

Large offspring syndrome

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

Zhiyuan Chen, Katherine Marie Robbins,[†] Kevin Dale Wells and Rocío Melissa Rivera*

Division of Animal Sciences; University of Missouri; Columbia, MO USA

[†]Current affiliation: Department of Biological Science; University of Delaware; Newark, DE USA

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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

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

Introduction

Beckwith-Wiedemann syndrome (BWS) (OMIM 130650) is a pediatric overgrowth condition with an occurrence of 1 in 13,700 natural births.^{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 spermatogenesis 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

*Correspondence to: Rocío Melissa Rivera; Email: riverarm@missouri.edu
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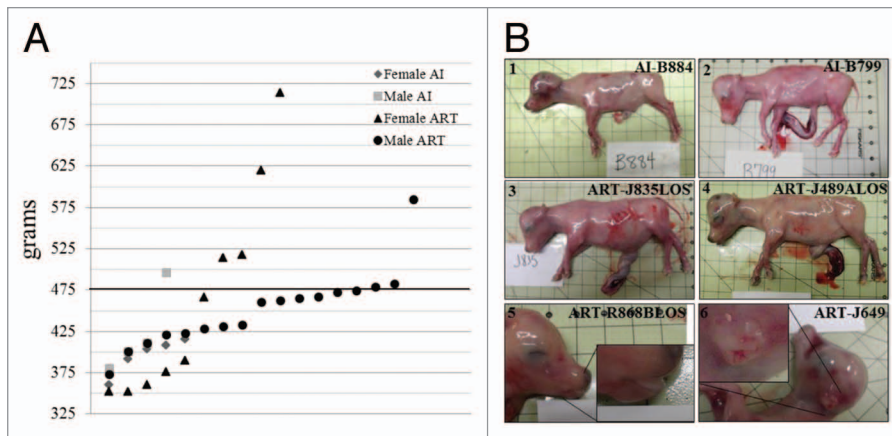


Figure 1. LOS bovine fetuses have similar phenotype characteristics as those reported in BWS patients. **(A)** Fetal weight at day ~105 gestation. Y axis represents the weight in grams. X axis has no actual implication and is used to scatter the spots representing each fetus for ease of visualization. The sex of the fetuses and the way they were generated is shown at the top-right side. The bold line represents the 97th percentile of control weight (i.e. 476.8 g). **(B)** Primary and secondary characteristics of BWS can be observed in LOS; **B1** = AI-B884 (control female weighing 400 g) and **B2** = AI-B799 (control male weighing 408 g which is the approximate average weight of the control fetuses). **B3** and **B4** show fetuses with macrosomia (ART-J835LOS: female weighing 714 g and ART-J489ALOS: female weighing 514 g). **B5** shows an example of macroglossia in a female weighing 620 g and **B6** shows an ear malformation in a female weighing 320 g. Each square on the background = 2.54 cm². LOS, large offspring syndrome; BWS, Beckwith-Wiedemann syndrome; AI, artificial insemination; ART, assisted reproductive technologies.

non-coding RNA *Kcnq1ot1*, which recruits the Polycomb group proteins (such as Ezh2 and Rnf2) and repressive histone marks (such as H3K27me3 and H2AK119U1) to create a repressive chromatin conformation where maternally-expressed genes are located and repressed on the paternal allele.^{8,9} However, on the maternal allele, methylation of the KvDMR1 prevents the transcription of the *Kcnq1ot1* gene; therefore, flanking maternally-expressed genes such as *Cdkn1c*, *Kcnq1*, and *Phlda2* are transcribed.¹⁰⁻¹² About 50% of naturally occurring BWS cases present with loss of methylation at KvDMR1 on the maternal allele, which is coupled with biallelic expression of *KCNQ1OT1* and downregulation of *CDKN1C*.^{1,2} Reports estimated a 3- to 9-fold increased likelihood of BWS in children conceived with the use of assisted reproductive technologies (ART).¹³⁻¹⁸ Currently, determination of the methylation status of the KvDMR1 is the most often used procedure to associate the syndrome to a molecular lesion.

IC1 contains the maternally-expressed ncRNA *H19*¹⁹ and the paternally-expressed fetal growth factor, *IGF2*.²⁰ The imprinting mechanism of this locus has been elucidated in a series of mouse experiments. On the maternal allele, *H19/Igf2* ICR is unmethylated, allowing for the binding of the insulator protein CTCF (CCCTC binding factor). This binding results in a chromosome boundary which prevents the interaction of the *Igf2* promoter with the downstream enhancers. Thus, *H19* has access to the enhancer and is transcribed. However, on the paternal allele, *H19/Igf2* ICR is methylated which avoids the binding of CTCF protein,²¹ therefore, allowing the *Igf2*'s promoter to interact with the downstream enhancers. Approximately 2–7% of naturally-conceived BWS cases have a gain of methylation on *H19/IGF2* ICR, which is associated with the increased expression level of *IGF2*.^{1,2}

Large offspring syndrome is an overgrowth disorder in ruminants which is phenotypically similar to BWS.²² The features of LOS include: excessive birth weight, large tongue, umbilical hernia, hypoglycemia, and visceromegaly.²²⁻²⁸ As in BWS, LOS can result from ART.²²⁻²⁸ Previous studies observed hypomethylation of the KvDMR1 and biallelic expression of *KCNQ1OT1* in somatic nuclear transfer (SCNT) and ART-produced bovine conceptuses.^{29,30} However, ascertainment of parental origin to the alleles during methylation studies has been difficult as a result of the polymorphic nature of cattle, which is similar to the situation in humans. A previous study performed in our laboratory showed that allelic expression of *KCNQ1OT1*, *CDKN1C*, and *H19* and DNA methylation of the KvDMR1 and *H19/IGF2* ICR in day 65 bovine conceptuses is conserved to humans.³¹

Given the similarities between BWS and LOS, and together with previous studies²⁹⁻³¹ 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 identified³¹ 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 *CDKN1C*.

