DNA methylation modulates H19 and IGF2 expression in porcine female eye

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Abstract

The sexually dimorphic expression of H19/IGF2 is evolutionarily conserved. To investigate whether the expression of H19/IGF2 in the female porcine eye is sex-dependent, gene expression and methylation status were evaluated using quantitative real-time PCR (qPCR) and bisulfite sequencing PCR (BSP). We hypothesized that H19/IGF2 might exhibit a different DNA methylation status in the female eye. In order to evaluate our hypothesis, parthenogenetic (PA) cells were used for analysis by qPCR and BSP. Our results showed that H19 and IGF2 were over-expressed in the female eye compared with the male eye (3-fold and 2-fold, respectively). We observed a normal monoallelic methylation pattern for H19 differentially methylated regions (DMRs). Compared with H19 DMRs, IGF2 DMRs showed a different methylation pattern in the eye. Taken together, these results suggest that elevated expression of H19/IGF2 is caused by a specific chromatin structure that is regulated by the DNA methylation status of IGF2 DMRs in the female eye.

Keywords: H19/IGF2, gene expression, DNA methylation, parthenogenetic, pig.
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Introduction

The imprinted gene IGF2 has been reported to be paternally expressed and it is a growth factor in mammals (Barlow et al., 1991). The H19 gene, which is maternally expressed, encodes a non-coding RNA (Bartolomei et al., 1991; Brunkow and Tilghman, 1991; Gabory et al., 2006). Numerous studies have demonstrated that the parent-specific expression of H19/IGF2 depends on differentially methylated regions (DMRs) (Park et al., 2011; Thorvaldsen et al., 2006). Studies have also shown that DMRs play an important role in the regulation of gene expression in the H19/IGF2 cluster within promoters and enhancers (Braunschweig et al., 2011).

It has been reported that the H19 DMRs in mice contain three CTCF-binding motifs (Han et al., 2008). Through CTCF binding to the DMRs, IGF2 is inactivated and maternal H19 is expressed. However, when that binding is prevented, paternal IGF2 is expressed. This is known as parent-specific chromatin loops (Wei et al., 2005), and, as part of this mechanism, IGF2 DMRs are considered an epigenetic switch that regulates H19 and IGF2 expressions. H19/IGF2 are reciprocally imprinted in most tissues, although they are expressed at different levels, a fact which contributes to sex-bias in the female mouse eye, but not in other tissues (Hudson et al., 2010; Park et al., 2009; Reinius and Kanduri, 2013). In this study, we investigated the expression of H19 and IGF2 in the eye of female pigs to detect sex-specific imprinting effects. We hypothesized that the DNA methylation patterns of the H19 DMRs and IGF2 DMRs interact in the eye, and that this might regulate H19 and IGF2 expression through a specific chromatin structure. To test this hypothesis, we analyzed the expression pattern and methylation status of H19/IGF2 in parthenogenic (PA) cells by qPCR and bisulfite sequencing PCR (BSP).

Materials and Methods

Ethics statement

Pig experiments were carried out in accordance with the guidelines on animal care and use of animals in research, which were approved by the Animal Care and Use Committee of Jilin University, Changchun, China.
Sample collection

The tissues of porcine eye were obtained from three female and three male newborn piglets. They were, rinsed in Dulbecco’s phosphate-buffered saline (PBS, Sigma, St. Louis, MO, USA), immediately frozen in liquid nitrogen and stored in -80 °C until use.

The protocol for cell harvesting was previously described in detail (Han et al., 2013; Wang et al., 2014b). Briefly, matured eggs were electrically and parthenogenetically activated (PA) by two direct current pulses of 1.2 kV/cm for 30 μs using a BTX Electro Cell Manipulator 2001 (BTX, San Diego, CA) and then cultured in cytochalasin B to suppress the extrusion of the second polar body. PA embryos were then transferred into the oviduct of a surrogate sow on the next day. PA fetuses were collected from each uterine horn at day 28.

