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Significance and Relevance of Spermatozoal RNAs to Male Fertility in Livestock

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Livestock production contributes to a significant part of the economy in developing countries. Although artificial insemination techniques brought substantial improvements in reproductive efficiency, male infertility remains a leading challenge in livestock. Current strategies for the diagnosis of male infertility largely depend on the evaluation of semen parameters and fail to diagnose idiopathic infertility in most cases. Recent evidences show that spermatozoa contains a suit of RNA population whose profile differs between fertile and infertile males. Studies have also demonstrated the crucial roles of spermatozoal RNA (spRNA) in spermatogenesis, fertilization, and early embryonic development. Thus, the spRNA profile may serve as unique molecular signatures of fertile sperm and may play pivotal roles in the diagnosis and treatment of male fertility. This manuscript provides an update on various spRNA populations, including protein-coding and non-coding RNAs, in livestock species and their potential role in semen quality, particularly sperm motility, freezability, and fertility. The contribution of seminal plasma to the spRNA population is also discussed. Furthermore, we discussed the significance of rare non-coding RNAs (ncRNAs) such as long ncRNAs (lncRNAs) and circular RNAs (circRNAs) in spermatogenic events.

Keywords: circRNA, lncRNA, ncRNA, piRNA, spermatozoa, spermatozoal RNA, spRNA, transcriptome

INTRODUCTION

Spermatogenic defects and sperm abnormalities are responsible for high incidence of male infertility cases in both animals and human. The diagnosis and treatment of spermatogenic failure remain to be a thrilling challenge to veterinarians and medical practitioners despite significant research progress in the investigation and treatment of infertility. Male infertility in livestock is commonly evaluated from semen quality assessment parameters such as sperm concentration, forward progressive motility, morphological defects, acrosomal abnormalities, hypo-osmotic swelling test (HOST) for membrane integrity, etc. Numerous functional assays have also been developed to evaluate the competence of spermatozoa within the female genital tract and include assessment of *in vitro* capacitation, acrosomal reaction and hypermotility, cervical mucus penetration test, zona-free hamster egg penetration assay, and *in vitro* fertilization (IVF) (Saacke et al., 2000; Foxcroft et al., 2008). Assessment of DNA fragmentation in the sperm nucleus has been a relatively recent addition to the semen evaluation parameters in farm animals (Kumaresan et al., 2020). However, these procedures fail to diagnose spermatogenic failure and, several cases remain undiagnosed and declared as idiopathic. Heterogeneous sperm population within a single

ejaculate, seasonal variation, influence of semen freezing protocol, etc., further adds to the variability of results (Yang et al., 2010; Varona et al., 2019). Thus, there is a need to develop newer molecular and biochemical methods for rapid and reliable diagnosis of spermatogenic failure and male infertility.

Spermatozoa contain a suite of RNA species, including both coding RNAs and non-coding RNAs such as microRNAs (miRNAs), intranuclearRNAs, small nucleolar RNAs (snRNAs), etc. The concentration of spermatozoal RNA (spRNA) may be as much as 0.015 pg per sperm (in humans), which is conspicuously high considering the high nuclear: cytoplasmic ratio and very low volume of cytoplasm in sperm (Miller et al., 2005). Microarray analysis has revealed that more than 3,500 unique mRNAs were present in ejaculated human spermatozoa (Ostermeier et al., 2002). However, these spRNAs have traditionally been considered a residual of the spermatogenesis process due to transcriptionally dormant nuclear genome (Hecht, 1998) and lack of 28S rRNA and 18S rRNA in the cytoplasm of mammalian spermatozoa (Miller et al., 1999). The lack of rRNAs eliminates the possibility of translation within sperm and hence, *de novo* protein synthesis (Miller et al., 1999).

More recent studies have shown that, during fertilization, at least six sperm-specific, developmentally related mRNAs are delivered to the zygote (Ostermeier et al., 2004). Studies have further shown that protein-coding spRNAs undergo spatio-temporally regulated degradation during early embryonic development (Ziyyat, 2001; Hayashi, 2003; Ostermeier et al., 2004). Thus, spRNAs may have specific functions during early embryonic development, although their exact role or mechanism of action remains elusive. (Wykes et al., 1997) observed that spRNA were localized at the periphery of the sperm nucleus and co-localized with spermatozoal histone. Since imprinted genes such as *IGF2* are bound with histone protein in the sperm nucleus, it has been suggested that spRNAs may have a role in meditating imprinting mechanism or chromatin repackaging following fertilization (Miller et al., 2005). Microarray profiling of spRNAs in infertile men revealed a distinct RNA profile that could be used as a molecular signature of infertile man's sperm (Ostermeier et al., 2002) (Wang et al., 2004). Thus, knowing the identity, functions, and regulation of spRNAs may have direct relevance to the diagnosis of male infertility and developing RNA-based therapies or contraceptives. The aberrance or absence of spRNA may be a contributing factor for idiopathic infertility and may bear on the poor performance of livestock. However, while many studies have analyzed the spRNA population in human spermatozoa, there are extremely limited results from livestock species. This review article provides a detailed review of known spRNA populations in various livestock species, their possible role in male fertility and early embryonic development, and their significance in the diagnosis and/or treatment of idiopathic infertility. A brief on RNA populations in seminal plasma is also discussed. Owning to very limited data on spRNAs in livestock, suitable relevance is also taken from humans.

Spermatogenesis and Spermatozoa

Spermatogenesis is a complex set of events initiated at the germinal epithelium of seminiferous tubules. The spermatogonial stem cells (SSCs), the stem cells at the basement of germinal epithelium, are responsible for the maintenance of spermatogenesis throughout the adulthood of males. They possess the ability to self-renew themselves and differentiate into haploid spermatozoa through the well-orchestrated spermatogenesis process. Upon molecular cues to differentiate, the SSCs initially divide mitotically to produce spermatogonia and subsequently undergo a meiotic process to produce spermatocytes, followed by the production of haploid spermatids (Gomes et al., 2013). The spermatids undergo sequential morphological changes by spermiogenesis to develop into haploid spermatozoa. The spermatozoa in seminiferous tubules are immature and undergo a maturation process in the epididymis. The epididymal maturation of spermatozoa is characterized by several physiological and molecular changes in the plasma membrane (e.g., lipid composition, surface proteins, etc.) and nucleus (e.g., DNA condensation, protamine formation, chromatin rearrangement, etc.) (Légaré et al., 2017), formation of acrosome and development of flagella, etc. before spermiation (Griswold, 2016; Neto et al., 2016). Due to cytoplasmic expulsion, mature spermatozoa are produced with very little-to-no cytoplasm. During sperm maturation, sperm transition proteins (TNP1 and TNP2) replaces the majority of the histone proteins of the spermatids, followed by the deposition of highly basic protamines (PRM1 and PRM2) in elongated spermatids and spermatozoa (Jambor et al., 2017). The complex chromatin packaging makes the sperm epigenome highly stable and makes it transcriptionally inactive (Lambard et al., 2004). However, transcriptional and translational activities have been observed during the early stages of spermiogenesis (Miller, 2007; Yang et al., 2010) until the development of round spermatids. Translation of specific mRNAs also continues for several days after discontinuing the transcription process and completing spermiogenesis. The latter is believed to occur from those spRNAs that are associated with leftover histone protein in the nucleus and are potentiated for transcription (Miller, 2007).

The spRNAs Population in Spermatozoa

In last 1 decade, a number of studies have prepared cDNA libraries and performed high-thoroughput RNA sequencing (RNA-seq) or microarray analysis of spermatozoal samples from epididymis and ejaculates. The spermatozoal samples have been compared between fertile and infertile males for spRNA profiling (**Figure 1**) and reported to have several coding and non-coding mRNA transcripts (Li and Zhou, 2012). The concentration of spRNA varied from picograms to femtograms per cell ranging from 100 fg in mice (Miller, 2014), 10–20 fg in humans (Zhang et al., 2017), two fg in bull (Sellem et al., 2020), 5 fg in swine (Hamatani, 2012), to 2–20 fg in stallions (Das et al., 2010). However, earlier reports on spRNAs have suggested their inertness for transcriptional and translational activities in spermatozoa (Zhang et al., 2017). The mature spermatozoa contain insufficient 28S or 18S rRNAs to support

