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1 **Invited Review Article: Congenital anomalies**

2

3 **Title: Epigenetic alterations in sperm associated with male infertility**

4 **Short title: Epigenetics and male infertility**

5

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18

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

1 **Abstract**

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

1 adult diseases in the future.

2

1 **Introduction**

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

15 Indirect evidence for a role for aberrant epigenetic processes in
16 oligozoospermia comes from studies on human assisted reproductive technology (ART),
17 in which the eggs and/or sperm are manipulated in the laboratory to help infertile
18 persons of reproductive age conceive. Recent reports identified an increased incidence
19 of normally rare imprinting disorders, especially Beckwith-Wiedemann syndrome
20 (BWS; OMIM 130650), Angelman syndrome (AS; OMIM 105830) and Silver-Russell
21 syndrome (SRS; OMIM 180860), in babies conceived after ART (DeBaun et al. 2003;
22 Gosden et al. 2003; Maher 2005). Several reports have suggested that imprint

1 methylation errors occur during the process of ART, both in *in vitro* fertilization (IVF)
2 and intracytoplasmic sperm injection (ICSI) (Cox et al. 2002; DeBaun et al. 2003;
3 Gicquel et al. 2003; Maher et al. 2003; Moll et al. 2003; Orstavik et al. 2003; Ludwig et
4 al. 2005; Rossignol et al. 2006; Bowdin et al. 2007; Kagami et al. 2007) which may be
5 due to *in vitro* embryo transfer procedures performed at the time of epigenetic fluidity
6 (Lucifero et al. 2004; Niemitz and Feinberg 2004; Thompson and Williams 2005;
7 Horsthemke and Buiting 2006). However, our work and that of others suggests that
8 epigenetic risks linked to ART techniques can also originate in the use of sperm with
9 preexisting epigenetic errors (Kobayashi et al. 2007; Kobayashi et al. 2009). This
10 review provides an overview of the current state of knowledge of human sperm
11 epigenetics and what is known regarding the effects of environmental and nutritional
12 factors on the sperm epigenome.

13

14 **Genomic imprinting**

15 Genomic imprinting is an epigenetic phenomenon that describes
16 parent-of-origin patterns of monoallelic gene expression reported in mammals and some
17 plant species (Barlow and Bartolomei 2014). The term genomic imprinting was first
18 used to describe the failure of mono-parental embryos to develop appropriately *in utero*
19 despite their diploid DNA content (Barton et al. 1984; McGrath and Solter 1984; Surani
20 et al. 1984). We now know that there are over one hundred genes in mammals that are
21 regulated by genomic imprinting and many of these have critically important roles in
22 early development and also later life process, both metabolic and behavioural (Surani

1 1998; Tilghman 1999; Cleaton et al. 2014).

2 Differences in the parental genomes are first established in the germline when
3 the two parental genomes are physically separate. Discrete DNA regions are marked by
4 DNA methylation in one or other germline. After fertilisation these marks are
5 maintained despite the extensive epigenetic reprogramming that takes place early in
6 development (Morgan et al. 2005), to generate regions of the genome that have DNA
7 methylation present on one parental allele and absent on the other allele. These regions
8 are termed gametic differentially methylated regions (gDMR; **Figure 1**). These gDMRs
9 act as the catalyst for a further series of epigenetic changes including both the
10 modification of histones and somatic DNA methylation events, which generate
11 extensive domains of imprinted chromatin some of which span several megabases.
12 Within these domains certain genes are silenced on one parental allele and active on the
13 other parental allele with most imprinted domains containing both paternally- and
14 maternally-expressed genes. While these gDMRs are maintained for the lifetime of the
15 individual, the monoallelic expression status of imprinted genes can vary with tissue
16 type and developmental stage suggesting that functional imprinting is important at
17 different times for different genes. In the mouse female germline, gDMRs acquire DNA
18 methylation after birth during the transition from primordial to antral follicles in the
19 postnatal growth phase (post-pachytene) (Obata and Kono 2002; Lucifero et al. 2004;
20 Hiura et al. 2006). In the human female germline, maternal methylation of gDMRs
21 has already been initiated to some extent in adult non-growing oocytes but not in
22 neonatal oocytes (Sato et al. 2007). In mouse male germline, methylation at three sites

1 (*H19*, *Rasgrf1* and *Gtl2*) is present prenatally before meiosis and completed by the
2 pachytene phase of postnatal spermatogenesis (Davis et al. 1999; Davis et al. 2000;
3 Ueda et al. 2000; Li et al. 2004) with complete loss of methylation of maternal DMRs.
4 While gDMRa are established in the germline, some imprinted domains also contain
5 somatic DMRs (sDMR) which are not inherited via the germline but which appear
6 during embryogenesis either before or after monoallelic expression is established and
7 which are also important for maintaining monoallelic gene expression (John and
8 Lefebvre 2011). In addition to the establishment and maintenance of allele-specific
9 epigenetic marks, imprints must be erased in the developing germline and reset for the
10 next generation (**Figure 2**). Establishment, maintenance and erasure of imprints all
11 involve dynamic changes in epigenetic marks that take place at different stages of
12 development in males and females. In summary, genomic imprinting is a dynamic
13 epigenetic process both in the germline and during early development. Epigenetic errors
14 at any stage in the process of establishment, maintenance or erasure of imprints can
15 have a catastrophic consequence for the next generation, as evidenced by the genomic
16 imprinting disorders.

