

# Differentiation status dependent function of FOG-1

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The molecular interactions between transcription factors and cofactors play crucial roles in various biological processes, including haematopoiesis. FOG-1 is a cognate cofactor of GATA-1, and the FOG-1/GATA-1 complex is essential for the haematopoietic differentiation of erythroid cells and megakaryocytes. In order to elucidate the biological functions of FOG-1 in the different contexts of cell differentiation, we analysed the effects of FOG-1 expression on haematopoietic cell differentiation, using a combination of *in vitro* differentiation of mouse embryonic stem (ES) cells and conditional gene expression. FOG-1 suppressed the proliferation of primitive and definitive erythroid cells in all stages of differentiation. However, FOG-1 inhibited and enhanced megakaryopoiesis in the early and late differentiation stages, respectively, through different molecular mechanisms. In addition, FOG-1 inhibited the proliferation of ES cells, the molecular mechanism of which differs from those of erythroid and megakaryocytic cells. These results suggest that FOG-1 functions in a cell differentiation context-dependent manner.

## Introduction

Haematopoiesis is a cell differentiation process that proceeds from immature haematopoietic stem/progenitor cells to more than eight distinct lineages of mature blood cell (Papayannopoulou & Lemischka 2001). Complex and well-orchestrated transcriptional regulation governs the differentiation process. Gene-targeting experiments have revealed that many haematopoietic cell lineage-specific and differentiation stage-specific transcription factors play crucial roles during this process (Shivdasani & Orkin 1996; Orkin 2001). The zinc-finger transcription factor GATA-1 is one of the best-characterized haematopoietic transcription factors, and is essential for erythroid and megakaryocytic differentiation (Pevny *et al.* 1995; Fujiwara *et al.* 1996; Takahashi *et al.* 1997, 1998; Vyas *et al.* 1999). GATA-1 functions in co-operation with two classes of transcription cofactor, CBP/P300 and Friend of GATA-1 (FOG-1) (Tsang *et al.* 1997; Boyes *et al.* 1998; Nichols *et al.* 2000). CBP and P300 are transcription cofactors for various transcription factors; their functions in haematopoiesis are not defined. On the other hand, FOG-1 expression is mainly restricted to haematopoietic cells.

The FOG family is a novel class of multiple zinc finger proteins that interact with GATA factors (Fox *et al.* 1998,

1999; Cantor *et al.* 2002; Chang *et al.* 2002). The founding member of this family, FOG-1, was cloned by yeast two-hybrid screening, using the amino terminal zinc finger of GATA-1 as bait (Tsang *et al.* 1997). FOG-1 is a polypeptide of 998 amino acids that contains nine zinc fingers; four of the fingers interact independently with GATA-1 (Fox *et al.* 1999). FOG-1 is expressed abundantly in erythroid and megakaryocytic cells, which correlates with the expression pattern of GATA-1. FOG-1-null mice die of anaemia by embryonic day 11.5 (Tsang *et al.* 1998). Erythroid cells of the mutant mice show a marked but partial blockage, which is reminiscent of GATA-1-null erythroid precursors. In contrast to erythropoiesis, megakaryocytes do not develop in FOG-1-null mice. Null mutations in FOG-1 exhibit more profound effects on megakaryocytes than on erythrocytes.

The *Xenopus laevis* homologue of FOG (xFOG) has been cloned, and its structure has been found to be highly similar to FOG-1. Ectopic expression and explant assays have demonstrated that the xFOG protein acts as a repressor of erythroid lineage cells *in vivo* (Deconinck *et al.* 2000). In addition, the *Drosophila* FOG homologue U-shaped acts as a negative regulator of blood cells (Haenlin *et al.* 1997; Fossett *et al.* 2001). However, in mammals, FOG-1 over-expression in the erythroid lineage cell line G1E and myeloid cell line 416B promoted erythroid and megakaryocytic differentiation, respectively, when coexpressed with GATA-1 (Tsang *et al.* 1997;

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Crispino *et al.* 1999). This discrepancy between mammals and the other species may be attributable to species differences or to the fact that the data on mammalian erythroid and megakaryocytic differentiation have been derived from established cell lines, which do not necessarily reflect the physiological differentiation process.