Gene expression analysis

Total RNA was isolated from porcine eye cells using the TRNzol reagent (TIANGEN, Beijing, China) according to the instructions of the manufacturer. The RNA was treated with DNase I (Fermentas) and reverse transcribed to cDNA using the BioRT cDNA first strand synthesis kit (Bioer Technology, Hangzhou, China). Quantitative real-time PCR was performed to determine the expression of H19 and IGF2. The primer sequences are listed in Table 1. Quantitative gene expression analysis was carried out according to the manufacturer’s instructions using the BIO-RAD iQ5 Multicolor Real-Time PCR Detection System with the BioEasy SYBR Green I Real Time PCR Kit (Bioer Technology, Hangzhou, China). The thermal cycling conditions were 95 °C for 3 min, followed by 40 cycles of denaturation at 95 °C for 10 s, annealing at 55 °C for 15 s, and extension at 72 °C for 30 s. The 2ΔΔCT formula was used to determine relative gene expression, which was normalized to the quantity of GAPDH mRNA. All experiments were repeated three times for each gene. Data are expressed as means ± S.E.M.

Methylation pattern of H19 DMR3, IGF2 DMR1/2

The procedure for BSP was previously described by Clark et al. (1994). Briefly, genomic DNA of porcine eye cells was isolated using the TIANamp Genomic DNA Kit (TIANGEN, Beijing, China) and treated with the CpGenome Turbo Bisulfite Modification Kit (Millipore) according to the manufacturer’s instructions. Nested PCR using Taq Plus PCR Master Mix (TIANGEN, Beijing, China) was performed for the amplification of the H19 DMR3 and IGF2 DMR1/2. The primer sequences are listed in Table 2. PCR products were purified and subjected to BSP (10 positive clones) and Combined Bisulfite Restriction Analysis (COBRA), which have been described in a previous study (Watanabe et al., 2010; Huntriss et al., 2013).

Statistical analysis

The obtained qRT-PCR and BSP data were analyzed by t-tests using SPSS 16.0 software (SPSS Inc., Chicago, IL, USA). A value of p < 0.05 was considered statistically significant. The methylation status was analyzed using the online BiQ Analyzer software (http://biq-analyzer.bioinf.mpi-inf.mpg.de/tools/MethylationDiagrams/index.php).

Results

Imprinted gene expression analysis of H19 and IGF2

The qPCR results showed that the expression of IGF2 was up-regulated 2-fold in female eyes compared with male eyes. Furthermore, H19 expression was also up-regulated 3-fold (Figure 1A). In order to investigate if the elevated expression was regulated by a specific chromatin structure in H19/IGF2 clusters in female eyes, PA cells were analyzed using qPCR. As expected, the expression of H19 in PA cells was up-regulated, while IGF2 expression was significantly reduced compared with porcine embryonic fibroblast (PEF) cells (Figure 1B).

Methylation analysis of H19 DMR3 and IGF2 DMR1/2

The methylation level of H19 DMR3 was lower in the female eye compared to the male eye (45.2 ± 0.8% and 51.3 ± 4.4%, respectively) (Figure 2A,B). The PCR products were subjected to COBRA analysis, which confirmed

Table 1 - Primers for qRT-PCR analysis.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Annealing (°C)</th>
<th>Primer sequences (5' → 3')</th>
<th>Size (bp)</th>
<th>Reference/accession</th>
</tr>
</thead>
<tbody>
<tr>
<td>H19</td>
<td>55</td>
<td>F: CTCAAACGACAAGAGATGGT</td>
<td>122</td>
<td>(Park et al., 2011)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: AGTGTAGTGCTCCAGAATG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IGF2</td>
<td>55</td>
<td>F: AAGAGTGCTCTTCTCCGTA</td>
<td>156</td>
<td>(Park et al., 2011)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: TGTCATAGCGGAAGAACTTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>55</td>
<td>F: ATTCACCGCAGACTGTAAGG</td>
<td>120</td>
<td>NM_001206359.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: ACATACTCAGCACCAGCCTCG</td>
<td></td>
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</tbody>
</table>
our results (Figure S1A). Statistical analyses confirmed the significant difference (Figure 4A). These results suggested that the H19 DMRs were hemi-methylated in both female and male eyes.

