

**LARGE OFFSPRING SYNDROME, A BOVINE MODEL
FOR THE HUMAN LOSS-OF-IMPRINTING
OVERGROWTH SYNDROME BECKWITH-WIEDEMANN**

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By

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The undersigned, appointed by the dean of the Graduate School, have examined the thesis entitled

**LARGE OFFSPRING SYNDROME, A BOVINE MODEL FOR THE
HUMAN LOSS-OF-IMPRINTING OVERGROWTH SYNDROME
BECKWITH-WIEDEMANN**

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Preface

Beckwith-Wiedemann syndrome (BWS) is a pediatric overgrowth condition with increased likelihood to develop embryonic tumors. Children conceived with the use of assisted reproductive technologies (ART) have an increased frequency to have BWS compared to naturally-conceived individuals. Genomic imprinting is defined as parental allele-specific gene expression which is regulated by epigenetic modifications including DNA methylation. The molecular alterations associated with BWS have been mapped to human chromosome 11p15 region. This region harbors two imprinting loci which are known as imprinting center 1 (IC1) and IC2.

Currently, only associations exist between the 11p15 regions and BWS. No direct evidence is available to demonstrate how the alterations of 11p15 regions could lead to the variable BWS phenotypes. Importantly, no animal model exists that faithfully recapitulates the various phenotypes of BWS. In ruminants, there is a similar overgrowth condition that recapitulates the features of BWS which is known as large offspring syndrome (LOS). LOS is the result of ART in ruminants. A previous study in our laboratory found that the DNA methylation and allelic expression of imprinted genes in the IC1/IC2 are conserved between human and bovine. The goal of the current study is to determine if LOS in bovine displays the same epigenetic alterations of IC1 and IC2 as BWS in human.

In this thesis, chapter 1 will introduce genomic imprinting and discuss in detail how genomic imprinting is regulated by epigenetic modifications including DNA

methylation, histone modifications, and non-coding RNAs. Chapter 1 will also introduce BWS, BWS-associated imprinted loci and will present associations between ART and BWS. LOS in bovine will also be described in chapter 1. Chapter 2, the research chapter, shows that similar loss-of-imprinting (LOI) of IC2 is observed in our LOS conceptuses generated with the use of ART. This chapter has been recently accepted in the Landes Bioscience Journal: Epigenetics.

Nomenclature

AI	Artificial insemination
AID	Activation Induced Deaminase
<i>Airn</i>	Antisense of <i>Igf2r</i> non-protein coding RNA
APOBEC	Apolipoprotein B mRNA editing enzyme, catalytic polypeptide
ART	Assisted Reproductive Technologies
AS	Angelman syndrome
BER	Base Excision Repair
<i>B. t. indicus</i>	<i>Bos taurus indicus</i>
<i>B. t. taurus</i>	<i>Bos taurus taurus</i>
BWS	Beckwith-Wiedemann syndrome
cDNA	Complementary DNA
<i>Cdkn1c</i>	Cyclin Dependent Kinase Inhibitor 1C
COBRA	Combined bisulfite restriction analysis
CTCF	“CCCTC” binding factor
CGIs	CpG islands
DMR/DMD	Differentially Methylated Region/Domain
Dnmt	DNA methyltransferase
ESCs	Embryonic Stem cells
5caC	5' carboxylcytosine
5fC	5' formylcytosine
5mC	5' methylcytosine
5hmC	5' hydroxymethylcytosine

<i>Gnas</i>	Guanine nucleotide binding protein, alpha stimulating
<i>H19</i>	cDNA clone No. 19 isolated from a fetal hepatic library
H3K9/27/36	Histone 3 lysine 9/27/36
IAP	Intracisternal A partical
IC1/IC2	Imprinting Center 1/2
ICM	Inner cell mass
ICR/ICE	Imprinting Control Region/Domain
ICSI	Intracytoplasmic Sperm Injection
<i>Igf2</i>	Insulin-like growth factor 2
<i>Igf2r</i>	Insulin-like growth factor type 2 receptor
IVC	<i>in vitro</i> culture
IVF	<i>in vitro</i> fertilization
IVM	<i>in vitro</i> maturation
<i>Kcnq1</i>	Potassium voltage-gated channel, KQT-like subfamily member 1
<i>Kcnq1ot1</i>	<i>Kcnq1</i> opposite Transcript 1
LINES	Long Interspersed Nuclear Elements
LOI	Loss-of-imprinting
LOS	Large offspring syndrome
Mecp2	Methyl-CpG binding protein 2
MBD	Methyl-CpG binding proteins
ncRNA	Non-coding RNA
OCT	Octamer binding transcription factor

OTB	Ovarian Time Bomb
PAGE	Polyacrylamide gel electrophoresis
PcGs	Polycomb group proteins
PCR	Polymerase Chain Reaction
<i>Peg1/3/5/10</i>	Paternally-expressed gene 1/3/5/10
PGC	Primordial germ cells
<i>Phlda2</i>	Pleckstrin homology-like domain, family A, member 2
PTMs	Post-translational modifications
PWS	Prader-Willi syndrome
RFLP	Restriction fragment length polymorphism
RSS	Russell-Silver syndrome
RT	Reverse transcription
SCNT	Somatic Cell Nuclear Transfer
<i>Slc22a2/Slc22a3</i>	Solute Carrier family 22c member 2/3
SNP	Single Nucleotide Polymorphism
<i>Snrpn</i>	Small Nuclear Ribonucleoprotein Polypeptide N
SSCP	Single strand conformation polymorphism
TDG	Thymine DNA Glycosylase
TE	Trophectoderm
Tet	Ten eleven translocation/Tet methylcytosine dioxygenase
TNDM	Transient Neonatal Diabetes Mellitus
UTR	Untranslated region
UPD	Uniparental disomy

Chapter 1 Literature review

1.1 Genomic Imprinting

1.1.1 Concepts

In mammals, each somatic cell has two sets of matched chromosomes; one is inherited from the mother through the oocyte, and the other is from the father by the sperm. Most of the genes are diploid active, which means both the maternal copy and the paternal copy are transcribed. Genomic imprinting is a series of processes that lead to parent-specific gene expression in diploid cells. Put another way, it is a phenomenon in which a few genes, known as imprinted genes, are only haploid active, which means that only one copy, either maternal or paternal, is transcribed. It should be noted that the imprinted genes are not subject to random monoallelic expression, which has been described in odorant receptors and some of the T-cells and B-cells in mouse and humans (Bix and Locksley, 1998; Hollander et al., 1998; Song et al., 2012), but rather dependent on the allele's parental origin. Imprinted genes with an active maternal copy are maternally-expressed genes (paternally imprinted), and the imprinted genes with an active paternal copy are paternally-expressed genes (maternally imprinted). Currently, about 150 imprinted genes have been identified in the mouse (Williamson et al., 2013) and at least 59 in humans (reviewed in Ishida and Moore, 2012; Morison et al., 2005). Imprinted genes are essential for multiple physiological processes including pre- and postnatal growth, regulation of placentation, neural and behavior development (reviewed in Bartolomei and Ferguson-Smith, 2011).

1.1.2 Historical Overview

Both genomic imprinting and X chromosome inactivation are processes that lead to functionally inequivalent contribution of the parental genomes. Findings in the past 40 years demonstrated that both maternal and paternal genomes are required for normal embryonic growth and development.

X chromosome inactivation was one of the earliest findings about dosage balance of sex chromosomes between males (heterogametic sex; XY) and females (homogametic sex; XX). One of X chromosomes in females is selected to be inactivated. Thus both males and females have only one copy of X-linked genes active in any one cell. In mice, there are two types of X-inactivation: random and imprinted. Random X-inactivation occurs in the inner cell mass (ICM) while imprinted X-inactivation occurs in the trophectoderm (TE). That is, always the paternal X-chromosome is inactivated in the extraembryonic-tissue of females (Cooper et al., 1971).

Other early work which hinted at the requirement of both parental alleles for normal development comes from the “Hairpin-tail” mouse mutants. In those mouse, mice mutants with a maternal deletion of part of chromosome 17 died before birth, but mice mutants with the deletion of identical paternal copy were viable and fertile (Johnson, 1974; Lyon and Glenister, 1977). In the 1980s, progress in nuclear transfer technology allowed for the production of androgenic embryos (i.e. embryos with two paternal genomes) and gynogenic embryos (i.e. embryos with two maternal genomes). Both types

of embryos died at mid-gestation but exhibited different phenotypes: androgenic conceptuses had a severely retarded fetus but a well-developed placenta while gynogenic embryos showed a normal fetus, except of a smaller size, but an undeveloped placenta (McGrath and Solter, 1984; Surani et al., 1984). In 1985, genetic experiments performed by Cattanach and colleagues showed that mice with uniparental disomy (UPD; either two maternal copies or two paternal copies) for part of chromosome 2 and 11 exhibited opposite phenotypes away from the normal depending on the type of UPD (maternal UPD led to smaller body size; paternal UPD led to larger body size; Cattanach and Kirk, 1985). In human studies, Nicholls and colleagues proved that the paternal copy of chromosome 15q11-q13 was essential to prevent developing Prader-Willi syndrome (PWS), a mental retardation condition in humans (Nicholls et al., 1989). However, maternal UPD of chromosome 15q11-q13 was associated with PWS (Nicholls et al., 1989).

Final proof of genomic imprinting was the identification of three imprinted genes in mouse: Insulin-like growth factor type 2 receptor (*Igf2r*; Barlow et al., 1991), Insulin-like growth factor (*Igf2*; DeChiara et al., 1991; Ferguson-Smith et al., 1991), and *H19* (cDNA clone No. 19 isolated from a fetal hepatic library; Bartolomei et al., 1991). *Igf2r* was found by identifying genes within the deletion of chromosome 17 in “Hairpin-tail” mice mutants. *Igf2r* is one of the genes that located in the deletion region in mice mutants. Mice with the maternal deletion of “Hairpin-tail” region had no expression of *Igf2r*, but the paternal deletion had the similar expression level of *Igf2r* to wild type mice (Barlow et al., 1991), which proved that *Igf2r* is a maternally-expressed gene. For *Igf2*,

gene targeting strategy was used to determine the role of *Igf2* in embryonic development. Mice with maternal transmission of the non-functional *Igf2* showed normal growth, but paternal transmission of mutant *Igf2* had deficient growth similar to *Igf2* deficient mice in both alleles (DeChiara et al., 1991). At the same time, *Igf2* was identified as a paternally-expressed gene using the UPD approach. *Igf2* was absent in mice with the maternal UPD of part of the distal chromosome 7, but had the normal level in mice of the paternal UPD of part of the chromosome 7 (Ferguson-Smith et al., 1991). These two experiments demonstrated that *Igf2* is a paternally-expressed gene. *H19* is located close to *Igf2* in chromosome 7. The imprinting status of *H19* was tested based on the hypothesis that imprinted genes are in clusters (Bartolomei et al., 1991). Single nucleotide polymorphism (SNP) within the *H19* RNA between parental alleles was used to determine the parental allele-specific expression of *H19*. Since genetic background of multiple mouse strains are known or early identified and SNPs among the strains are available, allele-specific expression based SNPs now are commonly used in genomic imprinting studies. Recently, Kono and colleagues confirmed the idea that genomic imprinting is the only barrier for viability of bi-maternal mice (Kono et al., 2004). They generated the viable mice with only maternal origin genomes by combing the nuclei from a mature oocyte and a non-growing oocyte which had deletions of two different imprinted loci (Kawahara et al., 2007; Kono et al., 2004). Currently, genomic imprinting has become an essential area to clarify the mechanisms of epigenetic regulations.

1.1.3 Evolution of Genomic Imprinting

Diploid organisms with two sets of homologous chromosomes have the advantage of survival with lethal recessive mutations in one of their chromosomes. In an evolutionary perspective, genomic imprinting is a paradox because it evolved to decrease the probability to mask deleterious recessive mutations (Wilkins and Haig, 2003). Currently, there are several hypotheses to explain the evolution advantage of genomic imprinting in mammals.

Previous studies of mice with UPD and haploid mutation of imprinted genes suggested that paternally-expressed genes enhance conceptus growth while maternally-expressed genes inhibit conceptus growth, which led to the “Kinship” or “Conflict” theory (Haig, 1993; Haig and Westoby, 1989). According to this theory, paternally-expressed genes are involved in enhancing growth of individuals. Oppositely, maternally-expressed genes develop to restrict the growth of the conceptus because of the limited maternal resources. For example, in mice, experimental deletion of the paternally-expressed fetal growth factor *Igf2* resulted in offspring with deficient growth. In those studies, the fetus and placenta were smaller in the *Igf2* knockout group when compared to wild type control (Constancia et al., 2002; DeChiara et al., 1991). The opposite examples used as the basis of the “Kinship” hypothesis come from the deletion of two maternally-expressed genes, the tumor suppressor *H19* (Leighton et al., 1995a) and the cyclin dependent cell cycle inhibitor *Cdkn1c* (Tunster et al., 2011). In addition, competition of parental interests mainly takes place during pregnancy through the placenta, which substantiates the “Conflict” theory because many imprinted genes are highly active in the

placenta and affect fetal growth and placentation (Coan et al., 2005; Tycko, 2006).

However, the strengths of this hypothesis are weakened because not all imprinted genes play a role in controlling growth.

The alternative explanation for the evolutionary importance of genomic imprinting is the ovarian time bomb theory (OTB; Varmuza and Mann, 1994). Ovarian teratomas, which progress from unfertilized oocyte, are nonthreatening because they are unable to differentiate into invasive trophoblast although they could generate most types of tissues. Thus, Varmuza and Mann proposed that failed development of invasive trophoblast in the OTB was because in oocytes, the genes responsible for trophoblast invasiveness were silenced to protect females from teratomas and the active copies of these genes must be inherited from father through the sperm. The OTB theory explained well why some genes are inactive in oocytes but not for the silencing of maternally-expressed genes in the paternal genome. An improved OTB theory suggested that paternally-expressed genes are involved in enhancing invasive trophoblast formation while silenced genes in the paternal genome (i.e. maternally-expressed genes) mainly encode invasive trophoblast suppressors (Weisstein et al., 2002).

Yet another theory for the evolution of genomic imprinting proposed that haploid inactive genes are beneficial for increased evolvability (Beaudet and Jiang, 2002). In their view, DNA regions within imprinted genes could accumulate mutations without causing deleterious phenotypes. Through generations, hidden mutations provide a genetic variation pool to enable the population to adapt to certain environmental change.

However, why only a small group of genes is subject to this evolution strategy is not well answered in this hypothesis.

1.2 Genomic imprinting is regulated by epigenetic modifications

Epigenetics, literally meaning “above genetics”, refers to gene expression patterns that could be transmitted through mitosis and meiosis without involving the primary DNA sequence. The faithful inheritance of gene expression information is realized by transmissible epigenetic marks which determine chromatin state by affecting the way DNA is packaged, and the transcripts abundance by impacting the stability of RNAs. The epigenetic marks include DNA methylation, histone variants, covalent modification of histone tails, and non-coding RNAs. Establishment and maintenance of epigenetic marks play an essential role in embryo development, cell lineage specification as well as correct monoallelic expression of imprinted genes and X chromosome inactivation. How epigenetic modifications are involved in the establishment, maintenance, and erasure of genomic imprints will be discussed in the sections of DNA methylation, histone modifications, and non-coding RNAs. Since the majority of reports use the mouse model to study imprinting, the following sections will present what is known for this species except when otherwise indicated.

1.2.1 DNA methylation

In mammals, DNA methylation is an epigenetic modification where a methyl group ($-\text{CH}_3$) is covalently attached to the 5th carbon of cytosine mostly in a CpG context (Bernstein et al., 2007). This reaction is catalyzed by the DNA methyltransferase (Dnmts;

ref). Methylated cytosines can also be found in other contexts such as CpHpG or CpHpH (H=A or T or C) in human and mouse embryonic stem cells (Lister et al., 2009; Ramsahoye et al., 2000). Methyl groups on the DNA change the transcription state by interacting with chromatin binding proteins, such as methyl binding domain proteins (MBDs) or insulator binding proteins (Robertson, 2005). DNA methylation pattern can be faithfully copied from parent strands to daughter strands during DNA replication, which makes it a perfectly heritable epigenetic mark.

CpG islands (CGIs)

CpG dinucleotides are distributed throughout the genome. The incidence of CpG sites is lower than expected in both human and mouse genomes (Rollins et al., 2006; Saxonov et al., 2006; Waterston et al., 2002). This is believed to result from the spontaneous deamination of methylated cytosines (C) to thymine (T; Duncan and Miller, 1980). The T:G mismatch is not always fixed by the base excision repair (BER) system (Poole et al., 2001).

CpG sites are not evenly distributed in the genome. CpG islands (CGIs) are DNA regions that are highly enriched with CpG dinucleotides. The definition of CGIs based on computational prediction is a DNA region that fulfill three criteria: (1) at least 500 base pairs; (2) with at least 55% GC content; (3) ratio of observed to expected CpG sites is at least 0.6 (Suzuki and Bird, 2008). According to this description, there are ~ 23,000 CGIs in the mouse genome (29,000 in the human genome) with ~60% located at promoter

regions of annotated genes and the remaining at gene bodies or intergenic regions (Maunakea et al., 2010; Probst et al., 2009).

CGIs associated with promoter regions are mostly (>97%) unmethylated in all tissue types throughout development (Antequera and Bird, 1993; Bird, 1986; Maunakea et al., 2010). In contrast, CGIs in gene bodies and intergenic regions are more frequently methylated. Gene body methylation may be involved in gene expression as highly expressed genes contain methylation from the second exon until the 3' untranslated region (UTR; Brenet et al., 2011). Alternatively, gene body methylation may be involved in alternative splicing or silencing intragenic alternative promoters (Lyko et al., 2010; Maunakea et al., 2010). Methylation of CGIs in intergenic regions are mainly to silence repetitive elements such as retrotransposons and long interspersed nuclear elements (LINEs) (Yoder et al., 1997). In addition, some intergenic CGIs are differentially methylated, which is essential for genomic imprinting (Edwards and Ferguson-Smith, 2007).

Imprinting Control Regions (ICR) in clusters of imprinted genes

Of the identified imprinted genes, most are found in clusters with two or more imprinted genes and regulated synchronously (reviewed in Edwards and Ferguson-Smith, 2007; Wan and Bartolomei, 2008). Presence of solitary imprinted genes could be partially explained by the not identified imprinted genes in the flanking regions. A typical imprinting cluster contains both maternally- and paternally-expressed genes, at least one imprinted non-coding RNA, as well as non-imprinted genes in some cases (Edwards and

Ferguson-Smith, 2007) and can be as large as 3700kb (i.e. *Snrpn* cluster; Bressler et al., 2001). An example of an imprinted cluster is represented by the *Airn/Igf2r* cluster. This cluster contains three maternally-expressed protein coding genes *Igf2r*, *Slc22a2*, and *Slc22a3*, and one paternally-expressed long non-coding RNA *Airn* (Zwart et al., 2001).

Regulation of the imprinted genes as clusters suggests that a key *cis*-factor controls the imprinting at a long-range element level instead that at the single gene level. The *cis*-factor is known as imprinting control region (ICR), imprinting center (IC), or imprinting control elements. This discrete region of DNA is able to regulate the monoallelic expression of more than one imprinted gene many kilobases away within the imprinting cluster (Buiting et al., 1995; Ripoche et al., 1997; Sutcliffe et al., 1994; Zwart et al., 2001). Of the 21 known clusters of imprinted genes in mice, seven have been identified by ICR deletion studies. These include *Kcnqlot1* cluster (Fitzpatrick et al., 2002), *H19/Igf2* cluster (Thorvaldsen et al., 1998), *Airn/Igf2r* cluster (Wutz et al., 1997), *Gnas* cluster (Williamson et al., 2006), *Dlk1/Gtl2* cluster (Lin et al., 2003), *Grb10* cluster (Shiura et al., 2009), and *Snrpn* cluster (Bressler et al., 2001). ICRs are CpG islands that are always differentially methylated regions (DMRs). Put another way, ICRs are methylated on one of the parental allele, but unmethylated on the other allele in a non-random manner. Most ICRs (17 ICRs of the 21) are maternally methylated, such as KvDMR1 for the *Kcnqlot1* cluster and Region 2 for the *Airn/Igf2r* cluster (Chotalia et al., 2009; Kobayashi et al., 2006; Tomizawa et al., 2011). On the contrary, only a few ICRs (4 ICRs of the 21) are paternally-methylated including *H19* ICR for the *H19/Igf2* cluster and IG-DMR for the *Dlk1/Gtl2* cluster (reviewed in Bartolomei and Ferguson-

Smith, 2011). Only maternal deletion of the ICRs that are maternally-methylated results in loss-of-imprinting (LOI) of the cluster (imprinted genes become biallelically-expressed or silenced), whereas deletion of the paternal copy of the ICRs had no effect (Fitzpatrick et al., 2002; Wutz et al., 1997).

Distinct DNA methylation between parental alleles indicates that they are established when maternal and paternal alleles are found in separate compartments (i.e. spermatozoa and oocyte). In fact, imprints are established during germline formation and maintained through development. However, the established imprints are not always faithfully maintained in every tissues (Smith et al., 2012). The maternal imprints that are established through female germline in the growing oocytes and the paternal imprints that are acquired during male germline before birth are known as primary or gametic imprints (Hiura et al., 2006; Lucifero et al., 2004; Sasaki and Matsui, 2008). Secondary or somatic imprints are DMRs that are established at post-implantation stages (John and Lefebvre, 2011). Somatic imprints are believed to be deposited as a result of the regulation of nearby gametic imprints (John and Lefebvre, 2011).

DNA methyltransferases (Dnmts)

The establishment (*de novo*) and maintenance of DNA methylation are catalyzed by a class of enzymes known as DNA methyltransferase (Dnmts). Dnmts are able to transfer a methyl group from the methyl donor S-adenosyl methionine to the 5th carbon of cytosine in DNA (Chen and Li, 2004). In mammals, the Dnmt family includes the following members: Dnmt1 for maintenance of DNA methylation (Bestor, 1988; Yoder

et al., 1996), Dnmt3a/3b for *de novo* DNA methylation (Okano et al., 1998a; Xie et al., 1999), Dnmt3l is a non-catalytic partner of Dnmt3a/3b which enhances their activity (Aapola et al., 2000), and Dnmt2 for RNA methylation (Goll et al., 2006; Okano et al., 1998b).

Dnmt1. The somatic Dnmt1 (Dnmt1s) is responsible for reproducing the DNA methylation pattern of the parent DNA strand onto the daughter DNA strands during DNA replication. In dividing cells, Dnmt1 is recruited to DNA replication forks by directly interacting with proliferating cell nuclear antigen (PCNA) and Np95 (also known as Uhrf1) (Bostick et al., 2007; Chuang et al., 1997; Sharif et al., 2007). Np95 recognizes the methylated parent strand and guides Dnmt1 to methylate the symmetric CpG sites on the newly synthesized DNA strand. Dnmt1 and Np95 have been shown essential for genomic imprinting and normal embryonic development. For example, *Dnmt1*^{-/-} mouse embryos show a prominent reduction of DNA methylation and do not survive beyond mid-gestation (Li et al., 1992). Specifically for genomic imprinting, loss of Dnmt1 results in loss of methylation of ICRs and leads to LOI (Li et al., 1993). *Np95*^{-/-} embryos show similar phenotype to *Dnmt1*^{-/-} embryos even though they have comparative Dnmt1 level to wild type embryos (Sharif et al., 2007).

Dnmt1o is an oocyte-specific isoform of Dnmt1. Dnmt1o shares homology with Dnmt1s in all exons except the first exon (Mertineit et al., 1998). The oocyte-specific first exon encodes 118 amino acids, and this distinct sequence might be involved in the stability of Dnmt1o in the cytoplasm (Latham et al., 2008). Dnmt1o is subject to intriguing subcellular localization during early embryo development. Dnmt1o is localized

in the cytoplasm in oocytes and preimplantation embryos except the transient nuclear localization at the eight-cell stage (Cardoso and Leonhardt, 1999). This nuclear movement of Dnmt1o is responsible for maintaining DNA methylation at specific sites in the midst of an ongoing global DNA demethylation (see following sections; Howell et al., 2001). Conditional knockout of oocyte-specific and zygotic Dnmt1 (Dnmt1o and Dnmt1s, respectively) shows that Dnmt1 is essential for maintaining the methylation imprints during pre-implantation development (Hirasawa et al., 2008). In pachytene spermatocytes, the sperm-specific Dnmt1 isoform encodes a non-translated *Dnmt1* which is thought to regulate *Dnmt1s* expression level during spermatogenesis (Mertineit et al., 1998).

Dnmt2. Dnmt2 shares the conserved C-terminal catalytic domain with Dnmt1, but lacks a large piece of N-terminal domain (Chen and Li, 2004; Van den Wyngaert et al., 1998). Dnmt2 displays weak DNA methylation activity both *in vivo* and *in vitro* (Hermann et al., 2003; Liu et al., 2003; Tang et al., 2003). *Dnmt2*^{-/-} mouse embryonic stem cells (ESCs) exhibits normal DNA methylation level, and mutant ESCs are able to methylate integrated retrovirus as efficiently as wild type ESCs, which indicates that Dnmt2 is not required for *de novo* or maintaining DNA methylation (Okano et al., 1998b). Actually it has been reported that Dnmt2 is responsible for the methylation of aspartic acid transfer RNA (tRNA; Goll et al., 2006).

