

Assisted reproduction treatment and epigenetic inheritance

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BACKGROUND: The subject of epigenetic risk of assisted reproduction treatment (ART), initiated by reports on an increase of children with the Beckwith–Wiedemann imprinting disorder, is very topical. Hence, there is a growing literature, including mouse studies.

METHODS: In order to gain information on transgenerational epigenetic inheritance and epigenetic effects induced by ART, literature databases were searched for papers on this topic using relevant keywords.

RESULTS: At the level of genomic imprinting involving CpG methylation, ART-induced epigenetic defects are convincingly observed in mice, especially for placenta, and seem more frequent than in humans. Data generally provide a warning as to the use of ovulation induction and *in vitro* culture. In human sperm from compromised spermatogenesis, sequence-specific DNA hypomethylation is observed repeatedly. Transmittance of sperm and oocyte DNA methylation defects is possible but, as deduced from the limited data available, largely prevented by selection of gametes for ART and/or non-viability of the resulting embryos. Some evidence indicates that subfertility itself is a risk factor for imprinting diseases. As in mouse, physiological effects from ART are observed in humans.

In the human, indications for a broader target for changes in CpG methylation than imprinted DNA sequences alone have been found. In the mouse, a broader range of CpG sequences has not yet been studied. Also, a multigeneration study of systematic ART on epigenetic parameters is lacking.

CONCLUSIONS: The field of epigenetic inheritance within the lifespan of an individual and between generations (via mitosis and meiosis, respectively) is growing, driven by the expansion of chromatin research. ART can induce epigenetic variation that might be transmitted to the next generation.

Key words: assisted reproduction / epigenetics / human / genomic imprinting / transgenerational epigenetic inheritance

Introduction

In 2009, Katari *et al.* have reported that some genes from babies conceived by means of IVF show a gene expression pattern that is different from naturally conceived children (Katari *et al.*, 2009). The observed changes were associated with the mechanism that switches genes on and off, which is heritable to forthcoming cell generations, hence under epigenetic control. In their study, Katari *et al.* have stated that this mechanism could put children conceived by means of assisted reproduction treatment (ART) at a greater risk of diseases, such as diabetes and obesity, later in life.

Epigenetic deregulation already received increasing attention as a possible common cause of adverse ART outcomes, since the incidence of disorders that involve imprinted genes, especially the Beckwith–Wiedemann syndrome (BWS), is often reported to be increased in the offspring (Amor and Halliday, 2008, Ceelen *et al.*, 2008b, Manipalviratn *et al.*, 2009). This led to an expansion of ART literature on epigenetic effects, also including mouse model studies. The subfertility of one or both parents as a causative factor has to be taken into account, which often is difficult to achieve. Moreover, mouse models in this area are almost absent. Another question is if the assumed epigenetic effects of ART can be transmitted to the next generation.

Besides classical Mendelian inheritance of information stored in the DNA sequence, other mechanisms are active in the transmission of phenotypic traits across generations too. No insight into underlying molecular mechanisms was available when the theory of non-genetic transmission was first put forward by Waddington (1953) to describe the acquired characteristics in the offspring of *Drosophila* exposed to heat (McLaren, 1999). In the broad sense coined by Waddington, these observations are termed transgenerational epigenetic effects (Youngson and Whitelaw, 2008). When these phenotypic alterations are caused by transfer of chromosome/chromatin modifications through the gametes, the term transgenerational epigenetic ‘inheritance’ is used (Youngson and Whitelaw, 2008; Jablonka and Raz, 2009). In this definition, the word epigenetic refers to the mechanisms involved in the mitotic and meiotic transfer of non-genetic (i.e. not DNA sequence based) information. Transgenerational epigenetic inheritance has been proved in organisms ranging from bacteria and plants to the mouse and humans (Jablonka and Raz, 2009).

Epigenetic mechanisms exist as an interplay between DNA methylation, RNA-mediated chromatin modifications, histone modifications and histone variants, but likely also less well-studied mechanisms, such as the organization of nuclear structure including chromosome replication behaviour (Jablonka and Raz, 2009; Margueron and Reinberg, 2010). In the context of this review, inheritance via DNA methylation will be mainly discussed.

The molecular epigenetic mechanisms are instrumental in the specification of cell identity and potency within generations. Epigenetic mechanisms are thought to concertedly orchestrate the spatial and temporal regulation of cell differentiation throughout development (Goldberg *et al.*, 2007; Margueron and Reinberg, 2010; Zaidi *et al.*, 2010). Since all cells of an organism have the same genotype, epigenetic marks are deposited to alter transcription and achieve cell-type specific gene expression patterns in different tissues. In fact, the definition of epigenetics given by Jablonka and Raz (2009) as ‘the study of the processes that underly developmental plasticity and canalization and that bring about persistent developmental effects in both prokaryotes and eukaryotes’ underlines its role in embryogenesis and cell

differentiation. Sex-specific genomic imprinting and stable female X-inactivation are also under epigenetic control.

Between generations, the germ line is subjected to two distinct reprogramming events [one in the primordial germ cells (PGCs) and one in the preimplantation embryo], in order to prepare the cells for pluri- and totipotency and down-regulate the inheritance of epigenetic information between generations [reviewed in (Reik *et al.*, 2001; Morgan *et al.*, 2005; Feng *et al.*, 2010)]. Both phases are pertinent to ART and especially the second one (in the preimplantation embryo) owing to the *in vitro* circumstances at this epigenetically crucial phase of development.

The discovery that some loci, notably imprinted ones, escape reprogramming in the early embryo, provided a first hint regarding the mechanism behind epigenetic inheritance as this brought into question the rigidity of epigenetic erasure between generations as a principle. Epigenetic marks are generally thought to be stable through rounds of somatic mitosis after initial deposition in development (Margueron and Reinberg, 2010). This careful balance between somatic maintenance of epigenetic marks and dynamic reprogramming in the germline has led epigenetic mechanisms to be put forward as a vehicle for ‘soft inheritance’ (Youngson and Whitelaw, 2008), a term first introduced to describe a more pliable system of inheritance, which would allow organisms to quickly adapt to fluctuations in nutrition, predation or disease (Mayr and Provine, 1980; Mayr, 1982).

The question we will pursue here is whether the conditions during gametogenesis and the *in vitro* phases intrinsic to ART could elicit epigenetic effects and, if so, whether these could be transmitted to the next generation. We will first present the processes of mitotic epigenetic inheritance. Next, we will describe the known molecular mechanisms involved in the escape of germline reprogramming and present environmental and hormonal cues that induce alterations in the epigenome to be passed on to the next generation.

In the second part of this review, observations of a molecular epigenetic nature made in mouse and human ART will be presented. Finally, we will attempt to integrate these observations, including the likelihood of transgenerational epigenetic inheritance, and designate the areas of human reproduction in the context of ART where insight is lacking most.

Methods

In order to gain information on transgenerational epigenetic inheritance and epigenetic effects induced by ART, literature databases (Pubmed, Medline) were thoroughly searched for papers on this topic by using relevant keywords.

Epigenetic inheritance and germline reprogramming

Mitotic inheritance of epigenetic marks

To address the mechanisms involved in transgenerational epigenetic inheritance, DNA must be viewed in the context of chromatin and its modifications (see Box I). The epigenetic chromatin state is tightly linked to transcription and as cells differentiate they acquire tissue-specific patterns of DNA methylation, histone modifications and other epigenetic chromatin marks (Lange and Schneider, 2010; Zaidi *et al.*, 2010). In this context, the faithful transmission of this epigenetic signature to daughter cells is essential for lineage commitment. Mitotic transmission of epigenetic marks is observed throughout somatic cellular development (Zaidi *et al.*, 2010) and insight into the mechanisms involved is gradually increasing (Cheng and Blumenthal,

2008; Probst *et al.*, 2009; Kaufman and Rando, 2010; Margueron and Reinberg, 2010).

Box I: Chromatin and epigenetic mechanisms.

Chromatin is the structure in which DNA is packaged inside the cell nucleus. The fundamental unit of chromatin is the nucleosome, which consists of 147 base pairs of DNA wrapped around a histone octamer that contains two duplicates of histone H3, H4, H2A and H2B (Kouzarides, 2007). Nucleosomes are subsequently compacted further into higher-order chromatin structures. The degree of chromatin packing is dynamically regulated and can be either heterochromatic (densely packed chromatin, transcriptionally repressed) or euchromatic (accessible chromatin, transcriptionally active).

An intricate network of epigenetic mechanisms operates to regulate the chromatin state and thereby transcription (Fig. 1). The best-studied chromatin modifications are the methylation of DNA, which occurs at CpG sites in mammals (Doerfler, 2008), and the modification of the N-terminal histone tails (Kouzarides, 2007).

In DNA, the cytosine member of a CG dimer when methylated at carbon 5 acts as a fifth base. Depending on density and position, this mark is associated with gene inactivation, and the formation of heterochromatin. Once methylated, the 5methyl CpG is copied during S phase. Hence, the deposition of methyl groups to DNA in mammals is catalysed by two families of DNA methyltransferases (DNMTs) that exhibit *de novo* (DNMT3) and predominantly maintenance (DNMT1) activity (Cheng and Blumenthal, 2008), whereas no demethylases have been described (Lucifero *et al.*, 2004, Popp *et al.*, 2010). In PGCs, effects on the conversion of 5methyl CpG to CpG by ablation of the cytidine deaminase AID has been found (Popp *et al.*, 2010) that can achieve this effect in combination with base excision repair that is indicated to be active at this stage (Hajkova *et al.*, 2010). Another pathway may make use of the Tet1,2,3 family of enzymes that convert 5methylCpG into 5hydroxymethylCpG. This pathway is preferentially active in the male pronucleus of the zygote (Wossidlo *et al.*, 2011) and Tet enzymes have been shown to be present in PGCs (Hajkova *et al.*, 2010). The epigenetic effect of this conversion is currently under investigation (Szulwach *et al.*, 2011).

Post-translational modifications of histone tails include methylation, acetylation, phosphorylation, ubiquitination and sumoylation and are thought to be of a more dynamic nature than DNA methylation as they are deposited and removed by a wide variety of enzymes (Kouzarides, 2007). As opposed to DNA methylation, which is generally involved in the repression of transcription, histone tail modifications display a wide variety of functions, both repressive and activating, and are often useful for describing the functional state at the level of a single gene to large chromatin domains (Filion *et al.*, 2010). Histone marks exert their influence either by directly changing the structure of chromatin or via the recruitment of chromatin-binding factors like transcription factors and ATP dependent chromatin-remodelling complexes (Clapier and Cairns, 2009). Together with the incorporation of histone variants and the use of a large variety of non-coding long and small RNAs (Moazed, 2009), these modifications interact in a complex and delicate web to regulate structural aspects of chromatin domains, such as chromatin compaction, nucleosomal occupancy and the localization inside the nucleus. Insight in what constitutes these domains and how this affects transcription is slowly becoming available (Filion *et al.*, 2010), unravelling a web of dazzling complexity. Most of the elements touched upon appear in Fig. 1.

To enable replication of DNA during the S-phase of the cell cycle, the chromatin structure is severely disrupted, which results in a partial loss of epigenetic marks. Thus, special mechanisms need to be in place to ensure the mitotic propagation of epigenetic information. Although some insight into mitotic inheritance of DNA methylation and histone modifications exists, the propagation of other epigenetic properties, such as histone occupancy and histone variants, remains largely elusive.

DNA methylation is transmitted semi-conservatively through DNA replication via the two hemimethylated DNA double helices. The DNA methyltransferase DNMT1 interacts with PCNA (proliferating cell nuclear antigen), a processivity factor of the replication machinery that forms a clamp around the DNA template (Chuang *et al.*, 1997), and is able to specifically recognize hemimethylated DNA (Hermann *et al.*, 2004) catalysing the addition of a methyl group to complementary CpGs of the newly synthesized strand. The error rate in base pairing is 1 in 10^9 . The error rate in adding the CH₃ to the inserted C opposite a methylated CpG is one in 40 in somatic cell (leukocyte) divisions (Fu *et al.*, 2010).

In contrast, the propagation of the more dynamic histone marks is more complex and not completely understood. As modified histones are removed to enable passage of the replication fork, no template is available to regulate the reassembly of histones and their marks on the daughter strands. In the leading current model, newly synthesized and old histones (the latter containing their original modifications) are incorporated into daughter-chromatin randomly, thus severely diluting the modifications (Margueron and Reinberg, 2010). To prevent the gradual fading of histone marks over cell divisions, it has been proposed that these diluted modifications are able to recruit histone-modifying enzymes directly after replication, which would catalyse further deposition of the mark in a positive-feedback loop (Lange and Schneider, 2010, Margueron and Reinberg, 2010). The limited reliability of the transmission of histone marks that follows from this model combined with the fact that, in contrast to CpG methylation, histone modifications can easily be removed by a wide range of enzymes, illustrates the notion that the methylation status of DNA is more faithfully inherited over mitotic divisions than are histone modifications. Hence, CpG methylation is a more indicative tool to predict the functionality of a certain locus over forthcoming mitotic generations (Peters and Schubeler, 2005; Feng *et al.*, 2006).

Another mechanism for the mitotic propagation of epigenetic marks is the relation between the timing of replication of a certain locus during S-phase and gene activity (Gondor and Ohlsson, 2009). Transcriptionally active genes or alleles are replicated earlier in S-phase than inactive genes or alleles. This timing pattern of DNA replication changes throughout development as cells differentiate and is intimately linked to the chromatin state. A striking example is provided by the imprinted genes (see Box II) *H19* and insulin growth factor 2 (*Igf2*) (Fig. 4). Imprinting is maintained on the maternal allele by CpG methylation-sensitive binding of the chromatin insulator CTCF to the imprint control region (DMR) and disturbance of CTCF-binding by mutating the binding sites of the maternal DMR caused the complete maternal *H19/Igf2* domain to be replicated in early, instead of late, S-phase (Bergstrom *et al.*, 2007).

