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IMPRINTING IN MAMMALS

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1. ABSTRACT

Genomic imprinting is an epigenetic gene-marking process which allows a subset of mammalian genes to be monoallelically expressed in a parent-of-origin specific manner. The regulation of this imprinted gene expression involves DNA methylation and chromatin modifications, marking parental alleles differentially. Imprints are established in accordance to the sex of the parent in the germline, and are subsequently maintained throughout development and adult life, and are erased prior to establishment during germ cell development. As imprinted gene expression plays an essential role in normal growth and development, genetic and epigenetic disruption altering the dosage of imprinted genes are associated with several developmental defects and diseases. Over the last few decades, after the discovery of the first imprinted gene, many different mechanisms have been implicated in the regulation of imprinted gene expression. This review summarizes the current understanding of the main molecular players and mechanisms in the life cycle of imprinting, and the regulation of the imprinted genes and clusters described so far, the diseases associated with imprinting, and finally the relevance of imprinting in assisted reprogramming technology (ART) or cell reprogramming.

Keywords: life cycle of imprinting, DNA methylation, histone modifications, imprinted genes, imprinting clusters, imprinting disorders, assisted reproductive technology (ART), cell reprogramming

2. INTRODUCTION

Genomic imprinting is an epigenetic phenomenon which results in differential expression of genes based on their parent of origin (Reviewed in MacDonald, 2012). Although most autosomal genes are expressed biallelically in mammals, a certain number of genes show biased or even monoallelic expression dependent on the parent of origin. This specific way of expression is brought by differences in epigenetic marks between two parental copies (Reviewed in Tomizawa and Sasaki, 2012). The epigenetic marks are established in the parental germline, maintained in early embryos, and erased during the early stages of germ cell development (Reviewed in Radford et al., 2011; Reviewed in Sasaki and Matsui, 2008).

Parental imprinting was initially discovered in mice via embryological experiments. Diploid mouse embryos with two female pronuclei or two male pronuclei were not able to complete normal embryogenesis as opposed to control nuclear transplant embryos (McGrath and Solter, 1984; Surani et al., 1984). In genetic studies where uniparental disomies were studied, maternally or paternally derived chromosome regions showed differential gene activity in mice (Cattanach and Kirk, 1985). These studies indicated the presence of genomic imprinting leading to parental specific gene expression in mammals (Reviewed in Tomizawa and Sasaki, 2012).

In 1991, the first imprinted gene *Igf2r* was identified (Barlow et al., 1991), followed by the identification of *H19-Igf2* gene (Bartolomei et al., 1991; DeChiara et al., 1991). Currently, the number of imprinted genes identified is approximately 100. Imprinted genes are found in clusters rather than being uniformly distributed in the genome. Differential expression of genes located within these clusters is in many cases regulated by imprinting control regions (ICRs). The molecular mechanisms directing imprinted gene expression involve DNA methylation and other chromatin modifications. These epigenetic marks differ on the two parentally inherited chromosomes, and ICRs are characterized by such differing epigenetic marks (Edwards and Ferguson-Smith, 2007; Reviewed in Radford et al., 2011). All ICRs identified so far are also known as differentially DNA methylated regions (DMRs) on the two parental chromosomes (Reviewed in Radford et al., 2011).

Genomic imprinting has been intensively studied during the last 30 years. Many imprinted genes as well as ICRs have been identified in mice and human and more are expected to follow. The mechanism of imprint establishment, maintenance, and regulation of these processes are crucial for proper embryonic growth and development in mammals. In addition to mainly studied DNA methylation mechanism, other chromatin modifications may be involved in imprinting. Abnormalities at several levels of the molecular processes directing chromatin modifications are associated with imprinting disorders in human. Regarding the importance of genomic imprinting during development, and current techniques applied in cloning, induced pluripotent stem cell (iPSC) technology, and assisted reproductive technology (ART); it is important to note that the epigenetic mechanism directing imprinted gene expression is very fragile and subject to somatic changes. Therefore studies to understand the establishment, maintenance and loss of imprinted gene expression are crucial

to assess the risks of these new techniques. Last but not least, the origin of the evolution of imprinting is not clear yet.

In this thesis, firstly I will address the key mechanisms in imprint life cycle, the molecular players involved in imprinting, and a description of imprinted genes and gene loci known so far. Then, I will lay out the findings about diseases associated with imprinting, and finally I will comment on the role and consequences of imprinting in cloning, iPSC studies, and ART.

3. LIFE CYCLE OF IMPRINTING

Parental specific imprints are stably inherited to somatic daughter cells through cell division; however, they must be reset in each generation (**Figure 1**). Hence, the life cycle of genomic imprints in mammals can be examined in three main steps: (i) establishment of the genomic imprints, (ii) maintenance of genomic imprints, and (iii) erasure of genomic imprints.

3.1. Imprint Establishment

The initial step of the cycle of genomic imprints is the establishment which is accomplished in male and female gametogenesis (**Figure 1**). In males, *de novo* DNA methylation occurs in the germline where ICRs progressively acquire paternal specific epigenetic marks in mitotically arrested (G1/G0) prospermatogonia after embryonic day 14.5 (E14.5). The paternal imprints are completely established in the prospermatogonia by the neonatal stage (Davis et al., 2000; Hiura et al., 2010; Kato et al., 2007; Li et al., 2004; Reviewed in Li and Sasaki, 2011; Ueda et al., 2000). In females, *de novo* DNA methylation of ICRs happens in growing oocytes which are in diplotene stage of meiotic prophase I, and comparison of different ICRs indicates that the establishment is asynchronous. The maternal imprints are fully established in fully grown oocytes (Hiura et al., 2006; Reviewed in Li and Sasaki, 2011; Lucifero et al., 2004).

The Mechanism of Imprint Establishment

Recent studies revealed mechanistic details about imprint establishment in mice. DNA methylation plays a crucial role in imprint establishment and maintenance. In mammals different methyltransferases have been identified. Dnmt3a and 3b are *de novo* methyltransferases, whereas Dnmt1 encodes the maintenance methyl transferase that copies a methyl group on the newly synthesized strand of hemi-methylated DNA in S-phase. Dnmt3a is required for imprint establishment in male and female germlines (Kaneda et al., 2010; Kaneda et al., 2004; Kato et al., 2007; Reviewed in Tomizawa and Sasaki, 2012). Dnmt3a enzyme forms a complex with a germ cell specific cofactor, Dnmt3L, which increases the catalytic activity of the enzyme and is crucial for imprint establishment (Arnaud et al., 2006; Bourc'his et al., 2001; Hata et al., 2002; Kato et al., 2007; Reviewed in Tomizawa and Sasaki, 2012). Although Dnmt3L shows homology to the other *de novo* methyltransferases, this enzyme lacks the catalytic domain. Nevertheless, the phenotype of Dnmt3a conditional knockout mice is similar to that of Dnmt3L knockout mice, and both knockouts show imprinting phenotypes (Reviewed in Kaneda et al., 2010; Kaneda et al., 2004; Webster et al., 2005). Another *de novo* DNA methyltransferase, Dnmt3b, is not involved in imprint establishment except for the *Rasgrf1* ICR where both Dnmt3a and Dnmt3b are required

(Reviewed in Abramowitz and Bartolomei, 2012; Kato et al., 2007; Reviewed in Tomizawa and Sasaki, 2012). Although the main DNA methyltransferases in imprint establishment are known, the mechanism of recruitment of these DNA methyltransferases to the targets is not well understood yet (Reviewed in Tomizawa and Sasaki, 2012).

In oocytes, several mechanisms and features have been identified as determinants instructing genomic imprinting: (i) transcription traversing ICRs (Reviewed in Abramowitz and Bartolomei, 2012; Chotalia et al., 2009), (ii) unmethylated lysine-4 of histone H3 (H3K4) (Ooi et al., 2007), (iii) 10-bp CpG spacing between two CpG sites (Jia et al., 2007), (iv) a histone H3K4 demethylase Kdm1b (Ciccone et al., 2009), and (v) a Krüppel-associated box (KRAB) zinc finger protein Zfp57 (Li et al., 2008b; Reviewed in Tomizawa and Sasaki, 2012). Among these, the first three seem to be common for all methylated ICRs in oocytes. Transcription through ICRs may enable the chromatin to be more accessible by the Dnmt3a–Dnmt3L complex; unmethylated H3K4 acts as the high-affinity binding target of Dnmt3L; and two CpG sites located 10-bp apart fit well with the catalytic centers of the heterotetrameric Dnmt3a–Dnmt3L complex. Nonetheless, these features are not specific to ICRs, and can be found in other places in the genome (Reviewed in Tomizawa and Sasaki, 2012). On the other hand, Kdm1b seems to act only in a subset of ICRs (i.e. ICRs at *Mest, Grb10, Plagl1,* and Impact loci) (Ciccone et al., 2009), and Zpf57 acts only at the ICR associated with the *Snrpn* locus in oocytes (Li et al., 2008b; Reviewed in Tomizawa and Sasaki, 2012). Even though the exact mechanism of specificity determination is not clear, the combination of common and ICR specific factors may determine the specificity (Reviewed in Tomizawa and Sasaki, 2012). Alternatively, it is also possible that the targeting of methylation at ICRs may not be specific and ICRs may be a subset of CpG islands methylated through general mechanisms of methylation (Smallwood and Kelsey, 2012; Reviewed in Tomizawa and Sasaki, 2012).

In prospermatogonia, methylation of the *Rasgrf1* ICR has been shown to involve the PIWI-interacting RNA machinery (Reviewed in Tomizawa and Sasaki, 2012; Watanabe et al., 2011). It has been shown that an interaction between the non-coding RNA produced by the RNA polymerase traversing the ICR and PIWI-interacting RNAs complementary to this RNA is required for the recruitment of Dnmt3a–Dnmt3L complex. The *Rasgrf1* associated ICR is the only ICR involving the PIWI-interacting RNA pathway, and it is interesting to point out that Dnmt3b is unique to this ICR (Kato et al., 2007; Reviewed in Tomizawa and Sasaki, 2012). Methylation of *H19* ICR in prospermatogonia involves CTCFL (also known as BORIS), and a protein arginine methyltransferase Prmt7 (Jelinic et al., 2006; Reviewed in Tomizawa and Sasaki, 2012). CTCFL, the paralog of the insulator protein CTCF, is highly expressed in male germline. In addition to the *H19/Igf2* locus, CTCF binds to ICRs at several imprinted loci including *Gtl2, Kcnq1/Kcnq1ot1, Grb10,* and *Rasgrf1*. Although CTCF binding seems to be methylation sensitive at the *Kcnq1/Kcnq1ot1,* the *Grb10,* and *Rasgrf1* loci, it is not completely understood whether CTCF has insulator function in other loci than *H19/Igf2* (Lin et al., 2011). Furthermore, histone modifications can act as imprint as histones have recently been found to be retained in mature sperm (Hammoud et al., 2009; Reviewed in Tomizawa and Sasaki, 2012). Particularly, the H3K4me3 modification is enriched at regions

with no methylation in sperm, indicating that it might protect the oocyte-methylated ICRs from methylation in the male germline (Delaval et al., 2007; Hammoud et al., 2009; Reviewed in Tomizawa and Sasaki, 2012).

3.2. Imprint Maintenance

Epigenetic imprints are established in the germline and passed on to the zygote, and maintained throughout the development and adult life (**Figure 1**). After fertilization, several epigenetic modifications are erased. In the paternal genome, protamines replace most histones during sperm maturation, and after fertilization these protamines are replaced by 'native' chromatin. In addition, the paternal genome is subject to active demethylation (Reviewed in Li and Sasaki, 2011; Oswald et al., 2000), in contrast to the maternal genome which is subject to passive demethylation (Reviewed in Li and Sasaki, 2011; Rougier et al., 1998; Santos et al., 2002). However, differential methylation at ICRs is protected from this reprogramming (Reviewed in Li and Sasaki, 2011; Morgan et al., 2005; Reik and Walter, 2001; Sasaki and Matsui, 2008).

After implantation, the non-methylated copies of the ICRs survive a wave of *de novo* DNA methylation. Even if many genes (i.e. pluripotency genes and germ-cell-specific genes) become highly methylated in early post-implantation embryos, the unmethylated allele of the ICR has to escape from this *de novo* DNA methylation. Especially, the maintenance of imprints is important for the imprinted genes having parent-of-origin specific mono-allelic expression during development (Reviewed in Li and Sasaki, 2011).

The Mechanism of Imprint Maintenance

Epigenetic imprints formed in the germline remain after genome-wide reprogramming including DNA demethylation during early embryogenesis (Reviewed in Tomizawa and Sasaki, 2012). The Dnmt3a-Dnmt3L complex has been shown to be involved in DNA methylation at more than a thousand CpG islands in oocytes (Smallwood et al., 2011; Reviewed in Tomizawa and Sasaki, 2012). However, most of the CpG islands lose DNA methylation during preimplantation development, and most of these CpG islands are not related to imprinting. Hence, this is an indication of the presence of protection mechanisms specific to ICRs (Reviewed in Tomizawa and Sasaki, 2012). In preimplantation embryos, the maintenance DNA methyltransferases Dnmt1o (oocyte-stored isoform of Dnmt1) and Dnmt1s (zygotically expressed isoform of Dnmt1) copies the hemi-methylated DNA strand of ICRs, respectively in S-phase (Hirasawa et al., 2008; Reviewed in Tomizawa and Sasaki, 2012). After implantation, Dnmt1s is required for the maintenance of imprints in somatic lineages (Reviewed in Li and Sasaki, 2011). The maintenance of DNA methylation is not only influenced by Dnmt1 but also by other factors (i.e. Dppa3, Zfp57, and MBD3). In addition to DNA methylation, histone modifications have also been shown to be crucial for the maintenance of imprinting.

