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DNA METHYLATION AND MALE INFERTILITY

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1. ABSTRACT

Male infertility is one of the biggest concerns of today's health care community. In the US and other developed countries, approximately 70% of infertility among couples is attributed to male reproductive failure. Alterations in reproductive organ development and sperm production have been listed as the major causes of this phenomenon. Sex determination and differentiation, X chromosome inactivation, gene imprinting and normal germ cell development are important biological processes that, in turn, control mammalian reproduction. Specific patterns of gene expression and repression are important in such processes. The strong correlation between DNA methylation, a major epigenetic modification of the genome, and gene expression patterns is well documented. The effects of DNA methylation on the expression of genes affecting male reproductive organ development, spermatogenesis, and male sexual behavior have been reported, suggesting that alterations in DNA methylation could induce abnormal male sexual development and reproductive performance. Inheritance of epigenetic processes and changes in DNA methylation patterns induced by certain diets have been demonstrated in recent years. However, the effects of DNA methylation on male fertility have not been well studied. Since inherited altered DNA methylation patterns could be a cause of increased susceptibility to xenobiotics or abnormal phenotype in future generations, multigenerational studies oriented to determine the effects of xenobiotics affecting DNA methylation in male fertility are recommended.

2. INTRODUCTION

DNA methylation is a major epigenetic modification of the genome (1). It is a heritable (2-4) enzymatic process (5-9) capable of being modified by dietary manipulations (10,11). In mammals, methylation of the 5' position of cytosine residues is a covalent modification of DNA in the sequence 5'-CpG-3' (12). In most eukaryotic DNA, 60 to 90% of methylcytosine residues occur at CpG dinucleotide sequences (13-17). DNA methylation occurs primarily in areas where CpG density is low. Most CpG islands are completely unmethylated, at least in young people (18,19). CpG islands often function as strong gene promoters and have also been proposed to function as replication origins (20). The strong correlation between DNA methylation and gene expression, silencing some genes and activating others, is well documented (1). Many very important biological processes regulating sex differentiation such as genomic imprinting (21), X chromosome inactivation (22) and embryonic development (21) have been associated with DNA methylation. Although the mechanisms that establish methylation patterns during development are not completely understood (5), programmed DNA methylation and demethylation regulates the expression of genes during mammalian development (23).

Male infertility is one of today's biggest global concerns (24). In the US and other developed countries, 15% of reproductive age couples are unable to conceive (25). Infertility of the male contributes up to 70% of all couples unable to conceive spontaneously (26). Many causes of male infertility including ejaculatory dysfunction, varicocele, obstructions, and infections have been substantiated (27,28).

Although a considerable number of adequately expressed genes have essential roles in human reproductive

function, altered expression of just one of them can induce deleterious effects in male reproductive capacity (29). The role of mechanisms controlling gene expression, such as DNA methylation, has not been investigated in this context. Because the hypothalamic-pituitary-gonad axis controls reproduction in mammals, in addition to the effects of DNA methylation in spermatogenesis and male reproductive organ development, other components of this axis are briefly discussed in this review.

Since many genes expressed at the appropriate time and levels are required for male reproductive organ development, correct sperm production, and reproductive performance, more studies addressing the effects of mechanisms of gene expression, such as DNA methylation and chromatin modification, in these biological processes are necessary. In addition, since diet-induced DNA methylation changes and DNA methylation inheritance (suggesting abnormal susceptibility and phenotype in future generations) have been reported, multigenerational studies are recommended (30).

3. DNA METHYLATION

In mammals, DNA methylation occurs after DNA replication and involves the transfer of a methyl group from S-adenosyl-methyonine (SAM) to the 5' position of cytosine residues, in a reaction catalyzed by the enzyme DNA methyltransferase (Dnmt) (31).

A number of key biological functions have been associated with DNA methylation. Genome organization, chromosome stability (32-34), gene expression (1), cell differentiation (35), genomic imprinting (21), X chromosome inactivation (22) and chromatin modification (36) were some of the first to be associated with DNA methylation. Earlier, specific pathologies and degenerative processes including cancer (32,37) and aging (38,39) were also associated with methylation. Finally, the role of DNA methylation in embryonic development has been substantiated (21,36,40-45).

