Invited Review Article: Congenital anomalies

Title: Epigenetic alterations in sperm associated with male infertility

Short title: Epigenetics and male infertility

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Key words: Male infertility, Genomic imprinting, DNA methylation, Genomic imprinting disorders, Assisted reproductive technologies (ART)
Abstract

The most common form of male infertility is a low sperm count, known as oligozoospermia. Studies suggest that oligozoospermia is associated with epigenetic alterations. Epigenetic alterations in sperm, which may arise due to the exposure of gametes to environmental factors or preexist in the sperm of infertile individuals, may contribute to the increased incidence of normally rare imprinting disorders in babies conceived after assisted reproductive technology using the sperm of infertile men. Genomic imprinting is an important developmental process whereby the allelic activity of certain genes is regulated by DNA methylation established during gametogenesis. The aberrant expression of several imprinted genes has been linked to various diseases, malignant tumors, lifestyle and mental disorders in humans. Understanding how infertility and environmental factors such as reproductive toxicants, certain foods, and drug exposures during gametogenesis contribute to the origins of these disorders via defects in sperm is of paramount importance. In this review, we discuss the association of epigenetic alterations with abnormal spermatogenesis and the evidence that epigenetic processes, including those required for genomic imprinting, may be sensitive to environmental exposures during gametogenesis, fertilization and early embryonic development. In addition, we review imprinting diseases and their relationships with environmental factors. While the plasticity of epigenetic marks may make these more susceptible to modification by the environment, this also suggests that aberrant epigenetic marks may be reversible. A greater understanding of this process and the function of epidrugs may lead to the development of new treatment methods for many
adult diseases in the future.
Introduction

Approximately half of human infertility can be explained by abnormal spermatogenesis. Disturbingly, the incidence of abnormal spermatogenesis has increased in developed countries, including Japan (Japan Society of Obstetrics and Gynecology Registry). Oligozoospermia is the most common disorder of male infertility characterized by abnormally low concentrations of spermatozoa in the semen. Although different genetic causes are known, they account only for a fraction of the cases of aberrant spermatogenesis (Dohle et al. 2002; Fernandes et al. 2002; Gianotten et al. 2003). Epigenetic factors, including DNA methylation, histone modifications and chromatin remodeling, have been studied extensively during gametogenesis and germ cell maturation and it is clear that germ cells undergo extensive epigenetic reprogramming in a sex-specific manner (Dada et al. 2012; van Montfoort et al. 2012; Boissonnas et al. 2013). Consequently, aberrant epigenetic alterations may underlie some cases of oligozoospermia.

Indirect evidence for a role for aberrant epigenetic processes in oligozoospermia comes from studies on human assisted reproductive technology (ART), in which the eggs and/or sperm are manipulated in the laboratory to help infertile persons of reproductive age conceive. Recent reports identified an increased incidence of normally rare imprinting disorders, especially Beckwith-Wiedemann syndrome (BWS; OMIM 130650), Angelman syndrome (AS; OMIM 105830) and Silver-Russell syndrome (SRS; OMIM 180860), in babies conceived after ART (DeBaun et al. 2003; Gosden et al. 2003; Maher 2005). Several reports have suggested that imprint
methylation errors occur during the process of ART, both in in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) (Cox et al. 2002; DeBaun et al. 2003; Gicquel et al. 2003; Maher et al. 2003; Moll et al. 2003; Orstavik et al. 2003; Ludwig et al. 2005; Rossignol et al. 2006; Bowdin et al. 2007; Kagami et al. 2007) which may be due to in vitro embryo transfer procedures performed at the time of epigenetic fluidity (Lucifero et al. 2004; Niemitz and Feinberg 2004; Thompson and Williams 2005; Horsthemke and Buiting 2006). However, our work and that of others suggests that epigenetic risks linked to ART techniques can also originate in the use of sperm with preexisting epigenetic errors (Kobayashi et al. 2007; Kobayashi et al. 2009). This review provides an overview of the current state of knowledge of human sperm epigenetics and what is known regarding the effects of environmental and nutritional factors on the sperm epigenome.