Compared with H19 DMRs, IGF2 DMR1/2 was found to be the hypermethylated in porcine eyes. The results of BSP showed that 75.4 ± 1.1% of IGF2 DMR1 were hypomethylated in the female eye compared with 89 ± 1.1% in the male eye (Figure 3A,B), while 76.3 ± 0.6% of IGF2 DMR2 were hypermethylated in the female eye compared with 64.1 ± 1% in the male eye (Figure 3C,D). The

Table 2 - Primers for bisulfite sequencing PCR analysis.

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Annealing (°C)</th>
<th>Primer sequences (5’ → 3’)</th>
<th>Size (bp)</th>
<th>Reference/accession</th>
</tr>
</thead>
<tbody>
<tr>
<td>H19 DMR1</td>
<td>55 °C</td>
<td>Outer F:AGGAGATTAGGTTAGGGGAAT R: CTACCTCTCCTCTAATACCTAA Inner F: AGCTTGGGAGGTTTTTTTTTTT R: CACCCTCACCTAAATACCTAA</td>
<td>260</td>
<td>(Park et al., 2011)</td>
</tr>
<tr>
<td>H19 DMR2</td>
<td>55°C</td>
<td>Outer F:TATGTGATGGGGTATAAGT R: CCCACTCTCATAATCAAC Inner F: AGGTGTTTTGTGGTTGGT R: ATAAAAACTCTAAAAACTCAA</td>
<td>216</td>
<td>(Park et al., 2011)</td>
</tr>
<tr>
<td>H19 DMR3</td>
<td>55 °C</td>
<td>Outer F:TTAAAAACATCTACATAC R: GATTGTAGTTTGTATTATTTT Inner F: GAAAAATATATCTAACAA</td>
<td>208</td>
<td>(Park et al., 2011)</td>
</tr>
<tr>
<td>IGF2 DMR1</td>
<td>55°C</td>
<td>Outer F:GGAAGTTTTGTTAGTTGGTTTTT R: AAACCAAAAAGGAAACAAAAACACCA Inner F: GATGTTTTGTTTAGTTGTTTTT R: TACCAAAACTCCTACCTCAA</td>
<td>389</td>
<td>(Braunschweig et al., 2011)</td>
</tr>
<tr>
<td>IGF2 DMR2</td>
<td>55 °C</td>
<td>Outer F:GTTAGGGGGGGGTTTTTGTAGTTTTTA R: CTGCCCTTAACCTCTAATAACCTCC Inner F: GGTAGTATTTGAAGTTAAGGAG R: CTAAAAACTCTCAAAACACC</td>
<td>268</td>
<td>(Braunschweig et al., 2011)</td>
</tr>
</tbody>
</table>

Figure 1 - Relative expression levels of H19/IGF2. qRT-PCR analysis of H19/IGF2 in (A) male and female eye, (B) porcine embryonic fibroblast (PEF) and parthenogenetic (PA) cells. F: female; M: male. Data are reported as means ± SEM (n = 3). * p < 0.05, ** p < 0.01.

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PCR products were subjected to COBRA analysis, which confirmed the results (Figure S1B). Statistical analyses revealed that there was a significant difference in IGF2 DMR1 and DMR2 between female and male eyes (Figure 4A). Taken together, these results suggested that the IGF2 DMRs methylation status play a crucial role in the regulation of imprinted gene expression in the female eye.