FIGURE 1 | A layout of whole transcriptome studies in four major livestock species.
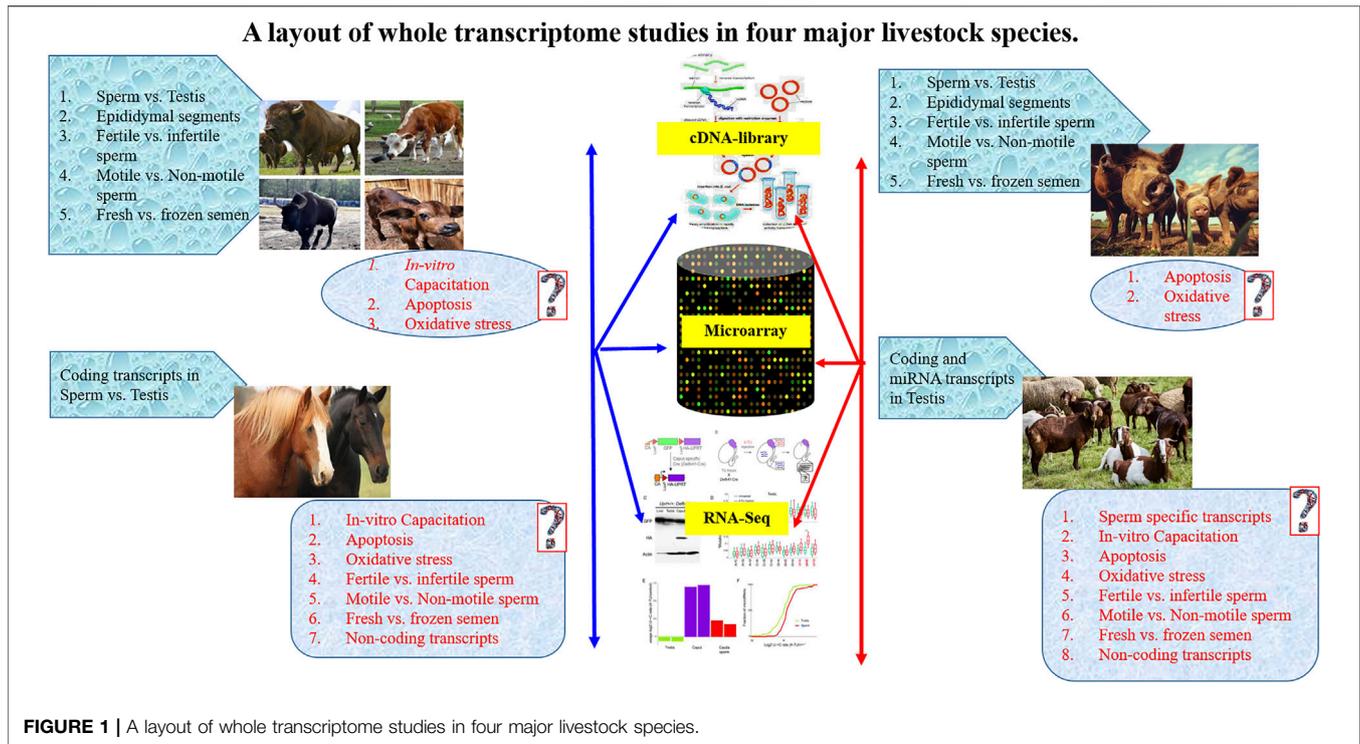
translation (Miller et al., 1999). The absence of essential components of translational machinery raised scepticism on *de novo* translation or any possible alternative roles of spRNAs in spermatozoa beyond the delivery of a paternal genome. Thus, the presence of spRNA not only aroused controversies but is intriguing to date from the discovery of sperm-borne RNAs in zygotes (Krawetz et al., 2011). The presence of RNAs in the male gamete is traditionally assumed to be either degraded leftover or spermatogenic expulsion of the residual body (Zhang et al., 2017). It was believed that *de novo* gene expression could not occur in mature spermatozoa due to highly compacted DNA by protamine that substitutes histone proteins during spermiogenesis (Balhorn, 2007). Conversely, spermatozoa rely upon pre-formed spRNAs and proteins for chromatin re-compaction, completion of the spermatogenesis process, and subsequent fertilization events (Miller, 2014).

Although sperm RNA population consists of various classes of coding RNAs, non-coding RNAs (miRNAs, piRNAs, siRNAs, lncRNAs), mitochondrial RNAs, ribosomal RNAs (rRNAs), and some intronic retained elements (Miller, 2007), the accuracy in determining its quantity and functional significance has been challenging (Dadoune, 2009). It was suggested that the variable amount of RNA in spermatozoa in different species might be due to differences in RNA isolation protocol or contamination of somatic cells in the semen. Thus, an optimized protocol for sperm purification and RNA extraction is required before any conclusion being drawn on their relevancy to male fertility (Gòdia et al., 2018). Nevertheless, evidence of both coding and non-coding spRNAs were proclaimed in different livestock species, including cattle, pigs, and stallion (Kempisty et al., 2008). Notably, full-length mRNA transcripts have also been

reported (Sun et al., 2021) that had the potential to be translated *de novo* under certain circumstances (Miller and Ostermeier, 2006).

The spRNA was also suggested to have the potential to modulate phenotype through epigenetic alternations in gene expression (Pantano et al., 2015) and imprinting of IGF2 expression (Boerke et al., 2007). It was shown that spRNAs were located at the periphery of the nucleus at the boundary of histone-bound and protamine-bound DNA in the sperm. Thus, they may have an association with potentiated DNAs for gene expression (Jodar et al., 2013). Further, the sperm transcriptome profiling using Microarray (Das et al., 2013; Zhang Y. et al., 2017), and RNA-seq (Gòdia et al., 2018) have identified a suite of RNAs in spermatozoa (Sendler et al., 2013) that included both coding and non-coding transcripts and were associated with regulation of various biological functions such as chromatin repackaging, genomic imprinting, early embryonic development (Das et al., 2013), and post-fertilization events (Prakash et al., 2021). The spRNA profile also differed between low-motile and high-motile spermatozoa (Lambard et al., 2004) and between fertile and infertile men (Wang et al., 2004; Ostermeier et al., 2005). Yatsenko and co-workers detected abnormal *UBE2B* (Yatsenko et al., 2013), *ZPBP1* (Yatsenko et al., 2012) and *KLHL10* (Yatsenko et al., 2006) genes in spRNA and associated it with impaired fertility. Wu et al. (Wu et al., 2012) reported that miR-19b and miR-let7a could serve as specific and sensitive biomarkers for spermatogenic status in idiopathic infertile males with oligozoospermia and non-obstructive azoospermia. Altered miRNAs expression has also been found in the testis with non-obstructive azoospermia (Lian et al., 2009). Thus, the expression level of spRNAs and

TABLE 1 | Important protein coding transcripts reported in cattle spermatozoa by transcriptome analysis.

Species	Study type	Groups	Transcripts (Gene symbol)	Function	References
Cattle	RNA-Seq	Whole transcriptome	ZBTB20	Spermatogenesis	Raval et al. (2019)
	RNA-Seq	Whole transcriptome	YWHAZ	Spermatogenesis and acrosomal reactions	Selvaraju et al. (2017)
			KIF5C and KCNJ6	Microtubule function	
			TNP1, RAD21, UBE2B and RAN	Spermatid development	
			TEKT1	Acrosome and on flagella and could play a key role during fertilization	
			CAPZA3	Spermatozoa capacitation and spermatozoa egg fusion	
			MAP7, PTK2, PLK1S1, MYH9 and PRKCZ	Spermatogenesis and sperm function	
			PLCZ1 and PLCB1	Calcium signalling and the spermatozoon-induced activation of an oocyte leading to its final maturation	
			ADAM1B, ADAM2 and ADAM32	Spermatozoal motility	
			ZP3 and FOXG1	Embryogenesis-associated transcript	
			PAG5, PAG7 and PAG10	Implantation and placentation	
	RNA-Seq	High vs Low fertile	PFN1	Oocyte maturation, fertilization, embryo development, spermatogenesis	Prakash et al. (2021)
			PRKCA 1	Acrosome formation	
			ODF1, BCL2L11, PRM2, TNP2, ODF2, SPEM1, and MEA1	Spermatogenesis	
			YBX1, UBE2B, BCL2L11, MYH10, and RBBP6	In-vitro embryonic development	

seminal RNAs may have potential use in studying the post-spermatogenesis events and detecting defects in sperm function (Prakash et al., 2021). A layout of whole transcriptome studies in major livestock species is presented in **Figure 1**.

Coding RNAs in Spermatozoa of Livestock

The spRNA population has been described in various farm animals such as cattle, horses, and pigs (Card et al., 2013; Das et al., 2013). The coding RNAs were limited mainly to mRNA transcripts, whereas ncRNAs belonged to different types such as miRNAs, lncRNA, rRNA, tRNA, tsRNA, circRNA, etc. The presence of fragmented rRNAs (Cappallo-Obermann et al., 2011), 5,000–6,000 mRNAs, and other classes of RNAs such as tRNA, small RNA, and miRNA have also been described (Parthipan et al., 2017). Among protein-coding RNAs, Hydrolases, SP-40, Sulfated glycoprotein 2, Calmegin, Heat shock proteins (HSPs) were highly abundant. At the same time, non-coding RNA (ncRNA) mainly included miRNAs and siRNAs whose targets were signaling pathways such as the Wnt signaling pathway that is known to be regulators of early embryonic development and differentiation (Peifer, 2000).