Box II: Genomic imprinting.

Genomic imprinting operates to silence the maternal or paternal alleles of genes that often are organized in clusters. The gene clusters/genes subjected to genomic imprinting are regulated by an imprinting control region (ICR, also called a germline differentially methylated region or germline DMR). In a cluster, genes that are exclusively expressed from the paternal allele can co-exist with genes that are exclusively expressed from the maternal allele. However, the DMR is marked by CpG methylation in one of the two germ lines (Reik and Walter, 2001). Hence methylation occurs in a sex-specific manner and is maintained throughout fertilization, embryonic and subsequent development (Fig. 2; Reik and Walter, 2001). Of the 15 human germline DMRs listed by Morison et al. (2001) (update Jan 2011) only two are methylated in the male germline. These DMRs are always located in an intergenic region, as with the DMR between *H19* and *IGF2* (=H19 DMR) and between *DLK1* and *MEG3* (=GTL2) (=IG-DMR). In mice a third intergenic paternally methylated DMR is located between *Rasgrf1* and *A19* (*Rasgrf1* DMR) (Thorvaldsen and Bartolomei, 2007). In the female germline, the DMR is always located in a promoter region. When more than one gene is regulated by this DMR, the promoter often involves a long non-coding RNA (i.e. no protein product) that is instrumental in the allelic exclusion by inducing a local chromatin change in cis, as with the KvDMR1 in the *KCNQ1OT1* (=LIT1) promoter (Kacem and Feil, 2009). For other imprinted genes like *MEST* (=PEG1) or *PLAGL1* (=ZAC1) the imprint mechanism is unknown yet.

At the start of germ cell development when the PGCs enter the developing gonad (Fig. 3) the imprints are erased and will be re-established later according to the sex of that germline. The deposition of new imprinting methylation marks has been associated with DNMT3a (Lucifero et al., 2004; Kaneda et al., 2010) and starts at E15 (mouse prenatal Day 15) in the male germ cells (prospERMatogonia). Remethylation in the oocyte follows over a wider timeframe during post-natal follicle development. The silencing of alleles within an imprinted gene cluster requires a number of additional epigenetic changes that include the methylation of promoters, antisense transcription and the loss of permissive (Ciccone et al., 2009) and acquisition of repressive histone modifications (Terranova et al., 2008). The total number of human imprinted genes is assessed at 70 (Morison et al., 2001) and new discoveries with a profound physiological impact, such as involvement in type II diabetes and high-density lipoprotein cholesterol metabolism (Small et al., 2011) and social behaviour (Garfield et al., 2011) are currently being made.

Reprogramming the genome towards totipotency

As already touched upon in the previous paragraphs, in contrast to the mitotic transmission of epigenetic marks within the lifespan of an individual, the inheritance of epigenetic information between generations is generally actively prevented. The classical view is that to restore the germline to the totipotent state of the early preimplantation embryo, differentiating epigenetic marks are removed by two phases of epigenetic reprogramming: at the PGC stage until just after their entry in the

incipient gonad and after fusion of sperm and oocyte in the zygote and during the first cleavage divisions (Morgan et al., 2005; Feng et al., 2010). Although reprogramming also entails dynamic changes in histone modifications and variants (Hajkova et al., 2008), the erasure of DNA methylation has been studied most extensively (Popp et al., 2010).

Box III: Retrotransposons in the Mouse and Human.

Almost half of the genome of the mouse (~50%) and humans (~40%) consists of transposable elements (TEs): mobile DNA elements which had the ability to allocate cellular transcription machinery for their replication. The majority of these fall into the category of retrotransposons, which require an RNA intermediate to duplicate themselves. Two types of retrotransposons can be discerned, based on whether or not they have long terminal repeats (LTRs) at their ends. LTR-retrotransposons that make up about 8–10% of the genome are also known as endogenous retroviruses as they are believed to be remnants of infectious retroviruses that have lost the 'envelope' gene necessary for capsid production. They do still possess Gag and Pol proteins, allowing them to reverse-transcribe their RNA in the cytoplasm and re-integrate into a new genomic locus. Non-LTR retrotransposons are most abundant in the mammalian genome and consist of short and long interspersed nucleotide elements (SINEs and LINEs) of which the LINE-1 (L1) subclass represents 17–20% of the human and mouse genome mass (Brouha et al., 2003). SINEs have no autonomous duplication potential but instead are mobilized *in trans* by the LINE encoded machinery (Goodier and Kazazian, 2008).

The presence of retrotransposons in the genome has various structural effects ranging from simple disruption of genes by insertional mutagenesis to chromosome rearrangements caused by homologous recombination (Goodier and Kazazian, 2008). Although these genomic alterations have mostly negative effects in the short-term, retrotransposons are involved in the expansion and structural evolution of the genome and are crucial in the evolution of new proteins and regulatory sequences (Volff, 2006). In mammals, retrotransposons are mostly located in euchromatic regions scattered around genes (Goodier and Kazazian, 2008). The strong, constitutive promoters of retrotransposons can disturb expression of nearby genes, either directly or via the spread of repressive epigenetic marks associated with retrotransposons.

Although most retrotransposons are rendered inactive by truncation or mutation, some are still potentially able to duplicate and re-integrate in the genome, of which the LTR-containing intracisternal A particles (IAPs) in the mouse are an excellent example. These elements are silenced throughout the germline by means of epigenetic mechanisms that include DNA methylation, chromatin modifications and RNAi pathways (Zamudio and Bourc'his, 2010).

The start of germ cell development from epiblast cells around E7.25 (Figs 3 and 5A) announces the first phase of epigenetic reprogramming (demethylation) in the mouse, which continues after these PGCs have reached the gonad at E10.5 and lasts until E13.5 (Seki et al., 2005) just before female meiosis commences. At this point, <10% of CpGs retain their methylation mark, whereas

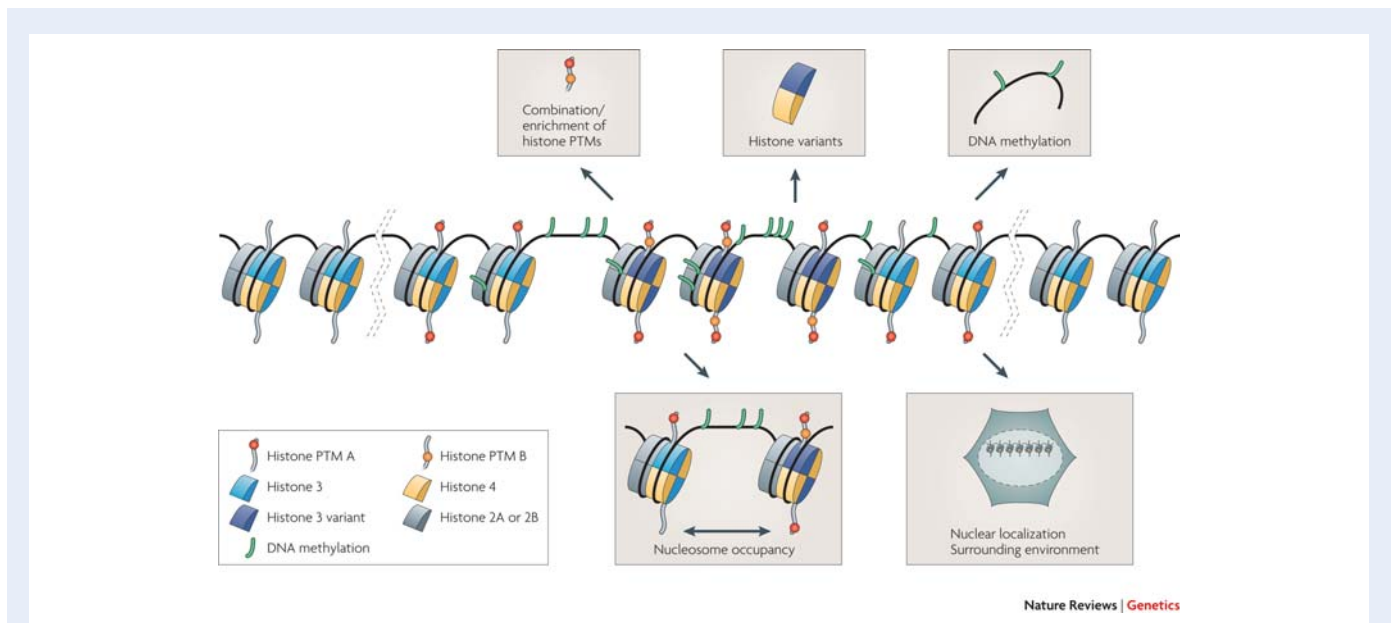


Figure 1 Characteristics of a chromatin domain. Schematic representation of the covalent and structural features that define a certain chromatin domain. Different contributing factors are highlighted in shaded boxes. The dashed line represents a separation between two adjacent domains. Figure from Margueron and Reinberg (2010).

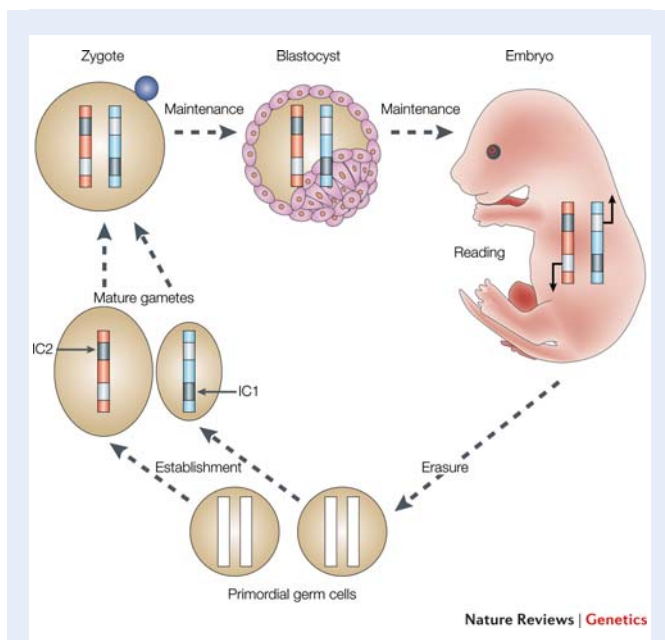


Figure 2 Imprinting in the germline. Erasure, establishment and maintenance of methylation imprints at imprinting control regions during germ cell and embryonic development. Imprinting control regions (IC1) and IC2 are shown as examples. Grey indicates modification and white indicates no modification at the corresponding alleles. Parental chromosomes are marked according to their sex in blue (male) or red (female). The reading in the developing embryo is indicated by arrows. Figure from Reik and Walter (2001).

70–80% of CpG sites are methylated in embryonic stem (ES) and somatic cells indicating the magnitude of reprogramming (Popp *et al.*, 2010). Demethylation is observed at nearly all sequence

elements including promoters and genic, intergenic and transposon sequences (see Box III) and is thought to be active, as maintenance DNMT1 is present in the nucleus throughout the process (Hajkova *et al.*, 2002; Morgan *et al.*, 2005). The allele-specific methylated DMRs of imprinted regions are also demethylated between E10.5 and E12.5, with the precise timing individually controlled for each gene cluster (Lee *et al.*, 2002; Hajkova *et al.*, 2008). However, at the DMRs of the *H19* and *Snrpn* gene clusters, the originally methylated sequences are *de novo* methylated at an earlier stage in subsequent spermatogenesis (*H19*) and oogenesis (*Snrpn*) than the originally unmethylated ones, suggesting that some kind of memory is conferred (Davis *et al.*, 1999; Lucifero *et al.*, 2004). The *de novo* methylase Dnmt3a in collaboration with Dnmt3L is responsible for resetting the sex specific germline DMR imprint (Shovlin *et al.*, 2007; Kaneda *et al.*, 2010).

The most evident exception to genome-wide demethylation in mouse PGCs known to date is formed by a class of retrotransposons called IAPs (see Box III), which show only partial demethylation (Popp *et al.*, 2010). As PGCs proceed into gametogenesis, the acquisition of specific male and female methylation patterns (Fig. 5A) is accompanied by a range of other epigenetic changes. In spermatogenesis, considerable chromatin-remodelling occurs, especially during meiosis in the adolescent and adult male, involving the incorporation of testis-specific histone variants (Gaucher *et al.*, 2010). Ultimately, histones are exchanged for protamines as a stress resistant packaging tool in spermatogenesis. This was long thought to limit the potential for male transgenerational epigenetic inheritance, despite the sizable portion of histones that, especially in the human, remains (Tanphai-chitr *et al.*, 1978; Gatewood *et al.*, 1987). Recent studies provide strong indications that histone modifications of these remnant nucleosomes play a role in embryonic development (Hammoud *et al.*, 2009; Brykczynska *et al.*, 2010).

