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Key Features of Genomic Imprinting during Mammalian Spermatogenesis: Perspectives for Human assisted Reproductive Therapy: A Review

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Abstract

Increasing influence of epigenetics is obvious in all medical fields including reproductive medicine. Epigenetic alterations of the genome and associated post-translational modifications of DNA binding histones equally impact gamete development and maturation, as well as embryogenesis. Relationships between methylation and acetylation of histones and involvement of DNA methylation are important not only for chromatin remodelling but also significant to gene imprinting and thus affecting the gene expression in the embryo. Therefore, gene silencing accompanied with methylation of histones and DNA is a result of heterochromatin establishment in haploid male germ cell, the spermatid. Complex epigenetic changes leading to the establishment of histone code and heterochromatin are regulated by a broad range of factors, such as histone deacetylases, histone methyltransferases, non-coding RNAs, and small protein modifiers ubiquitin and SUMO. These factors are candidate diagnostic targets for reproductive medicine when anomalous gene imprinting or histone modification of the gametes may disrupt embryo development or cause developmental disorders in the offspring. Using advanced non-invasive techniques for sperm selection based on testing of epigenetic markers is a possible approach to more successful assisted reproductive therapy (ART) as well as prevention of epigenetic-origin disorders in ART babies.

Keywords: Sperm; Epigenetics; Histone methylation; Gene imprinting

Introduction

Spermatogenesis is the process of proliferation and differentiation of spermatogonial stem cells into adult spermatozoa within seminiferous tubules. In the first, diploid phase, spermatogonial stem cells multiply by mitosis and subsequently undergo meiotic division to reach the haploid phase, thus becoming spermatids. During this second phase, called spermiogenesis, spermatids differentiate to spermatozoa capable of acquiring potential for motility and fertilization [1].

Spermatogenesis encompasses distinct epigenetic events that are characterised as heritable changes in gene expression without changes of nucleotide sequence, but with an influence on cell phenotype [2]. Epigenetic events of spermatogenesis include DNA imprinting and chromatin remodelling leading to its condensation. These changes are defined by post-translational modifications of histone proteins, such as the acetylation, methylation, phosphorylation and ubiquitination, and gradual replacement of histones in the nucleosome by protamines [3-7].

Protamines are basic proteins that wrap around DNA more stringently than histones and create compact toroidal structure, which protects DNA. Protamines are also subject to post-translational modification such as phosphorylation of serine, threonine and tyrosine residues, the purpose of which is poorly understood. Another post-translational modification of protamines is the formation of disulphide bonds that prevent dislocation from DNA [8-10]. Post-translational modifications either stimulate or repress developmentally regulated gene expression and are crucial for male fertility and paternally influenced aspects of embryo development. An incorrect replacement of histones, mutations in genes that encode for enzymes necessary for DNA methylation, and protein methylation and/or acetylation disrupt spermatogenesis and spermiogenesis [11,12]. Apart from these processes, noncoding RNAs also have an indispensable role in spermatogenesis [6,7].

Histone-protamine replacement is a gradual process confined to distinct areas of the spermatid genome. However, about 15% of original spermatocyte histones remain associated with the sperm DNA [13]. Originally considered a carryover from incomplete histone-protamine exchange, these retained histones now appear to have an essential regulatory role, conveyed by their post-translational modifications, such as acetylation and methylation. In the next step of histone-protamine exchange, hyperacetylated testicular histones allow for DNA relaxation in the nucleosome, and are gradually replaced. First, testis-specific histone variants (H2B and TH2B) are incorporated into spermatid chromatin by transition protein 1 and 2 (TNP1, TNP2) which are then supplanted by protamines 1 and 2 (PRM1, PRM2) [5,11]. Initially, protamines are phosphorylated by serine/arginine protein specific kinase 1 (SRPK1) and calcium/calmodulin-dependent protein kinase 4 (CAMK4), targeting PRM1 and PRM2, respectively. Rapid dephosphorylation follows, allowing for the formation of disulphide bonds between the unmasked cysteine residues of dephosphorylated protamines [14,15]. These events take place during spermatid elongation [5,11].

The PRM1:PRM1 ratio of the mammalian sperm genome is close to 1:1 [16,17]. The shift of this ratio has been associated with male reproductive disorders [11,18]. After protamine-rich chromatin establishment, spermatid nucleus becomes more compact. Such a hypercondensed state of sperm chromatin conveys hydrodynamic sperm phenotype and protects sperm DNA from damage during sperm transport in both the male and female reproductive tracts [12].

