Integrative Analysis of the Acquisition of Pluripotency in PGCs Reveals the Mutually Exclusive Roles of Blimp-1 and AKT Signaling

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SUMMARY

Primordial germ cells (PGCs) are lineage-restricted unipotent cells that can dedifferentiate into pluripotent embryonic germ cells (EGCs). Here we performed whole-transcriptome analysis during the conversion of PGCs into EGCs, a process by which cells acquire pluripotency. To examine the molecular mechanism underlying this conversion, we focused on Blimp-1 and Akt, which are involved in PGC specification and dedifferentiation, respectively. Blimp-1 overexpression in embryonic stem cells suppressed the expression of downstream targets of the pluripotency network. Conversely, Blimp-1 deletion in PGCs accelerated their dedifferentiation into pluripotent EGCs, illustrating that Blimp-1 is a pluripotency gatekeeper protein in PGCs. AKT signaling showed a synergistic effect with basic fibroblast growth factor plus 2i+A83 treatment on EGC formation. AKT played a major role in suppressing genes regulated by MBD3. From these results, we defined the distinct functions of Blimp-1 and Akt and provided mechanistic insights into the acquisition of pluripotency in PGCs.

INTRODUCTION

Germ cells are the only cells that continue to be reprogrammed throughout their lifetime in order to transfer genetic information to subsequent generations (Sasaki and Matsui, 2008). Germ cells have unique characteristics such as genome-wide epigenetic reprogramming and the potential to become pluripotent (Saitou and Yamaji, 2012). Primordial germ cells (PGCs) are specified at embryonic day (E) 7 in the epiblast. Blimp-1, Pdml1, and Tfr2c have critical roles in the specification of PGCs. A functional study of knockout embryos showed that BLIMP-1 represses somatic genes (Ohinata et al., 2005), whereas PRDM14 activates germ cell development genes (Yamaji et al., 2008). Tfr2c is thought to be a functional downstream target of BLIMP-1 (Weber et al., 2010). These three factors are sufficient to induce PGCs in vitro (Nakaki et al., 2013). Germ cell development, especially PGC specification, shares similarities with somatic cell reprogramming. Factors involved in germ cell development also function in the reprogramming of somatic cells (Nagamatsu et al., 2011). Moreover, PGCs have the potential to dedifferentiate into pluripotent embryonic germ cells (EGCs) without exogenous gene activation (Matsui et al., 1992). Although pluripotent stem cells and PGCs share many common features, PGCs are unipotent germ lineage-restricted cells and are distinct from pluripotent stem cells. When injected into blastocysts, PGCs do not give rise to any cell lineages (Leitch et al., 2014).

Originally, EGCs were established thorough screening of the culture conditions required for PGC proliferation (Matsui et al., 1992). Basic fibroblast growth factor (bFGF), leukemia inhibitory factor (LIF), and membrane-bound stem cell factor (mSCF) are present under these culture conditions. Because activation of phosphoinositide-3-kinase and AKT signaling negates the requirement for bFGF in such cultures, Akt is known to be involved in the induction of pluripotency in PGCs (Kimura et al., 2008). Recently, it was reported that a combination of signaling inhibitors enhances the efficiency of EGC formation (Leitch et al., 2010; Nagamatsu et al., 2012a). These inhibitors consist of 2i inhibitors (inhibitors of mitogen-activated protein kinase [MEK] and glycogen synthase kinase-β), which maintain pluripotency, and A83 (an inhibitor of transforming growth factor-β receptor), which enhances somatic cell reprogramming (Ying et al., 2008; Yuan et al., 2011). However, the mechanisms underlying the induction of pluripotency in PGCs remain largely elusive. While only germ cells can give rise to pluripotent cells following implantation, induced pluripotent stem cell (iPSC) technology enables pluripotent cells to be established from somatic cells (Takahashi and Yamanaka, 2006). Methyl-CpG binding domain protein 3 (Mbd3) was recently identified as a roadblock of somatic cell reprogramming (Rais et al., 2013). MBD3 is a component of the nucleosome remodeling deacetylase (NuRD) complex, which has histone
deacetylase activity and serves to close the chromatin structure (Hu and Wade, 2012).

In this study, we performed extensive gene expression analysis during the dedifferentiation of PGCs into EGCs and combined these data with the data for previously published target gene sets. Extensive analysis of transcription profiles revealed that BLIMP-1 suppressed pluripotency network genes and was therefore a pluripotency gatekeeper protein in PGCs. Moreover, there was a synergistic effect of AKT activation in the presence of bFGF and 2i+A83 on EGC formation. AKT activation suppressed genes regulated by MBD3. The targets of AKT and BLIMP-1 were different. Taken together, these results provide insight into the mechanism by which PGCs are converted into EGCs.

RESULTS

Transcriptome Analysis during the Conversion of PGCs into Pluripotent Stem Cells

To elucidate the molecular mechanisms by which PGCs become pluripotent cells, we performed whole-transcriptome analysis during the conversion of PGCs into EGCs. To obtain precise data, we used specific culture conditions for purified pluripotent candidate cells as previously reported (Figure 1A) (Nagamatsu and Suda, 2013). Heatmap and principal component analysis (PCA) indicated that the acquisition of pluripotency in PGCs is a stepwise process (Figures 1B and 1C). Table S1 shows the various genes that are gradually upregulated and downregulated during the conversion process. Gene Ontology (GO) analysis indicated that the transcription of genes involved in processes such as “transcription, DNA-dependent” and “regulation of transcription, DNA-dependent” was upregulated, while the transcription of genes involved in “protein-chromophore linkage” was downregulated (Table S1). To understand the global changes in gene expression during the acquisition of pluripotency, we compared the numbers of differentially expressed genes at each time point of the culture (Figure 1D). There were two waves observed by differential expression analysis. The first wave was from day 0 to day 1, and the second wave was from day 6 to day 10. When these differentially expressed genes were divided into upregulated or downregulated genes, most genes in the first wave were upregulated, and most genes in the second wave were downregulated (Figure 1E). Moreover, more than half of the genes upregulated in the first wave were downregulated in the second wave (Figure 1F; Table S1), indicating that they were oppositely regulated during these time periods. When we focused on these genes, GO analysis revealed the enrichment of terms associated with the “cell cycle,” “development,” and “metabolism” (Table S1).

