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Authors: Kamimura, Satoshi, Hatanaka, Yuki, Hirasawa, Ryutaro, Matsumoto, Kazuya, Oikawa, Mami, et al.

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Establishment of Paternal Genomic Imprinting in Mouse Prospermatogonia Analyzed by Nuclear Transfer[1]

Satoshi Kamimura,[3,4,5] Yuki Hatanaka,[3,4] Ryutaro Hirasawa,[3,4] Kazuya Matsumoto,[3,6] Mami Oikawa,[4,6] Jiyoung Lee,[6,7,8] Shogo Matoba,[4] Eiji Mizutani,[4] Narumi Ogonuki,[4] Kimiko Inoue,[4,5] Takashi Kohda,[6] Fumitoshi Ishino,[6,7,8] and Atsuo Ogura[2,4,5,9]

[4]*RIKEN BioResource Center, Tsukuba, Ibaraki, Japan*
[5]*Graduate School of Life and Environmental Science, University of Tsukuba, Ibaraki, Japan*
[6]*Department of Epigenetics, Medical Research Institute, Tokyo Medical and Dental University, Tokyo, Japan*
[7]*Global Center of Excellence Program for International Research Center for Molecular Science in Tooth and Bone Diseases, Tokyo Medical and Dental University, Tokyo, Japan*
[8]*PRESTO, Japan Science and Technology Agency, Saitama, Japan*
[9]*Center for Disease Biology and Integrative Medicine, Faculty of Medicine, University of Tokyo, Tokyo, Japan*

ABSTRACT

In mice, the establishment of paternal genomic imprinting in male germ cells starts at midgestation, as suggested by DNA methylation analyses of differentially methylated regions (DMRs). However, this information is based on averages from mixed populations of germ cells, and the DNA methylation pattern might not always provide a full representation of imprinting status. To obtain more detailed information on the establishment of paternal imprinting, single prospermatogonia at Embryonic Days 15.5 (E15.5), E16.5, and E17.5 and at Day 0.5 after birth were cloned using nuclear transfer; previous reports suggested that cloned embryos reflected the donor's genomic imprinting status. Then, the resultant fetuses (E9.5) were analyzed for the DNA methylation pattern of three paternal DMRs (IG-DMR, *H19* DMR, and *Rasgrf1* DMR) and the expression pattern of imprinted genes therein. The overall data indicated that establishment of genomic imprinting in all paternally imprinted regions was completed by E17.5, following a short intermediate period at E16.5. Furthermore, comparison between the methylation status of DMRs and the expression profiles of imprinted genes suggested that methylation of the IG-DMR, but not the *H19* DMR, solely governed the control of its imprinted gene cluster. The *Rasgrf1* DMR seemed to be imprinted later than the other two genes. We also found that the methylation status of the *Gtl2* DMR, the secondary DMR that acquires DNA methylation after fertilization, was likely to follow the methylation status of the upstream IG-DMR. Thus, the systematic analyses of prospermatogonium-derived embryos provided additional important information on the establishment of paternal imprinting.

DNA methylation, genomic imprinting, mouse, nuclear transfer, prospermatogonium

INTRODUCTION

Genomic imprinting is a mechanism that ensures parent-specific allelic expression of particular genes (imprinted genes) in eutherian mammals [1, 2]. Therefore, imprinted genes can be defined by the functional nonequivalence of their corresponding maternal and paternal alleles, caused by epigenetic marks imposed on one of these alleles. There are at least 100 imprinted genes, based on the accumulated information in mammalian species so far studied [3]. Therefore, imprinted genes might comprise only a small subset of the functional genes on the genome. However, most imprinted genes are expressed in fetuses and/or placentas during development and are essential for proper embryonic development to term, as has been revealed by gene knockout or transgenic experiments in mice and from the pathology of uniparental disomy or genomic imprinting disorders in humans [2, 4]. The major epigenetic changes in imprinting status occur in germline cells. The imprinting memories of zygotes and embryos are erased during the middle stage of primordial germ cell (PGC) development [5, 6]. Subsequently, during the sex-specific development of germ cells, the primary imprinting memories are imposed on certain chromosomal regions, which are transmitted to oocytes or spermatozoa and then to developing embryos [1, 2, 7].

Imprinted genes are not randomly distributed in the genome but tend to accumulate in clusters in certain genomic regions [8]. Each cluster is controlled by an imprinting control region containing differentially methylated regions (DMRs), in which DNA is methylated during gametic imprinting in a sex-specific manner. One or more DMRs have been identified in each imprinting cluster by DNA methylation analysis, and their methylation or demethylation during germ cell development is thought to control the establishment or erasure of genomic imprinting, respectively.

Until now, the most detailed analyses of the DNA methylation status of DMRs have been performed successfully in mice, so there is a broad consensus on the timing of erasure and establishment of imprinting in this species. According to these DNA methylation studies, paternal imprinting is established in premeiotic male germ cells, whereas maternal imprinting is established during oogenesis at the prophase of

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[2]Correspondence: Atsuo Ogura, RIKEN BioResource Center, 3-1-1, Koyadai, Tsukuba, Ibaraki 305-0074, Japan.
E-mail: ogura@rtc.riken.go.jp
[3]These authors contributed equally to this work.

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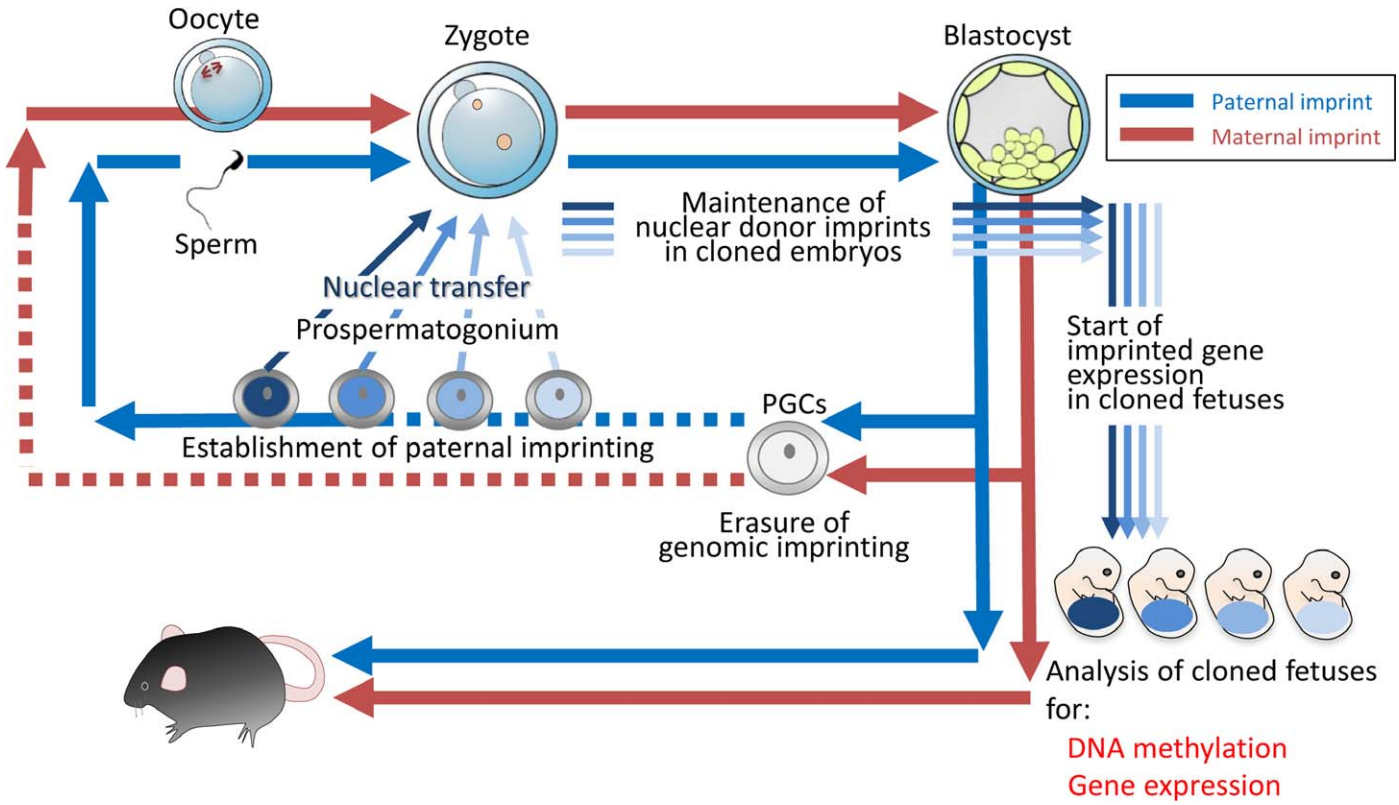

