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Molecules and Cells

H19 Gene Is Epigenetically Stable in Mouse Multipotent Germline Stem Cells

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Testis-derived germline stem (GS) cells can undergo reprogramming to acquire multipotency when cultured under appropriate culture conditions. These multipotent GS (mGS) cells have been known to differ from GS cells in their DNA methylation pattern. In this study, we examined the DNA methylation status of the *H19* imprinting control region (ICR) in multipotent adult germline stem (maGS) cells to elucidate how epigenetic imprints are altered by culture conditions. DNA methylation was analyzed by bisulfite sequencing PCR of established maGS cells cultured in the presence of glial cell line-derived neurotrophic factor (GDNF) alone or both GDNF and leukemia inhibitory factor (LIF). The results showed that the *H19* ICR in maGS cells of both groups was hypermethylated and had an androgenetic pattern similar to that of GS cells. In line with these data, the relative abundance of the *Igf2* mRNA transcript was two-fold higher and that of *H19* was three fold lower than in control embryonic stem cells. The androgenetic DNA methylation pattern of the *H19* ICR was maintained even after 54 passages. Furthermore, differentiating maGS cells from retinoic acid-treated embryoid bodies maintained the androgenetic imprinting pattern of the *H19* ICR. Taken together these data suggest that our maGS cells are epigenetically stable for the *H19* gene during *in vitro* modifications. Further studies on the epigenetic regulation and chromatin structure of maGS cells are therefore necessary before their full potential can be utilized in regenerative medicine.

INTRODUCTION

Genomic imprinting is a gene regulatory mechanism whereby only one of the two parental alleles is expressed while the other is suppressed (Carr et al., 2007). It is regulated by epigenetic mechanisms such as DNA methylation and histone modifications (Bernstein et al., 2007; Zhu et al., 2008). The DNA methylation of differentially methylated regions (DMRs) in imprinted genes determine paternal- or maternal-allele specific gene expression (Lucifero et al., 2002), which is important for normal foetal development, placental function, and post-natal behaviour (Murphy et al., 2001). Both gain and loss of imprinting are associated with various diseases (Reik and Walter, 2001).

Insulin-like growth factor 2 (*Igf2*) and *H19* are the best characterized imprinted genes in mice. *Igf2*, a foetal growth factor, is exclusively expressed from the paternal allele while *H19*, a non-coding RNA, is active only in the maternal allele (Grandjean et al., 2001). The two genes are located adjacent to each other as the *Igf2-H19* cluster on mouse chromosome 7 and share a common enhancer located in the 3′ region of the *H19* gene. The expression of both genes is regulated by DNA methylation at imprinting control region (ICR) located 2-4 kb upstream of the *H19* transcription start site (Thorvaldsen et al., 1998). This ICR region contains four binding sites for the CTCF (CCCTC-binding factor) transcriptional insulator (Yang et al., 2003) and its methylation allows the enhancer to bind *Igf2*. On the contrary, when the ICR is unmethylated, it forms a complex with CTCF protein that act as a chromatin boundary to block the binding of enhancer to the *Igf2* (Han et al., 2008; Reik et al., 2000; Sasaki et al., 2000; Yang et al., 2003).

Multipotent germline stem (mGS) cells generated from *in vitro* culture of testis-derived germline stem (GS) cells have recently been shown to have potential in regenerative medicine (Guan et al., 2006; Kanatsu-Shinohara et al., 2004). The GS cells, isolated from the adult mouse, can also acquire a pluripotent state when cultured under standard embryonic stem (ES) cell culture conditions (Guan et al., 2006; 2007; Zovoilis et al., 2008). The mGS cells were also shown to have undergone changes in their DNA methylation pattern contrary to GS cells which show a complete androgenetic DNA methylation pattern similar to their *in vivo* counterpart, spermatogonial stem cells (Kanatsu-Shinohara et al., 2004; 2005). However, detailed information on imprinting at the *Igf2-H19* cluster in maGS cells is still unclear.

