D, E, F, H, G**, respectively).

Reproductive maturation in Leydig cells increased transcription of the main regulator of mitochondrial crista architecture and profusion gene, *Opa1* (**Figure 4I**), without effect on other profusion genes, *Mfn1* (**Figure 4J**) and *Mfn2* (**Figure 4K**). The profission *Fiss1* (**Figure 4L**) and *Drp1* (**Figure 4M**) were not changed during Leydig cell maturation. However, the LL regime changed the mitochondrial dynamics by reducing *Opa1* and *Mfn2* expression during puberty and adulthood without effect on *Mfn1*, *Fiss1*, and *Drp1* (**Figures 4I–M**).

Further, sexual maturation has affected Leydig cells' mitophagy by reducing transcription of *Prkn* (**Figure 4O**) without effect on *Pink1* (**Figure 4N**). However, life under LL conditions stimulated *Pink1* (**Figure 4N**) in Leydig cells from P35 and P42 and P49 rats. The obtained results suggest that growing up under LL conditions stimulates mitophagy and inhibits mitochondrial biogenesis in Leydig cells leading to decreased mitochondrial mass.

Constant Light Changed the Expression of Clock Genes in Leydig Cells

Leydig cells are known to have a rhythmic endocrine function in addition to the rhythmic expression of clock genes (4, 27). To estimate the effect of LL on the Leydig cells' clock, qRT-PCR analysis of the canonical clock gene expression was done (**Figure 5**).

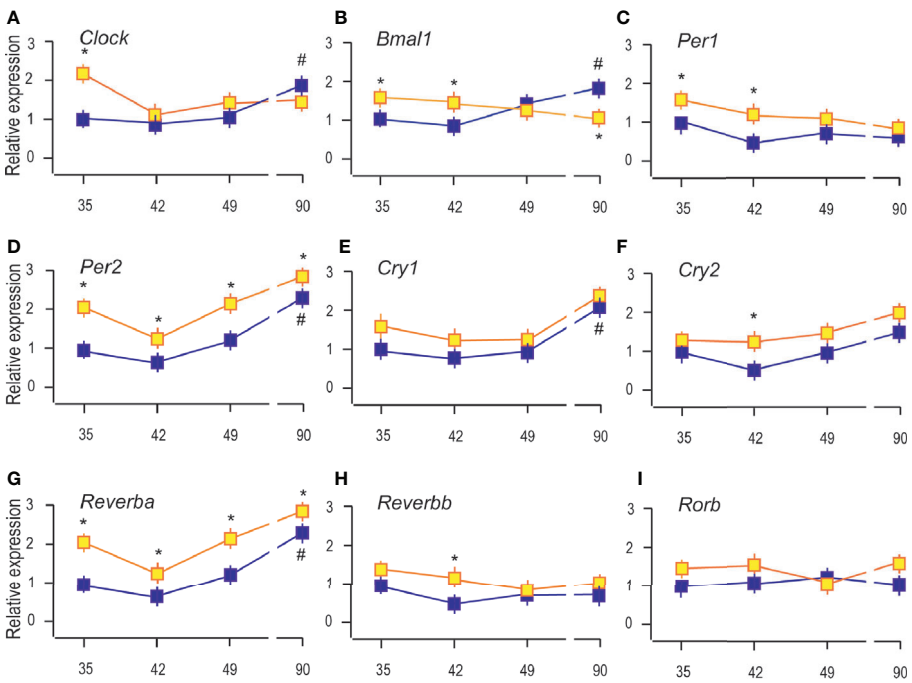

FIGURE 5 | Constant light changed the expression of clock genes in Leydig cells. RNAs were isolated from Leydig cells obtained from rats of different ages of both (LD and LL) groups and transcription of clock genes were estimated by qRT-PCR. The transcription of positive clock elements **(A, B)** and negative elements **(C–F)** from primary clock loop together with elements from the secondary loop **(G–I)** was shown. Data points represent group means ± SEM values (n = 5). *Statistical significance at level $p < 0.05$ compared to the corresponding LD group; #Statistical significance at level $p < 0.05$ compared to the P35 LD group.

Clock genes' expression was estimated 1 h after lights were turned on in the LD group. The gene expression in Leydig cells from P42, P49, and P90 was compared with P35 rats.

The results showed that reproductive maturation increased transcription of clock positive elements (*Clock* and *Bmal1*; **Figures 5A, B**) but also of negative elements (*Per2*, *Cry1*, and *Reverba*) (**Figures 5D, E, G**), suggesting that clockwork in Leydig cells depends on cell maturation. However, *Per1*, *Cry2*, *Reverbb*, and *Rorb* were not significantly changed during Leydig cell maturation (**Figures 5C, F, H, I**). The LL increased the expression of *Clock* and *Bmal1* in the Leydig cells from P35 and P42, but *Bmal1* was reduced in adult Leydig cells from P90 rats (**Figures 5A, B**). Transcriptional level of *Per2* and *Reverba* was increased in all the investigated age-categories (**Figures 5D, G**) while *Cry2* and *Reverbb* increased in Leydig cells from pubertal P42 rats (**Figures 5F, H**). The results indicate the stimulatory effect of LL on clock repressive elements *Per2*, *Cry1*, and *Reverba* in immature and adult Leydig cells and inhibitory on positive *Bmal1* in adult Leydig cells and propose a disruption of the clock and endocrine rhythm in Leydig cells.

Growing Up Under Constant Light Increased Blood Corticosterone and Changed the Transcriptional Pattern of Glucocorticoid-Signaling Elements

Since living in constant light could activate chronic stress-response, the blood corticosterone was measured. The results revealed increased corticosterone levels in the blood of rats which lived under continuous lighting (**Figure 6A**). The ratio between testosterone and corticosterone (T/C) in LL decreased (**Figure 6B**) suggesting possible connection with registered decreased activity (**Figure 1C**). Also, the results indicated higher T/C in the course of growth in both groups (**Figure 6B**).

Further, since corticosterone action is mainly mediated by the glucocorticoid receptor (GR), the transcription of gene-encoded GR (*Nr3c1*) was monitored in the pituitary and Leydig cells. Pituitary Nr3c1 was increased in P35–P49 under constant light, while in P90 the difference in respect to controls was not observed (**Figure 6C**). The Leydig cells' *Nr3c1* was increased in P35 and decreased in P90 (**Figure 6D**).

Nevertheless, the dehydrogenase activity of enzymes 11BHSD1/2 has been showing to protect Leydig cells from the harmful effects of excessive glucocorticoid exposures (28, 29). In that respect, transcription analysis showed increased *Hsd11b1* in Leydig cells during rat growing up, so that it reached the highest level in P90 (**Figure 6E**). LL increased *Hsd11b1* in P35 and P42, but it was decreased in P90 (**Figure 6E**). The *Hsd11b2* transcription did not change during the course of growing up and living in LL conditions (**Figure 6F**).

Finally, we examined the effects of LL on the expression of genes encoding MNTR1A in the pituitary and Leydig cells. Our previous work showed that the *Mntr1a* and *Mntr1b* were transcribed in the hypothalamus and pituitary, although *Mntr1b* is less prevalent. In adult Leydig cells, transcription of *Mntr1a* was at the level of significance, although low transcription of *Mntr1a/b* was detected in testicular tissue (4). Living in LL decreased *Mntr1a* in the pituitary in the peripubertal period (P42 and P49) (**Figure 6G**), while in Leydig cells, it was increased in P35, P42, and P49 (**Figure 6H**).

DISCUSSION

It is enthroned knowledge that temporal organization is essential for maintaining good body physiology and health (1). The circadian system needs to be reset every day by environmental cues such as light/dark cycle, temperature changes, or food availability to synchronize body function with habitat conditions. Without external signals, in constant conditions, the circadian system will oscillate with its endogenous period, the so-called

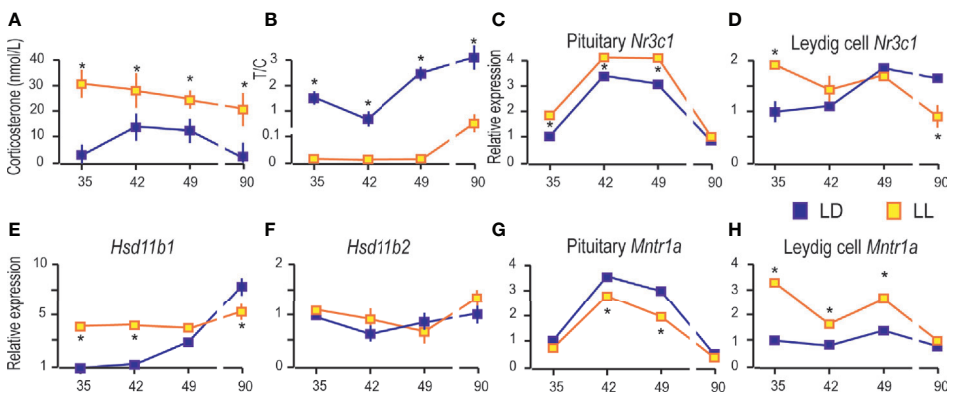

FIGURE 6 | Growing up under constant light increased blood corticosterone and changed the transcriptional pattern of glucocorticoid-signaling elements. Corticosterone levels were monitored in serum from rats that lived in LD or LL regime **(A)**. The blood testosterone/corticosterone (T/C) ratio is presented during growing up **(B)**. At a certain age Leydig cells were purified, RNA isolated, and qRT-PCR performed in order to measure expression of genes involved in corticosterone signaling: pituitary *Nr3c1* **(C)**, Leydig cells' *Nr3c1* **(D)** and genes encoded local regulators of glucocorticoid levels **(E, F)**. Relative mRNA expression of pituitary **(G)** and Leydig cells *Mntr1a* **(H)**. Data points represent group means ± SEM values (n = 5–8). *Statistical significance at level $p < 0.05$ compared to the corresponding LD group.

free-running period (1). Herein the consequences of growing up in constant conditions, *i.e.*, without temporal cues, on the development of Leydig cells' endocrine capacity were studied. Our results indicate delayed maturation of Leydig cells in free-running conditions. The effect is most pronounced during puberty, although the consequences are also observed in adulthood.

Reproduction is essential for the survival and perpetuation of species. Hormones of the neuroendocrine regulatory circuit govern reproductive development with hierarchical cascades of regulatory feedback loops (30). In male reproductive development, puberty is a critical period that entirely depends on Leydig cells' steroidogenic capacity increased by the awakening of the reproductive axis. Increased gradual expression of *Lhcgr* sensitizes cells' to LH to recruit steroidogenic regulators, whose expression is also growing during puberty (*Creb1a*, *Sf1, Nur77*), to stimulate steroidogenic genes (*Star, Cyp11a1, Hsd3b1/2, Hsd17b4*). On the other side, repression of steroidogenic genes decline, associated with *Arr19* lowering. Besides steroids, the Leydig cell lineage transitions are also associated with increased *Insl3*, a critical biomarker reflecting Leydig cell functional capacity (23). Additionally, the enlarged transcription of clock genes in the adult Leydig cells points to the regulatory role of reproductive axis hormones on the Leydig cells' circadian clock (31). Altogether, the Leydig cells' maturation increases the steroidogenesis (**Figure 7A**) and the level of androgens in the circulation, enabling the development of sex accessory organs and male phenotype.

It is known that exposure to constant light causes rhythmic clock activity changes in suprachiasmatic neurons and subsequently peripheral clocks in different body cells (32–34). The suprachiasmatic neurons sense the environmental light through the retinohypothalamic pathway and send information to peripheral clocks, including the Leydig cell clock, through many hormones such as LH and melatonin (4, 31). However, an integrated network of signals linking the suprachiasmatic neurons and peripheral oscillators, including Leydig cells, is presently not well understood. This study confirms that growing up in constant environmental conditions changed body rhythm, activated free-run activity model with period longer than 24 h, and altered the transcriptional pattern of Leydig cell's clock genes. In adult Leydig cells from rats growing up in LL, positive clock element *Bmal1* was down-regulated. Still, negative regulators, *Per2* and *Reverba*, were up-regulated, suggesting different clockwork in constant conditions and possibly changing the transcriptional pattern of clock-regulated genes. It is shown that BMAL1 (35) or REVERBa (36) could regulate the transcription of *Star* in steroidogenic cells.

In free-running conditions, the inhibition of pituitary *Gnrhr* and genes encoding gonadotropic hormones (*Cga, Lhb, Fshb*) in juvenile and peripubertal pituitary was detected. Accordingly, decreased serum LH was observed after prolonged exposure of rats to LL conditions (37). The observed reduction of pituitary *Cga/Lhb* is associated with decreased transcription of essential steroidogenic-related genes including *Scarb1*, *Star*, *Cyp11a1*, *Hsd3b*, all connected with cholesterol metabolism in the first steps of steroidogenesis (12) mostly operated in the mitochondria. Indeed, our results supported the significant role of the mitochondria in Leydig cell maturation.

However, despite the importance of the mitochondria in cellular and metabolic health, including steroidogenesis, the details about their organization and synchronization, especially during Leydig cells development, are not well characterized. Our results indicate increased mitochondrial biogenesis during Leydig cell maturation illustrated by increased primary mitochondrial biogenesis markers (*Ppargc1a, Tfam, Cytc*), namely, the *Ppargc1a*/PGC1a plays a crucial role in mitochondrial biogenesis through transcriptional regulation of its downstream genes such as *Nrf1*, *Nrf2*, and *Tfam*, leading to the synthesis of mitochondrial DNA, proteins, and generation of new mitochondria (38). Indeed, at puberty, the enlarged mtDNA content was observed coupled with increased mitochondrial membrane potential and likely increased fusion needed for efficient import necessary for steroidogenesis. However, ATP level in Leydig cells did not follow this upward trend.

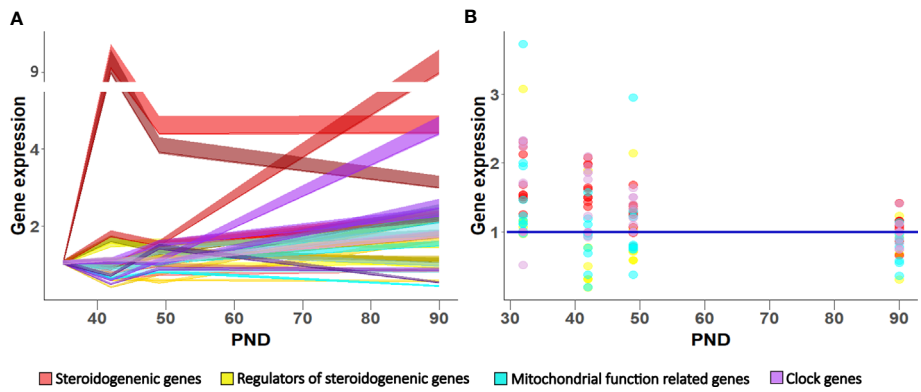

FIGURE 7 | Gene expression pattern in Leydig cells during maturation in LD **(A)** and LL condition **(B).** Data shown represent expression pattern of genes important for steroidogenesis, mitochondrial function and clock during Leydig cell maturation **(A)** relative to P35. Ribbons represent 95% confidence intervals. Effect of growing up in constant light regime on Leydig cell gene expression **(B)**. Points represent a deviation in the expression of a particular gene in LL condition in respect to corresponding LD (control value = 1; blue line).

On the contrary, the transitions along the Leydig cell lineage are associated with decreased cell energy capacity. Since for efficient steroidogenesis high polarized mitochondrial membrane is needed (24), results of increased steroidogenesis and decreased ATP production suggests dissociation of energetic and steroidogenic mitochondrial function. Progenitor Leydig cells are proliferative (9), having high metabolic energy requirements; with maturation, the number of divisions decreases, so the adult cells no longer divide, consequently associated with lower energy demand.

The mitochondrial fusion and fission regulate the formation of the mitochondrial network responsible for energetic and steroidogenic mitochondrial function (39). Both processes are enabled by the activity of several essential genes and their products, such are *Mfn1*/MFN1, *Mfn2*/MFN2, *Opa1*/OPA1, *Drp1*/DRP1, and *Fis1*/FIS1 (25). Mitofusion is a crucial step that allows transport of intermediate products in/out mitochondria and is essential for cholesterol import into mitochondria and steroid formation (13, 14, 40). When the reproductive axis wakes up or is active, in Leydig cells, transcription of *Opa1* increased likely involved in mitochondrial remodeling, including cristae shaping and serving as a regulator of cholesterol shuttling (41).

Living in LL, in addition to overall constant conditions, profoundly affects mitochondrial function in peri/pubertal and adult Leydig cells. The mtDNA decreases as well as *Cytc* (encoding subunit of respiratory protein), mitochondrial membranous potential, and ATP production. Since mitochondrial respiration produces around 80% of ATP in adult Leydig cells (22), decreased mitochondrial function significantly affects energy cell status. Decreased mitochondrial activity observed in Leydig cells from peripubertal and adult rats is supported by decreased *Opa1* and *Mfn2* with the potential to increase mitophagy due to increased *Pink1* suggesting unbalanced mitochondrial dynamics connected with lower steroidogenesis. Observed disturbed mitochondrial function in Leydig cells could arise as decreased LH signaling in LL condition, having a substantial effect on mitochondrial physiology (15).

However, living in LL is associated with cumulative impacts of the disturbed rhythmicity of many hormones important for reproductive function. Indeed, in LL the sustained increased levels of blood corticosterone were observed, reflecting chronic stress condition. Long-term glucocorticoid exposure becomes maladaptive, leading to a broad range of disorders, including metabolic syndrome and obesity (42). Our results pointed to decreased T/C ratio in the LL regime, indicating an altered behavior and reduced activity. The balance of testosterone as anabolic and corticosterone as a catabolic hormone could be used as a physiological stress biomarker. In humans, the increased T/C has been associated with aggression and social dominance (43). Anyway, it has long been recognized that increased corticosterone may reduce testosterone production by inhibiting steroidogenic enzyme expression and activity (44) in addition to Leydig cell apoptosis activation (45). Still, Leydig cells from juvenile and peripubertal rats could be protected from the adverse corticosterone effect due to the increased level of *Hsd11b1*. The HSD11B1 is a bi-directional oxidoreductase that inactivates biologically active glucocorticoid or activates inert metabolite into functional form and thus acts as a local regulator of glucocorticoid levels (29). However, in rat Leydig cells HSD11B1 changes from a primary reductase to predominant dehydrogenase during pubertal maturation (46). In adult Leydig cells from LL rats, the observed lower *Hsd11b1*expression may contribute to decreased testosterone production.

Additionally, melatonin is a principal darkness hormone with various physiological and metabolic functions, including influence on body weight, plasma insulin and leptin levels (47), and modulation of energetic metabolism (48). In rat males kept under LL conditions, low melatonin levels have been shown regardless of circadian time (49). Low blood melatonin is associated with decreased activity, increased visceral adiposity, and disturbed circadian rhythm and behavioral parameters. Interestingly, such effects, including melatonin secretion and activity pattern, were not observed in rats living in the continuous dark (49). Our previous results did not support direct melatonin effect on Leydig cells through melatonin receptors, but melatonin deprivation exerted a positive effect on steroidogenic and Leydig cells' clock genes (4). In the present study, increased *Mntr1a* transcription was detected in Leydig cells from immature and peripubertal rats that lived in LL conditions.

In conclusion, the data showed that during Leydig cell differentiation, the increased mitochondrial biogenesis occurred together with the cells' increased ability to produce testosterone. However, up-regulated mitochondrial biogenesis is not related to increased energetic cell capacity, suggesting dissociation of the mitochondria's energetic and steroidogenic function during Leydig cell maturation. Growing up in a constant (LL) environment changed the circadian system and slowed down Leydig cells' maturation by reducing the endocrine and energy capacity of cells, which led to a delay in reproductive development. Leydig cells responded to the free-run challenge by altered expression patterns of genes related to steroidogenesis, mitochondrial dynamics, and clock (**Figure 7B**), leading to unbalanced steroidogenesis, especially in the mitochondrial portion.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by the Local Ethical Committee on Animal Care and Use of the National Council for animal welfare and the National Low for Animal Welfare (No. 323-07-0-08975/2019-05).

