Primordial Germ Cell Specification from Embryonic Stem Cells

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Abstract

**Background:** Primordial germ cell (PGC) specification is the first crucial step in germ line development. However, owing to significant challenges regarding the in vivo system, such as the complex cellular environment and potential problems with embryo manipulation, it is desirable to generate embryonic stem (ES) cells that are capable of overcoming these aforementioned limitations in order to provide a potential in vitro model to recapitulate the developmental processes in vivo.

**Methodology and Principal Findings:** Here, we studied the detailed process of PGC specification from stella-GFP ES cells. We first observed the heterogeneous expression of stella in ES cells. However, neither Stella-positive ES cells nor Stella-negative ES cells shared a similar gene expression pattern with either PGCs or PGC precursors. Second, we derived PGCs from ES cells using two differentiation methods, namely the attachment culture technique and the embryoid body (EB) method. Compared with PGCs derived via the attachment culture technique, PGCs derived via the EB method that had undergone the sequential erasure of Peg3 followed by Ifg2r resulted in a cell line in which the expression dynamics of T, Fgf8 and Sox17, in addition to the expression of the epiblast markers, were more similar to the in vivo expression, thus demonstrating that the process of PGC derivation was more faithfully recapitulated using the EB method. Furthermore, we developed an in vitro model of PGC specification in a completely chemically defined medium (CDM) that indicated that BMP4 and Wnt3a promoted PGC derivation, whereas BMP8b and activinA had no observable effect on PGC derivation.

**Conclusions and Significance:** The in vitro model we have established can recapitulate the developmental processes in vivo and provides new insights into the mechanism of PGC specification.

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Introduction

The investigation of primordial germ cell (PGC) specification is the first essential step in the process of elucidating the mechanisms involved in the development of a germ cell lineage. However, significant difficulties exist with regard to research into the process of PGC specification in vivo. First, the complex in vivo environment of the cell has led to controversies over the mechanism of PGC development [1,2]. In addition, PGCs are difficult to study because they are limited in number, deeply embedded within the embryo, and are known to migrate during development [3–5], which mitigates the degree to which they can be effectively studied. Moreover, large-scale screens of potential inducers of the PGC specification process are difficult to implement. Hence, embryonic stem (ES) cells, which have overcome these aforementioned difficulties, provide promising candidates to recapitulate the developmental process in vitro and thus serve as a model to complement studies in vivo.

Previous studies have demonstrated that ES cells are capable of differentiating into germ cells in either the attachment culture technique or the EB method [6–12]. Nayernia et al. showed that live-birth mice could be obtained from spermatozoa that were completely derived in vitro from ES cells [10]. In addition, oocytes were derived from gcOct4-GFP ES cells in a study reported by Hubner et al. [6]. Although such reports have indicated the ability to successfully study germ cell development in vitro, the process of PGC specification is poorly understood. First, the parental imprints—which must be erased and reset during gametogenesis, reflecting the sex of the individual, and must be maintained in somatic cells after fertilization [13]—have been examined only in derived embryonic germ cells [8]. However, no derived PGCs have been tested for this property [6–12]. Second, the BMP...
pathway, which is confirmed to induce PGC specification of the proximal epiblast in vivo [14], has proven to function in an obscure fashion [7]. PGCs were rapidly derived from ES cells by co-aggregating the PGCs with BMP4 producing cells, whereas neither the direct addition of BMP4 to the medium nor the preparation of BMP4-producing feeder cells could obtain this effect. Moreover, the fundamental question of how PGCs are derived in vitro remains to be answered, although three current hypotheses exist. These hypotheses include the ideas that ES cells may already include PGCs, that ES cells may directly differentiate into PGCs, and, finally, that PGCs develop through an intermediate state, such as an epiblast-like stage [15].

Due to the fact that a significant number of markers are shared between PGCs and ES cells, the careful study of PGC specification in vitro is difficult. Pluripotent markers, such as Oct4 and SSEA1, between PGCs and ES cells, the careful study of PGC specification intermediate state, such as an epiblast-like stage [15]. May already include PGCs, that ES cells may directly differentiate into PGCs, and, finally, that PGCs develop through an intermediate state, such as an epiblast-like stage [15].