Dnmt3a/3b and Dnmt3l. Since Dnmt1 can exhibit *de novo* DNA methylation activity *in vitro* besides the methylation maintenance activity (Okano et al., 1998a), it was unknown if a single gene controlled both establishment and maintenance of DNA

methylation. However, *Dnmt1*^{-/-} mouse ESCs presented substantially reduced but stably maintained DNA methylation level, which indicated that additional methyltransferase existed for *de novo* DNA methylation activity (Lei et al., 1996). In 1998, *Dnmt3a/3b* were cloned in human and mouse (Okano et al., 1998a; Van den Wyngaert et al., 1998). *Dnmt3a/3b* share the common C-terminal catalytic domain with *Dnmt1* but the N-terminal domain of *Dnmt3a/3b* is distinct from *Dnmt1* (Okano et al., 1998a; Van den Wyngaert et al., 1998). *Dnmt3l* has no DNA methylation activity, but it is essential to stimulate the full enzyme activity of *Dnmt3a/3b* (Chedin et al., 2002; Gowher et al., 2005).

Two waves global *de novo* methylation occur during mammalian development. One takes place during gametogenesis and the other takes place after implantation (reviewed in Reik et al., 2001). The *Dnmt3* family is essential for the two waves of *de novo* methylation. In fact, *Dnmt3a* knockout mice are able to develop to term but die shortly after birth (Okano et al., 1999). On the other hand *Dnmt3b* knockout mice do not survive beyond day 16.5 (Okano et al., 1999). Different knockout defects indicated that *Dnmt3a* and *Dnmt3b* had the distinct target sequence for DNA methylation. *Dnmt3b*, but not *Dnmt3a*, is responsible for the methylation of centromeric major and minor satellite repeats (Okano et al., 1999). To this effect, mutation of *Dnmt3b* is associated with human ICF syndrome (immunodeficiency, centromeric instability, facial anomalies) which was characterized as centromeric heterochromatin instability and hypomethylation of centromeric satellites (Xu et al., 1999). Conditional knockout of *Dnmt3a/3b* in germ cells showed that *Dnmt3a*, but not *Dnmt3b*, is essential for the *de novo* methylation of

both maternal and paternal ICRs (Kaneda et al., 2004). Dnmt3l knockout mice are viable but with infertility. Dnmt3l knockout male exhibit impaired spermatogenesis and severe meiosis defects (Hata et al., 2002). The possible reason for meiotic chaos could be the reactivation of repeated sequence such as L1 repetitive element (LINE1) and intracisternal A particle (IAP) in prospermatogonia caused by the failure of *de novo* methylation of the repeated sequences (Bourc'his and Bestor, 2004). For Dnmt3l knockout females, their offspring failed to develop to term because of neural tubal defects, which could be the consequence of placenta abnormalities caused by the failure establishment of maternal imprints (Bourc'his et al., 2001).

DNA methylation reprogramming

The distinct transcriptome and proteome among different cell types in mammals is controlled by their epigenetic program. Epigenetic reprogramming is required to provide a “blank” epigenetic state of the genome to start a new development cycle. DNA methylation is one of the epigenetic marks that undergo two waves of global reprogramming: one occurs during primordial germ cells (PGCs) determination (Brandeis et al., 1993; Kafri et al., 1992), and the other takes place during pre-implantation embryonic development (reviewed in Reik et al., 2001; Saitou et al., 2012).

In mice, PGCs specification from the epiblast begins on day ~6 (post-fertilization) and PGCs start to migrate on day ~7.25. PGCs arrive to the genital ridge and colonize by day ~10.5 (reviewed in Saitou et al., 2012). During this process, PGCs undergo global DNA demethylation. As a result, methylation of parental imprints is erased, most

transposable elements are demethylated, and the inactivated X-chromosome in females is reactivated (reviewed in Hayashi and Surani, 2009). Erasure of DNA methylation at imprinted loci is essential to establish the new paternal and maternal imprints based on the sex of the PGCs (Brandeis et al., 1993; Kafri et al., 1992). Re-establishment of DNA methylation patterns in gametes requires the Dnmts (Bourc'his et al., 2001; Hata et al., 2002; Kaneda et al., 2004).

The second round of global DNA demethylation occurs during pre-implantation development, which is crucial to convert the DNA methylation pattern of gametes (i.e. sperm and oocyte) to the totipotent methylation pattern of a zygote. After fertilization, the paternal pronuclei undergo active demethylation before DNA replication (i.e. within 4-8 hours post-fertilization). However, the demethylation of the maternal pronuclei is passive and dependent on cell division as a result of the exclusion of Dnmt1o from the nucleus (Mayer et al., 2000; Oswald et al., 2000). Unlike the first global DNA demethylation, the second is more selective. For example, DNA methylation of imprinted loci, IAP, and centromeric repeats escape the second round of global DNA demethylation (reviewed in Reik et al., 2001).

Then at least two key questions could be asked about the DNA methylation reprogramming: 1) how do imprinted loci, IAP and centromeric repeats escape the second round of global demethylation? And 2) how could the demethylation machinery differ the parental genomes in their respective pronuclei to make them undergo asymmetrical reprogramming?

For the first question, several factors have been identified to be essential to maintain the DNA methylation of imprinted loci during preimplantation development. Zinc finger protein 57 homolog (*Zfp57*) was the first maternal factor identified to be related to imprinting control (Li et al., 2008). *Zfp57* directly binds to a methylated hexanucleotide in mouse ES cells and the bound *Zfp57* recruits Tripartite motif-containing 28 (*Trim28*, also known as *Kap1*; Quenneville et al., 2011; Zuo et al., 2012). *Trim28* is able to interact with *Dnmts* family including *Dnmt1o* which is essential for maintaining DNA methylation at some imprinted loci during the pre-implantation stage (Zuo et al., 2012). Abolishing maternal and zygotic *Zfp57* leads to loss of methylation of multiple ICRs such as *Snrpn* ICR, *H19* ICR, *IG* DMR (Li et al., 2008). In addition, hypomethylation of multiple imprinted loci in human is also associated with the *ZFP57* mutation (Mackay et al., 2008). Further, Maternal deletion of *Trim28* in mice is lethal and show an epigenotype of loss of methylation of several ICRs (Messerschmidt et al., 2012). However, differently from *Zfp57*^{-/-} which causes consistent loss of methylation of the imprinted loci, abolishment of maternal of *Trim28* resulted in variable epimutations and phenotype defects including lethality (Messerschmidt et al., 2012).

For the question of asymmetric demethylation of parental pronuclei after fertilization, a recent study found that *PGC7/Stella* binds to H3K9me2 which is a histone modification that mainly presents on the maternal genome (Nakamura et al., 2007; Santos et al., 2005). The presence of *PGC7/Stella* on the maternal genome prevents the *Tet3* binding, which is responsible for active demethylation and will be discussed subsequently (Nakamura et al., 2007; Nakamura et al., 2012). Intriguingly, *PGC7/Stella* is also

responsible for maintenance the DNA methylation of several but not all the ICRs. For example, maternal deletion of PGC7/Stella causes loss of methylation of ICRs for imprinted genes paternally expressed gene 1, 3, 10 (*Peg1*, *Peg3*, *Peg10*), *H19* and *Rasgrf1*, but has no effect on DMRs of *Peg5* and *Snrpn* (Nakamura et al., 2007).

DNA demethylation

DNA methylation is a relatively stable epigenetic mark, but rapid changes of methylation level involving active DNA demethylation are evident during development as mentioned above. The mechanism of DNA demethylation is not fully understood although several potential pathways have been proposed. These mechanisms include oxidative demethylation, base excision repair (BER) system for T:G mismatch (reviewed in Wu and Zhang, 2010).

Deamination of 5mC to T followed by BER. In this system, 5mC could be deaminated to T by deamination enzymes such as activation-induced deaminase (AID) and apolipoprotein B mRNA editing enzyme, catalytic polypeptide (APOBEC). The resulted T:G mismatch could be repaired by T glycosylases such as thymine DNA glycosylase (TDG) and methyl-CpG-binding protein 4 (MBD4; reviewed in Wu and Zhang, 2010). Deletion of AID results in elevated methylation level in PGCs in mice (Popp et al., 2010). Abolishment of TDG is lethal and leads to gain of methylation of imprinted loci such as *H19 ICR* and *Igf2 DMR*, which suggests that TDG plays a role in preventing the aberrant *de novo* methylation of imprinted loci (Cortellino et al., 2011).

Oxidative demethylation. Oxidative demethylation is mediated by ten-eleven translocation (Sharif et al., 2007) family which can convert 5mC to 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) in DNA (Ito et al., 2011; Wu and Zhang, 2010). The function of 5hmC is currently unclear. 5hmC is probably another epigenetic mark different from 5mC since methylcytosine binding proteins do not recognize 5hmC (Valinluck and Sowers, 2007). Alternatively 5hmC may be just an intermediate of 5mC. 5hmC can be either diluted through cell division as it is poorly recognized by Dnmt1 or 5hmC could be deaminated to 5hmU followed by DNA repair system (reviewed in Wu and Zhang, 2010).

Tet family contains three enzymes: Tet1, Tet2, and Tet3. Deletion of Tet1 and Tet2 at the same time partially compromises the demethylation of the ICRs in PGCs and the maintenance of the unmethylated status of the ICRs (Dawlaty et al., 2013). Most progeny of Tet1 and Tet2 double knockout females exhibited gain of methylation of *H19* ICR, which is normally unmethylated on the maternal allele. This suggested the incomplete erasure of DNA methylation due to deficiency of Tet proteins during female germline formation (Dawlaty et al., 2013). Also some but not all progeny of Tet1 and Tet2 double knockout females showed gain of methylation of *Igf2r* ICR, which is normally unmethylated on the paternal allele (Dawlaty et al., 2013), suggesting that Tet1 and Tet2 also play roles in maintaining the unmethylated status of *Igf2r* ICR through development. The effect on Tet3 on the imprinted gene has not been determined. However, mice with maternal Tet3 deficiency display an increased frequency of

development failure. In addition, active demethylation of paternal pronuclei fails to occur in Tet3^{-/-} zygotes (Gu et al., 2011).

1.2.2 Histone modifications

The DNA in the nucleus is packaged with histone proteins. About 146 bp of DNA wraps around a core octamer of histones forming the nucleosome which is the basic unit of chromatin. The octamer is composed of a H3-H4 tetramer with two interacted H3-H4 dimers, and two separate H2A-H2B dimers. In addition, a linker histone component, H1, interacts with the DNA octamer's entry-exit point (reviewed in Campos and Reinberg, 2009). For each nucleosome, both amino-terminal and carboxyl-terminal domains of histones could protrude outside of the core complex and are subject to multiple posttranslational modifications, such as methylation, acetylation and ubiquitylation on lysine (K) residues, phosphorylation on serine (S) residues, methylation on arginine (R) residues and ubiquitylation on lysine (K) residues (reviewed in Campos and Reinberg, 2009). Transcription status of chromatin is determined or reflected by posttranslational modifications of histone tails (PTMs) (Bernstein et al., 2007). Extensive research has been performed to study the association of PTMs and transcription outcomes. For example, histone acetylation is associated with transcription activation. Neutralized lysine residues (originally positive) by acetylation decreases its interaction with negatively charged DNA which leads to an "open" state of DNA (Vettese-Dadey et al., 1996). Histone acetylation could be also recognized by histone "readers" containing bromodomains. Additional transcription activation factors are recruited by the histone "reader" to the region and help keep the active status (Dhalluin et al., 1999). Some PTMs

are associated with transcriptional repression such as histone 3 lysine 27 trimethylation (H3K27me₃; Barrero and Belmonte, 2013; Cao et al., 2002). Nucleosomes can be further packaged into higher order chromatin but in a heterogeneous pattern. Some chromatin (euchromatin) is less dense and accessible to factors such as RNA polymerase for transcription. The package of some chromatin (heterochromatin) is dense which usually results in gene silencing. Euchromatin is correlated with certain modifications such as histone acetylation (i.e. H3K9ac and H3K14ac), while heterochromatin is associated with modifications such as H3K9me₃ (reviewed in Campos and Reinberg, 2009).

Role of histone modifications in the establishment of the imprints

Links between DNA methylation and histone modification have been established in recent studies. DNA methylation is known to have an effect on the histone modification patterns, which is mediated by methyl-cytosine-binding proteins such as Mecp2 and Mbd2. Mecp2 binds to methylated DNA and recruits histone deacetylase complexes, which leads to transcriptional repression (Jones et al., 1998). On the other hand, *de novo* DNA methylation has also been shown to be guided by histone modifications, which indicates that in some circumstances histone modifications are established before DNA methylation. For example, binding of Dnmt3l to the target sequence which is essential for the establishment of both maternal and paternal imprint (Bourc'his and Bestor, 2004; Bourc'his et al., 2001), is inhibited by the methylation of H3K4 (histone 3 lysine 4; Ooi et al., 2007). H3K4 trimethylation is associated with permissive state of chromatin (Bernstein et al., 2002). Further, oocytes deficient of Kdm1b (also known as Aof1 or Lsd2), a H3K4 demethylase, fail to establish several

maternal imprints including the ICRs of *Mest*, *Grb10*, *Zac1* and *Impact* (Ciccone et al., 2009). However, why some ICRs imprints like KvDMR1 and *H19/Igf2*DMR are not affected is still unclear (Ciccone et al., 2009; Smallwood and Kelsey, 2012). Unlike H3K4me1/2/3 which inhibits the binding of Dnmt3l, H3K36me3 could be recognized by Dnmt3a, one of the *de novo* DNA methyltransferase (Dhayalan et al., 2010).

Since many maternal imprints are located in the gene bodies which could act as alternative promoters (Chotalia et al., 2009), it was proposed that transcription may also play a role in the establishment of imprints. In the *Gnas* cluster, transcription of a protein-coding gene *Nesp* in oocytes overlaps the ICR (Liu et al., 2005; Williamson et al., 2006). Truncation of the transcript of *Nesp* led to the failure of maternal methylation establishment (Chotalia et al., 2009). “Transcription” hypothesis may provide a potential mechanism to explain how imprints are established in the absence of DNA replication (i.e. maternal imprints are established during oocyte growth). The transcription process is always coupled with chromatin remodeling, which allows for the engagement of Dnmt3a and Dnmt3l complex to the target sequence (Smallwood and Kelsey, 2012). In addition, some histone modifications associated with transcription activation have shown to interact with Dnmts. For example, H3K36me3, which is associated with transcript elongation (Mikkelsen et al., 2007), can recruit Dnmt3a to target region (Dhayalan et al., 2010). The function of chromatin remodelers in the imprints establishment is further supported by the evidence that chromatin remodelers Lsh is required for DNA methylation of *Cdkn1c* (a maternally-expressed gene) DMR (Fan et al., 2005). Lsh is a member of SNF2 family of chromatin remodeling proteins (Huang et al., 2004).

Differential DNA methylation of *Cdkn1c* DMR is established post-implantation (Bhogal et al., 2004). Mice that are deficient of Lsh fail to establish the methylation of *Cdkn1c* DMR on the paternal allele, but other imprints such as *H19/Igf2* ICR and *Igf2r/Air* ICR are not affected. This indicates that Lsh is required for the establishment of imprints at distinct DMRs.

Role of histone modifications in the maintenance of imprints

Besides the role of histone modifications in the establishment of the imprints during gametogenesis, it was also found that histone modifications show allele-specificity in the clusters of imprinted genes during the embryo development. Therefore, it was proposed that allele-specific histone modifications are involved in the maintenance and/or establishment of the imprinted expression in an imprinting cluster.

The asymmetric distribution of histone modifications provides a potential clue to explain the tissue-specific genomic imprinting. A good example for tissue-specific imprinted expression is *Airn/Igf2r* cluster in mice. In this cluster, *Igf2r* is a protein-coding gene that is expressed maternally while an antisense noncoding RNA starts the transcription in intron 2 of *Igf2r* on the paternal allele. *Igf2r* and *Airn* are imprinted in both embryonic and extraembryonic tissues, which is also known as “ubiquitous” imprinted gene expression (Sleutels et al., 2002). There are two other maternally-expressed genes, *Slc22a2* and *Slc22a3*, located downstream of *Igf2r*. *Slc22a2* and *Slc22a3* only maintain the maternal expression in the extraembryonic tissue and exhibit biallelic-expression in the embryos (Regha et al., 2006). No DMRs were identified to

regulate the tissue-specific imprinting of *Slc22a2* and *Slc22a3*. However, it has been shown that in the placenta *Airn* recruits G9a, a H3K9 methyltransferase, to the promoter of *Slc22a2* locus on the paternal allele. The locus is then marked with repressive chromatin mark preventing transcription (Nagano et al., 2008). The similar mechanism was also found in the KvDMR1 cluster. H3K9me2 and H3K27me3, the repressive histone modifications, are highly enriched on the paternal allele of *Osbp15*, *Ascl2*, and *Cd81* in the placenta but less in the embryo, which are maternally-expressed only in the placenta (Lewis et al., 2006; Lewis et al., 2004). Deletion of *G9a* in mice leads to biallelic expression of *Osbp15*, *Ascl2*, and *Cd81* in the placenta, but had no effect on ubiquitously imprinted genes in the cluster such as *Cdkn1c*, *Kcnq1*, and *Phlda2* (Wagschal et al., 2008). Deletion of the KvDMR1 cluster on E5.5 or E8.5 resulted in the relaxation of imprinting of ubiquitously imprinted genes but not placental-specific imprinted genes, suggesting that histone modifications maintain the placental-specific imprinted expression independently of DNA methylation once it has been established (Mohammad et al., 2012). However, the repressive histone modification to regulate the extraembryonic-specific imprinting is not a general mechanism since no repressive marks were found in the *Scg3/Peg10* cluster which contains four placental-specific imprinted genes (Monk et al., 2008).

In addition, it has also been shown that ICRs are subject to allele-specific histone modifications depending on their methylation status. For example, the ICR of the *Airn/Igf2r* cluster, carries repressive modifications on the methylated maternal allele such as H3K9me3 and H4K20me3. However, on the unmethylated paternal allele, the ICR is

enriched with active modifications including H3K4me3, H3k4me2 and H3K9ac (Regha et al., 2007). The similar allele-specific histone modification patterns have been confirmed in other 15 ICRs (Mikkelsen et al., 2007), but the general role for this phenomenon is still unclear.

1.2.3 Non-coding RNAs

Genome wide studies have shown that most of the mammalian genome is transcribed and most of the transcripts are not protein-coding or non-coding RNAs (ncRNA; Amaral and Mattick, 2008; Carninci et al., 2005; Okazaki et al., 2002). At first, the large amount of ncRNAs were thought to be “transcription noise”, but recent studies indicated that these ncRNAs play critical roles in the regulation of gene expression involved in multiple physiology processes including but not limited to genomic imprinting, X-inactivation, gametogenesis, and embryonic development (Amaral and Mattick, 2008; Bartolomei and Ferguson-Smith, 2011; O'Neill, 2005).

According to the way they act, ncRNAs can be characterized into two classes: *trans*-acting RNA and *cis*-acting RNA. *Trans*-acting RNA can further divided into subclasses based on the way they mature and the length including short interfering RNAs (siRNA; 21bp), micro RNAs (miRNA; ~22bp), piwi-interacting RNA (piRNA: 26-31bp), and short nucleolar RNA (snoRNAs; 60-300bp). *Cis*-acting RNA so far has been only characterized as the long ncRNAs (lncRNA) which could be as long as hundreds thousands nucleotides. One of the best characterized lncRNA is *Xist* (inactive X-specific

transcript), an essential transcript for X-inactivation in female mammals (reviewed in Barakat and Gribnau, 2012).

The lncRNAs have also been linked to the regulation of genomic imprinting. Most imprinted genes are clustered and each cluster at least contains one lncRNA (Barlow, 2011; Bartolomei and Ferguson-Smith, 2011). The best studied lncRNAs in clusters of imprinted genes are *Airn* in the *Igf2r/Airn* cluster, *Kcnq1ot1* in the *Kcnq1* cluster, and *H19* in the *H19/Igf2* cluster. The *Igf2r* Region2 and KvDMR1 ICRs encompass the promoter of the lncRNA *Airn* and *Kcnq1ot1*, respectively, and are methylated on the maternal allele (Fitzpatrick et al., 2002; Wutz et al., 1997). *Airn* (108kb) and *Kcnq1ot1* (91kb) are expressed from the paternal allele where the ICR is not methylated (Lyle et al., 2000; Pandey et al., 2008). Deletion of the paternal ICRs abolished the paternal expression of *Airn* and *Kcnq1ot1*, which lead to the biallelic expression of flanking maternally-expressed genes on the paternal allele. However, deletion of the maternal ICRs had no effect on the imprinted expression of genes in the clusters (Fitzpatrick et al., 2002; Wutz et al., 1997). In the *H19/Igf2* cluster, the ICR is located 2kb upstream of the *H19* (2.2kb) transcription start site and approximately 90kb downstream of *Igf2*. *H19* is expressed from the maternal allele where the *H19* ICR is not methylated (Thorvaldsen et al., 1998). Although the regulation mechanism of each imprinting cluster is complex and different, they follow several general roles: (1) lncRNAs usually express from the parental allele where the ICR is unmethylated; (2) the unmethylated ICR is required for the expression of lncRNA; and (3) most protein-coding imprinted genes not expressed from the allele in which the lncRNA is expressed (Koerner et al., 2009).

The lncRNAs are required to initiate imprinted gene expression in an imprinting cluster. Truncation of the *Airn* or *Kcnq1ot1* transcripts leads to loss of imprinted gene expression of the maternally-expressed flanking genes regardless of an intact ICR (Mancini-Dinardo et al., 2006; Sleutels et al., 2002). Both *Kcnq1ot1* and *Airn* are known to recruit the histone modifiers Polycomb proteins (PcGs) which catalyze their repressive chromatin marks to organize a compacted nuclear compartment of the locus (Redrup et al., 2009; Terranova et al., 2008). Chromatin contraction brought about by the PcGs is able to exclude RNA polymerase II and leads to transcriptional silencing. *Kcnq1ot1* itself could escape chromatin contraction silencing probably because the enrichment of enhancer-specific modifications such as H3K27ac and H3K4me1 (Mohammad et al., 2012).

Although the essential role of lncRNAs in the initiation of imprinted gene expression has been well investigated, whether or not lncRNAs are required to maintain the imprinted expression is not fully known. Recent studies pointed out that maintenance of imprinting requires the continuous transcription of lncRNA. In *Airn/Igf2r* cluster, *Igf2r* silencing on the paternal allele is ubiquitous. Transcription of *Airn* overlaps the promoter of *Igf2r* but not the promoters of the other two imprinted genes *Slc22a2* and *Slc22a3* (Sleutels et al., 2002). In a recent study, different truncations of *Airn* were made to shorten the endogenous *Airn* transcript in ES cells (Latos et al., 2012). In that study, it was found that only *Airn* transcription overlaps of the *Igf2r* promoter is required for *Igf2r* silencing and this is independent of *Airn* product size and the nuclear compartment, which suggested that *Igf2r* silencing is mediated by the interference of RNA polymerase

instead of by repressive chromatin (Latos et al., 2012). In another study, the promoter of *Kcnq1ot1* was conditionally knocked out during different stages in embryonic development (Mohammad et al., 2012). The establishment of the ubiquitous and placental-specific imprinted expression in *Kcnq1* cluster is completed by E5.5. Deletion of *Kcnq1ot1* promoter on either E5.5 or E8.5 was able to lead to the relaxation of imprinting of ubiquitously but not placental-specific imprinted genes (Mohammad et al., 2012). This study indicated that continuous transcription of *Kcnq1ot1* is required to maintain the ubiquitously imprinted expression during the development and placental-specific imprinting might be maintained by the heritable repressive chromatin marks such as Polycomb proteins (Mohammad et al., 2012).

1.3 Genomic imprinting and human disease

1.3.1 General overview

In humans, misregulation of several clusters of imprinted genes has been found to be associated with one or more syndromes. The loss-of-imprinting (LOI) syndromes could be related to either genetic or epigenetic aspects.

For the epigenetic aspect, changes in DNA methylation or histone modifications at imprinting control regions lead to the altered expression of imprinted genes. It should be noted that more than one imprinting disorder could be related to a single imprinting cluster. For example, gain of methylation of the *H19* ICR is associated with the overgrowth condition Beckwith-Widemann syndrome but the loss of methylation of the

locus leads to the growth retardation condition Russell-Silver syndrome (Bartholdi et al., 2009; Blik et al., 2009; Smith et al., 2007).

The overall consequence of genetic disruption of an imprinting region is the misregulation of imprinted genes. The known genetic mutations include duplication/deletion, translocation/inversion, uniparental disomy (UPD), and mutation of imprinted genes. Duplication or deletion of chromosome 15q11-13 (also known as PWS/AS region) has been frequently observed in Prader-Willi syndrome (PWS) and Angelman syndrome (AS; Butler, 2011). Translocation/inversion are rare events but have been reported in imprinting disorders such as Beckwith-Wiedemann syndrome (Chiesa et al., 2012). UPD could also lead to a loss or gain of imprinted gene expression. For PWS/AS domain, paternal disomy is associated with AS, but maternal disomy is associated with PWS (Butler, 2011; Nicholls et al., 1989). In addition, imprinting disorders can be associated with mutation of imprinted genes. This molecular alteration could be found in familial pedigrees in which only one parental transmission of the mutation causes the imprinting syndromes.