**Genomic imprinting**

Genomic imprinting is an epigenetic phenomenon that describes parent-of-origin patterns of monoallelic gene expression reported in mammals and some plant species (Barlow and Bartolomei 2014). The term genomic imprinting was first used to describe the failure of mono-parental embryos to develop appropriately in utero despite their diploid DNA content (Barton et al. 1984; McGrath and Solter 1984; Surani et al. 1984). We now know that there are over one hundred genes in mammals that are regulated by genomic imprinting and many of these have critically important roles in early development and also later life process, both metabolic and behavioural (Surani
Differences in the parental genomes are first established in the germline when the two parental genomes are physically separate. Discrete DNA regions are marked by DNA methylation in one or other germline. After fertilisation these marks are maintained despite the extensive epigenetic reprogramming that takes place early in development (Morgan et al. 2005), to generate regions of the genome that have DNA methylation present on one parental allele and absent on the other allele. These regions are termed gametic differentially methylated regions (gDMR; Figure 1). These gDMRs act as the catalyst for a further series of epigenetic changes including both the modification of histones and somatic DNA methylation events, which generate extensive domains of imprinted chromatin some of which span several megabases. Within these domains certain genes are silenced on one parental allele and active on the other parental allele with most imprinted domains containing both paternally- and maternally-expressed genes. While these gDMRs are maintained for the lifetime of the individual, the monoallelic expression status of imprinted genes can vary with tissue type and developmental stage suggesting that functional imprinting is important at different times for different genes. In the mouse female germline, gDMRs acquire DNA methylation after birth during the transition from primordial to antral follicles in the postnatal growth phase (post-pachytene) (Obata and Kono 2002; Lucifero et al. 2004; Hiura et al. 2006). In the human female germline, maternal methylation of gDMRs has already been initiated to some extent in adult non-growing oocytes but not in neonatal oocytes (Sato et al. 2007). In mouse male germline, methylation at three sites
(H19, Rasgrf1 and Gtl2) is present prenatally before meiosis and completed by the pachytene phase of postnatal spermatogenesis (Davis et al. 1999; Davis et al. 2000; Ueda et al. 2000; Li et al. 2004) with complete loss of methylation of maternal DMRs. While gDMRa are established in the germline, some imprinted domains also contain somatic DMRs (sDMR) which are not inherited via the germline but which appear during embryogenesis either before or after monoallelic expression is established and which are also important for maintaining monoallelic gene expression (John and Lefebvre 2011). In addition to the establishment and maintenance of allele-specific epigenetic marks, imprints must be erased in the developing germline and reset for the next generation (Figure 2). Establishment, maintenance and erasure of imprints all involve dynamic changes in epigenetic marks that take place at different stages of development in males and females. In summary, genomic imprinting is a dynamic epigenetic process both in the germline and during early development. Epigenetic errors at any stage in the process of establishment, maintenance or erasure of imprints can have a catastrophic consequence for the next generation, as evidenced by the genomic imprinting disorders.

Genomic Imprinting disorders

The importance of correct genomic imprinting in humans is best illustrated by a number of rare but striking childhood developmental disorders associated with imprinted loci. Prader-Willi syndrome (PWS; OMIM 176270) and Angelman syndrome (AS; OMIM 105830) are two clinically distinct imprinting disorders linked to the same
imprinted region on chromosome 15q11-q1 (Buiting 2010). PWS is characterized by endocrine and neural abnormalities and malformation and is mainly associated with maternal uniparental disomy of 15q11-q1 (70%) and methylation defects (2-5%). In contrast, AS, which is characterized by global developmental delay, convulsions, scoliosis, excessive laughter, and movement, balance and sleep disorders, is associated with loss of function of the maternally expressed $UBE3A$ gene either through deletions (70%), paternal uniparental disomy (0-20%) or aberrant methylation (2-5%) of the maternal allele. Beckwith-Wiedemann syndrome (BWS; OMIM 130650) and Silver-Russell syndrome (SRS; OMIM 180860) are similarly clinically distinct syndromes associated with a single chromosomal region at 11p15.5 (Jacob et al. 2013). BWS is a fetal overgrowth disorder characterized by exomphalos, macroglossia, gigantism and an increased risk of developing embryonal tumors in childhood. BWS is associated with a number of genetic and epigenetic alterations. The most frequent alteration observed in BWS is hypomethylation of a gDMR located over the promoter of a long, non-coding RNA called $LIT1$ or $Kcnqtot1$, which is found in >60% of sporadic BWS patients. Animal studies suggest that this gDMR regulates expression of the maternally-expressed $CDKN1C$ gene known to play a key role in limiting fetal growth (Andrews et al. 2007; Tunster et al. 2011). SRS is a similarly clinically heterogeneous condition characterized by severe intrauterine growth retardation, poor postnatal growth, craniofacial features such as a triangular-shaped face and a broad forehead, body asymmetry, and a variety of minor malformations. The most frequent alteration in SRS is hypomethylation of the gDMR spanning the promoter of a
non-coding RNA called *H19* apparent in 40% of cases (Bliek et al. 2006). This gDMR regulates the imprinted expression of the fetal growth factor gene *IGF2* (Insulin like growth factor 2) (DeChiara et al. 1991; Leighton et al. 1995). However, rare SRS patients have been reported with maternal microduplications spanning *CDKN1C* (Bonaldi et al. 2011). Furthermore, additional loci on various chromosomes have been implicated as having a role in this syndrome (Davis et al. 2000; Ueda et al. 2000; Gicquel et al. 2003; Maher et al. 2003; Sato et al. 2007). These disorders highlight the necessity of appropriately regulated gene dosage at imprinted loci mediated by epigenetic processes, which might consequently be subject to external influences acting on the epigenome.

