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Large offspring syndrome
A bovine model for the human loss-of-imprinting overgrowth syndrome Beckwith-Wiedemann

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Keywords: BWS, LOS, KvDMR1, KCNQ1OT1, epigenetics, genomic imprinting

Abbreviations: BWS, Beckwith-Wiedemann syndrome; LOS, large offspring syndrome; ART, assisted reproductive technologies; AI, artificial insemination; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; SSCP, single strand conformation polymorphism; SNP, single nucleotide polymorphism; RFLP, restriction fragment length polymorphism; B. t. taurus, Bos taurus taurus; B. t. indicus, Bos taurus indicus; COBRA, combined bisulfite restriction analysis

Introduction

Beckwith-Wiedemann syndrome (BWS) (OMIM 130650) is a pediatric overgrowth condition with an occurrence of 1 in 13,700 natural births.1,2 BWS is a complex syndrome and has highly variable clinical features.1,2 The primary features of BWS include macrosomia (overgrown bodyweight > 97th percentile), macroglossia (enlarged tongue), and abdominal wall defects (umbilical hernia).1,2 Secondary characteristics such as ear malformations, visceromegaly, neonatal hypoglycemia, and nevus flammeus are less frequently observed in BWS patients.1,2 In addition, BWS is associated with increased risk of childhood tumors (rate ranges from 4% to 21%), with Wilms’ tumor of kidney and hepatoblastoma being the two most commonly observed.2,3

Genomic imprinting is a series of epigenetic processes that lead to parental-allele-specific gene expression in mammals.4-6 Because of genomic imprinting, both maternal and paternal genomes are required for embryonic growth and development. Of the identified imprinted genes, most are found in clusters containing two or more imprinted genes in an imprinting domain which is in turn regulated by a differentially methylated region of DNA known as the imprinting control region (ICR).4-6 The parental-allele-specific DNA methylation of the ICRs is erased in primordial germ cells and re-established during prospermatogenesis in male and oocyte growth in female.4,6

The molecular alterations responsible for BWS have been mapped to chromosome region 11p15 (syntenic to mouse chromosome 7) which has two imprinting clusters: imprinting center 1 (IC1) and IC2.1,2 In humans, IC2 contains one paternally-expressed non-coding RNA (ncRNA) and at least six maternally-expressed protein-coding genes,7 and this cluster is regulated by the ICR referred to as KvDMR1. The KvDMR1 is unmethylated on the paternal chromosome. In mice, it has been shown that unmethylated KvDMR1 permits the transcription of long
non-coding RNA *Kcnq1ot1*, which recruits the Polycomb group proteins (such as Ezh2 and Rnf2) and repressive histone marks (such as H3K27me3 and H2AK119Ub) to create a repressive chromatin conformation where maternally-expressed genes are located and repressed on the paternal allele. However, on the maternal allele, methylation of the KvDMR1 prevents the interaction of the *Igf2* enhancer and is transcribed. However, on the paternal allele, *H19* / *IGF2* promoter with the downstream enhancers. Thus, *H19* has access to the enhancer and is transcribed. However, on the paternal allele, *H19* / *IGF2* ICR is methylated which avoids the binding of CTCF protein, therefore, allowing the *Igf2* promoter to interact with the downstream enhancers. Approximately 2–7% of naturally-conceived BWS cases have a gain of methylation on *H19* / *IGF2* ICR, which is associated with the increased expression level of *IGF2*. Large offspring syndrome is an overgrowth disorder in ruminants which is phenotypically similar to BWS. The features of LOS include: excessive birth weight, large tongue, umbilical hernia, hypoglycemia, and visceromegaly. As in BWS, LOS can result from ART. Previous studies observed hypomethylation of the KvDMR1 and biallelic expression of *KCNQ1OT1* in somatic nuclear transfer (SCNT) and ART-produced bovine conceptuses. However, ascription of parental origin to the alleles during methylation studies has been difficult as a result of the polymorphic nature of cattle, which is similar to the situation in humans. A previous study performed in our laboratory showed that allelic expression of *KCNQ1OT1*, *CDKNIC*, and *H19* and DNA methylation of the KvDMR1 and *H19* / *IGF2* ICR in day 65 bovine conceptuses is conserved to humans.

