



Sperm Functional Genome Associated With Bull Fertility

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Bull fertility is an important economic trait in sustainable cattle production, as infertile or subfertile bulls give rise to large economic losses. Current methods to assess bull fertility are tedious and not totally accurate. The massive collection of functional data analyses, including genomics, proteomics, metabolomics, transcriptomics, and epigenomics, helps researchers generate extensive knowledge to better understand the unraveling physiological mechanisms underlying subpar male fertility. This review focuses on the sperm phenomes of the functional genome and epigenome that are associated with bull fertility. Findings from multiple sources were integrated to generate new knowledge that is transferable to applied andrology. Diverse methods encompassing analyses of molecular and cellular dynamics in the fertility-associated molecules and conventional sperm parameters can be considered an effective approach to determine bull fertility for efficient and sustainable cattle production. In addition to gene expression information, we also provide methodological information, which is important for the rigor and reliability of the studies. Fertility is a complex trait influenced by several factors and has low heritability, although heritability of scrotal circumference is high and that it is a known fertility maker. There is a need for new knowledge on the expression levels and functions of sperm RNA, proteins, and metabolites. The new knowledge can shed light on additional fertility markers that can be used in combination with scrotal circumference to predict the fertility of breeding bulls. This review provides a comprehensive review of sperm functional characteristics or phenotypes associated with bull fertility.

Keywords: proteomics, metabolomics, transcriptomics, DNA methylome, chromatin dynamics, bull fertility

INTRODUCTION

Projections indicate that the world population will rise to 9 billion people by 2,050, requiring a 50% increase in food production (1) to satisfy the demands of a growing population. Animal agriculture will benefit from technological advances to produce livestock and their byproducts more efficiently and economically. Biotechnology is crucial in promoting sustainability of livestock production in order to meet these demands for high-quality food products with less environmental impact. Important advances in livestock production have been achieved through reproductive biotechnology (2). Bull fertility, defined as the ability of a spermatozoa to fertilize an oocyte and support embryonic development (3), and accurate evaluation of semen quality parameters

used as predictors of bull fertility, remain as important research imperatives to further enhance improvements in genetic selection in cattle (4). Generally, in studies, bull fertility is calculated based on the conception rate. For each bull, at least 100 insemination records are considered reliable data to evaluate the correct conception rate. Pregnancy diagnosis is controlled by transrectal ultrasonographic examination within 45–50 days following artificial insemination. The conception rate of each bull was plotted in a graph, and the standard deviation (SD) and the mean were calculated. The criterion for selecting high-fertile bulls was conception rate more than “mean + 1 or 2 SD,” while those below “mean – 1 or 2 SD” were considered as low-fertile bulls (5, 6).

Bulls are evaluated based on a breeding soundness exam (BSE) that is composed of an inspection of semen characteristics (phenotypes) combined with phenotypic features. Despite great efforts put into evaluating bulls using BSE, bull fertility is deemed suboptimal under field conditions, with a conception rate varying from 20 to 40% (7). Such differences may be due to the presence of subtle sperm abnormalities that might not be determined using current, established techniques. Semen evaluation tests, such as abnormalities, concentration, volume, membrane integrity, and motility, are now being conducted to predict the quality of semen samples for cryopreservation and subsequent use for artificial insemination. Although the standard semen evaluation procedures can help visually recognize poor-quality sperm, they are not enough to detect potential markers of subfertile bulls (7, 8). Since spermatogenesis in the bovine bull takes 61 days from spermatogonia to fully matured spermatozoa (9), there is ample time for molecular, cellular, and physiological errors to occur that can hamper sperm production and render infertility. Defects in the male germ cells during fetal life may be more probable causes of infertility than defects incurred in later phases of development, such as neonatal and postnatal periods (10). Therefore, more comprehensive studies spanning developmental stages and robust methods are needed to accurately ascertain semen quality and predict bull fertility for precision animal agriculture (11).

Genome-wide association studies (GWAS) have been effective in applying dense genetic markers, such as single-nucleotide polymorphism (SNP) markers, to determine genomic regions associated with economically important phenotype such as fertility (12). There are several studies showing a relationship between genomic regions and quantitative trait loci (QTL) and male reproductive traits in cattle (**Table 1**). Using a comprehensive genomic analysis on bulls, Han and Peñagaricano (25) demonstrated approximately eight genomic regions that are highly associated with bull fertility where most of these genomic regions contain genes including *Ckb*, *Kat8*, *Igf1r*, and *Tdrd9*, which are associated with sperm physiology, such as sperm motility and sperm–egg interaction. Feugang et al. (15) reported that polymorphisms in two bovine genes encoding sperm head proteins, collagen type I alpha 2 chain, and integrin subunit beta 5 are associated with bull fertility. In addition, Tüttelmann et al. (26) showed that polymorphisms in *Prm1* and *Prm2* genes were associated with human sperm quality. An SNP in *Spata1*, a gene implicated in sperm head structure, has been shown to be related to stallion fertility (27). Because the inheritance of fertility is low

and is influenced by environmental and epigenetic factors, there are fewer genetic markers associated with fertility.

Epigenetics refers to molecular processes that may regulate gene expression (active vs. inactive genes) without alterations in the DNA sequence. Epigenetic modifications, including DNA methylation, histone modifications, and nucleosome positioning, can be transmitted to the daughter cells through cell divisions. Aberrant alterations in the epigenetic profiles may give rise to abnormal gene silencing or activation (28). Transformation of male germ cells into functional spermatozoa requires a specific order involving the accumulation of specific non-coding RNA, substitution of protamines for most histones, and large-scale DNA methylation changes (29, 30). Although transcription is hardly observable in the mature sperm cells, the differentiation program in the male germline is regulated through a series of transcriptional modulations that depend directly on epigenetic reprogramming (31, 32).

PROTEOMICS, TRANSCRIPTOMICS, AND METABOLOMICS OF SPERM CELLS

Sperm Proteins and Bull Fertility

Sperm contains diverse proteins present in the sperm membrane, flagellum, cytoplasm, acrosome, and nucleus that play key roles in sperm physiology (33). Of these proteins, some are energy-related enzymes involved in sperm motility, both signaling and structural. For example, the outer dense fiber protein (ODF) has been implicated in the protection of the sperm tail against shear forces and motility in the mouse (34). Zhao et al. (35) stated that ODF2 might bind to and maintain acetylated levels of α -tubulin in HEK293T cell lines exposed to cold environment. In humans, energy-related proteins isocitrate dehydrogenase subunit alpha and phosphoglycerate mutase 2 are down- or upregulated in asthenozoospermia, respectively (36). Sperm postacrosomal sheath WW domain-binding protein (PAWP) and PLC zeta are involved in oocyte activation and embryogenesis in mice and humans (37, 38). However, Satouh et al. (39), using real-time PCR, immunoblotting, and electron microscopy, asserted that PAWP does not play an essential role in the formation of mouse sperm head or spermatogenesis in PAWP null mice. Compared with other studies, the differences in the findings of Satouh et al. (39) may arise from methodological approaches used and, perhaps, the functional interaction of PAWP with other proteins.

Using Western blotting and real-time PCR, Velho et al. (40) postulated that expression of integrin subunit beta 5 (ITG β 5) in germ cells and resultant embryos is important for fertilization and embryonic development in bovine. The fertility prediction for each bull was obtained using the Probit.F90 software (41) and expressed as the percent deviation of its conception rate from the average conception rate of all bulls. Moreover, IZUMO and fertilin subunit beta (ADAM 2) is considered to play a crucial role in the interactions between the sperm and zona pellucida, and in acrosome reactions. IZUMO1 binds to Juno, a receptor present on the egg, and facilitates gamete recognition during fertilization (42). Using 2D-PAGE, Park et al. (42) showed that ATP synthase H⁺ transporting mitochondrial

TABLE 1 | Genomic regions and quantitative trait loci demonstrated to be associated with bovine male reproductive traits.

