

Epigenetics and imprinting in human disease

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ABSTRACT Most genes are expressed from both parental chromosomes; however, a small number of genes in mammals are imprinted and expressed in a parent-of-origin specific manner. These imprinted genes play an important role in embryonic and extraembryonic growth and development, as well as in a variety of processes after birth. Many imprinted genes are clustered in the genome with the establishment and maintenance of imprinted gene expression governed by complex epigenetic mechanisms. Dysregulation of these epigenetic mechanisms as well as genomic mutations at imprinted gene clusters can lead to human disease.

KEY WORDS: *genomic imprinting, DNA methylation, Beckwith-Wiedemann syndrome, Russell-Silver syndrome*

Introduction

Genes that are subject to genomic imprinting are expressed exclusively or predominately from a single parental chromosome (Bartolomei, 2009). Among animals, this curious phenomenon has been described only in mammals, although plants such as *Arabidopsis* have imprinted genes, and other organisms, including arthropods, exhibit parental-specific behavior of entire chromosomes. The murine genome contains ~150 imprinted genes, (a complete up-to-date list of imprinted genes can be found here: <http://www.mousebook.org/catalog.php?catalog=imprinting>; (Williamson *et al.*, 2014)). Importantly, imprinting is well-conserved across mammals, with many imprinted genes and most imprinting mechanisms conserved between mouse and human (Lee and Bartolomei, 2013).

Most imprinted genes are present in distinct clusters that are about 1 Mb in length and contain both maternally and paternally expressed genes (Fig. 1). In addition to protein-coding genes in these clusters, there are typically long noncoding RNAs (ncRNA), some of which regulate the imprinting of the nearby genes. Regulation of the clustered genes is coordinated through short DNA sequences called imprinting control regions (ICRs). All ICRs identified thus far are differentially methylated regions (DMRs) in which DNA is methylated on one parental allele. As described in more detail below, DNA methylation usually represses either a long ncRNA or an insulator, which mediates the imprinting across the locus.

A significant consequence of imprinting is that mammalian development requires genetic contributions from both a mother and a father (McGrath and Solter, 1984, Solter, 1988). In humans, uniparental conceptuses arise at a very low frequency and have distinct phenotypes. Embryos with two paternal genomes and no

maternal contribution (androgenotes) produce hydatidiform moles that are comprised of extraembryonic membranes while embryos with only maternal genomes (gynogenotes) result in ovarian teratomas that are comprised of embryonic cell types. Several live-born individuals have been reported with mosaic genome-wide paternal uniparental isodisomy (Gogiel *et al.*, 2013, Inbar-Feigenberg *et al.*, 2013, Kalish *et al.*, 2013). In these cases, in some cells the entire maternal haplotype is lost and the paternal haplotype is duplicated, resulting in paternal uniparental isodisomy for the entire genome. These individuals have a mixture of normal biparental lineage cells and paternal uniparental cells in each tissue. Most of these individuals had enlarged extraembryonic tissues and were large conceptuses (phenotype is described in more detail below).

Experimental manipulation in the mouse using nuclear transfer showed that embryos reconstructed from two maternal pronuclei (gynogenetic embryos) or two paternal pronuclei (androgenetic embryos) failed to survive; whereas embryos reconstructed from one maternal and one paternal pronucleus produced viable and fertile offspring (McGrath and Solter, 1984, Solter, 1998). Gynogenetic embryos at the time of death were once again defective in extraembryonic tissues that contribute to the placenta, whereas androgenetic embryos were defective in embryonic tissue. These

Abbreviations used in this paper: ART, assisted reproductive technologies; AS, Angelman syndrome; BWS, Beckwith-Wiedemann syndrome; CTCF, CCCTC-binding factor; DMR, differentially methylated region; DNMT, DNA methyltransferase; GOM, gain of methylation; IC1, imprinting control region 1; IC2, imprinting control region 2; ICR, imprinting control region; LOM, loss of methylation; ncRNA, noncoding RNA; PWS, Prader-Willi Syndrome; RSS, Russell-Silver syndrome; TET, ten-eleven translocation; UPD, uniparental disomy.

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outcomes led to the hypothesis that embryonic development requires imprinted genes expressed from the maternal genome, whereas the paternal genome expresses imprinted genes required for extraembryonic development (Barton *et al.*, 1984). However, subsequent identification of imprinted genes in the mouse did not confirm such a bias in the function of imprinted genes, suggesting a less simple explanation for uniparental developmental outcomes. In fact, while imprinted genes have a prominent role in embryonic growth and placental development, they also play central roles in postnatal energy homeostasis and behavior (Fig. 2). Nevertheless, numerous imprinted genes have been identified that are placenta-specific, suggesting independent requirements for imprinted genes in embryonic versus extraembryonic lineages.