Given the similarities between BWS and LOS, and together with previous studies, we hypothesized that bovine conceptuses with the overgrowth phenotype would have similar misregulation of imprinted loci as those reported for the human overgrowth condition BWS. In the present study, we used *B. t. indicus* × *B. t. taurus* F1 hybrid conceptuses produced by ART. We determined the allele-specific DNA methylation and expression of imprinted genes in IC1 and IC2 by using the identified fixed polymorphisms between the two subspecies of cattle. We show that LOS conceptuses at day -105 resemble the phenotype of BWS. Most importantly, two LOS conceptuses display biallelic expression of the ncRNA *KCNQ1OT1*, which is coupled with loss of methylation of KvDMR1 and downregulation of *CDKNIC*.

**Results**

**Generation of LOS conceptuses.** To determine if the IC2 and IC1 are misregulated in LOS as in BWS, we generated LOS conceptuses with the use of ART procedures known to induce the syndrome in bovine. Based on published observations, we expected that 16% of our ART conceptuses would be of the overgrowth phenotype would have those alleles ascription of parental origin to the alleles during methylation studies has been difficult as a result of the polymorphic nature of cattle, which is similar to the situation in humans. A previous study performed in our laboratory showed that allelic expression of *KCNQ1OT1*, *CDKNIC*, and *H19* and DNA methylation of the KvDMR1 and *H19* / *IGF2* ICR in day 65 bovine conceptuses is conserved to humans. Given the similarities between BWS and LOS, and together with previous studies, we hypothesized that bovine conceptuses with the overgrowth phenotype would have similar misregulation of imprinted loci as those reported for the human overgrowth condition BWS. In the present study, we used *B. t. indicus* × *B. t. taurus* F1 hybrid conceptuses produced by ART. We determined the allele-specific DNA methylation and expression of imprinted genes in IC1 and IC2 by using the identified fixed polymorphisms between the two subspecies of cattle. We show that LOS conceptuses at day -105 resemble the phenotype of BWS. Most importantly, two LOS conceptuses display biallelic expression of the ncRNA *KCNQ1OT1*, which is coupled with loss of methylation of KvDMR1 and downregulation of *CDKNIC*.

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

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

Expression analysis of imprinted genes. To test if BWS-associated imprinted genes are similarly misregulated in LOS, we determined allelic expression of six imprinted genes in liver, muscle, brain, tongue, heart, lung, kidney, and placenta (Fig. 2). Four of these genes, CDKN1C, KCNQ1, PHLDA2, and H19 are expressed from the maternal chromosome, whereas KCNQ1OT1 and IGF2 are expressed from the paternal chromosome. Fifty percent of naturally-conceived BWS patients show loss of methylation on the maternal allele of the differentially methylated region known as KvDMR1, and this loss-of-imprinting is correlated with biallelic expression of KCNQ1OT1.1,2 KCNQ1OT1 was biallelically-expressed in several tissues in two (ART-J835LOS and ART-J489ALOS) of the seven overgrown conceptuses from the ART group, but showed monoallelic expression in all tissues of the AI conceptuses (Fig. 3; Fig. S1 and Table S1.1). H19, IGF2, and CDKN1C were imprinted for both ART and AI groups in liver, muscle, tongue, heart, lung, kidney, and placenta. However, CDKN1C and IGF2 were expressed from both parental alleles in the brain of fetuses from both groups (Table S1.2, S1.4 and S1.5). PHLDA2 only showed monoallelic expression in liver and placenta in both groups, but was biallelically-expressed in other tissues in both conditions (Table S1.4). KCNQ1 showed global biallelic expression with a bias toward the maternal allele in both groups (Table S1.3).

