Effect of ectopic expression of homeoprotein EGAM1C on the cell morphology, growth, and differentiation in a mouse embryonic stem cell line, MG1.19 cells

Momoe Iha¹, Masafumi Watanabe¹, Yumi Kihara¹, Saiko Sugawara¹, Koichi Saito¹, Miki Soma¹, Sho Sato¹, Yuki Mori¹, Kano Kasuga², Ikuo Kojima², Ryo Sasamura³, Jun Murata³ and Masayuki Kobayashi¹

¹Laboratory for Advanced Animal Cell Technology, ²Laboratory for Biological Chemistry and ³Laboratory for Molecular Biology, Graduate School of Bioresource Sciences, Akita Prefectural University, Akita 010-0195, Japan

Correspondence should be addressed to M Kobayashi; Email: makoba@akita-pu.ac.jp

M Iha and M Watanabe contributed equally to this work

Abstract

The homeoprotein EGAM1C was identified in preimplantation mouse embryos and embryonic stem (ES) cells. To explore the impact of EGAM1C on the hallmarks of mouse ES cells, MG1.19 cells stably expressing EGAM1C at levels similar to those in blastocysts were established using an episomal expression system. In the presence of leukemia inhibitory factor (+LIF), control transfectants with an empty vector formed flattened cell colonies, while *Egam1c* transfectants formed compacted colonies with increased E-CADHERIN expression. In *Egam1c* transfectants, the cellular contents of POU5F1 (OCT4), SOX2, TBX3, and NANOG increased. Cell growth was accelerated in an undifferentiated state sustained by LIF and in the course of differentiation. During clonal proliferation, EGAM1C stabilized the undifferentiated state. In adherent culture conditions, EGAM1C partly inhibited the progression of differentiation at least within a 4-day culture period in the presence of retinoic acid by preventing the downregulation of LIF signaling with a robust increase in TBX3 expression. Conversely, EGAM1C enhanced the expression of lineage marker genes *Fgf5* (epiblast), *T* (mesoderm), *Gata6* (primitive endoderm), and *Cdx2* (trophectoderm) in –LIF conditions. In embryoid bodies expressing EGAM1C, the expression of marker genes for extraembryonic cell lineages, including *Tpbpa* (spongiotrophoblast) and *Plat* (parietal endoderm), increased. These results demonstrated that the ectopic expression of EGAM1C is capable of affecting the stabilization of an undifferentiated state and the progression of differentiation in MG1.19 ES cells, in addition to affecting cellular morphology and growth.

Reproduction (2012) 143 477–489

Introduction

In preimplantation mouse embryos, the first developmental decisions occur during the morula stage. The outer cells of the morulae, which have enhanced cell–cell contacts mediated by CDH1 (E-CADHERIN), give rise to the trophoctoderm (TE), whereas the inner cells generate the pluripotent inner cell mass (ICM) in blastocysts (Pedersen *et al.* 1986, Fleming 1987, Riethmacher *et al.* 1995). In late blastocysts, primitive endoderm (PrE), the precursor cells of the extraembryonic endoderm lineage, forms on the blastocoel-exposed surface of the ICM. Subsequently, PrE differentiates into the extraembryonic endoderm lineage, including visceral endoderm (VE) and parietal endoderm (PE). Cells constituting the ICM are capable of differentiating into all fetal and adult cell lineages and thus are the founders of embryonic stem (ES) cells. Leukemia inhibitory factor

(LIF) can maintain the self-renewal of mouse ES cells through activation of the LIF signaling pathway (Niwa *et al.* 1998, 2009, Matsuda *et al.* 1999). LIF stimulation leads to phosphorylation of transcription factor STAT3 and results in its activation.

POU5F1 (OCT4) (Palmieri *et al.* 1994), SOX2 (Avilion *et al.* 2003), and NANOG (Chambers *et al.* 2003, Mitsui *et al.* 2003) are expressed in the ICM of blastocysts and ES cells, thereby maintaining the pluripotency of these cells. CDX2 is indispensable for segregation of the ICM and TE lineages from the morula stage to the blastocyst stage by ensuring the repression of *Pou5f1* and *Nanog* in both the outer cells of the morula and the TE in the blastocyst (Niwa *et al.* 2005, Strumpf *et al.* 2005, Ralston & Rossant 2008). In late blastocysts, GATA6 is expressed in a portion of cells in the ICM to promote the segregation of the extraembryonic endoderm lineages,

such as PrE and its derivatives, VE and PE (Morrisey *et al.* 1998, Shimosato *et al.* 2007). It can be concluded that these pluripotency-maintaining and differentiation-inducing genes are expressed to coordinately regulate cell fates during the preimplantation development of mammalian embryos.

The mouse *Crxos* gene was demonstrated to be expressed in the retina (Alfano *et al.* 2005). Recently, we have identified three kinds of mRNAs that are transcribed from the *Crxos* gene as splicing or transcription variants in preimplantation mouse embryos. These

transcripts encode structurally related homeoproteins, EGAM1 (also known as CRXOS1), EGAM1N (CRXOS1 sv2), and EGAM1C (CRXOS1 tv3), and are also expressed in mouse ES cells (Saito *et al.* 2010). The function of these homeoproteins was estimated by forced expression in mouse ES cells without isolation of independent transfectants: EGAM1 and EGAM1N may act as positive and negative regulators, respectively, in the differentiation of ES cells. On the other hand, we have recently reported that EGAM1C is likely to play a role in the expression of members of the placental

Figure 1 Contents of POU5F1 and NANOG and the morphology of MG1.19 ES cells expressing exogenous EGAM1C in the presence of LIF. Control clones transfected with an empty vector (E, total 14 clones) and clones transfected with an *Egam1c*-expressing vector (C, total six clones) were established. (A) Morphology of a representative control (E2) or *Egam1c* (C4) transfectant cultured in the presence of LIF (+LIF culture). Scale bar: 50 μ m. (B) Comparison of the cellular contents of EGAM1C, POU5F1, and NANOG in control and *Egam1c* transfectants in +LIF culture conditions by western blotting. Five representative clones of the control and all clones stably expressing EGAM1C were analyzed. ACTB was detected as a loading control. The numbers under each panel represent the relative densities of specific signals normalized to the density of ACTB. The mean value of the control clones was set as 1. Vertical bars on the right side of the panels indicate the areas quantified. (C) qRT-PCR analysis of selected genes associated with ES cell pluripotency or differentiation in +LIF culture conditions. The expression levels of these genes were quantified and normalized with that of *Hmbs*, a housekeeping gene. Data are expressed as mean $\pm$ s.e.m. and mean value of control clones (E) was set as 1. E, 14 clones and C, six clones. ** $P < 0.01$, *** $P < 0.001$. (D) Subcellular localization of CDH1 in a representative control or *Egam1c* transfectant in +LIF culture conditions. CDH1 was detected using FITC (green)-labeled secondary antibodies by immunofluorescence microscopy. Concurrently, POU5F1, as a pluripotent marker, and cell nuclei were also detected using DyLight594 (red)-labeled secondary antibodies and DAPI (blue) respectively. Scale bar: 25 μ m. (E) Cellular content of CDH1 in representative control (E2 and E8) and *Egam1c* (C4 and C28) transfectants in +LIF culture conditions determined by western blotting.

prolactin gene family in the mouse placenta immediately before partum (Saito *et al.* 2011). However, the effect of exogenous EGAM1C expression on phenotypes of ES cells is still obscure.

ES cells have drawn the attention of researchers as a renewable source of material in regenerative medicine; therefore, the action of EGAM1C in ES cells is also intriguing. In this study, in order to investigate the effects of EGAM1C on the hallmarks of mouse ES cells, we isolated ES cells expressing exogenous EGAM1C protein using an episomal expression system.

