Epigenetic regulation in male germ cells

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Abstract

In recent years, it has become increasingly clear that epigenetic regulation of gene expression is critical during spermatogenesis. In this review, the epigenetic regulation and the consequences of its aberrant regulation during mitosis, meiosis and spermiogenesis are described. The current knowledge on epigenetic modifications that occur during male meiosis is discussed, with special attention on events that define meiotic sex chromosome inactivation. Finally, the recent studies focused on transgenerational and paternal effects in mice and humans are discussed. In many cases, these epigenetic effects resulted in impaired fertility and potentially long-ranging effects underlining the importance of research in this area.

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Introduction

Male infertility is a very common problem affecting 1 in 20 men in western populations. Although there is a large amount of research in the field, the underlying causes in many cases are unknown. Many studies have explored the genetic causes of human male infertility using mouse models (O'Bryan & de Kretser 2006) or through candidate gene sequencing approaches using genomic DNA from infertile men (Miyamoto *et al.* 2003). As with other conditions including cancer, neurological and immune disorders, and even ageing (Egger *et al.* 2004, Rodenhiser & Mann 2006), it is also likely that epigenetic changes will contribute to cases of male infertility.

Spermatogenesis is a highly regulated process; it is not surprising that epigenetic mechanisms are involved and that perturbations in these mechanisms can result in male infertility. There have been various links drawn between epigenetic mechanisms and important events during spermatogenesis, including chromosome condensation, XY body formation, retrotransposon silencing and packaging of DNA into the spermatid nucleus; however, the underlying molecular mechanisms are only now becoming clear. While epigenetic regulation is crucial in many systems within the body, this review will concentrate on spermatogenesis, male fertility and the consequences of aberrant regulation for offspring.

Epigenetic modifications

Epigenetics is the study of mitotically and/or meiotically heritable changes in gene expression or cellular

phenotype, which occur without changes to the underlying DNA sequence (Goldberg *et al.* 2007). Epigenetic changes such as DNA methylation, histone methylation, histone acetylation and histone phosphorylation, and chromatin remodelling such as altering the position of nucleosomes all have a direct effect on chromatin structure. The ability to regulate chromatin structure is essential in eukaryotic organisms in order to organise the enormous sequence content of DNA into a compact arrangement and for defining which pieces of DNA are available for transcription. Indeed, chromatin structure is integral in defining both the phenotype of a cell and an organism as a whole.

Chromatin is made-up of histones and DNA, both of which may be modified to affect the way in which they interact. Histones are composed of predominantly positively charged amino acids, which allow them to bind with a high affinity to negatively charged DNA (Alberts *et al.* 1989). There are four types of nucleosomal histones: H2A, H2B, H3 and H4. Histones are responsible for the coiling of DNA into nucleosomes. Histone variants exist for histones H3 (CENP-A, H3.3), H2A (H2AZ, H2AX, macroH2A) and H2B (TH2B). Each has a different role in maintaining chromatin structure. Two copies of each nucleosomal histone (H2A, H2B, H3 and H4) form a histone octamer core around which 147 bp of double-stranded DNA is wound creating a nucleosome (reviewed in Zhang & Reinberg 2001; Fig. 1). Nucleosomes are the basic subunit of chromatin. Linker DNA as well as histone H1 links all the nucleosomes together to create a 30 nm chromatin

Figure 1 Nucleosome structure. (A) A model of a nucleosome representing the histone octamer core around which 147 bp of DNA is wrapped. (B) Schematic of a nucleosome. Black line represents DNA wrapped around octamer core. Amino-terminal tails extend out from the globular domain of each core histone (Allis *et al.* 2007).

fibre. Epigenetic mechanisms regulate the association between the DNA and histones in such a way that genes are made transcriptionally active or inactive.

Heterochromatin is chromatin that is transcriptionally inactive; that is, the genes within it are repressed. Heterochromatin involves a very tight association between the DNA and histones (Allis *et al.* 2007). For example, a compacted heterochromatic DNA structure is needed during meiosis for the chromosomes to align and recombine efficiently. Euchromatin is chromatin that is transcriptionally active and contains DNA bound loosely to histones (Allis *et al.* 2007). Relaxed, euchromatic structures are needed throughout the majority of spermatogenesis, or at least up until the elongated spermatid stage of spermatogenesis, in order to orchestrate the temporal expression of mRNAs that underlie the changes in nuclear and cytoplasmic morphology of the germ cells.

DNA methylation

DNA methylation is one of the most well-studied epigenetic genomic modifications. DNA methylation involves the transfer of a methyl group to the five-positioned carbon of a cytosine within a CpG dinucleotide (Nakao 2001, Aapola *et al.* 2004; Table 1). Approximately 60–90% of all CpG sequences in the genome are methylated, while unmethylated CpG dinucleotides are mainly clustered in the CpG-rich

Table 1 Types of epigenetic modifications and their generalised functions.

Epigenetic modification	Role in transcription
DNA methylation	Gene repression
Histone methylation	Gene repression and activation
Histone demethylation	Gene repression and activation
Histone acetylation	Gene activation
Histone deacetylation	Gene repression
Histone phosphorylation	Gene activation and repression
Histone ubiquitylation	Gene repression and activation
Histone sumoylation	Gene repression

sequence, termed CpG island, of the gene promoter region (Ng & Bird 1999). DNA methylation at promoter regions is involved with gene silencing. DNA methylation is also used to silence retrotransposons and imprinted genes (see below).

DNA methylation is performed by a group of proteins termed DNA (cytosine-5) methyltransferases (DNMTs). In the mouse, there are four methyltransferases: DNMT1, DNMT2 and the two closely related methyltransferases DNMT3A and DNMT3B (reviewed in Goll & Bestor 2005). DNMT3A and DNMT3B are *de novo* enzymes that establish methylation patterns. DNMT1 is a maintenance enzyme involved in preserving methylation patterns already acquired (Table 2). DNMT2 has been found to methylate RNA in humans (Goll *et al.* 2006). It is unknown whether it has a similar role in the mouse. The mouse and several other species, including humans, also contain a closely related sequence, DNMT3L. DNMT3L lacks a catalytic domain, but can interact with both DNMT3A and DNMT3B (Ooi *et al.* 2007). Both *in vitro* and *in vivo* studies have shown that DNMT3L facilitates the *de novo* methyltransferase activity of DNMT3A (Nimura *et al.* 2006, Suetake *et al.* 2006). There is a lack of data showing that DNMT3L stimulates DNMT3B; however, *in vitro* studies have shown that interactions occur between the two proteins (Suetake *et al.* 2004).

Histone modifications

Histone modifications are essential during many cellular processes including mitosis and spermatogenesis. They assist in the formation of either condensed heterochromatic states or open euchromatic states. Histones are composed of a globular domain and amino-terminal extensions known as histone tails. Histone tails contain residues that can be post-translationally modified by methylation, acetylation, phosphorylation, ubiquitylation and sumoylation (reviewed in Lachner & Jenuwein 2002, Fuks 2005; Fig. 2). These post-translational modifications (PTMs) alter chromatin structure thereby allowing the underlying gene to be activated or repressed.