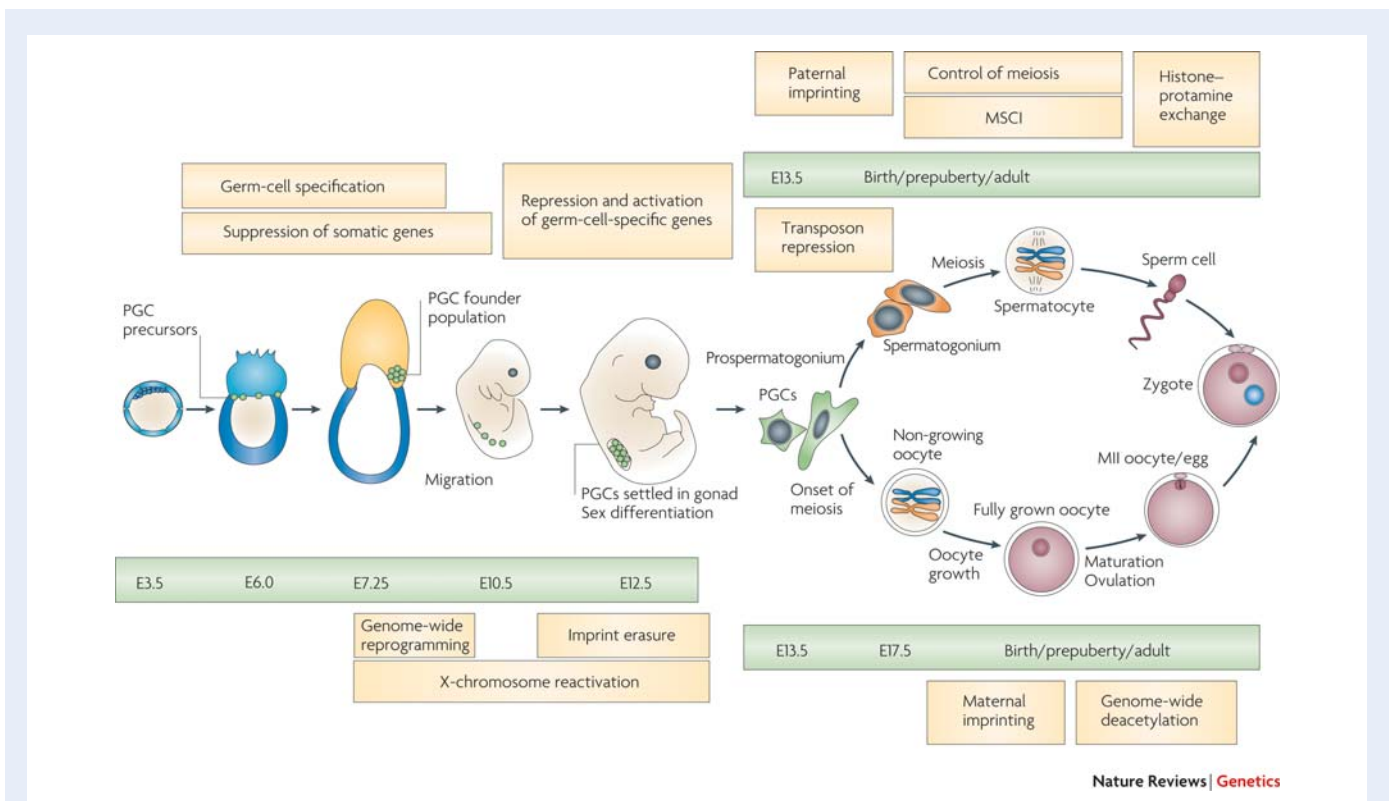


Figure 3 Chronology of mouse germ cell development and the main epigenetic events that occur. PGCs first emerge at embryonic Day 7.25 (E7.25) after which they migrate to the gonad where they enter the process of sex differentiation and eventually develop into full-grown gametes. Green bars indicate the developmental stage and yellow bars indicate the epigenetic processes occurring at these points in the germ line. MSCI stands for meiotic sex chromosome inactivation. Figure from Sasaki and Matsui (2008).

The chromatin changes that prepare the oocyte for driving embryonic development have hardly been characterized, likely by limitations in cell numbers that limit technical approaches and lack of theoretical concepts but molecular biology will also tackle this very interesting subject.

A second phase of reprogramming (Fig. 5B) starts early after gamete fusion with the rapid, active conversion of the five cytosine methyl group of CpG dimers into a hydroxymethyl group on mainly paternal DNA (Wossidlo et al., 2011). At this point, protamines are already exchanged for hyperacetylated histones from the maternal pool in an unknown but active process. In the zygote, the female genome also passively loses methylation related to a decrease in DNMT1 activity in the pronuclei (Cirio et al., 2008). Passive demethylation is continued until the morula stage after which the inner cell mass is differentially methylated from extra-embryonic lineages (Santos et al., 2002). Both paternally and maternally imprinted DMRs retain methylation during this phase (Howell et al., 2001; Hirasawa et al., 2008), along with IAPs (Lane et al., 2003) and regions of centromeric heterochromatin (Rougier et al., 1998). At implantation, DNMT3b catalyses *de novo* methylation to repress the germline expression programme and mediate the transition to terminal differentiation programmes (Borgel et al., 2010). This recent study identified a number of non-imprinted genes that inherited promoter DNA methylation from the parental gametes, suggesting that even more sequence elements are capable of escaping early embryonic reprogramming (Borgel et al., 2010). Also in this area, a more comprehensive insight of chromatin reprogramming at the onset of embryonic development is still lacking. However, there is no doubt about the

concept as such, as is amply demonstrated by the poor and variable outcomes of reproductive cloning from somatic cell nuclei (Hochedlinger and Jaenisch, 2002).

One other aspect, which is pertinent in this context, is that of epigenetic asymmetry between male and female chromatin in the early embryonic phase of reprogramming originating from the grossly different chromatin states at the onset of fertilization: a protamine-dominated chromatin in sperm versus an exclusively nucleosomal chromatin organization of the female meiotic chromosomes. Male and female pronuclei at their formation both differ in histone modifications on the N-terminal tail and in histone variants, notably of H3 (van der Heijden et al., 2005, 2009). During the first cleavage divisions, paternal and maternal chromosomes will become more similar as to their gross histone modification pattern (Puschendorf et al., 2008).

Transgenerational epigenetic inheritance: escaping reprogramming in the mouse occurs in both male and female germline

When looking at transgenerational epigenetic inheritance, sequence elements that escape reprogramming are of special interest as these might retain information over multiple generations. IAPs are observed to escape demethylation in the mouse preimplantation embryo, although stable methylation of both elements serves different purposes. IAPs are among the most active retrotransposons in the mouse genome and silencing of these elements is thought to be the original

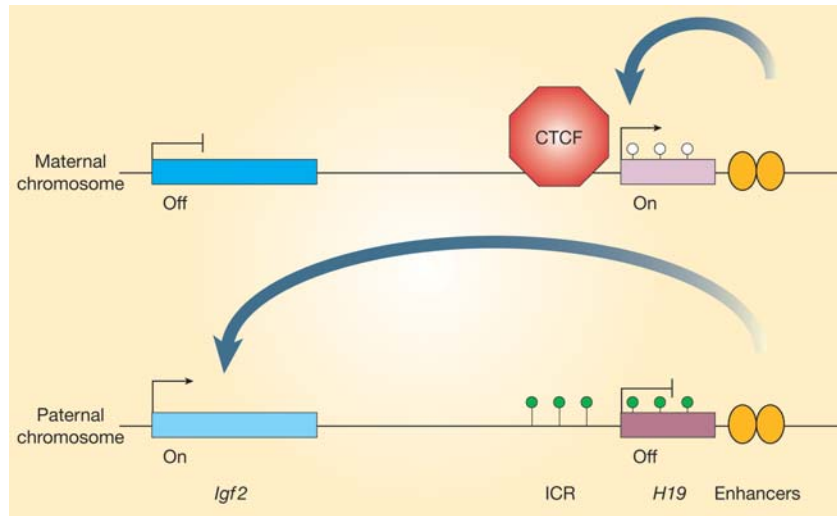


Figure 4 Epigenetic state of the imprinted *Igf2/H19* domain on the maternal and paternal genome. On the paternal chromosome, the *H19* gene and the adjacent DMR are methylated preventing *H19* expression and the binding of the insulator CTCF, thus allowing enhancers access to the *Igf2* gene promoting its expression. In the absence of DMR methylation on the maternal chromosome, bound CTCF prevents enhancer activity reaching *Igf2*, effectively silencing the gene. Instead, enhancer activity is limited to the unmethylated *H19* gene resulting in its expression on the maternally contributed chromosome. Figure from Reik and Murrell (2000).

evolutionary function of repressive CpG methylation and crucial for the prevention of retrotransposon induced mutations (Walsh *et al.*, 1998; Zamudio and Bourc'his, 2010). Imprinting has only been described in mammals, plants and insects as part of the sex-specific developmental programme and is in this sense a much more recent phenomenon in evolution, indicating that imprinting mechanisms might be originally derived from those involved in retrotransposon silencing, also suggesting mechanistic similarities (Ideraabduallah *et al.*, 2008). Indeed, the fact that both DMRs of imprinted loci and the LTRs of IAP retrotransposons behave similarly in the targeting of *de novo* methylation in the developing oocyte after PGC reprogramming (Lucifero *et al.*, 2004), and in the protection from active demethylation in the zygote (Lane *et al.*, 2003) are strong hints in this direction. The repeat-like nature of both sequence elements has been suggested to be an important factor in the acquisition of CpG methylation (Lucifero *et al.*, 2004). In the mouse, IAPs are still the best candidate for building up epigenetic inheritance as their methylation levels are relatively high at the time of active demethylation in early PGCs, in contrast to DMRs (Popp *et al.*, 2010).

The most striking evidence for transgenerational epigenetic inheritance in the mouse comes from experiments studying the *Agouti* (*A*) locus, a determinant of coat colour (Morgan *et al.*, 1999). *Agouti* alleles carrying an upstream IAP element exist of which *agouti viable yellow* (A^{VY}) has been most extensively studied. The cryptic promoter in the LTR of the IAP causes aberrant expression of the *Agouti* locus dependent on its level of CpG methylation. A gradient of yellow via mottled to pseudo agouti (wildtype) fur phenotypes exists caused by a similar gradient from an undermethylated LTR promoter (gene expression, yellow) to a fully methylated LTR (restricted expression, pseudo agouti) (Morgan *et al.*, 1999). It was observed that the average coat colour of the offspring was correlated to the coat colour of the mother, excluding the possibility of a maternally

contributed environment (Wolff *et al.*, 1998; Morgan *et al.*, 1999). Thus, this effect had to be generated by the gametic transfer of epigenetic information. Epigenetic transmission of coat colour via the father is only observed in certain genetic backgrounds (Rakyan *et al.*, 2003). A parallel example of inheritance of epigenetic marks in the mouse is provided by the *Axin-fused* ($Axin^{Fu}$) allele, which is involved in embryonic axis formation (Rakyan *et al.*, 2003). $Axin^{Fu}$ also contains an inserted IAP retrotransposable element that causes aberrant expression of *Axin* leading to a kinked tail. Again, the allele is differentially expressed dependent on the methylation status of the LTR promoter and epigenetic transmission of the kinky tail phenotype is observed in both the male and female germline (Rakyan *et al.*, 2003). Thus, it seems that the evolutionary conserved ability of IAPs to resist epigenetic reprogramming between generations renders their epigenetic state transgenerationally relatively stable, facilitating a mechanism of epigenetic inheritance. Typical for this mode of inheritance is the inter-individual variation that is shown in each generation. Pseudo agouti mums will always produce yellow as well as pseudo agouti offspring, the distribution of which depends on the phenotype of the mother.

The mechanism by which sequence elements like the LTRs of IAPs and the DMRs of imprinted regions are able to conserve their methylation status through reprogramming events is not yet fully clear. In the early mouse embryo, Dnmt1 can occur in a somatic form (Dnmt1s) and a more stable maternal form, inherited from the oocyte (Dnmt1o). Dnmt1o is not present in the nucleus during the first three cleavage divisions and its migration to the nucleus at the 8-cell stage has been deemed necessary for the maintenance of imprints (Howell *et al.*, 2001). More recently, Dnmt1s has been shown to be present in the preimplantation embryo from the 2-cell stage on (Cirio *et al.*, 2008), providing the necessary tools for selective preservation of DNA methylation in the germline. The involvement of Dnmt1o in the preservation of methylation was not only shown for

