Downregulation of *H19* Improves the Differentiation Potential of Mouse Parthenogenetic Embryonic Stem Cells

Neli P. Ragina,¹ Karianne Schlosser,² Jason G. Knott,^{3,4} Patricia K. Senagore,⁵ Pamela J. Swiatek,⁶ Eun Ah Chang,¹ Walid D. Fakhouri,⁷ Brian C. Schutte,^{7,8} Matti Kiupel,⁹ and Jose B. Cibelli^{1,5,10}

Parthenogenetic embryonic stem cells (P-ESCs) offer an alternative source of pluripotent cells, which hold great promise for autologous transplantation and regenerative medicine. P-ESCs have been successfully derived from blastocysts of several mammalian species. However, compared with biparental embryonic stem cells (B-ESCs), P-ESCs are limited in their ability to fully differentiate into all 3 germ layers. For example, it has been observed that there is a differentiation bias toward ectoderm derivatives at the expense of endoderm and mesoderm derivatives—muscle in particular—in chimeric embryos, teratomas, and embryoid bodies. In the present study we found that *H19* expression was highly upregulated in P-ESCs with more than 6-fold overexpression compared with B-ESCs. Thus, we hypothesized that manipulation of the *H19* gene in P-ESCs would alleviate their limitations and allow them to function like B-ESCs. To test this hypothesis we employed a small hairpin RNA approach to reduce the amount of *H19* transcripts in mouse P-ESCs. We found that downregulation of *H19* led to an increase of mesoderm-derived muscle and endoderm in P-ESCs teratomas similar to that observed in B-ESCs teratomas. This phenomenon coincided with upregulation of mesoderm-specific genes such as *Myf5*, *Myf6*, and *MyoD*. Moreover, *H19* downregulated P-ESCs differentiated into a higher percentage of beating cardiomyocytes compared with control P-ESCs. Collectively, these results suggest that P-ESCs are amenable to molecular modifications that bring them functionally closer to true ESCs.

Introduction

PARTHENOGENETIC DEVELOPMENT starts when an oocyte is artificially activated and develops in the absence of a sperm. Parthenogenetic embryos have been used to derive parthenogenetic embryonic stem cells (P-ESCs) in mouse [1,2], monkey [3,4], rabbit [5], and human [6,7]. Importantly, in mammals, parthenogenetic embryos fail to develop past the early to mid-stages of gestation [8–10].

P-ESCs provide an alternative source of stem cells for generation of autologous tissues and organs. This source of cells is mitochondria and major histocompatibility complex (MHC)-matched to the recipient [6,7,11–16]. Thus, the application of human P-ESCs in regenerative medicine is feasible and would bypass many of the ethical concerns associated with destroying fertilized embryos for biparental ESC (B-ESC) derivation.

P-ESCs, like B-ESCs derived from fertilized embryos, can differentiate—in vitro as embryoid bodies (EBs) and in vivo as teratomas—into cells from the 3 major germ layers: ecto-, meso-, and endoderm [17,18]. Although these cells have the potential to differentiate in a manner similar to their biparental counterparts, a differentiation bias toward ectoderm derivatives at the expense of endoderm and mesoderm derivatives—and muscle in particular—has been observed in chimeric embryos, teratomas, and EBs [10,19,20]. The major reason for failure of the PG embryos to develop to term, and the limited differentiation potential of P-ESCs in teratomas and EBs, has been attributed to defects in establishing correct expression patterns of the imprinted genes [17,19,21–24]. For example, the *H19* gene has been reported to be highly downregulated in PG embryos and P-ESCs [25,26]. *H19* is a maternally expressed (paternally imprinted) gene and codes for an untranslated mRNA. It has been reported that *H19* can

¹Cellular Reprogramming Laboratory, Department of Animal Science, Michigan State University, East Lansing, Michigan.

²Division of Hematology/Oncology, Children's Hospital, Boston, Massachusetts.

³Developmental Epigenetics Laboratory, Department of Animal Science, ⁴Department of Biochemistry and Molecular Biology, and ⁵Department of Physiology, Michigan State University, East Lansing, Michigan.

⁶Division of Biology and Medicine, Brown University, Providence, Rhode Island.

⁷Department of Microbiology and Molecular Genetics, ⁸Department of Pediatrics and Human Development, College of Human Medicine, and ⁹Department of Pathobiology and Diagnostic Investigation, Michigan State University, East Lansing, Michigan.

¹⁰Programa Andaluz de Terapia Celular y Medicina Regenerativa, Andalucía, Spain.

function as a long antisense RNA and as a micro RNA (mir675) [27,28]. In uniparental mouse fetuses the *H19* gene was found to be overexpressed up to 400 times compared with biparental concepti [21,29]. Moreover, deregulation of *H19* acts as a major barrier toward proper development of parthenogenetic embryos [21,22,30,31]. Kono and co-workers showed that a 13 kb deletion encompassing the *H19* coding region and the upstream differentially methylated region (DMR) allows parthenogenetic embryos to develop to term [22]. The efficiency of full-term development significantly increases when the *H19/Igf2* DMR together with the DMR found within a second imprinted gene cluster *Dlk1/Dio3* on chromosome 12 are deleted [23,32]. Most recently Hikichi et al. showed that the development of PG embryos can be extended following serial nuclear transfer (NT) and is associated with a decrease in *H19* expression [33]. Altogether, these results suggest that the correct expression of *H19* is important for normal embryonic development.

The results of the aforementioned studies led us to hypothesize that correct expression of *H19* is required for proper differentiation of mouse P-ESCs into the 3 germ layers. To test this we utilized a retroviral-mediated small hairpin RNA (shRNA) approach to silence *H19* gene expression in mouse P-ESCs. Further, we developed a method to quantify the abundance of the tissue derivatives of the 3 main germ layers in teratomas. We found that down-regulation of *H19* led to an increase of mesoderm-derived muscle and endoderm in P-ESCs teratomas similar to that observed in B-ESCs teratomas. This phenomenon coincides with upregulation of the mesoderm-specific genes such as *Myf5*, *Myf6*, and *MyoD*. These results suggest that P-ESCs are amenable to molecular modifications that bring them functionally closer to true ESCs.