**ART and congenital imprinting disorders**

A number of publications have suggested an association between ART and genomic imprinting disorders (*Table 1*) (Chiba et al. 2013; Hiura et al. 2014). The first report linking ART to AS in 2002 highlighted loss of DNA methylation on chromosome 15 (Cox et al. 2002). In 2004 an increased frequency of BWS after ART was reported, again linked to changes in DNA methylation (DeBaun et al. 2003). In 2007 SRS was linked to ART and hypermethylation at an imprinted loci (Kagami et al. 2007). ART does not, however, appear to be a risk factor in PWS (Gold et al. 2014).

There are several proposed mechanisms which may underlie the increased frequency of imprinting disorders in ART including the exposure of gametes and early embryos to culture conditions, the superovulation of oocytes and the presence of
preexisting imprinting mutations in sperm. Some studies have shown that exposure of
mouse embryos to different culture conditions can alter the expression and imprinting of
various genes, which could result in abnormal development (DeBaun et al. 2003;
Gicquel et al. 2003; Maher et al. 2003; Lucifero et al. 2004). We, and others, have
demonstrated that superovulation (artificial induction of ovulation with high doses of
gonadotrophins) affects imprint methylation (Chang et al. 2005; Ligon 2005; Sato et al.
2007). Embryo freezing may also be an issue as this has been found to have deleterious
effects on DNA, embryonic gene expression, telomeres and plasma and nuclear
membranes (Emiliani et al. 2000; Honda et al. 2001). Furthermore, the timing of
embryo transfer may be an issue. Case reports of monochorionic dizygotic twins and
conjoined twins with BWS resulting from transfer at the blastocyst stage (Shimizu et al.
2004; Miura and Niikawa 2005) reported demethylation of \textit{LIT1} (\textit{KCNQ1OT1}),
suggesting that this demethylation occurs at a critical stage of preimplantation
development. In addition to epigenetic errors induced by the process of ART, there is
evidence that sperm from men with fertility issues carry preexisting epigenetic errors.

\textbf{Sperm from infertile men and epigenetic errors}

Studies have shown that disturbed spermatogenesis is associated with
incorrect DNA methylation at gDMRs (Table 2). In spermatozoa from oligozoospermic
men, the occurrence of hypermethylation of several maternally imprinted DMRs or
hypomethylation of paternally imprinted DMRs is increased (Marques et al. 2004;
Boissonnas et al. also reported the association between methylation and sperm concentration in teratozoospermic (TZ) and oligoasthenoteratozoospermic (OAT) patients (Boissonnas et al. 2010). In the TZ group, 11 of 19 patients displayed loss of methylation of the \textit{IGF2} DMR or of both the \textit{IGF2} DMR and the \textit{H19} DMR. In the OAT group, 16 of 22 patients displayed a severe loss of methylation of the \textit{H19} DMR, and this closely correlated with sperm concentration. Marques et al. suggested an association between aberrant epigenetic sperm modifications and oligozoospermia (Marques et al. 2004). Normozoospermic individuals (0.13%), Moderate (17%) and Severe (30%) oligozoospermic patients all showed abnormal methylation of \textit{H19}. We examined the DNA methylation status of seven imprinted genes in spermatic DNA obtained from infertile men and also found abnormal maternal and paternal DNA methylation at several imprinted loci (Figure 3). Samples (10/96 cases) with both maternal and paternal defects were primarily from men with severe oligospermia. Importantly, the outcome of ART (fertility rates and implantation rates) with sperm shown to have an abnormal DNA methylation pattern is generally poor (Kobayashi et al. 2007).