Next, we analyzed the characteristic gene expression of PGCs. Gene set enrichment analysis (GSEA) showed that PGC markers were downregulated from day 2 of the culture (Figure 1G). This indicated that the PGC characteristics were lost in the early phase of pluripotency acquisition.

Analysis of the Core Transcription Network Involved in the Conversion of PGCs into Pluripotent Stem Cells

To analyze gene expression in pluripotent candidate cells (Nagamatsu et al., 2012a), we focused on downstream targets of core transcription network (Kim et al., 2008). Because PGCs express key transcription factors of pluripotent stem cells, such as Oct3/4, Sox2, and Nanog (Nagamatsu et al., 2013), it would be difficult to understand the differences between pluripotent stem cells and PGCs from the expression of these key factors themselves. In fact, with the exception of Dax1, the expression levels of the key pluripotency core network did not fluctuate during the acquisition of pluripotency (Figure S1). Therefore, we focused on the gene expression changes in the downstream targets of the core transcription network. We collected target gene sets from previous reports and applied these data to the GSEA of our time course gene expression profiles (Figure 2A) (Kim et al., 2008). Whereas the target genes of OCT3/4 tended to be repressed until EGCs formed, the targets of other core network factors were maintained or gradually upregulated during the conversion of PGCs into EGCs (Figure 2A). To evaluate the changes of this network, we calculated the numbers of genes commonly regulated by various network factors (Figure 2B). Some of these commonly regulated genes were involved in the process of pluripotency acquisition. Genes regulated by multiple factors of the core network are generally active in embryonic stem cells (ESCs), and these factors may be important in self-renewal and lineage commitment (Kim et al., 2008; Jeong et al., 2001). Therefore, we focused on genes that shared more than seven of nine regulatory factors in common (Table S2). Most of these genes that were activated in the early stage of the culture were also implicated in the late stage. Seven of ten genes involved in both the early (before day 3) and late (after day 6) culture stages encoded DNA-binding proteins involved in the control of transcription and/or chromatin remodeling (Klf9, Dido1, Rarg, Trim8, Mybl2, Zfp207, and Chd9). Such factors might function as a hub for the gene expression of other components of the core network.

The PGC-Specific Gene Blimp-1 Represses Downstream Targets of Pluripotency Network Genes

Microarray data of the conversion of PGCs into EGCs revealed an inverse correlation between certain PGC characteristics and pluripotent characteristics. We speculated that there is a mechanism that suppresses pluripotent
characteristics in PGCs. To investigate this possibility, we performed microarray analysis of ESCs, which expressed each germ gene. First, we selected six genes that are preferentially expressed in PGCs rather than in ESCs, namely, Prdm14, Vasa, Nanos2, Nanos3, Dnd, and Blimp-1 (Figure 3A) (Yamaji et al., 2008; Tanaka et al., 2000; Suzuki et al., 2007, 2008; Youngren et al., 2005; Ohinata et al., 2005). We generated ESCs that expressed PGC genes under the control of doxycycline (Masui et al., 2005). We could not obtain a stable clone of inducible Blimp-1-expressing

Figure 1. Microarray Analysis of the Acquisition of Pluripotency in PGCs
(A) Summary of the procedure used to culture cells and isolate samples. Gonads were surgically isolated from E11.5 embryos. After making a single cell suspension, PGCs were isolated based on SSEA-1 expression using a FACSAriaII cell sorter. Purified PGCs were seeded onto m220 feeder cells in ESC medium containing bFGF. One day after seeding, Z1+AB3 was added to the culture. At day 3 of culture, the cells were reseeded onto STO feeder cells. At day 7 of culture, bFGF and Z1+AB3 were removed by changing the medium. Colonies were picked, and EGC lines were established. At each time point, pluripotent candidate cells were sorted based on stage-specific embryonic antigen-1 (SSEA-1) expression for microarray analysis (Nagamatsu and Suda, 2013). Z1+AB3 was composed of inhibitors of MEK (PD325901), glycogen synthase kinase-3 (CHIR99021), and transforming growth factor-β type 1R (A83-01).
(B) Array heatmap of differentially expressed genes according to the culture period.
(C) Principle component analysis during the acquisition of pluripotency in PGCs.
(D) The number of differentially expressed genes at each adjacent time point.
(E) The numbers of upregulated and downregulated genes at each adjacent time point.
(F) The numbers of oppositely regulated genes at day 0 versus day 1 and day 6 versus day 10 during the acquisition of pluripotency. The bars indicate the total number of genes at each time point. Red indicates common genes at day 0 versus day 1 or day 6 versus day 10. The percentage of common genes is shown below each bar. DN, downregulated; UP, upregulated.
(G) GSEA of the PGC markers.
See also Table S1.
ESCs; therefore, Blimp-1-overexpressing cells were analyzed by transfection of ESCs with the Blimp-1-IRES-AcGFP vector and sorting of GFP-positive cells by fluorescence-activated cell sorting (FACS). Gene expression levels were confirmed by RT-PCR (Figure 3B). Microarray data were integrated with previously reported pluripotency network targets as shown in Figure 2A. Prdm14 is essential for germ cell specification and the maintenance of ESC pluripotency (Yamaji et al., 2013). When Prdm14 was overexpressed in ESCs, downstream targets of the pluripotency network were activated (Figure 3C). Similarly, expression of the other four germ cell-specific genes (Vasa, Nanos2, Nanos3, and Dnd) activated these downstream targets (Figure 3C). However, when Blimp-1 was overexpressed in ESCs, the downstream targets were clearly repressed (Figure 3C). These results suggest that pluripotency suppression is not achieved by the cooperation of multiple germ cell factors; rather, Blimp-1 appears to function as a pluripotency gatekeeper protein in PGCs.