The specificity of Dnmt1 isoforms for ICRs rather than any other region may be attributed to several mechanisms. According to a study, a mammalian-specific disordered region close to the amino terminus of Dnmt1 may be responsible for ICR specificity (Borowczyk et al., 2009;

Reviewed in Li and Sasaki, 2011). Another recent study proposed an autoinhibitory mechanism, in which unmethylated CpG dinucleotides are excluded from the active site of Dnmt1 to ensure the methylation of only hemimethylated CpG dinucleotides. In other words, when unmethylated CpG sites are protected from *de novo* methylation through binding by CXXC domain of Dnmt1, the efficiency of maintenance methylation is increased (Song et al., 2011). It was also supported by structural studies about Dnmt1 (Song et al., 2012). However, a different study indicated that the autoinhibitory mechanism does not apply to full-length Dnmt1. They also showed that the catalytic domain of Dnmt1 recognizes the hemimethylated CpG sites (Bashtrykov et al., 2012). Both active and autoinhibitory mechanisms seem to be involved in the maintenance DNA methylation mediated by Dnmt1.

In addition to Dnmt1, there are also other factors such as Dppa3, Zfp57, and MBD3 involved in imprint maintenance. The first factor, Dppa3 (also called Pgc7 or Stella), protects the maternal imprints from demethylation (Nakamura et al., 2007; Reviewed in Tomizawa and Sasaki, 2012). This protection is likely to act by protection against hydroxylation of 5-methylcytosine catalyzed by the Tet3 protein (Reviewed in Tomizawa and Sasaki, 2012; Wossidlo et al., 2011). Dnmt1 does not recognize 5-hydroxymethylcytosine. Therefore, maternal imprints may go through hydroxylation -replication-dependent passive demethylation if the protection does not exist. Another factor involved in preimplantation maintenance of both paternal and maternal imprints is the oocyte-derived maternal factor Zfp57. It is involved in the maintenance of imprints of the paternally methylated intergenic ICR (IG-DMR) within the *Dlk1-Meg3* locus (*Meg3* is also referred to as *Gtl2*) and the maternally methylated ICRs associated with the *Mest*, *Peg3* and *Snrpn* loci. Nevertheless, some ICRs such as *H19* and *Igf2r* ICRs are not affected in Zfp57 mutant mice (Li et al., 2008b; Reviewed in Tomizawa and Sasaki, 2012). Another factor involved in imprint maintenance may be a methyl-CpG-binding protein, MBD3, which is involved in the maintenance of the paternal methylation imprint specifically at the *H19* DMR in pre-implantation embryos (Reviewed in Li and Sasaki, 2011; Reese et al., 2007). This mechanism involves the recruitment of the Mi-2/NuRD repression complex to the highly methylated paternal allele of the H19 ICR (Reviewed in Li and Sasaki, 2011).

Interestingly, DNA methylation seems to be less important for the imprint maintenance in trophoblast. This was initially shown in a study where maternal-specific expression of *Ascl2* was maintained in the trophoblast lacking Dnmt1. In addition, mutations in Dnmt1 did not result in loss of imprinting of the placenta-specific genes in an imprinted cluster on mouse chromosome 7. Other studies demonstrated that silent paternal alleles have repressive histone modifications (H3K9me2 mediated by G9a, and H3K27me3 mediated by Polycomb repressive complex 2, PRC2). In the case of mice lacking G9a, mono-allelic expression patterns are lost in placenta-specific genes. Similarly, embryos lacking Eed, a component of the PRC2 complex, some of paternally repressed genes are abnormally activated in the trophoblast. These studies show the importance of histone modifications for imprint maintenance; however, it is unknown if these marks are present at imprinted regions in germ cells (Reviewed in Li and Sasaki, 2011).

Based on the current knowledge on imprint maintenance, the exact mechanistic details about specific protection at different loci are not fully understood and appear to involve different sometimes overlapping mechanisms to maintain genomic imprints (Reviewed in Tomizawa and Sasaki, 2012).

3.3. Imprint Erasure

Before the establishment of parental origin dependent imprints during germ cell development, epigenetic imprints need to be erased which happens in primordial germ cells (PGCs). Between E7.25 and E10.5, PGCs proliferate actively, and then migrate to the genital ridge. Epigenetic reprogramming involving DNA methylation (**Figure 1**), histone modifications, and X-chromosome reactivation occurs in PGCs during this period to restore pluripotency (Reviewed in Li and Sasaki, 2011; Sasaki and Matsui, 2008; Seki et al., 2007; Surani et al., 2007) even though the functional imprints remain at most ICRs (Reviewed in Li and Sasaki, 2011; Szabó et al., 2002). From E10.5 till E12.5, the erasure of the parental-origin-specific DNA methylation is observed at different germline ICRs. As a consequence, imprinted genes are either biallelically expressed or silenced (Reviewed in Hajkova et al., 2002; Lee et al., 2002; Li and Sasaki, 2011). Consistently, the parental-origin-specific DNA methylation at most ICRs has been lost in the male and female embryonic germ cells derived at this stage (Reviewed in Li and Sasaki, 2011; Tada et al., 1998).

The Mechanism of Imprint Erasure

The mechanism of imprint erasure in PGCs is not completely understood, and includes either active and/or passive DNA demethylation. As imprint erasure is a rapid process, it is likely to involve active DNA demethylation (Hajkova et al., 2002; Reviewed in Li and Sasaki, 2011). Several mechanisms have been suggested for DNA demethylation (Reviewed in Li and Sasaki, 2011; Wu and Zhang, 2010), but activation-induced cytidine deaminase (AID) has recently been shown to be involved in demethylation in PGCs (Reviewed in Li and Sasaki, 2011; Morgan et al., 2004). AID is involved in global demethylation as well as locus specific demethylation at some imprinted ICRs (*H19* and *Kcnq1ot1*) in both male and female PGCs (Reviewed in Li and Sasaki, 2011; Popp et al., 2010). AID catalyzes deamination of the 5-methylcytosine (5mC) to thymidine (T) in DNA, and it is expressed in tissues where DNA demethylation occurs (Reviewed in Li and Sasaki, 2011; Morgan et al., 2004). As a result of this action, T-G mismatch forms, and the resulting mismatch triggers the DNA repair machinery to enable the loss of 5mC. Nonetheless, AID deficient cells still show considerable demethylation, indicating that there are other mechanisms of demethylation (Reviewed in Li and Sasaki, 2011). 5-hydroxymethylcytosine (5hmC) may also act as an intermediate in an active demethylation pathway independent of the DNA-repair pathway (Reviewed in Li and Sasaki, 2011; Wu and Zhang, 2010) or 5hmC may be involved in passive demethylation via exclusion of Dnmt1 (Reviewed in Li and Sasaki, 2011; Valinluck and Sowers, 2007). The 10-11 translocation family proteins (TET1, TET2, and TET3) convert 5mC to 5hmC *in vitro* and *in vivo* (Ito et al., 2010; Reviewed in Li and Sasaki, 2011; Tahiliani et al., 2009), and TET1 and TET2 are expressed in PGCs between E11.5 and E12.5 (Hajkova et al., 2010; Reviewed in Li and Sasaki, 2011). Therefore, the TET family proteins may play role in DNA

demethylation. Nevertheless, further investigation is required to better understand imprint erasure in PGCs (Reviewed in Li and Sasaki, 2011).

Many questions remain related to the mechanism of imprinting life cycle in mammals. Current evidence shows that locus-specific sequences, transcription, histone modifications and chromatin structure influence these processes alone or in combinations. If the difficulty in obtaining large numbers of germ cells is overcome, it will be very informal to examine chromatin structure and dynamics together with imprint establishment in germ cells. (Reviewed in Abramowitz and Bartolomei, 2012). Moreover, chromatin modifications other than DNA methylation should be studied more in relation to genomic imprinting.

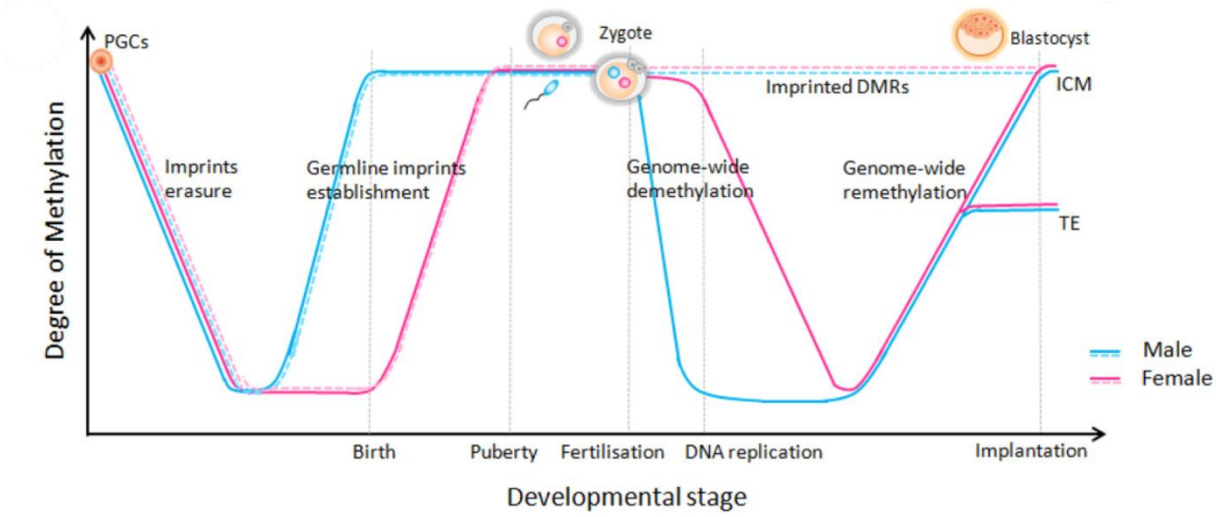

Figure 1. Genome-wide and imprint methylation programming during gametogenesis and early embryonic development in mice. As primordial germ cells (PGCs) enter the genital ridge, genome-wide (solid lines) demethylation occurs that erases the imprints (dashed lines) on the maternal and paternal chromosomes. This is followed by *de novo* methylation which establishes the new parent-of-origin specific imprints during gametogenesis. After fertilization, global demethylation is observed. The paternal pronucleus undergoes active demethylation that is completed before the first DNA replication, while the maternal chromosome is demethylated through a DNA replication-dependent passive mechanism. Once the imprints are established in the parental gametes, they are maintained. Around the time of implantation, genome-wide re-methylation occurs, and these marks are faithfully transferred to the daughter cells throughout the development of individuals. The inner cell mass (ICM; forming the fetus) of an embryo carries higher methylation compared to the trophectoderm (TE; forming the placenta) (Adapted from Ishida and Moore, 2012).

4. MOLECULAR PLAYERS IN IMPRINTING

ICRs have distinct epigenetic marks on the two parentally inherited chromosomes. These epigenetic marks are DNA methylation as well as other chromatin modifications including post-translational modification of histones. Both DNA methylation and specific histone modifications are enriched on one parental chromosome at a number of imprinted loci in the mice and human (Reviewed in Delaval and Feil, 2004; Edwards and Ferguson-Smith, 2007; and McEwen and Ferguson-Smith, 2010). In addition to DNA methylation and histone modifications, non-coding RNAs as well as higher order chromatin formation act in establishment and maintenance of the imprint state of ICRs (Reviewed in MacDonald, 2012).

4.1. DNA Methylation

DNA methylation is the first epigenetic mechanism associated with imprinting. DNA methyltransferases (DNMTs) act in the transfer of methyl group onto cytosine-C5 of CG (also called CpG) dinucleotides, and these enzymes are highly conserved in mammals and plants (Herman and Baylin, 2003), while being dispensable in *Drosophila* (Lyko et al., 2000; Reviewed in MacDonald, 2012). CpG dinucleotides are underrepresented in the genome through spontaneous de-amination of methylated cytosines resulting in thymidine residues. However, CpG dense regions are found clustered in small stretches of DNA, so called CpG islands. CpG islands are mostly associated with promoter regions. Most CpG islands at the gene promoters are unmethylated, enabling active transcription while most CpG sites outside of CpG islands are methylated, suggesting a role in global genome maintenance (Herman and Baylin, 2003; Reviewed in Koukoura O, 2012; Weber and Schübeler, 2007). In general, hypermethylated promoter associated CpG islands result in gene silencing as opposed to hypomethylated CpG islands (Reviewed in Koukoura O, 2012; Maccani and Marsit, 2009). The CpG methylation of important motifs in promoters prevents their recognition by transcription factors and RNA polymerase (Costello and Plass, 2001; Reviewed in Koukoura O, 2012), thus gene silencing is observed.

Differential DNA methylation between the parental chromosomes has been observed at many (though not all) imprinted gene promoters, at which inactive allele has been methylated (Reviewed in Edwards and Ferguson-Smith, 2007; and McEwen and Ferguson-Smith, 2010). As described above, these DNA methylation profiles are established in the maturing oocyte and in spermatogonia, and maintained throughout the development of embryo and adult life. Differentially DNA methylated regions (DMRs) are present at all ICRs identified so far. In addition to primary or germline ICRs, there are also secondary or somatic DMRs found at some imprinted promoter regions. Secondary DMRs gain parent of origin specific methylation after fertilization (Reviewed in Radford et al., 2011). In spite of the importance of DMRs in genomic imprinting, DNA methylation is not the only molecular player involved in imprinting.

4.2. Histone Modifications

Histone proteins and their modifications are highly conserved across all taxa. DNA is wrapped around nucleosomes which are octameric structures composed of H2A, H2B, H3, and H4. The covalent modifications of the core histones in nucleosomes, and histone variants have important roles in chromatin conformation and gene regulation. In general, histone acetylation results in accessible chromatin while histone deacetylation and histone methylation are associated with a compressed chromatin structure, known as heterochromatin correlated with gene silencing (Berger, 2002; Reviewed in MacDonald, 2012). Depending on the methylated lysine residue, histone methylation might represent an active or repressed state. Histone 3 lysine 9 (H3K9), histone 4 lysine 20 (H4K20), and histone 3 lysine 27 (H3K27) are involved in silencing as opposed to histone 3 lysine 4 (H3K4) methylation which is associated with active chromatin (Cheung and Lau, 2005; Reviewed in MacDonald, 2012).

