

Loss of imprinting control of the lncRNA *H19*-fetal mitogen *IGF2* gene cluster in the decidua microenvironment of patients with idiopathic spontaneous miscarriages

Xue Wen

Jilin University First Hospital

Qi Zhang

Jilin University First Hospital

Lei Zhou

Jilin University First Hospital

Zhaozhi Li

Jilin University First Hospital

Xue Wei

Jilin University First Hospital

Jiaomei Zhang

Jilin University First Hospital

Hui Li

Jilin University First Hospital

Yongchong Chen

Jilin University First Hospital

Chao Niu

Jilin university first hospital

Ji Qu

JILIN University first hospital

Min Li

Jilin university first hospital

Jianting Xu

Jilin University First Hospital

Zijun Xu

Jilin University First Hospital

Xueling Cui

Jilin University

Songling Zhang

Jilin University First Hospital

Yufeng Wang

Jilin University First Hospital

Wei Li

Jilin University First Hospital

Andrew R. Hoffman

Stanford University School of Medicine

Zhonghui Liu

Jilin University

Jifan Hu (✉ jifan@stanford.edu)

Stanford University Medical School <https://orcid.org/0000-0002-2174-0361>

Jiuwei Cui

Jilin University First Hospital

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Abstract

Miscarriage, the spontaneous loss of a pregnancy before the fetus achieves viability, is a common complication of pregnancy. Decidualization plays a critical role in the implantation of the embryo. To search for molecular factors underlying miscarriage, we explored the role of long noncoding RNAs (lncRNAs) in the decidual microenvironment, where the molecular crosstalk at the feto-maternal interface occurs. By integrating RNA-seq data from recurrent miscarriage patients and decidualized endometrial stromal cells, we identified H19, a noncoding RNA that exhibits paternally imprinted monoallelic expression in normal tissues, as the most upregulated lncRNA associated with miscarriage. Aberrant upregulation of H19 lncRNA was observed in decidual tissues derived from patients with spontaneous miscarriage as well as decidualized endometrial stromal cells. The maternally imprinted fetal mitogen Igf2, which is usually reciprocally co-regulated with H19 in the same imprinting cluster, was also upregulated. Notably, both genes underwent loss of imprinting, as H19 and IGF2 were actively transcribed from both parental alleles in decidual tissues. Mechanistically, this loss of imprinting in decidual tissues was associated with the loss of the H3K27m3 suppression marker in the IGF2 promoter, CpG hypomethylation at the central CTCF binding site in the imprinting control center (ICR) that is located between IGF2 and H19, and the loss of CTCF-mediated intrachromosomal looping. These data provide the first evidence that aberrant control of the ICR epigenotype-intrachromosomal looping-H19/IGF2 imprinting pathway may be a critical epigenetic risk factor in the abnormal decidualization related to miscarriage.

Introduction

Miscarriage is the most common complication of pregnancy, affecting >20% of recognized pregnancies in fertile women (1,2). Most miscarriages are sporadic and occur prior to the second trimester of pregnancy (3,4). A sub-set of women may suffer from recurrent miscarriage, defined as three or more consecutive miscarriages. This common gynaecological emergency poses significant challenges in regard to fertility and general psychological health.

A successful pregnancy depends upon complex crosstalk between the developmentally competent embryo and the receptive maternal endometrium (5,6). Upon implantation, embryos elicit a complex response in the decidua, characterized by transformation of stromal fibroblasts into secretory, epithelioid-like decidual cells, accompanied by the influx of specialized uterine immune cells and vascular remodeling. Decidual cells produce growth factors and cytokines (7,8), including insulin-like growth factor binding protein1 (IGFBP1) and prolactin (PRL), which are widely used as biomarkers of decidualized cells. Abnormal endometrial receptivity is a key factor leading to implantation failure. However, the molecular factors that regulate this crosstalk in decidualization reactions remains largely uncharacterized.

Long non-coding RNAs (lncRNAs) act as prominent epigenetic factors in normal development and numerous diseases, often by interacting with chromatin remodeling complexes (9-11). However, little is known about the functions of lncRNAs in miscarriage. Decidualization of the endometrium plays an

essential role for the establishment of a successful pregnancy. In order to identify key RNA molecules that mediate the molecular crosstalk at the feto-maternal interface, we integrated two RNA transcriptome sequencing datasets from RSA patients and decidualized human endometrial stromal cells. Notably, we identified *H19*, a paternally imprinted lncRNA (12,13), and its reciprocally co-regulated gene, the maternally imprinted fetal mitogen *Igf2* (14,15), were highly upregulated in decidual tissues. Aberrant DNA methylation in the *H19* imprinting control center (ICR) correlates with the risk of abortion (16). Decidual tissues derived from patients with spontaneous miscarriage showed that *H19-IGF2* expression was significantly increased in decidual tissues compared with healthy decidual tissues, suggesting abnormal *H19-IGF2* expression in decidualization was directly related to recurrent miscarriage. Allelic analysis revealed loss of *H19* and *IGF2* imprinting in decidual tissues of patients who had suffered a miscarriage. These data implicate the involvement of abnormal *H19-IGF2* imprinting in the decidual microenvironment related to miscarriage.

Results

Identification of H19 as miscarriage-associated lncRNA

To search for key factors that might be involved in the feto-maternal regulatory crosstalk, we integrated two RNA transcriptome sequencing datasets: GSE178535 (RNA-seq data from three RSA patients and three healthy control subjects) and GSE160702 (RNA-seq data from decidualized human endometrial stromal cells) (17). The integration using the VENN program identified a total of 745 differentially expressed genes (**Figs.1A**), including 673 protein-coding genes and 62 lncRNAs. The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis showed the association with cytokine-cytokine receptor interaction, pathways in cancer, basal cell carcinoma, proteoglycans in cancer, signaling pathways in the regulation of stem cell pluripotency (**Fig.1B, Table S2**).

Among 62 identified lncRNAs ($\log_2\text{FoldChange} > 2$, $\text{VALUE} < 0.001$), *H19* was the most upregulated lncRNA (**Figs.1C, Table S3**). *H19* is a well-known imprinted lncRNA. In most tissues, *H19* is expressed only from the maternal allele, while the paternal allele is imprinted and not expressed.

Dysregulation of H19 in patients with idiopathic spontaneous miscarriages

We then quantitated the expression of *H19* in decidual tissues collected from 32 patients with first-trimester miscarriage. For comparison, decidual tissues were also collected from 57 healthy adult women at 7-10 weeks of gestation who were undergoing early pregnancy termination. Using RT-qPCR, we found that the expression of *H19* was significantly higher in decidual tissues from the patients with spontaneous miscarriages than in decidual tissues of healthy female subjects (**Fig.2A**, $p < 0.05$).

The *H19* gene is located in an imprinting cluster on human chromosome 11 and is co-regulated with *IGF2*, a gene that encodes a mitogen that is required for normal fetal growth. Therefore, we also quantitated the mRNA abundance of *IGF2* in decidual tissues using quantitative PCR and found that, like

H19, *IGF2* was also significantly upregulated in decidual tissues derived from patients who had suffered a miscarriage (**Fig.2B**, p<0.01).