1.3.2 Characteristics of Beckwith-Wiedemann syndrome

Beckwith-Wiedemann syndrome (BWS; OMIM 130650) was first reported by Drs. Beckwith and Wiedemann independently and was first known as EMG syndrome, which presented with exomphalos, macroglossia and gigantism (Beckwith, 1963; Wiedemann, 1964). BWS is a complex overgrowth condition in both phenotypical and genetic/epigenetic aspects.

BWS is the most common pediatric overgrowth syndrome which occurs in 1/13,700 individuals (Choufani et al., 2010; Pettenati et al., 1986; Thorburn et al., 1970). BWS is equally represented in males and females, but monozygotic twin females have a higher frequency for BWS. BWS is a heterogeneous syndrome that presents with primary and secondary features based on their rate of appearance (reviewed in Weksberg et al., 2005). The primary features of BWS includes macrosomia (gigantism; >97 percentile), macroglossia (enlarged tongue), and abdominal wall defects such as exomphalos and umbilical hernia. Exomphalos is a symptom that infant's intestines protrude out of belly button and are only covered by a thin layer of tissue. Umbilical hernia is a similar abnormality characterized as protrusion of part of abdominal organ around the belly button area. Macroglossia and abdominal wall defects could be corrected by surgery.

The secondary characteristics of BWS include hemihyperplasia (asymmetric overgrowth of part bodies), adrenocortical cytomegaly (abnormal enlargement of cells in adrenal cortex), visceromegaly (enlargement of organs such as liver, heart, and kidney), congenital renal abnormalities (kidney dysfunction), neonatal hypoglycemia (low level blood glucose associated with high level of insulin several hours after birth), ear anomalies (i.e. ear creases), and embryonic tumors (reviewed in Choufani et al., 2010; Weksberg et al., 2005).

BWS patients have an increased frequency (ranges from 4%-21% based on different studies) to develop tumors during the first five years of age including Wilms tumors of the kidney (67% of tumor cases), hepatoblastoma, rhabdomyosarcoma, neuroblastoma, and adrenocortical carcinoma (Rump et al., 2005). The highly variable

phenotypic features of BWS increase the difficulty for the diagnosis of BWS. A newborn child would be diagnosed as BWS if three primary features or at least two primary features and one secondary feature present (reviewed in Choufani et al., 2010; Weksberg et al., 2005)

1.3.3 Molecular alterations of BWS

Despite of the complex genetic/epigenetic features of BWS, the main genes implicated in the BWS have been mapped to the chromosome 11p15.5 which contains two imprinting domains regulated by their own imprinting centers (IC). The following will detail the molecular alterations in IC1 and IC2 observed in BWS. Discussion will begin with IC2 as it is the imprinted domain that most commonly associated with BWS.

IC2

IC2 contains one paternally-expressed noncoding RNA *KCNQ1OT1*, and up to six maternally-expressed protein coding genes including *CDKN1C*, *PHLDA2*, *KCNQ1*, *SLC22A18*, *MTR1* and *TSSC5* (Fig.1; Beatty et al., 2006; Horike et al., 2000). The ICR for this imprinting locus was first identified based on the studies of cells from BWS patients (Lee et al., 1999; Smilnich et al., 1999). The ICR, known as KvDMR1, is located in the 10th intron of the *KCNQ1* gene (Lee et al., 1999; Smilnich et al., 1999).

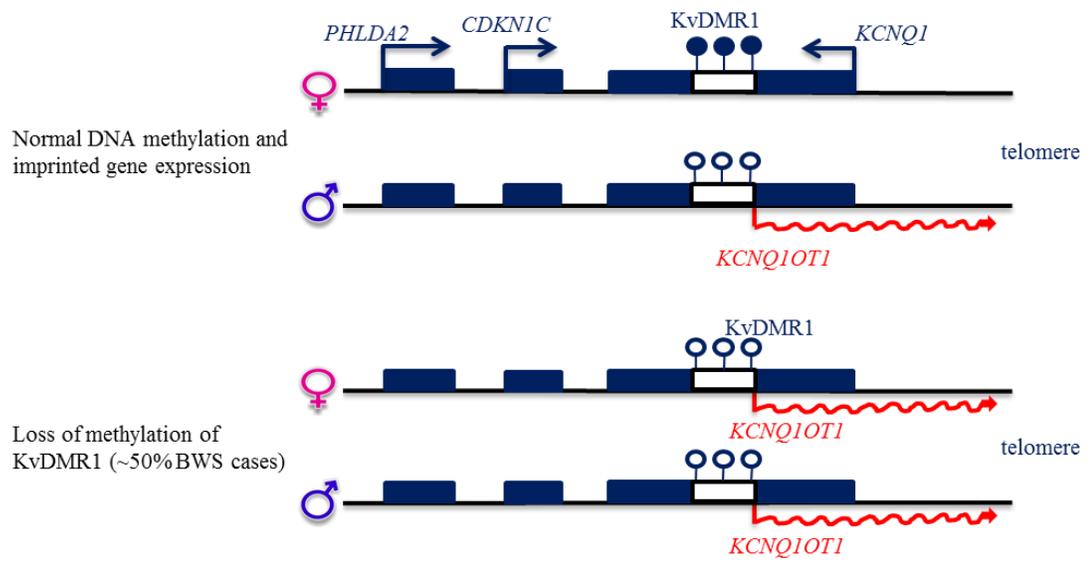


Figure 1.

Figure 1. The KvDMR1 domain (or IC2) on human chromosome 11p15. Female and male symbols represent maternal and paternal alleles, respectively. White box represents the differentially methylated imprinting control region KvDMR1 (closed lollipops = methylated CpGs; open lollipops = unmethylated CpGs). Not all the imprinted genes and non-imprinted genes in this region are shown here. The diagram is not drawn to scale. Normally, on the paternal allele KvDMR1 is unmethylated, and transcription of *KCNQ1OT1* represses the flanking maternally-expressed. *KCNQ1OT1* is transcribed in the antisense direction of the maternal gene *KCNQ1*. On the maternal allele, KvDMR1 is methylated, therefore *KCNQ1OT1* is not expressed and flanking maternal genes such as *CDKN1C* is expressed. In 50% BWS patients, KvDMR1 at both alleles are unmethylated, thus *KCNQ1OT1* is biallelically-expressed and flanking maternally-expressed genes are repressed. (Arrows = protein-coding gene; red scribbles = non-coding RNA)

The KvDMR1 is the promoter of *KCNQ1OT1* which is an antisense transcript to *KCNQ1*. On the paternal allele, KvDMR1 is unmethylated, and *KCNQ1OT1* is transcribed. However, on the maternal allele KvDMR1 is methylated, which is coupled with the silencing of *KCNQ1OT1* and the expression of neighboring maternally-expressed genes (Du et al., 2004; Lee et al., 1999; Smilnich et al., 1999). A targeted deletion of human KvDMR1 in recombination-proficient chicken DT40 cells led to the de-repression of the maternal genes such as *KCNQ1* and *CDKN1C* on the paternal chromosome (Horike et al., 2000). The syntenic region of IC2 is located on mouse chromosome 7, which is known as *Kcnq1* locus. The *Kcnq1* locus has been extensively studied to elucidate the imprinting mechanism. It should be noted that some genes in this locus such as *Ascl2*, *Tssc4*, and *Osbpl5* are imprinted in mouse but not in humans, but *MTR1* is imprinted in human but not mouse (Beatty et al., 2006; Fitzpatrick et al., 2002).

BWS associated alterations in IC2

The most frequent molecular alteration in BWS is the loss of methylation of the KvDMR1 on the normally methylated maternal allele, which is seen in 50% of sporadic BWS cases (Lee et al., 1999; Weksberg et al., 2001). In cells lines established from BWS patients with loss of methylation at the KvDMR1, biallelic expression of *KCNQ1OT1* is coupled with repressed *CDKN1C* expression (Diaz-Meyer et al., 2003). Similarly in mice, loss of methylation of KvDMR1 caused by targeted disruption of *Dnmts* is associated with biallelic expression of long noncoding RNA *Kcnq1ot1* and silencing of *Cdkn1c* (Kaneda et al., 2004; Sharif et al., 2007).

BWS has also been associated with genetic disruption of IC2. Paternal uniparental disomy for part of the chromosome 11p15 occurs in ~15-20% BWS cases. UPD usually affects both IC1 and IC2 resulting increased expression of *IGF2* and decreased level of *CDKN1C* (Weksberg et al., 2005). The *CDKN1C* mutation accounts for 5% sporadic BWS cases (Hatada et al., 1996; Lam et al., 1999; Li et al., 2001). In a familial BWS, only when mutated *CDKN1C* is inherited from the mother, the child exhibits BWS (Li et al., 2001). Recently, a BWS pedigree case was reported with an inverted duplication of 160kb IC2 containing the whole KvDMR1 and 5' end of *KCNQ1OT1* (Chiesa et al., 2012). Maternal transmission of the inverted duplication of 160kb was concomitant with discordant DNA methylation of KvDMR1 (i.e. the normal one is methylated, but the inverted one is unmethylated on the maternal allele. The discordant methylation of KvDMR1 led to the transcription of a truncated *KCNQ1OT1* on the maternal allele and resulted in the reduced expression of *CDKN1C* (Chiesa et al., 2012).

A series of studies in mice pointed out that *CDKN1C* is a key regulator in BWS cases. *CDKN1C* encodes a cyclin-dependent kinase inhibitor that negatively regulates cell division (Matsuoka et al., 1995; Tsugu et al., 2000). Genetic targeting of *Cdkn1c* in mice can lead to some characteristics seen in BWS such as abdominal muscle defects and cytomegaly (enlargement of part of body; Zhang et al., 1997).

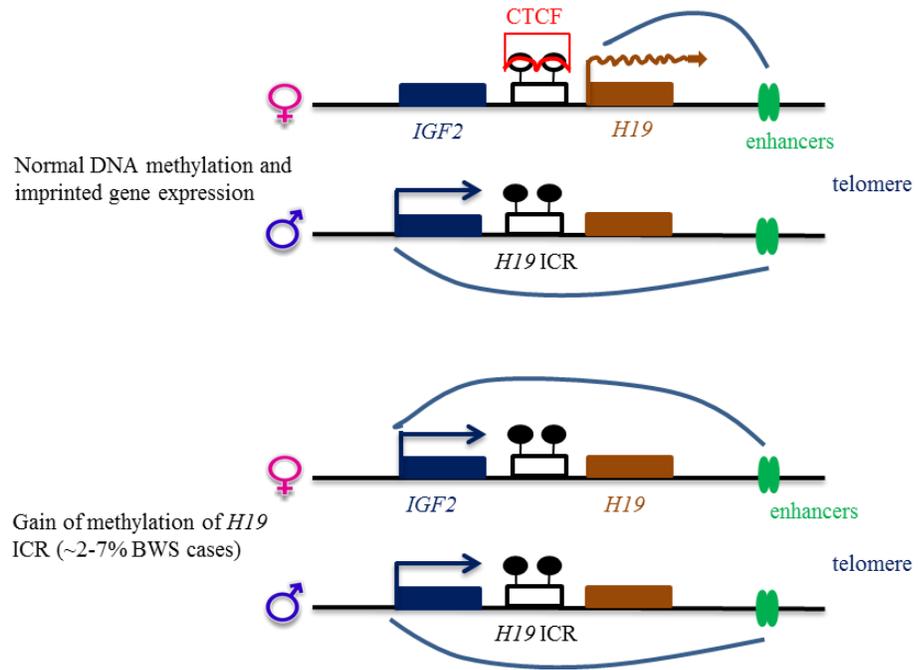


Figure 2.

Figure 2. *H19/IGF2* domain (or IC1) on human chromosome 11p15. Female and male symbols represent maternal and paternal alleles, respectively. White box represents the differentially methylated region *H19* ICR (closed lollipops = methylated CpGs; open lollipops = unmethylated CpGs). Normally, on the maternal allele *H19* ICR is unmethylated, which allows the binding of CTCF protein. CTCF acts as an insulator that prevents the interaction of the enhancers to the *IGF2* promoter. Thus *H19* is expressed while *IGF2* is repressed on the maternal allele. On the paternal allele, *H19* ICR is methylated, and the downstream enhancer interacts with *IGF2* instead of *H19*. Therefore *IGF2* is transcribed while *H19* is repressed. In 2-7% BWS patients, *H19* ICR at both alleles are methylated, thus *IGF2* is biallelically-expressed and *H19* is repressed. (Arrows = protein-coding gene; scribbles = non-coding RNA; green ovals = enhancers; curved lines = the intra-chromosome interaction). The diagram is not drawn to scale.

IC1

IC1 contains two main imprinted genes which are the paternally-expressed *IGF2* and the maternally-expressed *H19* (Fig.2). The ICR is located 2kb upstream of the *H19* transcription start site and ~120 kb downstream of *IGF2* (Thorvaldsen et al., 1998). *H19* is a spliced, RNA polymerase II transcribed non-coding RNA with five exons (Brannan et al., 1990; Juan et al., 2000). In mice, *H19* is highly expressed during embryogenesis and becomes repressed after birth. *H19* is only expressed in skeletal muscle and heart in adult mice (Poirier et al., 1991). The defined function of *H19* is not fully elucidated, although *H19* is one of the first identified imprinted genes in mice and human (Bartolomei et al., 1991; Zhang and Tycko, 1992).

The cytoplasm's localization of *H19* RNA excludes the possibility of its role in imprinting regulation *in cis* (Brannan et al., 1990). *H19* exhibited tumor suppressor activity in cultured human cancer cell lines derived from childhood kidney tumor and embryonic rhabdomyosarcoma (Hao et al., 1993). Moreover, repression of *H19* was observed in Wilms tumors (Dao et al., 1999; Frevel et al., 1999). Recently, a knock-out study of *H19* in mice suggested *H19* might regulate an imprinted gene network *in trans* (Gabory et al., 2009). The regulation activity of *H19* might be mediated by a microRNA cluster miR-675 located in *H19* exon1 (Keniry et al., 2012).

IGF2 plays vital roles in fetal growth and development. Misregulation of *IGF2* has been associated with growth disorders, cancer, and cardiovascular diseases (Bergman et al., 2012). In human, *IGF2* is encoded by 10 exons, which are modulated by four

promoters (P1-P4; Murrell et al., 2008; Vu and Hoffman, 1994). *IGF2* transcript from P1 is not imprinted which occurs in adult liver and chondrocyte, whereas transcripts starting from P2, P3, and P4 are always expressed from the paternal allele (Murrell et al., 2008; Vu and Hoffman, 1994). Similar to the *H19* expression pattern in mice, *IGF2* is highly expressed (predominantly with P3 and P4) during prenatal development, but it is down-regulated after birth in most tissues (Bergman et al., 2012). Tissue-specific imprinted expression of *IGF2* is regulated by three somatic DMRs (DMR0, DMR1, and DMR2) in which the differential DNA methylation is established post implantation (Murrell et al., 2008; Vu and Hoffman, 1994).

The reciprocal imprinted expression of *H19* and *IGF2* is regulated by an enhancer-competition model. The mechanism was established in a series of graceful mouse studies. In these experiments several key regions were identified including the ICR (Constancia et al., 2000; Leighton et al., 1995a; Sun et al., 1997; Thorvaldsen et al., 1998), the downstream enhancers located 3' of *H19* (Leighton et al., 1995c), and a *trans-acting* factor the "CCCTC" binding protein (CTCF) (Bell and Felsenfeld, 2000; Hark et al., 2000). On the maternal allele, the unmethylated ICR allows for the binding of CTCF protein, which prevents the access of the *Igf2* promoter to interact with downstream enhancers. The maternal *H19* accesses the enhancers and gets transcribed (Kurukuti et al., 2006). On the paternal allele, the ICR is methylated, blocking binding of the CTCF protein. The absence of CTCF protein on the paternal allele permits access of the *Igf2* promoter to the downstream enhancers and is transcribed while *H19* is repressed.

Recently, this “enhancer competition” model has been improved by identifying three other CTCF sites located *in cis* of the *H19/Igf2* cluster in both mice and humans. The CTCF sites are CTCF AD (CTCF binding upstream of 5’ end of *IGF2*), CTCF CCD (Centrally Conserved DNase I hypersensitive Domain) located between *IGF2* and the ICR, and CTCF DS (CTCF binding Downstream Site) located downstream the shared enhancers (Nativio et al., 2011; Nativio et al., 2009). The CTCF sites are not CpG rich and absent of DNA methylation, which allows for CTCF binding. Chromatin Conformation Capture (3C) assays indicated that co-localization of these CTCF sites brings these regions together and causes distinct chromatin loops on parental alleles (Engel et al., 2008; Nativio et al., 2011; Nativio et al., 2009).

BWS associated with alterations in ICI

Increased *IGF2* level caused by biallelic expression occurs in BWS cases. The molecular alteration could be either epigenetic or genetic. For the epigenetic aspect, gain of DNA methylation of the ICR occurs in approximately 2-7% BWS patients (Bliet et al., 2001; Cooper et al., 2005; Gaston et al., 2001; Weksberg et al., 2001). This epigenetic change could also be observed in sporadic Wilms tumor (Bjornsson et al., 2007; Steenman et al., 1994). As elucidated by the enhancer-competition model, this epimutation results in the biallelic expression of *IGF2* and repression of *H19*. In some cases, dysregulation of *IGF2* is independent of the regulation of *H19* ICR (Murrell et al., 2008), which might be explained by the somatic DMRs of the *IGF2* (Constancia et al., 2000) or an additional regulatory element that plays a role in controlling *IGF2* expression (Jones et al., 2001).

For the genetic aspect, maternal microdeletions of the ICR is relatively rare in BWS cases (<1%; Choufani et al., 2010). For these BWS cases, maternal CTCF binding sites are deleted which abolish the CTCF binding even the CpG-rich region is unmethylated (De Crescenzo et al., 2011; Sparago et al., 2004). CTCF binding site knock-out mice exhibited hypermethylation of the maternal alleles, which indicates that CTCF is required to maintain the unmethylated status (Engel et al., 2006; Schoenherr et al., 2003; Szabo et al., 2004). Similarly, in BWS patients with the microdeletions on the maternal allele also showed hypermethylation of the ICR (De Crescenzo et al., 2011; Sparago et al., 2004; Sparago et al., 2007). Recently, the ICR point mutation that disrupts octamer-binding transcription factor (OCT)-binding site but not CTCF binding site has been associated with BWS in *H19/IGF2* locus (Demars et al., 2010; Poole et al., 2012).

1.3.4 Mouse model for BWS

Several lines of genetically engineered mouse at the *Kcnq1* and *H19/Igf2* loci has been used extensively to explore the regulation mechanisms of genomic imprinting. Some mouse mutants with misregulated *Cdkn1c* and *Igf2* display various phenotypes of BWS. It was therefore proposed that these mouse mutants could be used as an animal model for BWS since the *CDKN1C* and *IGF2* are key regulators in the etiology of BWS in humans.

Mouse model with Cdkn1c deletion

Heterozygous mice with maternal deletion of *Cdkn1c* had the similar phenotypic defects to the homozygous *Cdkn1c* mutants (Yan et al., 1997; Zhang et al., 1997). This

was not observed when the deletion was inherited paternally. Most of the *Cdkn1c* mutant mice died around the time of birth. Many of the BWS defects including abdominal wall defects, cleft palate, and adrenal cytomegaly were observed in *Cdkn1c* mutants (Yan et al., 1997; Zhang et al., 1997). However, key features of BWS such as somatic overgrowth and macroglossia were not seen in these mice mutants, as well as embryonic tumors, neonatal hypoglycemia, and visceromegaly (Yan et al., 1997; Zhang et al., 1997). A recent *Cdkn1c* knock out study pointed out that intrauterine nutrient competition of large litter size might eliminate the potential for *Cdkn1c* null pups to develop somatic overgrowth since they found that *Cdkn1c* mutants can reach the intrinsic growth potential when the litter size is low (Tunster et al., 2011).

Mouse model with the overexpression of Igf2

R/H mice. Two loci were targeted in this mouse model. “R” refers to deletion of *Igf2r* which degrades excess *Igf2* via receptor-mediated endocytosis (Ludwig et al., 1996). “H” refers to deletion of the maternal *H19* and 10kb upstream region of *H19* which led to the de-repression of *Igf2* from the maternal alleles (Leighton et al., 1995a). On E12.5, approximately 11 fold higher level of *Igf2* in serum was present in R/H mice compared to controls (Eggenchwiler et al., 1997). For this mouse mutant, no pups survived beyond E18.5. This mouse model exhibited most BWS characteristics including somatic overgrowth, abdominal wall defects, visceromegaly, skeletal abnormalities, and cleft palate except macroglossia, renal dysplasia, embryonic tumors and adrenal cytomegaly (Eggenchwiler et al., 1997).

Igf2 transgenic mice. To produce mice with overexpression of *Igf2*, ES cell lines with exogenous *Igf2* were injected into the cavity of blastocyst to make chimeras (Sun et al., 1997). As the chimera developed, no transgenic *Igf2* was detected on E12.5 which was tagged with a marker sequence in 3' UTR, but endogenous *Igf2* became hyperactivated compared to the *Igf2* in control animals. This reason for this phenomenon is still unclear. Similar to other models, *Igf2* transgenic mice died around the time of birth. On E13.0, transgenic mice with 40-50% chimerism exhibited 116-131% overgrowth compared to controls. In addition to the somatic overgrowth, this mouse model also exhibited BWS features including macroglossia, visceromegaly. However, no abdominal wall defects, neonatal hypoglycemia, and hemihypertrophy were seen in this mouse model (Sun et al., 1997).

Mouse model with the loss of imprinting of Igf2 and null mutation of Cdkn1c

To better elucidate why *Igf2* overexpression mouse and null mutation of *Cdkn1c* mouse displayed distinct phenotypes, a double mutants with disruptions of both *Igf2* and *Cdkn1c* were generated, known as *p57H19* mutant. The *p57H19* mutant maternally inherited the deletion of *H19* region including the *H19 ICR* and a mutated *Cdkn1c*, which resulted in a gain of function of *Igf2* and loss of function of *Cdkn1c* (Caspary et al., 1999). This double mutant exhibited more severe phenotype than each single mutant. For example, no live offspring were recovered, and the parturition date was advanced at least one day earlier than *Cdkn1c* single mutant. The *p57H19* exhibited 20% increase in body weight at E16.5-17.5, but the body weight was indistinguishable from wild type offspring on E18.5. Other features such as macroglossia (122% of wild type littermates),

placentomegaly (190% of wild type littermates), kidney dysplasia and abdominal wall defects were observed in the double mutants.

In summary, these mouse models for BWS strongly support that imprinted genes are essential for normal embryonic development. However, these mouse models do not perfectly fulfill the criteria of an animal model for BWS. First, not all the main symptoms are present in each mouse model system, especially for embryonic tumors, which have an increased likelihood to be developed within the five years of age in BWS. The absence of tumors might be caused by early lethality of these mouse models in which most pups die around the time of birth. Second, the somatic overgrowth of *Igf2*-expressed mice is moderate, and most were determined before birth which is not strictly correlated to the birth weight since it was seen that prenatal overgrown phenotype could be compromised by intrauterine nutrient competition in large litter size (Tunster et al., 2011). Third and most importantly, these phenotypes are only observed by genetic manipulations (i.e. gene knockout) which do not mimic the epimutations naturally occurring in BWS.

1.3.5 Assisted reproductive technologies and BWS

Assisted reproductive technologies (ART)

Fertilization of a mammalian oocyte *in vitro* was first successful in rabbit (Chang, 1959). Later on, Professor Robert Edwards developed the *in vitro* fertilization techniques in human and gave rise to the first “test tube” baby Louis Brown in 1978. Since then, this technique has led to the birth of 4 million babies, and now ART accounts for ~1-5% births each year in developed countries (de Mouzon et al., 2010; Ferraretti et al., 2012).

ART is primarily used to overcome infertility/subfertility in order to help conceive and establish a successful pregnancy with the ultimate goal of giving rise to a healthy child. Generally, ART procedures include ovarian hyperstimulation (also known as superovulation), *in vitro* fertilization (IVF), intracytoplasmic sperm injection (ICSI), *in vitro* culture (IVC), and embryo transfer (ET). Ovarian hyperstimulation is used to increase the number of ovulations in order to improve the rate of pregnancy in each reproductive cycle. Ovarian hyperstimulation is accomplished in human by performing gonadotropin treatment. ICSI is a procedure in which a sperm head is injected into the cytoplasm of an oocyte, which circumvents the inability of the sperm to fertilize an egg due to morphological abnormalities. ICSI is the most used ART in human reproduction clinics and accounts for 60-80% ART procedures (Schultz and Williams, 2002). Following fertilization, embryos are subjected to be cultured *in vitro* for two or three days to cleavage stages and the “best” embryos based on morphology are selected for embryo transfer. A more recent trend is to culture the embryo for five or six days to blastocysts stage for prior to embryo transfer, which allows for the selection of the most competent embryos for further development based on their ability to reach the blastocyst stage (Iliadou et al., 2011; Maher, 2005). However, the concern for this strategy is that the overexposure of embryos *in vitro* may cause more detrimental damage of the embryos (Iliadou et al., 2011; Maher, 2005).