As spermatogenesis progresses, the genome undergoes major changes that not only influence genetic and epigenetic information but also alter the nuclear structure. It is consequently important to understand how the specific nucleoprotamine/histone structure of the sperm nucleus conveys epigenetic information and how this might control early embryonic growth. In most cell types, DNA is wrapped around histone but in sperm, protamines, which are small arginine-rich nuclear proteins, replace histones
late in the haploid phase of spermatogenesis and these proteins are essential for spermatic function (Cho et al. 2001)(Figure 2). Both the phosphorylation of protamines and the ratio of the two human protamines, protamine (P1) and protamine 2 (P2), are important for optimal sperm function. The P1/ P2 ratio in fertile men ranges from 0.8 to 1.2 (Carrell and Liu 2001). Perturbation of this ratio, either higher or lower than normal, has been reported to be associated with poor semen quality, increased DNA damage and/or decreased fertility (Chevaillier et al. 1987; Balhorn et al. 1988; Belokopytova et al. 1993; Carrell et al. 1999; Razavi et al. 2003; Aoki et al. 2005). An increasing number of reports now support the hypothesis that sperm DNA is not homogeneously packed with these protamines and that histones are still present at some sites (Rousseaux et al. 2005). While some investigators have suggested that this is due to inefficient protamine replacement, the persistence of histones at certain sites may play a functional role in supporting the epigenetic code in the sperm (Weber et al. 2007). Protamine replacement occurs in the spermatid stage of spermatogenesis after the completion of meiosis (Baarends et al. 1999). The elongating spermatid also undergoes other maturational events that affect motility and fertilization ability during the period of protamine replacement. The association between abnormal protamine replacement and generally diminished semen quality may be a defect in the unique gene regulation system of temporal uncoupling of transcription and translation during spermatogenesis (Carrell et al. 2007).

Alteration of the P1 to P2 ratio generally denotes abnormal spermatogenesis and is a possible direct cause of abnormal methylation of maternal and paternal gDMRs.
Azoospermia caused by anejaculation and secondary inflammatory obstruction is related to an increase of methylation level in maternal DMRs (Marques et al. 2010). Male infertility may also be related to the improper erasure of DNA methylation during spermatogenesis at many non-imprinted genes in addition to abnormal methylation levels at gDMRs (Houshdaran et al. 2007). There are some significant implications for sperm with abnormal protamine replacement, and for the use of such sperm for ICSI. Further research should be done to classify the role of retained histones throughout the spermatic genome in mature sperm from men with normozoospermia as well as in patients with known chromatin abnormalities.

Teratological environmental factors (endocrine disruptors) and epigenetic modifications

Abnormal sperm development may originate from exposure of the male germline to environmental factors. Persistent organic pollutants (POPs), which were used intensively worldwide for several decades until the 1980s, have been implicated in reproductive disorders. Because of the stability and bioaccumulation of these compounds in the environment, human populations are simultaneously exposed to a variety of those contaminants through the consumption of food. Several POPs have been shown to have toxic effects on reproductive and endocrine functions in humans (Govarts et al. 2012) and a number of human epidemiological studies have demonstrated the adverse effects of POPs exposure on markers of reproduction,
including semen quality (sperm concentration, motility, and morphology) (Guo et al. 2000; Richthoff et al. 2003; Toft and Guillette 2005; Meeker et al. 2010), spermatic DNA integrity (Bonde et al. 2008; McAuliffe et al. 2012), and circulating reproductive hormone levels (Richthoff et al. 2003), though some studies found only marginal effects (Toft et al. 2006; Haugen et al. 2011). In general, however, these reports suggest that POPs have adverse effects on reproductive health outcomes.