Since biallelic expression of KCNQ1OT1 is associated with the repressed expression of CDKN1C from the maternal allele, we then performed quantitative RT-PCR to determine CDKN1C mRNA levels in tissues with biallelic expression of KCNQ1OT1. We compared CDKN1C expression level in each of the two LOS conceptuses (ART-J835LOS and ART-J489ALOS) with the average level of transcript of eight AI conceptuses. To get a better understanding of whether CDKN1C’s expression is directly affected by the expression of KCNQ1OT1 from the maternal allele or if it is an artifact of the ART procedures we compared the CDKN1C expression of ART-J835LOS and ART-J489ALOS to the five remaining LOS conceptuses. We found that the level of the CDKN1C RNA of ART-J835LOS and ART-J489ALOS was lower when compared with the average expression of the AI controls and the average expression of the monoallelic LOS group (Fig. 4A). CDKN1C expression level in the AI group and LOS group with correct imprinting of KCNQ1OT1 was comparable.
in most tissues except in the placenta where the LOS group is higher (Fig. 4A; p < 0.05). It should be noted that, the AI-C010 fetus (bodyweight > 97 percentile) was included in the eight fetuses analyzed and its CDKN1C expression distributed randomly among the AI controls and never occupied an extreme position. We next queried about the level of CDKN1C expression between tissues in control fetuses and we found that this gene is expressed at lower levels in brain and heart when compared with other tissues (Fig. 4B). We also analyzed the expression level of PHLDA2, which is a maternally-expressed gene and expected to be regulated similar to CDKN1C. ART-J835LOS showed down-regulation of PHLDA2 in all tissues except muscle, while expression of this gene was only downregulated in liver, heart and lung in ART-J489ALOS (Fig. S2A). In contrast to what we observed for CDKN1C, PHLDA2 showed different expression level in the various tissues analyzed with highest expression observed in the kidney and placenta (Fig. S2B). As 2–7% of BWS cases are associated with biallelic expression of IGF2, we also determined IGF2 expression level in LOS conceptuses. ART-J835LOS showed down-regulation of IGF2 in all tissues except muscle, while expression of this gene was only downregulated in liver, heart and lung in ART-J489ALOS (Fig. S2A). We then asked if DNA methylation is involved in the regulation of CDKN1C imprinting. Currently, DNA sequence information in the upstream region of CDKN1C in bovine (GenBank accession number NW_003104648.1: 2774900–2775500) harbors a sequencing gap, and we were unable to amplify the 5’ end of CDKN1C.

Methylation analyses of KvDMR1, H19/IGF2 ICR and CDKN1C exon 2. Loss of methylation of KvDMR1 is the most common epimutation in BWS. Sodium bisulfite mutagenesis was used to investigate the methylation status of the KvDMR1 in the tissues of fetuses that had maternal KCNQ1OT1 expression. We determined methylation status of a 385 bp region containing 37 CpGs. We show that in most tissues, loss of methylation on the maternal allele was coupled with biallelic expression of KCNQ1OT1 in these fetuses (Fig. 5; Fig. S3). Interestingly, the placental tissue of AI-C010, the largest AI conceptus in the control group, also showed reduced methylation of the KvDMR1 on the maternal allele (Fig. 5).

The H19/IGF2 ICR is normally unmethylated on the maternal allele but methylated on the paternal allele. We then asked if the biallelic expression of IGF2 in the brain of the fetuses studied was associated with gain of methylation of H19/IGF2 ICR on the maternal alleles. Here we show that H19/IGF2 ICR had differential methylation in brain samples where IGF2 was biallelically-expressed (Fig. S5).

In mice, differential methylation of Cdkn1c region was observed from −600 bp from the transcription start site to exon 2. Sodium DMR is a somatic imprint, and therefore is established after implantation in mice. However, the homologous region in humans is unmethylated on both alleles. In the present study, biallelic expression of CDKN1C was observed in brain in both ART and AI conditions. We then asked if DNA methylation is involved in the regulation of CDKN1C imprinting. Currently, DNA sequence information in the upstream region of CDKN1C in bovine (GenBank accession number NW_003104648.1: 2774900–2775500) harbors a sequencing gap, and we were unable to amplify the 5’ end of CDKN1C. Therefore, we focused on the CDKN1C region encompassing exon 1 to exon 2. This region in the bovine (GenBank accession

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**Figure 3.** Biallelic expression of KCNQ1OT1 in LOS fetuses. Shown is Sanger sequencing data of KCNQ1OT1 RT-PCR product in tissues analyzed in AI-B799 (control), ART-J835LOS and ART-J489ALOS fetuses. The columns show the chromatograph for each tissue of each fetus. Values below the chromatograph are the percentage of KCNQ1OT1 expressed from the maternal allele. Arrows show the double peaks of SNP1 site (refer to Fig. 2) in ART-J835LOS and ART-J489ALOS fetuses. For clarity of depiction, SNP2 site is shown here (Fig. S1). LOS, large offspring syndrome; RT-PCR, reverse transcription-polymerase chain reaction; SNP, single nucleotide polymorphism.
amplified because of a bias introduced during PCR amplification. To exclude this possibility, we co-incubated genomic DNA with the methyltransferase Sss1 prior to performing bisulfite mutagenesis. Sss1 treatment combined with COBRA showed that no bias was introduced during PCR amplification as both methylated and unmethylated DNA were similarly amplified (Fig. 6C).

