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Methylation status of differentially methylated regions at *Igf2/H19* locus in porcine gametes and preimplantation embryos

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ABSTRACT

The aim of this study was to demonstrate how differential methylation imprints are established during porcine preimplantation embryo development. For the methylation analysis, the primers for the three *Igf2/H19* DMRs were designed and based upon previously published sequences. The methylation marks of *Igf2/H19* DMRs were analysed in sperm and MII oocytes with our results showing that these regions are fully methylated in sperm but remain unmethylated in MII oocytes. In order to identify the methylation pattern at the pronuclear stage, we indirectly compared the methylation profile of *Igf2/H19* DMR3 in each zygote derived by *in vitro* fertilization, parthenogenesis, and androgenesis. Interestingly, this region was found to be differently methylated according to parental origins; DMR3 was hemimethylated in *in vitro* fertilized zygotes, fully methylated in parthenogenetic zygotes, and demethylated in androgenetic zygotes. These results indicate that the methylation mark of the paternal allele is erased by active demethylation, and that of the maternal one is *de novo* methylated. We further examined the methylation imprints of *Igf2/H19* DMR3 during early embryonic development. The hemimethylated pattern as seen in zygotes fertilized *in vitro* was observed up to the 4-cell embryo stage. However, this mark was exclusively demethylated at the 8-cell stage and then restored at the morula stage. These results suggest that methylation imprints are established via dynamic changes during early embryonic development in porcine embryos.

Introduction

Genomic imprinting is an epigenetic mechanism by which certain loci are reciprocally expressed according to their parental origins. The developmental failure of uni-parental (bi-maternal and bi-paternal) embryos as evidenced by pronuclear transfer has outlined the functional importance of both parental genomes for complete development, it is believed therefore that imprinting mechanisms play a crucial role in normal development [1–3].

DNA methylation is one of the key physiological processes for regulating gene expression and is known to be an indispensable mechanism in the differential expression of imprinted genes. Many imprinted genes contain one or more differentially methylated regions (DMRs) exhibiting allelic methylation differences [3,4].

To date more than 70 imprinted genes have been identified in the mouse, with these genes tending to be clustered together within the genome. Of those identified, *Igf2* and *H19* have been the most widely studied as part of a well known cluster of imprinted genes; *Igf2* is expressed by the paternal allele whilst *H19* is transcribed from the maternal allele [5]. Both of these genes share enhancers that reside downstream of H19 and the reciprocal expression of both genes is regulated through *Igf2*|*H19* DMR situated 2–4 kilobases (kb) upstream from the *H19* transcription start site. The imprinting mechanism by which DNA methylation can control monoallelic expression at this locus is through the prevention of zinc finger protein CTCF (CCCTC-binding factor) binding which prevents promoter regions to access with enhancers [6,7]. Numerous deletional and mutational studies in mice have demonstrated the central role of the *Igf2*|*H19* DMR in regulating allelic expression and DNA methylation at the *Igf2*|*H19* locus, thus the methylation status of this region makes it an excellent candidate for identifying epigenetic imprints in development [8,9].

Furthermore the methylation dynamics of the *Igf2*|*H19* DMR have been studied in gametes and preimplantation embryos [10–12]. These studies suggest that imprinting marks are established during gametogenesis according to parental-specific mechanisms, and that thereafter these distinct marks are retained during the preimplantation stages. Such parental-specific marks in other imprinted genes such as *Snrpn*, *Mest*, *Peg3*, and *Igf2R* have also been found [13–15].

Since it has been shown that the abnormal expression of imprinted genes results in abnormal development, methylation analysis of imprinted genes has been used to study nuclear reprogramming during cloning and has also been widely used as a diagnostic tool to assess embryo normality or quality in assisted reproductive technologies [16–18]. Previous study into the methylation patterns of the repetitive elements in cloned and fertilized pig embryos suggests that

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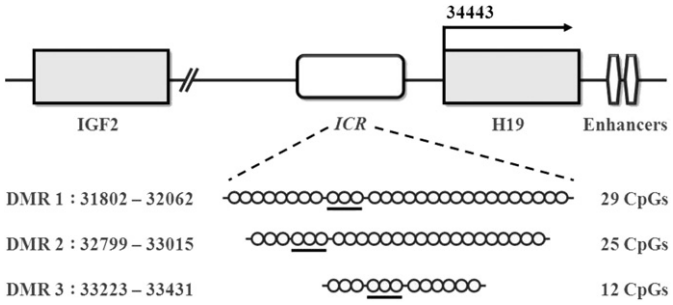

Fig. 1. Genomic structure of porcine *Igf2/H19* locus (not to scale). The schematic diagram of the *Igf2/H19* construct is as described in Amarger et al. (2002). Nucleotide numbers refer to GenBank acc. nos. (AY044827); The arrow above the light grey box indicates the transcription start site of *H19*; the white box represents the *ICR* containing three DMRs that are enlarged below; open circles indicate individual CpG dinucleotides within the amplified region; CTCF binding-sites are shown as bars below the CpGs.