Methylation is one of the most common histone modifications. There are several arginine and lysine residues in the N-termini of histones that are known to be methylated (Lachner *et al.* 2003; Fig. 2). Histone methylation is mediated by histone methyltransferases (HMTases). Histone arginine methylation is involved in both gene activation and silencing (reviewed in Berger 2002; Table 1). Lysine residues can be mono-, di- and trimethylated and have been associated with both transcriptional silencing and activation during spermatogenesis (reviewed in Lachner & Jenuwein 2002; Table 1). Histone demethylases mediate the removal of methyl groups from both arginine and lysine residues and are involved in both gene activation and silencing

Table 2 Testis phenotypes of mouse models with epigenetic regulators knocked-out.

Gene/protein	Function	Phenotype	References
<i>Dnmt3l</i>	Facilitates DNA methylation	Deficient pairing of homologous chromosomes at the zygotene stage. Loss of germ cells evident at 6 days <i>post partum</i> . Increased retrotransposon expression from the gonocyte stage. Loss of paternal imprints.	Bourc'his & Bestor, (2004), Webster <i>et al.</i> (2005) and La Salle <i>et al.</i> (2007)
<i>Dnmt1</i>	DNA methylation	Loss of genomic methylation, improper expression of imprinted genes and increased expression of the IAP transposons in mutant embryos.	Li <i>et al.</i> (1992), Lei <i>et al.</i> (1996), Panning & Jaenisch, (1996) and Walsh & Bestor (1999)
<i>RAD54 (Rad54l)</i> <i>Suv39h1</i> and <i>Suv39h2</i>	Chromatin remodelling Histone methylation	Defects in DNA DSB repair during meiosis Chromosome pairing defects, loss of spermatocytes at pachytene	Essers <i>et al.</i> (1997) Peters <i>et al.</i> (2001)
<i>G9a (Ehmt2)</i>	Histone methylation	Improper synaptonemal complex formation, major loss of spermatocytes	Tachibana <i>et al.</i> (2007)
<i>Meisetz (Prdm9)</i>	Histone methylation	Deficient pairing of homologous chromosomes during zygotene, impaired XY body formation	Hayashi <i>et al.</i> (2005)
<i>HR6B (Ube2b)</i>	Histone ubiquitylation	Major defects in meiotic prophase, synaptonemal complexes do not form	Baarends <i>et al.</i> (2003)
<i>H2ax (H2afx)</i>	Chromatin remodelling	Impaired XY body formation leading to spermatocytes arresting at the pachytene stage.	Fernandez-Capetillo <i>et al.</i> (2003)
<i>TP1 (Tnp1)</i> and <i>TP2 (Tnp2)</i>	Chromatin condensation and remodelling	Major abnormalities in condensation of sperm nuclei, presence of DNA strand breaks in spermatids	Zhao <i>et al.</i> (2004)
<i>Prm1</i> or <i>Prm2</i>	Chromatin compaction	In the haploinsufficient state, morphologically abnormal sperm (e.g. elongated heads) are produced with altered sperm chromatin integrity.	Cho <i>et al.</i> (2003)
<i>Camk4</i>	Protamine phosphorylation	Impairment of spermiogenesis due to loss of elongating spermatids and spermatozoa in the testis.	Wu <i>et al.</i> (2000)
<i>Snf2h (Smarca5)</i>	Chromatin remodelling	In the haploinsufficient state, a loss of methylation at the A ^γ IAP retrotransposon in wild-type offspring.	Chong <i>et al.</i> (2007)
<i>Jhdm2a (Jmjd1a)</i>	Histone demethylation	Post-meiotic germ cells display chromatin condensation defects. Reduced expression of <i>Tnp1</i> and <i>Prm1</i> .	Okada <i>et al.</i> (2007)

(reviewed in Agger *et al.* 2008; Table 1). The HMTases suppressor of variegation 3–9 homolog 1 (SUV39H1) and SUV39H2, the methylation of histones H3 and H4, and the demethylation of histone H3 are crucial during spermatogenesis; these modifications will be discussed later in this review.

Histone acetyltransferases (HATs) can acetylate specific lysine residues in the N-termini of all core histones and are associated with gene activation (An 2007; Table 1, Fig. 2). Histone deacetyltransferases reverse the action of HATs and are associated with gene repression (Table 1). The acetylation and deacetylation of several lysine residues on histones H3 and H4 are important during male meiosis and will be discussed later in this review.

Histone phosphorylation occurs on several serine residues of all the histones and is generally associated with transcriptional activation (reviewed in Berger 2002; Table 1, Fig. 2). By contrast, phosphorylation of the histone variant H2AX (forming γ H2AX) correlates with chromosome condensation and gene silencing during male meiosis (Fernandez-Capetillo *et al.* 2003; Table 1).

Ubiquitylation on specific lysine residues can either enhance or repress transcription (reviewed in Conaway

et al. 2002; Table 1). Ubiquitylation is the modification of a protein by the covalent attachment of an ubiquitin monomer to lysine residues. Histone H2A can be mono-ubiquitylated and its presence is linked to transcriptional repression during male meiosis (Baarends *et al.* 2005; Fig. 2). Conversely, H2B mono-ubiquitylation is linked to transcriptional activation (Zhu *et al.* 2005; Fig. 2).

Histone lysine residues may also be sumoylated and have been linked to gene repression (Shiio & Eisenman 2003; Table 1). Sumoylation involves the covalent attachment of small ubiquitin-related modifier (SUMO) proteins to lysine residues (Shiio & Eisenman 2003). Sumoylation generally prevents the activation of PTMs such as acetylation. There are four known SUMO proteins (SUMO1–4). To date, SUMO1 is the only one associated with spermatogenesis specifically, in silencing the XY body during meiosis (Rogers *et al.* 2004).

ATP-dependent chromatin remodelling

Unlike DNA methylation and histone PTMs, ATP-dependent chromatin remodelling complexes are correlated with non-covalent chromatin remodelling.

Figure 2 Histone tail modifications. Some arginine residues (R) can be methylated. Some lysine residues (K) can be either acetylated, methylated or ubiquitylated. Some serine residues (S) can be phosphorylated. Acetylation, orange; methylation, green; phosphorylation, red; ubiquitylation, purple.

ATP-dependent complexes use the energy of ATP hydrolysis to change the position or structure of the nucleosomes and therefore affect the accessibility of transcription factors and other regulatory elements to the DNA (reviewed in Narlikar *et al.* 2002). ATP-dependent chromatin remodelling has been implicated in both transcriptional activation and repression. The predominant mechanism of ATP-dependent chromatin remodelling involves sliding the histones along the DNA. In addition, these complexes may twist, spool and bulge DNA (Fan *et al.* 2003). There are currently three known families of ATP-dependent chromatin remodelling complexes, the SWI/SNF (switch/sucrose nonfermenter) family, the ISWI (imitation switch) family and the MI-2 (myositis specific autoantigen 2) family, all of which are defined by the presence of a conserved catalytic ATPase subunit (Wang & Zhang 2001, Fan *et al.* 2005).