Endocrine disruptors are another potential environmental factor driving abnormal sperm development. Male gonadal development occurs around midgestation in humans initiated by the differentiation of precursor Sertoli cells in response to the testis-determining factor SRY. The fetal testis contains steroid receptors and is a target for endocrine hormones. The androgen receptor and estrogen receptor-$\beta$ are present in both Sertoli cells and germ cells. Although the testis does not produce steroids at this stage of development, estrogens and androgens can affect testis cellular functions. Treatment with endocrine disruptors at a critical time of gonadal sex determination promotes an adult testis phenotype with decreased spermatogenic capacity in rat and, as a result, male infertility. External factors could induce an epigenetic transgenerational phenotype through apparent reprogramming of the male germ line (Anway et al. 2005). However, it is still unclear whether steroids acting inappropriately during the time of gonadal sex determination act to reprogram the germ line via epigenetic DNA methylation to cause this transgenerational transmission of an altered phenotype.

Seminal tract infection, one of the most common causes of infertility in men (Keck et al. 1998), may also contribute to abnormal sperm development. The presence
of leukocytes in semen, also known as leukocytospermia, (Korrovits et al. 2008; Cumming and Carrell 2009), is an indicator of seminal tract infection although this correlation remains controversial (Bezold et al. 2007). Asthenozoospermia is often associated with the presence of infection or leukocytes in semen although it is not known whether infection plays a causative role (Wolff 1995). The association between epigenetic changes and such sperm abnormalities as asthenozoospermia and leukocytospermia is unknown. However, there is a precedent for infection inducing epigenetic alterations in other cell types. In gastric carcinogenesis, H. pylori infection induces aberrant promoter methylation in tumor-suppressor genes, including p16\(^{INK4A}\), LOX, and CDH1 (Kaneda et al. 2004; Ushijima et al. 2006). Further work is required to establish whether epigenetic alterations in sperm are induced by seminal tract infections.

Social stress, acting through hormone signalling pathways, is another recent addition to the group of environmental factors that are known to induce epigenetic changes. The extent and type of maternal care very early in life in rodents has been shown to influence epigenetic marks at the glucocorticoid receptor in the neonatal hippocampus, and this may influence later life stress responses in the offspring (Weaver et al. 2004; Meaney et al. 2007). Furthermore, in another rodent model, Roth et al. found that psychosocial stress (comparable to human post-traumatic stress disorder (PTSD)) led to an increase in Bdnf methylation in the dorsal hippocampus and downregulation of Bdnf expression in the dorsal and ventral hippocampus, but not in other PTSD-relevant regions (Roth et al. 2011). The induction of region-specific
epigenetic changes in response to traumatic stress during adulthood demonstrates that DNA methylation remains an active process that can be shaped by environmental factors even in the adult nervous system. Again, the effect of stress on the sperm epigenome has not been investigated. However, stress is also a cause of male infertility this may occur through epigenetic alterations in the germline (Bale 2014).

7 Nutrition and epigenetic regulation

Epigenetic marks are tightly regulated, both temporally and spatially, during fetal development and lactation (Lee et al. 2002; Allegrucci et al. 2005; Morgan et al. 2005) but can be influenced at key stages by diet. Agouti viable yellow (Avy) is a fascinating animal model whereby the environmental influences on the epigenome can be monitored via a coat colour phenotype (Wolff et al. 1999). A gene alteration, which involves an intra-cisternal A particle (IAP) retrotransposon insertion upstream of the agouti gene (A), leads to ectopic expression of the agouti protein and a change of hair color from agouti to yellow. The extent of this coat colour change is influenced by the degree of methylation of the IAP element, which can be influenced by methyl donor supplementation of the maternal diet (Waterland and Jirtle 2003). Dietary supplementation with a methyl donor during pregnancy increases the proportion of pups carrying a methylated IAP sequence and thus the number with a yellow coat colour (Rakyan et al. 2003; Waterland and Jirtle 2004). Maternal and post-weaning high fat diets can also alter epigenetic regulation of the hedonic reward pathways and metabolic regulation of the energy balance in mice (Vucetic et al. 2011), and alter methylation of
the leptin promoter in rats (Milagro et al. 2009). These data provide compelling
evidence that diet alone can alter the epigenome.