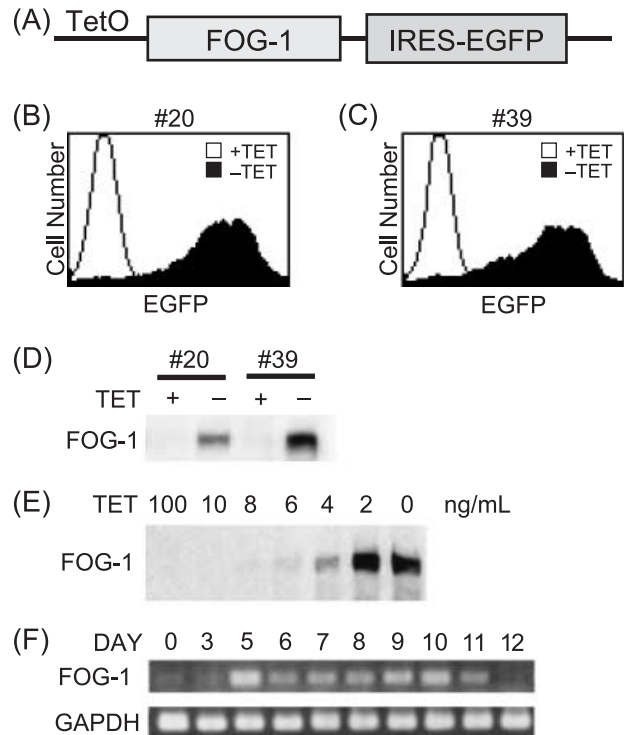
*In vitro* differentiation from mouse embryonic stem (ES) cells is a powerful tool for the analysis of the development and differentiation of various biological systems (Nakano 2003). Two methods have been reported for haematopoietic differentiation in ES cells: (1) embryoid body formation; and (2) coculture with the macrophage-colony stimulating factor (M-CSF)-deficient stroma cell line OP9. We have utilized the OP9 system to analyse various aspects of haematopoietic development and differentiation (Nakano *et al.* 1996, 1997). The OP9 system recapitulates the early phase of haematopoietic development and the differentiation from multipotential haematopoietic progenitors to all of the myeloid lineage cells, B lineage cells, and NK cells. It is noteworthy that differentiation is induced only once in the OP9 system, and that differentiation proceeds in a highly synchronous manner. Thus, at one distinct time point after the initiation of differentiation induction, most cells belong to a single differentiation stage.

Recently, the tetracycline-based conditional gene expression method has been used successfully with the OP9 system (Era & Witte 2000; Era 2002; Kitajima *et al.* 2002). The combination of conditional gene expression and OP9 cells enables us to analyse the function of various genes at distinctive differentiation stages of haematopoietic development and differentiation. In the present study, we used this method to analyse the functions of FOG-1 in erythroid cells, megakaryocytic cells, and ES cells. Unexpectedly, the effects of FOG-1 on the different stages of megakaryocyte differentiation varied significantly. Furthermore, experiments using the GATA-1-null haematopoietic cells, or the conditional expression of a mutant form of FOG-1 that could not bind to the transcriptional corepressor protein C-terminal binding protein (CtBP), revealed that different binding proteins were necessary for the distinctive effects of FOG-1. These data clearly show that the functions of FOG-1 should be interpreted in a differentiation context-dependent manner.

## Results

### Establishment of ES cell lines that conditionally express FOG-1

In order to understand the biological roles of FOG-1 in stage- and lineage-specific haematopoietic cells, we



**Figure 1** Conditional expression of FOG-1 and EGFP. (A) Construction of TetO-CMV-FOG-1/IRES-EGFP; TetO represents the tetracycline-responsive element. (B, C) FACS analyses of EGFP expression in ES cell clones no. 20 (B) and no. 39 (C) 24 h after tetracycline deprivation. (D) Western blot analysis of FOG-1 expression 24 h after tetracycline deprivation in the representative ES cell clones nos. 20 and 39 that conditionally express FOG-1. (E) The effect of TET concentration on FOG-1 expression. Western blot analysis of FOG-1 expression 24 h after the concentration of tetracycline was changed; clone no. 39 was used for this analysis. (F) RT-PCR analysis of FOG-1 during the course of differentiation from control ES cells in the presence of TPO.