Histone modifications and DNA methylation should be considered together as each epigenetic mark may influence the other's recruitment to reinforce differential epigenetic states (Cedar and Bergman, 2009; Reviewed in MacDonald, 2012). Some ICRs and imprinted gene promoters or transcription start sites (TSSs) have enrichment of several asymmetric histone modifications on two parental chromosomes. These histone marks include H3 acetylation, H4 acetylation, H3K4me2 and H3K4me3, which are enriched on the CpG hypomethylated ICR; and H3K27me2, H3K27me3, H3K9me2 and H3K9me3, which are enriched on the CpG methylated ICR (Reviewed in McEwen and Ferguson-Smith, 2010). Moreover, H4K20me3 has been shown to be enriched on the CpG methylated copy of eight ICRs (Reviewed in McEwen and Ferguson-Smith, 2010). On the other hand, two non-allele specific studies indicated the co-enrichment of active marks (i.e. H3K4me2/3), and repressive marks (i.e. DNA methylation and H3K9me3) at imprinted loci compared to other loci (Dindot et al., 2009; Wen et al., 2008). According to one of these studies, H3K4me2 and DNA methylation are enriched in imprinted gene regions in human genome (Wen et al., 2008). Other study showed that ICRs and secondary DMRs have overlapping pattern of H3K4me3 and H3K9me3 despite differential DNA methylation at non-CpG islands in imprinted gene clusters in mice (Dindot et al., 2009). It is important to consider that different combinations of histone modifications can be enriched at individual genes and individual ICRs in a cell type dependent manner (Reviewed in McEwen and Ferguson-Smith, 2010).

In order to differentiate epigenetic marks associated with imprinting regulation and those associated with more canonical developmental regulation, high throughput data extraction has been applied to the data derived from whole genome expression and epigenetic profiling studies in cellular material. As a result, it is found that both H3K9me3 and H4K20me3 are enriched at imprinted genes with a promoter DMR as compared to those without a promoter DMR, and both histone marks do not correlate with developmental regulation. In contrast, H3K27me3 and H3K4me3 are mostly associated with developmental regulation. This study showed that imprinted genes are developmentally regulated through bivalency with H3K4me3 and H3K27me3 enrichment on the same allele. Additionally, in the same study, a specific three mark signature (H3K4me3, H3K9me3 and H4K20me3) has been identified at all imprinting control regions, indicating an epigenetic signature for ICRs (McEwen and Ferguson-Smith, 2010). According to another study, demethylation of H3K4 has been identified to be required for CpG methylation at some ICRs in female germline (Ciccone et al., 2009; Reviewed in Radford et al., 2011). In support of this, the DNMT3A– DNMT3L complex showed lower affinity for H3K4me3, suggesting that the H3K4me3 modification may shield DNA from becoming methylated (Reviewed in Ferguson-Smith, 2011; Ooi et al., 2007). Therefore, it seems likely that histone modifications contribute to genomic imprinting; however, further investigation is required to understand the functional role of histone modifications in imprint life cycle as well as in other processes associated with unique imprinted genes and ICRs.

4.3. Higher Order Chromatin Structure

Higher order chromatin structure contributes to the imprint state of ICRs, and histone modifications at imprinted regions can facilitate the formation of higher order chromatin

structure. Heterochromatin structure is often involved in the maintenance of transcriptional inactivation at imprinted regions. Heterochromatin structure is a compacted chromatin structure which is associated with transcriptional silencing and can spread in *cis*. Such heterochromatic structures are stable throughout the development and passed to daughter cells through late replication in cell cycle (Kamakaka, 2003; Reviewed in MacDonald, 2012). Heterochromatic protein 1 (HP1) is a highly conserved non-histone chromatin protein which can recruit other heterochromatic proteins as well as accessory factors (i.e. histone methyltransferases), thereby reinforcing the structure of heterochromatin and initiating spreading in cis (Eissenberg and Elgin, 2000; Grewal and Elgin, 2002; James and Elgin, 1986; Reviewed in MacDonald, 2012). HP1 is associated with centromeric heterochromatin and other loci (Kamakaka, 2003). In addition to heterochromatic silencing, there are also Polycomb group (PcG) proteins generating a silencing pathway parallel to heterochromatic silencing (Reviewed in MacDonald, 2012; Orlando, 2003). Although both silencing pathways involve histone deacetylases and histone methyltransferases, the overlap between Polycomb group and heterochromatic silencing is not high (Reviewed in MacDonald, 2012).

The higher order chromatin structure also plays an important role in imprinted gene-expression. This became clear from studies involving the function of CTCF, which can bind to many sites within mammalian genomes where it may act both as a transcriptional regulator and a chromatin insulator. CTCF is able to block the spread of heterochromatin, and mediate long-range chromosomal interactions (Filippova, 2007; Reviewed in MacDonald, 2012). The association of CTCF with imprinting was established in studying its role in parent-specific expression of *Igf2* and *H19*. Chromatin conformation capture studies indicated the presence of intrachromosomal loop formed due to self-interaction between CTCF proteins at ICRs which isolate *H19* to maintain maternal expression, and silencing *Igf2* via a repressive domain (Li et al., 2008a; Reviewed in MacDonald, 2012). In spite of the importance of CTCF-mediated intrachromosomal looping in the regulation of the maternal *Igf2*/*H19* imprinted region (Kurukuti et al., 2006; Reviewed in MacDonald, 2012), it is still unknown if the formation of higher-order chromatin structures via CTCF-mediated intrachromosomal looping is common among other imprinted domains (Reviewed in MacDonald, 2012).

4.4. Noncoding RNAs (ncRNA), RNA Interference (RNAi), microRNAs (miRNAs), and PIWI-interacting RNAs (piRNAs)

In mammals and plants, expression of noncoding RNA (ncRNA) has been described at multiple imprinted regions. Imprinted ncRNA genes represent a family of untranslated transcripts which are mono-allelically expressed in a parent of origin specific manner. Each imprinting gene cluster expresses one or even several large ncRNA gene(s) in addition to containing many small RNAs. The large ncRNA genes show reciprocal imprinted expression with respect to the neighboring protein-coding genes. Moreover, some of these ncRNA genes are transcribed in antisense orientation relative to the protein-coding genes. On the other side, the small RNAs belong to the mammalian small regulatory RNA genes which mainly function through sequence-specific recognition of other cellular RNAs including microRNAs (miRNAs), and probably PIWI-interacting RNAs (piRNAs) (Reviewed in MacDonald, 2012; Royo and Cavaillé, 2008; Zhang and Qu, 2009). *H19* and *Airn* RNAs are known to be

associated with imprinted regions in mammals. In mice *Igf2r/Airn* cluster, *Igf2r* is maternally expressed whereas the overlapping *Airn* antisense transcript is paternally expressed. The *Airn* transcript can maintain paternal silencing in this gene cluster when the paternal *Igf2r* promoter is artificially activated (Reviewed in MacDonald, 2012; Sleutels et al., 2003) or when CpG methylation of DMR2 *Airn* promoter is lost (Barlow et al., 1991; Reviewed in MacDonald, 2012). This silencing function of *Airn* may be partly through the recruitment of H3K9 methyltransferase G9a in mouse (Reviewed in MacDonald, 2012; Nagano et al., 2008). Such a mechanism would be similar to silencing invoked by the ncRNA *Xist* which plays a key role in silencing of paternal X chromosome in extra-embryonic tissues of female mice. The spreading of transcription of *Xist* results in coverage of most of the paternal X chromosome, allowing the recruitment of epigenetic silencing factors (i.e. histone methyltransferases and heterochromatic proteins) (Andersen and Panning, 2003; Reviewed in MacDonald, 2012). However, ncRNAs may not always be functional themselves, but the act of transcription could also play a crucial role in the regulation of imprinted genes. For instance, the transcription of *Airn* through neighboring genes may be instrumental in gene silencing (Reviewed in MacDonald, 2012; Seidl et al., 2006). In support of this, another study pointed out that it is the act of transcription resulting in *Kcnq1ot1* ncRNA that contributes to the silencing rather than the presence of *Kcnq1ot1* transcript (Golding et al., 2011; Reviewed in MacDonald, 2012).

RNA interference (RNAi) is a conserved posttranscriptional silencing mechanism in which double-stranded RNA guide the degradation of complementary RNA transcripts through an RNA silencing complex (RISC) (Filipowicz, 2005; Reviewed in MacDonald, 2012; Tang, 2005). The components of RNAi silencing pathway are known to be involved in the recruitment DNA methyltransferases and other factors instructing chromatin changes in many organisms (Djupedal and Ekwall, 2009; Reviewed in MacDonald, 2012). Involvement of the RNAi machinery has been observed in the destruction of maternal *PEG11* transcript, where maternal antisense miRNAs acts to guide turnover (Davis et al., 2005; Reviewed in MacDonald, 2012). In addition, *miR-127* and *miR-136* genes have been shown to be involved in imprinting of the retrotransposon-like gene *Rtl1* in mice and the orthologous *PEG11* gene in sheep and humans (Charlier et al., 2001; Reviewed in MacDonald, 2012; Seitz et al., 2003). However, it is not clear if processing of *PEG11* transcript by RNAi machinery recruits chromatic remodeling enzymes regulating the maternal allele expression (Reviewed in MacDonald, 2012).

PIWI-interacting RNAs (piRNAs) are about 26–31 nt long RNAs corresponding to a novel class of germ-line-specific small RNAs. piRNAs specifically interact with members of the PIWI (P-element-induced wimpy testis) family proteins, a subset of the Argonaute proteins expressed predominantly in the germlines of a variety of organisms, to protect the mammalian germline from transposon activity by so far poorly understood mechanisms (Royo and Cavaillé, 2008; Siomi et al., 2010). piRNAs are involved in silencing of mobile genetic elements in many eukaryotic organisms (Reviewed in Daxinger and Whitelaw, 2012; Saito and Siomi, 2010), and PIWI has been shown to affect position effect variegation (PEV) in *D. melanogaster* (Reviewed in Daxinger and Whitelaw, 2012; Pal-Bhadra et al., 2004).

Interestingly, piRNAs have recently been shown to play role in the establishment of parental imprints in mammals. In this study, the requirement of piRNAs for the establishment of the CpG methylation at the *Rasgrf1* locus has been shown in the paternal germline in mice. piRNA generated from a different locus targets a retrotransposon sequence within a non-coding RNA spanning the DMR of this imprinted gene (Reviewed in Daxinger and Whitelaw, 2012; Watanabe et al., 2011). This study demonstrates the involvement of piRNAs in the establishment of DNA methylation imprints during reprogramming in PGCs, but it is not known whether piRNAs are carried in the gametes and influence DNA methylation in the offspring (Reviewed in Daxinger and Whitelaw, 2012).

Specific imprinted genes are not often conserved between diverse species; however, epigenetic mechanism and general structural features of imprinted regions are often similar (Reviewed in MacDonald, 2012). Hence, the novel characterization of imprinted domains in diverse organisms may enable better understanding of the regulatory mechanisms involving ncRNAs in the context of genomic imprinting. Considering all the molecular players mentioned here, it is still possible that there may be more unidentified epigenetic mechanisms playing role in imprinting.

5. IMPRINTED GENES

Genomic imprinting changes a biallelic gene expression to monoallelic, but silencing of the inactive copy is only partial for many imprinted genes (Morison et al., 2005). Since the discovery of the first imprinted gene, *Igf2r*, identified in 1991 (Barlow et al., 1991), more than 100 genes have been identified in mice. Approximately 50 of them are also imprinted in human (Reviewed in Ishida and Moore, 2012; Morison et al., 2005). A regularly updated list of mammalian imprinted genes (University of Otago's Catalogue of Parent of Origin Effects, http://igc.otago.ac.nz/; and Harwell mouse database, http://www.har.mrc.ac.uk/) has been derived from the published literature (Reviewed in Ishida and Moore, 2012; Morison et al., 2001; Reviewed in Morison et al., 2005).

All imprinted mammalian genes are represented either in human or mice, and findings in other species did not reveal new imprinted genes so far (Murphy and Jirtle, 2003). Work in the early nineties indicated that 0.2% of the CpG islands to retain parent of origin specific differential methylation (Hayashizaki et al., 1994). According to the genome-wide count of 29 000 CpG islands (Lander et al., 2001), ~100 differentially methylated imprinted loci were predicted (Reviewed in Morison et al., 2005). Recently, transcriptome sequencing studies indicated more than 1000 potential imprinted genes in the mouse brain (Gregg et al., 2010a; Gregg et al., 2010b). However, most of these findings could not be confirmed by other independent studies (DeVeale et al., 2012; Reviewed in Ishida and Moore, 2012). These studies revealed only 3–6 new putative imprinted genes (Babak et al., 2008; Reviewed in Ishida and Moore, 2012; Wang et al., 2008). Based on this evidence, the total number of imprinted genes is likely to range around the initial estimate of a few hundred genes (Barlow, 1995; Reviewed in Ishida and Moore, 2012). This is supported by the fact that the numbers are not expected to increase so much due to the restricted number of human and animal phenotypes with imprinted diseases. Nevertheless, some imprinted genes with subtle

phenotypic effects may remain to be discovered (Morison et al., 2005). Additionally, it should be pointed out here that about 900 X chromosomal genes are silenced paternally during early murine development (Brown and Greally, 2003; Mak et al., 2004; Ross et al., 2005); although this seems to be a mouse specific phenomenon (Reviewed in Morison et al., 2005).

Most of the imprinted genes are known encode proteins involved in growth and development. In addition to protein coding genes, imprinted genes can be examined in other specific categories known as intronic and retrotransposed genes, and non-coding RNAs.

5.1. Protein Coding Genes

Imprinted genes have crucial roles in reproductive and maternal behavior, embryonic growth, and the development and function of key metabolic axes in the embryo. The majority of imprinted genes are protein coding genes (Radford et al., 2011). Recently, the association of imprinted genes with individual birth size variation has been examined in healthy populations (Reviewed in Ishida and Moore, 2012).