BWS associated with ART

Several retrospective studies have showed that children conceived with the use of ART have a 3 to 9 fold higher likelihood of being affected with BWS. These studies were

performed by different research groups in United States, United Kingdom, and Australia (DeBaun et al., 2003; Gicquel et al., 2003; Halliday et al., 2004; Lim et al., 2009; Maher et al., 2003). The common finding of these studies is the higher percentage of ART cases in BWS patients compared to the ART percentage in general populations. For example, Debaun *et al.* found that the prevalence of ART in BWS patients was 4.6% (3 of 65), which is almost 6 times higher compared to the ART rate in the general population (0.8%) in the United States (DeBaun et al., 2003). It should be noted that the majority (83%-100%) of ART-conceived BWS patients presented the hypomethylation of KvDMR1, which only occurs in about 50% sporadic BWS cases (Choufani et al., 2010). For example, Lim et al found that 24/25 ART-conceived BWS patients had a loss of methylation at the KvDMR1 on the maternal allele (Lim et al., 2009). More importantly, two of the 25 patients developed hepatoblastoma or rhabdomyosarcoma at one year of age, whereas no embryonic tumors were observed in non-ART BWS patients (Lim et al., 2009).

An alternative hypothesis for the increased incidence of BWS in ART might result from the infertility/subfertility of the parents rather than the ART procedures themselves (Iliadou et al., 2011; Maher, 2005). This is supported by an infertility mouse model which lacks the essential connections between the oocyte and granulosa cells (Carabatsos et al., 2000). In this model, maternal imprints acquisition of a paternally-expressed gene *Peg1* was disrupted (Denomme et al., 2012). However, a recent BWS case suggested that ART is still potentially responsible for the BWS (Kuentz et al., 2011). In that case, a human immunodeficiency virus serodiscordant couple sought for ART

assistance to conceive a child without the risk of HIV. The mother had conceived before, and the normal sperm morphology indicated that the father had no infertility problems. Both hyperstimulation and IVF protocols were used, and the child was born with macrosomia, macroglossia, visceromegaly, hypoglycemia, and hypomethylation of KvDMR1 (Kuentz et al., 2011).

Loss-of-imprinting associated with ART

Although there is still debate about association of ART and imprinting disorders in human, a large body of experiments demonstrated that ART could induce the misregulation of imprinted genes in animals including mice and ruminants (Denomme and Mann, 2012; Hori et al., 2010; Young et al., 2001). It is evident that superovulation contributes to the increased incidence of misregulation of imprinted genes in *in vitro*-derived mice (de Waal et al., 2012; Fortier et al., 2008; Market-Velker et al., 2010). Currently there are two hypotheses about how superovulation leads to loss-of-imprinting. The first hypothesis is that hormone hyperstimulation accelerates the oocyte growth and rescue the low quality oocytes that might otherwise never ovulate. The second hypothesis is that exposure oocytes to excess gonadotropins alters the molecular environment of oocyte and affects the imprints maintenance during later development (de Waal et al., 2012; Fortier et al., 2008; Market-Velker et al., 2010).

Support for the first hypothesis comes from the evidence that an altered DNA methylation pattern was observed in growing human and mouse oocytes subjected to gonadotropin treatment (Khoueiry et al., 2008; Sato et al., 2007). Support for the second

hypothesis comes from studies performed by de Waal *et al.*, In that study, it was observed that loss of methylation at the *H19* DMR (a primary imprint on the paternal allele), had occurred in offsprings from superovulated mice (de Waal *et al.*, 2012). Another report supporting the second hypothesis derives from a study in which oocytes subjected to gonadotropin treatment initially exhibited normal imprinting, but gave rise to embryos with loss-of-imprinting (Denomme *et al.*, 2011).

Besides the superovulation, procedure *in vitro* culture can also exacerbate the misregulation of imprinted genes (Rivera *et al.*, 2008). In addition, *in vitro* culture could have long term effect on the animal behavior such as spatial learning, and anxiety (Ecker *et al.*, 2004). It should be noted that imprinted genes in extraembryonic lineages such as the placenta are more likely to be affected than the fetus (Fortier *et al.*, 2008; Mann *et al.*, 2004; Rivera *et al.*, 2008). It's possible that the severe loss-of-imprinting in the placenta is because of the outer position of trophoblast in blastocyst (Mann *et al.*, 2004). However, similar loss-of-imprinting was also observed in another extraembryonic tissue, Yolk sac (Rivera *et al.*, 2008). Yolk sac is initially derived from primitive endoderm that resides in the inner space of the blastocyst, which could not be well explained by the "position" hypothesis. Another explanation hypothesized by Rivera *et al.*, is that imprinted genes are regulated differently in extraembryonic tissue compared to the fetus (Rivera *et al.*, 2008). For example, histone modification is required for the maintenance of the imprinted expression of *Kcnq1* domain on chromosome 7 in the placenta, whereas in the embryo, the repression is dependent on DNA methylation (Lewis *et al.*, 2004; Umlauf *et al.*, 2004).

1.4 Large offspring syndrome

Progresses in assisted reproduction in farm animals such as bovine and ovine have facilitated the genetic improvement rate by increasing the selection intensity and shortening generation intervals. Somatic cell nuclear transfer (SCNT), also known as cloning, has provided considerable advantages to generate genetic-engineered farm animals for agriculture and human medicine use. However, it has been increasingly reported that the manipulations of oocytes or preimplantation embryos, which are essential steps in these technologies, could result in unusually large offsprings in ruminants with various congenital abnormalities, known as large offspring syndrome (Young et al., 1998). It has been recognized that higher frequency of LOS is observed in cloning offsprings, and LOS occurs less frequently in individuals conceived with the *in vitro* embryo production systems. The severity phenotypes of LOS could potentially threaten the broad wide applications of the assisted reproductive technologies (cloning may not be included) in ruminants.

1.4.1 Characteristics of LOS

The major feature of LOS is an oversized offspring when compared to individuals conceived naturally. The average increase in birth weight of offsprings conceived with the use of ART ranges from 8% to 50% compared to controls. The variance depends on the ART procedures used (i.e. IVM, IVF, IVC), different types of controls used (i.e. natural mating, artificial insemination), and experiments performed in different laboratories (Young et al., 1998). It is not uncommon that offspring has more than twice

bodyweight than the mean of controls (Sinclair et al., 2000; Young et al., 1998). The overgrowth phenotype usually resulted in dystocia condition; therefore Caesarean section was necessary for offspring delivery. LOS offspring tend to have extended gestation, but the overgrowth was proved to be independent of gestation length (Walker et al., 1996). For offsprings from *in vitro* produced embryos with serum supplementation of culture media (no cloning procedure was used), 16% exhibited overgrown phenotype, but the rest of individuals appeared to be normal, which indicates that not all the offspring are equivalently affected (Hiendleder et al., 2004; Hiendleder et al., 2006). Indeed, it has been reported that full siblings from the same culture micro-drop could express different degrees of overgrown phenotype (Sinclair et al., 2000). At one year of age, the LOS calves had lost the advantage in bodyweight but continue to have abnormally large hearts when compared to controls (McEvoy et al., 1998).

One report shows that LOS can be already observed on day 12 of gestation during the time of conceptus elongation (Lazzari et al., 2002). The length and width of day 12 bovine conceptuses derived from *in vitro* production (pre-implantation embryos cultured in synthetic oviduct fluid (SOF) medium system supplemented with either BSA or human serum) were significantly larger than the sizes of embryos in *in vivo* group (pre-implantation embryos cultured in ligated oviducts of sheep; Lazzari et al., 2002). After these conceptuses were transferred into synchronized recipients and developed to term, the birth weight of SOF-serum and SOF-BSA group was significantly higher than the weight of the offspring from the “sheep oviduct” control group or the artificial insemination (AI) control group (Lazzari et al., 2002).

Another feature of LOS is visceromegaly (internal enlargement of organs in the abdomen), and the affected organs usually are liver and heart (Sinclair et al., 2000). It was first reported in ovine that a period of *in vitro* culture could result in the allometric growth of liver and heart (different growth rate of the different part in the same organism) (Sinclair et al., 1999). Similar enlargement of liver and heart was also seen in mice with double mutation of *H19* and *Igf2r*, and visceromegaly is also a common feature of BWS cases (Eggenschwiler et al., 1997; Weksberg et al., 2005).

A series of adverse peri-natal outcomes has been reported in LOS. In a bovine study, 85% of offspring generated with the use of nuclear transfer exhibited misregulation of metabolic activity including hypoglycemia (Garry et al., 1996). During the normal process of delivery, the plasma insulin concentration decreases (Symonds, 1995). However, plasma insulin concentrations of cloned calves during the first several hours after birth were almost four times higher than those controls (Garry et al., 1996). The proportion of peri-natal death associated with LOS varies from 9-50%, potentially due to complications of overgrowth caused physiology problems (Behboodi et al., 1995; Hasler et al., 1995; Sinclair et al., 2000; van Wagendonk-de Leeuw et al., 1998). In addition, a range of congenital abnormalities were observed in LOS including cleft palates (Schmidt et al., 1996), skeletal defects (Garry et al., 1996; Wells et al., 1997), and cerebellar hypoplasia (missing or smaller cerebellum; Garry et al., 1996).

LOS has also been associated with placental dysfunctions such as polyhydraminos (Sinclair et al., 1999). Polyhydraminos is described as excess fluid in the amnionic sac, which is also commonly associated with BWS in human (Choufani et al., 2010). In sheep

LOS, the incidence of polyhydraminos is about 36-62% in sheep pregnancies established by *in vitro* produced embryos (Sinclair et al., 1999).

1.4.2 Embryo culture can induce LOS.

General procedures of *in vitro* embryos production in ruminant species includes: oocyte *in vitro* maturation (IVM; ~22 hours in bovine), *in vitro* fertilization (IVF; ~18 hours in bovine) and embryo culture (IVC), and embryo transfer at the blastocyst stage (7-9 day after fertilization for bovine; Rivera et al., 2003). The exposure of oocytes or early embryos at certain stages to the environment inequivalent to *in vivo* condition could be the main cause of LOS. Many studies have reported that embryos cultured with serum supplementation and /or co-culture with somatic cells (i.e. ovarian epithelial cells) tended to develop LOS (Holm et al., 1996; McEvoy et al., 2000; Sinclair et al., 1999; Thompson et al., 1995). It was suggested that ruminant blastocysts in culture media with serum or somatic cells appeared darker due to excess lipid accumulation in the cytoplasm (Ferguson and Leese, 1999; Thompson et al., 1995). The accumulation of the lipid droplet was considered to be harmful embryos and disrupt osmotic regulation (Thompson, 1997). Besides the effect of media components, the time period of exposure of embryos to the serum/somatic cells is critical for the induction of LOS. An ovine experiment showed that ovine embryos are more sensitive to the presence of serum in culture medium before rather than after the compaction (Rooke et al., 2007). Overall, LOS exhibits unpredictable phenotypes and the broad range characteristics even the similar protocols and media were used to produce embryos. How these ART procedures manipulations cause the LOS is still unknown (Farin et al., 2004; Farin et al., 2006;

Sinclair et al., 2000; Wrenzycki et al., 2004). Similar to LOS, BWS also displays unpredictable occurrence. Currently, there is no substantiated strategy to diagnose BWS during early stages (i.e. pre-implantation stage) due to the “random” property of BWS.

1.4.3 Potential molecular alterations of LOS.

To elucidate the potential molecular alterations responsible for LOS, a large body of experiments has been performed to compare the difference of gene expression in *in vitro*/SCNT ruminant embryos with *in vivo*-derived embryos, and it was found that both imprinted and non-imprinted genes are affected (Bertolini et al., 2002; Lazzari et al., 2002; Niemann et al., 2002; Rizos et al., 2002; Wrenzycki et al., 1998; Wrenzycki et al., 2001; Wrenzycki et al., 2002). For the non-imprinted genes affected by *in vitro* procedures, they are involved in numerous of biological processes such as morula compaction/cavitation, metabolism, trophoblastic function and growth factor signaling (Wrenzycki et al., 2004).

For the imprinted genes, *IGF2* pathway was extensively studied since misregulation of *IGF2* in human can result in a similar overgrowth condition Beckwith-Wiedemann (Shuman et al., 1993). Compared to *in vivo* derived bovine blastocysts, the *in vitro* group had lower expression level of *IGF2* and *IGF2r* (Bertolini et al., 2002). However, another group reported that there was no difference of *IGF2r* expression level between *in vivo* and *in vitro* derived bovine blastocysts (Wrenzycki et al., 2001). In addition, on day 70 of development (~25% of gestation length), *in vitro* produced bovine

fetuses had higher *IGF2* mRNA level in the liver and lower level in skeletal muscle when compared to counterpart control fetuses (Blondin et al., 2000).

Since not every offspring from the *in vitro* group develop into LOS, it is more appropriate to divide individuals into LOS group and non-LOS group and compare the gene expression level instead of simply comparing the difference between *in vivo* and *in vitro* derived embryos. In an ovine study, late gestation (day 125; ovine gestation=145 days) fetuses with LOS (bodyweight >5.5kg; bodyweight of the largest *in vivo* fetus is 4.5kg) were compared to controls (Young et al., 2001). It was found that LOS fetuses had lower *IGF2r* mRNA and protein level in the liver and muscle tissues compared to controls (Young et al., 2001). However, there was no difference of *IGF2* mRNA level between LOS and control fetuses. The *in vitro* derived non-LOS ovine fetuses exhibited no difference of *IGF2r* mRNA and protein level compared to the controls (Young et al., 2001). Recently, Hori, *et al* observed that hypomethylation at the KvDMR1 and decreased expression of *CDKN1C* in two LOS calves, one is a cloned calf, and the other is derived by *in vitro* production (Hori et al., 2010). This study highlighted the similar molecular alteration of KvDMR1 in LOS and BWS, although parental-specific epimutations were not determined in their experiment.

In summary, LOS is an overgrowth condition in ruminants with variable abnormalities, which could be induced by *in vitro* embryo culture or SCNT. The molecular mechanism of LOS is unknown, although some evidences suggested a potential role of imprinted genes in the programming of LOS.

1.5 Rationale for Thesis

BWS is one of the most common pediatric overgrowth conditions in humans. Children conceived with the use of ART have an increased likelihood of having BWS. The molecular alterations of BWS have been mapped to human chromosome 11p15. Loss of methylation at the KvDMR1 is the most frequent molecular lesion in BWS which is observed in 50% of patients. Gain of methylation of *H19* ICR accounts for up to 7% of BWS. However, only associations exist between these LOI and BWS, and no direct evidence exists that points to how these LOI triggers the variable BWS phenotypes. In addition, there is no animal model available to study the etiology of BWS.

LOS is an overgrowth condition in ruminant that recapitulates the phenotypes of BWS. Similarly, LOS is known to be induced by ART procedures. Although it was hypothesized that LOS shares the similar LOI as BWS, no direct evidence is available for the hypothesis. Previously, Hori *et al.*, observed hypomethylation of KvDMR1 in two LOS calves. However, in that experiment, results were shown without allele specificity. Previous study conducted in our laboratory showed that DNA methylation and expression of imprinted genes associated with BWS are conserved between human and bovine.

In the current study, the main goal is to determine the allele-specific methylation and expression of imprinted genes associated with BWS in LOS. This will allow us to determine the parental origin of the allele with the imprinting abnormalities and to test whether in LOS bovine is an appropriate animal model to study BWS.

Chapter 2 Large offspring syndrome, a bovine model for human loss-of-imprinting overgrowth syndrome Beckwith-Wiedemann

Abstract

Beckwith-Wiedemann syndrome (BWS) is a human loss-of-imprinting syndrome primarily characterized by macrosomia, macroglossia, and abdominal wall defects. BWS has been associated with misregulation of two clusters of imprinted genes. Children conceived with the use of assisted reproductive technologies (ART) appear to have an increased incidence of BWS. As in humans, ART can also induce a similar overgrowth syndrome in ruminants which is referred to as large offspring syndrome (LOS). The main goal of our study is to determine if LOS shows similar loss-of-imprinting at loci known to be misregulated in BWS. To test this, *Bos taurus indicus* × *Bos taurus taurus* F1 hybrids were generated by artificial insemination (AI; control) or by ART. Seven of the 27 conceptuses in the ART group were in the > 97th percentile body weight when compared to controls. Further, other characteristics reported in BWS were observed in the ART group, such as large tongue, umbilical hernia, and ear malformations. *KCNQ1OT1* (the most-often misregulated imprinted gene in BWS) was biallelically-expressed in various organs in two out of seven overgrown conceptuses from the ART group, but shows monoallelic expression in all tissues of the AI conceptuses. Furthermore, biallelic expression of *KCNQ1OT1* is associated with loss of methylation at the KvDMR1 on the maternal allele and with down-regulation of the maternally-expressed gene *CDKN1C*. In conclusion, our results show phenotypic and epigenetic similarities between LOS and

BWS, and we propose the use of LOS as an animal model to investigate the etiology of BWS.

Introduction

Beckwith-Wiedemann syndrome (BWS) (OMIM 130650) is a pediatric overgrowth condition with an occurrence of 1 in 13,700 natural births (Choufani et al., 2010; Weksberg et al., 2009). BWS is a complex syndrome and has highly variable clinical features (Choufani et al., 2010; Weksberg et al., 2009). The primary features of BWS include macrosomia (overgrown bodyweight > 97th percentile), macroglossia (enlarged tongue), and abdominal wall defects (umbilical hernia; Choufani et al., 2010; Weksberg et al., 2009). Secondary characteristics such as ear malformations, visceromegaly, neonatal hypoglycemia, and nevus flammeus are less frequently observed in BWS patients (Choufani et al., 2010; Weksberg et al., 2009). In addition, BWS is associated with increased risk of childhood tumors (rate ranges from 4% to 21%), with Wilms' tumor of kidney and hepatoblastoma being the two most commonly observed (Choufani et al., 2010; Rump et al., 2005).

Genomic imprinting is a series of epigenetic processes that lead to parental-allele-specific gene expression in mammals (Edwards and Ferguson-Smith, 2007; Schulz et al., 2008; Verona et al., 2003). Because of genomic imprinting, both maternal and paternal genomes are required for embryonic growth and development. Of the identified imprinted genes, most are found in clusters containing two or more imprinted genes in an imprinting domain which is in turn regulated by a differentially methylated region of DNA known as the imprinting control region (ICR; Edwards and Ferguson-Smith, 2007;

Schulz et al., 2008; Verona et al., 2003). The parental-allele-specific DNA methylation of the ICRs is erased in primordial germ cells and re-established during prospermatogenesis in male and oocyte growth in female (Edwards and Ferguson-Smith, 2007; Schulz et al., 2008; Verona et al., 2003).

The molecular alterations responsible for BWS have been mapped to chromosome region 11p15 (syntenic to mouse chromosome 7) which has two imprinting clusters: imprinting center 1 (IC1) and IC2 (Choufani et al., 2010; Weksberg et al., 2009). In humans, IC2 contains one paternally-expressed non-coding RNA (ncRNA) and at least six maternally-expressed protein-coding genes (Weksberg et al., 2005), and this cluster is regulated by the ICR referred to as KvDMR1. The KvDMR1 is unmethylated on the paternal chromosome. In mice, it has been shown that unmethylated KvDMR1 permits the transcription of long non-coding RNA *Kcnq1ot1*, which recruits the Polycomb group proteins (such as Ezh2 and Rnf2) and repressive histone marks (such as H3K27me3 and H2AK119U1) to create a repressive chromatin conformation where maternally-expressed genes are located and repressed on the paternal allele (Pandey et al., 2008; Terranova et al., 2008). However, on the maternal allele, methylation of the KvDMR1 prevents the transcription of the *Kcnq1ot1* gene; therefore, flanking maternally-expressed genes such as *Cdkn1c*, *Kcnq1*, and *Phlda2* are transcribed (Fitzpatrick et al., 2002; Horike et al., 2000; Thakur et al., 2003). About 50% of naturally occurring BWS cases present with loss of methylation at KvDMR1 on the maternal allele, which is coupled with biallelic expression of *KCNQ1OT1* and down-regulation of *CDKN1C* (Choufani et al., 2010; Weksberg et al., 2009). Reports estimated a 3- to 9- fold increased likelihood of

BWS in children conceived with the use of assisted reproductive technologies (ART) (DeBaun et al., 2003; Gicquel et al., 2003; Halliday et al., 2004; Lim et al., 2009; Maher et al., 2003; Sutcliffe et al., 2006). Currently, determination of the methylation status of the KvDMR1 is the most often used procedure to associate the syndrome to a molecular lesion.

IC1 contains the maternally-expressed ncRNA *H19* (Bartolomei et al., 1991) and the paternally-expressed fetal growth factor, *IGF2* (DeChiara et al., 1991). The imprinting mechanism of this locus has been elucidated in a series of mouse experiments. On the maternal allele, *H19/Igf2* ICR is unmethylated, allowing for the binding of the insulator protein CTCF (CCCTC binding factor). This binding results in a chromosome boundary which prevents the interaction of the *Igf2* promoter with the downstream enhancers. Thus, *H19* has access to the enhancer and is transcribed. However, on the paternal allele, *H19/Igf2* ICR is methylated which avoids the binding of CTCF protein (Hark et al., 2000), therefore, allowing the *Igf2*'s promoter to interact with the downstream enhancers. Approximately 2-7% of naturally-conceived BWS cases have a gain of methylation on *H19/IGF2* ICR, which is associated with the increased expression level of *IGF2* (Choufani et al., 2010; Weksberg et al., 2009).

Large offspring syndrome is an overgrowth disorder in ruminants which is phenotypically similar to BWS (Young et al., 1998). The features of LOS include: excessive birth weight, large tongues, umbilical hernia, hypoglycemia, and visceromegaly (Bertolini and Anderson, 2002; Bertolini et al., 2002; Farin et al., 2006; Hiendleder et al., 2004; Hiendleder et al., 2006; Miles et al., 2004; Young et al., 1998).

As in BWS, LOS can result from ART (Bertolini and Anderson, 2002; Bertolini et al., 2002; Farin et al., 2006; Hiendleder et al., 2004; Hiendleder et al., 2006; Miles et al., 2004; Young et al., 1998). Previous studies observed hypomethylation of the KvDMR1 and biallelic expression of *KCNQ1OT1* in somatic nuclear transfer (SCNT) and ART-produced bovine conceptuses (Couldrey and Lee, 2010; Hori et al., 2010). However, ascription of parental origin to the alleles during methylation studies has been difficult as a result of the polymorphic nature of cattle, which is similar to the situation in humans. A previous study performed in our laboratory showed that allelic expression of *KCNQ1OT1*, *CDKN1C*, and *H19* and DNA methylation of the KvDMR1 and *H19/IGF2* ICR in day 65 bovine conceptuses is conserved to humans (Robbins et al., 2012).

Given the similarities between BWS and LOS, and together with previous studies (Couldrey and Lee, 2010; Hori et al., 2010; Robbins et al., 2012) we hypothesized that bovine conceptuses with the overgrowth phenotype would have similar misregulation of imprinted loci as those reported for the human overgrowth condition BWS. In the present study, we used *B. t. indicus* × *B. t. taurus* F1 hybrid conceptuses produced by ART. We determined the allele-specific DNA methylation and expression of imprinted genes in IC1 and IC2 by using the identified (Robbins et al., 2012) fixed polymorphisms between the two subspecies of cattle. We show that LOS conceptuses at day ~105 resemble the phenotype of BWS. Most importantly, two LOS conceptuses display biallelic expression of the ncRNA *KCNQ1OT*, which is coupled with loss of methylation of KvDMR1 and down-regulation of *CDKN1C*.

Results

Generation of LOS conceptuses

To determine if the IC2 and IC1 are misregulated in LOS as in BWS, we generated LOS conceptuses with the use of ART procedures known to induce the syndrome in bovine (Bertolini and Anderson, 2002; Bertolini et al., 2002; Farin et al., 2006; Hiendleder et al., 2004; Hiendleder et al., 2006; Miles et al., 2004; Young et al., 1998). Based on published observations (Hiendleder et al., 2004; Hiendleder et al., 2006) we expected that 16% of our ART conceptuses would be of the overgrown phenotype. LOS conceptuses were collected at day ~105; when features of LOS can be first characterized (Breukelman et al., 2004). We used *B. t. indicus* × *B. t. taurus* F1 hybrid conceptuses which allowed us to distinguish paternal and maternal alleles based on fixed polymorphisms at these loci between the two subspecies of cattle (Robbins et al., 2012). *B. t. indicus* × *B. t. taurus* F1 hybrid control conceptuses were produced by artificial insemination (AI). Twenty-seven conceptuses were collected from the ART group and nine from the AI group (Fig. 1A). Average bodyweight, organ weight (liver, heart, lung, kidney, spleen, and tongue), crown-rump length, foreleg length, and head width were not significantly different ($P > 0.05$) between groups (data not shown). Body weight did not differ between singletons and twins. However, heart girth (an indirect measure of body weight) of ART conceptuses was significantly larger than AI conceptuses ($P < 0.03$; means \pm SEM = 16.42 ± 0.19 vs. 15.53 ± 0.32 cm, for ART and AI group, respectively).

Children with at least three primary features or two primary features and one or more secondary features are diagnosed as BWS patients (Choufani et al., 2010; Weksberg

et al., 2009). In the present study, we used the overgrown feature (bodyweight > 97th percentile) as a major criterion to diagnose fetuses with LOS. The 97th percentile was calculated based on the bodyweight of AI conceptuses, and this explains why one AI conceptus (AI-C010) was also above the bodyweight 97th percentile (Fig. 1A). For the ART conceptuses, seven out of 27 (26%) were above 97th percentile, and females showed a greater variability in bodyweight than males (range – male = 372-584 g and female = 352-714 g; Fig. 1A). Besides increased bodyweight, other features of LOS were also observed in the ART conceptuses (Fig. 1B) including enlarged tongue (macroglossia; n=3), umbilical hernia (n=2; data not shown), and ear malformation (n=1).