In addition to the necessity of both parental complements for appropriate development, deletions or mutations in specific imprinted genes cause a number of human imprinting disorders (Table 1). For example, failure to express the paternal allele or maternal allele of genes within the *SNRPN* imprinted domain results in Prader-Willi Syndrome (PWS) and Angelman Syndrome (AS), respectively. Moreover, genetic or epigenetic abnormalities in the *H19/IGF2* or *KCNQ1* domains result in Beckwith-Wiedemann Syndrome (BWS) or Russell-Silver Syndrome (RSS), depending on which parental allele is affected.

Establishment and maintenance of imprints

The key to the imprinting of genes in clusters is the consistent parental-specific epigenetic marking of the ICR as well as the

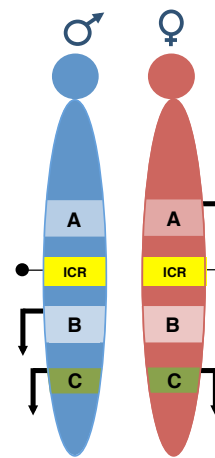


Fig. 1. Imprinted gene expression. Paternal (blue) and maternal (red) chromosomes are shown with expressed alleles indicated with an arrow. Methylation as designated by the filled circle at the imprinting control region (ICR, yellow box) leads to repression of gene A and expression of gene B from the paternal allele. Absence of methylation (open circle at ICR) on the maternal allele leads to expression of gene A and repression of gene B. Gene C is biallelically expressed.

subsequent maintenance of allele-specific epigenetic modifications. As described in more detail below, ICR deletions and aberrant allele-specific DNA methylation are associated with loss of imprinting of the linked genes in the clusters and, in the case of humans, imprinting disorders.

Although other epigenetic mechanisms such as post-translational histone modifications may also play a role in the parental-specific epigenetic mark, differential DNA methylation is the best recognized modification for conferring parental identity. DNA methyltransferases (DNMTs) have been demonstrated to have a role in both establishment and maintenance of DNA methylation based on mouse models where mutations in these genes lead to loss of ICR

TABLE 1

IMPRINTED GENE CLUSTERS IN HUMANS

Imprinted Gene Loci	Associated Imprinted ncRNAs	Other Associated Imprinted Genes	Germline DMRs	Imprinting Mechanism	Human Locus (Mouse Locus)	Association with Human Disease or Syndrome
<i>IGF2</i>	<i>H19</i>	<i>INS2</i>	H19 ICR (IC1 in humans)	CTCF-dependent insulator	11p15.5 (7qF5)	Beckwith-Wiedemann syndrome (BWS), Russell-Silver syndrome (RSS) ¹
<i>CDKN1C</i>	<i>KCNQ1OT1</i>	<i>KCNQ1</i> , <i>OSBP15</i> , <i>NAP114</i> , <i>PHLDA2</i> , <i>SLC22A18</i> , <i>MSUIT1</i> , <i>CD81</i> , <i>ASCL2</i> , <i>TSSC4</i>	KCNQ1OT1 promoter: KvDMR1 (IC2 in humans)	long ncRNA transcription	11p15.4 (7qF5)	Beckwith-Wiedemann syndrome (BWS) ¹
<i>SNRPN</i>	<i>UBE3AATS/snoRNAs</i>	<i>ATP10A</i> , <i>UBE3A</i> , <i>SNURF</i> , <i>NDN</i> , <i>MAGEL2</i> , <i>MKRN3</i> , <i>PEG12</i>	SNRPN exon 1 and putative transcription start site SNRPN promoter and exon 1: PWSIC (ICR)	long ncRNA transcription?	15q11.2 (7qB5)	Prader-Willi syndrome (PWS), Angelman syndrome (AS) ²
<i>ZAC (PLAGL1)</i>	<i>HYMAI</i>		HYMAI exon 1	ND	6q24.2 (10qA2)	Transient neonatal diabetes mellitus, neoplasia ³
<i>GNAS (NESP55)</i>	<i>GNAS</i> exon 1A/B, <i>GNAS (XLAS)</i> , <i>GNASAS</i> , <i>NESPAS</i>	<i>GNASX1</i> , <i>NESP</i>	GNASX1 and NESPAS promoter DMR (primary ICR), <i>GNAS</i> exon 1a promoter DMR (secondary ICR)	ND	20q13.32 (2qH4)	Cushing's syndrome, McCune-Albright syndrome, progressive osseous heteroplasia, pseudohypoparathyroidism (Ia, Ib, Ic), somatotroph adenoma, Albright hereditary osteodystrophy ⁴
<i>DLK1</i>	<i>MEG3 (GTL2)</i> , anti- <i>RTL1</i> microRNAs, <i>Rian</i> snoRNAs, <i>Mirg</i> microRNAs	<i>RTL1</i> , <i>DIO3</i>	GTL2 DMD/ICR Intergenic DMR: IG-DMR (ICR)	CTCF-dependent insulator ND	14q32.2 (12qF1)	UPD14, facial dysmorphisms, skeletal abnormalities ⁵
<i>PEG3</i>		<i>ZIM2</i> , <i>ZIM1</i> , <i>USP29</i> , <i>ZIM3</i> , <i>ZFP264</i>	PEG3 promoter and exon 1	ND	19q13.43 (7qA1)	Ovarian cancer, choriocarcinomas, oligodendrogliomas, hydatidiform moles ⁶
<i>PEG10</i>		<i>SGCE</i> , <i>PPP1R9A</i> , <i>ASB4</i>	PEG10 and SGCE promoter	ND	7q21.3 (6qA1)	Myoclonus-dystonia syndrome, hepatocellular carcinoma ⁷
<i>MEST (PEG1)</i>	<i>COPG2AS</i>	<i>COPG2</i> , <i>KLF14</i>	MEST-promoter exon 1	ND	7q32.2 (6qA3.3)	Russell-Silver syndrome (RSS)? ⁸
<i>RASGRF1</i>	<i>4930524O08Rik</i> (mouse)		-30 kb RASGRF1 DMR-Repeat (ICR)	CTCF-dependent insulator?	15q25.1 (9qE3.1)	alveolar rhabdomyosarcoma, myopia, epilepsy, gastric cancer ⁹
<i>GRB10</i>		<i>GRB10</i> brain isoform	GRB10 CpG Island 2	ND	7p12.2 (11qA1)	Russell-Silver syndrome (RSS)?, Albright's hereditary osteodystrophy, Hirschsprung disease, squamous cell cancers ¹⁰