Results

Expression of EGAM1C protein in MG1.19 cells and cell morphology

A feeder-free mouse ES cell line, MG1.19, expressing polyoma large T antigen (Gassmann *et al.* 1995) was supertransfected using an episomal expression system (Fujikura *et al.* 2002) with pMN1Pur/Egam1c (Saito *et al.* 2011) harboring polyoma *ori*. This vector was replicated episomally in MG1.19 cells, thereby the stable expression of EGAM1C protein would be expected. Transfection with an empty or *Egam1c*-expressing vector into MG1.19 cells led to the establishment of 14 control clones and six clones stably expressing EGAM1C protein.

Morphological changes were evident in the *Egam1c* transfectants (Fig. 1A). In general, all the control transfectants examined and parental MG1.19 cell (data not shown) colonies formed mainly comprised flattened cells. On the other hand, all the established *Egam1c* transfectants formed tightly aggregated, compacted colonies. As shown in Fig. 1B, the expression levels of EGAM1C in *Egam1c* transfectants were 5- to 13-fold higher than those in the control and were similar to those in blastocysts (as demonstrated later in Fig. 5). Cellular contents of POU5F1 and NANOG increased by 2.4 ± 0.3 -fold (mean $\pm$ S.E.M., $P < 0.001$) and 1.9 ± 0.3 -fold ($P < 0.05$) of the control respectively. Next, the expression level of *Pou5f1* and *Nanog* was analyzed (Fig. 1C). Interestingly, the expression levels were almost unchanged. On the other hand, basal expression levels of lineage-specific markers, such as *Gata6*, a PrE marker, *Cdx2*, a TE marker, and *T*, a mesoderm (MES) marker, decreased significantly in *Egam1c* transfectants. Two representative clones of each transfectant (control clones E8 (data not shown) and E2, and *Egam1c* clones C28 (data not shown) and C4) were analyzed immunofluorochemically for the expression of POU5F1 and CDH1 (Fig. 1D). Nevertheless, their cell morphology was quite different; both transfectant lines examined were confirmed as positive for immunostaining of POU5F1. Although limited expression levels of CDH1 protein were detected in control clones, as judged by western blotting (Fig. 1E), its cellular contents and subcellular

localization on the cell surface (Fig. 1D) were clearly increased in clones C4 and C28.

Cell growth in undifferentiated state-maintaining and differentiation-inducing cultures

To examine the proliferative potential of *Egam1c* transfectants, serial passage experiments were conducted in +LIF conditions (Fig. 2A). The population doubling times of the control and *Egam1c* transfectants in the presence of puromycin (2 μ g/ml) and G418 (250 μ g/ml) were 24.0 ± 0.3 h (mean $\pm$ S.E.M., $n = 14$ clones) and 16.4 ± 0.3 h ($n = 6$ clones), respectively, indicating that cell growth was accelerated significantly $P < 0.001$ in *Egam1c* transfectants. We also isolated three other clones expressing EGAM1C at 26- to 45-fold higher levels than the control transfectants (data not shown). All of them exhibited flattened cell types and

Figure 2 Effect of exogenous EGAM1C expression on the proliferation of MG1.19 ES cells. (A) Growth curves of control and *Egam1c* transfectants in the presence of LIF. A total of 1×10^5 cells for control (14 clones) and *Egam1c* (six clones) transfectants were plated into gelatin-coated, 60 mm dishes and passaged at the same density every 3 days in the presence of puromycin (2 μ g/ml) and G418 (250 μ g/ml). Data are expressed as mean $\pm$ S.E.M., but the SE bars are behind the symbols. (B) Growth curves of representative control and *Egam1c* transfectants with or without LIF. A total of 1×10^4 cells per well for the control (E2 and E8) and *Egam1c* (C4 and C28) transfectants were plated with +LIF medium (+LIF), -LIF medium (-LIF), or -LIF+RA medium (-LIF+RA) in the absence of puromycin and G418 in gelatin-coated, 24-well plates. Subsequently, cell numbers were counted. Data are expressed as mean $\pm$ S.E.M. ($n = 6$ independent wells), but SE bars are behind symbols. ** $P < 0.01$.

similar growth characteristics to the control. However, their expression of EGAM1C was unstable; after the induction of differentiation, the expression of EGAM1C became undetectable by western blotting (data not shown). Therefore, we excluded these high expressers from this study.

For subsequent detailed analyses in this study, two representative clones of control transfectants, E2 and E8, and *Egam1c* transfectants, C4 and C28, were selected. As shown in Fig. 2B, the cell growth of the control and *Egam1c* transfectants was compared by determining cell numbers at ~12 and 69 h after plating without puromycin and G418. Regardless of culture conditions, forced expression of *Egam1c* yielded an obvious increment in cell number ($P < 0.01$). It is noteworthy that the growth of EGAM1C transfectants was almost unchanged between +LIF and -LIF+RA culture conditions. Doubling times calculated from the data of control and *Egam1c* transfectants in the absence of puromycin and G418 were 14.8 h (mean of two clones) and 12.6 h, respectively, in +LIF conditions. It was demonstrated that cell growth was hampered in the presence of puromycin and G418.

The expression levels of proliferation regulatory genes were analyzed (Fig. 3). Among these, the expression of *Myc* (c-Myc) increased significantly in +LIF and -LIF culture conditions in *Egam1c* transfectants. In contrast, the expression level of *Cdkn1a* (*p21^{Cip1}*), a universal inhibitor of cyclin-dependent kinase, was downregulated markedly, but conversely, the expression level of *Ccnd1* was upregulated in -LIF and +1/10 LIF+RA culture conditions. Upregulation of *Ccne1* was not observed in *Egam1c* transfectants throughout the experiment.

Localization of anti-EGAM1C-positive cells in blastocysts

Before comparing the expression levels of EGAM1C protein between *Egam1c* transfectants and blastocysts, the localization of anti-EGAM1C-positive cells in blastocysts was investigated by immunofluorescence staining. Almost all the primary amino acid sequences for EGAM1C protein (17 kDa) are shared with EGAM1 protein (29 kDa) in its carboxyterminal region (Saito *et al.* 2010). Therefore, anti-EGAM1C, which was raised against the complete amino acid sequence of EGAM1C as a GST-fusion protein (Saito *et al.* 2011), recognized both proteins in blastocysts, two-thirds of them were large expanded, hatching, or hatched blastocysts, as judged by western blotting (Fig. 4A). However, signals for EGAM1C were substantially stronger than those for EGAM1, suggesting that the EGAM1C content was much larger than EGAM1 in blastocysts. In these conditions, immunofluorescence staining of blastocysts was performed. As shown in Fig. 4B, a distinct cell cluster both in a single blastocyst and a single, hatching blastocyst was clearly visualized with anti-POU5F1, indicating that the cluster

Figure 3 Expression levels of mRNA for growth regulatory genes. Control (E2 and E8) and *Egam1c* (C4 and C28) transfectants were plated with +LIF medium (+LIF), -LIF medium (-LIF), or +1/10 LIF+RA medium (+1/10 LIF+RA) in the presence of puromycin (0.2 µg/ml) and G418 (25 µg/ml) in gelatin-coated, 90 mm dishes. After 2- and 4-day culture periods, the expression levels of the indicated genes were quantified by qRT-PCR and normalized with that of *Hmbs*. Data are expressed as mean ± s.d. ($n=2$ independent dishes) and mean value of control clones in +LIF culture conditions was set as 1. * $P < 0.05$, ** $P < 0.01$.

was the ICM or the epiblast (EPI). On the other hand, almost all the nuclei in both types of blastocysts, probably composed of ICM, EPI, TE, or PrE, were positively stained with similar intensities by anti-EGAM1C.