AUTHOR CONTRIBUTIONS

DM—acquisition of the data, analysis and interpretation of the data, revising manuscript critically for important intellectual

content. MM—acquisition of the data, analysis and drafting the figure, revising manuscript critically for important intellectual content. AB—acquisition of the data, analysis and interpretation of the data. SA—acquisition of the data, analysis and interpretation of the data, revising manuscript critically for important intellectual content. TK—the conception and design of the research, acquisition of the data, analysis and interpretation of the data. drafting the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2021.653602/full#supplementary-material

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REVIEW
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Dynamic Interactions Between the Immune System and the Neuroendocrine System in Health and Disease

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The immune system and the neuroendocrine system share many common features. Both consist of diverse components consisting of receptors and networks that are widely distributed throughout the body, and both sense and react to external stimuli which, on the one hand control mechanisms of immunity, and on the other hand control and regulate growth, development, and metabolism. It is thus not surprising, therefore, that the immune system and the neuroendocrine system communicate extensively. This article will focus on bi-directional immune-endocrine interactions with particular emphasis on the hormones of the hypothalamus-pituitary-thyroid (HPT) axis. New findings will be discussed demonstrating the direct process through which the immune system-derived thyroid stimulating hormone (TSH) controls thyroid hormone synthesis and bone metamorphosis, particularly in the context of a novel splice variant of TSHβ made by peripheral blood leukocytes (PBL). Also presented are the ways whereby the TSHβ splice variant may be a contributing factor in the development and/or perpetuation of autoimmune thyroid disease (AIT), and how systemic infection may elicit immune-endocrine responses. The relationship between non-HPT hormones, in particular adipose hormones, and immunity is discussed.

Keywords: systemic regulation and adaptation, Hashimoto disease, hematopoiesis, thyroid hormones, integrated

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INTRODUCTION

In its most elemental form, homeostasis can be viewed as an integrated state of equilibrium between various physical and chemical processes, not only within individual organ systems, but also throughout the body overall. Whereas most biomedical research is conducted from a highly-focused reductionist perspective given the inherent difficulties in attempting to measure and quantify multifaceted processes, there is nonetheless a need to continually reflect on the vast cross-talk of organ systems in the body.

The immune system and the neuroendocrine system both consist of widely-distributed tissues, cells, receptors, ligands, and molecules. Moreover, both systems are highly adapted to sense external signals from the environment, and to communicate information regarding those throughout the body. It is perhaps not surprising, therefore, that the immune system and the neuroendocrine

system interact broadly at many levels. In fact, the immune system and the neuroendocrine system collectively have been referred to as a "sixth sense" based on shared neuropeptides and neurotransmitters used by the immune system (1). One of many examples of this is the dynamic set of interactions between the immune system and the neuroendocrine system in the gut. In fact, there are at least three mechanisms for detecting changes in the intestinal wall, consisting of neural sensation delivered by extrinsic and intrinsic afferent neurons, more than twenty endocrine hormones produced by the cells of the mucosal epithelium, and immune responses to local and systemic antigens (2). Collectively, these form a web of communication and defense at the level of the gut. However, many other examples of this exist, as will be discussed in the following sections.

That TSH is produced by cells of the immune system was first reported almost forty years ago (3, 4). TSH is also produced by mouse intestinal crypt enterocytes and intestinal leukocytes, particularly in "hotblocks" of experimental rotavirus and reovirus infection (5, 6). Two sets of findings opened the way for understanding a potential role for immune system TSH. First, hypophysectomized mice that are unable to make pituitary TSH had elevated levels of T4 following alloantigen priming similar to that of non-hypophysectomized animals (7). Second, bone marrow (BM) hematopoietic cells and PBL were found to produce a novel splice variant of TSHβ (8), as discussed in detail below.

BIDIRECTIONAL IMMUNE-ENDOCRINE INTERACTIONS OF THE HPT AXIS

The HPT axis is a critically-important hormone network for maintaining basal metabolism, growth, development, mood, and cognition. TSH is released into the circulation from the anterior pituitary followings thyrotropin releasing hormone (TRH) stimulation from the hypothalamus. TSH binds to and induces the release of the thyroid hormones (TH) thyroxine (T4) and triiodothyronine (T3) from the thyroid after binding to TSH receptors (TSHR), a seven-transmembrane domain G-protein coupled molecule on thyroid follicular cells. The majority of T4 is converted into the more biologically active T3 form following deiodination in target tissues after binding to thyroid hormone transporters (9, 10). The TSHR is also widely-distributed across many tissues outside the HPT axis (11).

Thyroid hormones have been shown to exert pleiotropic effects on PBL and on the inflammatory response. Early studies demonstrated that thymic peptides such as thymopoietin, thymulin, and thymosin produced by the thymic epithelium can have a positive effects on the secretion of hormones from the adenohypophysis (12). It was demonstrated in a series of studies that the thyroid is extensively involved in the maturation of the thymus (13–15). Conversely, THs have been shown to upregulate thymulin secretion (14). Exposure of T cells to TH has time dependent effects in that short-term exposure results in suppressed proliferation and apoptosis, whereas long-term exposure induces T cell proliferation. This appears to be regulated at least in part by activation of inducible nitric oxide synthetase (iNOS) (16–19). B cells respond differently to THs in that exposure induces development and cell-proliferation *in vivo* (20). T3 has direct effects on the maturation of macrophages into the M1 and M2 forms (21). T4 also has beneficial effects on the recovery from *Neisseria meningitidis* infection, mediated by iNOS production and nitric oxide mobilization (22). T4 blocks macrophage inhibitory factor proinflammatory activity *in vivo* and enhances survival of mice with induced sepsis (23, 24). The TSHR is expressed at high levels on a subset of murine dendritic cells (DCs), though it is minimally expressed on T cells and B cells. However, for reasons that are unclear, the TSHR is expressed on more lymph node T cells and B cells than on spleen cells (25). TSH enhances the phagocytic activity of DCs (25). TH have complex effects on the development and function of DCs, macrophages, and monocytes. Studies in which hypothyroid patients were treated with exogenous TH had increases in both plasmacytoid and myeloid DCs (26).

Adipose hormones such as adiponectin and leptin, which regulate metabolism and energy efficiency, also influence immunological function *via* receptors expressed on immune cells, particularly on M2-differentiated macrophages (27). Adiponectin has direct immunoregulatory activity by inhibiting the secretion of proinflammatory cytokines and increasing immunosuppressive cytokines (28, 29). Mice deficient in adiponectin fail to effectively modulate metabolic homeostasis (30). Leptin increases immune cell development, chemotaxis, and cytokine secretion (31, 32). Moreover, M1 and M2 macrophages in adipose tissues have opposing effects on insulin responses in that M1 macrophages promote insulin resistance whereas M2-macrophages enhance insulin sensitivity (33, 34). Invariant NKT (iNKT) cells and mast cells are present in adipose tissues (35, 36). Both of those are distinguished by their ability to rapidly respond to danger signals and to produce proinflammatory and regulatory cytokines. iNKT cells, in particular, are known to be a significant source of IFN-γ, IL-2, IL-4, IL-13, IL-17, and IL-21, as well as TNFα and GM-CSF, among others (37), all of which have important immunoregulatory activities and functions.

A NOVEL TSHB ISOFORM PRODUCED BY THE CENTRAL AND PERIPHERAL IMMUNE SYSTEM

TSH is one of three glycoprotein hormones made in the anterior pituitary. All glycoprotein hormones share a common α-chain molecule and a unique hormone-specific β-chain component. TSHβ is highly conserved across many mammalian species. Until recently, no functional isoforms of TSHβ had been identified. We characterized a unique in-frame splice variant of TSHβ (referred to as TSHβv), which is copiously made by PBL and BM hematopoietic cells, in particular though not exclusively on myeloid cells (8, 38–40). Notably, TSHβv is stored in intracellular secretory vesicles in macrophages (39), a property that would facilitate rapid release under appropriate conditions. In that context, it will be interesting to define the signals that drive the release of intracellular TSHβv.

TSHβ is coded for by exons 2 and 3 in humans and exons 4 and 5 in mice. The splice variant is unique, however, in that in both species only the second of the two exons is used to code for TSHβv, with a small portion of the upstream intron coding for a signal peptide (**Figure 1**). Predictions as to the mechanisms of alternative splicing of TSHβ in leukocytes leading to the generation of TSHβv are derived from putative donor and acceptor splice sites in human intron 1 and intron 2, respectively, resulting in the elimination of exon 2 and the retention of an intron 2 associated signal peptide (**Figure 2**) (41).

TSHβv has been shown to be present in the human circulation (42), and to be functionally active based on cAMP signaling (8, 39) as well as to successfully couple to TSHα (42), a condition considered to be essential to achieve full biological activity (43). Moreover, TSHβv has been shown to induce TH synthesis *in vivo* and *in vitro*. T3 and T4 were elevated in the circulation of mice within one hour of injection of recombinant TSHβv, and to induce the secretion of T3 and T4 from thyroid follicular cells *in vitro* (44). What's more, levels of thyroglobulin, thyroid peroxidase, and sodium-iodide supporter were elevated in thyroid follicular cells following TSHβv stimulation. Of particular interest, injection of mice with T3 and TRH caused a transient drop followed by an increase in native TSHβ though not in TSHβv in the pituitary (44).

Expression of TSHβv has been linked to the inflammatory response in AIT, in particular in Hashimoto's thyroiditis (HT), as demonstrated by elevated transcript levels of TSHβv in PBL of

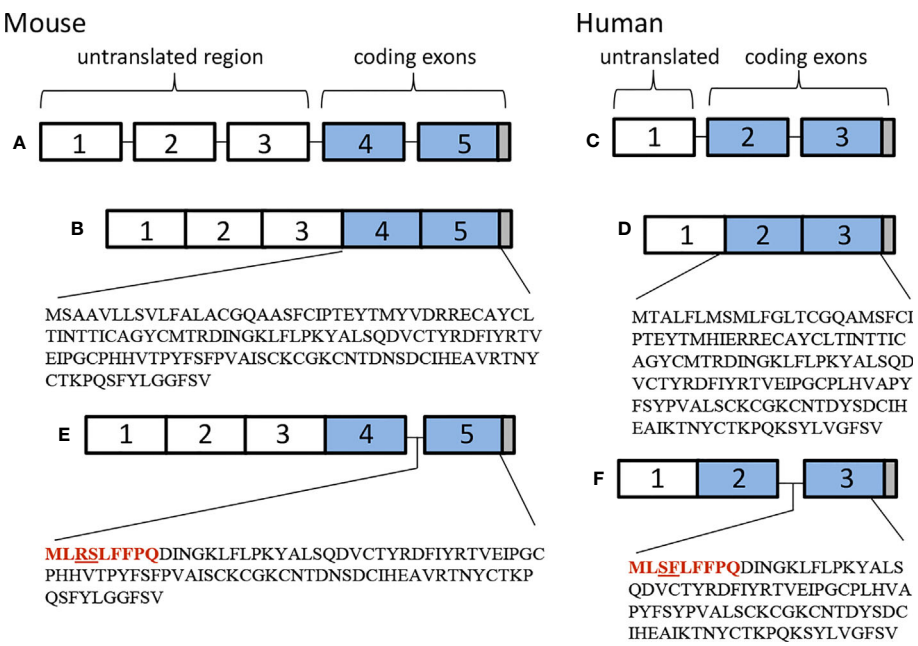

FIGURE 1 | Genetic organization of **(A, B)** mouse and **(C, D)** human native TSHβ, and **(E)** mouse and **(F)** human TSHβv.

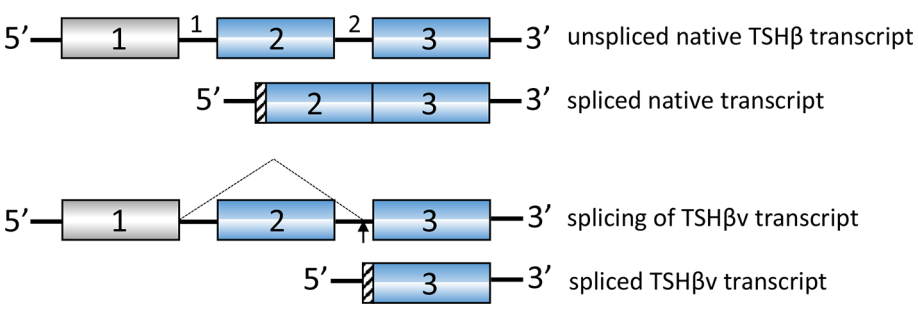

FIGURE 2 | Putative splicing mechanism used to generate human TSHβv in cells of the immune system. Donor splice sites in intron 1 and acceptor splice sites in intron 2 remove exon 2. A portion of intron 2 is used for the signal peptide as shown in **Figure 1**.

patients with HT compared to normal controls (42). Treatment of patients with prednisone reduced TSHβv transcript levels in persons with short duration of disease compared to persons with long duration. Additionally, TSHβv-producing plasma cells infiltrated the thyroid in HT patients (40). Recent studies demonstrate that immune system TSHβv in humans operates independently of the HPT axis and is capable of inducing TH synthesis from PBL in times of immune stress, such as during systemic infection (44). Those possibility conforms to finding in mice showing that TSHβv-producing inflammatory cells traffic to the thyroid following *L. monocytogenes* infection (38). Moreover, spleen cells from bacteria-infected mice, but not from non-infected mice, trafficked to the thyroid of normal non-infected mice at high density 48 hours post-transfer (**Figure 3**) (38). The connection between infection and AIT, while interesting, is unclear due in part to a lack of sufficient studies to draw definitive conclusions (45). Taken together, however, these findings suggest that under certain conditions TSHβv may contribute to the pathogenesis of HT and possibly other forms of AIT.

TSH has been shown to directly influence bone remodeling *via* TSHR expressed on osteoclasts by preventing bone resorption (46) and stimulating osteoblastic bone formation (47). In humans, there is an increased risk of bone fracture in women with low circulating TSH (48). Using *Tshr*$^{-/-}$ mice, which are incapable of TSH signaling, and WT mice that were induced to a state of hyperthyroidism by implantation of T4 pellets, *Tshr*$^{-/-}$ mice had significantly greater bone loss (49), further suggesting a role for TSH in bone restructuring. Moreover, expression of TSHβv in BM CD11b^{+} cells was positively rather than negatively regulated by *in vivo* T3 supplementation (49). This was further confirmed using human BM-derived macrophages, which had an increase in TSHβv following exposure to T3 in a dose-dependent manner (50). Those findings further indicate that the regulation of TSHβv by TH occurs independently of HPT axis control.

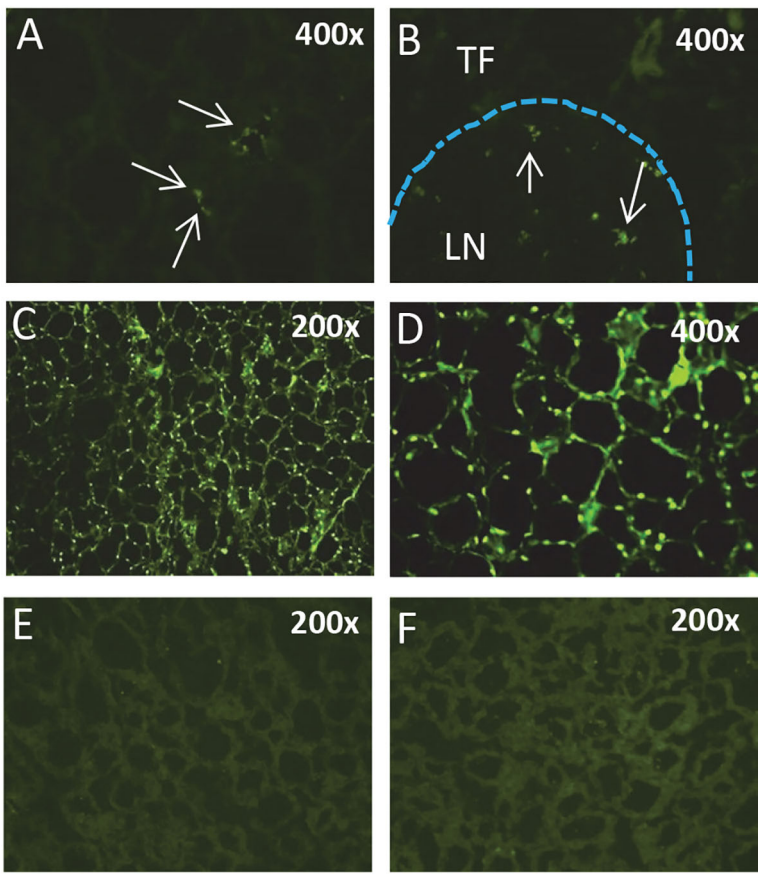

FIGURE 3 | Splenic leukocytes from *L. monocytogenes*-infected mice but not normal mice traffic to the thyroid. Immunofluorescence analysis of **(A)** the thyroid and **(B)** a thyroid perivascular lymph node from a non-infected mouse 24 hours post-cell transfer of CFSE-labeled splenic leukocytes from a *L. monocytogenes*-infected mouse. **(C, D)** Thyroid of a non-infected mouse 48 hours post-transfer of spleen cells from a *L. monocytogenes*-infected mouse. CFSE-labeled leukocytes are present surrounding thyroid follicles. **(E, F)** Thyroid of a non-infected mouse injected with CFSE-labeled spleen cells from a non-infected mouse. TF, thyroid follicle; LN, lymph node.

SUMMARY AND CONCLUSIONS

Over the past forty years, a large body or information has come forth defining an intricate nexus between the immune system and the endocrine system. Immune-endocrine pathways have effects on normal as well as pathophysiological processes, some of which is mediated by a novel alternatively-spliced form of TSHβ produced by the hematopoietic system. Indeed, a number of studies remain to be done to fully understand the biological implications of immune system TSHβ cell signaling in the thyroid and bone. For example, the extent to which native TSH and TSHβv work synergistically or antagonistically in delivering TSHR-mediated signals may provide important information into the specific role of TSHβ in AIT and osteoporosis.

AUTHOR CONTRIBUTIONS

JK is the sole author to all aspects of this article.

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Neuroimmune Interactions and Rhythmic Regulation of Innate Lymphoid Cells

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The Earth's rotation around its axis, is one of the parameters that never changed since life emerged. Therefore, most of the organisms from the cyanobacteria to humans have conserved natural oscillations to regulate their physiology. These daily oscillations define the circadian rhythms that set the biological clock for almost all physiological processes of an organism. They allow the organisms to anticipate and respond behaviorally and physiologically to changes imposed by the day/night cycle. As other physiological systems, the immune system is also regulated by circadian rhythms and while diurnal variation in host immune responses to lethal infection have been observed for many decades, the underlying mechanisms that affect immune function and health have only just started to emerge. These oscillations are generated by the central clock in our brain, but neuroendocrine signals allow the synchronization of the clocks in peripheral tissues. In this review, we discuss how the neuroimmune interactions create a rhythmic activity of the innate lymphoid cells. We highlight how the disruption of these rhythmic regulations of immune cells can disturb homeostasis and lead to the development of chronic inflammation in murine models.