Results

Generation of LOS conceptuses. To determine if the IC2 and IC1 are misregulated in LOS as in BWS, we generated LOS conceptuses with the use of ART procedures known to induce the syndrome in bovine.²²⁻²⁸ Based on published observations^{27,28} we expected that 16% of our ART conceptuses would be of the overgrown phenotype. LOS conceptuses were collected at day ~105; when features of LOS can be first characterized.³² 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.³¹ *B. t. indicus* × *B. t. taurus* F1 hybrid control conceptuses were produced by artificial insemination (AI). Twenty-seven conceptuses were collected from the ART group and nine from the AI group (Fig. 1A). Average bodyweight, organ weight (liver, heart, lung, kidney, spleen, and tongue), crown-rump length, foreleg length, and head width were not significantly different ($p > 0.05$) between groups (data not shown). Body weight did not differ between singletons

and twins. However, heart girth (an indirect measure of body weight) of ART conceptuses was significantly larger than AI conceptuses ($p < 0.03$; means \pm SEM = 16.42 ± 0.19 vs. 15.53 ± 0.32 cm, for ART and AI group, respectively).

Children with at least three primary features or two primary features and one or more secondary features are diagnosed as BWS patients.^{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).

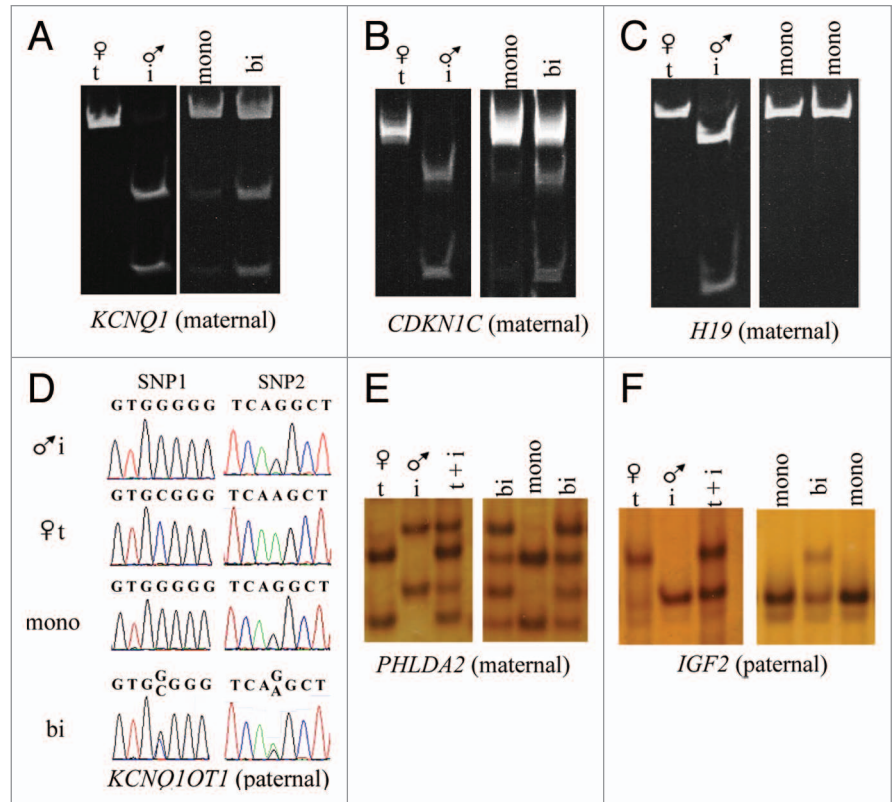


Figure 2. Example of assays used to determine allelic expression in tissues from *B. t. indicus* × *B. t. taurus* F1 hybrid conceptuses. Shown are examples of allelic determination by RT-PCR followed by RFLP and PAGE (A–C), Sanger sequencing (D) or SSCP analysis (E and F). The left portion of the panels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