To investigate PGC derivation in vitro, we differentiated stella-GFP ES cells by implementing either the attachment culture technique or the EB method. Because stella is specifically expressed in PGCs during PGC specification in vivo [18], we wanted to determine whether the derived GFP-positive cells from a day 7 attachment culture (Att+) and a day 4 EB culture (EB+) contained PGCs (Fig. 2A). We first confirmed this after observing the strong expression of PGC markers, such as Oct4, Sox2 and Blimp1, in Att+ and EB+ by RT-PCR (Fig. 2B), and the protein expression of Oct4, Mvh, c-Kit and SSEA1 by Immunocytochemical staining (Fig. S1). In addition, Evas and Dppa3, which are repressed in PGCs [17], were downregulated in Att+ and EB+, indicating the presence of PGCs in these samples (Fig. 2B). Moreover, we analyzed whether Att+ and EB+ were able to procure the erasure of parental imprints in a manner similar to that shown in PGCs in vivo [13]. The imprinted genes we chose were Peg3 (5’ upstream region of the paternally expressed 3 gene), which is a paternally imprinted gene, and Igf2r (region 2 of the insulin-like growth factor 2 receptor gene), which is a maternally imprinted gene. Both imprints have been shown to exhibit early imprint erasure, with Peg3 initiating the erasure earlier than Igf2r in vivo [13]. This enabled us to determine the change in early imprinting in derived cells and to follow the time course of imprinting erasure by detecting their methylation status. Thus, we examined the DNA methylation state of differentially methylated regions (DMRs) of Peg3 and Igf2r in vivo [18]. Upon comparison of Att+ to EB+, Att+ showed partial erasure of Igf2r, whereas EB+ displayed partial erasure of Igf2r and Peg3 (Fig. 2C), demonstrating that both Att+ and EB+ contained PGCs. In addition, in EB+ the number of methylated CpG sites was significantly fewer in Peg3 than in Igf2r, suggesting that Peg3 was erased prior to Igf2r. The EB method, as opposed to the attachment culture technique, was able to recapitulate the erasure pattern of gene imprinting in the same sequential manner as that observed in vivo. Thus, PGCs were derived from ES cells by both the attachment culture technique and the EB method.

To explore the process of PGC derivation in vitro using these two methods, the gene expression dynamics of GFP-positive cells from days 4 to 8 in the attachment culture technique (Att+) and days 2 to 4 in the EB method (EB+) were analyzed by Quantitative RT-PCR (Q-PCR) (Fig. 3). First, the six genes that can distinguish ES cells and PGCs of different stages were analyzed. In both Att+ and EB+ cells, a decrease in the expression of Evas and an increase in the expression of stella further confirmed that PGCs were derived from ES cells using these two methods (Fig. 3A). The expression of Mhc was also shown to decrease in both Att+ and EB+ (Fig. 3A) in a manner that is similar to its expression pattern in vivo [17]. Although both Att+ and EB+ showed increased expression of T and Fgf8, the expression level of T peaked on day 2, whereas Fgf8 expression peaked on day 3 in the EB method (Fig. 3A). This suggested that its expression pattern resembled that of the in vivo process at roughly E7.25 when gene upregulation is followed by a subsequent downregulation and the change in the expression of T
is earlier than that observed for Fgf8 [17]. In comparison, the expression of both genes peaked on day 5 in the attachment culture technique.

Sox17 expression increased after day 3 in the EB method, mimicking the in vivo process [17], whereas it remained at low levels in the attachment culture technique (Fig. 3A). In addition, the expression pattern of some other PGC and germ cell markers, which are expressed in both PGCs and ES cells, such as Oct4, Sox2 and Blimp1, fluctuated during PGC specification (Fig. 3B). Notably, in Att+ and EB+, the expression of Blimp1 was downregulated followed by a upregulation, indicating the differentiation of ES cells followed by PGC specification (Fig. 3B).

