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Epigenetic asymmetry in the zygote and mammalian development

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ABSTRACT In mammals, the maternal and the paternal genome are not functionally equivalent and are both required for embryonic and postnatal development. The genome is organised differently in the oocyte as compared to sperm, in which the DNA is tightly packaged with protamines rather than with histones. The requirement of both the parental genomes for normal development is a consequence of differential epigenetic marking in oogenesis and spermatogenesis, at the regulatory elements that control genomic imprinting. These germ line-derived marks of DNA methylation are resistant to the global waves of demethylation that occur following fertilisation, and bring about the parental allele-specific expression of imprinted genes during development and after birth. Perturbation of the differential organisation of the maternally and paternally derived genomes, before fertilisation, or in the early embryo, can give rise to aberrant growth and developmental disorders in humans.

KEY WORDS: *spermatogenesis, oogenesis, epigenetics, DNA methylation, imprinting*

Introduction

In mammals, as opposed to other groups of animals, there is the requirement of both a maternal and a paternal genome for embryonic and foetal development. This raises the question as to how it comes that the maternal genome in the egg is not functionally equivalent to the paternal genome of the sperm. This is the theme of the current review, which discusses the organisation of the genome in sperm and oocytes, and how the maternal and paternal sets of chromosomes undergo genome-wide reprogramming after the formation of the zygote. As will be outlined below, differential organisation of chromatin at a subset of genes leads to these becoming expressed from only one of the two parental alleles. This epigenetic phenomenon is called 'genomic imprinting' and is a direct consequence of the sperm and egg being epigenetically non-equivalent. Disruptions in the epigenetic reprogramming of the genome in the male or female germ line, and in the way the parental genomes are remodelled during early development, can have long-lasting effects on development and well-being. Various developmental and clinical phenotypes have emphasized important pathological implications of the asymmetry between the parental genomes in mammals.

The first insight that both the parental genomes are required for normal development has come from the observation, in mice, that parthenogenetic embryos die *in utero*. These embryos have two maternal genomes, and no paternal genome (Graham, 1974). Elegant studies have shown that the developmental failure from not having both the parental genomes is due to the nuclear compartment, rather than the cytoplasmic one. Specifically, by transfer of male and female pronuclei directly following fertilisation of the egg, zygotes were constituted that had either two maternal pronuclei (gynogenotes), or two paternal pronuclei (androgenotes). These showed grossly abnormal development and did not proceed beyond day 10 of gestation (McGrath and Solter, 1984; Surani *et al.*, 1984). Detailed studies on mice that were uniparentally disomic for individual chromosomes refined the notion that both the parental genomes are required for development to proceed to term (Cattanach and Kirk, 1985).

In mammals, there is a marked difference in the way the genomes are organised in the oocyte compared to sperm. Whereas

Abbreviations used in this paper: ART, assisted reproduction technology; BWS, Beckwith-Wiedemann syndrome; DMR, differentially methylated region; DNMT3A, DNA methyltransferase 3A; DNMT3L, DNMT3-like; H2AL, histone H2A-like; ICR, imprinting control region; ICSI, intracytoplasmic sperm injection; IUGR, intra-uterine growth restriction; ncRNA, non-coding RNA; PGC, primordial germ cell; SRS, Silver-Russell syndrome; TNDM, transient neonatal diabetes mellitus; UPD, uniparental disomy.

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most of the structural differences between the maternal and the paternal genomes disappear during the first cell cycles after formation of the zygote, some persist, leading to the functional differences between the parental genomes. A hallmark feature which distinguishes the sperm genome from the egg genome is that it is globally compacted with protamine proteins rather than with histone proteins (Rousseaux *et al.*, 2005; Kimmins *et al.,* 2005; Sasaki and Matsui, 2008). It is during the final stage of spermatogenesis that a global exchange of the histone proteins for the more basic protamines takes place, and this leads to the exceptionally high compaction of the genomic DNA in sperm (Rousseaux *et al.*, 2005). Directly following fertilisation, however, the protamines are removed and replaced by nucleosomal histones. These new histones acquire specific modifications after they are incorporated into the chromatin. Thus, globally, the paternal genome becomes nucleosomally organised shortly after fertilisation (as is the egg genome) and then undergoes many changes onto the newly-incorporated histones. An important question is whether all DNA in sperm is packaged with protamines, or whether certain sequence elements remain nucleosomally organised throughout spermatogenesis. The latter could have functional consequences, since these regions would possibly not undergo the global re-organisation into nucleosomal chromatin following fertilisation.