Keywords: circadian rhythm, neuroimmune interactions, homeostasis, inflammation, neuropeptide

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INTRODUCTION

The innate immune system is often seen as the first line of defense against invading pathogens, but it is now evident that they also carry out homeostatic functions by regulating essential pathways involved in tissue repair, mucosal barrier function, and metabolism. These functions have been particularly highlighted with the discovery of the innate lymphoid cells (ILCs) in early 2010's (Vivier et al., 2018). In contrast to B and T lymphocytes, ILC activity is not modulated by antigen-specific receptors but by a dynamic integration of host-derived physiological signals (Seillet and Jacquelot, 2019). The ILC family comprises NK cells, ILC1, ILC2, and ILC3. Their constitutive activity at the body's barrier surfaces ensures the maintenance of tissue homeostasis and immune protection (Vivier et al., 2018). ILC1 and NK cells are mainly involved in responses against intracellular pathogens and tumor surveillance (Seillet et al., 2020a). ILC2 initiate type-2 immune responses which are critical to allergy and anti-parasite responses (Fallon et al., 2006). They also mediate tissue repair through the secretion of amphiregulin (Monticelli et al., 2011). Enhanced ILC2 function in the lung is associated with asthma (Chang et al., 2011) while in adipose tissue, decreased ILC2 cytokine production is associated with obesity and insulin resistance

(Molofsky et al., 2013). ILC3 are greatly enriched in the intestine where they mediate anti-bacterial responses (Sawa et al., 2011). They produce the interleukin (IL)-22 which promotes colonization of the gut by beneficial commensal bacteria that protect against intestinal inflammation (Pickard et al., 2014). Decreased ILC3 functions are associated with impaired capacity to maintain the mucosal barrier intact (Satoh-Takayama et al., 2014). Recent studies have shown that ILC responses are modulated by rhythmically expressed neuropeptides. These recent advances could contribute to the understanding of the mechanisms that leads to increased incidence of chronic inflammatory diseases when circadian rhythms are disrupted.

Circadian rhythms are endogenous oscillations with a period close to 24 h. They are found in almost all living organisms. The temporal alignment of the internal physiology with the external environment is critical for survival and the evolution of species. Circadian rhythms are found in virtually all cells of the body and function autonomously. However, these oscillations need to be synchronized with the environment, and external signals such as the light, temperature, and food intake (Reppert and Weaver, 2002). Therefore, our lifestyle, physical activity, and feeding times are important components for the robustness of these rhythms. Light sensed by the retina is connected to the suprachiasmatic nucleus (SCN) in the hypothalamus. The SCN is also known as the master clock and is responsible for entraining peripheral circadian clocks distributed across the organism. All peripheral clocks are synchronized daily and coordinated by the SCN via the hypothalamic pituitary adrenal (HPA) axis and the autonomic nervous system (ANS; Gnocchi and Bruscalupi, 2017; **Figure 1**). Food intake can be aligned to natural feeding rhythms induced by the SCN and contribute to synchronize the peripheral clocks. Food intake can also be desynchronized with the SCN due to environmental changes, such as food restriction or temporally altered behavior.

Circadian rhythms are generated in SCN neurons using transcriptional feedback loops that take 24 h to complete. In mammals, the main loop is initiated by the transcription factors CLOCK and BMAL1 (Reppert and Weaver, 2002). These proteins induce the expression of period genes (Per1, Per2, and Per3) and cryptochrome genes (Cry1 and Cry2) that will then inhibit the expression of CLOCK and BMAL1 genes (Xu et al., 2015). This core feedback loop is modulated by additional transcription factors involving Rev-Erbα and the retinoic acid receptor (RAR)-related orphan receptor (ROR) family that ensures the rhythmic expression of BMAL1 (Sato et al., 2004; Yanofsky et al., 2013). Finally, the D-box binding protein (DBP) can activate BMAL1 while Nfil3 will inhibit the expression of clock genes (Mitsui et al., 2001). These transcription factors not only regulate the expression of their own inhibitors but also drive the rhythmic accumulation of target genes also known as clock-controlled genes (Zhang et al., 2014).

In addition to this molecular regulation, the central clock acts as a pacemaker that temporally aligns the peripheral clocks through neural outputs from the ANS, glucocorticoid (GC) hormones and a variety of neuropeptides released by nerves within tissues. While the regulation of ILC by the circadian molecular clock genes have been recently reviewed elsewhere (Wang and Colonna, 2020), we will highlight how the nervous and immune systems interact together. We will discuss the impact of neurohormones on immune cell activity and how they can potentially rhythmically modulate ILC responses.

SYMPATHETIC AUTONOMOUS NERVOUS SYSTEM AND THE HYPOTHALAMIC–PITUITARY–ADRENAL AXIS

Adrenergic System

The adrenergic system is regulated by the sympathetic nervous system (SNS), through the production of catecholamines [epinephrine (EP), norepinephrine (NE)], and via HPA axis. Circulating catecholamine levels exhibit circadian rhythmicity, with EP levels typically rising during the day and falling at night in humans (Linsell et al., 1985) and in an opposite way in rats (De Boer and Van der Gugten, 1987; Schiffer et al., 2001) while data in mice are lacking. EP and NE engage β1- and β2-adrenergic receptors (β2AR), which are commonly found on bronchial smooth and cardiac muscles. Modulating β-adrenergic receptor signaling is, therefore, a common pharmacological strategy used for treatment of asthma (Sears et al., 1990) and cardiovascular conditions (Milano et al., 1994).

The activation of β2AR in human NK cell line inhibits TNF-α, IFN-γ, granzyme B, and perforin expressions (Sun et al., 2018). In humans, EP induces the mobilization of cytotoxic lymphocytes including the NK cells in the circulation (Dimitrov et al., 2010) which could explain the increased number of circulating NK cells during the active period when EP peaks (Dimitrov et al., 2007). In mice, adrenergic signaling inhibits the cytotoxic activity of the NK cells (De Lorenzo et al., 2015), controls NK cell expansion during viral infection (Diaz-Salazar et al., 2020), and inhibits IFN-γ production in hepatic NK cells (Wieduwild et al., 2020). This inhibition was not observed in ILC1 in the liver or splenic NK cells, suggesting a tissue and cell-specific control. In the absence of β2AR signaling, liver NK cells had higher IFN-γ production that resulted in increased resistance to infections associated with better control of viral replication and reduced tissue damage (Wieduwild et al., 2020). A rhythmic expression of TNF-α, IFN-γ, and granzyme B has been observed in NK cells (Logan et al., 2011). Logan et al. reported that levels of NE peaked in the morning in the spleen of rats, and found that the levels of TNF-α, IFN-γ, and granzyme B expression was inhibited during the light phase (resting phase in rodents), while during dark phase, when levels of norepinephrine are reduced, their transcripts were increased (Logan et al., 2011). It is interesting to note that in rats, peak of NE in the spleen is different from the EP level found in the blood, peaking during the active period at night (De Boer and Van der Gugten, 1987; Schiffer et al., 2001). This highlights how neurotransmitters can locally modulate the activity of immune cells. Following splenic sympathectomy, oscillation of granzyme-B, and TNF-α expression in NK cells are abolished, demonstrating the role of the SNS in the entrainment of their rhythmic expression in these

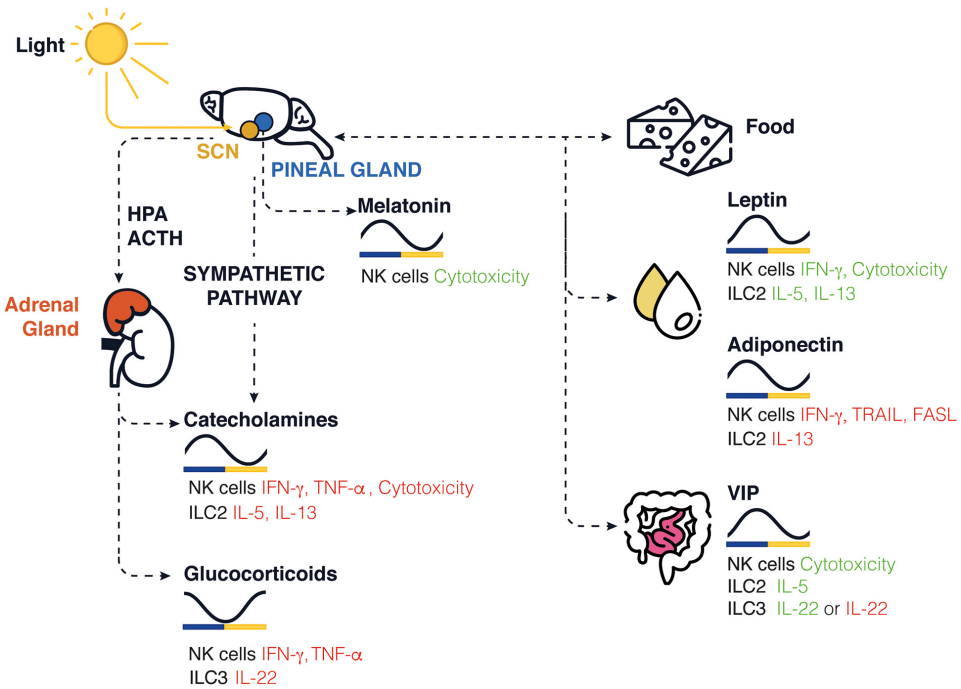

FIGURE 1 | Schematic representation of circadian clock-mediated control of innate immune cells in rodents. In the brain, the SCN controls the rhythmic expression of GCs and catecholamines released in periphery, while the pineal gland controls the release of melatonin. The SCN controls the daily feeding/fasting (activity/rest) cycles, whereas food intake, stress hormone, sleep, and locomotor activity entrains and synchronizes peripheral clocks and the local release of neuropeptides such as leptin, adiponectin, and VIP. The levels found in nocturnal rodents for each molecule are shown across a 24-h period. Green/red represents an increase/decrease in cytokine production or cytotoxicity function.

cells. This circadian regulation of NK cells by adrenergic signaling could explain the reduced cytotoxicity of NK cells in animals under chronic shift-lag (Logan et al., 2012) and sleep deprivation (De Lorenzo et al., 2015).

Norepinephrine also inhibits type 2 immune responses by impairing ILC2 proliferation and function through its binding to β2AR (Moriyama et al., 2018). After infection with *N. brasiliensis*, β2AR-deficient mice have increased intestinal ILC2 infiltration and ILC2-derived IL-13 production. Thus, β2AR acts as a molecular rheostat to control innate immune responses to prevent excessive tissue damage and the development of chronic inflammation (Moriyama et al., 2018). However, a direct link between β2AR signaling and the circadian regulation of ILC2 remains to be established.

Glucocorticoids

Adrenal glands have a key role in synchronizing peripheral clocks downstream of the SCN through the rhythmic secretion of GC. The GC are steroid hormones and their concentrations in the blood oscillate in a circadian manner, peaking in the morning and nadir at night, in diurnal animals. GC can also be released in pulsatile rhythm. While the circadian expression of GC relies on the SCN and the HPA axis (Spiga et al., 2014), the ultradian rhythm occurs independently of SCN (Waite et al., 2012). GC can regulate the expression of clock genes (Balsalobre et al., 2000). GC-responsive elements are found in Per1/2 genes and Rev-erbα and Rorα are negatively regulated by GC in mice (Surjit et al., 2011).

The GC are well known immunosuppressors of the innate immune responses and are widely used in clinics to treat chronic inflammatory disorders. They have been shown to inhibit the synthesis of various cytokines including TNF-α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-12, GM-CSF, and IFN-γ in both human and mice (Brattsand and Linden, 1996; Bhattacharyya et al., 2007; Li et al., 2015; Quatrini et al., 2018). GC limits the inflammation and therefore prevents tissue damages. Oscillations in GC concentrations directly impact the rhythmic regulation of the expression of these pro-inflammatory cytokines. Consequently, GC are partially responsible for the observed circadian variations of inflammatory symptoms that suffer patients with asthma and rheumatoid arthritis and many of whom have worsening of symptoms in early morning (Petty, 1988; Cutolo et al., 2003).

In mice, IL-7R expression is regulated by GR in a diurnal rhythm manner and has been shown to promote T lymphocyte survival and recruitment in lymph nodes (Shimba et al., 2018). ILC are strongly dependent on IL-7R signaling for their development and survival but it is still unclear whether GC similarly regulate IL-7R signaling on ILC in a circadian manner. In NK cells, GR deficiency promotes IFN-γ production, reducing mouse survival in response to toxoplasma and mouse cytomegalovirus infections (Quatrini et al., 2018). Similarly, GCs inhibit murine and human ILC3 function as reduced IL-22 production was observed

when ILC3 were stimulated with the steroid hormones (Seshadri et al., 2018).

Interestingly, in patients with adrenal insufficiency who require lifelong GC replacement, the circadian administration of GC can partially restore the number of NK cells in circulation (Venneri et al., 2018), while acute administration of cortisol has no effect (Olnes et al., 2016). This suggesting that the timing of GC expression is important to regulate NK cell trafficking.

Melatonin

At night, the SCN acts on the pineal gland to induce the synthesis of melatonin. Consequently, high plasmatic levels are found in the middle of the night and minimal during the day in mice and humans. Melatonin production is not restricted to the pineal gland, but can also be secreted by the retina, kidneys and the digestive tract in humans (Messner et al., 2001), however, most of the mouse strains do not produce significant amounts of melatonin (Kennaway, 2019). We therefore need to be careful when translating mouse studies to humans. This neurohormone mediates its effects through specific membrane receptors, named melatonin-1 receptors (MT1), MT2, and MT3. In the absence of the pineal gland, the murine NK cell cytotoxic function is decreased. Surprisingly, a single injection of melatonin is able to restore NK cell activity but not when melatonin is administered chronically for 9 days (del Gobbo et al., 1989). Studies have shown a time-dependent influence of melatonin as *in vivo* administration of melatonin induces a significant increase of murine NK cells in spleen and bone marrow (Currier et al., 2000). In pinealectomized rats, the frequency of NK cells in the blood and spleen is increased during the day compared to sham controls (McNulty et al., 1990). Human peripheral lymphocytes cultured in the presence of melatonin show an inhibition of NK cell activity (Lewinski et al., 1989), but chronic administration of melatonin augmented the cytolytic activity and the circulating number of NK cells (Angeli et al., 1988).

NEUROPEPTIDERGIC PATHWAYS

Vasoactive Intestinal Peptide

The vasoactive intestinal peptide (VIP) is a neurotransmitter expressed in neurons found in brain and peripheral tissues such as the lung and gut. In the SCN, VIP is essential for the normal circadian rhythmicity in clock neurons (Aton et al., 2005; Todd et al., 2020) and can induce the expression of PER1/2 (Hamnett et al., 2019). In tissues, VIP is a potent vasodilator but is also involved in other physiological processes, including coordination of gastrointestinal motility, mucus, and enzymatic secretions in response to feeding (Aton et al., 2005; Todd et al., 2020). In mice, food intake induces the release of VIP from enteric neurons creating a rhythmic expression of VIP in the gut (Talbot et al., 2020; Seillet et al., 2020b). The release of VIP in gut and lungs stimulates ILC2 through VIP receptor type 2 (VIPR2) to induce IL-5 production (Nussbaum et al., 2013). The cyclic release of VIP in response to feeding induces a rhythmic production of IL-5 by ILC2s. This circadian expression of IL-5 is detectable in the blood circulation and appears to regulate systemic eosinophil numbers (Nussbaum et al., 2013). In the intestine, VIP-VIPR2 signaling regulates ILC3-derived IL-22 expression (Talbot et al., 2020; Seillet et al., 2020b). While we observed a positive effect of VIP on IL-22 secretion (Seillet et al., 2020b), Talbot et al. (2020) made opposite observations. Despite these, yet unresolved, discrepancies, both studies demonstrated the importance of circadian regulations for ILC function to maintain intestinal homeostasis. While we showed that VIP signaling protects against exacerbated gut inflammation, Talbot and colleagues found that the inhibition of IL-22 by VIP in ILC3 allows the optimal absorption of nutrients (Talbot et al., 2020).

Additional studies have revealed that VIP increased human NK cell cytotoxic function after viral infection (Azzari et al., 1992) and polarized T cell responses by regulating dendritic cells functions. Collectively, these data suggest that VIP can differentially promote inflammation or its resolution in a circadian dependent manner by promoting anti-inflammatory type 2 immune responses, and preventing Th1 infiltration in inflammatory sites.

Adiponectin

The adiponectin is exclusively secreted by adipocytes, and regulates body energy homeostasis, lipid storage, and adipogenesis (Stern et al., 2016). Its expression is controlled by the clock machinery and peaks at the onset of the feeding phase in mice (Barnea et al., 2015) or early/late morning for human (Gomez-Abellan et al., 2010). Adiponectin induces hypothalamic and peripheral clock genes expression and enhances food intake (Hashinaga et al., 2013; Tsang et al., 2020) as adiponectin-deficient mice have reduced *Bmal1* and *Per2* expressions and reduce food intake during the dark phase (active phase) but experience increase food intake during light phase (resting phase; Tsang et al., 2020). In contrast, overexpression of the adipokine in the liver induces local expression of the clock genes *Bmal1*, *Dbp*, *Cry2,* and *Per2* (Hashinaga et al., 2013), indicating a direct effect of this adipokine on the cell-intrinsic circadian rhythm.

The adiponectin differentially impacts immune cells and can trigger both pro- and anti-inflammatory responses (Luo and Liu, 2016). High levels of adiponectin in mice result in an alteration of the adipose tissue immune cell composition, and a shift operates from a pro- to an anti-inflammatory immune profile leading to an improvement of insulin resistance in models of type 2 diabetes (Kim et al., 2007). NK cells also play a critical role in murine adipose tissue homeostasis, fine-tuning macrophage functions, and dysregulated NK cells are found in obesity (O'Shea and Hogan, 2019; Ferno et al., 2020). Both murine and human NK cells express the adiponectin receptors (Wilk et al., 2013; Jasinski-Bergner et al., 2017). Human NK cells stimulated with various TLR ligands and treated with adiponectin showed reduced IFN-γ production and degranulation capacities (Wilk et al., 2013). Conversely, in adiponectin-deficient mice, while an accumulation of mature NK cells ($CD27^{low}CD11b^{hi}$) is found in the spleen, impaired NK cell degranulation and cytotoxicity are observed and are associated with decreased expression of the activating

ligand NKG2D (Wilk et al., 2013). The addition of adiponectin to IL-2 stimulated NK cells leads to impaired cytotoxicity associated with reduced surface expression of FasL and TRAIL, and IFN-γ production (Kim et al., 2006). Recently, adiponectin was shown to suppress ILC2 proliferation and cytokine production and to decrease IL-33-driven ILC2 activation, thus acting as a negative regulator of ILC2 function in adipose tissue (Wang et al., 2021). While no direct link has demonstrated an influence of adiponectin on ILC function in a circadian manner, indirect evidence would suggest a rhythmically regulation of NK cell function by this adipokine. Indeed, adiponectin controls the expression of clock genes which are known to directly influence NK cell activity in rodents (Arjona and Sarkar, 2005, 2006, 2008). The disruption of the *Per2* or *Bmal1* in NK cells reduced IFN-γ, TNF-α, granzyme B, and perforin expressions (Liu et al., 2006; Arjona and Sarkar, 2008). Furthermore, murine NK cell cytotoxic function peaks during the active phase (Arjona et al., 2004; Arjona and Sarkar, 2005, 2006) which coincides with feeding time and higher levels of adiponectin (Barnea et al., 2015; Tsang et al., 2020), however, this interplay remains to be confirmed.

Further studies are warranted to ascertain the role and function of adiponectin on ILC subsets at steady state, over the course of metabolic syndromes, and during circadian misalignment.