Coding spRNAs in Cattle

The whole transcriptome profiling of bulls' spermatozoa revealed a wide range of potential transcripts (**Table 1**) that regulate DNA packaging, cytoskeletal organization, acrosomal reactions (e.g., PLCB1, YWHAZ) (Selvaraju et al., 2017), oocyte activation, embryogenesis, placental development (e.g., PAG5, PAG7, and PAG10), embryonic morphogenesis, microtubule

function (e.g., KIF5C and KCNJ6), mitochondrial function (e.g., COX5A and COXI1), calcium signaling (e.g., PLCZ1 and PLCB1), and centrosome organization (e.g., MAP7, MYH9, PLK1S1, PRKCZ, and PTK2). Bovine cDNA microarray analysis of spermatozoa from different segments of epididymis further revealed segmental differences in the transcriptome. Among the differentially expressed genes (DEGs), the top 10 DEGs were related to reproductive function (ADAM28, AKAP4, CTCFL, FAM161A, ODF1, SMCP, SORD, SPATA3, SPATA18, and TCP11) and five DEGs (DEAD, CYST11, DEFB119, DEFB124, and MX1) were related to the immune response and cellular defense (Légaré et al., 2017). Notably, most up-regulated transcripts of the caput epididymis are known to regulate spermatogenesis and sperm morphology and included AKAP4, ODF1 CTCFL, SMCP, SPATA3, SPZ1, and SPATA18. These genes in spermatozoa have been associated with sub-fertility of bulls with incomplete spermiogenesis (Hermo et al., 2010). The sperm transcripts related to sperm maturation were ADAM28, CRISP2, CST11, LCN9S, and TCP11. On the other hand, dysregulated immune defense-related genes were CATGL4 and GSTA2 in the caput epididymis; GPX5, MX1, and DEFB124 in the corpus epididymis and; DEFB7 and DEFB119 in the cauda epididymis (Selvaraju et al., 2017).

Various intact rRNA (e.g., RPL23, RPL27A, and RPS18) and degraded rRNAs (RPL6, RPL36AL, and RPL37) have also been reported in bull spermatozoa (Montjean et al., 2012), which might suggest their potential role in spermatogenesis. The comparative RNA profiles of semen from high- and low-fertility bulls using microarray revealed 415 DEGs (out of 24,000 analyzed genes) with a significant number of fertility-

associated markers (Feugang et al., 2010). The study showed higher expression of membrane and extracellular matrix genes in spermatozoa from high-fertility bulls, while transcripts of transcriptional and translational factors were lower in the low-fertility bulls. Increased expression of CSN2 and PRM1 in spermatozoa of high-fertility bull and lower expression of CD36 in low -fertility bull were suggested as possible fertility markers (Feugang et al., 2010).

The role of coding spRNA transcripts was also indicated in the motility of sperm flagellum (AKAP) and DNA packaging (PRM2) (Singh et al., 2019). Differences in spRNA transcripts among high- and low-fertility dairy bulls have been reported with 805 unique transcripts in high-fertility and 2,944 unique transcripts in the low-fertility bulls (Card et al., 2017). Expression of cytochrome oxidase subunit (COX7C) was negatively correlates with male fertility (Card et al., 2017). In another study, a combination of five genes (AK1, ITGB5, TIMP, SNRPN2, and PLCz1) accounted for 97.4% of the variation of conception rate from frozen-thawed Holstein bulls, which was indicative of the sire fertility index (Kasimanickam et al., 2012). The upregulated sperm transcripts (e.g., RPL3, PABPC1, TPT1, RPL14, RPS8, PFN1, DDX39B, and CD74) in low-fertile crossbred bulls annotated to biological functions like apoptosis, cellular differentiation, apoptosis of germ cells, spermatogenesis, fertilization, and early embryo development (Arcuri et al., 2004; Rawe et al., 2006; Selvaraju et al., 2018) whereas downregulated sperm transcripts (e.g., RUNDC3A, LYRM4, FAM71F1, ZFN706, PICK1, LUZP1, ANKRD9, and EPOP) were found to be modulating the cytoskeletal organization and acrosome formation (Selvaraju et al., 2017). The TSSK6, C12H13orf46, FABP3, and IQCF1 genes were the top spRNA transcripts that were unique to high-fertility bull spermatozoa and were associated with biological functions of protein phosphorylation, sperm chromatin condensation during spermiogenesis, sperm motility, acrosome reaction, and gamete fusion during fertilization (Bissonnette et al., 2009; Sosnik et al., 2009; Fang et al., 2015; Selvaraju et al., 2018). The upregulated sperm transcripts that were unique to the low-fertility bulls included ribosomal proteins and thymosin beta 10 (TMSB10), which are involved in sperm capacitation, fertilization, and cellular remodeling during trophoblast adhesion (Cammas et al., 2005; Selvaraju et al., 2017). These studies suggest an association between coding spRNAs in bulls sperm and their fertility.

Coding spRNAs in Pigs

The significance of spRNA functions has also been recognized in pigs (**Table 2**). Using RNA-Seq, several genes linked to spermatogenesis (e.g., FGF-14 and BAMBI), energy metabolism (e.g., ND6 and ACADM), protein phosphorylation (e.g., PTPRU and PTPN2), autophagy (e.g., RAB33B), inflammation, and apoptosis (e.g., EAF2, FOS, ITGAL, NFATC3, and ZDHHC14) have been reported to be significantly up-regulated in poor freezable ejaculates of boar semen (Fraser and Adeoya-Osiguwa, 2001). Thus, the spRNA population may serve as a molecular signature or marker of

sperm freezability. Comparison of spRNAs in fresh vs. frozen-thawed boar sperm resulted differential expression of 567 protein coding mRNA and 135 non-coding miRNA (Card et al., 2017; Dai et al., 2019). The KEGG pathway analysis of DEGs revealed several signaling pathways governing the spermatogenesis process, such as chemokines signaling, PI3K-AKT, cGMP-PKG signaling, JAK-STAT signaling, calcium signaling, TNF signaling, MAPK, calcium signaling, NF-kappa B signaling, and AMPK signaling pathways. Similarly, microarray analysis of spRNA population in differentially fertile pigs revealed significant involvement of major signaling pathways associated with JAK2 and STAT3 pathways, cytokine receptor activity, activation of B-and T-lymphocytes (Yang et al., 2009). High fertile groups were also marked with downregulation of cell adhesion and proliferation genes (e.g., CDH10, CDSN, ITGB8, ANGPTL1, and CTNNA3) and upregulation of genes associated with the cellular component organization (e.g., KRI1 and ZNHIT6), endocytic receptor activity (e.g., CXCL16), membrane channels (e.g., KCNA3, KCNIP3, KCNH4, and KCTD9) and translation regulator activity (e.g., CPEB3). Transcripts regulating sperm motility during in vitro capacitation, such as CATSPERG (CatSper channel auxiliary subunit gamma) was upregulated, and CATSPERB (CatSper channel auxiliary subunit beta) was down-regulated in high-fertility pigs. The DEGs annotating to zinc finger nucleases (ZFNs) such as LOC100739821, ZNRF4, PLAGL2, ZFN25, and ZDHHC7 were primarily upregulated in high-fertility boars (Alvarez-Rodriguez et al., 2020). Some of the ZFN transcripts (e.g., ZFN283, FEZF2, and GLI1) were downregulated in high fertile groups and were involved with sonic hedgehog signaling pathway (Kroft et al., 2001). Apart from this, transcripts of NFYA, TCF21, MBTPS2, MTF2, IRF3, and ISG20L2 were significantly overexpressed, and UBTFL1, ADAM7, ADAM29, IL23R, IFN-DELTA-4, IFNK, and IFIT3 were under-expressed in high fertile boars (Turner et al., 2006; Wei et al., 2011).

The spRNAs enriched in cell-cell adhesion, endometrial epithelial cell receptivity, lipid and glucose metabolism, inflammation, autophagy, matrix metalloproteases, mitochondrial apoptosis, and immune-related signaling pathways have also been reported (Alvarez-Rodriguez et al., 2020). The EST library of ejaculated spermatozoa from Landrace pig deciphered some of the putative homologs of transcripts regulating embryogenesis (e.g., HSP70.2, SSFA2, and SESN1). These transcripts were also marked as potential regulators of spermiogenesis, oocyte fertilization, cellular growth, and cleavage (Yang et al., 2009). Seasonal variation in RNA-seq profiling of spRNAs was also reported in pigs (Yang et al., 2010) and included transcripts having functional similarity with those previously reported in cattle (Selvaraju et al., 2017). The mRNA transcripts regulating spermatogenesis (e.g., ODF2 and SPATA18), nuclear genome structure (e.g., PRM1, OAZ3, HSPB9, and NDUFS4), mitochondrial function (e.g., COX1ATP8), and fertilization (e.g., HSPA1L and PRSS37) were observed to be associated with compaction of sperm chromatin, energy metabolism, oxidative stress, apoptosis, and early embryo

TABLE 2 | Important protein coding transcripts reported in pig spermatozoa by transcriptome analysis.