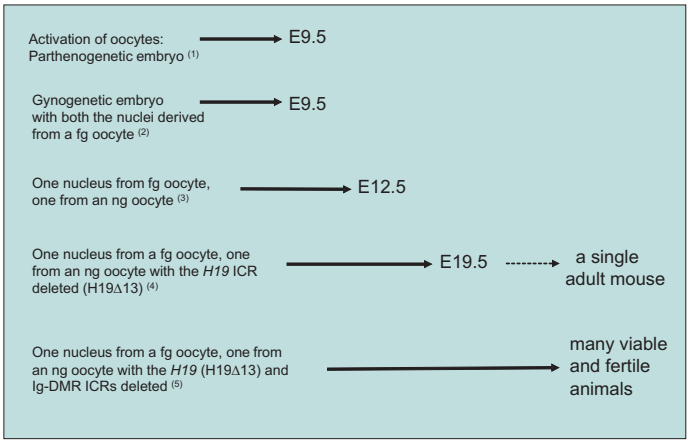

Fig. 1. Generation of mice with maternally-derived chromosomes only. *Artificial activation of fully-grown oocytes leads to parthenogenetic development which, in the mouse, proceeds only to embryonic day 9.5 (E9.5) due to aberrant imprinting (with maternal imprints only). A similar phenotype is observed in gynogenetic embryos, which are derived by transfer of pronuclei after fertilisation. When using one genome from a fully-grown (fg) ocyte (with maternal imprints) and one from a non-growing (ng) ocyte (with no maternal imprints yet), development proceeds a little further, till E13.5. Kono and co-workers (2004, 2007) showed that when they restored normal expression of the* Igf2 *gene (by deletion of the* H19 *ICR in the ng oocte) development proceeded even further, till birth, and, very rarely, till adulthood. Remarkably, when they restored normal expression levels at both the* Igf2-H19 *and the* Dlk1-Gtl2 *domains (by deletion of the two corresponding ICRs in the ng oocyte), many live-born animals were obtained that were viable and fertile. Combined, these studies indicate that the failure of parthenogenetic development is caused by the aberrant expression of genes at the paternally-imprinted domains (because of the absence of paternal imprints). These studies also show that sperm-derived proteins and RNAs are not required for full development to term. The format of this figure was adapted from Kono, 2006. 1, Surani and Barton, 1983; Sturm* et al. *1994; 2, Surani* et al., *1983; 3, Kono* et al., *1996; 4, Kono* et al. *2004, 2006; 5, Kawahara* et al., *2007.*

From studies in different mammalian species it has become evident that the sperm genome undergoes active demethylation shortly after fertilisation of the egg. This is brought about by yet-unknown factors present in the egg's cytoplasm. The process is thought to occur directly after the removal of the protamines and the incorporation of histones into the chromatin. The maternal genome is not subject to this active demethylation process, but undergoes a passive loss of DNA methylation during pre-implantation development, such that at the blastocyst stage, it becomes largely unmethylated (Reik *et al.,* 2001). Not all DNA sequences undergo active (paternal genome) and passive (both genomes) removal of DNA methylation. This raises the question as to why certain DNA sequences are protected against the global waves of DNA demethylation. Unravelling what protects these exceptional regions against demethylation after fertilisation, and against the acquisition of new DNA methylation at later developmental stages, may provide novel insights into the asymmetry between the parental genomes in mammals.

The functional non-equivalence of the parental genomes

In different groups of animals, parthenogenesis is compatible with normal development to adulthood. In different bird species, including chickens and turkeys, healthy adult animals have been obtained by parthenogenesis through spontaneous activation of the egg (Olsen, 1961; Sarvella, 1973). Also in several insect species, such as in aphids, parthenogenesis is commonly observed, as a means of asexual reproduction (Hales *et al.*, 2002). Furthermore, parthenogenesis is suspected to sometimes give rise to live-born offspring in different species of snakes, and has been observed in sharks in captivity as well (Groot *et al.,* 2003; Edwards, 2007).

In mammals, in contrast, parthenogenesis is not compatible with normal development (Fig. 1). Parthenogenetic mouse conceptuses, obtained by artificial activation of oocytes, develop till about mid-gestation only. They show gross developmental abnormalities, including reduced embryo size and an almost complete lack of placental tissues (Graham 1974; Surani and Barton, 1983; Mann and Lovellbadge, 1984). Such monoparental embryos can be obtained by nuclear transplantation as well, immediately following the fertilization of the egg by the sperm. By replacing the male pronucleus by a female one, for instance, so-called gynogenetic embryos can be obtained (carrying two maternal genomes). Developmentally, these are similarly affected as the parthenogenetic embryos due to the absence of a paternal genome (Surani and Barton, 1983). Androgenetic embryos have two paternal genomes, and no maternal genome, and show a completely different phenotype. Whereas their extra-embryonic membranes are relatively normal, the embryo proper is retarded and progresses rarely beyond the four to six-somite stage (McGrath and Solter, 1984; Surani *et al.*, 1984). Similar phenotypes have been observed in a ruminant species, the sheep (Feil *et al.*, 1998; Hagemann *et al.*, 1998). Combined, the studies in different species established that both a maternal and a paternal genome are required for mammalian development to proceed to term.

From studies during the last few years, it seems that the

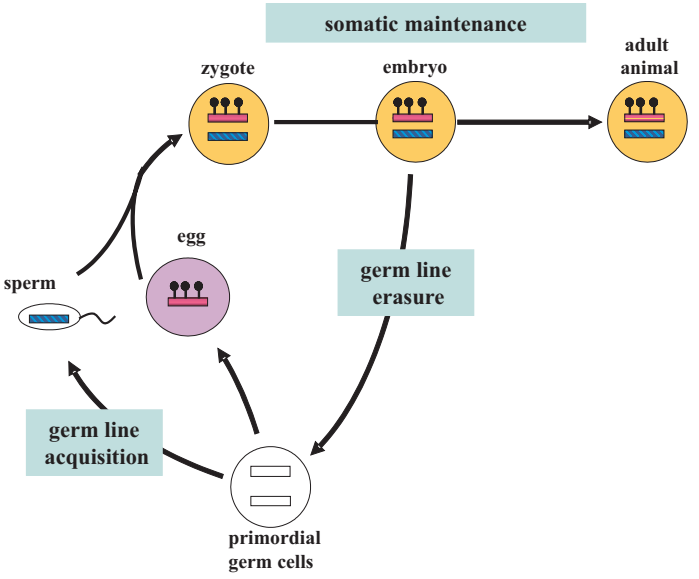

Fig. 2. The developmental cycle of DNA methylation at imprinted gene loci. *Methylation imprints are established at individual imprinting control regions (ICRs) during either oogenesis, or spermatogenesis. Specifically, some ICRs have the maternal imprints, which are established during oogenesis, after the first meiotic division, in growing oocytes. Only a few ICRs have patenal imprints. These are established during spermatogenesis, before meiosis, at a foetal stage of development. After fertilisation of the egg by the sperm, the allelic methylaton imprints at ICRs are maintained in all somatic lineages throughout development. Shown is one example, of an ICR which acquires its methylation in the female germ line. The DNA methylation imprint (black lollypops) is indicated. In the newly-formed primordial germ cells (PGCs) of the developing embryo, there is erasure of the methylation imprint, to allow novel imprints to be established subsequently for the next generation.*

aberrant phenotypes of androgenetic and parthenogenetic conceptuses are caused by certain genes that are expressed from the maternal or the paternal genome only (Fig. 1). The differential expression of genes according to their parental origin is called 'genomic imprinting' (Fig. 2) and will be discussed below.

