**MOLECULAR CHARACTERIZATION OF
A CONGENITAL OVERGROWTH SYNDROME INDUCED
BY ASSISTED REPRODUCTION**

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OVERGROWTH SYNDROME INDUCED BY ASSISTED
REPRODUCTION**

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*Due to space limit, Table S2-S5 can only be accessed through the web links, which are directed to the online *Epigenetics* paper
(<http://www.tandfonline.com/doi/full/10.1080/15592294.2016.1184805>).

Preface

Beckwith-Wiedemann syndrome (BWS) is a congenital overgrowth condition with increased likelihood to develop childhood tumors. Children conceived with the use of assisted reproductive technologies (ART) have an increased frequency to have BWS compared to naturally conceived individuals. In ruminants, the use of ART can induce a similar overgrowth condition that phenotypically recapitulates BWS, which is referred to as large offspring syndrome (LOS). Previously we have shown that LOS is both phenotypically and epigenetically similar to BWS. The overall goal of our research is to use bovine LOS as an animal model to understand the molecular etiology of congenital overgrowth syndromes. As a step to achieve the overall goal, the current project is to comprehensively characterize the bovine LOS at the molecular level.

In this dissertation, chapter 1 reviews the mechanisms of genomic imprinting and describes the current progress in understanding the etiology of the loss-of-imprinting conditions including BWS and LOS. In chapter 2, we determined the allelic expression of imprinted genes previously identified in human and/mouse in bovine control and LOS fetuses. We demonstrated that LOS is a multi-locus-loss-imprinting syndrome, which is similar to what has been reported for a subset of BWS individuals. This study was published in Proceedings of the National Academy of Sciences (PMID: 25825726). In chapter 3, we showed global misregulation of non-imprinted genes in LOS, indicating that loss-of-imprinting is not the only molecular lesion of LOS. Further, most differentially expressed genes in LOS cannot be directly associated with the aberrant DNA methylation, which highlights that caution should be exercised when interpreting time-point-specific DNA methylation data. The manuscript of this study is currently

under peer review. In chapter 4, for the purpose of *de novo* identification of novel imprinted genes, we determined global allele-specific gene expression in bovine conceptuses. We identified eight novel imprinted genes in bovine and demonstrated that monoallelic gene expression can be the result of *cis*-eQTL effect. Further, we expanded the potential adenosine-to-inosine RNA editing sites from ~20 to 671 in bovine. This chapter was published in the Landes Bioscience Journal: Epigenetics (PMID: 27245094). Lastly, chapter 5 discusses the key questions that remain to be addressed in the field and concludes the dissertation.

Nomenclature

ART	Assisted reproductive technologies
AS	Angelman syndrome
ASM	Allele-specific DNA methylation
A-to-I RNA editing	Adenosine-to-Inosine RNA editing
bp	Base pair
<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
CDS	Coding sequences
CPM	Counts per million
CTCF	“CCCTC” binding factor
DEG	Differentially expressed gene
DMR	Differentially methylated region
Dnmt	DNA methyltransferase
E #	Embryonic day #
5caC	5' carboxylcytosine
5fC	5' formylcytosine
5hmC	5' hydroxymethylcytosine
5mC	5' methylcytosine
gDMR	Gametic differentially methylated region
HCP	High CpG density promoter
ICP	Intermediate CpG density promoter
ICR	Imprinting control region
ICSI	Intracytoplasmic sperm injection
IGV	Integrative Genomic Viewer
IVC	<i>in vitro</i> culture
IVF	<i>in vitro</i> fertilization
Kb	Kilo base pairs

KEGG	Kyoto Encyclopedia of Genes and Genomes
LCP	Low CpG density promoter
LOI	Loss-of-imprinting
LOS	Large offspring syndrome
Mb	Mega base pairs
ncRNA	Non-coding RNA
PAGE	Polyacrylamide gel electrophoresis
PCA	Principle component analysis
PCR	Polymerase chain reaction
PGC	Primordial germ cell
PWS	Prader-Willi syndrome
RDD	RNA-DNA differences
RFLP	Restriction fragment length polymorphism
RPKM	Reads per kilobase of transcript per million mapped reads
RT	Reverse transcription
SCNT	Somatic cell nuclear transfer
SNP	Single nucleotide polymorphism
SRS	Silver-Russell syndrome
TES	Transcription end site
TSS	Transcription start site
WGBS	Whole genome bisulfite sequencing
WGCNA	Weighted gene correlation network analyses

Chapter 1 Literature review

1.1 General overview of genomic imprinting

Genomic imprinting is a series of precisely regulated epigenetic processes that lead to parental-allele-specific expression of a subset of genes in mammals (reviewed in Bartolomei and Ferguson-Smith, 2011). Since the discovery of the first three imprinted genes, namely, *Igf2*, *Igf2r*, and *H19* in 1991 (Barlow et al., 1991; Bartolomei et al., 1991; DeChiara et al., 1991), more than 100 genes have been identified to be imprinted in mice and humans (<http://www.mousebook.org/mousebook-catalogs/imprinting-resource>; <http://www.geneimprint.com/>). Most of the identified imprinted genes are located in clusters with two or more imprinted genes that spread over 20kb to 3.7Mb of DNA (reviewed in Edwards and Ferguson-Smith, 2007). Within each cluster, a discrete region of DNA, termed as imprinting control region (ICR), regulates the proper allele-specific expression of imprinted genes (reviewed in Bartolomei and Ferguson-Smith, 2011). ICRs are marked with parental-allele-specific DNA methylation, which distinguishes the parental alleles in the same nucleus (reviewed in Bartolomei and Ferguson-Smith, 2011). The allele-specific DNA methylation at ICRs is established in a sex-specific manner in the germline. In mice, male germ line methylated ICRs are established before birth, while acquisition of the maternal-specific DNA methylation occurs postnatally during oocyte growth. After fertilization, the gametic DNA methylation imprints escape the wave of global DNA demethylation and are maintained in somatic cells throughout development. Further, DNA methylation is erased in primordial germ cells in order to re-establish the imprints appropriately for next generation (reviewed in Bartolomei and

Ferguson-Smith, 2011). Over the past ~26 years, much work has been done to elucidate how DNA methylation is erased and acquired in the germ line and how allele-specific DNA methylation at ICRs are retained when global DNA demethylation occurs post-fertilization. Currently, it is recognized that highly coordinated epigenetic processes govern DNA methylation at ICRs to ensure parental-specific expression of imprinted genes.

The importance of genomic imprinting in mammalian development was first demonstrated by the pronuclear transplantation experiments, in which androgenetic and gynogenetic mouse embryos resulted in embryonic lethality (Barton et al., 1984; McGrath and Solter, 1984). Androgenetic conceptuses (derived from two paternal pronuclei) had a severely retarded fetus but a well-developed placenta while gynogenetic fetuses (derived from two maternal pronuclei) were smaller than the bi-parental fetuses and had an undeveloped placenta (Barton et al., 1984; McGrath and Solter, 1984). Later it was confirmed that genomic imprinting is the only barrier for viability of the gynogenetic embryos (Kono et al., 2004). In these experiments, alteration of maternal imprinting by oocyte reconstruction using non-grown oocytes that had a deletion of the *H19* region (including the ICR and the *H19* transcription unit) allowed gynogenetic embryos to develop to term (Kono et al., 2004). Investigations of imprinted gene function also demonstrated the significance of genomic imprinting in mammalian development. For example, mice with maternal transmission of the non-functional allele of *Igf2*, a paternally expressed gene, showed normal growth, but paternal transmission of this mutant allele had deficient growth that was similar to *Igf2* null mice (DeChiara et al., 1991). In addition to mouse models, the critical roles of imprinted genes in

development are also evident in human, cow, and sheep. For instance, Beckwith-Wiedemann syndrome (BWS) in humans and large offspring syndrome (LOS) in ruminants (*i.e.*, cattle and sheep), two overgrowth conditions that share many phenotypic features, are partially associated with the loss of DNA methylation at the maternal ICR of the imprinted *KCNQ1* locus (Chen et al., 2013; reviewed in Weksberg et al., 2010). Although less effort has been focused on determining the function of imprinted gene products when compared to the imprinting mechanisms, these genes emerge as important players in numerous biological processes including fetal growth, placental development, neural development, and behavior (reviewed in Plasschaert and Bartolomei, 2014).

In this chapter, the epigenetic properties of imprinted genes are reviewed with major focus on the dynamic regulation of DNA methylation imprints from germ cells to pre-implantation embryos in mice. Further, the functional roles of imprinted genes in mammalian development are reviewed with detailed description of two loss-of-imprinting disorders: BWS in humans and LOS in cattle.

1.2 Epigenetic properties of imprinted genes

1.2.1 Mechanism of imprinted gene regulation

Imprinted genes are usually found in clusters containing 2 to 12 genes (reviewed in Edwards and Ferguson-Smith, 2007). These clusters can span 20kb to 3.7Mb of DNA. Although not as common, examples of solitary imprinted genes do exist. A typical imprinting cluster contains maternally and paternally expressed protein-coding genes as

well as non-coding RNAs (ncRNAs). Within each cluster, the proper allele-specific expression of imprinted genes is regulated by the core *cis*-element ICR, which exhibits parental-specific epigenetic modifications such as DNA methylation and histone modifications (reviewed in Bartolomei and Ferguson-Smith, 2011). Deletion of the ICRs can lead to loss-of-imprinting of multiple imprinted genes within a cluster, demonstrating the critical role of ICRs in the regulation of imprinted gene expression (Fitzpatrick et al., 2002; Lin et al., 2003; Thorvaldsen et al., 1998; Williamson et al., 2006; Wutz et al., 1997; Yang et al., 1998). There are several mechanisms by which ICRs initiate and maintain the monoallelic expression of the imprinted genes within each cluster. The best-defined two mechanisms are the insulator model and the ncRNA model (reviewed in Lee and Bartolomei, 2013).

The insulator model is best exemplified by the *H19/Igf2* imprinted domain in mice. In this cluster, *Igf2* is a paternally expressed protein-coding gene, while *H19* is a maternally expressed ncRNA (Bartolomei et al., 1991; DeChiara et al., 1991). The ICR is located approximately 2kb upstream of the *H19* transcription start site and is paternally methylated while maternally unmethylated (Thorvaldsen et al., 1998; Tremblay et al., 1995). On the maternal allele, the insulator protein CTCF binds to the unmethylated ICR and prevents the *Igf2* promoter from accessing the downstream enhancers (Bell and Felsenfeld, 2000; Hark et al., 2000; Leighton et al., 1995b). Thus, the permissive chromatin conformation allows the maternal *H19* to access the enhancers and gets transcribed. On the paternal allele, however, DNA methylation of the ICR prevents binding by CTCF. Absence of the insulator leads to a chromatin conformation in which the *Igf2* promoter engages with the downstream enhancers (Bell and Felsenfeld, 2000;

Hark et al., 2000; Leighton et al., 1995b). Therefore, *Igf2* is activated on the paternal allele by the downstream enhancers while *H19* is repressed. As illustrated by this example, the DNA methylation status of the ICR directs the allelic regulation of imprinted genes.

The other major imprinting mechanism is the ncRNA model, which can be applied to the *Kcnq1/Kcnq1ot1* and *Igf2r/Airn* imprinted clusters. The promoter of the long ncRNA can serve as the ICR for the imprinted cluster (Fitzpatrick et al., 2002; Mancini-DiNardo et al., 2003; Smilnich et al., 1999; Stoger et al., 1993; Wutz et al., 1997). For the *Kcnq1/Kcnq1ot1* imprinted domain the unmethylated promoter/ICR on the paternal allele allows the transcription of the long ncRNA *Kcnq1ot1*, which can recruit and expand repressive histone modifications to silence the flanking paternally imprinted genes (Pandey et al., 2008; Terranova et al., 2008). However, on the maternal allele, the ICR is methylated and the *Kcnq1ot1* is not activated, allowing the transcription of the proximal maternal genes. For the *Igf2r/Airn* imprinted cluster, the unmethylated ICR activates the long ncRNA *Airn* on the paternal allele, which transcribes antisense through the *Igf2r* transcription start site and interferes with the interaction between the *Igf2r* promoter and the RNA polymerase II (Latos et al., 2012; Sleutels et al., 2002). In contrast, the ICR is methylated on the maternal allele and *Airn* is silenced by DNA methylation, and this allows the transcription of *Igf2r* (Latos et al., 2012; Sleutels et al., 2002). Notably, the allelic regulation process initiated by ICRs, also known as “primary imprints”, can set up additional epigenetic differences in the imprinting clusters, such as “secondary DMRs” and allele-specific histone modifications (reviewed in Sanli and Feil, 2015). These additional epigenetic differences stably propagate during cell divisions and

could act as a candidate mechanism for tissue-specific imprinting. For example, the allele-specific histone modifications established by the long ncRNA *Kcnq1ot1* at the maternally expressed genes (*e.g.*, *Ascl2* and *Osbp15*) are lost in embryonic lineages while maintained in extra-embryonic lineages (Lewis et al., 2004; Umlauf et al., 2004), resulting in the placental-specific imprinting of these genes.

1.2.2 Dynamic regulation of genomic imprints

Erasure of imprints in primordial germ cells

In mice, specification of primordial germ cells (PGCs) from the epiblast begins on embryonic day 6.5-7.5 (E6.5-7.5) (Ginsburg et al., 1990; Saitou et al., 2002; Sato et al., 2002). Following specification, PGCs then proliferate and migrate along the hindgut to the genital ridge where they colonize by E11.5 (Molyneaux et al., 2001; Tam and Snow, 1981). Before migration, PGCs exhibit similar global DNA methylation profiles to their somatic cell origins (Guibert et al., 2012; Seisenberger et al., 2012). For instance, the germline-specific genes (*e.g.*, *Mvh* and *Dazl*) and the pluripotent genes (*e.g.*, *Nanog*) are repressed by DNA methylation (Maatouk et al., 2006; Seisenberger et al., 2012). However, by E12.5-13.5, the global DNA methylation is very low and the pluripotent genes and germline-specific genes are activated (Maatouk et al., 2006; Seisenberger et al., 2012). Further, parental imprints are erased and the silenced X chromosome is reactivated in the process of PGC reprogramming (Hajkova et al., 2002; Monk and McLaren, 1981; Tam et al., 1994).

In mice, it is clear that both passive and active DNA demethylation are involved in this process. Currently, it is recognized that the epigenetic reprogramming in PGCs

begins with a passive DNA demethylation during E8.0-10.5 and is then followed by a wave of active DNA demethylation from E10.5 to 13.5 (Guibert et al., 2012; Seisenberger et al., 2012; Yamaguchi et al., 2013a). For the passive DNA demethylation, the 5-methylcytosines (5mC) are diluted by the rapid proliferation of PGCs (~12h per cell cycle) when the DNA methylation machinery is repressed (Kagiwada et al., 2013). Specifically, the *de novo* DNA methyltransferases (Dnmt3a/3b) and their cofactor Dnmt3l are silenced in PGCs (Hajkova et al., 2002; Kagiwada et al., 2013; Seki et al., 2005). Further, although the maintenance DNA methyltransferase Dnmt1 is abundant in PGCs, its essential cofactor Uhrf1 (also known as Np95) is excluded from the nucleus (Kagiwada et al., 2013). In addition, in support of the replication-dependent DNA demethylation, hairpin bisulfite sequencing can detect increased incidence of hemi-methylated DNA strands in PGCs in E9.5-10.5 (Arand et al., 2012).

Following passive dilution of 5mC, active DNA demethylation occurs in PGCs involving the ten-eleven translocation family of dioxygenases Tet1/Tet2 (Ito et al., 2010; Tahiliani et al., 2009) and the thymine-DNA glycosylase Tdg (He et al., 2011; Shen et al., 2013). In the wave of active demethylation, Tet1/Tet2 sequentially oxidize 5mC to 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5faC), or 5-carboxycytosine (5caC) (Ito et al., 2011). In support of these mechanisms, the level of 5hmC has a temporal increase during E9.5-10.5 accompanied by decreasing 5mC levels, which is then followed by a reduction of 5hmC levels at E11.5-12.5 (Hackett et al., 2013). The 5mC derivatives (*i.e.*, 5faC and 5caC) can be either diluted through cell proliferation or targeted by Tdg-mediated base excision repair (He et al., 2011; Shen et al., 2013). This is

exemplified in *Tdg* mutant E11.5 PGCs where the secondary imprints at *Igf2* DMR2 exhibit hypermethylation (Cortellino et al., 2011).

Two-phase DNA demethylation process in PGCs suggests that distinct DNA demethylation mechanisms (*i.e.*, passive and active demethylation) affect different genomic sequences. Indeed, the passive DNA demethylation in the early phase affects the bulk genome, while specific sequences including imprinted genes become fully unmethylated only after the Tet1/2-mediated active demethylation phase (Seisenberger et al., 2012). Notably, *Tet1* knockout males exhibit hypermethylation of the imprinted genes *Peg3*, *Peg10*, *Impact*, *Igf2r*, *Gnas*, *Grb10*, and *Kcnq1ot1* DMRs in both sperm and E13.5 PGCs, demonstrating the important role of Tet1 in erasure of the DNA methylation imprints at the late reprogramming stage in PGCs (Yamaguchi et al., 2013b). However, it should be noted that normal imprint erasure could occur in a fraction of the *Tet1/Tet2* mutant mice progenies (Dawlaty et al., 2013; Yamaguchi et al., 2013b), suggesting that replication-dependent dilution of 5mC is probably sufficient for DNA methylation reprogramming in PGCs while Tet proteins act as a “safety check” for this process.

Establishment of imprints in gametogenesis

The re-establishment of DNA methylation in germ cells occurs at different times in development in male and female gonads (reviewed in Stewart et al., 2016). In males, global *de novo* DNA methylation occurs in prospermatogonia from ~E14.5 until birth (Davis et al., 1999; Li et al., 2004). However, in females, *de novo* methylation initiates after follicle activation in the postnatal ovary and is complete in fully-grown oocytes (Lucifero et al., 2002). Notably, the acquisition of DNA methylation imprints in growing

oocytes is asynchronous for different maternally methylated gametic DMRs (gDMRs) (Lucifero et al., 2004; Obata and Kono, 2002). For example, of the analyzed maternal gDMRs, the *Snrpn* DMR is the first to acquire DNA methylation, followed by *Peg1*, *Peg3*, and *Impact* DMRs (Lucifero et al., 2004; Obata and Kono, 2002). This asynchronous acquisition of DNA methylation at maternal gDMRs suggests that different factors affect the imprint establishments at different loci. In agreement with this, absence of the Zfp57 (a KRAB zinc finger protein) in oocytes abolishes the acquisition of DNA methylation at *Snrpn* DMR but not *Peg1* and *Peg3* DMRs (Li et al., 2008).

Besides the asynchronous acquisition of DNA methylation for different gDMRs, the same gDMR can exhibit different rates of *de novo* DNA methylation for the maternally and paternally inherited alleles (Davis et al., 2000; Lucifero et al., 2004). For example, in prospermatogonia, *H19/Igf2* DMR acquires 5mC on the paternally inherited allele prior to maternally inherited allele (Lee et al., 2010). This phenomenon suggests that other epigenetic modifications still recognize the parental alleles in PGCs despite both alleles being unmethylated. It has been found that the insulator CTCF binds to the maternal allele in prospermatogonia prior to *de novo* methylation, potentially delaying the acquisition of methylation on this allele (Lee et al., 2010). Further, the chromatin composition of the parental alleles at *H19/Igf2* DMR is different prior to acquisition of DNA methylation with the maternal allele being enriched with H3K4me3 compared to the paternal allele, potentially also contributing to the delayed *de novo* DNA methylation as this histone modification inhibits the binding of Dnmt3l to the Histone 3 tail (Lee et al., 2010; Ooi et al., 2007).

The establishment of DNA methylation imprints in both male and female germ lines requires the *de novo* DNA methylation machinery, which consists of the *de novo* DNA methyltransferases Dnmt3a/3b/3c and the non-catalytic cofactor Dnmt3l. Dnmt3a and Dnmt3l are required for both maternal and paternal imprint establishment (Bourc'his et al., 2001; Kaneda et al., 2004; Kato et al., 2007). Female mice with *Dnmt3l* knockout or conditional knockout of *Dnmt3a* in oocytes fail to establish DNA methylation for all the maternal gDMRs examined and their progeny die around mid-gestation largely due to the defects of maternal imprinting (Bourc'his et al., 2001; Kaneda et al., 2004). For males, deficient Dnmt3a or Dnmt3l results in meiotic failure in spermatocyte most likely due to the derepression of retrotransposons, precluding the further analyses of paternal imprints in sperm or offspring (Bourc'his and Bestor, 2004; Kaneda et al., 2004; Webster et al., 2005). However, examination of the spermatogonia from the newborn testes of *Dnmt3a* conditional knockouts indicates that Dnmt3a is essential for the *de novo* methylation of *H19/Igf2* and *Gtl2/Dlk1* DMR, but not *Rasgrfl* DMR (Kaneda et al., 2004). Unlike *Dnmt3a* and *Dnmt3l* mutants, mice with conditional *Dnmt3b* knockout in germ cells and their offspring show no apparent abnormalities with most imprints correctly established except that the *Rasgrfl* DMR is moderately affected (Kato et al., 2007). Recently, *Dnmt3c*, which was previously annotated as a pseudogene, has been identified as a *de novo* methyltransferase and is required for the methylation of evolutionarily young repetitive elements and the imprint establishment only at *Rasgrfl* DMR in male germ cells (Barau et al., 2016).

Mechanisms of establishment of differential imprints in sperms and oocytes

Prior to determination of the DNA methylome of sperm and oocytes, it was hypothesized that the imprinted gDMRs are specifically targeted by the *de novo* DNA methylation machinery. However, recent whole genome wide DNA methylation profiling has led to the reconsideration of this assumption. Specifically, sperm cells have very high levels of global DNA methylation (~80-90%) with methylated CpGs being evenly distributed throughout the genome except for a few encountered within unmethylated CpG islands (Kobayashi et al., 2012; Smallwood et al., 2011; Smith et al., 2012). However, oocytes exhibit a lower level of genome wide DNA methylation (~30-40%) with alternating hypermethylated and hypomethylated domains (Kobayashi et al., 2012; Smallwood et al., 2011; Smith et al., 2012). The disparity in global DNA methylation between sperm cells and oocytes led to the discovery of gDMRs beyond the classical imprinted gDMRs (Kobayashi et al., 2012; Smallwood et al., 2011; Smith et al., 2012). Unlike the imprinted gDMRs, the majority of the newly discovered gDMRs are transient and not maintained throughout development (Kobayashi et al., 2012; Smallwood et al., 2011; Smith et al., 2012). Therefore, the imprinted gDMRs are selectively maintained after fertilization (discussed in detail later, page 17) instead of specifically targeted by *de novo* DNA methylation machinery over non-imprinted gDMRs during gametogenesis. To date, 26 definitive imprinted gDMRs have been identified in mice with 23 being maternally methylated and 3 being paternally methylated (reviewed in Stewart et al., 2016).

Although the mechanism that involves the establishment of different patterns of DNA methylation in sperm cells and oocytes remains elusive, the recent findings

highlight that the acquisition of DNA methylation, at least in oocytes, is coordinately regulated by transcription and histone modifications (reviewed in Hanna and Kelsey, 2014). The first piece of evidence that connects histone modification and DNA methylation comes from the finding that the specific interaction between the amino terminus of histone H3 and Dnmt3l is strongly inhibited by methylation at lysine 4 of H3 (Ooi et al., 2007). Further, it was found that histone H3, but not H3K4me3, can release the catalytic domain of Dnmt3a from the auto-inhibition by its Atrx-Dnmt3-Dnmt3l (ADD) domain and stimulate the enzymatic activity of Dnmt3a (Guo et al., 2015). In agreement with this, DNA methylation in oocytes is strongly anti-correlated with the broad H3K4me3 domain that covers ~22% of the genome (Dahl et al., 2016). Further, oocytes deficient of Kdm1b (also known as Aof1 or Lsd2), a H3K4 demethylase, fail to establish DNA methylation at hundreds of CpG islands including several maternal gDMRs such as *Mest*, *Grb10*, *Zac1* and *Impact* DMR (Ciccone et al., 2009; Stewart et al., 2015). Most importantly, profiling of H3K4me2/3 enrichment in primary and growing oocytes indicates that removal of H3K4me2/3 occurs prior to the onset of *de novo* DNA methylation (Stewart et al., 2015), suggesting that the methylation machinery is directed by pre-existing chromatin marks in female germ cells. Lastly, H3K4me2/3-enriched regions in male germ cells are resistant to acquiring DNA methylation, highlighting the critical role of H3K4me2/3 in protection against *de novo* DNA methylation (Singh et al., 2013).

Besides histone modifications, several lines of evidence indicate that transcription plays a role in the establishment of maternal imprints in oocytes (reviewed in Hanna and Kelsey, 2014). For example, in the *Gnas* cluster, truncation of a transcript *Nesp*, which

is transcriptionally active in oocytes and overlaps with the ICR, leads to the failure of maternal DNA methylation establishment at this locus (Chotalia et al., 2009). In addition, similar results were obtained for the maternal imprinted gDMRs *Snrpn* and *Plagl1* (Smith et al., 2011; Veselovska et al., 2015). Notably, comprehensive profiling of the oocyte transcriptome and DNA methylome indicates that transcription events correlate with ~90% of the DNA methylation established in the oocyte, including the methylated CpG islands and imprinted gDMRs (Veselovska et al., 2015). Although a small portion of actively transcribed genes escape DNA methylation, it is likely that transcription is a universal mechanism that contributes to the acquisition of DNA methylation at both imprinted and non-imprinted loci in female germ cells (Veselovska et al., 2015).

A question that remains to be answered is how exactly transcription and histone modifications coordinately direct *de novo* DNA methylation in oocytes. Current understanding is that activation of oocyte transcriptome accumulates H3K36me3 at gene bodies, a histone modification that is associated with transcript elongation and can recruit Dnmt3a to target regions (Dhayalan et al., 2010). Further, prior to the onset of *de novo* methylation, the H3K4 methylation at regions destined for DNA methylation has to be removed by Kdm1b, a process that appears to be also dependent on transcription (Stewart et al., 2015). In support of this, truncation of a transcript that overlap with the imprinted *Plagl1* DMR leads to the failure of DNA methylation establishment, which is coupled with the absence of H3K36me3 and retention of H3K4me2 at the imprinted gDMR (Stewart et al., 2015). Further, RNA-seq profiling of *Kdm1b* mutant oocytes indicates

that the transcriptome is largely normal, suggesting Kdm1b removal of H3K4 methylation is downstream of transcription (Stewart et al., 2015).

The *Rasgrfl* DMR is distinct from the other two paternal imprinted gDMRs because it relies on *Dnmt3b* and *Dnmt3c* (Barau et al., 2016; Kato et al., 2007). *Dnmt3c* is evolved as a duplication of *Dnmt3b*, and both are responsible for the methylation of retrotransposons (Barau et al., 2016; Kato et al., 2007). In male germ cells, DNA methylation of retrotransposons also relies on a class of small RNA called PIWI-interacting RNAs, or piRNAs (reviewed in Weick and Miska, 2014). piRNA can silence repetitive elements transcriptionally by recruiting machinery for *de novo* DNA methylation to the target regions through sequence complementarity (Aravin et al., 2008; Carmell et al., 2007; Itou et al., 2015; Kuramochi-Miyagawa et al., 2008). It has been shown that the *Rasgrfl* DMR contains repetitive elements that can be targeted by piRNAs (Watanabe et al., 2011), suggesting that *de novo* DNA methylation at the *Rasgrfl* DMR depends on the piRNA pathway. In agreement with this, disruption of key piRNA pathway components such as *Mili*, *Miwi2*, and *Mitopl1d* leads to reduction of DNA methylation of *Rasgrfl* DMR in sperm cells (Watanabe et al., 2011). Although other piRNA dependent gDMRs exist in the male germline, they do not maintain their methylation status after fertilization suggesting that other mechanisms exist to maintain the imprinted *Rasgrfl* DMR during development (Watanabe et al., 2011).

Maintenance of imprints during pre-implantation development

Following fertilization, both oocyte- and sperm-derived genomes undergo global DNA demethylation but the demethylation mechanism is distinct between the parental

genomes (reviewed in Messerschmidt et al., 2014). The maternal genome undergoes replication-dependent passive DNA demethylation as the methylation maintenance enzyme Dnmt1o is excluded from the nucleus (Howell et al., 2001; Inoue et al., 2011; Inoue and Zhang, 2011). Conversely, the DNA demethylation process on the paternal genome occurs before DNA replication, indicating an active mechanism. It was previously suggested that this active DNA demethylation is the result of Tet3-mediated oxidation of 5mC (Gu et al., 2011; Inoue and Zhang, 2011). In support of this, deletion of Tet3 in oocytes leads to undetectable 5hmC and results in the hypermethylation of pluripotent genes such as *Oct4* and *Nanog* in zygotes, demonstrating the role of Tet3 in active DNA demethylation (Gu et al., 2011). Recently, it was reported that DNA replication is a major contributor of DNA demethylation of both parental genomes, with Tet3 being critical for demethylation only at certain loci (Shen et al., 2014), raising the question whether active demethylation by Tet3 for paternal pronuclei is required for development. Indeed, live offspring can be derived from round spermatid-injected oocytes, in which the paternal DNA is bound by histones and does not undergo active demethylation in zygotes (Polanski et al., 2008). Further, it has been demonstrated that haploinsufficiency, instead of defective paternal 5mC oxidation, contributes to the developmental defects previously ascribed to lack of maternal Tet3 in the zygote (Inoue et al., 2015). In fact, a similar rate of neonatal sublethality is observed when Tet3 is deleted from either parental germline (Inoue et al., 2015). In addition, reconstructed zygotes with either oxidized or non-oxidized paternal genome can equally develop to term, indicating that the paternal 5mC oxidation is dispensable for mouse development (Inoue et al., 2015). Thus, Tet3-mediated active demethylation may function as an

“accelerator” for DNA demethylation, while replication-dependent dilution of 5mC alone is probably sufficient for the DNA methylation reprogramming purpose in early embryos.

Unlike in PGCs, the imprinted gDMRs and some repetitive elements such as IAP and centromeric repeats can retain their DNA methylation patterns in the wave of global DNA demethylation in early embryos (reviewed in Messerschmidt et al., 2014). How imprinted gDMRs escape the DNA methylation reprogramming is not completely understood, but several factors have been identified to be responsible for the maintenance of the DNA methylation imprints in pre-implantation embryos. In zygotes, it is well defined that the protein Stella (also known as Pgc7 or Dppa) specifically protects the maternal genome from Tet3-mediated oxidation (Nakamura et al., 2007; Nakamura et al., 2012). The Stella protein distinguishes the maternal genome from the paternal genome by interacting with H3K9me2, which is enriched in the maternal genome but not paternal pronuclei (Nakamura et al., 2012). Aside from its protection of global maternal genome from active demethylation, Stella is also required to maintain the allele-specific DNA methylation at several maternal gDMRs such as *Peg1*, *Peg3*, and *Peg10* and paternal gDMRs such as *H19/Igf2* and *Rasgrf1* DMR (Nakamura et al., 2007). However, not all imprinted gDMRs are equally affected by the deletion of Stella, suggesting that the imprinted gDMRs are differentially enriched with H3K9me2 (Nakamura et al., 2007), or other mechanisms may act in partial redundancy with Stella to maintain DNA methylation imprints in zygotic stage.

The exclusion of Dnmt1 from the nucleus for passive DNA demethylation in pre-implantation embryos poses a challenge for the maintenance of DNA methylation imprint (Howell et al., 2001). It has been found that Dnmt1o, the oocyte-specific isoform of

Dnmt1, is detected mostly in the cytoplasm with transient localization in the nucleus at the eight-cell stage, suggesting its role in maintenance of genomic imprints at this cell cycle (Howell et al., 2001). Indeed, Dnmt1o deficient embryos exhibit loss of DNA methylation at certain imprinted gDMRs including *H19/Igf2*, *Snrpn*, and *Peg3* (Howell et al., 2001). Further investigation demonstrated that disruption of both oocyte-specific and zygotic Dnmt1 completely eliminates the DNA methylation imprints, indicating that Dnmt1 contributes to DNA methylation maintenance at imprinted gDMRs during pre-implantation even though most of the proteins are not located in the nucleus (Hirasawa et al., 2008).

The scarce amount of Dnmt1 in the nucleus during pre-implantation development suggests that a non-canonical mechanism attracts Dnmt1 to imprinted gDMRs in early embryos in order to maintain the methylation imprints. *Zfp57* was the first maternal factor that was identified to target and maintain DNA methylation at imprinted gDMRs in early embryos (Li et al., 2008; Quenneville et al., 2011). Embryos with disruption of both maternal and zygotic *Zfp57* die prenatally and exhibit loss of methylation of multiple imprinted gDMRs such as *Snrpn* ICR, *H19* ICR, and *Gtl2/Dlk1* ICR (Li et al., 2008). Further, as *Zfp57* specifically binds to a methylated hexanucleotide, which can be found in most imprinted gDMRs (MacDonald and Mann, 2014; Quenneville et al., 2011), it is believed that *Zfp57* selects the imprinted gDMRs out of the thousands CpG islands with differential DNA methylation between sperm cells and oocytes (reviewed in Hanna and Kelsey, 2014). After binding to imprinted gDMRs, *Zfp57* recruits its co-factor Trim28, which is a component of a multifunctional complex containing H3K9 methyltransferase Setdb1, Dnmt1, and Dnmt3a/3b (Messerschmidt et al., 2012; Zuo et

al., 2012). Therefore, Trim28 could be a mediator for Zfp57 to recruit Dnmts to the imprinted gDMRs. In support of this, embryos derived from Trim28-deficient oocytes exhibit variable defects with hypomethylation of several maternal and paternal imprinted gDMRs (Messerschmidt et al., 2012). In summary, it is proposed that Zfp57/Trim28-mediated recruitment of DNA methylation maintenance machinery to imprinted gDMRs compensates for the scarce amount of Dnmt1 in the nucleus and ensures the faithful maintenance of methylation imprints in pre-implantation embryos (reviewed in Messerschmidt et al., 2014).

Maintenance of unmethylated ICRs in post-implantation development

Around the time of implantation, parental genomes undergo another wave of genome-wide *de novo* DNA methylation to establish cell lineage-specific methylation patterns (reviewed in Smith and Meissner, 2013). In this process, the parental genomes, now in the same compartment, are treated equally by the *de novo* DNA methylation machinery. Therefore, it is important to protect the unmethylated alleles of imprinted gDMRs from acquisition of *de novo* DNA methylation. For imprinted gDMRs, the unmethylated alleles are usually characterized by active chromatin marks including H3K4me2/me3, histone modifications that can prevent the interaction of Dnmt3a/3l complex with the H3 tails (Ooi et al., 2007). Therefore, active chromatin marks on the unmethylated allele could be a general mechanism that protects against *de novo* DNA methylation. Recent findings also indicate that different imprinted DMRs may apply distinct mechanisms to prevent *de novo* DNA methylation on the unmethylated allele. For *H19/Igf2* ICR, ablation of the CTCF binding sites leads to hypermethylation of this locus in neonatal mice but not in oocytes and blastocysts (Schoenherr et al., 2003; Szabo

et al., 2004), indicating that CTCF binding plays a role in protection against *de novo* methylation on the unmethylated allele post-fertilization. In addition, for *Gtl2/Dlk1* gDMR, it has been demonstrated that polycomb repressive complex 2 (PRC2), a complex that catalyzes the repressive chromatin mark H3K27me3, is essential to prevent recruitment of Dnmt3a, Dnmt3b, and Dnmt3l to the DMR for *de novo* DNA methylation in embryonic stem cells (Das et al., 2015).

1.3 Functional roles of imprinted genes in mammalian development

1.3.1 Imprinted gene network and pre- and post-natal development

The distinct phenotypes of androgenetic and gynogenetic embryos indicate that parental genomes play different roles in embryonic development (Barton et al., 1984) and the discovery of imprinted genes (*i.e.*, *Igf2*, *Igf2r*, and *H19*) demonstrated that imprinted genes are responsible for the functional asymmetry of parental alleles (Barlow et al., 1991; Bartolomei et al., 1991; DeChiara et al., 1991). *Igf2* is a paternally expressed fetal growth factor (DeChiara et al., 1991). Biallelic expression of *Igf2* leads to embryonic overgrowth while its reduction results in growth restriction (DeChiara et al., 1991). Contrary to *Igf2*, its scavenger mannose receptor *Igf2r* is maternally expressed and its deletion leads to overgrowth and embryonic death (Barlow et al., 1991; Ludwig et al., 1996). It has been shown that the lethal phenotype of *Igf2r* mutants can be rescued when they carry a mutation that abolishes *Igf2*, indicating an antagonistic relationship between *Igf2* and *Igf2r* in regulation of embryonic development (Ludwig et al., 1996). Unlike *Igf2* and *Igf2r*, the functional role of the maternally expressed ncRNA *H19* is still not

completely understood. *H19* was first proposed as a tumor suppressor gene (Hao et al., 1993) and the tumor suppressor activity of *H19* was suggested to be mediated by its regulation of *Igf2* in *cis*. However, transgenic experiments indicate that *H19* can act as a *trans* factor to control embryonic growth and *Igf2* is not the only target of *H19* (Gabory et al., 2009). In support of this, deletion of the *H19* gene leads to overgrowth while transgenic mice that overexpress *H19* exhibit reduced bodyweight (Gabory et al., 2009; Ripoche et al., 1997). Further, the expression of multiple imprinted genes that are important embryonic growth regulators such as *Igf2*, *Igf2r*, *Cdkn1c*, *Dlk1*, and *Rtl1* are altered when *H19* is overexpressed or deleted (Gabory et al., 2009). Therefore, it has been proposed that *H19* is a regulator for an imprinted gene network in which multiple imprinted genes are coordinately regulated to precisely control embryonic growth (Gabory et al., 2009).

As the *H19* gene expresses both a 2.3kb ncRNA and a microRNA (miR-675) (Brannan et al., 1990; Cai and Cullen, 2007), it is still not completely clear whether imprinted gene network is regulated by the ncRNA or the miRNA or both. Overexpression of miR-675 in a variety of embryonic cell lines can lead to reduced proliferation (Keniry et al., 2012). However, miR-675 can only be detected with high abundance in placenta, suggesting that only in placenta this miRNA mediates the *H19*'s growth repressive properties (Keniry et al., 2012). Recently, it has been shown that *H19* ncRNA controls gene expression of the imprinted gene network through recruiting the methyl-binding protein 1 (Mbd1) (Monnier et al., 2013). Specifically, the *H19*-Mbd1 complex can bring repressive histone mark H3K9me3 to the DMRs of the *Igf2*, *Peg1*, and *Slc38a4* loci and regulate the expression of imprinted genes in an allele-specific-

independent manner (Monnier et al., 2013). However, how *H19* ncRNA specifically recognizes the DMR sequences and why only these DMRs are targeted by *H19*-Mbd1 complex remain to be addressed. In addition to *H19*, a paternally expressed transcription factor *Plagl1* has also been demonstrated as a critical regulator of an imprinted gene network to control embryonic growth (Arima et al., 2005; Varrault et al., 2006). Paternal deletion of *Plagl1* leads to intrauterine growth restriction and affects embryonic bone development (Varrault et al., 2006). Further, misregulation of *Plagl1* can alter the expression of multiple imprinted genes including *Igf2*, *H19*, *Cdkn1c*, and *Dlk1* (Varrault et al., 2006). In addition, the *Plagl1* protein can directly regulate the *Igf2/H19* locus through binding the downstream enhancers shared by *H19* and *Igf2* (Varrault et al., 2006). In summary, the imprinted gene network may serve to fine-tune the expressed allele of imprinted genes for precise control of embryonic growth.

In addition to controlling embryonic growth, the imprinted gene network can also be an important indicator for postnatal obesity (Dalgaard et al., 2016). It has been shown that the isogenic mice with *Trim28* haploinsufficiency (*i.e.*, *Trim28*^{+/-}) can randomly develop an obese phenotype, which is associated with the reduced expression of an imprinted gene network including *Nnat*, *Peg3*, *Cdkn1c*, and *Plagl1* (Dalgaard et al., 2016). Notably, the fact that no altered DNA methylation is detected at the ICRs of these imprinted genes is consistent with the findings that imprinted gene network regulation is not dependent on alteration of DNA methylation or loss of allele-specific expression of imprinted genes (Al Adhami et al., 2015; Dalgaard et al., 2016; Monnier et al., 2013).

1.3.2 Roles of imprinted genes in placenta

Numerous mouse models with deletion or overexpression of imprinted genes have demonstrated that precise control of genomic imprinting is essential for normal placental development. For instance, embryos with deletion of the maternally expressed *Ascl2* fail to develop spongiotrophoblast and cannot survive beyond embryonic day 10.5 (Guillemot et al., 1994). Conversely, another maternally expressed imprinted gene *Phlda2*, when deleted, causes the expansion of spongiotrophoblast and increased placental weight (Frank et al., 2002). Further, deletion of *Sfmbt2* (paternally expressed) leads to reduction of all trophoblast cell types and embryos die before E12.5 (Miri et al., 2013). Notably, a retrotransposon-derived imprinted gene *Rtl1* (paternally expressed) is essential to maintain the fetal capillaries in placenta and both its deletion and its overexpression result in late-fetal and/or neonatal lethality in mice (Sekita et al., 2008). In addition to regulating placental development, imprinted genes can control the nutrient supply to the fetus through the placenta. For example, deletion of *Igf2 P0*, a placental-specific isoform of *Igf2*, inhibits the nutrient uptake by placenta and leads to embryo growth retardation (Constancia et al., 2002). Lastly, imprinted gene products may act as endocrine factors and modulate maternal metabolic processes. For instance, the protein product of the paternally expressed *Dlk1* gene is the source of maternal circulating Dlk1 and pregnancies lacking Dlk1 exhibit compromised lipid metabolism (Cleaton et al., 2016).

1.3.3 Roles of imprinted genes in behavior

Many imprinted genes are expressed in the brain and the significance of genomic imprinting in neurodevelopment is featured by the evidence that mis-expression of

imprinted genes can cause abnormal behaviors. For example, *Peg3* null females exhibit defects in maternal care behaviors such as milk release and nest building (Champagne et al., 2009). In addition, *Peg3* null newborns show suckling defects (Champagne et al., 2009). Similar to *Peg3*, females with *Peg1* deletions also exhibit deficient maternal care behaviors such that they do not respond to the neonates appropriately and do not ingest their extra-embryonic tissues afterbirth (a normal mammalian behavior termed placentophagia) (Lefebvre et al., 1998). Recent work also indicates that deletion of the paternal *Grb10* results in social dominance and aggression behaviors while deletion of the maternal *Grb10* leads to fetal and placental overgrowth (Garfield et al., 2011). *Grb10* is imprinted on both alleles, however, unlike the maternal isoform, the paternal *Grb10* transcribes from an alternative promoter and is only expressed in a subset of neurons (Garfield et al., 2011). This indicates distinct physiological and behavioral functions of parental alleles of the *Grb10* gene. The other imprinted genes known to control social behavior and communication include *Ube3a*, *Snord116*, and *Ndn*, which are described in detail in the following section titled “human loss-of-imprinting disorders” as these genes in humans are associated with Prader-Willi syndrome and Angelman syndrome.

1.4 Human loss-of-imprinting disorders

1.4.1 Prader-Willi syndrome (PWS) and Angelman syndrome (AS)

Both PWS (OMIM ID: 176270) and AS (OMIM ID: 105830) are complex neurodevelopmental disorders with an occurrence of 1 in 15,000 to 25,000 live births

(reviewed in Buiting, 2010). Individuals with PWS are typically characterized by low birth weight and hypotonia with poor suckling at pre-weaning stage and hyperphagia and obesity at post-weaning stage. Other main features observed in PWS include short stature with small hands and feet, hypogonadism, mild intellectual disability, and abnormal behavior such as skin picking. AS, which is also known as “happy puppet syndrome” due to a happy disposition, are also characterized by developmental delay, severe intellectual disability, speech impairment, seizures, hyperactivity and restless behavior (reviewed in Buiting, 2010). Both PWS and AS are associated with the imprinted *SNRPN/UBE3A* domain at chromosome 15q11-13 region but exhibit distinct genetic/epigenetic alterations (reviewed in Clayton-Smith and Laan, 2003; reviewed in Ohta et al., 1999). Most PWS individuals (~70%) have large deletions (~4Mb) at the *SNRPN/UBE3A* imprinted locus on the paternally inherited chromosome, which includes multiple paternally expressed protein coding genes (*e.g.*, *SNRPN/SNURF*, *MAGEL2*, *NDN*) and ncRNAs (*e.g.*, *SNORD115* and *SNORD116*). The other PWS individuals are characterized by either maternal uniparental disomy of the imprinted locus (~25%) or epimutations involving the alteration of DNA methylation at the ICR (~3%) (reviewed in Ohta et al., 1999). For AS, approximately ~60-70% patients also have a large deletion at the imprinted *SNRPN/UBE3A* locus, but unlike PWS, the deletion is always on the maternally inherited chromosome (reviewed in Clayton-Smith and Laan, 2003). Further, approximately 10% of individuals with AS have a mutation in the maternally expressed gene, ubiquitin protein ligase E3A (*UBE3A*) and 1-5% exhibit either paternal uniparental disomy or imprinting defects at the imprinted domain (reviewed in Clayton-Smith and Laan, 2003).

It is well established that the *UBE3A* gene is responsible for AS (Kishino et al., 1997; Matsuura et al., 1997). *UBE3A* is maternally expressed in a subset of neurons while biallelically expressed in peripheral tissues (Yamasaki et al., 2003). Mice that carry a non-functional maternal *Ube3a* exhibit many phenotypes that recapitulate AS such as seizures, learning and gait deficit (Jiang et al., 1998; Miura et al., 2002). Further, activation of the normally silent paternal *Ube3a* in neurons can partially restore the Ube3a protein and alleviate some cognitive defects associated with AS (Meng et al., 2012; Meng et al., 2015). Recently, it has been shown that *Ube3a* can rescue more symptoms in a mouse model of AS when it is re-expressed at earlier stage (*i.e.*, newborns) compared to a later stage (*i.e.*, juvenile or adulthood), suggesting the importance of timing for *Ube3a* expression in normal neurodevelopment (Silva-Santos et al., 2015). In humans, the severity of AS symptoms is associated with different types of mutations in *UBE3A* with full deletion of chromosome 15q11-13 most severe and point mutation of *UBE3A* less severe (Gentile et al., 2010; Valente et al., 2013). Unlike AS, PWS is caused by the loss of multiple, rather than a single paternally expressed gene (reviewed in Bervini and Herzog, 2013). Deletion of *Ndn*, a paternally expressed gene in the *Snrpn/Ube3a* imprinted cluster, leads to behavior defects that recapitulate PWS such as skin scraping and modified spatial learning (Muscatelli et al., 2000). However, *Ndn* null mice do not exhibit obesity, indicating that *Ndn* gene alone does not account for all the symptoms of PWS. Further, deletion of the *Snord116* gene cluster can lead to hyperphagia, but none of these mutant mice exhibit obesity (Ding et al., 2008). Therefore, these genetically modified mouse model systems do not recapitulate all PWS phenotypes.