the demethylation process establishes normally in cloned embryos [19]. On the contrary, it has been demonstrated that these repetitive elements remain in a highly methylated state in cloned bovine embryos [20]. It has also been shown that during mouse germ-cell development epigenetic demethylation occurs for imprinted genes as well as non-imprinted genes, while the repetitive elements remain in a highly methylated state [21]. It is clear therefore, that in order to more accurately determine the processes of epigenetic reprogramming during the preimplantation stages of pig embryos, more extensive analysis of the gene sequences that play an important role in development is still needed.

Recently, Amarger et al. have shown that *Igf2* and *H19* genes containing exon/intron and potential regulatory regions are highly conserved in mice, humans, and pigs. It was also found that a sequence identity exists between humans and pigs and that this occurs upstream of *H19* in about 100 bp, this sequence contains several 12-bp consensus motifs, and is a known CTCF binding site [22]. These CTCF sites are thought to be involved in insulating against *Igf2* promoters, which suggests that they may perform to regulate imprinting through a similar mechanism in various species [23].

This study aims to address two main questions; firstly, whether or not imprinting involves an allele-specific pattern in porcine gametes, and secondly, whether these gametic methylation patterns are maintained during the global demethylation event that occurs during

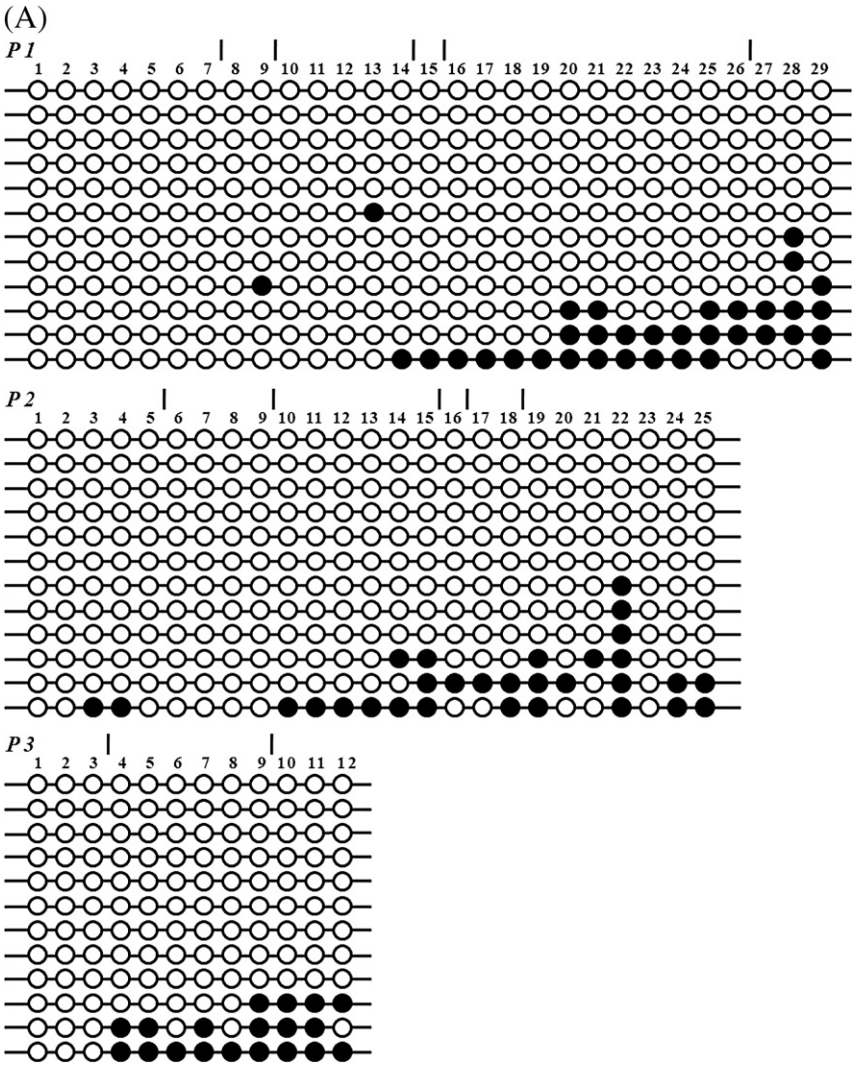

Fig. 2. The methylation pattern of three DMRs in porcine MII oocytes (A), sperm (B), and adult lung tissue (C). Individual circles indicate individual CpG dinucleotides. Open and solid circles represent unmethylated and methylated CpGs, respectively. Each horizontal line represents one individual clone from three independently amplified PCR products. Additionally, restriction sites of the BstUI enzyme in their regions for the combined bisulfite treatment and restriction analysis (COBRA) are also depicted as a vertical bar on the number of CpG site. (A).