3.1. Enzymes associated with DNA methylation

Three enzyme families associated with DNA methylation have been identified. Each of them plays different roles in establishing and maintaining specific methylation patterns. DNA methyltransferase 1 (Dnmt1), first cloned in 1988 (46), primarily mediates maintenance methyltransferase activity during the S phase (9).

On the basis of their preference for hemimethylated or unmethylated substrates, several forms of Dnmt1 have been identified, each differing in their translation start site (47). The existence of other DNA methyltransferases in mammalian cells was suggested by the ability of Dnmt1 knockout embryonic stem cells to *de novo* methylate viral DNA (48).

A search of expressed sequence tags (EST) databases revealed a second Dnmt (Dnmt2) (49,50). Although the biological role of any of the Dnmt2 family members remains to be demonstrated, a role of Dnmt2 in some aspects of centromere function has been suggested (14).

Dnmt3a and Dnmt3b were discovered by EST database screening using full length bacterial type II cytosine-5 methyltransferase sequences as queries (51). The essential requirement of Dnmt3a or Dnmt3b for *de novo* methylation *in vivo* and their importance for survival has been confirmed by inactivation of both genes (36). Methylation of a recombinant retrovirus transfected into embryo-derived stem (ES) cells required either Dnmt3a or Dnmt3b, but not both, suggesting redundancy of *de novo* methylation function of Dnmt3a and Dnmt3b; however, their unique patterns of tissue expression in the embryo suggest that they have separated functions (18). No evidence for the role of *de novo* methylation in normal regulation of gene expression apart from embryonic tissue has been reported (18). However, abnormal DNA and gene methylation and gene expression have been described during carcinogenesis (52-55).

3.2. DNA demethylation

Although the DNA methylation process is well understood, the mechanisms of DNA demethylation remain to be elucidated. Two types of demethylation process have been postulated: 1) Genome-wide demethylation, which occurs during gametogenesis, early embryonic development, and in some differentiating cells (56,57). 2) Site-specific demethylation, which in many vertebrates affects tissue specific genes at the time and site where they are expressed. It may occur in absence of CpG islands methylation (58).

Concomitantly, two possible mechanisms have been suggested:

1) Since proteins with demethylase activity have been isolated (59,60), a global process acting on the cellular pool of enzymes involved in controlling DNA methylation, followed by either activation of demethylating enzymes or inactivation of Dnmts, is suggested (61). Two enzymatic processes of genomic DNA demethylation have been suggested. First, via passive demethylation, DNA demethylation could simply result from an absence of maintenance methylation after replication (62). It is thought to be the primary mechanism of demethylation mediated by cytosine analogs such as 5-AZA-2-Deoxycytidine (5-AZA CdR). Certain DNA-binding factors may block cytosine methylation that normally occurs during S phase (63). Second, active demethylation, DNA demethylation occurs independently of DNA synthesis involving demethylases. The classical example could be DNA repair.

2) Local demethylation targeting specific sequences could either develop specific mechanisms adopted to a precise action, or use the activities involved in genome-wide demethylation (17).

3.3. DNA methylation and gene expression

CpG islands often function as strong promoters and have also been proposed to function as replication origins (20). It is estimated that CpG islands are associated

with about half of all mammalian genes (64). Unmethylated CpG islands are associated with housekeeping genes, while the CpG islands of many tissue-specific genes are methylated, except in the tissue where they are expressed (65).

Interactions between proteins and DNA are changed by methylation, leading to alterations in chromatin structure and a transcription rate change (66). In absence of DNA methylation, DNA methyltranferases (DNMTs) can function as transcriptional suppressors in collaboration with other proteins such as histone deacetylases (HDACs) (13). Therefore, the suggested DNA methylation mechanisms for gene regulation can be summarized as: 1) preventing the binding of transcription factors to their target sequences via proteins that bind preferentially to methylated promoters (67), 2) interfering with the binding of transcription factors to the methylated cytosine (68), and 3) altering chromatin structure leading to a change in the rate of transcription (66).