1.4.2 Silver-Russell syndrome (SRS)

SRS (OMIM ID: 180860) is a rare loss-of-imprinting disorder with an estimated frequency of 1 in 300,000 and is mainly characterized by pre- and postnatal growth restriction, triangular shaped face, and skeletal asymmetry (reviewed in Azzi et al., 2014). SRS is associated with the *H19/IGF2* imprinted domain on chromosome 11p15.5. Approximately 35-65% SRS patients exhibit hypomethylation of the *H19/IGF2* ICR and about 10% have maternal uniparental disomy of the same imprinted locus (reviewed in Abu-Amro et al., 2008). Of the individuals with epimutation at *H19/IGF2* domain, ~73% of them also exhibit hypo- or hypermethylation at other imprinted loci, suggesting a general defect in DNA methylation imprint establishment or maintenance in these individuals (Kannenbergh et al., 2012). Recently, a mouse model with the endogenous paternal *H19/IGF2* ICR replaced by the human orthologous ICR exhibited impaired DNA methylation acquisition at the locus, resembling the growth restriction phenotype of SRS (Hur et al., 2016). Thus, this mouse model could be used to elucidate some molecular mechanisms that underlie SRS (Hur et al., 2016).

1.4.3 Beckwith-Wiedemann syndrome (BWS)

BWS (OMIM ID: 130650) is the most common loss-of-imprinting disorder with occurrence of one in 11,000 live births, although this number is likely underestimated (reviewed in Mussa et al., 2016; Weksberg et al., 2010). BWS is equally represented in males and females (reviewed in Pettenati et al., 1986). The primary features of BWS include pre- and postnatal overgrowth, enlarged tongue, ear malformation, and abdominal wall defects such as exomphalos and umbilical hernia. The less common characteristics

of BWS include hemihyperplasia, adrenocortical cytomegaly, visceromegaly, congenital renal abnormalities, placentomegaly, and neonatal hypoglycemia (reviewed in Weksberg et al., 2010). Individuals with BWS also have an increased frequency to develop childhood tumors including Wilms' tumors of the kidney, hepatoblastoma, rhabdomyosarcoma, neuroblastoma, and adrenocortical carcinoma (reviewed in Choufani et al., 2013).

Most individuals with BWS (~85%) are associated with genetic and/or epigenetic alterations at chromosome 11p15.5 region, which harbors two imprinted clusters, the *KCNQ1* locus and the *H19/IGF2* locus (reviewed in Choufani et al., 2013). Hypomethylation of the ICR (*i.e.*, KvDMR1) of the *KCNQ1* imprinted domain is the most frequent molecular alteration of BWS and is seen in approximately 50% BWS cases (reviewed in Choufani et al., 2013). Aberrant DNA hypomethylation of KvDMR1 causes derepression of the maternal ncRNA *KCNQ1OT1*, which leads to repression of the flanking maternally expressed genes such as *CDKN1C* (Diaz-Meyer et al., 2003; Fitzpatrick et al., 2002; Smilnich et al., 1999). *CDKN1C* is a cyclin-dependent kinase inhibitor of G1 cyclin complex that negatively regulates cell proliferation and the maternal *CDKN1C* mutations accounts for ~10% of individuals with BWS (reviewed in Choufani et al., 2013). Hypermethylation of the *H19/IGF2* ICR leads to increased abundance of the fetal growth factor of *IGF2* and is seen in ~5-10% BWS cases (reviewed in Choufani et al., 2013). It has been reported that a subset of BWS individuals with hypermethylation of the *H19/IGF2* ICR has micro-deletions that disrupt the CTCF binding sites at the locus (Beygo et al., 2013; Sparago et al., 2004). In addition, point mutations of OCT4/SOX2 binding sites at the *H19/IGF2* ICR have been

reported in BWS patients (Abi Habib et al., 2014). Lastly, approximately 20% BWS cases exhibit paternal uniparental disomy of chromosome 11p15.5 region, which affects both the *H19/IGF2* and *KCNQ1* imprinted loci (reviewed in Choufani et al., 2013).

Besides the *KCNQ1* and *H19/IGF2* imprinted loci, approximately 22% BWS patients with KvDMR1 hypomethylation exhibit DNA methylation defects at other imprinted loci such as *PLAGL1* DMR, *PEG1* DMR, *IGF2R* DMR, *GNAS* DMR (Azzi et al., 2009; Blik et al., 2009; Eggermann et al., 2016). Of these imprinted genes, *PLAGL1* can bind to the KvDMR1 and induce the transcription of *KCNQ1OT1* in a DNA methylation-dependent manner (Arima et al., 2005). It is still unclear what causes the DNA methylation defects at multiple imprinted loci in BWS patients. It has been reported that two affected BWS siblings with KvDMR1 hypomethylation have a frameshift mutation of the *NLRP2* gene, suggesting that *NLRP2* is directly or indirectly implicated in the establishment and/or maintenance of DNA methylation in humans (Meyer et al., 2009).

1.4.4 Mouse models for BWS

Mouse models with Cdkn1c deletion

Several mouse models of BWS have been created by deletion of the *Cdkn1c* gene. Maternal deletion of *Cdkn1c* leads to neonatal lethality and the *Cdkn1c* null fetuses have some defects that resemble BWS, such as abdominal wall defects, cleft palate, and adrenal cytomegaly (Yan et al., 1997; Zhang et al., 1997). However, only prenatal overgrowth but not neonatal overgrowth, is observed in the *Cdkn1c* null mice (Tunster et al., 2011). It has been suggested that the prenatal overgrowth of *Cdkn1c* null mice is

compromised by the intrauterine nutrient competition between fetuses during late gestation, as these mutants show larger bodyweight when the litter size is small (Tunster et al., 2011). Further, no neonatal lethality has been reported for BWS, suggesting that, unlike the *Cdkn1c* null mouse models, BWS patients with *CDK1C* mutation or hypomethylation of KvDMR1 still retain partial *Cdkn1c* activity (Chiesa et al., 2012).

Mouse model with the overexpression of Igf2

R/H mice. In this mouse model, both *Igf2r* and *H19/Igf2* ICR are targeted in order to increase Igf2 activity. “R” refers to the deletion of *Igf2r*, which degrades excess *Igf2* via receptor-mediated endocytosis (Ludwig et al., 1996). “H” stands for deletion of the maternal *H19* gene region (including the *H19/Igf2* ICR), which leads to the de-repression of *Igf2* from the maternal alleles (Leighton et al., 1995a). On E12.5, approximately 11-fold higher level of Igf2 is present in R/H mice in serum compared to controls (Eggenchwiler et al., 1997). This mouse model exhibits many BWS characteristics including somatic overgrowth, abdominal wall defects, visceromegaly, skeletal abnormalities, and cleft palate (Eggenchwiler et al., 1997). However, some BWS features such as macroglossia, renal dysplasia, embryonic tumors and adrenal cytomegaly were not observed in this BWS model. For this mouse model, no pups survive beyond E18.5.

Igf2 transgenic mice. In this model, ES cell lines with exogenous *Igf2* are injected into the cavity of blastocyst to make chimeras (Sun et al., 1997). The *Igf2* overexpressing chimeras died within 24 hours of birth. On E13.0, transgenic mice with 40-50% chimerism exhibit 116-131% overgrowth compared to controls. In addition to

the somatic overgrowth, this mouse model also has some BWS features including macroglossia, visceromegaly. However, no abdominal wall defects, neonatal hypoglycemia, and hemihypertrophy are seen in this mouse model (Sun et al., 1997).

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

In this mouse model, both the maternal *H19* region (including the *H19/IGF2* ICR) and the maternal *Cdkn1c* are deleted to increase the *Igf2* activity and to deplete *Cdkn1c*, respectively (Caspary et al., 1999). These double mutants exhibit more severe phenotype than each single mutant. For example, no live offspring can be recovered, and the parturition date of the double mutants is advanced by at least one day when compared to *Cdkn1c* null mutants. The double mutants exhibit 20% increase in bodyweight at E16.5-17.5, but are indistinguishable from wild type offspring on E18.5. Other BWS characteristics such as macroglossia (122% of wild type littermates), placentomegaly (190% of wild type littermates), kidney dysplasia and abdominal wall defects are observed in the double mutant model (Caspary et al., 1999).

In summary, these mouse models for BWS strongly support that imprinted genes are essential for normal embryonic development and provide insights to the mechanisms of imprinted gene regulation. However, these mouse models do not perfectly fulfill the criteria of an animal model for BWS. First, not all the main BWS symptoms are present in each mouse model system. In particular, childhood tumors, which have an increased likelihood to be developed within the five years of age in BWS, are not present in the mouse models. The absence of tumors might be due to embryonic/neonatal lethality of these mouse models, which is also not seen in BWS patients. Second, the BWS

characteristics observed in these mouse models are induced by genetic manipulations (*i.e.*, gene knockout), which do not mimic the epimutations naturally occurring in BWS, such as aberrant DNA methylation at the *KCNT1* locus, *H19/IGF2*, and/or other imprinted loci.

1.5 Assisted reproductive technologies/therapies (ART) and genomic imprinting

1.5.1 ART can induce loss-of-imprinting in animals

In humans, ART refers to a series of procedures that are used to overcome infertility/subfertility in order to help establish a successful pregnancy with the ultimate goal of giving rise to a healthy child. In animal industry, ART is commonly used to facilitate the rapid multiplication of genetically superior animals. Common ART procedures include ovarian hyperstimulation (also known as superovulation), *in vitro* fertilization (IVF), intracytoplasmic sperm injection (ICSI), *in vitro* culture (IVC), and embryo transfer. ART manipulations overlap with the critical period for acquisition and maintenance of genomic imprints. For example, the ovarian hyperstimulation coincides with the oocyte-growing phase when the maternal gDMRs are acquiring DNA methylation (Lucifero et al., 2004; Lucifero et al., 2002). Further, IVF and IVC are implemented when preimplantation embryos undergo global DNA demethylation, in which DNA methylation imprints need to be faithfully maintained (reviewed in Messerschmidt et al., 2014). Therefore, ART manipulations take place during the sensitive epigenetic reprogramming period and may lead to epigenome perturbations.

Indeed, a large body of experiments has demonstrated that ART can induce loss-of-imprinting in mice, sheep, and cattle (reviewed in Denomme and Mann, 2012).

How ART procedure(s) cause loss-of-imprinting is still unclear. However, it is well established that the multiple ART procedures induce more severe loss-of-imprinting when compared to the use of a single ART manipulation. For example, embryo culture can exacerbate the adverse effects of embryo transfer on imprinted gene expression in E9.5 embryo and placenta (Rivera et al., 2008). Further, it has been shown that high dosage hormone hyperstimulation leads to more frequent imprinted methylation defects in mouse blastocysts when compared to the low dosage (Market-Velker et al., 2010). Notably, superovulation interrupts the paternal *H19* methylation in blastocysts, suggesting that superovulation not only disrupts acquisition of imprints in growing oocytes, but also affects the maternal-effect gene products required for imprint maintenance in early embryos (Market-Velker et al., 2010).

It is also intriguing that the placenta is more susceptible than the fetus to loss-of-imprinting when the ART procedures are applied (de Waal et al., 2014; de Waal et al., 2015; Mann et al., 2004; Rivera et al., 2008). It was first suggested that severe loss-of-imprinting in the placenta could be due to the outer position of trophectoderm, which is directly exposed to the artificial environment (*e.g.*, petri-dish) (Mann et al., 2004). The trophectoderm gives rise to tissues in the placenta (reviewed in Rossant and Cross, 2001). However, the yolk sac, which is derived from primitive endoderm that resides in the inner space of the blastocyst, also exhibits similar severity of loss-of-imprinting, suggesting that the “position” hypothesis alone cannot explain the differential loss-of-imprinting susceptibility between embryonic and extra-embryonic tissues (Rivera et al., 2008). It

also remains to be addressed why isogenic mouse embryos and/or placenta generated with the use of ART exhibit variable and stochastic loss-of-imprinting and why the occurrence of epimutation can be detected ubiquitously or in specific tissues in an individual (de Waal et al., 2014; de Waal et al., 2012a). It is likely that Trim28 activity is impaired in embryos generated with the use of ART as the variability of loss-of-imprinting observed in the ART-conceived offspring can also be found in mice lacking the maternal Trim28 (Messerschmidt et al., 2012).

1.5.2 ART and BWS

Several studies have suggested that children conceived with the use of ART have a 3- to 9-fold higher likelihood of being affected with the congenital overgrowth condition BWS (DeBaun et al., 2003; Gicquel et al., 2003; Halliday et al., 2004; Lim et al., 2009; Maher et al., 2003). Recently, a meta-analysis of eight epidemiologic studies indicated that ART-conceived children have a 5.2-fold increased likelihood of developing BWS (Vermeiden and Bernardus, 2013). It is possible that the increased incidence of BWS in ART-conceived offspring could result from the infertility/subfertility of the parents. However, the use of ART can lead to at least some of the imprinting errors as ART-induced loss-of-imprinting has been reported in numerous animal models that have no infertility issues (reviewed in Denomme and Mann, 2012). In agreement with this, a human immunodeficiency virus serodiscordant couple that had no infertility problems sought for ART to conceive a child without the risk of HIV and had a BWS baby after the ovarian hyperstimulation and IVF protocols were applied (Kuentz et al., 2011).

1.6 Large offspring syndrome

1.6.1 Characteristics of large offspring syndrome

The use of assisted reproduction in farm animals such as bovine and ovine has facilitated the rapid multiplication of genetically superior animals. However, the use of ART procedures can also induce unwanted pregnancy outcomes. For example, manipulations of oocytes or preimplantation embryos can result in unusually large offspring in ruminants with various congenital abnormalities and the overgrowth condition is referred to as large offspring syndrome (LOS) (reviewed in Young et al., 1998). During the late 1980s and 1990s, *in vitro* embryo culture techniques typically used a medium supplemented with serum or co-cultured with oviduct epithelia cells (reviewed in Young et al., 1998). At the time, a significant portion of the offspring generated with the use of ART exhibited aberrant phenotypic features including large size, large internal organs, abnormal placenta, and excess placental fluids (reviewed in Young et al., 1998). By the early 1990s, it was recognized that the addition of serum to the medium and the somatic cell co-culture system are major contributors to LOS (reviewed in Young et al., 1998). In addition, offspring produced by somatic cell nuclear transfer (SCNT) can be afflicted with LOS (reviewed in Hill, 2014).

The major feature of LOS is the increased birthweight. It is not uncommon for LOS offspring to have twice the mean bodyweight compared to controls (reviewed in Young et al., 1998). The largest ovine LOS fetus reported so far was five times larger than the normal bodyweight (Walker et al., 1996). It should be noted that the extent of overgrowth is highly variable among LOS individuals, which may depend on the

different ART procedures used, different animal breeds, and/or different embryo handling practices (reviewed in Young et al., 1998). Further, the full siblings from the same culture micro-drop can express different degrees of overgrown phenotype (Sinclair et al., 2000), suggesting that the induction of LOS by the use of ART is a stochastic process. The overgrowth phenotype can be detrimental for both the dam and the offspring. For the dam, the over-sized nature of the fetus may cause difficult delivery; therefore Caesarean section is commonly used for LOS offspring delivery. For the offspring, the newborns tend to experience difficulties to suckle and breathe.

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., 1999; Sinclair et al., 2000). Similar enlargement of liver and heart is also a common feature of individuals with BWS (reviewed in Choufani et al., 2010). LOS has also been associated with placental dysfunctions such as polyhydramnios (Sinclair et al., 1999), which is commonly seen in human BWS (reviewed in Choufani et al., 2010). In sheep LOS, the incidence of polyhydramnios is about 36-62% in embryos generated with the use of ART (Sinclair et al., 1999). In addition, a range of congenital abnormalities can be observed in LOS, including cleft palates, skeletal defects, high level insulin at birth, and hypoglycemia (Garry et al., 1996) (Schmidt et al., 1996).

It is still unclear how ART procedures cause LOS. It was suggested that ruminant blastocysts in culture media supplemented with serum or somatic cells appeared darker due to excess lipid accumulation in the cytoplasm (Ferguson and Leese, 1999; Thompson, 1997). The accumulation of the lipid droplet was considered to be harmful to 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 appears 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 compaction (Rooke et al., 2007). It is also unclear why induction of LOS by ART is variable even though the same *in vitro* protocol is used in the same laboratory (reviewed in Young et al., 1998). Overall, LOS exhibits unpredictable and broad range characteristics even when similar protocols and medium are used to produce embryos.

1.6.2 Molecular alterations of LOS.

To elucidate the potential molecular alterations responsible for LOS, a large body of experiments has been performed to identify differentially expressed genes between *in vitro* and *in vivo*-derived embryos (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). It has been reported that genes affected by the *in vitro* procedures are involved in numerous biological processes such as morula compaction/cavitation, metabolism, trophoblastic function and growth factor signaling (reviewed in Wrenzycki et al., 2004). However, pooled embryos are used in these studies and the gene expression levels of the *in-vitro* group represent the average of the embryos that will develop LOS and those that will be normal, which may not accurately characterize the molecular signatures of LOS.

Because not every fetus in the *in vitro* group exhibits an overgrowth phenotype, it is more appropriate to compare the gene expression differences between LOS and non-LOS fetuses instead of between the *in vivo* and *in vitro* derived fetuses. In an ovine

study, late gestation (day 125; ovine gestation=145 days) fetuses with bodyweight greater than 5.5kg were considered as LOS (bodyweight of the largest *in vivo* fetus is 4.5kg) and were compared to controls (Young et al., 2001). It was found that LOS calves have lower *IGF2R* mRNA and protein level in the liver and muscle tissues compared to the controls (Young et al., 2001). In a bovine study, it was observed that two LOS calves were characterized by hypomethylation at the KvDMR1 and decreased expression of *CDKN1C* (Hori et al., 2010), highlighting the similar molecular alteration of KvDMR1 in LOS and BWS, although parental-specific epimutations were not determined in this experiment. Recently, we have determined the allelic DNA methylation and the allelic expression of the imprinted genes in the *KCNQ1* and the *H19/IGF2* loci in day ~105 *Bos indicus taurus* × *Bos taurus taurus* F₁ control and LOS fetuses (Chen et al., 2013). We observed biallelic expression of the ncRNA *KCNQ1OT1*, loss of methylation of KvDMR1 on the maternal allele, and decreased expression of *CDKN1C* in two out of seven LOS fetuses (Chen et al., 2013). Our study demonstrated that LOS is both phenotypically and epigenetically similar to human BWS (Chen et al., 2013). From that work we proposed to use LOS as an animal model to study the molecular mechanism that underlie BWS (Chen et al., 2013).

1.7 Rationale

Most individuals with BWS are associated with genetic and/or epigenetic alterations at the *KCNQ1* locus and the *H19/IGF2* locus, and a subset of BWS individuals with hypomethylation at the *KCNQ1* locus also exhibit aberrant DNA methylation at

other imprinted loci (Azzi et al., 2009; Bliet et al., 2009; Eggermann et al., 2016). While we have previously described the hypomethylation of the *KCNQ1* locus in LOS fetuses (Chen et al., 2013), studies have not yet determined the extent of loss-of-imprinting in this overgrowth condition. Due to the fact that LOS is both phenotypically and epigenetically similar to human BWS, we hypothesize that LOS exhibits loss-of-imprinting beyond the *KCNQ1* locus. To test the hypothesis, we determined the allelic expression of imprinted genes previously identified in human and/or mouse in somatic tissues of day ~105 bovine control and LOS fetuses using high-throughput RNA sequencing (Chapter 2). In this study, we identified 20 imprinted genes exhibiting monoallelic expression in control fetal tissues and found that approximately half were biallelically expressed in at least one of the tissues analyzed in LOS. Thus, we conclude that LOS is a multi-locus loss-of-imprinting syndrome, which is similar to what has been observed in some BWS individuals (Azzi et al., 2009; Bliet et al., 2009; Eggermann et al., 2016).

Although it is well accepted that loss-of-imprinting can contribute to BWS and LOS, and as such both conditions have been coined “loss-of-imprinting” syndromes, it remains unknown whether aberrant gene expression and DNA methylation occur at non-imprinted loci and to what extent these molecular alterations contribute to the variable phenotypes observed in these conditions. To address these questions, we examined the transcriptome and DNA methylome of somatic tissues of day ~105 bovine control and LOS fetuses using high-throughput RNA sequencing and whole genome bisulfite sequencing (Chapter 3). We found that global misregulation of non-imprinted genes in addition to loss-of-imprinting characterizes the ART-induced overgrowth syndrome.

Further, most identified DEGs could not be directly associated with altered DNA methylation, highlighting that caution should be exercised when making conclusions about the etiology of such syndromes by interpreting time-point-specific DNA methylation data.

Since discovery of the first three imprinted genes *Igf2*, *Igf2r*, and *H19* in 1991 (Barlow et al., 1991; Bartolomei et al., 1991; DeChiara et al., 1991), more than 100 genes have been identified to be imprinted in mice and humans (<http://www.mousebook.org/mousebook-catalogs/imprinting-resource>; <http://www.geneimprint.com/>). Compared to mice and humans, many fewer imprinted genes (a total of 34) have been reported in domestic farm animal species (reviewed in Tian, 2014). Further, most imprinted genes in cow, pig, sheep, and rabbit have been characterized in studies designed to corroborate the allele-specific expression of genes previously described as imprinted in human and/or mouse (Bischoff et al., 2009; Chen et al., 2015; Chen et al., 2013; Robbins et al., 2012; Young et al., 2003; Zaitoun and Khatib, 2008). For the purpose of *de novo* identification of imprinted genes in bovine, we determined global monoallelic gene expression in fetal and placental tissues of day ~105 bovine conceptuses using RNA sequencing (Chapter 4). In this study, we expand the imprinted gene list in bovine and demonstrate that monoallelic gene expression can be the result of *cis*-eQTL effects (Chen et al., 2016).

The following three chapters are the full texts of three manuscripts that have been published or under review. **Chapter 2** was published in 2015 in “*PNAS*” (PMID: 25825726), **Chapter 3** is under review, and **Chapter 4** was published in 2016 in “*Epigenetics*” (PMID:27245094). The text presented is exactly as in the published work.

For each chapter, the main figures and tables are included in the body of the text while the supplemental figures and tables are included at the end of each chapter immediately after the acknowledgements.

Chapter 2 Characterization of global loss of imprinting in fetal overgrowth syndrome induced by assisted reproduction

2.1 Abstract

Embryos generated with the use of assisted reproductive technologies (ART) can develop overgrowth syndromes. In ruminants, the condition is referred to as large offspring syndrome (LOS) and exhibits variable phenotypic abnormalities including overgrowth, enlarged tongue, and abdominal wall defects. These characteristics recapitulate those observed in the human loss-of-imprinting (LOI) overgrowth syndrome Beckwith-Wiedemann (BWS). We have recently shown LOI at the *KCNQ1* locus in LOS, the most common epimutation in BWS. Although the first case of ART-induced LOS was reported in 1995, studies have not yet determined the extent of LOI in this condition. Here, we determined allele-specific expression of imprinted genes previously identified in human and/or mouse in day ~105 *Bos taurus indicus* × *Bos taurus taurus* F₁ hybrid control and LOS fetuses using RNAseq. Our analysis allowed us to determine the monoallelic expression of 20 genes in tissues of control fetuses. LOS fetuses displayed variable LOI when compared to controls. Biallelic expression of imprinted genes in LOS was associated with tissue-specific hypomethylation of the normally methylated parental allele. In addition, a positive correlation was observed between bodyweight and the number of biallelically expressed imprinted genes in LOS fetuses. Further, not only was there loss of allele-specific expression of imprinted genes in LOS, but we also observed differential transcript amounts of these genes between control and overgrown fetuses. In summary, we characterized previously unidentified imprinted genes in bovine and

identified misregulation of imprinting at multiple loci in LOS. We conclude that LOS is a multi-locus LOI syndrome, as is BWS.

2.2 Introduction

Genomic imprinting is a series of precisely regulated epigenetic processes that lead to parental allele-specific expression of a subset of genes in mammals (Bartolomei and Ferguson-Smith, 2011). Proper allelic expression of imprinted genes plays an important role in embryonic and neonatal growth, placental function, and postnatal behavior (Miyoshi et al., 2006). Allele-specific DNA methylation at discrete regions established during gametogenesis defines the functional asymmetry of parental alleles (Bartolomei and Ferguson-Smith, 2011). These regions, termed as differentially methylated regions (DMRs), are required to regulate the imprinted expression of these genes. DNA methylation of DMRs is erased in primordial germ cells, re-established during gametogenesis, and maintained when the global DNA demethylation occurs during preimplantation development (Bartolomei and Ferguson-Smith, 2011). In addition to DNA methylation, other epigenetic modifications and mechanisms such as ncRNAs and histone post-translational modifications may contribute to parental-allele specific expression of these genes (Bartolomei and Ferguson-Smith, 2011).

Because of the dynamic epigenetic reprogramming that occurs during oocyte growth and preimplantation development (Morgan et al., 2005), environmental perturbations during this time period, such as the use of assisted reproductive technologies (ART), can affect imprint establishment and maintenance (Denomme and

Mann, 2012). Numerous prospective studies in animals (de Waal et al., 2012a; de Waal et al., 2012b; Market-Velker et al., 2010; Rivera et al., 2008) and retrospective studies in humans (Vermeiden and Bernardus, 2013) have shown that ART can induce improper regulation of genomic imprinting. ART is commonly used in clinics to treat subfertility and infertility, and each year as high as 5.9% of infants born in developed countries are conceived by the use of these technologies (CDC, 2013; Kupka et al., 2014). In agriculture, ART is also widely used to increase the number of offspring produced from genetically superior individuals in a shortened period of time (IETS, 2013). Multiple reports have indicated that ART-conceived offspring are more likely to develop imprinting disorders such as Beckwith-Wiedemann syndrome (BWS) in human (Halliday et al., 2004; Lim et al., 2009; Vermeiden and Bernardus, 2013) and large offspring syndrome (LOS) in ruminants (Behboodi et al., 1995; Bertolini et al., 2002; Farin and Farin, 1995; Farin et al., 2006).

BWS is the most common pediatric overgrowth syndrome characterized by complex and variable symptoms such as prenatal and postnatal overgrowth, ear creases, macroglossia, umbilical hernia, and predisposition to develop childhood tumors (Weksberg et al., 2010). BWS has an estimated worldwide frequency of 1 in 13,700 live births (Weksberg et al., 2010) with no noted sex bias (Pettenati et al., 1986) and a weighted relative risk of 5.2 in children conceived with the use of ART (Vermeiden and Bernardus, 2013). Most BWS cases are sporadic and are associated with epimutations in human chromosome 11p15.5, a region that harbors *KCNQ1* and *H19/IGF2* imprinted loci (Weksberg et al., 2010). Approximately 50% of BWS cases are associated with the loss of methylation at the KvDMR1 (*i.e.* *KCNQ1* locus) and 2-7% are associated with the

gain-of-methylation of the DMR at the *H19/IGF2* locus (Weksberg et al., 2010). Recent studies have shown that a subset of BWS individuals with hypomethylation at the *KCNQ1* locus also exhibited aberrant DNA methylation at other imprinted loci (Azzi et al., 2009; Blik et al., 2009; Court et al., 2013; Eggermann et al., 2014; Lim et al., 2009; Maeda et al., 2014; Poole et al., 2013; Tee et al., 2013).

LOS in ruminants exhibits a variable combination of anomalies that recapitulate the phenotypes commonly observed in BWS (Young et al., 1998). These anomalies can have detrimental effects on both the dam and offspring including difficult delivery due to the oversized nature of the fetus and the inability of the newborn to suckle and breathe. We have recently reported that phenotypic and epigenetic similarities exist between LOS and BWS, such as macroglossia, macrosomia, and ear malformation, as well as loss of methylation at the KvDMR1 (*i.e.* *KCNQ1* locus) on the maternal allele (Chen et al., 2013). These parallels make LOS an appropriate animal model for the study of BWS and the understanding of the etiology of these overgrowth syndromes (Chen et al., 2013).

The incidence of LOS complications is variable and little is known about the molecular cause(s) of this complex phenotype. Given the variable phenotypes of BWS and LOS and the fact that only 50% of individuals exhibit LOI at the *KCNQ1* locus (Chen et al., 2013; Weksberg et al., 2010), we hypothesize that these overgrowth syndromes exhibit LOI at loci beyond those primarily used for diagnosis of BWS, namely *KCNQ1* and *H19/IGF2* DMRs.

In this study, we assessed the allelic expression of imprinted genes previously identified in human and/or mouse in somatic tissues of day ~105 (d105) bovine control

and LOS fetuses (term $\approx$ 280 days). We identified 20 genes exhibiting monoallelic expression in control fetal tissues and found that approximately half were biallelically expressed in at least one tissue in LOS. Furthermore, we observed that biallelic expression of imprinted genes was associated with loss of DNA methylation at DMRs in a tissue-specific manner. Lastly, our data show that misregulation of imprinted genes goes beyond loss of allele-specific expression and provide insights into the variable gene expression observed in this condition.

2.3 Results

Control and LOS bovine fetal tissues preparation and RNA sequencing reads processing

In the current study, we used high throughput RNA sequencing (from here on referred to as RNAseq) to determine allelic expression of imprinted genes previously identified in human and/or mouse in somatic tissues of $\sim$ 105 control and LOS bovine female fetuses (Fig. 1A). Fetuses used in this study were *Bos taurus indicus* (*B. t. indicus*) $\times$ *Bos taurus taurus* (*B. t. taurus*) F₁ hybrids (Chen et al., 2013). Only females were used in this study to avoid any potential sex-specific effects on gene expression (Ingleby et al., 2014a). On average, control fetuses weighed 405g while LOS fetuses weighed 592g ($P = 0.008$, Fig. S1 and Table S1). Kidney, brain, skeletal muscle (from here on referred to as muscle), and liver from each fetus were used for RNAseq analysis. These tissues were selected as they are representative of the primary germ cell lineages:

Figure 1. Loss-of-imprinting in large offspring syndrome (LOS). (A) Flowchart of methodology. (B) Allele-specific expression analysis of imprinted genes identified by RNAseq in day ~105 *B. t. indicus* × *B. t. taurus* fetal kidney. The results for brain, muscle, and liver may be found in Figure S2-4. Gene symbols are ordered from top to bottom in alphabetical order. The parental origin of imprinted gene expression (IGE) is defined according to the known imprinting status in human and/or mouse (maternal = white box; paternal = black box). Samples are arranged from left to right in ascending order by weight (Figure S1) and by treatment group. DNA polymorphisms identified by RNAseq analysis of each F₁ hybrid were used to discriminate between the parental alleles in control samples in order to establish baseline expression of each gene. A sample with at least 15% expression from the repressed allele was considered biallelically expressed (grey box). Genes that showed biallelic expression in LOS but not in control fetuses are indicated by red boxes. Missing data for *RTL1* indicate lack of discriminating SNPs between parental alleles in the reads. We obtained the allelic expression of the long noncoding RNA *KCNQ1OT1* (indicated by asterisk) in a previous study (Chen et al., 2013). (C) Heat map illustrating the degree of loss-of-imprinting for the genes indicated by red boxes in panel (B). Numbers represent the percentage of transcripts expressed from the normally repressed allele. 0 represents <15% expression from the repressed allele (actual percentages may be found in Table S3). A summary of the number genes with loss-of-imprinting may be found in Figure S5.

ectoderm (brain), mesoderm (kidney and muscle) and endoderm (liver). Moreover, kidney and liver are organs that have been documented to be susceptible to tumors in children affected by BWS (Weksberg et al., 2010).

We first aligned the reads to the bovine whole genome assembly *B. t. taurus* UMD3.1 (Table S2). The reads that aligned to the known imprinted genes were then compared between all four tissues of each fetus and genes with at least one consistent variant nucleotide in at least two tissues were used for subsequent analysis. Of the 105 known imprinted genes detected by RNAseq in our study, 72 genes (Table S4) had at least one single nucleotide polymorphism (SNP) with sufficient reads depth (≥ 10) to assess allelic expression. A gene was considered to be monoallelically expressed if the expression of the allele with the fewer reads accounted for less than 15% of the total reads. The other 33 genes were not used for further analysis as the heterozygous nature of the bovine makes it impossible for us to determine with any certainty if the reads originated from one or both alleles. For example, if all reads have a base which differs from the reference genome two scenarios are possible; 1) the gene is monoallelically expressed or 2) the gene is biallelically expressed and both alleles contain the same base.

Allelic determination of imprinted genes in ~d105 control fetal tissues

Of the 72 genes assessable for allelic expression, 52 (Table S4) were biallelically expressed in ~d105 fetal kidney, brain, muscle, and liver. The remaining 20 genes were observed to be imprinted in at least one tissue from any fetus. Of these, 18 were expressed monoallelically in the kidney and 14, 17, and 14 in brain, muscle, and liver, respectively (Fig. 1B and Table S3 and Figs. S2-S4). Thirteen genes (*i.e.* *DIRAS3*, *DLK1*, *GNAS*, *GTL2*, *H19*, *MAGEL2*, *NAPIL5*, *NNAT*, *PEG3*, *PEG10*, *PLAGL1*, *RTL1*, and

SNRPN) exhibited monoallelic expression in all the tissues analyzed where the genes were expressed, whereas seven genes (*i.e. BEGAIN, CDKN1C, IGF2, IGF2R, INPP5F, PHLDA2, and SGCE*) showed tissue-specific imprinting (Fig. 1B and Figs. S2-S4 and Table S3).

Allelic expression of imprinted genes in LOS

Allelic expression of the 20 genes identified to be imprinted in control tissues was then determined in tissues of LOS fetuses. For a gene to be described as experiencing loss-of-imprinted gene expression (as defined by our 15% cutoff), that gene must be monoallelically expressed in the same tissue in all control fetuses. We observed loss-of-imprinted gene expression in 9, 8, 8, and 6 genes in kidney, brain, muscle and liver, respectively (Fig. 1B, 1C and Figs. S2-S4). Of these, *IGF2R* showed universal LOI in LOS kidney, muscle, and liver while *INPP5F* was biallelically expressed in the brain of all LOS fetuses. In other tissues, however, these genes had variable allelic expression in control and/or LOS fetuses (Table S3). Fetuses LOS #3 and #4 displayed LOI of *NNAT* in all the tissues analyzed while LOS #4 had loss-of-imprinted expression of *PLAGL1* in brain, kidney and muscle. Notably, there is a positive correlation between the number of imprinted genes showing LOI in each LOS fetus and their bodyweights (Fig. S5).

Confirmation of SNPs identified by RNAseq

We first performed Sanger sequencing of genomic DNA from eight *B. t. taurus* animals, the *B. t. indicus* sire and several of the *B. t. indicus* × *B. t. taurus* F₁ hybrids to verify 37 of the SNPs identified by RNAseq. For 35 SNPs, both the chromosome position and allele variants matched between RNAseq and Sanger sequencing (Table S6).

The other two SNPs were not confirmed by Sanger sequencing possibly due to RNAseq alignment errors. The confirmed SNPs indicated that *SGCE*, *PEG10*, *NAPIL5*, *SNRPN*, *PEG3*, and *MAGEL2* were paternally expressed in the ~d105 bovine fetal tissues analyzed, showing conservation of maternal-allele silencing of these genes in bovine, human and mouse. We were not able to determine parental allele-specific expression of *NNAT*, since the five SNPs within the exons are heterozygous in both *B. t. indicus* and *B. t. taurus* (Fig. 2B and Table S6).

Confirmation of loss-of-imprinted expression identified by RNAseq

To exclude potential false positive imprinted expression caused by RNAseq systemic errors (DeVeale et al., 2012), we verified allele-specific expression of *PEG3*, *NNAT*, *SNRPN*, and *PLAGL1* by conventional methods (Fig. 2). Paternal expression was validated for *PEG3*, *SNRPN*, and *PLAGL1* in control fetuses. In addition, imprinted expression was also confirmed for *NNAT* although parental origin of the transcript was not assessable due to the lack of subspecies specific polymorphisms (Fig. 2B). In contrast to controls, *PEG3*, *SNRPN*, *NNAT*, and *PLAGL1* were biallelically expressed in LOS fetuses (Fig. 2 and Fig. S6).

Association between allelic expression and transcript abundance of imprinted genes

To assess if the biallelic expression of imprinted genes was associated with the increased amount of transcripts, we normalized the read counts aligned to each gene locus to determine the transcript abundance. We found that loss of imprinted gene

Figure 2. Verification of the allelic expression of *PEG3*, *NNAT*, *SNRPN*, and *PLAGL1* in control and LOS fetuses. The top left parts of (A), (B), (C), and (D) are genotyping results obtained by Sanger sequencing of *B. t. indicus*, *B. t. taurus* and/or F₁ hybrid fetuses for the SNPs identified by RNAseq analysis. SNP positions are indicated by arrows. Pedigree diagrams indicate the SNPs identified by Sanger sequencing for *B. t. indicus* (square; *i.e.* paternal allele in F₁), *B. t. taurus* (top circle; maternal allele in the F₁), and female F₁ ~d105 fetus (bottom circle). The bottom parts of (A) and (C) are Sanger sequencing results of RT-PCR amplicons of *PEG3* and *SNRPN*, respectively. Double peaks demonstrate biallelic expression. The bottom parts of (B), and (D) show allele-specific restriction of RT-PCR amplicons followed by acrylamide gel electrophoresis for *NNAT* and *PLAGL1*, respectively. Restriction enzyme (*i.e.* SfcI for *NNAT*, and BstUI for *PLAGL1*), restriction site and fragment length are illustrated on the bottom left of each panel.

expression in LOS tissues did not always correlate with the amount of transcript (Fig. S7). For example, increased number of transcripts of *SNRPN*, *NNAT* and *PLAGL1* were associated with biallelic expression in kidney and muscle ($P < 0.01$), but not in brain. Further, *IGF2R* had lower total level of expression in both liver and muscle of all LOS fetuses, although *IGF2R* was biallelically expressed in both tissues.

DNA methylation analysis of PLAGL1, SNRPN, and NNAT DMRs

As described above, *SNRPN*, *NNAT* and *PLAGL1* exhibited LOI in several of the LOS fetuses. These genes have also been demonstrated to be associated with BWS (Azzi et al., 2009; Blik et al., 2009; Court et al., 2013; Tee et al., 2013). Thus we assessed allele-specific DNA methylation of the three DMRs.

DNA methylation of *PLAGL1* was examined in a region within the DMR (O'Doherty et al., 2012) that contained 28 CpGs on the paternal (*B. t. indicus*) and 29 CpGs on the maternal (*B. t. taurus*) allele (Fig. 3A and Table S7). *PLAGL1* is maternally repressed and acquires maternal methylation during oocyte growth in bovine (O'Doherty et al., 2012). As expected, we observed methylated maternal alleles and unmethylated paternal alleles in kidney, brain, muscle, and liver of the control fetus analyzed (Fig. 3A and Fig. S8A). Methylation of CpGs within this region was substantially reduced on the maternal allele in the kidney of LOS #4 (Fig. 3A) coincident with the biallelic expression of this gene in this tissue (Fig. 1B). However, biallelic expression of *PLAGL1* did not coincide with a reduction in DNA methylation in brain and muscle (Fig. S8A).

We next examined the methylation at the DMR (O'Doherty et al., 2012) of the maternally imprinted gene *SNRPN* (Fig. 3B). The *SNRPN* DMR displayed differential

methylation in the brain of the control fetus analyzed (Fig. 3B and Table S7) but showed a substantial loss of methylation in the brain of LOS #4 fetus. Loss of methylation of this DMR in this tissue (Fig. 3B) is associated with the gain of maternal expression of this gene (Fig. S2).

Lastly, we analyzed DNA methylation at *NNAT* in control and LOS fetuses. Since the *NNAT* DMR has not been previously reported in bovine, we chose to analyze a CpG island at the *NNAT* promoter region (Fig. 3C) that was syntenic to the human *NNAT* DMR (Hubertus et al., 2013). We examined a region within the CpG island that contained 33 CpGs on the paternal and 34 CpGs on the maternal allele (Fig. 3C and Table S7). Control fetus #2 displayed an unmethylated paternal allele and methylated maternal allele in both kidney and brain (Fig. 3C and Fig. S8B), which strongly suggests that *NNAT* is maternally imprinted in bovine as in human and mouse (Evans et al., 2001; Kagitani et al., 1997). LOS fetus #4 showed reduced DNA methylation on the maternal allele in kidney but not in brain (Fig. 3C and Fig. S8B).

Determination of the transcript level of imprinted genes previously identified in human and/or mouse in bovine ~d105 fetuses.

Given the diverse phenotype and various loss-of-imprinted gene expression in LOS, we compared imprinted gene transcript abundance of each LOS fetus to the average of four controls to identify differentially expressed imprinted genes for each LOS individual using edgeR (McCarthy et al., 2012; Robinson et al., 2010). With the threshold of false discovery rate set at < 0.05 we identified 53, 21, 47, and 35 imprinted genes as being differentially expressed in kidney, brain, muscle, and liver, respectively, in at least

Figure 3. Allele-specific DNA methylation of the *PLAGL1*, *SNRPN*, and *NNAT* DMRs in control and LOS fetuses. The top parts of (A), (B), and (C) are schematic representation of regions analyzed by allele-specific bisulfite sequencing. The bent arrows and black boxes represent the transcription start site and first exon of each gene, respectively. Vertical lines illustrate the CpGs distributed across the region (drawn to scale). The positions of bisulfite-converted DNA specific PCR primers are shown as arrows facing each other. Bisulfite map represents of the number of CpGs contained within each amplicon. Circles represent the CpGs examined within the region of interest (the unmethylated *B. t. taurus* maternal allele is shown as an example). SNPs (shown in bold) used to determine parental origins are indicated by vertical arrows. The bottom parts of (A), (B), and (C) show the allele-specific bisulfite sequencing of *PLAGL1*, *SNRPN*, and *NNAT* DMRs, respectively. Open circles represent unmethylated CpG sites and filled circle represent methylated CpG sites. Missing circles are due to SNPs or undetermined bases because of sequencing issues. Each row represents one strand of DNA.

one LOS fetus (Fig. S9). The fetuses differed in the type of tissue which displayed the highest number of misregulated genes with the kidney of LOS #3 exhibiting inappropriate levels of expression in 50 of the 53 genes and the muscle of LOS #4 showing altered expression in 40 of the 47 genes (Fig. S9).

2.4 Discussion

Beckwith-Wiedemann syndrome and large offspring syndrome are similar fetal overgrowth conditions (Chen et al., 2013; Kalish et al., 2014). Phenotypically, these are heterogeneous syndromes characterized by variable developmental anomalies including macrosomia, macroglossia, abdominal wall defect, and ear malformations. These syndromes also share LOI at the *KCNQ1* locus (Chen et al., 2013), the most common epimutation in BWS, observed in 50% of patients (Weksberg et al., 2010). Even though reports describing BWS date back to the early 1960's (Weksberg et al., 2005) and the first cases of ART-induced LOS were reported in 1995 (Behboodi et al., 1995; Farin and Farin, 1995), no molecular signature has been identified that can consistently and reliably predict and diagnose these syndromes and/or their varied phenotypes. To date, only a small set of imprinted genes have been queried in LOS (Chen et al., 2013; Hori et al., 2010; Young et al., 2001). With the current study, we advance the field by describing the imprinted signature of ~d105 unaffected bovine fetuses and identifying misregulated imprinted loci in LOS.

Approximately 200 imprinted genes have been identified in human and mouse [<http://igc.otago.ac.nz/Search.html> and <http://www.mousebook.org/imprinting-gene-list>,

respectively], and little is known about the allele and tissue specificity of these genes in bovine. In our study we detected expression of 105 of these genes with 52 being biallelically expressed and 20 having monoallelic expression in the fetal tissues analyzed. The remaining 33 genes were not assessable in our system. RNAseq analysis confirmed the maternal expression of *CDKN1C*, *H19*, *PHLDA2* and the paternal expression of *IGF2* and *PLAGL1* (Chen et al., 2013; Robbins et al., 2012). In addition, we demonstrate paternal expression of *NNAT*, *NAPIL5*, *MAGEL2*, *PEG3*, *PEG10*, *SGCE*, and *SNRPN*, which is similar to what has been reported for human and mouse (Morison et al., 2005). Further, we also show that *BEGAIN*, *DIRAS3*, *DLK1*, *GNAS*, *GTL2*, *IGF2R*, *INPP5F*, and *RTL1* are monoallelically expressed, although our analysis precludes us from ascribing the allelic origin of the transcripts. Since monoallelic expression of imprinted genes is tissue- and stage-specific (Bartolomei and Ferguson-Smith, 2011), it is possible that the biallelically expressed genes in the present study are imprinted in other tissues and/or developmental stages. An example is *TSSC4* which has been previously reported to be imprinted in bovine placenta (Zaitoun and Khatib, 2008), however, this gene exhibited biallelic expression in fetal ~d105 kidney, brain, muscle, and liver.

We demonstrate that half of the imprinted genes in controls displayed biallelic expression in LOS, making this a multi-locus LOI syndrome, as is BWS (Azzi et al., 2009; Bliet et al., 2009; Court et al., 2013; Eggermann et al., 2014; Lim et al., 2009; Maeda et al., 2014; Poole et al., 2013; Tee et al., 2013). The profile of LOI was dependent on the tissue, fetus and degree of macrosomia and may provide insights into the understanding of the etiology of the anomalies observed in LOS and BWS. For instance, Wilm's tumor of the kidney, a high-risk childhood cancer in BWS, displayed

hypomethylation at the *NNAT* promoter, which coincided with upregulation of *NNAT* transcript (Hubertus et al., 2013). In our study we observed a similar situation where biallelic expression and increased transcript amount of *NNAT* was associated with hypomethylation of its DMR in LOS kidney. Further, we detected biallelic expression of the paternal gene *PLAGL1*, which corresponded with the increased transcript amount of this gene in the muscle and kidney of the largest LOS fetus. *PLAGL1* is a known regulator of embryonic growth and mice lacking the paternal allele of this gene exhibit growth retardation (Varrault et al., 2006). Interestingly, *PLAGL1* has been documented to bind the DMR of the *KCNQ1* locus in a methylation dependent manner suggesting the involvement of this molecule in the misregulation of this locus, as has been suggested by others (Arima et al., 2005).