It has previously been shown that *Smarca5* (encoding SNF2H) from the ISWI family is expressed in the testis where it may have a role in DNA repair and recombination (Lazzaro & Picketts 2001, Chong *et al.* 2007). As discussed later in this review, a hypomorphic allele of *Smarca5* has been generated through an *N*-ethylnitrosourea (ENU) screen for epigenetic modifiers and has revealed that haploinsufficiency for *Smarca5* shows paternal-specific effects on the phenotype of offspring (Chong *et al.* 2007; Table 2). The ablation of *Smarca5*

results in embryonic lethality at the peri-implantation stage (Stopka & Skoultschi 2003). RAD54 from the SWI/SNF family has also been implicated in DNA repair during spermatogenesis. Disruption of *Rad54* results in aberrant homologous recombination and DNA repair defects (Essers *et al.* 1997; Table 2).

The epigenetic state during spermatogenesis

Spermatogenesis is a highly regulated sequence of events that result in the formation of spermatozoa from precursor cells (Clermont 1972). There are three major phases that together constitute spermatogenesis: stem cell renewal by the process of mitosis, reduction of chromosomal number by meiosis and the metamorphic process termed spermiogenesis involving the transformation of the haploid germ cell into a spermatozoon (de Kretser & Kerr 1994). Within male germ cells, changes in epigenetic state are critical for the silencing of transposable elements (TEs), the imprinting of paternal genes, several aspects of meiosis, post-meiotic gene silencing and DNA compaction.

Transposable elements

In the prenatal testis, the only germ cells present are gonocytes. The gonocytes proliferate for a few days and then become arrested (Huckins & Clermont 1968).

Shortly after birth, the gonocytes move to the basement membrane to become spermatogonia and resume proliferation (Huckins & Clermont 1968). Spermatogonia divide by mitosis and constitute the pool of stem cells from which meiosis and spermatogenesis proceed.

Prior to spermatogenesis the silencing of TEs takes place in the gonocytes and prospermatogonia. TEs are pieces of mobile DNA and include DNA transposons, long terminal repeat (LTR) retrotransposons, long interspersed nuclear elements (LINEs) and short interspersed nuclear elements (reviewed in Deininger & Batzer 2002). TEs comprise 45% of human and 37% of mouse DNA (Deininger *et al.* 2003). Their movement can be mutagenic and can cause chromosome breakage, improper recombination and genome rearrangement if not silenced via methylation (Deininger *et al.* 2003). TEs can amplify and re-insert into the genome causing ectopic activation or inactivation of genes. Normally, DNA methylation is established in the gonocytes to silence TEs thereby preventing propagation.

DNMT3L facilitated methylation is involved in the silencing of TEs in the testis as evidenced by an absence of DNMT3L causing a loss of methylation at LINE-1 and the intracisternal A-particle (IAP) transposons (Bourc'his & Bestor 2004, Webster *et al.* 2005, Hata *et al.* 2006; Table 2). Consistent with such a role, DNMT3L is expressed in the gonocytes at 14–18 days *post-coitum* (dpc) when global DNA methylation is occurring (Bourc'his & Bestor 2004). Indeed, it has been proposed that the absence of TE silencing in this model contributes directly to meiotic arrest and infertility in the adult mouse.

Paternal imprinting

Paternally imprinted genes are genes that are subject to monoallelic expression. That is, expression occurs only from the allele inherited from the maternal parent. Paternally imprinted genes are methylated, and silenced, in male germ cells. Paternal imprinting of genes starts to be established in the gonocytes at about 15.5 dpc, and continues on into the spermatogonia (Fig. 3). This methylation and gene silencing are carried through the remainder of spermatogenesis and into any resultant sperm and offspring. Only three paternally imprinted regions, *H19-Igf2*, *Rasgrf* and *Dlk1-Gtl2* have been identified to date (Davis *et al.* 1999, Li *et al.* 2004). Many more maternally imprinted genes have been identified.

In male germ cells, the establishment of the paternal imprints involves a factor named BORIS (brother of the regulator of imprinted sites; Klenova *et al.* 2002) and the DNA methyltransferases, DNMT3A, DNMT3B and the closely related DNMT3L (Kato *et al.* 2007). Deletion of *Dnmt3l* results in a loss of methylation at paternally imprinted regions (Table 2). Spermatogonia deficient in *Dnmt3a* and *Dnmt3b* displayed variations in methylation patterns at paternally imprinted regions (Kato *et al.* 2007).

DNMT1 is also important in the imprinting process. *Dnmt1*-deficient embryos displayed a loss of genomic methylation to less than 30% of that found in heterozygous or wild-type embryos; furthermore, aberrant methylation patterns were observed at paternally and maternally imprinted regions (Li *et al.* 1992, 1993; Table 2).

While the critical importance of appropriate paternal imprinting has been established for offspring inheriting such alleles naturally (see below), a recent paper showing an increased prevalence of abnormal methylation in a set of seven imprinted genes in infertile men with oligospermia (decreased sperm count) compared with normospermic men from infertile relationships (Marques *et al.* 2004, Kobayashi *et al.* 2007) raises the possibility that a failure of imprinting may actually cause infertility. Alternatively, the observed hypomethylation of paternally imprinted alleles may be indicative of inappropriate global methylation, and as such inappropriate gene expression. Comfortingly, the study by Kobayashi *et al.* (2007) suggests that at least in their study, global methylation defects had not occurred. Normally, of course, such situations would be self-limiting because of the resultant infertility; however, with increasing access to intracytoplasmic sperm injection (ICSI) there is a possibility that such hypomethylated sperm are used to conceive children wherein the affects may be much more debilitating. It should be noted, however, that at the time of writing this review this possibility was only theoretical as there is no published evidence of an increase in paternally imprinted diseases in children conceived via ICSI compared with standard *in vitro* fertilisation where embryos have been cultured under the same conditions.