Species	Study type	Groups	Transcripts (Gene symbol)	Function	References
Pigs	cDNA-library	Ejaculated spermatozoa	Hsp70.2, SSFA2, SESN1	Embryogenesis	Yang et al. (2009)
	RNA-Seq	Capacitated sperm	MAPK1, PGK1, PPM1B, and PGAM1	Capacitation	Li et al. (2018)
	RNA-Seq	Fresh vs frozen semen	VEGFA	Self-renewal and maintenance of male spermatogonial stem cells, activate the AKT signalling, improve sperm motility	Dai et al. (2019)
			DNMT3A, DNMT3B, JHDM2A, KAT8, and PRM1	Epigenetic regulation	
			PLCZ1 and CRISP2	Calcium ion pump, sperm–egg interaction, regulation of sperm intake, and fertilization process	
			MTHFR, PAX8, IGF2, LIT1, and SNRPN	Epigenetic regulation	
	Microarray	High fertile vs Low fertile	AKAPs	Capacitation, motility	Alvarez-Rodriguez et al. (2020)
			CATSPER	Mammalian fertilization by Calcium signalling	
			Zinc finger proteins	Transcriptional regulation, ubiquitin-mediated protein degradation, signal transduction, actin targeting, DNA repair and cell migration	
			Matrix metallo-proteases	Activation of TNF-alfa and generation of Epidermal Growth Factor Receptor	

development (Sendler et al., 2013; Sakurai et al., 2016; Selvaraju et al., 2017). It was also suggested that the *TSARG1* transcript in spermatozoa might be involved in inhibiting apoptosis (Yang et al., 2005). The study reported that the expression level of *TSARG1* and testis-specific kinase 1 (*TESK1*), which are members of the DnaJ-like protein family and serine/threonine kinases, respectively, were significantly higher in the sperm pools collected in winter than in summer. The transcriptome analysis of pig testis and epididymis revealed several transcripts associated with different regions of the epididymis (Guyonnet et al., 2009). Several lipocalins were observed in different epididymal segments such as *LCN6, LCN8, LCN9, LCN10, PTGDS,* but their role is not yet evident in response to epididymal functionalities.

Coding spRNAs in Stallion
In order to establish the significance of spRNAs as potential biomarkers of stallion fertility, global transcriptome profiling of semen has been explored (Das et al., 2013; Suliman et al., 2018). The abundance of spermatozoal mRNAs (e.g., *ADIPO, AK1, CRISP2, DOPPEL, ITGB5, NGF, PEBP1, PLCZ1,* and *TIMP2*) were found to be positively correlated with fertility, while mRNA transcripts *of CCT8* and *PRM2* were found to be negatively correlated (Kasimanickam et al., 2012). The stallion sperm transcripts were predicted to regulate critical biological functions, viz., the integrity of plasma membrane, RNA processing, transcription regulation, mitochondrial ribosomal protein, ion binding, chemokine receptor DNA packaging, protein folding, cytoskeleton, GTPase activator, chromatin assembly complex, and protein transport (Suliman et al., 2018). The cysteine-rich secretory protein (CRISP), found in seminal plasma proteins, was involved in gamete fusion (Töpfer-Petersen et al., 2005). Some ribosomal binding proteins (e.g., *GRTH, SAM68, MSY2*, and *DAZAP1*) were also observed that are known to promote the translation of

mRNAs in germ cells. The latter may thus, suggest the importance of spRNAs in regulating protein translation during spermatogenesis (Bettegowda and Wilkinson, 2010). Comparative transcriptomics of stallion sperm identified 149 DE miRNAs between dense and less-dense spermatozoa (Ing et al., 2020) wherein *BC O 1, GLRA4, OTOL1, PRM1, SCP2D1,* and *SPATA31D1* were highly expressed in dense spermatozoa compared to those of less-dense spermatozoa. This study also found that expression of *PRM1* transcripts was significantly higher in morphologically normal spermatozoa from sub-fertile stallions and in spermatozoa with abnormal morphology (Paradowska-Dogan et al., 2014). The association of protamines expression levels in frozen-thawed semen had implications in stallion fertilization (Kadivar et al., 2020).

Non-Coding RNAs in Spermatozoa of Livestock
Two groups of ncRNAs are the short and long ncRNAs (Watson et al., 2019). Transcripts <200 nucleotides are the small ncRNA (sncRNA) that include piwi-interacting RNA (piRNAs), small interfering RNA (siRNAs), miRNA, rRNA, tRNA, snoRNAs, and small nuclear RNA (snRNAs) (Grivna et al., 2006). Transcripts >200 nucleotides are generally categorized as long ncRNA (lncRNA) (Fort et al., 2021). Deep sequencing of cattle spRNA revealed that sncRNA population comprises the most abundant rRNA followed by piRNAs, miRNAs, and tRNA fragments (tsRNA), contradictory to sperm-based data where tRNAs represented most of the reads (Sellem et al., 2020). Microarray analysis of spRNAs in humans revealed that spRNA contains an array of ncRNAs that included miRNAs and siRNAs. Interestingly, 68 of these siRNAs had protein targets which were regulators of development and differentiation in *C. elegans* (Martins and Krawetz, 2005). The function of these

sperm-derived miRNA was further demonstrated by (Amanai et al., 2006). Using anti-miRNA microinjection to oocytes, (Amanai et al., 2006), showed that spRNA has a limited function during early embryonic development in pigs. Nevertheless, direct proof of the function of these RNAs is still elusive. The presence of ncRNAs in livestock has been associated with spermatogenesis, fertilization, early embryogenesis (Prakash et al., 2020), and epigenetic modification of the sperm genome (Jodar et al., 2013; Prakash et al., 2020).

Spermatozoal miRNA

The miRNAs are short single-stranded RNAs of ~18–22 nucleotide length that are found abundantly in mammals and are known to post-transcriptionally regulate the function of mRNAs through inhibition or suppression of translation or degradation of mRNA themselves. The significance of miRNAs in male reproduction has been documented in the maintenance of self-renewal and differentiation of SSCs into spermatozoa as well as during the spermiogenesis process (Chen X. et al., 2017). We previously showed that the let-7 family of miRNA is involved in regulating germ cell proliferation and may be used as a marker of male germ cells (Jung et al., 2010) in addition to imprinted miRNAs (Shin et al., 2011). Similarly, miR-21, miR-34c, miR-183, miR-465a, etc., were also found to be vital for SSC self-renewal through regulation of *Etv5, Cdnd1, and Stat3* expression in mice (Niu et al., 2011; He et al., 2013). On the other hand, miR-224 and miR-322 regulate the Wnt signaling pathway for self-renewal of mice SSCs by increasing the expression of *Gfrα1, Plzf,* and *Rassf8* (Cui et al., 2016; Wang Y. et al., 2019) whereas miR-100 and miR-10b promoted the proliferation of SSCs through *Stat3* and *Klf4*, respectively (Huang et al., 2017; Li et al., 2017). The miRNAs were also shown to play crucial roles in the differentiation of mice SSCs and spermatogenesis by targeting the *Dmrt1* (Cui et al., 2016) and through the retinoic acid pathway (Bellvé et al., 1977).

The complexity of the miRNA population has also been seen in spermatozoa of several mammalian species, including cattle, pigs, stallion, mice, and humans (Capra et al., 2017; Castillo et al., 2018; Gòdia et al., 2018). However, the precise functions of these sperm miRNAs in spermatogenesis or post-fertilization events are yet to be established. Spermatozoal miRNAs are believed to play essential roles during fertilization of oocytes and early embryonic development (Castillo et al., 2018). They may also affect the abundance and epigenetic status of maternal mRNAs in oocytes and zygotes (Capra et al., 2017).

Spermatozoal miRNAs in Cattle

Spermatozoal miRNAs have been reported in healthy bulls and those differeing in their spermatozoal motility or fertility (**Table 3**). The whole miRNA profiling of bull spermatozoa through deep sequencing identified 2022 miRNAs and included a large number of isomiRs across different species (Turri et al., 2021). RNA-seq analysis of high fertility and low fertility bulls reported nine DE miRNAs (miR-2285n, miR-378, miR-423-3p, miR-19, miR-2904, miR-378c, miR-431, miR-486, and miR-2478) in which miR-2285n, miR-378, and miR-486 were observed to have altered expression levels between low and high

motile sperm. The highest expression of miR-2285n and miR-486 was seen in low motile spermatozoa and the semen samples from low fertility bulls. The miR-486 plays a crutial role in regulating stemness and cell proliferation of SSCs. The miR-378 targets mRNAs involved in metabolism and sperm motility (Liu et al., 2019). Differential expression of spermatozoal miRNAs was also observed upon microarray analysis of spermatozoal miRNA in low vs. high motility spermatozoal and low vs. high fertility bull (Sellem et al., 2020; Keles et al., 2021).