Therefore, this study was designed to investigate the effect of culture conditions on the imprinting status of the *Igf2-H19* cluster in maGS cells. The established maGS cells were cultured in the presence of glial cell line-derived neurotrophic factor (GDNF) alone or both GDNF and leukemia inhibitory factor (LIF) and were analyzed for their imprinting status by bisulfite sequencing PCR (BS-PCR) for the four CTCF binding sites within the *H19* ICR. We further investigated whether long term culture or *in vitro* differentiation of maGS cells could alter their imprinting status.

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Table 1. Details of primer pairs used for DNA methylation analysis

Gene name	Primer sequence (5′ to 3′)[a]	Annealing temp. (°C)	Amplicon size (bp)	GenBank no.
H19 ICR CTCF 1/2 - 1st	GAGTATTTAGGAGGTATAAGAATT AGGGGTTTATGTTAGTTTTTGAT	50	422	MMU19619
H19 ICR CTCF 1/2 - 2nd	GTAAGGAGATTATGTTTATTTTTGG CCTCATTAATCCCATAACTAT	50		
H19 ICR CTCF 3 - 1st	GGGTTTTTTTGGTTATTGAATTTTAA ACACAAATACCTAATCCCTTTATTAAAC	50	224	MMU19619
H19 ICR CTCF 3 - 2nd	GGGTTTTTTTGGTTATTGAATTTTAA AATACACACATCTTACCACCCCTATA	58		
H19 ICR CTCF 4 - 1st	GGGTTTTTTTGGTTATTGAATTTTAA ACACAAATACCTAATCCCTTTATTAAAC	50	457	MMU19619
H19 ICR CTCF 4 - 2nd	GGGTGGTAAGATGTGTGTATTTTTG CAAATACCTAATCCCTTTATTAAACCTAAA	58		
Peg3 - 1st	TTTTTAGATTTTGTTTGGGGGTTTTTAATA AATCCCTATCACCTAAATAACATCCCTACA	50	452	AF105262S1
Peg3 - 2nd	TTGATAATAGTAGTTTGATTGGTAGGGTGT ATCTACAACCTTATCAATTACCCTTAAAAA	50		

[a]Primers for *H19* ICR CTCF 1/2 and *Peg3* were originally described in References (Nakamura et al., 2007).

MATERIALS AND METHODS

ES cells and maGS cells culture

Mouse ES cells were cultured on STO feeder cell layers in high glucose Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, USA) supplemented with 15% (v/v) foetal bovine serum (FBS; Hyclone, USA), 1-mM MEM nonessential amino acids (Gibco BRL), 100 units/ml penicillin-100 μg/ml streptomycin (Gibco BRL), 0.1 mM 2-mercaptoethanol (Sigma, USA) and 1,000 units/ml LIF (Chemicon, USA). The maGS cells were isolated from testes of 4-6-week-old DBA/2 mice (Orient Bio, Korea) as described earlier (Guan et al., 2006). Briefly, testes were decapsulated and the isolated seminiferous tubules were washed with serum-free DMEM. Testicular cells were then dissociated by chopping and strained through a cell strainer (40 μm, Falcon BD, USA) without enzyme treatment. The dissociated cells were then cultured on ES cell culture medium further supplemented with 10 ng/ml recombinant human GDNF (R&D, USA). Cells were sub-cultured every three to four days for 4-5 passages to establish maGS cell lines. The established maGS cells were subsequently cultured either in the absence (GDNF alone group) or presence (GDNF plus LIF group) of LIF. Cells in both groups were cultured for at least 10 passages before any experimental investigation. All cells were maintained at 37°C in a humidified 5% CO_2 atmosphere.

Alkaline phosphatase (AP) staining

Cultured cells were stained for AP activity according to the manufacturer's protocol (Sigma). Briefly, cells were fixed with 4% paraformaldehyde for 30 s at room temperature, washed twice with distilled water and incubated with staining solution for 15 min at room temperature in dark conditions.

In vitro differentiation of maGS cells

In vitro differentiation of maGS cells was performed as described earlier (Bain et al., 1996). Briefly, maGS cells were induced to form embryoid bodies (EBs) by hanging drop culture for 4 d. The EBs were then further cultured in the presence of 1-μM *all-trans* retinoic acid (RA; Sigma) for 4 next days (Andollo et al., 2006).