17

18 **Genomic Imprinting disorders**

19 The importance of correct genomic imprinting in humans is best illustrated by
20 a number of rare but striking childhood developmental disorders associated with
21 imprinted loci. Prader-Willi syndrome (PWS; OMIM 176270) and Angelman syndrome
22 (AS; OMIM 105830) are two clinically distinct imprinting disorders linked to the same

1 imprinted region on chromosome 15q11-q1 (Buiting 2010). PWS is characterized by
2 endocrine and neural abnormalities and malformation and is mainly associated with
3 maternal uniparental disomy of 15q11-q1 (70%) and methylation defects (2-5%). In
4 contrast, AS, which is characterized by global developmental delay, convulsions,
5 scoliosis, excessive laughter, and movement, balance and sleep disorders, is associated
6 with loss of function of the maternally expressed *UBE3A* gene either through deletions
7 (70%), paternal uniparental disomy (0-20%) or aberrant methylation (2-5%) of the
8 maternal allele. Beckwith-Wiedemann syndrome (BWS; OMIM 130650) and
9 Silver-Russell syndrome (SRS; OMIM 180860) are similarly clinically distinct
10 syndromes associated with a single chromosomal region at 11p15.5 (Jacob et al. 2013).
11 BWS is a fetal overgrowth disorder characterized by exomphalos, macroglossia,
12 gigantism and an increased risk of developing embryonal tumors in childhood. BWS is
13 associated with a number of genetic and epigenetic alterations. The most frequent
14 alteration observed in BWS is hypomethylation of a gDMR located over the promoter
15 of a long, non-coding RNA called *LIT1* or *Kcnqtot1*, which is found in >60% of
16 sporadic BWS patients. Animal studies suggest that this gDMR regulates expression of
17 the maternally-expressed *CDKN1C* gene known to play a key role in limiting fetal
18 growth (Andrews et al. 2007; Tunster et al. 2011). SRS is a similarly clinically
19 heterogeneous condition characterized by severe intrauterine growth retardation, poor
20 postnatal growth, craniofacial features such as a triangular-shaped face and a broad
21 forehead, body asymmetry, and a variety of minor malformations. The most frequent
22 alteration in SRS is hypomethylation of the gDMR spanning the promoter of a

1 non-coding RNA called *H19* apparent in 40% of cases (Bliek et al. 2006). This gDMR
2 regulates the imprinted expression of the fetal growth factor gene *IGF2* (Insulin like
3 growth factor 2) (DeChiara et al. 1991; Leighton et al. 1995). However, rare SRS
4 patients have been reported with maternal microduplications spanning *CDKN1C*
5 (Bonaldi et al. 2011). Furthermore, additional loci on various chromosomes have been
6 implicated as having a role in this syndrome (Davis et al. 2000; Ueda et al. 2000;
7 Gicquel et al. 2003; Maher et al. 2003; Sato et al. 2007). These disorders highlight the
8 necessity of appropriately regulated gene dosage at imprinted loci mediated by
9 epigenetic processes, which might consequently be subject to external influences acting
10 on the epigenome.

11

12 **ART and congenital imprinting disorders**

13 A number of publications have suggested an association between ART and
14 genomic imprinting disorders (**Table 1**) (Chiba et al. 2013; Hiura et al. 2014). The first
15 report linking ART to AS in 2002 highlighted loss of DNA methylation on chromosome
16 15 (Cox et al. 2002). In 2004 an increased frequency of BWS after ART was reported,
17 again linked to changes in DNA methylation (DeBaun et al. 2003). In 2007 SRS was
18 linked to ART and hypermethylation at an imprinted loci (Kagami et al. 2007). ART
19 does not, however, appear to be a risk factor in PWS (Gold et al. 2014).

20 There are several proposed mechanisms which may underlie the increased
21 frequency of imprinting disorders in ART including the exposure of gametes and early
22 embryos to culture conditions, the superovulation of oocytes and the presence of

1 preexisting imprinting mutations in sperm. Some studies have shown that exposure of
2 mouse embryos to different culture conditions can alter the expression and imprinting of
3 various genes, which could result in abnormal development (DeBaun et al. 2003;
4 Gicquel et al. 2003; Maher et al. 2003; Lucifero et al. 2004). We, and others, have
5 demonstrated that superovulation (artificial induction of ovulation with high doses of
6 gonadotrophins) affects imprint methylation (Chang et al. 2005; Ligon 2005; Sato et al.
7 2007). Embryo freezing may also be an issue as this has been found to have deleterious
8 effects on DNA, embryonic gene expression, telomeres and plasma and nuclear
9 membranes (Emiliani et al. 2000; Honda et al. 2001). Furthermore, the timing of
10 embryo transfer may be an issue. Case reports of monozygotic dizygotic twins and
11 conjoined twins with BWS resulting from transfer at the blastocyst stage (Shimizu et al.
12 2004; Miura and Niikawa 2005) reported demethylation of *LIT1 (KCNQ1OT1)*,
13 suggesting that this demethylation occurs at a critical stage of preimplantation
14 development. In addition to epigenetic errors induced by the process of ART, there is
15 evidence that sperm from men with fertility issues carry preexisting epigenetic errors.