Single nucleotide polymorphisms (SNPs) in the *IGF2R* (rs8191754; maternal genotype), *IGF2* (rs3741205; newborn genotype) and in the 5' region of the *H19* (rs2067051, rs2251375, and rs4929984) genes were associated with birth weight. As consistent with imprinting, another study indicated that common polymorphic variation in fetal *H19* alleles transmitted only from the mother are associated with birth weight and other markers of size at birth (Adkins RM et al., 2010; Reviewed in Ishida and Moore, 2012; Petry et al., 2011). Moreover, maternal inheritance of a minor 15 bp repeat sequence variant (RS1) in the promoter of maternally expressed *PHLDA2* has recently been found to be associated with increased birth weight. Indeed, increased expression of the maternal *PHLDA2* allele has been correlated with lower birth weight, suggesting a growth suppressive role. However, RS1 allele was found to be associated with lower transcriptional efficiency, and homozygous RS1/RS1 mothers had heavier babies (Ishida et al., 2012; Reviewed in Ishida and Moore, 2012). Interestingly, while the minor RS1 allele is conserved in monkeys, a common duplicated RS2 allele seems to be specific to humans, and RS2 does not promote fetal growth. This suggests that there may be selection on RS2 allele to support human reproductive success because larger size at birth may be detrimental for the mother during the birth. Importantly, *PHLDA2* has been found to be expressed in the placenta of babies with a lower birth weight in a healthy human cohort, consistent with the conflict theory (Reviewed in Ishida and Moore, 2012; Moore and Haig, 1991). According to this conflict theory, mothers suppress fetal growth enhancing genes passed on to their offspring while fathers enable their offspring to get enough resources to maximize the survival of offspring, even at the expense of half-sibs and the mother (Moore and Haig, 1991; Reviewed in Morison et al., 2005). The well-known supportive example of the conflict theory, interaction between *Igf2* and *Igf2r* genes, will be described in the next part about gene clusters.

5.2. Intronic and Retrotransposed Genes

Intronic genes refer to the genes found in intronic regions of other genes. Some protein coding imprinted genes (i.e. *ARHI, Nap1l5, TCEB3C, NNAT*) are not associated with a cluster, and

they reside in the intronic region of other protein coding genes. *U2af1-rs1* also resides within the *Commd1* gene, and *Commd1* seems to have gained imprinting as a consequence (Reviewed in Morison et al., 2005).

The imprinted status of retrotransposed imprinted genes seems to have originated as a consequence of the retrotransposition event (Walter and Paulsen, 2003). Four imprinted protein coding genes (*DRLM/Nap1l5, MKRN3, TCEB3C, and U2af1-rs1*) and one pseudogene (*Mkrn1-ps1*) are single exon genes derived by retrotransposition events. Moreover, some imprinted genes (i.e. *PEG10, Peg12, and Rtl1*) certainly arose through retrotransposition. These imprinted genes are mostly in antisense orientation with respect to the genes they reside in. Based on these findings, imprinting is hypothesized to occur as a by-product of a defense mechanism against foreign DNA (Barlow, 1993; Reviewed in Morison et al., 2005; Yoder et al., 1997). There are also examples (i.e. *MKRN3, Peg12*), where retrotransposed genes acquire the imprint of the flanking region (Chai et al., 2001; Reviewed in Morison et al., 2005). Alternatively, imprint regulatory elements might have been carried over with retrotransposition of the gene, but detailed phylogeny analysis of the origin of such imprinted genes and retrotransposition events is required to better understand this phenomenon (Reviewed in Morison et al., 2005; Walter and Paulsen, 2003).

5.3. Imprinted Non-coding RNAs

Non-coding RNAs include antisense transcripts, small nucleolar RNAs (snoRNAs), microRNAs, pseudogenes, and other RNAs with unknown function. Some imprinted noncoding genes (e.g. *Airn, KCNQ1OT1, UBE3A-AS, SANG, Tsix*) express overlapping antisense transcripts, and are involved in imprinting. There are also other imprinted noncoding genes (e.g. *H19, Airn, Kcnq1ot1 MEG3, Xist*), which exert the regulatory effect in imprinting in cis over distances that range between kilobases up to megabases in case of *Xist* which is involved in silencing of most of the X chromosome (Reviewed in Morison et al., 2005; O'Neill, 2005). Adjacent protein coding genes are reciprocally imprinted in all cases except for some *GNAS* locus transcripts. Importantly, the mechanism of conveying the imprint signal by noncoding transcripts is not clear (Morey and Avner, 2004; Reviewed in Morison et al., 2005; Ogawa and Lee, 2002; Sleutels et al., 2003; Thakur et al., 2004). In case of *Xist*, it is known that silencing in cis occurs via chromosome coating by *Xist* RNA which recruits chromatin remodeling enzymes involved in the silencing process (Reviewed in Morison et al., 2005; Plath et al., 2002). Similarly, other long noncoding transcripts have roles in gene repression in cis acting on a single gene locus (*Tsix*), and a chromosomal domain (*Airn* and *Kcnq1ot1*) (Wutz, 2011).

For several noncoding antisense RNA transcripts (i.e. *MESTIT1, COPG2IT1, IGF2AS, WT1AS, Zfp127as, anti-Rtl1*) which are paired with imprinted sense protein coding transcripts, the role in the imprinting process is not established. Even though antisense transcripts are suggested to be particularly common for imprinted genes (Reviewed in Morison et al., 2005; Reik and Walter, 2001), it has been shown that previously underestimated sense-antisense transcription units have been estimated to be present in 10–20% of all autosomal genes. Indeed, the overall proportion of antisense transcripts may be

even higher owing to a database bias in favor of processed antisense RNAs (Kiyosawa et al., 2003; Morey and Avner, 2004; Reviewed in Morison et al., 2005; Yelin et al., 2003).

Imprinted snoRNAs and microRNAs have also been identified, and it has been speculated based on their location within imprinted gene clusters that the expression of small RNA genes contributes to the control of imprinting (Reviewed in Morison et al., 2005; Seitz H, 2004). For the remaining imprinted RNA transcripts, no known function has been identified except for few expressed pseudogenes such as *Mkrn1-ps1, Zim3,*and *Zfp264* (Reviewed in Morison et al., 2005).

6. IMPRINTED GENES IN CLUSTERS

The majority of imprinted genes are found in clusters located in the genome. There are some exceptions such as *Nap1l5, Nnat,* and *Inpp5f_v2* which are isolated imprinted genes (Choi et al., 2005; Reviewed in Edwards and Ferguson-Smith, 2007; Kagitani et al., 1997; Kikyo et al., 1997; Smith et al., 2003). A general feature of most imprinted gene clusters is that they contain several protein coding genes and at least one noncoding RNA (ncRNA) gene. Imprinting control regions (ICRs) are regulatory units that control these clusters. In fact, each cluster is controlled by a single ICR even though there may be other modulators affecting the function of ICRs. ICRs are known to be differentially methylated between the two parental copies in the germ cells, and the presence of CpG methylation is associated with repression or activation of flanking genes (Reviewed in Edwards and Ferguson-Smith, 2007). Differentially methylated promoters of imprinted genes usually function as their ICRs (Choi et al., 2005; Reviewed in Edwards and Ferguson-Smith, 2007; Kikyo et al., 1997). According to a survey of human and mouse genomes, more tandem repeats are found in methylated regions of imprinted genes compared to methylated regions of nonimprinted genes (Hutter et al., 2006; Reviewed in MacDonald, 2012). These repeats may act as structural elements of ICRs, which could direct chromatin alterations or recruit epigenetic mechanisms (Reviewed in MacDonald, 2012).

Imprinted gene clusters can be divided in two groups; (i) the clusters whose ICR is methylated in the maternal germline (**Figure 2a-d**), and (ii) clusters whose ICR is methylated in the paternal germline (**Figure 2e-g**) (Reviewed in Edwards and Ferguson-Smith, 2007). An important study showed that mouse parthenogenetic embryos (ng/fg PE) which contain haploid sets of genomes from nongrowing (ng) oocytes derived from newborn fetuses and fully grown (fg) oocytes derived from adults were able to develop into 13.5 day old fetuses, while mouse parthenotes normally resulted in death before 10 days of gestation. This indicated that the prolonged development is because of a lack of genomic imprinting in ng oocytes. After the establishment of maternal imprints was confirmed in oocytes derived from ng/fg PE, it was also demonstrated that matured oocytes containing a haploid genome derived from ng/fg PE oocytes were able to form live pups through *in vitro* fertilization. This indicated that most imprints are formed in the female germline (Niwa et al., 2004).

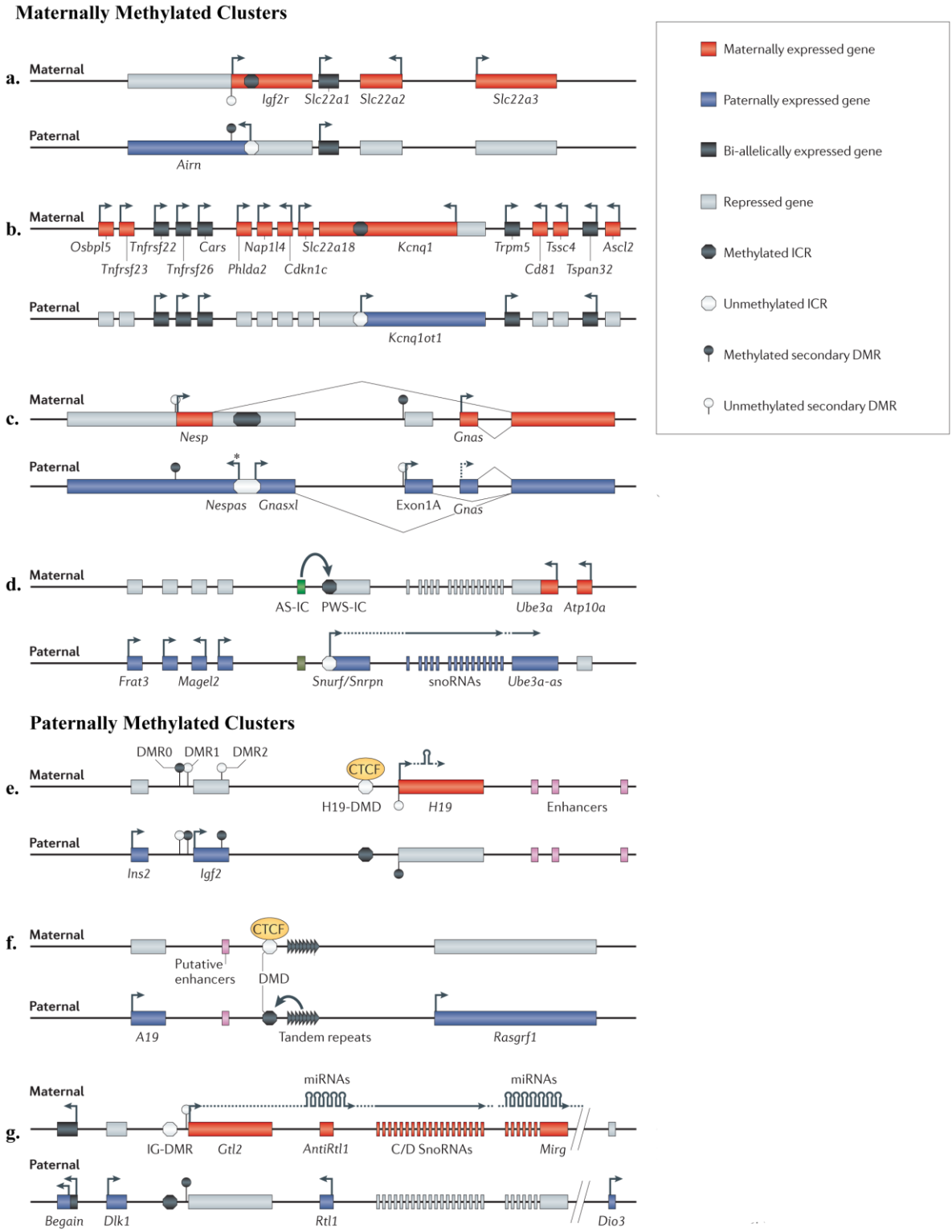

Figure 2. Imprinted gene clusters in mammals. Schematic representations of four imprinted clusters regulated by maternal methylation of ICRs in mouse **(a–d)** and three imprinted clusters regulated by paternal methylation in mouse **(e–g)**. In all of these clusters, ICR controls the parental-of-origin specific gene expression. **(a)** *Igf2r*/*Airn* cluster, (**b**) IC2/*Kcnq1* cluster, (**c**) *Gnas* cluster, (**d**) PWS/AS cluster (*Snrpn* cluster), (**e**) *Igf2*/*H19* cluster, (**f**) *Rasgrf1* cluster, (**g**) *Dlk1-Dio3* cluster. The genes and clusters are not drawn to scale. CTCF indicates CCCTC-binding factor (Reviewed in Edwards and Ferguson-Smith, 2007; Adapted from Ferguson-Smith, 2011).

In this part, imprinted gene clusters will be discussed in general. Additionally, a maternally methylated cluster *Igf2r/Airn* and a paternally methylated cluster *Igf2/H19* will be described in detail as examples of the complex regulatory mechanisms directing imprinted gene expression.

6.1. Maternally Methylated Imprinted Gene Clusters

Most of the imprinted clusters are controlled by maternally inherited methylation marks. In these maternally methylated clusters (*Igf2r/Airn*, IC2/*Kcnq1*, *Gnas*, and PWS/AS) in **Figure 2a-d**, the ICR acts as a promoter for a paternally expressed ncRNA, that is antisense to at least one of the genes in the cluster (Reviewed in Edwards and Ferguson-Smith, 2007).