Discussion

In the present study we show that the bovine model of LOS has extensive similarities with BWS. Phenotypically, LOS exhibited macrosomia, macroglossia, and umbilical hernia, which are primary characteristics of BWS. Additionally, a secondary number NW_003104648.1: 2775692–2776645) has 78% identity to the locus in humans (GenBank accession number NT 009237.18: 2847003–2846299). We determined methylation status of a 363 bp region located in the second exon which contains 48 CpG dinucleotides. Since no SNPs between B. t. indicus and B. t. taurus are available at this locus, we used Sanger sequencing for direct sequencing of the PCR product in order to investigate the potential of differential methylation which would be identifiable as a double peak (i.e. one for C and one for T) in the chromatogram. Sequencing results showed hypomethylation in both muscle, in which CDKNIC is normally imprinted, and brain, where CDKNIC is biallelically-expressed (Fig. 6A–B and Table S1.2). It is conceivable that only the unmethylated maternal alleles were amplified because of a bias introduced during PCR amplification. To exclude this possibility, we co-incubated genomic DNA with the methyltransferase Sss1 prior to performing bisulfite mutagenesis. Sss1 treatment combined with COBRA showed that no bias was introduced during PCR amplification as both methylated and unmethylated DNA were similarly amplified (Fig. 6C).
**IGF2** was exclusively expressed from the paternal chromosome except in brain where it had biallelic expression. This is in accordance with previous studies where **IGF2** showed biallelic expression in brain in both mice and cattle. \(^{40,41}\) Similarly, in our study, **KCNQ1** was biallelically-expressed in all tissues analyzed which is consistent with the situation in the mouse, where even though **Kcnq1** is maternally-expressed at midgestation, it is globally biallelically-expressed at birth. \(^{42,43}\) Even though it is accepted \(^{1,2}\) that BWS is associated with misregulation of one or more imprinted genes in one or more imprinting clusters, several pivotal questions remain unanswered about this overgrowth syndrome. First, what are the cellular and molecular alterations causing loss-of-imprinting at the specified loci? Second, how does misregulation of imprinted gene expression translates into the highly variable and complex phenotypes of BWS?

At present, only associations exist between loss of methylation at specific imprinting centers and BWS. No evidence exists that points to any particular genomic region which when epimutated, triggers the overgrowth phenotype and associated developmental

**Figure 5.** Loss of methylation of KvDMR1 on the maternal allele is associated with biallelic expression of **KCNQ1OT1** in LOS fetuses. DNA was treated with sodium bisulfite prior to PCR, and PCR product was cloned before sequencing. Sequencing data was used to determine the DNA methylation status at the KvDMR1. Shown on top is a depiction of the 10th intron of the maternally-expressed gene **KCNQ1** and its direction of transcription is shown with an arrow. The region harbors the promoter of the antisense long ncRNA **KCNQ1OT1** (shown as dashed arrow), which is also an imprinting control region known as KvDMR1. A 385 bp region of the KvDMR1 was used to determine the DNA methylation status of 37 CpG sites (ovals). A SNP (vertical arrow) between B. t. indicus and B. t. taurus was used to determine the parental origin of the alleles and only maternal alleles are shown here. Five tissues from two fetuses are shown. Filled and open circles represent methylated and unmethylated CpG dinucleotides, respectively. Missing circles are sequencing data of low quality. Each line denotes an individual DNA strand. The level of maternal **KCNQ1OT1** expression is shown in the center and next to the strands. Tail tissues were collected for the purpose of DNA analysis, precluding its use for gene expression determinations. NA, not available.
Several reasons exist to propose the use of LOS as an adequate animal model to study BWS. First, only ruminants and humans have been reported to display the overgrowth and excessive weight at birth as a result of minimal ART manipulations. Second, loss of methylation at KvDMR1 and biallelic expression of KCNQ1OT1 were observed in both human and LOS. Third, here we show lack of DNA methylation at the bovine CDKN1C exon 2 which is in accordance to what has been reported for humans but is in stark contrast to the situation in the mouse where differential methylation is evident. Fourth, females of both species carry primarily singleton pregnancies (monotocous). It has been suggested that variance for growth regulation exist between litter bearing and non-litter bearing species. Fifth, both human and bovine have a nine month gestation period. This is important because sequential events that lead to molecular lesions resulting in the overgrown phenotype or other features of BWS may occur at similar times during pregnancy and the potential exists to evaluate the timing of intervention strategies.