The expression of the epiblast markers, Cerl, Fgf5, Gata6 and Left-b, increased significantly with the use of the EB method, whereas the expression of only Left-b increased clearly in the attachment culture

Table 1. Summary of gene expression in ES cells and different stages of PGCs.

|       | Eras | Hoxa1 | Nanog | Oct4 | Myc | c-Kit | Sox2 | Sox17 | Fgf8 | T      | stella |
|-------|------|-------|-------|------|-----|-------|------|-------|------|--------|--------|
| E6.75*| -    | -     | +     | +    | +   | +/-   | -    | +     | -    | +      | -      |
| E7.25 | -    | -     | +     | +    | -   | +     | +    | +     | +    | +      | +      |
| E7.75 | -    | -     | +     | +    | -   | +     | +    | +     | +/-  | +      | +      |
| E8.25 | -    | -     | +     | +    | -   | +     | +    | +     | -    | -      | -      |
| ES+   | +    | -     | +     | +    | +   | +     | +    | +     | +/-  | -      | -      |
| ES   | -    | -     | +     | +    | +   | +     | +    | +     | +/-  | -      | -      |

*E = embryonic day.

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Figure 2. The derivation of PGCs from ES cells. (A) Merged view of the phase contrast images and the fluorescence images of stella-GFP expression (green) in day 7 of the attachment culture technique (upper panel) and day 4 of EB (lower panel); bar = 50 μm. GFP-positive cells in the attachment culture technique at day 7 accounted for 15% of all cells and formed clusters, whereas the cells in the EB method at day 4 accounted for 6% and were dispersed in the EB. (B) Gene-expression analysis of GFP-positive cells (+) and GFP-negative cells (−) in day 7 of the attachment culture technique (Att) and day 4 of the EB (EB). All of the detected genes are highly expressed during PGC specification, with the exception of Eras and Dppa5, which are repressed in PGCs [17]. (C) DNA methylation patterns for the differentially methylated regions (DMRs) of Peg3 and for the DMRs of Igf2r. The oocytes on day 14 served as a control. The percentage of methylated CpG sites in the GFP-positive cells of the EScells, the day 7 attachment culture, and the day 4 EB (ES+, Att+, and EB+, respectively) were as indicated.

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Derivation of PGCs In Vitro

A

- **Eras**
  - Relative expression
  - ES 4A 5A 6A 7A 8A 2E 3E 4E

- **Stella**
  - Relative expression
  - ES 4A 5A 6A 7A 8A 2E 3E 4E

- **Myc**
  - Relative expression
  - ES 4A 5A 6A 7A 8A 2E 3E 4E

B

- **Oct4**
  - Relative expression
  - ES 4A 5A 6A 7A 8A 2E 3E 4E

- **Nanog**
  - Relative expression
  - ES 4A 5A 6A 7A 8A 2E 3E 4E

- **Sox2**
  - Relative expression
  - ES 4A 5A 6A 7A 8A 2E 3E 4E

- **Blimp1**
  - Relative expression
  - ES 4A 5A 6A 7A 8A 2E 3E 4E

C

- **Cerl**
  - Relative expression
  - ES 4A 5A 6A 7A 8A 2E 3E 4E

- **Fgf5**
  - Relative expression
  - ES 4A 5A 6A 7A 8A 2E 3E 4E

- **Gata6**
  - Relative expression
  - ES 4A 5A 6A 7A 8A 2E 3E 4E

- **Left-b**
  - Relative expression
  - ES 4A 5A 6A 7A 8A 2E 3E 4E
technique (Fig. 3C), suggesting that an intermediate stage, perhaps an epiblast stage, existed during the process of PGC specification in vitro when the EB method was implemented. Thus, the process of PGC derivation was more faithfully recapitulated in the EB method.

**In vitro model of PGC specification**

To determine the signals that promote the derivation of PGCs, we attempted to develop an in vitro model of PGC specification. First, because the two subpopulations of stella-GFP ES cells were interchangeable regarding stella expression (Figs. 1B and C), we did not sort either of them to establish the model. Second, because the process of PGC derivation was more faithfully recapitulated in the EB method than in the attachment culture technique, we employed the EB method. Third, because unknown components coupled with the inherent variability in the quality of the serum are known to hamper the accuracy of the results [15], we decided to develop a completely chemically defined medium (CDM). In this respect, several basic media (including DMEM, DMEM/F12, Ham’s F12, X-vivo, 1640, and IMDM) were tested, and we found that a combination of Ham’s F12 with IMDM supported the survival of cells most effectively. Because the relative percentage of GFP-positive cells was extremely low in EBs formed in the CDM (0.78%) (Fig. 4A), the CDM model provided a strategy to study the signals that trigger PGC derivation.