FIG. 1. Experimental scheme of this study. Prospermatogonia at different stages were cloned by nuclear transfer, and the resultant fetuses were analyzed at E9.5 for the DNA methylation status of their DMRs and expression of imprinted genes. There is a consensus that embryos cloned by nuclear transfer retain the imprinting status of the donor genome [6, 14]. By this strategy, we can obtain systematic information about paternal imprinting based on the DNA methylation pattern of DMRs, the expression profiles of imprinted genes therein, and the relationships between them. For detailed experimental methods and materials, see Supplemental Figure S1.

the first meiosis [7]. However, the definition of genomic imprinting is parent-specific allelic expression rather than DNA methylation status. Indeed, the expression of some imprinted genes, especially those expressed in the placenta, might be controlled by histone modifications instead of DNA methylation [9]. Therefore, for more detailed information on the imprinting status of germline cells of interest, it would be desirable to examine their gene expression patterns as well as the DNA methylation levels of DMRs. However, there is no or little expression of imprinted genes in developing germ cells, because most imprinted genes are expressed from fetal somatic cells and/or placental cells. This limitation can be overcome by analyzing fetuses reconstructed with the germ cell nuclei by nuclear transfer (Fig. 1).

Fetuses generated from germ cells are expected to reflect the donor's genomic imprinting status, according to the principle that the mature ooplasm (usually used as the recipient for nuclear transfer) does not alter genomic imprinting [10]. This strategy has been used successfully for elucidating the erasure of imprinting in middle-stage PGCs [6] and the establishment of maternal imprinting in oocytes [11]. Similarly, the timing of the establishment and erasure of the imprints for imprinted X chromosome inactivation was defined by using a broad range of germ cells as donors [12]. For the erasure of imprinting, PGC-derived cloned fetuses were analyzed in detail for their expression of imprinted genes [6]. Results of allele-specific expression analyses revealed that the erasure process proceeds in Embryonic Day 10.5 (E10.5) to E11.5 PGCs, with the timing precisely controlled for each imprinted gene [6]. For the establishment of maternal imprinting, as the nucleus (germinal vesicle) of growing oocytes is at prophase I, parthenogenetic embryos were reconstructed using one haploid genome from a growing oocyte and another from a fully grown oocyte [11]. By analyzing the expression pattern of imprinted genes in such parthenogenetic fetuses, the oocyte size-specific imprinting scenario for each maternally imprinted gene was demonstrated clearly [11]. Therefore, one remaining issue to be determined is the pattern of establishment of paternally imprinted genes, which is thought to be completed in developing prospermatogonia before birth in the mouse [7]. As far as has been studied to date, the paternally imprinted group consists of three regions with DMRs: *H19–Igf2* (*H19* DMR), *Dlk1–Gtl2* (IG-DMR), and *Rasgrf1* (*Rasgrf1* DMR). The present study was undertaken to obtain detailed and expression-based information on the establishment of paternal imprinting by reconstructing cloned embryos using prospermatogonia at defined stages (Fig. 1 and Supplemental Fig. S1; supplemental data are available online at www.biolreprod.org).

MATERIALS AND METHODS

Animals

Eight- to 10-wk-old B6D2F1 strain (C57BL/6 ×DBA/2 hybrid) female mice (Japan SLC, Inc.) and 8- to 12-wk-old ICR strain female mice (CLEA Japan, Inc.) were used as donors of recipient oocytes for nuclear transfer and as embryo transfer recipients, respectively. Nuclear donor prospermatogonia with the genetic background of (129×JF1)F1 were prepared by in vitro fertilization (IVF) [13] using oocytes from 129X1/SvJ female mice (Japan SLC, Inc.) and spermatozoa from JF1/Msf male mice (RIKEN BioResource Center). A part of donor cells were derived from (C57BL/6×JF1)F1 (corresponding to samples 12 and 13 in Figs. 3–7 and Supplemental Fig. S7). The animals were housed under controlled lighting conditions (daily light 0700–2100 h) and were maintained

under specific-pathogen-free conditions. All animal experiments were conducted in accordance with guidelines of the RIKEN BioResource Center.

Donor Cell Preparation

To obtain prospermatogonia for nuclear donors, the gonads of E15.5, E16.5, and E17.5 fetuses and Postnatal Day 0.5 (P0.5) mice were produced by IVF as described above. Prospermatogonia for nuclear donors were collected from the gonads shortly before nuclear transfer. One or two gonads were placed in a 3-μl drop of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)–potassium simplex optimized medium (KSOM) containing 10% polyvinylpyrrolidone in a micromanipulation chamber and punctured using a fine disposable needle to allow the gonadal cells, including prospermatogonia, to spread into the medium. Immature Sertoli cells were collected from (129×JF1)F1 P3.5 male neonates [14]. Briefly, seminiferous tubules were treated with 0.1 mg/ml collagenase (Sigma-Aldrich) and 0.01 mg/ml DNase (Sigma-Aldrich) in phosphate-buffered saline (PBS) for 30 min at 37°C, followed by 0.2 mg/ml trypsin (Sigma-Aldrich) in PBS for 5 min at 37°C. The cell suspension was washed 3 or 4 times with PBS containing 4 mg/ml bovine serum albumin (Calbiochem), and the cells were used as nuclear donors.

Donor prospermatogonia at E15.5–E17.5 were also analyzed for their DNA methylation status by bisulfite sequencing. We analyzed IG-DMR and *Gtl2* DMR as representative primary and secondary DMRs, respectively. Prospermatogonia were picked up using a fine glass pipette under an inverted microscope as nuclear transfer (see below) and injected into the empty zona pellucida (Supplemental Fig. S2). Thus, a precise number of prospermatogonia (50–100 cells per zona) could be processed for bisulfite sequencing. Between 300 and 400 prospermatogonia were used for a single DMR at E15.5, E16.5, or E17.5.

Nuclear Transfer

Nuclear transfer was carried out as described elsewhere [15–17]. Briefly, B6D2F1 female mice were superovulated by injection of 7.5 IU of equine chorionic gonadotropin (Sankyo Yell Yakuhin) and 7.5 IU of human chorionic gonadotropin (hCG; Aska-Pharmaceutical) at 46- to 52-h intervals. At 14–17 h after hCG injection, cumulus-oocyte complexes were collected from the oviducts, and cumulus cells were removed by washing in KSOM containing 0.1% bovine testicular hyaluronidase (Calbiochem). Oocytes were enucleated in HEPES-KSOM containing 7.5 μg/ml cytochalasin B, using a piezo-driven micropipette (product no. PMM-150FU; Prime Tech, Ltd.). After donor nuclei were washed and cultured in fresh KSOM for more than 1 h, they were injected into enucleated oocytes by using a piezo-driven micropipette. Subsequent steps of the nuclear transfer experiments were performed under 5% CO_2 in air at 37.5°C. The injected oocytes were cultured in KSOM for 1 h and then activated in Ca^{2+}-free KSOM containing 2.5 mM $SrCl_2$, 5 μg/ml cytochalasin B, and 50 nM trichostatin A (TSA) for 1 h. Oocytes were incubated in KSOM containing 5 μg/ml cytochalasin B and 50 nM TSA for 5 h and then incubated in KSOM containing 50 nM TSA for 2 h. After being washed, the oocytes were cultured in KSOM. After 12 h, reconstructed embryos that had reached the two-cell stage were transferred into the oviducts of pseudopregnant ICR female mice at E0.5. Pregnant mice were euthanized by cervical dislocation at E9.5, and fetuses were collected from their uteri. The experimental scheme in this study is shown in Figure 1 and Supplemental Figure S1.