BS-PCR

ES cells and maGS cells were lysed in lysis buffer containing 0.5% SDS, 0.1-M EDTA, 10-mM Tris-Cl (pH 8.0) and 200 μg/ml proteinase K (Roche Applied Science, USA), and incubated at 55°C for 16 h. Genomic DNA was isolated using the phenol/chloroform extraction method and treated with sodium bisulfite to convert all unmethylated cytosine to uracil using the One Day MSP kit (In2Gen, Korea), according to the manufacturer's protocol. Briefly, genomic DNA (~1 μg) was denatured by 3-N NaOH for 10 min at 37°C and treated with sodium bisulfite for 16 h at 50°C in the dark. After desulfonation, neutralization and desalting, the modified DNA was dissolved in 20 μl of distilled water and amplified by nested or seminested PCR (Bioneer, Korea) using primer sets shown in Table 1 (Nakamura et al., 2007). The amplified DNA fragments were verified by electrophoresis on 1.0% agarose gel and purified (GeneAll, Korea). Successful PCR products were then subcloned into the pGEM-T Easy vector (Promega, USA), according to the manufacturer's protocol. Cloned plasmid DNAs were purified from individual clones using a Mini kit (GeneAll) and sequenced (Macrogen, Korea). Only clones with ≥ 90% conversion rate were accepted for analysis.

RT-PCR

Total RNA was extracted using the RNeasy kit (Qiagen, Germany) according to the manufacturer's protocol. RNA (500 ng) was then reverse-transcribed to cDNA using RT Premix (Bioneer) in 20 μl of reaction mixture for 60 min at 42°C. To analyze the expression of specific marker genes, PCR was performed with 1 μl of cDNA using the primer sequences shown in Table 2.

Real time quantitative RT-PCR

The quantitative expression analyses of imprinted genes were performed by real-time RT-PCR using a 7500 Real-Time PCR System (Applied Biosystems). The *H19* and *Igf2* primers were used as previously described (Eckardt et al., 2007; Horii et al., 2008). The expression of each gene was evaluated relative to

Table 2. Details of primer pairs used for RT-PCR

Gene name	Primer sequence (5′ to 3′)	Annealing temp. (°C)	Amplicon size (bp)	GenBank no.
Gapdh	CTCACTCAAGATTGTCAGCA CATACTTGGCAGGTT	58	326	XM 001473623.1
β-actin	GACAACGGCTCCGGCATGTGCAAAG TTCACGGTTGGCCTTAGGGTTCAG	55	321	NM 007393.3
Oct4	GGCGTTCTCTTTGGAAAGGTGTTC CTCGAACCACATCCTTCTCT	55	313	NM 013633
Vasa	CCAAAAGTGACATATATACCC TTGGTTGATCAGTTCTCGAG	50	416	NM 010029.1
Stra8	GCCAGAATGTATTCCGAGAA CTCACTCTTGTCCAGGAAAC	58	631	NM 009292.1