Chromosome	Positions ^a (Mb BTAU4.0)	N Markers ^b	Phenotype	N Animals ^c	Breed	References
8	93 cM (MCM64–71 Mb)	263 MS	Dystocia and stillbirth	888	Holstein	(13)
5	70 cM	130	FSH serum concentration	126	MARC herd	(14)
29	44 cM	130	Age at puberty in males	126	MARC herd	(14)
29	44 cM	130	Testicular weight and volume	126	MARC herd	(14)
1	70.3 Mb	8,207 SNP	Noncompensatory fertility in bulls (semen)	221	Holstein	(15)
4	12.0 Mb	8,207 SNP	Noncompensatory fertility in bulls (semen)	221	Holstein	(15)
14	22 Mb	43,863 SNP	Paternal calving ease	1,800	German Fleckvieh	(16)
21	3.1 Mb	43,863 SNP	Paternal calving ease	1,800	German Fleckvieh	(16)
15	74.7 Mb	45,878 SNP	Daughter stillbirth	1,654	Holstein	(17)
3	30.28 Mb	38,416 SNP	Noncompensatory fertility in bulls (semen)	795	Holstein	(18)
4	17.35 Mb	38,416 SNP	Noncompensatory fertility in bulls (semen)	795	Holstein	(18)
4	76.89 Mb	38,416 SNP	Noncompensatory fertility in bulls (semen)	795	Holstein	(18)
4	109.13 Mb	38,416 SNP	Noncompensatory fertility in bulls (semen)	795	Holstein	(18)
5	47.38 Mb	38,416 SNP	Noncompensatory fertility in bulls (semen)	795	Holstein	(18)
6	39.71 Mb	38,416 SNP	Noncompensatory fertility in bulls (semen)	795	Holstein	(18)
8	89.91 Mb	38,416 SNP	Noncompensatory fertility in bulls (semen)	795	Holstein	(18)
10	81.45 Mb	38,416 SNP	Noncompensatory fertility in bulls (semen)	795	Holstein	(18)
12	29.53 Mb	38,416 SNP	Noncompensatory fertility in bulls (semen)	795	Holstein	(18)
12	45.01 Mb	38,416 SNP	Noncompensatory fertility in bulls (semen)	795	Holstein	(18)
13	36.68 Mb	38,416 SNP	Noncompensatory fertility in bulls (semen)	795	Holstein	(18)
14	5.93 Mb	38,416 SNP	Noncompensatory fertility in bulls (semen)	795	Holstein	(18)
15	46.78 Mb	38,416 SNP	Noncompensatory fertility in bulls (semen)	795	Holstein	(18)
17	58.73 Mb	38,416 SNP	Noncompensatory fertility in bulls (semen)	795	Holstein	(18)
19	62.00 Mb	38,416 SNP	Noncompensatory fertility in bulls (semen)	795	Holstein	(18)
22	38.91 Mb	38,416 SNP	Noncompensatory fertility in bulls (semen)	795	Holstein	(18)
22	58.96 Mb	38,416 SNP	Noncompensatory fertility in bulls (semen)	795	Holstein	(18)
X	31.67 Mb	38,416 SNP	Noncompensatory fertility in bulls (semen)	795	Holstein	(18)
X	43.13 Mb	38,416 SNP	Noncompensatory fertility in bulls (semen)	795	Holstein	(18)
X	101.55 Mb	38,416 SNP	Noncompensatory fertility in bulls (semen)	795	Holstein	(18)
1	95 cM (BMS4031–91.3 Mb)	390 MS	Scrotal circumference	1,769	Angus	(19)
4	46 cM (BMS1840–51 Mb)	390 MS	Scrotal circumference	1,769	Angus	(19)
4	96 cM (RM088–108.5 Mb)	390 MS	Scrotal circumference	1,769	Angus	(19)
5	12 cM (BMS610–13 Mb)	390 MS	Scrotal circumference	1,769	Angus	(19)
5	101 cM (BM315–104 Mb)	390 MS	Scrotal circumference	1,769	Angus	(19)
5	127 cM (BMS597)	390 MS	Scrotal circumference	1,769	Angus	(19)
6	102 cM (BM8124)	390 MS	Scrotal circumference	1,769	Angus	(19)
7	10 cM (RM012–0.5 Mb)	390 MS	Scrotal circumference	1,769	Angus	(19)
7	28 cM (RM006–16 Mb)	390 MS	Scrotal circumference	1,769	Angus	(19)
7	41 cM (BM6105–22 Mb)	390 MS	Scrotal circumference	1,769	Angus	(19)
8	12 cM (IDVGA11–10 Mb)	390 MS	Scrotal circumference	1,769	Angus	(19)
9	68 cM (BMS2377–72.7 Mb)	390 MS	Scrotal circumference	1,769	Angus	(19)
9	110 cM (BMS1967–92 Mb)	390 MS	Scrotal circumference	1,769	Angus	(19)
10	99 cM (BMS614–94 Mb)	390 MS	Scrotal circumference	1,769	Angus	(19)
10	118 cM (BL1134–102 Mb)	390 MS	Scrotal circumference	1,769	Angus	(19)
11	12 cM (INRA044–6 Mb)	390 MS	Scrotal circumference	1,769	Angus	(19)
11	29 cM (BMS2325–11.8 Mb)	390 MS	Scrotal circumference	1,769	Angus	(19)
11	93 cM (BMS989–86.2 Mb)	390 MS	Scrotal circumference	1,769	Angus	(19)
12	13 cM (BMS2252–10.4 Mb)	390 MS	Scrotal circumference	1,769	Angus	(19)
13	41 cM (BMS1352–28.1 Mb)	390 MS	Scrotal circumference	1,769	Angus	(19)

(Continued)