The miRNA profiling of bull sperm using microarray presented an abundant quantity of miRNA along with DE miRNA target genes from high vs. low fertility groups (Dai et al., 2019) suggests the roles of miRNA in regulatory mechanisms. Seven important miRNAs (hsa-aga-3155, 8197, 6,125, 6,727, 11,796, 13,659 and 14,189) were DE (using human microarray probe set) and validated by qRT-PCR. The target genes of these miRNAs included *AQP7P1, CHN2-EPHA1-EFNA2, DNM2, IFT80, TOB2, CHN2, CLUL1, BC035897, BTBD2,* possibly regulating gametogenesis, acrosome integrity, and fertilization. An interactome model of miRNA target genes has also been proposed to identify the master regulator of a set of genes with a single miRNA molecule (Govindaraju et al., 2012). The miR-26a and miR-455-5p were significantly up-regulated in highly motile spermatozoa, whereas levels of miR-10a and miR-1 were significantly down-regulated. The miR-26a also participates in PTEN and PI3K/AKT signaling pathways, affecting sperm viability and motility (Dai et al., 2019). In another study, miR-34b-3p and miR-100-5p were also significantly over-expressed in spermatozoa of high fertility bull compared to those of low fertility bull (Keles et al., 2021). The miRNAs, previously known to be expressed testis, were also detected in bull spermatozoa and included miR-34b/c miRNA cluster, which plays vital roles in chromatin condensation during spermiogenesis (Capra et al., 2017). Thus, miRNA profile could be a useful indicator for high fertility spermatozoa.

The miRNA profiling has also found its application in assessing the success of semen cryopreservation protocols. Increased expression of miR-34c was seen in the highly motile cryopreserved bull spermatozoa purified through Percoll density gradient centrifugation. The miRNA profiling of differentially motile cryopreserved spermatozoa revealed that spermatozoal miRNAs target STAT3, PI3K/AKT, and PTEN signaling pathways that play essential roles during mammalian spermatogenesis, mitochondrial membrane potential, sperm maturation, and fertilization. PTEN, the target of multiple miRNAs (e.g., miR-17-5p, miR-26a-5p, and miR-486-5p), inhibits AKT signaling and activates RAF1/ERK signaling to initiate sperm maturation. Two miRNA *viz.* miR-122 and miR-184 were upregulated in low motile fractions of semen, which targets the AKT signaling pathway to cause apoptosis. In addition, miR-17-5p and miR-20a- 5p, which were found to be under-expressed in the low motile fraction of semen, targets PTEN and STAT signaling pathways to trigger apoptosis (Capra et al., 2017).

The spermatozoal miRNA have also been shown to differ in gonadotoxicity, heat stress, and impaired spermatogenesis. Differentially expressed miRNAs have been reported between

TABLE 3 | Important non-coding microRNAs reported in cattle spermatozoa by transcriptome analysis.

Species	Study type	Groups	MicroRNAs	Function	References
Bovine	cDNA library	High fertile vs Low fertile	miR-34b/c	Sperm chromatin condensation	Lian et al. (2021)
			miR-17-92 and miR-106b-25	Spermatogenesis	
			miR-10a-5p	Motility	
	MicroRNA microarray	High and low fertile	miR-3155, miR-8197, miR-6727, miR-11796, miR-14189, miR-6125, and miR-13659	Sperm fertilization	Govindaraju et al. (2012)
	RNA-Seq	Semen sample	miR-365-2	Oocyte fertilization and cleavage	Selvaraju et al. (2017)
	RNA-Seq	Small RNA sequencing	bta-miR-103, bta-miR-30b-5p, bta-miR-17-5p, bta-miR-106b, bta-miR-142-3p, bta-miR-34b, bta-miR-18a, bta-miR-34c, bta-miR-455-5p, bta-miR-10b, bta-miR-99b, bta-miR-1246, bta-miR-99a-5p, bta-miR-1388-5p	Motility	Capra et al. (2017)

normal and abnormal spermatozoa of bulls affected by Fescue toxicosis (Stowe et al., 2014). An increased expression of let-7a and miR-22 was shown in spermatozoa with abnormal morphology due to toxicosis. Further, among the most abundant miRNAs, the let-7 family and miRNA-146 were found to regulate the differentiation of spermatogonia and impaired blastocyst implantation in mice whereas, miRNA-16 is known to coordinate cell-cycle (Zhang et al., 2012). A recent study suggests miRNA (e.g., miR-126-5p) accumulates during the final stage of spermatogenesis, and epidydimal transit and their expression is affected during heat stress, which might explain reduced fertility in heat-stressed bulls due to impaired spermatogenesis and maturation (Dos Santos da Silva et al., 2021). The miR-17-92 and miR-106b-25 are two miRNA clusters whose target genes were reported to be essential for the progression of spermatogenesis (Tong et al., 2012). It was reported that spermatozoa that exhibited normal morphology had mutant miR-92a, implying that miR-92a might affect male fertility. The miR-10a and miR-9-5p have been frequently reported in cattle and pig spermatozoa. Both of these miRNAs were observed to be upregulated in cryopreserved spermatozoa. On the other hand, miR-10a-5p was found to be overexpressed in low motility spermatozoa and was associated with spermatogenesis and DNA repair capacity.

Spermatozoal miRNAs in Pig

The miRNAome of boar spermatozoa has been described in several studies (**Table 4**) and may vary with season (Varona et al., 2019). The miR- 34c, miR-191, miR-30d, miR-10b, and let-7a were the most abundant spermatozoal miRNAs associated with spermatogenesis in pigs. The differential expression of several miRNAs is seen in low vs. high fertility boars, low vs. high motility, and fresh vs. cryopreserved spermatozoa. The small RNA libraries of fresh and frozen-thawed boar spermatozoa revealed several potential cell signaling pathways governed by spermatozoal miRNAs (Dai et al., 2019). For example, miR-17-5p, miR-20a-5p, miR-26a-5p, miR-122-5p, miR-184, and miR-486-5p were involved in regulating PTEN, PI3K/AKT, and STAT signaling pathways that influence sperm motility, viability, and apoptosis in frozen-thawed spermatozoa (Zhang Y. et al., 2017) The increased expression of miRNAs such as let-7a, 7d, 7e, miR-22, let-7d, and let-7e were seen in frozen-thawed spermatozoa with morphological abnormalities or low motility. The

cryopreservation of boar sperm also led to reduced expression of let-7c, miR-22, miR-26a, miR-186, and miR-450b-5p in frozen-thawed boar spermatozoa (Capra et al., 2017). The differential expression of these spermatozoal miRNAs may, thus, have implications in sperm fertility as they are associated with motility and apoptosis in frozen-thawed boar spermatozoa.

The miRNA microarray profile of high and low fertile boars found 326 pig-specific miRNAs (Alvarez-Rodriguez et al., 2020), among which miR-1285 was found to be related to sperm production, promoting AMPK phosphorylation and regulating oxidative stress (Jiao et al., 2015). Interestingly, miR-15/miR-16 was the most abundant miRNA and is known to suppress the TGF-β signaling pathway (Ayaz and Dinç, 2018) and enhance spermatogonial proliferation and spermatogenesis in gonadotoxic patients (Moraveji et al., 2019). The mRNA targets of these miRNA are involved in various essential cellular functions such as lipid metabolism (e.g., miR-4332), cell proliferation, and apoptosis (e.g., miR-671-5p, mir-425-5p) (Qiu et al., 2018). Moreover, miR-425 and IL-23 were downregulated in high fertile bulls, whereas its receptor (IL-23R) was down-regulated in high-fertility boars (Lu et al., 2019). The overexpressed miRNAs in high fertile boars included miR-191, miR-42, which play significant role in regulating the motility of spermatozoa inside the female reproductive tract (Zhang et al., 2018). It has also been indicated as a key activator of the NF-κB signaling pathway, the regulator of estrogen receptors, and an inhibitor of the Wnt pathway (Alvarez-Rodriguez et al., 2020). Two down-regulated miRNAs, viz. miR-615, miR-221 are known to act as potential targets for EGFR and regulate acrosome reaction (Michailov et al., 2014) and PI3K-AKT and the estrogen signaling pathways (Alvarez-Rodriguez et al., 2019).

The pioneer report on sperm capacitation-specific miRNA profiling in boar was elucidated by (Li et al., 2018). This study identified DE miRNAs in fresh non-capacitated and capacitated spermatozoa. Some of the upregulated miRNAs in capacitated spermatozoa included, miR-148a-3p, miR-151-3p, miR-425-5p, miR-132, miR-451, miR7136-5p, miR-489, miR-1343, miR-1306-3p and fresh spermatozoa were enriched for miR-378b-3p, miR493-5p, miR-133a-3p, miR-362, and miR-214. Pathway analysis of miRNA targets revealed their biological significance in PI3K-AKT, MAPK, cAMP-PKA, and calcium signaling pathways, which are

TABLE 4 | Important non-coding microRNAs reported in pig spermatozoa by transcriptome analysis.