Figure 6. Imprinted expression of CDKN1C is not regulated by somatic DNA methylation. (A) Shown on top is a depiction of the first two exons and the first intron of the maternally-expressed gene CDKN1C. No DNA polymorphisms were identified in this region. Bisulfite converted specific primers for a 363 bp region of the exon 2 were used to determine the DNA methylation status of 48 CpG sites (ovals). (B) The PCR product was sequenced without cloning. Two tissues from five fetuses are shown. Filled and open circles represent methylated and unmethylated CpG dinucleotides, respectively. Each line denotes the DNA methylation pattern of each sample. Only the first 39 CpGs are shown here because of the low quality sequencing of last 9 CpGs due to primer binding. The level of paternal CDKN1C expression is shown in the center and next to the strands (based on a SNP in exon 4). (C) COBRA performed to ensure ability of primers to equally amplify methylated and unmethylated alleles. The restriction enzyme HincII recognized and cleaved the methylated amplicon. Sss1 = genomic DNA co-incubated with the methyltransferase Sss1 prior to bisulfite conversion, U = bisulfite converted DNA with no Sss1 treatment, Sss1 + U = equal portions of methylated and unmethylated DNA were used for PCR amplification. The digested products were resolved by PAGE.

Errors in humans. It is not known if loss-of-imprinting is the cause or a symptom of BWS. Currently, no animal model exists that faithfully recapitulates the various phenotypic and epigenetic singularities of BWS. Several mouse models for this syndrome have been generated by introducing genetic mutations into IC1 and/or IC2. The genetic mutations include: maternal mutation of Cdkn1c, double mutation including H19Δ13 (deletion of H19/Igf2 ICR and H19) and Cdkn1c, maternal H19Δ13 which leads to higher expression level of Igf2 and overexpression of Igf2. All these mouse models provided fundamental understanding of the essential function of imprinted genes in embryonic development as well as mechanisms of genomic imprinting regulation. However, these models did not phenocopy the overgrowth as well as other the primary and secondary characteristics of BWS. For example, maternal mutant Cdkn1c mice exhibited 20% overgrowth during prenatal period, but the increased bodyweight was not seen at birth which can probably be explained by intrauterine competition for maternal nutrients in litter bearing species. Several reasons exist to propose the use of LOS as an adequate animal model to study BWS. First, only ruminants and humans have been reported to display the overgrowth and excessive weight at birth as a result of minimal ART manipulations. Second, loss of methylation at KvDMR1 and biallelic expression of KCNQ1OT1 were observed in both human and LOS. Third, here we show lack of DNA methylation at the bovine CDKN1C exon 2 which is in accordance to what has been reported for humans but is in stark contrast to the situation in the mouse where differential methylation is evident. Fourth, females of both species carry primarily singleton pregnancies (monotocous). It has been suggested that variance for growth regulation exist between litter bearing and non-litter bearing species. Fifth, both human and bovine have a nine month gestation period. This is important because sequential events that lead to molecular lesions resulting in the overgrown phenotype or other features of BWS may occur at similar times during pregnancy and the potential exists to evaluate the timing of intervention strategies.
BWS has more recently been associated with misregulation at loci other than the IC2 and IC1 such as MEST (PEG1), PLAGL1 (ZAC1) and GNAS. Misregulation of multiple imprinting clusters in BWS speculate that highly variable clinical features of BWS may result from diverse combinations of epimutation of each imprinting center. Future work is planned to determine if the same is observed in the LOS model.

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

Materials and Methods

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

Experimental groups. Control conceptuses. The estrous cycle of B. t. taurus (Holstein breed) females was synchronized and the females were artificially inseminated (AI) with semen from one B. t. indicus bull (Nelore breed; ABS CSS MR N OB 425/1 677344 29NE0001 97155). Four males and five female B. t. indicus × B. t. taurus F1 conceptuses (fetus + placenta) were collected on day -105 (104–106). This time was chosen because phenotypic characteristics of LOS can be recognized at this stage. Conceptuses were retrieved from the gravid uterus at caesarean section in order to preserve nucleic acid integrity. At collection, crown-rump length, heart girth, foreleg length and head width were measured, as well as body and organ weight. The following tissues were collected: liver, muscle, brain, tongue, heart, lung, kidney, spleen, reproductive tract, intestine, skin and placenta. Tissues were diced and mixed at collection and were snap frozen in liquid nitrogen and stored at −80°C until use.