Expression levels of crucial genes for maintenance of the undifferentiated state and progression of differentiation during adherent cultures in MG1.19 cells expressing exogenous EGAM1C

Next, the effects of EGAM1C on the expression of crucial transcription factors involved in differentiation from ES cells into EPI and its derivative MES, and PrE and its derivative VE, or TE were investigated. In order to avoid

Figure 4 Localization of anti-EGAM1C-positive cells in blastocysts. (A) Cellular contents of EGAM1 and EGAM1C proteins in mouse blastocysts. Expression levels of EGAM1C in 200 blastocysts, two-thirds of them were large expanded, hatching, or hatched blastocysts, were analyzed by western blotting. A vertical bar on the right side of the panels indicates the position of a contaminating band. (B) Localization of anti-EGAM1C-positive cells in blastocysts and hatching blastocysts. Signals as a mixture of EGAM1 and EGAM1C proteins were detected using FITC-labeled secondary antibodies by immunofluorescence microscopy. At the same time, POU5F1 and cell nuclei were also detected using DyLight594-labeled secondary antibodies and DAPI respectively. White arrowheads indicate embryonic cells hatching from the zona pellucida. Scale bar: 100 μ m.

the enrichment of cells harboring higher growth activities, gene expression levels within 4 days after the induction of differentiation were analyzed without cell passages. As indicated in Fig. 5A, the expression levels of EGAM1C in *Egam1c* transfectants (C4 and C28) and blastocysts were compared: the forced expression levels were calculated to be similar to those in blastocysts (0.5- to 3-fold of blastocysts by calculating the number of cells as 104 cells/blastocyst (Kobayashi *et al.* 1997)) from two independent trials. After the induction of differentiation, the overexpression of EGAM1C was maintained. In $-$ LIF culture conditions, the expression level of *Nanog* decreased rapidly, but conversely, the expression level of *Fgf5*, a marker for EPI, *T*, a marker for MES, and *Gata6*, a marker for PrE, in clones stably expressing EGAM1C increased (Fig. 5B). Meanwhile, in $+1/10$ LIF+RA culture conditions, the control clones expressed *Gata6* and α -fetoprotein (*Afp*), a VE marker. In *Egam1c* transfectants, the expression levels of *Nanog* and *Pou5f1* were roughly maintained. The upregulation of *Gata6* and *Afp* was inhibited to below half the levels of the control at 4 days after the induction of differentiation. As shown in Fig. 5C, the expression levels of *Cdx2* and *Eomes*, crucial transcription factors for the establishment and maintenance of TE and TS cells (Niwa *et al.* 2005), were compared with those in a mouse TS cell line, TS7. Four days after the

induction of differentiation, remarkable upregulation of *Cdx2* was observed in *Egam1c* transfectants. The expression level of *Cdx2* was significantly higher ($P < 0.01$) than that of TS7 cells.

In these conditions, expression levels of mRNAs and their encoded proteins for crucial transcription factors downstream of the LIF signaling (Niwa *et al.* 2009) were compared (Fig. 6A and B). In addition to POU5F1 and NANOG, the expression levels of *Sox2*, *Tbx3*, and their encoded proteins were increased robustly in *Egam1c* transfectants in $+LIF$ culture conditions. Three days after the induction of differentiation in $-LIF$ culture conditions, the POU5F1 protein level was roughly maintained without an apparent upregulation of *Pou5f1*. In $+1/10$ LIF+RA culture conditions, a significant decrease in *Stat3* expression (total of *Stat3 α* and β) was detected, although the cellular content of STAT3 protein was almost maintained. The down-regulation of LIF signaling, including phosphorylated STAT3 protein level, expression levels of relevant genes (*Klf4*, *Sox2*, *Nanog*, and *Pou5f1*), and their encoded proteins, was prevented in *Egam1c* transfectants. In particular, the expression of *Tbx3* and its encoded protein increased obviously. The cell morphology of *Egam1c* transfectants (Fig. 6C) became flattened with a similar cellular CDH1 content to the control (data not shown). However, alkaline phosphatase (ALP) activity was apparently positive in $+1/10$ LIF+RA culture conditions without the upregulation of endogenous *Lif* expression, which was at a level similar to that of the undifferentiated state ($+LIF$). Therefore, the progression of differentiation in *Egam1c* transfectants in $+1/10$ LIF+RA culture conditions seemed, at least in part, to be inhibited.

Effect of EGAM1C on the expression of lineage-specific marker genes in embryoid bodies

In order to clarify the effect of EGAM1C on cell fate in long-term, differentiation-inducing culture conditions, embryoid bodies (EBs) were generated. After 11 days from the initiation of EB formation, the stable expression of EGAM1C protein was also detected (Fig. 7A); the expression level was maintained with that in $+LIF$, adherent culture conditions (data not shown). Expression levels of *Pou5f1* and *Nanog* in EBs expressing EGAM1C were significantly below those in the control and also lower than those in an undifferentiated state (Fig. 7B). In *Egam1c* transfectants, the expression of *Fgf5* and *T* disappeared almost completely ($P < 0.01$), although that of *Ascl1* (*Mash1*), a marker for neuroectoderm, remained at a similar expression level (Fig. 7C). Expression of marker genes for extraembryonic cell lineages, including *Tpbpa* for spongiotrophoblast and *Plat* ($P < 0.01$) for PE, increased with decreasing expression in *Afp*.

Figure 5 Expression of genes encoding lineage-specific transcription factors. (A) Cellular contents of EGAM1C protein in *Egam1c* transfectants and mouse blastocysts. Control (E2 and E8) and *Egam1c* (C4 and C28) transfectants were plated with the respective culture media as indicated in the legend for Fig. 3. After a 3-day culture period, expression levels of EGAM1C in $\sim 1.6 \times 10^4$ cells were compared by western blotting. The mean value of control clones in +LIF culture conditions was set as 1. Expression levels of EGAM1C in varying numbers of blastocysts (34, 67, 100, and 200 embryos) were also analyzed in two separate trials. The data for 200 blastocysts were taken from another blot and combined carefully. (B) qRT-PCR analysis of selected genes associated with ES cell pluripotency or differentiation. Control and *Egam1c* transfectants were plated with the respective culture media as indicated in the legend for Fig. 3. After 2- and 4-day culture periods, the expression levels of the indicated genes were quantified and normalized with those of *Hmbs*. Data were processed as indicated in the legend for Fig. 3. In the case of *T* and *Afp*, mean values of the control clones in -LIF (D4) and +1/10 LIF+RA (D4) culture conditions, respectively, were set as 1. * $P < 0.05$, ** $P < 0.01$. (C) Comparison of expression levels of trophoblast markers with those in a mouse TS cell line, TS7 cells. Data for TS7 cells are expressed as mean $\pm$ s.d. ($n = 4$ independent dishes). * $P < 0.05$, ** $P < 0.01$.