3.4. DNA methylation, genomic imprinting and X chromosome inactivation

DNA methylation plays a key role in genomic imprinting and X chromosome inactivation (14). In both processes, gene silencing is strongly associated with hypermethylation of CpG islands within the promoter region of silenced genes (18). Genomic imprinting is a process whereby only one of the two parental alleles is expressed, while the other gene is imprinted or silenced by DNA methylation (69). Therefore, epigenetic reprogramming in germ cells and the early embryo is critical for imprinting. In addition, this imprinting has crucial roles in protecting the genome integrity (21), thus, establishing nuclear totipotency (3) and stem cell differentiation in animal development. Imprinted genes are germline derived and inherited from either the maternal or paternal gamete. CpG islands have been observed in about 88% of approximately 45 identified imprinted genes in mice (70).

X chromosome inactivation is a related methylation-dependent phenomenon and consists of the transcriptional silencing of one of the two X chromosomes in mammalian females (18). This process insures equivalent levels of gene expression from the sex chromosomes in males and females (22,71).

3.5. DNA methylation patterns and embryonic development

DNA methylation is an epigenetic mechanism that contributes to the coordination of gene regulation during vertebral development (36,42,67,72-76). It has been proposed that the methylation pattern of the mammalian embryo genome is established during gametogenesis (76) and changes through development (77). It involves genome-wide demethylation and *de novo* methylation, followed by selective demethylation of regulatory elements occurring in parallel with their activation (57,78). During mammalian development, programmed DNA methylation and demethylation regulates the expression of genes (23,78,79) controlling the interaction of regulatory sequences with transcription factors (38).

Mammalian development is accompanied by two major waves of genome-wide demethylation and remethylation: one during germ-cell development and the other after fertilization (32,62,72,77,80-82). During embryogenesis, methylation patterns are reprogrammed genome wide, generating cells with a broad developmental potential. Passive genome-wide demethylation after fertilization (by the lack of maintenance methylation following DNA replication and cell division (62,82)) and replication-independent demethylation during early embryogenesis have been suggested (43).

The mechanisms that establish methylation patterns during development are not completely understood (5). However, it is clear that the complete process in mammals involves demethylation, *de novo* methylation and maintenance methylation in which genome wide reprogramming of methylation patterns takes place (21). It has been suggested that methylation patterns in males are established by *de novo* methylation in prospermatogonia at 14-20 days post-coitum and in females during growth of dictyate oocytes at 5 days post partum (83). In adults, methylation patterns are reproduced at each round of cell division (84) and vary according to cell type and developmental stage (73).

3.6. DNA methylation and diet

Numerous studies suggest diet as a key modulator of DNA (85) and gene (52,54,55) methylation. Such modifications can be carried to the offspring affecting DNA methylation, epigentic variations, phenotype and likely long-term health (86). The exact mechanisms have yet determined. However, the key role of Dnmts has been suggested by the altered Dnmt activity observed in tumors of rats fed methyl-deficient diet (53).

Folic acid, vitamin B12, vitamin B6, methionine, choline and other nutrients influence the function of enzymes that participate in various methylation processes by affecting the supply of methyl groups that are incorporated into a wide variety of molecules. Variations of any of the factors that feed into the methylation pathway could alter DNA methylation efficiency (10,11). Abnormal methylation patterns are commonly found in cancer (87-89). It is believed that diet alone contributes approximately 35% of all cancer deaths in humans (90).

3.7. DNA methylation inheritance

It was believed that acquired epigenetic modifications such as DNA methylation or stable chromatin structures could not be inherited through the germline to future generations of mammals because they were cleared and reset on passage through the germline (91,92). However, in recent years it has been established that in somatic differentiated cells, genomic methylation patterns are generally stable and heritable (2-4,21).

Numerous recent publications indicate that specific manipulations of early embryos, such as nuclear

transplantation, can result in heritable altered patterns of gene expression and induce phenotypic alterations at later stages of development (93-96). Concomitantly, inherited phenotypic changes can be induced by methyl group supplementation (97). Roemer and collaborators reported that DNA methylation and repression of genes encoding major urinary proteins, olfactory marker protein, and reduced body weight can be experimentally induced by nuclear transplantation in early embryos (95). Those acquired phenotypes are transmitted to most of the offspring of manipulated parent mice (4). Recently, Morgan and collaborators described the inheritance of an epigenetic modification at the agouti locus in mice (94). Sutherland and collaborators reported the germline inheritance of transcriptional silencing in mice and reversion to activity after as many as three generations in the silent state (3). Cisneros observed that altered global DNA methylation patterns induced by a single dose of the demethylating agent, 5-AZA-CdR, persisted to the F3 generation of treated mice (30). These studies support the theory that silent genetic information is inherited and later reactivated in mammals implying a mode of phenotypic inheritance less stable than Mendelian inheritance (3).