DNA Methylation Analysis by Bisulfite Sequencing

Each DNA sample was mixed directly into a solution of 10 M sodium bisulfite (to obtain a 9 M final solution, as described previously [18]), denatured at 98°C for 1 min and incubated for 1 h at 70°C. Desulfonation and purification of bisulfite-treated DNA were performed using a bisulfite DNA purification kit (Zymo Research). Following PCR amplification, the amplified DNA was cloned into a pGEM T-Easy vector (Promega) and transformed into DH5α-competent bacterial cells. Individual clones were amplified using TempliPhi DNA amplification kits (GE Healthcare) and sequenced. Primer sequences are provided in Supplemental Table S1. Maternal and paternal alleles were distinguished using single nucleotide polymorphism (SNP) analyses.

Quantitative Reverse Transcription PCR

Total RNA was extracted from E9.5 fetuses by using Isogen (Nippon Gene). Complementary DNA (cDNA) was synthesized from 1 μg of total RNA, using SuperScript III reverse transcriptase (Invitrogen) with oligo(dT) primers. Quantitation of cDNA was performed with QuantiTect SYBR Green PCR kits (Qiagen) and an ABI Prism 7900HT sequence detection system (Life Technologies). Seven imprinted genes, 3 paternally expressed genes (Pegs) and 4 maternally expressed genes (Megs), were selected from 2 paternally imprinted regions and 2 maternally imprinted regions: *Igf2* (distal chr 7), *H19* (distal chr 7). *Peg3* (proximal chr 7), *Igf2r* (proximal chr 12), *Dlk1* (distal chr 12), *Gtl2* (distal chr 12), and *Rian* (distal chr 12). The amount of imprinted gene mRNA was determined from the appropriate standard curve and controlled relative to the amount of *Actb* (β-actin) mRNA. All expression data are presented as values relative to the mean expression levels of IVF-derived fetuses at the same age (E9.5). Primer sequences are listed in Supplemental Table S1.

Rasgrf1 *Expression Analysis*

Mice or fetuses with the (129×JF1)F1 genotype were produced by IVF [13]. Total RNA was isolated from whole E9.5 fetuses and adult brains and livers (57 days of age), using Isogen. Reverse transcription (RT) was performed according to manufacturer's instructions, using 1 μg of RNA, SuperScript III, and oligo(dT) primers. PCR amplification was performed using Ex Taq Hot Start version (TaKaRa Bio, Inc.) with 50 ng of the RT product (complementary DNA) and 0.8 μM primers for *Rasgrf1* and *Actb* (β-actin; Supplemental Table S1). PCR cycling conditions were 5 min at 95°C, followed by 40 cycles of 20 sec at 95°C, 30 sec at 58°C, and 30 sec at 72°C; followed by 7 min at 72°C. PCR products were analyzed using 2% agarose gel electrophoresis.

Statistical Analysis

DNA methylation levels (%) determined by bisulfite sequencing were analyzed using arcsine transformation followed by 1-way ANOVA analysis. Where appropriate, a post hoc procedure using Scheffe F test was adopted for multiple comparisons between the groups. The gene expression levels of cloned fetuses determined by quantitative RT-PCR performed in triplicate were analyzed by Dunnett multiple comparison procedure using 1 of the 3 IVF fetuses of the intermediate level as a control. For multiple genes within the same DMR domains, paternal:maternal gene expression ratio levels were analyzed. Relationships between the methylation levels in IG-DMR and *Gtl2* DMR were evaluated by Pearson product-moment correlation. P values < 0.05 were considered significant.

RESULTS

Development of Spermatogonium-Derived Cloned Embryos

Prospermatogonia can be easily distinguished from gonadal somatic cells by their high nucleus:cytoplasm ratio and characteristic pseudopodia [19] (Fig. 2A). The prospermatogonia were very soft cells and were picked up easily by using an injection pipette with a 5- to 7-μm-inner diameter (Fig. 2B). When a gonadal somatic cell was picked up by the same pipette, the cell stuck to the tip of the pipette because of its physical rigidity (Fig. 2C). Thus, we were able to select prospermatogonia for nuclear transfer with nearly 100% accuracy.

Most (>85%) embryos reconstructed with prospermatogonium nuclei developed into 2-cell embryos by 24 h, except for those derived from P0.5 prospermatogonia (Table 1). However, the efficiency of postimplantation development varied greatly between experiments, giving a range of 0.7%–9.5% for the rates of fetal development in the various experimental groups (Table 1). The retrieved fetuses had a beating heart but often showed retarded development compared with IVF-derived fetuses of the same age (Supplemental Fig. S3).

DNA Methylation Analysis of DMRs in Prospermatogonium-Derived Embryos

DMRs were subjected to bisulfite sequence analysis to determine their DNA methylation status. This was combined with SNP analysis between laboratory mice (strains 129 and C57BL/6) and wild-derived mice (JF1) to determine the parental allele-specific methylation status. The IG-DMR in fetuses derived from E15.5 prospermatogonia was largely

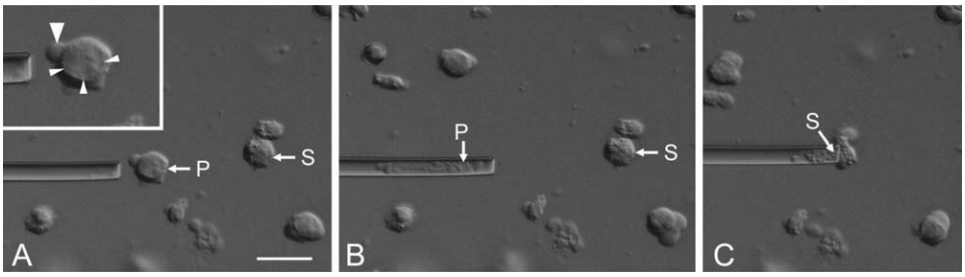

FIG. 2. Selection of prospermatogonia for nuclear transfer from the gonadal cell suspension. **A**) Prospermatogonia could be distinguished easily from other gonadal somatic cells by their high nucleus:cytoplasm ratio and characteristic pseudopodium (large arrowhead) and nuclear membrane (small arrowhead [inset]). **B**) Prospermatogonia were very soft cells and were easily picked up using an injection pipette with an inner diameter (5–7 μm) that was narrower than the cell diameter (10–15 μm). **C**) When a gonadal somatic cell was picked up by the same pipette, the cell stuck to the tip of the pipette because of the physical rigidity of the somatic cells. P = prospermatogonium; S = gonadal somatic cell. Bar = 20 μm.

unmethylated, with occasional methylation of the paternal allele in 1 of the 4 embryos analyzed (Fig. 3A, embryo 9). This DNA methylation pattern was also found in 4 embryos from E16.5 prospermatogonia (Fig. 3A, embryos 11–14). Embryo 15 showed moderate methylation, and embryos 16 and 17 showed higher methylation, especially in the maternal allele. The IG-DMR of 2 embryos (Fig. 3A, embryos 18 and 19) from E17.5 prospermatogonia was fully methylated in both alleles. Thus, methylation of the IG-DMR started at E16.5 and was completed by E17.5.

The DNA methylation status of the *H19* DMR was very similar to that of the IG-DMR. The *H19* DMR of all embryos from E15.5 prospermatogonia examined and 4 embryos from E16.5 prospermatogonia was largely unmethylated (Fig. 4A, embryos 11–14). It was slightly methylated in embryo 15 and highly methylated in embryo 16 (unfortunately, we lost the embryo 17 sample before *H19* DMR, *Rasgrf1* DMR, and *Gtl2* DMR analyses). The embryos from E17.5 prospermatogonia had methylated *H19* DMRs (Fig. 4A), indicating that methylation of the *H19* DMR was completed by E17.5.

The *Rasgrf1* DMR was consistently undermethylated in embryos from E15.5 and E16.5 prospermatogonia, including embryo 16, in which the IG-DMR and *H19* DMR were highly methylated (Fig. 5). The two embryos from E17.5 prospermatogonia showed a highly methylated pattern. These findings indicate that the *Rasgrf1* DMR acquired methylation later than the other two paternal DMRs.

We analyzed the DNA methylation levels of these three paternal DMRs by using one-way ANOVA. Results were essentially the same for the three DMRs: there was a significant effect of the day of the donor spermatogonia on the methylation levels of the DMR of the cloned fetuses. The multiple comparison test (Scheffe F test) revealed significant differences between E15.5 and E17.5 and between E16.5 and E17.5 (Supplemental Table S2).