Loss of genomic imprinting in decidual tissues from miscarriage patients

To examine the status of *H19* and *IGF2* imprinting in decidual tissues, we genotyped genomic DNA using two single nucleotide polymorphisms (SNPs) in *H19* and *IGF2*. Heterozygous SNPs were used to distinguish the two parental alleles, and the imprinting status was examined in those tissues that were SNP-informative. Twenty-one of the decidual tissues derived from patients who had suffer a miscarriage were informative for *H19* heterozygosity and 20 were informative for *IGF2* heterozygosity. We found that the *H19/IGF2* imprinting was lost in 39% (11/28) of *H19/IGF2* informative decidual tissues from the miscarriage cases (**Fig.3A**). Among them, 2 out of 21 samples (9.5%) showed the loss of *H19* imprinting, and 7 out of 20 samples (35%) exhibited *IGF2* LOI. Two samples that were *H19/IGF2* informative also showed the loss of imprinting of both *H19* and *IGF2* (**Table 1**).

As an example, the decidual tissue from Control #13 showed normal imprinting of *H19* (maintenance of imprinting, MOI) (**Fig.3B**, panel 2). The genomic DNA carried both the “A” and “C” alleles, but the cDNA showed the exclusive expression of the “A” allele. The “C” allele was silenced. The decidual tissues from two cases (#U18 and #M22) were also informative for the SNP (**Fig.3B**, panels 3-4). However, both the “A” and “C” alleles were detected in their cDNA samples, demonstrating loss of imprinting (LOI) (**Fig.3B**).

Similarly, the genotyping of a SNP at the 3'-UTR of *IGF2* showed the presence of the “C/T” alleles. In normal informative decidual tissues, only the “T” allele was expressed (**Fig.3C**, top right panel). However, in two cases of miscarriage (U11, M22), the normally silenced C allele was expressed in decidual tissues (**Fig.3C**, right panels 2-3).

Loss of *IGF2/H19* imprinting is an early oncogenic event being detected in tumor-paired adjacent normal tissues (18). Thus, we also examined the allelic expression of *IGF2/H19* in decidual samples of control subjects. Notably, we also detected the presence of *IGF2/H19* LOI in the decidua of some control subjects (**Tables S4-S5**), suggesting epigenetic vulnerability in the decidual microenvironment of early embryo development.

Loss of genomic imprinting following decidualization in primary cells

In vitro cell-induced decidualization has provided a good model for studying the complex process of implantation (11,19,20). We thus examined if genomic imprinting would be altered following induction of decidualization in human U29 primary endometrial stromal cells that kept normal *H19-IGF2* imprinting (MOI). We induced decidualization *in vitro* by treating U29 cells with 10 nM E2, 1 µM P4 and 0.5 mM 8-Br-cAMP for 96 h. Following the treatment, cell morphology changed from elongated to rounded, and proliferation increased (**Fig.4A**). The treated cells exhibited elevated expression of decidualization markers PRL and IGFBP1 (**Fig.4B**). *H19* and *IGF2* were significantly upregulated in the decidualized cells (**Fig.4C**).

The untreated cells maintained normal imprinting, with only the “C” allele expressed (**Fig.4D**, right top panel). The imprinting status of *H19* was maintained in the *in vitro* induced decidualized cells (right bottom panel), but *IGF2* imprinting was lost, with both parental alleles (C/T) expressed in the decidualized cells (**Fig.4E**, right bottom panel). *IGF2* and *H19* expression are normally tightly coordinated and reciprocally controlled by an “enhancer competition” mechanism [64]. The data from these treated primary endometrial stromal cells, however, suggest that the imprinting control of *IGF2* and *H19* can be uncoupled.

Loss of imprinting is associated with aberrant histone H3K27 methylation

We then examined the epigenetic mechanisms underlying the loss of imprinting by focusing on the histone 3 lysine 27 (H3K27) suppression marker in the *IGF2* promoter (**Fig.5A**) (21). Using a ChIP assay with antibodies specific for H3K27me3, we found that H3K27 methylation in the first two *IGF2* imprinted promoters (hP2, hP3) was significantly reduced in 8-Br-cAMP-treated primary U29 decidual cells, where the *IGF2* imprinting status was lost (**Fig.5B**). No significant change of the H3K27me3 suppression mark was observed in the hP4 promoter.

Aberrant H3K27 imprinting is accompanied with the loss of intrachromosomal looping

The status of histone 3 lysine 27 (H3K27) is determined by the CTCF-orchestrated intrachromosomal looping (22,23). CTCF binds to unmethylated DNA motifs in the imprinting control region (ICR) located between the *H19* and *IGF2* genes, and orchestrates the formation of an intrachromosomal loop, where polycomb repressive complex 2 (PCR2) is recruited via the docking factor SUZ12, leading allelic H3K27 methylation which then silences the imprinted allele (24).

We used chromosome conformation capture (3C) methodology to examine the chromatin three-dimensional (3D) structure surrounding the *IGF2/H19* locus, with the focus on the CTCF-binding site in the ICR (25). As expected, we detected an intrachromosomal loop structure between the ICR-enhancers and ICR-*IGF2* promoters in untreated U29 primary decidual cells (**Fig.6A**). The 3C products were purified and DNA sequencing confirmed the loop joint separated by the Bgl2/BamH1, Bgl2/Bgl2, BamH1/BamH1 ligation sites (**Fig.6B**). However, after induced decidualization *in vitro* with 8-Br-cAMP, all three intrachromosomal loops were abolished (**Fig.6C**), in parallel with the loss of *IGF2* imprinting. As was previously reported in cancer cells with LOI (22), CTCF-orchestrated intrachromosomal looping may be essential for maintaining normal imprinting of *IGF2* in decidual tissues.

Loss of imprinting is associated with de novo DNA methylation in the imprinting control region

The methylation status of CpG islands in the imprinting control region (ICR) located upstream of the *H19* gene plays a pivotal role in the formation of intrachromosomal loops. The ICR contains seven CTCF binding sites. Among them, the 6th CTCF is differentially methylated (26) and serves as a CTCF “boundary insulator” (27). Specific binding of CTCF to the unmethylated maternal allele creates a physical boundary that blocks the interaction of downstream enhancers with the *IGF2* promoters and

thus silences the maternal *IGF2* allele. On the other hand, methylation of the ICR prevents CTCF binding and permits expression of *IGF2* and silencing of *H19* from the paternal allele. As a result, differential methylation at the CTCF binding sites ensures the reciprocal imprinting of these two neighboring genes (18).