Differential chromatin remodelling after fertilisation

At the time of fertilisation, the sperm and egg genomes are differently organised, as a consequence of the differential epigenetic reprogramming during male and female gametogenesis (Sasaki and Matsui, 2008). The paternal genome in the sperm is haploid and is packaged with protamines. The egg, in contrast, is diploid at fertilisation (with one genome present in the extruded first polar body) and its genome is packaged with nucleosomes. Although the oocyte completes meiosis only following fertilisation, with extrusion of the second polar body, its genome remains nucleosomally organised throughout. At the sperm genome, in contrast, following fertilisation there is a rapid replacement of protamines by histone proteins (Morgan *et al.*, 2005). Protamine-to-histone substitution precedes the apparently genome-wide loss of DNA methylation that is observed specifically at the sperm-derived paternal pronucleus, several hours following fertilisation. Interestingly, this demethylation occurs before the onset of replication leading to the first cell division, and is therefore thought to be an active process. The process has been observed most clearly in mouse studies, but has also been reported to occur in humans, cattle and other mammalian species (Mayer *et al.*, 2000; Oswald *et al.*, 2000; Barton *et al.*, 2001; Dean *et al.*, 2001; Fulka *et al.*, 2004). It remains to be confirmed, however, to which extent this demethylation process is conserved in all groups of mammals (Beaujean *et al.,* 2004; Shi *et al.*, 2004; Lepikhov *et al.,* 2008), and what could be its biological role. Studies are also required to more comprehensively assess the range of sequences that become demethylated.

Which enzymatic factors are involved in the active removal of DNA methylation from the paternal genome is not known, but candidate mechanisms have been discussed, including glycosylation occurring specifically at methylated cytosines followed by a DNA repair process with inclusion of non-methylated cytosines (Morgan *et al.*, 2004). From several studies, it appears that the active demethylation occurs in the context of DNA which is (becoming) nucleosomally organised, at a point in time however at which histone modifications are still grossly different at the paternal and maternal pronuclei (Adenot *et al.*, 1997; Santos *et al.*, 2002; Erhardt *et al.*, 2003). It is unknown what protects the maternal genome against the active process of demethylation, but this could well be related to the fact that its core histones are marked by different modifications than on the paternal genome. During the pronuclear stages that follow fertilisation, it has indeed been observed for several histone modifications that they display pronounced differences in the maternal and the paternal pronucleus. For instance, whereas H3 lysine-9 (di and tri) methylation is strongly detected at the maternal pronucleus, at the early pronuclear stages, it is virtually absent from the paternal pronucleus. The same was reported for H3 lysine 27 di and trimethylation (Erhardt *et al.*, 2003; Morgan *et al.*, 2005; van de Heijden *et al.*, 2005). H3 lysine-9 methylation, possibly in combination with other methylation marks, such as H3 K27 methylation, could protect the genome against the active process of DNA demethylation. Although such a protective mechanism is still to be demonstrated, it is interesting to note that some sequences do not undergo active demethylation on the paternal genome, and chromatin at these regions seems to be enriched in H3 lysine-9 methylation (Lepikhov and Walter, 2004; Santos *et al.*, 2005).

Another element that could be important is that the new histones on the paternal genome are incorporated before DNA replication occurs. Some of these incorporated histones are therefore thought to be non-canonical replacement variants. The best studied of these is H3.3, an H3 variant which is incorporated into the chromatin independently of DNA replication by a mechanism using specific chaperone proteins including the assembly factor HIRA. Interestingly, H3.3 shows a dynamic distribution in the mouse oocyte and the early embryo, with a preferential incorporation into the paternal pronucleus following fertilisation (van der Heijden *et al.*, 2005; Torres-Padilla *et al.,* 2006). Whether replacement histone variants such as H3.3 are involved in steering the subsequent active DNA demethylation process, is unknown, but this would represent another putative mechanism that could provide specificity to the process and explain why the maternal genome is protected against loss of methylation at this post-fertilisation stage.

Finally, it is likely that specific non-histone proteins produced during oogenesis and early development participate in the protection against the active DNA demethylation following fertilisation. One such a protective maternal protein has been identified so far. It is Stella (also called PGC7), a protein with a SAP domain (scaffold attachment factor/acinus/PIAS) which is expressed in primordial germ cells, in oocytes, and in pre-implantations embryos. Initial studies had shown that maternal deletion, leading to lack of the protein in the oocyte, has a strong maternal effect with embryos rarely reaching the blastocyst stage (Payer *et al.* 2003). Interestingly, a recent study shows that Stella is important for the epigenetic asymmetry in the zygote and protects the DNA methylation state at several imprinted gene loci. Specifically, Stella protects the maternal genome against demethylation after the protein becomes localised to the female pronucleus, where it protects several of the maternal "imprinting control regions" (see below) against demethylation. The protective mechanism remains to be uncovered though, but may involve direct binding of Stella/PGC7 to the chromatin given that the protein has a high binding affinity to DNA (Nakamura *et al.* 2007).