Materials and Methods

Mouse ESCs derivation and culturing

Mouse P-ESCs and B-ESCs cells were derived from parthenogenetically activated and fertilized oocytes, respectively. P-ESCs and B-ESCs were derived from TgR (ROSA26)26Sor strain of mice (The Jackson Laboratory) expressing a constitutive β -galactosidase gene in all tissues and organs of the animal [34]. P-ESCs cells were produced by artificial activation of metaphase II eggs preventing the extrusion of second polar body [4,35]. Briefly, for P-ESCs derivation, matured MII oocytes were collected around 16 h post-human chorionic gonadotrophin injection. The matured oocytes were activated by 5 mM SrCl₂ in 2 mM Ca²⁺-free mCZB medium in the presence of 5 μ g/mL cytochalasin B for 6 h and cultured at 37°C in 5% CO₂ balance air up to 4 days in Ca²⁺ included mCZB medium [4]. Blastocysts were then treated with Tyrode's solution to remove the zona pellucida and placed in 96-multi well dishes coated with 90% confluent mytomycin C-inactivated mouse embryonic fibroblast (MEFs) until attachment. From the plated embryos that exhibited sufficient inner cell mass out-growth and colony formation, the colonies were manually cut, pipetted gently, and replated onto fresh mytomycin C-inactivated MEFs. The cells were sent for karyotyping to Cell Line Genetics. The P-ESCs line 1 and line 2 and 1 B-ESCs line used in this study have been karyotyped. Twenty cells at metaphase state have

been karyotyped from each of the 3 cell lines mentioned above. The karyotype analysis is as follows: The P-ESCs line1 (also called mPGESR26-7) was karyotyped at passage 5 (p5) and found to contain 40XX normal female karyotype with a single cell 39, X, -X, +3, -14, and a single cell 41, XX, +marker chromosome. The P-ESCs line 2 (also called mPGESR26-1) was karyotyped at p4 and found to contain a normal 40XX female karyotype and no abnormal cells with trisomy 12 and/or 17 were detected. The B-ESC (also called mBPESR26-9) was karyotyped at p7 and found to possess normal 40,XY karyotype with a single cell 39,XY, -12. The cells were grown with complete medium consisting of KO DMEM (Invitrogen), knockout serum, 10% (Invitrogen), L-glutamine 2 mM (Invitrogen), nonessential amino acids 1 $\times$ solution (Invitrogen), FGF2, 4 ng/mL, 2-mercaptoethanol (Sigma), penicillin-streptomycin, 1 $\times$ final volume, and 10³ U of leukemia inhibitory factor (LIF) (Millipore). Normal fertilized B-ESCs controls were derived from fertilized blastocysts at 3.5 dpc [36] and cultured under the same conditions as the P-ESCs described above.

RNA isolation and cDNA synthesis from ESCs

Total mRNA from mouse P-ESCs and B-ESCs was isolated using the RNeasy Isolation Kit (Qiagen) following the manufacturer's instructions. Total mRNA amount was measured using Nanodrop. One microgram of total RNA with OD260/280 > 2.1 was used for first-strand cDNA synthesis. cDNA was synthesized with Superscript II (Invitrogen) using anchored Oligo (dT₁₂₋₁₈) primers (Invitrogen) and following the manufacturer's instructions.

RNA isolation and cDNA synthesis from formalin-fixed, paraffin-embedded teratomas

RNA was isolated from ectoderm and endoderm derivatives and from whole teratoma sections following strict RNase and DNase free techniques. Ectoderm and endoderm derivatives were isolated using laser capture microdissection system (LCM) (Supplementary Fig. S1A, B; Supplementary Data are available online at www.liebertonline.com/scd). Total mRNA was extracted using High Pure RNA paraffin Kit (Roche) and after each RNA extraction procedure, the RNA was further purified by using NucleoSpin RNA II protocol for RNA purification (Machinery-Nagel). A validation of this method has been described by Hoshida et al. [37]. Total RNA (tRNA) was measured by RNA picochip on Agilent 2100 Bioanalyzer. For the LCM obtained samples, 1 ng of total RNA was converted to cDNA using SuperScript II first strand cDNA kit (Invitrogen) using random oligo primers. For the tRNA extracted from whole sections, 1 μ g was used for cDNA synthesis following the same protocol as mentioned above.

Quantitative real-time RT-PCR

The quantification of all gene transcripts was performed by reverse transcription of total RNA followed by absolute real-time quantitative RT-PCR using SYBR Green PCR Master Mix (Applied Biosystems). Absolute quantification using this method is described elsewhere [38,39]. Primers for absolute real-time PCR were designed using Primer Express program (Applied Biosystems) and derived from mouse

sequences found in GeneBank (Supplementary Table S1). A primer matrix was performed for each gene tested to determine optimal concentrations. Each reaction mixture consisted of 2 μ L of cDNA, optimum concentration of each forward and reverse primer, nuclease-free water, and 12.5 μ L of SYBR Green PCR Master Mix in a total reaction volume of 25 μ L (96-well plates). Reactions were performed in triplicate for each sample using an ABI Prism 7000 Sequence Detection System (Applied Biosystems). The thermal cycle consisted of 40 cycles of 95°C for 15 s and 60°C for 1 min. Standard curves for each gene and controls were constructed using tenfold serial dilution and run on sample plates as standards. For gene expression normalization (except for the expression analysis done on the LCM obtained endoderm and ectoderm derivatives from fixed tumors) expression levels of β -actin mRNA was used. For the normalization of gene expression in the LCM obtained endoderm and ectoderm derivatives from fixed tumors, *Gapdh* expression levels were used. Copies of β -actin and *Gapdh* RNA in each pool were determined using standard curves constructed from the plasmid PCR2.1. Partial cDNA sequences for β -actin, *Gapdh*, *H19*, *IGF2*, and other genes tested were amplified from mouse P-ESCs, cloned into pCR2.1 Topo vector (Invitrogen), and subjected to fluorescent dye primer sequencing to confirm identity. Resulting plasmids were used to construct standard curves. Representative R^2 for β -actin, *Gapdh*, *H19*, and the rest of the genes were estimated and only the one ≥ 0.98 used. For each measurement, threshold lines were adjusted to intersect amplification lines in the exponential portion of amplification curve [40].