The promoter of *Peg3*, a maternally imprinted DMR, was totally unmethylated in 11 embryos from E15.5 and E16.5 prospermatogonia, as expected (Fig. 6A). This also supports our idea that the nuclear transfer procedure does not alter the imprinting status of DMRs.

We also analyzed the DNA methylation status of the *Gtl2* DMR, the secondary DMR within the IG-DMR region [20, 21] to see their relationship during the methylation process. The methylation status in each prospermatogonium-derived embryo was essentially similar to that of the IG-DMR of the same embryo, with the exception of a high methylation status of the paternal allele of embryo 16 (Fig. 7). Indeed, there was a high correlation ($r = 0.97$; $P < 1.82 \times 10^{-7}$, Pearson product-moment correlation coefficient) (Supplemental Fig. S4). Embryos cloned from somatic (Sertoli) cells maintained maternally unmethylated and paternally methylated DMRs, indicating that they maintained the somatic cell pattern in the methylation status of the *Gtl2* DMR (Supplemental Fig. S5).

Gene Expression Analysis of Cloned Mouse Embryos Derived from Prospermatogonia

The expression levels of imprinted genes in E9.5 embryos derived from prospermatogonia were analyzed by quantitative RT-PCR using specific primers. For the IG-DMR domain, 2 Megs, *Gtl2* and *Rian*, and 1 Peg, *Dlk1*, were analyzed. All embryos from E15.5 prospermatogonia (Fig. 3B, embryos 1–10) showed predominant expressions of the two Megs *Gtl2* and *Rian* compared with the Peg *Dlk1*. This pattern most likely represented the nonimprinted (default) status of these genes, because the same pattern was observed in embryos cloned from PGCs at E12.5 or E13.5 [6]. By contrast, embryos cloned from E16.5 prospermatogonia showed diverse patterns. Among the 7 embryos analyzed, 4 (Fig. 3B, embryos 11–14) showed Meg-predominant, 2 (Fig. 3B, 16 and 17) showed Peg-predominant, and 1 (Fig. 3B, embryo 15) showed intermediate expression patterns. Two embryos from E17.5 prospermatogonia and 4 embryos from P0.5 prospermatogonia showed Peg-dominant expression (Fig. 3B). Dunnett multiple comparison test also noted a shift in the gene expression pattern between embryos 15 and 16 (Fig. 3B). Thus, overall, the expression patterns of

TABLE 1. In vitro and in vivo development of embryos cloned from prospermatogonia at different developmental days.

Age of donors	Donor strain	No. of embryos cultured	No. of 2-cell embryos at 24 h (%)	No. of embryos transferred	No. of embryos implanted (%)	No. of embryos that developed to fetuses at E9.5 (%)
E15.5	129×JF1	208	187 (89.9)	175	54 (30.9)	11 (6.3)
E16.5	C57BL/6×JF1	326	298 (91.4)	270	79 (29.3)	2 (0.7)
	129×JF1	390	338 (86.7)	327	114 (34.9)	6 (1.8)
E17.5	129×JF1	152	144 (94.7)	100	81 (81.0)	2 (2.0)
P0.5	129×JF1	148	95 (64.2)	74	24 (32.4)	7 (9.5)

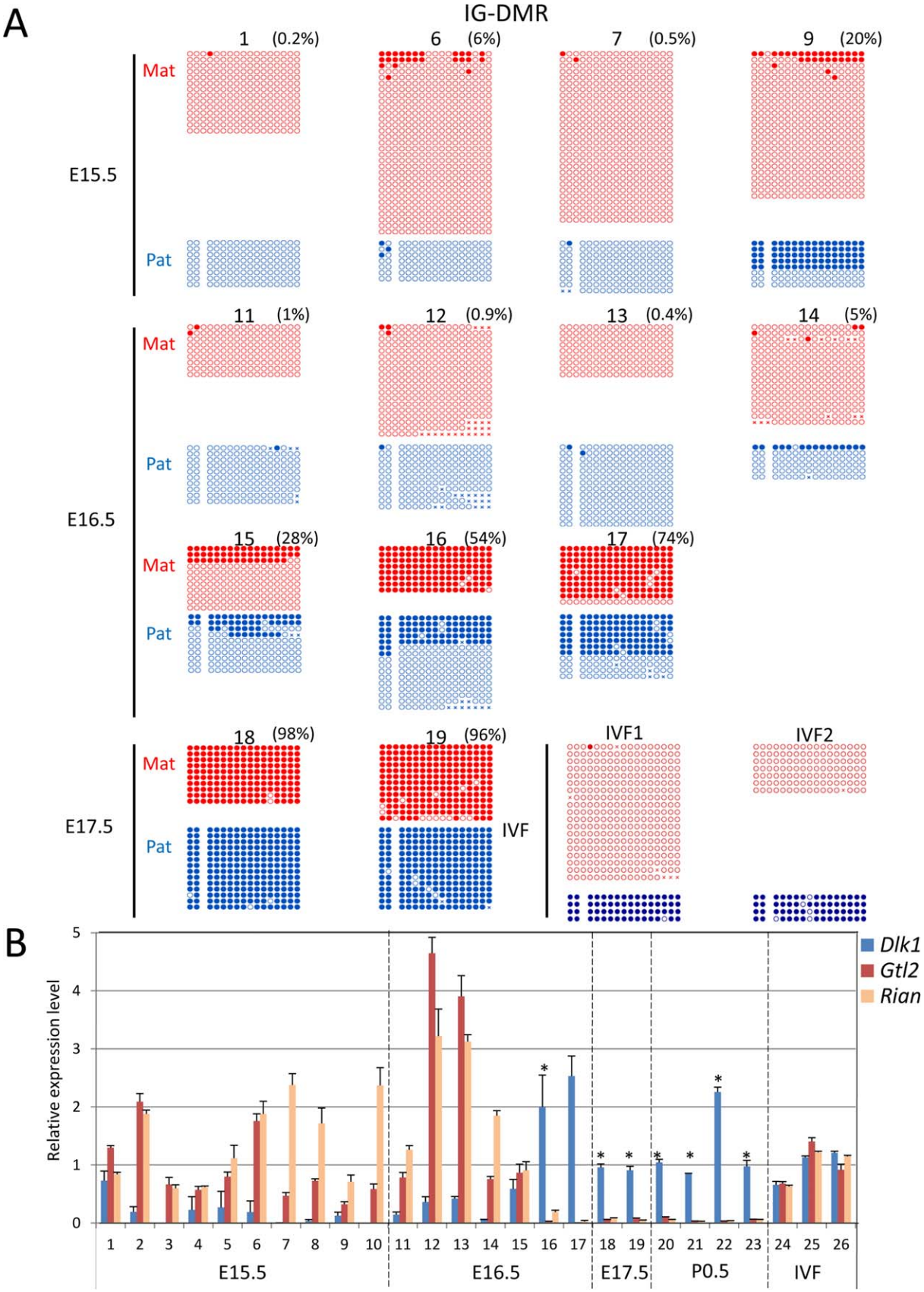

FIG. 3. DNA methylation of the IG-DMR (**A**) and expression levels of *Dlk1*, *Gtl2*, and *Rian* (**B**) in embryos cloned from prospermatogonia at different stages. The numbers associated with the methylation and gene expression data represent the identity numbers of embryos (the same applies to the following figures). **A**) The IG-DMR of all embryos derived from E15.5 prospermatogonia and four embryos from E16.5 prospermatogonia (embryos 11–14) was largely unmethylated. Embryo 15 showed moderate methylation, whereas embryos 16 and 17 showed higher methylation. E17.5 prospermatogonia-derived embryos (18 and 19) were fully methylated. **B**) Expression patterns of imprinted genes were well correlated with the methylation status of the