Of particular importance relative to the paternal pronucleus is the discovery that not the entire genome is subject to the active removal of methylation following fertilisation. For instance, the satellite DNA sequences of the compacted chromatin surrounding centromeres and also certain intracisternal-A particle (IAP) retrotransposons seem to be relatively resistant to demethylation (Rougier *et al.,* 1998; Lane *et al.,* 2003). Why the DNA of heterochromatin does not lose its methylation is unknown. To address this key question, it should be important to unravel its organisation in mature sperm. Amongst other questions, it should be pertinent to determine whether the pericentric regions are packaged with protamines, or rather, have a nucleosomally organised structure in sperm. Given the highly compacted state of the genome in mature sperm, this question has been extremely difficult to address. One preliminary study in which human sperm was decompacted with different concentrations of salt has attempted to address this question, and it reported that the imprinted locus comprising the *Insulin-like growth factor-2* (*IGF2*) gene (Fig. 3) had a relative enrichment in histone-compacted DNA (Wykes and Krawetz, 2003). Other studies have taken another angle, and explored the process of spermiogenesis, the final stages of male germ cell development during which the global histone-to-protamine exchange takes place (Govin *et al.*, 2007; Delaval *et al.*, 2007). At these specific stages, there is expression of certain histone variants that are only present during spermatogenesis (Govin *et al.*, 2004; Rousseaux *et al.*, 2005; Kimmins and Sassone-Corsi, 2005). Elegant recent work shows that two histone H2A variants, H2A-like 1 and 2 (H2AL1 and 2), become specifically incorporated into heterochromatin at the elongating spermatid stage and that this correlates with the formation of an unusual, nucleosome-like, structure (Govin *et al.*, 2007). This remarkable finding raises the possibility that besides specific histone modifications, also the incorporation of specialised histone variants could impact on whether or not a chromosomal region undergoes histone-to-protamine exchange. Whether the non-canonical nucleoprotein structures comprising H2AL1 and H2AL2 persist in mature sperm, and whether they interfere with the DNA demethylation occurring after fertilisation, remains to be explored.

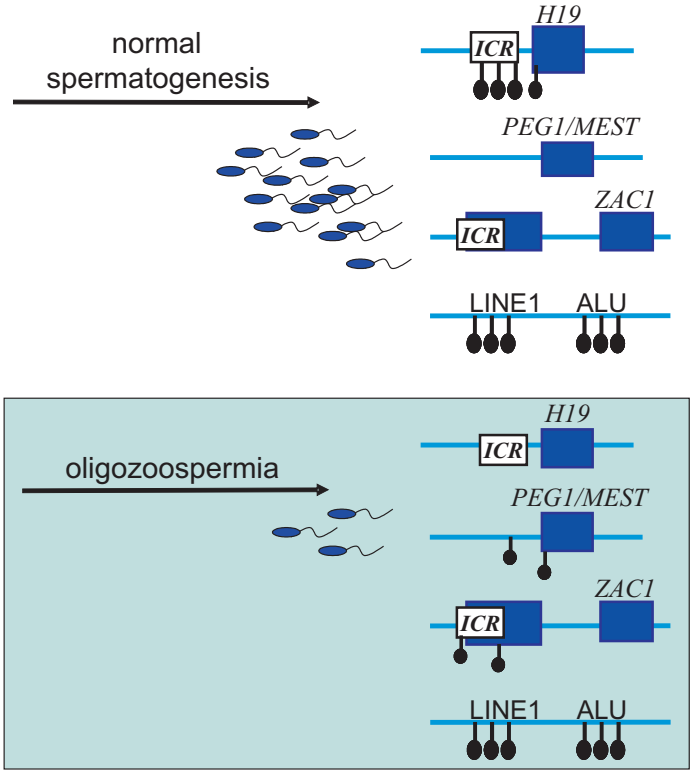

Fig. 3. Altered methylation patterns at imprinting control regions in sperm of oligospermic men. *Oligozoospermia is characterised by a strongly reduced concentration of spermatozoa in sperm. In several recent studies (Marques* et al. *2004, 2008; Kobayashi* et al. *2007), cases were identified in which the reduced sperm counts were associated with a high frequency of altered DNA methylation at imprinting control regions (ICRs). Loss of methylation was observed at the* IGF2-H19 *ICR. The ICRs controlling the ZAC and PEG1/MEST imprinted loci are fully unmethylated in normal sperm, but showed frequent gain of methylation in sperm of oligospermic men. Interestingly, unaltered levels of DNA methylation were observed at LINE1 and ALU repeat elements.*

Imprinting control regions resist reprogramming after fertilisation

To date, some eighty genes have been identified to be subject to genomic imprinting in mammals. Many these play key roles in foetal growth development, whereas others are involved in postnatal fitness and behaviour (Morison *et al.*, 2005). Imprinted genes are organised in chromosomal domains, which are broadly conserved between mice and humans. Imprinted domains are unusual in that the specific sequence elements which control their expression are marked by mono-allelic DNA methylation. These so-called 'imprinting control regions' (ICRs) convey the parental allele-specific expression of imprinted genes (Li *et al.*, 1993). Specifically, ICRs are elements of up to several kilobases in size that are rich in CpG dinucleotides. Many correspond to CpG islands (Hutter *et al.*, 2006; Kobayashi *et al.*, 2006). At most ICRs, the allelic methylation is established during oogenesis. This occurs in the adult animal at a late stage of oogenesis, during the growth and maturation of the oocyte, and coincides with global chromatin condensation and the shut-off of transcription (Obata

et al. 2002; Lucifero *et al.* 2004). At only three of the known ICRs in the mouse, methylation is established during spermatogenesis. Acquisition of methylaton imprints in this germ line occurs at late foetal stages, before the onset of meiosis, and coincides with a genome-wide increase in DNA methylation (Schaefer *et al*., 2007; Oakes *et al.* 2007). After the establishment of the methylation marks at ICRs, in either the female or the male germ line, these imprints are maintained during the subsequent development of the germ cells. Consequently, some ICRs are methylated in the mature oocyte, and not in spermatozoa (Fig. 2). Others are methylated in sperm only. The DNA methyltransferase DNMT3A, together with a DNMT3-like protein, DNMT3L, is required for putting new methylation imprints onto the ICRs (Bourc'his *et al*., 2001; Hata *et al*., 2002; Kaneda *et al*., 2004). It remains to be discovered why at some ICRs this occurs in the female germ line only, whereas at others, acquisition of methylation occurs in the male germ line only. Possibly, the 'choice' could relate to the finding that ICRs that acquire their DNA methylation in the oocyte correspond all to promoter regions, whereas the sperm-methylated ICRs are not promoters and are located many kilobases away from genes. Paternal ICRs are also less rich in CpG dinucleotides than the maternal ICRs (Kobayashi *et al.* 2006). For some ICRs it was found that, besides the ICR itself, close-by sequences are essential for the germ line specific establishment of their DNA methylation. This raises the possibility that specific DNA binding proteins could be involved by interacting with the ICR and nearby sequences (Yoon *et al*., 2002; Perk *et al*., 2002). Interestingly, for two of the paternal ICRs, a recent *in vitro* study suggests that the zinc-finger protein CTCF-like (also called BORIS), expressed during spermatogenesis only, could be involved in the establishment of their methylation imprints (Jelinic *et al.* 2006). Finally, it seems important to note that many of the ICRs comprise imperfect tandemly-repeated sequences, of which the significance in imprint establishment remains to be discovered (Hutter *et al*., 2005).