**Identification of a Blimp-1 Module in ESCs**

To understand the mechanism by which BLIMP-1 suppresses downstream targets of the pluripotency network, we attempted to identify BLIMP-1 targets in ESCs. Recently, BLIMP-1-regulated genes were identified by chromatin immunoprecipitation (ChIP)-sequencing analysis of BLIMP-1 in PC19 pluripotent embryonic carcinoma cells (Magnúsdóttir et al., 2013). These BLIMP-1-regulated genes were projected to pluripotency controlling modules of CoORE, Polycomb (PRC), and MYC (Kim et al., 2010). In total, 4,808 unique genes were identified from BLIMP-1 ChIP-sequencing analysis and were included in the modules. We referred to these putative BLIMP-1 targets in ESCs as the BLIMP-1 modules (Figure 4A). Among the
CORE, PRC, and MYC modules, about 40% of genes belonged to the BLIMP-1 module (Figures 4B and 4C). Next, the BLIMP-1 module was applied to our microarray data from Blimp-1-overexpressing ESCs. When Blimp-1 was overexpressed in ESCs, the majority of BLIMP-1 module genes were downregulated, while about 10% of genes were upregulated (Figure 4D). Most of these upregulated genes were collectively classified as a PRC module (Figures 4E–4G). In ESCs, whereas CORE and MYC modules are activated, PRC modules are suppressed (Kim et al., 2010). Therefore, Blimp-1 likely suppressed pluripotency through a BLIMP-1 module.

**Blimp-1 Depletion Induces Pluripotency in PGCs**

To clarify the functional role of Blimp-1 in pluripotency acquisition in PGCs, we deleted Blimp-1. First, we purified PGCs from Blimp-1-floxed mouse embryos and then used recombinant CRE protein to delete Blimp-1 (Ohinata et al., 2005). CRE-treated PGCs were seeded onto STO or m220 feeder cells without bFGF. In the absence of bFGF,
PGCs could not convert to EGCs (Durcova-Hills et al., 2006). m220 feeder cells express mSCF, which is an important signal for EGC formation (Matsui et al., 1992). After culture, ESC-like colonies formed from Blimp-1-deleted PGCs grown on both types of feeder cells (Figures 5A–5C). Blimp-1 deletion was confirmed by genomic PCR (Figure S2 A). Additional treatment with 2i+A83, which enhances EGC generation (Nagamatsu et al., 2012a), also enhanced the efficiency with Blimp-1 deletion. On the other hand, in the presence of bFGF, the efficiency was decreased. These results indicated that there is no synergistic effect between Blimp-1 deletion and bFGF. The gene expression pattern in Blimp-1-deleted ESC-like colony cells indicated that the lack of Blimp-1 induced the upregulation of Klf4 and Eras (Figure 5D). When injected into nude mice, these established ESC-like cells formed teratomas containing three germ layers (Figures 5E and 5F), reminiscent of pluripotent stem cells. These results indicate that Blimp-1 deletion causes PGCs to becoming pluripotent. In the next experiment, rather than deleting Blimp-1, we induced the forced expression of Blimp-1 in PGCLCs during EGC formation. PGCLCs are in vitro-induced PGCs from ESCs (Hayashi et al., 2011). In this study, we established these cells using doxycycline inducible Blimp-1-expressing
ESCs (Nakaki et al., 2013). When Blimp-1 was induced at the early phase of the EGC formation, PGCLCs could not convert to EGCs (Figure S2B). To confirm the effect of BLIMP-1 modules during the acquisition of pluripotency in PGCs, we analyzed expression changes of BLIMP-1 modules in the time course transcriptome data. Whereas the BLIMP-1 modules of CORE and MYC were activated, the BLIMP-1 module of PRC was suppressed (Figure 5G). These changes correspond to the regulation in ESCs, suggesting that the BLIMP-1 modules are of functional relevance during EGC formation (Kim et al., 2010). Taken together, these results show that Blimp-1 acted as a gatekeeper of pluripotency in PGCs.

**AKT Has a Synergistic Effect with bFGF and 2i+A83 on the Acquisition of Pluripotency**

Next, we analyzed the key signaling required for the acquisition of pluripotency in PGCs. Whereas bFGF is an
essential signal for the generation of EGCs from PGCs (Ducovska-Hills et al., 2006), AKT activation has been reported to be sufficient to induce EGCs (Kimura et al., 2008). To analyze the effect of AKT activation, we used PGCs from Akt-mer Tg mice (Kimura et al., 2008). In these mice, AKT is activated by adding 4OHT. We found that AKT activation induced EGCs from PGCs in the absence of bFGF (Figure 6A). To analyze the AKT signal, we collected AKT target molecules in ESCs from a previous report (Yamano et al., 2010). When the expression profiles of AKT targets were applied to our time course gene expression data, the AKT targets were shown to be activated from day 1 of the culture.

