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Exposure of Preimplantation Embryos to Insulin Alters Expression of Imprinted Genes

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Insulin promotes early embryonic development, but whether this action affects postimplantation fetal development and alters the expression of imprinted genes remain to be determined. This study analyzed the expression and methylation levels of the growth-related imprinted genes *H19* and *insulin-like growth factor 2* (*Igf2*) in fetuses exposed to insulin before implantation. We cultured 2-cell embryos in either 0 or 0.25 μg/ml insulin until the blastocyst stage and then transferred them into pseudopregnant recipient mice. The number of embryos developing to blastocysts after insulin exposure was 16.4% higher than that of the control, and the birth body weight of the insulin-exposed group was 17.8% higher than that of the control group. Real-time reverse transcription–polymerase chain reaction analysis revealed that exposure of preimplantation embryos to insulin increased the mRNA expression of both *Igf2* and *H19* in embryonic day (E) 14 fetuses. Bisulfite genomic sequencing demonstrated that the methylation level of the *H19–Igf2* imprint control region was 19.3% lower in insulin-exposed E14 fetuses than in controls. The present study indicates that insulin exposure during the preimplantation stage alters the expression of imprinted genes and affects fetal development.

Abbreviations: E, embryonic day; Igf2, insulin-like growth factor 2; PCR, polymerase chain reaction

Insulin enhances growth in preimplantation mouse embryos. Addition of insulin to mouse embryos in culture results in an increase in protein synthesis in the morulae, blastocysts, and expanded blastocysts, as well as an increase in cell number and the proportion of embryos that develop to the blastocyst stage.[14,15] Insulin also stimulates amino-acid transport[17] and enhances cleavage and morphologic development[12] in preimplantation mouse embryos. The role of insulin at the preimplantation stage of development is supported further by studies[21,25] showing that insulin was capable of binding to zona pellucida-stripped mouse pre-embryos beginning at the morula stage of development. Furthermore, insulin can inhibit protein degradation completely while stimulating protein synthesis, thus leading to an increase in total protein.[9]

Regulation of growth in early mammalian embryos can be modulated by genomic imprinting.[28] Imprinted genes play important roles in fetal growth and development.[18] *H19* and *Igf2* are neighboring imprinted genes on mouse distal chromosome 7.[3,6] Expression of *Igf2* is from the paternal allele, whereas is that of *H19* is from the maternal allele. These 2 genes are expressed widely during embryonic development in the same tissues and are downregulated shortly after birth.[1] Alterations in the expression of *Igf2* severely affect fetal growth in the mouse;[5] in particular, overexpression of mouse *Igf2* gives rise to fetal overgrowth.[29] *H19* encodes an untranslated RNA, and its function remains unknown. Some experiments suggest that *H19* plays a role in the control of growth (possibly by controlling *Igf2* expression) during embryogenesis. Deletion of *H19* leads to the birth of pups that are 27% heavier than their wild-type littermates and that show increased *Igf2* expression.[19]

The allelic methylation status of the imprint control region upstream of the *H19* gene is critical to imprinted expression of both *H19* and *Igf2*.[30,31] During early embryonic development, extensive changes in genome-wide methylation take place, and several imprinted genes undergo allele-specific changes in the DNA methylation during both gametogenesis and early embryogenesis.[10] Any change in methylation status could result in deregulation of development at later stages; therefore imprinted genes are perhaps most vulnerable to alterations induced by exogenous factors and other in vitro manipulations.

The methylation patterns of *H19* and *Igf2* are tissue-specific and are thought to be established during early embryogenesis.[4,11,16,24,36] The genomic DNA methylation status of the imprint control region of *H19/Igf2* is influenced by nutritional factors; culture medium; exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin, a toxic environmental contaminant; and radiation.[18,34,37,38] Such alterations would be maintained somatically and might affect gene expression at later stages of development.[18] However, whether insulin's action on embryonic development involves epigenetic modification of imprinted genes by means of changes in DNA methylation remains to be determined.

Therefore, the objectives of the present study were to determine whether exposure of preimplantation mouse embryos to insulin in vitro would affect fetal development after transfer of the embryos to unexposed recipient mice and to clarify whether this effect was accompanied by alterations in mRNA expression and genomic methylation status in the imprint control region of *H19–Igf2*.