Methylation status analysis of PA cells

The expression pattern and methylation status of H19/IGF2 led us to hypothesize that a specific chromatin structure was located in the porcine female eye (Figure 5). To further investigate whether the expression of H19 and IGF2 were associated with their respective DMR patterns, H19 DMRs and IGF2 DMRs were analyzed in PA cells. The BSP data showed that H19 DMRs in PA cells were significantly demethylated ($p < 0.05$) compared with those in PEF cells (Figure 2C,D), while IGF2 DMR1/2 were found to be hypermethylated in both PA and PEF cells (Figure 3E-H). The PCR products were subjected to COBRA analysis, which confirmed our BSP results (Figure S1C,D). Statistical analyses revealed that there was no difference in the

**Figure 2** - Methylation pattern of H19 DMR3. CpG methylation profiles of H19 DMR3 in male eye (A), female eye (B), porcine embryonic fibroblast (PEF) cells (C) and parthenogenetic (PA) cells (D). The black and white circles indicate methylated and unmethylated CpGs, respectively.

**Figure 3** - Methylation pattern of IGF2 DMRs. CpG methylation profiles of IGF2 DMR1 and DMR2 in (A-D) male eye and female eye, (E, G) porcine embryonic fibroblast (PEF) cells (F, H) parthenogenetic (PA) cells. The black and white circles indicate methylated and unmethylated CpGs, respectively.
methylated DMR2 of IGF2 closely interacts with H19 DMRs on the paternal chromosome in the female eye. This forces IGF2 over-expression (Figure 5D).

Due to the fact that H19 DMRs were found to be hemi-methylated in both male and female eyes, we explored whether the hypomethylation status of DMRs closely interacted with each other on the maternal chromosome. In this study, we used PA cells to determine if the elevated expression of H19/IGF2 in the porcine female eye was regulated by the DNA methylation interaction. Indeed, evolutionarily conserved sexually dimorphic expression of H19/IGF2 suggests a functional sex difference. PA cells contain exclusively maternal chromatin, thus, H19/IGF2 expression from paternal chromatin was not a confounding factor in the analyses (Zhu et al., 2003).

In our previous study, BSP results suggested that there was no difference in IGF2 DMR1 and DMR2 between PA and normal fetuses (Wang et al., 2014a). These data are in accordance with the present results from PA cells. In addition, the H19 DMRs were hypomethylated in PA cells, confirming previous reports of aberrant methylation profiles in oocytes, PA embryos and fetuses (Park et al., 2009; Wang et al., 2014a). Our results indicate that H19 DMRs closely interact with IGF2 DMR1 on the maternal chromosome in PA samples (Figure 5E,F). Due to the hypomethylated DMRs, H19 expression was higher in PEF cells, while IGF2 was not expressed. The results from the PA cells indirectly show that IGF2 was over-expressed by the hypermethylation status of IGF2 DMR2 on the paternal chromosome in the female eye.
Figure 5 - Schematic representations of H19/IGF2 DNA methylation interactions of H19 DMD and IGF2 DMRs on the maternal (A) and paternal allele (B) in the male eye. The specific chromatin structure is described on the maternal (C) and paternal allele (D) in the female eye. As expected, the specific chromatin structures are established as in parthenogenetic (PA) cells (E and F). Ex: exon; E: enhancer; Red dotted line: active domain; CTCF: CCCTC-binding factor - binding sites; PPF: putative protein factors.
DNA methylation affects \textit{H19} and \textit{IGF2}

In summary, the results of the present study demonstrate the elevated expression of \textit{H19}/\textit{IGF2} in female porcine eye, which was associated with the methylation patterns of \textit{IGF2} DMRs. Our detailed analysis of PA and PEF cells suggest that a specific chromatin structure was formed due to the methylation of DMRs, which regulated the expression of \textit{H19}/\textit{IGF2}.

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References


Supplementary Material

The following online material is available for this article:

Figure S1 - Methylation pattern of \textit{H19} DMD and \textit{IGF2} DMRs.

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