Figure 6. Synergistic Effect of AKT Activation Together with bFGF and 2i+E83 Treatment
(A) E11.5 PGCs were purified based on SSEA-1 expression. PGCs were seeded onto STO feeder cells in ESC medium containing 4-hydroxytamoxifen (4OHT) with or without 2i+E83. The numbers of ESC-like colonies at day 10 are shown. Data represent the mean ± SD of independent experiments. Statistical significance was determined using Student’s t test (n = 3). *p = 0.047.
(B) GSEA of AKT targets for the microarray time course data. AKT targets were identified by comparison with the data of Yamano et al. (2010).
(C) Number of AKT targets in Blimp-1 modules. The bars indicate the total number of genes in each Blimp-1 module (Figure 4A). Red indicates AKT target genes.
(D) Number of AKT targets among all BLIMP-1 targets (Magnúsdóttir et al., 2013).
(E) Number of AKT targets in regulated modules of ESCs (Kim et al., 2010).
(F) The number of colonies at day 10 of the culture (top) and alkaline phosphatase (ALP) staining (bottom) are shown. Scale bar represents 7 mM (top). E11.5 PGCs were cultured in N2B27 medium containing bFGF, 4OHT, and 2i or 2i+E83 without feeder cells or serum replacement (KSR). The lower panels are higher magnification images of the upper panels. Scale bars represent 250 μM (top) and 100 μM (bottom). (Right) The number of colonies at day 8. Data represent the mean ± SD of independent experiments. Statistical significance was determined using Student’s t test (n = 4). *p = 0.088.
transduced with four reprogramming factors (Oct3/4 untreated mouse embryonic fibroblasts (MEFs) and those identified the target genes of MBD3 by ChIP sequencing in treatments, we found that AKT activation was not correlated with the changes in BLIMP-1 modules that accompanied EGC generation, namely, activation of the Core and Myc modules and suppression of the PRC module (Figure 7F). Taken together, these findings showed that AKT activation suppressed the MBD3 targets of OSKM-transduced MEFs in PGCs, which were different from the downstream targets of the BLIMP-1 and ESC modules. Finally, we analyzed whether AKT activation also downregulates MBD3 during somatic cell reprogramming. We found that, following the activation of AKT, MBD3 expression was suppressed in MEFs (Figures 7F and S3E). Furthermore, AKT activation at the early phase of somatic cell reprogramming enhanced the efficiency of this process (Figure 7H). Therefore, AKT activation enhanced pluripotency acquisition in MEFs via the suppression of MBD3-regulated genes.

**DISCUSSION**

We have identified that Blimp-1 functions as a gatekeeper of pluripotency in PGCs. Blimp-1 was originally identified as a transcriptional repressor in B cell maturation (Turner et al., 1994). During PGC specification, BLIMP-1 is important for the suppression of somatic cell programming (Kurimoto et al., 2008). The targets of BLIMP-1 may differ according to the situation. Transcription factors change the targets in a cell state-dependent manner. For example, Niwa et al. reported that the targets of SOX2 differed between ESCs and trophoblast stem cells because of different binding partners (Adachi et al., 2013). It is reported that BLIMP-1 binds the histone deacetylases TLE1 and EHMT2 in a context-dependent manner (Bikoff et al., 2009). It would be interesting to analyze the binding partners of BLIMP-1 during the specification of PGCs and induction of EGCs.

Whereas we found that Blimp-1 deletion induced pluripotency in PGCs, the effects of Blimp-1 overexpression in pluripotent cells appear to be more complicated. Forced expression of Blimp-1 in ESCs induces growth retardation (Nagamatsu et al., 2011). During PGC induction from ESCs, induction of an intermediate cell state, namely epiblast-like cells (EpilCs), is important (Hayashi et al., 2011). The combination of transcription factors Prdm14, Blimp-1, and Tfp2c is critical to induce PGCs from EpilCs (Nakaki et al., 2013). However, forced induction of these three factors cannot induce PGCs directly from ESCs. Furthermore, whereas Prdm14 alone can induce PGCs with low efficiency, Blimp-1 alone cannot even induce PGCs from EpilCs. Overexpression of Prdm14 in ESCs enhances pluripotency maintenance but does not induce PGC differentiation (Okashita et al., 2014). These facts reveal that there are important differences between ESCs and EpilCs in relation to PGC induction. One such
Figure 7. AKT Activation Suppresses Mbd3

(A–F) Summary of the procedure used to culture cells and isolate samples for microarray analysis (B–F). Gonads were surgically isolated from E11.5 AKT-mer embryos. After making a single cell suspension, PGCs were isolated based on SSEA-1 expression using a FACS AriaII cell sorter. Purified PGCs were seeded onto m220 feeder cells in ESC medium containing bFGF. One day after seeding, the indicated chemicals were added. On day 2, pluripotent candidate cells were sorted based on SSEA-1 expression for microarray analysis (Nagamatsu and Suda, 2013). 2i comprised inhibitors of MEK and glycogen synthase kinase-3β (GSK3-β). A83 indicates the transforming growth factor-β (TGF-β) type 1R inhibitor. (B) Array heatmap analysis for pluripotent candidate cells at day 2 of each treatment. Mbd3 and Mbd3 targets of MEFs in which four reprogramming factors (Oct3/4, Sox2, Klf4, and c-Myc [OSKM]) were transduced are shown. These target genes are from Rais et al. (2013). (C) The number of Mbd3 targets of OSKM-transduced MEFs in BLIMP-1 modules. Bars indicate the total number of genes in each BLIMP-1 module (Figure 4A). Red indicates Mbd3 target genes of OSKM-transduced MEFs. (D) Number of Mbd3 targets of OSKM-transduced MEFs among all BLIMP-1 targets (Magnúsdóttir et al., 2013). (E) Number of Mbd3 targets of OSKM-transduced MEFs in ESC module (Kim et al., 2010). Bars indicate the total number of genes in each regulated module of ESCs. Red indicates Mbd3 target genes of OSKM-transduced MEFs. (F) GSEA of BLIMP-1 targets for pluripotent candidate cells at day 2 of each treatment. BLIMP-1 modules were the gene sets identified in Figure 4.

(G) Western blotting of MBD3 after 4-hydroxytamoxifen (4OHT) treatment of MEFs isolated from WT and Akt-mer embryos. MEFs were treated with 4OHT (100 nM). At the indicated time points, MEFs were harvested and MBD3 expression was analyzed by western blot. (H) The number of 3F (Oct3/4, Sox2 and Klf4)-induced ESC-like colonies formed from Akt-mer MEFs, with or without 4OHT treatment at day 22. 4OHT was added at day 2 and allowed to react until day 6 after the 3F induction. Data represent the mean ± SD of independent experiments. Statistical significance was determined using Student’s t test (n = 6). **p < 0.01.