Materials and Methods

Animal. ICR strain mice (specific pathogen-free; age, 6 to 8 wk) were purchased from Shanghai Laboratory Animals Center, Chinese Academy of Sciences (Shanghai, PR China). Female

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Table 1. Nucleotide sequences of specific primers and PCR conditions

Target	GenBank accession no.	Length of PCR product (bp)	Primer sequences	PCR conditions
Igf2	U71085	253	F: gtgtgtgtcagccaagcatg R: caaatgtggggacacagagg	95 °C × 15 min 95 °C × 15 s, 56 °C × 20 s, 72 °C × 40 s 28 cycles
H19	Af049091	185	F: taccccgggatgacttcatc R: tatctccgggactccaaacc	95 °C × 15 min 95 °C × 15 s, 56 °C × 20 s, 72 °C × 40 s 28 cycles
Imprint control region of *Igf2–H19*	U19619	498	F: gtataagaattttgtaaggagattatgttt R: ataaatcaaatacctaaaataactcttaaa	95 °C × 5 min 95 °C × 1 min, 55°C × 1 min, 72 °C × 1 min 40 cycles
Imprint control region of *Igf2–H19*	U19619	430	F: tttgtaaggagattatgttttatttttgga R: ccctaacctcataaaacccataactataaa	95 °C × 5 min 95 °C × 1 min, 55 °C × 1 min, 72 °C × 1 min 35 cycles

F, forward; R, reverse.

mice were superovulated by intraperitoneal injection with 8 IU of pregnant mare serum gonadotropin followed 48 h later by intraperitoneal injection of 8 IU of human chorionic gonadotropin. The superovulated female mice were allowed to mate with male mice. The presence of a vaginal plug the morning after mating indicated gestational day 0.5.

The animals had free access to food and water and were maintained at an ambient temperature of 22 °C with 12:12-h light:dark cycle. All animal protocols were approved by the Shanghi Laboratory Animal Care and Ethics Committee.

Embryos collected. On gestational day 3.0, approximately 45 to 48 h after administration of human chorionic gonadotropin, mice were killed by cervical dislocation after light ether anesthesia. Embryos at the 2-cell stage were collected from the oviduct; a total of 1300 2-cell embryos were collected. The 2-cell embryos from the same donor mouse were allocated equally into control and insulin-exposed groups (n = 650 embryos each). The insulin concentration in the media ranged between 0.1 and 1 µg/ml previously;[2] we used 0.25 µg/ml in the present study. The embryos in the control group were cultured in 1-ml drops of KSOM medium (45 to 50 embryos/drop),[22] whereas for the insulin-exposed groups, the same number of embryos was cultured in KSOM supplemented with 0.25 µg/ml insulin; both groups of embryos were cultured to the blastocyst stage in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. After a 48-h incubation, blastocyst embryos with clearly visible blastocoel cavities that had developed from the 2-cell stage were washed. The insulin-exposed and control blastocysts were transferred into estrus-synchronized pseudopregnant recipient mice (10 blastocysts uterine per horn). Fetuses were dissected on embryonic day (E) 14, and total RNA and DNA were purified from each fetus. Four pregnancies for each group were used for the experiment; several dams were maintained until the pups were born (E21) and weighed.

Reverse transcription. E14 fetuses were collected, and total RNA was extracted from whole fetuses by using RNeasy Mini Kits (74104, Qiagen, Hilden, Germany). RNA concentration was then quantified by measuring the absorbance at 260 nm in a photometer (Biophotometer, Eppendorf, Hamburg, Germany); ratios of absorption (260:280 nm) of all preparations were between 1.9 and 2.0. Aliquots (1 µg) of total RNA were reverse-transcribed by incubation at 37 °C for 1 h in a 30-µl reaction volume consisting of 10 U AMV Reverse Transcriptase (Promega, Madison, WI), 40 U RNase inhibitor, 0.17 µmol/l random primers (9 basepairs), 250 mmol/l Tris-HCl (pH 8.3), 50 mmol/l $MgCl_2$, 250 mmol/l KCl, 2.5 mmol/l spermidine, 50 mmol/l dithiothreitol, and 1.0 mmol/l each dNTP. The reaction was terminated by heating at 95 °C for 5 min and quickly cooling on ice.