We examined allele-specific DNA methylation in decidual tissues that were informative for two SNPs in the ICR and one SNP in the *H19* promoter (**Fig.7A**). The status of CpG DNA methylation was examined using sodium bisulfite sequencing. After converting the unmethylated cytosines into uracils by sodium bisulfite, the ICR and *H19* promoter regions were amplified with DNA methylation-specific primers, and cloned into a pJet vector for DNA sequencing. Case #M22 tissue derived from a patient with miscarriage, was homozygous for two SNPs, and therefore we were not be able to distinguish the two parental alleles. However, we detected hyper-methylation in the ICR and the *H19* promoter (**Fig.7B**, top panel). Case U11, which was heterozygous for the ICR SNP, had a hyper-methylated “AA” allele and an increased DNA methylation in the “AG” allele (36.5%)(left top panel). As expected, a typical semi-methylated pattern was observed in control #C4 that had normal mono-allelic expression of *H19* and *IGF2* (**Fig.S2**).

We also observed increased CpG DNA methylation at the ICR CTCF6 site and *H19* promoter (19.2% and 63.1%) in decidualized cells, as compared with the control cells (4.6% and 47%) (**Fig.S3**). These data suggest that aberrant imprinting of *H19/IGF2* may be associated with CpG DNA epimutations in the ICR region.

Discussion

The molecular mechanisms underlying the spontaneous loss of a pregnancy are unknown(28). Decidualization plays a critical role in the implantation of the embryo through a regulatory network that coordinates trophoblast invasion of the maternal decidua-myometrium and remodeling of maternal uterine **spiral arteries** (29,30). Many factors, including locally secreted cytokines and growth factors, are involved in this complicated network. We have identified the lncRNA *H19* as the most upregulated RNA molecule in decidual tissue, where the molecular crosstalk at the feto–maternal interface occurs. *H19* is also significantly upregulated in the decidua derived from patients with miscarriage. *IGF2*, a gene which encodes an important fetal mitogen, is located at the same chromosomal locus, and it is also increased in the decidua in patients who have suffered a miscarriage. In most normal tissues, the *H19/IGF2* locus is imprinted. In this study, we demonstrate that there is loss of *H19* and *IGF2* imprinting in decidual tissues of miscarriage patients. Loss of imprinting also occurs following induced decidualization in primary endometrial stromal cells. These data suggest that dysregulation of *IGF2/H19* imprinting may be related to poor decidualization in patients with miscarriage. Mechanistically, we show that this aberrant imprinting in decidual tissues was associated with the loss of the H3K27m3 suppression marker as well as the loss of intrachromosomal looping and CpG demethylation in the imprinting control center (ICR). These studies suggest the involvement of abnormal *H19/IGF2* epigenetic regulation in the decidual microenvironment, which may be a risk factor for the development of early unexplained spontaneous abortion (**Fig.7C**).

Both the maternal and paternal genomes are necessary for normal embryogenesis and fetal development (31,32). *H19* is a maternally-expressed imprinted gene and its transcription gives rise to a fetal lncRNA that also functions as a precursor to miR675 (33), which negatively affects cell proliferation and tumor metastasis (34). *H19* is abundantly expressed prior to implantation or shortly thereafter, and its expression is specifically confined to progenitor cells of the placenta and extraembryonic tissues (35,36). *H19* is expressed coordinately with its neighboring gene *Igf2*, a gene that plays a key role in regulating feto-placental development (37,38). Genomic deletion of *Igf2* causes placental and fetal growth restriction. In contrast, overexpression of *Igf2* induces placental and fetal overgrowth via paracrine and/or autocrine IGF pathways. The serum levels of IGF-II have been positively linked to infant birth weight. *H19* and *Igf2* regulate embryonic development (39,40). The allelic expression of *IGF2/H19* is coordinately controlled by a differentially methylated imprinting control region (ICR) in the upstream of the *H19* promoter (18,41). In this study, we demonstrate that both *H19* and *IGF2* are upregulated in decidual tissues of miscarriage patients. Moreover, there is loss of imprinting of both genes in decidual tissues. Our study suggests that aberrant allelic expression of *H19/IGF2* genes may lead to abnormal fetal development and spontaneous miscarriage in these patients.

Major epigenetic events take place in the embryo both in pre-implantation development and in post-implantation stages, including the genome-wide resetting of imprints in the PGCs (42,43). Aberrant methylation of imprinted genes correlates with the risk of abortion (16). Specifically, CpG hypomethylation in *H19* ICR is correlated with recurrent pregnancy loss (44). As a result, the periconceptional stage is very sensitive to environmental stressors, leading to epigenetic disturbances. Our data also suggest that aberrant resetting of imprints in pre-implantation development and post-implantation stages may be mechanistically associated with the onset of early spontaneous miscarriage.

In summary, this study demonstrates that loss of *H19/IGF2* imprinting in decidua may be a critical risk factor related to early miscarriage. Increased abundance of *H19* lncRNA in association with the high abundance of IGF-II mitogen in the human fetal decidua may alter normal fetal-placental development. Dynamic regulation of the *H19/IGF2* cluster is critical for normal fetal growth and development. It is noteworthy that aberrant imprinting can be epigenetically corrected (18). It would be interesting to explore whether epigenetic targeting of the *H19/IGF2* epimutation may provide a novel alternative strategy for the prevention and therapy of recurrent miscarriage.

Materials And Methods

Identification of miscarriage-associated lncRNAs using RNA-Seq data

To identify miscarriage-associated lncRNAs, we downloaded two datasets (GSE178535 and GSE160702) from the NIH GEO database website. The GSE178535 dataset contained the RNA-seq data of decidual tissues from three recurrent miscarriage patients and three healthy control subjects (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE178535>).

The GSE160702 dataset was the RNA-seq data from decidualized human endometrial stromal cells (ESCs) (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE160702>). The *in vitro* decidualization of ESCs was induced using differentiation media containing 0.3 mM dibutyryl cAMP, 1 μ M medroxyprogesterone 17-acetate and 10 nM β -estradiol. Decidualized cells were used for RNA-seq (17).

Differentially expressed RNAs were calculated as the log2-transformed gene expression values (Fold Change). The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis (KEGG_PATHWAY) was carried out using DAVID Bioinformatics Resources 6.8 (<https://david.ncifcrf.gov>). Hierarchical Cluster Heatmap was generated using HIPILOT (<https://hiplot.com.cn>). The above two RNA-Seq datasets were merged using the VENN program (<http://bioinformatics.psb.ugent.be/webtools/Venn/>). Venn diagrams were constructed to visualize the overlap RNAs between the two datasets. The overlapping RNAs with the fold-change > 2 and $p < 0.001$ were chosen for further functional characterization.

Human decidual samples

Decidual tissue samples were collected from The First Hospital of Jilin University between 2017-2019. A total of 32 decidual tissues were collected from women with unexplained miscarriage. In addition, 57 decidual samples were obtained as the control group from healthy adult women at 7-10 weeks of gestation undergoing legal elective termination. Ethical approval for this study was provided by the Research Ethics Board of the First Hospital of Jilin University, and written informed consent was obtained from all patients prior to sample collection.