Nutrition during early growth and development may influence DNA
methylation because one-carbon metabolism is dependent on dietary methyl donors and
on cofactors such as methionine, choline, folic acid and vitamin B-12 (MacLennan et al.
2004). The limited availability of acetyl-CoA for HAT activity and methyl donors of
SAM (S-adenosylmethionine) provided via the folate-methionine pathway may
therefore play a role in the establishment of inappropriate epigenetic patterns.
Conversely, dietary supplementation may provide a route to attenuating inappropriate
epigenetic patterns as the changes in DNA methylation which result from a decrease in
DNMT1 (DNA methyltransferase) activity can be partially prevented by folate
supplementation (Lillicrop et al. 2005; Lillicrop et al. 2007).

The influences of poor nutrition on epigenetic marks is not limited to the fetal
stage. Nutrition during postnatal development can permanently alter the epigenetic
regulation of some imprinted genes. Methyl-donor-deficient diet in postnatal life is
associated with altered epigenetic regulation of IGF2 and growth retardation (Waterland
et al. 2006). In humans, diet has been shown to affect the DNA methylation status of
patients with hyperhomocysteinaemia. This disease is caused by the accumulation of
S-adenosylhomocysteine (an inhibitor of DNA methyltransferases)(Waterland et al.
2006).

Given the consequences of altered nutrient availability in a number of situations, it is
possible that changes may be also occur in the male germline in response to diet. One very
A compelling study demonstrated that a low protein diet in male rats results in altered chromatin packing in sperm and changes in DNA methylation in the offspring (Carone et al. 2010). These data all suggest that something as seemingly innocuous as a dietary imbalance can have a detrimental effect on the epigenome at certain critical stages.

In addition to the availability of specific nutrients, alterations in the expression, localization and/or activity of epigenetic modifiers, such as the DNA methyltransferases, the histone-modification enzymes and their associated proteins, may play a role in driving abnormalities in the sperm epigenome. Some modifiers are specifically expressed in germ cells and the crucial roles of germ-cell-specific genes such as $Dnmt3L$ and $Prdm9$ has been highlighted in conventional mouse gene knockout studies (Bourc'his et al. 2001; Hata et al. 2002; Hayashi et al. 2005). We reported DNA sequence variations in the gene encoding $DNMT3L$ associated with imprinting errors and oligospermia (Kobayashi et al. 2009). A recent report suggests that gestational diet can alter the expression of histone demethylases and $Dnm3L$, at least in the exposed placenta (Gabory et al. 2012). Consequently both poor sperm quality and imprinting errors may be linked by both genetic and dietary-driven alterations in epigenetic regulators.
Conclusions

Mounting evidence from both human studies and animal models suggests that epigenetic modifications provide a link between the environment and alterations in gene expression that might lead to disease phenotypes. Importantly, direct evidence from animal studies supports the role of environmental epigenetics in male infertility and suggests the possibility that the use of ART to treat male infertility may lead to disease later in life. However, ART is a relatively recent technology and the longer term consequences of ART treatments such as ICSI and embryo freezing before transfer have not yet been manifested due to the young age of the majority of ART children.

Environmental exposures to nutritional, chemical and physical factors all have the potential to alter gene expression and, therefore, modify sperm quality in various ways through changes in the epigenome. A summary of the factors known to influence DNA methylation is presented in Figure 4.

It is still unknown when imprinting epigenetic errors related to male infertility arise and what factors may predispose to epigenetic changes. Hormonal stimulation of oocytes, in vitro culture, cryopreservation, and the timing of embryo transfer have all been shown to influence the proper establishment and maintenance of genomic imprints. Some infertile males, particularly those with oligozoospermia, carry preexisting imprinting errors in their sperm. Therefore the process of ART and infertility itself might increase the risk of imprinting disorders.

The developmental origins of health and disease (DOHaD) paradigm, first proposed by Prof. David Barker, postulates that suboptimal growth early life can
program changes which affect life long health, increasing the risks for various diseases.

There is evidence both from human studies and experimental models that this programming may be mediated via changes in the epigenome. Epigenetic changes likely occur during the fetal and infant periods but it is clear that oocytes and sperm are also vulnerable to environmentally-induced epigenetic alterations, and that the newly fertilised zygote is at a particular susceptible stage.