Imprinted domains are thus far the only endogenous chromosomal regions at which there is differential DNA methylation between the sperm and the egg. Importantly, in the context of this review, all ICRs are fully resistant to the chromatin remodelling that occurs after fertilisation (Fig. 2). Later in development, they induce parental allele-specific expression at nearby genes (Delaval and Feil, 2004). It is because of the imprinting mechanism that the maternal and the paternal genome are functionally different (Latham *et al.,* 1994; Kono *et al*., 2004; Niwa *et al*., 2005; Kowahara *et al.,* 2007). In placental mammals, therefore, there is an absolute requirement for normal embryonic and postnatal development to have both a maternally and a paternally derived genome. As mentioned earlier in the text, having just maternal, or paternal, genomes leads to gross developmental abnormalities due to the aberrant expression of imprinted genes. So far, more than eighty imprinted genes have been discovered in the mouse. Many of these show expression from only one of the two parental alleles in humans as well. Several imprinted genes play key roles in cellular proliferation and growth of the embryonic and extra-embryonic lineages, whereas others influence postnatal fitness and behaviour (Morison *et al.,* 2005). Imprinted genes are organised in chromosomal domains, each of which comprises an ICR that controls the allele-specific expression of the domain's genes. How, precisely, the differentially methylated ICRs bring about the allelic gene expression of nearby genes, differs between different imprinted domains. At some domains, on its non-methylated allele the ICR transcribes a non-coding RNA (ncRNA) which conveys chromatin repression *in cis* during early development. At other domains, the ICR brings about an allelic chromatin configuration which prevents the activation of promoters by distant enhancer sequences. The different scenarios *via* which the epigenetic imprints at ICRs are 'read' and interpreted to give rise to allelic expression of nearby genes are not presented in this review, but are discussed in detail elsewhere (Delaval and Feil, 2004; Edwards and Ferguson-Smith, 2007; Pauler *et al.,* 2007).

The insight that the egg and sperm have a different epigenetic regulation had emerged several years before the discovery of the first imprinted genes, based on studies on transgenes. Swain and co-workers (1987) provided a first indication of a parent-of-origin specific effect on the expression and methylation of a transgene. They had a mouse transgene in which the LTR from the Rous sarcoma virus was linked to *cMyc*. This transgene was found to be unmethylated and expressed upon paternal transmission, but was methylated and silent upon maternal transmission. It was shown subsequently, that the methylation reprogramming of this 'imprinted' transgene, and hence the reprogramming of its transcription potential, occurred in the primordial germ cells of the developing gonads (Chaillet *et al*., 1991). Other transgenic constructs were found to be subjected to parent-of-origin specific DNA methylation and gene silencing as well (Reik *et al*., 1987), indicating that the transgene at the insertion site conveyed differential epigenetic regulation. The first endogenous imprinted genes that were shown to have such an unusual behaviour were the *Insulin-like growth factor 2* (*Igf2*) (DeChiara *et al*., 1991), the *Igf2-receptor* gene (*Igf2r*) (Barlow *et al.,* 1991), and the ncRNA *H19* gene located close to *Igf2* (Bartolomei *et al*., 1991).

Methylation imprints at ICRs are maintained in all somatic cells throughout development (Fig. 2). During preimplantation development, this maintenance involves both the maternal and the somatic form of DNA methyltransferase-1 (Dnmt1) (Kurihara *et al*., 2008; Hirasawa *et al.,* 2008). Strikingly, ICRs are not subject to the global waves of DNA demethylation following fertilisation, and do also not acquire novel DNA methylation during and after implantation of the embryo in the uterus. This full protection against epigenetic changes is most remarkable for the three known ICRs which have paternal DNA methylation. These ICRs acquire their DNA methylation in pre-meiotic spermatogenic cells. Similarly as for methylation at pericentric heterochromatin, these methylation imprints persist to mature sperm, and are protected against the global waves of DNA demethylation after fertilisation of the egg (Olek and Walter, 1997). How paternally methylated ICRs are organised in mature sperm is not known and this makes it difficult to speculate on what might provide their specific protection. However, during early spermatogenesis the paternally imprinted regions are associated with different histone methylation patterns than the ICRs that are methylated in the oocyte. Particularly, chromatin at the paternal ICRs is devoid of histone H3 lysine-4 methylation (Delaval *et al*., 2007). This covalent H3 modification is thought to be important in the protection against DNA methylation, by preventing recruitment of the DNMT3L-DNMT3A protein complex (Ooi *et al*., 2007). After the first cell division, and at later stages of embryonic development, when the maternal and the paternal genomes become nucleosomally

organised in a similar way (Morgan *et al.*, 2005), ICRs are protected against changes in DNA methylation as well. It is not understood how it comes that the methylated allele of the ICR remains methylated throughout development, whereas the unmethylated is protected against *de novo* methylation. However, the chromatin organisation at the methylated allele was found to be comparable to that at pericentric heterochromatin (Martens *et al.* 2005; Delaval *et al.*, 2007), which shows constitutive maintenance of DNA methylation as well. Particularly, DNA methylation at ICRs is associated with chromatin that has constitutive H3 lysine-9 di and trimethylation and H4 lysine-20 trimethylation, and a consistent absence of H3 acetylation (Vu *et al.*, 2004; Wu *et al.*, 2006; Delaval *et al.*, 2007; Regda *et al.*, 2007). The unmethylated alleles of ICRs, in contrast, are always associated with H3 lysine-4 methylation (Fournier *et al.*, 2002; Umlauf *et al.*, 2004; Delaval *et al.*, 2007), a mark which could prevent acquisition of new DNA methylation by interfering with the binding of the DNMT3A-DNMT3L complex (Ooi *et al.*, 2007). One of the challenges will be to unravel which histone methyltransferases are involved in the differential histone methylation at ICRs and how these could be involved in the imprinting process (Pannetier *et al.,* 2008).