16

17 **Sperm from infertile men and epigenetic errors**

18 Studies have shown that disturbed spermatogenesis is associated with
19 incorrect DNA methylation at gDMRs (Table 2). In spermatozoa from oligozoospermic
20 men, the occurrence of hypermethylation of several maternally imprinted DMRs or
21 hypomethylation of paternally imprinted DMRs is increased (Marques et al. 2004;
22 Kobayashi et al. 2007; Marques et al. 2008; Hammoud et al. 2010; Sato et al. 2011).

1 Boissonnas et al. also reported the association between methylation and sperm
2 concentration in teratozoospermic (TZ) and oligoasthenoteratozoospermic (OAT)
3 patients (Boissonnas et al. 2010). In the TZ group, 11 of 19 patients displayed loss of
4 methylation of the *IGF2* DMR or of both the *IGF2* DMR and the *H19* DMR. In the
5 OAT group, 16 of 22 patients displayed a severe loss of methylation of the *H19* DMR,
6 and this closely correlated with sperm concentration. Marques et al. suggested an
7 association between aberrant epigenetic sperm modifications and oligozoospermia
8 (Marques et al. 2004). Normozoospermic individuals (0.13%), Moderate (17%) and
9 Severe (30%) oligozoospermic patients all showed abnormal methylation of *H19*. We
10 examined the DNA methylation status of seven imprinted genes in spermatic DNA
11 obtained from infertile men and also found abnormal maternal and paternal DNA
12 methylation at several imprinted loci (**Figure 3**). Samples (10/96 cases) with both
13 maternal and paternal defects were primarily from men with severe oligospermia.
14 Importantly, the outcome of ART (fertility rates and implantation rates) with sperm
15 shown to have an abnormal DNA methylation pattern is generally poor (Kobayashi et
16 al. 2007).

17 As spermatogenesis progresses, the genome undergoes major changes that not
18 only influence genetic and epigenetic information but also alter the nuclear structure. It
19 is consequently important to understand how the specific nucleoprotamine/histone
20 structure of the sperm nucleus conveys epigenetic information and how this might
21 control early embryonic growth. In most cell types, DNA is wrapped around histone but
22 in sperm, protamines, which are small arginine-rich nuclear proteins, replace histones

1 late in the haploid phase of spermatogenesis and these proteins are essential for
2 spermatogenic function (Cho et al. 2001)(**Figure 2**). Both the phosphorylation of protamines
3 and the ratio of the two human protamines, protamine (P1) and protamine 2 (P2), are
4 important for optimal sperm function. The P1/ P2 ratio in fertile men ranges from 0.8 to
5 1.2 (Carrell and Liu 2001). Perturbation of this ratio, either higher or lower than normal,
6 has been reported to be associated with poor semen quality, increased DNA damage
7 and/or decreased fertility (Chevallier et al. 1987; Balhorn et al. 1988; Belokopytova et
8 al. 1993; Carrell et al. 1999; Razavi et al. 2003; Aoki et al. 2005). An increasing number
9 of reports now support the hypothesis that sperm DNA is not homogeneously packed
10 with these protamines and that histones are still present at some sites (Rousseaux et al.
11 2005). While some investigators have suggested that this is due to inefficient protamine
12 replacement, the persistence of histones at certain sites may play a functional role in
13 supporting the epigenetic code in the sperm (Weber et al. 2007). Protamine replacement
14 occurs in the spermatid stage of spermatogenesis after the completion of meiosis
15 (Baarends et al. 1999). The elongating spermatid also undergoes other maturational
16 events that affect motility and fertilization ability during the period of protamine
17 replacement. The association between abnormal protamine replacement and generally
18 diminished semen quality may be a defect in the unique gene regulation system of
19 temporal uncoupling of transcription and translation during spermatogenesis (Carrell et
20 al. 2007).

21 Alteration of the P1 to P2 ratio generally denotes abnormal spermatogenesis
22 and is a possible direct cause of abnormal methylation of maternal and paternal gDMRs

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

10

11

12 **Teratological environmental factors (endocrine disruptors) and epigenetic**
13 **modifications**

14 Abnormal sperm development may originate from exposure of the male
15 germline to environmental factors. Persistent organic pollutants (POPs), which were
16 used intensively worldwide for several decades until the 1980s, have been implicated in
17 reproductive disorders. Because of the stability and bioaccumulation of these
18 compounds in the environment, human populations are simultaneously exposed to a
19 variety of those contaminants through the consumption of food. Several POPs have
20 been shown to have toxic effects on reproductive and endocrine functions in humans
21 (Govarts et al. 2012) and a number of human epidemiological studies have
22 demonstrated the adverse effects of POPs exposure on markers of reproduction,

1 including semen quality (sperm concentration, motility, and morphology) (Guo et al.
2 2000; Richthoff et al. 2003; Toft and Guillette 2005; Meeker et al. 2010), spermatric
3 DNA integrity (Bonde et al. 2008; McAuliffe et al. 2012), and circulating reproductive
4 hormone levels (Richthoff et al. 2003), though some studies found only marginal effects
5 (Toft et al. 2006; Haugen et al. 2011). In general, however, these reports suggest that
6 POPs have adverse effects on reproductive health outcomes.