Future perspective

While genomic imprinting disorders are very rare, it is increasingly apparent that the bulk of common human diseases do not arise solely from genetic or environmental causes but also have an epigenetic component. Our knowledge that the epigenomes of gametes and newly fertilized embryos are susceptible stages for environmentally-induced epigenetic changes has particularly important implications as changes in lifestyle and modes of reproduction may have long term implications for human health that are not yet fully appreciated. Recent work identifies advanced paternal age as a risk factor for autism, depression, epilepsy and prostate cancer in children (Kondrashov 2012; Sun et al. 2012). While there are a number of possible explanations for these associations, the accumulation of epigenetic errors in the sperm may be a contributory factor. As the human population ages and the use of ART increases worldwide, it will become increasingly important to determine the extent to which environmentally-induced epigenetic changes contribute to disease. A detailed characterisation of the normal epigenetic process that take place in the germline and
during very early development will be important in achieving this goal. Understanding how and when environmental factors can influence the epigenome to cause disease, identifying ways in which to modulate aberrant epigenetic marks, and also determining the best timeframe to reverse aberrant epigenetic marks all have the potential to lead to improved human health.
Acknowledgments

We would like to thank all the members of our laboratory for their technical assistance, support and valuable suggestions. In particular, we thank Prof. Kazuyoshi Arishima of Azabu University and Prof. Kentaro Tanemura of Tohoku University for giving us the opportunity to write this review.

Funding

This work was supported by Grants-in-Aid from the Ministry of Health, Labour and Welfare of the Japanese government (Health Labour Sciences Research Grant, Research on Measures for Intractable Diseases 26310301), Scientific Research (KAKENHI) (Exploratory Research 25670691, Scientific Research (C) 24613001, Grant-in-Aid for Young Scientists (B), 26870036, 26870033), as well as the Takeda Science Foundation (TA).

Conflict of interest statement: Disclosure none.
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Figure legends

Figure 1. The regulation of imprinted genes by DNA methylation. Genomic imprinting describes the differential expression of the two parental alleles in mammals (and some plants). This differential expression is initiated within the germline when discrete regions of the genome acquire DNA methylation in one germline but not the other. These differentially methylated regions (DMRs) are present within all well characterised imprinted loci are key to establishing and, in some cases, maintaining imprinted gene expression. Paternal: paternal allele; Maternal: maternal allele; ICR: imprint control region; TF: transcriptional factor.

Figure 2. Imprints in gametogenesis and ART procedure. (Upper: Oogenesis) During the transition from primordial to antral follicles in the postnatal growth phase (post-pachytene) methylation is acquired asynchronously in a gene-specific manner in mouse oogenesis. In sperm, imprint methylation is initiated prenatally before meiosis and is completed by the pachytene phase of postnatal spermatogenesis. The imprints of gametes are maintained stably in the early embryo despite overall epigenetic reprogramming. (Lower: Spermatogenesis) ART results from the use of sperm with incomplete reprogramming and from in vitro embryo procedures performed at the time of epigenetic reprogramming. IVM: in vitro oocyte maturation; GIFT: gamete intrafallopian transfer; ZIFT: zygote intrafallopian transfer; PGD: preimplantation genetic diagnosis; IVF: in vitro fertilization; ICSI: intracytoplasmic sperm injection; ROSI: round spermatid injection; PGC: primordial germ cell; Oog: oogonium; POo:
primary oocyte; ProSpg: pro spermatogonium; Spg: sper matogonium; PSp: primary spermatocyte; SS pint spermatocyte.

Figure 3. Aberrant DNA methylation of imprinted loci in sperm from infertile male. (A) Frequency of imprint methylation errors (B) Abnormal imprinted loci (C) Abnormal methylation imprinting and sperm concentrations, morphology and motility. Methylation errors at maternal and paternal imprinted loci specific to oligozoospermic men. (D) Model comparing oligozoospermia and epigenetic errors (described in detail by Kobayashi et al. HMG 2007).

Figure 4. Factors influencing DNA methylation. DNA methylation is influenced by a number of external factors including nutrition, aging and hormones. Preventive and promotive factors are shown.

Table 1. ART and imprint-associated disorders.

