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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 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 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.
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. However, on the maternal allele, methylation of the KvDMR1 promoter prevents the interaction of the Igf2 enhancer and is transcribed. However, on the paternal allele, H19 and IC1 contains the maternally-expressed ncRNA H19 and DNA methylation of the KvDMR1 and H19/IGF2 ICR in day 65 bovine conceptsces is conserved to humans.31

Given the similarities between BWS and LOS, and together with previous studies29,31 we hypothesized that bovine conceptsces 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 conceptsces 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 conceptsces at day -105 resemble the phenotype of BWS. Most importantly, two LOS conceptsces display biallelic expression of the ncRNA KCNQ1OT1, which is coupled with loss of methylation of KvDMR1 and downregulation of CDKNIC.

Results

Generation of LOS conceptsces. To determine if the IC2 and IC1 are misregulated in LOS as in BWS, we generated LOS conceptsces with the use of ART procedures known to induce the syndrome in bovine.22-28 Previous studies observed hypomethylation of the KvDMR1 and biallelic expression of Kcnq1ot1 in somatic nuclear transfer (SCNT) and ART-produced bovine conceptsces.29,30 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 conceptsces is conserved to humans.31

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.
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. 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 $KCNQ1OT1$. $KCNQ1OT1$ 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 biallelically-expressed in both ART and AI groups in liver, muscle, tongue, heart, lung, kidney, and placenta. $PHLDA2$ was biallelically-expressed in both ART and AI groups in liver, muscle, tongue, heart, lung, kidney, and placenta. $KCNQ1$ 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.](http://www.landesbioscience.com/journals/epigenetics/article/593/)

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.
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. Cdkn1c 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.

Figure 3. Biallelic expression of KCNQ1OT1 in LOS fetuses. Shown is Sanger sequencing data of KCNQ1OT1 RT-PCR product in tissues analyzed in Al-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 methylation. 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
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.\textsuperscript{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 biallelically-expressed at birth.\textsuperscript{42,43}

Even though it is accepted\textsuperscript{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

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**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.
 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,44 double mutation including H19Δ13 (deletion of H19/ Igf2 ICR and H19) and Cdkn1c, maternal H19Δ13 which leads to higher expression level of Igf2,46,47 and overexpression of Igf2.48

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 intruterine competition for maternal nutrients in litter bearing species.44 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.13-18,22-28,49-51 

Second, loss of methylation at KvDMR1 and biallelic expression of KCNQ1OT1 were observed in both human1,2 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 humans35,36 but is in stark contrast to the situation in the mouse where differential methylation is evident.33 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.44 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 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.

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/hansen/ivf/. 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 F1 hybrid progenies. The use of B. t. indicus × B. t. taurus to produce F1 hybrid progenies. The use of B. t. indicus × B. t. taurus to produce F1 hybrid progenies.

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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 KCNQIOT1, and the LOS conceptuses with monoallelic expression of KCNQIOT1. 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 KCNQIOT1 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 KCNQIOT1. 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_{\text{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) 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 KCNQIOT1 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
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