Pathological disruption of parental chromatin organisation

The methylation asymmetry becomes established during spermatogenesis and oogenesis (Fig. 2). This raises the question of whether defects in gametogenesis could interfere with the epigenetic marking of the male and female gametes. Several recent studies demonstrate for spermatogenesis, that this is indeed the case. Oligospermic men produce sperm which is often morphologically normal, but sperm counts are severely reduced and this is associated with infertility. An initial study (Marques *et al.* 2004) on twenty five moderate to severe cases described that about one-fourth of these men had abnormal patterns of methylation at the imprinted *IGF2* domain at which the ICR has sperm-derived DNA methylation. In the oligospermic patients, the sperm showed loss of DNA methylation at specific CpG dinucleotides of this ICR (Marques *et al.*, 2004, 2008). This finding indicates a potential risk of transmitting perturbed imprinted *IGF2* and *H19* expression to the next generation. This conceptually important finding was confirmed recently in a study of 97 infertile men, in which DNA methylation was analysed at seven imprinted loci (Kobayashi *et al.*, 2007). Abnormal paternal methylation imprints were detected in 14% of patients in this cohort. Cases were identified in which there was an almost complete absence of DNA methylation at the *IGF2-H19* ICR in sperm. Interestingly, this and another study (Marques *et al.*, 2008) revealed abnormal imprints at maternal ICRs as well, with varying degrees of methylation at several ICRs that are normally not methylated in sperm (Fig. 3). These epigenetic defects are most likely specific to imprinted genes, since levels of DNA methylation at LINE-1 and ALU repeat sequences were unaltered (Fig. 3). Interestingly, it was noted in one of the studies that the outcome of assisted reproduction by intra-cytoplasmic sperm injection (ICSI) was poor when sperm from these oligospermic males was used. However, so far, no cases have

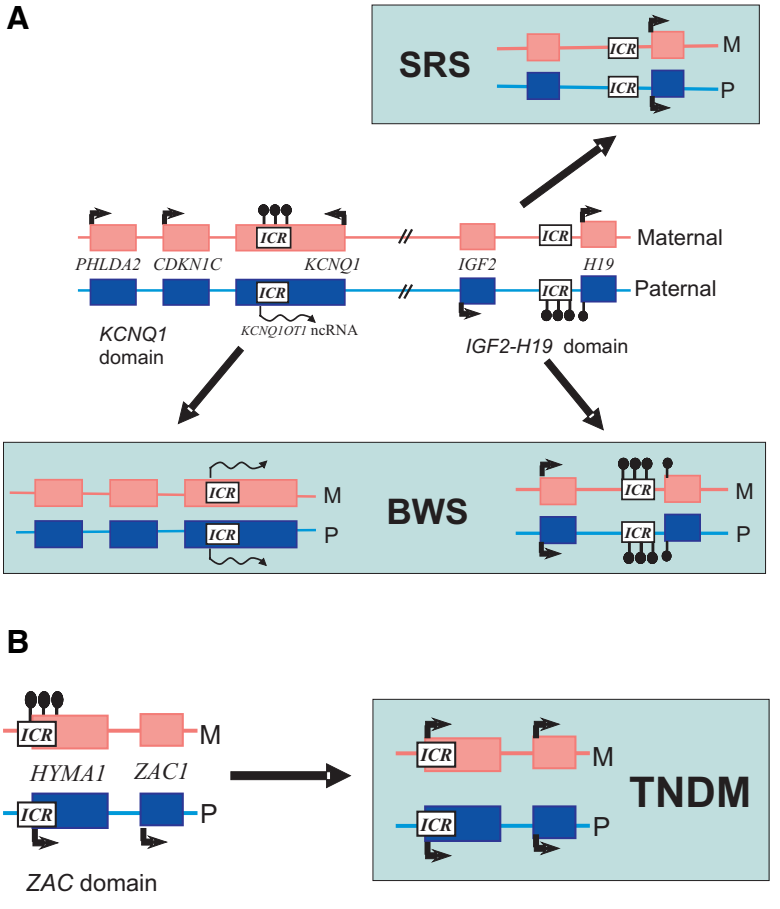

Fig. 4. Altered parental imprints in human perinatal disorders of aberrant growth. (A) *Loss of imprinting at human chromosome 11p15 in Silver-Russell Syndrome (SRS) and Beckwith-Wiedemann Syndrome (BWS). The* H19 *ICR (open box) is methylated (lollipops) exclusively on the paternal allele (P) and conveys paternal expression of the* IGF2 *growth-factor gene, and maternal expression of the* H19 *gene. SRS is characterised by reduced foetal growth and in about half of the patients, there is loss of methylation at the* H19 *ICR. This causes a strong reduction in* IGF2 *expression, and biallelic expression of* H19. *The opposite epimutation is observed in ten percent of patients with the foetal overgrowth syndrome BWS. Here, biallelic methylation at the* H19 *ICR (and* H19*) causes biallelic* IGF2 *expression. The ICR regulating the flanking* KCNQ1 *domain is methylated only on the maternal allele (M). On the unmethylated paternal allele, it produces an ncRNA (*KCNQ1OT1*) that mediates repression on the paternal chromosome. The KCNQ1 domain encompasses the negative growth regulator* CDKN1C, *and* PHLDA2, *a placental gene whose expression is perturbed in intra-uterine growth restriction (McMinn* et al. *2006)..In half the BWS patients, there is loss of methylation at the ICR. This correlates with biallelic expression of the ncRNA, and biallelic repression of genes along the domain, including that of* CDKN1C. **(B)** *Loss of imprinting in Transient Neonatal Diabetes Mellitus (TNDM). The imprinted* ZAC *and* HYMA1 *genes on chromosome 6q24 are regulated by a putative ICR, which is methylated (lollipops) on the maternal allele (M) only. In TNDM, there is loss of DNA methylation. This causes biallelic* ZAC *expression and TNDM, a syndrome frequently associated with intra-uterine growth restriction.*