TABLE 1 | Continued

Chromosome	Positions ^a (Mb BTAU4.0)	N Markers ^b	Phenotype	N Animals ^c	Breed	References
15	21 cM (ADCY2-BTA20 at 69.2 Mb)	390 MS	Scrotal circumference	1,769	Angus	(19)
15	34 cM (JAB8–29.3 Mb)	390 MS	Scrotal circumference	1,769	Angus	(19)
16	73 cM (INRA048)	390 MS	Scrotal circumference	1,769	Angus	(19)
17	94 cM (BM1233-BTA18 54.7 Mb)	390 MS	Scrotal circumference	1,769	Angus	(19)
18	77 cM (BM2078–62.0 Mb)	390 MS	Scrotal circumference	1,769	Angus	(19)
19	12 cM (BMS745–11.8 Mb)	390 MS	Scrotal circumference	1,769	Angus	(19)
19	56 cM (BMS650–36.2 Mb)	390 MS	Scrotal circumference	1,769	Angus	(19)
19	80 cM (IDVGA44–56.7 Mb)	390 MS	Scrotal circumference	1,769	Angus	(19)
19	98 cM (RM388–59.4 Mb)	390 MS	Scrotal circumference	1,769	Angus	(19)
20	1 cM (RM106–1.2 Mb)	390 MS	Scrotal circumference	1,769	Angus	(19)
21	30 cM (BM103–20.0 Mb)	390 MS	Scrotal circumference	1,769	Angus	(19)
22	27 cM (DIK2694–21.1 Mb)	390 MS	Scrotal circumference	1,769	Angus	(19)
22	65 cM (BMS875–46.1 Mb)	390 MS	Scrotal circumference	1,769	Angus	(19)
23	35 cM (BOLADRB1–26.3 Mb)	390 MS	Scrotal circumference	1,769	Angus	(19)
25	59 cM (BMS1353–32.9 Mb)	390 MS	Scrotal circumference	1,769	Angus	(19)
26	15 cM (FASMC2–11.1 Mb)	390 MS	Scrotal circumference	1,769	Angus	(19)
27	61 cM (BMS1675–46.2 Mb)	390 MS	Scrotal circumference	1,769	Angus	(19)
28	30 cM (BMS510–21.8 Mb)	390 MS	Scrotal circumference	1,769	Angus	(19)
28	49 cM (BMS1714–34.6 Mb)	390 MS	Scrotal circumference	1,769	Angus	(19)
29	13 cM (BMS764–10.0 Mb)	390 MS	Scrotal circumference	1,769	Angus	(19)
2	108–109 Mb	43,821 SNP	Serum inhibin at 4 months	786	Brahman	(20)
14	22–26 Mb	43,821 SNP	Scrotal circumference at 12 months	1,112	Brahman	(20)
28	18 Mb	43,821 SNP	Luteinizing hormone levels at 4 months	537	Brahman	(20)
X	4 Mb	43,821 SNP	Percent normal sperm at 24 months	964	Brahman	(20)
X	40–55 Mb	43,821 SNP	Percent normal sperm at 24 months	964	Brahman	(20)
X	97 Mb	43,821 SNP	Percent normal sperm at 24 months	964	Brahman	(20)
X	62–96 Mb	43,821 SNP	Scrotal circumference at 12 months	1,112	Brahman	(20)
14	22–28 Mb	43,821 SNP	Age at puberty	1,118	Brahman	(21)
X	86 Mb	43,821 SNP	Age at puberty	1,118	Brahman	(21)
2	25.6 Mb	38,650 SNP	Sire conception rate	1,755	Holstein	(22)
5	119.4 Mb	38,650 SNP	Sire conception rate	1,755	Holstein	(22)
18	54.3 Mb	38,650 SNP	Sire conception rate	1,755	Holstein	(22)
25	1.4 Mb	38,650 SNP	Sire conception rate	1,755	Holstein	(22)
25	2.8 Mb	38,650 SNP	Sire conception rate	1,755	Holstein	(22)
25	4.8 Mb	38,650 SNP	Sire conception rate	1,755	Holstein	(22)
13	8.42 Mb	46,035 SNP	Tail stump sperm defect	321	Swedish Red	(23)
25	2.98 Mb	54,001 SNP	Asthenospermia		Nordic Red	(24)

^aChromosomal positions are represented in centiMorgans (cM). The microsatellite marker location was employed to translate cM into Mb positions, according to the BTAU4.0 assembly.

^bIndicates the number of gene markers used in the relevant study (SNP, single nucleotide polymorphisms; MS, microsatellite).

^cRepresents how many experimental animals were used.

F1 complex beta subunit (ATP5B), alpha-2-HS-glycoprotein 2 (AHSG), enolase 1 (ENO1), apoptosis-stimulating of p53 protein (ASPP2), and phospholipid hydroperoxide glutathione peroxidase (GPx4) were more abundant in sperm from high-fertility bulls, whereas ubiquinol–cytochrome c reductase complex core protein 2 (UQCRC2), ropporin-1, and voltage-dependent anion channel 2 (VDAC2) were in greater amounts in sperm from low-fertility bulls (Table 2).

Currently, proteomic approaches are widely used to explore male reproductive physiology (43). Aslam et al. (5) analyzed the bull sperm proteome using 2D-DIGE and MALDI-TOF-MS techniques, and validated these proteomic studies using Western blotting. The authors reported that malate dehydrogenase 2 (MD2), enolase 1 (ENO1), calpain-7 like protein (CAPN7), N-acetyllactosaminide beta-1,6-N-acetylglucosaminyl transferase isoform C (GCNT2), RIB43A

domain with coiled-coils 1 (RIBC1), condensin-2 complex subunit D3 (NCAPD3), 2,4-dienoyl CoA reductase-1 (DEC1), beta galactosidase-1-like protein-2 like (LacA-like protein-2 like), GDP dissociation inhibitor 2 (GDI2), chain D, F-1 ATPase (ATP5D), ubiquitin carboxyl terminal hydrolase-12 (USP12), and thimet oligopeptidase-1 (TOP) are over expressed in sperm from high-fertility bulls, whereas binder of sperm-1 (BSP1), transmembrane protein-43 (TMEM43), and dystonin-like isoform-1 (DST like isoform 1) are more abundant in sperm from low-fertility bulls (Table 2).

The MDH2 catalyzes the reversible oxidation of malate to oxaloacetate using NAD⁺/NADH as a cofactor in the citric acid cycle (45). Aslam et al. (5) suggested that the reduction of MDH2 has a negative impact on energy metabolism of spermatozoa, disrupting sperm motility, capacitation, and ultimately fertilizing ability. ENO1, a multifunctional enzyme, is found mainly in the motile sperm tail. In addition to regulating the constant provision of energy for motility, it assists in the protection of the sperm from oxidative stress (42). The RibC is a ribbon protein that is vital for sperm motility and structural integrity of sperm tails, suggesting that low expression of RibC in bull sperm reduces fertility by disrupting sperm motility (5). Calpains in mammalian sperm are involved during the acrosome reaction and capacitation (46). The Rab are small GTP-binding proteins that are critical in vesicular trafficking of molecules. The GDI keeps the function of Rab proteins under control by freeing it from membranes and preventing the GDP dissociation (47). The USP12 plays a crucial role in maintaining the androgen receptors steady and improving their cellular functions (48). The TOP are highly expressed enzymes in testes and exert their functions by catalyzing the hydrolysis of gonadotropin-releasing hormone (49). The LacA-like protein-2, which is produced and secreted from the epididymis, binds to sperm membranes during the maturation process in rats (50). Aslam et al. (5) suggested that low levels of expression of this enzymatic protein are considered to have a significant role in sperm physiology and led to a reduction in functional competence of the sperm in low-fertility bulls.

Using 2D-DIGE analysis of bull sperm, it was shown that ALB, TIMP, spermadhesin-1, and binder of sperm proteins (BSP)-1, 3, and 5, PEBP1, and AKI in sperm and seminal plasma were more abundant in sperm from high fertility bulls, while PSMA6, ELSPbP1, CCT5, CCT8, and CLU were in greater amounts in seminal plasma and in sperm from low-fertility bulls. The expression levels of ZFP34, HSP90, BCL62, IFNRF4, NADHD, histone H1, and TUBB3 were higher in high-fertility bull sperm (44) (Table 2). Matrix metalloproteinases (MMPs) belonging to a group of proteolytic zinc-dependent enzymes are crucial components of semen (51). The MMPs and other proteases participate in semen liquefaction in the female genital tracts, and they are needed for sperm viability during capacitation in humans (52).

Spermadhesin family members interact with carbohydrates, phospholipids, and zona pellucida glycoproteins and participate in sperm-egg binding (53–55). Spermadhesin-1 is a nonglycosylated protein produced by the epithelium of the epididymis, ampulla, and seminal vesicle, and is secreted into the seminal fluid (56, 57). Furthermore, it has been suggested that

recombinant spermadhesin-1 influences sperm mitochondrial activity through its binding ability to the sperm midpiece (58).

Albumin has been reported to facilitate cholesterol outflow from sperm membranes and mediates sperm capacitation in the female reproductive tract (59, 60). Moreover, albumin preserves sperm against lipid peroxidation by binding to free radicals (44). Adenylate kinase isoenzyme 1 (AK1), a ubiquitous enzyme related to cellular energy homeostasis, is expressed in murine and bovine sperm flagella, suggesting its participation in sperm motility (61–63). Furthermore, AK1 has been reported to be active when spermatozoa are highly motile (62). Phosphatidylethanolamine-binding protein 1 (PEBP1) is an evolutionarily conserved protein in mammals and reported to be present in the acrosome, the postacrosomal region, and the tail of both human and mouse sperm. The PEBP1 seems to promote inhibition of sperm capacitation because it serves either as a decapacitation factor released throughout capacitation or as a membrane-bound, glycosylphosphatidylinositol (GPI)-anchored receptor for a decapacitation factor (64, 65). Binder of sperm proteins (BSP) are synthesized in the male accessory sex glands and bind to sperm via choline phospholipids upon ejaculation, which prevent premature initiation of the capacitation and acrosome reaction (66). Among the BSP proteins, BSP1, BSP3, and BSP5 are predominant proteins secreted into bovine seminal plasma, all of which contain two tandem repeated fibronectin type 2 (Fn2) domains (67, 68).