In the current study, we also determined the transcript amount of imprinted genes to determine if their misregulation in LOS transcends parental-allele specific expression. Similar to what others have reported in the mouse, biallelic expression of imprinted genes does not always correlate with increased transcript level in bovine (de Waal et al., 2012a; Susiarjo et al., 2013). For example, even though *IGF2R* was biallelically expressed in liver and muscle of LOS fetuses, the total transcript amount was lower than in the controls where monoallelic expression of this gene was observed. Lower *IGF2R* levels have been previously observed in LOS (Young et al., 2001).

Further, differentially expressed imprinted genes in each LOS fetus were analyzed in the GeneCard database [<http://www.genecards.org>, (Table 1)] in order to assess the attributes of these genes (e.g. gene ontology, disorders, phenotypes, expression patterns).

The analysis showed that these genes were most significantly enriched with the descriptors “Beckwith-Wiedemann syndrome”, “growth size and body phenotype”, “cellular phenotypes”, and “tumors” indicating similarities in the types of imprinted genes misregulated in LOS and BWS. In addition, differentially expressed genes were implicated in tumor and/or body size control, which suggests that misregulation of these genes may contribute to the overgrowth phenotype of LOS. The analysis not only allowed us to confirm the imprinted genes previously known to be associated with LOS such as *IGF2R* (Young et al., 2001) and *CDKN1C* (Chen et al., 2013), but also to identify new candidates that can be used to predict or diagnose LOS at the molecular level. A gene of interest, for example, is *FBXO40*, which was downregulated by at least 2 fold in the muscle of all LOS fetuses. Recently, Shi and coworkers demonstrated that *FBXO40* is a negative regulator of *IGF1* signaling in muscle differentiation and observed increased bodyweight and muscle mass in *FBXO40* null mice at six weeks of age (Shi et al., 2011). Other genes with a potential link to the overgrowth seen in LOS are *MKRN3* and *NNAT* which were upregulated in the muscle of the two largest LOS fetuses by >3.5 and 2.37 folds, respectively, when compared to controls. *MKRN3* and *NNAT* are protein-coding genes with currently undefined function in muscle.

In summary, our study characterizes previously unknown expression of imprinted genes in unaffected and LOS bovine fetuses. We conclude that LOS is a multi-locus LOI syndrome, as is BWS. Future studies will assess if genes identified in this study are similarly misregulated in BWS.

Table 1. Shared descriptors of differentially expressed imprinted genes in LOS fetuses

Rank	Descriptor	# of Genes	Associated imprinted genes	P-value
1	Beckwith-Wiedemann syndrome	13	CDKN1C, H19, IGF2, IGF2R, KCNQ1, NAP1L4, PEG3, PHLDA2, PLAGL1, SLC22A18, SNRPN, TSPAN32, TSSC4	1.00 X 10 ⁻¹⁶
2	Growth/size/body phenotype	34	ANO1, CD81, CDKN1C, COMMD1, DCN, DHCR7, DIO3, DLK1, DNMT1, GATM, GLIS3, GNAS, H19, IGF2, IGF2R, KCNQ1, LIN28B, MAGEL2, MEST, PDE10A, PDE4D, PEG10, PEG3, PHLDA2, PLAGL1, PON3, RASGRF1, RB1, RTL1, SLC22A3, SNRPN, TCEB3, TP73, UBE3A	1.00 X 10 ⁻¹⁶
3	Cellular Phenotype	30	AMPD3, AXL, CDKN1C, DCN, DIO3, DLK1, DNMT1, GNAS, H19, IGF2, IGF2R, L3MBTL1, MAGEL2, MEST, NDN, PDE4D, PEG10, PEG3, PHLDA2, PLAGL1, PON2, PON3, RASGRF1, RB1, SGCE, SLC22A3, SNRPN, TCEB3, TP73, UBE3A	2.11 X 10 ⁻¹⁵
4	Tumors	32	AMPD3, ANO1, AXL, BLCAP, CALCR, CD81, CDKN1C, DCN, DIO3, DLK1, DNMT1, GNAS, H19, IGF2, IGF2R, L3MBTL1, NAP1L4, NDN, NNAT, NTM, PEG3, PLAGL1, QPCT, RB1, SLC22A18, SLC22A3, TCEB3, TFPI2, TP73, TSPAN32, UBE3A, WIF1	1.02 X 10 ⁻¹⁴

2.5 Materials and Methods

Fetal Tissue Collection and Illumina RNAseq

Day ~105 *B. t. indicus* X *B. t. taurus* F1 hybrid control and LOS fetuses were used to analyze allele-specific expression and DNA methylation of imprinted genes. Fetuses used in this study were produced by us as part of a previous study (Chen et al., 2013). Serum was purposely used in that study to supplement the culture media in order to increase the incidence of the overgrowth phenotype. This method enables us to have a reliable system to study the etiology and progression of LOS. In our previous study, we generated 9 controls fetuses (5 females and 4 males) and 7 LOS fetuses (4 females and 3 males). In the current study, all four females in the LOS group were used. For the control group, we randomly chose four of the five females to analyze the same number of samples in each treatment group.

Total RNA was isolated from kidney, brain, muscle, and liver of four control and four LOS females using Trizol Reagent (Invitrogen) according to manufacturer's instructions. RNA quality was assessed using spectrometry and agarose gel electrophoresis. RNAseq libraries were prepared using standard Illumina protocol and sequenced on Illumina HiSeq 2000 platform as single-end reads. The raw FASTQ files are publically available at Gene Expression Omnibus (GEO; accession no. GSE63509).

Bioinformatics Analysis

After quality trimming, RNAseq reads were aligned to the bovine genome reference (UMD3.1) using TopHat2 (Kim et al., 2013). Only reads that had >95% identity with the reference genome were used for the analyses. Uniquely aligned RNAseq

reads were normalized to the library size to compute counts per million (CPM). Known imprinted genes were selected based on the human and mouse imprinted gene databases (<http://igc.otago.ac.nz/Search.html>; <http://www.geneimprint.com/site/genes-by-species>; <http://www.mousebook.org/mousebook-catalogs/imprinting-resource>). Imprinted genes annotated by RefSeq or Ensembl were analyzed.

SNPs between *B. t. indicus* and *B. t. taurus* were identified by the following criteria: 1) at least 10 reads coverage at a given chromosome position; 2) presence of only one variant allele (different from the reference allele); 3) at least 3 reads for the lower expressed allele. In order to further reduce the false positive SNPs, only the SNPs that exist in at least two of the four tissues (*e.g.* liver and muscle) from a single individual were kept for further analysis. Only genes that had one or more SNPs and at least 10 SNP-containing read counts were analyzed. Only genes with at least 15% of reads from the repressed allele were considered biallelically expressed.

SNP validation by Sanger sequencing

DNA was isolated from *B. t. taurus*, *B. t. indicus*, and *B. t. indicus* $\times$ *B. t. taurus* F₁ hybrid tissues by phenol-chloroform extraction. PCR amplifications were performed using GoTaq Hot Start polymerase (Promega) and amplicons were sequenced at the University of Missouri DNA core using the 96-capillary applied Biosystems 3730 DNA analyzer with Big Dye Terminator.

Analysis of allelic expression

Allele-specific expression of *PEG3* and *SNRPN* were validated using RT-PCR followed by Sanger sequencing. The PCR reactions were prepared and sequenced as

described above. Allelic expression of *NNAT* and *PLAGL1* were determined using RT-PCR followed by RFLP and PAGE.

DNA methylation analysis

Genomic DNA was mutagenized with sodium bisulfite using the Imprint DNA Modification Kit (Sigma) according to the manufacturer's instructions. Amplified PCR products were ligated with pCC1 vector and cloned using CopyControl PCR cloning kit according to manufacturer's instructions except all the cloning incubation procedures were performed at room temperature. Colonies were usually visible within two days. Positive colonies were sequenced as described above.

2.6 Acknowledgements

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2.7 Supplemental information for Chapter 2

Supplemental material and methods

Fetal tissue collection

Day ~105 control and ART-induced LOS fetuses were generated in a previous study by our laboratory (Chen et al., 2013). Briefly, for the ART group, cumulus-oocyte complexes (COCs) from *B. t. taurus* (Holstein breed) were matured *in vitro* and fertilized *in vitro* using the semen from one *B. t. indicus* bull (Nelore breed; ABS CSS MR N OB 425/1677344 29NE0001 97155). Five days post fertilization, embryo culture medium was supplemented with 10% estrus cow serum. On day 7 post-fertilization, blastocysts were transferred into synchronized *B. t. taurus* recipients (two blastocysts per recipient; one blastocyst per uterine horn). For control group, *B. t. taurus* females were artificially inseminated with the semen from the same *B. t. indicus* bull used in the ART group. On day ~105, *B. t. indicus* × *B. t. taurus* F₁ hybrid conceptuses (fetus + placental tissues) from both control and ART groups were collected from gravid uteri at caesarean section. For the control group, all fetuses were singleton pregnancies. For the ART group, 11 fetuses were singleton pregnancies and 16 were twins (Chen et al., 2013). Two of the LOS females used here were twins (one fetus/uterine horn; LOS #1 and LOS #3) and two were singletons (LOS #2 and LOS #4). Statistical analysis showed that bodyweight was not different between twins and singletons (P= 0.9 between ART-produced singletons and twins). At collection, liver, skeletal muscle (from here referred to as muscle), brain, and kidney were well diced, mixed, and snap frozen in liquid nitrogen and stored at -80°C. All animal surgery procedures were performed at TransOva Genetics (IA) by veterinarians and in accordance with TransOva's animal care and use committee.

Illumina RNA sequencing (RNAseq)

Liver, muscle, brain, and kidney RNA samples from four control females and four LOS females were subjected to RNAseq at the University of Missouri-Columbia's DNA core. Total RNA from fetal tissues was isolated using Trizol Reagent (Invitrogen) according to the manufacturer's instructions. RNA quality was determined by both spectrometry and agarose gel electrophoresis. For each sample, 5µg RNA was submitted to the DNA core for RNAseq library preparation using Illumina TruSeq RNA sample preparation kit. Briefly, poly-A containing RNA was purified using magnetic beads with oligo-dT attached. The purified RNA was fragmented and used as a template for cDNA synthesis using random primers. In the following end-repair procedure, an "A" was added to the 3' end of the cDNA. The cDNA fragments with A-tailing were then ligated with T-tailing adaptors. PCR amplifications of cDNA fragments were followed to generate the final RNAseq libraries.

Initially, liver, muscle, and brain RNAseq libraries from one control fetus (i.e. Control #2) were sequenced in one lane on Illumina HiSeq 2000 platform to test the sequencing quality and sequencing depth with our RNA samples. We obtained 223.7 million single-end reads (read length 50 bp) for the three samples. Subsequent RNAseq libraries were sequenced using Illumina HiSeq 2000 platform with single-end read length as 100 bp.

RNAseq reads quality trimming

Raw RNAseq reads were subjected to quality trimming before being aligned to the reference genome. Briefly, adaptor sequences of the reads were removed with the use

of FastqMcf program (version 1.04.636; ref Aronesty, 2011). Low quality reads (*i.e.* reads with high percentage of ambiguous base calls) were filtered out using DynamicTrim (version 1.13; ref Cox et al., 2010). DynamicTrim generates the longest contiguous segment of a read in which the phred sequencing score of each base is greater than 20 (base call accuracy > 99%). Only reads with a length above 30 bp were kept for further analysis. Lastly, read quality was confirmed by FastQC (version 0.10.1; <http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). The number of reads before and after trimming for each sample is summarized in Table S2.

RNAseq reads alignment

RNAseq reads were aligned to the bovine reference genome assembly UMD3.1 using TopHat2 (version 2.0.10; Kim et al., 2013). By default, TopHat first aligns the reads to the reference genome allowing for a maximum of two mismatches. Reads that span exon-exon junctions would not be aligned and set aside at this step. TopHat then breaks up the exon-exon junction reads into smaller segments and aligns them to the reference genome independently. The aligned smaller segments are then assembled together to generate a final read alignment. The *de novo* identification of splicing junction sites by TopHat is based on the aligning of exon-exon junction reads. Any two segments pairs that are closer or farther than these parameters are not considered as exon-exon junction reads.

In our analysis, we adjusted the number of allowed mismatches based on read length (*i.e.* shorter reads were allowed fewer mismatches than longer reads) by passing Bowtie2 scoring option (--score-min L,0,-0.2 --mp 6,6; <http://bowtie->

bio.sourceforge.net/bowtie2/manual.shtml - Scoring-options) through Tophat2 (Kim et al., 2013; Langmead and Salzberg, 2012). This allows us to achieve unique alignment of any read length at greater than 96.7% identity. In this method, the score for a perfect alignment (i.e. 100% identity) is 0 while a single nucleotide mismatch within the aligned fragment will be penalized by -6 units. Therefore, the closer the number is to 0 the greater the similarity between the read and the reference genome. For an alignment to be considered valid, then the minimum score cannot be less than $(-0.2) \times (\text{read length})$.

For example; for a 100bp read, the minimum required mapping score is $(-0.2) \times (100) = -20$. Since each mismatch receives a penalty of -6, then $-20/-6 = 3.33$ mismatches allowed. At this level, the alignment identity is $>97\%$. So using the formula above, the score for a 30 bp read = -6 thus only 1 mismatch is allowed for such a read length.

Furthermore, gene and exon models from the Ensembl (<http://www.ensembl.org>) and the RefSeq (<http://www.ncbi.nlm.nih.gov/refseq/>) databases were provided to TopHat in order to increase mapping depth at any base. When known gene and exon models are provided, TopHat extracts the transcripts sequences and align the reads to the transcriptome first. This step improves the alignment accuracy of exon-exon junction reads by taking advantage of known splice junctions. The reads that were not aligned to the transcriptome are then aligned to the genome as described above.

Only reads that were uniquely aligned to the reference genome were kept for the subsequent analysis. Percentage of uniquely aligned reads for each sample is summarized in Table S2.

Transcript assembly from aligned RNAseq reads

Cufflinks (version 2.1.1; Trapnell et al., 2010) was used to assemble transcripts from the aligned reads for each sample. Assembled transcripts of each sample (*i.e.* total 32 samples) were merged into a single set of genes and transcripts using Cuffmerge (version 2.1.1; Trapnell et al., 2010) for gene expression analysis. Known gene and exon models from the Ensembl and the RefSeq databases were provided to Cuffmerge to maximize the assembly accuracy. Cuffmerge also performs Cuffcompare (version 2.1.1; Trapnell et al., 2010) to compare the merged transcripts to known gene models. Cuffcompare classifies each transcript into different categories depending on how they match the gene annotation (*e.g.* class code “=” stands for complete match of a transcript to a known gene intron chain; “u” stands for unknown, intergenic transcript, etc.). Merged transcripts that completely match a known gene intron chain (*i.e.* class code “=”) were assigned with either the Ensembl gene ID or the RefSeq ID if it is not annotated in Ensembl database. Both overall gene expression and allele-specific gene expression were analyzed based on the gene models generated from Cuffmerge.

RNAseq based gene expression analysis

Gene expression was determined by the total read counts from a gene locus. Total read counts for each gene was generated using the Htseq (version 0.5.4) with default union-counting mode (Anders et al., 2013). In union-counting mode, reads that are located at the overlapping regions of different genes are discarded (Anders et al., 2013). The rationale for this action is that true differential expression of one gene may inaccurately appear as differential expression another gene with overlapped regions.

Bioconductor package edgeR is a read-counts based RNAseq differential expression analysis tool and determines expression at a gene level based on the total read counts from a gene locus (McCarthy et al., 2012; Robinson et al., 2010). EdgeR normalizes the raw read counts of genes with several parameters (*e.g.* sequencing depth) excluding gene length (McCarthy et al., 2012; Robinson et al., 2010). Thus edgeR presents gene expression unit as counts per million (CPM) instead of reads per kilobase per million (RPKM) (McCarthy et al., 2012; Robinson et al., 2010). Genes that have less than 10 CPM for the sum of all 8 samples (*i.e.* control group: n=4 and LOS group: n = 4) were considered as low expression and not included in the subsequent analysis.

About 100 known imprinted genes in humans and 150 in mouse (~50 overlapped with humans') were obtained from human (<http://igc.otago.ac.nz/Search.html>; <http://www.geneimprint.com/site/genes-by-species>) and mouse imprinted gene database (http://www.har.mrc.ac.uk/research/genomic_imprinting/). Approximately 130 known imprinted genes are annotated in bovine Ensembl and/or RefSeq databases and included in Cuffmerge gene models. In the annotated known imprinted gene list, 105 genes were expressed in at least one of the tissues analyzed (*i.e.* liver, muscle, brain, and kidney) based on the criteria described above.

Gene expression for each tissue from each LOS fetus was compared to the average gene expression of control fetuses. Differential gene expression between each LOS fetus and the average of the controls was identified using edgeR (McCarthy et al., 2012; Robinson et al., 2010). Differentially expressed imprinted genes were uploaded to GeneCards database (<http://www.genecards.org/>) to identify their shared descriptors.

RNAseq based allele-specific expression analysis of known imprinted genes

Single nucleotide polymorphisms (SNPs) between *B. t. indicus* and *B. t. taurus* are needed to determine parental-allele specific gene expression. In order to ascertain the allele-specific expression in F₁ fetuses, we identified SNPs from the *B. t. indicus* × *B. t. taurus* F₁ hybrid fetal RNAseq data.

Reads that were uniquely aligned to the UMD3.1 reference genome (*B. t. taurus*) were used to identify SNPs. The following criteria were initially applied to identify heterozygous SNPs for each sample: 1) coverage by at least 10 reads at a given chromosome position; 2) presence of only one variant allele (different from the reference allele); 3) at least 3 reads for the lower expressed allele. In order to further reduce the false positive SNPs, only the SNPs that exist in at least two of the four tissues (*e.g.* liver and muscle) from a single individual were kept for further analysis. In addition, SNPs that could not be assigned to a single gene (*i.e.* a SNP that is located in a region with two different genes with sense and antisense transcription) were not used for allele-specific expression analysis.

With the identified SNPs and SNP-containing read counts, we determined the allele-specific expression of the known imprinted genes by the relative counts of reference and alternative alleles. Only genes that had one or more SNPs and at least 10 SNP-containing read counts were analyzed.

Determination of Loss-of-imprinting

Eleven imprinted genes (i.e. *PEG3*, *SNRPN*, *CDKN1C*, *H19*, *IGF2*, *SGCE*, *PEG10*, *NAP1L5*, *PLAGL1*, *PHLDA2*, *MAGEL2*) were selected to compute the standard deviation of the expression from the repressed allele. These were chosen because Sanger sequencing was performed on this subset of genes as part of validations performed in this and our previous study (Chen et al., 2013) to confirm allelic parental origin.

We assumed that an imprinted gene is monoallelically expressed and hence has no expression from the repressed allele. Therefore, the low level of expression detected from the repressed allele of these genes was categorized as noise, which is expected to follow a normal distribution. We calculated $2 \times \text{SD}$ using all of the aforementioned genes. That number was ~12%. We decided to use the more stringent cutoff of 15% expression from the repressed allele to ascribe an imprinted gene as being biallelically expressed. Further, we used Pearson correlation to determine associations between allelic expression and transcript amount.

SNPs validation by Sanger sequencing

DNA was isolated using the phenol-chloroform method. For eight *B. t. taurus* animals (four Holstein breed and four Angus breed), DNA was isolated from the uterine tissues. DNA of the *B. t. indicus* bull and *B. t. indicus* $\times$ *B. t. taurus* F₁ hybrid fetuses was isolated from semen and tails, respectively. PCR primers were designed to amplify 14 genomic regions that contain 37 SNPs identified by RNAseq using IDT primer design program (<http://www.idtdna.com/Primerquest/Home/Index>). PCR in a 20 μ l reaction containing 1 $\times$ colorless GoTaq Flexi Buffer (Promega), 0.3 μ M forward and reverse

primer (IDT), 2.5mM MgCl₂ (Promega), 200μM dNTP (Invitrogen), and 0.5U GoTaq Hot Start polymerase (Promega) were under the following cycling program: initial denaturation at 94.0°C for 2 minutes 15 seconds, 35 cycles of 94.0°C for 15 seconds, 58.4-62.3°C for 30 seconds, and 72.0°C for 1 minute, and final extension at 72.0°C for 5 minutes (For detailed PCR and primer condition information see Table S8). PCR products were resolved by 1% agarose gel electrophoresis and products at expected size were excised and cleaned using Wizard DNA Clean Up system (Promega). Cleaned PCR products were sequenced at the University of Missouri DNA core using the 96-capillary applied Biosystems 3730 DNA analyzer with Big Dye Terminator. Sequencing data was aligned to references using MacVector software.

Validation of allele-specific expression of imprinted genes by Sanger sequencing and restriction fragment length polymorphism (RFLP)

RNA was treated with RNase-free DNase (Fisher Scientific) before cDNA synthesis. First strand cDNA synthesis was performed with 80ng RNA in a 20μl reaction; 10mM DTT (Invitrogen), 1× First Strand Buffer (Invitrogen), 0.5μg random primer (Promega), 1mM dNTP (Invitrogen), 100U Superscript II reverse transcriptase (Invitrogen), and 20U RNase Inhibitor (Fisher Scientific). The cDNA synthesis reaction was first incubated at 42°C for 1 hour, followed by 95°C for 10 minutes, and stored at -20°C until further use. In order to ensure no genomic DNA contamination, a cDNA synthesis reaction without reverse transcriptase (RT) for each sample was also included.

Allele-specific expression of *PEG3* and *SNRPN* were validated using RT-PCR followed by Sanger sequencing (Chen et al., 2013). The PCR reactions were prepared and

sequenced as described above. Allelic expression of *NNAT* and *PLAGL1* were determined using RT-PCR followed by RFLP and polyacrylamide gel electrophoresis (PAGE). Briefly, SNPs in *NNAT* and *PLAGL1* exons were used to discriminate parental alleles by applying allele-specific restriction enzyme digestion (*i.e.* SfcI for *NNAT*, and BstUI for *PLAGL1*). Detailed PCR and primer information is included in Table S8.

DNA methylation analysis of PLAGL1, SNRPN, and NNAT DMRs

Bisulfite mutagenesis of DNA was conducted using the Imprint DNA Modification Kit (Sigma) according to the manufacturer's instructions. During this process, unmethylated cytosines are converted into uracil, and replaced by thymine during PCR amplifications, whereas methylated cytosines remain cytosines. *PLAGL1* DMR (ref|AC_000166.1: 82473331-82474137) and *SNRPN* DMR (ref|AC_000178.1: 25826-26186) have been reported as gametic DMRs in bovine (O'Doherty et al., 2012). PCR Primers for these regions were designed using the Methyl Primer Express Software (version 1.0, Applied Biosystems). Since *NNAT* DMR had not been previously reported in bovine, a CpG island (ref|AC_000170.1: 67118285-67119013) at *NNAT* promoter region identified by MethPrimer (Li and Dahiya, 2002) was used to design primers. All the amplicons cover SNPs between *B. t. indicus* and *B. t. taurus* identified by Sanger sequencing. Primer information is included in Table S8.

Bisulfite PCR conditions were similar to those described above except that 4mM MgCl₂ was used. For *PLAGL1* DMR, the PCR cycling program was as follows: initial denaturation at 94.0°C for 2 minutes 15 seconds, 45 cycles of 94.0°C for 30 seconds, 60.5°C for 45 seconds, and 72.0°C for 1 minute, and final extension at 72.0°C for 5

minutes. For *NNAT* and *SNRPN* DMRs, touchdown PCR cycling program was used to optimize the amplification of product. Briefly, after initial denaturation, the annealing temperature was gradually decreased by 1°C/cycle for five cycles to the optimized temperature (*i.e.* 60.5°C). The optimized temperature was used as the annealing temperature for the subsequent 40 cycles.

PCR products were resolved by 1% agarose gel electrophoresis and isolated as described above. *PLAGL1* DMR amplicon (298bp contains 29 CpGs; ref|AC_000166.1: 82473614-82473911), *SNRPN* DMR amplicon (566bp contains 37 CpGs; ref|AC_000178.1: 25757-26322), and *NNAT* DMR amplicon (342bp contains 34 CpGs; ref|AC_000170.1: 67118612-67118953) were ligated with pCC1 vector and cloned using CopyControl PCR cloning kit according to manufacturer's instructions except all the cloning incubation procedures were performed at room temperature. Colonies were usually visible within two days. Individual colonies were picked up and diluted with 10µl H₂O, from which 2µl mix was used as template for colony PCR. PCR products were then precipitated with isopropanol. Briefly, 20µl PCR product was added with 4µl 3M sodium acetate and 20ul 100% isopropanol and placed at -80°C for at least 15 minutes. After centrifuging at 16,000g for 20 minutes, supernatant was removed and pellet (generally invisible) was washed with 70% ethanol. After repeating the centrifuge step, pellet was suspended in H₂O and sent for Sanger sequencing as described above.

Supplemental figures and tables for Chapter 2

Figure S1. Day ~105 fetuses used in this study. Each fetus is a *B. t. indicus* and *B. t. taurus* hybrid females. Fetus number and bodyweight are shown. Each square on the background = 2.54 cm². Information on the fetus beyond that obtained in this study may be found in the publication (Chen et al., 2013).

Figure S2. Allele-specific expression analysis of known imprinted genes using RNAseq in brain of the fetuses. Details are as in Figure 1. LE=low expression (fewer than 10 reads).

Figure S3. Allele-specific expression analysis of known imprinted genes using RNAseq in liver of the fetuses. Details are as in Figure 1. LE=low expression (fewer than 10 reads).

Figure S4. Allele-specific expression analysis of known imprinted genes using RNAseq in skeletal muscle of the fetuses. Details are as in Figure 1. NA: not assessable due to lack of subspecies-specific SNPs.

Figure S5. Positive correlation between number of genes showing LOI and bodyweight of LOS fetuses. Each bar represents the number of imprinted genes showing abnormal allelic expression in kidney, brain, liver and muscle of each LOS fetus. The bodyweight of each LOS fetus is illustrated by the connected black dots. The Pearson correlation coefficient between the number of genes showing LOI and the bodyweight of LOS fetuses is 0.90 ($P = 0.049$). Allelic expression of *KCNQ1OT1* was also included in the analysis.

Figure S6. Verification of the allelic expression of *PEG3*, and *SNRPN* in control and LOS fetuses. Shown is Sanger sequencing of RT-PCR amplicons of *PEG3* and *SNRPN*. Double peaks pointed by arrows demonstrate biallelic expression. *PEG3* in LOS #3 and #4 liver, and *SNRPN* in LOS#3 kidney were not considered biallelically expressed because the ratios expressed from the repressed allele were below 15% according to RNAseq data. Other details are the same as described in Figure 2.

Figure S7.

Figure S7. Lack of association between allelic expression and transcript abundance of imprinted genes. Transcript abundance was determined by the normalization of total RNAseq reads that aligned to the gene locus. Black dots represent genes with monoallelic expression and red dots represent genes with biallelic expression.

Figure S8. Allele-specific DNA methylation analysis of *PLAGL1* and *NNAT* DMRs. (A). Methylation profile of *PLAGL1* DMR in liver, muscle, and brain of Control #4 and LOS #4 fetus. **(B).** Methylation profile of *NNAT* DMR in brain of Control #2 and LOS #4 fetus. Other details are the same as described in Figure 3.

Figure S9. Differential expression of imprinted genes in LOS compared to controls. Heat map illustrating the differentially expressed imprinted genes identified using edgeR (false discovery rate <0.05) in each tissue of each LOS fetus (Blue = downregulated; Red = upregulated). Relative intensity of the color represents the level of fold change. Numbers in the table represent the transcript level relative to the average of controls.

Table S1. *B. t. indicus* × *B. t. taurus* F₁ hybrid fetus information

Fetus	Sex	Fetal age	BW (g)	Organ Weight (g)			
				Liver	Muscle	Brain	Kidney
Control #1	Female	d104	392	13.4 (3.4%)	ND	12.8 (3.3%)	5.6 (1.4%)
Control #2	Female	d105	404	14.9 (3.7%)	ND	11.3 (2.8%)	6.0 (1.5%)
Control #3	Female	d106	408	13.9 (3.4%)	ND	12.0 (2.9%)	5.2 (1.3%)
Control #4	Female	d106	416	14.1 (3.4%)	ND	11.7 (2.8%)	5.6 (1.3%)
LOS #1	Female	d106	514*	23.3 (4.5%)	ND	11.6 (2.3%)	8.7 (1.7%)
LOS #2	Female	d105	518*	19.4 (3.7%)	ND	11.6 (2.2%)	6.7 (1.3%)
LOS #3	Female	d106	620*	27.7 (4.5%)	ND	11.7 (1.9%)	6.2 (1.0%)
LOS #4	Female	d105	714*	26.8 (3.8%)	ND	11.2 (1.6%)	13.3 (1.9%)

LOS: large offspring syndrome; BW: body weight (student t-test: LOS vs. Control *P=0.008). Organ weights were also presented as the percentage of the bodyweight (as shown in parentheses), which were not statistically different between LOS and control fetuses. ND: not determined, only a piece of skeletal muscle was collected for analysis.

Table S2. Control and LOS fetal tissues RNAseq summary

Fetus ID	Organ	Read length	# reads before quality trimming	# reads after quality trimming	# total reads mapped to UMD3.1	% total reads mapped	# uniquely mapped to UMD3.1	% uniquely mapped reads
Control #1	Liver	100bp	49,324,460	39,771,401	37,440,644	94.14	34,805,066	87.51
Control #1	Muscle	100bp	35,012,854	28,164,307	25,873,674	91.87	24,593,143	87.32
Control #1	Brain	100bp	39,114,786	31,602,386	28,431,993	89.97	27,434,118	86.81
Control #1	Kidney	100bp	44,168,361	35,748,305	32,663,416	91.37	31,188,516	87.24
Control #2	Liver	50bp	89,453,260	45,661,628	43,683,972	95.67	37,402,160	81.91
Control #2	Muscle	50bp	56,538,563	29,854,108	28,126,835	94.21	26,296,827	88.08
Control #2	Brain	50bp	77,691,951	38,651,699	36,395,229	94.16	33,913,437	87.74
Control #2	Kidney	100bp	60,857,482	43,334,190	39,760,139	91.75	37,999,230	87.69
Control #3	Liver	100bp	47,032,906	37,570,762	35,616,256	94.80	32,918,922	87.62
Control #3	Muscle	100bp	36,876,793	29,969,941	27,970,927	93.33	26,636,566	88.88
Control #3	Brain	100bp	45,716,097	37,136,173	34,133,616	91.91	32,718,553	88.10
Control #3	Kidney	100bp	60,174,902	41,681,288	38,143,750	91.51	36,302,479	87.10
Control #4	Liver	100bp	48,359,824	39,244,627	36,949,505	94.15	34,378,296	87.60
Control #4	Muscle	100bp	32,704,016	26,326,185	23,929,859	90.90	22,866,008	86.86
Control #4	Brain	100bp	47,403,478	38,145,390	34,476,641	90.38	33,229,226	87.11
Control #4	Kidney	100bp	57,133,460	40,562,200	36,830,958	90.80	35,088,062	86.50
LOS #1	Liver	100bp	43,702,500	35,039,540	32,617,194	93.09	29,689,819	84.73
LOS #1	Muscle	100bp	36,677,201	29,433,490	26,969,294	91.63	25,400,463	86.30
LOS #1	Brain	100bp	34,368,056	27,188,866	25,718,282	94.59	24,477,407	90.03
LOS #1	Kidney	100bp	52,656,189	39,385,040	35,992,962	91.39	34,353,786	87.23
LOS #2	Liver	100bp	38,657,402	31,204,376	29,457,242	94.40	27,117,333	86.90
LOS #2	Muscle	100bp	46,224,766	37,352,304	34,653,583	92.77	32,965,587	88.26
LOS #2	Brain	100bp	43,052,038	34,693,252	32,524,533	93.75	31,100,002	89.64
LOS #2	Kidney	100bp	62,356,428	43,043,803	39,206,473	91.09	37,295,278	86.64
LOS #3	Liver	100bp	67,410,973	49,344,697	46,497,196	94.23	43,052,525	87.25
LOS #3	Muscle	100bp	66,164,453	48,644,403	45,352,608	93.23	43,488,821	89.40
LOS #3	Brain	100bp	76,645,582	56,760,249	52,959,888	93.30	50,911,766	89.70
LOS #3	Kidney	100bp	44,825,859	32,386,152	29,141,781	89.98	27,305,679	84.31
LOS #4	Liver	100bp	53,967,229	43,237,304	40,707,119	94.15	37,594,353	86.95
LOS #4	Muscle	100bp	56,742,110	45,144,209	41,889,305	92.79	40,379,885	89.45
LOS #4	Brain	100bp	79,150,645	62,861,848	58,386,058	92.88	56,343,425	89.63
LOS #4	Kidney	100bp	56,302,574	38,654,962	35,183,985	91.02	33,495,182	86.65

LOS: large offspring syndrome; bp: base pairs

Table S3. Allelic expression of the monoallelically expressed imprinted genes in ~d105 bovine fetuses. For each tissue, left table shows the actual percentage of the expression from the repressed allele for each gene in each fetus. Right table shows the number of the reads covering the SNPs used for ascribing allelic expression of the imprinted gene in each fetus. Genes with biallelic expression in LOS but not in controls are delineated by a red box. LE = low expression (fewer than 10 reads); ND = not detectable; NA = not assessable due to lack of subspecies-specific SNPs.

Brain (% of the repressed allele)									Brain (# of reads containing the SNPs used to ascribe the allelic expression)								
Control					LOS				Control					LOS			
#1	#2	#3	#4	#1	#2	#3	#4	#1	#2	#3	#4	#1	#2	#3	#4		
DIRAS3	0.1	0.0	0.3	0.2	6.1	6.3	33.4	31.9	DIRAS3	3627	696	2777	4629	783	821	2022	2993
DLK1	0.0	0.0	0.0	0.0	LE	LE	0.0	0.0	DLK1	28	18	59	62	3	7	11	191
GNAS	0.5	1.3	0.4	0.5	0.1	0.4	1.5	1.6	GNAS	1932	1012	2071	2052	1425	1390	1579	2326
GTL2	0.0	0.2	0.1	0.1	0.0	0.1	0.0	0.5	GTL2	877	929	885	719	508	735	1180	1959
H19	12.1	6.7	14.2	4.0	0.0	LE	6.9	9.2	H19	41	30	21	25	9	20	160	577
MAGEL2	0.0	0.0	0.0	0.6	6.7	3.2	17.7	26.5	MAGEL2	109	50	99	165	60	62	96	177
NAP1L5	0.0	2.3	0.1	0.4	9.8	9.3	16.5	9.5	NAP1L5	2004	308	805	1715	102	140	309	979
NNAT	0.9	0.1	0.6	0.4	20.7	1.5	51.6	28.4	NNAT	3390	1037	3804	11825	733	4883	2355	13430
PEG10	0.6	0.0	0.2	0.1	0.1	0.1	0.0	0.1	PEG10	5225	3585	6097	7762	6327	5430	7472	11290
PEG3	0.0	0.6	0.4	0.0	0.0	7.8	18.0	28.0	PEG3	262	154	230	225	137	154	900	1770
PLAGL1	1.2	1.8	1.1	2.7	2.5	1.5	0.8	23.1	PLAGL1	81	57	95	73	40	67	124	221
RTL1	ND	ND	ND	ND	ND	ND	ND	ND	RTL1	0	0	0	0	0	0	0	0
SNRPN	0.0	0.0	0.0	0.0	6.2	5.3	18.0	24.5	SNRPN	1971	498	1758	2477	1823	1472	1760	2311
BEGAIN	23.8	46.7	38.8	32.9	58.6	14.6	43.1	40.3	BEGAIN	63	30	103	82	29	48	58	139
CDKN1C	11.4	11.1	20.0	13.1	18.2	18.7	34.8	32.0	CDKN1C	44	18	50	76	11	16	66	72
IGF2	38.9	19.0	LE	58.3	0.0	23.3	34.4	25.0	IGF2	72	79	7	36	17	43	122	776
IGF2R	51.1	41.6	45.8	59.4	54.2	49.7	47.5	41.5	IGF2R	223	173	194	180	306	316	547	695
INPP5F	3.4	11.2	2.7	1.6	21.0	24.6	46.5	20.3	INPP5F	5133	1013	589	733	2123	3055	6452	1132
PHLDA2	50.0	LE	LE	LE	LE	LE	LE	70.0	PHLDA2	14	3	6	6	3	4	6	10
SGCE	1.0	2.6	0.0	1.2	0.0	1.0	1.2	2.0	SGCE	186	77	265	254	119	198	257	350

Imprinted in all tissues analyzed

Tissue-specific imprinting

Kidney (% of the repressed allele)									Kidney (# of reads containing the SNPs used to ascribe the allelic expression)								
Control					LOS				Control					LOS			
#1	#2	#3	#4	#1	#2	#3	#4	#1	#2	#3	#4	#1	#2	#3	#4		
DIRAS3	1.5	0.0	0.5	0.0	3.0	3.5	51.3	41.2	DIRAS3	132	202	211	105	199	144	386	262
DLK1	0.2	0.0	0.3	0.1	0.2	0.1	0.1	0.1	DLK1	2391	2564	2672	2817	3352	3324	8785	3686
GNAS	1.1	0.8	1.3	0.9	1.7	1.2	1.0	1.2	GNAS	1184	1470	1351	1322	1295	1691	1240	1836
GTL2	1.0	0.7	0.5	0.5	1.1	0.6	1.6	1.2	GTL2	706	839	547	650	797	637	124	650
H19	3.3	3.3	3.5	3.6	3.8	3.8	3.8	3.8	H19	10705	13994	12275	16069	14507	13336	15148	15991
MAGEL2	0.0	0.0	0.0	0.0	10.2	3.9	8.7	35.6	MAGEL2	53	67	65	38	59	76	57	101
NAP1L5	0.0	13.0	0.0	2.4	25.8	6.5	6.5	15.5	NAP1L5	52	46	46	42	93	61	62	71
NNAT	0.9	5.3	2.2	2.5	7.3	0.6	43.1	41.8	NNAT	3390	19	185	276	41	326	65	491
PEG10	0.1	0.2	0.1	0.1	0.1	0.1	0.4	0.1	PEG10	4571	5922	4568	4776	5395	4591	972	4311
PEG3	0.1	0.4	0.3	0.4	0.6	13.6	52.4	47.6	PEG3	1755	1855	2048	1091	1316	1541	248	2195
PLAGL1	0.6	0.4	0.6	1.3	0.3	0.7	0.5	35.5	PLAGL1	1257	1547	1396	1076	1263	1225	425	2966
RTL1	2.1	0.0	4.5	7.5	NA	2.2	12.0	3.4	RTL1	48	18	44	40	NA	46	67	89
SNRPN	0.0	0.0	0.0	0.0	5.1	9.1	11.8	33.9	SNRPN	348	433	379	355	375	482	382	613
BEGAIN	0.0	7.4	3.0	5.9	3.6	0.0	21.3	1.8	BEGAIN	22	27	33	34	28	24	47	55
CDKN1C	3.1	3.9	1.7	4.3	6.3	4.1	3.5	8.5	CDKN1C	292	281	290	276	159	318	517	200
IGF2	0.2	0.1	0.2	0.1	0.4	0.1	0.1	0.1	IGF2	9227	11520	11107	11709	9578	10549	8825	10075
IGF2R	9.0	7.7	9.5	11.3	17.7	15.1	32.2	21.7	IGF2R	2674	3459	3002	2706	1754	1661	556	1601
INPP5F	36.5	36.5	2.0	3.4	49.7	38.6	50.6	13.4	INPP5F	356	389	50	29	541	485	259	67
PHLDA2	17.6	18.6	11.2	9.7	15.7	8.7	5.8	12.7	PHLDA2	262	306	259	421	210	392	1003	291
SGCE	0.2	0.2	0.0	0.0	0.6	0.0	2.1	0.9	SGCE	556	510	569	465	514	663	517	633

Imprinted in all tissues analyzed

Tissue-specific imprinting

Table S3. Allelic expression of the monoallelically expressed imprinted genes in ~d105 bovine fetuses.