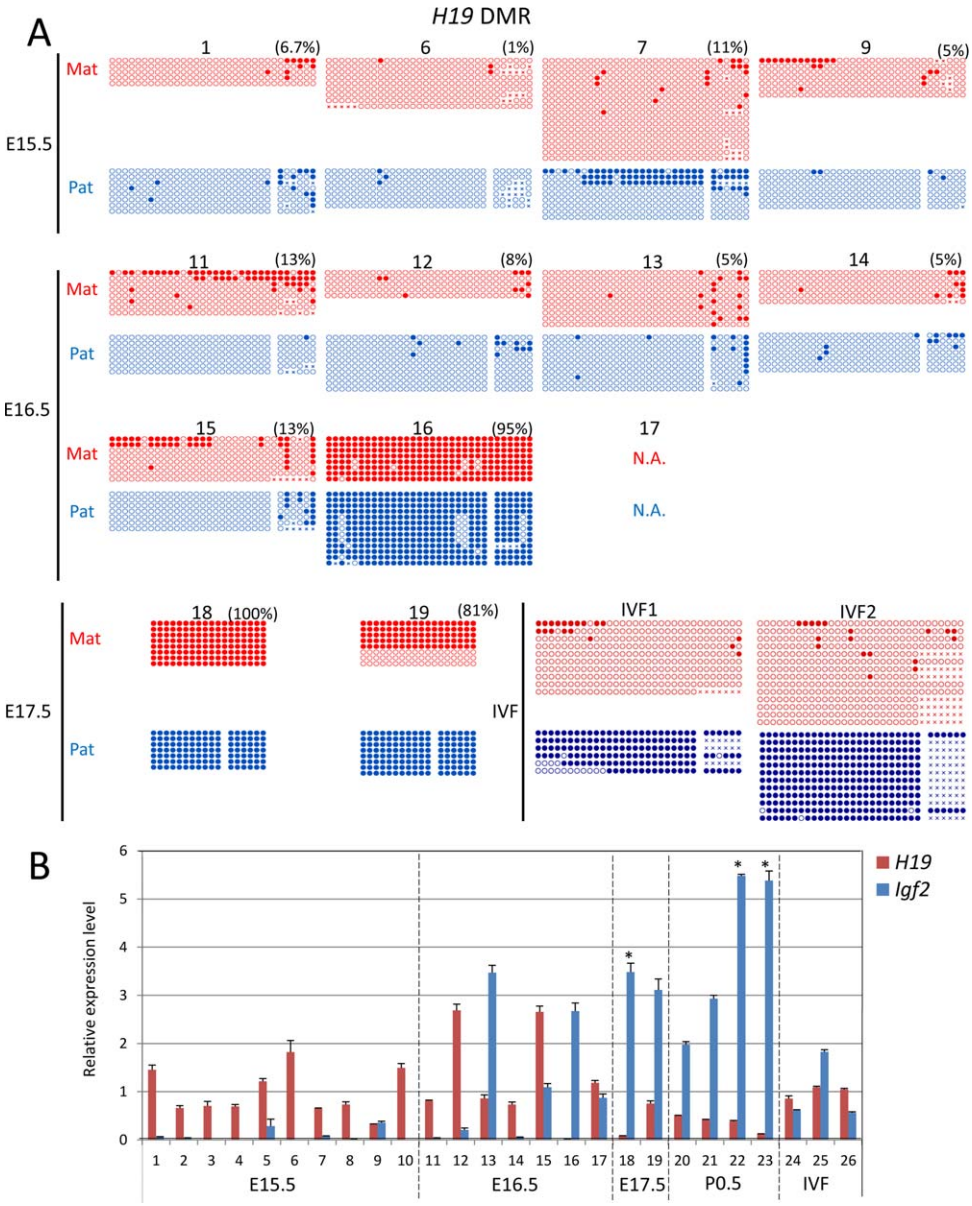

FIG. 4. DNA methylation of the *H19* DMR (**A**) and expression levels of *H19* and *Igf2* (**B**) in embryos cloned from prospermatogonia at different stages. **A**) The DNA methylation status of the *H19* DMR was very similar to that of the IG-DMR shown in Fig. 2A. The *H19* DMR of all embryos from E15.5 prospermatogonia and 4 embryos from E16.5 prospermatogonia (11–14) was undermethylated. It was slightly methylated in embryo 15 and highly methylated in embryo 16. Embryos from E17.5 prospermatogonia (18 and 19) had fully methylated *H19* DMR. N.A. = not analyzed. **B**) Expression of the *H19* and *Igf2* genes in all embryos from E15.5 prospermatogonia followed an *H19*-predominant pattern, as expected from the DMR methylation patterns. E16.5 prospermatogonia-derived embryos showed diverse patterns, which were not always consistent with methylation levels of the DMRs (see *Results* and *Discussion*). The *Igf2* gene was expressed predominantly in embryos cloned from E17.5 or P0.5 prospermatogonia, as expected. Asterisks above the bars indicate that the paternal:maternal gene expression level ratios differed significantly from those of IVF fetuses (Dunnett test). Error bars = SEM.

the three genes within the IG-DMR were highly correlated with the DNA methylation status (unmethylated, moderately methylated, and highly methylated) of the IG-DMR shown above (Fig. 3A).

For the *H19* DMR genes *H19* and *Igf2*, the Meg and Peg patterns of this region were analyzed, respectively. All 10 embryos cloned from E15.5 prospermatogonia showed Meg-predominant patterns (Fig. 4B), consistent with the default status reported previously [6]. In E16.5 prospermatogonia-derived embryos, *H19* and *Igf2* showed diverse patterns, as did the genes within the IG-DMR regions. However, unlike the genes within the IG-DMR, the expression patterns of *H19* and *Igf2* were not always consistent with the DNA methylation level of the *H19* DMR. For example, embryo 13 showed an

associated DMRs, from the Meg (*Gtl2* and *Rian*)-predominant pattern in the E15.5 embryos to the Peg (*Dlk1*)-predominant pattern in the E17.5 prospermatogonia-derived embryos. E16.5 prospermatogonia-derived embryos showed diverse patterns, consistent with the methylation levels of the DMRs. Asterisks above the bars indicate that the Peg:Meg expression level ratios differed significantly from those of IVF fetuses (Dunnett test). Embryo 17 was excluded from statistical analysis because its value was an outlier (Grubbs outlier test, $P < 0.01$). Error bars = SEM.

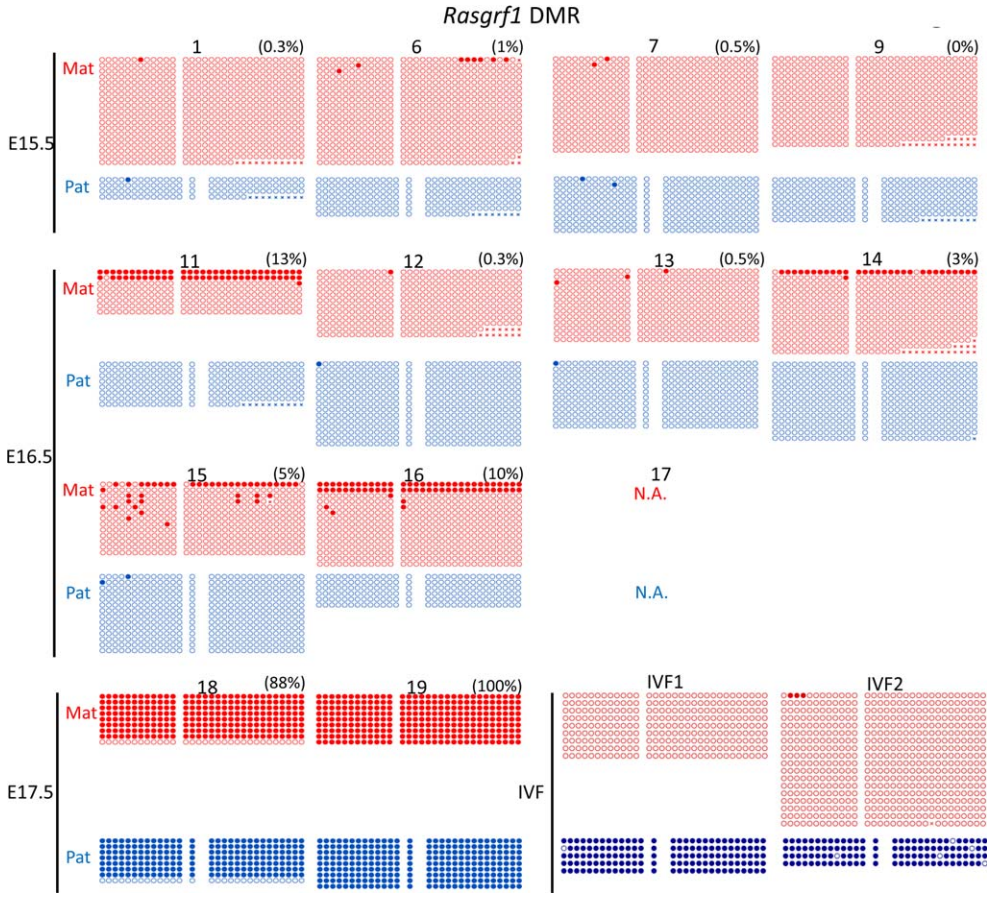

FIG. 5. DNA methylation patterns of the *Rasgrf1* DMR in embryos cloned from prospermatogonia at different stages. This DMR was consistently undermethylated in embryos cloned from E15.5 and E16.5 prospermatogonia, including embryo 16, in which both the IG-DMR and *H19* DMR were highly methylated (Figs. 2A and 3A). The two embryos cloned from E17.5 prospermatogonia showed a fully methylated pattern. N.A. = not analyzed. Unlike other paternally imprinted genes, *Rasgrf1* mRNA was not detected in E9.5 fetuses (see Supplemental Fig. S6). Therefore, quantitative RT-PCR for *Rasgrf1* was not performed in this study.