Stabilization of the undifferentiated state in *Egam1c* transfectants during clonal proliferation

One of the major characteristics of MG1.19 cells expressing exogenous EGAM1C in +LIF conditions was upregulation of pluripotency factors POU5F1, SOX2, NANOG, and TBX3. This phenotype led us to examine the stability of the undifferentiated state in *Egam1c* transfectants. To this end, transfectants were cultured at a clonal density in the presence of LIF for a 7-day period (Fig. 8), which would allow the spontaneous differentiation. The total number of colonies per

well with *Egam1c* transfectants (292 ± 13 and 318 ± 11 colonies, mean $\pm$ s.e.m., for C4 and C28, respectively, $n = 3$ independent wells) was significantly larger ($P < 0.01$) than that for the control (204 ± 14 and 207 ± 7 colonies for E2 and E8 respectively), suggesting that EGAM1C promotes the survival of ES cells during clonal proliferation. As shown in Fig. 8B, more than 80% of colonies generated from *Egam1c* transfectants (C4 and C28) formed tightly aggregated colonies with strong ALP activity (undifferentiated), but almost all colonies showed flattened cells with weakened or an absence of

Figure 6 Expression of genes encoding crucial transcription factors regulating maintenance of the undifferentiated state and their protein levels. (A) qRT-PCR analysis of mRNAs for selected transcription factors downstream of the LIF signaling pathway. Control (E2 and E8) and *Egam1c* (C4 and C28) transfectants were plated with the respective culture media as indicated in the legend for Fig. 3. Data were processed as indicated in the legend for Fig. 3. * $P < 0.05$, ** $P < 0.01$. (B) Cellular contents of selected transcription factors downstream of the LIF signaling pathway. Control (E2 and E8) and *Egam1c* (C4 and C28) transfectants were plated with the respective culture media as indicated in the legend for Fig. 3. After a 3-day culture period, the cellular contents of specific proteins were analyzed by western blotting. Data were processed as indicated in the legend for Fig. 5A. Note that the upper and lower protein bands in the STAT3 panel were STAT3 α and β respectively. Changes in intensity of signals for these two variant proteins were very similar. (C) Cell morphology and ALP activity of control (E2) and *Egam1c* (C4) transfectants under respective culture conditions. After a 2-day culture period, ALP staining was carried out. Scale bar: 25 μ m.

activity in the control (E2 and E8, 'partially differentiated' or 'differentiated'). A relationship between the cellular ALP activity of transfectants and the reactivity with anti-NANOG or anti-POU5F1 antibodies in immunofluorescence staining was confirmed (data not shown). These results indicated that enforced EGAM1C expression stabilized the undifferentiated state of MG1.19 ES cells during clonal propagation.

Discussion

Because the effect of enforced EGAM1C expression in ES cells remains to be clarified (Saito *et al.* 2010), we aimed to establish mouse ES cells expressing exogenous EGAM1C. It is noteworthy that the expression level was considered as moderate because the level was similar to that in blastocysts. The estimated effects of forced

Figure 7 Expression of lineage-specific genes in embryoid bodies (EBs). EBs were generated from transfectants by suspension culture for a 5-day period. Then, EBs were transferred to adherent culture. (A) Cellular contents of EGAM1C in control (E2 and E8) and *Egam1c* (C4 and C28) transfectants after the generation of EBs. EGAM1C was detected by western blotting after adherent culture for a 4-day period. (B) Expression levels of mRNA for *Pou5f1* and *Nanog* quantified by qRT-PCR after adherent culture for a 6-day period. Data normalized with that of *Hmbs* were processed as described in the legend for Fig. 3. The expression levels of these genes in an undifferentiated state sustained by LIF were almost equivalent in these transfectants; therefore, the mean value during the undifferentiated state was set as 1. ** $P < 0.01$. (C) Expression levels of mRNA for lineage-specific marker genes in EBs during adherent culture. Expression levels of mRNAs were quantified by qRT-PCR after adherent culture for a 6-day period and normalized with that of *Hmbs*. Data were processed as described in the legend for Fig. 3. ** $P < 0.01$.

EGAM1C expression in mouse ES cells are summarized in Fig. 9 in combination with the transcriptional factor network reviewed by Niwa (2007) and Niwa *et al.* (2009). Results obtained herein can be summarized as follows: *Egam1c* transfectants 1) showed stabilization of an undifferentiated state in +LIF conditions, 2) promoted differentiation in -LIF conditions, but partially inhibited differentiation in the presence of RA in adherent culture, 3) showed enhanced expression of

specific markers for the extraembryonic cell lineages by generation of EBs, 4) exhibited accelerated cell growth, and 5) formed aggregated colonies with increased CDH1 expression in +LIF conditions. These results demonstrated that EGAM1C is capable of affecting hallmarks of mouse ES cells. In our previous study (Saito *et al.* 2010) using a random integration system of expression vectors, no obvious effect of EGAM1C was shown in a mouse ES cell line, EB3. Forced expression levels of EGAM1C protein in EB3 cells in the previous study were evaluated as being less than half of those in MG1.19 clones C4 and C28 (data not shown). These could relate to our previous unclear observations in EB3 cells expressing EGAM1C.

Pou5f1 and *Nanog* are crucial downstream targets of LIF signaling for maintaining self-renewal and pluripotency of mouse ES cells (Niwa *et al.* 2009). In this study, the obvious elevation in the cellular contents of POU5F1 and NANOG was shown in *Egam1c* transfectants in +LIF conditions. Interestingly, an unremarkable activation of the LIF signaling pathway, such as phosphorylation levels of STAT3 and expression levels of *Pou5f1* and *Nanog*, was induced by EGAM1C in +LIF conditions. It is reported that the cellular contents of transcription factors, such as POU5F1, NANOG, and STAT3, are regulated at posttranscriptional levels, including degradation-dependent pathways (Daino *et al.* 2000, Xu *et al.* 2004, Zhang *et al.* 2007, Fujita *et al.* 2008, Moretto-Zita *et al.* 2010, Musch *et al.* 2010). The exact mechanisms underlying the metabolism of these transcription factors in *Egam1c* transfectants remain to be clarified.

It is well known that POU5F1, SOX2, TBX3, or NANOG play a role in preventing the differentiation of ES cells, such as into TE (Niwa *et al.* 2000), PrE (Mitsui *et al.* 2003), EPI (Rao & Orkin 2006), MES (Suzuki *et al.* 2006, Bourillot *et al.* 2009), or endoderm (Bourillot *et al.* 2009). In the presence of a sufficient amount of LIF, the resultant increases in the cellular content of POU5F1, SOX2, TBX3, and NANOG are likely to contribute to the inhibition of spontaneous differentiation in *Egam1c* transfectants during clonal proliferation. Indeed, a significant repression of basal expression levels of lineage-specific markers was observed in *Egam1c* transfectants in +LIF culture conditions. It was also demonstrated that ectopic expression of EGAM1C impairs, at least in part, cell differentiation with +1/10 LIF+RA because certain cellular contents of pluripotency factors, ALP activity, and cell growth were maintained, and the induction of the differentiation marker genes examined was partially repressed in *Egam1c* transfectants. An increase in the expression level of *Tbx3* was also induced, as shown in the +LIF conditions, with an accompanying increase in STAT3 protein levels without upregulation of its mRNA. These results indicated a role of EGAM1C in inhibiting differentiation in +1/10 LIF+RA conditions. During

Figure 8 Effect of exogenous EGAM1C expression on the stabilization of the undifferentiated state in clonal proliferation. A single-cell suspension of the control (E2 and E8) and *Egam1c* (C4 and C28) transfectants was plated at 500 cells/well in gelatin-coated, 6-well plates and cultured with +LIF medium in the absence of puromycin and G418. A week after plating, cell colonies were stained with NBT/BCIP solution for the detection of ALP activity. (A) ALP staining of cell colonies derived from the control (E2) and *Egam1c* (C4) transfectants. A black arrowhead indicates a representative 'undifferentiated' colony of tightly aggregated cells and stained for ALP activity. Gray arrowheads indicate representative 'partially differentiated' colonies surrounded by flattened cells without ALP activity. White arrowheads indicate representative 'differentiated' colonies almost fully composed of flattened cells without ALP activity. Scale bar: 500 μ m. (B) Effect of EGAM1C on the maintenance of ALP-positive colonies. The number of colonies was counted in accordance with the classification indicated in (A). Data are expressed as mean $\pm$ S.E.M. ($n=3$ independent wells); #no colonies; ** $P<0.01$.

the first 3 days after the induction of differentiation, a 1/10 amount of LIF (the original amount was sufficient for sustaining ES cells in an undifferentiated state) was added to the culture medium. It is likely that the sensitivity to exogenous and endogenous LIF was augmented in *Egam1c* transfectants because endogenous *Lif* expression remained at a lower level. Analysis with respect to the relationship between EGAM1C and LIF signaling, RA signaling, or expression of transcription factors possibly targeted by EGAM1C will provide valuable information concerning the regulatory mechanisms involved.