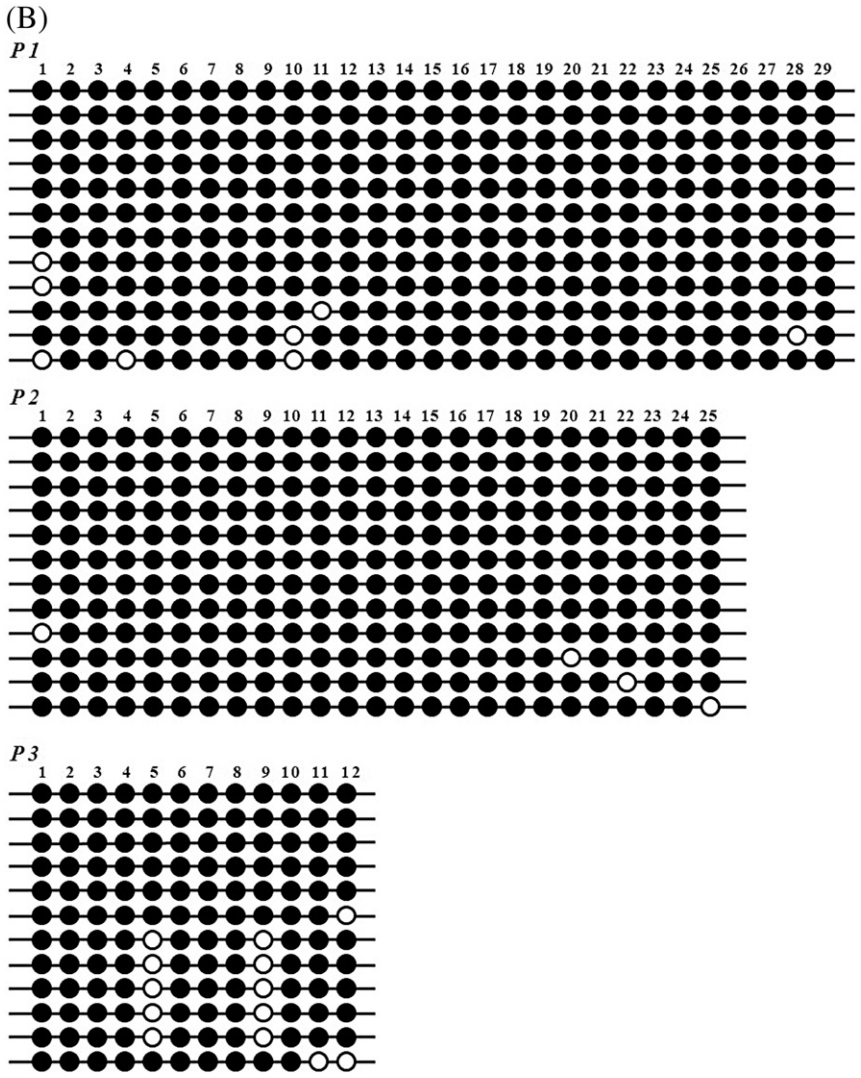

Fig. 2 (*continued*).

preimplantation development. In order to investigate the methylation status of *Igf2*/*H19* DMRs using the bisulfite sequencing technique, porcine gametes were collected and tested along with zygotes (*in vitro* fertilized, parthenogenetic, and androgenetic) and *in vitro* fertilized embryos (from two cell stage to morula stage embryos).

Results

The methylation patterns of Igf2/H19 DMRs in gametes and adult lung tissue

For methylation analysis, the primers for the three DMRs were designed based on the porcine *Igf2–H19* genomic region which was the previously published sequence [22]. As shown in Fig 1, each sequence (DMR1, 2, and 3) has 81, 83, and 55 cytosine residues, and contains 29, 25, and 12 CpG sites, respectively. Restriction sites of the BstUI enzyme in their regions for the combined bisulfite treatment and restriction analysis (COBRA) are also depicted. The bisulfite conversion method reported by Frommer et al. is widely used in investigating the methylation of genomic DNA [24]. Under the following conditions, genomic DNA is treated with 3 M sodium bisulfite and incubated at 55 °C for 4 to 16 h [25,26]. However, satisfactory results were not obtained with porcine genomic DNA under these conditions; using this method many non CpG cytosines in individual strands remained unconverted. The distinct features of the pig sequence itself may have been largely responsible for this result. The porcine target regions may indeed have a higher GC content and larger numbers of CpG islands than those found in humans and mice [22]. This feature of porcine sequences may have been responsible for the lack of complete conversion in CpG sites as well as in non CpG cytosines which are located around CpG sites [26]. Therefore, the bisulfite conversion conditions were slightly modified, as described in Materials and methods. Consequently, >98% of the conversion rate was consistently obtained.