Igf2-predominant pattern, and embryo 15 showed a significant *Igf2* expression (Fig. 4B), which differed from those expected from the DNA methylation of the *H19* DMR (embryo 17 also seemed to be out of order, but we could not confirm it) (Fig. 4A). In embryos from E17.5 or P0.5 prospermatogonia, *Igf2* was predominantly expressed (Fig. 4B), as expected from the DNA methylation pattern (Fig. 3A). Dunnett multiple comparison test showed that only three embryos (18, 22, and 23) had expression patterns that different from those of a control IVF embryo (Fig. 4B).

For *Rasgrf1*, we did not analyze the expression levels by quantitative RT-PCR because this gene was not expressed in the E9.5 fetuses examined (Supplemental Fig. S6). We confirmed the accuracy of our RT-PCR method in positive (brain) and negative (liver) samples from adult mice [22, 23] (Supplemental Fig. S6).

We also analyzed expression levels of two maternally imprinted genes, 1 Peg (*Peg3*) and 1 Meg (*Igf2r*), in prospermatogonium-derived embryos. As expected, these genes consistently showed upregulated and downregulated expression patterns, respectively, throughout the stages analyzed (prospermatogonia from E15.5 to P0.5 as donor cells) (Fig. 6B and Supplemental Fig. S7). Results of the Dunnett multiple comparison test supported these tendencies, although two exceptional embryos (16 and 18) showed significantly lower *Peg3* expression levels (Fig. 6B and Supplemental Fig. S7).

DNA Methylation Analysis of DMRs in Donor Prospermatogonia

Finally, we performed bisulfite sequencing analysis to determine the DNA methylation status of the donor spermatogonia. IG-DMR in E15.5 prospermatogonia was hypomethylated (33%), methylated further (60%) in E16.5 prospermatogonia, and fully methylated (98%) in E17.5 prospermatogonia (Fig. 8). This result was consistent with that reported previously [24]. By contrast, *Gtl2* DMR, a secondary DMR within the IG-DMR domain, was hypomethylated (<12%) throughout the stages examined (Fig. 8). This result, together with that from the prospermatogonium-derived embryos (Fig. 7), supports the idea that *Gtl2* DMR is methylated after fertilization but not during germ cell development [20, 21].

DISCUSSION

Genomic imprinting memory in early PGCs starts to be erased at E11.5 and is erased completely by E12.5 in both sexes, as demonstrated by the DNA methylation analysis of DMRs and gene expression analysis of imprinted genes in PGC-derived embryos in mice [5, 6, 25]. Later, a new set of imprints is reestablished in germ cells at specific stages according to sex [7]. Studies of DNA methylation status in paternal DMRs during male germ cell development revealed that significantly hypermethylated DMRs were found in

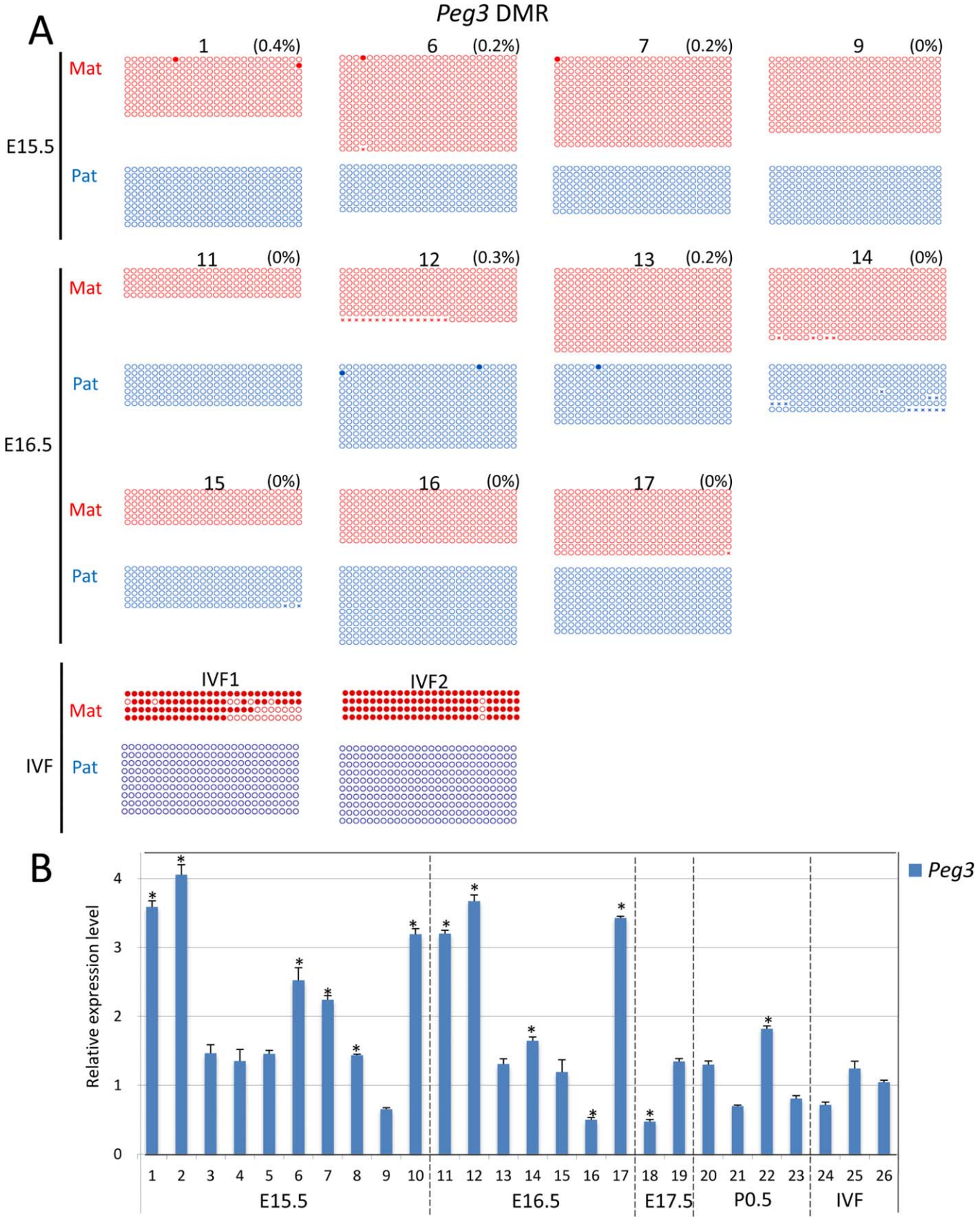

FIG. 6. DNA methylation (**A**) and expression levels (**B**) of *Peg3* in embryos cloned from prospermatogonia at different stages. Because *Peg3* is a maternally imprinted gene, all embryos showed undermethylated DNA and high gene expression levels throughout the stages examined. Asterisks above the bars (**B**) indicate that the paternal:maternal gene expression level ratios differed significantly from those of IVF fetuses (Dunnett test). Error bars = SEM.