The differentiation of ES cells can be induced by the ectopic expression of certain transcription factors such as *Pou5f1*, *Gata6*, or *Gata4* toward the PrE cell lineage

(Niwa *et al.* 2000, Fujikura *et al.* 2002) and *Cdx2* or *Eomes* toward the TE cell lineage (Niwa *et al.* 2005). After a relatively short period (4 days) following the removal of LIF in adherent conditions, obvious increases in lineage-specific marker genes for at least four kinds of cell lineage, including *Fgf5* for EPI, *T* for MES, *Gata6* for PrE, and *Cdx2* for TE, were found. Of these genes, it is noteworthy that the expression level of *Cdx2* was significantly higher than that of TS cells. Interestingly, after a long period (11 days) from the induction of differentiation by EB formation, ectopic expression of EGAM1C probably led to differentiation into the extraembryonic TE cell lineage, such as spongiotrophoblast expressing *Tpbpa*, and the extraembryonic PrE cell lineage, including PE expressing *Plat*. These results demonstrated that EGAM1C is capable of affecting the progression of differentiation in ES cells via the induction of lineage-specific transcription factors, although it is incapable of activity in +LIF conditions. In the presence of LIF, sufficient amounts of pluripotency factors POU5F1, SOX2, TBX3, and NANOG possibly prevented the activation of differentiation-promoting genes by EGAM1C.

Other remarkable features of *Egam1c* transfectants are their cell morphology and growth activity. CDH1 is well known for being required in the intercellular adhesion of ES cells (Larue *et al.* 1996) and morulae (Riethmacher *et al.* 1995). Thus, increased CDH1 levels in *Egam1c* transfectants probably led to morphological changes to form compacted colonies. Concerning cell growth, the *Myc* (Cartwright *et al.* 2005) and *Ccnd1* (Bowman *et al.* 2000) genes are direct targets of STAT3. In particular, *Myc* is known for maintaining self-renewal, pluripotency, and growth of mouse ES cells (Cartwright *et al.* 2005). However, the expression of *Myc* and *Ccnd1* was upregulated without the augmentation of LIF signaling, as indicated by the level of pSTAT3 in *Egam1c* transfectants. This suggested that the elevation of *Myc* and *Ccnd1* expression mediated by other pathways resulted in the acceleration of cell growth. Furthermore, because possible effector genes, including *Myc*, *Ccnd1*, and *Cdkn1a*, for stimulation of cell growth varied in each culture condition, the mechanism underlying the control of cell growth in *Egam1c* transfectants was probably different for the respective culture conditions.

As summarized in Fig. 9, this study predicted that the ectopic expression of EGAM1C extensively affects genes encoding not only pluripotency and lineage-specific factors but also growth regulatory or intercellular adhesion molecules in self-renewing and differentiating ES cells. In contrast to this feature of EGAM1C, the transcriptional core, pluripotency factors POU5F1, SOX2, and NANOG modulate the expression of specific genes that are required for either pluripotency or differentiation events. In subsequent experiments, knockout or knockdown analysis in embryos will be critical for clarifying the functions of this protein in embryonic development.

Figure 9 Diagrams of the relationship among the ectopic expression of EGAM1C, the transcription factor network, and the maintenance and differentiation of mouse MG1.19 ES cells in the respective culture conditions as well as cell morphology and growth. (A) Estimated effects of ectopic expression of EGAM1C on the maintenance and differentiation of mouse ES cells are summarized in combination with the transcriptional factor network and the determination of cell fates reviewed by Niwa (2007) and Niwa *et al.* (2009). See Discussion for details. Red arrows and red bars from 'EGAM1C' indicate 'stimulatory effect' and 'inhibitory effect', respectively, observed in this study. Black arrows indicate 'stimulatory effect' (Niwa 2007, Niwa *et al.* 2009). Dashed gray arrows indicate a pathway. The degradation pathway is a possible pathway and is indicated as 'Degradation?'. Gray arrows indicate a possible cell fate (Niwa 2007). Boxes indicate a short summary of the EGAM1C effect in each culture condition. PrE, primitive endoderm; EPI, epiblast; MES, mesoderm; TE, trophectoderm. (B) Effects of ectopic expression of EGAM1C on ES cell morphology and growth. Upward and downward arrowheads indicate the upregulation and downregulation of gene or protein expression respectively. (C) Effects of ectopic expression of EGAM1C on the expression of lineage-specific markers in embryoid bodies. Spongiotrophoblast (SpT) is derived from TE. Visceral endoderm (VE) and parietal endoderm (PE) are from PrE.

Materials and Methods

Embryo collection and culture

Induction of superovulation and subsequent mating of 6- to 12-week-old virgin female CD-1 mice (random bred, Swiss, Charles River Japan, Yokohama, Japan) were performed as described previously (Kobayashi *et al.* 1996, 2011). Two-cell embryos were flushed in M2 medium from excised oviducts 45–46 h after hCG injection and cultured to the blastocyst stage in 1 ml Whitten's medium in the presence of 3 mg/ml BSA

in 4-well dishes (Nunc, Roskilde, Denmark). All animal procedures conformed to the Guidelines for the Care and Use of Laboratory Animals of Akita Prefectural University.

Culture of established cell lines

Feeder-free mouse MG1.19 ES cells (Gassmann *et al.* 1995), expressing polyoma large T antigen and neomycin resistance gene, were routinely maintained on gelatin-coated plates in Glasgow modified Eagle's medium (GMEM; Sigma) containing

10% FCS (defined for mouse ES cells, Biological Industries, Haemek, Israel), 10^{-4} M 2-mercaptoethanol (Nacalai Tesque, Kyoto, Japan), 1 mM sodium pyruvate (Sigma), 1 mM nonessential amino acids (Sigma), and recombinant human LIF (100 IU/ml, made in-house (Smith 1991)) (+LIF medium) at 37 °C with 5% CO₂/air. Differentiation of ES cells was induced by either the removal of LIF from the culture medium (–LIF medium) or the addition of retinoic acid (RA; 10^{-6} M, all-*trans* type, Sigma) to the medium. In order to prevent the appearance of detrimental effects induced by RA, a 1/10 amount of LIF (mouse LIF, ESGRO, Chemicon, Temecula, CA, USA; the original amount was sufficient for maintaining the undifferentiated state in ES cells) was added to the culture medium (+1/10 LIF+RA medium) (Smith 1991). After a 3-day culture period with +1/10 LIF+RA medium, the medium was changed to –LIF medium. In some experiments within a 3-day period, 10^{-6} M RA was added to –LIF medium (–LIF+RA medium). Culture of a mouse trophoblast stem (TS) cell line, TS7 cells (Hosoi *et al.* 2010, Saito *et al.* 2011), was performed in accordance with the method reported previously (Hosoi *et al.* 2011).