Bisulfite sequencing analysis

Bisulfite sequencing of genomic DNA was performed using EZ DNA Methylation-Direct™ (ZymoResearch) and following the manufacturer's recommendations. The primers used for the bisulfite sequencing and the amplification conditions were as described elsewhere [41].

Quantification of endoderm, ectoderm, and mesoderm derivatives in teratomas

Teratomas from B-ESCs, P-ESCs, and P-ESCs infected with *H19*shRNA-expressing retroviral vector (*H19*shRNA cells) and P-ESCs infected with the control viral vector (Con.vector cells), were derived by subcutaneous injection into immune-deficient Nude mice, CD-1 (Charles River Laboratories). The teratomas were formalin-fixed, sectioned, and embedded in paraffin blocks; each was entirely submitted for histological analysis. Initially, 1–3 gross tissue samples per tumor were obtained for this study. The College of American Pathologists guidelines for gross sampling of solid tumors states that 1 sample for each centimeter of tumor or 3 samples, whichever is greater, should be used for tumor grading. Histological grading was performed utilizing the modified Thurlbeck-Scully histological grading system for solid ovarian teratomas proposed by Norris et al. [42] and successfully implemented by O'Connor and Norris [43] and Steeper and Mukai [44]. This system, which is widely employed clinically, estimates percentage of neuroepithelium in the total tumor mass by counting the number of microscopic 10 $\times$ low-power fields (LPFs) to dif-

ferentiate between tumors grades I, II, and III. We estimated that from our teratoma samples about 12–16 LPFs could be evaluated per full tissue section on 1 glass microscopic slide and that 3 tissue sections would yield ~36–48 LPFs for examination, and thus comply with the modified Thurlbeck-Scully histological grading system. However, to increase our quantification precision in an inherently heterogeneous tumor and adjust for variability in tumor size (8–10 mm in diameter), we obtained consecutive additional deep level sections 15, 30, and 45 μ m apart from each tumor sample paraffin block. Thus, the number of sections was increased to 10–18 per tumor, the number of LPFs evaluated was increased to 120–288 because the tumors were different size, and sections reflected whole tumor. The sections identity was hidden and the sections were also given to an independent histopathologist for evaluation.

Generation pMSCV-Hygro-H19shRNA and pMSCV-Hygro-EGFP-expressing viral vectors and infection of mouse P-ESCs

For generation of *H19* shRNA-expressing plasmid, a fragment containing *H1/T0* promoter and PolIII termination site was excised from pENTR/*H1/T0* plasmid (Invitrogen) and was sub-cloned into a unique *Xho*I site of pMSCV-Hygro viral vector (Clontech). Control viral vector was designed by inserting the β -actin-CMV-IE Enhancer-EGFP fragment derived from pCX-EGFP plasmid into a unique *Xho*I site. Both viruses were packaged and infected into B-ESCs and P-ESC1 according to the manufacturer's recommendation (Clontech). Cells infected with the shRNA-expressing and control viral vectors were subjected to 200 μ g/mL hygromycin selection for 10–14 days. Single transgenic clones were selected and expanding on fresh mitomycin C (Invitrogen)-treated MEFs.

Statistics

qRT-PCR experimental data and teratoma quantification data were analyzed by analysis of variance procedure using the mixed procedure of SAS. Differences of $P < 0.05$ were considered statistically significant.

Results

Generation of mouse P-ESCs and B-ESCs

Mouse P-ESC and B-ESC lines were derived from parthenogenetic and biparental blastocysts of Rosa 26 strain of mice (see Materials and Methods section). All lines (P-ESC1, P-ESC2, and 1 B-ESC) were found to possess a normal karyotype when cultured under normal growth conditions (see Materials and Methods section). Morphologically, the P-ESCs and B-ESCs displayed small cytoplasmic/nuclear ratio and grew as small compact colonies with prominent nucleoli (Supplementary Fig. S2A, B). These cells were extensively propagated in vitro and maintained their ESC morphology. The P-ESCs and B-ESCs stained positive for β -galactosidase (Supplementary Fig. S2C, D) and the pluripotency markers OCT4 and SSEA1 (Supplementary Fig. S3). Moreover, after injection into immunodeficient Nude mice-CD-1 the P-ESC lines were capable of forming teratomas (Supplementary Fig. S4).

Alteration of key imprinted genes in P-ESCs

In vitro culture of B-ESCs and P-ESCs can lead to alterations in DNA methylation and covalent modifications of histones such as histone acetylation and methylation [19,33,45–48]. Moreover, in P-ESCs the expression of several imprinted genes is altered due to absence of a paternal chromosomal complement [19,46,47]. We analyzed the expression profile of the paternally imprinted gene *H19* and the maternally imprinted gene *Igf2*. Both *H19* and *Igf2* share the same DMR as the main regulator of their expression [54]. We found that expression levels of the paternally imprinted gene (i.e., maternally expressed gene) *H19* was upregulated greater than 6-fold in P-ESCs compared with control B-ESCs ($P < 0.05$; Fig. 1A). Contrary to its maternal imprinted status, there were significantly higher levels of *Igf2* in P-ESCs compared with B-ESCs (Fig. 1A). These results demonstrate that the expression of key imprinted genes such as *H19* and *Igf2* is deregulated in P-ESCs.

Partial CpG methylation of the *H19/Igf2* DMR in P-ESCs

The methylation status of the *H19/Igf2* DMR has been viewed as the main regulator of *H19* and *Igf2* gene expression [49]. On the maternal allele the CpG dinucleotides are not methylated, which allows for a CTCF insulator protein to bind, preventing the enhancer downstream of *H19* to reach *Igf2* gene [49]. This leads to *H19* being expressed on the maternal allele and *Igf2* silenced. On the paternal allele the DMR is methylated, which inhibits binding of the CTCF insulator protein and allows for the expression of *Igf2* from the paternal allele while *H19* remains silenced. Since we were able to detect expression of *Igf2* in our P-ESCs, and the expression of *H19* and *Igf2* is coordinately regulated, we decided to analyze the methylation status of the *H19/IGF2* DMR. It has been previously reported that this region in CD-1 mice contains 16 critical CpG sites important for CTCF binding [41]. After bisulfite sequencing analysis we found that in the Rosa26 strain of mice there are 15 CpG sites, the fourth of which seems not to be methylated in normal fertilized B-ESCs (Fig. 1B, C). The DMR methylation status in both P-ESC lines (P-ESC1 and P-ESC2) revealed a partial methylation of the CpG sites analyzed with P-ESC1 and P-ESC2 having 7.3% and 19.3% methylation, respectively (Fig. 1B, C). These results demonstrate that the DMR of the *H19/Igf2* genes is not completely demethylated and that this may account for the elevated levels of *Igf2* expression in mouse P-ESCs.