Culture of human primary endometrial stromal cells

Primary endometrial stromal cells were cultured from U29 decidual tissues that were *H19-IGF2* informative and kept normal imprinting. Fresh tissues were cut into approximately 2 mm³ fragments, washed in DMEM (high glucose; Sigma), and directly cultured at 37°C in 5 % CO₂ by attaching to the substratum in a 10-cm dish with complete medium consisting of DMEM medium (Sigma, MO) supplemented with 10% (v/v) fetal bovine serum (Sigma, MO), 100 U/ml of penicillin sodium, and 100 μ g/ml of streptomycin sulfate (Invitrogen, CA). After approximately 12 days in culture, cells migrated out from the edges. Migrating cells were collected with 0.1% trypsin and 0.25 mM EDTA and passaged for allelic study and *in vitro* decidualization assays (Fig.S1).

In vitro decidualization

In vitro artificially-induced decidualization was performed following the method as described (19). Briefly, U29 primary endometrial stromal cells were cultured in complete medium containing 10 nM E2, 1 μ M P4 and 0.5 mM 8-Br-cAMP. Culture medium was changed every 2 days. Cells were harvested for subsequent experiments 96 h after the treatment.

RT-PCR quantitation

Decidual tissues and cells were collected and total RNA was extracted by TRIzol reagent (Sigma, CA) and stored at -80°C. cDNA was synthesized using RNA reverse transcriptase (Invitrogen, CA), and target amplification was performed with a Bio-Rad Thermol Cycler. PCR of 1 cycle at 95°C for 2 min, 32 cycles at 95°C for 15 sec, 60°C for 15 sec, and 72°C for 15 sec, and 1 cycle at 72°C for 10 min; β-actin was used as the control. Quantitative real-time PCR was performed using SYBR GREEN PCR Master (Applied Biosystems, USA); the threshold cycle (Ct) values of target genes were assessed by quantitative PCR in triplicate using a sequence detector (ABI Prism 7900HT; Applied Biosystems) and were normalized over the Ct of the β-actin control. Primers used for PCR quantitation are listed in **Table S1**.

Allelic expression of IGF2 and H19

Genomic DNA and total RNA extraction from decidual tissues and cDNA synthesis were performed as previously described. Decidual tissues were first genotyped for heterozygosity of SNPs in *IGF2* exon 9 and *H19* exon 5 (Fig2A). Target amplification was performed with a Bio-Rad Thermol Cycler. PCR of 1 cycle at 95°C for 2 min, 32 cycles at 95°C for 15 sec, 60°C for 15 sec, and 72°C for 15 sec, and 1 cycle at 72°C for 10 min using primers specific for two polymorphic restriction enzymes (Apal, AluI) in the last exon of human *IGF2* and *H19* exon 5. To determine the status of *IGF2* imprinting, the amplified products were sequenced by Comate Bioscience Co, Ltd (Changchun, China). Decidual tissues that maintain normal imprinting (MOI) express a single parental allele, while the LOI showed biallelic expression of *IGF2* and *H19*. PCR primers used for *IGF2* imprinting are listed in Supplementary Table S1.

DNA methylation analysis

Genomic DNA collected from tissues or cells, using dBIOZOL Genomic DNA Extraction Reagent (BioFlux, BSC16M1) following the manufacturer's instructions. DNA was treated with EZ DNA Methylation-Gold™ Kit (ZYMO RESEARCH, D5005), and PCR was performed using DNA methylation-specific primers designed for the promoter of *H19* and CTCF binding sites (**Table S1**). To examine the status of DNA methylation in every CpG site, the amplified PCR DNAs were cloned into pJET1.2/blunt cloning vector (Thermo, K1231) and transformed into TOP10. Plasmid DNA was collected by Wizard® Plasmid DNA Purification kit (Promega, A1223) and sequenced.

Chromosome conformation capture (3C)

The 3C assay was performed to determine long-range intrachromosomal interactions as previously described (23,45-47). Briefly, 1.0×10^7 cells were cross-linked with 2% formaldehyde and lysed with cell lysis buffer (10 mM Tris [pH 8.0], 10 mM NaCl, 0.2% NP-40, supplemented with protease inhibitors). Nuclei were collected, suspended in 1× restriction enzyme buffer. An aliquot of nuclei (2×10^6) was digested with 800U of restriction enzyme BamH1 / Bgl2 at 37°C overnight. After stopping the reaction by adding 1.6% SDS and incubating the mixture at 65°C for 20 min, chromatin DNA was diluted with NEB ligation reaction buffer, and 2μg DNA was ligated with 4000U of T4 DNA ligase (New England BioLabs, CA) at 16°C for 4 h (final DNA concentration, 2.5μg/ml). After treatment with 10mg/ml proteinase K at 65°C for 4 h to reverse cross-links and with 0.4μg/ml RNase A for 30 min at 37°C, DNA was extracted

with phenol-chloroform, ethanol precipitated and detected by PCR amplification of the ligated DNA products. 3C PCR products were cloned and sequenced to validate the intrachromosomal interactions by assessing for the presence of the BamH I/Bgl II ligation site. The 3C interaction was quantitated by qPCR and was standardized over the 3C ligation control. For comparison, the relative 3C interaction was calculated by setting the control as 1. Primers used for 3C assay are listed in Supplementary **Table S1**.

Histone methylation by chromatin immunoprecipitation (ChIP) assay

A ChIP assay was used to quantitate the status of histone modifications, following the manufacturer's protocol (Upstate Biotechnology, Lake Placid, NY, USA). Briefly, 1.0×10^7 cells were fixed with 1% formaldehyde and then sonicated for 180 s (10 s on and 10 s off) on ice with a sonicator with a 2-mm microtip at 40% output control and 90% duty cycle settings. The sonicated chromatin was collected by centrifugation, aliquoted and stored at -80°C. Protein A/G Magnetic Beads and a specific anti-trimethyl-histone H3 (Lys27) antibody (Merck Millipore, Darmstadt, Germany) were incubated with rotation for 30min at room temperature. The sonication supernatant and beads were incubated with antibody at 4°C on a rotating rack for 4-16 hours or overnight. To reduce the ChIP background, we modified the manufacturer's protocol by adding two washing steps following immunoprecipitation. As previously reported (23), anti-IgG was used as the ChIP control in parallel with testing samples. Precipitated DNA was subjected to qPCR and expressed as fold-enrichment compared to the IgG chromatin input.

Statistical Analysis

The experimental data are expressed as mean \pm SD and were performed in triplicate. Data were analyzed using SPSS software (version 16.0; SPSS, IL). Student's t test or one-way ANOVA (Bonferroni test) was used to compare statistical differences for variables among groups. Results were considered statistically significant at $p < 0.05$.

Declarations

Ethics approval and consent to participate

Ethical approval for this study was provided by the Research Ethics Board of the First Hospital of Jilin University, and written informed consent was obtained from all patients before sample collection.

Consent for publication

Not applicable.