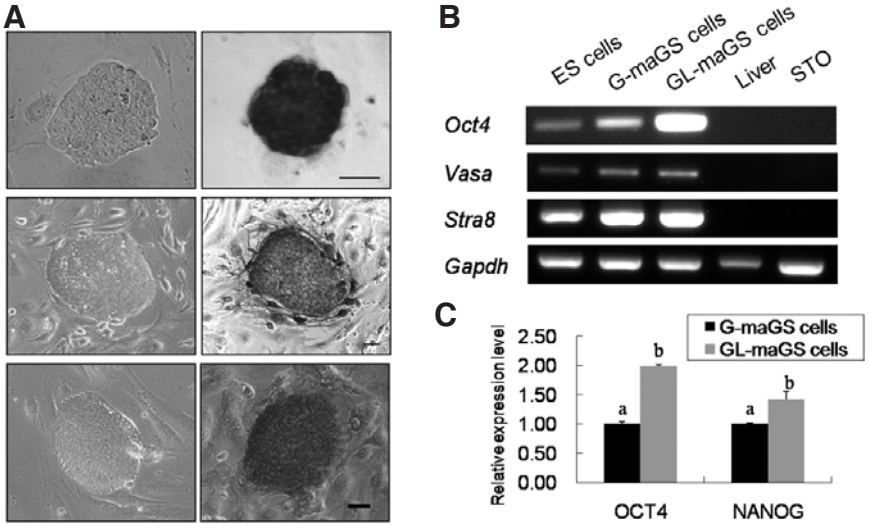

Fig. 1. Alkaline phosphatase (AP) activity and expression of marker genes in ES and maGS cells. (A) Left, phase contrast; right, AP staining; top, ES cells; middle, maGS cells cultured in the presence of GDNF alone; bottom, maGS cells cultured in the presence of both GDNF and LIF. Scale bars, 50 μm. (B) RT-PCR analyses. Specific primers were used to amplify cDNA from ES cells and maGS cells cultured in the presence of GDNF alone or both GDNF and LIF. Liver and STO cells were used as controls. (C) Real time quantitative RT-PCR analysis of pluripotent marker genes in maGS cells. Different letters on the bars indicate significant differences ($P < 0.05$).

the *Gapdh* expression in the individual samples and presented as a ratio to that in controls.

Statistical analysis

The imprinted gene expression levels were compared using Student's *t*-test. P values of less than 0.05 ($p < 0.05$) were considered significant.

RESULTS

AP activity and expression of specific marker genes in maGS cells

The results showed that maGS cells cultured in the presence of GDNF alone or both GDNF and LIF did not differ in their morphology and AP activity (Fig. 1A). The maGS cells in both groups also showed positive expression of *Oct4* (pluripotent marker), *Vasa* (germ cell marker), and *Stra8* (spermatogonia marker) following RT-PCR analysis (Fig. 1B). However, real time quantitative RT-PCR revealed that the expression of the pluripotent marker genes *Oct4* and *Nanog* was significantly higher in maGS cells cultured in the presence of both GDNF and LIF than in those cultured in the presence of GDNF alone (Fig. 1C).

DNA methylation at *H19* ICR in maGS cells

We investigated the DNA methylation status of the four CTCF binding sites of the *H19* ICR that are separated into three regions - CTCF1/2, CTCF3, and CTCF4 (Fig. 2A). Additionally, we investigated the DNA methylation status of *Peg3* (paternally expressed gene 3), which is well known to be maternally imprinted and hence, undergoes DNA hemizygotic methylation in ES cells. The DNA methylation pattern of *Peg3* served as a control, and as expected, we found hemizygotic DNA methylation in ES cells but hypomethylation in maGS cells (Figs. 2B and 2D). Similarly, we found hemizygotic DNA methylation patterns at all CTCF binding sites in ES cells (Fig. 2B). However, all CTCF binding sites in maGS cells were hypermethylated, suggesting an androgenetic type of DNA methylation pattern at *H19* ICR (Fig. 2C). Consistent with this androgenetic DNA methylation pattern at *H19* ICR in maGS cells, real time quantitative RT-PCR showed that the relative abundance of *Igf2* transcript was ~2.5-fold higher, while that of *H19* was ~3-fold lower in maGS cells than in ES cells (Fig. 2E).

The results further showed that the hypermethylated state of CTCF did not differ between maGS cells cultured in the presence of GDNF alone or both GDNF and LIF, although CTCF3 showed a reduction in DNA methylation level (66.7%) when cultured in the presence of both GDNF and LIF (Fig. 2D). The relative abundance of *Igf2* was ~1.7-fold higher while that of *H19* was ~3-fold lower in maGS cells cultured in the presence of GDNF and LIF (Fig. 2E). The androgenetic DNA methylation