Cholesterol and phospholipids contribute to the regulation of sperm membrane bilayer stability and fluidity. The BSP proteins promote efflux of phospholipids and cholesterol from sperm membranes, thereby, disrupting sperm membrane architecture, resulting in capacitation (66). Moreover, BSP proteins promote the binding of sperm to the epithelium of the oviduct, contributing to maintain sperm viability and motility in the oviduct (69). Studies on BSPs have reported different results. Some studies (5, 68, 69) showed that BSP protein expression in semen was negatively correlated with bull fertility, unlike the findings of Kasimanickam et al. (44) who reported a positive correlation between BSP expression and bull fertility. These differences were attributed to degenerated and fragmented sperm membrane wastes in semen. A single-cell analysis approach may be required to obtain a reliable result. Furthermore, due to structural similarities to BSP, epididymal sperm-binding protein E12 (ELSPbP1) can induce lipid efflux and perturb the membrane stability (70).

Proteasome subunit alpha type-6 (PSMA6) belongs to proteasome multicatalytic protease degrading polyubiquitinated proteins into small peptides and amino acids (71). Proteasomes are localized in the acrosomal region, connecting head and tail (33, 72). The presence and expression levels of PSMA6 are associated with sperm DNA fragmentation in bulls (68). T-complex protein 1 subunit 3 (CCT3) and 8 (CCT8) are parts of the class II chaperonins (73). Cytoplasmic CCT expression has been shown to localize in the centrosomes and microtubules of the manchette during spermatogenesis and assumed to be discarded during spermiation. Hence, it is considered that the abundance of CCT subunits in sperm from low-fertility bulls reflects uncompleted developmental processes

TABLE 2 | Fertility-associated proteins of sperm from low- and high-fertility bulls.

Protein name	Abbreviation	High fertility	Low fertility	Function	Methods	Breed	References
ATP synthase H ⁺ -transporting mitochondrial F1 complex beta subunit	ATP5B	Upregulated		Energy metabolism	2D-PAGE	Hanwoo	(42)
Alpha-2-HS-glycoprotein 2	AHSG	Upregulated		Immune system	2D-PAGE	Hanwoo	(42)
Enolase 1	ENO1	Upregulated		Energy metabolism	2D-PAGE	Hanwoo	(42)
Apoptosis-stimulating of p53 protein	ASPP2	Upregulated		Oxidative stress	2D-PAGE	Hanwoo	(42)
Phospholipid hydroperoxide glutathione peroxidase	GPx4	Upregulated		Oxidative stress	2D-PAGE	Hanwoo	(42)
Ubiquinol-cytochrome c reductase complex core protein 2	UQCRC2		Upregulated	Oxidative stress	2D-PAGE	Hanwoo	(42)
Ropporin-1			Upregulated	Cell signaling	2D-PAGE	Hanwoo	(42)
Voltage-dependent anion channel 2	VDAC2		Upregulated	Ion transport	2D-PAGE	Hanwoo	(42)
Malate dehydrogenase 2	MD2	Upregulated		Energy metabolism	2D-DIGE and MALDI-TOF-MS	Holstein x Tharparkar crossbred	(5)
Enolase 1	ENO1	Upregulated		Energy metabolism	2D-DIGE and MALDI-TOF-MS	Holstein x Tharparkar crossbred	(5)
Calpain-7-like protein	CAPN7	Upregulated		Acrosome reaction and capacitation	2D-DIGE and MALDI-TOF-MS	Holstein x Tharparkar crossbred	(5)
N-Acetyllactosaminide beta-1,6N acetylglucosaminyl transferase isoform C	GCNT2	Upregulated		Development and maturation of erythroid cells	2D-DIGE and MALDI-TOF-MS	Holstein x Tharparkar crossbred	(5)
RIB43A domain with coiled-coils 1	RIBC1	Upregulated		Sperm motility and the structural integrity of sperm tail	2D-DIGE and MALDI-TOF-MS	Holstein x Tharparkar crossbred	(5)
Condensin-2 complex subunit D3	NCAPD3	Upregulated			2D-DIGE and MALDI-TOF-MS	Holstein x Tharparkar crossbred	(5)
2,4-Dienoyl CoA reductase-1	DECR1	Up regulated		Energy metabolism	2D-DIGE and MALDI-TOF-MS	Holstein x Tharparkar crossbred	(5)
Beta galactosidase-1-like protein-2	LacA like protein-2	Upregulated		Maturation of spermatozoa	2D-DIGE and MALDI-TOF-MS	Holstein x Tharparkar crossbred	(5)
GDP dissociation inhibitor 2	GDI2	Upregulated		Preventing membrane integrity	2D-DIGE and MALDI-TOF-MS	Holstein x Tharparkar crossbred	(5)
Chain D, F-1 ATPase	ATP5D	Upregulated		Energy metabolism	2D-DIGE and MALDI-TOF-MS	Holstein x Tharparkar crossbred	(5)
Ubiquitin carboxyl terminal hydrolase-12	USP12	Upregulated		Cell signaling	2D-DIGE and MALDI-TOF-MS	Holstein x Tharparkar crossbred	(5)
Thimet oligopeptidase-1	TOP	Upregulated		Catalyze the hydrolysis of gonadotropin-releasing hormone	2D-DIGE and MALDI-TOF-MS	Holstein x Tharparkar crossbred	(5)

(Continued)

TABLE 2 | Continued

Protein name	Abbreviation	High fertility	Low fertility	Function	Methods	Breed	References
Binder of sperm-1	BSP1		Upregulated	Prevent premature acrosome reaction and capacitation	2D-DIGE and MALDI-TOF-MS	Holstein x Tharparkar crossbred	(5)
Transmembrane protein-43	TMEM43		Upregulated	Maintain nuclear envelope structure	2D-DIGE and MALDI-TOF-MS	Holstein x Tharparkar crossbred	(5)
Dystonin-like isoform-1	DST like isoform 1		Upregulated	An integrator of intermediate filaments, actin, and microtubule cytoskeleton networks	2D-DIGE and MALDI-TOF-MS	Holstein x Tharparkar crossbred	(5)
Albumin	ALB	Upregulated		Ease cholesterol outflow from sperm membranes and preserves sperm against lipid peroxidation	2D-DIGE	Holstein	(44)
The tissue inhibitors of metalloproteinase	TIMP	Upregulated		Inhibit MMPs by binding to their catalytic Zn cofactor	2D-DIGE	Holstein	(44)
Spermadhesin-1	SPADH1	Upregulated		Participate in sperm-egg binding	2D-DIGE	Holstein	(44)
Binder of sperm proteins 1, 3, and 5	BSP1, 3, 5	Upregulated		Prevent premature acrosome reaction and capacitation	2D-DIGE	Holstein	(44)
Phosphatidylethanolamine-binding protein 1	PEBP1	Upregulated		Promote inhibition of early sperm capacitation	2D-DIGE	Holstein	(44)
Adenylate kinase isoenzyme 1	AK1	Upregulated		Energy metabolism	2D-DIGE	Holstein	(44)
Heat shock protein 90	HSP90	Upregulated		Stabilizes proteins against heat stress	2D-DIGE	Holstein	(44)
B-cell lymphoma-62	BCL62	Upregulated		Antiapoptotic	2D-DIGE	Holstein	(44)
NADH dehydrogenase	NADHD	Upregulated		Energy metabolism	2D-DIGE	Holstein	(44)
Interferon regulatory factor 4	IFNRF4	Upregulated		Immune system	2D-DIGE	Holstein	(44)
Class III β -tubulin	TUBB3	Upregulated		Sperm motility	2D-DIGE	Holstein	(44)
Proteasome subunit alpha type-6	PSMA6		Upregulated	Associated with sperm DNA fragmentation	2D-DIGE	Holstein	(44)
Phosphatidylethanolamine-binding protein 1	PEBP1		Upregulated	Inhibition of sperm capacitation	2D-DIGE	Holstein	(44)
T-complex protein 1 subunits 3 and 8	CCT3, CCT8		Upregulated	Reflecting incomplete developmental processes	2D-DIGE	Holstein	(44)
Clusterin	CLU		Upregulated	Oxidative stress	2D-DIGE	Holstein	(44)
The tissue inhibitors of metalloproteinase-2	TIMP-2	Upregulated		Inhibit MMPs by binding to their catalytic Zn cofactor	Mass spectrometry coupled with Nano HPLC	Holstein	(4)
C-type natriuretic peptide	NPPC	Upregulated		Stimulating intracellular cGMP and sperm motility	Mass spectrometry coupled with Nano HPLC	Holstein	(4)