Meiosis

Spermatogonia undergo differentiation and continuous rounds of mitosis to generate primary spermatocytes, which enter meiosis (de Kretser & Kerr 1994). Meiosis is characterised by two cell divisions, during which chromosome numbers are halved. In the first division, primary spermatocytes duplicate DNA content then divide to become secondary spermatocytes (Clermont 1972). In the second division, secondary spermatocytes divide to become round spermatids (Clermont 1972). A large part of meiosis is spent in prophase of the first division. Prophase accounts for over 90% of the duration of meiosis and lasts about 3 weeks in the mouse (Cobb & Handel 1998). Prophase 1 is further broken up into four stages: leptotene, zygotene, pachytene and diplotene. During leptotene of meiosis, the chromosomes start condensing and double-strand breaks (DSBs) begin forming. DSBs are essential for the recombination between all homologous chromosomes that occur later in meiosis (Roeder 1997, Cobb & Handel 1998). During zygotene, sister chromatids begin to pair and form synaptonemal complexes (SCs). SCs are proteinaceous

Figure 3 Epigenetic modifications occurring during spermatogenesis. In mitotic germ cells, DNA methylation occurs to set up the paternal-specific imprints. In meiotic cells, phosphorylation occurs to assist in both recombination and XY body formation. Ubiquitylation, sumoylation and incorporation of the H2AZ and H3.3 variants are all involved in XY body formation. Hyperacetylation occurs during spermiogenesis to assist in the histone–protamine transition.

meiosis-specific structures composed of two axial elements, which form along the length of the sister chromatids and are joined by a central element which 'zips up' the two axial elements (now called lateral elements; [Morelli & Cohen 2005](#)). Synapsis is completed during pachytene. Homologous recombination also occurs in pachytene spermatocytes. Prophase ends at diplotene when chromosome separation is initiated and SCs start disappearing (reviewed in [Baarends & Groote-god 2003](#)). Following prophase, the spermatocytes undergo metaphase, anaphase and telophase of the first division to produce two secondary spermatocytes per primary spermatocyte. During metaphase, the homologous chromosomes condense further and become attached to spindle fibres. The chromosomes separate,

but sister chromatids remain attached and move towards opposite poles during anaphase. The spindle is squeezed and the nuclei and cytoplasm start dividing during telophase.

The secondary spermatocytes contain a single set of chromosomes but have a 2n DNA content. During the second meiotic division, the chromosomes condense and the spermatocytes undergo prophase, metaphase, anaphase and telophase of meiosis II. During meiosis II, sister chromatids separate and divide to produce round spermatids which have a 1n content ([de Kretser & Kerr 1994](#)). The end products of meiosis are four haploid round spermatids per spermatocyte. Several aspects of meiosis are critically regulated by epigenetic modifications.

Chromatin remodelling and methylation during meiosis

As evidenced in several studies, epigenetic modifications are essential for several aspects of meiotic chromosome packing, pairing and recombination (Fig. 3). DNMT3L is involved in facilitating the methylation pattern required during male meiosis. In the absence of DNMT3L, meiotic chromosomes fail to form heterochromatin appropriately and fail to pair at the zygotene stage (Bourc'his & Bestor 2004, Webster *et al.* 2005; Table 3). Leptotene and zygotene spermatocytes displayed non-homologous synapsis and spermatocytes were unable to reach the full pachytene stage (Bourc'his & Bestor 2004). As a consequence, all germ cells eventually die by apoptosis or sloughing at the zygotene–pachytene stage (Bourc'his & Bestor 2004, Webster *et al.* 2005). It is believed that in *Dnmt3l* knockout males the loss of methylation resulted in an inappropriate euchromatic state during meiosis and genes that should be repressed, including imprinted regions, retrotransposons and repeat elements, remained active resulting in what the Bestor lab described as a 'meiotic catastrophe' (Bourc'his & Bestor 2004, Webster *et al.* 2005). It is clear that DNMT3L-mediated methylation is needed for meiotic cells to progress through spermatogenesis.

The HMTases SUV39H1 and SUV39H2 have also been implicated in epigenetic regulation during male meiosis (Peters *et al.* 2001; Table 2). *Suv39h1* is expressed in many tissues, while *Suv39h2* is mainly expressed in the testis (O'Carroll *et al.* 2000). SUV39H1 and SUV39H2 are responsible for tri-methylating lysine residue 9 on histone 3 (H3K9) at pericentric heterochromatic regions. H3K9 tri-methylation (H3K9me3) leads to the binding of the heterochromatin proteins HP1 α , PP β and HP1 γ (Lachner *et al.* 2001), and the subsequent binding of the HMTase SUV420H and the methyltransferases DNMT3A/DNMT3B and the di- and tri-methylation of H4K20. Cumulatively, this leads to the establishment of a transcriptionally repressed state (Lehnertz *et al.* 2003, Schotta *et al.* 2004). *Suv39h*-deficient mice displayed impaired spermatogenesis. Spermatocytes undergo apoptosis in the pachytene

stage as a consequence of incomplete homologue pairing and synapsis (Peters *et al.* 2001).

More recently, the HMTase G9A, which in somatic cells is involved in H3K9 mono- and di-methylation at euchromatic regions, has been shown to be important in male meiosis. Deletion of *G9a* resulted in embryonic lethality at mid-gestation, for this reason germ lineage-specific conditional *G9a* knockout mice were generated. These mice were sterile as spermatocytes failed to progress past the pachytene stage (Tachibana *et al.* 2007). Many spermatocytes were unable to form SCs. It was proposed that this perturbation in SC formation may be due to the chromosomes failing to find and pair with a homologue or to the overexpression of G9A-regulated genes (Tachibana *et al.* 2007).

In addition to dynamic changes in histone modifications, successful male meiosis also requires the use of specialised histone variants. The histone variant H2AX is an important part of the nucleosome of meiotic cells. In response to DNA DSBs, H2AX is phosphorylated at serine residue Ser139 to form phosphorylated histone H2AX (γ H2AX; Mahadevaiah *et al.* 2001). On autosomes during meiosis, γ H2AX localises to sites of DSBs and is involved in the chromatin condensation which occurs during meiotic recombination in leptotene and zygotene spermatocytes (Redon *et al.* 2002). It has been proposed that γ H2AX may act to recruit members of the DNA repair machinery to DSB sites (Baarends & Grootegeed 2003). Once DSBs disappear and synapsis is achieved, γ H2AX is no longer localised to DSB sites.

Other DNA repair proteins that are present during meiosis are RAD50 and RAD51. RAD50 is the first to be recruited to the site of DNA DSBs on the autosomes and is present from preleptotene until the end of zygotene (Eijpe *et al.* 2000). RAD51 is associated with the axial elements formed during leptotene (Tarsounas & Moens 2001). RAD54, as outlined above, is a DNA-dependent ATPase needed for recombination and DNA repair (Table 2). RAD54 interacts with RAD51 during DNA repair, together these proteins are believed to promote chromatin remodelling by displacing nucleosomes during homologous recombination (Alexeev *et al.* 2003).

Table 3 Epigenetic modifications associated with meiotic sex chromosome inactivation (MSCI) in the mouse.