4. MALE INFERTILITY

Infertility, defined as the incapacity of achieving pregnancy over a considerable period of time (usually a year) in spite of determined attempts by intercourse without contraception (27), is a major health problem today (24). In the US and other developed countries, approximately 15% of couples who attempt to conceive are infertile (25). Male failure causes approximately 70% of couple infertility (26). A male is consider infertile if after 12 months of regular intercourse without contraception he is not able to induce pregnancy (98).

Ejaculatory dysfunction, varicocele, obstructions, and infections have been substantiated as causes of male infertility (27,28). The role of genetics in such phenomena has been elucidated (25,28,99-103). However, for up to 23% of infertile males no specific cause (idiopathic) can be found (104). Such infertile men are usually healthy, suggesting the genes involved might be only expressed in spermatogenesis, or be functionally required only for spermatogenesis and/or reproduction (105).

Multiple factors, including normal spermatogenesis, genital tract integrity, erection and ejaculation potential are important for normal male reproductive function. In addition, sperm transit, capacitation, egg penetration, fertilization and early embryonic development are determinant conditions for male fertility (28). Genetic or epigenetic alterations affecting any of these factors and conditions could alter male reproductive capacity.

Although it is known that a considerable number of genes have an essential function in human reproduction and their alterations can induce pathology in the male reproductive performance (29), the mechanisms controlling gene expression such as DNA methylation have not been fully elucidated. This review details the current information emphasizing the role of DNA methylation in male infertility. Spermatogenesis and the development of the male reproductive system are the focus; however, other aspects of male sexual failure are also considered.

4.1. Spermatogenesis

Adequate production of viable sperm is necessary to ensure a healthy male reproductive life. Many reports have indicated decreasing sperm counts and increasing reproductive problems in wildlife and humans as a consequence of toxicant exposure (106,107).

Spermatogenesis, the biological process in which sperm is produced, involves cell division, cell differentiation and cell-cell interactions (105). Many genes expressed in a specific amount and at the appropriate time are required for such a complex developmental process. It has been suggested that over 150 genes are associated with mammalian spermatogenesis (28,108). If the required expression of any of these genes were altered, the reproductive capacity of males could be compromised. The reproductive incapacity could be caused by a diminished sperm production (oligospermia/asthenospermia) or no sperm production (azoospermia) (98).

In addition to the well known male and female genomic methylation pattern differences (42), DNA methylation patterns in males undergo changes in both mitotic spermatogonia and post-replicative germ cells (109). In the rat, a significant increase in methylation levels of testis DNA occurs from 30 to 150 postnatal days, while at 2 years of age a significantly decrease of methylation levels take place. At cellular level, Pachytene spermatocyte DNA contains significantly higher methylation levels than spermatogonia, while elongated spermatids contains significantly lower methylation levels than pachytene spermatocyte and mature sperm DNA (110).

The role of DNA methylation in male gametogenesis has been substantiated in part because Dnmt1 is highly regulated during spermatogenesis (111,112). Expression of high levels of Dnmt1 messenger RNA and protein in mitotic and early meiotic male germ cells (111,113,114) consistent with its maintenance and possibly *de novo* methylation activities have been reported. Dnmt1 down-regulation in pachytene spermatocytes (111,112) and up-regulation in post-replicative leptotene/zygotene spermatocytes suggest a role for Dnmt1 during meiotic prophase in male germ cell development (112,115).

In the Y chromosome, two gene families that show testis specific expression, RBM (RNA Binding Motif) and DAZ, have been associated with spermatogenesis (116). In a recent study, Dasari and collaborators reported that expression of the Y chromosome specific genes DAZ, SRY, RBMY1A, RBMY1H, RBMII, BPY1, PRY and TSPY is regulated by DNA methylation in prostate cancer (117). Although the role of methylation in these genes in testis or spermatogenesis has not been studied, it is likely that DNA

methylation governs their expression in all tissues and processes where they are present.