Liver (% of the repressed allele)										Liver (# of reads containing the SNPs used to ascribe the allelic expression)										Imprinted in all tissues analyzed	
Control					LOS					Control					LOS						Tissue-specific imprinting
#1	#2	#3	#4	#1	#2	#3	#4	#1	#2	#3	#4	#1	#2	#3	#4						
DIRAS3	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND		
DLK1	0.2	1.4	0.5	0.5	0.4	0.3	0.0	0.1	4330	1898	4290	3919	3883	2570	4452	3875					
GNAS	1.1	3.7	1.0	0.6	2.1	0.7	0.7	1.7	807	815	894	837	807	568	1069	922					
GTL2	0.0	0.6	0.5	0.5	0.0	0.2	0.3	1.1	628	787	424	650	185	432	953	1008					
H19	3.6	1.8	5.8	6.2	5.5	7.1	5.8	5.4	7771	4854	7997	8778	6513	4520	22867	24216					
MAGEL2	0.0	0.0	0.0	0.0	3.8	3.8	19.6	23.5	44	23	11	43	26	26	56	34					
NAP1L5	0.0	4.3	0.0	0.0	21.9	5.4	27.0	3.0	240	116	251	175	333	204	392	305					
NNAT	0.1	0.1	0.9	0.3	1.0	0.4	34.8	19.5	12020	4039	9643	20024	2822	14834	7381	30639					
PEG10	0.0	0.0	0.1	0.1	0.2	0.3	0.1	0.1	15788	10197	15286	16663	7629	12360	17495	12801					
PEG3	0.1	0.0	0.1	0.1	1.1	0.1	14.1	12.5	1366	1232	1648	1430	179	1125	5778	4650					
PLAGL1	0.1	0.1	0.1	0.1	0.1	0.1	3.7	8.6	3864	3815	2727	2890	1660	2896	5092	6560					
RTL1	ND	ND	ND	ND	ND	ND	ND	ND	0	0	0	0	0	0	0	0					
SNRPN	0.0	0.0	0.0	0.0	7.9	4.5	18.7	30.1	296	125	272	294	302	262	417	418					
BEGAIN	13.3	0.0	21.1	13.3	LE	0.0	21.7	0.0	15	12	19	15	5	10	23	14					
CDKN1C	5.0	5.0	7.3	9.9	36.1	7.5	9.4	6.0	219	100	246	242	61	133	394	283					
IGF2	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.1	6849	6880	7332	7043	3167	4655	12894	11801					
IGF2R	14.2	13.6	14.8	14.0	42.0	23.2	27.4	21.7	2461	1693	2580	2357	295	1159	1842	2314					
INPP5F	32.4	37.4	2.0	3.4	50.3	35.5	44.1	19.0	278	99	18	23	260	214	383	90					
PHLDA2	5.0	LE	0.0	0.0	LE	LE	LE	21.1	20	4	17	15	1	8	5	19					
SGCE	25.2	33.1	24.0	30.1	36.2	24.1	26.8	16.3	856	293	890	730	828	605	761	576					

Muscle (% of the repressed allele)										Muscle (# of reads containing the SNPs used to ascribe the allelic expression)										Imprinted in all tissues analyzed	
Control					LOS					Control					LOS						Tissue-specific imprinting
#1	#2	#3	#4	#1	#2	#3	#4	#1	#2	#3	#4	#1	#2	#3	#4						
DIRAS3	ND	ND	ND	ND	ND	ND	ND	ND	0	0	0	0	0	0	0	0					
DLK1	0.4	1.0	0.3	0.2	0.5	0.4	0.1	0.1	5274	1868	4470	4261	3925	5722	7932	7287					
GNAS	0.4	2.8	0.4	0.6	1.0	0.2	1.4	1.6	1702	1185	1780	1350	1517	2408	3476	3336					
GTL2	0.2	0.2	0.5	0.3	0.5	0.2	0.4	0.3	3663	5394	3597	4144	1348	4016	5536	5248					
H19	3.8	4.5	4.6	5.7	4.7	4.4	4.4	5.3	8841	4663	8245	6604	7834	8613	32769	34196					
MAGEL2	0.0	0.0	0.0	4.2	10.3	10.9	24.7	36.1	50	31	50	47	97	73	89	119					
NAP1L5	0.0	2.5	0.0	0.0	19.3	10.0	14.1	16.2	59	39	64	48	62	100	71	74					
NNAT	6.3	3.4	4.0	13.5	31.0	2.6	45.7	45.7	288	59	273	297	116	699	341	1862					
PEG10	0.2	0.1	0.2	0.2	0.2	0.3	0.1	0.8	9361	7093	12023	11439	9742	11932	18427	12250					
PEG3	0.6	0.0	0.3	0.0	0.0	7.2	20.5	39.2	173	202	341	203	86	293	1457	1972					
PLAGL1	1.9	1.1	0.6	1.9	0.5	1.2	0.4	39.2	881	541	1056	619	854	1109	1700	4509					
RTL1	2.1	2.7	3.1	7.0	NA	2.8	5.4	1.4	47	37	64	57	NA	72	242	142					
SNRPN	0.0	0.0	0.0	0.0	18.6	11.4	21.3	41.6	234	101	269	205	397	525	684	730					
BEGAIN	0.0	0.0	0.0	5.3	3.3	3.2	24.5	10.8	33	15	21	19	30	31	57	92					
CDKN1C	5.9	4.2	6.0	5.3	4.7	2.7	4.2	11.7	603	237	547	436	360	563	970	468					
IGF2	0.5	0.5	0.6	0.5	0.4	0.2	0.4	1.0	9937	10007	9942	8423	8188	11964	25527	23919					
IGF2R	8.9	7.7	11.1	8.6	20.7	20.7	24.0	20.9	2109	1510	2537	1989	807	1599	1899	2887					
INPP5F	47.2	52.5	1.1	1.4	51.9	46.3	53.5	19.0	593	312	88	71	839	973	976	121					
PHLDA2	LE	LE	LE	ND	LE	LE	ND	LE	2	1	2	0	1	1	0	6					
SGCE	1.6	2.2	1.8	0.9	2.2	0.8	2.4	10.3	872	214	841	697	694	1197	970	1014					

Table S4 Transcript amount and allelic expression of the imprinted genes in d105 bovine fetuses																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																
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1	Brain	AIM1	ENSBTAG000000017527	14.92	13.55	10.35	27.33	16.54	8.77	11.33	11.84	16.02	0.53	0.69	0.72	0.97	0.41	0.90	0.73	0.97																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																												

Table S4 Transcript amount and allelic expression of the imprinted genes in d105 bovine fetuses																						
Gene #	Tissue	Gene Name	Weight → Fetus → Gene ID ↓	392g 404g 408g 416g				Mean	514g 518g 620g 714g				Fold Change				FDR (False Discovery Rate)				Allelic Exression in Control	
				Control #1	Control #2	Control #3	Control #4		LOS #1	LOS #2	LOS #3	LOS #4	LOS #1	LOS #2	LOS #3	LOS #4	LOS #1	LOS #2	LOS #3	LOS #4		
23	Brain	DIO3	ENSBTAG000000043578	8.23	6.76	9.40	7.42	7.95	7.64	7.43	27.82	9.94	0.96	0.93	3.50	1.25	1.00	1.00	0.00	0.66	NE	
	Kidney	DIO3																		NE		
	Liver	DIO3																		NE		
	Muscle	DIO3																		NE		
24	Brain	DIRAS3	ENSBTAG00000012182	676.74	254.81	459.65	688.40	519.90	209.89	146.21	221.82	283.04	0.40	0.28	0.43	0.54	0.01	0.00	0.03	0.17	Paternal	
	Kidney	DIRAS3	ENSBTAG00000012182	19.17	28.65	27.23	15.38	22.61	29.93	22.66	63.75	37.27	1.32	1.00	2.82	1.65	1.00	1.00	0.00	0.13		
	Liver	DIRAS3	ENSBTAG00000012182																	NE		
	Muscle	DIRAS3	ENSBTAG00000012182																	NE		
25	Brain	DLGAP2	100847272	17.61	32.88	32.78	24.15	26.85	42.33	27.31	37.75	31.39	1.58	1.02	1.41	1.17	0.38	1.00	0.57	0.93	NE	
	Kidney	DLGAP2	100847272																	NE		
	Liver	DLGAP2	100847272																	NE		
	Muscle	DLGAP2	100847272																	NE		
26	Brain	DLK1	ENSBTAG00000037899	13.34	12.85	4.61	14.13	11.23	2.10	2.17	2.88	38.61	0.19	0.19	0.26	3.44	0.05	0.11	0.18	0.08	Paternal	
	Kidney	DLK1	ENSBTAG00000037899	485.84	478.26	505.79	543.00	503.22	605.83	561.60	1929.80	714.54	1.20	1.12	3.83	1.42	0.76	1.00	0.00	0.01		
	Liver	DLK1	ENSBTAG00000037899	834.07	1091.22	893.59	808.18	906.76	979.05	702.84	1058.41	945.46	1.08	0.78	1.17	1.04	0.33	0.75	0.16	0.53		
	Muscle	DLK1	ENSBTAG00000037899	1367.87	1637.22	1103.25	1176.30	1321.16	1118.00	1263.38	1827.77	1521.74	0.85	0.96	1.38	1.15	0.73	1.00	0.06	0.32		
27	Brain	DLX5	ENSBTAG00000018645	0.94	9.70	13.07	0.99	6.17	3.33	3.79	10.17	6.99	0.54	0.61	1.65	1.13	0.85	1.00	0.72	0.96	NE	
	Kidney	DLX5	ENSBTAG00000018645																	NE		
	Liver	DLX5	ENSBTAG00000018645																	NE		
	Muscle	DLX5	ENSBTAG00000018645																	NE		
28	Brain	DNMT1	ENSBTAG00000002736	80.07	91.01	69.29	76.29	79.17	84.72	82.66	86.36	93.53	1.07	1.04	1.09	1.18	0.90	1.00	0.86	0.91	Biallelic	
	Kidney	DNMT1	ENSBTAG00000002736	98.62	94.18	100.19	92.28	96.32	95.58	90.64	69.96	94.35	0.99	0.94	0.73	0.98	1.00	1.00	0.00	0.97		
	Liver	DNMT1	ENSBTAG00000002736	113.14	125.51	110.20	108.23	114.27	78.12	126.09	135.13	131.78	0.68	1.10	1.18	1.15	0.00	0.79	0.10	0.13		
	Muscle	DNMT1	ENSBTAG00000002736	69.85	91.31	67.66	71.37	75.05	60.76	79.17	100.73	107.51	0.81	1.05	1.34	1.43	0.45	0.99	0.01	0.00		
29	Brain	DRD1A	ENSBTAG00000047719	7.28	13.51	35.83	13.65	17.57	73.37	13.81	30.15	13.98	4.18	0.79	1.72	0.80	0.03	0.98	0.70	0.92	NE	
	Kidney	DRD1A	ENSBTAG00000047719																	NE		
	Liver	DRD1A	ENSBTAG00000047719																	NE		
	Muscle	DRD1A	ENSBTAG00000047719																	NE		
30	Brain	FBXO40	ENSBTAG00000014508																	NE		
	Kidney	FBXO40	ENSBTAG00000014508	28.13	23.55	25.98	28.77	26.61	29.96	25.77	9.72	28.68	1.13	0.97	0.37	1.08	1.00	1.00	0.00	0.85		
	Liver	FBXO40	ENSBTAG00000014508																	NE		
	Muscle	FBXO40	ENSBTAG00000014508	4.01	3.72	3.22	3.48	3.61	1.08	0.84	1.32	1.61	0.30	0.23	0.37	0.45	0.00	0.00	0.02	0.02		
31	Brain	GATM	ENSBTAG00000005586	191.12	91.15	108.32	101.40	123.00	123.99	119.18	81.65	70.08	1.01	0.97	0.66	0.57	0.91	1.00	0.46	0.38	Biallelic	
	Kidney	GATM	ENSBTAG00000005586	406.54	375.18	380.69	347.31	377.43	450.09	331.96	219.69	466.10	1.19	0.88	0.58	1.23	0.86	1.00	0.00	0.24		
	Liver	GATM	ENSBTAG00000005586	1228.00	943.43	1081.66	1223.62	1119.18	1834.72	1426.25	1036.80	1110.94	1.64	1.27	0.93	0.99	0.00	0.72	0.59	0.84		
	Muscle	GATM	ENSBTAG00000005586	17.66	11.15	17.34	14.72	15.22	14.48	14.11	12.67	7.27	0.95	0.93	0.83	0.48	0.61	1.00	0.32	0.00		
32	Brain	GDAP1L1	ENSBTAG00000002593	53.31	63.78	64.79	63.36	61.31	96.71	75.97	78.22	64.26	1.58	1.24	1.28	1.05	0.32	0.97	0.70	0.99	Biallelic	
	Kidney	GDAP1L1	ENSBTAG00000002593																	NE		
	Liver	GDAP1L1	ENSBTAG00000002593																	NE		
	Muscle	GDAP1L1	ENSBTAG00000002593																	NE		
33	Brain	GLIS3	ENSBTAG00000014467	10.17	6.72	2.69	5.51	6.27	3.12	3.64	3.19	7.65	0.50	0.58	0.51	1.22	0.58	0.94	0.65	0.96	NE	
	Kidney	GLIS3	ENSBTAG00000014467	12.02	14.39	10.06	11.77	12.06	11.93	10.48	1.89	8.80	0.99	0.87	0.16	0.73	1.00	1.00	0.00	0.49		
	Liver	GLIS3	ENSBTAG00000014467																	NE		
	Muscle	GLIS3	ENSBTAG00000014467	1.70	3.63	2.92	2.10	2.59	2.86	2.43	3.58	5.60	1.10	0.94	1.39	2.17	0.63	1.00	0.28	0.01		
34	Brain	GNAS	ENSBTAG00000017475	2080.31	1323.53	1991.16	2068.06	1865.77	1859.84	1440.89	1221.72	1465.72	1.00	0.77	0.65	0.79	0.88	0.83	0.43	0.77	Maternal	
	Kidney	GNAS	ENSBTAG00000017475	2550.90	2332.40	2537.77	2283.96	2426.26	2584.77	2843.09	4547.49	3119.53	1.07	1.08	1.87	1.29	1.00	1.00	0.00	0.12		
	Liver	GNAS	ENSBTAG00000017475	1038.77	1078.07	1102.19	1040.60	1064.91	1600.39	924.31	1103.26	985.37	1.50	0.87	0.14	0.93	0.00	0.92	0.86	0.77		
	Muscle	GNAS	ENSBTAG00000017475	3009.67	2521.03	2774.96	2665.60	2742.81	3504.88	3111.15	3115.71	3088.79	1.28	1.13	1.14	1.13	0.29	1.00	0.73	0.78		
35	Brain	GPR1	ENSBTAG00000017043	0.49	0.83	0.58	0.86	0.69	0.22	2.01	0.81	14.70	0.31	2.92	1.18	21.35	0.41	0.32	0.90	0.00	NE	
	Kidney	GPR1	ENSBTAG00000017043																	NE		
	Liver	GPR1	ENSBTAG00000017043																	NE		
	Muscle	GPR1	ENSBTAG00000017043																	NE		
36	Brain	GRB10	ENSBTAG00000017086	18.26	23.73	21.31	30.48	23.44	35.82	25.61	35.73	31.86	1.53	1.09	1.52	1.36	0.54	1.00	0.56	0.88	Biallelic	
	Kidney	GRB10	ENSBTAG00000017086	28.97	29.07	28.64	29.58	29.06	33.21	27.91	36.47	32.30	1.14	0.96	1.25	1.11	1.00	1.00	0.32	0.72		
	Liver	GRB10	ENSBTAG00000017086	37.43	42.72	41.67	38.32	40.04	42.65	41.71	49.72	47.59	1.07	1.04	1.24	1.19	0.63	1.00	0.12	0.20		
	Muscle	GRB10	ENSBTAG00000017086	37.64	40.81	32.24	38.09	37.20	30.97	35.12	44.87	46.06	0.83	0.94	1.21	1.24	0.48	1.00	0.25	0.18		
37	Brain	GTL2	100335527	2942.28	3185.48	2997.76	2675.69	2950.30	1974.43	2183.02	1977.22	2706.53	0.67	0.74	0.67	0.92	0.50	0.93	0.63	0.97	Maternal	
	Kidney	GTL2	100335527	2743.86	2951.02	2688.35	2852.13	2808.79	3404.54	2508.46	1347.47	3304.71	1.21	0.86	0.48	1.18	0.53	1.00	0.00	0.40		
	Liver	GTL2	100335527	1737.81	2498.12	1587.00	1992.35	1953.82	728.59	1696.79	1926.90	2177.65	0.37	0.87	0.99	1.11	0.00	1.00	0.74	0.32		
	Muscle	GTL2	100335527	8618.49	12651.22	7314.21	10408.91	9748.21	3701.30	7428.26	6869.11	8211.05	0.38	0.76	0.70	0.84	0.00	1.00	0.54	0.80		
38	Brain	H13	ENSBTAG00000008840	40.10	37.38	28.97	33.66	35.03	34.59	32.18	33.92	39.52	0.99	0.92	0.97	1.13	0.99	1.00	0.99	0.96	NE	
	Kidney	H13	ENSBTAG00000008840	57.14	57.48	58.03	65.23	59.47	57.38	62.91	92.60	63.22	0.96	1.06	1.86	1.06	1.00	1.00	0.00	0.85		
	Liver	H13	ENSBTAG00000008840	47.37	57.02	42.53	46.39	48.33	64.86	39.19	56.86	56.44	1.34	0.81	1.18	1.17	0.00	0.90	0.14	0.13		
	Muscle	H13	ENSBTAG00000008840	38.30	43.42	35.29	33.85	31.71	39.64	41.03	50.35	51.18	1.05	0.99	1.34	1.36	0.62	1.00	0.04	0.03		
39	Brain	H19	100126192	41.91	77.74	18.75	29.50	43.97	17.94	24.10	48.16	118.40	0.43	0.57	1.15	2.82	0.42	0.97	0.50	0.00	Maternal	
	Kidney	H19	100126192	13054.53	12685.42	13964.67	16180.77	13971.35	13330.67	13092.79	21343.08	13265.92	0.97	0.94	1.53	0.95	1.00	1.00	0.00	0.91		
	Liver	H19	1001																			

Table S4 Transcript amount and allelic expression of the imprinted genes in d105 bovine fetuses																				
Gene #	Tissue	Gene Name	Weight -- Fetus Gene ID	392g 404g 408g 416g				Mean	514g 516g 520g 714g				Fold Change				FDR (False Discovery Rate)			
				Control #1	Control #2	Control #3	Control #4		LOS #1	LOS #2	LOS #3	LOS #4	LOS #1	LOS #2	LOS #3	LOS #4	LOS #1	LOS #2	LOS #3	LOS #4
	Kidney	INPP5F	ENSBTAG000000021801	64.64	63.75	63.79	54.91	61.77	77.16	69.77	48.71	69.68	1.25	1.13	0.79	1.13	0.63	1.00	0.01	0.64
	Liver	INPP5F	ENSBTAG000000021801	35.91	28.54	35.22	36.22	33.97	43.09	40.84	39.78	38.98	1.27	1.20	1.17	1.15	0.19	0.91	0.57	0.62
	Muscle	INPP5F	ENSBTAG000000021801	120.81	118.40	149.23	131.34	129.94	168.55	143.70	114.48	109.12	1.30	1.11	0.88	0.84	0.18	1.00	0.44	0.14
46	Brain	JADE1	ENSBTAG000000017493	40.42	54.53	41.96	35.37	43.07	94.24	55.43	81.67	57.09	2.19	1.29	1.90	1.33	0.02	0.85	0.07	0.76
	Kidney	JADE1	ENSBTAG000000017493	55.26	63.07	52.92	52.52	55.94	58.15	51.08	22.28	51.11	1.04	0.91	0.40	0.91	1.00	1.00	0.00	0.79
	Liver	JADE1	ENSBTAG000000017493	27.65	28.77	23.98	24.81	26.31	15.11	28.26	30.80	26.69	0.57	1.07	1.17	1.01	0.00	0.99	0.32	0.82
	Muscle	JADE1	ENSBTAG000000017493	50.96	76.00	52.81	59.77	59.88	32.25	52.50	64.10	62.52	0.54	0.88	1.07	1.04	0.00	1.00	0.37	0.49
47	Brain	KCNK9	ENSBTAG000000047488	11.83	13.41	6.82	6.98	9.76	22.81	13.38	27.20	16.27	2.34	1.37	2.79	1.67	0.10	0.90	0.03	0.66
	Kidney	KCNK9	ENSBTAG000000047488																	
	Liver	KCNK9	ENSBTAG000000047488																	
	Muscle	KCNK9	ENSBTAG000000047488																	
48	Brain	KCNQ1	ENSBTAG000000010986	15.94	11.88	6.72	7.83	10.59	6.56	6.81	8.60	12.60	0.62	0.64	0.81	1.19	0.68	0.95	0.92	0.96
	Kidney	KCNQ1	ENSBTAG000000010986	21.32	27.15	19.20	18.38	21.51	23.75	15.45	12.65	28.61	1.10	0.72	0.59	1.33	1.00	1.00	0.00	0.37
	Liver	KCNQ1	ENSBTAG000000010986	6.51	14.09	3.92	5.69	7.55	3.17	5.34	8.28	8.13	0.42	0.71	1.10	1.08	0.15	1.00	0.24	0.23
	Muscle	KCNQ1	ENSBTAG000000010986	34.24	47.85	27.10	35.12	36.08	16.35	23.01	34.70	69.03	0.45	0.64	0.96	1.91	0.00	0.59	0.75	0.00
49	Brain	KLF14	785436																	
	Kidney	KLF14	785436	3.79	2.63	2.92	3.64	3.24	2.55	3.05	3.47	1.07	0.78	0.94	1.07	0.33	1.00	1.00	0.96	0.03
	Liver	KLF14	785436																	
	Muscle	KLF14	785436	5.76	4.51	2.79	4.60	4.42	5.22	4.19	4.29	2.86	1.18	0.95	0.97	0.65	0.73	1.00	0.99	0.30
50	Brain	L3MBTL1	529298	39.61	54.98	43.09	41.40	44.77	47.28	37.87	38.89	40.61	1.06	0.85	0.87	0.91	0.89	1.00	0.95	0.98
	Kidney	L3MBTL1	529298	13.28	16.60	16.42	16.39	15.67	16.67	12.62	12.47	16.70	1.06	0.81	0.80	1.07	1.00	1.00	0.09	0.91
	Liver	L3MBTL1	529298	6.14	8.87	7.61	7.37	7.49	2.20	5.42	5.79	7.91	0.29	0.72	0.77	1.06	0.00	0.94	0.67	0.76
	Muscle	L3MBTL1	529298	16.15	20.09	12.37	13.75	15.59	6.35	11.89	14.62	21.10	0.41	0.76	0.94	1.35	0.00	1.00	0.90	0.05
51	Brain	LIN28B	ENSBTAG000000043973	7.16	4.16	5.23	9.75	6.57	1.94	1.70	2.12	2.60	0.29	0.26	0.32	0.40	0.13	0.16	0.20	0.45
	Kidney	LIN28B	ENSBTAG000000043973																	
	Liver	LIN28B	ENSBTAG000000043973	32.61	22.49	27.11	29.34	27.89	26.83	25.37	19.04	23.44	0.96	0.91	0.68	0.84	0.58	0.92	0.01	0.22
	Muscle	LIN28B	ENSBTAG000000043973																	
52	Brain	LRRTM1	ENSBTAG000000002967	24.89	33.47	27.15	33.80	29.83	49.43	28.93	28.46	30.38	1.66	0.97	0.95	1.02	0.29	1.00	1.00	0.99
	Kidney	LRRTM1	ENSBTAG000000002967																	
	Liver	LRRTM1	ENSBTAG000000002967																	
	Muscle	LRRTM1	ENSBTAG000000002967																	
53	Brain	MAGEL2	ENSBTAG000000045998	34.24	29.90	31.87	45.23	35.31	23.56	19.46	27.08	42.20	0.67	0.55	0.77	1.20	0.43	0.38	0.69	0.96
	Kidney	MAGEL2	ENSBTAG000000045998	19.94	22.02	16.19	18.35	19.12	16.29	23.98	28.32	32.27	0.85	1.25	1.48	1.69	1.00	1.00	0.07	0.01
	Liver	MAGEL2	ENSBTAG000000045998	8.97	9.34	5.67	9.08	8.26	5.24	7.12	13.10	12.69	0.63	0.86	1.59	1.54	0.23	1.00	0.11	0.10
	Muscle	MAGEL2	ENSBTAG000000045998	17.66	20.85	15.12	17.13	17.69	24.67	16.02	24.19	34.37	1.39	1.02	1.37	1.94	0.04	1.00	0.07	0.00
54	Brain	MAGI2	100298336	51.52	47.46	42.54	46.32	46.96	117.37	67.19	67.33	59.42	2.50	1.43	1.43	1.27	0.01	0.82	0.54	0.90
	Kidney	MAGI2	100298336	20.63	21.89	20.08	19.50	20.52	20.44	17.21	4.28	16.84	1.00	0.84	0.21	0.82	1.00	1.00	0.00	0.56
	Liver	MAGI2	100298336																	
	Muscle	MAGI2	100298336																	
55	Brain	MEST	ENSBTAG000000017223	118.33	147.79	109.91	109.68	121.43	98.22	91.71	101.39	108.28	0.81	0.76	0.83	0.89	0.81	0.94	0.90	0.97
	Kidney	MEST	ENSBTAG000000017223	213.51	233.19	194.26	183.92	206.20	238.63	227.88	218.52	332.13	1.16	1.11	1.06	1.61	1.00	1.00	0.76	0.90
	Liver	MEST	ENSBTAG000000017223	22.11	20.05	20.46	19.35	20.49	31.51	18.12	19.48	42.14	1.54	0.88	0.95	2.06	0.00	0.96	0.86	0.00
	Muscle	MEST	ENSBTAG000000017223	1136.59	969.63	1173.48	1205.39	1121.27	1308.81	1385.65	931.59	1229.94	1.17	1.24	0.83	1.10	0.59	0.97	0.33	0.90
56	Brain	MKRN3	517464	14.31	10.46	17.68	16.59	14.76	14.46	12.53	16.69	14.64	1.01	1.20	0.94	0.88	0.90	0.97	0.98	0.96
	Kidney	MKRN3	517464																	
	Liver	MKRN3	517464																	
	Muscle	MKRN3	517464																	
57	Brain	MST1R	ENSBTAG000000015046	0.37	3.40	2.29	0.89	1.73	2.53	1.74	1.57	2.17	1.46	1.00	0.91	1.25	0.57	0.98	0.89	0.84
	Kidney	MST1R	ENSBTAG000000015046	3.75	2.73	3.60	3.95	3.51	3.87	3.30	5.27	5.04	1.10	0.94	1.50	1.44	1.00	1.00	0.29	0.52
	Liver	MST1R	ENSBTAG000000015046	1.43	2.20	1.75	1.23	1.95	0.48	1.32	1.73	1.43	0.29	0.80	1.05	0.86	0.02	1.00	0.81	0.98
	Muscle	MST1R	ENSBTAG000000015046																	
58	Brain	NAA60	ENSBTAG000000004875	66.16	59.55	60.33	62.44	62.12	74.34	60.73	64.12	65.48	1.20	0.98	1.03	1.05	0.83	1.00	0.99	1.00
	Kidney	NAA60	ENSBTAG000000004875	20.96	28.45	28.05	31.81	28.35	29.79	31.57	40.07	32.34	1.02	1.08	1.37	1.11	1.00	1.00	0.04	0.76
	Liver	NAA60	ENSBTAG000000004875	19.87	23.37	19.87	20.13	20.81	14.89	18.66	25.69	23.95	0.72	0.90	1.23	1.15	0.05	1.00	0.14	0.29
	Muscle	NAA60	ENSBTAG000000004875	34.48	31.60	26.58	29.35	30.50	29.74	36.24	43.03	38.39	0.98	1.19	1.41	1.26	0.97	0.95	0.07	0.25
59	Brain	NAP1L4	ENSBTAG000000022160	145.90	135.46	150.93	152.58	146.22	221.67	187.02	153.10	129.37	1.52	1.28	1.05	0.88	0.41	0.96	0.98	0.91
	Kidney	NAP1L4	ENSBTAG000000022160	114.39	118.41	119.52	122.50	118.81	113.72	121.16	163.15	131.86	0.96	1.02	1.37	1.11	1.00	1.00	0.04	0.66
	Liver	NAP1L4	ENSBTAG000000022160	111.30	99.56	101.96	114.43	106.81	113.24	119.17	102.70	107.55	1.06	1.12	0.96	1.01	0.82	0.97	0.78	0.97
	Muscle	NAP1L4	ENSBTAG000000022160	158.73	147.17	158.90	137.07	150.47	156.19	174.63	150.81	144.25	1.04	1.16	1.00	0.96	0.89	0.96	0.99	0.69
60	Brain	NAP1L5	ENSBTAG000000010128	218.53	50.34	74.38	156.82	125.02	15.44	18.41	21.72	50.73	0.12	0.15	0.17	0.41	0.00	0.00	0.00	0.12
	Kidney	NAP1L5	ENSBTAG000000010128	6.08	5.10	4.55	4.01	4.94	9.07	6.48	7.92	8.90	1.84	1.31	1.60	1.80	0.27	1.00	0.16	0.13
	Liver	NAP1L5	ENSBTAG000000010128	20.65	15.82	22.11	19.84	19.80	35.25	24.04	24.90	21.26	1.80	1.23	1.27	1.08	0.00	0.90	0.38	0.92
	Mus																			

Table S4 Transcript amount and allelic expression of the imprinted genes in d105 bovine fetuses																																				
Gene #	Tissue	Gene Name	Weight -- Fetus -- Gene ID	392g				404g				408g				416g				514g				518g				620g				714g				Allelic Expression in Control
				Control #1	Control #2	Control #3	Control #4	Mean	LOS #1	LOS #2	LOS #3	LOS #4	Control #1	Control #2	Control #3	Control #4	Mean	LOS #1	LOS #2	LOS #3	LOS #4	Control #1	Control #2	Control #3	Control #4	Mean	LOS #1	LOS #2	LOS #3	LOS #4	Control #1	Control #2	Control #3	Control #4	FDR (False Discovery Rate)	
68	Liver	PEG10	ENSBTAG00000038093	1625.91	1897.99	1532.44	1528.99	1646.33	649.28	1581.88	1868.60	1608.91	649.28	1581.88	1868.60	1608.91	0.39	0.96	1.14	0.98	0.00	1.00	0.35	0.89	0.63	0.78	1.38	1.07	0.07	1.00	0.09	0.62	Paternal			
	Muscle	PEG10	ENSBTAG00000038093	1225.38	1988.17	1701.77	1572.22	1621.89	1014.20	1259.79	2231.20	1727.38	0.63	0.78	1.38	1.07	0.07	1.00	0.09	0.62	Paternal															
	Brain	PEG3	444864	612.49	583.69	513.81	618.23	582.05	466.47	353.66	553.70	945.75	0.80	0.61	0.95	1.62	0.70	0.48	0.96	0.55	Paternal															
	Kidney	PEG3	444864	357.10	3799.82	3347.71	3016.32	3420.24	3140.71	3248.56	4049.62	4469.42	0.92	0.95	1.18	1.31	1.00	1.00	0.59	0.13	Paternal															
69	Liver	PEG3	444864	3104.77	4228.20	3177.00	3333.73	3460.92	1700.85	2859.57	4242.07	3987.01	0.49	0.83	1.28	1.15	0.00	0.99	0.08	0.29	Paternal															
	Muscle	PEG3	444864	625.59	880.22	632.82	688.56	706.80	583.20	613.41	1180.10	1588.45	0.83	0.87	1.61	2.25	0.58	1.00	0.00	0.00	Paternal															
	Brain	PHACTR2	ENSBTAG00000016498	86.78	49.16	58.04	74.85	67.21	31.95	38.68	35.89	72.60	0.48	0.58	0.53	1.08	0.05	0.30	0.16	0.99	Biallelic															
	Kidney	PHACTR2	ENSBTAG00000016498	208.34	207.99	214.45	175.39	201.54	242.75	182.12	85.90	139.46	1.20	0.90	0.43	0.69	0.84	1.00	0.00	0.02	Biallelic															
70	Liver	PHACTR2	ENSBTAG00000016498	19.81	21.05	20.72	23.10	21.17	17.54	19.32	22.59	18.44	0.83	0.91	1.09	0.87	0.26	1.00	0.73	0.54	Biallelic															
	Muscle	PHACTR2	ENSBTAG00000016498	104.14	101.49	138.34	141.06	121.26	180.56	125.68	127.39	152.46	1.49	1.04	1.05	1.26	0.05	1.00	0.98	0.36	Biallelic															
	Brain	PHLDA2	ENSBTAG000000031194	43.21	42.74	50.00	59.84	48.95	27.38	44.97	150.95	41.41	0.56	0.92	3.08	0.85	0.01	1.00	0.00	0.63	NE															
	Kidney	PHLDA2	ENSBTAG000000031194	1.78	2.29	2.24	1.94	2.06	0.31	1.49	1.04	2.26	0.15	0.72	0.50	1.10	0.00	0.99	0.22	0.81	NE															
71	Liver	PHLDA2	ENSBTAG000000031194	21.10	22.07	27.59	23.71	23.62	15.33	16.59	21.39	25.00	0.65	0.70	0.91	1.06	0.47	0.85	0.91	1.00	Paternal															
	Brain	PLAGL1	ENSBTAG00000026523	470.11	431.33	527.64	504.18	483.32	589.16	460.04	365.83	794.09	1.22	0.95	0.76	1.64	0.78	1.00	0.00	0.00	Paternal															
	Kidney	PLAGL1	ENSBTAG00000026523	860.67	914.60	945.27	929.01	912.39	838.13	1056.23	960.16	976.15	0.92	1.16	1.05	1.07	0.60	0.86	0.81	0.71	Paternal															
	Muscle	PLAGL1	ENSBTAG00000026523	352.32	333.15	420.19	400.83	376.62	526.53	386.30	368.13	712.79	1.40	1.03	0.98	1.89	0.05	1.00	0.83	0.00	Paternal															
72	Brain	PON2	ENSBTAG00000008361	70.84	36.55	43.67	58.91	52.49	12.96	17.56	15.01	26.36	0.25	0.33	0.29	0.50	0.00	0.02	0.00	0.22	Biallelic															
	Kidney	PON2	ENSBTAG00000008361	43.74	45.79	44.43	45.23	44.80	41.44	50.16	56.86	46.00	0.93	1.12	1.27	1.03	1.00	1.00	0.24	0.95	Biallelic															
	Liver	PON2	ENSBTAG00000008361	76.11	64.33	80.75	79.06	75.06	115.93	80.24	66.38	72.15	1.54	1.07	0.88	0.96	0.00	1.00	0.31	0.66	Biallelic															
	Muscle	PON2	ENSBTAG00000008361	89.40	55.68	86.53	85.94	79.39	104.54	93.21	66.53	67.08	1.32	1.17	0.84	0.84	0.27	1.00	0.12	0.05	Biallelic															
73	Brain	PON3	ENSBTAG00000034645	89.40	55.68	86.53	85.94	79.39	104.54	93.21	66.53	67.08	1.32	1.17	0.84	0.84	0.27	1.00	0.12	0.05	NE															
	Kidney	PON3	ENSBTAG00000034645	1.38	0.91	4.26	0.24	1.70	0.87	4.50	3.06	1.73	0.51	2.65	1.81	1.02	1.00	1.00	0.46	0.98	NA															
	Liver	PON3	ENSBTAG00000034645	5.08	4.49	39.47	7.72	14.19	12.38	7.99	21.41	20.65	0.87	0.56	1.51	1.46	0.69	0.88	0.86	0.87	Biallelic															
	Muscle	PON3	ENSBTAG00000034645	4.68	3.23	1.39	3.68	3.25	4.44	7.74	0.87	10.92	0.14	2.39	0.27	3.36	0.00	0.38	0.64	0.00	Biallelic															
74	Brain	PPP1R9A	ENSBTAG00000024426	252.12	259.73	272.02	291.55	268.86	311.55	285.31	284.55	271.60	1.16	1.06	1.06	1.01	0.87	1.00	0.96	0.99	Biallelic															
	Kidney	PPP1R9A	ENSBTAG00000024426	157.79	179.89	158.19	156.67	163.13	147.03	139.29	73.79	120.44	0.90	0.85	0.45	0.74	1.00	1.00	0.00	0.05	Biallelic															
	Liver	PPP1R9A	ENSBTAG00000024426	40.45	49.59	46.22	36.06	43.08	26.92	39.48	35.08	32.19	0.62	0.92	0.81	0.75	0.01	1.00	0.51	0.22	Biallelic															
	Muscle	PPP1R9A	ENSBTAG00000024426	234.87	307.48	308.65	316.98	292.00	221.04	233.35	252.65	240.67	0.76	0.80	0.87	0.82	0.17	0.91	0.61	0.28	Biallelic															
75	Brain	QPCT	ENSBTAG00000013923	36.19	11.85	30.60	35.07	28.43	17.43	15.09	8.79	10.62	0.61	0.53	0.31	0.37	0.21	0.19	0.01	0.04	Biallelic															
	Kidney	QPCT	ENSBTAG00000013923	18.84	10.53	15.16	16.62	15.29	17.78	27.55	9.38	5.80	1.16	1.80	0.61	0.38	0.86	0.06	0.02	0.00	NE															
	Liver	QPCT	ENSBTAG00000013923	68.36	70.81	79.02	96.85	78.76	34.26	37.79	25.41	70.99	0.44	0.48	0.32	0.90	0.03	0.13	0.01	0.93	NE															
	Muscle	QPCT	ENSBTAG00000013923	18.84	10.53	15.16	16.62	15.29	17.78	27.55	9.38	5.80	1.16	1.80	0.61	0.38	0.86	0.06	0.02	0.00	Biallelic															
76	Brain	RASGRF1	ENSBTAG00000019940	5.78	6.14	6.45	6.51	6.22	4.19	5.10	4.46	7.14	0.67	0.82	0.72	1.15	1.00	1.00	0.11	0.83	NE															
	Kidney	RASGRF1	ENSBTAG00000019940	47.37	55.67	59.86	46.29	52.30	56.43	49.43	54.38	43.70	1.08	0.95	1.04	0.84	0.92	1.00	0.95	0.92	NE															
	Liver	RASGRF1	ENSBTAG00000019940	14.01	14.81	14.58	14.03	14.36	19.39	15.26	9.27	16.12	1.35	1.06	0.65	1.12	0.69	1.00	0.00	0.79	NE															
	Muscle	RASGRF1	ENSBTAG00000019940	55.81	49.91	57.98	54.76	54.62	55.34	62.73	55.13	51.53	1.01	1.15	1.01	0.94	0.93	0.94	0.97	0.68	Biallelic															
77	Brain	RB1	ENSBTAG00000006640	39.44	46.08	42.79	44.63	43.23	45.20	35.85	34.62	36.72	1.05	0.83	0.80	0.85	0.75	0.99	0.34	0.35	Biallelic															
	Kidney	RB1	ENSBTAG00000006640	14.01	14.81	14.58	14.03	14.36	19.39	15.26	9.27	16.12	1.35	1.06	0.65	1.12	0.69	1.00	0.00	0.79	Biallelic															
	Liver	RB1	ENSBTAG00000006640	55.81	49.91	57.98	54.76	54.62	55.34	62.73	55.13	51.53	1.01	1.15	1.01	0.94	0.93	0.94	0.97	0.68	Biallelic															
	Muscle	RB1	ENSBTAG00000006640	39.44	46.08	42.79	44.63	43.23	45.20	35.85	34.62	36.72	1.05	0.83	0.80	0.85	0.75	0.99	0.34	0.35	Biallelic															
78	Brain	RBP5	ENSBTAG00000008127	6.17	5.99	5.77	9.01	6.73	6.65	3.56	5.96	8.05	0.99	0.53	0.88	1.20	0.92	0.32	0.78	0.74	NE															
	Kidney	RBP5	ENSBTAG00000008127	17.11	20.59	13.53	32.31	20.89	18.38	13.63	36.42	21.53	0.88	0.65	1.74	1.03	1.00	1.00	0.07	0.97	Biallelic															
	Liver	RBP5	ENSBTAG00000008127	6.17	5.99	5.77	9.01	6.73	6.65	3.56	5.96	8.05	0.99	0.53	0.88	1.20	0.92	0.32	0.78	0.74	Biallelic															
	Muscle	RBP5	ENSBTAG00000008127	6.17	5.99	5.77	9.01	6.73	6.65	3.56	5.96	8.05	0.99	0.53	0.88	1.20	0.92	0.32	0.78	0.74	NE															
79	Brain	RTL1	ENSBTAG00000046585	6.59	3.26	2.76	4.00	4.15	0.27	0.43	0.36	2.54	0.06	0.10	0.09	0.61	0.01	0.03	0.01	0.82	LE															
	Kidney	RTL1	ENSBTAG00000046585	62.07	46.90	38.79	41.55	47.33	54.59	41.10	65.68	69.57	1.15	0.87	1.39	1.47	1.00	1.00	0.18	0.13	Paternal															
	Liver	RTL1	ENSBTAG00000046585	3.08	7.72	2.67	2.65	4.03	1.85	1.49	13.62	4.55	0.46	0.37	3.38	1.13	0.32	0.56	0.00	0.19	Paternal															
	Muscle	RTL1	ENSBTAG00000046585	97.86	147.74	95.68	92.38	108.42	57.41	74.95	127.44	86.34	0.53	0.69	1.18	0.80	0.00	0.63	0.049	0.46	Paternal															
80	Brain	SCIN	ENSBTAG00000009786	2.03	1.73	1.38	3.08	2.06	0.70	2.05	2.00	3.03	0.34	1.00	0.97	1.47	0.30	1.00	0.97	0.93	NA															
	Kidney	SCIN	ENSBTAG00000009786	55.99	53.56	60.42	67.02	59.25	60.70	65.43	66.13	59.12	1.02	1.10	1.12	1.00	1.00	1.00	0.95	1.00	NE															
	Liver	SCIN	ENSBTAG00000009786	2.03	3.23	4.01	5.21	3.62	3.00																											

Table S4 Transcript amount and allelic expression of the imprinted genes in d105 bovine fetues

Gene #	Tissue	Gene Name	Gene ID	Weight → Fetus →				Mean	416g				514g	518g	620g	714g	Fold Change				FDR (False Discovery Rate)				Allelic Exsrssion in Control
				Control #1	Control #2	Control #3	Control #4		LOS #1	LOS #2	LOS #3	LOS #4					LOS #1	LOS #2	LOS #3	LOS #4	LOS #1	LOS #2	LOS #3	LOS #4	
Tissue			Gene	read counts/million	read counts/million	read counts/million	read counts/million	read counts/million	read counts/million	read counts/million	read counts/million	read counts/million	read counts/million	read counts/million	read counts/million	read counts/million	read counts/million	read counts/million	read counts/million	read counts/million	read counts/million	read counts/million	read counts/million		
90	Brain	TCEB3	ENSBTAG000000026585	38.87	38.29	45.79	46.42	42.34	54.41	43.60	37.86	48.72	1.29	1.03	0.89	1.15	0.21	1.00	0.55	0.63					
	Kidney	TCEB3	ENSBTAG000000026585	80.72	112.35	87.08	69.59	87.43	108.98	110.86	126.90	126.96	1.25	1.27	1.45	1.45	0.57	0.82	0.32	0.55					
	Liver	TCEB3	ENSBTAG000000026585	129.20	132.90	120.05	110.36	123.13	125.65	118.05	82.97	123.00	1.02	0.96	0.67	1.00	1.00	1.00	0.00	1.00					
	Muscle	TCEB3	ENSBTAG000000026585	72.69	72.37	67.80	69.95	70.70	45.83	64.84	75.78	85.25	0.65	0.92	1.07	1.21	0.00	1.00	0.65	0.14					
91	Brain	TFPI2	ENSBTAG000000015844	86.81	115.21	93.81	91.87	96.92	85.19	90.92	110.45	133.59	0.88	0.94	1.14	1.38	0.75	1.00	0.21	0.00					
	Kidney	TFPI2	ENSBTAG000000015844	46.38	49.66	30.34	33.93	40.08	31.64	58.10	36.92	26.26	0.79	1.45	0.92	0.66	1.00	0.69	0.36	0.20					
	Liver	TFPI2	ENSBTAG000000015844	42.26	28.74	56.01	47.59	43.65	53.71	42.66	34.36	26.07	1.23	0.98	0.79	0.60	0.60	0.97	0.07	0.00					
	Muscle	TFPI2	ENSBTAG000000015844	31.17	24.74	28.76	62.37	36.76	45.89	46.59	34.15	19.01	1.25	1.27	0.93	0.52	0.77	1.00	0.72	0.01					
92	Brain	TH	ENSBTAG000000026768																						
	Kidney	TH	ENSBTAG000000026768																						
	Liver	TH	ENSBTAG000000026768	1.03	3.11	1.58	3.17	2.22	1.28	3.19	2.15	1.62	0.58	1.43	0.97	0.73	0.51	0.88	0.91	0.85					
	Muscle	TH	ENSBTAG000000026768																						
93	Brain	TNFRSF23	ENSBTAG0000000039129	7.16	6.44	3.88	2.67	5.04	3.01	3.95	5.22	5.05	0.60	0.78	1.04	1.00	0.76	1.00	0.94	0.99					
	Kidney	TNFRSF23	ENSBTAG0000000039129	3.33	2.99	3.08	2.56	2.99	2.48	2.61	1.76	2.69	0.83	0.87	0.59	0.90	1.00	1.00	0.07	0.93					
	Liver	TNFRSF23	ENSBTAG0000000039129	5.95	11.27	7.87	0.90	6.50	1.63	1.74	2.65	2.12	0.25	0.27	0.41	0.33	0.14	0.68	0.55	0.35					
	Muscle	TNFRSF23	ENSBTAG0000000039129	4.06	7.17	4.53	2.76	4.63	2.22	3.48	2.85	4.64	0.48	0.75	0.61	1.00	0.23	1.00	0.57	0.65					
94	Brain	TP73	ENSBTAG000000005812	2.52	6.69	5.52	1.40	4.03	4.20	6.54	3.60	14.09	1.04	1.62	0.89	3.49	0.86	0.81	0.95	0.12					
	Kidney	TP73	ENSBTAG000000005812																						
	Liver	TP73	ENSBTAG000000005812																						
	Muscle	TP73	ENSBTAG000000005812	4.86	8.68	3.96	4.60	5.53	6.01	4.96	2.37	1.50	1.09	0.90	0.43	0.27	0.42	1.00	0.10	0.00					
95	Brain	TRAPPC9	ENSBTAG000000013955	53.60	78.12	58.01	64.90	63.66	57.07	53.61	75.17	67.63	0.90	0.84	1.18	1.06	0.95	1.00	0.72	0.96					
	Kidney	TRAPPC9	ENSBTAG000000013955	28.78	29.33	27.82	32.14	29.52	25.78	27.66	33.36	29.54	0.87	0.94	1.13	1.00	1.00	1.00	0.89	1.00					
	Liver	TRAPPC9	ENSBTAG000000013955	15.85	28.95	18.61	16.90	20.08	13.00	15.72	27.12	25.06	0.65	0.78	1.35	1.25	0.10	1.00	0.00	0.01					
	Muscle	TRAPPC9	ENSBTAG000000013955	11.24	15.58	10.24	7.82	11.22	8.52	10.06	17.26	17.68	0.76	0.90	1.54	1.58	0.69	1.00	0.02	0.01					
96	Brain	TSPAN32	ENSBTAG000000002702	3.09	2.84	1.23	1.61	2.19	1.18	1.86	3.05	2.64	0.54	0.85	1.39	1.20	0.72	1.00	0.77	0.95					
	Kidney	TSPAN32	ENSBTAG000000002702																						
	Liver	TSPAN32	ENSBTAG000000002702	67.95	94.77	61.31	66.62	72.66	46.13	73.62	88.21	72.90	0.63	1.01	1.21	1.00	0.01	0.90	0.02	0.47					
	Muscle	TSPAN32	ENSBTAG000000002702	1.46	1.77	1.26	2.05	1.64	0.69	1.30	1.95	1.58	0.42	0.80	1.19	0.97	0.11	1.00	0.70	0.98					
97	Brain	TSSC4	ENSBTAG000000004793	11.10	12.16	12.16	12.21	11.91	8.23	9.44	12.05	12.00	0.69	0.79	1.01	1.01	0.69	0.98	0.99	1.00					
	Kidney	TSSC4	ENSBTAG000000004793	11.52	12.60	11.17	13.76	12.26	11.37	12.34	30.57	14.05	0.93	1.01	2.49	1.15	1.00	1.00	0.00	0.78					
	Liver	TSSC4	ENSBTAG000000004793	17.50	22.49	17.76	17.09	18.71	19.83	14.40	22.97	22.38	1.06	0.77	1.23	1.20	0.47	0.82	0.09	0.12					
	Muscle	TSSC4	ENSBTAG000000004793	14.07	14.25	12.85	10.89	13.02	11.47	11.96	14.28	13.13	0.88	0.92	1.10	1.01	0.76	1.00	0.67	0.95					
98	Brain	UBE3A	ENSBTAG000000002487	128.62	180.79	184.29	184.04	159.43	164.97	176.93	173.73	138.96	1.03	1.11	1.09	0.87	0.99	1.00	0.92	0.93					
	Kidney	UBE3A	ENSBTAG000000002487	160.93	155.44	160.94	151.75	157.26	163.88	154.05	108.09	144.12	1.04	0.98	0.69	0.92	1.00	1.00	0.00	0.72					
	Liver	UBE3A	ENSBTAG000000002487	92.18	82.03	95.97	94.34	91.13	110.42	101.79	87.15	86.51	1.21	1.12	0.96	0.95	0.17	0.98	0.69	0.64					
	Muscle	UBE3A	ENSBTAG000000002487	187.78	192.36	203.43	189.78	193.34	225.87	184.13	165.67	142.19	1.17	0.95	0.86	0.74	0.36	1.00	0.38	0.01					
99	Brain	WIF1	ENSBTAG0000000014758																						
	Kidney	WIF1	ENSBTAG0000000014758	10.26	8.96	10.06	11.84	10.28	10.53	13.25	18.50	14.70	1.02	1.29	1.80	1.43	1.00	1.00	0.01	0.30					
	Liver	WIF1	ENSBTAG0000000014758																						
	Muscle	WIF1	ENSBTAG0000000014758	0.94	1.02	0.39	0.77	0.78	2.02	1.13	1.58	4.39	2.59	1.44	2.03	5.62	0.02	0.96	0.12	0.00					
100	Brain	WT1	ENSBTAG0000000047268																						
	Kidney	WT1	ENSBTAG0000000047268	92.27	90.22	110.52	108.20	100.30	113.23	95.80	108.68	99.63	1.13	0.96	1.08	0.99	1.00	1.00	0.89	1.00					
	Liver	WT1	ENSBTAG0000000047268																						
	Muscle	WT1	ENSBTAG0000000047268																						
101	Brain	ZC3H12C	ENSBTAG000000008732	39.53	49.26	51.11	46.56	46.62	39.32	41.39	52.26	49.21	0.84	0.89	1.12	1.06	0.82	1.00	0.88	0.99					
	Kidney	ZC3H12C	ENSBTAG000000008732	41.14	36.60	43.61	41.55	40.73	47.58	39.18	26.65	34.79	1.17	0.96	0.65	0.85	1.00	1.00	0.00	0.58					
	Liver	ZC3H12C	ENSBTAG000000008732	10.74	11.77	10.77	11.34	11.16	12.69	11.75	11.37	10.51	1.14	1.05	1.02	0.94	0.53	1.00	0.92	0.93					
	Muscle	ZC3H12C	ENSBTAG000000008732	36.41	36.07	41.04	43.76	39.32	43.87	33.50	35.20	34.03	1.12	0.85	0.90	0.87	0.70	0.95	0.58	0.29					
102	Brain	ZFAT	ENSBTAG000000017731	9.23	15.14	11.65	11.05	11.77	18.83	19.11	18.01	16.79	1.60	1.62	1.53	1.43	0.42	0.66	0.51	0.80					
	Kidney	ZFAT	ENSBTAG000000017731	13.28	15.95	13.66	14.13	14.26	13.60	16.65	12.74	15.81	0.95	1.17	0.89	1.11	1.00	1.00	0.29	0.83					
	Liver	ZFAT	ENSBTAG000000017731	3.46	4.29	3.33	3.62	3.67	2.66	4.76	4.84	5.39	0.70	1.30	1.35	1.47	0.45	0.83	0.39	0.24					
	Muscle	ZFAT	ENSBTAG000000017731	8.84	10.76	9.24	11.01	9.96	7.48	10.03	12.01	14.03	0.75	1.01	1.21	1.41	0.42	1.00	0.46	0.14					
103	Brain	ZFP64	ENSBTAG000000001512	25.85	39.15	33.25	37.29	34.56	46.96	41.70	47.87	52.03	1.36	1.21	1.39	1.51	0.58	0.97	0.57	0.66					
	Kidney	ZFP64	ENSBTAG000000001512	32.49	34.78	35.03	33.59	33.37	38.23	27.82	24.63	30.78	1.13	0.82	0.72	0.91	1.00	1.00	0.00	0.75					
	Liver	ZFP64	ENSBTAG000000001512	8.05	10.11	7.35	7.46	8.21	7.31	6.25	9.79	9.86	0.89	0.76	1.19	1.20	0.91	0.98	0.46	0.38					
	Muscle	ZFP64	ENSBTAG000000001512	28.05	28.55	27.01	25.61	27.31	30.38	25.09	34.54	33.41	1.11	0.92	1.26	1.22	0.57	1.00	0.21	0.29					
104	Brain	ZNF215	ENSBTAG000000003638	13.13	20.96	9.84	14.57	14.63	6.62	8.08	6.76	7.22	0.45	0.55	0.46	0.49	1.38	0.86	0.48	0.70					
	Kidney	ZNF215	ENSBTAG000000003638	11.21	11.33	11.14	11.20	11.22	9.77	10.67	11.61	8.83	0.87	0.95	1.04	0.79	1.00	1.00	0.79	0.57					
	Liver	ZNF215	ENSBTAG000000003638	4.77	6.90	2.70	4.16	4.74	4.67	3.27	3.12	2.29	0.99	0.69	0.66	0.48	0.76	1.00	0.67	0.25					
	Muscle	ZNF215	ENSBTAG000000003638	6.71	13.54	5.14	7.16	8.34	4.09	4.54	10.80	3.76	0.49	0.54	0.58	0.45	0.22	0.95	0.49	0.08					
105	Brain	ZNF331</																							