Leptin

Leptin is mainly secreted by adipocytes and follows diurnal variations. In both humans and rodents, the leptin plasma levels peak at night before progressively decreasing, reaching a nadir during the day (Langendonk et al., 1998; Gavrila et al., 2003; Bodosi et al., 2004; Arble et al., 2011). In rodents, starvation induced a decrease in leptin levels and timed-restricted food availability inverted leptin plasma concentrations (Ahima et al., 1996; Bodosi et al., 2004). Furthermore, in humans, circadian misalignment decreases the leptin plasma levels compared to normal alignment (Scheer et al., 2009). Circadian disruptions through thermal lesions of the hypothalamic SCN or in $Cry^{-/-}$ and $Per^{-/-}$ deficient mice completely abolish the diurnal variation of leptin plasma levels (Kalsbeek et al., 2001; Kettner et al., 2015), indicating that circadian clocks control the rhythmic oscillation of the leptin plasma, independently of external food cues, potentially through direct regulation of gene expression. Indeed, the heterodimer BMAL1:CLOCK is capable of binding to the promoter of the leptin gene following a circadian rhythm and regulating C/EBP-α mediated *leptin* transcription (Kettner et al., 2015). Finally, chronic circadian disruption promotes leptin resistance in murine CNS (Kettner et al., 2015) which is known to be associated with obesity (Flier, 2004).

Mice lacking leptin (*ob*/*ob*) or its receptor (*db*/*db*) expression show immune deficiencies suggesting a direct role of the leptin signaling on the immune system (Bennett et al., 1996; Lord et al., 1998; Howard et al., 1999; Sanchez-Margalet et al., 2003; De Rosa et al., 2007). Both murine and human NK cells express variable levels of the short and long forms of the leptin receptor (Zhao et al., 2003; Lamas et al., 2013; Laue et al., 2015; Keustermans et al., 2017; Bahr et al., 2018). Particularly, obese patients who experience high leptin plasma levels have impaired NK cell phenotype and function, a reversible compromised state when there is a diminution of leptin plasma levels-associated with fat mass reduction (Jahn et al., 2015; Laue et al., 2015; Bahr et al., 2018). In leptin receptor deficient *db*/*db* mice, NK cell numbers in blood, spleen, liver and lungs are all reduced and cells have impaired cytolytic capacities compared to wild type control animals (Tian et al., 2002). In addition, leptin signaling-deficient mice injected with B16 melanoma or LLC cells have increased number of lung metastases compared to control mice (Mori et al., 2006). Stimulation of human NK cells with leptin promotes NK cell metabolism, proliferation, and cytotoxic functions (Zhao et al., 2003; Wrann et al., 2012; Lamas et al., 2013). Interestingly, while short term exposure of NK cells to physiological doses of leptin stimulates NK cells, long-term stimulation inhibits NK cell cytotoxicity and cytokine production (Wrann et al., 2012). Thus, dependent on the levels and duration of the stimulation, leptin may differently influence immune cell responses. The impact of leptin on other ILC subsets is only beginning to emerge. Leptin enhances type-2 responses in allergic rhinitis and stimulates ILC2 proliferation and IL-13 production in both humans and mice (Zheng et al., 2016; Wen et al., 2020; Zeng et al., 2020). Given the role of ILC2 on adipose tissue homeostasis and allergy reactions, leptin and other adiponectin would certainly modulate widely the function of these innate immune cells in health and diseases. Thus, further studies are warranted to delineate the role of these hormones on non-NK cell ILC.

CONCLUDING REMARKS

In our modern societies, the prevalence of circadian rhythm disruption is rising due to our lifestyle or might be imposed by extending working hours and night shift. Nonetheless, circadian rhythm disruption is yet to be recognized as a major public health issue; but accumulating evidence shows that circadian rhythms are an important part of our healing process and control inflammation. As medicine is evolving toward a more personalized approach, circadian regulation of immune responses will be an aspect to consider. Studying circadian rhythms at a global physiological lens will help us to understand how alteration of our natural rhythms by environmental cues or our behavior will impact the metabolism, hormonal signaling and immune function. It is crucial to increase our understanding of the circadian patterns of immune responses and how they are regulated by central and peripheral clocks to enable discovery of chronotherapeutic approaches for optimal timing of therapy administration toward effective measures for treating inflammatory diseases, allergies, and infections. Because animal studies are used to reduce the complexity of parameters influencing circadian rhythms (e.g., temperature, food availability, and external stressor), it will be important to optimize the experimental setups, including the control of external circadian rhythm perturbations (such as, manipulation of light-dark cycles, sleep restriction, or time-restricted feeding) to develop the best translational approaches.

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Immune Checkpoint Inhibitors-Related Thyroid Dysfunction: Epidemiology, Clinical Presentation, Possible Pathogenesis, and Management

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Immune checkpoint inhibitors (ICIs) are a group of drugs employed in the treatment of various types of malignant tumors and improve the therapeutic effect. ICIs blocks negative co-stimulatory molecules, such as programmed cell death gene-1 (PD-1) and its ligand (PD-L1) and cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4), reactivating the recognition and killing effect of the immune system on tumors. However, the reactivation of the immune system can also lead to the death of normal organs, tissues, and cells, eventually leading to immune-related adverse events (IRAEs). IRAEs involve various organs and tissues and also cause thyroid dysfunction. This article reviews the epidemiology, clinical manifestations, possible pathogenesis, and management of ICIs-related thyroid dysfunction.

Keywords: immune checkpoint inhibitors, immune-related adverse events, thyroid dysfunction, epidemiology, clinical manifestations, pathogenesis, management

INTRODUCTION

The immune system plays an important role in the occurrence, development, and prognosis of most tumors and forms a specific tumor immune microenvironment. The immune system can recognize, kill, and resist tumor cells. However, tumor cells can escape the killing or clearance effect of the immune system through various escape mechanisms. For example, the immune checkpoint pathway

Abbreviations: ICIs, immune checkpoint inhibitors; IRAEs, immune-related adverse events PD-1, programmed cell death gene-1; PD-L1, programmed cell death gene-1 ligand; CTLA-4, cytotoxic T-lymphocyte-associated antigen-4; APCs, antigen-presenting cells; DCs, dendritic cells; RCT, randomized controlled trial; LUAD, lung adenocarcinoma; SCLC, small cell lung cancer; NSCLC, non-small cell lung cancer; SCCA, squamous cell carcinoma of the anal canal; cSCC, cutaneous squamous cell carcinoma; TNBC, triple-negative breast cancer; UTUC, urothelial carcinoma; Th cells, helper T cells; CTL, cytotoxic T lymphocyte cell; Treg, T-regulatory cells; AITDs, autoimmune thyroid diseases; HT, Hashimoto's thyroiditis; GD, Graves' disease; FT3, free triiodothyronine; FT4, free thyroxine; TSH, thyroid-stimulating hormone; TPO-Ab, thyroperoxidase antibodies; TG-Ab, thyroglobulin antibody; Tg, thyroglobulin; CTCAE, the Common Terminology Criteria for Adverse Events; HLA, human leukocyte antigen; MHC, major histocompatibility complex; ACTH, Adrenocorticotropic hormone; LH, Luteinizing hormone; FSH, Follicle-stimulating hormone; TRH, Thyrotropin-releasing hormone; TRAb, TSH receptor antibodies.

could be activated to inhibit the anti-tumor immune response (1). Some cell surface receptors play a significant role in the process. Programmed cell death gene-1 (PD-1) and its ligand (PD-L1), known as negative co-stimulatory molecules, are the second signal of T cell activation in cellular immune response (2). Cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4) is another inhibitory receptor of active T cells by high-affinity binding to natural B7 family ligands, which plays a similar role (3). They work together with the first signal to inhibit T cells and regulate the immune response. The original function of the immune checkpoint is to maintain immune homeostasis and prevent autoimmunity (**Box 1**) (2, 3). However, those pathways are activated to escape the cytotoxic T-lymphocyte cell (CTL)-mediated immune killing effect in most malignant tumor cells (4, 5).

Some immunotherapeutic agents can block those intercellular signal transductions, and thus eliminate the inhibitory effect of T cells, which restore the anti-tumor response (6). In recent years, immune checkpoint inhibitors (ICIs) are reported to be novel agents for the treatment of malignant tumors, which show promising therapeutic effects and potential (7–9). Although ICIs are often described as well tolerated, sometimes, they still produce inevitable immune-related adverse events (IRAEs). ICIs activate the immune system, affect normal organ tissues, and lead to cell death in addition to targeting tumor cells, eventually leading to IRAEs (6). IRAEs involve various organs and systems of the whole body and also cause thyroid dysfunction, which needs clinical attention (10–14).

Thyroid dysfunction is a common pathological state of thyroid hormone disorder, most commonly hypothyroidism (15). It needs active surveillance and treatment; otherwise, severe thyroid dysfunction may seriously affect health in some cases (16, 17). The specific mechanism of hypothyroidism is still unclear and warrants further laboratory and clinical exploration. Currently, the diagnosis of thyroid dysfunction depends primarily on the identification of biochemical indicators due to a lack of special symptoms (**Figure 1**) (18). Although thyroid dysfunction is mild among all IRAEs, they have considerable morbidity (19, 20). Better characterization of thyroid IRAEs and their underlying mechanisms could improve clinical identification, management, and care of these patients and assist in choosing a more effective treatment.

ICIs AND IRAEs

The common ICIs, approved by the US Food and Drug Administration (FDA), include Ipilimumab for anti-CTLA-4 therapy (5), Nivolumab, Pembrolizumab and Cemplimab for anti-PD-1 therapy (22–25), Durvalumab, Atezolizumab and Avelumab for anti-PD-L1 therapy (**Table 1**) (10, 26–28). Currently, ICIs have been widely used in cutaneous squamous cell carcinoma (cSCC), triple-negative breast cancer (TNBC), urothelial carcinoma (UTUC), squamous cell carcinoma of the anal canal (SCCA), malignant melanoma, renal cell carcinoma (RCC), non-small cell lung cancer (NSCLC), small cell lung cancer (SCLC), lung adenocarcinoma (LUAD) etc. (25, 27–41).

ICIs are often accompanied by IRAEs, including hypophysitis, thyroid dysfunction, and autoimmune diabetes, which can occur alone or concurrently (10–12, 23, 41). So far, numerous articles have reviewed the incidence rate of IRAEs, among which thyroid IRAEs was found to be the most common (19, 20). However, it is less likely to accurately predict the system or organ to be affected by IRAEs. Therefore, more prospective studies are needed to explore the predictive biomarkers of IRAEs.

SPECIFIC EFFECTS OF IRAES ON THE THYROID

Epidemiology

Some scholars have reported that most patients on ICIs for malignancies are at risk of developing thyroid dysfunction. Thyroid IRAEs present mainly as hypothyroidism, hyperthyroidism, and transient thyroiditis (10, 13, 14, 22). Transient thyroiditis was diagnosed as noticeable hyperthyroidism or subclinical hyperthyroidism at the time of diagnosis and subsequently progressing to hypothyroidism (22). The latest review and meta-analyze have reported high thyroid IRAEs frequencies, especially relatively high risk for hypothyroidism (42). Notably, ICIs-related thyroid dysfunction incidences lie on the type of malignant tumor and ICIs employed (**Table 1**). Stelmachowska-Banas et al. (42) summarized that combination therapy has been associated with the highest estimated incidence of high thyroid dysfunction frequencies, ranging from 8.0 to 16.4%, remarkably higher than monotherapy with anti-PD-1 drugs (2.8-8.5%) or anti-PD-L1 drugs (0.6-6.0%) or anti-CTLA-4 (0.2-5.2%). The combination of multiple immunotherapies can increase the risk of thyroid dysfunction (23, 43–46). What's more, the incidence of hypothyroidism, hyperthyroidism, and thyroiditis was statistically significant between different drugs (47, 48). Previous researchers found that the probability of thyroid dysfunction in the anti-PD-1 treatment group was higher than that in the anti-PD-L1 and anti-CTLA-4 treatment group (45, 49). Furthermore, although both Nivolumab and Pembrolizumab are anti-PD-1 drugs, patients using the former are more likely to develop hypothyroidism, whereas those using the latter are more likely to develop hyperthyroidism (**Table 1**) (49).

Box 1 | PD-1/PD-L1 and CTLA-4 play a role as the immune checkpoint.
The receptor on the surface of the T cell (TCR) binds to an antigen, acting as the first signal to activate T cells (1). The second signal of T cell activation in cellular immunity is composed of costimulatory molecules on the surface of T cells, antigen-presenting cells (APCs), and target cells. There are numerous costimulatory molecules on the T cell surface, including positive and negative costimulatory molecules such as CD28, PD-1, and CTLA-4 (2, 3, 19, 21). PD-L1 is found on tumor cells and APCs, such as B cells, dendritic cells (DCs), and macrophages (2, 21). PD-1 binds to PD-L1, working together with the first signal to inhibit T cells and regulate the immune response (2). CTLA-4, which is similar to its homologous stimulatory receptor CD28, combines with natural B7 family ligands, CD80 and CD86, and exerts an immunomodulatory role (3). To conclude, the PD-1/PD-L1 pathway and CTLA-4 play a vital role as immune checkpoints, which interact with positive costimulatory molecules, so that immune response can start effectively, play a role properly, and terminate in time (**Figure 2**).

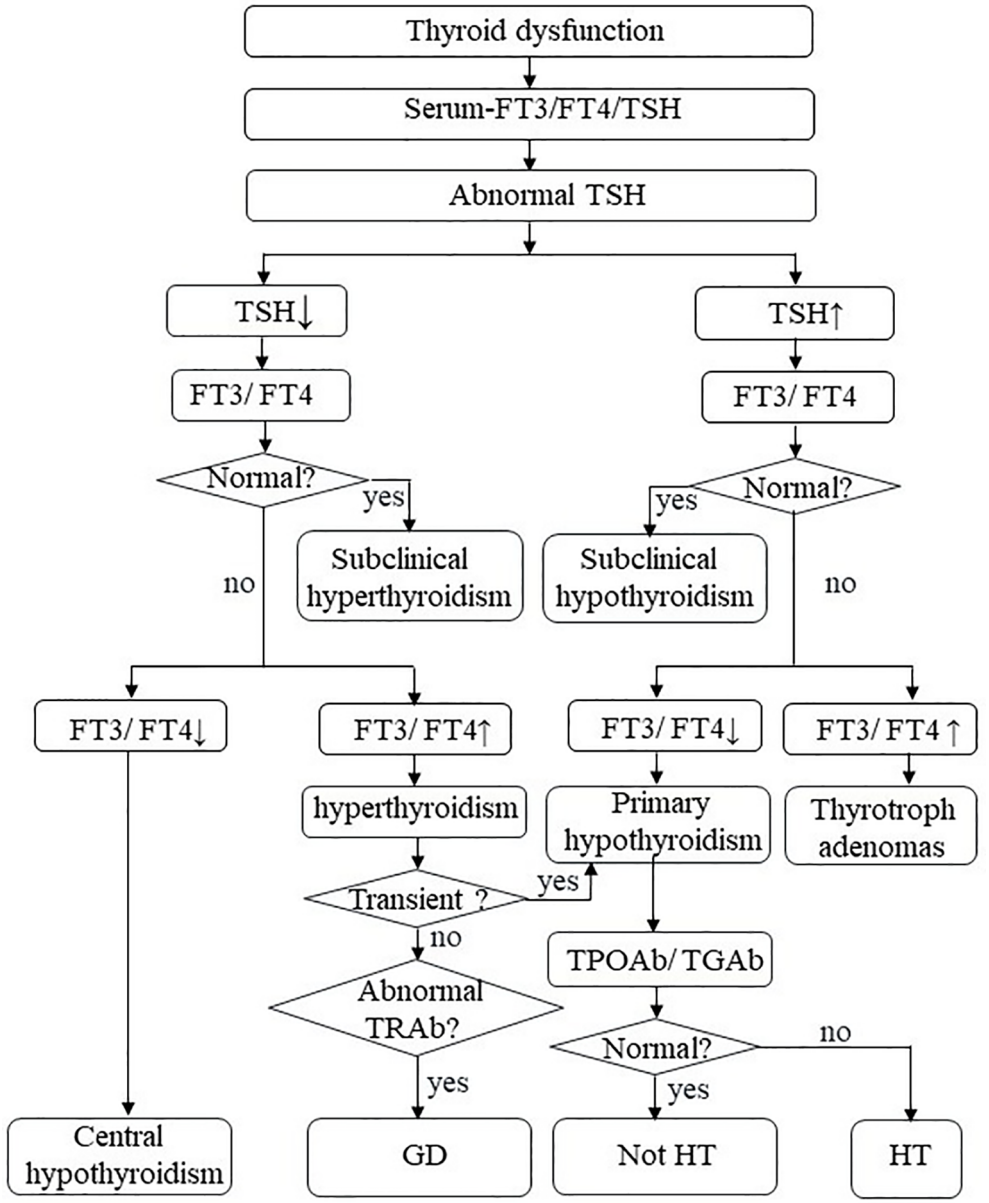

FIGURE 1 | Thyroid dysfunction. FT3, free triiodothyronine; FT4, free thyroxine; TSH, thyroid-stimulating hormone; TPO-Ab, thyroperoxidase antibodies; TG-Ab, thyroglobulin antibody; TRAb, TSH receptor antibodies; HT, Hashimoto's thyroiditis; GD, Graves' disease.

Notably, the type of ICIs-related thyroid dysfunction was not completely identical among different tumors. We can be implied from several prospective studies that malignant melanoma and TNBC patients have a certain risk of hyperthyroidism incidence (26, 27, 30, 32), while malignant melanoma and NSCLC patients also have a risk of transient thyroiditis (9, 32). To concluded, patients on combination therapy were significantly more prone to develop thyroid dysfunction than those receiving monotherapy. And patients treated with anti-CTLA-4 drugs had a significantly lower risk for thyroid dysfunction compared to those with anti-PD-1 and anti-PD-L1.

Clinical Manifestations

The biochemical behavior of thyroid dysfunction is different between tumor types as well as immunosuppressive therapy (**Table 1**). Ohara et al. (22) reported that a 69-years-old patient with LUAD developed painless thyroiditis during a 3-month nivolumab treatment. The patient had a mild and soft goiter but had no symptoms of thyrotoxicosis or exophthalmos. She did not present any fever or pain. Serum-free thyroxine (FT4) was elevated and thyroid-stimulating hormone (TSH) was decreased; shortly after, primary hypothyroidism began to appear. Another patient had the same thyroid disorder after

TABLE 1 | Comparison of ICIs-related thyroid dysfunction

Author & year	Study type	Target tumor	ICIs	Thyroid dysfunction	Incidence	
					Any Grade (%)	Grade 3-5 (%)
Migden et al., 2020 (24)	RCT	cSCC	Cempilimab	Hypothyroidism	10.0	0
Migden et al., 2018 (25)	RCT	cSCC	Cempilimab	Hypothyroidism	8.0	0
Loibl et al., 2019 (26)	RCT	TNBC	Durvalumab	Hypothyroidism	7.6	0
				Hyperthyroidism	9.8	0
Mittendorf et al., 2020 (27)	RCT	TNBC	Atezolizumab	Hypothyroidism	6.7	0
				Hyperthyroidism	3.0	0
Powles et al., 2020 (28)	RCT	UTUC	Avelumab	Hypothyroidism	11.6	0.3
Morris et al., 2017 (29)	RCT	SCCA	Nivolumab	Hypothyroidism	6.0	3.0
Wolchok et al., 2017 (30)	RCT	melanoma	Ipilimumab	Hypothyroidism	5.0	0
				Hyperthyroidism	1.0	0
			Nivolumab	Hypothyroidism	11.0	0
				Hyperthyroidism	4.0	0
			Nivolumab + Ipilimumab	Hypothyroidism	17.0	<1.0
				Hyperthyroidism	11.0	1.0
Ascierto et al., 2020 (31)	RCT	melanoma	Nivolumab	Hypothyroidism	<1.0	0
			Ipilimumab		<1.0	0
Eggermont et al., 2018 (32)	RCT	melanoma	Pembrolizumab	Hypothyroidism	14.3	0
				Hyperthyroidism	10.2	0.2
				Transient thyroiditis	3.1	0
Motzer et al., 2018 (33)	RCT	RCC	Nivolumab + Ipilimumab	Hypothyroidism	16.0	<1.0
Koshkin et al., 2018 (34)	RCT	RCC	Nivolumab	Hypothyroidism	7.0	0
McDermott, et al., 2021 (35)	RCT	RCC	Pembrolizumab	Hyperthyroidism	5.5	0
Osorio et al., 2017 (9)	RCT	NSCLC	Pembrolizumab	Hypothyroidism	8.0	NA
				Transient thyroiditis	13.0	NA
Hellmann et al., 2019 (36)	RCT	NSCLC	Nivolumab + Ipilimumab	Hypothyroidism	12.0	<1.0
Hellmann et al., 2018 (37)	RCT	Lung Cancer	Nivolumab + Ipilimumab	Hypothyroidism	11.6	0.3
			Nivolumab		6.4	0.3
Patel et al., 2020 (38)	RCT	Neuroendocrine Neoplasms	Ipilimumab + Nivolumab	Hypothyroidism	31.3	0

ICIs, immune checkpoint inhibitors; RCT, a randomized controlled trial; cSCC, cutaneous squamous cell carcinoma; TNBC, triple-negative breast cancer; UTUC, urothelial carcinoma; SCCA, squamous cell carcinoma of the anal canal; RCC, renal cell carcinoma; NSCLC, non-small cell lung cancer; NA, not available.