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

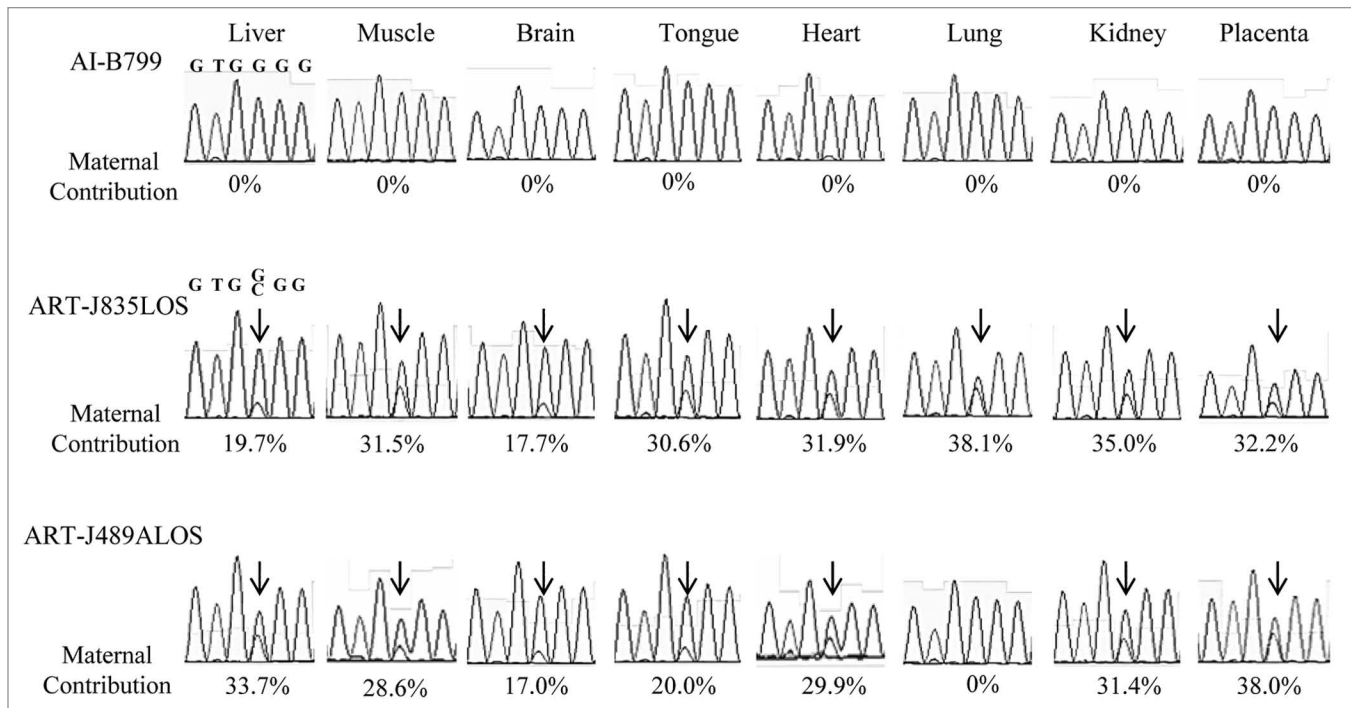


Figure 3. Biallelic expression of *KCNQ1OT1* in LOS fetuses. Shown is Sanger sequencing data of *KCNQ1OT1* RT-PCR product in tissues analyzed in AI-B799 (control), ART-J835LOS and ART-J489ALOS fetuses. The columns show the chromatograph for each tissue of each fetus. Values below the chromatograph are 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.

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 downregulation 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*, which leads to increased mRNA level of *IGF2*,^{1,2} we also determined *IGF2* expression level in LOS conceptuses. *IGF2* transcript levels were similar between LOS and the AI control group (data not shown).

Methylation analyses of KvDMR1, *H19/IGF2* ICR and *CDKN1C* exon 2. Loss of methylation of KvDMR1 is the most common epimutation in BWS.^{1,2} 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 2.³³ *Cdkn1c* DMR is a somatic imprint,³³ and therefore is established after implantation in mice.^{33,34} However, the homologous region in humans is unmethylated on both alleles.^{35,36} 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