Understanding sperm epigenetics will be beneficial for human assisted reproductive therapy (ART), wherein fertilization protocols do not approximate *in vivo* conditions. Currently, we do not know the crucial sperm epigenetic factors, and broad experiments elucidating their role in fertilization process and pre-implantation embryonic development are needed.

Histone Acetylation: Residual Sperm Histones Carry Developmentally Relevant Information

Acetylation of protein is characterised by transfer of an acetyl moiety from acetyl CoA to a free amino-group of the target protein. Histone acetylation is catalyzed by specific enzymes, namely histone acetyltransferases (HATs) and histone deacetylases (HDACs). Based on the binding site for acetyl group, there are two types of acetylation: lysine acetylation and N-terminal acetylation [19-21].

Sperm histone H3 is acetylated on lysine (K) residues K9, K18 and K23. The level of H3 acetylation changes throughout spermatogenesis, influencing both the proliferation and differentiation of male germ cells. Hyperacetylation of H3K9, H3K18 and H3K23 peaks in spermatogonia, and later tapers off to recur in elongating spermatids and dissipate in fully differentiated spermatozoa [22,23]. Contrary to H4 acetylation, the role of H3 acetylation is not known, and it awaits further examination [24].

The main reason for histone acetylation in spermatids is to facilitate chromatin remodelling, leading to genome-wide cessation of gene expression in the spermatid nuclei. Histone acetylation levels change during spermatogenesis. As a result, spermatozoa display acetylation of histone H4 on lysine residues K5, K8, K12 and K16. Hyperacetylation of histone H4 yields sites for binding of bromodomain testis associated proteins (BRDT) of the BET subfamily, including BRDT, BRD2, BRDT3 and BRDT4 [25-27]. During spermiogenesis, the BRDT proteins participate in chromatin remodelling required for successful spermatid differentiation [23,24]. Mice homozygous for BrdtΔBD1/ΔBD1 mutation lacked BRDT from their spermatids and were sterile due to a defect in chromatin remodelling causing chromocenter fragmentation in spermatid nuclei during spermiogenesis [28,29]. With regard to other bromodomain proteins, there is little information about their function in spermatogenesis. However, there is a proven link between BRD2, BRD4 and mouse embryo lethality [30-32]. It is known that BRD2 has an impact on the development of the neural system, and its presence was also shown in oocytes and early embryos. The activity of BRD4 influences embryo development: the mutation of this gene has a negative effect on an embryo, and it is associated with epilepsy and neural developmental defects [31,33]. Physiological roles of BRDT proteins have mainly been studied in mice and rats [30-32]. Altogether, BRDT-driven changes on the genomic or epigenomic level impact fertilization, embryo implantation and development to term in rodents, supporting an assumption that similar effects are conveyed by them in human [34].

Berkovits with collective proposed that in normal healthy spermatids, the BRDT protein keeps SIRT1 (sirtuin-1, NAD+-dependent HDAC) out of the chromocenter, the pericentromeric heterochromatin-based structure. Ablation of the murine *Sirt1* gene did not affect post-meiotic male germ cells, but pre-meiotic *Sirt1* inactivation delayed pre-meiotic germ cell differentiation, conveyed abnormal shape of spermatids with higher levels of DNA fragmentation and an overall smaller testis size in the mutant males [29]. Also noticeable was abnormal histones-protamine replacement and defects of chromatin condensation in spermatids [1,29].

Compared to wild type, disruption of Sirt1 gene results in smaller (in diameter), abnormally shaped seminiferous tubules, a reduced number of fully differentiated spermatozoa, a higher level of sperm DNA damage and compromised genome integrity. Consequently, mutant spermatozoa have a reduced ability to produce viable zygotes and offspring by *in vitro* fertilization (IVF) and embryo transfer, most likely due to high incidence of implantation failure [35].

Histone Methylation as a Regulator of Spermatogenesis

Histone acetylation is counterbalanced by methylation events that in general have an opposite effect on chromatin structure and gene expression. The DNA methyltransferases (DNMTs) perform both *de novo* methylation and maintenance methylation [36,37]. Cellular content of DNMTS reflects the level of methylation in genome. Apart from histone methylation, genomic DNA methylation also takes place [37,38].