- ***Igf2r/Airn*** is a cluster located on mouse chromosome 17 (human 6q26) and contains three maternally expressed protein coding genes (*Igf2r*, *Slc22a2* and *Slc22a3* (Barlow et al., 1991; Zwart et al., 2001) in addition to biallelically expressed *Slc22a1*, and paternally expressed antisense transcript *Airn* (previously known as *Air*). The ICR of this domain is maternally methylated, maps to the second intron of *Igf2r*, and contains *Airn* promoter (Reviewed in Edwards and Ferguson-Smith, 2007).
- **IC2/*Kcnq1*** is a cluster located on mouse chromosome 7 (human 11p15.5) which contains several maternally expressed protein coding genes as well as one paternally expressed ncRNA (*Kcnq1ot1*) which is antisense to the protein coding gene *Kcnq1* (Smilinich et al., 1999). Similar to *Igf2r/Airn* domain, KvDMR1 ICR is also the promoter for the ncRNA (Reviewed in Edwards and Ferguson-Smith, 2007).
- ***Gnas*** cluster on mouse distal chromosome 2 (human 20q13.11) consists of *Gnas* gene having different transcripts regulated in a tissue-specific manner, *Nesp* gene encoding Nesp55, and a paternally expressed antisense transcript *Nespas* (Reviewed in Edwards and Ferguson-Smith, 2007; Kelsey et al., 1999). Three different ICRs have been identified in *Gnas* cluster (Reviewed in Edwards and Ferguson-Smith, 2007), but *Nespas* ICR has been reported to be the principal ICR for the entire *Gnas* cluster (Reviewed in Ishida and Moore, 2012).
- **Prader-Willi/Angelman Syndrome (PWS/AS**) or ***Snrpn*** cluster on mouse chromosome 7 (human 15q11-13) contains a number of paternally expressed genes such as *Frat3* (only in mice), *Mkrn3*, *Magel2*, *Ndn* and *Snrpn* (Nicholls and Knepper, 2001), paternally expressed ncRNAs (e.g. *Ube3a-as* and several snoRNAs), and maternally expressed genes (*Ube3a* and *Atp10a*) (Reviewed in Edwards and Ferguson-Smith, 2007). The ICR for this domain seems to be a bipartite element comprising the PWS-IC and the AC-IC (Buiting et al., 1999; Reviewed in Edwards and Ferguson-Smith, 2007; Ohta et al., 1999).

6.2. Paternally Methylated Imprinted Gene Clusters

Three paternally methylated germline-derived ICRs (*Igf2/H19* ICR, *Dlk1-Dio3* ICR, and the ICR regulating the *Rasgrf1* locus) have been identified to regulate imprinted gene clusters. In the paternally methylated clusters (*Igf2/H19*, *Rasgrf1* and *Dlk1–Dio3*) shown in **Figure 2e-g**, ICR is not acting as a promoter for the ncRNAs, and ncRNAs are expressed from different

locations within the maternal clusters. The *Igf2/H19* gene locus is controlled by the interactions between enhancers and DMRs; however, the mechanism by which the IG-DMR controls imprinting in the *Dlk1-Dio3* region is not clear (Reviewed in Edwards and Ferguson-Smith, 2007), and a separate study has indicated that the mechanism of action of IG-DMR is different in embryo and placenta (Lin et al., 2007).

- ***Igf2/H19*** imprinting cluster on mouse chromosome 7 (human 11) contains paternally expressed *Igf2* and *Ins* protein-coding genes as well as a maternally expressed ncRNA, *H19* (Bartolomei et al., 1991; DeChiara et al., 1991; Reviewed in Edwards and Ferguson-Smith, 2007; Giddings et al., 1994). Four DMRs (DMR1, DMR2, DMR0, and *H19*-DMD) have been identified in mice and *H19*-DMD acts as the ICR of the cluster (Reviewed in Edwards and Ferguson-Smith, 2007).
- ***Rasgrf1*** imprinting cluster on mouse chromosome 9 (human 15) contains paternally expressed *RasGrf1* (The RAS protein-specific guanine nucleotide releasing factor 1) gene, *A19* gene having non-coding transcript, and the tandem repeats required for the paternal germline methylation of the ICR regulating *Rasgrf1* cluster (Reviewed in de la Puente et al., 2002; Ferguson-Smith, 2011).
- ***Dlk1–Dio3*** cluster on mouse chromosome 12 (human 14q) contains three paternally expressed protein coding genes (*Dlk1, Dio3* and *Rtl1*) (Takada et al., 2000; Tsai et al., 2002), and multiple ncRNA genes (e.g. *Gtl2*, anti*Rtl1*, C/D snoRNAs and microRNAs) (Reviewed in Edwards and Ferguson-Smith, 2007). Four DMRs (*Gtl2* promoter, *Dlk1*-DMR, DMR-0, and IG-DMR) have been identified in this region, and IG-DMR acts as the ICR for the *Dlk1-Dio3* region (Reviewed in Edwards and Ferguson-Smith, 2007).

6.3. Mechanisms of Gene Regulation in *Igf2/H19* and *Igf2r/Airn* Clusters

Among imprinted clusters, two dominating mechanisms have been described to explain the gene regulation; (i) the insulator model in which imprinted genes share regulatory elements and insulator controls the access to these elements, and (ii) ncRNA model where the associated genes are silenced by the ncRNAs. However, it is crucial to note that there may be other mechanisms of regulation such as the involvement of alternative polyadenylation sites in an allele-specific manner in the case of a new imprinted locus, *H13*. The insulator model of imprinting has been well studied for *Igf2/H19* cluster, while ncRNA model has been well described for *Igf2r/Airn* cluster as shown in **Figure 3** (Reviewed in Abramowitz and Bartolomei, 2012; Barlow, 2011; Reviewed in Bartolomei, 2009; Ferguson-Smith, 2011).

Igf2/H19 Cluster

The most evolutionarily ancient (Smits et al., 2008) mechanism is the insulator model utilized in the *Igf2/H19* imprinted locus (Reviewed in Abramowitz and Bartolomei, 2012). In this locus, maternally expressed *H19* gene and paternally expressed *Igf2* gene have common enhancers and the reciprocal imprinting is achieved by a CTCF-dependent insulator located within the ICR which is found between the genes. In addition, human insulin gene (*INS*) and mouse homologue (*Ins2)* in the downstream of *Igf2* are also imprinted, and paternal alleles for both genes are active in the yolk sac (Giddings et al., 1994); which is not shown in **Figure 3a**.

CTCF is a DNA methylation sensitive zinc finger protein which binds to 4 binding sites in the ICR on the maternally inherited unmethylated allele in mouse. CTCF acts as an insulator which prevents *Igf2* from accessing the shared enhancers located downstream of *H19*. DNA methylation of the CTCF binding motifs within the ICR of the paternally inherited allele prevents CTCF from binding, and allows *Igf2* to interact the enhancers. In addition, ICR mediated paternal specific DNA methylation of the *H19* promoter is required for proper silencing of *H19* (Reviewed in Abramowitz and Bartolomei, 2012). Strikingly, intra-chromosomal loops created by self-association between CTCF proteins bound to *H19* ICR and *Igf2* DMRs isolates *H19* to maintain maternal expression, while reinforcing *Igf2* silencing through the formation of a repressive domain (Li et al., 2008a; Reviewed in MacDonald, 2012). At the same time, this intra-chromosomal loop formed through CTCF recruitment prevents DNA methylation of the *H19* ICR and *Igf2* DMRs (DMR1 and DMR2). When CTCF binding at the maternal *H19* ICR is disrupted, *de novo* DNA methylation of maternal *Igf2* DMR1 and DMR2 have been observed, suggesting that intrachromosomal looping regulates the entire maternal *Igf2*/*H19* imprinted region (Kurukuti et al., 2006; Reviewed in MacDonald, 2012). Furthermore, CTCF has recently been reported to interact with cofactors such as cohesins, the DEAD-box RNA-binding protein p68 (p68), and the steroid receptor RNA activator (SRA) at the *H19* ICR. This may facilitate higher order chromatin at the *H19* locus (Reviewed in Abramowitz and Bartolomei, 2012). Although *Igf2/H19* domain is the most studied domain, there remains much to be understood about the involvement of DNA methylation at this domain. Last but not least, it needs to be investigated if CTCF-mediated intrachromosomal looping is a common mechanism in imprinted clusters (Reviewed in MacDonald, 2012).

Igf2r/Airn Cluster

The less evolutionarily ancient, but more commonly used mechanism is the ncRNA model which was initially observed, and perhaps best described, in *Igf2r/Airn* cluster (**Figure 3b**). Two additional well-defined clusters are the IC2/Kcnq1 cluster, and the Gnas cluster. It is also likely that the PWS/AS and Dlk1/Dio3 clusters employ ncRNA model, but more work is required to understand the role and properties of the ncRNAs in these clusters (Reviewed in Abramowitz and Bartolomei, 2012; and Bartolomei, 2009).

In ncRNA model, the differentially methylated promoter within the ICR regulates the expression of an ncRNA. When the ICR is unmethylated, the ncRNA is expressed, and represses cis-linked genes. On the other hand, when the ICR is methylated, the ncRNA is repressed, and the cis-linked genes are expressed. In *Igf2r/Airn* cluster, the ncRNA *Airn* represses *Igf2r* ubiquitously, and *Slc22a2* and *Slc22a3* in the placenta. Even though the mechanism of this repression is unclear, it has been proposed that the interaction of *Airn* with the *Slc22a3* promoter and the H3K9 histone methyltransferase G9a in the placenta may cause epigenetic silencing of the transcription (Reviewed in Abramowitz and Bartolomei, 2012; Nagano et al., 2008). Alternatively, transcription through the domain has been associated with silencing of the genes in cis (Reviewed in Abramowitz and Bartolomei, 2012; Barlow, 2011). Both mechanisms may be used in a tissue specific manner (Reviewed in Abramowitz and Bartolomei, 2012).

There are some differences in the mechanism of regulation in *Igf2r/Airn* cluster in mice versus human. In mice, *Igf2r* is maternally expressed as opposed to *Airn* which is paternally expressed. The expressed maternal *Igf2r* allele and paternal *Airn* allele are both marked by H3K4 di- and trimethylation, whereas the repressed paternal *Igf2r* allele and maternal *Airn* allele are both marked by H3K9 trimethylation within the promoter regions (DMR1 and DMR2). The *Airn* transcript overlaps the *Igf2r* promoter, and the act of transcription or the RNA contribute to the silencing of the paternal *Igf2r* allele which involves DNA methylation and histone H3K9 trimethylation. In contrast, in human, *Igf2r* is biallelically expressed, and both maternal and paternal promoter regions of *Igf2r* are enriched for activating H3K4 methylation. The maternal DNA methylation of DMR2 is maintained, while no activating H3K4 methylation is present on the paternal DMR2, enabling the silencing of the *Airn* transcription (Reviewed in MacDonald, 2012).

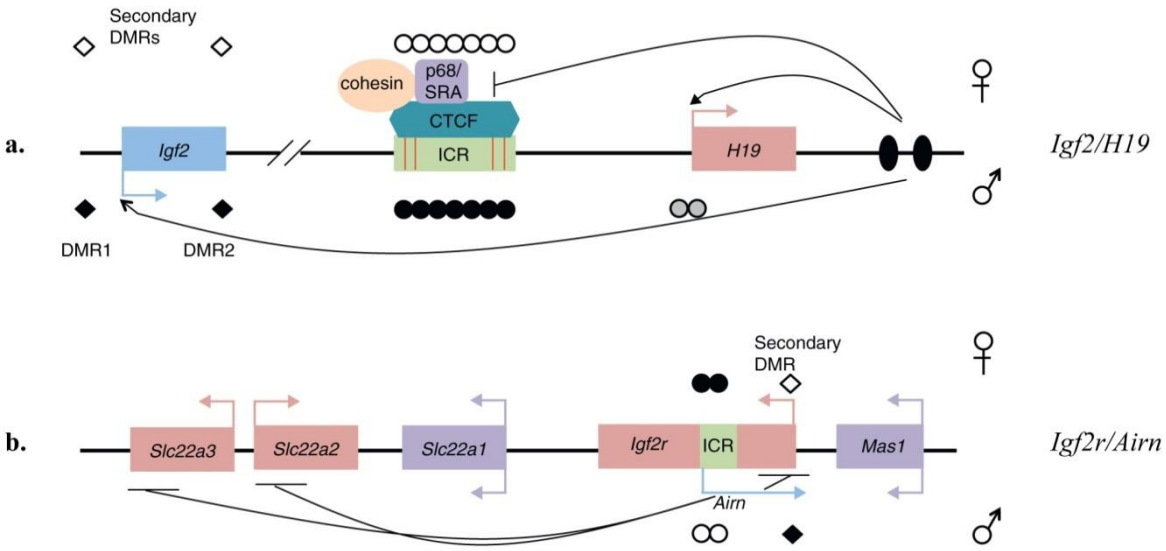

Figure 3: Imprinted regulation of *Igf2/H19* and *Igf2r/Airn* loci. (a) Insulator mediated imprinting of the *Igf2/H19* locus. The maternally expressed *H19* and paternally expressed *Igf2* genes are shown in the figure. Note that *Ins2* adjacent to *Igf2* is not shown here. The ICR is unmethylated (open circles) on the maternal allele, allowing CTCF and its cofactors (cohesins and p68/SRA) to bind. This interaction results in the blocking of downstream enhancers (black ovals), allowing them to access the *H19* promoter. On the paternal allele, CpG methylation prevents CTCF binding at the ICR (black circles), and together with *H19* promoter methylation (grey circles) allows the access of enhancers to *Igf2*. Diamonds represent the paternal methylation at secondary DMRs (DMR1 and DMR2), which occurs after the fertilization. **(b)** The ncRNA mediated imprinting of *Igf2r/Airn* locus. The maternally expressed *Slc22a3, Slc22a2, Igf2r*, the paternally expressed ncRNA *Airn* and non-imprinted *Mas1* and *Slc22a1* are indicated in the figure. The ICR includes the *Airn* promoter, and is hypermethylated on the maternal allele (black circles). The hypomethylated ICR (open circles) allows *Airn* expression on the paternal allele, which represses *Slc222a2, Slc22a3* and *Igf2r* in cis. The *Igf2r* promoter harbors a secondary paternally methylated DMR (diamonds) whose methylation occurs after transcription through the region. The loci are not drawn to scale. The maternal alleles are shown above the line whereas the paternal alleles are below the line (Adapted from Abramowitz and Bartolomei, 2012).