Table S5 Imprinted gene expression profile in each tissue of control fetuses

Gene Name	CPM ratio to the average						
	Mean CPM	Min CPM	Max CPM	Brain	Kidney	Liver	Muscle
AIM1	26.27	10.35	58.91	0.63	2.11	0.73	0.54
AMPD3	9.06	4.42	15.79	1.48	0.52	0.00	0.00
ANO1	10.32	1.25	27.72	0.22	1.40	2.13	0.25
ART5	16.38	1.74	65.16	0.00	0.00	1.87	0.13
ASB4	13.73	0.82	35.95	0.00	0.00	0.12	1.88
ATP10A	9.26	2.18	15.19	0.32	1.51	0.00	1.17
AXL	40.05	16.94	81.93	0.95	1.02	0.47	1.55
BEGAIN	28.35	10.09	53.25	1.65	0.85	0.48	1.01
BLCAP	175.00	59.01	362.57	1.55	0.34	0.82	1.29
CALCR	18.11	8.49	39.69	1.02	0.00	0.00	0.98
CASD1	110.16	15.80	179.98	1.33	1.17	0.17	1.33
CD81	162.45	120.49	227.68	1.05	0.93	0.86	1.16
CDKN1C	90.09	8.24	252.95	0.17	1.03	0.77	2.02
CMAH	19.00	1.45	39.89	0.00	0.10	1.90	0.00
CNTN3	26.01	6.41	53.32	0.99	0.30	0.00	1.71
COBL	53.27	6.72	165.52	0.29	0.64	0.17	2.91
COMMD1	33.42	14.09	55.81	0.82	1.22	0.48	1.48
COPG2	29.44	9.51	56.79	1.61	0.65	0.33	1.42
CPA4	32.79	1.13	114.68	0.35	0.50	0.05	3.10
DCN	580.25	21.16	2079.08	0.09	0.84	0.13	2.95
DDC	12.61	5.77	20.23	0.00	0.56	1.44	0.00
DHCR7	66.42	19.53	135.74	1.58	0.59	1.42	0.41
DIO3	7.95	6.76	9.40	0.00	1.00	0.00	0.00
DIRAS3	271.25	15.38	688.40	1.92	0.08	0.00	0.00
DLGAP2	26.85	17.61	32.88	1.00	0.00	0.00	0.00
DLK1	685.60	4.61	1637.22	0.02	0.73	1.32	1.93
DLX5	6.17	0.94	13.07	1.00	0.00	0.00	0.00
DNMT1	91.20	67.66	125.51	0.87	1.06	1.25	0.82
DRD1A	17.57	7.28	35.83	1.00	0.00	0.00	0.00
FBXO40	15.11	3.22	28.77	0.00	1.76	0.00	0.24
GATM	408.71	11.15	1228.00	0.30	0.92	2.74	0.04
GDAP1L1	61.31	53.31	64.79	1.00	0.00	0.00	0.00
GLIS3	6.97	1.70	14.39	0.90	1.73	0.00	0.37
GNAS	2024.94	1038.77	3009.67	0.92	1.20	0.53	1.35
GPR1	0.69	0.49	0.86	1.00	0.00	0.00	0.00
GRB10	32.43	18.26	42.72	0.72	0.90	1.23	1.15
GTL2	4365.28	1587.00	12651.22	0.68	0.64	0.45	2.23
H13	45.13	28.97	65.23	0.78	1.32	1.07	0.84
H19	7243.57	18.75	16180.77	0.01	1.93	0.84	1.23
HTR2A	7.11	1.26	20.00	2.05	0.75	0.20	0.00
HTRA3	22.53	6.91	47.36	0.00	0.35	0.00	1.65
IGF2	6128.87	14.96	12219.01	0.01	1.62	0.75	1.63
IGF2R	495.40	37.50	868.58	0.11	1.43	1.03	1.43
IMPACT	68.55	29.16	98.52	1.11	1.05	0.46	1.39
INPP5F	224.82	28.54	837.85	3.00	0.27	0.15	0.58
JADE1	46.30	23.98	76.00	0.93	1.21	0.57	1.29
KCNK9	9.76	6.82	13.41	1.00	0.00	0.00	0.00
KCNQ1	18.93	3.92	47.85	0.56	1.14	0.40	1.91
KLF14	3.83	2.63	5.76	0.00	0.85	0.00	1.15
L3MBTL1	20.88	6.14	54.98	2.14	0.75	0.36	0.75
LIN28B	17.23	4.16	32.61	0.38	0.00	1.62	0.00
LRRTM1	29.83	24.89	33.80	1.00	0.00	0.00	0.00
MAGEL2	20.10	5.67	45.23	1.76	0.95	0.41	0.88
MAGI2	33.74	19.50	51.52	1.39	0.61	0.00	0.00
MEST	367.35	19.35	1205.39	0.33	0.56	0.06	3.05
MKRN3	14.76	10.46	17.68	1.00	0.00	0.00	0.00
MST1R	2.30	0.37	3.95	0.75	1.53	0.72	0.00
NAA60	35.69	19.87	66.16	1.74	0.82	0.58	0.85
NAP1L4	130.58	99.56	158.90	1.12	0.91	0.82	1.15
NAP1L5	38.92	4.01	218.53	3.21	0.13	0.50	0.16
NDN	44.19	12.27	96.34	1.73	0.96	0.31	1.00
NNAT	975.64	33.09	3377.11	1.14	0.04	2.75	0.07
NTM	80.94	7.99	273.62	2.47	0.10	0.00	0.43
OSBPL5	27.82	14.41	45.03	1.32	1.42	0.62	0.63
PDE10A	46.49	8.37	204.76	2.49	0.55	0.28	0.68
PDE4D	80.33	10.74	185.36	1.74	0.98	0.18	1.09
PEG10	1128.54	405.93	1988.17	0.67	0.43	1.46	1.44
PEG3	2042.50	513.81	4228.20	0.28	1.67	1.69	0.35
PHACTR2	102.79	19.81	214.45	0.65	1.96	0.21	1.18
PHLDA2	25.50	1.78	59.84	0.00	1.92	0.08	0.00
PLAGL1	448.99	21.10	945.27	0.05	1.08	2.03	0.84
PON2	62.94	36.55	89.40	0.83	0.71	1.19	1.26
PON3	6.38	0.24	39.47	0.00	0.27	2.23	0.51
PPP1R9A	191.77	36.06	316.98	1.40	0.85	0.22	1.52
QPCT	21.86	10.53	36.19	1.30	0.00	0.00	0.70
RASGRF1	42.49	5.78	96.85	1.85	0.15	0.00	0.00
RB1	41.13	14.01	59.86	1.27	0.35	1.33	1.05
RBP5	13.81	5.77	32.31	0.00	1.51	0.49	0.00
RTL1	40.98	2.65	147.74	0.10	1.15	0.10	2.65

CPM: counts per million

Mean CPM: average of CPM of all tissue analyzed in control fetuses

Min: minimum CPM in tissues of control fetuses

Max: maximum CPM in tissues of control fetuses

CPM ratio: the average CPM of each tissue was divided by Mean CPM

Boxed = not expressed

Table S5 Imprinted gene expression profile in each tissue of control fetuses

Gene Name	Mean CPM	Min CPM	Max CPM	CPM ratio to the average			
				Brain	Kidney	Liver	Muscle
<i>SCIN</i>	21.64	1.38	67.02	0.10	2.74	0.00	0.17
<i>SFMBT2</i>	28.06	10.21	50.46	1.28	1.17	0.39	1.16
<i>SGCE</i>	104.75	29.34	192.69	0.37	0.99	1.04	1.60
<i>SGK2</i>	14.74	12.05	18.08	0.00	1.00	0.00	0.00
<i>SLC22A18</i>	27.80	1.00	66.50	0.00	1.60	1.34	0.06
<i>SLC22A2</i>	33.25	2.20	94.98	0.00	1.82	0.18	0.00
<i>SLC22A3</i>	11.12	4.43	19.32	1.17	1.14	0.47	1.22
<i>SLC38A4</i>	71.99	1.34	315.65	0.04	0.08	3.79	0.08
<i>SNRPN</i>	139.44	51.29	416.35	2.44	0.63	0.38	0.55
<i>TBC1D12</i>	38.16	9.49	61.83	1.14	1.49	0.26	1.11
<i>TCEB3</i>	94.55	67.80	132.90	0.92	1.30	0.75	1.03
<i>TFPI2</i>	40.16	24.74	62.37	0.00	1.00	1.09	0.92
<i>TH</i>	2.22	1.03	3.17	0.00	0.00	1.00	0.00
<i>TNFRSF23</i>	4.79	0.90	11.27	1.05	0.62	1.36	0.97
<i>TP73</i>	4.78	1.40	8.68	0.84	0.00	0.00	1.16
<i>TRAPPC9</i>	31.12	7.82	78.12	2.05	0.95	0.65	0.36
<i>TSPAN32</i>	25.50	1.23	94.77	0.09	0.00	2.85	0.06
<i>TSSC4</i>	13.97	10.89	22.49	0.85	0.88	1.34	0.93
<i>UBE3A</i>	150.29	82.03	203.43	1.06	1.05	0.61	1.29
<i>WIF1</i>	5.53	0.39	11.84	0.00	1.86	0.00	0.14
<i>WT1</i>	100.30	90.22	110.52	0.00	1.00	0.00	0.00
<i>ZC3H12C</i>	34.46	10.74	51.11	1.35	1.18	0.32	1.14
<i>ZFAT</i>	9.91	3.33	15.95	1.19	1.44	0.37	1.00
<i>ZFP64</i>	26.01	7.35	39.15	1.33	1.31	0.32	1.05
<i>ZNF215</i>	9.73	2.70	20.96	1.50	1.15	0.49	0.86
<i>ZNF331</i>	20.97	5.01	39.42	1.63	1.51	0.26	0.60

Table S6. Validation of SNPs identified by RNAseq using Sanger sequencing

Gene symbol	Chr	Position	Reference (UMD3.1)	Sanger Sequencing of genomic DNA			RNAseq (F1 hybrid fetuses)		SNP flanking Sequence
				<i>B. t. taurus</i>	<i>B. t. indicus</i>	F1 hybrid fetuses	Reference allele	Alternative allele	
<i>MEST*</i>	4	95073135	A	A	A	A/A(LOS #4)	A	G	
<i>SGCE</i>	4	11856585	C	C	T	NA	C	T	atcagggtga(C/T)jagaacaaagc
<i>PEG10</i>	4	11921764	T	T	C	NA	T	C	caccttgacc(T/C)ggaacttcag
<i>PEG10</i>	4	11921800	T	T	C	NA	T	C	gcattgttca(T/C)tgctggaag
<i>PEG10</i>	4	11921824	G	G	A	NA	G	A	gccacctgaa(G/A)tcctgggagc
<i>PEG10</i>	4	11922094	T	T	C	NA	T	C	ctgacttctc(T/C)tagcctggcc
<i>PEG10</i>	4	11922259	G	G	C	NA	G	C	ctgggagcat(G/C)tggttcaaca
<i>PEG10</i>	4	11922533	A	A	C	NA	A	C	tcctaaatca(A/C)ttaaaattga
<i>NAP1L5</i>	6	37510035	C	C	T	NA	C	T	taaaatacta(C/T)gaaattgacg
<i>NAP1L5</i>	6	37510055	C	C	T	NA	C	T	gcaatcagat(C/T)atgcccagcg
<i>NAP1L5</i>	6	37510166	A	A	G	NA	A	G	tgtgaggact(A/G)gagttgagac
<i>NAP1L5</i>	6	37510180	C	C	A	NA	C	A	ttgagacta(C/A)taaggggtgc
<i>NAP1L5</i>	6	37510241	A	A	G	NA	A	G	tggtcagat(A/G)tcgcactagg
<i>NAP1L5</i>	6	37510307	G	G	C	NA	G	C	tcctcattt(G/C)ttttgattt
<i>NAP1L5</i>	6	37510534	C	C	T	N/A	C	T	atgacctgta(C/T)aatattgtc
<i>NAP1L5</i>	6	37510546	T	T	C	NA	T	C	atattgtcaa(T/C)gtgtgtgttc
<i>NNAT</i>	13	67120130	A	A/T	A/T	A/T(LOS #4)	A	T	cagcaccgac(A/T)atgacgaca
<i>NNAT</i>	13	67120164	C	C/T	C/T	C/T(LOS #4)	C	T	ccccaccct(C/T)tgcccctc
<i>NNAT</i>	13	67120187	G	G/T	G/T	G/T(LOS #4)	G	T	actcggggtg(G/T)cctagaggga
<i>NNAT</i>	13	67120502	G	G/A	G/A	G/A(LOS #4)	G	A	ctttgctac(G/A)gcatgtgcat
<i>NNAT</i>	13	67120662	C	C/T	C/T	C/T(LOS #4)	C	T	ccctcactga(C/T)cttgaatccc
<i>PEG3</i>	18	64275301	C	T	C	NA	C	T	agaagaacat(C/T)gagcgcaaga
<i>PEG3</i>	18	64275316	C	C	G	NA	C	G	aggcacgtgc(C/G)aaagaaga
<i>PEG3</i>	18	64275553	A	A	G	NA	A	G	gcctcaggga(A/G)gacgggagcc
<i>PEG3</i>	18	64275646	T	T	C	NA	T	C	gccccgaaga(T/C)ggcaggagg
<i>PEG3</i>	18	64275685	C	C	C/T	NA	C	T	aggatgagga(C/T)gaagagaagg
<i>PEG3</i>	18	64275732	G	G	G/A	NA	G	A	gtccatgcc(G/A)gctctcagaa
<i>PEG3</i>	18	64281735	T	T	C/T	C/T(LOS #3, #4, and Control #4)	T	C	aggcgatga(T/C)gagcgggaag
<i>PEG3</i>	18	64281750	T	T	C/T	C/T(LOS #3, #4); T/T(Control #4)	T	C	tcctggaaga(T/C)tcagaggcga
<i>MAGEL2</i>	21	783853	A	A	C	A/C(LOS #4)	A	C	aggtgcaaac(C/T)ccatccaat
<i>MAGEL2</i>	21	784015	C	C	T	C/T(LOS #4)	C	T	gggcccctggg(C/T)ctctgggaaa
<i>MAGEL2</i>	21	784070	C	C	T	C/T(LOS #4)	C	T	gctccctact(C/T)ttctcaggg
<i>MAGEL2</i>	21	784354	T	T	C	T/C(LOS #4)	T	C	tcaacaagg(T/C)agaaagggtg
<i>MAGEL2</i>	21	784789	T	T	C	T/C(LOS #3)	T	C	ataaaaccga(T/C)gaccccacca
<i>SNRPN</i>	21	8725	A	A	G	NA	A	G	agcttgatt(A/G)tttgtaagag
<i>SNRPN</i>	21	19911	A	A	G	NA	A	G	gcctcactga(A/G)caaccaggag
<i>SNRPN*</i>	21	25855	C	C	C	C/C(LOS #4)	C	A	

Two SNPs (indicated by asterisk) were not confirmed by Sanger sequencing. Chr: Chromosome. UMD3.1 reference: *B. t. taurus* reference genome assembly; LOS: large offspring syndrome; NA: not analyzed; SNP: single nucleotide polymorphism;

Table S7. Identification of SNPs at DMRs by Sanger sequencing

DMRs	Chr	Position	<i>B. t. taurus</i>	<i>B. t. indicus</i>	F1 hybrid fetus	SNP flanking sequence
<i>PLAGL1</i> DMR	9	82473774	G	T	G/T(LOS #4, Control #4)	acgggcggaac(G/T)gggaggctct
<i>SNRPN</i> DMR	21	26268	A	G	A/G(LOS #4, Control #2, #4)	ttacaacaaa(A/G)gaaatacca
<i>NNAT</i> DMR	13	67118693	G	A/G	G/G(Control #4), G/A (Control #2, LOS #3, #4)	ttctggggac(G/A)cgcatcgga

DMR: differentially methylated region; Chr: Chromosome; The chromosome positions are based on UMD3.1 reference assembly.

Table S8. PCR primer information

Genes	Amplicon locations	Primer types	Primer sequences (5'-3')	Annealing temperature	Amplicon size	Assays
<i>MEST</i>	Chr 4: 95072724-95073233	Forward	CTAGATGGTACAGCGTTGAG	59.1°C	510bp	gDNA-PCR for SNP validation
		Reverse	CGTCACTGCCGTTTCTAA			
<i>SGCE</i>	Chr 4: 11856803-11856067	Forward	GGACCTAGAAAGGCCAATATAA	59.1°C	737bp	gDNA-PCR for SNP validation
		Reverse	GTTCACAGTCTCTGCTTAC			
<i>PEG10</i>	Chr 4: 11921664-11922608	Forward	CAGTCATGTATGAGGGCTAATG	62.3°C	945bp	gDNA-PCR for SNP validation
		Reverse	GCCTCTTATTCACAGTGTGTA			
<i>NAP1L5</i>	Chr 6: 36808477-36809288	Forward	CTGCCGCCAAAGATGAG	59.1°C	812bp	gDNA-PCR for SNP validation
		Reverse	GGGATTTAATGCTTAAACAATTGGG			
<i>NNAT</i>	Chr 13: 67119624-67120409	Forward	CCATTGGGAGAAGTGAGGTAT	58.4°C	804bp	gDNA-PCR for SNP validation
		Reverse	ACACAGGCACAGAGTTGTC			
<i>NNAT</i>	Chr 13: 67120338-67120931	Forward	TACACCGCCAGCAGAATG	58.4°C	615bp	gDNA-PCR for SNP validation
		Reverse	ACCCAGACCGAGTCTTAGAAGTA			
<i>PEG3</i>	Chr 18: 64275728-64275179	Forward	CTCAGAAGAGTCACACGATCAC	59.8°C	550bp	gDNA-PCR for SNP validation
		Reverse	GAGCTACGGACAAGAAGCTCTC			
<i>PEG3</i>	Chr 18: 64276162-64275271	Forward	CCTGATTGAGCACCAGAAGAT	59.8°C	892bp	gDNA-PCR for SNP validation
		Reverse	CATCATGGAGGCGTCGTAAT			
<i>PEG3</i>	Chr 18: 64282227-64281459	Forward	GGGACATGTGTGCTCCTTAC	59.8°C	770bp	gDNA-PCR for SNP validation
		Reverse	GCTGCTGTTGCTCTCTGA			
<i>MAGEL2</i>	Chr 21: 783758-784573	Forward	GAGGAGGATAGTAGTGCCAAGA	58.4°C	816bp	gDNA-PCR for SNP validation
		Reverse	CTCCAGGTACTTCTCCCTTACA			
<i>MAGEL2</i>	Chr 21: 784158-784946	Forward	TGCAGTTCCTCTTGGTTAAGG	58.4°C	789bp	gDNA-PCR for SNP validation
		Reverse	GAAATCCAACTTACACACACAGA			
<i>SNRPN</i>	Chr 21: 8930-8548	Forward	TGGGAGATAAGGTCATCAAATAG	59.1°C	383bp	gDNA-PCR for SNP validation
		Reverse	CACCTCACACTCTGGAAATC			
<i>SNRPN</i>	Chr 21: 20218-19677	Forward	GGGCTAGATTCTGACCATTG	59.1°C	542bp	gDNA-PCR for SNP validation
		Reverse	GCTGAAGGAGGTAGAACTTTAT			
<i>SNRPN</i>	Chr 21: 26236-25797	Forward	ACCACTCTGGGTTGAAGTA	59.1°C	440bp	gDNA-PCR for SNP validation
		Reverse	CTGGAAGCTCAGGATGATATAA			
<i>PLAGL1</i>	Chr 9: 82419707-82420135	Forward	CTGGACAGATGCTTCTAC	60.7°C	429bp	RT-PCR for allelic expression
		Reverse	GCCTGTACTTGTGATTCT			
<i>PEG3</i>	Chr 18: 64280579-64281817	Forward	GGATCAAGCCTTGGGTGTA	64.7°C	299bp	RT-PCR for allelic expression
		Reverse	GTCCCGTGACTCCATGTC			
<i>SNRPN</i>	Spanning 2 introns Chr 21: 8676-19987	Forward	CACTTGAGACGGACTACAGAAC	63.7°C	302bp	RT-PCR for allelic expression
		Reverse	ACCTAAGGGAAGGCTACAGATA			
<i>NNAT</i>	Spanning 2 introns Chr 13: 67120319-67120605	Forward	CCACAGAGATCCAGACAACTAC	63.1°C	287bp	RT-PCR for allelic expression
		Reverse	CGTGCTCAGTAGGTCCAAT			
<i>PALGL1</i> DMR	Chr 9: 82473614-82473911	Forward	GTTGAGGYGGTAAAGTTTA	60.5°C	298bp	DNA methylation assay
		Reverse	CRACTACAACCTCCCCAC			
<i>SNRPN</i> DMR	Chr 21: 25757-26322	Forward	TGGAAAGTTTGAGGAAATTTGA	60.5°C Touchdown PCR	566bp	DNA methylation assay
		Reverse	AAAACCCAAATCCCCAATAAAT	See SI methods		
<i>NNAT</i> DMR	Chr 13: 67118612-67118953	Forward	ATTTGGTGATAGGATTTTTTAT	60.5°C Touchdown PCR	342bp	DNA methylation assay
		Reverse	CTCATCATCRACTAATACATTTCC	See SI methods		

Chapter 3 Global misregulation of genes largely uncoupled to DNA methylome epimutations characterizes a congenital overgrowth syndrome

3.1 Abstract

Assisted reproductive therapies (ART) have become increasingly common worldwide and numerous retrospective studies have indicated that ART-conceived children are more likely to develop the overgrowth syndrome Beckwith-Wiedemann (BWS). In bovine, the use of ART can induce a similar overgrowth condition, which is referred to as large offspring syndrome (LOS). Both BWS and LOS involve misregulation of imprinted genes. However, it remains unknown whether molecular alterations at non-imprinted loci contribute to these syndromes. Here we examined the transcriptome of skeletal muscle, liver, kidney, and brain of control and LOS bovine fetuses and found that different tissues within LOS fetuses have perturbations of distinct gene pathways. Notably, in skeletal muscle, multiple pathways involved in myoblast proliferation and fusion into myotubes are misregulated in LOS fetuses. Further, characterization of the DNA methylome of skeletal muscle demonstrates numerous local methylation differences between LOS and controls; however, only a small percent of differentially expressed genes (DEGs), including the imprinted gene *IGF2R*, could be associated with the neighboring differentially methylated regions. In summary, we not only show that misregulation of non-imprinted genes and loss-of-imprinting characterize the ART-induced overgrowth syndrome but also demonstrate that most of the DEGs is not directly associated with DNA methylome epimutations.

3.2 Introduction

The use of ART has become increasingly common worldwide and each year approximately six percent of infants born in developed countries are conceived employing these technologies (European et al., 2016). The use of ART involves manipulation of gametes and/or embryos in an artificial environment, and it has been reported that ART-conceived children have increased risk for birth defects (Grafodatskaya et al., 2013). For example, a meta-analysis of eight epidemiologic studies indicates that ART-conceived children have a 5.2-fold increased likelihood of developing the congenital overgrowth condition BWS (Vermeiden and Bernardus, 2013). However, it is still unresolved whether the use of ART or the infertility per se is the main cause of the loss-of-imprinting disorders observed in ART-conceived humans (Vermeiden and Bernardus, 2013). BWS is characterized by complex and variable symptoms including pre- and post-natal overgrowth, enlarged tongue, ear malformation, umbilical hernia, and predisposition to develop childhood tumors (Weksberg et al., 2010). In ruminants, gametes and embryos subjected to *in vitro* manipulations can develop into unusually large offspring that share phenotypes with BWS (Chen et al., 2013; Young et al., 1998). LOS, as the congenital overgrowth syndrome is referred to in ruminants, can cause detrimental effects to both the dam and offspring such as delivery difficulty due to the oversized fetus and inability for the newborn to stand and to suckle (Young et al., 1998). It is not clear what triggers the development of these congenital overgrowth conditions and associated phenotypes and why ART potentiates the syndromes. Numerous animal studies have indicated that the use of ART can alter the epigenome of the gametes and embryos (de Waal et al., 2012a; de Waal et al., 2012b; Market-Velker et al., 2010; Rivera

et al., 2008) and this can contribute to the etiology of the ART-induced overgrowth conditions (Kuentz et al., 2011; Vermeiden and Bernardus, 2013).

DNA methylation is an epigenetic modification involving the addition of a methyl group to the 5th carbon of cytosine (Smith and Meissner, 2013). In mammals, DNA methylation typically occurs in a CpG context with the exception of CpH methylation, which is mostly observed in neural tissues, oocytes and embryonic stem cells (He and Ecker, 2015; Shirane et al., 2013). DNA methylation plays a key role in many biological processes such as regulation of tissue-specific gene expression, suppression of parasite DNA in the genome, X-chromosome inactivation, and genomic imprinting (Smith and Meissner, 2013). Genomic imprinting is an epigenetic phenomenon in which a subset of genes, known as imprinted genes, are transcribed monoallelically in a parental-origin-dependent manner (Bartolomei and Ferguson-Smith, 2011). The transcriptional asymmetry of the parental alleles is usually directed by allele-specific DNA methylation (ASM) at imprinted loci established during gametogenesis (Bartolomei and Ferguson-Smith, 2011). Proper allele-specific DNA methylation and allele-specific expression of imprinted genes play important roles in the regulation of embryonic and neonatal growth, placental function, postnatal behavior, and metabolism (Plasschaert and Bartolomei, 2014). Altered gene expression and DNA methylation at imprinted loci have been associated with congenital overgrowth disorders such as BWS in human (Kalish et al., 2014) and LOS in bovine (Chen et al., 2015; Chen et al., 2013).

Most BWS cases are sporadic and have been linked to two imprinted loci on chromosome 11p15.5, the *KCNQ1* locus and the *H19/IGF2* locus (Weksberg et al., 2010). Approximately 50% of the BWS cases are associated with the hypomethylation of

KvDMR1 at the *KCNQ1* locus and 2-7% are linked to the hypermethylation of the differentially methylated region (DMR) at the *H19/IGF2* locus (Weksberg et al., 2010). In addition, studies have shown that a subset of BWS patients with epimutation at the *KCNQ1* locus also exhibit abnormal DNA methylation at other imprinted loci (Eggermann et al., 2016). We have observed loss of methylation of the KvDMR1 on the maternal allele in LOS (Chen et al., 2013) and have reported that LOS is a multi-locus loss-of-imprinting condition in which aberrant imprinted gene expression is associated with tissue-specific loss of imprinted DNA methylation (Chen et al., 2015).

Although it is well accepted that loss-of-imprinting can contribute to these overgrowth syndromes, and as such have been coined “loss-of-imprinting syndromes”, it remains unknown whether aberrant gene expression and DNA methylation occur at non-imprinted loci and to what extent these molecular alterations contribute to the variable phenotypes observed in these conditions. To address this question, we examined the transcriptome of skeletal muscle, liver, kidney, and brain of four control and four LOS day ~105 *Bos taurus indicus* (*B. t. indicus*) $\times$ *Bos taurus taurus* (*B. t. taurus*) F₁ fetuses (Chen et al., 2015). We found that different LOS fetuses exhibit different numbers of DEGs and that each tissue within LOS fetuses have perturbations of distinct gene pathways. Notably, in skeletal muscle, multiple pathways involved in myoblast proliferation and fusion into myotubes are misregulated in LOS fetuses. Further, characterization of the DNA methylome of skeletal muscle revealed numerous local methylation differences between LOS and controls; however, very few DEGs could be linked to neighboring identified DMRs. This study indicates that global misregulation of non-imprinted genes in addition to loss-of-imprinting characterizes the ART-induced

overgrowth syndrome. The observation that most identified DMRs could not be directly associated with aberrant gene expression suggests that caution should be exercised when making conclusions about the etiology of such syndromes by interpreting time-point-specific DNA methylation data.

3.3 Results

Identification and characterization of DEGs in LOS fetuses

In order to determine to what extent the transcriptome is altered in bovine LOS fetuses, we analyzed RNA sequencing (RNAseq) data of skeletal muscle, liver, kidney, and brain from four control and four LOS day ~105 (d104-106) *B. t. indicus* × *B. t. taurus* F₁ fetuses that we generated in a previous study (Chen et al., 2015) (Table 1). These tissues were selected because they represent the three primary germ layers (*i.e.*, liver for endoderm, kidney and skeletal muscle for mesoderm, and brain for ectoderm). Only females were used in this study to avoid any potential sex-specific gene expression (Ingleby et al., 2014b). The average bodyweight for control and LOS fetuses was 405g [standard deviation (SD) = 10g, range = 24g] and 592g (SD = 95g, range = 200g), respectively (p = 0.008) (Table 1). The bodyweight differences between the control and LOS fetuses are not expected to be due to paternal effects because all fetuses were sired by one Nelore bull [ABS CSS MR N OB 425/1 677344 29NE0001 97155 (*i.e.*, *B. t. indicus*)](Chen et al., 2013).

As LOS fetuses exhibited dramatic difference in bodyweight, each LOS fetus was considered individually and was compared to the mean of the four controls to identify

DEGs. To ensure the validity of grouping four control fetuses for DEG identification, principle component analyses (PCA) were performed using the normalized RNAseq read counts. For RNAseq the expression libraries of kidney, skeletal muscle, and liver, control fetuses clustered while the libraries from the LOS fetuses did not (Fig. S1). It should be noted that the RNAseq libraries of skeletal muscle and liver from Control #2 fetus were sequenced with 50bp read length; while the RNAseq reads of other fetuses were of 100bp. The read length differences may explain why Control #2 is segregated from other control fetuses in these two tissue types. In brain, both control and LOS fetuses exhibited a segregated pattern in the PCA plot (Fig. S1). In order to minimize the false positive DEGs caused by natural biological variation and technical variation, the DEGs were identified with the consideration of variance among the controls and the RNAseq read length differences using the edgeR package as we previously described (Anders et al., 2013; Chen et al., 2015; Robinson et al., 2010). The number of DEGs varied between tissues for different LOS fetuses (Fig. S2). For example, in the largest LOS fetus analyzed (weight = 714g), the number of DEGs was 3868, 1581, 441, and 276 for skeletal muscle, liver, kidney, and brain, while in the smallest LOS fetus (weight = 514g), the number of DEGs for those tissues were 2270, 3232, 139, and 1106 (Fig. S2). The number of DEGs was not associated with fetal weight, but the fetuses with loss-of-imprinting at the *KCNQ1* locus (*i.e.*, LOS #1 and #4) had more DEGs in liver and skeletal muscle than LOS fetuses with correct imprinting at this locus (Fig. S2). Next, we performed Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses (Huang da et al., 2009) to gain biological insights of the DEGs identified in the LOS fetuses. We found that several KEGG pathways were shared by DEGs in different

Figure 1. KEGG pathway analyses of DEGs in somatic tissues of LOS fetuses. A). KEGG pathways enriched for DEGs in skeletal muscle and liver ($\text{FDR} < 0.05$). Shown here are examples of tissues with the most DEGs. **B).** KEGG pathways enriched for upregulated and downregulated genes in skeletal muscle and liver ($\text{FDR} < 0.05$). DEG: differentially expressed genes; LOS: large offspring syndrome.

tissues while others were unique for a particular tissue (Fig. 1A and Fig. S2). For example, “ribosome” and “oxidative phosphorylation” were enriched in DEGs in skeletal muscle, liver, and kidney, while “pathways in cancer” was unique for skeletal muscle (Fig. 1A and Fig. S2), suggesting the over-proliferation of the muscle cells in LOS fetuses. Further analyses revealed that even the common pathways could be differentially represented in different tissues. For instance, “ribosome” and “oxidative phosphorylation” were enriched with downregulated genes in skeletal muscle but enriched with upregulated genes in liver and kidney (Fig. 1B and Fig. S2). For brain, however, misregulated genes were only found to enrich two KEGG pathways (Fig. S2). Overall, we found that numbers of DEGs varied among LOS fetuses and different tissues within LOS fetuses have perturbations of distinct gene pathways.

Multiple pathways involved in myoblast proliferation and fusion into myotubes are misregulated in LOS

To identify DEGs correlated with the overgrowth phenotype of the LOS fetuses, weighted gene correlation network analyses (WGCNA) (Langfelder and Horvath, 2008) were used to identify clusters of genes that were highly interconnected, namely modules, and to determine which modules were correlated with bodyweight of the fetuses. For all the tissues analyzed, three modules [*i.e.*, turquoise, red, and green (Fig. S3)] in skeletal muscle were identified to be significantly correlated ($p\text{-value} < 0.05$) with bodyweight and were enriched with the KEGG pathways associated with overgrowth phenotype (Fig. 2A and B). In both turquoise and red clusters, the enriched pathways were involved in cell proliferation and differentiation (*e.g.*, “pathways in cancer” and “cell cycle”), and cell-cell adhesion and fusion (*e.g.*, “focal adhesion”, “cell adhesion molecules”, and

“adherens junction”) (Fig. 2A and B), while the green module was only enriched with genes associated with the “spliceosome” pathway (Fig. S3).

Fetal myogenesis involves myoblast proliferation/differentiation and fusion of myoblasts into multinucleated muscle fibers (Biressi et al., 2007). Characterization of genes that belong to “pathways in cancer” led us to identify multiple upregulated genes in LOS fetuses that are known to be involved in myoblast proliferation and differentiation (Fig. 2C). For example, IGF1 is a major regulator for skeletal muscle growth and both *IGF1* (Schiaffino and Mammucari, 2011) and its downstream signaling component *PIK3CD* (Schiaffino and Mammucari, 2011) exhibited increased expression levels in LOS #3 and/or #4, the two largest fetuses. Besides *IGF1*, transcript abundance of several other growth factors and their receptors such as *FGF1*, *FGF18*, *FGFR1*, *FGFR2*, *PDGFB*, *PDGFRA*, *PDGFRB*, *TGFB1*, and *TGFBR2* were also increased in LOS #3 and/or #4 (Fig. 2C). These growth factors may work in synergy to promote myoblast proliferation and differentiation (Biressi et al., 2007). In addition, numerous upregulated genes in LOS that belong to the “focal adhesion” and the “cell adhesion molecules” pathways have been reported to play a role in myoblast adhesion and fusion. For example, we found *DOCK1*, a prototypical member of the family of Rho GTPase activator that is essential for myoblast fusion (Laurin et al., 2008), to be upregulated in both LOS #3 and #4 (Fig. 2C). Further, a number of extra cellular matrix genes, reported to be involved in myoblast migration, adhesion, and fusion (*e.g.*, *ADAM12*, *FN1*, *ICAM1*, *ITGA3*, and *ITGA5*) (Abmayr and Pavlath, 2012; Thorsteinsdottir et al., 2011) exhibited increased transcript abundance in LOS #3 and/or #4 (Fig. 2C). Although it is likely that some of the gene expression changes could be the consequential events of the altered

Figure 2. Multiple pathways involved in myogenesis are misregulated in skeletal muscle of LOS fetuses. **A).** Heat map profiling of the genes of the WGCNA network module red and turquoise. Red and turquoise modules represent clusters of co-expressed genes that are correlated with bodyweight of the fetuses (details may be found in Figure S2). Shown in the parentheses are the number of genes in each module and p-value of the correlation between the module and bodyweight. The z-score scale represents the mean-subtracted regularized log-transformed read counts. **B).** Enriched KEGG pathways for module turquoise and red (FDR < 0.05). **C).** Heat map profiling of the genes that belong to the "pathways in cancer" and the "focal adhesion/adherens junction/cell adhesion molecules" pathways. Bolded genes are those known to be involved in myoblast proliferation and differentiation or fusion of myoblasts into myotubes.

fetal myogenesis, these data suggest that the misregulated pathways may be responsible for the increased muscle mass and overgrowth phenotype of the LOS fetuses. In summary, gene co-expression network analyses revealed that multiple pathways involved in fetal skeletal muscle development were disrupted in LOS.

Whole genome bisulfite sequencing (WGBS) and read processing

As differential gene expression can be regulated by differential DNA methylation (Smith and Meissner, 2013) and DNA methylation can be altered by the use of ART (de Waal et al., 2012a; de Waal et al., 2012b; Market-Velker et al., 2010; Rivera et al., 2008), we next sought to address whether altered DNA methylation could be the molecular mechanism responsible for the misregulated gene pathways in skeletal muscle of LOS fetuses. To compare the methylome between control and LOS, we generated WGBS data of skeletal muscle of the fetuses used for RNAseq analyses. For each individual, we sequenced ~400 million 100bp paired-end reads, which is approximately 26× coverage of the bovine reference genome (Table S1). The average bisulfite conversion rate was 99.31% (Table S1). Of note, strong methylation bias was detected at the 5' end of read mate 1 and both ends of read mate 2 (Fig. S4). As these biases could be introduced during the library preparation and/or sequencing steps (Hansen et al., 2012), the first 3 base pairs (bp) and last 2bp of each WGBS read were trimmed and not used for determination of CpG methylation levels. Following a series of quality filtering steps, ~80% CpGs in the reference genome were covered by ≥ 5 WGBS reads (Table S2).

Genome-wide CpG methylation landscape and its relationship with transcriptome in fetal skeletal muscle

Prior to making comparisons between LOS and controls, we sought to establish the DNA methylation baseline in bovine fetal skeletal muscle. In controls, the distribution of CpG sites across different methylation levels exhibited a bimodal pattern (Fig. 3A), which is similar to what have been observed in somatic tissues of human and mouse (Messerschmidt et al., 2014). Further, consistent with the reports that CpG islands are usually unmethylated (Messerschmidt et al., 2014), the majority of the CpGs within CpG islands in our study were hypomethylated (Fig. 3A and B). In addition, we explored the relationship between the DNA methylome and the transcriptome. We first plotted the average methylation of all the annotated genes along the gene body and 3kb upstream of the transcription start site (TSS) and 3kb downstream of the transcription end site (TES). The 3kb range was chosen because the average DNA methylation beyond this distance was ~0.6-0.7, which was the overall average methylation of bovine fetal skeletal muscle. As shown in Figure 3C, we found a negative correlation (Spearman rank test, $p < 0.02$) between the gene expression level and the level of DNA methylation at regions near the TSS (-500bp to TSS) (Fig. 3C). Furthermore, we queried the relationship between DNA methylation and transcript abundance for genes with different levels of CpG density at their promoter regions. For genes with high (HCP) and intermediate (ICP) CpG density promoters, there was a negative correlation (Spearman rank test, $p < 0.02$) between the methylation level at promoter regions and the transcript abundance (Fig. 3C). However, low CpG density promoters (LCP) were usually methylated and had no apparent correlation with gene expression levels (Fig. 3C).

Figure 3. Genome-wide CpG methylation landscape and its relationship to the transcriptome in day ~105 skeletal muscle of control fetuses. **A).** Distribution of CpGs sites across different methylation levels. The methylation level of each CpG site was calculated as the ratio of the “C” reads to the sum of the “C” and “T” reads. CpGs from all four controls were combined for the generation of this plot. **B).** Percent of CpGs at each methylation level in control fetuses. **C).** Averaged DNA methylation of annotated bovine genes along the gene body and 3kb upstream of the TSS and 3kb downstream of the TES. Genes were classified into five groups based on their expression levels. HCP: high CpG density promoter; ICP: intermediate CpG density promoter; LCP: low CpG density promoter; TSS: transcription start site; TES: transcription end site.

Notably, we observed a positive correlation (Spearman rank test, $p < 0.02$) between DNA methylation at gene bodies and transcript abundance, especially with HCP genes (Fig. 3C). Overall, in our study, the associations between global DNA methylation and global gene expression were consistent with the findings in human and mouse (Messerschmidt et al., 2014).

Identification and characterization of the ASM regions in bovine fetal skeletal muscle

As only a limited number of imprinted ASM regions have been characterized in bovine (Chen et al., 2015; Chen et al., 2016; Chen et al., 2013; O'Doherty et al., 2012; Robbins et al., 2012), one objective of our study was to identify ASM regions at base resolution in bovine fetal skeletal muscle using WGBS. To distinguish the parental origins of the alleles in the *B. t. indicus* $\times$ *B. t. taurus* F₁ progenies, we obtained heterozygous SNPs of the fetuses from two sources: 1) SNPs identified from the WGBS data using Bis-SNP (Liu et al., 2012) and 2) SNPs identified from the RNAseq data of the F₁ hybrids (Chen et al., 2016) (Fig. S5). We assigned the WGBS reads overlapping the SNPs to their parental origins based on the genotype of the *B. t. indicus* sire (Chen et al., 2016) of the F₁ fetuses. As an initial step to screen CpGs with ASM, allelic WGBS reads were combined from the four controls and CpGs with at least 4 $\times$ coverage of each allele ($n=4,798,414$; 17.4% of CpGs in the bovine genome) were subject to Fisher's exact test to determine whether DNA methylation level was significantly different between alleles. In total, 109,794 ASM candidate CpGs were identified with a p-value cutoff of 0.01 [false discovery rate (FDR) $\approx$ 0.05, Fig. S5]. To further minimize the false positive ASM sites, the ASM candidate CpGs were merged into regions using a clustering method

Figure 4. Identification and characterization of ASM regions in skeletal muscle of control fetuses. **A).** Summary of the ASM regions identified at imprinted and non-imprinted loci. Red color: DMRs with maternal allele-specific methylation; blue color: DMRs with paternal allele-specific methylation. **B-D).** Genome browser views of the imprinted ASM loci: *GNAS*/*GNASXL* and *NAP1L5*/*HERC3* and the non-imprinted *CPOX*. The $-\log_{10}(\text{p-value})$ for ASM significance (black) and the CpG methylation levels for total (green), *B. t. indicus* allele (blue, paternal), and *B. t. taurus* allele (red, maternal) are also shown. Each bar represents a single CpG site. **E).** Allelic expression percentage of *CPOX* in skeletal muscle of the control fetuses. ASM: allele-specific DNA methylation; DMR: differentially methylated region; Mat: maternal allele; Pat: paternal allele. More examples may be found in Figure S7-S8.

as previously described (Xie et al., 2012). In total, 1,070 ASM CpGs were grouped into 86 discrete genomic regions (Fig. 4A and Table S3).

We manually annotated the identified 86 ASM regions (Table S3) and found that 18 of them were located at known imprinted loci, while others were located >1Mb away from any known imprinted gene in bovine (Fig. 4A). The two types of ASM regions are of comparable size; however, the imprinted ASMs have a higher density of CpGs (Fig. S6) located within CpG islands. Both imprinted and non-imprinted ASM loci have overall DNA methylation level around 0.5, but ASM regions at imprinted loci exhibit less variation (range = 0.43 [imprinted] vs 0.8 [non-imprinted] and SD = 0.11 [imprinted] vs. 0.19 [non-imprinted]; Fig. S6). Lastly, the methylation differences between the parental alleles for the ASM regions at imprinted loci are also more dramatic when compared to the ASM regions that are not close to any known imprinted loci (mean = 0.73 [imprinted] vs. 0.51 [non-imprinted]; Fig. S6).

Of the 18 imprinted ASM regions, seven have been previously described in bovine by us and others including the paternal DMRs: *GNAS* DMR, *H19/IGF2* DMR (Chen et al., 2016; Robbins et al., 2012) and the maternal DMRs: *GNASXL* DMR, *IGF2R/AIRN* DMR, KvDMR1, *MEST* DMR, and *PLAGL1* DMR (Chen et al., 2015; Chen et al., 2016; O'Doherty et al., 2012; Robbins et al., 2012) (Fig. 4B and Fig. S7). Of the novel imprinted ASM regions identified in bovine fetal skeletal muscle, eight of them, including *NAPIL5* DMR, *DIRAS3* DMR, *GTL2* DMR and *DLK1* DMR (Fig. 4C, Fig. S7), have been previously reported in human and/or mouse (Table S4). The other three novel ASM regions namely, *BEGAIN*, *MEG8*, and *MGC157368* DMRs, were located within the gene bodies of the known imprinted genes in bovine (Table S3). All

the ASM regions described above could be associated with the nearby imprinted transcriptional activities previously reported by us (Chen et al., 2016) with the exception of *MEST* DMR, for which no heterozygous SNP was identified to enable allelic gene expression analyses.

We also manually inspected the imprinted ASM regions previously described in human and/or mouse in our bovine WGBS data. We found that five ASM regions (*i.e.*, *GRB10* DMR, *H13* DMR, *NNAT* DMR, *PEG3* DMR, and *PEG13* DMR) were absent in this study due to either poor SNP coverage or low read coverage (Table S4). A less stringent criterion (*i.e.*, 3× coverage per allele instead of 4×) for ASM calling could lead to the identification of another three DMRs (*i.e.*, *INPP5F* DMR, *PEG10* DMR, and *SNRPN* DMR) (Table S4). Lastly, we found that some imprinted DMRs in human and/or mouse were simply not subject to ASM in bovine fetal skeletal muscle as these loci were either hypomethylated (*e.g.*, *RASGRF1* DMR, *ZRSR1/IMPACT1* DMR, and *COMMD1* DMR) or partially methylated on both parental alleles (*e.g.*, *MKRN3* DMR and *GPR1/ZDBF2* DMR) (Table S4).