Expression analysis of imprinted genes

To test if BWS-associated imprinted genes are similarly misregulated in LOS, we determined allelic expression of six imprinted genes in liver, muscle, brain, tongue, heart, lung, kidney, and placenta (Fig. 2). Four of these genes, *CDKN1C*, *KCNQ1*, *PHLDA2*, and *H19* are expressed from the maternal chromosome, whereas *KCNQ1OT1* and *IGF2* are expressed from the paternal chromosome. Fifty percent of naturally-conceived BWS patients show loss of methylation on the maternal allele of the differentially methylated region known as KvDMR1, and this loss-of-imprinting is correlated with biallelic expression of *KCNQ1OT1* (Choufani et al., 2010; Weksberg et al., 2009). *KCNQ1OT1* was biallelically-expressed in several tissues in two (ART-J835LOS and ART-J489ALOS) of the seven overgrown conceptuses from the ART group, but showed monoallelic expression in all tissues of the AI conceptuses (Fig. 3, Supplemental Fig. 1 and Table S1.1). *H19*, *IGF2*, and *CDKN1C* were imprinted for both ART and AI groups

in liver, muscle, tongue, heart, lung, kidney, and placenta. However, *CDKN1C* and *IGF2* was expressed from both parental alleles in the brain of fetuses from both groups (Supplemental Table S1.2, S1.4-S1.5). *PHLDA2* only showed monoallelic expression in liver and placenta in both groups, but was biallelically-expressed in other tissues in both conditions (Supplemental Table S1.4). *KCNQ1* showed global biallelic expression with a bias towards the maternal allele in both groups (Supplemental Table S1.3).

Since biallelic expression of *KCNQ1OT1* is associated with the repressed expression of *CDKN1C* from the maternal allele, we then performed quantitative RT-PCR to determine *CDKN1C* mRNA levels in tissues with biallelic expression of *KCNQ1OT1*. We compared *CDKN1C* expression level in each of the two LOS conceptuses (ART-J835LOS and ART-J489ALOS) with the average level of transcript of eight AI conceptuses. To get a better understanding of whether *CDKN1C*'s expression is directly affected by the expression of *KCNQ1OT1* from the maternal allele or if it is an artifact of the ART procedures we compared the *CDKN1C* expression of ART-J835LOS and ART-J489ALOS to the five remaining LOS conceptuses. We found that the level of the *CDKN1C* RNA of ART-J835LOS and ART-J489ALOS was lower when compared to the average expression of the AI controls and the average expression of the monoallelic LOS group (Fig. 4A). *CDKN1C* expression level in the AI group and LOS group with correct imprinting of *KCNQ1OT1* was comparable in most tissues except in the placenta where the LOS group is higher (Fig. 4B; $P < 0.05$). It should be noted that, the AI-C010 fetus (bodyweight > 97 percentile) was included in the eight fetuses analyzed and its *CDKN1C* expression distributed randomly among the AI controls and never occupied an

extreme position. We next queried about the level of *CDKN1C* expression between tissues in control fetuses and we found that this gene is expressed at lower levels in brain and heart when compared to other tissues (Fig. 4B). We also analyzed the expression level of *PHLDA2*, which is a maternally-expressed gene and expected to be regulated similar to *CDKN1C*. ART-J835LOS showed down-regulation of *PHLDA2* in all tissues except muscle, while expression of this gene was only down-regulated in liver, heart and lung in ART-J489ALOS (Supplemental Fig. 2A). In contrast to what we observed for *CDKN1C*, *PHLDA2* showed different expression level in the various tissues analyzed with highest expression observed in the kidney and placenta (Supplemental Fig. 2B). As 2-7% of BWS cases are associated with biallelic expression of *IGF2*, which leads to increased mRNA level of *IGF2* (Choufani et al., 2010; Weksberg et al., 2009), we also determined *IGF2* expression level in LOS conceptuses. *IGF2* transcript levels were similar between LOS and the AI control group (data not shown).

Methylation analyses of KvDMR1, *H19/IGF2* ICR and *CDKN1C* exon 2

Loss of methylation of KvDMR1 is the most common epimutation in BWS (Choufani et al., 2010; Weksberg et al., 2009). Sodium bisulfite mutagenesis was used to investigate the methylation status of the KvDMR1 in the tissues of fetuses that had maternal *KCNQ1OT1* expression. We determined methylation status of a 385 bp region containing 37 CpGs. We show that in most tissues, loss of methylation on the maternal allele was coupled with biallelic expression of *KCNQ1OT1* in these fetuses (Fig. 5 and Supplemental Fig. 3). Interestingly, the placental tissue of AI-C010, the largest AI

conceptus in the control group, also showed reduced methylation of the KvDMR1 on the maternal allele (Fig. 5).

The *H19/IGF2* ICR is normally unmethylated on the maternal allele but methylated on the paternal allele. We then asked if the biallelic expression of *IGF2* in the brain of the fetuses studied was associated with gain of methylation of *H19/IGF2* ICR on the maternal alleles. Here we show that *H19/IGF2* ICR had differential methylation in brain samples where *IGF2* was biallelically-expressed (Supplemental Fig. 5).

In mice, differential methylation of *Cdkn1c* region was observed from -600 bp from the transcription start site to exon 2 (Bhogal et al., 2004). *Cdkn1c* DMR is a somatic imprint (Bhogal et al., 2004), and therefore is established after implantation in mice (Bhogal et al., 2004; John and Lefebvre, 2011). However, the homologous region in humans is unmethylated on both alleles (Chung et al., 1996; Monk et al., 2006). In the present study, biallelic expression of *CDKN1C* was observed in brain in both ART and AI conditions. We then asked if DNA methylation is involved in the regulation of *CDKN1C* imprinting. Currently, DNA sequence information in the upstream region of *CDKN1C* in bovine (GenBank accession no. NW_003104648.1: 2774900-2775500) harbors a sequencing gap, and we were unable to amplify the 5' end of *CDKN1C*. Therefore, we focused on the *CDKN1C* region encompassing exon 1 to exon 2. This region in the bovine (GenBank accession no. NW_003104648.1: 2775692-2776645) has 78% identity to the locus in humans (GenBank accession no. NT_009237.18: 2847003-2846299). We determined methylation status of a 363 bp region located in the second exon which contains 48 CpG dinucleotides. Since no SNPs between *B. t. indicus* and *B. t.*

taurus are available at this locus (Robbins et al., 2012), we used Sanger sequencing for direct sequencing of the PCR product in order to investigate the potential of differential methylation which would be identifiable as a double peak (*i.e.* one for C and one for T) in the chromatograph. Sequencing results showed hypomethylation in both muscle, in which *CDKN1C* is normally imprinted, and brain, where *CDKN1C* is biallelically-expressed (Fig. 6A-B and Supplemental Table S1.2). It is conceivable that only the unmethylated maternal alleles were amplified because of a bias introduced during PCR amplification. To exclude this possibility, we co-incubated genomic DNA with the methyltransferase Sss1 (Matsuo et al., 1994) prior to performing bisulfite mutagenesis. Sss1 treatment combined with COBRA showed that no bias was introduced during PCR amplification as both methylated and unmethylated DNA were similarly amplified (Fig. 6C).

Discussion

In the present study we show that the bovine model of LOS has extensive similarities with BWS. Phenotypically, LOS exhibited macrosomia, macroglossia, and umbilical hernia, which are primary characteristics of BWS (Choufani et al., 2010; Weksberg et al., 2009). Additionally, a secondary feature of BWS, namely ear malformations, was also observed in LOS (Choufani et al., 2010; Weksberg et al., 2009). At the epigenetic level, two LOS conceptuses showed loss of methylation at the KvDMR1 on the maternal allele. This epimutation is the major molecular signature of BWS (Choufani et al., 2010; Weksberg et al., 2009) and is observed in 50% of individuals afflicted with this syndrome (Choufani et al., 2010; Weksberg et al., 2009). Furthermore, down-regulation of *CDKN1C* was observed in conceptuses with biallelic

expression of *KCNQ1OT1*. This is a finding similar to what have been reported using skin fibroblasts from BWS patients with loss of methylation at the KvDMR1 (Chiesa et al., 2012).

It should also be noted that in our study, female conceptuses showed greater variability in bodyweight when compared to males. In humans, it has been observed that monozygotic female twins have a higher frequency of BWS cases than monozygotic male twins (Weksberg et al., 2002). Likewise, the monozygotic female twins with BWS were generally associated with loss of methylation of the KvDMR1 on the maternal allele (Weksberg et al., 2002). Weksberg and others (Weksberg et al., 2002) suggested that the higher frequency of BWS in monozygotic female twins might be partially explained by the overlap in timing of X-inactivation and global DNA methylation reprogramming in the preimplantation period.

IGF2 was exclusively expressed from the paternal chromosome except in brain where it had biallelic expression. This is in accordance with previous studies where *IGF2* showed biallelic expression in brain in both mice and cattle (Curchoe et al., 2005; Hemberger et al., 1998). Similarly, in our study, *KCNQ1* was biallelically-expressed in all tissues analyzed which is consistent with the situation in the mouse, where even though *Kcnq1* is maternally-expressed at midgestation, it is globally biallelically-expressed at birth (Gould and Pfeifer, 1998; Paulsen et al., 1998).

Even though it is accepted (Choufani et al., 2010; Weksberg et al., 2009) that BWS is associated with misregulation of one or more imprinted genes in one or more imprinting clusters, several pivotal questions remain unanswered about this overgrowth

syndrome. First, what are the cellular and molecular alterations causing loss-of-imprinting at the specified loci? Second, how does misregulation of imprinted gene expression translates into the highly variable and complex phenotypes of BWS?

At present, only associations exist between loss of methylation at specific imprinting centers and BWS. No evidence exists that points to any particular genomic region which when epimutated, triggers the overgrowth phenotype and associated developmental errors in humans. It is not known if loss-of-imprinting is the cause or a symptom of BWS. Currently, no animal model exists that faithfully recapitulates the various phenotypic and epigenetic singularities of BWS. Several mouse models for this syndrome have been generated by introducing genetic mutations into IC1 and/or IC2. The genetic mutations include: maternal mutation of *Cdkn1c* (Tunster et al., 2011), double mutation including *H19* Δ 13 (deletion of *H19/Igf2* ICR and *H19*) and *Cdkn1c* (Casparly et al., 1999), maternal *H19* Δ 13 which leads to higher expression level of *Igf2* (Eggenchwiler et al., 1997; Leighton et al., 1995b), and overexpression of *Igf2* (Sun et al., 1997). All these mouse models provided fundamental understanding of the essential function of imprinted genes in embryonic development as well as mechanisms of genomic imprinting regulation. However, these models did not phenocopy the overgrowth as well as other the primary and secondary characteristics of BWS. For example, maternal mutant *Cdkn1c* mice exhibited 20% overgrowth during prenatal period, but the increased bodyweight was not seen at birth which can probably be explained by intrauterine competition for maternal nutrients in litter bearing species (Tunster et al., 2011).

Several reasons exist to propose the use of LOS as an adequate animal model to study BWS. First, only ruminants and humans have been reported to display the overgrowth and excessive weight at birth as a result of minimal ART manipulations (Bertolini and Anderson, 2002; Bertolini et al., 2002; Blondin et al., 2000; DeBaun et al., 2003; Farin et al., 2006; Gicquel et al., 2003; Halliday et al., 2004; Hiendleder et al., 2004; Hiendleder et al., 2006; Kuentz et al., 2011; Lim et al., 2009; Maher et al., 2003; Miles et al., 2004; Sutcliffe et al., 2006; Young et al., 2001; Young et al., 1998). Second, loss of methylation at KvDMR1 and biallelic expression of *KCNQ1OT1* were observed in both human (Choufani et al., 2010; Weksberg et al., 2009) and LOS. Third, here we show lack of DNA methylation at the bovine *CDKN1C* exon 2 which is in accordance to what has been reported for humans (Chung et al., 1996; Monk et al., 2006) but is in stark contrast to the situation in the mouse where differential methylation is evident (Bhogal et al., 2004). Fourth, females of both species carry primarily singleton pregnancies (monotocous). It has been suggested that variance for growth regulation exist between litter bearing and non-litter bearing species (Tunster et al., 2011). Fifth, both human and bovine have a nine month gestation period. This is important because sequential events that lead to molecular lesions resulting in the overgrown phenotype or other features of BWS may occur at similar times during pregnancy and the potential exists to evaluate the timing of intervention strategies.

BWS has more recently been associated with misregulation at loci other than the IC2 and IC1 such as *MEST* (*PEG1*), *PLAGL1* (*ZAC1*) and *GNAS* (Bliek et al., 2009; Rossignol et al., 2006). Misregulation of multiple imprinting clusters in BWS speculates

that highly variable clinical features of BWS may result from diverse combinations of epimutation of each imprinting center. Future work is planned to determine if the same is observed in the LOS model.

In conclusion, our results show phenotypic and epigenetic similarities between LOS and BWS, and we propose the use of LOS as an animal model to investigate the etiology of BWS.

Materials and Methods

Animals

We used *B. t. indicus* and *B. t. taurus*, two subspecies of cattle, to produce F1 hybrid progenies. The use of *B. t. indicus* × *B. t. taurus* F1 individuals allowed us to determine allele-specific expression and DNA methylation of imprinted genes by the use of polymorphisms between the two subspecies. A previous study in our laboratory identified DNA polymorphisms between the two subspecies in IC1 and IC2 imprinting domains (Robbins et al., 2012).

Experimental groups:

Control conceptuses: The estrous cycle of *B. t. taurus* (Holstein breed) females was synchronized and the females were artificially inseminated (AI) with semen from one *B. t. indicus* bull (Nelore breed; ABS CSS MR N OB 425/1 677344 29NE0001 97155). Four males and five female *B. t. indicus* × *B. t. taurus* F1 conceptuses (fetus + placenta) were collected on day ~105 (104-106). This time was chosen because phenotypic characteristics of LOS can be recognized at this stage (Breukelman et al.,

2004). Conceptuses were retrieved from the gravid uterus at caesarean section in order to preserve nucleic acid integrity. At collection, crown-rump length, heart girth, foreleg length and head width were measured (O'Rourke P et al., 1991), as well as body and organ weight. The following tissues were collected: liver, muscle, brain, tongue, heart, lung, kidney, spleen, reproductive tract, intestine, skin and placenta. Tissues were diced and mixed at collection and were snap frozen in liquid nitrogen and stored at -80°C until use.

ART conceptuses: *In vitro* production of bovine embryos was performed as previously described by us ((Rivera et al., 2003) and <http://www.animal.ufl.edu/hansen/ivf/>). All media (Hepes-TL, IVF-TL, SP-TL) were purchased from Caisson Laboratorys (Logan, UT). All chemicals used to prepare media were purchased from Sigma (St. Louis, MO). Briefly, *B. t. taurus* (Holstein) cumulus-oocyte complexes (COCs) were shipped overnight in maturation medium from TransOva Genetics (Long Praire, MN). At receipt, the oocytes were rinsed in Hepes-TALP (Tyrode's Albumin Lactate Pyruvate) and immediately placed in IVF-TALP. Semen from the same *B. t. indicus* bull used to generate control conceptuses was used for IVF. The semen in one straw was thawed at 37.0°C. The semen straw contents were added into a 15 ml centrifuge tube containing 13 ml SP-TALP and centrifuged for 15 minutes at 200×*g*. The sperm pellet was suspended in 300 µl of IVF-TALP and this volume was pipetted into a glass wool column in order to separate live sperm cells from debris and dead sperm. Note: this bull does not perform well *in vitro* when purified by Percoll gradient. The COCs and the sperm cells were co-incubated in IVF-TALP at 38.5°C in

humidified air containing 5% CO₂ for 18 hours (Rivera et al., 2003). Putative zygotes were cultured in KSOM-BE (Rivera et al., 2003) in groups of 25-30. The embryos were cultured at 38.5°C in humidified atmosphere containing 90% N₂, 5% CO₂ and 5% O₂. On day 5 after fertilization, embryo culture drops were supplemented with 10% estrus cow serum. On day 6, 20-30 early blastocysts were placed in 2.0 ml tubes containing 1.8 ml KSOM-BE supplemented with 180 µl (10%) estrus cow serum and covered with mineral oil. The embryos were shipped overnight to TransOva Genetics (Sioux Center, IA) in a portable incubator maintained at 38.5°C. Upon receipt, the embryos were transferred into synchronized *B. t. taurus* recipients (2 blastocyst per recipient; one/uterine horn). Embryo transfers and conceptus retrievals were performed by theriogenologists at TransOva Genetics. On day ~105 (104-106), conceptuses were collected and processed as described for the control conceptuses. Twenty-seven conceptuses were collected from 19 recipients (8 recipients had twins).

All animal procedures were performed at TransOva Genetics by veterinarians, and all procedures were approved by TransOva's animal care and use committee.

RNA isolation, cDNA synthesis and reverse transcriptase polymerase chain reaction

RNA was isolated from fetal tissues with the use of Trizol Reagent (Invitrogen; Carlsbad, CA) according to the manufacturer's instructions. RNA amount and quality were confirmed by spectrometry and agarose gel electrophoresis, respectively. RNA was treated with DNase (Fisher Scientific; Pittsburgh, PA) before cDNA synthesis. 100 ng RNA was used as template to synthesize cDNA in a 20µl reaction with: 10 mM DTT

(Invitrogen; Carlsbad, CA), 1x First Strand Buffer (Invitrogen; Carlsbad, CA), 0.5µg random primer (Promega; Madison, WI), 1mM dNTPs (Fisher Scientific; Pittsburgh, PA), 100 U SuperscriptII reverse transcriptase (Invitrogen; Carlsbad, CA), and 20 U of Optizyme RNase Inhibitor (Fischer Scientific; Pittsburgh, PA). The samples were incubated at 42°C for 1 hour, at 95°C for 10 minutes, and stored at -20°C until use. To ensure no genomic DNA contamination, a minus reverse-transcriptase control was also included. For PCR amplification, 2µl cDNA (~10ng) was added into the PCR mix [1× colorless GoTaq Flexi Buffer (Promega; Madison, WI), 0.3µM forward and reverse primer (IDT; Coralville, IA), 2.5 mM MgCl₂ (Promega; Madison, WI), 200 µM dNTP (Fisher Scientific; Pittsburgh, PA) and 0.5 U GoTaq Hot Start polymerase (Promega Madison, WI)]. For each assay, at least 5 sets of primers were tested to get specific amplification. The PCR conditions were as follows: denaturation at 94°C for 2 minutes 15 s, then 35 cycles of 94°C for 15 s, 59.5-62.8°C for 20 s and 72°C for 30 s, and final extension at 72°C for 5 minutes (Supplemental Table S2.1).

Allele-specific expression analysis of imprinted genes

The six imprinted genes analyzed in this study were the maternally-expressed genes: *CDKN1C*, *PHLDA2*, *H19*, and *KCNQ1* and the paternally-expressed genes: *KCNQ1OT1* and *IGF2*. These genes were analyzed in eight tissues; namely, liver, muscle, brain, tongue, heart, lung, kidney, and placenta. Expression of *KCNQ1OT1* was determined by RT-PCR followed by Sanger sequencing. The samples were sequenced at the University of Missouri's DNA core using the 96-capillary applied Biosystems 3730 DNA analyzer with Big Dye Terminator. Sequencing data was aligned to a reference with

the use of MacVector software (Cary, NC). Allelic expression of *CDKN1C*, *H19*, and *KCNQ1* were determined by RT-PCR followed by allele-specific restriction enzyme digests (Supplemental Table S2.1). The digested PCR products were resolved by polyacrylamide gel electrophoresis (Dawlaty et al.). The assay used to determine allele-specific expression of *H19* was previously described (Robbins et al., 2012). Allelic expression of *PHLDA2* and *IGF2* were determined by RT-PCR-SSCP (single strand conformation polymorphism) because restriction enzymes that recognized the sequence of interest were not available. Briefly, SSCP was conducted on an 8% polyacrylamide gel and run at 110V overnight (~14 hours). The SSCP gel was then subjected to silver staining (Bio-Rad; Hercules, CA) and dried by Gel-Dry (Invitrogen; Carlsbad, CA). The contribution of each parental allele to the total expression was determined by Image J (Sharif et al.). Only samples with at least 10% expression from the repressed allele were considered biallelic (Rivera et al., 2008).

Quantitative RT-PCR of CDKN1C, PHLDA2 and IGF2

Taqman gene expression assays (Applied Biosystems; Supplemental Table S2.1) were used to determine if *CDKN1C*, *PHLDA2*, and *IGF2* showed different expression levels among control conceptuses, the LOS conceptuses with biallelic expression of *KCNQ1OT1*, and the LOS conceptuses with monoallelic expression of *KCNQ1OT1*. The assay was conducted in the eight tissues described in the allele-specific expression analysis section. The *CDKN1C* level of expression of the eight tissues of the two LOS conceptuses with biallelic expression of *KCNQ1OT1* were compared to the tissues of the eight control conceptuses (5 females and 3 males) and five LOS conceptuses (2 females

and 3 males) with monoallelic *KCNQ1OT.1*. The samples were analyzed in triplicates, and the threshold cycle was normalized to the housekeeping gene *GAPDH* using an ABI Real-time 7500 system. The expression level for each gene in each tissue was calculated using the comparative C_T method. The expression levels of our bovine samples were plotted as described before in human (Chiesa et al., 2012).

DNA isolation and bisulfite conversion

DNA from *B. t. indicus* × *B. t. taurus* F1 individuals was isolated using phenol-chloroform. Bisulfite mutagenesis was conducted with the Imprint DNA Modification Kit (Sigma; St. Louis, MO) according to manufacturer's instructions. During this procedure, unmethylated cytosines are converted into uracils, but methylated cytosines remain cytosines. After PCR amplification, uracils are replaced by thymines. Primers for the bisulfite-converted DNA were designed for KvDMR1, *H19/IGF2* ICR, and *CDKN1C* exon 2 (Supplemental Table S2.2). The PCR conditions were as follows: denaturation at 94°C for 2 min 15 s, then 45 cycles at 94°C for 30 s, 53.5-62.1°C for 45 s and 72°C for 1 min 30 s, and final extension at 72°C for 5 minutes (Supplemental Table S2.2). Note that 1M Betaine was necessary for amplification of the *H19/IGF2* ICR.

DNA methylation analysis of KvDMR1 and H19/IGF2 ICR

The PCR product of the bisulfite-converted regions of interest was isolated from a 1% agarose gel with Wizard SV gel and PCR Clean-Up System (Promega; Madison, WI). KvDMR1 (385 bp containing 37 CpGs; GenBank accession no. NW_003104648.1: 2960086-2960470) and *H19/IGF2* ICR (318 bp containing 20/21 CpGs; GenBank

accession no. NW_003104648.1: 3556002-3556319) amplicons were inserted into pCC1 vector with chloramphenicol resistance gene and cloned using CopyControl PCR cloning kit with TransforMax EPI300 electrocompetent *E. coli* cells (Epicenter Biotechnologies; Omaha, NE) (Rivera et al., 2008) according to the manufacturer's instructions except that all the cloning incubation procedures were done at 25°C. Note: it took approximately 2 days to form visible colonies at this temperature. The individual clones were sequenced and analyzed as described for *KCNQ1OT1* sequencing.

DNA methylation analysis of CDKN1C exon 2

In mice, *Cdkn1c* DMR starts from 600 bp upstream of transcription start site of *Cdkn1c* and extends through exon 2 (Bhogal et al., 2004). The homologous region in humans is, however, unmethylated (Chung et al., 1996; Monk et al., 2006). A 363 bp region of bisulfite-converted exon 2 (containing 48 CpGs and no SNPs; GenBank accession no. NW_003104648.1: 2776175-2776537) was amplified by PCR. The PCR product was processed and sequenced as described above. Primer information can be found in Supplemental Table S2.2.

Sequencing data showed hypomethylation of *CDKN1C* exon 2. To ensure that the primers used were equally able to amplify bisulfite converted methylated and unmethylated DNA, we did the following; 1) an aliquot of DNA was bisulfite converted with no Sss1 treatment; 2) another aliquot of DNA was treated with Sss1 methyltransferase (New England BioLabs; Ipswich, MA) prior to bisulfite conversion; 3) samples were mixed with a 1:1 ratio. The three types of template were analyzed

separately by combined bisulfite restriction analysis (COBRA). The enzyme used for COBRA was HincII (New England BioLabs; Ipswich, MA) which only cuts the methylated amplicons.

Statistical analysis

Bodyweight, organ weight, crown-rump length, heart girth, foreleg length and head width were analyzed by using standard General Linear Model procedure of SAS with fixed factors: ART/AI and sex. The significance level is $P < 0.05$.