Fluorescent real-time quantitative polymerase chain reaction analysis. The mRNA expression of *Igf2* and *H19* was quantified relative to that of *18S rRNA* by using the Quantum RNA 18S Internal Standards kit (Ambion, Austin, TX). Quantitative real-time polymerase chain reaction (PCR) analysis was performed by using a thermocycler (Opticon 2, MJ Research, Miami, FL) and the iQ SYBR Green Supermix (Bio-Rad, Hercules, CA). One µl of reverse transcription reaction mix was used for PCR in a 20-µl volume containing 2 µl iQ SYBR Green Supermix and 0.2 µmol/l of the primer pair specific for Igf2, H19, or 18S rRNA. After completion of the final cycle, a melting curve analysis was performed to monitor the purity of the PCR products. We used this information to calculate the differential expression of genes of interest. Gene expression levels were calculated and presented as $2^{-\Delta\Delta c(t)}$ values.[20]

The primer pairs for *H19* and *Igf2* were designed as described[37] and were synthesized (Shengneng Bicolor Biotech, Shanghai, PR China). The nucleotide sequences of these primers and the PCR conditions set for the genes of interest are shown in Table 1.

DNA isolation and bisulfite treatment. Genomic DNA of E14 fetuses from both insulin-exposed and control groups was isolated by using the DNeasy kit (Qiagen) according to the manufacturer's protocol. The DNA concentration and purity were evaluated spectrophotometrically. DNA samples were digested overnight with *Not*I and then subjected to sodium bisulfite treatment by using the CpGenome DNA Modification Kit (Chemicon, Billerica, MA) according to the manufacturer's protocol.

PCR amplification, cloning, and sequencing. The 430-basepair imprint control region of the *H19–Igf2* locus was amplified by PCR with Ampli*Taq* Gold polymerase (Applied Biosystems, Foster, CA). Two rounds of PCR were performed with the fully nested primer pairs shown in Table 1. Each reaction mixture for the first-round PCR (product, 498 basepairs) contained 2 µl of sodium bisulfite-treated DNA, 2 µl of 10 × PCR buffer II (provided with the polymerase), 2 mmol/l $MgCl_2$, 200 µmol/l dNTPs, 0.2 µmol/l of each primer, and 0.4 µl AmpliTaq Gold polymerase (5 U/µl) in a total volume of 20 µl.