Epigenetic modification	Reference
Ubiquitylation of histone H2A (forming uH2A)	Baarends <i>et al.</i> (2005)
Phosphorylation of histone H2AX (forming γ H2AX)	Fernandez-Capetillo <i>et al.</i> (2003)
Small ubiquitin-related modifier-1 (SUMO1) involved in sumoylation	Rogers <i>et al.</i> (2004)
Methylation of H3 (K27)	van der Heijden <i>et al.</i> (2007)
Di-methylation of H3 (K9, K27, K79)	Khalil <i>et al.</i> (2004) and van der Heijden <i>et al.</i> (2007)
Di-methylation of H4 (K20)	van der Heijden <i>et al.</i> (2007)
Tri-methylation of H3 (K9)	van der Heijden <i>et al.</i> (2007)
Tri-methylation of H4 (K20)	van der Heijden <i>et al.</i> (2007)
Deacetylation of H3 (K9)	Khalil <i>et al.</i> (2004)
Deacetylation of H4 (K12 and K16)	Khalil <i>et al.</i> (2004)
Acetylation of H4 (K5 and K8)	Khalil <i>et al.</i> (2004)

The ubiquitin-conjugating enzyme HR6B is also involved in DNA damage repair and has been implicated in the regulation of chromatin structure during meiotic recombination. In *Hr6b* knockout mice, spermatogenesis was impaired due to major changes found in the structure of the SCs. These mice also displayed an increased frequency of meiotic recombination (Baarends *et al.* 2003; Table 2).

Meiotic sex chromosome inactivation (MSCI)

In the pachytene stage of meiosis, the X and Y chromosomes form an XY (or sex) body and become transcriptionally silent in a process known as MSCI (Handel 2004, Turner 2007; Fig. 3). Unlike the autosomes, in males sex chromosomes are heterologous and only synapse at their pseudoautosomal regions. The development of the XY body and the maintenance of its heterochromatic state have been the focus of several recent studies (Baarends *et al.* 2007, van der Heijden *et al.* 2007). There are now a large number of known epigenetic modifications that are associated with MSCI, although the exact order of events are unclear (Table 3). Several of these modifications and modifiers are detailed below.

During pachytene when the autosomes synapse, γ H2AX localises to the XY body independently of DSBs occurring (Fernandez-Capetillo *et al.* 2003). The importance of H2AX in this process is indicated in *H2ax*-deficient spermatocytes which are unable to form an XY body and arrest at the pachytene stage (Fernandez-Capetillo *et al.* 2003; Tables 2 and 3). H2AX phosphorylation is dependent on the DNA repair protein ataxia telangiectasia and Rad3 related (ATR). ATR localises to the XY body at the onset of MSCI (Turner *et al.* 2004). The recruitment of ATR to the XY body depends on the tumour suppressor protein breast cancer 1, early onset (BRCA1; Turner *et al.* 2004). Meiotic cells of mice hypomorphic for *Brca1* do not undergo H2AX phosphorylation and the pachytene cells do not undergo MSCI (Turner *et al.* 2004). H2AX phosphorylation, ATR and BRCA1 all act together to initiate MSCI. This is one of the earliest events which takes place on the XY body during MSCI.

In order for MSCI to be maintained throughout pachytene, there are many other epigenetic modifications that occur on the XY body (reviewed in Hoyer-Fender 2003, Handel 2004, Turner 2007). They include ubiquitylation of H2A (forming uH2A), sumoylation (involving SUMO1), methylation of H3K27, di-methylation of H3K9, H4K20, H3K79 and H3K27, tri-methylation of H3K9 and H4K20, and deacetylation of H3K9, H4K12 and H4K16 (Table 3). Modifications known to be involved with gene activation such as H4K5 and H4K8 hyperacetylation are also found on the XY body. The exact function each performs, however, are unknown.

In addition, it is hypothesised that meiosis-induced factor containing a PR/SET domain and zinc-finger motif (MEISETZ), which is involved in H3K4 methylation, may have a role in activating genes essential for meiosis via XY body formation. *Mei* is expressed in the germ cells of the foetal female gonad and in the post-natal testis (Hayashi *et al.* 2005). *Mei* knockout males and females have an impaired DSB repair pathway and homologous chromosomes failed to pair (Hayashi *et al.* 2005; Table 2). Of relevance here, in the males, the sex body does not form and the spermatocytes displayed a meiotic arrest.

The appearance of the H2A variant H2AZ is also associated with MSCI in very late pachytene spermatocytes when many other heterochromatic marks are disappearing from the XY body (Greaves *et al.* 2006). At the completion of MSCI, H2AZ is incorporated into the XY body where it is thought to at least partially maintain the inactive state of the XY body in round and elongating spermatids (Greaves *et al.* 2006).

The H3 histone variant H3.3 has been implicated in MSCI. H3.3 is incorporated into the XY body during MSCI and coincides with the loss of not only the histone variants H3.1 and H3.2 but also most of the histone PTMs (van der Heijden *et al.* 2007). PTMs, such as mono-, di- and tri-methylated H3K4; di- and tri-methylated H3K9; mono- and di-methylated H3K27; di-methylated H3K79; and di- and tri-methylated H4K20, were all lost after H3.3 incorporation into the XY body (van der Heijden *et al.* 2007). In late meiotic and post-meiotic (i.e. round spermatid) stages, a select number of these histone modifications, predominantly those involved in gene silencing, are re-acquired (van der Heijden *et al.* 2007). H3.3 is known to be associated with modifications involved in gene activation including acetylation of K9, K14 and K18 (McKittrick *et al.* 2004). The exact role H3.3 performs in MSCI is unclear, although it is evident that H3.3 incorporation into the XY body promotes extensive chromatin remodelling and is essential for gene silencing on the XY body during the later stages of MSCI and the post-meiotic stages of spermatogenesis (van der Heijden *et al.* 2007).

In addition, SCMH1 (the mammalian homologue of *Drosophila* sex comb on midleg) has recently been shown to be important in regulating some of the chromatin modifications on the XY body. SCMH1 is part of the mammalian Polycomb repressive complex 1 (PRC1) and is involved in the repression of numerous genes (Levine *et al.* 2002). Within the testis, SCMH1 is expressed in the zygotene–pachytene spermatocytes and round spermatids where it is involved in the exclusion of the PRC1 complex from the XY body. As illustrated by a recent *Scmh1* knockout mouse line (missing the SPM domain), a loss of *Scmh1* leads to male sub-fertility characterised by a loss of spermatocytes by apoptosis (Takada *et al.* 2007). *Scmh1*-deficient pachytene spermatocytes failed to maintain exclusion of the PRC1

complex from the XY body leading to the XY body H3K27me3. Interestingly, a double knockout of *Scmh1* and *Phc2* (another polycomb protein) corrected this phenotype. SCMH1 binds to PHC2, via its SPM domain (Isono *et al.* 2005). These data indicate the SCMH1 is a key regulatory component of the PRC1 in maintaining exclusion from the XY body. PHC2 on the other hand may promote such an association (Takada *et al.* 2007). Gene expression data also indicated that inappropriate PRC1 exclusion from the XY body did not affect MSC1 (Takada *et al.* 2007).