Species	Study type	Groups	MicroRNAs	Function	References
Pigs	qRT-PCR	Ejaculated spermatozoa	let-7a, -7d, and -7e miR-22	Spermatogenesis Sperm structure	Curry et al. (2011)
		Epididymal and ejaculated sperm	let-7a and miR-92a	Calcium and camp signalling	Chang et al. (2016)
			ssc-let-7a, ssc-let-7d, ssc-let-7e and ssc-miR-98 regulate	Sperm apoptosis	
			miR-19 and miR-26a	AKT/PKB signalling pathway	
			miR-224, miR-19b, miR-504 and miR-676	Cell apoptosis and cell proliferation	
	RNA-Seq	Fresh vs frozen spermatozoa	miR-26a, and miR-455-5p	PTEN, and PI3K/AKT signalling pathway	Dai et al. (2019)
	RNA-Seq	Capacitated sperm	miR-127, miR-1343, miR-151-3p	Calcium signalling pathway and MAPK signalling pathway	Li et al. (2018)
	RNA-Seq	Fresh vs frozen semen	miR-17-5p, miR-26a-5p, miR-486-5p, miR-122-5p, miR-184, and miR-20a-5p	Regulate PTEN, PI3K/AKT, and STAT signalling influence motility, viability, and sperm apoptosis	Dai et al. (2019)
			let-7a, -7d, -7e, miR-22, let-7d, and let-7e	Sperm motility	
			miR-3155, miR-8197, miR-6727, miR-11796, miR-14189, miR-6125, and miR-13659	Bovine sperm fertilization	
			miRNAs, miR-26a, and miR-455-5p	Sperm motility	
			miR-10a and miR-1 were	Sperm motility	
	Microarray	High fertile vs Low fertile	miR-615	Capacitation	Alvarez-Rodriguez et al. (2020)
			miR-221	Wnt2, BDNF, CREB-related genes, PI3K-Akt and the estrogen signalling pathway	

considered necessary for protein tyrosine phosphorylation and sperm capacitation. These miRNA targets also play a significant role in cell proliferation, differentiation, sperm motility, hyper-activation, and acrosome reaction, primarily *via* the ERK (Ras/Raf/MEK/ERK) signaling pathway (Li et al., 2018). The cAMP-PKA signaling activates the Ca^{2+} channel and is considered a crucial step for sperm capacitation. The miR-134 was up-regulated in capacitated spermatozoa and targets *COL11A1* and *PDE4A* genes in PI3K-AKT and cAMP-PKA signaling pathways. Some other miRNA targets such as *AKAP3* for miR-1285, *VDAC1* and *HSPA2* for miR-127, *CATSPER4* for miR-151-3p regulate sperm motility, ATPase activity, sperm capacitation (Alvarez-rodriguez, 2017). *CABYR* and *ACRBP* regulate tyrosine phosphorylation during capacitation processes (Dong et al., 2015).

The abundance of miRNAs has also been documented in epididymal spermatozoa and seminal plasma of boar semen (Chen C. et al., 2017). A total of 221, 259, and 136 miRNAs were DE between ejaculated spermatozoa and epididymal spermatozoa; seminal plasma and epididymal spermatozoa; and seminal plasma and ejaculated spermatozoa, respectively. Further, three miRNAs *viz.* let-7a, miR-26a, and miR-10b were among the top ten most abundant miRNAs in ejaculated spermatozoa, epididymal spermatozoa, and seminal plasma. The most abundant spermatozoal miRNAs targeted mRNA transcripts of binding proteins, including metal ion binding, ATP binding, and nucleotide-binding. However, caution is to be exercised in analyzing spermatozoal miRNAs in boar spermatozoa collected at different seasons. Studies have shown that spermatozoal miRNAome may show seasonal variations in boars (Varona et al., 2019). For example, miR-34c, miR-221-3p, miR-362, miR-378, miR-106a, and miR-34c were down-regulated, whereas miR-1306-5p and miR-1249 were upregulated in boar spermatozoa collected during the winter seasons. These miRNA targets primarily regulate fatty acid metabolism and oxidative stress (Wu et al., 2017), suggesting their role during the winter season. On the other hand, miR-106b, miR-378, miR-221 were previously reported to regulate autophagy machinery in different cell lines (Zhai et al., 2013; Tan et al., 2018).

Spermatozoal miRNAs in Stallion

A few reports have also documented the spermatozoal miRNAs in stallion spermatozoa. Direct sequencing of spermatozoal miRNAs in stallion sperm revealed 82 sperm-specific miRNAs (Das et al., 2013), out of which 68 miRNAs were previously reported in human spermatozoa (He et al., 2009). Several spermatozoal miRNAs of stallion were the same as identified in the sperm of men (Krawetz et al., 2011), boars (Curry et al., 2009), and mice (Liu et al., 2012). These spermatozoal miRNAs are noteworthy because they were absent in oocytes but were present in zygotes and were involved in regulating first cleavage division in mice (Krawetz et al., 2011; Liu et al., 2012). Three highly abundant miRNAs, *viz.* miR34B, miR34C, and miR449A, regulate early embryonic development either by direct interaction with mRNAs or *via* epigenetic mechanisms in humans (Liu et al., 2012). A total of 66 new miRNAs were reported in epididymal (cauda epididymis) spermatozoa, whose predicted pathways suggested their role in sperm motility, sperm viability, and early embryonic development (Das et al., 2013).

Other Spermatozoal ncRNAs
Spermatozoal sncRNAs and piRNAs

Recent studies have documented the presence of thousands of sncRNAs in spermatozoa of several animal species. These

sncRNAs comprise rRNAs, tRNAs, tsRNAs, snoRNAs, piRNA, etc. (Sellem et al., 2020) and are believed to play essential regulatory roles in spermatogenesis and determining male fertility. The piRNAs are 26–32 nucleotide-long sncRNAs that exclusively represent the germline population and are considered "guardian of germline" by transposon surveillance to protect genome integrity (Krawetz et al., 2011). At tissue-specific levels, piRNA modulates key signaling pathways both at transcription and translational levels. These sncRNAs are characterized to maintain transposon silencing and genome stability during spermatogenesis (Luo et al., 2016). The piRNAs represent the most abundant sncRNA population in human sperm (Pantano et al., 2015) associated with sperm concentration and fertilization rate. Some reports evaluated their significance as semen quality parameters for cattle spermatozoa (Capra et al., 2017).

Several growing pieces of evidences suggest that piRNAs may regulate protein-coding genes in germ cells and engage in determining sperm fertility and early embryonic development (Suh and Blelloch, 2011). Although piRNAs are generally produced from repeat-associated regions or transposons by PIWIL2/PIWIL4-directed pathways, the PIWIL1-directed pathway is the primary production pathway in mature bull spermatozoa. In pig spermatozoa, piRNAs have been annotated to sperm morphology, spermatogenesis, acrosomal reaction, sperm hyperactivation, and male fertility (Ablondi et al., 2021). Some potential coding transcripts falling within the 5 kb region of centered piRNA are *CATSPER2, CATSPERG, OAZ3, ODF1, ODF2, PRM1, TEX14, TSSK2, TSSK3,* and *TSSK6.* The DE piRNAs in boar spermatozoa regulate spermatogenesis-related cell signaling pathways such as cAMP, cGMP, MAPK, and PI3K–AKT signaling pathways (Wang et al., 2021). Thus, taken together, the involvement of piRNA in the maintenance of genomic integrity during spermatogenesis and fertility seems to be a potential molecular parameter for evaluating male fertility in livestock animals.

Spermatozoal lncRNAs

Preliminary reports on lncRNAs considered these classes of RNAs as noise in the RNA content of a species due to lack of any protein-coding open coding frames (ORF) (Brownmiller et al., 2020). Later on, collective evidences supported their roles in epigenetic regulation, controlling transcription, and post-transcriptional mechanism (Peris-Frau et al., 2019). The role of lncRNA on spermatogenesis has been predicted by several researchers (Wallrapp et al., 2001; Bianchi et al., 2006; Forsberg et al., 2014; Falchi et al., 2018; Peris-Frau et al., 2019; Xu X. et al., 2020), but functional studies exploring their mechanism of action and relevance to spermatozoal fertility are lacking. Many targets of spermatozoal lncRNAs have been predicted (Gao et al., 2019) and were found to be enriched in apoptosis (e.g., PI3K-AKT, p53) (Xiong et al., 2019) and capacitation-related pathways (e.g., Calcium, cAMP, and MAPK signaling) (Gao et al., 2017). In stallion, comparative transcriptomics of dense and less-dense spermatozoa revealed 1,492 bp lncRNA as the most prevalent RNA (Ing et al., 2020). Further, the expression of 159 RNAs was higher in dense spermatozoa than in less-dense spermatozoa. Importantly, the dense spermatozoa resulted in a higher

pregnancy rate than those achieved by unfractionated stallion spermatozoa (Morrell et al., 2011).