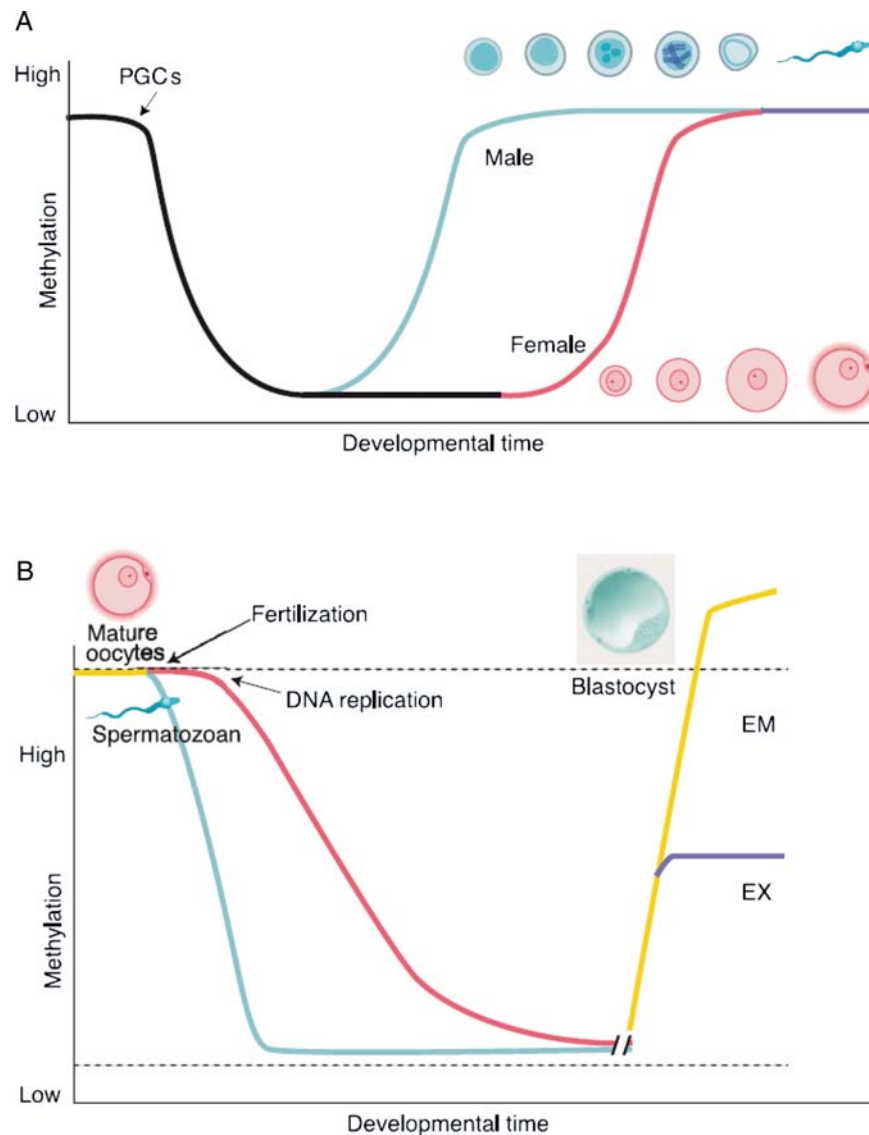


Figure 5 (A) Methylation reprogramming in the germ line. PGCs in the mouse become demethylated early in development, between E7.5 and E13.5. Remethylation begins in prospermatogonia in male germ cells, and after birth in growing oocytes. (B) Methylation reprogramming in preimplantation embryos. The paternal genome (blue) is demethylated by an active mechanism immediately after fertilization. The maternal genome (red) is demethylated by a passive mechanism. Both are remethylated around the time of implantation to different extents in embryonic (EM) and extraembryonic (EX) lineages. Methylated imprinting genes and some repeat sequences (dashed line) do not become demethylated. Unmethylated imprinting genes (dashed line) do not become methylated. Figure from Reik et al. (2001).

a cryptic promoter such as an IAP (Gaudet et al., 2004) but also for DMRs. Loss of maternal Dnmt1o led to loss of imprinting at many loci, including paternally imprinted *H19* and maternally imprinted *Snrpn* (Howell et al., 2001), resulting in profound phenotypic variation in the offspring expressed as a retarded development at mid gestation in 60% of the embryos owing to the deficiency in the maintenance of imprinting marks (Toppings et al., 2008). In addition to Dnmt1, various other proteins have been suggested to play a role in the prevention of demethylation. A poorly understood aspect of the rescue of certain sequence elements from demethylation is how these target sequences are recognized. Several proteins have been suggested to be involved in conferring chromatin specificity. These include the CpG binding

protein Mbd3 (Reese et al., 2007) and the maternal-effect proteins Zfp57 (Li et al., 2008), Stella (Payer et al., 2003) and the RNA elongator factor Elp3 (Okada et al., 2010). These results show that the oocyte is involved in the maintenance of transgenerational epigenetic marks.

Although DNA methylation has been proved to be an important tool for silencing imprinting alleles and IAPs in the germline, other epigenetic mechanisms are almost certainly at play. Despite the observed epigenetic inheritance at the A^{VY} and $Axin^{Fu}$ alleles, the LTR of the IAP of these has been shown to be demethylated at the blastocyst stage (Blewitt et al., 2006; Fernandez-Gonzalez et al., 2010) thus indicating that other mechanisms might contribute to conferring memory of

the repressed epigenetic state. Additional evidence for this is provided by a recent study in mouse ES cells carrying mutations for *Dnmt1*, in which no increase in IAP transcripts was detected despite the absence of DNA methylation at IAP loci (Hutnick *et al.*, 2010). To explain these observations, different epigenetic mechanisms have been proposed to operate concertedly with CpG methylation in early embryonic silencing. A recent study showed active and inactive alleles of *Axin^{Fu}* to be differentially modified at the blastocyst stage with activating and inactivating histone marks, respectively (Fernandez-Gonzalez *et al.*, 2010), and repressive histone modifications were found to be involved in the paternal marking of the *Kcnqlot1* imprinted cluster from the zygote stage on (Terranova *et al.*, 2008).

In addition to these chromatin-based epigenetic marks, regulatory small RNAs have recently been implicated to be directly involved in the transfer of epigenetic variation via sperm in the mouse (Rassoulzadegan *et al.*, 2006; Wagner *et al.*, 2008; Grandjean *et al.*, 2009). The observation that human sperm also contains a wide variety of RNA-species led to the speculation that similar RNA-based mechanisms play a role in epigenetic inheritance in humans (Lalancette *et al.*, 2008; Cuzin and Rassoulzadegan, 2010). It is becoming more and more clear that RNAs play a major role in fertility (Bourc'his and Voinnet, 2010; Cuzin and Rassoulzadegan, 2010). A hint of the mechanisms by which they facilitate transgenerational epigenetic effects is provided by the observation that different species of small RNAs are involved in the suppression of retrotransposons. In the male mouse germline, piRNAs (PIWI interacting RNAs) assist in the degradation of retrotransposon RNA transcripts, thus preventing reintegration in the genome, and guide *de novo* methylation of partially demethylated TEs during epigenetic reprogramming in the embryo and during spermatogenesis (Aravin *et al.*, 2008, 2009; van der Heijden *et al.*, 2010). In the female germline, other classes of small interfering RNAs (miRNAs and siRNAs) have also been demonstrated to assist in retrotransposon silencing (Murchison *et al.*, 2007; Watanabe *et al.*, 2008). Experimental results (Lykke-Andersen *et al.*, 2008) and theoretical considerations (Bourc'his and Voinnet, 2010) predict small RNAs to be involved in early embryonic development as well. The recently observed dynamic changes in populations of small RNA species from the mature secondary oocyte to the blastocyst might facilitate the necessary requirements for developmental gene regulation as well as retrotransposon silencing (Ohnishi *et al.*, 2010).

As demonstrated by the discussed observations that RNA as well as modified histones retained in sperm likely are involved in early embryonic development, the belief that the male germline contributes no more than the paternal genome sequence to the zygote is less and less valid (Yamauchi *et al.*, 2011). In line with this growing comprehension and in addition to the above-introduced 'maternal-effect' chromatin involved genes (such as *Zfp57*, *Stella* and *Elp3* contributed in either RNA or protein by the oocyte), evidence for 'paternal-effect' genes has been presented (Chong *et al.*, 2007). Here it was shown that 'chromatin metabolism' during spermatogenesis influences paternal gene expression in the next generation. Differences between female and male inheritance of epigenetic variation might be partially explained by these chromatin alterations during gametogenesis mediated by maternal- and paternal-effect genes.

In summary, both the maternal and paternal germline possess the tools necessary for the transmission of epigenetic marks. However,

differences in the maternal and paternal genotype, expressed before, at and after fertilization, are likely to influence the inheritance of epigenetic variation in a sex-specific manner. The epigenetic pathways outlined here likely also have a bearing on the recently discovered phenomenon of transgenerational genetic effects (Nadeau, 2009). As an example, a paternal ancestral genotype determines food intake and body weight for multiple generations in offspring not carrying the obesity resistance conveying ancestral genetic variant (Yazbek *et al.*, 2010). As more conditional mutants (i.e. their expression is restricted to the germline) for chromatin involved genes become available in the mouse, our insight into these very intriguing aspects of transmission biology will undoubtedly grow.

Stress, hormone and nutrition-induced transgenerational epigenetic variation

In experimental animals, such as mice and rats, a range of external influences, including irradiation stress, exposure to hormones and nutrition, has been shown to induce variation in the epigenome (Jirtle and Skinner, 2007; Kovalchuk and Baulch, 2008). Some studies have focused on investigating the possibility that this variation might be transmitted to subsequent generations. When F0 pregnant females carrying F1 embryos that already contain germ cells that will produce the F2 are treated with an inducing agent, observations have to be extended into the F3 generation in order to exclude a direct effect of the treatment on the germ cells, observed in the F2 individuals (Jablonka and Raz, 2009). When F0 adult males are treated, a transmissible epigenetic effect can be concluded at the earliest in the gametes of the F1. Evidence for the induction of heritable epigenetic variation by hormones, nutrition and stress will be briefly evaluated.

Genotoxic stress-induced transgenerational epigenetic variation

Currently known examples of stress-induced transgenerational epigenetic effects in the mouse mostly involve irradiation as the triggering event and these have recently been reviewed (de Boer *et al.*, 2010). In addition to the direct genotoxic effects of radiation on cells, notably the nucleus, it has been recognized that radiation can induce an increased rate of DNA breaks and mutations in descendent cells and even across generations (Morgan, 2003a, b). This phenomenon is termed radiation-induced genomic instability. Germline transmission has been shown to occur exclusively after male exposure although subsequent transmission is observed via both sexes. The most widely used experimental setup to detect transmission of delayed effects of irradiation uses the mutation frequency at expanded simple tandem repeat (ESTR) loci as the readout of genomic instability (the mutation is read as a change in repeat number; Dubrova *et al.*, 1993). The fact that non-Mendelian inheritance of an increased ESTR mutation rate is usually observed led epigenetic disturbances to be suggested as a causative factor (Barber *et al.*, 2006). This notion was further supported by the observation that irradiation results in a quickly induced global hypomethylation of DNA (Koturbash *et al.*, 2005; Pogribny *et al.*, 2005; Loree *et al.*, 2006). After testis irradiation, hypomethylation of retrotransposed interspersed repeat elements (LINEs and SINEs) was found in the offspring (Filkowski *et al.*, 2010) next to lower levels of the methylated CpG binding protein MeCP2 and the DNA homologous recombination

repair protein RAD51 in the thymus (Koturbash et al., 2006). These experiments show that genotoxic stress in the male germline can induce genetic and epigenetic variation in the offspring.

Nutrition, hormones and epigenetic variation

Epigenetic effects caused by nutrition as well as hormones have mainly been described after induction in late embryos and early fetuses, when PGCs are arising and migrate to the early gonad. Recent publications have demonstrated that the adult male is also capable of acquiring nutrition-induced epigenetic variation in the germline (Carone et al., 2010; Ng et al., 2010). The first hint was provided by the discovery that pre-mating fasting of male mice led to altered serum glucose levels in offspring (F1) (Anderson et al., 2006). Chronic exposure of male rats to a high-fat diet was associated with pancreatic beta cell dysfunction in the offspring (Ng et al., 2010), while a low-protein diet in the male mouse affected the hepatic expression of genes involved in proliferation and cholesterol biosynthesis (Carone et al., 2010). In the mouse, the diet was found to be correlated with differential methylation of several lipid metabolism-related genes in the liver of both male and female offspring. These results show that, next to the oocyte and the developing embryo in the female, sperm is sensitive to nutrition-induced epigenetic variation. The next step in this line of research is to verify that these effects are transmitted to subsequent generations (F2) and thus elicit true long-lasting heritable epigenetic variation.

Current evidence suggests that humans are also sensitive to the effects of environmental influences on the deposition of epigenetic marks during human embryonic development (Heijmans et al., 2008). This evidence was obtained by studying the level of DNA methylation at the imprinted *IGF2* gene in individuals who were prenatally exposed to famine during the Dutch Hunger Winter (1944–1945). For individuals who were conceived during the famine and thus were exposed in the earliest stages of development when epigenetic reprogramming events occur, the DMR of *IGF2* was significantly hypomethylated compared with non-exposed siblings (Heijmans et al., 2008). This was not the case for individuals who were exposed at a later gestational stage. Although this study only described epigenetic effects within one generation, it shows that nutrition can also induce epigenetic variation in humans. In another study, it has been observed that the mortality rate of tested individuals related to cardiovascular disease and diabetes was significantly reduced if their grandfather experienced scarcity of food during prepuberty (Kaati et al., 2002). Subsequent analysis led to the conclusion that transgenerational epigenetic effects had to be the cause of these observations (Kaati et al., 2002; Pembrey, 2002, 2006). The study of the mechanisms behind transgenerational epigenetic inheritance in human populations is hindered by the genetic variation that exists within the population, this being the natural cause of most variation in DNA methylation (Heijmans et al., 2007). Although the use of siblings of the same sex can eliminate these genetic factors in cohort studies within one generation, this advantage is lost when looking at transgenerational epigenetic effects.

After the exposure of mid-gestation rats to the anti-androgenic compound vinclozolin, a decreased spermatogenic capacity and increased incidence of male infertility up to F4 was found (Anway et al., 2005) and recently evidence for persistent CpG methylation changes in selected gene promoters of F3 sperm was obtained (Guerrero-Bosagna et al., 2010). As further testimony to its epigenetic activity,

vinclozolin was shown to increase and decrease DMR methylation levels of respective maternally and paternally imprinted gene clusters in the male germline (Stouder and Paoloni-Giacobino, 2010). The effect was transgenerational, as hypo- and hypermethylation were not yet completely lost in the sperm of F3 males.