Considering that BMP4, a mesoderm inducer, plays an important role in PGC generation in vivo [14], we applied BMP4 to our differentiation model. Interestingly, the percentage of GFP-positive cells was higher in day 4 EBs supplemented with BMP4 than in EBs with CDM alone, 2.96% and 0.78%, respectively (Fig. 4A). To detect whether the GFP-positive cells in the CDM supplemented with BMP4 included PGCs, the expression of the PGC-related genes, the 6 main genes and also Nanog, Sox2 and Blimp1, was tested (Fig. 4B). As expected, in the presence of BMP4, the expression patterns of these genes were similar to that observed in BMP4 alone (Figs. 5A,B). Because BMP8b shows no additive effect with either BMP4 or BMP2 in vivo [20–22], suggesting different roles of BMP8b in PGC specification, BMP8b alone was added at day 2 to the EBs in the CDM. Both the overall percentage of GFP-positive cells (1.15%) and the gene expression pattern of these cells were similar to that in the CDM, indicating that BMP8b was not sufficient to promote the generation of PGCs (Figs. 5A and B). Furthermore, because the absence of BMP8b is known to result in an absence of PGC generation in vivo [20,21], we examined whether BMP4 induced the expression of BMP8b in the CDM. The expression of BMP8b was detected in the GFP-negative cells in day 4 EBs in the CDM with BMP4 but not in the GFP-positive cells, ES cells, or the day 4 EBs in the CDM (Fig. 5C). These results demonstrated that no synergic effect was detected by adding BMP8b or BMP2 with BMP4, while BMP4 can stimulate the expression of BMP8b in non-PGC cells in the CDM.

Because BMP4 is also a member of a group of proteins known as mesoderm inducers [23], Wnt3a, another factor promoting mesoderm lineage [24], was tested. As expected, Wnt3a has a positive effect on PGC derivation similar to that observed for BMP4 (Figs. 5A and B). Subsequently, activinA, which induces the generation of both the mesoderm and the endoderm [25], was shown to act in a similar fashion to BMP8b (Figs. 5A and B) with no induction of PGC specification. Thus, Wnt3a stimulated PGC specification in a manner similar to BMP4, whereas BMP8b and activinA failed to stimulate PGC specification.

**Discussion**

In this study, we successfully derived PGCs from mouse ES cells using stella as a selective marker. Interestingly, our data demonstrated that although ES cells were heterogeneous, they may not contain cells that are equivalent to PGC precursors or PGCs prior to E7.75. In addition, we found that the process of PGC differentiation from ES cells underwent complicated changes in the patterns of gene expression and methylation status, therefore mimicking the in vivo PGC generation process. Moreover, this process was more faithfully recapitulated when implementing the EB method than when using the attachment culture technique, suggesting a significant influence of the precise method on the ES cell differentiation process. Furthermore, to our knowledge, this is the first report demonstrated that PGCs were capable of being derived in a completely CDM, and our results showed that BMP4 and Wnt3a promoted PGC derivation from ES cells, whereas BMP8b and activinA were unable to promote PGC derivation.

Our data showed that ES cells were heterogeneous regarding their ability to express stella-GFP and that the two populations...
Derivation of PGCs In Vitro

A

FLH-2

CDM  BMP4+noggin  noggin

BMP4 50ng/ml  BMP4 100ng/ml  BMP4 20ng/ml

GFP

B

Relative expression

Eras  stella  Myc  Sox17  T  Fgf8  Nanog  Sox2  Blimp1

ES  CDM  noggin  BMP4+noggin  BMP4

C

BMP4+  BMP4-  RT-

Foa2  Mix1  Fk1  Mesp1  Evx1  Tbx6  Gapdh
were capable of generating one another. This phenomenon is similar to that reported for Rex1 in ES cells [26] and thus serves to confirm their reported heterogeneity. We also found that the two subpopulations of ES cells not only differed in the gene expression pattern (Figs. 1D and E), but also showed different DNA methylation patterns in imprinted genes (Figs. 2C and S3), consistent with previous studies indicating that ES cells are epigenetically unstable [27]. However, neither of the subpopulations possessed expression patterns similar to PGC precursors or PGCs prior to E7.75 (Fig. 1E, Table 1). Recently, Hayashi et al. also reported on the heterogeneity of ES cells regarding the expression of stella [28]. Through single cell Q-PCR analysis, they found that Stella-positive ES cells are closely related to the inner cell mass and not related to the epiblast or PGCs, whereas Stella-
negative ES cells are more similar to the epiblast cells [28]. Taken together, these results suggest that ES may not contain either PGCs or PGC precursors.