established ES cell lines that express FOG-1 under the control of the TET-Off conditional gene expression system. The Tet-O CMV-FOG-1-IRES-EGFP was introduced by electroporation into an ES cell line that expressed the Tet-regulatable transcription factor  $\text{tTA-2}$  (Fig. 1A). Forty-eight clones were selected, and analysed for conditional regulation of EGFP expression. Eight clones showed EGFP expression that was strictly regulated, i.e. these clones expressed EGFP only in the absence of tetracycline. Clones nos. 20 and 39 were chosen for further analysis, as a high percentage of these cells expressed EGFP (96.5% and 94.3% for clones nos. 20 and 39, respectively) 24 h after tetracycline deprivation (Fig. 1B,C). The expression of FOG-1 was confirmed by Western blotting (Fig. 1D), and the expression levels

of induced FOG-1 correlated inversely with the concentrations of tetracycline (Fig. 1E). Since these two clones showed essentially similar results in subsequent experiments, the results for clone no. 39 are presented as representative data.

### Inhibitory effects of FOG-1 on haematopoietic progenitor cells and erythroid cells

In order to define in detail the roles of FOG-1 during haematopoietic differentiation, *in vitro* haematopoietic differentiation of the ES cells was performed, so that conditional FOG-1 expression could be achieved. As previously described, multipotential haematopoietic progenitors began to appear on day 5 of OP9 *in vitro* haematopoietic differentiation. At day 7 of induction, ES cells gave rise to immature haematopoietic colonies. Primitive embryonic and definitive adult erythroid cells appeared between days 6 and 8 and between days 12 and 15, respectively, in the presence of erythropoietin (EPO). FOG-1 expression during the differentiation from control ES cells in the presence of TPO was examined by RT-PCR analysis (Fig. 1F). FOG-1 was detectable between day 5 and day 11. To reveal the activity of FOG-1 on immature progenitors, FOG-1 was over-expressed from day 5, and the colonies were counted on day 7 (Fig. 2A). The number of haematopoietic colonies decreased to < 20% as the result of FOG-1 over-expression. The development of primitive erythrocytes was analysed on day 8 of differentiation induction in the presence of EPO. The total number of cells was drastically reduced (Fig. 2B), and the vast majority of the cells expressed neither TER-119 nor an earlier erythroid marker, the CD71/transferrin receptor (Fig. 2C). These data show that FOG-1 over-expression impairs the development of immature haematopoietic progenitors and primitive erythrocytes.

Next, the cells were counted on day 12 of differentiation, to examine the effects of FOG-1 over-expression on definitive erythropoiesis. As shown in Fig. 2D, when FOG-1 was over-expressed between days 5 and 12, the number of cells decreased to about 10% of the control number. This effect could be attributed to the effect of FOG-1 on immature haematopoietic progenitors (Fig. 2A). FOG-1 was then expressed for a restricted period of time, to examine in greater detail the function of FOG-1. As shown in Fig. 2D, FOG-1 over-expression during both the early period (between days 5 and 8) and late period (between days 8 and 12) inhibited definitive erythropoiesis. The TER-119 expression patterns and the morphologies of the FOG-1-expressing cells were essentially the same as those of control cells