Availability of date and materials

GSE178535 and GSE160702 downloaded from the NIH GEO database website. The GSE178535 dataset contained the RNA-seq data of decidua tissues from three recurrent miscarriage patients and three healthy control subjects (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE178535>).

The GSE160702 dataset was the RNA-seq data from decidualized human endometrial stromal cells (ESCs) (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE160702>).

Competing interests

The authors declare no competing interests.

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Authors' contributions

J.F.H., J.C., and Z.L. conceived and designed the study; W.L., S.Z., X.C., and Y.W. supervised the project; W.X. and Q.Z. performed most of the experiments and organized the data; L.Z., Z.L., X.W., J.Z., H.L., Y.C., C.N., J.Q., M.L., and J.X. conducted cell assays; J.F.H. wrote the paper; A.R.H. edited the manuscript. All authors read and approved the manuscript.

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Tables

Table 1. Loss of *H19* and *IGF2* imprinting in miscarriage decidua

		<i>H19</i>		<i>IGF2</i>	
Cases (ID)		Genotype	cDNA	Genotype	cDNA
Loss of imprinting of <i>H19</i> (9.5%)*					
1	U18	A/B	a/b	A/B	b
2	U21	A/B	a/b	B/B	-
Loss of imprinting of <i>IGF2</i> (35%)**					
1	8	A/B	a	A/B	a/b
2	E1	A/B	b	A/B	a/b
3	E3	A/A	-	A/B	a/b
4	E5	A/B	b	A/B	a/b
5	U11	A/A	-	A/B	a/b
6	U14	A/A	-	A/B	a/b
7	U17	A/A	-	A/B	a/b
Loss of imprinting of <i>H19</i> and <i>IGF2</i>***					
1	M22	A/B	a/b	A/B	a/b
2	U20	A/B	a/b	A/B	a/b

* After genotyping, 21 informative samples were used for *H19* allelic analysis

** 20 *IGF2*-informative samples were used to examine *IGF2* imprinting

*** Informative for both *H19* and *IGF2*

- Tissues that are not informative for allelic analysis of either *H19* or *IGF2*