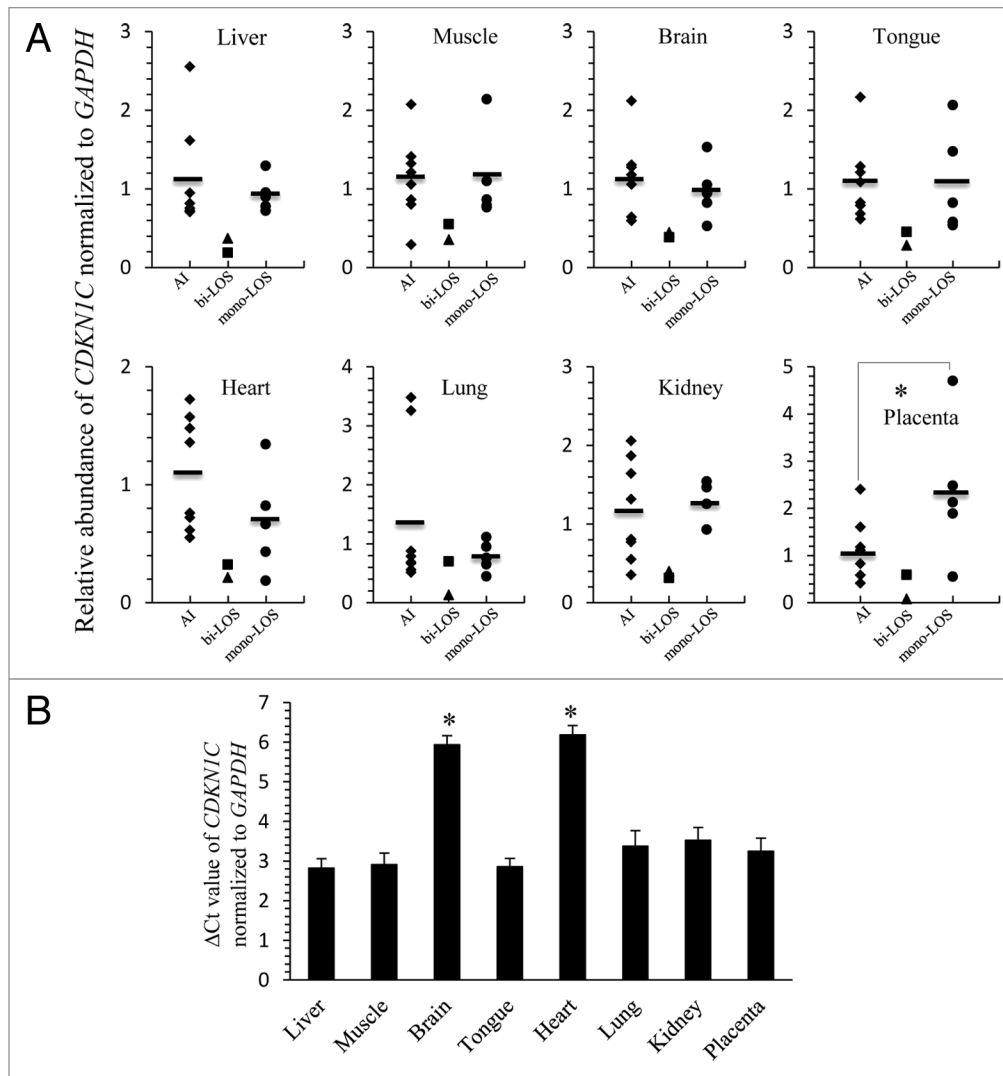


Figure 4. LOS fetuses with biallelic expression of *KCNQ1OT1* show downregulation of *CDKN1C*. **(A)** The *CDKN1C* RNA was determined by quantitative RT-PCR in several tissues from eight AI fetuses (diamonds, AI), two LOS fetuses with biallelic expression of *KCNQ1OT1* (triangle = ART-J835LOS; square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

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,³¹ we used Sanger sequencing for direct sequencing of the PCR product in order to investigate the potential of differential methylation which would be identifiable as a double peak (*i.e.* one for C and one for T) in the chromatograph. Sequencing results showed hypomethylation in both muscle, in which *CDKN1C* is normally imprinted, and brain, where *CDKN1C* is biallelically-expressed (Fig. 6A–B and 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 Sss1³⁷ 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.^{1,2} Additionally, a secondary

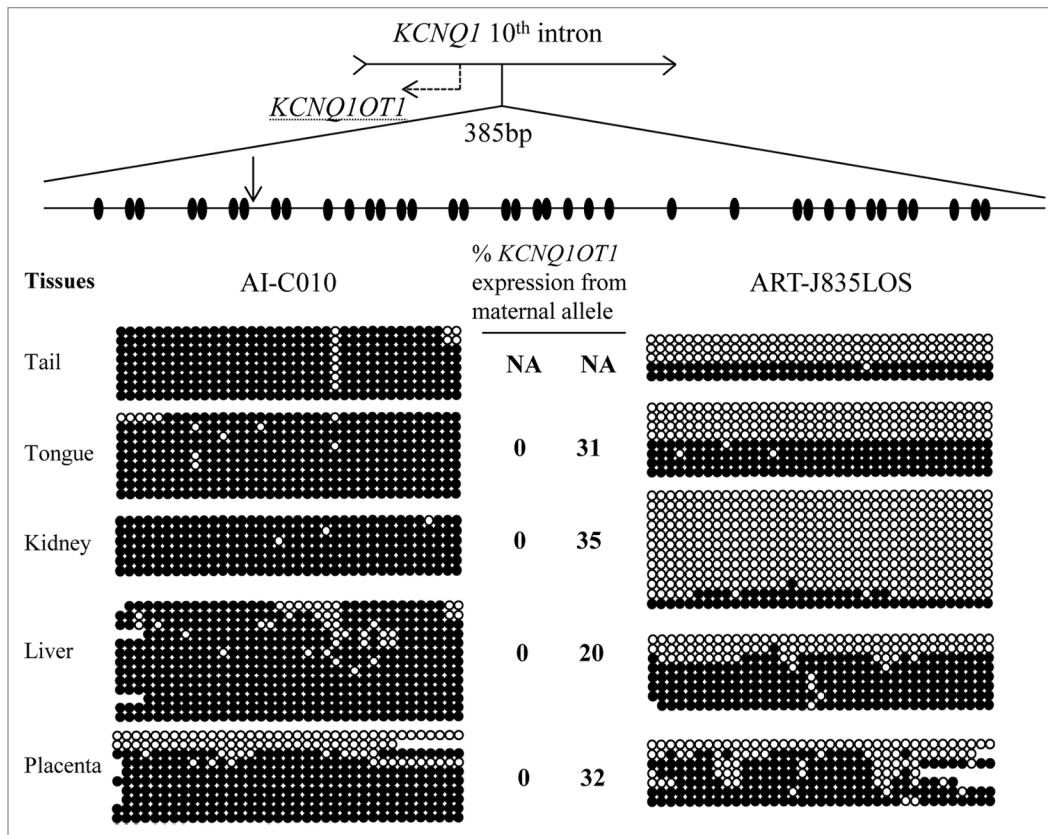


Figure 5. Loss of methylation of KvDMR1 on the maternal allele is associated with biallelic expression of *KCNQ1OT1* in LOS fetuses. DNA was treated with sodium bisulfite prior to PCR, and PCR product was cloned before sequencing. Sequencing data was used to determine the DNA methylation status at the 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.

feature of BWS, namely ear malformations, was also observed in LOS.^{1,2} At the epigenetic level, two LOS conceptuses showed loss of methylation at the KvDMR1 on the maternal allele. This epimutation is the major molecular signature of BWS^{1,2} and is observed in 50% of individuals afflicted with this syndrome.^{1,2} Furthermore, downregulation of *CDKN1C* was observed in conceptuses with biallelic expression of *KCNQ1OT1*. This is a finding similar to what have been reported using skin fibroblasts from BWS patients with loss of methylation at the KvDMR1.³⁸

It should also be noted that in our study, female conceptuses showed greater variability in bodyweight when compared with males. In humans, it has been observed that monozygotic female twins have a higher frequency of BWS cases than monozygotic male twins.³⁹ Likewise, the monozygotic female twins with BWS were generally associated with loss of methylation of the KvDMR1 on the maternal allele.³⁹ Weksberg and others³⁹ suggested that the higher frequency of BWS in monozygotic female twins might be partially explained by the overlap in timing of X-inactivation and global DNA methylation reprogramming in the preimplantation period.