(Continued)

TABLE 2 | Continued

Protein name	Abbreviation	High fertility	Low fertility	Function	Methods	Breed	References
Sulfhydryl oxidase	QSOX1	Upregulated		Oxidative stress	Mass spectrometry coupled with Nano HPLC	Holstein	(4)
Binder of sperm-5	BSP5	Upregulated		Participate in sperm-egg binding	Mass spectrometry coupled with Nano HPLC	Holstein	(4)
Galectin-3-binding protein	LGALS3BP		Upregulated	Inhibiting cell signaling	Mass spectrometry coupled with Nano HPLC	Holstein	(4)
Tissue factor pathway inhibitor 2	TFPI2		Upregulated		Mass spectrometry coupled with Nano HPLC	Holstein	(4)
Clusterin	CLU		Upregulated	Oxidative stress	Mass spectrometry coupled with Nano HPLC	Holstein	(4)

throughout spermatogenesis (74). Clusterin (CLU), a 75- to 80-kDa disulfide-linked heterodimeric protein, is produced in the testis, epididymis, and seminal vesicles and has been speculated to be an alternative oxidative stress marker for seminal plasma in humans (75). The CLU is localized mainly on the abnormal sperm surface (76). Furthermore, increased levels of CLU expression in semen are positively correlated with sperm DNA defects (75).

Using mass spectrometry coupled with nano HPLC, a total of 1,159 proteins were detected in the bull seminal plasma, of which 29 were abundant in low-fertility bulls, whereas 50 were abundant in high-fertility bulls (77). While TIMP-2, C-type natriuretic peptide, sulfhydryl oxidase, and BSP5 revealed a relationship with high-fertility bulls, galectin-3-binding protein, tissue factor pathway inhibitor 2, clusterin, and 5'-nucleotidase were associated with low-fertile bulls based on multivariate analysis. Furthermore, high levels of transmembrane protein 2, prosaposin, and NAD (P) (+)-arginine ADP ribosyltransferase proteins had the highest positive correlations with fertility ranking, whereas quantities of nucleotide exchange factor SIL1, galectin-3-binding protein, and vitamin D-binding protein exhibited the highest negative correlations with fertility ranking (77) (Table 2). The C-type natriuretic peptide (NPPC) is a member of natriuretic peptides that exerts its physiological functions through binding to two distinct membrane-bound guanylyl cyclases and activating cyclic guanosine monophosphate signaling pathways (78, 79). In addition to being synthesized by cardiomyocytes and known to modulate vascular permeability and dilation/constriction, NPPC is also produced locally by Sertoli cells in the testis and serves in an autocrine manner (80). Also, NPPC is more abundantly expressed in male reproductive tissues than in other tissues (81, 82). In rats, NPPC was intensely expressed in Leydig cells and epididymal epithelium, and its expression dramatically increased after puberty (83). Furthermore, NPPC receptor (NPR-B) has been shown to localize in the acrosome and tail of human sperm, suggesting that NPPC binds to NPR-B, thus, stimulating intracellular cGMP and sperm motility (84). In the

male reproductive tract, QSOX1 maintains the structure and function of sperm through the oxidization of sulfhydryl groups that might damage the cell (85). Sulfhydryl oxidase (QSOX1) is involved in the reduction of an oxygen molecule to hydrogen peroxide; thus, it creates disulfide bonds in peptides and proteins (86). It has been proposed that QSOX is essential for sperm physiology, and its dysregulation is attributed to defects that may occur during spermatogenesis in hamsters (87) and rats (88).

Galectins (Gals) belong to members of β -galactoside-binding lectins, which can be localized in extracellular spaces and in cellular components such as cell membrane, cytoplasm, and nucleus (89). They are implicated in cell-to-cell interactions, cell-extracellular matrix interactions, receptor crosslinking or lattice formation, intracellular signaling, and posttranscriptional splicing (90). Gal-3 has antiapoptotic effects, unlike most members of the galectin family (91). Gal-3 expression has been observed in the epithelium of corpus and cauda epididymis but not in initial segment and caput epididymis, suggesting that Gal-3 participates in maturation and storage of rat sperm (92). Previously, Gal-3-binding protein has been observed in bovine epididymal fluid (57) and shown to participate in sperm motility, semen liquefaction, and angiogenesis in the female reproductive tract (93).

Gomes et al. (94) examined the proteome and posttranslational modifications in bovine seminal plasma with the aid of a top-down mass spectrometry (TDMS) strategy to uncover more comprehensive information. They separated plasma proteins using sheathless capillary zone electrophoresis (CZE)-MS and reversed-phase liquid chromatography (LC)-MS. Then, the proteins were fragmented using electron-transfer/higher-energy collisional dissociation and 213-nm ultraviolet photodissociation. The use of the sheathless CZE-MS method helped identify 417 proteoforms, including 170 unique species, whereas 3,090 proteoforms, including 1,707 unique species were detected by using LC-MS. The researchers identified 1,433 proteoforms (ETHcD) and 2,151 proteoforms (213 nm UVPD) with 612 species for ETHcD and 1,021 for 213-nm UVPD (94).

Sperm Transcriptome and Bull Fertility

Sperm delivers, not just the paternal DNA, but other factors, such as cell signaling molecules, RNA, and transcription factors, into the oocyte at the time of fertilization (95). New cutting-edge technologies, such as RNA sequencing (RNA-seq) and microarray analysis have enabled characterization of various types of sperm RNAs, including transfer RNA (tRNA), ribosomal RNA (rRNA), messenger RNA (mRNA), small nucleolar RNA (snoRNA), small nuclear RNA (snRNA), small non-coding RNA (sncRNA), long non-coding RNA (lncRNA), and mitochondrial RNA (mt-RNA), which are present in bovine spermatozoa (96). The miRNAs, piRNAs, and tRNAs are grouped as “small non-coding” RNAs (sRNAs) (97).