To validate these assays, the methylation status of adult lung tissue was analysed using bisulfite sequencing. This showed that methylated and unmethylated strands were obtained at similar level of 50% in all DMRs, indicating that there were no potential artifacts or bias (Fig. 2C). Further methylation analysis was then used to determine whether a gamete-specific methylation imprint at these regions really occurs in porcine gametes. The results presented in Fig. 2A show that all of the regions in sperm were fully methylated (96.7%), whereas these regions in MII oocytes were unmethylated (89.9%) (Fig. 2B). This gamete-specific methylation pattern was also evident in results of COBRA analysis, which is consistent with bisulfite sequencing results (data not shown).

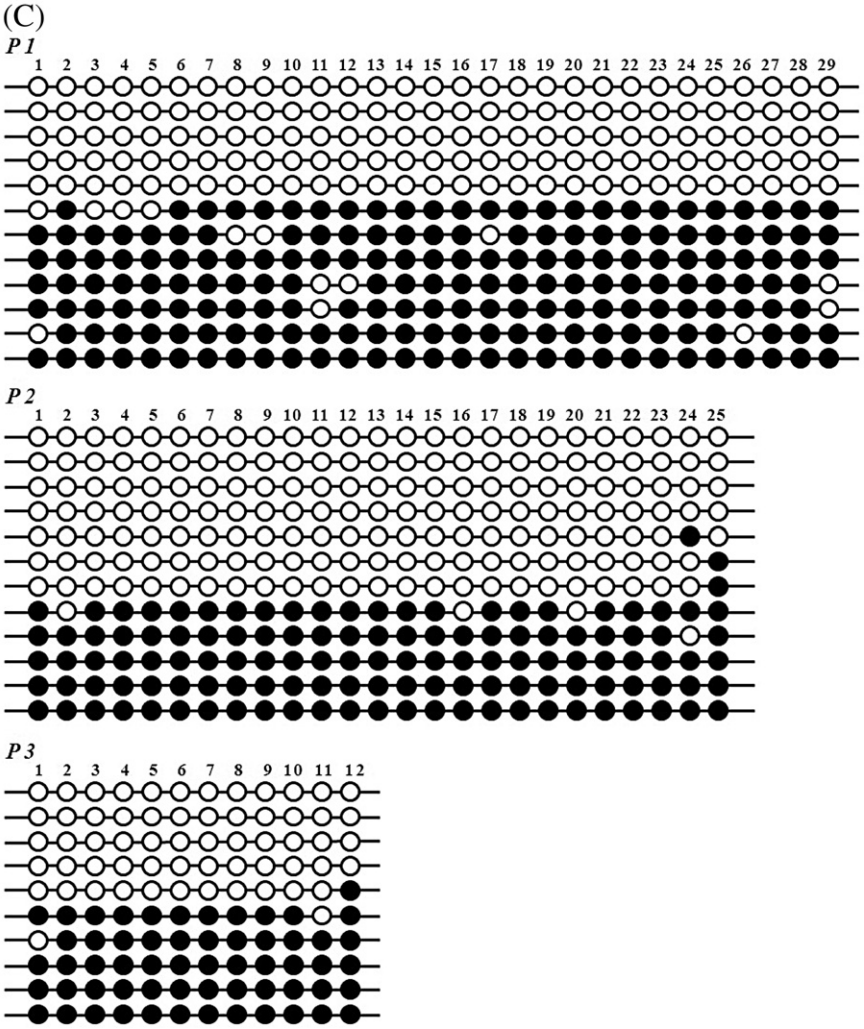

Fig. 2 (*continued*).

The methylation pattern of Igf2/H19 DMR3 in in vitro fertilized, parthenogenetic, and androgenetic zygotes

In order to determine the methylation status of *Igf2*/*H19* DMR3 in the zygotic stage embryo in detail, a genetic marker was needed to distinguish parental alleles independently. For this experiment, *in vitro* fertilized, parthenogenetic, and androgenetic zygotes were produced. After 12 to 14 h following fertilization or parthenogenesis, genomic DNA was isolated from pooled embryos containing two pronuclei (IVF and androgenesis) or a single pronucleus (parthenogenesis). Results presented in Fig. 3 show that *Igf2*/*H19* DMR3 was 43.2% methylated in the *in vitro* fertilized zygotes. This region revealed a high methylation level (78.5%) in parthenogenetic zygotes but was only 14.5% methylated in androgenetic zygotes.