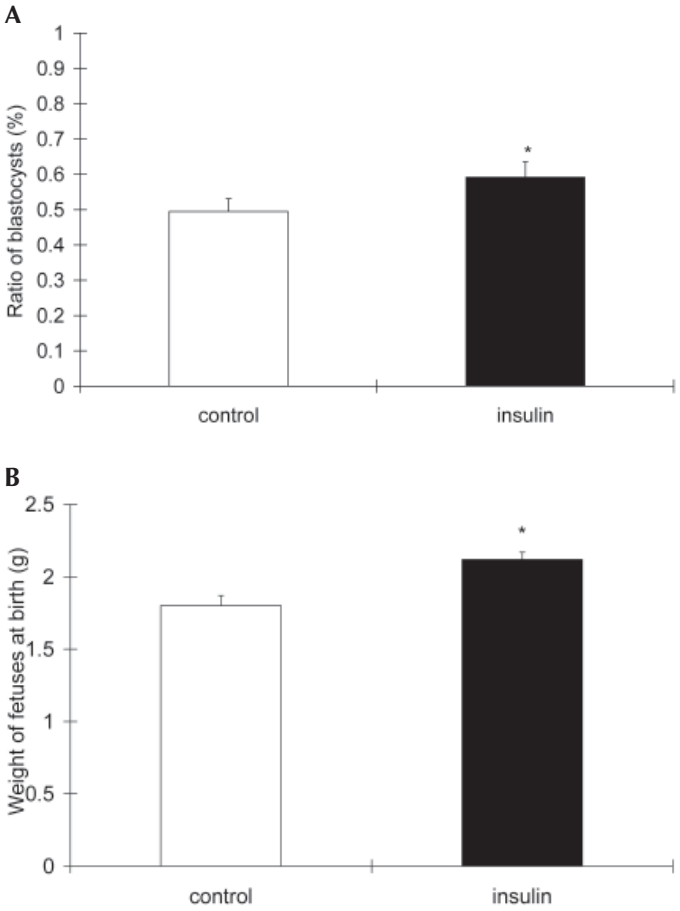

Figure 1. Effect of insulin on pre- and postimplantation embryonic development. (A) Ratios of developed blastocysts in insulin-exposed and control groups. Data for insulin-exposed and control embryos (n = 650 embryos each) were compared. (B) Body weight of E21 fetuses in insulin-exposed and control groups. Data for insulin-exposed and control fetuses (n = 24 fetuses each) from 4 recipients were compared. Results are expressed as mean ± standard error of the mean. *, $P < 0.05$.

For the second-round PCR, 1 µl of the first-round products was used as template. PCR products (430 basepairs) were separated on a 1% agarose gel, and the bands were purified with agarose Gel DNA Purification Kit (Qiagen). The purified DNA was subcloned into pGEM-T vector (Promega) by using T4 DNA ligase and transformed into JM109 cells. Colonies were picked up and DNA was amplified, positive colonies were sequenced (BigDye Terminator Cycle Sequencing Kit, version 3.1, Applied Biosystems, Foster, CA) with standard primers (M13 forward and reverse) on an automated sequencer (Prism 3700, Applied Biosystems). A total of 16 CpG sites in the 5′ end of the imprint control region of the *H19–Igf2* gene were examined. On E14, 4 clones corresponding to each of 8 fetuses in the insulin-exposed group and 4 or 5 clones corresponding to each of 5 fetuses in the control group were selected; therefore 32 clones from the insulin-exposed group and 22 clones from control group were sequenced.

Statistical analysis. SPSS 13.0 for windows (StatSoft, Tulsa, OK) was used for the statistical analysis. All results were expressed as mean ± standard error of the mean. Differences in the development ratios of blastocysts were analyzed by paired *t* tests, differences in methylation levels were analyzed by the chi-squared test, and differences in birth weight and gene expression were analyzed by 1-way analysis of variance. Statistical significance was indicated by a *P* value of less than 0.05.

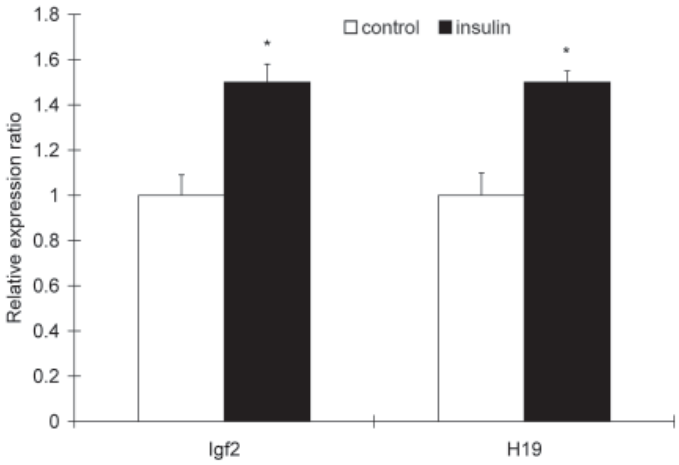

Figure 2. Relative gene expression analysis by fluorescent quantitative reverse transcription–PCR. The $2^{-\Delta\Delta Ct}$ method was used to analyze gene expression in E14 mouse fetuses. 18S rRNA was used as a reference gene. Data for insulin-exposed and control fetuses (n = 8 fetuses each) from 4 recipients were compared. *, $P < 0.05$.

Results

Embryonic development before and after implantation. The proportion of preimplantation embryos that developed into blastocysts in the insulin-exposed group was 16.4% ($P < 0.05$, paired *t* test) higher than that of the control group (Figure 1 A). The data were collected from 20 experiments, with a total of 1300 control and insulin-exposed embryos. In addition, the birth weight of pups from the insulin-exposed group was 17.8% ($P < 0.05$) higher than that of the control group (Figure 1 B). The data were obtained from 24 control fetuses and 24 insulin-exposed fetuses of 4 recipients for each treatment.