ART conceptuses. In vitro production of bovine embryos was performed as previously described by us and http://www.animal.ufl.edu/hansens/ivfi/. All media (Hepes-TL, IVF-TL, SP-TL) were purchased from Caisson Laboratories. All chemicals used to prepare media were purchased from Sigma. Briefly, B. t. taurus (Holstein) cumulus-oocyte complexes (COCs) were shipped overnight in maturation medium from TransOva Genetics. At receipt, the oocytes were rinsed in Hepes-TALP (Tyrode’s Albumin Lactate Pyruvate) and immediately placed in IVF-TALP. Sperm from the same B. t. indicus bull used to generate control conceptuses was used for IVF. The semen in one straw was thawed at 37.0°C. The sperm pellet was suspended in a 15 ml centrifuge tube containing 13 ml SP-TALP and centrifuged for 15 min at 200 × g. The sperm pellet was suspended in 300 μl of IVF-TALP and this volume was pipetted into a glass wool column in order to separate live sperm cells from debris and dead sperm. Note: this bull does not perform well in vitro when purified by Percoll gradient. The COCs and the sperm cells were co-incubated in IVF-TALP at 38.5°C in humidified air containing 5% CO2 for 18 h. Putative zygotes were cultured in KSOM-BE55 in groups of 25–30. The embryos were cultured at 38.5°C in humidified atmosphere containing 90% N2, 5% CO2 and 5% O2. On day 5 after fertilization, embryo culture drops were supplemented with 10% estrus cow serum. On day 6, 20–30 early blastocysts were placed in 2.0 ml tubes containing 1.8 ml KSOM-BE supplemented with 180 μl (10%) estrus cow serum and covered with mineral oil. The embryos were shipped overnight to TransOva Genetics in a portable incubator maintained at 38.5°C. Upon receipt, the embryos were transferred into synchronized B. t. taurus recipients (2 blastocyst per recipient; one/uterine horn). Embryo transfers and conceptus retrievals were performed by theriogenologists at TransOva Genetics. On day -105 (104–106), conceptuses were collected and processed as described for the control conceptuses. Twenty-seven conceptuses were collected from 19 recipients (8 recipients had twins).

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

RNA isolation, CDNA synthesis and reverse transcriptase polymerase chain reaction. RNA was isolated from fetal tissues with the use of Trizol Reagent (Invitrogen) according to the manufacturer’s instructions. RNA amount and quality were confirmed by spectrometry and agarose gel electrophoresis, respectively. RNA was treated with DNase (Fisher Scientific) before cDNA synthesis. 100 ng RNA was used as template to synthesize cDNA in a 20 μl reaction with: 10 mM DTT (Invitrogen), 1× First Strand Buffer (Invitrogen), 0.5 μg random primer (Promega), 1 mM dNTPs (Fisher Scientific), 100 U SuperscriptII reverse transcriptase (Invitrogen), and 20 U of Optizyme RNase Inhibitor (Fischer Scientific). The samples were incubated at 42°C for 1 h, at 95°C for 10 min, and stored at −20°C until use. To ensure no genomic DNA contamination, a minus reverse-transcriptase control was also included. For PCR amplification, 2 μl cDNA (~10 ng) was added into the PCR mix [1 × colorless GoTaq Flexi Buffer (Promega), 0.3 μM forward and reverse primer (IDT), 2.5 mM MgCl2 (Promega), 200 μM dNTP (Fisher Scientific) and 0.5 U GoTag Hot Start polymerase (Promega)]. For each assay, at least 5 sets of primers were tested to get specific amplification. The PCR conditions were as follows: denaturation at 94°C for 2 min 15 sec, then 35 cycles of 94°C for 15 sec, 59.5–62.8°C for 20 sec and 72°C for 30 sec, and final extension at 72°C for 5 min (Table S2.1).