6 months of treatment with nivolumab for melanoma (50). An 85-year-old male suffered hypothyroidism coexisting with various autoimmune diseases after the administration of pembrolizumab for advanced melanoma (23). In another case, after 4 months of Durvalumab immunotherapy, the level of FT4 decreased and that of TSH increased in a 49 years old female patient with LUAD (10). However, thyroperoxidase antibodies (TPO-Ab) and thyroglobulin antibody (TG-Ab) were negative (22, 50). In summary, most of the patients were diagnosed with transient thyroiditis or hypothyroidism during immune checkpoint blockade, which can be verified in many retrospective studies (51, 52). Even though many studies have viewed the highest incidence rate of hypothyroidism, thyroiditis is not uncommon (51, 53, 54). According to the cause of hypothyroidism, among which ICIs-related hypothyroidism is categorized as the primary hypothyroidism. Primary hypothyroidism is defined as TSH level higher than the reference range and FT4 level lower than the reference range (15). In turn, central hypothyroidism is defined as low or low-to-normal TSH level and a disproportionately low FT4 level, owing to dysfunction of the hypothalamus or the pituitary gland, or both (55). Generally, most patients present with primary hypothyroidism, and only a few cases with hypothalamic or pituitary dysfunction have secondary central hypothyroidism (**Figures 1** and **3**) (18, 56). Patients may be asymptomatic or only show non-specific symptoms, such as fatigue, dizziness, weight changes, and emotional or behavioral changes (18). Thyroid disorders are often neglected because their presentation is often inconspicuous and only a few patients have thyroid storms (16). It can also be easily deduced from the aforementioned literature that the median time from the beginning of drug commencement to the development of thyroid dysfunction varies in different immunotherapies. Thyroid dysfunction has been reported to mostly occurs in 5-36 weeks after immunotherapy (13, 48, 57). In a retrospective study, the median occurrence time and the duration time of thyroiditis was 5.3 weeks (range 0.6-19.6 weeks) and 6 weeks (range 2.6-39.7 weeks), and the median occurrence time of hypothyroidism was 10.4 weeks (range 3.4-48.7 weeks) (54). Although a few patients develop permanent hypothyroidism, most of them can be relieved after suspending immunotherapy or undergoing thyroid hormone replacement therapy (22, 23). Finally, the recovery time of thyroid dysfunction among patients with combination therapy was significantly longer than that of patients with monotherapy (13, 14).

To conclude, patients can present with hypothyroidism or transient thyroiditis during the commencement of ICIs (**Table 1**). However, these patients are mostly detected during routine hormone monitoring because of a lack of clinical symptoms. The dynamic changes of free triiodothyronine (FT3), FT4, and TSH can be detected but there are few reports of positive TPO-Ab and TG-Ab. Additionally, based on the Common

Terminology Criteria for Adverse Events (CTCAE) Version 5.0, recommended by the National Cancer Institute (58), thyroid IRAEs are mostly graded from level 1 to 3 (**Tables 1** and **2**) (9, 42, 45, 59).

MECHANISM OF ICIs-RELATED THYROID DYSFUNCTION

The underlying mechanism for ICIs-related thyroid dysfunction remains unknown. In terms of clinical presentation, hypothyroidism or hypothyroidism after transient thyrotoxicosis is the most common and consistent characteristic of patients with ICIs. Thyroid IRAEs seem to overlap with that of autoimmune thyroid diseases (AITDs), such as Graves' disease (GD) (60), Hashimoto's thyroiditis (HT) (61). The thyroid gland is known to be more susceptible to autoimmune attacks than any other organ (62). Hypothyroidism often occurs after hemithyroidectomy, radioiodine therapy, and neck radiotherapy (15, 63). Whether thyroid IRAEs have the same mechanism as AITDs, warrants further elucidation.

Link Between the Immune System and HT and GD

HT, widely seen as the common cause of hypothyroidism (15), is caused by impaired immune tolerance of autoantigens, the destruction of thyroid cells (64, 65). The pathogenesis of HT is considered to be a complex autoimmune process. Various T lymphocytes (**Box 2**) activate and infiltrate thyroid follicular cells, and then induce a cellular immune response leading to direct thyroid injury and further thyroid antigen exposure (65). However, B lymphocytes participate in humoral immune response and secrete specific TPO-Ab and TG-Ab against thyroid auto-antigen (61, 65). Besides, natural killer (NK) cells, macrophages, and various cytokines, such as Type 1 T helper (Th1) cytokines (interleukin-2 (IL-2) and interferon-gamma (IFN-γ)), T-regulatory cells (Treg) cytokine (IL-10), and Th17 cytokine (IL-17), participate in the autoimmune process (62). Meanwhile, CTL and Th1-mediated immune responses play a leading role in the development of autoimmune diseases (43). However, the body does not allow the immune system's unrestricted self-attack on the thyroid gland. Treg and PD-1 pathways may be triggered and activated because of persistent autoimmunity. Treg has an inhibitory effect on autoimmunity, which is inhibited in HT until it is reactivated by uncontrolled autoimmunity (62). Álvarez-Sierra et al. (67) detected the expression of PD-1 in peripheral blood and thyroid gland among HT patients and found that the expression of $CD4^+$ and $CD8^+$ PD-1 positive T cells in the thyroid gland was increased. In the background of lymphocytic thyroiditis and hyperthyroidism, PD-L1 expression in benign follicular epithelial cells was also increased (67). Although the PD-1/PD-L1 pathway cannot stop the autoimmune reaction, it can inhibit the autoimmune response by inhibiting T cells. However, whether it achieves the effect of complete inhibition of disease or not remains unknown. It is reasonable to speculate that HT does not progress as rapidly as acute thyroiditis, which may be due to the negative regulatory effect of Treg and the PD-1/PD-L1 pathway (**Figure 2**).

TABLE 2 | Thyroid IRAEs grade in the CTCAE Version 5.0.

	Thyroid IRAEs				
Term	**Grade 1**	**Grade 2**	**Grade 3**	**Grade 4**	**Grade 5**
Hypothyroidism	Asymptomatic; clinical or diagnostic observations only;intervention not indicated	Symptomatic; thyroid replacement indicated; limiting instrumental ADL	Severe symptoms; limiting self-care ADL; hospitalization indicated	Life-threatening consequences; urgent intervention indicated	Death
Hyperthyroidism	Asymptomatic; clinical or diagnostic observations only; intervention not indicated	Symptomatic; thyroid suppression therapy indicated; limiting instrumental ADL	Severe symptoms; limiting self-care ADL; hospitalization indicated	Life-threatening consequences; urgent intervention indicated	Death
Thyroiditis	Asymptomatic; clinical or diagnostic observations only; intervention not indicated	Symptomatic; thyroid suppression therapy indicated; limiting instrumental ADL	Severe symptoms; limiting self-care ADL; hospitalization indicated	Life-threatening consequences; urgent intervention indicated	Death

Hypothyroidism: a disorder characterized by a decrease in the production of thyroid hormone by the thyroid gland.
Hyperthyroidism: a disorder characterized by excessive levels of thyroid hormone in the body. Common causes include an overactive thyroid gland or thyroid hormone overdose.
Thyroiditis: a disorder characterized by transiently obvious hyperthyroidism or subclinical hyperthyroidism and subsequently hypothyroidism.
IRAEs, immune-related adverse events.

Box 2 | T cells and human leukocyte antigen (HLA).
T cells can be divided into naïve T cells, effector T cells, and memory T cells based on the activation stage. T cells can also be divided into $CD4^+$ and $CD8^+$ T cells. Further, T cells are divided into helper T (Th) cells, CTL, and Treg based on their functions. There exist a Th1/Th2 balance that transforms depending on the status of the immune response (64). T cells promote organ and tissue autoimmunity mainly through the following ways: activated T cells proliferate and differentiate, then transform into effector T cells, such as Th1 and CTL; Th2 cell-dependent B cells produce and secrete auto-antibodies; various inflammatory factors (21, 64). Furthermore, tissue damage in turn gives rise to further exposure of tissue self-antigen, which leads to more active T cells in the positive feedback loop (64). Then Treg, immune checkpoint, and other inhibitory pathways will be induced to eliminate self-immune (**Figure 2**) (21).

HLA gene complex, also called the major histocompatibility complex (MHC), is closely associated with immune response (66). With a complex structure, HLA gene regions are mainly divided into class I and class II, which are both directly involved in the activation and differentiation of T cells and regulation of adaptive immune response by binding to a specific antigen peptide (**Figure 2**) (21). HLA I is distributed on the surface of all nucleated cells, but HLA II only expresses surfaces specific cells, such as activated T cells and professional APCs. HLA II binds to antigen peptides and then to CD4 Th T cells receptor to accurately recognize Th T cells (21).

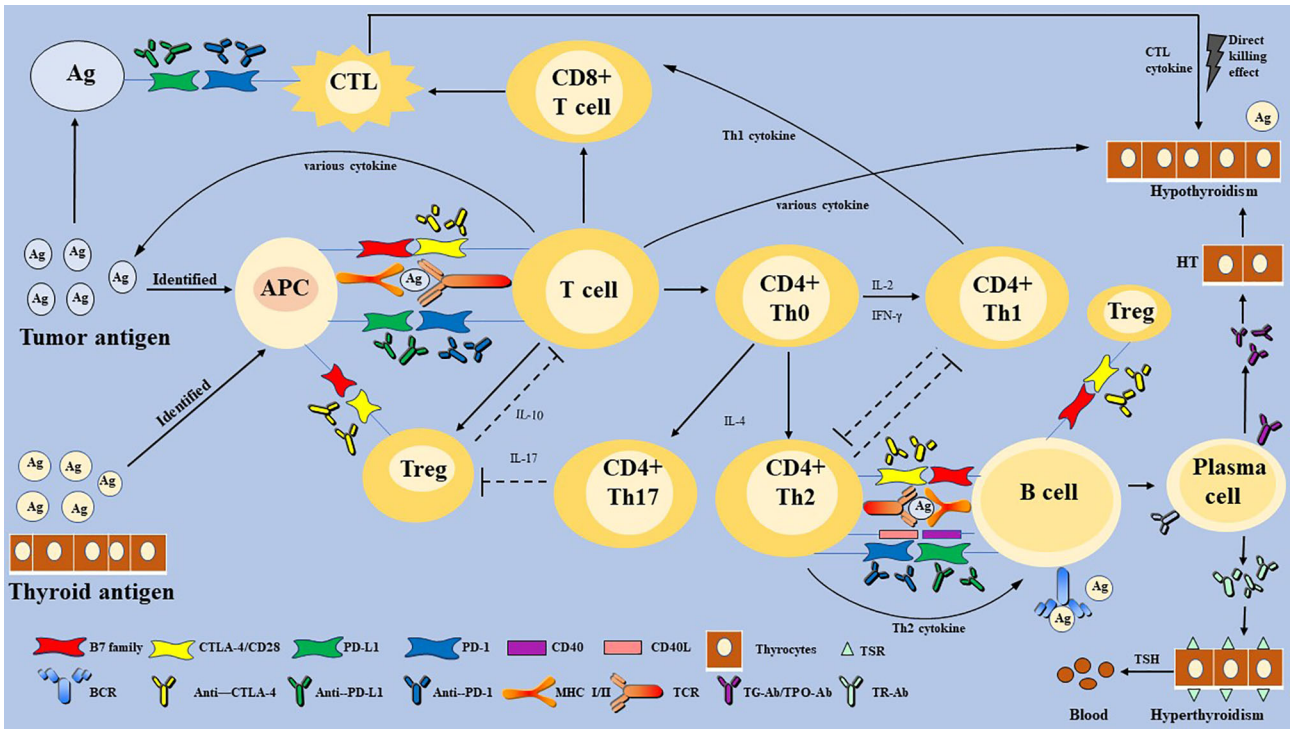

FIGURE 2 | The proposed mechanism of immune checkpoint inhibitors-related thyroid dysfunction. Thyroid IRAEs may involve T and B-lymphocytes, multiply cytokines, and diverse factors. Immune checkpoints are activated to escape the immune killing and clearance effect in most malignant tumor cells. Some immunotherapeutic agents can eliminate the inhibitory effect of T cells, which restore the anti-tumor response. However, activation of the immune system can also affect normal organ tissues, and lead to cell death, eventually leading to organ IRAEs. Thyroid IRAEs present mainly as hypothyroidism, hyperthyroidism, and transient thyroiditis, seem to overlap with AITDs. HT and GD are AITDs that cause hypothyroidism and hyperthyroidism, respectively. HT is caused by impaired immune tolerance of autoantigens, the destruction of thyroid cells. The pathogenesis of HT is considered to be a complex autoimmune process involving various activate and infiltrate T lymphocytes, B lymphocytes, and various cytokines. Then a cellular immune response and humoral immune response are induced, leading to direct thyroid injury and further thyroid antigen exposure. The main pathogenesis of GD can be understood as the combination of TSH receptor and TR-Ab secreted and released by Th2 cell-dependent B cells. Immune checkpoints are proposed to play a role in inhibiting the autoimmune process by inhibiting various immune cells. Whether thyroid IRAEs have the same mechanism as AITDs, warrants further elucidation. PD-1, programmed cell death gene-1; PD-L1, programmed cell death gene-1 ligand; CTLA-4, cytotoxic T-lymphocyte-associated antigen-4; MHC, major histocompatibility complex; Th cells, helper T cells; CTL, cytotoxic T lymphocyte cell; Treg, T-regulatory cells; APCs, antigen-presenting cells; TSH, thyroid-stimulating hormone; TPO-Ab, thyroperoxidase antibodies; TG-Ab, thyroglobulin antibody; TRAb, TSH receptor antibodies; AITDs, autoimmune thyroid diseases; HT, Hashimoto's thyroiditis; GD, Graves' disease.

GD is an AITDs that causes hyperthyroidism. Its main pathogenesis can be understood as follows: the combination of TSH receptor on the thyroid cells surface and TSH receptor antibody (TR-Ab) secreted and released by Th2 cell-dependent B cells results in thyroid cell damage and a series of symptoms of hyperthyroidism caused by the release of thyroid hormone. Interestingly, hypothyroidism does not occur in patients with GD because Th2 is dominant (**Figure 2**) (60, 64).

Link Between the Immune System and ICIs-Related Thyroid Dysfunction

Role of T Cell-Mediated Cellular Immune in Thyroid Dysfunction

Until now, the immune system activated by ICIs not only targets tumor cells but also leads to the death of normal organs, tissues, and cells, which is recognized as the most possible mechanism. Numerous studies have reported that increasing infiltrating CD4+ and CD8+ T cells represent a higher response rate and a better clinical outcome of ICIs because it also represents the activity of anti-tumor immunity (7–9, 68). Intriguingly, previous studies reported that increased circulating $CD4^+$ and $CD8^+$ T cells also presented a relatively higher incidence of IRAEs (69). However, a large amount of clinical and experimental data is needed to confirm the authenticity of this phenomenon.

ICIs may trigger T cell-mediated pathways that induce subsequent thyroid dysfunction (65, 70). Generally, anti-CTLA-4 treatment is more likely to trigger IRAEs than anti-PD-1 or PD-L1 treatment, because anti-CTLA-4 is more likely to lead to extensive T cell activation but blocking PD-1 or PD-L1 is likely to trigger pre-existing $CD8^+$ T cell activation (6). However, in terms of thyroid IRAEs, the probability of anti-PD-1 or anti-PD-L1 agents was higher than that of anti-CTLA-4 agents. Additionally, blocking PD-1 is more likely to lead to the activation of pre-existing $CD8^+$ T cells than PD-L1 and CTLA-4 inhibition (1), which also well explains why thyroid dysfunction in the anti-PD-1

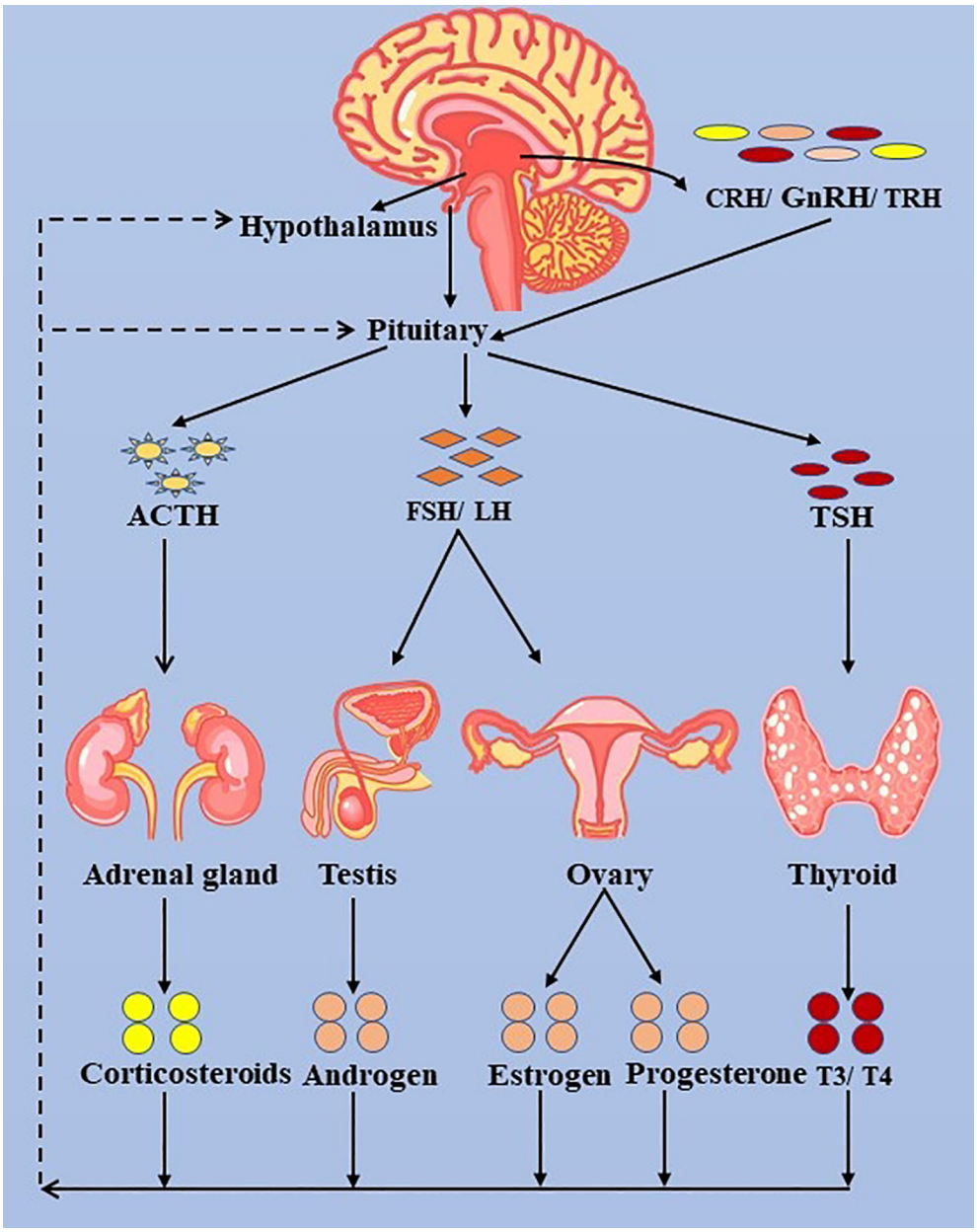

FIGURE 3 | The hypothalamus-pituitary-thyroid/adrenal gland/ovary/testis axis. ACTH, adrenocorticotropic hormone; LH, luteinizing hormone; FSH, follicle-stimulating hormone; FT3, free triiodothyronine; FT4, free thyroxine; TSH, thyroid-stimulating hormone; TRH, thyrotropin-releasing hormone.

treatment group was higher than that of the anti-PD-L1 and anti-CTLA-4 treatment group (45, 49). Besides, several patients receiving immunosuppressive therapy showed symptoms of hyperthyroidism before hypothyroidism (9). It is suspected that GD occurs first, and then thyroid cell antigen is exposed, which leads to autoimmunity and hypothyroidism because Th1 cells are dominant. Treg plays a role in the inhibitory effect through cell-cell contact and secreting a regulatory cytokine IL-10. Some studies have shown that a higher baseline IL-10 level can improve anti-PD-1 therapy response, indicating that PD-1 is involved in regulating the proliferation and differentiation of Treg (71). ICIs may cause the loss of Treg energy, inducing self-immune on the thyroid (72, 73). Taken together, T cell-mediated cellular immune is the main cause of thyroid IRAEs (**Figure 2**).