Table 2. DNA methylation errors in the human spermatozoa
OAT: patients presenting with combined oligozoospermia, asthenozoospermia and teratozoospermia, ANJ: Anejaculation, OAZI: secondary inflammatory obstructive azoospermia, CBAVD: obstructive azoospermia due to congenital bilateral absence of
the vas deferens, HP: secretory azoospermia due to hypospermatogenesis.
Abbreviations

ANJ: Anejaculation,
ART: assisted reproductive technologies
AS: Angelman syndrome
Avy: Agouti viable yellow
BS: Bisulphite PCR sequence method
BWS: Beckwith-Wiedemann syndrome
CBAVD: obstructive azoospermia due to congenital bilateral absence of the vas deferens
COBRA: combined bisulphite PCR restriction analysis
DNMT: DNA methyltransferase
DOHaD: The developmental origins of health and disease
gDMRs: gametic differentially methylated regions
GIFT: gamete intrafallopian transfer
HP: secretory azoospermia due to hypospermatogenesis
IAP: intra-cisternal A particle
ICRs: imprinting control regions
ICSI: intracytoplasmic sperm injection
IVF: in vitro fertilization
IVM: in vitro oocyte maturation
OAT: oligoasthenoteratozoospermic
<table>
<thead>
<tr>
<th></th>
<th>Term</th>
<th>Definition</th>
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<tbody>
<tr>
<td>1</td>
<td>OAZI</td>
<td>secondary inflammatory obstructive azoospermia</td>
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<tr>
<td>2</td>
<td>Oog</td>
<td>oogonium</td>
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<td>3</td>
<td>PGC</td>
<td>primordial germ cell</td>
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<td>4</td>
<td>PGD</td>
<td>preimplantation genetic diagnosis</td>
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<tr>
<td>5</td>
<td>POPs</td>
<td>Persistent organic pollutants</td>
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<td>6</td>
<td>POo</td>
<td>primary oocyte</td>
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<tr>
<td>7</td>
<td>PSp</td>
<td>primary spermatocyte</td>
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<td>8</td>
<td>PTSD</td>
<td>post-traumatic stress disorder</td>
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<td>ProSpg</td>
<td>prospermatogonium</td>
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<td>10</td>
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<td>Prader-Willi syndrome</td>
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<td>13</td>
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<td>15</td>
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<td>16</td>
<td>TF</td>
<td>transcriptional factor</td>
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<td>17</td>
<td>TZ</td>
<td>teratozoospermic</td>
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<td>18</td>
<td>ZIFT</td>
<td>zygote intrafallopian transfer</td>
</tr>
</tbody>
</table>
Figure 1
Figure 3

A. Abnormal Methylation
- 2 or more loci (11.3%)
- Single locus (13.4%)
- Normal (75.3%)

B. Paternal DMRs
- SNRPN (8.3%)
- H19 (10.6%)
- GTL2 (25.1%)
- PEG1 (30.1%)
- ZAC (7.1%)
- LIT1 (6.1%)
- PEG3 (7.8%)

Maternal DMRs

C. Concentration (count) (%)

- Concentration (く
- Concentration (％)
- Concentration (%)}

- Normal
- Moderate
- Severe

- < 50
- 50 - 65
- 65 -

- < 10
- 10 - 40
- 40 -

- < 0.05
- ** < 0.01
Figure 4

Prevention

Promotion

Growth hormone
Drug (anti-methyl substrates)
Chromatin remodeling factors
Histone acetylation

Nutrients (folic acid, vitamin B6)
Virus
Aging
Food additives
Endocrine disrupter

Histone methylation
Inflammation
Drug (methyl-substrates)
Figure 2

Oogenesis

Paternal DMPs → Maternal DMPs

Establishment → Maintenance

Gonadotrophs

Diplasmic transfer

IVM

Spermatogenesis

Methylation for imprinting

H19
G6δ2
Razphil

Establishment → Maintenance

ProSpg
Spg
PSp
SSp
Spermaids
Spermaioza

Methylation → Core Histone Activation → Core Histone Deacetylation → Phosphorylation → Protamine Catcallivalence

ICSI
Cryopreservation
ROS1

IVF
GIFT, ZIFT
Extended culture
Cryopreservation
PGD