Another gene with a high degree of homology to the DAZ gene family, frequently deleted in males with azoospermia or severe oligospermia (118,119), is the DAZLA (DAZ Like Autosomal) gene on human chromosome 3, which has been suggested to be involved in spermatogenesis. Like the DAZ gene family, this gene is a homologue of the Drosophila male infertility gene, boule, and is expressed specifically in testis. Chai and collaborators reported that the 5' end of both DAZLA and DAZ genes are hypomethylated in spermatozoa where they are expressed, but not in leukocytes or placenta where they are repressed (108).

While studying the role of DNA methylation in proliferation and differentiation of germ cells in testis, Raman and Narayan found that DNA methylation plays a critical role in the differentiation of gonia into primary spermatocytes. Administration of 5-AZA-CdR to neonatal mice having only spermatogonial (premeiotic) cells induced considerable loss of methylation, complete inhibition of differentiation into spermatocytic stage, and altered expression of at least 5 polypeptides (120).

A significant decrease of pregnancy rates, and increase of subfertility and time to pregnancy has been associated with aged males (121). Reported studies indicate that sperm motility rather than semen volume or sperm number is most strongly affected by age (122), suggesting altered sperm maturation in aged males. Ariel and collaborators concluded that remethylation is part of the process of sperm maturation that takes place in the epididymis. These authors studied the DNA methylation status of three spermatogenesis-specific genes, Pgk-2, ApoA1 and Oct-3/4, throughout the development and differentiation of male germ cells in the mouse. Although all these genes were unmethylated in adult spermatogenic cells in the testis, they were remethylated in mature spermatozoa in the vas deferens (123). In addition, a preferentially age methylated DNA ribosomal locus region in spermatozoa has been reported (124).

In vivo and *in vitro* age-related altered DNA methylation patterns have been reported (125). Recently, Doerksen and collaborators reported different degrees of susceptibility to 5-azacytidine (5-AZA) (126). Male rats treated with the demethylating agent resulted in abnormal embryo development when germ cells were exposed throughout spermatogenesis, encompassing mitotic, meiotic, and postmeiotic development, but not if they were only exposed postmeiotically. This suggests that the hypomethylating effects of 5-azacytidine were stronger in spermatogonia than in spermatocytes. In addition, histological testicular alterations were dependent on the time of exposure and degree of germ cell DNA demethylation. Altered testicular histology was observed only after 11 weeks of exposure.

It has been suggested that a gene in the Prader-Willi syndrome (PWS) critical region, "chromosome 15 open reading frame 2" (C15orf2), may play a role in primate spermatogenesis. C15orf2 is an intronless gene located between MAGEL2 and SNURF-SNRPN associated with a CpG island, which is hypomethylated in germ cells (127).

The role of DNA methylation in testis-specific H2B (TH2B) histone gene of rat transcriptional repression has been reported. During spermatogenic differentiation, TH2B is expressed in the meiotic events as early as spermatogonia type A and continuing on to sperm. During this time, CpG sites in the promoter region are unmethylated (128).

The spermatogenic capacity of the testis is regulated in part by follicle-stimulating hormone (FSH). The Sertoli cells response to FSH is controlled by the FSH receptor (FSHR) gene expression. CpG dinucleotides in rodent FSHR promoter are methylated in non-expressing cells and tissue but unmethylated in Sertoli cells (129). DNA methylation in testes is differentially regulated during development and is controlled by gonadotropic hormones. Hypomethylation of seminiferous tubular and Leydig cells DNA can be achieved by administration of FSH and Luteinizing hormone (LH), respectively (110).

Hata and collaborators, while investigating the function of Dnmt3L in reproduction observed that the male Dnmt3L -/- mice were completely infertile (5). At birth, mutant mice testis size appeared normal but it was significantly reduced at 8 weeks of age compared to the controls. Very few differentiated spermatids or spermatozoa were found in the testes of mutant adult mice, suggesting that Dnmt3L is probably required for the differentiation of spermatogonia. This was consistent with the expression pattern of Dnmt3L in the testis. This observation lead the authors to conclude that Dnmt3a and Dnmt3L are required for spermatogenesis.