¹ (Azzi *et al.*, 2014); ² (Cassidy *et al.*, 2012, Mabb *et al.*, 2011); ³ (Anolkar *et al.*, 2013, Docherty *et al.*, 2010, Iglesias-Platas *et al.*, 2013, Kamikihara *et al.*, 2005); ⁴ (Lecumberri *et al.*, 2010, Linglart *et al.*, 2013, Turan and Bastepe, 2013); ⁵ (Huang *et al.*, 2007, Jorgensen *et al.*, 2013, Kawakami *et al.*, 2006, Sutton and Shaffer, 2000); ⁶ (Dowdy *et al.*, 2005, Trouillard *et al.*, 2004, Van den Veyver *et al.*, 2001); ⁷ (Gao *et al.*, 2010, Tsou *et al.*, 2003); ⁸ (Kobayashi *et al.*, 1997, Lee and Bartolomei, 2013); ⁹ (Hysi *et al.*, 2010, Takamaru *et al.*, 2012, Tarnowski *et al.*, 2012, Zhu *et al.*, 2013); ¹⁰ (Angrist *et al.*, 1998, Arnaud *et al.*, 2003, Okino *et al.*, 2005).

methylation and biallelic expression of imprinted genes (Kaneda *et al.*, 2004). Through the use of the *de novo* DNA methyltransferases DNMT3A and DNMT3B and the accessory protein DNMT3L, ICRs and DMRs are specifically methylated in the male or female germline (Bartolomei and Ferguson-Smith, 2011). Curiously, most of these regions are methylated in the oocyte postnatally during oocyte growth prior to ovulation. In contrast, a few ICRs, including the *H19/Igf2* ICR, are methylated in the male germline prenatally.

The differential epigenetic modifications that are placed on the ICRs in the germline must be maintained following fertilization, despite the extensive reprogramming that takes place to prepare the genomes for embryonic development (Weaver *et al.*, 2009). Here, the paternal genome undergoes active demethylation, in part through the action of the ten-eleven translocation (TET) gene family member TET3, which converts 5-methylcytosine to 5-hydroxymethylcytosine (Gu *et al.*, 2011), while the maternal genome undergoes passive demethylation with the pattern being lost through multiple cell divisions.

One of the least understood aspects of imprinting is how ICRs maintain their differential methylation during the post-fertilization reprogramming period. It is likely that a combination of *cis*-acting sequences and *trans*-acting factors mediates the protection. One maternal factor, PGC7/STELLA, appears to have a general role in maintaining DNA methylation in the early mouse embryo through interactions with dimethylated histone 3, lysine 9 (Nakamura *et al.*, 2012). However, a factor that may be more specific for imprinted genes is ZFP57. Studies have demonstrated that *ZFP57* mutations identified in transient neonatal diabetes patients are associated with defects in DNA methylation at multiple imprinted loci (Mackay *et al.*, 2008). Additionally, *Zfp57* null mice exhibit embryonic lethality

and loss of imprinting at many (but not all) loci (Li *et al.*, 2008). It has recently been shown that ZFP57 binds to KAP1, which can then recruit other epigenetic regulators (Quenneville *et al.*, 2011). Thus, sequence- and DNA methylation-dependent binding of ZFP57, could act as an anchor to specify allelic binding of KAP1, which would subsequently recruit other major epigenetic regulators. It is possible that other yet-to-be-identified proteins also maintain DNA methylation at imprinted loci in the early embryo.