Transfection of *Egam1c*-expressing vector and selection of stable transfectants

MG1.19 ES cells were supertransfected (Fujikura *et al.* 2002) with pMN1Pur/Egam1c (Saito *et al.* 2011) harboring polyoma *ori* (Gassmann *et al.* 1995) by the lipofection method (Saito *et al.* 2010). This vector contains a powerful and versatile chicken *Hbb* promoter with the CMV immediate early enhancer and rabbit *Actb* intron II acceptor site (CAG promoter (Niwa *et al.* 1991, Abe *et al.* 2011)). The vector expresses a single mRNA concomitantly encoding a gene of interest, an internal ribosomal entry site (IRES) derived from the encephalomyocarditis virus, and a puromycin-resistant gene. In cells transfected with this vector, the high degree of secondary structure of the IRES in the transcript is recognized by ribosomes, and the puromycin-resistant gene placed downstream of the IRES is translated IRES dependently. Such a dicistronic vector is advantageous to ensure the co-expression of the desired gene in cells expressing a selectable marker gene. This vector was replicated episomally in transfected cells expressing polyoma large T antigen, such as MG1.19 cells.

For the isolation of stable transfectants, after an additional 18 h incubation after transfection, the cells were diluted and divided into new culture dishes (90 mm diameter) with fresh +LIF medium in the presence of 6 µg/ml puromycin (Sigma) and 250 µg/ml G418 (Sigma). After 3 days, the medium was changed to +LIF medium in the presence of 2 µg/ml puromycin and 250 µg/ml G418. Stable transfectants were selected by cultivation with selection medium for ~8 days and independent clones were picked-up using a micropipette and further cultured in the presence of 2 µg/ml puromycin and 250 µg/ml G418. Doubling times of transfectants were determined as follows: a total of 1×10^5 cells were plated into gelatin-coated dishes (60 mm diameter) with +LIF medium containing 2 µg/ml puromycin and 250 µg/ml G418 and cultured for 30 days with passaging at the same density every 3 days.

Generation of EBs

Aliquots of the cell suspension (4000 cells/200 µl) were transferred to a 96-well plate (U bottom, Lipidure-coat plate A-U96, NOF, Tsukuba, Japan) with puromycin (0.2 µg/ml) and G418 (25 µg/ml) in –LIF medium. The cell suspension was cultured for a 5-day period at 37 °C with 5% CO₂/air to form EBs. Subsequently, EBs were transferred into a 24-well plate coated with gelatin (four EBs/well) for adherent culture, and the culture was continued for a 6-day period.

Clonal proliferation analysis

A single-cell suspension was prepared gently using trypsin–EDTA solutions, seeded at 500 cells/well in gelatin-coated, 6-well plates, and cultured with +LIF medium in the absence of puromycin and G418. A week after seeding, ES cell colonies were stained with a nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) solution for the detection of

Table 1 Sequences of primers for quantitative RT-PCR.

Gene	Primer sequence	Product size (bp)
<i>Hmb</i> s	5'-CCCTTGATGCTGTTGTCTTTCA-3' 5'-AGGCGGGTGTGAGGTTTC-3'	168
<i>Pou5f1</i>	5'-GCCGTGAAGTTGGAGAGGTG-3' 5'-CGAAGCGACAGATGGTGGTC-3'	196
<i>Nanog</i>	5'-TCTGGGAACGCCTCATCA-3' 5'-GCTTTTGTGGGACTGGTAGAA-3'	169
<i>Gata6</i>	5'-TCACCCGACCTACTCGCCTACA-3' 5'-TTCACGCACTCGCGGCTCTC-3'	177
<i>Cdx2</i>	5'-TTTAGTCGATACATCACCATCAGGAGGA-3' 5'-TCCTTGGCTCTGCGGTTCTG-3'	104
<i>T</i>	5'-TGCCAAAGAAAGAAACGACCA-3' 5'-TGCCAGCACCAGGAACAAG-3'	101
<i>Myc</i>	5'-TCCGATTCCACGGCTTCTC-3' 5'-TTGCCTCTTCTCCACAGACACCA-3'	180
<i>Ccnd1</i>	5'-CAGAGGCGGATGAGAACAAG-3' 5'-CGGGTAGCAGGAGGAAG-3'	179
<i>Ccne1</i>	5'-GGAAGGCAATGTGGCCGTGT-3' 5'-TCTTTGTAGGGGTGGGGATGAAAG-3'	151
<i>Cdkn1a</i>	5'-GAGTCAGCGCAGATCCACA-3' 5'-CACGGGACCGAAGAGACAAC-3'	124
<i>Fgf5</i>	5'-GTGTCTCAGGGGATGTAGGAATCAGAG-3' 5'-CGGCCTGTCTTTTCAGTTCTGTGG-3'	182
<i>Ascl1</i>	5'-GGCTCTCCTGGGAATGGACTTTGG-3' 5'-GCCCTGTAGGTTGGCTGTCTGG-3'	154
<i>Afp</i>	5'-TGCAATGAAGAAAACCTGGCGATG-3' 5'-TGGAACCTGGAAGGGTGGGACAGA-3'	199
<i>Plat</i>	5'-CGCAGACAACCTACCAACAGCACCA-3' 5'-GTCACAGCGTTTCCCTACAAATCCATC-3'	224
<i>Eomes</i>	5'-CCTGGTGGTGTGTTTGTGTG-3' 5'-TTTAATAGCACCGGGCACTC-3'	292
<i>Tpbpa</i>	5'-CCAGCAGAGCTTTGGACATCAC-3' 5'-CGTCTCCTGGTCATTTTCGCTAC-3'	218
<i>Lif</i>	5'-ACGGCAACCTCATGAACCA-3' 5'-GGAAACGGCTCCCCTTGA-3'	103
<i>Stat3</i>	5'-TCCAATGTCCTCTATCAGACAACC-3' 5'-GAGGTTCTCCACCACCTTCATTTTC-3'	282
<i>Klf4</i>	5'-GATGCAGTCACAGATGCCCTCTCTC-3' 5'-CCTCTTCATGTGTAAGGCAAGGTGGT-3'	367
<i>Sox2</i>	5'-GGACAGCTACGCGCATGAA-3' 5'-TGGCCTCGGACTTGACCACA-3'	275
<i>Tbx3</i>	5'-GGCAATGGCAGGAGAGAAAAA-3' 5'-GCCGTAGTGGTGGAAATCTTGG-3'	275

ALP activity (Saito *et al.* 2010). The number of colonies was counted by microscopic observation.

RNA extraction and quantitative RT-PCR analysis

Extraction of total RNA, synthesis of cDNA, and quantitative (q) PCRs were carried out in accordance with the method reported previously (Soma *et al.* 2011). The set of gene-specific primers for qRT-PCR is shown in Table 1. Hydroxymethylbilane synthase (*Hmbs*) was analyzed as a housekeeping gene.

Western blotting

Cellular proteins separated by SDS–PAGE followed by transfer onto a PVDF membrane were incubated with a PVDF Blocking Reagent (Toyobo, Osaka, Japan). Subsequently, the membrane was incubated with anti-EGAM1C antiserum (1:20 000 (Saito *et al.* 2011)), anti-POU5F1 (1:3000, sc-5279; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-NANOG (1:2000, RCAB0001P; ReproCELL, Yokohama, Japan), anti-SOX2 (1:75 000, sc-20088; Santa Cruz Biotechnology), anti-KLF4 (1:75 000, sc-20691; Santa Cruz Biotechnology), anti-TBX3 (1:5000, sc-17871; Santa Cruz Biotechnology), anti-CDH1 (1:75 000, AF748; R&D Systems, Minneapolis, MN, USA), anti-STAT3 (1:5000, sc-482; Santa Cruz Biotechnology), anti-pSTAT3 (1:1500, sc-7993; Santa Cruz Biotechnology), or anti-ACTB (1:20 000, IMG-5142A; IMGEX, San Diego, CA, USA) antibodies. The membrane was incubated with HRP-conjugated anti-rabbit IgG (AP132P; Chemicon), anti-goat IgG (sc-2768; Santa Cruz Biotechnology), or anti-mouse IgG (NA931V; GE Healthcare Bio-Sciences, Piscataway, NJ, USA) as secondary antibodies at appropriate concentrations. The enzyme reaction was detected using ECL plus Western Blotting Reagent (GE Healthcare Bio-Sciences) and a LAS-4000 image analyzer (Fujifilm, Tokyo, Japan).