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

21 Seminal tract infection, one of the most common causes of infertility in men
22 (Keck et al. 1998), may also contribute to abnormal sperm development. The presence

1 of leukocytes in semen, also known as leukocytospermia, (Korrovits et al. 2008;
2 Cumming and Carrell 2009), is an indicator of seminal tract infection although this
3 correlation remains controversial (Bezold et al. 2007). Asthenozoospermia is often
4 associated with the presence of infection or leukocytes in semen although it is not
5 known whether infection plays a causative role (Wolff 1995). The association between
6 epigenetic changes and such sperm abnormalities as asthenozoospermia and
7 leukocytospermia is unknown. However, there is a precedent for infection inducing
8 epigenetic alterations in other cell types. In gastric carcinogenesis, *H. pylori* infection
9 induces aberrant promoter methylation in tumor-suppressor genes, including *p16^{INK4A}*,
10 *LOX*, and *CDHI* (Kaneda et al. 2004; Ushijima et al. 2006). Further work is required to
11 establish whether epigenetic alterations in sperm are induced by seminal tracts
12 infections.

13 Social stress, acting through hormone signalling pathways, is another recent
14 additions to the group of environmental factors that are known to induce epigenetic
15 changes. The extent and type of maternal care very early in life in rodents has been
16 shown to influence epigenetic marks at the glucocorticoid receptor in the neonatal
17 hippocampus, and this may influence later life stress responses in the offspring (Weaver
18 et al. 2004; Meaney et al. 2007). Furthermore, in another rodent model, Roth et al.
19 found that psychosocial stress (comparable to human post-traumatic stress disorder
20 (PTSD)) led to an increase in *Bdnf* methylation in the dorsal hippocampus and
21 downregulation of *Bdnf* expression in the dorsal and ventral hippocampus, but not in
22 other PTSD-relevant regions (Roth et al. 2011). The induction of region-specific

1 epigenetic changes in response to traumatic stress during adulthood demonstrates that
2 DNA methylation remains an active process that can be shaped by environmental
3 factors even in the adult nervous system. Again, the effect of stress on the sperm
4 epigenome has not been investigated. However, stress is also a cause of male infertility
5 this may occur through epigenetic alterations in the germline (Bale 2014).

6

7 **Nutrition and epigenetic regulation**

8 Epigenetic marks are tightly regulated, both temporally and spatially, during
9 fetal development and lactation (Lee et al. 2002; Allegrucci et al. 2005; Morgan et al.
10 2005) but can be influenced at key stages by diet. Agouti viable yellow (A^{vy}) is a
11 fascinating animal model whereby the environmental influences on the epigenome can
12 be monitored via a coat colour phenotype (Wolff et al. 1999). A gene alteration, which
13 involves an intra-cisternal A particle (IAP) retrotransposon insertion upstream of the
14 agouti gene (A), leads to ectopic expression of the agouti protein and a change of hair
15 color from agouti to yellow. The extent of this coat colour change is influenced by the
16 degree of methylation of the IAP element, which can be influenced by methyl donor
17 supplementation of the maternal diet (Waterland and Jirtle 2003). Dietary
18 supplementation with a methyl donor during pregnancy increases the proportion of pups
19 carrying a methylated IAP sequence and thus the number with a yellow coat colour
20 (Rakyan et al. 2003; Waterland and Jirtle 2004). Maternal and post-weaning high fat
21 diets can also alter epigenetic regulation of the hedonic reward pathways and metabolic
22 regulation of the energy balance in mice (Vucetic et al. 2011), and alter methylation of

1 the leptin promoter in rats (Milagro et al. 2009). These data provide compelling
2 evidence that diet alone can alter the epigenome.

3 Nutrition during early growth and development may influence DNA
4 methylation because one-carbon metabolism is dependent on dietary methyl donors and
5 on cofactors such as methionine, choline, folic acid and vitamin B-12 (MacLennan et al.
6 2004). The limited availability of acetyl-CoA for HAT activity and methyl donors of
7 SAM (*S* - adenosylmethionine) provided via the folate-methionine pathway may
8 therefore play a role in the establishment of inappropriate epigenetic patterns.
9 Conversely, dietary supplementation may provide a route to attenuating inappropriate
10 epigenetic patterns as the changes in DNA methylation which result from a decrease in
11 DNMT1 (DNA methyltransferase) activity can be partially prevented by folate
12 supplementation (Lillycrop et al. 2005; Lillycrop et al. 2007).

13 The influences of poor nutrition on epigenetic marks is not limited to the fetal
14 stage. Nutrition during postnatal development can permanently alter the epigenetic
15 regulation of some imprinted genes. Methyl-donor-deficient diet in postnatal life is
16 associated with altered epigenetic regulation of *IGF2* and growth retardation (Waterland
17 et al. 2006). In humans, diet has been shown to affect the DNA methylation status of
18 patients with hyperhomocysteinaemia. This disease is caused by the accumulation of
19 S-adenosylhomocysteine (an inhibitor of DNA methyltransferases)(Waterland et al.
20 2006).