Intriguingly, the extraembryonic and embryonic tissues may use different mechanisms to maintain imprinting, as demonstrated by experiments assaying imprinted gene expression in mice that are deficient for the maintenance DNA methyltransferase, DNMT1 (Lewis *et al.*, 2004, Umlauf *et al.*, 2004, Weaver *et al.*, 2010). These experiments show that placenta-specific imprinted genes in the *Kcnq1* cluster, including *Osbpl5*, *Tssc4*, *Cd81*, and *Ascl2*, maintain imprinting in the absence of DNMT1. These genes are differentially marked by histone modifications in the placenta, with active histone modifications on the expressed maternal allele and repressive marks on the silent paternal allele (Lewis *et al.*, 2004, Umlauf *et al.*, 2004). These observations have led to the proposal that somatic DNMT1 is not required for maintenance of imprinting of these genes, which are instead regulated by post-translational histone modifications.

Regulation of imprinting in clusters

Two main regulatory mechanisms have been described for mediating imprinting in clusters (Bartolomei, 2009, Lee and Bartolomei, 2013). The first is the insulator model of imprinting, which is employed by the *H19/Igf2* imprinted locus (Fig. 3A). The maternally

Gene	Expressed Allele	Function of Gene Product	Role in Embryo
<i>IGF2</i>	Paternal	Positive regulator of general growth	Growth
<i>H19</i>	Maternal	Negative regulator of general growth	Suppression of growth
<i>IGF2R</i>	Maternal	Negative regulator of general growth	Suppression of growth
<i>GRB10</i>	Maternal	Negative regulator of general growth	Suppression of growth
<i>GRB10</i>	Paternal	Signal adaptor	Aggression
<i>UBE3A</i>	Maternal	Ubiquitin ligase & transcriptional co-activator	Memory, learning, motor function
<i>PEG3</i>	Paternal	Zinc finger protein; control of apoptosis	Sex-specific behavior
<i>NDN</i>	Paternal	Regulator of neuronal growth and differentiation	Spatial learning; socialization
<i>NESP</i>	Maternal	Secretory pathway	Exploratory behavior
<i>GNAS</i>	Maternal	Signal transduction	Cognition & sleep

Gene	Expressed Allele	Function of Gene Product	Role in Placenta
<i>PEG3</i>	Paternal	Zinc finger protein; control of apoptosis	Growth
<i>PEG1</i>	Paternal	Hydrolase	Growth
<i>MASH2</i>	Paternal	Helix-loop-helix transcription factor	Songiotrophoblast development
<i>PHLDA2</i>	Maternal	Pleckstrin homology domain protein	Songiotrophoblast restriction
<i>CDKN1C</i>	Maternal	Cell cycle regulator	Songiotrophoblast restriction
<i>SLC22A3</i>	Maternal	Cation transporter	Nutrient transfer

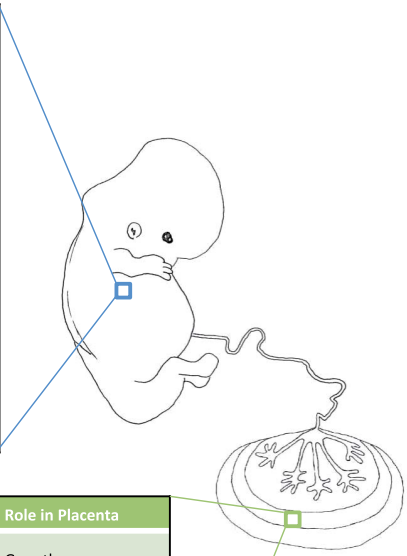


Fig. 2. Examples of the functions of imprinted genes. Shown is a non-exhaustive list of imprinted genes and their functions during development in the embryo (top panel) and the placenta (bottom panel).

expressed *H19* gene and paternally expressed *Igf2* gene share enhancers and their reciprocal imprinting is governed by the CCCTC binding factor (CTCF)-dependent insulator that is located between the genes. On the maternal allele in mouse, CTCF binds to 4 binding sites within the ICR, generating an insulator that prevents *Igf2* from accessing the shared enhancers that are located on the *H19* side of the insulator. On the paternal chromosome, methylation at the ICR prevents CTCF from binding, allowing *Igf2* to engage

the enhancers. DNA methylation also silences the *H19* promoter on the paternal allele. Note that this locus is similarly regulated in human, with the main difference being that the ICR (designated IC1) is larger and contains 7 CTCF sites.