For the 68 ASM regions not adjacent to any known imprinted locus, we sought to identify genes subject to allele-specific expression in the nearby regions. As shown in Table S3, of the 68 ASM regions, 20 were intergenic (> 5kb away from any annotated gene) while the other 48 regions could be associated with nearby genes. For the 48 genes, parental-allele-specific read counts for 24 genes could be obtained from our previous study (Chen et al., 2016). We found that all of these genes exhibited biallelic gene expression and had no association with the nearby ASM regions (shown in in Fig. 4D and E, and Fig. S8 are five examples). The remaining 24 genes were either lowly

expressed in skeletal muscle or had no heterozygous SNPs available to ascertain the parental alleles. It is possible that some of these ASM regions are associated with tissue-specific or transcript-isoform-specific monoallelic gene expression.

Lack of direct associations between DMRs and DEGs in LOS

As for the DEG analysis, each LOS fetus was analyzed independently and compared to the average of all four controls to identify DMRs using the Bsseq R package (Hansen et al., 2012). The hierarchical clustering of CpG methylation within DMRs showed that control fetuses clustered together and were separated from the LOS fetuses (Fig. 5A). The numbers of DMRs identified were similar for the four LOS fetuses with most DMRs identified in LOS #1 (Fig. 5B). Notably, we identified loss of DNA methylation of KvDMR1 in LOS#1 and #4 (Fig. 5C), indicating the validity of the statistical method used for DMR determination, as we have previously identified these specific epimutations by the bisulfite polymerase chain reaction (PCR), cloning, and sequencing (Chen et al., 2013). However, our previous study only reported a DMR with a size of only 385bp (37 CpGs) while the WGBS analyses allowed us to identify methylation differences between control and LOS across the entire CpG island (1957bp, 207 CpGs, UMD3.1 Chr29: 49,552,845-49,554,801). Further characterization of the DMRs revealed that most of them contained less than 20 CpGs and were of methylation differences between 0.2-0.5 (Fig. 5D).

We then characterized the genomic distances between the identified DMRs and the DEGs, in order to determine whether any of the DMRs could be linked to aberrant gene expression in LOS. We first queried the genomic regions within 5kb range of the

Figure 5. Identification and characterization of DMRs in fetal skeletal muscle between control and LOS (A). Hierarchical clustering of CpG methylation within DMRs between control and LOS using 1-Pearson's correlation distance. Bodyweight for each fetus is also shown. (B). Number of DMRs identified by the Bseq R package between each LOS fetus and all four controls. (C). Smoothed methylation profiles of the F₁ fetuses at KvDMR1 with the pink area representing the DMR identified (size=520bp; ΔM =mean methylation difference of the DMR between all four LOS and the controls; grey=controls; blue=LOS #1; black=LOS #2; green=LOS #3; red=LOS #4). Ticks at bottom represent CpG sites. (D). Number of CpGs and the mean methylation difference of each DMR identified in each LOS fetus.

DEGs and found that on average only ~1.5% of DEGs in each LOS fetus could be associated with the identified DMRs (Fig. 6A). Increasing the range queried from 5kb to 20kb only added an additional ~0.5% of DEGs that associate with the identified DMRs (Fig. 6A). To determine whether the numbers of DEGs that are associated with the DMRs were greater than expected by chance, the identified DMRs were shuffled within each chromosome (excluding genome assembly gaps) 1000 times. For each dataset, DEGs were considered to be associated with altered DNA methylation if DMRs were within 5kb or 20kb range of the DEGs. The expected counts of DEGs were calculated as the average of the 1000 randomized datasets. As shown in Figure 6A, DEGs are associated with DMRs more often than expected by chance in LOS #1, #3, and #4 but not in LOS #2.

We found that 1407 DEGs are common between LOS #3 and #4 (the two largest LOS fetuses) and we sought to determine if differential DNA methylation can explain misexpression of these genes (Fig. 2C). Only 14 DMRs overlapped between LOS #3 and #4, and only one of these was located within 5kb of one common DEG (*C5H12orf45*) and another within 20Kb (*RWDD1*). We also noted that not all LOS fetuses with aberrant DNA methylation at the same locus had differential gene expression of the associated gene (an example shown in Fig. 6B-C), suggesting that differential DNA methylation is not the sole cause of differential gene expression. Lastly, minor methylation differences were detected in the TSS regions (-1.5kb to +500bp) of the DEGs between control and LOS (Fig. 6F and Fig. S9). In summary, only a very small percent DEGs in fetal skeletal muscle could be directly associated with the DMRs between control and LOS.

Figure 6. Lack of associations between the DEGs and the DMRs in skeletal muscle of the LOS fetuses. (A). Percentage of DEGs that are associated with the DMRs in LOS fetuses. A DMR is considered to be associated with a DEG if it is less than 5kb or 20kb away from the DEG. To determine whether the number of DEGs associated with DMRs were greater than expected by chance, DMRs were shuffled within each chromosome 1000 times and the expected numbers of DEGs associated with the DMRs were calculated as the average of 1000 datasets and were used in chi-square tests (* $p < 0.01$). (B-C). Examples of lack of association between DEGs and DMRs when the DMRs are within 5kb range of the DEGs. For (B), the details may be found in Fig. 5. Panel (C) shows the transcript abundance of *MZF1* in all fetuses (asterisk: edgeR FDR < 0.05). DEG: differentially expressed genes. (D). Mean methylation level differences of the DEGs between control and LOS fetuses at the TSS region (-1.5kb to +500bp). Only DEGs that had at least 2-fold change are shown here.

Association of aberrant DNA methylation and differential gene expression at IGF2R/AIRN and MAGEL2 imprinted loci

Insulin-like growth factor 2 receptor (*IGF2R*) is a scavenger receptor of the fetal growth factor *IGF2*, and is maternally expressed in cow (Bebbere et al., 2013; Chen et al., 2015) (Fig. 7A) and mouse (Barlow et al., 1991), but exhibits polymorphic imprinting in human (Xu et al., 1993). In this study, *IGF2R* exhibited decreased transcript abundance in three out of four LOS fetuses (Fig. 7C), and this differential gene expression was associated with the altered DNA methylation at DMR1 and DMR2 of the *IGF2R* locus (Fig. 7A-B, Fig. S9). In all four LOS fetuses, we found loss of methylation of DMR2, which is located in the second intron of *IGF2R* with loss of methylation in all four LOS fetuses (Fig 7B). Normally, DMR2 was methylated on the maternal allele and unmethylated on the paternal allele. Hypomethylation of DMR2 has also been reported in sheep LOS (Young et al., 2001), suggesting the *IGF2R* locus is similarly misregulated in these two species. DMR1, which is close to the promoter region of *IGF2R*, is normally partially methylated on the paternal allele and unmethylated on the maternal allele. In our study, DMR1 was hypermethylated in LOS (Fig. 7A-B).

In mice, paternal silencing of *IGF2R* is mediated by the paternally expressed long non-coding RNA *AIRN*, which is transcribed in the antisense direction to *IGF2R* and is regulated by DMR2 of *IGF2R* (Wutz et al., 1997). Thus, we reasoned that hypomethylation of DMR2 can lead to biallelic expression of *AIRN*, which represses the expression of *IGF2R* on both alleles and results in the decreased level of *IGF2R* in LOS fetuses. However, contrary to our expectation, *AIRN* was biallelically expressed in both control and LOS fetal skeletal muscle (Fig. 7D), suggesting that down-regulation of

Figure 7. DNA methylation and differential gene expression at *IGF2R/AIRN* and *MAGEL2* imprinted loci. **A).** Schematics of the *IGF2R/AIRN* locus. Bent arrows represent the transcription start sites. White boxes represent the ASM regions (white lollipops = unmethylated; grey lollipop = partially methylated; black lollipops = methylated). **B).** Smoothed methylation profiles for the two DMRs at *IGF2R* locus between control and LOS fetuses. The pink areas represent the DMR identified (ΔM =mean methylation difference of the DMR between all four LOS and the controls; grey=controls; blue=LOS #1; black=LOS #2; green=LOS #3; red=LOS #4). Ticks at bottom represent CpG sites. **C).** Transcript abundance of *IGF2R* in control and LOS fetuses (asterisk: edgeR FDR < 0.05, LOS vs control). **D).** Allelic expression analyses for *AIRN* by Sanger sequencing. The SNP site is indicated by an arrow. **E)** Smoothed methylation profile of *MAGEL2* locus. The labels are as panel B. **F).** Transcript abundance of *MAGEL2* in the F₁ fetuses (asterisk: edgeR FDR < 0.05).

IGF2R is not mediated by biallelic expression of *AIRN*. Nevertheless, decreased transcript abundance of *IGF2R* was associated with a hypomethylated DMR2 and hypermethylated DMR1 in LOS fetuses.

In addition to *IGF2R*, we detected an association between differential gene expression and differential DNA methylation at the *MAGEL2* locus. *MAGEL2* is a paternally expressed gene (Chen et al., 2015) with its promoter methylated on the maternal allele but unmethylated on the paternal allele (Table S3). As shown in Fig. 7E-F, the increased transcript abundance of *MAGEL2* in LOS #4 is associated with the loss of DNA methylation at its promoter region.

3.4 Discussion

Since BWS was first described in the early 1960s (Weksberg et al., 2005) and the first cases of ART-induced LOS were reported in 1995 (Farin and Farin, 1995), the main focus in both fields has been to understand how loss-of-imprinting alone can contribute to the complex and variable phenotypic features of these overgrowth conditions. In human, the epimutation and genetic alteration of the two imprinted loci (*i.e.*, *KCNQ1* and *H19/IGF2* loci) affect nearly 70% of BWS patients with the hypomethylation at KvDMR1 accounting for ~50% of all BWS cases (Eggermann et al., 2016). In addition, loss-of-imprinting at multiple loci was observed in ~25% of BWS cases with epimutation at the *KCNQ1* locus (Eggermann et al., 2016). Similarly in bovine, we have also shown that LOS exhibits loss-of-imprinting at multiple loci including the *KCNQ1* locus (Chen et al., 2015; Chen et al., 2013). However, to date, there has been no report of molecular

features that can consistently and reliably diagnose and predict these syndromes and the associated variable phenotypes. Here we advance the field by describing the altered transcript abundance of genes beyond imprinted loci in LOS fetuses, showing that loss-of-imprinting is not be the only molecular lesion of this condition.

In this study, WGCNA and KEGG pathway analyses identified that multiple pathways involved in myoblast proliferation and fusion of myoblasts into myotubes are misregulated in skeletal muscle of LOS fetuses. This finding is consistent with the previous report that short-term culture of ovine embryos can affect myogenic programming and the altered myogenesis may contribute to the large muscles observed in the oversized fetuses (Maxfield et al., 1998). In addition to skeletal muscle, altered transcript abundance of genes was also detected in liver, brain, and kidney of the LOS fetuses. Interestingly, tissue specific alterations in transcript abundance varied between LOS fetuses. For example, LOS #1 had the most DEGs in liver, LOS #3 in kidney and LOS #4 in muscle. This observation is supported by findings in BWS in which different individuals have different susceptibility for Wilms tumor of the kidney or hepatoblastoma (Eggermann et al., 2016). Future studies with in depth characterization of the histology and the transcriptome of the tissues at various developmental stages of LOS will better elucidate relationships between the altered gene pathways and the potential disturbed development of these organs.

An interesting finding of this study was that only a small percent of DEGs could be linked to the identified DMRs between control and LOS. Recently, it has been shown that histone modifications such as H3K27me3 at distal regions in oocytes can escape epigenetic reprogramming and maintain inheritance during the pre-implantation

development (Zheng et al., 2016). It is possible that molecular marks like H3K27me3 in bovine may act as an epigenetic memory of the use of ART procedures and mediate the differential gene expression and the aberrant development in later stages. Alternatively, the DEGs identified in this study could represent the downstream effect of the aberrant expression of developmentally important genes affected by ART during pre-implantation development. In summary, our study demonstrates that altered transcriptome in LOS skeletal muscle is largely uncoupled to DNA methylome epimutations and we submit that caution should be exercised in interpreting results from DNA methylation data alone when studying the etiology of these overgrowth syndromes.

In addition to providing novel insights on the etiology of LOS, our study characterized the cytosine methylation landscape at base resolution in bovine. Consistent to what has been previously reported for other mammals, CpG islands in bovine are hypomethylated and a negative association between DNA methylation and gene expression levels are observed at HCP and ICP, but not LCP promoters (Messerschmidt et al., 2014). Furthermore, we have identified and characterized novel ASM at both imprinted and non-imprinted loci in bovine. For the imprinted loci, we have expanded the number of imprinted ASM in bovine by describing several novel DMRs such as *DIRAS3* DMR, *GTL2* DMR, and *NAPIL5* DMR. Moreover, we have shown that some known imprinted DMRs in human and/or mouse (*e.g.*, the *L3MBTL* DMR in human and the primary DMRs *RASGRF1* and *IMPACT* in mouse) are not subject to ASM in fetal bovine skeletal muscle, suggesting species-specific imprinting of these genes. Lastly, we have identified ASM regions at non-imprinted loci which others (Tycko, 2010) have proposed may be the result of *cis*-acting regulatory polymorphisms. It should be noted

that ASM regions identified in this study are not comprehensive because we could not assign all WGBS reads covering CpGs to either parental alleles due to the lack of informative SNPs. In future studies, analyses of the F₁ hybrids of different crosses may further improve the identification of ASM CpG sites.

In conclusion, we have shown that misregulation of non-imprinted genes in addition to loss-of-imprinting characterizes the ART-induced overgrowth syndrome and have demonstrated that most of the aberrant gene expression in fetal skeletal muscle is not associated with aberrant DNA methylation.

3.5 Materials and Methods

RNAseq data analyses

The production of the *B. t. indicus* × *B. t. taurus* F₁ fetuses and collection of the fetal tissues were previously described by us (Chen et al., 2015). All animal procedures were performed at TransOva Genetics by veterinarians, and all procedures were approved by their animal care and use committee. RNAseq data of the control and LOS fetuses were obtained from one of our previous studies and were processed as previously described (Chen et al., 2015; Chen et al., 2016). DEGs between each LOS fetus and the mean of all four controls were determined with the consideration of variance among the controls using the edgeR package (Anders et al., 2013; Robinson et al., 2010). The WGCNA package (Langfelder and Horvath, 2008) was used to construct a weighted gene co-expression network for the skeletal muscle of the four control and four LOS fetuses. Further, the identified network modules were correlated with the bodyweight to identify

genes in modules most likely responsible for the altered bodyweight. In addition, KEGG pathway analyses for the significant network modules and the DEGs in LOS fetuses were performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID) (Huang et al., 2009).

WGBS library preparation and sequencing

Genomic DNA isolated from skeletal muscle of four control and four LOS day ~105 *B. t. taurus* × *B. t. taurus* F₁ female fetuses (Chen et al., 2015) were subject to WGBS analyses. The WGBS libraries were generated using the NEBNext Ultra DNA library Prep Kit for Illumina (NEB E7370) as per the manufacturer's instructions. Prior to sodium bisulfite mutagenesis, 0.5% unmethylated Lambda DNA was added to the bovine DNA to act as an internal control to monitor the bisulfite conversion rate. Each WGBS library was sequenced using Illumina sequencing technology to generate 100bp paired-end reads. The raw FASTQ files are publically available at Gene Expression Omnibus (GEO accession no. GSE93775).

WGBS data analyses

Following adaptor removal and quality trimming, WGBS read pairs were aligned to the bovine reference genome assembly UMD3.1 using Bismark (version: 0.15.0) (Krueger and Andrews, 2011) with the default parameters. Only uniquely aligned reads were used for subsequent analyses. In order to distinguish the C>T SNPs from C>T substitutions that were caused by sodium bisulfite conversion in the F₁ fetuses, Bis-SNP (version: 0.82.2) (Liu et al., 2012) was used to perform the SNP calling. Only CpG sites that showed consensus CpG context for both parental alleles (*i.e.*, do not overlap any

SNPs identified by Bis-SNP) were used for the subsequent analyses. The methylation level was determined using the reads from both forward and reverse strands covering the same symmetric CpG site. The methylation level was calculated as: (number of “C” reads)/(number of “C” reads + number of “T” reads).

In order to identify ASM regions, the WGBS read pairs overlapping SNPs were assigned to their parental origin based on the genotype of the *B. t. indicus* sire (Chen et al., 2016). Further, the allelic WGBS reads were pooled from four controls to determine the sequencing depth and only CpGs that had at least 4× coverage of each allele were used for Fisher’s exact test. To estimate the FDR, WGBS read pairs overlapping SNPs were randomly assigned to be either *B. t. indicus* or *B. t. taurus* in origin in order to generate a randomized dataset of ASM sites (Xie et al., 2012). ASM CpGs were identified using a p-value of 0.01, resulting in 109,794 ASM sites with a FDR of ~5% (compared to the 5507 CpGs identified as ASM sites in the permuted datasets). Lastly, the identified ASM sites were clustered into regions as previously described (Xie et al., 2012). MethylKit R package (Akalin et al., 2012) was used to perform the hierarchical clustering of the methylation profiles. The DMRs between each LOS fetus and all four controls were identified using the Bseq R package (Hansen et al., 2012).

To estimate the expected number of DEGs associated with DMRs, the identified DMRs were randomly shuffled within each chromosome using “shuffleBed” function from BedTools (Quinlan and Hall, 2010). The UMD3.1 genome assembly gaps from UCSC genome Browser were excluded from the potential shuffled positions. Shuffling was performed 1000 times and for each dataset, a DEG was considered to be associated with a DMR if the DMR falls into the DEG coordinates that had been extended by 5kb or

20kb. The averaged DEG counts of the 1000 datasets were used as the expected count for chi-square test.

Statistical analyses and data visualization

Statistical analyses were implemented with R (<http://www.r-project.org/>). Heat maps were generated with the R function “heatmap.2”. The averaged methylation level of the annotated genes were generated using deepTools (Ramirez et al., 2014). For ASM regions, bed files were converted to the bigwig format and visualized as custom tracks in the Integrative Genomics Viewer (IGV) (Robinson et al., 2011).

Allelic expression analyses of AIRN

Both DNA and cDNA of the F₁ fetuses and DNA of the sire were amplified by PCR followed by Sanger sequencing to ascribe the parental origin of the transcript. Nucleic acid isolation, cDNA synthesis, and PCR amplifications were as previously reported (Chen et al., 2015).

3.6 Acknowledgements

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3.7 Supplemental information for Chapter 3

Supplemental materials and methods

WGBS library preparation and sequencing

Genomic DNA isolated from skeletal muscle of four control and four LOS ~d105 *B. t. taurus* × *B. t. taurus* F₁ fetuses were subject to WGBS analyses. For library preparation, 1 µg bovine DNA was subject to sodium bisulfite mutagenesis using NEB EpiMark Bisulfite Conversion Kit (NEB E3318) according to the manufacturer's instructions. Prior to the sodium bisulfite reaction, 5ng (0.5%) unmethylated cl857 *Sam7* Lambda DNA (48,502bp, Promega D1521) was combined with bovine DNA to act as an internal control to monitor the bisulfite conversion rate. The bisulfite conversion rate was calculated as the percentage of thymines sequenced at the cytosine reference positions in the Lambda genome. The WGBS libraries were generated using the NEBNext Ultra DNA library Prep Kit for Illumina (NEB E7370) as per the manufacturer's instructions. Each WGBS library was sequenced in two lanes with 100bp paired-end reads on an Illumina HiSeq 2000 platform. For Control #4 fetus, one lane of the WGBS library was sequenced on an Illumina HiSeq 2500 platform.

WGBS reads trimming and alignment

Raw WGBS read pairs were subject to quality trimming prior to alignment to the reference genome. The Cutadapt program (version: 1.9.1) (Martin, 2011) was used to remove the adaptor sequences in the read pairs. For base quality trimming, DynamicTrim (version 3.1) (Cox et al., 2010) was used to obtain the longest segment of the read in which each base has <1% error rate. Following quality trimming, WGBS read

pairs were aligned to the bovine reference genome assembly UMD3.1 and the Lambda genome using Bismark (version: 0.15.0) (Krueger and Andrews, 2011) with the default parameters. Only uniquely aligned read pairs that had the expected read mate orientation and expected ranges of insert size were retained for further analyses.

Identification of SNPs using WGBS data

In order to distinguish the C>T SNPs in the F₁ fetuses from the C>T substitutions that are caused by sodium bisulfite conversion, Bis-SNP (version: 0.82.2)(Liu et al., 2012) was used to perform the SNP calling using the WGBS data. In brief, the uniquely aligned WGBS read pairs were subject to several post-alignment processes prior to the SNP calling including PCR duplicates removal, insertions/deletions re-alignment, and base quality recalibration. Known insertions/deletions (indel) and known SNPs downloaded from Ensembl and RefSeq FTP sites were supplied for the indel realignment and the base quality recalibration (ftp://ftp.ensembl.org/pub/release-78/variation/vcf/bos_taurus/; ftp://ftp.ncbi.nlm.nih.gov/snp/organisms/cow_9913/VCF/; ftp://ftp.ncbi.nlm.nih.gov/snp/organisms/cow_30522/). In addition, the SNPs identified from the DNA sequencing data (Chen et al., 2016) of the sire of the *B. t. indicus* X *B. t. taurus* F₁ fetuses were also supplied for the base quality recalibration process. After processing the uniquely aligned WGBS read pairs, Bis-SNP was used to identify SNPs with the exclusion of first 3bp and last 2bp of the both read mates using the option “-trim5 3 -trim3 2” as these read positions were associated with strong methylation bias (Fig. S1).

Determination of the methylation level of each CpG site

Only CpG site that show consensus CpG context for both parental alleles (*i.e.*, do not overlap any SNPs identified by Bis-SNP) were used for the subsequent analyses. As CpG methylation is symmetric, the methylation level was determined using the reads from both forward and reverse strands covering the same symmetric CpG site. The methylation level was calculated as: (number of “C” reads)/(number of “C” reads + number of “T” reads). For most analyses, only CpGs covered by at least by five reads were used for the calculation of the methylation level. The exception is the allele-specific methylation analyses, in which only CpGs covered at least by 8 reads (four reads per parental allele) were included for the statistical test. For reads with PCR duplicates, only one of them was randomly choosed for the determination of the methylation level.

Identification of the ASM regions

Heterozygous SNPs of the fetuses were obtained from two sources for the determination of parental allele origin: 1) SNPs identified from the WGBS data using Bis-SNP (Liu et al., 2012); 2) SNPs identified from the RNA-seq data of the F₁ hybrids (Chen et al., 2016). The WGBS read pairs overlapping SNPs were assigned to their parental origins based on the genotype of the *B. t. indicus* sire (Chen et al., 2016). If the SNP included a cytosine, its bisulfite converted form thymine was also considered. Further, the allelic WGBS reads were pooled from four controls to determine the sequencing depth and only CpGs that had at least 4× coverage of each allele were used for Fisher’s exact test. To estimate the FDR, WGBS read pairs overlapping SNPs were permuted for their parental origin by randomly assigning them to either *B. t. indicus* or

B. t. taurus alleles. The ASM for each CpG in the permuted dataset was assessed using Fisher's exact test. The permutation was performed five times and the numbers of identified ASM CpGs at different p-value cutoff were averaged. ASM CpGs were identified using a p-value of 0.01, resulting 109,794 ASM sites with a FDR of ~5% as simply by chance 5507 ASM sites can be identified in the permuted datasets. Lastly, the identified ASM sites were clustered into regions as previously described (Xie et al., 2012). In brief, significant ASM sites within 2kb were merged as DMR candidates. For each candidate, at least five ASM sites were required and at least 75% CpGs should show consistent methylation bias toward the same allele. The clustered DMRs were also manually inspected and annotated.

Association between DEGs and DMRs in LOS fetuses

Methylkit R package (Akalin et al., 2012) was used to perform the hierarchical clustering of the methylation profiles using 1-Pearson's correlation distance. The DMRs between each LOS fetus and all four controls was identified using Bsseq R package (Hansen et al., 2012) with the default parameters except that only CpGs with at least 5 reads coverage for each fetus were included for analyses. For comparison, Bsseq was also used to identify DMRs between all four LOS fetuses and the controls.

RNA-seq data of the control and LOS fetuses were obtained from one of our previous studies and were processed as previously described (Chen et al., 2015; Chen et al., 2016). DEGs between each LOS fetus and all four controls were determined using the edgeR package (McCarthy et al., 2012; Robinson et al., 2010). In brief, for each LOS fetus, a DGEList was created with the gene read counts of the four controls and the LOS

fetus. Following normalization (*i.e.*, function “calcNormFactors”) and parameters estimation (functions “estimateGLMTrendedDisp” and “estimateGLMTagwiseDisp”), functions “glmFit” and “glmLRT” were used to identify genes with differential transcript abundance between the LOS and the mean of the four controls (FDR < 0.05). To determine whether any DMRs are associated with DEGs, 5kb or 20kb regions surrounding the DEGs (including the gene body) were used to intersect with the DMRs using “intersectbed” from bedtools (Quinlan and Hall, 2010). The methylation level of the TSS region (-1.5kb to +500bp) was calculated by averaging the methylation level of all CpGs with at least five reads coverage.

WGCNA and KEGG analyses

A weighted gene co-expression network was constructed for the skeletal muscle of the four control and four LOS fetuses using “blockwiseModule” function from the WGCNA package (Langfelder and Horvath, 2008). Further, the identified network modules were linked to the external trait (*i.e.*, bodyweight) to determine which module is significantly correlated to the bodyweight. In addition, KEGG pathway analyses for the significant network modules were performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID) (Huang et al., 2007).

Genomic annotations

The TSS and TES of the annotated genes were obtained from Bovine Genome Database (<http://bovinegenome.org>)(Elsik et al., 2015) and were curated with the RNA-seq data of brain, kidney, liver, and skeletal muscle using cuffmerge (Trapnell et al., 2010). Annotation of CpG islands was obtained from UCSC genome browser

(Rosenbloom et al., 2015), in which the CpG islands are defined as genomic regions that fulfill the following criteria: 1) $> 200\text{bp}$, 2) GC content $\geq 50\%$ and 3) the ratio of the observed CpGs to the expected CpGs (O/E) > 0.6 .

Promoters are defined as -2kb to $+500\text{bp}$ of the TSS (Liao et al., 2015). High CpG Promoters (HCP) promoters contain a 500bp interval with: 1) GC content $\geq 55\%$ and 2) the O/E ratio ≥ 0.6 (Mikkelsen et al., 2007). Low CpG Promoters (LCP) promoters do not contain a 500bp interval with the O/E ratio ≥ 0.4 (Mikkelsen et al., 2007). The rest of the promoters are classified as Intermediate CpG Promoters (ICP) promoters (Mikkelsen et al., 2007). The formula used for calculation of the O/E ratio of a sequence segment is as follows: number of CpGs * sequence length / (number of C * number of G) (Gardiner-Garden and Frommer, 1987).

Allelic expression analyses of AIRN

Both DNA and cDNA of the F_1 fetuses and DNA of the sire were amplified by PCR followed by Sanger sequencing to ascribe the parental origin of the transcript. Nucleic acid isolation, cDNA synthesis, and PCR amplifications were as previously reported (Chen et al., 2015). The primers used are as follows: forward ($5'$ -AGAAACGGGCACATTCA- $3'$) and reverse ($5'$ -GGCAATATTAGAACAGCTAAGAG- $3'$).

Statistical analyses and data visualization

Statistical analyses such as Fisher's exact test were implemented with R (<http://www.r-project.org/>). Fig. 2A and C and Fig. S2C-D were generated with R function "heatmap.2". Fig. 3C and 5B were generated using the "computeMatrix" and

“plotProfile” functions in deepTools (Ramirez et al., 2014). For Fig. 4B-D, Fig. 5C, Fig. S5, Fig. S6A, C, and E, bed files were converted to the bigwig format using the “bedGraphToBigWig” from UCSC Genome Browser database and visualized as custom tracks in the Integrative Genomics Viewer (IGV) (Robinson et al., 2011).

Supplemental figures and tables for Chapter 3

Figure S1. Principle component analyses using all expressed genes in kidney, skeletal muscle, liver, and brain. In skeletal muscle, liver, and brain, RNAseq library of Control #2 (the grey dot circled with black) was sequenced with 50bp read length, while other fetuses were sequenced with 100bp read length. The read length differences may explain why Control #2 fetus is not closely clustered with other control fetuses in these tissues.

Panel B and C can be found in next page.

Figure S2. Identification and characterization of DEGs identified in the LOS fetuses. A). Differentially expressed genes identified between each LOS fetus and the mean of the four controls. **B).** KEGG pathways enriched for DEGs in kidney and brain (FDR < 0.05). **C).** KEGG pathways enriched for upregulated and downregulated genes in kidney and brain (FDR < 0.05).

Figure S2

Figure S3. WGCNA and KEGG pathway analyses in skeletal muscle. **A).** WGCNA cluster dendrogram on all control and LOS fetuses groups genes into 33 modules, namely, 33 co-expressed gene networks. **B).** Correlation of WGCNA modules and the bodyweight of the fetuses. Arrows indicate the three modules that show significant correlation with the bodyweight. **C).** Heat map profiling of the genes of module green. Module green includes genes that are mostly misregulated in LOS #1 and #4. **D).** Heat map profiling of genes that belong to the "Spliceosome" pathway.

Figure S4. Mean CpG methylation level at each WGBS read position of the skeletal muscle from control and LOS fetuses.

Figure S5. Pipeline used for the identification of ASM regions in skeletal muscle of control fetuses. A). Schematics pipeline. B). The number of ASM sites identified with various p-value cutoffs is plotted. The optimal cutoff chosen for identifying ASM sites is indicated.

Figure S6. Characterization of the ASM regions identified in bovine fetal skeletal muscle.

A) The size of the ASM regions. B) The number of CpGs in the ASM regions. Only CpGs identified with allele-specific DNA methylation were counted for each ASM region. C) Localization of the ASM regions in CpG islands. D) Overall methylation level for the ASM regions. E) Methylation differences between parental alleles for the ASM regions. CGI: CpG island.

Figure S7. Examples of imprinted ASM loci. Genome browser views of the imprinted ASM loci: *H19/IGF2*, *DIRAS3*, and *GTL2/DLK1*. The $-\log_{10}(\text{p-value})$ for ASM significance (black) and the CpG methylation levels for total (green), *B. t. indicus* allele (blue, paternal), and *B. t. taurus* allele (red, maternal) are also shown. Each bar represents a single CpG site. Mat: maternal allele; Pat: paternal allele.

Figure S8. Examples of ASM regions at non-imprinted loci. A), C), and E). Genome browser views of the non-imprinted ASM loci: *NRDE2*, *PLCL2*, and *SCARF1*. The $-\log_{10}(\text{p-value})$ for ASM significance (black) and the CpG methylation levels for total (green), *B. t. indicus* allele (blue, paternal), and *B. t. taurus* allele (red, maternal) are also shown. Each bar represents a single CpG site. B), D), and F). Allelic expression percentage in skeletal muscle of the control fetuses. Mat: maternal allele; Pat: paternal allele.

Figure S9. A). Mean methylation level differences of the DEGs between controls and LOS #2 and #3 at the TSS region (-1.5kb to +500bp). Only DEGs that have at least 2-fold change are shown here. **B).** Number of CpGs and the mean methylation difference of each DMR identified when comparing all LOS fetuses to the controls.

Table S1. Summary of the reads alignment and bisulfite conversion rate for each WGBS library

Libraries	Bodyweight	Gestational day	Raw reads	Aligned reads	Aligned reads (%)	Uniquely aligned reads	Uniquely aligned reads (%)	Bisulfite conversion rate (%)
Control #1	392g	Day 104	396,328,924	333,030,504	84.0	267,417,844	67.5	99.73
Control #2	406g	Day 105	428,296,878	331,841,555	77.5	266,934,993	62.3	99.77
Control #3	408g	Day 106	418,901,079	336,561,860	80.3	268,410,454	64.1	99.75
Control #4	416g	Day 106	355,810,944	289,745,138	81.4	229,015,740	64.4	99.56
LOS #1	514g	Day 106	424,025,153	338,241,972	79.8	268,667,106	63.4	99.77
LOS #2	518g	Day 105	431,258,953	333,939,082	77.4	260,337,387	60.4	99.75
LOS #3	620g	Day 106	397,474,455	320,530,155	80.6	254,012,990	63.9	96.48
LOS #4	714g	Day 105	421,457,025	338,500,780	80.3	269,718,690	64.0	99.74

Table S2. Reads coverage at CpG sites

Sample	≥1 read (%)	≥3 reads (%)	≥5 reads (%)	≥8 reads (%)	≥10 reads (%)
Control #1	25,422,282 (92.3)	24,523,876 (89.0)	22,835,084 (82.9)	18,144,557 (65.9)	13,953,964 (50.7)
Control #2	25,380,860 (92.2)	24,233,110 (88.0)	22,007,331 (79.9)	16,221,532 (58.9)	11,560,309 (42.0)
Control #3	25,420,791 (92.3)	24,414,185 (88.6)	22,484,754 (81.6)	17,326,183 (62.9)	12,984,193 (47.1)
Control #4	25,260,324 (91.7)	23,354,088 (84.8)	19,582,672 (71.1)	11,582,224 (42.0)	6,717,916 (24.4)
LOS #1	25,431,432 (92.3)	24,491,532 (88.9)	22,732,952 (82.5)	17,931,727 (65.1)	13,732,790 (49.9)
LOS #2	25,427,128 (92.3)	24,505,589 (89.0)	22,772,178 (82.7)	18,057,629 (65.6)	13,958,856 (50.7)
LOS #3	25,426,184 (92.3)	24,550,790 (89.1)	22,958,659 (83.4)	18,522,677 (67.3)	14,501,482 (52.7)
LOS #4	25,416,233 (92.3)	24,368,611 (88.5)	22,334,119 (81.1)	16,895,545 (61.3)	12,340,998 (44.8)

*Percentage of CpGs in the reference assembly UMD3.1 covered by at least 1, 3, 5, 8, and 10 reads.

Table S3. ASM regions identified in bovine fetal skeletal muscle using WGBS

# of DMRs	Chromosome	Start position	End position	Methylation bias toward:	Manual annotation	Close to known imprinted loci (<1MB)?	Described in cattle before?
1	1	42,531,131	42,531,182	Indicus	1st exon of <i>CPOX</i>	No	No
2	1	155,832,527	155,832,651	Taurus	last exon of <i>PLCL2</i>	No	No
3	2	108,192,408	108,192,519	Taurus	last exon of <i>CHPF</i>	No	No
4	2	133,907,427	133,907,504	Taurus	<i>CAPZB</i> intron	No	No
5	2	134,688,529	134,688,625	Indicus	Intergenic	No	No
6	3	14,945,751	14,945,783	Indicus	1st intron of <i>SYT11</i>	No	No
7	3	20,813,565	20,813,646	Taurus	upstream of <i>LOC516742</i>	No	No
8	3	77,572,982	77,573,138	Taurus	2nd exon of <i>DIRAS3</i>	Yes (<i>DIRAS3</i> domain)	No
9	3	92,054,107	92,054,197	Taurus	1st exon of <i>C3H1ORF177</i>	No	No
10	3	92,081,371	92,082,016	Indicus	last exon of <i>TTC22</i>	No	No
11	3	116,009,370	116,009,556	Indicus	<i>AGAP1</i> intron	No	No
12	4	95,065,424	95,065,580	Taurus	<i>MEF2</i>	Yes (<i>MEF2</i> domain)	Yes
13	4	95,422,095	95,422,142	Indicus	Intergenic	No	No
14	4	114,468,595	114,468,839	Taurus	<i>AGAP3</i> intron	No	No
15	4	119,913,118	119,913,256	Indicus	<i>PTPRN2</i> intron	No	No
16	5	118,121,533	118,121,692	Indicus	<i>TBC1D22A</i> intron	No	No
17	5	120,976,901	120,976,977	Taurus	~3.5kb downstream of <i>PIM3</i>	No	No
18	6	37,508,900	37,510,231	Taurus	<i>NAP1L5</i>	Yes (<i>NAP1L5</i> domain)	No
19	7	2,485,761	2,486,047	Taurus	<i>HIST3H2A</i>	No	No
20	7	44,766,662	44,767,403	Indicus	1st exon of <i>LOC101903623</i>	No	No
21	7	46,025,028	46,025,151	Taurus	<i>SOWAHA</i>	No	No
22	9	82,472,274	82,473,917	Taurus	1st exon of <i>PLAGL1</i>	Yes (<i>PLAGL1</i> domain)	Yes
23	9	97,663,961	97,664,174	Taurus	2nd intron of <i>IGF2R</i>	Yes (<i>IGF2R</i> domain)	Yes
24	10	28,299,731	28,300,013	Indicus	Intergenic	No	No
25	10	74,409,535	74,409,990	Taurus	1st intron of <i>SYT16</i>	No	No
26	10	102,886,935	102,887,023	Taurus	last exon of <i>KCNK13</i>	No	No
27	10	102,904,503	102,904,679	Indicus	Intergenic	No	No
28	10	102,947,738	102,948,378	Taurus	<i>NRDE2</i>	No	No
29	11	1,747,119	1,747,211	Indicus	1st exon of <i>MALL</i>	No	No
30	11	70,646,087	70,646,224	Indicus	last exon of <i>ALK</i>	No	No
31	11	78,385,525	78,386,123	Taurus	Intergenic	No	No
32	11	87,218,224	87,218,388	Taurus	<i>HPCAL1</i> intron	No	No
33	12	11,365,703	11,366,274	Taurus	1st exon of <i>KBTBD6</i>	No	No
34	12	16,102,307	16,102,589	Indicus	<i>ZC3H13</i> exon	No	No
35	13	39,608,336	39,608,402	Taurus	<i>SLC24A3</i> exon	No	No
36	13	43,033,891	43,034,188	Indicus	<i>ACSS1</i>	No	No
37	13	58,033,921	58,039,200	Taurus	<i>GNASXL</i> DMR	Yes (<i>GNASXL</i> / <i>GNAS</i> domain)	Yes
38	13	58,045,896	58,049,131	Indicus	<i>GNAS</i> DMR	Yes (<i>GNASXL</i> / <i>GNAS</i> domain)	Yes
39	13	58,056,771	58,057,077	Taurus	~8kb upstream of <i>GNAS</i>	Yes (<i>GNASXL</i> / <i>GNAS</i> domain)	No
40	13	64,131,680	64,131,870	Taurus	Intergenic	No	No
41	13	65,489,399	65,490,377	Taurus	Intergenic	No	No
42	13	75,947,946	75,948,064	Taurus	Intergenic	No	No
43	15	38,159,369	38,159,492	Taurus	~1.5kb upstream of <i>CALCA</i>	No	No

44	15	38,322,072	38,323,223	Taurus	Intergenic	No	No
45	15	38,342,218	38,342,321	Taurus	~2kb upstream of <i>CALCB</i>	No	No
46	15	38,411,342	38,412,590	Taurus	overlapping 1st exon of <i>LOC784945</i>	No	No
47	17	73,848,859	73,849,139	Indicus	downstream of <i>RTDR1</i> and <i>LOC531152</i>	No	No
48	18	2,490,132	2,490,721	Taurus	<i>LDHD</i>	No	No
49	18	44,226,616	44,226,818	Taurus	Intergenic	No	No
50	18	54,853,816	54,853,887	Indicus	last exon of <i>SLC8A2</i>	No	No
51	18	57,589,967	57,590,290	Taurus	<i>SIGLEC12</i>	No	No
52	18	58,130,552	58,130,966	Taurus	1st exon of <i>ZNF613</i>	No	No
53	18	64,223,889	64,224,080	Taurus	<i>MGC157368</i>	YES (<i>PEG3</i> domain)	No
54	18	64,916,693	64,917,122	Indicus	1st exon of <i>LOC101906235</i>	No	No
55	19	15,535,373	15,535,514	Taurus	Intergenic	No	No
56	19	23,300,236	23,300,388	Taurus	1st intron of <i>SLC24A2</i>	No	No
57	19	23,317,855	23,318,068	Taurus	<i>SCARF1</i> exon	No	No
58	19	28,879,630	28,880,069	Taurus	<i>CCDC42</i> intron	No	No
59	19	61,552,878	61,553,203	Taurus	Intergenic	No	No
60	19	64,006,701	64,006,992	Indicus	Intergenic	No	No
61	20	2,210,004	2,210,070	Taurus	1st intron of <i>KCNIP</i>	No	No
62	20	67,424,767	67,425,257	Taurus	Intergenic	No	No
63	20	70,536,577	70,536,666	Taurus	Intergenic	No	No
64	21	781,530	781,640	Taurus	<i>MAGEL2</i>	Yes (<i>SNRPN</i> domain)	No
65	21	67,079,426	67,079,951	Indicus	<i>BEGAIN</i>	Yes (<i>GTL2/DLK1</i> domain)	No
66	21	67,373,112	67,373,251	Indicus	~3.5kb upstream of <i>GTL2</i>	Yes (<i>GTL2/DLK1</i> domain)	No
67	21	67,274,019	67,274,308	Taurus	~0.5kb upstream of <i>DLK1</i>	Yes (<i>GTL2/DLK1</i> domain)	No
68	21	67,448,930	67,449,005	Taurus	<i>MEG8</i> intron	Yes (<i>GTL2/DLK1</i> domain)	No
69	21	67,717,546	67,717,714	Taurus	Intergenic	No	No
70	21	70,921,482	70,921,682	Taurus	<i>KIAA0284</i>	No	No
71	22	681,862	682,055	Indicus	~3.5kb downstream of <i>VOPP1</i>	No	No
72	22	53,236,543	53,236,607	Indicus	1st exon of <i>PRSS42</i>	No	No
73	22	58,919,867	58,920,123	Indicus	Intergenic	No	No
74	22	61,191,097	61,191,283	Indicus	<i>SLC41A3</i> intron	No	No
75	23	9,681,409	9,681,520	Taurus	1st intron of <i>ARMC12</i>	No	No
76	23	52,142,619	52,142,708	Taurus	1st intron of <i>DUSP22</i>	No	No
77	24	21,945,216	21,945,786	Taurus	Intergenic	No	No
78	25	39,692,219	39,692,665	Taurus	1st intron of <i>RADIL</i>	No	No
79	25	39,969,167	39,969,440	Indicus	1st exon of <i>LOC101907846</i>	No	No
80	25	42,378,294	42,378,354	Taurus	last exon of <i>SUN1</i>	No	No
81	26	30,643,478	30,643,604	Taurus	Intergenic	No	No
82	29	42,176,000	42,176,138	Indicus	Intergenic	No	No
83	29	49,552,793	49,554,961	Taurus	<i>KCNQ1</i> intron	Yes (<i>KCNQ1</i> domain)	Yes
84	29	50,147,554	50,148,349	Indicus	~2.5kb upstream of <i>H19</i>	Yes (<i>H19/IGF2</i> domain)	Yes
85	29	50,149,689	50,149,709	Indicus	~1kb upstream of <i>H19</i>	Yes (<i>H19/IGF2</i> domain)	No
86	29	50,151,516	50,151,573	Indicus	1st exon of <i>H19</i>	Yes (<i>H19/IGF2</i> domain)	No

Table S4. Methylation status of known imprinted DMRs in human and/or mouse in bovine fetal skeletal muscle

Known imprinted DMRs	ASM in mouse?	ASM in human?	ASM in cow?	ASM in cow supported by:			Methylation bias	DMR coordinates (based on allelic reads)	Notes
				ASM with ≥ 4 WGBS reads/allele	ASM with ≥ 3 WGBS reads/allele	ASM with ≥ 3 WGBS reads/allele			
<i>AIRN/IGF2R*</i>	✓	✓	✓	✓	✓	✓	Taurus (M)	Chr9: 97,663,961-97,664,174	
<i>CDKN1C promoter</i>	✓	✗	✗						
<i>DIRAS3^</i>	✗	✓	✓	✓	✓	✓	Taurus (M)	Chr3: 77,572,982-77,573,138	Hypomethylation around TSS
<i>DLK1</i>	✓	✓	✓	✓	✓	✓	Taurus (M)	Chr21: 67,274,019-67,274,308	
<i>DLK1-GTL2*</i>	✓	✓	✓	✓	✓	✓	Indicus (P)	Chr21: 67,373,112-67,373,251	
<i>FAM50B^</i>	✗	✓	No ortholog						Not annotated in UMD3.1, Blast analyses indicate no ortholog in cattle
<i>GNAS1A*</i>	✓	✓	✓	✓	✓	✓	Taurus (M)	Chr13: 58,056,771-58,057,077	
<i>GPR1/ZDBF2*</i>	✓	✓	✗						No evidence of ASM across the intergenic region between <i>GPR1</i> and <i>ZDBF2</i>
<i>GRB10*</i>	✓	✓	?						Poor SNPs coverage
<i>H19 promoter</i>	✓	✓	✓	✓	✓	✓	Indicus (P)	Chr29: 50,151,516-50,151,573	
<i>H19 ICR*</i>	✓	✓	✓	✓	✓	✓	Indicus (P)	Chr29: 50,147,554-50,148,349	
<i>HERC3/NAP1L5*</i>	✓	✓	✓	✓	✓	✓	Taurus (M)	Chr6: 37,508,900-37,510,231	
<i>IMPACT*</i>	✓	✗	✗				NA		Hypomethylation around TSS
<i>INPP5F*</i>	✓	✓	✓	✗	✓	✓	Taurus (M)	Chr26: 40,282,446-40,282,480	
<i>KvDMR1*</i>	✓	✓	✓	✓	✓	✓	Taurus (M)	Chr29: 49,552,793-49,554,961	
<i>L3MBTL1^</i>	✗	✓	✗				NA		Hypomethylation around TSS
<i>MAGE12</i>	✓	✓	✓	✓	✓	✓	Taurus (M)	Chr21: 781,530-781,640	
<i>METS2/H13*</i>	✓	✓	?						Poor SNPs coverage
<i>MEST (PEG1)*</i>	✓	✓	✓	✓	✓	✓	Taurus (M)	Chr4: 95,065,424-95,065,580	
<i>MKRN3</i>	✓	✓	✗						Both parental alleles are partially methylated around TSS
<i>NESP55 (GNAS)</i>	✓	✓	✓	✓	✓	✓	Indicus (P)	Chr13: 58,045,896-58,049,131	
<i>NESPAS/GNASXL*</i>	✓	✓	✓	✓	✓	✓	Taurus (M)	Chr13: 58,056,771-58,057,077	
<i>NDN</i>	✓	✓	✗						Both parental alleles are hypomethylated around TSS
<i>NNAT/BLCAP*</i>	✓	✓	?						Poor SNPs coverage
<i>PEG3/Us29*</i>	✓	✓	?						Poor SNPs coverage
<i>PEG10/SGCE*</i>	✓	✓	✓	✗	✓	✓	Taurus (M)	Chr13: 11,910,836-11,912,554	
<i>PEG12</i>	✓	No ortholog	✓						Not annotated in UMD3.1, Blast analyses indicate no ortholog in cattle
<i>PEG13/TRAPPC9*</i>	✓	✓	?						Poor SNPs coverage
<i>PLAGL1*</i>	✓	✓	✓	✓	✓	✓	Taurus (M)	Chr9: 82,472,274-82,473,917	
<i>RASGRF1*</i>	✓	✗	✓						Both parental alleles are hypomethylated around TSS
<i>RB1^</i>	✗	✓	✗						Both parental alleles are hypomethylated around TSS
<i>SNURF/SNRPN*</i>	✓	✓	✓	✗	✓	✓	Taurus (M)	Chr21: 25,687-26,167	
<i>SLC38A4*</i>	✓	?	✗						Hypomethylation around TSS
<i>ZN597/NA460^</i>	✗	✓	✗						Hypomethylation around TSS
<i>ZRSR1/COMMD1*</i>	✓	✗	✗						Hypomethylation around TSS

Note:

* Germline DMRs in mouse

^ ASM in human but not in mouse

Chapter 4 Global assessment of imprinted gene expression in the bovine conceptus by next generation sequencing

4.1 Abstract

Genomic imprinting is an epigenetic mechanism that leads to parental-allele-specific gene expression. Approximately 150 imprinted genes have been identified in humans and mice but less than 30 have been described as imprinted in cattle. For the purpose of *de novo* identification of imprinted genes in bovine, we determined global monoallelic gene expression in brain, skeletal muscle, liver, kidney and placenta of day ~105 *Bos taurus indicus* × *Bos taurus taurus* F₁ conceptuses using RNA sequencing. To accomplish this, we developed a bioinformatics pipeline to identify parent-specific SNP alleles after filtering A-to-I RNA editing sites. We identified 53 genes subject to monoallelic expression. Twenty three are genes known to be imprinted in the cow and an additional 7 have previously been characterized as imprinted in human and/or mouse that have not been reported as imprinted in cattle. Of the remaining 23 genes, we found that 10 are uncharacterized or unannotated transcripts located in known imprinted clusters, whereas, the other 13 genes are distributed throughout the bovine genome and are not close to any known imprinted clusters. To exclude potential *cis*-eQTL effects on allele expression, we corroborated the parental specificity of monoallelic expression in day 86 *Bos taurus taurus* × *Bos taurus taurus* conceptuses and identified eight novel bovine imprinted genes. Further, we identified 671 candidate A-to-I RNA editing sites and describe random X-inactivation in day 15 bovine extraembryonic membranes. Our results expand the imprinted gene list in bovine and demonstrate that monoallelic gene

expression can be the result of *cis*-eQTL effects.