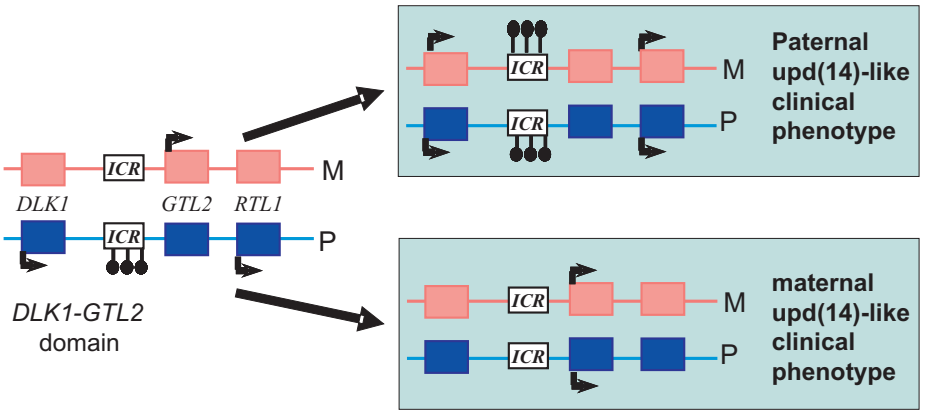

Fig. 5. Perturbed germ line imprints at the *GTL2-DLK1* domain on human chromosome 14q32. *The* DLK1-GTL2 *domain comprises both maternally and paternally expressed genes that are associated with specific developmental phenotypes (Kagami* et al. *2008). The domain is controlled by an intergenic ICR which has sperm-derived DNA methylation. A recent study identified patients that had a clinical phenotype that was comparable to that observed in paternal UPD of chromosome 14 (including the occurrence of a bell-shaped thorax), but in whom there was no evidence for genetic alterations. In these rare patients, there was biallelic DNA methylaton at the ICR, leading to over-expression of paternally expressed genes (Kagami* et al.*, 2008). The opposite epigenetic change has been reported relative to a clinical phenotype similar to that observed in maternal UPD for chromosome 14.*

been reported of children born after ICSI who had an imprinting-related disorder, or displayed aberrant DNA methylation at ICRs (Kobayashi *et al.*, 2007). It also remains to be discovered how defects in the production of spermatozoa could be mechanistically linked to the methylation status of ICRs in sperm. Besides the specific methylation changes in compromised spermatogenesis, it was discovered recently that there is considerable intra- and inter-individual variation in methylation patterns in sperm samples from fertile men (Flanagan *et al.*, 2006). Some of these variations could be due to an incomplete erasure of DNA methylation in the early germ cells (were methylation is normally completely erased), as observed at the mouse Agouti variable yellow (A^{vy}) locus (Morgan *et al.*, 1999). In addition, it was shown in this experimental model that nutritional supplementation with methyl donors can readily lead to altered methylation patterns in the sperm (Cropley *et al.*, 2006). It should be important, therefore, to further explore the influence of dietary and environmental factors (Feil, 2006; Jirtle and Skinner, 2007) on methylaton patterns in sperm, and to determine the possible consequences for the next generation.

Whether similar effects on DNA methylation occur during oogenesis is unknown. However, oocytes are produced early in life and are maintained in a non-mature state till fertilisation, which can be at quite an advanced age in humans. Although genetic defects are known to accumulate in oocytes with age, it remains to be explored if also epigenetic changes are likely to occur upon ageing, a question which could be particularly relevant for imprinting loci.

Several aberrant growth and developmental disorders in humans are caused by perturbation of methylation imprints at ICRs, and are thought to arise during the early stages of development (Fig. 4A). The underlying mechanisms are unknown, but these diseases could be linked to the parental genomes' reprogramming which occurs after fertilisation and during the pre-implantation stage of development (reviewed in Arnaud and Feil, 2005; Delaval *et al.*, 2006). Possibly, methylation patterns are not yet firmly fixed during early development in the non-committed cells, as they are in differentiated cells.

Silver-Russell Syndrome (SRS) is a mostly sporadic congenital disorder, characterised by intra-uterine and postnatal growth restriction, facial dysmorphism and minor, less frequently observed, abnormalities. Several recent studies on SRS show that in about half of the patients with this rare disease, there is loss of DNA methylaton at the ICR regulating *IGF2* and *H19* on chromosome 11p15 (Gicquel *et al.*, 2005; Eggermann *et al.*, 2006; Bliek *et al.*, 2006). This leads to an almost complete loss of expression of *IGF2*, and therefore reduced levels of this major growth factor, which explains the observed growth retardation (Fig. 4A).