By following these detailed differentiation dynamics, we have found that, in comparison to the attachment culture technique, the process of PGC specification was more faithfully recapitulated using the EB method. Our results indicated that the imprinted genes were able to procure the erasure in stella-positive cells when the EB method (EB+) was used, whereas only Igf2r was able to procure the erasure when the attachment culture (Att+) technique was implemented (Fig. 2C). The sequential erasure of Peg3 followed by Igf2r was detected in EB+ but not in Att+ (Fig. 2C). In addition, our results showed that the gene expression dynamics of specific PGC markers, such as T, Fgf8 and Sox17 in the EB method, resembled the expression of in vivo markers more closely than the markers observed using the attachment culture technique (Fig. 3A). Finally, our results indicated that all of the levels of the detected epiblast genes were remarkably higher in the EB+, whereas only the level of Left-b was higher in Att+, indicating the presence of an epiblast stage in the PGC specification process when analyzed using the EB method. This observation was similar to that shown for the PGCs derived from proximal epiblasts in vivo [14]. Thus, the EB method presented a process of PGC specification that more closely mimicked the in vivo process.

Our data demonstrated that BMP4 was sufficient to promote PGC specification in the CDM. Initially, we discovered that BMP4 functioned as a soluble protein. In contrast to our findings, Toyooka et al. indicated that the direct addition of BMP4 to the medium, or simply co-culturing cells with BMP4-producing cells as feeders, does not necessarily stimulate PGC production [7]. A possible reason for the latter observation is that the serum in their culture medium includes factors that perform functions similar to BMP4 or interfere with BMP4, while our CDM culture eliminated this complicated effect by providing a far superior strategy to study the signals in PGC specification. In addition, our data suggest that the dose of BMP4 regulates the efficiency of PGC specification. Upon increasing the dose of BMP4 from 50 to 100 ng/ml, the percentage of GFP-positive cells increased (Fig. 4A) in a manner that was consistent with previous studies demonstrating that mice heterozygous for the BMP4-null generate fewer PGCs than wild-type mice [14]. This aforementioned result was also consistent with a previous study demonstrating that the PGC number is regulated by BMP signaling in an organ culture [29]. Moreover, it is possible that BMP4 triggers PGC derivation by providing a favorable microenvironment in our model. BMP4 induced the expression of BMP9b in GFP-negative cells in the CDM, which are the nearby cells of our derived PGCs in the EBs (Fig. 5C), consistent with previous findings that BMP9b expressed in the extraembryonic ectoderm is necessary for PGC specification from an epiblast precursor [20]. However, because BMP9b alone is not sufficient to promote PGC derivation (Figs. 5A and B), BMP4 must have an additional effect on this process. Interestingly, BMP4 can induce the formation of the extraembryonic mesoderm [30,31] where PGCs form a cluster and undergo further development. Therefore, BMP4 may establish a proper microenvironment for PGC specification and development. Thus, the functions performed by BMP4 in PGC specification were represented in our in vitro model.

Our results suggested that the germ cell fate was related to the fate of the mesoderm. Here, we found that a majority of the detected mesoderm markers were confirmed in the GFP-positive cells of the CDM in the presence of BMP4, which is consistent with an in vivo study demonstrating that mesoderm markers are expressed in some nascent PGCs while being repressed in PGCs [18]. However, Wnt3a and activinA, both of which induce mesoderm production in vitro [23–25], were shown to have different effects on PGC specification (Fig. 5A,B). Recently, a study by Gadue et al. revealed that upon direct addition of the two factors individually, Wnt3a is responsible for the induction of a population of cells with Foxa2<sup>wat+</sup>+, which are cells in the posterior part of the primitive streak, whereas activinA induces cells with Foxa2<sup>wat-</sup>+, which are cells of the anterior part of the primitive streak [32]. Thus, the promotion effect of Wnt3a and negative effect of activinA in PGC specification suggest that either PGCs originate from the posterior part of the primitive streak or the germ cell fate is imposed on these cells in the primitive streak. Together, it is possible that, during gastulation the precursors of putative PGCs and nascent mesoderm cells, expressing mesoderm markers, such as Extl, Tbx1 and Mesp1, were segregated from other somatic cells with the induction of BMP4 and/or Wnt3a. Soon after this segregation, the upregulation of Blimp1 in some of these precursors repressed the expression of mesoderm markers and finally the Blimp1-positive cells destined for a germ cell fate. (Fig. 6). Hence, our model provides a novel method to screen for factors or small molecules that may be involved in PGC specification.