The mechanistic link between estrogen and androgen hormones and epigenetic variations in chromatin architecture might be mediated by nuclear receptors (Biddie, 2011). In humans, the best-described case of hormone-induced epigenetic variation is the exposure of pregnant women to the estrogen receptor agonist diethylstilbestrol (DES), leading to developmental abnormalities in uterus structure, increased cancer risk in the daughters of pregnant mothers exposed to DES (Li et al., 2003) and an abnormal methylation of the lactoferrin promotor (Li et al., 1997). In mice, these defects in uterine development and carcinogenesis can be observed up to the F3 generation (Walker and Haven, 1997; Newbold et al., 2006), hence showing transgenerational epigenetic inheritance. For the human this has not been proven as only modest effects were found in the F2 (Blatt et al., 2003; Titus-Ernstoff et al., 2006, 2008, 2010), despite the similar nature of the phenotypic effects of DES in humans and the mouse (Ruden et al., 2005). In this context, it was suggested that the stress-induced chaperone Hsp90, of which the estrogen receptor is one of many clients, might be involved in the induction of epigenetic variation (Ruden et al., 2005).

Epigenetic effects of ART

Studies on mice designed to evaluate epigenetic and physiological aspects of ART

In all publications on epigenetic and physiological, behavioural read-outs after ART in the mouse (Table I), effects have been noted. Most experiments address the effects of ovulation induction and pre-implantation embryo culture on maintenance of imprinting up to mid-gestation. Imprinting status is followed using the methylation status of the DMR, in which surveys the paternally imprinted *H19* is often studied. Imprinted gene expression has been determined as well. Examples of unaffected (Fauque et al., 2007) or slightly affected methylation (Market-Velker et al., 2010a, b) followed by affected expression, are given. Hence, all details of regulation by CpG methylation of imprinted gene expression are not yet available, which is a lacuna in our understanding.

There are clear indications for both an effect of ovulation induction and of *in vitro* embryo culture on maintenance of DMR methylation. Also, the physiological status of the maternal tract after a ovulation induction procedure adds to deregulation of imprinting. As the only paper devoted to DMR methylation in maturing and mature oocytes after ovulation induction did not find an effect of hormonal priming (Sato et al., 2007), a maternal 'zygotic' effect and/or an effect of the female tract after ovulation induction, and (and should not be removed) an effect of the *in vitro* environment, is indicated.

In the experiments reported upon, inbred strains or hybrids between inbred strains have been predominantly used. No experiments with oocytes from random bred stocks have been reported in the literature. So principally, the oocyte inbred genotype could already confer cell biological stress (Nadeau, 2009) that exacerbates after hormonal priming and during *in vitro* culture.

Table 1 Survey of the mouse experiments aimed at testing imprinting and physiological parameters after ART.

Reference	Genotype	Conditions						Readouts					
		In vivo	Superov in vivo	Superov in vitro cleavage	Superov IVF + cleavage	ET	Media comparison	Blastocyst gDMR methyl	Blastocyst Impr expr	Mid gest Embryo gDMR methyl	Mid gest Embryo Impr expr	Mid gest Placenta gDMR methyl	Mid gest Placenta Impr expr
Market-Velker <i>et al.</i> (2010a)	B6(CAST7)	x		x			x	x	x				
Market-Velker <i>et al.</i> (2010b)	B6(CAST7)	x	x					x					
Doherty <i>et al.</i> (2000)	B6(CAST7)		x	x			x	x	x				
Mann <i>et al.</i> (2004)	B6(CAST7)		x	x		x	x	x	x	x	x	x	x
Khosla <i>et al.</i> (2001)	B6CBA F1			x		x	x				x		
Fauque <i>et al.</i> (2007)	B6CBA F1 x F1	x	x	x	x		x	x	x				
Fauque <i>et al.</i> (2010a)	B6CBA F1 x F1		x	x	x	x	x			x	x	x	x
Fauque <i>et al.</i> (2010b)	B6CBA F1 x F1		x		x	x							a
Rivera <i>et al.</i> (2008)	B6(CAST7)	x	x	x		x				x	x	x	x
Fortier <i>et al.</i> (2008)	CD1 x B6(CAST7)	x	x			x				x	x	x	x
Li <i>et al.</i> (2005)	B6 x Spretus		x		x								x
Adult readout													
Morgan <i>et al.</i> (2005)	FVB/N x B6 A ^{vy} /a		x	x		x							Phenotype epiallele
Fernandez-Gonzalez <i>et al.</i> (2010)	B6CBA F1 x I29/Rr Axin1 ^{fl}		x	x			x						Phenotype epiallele
Ecker <i>et al.</i> (2004)	I29Sv x B6	x ^b				x	x						Behavioural testing
Fernandez-Gonzalez <i>et al.</i> (2004)	B6CBA F1			x		x	x						Behavioural testing
Watkins <i>et al.</i> (2007)	CBAB6 F1 x MFI	x		x		x							Blood pressure, serum angiotensin converting enzyme, hepatic phosphoenolpyruvate carboxykinase
Scott <i>et al.</i> (2010)	B6C3 F1	x			x ^c	x							Glucose parameters

Superov, superovulation; ET, embryo transfer; gDMR, germline differentially methylated region (DMR); Impr expr, Imprinted expression; Mid gest, mid gestation.

^aTranscriptome analysis.

^bIn vivo fertilization was followed by *in vivo* and *in vitro* development.

^cCulture to the 2-cell stage.

In general the placenta is much more vulnerable than is the embryo proper, which is an illustration of the great significance of imprinting regulation for placental gene expression (Kawahara et al., 2009). One reason for the increased sensitivity of the placenta for lack of maintenance of DMR methylation might be related to the overall lower level of 5methylCpG (Monk et al., 1987; Fig. 5). In the mouse at least, effects on placental imprinted gene expression of *H19*, the fine regulator of prenatal growth, translate into deregulation of the imprinted gene network (Gabory et al., 2009, 2010; Fauque et al., 2010a, b) most likely also influencing gene expression among non-imprinted genes.

Effects of *in vitro* culture have also been observed in the A^{vy} an *Axin^{fl}* genetic systems, leading to hypomethylation of the IAP cryptic promoters. In the mouse, ART also affects physiological parameters at adult age, such as insulin sensitivity and blood pressure. Effects on adult behaviour have been reported too. For more information regarding the effects in the mouse, the reader is referred to the Supplementary Data (available online).

Epigenetic aspects of ART in human

Imprinting disorders in children born after ART

Since 2002, a number of reports have shown an association between ART and the frequency of imprinting disorders, notably BWS (Table II). Risk, expressed as the relative abundance of ART in the BWS cases compared with relative abundance of ART in the general population, was estimated at between 3.1 and 16.1 (Table II). Cases occurred irrespective of cause of infertility and are reported after IVF and ICSI, after transfer of fresh and frozen embryos, after transfer on Day 2, 3 or Day 5 and after different levels of hormonal stimulation, with *in vitro* culture being the common denominator (Gicquel et al., 2003; Chang et al., 2005; Sutcliffe et al., 2006; Doornbos et al., 2007). However, also after intrauterine insemination that often involves gonadotrophin stimulation, or the use of fertility drugs alone, BWS cases have been reported (Chang et al., 2005; Sutcliffe et al., 2006; Doornbos et al., 2007), which led Doornbos et al. to speculate that

Table II Reports on the incidence of imprinting disorders after human IVF.

Reference	Type of study	N cases	% IVF in cases	% IVF in ref	Estimated risk	Type of IVF	Molecular defect
Beckwith–Wiedemann syndrome							
DeBaun et al. (2003)	Case series	65	4.6	0.76	6.1	IVF and ICSI	5/6 LOM KCNQ1OT1 gDMR ^c 1/6 GOM H19 DMR 1/6 no imprint defect
Maher et al. (2003)	Case series	149	4.0	0.997	4.0*	IVF (n = 3) and ICSI (n = 3)	2/6 LOM KCNQ1OT1 gDMR 4/6 not analysed
Gicquel et al. (2003)	Case series	149	4.0	1.3	3.1*	IVF (n = 4) and ICSI (n = 2)	6/6 LOM KCNQ1OT1 gDMR
Halliday et al. (2004)	Case control	37	10.8	0.67	16.1*	IVF (n = 3) and ICSI (n = 1)	3/4 LOM KCNQ1OT1 gDMR 1/4 not analysed
Chang et al. (2005)	Case series	341	5.6 ^a	–	–	IVF (n = 5) and ICSI (n = 5) ^a	NA
Sutcliffe et al. (2006)	Survey	209	2.9–7.6 ^b	0.8	3.6–9.5 ^{b*}	IVF (n = 1) and ICSI (n = 5)	6/6 LOM KCNQ1OT1 gDMR
Doornbos et al. (2007)	Survey	71	5.6	0.92	6.1*	IVF (n = 4)	4/4 LOM KCNQ1OT1 gDMR
Angelman Syndrome							
Cox et al. (2002)	Case series	2	–	–	–	ICSI (n = 2)	2/2 LOM SNRPN
Orstavik et al. (2003)	Case report	1	–	–	–	ICSI (n = 1)	1/1 LOM SNRPN
Ludwig et al. (2005)	Survey	79	3.8	–	–	ICSI (n = 3)	1/3 LOM SNRPN 2/3 maternal deletion 15q11
Sutcliffe et al. (2006)	Survey	75	0	0.8	–	–	–
Doornbos et al. (2007)	Survey	63	0	0.92	–	–	–

LOM, loss of methylation; GOM, gain of methylation; –, not analysed.

^aAll 19 ART cases are included, 10 after IVF (and ICSI), 2 after hormonal stimulation and insemination and 7 for which no data on type of ART were available.

^bRange takes into account the large number of lost to follow-up by assuming that all non-responders conceived naturally.

^cSix ART- Beckwith–Wiedemann syndrome cases were identified in a database. Three patients were from before 2001, when use of ART was not systematically assessed. This period was excluded from the risk assessment.

*Risk is significantly increased in IVF compared with non-IVF pregnancies.

not ART practice but subfertility is at the heart of this increase in BWS. Indications for a link between ART and epigenetic regulation are that in the general population BWS is caused by a DMR CpG methylation error in 50–60% of the cases (Manipalviratn *et al.*, 2009), while after ART almost all cases are related to hypomethylation of the maternal *KCNQ1OT1* DMR (Table II). Also, more often other maternally methylated regions are hypomethylated in ART-BWS than in non-ART BWS children (Lim *et al.*, 2009). To investigate whether children born after ART might have subclinical forms of BWS, Bowdin *et al.* (2007) analysed 1524 probands for clinical features linked to BWS. Four children had at least one of these signs, of which one was already diagnosed as having BWS. None of the other three children showed loss of methylation at *KCNQ1OT1*, suggesting that no milder forms of BWS have been missed in previous ART-BWS reports.

Angelman syndrome (AS) is caused by a shortage of maternal *UBE3A* expression in the *SNRPN* imprinting cluster. Less than 5% of cases are caused by an imprinting defect. Six cases of AS have been reported after ICSI (Table II; Cox *et al.*, 2002; Orstavik *et al.*, 2003; Ludwig *et al.*, 2005), of which the unexpected high number of four shows a methylation defect. In two other studies covering more than 400 AS cases, none were conceived by IVF or ICSI. Instead, seven AS cases originated from ovulation induction and/or intrauterine insemination (Sutcliffe *et al.*, 2006; Doornbos *et al.*, 2007).

To date, five cases of Silver–Russell Syndrome (SRS) have been published in children born after IVF or ICSI (Svensson *et al.*, 2005; Kagami *et al.*, 2007; Galli-Tsinopoulou *et al.*, 2008; Kallen *et al.*, 2010b). In one, hypermethylation of the paternal *MEST* DMR was reported (Kagami *et al.*, 2007). Generally, around 44% of the SRS cases is caused by *H19* DMR hypomethylation and 5–10% by maternal uniparental disomy of chromosome 7. Thus far, no imprinted candidate gene on chromosome 7 could be identified (Binder *et al.*, 2011). The number of cases involving ART is too small for a relation to be indicated.

Retinoblastoma (RB) and Prader–Willi syndrome (PWS) are two (epi)genetic disorders involving imprinting. In most cases the underlying molecular mechanism is a (point)mutation or a deletion and not an epimutation, just as in three reported PWS-ART cases (Sutcliffe *et al.*, 2006; Doornbos *et al.*, 2007) and two out of seven RB-ART cases (Marees *et al.*, 2009). For the other five cases no gene defect was found and methylation was not analysed. In the PWS/AS region, methylation was normal in 92 children born after ICSI (Manning *et al.*, 2000).

In a large follow-up study of children born after IVF in Sweden, one BWS, two SRS and four PWS patients were found ($n = 31\,850$) (Kallen *et al.*, 2005, 2010b). In the Danish National Cohort study (Lidegaard *et al.*, 2005) among 6052 children there were none with a genomic imprinting disease. Recently, the French follow-up association reported 6 BWS cases (and no PWS, AS or SRS) in a cohort of 15 162 IVF children (Viot *et al.*, 2010). With a spontaneous BWS incidence of 1 out of 13 700 (Amor and Halliday, 2008), the results of this study follow the tendency of the case series towards an increased risk after ART.