Allele-specific expression analysis of imprinted genes. The six imprinted genes analyzed in this study were the maternally-expressed genes: CDKN1C, PHLD2A, H19, and KCNQ1 and the paternally-expressed genes: KCNQ1OT1 and IGF2. These genes were analyzed in eight tissues; namely, liver, muscle, brain, tongue, heart, lung, kidney, and placenta. Expression of KCNQ1OT1 was determined by RT-PCR followed by Sanger sequencing. The samples were sequenced at the University of Missouri’s DNA core using the 96-capillary applied Biosystems 3730 DNA analyzer with Big Dye Terminator. Sequencing data was aligned to a reference with the use of MacVector software (Cary). Allelic expression of CDKN1C, H19, and KCNQ1 were determined by RT-PCR followed by allele-specific restriction enzyme digests (Table S2.1). The digested PCR products were
resolved by polyacrylamide gel electrophoresis (PAGE). The assay used to determine allele-specific expression of H19 was previously described.31 Alleric expression of PHLDA2 and IGF2 were determined by RT-PCR-SSCP (single strand conformation polymorphism) because restriction enzymes that recognized the sequence of interest were not available. Briefly, SSCP was conducted on an 8% polyacrylamide gel and run at 110 V overnight (~14 h). The SSCP gel was then subjected to silver staining (Bio-Rad) and dried by Gel-Dry (Invitrogen). The contribution of each parental allele to the total expression was determined by Image J (NIH). Only samples with at least 10% expression from the repressed allele were considered biallelic.56

### Quantitative RT-PCR of CDKNIC, PHLDA2 and IGF2.

Taqman gene expression assays (Applied Biosystems; Table S2.1) were used to determine if CDKNIC, PHLDA2, and IGF2 showed different expression levels among control conceptuses, the LOS conceptuses with biallelic expression of KCNQ1OT1, and the LOS conceptuses with monoallelic expression of KCNQ1OT1. The assay was conducted in the eight tissues described in the allele-specific expression analysis section. The CDKNIC level of expression of the eight tissues of the two LOS conceptuses with biallelic expression of KCNQ1OT1 were compared with the tissues of the eight control conceptuses (5 females and 3 males) and five LOS conceptuses (2 females and 3 males) with monoallelic KCNQ1OT1. The samples were analyzed in triplicates, and the threshold cycle was normalized to the housekeeping gene GAPDH using an ABI Real-time 7500 system. The expression level for each gene in each tissue was calculated using the comparative C τ method. The expression levels of our bovine samples were plotted as described before in human.38

### DNA isolation and bisulfite conversion.

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

### DNA methylation analysis of KvDMR1 and H19/IGF2 ICR.

The PCR product of the bisulfite-converted regions of interest was isolated from a 1% agarose gel with Wizard SV gel and PCR Clean-Up System (Promega). KvDMR1 (385 bp containing 37 CpGs; GenBank accession number NW_003104648.1: 2960086–2960470) and H19/IGF2 ICR (318 bp containing 20/21 CpGs; GenBank accession number NW_003104648.1: 3556002–3556319) amplicons were inserted into pCC1 vector with chloramphenicol resistance gene and cloned using CopyControl PCR cloning kit with TransforMax EPI300 electrocompetent E. coli cells (Epigenetik Biotechnologies)56 according to the manufacturer’s instructions except that all the cloning incubation procedures were done at 25°C. Note: it took approximately 2 d to form visible colonies at this temperature. The individual clones were sequenced and analyzed as described for KCNQ1OT1 sequencing.

### DNA methylation analysis of CDKNIC exon 2.

In mice, Cdkn1c DMR starts from 600 bp upstream of transcription start site of Cdkn1c and extends through exon 2.33 The homologous region in humans is, however, unmethylated.35,36 A 363 bp region of bisulfite-converted exon 2 (containing 48 CpGs and no SNPs; GenBank accession number NW_003104648.1: 2776175–2776537) was amplified by PCR. The PCR product was processed and sequenced as described above. Primer information can be found in Table S2.2.

Sequencing data showed hypomethylation of CDKNIC exon 2. To ensure that the primers used were equally able to amplify bisulfite converted methylated and unmethylated DNA, we did the following; (1) an aliquot of DNA was bisulfite converted with no Sss1 treatment; (2) another aliquot of DNA was treated with Sss1 methyltransferase (New England BioLabs) prior to bisulfite conversion; (3) samples were mixed with a 1:1 ratio. The three types of template were analyzed separately by combined bisulfite restriction analysis (COBRA). The enzyme used for COBRA was HincII (New England BioLabs) which only cuts the methylated amplicons.

### Statistical analysis.

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

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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### Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journals/epigenetics/article/24655


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