Immunofluorescence microscopy

Cells were fixed with 4% paraformaldehyde in PBS for 20 min at room temperature and permeabilized with 0.1 or 0.5% Triton X-100 for ES cells and blastocysts, respectively, in PBS at room temperature for 15 min. Primary and secondary antibodies were diluted to final working concentrations in 1% BSA in PBS. 4'-6-Diamidino-2-phenylindole (DAPI, 100 ng/ml) was added to a reaction mixture of secondary antibodies. Specimens were mounted with SlowFade Antifade Reagent for ES cells or SlowFade Gold Antifade Reagent for blastocysts (Invitrogen). Images of immunofluorescent samples were obtained by fluorescence microscopic observation. The final concentrations of antibodies were as follows: whole IgG fraction of rabbit anti-EGAM1C antiserum, 0.2 µg/ml for blastocysts; goat anti-CDH1, 1:500; mouse anti-POU5F1, 1:500 and 1:1000 for ES cells and blastocysts respectively; FITC-conjugated anti-goat IgG, 1:500 (A50-200F; Bethyl Laboratories, Montgomery, TX, USA); and DyLight594 conjugated anti-mouse IgG 1:500 to 1:1000 (ab96881; Abcam, Tokyo, Japan).

Statistical analysis

The statistical significance of the difference between sample means was determined using Student's *t*-test. Results obtained from the cell growth assay, the clonal proliferation assay, and qRT-PCR analysis for the representative two control and two *Egam1c* transfectants were subjected to one-way ANOVA followed by the Fisher's protected least squares difference (PLSD) test. Symbols representing statistical differences are indicated only in cases where both values for two representative transfectants were evaluated as significantly different.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was supported in part by a research grant from the President of Akita Prefectural University to M Kobayashi.

Acknowledgements

The authors thank Hitoshi Niwa (RIKEN Center for Developmental Biology, Kobe, Japan) for providing the human LIF expressing vector. The authors also thank Max Gassmann (University of Zurich, Switzerland) for providing the pMGD20neo vector and MG1.19 cells. The authors particularly thank Shujiro Sakaki (Akita National College of Technology, Akita, Japan) for invaluable advice on the generation of embryoid bodies from mouse ES cells and Satoshi Yamada (NOF Corporation, Tsukuba Corporate Research Laboratory, Tsukuba, Japan) for kindly supplying Lipidure coat plates (A-U96) for the generation of embryoid bodies.

References

- Abe H, Nakazawa M, Kasuga K, Kojima I & Kobayashi M 2011 The CAG promoter is more active than the CEF promoter for the expression of transgenes in a mouse ES cell line E14-derived EB3 cells. *Biotechnology & Biotechnological Equipment* **25** 2301–2304. (doi:10.5504/bbeq.2011.0013)
- Alfano G, Vitiello C, Caccioppoli C, Caramico T, Carola A, Szego MJ, McInnes RR, Auricchio A & Banfi S 2005 Natural antisense transcripts associated with genes involved in eye development. *Human Molecular Genetics* **14** 913–923. (doi:10.1093/hmg/ddi084)
- Avilion AA, Nicolis SK, Pevny LH, Perez L, Vivian N & Lovell-Badge R 2003 Multipotent cell lineages in early mouse development depend on SOX2 function. *Genes and Development* **17** 126–140. (doi:10.1101/gad.224503)
- Bourillot PY, Aksoy I, Schreiber V, Wianny F, Schulz H, Hummel O, Hubner N & Savatier P 2009 Novel STAT3 target genes exert distinct roles in the inhibition of mesoderm and endoderm differentiation in cooperation with Nanog. *Stem Cells* **27** 1760–1771. (doi:10.1002/stem.110)
- Bowman T, Garcia R, Turkson J & Jove R 2000 STATs in oncogenesis. *Oncogene* **19** 2474–2488. (doi:10.1038/sj.onc.1203527)
- Cartwright P, McLean C, Sheppard A, Rivett D, Jones K & Dalton S 2005 LIF/STAT3 controls ES cell self-renewal and pluripotency by a Myc-dependent mechanism. *Development* **132** 885–896. (doi:10.1242/dev.01670)
- Chambers I, Colby D, Robertson M, Nichols J, Lee S, Tweedie S & Smith A 2003 Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. *Cell* **113** 643–655. (doi:10.1016/S0092-8674(03)00392-1)