The methylation pattern of the Igf2/H19 DMR3 in preimplantation embryos

The results presented in Fig. 4 show that the establishment of differential methylation patterns in *Igf2*/*H19* DMR3 displays dynamic changes during preimplantation development. The hemimethylation pattern as seen in *in vitro* fertilized zygotes remained until the first two cleavage stages (Figs. 4A, B). However, this hemimethylated pattern rapidly changed to fully demethylated pattern at the 8-cell stage which continued up to the 16-cell stage (Figs. 4C, D). Eventually, this region returned to a hemimethylated pattern by the morula stage (Fig. 4E). These results show that the demethylation and *de novo* methylation processes in *Igf2*/*H19* DMR3 takes place temporarily during the stages of porcine preimplantation development.

Discussion

We present here the methylation status of *Igf2*/*H19* DMRs in porcine gametes and preimplantation embryos at various stages in order to evaluate whether imprint marks indeed occur in a sex-specific way and whether these imprinting marks are achieved during preimplantation development.

Our results showed that all *Igf2*/*H19* DMRs in adult lung tissue are hemimethylated, as is typical for a somatic methylation pattern. The *Igf2*/*H19* DMRs of MII oocytes where shown to be completely demethylated in MII oocytes but methylated in sperm. These findings are in agreement with previous data indicating that *Igf2*/*H19* DMRs are marked by allele-specific differences as seen in mice and humans [8,27,28]. This suggests that imprinting marks in porcine gametes are established via a sex-specific mechanism.

The methylation profiles for each DMR in MII oocytes show that the majority of sequenced clones are unmethylated, however it was unexpectedly discovered that several strands were partially methylated. In humans, it has been shown that some MII-rescued oocytes display a fully methylated pattern in the *Igf2*/*H19* DMR, whilst

generally most of these remain unmethylated, suggesting that these aberrant marks also occur during oocyte growth *in vivo* [12]. In the porcine *in vitro* maturation (IVM) system, incomplete or delayed maturation of oocytes caused by inadequate culture conditions can often lead to molecular and metabolic abnormalities as well as developmental failure of oocytes after fertilization [29]. Therefore, the partially methylated pattern observed in the MII oocytes could suggest either that the IVM system being used is still suboptimal, or that poor quality oocytes have not been rigorously eliminated before IVM.

In general, after fertilization, the paternal genome undergoes global demethylation whilst the maternal genome remains methylated [30]. DMRs of imprinted genes such as the *H19* gene sustain their methylation marks on both alleles, regardless of genome-wide events [10,11]. However, the opposite results have also shown that the methylation mark of *Igf2* DMR is lost on the paternal allele, while it is *de novo* methylated on the maternal side [31,32]. Thus, it is still uncertain as to whether imprinting marks would be protected from this event. To approach this question, a suitable genetic strategy to distinguish each of the parental alleles with allele-specific polymorphisms is needed. Within the limits of the experimental system used in this study such a strategy would have been impossible, therefore for this purpose uniparental zygotes (consisting entirely of either a paternally or maternally inherited genome) were used. It has been proposed that androgenetic and parthenogenetic embryos are a valuable resource for studying imprinting [32,33]. Although the successful production of porcine embryos derived from *in vitro* fertilization (IVF) and parthenogenesis has been extensively recorded [34,35], no report associated with androgenesis in this species has been published so far. We report here that porcine androgenetic embryo can be successfully produced by the method of IVF using enucleated oocytes. Additionally, this method may be suitable for studies that require large numbers of androgenetic embryo samples.

We have shown in this study that the methylation pattern of *Igf2*/*H19* DMR3 is significantly different in porcine zygotes of different parental origins. The hypomethylated pattern of *Igf2*/*H19* DMR3 observed in androgenetic embryos suggests that the demethylation occurs in the imprinted genes of the paternal alleles. Furthermore, *Igf2*/*H19* DMR3 in parthenogenetic zygotes appears to undergo *de novo* methylation. These results appear to be largely consistent with previously obtained results on active demethylation of imprinted genes occurring in paternal alleles as well as results obtained on *de novo* methylation for maternal imprints in mice [31]. It has been shown that that the paternal genome is preferentially demethylated after fertilization, whereas the maternal one remains methylated, this has been demonstrated in studies involving mice, rats, cows, and pigs [36,37]. The active demethylation also occurs in androgenetic zygotes but not in parthenogenetic zygotes [33]. Moreover, this differential demethylation is more prominently detected in porcine systems than that of other species [37]. Taken together, these results may suggest that methylation imprints of the paternal allele are erased by an active demethylation process, while for maternal allele *de novo* methylation occurs at the porcine zygotic stage.