Funding

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Conflicts of interest

None declared

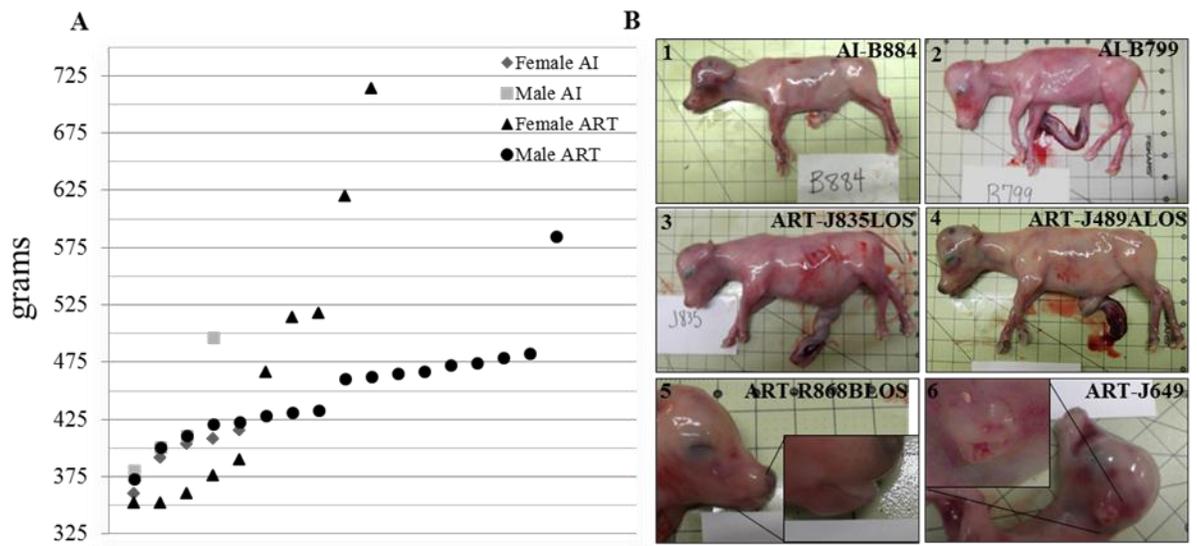


Figure 1

Figure 1. LOS bovine fetuses have similar phenotype characteristics as those reported in BWS patients. (A). Fetal weight at day ~105 gestation. Y axis represents the weight in grams. X axis has no actual implication and is used to scatter the spots representing each fetus for ease of visualization. The sex of the fetuses and the way they were generated is shown at the top-right side. The bold line represents the 97th percentile of control weight (*i.e.* 476.8 g). **(B).** Primary and secondary characteristics of BWS can be observed in LOS; **B.1** = AI-B884 (control female weighing 400 g) and **B.2** = AI-B799 (control male weighing 408 g which is the approximate average weight of the control fetuses). **B.3** and **B.4** show fetuses with macrosomia (ART-J835LOS – female weighing 714 g and ART-J489ALOS– female weighing 514 g). **B.5** shows an example of macroglossia in a female weighing 620 g and **B.6** shows an ear malformation in a female weighing 320 g. Each square on the background = 2.54 cm². LOS: large offspring syndrome; BWS: Beckwith-Wiedemann syndrome; AI: artificial insemination; ART: assisted reproductive technologies.

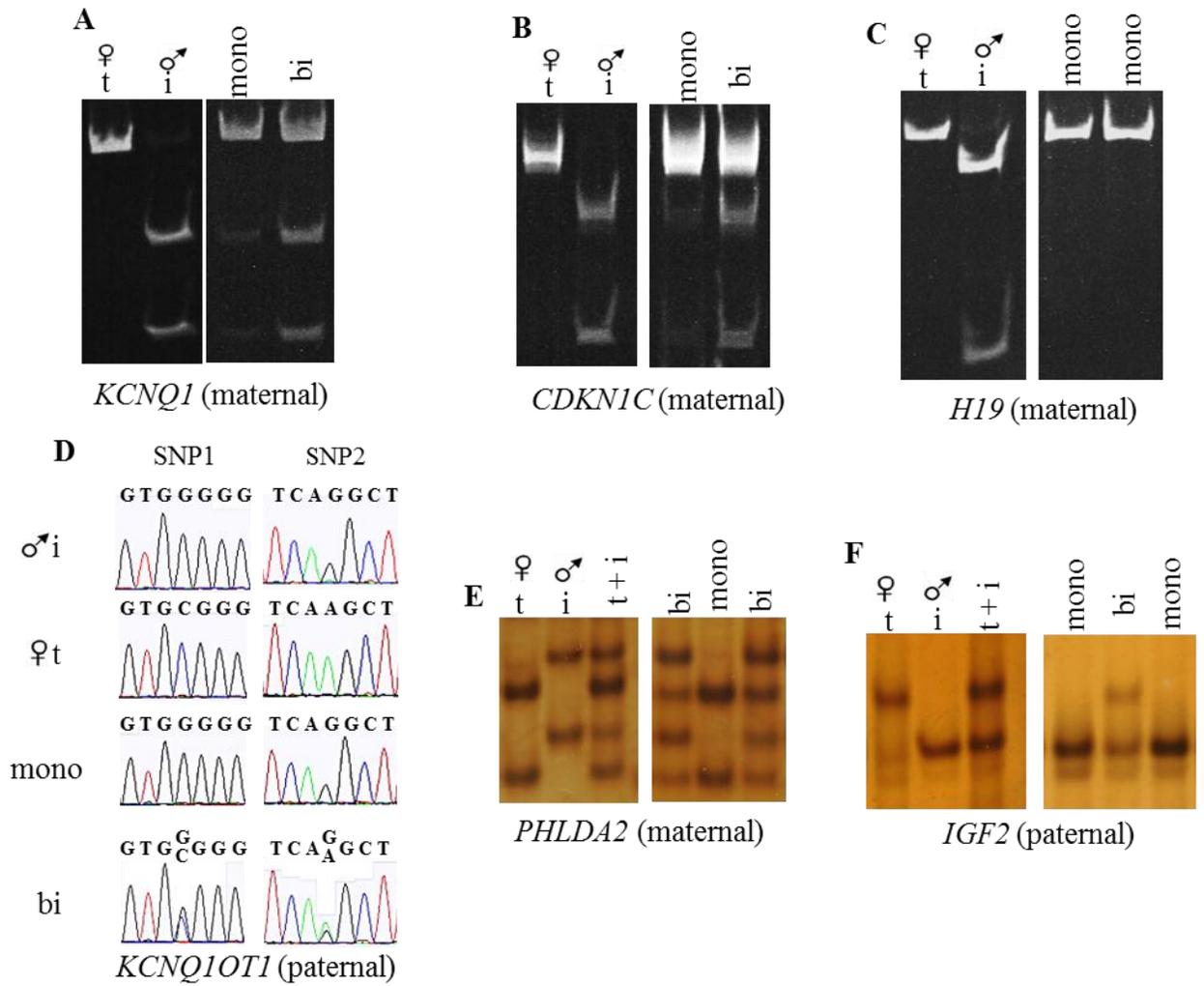


Figure 2

Figure 2. Example of assays used to determine allelic expression in tissues from *B. t. indicus* × *B. t. taurus* F1 hybrid conceptuses. Shown are examples of allelic determination by RT-PCR followed by RFLP and PAGE (A-C), Sanger sequencing (D) or SSCP analysis (E-F). The left portion of the panels (A, B, C, E, and F) shows the band pattern of *B. t. taurus* and *B. t. indicus* control tissues (liver) which was used as reference to determine parental expression of imprinted gene in tissues from *B. t. indicus* × *B. t. taurus* F1 hybrids. The right portion of the panels shows examples of monoallelic and biallelic expression of several imprinted genes in ~d105 conceptus. (D) is an example of the Sanger sequencing allelic assay for *KCNQ1OT1*, a paternally-expressed gene. Two SNPs were used in this assay; double peaks demonstrate biallelic expression. The contribution of each parental allele to the total expression was determined by the use of Image J (Sharif et al.). Only samples with at least 10% expression from the repressed allele were considered to be biallelically-expressed. t: *B. t. taurus*; i: *B. t. indicus*; Mono: monoallelic; Bi: biallelic; SNP: single nucleotide polymorphism; RFLP: restriction fragment length polymorphism; SSCP: single strand conformation polymorphism; PAGE: polyacrylamide gel electrophoresis.

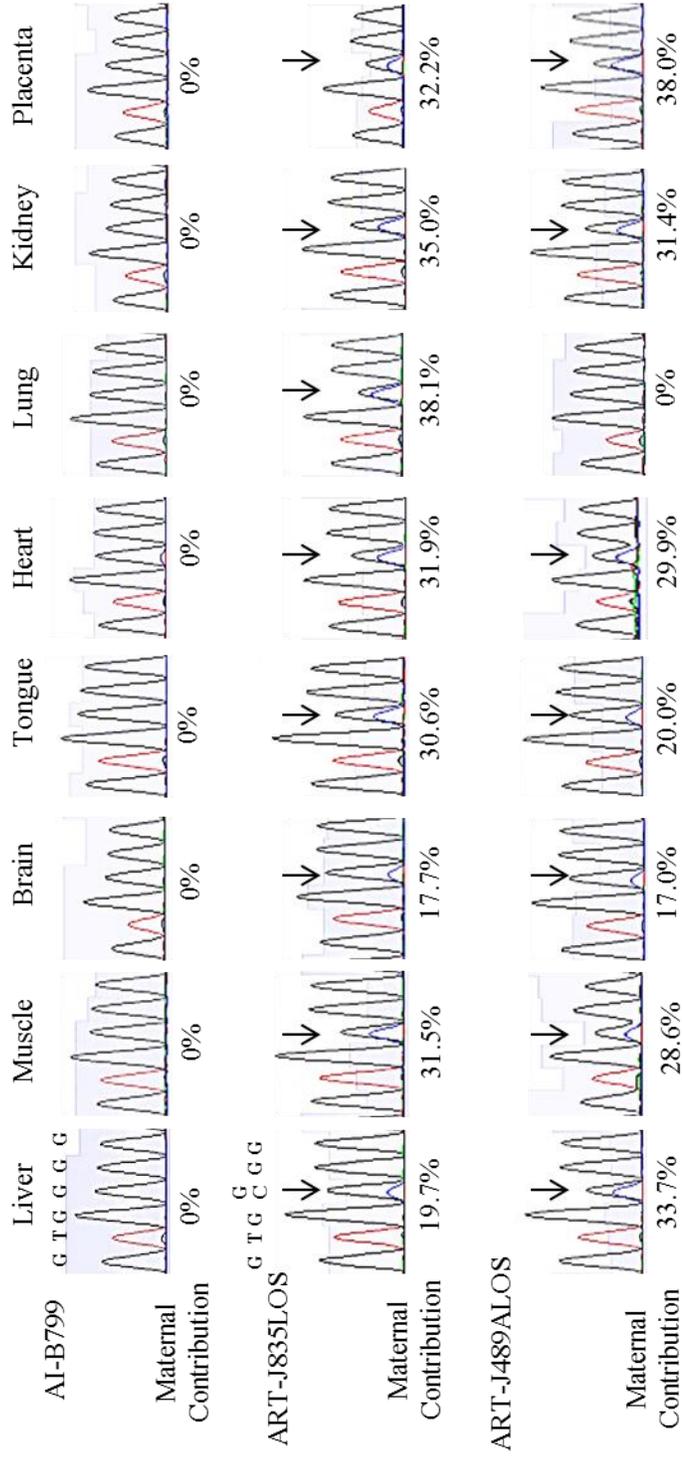


Figure 3

Figure 3. Biallelic expression of *KCNQ1OT1* in LOS fetuses. Shown is Sanger sequencing data of *KCNQ1OT1* RT-PCR product in tissues analyzed in AI-B799 (control), ART-J835LOS and ART-J489ALOS fetuses. The columns show the chromatograph for each tissue of each fetus. Values below the chromatograph are the percentage of *KCNQ1OT1* expressed from the maternal allele. Arrows show the double peaks of SNP1 site (refer to Figure 2) in ART-J835LOS and ART-J489ALOS fetuses. For clarity of depiction, SNP2 site is shown here (Supplemental Fig. 1). LOS: large offspring syndrome; RT-PCR: reverse transcription-polymerase chain reaction; SNP: single nucleotide polymorphism.

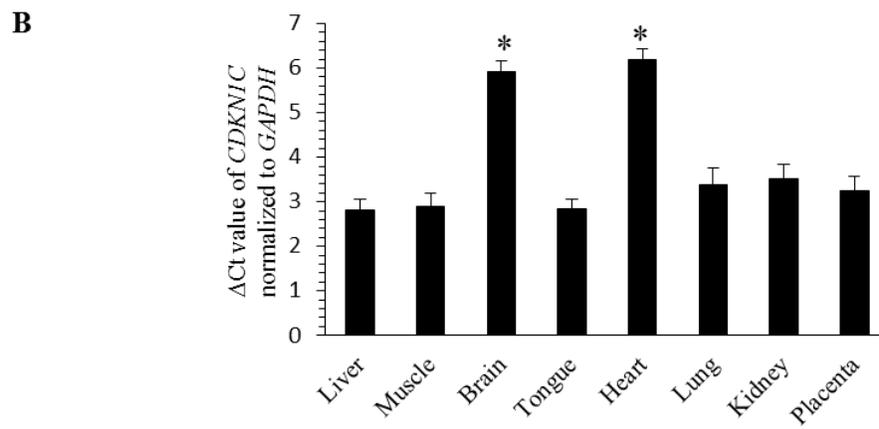
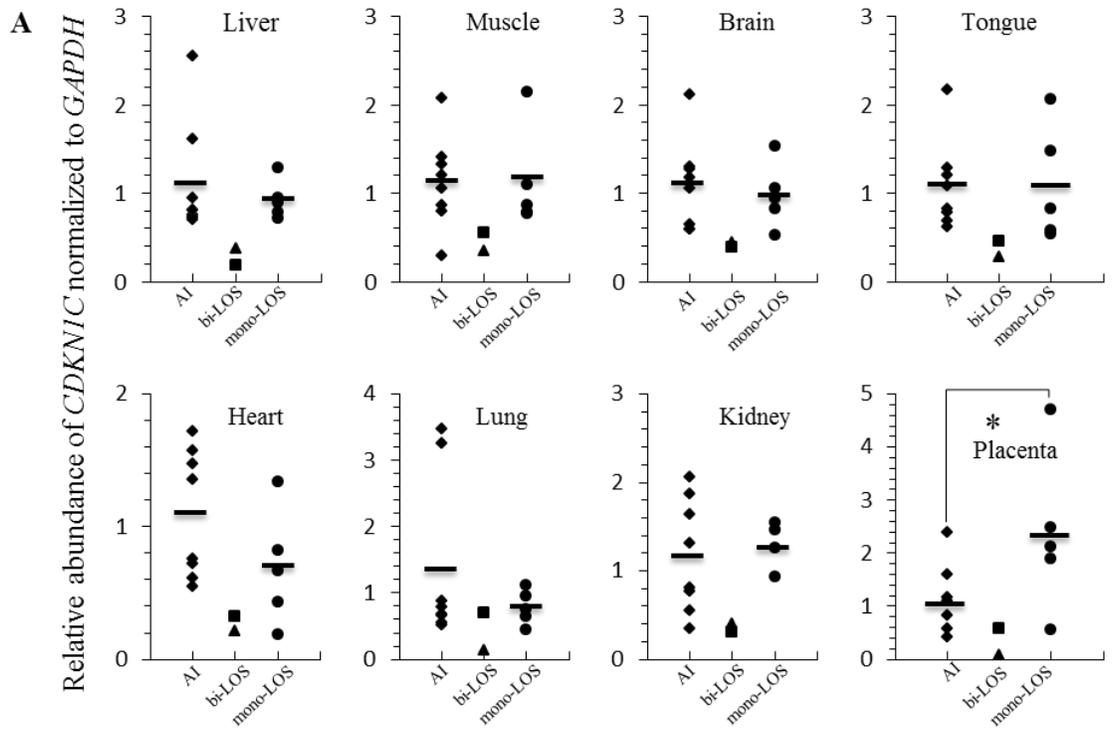


Figure 4

Figure 4. LOS fetuses with biallelic expression of *KCNQ1OT1* show down-regulation of *CDKN1C*. (A) The *CDKN1C* RNA was determined by quantitative RT-PCR in several tissues from eight AI fetuses (diamonds; AI), two LOS fetuses with biallelic expression of *KCNQ1OT1* (triangle = ART-J835LOS; square = ART-J489ALOS; bi-LOS), and five LOS fetuses with correct imprinting of *KCNQ1OT1* (circles; mono-LOS). The short line among the diamonds and circles represents the average level of the individuals. *CDKN1C* level was normalized to the expression of *GAPDH*. Note that for each tissue analyzed *CDKN1C* level in ART-J835LOS is at least 2fold lower than the average level found in AI fetuses and LOS fetuses with correct imprinting of *KCNQ1OT1*. It should be also noted that lung from ART-J489ALOS which had correct imprinting of *KCNQ1OT1* (Fig. 3) exhibits the comparative *CDKN1C* level with controls. (B) The threshold cycle (Ct) of *CDKN1C* was normalized to the reference gene *GAPDH* in each tissue from the eight AI fetuses. The data are expressed as mean \pm SEM. *P < 0.05 between the brain, heart and other tissues analyzed. LOS: large offspring syndrome;

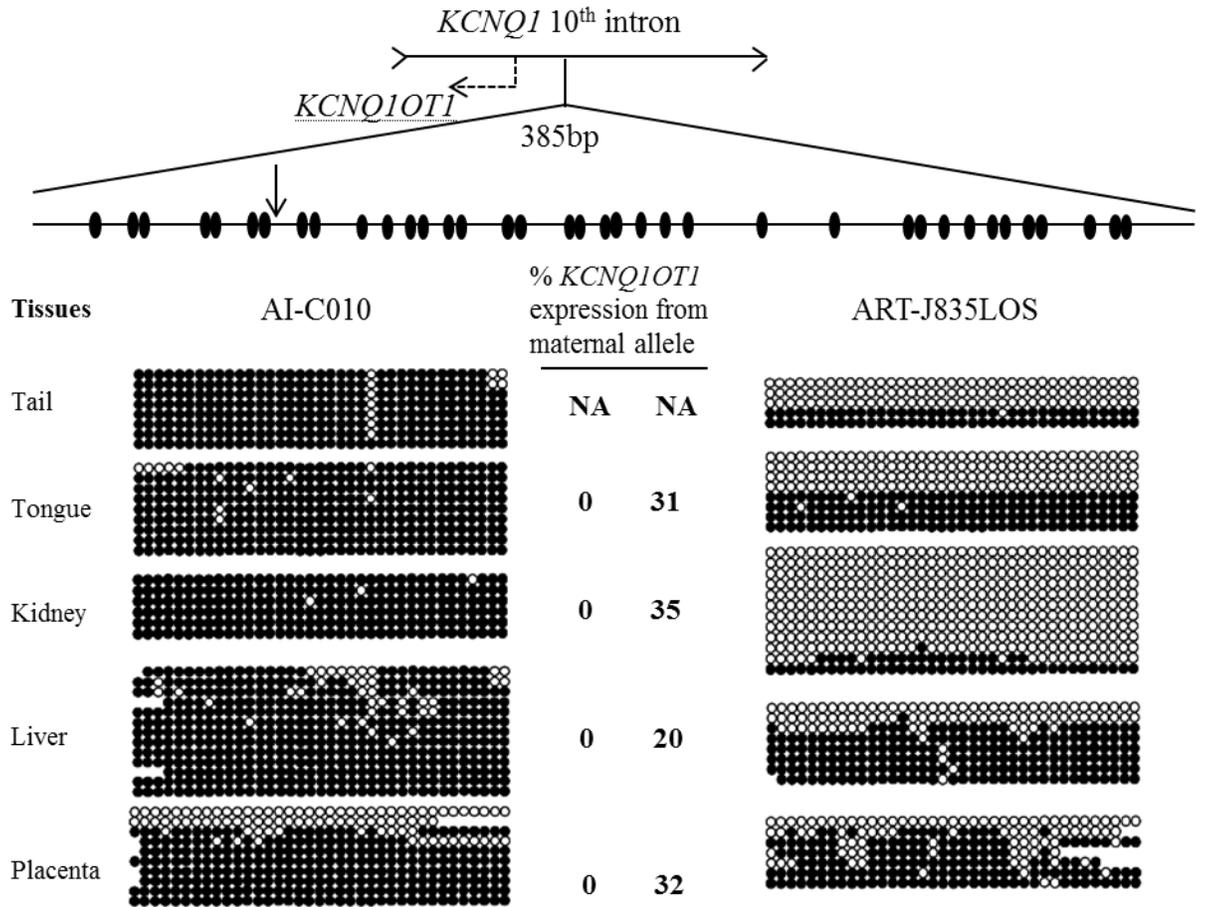
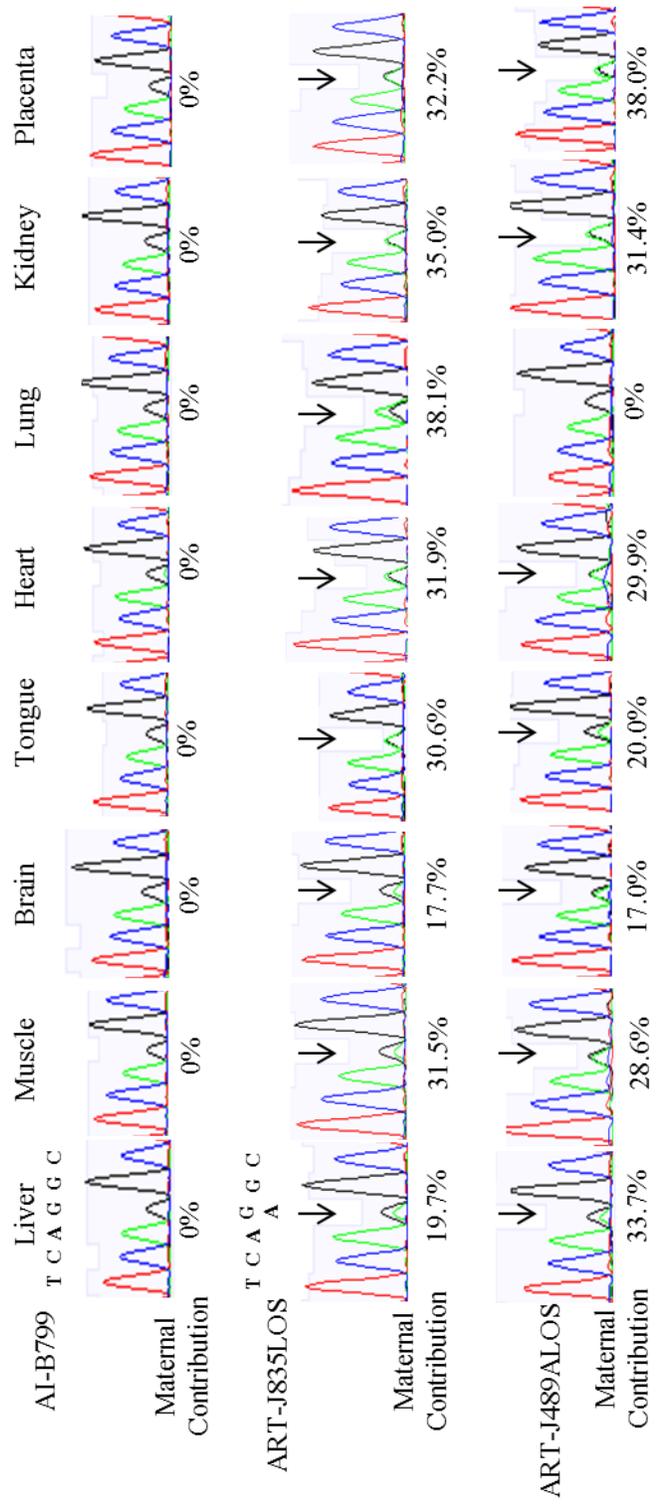


Figure 5

Figure 5. Loss of methylation of KvDMR1 on the maternal allele is associated with biallelic expression of KCNQ1OT1 in LOS fetuses. DNA was treated with sodium bisulfite prior to PCR, and PCR product was cloned before sequencing. Sequencing data was used to determine the DNA methylation status at the KvDMR1. Shown on top is a depiction of the 10th intron of the maternally-expressed gene KCNQ1 and its direction of transcription is shown with an arrow. The region harbors the promoter of the antisense long ncRNA KCNQ1OT1 (shown as dashed arrow), which is also an imprinting control region known as KvDMR1. A 385 bp region of the KvDMR1 was used to determine the DNA methylation status of 37 CpG sites (ovals). A SNP (vertical arrow) between *B. t. indicus* and *B. t. taurus* was used to determine the parental origin of the alleles and only maternal alleles are shown here. Five tissues from two fetuses are shown. Filled and open circles represent methylated and unmethylated CpG dinucleotides, respectively. Missing circles are sequencing data of low quality. Each line denotes an individual DNA strand. The level of maternal KCNQ1OT1 expression is shown in the center and next to the strands. Tail tissues were collected for the purpose of DNA analysis, precluding its use for gene expression determinations. NA: Not available.