Recently, the role of FKbp6 gene in male fertility has been reported. In the mouse, expression of Fkbp6 mRNA was restricted to the testes. Both mRNA and protein were found in the cytoplasm and nucleus of spermatocytes. While male Fkbp6 deficient mice were sterile, females were not. Reduced testis size with lack of spermatids was observed in Fkbp6-/- males. Mature spermatozoa were not observed in the caudal epididymis or seminiferous tubules. An increase in apoptosis in spermatocytes of mutant mice was evident via terminal deoxynucleotidyl transferase-mediated deoxuridine triphosphate nick-end labeling (TUNEL) stain. This suggested that a complete block in spermatogenesis and cell death of meiotic spermatocytes is the cause of the infertility observed in Fkbp6-deficient male mice (130). The mechanisms controlling the expression of this particular gene have not been reported; however, since this gene induced apoptosis and apoptotic genes have been correlated with DNA methylation (41,131), the potential role of DNA methylation must be considered.

4.2. Genital tract development

A coordinated expression of specific genes in a strict spatiotemporal manner is required for mammalian

gonadal development and sexual differentiation (132). Several of these genes are more crucial than others, but all play very important roles in maintaining these developmental pathways (133,134). Sexual development is a very complex process including gonadal determination and differentiation. After gonadal sex determination, an indifferent gonadal primordium becomes ovary or testis. In males, sex determination has been associated with testis determination. Several hormones such as antimullerian hormone (AMH) and sex steroids are imperative to develop the male phenotype. Therefore, altered hormone secretion induced by abnormal testicular development might result in hermaphrodites or even individuals presenting a complete female phenotype (135).

4.2.1. SRY gene

The sex determining region of the Y Chromosome (SRY), the gene that triggers the testis determining cascade (136), has been suggested to function as a transcriptional regulator (137). Complete and partial gonadal dysgenesis (GD) of 46 XY individuals has been associated with mutation in SRY or in its flanking regions (138). Complete and partial GD as a consequence of SRY 5' and 3' microdelitions, which might modify its expression, have been observe in cases of 46XY individuals (139). Although individuals with a mutation in SRY are infertile, suggesting that all mutations are *de novo*, several cases of familial mutations associated with 46 XY complete GD have been reported (140).

The existence of a threshold for the appropriate function of murine Sry (141,142) and human SRY (143,144) has been proposed since reduced levels may produce sex reversal. Therefore, a disruption of the timing and/or amount of SRY expression may result in an abnormal sex phenotype (135). Veitia and collaborators after analyzing the main components of testis development pathways conclude that almost all steps are dosage sensitive (135).

While in the somatic cells of the genitalial ridge (precursors of Sertoli cells) of mice, Sry transcripts appear between 10.5 and 12.5 dpc (days post coitus) peaking at 11.5 dpc (145). In man, expression of SRY mRNA is initiated at 41 dpo (days post ovulation), peaks at 44 dpo and persists there after at low levels throughout the entire individual life (146). The presence of SRY protein in the genital ridge after testis formation (147) and in the nuclei of Sertoli and germ cells in the testes of individuals of different ages (135) has been demonstrated. Therefore, any SRY expression changes could induce pathologies of male reproductive organs.

Complete inactivation of SRY protein induced by SRY (murine Sry) mutations has led many to believe that gonad determination is the main function of SRY gene. However, the presence of SRY protein in the nucleus of Sertoli and germ cells in developing and adult individuals suggests additional SRY male specific functions (148). The Y chromosome contains a number of genes that have an important role in the development and maintenance of male specific organs, including testis and prostate (117,149).