There are several transcriptomic studies on bull sperm using different techniques. Wang et al. (98) used strand-specific RNA sequencing to profile the semen transcriptome (lncRNA and mRNA) and to ascertain the functions of lncRNA and mRNA in bull sperm motility. They detected 20,875 transcripts of protein-encoding genes in semen and found 19 different mRNAs between high- and low-motility sperm. They also detected five differentially expressed genes, such as *Efn1*, *Rbmx*, *Mlph*, *Rpl30*, and *Aqp2*, which participate in “extracellular exosome” GO term. Among them, the ephrin A1 (*Efn1*) protein that is localized on cell surfaces participates in membrane integrity and sperm morphology, and it has been reported that *Efn1* is highly present in both seminal plasma and sperm (44, 99) and possibly influences sperm motility (98). Heterogeneous nuclear ribonucleoprotein G (*Rbmx*) has been proposed to be a possible splicing factor that modulates spermatogenesis (100). Based on immunohistochemical methods, aquaporin 2 (*Aqp2*) is expressed in male germ cells, seminiferous epithelium, Leydig cells, and in the male reproductive tract (101), suggesting that *Aqp2* directly or indirectly participates in male fertility.

Moreover, Wang et al. (98) also identified 11,561 lncRNA in bull sperm, of which 2,517 were distinctly expressed between the low- and high-motility sperm. They also determined that TCONS_00041733 lncRNA targets the node gene *ephrin A1* and participates in the physiology of the male reproductive system. Card et al. (102) detected 6,166 transcripts in bull sperm, most of which were full-length transcripts that *Plcz1* and *Crisp2* transcripts are associated with bull fertility. Furthermore, a comprehensive microarray analysis revealed 415 transcripts to be differentially expressed in sperm from high- and low-fertility bulls (103). Légaré et al. (104) showed that 10 mRNA transcripts (*Smcp*, *Akap4*, *Tcp11*, *Spata3*, *Ctcf1*, *Odf1*, *Adam28*, *Spata18*, *Fam161a*, and *Sord*) in bovine sperm were associated with reproductive system functions. They also found that five mRNA transcripts (*Cyst11*, *Dead*, *Mx1*, *Defb124*, and *Defb119*) are related to the immune defense response.

Sperm miRNA content is dynamic, and the factors affecting spermatogenesis and epididymal maturation influence sperm miRNA composition (105, 106). Microarray and RNA-Seq-based gene expression profiling studies showed that *miR-10a*, *miR-10b*, *miR-34c*, *miR-100*, *miR-103*, *miR-196b*, *miR-365-2*, and *miR-2478* consistently exist in bovine spermatozoa (96, 105, 107). Interestingly, RT-qPCR studies determined that *miR-19b-3p*,

miR-34c-3p, *miR-148b-3p*, *miR-320a*, and miRNAs *miR-1249* were detectable at low levels, whereas *miR-27a-5p* and *502-5p* were not detectable in sperm from most high-fertility bulls (108). These miRNA transcripts, such as *miR-34*, *miR-34b/c*, and *miR-449*, modulate spermatogenesis and possibly embryogenesis (109, 110). Liu et al. (111) have suggested that *miR-34c-5p* is involved in mRNA degradation and translational repression.

Two different RNA-seq platforms were Illumina and Ion Proton, and they provided evidence that the most abundant miRNA in the bovine sperm is *miR-196b* and is more abundantly expressed in the zygote than the oocyte. *miR-196b* targets transcripts of *Hoxa7*, *Hoxa9*, and *Hoxc8* genes. In addition, protein products of these genes play crucial roles in the meiotic phases of spermatogenesis and are present at high levels in spermatocytes (96, 111, 112). Menezes et al. (113) examined the dynamics of *miR-15a*, *miR-29b*, and *miR-34a* in low- and high-fertility bull sperm using RT-qPCR (113). They reported that *miR-15a* and *miR-29* were more abundantly present in sperm from low-fertility bulls than those of high fertility bulls. However, *miR-34a* expression levels did not differ in sperm from the two groups. In addition, results of several studies suggest that lncRNAs may be involved in the regulation of testis development and spermatogenesis. For example, Zhang et al. (114) showed that *Dmrt1* was involved in the transition of germ cells from mitosis to meiosis using transfection, Western blotting, and Northern and Southern blotting hybridizations. Based on proteomic, immunostaining, and microarray approaches, *HongrES2* has been reported to modulate sperm maturation, and *Mrhl* lncRNA influences spermatogenesis (115, 116). The *Tsx*, which is specifically expressed in pachytene spermatocytes, has a crucial role in the progression of spermatocyte meiosis (117).

Sperm Metabolome and Bull Fertility

Metabolites are also associated with physiological events via a cascade of complex biochemical networks (118, 119) and may provide insights of an individual's phenom (119). Metabolomic methods are used to detect low molecular weight compounds that may offer deep insights into the regulatory pathways within spermatozoa as well (120, 121). In this regard, the mounting evidence shows that mature sperm metabolize a wide range of exogenous substrates that modulate the signaling pathways implicated in key aspects of sperm physiology, including the acrosome reaction, capacitation, hyperactivation, motility, and fusion of spermatozoon and egg (122). The latest improvements in methods of metabolite profiling of infertile individuals offer better insights into the development of useful fertility markers (123). Several metabolite biomarkers have been discovered by untargeted metabolic profiling of sperm samples from healthy individuals and infertile patients using different analytical techniques such as nuclear magnetic resonance (NMR) (124) and mass spectrometry (MS) (125).

There were 22 distinct metabolites detectable in bull sperm employing gas chromatography-mass spectrometry (GC-MS) analysis (126) where major metabolites were fatty acids/conjugates and organic acids/derivatives. The researchers also showed that the levels of five sperm metabolites that differed between high- vs. low-fertility groups were benzoic

acid, gamma-aminobutyric acid (GABA), palmitic acid, carbamate, and lactic acid. In addition, four metabolic pathways were found to be associated with differential metabolites, namely, glycolysis or gluconeogenesis, aspartate and glutamate metabolism, pyruvate metabolism, alanine, and β -alanine metabolism. GABA plays an essential role in sperm physiology by inducing the acrosome reaction and sperm hyperactivation. Furthermore, benzoic acid participates in GABA regulation and is highly expressed in high-fertility bull sperm. Because of its participation in lipid metabolism to generate energy, palmitic acid production may be more abundant in high-fertility bull sperm. Higher levels of lactic acid in high-fertility bull sperm might be because anaerobic glycolysis is more efficiently utilized in high-fertility sperm compared with those in low-fertility sperm. Functions of carbamate are considered as potential regulators of intracellular pH in sperm (127).

Velho et al. (128) studied seminal plasma metabolomes of Holstein bulls using GC-MS. They reported that the most abundant metabolites were fructose followed by urea, citric acid, phosphoric acid, and lactic acid. Erythronic acid, 4-ketoglucose, 2-oxoglutaric acid, androstenedione, and D-xylofuranose represented the least predominant metabolites in bull seminal fluid. They demonstrated that levels of 2-oxoglutaric acid were low, whereas the levels of fructose were greater in high-fertility bulls compared with low-fertility bulls. Sperm metabolism can oxidize fructose and convert it to lactic acid (129), supporting both fructose and lactic acid as necessary for fertile sperm as energy sources. Therefore, in many species, fructose is the main monosaccharide abundantly present in semen (124, 130–132).

Citric acid influences the acrosome reaction, sperm transport, and fertilization by being an energy source and regulating semen pH as a chelator for calcium, magnesium, and zinc (133, 134), suggesting that citric acid is a candidate fertility marker in seminal plasma. However, roles of urea and phosphoric acid in seminal plasma on bull fertility remain mostly unclear. Velho et al. (128) speculated that phosphoric acid in seminal plasma may result from catalysis of inorganic phosphate. Hydrolysis of inorganic pyrophosphate to two phosphate ions yields energy (135) that may be utilized for sperm motility or fertilization. Urea in seminal plasma is considered as a metabolite resulting from protein degradation (136). High concentrations of urea in seminal plasma suggest that fertile sperm contain enough protein sources, and some of these proteins are metabolized for biological processes associated with fertility. Using MS, Soggiu et al. (137) demonstrated that isocitrate dehydrogenase, triose phosphate isomerase, and alpha enolase were fertility-associated molecules in bull sperm. Recently, amino acid contents in seminal plasma were shown to be associated with bull sperm freezability (138). Using GC-MS, the researchers also showed that the most abundant amino acid in bull seminal fluid was glutamic acid. Furthermore, phenylalanine concentration in seminal plasma was significantly associated with post-thaw viability.