This study has demonstrated that *Igf2*/*H19* DMR3 becomes temporarily demethylated at the 8 to 16 cell stages before returning to the allele-specific pattern by the morula stage. This finding is in part consistent with previous reports that methylation imprints are erased and re-established in the early embryo [32,38]. Therefore, these results indicate that methylation imprint of *Igf2*/*H19* DMR3 is achieved by means of dynamic changes during preimplantation development. However, conflicting results have also appeared showing that methylation imprints appear stable throughout preimplantation development [11,14]. Recently, it has been determined in mice that the allele-specific methylation of *H19* and *Rasgrf1* disappears at the 2-cell stage but returns by the morula stage in embryos cultured *in vitro*, whereas methylation is maintained in *in vivo* embryos [39]. It is known that methylation marks of some imprinted genes, in particular the *H19* gene, can be easily affected by the exogenous environments, such as culture conditions or manipulations [40,41]. Numerous studies on human, mouse, and ruminant have previously demonstrated that the methylation patterns and expression levels of several genes essential for normal embryonic development are altered by culture environments and differ between *in vitro*, cultured and *in vivo* embryos [42]. These studies emphasize that methylation defects observed in preimplantation development can be caused by suboptimal culture conditions. In addition, current IVP systems are known to be suboptimal because *in vitro* produced porcine embryos results in a decreased rate of pregnancy compared with embryos derived *in vivo* [34]. In this regard, the possibility that the alterations of methylation patterns of *Igf2*/*H19* DMR3 were triggered by the culture conditions cannot be excluded. Nonetheless, it still remains unclear whether the temporary changes of imprinting marks at this locus in porcine preimplantation embryos are caused by environmental factors or, indeed, whether there exists a difference in methylation reprogramming between species [30]. Consequently, additional study is required to clarify this issue.

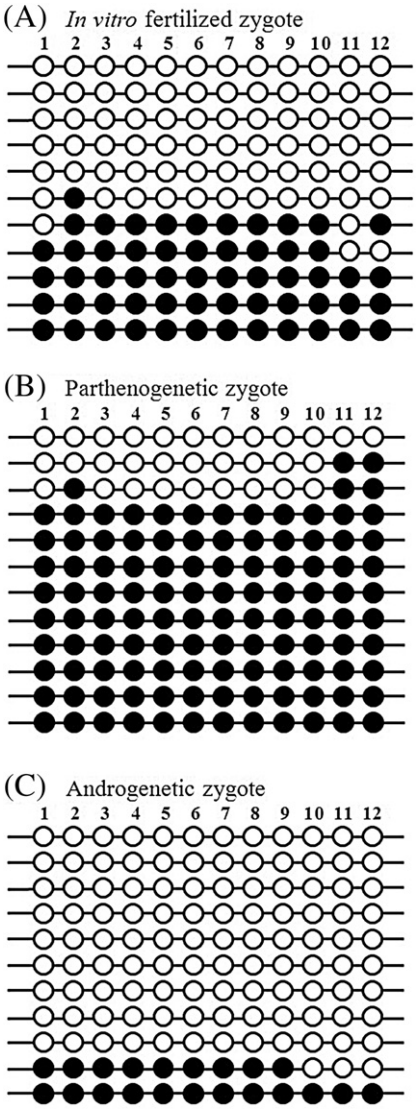

Fig. 3. The methylation pattern of *Igf2*/*H19* DMR3 in porcine *in vitro* fertilized and uniparental zygotes. The zygotes having two pronuclei or a single pronucleus were selected by Hoechst staining at 12 to 14 h after fertilization or parthenogenesis, respectively. Individual circles indicate a CpG dinucleotide. Open and solid circles represent unmethylated and methylated CpGs, respectively. Each horizontal line represents one individual clone from three independently amplified PCR products.