Role of Humoral Autoimmune Response in Thyroid Dysfunction

It is unclear whether PD-1 blockade induces B cell-mediated humoral autoimmune response. Whether patients with ICI-induced hypothyroidism have positive auto-antibodies is an

unknown problem (74). Several case reports found that there were negative auto-antibodies among patients with the treatment of ICIs, although thyroid dysfunction occurred (22, 50). A few data indicate that blocking PD-1 induces T cell-dependent B cells to produce and secrete auto-antibodies and the presence of thyroid auto-antibodies and an early increase in serum thyroglobulin (Tg) levels may result in an increased risk of thyroid IRAEs (9, 71, 75, 76). However, it remains to be determined whether there are any specific risks in subgroups with previous subclinical autoimmune thyroid disease (77). There was also another notion that thyroid auto-antibodies result from humoral immune response to release thyroid antigens in the process of destructive thyroiditis. Of greatest interest, a single-center, retrospective cohort study conducted by Delivanis et al. (65) showed that a minority of the patients had positive TPO-Ab among patients with thyroid IRAEs. However, Delivanis and colleagues (65) did not suspect that the mechanism of thyroid destruction is related to thyroid auto-antibodies. Therefore, whether auto-antibodies are the reason for thyroid dysfunction or the result of destructive thyroiditis when applying ICIs remains controversial, which may be the focus of future research (**Figure 2**). Moreover, Delivanis et al. (65) reported that NK cells or monocyte-mediated pathways may be involved in thyroid IRAEs due to the elevated HLA class (**Box 2**) surface expression in $CD56^{+}CD16^{+}$ NK cells and $CD14^{+}CD16^{+}$ monocytes, which needs data to ascertain its credibility.

Role of Individual Genetic Susceptibility in Thyroid Dysfunction

It is worth mentioning that autoimmune diabetes and AITDs, are associated with genetic susceptibility associated with overexpression of HLA-DR (human leukocyte antigen-DR isotype) (66, 78–80). More interestingly, hypothyroidism is more prevalent in patients with autoimmune diseases, such as type 1 diabetes and autoimmune gastric atrophy, and sometimes occurs as part of various autoimmune endocrine diseases (80). This phenomenon also exists in people who employ ICIs to treat malignant tumors (23). The mechanisms by which hypothyroidism may be linked to systemic autoimmune diseases have not yet been fully understood (64). ICIs may change the expression of HLA-DR, increasing the abnormal activation of T cells and thyroid autoimmunity susceptibility (**Figure 2**). Delivanis et al. (65) found that macrophage activation by up-regulating HLA-DR may be a potential mechanism of pembrolizumab-induced thyroiditis. Krieg et al. (77) have reported that the frequency of $CD14^{+}CD16^{-}$ HLA-DR^{hi} monocytes are a strong indicator for progression-free survival (PFS) and overall survival (OS) of anti-PD-1 immunotherapy.

Role of Various Cytokines in Thyroid Dysfunction

Besides T and B lymphocytes, various cytokines play an essential role in the development of thyroid disorders (**Figure 2**) (62). Firstly, a higher level of IL-2 can not only induce the binding between HLA-II with thyroid cell autoantigen, but also promote the killing effect of $CD8^{+}$ CTL on the thyroid (71, 81). Krieg et al. (77) found that the number of $CD4^{+}$ Th1 that express IFN-γ and IL-2 increased after anti-PD-1 treatment, indicating that PD-1 and PD-L1 are involved in the inhibition of T cell proliferation and the production of pro-inflammatory Th1 cytokines, including IFN-γ and IL-2. Kurimoto et al. (71) measured the changes of various cytokines before and after the treatment of ICIs and found that an increase of IL-2 and a decrease of granulocyte colony-stimulating factor (G-CSF) were seemly correlated with thyroid IRAEs. Th2 cytokine has a strong positive correlation with G-CSF, whose decrease may be related to the decrease of Th2 cytokine activity, which also indicates the increase of Th1 dominance in thyroid IRAEs (71). In summary, it is a plausible suspicion that Th1 cytokines (IFN-γ and IL-2) are involved in thyroid autoimmunity through blocking PD-1 and PD-L1. The decrease of IL-10 may be related to the increase of TPO-Ab, suggesting the loss of Treg energy and the development of thyroid IRAEs. Additionally, the toxicity mediated by IL-17 has been shown to contribute to anti-CLTA-4 induced enterocolitis, which also suggested the loss of Treg energy.

Role of Aging and Gender in ICIs Thyroid Dysfunction

Aging itself is conducive to an increased incidence of autoimmune diseases and malignant tumors because of immune function disorder and remodeling of the immune microenvironment (82, 83). The expression of PD-L1 is a critical mechanism by which aging tissues prevent their reactive T cells from infinitely participating in autoimmunity (2, 23). Some scholars believe that the immune system activated by ICIs is more likely to lead to thyroid self-immunity among the elderly (25). However, in an ICIs safety study among elderly patients with NSCLC, aging was not a high-risk factor for IRAEs (84). Sex hormones are also involved in the regulation of the immune system (85) but the relationship between gender and IRAEs remains unknown because of numerous contrary conclusions (72, 86, 87).

MANAGEMENT OF ICIs-RELATED THYROID DYSFUNCTION

In recent studies (17, 88–90), higher OS and PFS are observed among patients with thyroid dysfunction and positive auto-antibodies when they are treated with ICIs. Nevertheless, with the increasing use of immunosuppressive therapy, clinicians should identify and regulate thyroid IRAEs to prevent further rising incidence. At present, there is no prospective trial to determine the best treatment for thyroid IRAEs and the currently recommended treatment is based on the consensus of endocrinologists and guidelines (**Figure 4**) (18, 42, 91, 92).

Surveillance

The majority of patients with thyroid dysfunction after immunotherapy are asymptomatic (18). Therefore, it is unnecessary to expect patients to have positive and marked clinical manifestations when observing ICIs-related side effects on the thyroid. However, we should routinely assess the patient's symptoms and signs during the ICIs therapy. Moreover, we should focus on the thyroid biochemical indexes and imaging tests of patients to ascertain if it is hyperthyroidism, transient thyroiditis, or hypothyroidism, such as FT3, FT4, TSH, and cervical ultrasound (US) (92). The biochemical behavior of

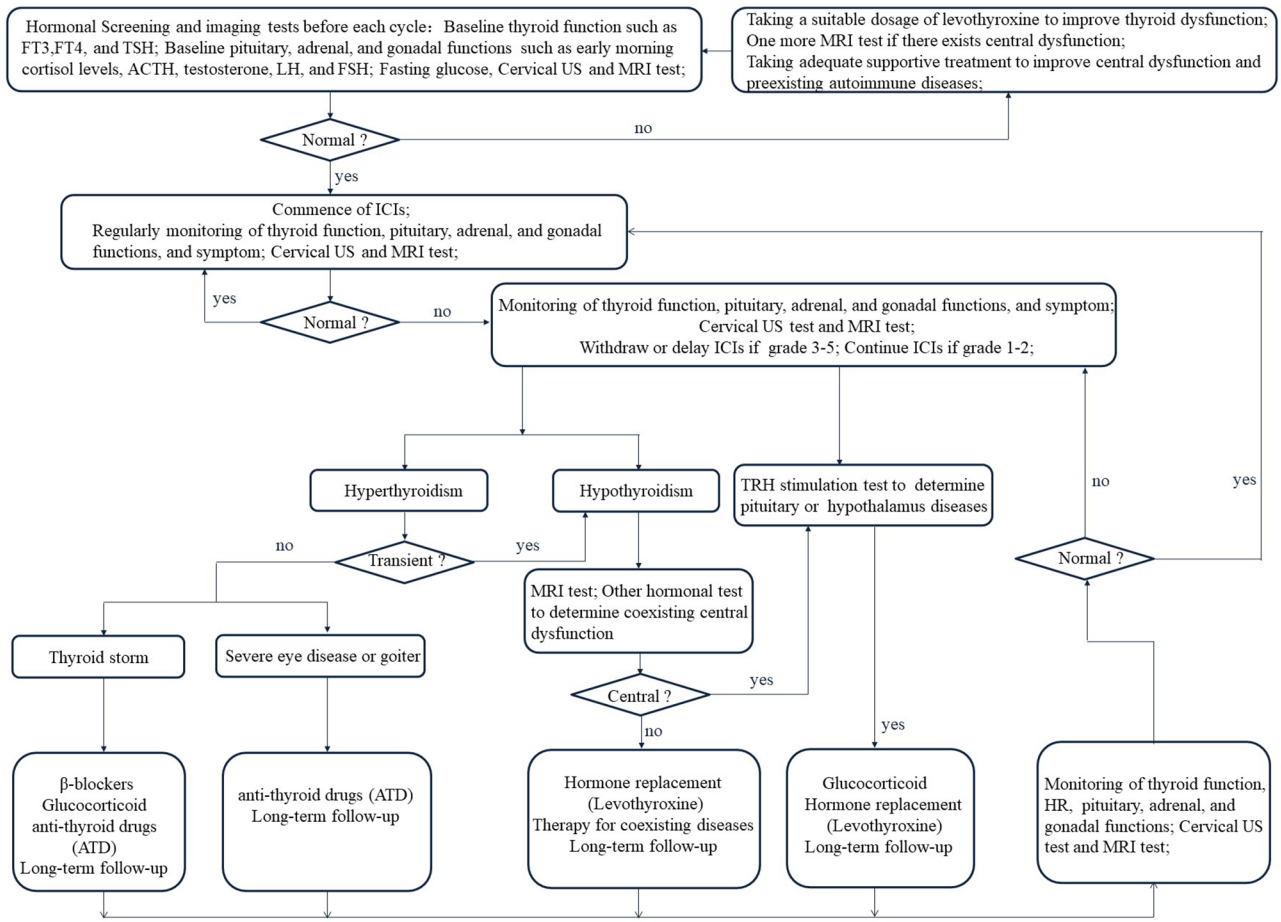

FIGURE 4 | Management flow chart for Immune checkpoint inhibitors-related thyroid dysfunction. FT3, free triiodothyronine; FT4, free thyroxine; TSH, thyroid-stimulating hormone; ACTH, adrenocorticotropic hormone; LH, luteinizing hormone; FSH, follicle-stimulating hormone.

thyroid dysfunction is discrepant in different tumor types on specific ICIs and cycles (13). Consequently, there is no consistent conclusion on monitoring time (92, 93). Routine biochemical function tests and screening can detect endocrinopathies before symptoms appear, promoting earlier treatment and a lower incidence rate. A comprehensively initial test should include a thyroid function test (FT3, FT4, TSH), fasting glucose, pituitary functions (early morning cortisol levels), adrenal function (plasma adrenocorticotropic hormone (ACTH)), and gonadal functions (testosterone, luteinizing hormone (LH), and follicle-stimulating hormone (FSH)) (42). In addition, the possibility of central hypothyroidism should be investigated by MRI test in the case of low FT4 with low TSH, including ICIs-induced hypophysitis, and then a systematic assessment of pituitary, adrenal, and gonadal functions is required (**Figures 1**, **3**, **4**) (18, 56). It is important to check coexisting hormonal disorders and thyroid function in case of hormonal replacement therapy. Besides, long-term follow-up for thyroid IRAEs is recommended (92).

In recent years, numerous scholars are trying to explore markers related to thyroid IRAEs (71, 94). There exists controversy about the exclusion of patients with autoimmune diseases from ICI therapy. There is increasing evidence that ICIs may be safe and effective in cancer patients with preexisting autoimmune diseases (95–97). Prospective studies to testify such novel strategies among patients with autoimmune diseases are needed. Nevertheless, the guidelines suggest that thyroid disease-related symptoms and signs, thyroid function test, other hormonal function tests and imaging tests should be detected before the beginning of immunotherapy and each treatment cycle (18). It is uncertain whether baseline assessment of thyroid antibodies will help identify the risk of thyroiditis because patients with a history of autoimmune diseases are mostly excluded from clinical ICIs trials (77). Currently, although there is insufficient data to recommend routine measurement of thyroid antibodies as a baseline standard, this may be a useful follow-up test to determine those who are more likely to have a persistent disease rather than transient drug-induced thyroiditis (17, 92). Most scholars suggested that close follow-up should be performed in patients who have high TPO-Ab at baseline or a history of hypothyroidism because they believe that it indicates an increased risk of hypothyroidism deterioration after the

employment of ICIs (9, 71, 75, 76). Test for TG-Ab and TSH receptor antibodies (TR-Ab) is necessary if there are clinical features and suspicions of GD (**Figure 1**) (92).

Treatment

As aforementioned, thyroid IRAEs has a relatively consistent pattern from the initial stage of transient hyperthyroidism to hypothyroidism or direct hypothyroidism (9, 13, 98). The presence of symptoms and the biochemical confirmation of evident subclinical hypothyroidism or hypothyroidism are the indications to start continuous thyroxine treatment (49). Taking a suitable dosage of levothyroxine in solid form on an empty stomach is the main choice (42, 92). However, elderly patients and patients with heart disease should receive low-dose levothyroxine (56, 91). Transient hyperthyroidism should not be treated because it usually subsides naturally and often transforms into hypothyroidism. However, when faced with serious thyrotoxicoses, such as thyroid storm (16), severe eye disease, or goiter (99), doctors should respond swiftly to ensure that patients get the best results. Supportive therapy of β-blockers, glucocorticoid, and anti-thyroid drugs (ATD) is helpful to relieve the symptoms of serious thyrotoxicosis (92, 100). Clinicians should decide whether ICIs should be stopped or delayed after the occurrence of thyroid dysfunction based on the grade in the CTCAE Version 5.0. (**Table 2**) (58). If there are coexisting hormonal disorders with thyroid function during hormonal replacement therapy, adequate supportive treatment should be considered in clinical practice (18, 42, 91, 92). Meanwhile, the employment of ICIs should be guided based on IRAEs grade (92). ICIs should be withdrawn or delayed if thyroid or other organ IRAEs are graded from level 3 to 5 but ICIs could be continued if thyroid or other organ IRAEs are graded from level 1 to 2 (18, 42, 91, 92). Of course, in case of IRAEs with adequate supportive treatment, ICIs can be continued or restarted (18, 42, 91, 92, 101, 102).

CONCLUSION

Thyroid dysfunction is the most common IRAEs, which warrants close attention from endocrinologists and oncologists. Thyroid IRAEs may involve T and B-lymphocytes, multiply cytokines, and diverse factors. With a limited understanding of the pathogenesis, it is not clear whether the immune cells responsible for IRAEs are the same as those involved in enhancing the anti-tumor immune response and HT. It is also controversial whether malignant tumor coexisting autoimmune diseases should be excluded from ICIs. We do not always exclude using ICIs for cancer patients with a preexisting autoimmune disease from the current understanding and consensus. Further clinical and laboratory researches should be conducted to improve the understanding of ICIs-related thyroid dysfunction. Additionally, the identification and management of thyroid IRAEs should be enhanced to avoid life-threatening complications and increasing mobility. Besides, the long-term effects of ICIs on thyroid function should be evaluated in future studies to better understand thyroid IRAEs and AITDs.

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 authors.

AUTHOR CONTRIBUTIONS

The manuscript was jointly written by LZ and H-FF. LZ, H-FF, X-LY, and S-RS contributed to the conception and design, the acquisition of data, the figure, and the drafting of the manuscript. H-QL, L-TG, CC, and X-LY collected and assembled the quantitative data. All authors contributed to the design and interpretation of the review, and reviewed and wrote the final paper.

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REVIEW
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The Molecular Mechanism of Sex Hormones on Sertoli Cell Development and Proliferation

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Sustaining and maintaining the intricate process of spermatogenesis is liable upon hormones and growth factors acting through endocrine and paracrine pathways. The Sertoli cells (SCs) are the major somatic cells present in the seminiferous tubules and are considered to be the main regulators of spermatogenesis. As each Sertoli cell supports a specific number of germ cells, thus, the final number of Sertoli cells determines the sperm production capacity. Similarly, sex hormones are also major regulators of spermatogenesis and they can determine the proliferation of Sertoli cells. In the present review, we have critically and comprehensively discussed the role of sex hormones and some other factors that are involved in Sertoli cell proliferation, differentiation and maturation. Furthermore, we have also presented a model of Sertoli cell development based upon the recent advancement in the field of reproduction. Hence, our review article provides a general overview regarding the sex hormonal pathways governing Sertoli cell proliferation and development.

Keywords: Sertoli cells, fertility, sex hormone, spermatogenesis, testis

BACKGROUND

Testes are destined to perform two important roles: to produce testosterone (steroidogenesis) and to maintain germ cell development (1). These functions are supported by the testicular somatic cells, Sertoli cells (SCs), which are located within the seminiferous tubules of testes (2, 3). Sertoli cells are considered as the most complex type of cells in an organism on the bases of their three-dimensional structure and their production of a microenvironment for germ cell development (3–5). Dependence of germ cells to obtain nutritional contents from Sertoli cells is owing to the presence of blood testes barrier (BTB) which physically portioned the seminiferous tubules into basal and adluminal compartments (6). The BTB is constituted by tight junction, ectoplasmic specialization (N-cadherin), desmosomes and gap junctions that are present in Sertoli cells (7–10). The SC–SC junctional complex has been studied and is known to undertake an indispensable job in testis directional morphogenesis (11, 12). Thus, Sertoli cells encompass all sorts of germ cells and have a chief assistive role in spermatogenesis.