SPERM EPIGENOME AND BULL FERTILITY

Sperm DNA Methylation and Bull Fertility

DNA methylation has been the most studied epigenetic mechanism in sperm and is presumed to fulfill a major role in the non-genetic information transfer across generations. Sperm DNA methylation participates in many physiological processes, such as silencing of transposable elements (139), paternal genomic imprinting (30), DNA compaction (140), and chromosome inactivation in females (141). In combination with histone modifications, DNA methylation has a fundamental role in modulating gene expression in germ cells by inhibiting the binding of transcription factors to enhancers or by recruiting the binding of proteins that facilitate the deacetylation or methylation of histones, thereby stabilizing the nucleosomes (142). Advances in technologies offered quantitative and base-level ultra-resolution methylome maps. DNA methylation involves the addition of a methyl group to the carbon-5 position of cytosine in the context of cytosine followed by guanine (CpG dinucleotides), referred to as 5-methylcytosine (5mC), although to a lesser extent, DNA methylation also occurs at cytosine bases in a non-CpG context (143, 144).

Gametic DNA methylation is archived in a progressive manner via the activity of the *de novo* methyltransferases DNMT3A, DNMT3B, and their cofactor DNMT3L. Significant levels of DNA methylation are present at birth and must be sustained by DNMT1 during adulthood across different phases of spermatogenesis (145, 146). However, DNA methylation can be reversible, mediated by the ten-eleven translocation (TET) family of DNA dioxygenases that progressively oxidize 5mC to 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC), and 5-carboxylcytosine (5caC) (147–151). The CpG islands experience differential methylation during gametogenesis and early embryonic development (152). Exposure to harmful environmental conditions may alter DNA methylation patterns in male germ cells and inhibit differentiation into functional mature spermatozoa, thereby causing infertility (153, 154).

Employing whole-genome bisulfite sequencing (WGBS) data (486 × coverage) from neighboring CpG sites among 28 distinct bull sperm samples, Liu et al. (155) identified 31,272 methylation haplotype blocks (MHB) based on the correlation analysis of methylation levels. Of these MHBs, they defined highly variably methylated, variably methylated, and conserved methylated regions. By integrating evidence from traditional and molecular quantitative trait loci, they revealed that highly variably methylated regions may play roles in transcriptional regulation and function in variations in complex traits. Furthermore, they detected 46 variably methylated regions significantly related to reproduction traits, nine of which were modulated by cis-SNP. These variably methylated regions were colocalized with fertility-associated genes, such as *Crisp2*, *Hgf*, and *Zfp361l*. Sperm protein CRISP2 has important roles in spermatogenesis, modulation of flagellar motility, acrosome reaction, and gamete fusion. Naz et al. (156) showed that HGF was distinctly expressed in the vas deferens and epididymis in mice. Moreover, Herness and Naz (157) implicated that HGF is involved in the process of

acquisition of the potential for sperm motility as sperm mature during epididymal transit, as when immotile mouse sperm from the caput epididymis were incubated with HGF, motility of these spermatozoa was increased by 5–15%. Therefore, it is plausible that there is a relationship between expression patterns of these genes and fertility.

Using GWAS, Fang et al. (158) compared sperm DNA methylomes between cattle and humans, finding that genes with conserved hypermethylated promoters (e.g., *Cd80* and *Tcap*) have been shown to be involved in immune responses, whereas genes with conserved non-methylated promoters (e.g., *Anks1a* and *Wnt7a*) participated in embryonic and fetal development. They also found that genes with cattle-specific hypomethylated promoters (e.g., *Dgat2* and *Ldhd*) predominantly engaged in lipid storage and metabolism (158). Using WGBS, Zhou et al. (159) compared methylomes of sperm DNA with those of three somatic tissues in bulls. They detected large differences in the methylation patterns of global CpGs, hypomethylated regions (HMR), partially methylated domains (PMD), common repeats, and pericentromeric satellites between sperm and somatic tissues. Moreover, they observed high methylation in the active gene bodies and low methylation in the promoter regions. Interestingly, meiosis-related genes including *Kif2b* and *Repin1* have been shown to be hypermethylated in somatic cells but hypomethylated in sperm. It has been reported that a broad range of kinesins have important functions in spermatogenesis. Kinesin-13 proteins, mitotic centromere-associated kinesin (MCAK), KIF2A, and KIF2B are involved in spindle bipolarity through induction of depolymerization of microtubules to modulate mitotic dynamics during spermatogenesis (160). In addition, REPIN1 could be regarded as the possible key transcription factors in spermatids (112). Therefore, previous studies support the positive correlation between hypomethylated *Kif2b* and *Repin1* genes with fertility. Therefore, there is a need for further studies on the functional associations between sperm DNA methylation and bull fertility and early development.

Sperm Chromatin Dynamics and Bull Fertility

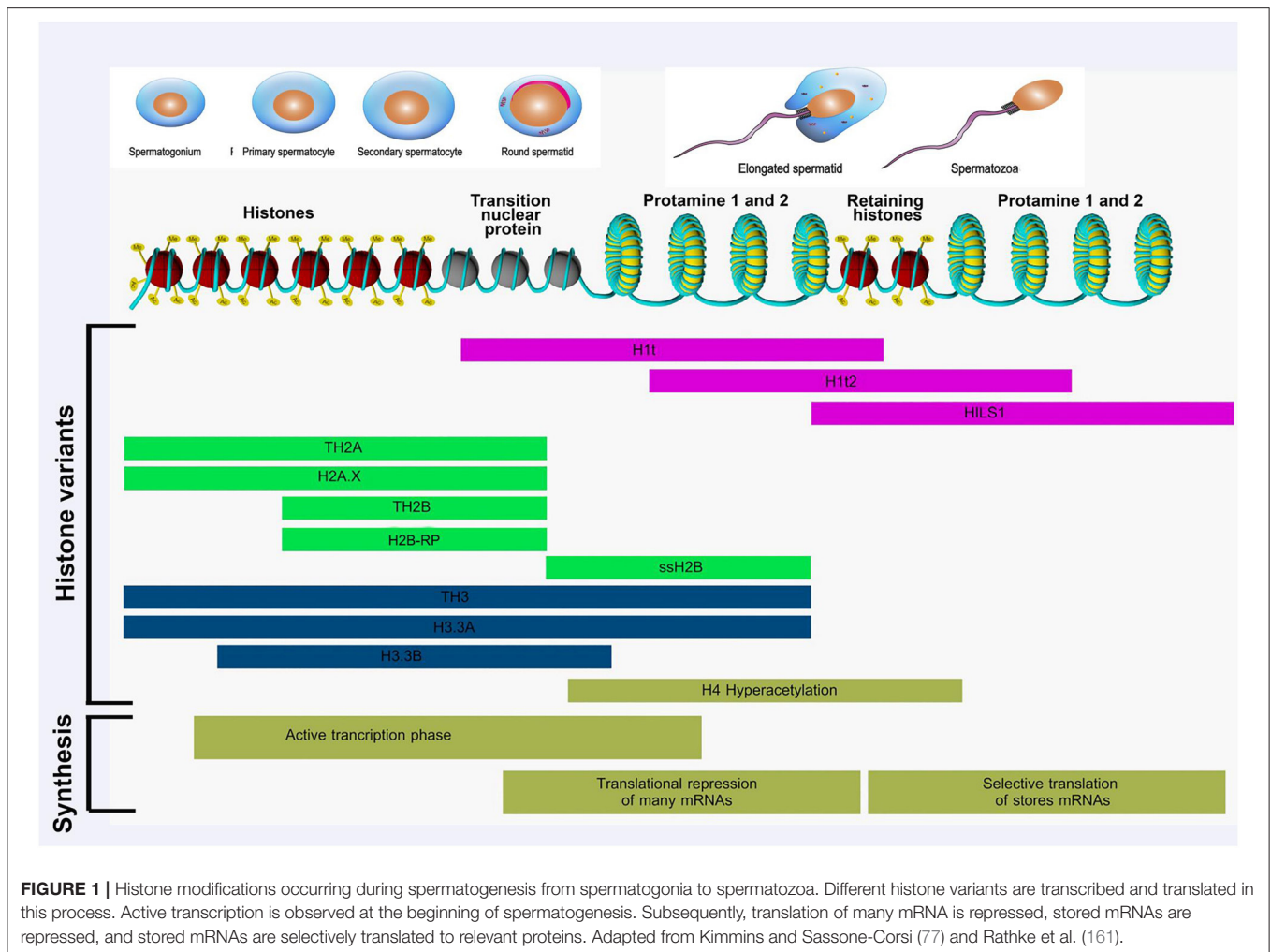
During spermiogenesis, chromatin structure and cellular morphology of round spermatids undergo dramatic reconfigurations, giving rise to an extremely condensed chromatin state and transcriptional quiescence in spermatozoa. During this period, histone hyperacetylation occurs increasingly in round and elongating spermatids, resulting in chromatin destabilization and loosening of chromatin structure to facilitate histone eviction (161, 162). In the early post-meiotic phase, most of the nucleosomal canonical histones are gradually replaced with testis-specific histone variants (noncanonical) (77). The linker histone H1 and H2A, H2B, and H3 have testis-specific histone variants. However, no histone H4 variant is known in mammals (161) (Figure 1).