Elevated levels of *H19* persist throughout P-ESCs differentiation in vivo

Next we wanted to determine the expression status of the *H19* gene throughout differentiation in vivo. We hypothesized that expression levels of *H19* remained higher in teratomas derived from P-ESCs compared with B-ESCs. To test this we injected P-ESCs and B-ESCs subcutaneously into immune-deficient Nude mice-CD1. Teratomas were recovered and stained for β -galactosidase expression, confirming that the teratomas originated from the injected cells (Supplementary Fig. S5A). The ectoderm, endoderm, and mesoderm derivatives were also stained for α -fetoprotein, β -

tubulin, and α -smooth muscle actin indicating the identity of the morphologically observed structures (Supplementary Fig. S5B). qRT-PCR data indicated that the expression levels of *H19* in ectoderm and endoderm derivatives and in whole teratoma sections from P-ESCs teratomas were significantly higher than those in controls ($P < 0.05$; Fig. 2A, B, respectively). Therefore, upon in vivo differentiation, the expression pattern of the imprinted gene *H19* remains faithful to the origin of the P-ESCs from which the teratomas were derived.

Teratomas derived from P-ESCs give rise to a lower proportion of mesoderm-derived muscle compared with teratomas derived from B-ESCs

Results of previous studies suggest that there is a differentiation bias of P-ESCs toward ectoderm derivatives at the expense of endoderm and mesoderm derivatives and muscle in chimeric embryos, teratomas, and EBs [19,20,50]. We hypothesized that correct expression of *H19* is required for normal germ layer formation in vivo. We quantified the percentage of ecto-, endo-, and mesoderm derivatives in teratomas derived from our P-ESCs and B-ESCs lines using our modified Thurlbeck-Scully grading system (see materials and Methods). We found that teratomas derived from P-ESCs consisted of predominantly ectoderm derivatives (70%) (Fig. 3A) compared with B-ESCs (50%). There was less endoderm (~20%) in P-ESC-derived teratomas compared with B-ESCs ones (~30%). Interestingly, we found that P-ESCs give rise to less mesoderm-derived muscle (~5%) compared with B-ESCs cell teratomas (~20%) (Fig. 3A).

We also performed qRT-PCR to analyze the expression pattern of genes specific to ectoderm (*Vimentin* and *Nestin*), endoderm [α -feto protein (*Afp*) and *Gata4*], and mesoderm (*Myf5*, *Myf6*, and *MyoD*) germ layers in these teratomas (Fig. 3B). In addition, we analyzed the expression pattern of the *Mier2* gene. *Mier2* is an early mesoderm-promoting gene that we identified as a target of mir675 by performing miRNA targets using TargetScan (Supplementary Table S2). qRT-PCR data indicated that there was no difference in expression of ectoderm and endoderm-specific transcript in P-ESC- and B-ESC-derived teratomas. In contrast, there was a significant decrease in the expression of *Myf5*, *Myf6*, and *MyoD* in P-ESCs teratomas compared with B-ESCs ones ($P < 0.05$; Fig. 3B). The expression of *Mier2* showed the same pattern (Fig. 3B) and coincided with high levels of *H19* gene expression ($P < 0.05$; see Fig. 2A, B). We also analyzed the expression pattern of *deltaNp63*. *DeltaNp63* is a product of the Tumor Protein 63 gene (TP63) and is involved in stem cell maintenance and commitment to ectoderm lineages [51,52]. Disruption of *deltaNp63* in mice results in failure of proper ectoderm development [53]. Overexpression of *deltaNp63* may induce the P-ESCs, when differentiated in vivo, to give rise to mostly ectodermal derivatives. We found the expression of *deltaNp63* to be 250-fold overexpressed in P-ESCs compared with B-ESCs control cells ($P < 0.05$; Supplementary Fig. S6A). These data suggest that upon differentiation in vivo P-ESCs exhibit limited developmental potential toward endoderm and mesoderm germ layer, muscle in particular. This phenotype coincides with overexpression of *H19* and downregulation of the muscle and mesoderm promoting genes—*Myf5*, *Myf6*, *MyoD*, and *Mier2*.

FIG. 1. Alterations in *H19/Igf2* expression and *DMR* methylation in P-ESCs. **(A)** Quantification of mRNA abundance of *H19* and *Igf2* imprinted gene expression in mouse B-ESCs and P-ESCs by qRT-PCR. **(B)** Methylation profile of *H19/Igf2* DMR in Rosa 26 strain of mice, on the bottom are indicated the CpG sites in nucleotides (nt), on the top is indicated the number of the CpG site. **(C)** Percent (%) methylation of the 15th CpG islands of the DMR in Rosa26 strain of mice. For all qRT-PCR experiments 3 biological replicates in triplicate were used in each run. $P < 0.05$ has been considered as statistical significant, unless stated otherwise. a and b indicate statistical significant difference ($P < 0.05$). B-ESCs, biparental embryonic stem cells; DMR, differentially methylated region; P-ESCs, parthenogenetic embryonic stem cells.

FIG. 2. Elevated levels of *H19* expression persist during P-ESCs differentiation in vivo. **(A)** Quantification of *H19* from laser capture microdissection-isolated primitive glandular structures (endoderm) and neural rosettes (ectoderm) by qRT-PCR. **(B)** Quantification of *H19* mRNA abundance in whole teratoma sections by qRT-PCR ($P < 0.05$). a and b indicate statistical significant difference ($P < 0.05$).