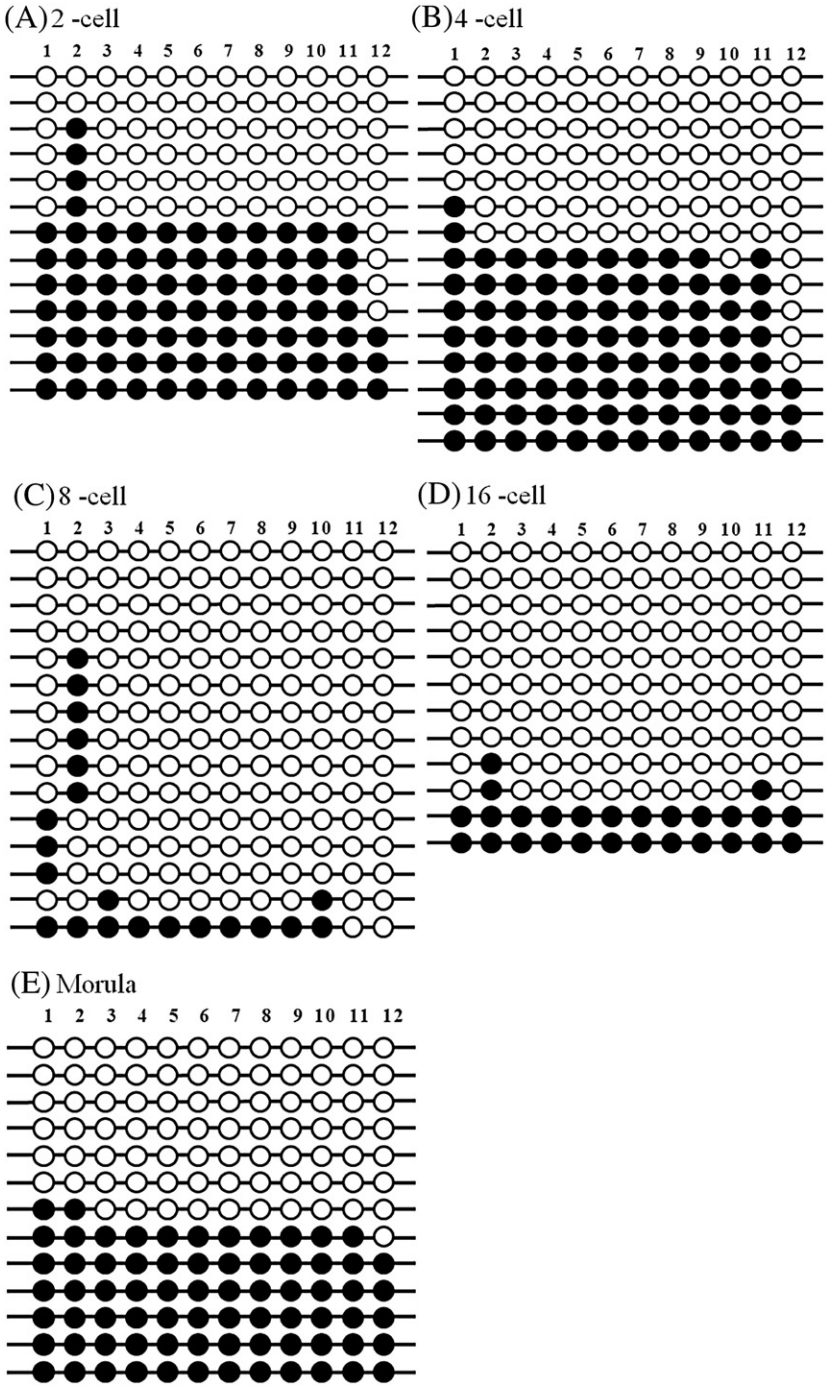

Fig. 4. The methylation pattern of *Igf2/H19* DMR3 in porcine cleavage-stage embryos. Individual circles indicate a CpG dinucleotide. Open and solid circles represent unmethylated and methylated CpGs, respectively. Each horizontal line represents one individual clone from three independently amplified PCR products. Each-stage embryo was selected by their morphological features.

Although methylation imprints have been extensively performed in other species, extensive porcine studies and published experimental results have been lacking so far. This study shows that the achievement of methylation imprints occurs through distinct changes during early porcine embryo development. Clearly, dynamic changes of differential methylation in imprinted genes should be differentiated from genome-wide demethylation events and repeat sequences which gradually diminish through preimplantation development [19,37]. However, it should be noted that demethylation and *de novo* methylation timing is not the same for all imprinted genes in preimplantation embryos [39]. It will therefore be important to elucidate how methylation marks are erased and re-established in other imprinted genes during porcine preimplantation development.

Materials and methods

Collection of sperm, MII oocytes and the production of in vitro embryos

The fresh ejaculate of the boar breed (Berkshire) was supported by DARBY A.I. Center (Chungju, South korea). The sperm was collected by centrifugation at 350 ×*g* for 3 min in DPBS (Gibco, USA) and then used directly for genomic DNA isolation. The removal of cumulus cells from

Table 1
Sequence-specific primers used for PCR

Regions		Primer sets (5′ to 3′)	Length (bp)
DMR1	Outside forward:	AGG AGATTA GGT TTA GGG GAA T	260
	Inside forward:	AGT GTT TGG GGA TTT TTT TTT T	
	Inside reverse:	CAC CCC ATC CCC TAA ATA ACC CTC	
	Outside reverse:	CTA CCA CTC CCC TCA TAC CTA A	
DMR2	Outside forward:	TAT GTT TAG GGG TGA TAA AAG T	216
	Inside forward:	AGG TGT TAT TTT GTT TGT TGG T	
	Inside reverse:	ATA AAA TAA CCT AAA AAA ACT CAA	
	Outside reverse:	CCC CAC TTC TAC AAT TCA AC	
DMR3	Outside forward:	GGT TTT AGG GGG ATA TTT TTT	208
	Inside forward:	GAT TTT TAG GTT TGT TAT TAT TT	
	Inside reverse:	CAA ATA TTC AAT AAA AAA ACC C	
	Outside reverse:	TTA AAA AAA CAT TAC TTC CAT ATA C	

oocytes was performed by mechanical pipetting in DPBS with 0.1% hyaluronidase (Sigma-Aldrich, USA). The procedures for *in vitro* embryo production including maturation (IVM), fertilization (IVF) and culture *in vitro* (IVC) were performed as reported previously [43]. To exclude the possible contamination of remaining cumulus cells, the polar body, or sperm DNA, the zona pellucida (ZP) of oocytes and embryos was eliminated by short-exposure to 0.5% Actinase E (Kaken, Japan).