Effect of ART on epigenetic parameters in human gametes and embryos

Oocytes

Spontaneous oogenesis. In humans, studies on imprinting directed epigenetic reprogramming during oogenesis are very limited for ethical

reasons. Only one study used immature oocytes from growing follicles in non-stimulated fertile patients after laparoscopy [(Sato *et al.*, 2007) Table III]. At the primary follicle stage almost 50% of the maternally imprinted *MEST*, *KCNQ1OT1* and *PLAGL1* DMR alleles was methylated. This level gradually increased in growing preant follicles and at the antral follicle stage, almost all alleles were methylated (Sato *et al.*, 2007), just as in mice (Obata and Kono, 2002; Lucifero *et al.*, 2004). The paternal imprint of the *H19* DMR was partially erased at the primary follicle stage. A remnant of around 10% methylation was found at the antral follicle stage, which is different from the expectation based on mice, where in E13.5 PGCs the imprint is already removed (Hajkova *et al.*, 2002).

Ovulation induction. Table III gives a numerical overview of the data on genomic imprinting in oocytes from ovulation induction in women. The analysis of a possible effect of hormonal priming on imprinting can be confounded with maternal age and/or general suboptimal oogenesis. Proper control oocytes are scarce but, nevertheless, important information can be obtained.

In contrast to the almost complete methylation at the antral follicle stage of non-stimulated oocytes, after ovarian stimulation only 10 of the 16 germinal vesicle (GV)/metaphase I (MI) oocytes were methylated at *MEST* (Sato *et al.*, 2007). The cause of subfertility in the couples donating the affected GV/MI primary oocytes was male factor or tuba obstruction, suggesting a genuine ovulation induction effect. The methylation status of two other maternally imprinted DMRs [*SNRPN* (Geuns *et al.*, 2003) and *KCNQ1OT1* (Geuns *et al.*, 2007b)] was in most cases undisturbed. However, in one of the 12 GV/MI oocytes *KCNQ1OT1* DMR was completely unmethylated (Table III). In the study of GV and MI oocytes of Khoueiry *et al.*, around 60–70% of the alleles was methylated at *KCNQ1OT1* DMR, while in metaphase II (MII) oocytes, which are mostly used for IVF treatment, the methylation level was around 90% (Khoueiry *et al.*, 2008). This would suggest that around 10% of the MII oocytes can lead to BWS, which is not in agreement with the true incidence of BWS in ART. In this study of (Khoueiry *et al.*, 2008), that included 52 ICSI cycles, some women suffered from polycystic ovary syndrome (PCOS, $n = 11$), endometriosis ($n = 4$) or dysovulation ($n = 3$). The methylation level of oocytes from women with or without PCOS was similar. Concerning *SNRPN*, El-Maarri *et al.* (2001) found a completely unmethylated DMR in a pool of 20–30 human MII oocytes obtained after ovarian stimulation. This is in agreement with neither the data from Geuns *et al.* (2003) where at the GV and MI stage *SNRPN* is already completely methylated, nor with the *SNRPN* methylation pattern in mouse MII oocytes (Lucifero *et al.*, 2002). Also, the relative low incidence of AS and PWS after ART is not what would be expected with a completely unmethylated DMR.

Regarding the expected paternal DMR demethylation in oocytes, two out of six GV/MI oocytes were erroneously methylated at *H19* after ovarian stimulation (Sato *et al.*, 2007), a finding not substantiated by Borghol *et al.* (2006) where the paternal methylation imprint at *H19* DMR was absent, as expected. Also, the paternally imprinted intergenic DMR of *DLK1* and *MEG3* (IG-DMR) was mainly unmethylated in superovulated oocytes at the GV and MI stages (Geuns *et al.*, 2007a).

Table III DNA CpG methylation in human oocytes at different stages of development with and without ovarian stimulation and IVM.

Reference	gDMR	Methylation	No ovarian stimulation			Ovarian stimulation, in vivo maturation			Ovarian stimulation, IVM of GV		
			Primary follicle	Pre-antral follicle	Antral follicle	GV	MI	MII	Arrested GV	Arrested MI	MII
Sato <i>et al.</i> (2007)	MEST	M	50.0	57.0	91.6	55.6 (5/9)	71.4 (5/7)				
	PLAGLI	M	40.0	10.4	83.3						
	KCNQ1OT1	M	55.5	66.6	87.5						
Geuns <i>et al.</i> (2007b)	KCNQ1OT1	M				100 (6/6)	83.3 (5/6)				100 ^c (4/4)
Khoueiry <i>et al.</i> (2008)	KCNQ1OT1	M			67.8 (GV) ^a	62.5	66.7	89.5	61.2	65.2	78.3
					70.3 (MI) ^a						
Geuns <i>et al.</i> (2003)	SNRPN	M				100 (7/7)	100 (3/3)				100 ^c (3/3)
El-Maarri <i>et al.</i> (2001)	SNRPN	M						0 ^b			
	NDN	M						33.3 ^b			
Sato <i>et al.</i> (2007)	H19	P	28.2	33.4	12.5	0 (0/3)	66.7 (2/3)				
Borghol <i>et al.</i> (2006)	H19	P				0 (0/5)			24.3	13.8	16.7
Geuns <i>et al.</i> (2007a)	DLKI	P				0 (0/4)	0 (0/6)				0 ^c (0/6)

Methylation is depicted as the percentage of methylated alleles (greater-than two-third of DMR is methylated) of the total number of alleles analysed.

M, maternal; P, paternal.

If known, the number of methylated oocytes and the total number of oocytes are put in parentheses.

^aGV and MI are obtained from non-stimulated patients with polycystic ovaries and after culture arrested in GV and MI.

^bUnfertilized oocytes.

^cMII obtained after culture of both GV and MI.

In vitro maturation. IVM of oocytes has been introduced to retrieve several oocytes for IVF treatment avoiding exogenous gonadotrophins, especially for patients at risk for the ovarian hyperstimulation syndrome and/or PCOS (Jurema and Nogueira, 2006). In general, small and medium-sized antral follicles are aspirated and the associated oocytes are cultured for 24–48 h before fertilization, depending on procedure at the clinic.

At the antral follicle stage, most DMR CpG methylation has been established although not completely so (see text above and Table III). Hence, in theory, IVM could interfere with imprint establishment or maintenance. To prove this point, GV and MI oocytes from women undergoing IVF treatment with hormonal stimulation were cultured in maturation medium. The normal maternal imprints of *SNRPN* and *KCNQ1OT1* in the GV and MI oocytes were stably maintained *in vitro* up to the secondary oocyte stage (Geuns *et al.*, 2003, 2007b; Table III). However, in a study comparing *in vivo* derived and IVM derived MII oocytes, the methylation level of *KCNQ1OT1* DMR in the second group was statistically lower (Khoueiry *et al.*, 2008). These authors point out that the maturation time (28 h when compared with ~36 h *in vivo*) might be too short to finish the methylation process but this is disputed by the correct methylation pattern in oocytes cultured overnight as reported in Geuns *et al.* (2003, 2007b).

The paternal imprint at the IG-DMR was correctly absent after IVM of GV and MI oocytes (Geuns *et al.*, 2007a). Borghol *et al.* (2006) obtained evidence that the *H19* DMR is more vulnerable to the environment. After GV maturation for 24 h, two of the six pools with a maximum of three MII oocytes showed complete methylation in at least one allele, while in MI-derived MII oocytes methylation was completely absent.

Spermatozoa

In the human male germline (Table IV and Supplementary data, Table), the imprints of both maternal (*MEST*) and paternal (*H19*) DMRs are completely erased in fetal prospermatogonia (Kerjean *et al.*, 2000). The maternally methylated *MEST* DMR remains unmethylated during spermatogenesis. For *H19*, the imprint is established during the adult spermatogonial stage or at least before the spermatocytes enter meiosis I, and is maintained thereafter (Kerjean *et al.*, 2000), resembling the reprogramming in the mouse (Ueda *et al.*, 2000). Hence in mature spermatozoa the paternally imprinted DMRs are completely methylated, while the maternally imprinted ones are unmethylated (see Table IV for references).

Effect of male subfertility on the epigenetic status of DMRs in spermatozoa.

ART as such is unlikely to affect methylation in spermatozoa since these patterns, including the paternal imprints, are established before any manipulation occurs, the normal sperm nucleus being metabolically inert. Several studies show that a disturbed spermatogenesis itself is associated with incorrect imprinting (Table IV, Supplementary data, Table). In spermatozoa from oligozoospermic men, the occurrence of hypermethylation of several maternally imprinted DMRs or hypomethylation of the *H19* and IG-DMR is increased, especially in ejaculates of $<10 \times 10^6$ /ml (Supplementary data, Table). The number of affected CpG sites ranges from only a few in normozoospermia to the whole DMR in severe azoospermia, only occurring in a minority of alleles sampled (Supplementary data, Table). Further evidence for an association between methylation and sperm

concentration comes from a study by Boissonnas *et al.* (2010) who analysed the *H19* DMR (CTCF6 region) in teratozoospermic (TZ) and oligo-astheno-teratozoospermic (OAT) patients. In spermatozoa from TZ patients, only 2 out of 16 CpGs were significantly hypomethylated. In OAT spermatozoa, methylation was drastically reduced for all CpGs, reaching significance in subgroups with a sperm concentration of $<10 \times 10^6$ /ml. Sperm concentration is positively correlated with *H19* methylation and negatively correlated with *MEST* methylation that is normally absent (Boissonnas *et al.*, 2010, Poplinski *et al.*, 2010). OAT spermatozoa also show reduced IG-DMR methylation (El Hajj *et al.*, 2011).

Alteration of the protamine 1 to protamine 2 ratio, which should be around 1, generally denotes affected spermatogenesis [either as cause or as consequence (Nanassy *et al.*, 2011)] and led to hypermethylation of several normally maternally methylated loci DMRs (*KCNQ1OT1*, *SNRPN*, *MEST*, *PEG3*, *PLAGL1*, *IGF2*) and to hypomethylation of the *H19* DMR (Hammoud *et al.*, 2010). Together, these data clearly indicate that DMR methylation defects are associated with poor spermatogenesis. Besides oligozoospermia, also other aetiologies of male subfertility are associated with epigenetic defects (Table IV, Supplementary data, Table). Azoospermia caused by anejaculation and secondary inflammatory obstruction was related to an increase in *MEST* methylation (Marques *et al.*, 2010), as was a sperm motility <40 , or $<5\%$ of sperm with normal morphology (Poplinski *et al.*, 2010).

Global DNA methylation of non-imprinted repetitive sequences, such as long and short interspersed nucleotide elements (LINE1 and SINE (Alu)), did not show a decrease in spermatozoa from oligozoospermic or OAT patients (Kobayashi *et al.*, 2007; Marques *et al.*, 2008; Boissonnas *et al.*, 2010; El Hajj *et al.*, 2011), except for Alu element methylation in the study by El Hajj *et al.* (Supplementary data, Table). The methylation of non-imprinted genes and a repetitive sequence was also affected (Houshdaran *et al.*, 2007), typically for sequences showing large intra- and interindividual methylation variation in spermatozoa from normozoospermic males (Flanagan *et al.*, 2006).

Recently, it has been shown that patients with OAT had an increased level of methylation in the promotor of the normally unmethylated germline regulator gene *DAZL*, the autosomal substitute of Y-linked *DAZ* that correctly remained unmethylated (Navarro-Costa *et al.*, 2010; Supplementary data, Table).

Effect of spermatozoa methylation defects on IVF outcome.

It is not known to what extent DMR CpG methylation in both degree and prevalence can be ablated before germline transmission of this mark suffers. Kobayashi *et al.* (2009) compared the methylation defect that was found in trophoblastic villi from ART-miscarriages between 6–9 weeks of gestation with the imprints in the semen from the father. In 7 out of the 17 ART pregnancies with a placental *H19* methylation defect, this was also found in the spermatozoa, suggesting transfer from the father. In a patient with hypospermatogenesis and with almost complete hypomethylation of the *H19* DMR, the embryos obtained after ICSI all showed developmental arrest (stage unknown) (Marques *et al.*, 2010). As in the human *H19* is not expressed up to the blastocyst stage (Salpekar *et al.*, 2001), a common paternal factor might be at stake. As no analysis of the embryos was undertaken, there is no formal proof of paternal

Table IV DNA CpG methylation studies on human spermatozoa from normal probands and subfertile patients.