IGF2 was exclusively expressed from the paternal chromosome except in brain where it had biallelic expression. This is in accordance with previous studies where *IGF2* showed biallelic expression in brain in both mice and cattle.^{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

BWS has more recently been associated with misregulation at loci other than the IC2 and IC1 such as *MEST* (*PEG1*), *PLAGL1* (*ZAC1*) and *GNAS*.^{52,53} Misregulation of multiple imprinting clusters in BWS speculates that highly variable clinical features of BWS may result from diverse combinations of epimutation of each imprinting center. Future work is planned to determine if the same is observed in the LOS model.

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

Materials and Methods

Animals. We used *B. t. indicus* and *B. t. taurus*, two subspecies of cattle, to produce F1 hybrid progenies. The use of *B. t. indicus* × *B. t. taurus* F1 individuals allowed us to determine allele-specific expression and DNA methylation of imprinted genes by the use of polymorphisms between the two subspecies. A previous study in our laboratory identified DNA polymorphisms between the two subspecies in IC1 and IC2 imprinting domains.³¹

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.³² 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,⁵⁴ 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 us⁵⁵ and <http://www.animal.ufl.edu/hansen/ivf/>. All media (Hepes-TL, IVF-TL, SP-TL) were purchased from Caisson Laboratorys. 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. Semen from the same *B. t. indicus* bull used to generate control conceptuses was used for IVF. The semen in one straw was thawed at 37.0°C. The semen straw contents were added into a 15 ml centrifuge tube containing 13 ml SP-TALP and centrifuged for 15 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.⁵⁵ 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 GoTaq 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*, *PHLDA2*, *H19*, and *KCNQ1* and the paternally-expressed genes: *KCNQ1OT1* and *IGF2*. These genes were analyzed in eight tissues; namely, liver, muscle, brain, tongue, heart, lung, kidney, and placenta. Expression of *KCNQ1OT1* was determined by RT-PCR followed by Sanger sequencing. The samples were sequenced at the University of Missouri's DNA core using the 96-capillary applied Biosystems 3730 DNA analyzer with Big Dye Terminator. Sequencing data was aligned to a reference with the use of MacVector software (Cary). 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 Allelic expression of *PHLDA2* and *IGF2* were determined by RT-PCR-SSCP (single strand conformation polymorphism) because restriction enzymes that recognized the sequence of interest were not available. Briefly, SSCP was conducted on an 8% polyacrylamide gel and run at 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 *CDKN1C*, *PHLDA2* and *IGF2*. Taqman gene expression assays (Applied Biosystems; Table S2.1) were used to determine if *CDKN1C*, *PHLDA2*, and *IGF2* showed different expression levels among control conceptuses, the LOS conceptuses with biallelic expression of *KCNQ1OT1*, and the LOS conceptuses with monoallelic expression of *KCNQ1OT1*. The assay was conducted in the eight tissues described in the allele-specific expression analysis section. The *CDKN1C* level of expression of the eight tissues of the two LOS conceptuses with biallelic expression of *KCNQ1OT1* were compared 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_T 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 are converted into uracils, but methylated cytosines remain cytosines. After PCR amplification, uracils are replaced by thymines. Primers for the bisulfite-converted DNA were designed for KvDMR1, *H19/IGF2* ICR, and *CDKN1C* exon 2 (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 (Epicenter 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 *CDKN1C* 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 *CDKN1C* exon 2. To ensure that the primers used were equally able to amplify bisulfite converted methylated and unmethylated DNA, we did the following; (1) an aliquot of DNA was bisulfite converted with no Sss1 treatment; (2) another aliquot of DNA was treated with Sss1 methyltransferase (New England BioLabs) 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