A more commonly utilized mechanism of imprinting employs long ncRNAs. An example of such a locus is the *Kcnq1* locus (Fig. 3A), which encodes the paternally expressed long ncRNA, *Kcnq1ot1*. Regulation of this cluster also appears to be similar in

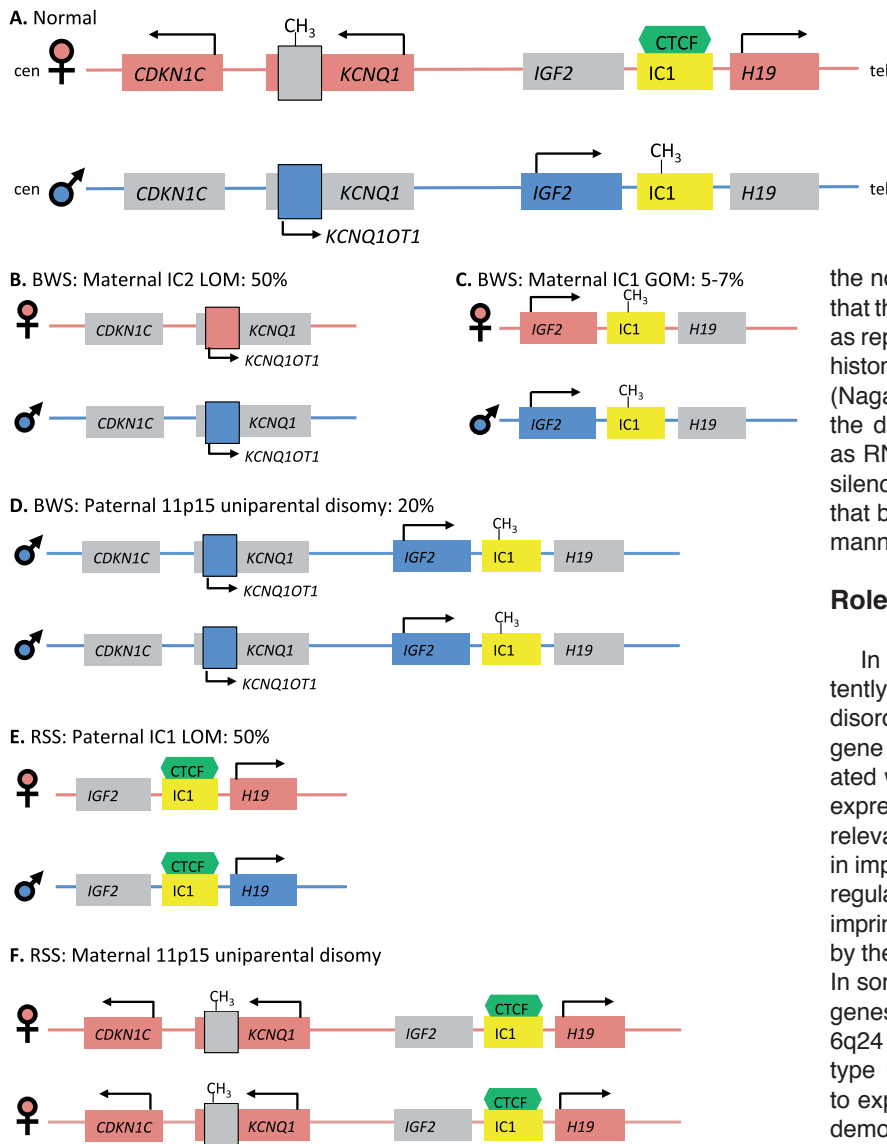


Fig. 3. Genetic and epigenetic alterations leading to Beckwith-Wiedemann syndrome (BWS) and Russell-Silver syndrome (RSS). (A) Normal imprinting and methylation at the 11p15 locus. (B) Hypomethylation at the human ICR (IC2) in the *KCNQ1* locus leading to BWS. (C) Hypermethylation at IC1 in the *H19/IGF2* locus leading to BWS. (D) Paternal uniparental disomy leading to BWS. (E) Hypomethylation at IC1 leading to RSS. (F) Maternal uniparental disomy leading to RSS. Additional alterations at this locus leading to BWS include maternally transmitted inactivating mutations in *CDKN1C*, paternally transmitted duplications of the whole region or of *IC1* alone, maternally transmitted microdeletions in *IC1*, and maternally transmitted deletions in *IC2*. RSS can also be due to activating mutations in *CDKN1C* or maternally transmitted duplications of the whole region or of *IC2* alone.

mouse and human, although the mechanism has been largely elucidated in mouse models. In this case, the ICR (designated KvDMR1 in mouse and IC2 in human) includes a differentially methylated promoter that regulates the expression of the ncRNA (*Kcnq1ot1*); when unmethylated, the ncRNA is expressed and represses *cis*-linked genes. In contrast, when the ICR is methylated, the ncRNA is repressed and the *cis*-linked genes are expressed. How the ncRNA silences genes *in cis* is unclear. One idea is that the ncRNA attracts repressive chromatin machinery, as reported for the interaction of the *Airn* ncRNA with the histone methyltransferase G9A at the *Igf2* imprinted locus (Nagano *et al.*, 2008). Alternatively, transcription through the domain, displacing transcriptional machinery such as RNA polymerase II, has likewise been suggested to silence genes *in cis* (Latos *et al.*, 2012). It is also possible that both mechanisms are used, but in a tissue-specific manner.