Reference	Conditions analysed					DMR	Methylation (M/P)	Technique	ART outcome analysed
	Normal	Concentration	Motility	Morphology	Other				
Geuns <i>et al.</i> (2007a)	+					IG-DMR	P	Bisulphite sequencing	
Geuns <i>et al.</i> (2003)	+					SNRPN	M	Bisulphite sequencing	
Geuns <i>et al.</i> (2007b)	+					KCNQ1OT1	M	Bisulphite sequencing	
Kerjean <i>et al.</i> (2000)	+				Spermatids, Testicular (fetal) spermatogonia and spermatocytes	H19 MEST	P M	Bisulphite sequencing	
Marques <i>et al.</i> (2004)	+	+				H19 MEST	P M	Bisulphite sequencing	
Marques <i>et al.</i> (2008)	+	+				H19 MEST LINE1	P M Global	Bisulphite sequencing	
Kobayashi <i>et al.</i> (2007)	+	+				H19 IG-DMR MEST KCNQ1OT1 PLAGL1 PEG3 SNRPN LINE1 Alu	P P M M M M M Global Global	Bisulphite sequencing + COBRA	+ (1 child)
Sato <i>et al.</i> (2011)	+	+				ZDBF2 H19 IG-DMR MEST KCNQ1OT1 PEG3 SNRPN PLAGL1	P P P M M M M M	Bisulphite polymerase chain reaction Luminex, bisulphite sequencing and COBRA	
Hammoud <i>et al.</i> (2010)	+	+			Protamine replacement defect	H19 KCNQ1OT1 MEST SNRPN PLAGL1 IGF2 PEG3	P M M M M M M	Bisulphite sequencing	

Poplinski <i>et al.</i> (2010)	+	+	+	+		H19 MEST	P M	Bisulphite sequencing	
Benchaib <i>et al.</i> (2003)	+	+	+	+		Overall	global	Immunostaining	
Houshdaran <i>et al.</i> (2007)	+	+	+	+		37 regions 1421 regions	– –	MethylLight Illumina array	
Boissonnas <i>et al.</i> (2010)	+			+	OAT	H19-CTCF3 H19 CTCF6 IGF2-DMR0 IGF2-DMR2 LINE1	P P P P Global	Pyrosequencing	+ (fertilization rate, cleavage and fragmentation)
Navarro-Costa <i>et al.</i> (2010)	+				OAT	DAZL DAZ	M+P M+P	Bisulphite sequencing	
El Hajj <i>et al.</i> (2011)	+				OAT	H19 IG-DMR MEST KCNQ1OT1 PEG3 SNRPN NESPAS LINE1	P P M M M M M Global	Pyrosequencing	+ (Fertilization rate, pregnancy rate, live birth rate, abortion rate)
Manning <i>et al.</i> (2001)	+				Testicular round spermatids, testicular elongated spermatids	SNRPN	M	Methylation sensitive PCR with fragment length analysis	
Hartmann <i>et al.</i> (2006)	+				Arrested spermatogonia Arrested spermatocytes	H19	P	Bisulphite PCR with single-strand conformation polymorphism (SSCP) analysis	
Marques <i>et al.</i> (2010)					Testicular spermatozoa from patients azoospermic due to ANJ, OAZI, CBAVD, HP	H19 MEST	P M	Bisulphite sequencing	+ (embryonic developmental arrest in HP patient)

COBRA = combined bisulphite-PCR restriction analysis, OAT = patients presenting with combined oligozoospermia, asthenozoospermia and teratozoospermia, ANJ = Anejaculation, OAZI = secondary inflammatory obstructive azoospermia, CBAVD = obstructive azoospermia due to congenital bilateral absence of the vas deferens, HP = secretory azoospermia due to hypospermatogenesis.

inheritance of *H19* DMR hypomethylation. In patients with OAT with a partial hypomethylation of *H19*, the fertilization rate after ICSI was reduced (Boissonnas et al., 2010). Developmental parameters, such as embryo quality, implantation rate, gestational age and birth-weight, were similar to normally methylated paternal controls (Boissonnas et al., 2010). In another case, with spermatozoa showing both a maternal and a paternal methylation imprinting error (*MEST* DMR was methylated in 60% of the alleles and *PLAGL1* in 20%, and *H19* DMR was unmethylated in 90% of the alleles), a normal pregnancy was achieved with normal methylation (Kobayashi et al., 2007).

Methylation analysis in 19 ICSI children born small for gestational age revealed that one of them had hypermethylation in *KCNQ1OT1* and *MEST*. As both parents had a normal methylation profile (and

hypermethylation can only refer to the normally hypomethylated paternal allele), the methylation must have appeared *de novo*, maybe in the male germline because of the oligozoospermia of the father (Kanber et al., 2009; Table V).

There is only one case report in which part of the methylation defect of the child was also detected in leucocytes from the father. In a child with SRS conceived by IVF, eight CpGs were hypermethylated in *PEG1/MEST* DMR, four of which were also hypermethylated in the father (Kagami et al., 2007).

The preimplantation embryo

Loss of 5methyl CpG immunostaining in human embryos after fertilization resembles that reported in mammalian embryos (Santos et al., 2002, 2010). Active loss of immuno-recognition of paternal

Table V DNA CpG methylation studies of mainly DMRs in human offspring following ART.

Reference	ART	n	Control	Sample	DMR	M/ P	Results
Kanber et al. (2009)	ICSI + SGA	19	Normal weight children after spontaneous conception	Buccal smear	<i>KCNQ1OT1</i> <i>MEST</i> <i>PEG3</i> <i>H19</i> <i>GTL2</i> <i>PLAGL1</i>	M M M P P M	1/19 children had hypermethylation of <i>KCNQ1OT1</i> and <i>MEST</i>
Manning et al. (2000)	ICSI	92	–	Blood	<i>SNRPN</i>	M	In all 92 children the expected methylation pattern was seen
Gomes et al. (2009)	IVF and ICSI	18	Naturally conceived children and BWS patients	Peripheral blood, UCB or placenta	<i>KCNQ1OT1</i>	M	3 of 18 IVF children showed hypomethylation at <i>KCNQ1OT1</i> , without BWS phenotype
Tierling et al. (2010)	IVF and ICSI	112	Naturally conceived children	UCB, amnion membrane	<i>KCNQ1OT1</i> <i>H19</i> <i>SNRPN</i> <i>MEST</i> <i>GRB10</i> <i>GTL2</i> <i>GNAS</i> locus	M P M M M P M+P	Only <i>MEST</i> was slightly hypermethylated in IVF compared with ICSI and control samples
Katari et al. (2009)	IVF	10	Naturally conceived children	UCB, placenta	1536 CpG sites	–	23% CpG sites differed in UCB and 16% in placenta. Imprinted genes are not extra vulnerable for deregulation. 4/11 tested genes with differential methylation also showed differential expression
Zechner et al. (2010)	IVF and ICSI	42	Abortions/stillbirths after spontaneous conception	Chorion villi	<i>H19</i> <i>MEG3</i> <i>KCNQ1OT1</i> <i>MEST</i> <i>NESP55</i> <i>PEG3</i> <i>SNRPN</i> <i>NANOG</i> <i>APC</i>	P P M M P M M+P M+P	IVF villi showed a hypomethylation (3% less) of <i>KCNQ1OT1</i>
Turan et al. (2010)	IVF (ICSI unknown)	45– 98	Naturally conceived children	UCB, cord, placenta	<i>H19</i>	P	After IVF the intra- and inter-individual variation in methylation is higher. The expression of <i>H19</i> and <i>IGF2</i> in placenta and UCB was reduced in the IVF group

M/P, maternally or paternally methylated; SGA, small for gestational age; UCB, umbilical cord blood.

5methylC also takes place in the human zygote indicating active demethylation (Beaujean *et al.*, 2004; Fulka *et al.*, 2004). After gamete fusion, a global passive maternal demethylation takes place, clearly visible at the 4-cell stage. At the end of the morula stage, remethylation starts (Fulka *et al.*, 2004; Santos *et al.*, 2010). In blastocysts, the methylation level of the trophectoderm and inner cell mass diverge, with more methylation in the inner cell mass (Santos *et al.*, 2010). Abnormal chromatin organization, as observed via DNA-specific YOYO staining and aberrant mCpG staining, seemed to be correlated in arrested IVF embryos, suggesting proper chromatin organization for early development (Santos *et al.*, 2010).

Transcripts of several imprinted genes like *SNRPN*, *MEST*, *UBE3A* and *IGF2* (but not *H19*) are already present at the preimplantation embryonic stages (Lighten *et al.*, 1997; Huntriss *et al.*, 1998; Salpekar *et al.*, 2001). The monoallelic expression starts from the 4 (*SNRPN*) and 8 (*IGF2*) cell stage, meaning that the primary imprints laid down during oogenesis and spermatogenesis are resistant to active and passive demethylation during the cleavage divisions.

Effects of IVF and embryo culture. Effects of IVF or subsequent development in culture medium alone are difficult to investigate in the human, since both are inevitably connected with each other. Moreover, the *in vivo* comparison cannot be made. *In vitro* conditions could affect maintenance of imprinting: at Day 3, 19% of human surplus embryos of low-quality (not suitable for transfer or for cryopreservation) showed hypomethylation of *H19* (Chen *et al.*, 2010). Paternal transmission was unlikely as none of the sperm samples showed hypomethylation. Similar results were obtained in a study where 8 of the 21 arrested surplus embryos showed loss of paternal methylation at *H19* DMR, while the corresponding sperm samples were normal (Ibala-Romdhane *et al.*, 2011). It is not known whether the hypomethylation (likely as a correlated response) leads to growth arrest or whether the growth arrest (induced by *in vitro* conditions) leads to loss of methylation. In the same study, eight arrested embryos showed methylation of the maternal allele (three of which also had hypomethylation at the paternal allele). After analysis of the unfertilized oocytes of two patients by using a distinguishing single nucleotide polymorphism, it was hypothesized that a defect in the erasure of the paternal imprint in the maternal germline led to this hypermethylation. Interestingly, five cryopreserved blastocysts that were donated for research after several years, all showed normal methylation (Ibala-Romdhane *et al.*, 2011).

As to the mode of IVF, there is no convincing evidence that ICSI elevates the risk for epigenetic abnormalities when compared with IVF or vice versa. Nucleus structure and methylation levels (immunofluorescence) seen in arrested embryos and fully grown blastocysts did not differ between IVF and ICSI (Santos *et al.*, 2010). An increased risk of imprinting disorders applies to pregnancies originating from both IVF and ICSI (Table II).

Recently, proof has been obtained of an effect of culture medium on offspring. IVF children derived from embryos that were cultured in two different media showed a significant difference in birthweight of almost 250 g (Dumoulin *et al.*, 2010). This resembles the animal studies where the addition of serum to the culture medium affects the growth of the fetus (Khosla *et al.*, 2001; Young *et al.*, 2001;

Fernandez-Gonzalez *et al.*, 2004), although the effect in the human (not involving serum) seems less severe and a causative epigenetic variable has not been found yet.

Epigenetic effect of IVF on offspring other than imprinting diseases

Except for the described imprinting disorders, induced epigenetic variants (Table V) that do not have clear phenotypical effects might be transmitted to the offspring.

Gomes *et al.* (2009) analysed *KCNQ1OT1*, that was hypomethylated in 3 out of 18 IVF children. These were all part of a dizygotic twin, with the co-twin showing normal methylation. The methylation level was reduced from 41.5% in naturally conceived children to around 14% in these three probands without clinical symptoms. BWS patients show 1% methylation.

Another group did not find a difference in *KCNQ1OT1* methylation in amnion/chorion membranes, umbilical cord blood and maternal peripheral blood of IVF and control conceptions (Tierling *et al.*, 2010). Eight other DMRs also showed a normal methylation pattern (*H19*, *SNRPN*, *GRB10*, *IG-DMR* and 4 DMRs in the *GNAS* region). Only *MEST* was slightly hypermethylated in IVF compared with ICSI and control samples. No correlation between the methylation level of any of these genes and birthweight was found.

In chorion villus samples from spontaneous miscarriages and stillbirths, a hypomethylation of *KCNQ1OT1* (significant) as well as *H19* (trend) was seen in samples derived after IVF ($n = 42$) (Zechner *et al.*, 2010). Five other DMRs did not show a difference compared with a control group.

The intra- and interindividual variation in methylation as assessed for *H19* makes comparison in humans harder (Turan *et al.*, 2010). The variation is higher in placental tissue compared with umbilical cord blood but also increased after IVF compared with *in vivo* fertilization, probably because IVF offspring result from embryos with fewer trophoblast stem cells (Turan *et al.*, 2010). The expression of both *IGF2* and *H19* was reduced in placental tissue of ART pregnancies. The expression of *IGF2* was not correlated with birthweight.

An extended DNA methylation analysis of more than 1500 genes, including DMRs, in placental tissue and umbilical cord blood from IVF and control pregnancies indicated that imprinted genes are not more vulnerable to methylation differences than non-imprinted genes (Katari *et al.*, 2009). Around 16% of the analysed CpG sites showed a difference (hypo- or hypermethylation) in placental tissue and 23% in umbilical cord blood. Four out of 11 tested genes that showed a difference in methylation between the two groups also showed a difference in level of transcription.

Zhang *et al.* (2010) analysed global gene expression patterns in placentae from three IVF and three control pregnancies. Twenty-six genes were differentially expressed, none of them imprinted.

In all the above mentioned studies that found a methylation effect in ART children, the methylation of the single investigated CpG within the analysed tissue was never completely (100%) methylated or demethylated (0%). This suggests that the methylation defects are not transmitted from the oocyte or sperm cell. Unfortunately, the type of analyses and the presentation of the results do not allow us to accurately specify mosaicism.

Perinatal, congenital and physiological outcome of IVF children; an epigenetic response?

Three meta-analyses with similar results on perinatal outcome have been published (Helmerhorst et al., 2004; Jackson et al., 2004; McDonald et al., 2009). The studies included in the analyses were selected either on the use of an appropriate control group (Helmerhorst et al., 2004), or whether they controlled for maternal age (McDonald et al., 2009) or maternal age and parity (Jackson et al., 2004). The substantial number of included singletons ranged from 5361 (Helmerhorst et al., 2004) to more than 31 000 (McDonald et al., 2009) and all three meta-analyses showed an increased risk in the IVF group for very preterm birth [relative risk (RR) ranged from 3.0 to 3.3], preterm birth (RR 1.9–2.0), very low-birthweight (RR 2.7–3.8), low-birthweight (RR 1.4–1.8), small for gestational age (RR 1.4–1.6), Caesarean section (RR 1.5–2.1), admittance to neonatal intensive care unit (RR 1.3–1.6) and mortality (RR 1.7–2.4).