Spermatozoal circRNAs

Circular RNAs (circRNAs) are a class of ncRNAs having a closed-loop structure formed by alternative back splicing of pre-mRNA in which the 3′-end of an exon is spliced to the 5′-end of an upstream exon (Gòdia et al., 2020). Depending on their genomic locations, they can be of exonic, intronic, and intergenic types (Zhou et al., 2019). The exonic circRNAs are preferentially located in the cytoplasm, whereas intronic and intergenic circRNAs are mainly found in the nucleus. Key circRNAs can participate in testis development or spermatogenesis (Xiong et al., 2019). CircRNAs Sry (circSry) is the first reported testicular circRNA in mice (Capel et al., 1993), whereas the first report on circRNA of spermatozoa was reported in boars (Gòdia et al., 2020). Gene Ontology of circRNA revealed their epigenetic functions such as histone modification, histone H3-K36 methylation, and chromatin organization during spermatogenesis, embryonic development. Four genes *viz. ATP6V0A2, PPA2, PAIP2,* and *PAXIP1* have been directly implicated in spermatozoal function and male fertility. Among these, the PPA2 is a pyrophosphatase enzyme located at the mitochondrial membrane and is involved in ATP production during sperm capacitation and motility (Asghari et al., 2017). On the other hand, *PAXIP1* plays crucial functions in genomic stability and chromatin condensation during spermiogenesis, and its knockout resulted in testicular atrophy and male infertility in mice (Schwab et al., 2013). The TESK2 is a protein kinase that is primarily expressed in round spermatids and is predicted to function during the early stages of spermatogenesis (Røsok et al., 1999). The SPATA19 is vital for mitochondrial function and ATP production for sperm motility and fertilization (Luo et al., 2014). Genes related to early embryonic development (e.g., *ANGPT1,* CDC73, *DHX36, IPMK, RICTOR,* etc.) have also been found to be influenced by circRNAs. Remarkably, four circRNAs host genes (*DENND1B, PTK2, SLC5A10*, and *CAMSAP1*) showed a significant correlation with the motility of spermatozoa.

The circRNAs reported in adult vs. piglet testis (Zhang et al., 2021) were involved in regulating spermatogenic events (e.g., circRNA 10979 derived from *POC1A* gene) and germ cell development (e.g., circRNA 18456 derived from the *TDRD1* gene). Due to their stability and spatiotemporal specificity, circRNAs such as circRNA 10187, circRNA 6,682, circRNA 10979, and circRNA 18456 could be used as biomarkers of boar sexual maturity. The circRNA 1774 (derived from *CDC42* gene) and circRNA 18184 (derived from *PTEN* gene) were significantly downregulated whereas circRNA 40370 (derived from the RICTOR gene) was significantly upregulated in the testis of the sexually mature boars. The circRNAs were involved in signaling pathways that regulate stem cell pluripotency (e.g., *AKT3, AVCVR2A, FGFR1, ACVR1, FZD3, SMAD4*), tight junction (*MYH15, PRKCA, AMOTL1, PPP2CB, CDC42, PTEN*), and adhesion connections (e.g., *LEF1, NECTIN3, SMAD2, AFDN*), hedgehog signaling pathways (e.g., *HHIP,*

GSK3B, PTCH1, SMO, RAB23), cAMP signaling pathways (e.g., *PLD1, TIAM1, GNAI1, ADCY1*), mTOR signaling pathways (e.g., *BRAF, RICTOR, HIF1A*), and phosphatidylinositol signaling systems (e.g., *CDS1, DGKH, PLCB1, DGKK*). The expression profile of circRNAs in neonatal and adult cattle testis revealed some of the potential target genes of circRNAs mainly involved in cell-to-cell junction, oocyte maturation, and TGFβ signaling pathway (Gao et al., 2018). These transcripts included *PIWIL1, DPY19L2, SLC26A8, IFT81, SMC1B, IQCG,* and *TTLL5*. The host genes were associated with spermatogenesis and included *PIWIL1, SPATA6, TGFβ2, TGFBR2, ACVR2A,* and *SMAD2*.

RNA Population in Seminal Plasma and Their Contribution to spRNA

Seminal plasma is the secretions of accessory glands containing proteins and RNAs and has been overlooked for decades. Seminal plasma components have no role in assisted reproductive technologies (ART) and, therefore, are excluded from IVF and intracytoplasmic injection (ICSI) of ejaculated sperm. The ARTs have resulted in successful fertilization, pregnancy, and live birth of offspring in humans (Marzano et al., 2020) and animals (Morgan et al., 2020) without the use of seminal plasma. However, recent studies have found that the spRNA population may be contributed by seminal plasma via exosome. Exosomes of seminal plasma are released by epididymis and other accessory sex organs, which interact with spermatozoa to unload RNA cargo to the sperm. Therefore, analyzing the exosome of the epididymis (epididymosomes) or seminal plasma may be used as a marker of reproductive disease and male infertility [reviewed in (Vickram et al., 2021)]. Further, each ejaculation contain trillions of exosomes that aids in fertilization in two ways, 1) exosome exerts immunosuppressive effects on cells of mucosa layer and, 2) transfer of RNAs to the spermatozoa (Jodar, 2019). In humans, exosomes contained miRNAs (21.7% of the total RNA), Y-RNA, mRNAs, mature piwi-RNAs, and tRNAs, suggesting that exosome delivers regulatory signals to the spermatozoa (Vojtech et al., 2014).

In pigs, sequencing of seminal plasma-derived extracellular vesicles showed diverse small RNAs, including mRNA (25% of the total reads), tsRNA (0.01% of the total reads), miRNA, and piRNA. There were 325 miRNAs, of which 37 novel miRNAs were identified in boar (Xu Z. et al., 2020). The study identified the adverse effects of miR-21-5p on boar sperm fertility. Likewise, miRNA and piRNA have also been identified in beef bulls' seminal plasma. Of 617 small RNA, nine miRNA were DE between high and low fertility beef bulls (Stephanie, 2016). The role of seminal plasma RNA in determining male fertility seems to be undeniable. In another study, expression of *PRM1* in seminal plasma was positively correlated with mitochondrial membrane potential, and lateral movement of sperm head was associated with expression of *BMP2, UBE2D3, TRADD,* and *CASP3* (Shilpa et al., 2017). In this study, investigators have found a positive association of high expression of nerve growth factor (NGF) in the maintenance of post-thaw integrity of

membrane of bull's spermatozoa. More studies need to be conducted in other livestock animals as well to ascertain the effectiveness of seminal plasma RNAs as markers of the reproductive performance of male animals.

Epigenetic Control of spRNA Expression

The terminal stages of spermatogenesis are accompanied by chromatin condensation and the replacement of histones by protamines. These changes are primarily driven by transition proteins and cleavage of rRNAs by nuclease activities and lead to the progressive shutdown of transcriptional and translational activities in mature spermatozoa (Jodar, 2019). Interestingly, however, some studies have documented post-translational modifications of histones in mature spermatozoa and included acetylation (Johnson et al., 2011), ubiquitination (Wang T. et al., 2019), methylation (Johnson et al., 2011), and phosphorylation (Dada et al., 2012) that are known to be involved in regulating chromatin remodeling. Thus, some canonical structures of histone variants appear to have remained unchanged throughout the final stages of spermatogenesis to maintain a hierarchical layer of genomic organization in paternal chromatin. Indeed, studies have shown that about 15% of the paternal histone remains associated with the genome in mature spermatozoa (Ward, 2009). Initially, these segments were considered a mark of partial/incomplete transition of histone-protamine exchange, but gradually these histone segments were noted with a significant regulatory network by post-translational modifications (Barrachina et al., 2018). Notably, the histone retention in spermatozoa was not randomly positioned on chromosomes, instead distinctly localized within the nucleus (Johnson et al., 2011). It is, therefore, believed that specific regions in sperm chromatin are differentially marked with modified histones to have the potential for transcription through epigenetic regulations (Krawetz et al., 2011).