The relation between *Igf2/H19* and *Igf2r/Airn* clusters

The conflict theory predicts that fetal growth is suppressed by maternally expressed genes transferred to offspring, while the fetal growth is being supported by paternally expressed

genes in the offspring. The mouse *Igf2* and *Igf2r* genes represent a nice paradigm supporting this theory. The paternally expressed *Igf2* gene encodes a potent growth enhancer, whereas the maternally expressed *Igf2r* gene encodes a transmembrane protein receptor involved in growth suppression. This receptor acts to sequester and internalize excess circulating IGF-II for lysosomal degradation (Berkowicz et al., 2012; Reviewed in Ishida and Moore, 2012; Scott and Weiss, 2000). In support of this intricate balance, mouse knockouts of these genes yielded in opposite growth phenotypes. In other words, *Igf2*-null mice are growth retarded as opposed to *Igf2r*-null mice showing overgrowth phenotypes (Reviewed in Ishida and Moore, 2012; Lau et al., 1994). In addition, mice deficient for the paternally expressed *Peg3* and *Mest* genes show an intrauterine growth restriction (IUGR) phenotype (Reviewed in Ishida and Moore, 2012; Lefebvre et al., 1998; Li et al., 1999), while mice harboring a homozygous (or maternally inherited) null mutation for maternally expressed *H19* and *Grb10* genes showed fetal overgrowth (Charalambous et al., 2003; Reviewed in Ishida and Moore, 2012; Leighton et al., 1995). These findings are also consistent with the conflict theory. Hence, it is argued in most cases that paternally expressed genes promote fetal growth while maternally expressed genes restrict fetal growth (Reviewed in Ishida and Moore, 2012).

6.4. Similarities and Differences in Imprinted Clusters

Imprinted clusters have common features as well as differences in parent of origin specific activation and repression of imprinted genes. The first well-known common property is the presence of a germline derived differential methylation generated by the *de novo* methyl transferase Dnmt3a, cooperating with Dnmt3l (Reviewed in Edwards and Ferguson-Smith, 2007).

Generally, when the DNA methylation is established on the maternally inherited chromosome, this methylation is found in a region which acts to repress a promoter for an antisense transcript. These antisense transcripts normally have an important role in repression of protein-coding genes in cis in the same imprinted cluster. It is generally the maternally methylated ICR which is associated with the expression of protein coding genes, except for the PWS/AS cluster where there are several paternally expressed protein coding genes (Reviewed in Edwards and Ferguson-Smith, 2007). However, the PWS/AS cluster has a complex evolutionary origin since there are also few protein coding genes expressed from the maternal alleles (Reviewed in Edwards and Ferguson-Smith, 2007; Rapkins et al., 2006).

In case of a paternally established DNA methylation at the ICR, the protein-coding genes are expressed from the paternally inherited chromosome. Unlike the maternally methylated ICRs, the ICR in this case is not a promoter for ncRNAs. These ncRNAs may not be involved in imprinting control. Perhaps, they only arose as a consequence of imprinting, and evolved functions as in the case of microRNAs at the *Dlk1–Dio3* cluster (Reviewed in Edwards and Ferguson-Smith, 2007).

Currently, how and why the epigenetic machinery active in the two parental germlines direct DNA methylation to different places at these particular loci is unknown. Interestingly, paternally methylated ICRs have fewer CpGs than maternal ICRs according to recent sequence analysis of ICRs (Reviewed in Edwards and Ferguson-Smith, 2007; Kobayashi et

al., 2006). Moreover, methylation imprints in the maternal germline appear to be more specific to sequences with promoter activity of regulatory ncRNAs (Reviewed in Edwards and Ferguson-Smith, 2007). Different mechanisms may have evolved in the male and female germlines, possibly recognizing retrotransposed genes or retrotransposons and silencing them, but this may only happen efficiently in one germline. Nevertheless, further investigation is necessary to understand the specificity and differences in paternal and maternal mechanisms.

7. DISEASES ASSOCIATED WITH IMPRINTING

In humans, the importance of imprinting is demonstrated by imprinting disorders, which are very rare, but often severe; indicating that the small subset of imprinted genes are indispensable for proper human development (Ishida and Moore, 2012).

Imprinting disorders are caused by abnormalities ranging from chromosomal and genetic disruptions to epigenetic disruptions. More crucially, one disorder may result from a failure of several molecular mechanisms. In the case of chromosomal and genetic abnormalities (i.e. uniparental disomies, chromosomal deletion, duplication, translocation, inversion, point mutation), the dosage of the imprinted gene is altered. Epigenetic abnormalities mostly refer to aberrant hypermethylation or hypomethylation at ICRs (Tomizawa and Sasaki, 2012); however, other epigenetic players described earlier may also contribute to the disorders. Imprinting disorders are mainly associated with growth-related problems and developmental abnormalities especially in fetal growth and neurological behavior due to the fact that imprinted genes are mainly involved in growth and development (Butler, 2009; Reviewed in Ishida and Moore, 2012; and Tomizawa and Sasaki, 2012).

Aberrant expression of imprinted genes has a causative role in the following disorders: Beckwith-Wiedemann Syndrome, Prader-Willi Syndrome, Angelman Syndrome, Silver-Russell Syndrome, Transient Neonatal Diabetes Mellitus, Pseudohypoparathyroidism Type 1b, Familial Nonchromaffin Paraganglioma, and Maternal and Paternal UPD14 Syndromes. In addition to these syndromes for which the causative genes are known, there are also other parent-of-origin specific syndromes, which may involve imprinted genes (Reviewed in Morison et al., 2005). Moreover, some complex genetic diseases have indicated parent-of-origin specific effects in linkage studies, but further confirmation is required in these linkage studies. All these disease cases mentioned have been listed in **Table 1** (Reviewed in Colhoun et al., 2003; Morison et al., 2005).

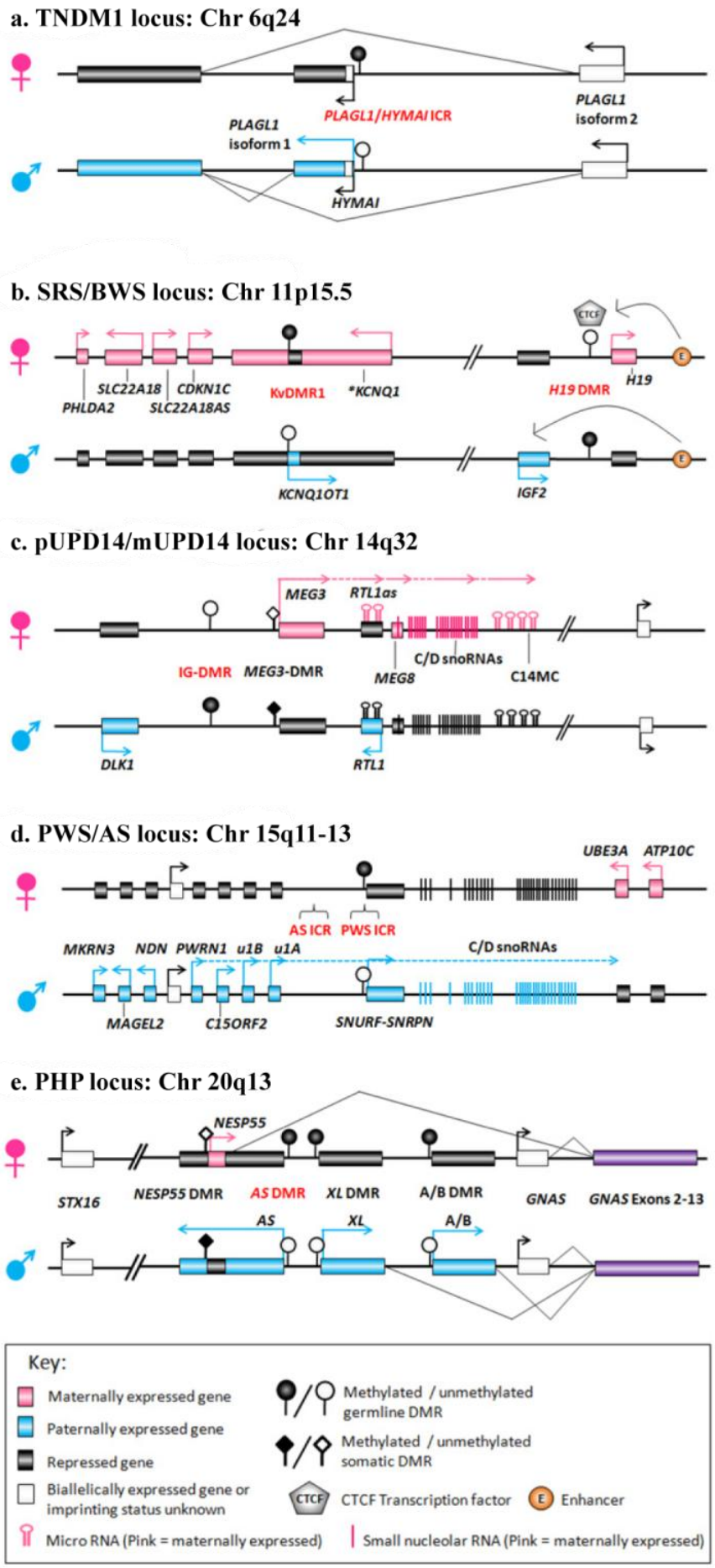
a. TNDM1 locus: Chr 6q24
PLAGL1/HYMAI ICR
PLAGL1 isoform 2
PLAGL1 isoform 1
HYMAI
b. SRS/BWS locus: Chr 11p15.5
CTCF
PHLDA2
SLC22A18
SLC22A18AS
CDKN1C
KvDMR1
*KCNQ1
H19 DMR
H19
KCNQ1OT1
IGF2
c. pUPD14/mUPD14 locus: Chr 14q32
MEG3
RTL1as
IG-DMR
MEG3-DMR
MEG8
C/D snoRNAs
C14MC
DLK1
RTL1
d. PWS/AS locus: Chr 15q11-13
UBE3A
ATP10C
AS ICR
PWS ICR
MKRN3
NDN
PWRN1
u1B
u1A
C/D snoRNAs
MAGEL2
C15ORF2
SNURF-SNRPN
e. PHP locus: Chr 20q13
NESP55
STX16
NESP55 DMR
AS DMR
XL DMR
A/B DMR
GNAS
GNAS Exons 2-13
AS
XL
A/B
Key:
Maternally expressed gene
Paternally expressed gene
Repressed gene
Biallelically expressed gene or imprinting status unknown
Methylated / unmethylated germline DMR
Methylated / unmethylated somatic DMR
CTCF Transcription factor
Enhancer
Micro RNA (Pink = maternally expressed)
Small nucleolar RNA (Pink = maternally expressed)

Figure 4. Human imprinted gene clusters associated with imprinting disorders. Schematic representations of the human imprinted clusters related to imprinting disorders **(a-e)**. DMRs indicated in red are the ICRs of each cluster, identified by targeted deletion in mice. **(a)** *PLAGL1* imprinted cluster is associated with transient neonatal diabetes mellitus 1 (TNDM1). *PLAGL1* isoform 1 is imprinted and expressed from the paternal allele that is unmethylated. **(b)** *KCNQ1* (potassium voltage-gated channel, KQT-like subfamily, member 1) and *IGF2/H19* imprinted domains are implicated in Silver–Russell syndrome (SRS) and Beckwith–Wiedemann syndrome (BWS). *KCNQ1* shows polymorphic maternal expression in term placenta. KvDMR1 (potassium voltage-gated channel differentially methylated region 1) functions dually as an ICR for the *KCNQ1* cluster and a promoter for the paternally expressed ncRNA *KCNQ1OT1* (*KCNQ1* overlapping transcript 1). The CTCF protein binds to the unmethylated *H19* DMR which blocks the access of *IGF2* promoters to the enhancers and activates *H19* expression. The methylation of *H19* DMR prevents the CTCF from binding, enabling the enhancers to interact with *IGF2* promoters. **(c)** The *DLK1-DIO3* locus is associated with paternal and maternal uniparental disomy 14 syndromes (pUPD14/mUPD14). Dotted lines represent the possible extension of the transcripts. **(d)** The *SNRPN* locus is implicated in Prader–Willi syndrome (PWS) and Angelman syndrome (AS). **(e)** The *GNAS* cluster is associated with Pseudohypoparathyroidism (PHP). The first exons of *GNAS, A/B, GNASXL* and *NESP55* are all spliced onto the *GNAS* downstream exons 2–13 in order to form different transcripts. Figures are not drawn to scale. The direction of transcription is indicated with colored arrows; black for biallelic, blue for paternal, and pink for maternal specific expression. Left side of the figure indicates centromeric orientation while right is for telomeric orientation (Adapted from Ishida and Moore, 2012).

Syndromes Involving Imprinted Genes

Abnormalities in the expression of imprinted genes have causative role in syndromes as mentioned before. In this section, some of the well-characterized syndromes will be described.

1. Prader–Willi Syndrome (PWS) and Angelman Syndrome (AS)

Prader-Willi syndrome (PWS) and Angelman syndrome (AS) were the first human imprinting disorders reported. Both PWS and AS are neurological diseases, each observed with an estimated frequency of one in 15,000 to 25,000 live births (Buiting, 2010; Reviewed in Ishida and Moore, 2012).

The clinical phenotypes of PWS include mild intellectual disability, low birth weight, poor suckling and hypotonia before weaning, which are followed by voracious appetite leading to obesity after weaning. About 70% of PWS patients have a *de novo* interstitial deletion on the paternal chromosome 15q11-13, approximately 25-30% show maternal uniparental disomy (UPD) of chromosome 15 (mUPD15) (Buiting, 2010; Reviewed in Ishida and Moore, 2012), about 1–3% of the cases occurs due to epimutations resulting in abnormal imprinting, and a small fraction of PWS patients carry microdeletion, indicating a bipartite ICR (Reviewed in Ishida and Moore, 2012; and Tomizawa and Sasaki, 2012). So far, it is unclear which genes in this region contribute to PWS. Nevertheless, paternally expressed *SNORD116* snoRNAs residing within the *SNURF/SNRPN* locus (see **Figure 4d**) have been suggested to play a role in the etiology of PWS (Reviewed in Ishida and Moore, 2012).