Downregulation of *H19* expression in P-ESCs promotes mesoderm differentiation in vivo

Proper *H19* regulation is central to normal development of parthenogenic embryos [22,54]. Moreover, *H19* may be important for proper muscle cell differentiation [55,56]. For example, chimeras between wild-type and P-ESCs result in restricted contribution of P-ESCs to muscle tissues in chimeric embryos [56]. Another study by Milligan et al. found a connection between *H19* and muscle differentiation in vitro [55]. In this study it was elegantly shown that *H19* ncRNA accumulates in the cytoplasm of C2C12 myoblastic cells during muscle differentiation and is protected from degradation by association with polysome complexes. The association with polysomes leads to stabilization of the *H19* ncRNA, which is important for proper muscle differentiation and is controlled by post-transcriptional, yet unknown, mechanism [55]. Because we found that teratomas derived from P-ESCs are composed of a very limited amount of muscle tissue (Fig. 3A), we hypothesized that deregulation of *H19* expression in P-ESCs is responsible for the decreased differentiation potential of these cells into muscle. To test this hypothesis we employed an *H19* shRNA-expressing retroviral vector system to chronically suppress *H19* gene expression in P-ESC1 cells, given that they had the highest expression of *H19* gene at the start of the experiments. P-ESC1 cells were infected with an *H19* shRNA retroviral vector or control vector (Fig. 4). qRT-PCR analysis revealed that *H19* was stably and efficiently downregulated in the *H19*shRNA cell lines compared with P-ESC1 cells infected with the control vector (Con.vector) cell lines ($P < 0.05$; Fig. 4). Moreover, low levels of *H19* transcripts were faithfully maintained in teratomas derived from *H19* shRNA P-ESC1 cells (Fig. 5A).

FIG. 3. Lack of mesoderm muscle derivatives and downregulation of mesoderm-specific transcripts in P-ESCs upon differentiation in vivo. **(A)** Quantification (%) of germ layer derivatives: ectoderm, endoderm, and mesoderm (the letters above indicate a statistical significant difference between the B-ESCs and P-ESCs in their ability to give rise to the specific germ layer lineage, $P < 0.05$) **(B)** qRT-PCR measuring the mRNA abundance of germ layer-specific transcripts in P-ESC- and B-ESC-derived teratomas ($P < 0.05$).

FIG. 4. Stable and efficient downregulation of *H19* mRNA levels in mouse P-ESCs. Quantification of *H19* mRNA abundance by qRT-PCR in *H19* downregulated P-ESCs (*H19*shRNA) and in P-ESCs infected with the control retroviral vector (Con.vector) ($P < 0.05$). a and b indicate statistical significant difference ($P < 0.05$). shRNA, small hairpin RNA.

To determine whether downregulation of *H19* expression improved the differentiation potential of P-ESCs in vivo, we used our modified Thurlbeck-Scully grading system to quantify the percentage of ecto-, endo-, and mesoderm derivatives in teratomas. We found that there was a significant increase in endoderm (15%) and mesoderm-muscle derivatives (11%) in *H19* shRNA-derived teratomas compared with Con.vector P-ESC1 cells ($P < 0.05$; Fig. 5B). This increase was similar to the percentage of endoderm and muscle derivatives in teratomas derived from B-ESCs ($P < 0.05$).

To further confirm our findings, we performed qRT-PCR analysis of mesoderm-specific transcripts—*MyoD*, *Myf6*, and *Mier2*, whose transcription levels were significantly altered in teratomas derived from wild-type P-ESC1 cells (see Fig. 3B). We found that upon downregulation of *H19*, the levels of *MyoD*, *Myf6*, and *Mier2* were significantly upregulated in *H19* shRNA-derived teratomas compared with control ones ($P < 0.05$; Fig. 5C). Moreover, we found that suppression of *H19* gene expression in P-ESC1 cells results in significant downregulation of the *deltaNp63*, which coincides with less propensity of these cells to differentiate to ectoderm lineage ($P < 0.05$; Supplementary Fig. S6B). Altogether, these results suggest that *H19* is required for proper mesoderm differentiation in vivo.

Induction of beating EBs from *H19* downregulated P-ESCs

To further compare and analyze the differential potential of B-ESCs, P-ESCs, Con.vector P-ESC1 cells, and *H19* shRNA P-ESC 1 lines, we employed an EB assay to examine cardiomyocyte formation. We adopted a protocol for derivation of beating cardiomyocytes by Yuasa et al. [57]. The incidence of beating EBs was recorded at days 8, 10, and 12 of EB formation. Beating EBs stained positive for cardiomyocyte-specific markers α -sarcomeric actin, α -sarcomeric myosin, and *Myf5* (Fig. 6A). Cardiomyocyte contractions were inhibited upon addition of 10 μ M Verapamil, which acts by specifically blocking L-type Ca^{2+} channels in smooth and cardiac muscle (Supplementary Movie S1). Our data indicated that P-ESCs have a lower potential to give rise to beating EBs compared with control B-ESCs. Remarkably,

FIG. 5. Downregulation of *H19* expression in P-ESCs promotes normal endoderm and mesoderm differentiation in vivo. (A) Quantification of *H19* by qRT-PCR in samples derived from whole teratoma sections ($P < 0.05$). (B) Quantification (%) of germ layer derivatives: ectoderm, endoderm, and mesoderm in teratomas derived from B-ESCs, *H19* downregulated P-ESCs (*H19*shRNA) and in P-ESCs infected with the control retroviral vector (Con.vector). (C) Quantification of mesoderm-specific transcripts: *MyoD*, *Myf6*, and *Mier2* by qRT-PCR in samples derived from whole teratoma sections ($P < 0.05$). a and b indicate statistical significant difference ($P < 0.05$).

upon downregulation of *H19*, this phenotype was completely reversed (Fig. 6B). The incidence of beating cardiomyocytes was similar between EBs derived from *H19*shRNA P-ESCs and B-ESCs ($P > 0.05$). In summary, downregulation of *H19* gene expression increases the differentiation potential of P-ESCs into cardiomyocytes and beating EBs in a manner similar to that of normal B-ESCs.