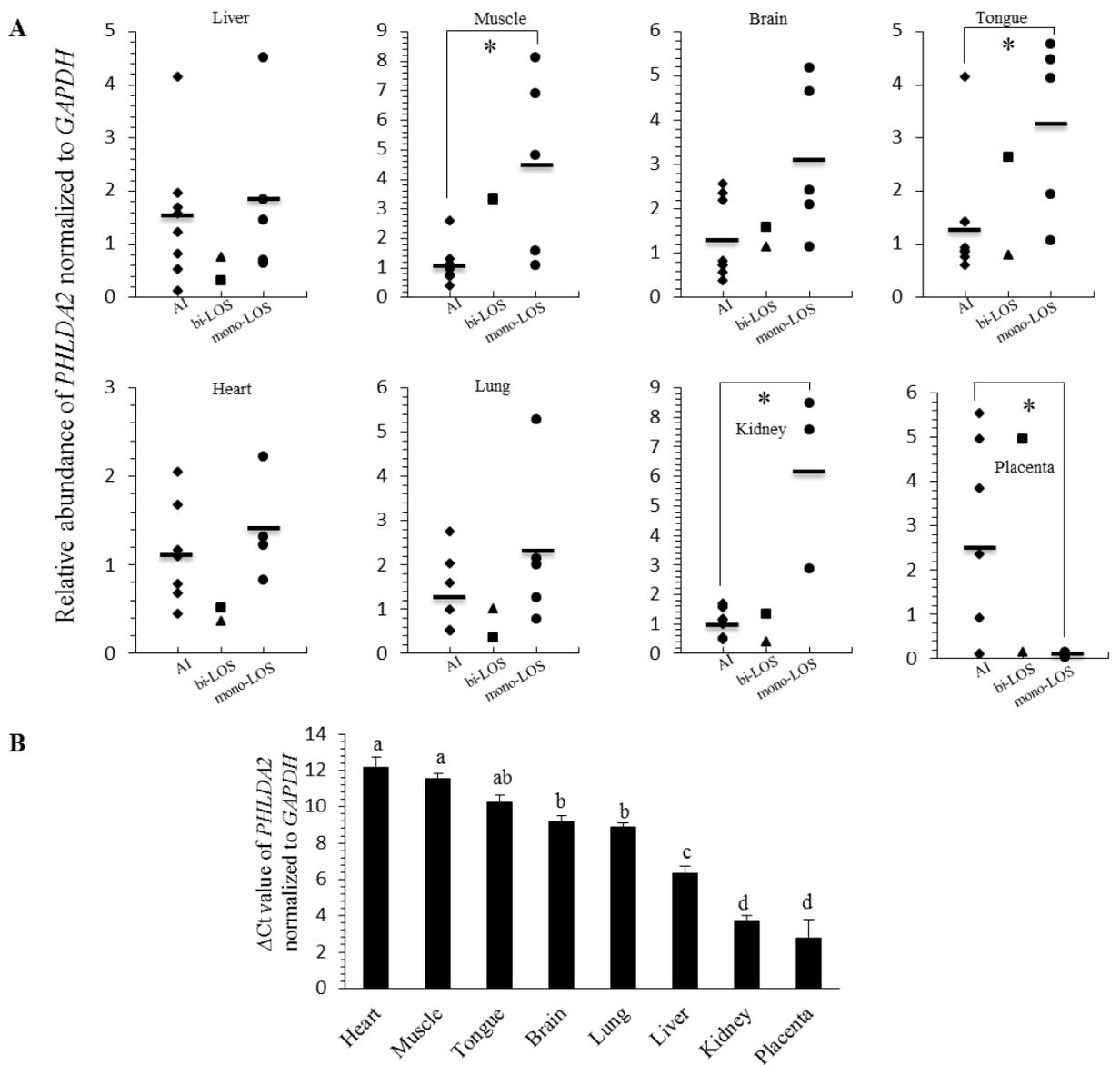
Figure 6. Imprinted expression of *CDKN1C* is not regulated by somatic DNA

methylation. (A) Shown on top is a depiction of the first two exons and the first intron of the maternally-expressed gene *CDKN1C*. No DNA polymorphisms were identified in this region. Bisulfite converted specific primers for a 363 bp region of the exon 2 were used to determine the DNA methylation status of 48 CpG sites (ovals). (B) The PCR product was sequenced without cloning. Two tissues from five fetuses are shown. Filled and open circles represent methylated and unmethylated CpG dinucleotides, respectively. Each line denotes the DNA methylation pattern of each sample. Only the first 39 CpGs are shown here because of the low quality sequencing of last 9 CpGs due to primer binding. The level of paternal *CDKN1C* expression is shown in the center and next to the strands (based on a SNP in exon 4). (C) COBRA performed to ensure ability of primers to equally amplify methylated and unmethylated alleles. The restriction enzyme HincII recognized and cleaved the methylated amplicon. Sss1 = genomic DNA co-incubated with the methyltransferase Sss1 prior to bisulfite conversion, U= bisulfite converted DNA with no Sss1 treatment, Sss1 + U = equal portions of methylated and unmethylated DNA were used for PCR amplification. The digested products were resolved by PAGE.



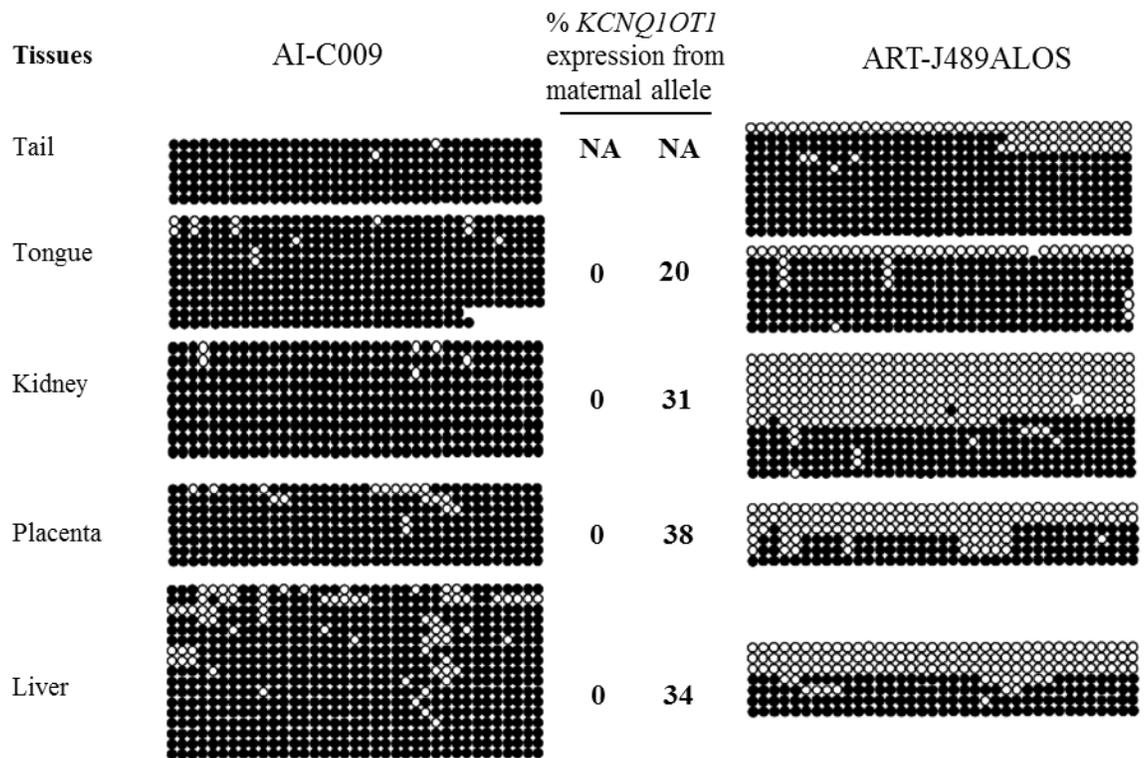
Supplemental Figure 1

Supplemental Figure 1. Biallelic expression of *KCNQ1OT1* in LOS fetuses. Shown is Sanger sequencing data of *KCNQ1OT1* RT-PCR product in tissues analyzed in AI-B799 (control), ART-J835LOS and ART-J489ALOS fetuses. The columns show the chromatograph for each tissue of each fetus. Values below the chromatograph are the percentage of *KCNQ1OT1* expressed from the maternal allele. Arrows show the double peaks of SNP2 site (refer to Figure 2) in ART-J835LOS and ART-J489ALOS fetuses. LOS: large offspring syndrome; RT-PCR: reverse transcription-polymerase chain reaction; SNP: single nucleotide polymorphism.



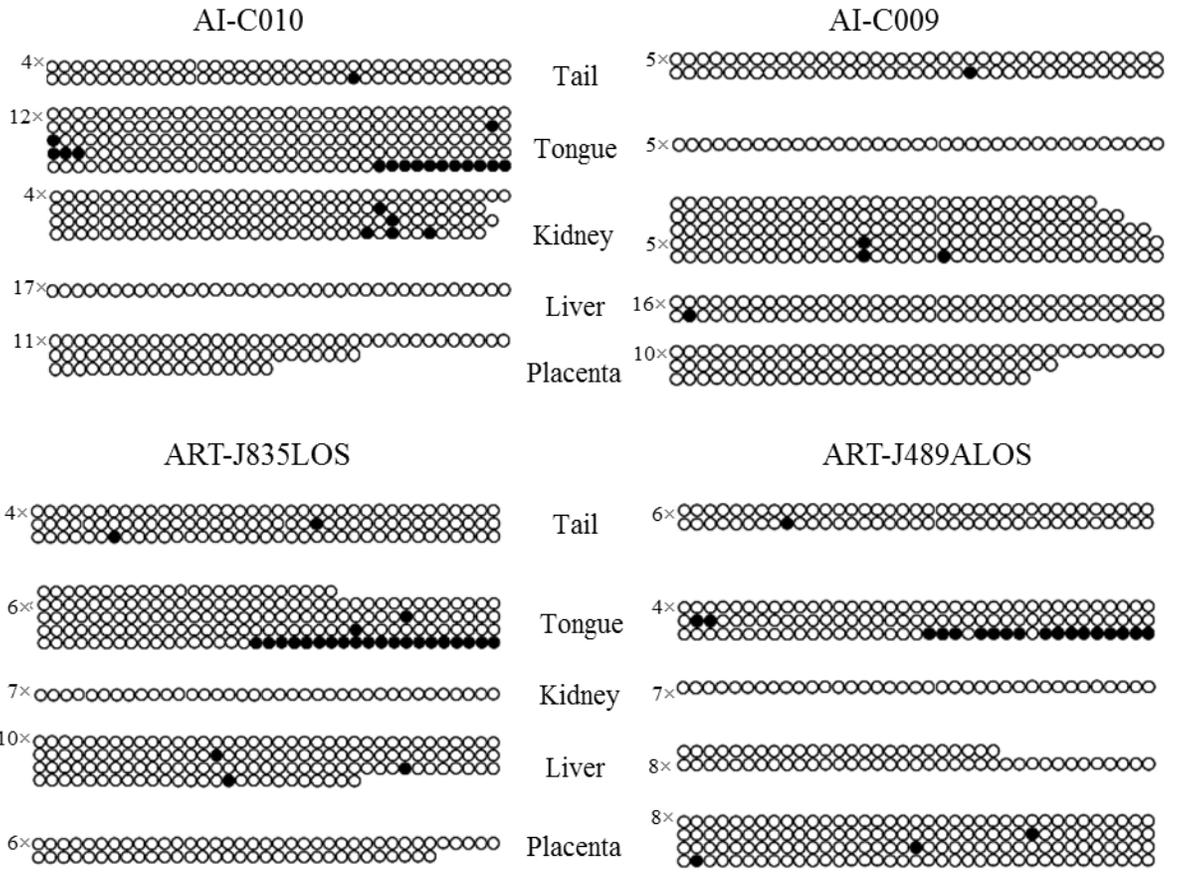
Supplemental Figure 2

Supplemental Figure 2. Analysis of *PHLDA2* expression. (A) The *PHLDA2* RNA was determined by quantitative RT-PCR in several tissues from eight AI fetuses (diamonds; AI), two LOS fetuses with biallelic expression of *KCNQ1OT1* (triangle = ART-J835LOS; square = ART-J489ALOS; bi-LOS), and five LOS fetuses with correct imprinting of *KCNQ1OT1* (circles; mono-LOS). The short line among the diamonds and circles represents the average level of the individuals. *PHLDA2* level was normalized to the expression of *GAPDH*. (B) The threshold cycle (Ct) of *PHLDA2* was normalized to the reference gene *GAPDH* in each tissue from the eight AI fetuses. The data are expressed as mean \pm SEM. Tissues that do not share the common characters have significant difference (* $P < 0.05$). LOS: large offspring syndrome;



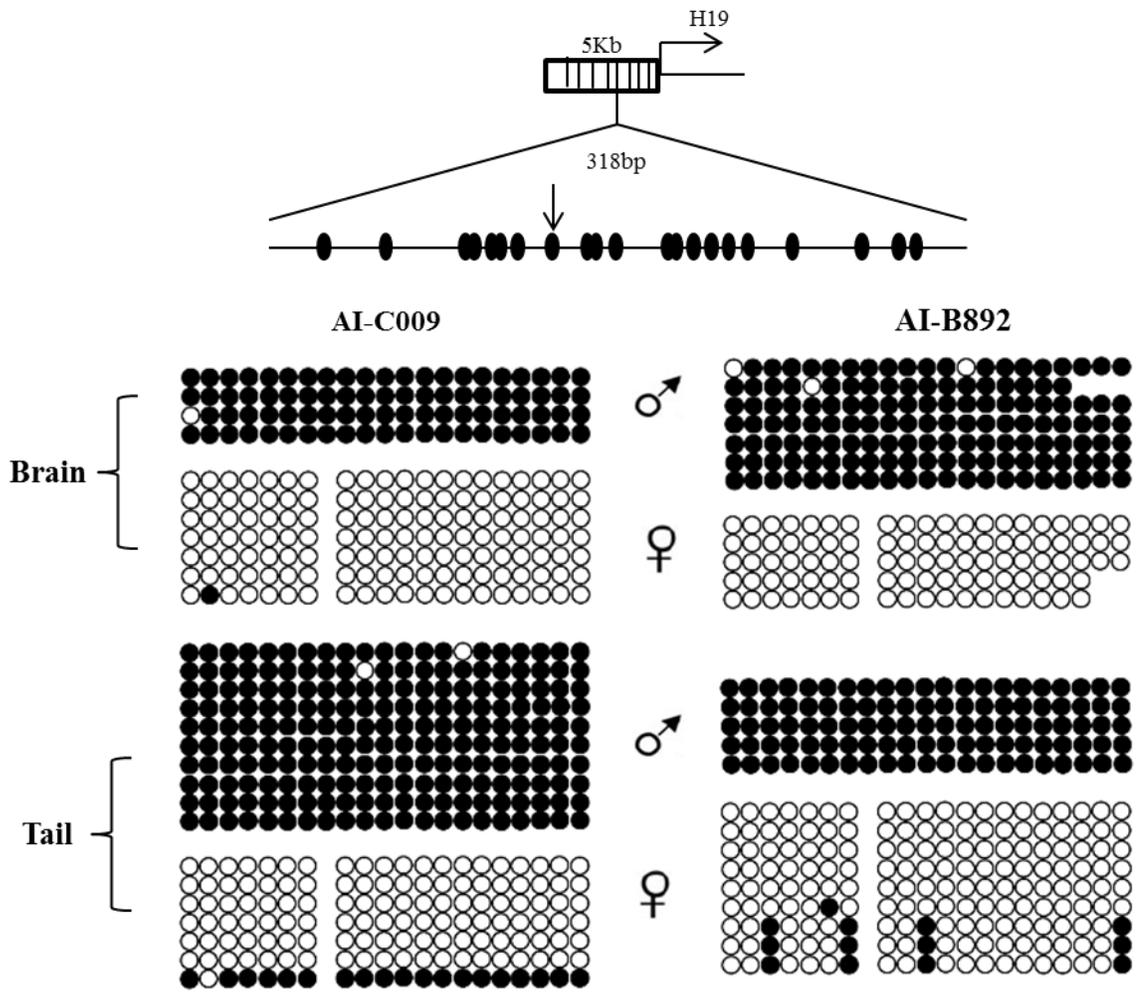
Supplemental Figure 3

Supplemental Figure 3. Loss of methylation of KvDMR1 on the maternal allele is associated with biallelic expression of *KCNQ1OT1* in LOS fetuses. DNA was treated with sodium bisulfite prior to PCR, and PCR product was cloned before sequencing. Sequencing data was used to determine the DNA methylation status at the KvDMR1. Shown on top is a depiction of the 10th intron of the maternally-expressed gene *KCNQ1* and its direction of transcription is shown with an arrow. The region harbors the promoter of the antisense long ncRNA *KCNQ1OT1* (shown as dashed arrow), which is also an imprinting control region known as KvDMR1. A 385 bp region of the KvDMR1 was used to determine the DNA methylation status of 37 CpG sites (ovals). A SNP (vertical arrow) between *B. t. indicus* and *B. t. taurus* was used to determine the parental origin of the alleles and only maternal alleles are shown here. Five tissues from two fetuses are shown. Filled and open circles represent methylated and unmethylated CpG dinucleotides, respectively. Missing circles are sequencing data of low quality. Each line denotes an individual DNA strand. The level of maternal *KCNQ1OT1* expression is shown in the center and next to the strands. Tail tissues were collected for the purpose of DNA analysis, precluding its use for gene expression determinations. **NA:** Not available.



Supplemental Figure 4

Supplemental Figure 4. KvDMR1 is unmethylated on the paternal alleles. DNA was treated with sodium bisulfite prior to PCR, and PCR product was cloned before sequencing. Sequencing data was used to determine the DNA methylation status. Only paternal alleles are shown here – maternal allele results can be found in Figure 5 and Supplemental Figure 3. Five tissues from 4 fetuses are shown. Filled and open circles represent methylated and unmethylated CpG dinucleotides, respectively. Missing circles are sequencing data of low quality. Each line denotes an individual DNA strand and the number to the left of the strands represents the number of strands with that phenotype when greater than one.



Supplemental Figure 5

Supplemental Figure 5. Differential methylation of the *H19/IGF2* ICR in tail and brain of AI control fetuses. DNA was treated with sodium bisulfite prior to PCR, and PCR product was cloned before sequencing. Sequencing data was used to determine the DNA methylation status. Shown on top is a depiction of the maternally-expressed gene *H19* (shown as an arrow) and the ICR is represented as a black box in which the vertical lines represent 8 predicted CTCF binding sites (Robbins et al., 2012). A 318 bp region which covers the 5th CTCF binding site was used to determine the DNA methylation status of 21/20 CpG sites (ovals). An extra CpG site on paternal alleles is a result of the SNP that “CG” in *B. t. indicus* (paternal) and “CA” in *B. t. taurus* (maternal). A SNP (vertical arrow) between *B. t. indicus* and *B. t. taurus* was used to determine the origin of the parental allele. Two tissues from 2 fetuses are shown. Filled and open circles represent methylated and unmethylated CpG dinucleotides, respectively. Missing circles are sequencing data of low quality. Each line denotes an individual DNA strand.

Supplemental Table 1

The five tables show the calculated allelic expression of *KCNQ1OT1*, *CDKN1C*, *KCNQ1*, *PHLDA2*, *H19* and *IGF2* imprinting status in each tissue for each fetus. *KCNQ1OT1* and *IGF2* are paternally-expressed; and *CDKN1C*, *PHLDA2*, *KCNQ1* and *H19* are maternally-expressed. The six genes are located on chromosome 29. Each table shows the data of one gene. Gene symbol and tissues are listed on the left side of the table (i.e. Liver, Muscle, Brain, Tongue, Heart, Lung, Kidney and Placenta). J835, R868B *et al.*, are fetus numbers, and they are arranged based on fetal body weight from large to small in ART group. First seven ART fetuses from left to right present with macrosomia (> 97th percentile body weight of controls). Sex of each fetus is listed above the fetus numbers. The numbers on the top are bodyweight in grams of each fetus. Two columns show the way (ART or AI) the fetuses were generated. The numbers in the tables are the percentage of mRNA expressed from the normally repressed allele. Only samples that had at least 10% expression from the repressed allele were considered biallelic. For example, the liver of the fetus J835 had biallelic expression of *KCNQ1OT1* (19.7% expression from the paternal allele (normally repressed)) and the liver of the fetus R868B had monoallelic expression of *KCNQ1OT1* (0% expression from the repressed allele). “NE” means expression not detectable based on current methods. A “--” means sample not analyzed. ART= assisted reproductive technologies; AI= artificial insemination; F=female; M=male.

Supplemental Table 2

1. RT-PCR primers, conditions, allelic expression assay restriction enzymes, digested product size and PAGE concentrations and Real-time PCR probes. NA: not applicable.

2. Primers and conditions for bisulfite converted KvDMR1, *CDKN1C* exon 2, and *H19/IGF2* ICR. NA: not applicable.

KCNQ10T1		ART																									
		<97% bodyweight																									
Bodyweight	714g	620g	584g	518g	514g	482g	478g	474g	472g	466g	464g	462g	460g	432g	430g	428g	422g	420g	410g	400g	390g	376g	372g	360g	352g	352g	
Sex	F	F	M	F	F	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	F	F	M	F	F	F
Fetus No#	J835	R868B	J183b	J355	J489A	K120	K290b	K169b	J183A	J498A	R893	K282	K075	J817A	R868A	K525	K169A	J566a	J566b	K290A	J498b	J817b	J619	K648	J649	6312	J489b
Liver	19.7	0	0	0	33.7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Muscle	31.5	0	0	0	28.6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Brain	17.7	0	0	0	17.0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Tongue	30.6	0	0	0	20.0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Heart	31.9	0	0	0	29.9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lung	38.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Kidney	35.0	0	0	0	31.4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Placenta	32.2	0	0	0	38.0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	-

KCNQ10T1		AI					
		>97%			<97%		
Bodyweight	496g	416g	410g	408g	404g	380g	
Sex	M	F	M	F	F	M	
Fetus No#	C010	C009	C002	B799	B892	B773	
Liver	0	0	0	0	0	0	
Muscle	0	0	0	0	0	0	
Brain	0	0	0	0	0	0	
Tongue	0	0	0	0	0	0	
Heart	0	0	0	0	0	0	
Lung	0	0	0	0	0	0	
Kidney	0	0	0	0	0	0	
Placenta	0	0	0	0	0	0	

Supplemental Table S1.1

CDKN1C	ART																					
	>97% bodyweight									<97% bodyweight												
	714g	620g	584g	518g	514g	482g	478g	474g	466g	466g	464g	462g	460g	430g	422g	420g	410g	390g	376g	372g	360g	352g
Bodyweight	F	F	M	F	F	M	M	M	F	M	M	M	M	M	M	M	M	F	F	M	F	F
Sex	J835	R868B	J183b	J355	J489A	K120	K290b	K169b	J498A	R893	K282	K075	J817A	K525	J566a	J566b	K290A	J817b	J619	K648	J649	6312
Fetus No#	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Liver	0	0	0	0	19.9	0	0	0	0	0	0	0	0	0	0	0	11.6	0	0	0	0	0
Muscle	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Brain	12.4	35.2	16.9	38.0	12.7	19.1	20.3	21.6	27.0	20.2	25.5	11.8	14.6	18.5	27.3	28.3	27.8	31.5	19.9	23.8	25.0	17.4
Tongue	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Heart	0	0	0	20.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lung	0	0	11.7	0	0	0	0	0	0	0	0	0	0	0	12.9	0	0	10.6	0	0	0	0
Kidney	16.7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Placenta	0	0	0	NE	0	0	0	0	0	0	0	0	0	0	0	0	0	NE	0	0	0	0

CDKN1C	AI							
	>97%				<97%			
	496g	416g	410g	408g	404g	380g		
Bodyweight	M	F	M	F	F	M		
Sex	C010	C009	C002	B799	B892	B773		
Fetus No#	0	0	0	0	0	0		
Liver	0	0	0	0	0	0		
Muscle	0	0	0	0	0	0		
Brain	22.0	16.1	15.7	23.2	23.2	27.7		
Tongue	0	0	0	0	0	0		
Heart	0	0	0	0	0	0		
Lung	15.5	0	0	15.7	0	0		
Kidney	0	0	0	0	0	0		
Placenta	0	0	0	0	0	0		

Supplemental Table S1.2

KCNQ1		>97% bodyweight												<97% bodyweight											
		714g	620g	584g	518g	514g	482g	478g	474g	466g	464g	462g	460g	430g	422g	420g	410g	390g	376g	372g	360g	352g	352g		
Bodyweight	F	J835	R868B	J183b	J355	J489A	K120	K290b	K169b	J498A	R893	K282	K075	J817A	K525	J566a	J566b	K290A	J817b	J619	K648	J649	6312	J489b	
Sex	F	M	F	M	F	M	M	M	M	F	M	M	M	M	M	M	M	M	F	F	F	M	F	F	F
Fetus No#	0	0	13.0	14.9	0	0	15.3	22.0	14.1	12.5	0	20.1	16.2	20.5	0	0	12.5	15.2	18.1	15.6	11.0	0	0	16.4	
Muscle	20.6	10.0	23.2	20.0	29.4	18.6	13.6	11.2	22.8	12.1	0	17.9	20.6	17.3	22.0	22.0	10.8	12.9	14.8	11.6	18.5	0	0	18.5	
Brain	29.9	19.7	41.1	36.2	42.9	41.3	37.6	38.8	35.2	28.5	20.9	15.2	38.0	44.9	31.4	16.7	44.4	30.4	34.8	43.8	35.8	23.8	33.0	33.0	
Tongue	13.7	21.6	17.6	25.2	27.0	17.0	26.7	19.9	22.2	14.4	10.6	23.8	10.0	24.8	0	17.3	20.9	17.7	9.8	18.3	14.6	16.8	28.0	28.0	
Heart	23.8	27.2	26.3	13.6	31.3	27.0	31.6	34.2	29.7	23.2	23.0	27.7	29.5	30.4	32.4	26.9	31.4	27.9	30.6	37.7	31.9	35.1	28.8	28.8	
Lung	13.1	0	28.0	0	17.8	16.3	16.2	20.0	0	10.9	14.1	20.8	18.4	19.0	16.7	15.6	15.2	0	19.7	0	21.3	0	17.5	17.5	
Kidney	20.7	0	0	17.3	35.4	20.0	18.1	16.0	13.9	22.7	13.3	0	23.0	12.5	18.5	20.3	20.7	0	11.9	0	28.2	17.8	11.3	11.3	
Placenta	22.3	0	0	NE	46.9	14.3	0	35.4	0	23.8	0	38.0	23.3	26.2	38.0	14.3	30.6	0	34.0	21.1	0	38.5	--	--	