(I) Gene expression regulation of cellular dynamics in the process of the acquisition of pluripotency in PGCs. Events identified in both the current study and a previous study (Nagamatsu et al., 2012a) are shown. The efficient dedifferentiation of Mbd3 deficient PGCs was previously reported (Rais et al., 2013).

See also Figure S3.
difference is the existence of a suppressive mechanism in ESCs. Recently, it was reported that inactivation of Myc induces upregulation of germ cell marker genes in ESCs (Maeda et al., 2013). This indicates that PGC induction is suppressed in ESCs. However, in that report, Vasa was expressed much earlier than in vivo, and early markers, such as Blimp-1, were not activated efficiently. Therefore, it is unclear whether Myc inactivation induces functional differentiation. It would be intriguing to analyze differences between ESCs and EpiLCs in relation to the prerequisites for PGC differentiation.

To our surprise, overexpression of Dnd enhanced downstream targets of pluripotency (Figure 3). When Dnd is inactivated, the number of PGCs is decreased but basal PGCs give rise to teratomas in vivo (Youngren et al., 2005). Teratomas contain three germ layers generated by pluripotent cells. Therefore, deletion of Dnd leads to pluripotency in PGCs. However, in the present work, Dnd did not appear to mediate the suppression of pluripotency in ESCs. Dnd is not a target gene of either BLIMP-1 or AKT (Dataset S1), indicating that Dnd is regulated differently from BLIMP-1 or AKT. The mechanisms underlying pluripotency acquisition upon Dnd deletion, and the relationship between Dnd and Blimp-1 or Akt in PGCs is an important issue to be investigated.

In this study, we found a synergistic effect of AKT activation in the presence of bFGF and 2i+A83 on the acquisition of pluripotency. AKT activation suppressed MBD3-regulated genes in PGCs (Figure 7B). Furthermore, AKT activation downregulated MBD3 in MEFs (Figure 7G). Both AKT activation and MBD3 inactivation have been shown to prevent differentiation of ESCs in the absence of LIF (Watanabe et al., 2006; Kaji et al., 2006). It is conceivable that AKT downregulates MBD3 and thereby maintains pluripotency in the absence of LIF. Mbd3 is a roadblock of pluripotency (Rais et al., 2013). However, the regulation of Mbd3 expression is poorly understood. It would be interesting to analyze how AKT signaling downregulates Mbd3.

It has been reported that a histone deacetylase (HDAC) inhibitor had a positive effect on EGC formation. We therefore analyzed AKT activation and HDAC target genes. For this purpose, we determined the genes that were upregulated in Hdac-deficient ESCs (Jamaladdin et al., 2014). GSEA analysis was performed at day 2 of the culture with or without AKT activation (Figure S3F). Whereas bFGF alone activated HDAC target genes, AKT activation suppressed this gene set, indicating that AKT enhances the formation of EGCs in a manner distinct from that of the HDAC pathway.

In this study, we used target gene set analysis. We considered that this approach would allow us to understand the gene network and epigenetic state, which are difficult to analyze based on the individual gene expressions. Whereas both PGCs and EGCs express Oct3/4, the downstream targets of OCT3/4 were repressed from days 1 to 10 (Figures 2 and S1A). This indicated that the region downstream of OCT3/4 might differ between PGCs and EGCs. It was previously shown that Oct3/4 plays a critical role in PGC specification (Okamura et al., 2008). It would thus be of interest to investigate the molecular interaction between Oct3/4 and the factors that are important for PGC specification, such as Blimp-1 and Prdm14. We also applied this approach to epigenetic modifications. First, we analyzed the histone modification-associated active genes (H3K36me3, H3K79me2, and H3K4me3) (Marson et al., 2008; Mikkelsen et al., 2007). The targets of these modifications were also gradually upregulated (Figure S1B). On the other hand, another set of modification targets consisting of H3K4me3, H3K27me3, or both (i.e., the Bivalent domain targets) showed intriguing change (Figure S1C). Whereas the targets of the Bivalent domain were upregulated in EGCs compared with early culture periods, the H3K27me3 targets were repressed. The targets of H3K4me3 were maintained in an active state. With respect to the targets of DNA methylation, three different gene sets of 5hmC and two different gene sets of 5mC were collected from three different papers (Pastor et al., 2011; Borgel et al., 2010; Guibert et al., 2012). Except for one time point (EGCs of Figure S1E), both the 5hmC and 5mC targets were activated from the early phase of the culture (Figures S1D–S1F). PGCs contain DNA with a low level of methylation (Seki et al., 2005). Therefore, it is feasible that the targets of DNA methylation in ESCs are already hypomethylated in PGCs and so easy for the early activation.