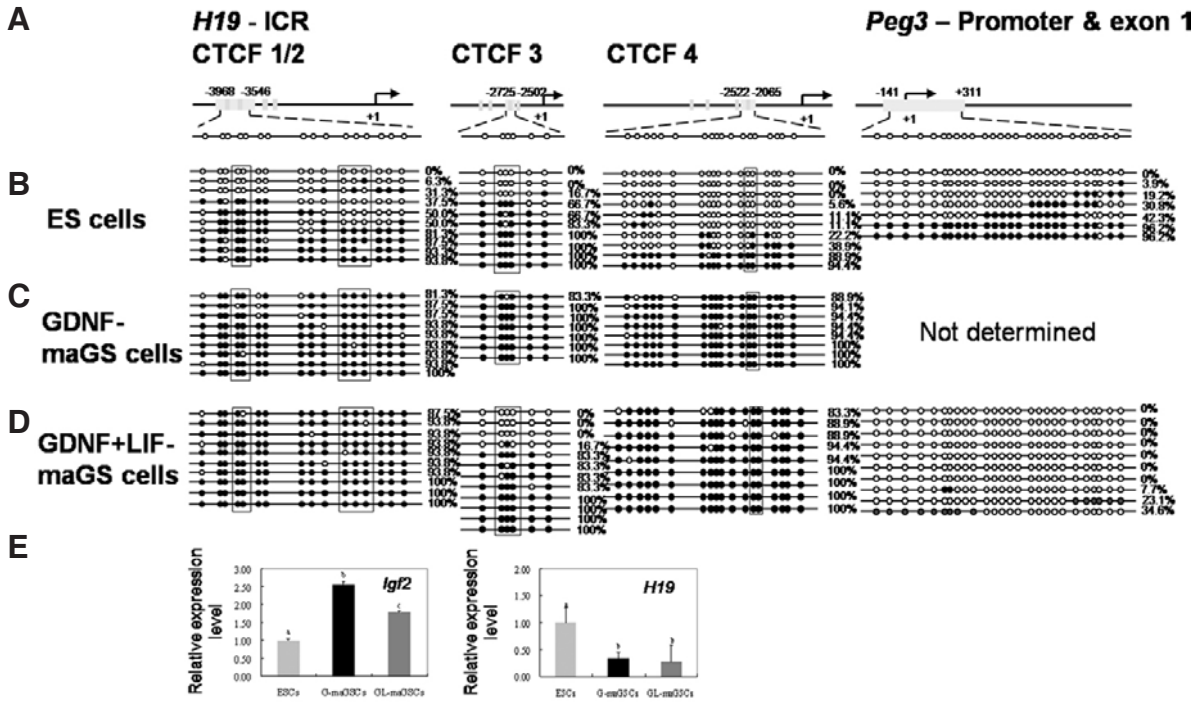

Fig. 2. DNA methylation at *H19* ICR in maGS cells. (A) Genomic structure of mouse *H19* ICR and *Peg3*. Arrows, transcription start site of the genes; boxes, DMRs of the genes examined; circles, individual CpG residues within the areas amplified. (B, C, and D) DNA methylation status in ES cells, maGS cells cultured in the presence of GDNF alone or GDNF plus LIF, respectively. Open and closed circles indicate the unmethylated and methylated CpG sites, respectively. Numbers in parentheses on the right of each strand represent the percentage of methylated CpG sites relative to total CpG sites examined in each strand. (E) Expression analysis of *Igf2* and *H19* genes. The different letters on the bars indicate significant differences ($p < 0.05$).

pattern at *H19* ICR did not change upon long term culture (upto at least 54 passages) (Fig. 3). These results clearly suggest that the androgenetic type of DNA methylation at *H19* ICR is epigenetically stable and does not change with *in vitro* culture conditions.

DNA methylation of *H19* ICR in differentiating maGS cells

To examine if the androgenetic type of genomic imprinting at *H19* ICR in maGS cells is maintained upon induction of differentiation, we induced *in vitro* differentiation of maGS cells by EB formation and subsequent RA treatment. As expected, RT-PCR analysis showed high expression of *Oct4* in Day 4 and Day 8 EBs, and downregulation upon RA treatment (Fig. 4A). Thus, we chose Day 4 EBs and RA-treated EBs for imprinting analysis at CTCF1/2. We also evaluated the DNA methylation of somatic tissue as a control. As expected, somatic tissue showed a hemizygotic DNA methylation pattern for *H19* ICR (Fig 4B). However, both Day 4 EBs and RA-treated EBs showed hypermethylation of *H19* ICR similar to that observed in undifferentiated maGS cells (Figs. 4C and 4D). Consistent with the DNA methylation data, the relative abundance of *Igf2* transcript was also higher while that of *H19* was lower in RA-treated maGS cells (Fig. 4E). These results, therefore, suggest that the hypermethylation and gene expression pattern of the imprinted *H19* gene is stably maintained in differentiating maGS cells.