4.2 Introduction

In mammals, approximately 150 genes have been shown to be expressed in a parental-allele-specific manner (Bartolomei and Ferguson-Smith, 2011). These genes, collectively known as imprinted genes, are essential for normal embryonic growth, placental function, and postnatal behavior (Peters, 2014) and their misregulation has been linked to several developmental syndromes and types of cancers (Kalish et al., 2014; Lim and Maher, 2010). Most imprinted genes are organized in clusters and are regulated by cluster-specific DNA elements termed imprinting control regions (ICR; Bartolomei and Ferguson-Smith, 2011). ICRs are marked with DNA methylation and histone post-translational modifications in a sex-specific manner during gametogenesis (Bartolomei and Ferguson-Smith, 2011). These epigenetic signatures are maintained during development and serve to differentiate the parental alleles in somatic cells, when both genomes are found within the same nucleus (Bartolomei and Ferguson-Smith, 2011). Since the discovery of the first three imprinted genes in 1991 (Barlow et al., 1991; Bartolomei et al., 1991; DeChiara et al., 1991), more than 100 imprinted genes have been identified in humans and mice (<http://igc.otago.ac.nz>; http://www.har.mrc.ac.uk/research/genomic_imprinting/). However, the list of imprinted genes is not yet comprehensive as their expression can vary by tissue and stage of development (Bartolomei and Ferguson-Smith, 2011; Peters, 2014).

Many fewer imprinted genes (n=34) have been reported in domestic farm animal species (Tian, 2014). In addition, most imprinted genes in cow, pig, sheep, and rabbit have been characterized in studies designed to corroborate the allele-specific expression of genes previously described as imprinted in human and/or mouse (Bischoff et al., 2009; Chen et al., 2015; Chen et al., 2013; Okamura et al., 2005; Robbins et al., 2012; Young et al., 2003; Zaitoun and Khatib, 2006). As in human and mouse, allele specificity in monoallelic expression of these genes is a requirement for proper embryonic and fetal growth; loss-of-imprinting (LOI) has been associated with abnormal development of the conceptus (Chen et al., 2013; Farin et al., 2006; Tian, 2014; Young et al., 2001). One such example is large offspring syndrome (LOS) in bovine and ovine (Young et al., 1998), a fetal overgrowth condition that is phenotypically and epigenetically similar to the loss-of-imprinting condition Beckwith-Wiedemann syndrome in human (Chen et al., 2013). In a previous study (Chen et al., 2015), we analyzed a group of genes known to have imprinted expression in human and/or mouse and showed loss of monoallelic expression and differential transcript abundance of multiple imprinted genes in LOS. We also found that some of the genes (*e.g.*, *COPG2*, *IMPACT*, *HTR2A*) do not express in the expected monoallelic fashion but rather are biallelically expressed in the bovine conceptus. The species specificity of genomic imprinting suggests that certain genes may only be imprinted in cattle; therefore, *de novo* identification of imprinted genes using genome-wide scale approaches is required in order to achieve a more thorough understanding of this epigenetic mechanism and its role in fetal development of this economically important species.

Advances in high throughput sequencing technology provide an unprecedented opportunity for the discovery of imprinted genes at the transcriptome level and has led to the discovery of additional imprinted genes in mice and humans (Wang and Clark, 2014). In mice, transcriptome wide identification of imprinted genes relies on RNA sequencing (from here on referred to as RNAseq) of F₁ offspring resulting from reciprocal crosses of distantly related inbred mice to identify parental-allele-specific gene expression (Babak et al., 2008; Wang et al., 2008). While this approach has been successful, bioinformatics analyses alone can suffer from a high false positive rate due to the potential technical artifacts of RNAseq (DeVeale et al., 2012). Thus, the use of next generation sequencing to identify imprinted genes requires independent validation through traditional methods (*i.e.*, Sanger sequencing). In human, systemic characterization of genomic imprinting using RNAseq has recently been performed in 46 tissues from 178 adults (Baran et al., 2015). This study highlights the widespread tissue-specificity of genomic imprinting as well as the species-specificity of imprinting (some genes imprinted in human are not imprinted in the mouse) (Baran et al., 2015).

Beyond genomic imprinting, X chromosome inactivation (XCI) is another epigenetic phenomenon that can regulate gene dosage by silencing one of the parental alleles in females (Lee and Bartolomei, 2013). Contrary to parental-origin-dependent silencing of imprinted genes, dosage compensation of X-linked genes in most organs is achieved by randomly silencing one of the X chromosomes (Lyon, 1961). However, in extra-embryonic tissues of mouse and rat, XCI is imprinted and it is always the paternal X chromosome that is inactivated (Huynh and Lee, 2001; Wake et al., 1976). It has not been resolved whether XCI is imprinted or random in the human placenta (Lee and

Bartolomei, 2013) and, in cattle, a study based on the allele-specific expression of the X-linked gene *MAOA* indicates that XCI is imprinted in the placenta with preferential silencing of the paternal X chromosome (Xue et al., 2002).

In this study, we determined global allele-specific gene expression in day ~105 *Bos taurus indicus* (*B. t. indicus*) $\times$ *Bos taurus taurus* (*B. t. taurus*) F₁ conceptuses (gestation length ~280 days) using RNAseq. We developed an analysis pipeline to identify monoallelically expressed genes by resolving several technical challenges such as correction of allelic mapping bias of RNAseq reads and filtering potential RNA editing sites in RNAseq data and then validated our findings by Sanger sequencing. Further, we corroborated the parental specificity of monoallelic expression in day 86 *B. t. taurus* $\times$ *B. t. taurus* (Holstein breed) conceptuses to exclude potential *cis*-eQTL effects on allele expression. Finally, we describe random X-inactivation in day 15 bovine extraembryonic membranes.

4.3 Results

Pipeline for identification of monoallelically expressed genes in bovine using RNAseq

The identification of imprinted gene expression requires the use of DNA polymorphisms to identify parental origin of the alleles in an F₁ individual. For bovids, it is not always possible to know the identity of the parental alleles, for example, if those individuals were conceived with oocytes obtained from abattoir ovaries. This fact, together with the fact that cattle are not inbred, makes the global identification of genes

subject to allele-specific expression using RNAseq a challenging task. Another challenge when working in bovine is encountered when aligning RNAseq reads to the publically available reference genome (*i.e.*, UMD3.1) because only one *B. t. taurus* female (*i.e.*, Hereford breed) is documented with only one of her alleles recorded. In the present study, we developed a bioinformatics pipeline (Fig. 1) to identify candidate imprinted genes in cattle using RNAseq data from four gestation day ~105 female bovine conceptuses (fetus and placenta) generated from the breeding of one Nelore bull (ABS CSS MR N OB 425/1 677344 29NE0001 97155 [*i.e.*, *B. t. indicus*]) to four Holstein breed (*B. t. taurus*) females of unknown genetics (Chen et al., 2013). We utilized this breeding scheme, which involves fertile mating between subspecies of cattle that diverged ~620,000 years ago (MacHugh et al., 1997), to increase the likelihood of finding informative polymorphisms with which to ascribe parental origin of the nucleic acid being studied.

First, the sire's DNA was subjected to next generation sequencing (DNAseq) to identify all single nucleotide polymorphisms (SNPs) between his genome and the *B. t. taurus* reference genome UMD3.1 build. In total, 347.3 million 2×100 bp paired-end reads (~20X coverage of the bovine reference genome) were obtained (Fig. S1A) and used to genotype the *B. t. indicus* sire. We used Genome Analysis Toolkit (GATK; McKenna et al., 2010) and SAMtools (Li, 2011) pipelines for SNP calling and only SNPs identified by both pipelines (*i.e.*, 93.11%; Fig. 1 and Fig. S1B and C) were used to edit the reference genome to generate a pseudo *B. t. indicus* genome. Aligning RNAseq reads to a non-identical reference genome has been shown to introduce alignment bias (Degner et al., 2009; Stevenson et al., 2013) so we combined the reference genome and the pseudo

Figure 1. Summary of the pipelines used in this study. Top left and right of figure show the bioinformatics methods used to identify monoallelically expressed genes in day ~105 *Bos taurus indicus* (*B. t. indicus*) × *Bos taurus taurus* (*B. t. taurus*) F₁ conceptuses. Bottom left shows the pipeline used to corroborate the findings of the bioinformatics analyses. SNP = single nucleotide polymorphism.

B. t. indicus genome to create a custom diploid genome. RNAseq reads from the *B. t. indicus* × *B. t. taurus* F₁ conceptuses were mapped to the diploid genome using both the HISAT2 (Kim et al., 2015) and BWA (Li and Durbin, 2009) pipelines and only variants identified by both methods were retained for further analyses (Fig. 1) to minimize the potential effects of false positives.

Genotyping the day ~105 B. t. indicus × B. t. taurus F₁ conceptuses using RNAseq

RNAseq data were obtained from brain, kidney, liver, skeletal muscle and placenta of the four conceptuses (*i.e.*, Control #1, #2, #3, and #4; Table S1) and were aligned to the custom *B. t. indicus/B. t. taurus* diploid genome. We adapted the SNPiR pipeline (Piskol et al., 2013) to identify SNPs using all RNAseq reads from each conceptus. To minimize false positive SNP calls, in addition to the default pipeline (BWA; Li and Durbin, 2009; Piskol et al., 2013), we also mapped the reads using HISAT2 (Kim et al., 2015) and only retained variants common to both alignment strategies (Fig. S2A, Table S1). The identified RNA variants in the F₁ conceptuses were compared to known bovine dbSNP entries (<http://www.ncbi.nlm.nih.gov/SNP>) and the identified *B. t. indicus* SNPs. We found that ~98% of the RNA variants were matched to the bovine dbSNP entries and/or were supported by the *B. t. indicus* DNaseq reads and we refer to them as “genomic variants” (Fig. S2B). The genomic variants exhibited a ~2.5 transition/transversion ratio (see methods; Fig. S2C) while the remaining ~2% RNA variants (Fig. S2B), hereinafter referred to as “RNA-DNA differences (RDDs)”, exhibited 14-18 transition/transversion ratios and were enriched for A>G and T>C mismatches (Fig. S2D).

Identification and validation of A-to-I RNA editing sites in B. t. indicus × B. t. taurus conceptuses

We reasoned that some of the RDDs with A>G and T>C mismatches resulted from adenosine-to-inosine (A-to-I) RNA editing, a tissue specific post-transcriptional mechanism in which adenosine is converted to inosine in RNA duplexes by adenosine deaminase acting on RNAs (ADARs; Nishikura, 2010). A-to-I RNA editing is detected as A>G mismatches in cDNA (Nishikura, 2010). To confirm the true identity of A-to-I RNA editing, we retrieved the variant information (*i.e.*, read counts for both the reference and alternative alleles) in the RNAseq data of each tissue from each individual (Fig. 2A). Further, we required that each RDD have at least 10 reads coverage per tissue per conceptus to discriminate rare SNPs or sequencing errors from true RNA editing sites. For brain, liver, and skeletal muscle, only RDDs shared by Controls #1, #3, and #4 were used to identify RNA editing sites since the RNAseq reads for these tissues from Control #2 were sequenced at 50 bp length instead of 100 bp length for the other samples (Table S1). We identified 404, 275, 266, 208, and 180 RNA variants in brain, kidney, liver, skeletal muscle, and placenta, respectively (Fig. 2A). Of these, the majority (> 72%) were A>G mismatches (Fig. 2A and B) and we considered them to putatively represent A-to-I editing sites (Table S2; link to Table S2: <http://www.tandfonline.com/doi/full/10.1080/15592294.2016.1184805>).

We analyzed 7 predicated editing sites in four genes (*i.e.*, *COPA*, *SEC16A*, *GRIK2*, and *GABRA3*) using Sanger sequencing to confirm that the editing site did not represent a heterozygous SNP in DNA and also to corroborate the editing of the RNA

Figure 2. Identification of adenosine-to-inosine (A-to-I) RNA editing sites in day ~105 *B. t. indicus* × *B. t. taurus* F₁ conceptuses. **A).** Pipeline used to identify which RNA-DNA differences (RDDs) were potential A-to-I RNA editing sites. RDDs refer to RNA variants not found in the bovine dbSNP database nor in the *B. t. indicus* sire DNaseq reads. RNA variants were identified using all the RNAseq reads of each conceptus. Only RDDs with ≥ 10 reads coverage per tissue per conceptus and had strand information of the transcript template were used to identify potential editing sites. *Note: for brain, liver, and skeletal muscle only RDDs shared by Control #1, #3, and #4 were used to identify RNA editing sites since the RNAseq reads for those tissues in Control #2 were sequenced at 50 bp length instead of 100 bp length for all other samples. **B).** Variant type distribution of the RDDs. “A>G” variants were considered candidate A-to-I RNA editing sites (detailed information of these sites may be found in Table S2). **C)** and **D).** Validation of the identified A-to-I RNA editing sites by Sanger sequencing. Red arrows indicate the editing sites. Panel **C** shows an example of A-to-I editing in the coding sequence (CDS) of *COPA*, which can cause an amino acid change (Ile→Val) while panel **D** shows two editing sites in the 3’ untranslated region (3’UTR) of *SEC16A*. Further verification of A-to-I RNA editing sites may be found in Figure S3.

molecules (a second “G” peak in the sequencing chromatogram). We were able to validate 6 of 7 to be true A-to-I editing sites (Fig. 2C and D, Fig. S3).

Characterization and species conservation of candidate A-to-I editing sites

Analysis of the putative A-to-I editing events indicated that ~96% (643/671) of these sites were within noncoding regions such as UTRs and introns and approximately two percent (15/671) were located in coding sequences (CDS; Fig. S4). This observation is consistent with findings in human and mouse that the majority of A-to-I RNA editing events occur in non-coding regions (Ramaswami et al., 2013). Of the sites located in CDS', 14 could cause an amino acid change (Table S2).

To determine whether the identified A-to-I RNA editing sites in bovine were conserved in human and/or mouse, we retrieved 80 bp of flanking sequences (40 bp upstream and 40 bp downstream; Pinto et al., 2014) and aligned the 81 nucleotides to the human and mouse genomes using BLAST . A valid BLAST hit with > 86% identity was required to ascribe a site as being conserved between species (Pinto et al., 2014). With this criterion, 20 of the sites were predicted to be conserved between bovine and human and/or mouse. Table 1 shows that 13 out of 20 editing sites are located in the CDS and 12 of those editing events can cause an amino acid change.

*Identification of genes with monoallelic expression in *B. t. indicus* × *B. t. taurus* conceptuses*

As the RDDs were enriched with A-to-I editing sites, we used only genomic variants for the identification of monoallelically expressed genes. We first assigned parental origin of each allele within the F₁ RNAseq reads by determining which SNPs

Table 1. A-to-I RNA editing sites in bovine that are conserved in human and/or mouse

Chromosome	Tissue type	Gene	Gene section	Outcome	Conservation
Chr3:9479773	Brain, kidney, liver, skeletal muscle, placenta	<i>COPA</i>	CDS	Nonsyn (Ile->Val)	Human
Chr4:114454773	Skeletal muscle, placenta	<i>FASTK</i>	Intron	Unknown	Human, mouse
Chr5:76189492	Brain	<i>ELFN2</i>	Intron	Unknown	Human
Chr7:71024261	Brain	<i>CYFIP2</i>	CDS	Nonsyn (Lys->Glu)	Human, mouse
Chr9:48598875	Brain	<i>GRIK2</i>	Intron	Unknown	Human, mouse
Chr9:48598884	Brain	<i>GRIK2</i>	Intron	Unknown	Human
Chr9:48598902	Brain	<i>GRIK2</i>	Intron	Unknown	Human, mouse
Chr9:48600509	Brain	<i>GRIK2</i>	Intron	Unknown	Human
Chr9:48600848	Brain	<i>GRIK2</i>	CDS	Nonsyn (Gln->Arg)	Human, mouse
Chr9:48600865	Brain	<i>GRIK2</i>	CDS	Syn (Gly->Gly)	Human
Chr9:48634035	Brain	<i>GRIK2</i>	CDS	Nonsyn (Tyr->Cys)	Human, mouse
Chr9:48634048	Brain	<i>GRIK2</i>	CDS	Nonsyn (Ile->Val)	Human, mouse
Chr12:15671536	Brain, kidney, liver, skeletal muscle, placenta	<i>COG3</i>	CDS	Nonsyn (Ile->Val)	Human, mouse
Chr13:67116409	Brain, skeletal muscle, placenta	<i>BLCAP</i>	CDS	Nonsyn (Lys->Arg)	Human
Chr13:67116439	Brain, kidney, skeletal muscle, placenta	<i>BLCAP</i>	CDS	Nonsyn (Gln->Arg)	Human, mouse
Chr13:67116448	Brain, kidney	<i>BLCAP</i>	CDS	Nonsyn (Tyr->Cys)	Human, mouse
Chr17:42806463	Brain	<i>GRIA2</i>	Intron	Unknown	Human, mouse
Chr17:42806724	Brain	<i>GRIA2</i>	CDS	Nonsyn (Gln->Arg)	Human, mouse
Chr22:39005364	Brain	<i>CADPS</i>	CDS	Nonsyn (Glu->Gly)	Human, mouse
ChrX:34866822	Brain	<i>GABRA3</i>	CDS	Nonsyn (Ile->Met)	Human, mouse

Nonsyn: nonsynonymous editing; Syn: synonymous editing

were found in the sire's DNaseq reads. As illustrated in Figure S5, the only SNPs that were used to assign parental alleles were those that were homozygous in the *B. t. indicus* sire and that had at least one allele different from the allele found in the sire in the F₁ RNAseq data. Following assignment of the parental alleles, allele-specific read counts covering each of the SNPs in the same gene were aggregated as previously described (DeVeale et al., 2012) and a binomial test was used to detect consistent allelic expression bias (false discovery rate (FDR) < 0.05; Table S3 and S4). As the annotation of the bovine genome is imperfect, we further manually annotated the candidate genes using the Integrative Genomic Viewer (IGV; Robinson et al., 2011). We identified a total of 53 genes subject to monoallelic expression in at least one of the analyzed tissues (*i.e.*, 33 in brain, 36 in kidney, 38 in liver, 36 in muscle, and 35 in placenta; Table S3 and S4; link to Table S3-4: <http://www.tandfonline.com/doi/full/10.1080/15592294.2016.1184805>). Of the identified 53 genes, 23 were known to be imprinted in the cow (also in human and/or mouse; <http://igc.otago.ac.nz/>; http://www.har.mrc.ac.uk/research/genomic_imprinting/) and 7 had previously been characterized as imprinted in human and/or mouse but had not been reported to be imprinted in cow (Table S5; link to Table S5: <http://www.tandfonline.com/doi/full/10.1080/15592294.2016.1184805>). Of the remaining 23 genes, 10 were uncharacterized or unannotated transcripts located in known imprinted clusters, whereas the other 13 genes were distributed throughout the bovine genome and were not located close to any known imprinted clusters (Table S5).

Validation of the imprinted genes previously identified in human and/or mouse

We used PCR followed by Sanger sequencing to validate the allele specific expression of *GNASXL* (*GNAS*/*GNASXL* domain), *COPG2IT1* (*MEST*/*COPG2* domain), *MEG8* and *MEG9* (*GTL2/DLK1* domain), *MIMT1*, *USP29*, *ZIM2* (*PEG3/USP29* domain), *PON3* and *PPP1R9A* (*PEG10/SGCE* domain), and *MKRN3* (*SNRPN* domain). The other 20 imprinted genes (Table S5) were not validated because we have previously described these genes to be imprinted (Chen et al., 2015). In the *GNAS* domain, we identified *GNASXL* as a paternally expressed transcript isoform which has a unique first exon but shares identical sequences from exon 2 to the end of the transcript with the maternally expressed transcript *GNAS* (also known as *NESP55* in cattle [NM_001271771.1], Fig. 3A). By using the SNP located in the last exon of these transcripts (Fig. 3A) we validated the paternal expression of *GNASXL* and maternal expression of *GNAS* (Fig. 3B). In addition, analyses of two other SNPs (one specific to the *GNASXL* first exon and the other specific to the *GNAS* first exon) confirmed this result (Fig S6A). Further, DNA methylation of the *GNASXL* and *GNAS* promoter regions was confirmed using bisulfite mutagenesis followed by cloning and sequencing. The *GNASXL* differentially methylated region (DMR; the locus' putative ICR) showed hypomethylation of the paternal allele and hypermethylation of the maternal allele, which was associated with the paternal expression of this transcript (Fig. 3C and Fig. S6B). To the contrary, the *GNAS* DMR was methylated on the paternal allele while unmethylated on the maternal allele, which was associated with the maternal expression of *GNAS* (Fig. 3C and Fig. S6B). In addition to *GNASXL* and *GNAS*, we verified the maternal expression of *MEG8*, *MEG9*, *PPP1R9A*, *PON3* and *ZIM2*, and paternal expression of

Figure 3.

Figure 3. Validation of the allele-specific gene expression in *GNAS/GNASXL* and *PEG3/USP29* imprinted clusters in day ~105 *B. t. indicus* × *B. t. taurus* F₁ conceptuses. **A).** Schematics of the *GNAS/GNASXL* loci (not all exons are shown and are not drawn to scale). Bent arrows represent the transcription start sites. Patterned boxes above and below the gene structure (in black) represent the *GNASXL* and *GNAS* transcripts, respectively. The red asterisk in the last exon represents the SNP used to discriminate the parental alleles for allele-specific expression analyses in panel **B**. The black ovals represent the CpGs found within the amplicon used for DNA methylation analyses (*i.e.*, panel **C**). **B).** Validation of the maternal expression of *GNAS* and paternal expression of *GNASXL* by Sanger sequencing. The SNP sites are indicated by red arrows. Left: genotyping results obtained by Sanger sequencing of the *B. t. indicus* sire and the F₁ individuals at the SNP site. Middle and right: Sanger sequencing results of RT-PCR amplicons of *GNAS* and *GNASXL*. Validation of the allelic expression of *GNAS* and *GNASXL* at other SNP sites may be found in Figure S6A. **C).** DNA methylation analyses of *GNASXL* (top) and *GNAS* (bottom) differentially methylated regions (DMRs). DNA methylation was determined using bisulfite mutagenesis, cloning, and Sanger sequencing. Parental-origin of each clone was determined using heterozygous SNPs in the F₁ individuals. Methylation of CpG sites for each parental allele was summarized from at least 7 clones and is presented with pie charts with black representing the percent of methylated cytosines and white representing the percent of unmethylated cytosines at each site. The complete bisulfite maps may be found in Figure S6B. **D).** Schematics of the *PEG3/USP29* imprinted cluster. Bent arrows represent the transcription direction of each gene. **E).** Validation of maternal expression of *MGC157368* and *LOC100298176*. Top panels for each gene are the genotyping results obtained by Sanger sequencing of the *B. t. indicus* sire and F₁ individuals. Bottom panels are the Sanger sequencing results of the RT-PCR amplicons of *MGC157368* and *LOC100298176*. The validation of the allelic expression of other genes in the *PEG3/USP29* imprinted cluster may be found in Figure S8. = genes previously identified to be imprinted in human, mouse, and bovine. = genes previously identified to be imprinted in human and/or mouse but not reported to be imprinted in bovine. = uncharacterized transcripts in known imprinted clusters.

COPG2IT1, *MIMT1*, *MKRN3*, and *USP29* in *B. t. indicus* × *B. t. taurus* F₁ hybrids (Fig. S7-S9).

Validation of the monoallelic expression of the uncharacterized/unannotated transcripts in previously known imprinted clusters

We identified 10 uncharacterized/unannotated transcripts with monoallelic expression in previously known imprinted clusters, of which four transcripts were located in the *PEG3/USP29* cluster and six transcripts were in the *SNRPN* domain. In the *PEG3/USP29* domain, the four uncharacterized transcripts (*i.e.*, *LOC508098*, *MGC157368*, *LOC104974975*, and *LOC100298176*) were located downstream of the paternally expressed gene *PEG3* and upstream of the maternally expressed gene *ZIM2* (Fig. 3D). We verified the maternal expression of *LOC508098*, *MGC157368*, *LOC104974975*, and *LOC100298176* (Fig. 3E, Fig. S8, Table S3 and S5). According to RefSeq gene annotation (Pruitt et al., 2014), *LOC508098*, *MGC157368*, and *LOC104974975* are protein-coding genes. In addition, *LOC100298176* has high protein-coding potential (0.78 coding probability) based on the Coding Potential Assessment Tool (CPAT; Wang et al., 2013). We considered a transcript with CPAT coding probability > 0.348 to possess protein-coding potential as previously reported for bovine (Billerey et al., 2014).

In the *SNRPN* domain, *LOC100848941* and *LOC101907203* were identified as two paternally expressed noncoding RNAs (ncRNAs) located downstream of the maternally expressed gene *UBE3A* (Fig. S9). In addition, two paternally expressed ncRNAs (*LOC100849023* and *LOC101907679*; Fig. 4A left panel) located upstream of

UBE3A were identified (Fig. S9). Finally, we verified paternal expression of an unannotated ncRNA (CPAT coding probability 0.06; Wang et al., 2013) with cuffmerge gene ID *XLOC_045114* (Chr21:350,550-552,040 bp, UMD3.1) which was located ~300kb upstream of the paternally expressed gene *SNRPN* (Fig. S9). To determine whether *XLOC_045114* has any human/mouse counterpart, we aligned its cDNA sequence to the human and mouse genomes using BLAST but were unable to confidently identify any matches.

Validation of monoallelically expressed genes not found in known imprinted clusters

We identified 13 genes with monoallelic expression that were located >15Mb distance from any known imprinted clusters. For these genes, nine (*i.e.*, *AOXI*, *APCS*, *AS3MT*, *C1R*, *C1S*, *CDA*, *KRT7*, *OOEP*, and *RDH16*) were annotated protein-coding genes, of which *APCS*, *AS3MT*, *C1R*, *C1S*, *CDA*, *OOEP*, and *RDH16* were monoallelically expressed from the *B. t. taurus* allele (maternal), while *AOXI* and *KRT7* were expressed from the *B. t. indicus* allele (paternal) (Fig. 4C and Fig. S10 left panels and Table S3). The remaining four genes were either uncharacterized ncRNA (*i.e.*, *LOC101905472*) or unannotated transcripts: *XLOC_009410* (Chr4:59,913,253-59,913,967, UMD3.1, CPAT coding probability: 0.38), *XLOC_012439* (Chr5:115,431,044-115,432,886, UMD3.1, CPAT coding probability: 0.36), and *XLOC_052524* (ChrX:113,053,268-113,055,164, UMD3.1, CPAT coding probability: 0.02). Consistent with RNAseq analysis, Sanger sequencing confirmed the maternal expression of *XLOC_052524*, and paternal expression of *LOC101905472*,

Figure 4.

Figure 4. Corroboration of the allele specificity in gene expression identified by RNAseq.

A-C). left panels show results obtained from the day ~105 *B. t. indicus* × *B. t. taurus* F₁ conceptuses. **A-C).** right panels show results obtained from the day 86 *B. t. taurus* × *B. t. taurus* individuals. For each gene, the top lines are the genotyping results of the F₁s and their sire while the bottom lines are the Sanger sequencing results of the RT-PCR amplicons of the F₁ conceptuses. **A).** An example of a gene that had parental-origin-dependent monoallelic expression. *LOC101907679*, an uncharacterized transcript in the *SNRPN* imprinted cluster was paternally expressed in both *B. t. indicus* × *B. t. taurus* and *B. t. taurus* × *B. t. taurus* individuals. **B).** An example of a gene that had sequence-dependent monoallelic expression. For *LOC101905472*, the “C” allele was always predominantly expressed, regardless of its parental-origin. **C).** An example of a gene (*i.e.*, *CDA*) that was monoallelically expressed in *B. t. indicus* × *B. t. taurus* F₁ hybrids but biallelically expressed in *B. t. taurus* × *B. t. taurus* individuals. Further validations of allele-specific expression in *B. t. indicus* × *B. t. taurus* F₁s and *B. t. taurus* conceptuses may be found in Figure S10-11.

XLOC_012439, and *XLOC_009410* in the *B. t. indicus* × *B. t. taurus* F₁ hybrids (Fig. 4B and Fig S10 left panels and Table S3).

Corroboration of the allele-specificity of gene expression in day 86 B. t. taurus × B. t. taurus conceptuses

Beyond genomic imprinting, *cis*-eQTL (*cis*-regulating expression quantitative trait loci) can also induce biased allelic gene expression (Wang and Clark, 2014). As we lack the ability to produce the reciprocal *B. t. taurus* × *B. t. indicus* F₁ conceptuses, we used tissues from nine day 86 *B. t. taurus* × *B. t. taurus* female conceptuses (Holstein breed all sired by bull: 507HO10723 DE-SU 527 SPUR-ET) to corroborate allelic specificity of transcription of genes not previously reported to be imprinted in bovine (n=33; Table S5). We used PCR followed by Sanger sequencing to identify informative SNPs in the *B. t. taurus* × *B. t. taurus* conceptuses. Of the analyzed 33 genes we found 20 genes with heterozygous SNPs in at least one conceptus (Fig 4, Fig S10 and S11 right panels). Of these, nine genes (*i.e.*, *COPG2IT1*, *GNAS*, *GNASXL*, *MEG8*, *LOC101907679*, *LOC100298176*, *LOC104974975*, *LOC1009849023*, *MEG8*, and *XLOC_052524*) showed consistent parental-allele-specific gene expression between the *B. t. indicus* × *B. t. taurus* and *B. t. taurus* × *B. t. taurus* conceptuses while *RDH16* and *CDA* were found to be biallelically expressed and *CIR*, *CIS*, *LOC101905472*, and *XLOC_012439* were monoallelically expressed but not imprinted (Fig 4, Fig S10 and S11). We were not able to assign parental origin to the monoallelically expressed *APCS*, *ZIM2*, *USP29*, *LOC508908*, and *XLOC_009410* alleles as the sire was heterozygous at these loci (Fig. 5, Fig. S10 and S11). Using this scheme, eight novel imprinted genes were identified (Fig. 5B) and imprinted expression of another 23 genes

Figure 5. Summary of the imprinted and monoallelically expressed genes identified in the current study. For A-C), different colors denote the type of expression of listed genes for each analyzed tissue. **A).** Genes previously reported to be imprinted in bovine. **B).** Genes not previously reported to be imprinted in bovine. **C).** Genes with monoallelic expression in the *B. t. indicus* × *B. t. taurus* F₁s but which parental origin of the transcript could not be corroborated due to lack of informative SNPs in the *B. t. taurus* × *B. t. taurus* conceptuses or as a result of the heterozygosity of their sire. # = genes with inter-individual variations in allele-specific gene expression. *LOC100848941*[^] is an isoform of *LOC100848941* (*SNRPN* imprinted cluster) only identified in brain. Allele-specific read counts and allelic expression ratios of each gene may be found in Tables S3.

in cattle was corroborated (Fig. 5A). RNAseq results indicated that 16 of these genes showed allele-specific expression in all analyzed tissues, seven had biallelic expression in at least one tissue, and eight were lowly expressed in some tissues (Fig. 5A and 5B). We also identified a paternal transcript in the *SNRPN* domain (referred here as “*LOC100848941 brain isoform*”) which connected the two paternal transcripts *LOC100848941* and *LOC101907203* (Fig. S9 top panel).

Allele-specific expression analyses of X-linked genes in bovine placenta

To determine whether X-chromosome inactivation (XCI) in bovine placenta is random or imprinted (Dindot et al., 2004; Xue et al., 2002), allele expression ratios for the annotated X-linked genes were analyzed in placenta of the four *B. t. indicus* × *B. t. taurus* F₁ female conceptuses. If the XCI is imprinted, we expect that most of the X-linked genes would be expressed from the maternal allele, except for those genes that escape XCI (Wang et al., 2012). However, if XCI is random, we expect that both parental alleles of the X-linked genes would be expressed and that allele expression ratios would be variable among individuals due to the sampling effects (Wang et al., 2012). We plotted the distribution of the allele expression ratios for both autosomal genes and X-linked genes in placenta of the four *B. t. indicus* × *B. t. taurus* F₁ conceptuses (Fig. 6A). For autosomal genes, the distribution of the allele expression ratios of all four individuals had an approximate 50:50 ratio. However, the X-linked genes showed biallelic expression but with inter-individual variation of the allele expression ratios (mean ranges from 29.0 to 66.7%; Fig. 6A), which is consistent with the distribution of the allele expression ratios for the X-linked genes in the mule and horse placenta (Wang et al., 2012). We further analyzed the allele expression ratios of 14 X-linked genes that have

Figure 6. Allele-specific expression analyses for autosomal and X-linked genes in placental membranes from day ~105 *B. t. indicus* × *B. t. taurus* F₁ hybrids. The placental membranes used for this study were the intercotyledonary region of the chorioallantois. **A).** Histograms of the allelic expression ratios for the autosomal genes (top) and X-linked genes (bottom) in placenta from each conceptus. The Y-axis represents the percent of genes with varied levels of expression from the *B. t. taurus* (maternal) allele (X axis). N = number of annotated genes with informative SNPs. Mean = the mean of the percent allelic expression. **B).** Plot of allelic expression ratios of *XIST* (filled circles), two pseudo-autosomal genes (gray diamonds), and 14 X-linked genes (open circles) from the placenta RNAseq data. The X-linked genes have previously been described as being subjected to imprinted X chromosome inactivation in mouse. The detailed allele-specific read counts of these genes may be found in Table S6. **C).** Sanger sequencing results of *XIST* allelic expression in the *B. t. indicus* × *B. t. taurus* F₁ placenta, in *in vivo*- (AI) and *in vitro*- (IVF)-produced day 15 (D15) bovine extraembryonic membranes (EEM), and CT1 trophoblast cell line. Top row is the genotyping results while the two bottom rows are sequencing results for the RT-PCR amplicons. Both random primers and oligo(dT) were used for cDNA synthesis.

previously been described as imprinted in mouse placenta (McGraw et al., 2013) and/or extra-embryonic cell lines (Merzouk et al., 2014), two genes (*CD99* and *ZBED1*) found in the pseudoautosomal region (PAR; Das et al., 2009), and the ncRNA *XIST* which is expressed from the inactive X (Lee and Bartolomei, 2013). We observed that similar to the X-linked genes, *XIST* was expressed from both parental alleles (Fig 6B and C, Table S6). Further, we found an anti-association between the allelic expression ratio of *XIST* and the X-linked genes in two of the samples (Fig 6B and Table S6). Conversely to *XIST* and the X-linked genes, the allele expression ratios of the PAR genes *CD99* and *ZBED1* centered toward 50:50 with no apparent association with X-linked genes or *XIST* expression (Fig. 6B). For this study the placental samples were taken from the intercotyledonary chorioallantois for which predecessor cells come from the trophectoderm and the epiblast (Bianchi et al., 1993; Luckett, 1978), therefore, the possibility exists that the randomness of X-linked gene allele expression observed here was simply the result of the expression observed in tissues derived from the inner cell mass (Lee and Bartolomei, 2013). To refine our findings, we turned to day 15 bovine extraembryonic membranes (EEM)(Dobbs et al., 2014) and CT1 cells (Talbot et al., 2000), a trophectoderm cell line derived from a single bovine female blastocyst. The EEM were dissected from the female conceptuses by removing the embryonic disk and should contain no epiblast derivatives(van Leeuwen et al., 2015). Sanger sequencing results showed that *XIST* was expressed from both parental alleles in EEM of the four day 15 conceptuses analyzed (Fig. 6C and Fig. S12), indicating random XCI in bovine extraembryonic membranes. However, in CT1 cells only one allele of *XIST* was

expressed. It is possible that this “monoallelic” *XIST* expression resulted from the clonal expansion of the cells that randomly inactivate one of the X chromosomes.

4.4 Discussion

To date, approximately 150 imprinted genes have been identified in humans and mice (<http://igc.otago.ac.nz>; http://www.har.mrc.ac.uk/research/genomic_imprinting/), but less than 30 genes have been described as imprinted in cattle (Tian, 2014). In this study, we advance the genomic imprinting field by the *de novo* identification of imprinted genes in bovine using RNAseq. To do so, we identified parent-specific SNP alleles in RNAseq data after filtering A-to-I RNA editing sites and determined global allele-specific gene expression in *B. t. indicus* × *B. t. taurus* F₁ conceptuses. We also corroborated the allele specificity of the identified monoallelically expressed genes using *B. t. taurus* × *B. t. taurus* progenies to exclude potential *cis*-eQTL effects on allelic expression. These analyses confirmed the allelic origin of 23 transcripts previously reported to be imprinted in bovine and identified eight novel imprinted genes in the bovine conceptuses. Of note, we were not able to corroborate the allele-specific expression of 16 genes in the *B. t. taurus* × *B. t. taurus* conceptuses because of a lack of informative SNPs. However, of these 16 genes, *PPP1R9A*, *PON3*, *MKRN3*, and *ZIM2* have previously been reported to be imprinted in human and/or mouse and showed consistent allele-specific expression in the *B. t. indicus* × *B. t. taurus* F₁s, which together suggest that these four genes are also imprinted in bovine.

Most imprinted genes are organized in megabase-sized clusters. Within the cluster is a differentially methylated imprinting control region that regulates the proper allelic expression of the imprinted genes across the entire domain (Edwards and Ferguson-Smith, 2007; Wan and Bartolomei, 2008). Of the 53 monoallelically expressed genes identified in this study, 30 genes have previously been reported to be imprinted in humans and/or mice and 24 are distributed in eight imprinted clusters namely; *GNAS/GNASXL* domain, *PEG3/USP29* domain, *GTL2/DLK1* domain, *PEG10/SGCE* domain, *MEST/COPG2* domain, *SNRPN* domain, *H19/IGF2* domain, and *KCNQ1* domain. Further, ten previously uncharacterized/unannotated transcripts were identified to be subject to allele-specific expression in two known imprinted clusters (*i.e.*, four transcripts in *PEG3/USP29* domain and six in the *SNRPN* domain) in the *B. t. indicus* × *B. t. taurus* conceptuses. Of these 10 transcripts, *LOC100298176* and *LOC1014974975* (within the *PEG3/USP29* domain) and *LOC101907679* and *LOC100849023* (within the *SNRPN* domain) were demonstrated to be imprinted in the bovine conceptuses. For the remaining six transcripts, parental-specificity could not be ascertained due to a lack of informative SNPs in the *B. t. taurus* × *B. t. taurus* conceptuses. However, we suggest that these genes are also imprinted in bovine because of the proximity of these uncharacterized/unannotated transcripts to known imprinted genes is less than 100 kb and they are within known and well characterized clusters. In addition, in the *SNRPN* domain, the last exon of the antisense *LOC100848941* brain isoform extends to the *UBE3A* gene. It is tempting to speculate that the paternal expression of this transcript may be involved in the repression of the paternal copy of

UBE3A as is observed for *UBE3A-ATS* in human (Rougeulle et al., 1998) and mouse (Meng et al., 2012).

Gene expression can be affected by genetic variants. *cis*-eQTL refers to the class of genetic variants that affect gene expression on the same DNA molecule thus leading to allele-specific or allele-biased gene expression (Rockman and Kruglyak, 2006). To determine whether monoallelic gene expression identified in the *B. t. indicus* $\times$ *B. t. taurus* F₁s was due to *cis*-eQTL effects or genomic imprinting, we corroborated the parent-specificity of the transcripts in *B. t. taurus* $\times$ *B. t. taurus* progenies as we lack the ability to produce reciprocal *B. t. taurus* $\times$ *B. t. indicus* crosses. Our findings confirmed that monoallelic gene expression is not necessarily the result of genomic imprinting (Wang and Clark, 2014). For the example of *LOC101905472*, it is always the “C” allele that is predominantly expressed regardless of its parental-origin in both *B. t. indicus* $\times$ *B. t. taurus* and *B. t. taurus* $\times$ *B. t. taurus* conceptuses. Further, *CDA* and *RDH16* showed monoallelic expression in *B. t. indicus* $\times$ *B. t. taurus* conceptuses but biallelic expression in *B. t. taurus* $\times$ *B. t. taurus* progenies. Our results demonstrate that strong *cis*-eQTL could lead to monoallelic gene expression and highlight the importance of the determination of allele-specific gene expression using different mating structures crosses prior to claiming that any monoallelically expressed genes are imprinted.

Genome-wide assessment of imprinted gene expression in non-inbred organisms such as cattle and humans can be challenging, as heterozygous SNPs are not always informative of parental-origin of an allele. We were unable to assign the allele-origin of a heterozygous SNP in the *B. t. indicus* $\times$ *B. t. taurus* F₁s if the *B. t. indicus* sire was heterozygous at the same site. Discovery of imprinted genes using RNAseq relies on

aligning reads to a reference genome, however, some bovine sequences have recently been shown to be absent or misassembled in the current bovine reference genome UMD3.1 (Whitacre et al., 2015) and perfection of the reference assembly will help improve the RNAseq-based identification of imprinted genes in cattle. Further, technical limitations of standard RNAseq may also preclude the comprehensive discovery of imprinted genes. For example, the identification of antisense imprinted transcripts such as *APEG3*, a paternally expressed antisense transcript in the *PEG3* locus (Choo et al., 2008), is not possible as our samples were not sequenced using a strand-specific RNAseq protocol (Levin et al., 2010). In addition, the procedures performed for RNA isolation for this study prevent us from identifying small imprinted RNAs as well as non-polyadenylated molecules (*e.g.*, some long ncRNAs). In future studies, the single-cell RNAseq technology will allow allele-specific gene expression analyses at exquisite resolution (Deng et al., 2014) and help identify genes that are only imprinted in specific cell types such as *Ube3a* in neurons (Yamasaki et al., 2003).

Advances in next generation sequencing technology have allowed the discovery of more than a million A-to-I RNA editing sites in human (Ramaswami et al., 2012; Ramaswami et al., 2013) and thousands of sites in mouse (Danecek et al., 2012; Neeman et al., 2006). Conversely to human and mouse, only 23 A-to-I RNA editing sites have been reported in cattle and these editing sites were identified as a result of searching evolutionarily conserved human/mouse A-to-I RNA editing sites in bovine brain RNAseq data (Pinto et al., 2014). In this study, we greatly expand the list by identifying 671 potential A-to-I RNA editing sites in the bovine conceptuses using RNAseq. Consistent with the observation that most A-to-I editing sites in human and mouse are not

located in coding regions (Li and Church, 2013; Ramaswami et al., 2013), ~96% of the identified editing sites in this study were in UTRs and introns. These editing events could either regulate RNA splicing processes (Rueter et al., 1999) or prevent the formation of double stranded RNA that may be recognized as non-self by the immune system (Liddicoat et al., 2015). We also found that ~3% (20/671) of the editing sites identified in bovine are conserved in human and/or mouse and 11 of these sites are within neurotransmitter receptor genes such as *GRIK2*, *GRIA2*, and *GABRA3*. One of the notable conserved A-to-I RNA editing sites is in *GRIA2* CDS region (Chr17:42,806,724; UMD3.1), which has ~99% edited transcripts in brain and can result in the replacement of a glutamine residue by arginine in the GRIA2 protein. This editing site has been demonstrated to be the physiologically most important editing substrate of Adar2 because the seizure and lethality phenotype of *Adar2* null mice can be reverted when the unedited *Gria2* transcripts are replaced with the edited allele (Higuchi et al., 2000).

Imprinted X-chromosome inactivation in extraembryonic membranes has been described in mouse (Huynh and Lee, 2001) and rat (Wake et al., 1976). In bovine, a report showed that the X-linked gene *MAOA* is preferentially expressed from the maternal X-chromosome in term placenta suggesting imprinted X-inactivation in this species (Xue et al., 2002). Here we show that *XIST* is expressed from both parental chromosomes in epiblast-free (van Leeuwen et al., 2015) extraembryonic membranes from day 15 bovine conceptuses (Dobbs et al., 2014). From our results, we favor the hypothesis that XCI is random in bovine extraembryonic membranes and not imprinted as previously suggested.