To understand how pluripotency is achieved, we compared somatic cell reprogramming with the acquisition of pluripotency in PGCs. Although these two phenomena are different, the obtained pluripotent stem cells have similar characteristics. Analysis of how pluripotent stem cells are generated from different cell types might help to clarify the mechanism of reprogramming. Of note, PGCs already have many similarities with pluripotent stem cells. Both processes showed two distinct waves of gene expression changes, in the early and late phases (Polo et al., 2012). During somatic cell reprogramming, both waves showed similar patterns of upregulated and downregulated genes. In contrast, during the acquisition of pluripotency in PGCs, the first and second waves were mainly composed of upregulated and downregulated genes, respectively (Figure 1E). These oppositely regulated genes are associated with the GO terms cell cycle, development, and metabolism (Table S1). In the early phase of somatic cell reprogramming, genes associated with “gain of proliferation”, “transient activation of developmental regulators”, and “metabolic changes” are regulated (Polo et al., 2012). Genes that are oppositely regulated during
the conversion of PGCs to EGCs might have an important role in somatic cell reprogramming. Furthermore, in both cases, cells lost their original characteristics during the early phase, after which genes in the pluripotency-associated network were upregulated. Comparison of these two types of pluripotency induction would improve our understanding of the reprogramming mechanisms and characteristics of PGCs. Taken together with the results of our previous study (Nagamatsu et al., 2012a), these findings summarize the process of acquisition of pluripotency in PGCs (Figure 7I). This study presents precise information on gene expression profiles during the acquisition of pluripotency in PGCs. This information, in turn, provides mechanistic insights into the difference between PGCs and pluripotent stem cells and can be used to investigate the mechanism underlying somatic cell reprogramming. In future studies, it would be useful to compare distinct cell types and mechanisms to better understand the germ cell characteristics and reprogramming machinery.

**EXPERIMENTAL PROCEDURES**

**Isolation and Culture of PGCs**
PGCs were purified and cultured as described previously (Nagamatsu and Suda, 2013), and the detail is described in the Supplemental Information.

**Microarray Analysis**
Microarray analysis was performed using Whole Mouse Genome Oligo Microarray 44K (Agilent Technologies), and the detailed information is described in the Supplemental Information.

**Generation and Isolation of ESCs Expressing Germ Cell Genes**
Germ cell factors were introduced into EBRTcH3 ESCs as described in a previous report (Masui et al., 2005), and the detail is described in the Supplemental Information.

**Teratoma Formation and Alkaline Phosphatase Staining**
Teratoma formation and alkaline phosphatase staining were performed as described previously (Nagamatsu et al., 2012a), and the detail is described in the Supplemental Information.

**Antibodies**
The monoclonal antibodies used for western blotting were rabbit anti-MBD3 (ab157464; Abcam) and rabbit anti-β-ACTIN (A-2066; Sigma).

**iPSC Generation**
AKT-mer MEFs were reprogrammed using Oct3/4, Sox2, and Klf4 as described previously (Nagamatsu et al., 2012b). 4OHT was added at day 2 and allowed to react to day 6 after the three-factor induction. The numbers of morphologically ESC-like colonies were counted at day 22.

**ACCESSION NUMBERS**
The accession number of the microarray data in this study is GEO: GSE67616.

**SUPPLEMENTAL INFORMATION**
Supplemental Information includes Supplemental Experimental Procedures, three figures, two tables, and one dataset and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2015.05.007.

**AUTHOR CONTRIBUTIONS**
G.N., K.T., and T.S. designed the project. G.N. performed the experiments and generated the figures. S.S. performed bioinformatics analysis, and G.N. and T.S. wrote the manuscript.

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Stem Cell Reports
Supplemental Information

Integrative Analysis of the Acquisition of Pluripotency in PGCs Reveals the Mutually Exclusive Roles of Blimp-1 and AKT Signaling

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Supplemental experimental procedures

Mice

Blimp-1^floXfloX mice were the kind gift of Dr. A. Tarakhovsky (Rockefeller University) (Ohinata et al., 2005). Akt-mer transgenic mice were the kind gift of Drs. T. Kimura and T. Nakano (Osaka University) (Kimura et al., 2008). Akt-mer transgenic mice were crossed with ICR for more than 6 generations. ICR and Balb/c nude mice were purchased from Japan SLC (Shizuoka, Japan). Animal care was performed in accordance with the guidelines established by Keio University for animal use and recombinant DNA experiments.

Isolation and culture of PGCs

PGCs were purified by SSEA-1 staining and using a BD FACS AriaII cell sorter (BD Bioscience). Sorted PGCs were cultured on Sl/SI^+m220 or STO feeder cells in Knockout DMEM (Invitrogen) supplemented with 15% KSR (Invitrogen), 2 mM glutamine, 1 mM non-essential amino acids, 2-β-mercaptoethanol, LIF, and bFGF. The chemical compounds used in this study were PD325901 (1 μM), CHIR99021 (3 μM), and A83-01 (250 nM), which respectively inhibit MEK, GSK3-β, and TGF-βR. At day 7 (on m220 cells) or day 6 (on STO cells), the bFGF-containing medium was replaced with fresh medium lacking bFGF. In the case of seeding on Sl/SI^+m220 cells, cells were collected and reseeded onto STO feeder cells on day 3 of culture. For deletion of floxed Blimp-1 in PGCs, sorted PGCs were incubated with recombinant Cre (the kind gift of Dr. K. Hosokawa) at 37°C for 30 min. After washing, the cells were seeded. In the case of AKT activation, 4-hydroxytamoxifen (Sigma) was added at a concentration of 100 nM. For the feeder cell-free method, the culture conditions were identical to those for the ESC
Microarray analysis

Total RNA was extracted by RNeasy Plus Micro kit (Qiagen) according to manufacturers' manual. The quality of purified total RNA was verified by an Agilent 2100 bioanalyzer (Agilent technologies). Isolated total RNA was converted into cDNA. Cyanine-3 labeled cDNA was prepared using Genomic Enzymatic Labeling Kit (Agilent technologies) according to the manufacturer's instructions, followed by MinElute column purification. Labeled cDNA was hybridized to Whole Mouse Genome Oligo Microarray 44K (Agilent Technologies) for 17 hr at 65°C. The microarray was scanned by an Agilent Scanner, and the scan image data was analyzed with Feature Extraction software (Agilent Technologies). To perform gene-level interpretation, the whole probes were collapsed into genes with Entrez Gene ID by taking the maximum intensity among probe sets targeting the same gene. Differential gene expression analysis was performed using the fold-change method. Gene expression changes of >2-fold were considered significant. Heat maps were produced by color-coding with the Z scores of samples. To confirm the existence of genes that are gradually expressed during the acquisition of pluripotency, genes that showed expression changes at each time point were extracted. PCA and GO analysis were performed with these gradually expressed genes using the prcomp function of the R statistical package. To characterise the molecular backgrounds of the genes that were oppositely regulated during the two waves, an enrichment analysis of canonical pathways and GO biological processes on the generic GO slim subset was performed using the GO Term Finder (Boyle et al., 2004). Thereafter, the family-wise error rate (FWER) was estimated. The Gene Expression
GSEA

GSEA was performed to determine whether the expression of downstream targets increased or decreased. GSEA was performed using GSEA version 2.0.10 from the Broad Institute according to the pre-ranked list protocol using the gene sets defined in the Dataset S1. The pre-ranked lists were calculated based on the fold-change in expression. The downstream expression status was judged using the crude nominal p value in the GSEA results.