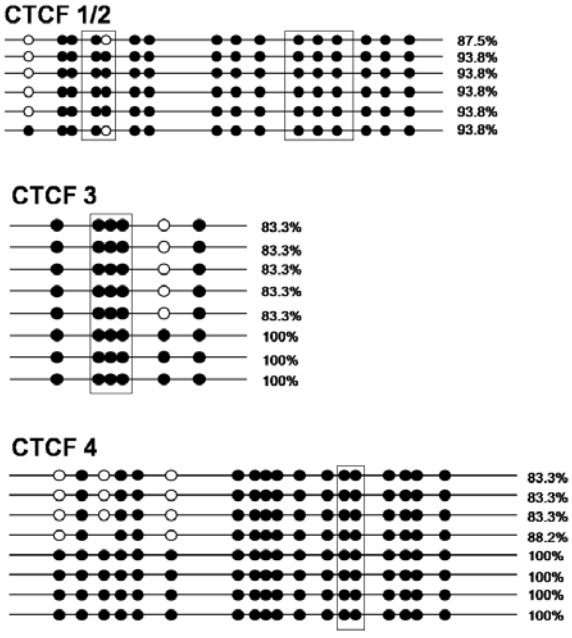

Fig. 3. DNA methylation at the *H19* ICR in long term cultured (passage 54) maGS cells cultured in the presence of both GDNF and LIF.

DISCUSSION

In this study we investigated the genomic imprinting at the *H19* ICR in mouse maGS cells by examining DNA methylation at four CTCF binding sites. First we examined the DNA methylation in maGS cells cultured in the presence of GDNF, which plays a crucial role in regulating their self-renewal (Kubota et al.,

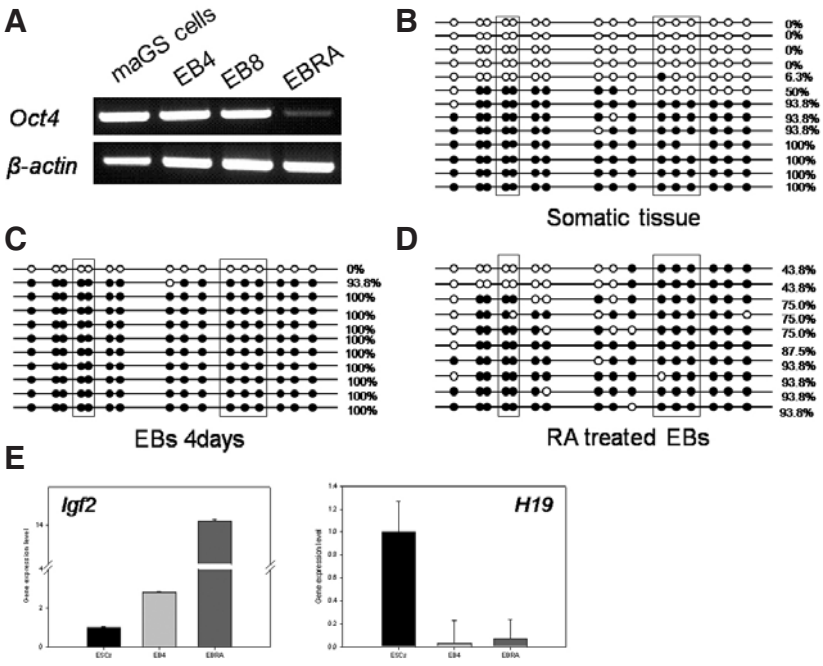

Fig. 4. DNA methylation at the *H19* ICR in differentiating maGS cells. (A) Expression of *Oct4* mRNA in differentiating maGS cells. The predicted sizes of PCR products are 313 and 321 bp for *Oct4* and *β-actin*, respectively. (B) Somatic tissue was used as a control. (C, D) DNA methylation status of the *H19* ICR in differentiating cells. (E) Expression of *Igf2* and *H19* in differentiating maGS cells. All groups showed statistically significant differences ($p < 0.05$).