Beckwith-Wiedemann Syndrome (BWS) is another rare congenital disorder that affects growth and is linked to chromosome 11p15. This mostly sporadic disease presents an opposite phenotype compared to SRS, with pre- and post-natal overgrowth, and developmental defects including macroglossia, abdominal wall defects, and organomegaly. In BWS, there is an increased risk of Wilms' tumour of the kidney as well. Interestingly, in about 10% of the patients with BWS there is the exact opposite epigenetic alteration at the *IGF2-H19* ICR as the one occurring in SRS. This class of BWS patients shows gain of DNA methylation at the ICR, on the parental allele which is normally not methylated. This methylation change leads to biallelic expression (and a double dose) of *IGF2*, which explains the observed foetal overgrowth. BWS can arise not only from epigenetic alterations at the ICR controlling *IGF2*, but also by altered DNA methylation at the ICR which regulates the growth-related *KCNQ1* domain next to the *IGF2-H19* locus. In about half of the patients, there is loss of methylation at this intronic ICR controlling the *KCNQ1* imprinted domain (Fig. 4A). This early-embryonic loss of methylation correlates with reduced expression (i.e., biallelic repression) of the *CDKN1C* gene located in the domain (Lee *et al.*, 1999; Smilinich *et al.*, 1999; Diaz-Meyer *et al.*, 2003). The loss of expression of this anti-proliferative protein is involved in the BWS syndrome in this subgroup of patients.

Epigenetic deregulation of imprinting is causally involved in transient neonatal diabetes mellitus (TNDM) as well (Fig. 4B), a rare congenital disease associated with intrauterine growth retardation, dehydration and lack of insulin. The disease involves an imprinted locus comprising the paternally expressed *ZAC* gene on chromosome 6q24. This locus is regulated by a putative ICR which has maternal methylation. In the majority of patients with TNDM, there is early embryonic loss of methylation at this ICR leading to biallelic expression of *ZAC* (Gardner *et al.*, 2000; Varrault *et al.*, 2001; Arima *et al.*, 2001). Interestingly, the ZAC transcription factor regulates the expression levels of many growth-

related genes, including *IGF2, H19* and *CDKN1C*. It also influences the transcriptional activity of KvDMR1, the ICR that controls the *KCNQ1* domain (Arima *et al*., 2005; Varrault *et al*., 2006). This interesting finding places the imprinted genes involved in TNDM, BWS and SRS in a common regulatory network that influences intra-uterine growth.

Recently, another ICR was identified to be subject to 'epimutations' in humans. It concerns the *DLK1-GTL2* imprinted domain on chromosome 14q32.2 (Fig. 5). This domain comprises both paternally and maternally expressed genes that are important for development and growth. Uniparental disomy for the paternal chromosome, UPD(14)pat, had been known to result in a typical phenotype with facial abnormalities, a small, bell-shaped thorax, polyhydramnios and abdominal wall defects. Kagami and co-workers (2008) identified several rare cases of people without uniparental disomy who showed a very similar phenotype, particularly the manifestation of a bell-shaped thorax with a 'coat-hanger appearance' of the ribs in the neonatal period. In three of these patients, there was also no evidence for gene deletions in this large domain, and the authors therefore focused their attention on the paternally methylated ICR (IG-DMR) of this domain. In the three patients without genetic defects the ICR showed methylation on the maternal allele as well. This biallelic methylation correlated with excessively high levels of expression of *RTL1*, one of the paternally expressed genes of the domain which is thought to be involved in the paternal up(14)-like clinical phenotype (Kagami *et al*., 2008).

Maternal UPD(14) is associated with a different set of phenotypic manifestations. A recent study identified a single patient with maternal UPD(14)-like clinical phenotypes in whom an epigenetic mutation had occurred at the ICR (Temple *et al.,* 2007). Interestingly, this epigenetic change was the exact mirror image of the epimutations leading to the UPD(14) pat-like phenotypes: a loss of the paternal methylation at the IG-DMR such that now both the alleles were unmethylated (Fig. 5). The resulting decreased expression of *DLK1* at the *DLK1-GTL2* domain is thought to contribute to this clinical phenotype (Kagami *et al.* 2008).

When do the pathological epimutations at ICRs arise? This is difficult to determine, but is thought to occur early in development. This idea follows firstly from the observed broad presence of epimutations throughout the body in patients, and from cases of monozygotic twins of whom only one of the two had the epimutation (Delaval *et al*., 2006). At a low frequency -these are rare sporadic diseases- maintenance of the differential DNA methylation at ICRs is perturbed during early pregnancy, presumably in a stochastic manner. In most cases, this process affects individual ICRs, but it was observed in cases of TNDM and BWS that multiple ICRs were affected at the same time (Mackay *et al*., 2006; Mackay *et al.,* 2008; Bliek *et al.,* 2008). Altered methylation states persist through subsequent development and this leads to different disease phenotypes depending on which imprinted domain(s) is affected.

The occurrence of epimutations is significantly increased when early embryos are taken from their natural environment, and are subjected to the stress of *in vitro* culture. It has been shown in the mouse, that pre-implantation embryo culture frequently leads to aberrant DNA methylation at the *IGF2-H19* ICR and other ICRs, and that this has phenotypic consequences for subsequent development *in vivo* (Khosla *et al*., 2001; Mann *et al*., 2004; Rivera *et al*., 2008). Similar culture effects have been reported in early ovine embryos (Young *et al*., 2001). It is not clear whether the mechanisms underlying the culture-induced perturbation of ICRs are the same as for the epimutations in BWS, SRS, and TNDM, and the thorax abnormalities linked to chromosome 14q32. Nevertheless, the *in vitro* work highlights the possibility that also in humans, culture and manipulation could lead to epimutations at ICRs with long-lasting phenotypic consequences. This question would be particularly pertinent for assisted reproduction technologies (ART). Based on initial studies on relatively small cohorts, it has been suggested that the frequency of imprinting-related diseases could be higher in babies conceived by ART as compared to naturally conceived babies (Arnaud and Feil, 2005). It should be important to determine whether this holds true in larger cohorts as well (Bowdin *et al.,* 2007). Given that couples seeking assisted reproduction are compromised in their natural fertility, an alternative hypothesis for the occurrence of imprinting-related diseases in ART babies would be that defects in gametogenesis and reproduction interfere with the epigenetic regulation of imprinting. The recent demonstration of aberrant ICR methylation in oligospermic males (Marques 2004; Kobayashi *et al*., 2007; Marques 2008) indicates that this should be a promising theme for future research in reproduction.

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