Methylation activates gene expression, which can then be repressed by demethylation; it is defined as the binding of the methyl unit from the S-adenosyl-L-methionine to the 5th position of cytosine residues in nucleotides using enzymes methyltransferases [39]. Methylation of DNA is mostly observed in cytosine-phosphate-guanine dinucleotides (CpGs) [40]. Key chromatin/histone methylation events occur on histones H3 and H4. Mono, di- and tri- methylated histones have been detected during spermatogenesis [41,42].

To date, methylation of lysine residues K4, K9, K27, K36 and K79 of histone H3 and lysine K20 of histone H4 have been described [43]. While methylations of H3K4, H3K36 and H3K79 are typical for euchromatin, methylations of H3K9, H3K27 and H4K20 are more common for transcriptionally silent heterochromatin associated regions of the genome [44]. Changing levels of histone methylation during spermatogenesis suggest its impact on germ cell differentiation. While H3K4me1, H3K4me3 and H3K27me2/3 methylations were increased in spermatogonia and round spermatids, but not in elongating spermatids, methylation of H4K20 was detected throughout spermatogenesis [22,45]. However, during differentiation of spermatogonia, the level of H4K20me3 was reduced and H4K20me1 was increased, underscoring the influence of histone methylation on differentiation of spermatogonia to spermatids [45,46]. Methylation of histone H4 is connected with the process of chromatin remodeling, mediated by the replacement of histones with protamines. At the corresponding steps of spermatid elongation, the level of H4K20 methylation is reduced and H4 acetylation is increased, collectively allowing for histone replacement [47].

The equilibrium of histone methylation during spermatogenesis is maintained by demethylase enzymes. Overexpression of H3K4 demethylase KDM1A reduced H3K4me2 in spermatozoa and caused transgenerational developmental defect of offspring [48]. Conversely, genetic ablation of H3K9 demethylase JMJD1A caused germ cells apoptosis and anomalous spermatid elongation [49,50]. From observations about effect of histone methylation, gene exposure to

translation or gene silencing for methylation of H3K4 and K9 we can assume the necessity of chromatin stability.

Histone Ubiquitination and Sumoylation: Epigenetic Factors or Mediators of Histone Degradation?

Protein ubiquitination is a stable, yet reversible post-translational modification by the covalent binding of the small chaperone protein ubiquitin to lysine residues of substrate proteins. This process is ATP-dependent and catalyzed by three different classes of enzymes, the ubiquitin-activating enzyme E1 (UBA1), ubiquitin-conjugating enzymes (UBE2) and ubiquitin ligases (UBE3) [51]. Ubiquitination during spermatogenesis serves the substrate specific, developmentally regulated degradation of various proteins by the ubiquitin proteasome system (UPS). Apart from protein turnover, ubiquitination participates in the regulation of transcription, protein transport and cell signaling, among others [52]. Within the male reproductive system, ubiquitination has been implicated both in spermatogenesis and in epididymal sperm maturation as quality control to eliminate defective spermatozoa and dead epididymal epithelial cells [53-55]. This proposed role is supported by the detection of high amounts of ubiquitin in epididymal epithelia and luminal fluid. Ubiquitin as well as the enzyme of ubiquitin-substrate conjugation machinery are secreted by epididymal epithelium to eliminate defective spermatozoa by subsequent phagocytosis. Nevertheless, some of the defective spermatozoa tagged by extracellular/cell surface ubiquitination are carried over into ejaculate [56,57].

Whereas polyubiquitination plays a central role in the protein turnover by ubiquitin proteasome system, monoubiquitination influences gene expression [58]. The other role of ubiquitin is probably in meiotic sex chromosome inactivation (MSCI), deduced from high level of enzyme ubiquitin ligase UBR2 in unpaired XY axes as well as ubiquitination of histone H2. Ubiquitination of histone H2 is one of the most common histone ubiquitinations in mammals and has been implicated in transcriptional silencing such as X inactivation. Deficiency in UBE2B is associated with impaired spermatogenesis and male sterility [58-61]. Testis specific E2 enzyme UBC4 (UBC4-testis isoform), that mediates the first step of histone degradation and replacement by protamines during spermatid elongation [62,63], has been assigned an important role in spermatid histone ubiquitination.

Furthermore, protein ubiquitination is related to sumoylation that involves the attachment of small ubiquitin-like modifier (SUMO) to lysine residues of substrate proteins [64,65]. There are four SUMO isoforms: SUMO1, SUMO2, SUMO3 and SUMO4. Since SUMO2 and SUMO3 have nearly identical amino acid sequences, a collective name SUMO2/3 applies [66-68]. The SUMO1 has been localized to the nucleus and midpiece of human spermatozoa [69].