Expression of *Igf2* and *H19* mRNAs. Real-time quantitative PCR analysis revealed that the ratio of mRNA expression in insulin-exposed fetuses to that in control fetuses was 1.47 for *Igf2* and 1.5 for *H19* ($P < 0.05$ for both comparisons; Figure 2). The data were obtained from 8 control fetuses and 8 insulin-exposed fetuses of 4 recipients for each treatment.

Methylation patterns of CpG islands in the imprint control region of H19/Igf2 in E14 mouse fetuses. We sequenced 22 clones from control E14 fetuses and 32 clones from insulin-exposed E14 fetuses; the nucleotide sequences of representative clones are shown in Figure 3 A, B. In the control group, 4 (18.2%) of the 22 clones assayed from 5 individual PCR products exhibited methylation at all CpG islands, whereas 4 (18.2%) clones exhibited no methylation at any of the CpG sites. In comparison, in the insulin-exposed group, 5 (15.6%) of the 32 clones assayed from 8 individual PCR products were fully methylated, whereas 11 (34.4%) of the clones lacked methylation at all CpG sites. On average, 57% of CpG sites were methylated in the control group compared with 46% CpGs in the insulin-exposed group ($P < 0.05$). Therefore the CpG methylation ratio in the targeted region of genomic DNA was decreased by 19.3% in the insulin-exposed group, compared with the control group.

Discussion

Insulin has both long-term growth effects and short-term metabolic effects on various cell types in vivo. Preimplantation mouse embryos present a unique system for studying insulin's action because they develop normally during culture in vitro in a defined medium and subsequently complete development into normal live offspring after transfer to recipients as blastocysts.[12] Previous studies demonstrated various effects of insulin on early embryos. For example, insulin was reported to upregulate the expression of insulin receptor mRNA prior to compaction,[23] to increase the cell number of the inner cell mass and stimulate blastocyst formation via insulin receptors,[15] to stimulate protein synthesis and regulate protein metabolism,[9,14] and to promote biosynthesis, proliferation, and morphologic development in early embryos.[8] Our results show that insulin accelerated blastocyst formation increased the number of blastocyst cells and enhanced birth weight. Insulin-exposed fetuses were 17.8% heavier at birth than were controls of the same age. This finding is in agreement with a previous report of a 10% increase in the weight of E19 fetuses subjected to 48-h insulin exposure from the 2-cell stage to blastocysts before transplantation.[12]

The present study found that the expression levels of *H19* and *Igf2* were increased in the insulin-exposed E14 mice fetuses and accompanied by decreased methylation of the imprint control region of *H19–Igf2*. Several lines of evidence have suggested that the expression of *H19* and *Igf2* and their DNA methylation status can be altered by exogenous factors. For example, deregulation of *H19* and *Igf2* imprinting has been reported in cultured rat fibroblast cells.[32] The aberrant *H19* and *Igf2* expression is associated with a gain of DNA methylation at an imprinting control region upstream of *H19* in the serum cultured embryo.[18] In other media, however, embryo culture can lead to heritable loss of *H19* methylation.[7,26] When mice were fed a methyl-donor-deficient diet (lacking folic acid, vitamin B12, and choline), methylation of the imprinted *Igf2* gene was downregulated,[35] whereas pregnant mice fed a methyl-supplemented diet showed increased DNA methylation in their A^{vy} agouti offspring.[33] In another study, exposure of preimplantation embryos to 2,3,7,8-tetrachlorodibenzo-p-dioxin tended to decrease the expression levels of *H19* and *Igf2* and altered the genomic DNA methylation status of imprinted genes.[37]

The regulation of imprinted genes correlates well with their DNA methylation.[13] DNA methylation is involved in transcriptional silencing of genes, leading to regulation of expression of imprinted genes.[27] Several studies have identified the changes in imprinted gene expression throughout the development associated with culture composition during the preimplantation period.[18,32] Therefore, global changes in DNA methylation have been observed in developing germ cells in mice and other animals.