References

- Weksberg R, Shuman C, Beckwith JB. Beckwith-Wiedemann syndrome. *Eur J Hum Genet* 2010; 18:8-14; PMID:19550435; <http://dx.doi.org/10.1038/ejhg.2009.106>
- Choufani S, Shuman C, Weksberg R. Beckwith-Wiedemann syndrome. *Am J Med Genet C Semin Med Genet* 2010; 154C:343-54; PMID:20803657; <http://dx.doi.org/10.1002/ajmg.c.30267>
- Rump P, Zeegers MP, van Essen AJ. Tumor risk in Beckwith-Wiedemann syndrome: A review and meta-analysis. *Am J Med Genet A* 2005; 136:95-104; PMID:15887271; <http://dx.doi.org/10.1002/ajmg.a.30729>
- Verona RI, Mann MR, Bartolomei MS. Genomic imprinting: intricacies of epigenetic regulation in clusters. *Annu Rev Cell Dev Biol* 2003; 19:237-59; PMID:14570570; <http://dx.doi.org/10.1146/annurev.cellbio.19.111401.092717>
- Schulz R, Woodfine K, Menhenniott TR, Bourc'his D, Bestor T, Oakey RJ. WAMIDEX: a web atlas of murine genomic imprinting and differential expression. *Epigenetics* 2008; 3:89-96; PMID:18398312; <http://dx.doi.org/10.4161/epi.3.2.5900>
- Edwards CA, Ferguson-Smith AC. Mechanisms regulating imprinted genes in clusters. *Curr Opin Cell Biol* 2007; 19:281-9; PMID:17467259; <http://dx.doi.org/10.1016/j.ccb.2007.04.013>
- Weksberg R, Shuman C, Smith AC. Beckwith-Wiedemann syndrome. *Am J Med Genet C Semin Med Genet* 2005; 137C:12-23; PMID:16010676; <http://dx.doi.org/10.1002/ajmg.c.30058>
- Pandey RR, Mondal T, Mohammad F, Enroth S, Redrup L, Komorowski J, et al. Kcnq1ot1 antisense noncoding RNA mediates lineage-specific transcriptional silencing through chromatin-level regulation. *Mol Cell* 2008; 32:232-46; PMID:18951091; <http://dx.doi.org/10.1016/j.molcel.2008.08.022>
- Terranova R, Yokobayashi S, Stadler MB, Otte AP, van Lohuizen M, Orkin SH, et al. Polycomb group proteins Ezh2 and Rnf2 direct genomic contraction and imprinted repression in early mouse embryos. *Dev Cell* 2008; 15:668-79; PMID:18848501; <http://dx.doi.org/10.1016/j.devcel.2008.08.015>
- Horike S, Mitsuya K, Meguro M, Kotobuki N, Kashiwagi A, Notsu T, et al. Targeted disruption of the human LIT1 locus defines a putative imprinting control element playing an essential role in Beckwith-Wiedemann syndrome. *Hum Mol Genet* 2000; 9:2075-83; PMID:10958646; <http://dx.doi.org/10.1093/hmg/9.14.2075>
- Fitzpatrick GV, Soloway PD, Higgins MJ. Regional loss of imprinting and growth deficiency in mice with a targeted deletion of KvDMR1. *Nat Genet* 2002; 32:426-31; PMID:12410230; <http://dx.doi.org/10.1038/ng988>
- Thakur N, Kanduri M, Holmgren C, Mukhopadhyay R, Kanduri C. Bidirectional silencing and DNA methylation-sensitive methylation-spreading properties of the Kcnq1 imprinting control region map to the same regions. *J Biol Chem* 2003; 278:9514-9; PMID:12511562; <http://dx.doi.org/10.1074/jbc.M212203200>
- DeBaun MR, Niemitz EL, Feinberg AP. Association of in vitro fertilization with Beckwith-Wiedemann syndrome and epigenetic alterations of LIT1 and H19. *Am J Hum Genet* 2003; 72:156-60; PMID:12439823; <http://dx.doi.org/10.1086/346031>
- Maher ER, Bructon LA, Bowdin SC, Luharia A, Cooper W, Cole TR, et al. Beckwith-Wiedemann syndrome and assisted reproduction technology (ART). *J Med Genet* 2003; 40:62-4; PMID:12525545; <http://dx.doi.org/10.1136/jmg.40.1.62>
- Halliday J, Oke K, Breheny S, Algar E, J Amor D. Beckwith-Wiedemann syndrome and IVF: a case-control study. *Am J Hum Genet* 2004; 75:526-8; PMID:15284956; <http://dx.doi.org/10.1086/423902>
- Sutcliffe AG, Peters CJ, Bowdin S, Temple K, Reardon W, Wilson L, et al. Assisted reproductive therapies and imprinting disorders—a preliminary British survey. *Hum Reprod* 2006; 21:1009-11; PMID:16361294; <http://dx.doi.org/10.1093/humrep/dei405>
- Lim D, Bowdin SC, Tee L, Kirby GA, Blair E, Fryer A, et al. Clinical and molecular genetic features of Beckwith-Wiedemann syndrome associated with assisted reproductive technologies. *Hum Reprod* 2009; 24:741-7; PMID:19073614; <http://dx.doi.org/10.1093/humrep/den406>
- Gicquel C, Gaston V, Mandelbaum J, Siffroi JP, Flahault A, Le Bouc Y. In vitro fertilization may increase the risk of Beckwith-Wiedemann syndrome related to the abnormal imprinting of the KCN10T gene. *Am J Hum Genet* 2003; 72:1338-41; PMID:12772698; <http://dx.doi.org/10.1086/374824>
- Bartolomei MS, Zemel S, Tilghman SM. Parental imprinting of the mouse H19 gene. *Nature* 1991; 351:153-5; PMID:1709450; <http://dx.doi.org/10.1038/351153a0>
- DeChiara TM, Robertson EJ, Efstratiadis A. Parental imprinting of the mouse insulin-like growth factor II gene. *Cell* 1991; 64:849-59; PMID:1997210; [http://dx.doi.org/10.1016/0092-8674\(91\)90513-X](http://dx.doi.org/10.1016/0092-8674(91)90513-X)
- Hark AT, Schoenherr CJ, Katz DJ, Ingram RS, Lovorse JM, Tilghman SM. CTCF mediates methylation-sensitive enhancer-blocking activity at the H19/Igf2 locus. *Nature* 2000; 405:486-9; PMID:10839547; <http://dx.doi.org/10.1038/35013106>
- Young LE, Sinclair KD, Wilmot I. Large offspring syndrome in cattle and sheep. *Rev Reprod* 1998; 3:155-63; PMID:9829550; <http://dx.doi.org/10.1530/ror.0.0030155>
- Farin PW, Piedrahita JA, Farin CE. Errors in development of fetuses and placentas from in vitro-produced bovine embryos. *Theriogenology* 2006; 65:178-91; PMID:16266745; <http://dx.doi.org/10.1016/j.theriogenology.2005.09.022>