Role of imprinting in human disease

In humans, six imprinted regions have been consistently associated with disease. Many of these imprinting disorders cannot be explained by absence of a single gene product. In fact, the phenotypic diversity associated with each syndrome is consistent with absence of expression or mis-expression of multiple genes in the relevant region. Mis-expression can be due to mutations in imprinted genes, methylation defects at ICRs or other regulatory regions, or uniparental disomy (UPD), where an imprinted chromosomal region from one parent is replaced by the same chromosomal region from the other parent. In some cases, overexpression of paternally expressed genes leads to disease, for example, on chromosome 6q24 resulting in transient neonatal diabetes mellitus type I (Docherty *et al.*, 2010). Alternatively, the failure to express the maternal alleles can lead to disease, as demonstrated by chromosome 20q13.32 where loss of maternal gene expression causes pseudohypoparathyroidism (Lecumberri *et al.*, 2010). In some cases the causal change - overexpression or loss of expression is unclear as demonstrated by paternal uniparental disomy on chromosome 14q32, which leads to facial dysmorphism and skeletal findings including a bell-shaped thorax and “coat-hanger” ribs (Sutton and Shaffer, 2000). In still other cases, paternal mis-expression or maternal mis-expression at the same genetic locus can cause distinct disorders. Failure to express the maternal allele of *UBE3A* on chromosome 15q11.2 leads to Angelman syndrome,

characterized by ataxic movements, developmental delay, intellectual disability, and epilepsy (Mabb *et al.*, 2011). Conversely, failure to express the paternal alleles in the same region leads to Prader-Willi syndrome, characterized by hypotonia, mental retardation, short stature, hypogonadotropic hypogonadism, small hands and feet, and obesity (Cassidy *et al.*, 2012). Additionally, there are two imprinting disorders caused by genetic or epigenetic changes in the same region of chromosome 11 that result in opposite growth phenotypes (Fig. 3 B,F). Russell-Silver syndrome (RSS), an undergrowth disorder, is due to overexpression of maternal alleles and loss of paternal gene expression for chromosome 11p15.5. For the same region on 11p15.5, overexpression of paternal alleles and loss of maternal gene expression leads to Beckwith-Wiedemann syndrome (BWS), an overgrowth disorder. Of note, RSS can also be due to maternal UPD for chromosome 7.

As stated above, the alterations at loci that cause imprinting syndromes are diverse and include gain or loss of methylation at either the ICR or another DMR, uniparental disomy, mutation on the active allele, or disruption of regulatory sequences. Each of these changes alters expression of the maternally or paternally expressed imprinted genes. Here we will focus on two of these disorders, BWS and RSS, which are linked in some cases to the same imprinted region at 11p15.5. These disorders demonstrate the opposing effects of imprinted genes on fetal and extraembryonic growth and development.

Beckwith-Wiedemann syndrome and Russell-Silver syndrome

BWS is the most commonly identified imprinting disorder with a reported incidence of 1/13700 live births and equal incidence in males and females (Pettenati *et al.*, 1986). BWS is characterized by both fetal and extraembryonic overgrowth including macrosomia, macroglossia, visceromegaly, mesenchymal dysplasia, placentomegaly, and increased incidence of embryonic tumors (Choufani *et al.*, 2013). Overgrowth is seen during fetal and postnatal development. Wilms tumors and hepatoblastomas are the most common embryonic tumors seen, with an overall risk of about 7.5% (DeBaun and Tucker, 1998). RSS is characterized by severe pre- and postnatal growth retardation including short stature with a normal head size, a triangular face with prominent forehead, and skeletal/limb asymmetry (Azzi *et al.*, 2014). Most cases of BWS and RSS are due to genetic and/or epigenetic changes on chromosome 11p15.5 (Fig. 3). Several of the genes in this region are growth regulators and, depending on the nature of the imprinting aberration, lead to either BWS or RSS. Both BWS and RSS are recognized as a spectrum of disorders ranging from mild to severe disease, suggesting that, for some alterations, the changes occur only in a subset of cells.