21 Given the consequences of altered nutrient availability in a number of situations, it is
22 possible that changes may be also occur the male germline in response to diet. One very

1 compelling study demonstrated that a low protein diet in male rats results in altered
2 chromatin packing in sperm and changes in DNA methylation in the offspring (Carone
3 et al. 2010). These data all suggest that something as seemingly innocuous as a dietary
4 imbalance can have a detrimental effect on the epigenome at certain critical stages.

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

17
18

1 **Conclusions**

2 Mounting evidence from both human studies and animal models suggests that
3 epigenetic modifications provide a link between the environment and alterations in gene
4 expression that might lead to disease phenotypes. Importantly, direct evidence from
5 animal studies supports the role of environmental epigenetics in male infertility and
6 suggests the possibility that the use of ART to treat male infertility may lead to disease
7 later in life. However, ART is a relatively recent technology and the longer term
8 consequences of ART treatments such as ICSI and embryo freezing before transfer have
9 not yet been manifested due to the young age of the majority of ART children.
10 Environmental exposures to nutritional, chemical and physical factors all have the
11 potential to alter gene expression and, therefore, modify sperm quality in various ways
12 through changes in the epigenome. A summary of the factors known to influence DNA
13 methylation is presented in **Figure 4**.

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

21 The developmental origins of health and disease (DOHaD) paradigm, first
22 proposed by Prof. David Barker, postulates that suboptimal growth early life can

1 program changes which affect life long health, increasing the risks for various diseases.
2 There is evidence both from human studies and experimental models that this
3 programming may be mediated via changes in the epigenome. Epigenetic changes likely
4 occur during the fetal and infant periods but it is clear that oocytes and sperm are also
5 vulnerable to environmentally-induced epigenetic alterations, and that the newly
6 fertilised zygote is at a particular susceptible stage.

7

8 **Future perspective**

9 While genomic imprinting disorders are very rare, it is increasingly apparent that the
10 bulk of common human diseases do not arise solely from genetic or environmental
11 causes but also have an epigenetic component. Our knowledge that the epigenomes of
12 gametes and newly fertilized embryos are susceptible stages for
13 environmentally-induced epigenetic changes has particularly important implications as
14 changes in lifestyle and modes of reproduction may have long term implications for
15 human health that are not yet fully appreciated. Recent work identifies advanced
16 paternal age as a risk factor for autism, depression, epilepsy and prostate cancer in
17 children (Kondrashov 2012; Sun et al. 2012). While there are a number of possible
18 explanations for these associations, the accumulation of epigenetic errors in the sperm
19 may be a contributory factor. As the human population ages and the use of ART
20 increases worldwide, it will become increasingly important to determine the extent to
21 which environmentally-induced epigenetic changes contribute to disease. A detailed
22 characterisation of the normal epigenetic process that take place in the germline and

1 during very early development will be important in achieving this goal. Understanding
2 how and when environmental factors can influence the epigenome to cause disease,
3 identifying ways in which to modulate aberrant epigenetic marks, and also determining
4 the best timeframe to reverse aberrant epigenetic marks all have the potential to lead to
5 improved human health.
6

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1 **Figure legends**

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

10

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

1 primary oocyte; ProSpg: prospermatogonium; Spg: spermatogonium; PSp: primary
2 spermatocyte; SSp: secondary spermatocyte.

3

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

10

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

14

15 **Table 1. ART and imprint-associated disorders.**

16 BWS: Beckwith-Wiedemann syndrome, AS: Angelman syndrome, SRS: Silver-Russell
17 syndrome, RB: Retinoblastoma.

18

19 **Table 2. DNA methylation errors in the human spermatozoa**

20 OAT: patients presenting with combined oligozoospermia, asthenozoospermia and
21 teratozoospermia, ANJ: Anejaculation, OAZI: secondary inflammatory obstructive
22 azoospermia, CBAVD: obstructive azoospermia due to congenital bilateral absence of

1 the vas deferens, HP: secretory azoospermia due to hypospermatogenesis.