The expression of genes dependent on the SRY gene, including WT-1, SF-1, SOX-9, DAX-1, and FGF-9 is very important for genital tract development. WT-1 knock-out mice die *in utero* lacking kidneys and gonads and present a female phenotype (150). SF-1 null mice are able to survive *in utero* but die shortly after birth. They lack adrenal glands and male gonads and consequently have female internal and external genitalia (151). Disruptions of human SOX-9 gene (SRY-related HMG box, 9) often result in XY sex reversal (152). Loss of function in DAX-1 causes hypogonadotropic hypogonadism (HHG) and congenital adrenal hypoplasia (CAH) (153). It appears that Sry promotes mesenchymal cell proliferation, mesonephric cell migration, and Sertoli cell differentiation in the XY gonad (testis) by activating Fgf9. Therefore, sex reversal has been observed in Fgf9 knock out mice (154). Since the role of DNA methylation in the expression of some of these genes has been already reported (117), the importance of DNA methylation in genital trat development is evident.

4.3. Hypothalamic-pituitary-gonadal axis

It is well known that the hypothalamic-pituitary-gonadal (HPG) axis controls male sexual and reproductive activity. Therefore, in addition to a well-developed male reproductive tract and appropriate production of viable sperm, there are other aspects to consider. Hormones, growth factors, their receptors, and other associated signal transduction proteins may be altered and could affect the HPG axis inducing male reproductive failure (155).

In addition to alterations of early primordial germ cell determination, gonadal differentiation, gametogenesis, development of external genitalia, various chemicals, and physical and biological conditions could affect critical signaling events regulating sexual behavior (107,156-159). These events may include male sexual behavior which is mediated in part by androgens and estrogens (160,161). These hormones act in the brain to influence male mating (161,162). The relationship between androgens, estrogens and DNA methylation has been substantiated (163-165).

In recent years, the analysis of mutant animals has demonstrated that a number of imprinted genes influence brain development and behavior (166-172). Recent studies indicate that the expression of genes affecting sexual behavior and reproduction such as Y chromosome specific genes (DAZ, SRY, RBMY1A, RBMY1H, RBMII, BPY1, PRY and TSPY) (117), IGF (173), and c-fos (174) is regulated by DNA methylation.

The insulin-like growth factors (IGF-I and IGF-II) are essential for normal growth and development in different species (175-179). IGF-I, a low molecular weight peptide that mediates the cell proliferating actions of growth hormone, has been implicated in a variety of reproductive processes (178,180,181). Dyck and collaborators reported that IGF-I expression is dependent on DNA methylation. Human IGF-I (hIGF-I) was expressed only in testicular tissue of hIGF-I trangenic mice, where it is hypomethylated relative to non-reproductive tissue. Its expression was evident in germ line, and

occurred during early spermatogenesis between day 10 and 15 of sperm development (181).

Recently Cisneros and collaborators, while studying the intra-embryonic exposure effects of 5-AZA-CdR, found that *in utero* exposed mice presented growth retardation and impaired male reproductive activity (30,182,183). The altered male reproductive activity, possibly an altered behavior (184), was correlated with low levels of serum IGF-1 and higher global DNA methylation levels (183). Therefore, alteration of DNA methylation patterns during development affects IGF-1 and male reproductive capacity.

Finally, the role of TRP2 gene in activating the mouse vomeronasal organ (VNO) that mediates social behaviors and neuroendrocrine changes elicited by pherohormone cues was reported. Indiscriminate sexual and courtship behavior toward both males and females and absence of normal male – male aggressive behavior was observed in TRP2 deficient male mice (185). Since the mechanisms of TRP2 expression are unknown, there is the possibility that DNA methylation could be one of the mechanisms controlling its expression.

5. PERSPECTIVE

Male infertility is a global health concern. In addition to the adequate time of expression, the need of expression threshold for genes controlling male reproductive organ development, spermatogenesis and normal male sexual activity has been substantiated. The role of DNA methylation in controlling the expression of several specific genes necessary for appropriate male reproductive processes has been reported. Therefore, the role of DNA methylation in male fertility is evident. If certain diets, drugs, or environmental pollutants are able to alter DNA methylation, the need for more studies examining the effects of such xenobiotics in male reproductive capacity is evident. Since many genes important for male reproductive organ development, spermatogenesis and sexual behavior are controlled at least in part by DNA methylation, all these aspects should be considered. In addition, the heritability of epigenetic mechanisms has been substantiated in recent years. Therefore, the effects of such xenobiotics should be investigated in multigenerational studies, because xenobiotic-induced DNA methylation changes could be the cause of altered disease susceptibility and abnormal phenotype in future generations.

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