2004). The results showed that maGS cells cultured in the presence of GDNF alone had complete DNA hypermethylation at *H19* ICR. These maGS cells also had ~2 fold higher expression of *Igf2* transcript and ~3 fold lower expression of *H19* transcript than ES cells. Given that the expression of the *Igf2* and *H19* genes is reciprocally regulated by DNA methylation at the *H19* ICR (Reik et al., 2000; Sasaki et al., 2000; Yang et al., 2003), the DNA methylation and gene expression pattern of *Igf2* and *H19* in our maGS cells suggest the androgenetic type of DNA methylation at their *H19* ICR.

We also observed that the androgenetic imprinting pattern of *H19* ICR in maGS cells was not altered in the presence of LIF (Fig. 2), which is a key regulator of ES cell self-renewal (Okita and Yamanaka, 2006). These data are consistent with those of Izadyar et al. who found an androgenetic imprinting pattern of several genes including *Meg3*, *Peg10*, *Igf2r*, and *Rasgrf1* in multipotent germ cell (mGC) lines (Izadyar et al., 2008). In contrast, however, Kanatsu-Shinohara et al. found that the DNA methylation pattern of *H19* ICR changed to a hypomethylated state when mGS cells were in the presence of LIF (Kanatsu-Shinohara et al., 2004). Interestingly, a later report from the same research group (Kanatsu-Shinohara et al., 2005) reported hypermethylation of *H19* ICR in mGS cells, which is similar to our results. Thus, it appears that imprinting at the *H19* ICR in maGS cells may be cell line-specific. This is further substantiated by several reports which showed differences in DNA methylation status of the *H19* ICR in different ES cell lines (Chang et al., 2009; Deng et al., 2007; Rugg-Gunn et al., 2005; Shovlin et al., 2008). If the latter is true, by extrapolation, detailed study on the epigenetic regulation and chromatin structure of maGS cells would seem to be essential before their clinical and research applications.

Preimplantation embryo, ES, and embryonic germ (EG) cells have been shown to undergo changes in imprinting pattern during *in vitro* culture (Dean et al., 1998; Doherty et al., 2000; Humpherys et al., 2001; Khosla et al., 2001). However, maGS cells showed stable imprinting status with androgenetic types of DNA methylation even after long-term culture (Fig. 3). This may be due to the postnatal origin of maGS cells as postnatal stem cells possess repair mechanisms to prevent germline transmission of epigenetic damage, while such mechanisms are not well established in cell types with embryonic origin (Kanatsu-Shinohara et al., 2005).

For application in regenerative medicine, maGS cells should acquire a hemizygotic DNA methylation pattern for imprinted genes in differentiating cells. Yamagata et al. (2007) found that the chromosomal centric and pericentric regions of both GS and mGS cells become hypermethylated upon induction of differenttiation by EB formation. Accordingly, we expected a hemizygotic DNA methylation pattern at the *H19* ICR in differentiating maGS cells in EB. However, contrary to our expectation, the DNA methylation of the *H19* ICR and the gene expression pattern of *H19* and *Igf2* in maGS cells were maintained in differentiating cells derived from maGS cells (Fig. 4). This difference may be due to differences in the regulatory mechanisms between imprinted genes and repetitive sequences (Lee et al., 2009). Although the mechanism responsible for the stable androgenetic pattern of maGS cells is yet to be elucidated, it is likely to be associated with their different factors such as genetic background, culture condition, cell line, and gene specificity.

In summary, our results show a conserved androgenetic imprinting pattern of the *H19* ICR in undifferentiated maGS cells cultured in the presence of GDNF alone or both GDNF and LIF. The androgenetic type of *H19* ICR imprinting was maintained even after induction of differentiation by EB formation and RA treatment. Taken together, these results suggest an epigenetic stability of *H19* ICR in our maGS cells during *in vitro* modification and differentiation. Since genomic imprinting is important for normal development and cell proliferation (Hernandez et al., 2003; Reik, 2007), our data also suggest the need for detailed study of epigenetic regulation and chromatin structure in maGS cells before their full potential can be utilized in regenerative medicine and developmental biotechnology.

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