2

1 **Abbreviations**

2

3 ANJ: Anejaculation,

4 ART: assisted reproductive technologies

5 AS: Angelman syndrome

6 Avy: Agouti viable yellow

7 BS: Bisulphite PCR sequence method

8 BWS: Beckwith-Wiedemann syndrome

9 CBAVD: obstructive azoospermia due to congenital bilateral absence of the vas
10 deferens

11 COBRA: combined bisulphite PCR restriction analysis

12 DNMT: DNA methyltransferase

13 DOHaD: The developmental origins of health and disease

14 gDMRs: gametic differentially methylated regions

15 GIFT: gamete intrafallopian transfer

16 HP: secretory azoospermia due to hypospermatogenesis

17 IAP: intra-cisternal A particle

18 ICRs: imprinting control regions

19 ICSI: intracytoplasmic sperm injection

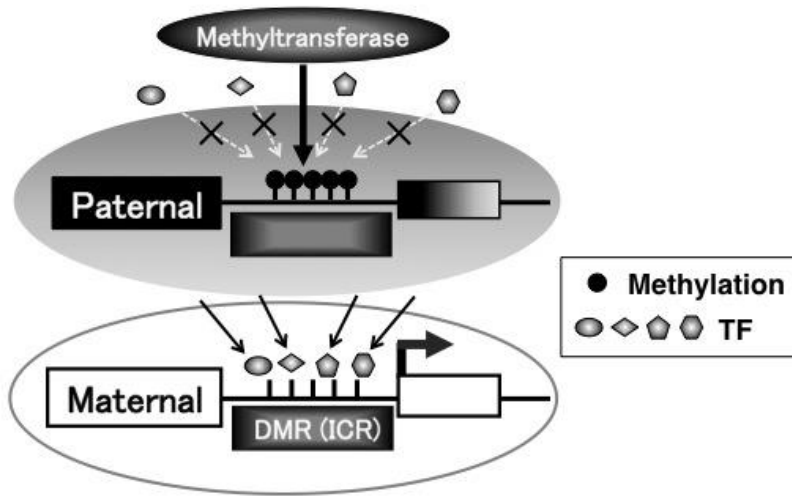
20 IVF: *in vitro* fertilization

21 IVM: *in vitro* oocyte maturation

22 OAT: oligoasthenoteratozoospermic

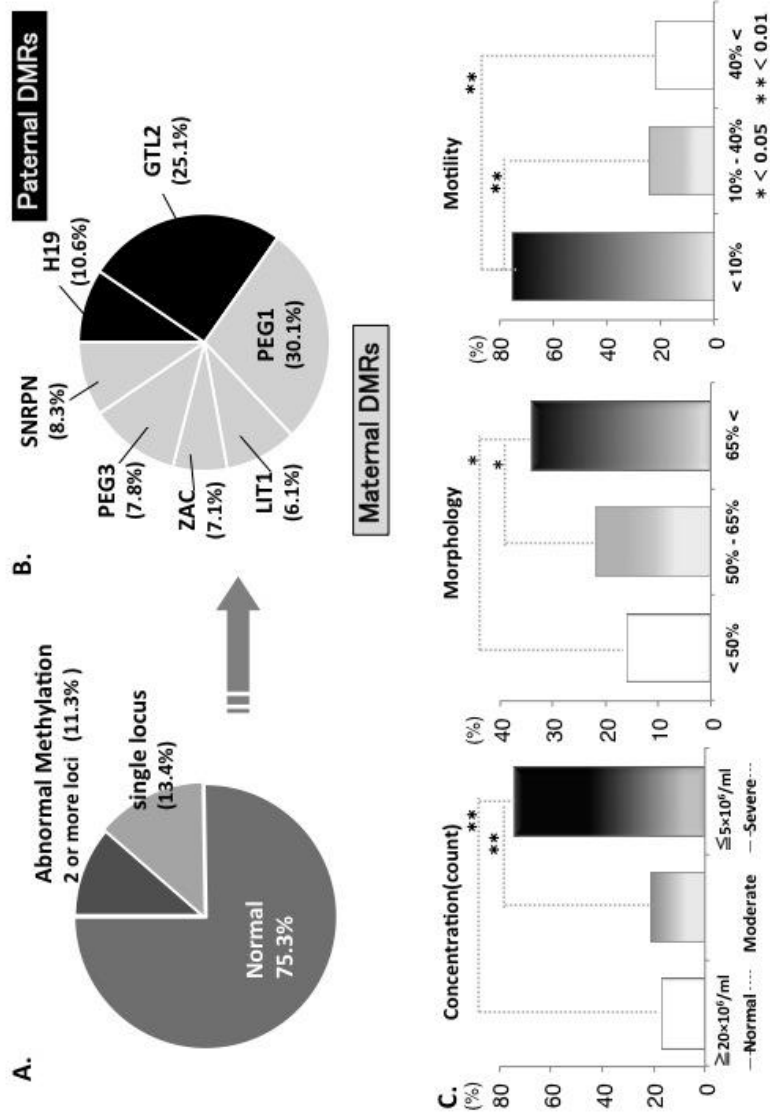
- 1 OAZI: secondary inflammatory obstructive azoospermia
- 2 Oog: oogonium
- 3 PGC: primordial germ cell
- 4 PGD: preimplantation genetic diagnosis
- 5 POPs: Persistent organic pollutants
- 6 POo: primary oocyte
- 7 PSp: primary spermatocyte
- 8 PTSD: post-traumatic stress disorder
- 9 ProSpg: prospermatogonium
- 10 PWS : Prader-Willi syndrome
- 11 ROSI: round spermatid injection
- 12 SAM: S - adenosylmethionine
- 13 SRS: Silver-Russell syndrome
- 14 SSp: secondary spermatocyte
- 15 Spg: spermatogonium
- 16 TF: transcriptional factor
- 17 TZ: teratozoospermic
- 18 ZIFT: zygote intrafallopian transfer
- 19