Furthermore, developing germ cells cannot metabolize macromolecules such as lipids, carbohydrates and proteins, and most preferable energy source for germ cells is lactate molecule

which is produced by Sertoli cells (13, 14). On the other hand, Sertoli cells not only provide lactate to the developing germ cells for energy production but they also supply other nutrients including amino acids, vitamins and metal ions (14–16). Another important task of Sertoli cells is to generate and produce signaling molecules including growth factors and inflammatory cytokines which are involved in a cascade of events that are necessary for the spermatogenic process (17–19). Thus, accurate establishment and proper functioning of Sertoli cells is crucial for the developing germ cells to sustain the process of spermatogenesis.

PROLIFERATION AND MATURATION OF SERTOLI CELLS

The proper proliferation of Sertoli cells takes place during their immature period and can be mediated by specific factors (20, 21). The proliferative phase of Sertoli cells varies between species and two periods of Sertoli cells proliferation (one during fetal or neonatal period and other before pubertal period) are generally observed in various species (4, 6). Furthermore, marked variations exist between mature and immature Sertoli cells especially in terms of morphological and biochemical aspects. Generally, immature Sertoli cells reside on the basement and possess cytoplasmic projections which fill up the space of seminiferous cords (20, 22). In addition to immature Sertoli cells, seminiferous tubules also contain peritubular and germ stem cells which give solid appearance with the absence of lumen (23). After puberty, the Sertoli cells start to elongate and BTB begins to establish (8). Finally, Sertoli cells switch from their immature stage to mature phase and their proliferative state is stopped (6). At this stage, mature Sertoli cells represent radical changes within their morphology and functions. Further changes occurred in the nucleus and nucleoli become large in size along with the completion of tight junction which makes the fluid filled lumen space. The whole process of Sertoli cell proliferation and maturation is regulated under strict control and any impairment in the process of Sertoli cell development or proliferation can causes pathological events which may lead to the reduction of sperm count and semen quality (6, 20, 24–26).

Sertoli cells can serve as the organizing center for testis differentiation and signalings from Sertoli cells also regulate the differentiation events of testicular cord formation and testis organogenesis (5, 27). The Sertoli cells also provide a means of canalizing gonadal fate to coordinate testis development (5). Interestingly, Sertoli cell fate, once specified, is not permanent but instead needs to be constantly reinforced (3, 5).

Testicular development and spermatogenesis are influenced by various hormones which are generally mediated by the hypothalamus–pituitary–gonad (HPG) axis (14, 27) (**Figure 1**). HPG axis establishes a connection between brain and testes (28, 29). The gonadotropin leuitinizing hormone (LH) and follicle stimulating hormone (FSH) are secreted by adenohypophysis which are considered to be the important regulators of testicular function (30). It has been demonstrated that FSH mainly controls the proliferation of Sertoli cells while LH regulates testosterone production (14, 27, 31). Thus, the pre-pubertal decrease of LH and subsequent FSH secretion tends to cause a disturbance in the pulsatile release of gonadotropin-releasing hormone (GnRH). This hypothalamic-releasing agent provides the main push to the gonadotropin-secreting cells of the anterior pituitary gland (32). What is more, the HPG axis also works in association with local endocrine system to mechanistically regulate the complete process of Sertoli cell maturation and testis development (30). Paracrine system intercedes with various types of cells including germ cells, peritubular myoid cells and Leydig cells. Thus, proper hormone levels and their regulation are necessary for these complex processes which further ensure the accurate and smooth development of Sertoli cells to support spermatogenic process.

Sex Hormones in Sertoli Cell Development and Proliferation

The complex process of reproduction is generally regulated by various factors including autocrine, paracrine, juxtracrine and endocrine environment within the gonads (33). Though these processes are well inter-connected, the major function is performed by sex hormones such as leuitinizing hormone, follicle stimulating hormone and prolactin that orchestrate and coordinate sexual development, sexuality and reproduction (34–36). Sex hormones are also playing key roles in development and maturation of Sertoli cells by modulating either Sertoli cell metabolism or influencing growth signaling pathways (14, 27, 31, 35–38). These hormones also create adequate ionic environment in Sertoli cells which is required for germ cell development. In this review, we have discussed the role of reproductive hormones in association with Sertoli cell development, proliferation and maturation (**Figure 2**).

Follicle Stimulating Hormone (FSH)

FSH plays a crucial role in fertility as it influences the proliferation of Sertoli cells during perinatal life and also stimulates the production of Sertoli cell derived factors that are required for the development of germ cells and testes (39). FSH, LH, thyroid-stimulating hormone (TSH) and chorionic gonadotropin (hCG), belong to pituitary glycoprotein hormone family and these hormones are known to perform important function during Sertoli cell development, thus, directly or indirectly influencing male reproductive health. These hormones are usually existed in the form of a heterodimer which consists of a α-subunit that has the ability to associate with β-subunit (40).

The mechanism of action in which FSH binds and stimulates membrane receptor belonging to the G protein-coupled receptor (GPCR) superfamily (41). It was noted that FSH receptor (FSHR) presents tissue specificity as it is majorly expressed in granulosa cells (female) and Sertoli cells (male) (42). Furthermore, FSHR has the capability to subordinate with other type of G proteins including Gαi to initiate signaling cascade events that modulate Sertoli cell function. Impaired secretion of FSH due to homozygous mutation in the gene encoding β-subunit leads to bilateral small and soft testicles, androgen deficiency, elevated level of LH in serum, low level of testosterone, as well as azoospermia in

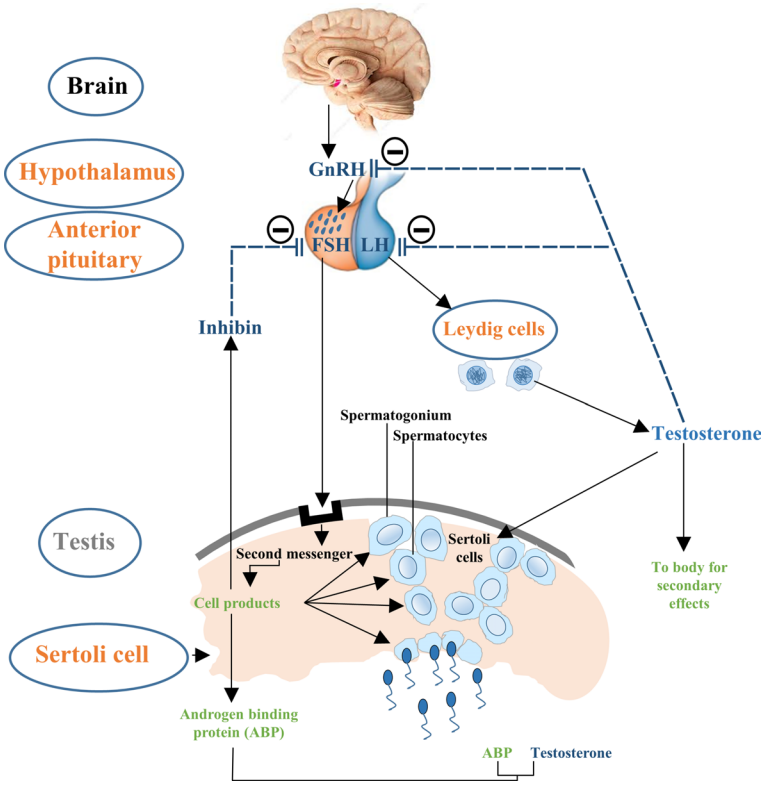

FIGURE 1 | Flow chart description the control of hypothalamus–pituitary–testis axis on Sertoli cell proliferation. The hypothalamic GnRH modulates the biosynthesis and secretion of pituitary hormones i.e., LH and FSH. LH induces secretion of testosterone in Leydig cells and is involved in the late Sertoli cell proliferation period, followed by negative feedback reducing GnRH and LH production. FSH primarily stimulates the seminiferous tubules to form steroid hormones such as inhibin and further sustain the process of spermatogenesis. Steroid hormones i.e., testosterone and inhibin exert negative feedback effects on GnRH.

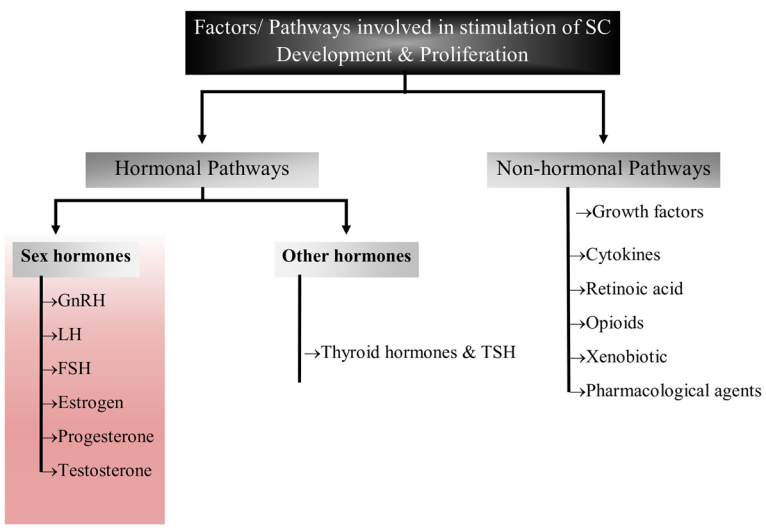

FIGURE 2 | Flow chart diagram representing the factors/pathways involved in Sertoli cell development and proliferation. This figure summarized the role of sex hormones, hormones other than sex hormones and non-hormonal pathways that have been implicated in Sertoli cell development.

human (43, 44). Furthermore, homozygous *FSHR* mutations lead to male infertility in few cases, while the A189V *FSHR* mutation in males is linked with subfertility but not azoospermia (45). Interestingly, *Fshr* knockout mice still had sperm production albeit sperm reduction was observed (46–48).

It is a well-known fact that FSH is the factor necessary for Sertoli cell mitogen which stimulates the expression of various Sertoli cell markers such as c-Myc, Cyclin A2, Cyclin D1, and proliferating cell nuclear antigen (PCNA) (39, 49). Moreover, it has been described that FSH level and FSHR expression become stable after puberty, however, a change has been observed in signaling pathways triggered by FSH during transition of Sertoli cells from proliferation to differentiation stage (50). Consistently, some pathways such as FSH-mediated ERK activation and calcium uptake are exclusively activated in immature Sertoli cells during proliferative phase. The opposite action of FSH in immature and mature Sertoli cells is related to the cAMP kinetics (51). It was found that cAMP level was low in immature rat Sertoli cells. On the other hand, higher basal concentration of cAMP was observed in 20 days old Sertoli cells along with almost 4-fold increased activity of phosphodiesterase and completely abolished in older rat Sertoli cells (52–55). Hence, it is assumed that diverse function of Sertoli cells in response to FSH might be responsible for robust onset of germ cell differentiation during prepubertal testicular maturation in rats. What is more, *Gαs* and *Ric8b*, which activate adenylate cyclase for supplementing cAMP production and gene transcription, can also cause constrained FSH action during infancy in primates (56). Thus, the FSH action on Sertoli cell development and maturation is complicated and it is still difficult to investigate the complete array of signaling events *in vivo*.

In fact, it is hard to differentiate the overlap signaling pathways *in vivo* that are triggered during Sertoli cell proliferation and maturation. Most of the studies are conducted *in vitro* and these studies have demonstrated some of the major signaling pathways that are stimulated by FSH. In this regard, a study described that FSH binds with its receptor (FSHR) to form Gα protein, which is further dissociated into two heteromeric molecules, Gα-subunit and Gβ/γ unit. This dissociation further stimulates a cascade signaling mechanism by activating mitogen-activated protein kinase (MAPK), or phosphoinositide 3-kinase (PI3K)/protein kinase B (PKB) and adenylate cyclase/cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) which cause a change of Sertoli cell membrane potential and calcium influx. During this process, each subunit of FSH heterodimer protein is destined to perform specific function such as Gα subunit is responsible for the activation of adenylate cyclase which further initiates the formation of cAMP and phosphorylation of PKA (57, 58). Furthermore, PKA activates structural proteins, transcription factors and enzymes which trigger diverse biological processes with varying effects on Sertoli cells (37). More specifically, FSH has biphasic effects on membrane potential of immature rat Sertoli cells, which are manifested by membrane hyperpolarization (59).

FSH was also found to stimulate cAMP/PKA which intercedes various protein phosphorylation to trigger calcium channels and their regulators. But the complete scenario of FSH stimulation of cAMP/PKA and subsequent voltage gated calcium channels (VDCC) modulation is still not clear. Previous reports described that PKA system phosphorylates α1-subunit of the VDCC resulting in calcium potentiation (60, 61). However, up till now, no research has been conducted to investigate this mechanism in Sertoli cells. The addition of PKA and adenylate cyclase inhibitors [MDL, (Bu)2cAMP and staurosporine] in cultured Sertoli cells can partially impede FSH mediated calcium uptake, indicating involvement of other mechanisms in calcium influx during Sertoli cell proliferation (62). Further evidence showed that Sertoli cell proliferation is not only depend upon AC/cAMP/PKA pathway, some alternative mechanisms also exist, such as FSH-mediated dissociation of the Gαi-GGβ/γ heterodimer which causes calcium influx through L-type VDCC and [^{14}C]-MeAIB transport system (63, 64). Moreover, FSH has the ability to transport small amino acids through activation of system A (which is basically designed for the transport of neutral amino acids with small side chains such as alanine, serine and glutamine). System A activation by FSH can provide nitrogen from alanine and other amino acids for biosynthesis of proteins and nucleotides which are essential for cellular growth (65, 66). Similarly, alanine is converted into pyruvate and is used as energy substrate by Sertoli cells. The presence of this alternative mechanism of Sertoli cell proliferation has been validated by inhibition of [^{14}C]-MeAIB transport system (67). FSH activates PI3K downstream target, PKB, which further stimulates enhanced uptake of glucose, calcium and small amino acids in cultured Sertoli cells (68). The active PI3K/AKT signaling pathway is required to stimulate the actions of FSH, whereas an active ERK/MAPK pathway can inhibit the expression of aromatase (such as Cyp19a1) (69). Altogether, these pathways are essential for proliferation and differentiation of immature Sertoli cells that pave the way for successful spermatogenesis (70). Taking consideration of all these studies, a comprehensive diagram explaining the role of FSH and other factors in Sertoli cell proliferation can be proposed (27) (**Figure 3**).

Androgens

Cessation of proliferative phase of Sertoli cells is mediated by changes in gene expression and establishment of BTB and finally Sertoli cells become able to sustain developing germ cells. Thus, it is imperative to investigate the factors that are involved in transition of Sertoli cells from proliferation to maturation phase. In this regard, some studies have demonstrated that androgens and their derivative products are key mediators for Sertoli cell proliferative phase cessation in diverse species (73, 74). In fact, androgens play important functions that reach far beyond the reproductive process, for example 5α-dihydrotestosterone (DHT) regulates glucose consumption and lactate production in cultured rat Sertoli cells (35, 75). Similarly, it is also reported that long time treatment of DHT in cultured human Sertoli cells can cause decrease expression of lactate dehydrogenase A and monocarboxylate transporter 4 (MCT4) levels (76).

The function of androgens is intensively investigated in terms of fertility and spermatogenesis while its role in Sertoli cell maturation and development generally remains elusive, instead of knowing that high amounts of androgens is produced by

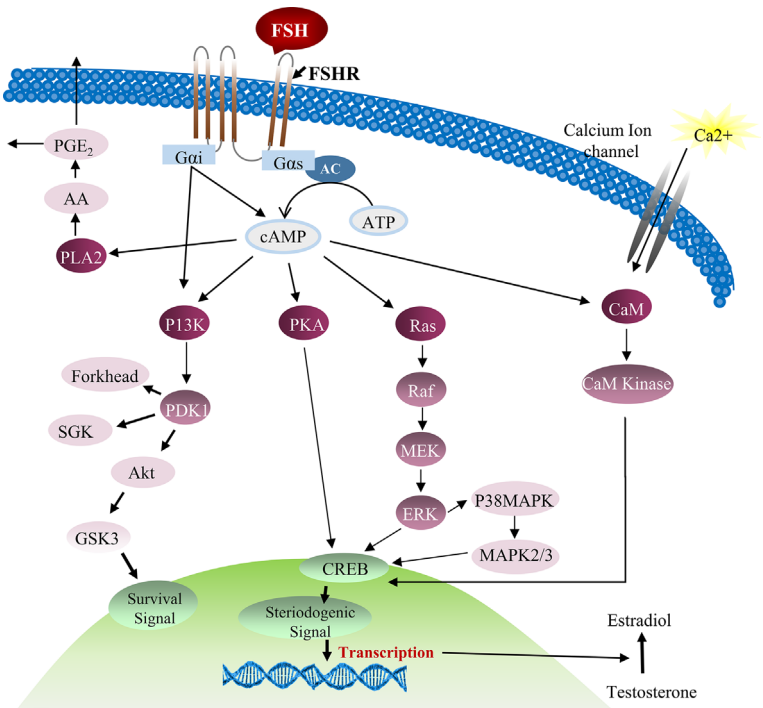

FIGURE 3 | FSH and testosterone signaling pathways in Sertoli cell proliferation. Initially FSH binding to the FSH receptor causes receptor coupled G proteins to activate adenylate cyclase (AC) and increase intracellular cAMP levels. Multiple factors can be activated by cAMP in Sertoli cells including PKA that can phosphorylate a number of proteins and also regulate the expression and activity of numerous transcription factors including CREB. FSH also causes Ca^{2+} influx into Sertoli cells that is mediated by cAMP and perhaps PKA modification of surface Ca^{2+} channels. Depolarization of the cell is also involved in Ca^{2+} influx. Elevated Ca^{2+} levels can activate calmodulin and CaM kinases that have multiple potential downstream effects including the phosphorylation of CREB. During puberty, FSH activates the MAP kinase cascade and ERK kinase in Sertoli cells most likely via cAMP interactions with guanine nucleotide exchange factors (GEFs) and activation of Ras-like G proteins. ERK is capable of activating transcription factors including SRF, c-jun and CREB. FSH and cAMP likely act through GEFs to activate PI3-K and then phosphoinositide dependent protein kinase (PDK1) and PKB in Sertoli cells. FSH also mediates the induction of PLA_2 and the subsequent release of arachadonic acid (71, 72).

Leydig cells in the form of testosterone. The dynamic level of testosterone is observed during different developmental stages of organism such as its concentration increases at the end of fetal life, and starts to decrease from birth until puberty, and then increases again (77, 78). Testosterone performs its function through classical and non-classical mechanism. Non-genomic signaling of testosterone can activate gene transcription through CREB mediated pathway (79, 80). Furthermore, zinc transporter ZIP9 subfamily protein that is localized on the plasma membrane also has ability to mediate testosterone level (81).

Testosterone can also function in a non-classical pathway through the androgen receptor to rapidly activate kinases. For example, by increasing testosterone levels, the MAP kinase cascade is rapidly activated in Sertoli cells. An inhibitor of non-classical testosterone signaling blocked meiosis in pubertal mice and caused germ cell loss in adult mouse testes, while a classical pathway inhibitor caused the premature release of immature germ cells. Thus, classical and nonclassical testosterone signaling have overlapping and distinct functions that are required for the spermatogenesis and male fertility. Furthermore, some findings suggested that the non-classical testosterone signaling can act *via* Src and ERK kinases to facilitate the adhesion of germ cells to Sertoli cells (82, 83). On the other hand, the non-classical signaling of androgens alter the cellular process within seconds to minutes (84). This system can increase calcium influx by activation of phospholipase C which causes hydrolysis of phosphatidylinositol 4,5-biphosphate (PIP2) (85). The absence of PIP2 decreases negative charges on membranes and causes closing of K^+ ATP channels and opening of the VDCC, which results in calcium influx (86). Similarly, the calcium influx stirred by testosterone may be involved in several other biological processes such as cytoskeleton rearrangement, gene transcription and cell proliferation (27, 42). Thus, it is believed that non-classical action of androgens is more closely related to Sertoli cell maturation and spermatogenesis.