Sumoylation has been implicated in chromatin inactivation and transcriptional repression, supported by the presence of SUMO1 in XY bodies of pachytene spermatocytes, and near the centromere [70,71]. It is suspected that sumoylation participates in MSCI. Vigodner [72] evaluated early presence of SUMO1 on sex chromosomes during MSCI which was followed by γH2AX accumulation [72]. SUMO1 is important in the regulation of meiotic division of spermatocytes, where it binds to synaptonemal proteins SCP1 and SCP2 that form the synaptonemal complex as a prerequisite for recombination [73]. Accumulation of SUMO1 in human spermatozoa coincides with reduced motility and abnormal sperm morphology. Specific targets of sumoylation in male germ cells include the dynamin-related protein 1 (DRP1) in the sperm tail mid-piece, and Ran GTPase-activating protein 1 (RANGAP1) and DNA Topoisomerase IIa (TOP2A) in the postacrosomal region of the sperm head. Expression of SUMO1 and its binding to the aforementioned substrate proteins was significantly higher in males with reduced sperm motility and abnormal sperm morphology. In addition, a positive correlation was found between sperm DNA fragmentation and SUMO1 [74].

Furthermore, SUMO1 is important for organization of constitutive chromatin because the regions rich in SUMO1 also carry the hallmark heterochromatin proteins HP1α/CBX5, trimethylated H3K9 and trimethylated H4K20. On the other hand, sumoylation may block histone H4 methylation, an important step for subsequent H4 acetylation and histone-protamine replacement [75].

Aforementioned post-translational modifications represent essential epigenetics factors with key roles during gene imprinting, fertilization and embryonic development. These modifications are considered to be in active cross-talk with another epigenetic phenomenon-non coding RNAs [76,77], important for sperm-driven signalling in the fertilized oocyte/zygote.

Non-coding RNAs - Big Device for Small Spermatozoon

Noncoding RNAs are not translated to protein but have important regulatory function in cellular gene expression. Their role in spermatogenesis [78] and fertilization [79] has already been established. The basic classification of non-coding RNAs defines long noncoding RNAs (lnRNAs) and small noncoding RNAs (snRNAs). The small noncoding RNA group includes small interfering RNAs (siRNAs), microRNAs (miRNAs/MiR) and PIWI-interacting RNAs (piRNAs) [80-82]. The piRNAs differ from the rest in their biogenesis but share similar regulatory functions [7].

The miRNAs are small RNAs of 20-24 nucleotides that are complementary to their target RNAs. Synthesis of miRNA starts in the nucleus and finishes in the cytoplasm; it is catalyzed by enzymes of the RNA interference machinery, including DROSHA and DICER. The function of miRNAs is to inhibit translation of mRNA to protein and thus silence mRNA manifestation [83,84]. During spermatogenesis, differential expression patterns and wide repertoires of miRNAs were observed. Liu with collective identified 559 miRNAs at distinct stages of spermatogenesis [85]. Spermatozoal miRNAs may also control expression of genes in early embryonic development after fertilization [86].

Likewise, siRNAs have an important role in gene silencing. The length of these molecules is also 20-24 nucleotides. Similar to miRNAs, biosynthesis of siRNA requires endoribonuclease DICER. Together, miRNAs with siRNAs participate in the RNA-induced silencing complex (RISC) that is the main cellular tool for silencing of gene expression in spermatogenesis [87]. The siRNAs are often used as an experimental tool for knocking down gene expression and to deduce gene function [88,89].

Piwi RNAs specifically interact with proteins from PIWI family, such as MIWI, MIWI2 and MILI [90]. In comparison to siRNAs and miRNAs, piRNAs have 26-31 nucleotides and are independent of DICER. The role of piRNAs is in silencing transposome elements in germ line during gametogenesis [91]. When mutated, the MIWI2 protein holds spermatogonia in the leptotene stage of meiosis [92]. MIWI knock out blocks spermatid differentiation, causing male

infertility [93]. Insufficiency of MILI and MIWI proteins is connected todefects of methylation that influence expression of retrotransposons in fetal male germ cells [94].

Long noncoding RNAs (lncRNAs) are longer than 200 bp and have an indispensable role in the regulation of spermatogenesis [95]; they inhibit binding of transcription factors to specific DNA sites and enhance DNA methylation [96,97]. The long noncoding RNAs can also be a resource for DROSHA and DICER to produce small noncoding RNA [98].