Figure.1



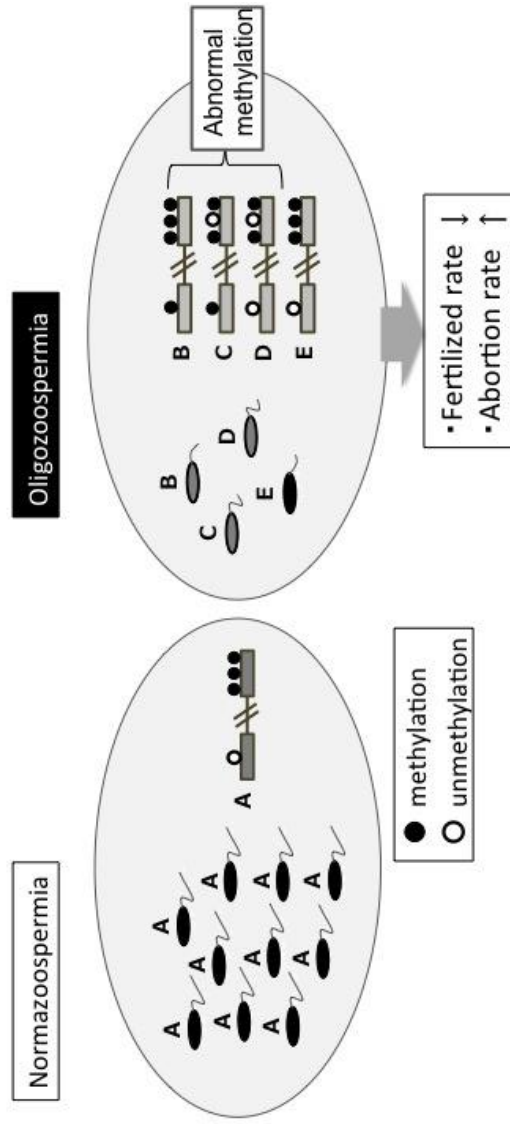
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Figure 3



1

D.



(Kobayashi et al. HMG 2007 Revised)

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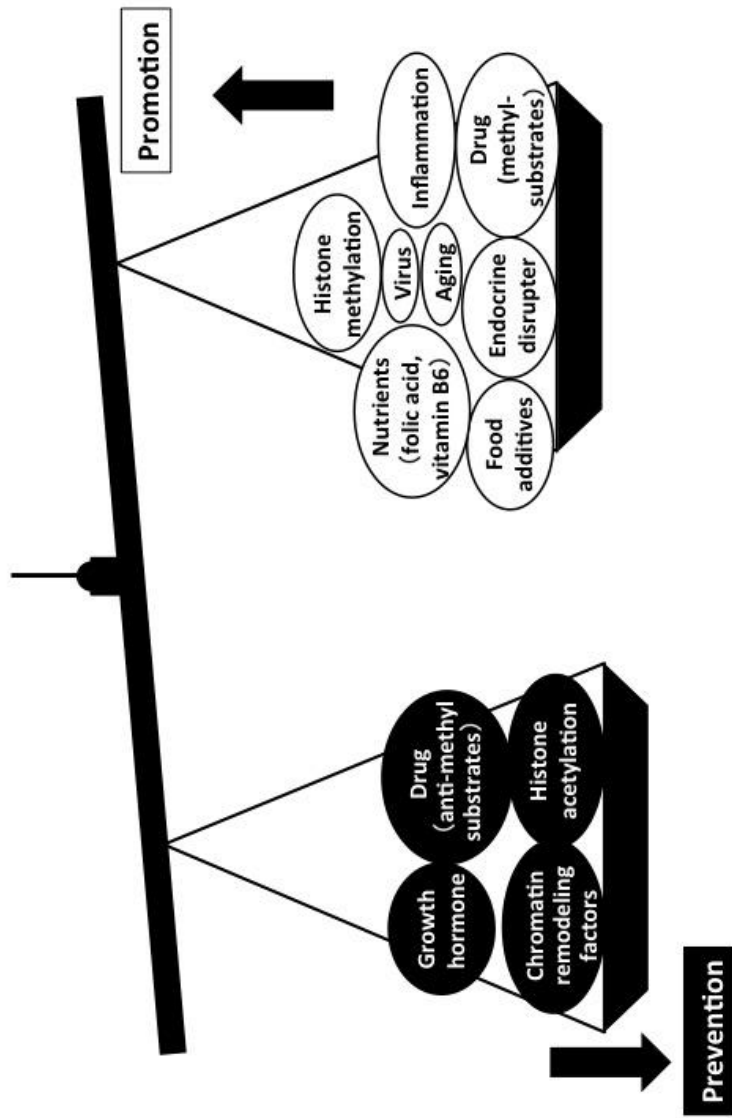