Studies that *in vivo* treatment of testosterone caused reduced incorporation of $[H^3]$ thymidine by Sertoli cells in some species suggesting that testosterone can inhibit the proliferation of Sertoli cells (35, 87). Further work by Buzzard and colleagues displayed that the addition of testosterone in cultured Sertoli cells leads to strong inhibition of proliferation as well as increased expression of cell cycle inhibitor markers such as p27Kip1 and p21Cip1, while it also induces the enhanced expression of GATA-1 which is a marker for Sertoli cell differentiation (88). By crossing hypogonadal (hpg) mice that lack gonadotrophins and intratesticular androgen with mice lacking ubiquitous AR (ARKO) or specifically in Sertoli cells (SCARKO), O'Shaughnessy et al. found that dihydrotestosterone

has no effect on germ cell numbers in hpgSCARKO and hpgARKO mice, while testosterone increased germ cell numbers in hpgSCARKO and hpgARKO mice, and this was associated with stimulation of FSH release (89). Thus, androgen stimulation of spermatogenesis requires direct androgen action on the Sertoli cells. However, some studies on mouse model demonstrated controversial results related to androgens involvement in Sertoli cell proliferation. For example, *Tfm* mutant mice lacking functional androgen receptor and *AR* knockout mice displayed reduced Sertoli cell number (90–92). But the observed phenotypes of *Tfm* and *AR* knockout mice could not be attributed entirely to the androgen effect on Sertoli cells since androgens are also known to be produced by peritubular cells. Nevertheless, specific deletion of *AR* in mouse did not show any aberration in Sertoli cell number as well as the expression of Sertoli cell maturation markers (90). These results demonstrated that androgens may affect Sertoli cell proliferation through an indirect way because peritubular cells secrete Activin A which also influences Sertoli cell physiology (93–95). Furthermore, SCAR KO mice showed minor changes which further suggests that the effect of androgen on number of Sertoli cells is not regulated by the direct action. Subsequently, *TgSCAR* (transgenic mouse with gain of function) mice showed reduced Sertoli cell proliferation which further lead to decreased testis size (96). Altogether, it can be deduced that AR expression in Sertoli cells is wisely orchestrated to avoid early maturation of Sertoli cells.

The synergistic actions of testosterone and FSH *via* testicular Sertoli cells regulate male fertility (53). FSH acts through receptors (FSHR) on the Sertoli cell to stimulate spermatogenesis while androgens promote testis growth through receptors (AR) on the Sertoli cells, Leydig cells and peritubular myoid cells. By examining the effects on testis development of ablating FSHRs (FSHRKO mice) and/or ARs ubiquitously (ARKO mice) or specifically on the Sertoli cells (SCARKO mice), results showed that development of most testicular parameters is more dependent on FSH action than androgen action mediated through the Sertoli cells prior to puberty. Post-pubertally, germ cell numbers and spermatogenesis are dependent on FSH and androgen action through the Sertoli cells (91). Furthermore, through the analysis of mice lacking both FSH receptors and androgen receptors in Sertoli cells (FSHRKO-SCARKO), Abel et al. found that FSH and androgen act through redundant, additive, and synergistic regulation in spermatogenesis and Sertoli cell activity (97). Additionally, in pubertal primate (*Macaca mulatta*) Sertoli cells, prolonged stimulation of testosterone significantly elevated the expression of genes involved in FSH signaling pathway such as FSHR, GNAS and RIC8B, and this was associated with a rise in cAMP production. Testosterone also augmented FSH induced expression of genes like SCF, GDNF, ABP and Transferrin. Such a coordinated network of hormonal signaling in Sertoli cells may facilitate the timely onset of the first spermatogenic wave in pubertal primates and is responsible for normal spermatogenesis (53). On the other hand, it has been reported that insufficient FSH and androgen are associated with azoospermia in infantile primate testes (98). Thus, it is assumed that infant primate Sertoli cells may have insufficient number of AR and the binding ability of testosterone to AR might be compromised during primate infancy.

Luteinizing Hormone

Luteinizing hormone (LH) belongs to the family of glycoproteins, with α subunit and hormone-specific β subunit. LH and FSH both were isolated as molecules in 1942 and these two gonadotrophins are involved in synthesis of estradiol and ultimately form the androgens. LH accelerates testosterone production in Leydig cells, thus, helping in spermatogenesis by directly impacting on Sertoli cells. Knockout mice for LH receptor (*Lhr*) have no testosterone production with disrupted spermatogenesis. This LH-dependent testosterone absence leads to azoospermia, however, in some cases absence of LH signaling does not disrupt the pathway fully and results in oligozoospermia with low testosterone production (99). But knockout mice for luteinizing hormone/choriogonadotropin receptor *(Lhcgr*) had elevated levels of *Wnt5a* (wingless-type MMTV integration site family member 5A) in Sertoli cells that favors cell proliferation. It was also noted that absence of LH caused alternations in genes associated with Sertoli cell development and proliferation (100, 101).

Three genetically modified mouse model were generated to study the effect of LH on Sertoli cell development by completely or partially reducing its activity. Two distinct strategies were used to generate these mutant models; one with LH-deficient hpg-(hypogonadal) mice to selectively study either pituitary-independent transgenic-(tg) FSH or ligand-independent activated tg FSH receptor (FSHR) expression, and second model used LH receptor (*Lhr*)-deficient mice in which their gonads were isolated to examine endogenous mouse FSH effects on gonad development. Analysis of these models showed subtle differences in gem cell maturation between *tg-hpg* and *Lhr*-null mouse models, indicating that the FSH cannot fully restore Sertoli cell number in absence of LH activity (102). Thus, the synergistic effect of both LH and FSH is important for proper proliferation and development of Sertoli cells.

Estrogens

Estrogens are steroid pleiotropic hormones, present in ovary and testis. These hormones act by cytosolic estrogen receptors (ERs). Alpha (α) and beta (β) receptors are found in animals while in fishes, ERγ has been discovered as the third type receptor. ERα and ERβ are located in the cell membrane; either as homodimers (ERα-ERα or ERβ-ERβ) or as heterodimers (ERα-ERβ). These hormones play their roles in production, regulation as well as maintenance of concentration of testicular liquid (36).

A study conducted by Royer et al. indicated that estradiol initiates the proliferation of Sertoli cells by activating classical estrogen receptors and G protein-coupled estrogen receptor which further induce a cascade of signaling events through CREB activation (103). It is important to mention that estrogen expression in testis is dynamic and varies from postnatal to adult life. Its concentration increases from 20 days of post-partum and continues to increase till 30 days old while aromatase transcripts has not been detected in adult rat Sertoli cells. However, in adult rats, aromatase expression is noted in Leydig, pachytene spermatocytes and round spermatids (104). It is suggested that Sertoli cells may produce estrogen in immature

animals while the source of estrogens in adult animals comes from Leydig or germ cells (105). Hence, complete understanding of physiological effects of estrogens is necessary to investigate its actual function in postnatal testis development.

Studies found that *ERα* knockout mice or rats are infertile while *ERβ* knockout mice or rats have no such abnormalities, which indicates that ERα subunit is essential for fertility and reproduction. This function is evident in 15 days old rat Sertoli cells in which ERα promotes cell proliferation by acting on NF-kB (nuclear factor-kB) in P13K and ERK1/2 (extracellular signal-regulated kinase 1/2) manner and ultimately increasing the levels of Cyclin D1. On the other hand, ERβ promotes cell cycle arrest by interacting with 17β-estradiol (E2) (106).

Progesterone

Progesterone is a major cholesterol-derivative steroid and is specifically involved in reproduction. The receptors of this hormone are localized in the nucleus and cytoplasm of spermatogenic cells, Sertoli cells and occasionally in the Leydig cells. Structurally, two isoform receptors of this hormone exist; namely PR-A and PR-B and these intracellular proteins belongs to nuclear receptor superfamily of transcription factor (107). High level of progesterone has inhibitory role in spermatogenesis by limiting the production of Leydig cells and Sertoli cells at developmental stage (108). The effect of progesterone was examined by generating progesterone receptor (*PR*) knockout mice. *PR* knockout mice displayed large testis size, increased total sperm contents and increased number of Sertoli cells. On the other hand, synthetic progestins such as levonorgestrel (LNG) in combination with testosterone caused suppression of spermatogenesis and increased germ cell apoptosis (108).

Prolactin

Prolactin is a type of polypeptide hormone that is involved in wide range of biological functions including lactation, osmoregulation, immune articulation and reproduction (109). Prolactin receptors (PLR) are present on Sertoli cells and prolactin through its receptors mediates proliferation of Sertoli cells (110). Various reports reveled its biological function in reproduction and elevated level of prolactin leads to hypogonadism and male infertility (111). It is highly recognized that prolactin regulates testicular function by two ways either altering pituitary function by inducing LH and FSH production or Leydig cells through modulation of testosterone hormone (1). Furthermore, targetted mutation of prolactin receptor in model organism displayed mild phenotype indicating that prolactin has partial effects on male reproductive health (112).

OTHER REGULATORY FACTORS INVOLVED IN SERTOLI CELL DEVELOPMENT AND PROLIFERATION

Besides the mentioned hormones, many other factors such as growth factors, cytokines, xenobiotic and pharmacological agents, have been identified and are involved in Sertoli cell development process. Opioids, such as α-melanocyte-stimulating hormone (αMSH), β-endorphin and proopiomelanocortin (POMC), mainly produced in Leydig cells, exert direct paracrine actions on Sertoli cell proliferation (113–115). The *in vitro* exposure of fetal human testis to ibuprofen does not modify the number of Sertoli cells but decreases AMH and SOX9 expression, suggesting a role in Sertoli cell maturation (116).

Insulin Receptor Signaling Family

The insulin receptor tyrosine kinase family consists of insulin receptor (IR), IGF-1R and insulin related receptor (Irr). These receptors are present in all types of cells in eutherian mammals (117). Mice lacking IR and IGF-1R die within four days after birth due to ketoacidosis and respiratory failure, respectively (118). A lot of studies had investigated the function of these hormones and new insights regarding their involvement in reproductive system (29, 50, 117, 119–121). Recently, a study investigated the *in vivo* function of IR and IGF-1R in which both factors work in a synergistic way to regulate the Sertoli cell number and testis size. Furthermore, the study also described that both receptors and their downstream molecules are critical for the development of male gonads and sexual differentiation (122). Similarly, the insulin-related peptide hormone relaxin *(Rlx*) has also been recognized to perform an essential role in reproduction and it precipitates in the regulation of the cyclic adenosine monophosphate and nitric oxide pathways that are implicated in Sertoli cell proliferation (123).

It has been recognized that insulin is involved in energy metabolism and also regulates cell proliferation and differentiation. Generally, the insulin function is interceded by IR through phosphorylation that further activates classical signaling mechanism involving adaptor protein such as insulin receptor substrate-1 (IRS-1) (28). Different studies have reported the function of insulin in testicular development, in modulating testicular cell function (38, 124, 125), or even influencing HPG axis function (28, 29). Furthermore, the compromised function of insulin is the leading cause of Diabetes Mellitus (DM) which is usually accompanied by aberrant testosterone levels (126). Thus, it can be deduced that insulin could regulate testosterone secretion in human and animal models. A study demonstrated that insulin directly influences Sertoli cell metabolism by affecting amino acid accumulation, glucose transport and lactate production either through the modification of glucose transporter expression or altering important glycolytic enzyme activity (121). Further studies indicated that cultured Sertoli cells can cause reduced lactate production and altered caspase-dependent apoptotic signaling (75, 127). Similarly, it has also been reported that insulin activate calcium-dependent membrane depolarization in immature Sertoli cells, which is mostly induced through IGF-1R activation (120). Altogether, these findings clearly indicate the importance of insulin function in regulating Sertoli cell metabolism which is further manifested by Sertoli cell proliferation.

Cytokines

Various studies reported that inflammatory cytokines are not only produced by macrophages in response to inflammatory signals but these cytokines are also secreted from Sertoli cells and

appear to take part in the regulation of Sertoli cell proliferation (1, 19). For example, interleukin-1, 6 (IL-1 and IL-6) and tumor necrosis factor α (TNF-α) are produced by Sertoli cells and *in vitro* studies demonstrated that all of these cytokines are involved in Sertoli cell metabolism by activating the production of transferrin. Furthermore, it was also noted that only IL-1, neither IL-6 nor TNF-α, enhanced lactate production and secretion during Sertoli cell proliferation (1). Notably, IL-1 activity in Sertoli cells can be specifically neutralized by IL-1α antiserum, implying that IL-1α is the major isoform of IL-1 in Sertoli cells (17). But the underlying pathophysiological mechanism is still not completely understood due to the lack of *in vivo* studies. Interestingly, animal model studies with disrupted interleukin or tumor necrosis factors displayed no obvious alterations in testicular development. Thus, the actual function of these cytokines in relevance to Sertoli cell development is still obscure and needs more investigations.

Thyroid Hormones

It has been described that thyroid hormones regulate lactate production, glucose transporter type 1 mRNA levels, aromatase activity, Sertoli cell proliferation and other processes of Sertoli cells in various mammalian species (128–130). The involvement of thyroid hormones in establishing the Sertoli cell population have been extensively investigated and results indicated that thyroid hormones can affect Sertoli cell proliferation through direct or indirect ways. Generally, indirect way of thyroid hormone on Sertoli cells is mediated by triiodothyronine (T3) that inhibits FSH production and leads to reduced Sertoli cell proliferation (128). Some studies demonstrated that T3 treatment can reduce Sertoli cell proliferation activity, as well as Sertoli cell proliferation period and Sertoli cell number (88, 128, 131). Similarly, it has been described that T3 also stimulates the maturation of Sertoli cells *in vitro* implying that T3 can terminates Sertoli cell proliferation and favors the terminal maturation of Sertoli cells (132). Thyroid hormones can halt Sertoli cell proliferation by accelerating the accumulation of cell cycle inhibitors p27Kip1 and p21Cip1 (88, 133, 134). To be noted, thyroid hormone, retinoic acid, and testosterone share similar suppressive effects on the rate of Sertoli cell division without any apparent additive effects (88). Another study displayed that *Connexins 43* (*Cx43*) could be an intermediate target of T3 in the inhibition of Sertoli cell proliferation (135). Thus, a balance level of thyroid hormones during early life of development is essential for the terminal differentiation of Sertoli cells.

WNT and BMP Signaling Pathways

The vertebrate WNT (Wingless-related integration site) family consists of 19 secreted cysteine-rich glycoproteins (136). Though WNT signaling exerts an antagonistic effect on testis-determining pathways in sex determination during the embryonic stage, it promotes sperm maturation in adult epididymis (137). During the development of seminiferous tubules, Wnt/β-catenin can play an important role in the differentiation of Sertoli cells. However, these findings appear to be inconsistent about the influence of Wnt/β-catenin signaling. For example, several studies have shown that β-catenin deletion does not induce aberration in Sertoli cells, but β-catenin stabilization results in immaturity, inadequate differentiation and irregular cellular interaction in Sertoli cells, as well as reduced proliferation and increased apoptosis of germ cells (138–141). Similar findings have also been observed when the Wnt/β-catenin pathway is activated in *APC* (adenomatous polyposis coli-conditional) knockout mice (142). Therefore, the suppression Wnt/β-catenin pathway is required to sustain normal maturation and proliferation of Sertoli cells (143).

Bone morphogenetic proteins (BMPs) and transforming growth factor-beta superfamily (TGF-β) also have pivotal roles in reproductive biology. Their roles have been established by various *in vivo* and *in vitro* studies. BMP2, BMP4, BMP8a and BMP8b are involved in specification of primordial germ cell (PGC), acceleration of spermatogonial proliferation as well as are responsible for adult spermatogenesis *in vivo* (144).

A study found that *Bmp4* was expressed in postnatal days 4 and 7 isolated Sertoli cells implying that *Bmp4* perform important role in early postnatal testis development. In testes, multiple BMP genes are expressed and BMP7 and BMP8 a/b transcripts were specifically found in germ cells at various stages of differentiation (145, 146), thus indicating that these molecules may mediate paracrine interactions which are secreted by Sertoli cells. Furthermore, mice lacking BMP8b had smaller testes, similar type of phenotype was also observed in *BMP8a* null mice (147, 148). A recent study explored the role of Sclerostin domain containing 1 protein (*Sostdc1*) in modulating the Sertoli cell gene expression and its possible outcomes on mouse spermatogenesis. Interestingly, Pradhan et al. found that *Sostdc1* is a negative regulator of spermatogenesis, and found that down regulation of *Sostdc1* during puberty is necessary for quantitatively and qualitatively normal spermatogenesis (149). Thus, it is argued that *Sostdc1* is a dual BMP/Wnt regulator and plays indirect role in mouse spermatogenesis by influencing Sertoli cells.

Activin and Inhibin

Activins are dimeric glycoproteins, consisting of β subunits and members of TGF-β superfamily. Activins mediate FSH production by a cascade of interacting proteins event (150). Their ability to bind with type II receptor causes phosphorylation of type I receptor, starting a series of phosphorylation of SMAD proteins (SMAD2, SMAD3, SMAD4) which ultimately triggers the transcription of FSHβ encoding gene (150, 151). On the other hand, inhibins and follistatin are considered as antagonists of activins (152). They are also glycoproteins but structurally different from activins. Inhibins compete at binding sites for activins which ultimately affects its activation. Thus, activins, inhibins and follistatin collectively form a complex autocrine network that plays a vital role in fertility. The interruptions of these can cause lower testis size, progressive sterility, delayed fertility as well as other fertility-related issues due to defective Sertoli cell development and proliferation (153, 154).

Retinoic Acid

Retinoic acid (biologically active component of vitamin A) is a major factor that control the complex process of spermatogenesis and is also important driven force of Sertoli cell development (155). RA induce the initiation of spermatogonia differentiation in the mammals and its activity is generally governed by FSH

(156). The functional role of RA was verified by generating vitamin A-deficient (VAD) mice that were infertile due to spermatogonia differentiation arrest at the $A_{aligned}$ stage and treating them with RA results in the complete recovery of spermatogenesis (157).

SUMMARY

Sex hormonal regulation of Sertoli cell proliferation, differentiation and maturation is an intricate process which requires synergistic effects of these hormones along with the regulatory factors including IGF-1R, insulin, thyroid hormones and cytokines. All these hormones and factors have been implicated in various stages of Sertoli cell development and their balanced action of mechanism is mandatory for ensuring accurate Sertoli cell number, establishment of BTB and maintaining spermatogenesis. Although, recent *in vivo* studies explained the involvement of FSH, androgen, estrogen and IGF-1R to be essential for Sertoli cell development, still the complete scenario of this complex process is unresolved. Thus, it is suggested that there are some additional factors needs to be elucidated in future. Similarly, *in vivo* description of some factors such as TGF-α family members, TGF-β, TNF-α, and IL-1 may shed light on complex process of Sertoli cell proliferation and testis development. Subsequently, the detailed mechanism of action of these hormones might give us insights into a better comprehension of hormonal regulation in Sertoli cell proliferation, as well as provide possible therapeutic molecules for human infertility.

AUTHOR CONTRIBUTIONS

RK and XJ conceived the review. WS, BS, AK, and SD collected the information. WS, RK, and XJ wrote the paper. JW, XJ, WL, and RK modified the manuscript. All authors contributed to the article and approved the submitted version.

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