AS is characterized by severe intellectual disability, microcephaly, delayed weaning by prolonged suckling period, and frequent laughter and smiling (Buiting, 2010; Reviewed in Ishida and Moore, 2012). AS patients fail to inherit a normal active maternal copy of ubiquitin protein ligase E3A (UBE3A) (Lalande and Calciano, 2007). Approximately 70% of AS

patients retain a chromosomal deletion on the maternal chromosome 15q11-13 (see **Figure 4d**), about 2–5% have paternal uniparental disomy of chromosome 15 (pUPD15), and around 10% have a mutation in the maternally expressed imprinted gene, *UBE3A* (Buiting, 2010; Reviewed in Ishida and Moore, 2012). In addition, epimutations are observed with a frequency of 2-4% of AS patients, and microdeletion is observed in small part of AS patients, showing a bipartite ICR (Reviewed in Ishida and Moore, 2012).

Most of PWS (85%) and AS (92%) patients with an imprinting defect represent primary epimutations. Such defective imprinting has been suggested to occur during maternal imprint establishment or maintenance in AS patients, while it is associated with failure in imprint erasure in the paternal germline in PWS patients (Buiting, 2010; Reviewed in Ishida and Moore, 2012).

2. Silver–Russell Syndrome (SRS)

In Silver-Russell syndrome (SRS), prenatal and postnatal growth retardation has been observed with heterogeneous clinical features (Reviewed in Ishida and Moore, 2012; and Tomizawa and Sasaki, 2012). SRS is observed in about 1 in 3000 to 100,000 people. SRS shows heterogeneity in terms of genetic and epigenetic causes of the disease. Maternal uniparental disomy of chromosome 7 (mUPD7) is found in 10% of SRS patients while hypo-methylation of the paternally methylated *H19* ICR at chromosome 11p15.5 is detected in around 35–65% of the patients. Furthermore, SRS patients with *H19* ICR hypomethylation have been reported to display hypomethylation and hypermethylation at other imprinted loci (so-called multilocus methylation defects, MLMD), suggesting that there may be a more general cause for the disease involving imprint establishment or maintenance (Reviewed in Ishida and Moore, 2012). Most of SRS patients with hypomethylation at multiple maternally methylated ICRs also show hypomethylation on the paternally methylated ICR of the *DLK1-MEG3* locus (IG-DMR, see **Figure 4b**). This suggests that post-fertilization maintenance has probably been affected (Reviewed in Tomizawa and Sasaki, 2012). Although two candidate regions within chromosome 7 (7q32 containing *MEST* and 7p12.2-3 containing *GRB10*) have been linked to SRS (Reviewed in Ishida and Moore, 2012), more conclusive experiments are required to identify the genes responsible for this disease (Reviewed in Tomizawa and Sasaki, 2012).

3. Beckwith–Wiedemann Syndrome (BWS)

Beckwith-Wiedemann syndrome (BWS) is characterized by macrosomia, macroglossia, organomegaly, omphalocele, neonatal hypoglycemia, ear creases or ear pits, and predisposition to childhood tumor (Reviewed in Tomizawa and Sasaki, 2012). BWS is phenotypically and genotypically opposite to SRS, and observed in approximately one in 13,700 live births. The chromosome region 11p15.5 is also responsible for BWS, and deregulation of this region holds for 85% cases of BWS (Reviewed in Ishida and Moore, 2012). The region contains a large cluster of imprinted genes regulated by the maternally methylated *KCNQ1OT1* ICR (also known as KvDMR1), and the paternally methylated *H19* ICR as shown in **Figure 4b** (Reviewed in Tomizawa and Sasaki, 2012). About 50% of BWS patients show hypomethylation of the maternal *KCNQ1OT1* ICR, and 5% are associated with

hypermethylation at the maternal *H19* ICR, possibly resulting in reduced *H19* expression and as a consequence increased *IGF2* expression. Approximately 22% of BWS cases with a hypomethylated *KCNQ1OT1* ICR have been shown to exhibit multilocus methylation defects (MLMD). In addition, 10% of BWS patients have been reported to have mutations in *CDKN1C* (cyclin-dependent kinase inhibitor 1C) gene in *KCNQ1* domain. Although most BWS cases are sporadic, about 15% are familial cases of which 40% are associated with a *CDKN1C* mutation. Approximately 20% of BWS patients have pUPD involving both *IGF2/H19* and *KCNQ1* clusters while chromosomal rearrangements occur less than 1% at the 11p15 locus (Reviewed in Ishida and Moore, 2012). Furthermore, *NLRP2* (NLR family, pyrin domain containing 2) gene has been suggested to act in trans to establish or maintain imprints at *KCNQ1OT1* ICR as the mother of two BWS affected siblings with hypomethylated *KCNQ1OT1* ICR was shown to carry a homozygous mutation at *NLRP2* (Reviewed in Ishida and Moore, 2012; Meyer et al., 2009; Reviewed in Tomizawa and Sasaki, 2012). Briefly, BWS is caused by epigenetic alterations or genomic imbalances at the chromosome 11p15.5 region which contains multiple imprinted genes divided into two domains; one domain containing *IGF2* and *H19*, and the other domain harboring three imprinted genes known as *CDKN1C*, *KCNQ1*, and *KCNQ1OT1* (Chen, 2012).

4. Paternal Uniparental Disomy 14 (pUPD14) and Maternal Uniparental Disomy 14 (mUPD14)

Maternal uniparental disomy 14 (mUPD14) is characterized by a prenatal and postnatal growth restriction, premature puberty and obesity. Clinical characteristics of paternal uniparental disomy 14 (pUPD14) are facial anomaly, small bell-shaped thorax, abdominal wall defects, placentomegaly (enlarged placenta) and polyhydramnios (excessive amniotic fluid) (Reviewed in Ishida and Moore, 2012). Both syndromes are associated with deregulation of the genes within the *DLK1-MEG3* imprinting cluster located on chromosome 14q32 (see **Figure 4c**). This cluster has two well characterized paternally methylated DMRs; the germline-derived primary *DLK1-MEG3* intergenic IG-DMR, and the postfertilization derived secondary *MEG3*-DMR (Reviewed in Ishida and Moore, 2012; and Kagami et al., 2010). Patients having mUPD14 and pUPD14 phenotypes have been reported to harbor epimutations and microdeletions at 14q32 on the paternal and maternal chromosomes, respectively (Reviewed in Ishida and Moore, 2012; Kagami et al., 2010; Kagami et al., 2008). In addition, pUPD14 patients having microdeletions of the IG-DMR have affected body and placenta weights while pUDP14 patients having *MEG3*-DMR microdeletion have an affected body weight only. These observations suggested that the IG-DMR and *MEG3*-DMR may function as the placenta and the body ICRs, respectively. It is also known that the methylation status of the *MEG3*-DMR in the body is controlled by that of IG-DMR. The hypomethylated *MEG3*-DMR acts as an essential imprinting regulator for both *PEGs* (protein-coding paternally expressed genes such as *DLK1* and *RTL1*) and *MEGs* (non-coding maternally expressed genes such as *MEG3*, *RTL1as*, *MEG8*, snoRNAs, and miRNAs) in the body, while the hypomethylated IG-DMR controls the imprinting pattern of *PEGs* and *MEGs* in the placenta. Interestingly, this is the first study showing an important long-range imprinting regulatory function for secondary DMR (Reviewed in Ishida and Moore, 2012; Kagami et al.,

2010). It has also been proposed that excessive *RTL1* expression and decreased *DLK1* and *RTL1* expression may play an important role in the development of pUPD14-like and mUPD14-like phenotypes, respectively. Nevertheless, the exact regulation of this cluster and biological functions of most *MEGs* remains to be elucidated (Ogata et al., 2008), which will enable better understanding of pUPD14 and mUPD14.

5. Transient Neonatal Diabetes Mellitus Type 1 (TNDM1)

Transient neonatal diabetes mellitus type 1 (TNDM1) is a rare type of diabetes characterized by severe intrauterine growth restriction (IUGR), and hyperglycemia that is manifested within the first 6 months of life. This is caused by over-expression of paternally expressed genes including *PLAGL1* and *HYMAI* located within the chromosome 6q24 region as depicted in **Figure 4a** (Reviewed in Ishida and Moore, 2012; and Tomizawa and Sasaki, 2012). Paternal uniparental disomy 6 (pUPD6) accounts for about 40% TNDM1 while paternal duplication of 6q24 is responsible for 32 % of TNDM1 cases (Reviewed in Ishida and Moore, 2012). About 20-30 % of TNDM1 patients show hypomethylation at normally maternally methylated *PLAGL1/HYMAI* ICR. *PLAGL1* has a biallelically expressed upstream promoter, and this major promoter is not imprinted. In addition, the ICR overlaps with another promoter shared by *PLAGL1* and *HYMAI* genes, from which the expression of both genes is monoallelic in normal individuals, but biallelic in the case of TNDM1. The major candidate of TNDM1 is *PLAGL1* gene, whose product is a zinc-finger transcriptional factor with activation or repression activity, depending on its binding partners. For instance, it has also been shown to be a co-activator of p53, which may account for its activity in cell-cycle arrest and induction of apoptosis. Given the presence of a strong nonimprinted promoter of *PLAGL1*, it has been hypothesized that the neonatal presentation of TNDM1 is associated with fetal overexpression of *PLAGL1* from its imprinted promoter, in a tissue and stage at which nonimprinted promoter is not used. In addition to *PLAGL1*, *HYMA1* is a non-coding RNA ubiquitously expressed in the same orientation as *PLAGL1*. Although the function of *HYMA1* is not clear yet, it is expected to essential for imprinted expression of *PLAGL1*. More than half of the patients with hypomethylated *PLAGL1/HYMAI* ICR also display hypo-methylation at other imprinted loci, some of which was recently found to be associated with mutations in *ZFP57* gene which is involved in post-fertilization maintenance of imprints in mice (Reviewed in Ishida and Moore, 2012; Mackay and Temple, 2010; and Tomizawa and Sasaki, 2012).

6. Pseudohypoparathyroidism Type Ib (PHP-Ib)

Pseudohypoparathyroidism type Ib (PHP-Ib) is an endocrine disorder characterized by renal resistance to parathyroid hormone (PTH), leading to hypocalcemia and hyperphosphatemia. There are both familial (autosomal dominant, AD-PHP-Ib), and sporadic forms of PHP-Ib. PHP-Ib is mostly associated with epigenetic defects in the *GNAS* cluster located on chromosome 20q13, which is shown in **Figure 4e** (Reviewed in Ishida and Moore, 2012; and Tomizawa and Sasaki, 2012). Hypomethylation is mainly observed at the maternal germline methylated *GNAS* exon A/B ICR, and sometimes additionally at the *GNAS XL*, *AS* (*NESP* antisense, known as *Nespas* in mice) and *NESP55* ICRs. On one side in AD-PHP-Ib patients, maternal microdeletions disrupt the non-imprinted *STX16* gene, resulting in hypomethylation

mostly confined to A/B ICR. Moreover, maternal microdeletions overlapping *NESP55* and *AS*, *AS* only and *NESP55* only, have also been reported to cause hypomethylation at A/B DMR, which is indicative of additional control regions for A/B ICR. In addition to A/B ICR, the former two deletions have been demonstrated to result in hypomethylation of other *GNAS* ICRs (*AS* and *XL*), indicating an important region for the control of the whole maternal *GNAS* allele (Reviewed in Ishida and Moore, 2012). Furthermore, another study indicated maternally transmitted deletions of an upstream exon of the *NESP55* transcript in two families, which were associated with methylation defects at all downstream *GNAS* ICRs (AS, XL and A/B). Based on mouse studies at *Gnas* locus, the absence of *NESP55* transcription leads to a failure in imprint establishment (Reviewed in Tomizawa and Sasaki, 2012). In mice, *Nespas* ICR has been shown to be the main ICR of this cluster. Nevertheless, in sporadic PHP-Ib patients, methylation defects have been observed at multiple *GNAS* ICRs, but the underlying mechanism is currently unknown (Reviewed in Ishida and Moore, 2012). Interestingly, there has been one case reported, in which pUPD20q has been found in a patient having similar features to PHP-Ib (Bastepe et al., 2001; Reviewed in Ishida and Moore, 2012).

Table 1. Human phenotypes associated with parent-of-origin effects

Phenotype	Chromosomal location	Gene locus for ICR with methylation defects	Candidate factor involved in methylation defects
Syndromes associated with imprinted genes			
PWS/AS	15q11-q12	*SNRPN*	Genomic deletion of AS-IC (AS) in PWS/AS locus
SRS	7p11-p13, 7q31-qter	*PLAGL1/HYMAI, IGF2R, MEST, KCNQ1OT1, SNRPN, H19, DLK1-MEG3* (IG-DMR)	*MEST* and *GRB10**
BWS	11p15	*PLAGL1/HYMAI, IGF2R, MEST, GRB10, KCNQ1OT1, SNRPN, PEG3, GNAS* (AS and A/B ICRs), *H19*	NLRP2
mUPD14 and pUPD14	14	*DLK1-MEG3* (IG-DMR and *MEG3*-DMR)*	
TNDM1	6q24	*PLAGL1/HYMAI, MEST, GRB10, KCNQ1OT1, PEG3, GNAS* (AS ICR)	ZFP57
PHP-1b	20q13	*GNAS* (AS, XL and A/B ICRs)	Genomic deletion of *NESP55* or *STX16*
Familial nonchromaffin paraganglioma	11q13		
Syndromes that are probably associated with imprinted genes			
Turner syndrome phenotypes	X		
Familial pre-eclampsia	10q22		
Maternal UPD2 syndrome	2		
Maternal UPD16 syndrome	16		
Complex genetic diseases with parent-of-origin effects			
Asthma, atopy	4q35, 11q13, 16q24, 16p12		
Autism	7q22-q31, 15q11-q13		
Hirschsprung disease	10q11		
Cornelia de Lange syndrome	3q26, 5p13		
Psoriasis	6p, 16q		
Handedness	2p12-q11		
Type I diabetes	6p21, 6q25-q27, 10p11-q11, 16q		
Type II diabetes	5p, 12q, 18p11		
Alcoholism	1, 2, 4, 8, 9, 16,		
Alzheimer disease	10q, 12q		
Bipolar affective disorder	1q, 2p, 2q, 6q, 13q, 14q, 16q, 18q		
Schizophrenia	2p12-q11, 22q12		

Table 1 is adapted from the following references (Morison et al., 2005; Tomizawa and Sasaki, 2012) Abbreviations: PWS/AS, Prader–Willi syndrome/Angelman syndrome; SRS, Silver–Russell syndrome; BWS, Beckwith–Wiedemann syndrome; mUPD14 and pUPD14, Maternal uniparental disomy of chromosome 14 and paternal uniparental disomy of chromosome 14; TNDM1, Transient neonatal diabetes mellitus type 1; PHP-1b, Pseudohypoparathyroidism type Ib; UPD, uniparental disomy. '*' indicated that the information has been reviewed in a different reference (Ishida and Moore, 2012).