ART

KCNQ1		>97%												<97%											
		496g	416g	410g	408g	404g	380g	496g	416g	410g	408g	404g	380g												
Bodyweight	M	C010	C009	C002	B799	B892	B773	0	10.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Sex	M	F	M	F	F	F	M	0	10.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Fetus No#	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Liver	0	16.7	16.4	14.3	0	13.5	0	16.7	16.4	14.3	0	13.5	0	16.7	16.4	14.3	0	13.5	0	16.7	16.4	14.3	0	13.5	
Muscle	23.5	15.9	14.7	26.7	28.1	37.8	0	23.5	15.9	14.7	26.7	28.1	37.8	0	23.5	15.9	14.7	26.7	28.1	37.8	0	23.5	15.9	14.7	
Brain	26.5	15.0	0	0	26.4	16.9	0	26.5	15.0	0	0	26.4	16.9	0	26.5	15.0	0	0	26.4	16.9	0	26.5	15.0	0	
Tongue	27.7	14.8	23.6	19.3	25.9	25.1	0	27.7	14.8	23.6	19.3	25.9	25.1	0	27.7	14.8	23.6	19.3	25.9	25.1	0	27.7	14.8	23.6	
Heart	11.2	19.4	0	0	20.6	15.0	0	11.2	19.4	0	0	20.6	15.0	0	11.2	19.4	0	0	20.6	15.0	0	11.2	19.4	0	
Lung	0	16.2	0	0	13.5	15.0	0	0	16.2	0	0	13.5	15.0	0	0	16.2	0	0	13.5	15.0	0	0	16.2	0	
Kidney	47.2	0	12.3	39.3	28.5	14.4	0	47.2	0	12.3	39.3	28.5	14.4	0	47.2	0	12.3	39.3	28.5	14.4	0	47.2	0	12.3	
Placenta	47.2	0	12.3	39.3	28.5	14.4	0	47.2	0	12.3	39.3	28.5	14.4	0	47.2	0	12.3	39.3	28.5	14.4	0	47.2	0	12.3	

AI

Supplemental Table S1.3

PHLDA2	ART						AI							
	>97% bodyweight						<97%							
Bodyweight	714g	620g	584g	518g	514g	482g	478g	390g	372g	416g	410g	408g	404g	380g
Sex	F	F	M	F	M	M	M	F	M	F	M	F	F	M
Fetus No#	J835	R868B	J183b	J355	J489A	K120	K290b	J817b	K648	C009	C002	B799	B892	B773
Liver	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Muscle	0	35.3	50.0	35.1	37.6	0	0	19.9	0	0	0	35.3	22.4	0
Brain	80.0	36.3	17.0	41.3	100	70.5	45.0	55.7	0	40.4	29.4	53.0	0	0
Tongue	100	0	0	0	0	0	39.3	29.5	100	0	0	41.4	0	32.2
Heart	0	0	0	20.1	0	0	35.1	34.7	0	100	0	100	48.9	100
Lung	51.9	51.3	0	55.3	0	0	100	0	0	64.4	0	0	0	0
Kidney	18.7	0	9.8	12.7	21.0	0	15.1	20.0	0	19.4	26.2	21.8	12.5	41.4
Placenta	0	0	0	0	0	0	0	NE	0	0	0	0	0	0

PHLDA2	ART													
	>97% bodyweight						<97%							
Bodyweight	714g	620g	584g	518g	514g	482g	478g	390g	372g	416g	410g	408g	404g	380g
Sex	F	F	M	F	M	M	M	F	M	F	M	F	F	M
Fetus No#	J835	R868B	J183b	J355	J489A	K120	K290b	J817b	K648	C009	C002	B799	B892	B773
Liver	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Muscle	0	35.3	50.0	35.1	37.6	0	0	19.9	0	0	0	35.3	22.4	0
Brain	80.0	36.3	17.0	41.3	100	70.5	45.0	55.7	0	40.4	29.4	53.0	0	0
Tongue	100	0	0	0	0	0	39.3	29.5	100	0	0	41.4	0	32.2
Heart	0	0	0	20.1	0	0	35.1	34.7	0	100	0	100	48.9	100
Lung	51.9	51.3	0	55.3	0	0	100	0	0	64.4	0	0	0	0
Kidney	18.7	0	9.8	12.7	21.0	0	15.1	20.0	0	19.4	26.2	21.8	12.5	41.4
Placenta	0	0	0	0	0	0	0	NE	0	0	0	0	0	0

IGF2	ART						AI								
	>97%						<97%								
Bodyweight	714g	620g	584g	518g	514g	482g	478g	474g	466g	466g	464g	462g	404g	392g	380g
Sex	F	F	M	F	M	M	M	M	M	F	M	M	F	F	M
Fetus No#	J835	R868B	J183b	J355	J489A	K120	K290b	K169b	J498A	R893	K282	K075	B892	B783	B773
Liver	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Muscle	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Brain	24.3	24.6	35.5	25.8	26.1	30.1	25.3	34.1	13.6	0	14.0	9.3	32.2	0	39.2
Tongue	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Heart	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lung	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Kidney	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Placenta	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

IGF2	ART														
	>97%						<97%								
Bodyweight	714g	620g	584g	518g	514g	482g	478g	474g	466g	466g	464g	462g	404g	392g	380g
Sex	F	F	M	F	M	M	M	M	M	F	M	M	F	F	M
Fetus No#	J835	R868B	J183b	J355	J489A	K120	K290b	K169b	J498A	R893	K282	K075	B892	B783	B773
Liver	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Muscle	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Brain	24.3	24.6	35.5	25.8	26.1	30.1	25.3	34.1	13.6	0	14.0	9.3	32.2	0	39.2
Tongue	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Heart	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lung	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Kidney	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Placenta	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Supplemental Table S1.4

H19	ART																			
	>97% bodyweight									<97% bodyweight										
	714g	620g	584g	518g	514g	482g	478g	474g	466g	464g	462g	460g	430g	422g	420g	410g	390g	372g	360g	352g
Bodyweight	F	F	M	F	F	M	M	M	F	M	M	M	M	M	M	M	F	M	F	F
Sex	J835	R868B	J183b	J355	J489A	K120	K290b	K169b	J498A	R893	K282	K075	J817A	K525	J566a	J566b	K290A	J817b	K648	J649
Fetus No#	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Liver	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Muscle	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Brain	0	0	0	0	0	NE	0	0	0	0	0	0	0	0	0	0	NE	0	0	NE
Tongue	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Heart	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lung	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Kidney	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Placenta	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

H19	AI					
	>97%			<97%		
	495g	416g	410g	408g	404g	380g
Bodyweight	M	F	M	F	F	M
Sex	C010	C009	C002	B799	B892	B773
Fetus No#	0	0	0	0	0	0
Liver	0	0	0	0	0	0
Muscle	0	0	0	0	0	0
Brain	0	0	0	0	0	0
Tongue	0	0	0	0	0	0
Heart	0	0	0	0	0	0
Lung	0	0	0	0	0	0
Kidney	0	0	0	0	0	0
Placenta	0	0	0	0	0	0

Gene Symbol	SNP information		Primers (5 → 3)	PCR annealing Temp (°C)	PCR amplicon size (bp)	Expressed allele	Restriction enzyme	Digested Indicus allele (bp)	Digested Taurus allele (bp)	PAGE(%)
	NCBI Accession No#	Bos. Indicus								
KCNQ10T1	NW_003104648.1 2971334, 2971399	G, G	C, A	Forward: TTCTCCCAAGGCTGCCAGAAA	60.7	460	Paternal	NA	NA	NA
				Reverse: TCTGCTCTTCCAGCTGTAGGTT						
CDKN1C	NW_003104648.1 2777626	T	C	Forward: ATGAGACCTCGTGTCAAAGA	60.7	443	Maternal	232, 133, 78	365, 78	7%
				Reverse: AGTGAGGTGCCTTTGGCATAACCA						
KCNQ1	NW_003104648.1 3083541	A	G	Forward: ATCTATGTCGGGAAGCCGTC	59.5	300	Maternal	142, 89, 50, 19	231, 50, 19,	7%
				Reverse: ATCCAGGTCCAAGTCTCTCA						
PHLDA2	NW_003104648.1 2737363	A	G	Forward: AATTGCTGACCAGGGCAAGGAT	61.6	207	Maternal	NA	NA	8%
				Reverse: CCGAGTCTTTATTGGAATGGTGG						
H19	NW_003104648.1 3559835	T	C	Forward: GATATGGTCCGGTGTGATGGAGAGAGCA	62.8	752	Maternal	609, 108, 35	609, 143	18%
				Reverse: TTCTGACCGCTCCAGACTCGGGT						
IGF2	NW_003104648.1 3471637	T	C	Forward: TTCTGACCGCTGGGTGCGCTT	62.0	172	Paternal	NA	NA	8%
				Reverse: AGCTCACTAGTCCGGGCTGC						

Real-Time Taqman Probe	
CDKN1C	Bt03250987_m1
PHLDA2	Bt03249477_g1
IGF2	Bt03259226_m1
GAPDH	Bt03210913_g1

Supplemental Table S2.1

DMR Symbol	SNP information			Primers (5 → 3)	PCR annealing Temp (°C)	Betaine	MgCl ₂	PCR amplicon size (bp)
	NCBI Accession No#	Bos. Indicus	Bos. Taurus					
KvDMR1	NW_003104648.1 2960379	C	G	Forward: GTGAGGAGTGGTATTGAGG Reverse: TCAACCTCTCAACCAATAAC	62.1	NA	4mM	385
CDKN1Cexon2	NA	NA	NA	Forward: GGGATTATAATTTTTAGTTGGATATG Reverse: GCCCCGAAATCCCTAAATAC	58.8	NA	4mM	363
H19/IGF2 ICR	NW_003104648.1 3556134	G	A	Forward: AGTGTGTAGAGGATATTGG Reverse: CTCCTCTCCTAACTTCAAC	53.6	1M	4mM	318

Supplemental Table S2.2

General discussion

In the current study, we compared LOS to BWS at both phenotypic and epigenetic level. To make LOS in bovine, we generated bovine embryos by using ART procedures known to induce LOS and collected them at 105 day of gestation. The conceptuses produced were F1 hybrids of two bovine subspecies *B. t. indicus* and *B. t. taurus*, which enables us to determine the parental-origin of gene expression and DNA methylation based on the polymorphisms between these two subspecies. Our LOS conceptuses recapitulate the major characteristics of BWS such as somatic overgrowth (> 97 percentile which was calculated from the AI controls), enlarged tongue, and abdominal wall defects. We also looked at two imprinting loci in LOS conceptuses, namely KvDMR1 and *H19/IGF2* domain, which are associated with BWS in human. We found that two out of seven LOS conceptuses showed loss of methylation at the KvDMR1 on the maternal alleles. This loss of methylation is correlated with the biallelic expression of the non-coding RNA *KCNQ1OT1* and decreased expression of the flanking maternally-expressed gene *CDKN1C*. Since these are primary epimutations in BWS patients, we concluded that LOS is the bovine counterpart of BWS and LOS could be used as an animal model to study the etiology of BWS.

LOI syndromes in humans are complex imprinting disorders with variable symptoms. It was considered that there is a given imprinting region for a particular syndrome. For example, chromosome 11p15 region is associated with BWS; *H19/IGF2* region for Russell-Silver syndrome (RSS; Smith et al., 2007); *PLAGL1* locus for transient neonatal diabetes mellitus (TNDM; Mackay et al., 2006). However, it was also reported

that a subgroup of BWS exhibited loss of methylation at ICRs other than KvDMR1 (Bliiek et al., 2009; Lim et al., 2009). In these studies, it was found that hypomethylation of other imprinting loci such as *PLAGLI* and *GNAS* loci were present in a subset of BWS patients with loss of methylation at the KvDMR1 (Bliiek et al., 2009; Lim et al., 2009). Hypomethylation of multiple imprinting loci occurred in both ART and non-ART BWS cases (Lim et al., 2009). The overlapping LOI at *PLAGLI* locus in BWS and TNDM might explain some shared clinical features of these two syndromes such as macroglossia and abdominal defects. Misregulated imprinted gene expression has also been reported in LOS ovine (Young et al., 2001). In that study, decreased expression of *IGF2R* and loss of methylation at the *IGF2R* DMR were observed in LOS ovine fetuses (Young et al., 2001). In our study, two out of seven LOS showed loss of methylation of KvDMR1 while other five displayed normal imprinting at both loci examined. The genomic imprinting study in BWS and ovine LOS highly suggested that LOI of some other imprinting loci may be associated with the LOS fetuses. Indeed, an ongoing RNA-seq experiment in our laboratory has shown that the ART-J835LOS (Chapter 2) displayed biallelic-expression of *PLAGLI*. Therefore, future determinations of the imprinted status of other imprinting loci such as *GNAS*, *PLAGLI*, and *IGF2R* in LOS bovine may help elucidate the potential LOI of other loci in LOS, and help explain the differences in phenotypes observed in this study.

Another intriguing phenomenon in BWS is the epigenetic “mosacism” of an individual. The epigenetic “mosacism” involves two type of phenomenon: 1) there is a different pattern of loss of methylation at a single locus across organs/tissues within the

same individual: some organ/tissues have a severe loss of methylation while some other tissues display a moderate loss of methylation; and 2) different imprinted loci exhibit different pattern of loss of methylation in the same organ/tissue within a BWS patient: some loci show complete loss of methylation whereas some other loci show incomplete pattern. For the first situation, similar scenario was observed in our LOS model. In ART-J835LOS (Fig. 5), kidney shows a 13% methylation at the KvDMR1 on the maternal allele (~98% methylation in control), whereas liver displays a 63% methylation of KvDMR1 (~92% methylation in control). It is still unclear how this “mosacism” occurs. It is possible that at certain stages of early development after fertilization, the DNA methylation of the ICR is not faithfully maintained through DNA replication. The incomplete DNA methylation maintenance leads to hemi-methylation of the ICR: one strand of DNA is methylated while the other is unmethylated. Subsequently, the continuous cell divisions and chromosome segregations give rise to the different DNA methylation pattern of ICR in different tissues. However, it is still unknown when this happens in embryo/fetal development. In mice, it has been shown that embryos with a null mutation of *Dnmt1o* can develop into an epigenotypic “mosacism” individual (Cirio et al., 2008). And *Dnmt1o* is believed to be important to maintain DNA methylation at the 8-cell stage (Howell et al., 2001). Since pre-implantation stage is a critical period for imprints maintenance when it is undergoing global DNA demethylation, it's possible that the time point of loss of methylation also occurs at the pre-implantation stage in BWS and LOS. Therefore, in future it will be worth determining if bovine pre-implantation embryo already exhibit LOI of KvDMR1 and if blastocysts with the LOI can lead to

LOS. For the second situation of “epigenetic mosaicism”, it was reported that one BWS patient showed complete loss of methylation of *PLAGL1* and *MEST* DMR but incomplete loss of methylation of KvDMR1 (Bliek et al., 2009). This suggests that different imprinting loci have different resistance to the loss of methylation, but the underlying mechanism is still unknown.

In the current study, we established a bovine LOS model for the human pediatric overgrowth condition BWS. Study on the LOS and associated LOI can help us understand the potential mechanisms that lead to the BWS symptoms. In addition, future studies of LOS could also contribute to our understanding of genomic imprinting in species beyond human and mice.

Bibliography

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Appendix I:

DNA methylation of KvDMR1 in day 65 *B. t. indicus* × *B. t. taurus* hybrid F1

The work in this part followed the results obtained in a previous study was performed to establish the baseline DNA methylation and imprinted gene expression in the KvDMR1 and *H19/IGF2* domain in bovine (Robbins et al., 2012). The day~65 *B. t. indicus* × *B. t. taurus* hybrid F1 were generated with the use of artificial insemination (AI). In that study, three F1 conceptuses were collected and various tissues from each conceptus were analyzed at these two imprinting domain.

The method used to analyze DNA methylation is bisulfite sequencing. Basically, genomic DNA is treated with sodium bisulfite in which unmethylated cytosines are converted to uracil while methylated cytosines remain cytosines. In the following polymerase chain reaction (PCR), uracil is replaced by thymine. Then the amplified region of interest from PCR is cloned into *E. coli*. In each *E. coli* colony, only a single PCR product is cloned, which allow us to separate individual PCR product and get parental allele-specific methylation pattern based on the polymorphisms between the two subspecies. Cloned bisulfite-converted DNA in each *E. coli* colony is then sequenced. DNA methylation of each CpG in the region can be obtained by comparing sequencing data to the genome reference.

Initial results using combined bisulfite restriction analysis (COBRA) showed that KvDMR1 and *H19* ICR are differentially. The bisulfite sequencing assay of the *H19* ICR also displayed normal differential methylation (methylated paternal alleles; unmethylated maternal alleles) in the three day~65 hybrid conceptuses although we found that *E. coli*

has a bias toward to copy the methylated DNA (more CpGs) and reject unmethylated DNA (more TpGs). However, we found that all the bacteria colonies for KvDMR1 we picked up were of maternal origin (methylated allele) based on the known polymorphisms (maternal allele: *B. t. taurus* = G; paternal allele: *B. t. indicus* = T). In these colonies, we also found several completely unmethylated alleles but with maternal genetic polymorphism.

At first, we thought it must be *E. coli*'s fault which rejected all the unmethylated paternal alleles, or maybe the bisulfite PCR primers were not equally amplifying both methylated and unmethylated alleles. Then we developed new bisulfite assay in which we still used the same polymorphic site. We tested this assay on the day~105 AI conceptuses and obtained beautiful differential DNA methylation data (half the colonies displayed methylated maternal alleles; the other half colonies showed unmethylated paternal alleles). Then we applied the new developed assay on the three day~65 conceptuses. To our great surprise, the colonies showed beautiful differential methylation like the data from day~105 AI conceptuses, however, all of them were maternal alleles based on the polymorphism. This led us to think it may be the polymorphism issue instead of *E. coli* or bisulfite primers.

We resequenced the KvDMR1 region of the three *B. t. taurus* animals, one *B. t. indicus* whose semen strains were used to produce both day~65 and day ~105 AI conceptuses, three day~65 conceptuses, and several day~105 AI conceptuses. We found that the *B. t. indicus* bull is heterozygous (T/G) at the polymorphism site used in the bisulfite assay, a fact that was no evident at first we sequenced the region. All the three

day ~65 conceptuses inherited the “G” allele from the bull, which “disguised” the actual paternal allele (supposed to be “T” instead of “G”) as the maternal allele (G). However, the two day~105 AI conceptuses inherited the “T” allele from the bull, which is an informative polymorphism that allows us to the parental origin.

Then we developed another assay to cover a different polymorphic sites in the KvDMR1 region. In the new assay, no single primer set was available to amplify the both alleles at the same time in the three day~65 conceptuses due to high GC content and additional polymorphisms. Thus we developed allele-specific bisulfite primers that were able to amplify each allele separately in the same reaction (Table 1). The success of this assay was finally confirmed by the bisulfite cloning sequencing (Figure 1). The table and figure have been published (Robbins et al., 2012).

Table 1. PCR primers and conditions used to determine DNA methylation at the KvDMR1

Gene/ICR Symbol	Primers (5'-3')		PCR Annealing T _m (°C)	PCR size (bp)	Primer [] μM	MgCl ₂ [] (mM)	# Cycles
KvDMR1	Forward	TGAGGAGTGTAGTTATGAGGA <i>(taurus)</i> TGAGGATTGTAGTTGTGAGGA <i>(indicus)</i>	59.2	419/422	0.3	4	45
	Reverse	CTACCACATCTACCCCAATC					

T_m = Temperature in °C, bp= base pairs, []= concentration

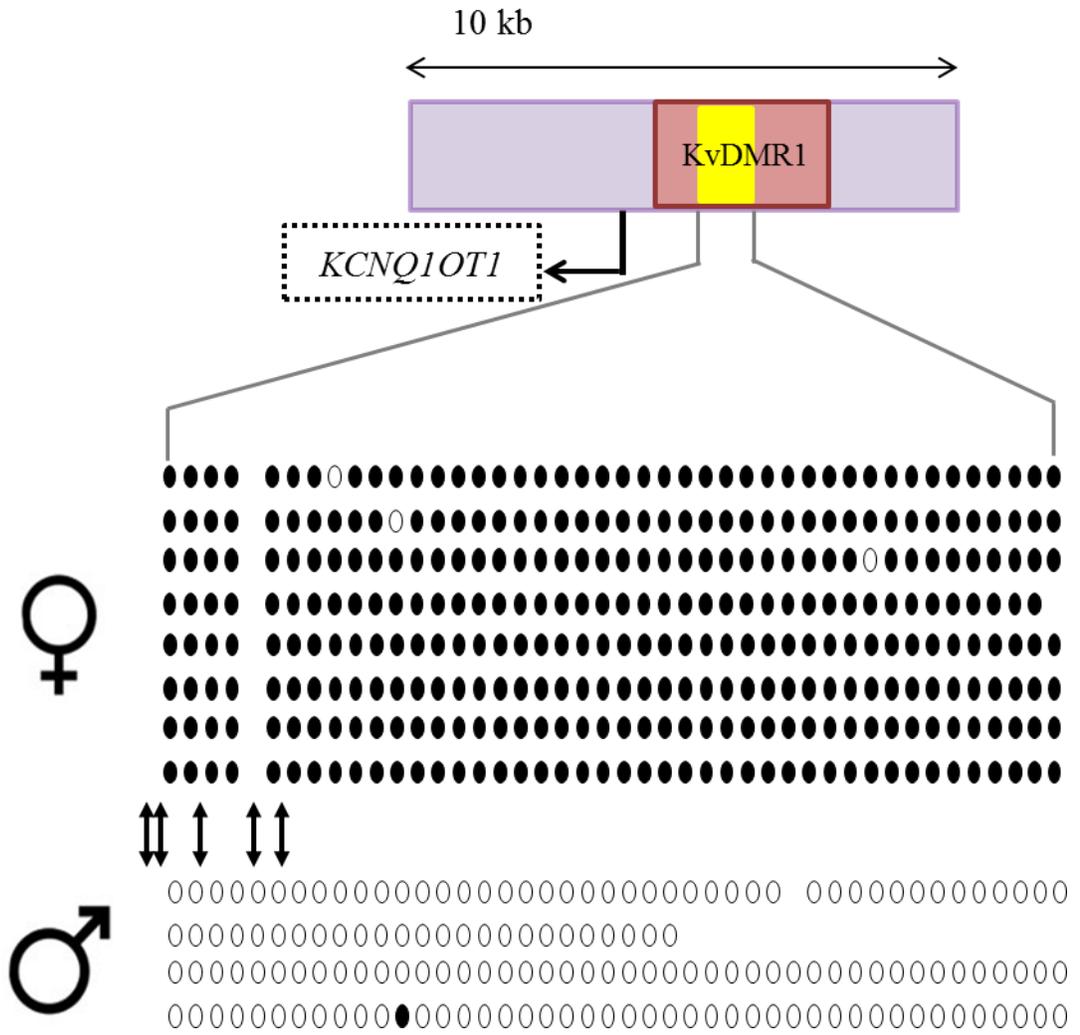


Figure 1

Figure 1. Differential methylation at the KvDMR1 in bovine

Top: Part of *Kcnq1* intron 10th is drawn to scale and depicted in light purple. Arrow depicts direction of *Kcnq1* transcription. The region amplified by the bisulfite specific primers is represented by a yellow box. Bottom: Shown is an example of bisulfite sequence data from an F1 individual. The bisulfite converted DNA was amplified and cloned prior to sequencing. Each line of circles represents individual alleles. Open circles represent unmethylated CpGs and closed circles represent methylated CpGs. Female symbol = maternal alleles, male symbol = paternal alleles. The position of the SNP used to differentiate between *B. t. indicus* and *B. t. taurus* alleles is shown by arrows. The insertion/deletion “GCG” SNP (Table 1) results in an additional CpG site on the paternal alleles compared to maternal alleles.

VITA

Zhiyuan Chen (people usually call him “Chen” for ease pronunciation) was born on April 23th, 1990 in a town in Shanxi Province, China (~ 400 miles south of Beijing). Chen grew up with his parents Cunliang (Dad) and Xiaoyin (Mom). Chen attended China Agricultural University in Beijing during 2007-2011. In June, 2011, Chen graduated with a bachelor degree in Animal Sciences. In August, 2011, Chen came to the University of Missouri, Columbia and joined Dr. Rivera’s laboratory to pursue a Master of Science degree in the area of developmental epigenetics. After he completes his M.S. degree in May 2013, Chen will continue his Ph. D work in Dr. Rivera’s laboratory.