- Daino H, Matsumura I, Takada K, Odajima J, Tanaka H, Ueda S, Shibayama H, Ikeda H, Hibi M, Machii T *et al.* 2000 Induction of apoptosis by extracellular ubiquitin in human hematopoietic cells: possible involvement of STAT3 degradation by proteasome pathway in interleukin 6-dependent hematopoietic cells. *Blood* **95** 2577–2585.
- Fleming TP 1987 A quantitative analysis of cell allocation to trophoblast and inner cell mass in the mouse blastocyst. *Developmental Biology* **119** 520–531. (doi:10.1016/0012-1606(87)90055-8)
- Fujikura J, Yamato E, Yonemura S, Hosoda K, Masui S, Nakao K, Miyazaki J & Niwa H 2002 Differentiation of embryonic stem cells is induced by GATA factors. *Genes and Development* **16** 784–789. (doi:10.1101/gad.968802)
- Fujita J, Crane AM, Souza MK, Dejosez M, Kyba M, Flavell RA, Thomson JA & Zwaka TP 2008 Caspase activity mediates the differentiation of embryonic stem cells. *Cell Stem Cell* **2** 595–601. (doi:10.1016/j.stem.2008.04.001)
- Gassmann M, Donoho G & Berg P 1995 Maintenance of an extra-chromosomal plasmid vector in mouse embryonic stem cells. *PNAS* **92** 1292–1296. (doi:10.1073/pnas.92.5.1292)
- Hosoi Y, Soma M, Saito K, Arakawa M, Ando Y, Morita S, Otsu A, Kasuga K, Kojima I & Kobayashi M 2010 Characterization of gene expression in trophoblast stem cells and extra-embryonic endoderm cells established from mouse blastocysts. *Tohoku Journal of Animal Science and Technology* **60** 6–12.
- Hosoi Y, Ando Y, Arakawa M, Kasuga K, Kojima I & Kobayashi M 2011 Production of mouse fibroblast growth factor 4 in *E. coli* and its application for isolation and maintenance of mouse trophoblast stem cells *in vitro*. *Journal of Reproduction and Development* **57** 650–654. (doi:10.1262/jrd.11-043H)
- Kobayashi M, Hirako M, Minato Y, Sasaki K, Horiuchi R & Domeki I 1996 Rat hepatoma Reuber H-35 cells produce a 2-cell stage-specific inhibitor of the cleavage of mouse embryos. *Biology of Reproduction* **54** 364–370. (doi:10.1095/biolreprod54.2.364)
- Kobayashi M, Hirako M, Minato Y, Sasaki K, Horiuchi R & Domeki I 1997 Rat hepatoma Reuber H-35 cells produce factors that promote the hatching of mouse embryos cultured *in vitro*. *Biology of Reproduction* **56** 1041–1049. (doi:10.1095/biolreprod56.4.1041)
- Kobayashi M, Saito K, Tamogami S, Takashima J, Kasuga K & Kojima I 2011 Identification of a 2-cell stage specific inhibitor of the cleavage of preimplantation mouse embryos synthesized by rat hepatoma cells as 5'-deoxy-5'-methylthioadenosine. *Zygote* **19** 117–125. (doi:10.1017/S0967199410000158)
- Larue L, Antos C, Butz S, Huber O, Delmas V, Dominis M & Kemler R 1996 A role for cadherins in tissue formation. *Development* **122** 3185–3194.
- Matsuda T, Nakamura T, Nakao K, Arai T, Katsuki M, Heike T & Yokota T 1999 STAT3 activation is sufficient to maintain an undifferentiated state of mouse embryonic stem cells. *EMBO Journal* **18** 4261–4269. (doi:10.1093/emboj/18.15.4261)
- Mitsui K, Tokuzawa Y, Itoh H, Segawa K, Murakami M, Takahashi K, Maruyama M, Maeda M & Yamanaka S 2003 The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. *Cell* **113** 631–642. (doi:10.1016/S0092-8674(03)00393-3)
- Moretto-Zita M, Jin H, Shen Z, Zhao T, Briggs SP & Xu Y 2010 Phosphorylation stabilizes Nanog by promoting its interaction with Pin1. *PNAS* **107** 13312–13317. (doi:10.1073/pnas.1005847107)
- Morrissey EE, Tang Z, Sigrist K, Lu MM, Jiang F, Ip HS & Parmacek MS 1998 GATA6 regulates HNF4 and is required for differentiation of visceral endoderm in the mouse embryo. *Genes and Development* **12** 3579–3590. (doi:10.1101/gad.12.22.3579)
- Musch T, Oz Y, Lyko F & Breiling A 2010 Nucleoside drugs induce cellular differentiation by caspase-dependent degradation of stem cell factors. *PLoS ONE* **5** e10726. (doi:10.1371/journal.pone.0010726)
- Niwa H 2007 How is pluripotency determined and maintained? *Development* **134** 635–646. (doi:10.1242/dev.02787)
- Niwa H, Yamamura K & Miyazaki J 1991 Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene* **108** 193–200. (doi:10.1016/0378-1119(91)90434-D)
- Niwa H, Burdon T, Chambers I & Smith A 1998 Self-renewal of pluripotent embryonic stem cells is mediated via activation of STAT3. *Genes and Development* **12** 2048–2060. (doi:10.1101/gad.12.13.2048)
- Niwa H, Miyazaki J & Smith A 2000 Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. *Nature Genetics* **24** 372–376. (doi:10.1038/74199)
- Niwa H, Toyooka Y, Shimosato D, Strumpf D, Takahashi K, Yagi R & Rossant J 2005 Interaction between Oct3/4 and Cdx2 determines trophoblast differentiation. *Cell* **123** 917–929. (doi:10.1016/j.cell.2005.08.040)
- Niwa H, Ogawa K, Shimosato D & Adachi K 2009 A parallel circuit of LIF signalling pathways maintains pluripotency of mouse ES cells. *Nature* **460** 118–122. (doi:10.1038/nature08113)
- Palmieri SL, Peter W, Hess H & Scholer HR 1994 Oct-4 transcription factor is differentially expressed in the mouse embryo during establishment of the first two extraembryonic cell lineages involved in implantation. *Developmental Biology* **166** 259–267. (doi:10.1006/dbio.1994.1312)
- Pedersen RA, Wu K & Balakier H 1986 Origin of the inner cell mass in mouse embryos: cell lineage analysis by microinjection. *Developmental Biology* **117** 581–595. (doi:10.1016/0012-1606(86)90327-1)
- Ralston A & Rossant J 2008 Cdx2 acts downstream of cell polarization to cell-autonomously promote trophoblast fate in the early mouse embryo. *Developmental Biology* **313** 614–629. (doi:10.1016/j.ydbio.2007.10.054)
- Rao S & Orkin SH 2006 Unraveling the transcriptional network controlling ES cell pluripotency. *Genome Biology* **7** 230. (doi:10.1186/gb-2006-7-8-230)
- Riethmacher D, Brinkmann V & Birchmeier C 1995 A targeted mutation in the mouse E-cadherin gene results in defective preimplantation development. *PNAS* **92** 855–859. (doi:10.1073/pnas.92.3.855)
- Saito K, Abe H, Nakazawa M, Irokawa E, Watanabe M, Hosoi Y, Soma M, Kasuga K, Kojima I & Kobayashi M 2010 Cloning of complementary DNAs encoding structurally related homeoproteins from preimplantation mouse embryos: their involvement in the differentiation of embryonic stem cells. *Biology of Reproduction* **82** 687–697. (doi:10.1095/biolreprod.108.075697)
- Saito K, Ogawa A, Toyofuku K, Hosoi Y, Soma M, Iha M, Kasuga K, Kojima I & Kobayashi M 2011 Relationships between homeoprotein EGAM1C and the expression of the placental prolactin gene family in mouse placenta and trophoblast stem cells. *Reproduction* **141** 259–268. (doi:10.1530/REP-10-0355)
- Shimosato D, Shiki M & Niwa H 2007 Extra-embryonic endoderm cells derived from ES cells induced by GATA factors acquire the character of XEN cells. *BMC Developmental Biology* **7** 80. (doi:10.1186/1471-213X-7-80)
- Smith A 1991 Culture and differentiation of embryonic stem cells. *Journal of Tissue Culture Methods* **13** 89–94. (doi:10.1007/BF01666137)
- Soma M, Iha M, Sato S, Mori Y, Kasuga K, Kojima I & Kobayashi M 2011 Factors affecting the expression of differentiation marker genes for the primitive endoderm lineage in a mouse extra-embryonic endoderm stem cell line, XEN26 cells. *Biotechnology & Biotechnological Equipment* **25** 2679–2682. (doi:10.5504/bbeq.2011.0094)
- Strumpf D, Mao CA, Yamanaka Y, Ralston A, Chawengsaksophak K, Beck F & Rossant J 2005 Cdx2 is required for correct cell fate specification and differentiation of trophoblast in the mouse blastocyst. *Development* **132** 2093–2102. (doi:10.1242/dev.01801)
- Suzuki A, Raya A, Kawakami Y, Morita M, Matsui T, Nakashima K, Gage FH, Rodriguez-Esteban C & Izpisua Belmonte JC 2006 Nanog binds to Smad1 and blocks bone morphogenetic protein-induced differentiation of embryonic stem cells. *PNAS* **103** 10294–10299. (doi:10.1073/pnas.0506945103)
- Xu HM, Liao B, Zhang QJ, Wang BB, Li H, Zhong XM, Sheng HZ, Zhao YX, Zhao YM & Jin Y 2004 Wwp2, an E3 ubiquitin ligase that targets transcription factor Oct-4 for ubiquitination. *Journal of Biological Chemistry* **279** 23495–23503. (doi:10.1074/jbc.M400516200)
- Zhang Z, Liao B, Xu M & Jin Y 2007 Post-translational modification of POU domain transcription factor Oct-4 by SUMO-1. *FASEB Journal* **21** 3042–3051. (doi:10.1096/fj.06-6914com)

Received 11 October 2011

First decision 21 November 2011

Revised manuscript received 25 January 2012

Accepted 24 February 2012