Figure.4

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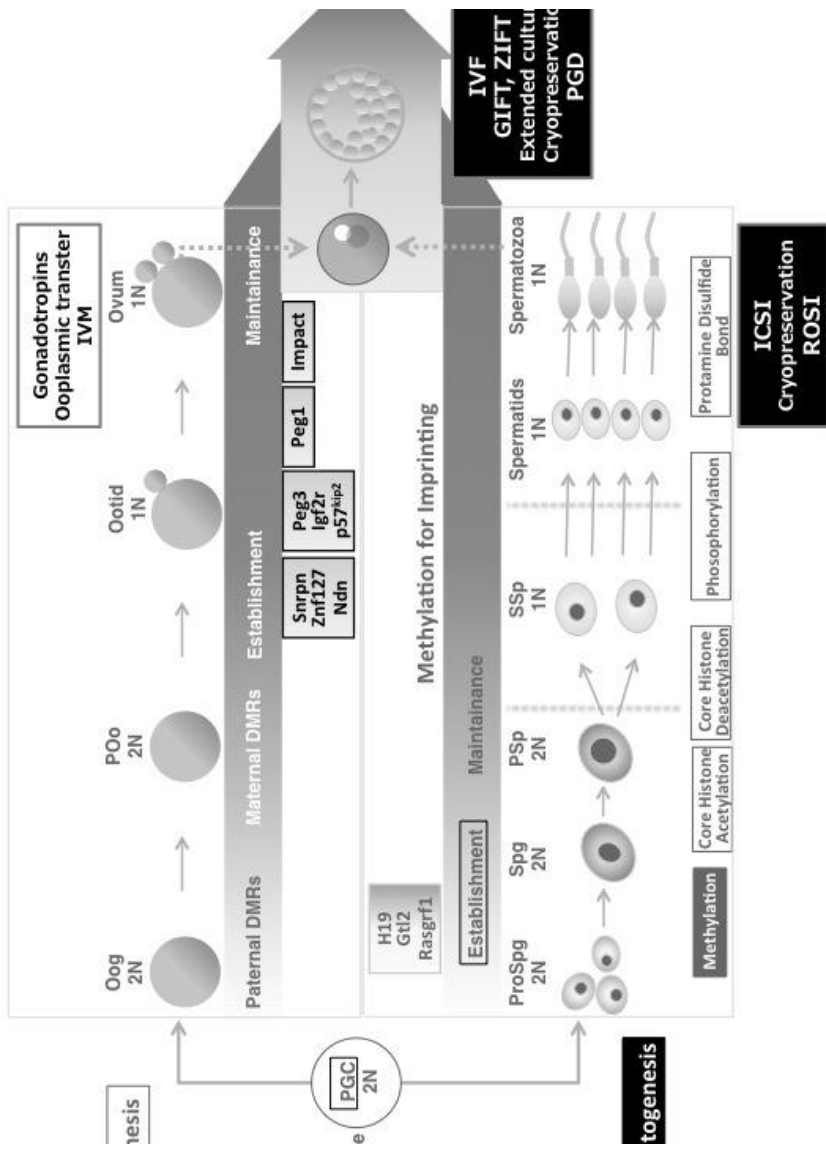
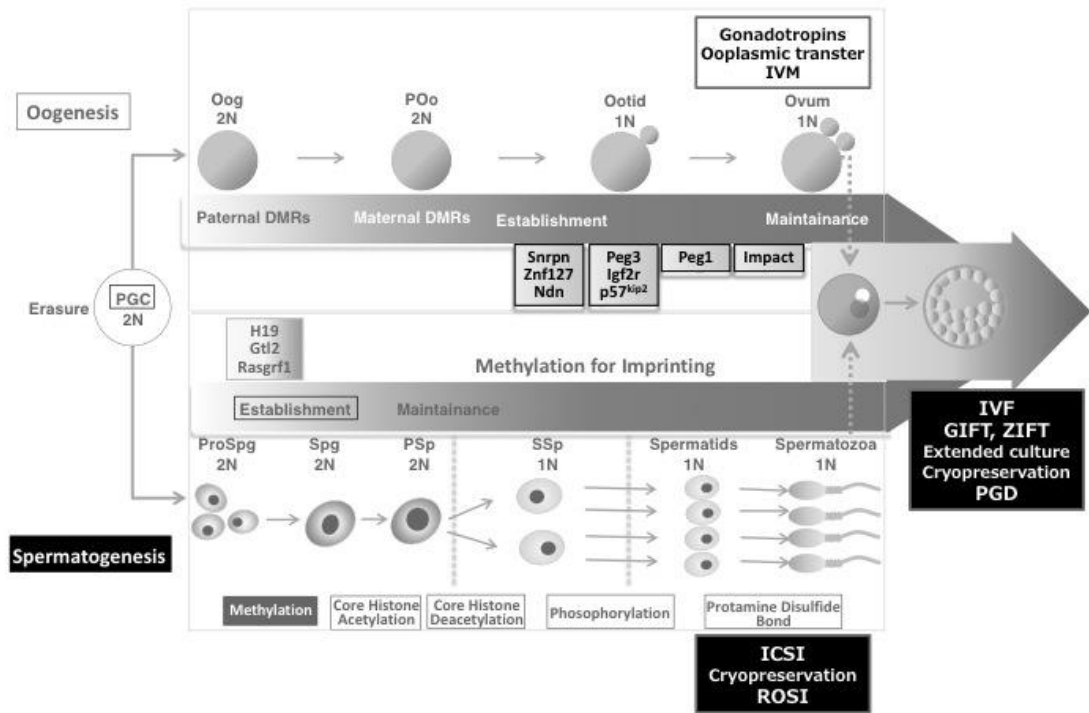


Figure.2



1