Our data showed that exposure of preimplantation mouse embryos to insulin in vitro did, in fact, affect fetal development, and suggested a link between alterations in *H19–Igf2* mRNA expression and genomic methylation status in the imprint control region of *H19–Igf2*. To our knowledge, this study is the first to provide evidence that insulin can alter the expression level of *H19–Igf2* and the genomic DNA methylation status of imprinted genes in preimplantation mouse embryos. The present findings may provide insights into early embryonic endocrine interventions in other mammalian species, including human. Any endocrine intervention or disruption induced by environmental or nutritional factors, clinical treatments, or disease condition during 'critical windows' of early embryonic development may cause epigenetic alterations, which may be transmitted to the next generations to induce phenotypic changes.[31] In conclusion, insulin stimulates

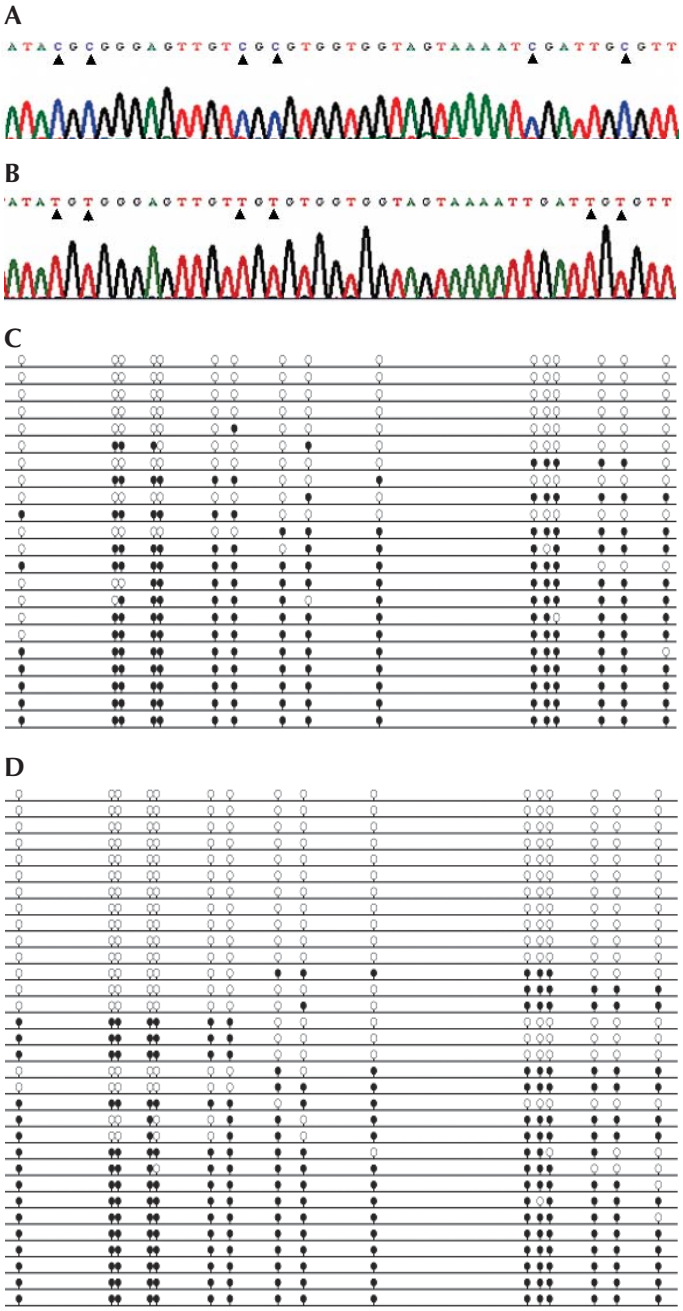

Figure 3. Summary of clone data for imprint control region of *H19-Igf2* in E14 mouse fetuses. The E14 DNA samples were treated with sodium bisulfite, and PCR products were subcloned into T vector and sequenced. (A) Nucleotide sequence of a representative clone in the control group. (B) Nucleotide sequence of a representative clone in the insulin-exposed group. (C) Methylation patterns of CpG islands in 22 clones of the control group. (D) Methylation patterns of CpG islands in 32 clones of the insulin-exposed group. Arrows indicate methylated to unmethylated cytosine through insulin treatment. (C, D) Each line represents a separate clone; a black circle indicates a methylated CpG, whereas an open circle indicates an unmethylated CpG. The differences in methylation between the insulin-exposed group and control group were significant ($P < 0.05$).

preimplantation embryo development by accelerating blastocyst formation and increasing blastocyst number and birth weight. Our data also suggest that there is a link between *H19–Igf2* mRNA expression and genomic methylation status in the imprint control region of *H19–Igf2*.

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