In addition to spermatogenesis, ncRNAs are essential for oocyte-cumulus complexes as well. Cumulus expansion is a hallmark of oocyte maturation [99,100]. During oogenesis, miR124 targets pentraxin 3 gene (Ptx3) that is important for cumulus expansion, and its silencing disrupts oogenesis [101]. In addition to this interaction, miR-378-aromatase [102] and miR-207-BDNF [103] interactions have been described, both significantly related to cumulus expansion and oocyte maturation. Contrary to miRNAs, the interactions and complex effects of ncRNAs, either sperm or oocyte derived, during fertilization and early embryogenesis remain unclear. Possible cross-talk between ncRNAs and other epigenetic regulatory mechanisms, such as DNA methylation and histone modifications remains to be characterized [79,104].

The Role of Sperm Histone Code in Fertilization and Early Embryogenesis

Immediately after fertilization, maternal and paternal pronuclei develop in the zygote. Rapid demethylation of DNA and histones is essential for the formation of a dedifferentiated embryo. Although rapid active DNA demethylation of paternal chromatin followed by paternal pronucleus development is observed [105,106], specific loci still remain methylated [107]. These epigenetic marks are necessary for further embryonic development. Aforementioned, locus-specific epigenetic modifications are closely tied with gene imprinting (Figure 1). Accurate parent-of-origin gene dosage is crucial for successful embryo development. Therefore, monoallelic gene activity, affecting about 150 genes, is observed during mouse embryonic development, respective of maternal or paternal allele-specific gene expression [108]. Both DNA methylation and the histone code established by posttranslational modifications regulate gene promoters and are required during gametogenesis, resulting in gene imprinting in the embryo (Figure 1) [109]. Accordingly, histone methylation, such as H3K9me3 and H3K20me2, is often associated with methylated DNA in the embryo and placenta [110]. Collectively, such parent-of-origin-specific epigenetic changes promote differential expression of select genes in the embryo, particularly those encoding for transcription and growth factors [111]. Although relatively few genes are paternally imprinted, the necessity of sperm-derived gene imprinting is obvious [107].

While genes are imprinted in a sex-specific manner, the regulatory mechanisms of gene imprinting seem to be shared by spermatogenesis and oogenesis [112]. Sperm gene imprinting depends on posttranslational modifications of residual sperm histones, such as H3K20me3 and H4K20me3 (Figure 1) [43,107]. Acquisition of DNA methylation by male germ cell stem cells, the pro-spermatogonia occurs during foetal development, e.g. on day 14.5-18.5 in the mouse; it is completed postnatally, in pachytene spermatocytes [113,114]. These epigenetic changes regulate a limited number of paternal loci, e.g. those affecting *Gtl2* and *Rasgrf1* genes [115,116]. The DNA methyltransferases (DNMT3A, DNMT3L) and histone methyltransferases are involved in male germline gene imprinting [117-120]. In spite of rapid paternal DNA demethylation after fertilization [106], paternally imprinted genes, such as *H19, Gtl2* and *Rasgrf1* are protected against demethylation of their promoters [110,121]. However, the molecular mechanisms of DNA methylation- and histone code-driven gene imprinting remain poorly understood.

Imprinted genes are often grouped in clusters and these are controlled by imprinting control regions. Within those clusters lie the differentially methylated regions (DMRs) [40,110,122]. The best known DMR is *H19-Igf2* in human locus 11p15, associated with imprinting disorders, that contains gene encoding Insulin-like growth factor 2 (IGF2) and H19 [123]. While the paternal H19 allele is methylated and the maternal one transcribed into a noncoding RNA, expression of *Igf2* gene is exclusively from paternal allele [124-126]. Apart from DNA methylation and histone modifications, regulation by noncoding RNA is also important for proper gene imprinting. Alterations of ncRNA profiles are connected with epigenetic developmental disorders such as Goiter, Kabuki, and Claes-Jensen X-linked mental retardation syndrome [127-129]. In general, abnormal imprinting has been associated with Angelman, Beckwith-Wiedemann, Prader-Willi and Silver-Russell syndromes [130-132].