**Materials and Methods**

**ES Cell Maintenance**

The *stella*-GFP ES cells (a gift from Prof. M. Azim Surani, also described as *stella*-GFP BAC ES cells in their publication [19]) were maintained in an ES medium that consisted of DMEM/F12 (Invitrogen) supplemented with 15% fetal calf serum (Hyclone), 1 mM glutamine (Invitrogen), 100 U/ml penicillin/streptomycin (Invitrogen), 0.1 mM b-mercaptoethanol (Sigma), and 1,000 U/ml LIF (Sigma) on mitomycin C-treated mouse embryonic fibroblasts.

**ES Cell Differentiation**

ES cells were differentiated in either a serum-containing medium (ES medium without LIF) or a serum-free medium, the components of which were described previously by Gadue et al. [32], containing 75% Iscove’s modified Dulbecco’s medium (Invitrogen), 25% Ham’s F12 medium (Invitrogen) with 0.5-fold of both N2 and B27 (without retinoic acid) (Invitrogen), 0.05% BSA (Sigma), 2 mM glutamax (Invitrogen), 0.5 mM ascorbic acid (Sigma) and 4.5×10<sup>-4</sup> M 1-thioglycerol (Sigma). The attachment culture technique and EB method were performed as previously described [6,32]. Briefly, after the ES cells were trypsinized, dissociated ES cells were plated on gelatin-treated plates for 40 min to remove the feeder cells. These cells were subsequently filtered through a 75-μm cell strainer. The ES cells were seeded at a density of 1 to 2.5×10<sup>4</sup> cells per cm<sup>2</sup> in a serum-containing medium in the attachment culture technique at a density of 0.5×10<sup>5</sup> cells/ml in serum-containing medium or at 1.5×10<sup>4</sup> cells/ml in a serum-free medium in the EB culture. After 48 h, the EBs were dissociated, filtered, and seeded as primary EBs to generate secondary EBs. The secondary EBs were cultured in either a serum-containing medium or a serum-free medium supplemented with BMP4, noggin, BMP2, BMP8b, Wnt3a or activinA, as indicated.

**Flow cytometry**

Cells were dissociated in trypsin-EDTA and resuspended in the medium used previously. The cells were then placed in a MoFlo High-Performance Cell Sorter (Dako Cytomation, Glostrup, Denmark) using Summit 4.0 Software (Dako Cytomation) for analysis and sorting.
Figure 6. A potential model of PGC specification. Some of the cells in the ICM (inner cell mass)/ES cells were Stella and/or Blimp1 positive cells. with the development of the embryo, the expression of Blimp1 was depressed in these cells with the help of signals from neighboring cells. Later, induced by WNT and/or BMP signaling, a subpopulation of these cells with the expression of mesoderm markers emerged, some of which acquired the expression of Blimp1. Then, Blimp1 functions to repress the expression of somatic markers that were normally down-regulated in PGCs [34]. Subsequently, cells with the expression of both Blimp1 and Stella were fated to germ-line development.

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Reverse Transcription Polymerase Chain Reaction (RT-PCR) analysis and Q-PCR analysis

The total RNA was extracted using an RNeasy Micro Kit (Qiagen). The RNA was then reverse-transcribed into cDNA using Sensiscript RT Kits (Qiagen). PCR was performed with Ex Taq polymerase (Takara) in a PCR buffer. The cycle conditions were as follows: 94°C for 5 min followed by 28–30 cycles of a 94°C denaturation period for 40 sec, a 56–60°C annealing period for 40 sec, and a 72°C elongation period for 40 sec, with a final elongation period at 72°C for 10 min. The primers used are listed in Table S1.