- Miles JR, Farin CE, Rodriguez KF, Alexander JE, Farin PW. Angiogenesis and morphometry of bovine placentas in late gestation from embryos produced in vivo or in vitro. *Biol Reprod* 2004; 71:1919-26; PMID:15286036; <http://dx.doi.org/10.1095/biolreprod.104.031427>
- Bertolini M, Anderson GB. The placenta as a contributor to production of large calves. *Theriogenology* 2002; 57:181-7; PMID:11775968; [http://dx.doi.org/10.1016/S0093-691X\(01\)00665-3](http://dx.doi.org/10.1016/S0093-691X(01)00665-3)
- Bertolini M, Mason JB, Beam SW, Carneiro GF, Sween ML, Kominek DJ, et al. Morphology and morphometry of in vivo- and in vitro-produced bovine concepti from early pregnancy to term and association with high birth weights. *Theriogenology* 2002; 58:973-94; PMID:12212896; [http://dx.doi.org/10.1016/S0093-691X\(02\)00935-4](http://dx.doi.org/10.1016/S0093-691X(02)00935-4)
- Hiendler S, Mund C, Reichenbach HD, Wenigerkind H, Brem G, Zakhartchenko V, et al. Tissue-specific elevated genomic cytosine methylation levels are associated with an overgrowth phenotype of bovine fetuses derived by in vitro techniques. *Biol Reprod* 2004; 71:217-23; PMID:15028629; <http://dx.doi.org/10.1095/biolreprod.103.026062>
- Hiendler S, Wirtz M, Mund C, Klemp M, Reichenbach HD, Stojkovic M, et al. Tissue-specific effects of in vitro fertilization procedures on genomic cytosine methylation levels in overgrown and normal sized bovine fetuses. *Biol Reprod* 2006; 75:17-23; PMID:16554415; <http://dx.doi.org/10.1095/biolreprod.105.043919>
- Hori N, Nagai M, Hirayama M, Hirai T, Matsuda K, Hayashi M, et al. Aberrant CpG methylation of the imprinting control region KvDMR1 detected in assisted reproductive technology-produced calves and pathogenesis of large offspring syndrome. *Anim Reprod Sci* 2010; 122:303-12; PMID:21035970; <http://dx.doi.org/10.1016/j.anireprosci.2010.09.008>
- Couldrey C, Lee RS. DNA methylation patterns in tissues from mid-gestation bovine foetuses produced by somatic cell nuclear transfer show subtle abnormalities in nuclear reprogramming. *BMC Dev Biol* 2010; 10:27; PMID:20205951; <http://dx.doi.org/10.1186/1471-213X-10-27>
- Robbins KM, Chen Z, Wells KD, Rivera RM. Expression of KCNQ1OT1, CDKN1C, H19, and PLAGL1 and the methylation patterns at the KvDMR1 and H19/IGF2 imprinting control regions is conserved between human and bovine. *J Biomed Sci* 2012; 19:95; PMID:23153226; <http://dx.doi.org/10.1186/1423-0127-19-95>
- Breukelman SP, Reinders JM, Jonker FH, de Ruigh L, Kaal LM, van Wageningen-de Leeuw AM, et al. Fetometry and fetal heart rates between Day 35 and 108 in bovine pregnancies resulting from transfer of either MOET, IVP-co-culture or IVP-SOF embryos. *Theriogenology* 2004; 61:867-82; PMID:14757473; <http://dx.doi.org/10.1016/j.theriogenology.2003.07.001>
- Bhogal B, Arnaudo A, Dymkowski A, Best A, Davis TL. Methylation at mouse Cdkn1c is acquired during postimplantation development and functions to maintain imprinted expression. *Genomics* 2004; 84:961-70; PMID:15533713; <http://dx.doi.org/10.1016/j.ygeno.2004.08.004>
- John RM, Lefebvre L. Developmental regulation of somatic imprints. *Differentiation* 2011; 81:270-80; PMID:21316143; <http://dx.doi.org/10.1016/j.diff.2011.01.007>
- Chung WY, Yuan L, Feng L, Hensle T, Tycko B. Chromosome 11p15.5 regional imprinting: comparative analysis of KIP2 and H19 in human tissues and Wilms' tumors. *Hum Mol Genet* 1996; 5:1101-8; PMID:8842727; <http://dx.doi.org/10.1093/hmg/5.8.1101>
- Monk D, Arnaud P, Apostolidou S, Hills FA, Kelsey G, Stanier P, et al. Limited evolutionary conservation of imprinting in the human placenta. *Proc Natl Acad Sci U S A* 2006; 103:6623-8; PMID:16614068; <http://dx.doi.org/10.1073/pnas.0511031103>
- Matsuo K, Silke J, Gramatikoff K, Schaffner W. The CpG-specific methylase SssI has topoisomerase activity in the presence of Mg²⁺. *Nucleic Acids Res* 1994; 22:5354-9; PMID:7816625; <http://dx.doi.org/10.1093/nar/22.24.5354>
- Chiesa N, De Crescenzo A, Mishra K, Perone L, Carella M, Palumbo O, et al. The KCNQ1OT1 imprinting control region and non-coding RNA: new properties derived from the study of Beckwith-Wiedemann syndrome and Silver-Russell syndrome cases. *Hum Mol Genet* 2012; 21:10-25; PMID:21920939; <http://dx.doi.org/10.1093/hmg/ddr419>
- Weksberg R, Shuman C, Caluseriu O, Smith AC, Fei YL, Nishikawa J, et al. Discordant KCNQ1OT1 imprinting in sets of monozygotic twins discordant for Beckwith-Wiedemann syndrome. *Hum Mol Genet* 2002; 11:1317-25; PMID:12019213; <http://dx.doi.org/10.1093/hmg/11.11.1317>
- Hemberger M, Redies C, Krause R, Oswald J, Walter J, Fundele RH. H19 and Igf2 are expressed and differentially imprinted in neuroectoderm-derived cells in the mouse brain. *Dev Genes Evol* 1998; 208:393-402; PMID:9732553; <http://dx.doi.org/10.1007/s004270050195>
- Curchoe C, Zhang S, Bin Y, Zhang X, Yang L, Feng D, et al. Promoter-specific expression of the imprinted IGF2 gene in cattle (*Bos taurus*). *Biol Reprod* 2005; 73:1275-81; PMID:16120826; <http://dx.doi.org/10.1095/biolreprod.105.044727>
- Paulsen M, Davies KR, Bowden LM, Villar AJ, Franck O, Fuermann M, et al. Syntenic organization of the mouse distal chromosome 7 imprinting cluster and the Beckwith-Wiedemann syndrome region in chromosome 11p15.5. *Hum Mol Genet* 1998; 7:1149-59; PMID:9618174; <http://dx.doi.org/10.1093/hmg/7.7.1149>