A substantial proportion of coding spRNAs detected in mature sperm are also considered as a spermatogenic leftover from transcription events during the spermatogenesis process (Jodar, 2019) and were under epigenetic control. During spermatid elongation, protamines undergo phosphorylation by serine/arginine protein-specific kinase 1 (*SRPK1*) and calcium/calmodulin-dependent protein kinase 4 (*CAMK4*), followed by rapid dephosphorylation to establish disulfide bonds between the unmasked cysteine residues of dephosphorylated protamines (Carrell et al., 2007). Histone acetylation was shown to be essential for the initiation of chromatin remodeling in spermatids (Stiavnicka et al., 2016). The hyperacetylated testicular histones were gradually replaced upon relaxation of the nucleosome complex. Testicular histone variants (*H2B and TH2B*) incorporate into spermatids with the help of transition proteins (*TNP1, TNP2*), which supersede with protamines (*PRM1, PRM2*) (Carrell et al., 2007). Histone methylation also has a significant role in the differentiation of spermatogonia, especially for H3me (H3 methylation) and H4me (H4 methylation) variants. It was reported that reduced H4me and hyperacetylation of H4 during spermatid elongation collectively mediated the histone-protamine replacement (SHIRAKATA et al., 2014). The significance of ubiquitination has also been
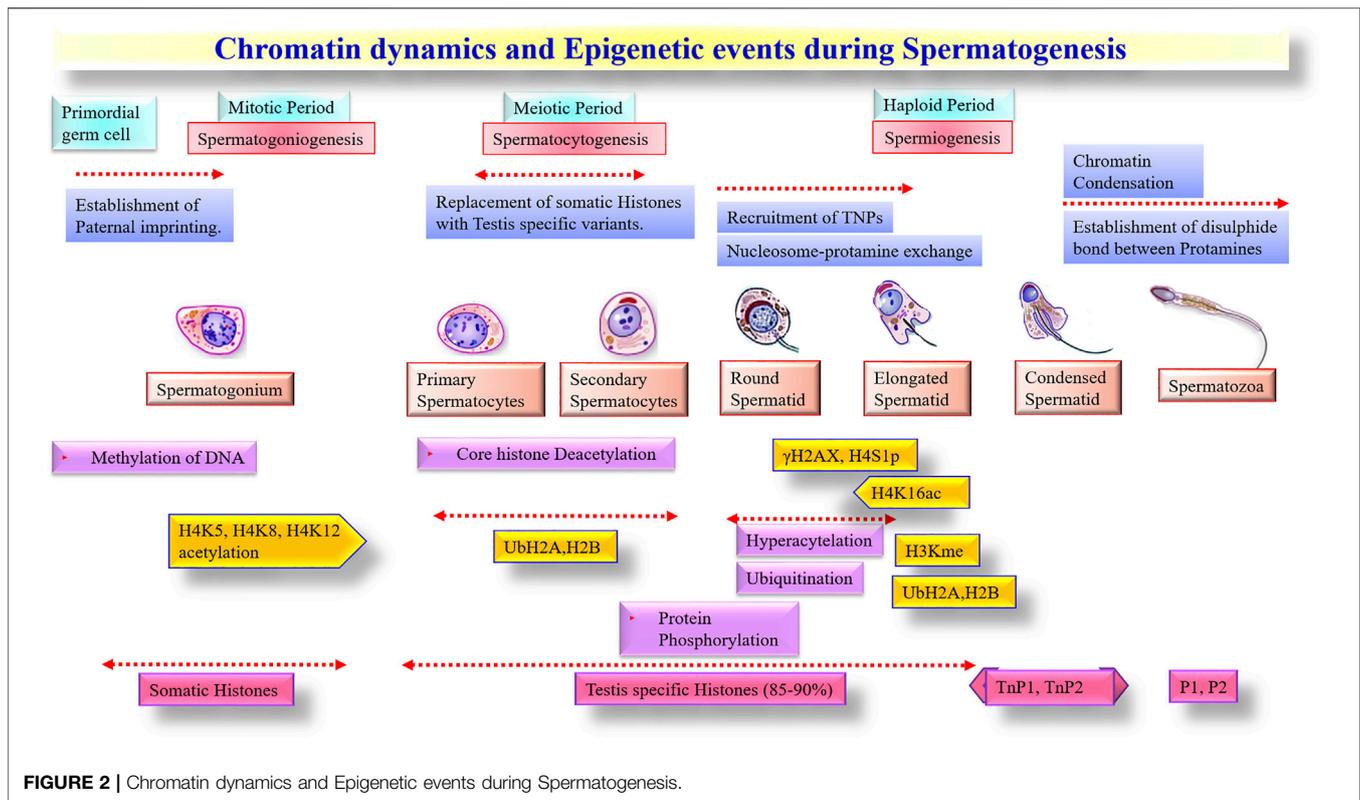
FIGURE 2 | Chromatin dynamics and Epigenetic events during Spermatogenesis.

implicated in spermatogenesis and sperm maturation to eliminate dead and defective spermatozoa and epididymal cells (Røsok et al., 1999). The stage-specific significance of epigenetic alternations during spermatogenesis is summarised (**Figure 2**).

Relevance of spRNAs in Sperm Function in Livestock Species

While recent literature on Microarray and high-throughput RNAseq data have provided novel information on the repertoire of RNA population in spermatozoa, their functional validation is largely missing in the literature. Moreover, common genes and pathways across different species are yet to be fully deciphered. Nevertheless, the involvement of spRNAs in the ontology of capacitation, motility, and fertilization are apparent in various species. Among all spRNAs, protamines were the most prominent sperm-specific transcript, which is known to affect fertility in cattle (Chen et al., 2015), pigs (Gòdia et al., 2018), horses (Kadivar et al., 2020), and human (Jodar et al., 2013). Similarly, the phospholipase C (PLC)-mediated pathway was the most common pathway affected by spRNA in cattle (Selvaraju et al., 2017), pigs (Kasimanickam and Kastelic, 2016), and horses (Varner et al., 1993). The PLC is an essential regulator of intracellular Ca^{2+} oscillations that play a critical role during oocyte activation. Several studies also identified Calcium ion channels such as *CatSper* in the spRNA population, which is essential in regulating capacitation and sperm motility in livestock species as well as humans (Jan et al., 2017; Li et al., 2018; Singh et al., 2019; Nicolas et al.,

2020). Besides these common sperm transcripts across various livestock species, there were flagellar specific spRNAs and sperm motility proteins such as *BSP* (de Souza et al., 2017; Pardede et al., 2020), AKAP (Gilbert et al., 2007; Chatterjee et al., 2010; Kasimanickam and Kastelic, 2016; Ing et al., 2020), *ODF* (Chen et al., 2015; Li et al., 2018; Pardede et al., 2020; Özbek et al., 2021), zinc finger nucleases (Card et al., 2013; Kasimanickam and Kastelic, 2016; Corral-Vazquez et al., 2021) and heat shock proteins. The heat shock proteins are a group of tyrosine regulatory elements that participate in hyperactivation and nitric oxide synthesis during fertilization (Feugang et al., 2010; Mohamad et al., 2018; Nicolas et al., 2020; Lian et al., 2021; Sun et al., 2021). The spRNAs such as calmegin (Guyonnet et al., 2009; Card et al., 2013), clusterin (Singh et al., 2019; Lian et al., 2021; Zhao et al., 2021), TSSK (Bissonnette et al., 2009; Li et al., 2020; Ablondi et al., 2021), and *CABYR* (Selvaraju et al., 2017) were also reported in cattle and pigs, are known to be essential for male fertility.

A few transcripts related to sperm capacitation were also reported in the spRNA population of cattle, pig, and horse spermatozoa. The important spRNAs related to sperm capacitation included CABYR (Bailey, 2010) and AKAP (Maciel et al., 2018), which regulate calcium-binding and tyrosine phosphorylation. On the other hand, *CatSper, VDAC1, and HSPA2* are involved in calcium signaling whereas, *COL11A1, PDE4A* can participate in PI3K-Akt and cAMP-PKA signaling pathways (Li et al., 2018), The *HSP70* (Mohamad et al., 2018) and *HSP90* (Jin and Yang, 2017), involved in calcium signaling, were also reported in spRNA

population. Thus, it appears that calcium signaling and tyrosine phosphorylation are important events of capacitation that spRNAs may mediate. Functional studies such as gain or loss-of function research are warranted to verify these findings further.

CONCLUSION

In conclusion, various types of protein-coding and non-coding spRNAs have been documented in the literature with their potential roles in regulating male reproduction and fertility. These spRNAs may be exploited *via* transcriptome analysis of spermatozoa to improve the conception rate of livestock by crossbreeding, artificial insemination, or ARTs. Microarray and RNA-seq have been extensively used as high throughput transcriptome analysis tools and have established sperm transcriptome as a subset yet distinct from testicular transcriptome with some uniquely expressed transcripts in spermatozoa. The spRNA profile, generated by microarray or RNA-seq, may serve as a molecular signature to identify semen with superior fertilizability, freezability, and fertility. Alternatively, exosome analysis of seminal plasma, which

contributes to the spRNA population, may be used as a proxy to spRNAs profiling. It is also emphasized that analysis of spRNA population by high sensitive high throughput sequencing technologies should involve stringent quality control measures to avoid somatic cell contamination and batch-to-batch variation in spRNA isolation protocols. Further, loss- and gain-of function studies are required to validate the function of spRNAs in spermatogenesis, fertilization, and early embryonic development. Profiling of spRNA may also prove helpful in understanding the mechanism of action of genotoxic agents, drugs, capacitation agents, motility enhancers, etc. The next generation sequencing (NGS) of spRNA may find its application in semen evaluation for diagnosing idiopathic male infertility and devising newer methods for treatment.

AUTHOR CONTRIBUTIONS

MG and RC designed the study and proofread the draft mansucript. BS, MG, PS, RC, and SC wrote the manuscript. All authors reviewed and approved the manuscript for submission.

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