Q-PCR analysis was performed on an ABI PRISM 7300 Sequence Detection System using the SYBR Green PCR Master Mix (TOYOBO). The PCR consisted of 12.5 μl of SYBR Green PCR Master Mix, 1 μl of 10 mM forward and reverse primers, 10.5 μl water, and 1 μl template cDNA in a total volume of 25 μl. Cycling was performed using the default conditions of ABI 7300 SDS Software 1.3.1: 2 min at 95°C, followed by 30–35 cycles of 15 sec at 95°C and 1 min at 60°C. The relative expression of each gene was first normalized against Gapdh. The expression results for each gene were subsequently normalized by the expression with respect to the selected sample in each group, as indicated in each figure. The primers used for Q-PCR are shown in Table S2.

DNA Methylation Analysis

Genomic DNA was extracted using the DNeasy kit (Qiagen). The DNA was then treated with a sodium bisulfite solution, as described previously [33]. Differentially methylated regions (DMRs) of Igf2r or Peg3 were amplified by Ex-Taq DNA polymerase (TaKaRa) via nested PCR. The conditions for the first round of cycling were as follows: 94°C for 5 min followed by 35 cycles of a 94°C denaturation period for 30 sec, a 55°C annealing period for 30 sec, and a 72°C elongation period for 60 sec, with a final elongation period at 72°C for 10 min. The second round of PCR cycling was as follows: 94°C for 5 min followed by 35 cycles of a 94°C denaturation period for 30 sec, a 58°C annealing period for 30 sec, and a 72°C elongation period for 60 sec, with a final elongation period at 72°C for 10 min. The PCR primers are listed in Table S3. Amplified fragments were cloned into the plasmid vector pGEM-T Easy (Promega), and 10 samples in each experiment were sequenced using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA).

Immunocytochemical analysis

The cells were treated as described previously [12]. Briefly, the cells were fixed in 1% paraformaldehyde and blocked with 10% normal goat serum and 0.2% Triton X-100 for 60 min at room temperature. The cells were then incubated overnight at 4°C with the primary antibody to Oct4 (rabbit polyclonal IgG, Abcam), Mvh (rabbit polyclonal IgG, a kind gift from Dr. Toshiaki Noce), SSEA-1 (mouse monoclonal IgG, Chemicon) or c-kit (rabbit polyclonal IgG, Chemicon). Further incubation with anti-rabbit tetramethylrhodamine isothiocyanate TRITC-conjugated IgG or anti-mouse TRITC (both from Santa Cruz) was performed for 45 min at room temperature. The cells with only secondary antibody staining served as negative controls. The nuclei were detected by DAPI (Roche) staining. The images were obtained with an Olympus phase contrast microscope (IX-71; Olympus).

Statistical analysis

All data presented are representative of at least three independent experiments unless indicated otherwise. The results are expressed as the mean±s.e.m. of at least three independent experiments. Statistical analysis was performed using one-way ANOVA, followed by the SNQ test if necessary. The data collected from Quantitative RT-PCR were analyzed with the original data normalized with Gapdh. The statistical significance was inferred at * P<0.05 and ** P<0.01.

Supporting Information

Figure S1 Immunostaining of PGC markers, Oct4, Mvh, SSEA-1 and c-Kit in differentiated cells derived from ES cells by attachment culture (four upper panels) or the EB method (cells were dissociated from EBs before staining, four lower panels). Nuclei were visualized by Dapi. Bar = 50 μm. Found at: doi:10.1371/journal.pone.0004013.s001 (1.87 MB TIF)

Figure S2 Immunostaining of PGC markers Oct4, Mvh, SSEA-1 and c-Kit in sorted GFP positive cells in day 4 EB in CDM with BMP4. Nuclei were visualized by Dapi. Bar = 50 μm. Found at: doi:10.1371/journal.pone.0004013.s002 (0.24 MB TIF)

Figure S3 DNA methylation patterns of Peg3 differentially methylated regions (DMRs) and Igf2r DMRs. The percentage of methylated CpG sites in GFP-negative cells in ES cells, day 7 attachment culture and day 4 EB (ES→, Att→, EB→, respectively) were as indicated. Found at: doi:10.1371/journal.pone.0004013.s003 (0.49 MB TIF)

Table S1

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Table S2

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Author Contributions

Conceived and designed the experiments: HD. Performed the experiments: WW TQ XY HL DZ WY. Analyzed the data: WW TQ XY HL DZ WY. Wrote the paper: WW TQ XY HD.
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