Post-meiotic sex chromosome repression (PMSR)

It is now evident that at least partial XY body silencing persists after meiosis in a process known as PMSR (Namekawa *et al.* 2006). Through microarray studies focusing on X chromosome-linked genes, Namekawa *et al.* (2006) found that 87% of 676 genes on the X chromosome remain suppressed post-meiotically. This was comparable with pachytene spermatocytes where 92% of genes were repressed. Many studies have found that modifications associated with a heterochromatic state such as trimethylation of H3K9 and deacetylation of H3K9 and H4K12 remain on the sex chromosomes post-meiotically (Khalil *et al.* 2004, van der Heijden *et al.* 2007). It has been suggested that this silencing persists into the zygote; that is, the paternally inherited X chromosome (Xp) is pre-inactivated in female pre-implantation embryos (Huynh & Lee 2003). This theory is, however, hotly debated by others who propose that the Xp is not pre-inactivated, but rather is inactivated solely within the zygote by expression of the *Xist* gene (Okamoto *et al.* 2005).

Spermiogenesis

In addition to extensive cytoplasmic metamorphoses, during this post-meiotic phase the haploid round spermatids undergo extensive chromatin remodelling to develop into mature spermatozoa (Fig. 3). This involves re-shaping and condensation of the nucleus to transcriptionally inactivate and protect the DNA. This is assisted by DSBs and the histone-to-protamine transition (reviewed in Doenecke *et al.* 1997, Govin *et al.* 2004).

The histone-to-protamine transition involves the replacement of the histones with transition nuclear proteins (TP1 and TP2), then subsequently the replacement of the TPs with protamines (PRM1 and PRM2). The histone-to-protamine transition is associated with hyperacetylation of histone H4 (Sonnack *et al.* 2002). Hyperacetylation promotes a looser nucleosomal structure, which allows for easy removal of the histones and incorporation of the TPs and subsequently the protamines (Sonnack *et al.* 2002; Fig. 3).

The replacement of histones for TPs then PRMs is essential as evidenced in several gene knockout models.

Mice lacking either TP1 or TP2 have subtle abnormalities in chromatin condensation during spermiogenesis and were fertile with normal numbers of sperm (Yu *et al.* 2000, Zhao *et al.* 2001). However, mice lacking both TPs exhibited irregular chromatin condensation in all spermatids, many showed DNA breaks, PRM2 was not post-translationally processed and male mice were sterile (Zhao *et al.* 2004; Table 2).

Protamines are small basic proteins rich in arginine and cysteine and are found only in spermatids (reviewed in Wouters-Tyrou *et al.* 1998). Most mammals produce two forms of protamine, PRM1 and PRM2, which are responsible for the DNA being packaged into a very compact arrangement. Disruption of one copy of either gene, i.e. haploinsufficiency for either *PRM1* or *PRM2*, disrupts nuclear formation, processing of PRM2 and sperm function (Cho *et al.* 2003; Table 2). Consequently, heterozygous males were sterile. The importance of protamines is also evident in humans where an absence of PRM2 or changes in the PRM1 to PRM2 ratio in the sperm have been associated with male infertility (Balhorn *et al.* 1988, de Yebra *et al.* 1993).

The importance of protamines and their regulation for fertility is further evidenced in the *Jhdm2a* knockout mouse line. Protamine expression is at least in part regulated by the H3K9me2/H3K9me1 demethylase JmjC-domain-containing histone demethylase 2A (JHDM2A). Expression of JHDM2A starts in the late pachytene stage and persists until the elongated spermatid period. Sperm do not contain JHDM2A (Okada *et al.* 2007). Disruption of *Jhdm2a* caused post-meiotic chromatin condensation defects including elongating spermatids with abnormal nuclear structure and elongation (Okada *et al.* 2007; Table 2). As a result, mutant males had reduced sperm counts and were infertile (Okada *et al.* 2007). JHDM2A functions to demethylate mono- and di-methylated H3K9 in the promoter regions of *Tnp1* and *Prm1* in round spermatids (Okada *et al.* 2007). An absence of JHDM2A results in promoter hyper-methylation and gene silencing and a lack of TP1 and PRM1, and infertility.

As indicated above, TP and PRM function, and as a consequence spermiogenesis, is regulated by epigenetic modifications. The loss of calmodulin-dependent protein kinase IV (CAMK4)-dependent serine/threonine phosphorylation of PRM2 results in sterility (Wu *et al.* 2000). *Camk4* knockout spermatids showed a loss of PRM2 and prolonged retention of TP2 (Wu *et al.* 2000). As a consequence, *Camk4* knockout males were infertile and displayed a reduced number of late elongating spermatids and no mature sperm in the epididymides (Wu *et al.* 2000; Table 2).

In addition to the histone to protamine transition, spermiogenesis is critically dependent on changes in H1 linker histones. H1T2 is a H1 variant specifically expressed in round through to elongated spermatids (Martianov *et al.* 2005, Tanaka & Baba 2005).

Knockout males were sterile, or severely sub-fertile, and epididymal sperm showed abnormal morphology, cytoplasmic retention and impaired motility. Interestingly, sperm showed only trace amounts of PRM1 and PRM2 suggesting that H1T2 is also involved in the histone to protamine transition (Martianov *et al.* 2005, Tanaka & Baba 2005).

At least two other H1 variants are also involved in spermiogenesis. H1T, which is a testis-specific variant, is first detected in pachytene spermatocytes where it is integrated into the genome and replaces the majority of somatic H1 linker histones. H1T is not detected beyond the early elongating spermatid stage concordant with its replacement by TPs and PRMs (Meistrich *et al.* 1985, Yan *et al.* 2003). Despite such a compelling expression pattern, an absence of H1T in the *H1t* knockout line did not result in male infertility, suggesting that H1T does not significantly affect chromatin structure or that there is redundancy with other H1 testis variants (Lin *et al.* 2000).

HILS1 is another H1 linker histone variant that is detected within elongating spermatids. HILS1 protein localisation within elongating spermatids overlaps only minimally with H1T, but substantially with the TPs and PRMs (Yan *et al.* 2003, Iguchi *et al.* 2004). At least one study indicates that decreased HILS1 expression in sperm is associated with asthenozoospermia (Jedrzejczak *et al.* 2007).

Transgenerational epigenetic inheritance

During gametogenesis and following fertilisation, epigenetic reprogramming occurs involving the erasing and resetting of DNA methylation at imprinted loci, TEs and repeat sequences in the genome (reviewed in Morgan *et al.* 2005). During germ cell development, it was originally thought that all epigenetic marks were erased in the early primordial germ cells and re-established in the early gonocytes (Monk *et al.* 1987). Following fertilisation, it was generally held that the paternal genome is reprogrammed and epigenetic marks are again erased and reset (Oswald *et al.* 2000). It is now clear that epigenetic marks are not always cleared between generations and as a result epigenetic marks can be inherited. This is known as transgenerational epigenetic inheritance and it provides an additional level of phenotypic variability between individuals. It has recently been shown that several genes show transgenerational epigenetic inheritance through the male germ line (Chong *et al.* 2007).