Gain of methylation (GOM) at IC1 leads to overexpression of the growth factor *IGF2* and downregulation of *H19*, which encodes a ncRNA and microRNA implicated in growth suppression, with a developmental consequence of overgrowth (Azzi *et al.*, 2014). *IGF2* encodes a growth factor that is highly expressed in the fetus and placenta from the paternal allele (Monk *et al.*, 2006). Postnatally, *IGF2* is biallelically expressed from the liver via a different promoter (Monk *et al.*, 2006). *H19* is a maternally expressed ncRNA that is expressed in the endoderm and mesoderm of the embryo and throughout the placenta. After birth, *H19* is silenced in most

tissues, except in heart and skeletal muscle. *H19* is evolutionarily conserved and has been speculated to have a role in both tumor formation and tumor suppression (Yoshimizu *et al.*, 2008). Additionally, a microRNA, miR-675, has been identified within the first exon of *H19*. In mice, this microRNA demonstrates distinct expression patterns from *H19* and is speculated to play a role in placental and postnatal growth (Keniry *et al.*, 2012). About 10% of BWS patients have GOM at IC1 and they have an increased risk of developing embryonic tumors (Choufani *et al.*, 2013). Loss of methylation (LOM) at IC1, with downregulation of *IGF2* expression and *H19* overexpression (i.e. biallelic *H19* expression) leads to undergrowth and occurs in about 50% of RSS patients (Azzi *et al.*, 2014). LOM at IC2 is reported in over 50% of BWS cases (Azzi *et al.*, 2014, Choufani *et al.*, 2013). Aberrant hypomethylation leads to derepression of the ncRNA *KCNQ1OT1* on the maternal allele and, as a consequence, loss of expression of *CDKN1C* and other protein coding genes that are normally expressed on the maternal allele (Azzi *et al.*, 2014, Choufani *et al.*, 2013). *CDKN1C* is a cyclin-dependent kinase inhibitor of G1 cyclin complexes and acts to negatively regulate cell growth and proliferation. *CDKN1C* is expressed in both embryo and placenta during development and continues to be expressed postnatally (Jacob *et al.*, 2013). Maternally-inherited loss of function mutations in *CDKN1C* are reported in about 10% of BWS patients and about 40% of familial cases of BWS (Choufani *et al.*, 2013). BWS patients with *CDKN1C* mutations are more likely to have polydactyly, genital abnormalities, cleft palate and are less likely to develop tumors compared with other molecular causes of BWS, suggesting that decreased *CDKN1C* expression disrupts development of these organ systems (Kantaputra *et al.*, 2013, Romanelli *et al.*, 2010). Activating mutations in *CDKN1C* have been reported in RSS patients (Azzi *et al.*, 2014). Chromosomal alterations such as paternal uniparental isodisomy of 11p15.5 leads to BWS in 20% of cases while maternal UPD has been reported in one case of RSS (Bullman *et al.*, 2008). Maternal UPD7 has been reported in 5-10% of RSS. More recently, maternally transmitted microdeletions in IC1 have been demonstrated to cause familial BWS and are associated with hypermethylation of IC1. Although the phenotype of these patients is similar to GOM at IC1 (Sparago *et al.*, 2004), the relationship between the microdeletion and gain of methylation is not clear. That is, it is not known whether the loss of imprinting at the locus (biallelic *IGF2* and reduced *H19* expression) is dependent upon the microdeletion, gain of methylation or both. However, anticipation with increased hypermethylation in successive generations correlating with increased severity of BWS phenotype has been reported (Berland *et al.*, 2013). Maternally transmitted IC2 mutations have also been described in one family, leading to hypomethylation and decreased *CDKN1C* expression (Algar *et al.*, 2011). Finally, paternal transmission of duplications of the entire IC1 and IC2 region can also lead to BWS (Azzi *et al.*, 2014).

Mouse models of the BWS and RSS orthologous regions have provided insight into the epigenetic regulation of this region and its role in embryonic and placental growth. Mouse models overexpressing *Igf2* with deletion of *Cdkn1c* or *Igf2r* showed fetal phenotypes similar to BWS (Caspary *et al.*, 1999, Eggenschwiler *et al.*, 1997, Sun *et al.*, 1997). Moreover, mice with deletions of the *H19/Igf2* ICR showed expression and growth trends similar to BWS and RSS depending on paternal or maternal inheritance of the deletions, respectively (Thorvaldsen *et al.*, 1998, Thorvaldsen *et al.*, 2006).

A model with CpG mutations preventing maintenance of methylation on the paternal ICR led to decreased *Igf2* expression and *H19* overexpression with resulting small size as seen in RSS (Engel *et al.*, 2004). It should be noted, however, that none of these models provides the complete BWS or RSS phenotypes, suggesting that either the mouse locus is distinct enough from the human to not demonstrate the full phenotype or there are other regulatory factors contributing to the human phenotypes. It has also been suggested that the rate of growth between human and mouse may account for the differences in phenotype (Casparly *et al.*, 1999).