For congenital malformations, more controversy exists, also mainly because of the relative small sample sizes in comparison with the frequency of the malformation. Recently, a Swedish group analysed two consecutive cohorts, each consisting of more than 15 000 singleton IVF children (Kallen et al., 2005, 2010b). Besides esophageal atresia (OR = 5.2) and urogenital defects (OR = 2.3) (Kallen et al., 2005), in both cohorts an increased risk for limb reduction (OR = 1.7–2.0), neural tube defects (OR = 2.9–4.2) cardiovascular malformations (OR = 1.3–1.7) and syndromes associated with imprinting defects like Prader–Willi (RR 4.0) was reported. These malformations were also found in smaller cohorts [e.g. (Ericson and Kallen, 2001; Hansen et al., 2002; Klemetti et al., 2005)]. Regarding cancer, Kallen et al. found a relative risk of 1.4 in a large cohort of almost 27 000 IVF children. Pertinent to this review is the aetiology of these malformations, i.e. the balance between genetic and epigenetic aberrations.

Ceelen et al. dedicated themselves to the physical development of IVF children aged 8–18 years. They investigated systolic and diastolic blood pressure, skinfold thickness, fasting glucose/insulin levels, fat, growth velocity, bone development and endocrine status during puberty. Systolic and diastolic blood pressure, peripheral skinfold thickness, fasting glucose level, weight and height gain between 3 months and 1 year and dehydroepiandrosterone sulphate (DHEAS) and LH level in pubertal girls were all higher in the IVF group compared with a control group (consisting of naturally conceived children from subfertile couples) even after adjustment for potential confounders, such as maternal BMI (Ceelen et al., 2007, 2008a, c, 2009). The higher blood pressure was confirmed in a cohort of 4–14-year-old IVF children, together with a higher triglyceride level. DHEAS levels did not differ from the control group (Sakka et al., 2010). In a younger IVF group of around 6 years old, the IVF children were taller and had a slightly more favourable lipid profile when compared with naturally conceived children (Miles et al., 2007).

For all these parameters, under the assumption of a random participation in ART among the genotypic variance present in our population, no genetic component is indicated. It may therefore well be the result of an epigenetic adaptive response to the (preimplantation) environment.

Conclusions

It is clear that a number of questions regarding possible epigenetic effects of ART can be answered (see Fig. 6).

In the mouse, an effect of ART, from imprinting maintenance to physiological homeostasis to behaviour, has generally been found. The placenta stands out as much more vulnerable to the influences of ART on imprinting compared with the embryo proper. This is likely related to the underlying theoretical basis of imprinting, the male–female conflict hypothesis in mammalian reproduction (Moore and Haig, 1991).

With respect to the information that is lacking in the mouse, the clearest omissions are the absence of an OAT model and the effects of ART at increased maternal age. Also, the effect of *in vitro* culture without ovulation induction has not yet been studied.

The effect of ovulation induction on maintenance of imprinting can be expressed at three levels. (i) That on maintenance of imprinting at recruitment of an antral follicle, (ii) that expressed as a maternal early embryonic ('zygotic') cellular effect on maintenance of the imprint after gamete fusion and (iii) that expressed via the maternal tractus. Not much work has been done on the first effect, for which some indication is found in the human but none in the mouse. It should be noted that in mice maturation of multiple oocytes in one cycle is natural, while in humans it is not. That might explain why in humans ovulation induction interferes with the maintenance of imprinting in the antral follicle while in the mouse the effect is only after *in vitro* pre-implantation development and/or through the maternal tractus. In both species, the unusual phenomenon of methylation of the *H19* DMR has been observed in oocytes, the etiology of which could be different as in the human germline, erasure is indicated to be later (during first meiotic prophase) compared with the mouse (before first meiotic prophase).

Since in the mouse no ovulation induction effect in oocytes was reported (except *H19*), and a mosaic pattern for DMR methylation status was observed in blastocysts and the placenta, a ovulation induction effect on imprinting must be based on the maternal cellular effect in combination with the maternal environment. In the mouse, a negative effect of ovulation induction on the maternal tractus is generally accepted (de Boer et al., 1991; Van der Auwera et al., 1999; Van der Auwera and D'Hooghe, 2001). After changing this environment to normal by transferring the embryo to a non-stimulated uterus, the ovulation induction effect on imprinting lessened (Fortier et al., 2008). At mid-gestation, *in vitro* culture aggravated the impact of ovulation induction on imprinting maintenance (Rivera et al., 2008). In all, enough evidence has been obtained in the mouse as to an effect of hormonal intervention and the *in vitro* steps (fertilization and subsequent culture) on maintenance of imprinting, especially for the placenta.

In the only study that analysed superovulated *in vivo* matured human MII oocytes, the number of the oocytes not methylated at *KCNQ1OT1* was much higher than the prevalence of BWS after ART. Most likely this suggests that the great majority of embryos derived from these oocytes are not viable. This non-viability of methylation disturbed embryos is substantiated by the hypo- and hypermethylation of *H19* DMR in arrested embryos, while non-arrested embryos showed normal methylation (Ibala-Romdhane et al., 2011).

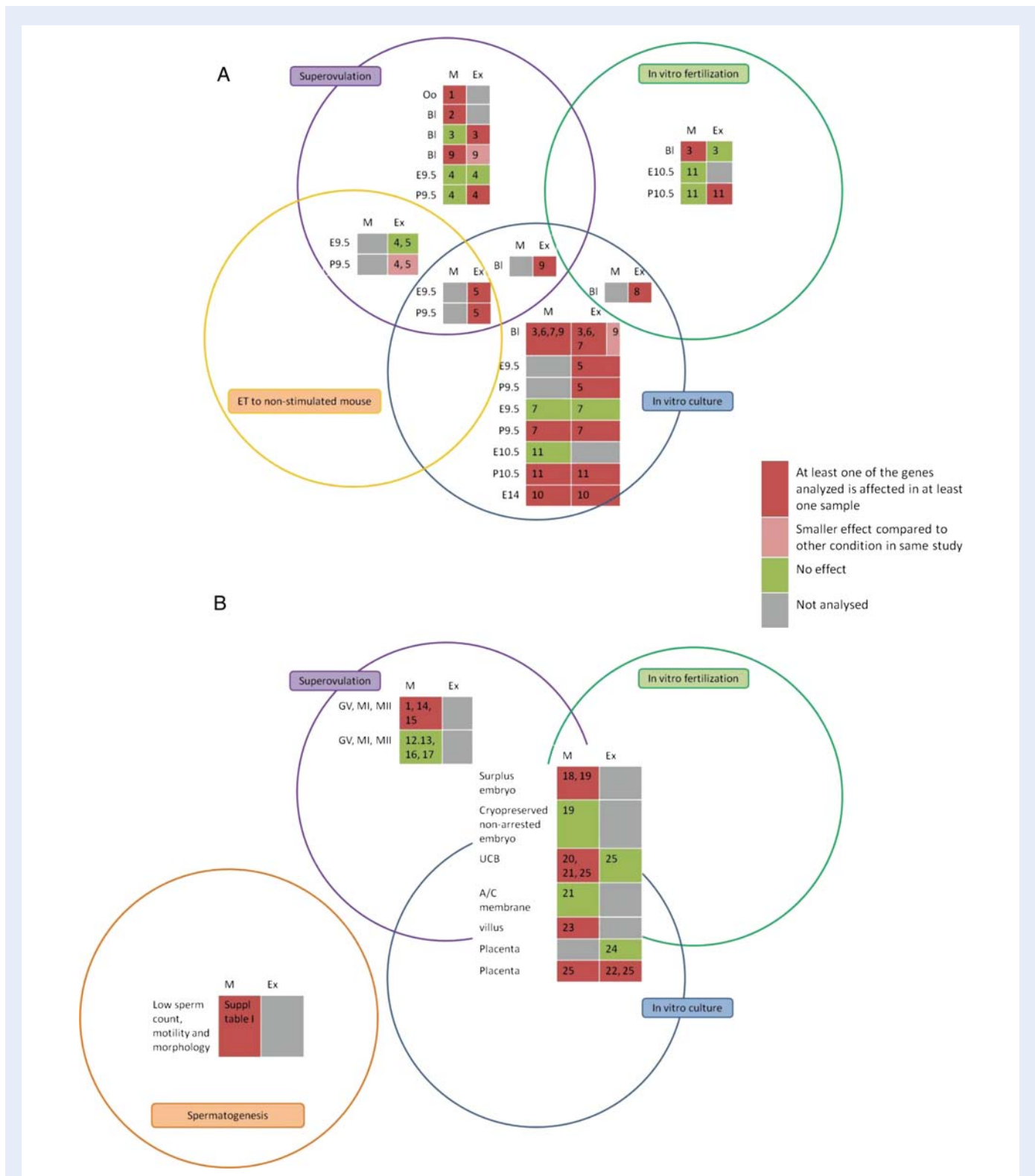


Figure 6 Overview of the results of studies on the effect of ART on methylation and expression of imprinted genes. **(A)** Overview of mouse data. **(B)** Overview of human data. A/C = Amnion/Chorion, Bl = Blastocyst, E = embryo with the age, Ex = Expression which indicates either the level of expression or the allelic expression, ET = embryo transfer, GV = germinal vesicle oocyte, M = methylation, MI = oocyte in meiosis I, MII = oocyte in meiosis II, P = placenta with the age, UCB = umbilical cord blood. The numbers refer to the studies: 1. Sato *et al.* (2007), 2. Market-Velker *et al.* (2010a), 3. Fauque *et al.* (2007), 4. Fortier *et al.* (2008), 5. Rivera *et al.* (2008), 6. Doherty *et al.* (2000), 7. Mann *et al.* (2004), 8. Li *et al.* (2005), 9. Market-Velker *et al.* (2010b), 10. Khosla *et al.* (2001), 11. Fauque *et al.* (2010a), 12. Geuns *et al.* (2003), 13. Geuns *et al.* (2007b), 14. Khoueiry *et al.* (2008), 15. El-Maarri *et al.* (2001), 16. Borghol *et al.* (2006), 17. Geuns *et al.* (2007a) 18. Chen *et al.* (2010), 19. Ibalá-Romdhane *et al.* (2011), 20. Gomes *et al.* (2009), 21. Tierling *et al.* (2010), 22. Turan *et al.* (2010), 23. Zechner *et al.* (2010), 24. Zhang *et al.* (2010), 25. Katari *et al.* (2009).

It would be of interest to know whether a milder ovarian stimulation would lead to a reduction of BWVS cases (single embryo transfer is increasingly applied and therefore fewer embryos are needed).

In the human, the influence of poor spermatogenesis on maintenance of imprinting methylation and methylation of other sequences is undisputed, making sperm a potential vehicle for transmitting paternal methylation abnormalities. However, the chance of transmission to the offspring appears rather small.

The type of IVF (conventional IVF or ICSI) does not seem to make a difference regarding methylation defects. *In vitro* culture conditions and methylation defects might be associated since in human arrested embryos, *H19* hypomethylation is reported, without methylation defects in the spermatozoa (Chen et al., 2010; Ibalá-Romdhane et al., 2011). An *in vivo* comparison can, however, not be made.

The first array-based analysis on CpG methylation at birth (Katari et al., 2009) gives the impression of an ART-induced vulnerability for CpG methylation disturbances for a considerable frequency of sites, with more CpGs differentially methylated in umbilical cord blood than in placental tissue. When a difference was observed, in cord blood most CpGs were hypermethylated in the *in vitro* group while in placental tissue most CpGs were hypomethylated.

An affected 5methyl CpG maintenance can be without any effect but might bring IVF progeny closer to a threshold, making them more vulnerable to physiological reported effects at adolescence (Ceelen et al., 2007, 2008a, c, 2009) or late-onset diseases, such as cardiovascular disease, cancer (Kallen et al., 2010a) or other minor effects that have not yet been observed. Differences in gene expression of metabolism-related genes set by embryonic and/or fetal programming are assumed to underlie the relation between low-birthweight [also reported in IVF neonates (Helmerhorst et al., 2004; Jackson et al., 2004; McDonald et al., 2009)] and chronic diseases in later life, like cardiovascular diseases and diabetes mellitus (Barker, 2006). This finding could also be one explanation of the parallel observation of an effect of ART on both systolic blood pressure and glucose tolerance in mice and men (Watkins et al., 2007; Ceelen et al., 2008a; Scott et al., 2010). The recently isolated maternally imprinted gene *KLF14* (Small et al., 2011) could well be the master regulator, at least for diabetes II and adipocyte-related metabolic disease risk.

In general, more evidence for an epigenetic effect of ART has been obtained in the mouse than in man. However, the definite answer to many questions in both mouse and human will await genome-wide epigenetic profiling in the different variants of ART. From the patient's perspective, those that contribute sperm from poor spermatogenesis stand out as very interesting for such an analysis as do the oocytes from older women.

Supplementary data

Supplementary data are available at <http://humupd.oxfordjournals.org/>.

Authors' roles

A.P.A.vM., L.H., P.dS., S.V., J.P.M.G. and P.dB. played a role in conception and design. A.P.A.vM., L.H., P.dS., S.V., J.P.M.G. and P.dB. were involved in acquisition, analysis and interpretation of the data.

A.P.A.vM., L.H. and P.dB. drafted the manuscript. A.P.A.vM., L.H., P.dS., S.V., J.P.M.G. and P.dB. contributed to the revision of the manuscript and approval of the final version.

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The publication of Karati et al. on CpG methylation in newly borns and their placentas was also communicated in the lay press. Thereafter the European Society of Human Reproduction and Embryology (ESHRE) was asked for expert advice. After consultation of the Special Interest Groups (SIG) Safety and Quality and Reproductive Genetics, it became clear that no document was available to which could be referred. For that reason it was decided to put together a working group consisting of members of both SIG's (J.G.; P.dS.; S.V.) and a few external experts (P.dB.; L.H.; A.P.A.vM.) to review the epigenetic state of ART as reported in the literature, supplemented by an update on epigenetic inheritance in mammals.

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