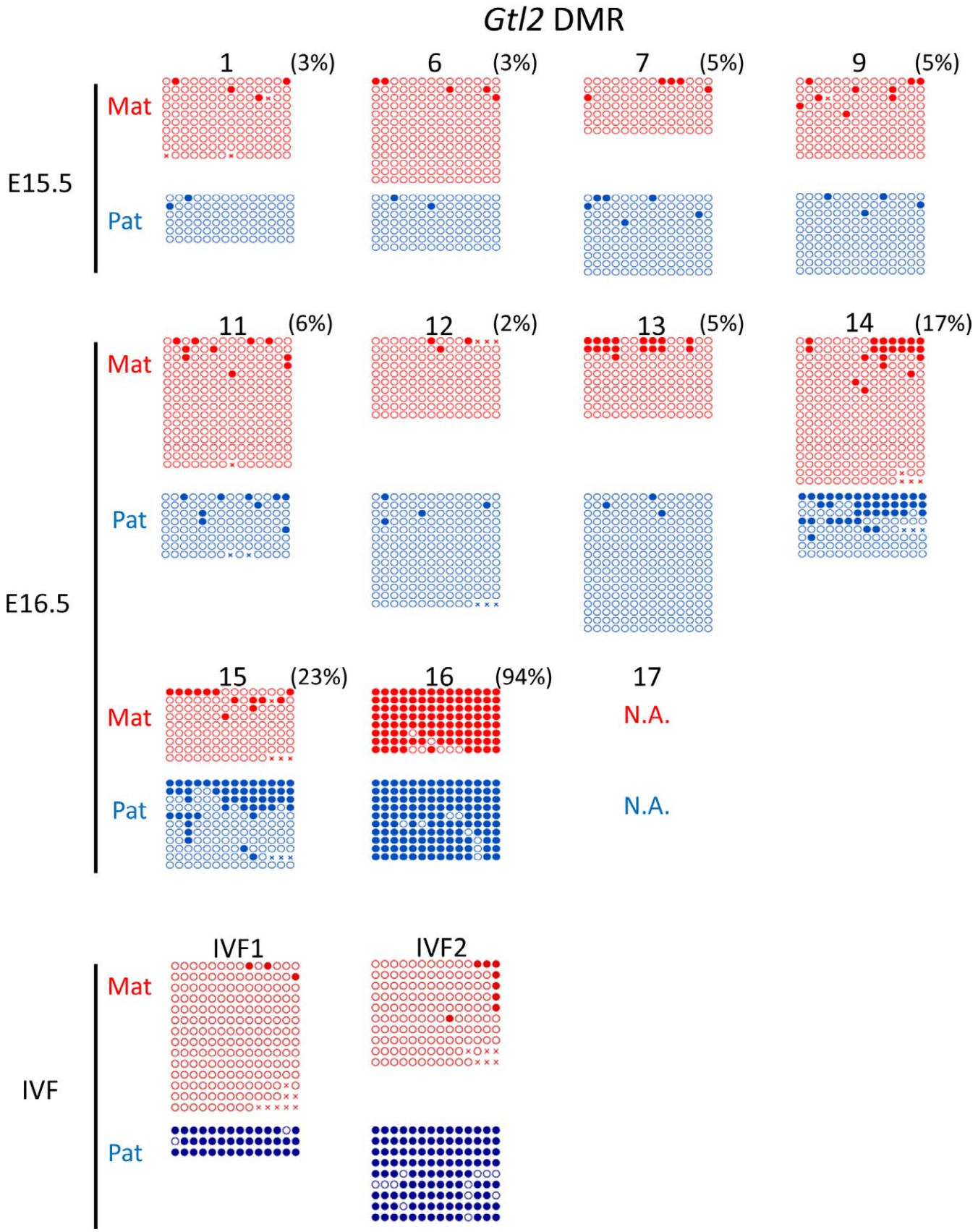

FIG. 7. DNA methylation of the *Gtl2* DMR in embryos cloned from prospermatogonia at different stages. Methylation levels of the *Gtl2* DMR were highly correlated with those of the IG-DMR shown in Figure 2A. Their correlation is shown graphically in Supplemental Figure S4. N.A. = not analyzed.

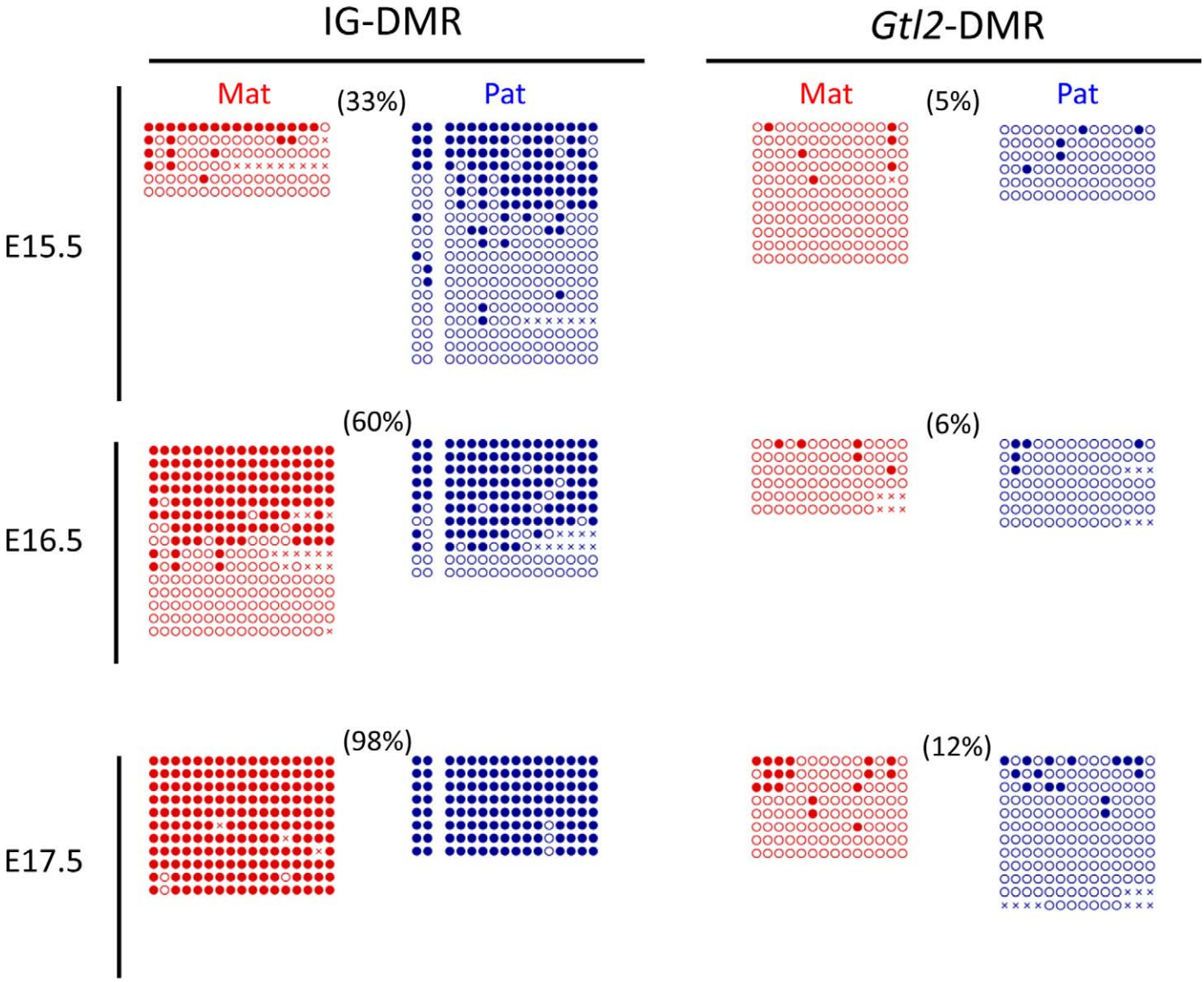

FIG. 8. DNA methylation of DMRs in spermatogonia used for nuclear transfer. We analyzed IG-DMR and *Gtl2* DMR as representative primary and secondary DMRs, respectively. The DNA methylation level of IG-DMR in prospermatogonia increased with age, and IG-DMR was fully methylated by E17.5. By contrast, *Gtl2* DMR was hypomethylated throughout the stages examined.