Only normally fertilized oocytes having two pronuclei and two polar bodies were selected. Suitable oocytes were determined via Hoechst. 33342 staining 12 to14 h after fertilization, and these oocytes were then cultured *in vitro* for 144 h. Embryo culture conditions were maintained at 39 °C in an atmosphere containing 5% CO_2, 5% O_2 and 100% humidity for all embryo cultures.

Production of parthenogenetic and androgenetic zygotes

Diploid parthenogenetic zygotes were produced by electrical activation followed by cytochalasin D treatment to suppress second polar body extrusion. Briefly, cumulus-free oocytes were treated with cytochalasin D prior to washing twice in a 280 mM mannitol solution containing 0.5 mM HEPES, 0.1 mM $CaCl_2$ and 0.1 mM $MgCl_2$. They were placed within an electrode-chamber and activated with a single DC pulse (2.0 kV/cm 30 μs) using a BTX Electro-cell Manipulator (BTX, CA, USA), they were then cultured for 12 to14 h.

As previously reported, androgenetic zygote was produced by the *in vitro* fertilization of enucleated oocytes [44]. Briefly, matured oocytes were enucleated by a squeezing enucleation method, enucleation was confirmed using a Hoechst 33342 dye under a UV light. The successfully enucleated oocytes were placed into 40 μl drops of a fertilization medium that had been covered with warm paraffin oil. Frozen semen samples were thawed and washed twice in DPBS (Gibco, Life Technologies, Grand Island, NY). A final sperm fraction (10 μl), with a concentration of 5.0×10^6 cells/ml, was added for insemination and then co-incubated for 6 h. The oocytes were then cultured *in vitro*. Those zygotes with two pronuclei were identified using Hoechst 33342 staining and were selected 12 to 14 h after fertilization.

DNA isolation and bisulfite treatment

Genomic DNA was isolated from 1×10^6 sperm cells, metaphase II oocytes (100 in number), 1 to 8 cell stages embryos (50), and embryos at later stages (30). Additionally, zygotes that had two pronuclei were selected by Hoechst staining 12 to 14 h after fertilization or parthenogenesis.

The isolation of genomic DNA from porcine samples was carried out using a commercial spin column (G-spin Genomic DNA extraction kit for Cell/Tissue, iNtRON, Korea), with an additional 6 M Urea (Amresco, USA) and 100 mM dithiothreitol (DTT; Sigma, USA) supplemented in a lysis buffer. The genomic DNA was digested with EcoRI (New England Biolabs, Germany). The Bisulfite treatment of DNA was performed as described [45] with some modifications. Briefly, 200 ng of denatured DNA was sulfonated with 5 M sodium bisulfite (pH 5.0; Sigma), 100 mM hydroquinone (Sigma) and 5 μg of *Escherichia coli* tRNA (Sigma) as a carrier in a thermo-cycler programmed for 6 cycles (3 min at 94 °C and 3 h at 60 °C). The bisulfite-treated DNA was purified using the Wizard DNA Clean-Up system (Promega, USA) and desulfonated in 0.3 M NaOH for 25 min at 37 °C. The DNA was purified again and then resuspended in distilled water. Subsequently, 5 μl of the aliquot was eventually used as a template for PCR.

PCR amplification, cloning, and sequencing

Nested PCR amplifications of bisulfite-treated DNA were performed using the primers as described in Table 1. The primers were designed from the sequence of the 5′ H19 region (GenBank: AY044827) using Methyl Primer software. The PCR amplification was performed with a 2× PCR master mix solution (iNtRON, Korea) containing 0.5 pmol of the primers. The first-round of PCR was performed as follows, 1 cycle of 94 °C for 10 min; 35 cycles of 95 °C for 45 s / 50 °C for 1 min / 72 °C for 1 min, 72 °C for 7 min. The nested PCR was carried out at 1 cycle of 94 °C for 10 min; 40 cycles of 95 °C for 45 s / 55 °C for 2 min / 72 °C for 2 min; 1 cycle of 72 °C for 7 min. PCR products were cloned into the pGEMT-Easy vector (Promega) and transformed into *E. coli* cells (Novagen, USA) and at least 10 insert-positive plasmid clones were sequenced by an ABI PRISM 3730 automated sequencer (Applied Biosystems). The methylation patterns were analyzed in sequences derived from clones with ≥98% cytosine conversions only. All experiments were repeated at least five times for each DMR for sperm, oocyte, and lung tissue and three times for each cleavage stage.

Acknowledgments

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