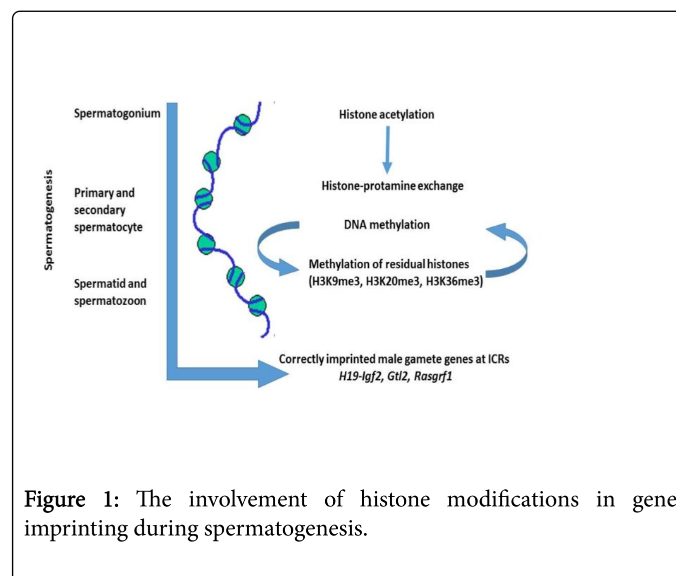

Figure 1: The involvement of histone modifications in gene imprinting during spermatogenesis.

Histone code is stable during proliferative (spermatogonium) and meiotic (primary and secondary spermatocyte) phases of spermatogenesis. Post-meiotically, histone acetylation promotes histone-protamine exchange, although some residual histones remain associated with spermatid DNA. At this time, post-translational modifications of core histones, particularly histone H3 on their lysine (K) residua, play an essential role in epigenetic imprinting of a small number of paternally-silenced loci at imprinting control regions (ICRs). Although DNA methylation within the imprinted genes (H19-Igf2, Gtl2, Rasgrf1) is well documented, the association of post-translationally modified histones with imprinted loci remains unclear. In addition to methylation and acetylation, other modifications are potentially capable of regulating gene expression. The code of residual histones seems to be more precise and more specific than DNA methylation. The failure of gene imprinting leads to severe developmental disorders, birth defects such as the Angelman, Beckwith-Wiedemann, Prader-Willi, and Silver-Russell syndromes.

Therefore, epigenetic changes during spermatogenesis represent a crucial process aimed at preventing epigenetic diseases in offspring.

Conclusion and Perspectives

By various estimates, 15-19% of reproductive age couples experience difficulties conceiving, with male factor infertility contributing to approximately one half of those cases. The most common option for infertile couples is using assisted reproductive therapy. Based on recent knowledge, the epigenetics seem to be impactful phenomenon for fertilization and early embryonic development. In addition to these processes, gametogenesis is affected by epigenetic changes as well. However, less is known about the consequences of gamete epigenetics for the success of embryonic development. Spermatogenesis is an attractive model system to study the epigenome because a large sperm output is continuously produced and various epigenetic changes occur throughout this process. Without a doubt, a number of epigenetic factors with crucial effect on sperm quality and male fertility are awaiting discovery.

Correct sperm chromatin structure gradually achieved during spermatogenesis is required for male fertility [133-135]. Accordingly, more detailed insights into spermatid DNA structure, histone modifications, sperm protamination and histone-protamine exchange have uncovered unique epigenetic mechanisms leading to the acquisition of sperm fertilization ability and sperm contribution to the regulation of embryonic development [11,18,48,110]. Indeed, spermatogenesis represents sensitive process of epigenetic regulation of sperm chromatin packaging as well as correct gene imprinting. Systematic study of epigenetics promises to solve some problems associated with clinical infertility and prevention of genetically determined syndromes.

Based on the body of knowledge reviewed above, identification of relevant biomarkers of sperm quality and male fertility will help clinicians differentiate between healthy and unhealthy spermatozoa and chart the treatment course for ART couples. Some biomarkers have already been validated in retrospective clinical studies, such as ubiquitin, post-acrosomal WW-domain binding protein (PAWP) and others, mainly by using flow cytometry [136].

Intense study and testing of markers of epigenetic defects using relevant biological models as well as clinical routine identify suitable approaches for epigenetics-based sperm analysis and selection. Some of recently utilized methods, such as SUTI assay [57,137] and ejaculate nanopurification [138] would be helpful for modification and epigenetic markers utilization. This approach can lead to the elimination of epigenome determined diseases, such as syndromes based on failure of gene imprinting. On the other hand, a more precise understanding of the relationship between sperm epigenome and the phenotype of both spermatozoon and embryo is needed. Although more research is necessary, the advanced sperm screening and selection methods will eventually be translated into assisted reproductive therapy.

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