Generation and isolation of ESCs expressing germ cell genes

Germ cell factors were cloned into the pZhc exchange vector. The exchange vectors and pCAGGS-Cre were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Two days after transfection, ESCs were selected using Zeocin and resistant colonies were isolated. The established lines were treated with doxycycline, and GFP-positive cells were purified using a FACS AriaII cell sorter (BD Bioscience) for microarray analysis. In the case of Blimp-1 overexpression, EBRTcH3 ESCs were transfected with pEF1a·IRES·AcGFP (TaKaRa) or pEF1a-Blimp-1·IRES·AcGFP using Lipofectamine 2000 according to the manufacturer’s instructions. Two days after transfection, AcGFP-positive cells were purified using a FACS AriaII cell sorter (BD Bioscience) for microarray analysis.

Gene expression analysis
Transcript levels were determined using the 7500 Fast Real-Time PCR system (Applied Biosystems) with a Taqman assay mix (Applied Biosystems). The assay mix used for each gene was following: Prdm14 (Mm01237813_m1), Vasa (Mm00802445_m1), Nanos2 (Mm02525720_s1), Nanos3 (Mm00808138_m1), Dnd (Mm00849348_s1), Blimp-1 (Mm01187285_m1), Klf4 (Mm00516104_m1) and Eras (Mm03053919_s1).

**Genomic PCR analysis for Blimp-1 floxed and deletion alleles**

Genotypes of Blimp-1 flox and deletion were determined by genomic PCR using three primers: a forward primer for both floxed and deleted alleles (5'-GCCCATGACTCAAGACCA -3'), a reverse primer for the floxed allele (5'-TATGGTCTTCTCATGGTGGG -3'), and a reverse primer for the deleted allele (5'-GTTGTCTGAAGAGCAAGCTG -3'). Genomic PCR was performed under the following conditions: 35 cycles of 94°C for 30 s, 59°C for 30 s, and 72°C for 30 s.

**Teratoma formation**

Cells (1.0 × 10⁶) were suspended in Matrigel (BD) and injected into nude mice. After 3–4 weeks, the tumours were fixed with 4% paraformaldehyde (PFA) prepared in phosphate-buffered saline (PBS), embedded in paraffin, sectioned, and stained with hematoxylin and eosin.

**Alkaline phosphatase staining**

ESCs were fixed at 4°C for 10 min with 4% PFA prepared in PBS, washed twice with PBS, and stained at 37°C for 30 min using the SCIP/NBT liquid substrate system (B-1911: Sigma).
Induction of EGCs from PGCLCs

Doxycycline-inducible Blimp-1 ESCs that contained Blimp-1-Venus and Stella-CFP were the kind gift of Dr. M. Saitou (Kyoto University) (Nakaki et al., 2013). PGCLCs were induced from ESCs according to the method in a previous report (Hayashi et al., 2011). At day 6 of induction, PGCLCs were sorted by Blimp-1-Venus fluorescence. Sorted PGCLCs were cultured as EGCs induced from E11.5 PGCs. One day after EGC induction, doxycycline (1.5 μg/ml) was added to the culture at the indicated time periods. The numbers of ESC-like colonies were counted at day 10 after EGC induction.

Quantification of western blot analysis

Chemiluminescence intensity of western blot analysis was numerically converted by using FusionCapt Advance Solo 4 S (VILBER LOURMAT) software.
**Supplementary Figure Legends**

**Supplementary Figure S1. a.** Gene expression changes of core transcription factors in ESCs during the acquisition of pluripotency from PGCs. **b-f** Gene Set Enrichment Analysis (GSEA) of downstream targets of epigenetic modification. **b, c.** GSEA of downstream targets of histone modifications from Marson A et al.(Marson et al., 2008) (b) and Mikkelsen TS et al.(Mikkelsen et al., 2007) (c). **d-f.** GSEA of downstream targets of DNA methylation from Pastor WA et al.(Pastor et al., 2011) (d), Borgel J et al.(Borgel et al., 2010) (e) and Guibert S et al.(Guibert et al., 2012) (f). 5hmC represents 5-(Hydroxy)methylcytosine and 5meC represents 5-methylcytosine.

**Supplementary Figure S2. a.** Genomic PCR analysis of EGCs generated from PGCs that were treated with PBS and cultured in the presence of bFGF or treated with rCre and cultured in the absence of bFGF. The Blimp-1 floxed allele (250 bp) and Blimp-1 deletion allele (410 bp) are shown. **b.** Forced expression of Blimp-1 during EGC formation from PGCLCs. The number of ESC-like colonies observed from PGCLCs at 10 days after EGCs induction. Blimp-1 was induced by adding doxycycline at the indicated time points. Data represent the mean ± SD of independent experiments. Statistical significance was determined using Tukey’s multiple comparison test (n = 4 for no treatment, n = 3 for day1-3 on m220, n = 4 for day1-7).