Genome-wide paternal uniparental disomy

Surprisingly, several rare cases of mosaic genome-wide paternal UPD have been reported (Gogiel *et al.*, 2013, Inbar-Feigenberg *et al.*, 2013, Kalish *et al.*, 2013). While no cases of live-born complete UPD cases have been documented, thirteen mosaic live-born cases have been reported to date. The predominant phenotype is similar to BWS with overgrowth, hemihyperplasia, hyperinsulinism, and a high incidence of tumor development (Kalish *et al.*, 2013). Most patients were premature and had placental overgrowth, which is consistent with the mostly paternally derived tissue observed in androgenetic conceptuses. Additional features of other paternal UPD disorders were observed in some of the cases and included pseudohypoparathyroidism (UPD20) and bell-shaped thoraces (UPD14). Few of the patients had developmental delays or other features of Angelman syndrome (Kalish *et al.*, 2013). All of these patients demonstrated a mosaic mixture of biparental and uniparental cells in each tissue type tested. In some patients, all cell types and tissues tested showed greater than 80% paternal UPD cells. Importantly, none of the patients showed 100% paternal UPD in any cell type, which raises the question of how much maternal contribution is needed for viable embryonic development. Moreover, although the basis for the observed phenotypes in the mosaic genome-wide UPD patients is not known, the expressed phenotype likely corresponds to the amount of paternal UPD cells present in a given target tissue (i.e. more paternal UPD in the neuronal cells of mosaic genome-wide paternal UPD patients expressing Angelman syndrome features).

Assisted reproductive technologies and imprinting disorders

Another growing patient population that questions our understanding of the maintenance and establishment of imprinting is assisted reproductive technologies (ART) conceptions. The timing of ART coincides with both the establishment and maintenance of imprinting. In ART, the egg donor undergoes hormonal hyperstimulation to facilitate release of multiple oocytes; this is the time at which the oocyte is in its growth phase and is being reprogrammed. With respect to imprinting, mouse studies have shown that maternally-methylated ICRs are methylated during oocyte growth, although these ICRs are not methylated simultaneously (Lucifero *et al.*, 2004). The subsequent *in vitro* fertilization, embryo culture and transfer to mothers also occur when the embryo is undergoing extensive reprogramming. In this case, the embryo undergoes a post-fertilization extensive loss of DNA methylation together with changes in post-translational histone modifications, which prepare the embryo for cleavage divisions and subsequent lineage differ-

entiation. Thus, ART manipulations take place during sensitive periods of mammalian development. Several small studies have suggested increased incidence of BWS and AS following ART; however, large studies to confirm the true incidence have not been completed to date (Chang *et al.*, 2005, Odom and Segars, 2010). A recent meta-analysis attempting to correlate the results of 8 studies of ART and BWS summarized that 6 of the studies found a positive correlation between BWS and ART and calculated an overall relative risk of 5.2 (Vermeiden and Bernardus, 2013). In several of the individual studies, when decreased fertility in the parents was taken into account, the increased incidence of imprinting disorders in ART was not significant. Increased incidences of RSS, AS, and PWS were not seen but the overall incidences of these disorders are much lower than BWS (Vermeiden and Bernardus, 2013). It should be noted that the vast majority of ART-associated cases of BWS and AS involve loss of ICR methylation. This is especially interesting for AS, where loss of methylation is extremely rare in the population.

Animal models have confirmed that techniques used in ART can cause epigenetic perturbations at imprinted (and other) loci (El Hajj and Haaf, 2013, Grace and Sinclair, 2009, Laprise, 2009). The animal models have the added attraction that infertility is not a confounding factor. Animal models have tested hormonal hyperstimulation, IVF, embryo culture and transfer, all of which have been associated with aberrant imprinting, including loss of imprinting and loss of ICR methylation. Bovine models demonstrate that ART leads to increased large offspring syndrome with macrosomia, macroglossia, and abdominal wall defects and biallelically expressed imprinted genes seen in BWS (Chen *et al.*, 2013). Interestingly, the ART conceptuses show a much greater imprinting perturbation in the placentas than in embryonic tissues (de Waal *et al.*, 2014). While there are a number of possible explanations for this result, one of the most compelling explanations is that imprinted genes have redundant mechanisms to maintain parental-specific imprinting, including DNA methylation and post-translational histone modifications, in the embryonic lineages whereas extraembryonic tissues are less likely to employ both sets of epigenetic machinery in the maintenance of imprinted gene expression.

Summary and future directions

Establishment and maintenance of imprinted gene expression is integral for normal embryonic and extraembryonic development. Mis-regulation of this process can occur at many levels and leads to clinical disease. The role of individual genes in each of these imprinted clusters is still being uncovered. Further understanding of the regulation of imprinted genes may lead to improvements in ART and improved management of human imprinting disorders.

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