The existence of transgenerational epigenetic inheritance has been most conclusively demonstrated using the agouti viable yellow (A^{vy}) mouse model (Bultman *et al.* 1992, Morgan *et al.* 1999). The agouti gene is responsible for the production of yellow pigment in hair and is normally controlled by a promoter which is active at only one stage in the hair growth cycle resulting in a

yellow band on black hair (Bultman *et al.* 1992). In the A^{vy} line, a retrotransposon IAP has integrated upstream of the agouti promoter (Duhl *et al.* 1994; Fig. 4A). As a consequence, expression at the A^{vy} locus is controlled by the LTR of the IAP retrotransposon. When the IAP retrotransposon is unmethylated, and therefore active, it overrides the endogenous promoter and results in constitutive agouti production which is visible as yellow fur. The IAP retrotransposon can be silenced (methylated), resulting in the production of agouti using the endogenous promoter and therefore brown fur. Genetically identical littermates carrying the A^{vy} allele display variable expressivity of the agouti gene ranging from completely yellow to brown depending on the methylation state of the IAP in the promoter (Morgan *et al.* 1999; Fig. 4B). Indeed, the agouti gene within different cells can be differentially methylated to produce mottled mice. The coat colour phenotype of the mice can be classified by a trained observer as either yellow, mottled or agouti.

Figure 4 Agouti viable yellow allele. (A) Map of the A^{vy} allele. A^{vy} has an IAP (red box) inserted into the locus upstream of the agouti gene. Transcription originating in a cryptic promoter (green arrowhead) in the LTR of the IAP drives constitutive expression of A^{vy} when it is active, leading to yellow coat colour. When this cryptic promoter is silenced, expression of A^{vy} occurs under the control of hair-cycle specific promoters, leading to brown coat colour. (B) Range of phenotypes in isogenic A^{vy} mice. Yellow mice have agouti expression driven by the inserted IAP. Mottled mice display variegation, some cells have expression driven by the IAP and some lack this expression. Agouti mice lack expression from the cryptic promoter, A^{vy} is regulated by hair-cycle specific promoters, these mice have the brown coat colour.

The A^{vy} allele shows parent-of-origin effects where the range of coat colours depends on whether A^{vy} is inherited from the mother or father. When A^{vy} is transmitted by a yellow A^{vy} dam, her offspring are more likely to be yellow than agouti (Morgan *et al.* 1999). This effect is not seen through paternal transmission. It was proposed that this maternal epigenetic effect resulted from incomplete clearing of epigenetic marks at the A^{vy} allele leading to inheritance of the epigenetic modification (Morgan *et al.* 1999). Maternal and paternal epigenetic inheritance has also been observed at the axin-fused ($Axin^{Fu}$) allele, another gene where expression is controlled by the LTR of an IAP retrotransposon (Rakyan *et al.* 2003).

Paternal effect genes in mice

The existence of mammalian paternal effect genes has also been recently confirmed. This was done via an ENU mutagenesis screen to identify regulators of the epigenetic state; specifically mutations that would alter the methylation status of a blood expressed transgene (Blewitt *et al.* 2005). This screen identified a mouse line carrying a point mutation in *Smarca5*: *Smarca5*^{MommeD4} (modifiers of murine metastable epialleles D4). This mutation not only resulted in altered expression of the transgene, but also displayed a paternal-specific transgenerational effect on the phenotype of the offspring (Chong *et al.* 2007). Paternal effect genes had previously been reported in *D. melanogaster* but had not previously been identified in mammals (Fitch *et al.* 1998).

Specifically, when *Smarca5* heterozygous males were mated to yellow A^{vy} heterozygous females, heterozygous offspring were more likely to be brown than their wild-type littermates (Blewitt *et al.* 2005). This suggested an increased incidence of methylation at the IAP retrotransposon associated with the A^{vy} allele in these offspring and is consistent with *Smarca5* being an enhancer of variegation (Fig. 5). As an extension of this, Chong *et al.* (2007) observed that the range of coat colours of wild-type offspring produced from a *Smarca5* heterozygous male was different to the range seen in wild-type offspring from a *Smarca5* wild-type male (Table 2). The wild-type offspring produced from the heterozygous fathers were more likely to be yellow than the wild-type offspring produced from the wild-type fathers (Chong *et al.* 2007; Fig. 5). This is consistent with hypomethylation of the IAP retrotransposon in wild-type pups from heterozygous fathers and suggests that while the majority of paternally inherited epigenetic modifications are erased in the zygote, not all are.

As such, *Smarca5* is a paternal effect gene; that is, a gene in which a mutation in the male parent affects the phenotype of wild-type offspring. This effect was not seen in pups from *Smarca5* heterozygous dams. Wild-type offspring from the *Smarca5* heterozygous and wild-type fathers were genetically identical (isogenic).

The only difference was that one group was sired by a *Smarca5* heterozygous male and the other from a wild-type male. Thus, the wild-type offspring produced from the heterozygous fathers were maintaining an epigenetic memory of their father's genotype. A similar result was observed with a hypomorphic allele of *Dnmt1* (termed *Dnmt1*^{MommeD2}) produced in the same ENU screen (Chong *et al.* 2007). This provides evidence that the epigenetic state of the paternal germ line may have a significant effect on the phenotype of offspring independently of the transmitted genotype. The number of paternal effect genes in the whole genome is currently unknown.

Transgenerational paternal effects of another type have been observed in the *Dnmt3l* knockout mouse line. In the *Dnmt3l* knockout mouse line, when heterozygous males were mated to wild-type females, spontaneous X chromosome aneuploidy (XO) occurred with an increased frequency in offspring. Humans with an XO chromosome content have Turner's syndrome and are outwardly female, but have early degeneration of the ovaries and oestrogen insufficiency. They usually have short stature and may have a high arched palate, neck webbing, broad chest, cardiac and renal abnormalities, impaired cognitive skills and spatial intelligence (Skuse 2005).

When *Dnmt3l* heterozygous males were mated to wild-type females the frequency of XO offspring was 2–2.4%, depending on genetic background, compared with 0% from wild-type males (Chong *et al.* 2007). Out of the three *Dnmt3l* XO offspring studied, two had lost the paternal X or Y chromosome while one had lost the maternal X chromosome. The loss of the paternal X or Y chromosome suggested that the loss occurred either in the male germ line during spermatogenesis or post-fertilisation, while the loss of the maternal X chromosome suggested that the X chromosome is lost post-fertilisation (Chong *et al.* 2007). The occurrence of XO in the *Dnmt3l* mouse line suggests a previously unrecognised role for DNMT3L in regulating X chromosome alignment and segregation during meiosis and of the epigenetic state in general in the generation of aneuploid gametes. Aneuploidy in humans occurs in at least 5% of all live births and probably accounts for a large number of miscarriages (Lamb & Hassold 2004). These data suggest that hypomorphic epigenetic modifiers in a potential father may be a cause of sub-fertility. Clearly, this is an area of research that requires additional work.