Discussion

Parthenogenetic embryonic stem cells: an alternative source of pluripotent stem cells in regenerative medicine

In light of an increased need for cell and tissue replacement therapy, a number of different strategies have been

FIG. 6. Derivation of beating EBs from B-ESCs, Control P-ESCs, and Control vector (Con.vector), and H19shRNA P-ESCs. **(A)** Immunostaining for cardiomyocyte-specific markers: α -sarcomeric actin (α SA), α -sarcomeric myosin (α SM), and Myf5. **(B)** Incidence of derivation of beating EBs in percentage (%) on y-axis vs. days in culture on the x-axis. These experiments were repeated 3 times and total of 100 B-ESCs, 176 Control P-ESCs, 140 H19shRNA P-ESCs, and 224 Control vector (Con.vector) P-ESC-derived EB were analyzed. EB, embryo body. Color images available online at www.liebertonline.com/scd

implemented to obtain an MHC-matched pluripotent source of stem cells. P-ESCs are unique in that they can potentially overcome the current limitations with the destruction of viable embryos, produced by fertilization or NT, during B-ESCs derivation. Also, the efficiency of derivation of P-ESCs is higher than the efficiency for derivation of B-ESCs from NT [14,15,58,59].

Moreover, P-ESCs with MHC antigens that are a complete match to the oocyte donor have been derived with high efficiency in mice, humans, and primates [6,14,15,59]. These cells are not only compatible with the oocyte donors' antigens, but also with the mother donor siblings.

The results reported in this study strongly suggest that H19 plays an important role for the P-ESCs development toward mesoderm germ layer, muscle in particular. We used teratomas as an experimental model for evaluating the differentiation potential of the P-ESCs instead of chimeras in an effort to avoid the potential effect of the host B-ESCs. During embryo development the P-ESCs in the chimeras are in constant cross-

talk with the B-ESCs of the host embryo. These cell-cell interactions could, in turn, influence the P-ESCs developmental potential in the chimeras. Thus, to evaluate the intrinsic differentiation potential of our mouse P-ESCs, we used teratoma formation and cardiomyocyte differentiation assays to analyze the sole ability of these cells to give rise to derivatives of all 3 germ layers, ectoderm, endoderm, and mesoderm, and to beating EBs. The results reported in this study demonstrate for the first time, to our knowledge, that downregulation of a single imprinted gene can increase the differentiation potential of P-ESCs toward mesoderm development and beating EBs and that this phenotype coincides with an up-regulation of the expression of mesoderm-specific genes—*MyoD*, *Myf6*, and *Mier2*. These findings are consistent with reported studies by Surani and Kono's groups revealing the significance of H19 in development and differentiation of parthenogenetic embryos and P-ESCs [30,31,56].

In an earlier study McKarney et al. used parthenogenetic ESCs in an EB differentiation assay to evaluate the

expression of *H19* and *Igf2* along with the muscle-specific genes *Myogenin*, *Myf5*, *Myf6*, and *MyoD1* [60]. The authors did not observe an association between expression of muscle-specific genes and the level of *H19* [60]. The apparent differences between our experimental findings and those by McKarney et al. may be due to the expression of *H19* and/or the differentiation assay used to assess muscle formation. Unlike McKarney et al.—where the levels of *H19* were not reported in the undifferentiated P-ESCs—we monitored the expression of *H19* before and after differentiation and were able to make an association with *H19* levels, the expression of muscle-specific genes, and muscle formation. We utilized an in vivo assay where we estimated the percentage of muscle contribution to whole tumor mass before and after down regulation of *H19*. We extended the findings of McKarney et al. by using a functional assay where the P-ESCs were induced to differentiate to beating cardiomyocytes after specific inhibition of BMP signaling; we demonstrated that P-ESCs with suppressed *H19* expression were capable of giving rise to a higher percentage of beating EBs [60]. Clearly, *H19* is not the only gene responsible for the observed phenotype. However, we have shown evidence indicating that *H19* participates in the cascade of molecular events leading to muscle and endoderm formation.

The exact mechanism through which *H19* exerts its role in the P-ESCs differentiation potential is currently not known. One suggestive mechanism could be that *H19* through its product mir675 regulates the expression of *MyoD*, *Myf6*, and *Mier2*. We already have identified *Mier2* as a potential target of mir675 based on miRNA TargetScan (Supplementary Table S2) [61]. In turn, mir675 can target *Mier2* either on transcriptional level by inducing *Mier2* mRNA degradation and/or on post-transcriptional level by repressing *Mier2* mRNA translation. Indeed, the potential of mir675 to induce translational repression has been recently shown in human cancer cell lines where RB protein was found to be a direct target of mir675 [62]. We are currently investigating the effect of mir675 on *Mier2* expression. This will help elucidate the mechanism that *H19* exerts on mesoderm layer development.

We also found the expression of *deltaNp63* to be 250-fold overexpressed in P-ESCs compared with B-ESC control cells. It is well known that long noncoding RNAs (ncRNA), such as *H19*, are involved in transcriptional regulation of multiple gene targets [63]. Therefore, one possible mechanism for overexpression of *deltaNp63* in P-ESCs may involve the *H19*ncRNA. *H19*ncRNA may act as transcriptional activator of *deltaNp63* expression, and therefore suppression of *H19* may result in decrease of the *deltaNp63* transcript. However, further evaluation of these proposed mechanisms is required to support these hypotheses.

In conclusion, these data demonstrate that elevated expression of *H19* in P-ESCs has a deleterious effect on the ability of these cells to give rise to mesoderm muscle derivatives. By suppressing *H19* expression P-ESC can indeed differentiate into muscle at similar rate when compared with B-ESC. Our data, together with others, support the rationale for using P-ESCs in the clinic. Studies in P-ESCs of primates, the closest species to human, have already shown the differentiation plasticity of these cells to give rise to all 3 germ layers in teratomas and to functional neurons when differentiated in vitro and transplanted into animal models [11–13]. Further studies of the role of the imprinted genes in

modulating the developmental potential of human P-ESCs are needed to assess the safety and efficacy of these cells to replace or regenerate the needed tissue in vivo.