**Supplementary Figure S3. a.** Array heat map analysis for pluripotent candidate cells at day 2 of each treatment. Mbd3 and MBD3 targets of mouse embryonic fibroblasts (MEFs) are shown. These target genes are from Rais et al.(Rais et al., 2013). **b.** The number of MBD3 targets of MEFs in BLIMP-1 modules. Bars indicate the total number
of genes in each BLIMP-1 module (Figure 4a). Red indicates MBD3 target genes of MEFs. c, Number of MBD3 targets of MEFs among all BLIMP-1 targets (Magnusdottir et al., 2013). d, Number of MBD3 targets of MEFs in ESC module (Kim et al., 2010). Bars indicate the total number of genes in each regulated module of ESCs. Red indicates MBD3 target genes of MEFs. e, Quantification of MBD3 expression of western blot shown in Figure 7g. The MBD3 expression level was normalized by the ACTIN expression level. Relative expression level was shown as Akt-mer/WT at each day of the culture. Dots represent the numbers of each experiment and the bar indicates the median. Statistical significance was determined using a one-sided t-test (independent experiments, n = 3). #p=0.0512. f, GSEA of upregulated genes in Hdac-deficient ES cells from Jamaladdin S et al. (Jamaladdin et al., 2014).
Supplementary Table S2. Genes shared by more than 7 of 9 regulatory factors

| Gene   | Day 1 | Day 2 | Day 3 | Day 6 | Day 10 | EGCs |
|--------|-------|-------|-------|-------|--------|------|
| Klf9   | *     | *     | *     | *     | *      | *    |
| Tmem131| *     | *     | *     | *     | *      | *    |
| Dido1  | *     | *     |       | *     | *      | *    |
| Rarg   | *     | *     | *     |       | *      | *    |
| Trim8  | *     | *     | *     | *     |        | *    |
| Mybl2  |       | *     | *     | *     |        | *    |
| Nid2   | *     |       | *     | *     |        | *    |
| Nolc1  | *     |       |       |        | *      | *    |
| Zfp704 |       |       |       | *     | *      | *    |
| Trim25 |       |       |       |        |        | *    |
| Chd9   |       |       |       |       |        | *    |
| Gpa33  |       |       |       |       |        | *    |
| Lrrc2  |       |       |       |       |        | *    |
| Tdgf1  |       |       |       |       |        | *    |
| Tgif1  |       |       |       |       |        | *    |
| Zscan10|       |       |       |       |        | *    |
| Gm13051|       |       |       | *     |        | *    |
| Tcea3  |       |       |       | *     |        | *    |
| Slec20a1|      |       |       |       | *      | *    |
| Asxl1  |       |       |       |       |        | *    |
| Cbx7   |       |       |       |       |        | *    |
| E2F4   |       |       |       |       |        | *    |
| Gm13154|       |       |       |       |        | *    |
| Gm13212|       |       |       |       |        | *    |
| Msh6   |       |       |       |       |        | *    |
| Pou5f1 |       |       |       |       |        | *    |
| Rest   |       |       |       |       |        | *    |
| Rlim   |       |       |       |       |        | *    |
| Rmnd5b |       |       |       |       |        | *    |
| Sfrp1  |       |       |       |       |        | *    |
| Sulf2  |       |       |       |       |        | *    |
| Zfp532 |       |       |       |       |        | *    |
| Zic2   |       |       |       |       |        | *    |
Zic5
2410137M14Rik
5730390G19Rik
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**Histone Modification**

- **a**
  - Relative expression (compared with E11.5 PGCs)
  - Day1, Day2, Day3, Day6, Day10, EGCs
  - Y-axis: Relative expression
  - X-axis: Days

- **b**
  - H3K36 me3
  - H3K79 me2
  - H3K4 me3
  - Day 1, Day 2, Day 3, Day 6, Day 10, EGCs

- **c**
  - H3K27 me3
  - H3K4 me3
  - Bivalent
  - Day 1, Day 2, Day 3, Day 6, Day 10, EGCs

**DNA Methylation**

- **d**
  - 5hmC (CMS)
  - 5hmC (GLIB)
  - 5mC (MEDIP)
  - Day 1, Day 2, Day 3, Day 6, Day 10, EGCs

- **e**
  - 5mC (Day 1)
  - Day 1, Day 2, Day 3, Day 6, Day 10, EGCs

- **f**
  - 5mC (EGCs)
  - Day 1, Day 2, Day 3, Day 6, Day 10, EGCs

Enrichment of Target Genes

- **d**
  - 0.25-0.00
  - 0.50-0.25
  - 0.75-0.50
  - 1.00-0.75
  - 0.75-0.50
  - 0.50-0.25
  - 0.25-0.00
  - <0.01

**Legend**

- Myc
- Dax1
- Klf4
- Nac1
- Nanog
- Oct3/4
- Rex1
- Sox2
- Tcf3
- Sux12

**Enrichment Levels**

- * <0.01
Figure S2: DNA mobility image showing deleted and floxed bands in lanes PBS and rCRE.

Bar graph showing the number of colonies per 400 PGCLCs under different conditions:
- none
- day1-3
- day1-7
- Dox
Supplementary Figure S3

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MBD3 expression in Akt-mer MEF

Relative expression of Mbd3

Day0  Day2  Day4
4OHT

Up in Hdac KO

Enrichment of Target Genes

bFGF
bFGF + 4OHT

BLIMP-1 modules
ESC modules

MBD3 Targets (MEF)

CORE  PRC  MYC

Number of genes

0  50  100  150  200  250  300

MBD3 Targets (MEF)

All BLIMP-1 Targets

Number of genes

0  100  200  300  400  500

MBD3 Targets (MEF)

CORE  PRC  MYC

Number of genes

0  100  200  300  400  500  600

No chemical
MEKi
GSKi
A83
2i+A83
4OHT
2i+A83+4OHT

MBD3 expression in Akt-mer MEF

Relative expression of Mbd3

Day0  Day2  Day4
4OHT

Enrichment of Target Genes

* <0.01