Evidence of transgenerational effects of environmental factors on the germ line in rodents

The effects described above are only thought to affect a potential father and his immediate offspring. However, several recent animal studies have suggested that embryonic exposure to some environmental factors

Figure 5 Pedigrees produced from *Smarca5* heterozygous males and *Smarca5* wild-type males. (A) Pedigree produced from *Smarca5* heterozygous males and yellow A^{vy} heterozygous females. When sired by a heterozygous father, offspring heterozygous for *Smarca5* demonstrated a shift in penetrance at A^{vy} towards mottled when compared with wild-type littermates ($P < 0.005$). (B) Pedigree produced from *Smarca5* wild-type males and yellow A^{vy} heterozygous females. The coat colour phenotypes of wild-type offspring from a wild-type sire were compared with the wild-type offspring from a *Smarca5* heterozygous sire. Wild-type offspring from *Smarca5* heterozygous sires showed a significant increase in the proportion of offspring with yellow coats when compared with the wild-type offspring from wild-type sires.

can result in diseases which may be inherited across multiple generations. For example, feeding pregnant rats a protein-restricted isocaloric diet increases the incidence of diabetes in the F_2 progeny (Zambrano *et al.* 2005). *In utero* and lactational exposure of the environmental chemical dioxin in rats affects the development of male gonads in offspring (F_1), leading to changes in the sex ratio (reduction in male pups) of the subsequent generation (F_2 ; Ikeda *et al.* 2005).

While it is clear that exposure of the grandmother in these circumstances did have affects on the grandchild, these affects could all be directly due to the initial exposure; that is, the foetus and its primordial germ cells were all directly exposed via the mother's (or grandmother's) circulation. For a compound or incident to truly have a transgenerational affect it should affect at least the F_3 generation. There is some evidence that such situations do occur. For example, exposure of pregnant mice to the carcinogen benzopyrene increased the risk of lung tumours in not only the F_1 generation but also in the F_2 – F_5 generations of mice (Turusov *et al.* 1990).

More recent studies have suggested transgenerational effects from the endocrine disruptor vinclozolin (Anway *et al.* 2005, Chang *et al.* 2006). Vinclozolin is a fungicide used on agricultural crops including in the wine industry and is an anti-androgenic (Kelce *et al.* 1994). Pregnant rats treated with vinclozolin at the time of gonadal sex determination (embryonic days 8–14) produced male offspring with reduced spermatogenic capacity which persisted in male offspring in the F_2 – F_4 generations (Anway *et al.* 2005). When male and female progeny in the F_1 – F_4 generations were maintained until adulthood they developed diseases or tissue abnormalities such as prostate disease, kidney disease, breast cancer, testis abnormalities and immune system abnormalities (Anway *et al.* 2006). These transgenerational disease states were only observed when colonies were maintained through the paternal line. When offspring were produced using an affected dam, subsequent generations of animals appeared normal.

The authors have proposed that these transgenerational affects were due to altered epigenetic state and involve aberrant regulation of the methyltransferases DNMT3A and DNMT3L (Anway *et al.* 2008). They propose that disease (and male sub-fertility) was the result of altered genome-wide methylation and as a consequence gene expression.

It is true to say that both the epigenetic mechanism and transgenerational consequences of vinclozolin are hotly debated. Nonetheless, these studies provide a fascinating area for future research with potentially widespread implications for public health.

Evidence of transgenerational effects of environmental factors on the germ line in humans

There are several diseases that are caused by aberrant expression of imprinted genes. These include the developmental disorders Prader–Willi syndrome (PWS), Angelman syndrome (AS) and Beckwith–Wiedemann syndrome (BWS). PWS and AS are caused by the disruption of several maternally and paternally imprinted genes located on chromosome 15 (Camprubi *et al.* 2007). The loss of *SNRPN* expression is most commonly associated with PWS, while the loss of *UBE3A* is most commonly associated with AS. All causes of BWS to date have been associated with alterations in the methylation state of one or more imprinted genes in the 11p15.5 imprinted gene cluster (reviewed in Maher & Reik 2000).

Assisted reproductive techniques (ART) have been associated with a statistically significant increased risk of AS and BWS in offspring (reviewed in Gosden *et al.* 2003). It is proposed that the frequency of imprinting disorders is increased as a consequence of *in vitro* culturing conditions, or chemical composition of the culture media, which can result in the loss of methylation on imprinted regions in either the oocyte or embryo as opposed to aberrant epigenetic state in male gametes (Khosla *et al.* 2001). However, as mentioned earlier, it has been recently shown that

males with oligospermia have an increased frequency of defective methylation compared with normospermic males (Kobayashi *et al.* 2007). This raises the possibility that hypomethylated paternally imprinted genes may contribute to imprinting errors and disease in ART-conceived children.

Similar to the exposure of rodents to dioxins or caloric restriction, in the human populations there have been many documented cases of changes in sex ratios following chemical exposure or after a natural disaster which may be consistent with altered epigenetic state across a population. For example in 1976, the population of Seveso in Italy were exposed to the herbicide dioxin. Exposed males produced a higher percentage of female offspring (65%) compared with male offspring (35%; Mocarelli *et al.* 1996). Pesticide workers in several countries are still exposed to high levels of dioxin. An investigation conducted on Russian pesticide workers also found that male exposure to dioxin was associated with an increase in the number of female births (62%; Ryan *et al.* 2002). It was proposed that exposure to dioxin affected sperm production in the testis leading to changes in the sex ratio (Jongbloet *et al.* 2002).

Similarly, after the Kobe earthquake in Japan (Fakuda *et al.* 1998), the 10-day war in Slovenia (Zorn *et al.* 2002) and in California following the terrorist attacks of September 11 (Catalano *et al.* 2005) a decline in the sex ratio (male:female) at birth was observed. It has been proposed that stress related to a catastrophic event may have a negative effect on sperm production or function resulting in a decline in sex ratio (Fakuda *et al.* 1998, Zorn *et al.* 2002). Alternatively, it could be interpreted that these studies reveal paternal effects similar to those documented in rodents (Anway *et al.* 2005, Ikeda *et al.* 2005). It is of note that within these populations there is no documented evidence of elevated levels of sex reversal or infertility, suggesting that the transmission ratio distortion is a true change in X- versus Y-bearing sperm.

Conclusion

This review underscores the importance of appropriate epigenetic regulation throughout all phases of spermatogenesis. Deviation from this program can lead to infertility or consequences for subsequent generations. During male germ cell development, epigenetic regulation is crucial in TE silencing, imprinting, chromatin remodelling, MSCI, the histone-protamine transition and PMSR. Although many of the epigenetic modifications regulating these events have been elucidated, there are still many questions to be answered. What are the exact functions of the epigenetic modifications associated with spermatogenesis? In what order do these modifications occur and what impact do these modifications have on human male fertility?

The studies discussed in this review suggest that environmental factors may influence the epigenetic state and that these epigenetic modifications may be inherited through the male germ line and passed onto more than one generation. The paternal effects described in this review have further highlighted the importance of research into epigenetic regulation and male fertility. The concept that untransmitted alleles passed through the male germ line can affect the phenotype of the next generation is a new and exciting area of research. The transgenerational and paternal effects displayed by these mice may be related to idiopathic cases of sporadic disease or infertility in humans.

Declaration of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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