Acknowledgments

We appreciate the support from the Michigan State University (MSU) Foundation, MSU's Vice President of Research, and the Michigan Agricultural Experiment Station (MAES), as well as the Naylor Family Foundation. This study was funded in part by U.S. National Institutes of Health grant DE13513 (B.C.S.). We would like to acknowledge the HistoPathology Laboratory at Michigan State University and especially Amy S. Porter and Kathy A. Joseph. We would like to acknowledge Dr. Hasan H. Out, Harvard University, Cambridge, MA, for his assistance with predicting mir675 target genes. We would like to acknowledge Dr. Juan Pedro Steibel, Animal Science Department, Michigan State University, for his assistance with the statistical data. We would like to acknowledge all past and present members of the Cellular Reprogramming Laboratory, Michigan State University. We would also like to acknowledge the "Van Andel Institute," Grand Rapids, MI, for their cooperation.

Author Disclosure Statement

No competing financial interests exist.

References

1. Kaufman MH, et al. (1983). Establishment of pluripotential cell lines from haploid mouse embryos. *J Embryol Exp Morphol* 73:249–261.
2. Robertson EJ, MJ Evans and MH Kaufman. (1983). X-chromosome instability in pluripotential stem cell lines derived from parthenogenetic embryos. *J Embryol Exp Morphol* 74:297–309.
3. Cibelli JB, et al. (2002). Parthenogenetic stem cells in non-human primates. *Science* 295:819.
4. Mitalipov SM, KD Nusser and DP Wolf. (2001). Parthenogenetic activation of rhesus monkey oocytes and reconstructed embryos. *Biol Reprod* 65:253–259.
5. Fang ZF, et al. (2006). Rabbit embryonic stem cell lines derived from fertilized, parthenogenetic or somatic cell nuclear transfer embryos. *Exp Cell Res* 312:3669–3682.
6. Dighe V, et al. (2008). Heterozygous embryonic stem cell lines derived from nonhuman primate parthenotes. *Stem Cells* 26:756–766.
7. Revazova ES, et al. (2007). Patient-specific stem cell lines derived from human parthenogenetic blastocysts. *Cloning Stem Cells* 9:432–449.
8. Kono T, et al. (1996). Epigenetic modifications during oocyte growth correlates with extended parthenogenetic development in the mouse. *Nat Genet* 13:91–94.
9. Barton SC, MAH Surani and ML Norris. (1984). Role of paternal and maternal genomes in mouse development. *Nature* 311:374–376.
10. Sturm KS, ML Flannery, RA Pedersen. (1994). Abnormal development of embryonic and extraembryonic cell lineages in parthenogenetic mouse embryos. *Dev Dyn* 201:11–28.
11. Ferrari D, et al. (2006). Transplanted dopamine neurons derived from primate ES cells preferentially innervate DARPP-32 striatal progenitors within the graft. *Eur J Neurosci* 24:1885–1896.