In summary, with this work we advance the field of genomic imprinting by expanding the list of imprinted genes in bovine and demonstrate that monoallelic gene expression can be due to *cis*-eQTL effects. In addition, our results greatly increase the number of identified A-to-I RNA editing sites in bovine conceptuses. We also show that XCI is random in bovine extraembryonic membranes. These findings will facilitate future studies to further dissect the molecular mechanisms and physiological roles of genomic imprinting and A-to-I RNA editing during fetal development of this economically important species.

4.5 Materials and methods

Whole genome sequencing of the B. t. indicus sire

Genomic DNA isolated from the semen of the *B. t. indicus* sire (Nelore breed; ABS CSS MR N OB 425/1 677344 29NE0001 97155) used to generate the *B. t. indicus* × *B. t. taurus* F₁ hybrids was subject to DNaseq. In brief, two DNaseq libraries (one with 350 bp target insert size, the other with 550 bp target insert size) were prepared using Illumina TruSeq DNA PCR-Free Sample Preparation Kit according to the manufacturer's instructions (Illumina). Each DNaseq library was sequenced in a single lane with 2 × 100 bp paired-end reads on an Illumina HiSeq 2000 platform. One percent PhiX DNA was spiked into the bovine genomic DNA sample prior to library preparation to act as an internal control and allow the HiSeq optical system optimal base calling. A total of 347.3 million raw read pairs were obtained for genotyping the *B. t. indicus* sire after removing the DNaseq reads derived from PhiX DNA (Fig. S1). The raw FASTQ files

are publically available at Gene Expression Omnibus (GEO accession no. GSE77389).

Identification of the SNPs of B. t. indicus sire using DNaseq

Raw DNaseq read pairs were subject to quality trimming prior to alignment to the reference genome (Fig. S1). The FastqMcf program (version: 1.04.636; <https://code.google.com/p/ea-utils/wiki/FastqMcf>) was used to remove the adaptor sequences in the reads. Further, DynamicTrim (version: 3.3.1; Cox et al., 2010) was used to obtain the longest segment of the read in which each base has <1% error rate. After quality trimming, DNaseq read pairs were aligned to the bovine reference genome assembly UMD3.1 using Bowtie2 (version: 2.2.3; Langmead et al., 2009). Only read pairs that had the expected read mate orientation and expected ranges of insert size were retained for further analyses.

Following Bowtie2 alignment, Genome Analysis Tool Kit (GATK; version: 3.3-0; DePristo et al., 2011; McKenna et al., 2010) was used to further process the aligned read pairs for SNP calling. Known insertions/deletions (indel) and known SNPs downloaded from Ensembl and RefSeq FTP sites were supplied for insertions/deletions re-alignment and base quality recalibration (ftp://ftp.ensembl.org/pub/release-78/variation/vcf/bos_taurus/; ftp://ftp.ncbi.nlm.nih.gov/snp/organisms/cow_9913/VCF/; ftp://ftp.ncbi.nlm.nih.gov/snp/organisms/cow_30522/). After processing the aligned read pairs, GATK HaplotypeCaller was used to identify SNPs using the default parameters. Aligned read pairs subjected to indel realignment were also used for SNP calling by GATK HaplotypeCaller and SAMtools mpileup (version: 0.1.19; Li, 2011; Li et al.,

2009) without the base quality recalibration step in order to improve the identification of true SNPs.

*Generation of the pseudo *B. t. indicus* genome and construction of a *B. t. indicus/B. t. taurus* diploid genome*

A pseudo *B. t. indicus* genome was generated by editing the reference allele (UMD3.1; *i.e.*, *B. t. taurus*) with the SNPs found in our *B. t. indicus* sire identified by the three SNP calling pipelines mentioned above (Fig. S1B, C, and D). The diploid genome was generated by combining the *B. t. taurus* and the pseudo *B. t. indicus* FASTA files.

*RNAseq of the day ~105 *B. t. indicus* × *B. t. taurus* F₁ conceptuses*

The four day ~105 *B. t. indicus* × *B. t. taurus* F₁ conceptuses used in this study (*i.e.*, Control #1, #2, #3, and #4) were previously described (Chen et al., 2013). These conceptuses shared the same *B. t. indicus* sire (ABS CSS MR N OB 425/1 677344 29NE0001 97155). RNAseq data of brain, kidney, liver, and skeletal muscle of the F₁ individuals were previously described (GEO accession number: GSE63509; Chen et al., 2015). In addition, for the current study, we generated RNAseq data from the placenta (intercotyledon regions) of these conceptuses following the RNA isolation, library preparation and sequencing procedures described previously (Chen et al., 2015). The raw FASTQ files are publically available at GEO database (accession no. GSE77389).

Alignment of RNAseq reads to the custom diploid genome

Prior to alignment, single-end RNAseq reads were processed as previously described (Chen et al., 2015). In brief, FastqMcf (version: 1.04.636;

<https://code.google.com/p/ea-utils/wiki/FastqMcf>) and DynamicTrim (version: 3.3.1; Cox et al., 2010) programs were used to remove adaptor sequences and low quality bases in the reads, respectively. Only reads with a length greater than 30 bp were used for alignment. The RNAseq reads were aligned to the custom diploid genome to minimize single reference genome alignment bias (Degner et al., 2009; Munger et al., 2014; Stevenson et al., 2013; Yuan et al., 2015).

HISAT2 (version: 2.0.0; Kim et al., 2015) was used to align RNAseq reads to the diploid genome using the following parameters: *--score-min L, 0, -0.2 -mp 6, 6 -known-splicesite-infile known-splicesite-RefSeq-Ensembl.txt*. These parameters require at least 96% sequence identity for the RNAseq reads to be aligned. The known splicing junctions in Ensembl and RefSeq databases were downloaded from the Bovine Genome Database (<http://www.bovinegenome.org>; Elsik et al., 2015). The alignment of the RNAseq reads to the diploid genome was then merged to identify: i) reads that were aligned to the same single genomic position in both the *B. t. taurus* reference genome and the pseudo *B. t. indicus* genome, and ii) reads that were uniquely aligned to either genome but not aligned to the other.

Transcript assembly and determination of transcript abundance

Cufflinks (version: 2.2.1; Trapnell et al., 2010) was used for transcript assembly of each RNAseq library. Cuffmerge (version: 2.2.1; Trapnell et al., 2010) was used to generate a unified gene annotation for all libraries. Gene models generated by Cuffmerge were then compared to Ensembl and RefSeq gene models using Cuffcompare (version: 2.2.1; Trapnell et al., 2010). If a Cuffmerge gene model matched one Ensembl/RefSeq

gene model (*i.e.*, shared the same intron chain; *class_code* “= ”), this Cuffmerge gene model was considered annotated. If a cuffmerge gene model did not match any Ensembl/RefSeq gene model (*e.g.*, *class_code* “u” or “x”), it was considered unannotated. In addition, if a single Cuffmerge gene model matched more than one Ensembl/RefSeq gene model, the Cuffmerge gene model was replaced by the corresponding Ensembl/RefSeq gene models.

RNAseq read counts for each gene model were generated using HTSeq-count (version: 0.5.4; Anders et al., 2013) with mode: *intersection-nonempty*. Read counts were then normalized to the library size using Bioconductor package edgeR (version: 3.8.6; McCarthy et al., 2012; Robinson et al., 2010). For each tissue type, genes with ≥ 4 counts/million (CPM) between all conceptuses were considered to be expressed and were retained for allele-specific expression analyses.

Identification of SNPs in the B. t. indicus $\times$ B. t. taurus F₁ conceptuses transcriptomes using RNAseq

The SNPiR pipeline (Piskol et al., 2013) was adapted to identify SNPs from the RNAseq data. The adapted SNPiR pipeline included 3 major steps: i) align RNAseq reads to the diploid UMD3.1/*B. t. indicus* genome using BWA mem (Li and Durbin, 2009), ii) call variants using GATK UnifiedGenotyper (McKenna et al., 2010), and iii) filter potential false positive variants (as described below). For read mapping, BWA mem (version: 0.7.7; Li and Durbin, 2009) was used to align the reads to the diploid genome using default parameters. As BWA does not align reads that span splice junctions, we next aligned the unmapped reads to the known splice junctions

(<http://www.bovinegenome.org>; Elsik et al., 2015). The RNAseq reads that were successfully aligned to the diploid genome were then merged with the splice junction alignments. The uniquely aligned RNAseq reads were subjected to PCR duplicates removal (only for the purpose of SNP identification), insertions/deletions re-alignment, and base quality recalibration. All RNAseq read alignments from each conceptus were then pooled for SNP calling. GATK UnifiedGenotyper was used to call variants using the following loose criteria: *-stand_call_conf 0 -stand_emit_conf 0 -mbq 20 -output_mode EMIT_ALL_CONFIDENT_SITES -U ALLOW_N_CIGAR_READS*. The last step of these analyses was to apply a series of stringent filtering steps to remove potential false positive variants. Specifically, variants were required to have quality $Q > 20$ and all variants that were only found at the end (< 6 bp to read ends) of aligned reads were discarded. Further, RNA variants in repetitive regions annotated by UCSC (<http://hgdownload.cse.ucsc.edu/goldenPath/bosTau6/database/>) were discarded and variants that were in a homopolymer runs ≥ 5 bp were also removed. In addition, intronic variants that were within 4 bp of splice junctions were discarded. Finally, to ensure that the reads supporting the variants were uniquely aligned, BLAT (version: 34; Kent, 2002) was used to re-align these reads to the reference genome. A read alignment was required to meet two criteria to be considered as a unique mapping: i) the best alignment hit overlapped the variant site, and ii) the second best hit resulted in a $< 95\%$ sequence identity to the reference genome. Only the variants for which at least half of the alignments were unique to the reference genome were retained for further analyses.

To minimize potential false positives, only the RNA variants identified by both the HISAT2 and BWA alignment strategies were used for further analyses. Further, the

RNA variants that were supported by the *B. t. indicus* DNaseq reads and/or were present in RefSeq and Ensembl dbSNP (downloaded from RefSeq and Ensembl FTP sites) were defined as genomic variants, while others were defined as RDDs. It should be noted that if a SNP in dbSNP was submitted with molecular type as “cDNA”, this SNP was considered to be an RDD (*i.e.*, a potential RNA editing site). The genomic variants were considered to be the result of SNPs and were used to assign RNAseq reads to parental alleles for allele-specific gene expression analyses, while the RDDs were used to identify A-to-I RNA editing sites. Finally, the transition/transversion (Ts/Tv) is the number of transition variants (purine-to-purine, pyrimidine-to-pyrimidine) to the number of transversion variants (purine-to-pyrimidine, pyrimidine-to-purine).

Identification of A-to-I RNA editing sites using the RNAseq data of B. t. indicus × B. t. taurus F₁ conceptuses

The RDDs were subjected to further filtering to identify A-to-I RNA editing sites. Only the RDDs that were detected in the same tissue for all four conceptuses (≥ 10 reads coverage/conceptus) were retained for further analyses. The rationale for this was that true A-to-I RNA editing sites should be present in all animals while low frequency SNPs should not (Ramaswami et al., 2013). It should be noted that the RNAseq reads for brain, liver, and skeletal muscle of Control #2 were of 50 bp in length and for these samples, the RDDs were kept for further analyses if they were present in Control #1, #3, and #4 conceptuses (all 100 bp reads). Finally, the RDDs were manually annotated to determine the variant type (*e.g.*, A>G or T>C) according to the DNA strand of the transcript template. For example, if a “T>C” variant was in a transcript that was transcribed from the antisense DNA strand then the variant type was converted from “T>C” to “A>G”.

Only the RDDs that had the “A>G” variant type were considered candidate A-to-I RNA editing sites.

To determine whether any of the A-to-I RNA editing sites identified in bovine day~105 conceptuses were conserved in human and/or mouse, 40 bp upstream and downstream sequences (Pinto et al., 2014) of the bovine A-to-I RNA editing site were BLAST against the human (hg19) and mouse builds (mm9). Both human and mouse genomes were downloaded from the UCSC database (<http://hgdownload.soe.ucsc.edu/goldenPath/hg19/bigZips/>; <http://hgdownload.soe.ucsc.edu/goldenPath/mm9/bigZips/>, respectively). For a site to be considered to be conserved between bovine, mouse, and/or human, 70 of the 81 nucleotides had to provide a perfect match (Pinto et al., 2014) and the site had to have previously been reported to be an A-to-I RNA editing site. The previously identified editing sites in human and mouse were downloaded from the RADAR database (<http://rnaedit.com>; Ramaswami and Li, 2014).

Allele-specific gene expression analyses using the RNAseq data of B. t. indicus × B. t. taurus F₁ conceptuses

The DNA variants of the *B. t. indicus* sire were used to determine the allele-origin of the RNAseq reads in the *B. t. indicus* × *B. t. taurus* F₁ hybrids. As illustrated in Fig. S5, only SNP sites that were homozygous in the *B. t. indicus* sire and for which the SNP was heterozygous or an alternate homozygote in the conceptuses were used to determine the allele-origin of the RNAseq reads. Following the allele-origin assignment, allele-specific read counts for SNPs in the same gene were aggregated for allele-specific gene

expression analyses, as this strategy has been shown to improve the sensitivity of prediction of imprinted genes using RNAseq (DeVeale et al., 2012). To identify genes with monoallelic expression in each tissue type, allele-specific read counts of the same gene were pooled for all four individuals and the significance of allelic expression bias was determined using the binomial test (see statistical analyses section). To further filter potential false positives, these candidate genes were manually inspected using the Integrative Genomic Viewer (IGV; Robinson et al., 2011) and genes with spurious alignment of the RNAseq reads or discordant allelic bias of the SNPs within the same exon were discarded.

Validation of the A-to-I RNA editing sites in $B. t. indicus \times B. t. taurus$ F_1 hybrids

DNA and cDNA of the $B. t. indicus \times B. t. taurus$ F_1 conceptuses were amplified by PCR and were subjected to Sanger sequencing to validate the A-to-I RNA editing sites identified by RNAseq. Nucleic acid isolation, cDNA synthesis, and PCR amplification were as previously reported (Chen et al., 2015; Chen et al., 2013). Primer information may be found in Table S7 (link to Table S7: <http://www.tandfonline.com/doi/full/10.1080/15592294.2016.1184805>).

Validation of the allele-specific gene expression in $B. t. indicus \times B. t. taurus$ F_1 hybrids

To validate the allele specificity of the monoallelically expressed genes, both DNA and cDNA of the F_1 hybrids and DNA of the sire were amplified by PCR and sequenced over the SNP sites used to ascribe parental origin of the transcripts. Nucleic

acid isolation, cDNA synthesis, and PCR amplifications were as previously reported (Chen et al., 2015; Chen et al., 2013). Primer information may be found in Table S7.

Corroboration of the allele-specific gene expression in day 86 B. t. taurus × B. t. taurus conceptuses

B. t. taurus females (*i.e.*, Holstein breed) were subjected to estrous synchronization and artificial insemination with sex-sorted semen from a single *B. t. taurus* bull (*i.e.*, Holstein breed; 507HO10723 DE-SU 527 SPUR-ET). Nine *B. t. taurus* × *B. t. taurus* female conceptuses were collected at day 86 of gestation. At collection, 1 × 1 mm² samples of internal organs including brain, kidney, liver, skeletal muscle and fetal placenta (intercotyledon area) were snap frozen in liquid nitrogen and stored at -80 °C. Both DNA and cDNA of the *B. t. taurus* × *B. t. taurus* conceptuses and DNA of the *B. t. taurus* sire were amplified by PCR to corroborate the parental-specific gene expression (*i.e.*, imprinted status) of the identified monoallelically expressed genes detected in the *B. t. indicus* × *B. t. taurus* F₁ hybrids. Nucleic acid isolation, cDNA synthesis, and PCR amplification were as previously reported (Chen et al., 2015; Chen et al., 2013). Primer information may be found in Table S7.

DNA methylation analysis at GNAS/GNASXL domain

Bisulfite mutagenesis of genomic DNA was performed following the One-Step Modification Procedure of the Imprint DNA Modification Kit (Sigma). Methyl Primer Express Software (version 1.0, Applied Biosystems) was used to design primers that amplify regions proximal to the *GNAS* and *GNASXL* promoters. Both amplicons spanned SNPs identified by the whole genome sequencing of the *B. t. indicus* sire. Bisulfite PCR

reactions were as previously reported (Chen et al., 2015; Chen et al., 2013). The cycling programs were as follows: initial denaturation at 94°C for 2 minutes and 15 seconds, 45 cycles of 94°C for 30 seconds, 56.1/58.7°C for 45 seconds (ramping rate: 1°C/second), and 72°C for 45 seconds, and final extension at 72°C for 5 minutes. The PCR products were resolved by agarose gel electrophoresis and isolated as previously described (Chen et al., 2013). The PCR amplicons were cloned using pGEM-T Easy Vector System (Promega) according to the manufacturer's instructions. Bacteria used were NEB 5-alpha F'I^q Competent *E. coli* (NEB). Bacteria colonies were screened by blue-white screen method and positive colonies were subject to Sanger sequencing as described above.

Statistical analyses

All RNAseq libraries were normalized by the trimmed mean of M-values method (Robinson and Oshlack, 2010). For each gene, a binomial exact test was applied to examine if the *B. t. indicus* or *B. t. taurus* allele represented more than 85% of all reads (Chen et al., 2015). Genes were then ranked by their *p*-values and adjusted to compute false discovery rates (FDRs) using the Storey method (Storey, 2002). Genes for which the FDR was less than 0.05 were considered to be monoallelically expressed.

Data access

The DNaseq reads from the *B. t. indicus* sire and the RNAseq reads from the *B. t. indicus* × *B. t. taurus* F₁ conceptuses are available in the NCBI Gene Expression Omnibus (GEO) database (<http://www.ncbi.nlm.nih.gov/geo/>) under accession numbers GSE77389 (DNaseq reads of the sire and RNAseq reads of the placenta) and GSE63509 (RNAseq reads of the brain, kidney, liver, and skeletal muscle). The sequences of the

unannotated transcripts identified in this study have been submitted to GenBank (<http://www.ncbi.nlm.nih.gov/genbank/>) under accession numbers KU530101-KU530107.

4.6 Acknowledgements

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4.7 Supplemental information for Chapter 4

Supplemental figures and tables for Chapter 4

Figure S1. Genotyping the *B. t. indicus* sire using whole genome sequencing. **A).** Summary of the *B. t. indicus* sire DNaseq results. **B).** Pipelines used for SNP identification. **C).** Venn diagram showing the overlapping of the SNPs identified using different pipelines. **D).** Number of heterozygous and homozygous SNPs identified in the *B. t. indicus* sire using different pipelines.

A

B

Conceptuses	Total RNA variants	RNA variants used for allele-specific expression analyses (genomic variants)			
		Variants supported by the DNaseq reads of the sire and dbSNP (%)	Variants only present in dbSNP (%)	Variants only supported by the sire DNaseq reads (%)	RNA-DNA differences (%)
Control #1	623,102	406,577 (65.3)	68,276 (11.0)	135,940 (21.8)	12,309 (2.0)
Control #2	552,389	364,609 (66.0)	59,461 (10.8)	119,426 (21.6)	8,893 (1.6)
Control #3	603,148	396,161 (65.7)	63,739 (10.6)	130,720 (21.7)	12,528 (2.1)
Control #4	596,973	392,030 (65.7)	63,251 (10.6)	129,604 (21.7)	12,088 (2.0)

C

Genomic variants (Ts/Tv: ~2.5)

D

RNA-DNA differences (Ts/Tv: 14-18)

Figure S2. Genotyping the *B. t. indicus* × *B. t. taurus* F₁ hybrids using RNAseq. A). Venn diagrams showing the overlapping of the RNA variants identified using the HISAT2 or BWA as reads aligner. **B).** Comparison of the identified RNA variants with bovine dbSNP entries and the *B. t. indicus* sire SNPs. **C).** Variant type distribution of the genomic variants (*i.e.*, variants matched to bovine dbSNP entries and/or supported by the *B. t. indicus* sire DNaseq reads). **D).** Variant type distribution of the RNA-DNA differences (RDDs). Ts = transition (purine-to-purine, pyrimidine-to-pyrimidine); Tv = transversion (purine-to-pyrimidine, pyrimidine-to-purine).

Figure S3. Validation of the identified A-to-I RNA editing sites in the *B. t. indicus* × *B. t. taurus* F₁ hybrids by Sanger sequencing. The red arrows indicate the editing sites. **A).** An example of A-to-I editing in coding sequences (CDS) of *COPA*. **B).** Two RNA editing sites in CDS of *GRIK2*. **C).** Two RNA editing sites in the CDS of *GABRA3*, one of which was not validated by Sanger sequencing (*i.e.*, ChrX:34,866,869).

Figure S4. Pie charts for the A-to-I RNA editing sites at different gene sections in each tissue of the *B. t. indicus* × *B. t. taurus* F₁ hybrids. CDS: coding sequence; UTR: untranslated region; ncRNA: noncoding RNA.

Figure S5. Illustration of the strategy used to assign parental alleles of RNAseq reads in the *B. t. indicus* × *B. t. taurus* F₁ hybrids.

Figure S6. Allele-specific gene expression and DNA methylation analyses at *GNAS*/*GNASXL* imprinted domain in the *B. t. indicus* × *B. t. taurus* F₁ hybrids. A). Schematics of *GNAS*/*GNASXL* loci and Sanger sequencing validation of the allele-specific gene expression of *GNAS* and *GNASXL*. Details are as Figure 3. Primers used for RT-PCR are shown as arrows facing each other. **B).** Full bisulfite maps of *GNAS* and *GNASXL* DMR. Each row = one strand of DNA; open circles, unmethylated CpG sites; filled circle, methylated CpG sites; missing circles are due to SNPs or undetermined bases because of sequencing issues.

Figure S7. Validation of the allele-specific expression of the genes that are previously described as imprinted in human and/or mouse in the *B. t. indicus* × *B. t. taurus* F₁ hybrids. A). *GTL2/DLK1* domain. B). *PEG10/SGCE* domain. C). *MEST/COPG2* domain. Details are as Figure 3.

Figure S8. Validation of the allele-specific gene expression in *PEG3/USP29* imprinted domain in the *B. t. indicus* × *B. t. taurus* F₁ hybrids. Details are as Figure 3.

Figure S9. Validation of the allele-specific gene expression in *SNRPN* imprinted domain in the *B. t. indicus* × *B. t. taurus* F₁ hybrids. For *LOC100848941* and *LOC101907203*, SNPs used for allelic expression analyses are indicated by asterisks. RT-PCR primers amplifying each transcript are indicated by arrows facing each other. Other details are as Figure 3.

Figure S10.

**No SNPs were identified
in the *B. t. taurus* × *B. t. taurus* conceptuses**

**No SNPs were identified
in the *B. t. taurus* × *B. t. taurus* conceptuses**

Figure S10.

Figure S10.

Figure S10.

Figure S10. Corroboration of the allele-specific expression of the singleton genes identified in the *B. t. indicus* × *B. t. taurus* F₁ hybrids and the *B. t. taurus* × *B. t. taurus* conceptuses. For each gene, the left panel is the Sanger sequencing validation of the monoallelic expression in the *B. t. indicus* × *B. t. taurus* F₁ hybrids while the right panel is the corroboration of the allelic expression in the *B. t. taurus* × *B. t. taurus* conceptuses. Other details are as in Figure. 4.

Figure S11.

Figure S11.

Figure S11.

Figure S11.

Figures S11. Corroboration of the allele-specific expression of the genes in the *B. t. taurus* × *B. t. taurus* conceptuses that are either known imprinted genes in human/mouse or are located in known imprinting clusters. Details are as Figure. 4.

Figure S12. Allelic expression analyses of *XIST*. Other details are as Figure 6.

Table S1. Day ~105 *B. t. indicus* × *B. t. taurus* F₁ hybrids RNAseq summary

Sample	Read length	Raw Reads	Trimmed reads	HISAT2			BWA		
				Aligned reads	Uniquely aligned reads	% of Uniquely aligned reads	Aligned reads	Uniquely aligned reads	% of uniquely aligned reads
Control #1 brain	100bp	39,114,786	31,602,386	28,413,929	27,357,626	86.57	29,550,576	25,822,816	81.71
Control #2 brain	50bp	77,691,951	38,651,699	35,048,015	33,074,719	85.57	36,493,705	31,235,176	80.81
Control #3 brain	100bp	45,716,097	37,136,173	34,111,919	32,606,337	87.8	35,177,243	30,360,585	81.75
Control #4 brain	100bp	47,403,478	38,145,390	34,464,587	33,071,983	86.7	35,769,254	31,068,139	81.45
Control #1 kidney	100bp	44,168,361	35,748,305	32,299,452	30,805,282	86.17	33,578,491	28,669,472	80.2
Control #2 kidney	100bp	60,857,482	43,334,190	39,316,489	37,600,764	86.77	40,746,834	35,023,122	80.82
Control #3 kidney	100bp	60,174,902	41,681,288	37,663,408	35,816,945	85.93	39,202,501	33,293,567	79.88
Control #4 kidney	100bp	57,133,460	40,562,200	36,410,608	34,643,342	85.41	37,897,769	32,186,983	79.35
Control #1 liver	100bp	49,324,460	39,771,401	37,328,595	34,817,515	87.54	38,208,277	32,447,015	81.58
Control #2 liver	50bp	89,453,260	45,661,628	41,663,939	37,295,881	81.68	43,796,587	34,650,017	75.88
Control #3 liver	100bp	47,032,906	37,570,762	35,529,147	33,040,542	87.94	36,221,968	30,672,340	81.64
Control #4 liver	100bp	48,359,824	39,244,627	36,845,734	34,370,485	87.58	37,704,738	32,101,460	81.8
Control #1 skeletal muscle	100bp	35,012,854	28,164,307	25,762,988	24,278,978	86.2	26,630,942	22,119,961	78.54
Control #2 skeletal muscle	50bp	56,538,563	29,854,108	27,054,443	25,451,742	85.25	28,209,667	24,052,141	80.57
Control #3 skeletal muscle	100bp	36,876,793	29,969,941	27,878,167	26,336,295	87.88	28,565,523	23,968,821	79.98
Control #4 skeletal muscle	100bp	32,704,016	26,326,185	23,849,544	22,665,183	86.09	24,733,881	20,964,080	79.63
Control #1 placenta	100bp	62,328,990	51,859,333	47,941,284	46,093,434	88.88	48,433,476	42,082,767	81.15
Control #2 placenta	100bp	44,606,067	36,839,904	34,015,595	32,433,673	88.04	34,560,192	30,092,603	81.68
Control #3 placenta	100bp	47,081,627	39,009,276	36,266,120	34,595,425	88.69	36,582,045	31,998,015	82.03
Control #4 placenta	100bp	51,401,719	42,579,644	38,981,770	37,353,644	87.73	39,744,045	34,259,796	80.46

RNAseq single-end reads were aligned to a custom diploid genome, which is a combination of the reference UMD3.1 and a pseudo *B. t. indicus* genome.

Table S6. Allele-specific read counts for *XIST*, X-linked genes, and genes in pseudoautosomal region

	Allele-specific read counts															
	Control #1 placenta				Control #2 placenta				Control #3 placenta				Control #4 placenta			
	<i>B. t. taurus</i>	<i>B. t. indicus</i>	% of		<i>B. t. taurus</i>	<i>B. t. indicus</i>	% of		<i>B. t. taurus</i>	<i>B. t. indicus</i>	% of		<i>B. t. taurus</i>	<i>B. t. indicus</i>	% of	
	<i>B. t. taurus</i>	<i>B. t. indicus</i>	<i>B. t. taurus</i>	<i>B. t. taurus</i>	<i>B. t. taurus</i>	<i>B. t. indicus</i>	<i>B. t. taurus</i>	<i>B. t. indicus</i>	<i>B. t. taurus</i>	<i>B. t. indicus</i>	<i>B. t. taurus</i>	<i>B. t. indicus</i>	<i>B. t. taurus</i>	<i>B. t. indicus</i>	<i>B. t. taurus</i>	<i>B. t. indicus</i>
<i>ATP7A</i>	38	111	25.5	37	32	53.6	70	24	74.5	43	46	48.3				
<i>ATRX</i>	18	89	16.8	39	50	43.8	58	12	82.9	31	26	54.4				
<i>APOOL</i>	15	39	27.8	50	47	51.5	52	30	63.4	21	22	48.8				
<i>ELF4</i>	67	287	18.9	79	88	47.3	140	61	69.7	92	80	53.5				
<i>AIFM1</i>	47	209	18.4	41	36	53.2	44	16	73.3	122	97	55.7				
<i>RLIM</i>	102	467	17.9	198	180	52.4	274	113	70.8	215	237	47.6				
<i>TAF1</i>	14	28	33.3	16	12	57.1	27	11	71.1	12	7	63.2				
<i>OGT</i>	18	55	24.7	85	71	54.5	89	51	63.6	27	26	50.9				
<i>FLNA</i>	213	899	19.2	641	510	55.7	1014	465	68.6	200	243	45.1				
<i>CENPI</i>	81	167	32.7	73	45	61.9	116	48	70.7	141	122	53.6				
<i>TFE3</i>	12	89	11.9	46	57	44.7	67	28	70.5	69	86	44.5				
<i>MECP2</i>	26	33	44.1	51	31	62.2	62	26	70.5	16	7	69.6				
<i>MAOA</i>	102	91	52.8	149	130	53.4	147	81	64.5	162	65	71.4				
<i>JARID1C</i>	28	86	24.6	64	44	59.3	68	31	68.7	56	32	63.6				
<i>XIST</i>	3739	828	81.9	1521	1551	49.5	1108	2253	33	643	876	42.3				
<i>CD99*</i>	1397	1719	44.8	10290	11461	47.3	11268	18896	37.4	2433	3651	40				
<i>ZBED1*</i>	51	53	49	61	62	49.6	111	83	57.2	45	41	52.3				

*Genes in pseudoautosomal region

Chapter 5 General discussion

In the 1960s, Drs. Beckwith and Wiedemann first described BWS as a somatic overgrowth disorder with predisposition of childhood cancer (Beckwith, 1969; Wiedemann, 1964). In the 1980s, genetic linkage studies indicated that BWS is related to chromosome 11p15.5 (Koufos et al., 1989; Ping et al., 1989), a region that turned out to harbor two imprinted clusters, namely, the *KCNQ1* locus and the *H19/IGF2* locus. Since then, with the help of several mouse models of BWS, the main focus of the field has been to address how loss-of-imprinting at 11p15.5 leads to the complex and variable phenotypic features of BWS. However, to date, there has been no report of molecular features that can consistently and reliably diagnose and predict BWS and the associated variable phenotypes.

The first cases of ART-induced LOS were reported in 1995 (Farin and Farin, 1995) and several reasons have prompted us to use LOS as an animal model to study the molecular etiology of human BWS. First, LOS recapitulates most BWS phenotypic features including the cardinal symptoms such as somatic overgrowth, enlarged tongue, ear malformation, and abdominal wall defects (Chen et al., 2013; Mussa et al., 2016; Young et al., 1998). Second, LOS fetuses exhibit the most common epimutation observed in BWS, namely, hypomethylation at the *KCNQ1* locus (Chen et al., 2013; Hori et al., 2010). Third, both BWS and LOS have a higher risk in the offspring born with the use of ART and both syndromes can also occur naturally (reviewed in Choufani et al., 2010; Personal communication with RMR; Young et al., 1998). Fourth, the development of LOS in bovine is more similar to BWS compared to the mouse models, which rely on

genetic manipulations of the imprinted genes and exhibit more severe phenotypes that are not observed in BWS (*e.g.*, embryonic/neonatal lethality).

We have advanced the field by providing the following insights. 1) LOS is a multi-locus loss-of-imprinting syndrome and the number of genes exhibiting loss-of-imprinting is positively correlated with bodyweight. 2) Loss of allele-specific expression of imprinted genes in LOS is associated with aberrant DNA methylation at the associated imprinted DMRs in a tissue-specific manner. 3) LOS fetuses not only exhibit loss of allele-specific expression of imprinted genes, but also display differential transcript amount of these genes compared to controls. 4) Different tissues within LOS fetuses have perturbations of distinct gene pathways and in particular, multiple pathways involved in myoblast proliferation and fusion into myotubes are mis-regulated in skeletal muscle of LOS fetuses. 5) Numerous local methylation differences exist between control and LOS. 6) Most of identified DEGs between control and LOS could not be directly associated with altered DNA methylation, suggesting that caution should be exercised when making conclusions about the etiology of such syndromes by interpreting time-point-specific DNA methylation data.

In addition to characterizing the transcriptome and methylome in LOS at day ~105 pregnancy (gestation = 280 days), we also identified eight novel bovine imprinted genes and showed that monoallelic gene expression can be the result of *cis*-eQTL effects. Further, during the *de novo* identification of novel imprinted genes, it was imperative to differentiate DNA polymorphisms from RNA editing sites in order to accurately assign the parental-origin of RNAseq read. By doing this, we identified 671 A-to-I RNA editing

candidate sites, thus increasing the list in bovine by 651 potential sites. It should be noted that no differences in A-to-I RNA editing were observed between LOS and control.

Despite the progress accomplished by my dissertation research, the molecular etiology of BWS/LOS is still not understood and several key points remain to be addressed in future studies. The following sections will present and address some of these points.

5.1 How does the use of ART lead to epigenetic instability?

5.1.1 Superovulation and loss-of-imprinting

Superovulation is commonly used to increase oocyte number for assisted reproduction and numerous studies have reported that superovulation results in loss-of-imprinting (reviewed in Denomme and Mann, 2012). For instance, superovulation can induce gain of methylation of the paternally methylated *H19/IGF2* DMR and loss of methylation of the maternally methylated *Snrpn* DMR in mouse blastocysts (Market-Velker et al., 2010). However, no DNA methylation defects at imprinted gDMRs (*i.e.*, *Snrpn* DMR, KvDMR1, *Peg3* DMR, and *H19* DMR) are detected so far in ovulated oocytes following hyperstimulation, which suggests that superovulation leads to loss-of-imprinting by affecting imprint maintenance in early embryos rather than imprint establishment in oocytes (Denomme et al., 2011).

Several maternal factors have been identified to maintain the epigenetic stability in early embryos such as the oocyte-specific DNA methyltransferase *Dnmt1o* and Kruppel-associated box zinc finger proteins *Zfp57* and *Trim28* (Howell et al., 2001; Li et

al., 2008; Messerschmidt et al., 2012). Absence of these maternal factors in oocytes leads to embryonic or neonatal lethality largely due to the defects in genomic imprints maintenance in early embryos (Howell et al., 2001; Li et al., 2008; Messerschmidt et al., 2012). It is possible that superovulation induces ovulation of oocytes that do not reach full maturity and do not have sufficient maternal factors such as Dnmt1o, Zfp57, and Trim28 to maintain DNA methylation at imprinted gDMRs in early embryos. In addition, superovulated oocytes may represent a rather heterogeneous population of cells with some oocytes reaching full maturity while some others with insufficient maternal epigenetic factors, which may explain the stochastic feature of superovulation-induced loss-of-imprinting (*i.e.*, only some embryos showing loss-of-imprinting while others are normal). In future studies, determination of the transcriptome of individual naturally- and superovulated oocytes will ascertain the molecular machinery that is responsible for the developmental incompetence of oocytes caused by hyperstimulation.

5.1.2 Embryo culture and loss-of-imprinting

Following fertilization, embryos are usually cultured *in vitro* to eight-cell or blastocyst stage before being transferred to a recipient. It is well established that embryo culture can result in loss-of-imprinting in the absence of superovulation (reviewed in Denomme and Mann, 2012). Notably, mouse embryos cultured in what is considered an optimal medium (*i.e.*, KSOM) are less susceptible to loss-of-imprinting and gene expression changes compared to a suboptimal medium like Whitten's (Mann et al., 2004; Rinaudo and Schultz, 2004). It may be speculated that embryos cultured *in vitro* undergo a stress response that consumes more energy otherwise would be used for imprint maintenance. In agreement with this, the glucose transporter gene *Slc2a1* that shuttles

glucose from the blastocoel cavity to ICM cells is downregulated in cultured embryos compared to *in vivo* embryos (Leppens-Luisier et al., 2001; Uechi et al., 1997). Thus, the decreased availability of glucose may lead to inadequate ATP generation, which could negatively affect the regulation of genomic imprinting as ATP powers a number of epigenetic factors such as chromatin remodelers (reviewed in Hargreaves and Crabtree, 2011). However, it remains unclear why every cultured embryo does not exhibit loss-of-imprinting and every imprinted gene is not equally affected.

5.1.3 Loss-of-imprinting in placenta induced by ART

The placenta is more susceptible to loss-of-imprinting when the conceptus is conceived with the use of ART (de Waal et al., 2014; de Waal et al., 2015; Mann et al., 2004; Rivera et al., 2008). Currently, a couple of hypotheses have been put forward to explain why epimutations are more frequent in the extra-embryonic lineages when ART procedures are applied. In the first hypothesis, it is thought that extraembryonic cells are in direct contact with the culture media and are more severely affected by *in vitro* culture (Mann et al., 2004). However, this hypothesis does not explain why yolk sac, which is derived from primitive endoderm that resides in the inner space of the blastocyst, also exhibits similar severity of loss-of-imprinting (Rivera et al., 2008). Further, administration of hyperstimulation hormones without embryo culture and embryo transfer can induce more severe loss-of-imprinting in placenta compared to the embryo proper (Fortier et al., 2008). Collectively, this evidence suggests that the direct contact of trophoblast to the culture medium is not the main reason for the differential frequency of epimutation between the embryo and the placenta. Another hypothesis is based on the fact that genomic imprinting is differentially regulated in embryo and placenta. For

instance, repression of the maternal flanking genes by the long ncRNA *Kcnq1ot1* at the *Kcnq1* locus depends on DNA methylation in the embryo but relies on histone modifications in the placenta (Lewis et al., 2004; Umlauf et al., 2004). It is possible that histone modifications are more dynamic and reversible compared to DNA methylation. Thus, the imprinted genes in placenta are more prone to epigenetic errors.

In addition to loss-of-imprinting, ART can also induce aberrant gene expression and DNA methylation at non-imprinted loci in placenta (de Waal et al., 2015). For example, the use of IVF results in decreased transcript abundance of glucose transporter *Glut3*, which is correlated with the increased DNA methylation level at a CpG island that is 1000 bp upstream of the transcription start site (de Waal et al., 2015). Currently, the connections between loss-of-imprinting and misregulation of non-imprinted genes remain unclear.

5.2 Molecular etiology of LOS

5.2.1 Loss-of-imprinting in LOS

LOS was first found to be associated with the epigenetic change in *IGF2R* in sheep (Young et al., 2001). This finding generated questions about the epigenetic likeliness of LOS and BWS as *Igf2r* is not often imprinted in human (Monk et al., 2006). Later, we observed biallelic expression of the long ncRNA *KCNQ1OT1* and loss of methylation of KvDMR1 on the maternal allele in bovine LOS fetuses (Chen et al., 2013), which epimutations account over 50% of BWS individuals (reviewed in Choufani et al., 2010). More recently, we demonstrated that bovine LOS is a multi-locus loss-of-

imprinting syndrome (Chen et al., 2015), which is in parallel with the fact that a subset of BWS individuals with hypomethylation at the *KCNQ1* locus also exhibits imprinting errors at other loci (Azzi et al., 2009; Blik et al., 2009; Eggermann et al., 2016). Of particular interest, the number of imprinted genes showing biallelic gene expression is correlated with the bodyweight of LOS fetuses, suggesting that severity of loss-of-imprinting could be an indicator for LOS overgrowth phenotype (Chen et al., 2015). Although some imprinted genes showing loss-of-imprinting in LOS fetuses are known fetal growth regulators such as *IGF2R* and *CDKN1C* (Barlow et al., 1991; Tunster et al., 2011), how misregulation of other imprinted genes contributes to variable phenotypes of LOS is still not understood. In future studies, detailed examination morphology, histology, and metabolic status of LOS fetuses will help establish the relationship between individual imprinted gene/domain and specific phenotypes of LOS.

It should be noted that biallelic expression of imprinted genes in LOS fetuses are not always associated with aberrant DNA methylation at the adjacent DMRs (Chen et al., 2015), which is consistent with the observations reported by several other groups (de Waal et al., 2014; de Waal et al., 2012a; Susiarjo et al., 2013). One possibility is that only specific cell types in a particular tissue express the imprinted gene and are responsible for the monoallelic or biallelic expression pattern detected in the tissue. Thus, abnormal DNA methylation in the specific cell types could be masked by the normal DNA methylation profiles of other cell types that do not express the imprinted gene. Alternatively, aberrant expression of imprinted genes can be due to either misregulation of other epigenetic modifications besides DNA methylation or misregulation of DNA methylation at other DMRs. Another interesting observation is

that biallelic expression of imprinted genes does not always lead to increased transcript abundance. For example, *IGF2R* is biallelically expressed in kidney, skeletal muscle and liver in all LOS fetuses but exhibits decreased transcript abundance in these tissues compared to controls (Chen et al., 2015). This observation is consistent with previous reports that misregulation of imprinted genes can be independent of allele-specific gene expression or allele-specific DNA methylation (Boucher et al., 2014; Dalgaard et al., 2016). It is likely that additional layer of regulatory factors exist to ensure the fine-tuning expression of imprinted genes.

It is unclear why the BWS and LOS individuals exhibit variable loss-of-imprinting and variable phenotype. Genetic variation between individuals may not completely explain the variability as the LOS fetuses in our studies shared the same sire and the dams are from the same population of *B. t. taurus* animals (Chen et al., 2015; Chen et al., 2013). Instead, stochastic epimutation originating from the *in vitro* oocyte maturation and embryo culture are more plausible causes for the variable loss-of-imprinting observed in LOS fetuses. To understand the mechanisms of imprinting errors induced by these ART procedures, it would be necessary to determine how early these epimutations can be detected in *in vitro*-derived bovine embryos. As not all embryos exhibit loss-of-imprinting following the use of ART procedures, individual embryos should be used for assessment of allele-specific expression and allele-specific DNA methylation of imprinted genes.

5.2.2 Aberrant gene expression at non-imprinted loci in LOS

Using high throughput RNA sequencing, we found that LOS fetuses exhibited different numbers of DEGs and that each tissue within LOS fetuses had perturbations of distinct gene pathways. These findings indicate that the molecular lesion of LOS is not limited to loss-of-imprinting and the variable phenotypes observed in LOS could be contributed by misregulation of both imprinted and non-imprinted genes. In agreement with this, we found that multiple pathways involved in myoblast proliferation and fusions into myotubes are misregulated in skeletal muscle of LOS fetuses. As proposed by a previous study in sheep LOS (Maxfield et al., 1998), these altered myogenesis pathways may contribute to the large muscles in the oversized LOS fetuses.

It is unclear how the use of ART procedures leads to aberrant gene expression at the non-imprinted loci. It is possible that the misregulation of non-imprinted genes is either an upstream or downstream effect of the ART-induced loss-of-imprinting. In support of this, it has been shown that imprinted genes regulate fetal development through an imprinted gene network in which many non-imprinted genes are also co-regulated (Varrault et al., 2006). However, it is also likely that abnormal expression of non-imprinted genes and loss-of-imprinting are independent events. For example, mouse conceptuses generated with the use of embryo transfer can exhibit placentomegaly and junctional zone overgrowth, but the DNA methylation appears normal at several imprinted genes that are associated with the similar phenotypes (de Waal et al., 2015). In future studies, transcriptome analyses of control and LOS fetuses at various developmental stages will help decipher these possibilities.

5.2.3 Lack of association between DMRs and DEGs in LOS

For the purpose of identifying the mechanistic causes of abnormal gene expression in LOS fetuses, we profiled the genome wide DNA methylation at single base resolution of control and LOS skeletal muscle using whole genome bisulfite sequencing. We found numerous local methylation differences between control and LOS fetuses. Surprisingly, only a small percent of DEGs could be directly associated with the identified DMRs between control and LOS, while altered DNA methylation may not be the direct cause of the large number of DEGs identified in LOS. One potential epigenetic modification that mediates the misregulation of the non-imprinted genes is the repressive histone modification H3K27me3. H3K27me3 is mostly enriched at the CpG island promoters of a number of developmentally important genes that are not regulated by DNA methylation (Boulard et al., 2015). It has been shown that Ezh2, the methyltransferase responsible for H3K27me3, regulates muscle gene expression and skeletal muscle differentiation (Caretto et al., 2004). Further, impaired reprogramming of H3K27me3 in early embryos compromises the blastocyst development in bovine (Canovas et al., 2012). Collectively, it is possible that H3K27me3 in bovine acts as an epigenetic memory for the use of ART and mediates the abnormal gene expression in later stages.

5.2.4 Challenges for studying LOS

Several challenges impede the complete understanding of the etiology of LOS. The most prominent one is that we are still not able to predict which *in vitro* cultured embryos will develop LOS. A current strategy is to supplement estrus serum in the

culture media to increase the frequency of LOS fetuses. However, generation of LOS fetuses using this method is not reproducible. For example, production of *in vitro* embryos at different occasions may yield different number of LOS fetuses or LOS fetuses at each time may show different abnormalities (reviewed in Young et al., 1998). If the molecular signature of LOS can be defined in early embryos, a pre-implantation screen for LOS prior to embryo transfer will increase the efficiency for production of LOS. In addition, it has been recognized that LOS fetuses exhibit a number of misregulated genes and a wide range of abnormalities. Thus, it is also challenging to establish the connection between the altered gene expression and the observed phenotypes in LOS. In future studies, experiments involving genetic manipulation may be necessary to ascertain the causal relationship between the aberrant gene expression and the phenotypic defects in LOS individuals.

5.3 Conclusions

The work in this dissertation demonstrates that bovine LOS is a multi-locus loss-of-imprinting syndrome, as in a subset of BWS patients. Further, we show that loss-of-imprinting is not the only molecular lesion for LOS and misregulation of both imprinted and non-imprinted genes may contribute to the abnormal phenotypes observed in LOS. Importantly, most aberrant gene expression in LOS is not directly associated with the altered DNA methylation. In addition to providing insights for the etiology of LOS, we also identify novel imprinted genes in bovine and expand the A-to-I RNA editing sites (from ~20 to 671) in bovine fetal tissues. In future studies, continued investigation of

LOS at various development stages will lay the foundation to predict the occurrence of LOS and help understand the molecular mechanisms of these congenital overgrowth conditions.

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VITA

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