8. THE RELEVANCE OF GENOMIC IMPRINTING IN CLONING, INDUCED PLURIPOTENT CELLS, AND ASSISTED REPRODUCTIVE TECHNOLOGY

8.1. Cloning and Induced Pluripotent Stem Cells (iPSCs)

Over the last few decades, different technologies have been applied to reprogram somatic cell nuclei of differentiated states into a pluripotent state. Reprogramming was either achieved by cell fusion technology, nuclear transfer or by using defined factors (Campbell et al., 1996; Reviewed in Li and Sasaki, 2011; Takahashi and Yamanaka, 2006). In this process, parental origin specific imprints need to be maintained, while other genes related to pluripotency, development, and tissue specificity need to be reprogrammed (Reviewed in Li and Sasaki, 2011).

Reprogramming by somatic cell nuclear transfer (SCNT) of a somatic cell which has lost cues for parental specific gene expression will affect the developmental capacity of mammalian clones. SCNT involves the transfer of a nucleus from an adult somatic cell into an enucleated donor egg, which is then triggered to start the cleavage divisions followed by a transfer into a surrogate uterus. Unfortunately, the success rate is low owing to large number of deaths during pre- and post-implantation development (Reviewed in Biliya and Bulla, 2010). Post-implantation developmental problems of SCNT embryos are attributed to problems related to genomic imprinting (Wei et al., 2011). Normally, the fertilized egg goes through a reprogramming round which involves global demethylation and other epigenetic changes (Reviewed in Biliya and Bulla, 2010). Interestingly, in cloned embryos, initiation of active demethylation is observed in the male gamete while passive demethylation is observed in the female gamete after fertilization as similar to normal embryos, but unlike normal embryos, further passive demethylation is minimal or lacking completely after the two-cell stage (Reviewed in Biliya and Bulla, 2010; Dean et al., 2001). Moreover, the cells of cloned embryos undergo *de novo* methylation at an earlier stage (at 4-8 cell stages) than usual. As a consequence, the trophoblastic layer of blastocyst becomes abnormally hypermethylated, whereas this layer is expected to be eventually hypomethylated in normal embryos. Starting from the time when normal embryos are implanted in uterus, the methylation patterns are set in inner cell mass, while the trophectoderm remains hypomethylated. In contrast, the main abnormality of cloned embryos is that the trophectoderm and the inner cell mass do not differ much in their methylation status even starting from the 32-64 cell stage (Reviewed in Biliya and Bulla, 2010). This clearly indicates disturbances in the regular timing of events resulting in problems in epigenetic reprogramming and subsequent defects in extra-embryonic lineages, which can be detrimental to the development of the cloned embryo (Reviewed in Biliya and Bulla, 2010; Yang et al., 2007). In cattle, in embryos obtained through SCNT, *H19* biallelic expression is tightly associated with a severe demethylation of the paternal *H19* domain, suggesting that these abnormalities could be directly responsible for low percentage of live offspring (Suzuki et al., 2011). Moreover, in the placenta of deceased transgenic calves after SCNT, aberrant methylation patterns were observed for *H19* DMR (hypermethylation), *XIST* DMR (hypomethylation), and *IGF2R* ICR (hypomethylation). In contrast, no difference was

observed in the expression and DNA methylation status of imprinted genes examined (except for the DNA methylation level of *XIST* DMR) in live transgenic calves after SCNT, suggesting that only embryo's with a proper methylation profile survive embryonic development and are born (Su et al., 2011). Examination of gene expression profiles of mice obtained through SCNT indicates that even live born mice show major defects in parental specific expression of many imprinted genes (Humpherys et al., 2001). In addition to the abnormal methylation patterns observed in mice and cattle, also in cloned swine genomes differences in the methylation status has been observed in putative DMRs of imprinted genes. Since there are only few reports about the methylation analysis of cloned pig genome, DMRs identified in this study have been referred to as putative (Shen et al., 2012). Unfaithful maintenance of parental specific methylation profiles at the ICRs in cloned porcine embryos is significantly influenced by the loss of Dnmt1 in the maternal nucleus during SCNT (Wei et al., 2011). In addition, parental specific methylation profiles tend to be unstable and are lost stochastically during aging of the animal. In order to apply cloning as a safe reproductive tool, loss of epigenetic information should be prevented by using the right donor material and by controlling the timing of events during fertilization and embryonic development.

Induced pluripotent stem cells (iPSCs) are obtained by forced expression of a different combination of stem cell specific transcription factors in somatic cells, thereby overruling the somatic cell specific program. IPSCs strongly resemble ES cells and have been used to generate mice underscoring the developmental potential of these cells. In SCNT derived embryos the parental specific methylation profiles have to be maintained throughout the pre- and post-implantation development and need to survive the wave of epigenetic reprogramming after the start of the first cell division. In contrast, in iPSCs, this first round of epigenetic reprogramming is omitted, which may lead to fewer defects in imprinted gene expression in iPSC clones. (Reviewed in Li and Sasaki, 2011). Nevertheless, recent work showed that expression of genes of the imprinted *Dlk1-Dio3* cluster is often altered in iPSCs, and this miss-expression can be used as a marker for evaluating pluripotency. In the affected mouse iPSCs, imprinted genes such as *Gtl2* (also known as *Meg3*) were abnormally silenced due to aberrant methylation of the ICR within the *Dlk1-Dio3* cluster (Reviewed in Li and Sasaki, 2011; Stadtfeld et al., 2010; Reviewed in Tomizawa and Sasaki, 2012). This indicated the failure to maintain the unmethylated state at the maternally derived ICRs (Reviewed in Li and Sasaki, 2011). In addition to hypermethylation, hypoacetylation was also detected at the ICRs within this cluster in other iPSC clones (Reviewed in Li and Sasaki, 2011; Stadtfeld et al., 2010). Moreover, these iPSC clones with silencing of imprinted genes (i.e. *Gtl2, Rian* and *Mirg*) in *Dlk1-Dio3* cluster contributed poorly to chimeras, and failed to support the development of entirely iPSC derived mice (all-iPSC mice), whereas embryos derived from iPSC clones with normal expression of these genes developed well (Reviewed in Li and Sasaki, 2011; Stadtfeld et al., 2010; Reviewed in Tomizawa and Sasaki, 2012). Importantly, treatment of an iPSC clone that had silenced *Dlk1–Dio3* with a histone deacetylase inhibitor (valproic acid, VPA) reactivated the *Gtl2, Rian* and *Mirg* genes in this locus, resulting in a rescue of the phenotype and full-term development of all-iPSC mice (Stadtfeld et al., 2010). Likewise, in a recent study, reprogramming in the presence of ascorbic acid inhibited *Dlk1-Dio3* silencing by interfering with reprogramming factor (Oct-4, Sox2, Klf4, c-Myc)-induced

loss of H3K4 methylation at the maternal IG-DMR through an unknown mechanism. By this way, maintained H3K4 methylation prevented the recruitment of the *de novo* DNA methyltransferase Dnmt3a, which was required for *Dlk1-Dio3* hypermethylation. Hence, all-iPSC mice were generated from mature B cells (Stadtfeld et al., 2012). Both studies not only show the importance of culture conditions during iPSC generation, but also indicate the possibility to prevent the loss of *Dlk1–Dio3* imprinting. Abnormalities described for iPSCs with regard to the *Dlk1-Dio3* cluster were not observed in embryonic stem cells (ESCs). Currently, the exact reason of the aberrant the *Dlk1-Dio3* silencing in iPSCs is unknown; however, it is likely to result from the reprogramming procedure itself (Reviewed in Li and Sasaki, 2011; Stadtfeld et al., 2010). This is supported by the finding that abnormal silencing of the *Dlk1-Dio3* cluster is not frequent in mice obtained through SCNT, and suggests that the oocyte cytoplasm may contain a factor which protects the ICRs of this cluster from *de novo* DNA methylation (Reviewed in Li and Sasaki, 2011). Additionally, clone- and gene-specific aberrations in imprinted gene-expression were also reported for human iPSCs. In this case, various genes such as *H19* and *KCNQ1OT1* were affected (Reviewed in Li and Sasaki, 2011; Pick et al., 2009; Reviewed in Tomizawa and Sasaki, 2012).

These studies clearly demonstrate the susceptibility of imprinted genes for loss of parental specific epigenetic information during nuclear reprogramming and highlight the importance for maintaining this information to obtain proper iPSCs which can be used in many fields including reproductive medicine.

8.2. Assisted Reproductive Technology (ART)

Assisted reproductive technology (ART), including in vitro fertilization (IVF) and intra-cytoplasmic sperm injection (ICSI), has been related to an increased risk of developing imprinting disorders (Reviewed in Ishida and Moore, 2012). The link between imprinting disorders and ART (i.e. IVF and ICSI) has been reported to be confined to epi-mutations (Reviewed in Amor and Halliday, 2008; Eroglu and Layman, 2012; and Ishida and Moore, 2012). As ART is performed during the critical periods of epigenetic reprogramming, external factors may influence the proper regulation of epigenetic modifications (Reviewed in Ishida and Moore, 2012).

To obtain oocytes for ART, women are treated with hormones to stimulate ovulation. In mice and women, such hormone stimulation has been reported to result in methylation defects. Hypomethylation has been observed at *MEST* in humans and *Snrpn*, *Peg3* and *Kcnq1ot1* in mice. In addition, hypermethylation has been reported at *H19* both in humans and mice (Reviewed in Ishida and Moore, 2012; Market-Velker et al., 2010; Sato et al., 2007; Reviewed in Tomizawa and Sasaki, 2012). These defects may be related to the embryonic culture conditions which have been shown to influence methylation pattern in the case of *H19* ICR in mice (Doherty et al., 2000; Fauque et al., 2007; Reviewed in Ishida and Moore, 2012). On the other hand, the condition of infertility/subfertility itself could also be considered as an increased risk of epigenetic abnormality, since patients receiving ART may differ from the general population with respect to parental age and fertility (Doornbos et al., 2007; Reviewed in Ishida and Moore, 2012). Indeed, in sperm from infertile patients with deficiencies such as

oligospermia or azoospermia, abnormal methylation patterns at ICRs have been reported, and may explain or partially explain the reported defects in parental specific DNA methylation profiles (Reviewed in Ishida and Moore, 2012; Kobayashi et al., 2007; Marques et al., 2008; Minor et al., 2011; Reviewed in Tomizawa and Sasaki, 2012).

Among the imprinting disorders described in this thesis, BWS, AS, and SRS have been overrepresented in ART-conceived offspring (Reviewed in Ishida and Moore, 2012; and Tomizawa and Sasaki, 2012). In spite of these reports, there are also other studies in which no increased risk have been observed for these diseases (Reviewed in Tomizawa and Sasaki, 2012). Unfortunately, these studies are difficult to compare because of the variability of ART protocols and the rarity of imprinting disorders (Eroglu and Layman, 2012). Also, commercial ART clinics are less willing to participate in these studies, and for most clinics and hospitals proper follow up studies are not installed. Therefore, the ART associated risk of imprinting disorders remains to be established in new studies involving larger research groups (Reviewed in Ishida and Moore, 2012; and Tomizawa and Sasaki, 2012).

9. CONCLUSION

In this review, the current knowledge on the epigenetic players and mechanisms involved in imprint establishment, maintenance, and erasure has been summarized. In addition, imprinted genes and clusters in mammals, and the association of imprinting with human disorders as well as assisted reproductive technology (ART) or cell reprogramming have been described. Recent molecular studies highlighted the importance of other epigenetic modifications in addition to DNA methylation in imprinted gene expression in the mouse. Therefore, future studies in human should also extend the view to chromatin modifications in genomic imprinting. Among more than 100 imprinted genes identified in mice, about half were also found to be imprinted in human, and future studies are not predicted to result in enormous changes in the imprinted gene number based on the limited number of human and animal phenotypes with imprinting disorders. It should also be noted that the mechanisms applied to regulate different clusters of imprinted genes may differ in spite of the insulator model and ncRNA model described here. This is why complex regulatory patterns in imprinting clusters and new imprinted genes continue to be discovered. Both epigenetic and genetic disruptions cause imprinting disorders that are particularly associated with problems in growth and development. Also, the association of defects in imprinted gene expression has been described in the context of cell reprogramming and assisted reproductive technologies (ARTs). Although loss of some imprints may have been already present in the donor cell, susceptibility of imprinted genes to lose epigenetic marks during reprogramming should be overcome. In addition, ART associated risk of imprinting disorders remains to be established in future. All in all, understanding of the processes involved in genomic imprinting will help to explain imprinting associated abnormalities, provide more insight in gene regulation in general, and may lead to improved and safer technologies regarding ART and nuclear reprogramming.

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