prospermatogonia at E14.5 (*H19* DMR [26]), at E15.5 (IG-DMR and *H19* DMR; [27]), or at E16.5 (IG-DMR; [24]). As far as we examined, based on the expression patterns of imprinted genes and the DNA methylation status of DMRs in cloned fetuses, the paternally imprinted E15.5 prospermatogonia maintained the default genomic status, most likely the consequence of the erased imprint at E12.5. Taking all the data into consideration, we conclude that the establishment of genomic imprinting in all paternal imprint regions is complete by E17.5, following a short intermediate period at E16.5. This timing of imprinting is consistent with the data obtained from DNA methylation analyses of donor prospermatogonia in this study (Fig. 8) and in previous studies [24, 27], although the imprinting occurred significantly later than that (E14.5) reported by Davis et al. [26]. Although we do not know the reasons for this discrepancy, there might have been a substrain-specific difference because only the *Mus musculus castaneus* allele, but not the C57BL/6 allele, was methylated at E14.5 [26]. The methylation levels of IG-DMR in fetuses cloned from E15.5 prospermatogonia was 0.2%–20% (Fig. 3A), which was lower than that in their donor prospermatogonia (33%) (Fig. 8), suggesting that the DMR at E15.5 was demethylated by nuclear transfer to some extent. It is reasonable to assume that DNA methylation alone is not enough to protect the imprints against global demethylation at fertilization or nuclear transfer. It is probable that they become more stable as they acquire other repressive epigenetic marks such as histone methylation. It is known that dimethylated or trimethylated histone H3 lysine 9 is enriched in the paternally imprinted DMRs of spermatozoa and somatic cells [28, 29].

We found that the three DMRs analyzed basically followed a common time course of imprinting, as mentioned above. However, the nuclear transfer technique enabled detailed comparison of the imprinting status of different DMRs within the same embryos, which suggested the presence of a slight DMR-specific difference in the timing of imprinting. We found that imprinting of the *Rasgrf1* DMR seemed to be established later than that of the other two. Although we do not know the exact mechanisms of this DMR-specific difference, there is evidence suggesting that imprinting of the *Rasgrf1* DMR might

differ from that of the IG-DMR or *H19* DMR in several respects. DNA methylation of the *Rasgrf1* DMR requires not only Dnmt3A but also Dnmt3B and Piwi-interacting (pi) RNAs [30, 31]. In human, unlike IG-DMR or *H19* DMR, there is no *Rasgrf1*-equivalent DMR [32].

Another important advantage of using nuclear transfer techniques for studying gene imprinting is the availability of expression data from germ cell-derived fetuses and placentas. In this study, we found a clear, appropriate correlation between the gene expression pattern and DNA methylation status for the IG-DMR cluster. This implies that the expression of imprinted genes in this cluster may be strongly or solely dependent on DNA methylation of this particular DMR. This assumption is consistent with our observation on the *Gtl2* DMR of the same cluster, which was likely to be under the control of the IG-DMR (see below). By contrast, the expression profiles of *H19* and *Igf2* within the *H19* DMR cluster could not always be explained by methylation of the *H19* DMR. It is unlikely that these unexpected profiles were caused by technical errors or clone-associated aberrations, because data from both E15.5 and E17.5 prospermatogonia were highly consistent. Presently, the best known scenario for the reciprocal expression of *Igf2* and *H19* during mouse development is that the *H19* DMR is located at a chromatin boundary upstream of *H19*. This allows binding of the zinc finger binding protein CCCTC-binding factor (CTCF), which then blocks the access of enhancers to the maternal *Igf2* promoters [33]. However, the real mechanisms could be more complex, because other elements can affect the expression of the *H19* and *Igf2* genes. There are tissue-specific enhancers (endoderm- and mesoderm-specific) downstream of *H19*, which might work in a differential CTCF-dependent manner. In the *Igf2* region, there are several tissue-specific promoters and a DMR silencer (DMR1) and activator (DMR2), which are methylated on the active paternal allele [34, 35]. For example, deletion of DMR1 resulted in the expression of *Igf2* irrespective of the methylation status of the *H19* DMR in mesodermal tissues [34]. The presence of these elements, probably modulating the fine tuning of *Igf2* and *H19* expression, might have caused the unexplained diverse expression patterns in some cloned embryos from E16.5 prospermatogonia. Later, all embryos cloned from E17.5 and P0.5 cells showed the expected expression patterns of *Igf2* and *H19*.

It is known that there are two types of DMRs in the imprinting clusters: primary DMRs methylated differentially in germ cells (gametic imprinting) and secondary DMRs that are methylated after fertilization. The *Gtl2* DMR (also known as *Meg3* DMR) is the most studied secondary DMR in mice and humans. The mouse *Gtl2* DMR is unmethylated throughout male germ cell development [20, 21]. We also confirmed this by analyzing the methylation pattern of prospermatogonia from E15.5 to E17.5 (Fig. 8). The paternal allele of the mouse *Gtl2* DMR in fertilized embryos acquires methylation between E3.5 and E6.5 [20, 21]. Our methylation analysis of the donor prospermatogonia and their cloned fetuses suggests that the *Gtl2* DMR acquired DNA methylation at some time after nuclear transfer but no later than E9.5. Importantly, our analysis of embryos with different imprinting status revealed a highly significant correlation ($r = 0.97$; $P < 1.82 \times 10^{-7}$) between the methylation level of the *Gtl2* DMR and that of the IG-DMR (Supplemental Fig. S4). This is consistent with an analysis of human patients carrying a microdeletion of the *MEG3* DMR, which showed a high dependency of its methylation on the IG-DMR methylation *in cis* [36]. Okae et al. [37] also reported a high correlation between the methylation level of the IG-DMR and that of the *Gtl2* DMR in somatically cloned fetuses. The *Gtl2* DMR is responsible for the imprinted expression of *Gtl2* at least after midgestation, as revealed by gene-targeting studies [38, 39]. Taken together, it is very likely that imprinted expression of *Gtl2* is ensured by hierarchical regulation of the *Gtl2* DMR by the IG-DMR.

In this study several cloned fetuses derived from E16.5 prospermatogonia showed moderate DNA methylation in both the IG-DMR and the *H19* DMR. This is intriguing because it implies that these cloned fetuses comprised a mixture of cells with methylated and unmethylated DMRs. This might be explained by the segregation of hemimethylated DNA sequences of the donor genome. Each clone was derived from a single donor cell at the G1 cell cycle [15, 16], which must have carried a 2n diploid genome, namely a double-stranded (ds) DNA for each parental allele. Therefore, in theory, if a DMR allele is hemi-methylated in the donor genome, it gives rise to methylated and unmethylated alleles at the S phase after nuclear transfer by the activity of Dnmt1, a maintenance methyltransferase [40]. These are then segregated into sister blastomeres at the first cleavage. As these two-cell blastomeres are later evenly distributed into embryos and tissues [41], the DNA methylation level of the DMR in cloned embryos as a whole will be around 50%. This should occur in the maternal and paternal alleles independently. For example, in the IG-DMR, the maternal and paternal alleles of embryo 15 and the paternal alleles of embryos 16 and 17 might have represented hemimethylated DMR of the donor genome (Fig. 2A). Supporting this hypothesis, previous studies reported similar observations in developing embryos following oocyte-specific depletion of Dnmt1 [40, 42]. In these embryos, hemimethylated DMRs arose from the methylated DMRs of the parental alleles because of Dnmt1 deficiency. When the Dnmt1 activity was restored by zygotic gene activation, these hemimethylated alleles gave rise to a methylated allele and an unmethylated allele in two daughter cells, respectively. This resulted in a mixture of cells with methylated and unmethylated alleles in later embryos and fetuses.

In conclusion, we completed a series of nuclear transfer studies of genomic imprinting in mice, following previous studies of imprinting erasure [6] and establishment of maternal imprinting [11]. Our nuclear transfer study suggests that genomic imprinting in all paternal imprint regions was established by E17.5, following a short intermediate period at E16.5. Additionally, our detailed analysis of cloned embryos allows us to propose that the *Rasgrf1* DMR is imprinted slightly later than the other two DMRs studied, and that the IG-DMR, but not *H19* DMR, solely governs the control of its imprinted gene cluster. The methylation status of the *Gtl2* DMR, the secondary DMR that acquires DNA methylation after fertilization, is likely to follow that of the upstream IG-DMR.

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