43. Gould TD, Pfeifer K. Imprinting of mouse *Kvlqt1* is developmentally regulated. *Hum Mol Genet* 1998; 7:483-7; PMID:9467008; <http://dx.doi.org/10.1093/hmg/7.3.483>
44. Tunster SJ, Van de Pette M, John RM. Fetal overgrowth in the *Cdkn1c* mouse model of Beckwith-Wiedemann syndrome. *Dis Model Mech* 2011; 4:814-21; PMID:21729874; <http://dx.doi.org/10.1242/dmm.007328>
45. Caspary T, Cleary MA, Perlman EJ, Zhang P, Elledge SJ, Tilghman SM. Oppositely imprinted genes *p57(Kip2)* and *igf2* interact in a mouse model for Beckwith-Wiedemann syndrome. *Genes Dev* 1999; 13:3115-24; PMID:10601037; <http://dx.doi.org/10.1101/gad.13.23.3115>
46. Leighton PA, Ingram RS, Eggenschwiler J, Efstratiadis A, Tilghman SM. Disruption of imprinting caused by deletion of the H19 gene region in mice. *Nature* 1995; 375:34-9; PMID:7536897; <http://dx.doi.org/10.1038/375034a0>
47. Eggenschwiler J, Ludwig T, Fisher P, Leighton PA, Tilghman SM, Efstratiadis A. Mouse mutant embryos overexpressing IGF-II exhibit phenotypic features of the Beckwith-Wiedemann and Simpson-Golabi-Behmel syndromes. *Genes Dev* 1997; 11:3128-42; PMID:9389646; <http://dx.doi.org/10.1101/gad.11.23.3128>
48. Sun FL, Dean WL, Kelsey G, Allen ND, Reik W. Transactivation of *Igf2* in a mouse model of Beckwith-Wiedemann syndrome. *Nature* 1997; 389:809-15; PMID:9349812; <http://dx.doi.org/10.1038/39797>
49. Young LE, Fernandes K, McEvoy TG, Butterwith SC, Gutierrez CG, Carolan C, et al. Epigenetic change in IGF2R is associated with fetal overgrowth after sheep embryo culture. *Nat Genet* 2001; 27:153-4; PMID:11175780; <http://dx.doi.org/10.1038/84769>
50. Blondin P, Farin PW, Crosier AE, Alexander JE, Farin CE. In vitro production of embryos alters levels of insulin-like growth factor-II messenger ribonucleic acid in bovine fetuses 63 days after transfer. *Biol Reprod* 2000; 62:384-9; PMID:10642577; <http://dx.doi.org/10.1095/biolreprod62.2.384>
51. Kuentz P, Bailly A, Faure AC, Blagosklonov O, Amiot C, Bresson JL, et al. Child with Beckwith-Wiedemann syndrome born after assisted reproductive techniques to an human immunodeficiency virus serodiscordant couple. *Fertil Steril* 2011; 96:e35-8; PMID:21550040; <http://dx.doi.org/10.1016/j.fertnstert.2011.04.030>
52. Rossignol S, Steunou V, Chalas C, Kerjean A, Rigolet M, Viegas-Pequignot E, et al. The epigenetic imprinting defect of patients with Beckwith-Wiedemann syndrome born after assisted reproductive technology is not restricted to the 11p15 region. *J Med Genet* 2006; 43:902-7; PMID:16825435; <http://dx.doi.org/10.1136/jmg.2006.042135>
53. Blik J, Verde G, Callaway J, Maas SM, De Crescenzo A, Sparago A, et al. Hypomethylation at multiple maternally methylated imprinted regions including *PLAGL1* and *GNAS* loci in Beckwith-Wiedemann syndrome. *Eur J Hum Genet* 2009; 17:611-9; PMID:19092779; <http://dx.doi.org/10.1038/ejhg.2008.233>
54. O'Rourke PK, Entwistle KW, Arman C, Esdale CR, Burns BM. Fetal development and gestational changes in *Bos taurus* and *Bos indicus* genotypes in the tropics. *Theriogenology* 1991; 36:839-53; PMID:16727053; [http://dx.doi.org/10.1016/0093-691X\(91\)90350-M](http://dx.doi.org/10.1016/0093-691X(91)90350-M)
55. Rivera RM, Kelley KL, Erdos GW, Hansen PJ. Alterations in ultrastructural morphology of two-cell bovine embryos produced in vitro and in vivo following a physiologically relevant heat shock. *Biol Reprod* 2003; 69:2068-77; PMID:12930717; <http://dx.doi.org/10.1095/biolreprod.103.020347>
56. Rivera RM, Stein P, Weaver JR, Mager J, Schultz RM, Bartolomei MS. Manipulations of mouse embryos prior to implantation result in aberrant expression of imprinted genes on day 9.5 of development. *Hum Mol Genet* 2008; 17:1-14; PMID:17901045; <http://dx.doi.org/10.1093/hmg/ddm280>