Figures

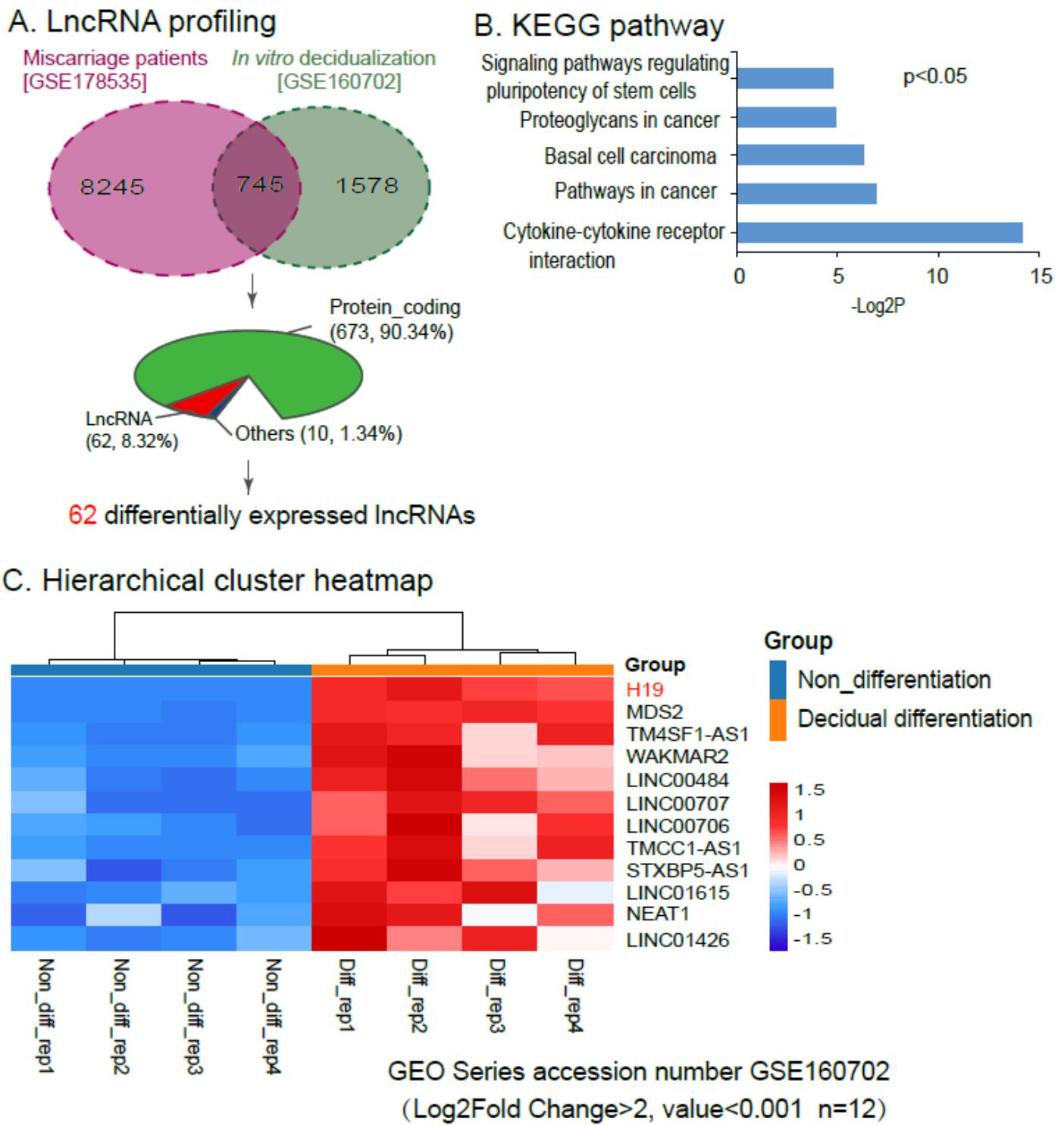


Figure 1

Differentially expressed lncRNAs in RSA patients by RNA-seq

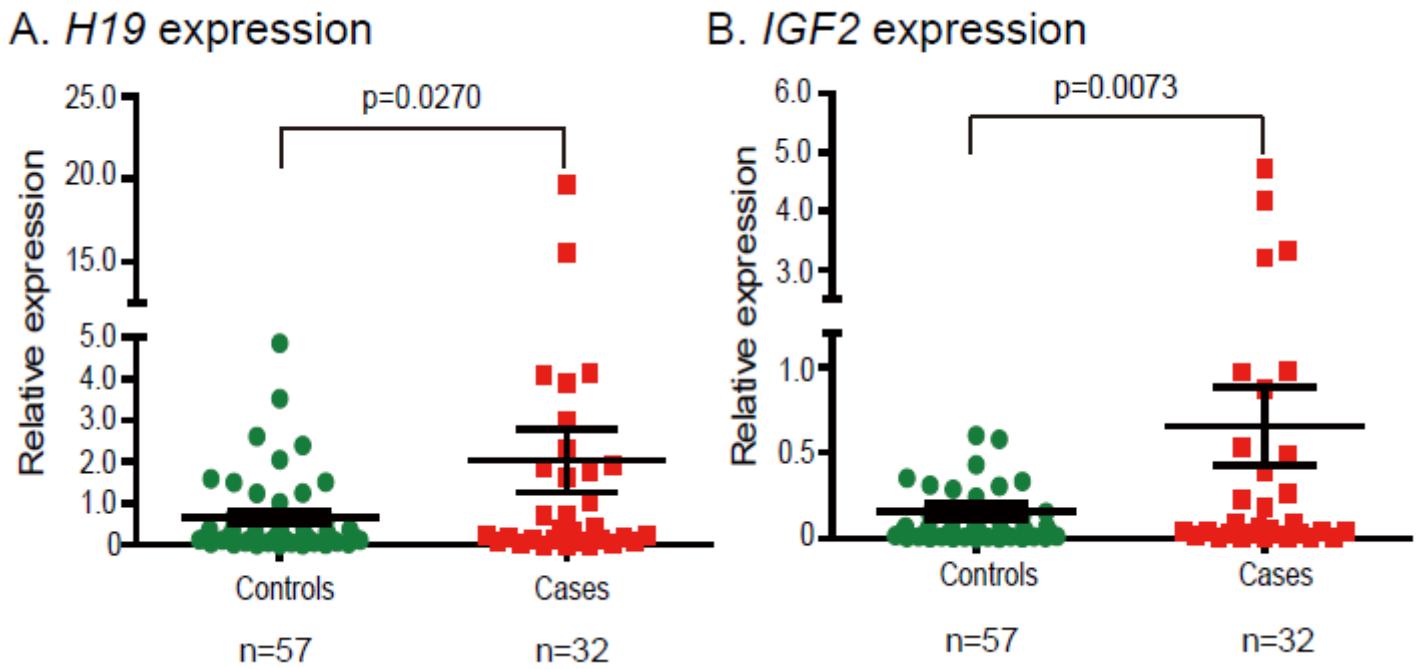
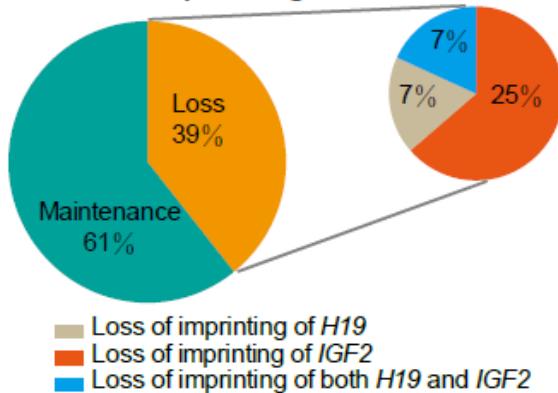


Figure 2

Upregulation of H19 and IGF2 in decidua of miscarriage patients

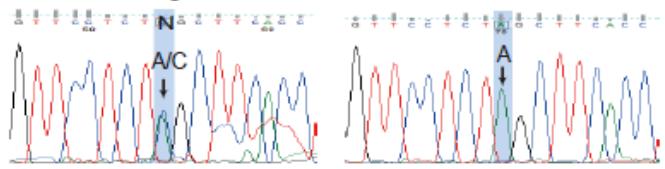
A. Loss of imprinting



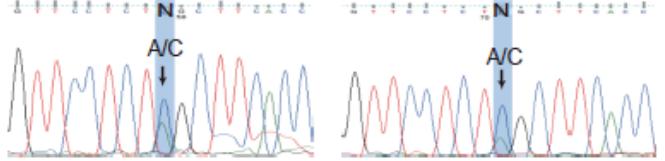
B. *H19* imprinting



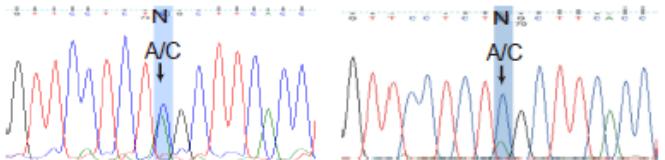
Control 13



Case U18



Case M22



C. Imprinting of *IGF2*

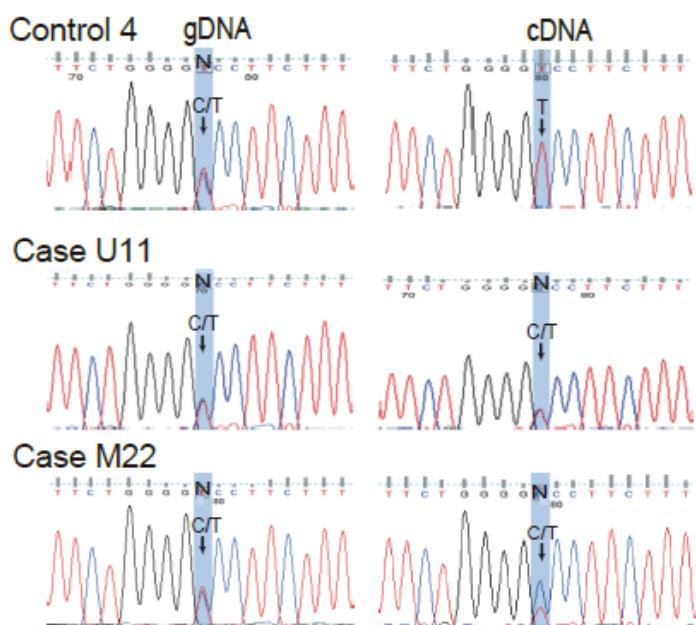
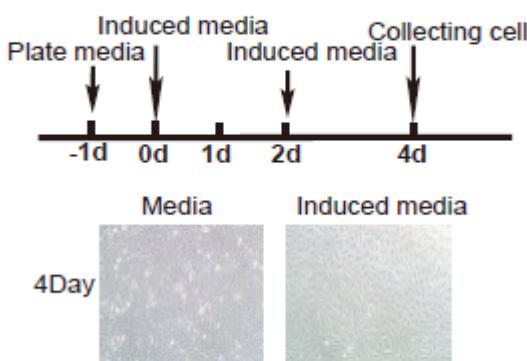


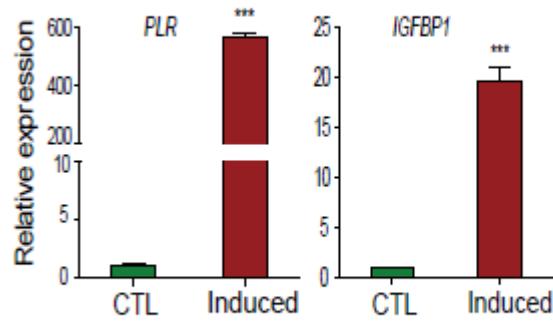
Figure 3

Loss of *H19/IGF2* imprinting in decidual tissues of miscarriage cases.