12. Sanchez-Pernaute R, et al. (2008). Parthenogenetic dopamine neurons from primate embryonic stem cells restore function in experimental Parkinson's disease. *Brain* 131:2127–2139.
13. Sanchez-Pernaute R, et al. (2005). Long-term survival of dopamine neurons derived from parthenogenetic primate embryonic stem cells (cyno-1) after transplantation. *Stem Cells* 23:914–922.
14. Kim K, et al. (2007). Histocompatible embryonic stem cells by parthenogenesis. *Science* 315:482–486.
15. Lengerke C, et al. (2007). Differentiation potential of histocompatible parthenogenetic embryonic stem cells. *Ann N Y Acad Sci* 1106:209–218.
16. Eckardt S, et al. (2007). Hematopoietic reconstitution with androgenetic and gynogenetic stem cells. *Genes Dev* 21:409–419.
17. Park JI, et al. (1998). Differentiative potential of a mouse parthenogenetic embryonic stem cell line revealed by embryoid body formation in vitro. *Jpn J Vet Res* 46:19–28.
18. Sturm KS, et al. (1997). Unrestricted lineage differentiation of parthenogenetic ES cells. *Dev Genes Evol* 206:377–388.
19. Allen N, et al. (1994). A functional analysis of imprinting in parthenogenetic embryonic stem cells. *Development* 120:1473–1482.
20. Onodera Y, et al. (2010). Differentiation diversity of mouse parthenogenetic embryonic stem cells in chimeric mice. *Theriogenology* 74:135–145.
21. Sotomaru Y, et al. (2002). Unregulated expression of the imprinted genes H19 and Igf2r in mouse uniparental fetuses. *J Biol Chem* 277:12474–12478.
22. Kono T, et al. (2004). Birth of parthenogenetic mice that can develop to adulthood. *Nature* 428:860–864.
23. Kawahara M, et al. (2007). High-frequency generation of viable mice from engineered bi-maternal embryos. *Nat Biotechnol* 25:1045–1050.
24. Ogawa H, et al. (2006). Disruption of parental-specific expression of imprinted genes in uniparental fetuses. *FEBS Lett* 580:5377–5384.
25. Liu J-H, et al. (2008). Diploid parthenogenetic embryos adopt a maternal-type methylation pattern on both sets of maternal chromosomes. *Genomics* 91:121–128.
26. Liu N, et al. (2010). Genome-wide gene expression profiling reveals aberrant MAPK and Wnt signaling pathways associated with early parthenogenesis. *J Mol Cell Biol* 2:333–344.
27. Schoenfelder S, et al. (2007). Non-coding transcripts in the H19 imprinting control region mediate gene silencing in transgenic *Drosophila*. *EMBO Rep* 8:1068–1073.
28. Berteaux N, et al. (2008). A novel H19 antisense RNA overexpressed in breast cancer contributes to paternal IGF2 expression. *Mol Cell Biol* 28:6731–6745.
29. Platonov ES, LI Penkov and DA New. (2002). [Effects of growth factors FGF2 and IGF2 on the development of parthenogenetic mouse embryos in utero and in vitro]. *Ontogenez* 33:60–67.
30. Kono T. (2006). Genomic imprinting is a barrier to parthenogenesis in mammals. *Cytogenet Genome Res* 113:31–35.
31. Kono T, et al. (2006). Paternal dual barrier by Ifg2-H19 and Dlk1-Gtl2 to parthenogenesis in mice. *Ernst Schering Res Found Workshop* 60:23–33.
32. Wu Q, et al. (2006). Regulated expression of two sets of paternally imprinted genes is necessary for mouse parthenogenetic development to term. *Reproduction* 131:481–488.
33. Hikichi T, et al. (2010). Functional full-term placentas formed from parthenogenetic embryos using serial nuclear transfer. *Development* 137:2841–2847.
34. Zambrowicz BP, et al. (1997). Disruption of overlapping transcripts in the ROSA beta geo 26 gene trap strain leads to widespread expression of beta-galactosidase in mouse embryos and hematopoietic cells. *Proc Natl Acad Sci U S A* 94:3789–3794.
35. Kono T, et al. (1996). A cell cycle-associated change in Ca²⁺ releasing activity leads to the generation of Ca²⁺ transients in mouse embryos during the first mitotic division. *J Cell Biol* 132:915–923.
36. Abbondanzo SJ, I Gadi and CL Stewart. (1993). Derivation of embryonic stem cell lines. *Methods Enzymol* 225:803–823.
37. Hoshida Y, et al. (2008). Gene expression in fixed tissues and outcome in hepatocellular carcinoma. *N Engl J Med* 359:1995–2004.
38. Li X and X Wang. (2000). Application of real-time polymerase chain reaction for the quantitation of interleukin-1[beta] mRNA upregulation in brain ischemic tolerance. *Brain Res Protoc* 5:211–217.
39. Whelan JA, NB Russell and MA Whelan. (2003). A method for the absolute quantification of cDNA using real-time PCR. *J Immunol Methods* 278:261–269.
40. Bettegowda A, et al. (2006). Quantitative analysis of messenger RNA abundance for ribosomal protein L-15, cyclophilin-A, phosphoglycerokinase, β -glucuronidase, glyceraldehyde 3-phosphate dehydrogenase, β -actin, and histone H2A during bovine oocyte maturation and early embryogenesis in vitro. *Mol Reprod Dev* 73:267–278.
41. Lucifero D, et al. (2002). Methylation dynamics of imprinted genes in mouse germ cells. *Genomics* 79:530–538.
42. Norris HJ, HJ Zirkin and WL Benson. (1976). Immature (malignant) teratoma of the ovary: a clinical and pathologic study of 58 cases. *Cancer* 37:2359–2372.
43. O'Connor DM and HJ Norris. (1994). The influence of grade on the outcome of stage I ovarian immature (malignant) teratomas and the reproducibility of grading. *Int J Gynecol Pathol* 13:283–289.
44. Steeper TA and K Mukai. (1984). Solid ovarian teratomas: an immunocytochemical study of thirteen cases with clinicopathologic correlation. *Pathol Annu* 19 Pt 1:81–92.
45. Dean W, et al. (1998). Altered imprinted gene methylation and expression in completely ES cell-derived mouse fetuses: association with aberrant phenotypes. *Development* 125:2273–2282.
46. Horii T, et al. (2008). Loss of genomic imprinting in mouse parthenogenetic embryonic stem cells. *Stem Cells* 26:79–88.
47. Jiang H, et al. (2007). Activation of paternally expressed imprinted genes in newly derived germline-competent mouse parthenogenetic embryonic stem cell lines. *Cell Res* 17:792–803.
48. Bibikova M, et al. (2006). Human embryonic stem cells have a unique epigenetic signature. *Genome Res* 16:1075–1083.
49. Sasaki H, K Ishihara and R Kato. (2000). Mechanisms of Igf2/H19 imprinting: DNA methylation, chromatin and long-distance gene regulation. *J Biochem* 127:711–715.
50. Jagerbauer EM, et al. (1992). Parthenogenetic stem cells in postnatal mouse chimeras. *Development* 116:95–102.
51. Honeycutt KA, MI Koster and DR Roop. (2004). Genes involved in stem cell fate decisions and commitment to differentiation play a role in skin disease. *J Invest Dermatol Symp Proc* 9:261–268.

52. Senoo M, et al. (2007). p63 is essential for the proliferative potential of stem cells in stratified epithelia. *Cell* 129:523–536.
53. Yang A, et al. (1999). p63 is essential for regenerative proliferation in limb, craniofacial and epithelial development. *Nature* 398:714–718.
54. Kono T, et al. (2002). Mouse parthenogenetic embryos with monoallelic H19 expression can develop to day 17.5 of gestation. *Dev Biol* 243:294–300.
55. Milligan L, et al. (2000). H19 gene expression is up-regulated exclusively by stabilization of the RNA during muscle cell differentiation. *Oncogene* 19:5810–5816.
56. Fundele RH, et al. (1990). Temporal and spatial selection against parthenogenetic cells during development of fetal chimeras. *Development* 108:203–211.
57. Yuasa S, et al. (2005). Transient inhibition of BMP signaling by Noggin induces cardiomyocyte differentiation of mouse embryonic stem cells. *Nat Biotechnol* 23:607–611.
58. Thuan NV, S Kishigami and T Wakayama. (2010). How to improve the success rate of mouse cloning technology. *J Reprod Dev* 56:20–30.
59. Hao J, et al. (2009). Human parthenogenetic embryonic stem cells: one potential resource for cell therapy. *Sci China C Life Sci* 52:599–602.
60. McKarney LA, ML Overall and M Dziadek. (1997). Myogenesis in cultures of uniparental mouse embryonic stem cells: differing patterns of expression of myogenic regulatory factors. *Int J Dev Biol* 41:485–490.
61. Friedman RC, et al. (2009). Most mammalian mRNAs are conserved targets of microRNAs. *Genome Res* 19:92–105.
62. Tsang WP, et al. (2010). Oncofetal H19-derived miR-675 regulates tumor suppressor RB in human colorectal cancer. *Carcinogenesis* 31:350–358.
63. Barrandon C, B Spiluttini and O Bensaude. (2008). Non-coding RNAs regulating the transcriptional machinery. *Biol Cell* 100:83–95.

Address correspondence to:

Prof. Jose B. Cibelli

Cellular Reprogramming Laboratory

Department of Animal Science

Michigan State University

East Lansing, MI 48824

E-mail: cibelli@msu.edu

Received for publication April 4, 2011

Accepted after revision July 26, 2011

Prepublished on Liebert Instant Online July 27, 2011