Oliveira et al. (163) examined the differences in expression of two core histones (H2B and H4) and a histone variant (H3.3) in bull sperm using immunocytochemistry staining and Western

blotting. However, they did not observe any differences in the levels of H2B, H3.3 or H4 in sperm from high-fertile vs. low-fertile bulls. Using immunofluorescence, Western blotting, and flow cytometry, Kutchy et al. (164) determined the associations between expression of the testis-specific histone variant 2B in sperm and bull fertility. Moreover, sperm chromatin damage and abnormal protamination were reported to be associated with reduced fertility in bulls using immunofluorescence, Western blotting, and chromatin dispersion tests (165, 166). In addition, using flow cytometry and immunocytochemistry, methylation and acetylation of sperm histone 3 lysine 27 (H3K27me3 and H3K27ac) were shown to be associated with bull fertility (167). Verma et al. (168) examined tri-methylated H3K27 (H3K27me3)- and di-methylated H3K4 (H3K4me2)-enriched genes in sperm of water buffalo bulls (*Bubalus bubalis*) with different fertility by using a custom ChIP on-chip array. For H3K27me3- and H3K4me2-enriched genes, they detected 80 and 84 genes, respectively. Among the H3K4me2-enriched genes, *Cct5*, *Cdc45*, *Dmc1*, *Meg3*, *Mlh1*, *Prdm14*, *Pax3*, *Sox4*, *Sox14*, and *Tbx15* have crucial roles in spermatogenesis and embryogenesis. While *Cct5*, *Cdc45*, *Dmc1*, *Mlh1*, *Prdm14*, *Pax3*, *Sox4*, *Sox14*, and *Tbx15* genes were in greater amounts in sperm from high fertility bulls, *Meg3* was enriched in sperm from subfertile bulls.

Considering that the H3K4me2 epigenetic modification activates gene transcription, the appearance of some H3K4me2-enriched genes in high-fertility bulls raises contradictions with previous studies. For example, CCT5 has been reported to be expressed in the microtubules of the manchette and centrosomes of spermatids and is discarded at later stages of development in mice (74). Also, CCT5 is highly expressed in sperm from low-fertility bulls (44). This might be due to the presence of seminal plasma in the samples analyzed because discarded CCT5 may be seen in the ejaculate. *Pax3*, *Sox4*, and *Sox14* are genes encoding for transcription factors that participate in supporting embryonic development (169, 170). The DMC1 and CDC45 are involved in meiotic recombination and initiation of chromosomal DNA replication, respectively (171, 172). Sancar (173) reported that MLH1 prevents exonuclease-mediated DNA degradation by repairing mismatched DNA pairs. Furthermore, Ji et al. (174) demonstrated that SNP in *Mlh1* gene gave rise to reduced fertility in humans. PRDM14 functions as a transcriptional regulator during germ cell development (175, 176). It also has a critical role in epigenetic modification by both recruiting DNA demethylases of the TET family and by repressing DNA methyltransferases in primordial germ cells and naïve pluripotent stem cells (177–179). Some studies reported that *Prdm14* knockout in mice gave rise to misregulation of H3K27me3 in primordial germ cells and embryonic stem cells, thereby being involved in histone modification (180, 181).

The product of *Meg3* (*Gtl2*) gene acts as a long non-coding RNA; therefore, it does not encode a protein. Researchers claimed that *Meg3* is involved in p53-mediated transactivation and its suppression of cell proliferation (182). Moreover, abnormal methylation of *Meg3* gene gave rise to deterioration of spermatogenesis (183). Nine of H3K27me3-enriched genes, including *Cdkn2c*, *Fancl*, *Foxa1*, *Gfra1*, *Lhx3*, *Rpl3*, *Six6*, *Sox4*,



and *Sox14*, were speculated to be participating in sperm function and embryonic development. While H3K27me3-enriched *Foxa1* gene was in greater amounts in sperm from subfertility bulls, the others were enriched in sperm from high-fertility bulls (168). Hammoud et al. (183) showed that increase in H3K27me3 in sperm genome gives rise to inactivation of gene promoters in early embryo development. Interestingly, *Sox4* and *Sox14* genes have bivalent chromatin structure marks, both of which bear H3K27me3 and H3K4me2. Bernstein et al. (184) reported that bivalent chromatin structure marks were critical in embryonic development. On the other hand, *Cdkn2c* gene suppression is required for effective modulation of spermatogenesis in mice (185), which agrees with the reports by Verma et al. (168). There is a need for further research aimed at demystifying the functional underpinnings of suppression of these genes associated with H3K27me3 modification and fertility.

CONCLUSIONS AND PROSPECTS

The major advancements in the *-omic* technologies (metabolomics, proteomics, transcriptomics, and genomics) have enabled high-throughput screening of a wide range of molecular

and cellular dynamics in fertility molecules. These approaches also provide means of detecting minute amounts of changes in molecules due to their higher sensitivity. Such attributes of the advanced methods are vitally important for innovative studies to produce new knowledge with transformational and translational values. However, an ejaculate contains many spermatozoa with different phenotypes. Therefore, each spermatozoon should be examined using new high technologies of single-cell analyses such as single-cell metabolomics, proteomics, transcriptomics, and genomics. In addition, these methods should be combined with conventional techniques, such as sperm chromatin structure assay, computer-assisted sperm analyses (CASA), integrity of membranes, flow cytometry, and reactive oxidation stress levels, to determine semen quality using system biology approaches. As an economically important trait, fertility has become more important as there is an urgent need for more efficient, sustainable, and profitable production for food animals to feed the ever-increasing human population in the world. The bull is a unique model for the study of male fertility because of the availability of large amounts of sperm from bulls with reliable fertility phenotypes, and the significant similarities between the bull and other mammals in both sperm biology and genetics.

AUTHOR CONTRIBUTIONS

All authors assisted in the conception of the study, contributed to manuscript revision, read, and approved the submitted version.

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

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