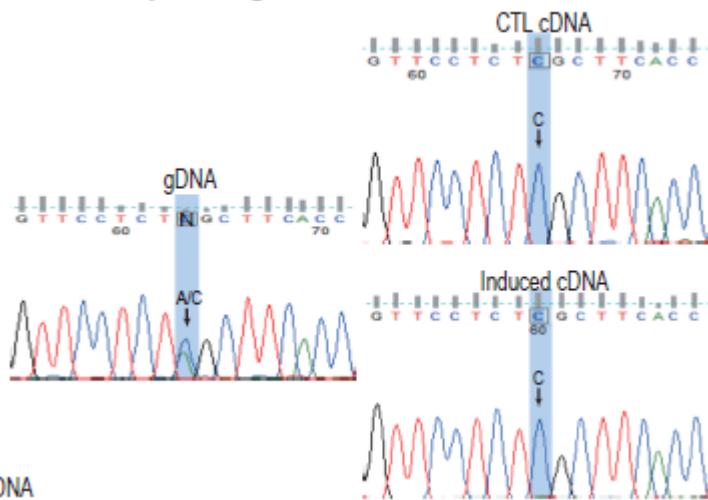
A. Drug-induced decidualization



B. Deciduation markers



D. *H19* Imprinting



E. *IGF2* Imprinting

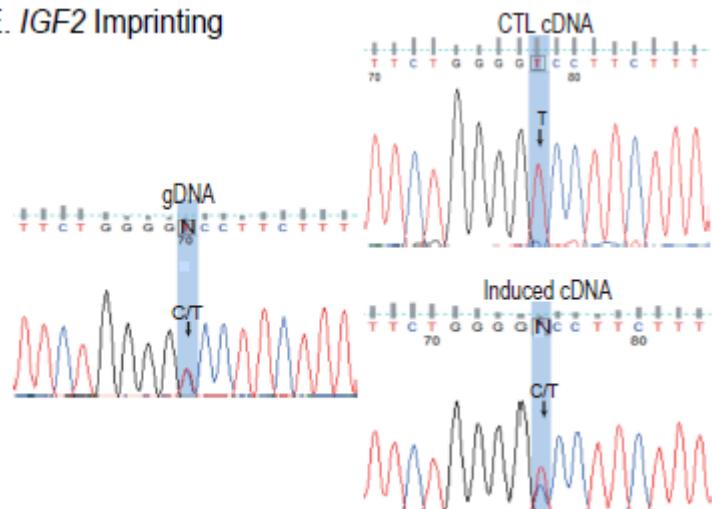
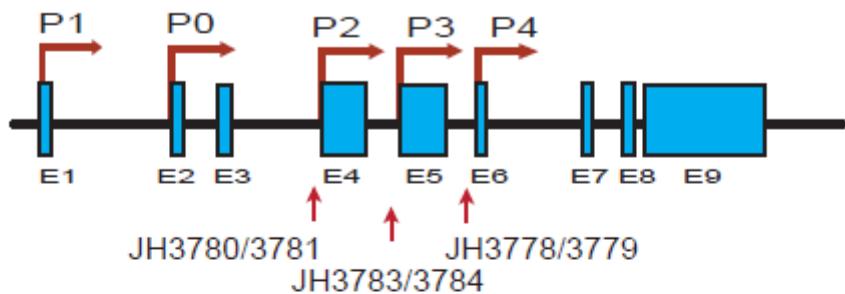


Figure 4

Aberrant *H19*/*IGF2* expression in primary endometrial cells following drug-induced decidualization.

A. ChIP primers



B. H3K27 methylation

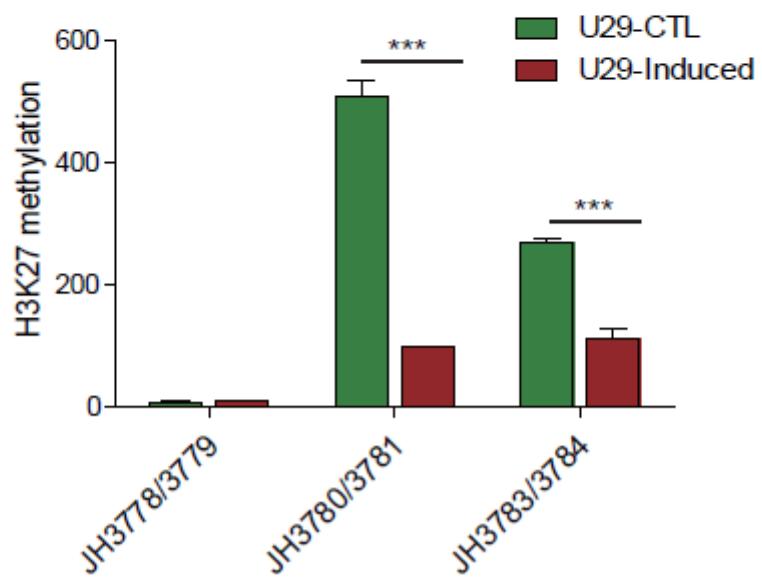
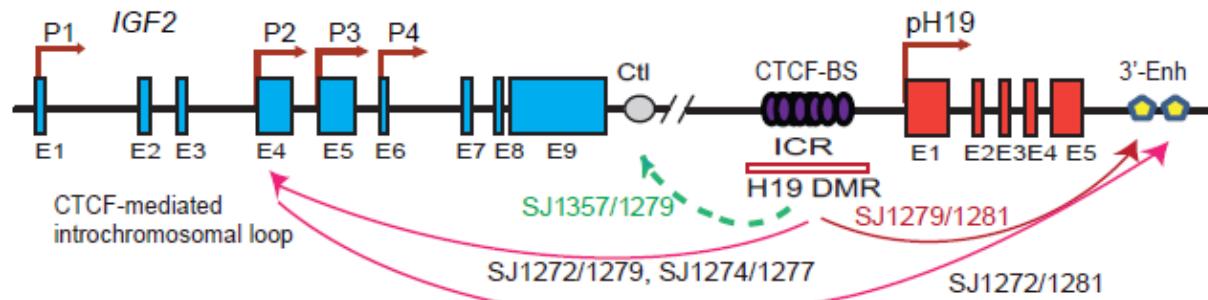


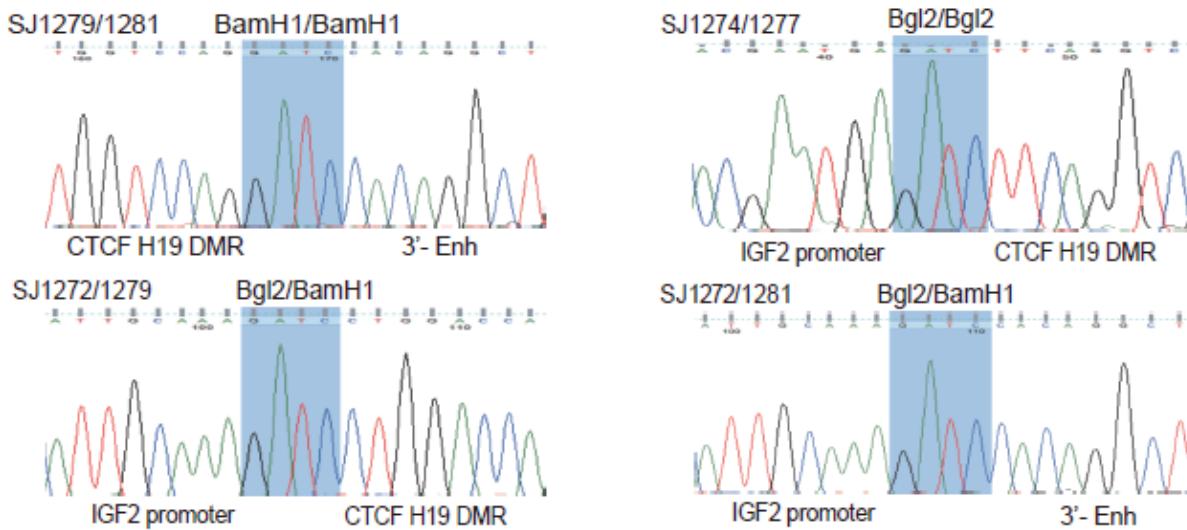
Figure 5

H3K27 methylation in the promoter of IGF2.

A. CTCF-mediated intrachromosomal loops in the *H19/IGF2* locus



B. *H19/IGF2* 3C sequencing



C. Intrachromosomal loop

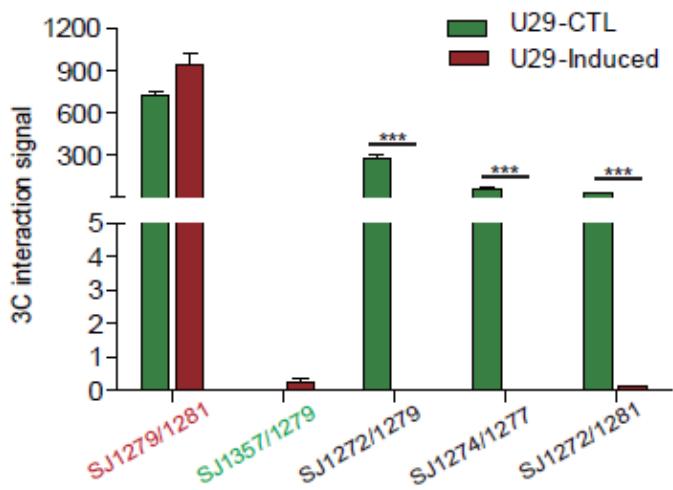
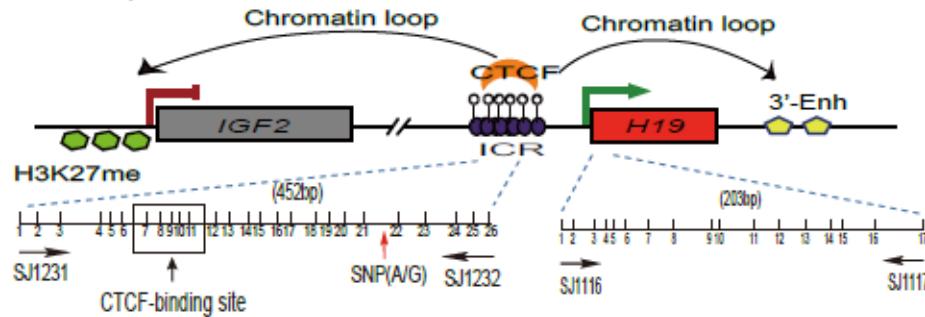


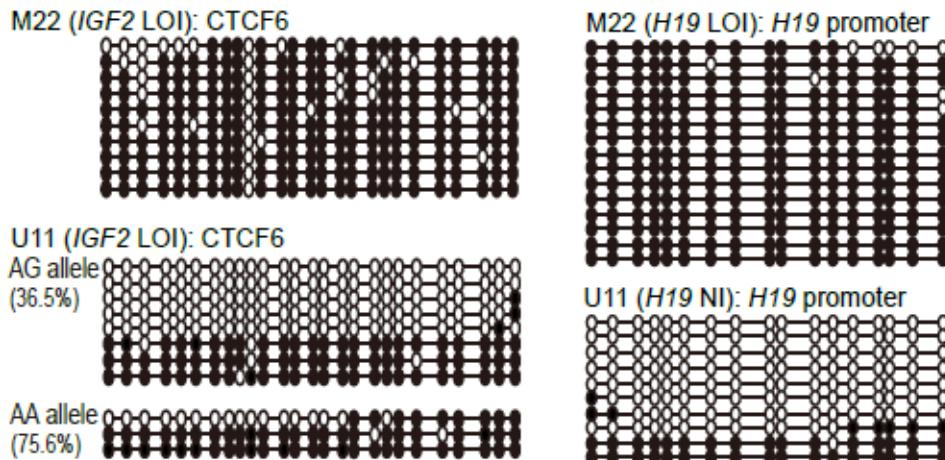
Figure 6

Intrachromosomal loop interactions in the *H19/IGF2* imprinting locus.

A. CTCF CpG sites in H19/IGF2 ICR



B. DNA methylation



C. Aberrant imprinting in miscarriage

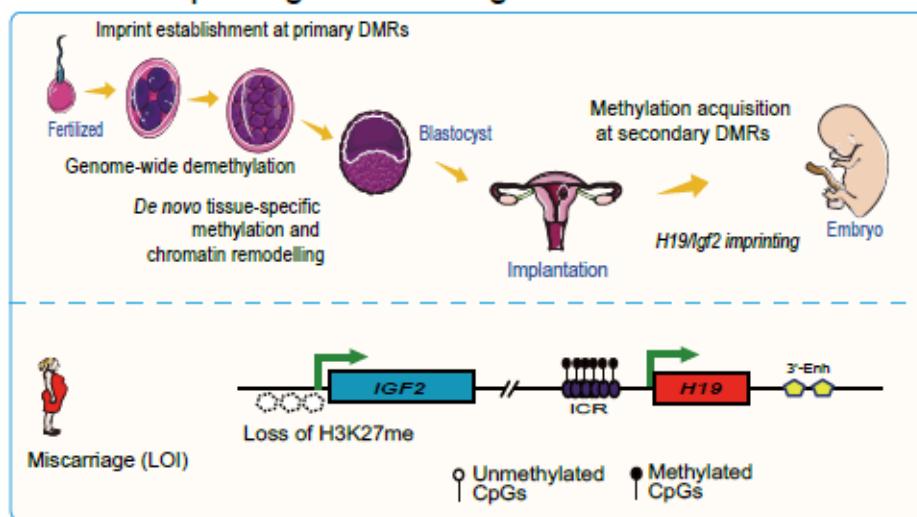


Figure 7

Abnormal DNA methylation in the imprinting control region (ICR)

Supplementary Files

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