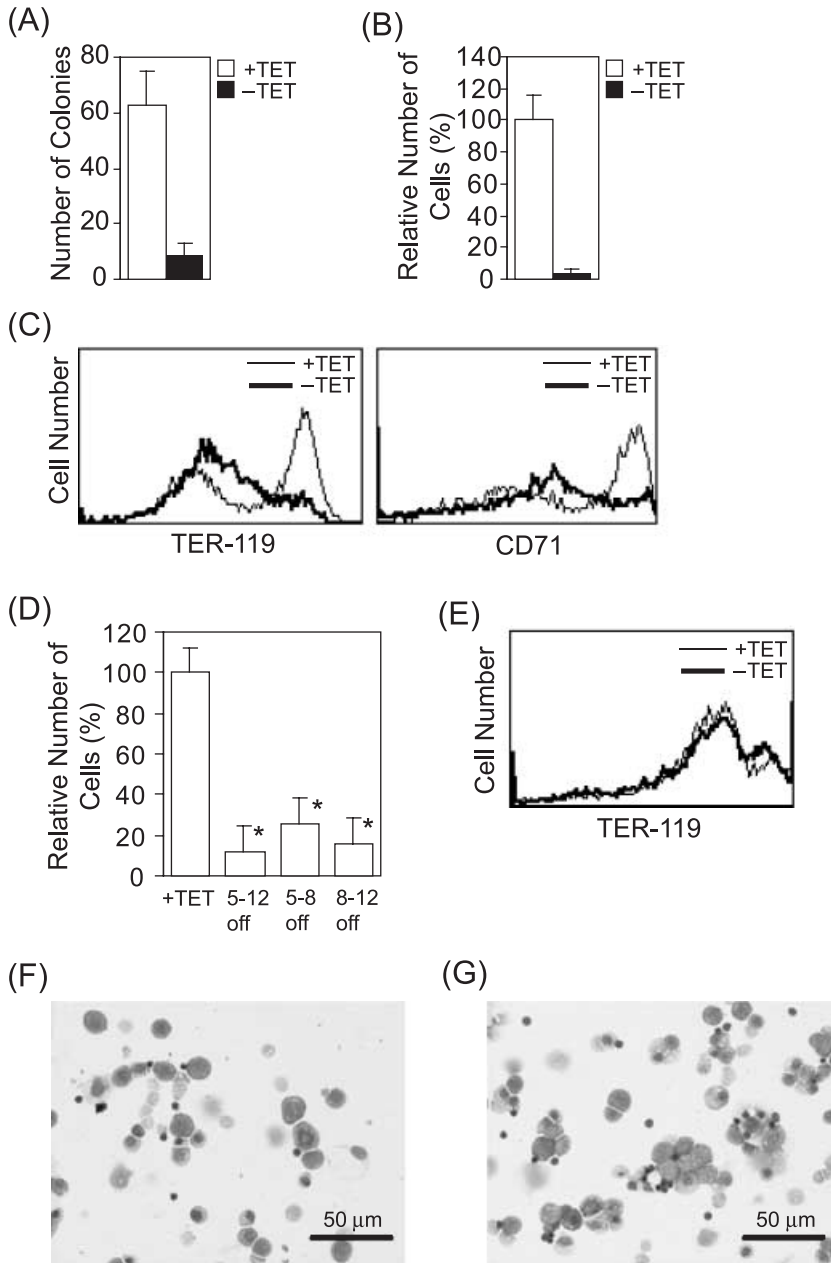
(Fig. 2E–G). Taken together, these results indicate that FOG-1 inhibits the proliferation of definitive erythrocytes throughout the differentiation process.

### Differentiation stage-dependent effects of FOG-1 on megakaryopoiesis

We also examined the effects of FOG-1 on the proliferation and differentiation of megakaryocytes following the addition of thrombopoietin (TPO). FOG-1 was expressed starting on day 5, and the number of colonies was counted on day 8 postinduction. As shown in Fig. 3A,B, FOG-1 over-expression reduced the numbers of immature megakaryocytic colonies and mature megakaryocytes on days 8 and 12, respectively. Compared to the effects of FOG-1 on erythroid cells (Fig. 2A,B), those on megakaryocytes were mild. Proliferation of erythroid cells was inhibited by FOG-1 throughout the differentiation, but that of megakaryocytes was enhanced at the late phase of differentiation as shown below. Therefore, at least in part, milder effect of FOG-1 can be explained by the proliferation of megakaryocytes at later phase of differentiation. FOG-1 did not alter significantly the expression pattern of either the megakaryocyte marker platelet glycoprotein V (Fig. 3C) or GPIIb (data not shown), both of which are expressed during the stages from immature to mature megakaryocytes. Thus, FOG-1 expression seems to suppress megakaryopoiesis, as well as erythropoiesis. Then, as in the erythropoiesis experiments, the expression of FOG-1 was induced for restricted periods of time, to allow the analysis of the megakaryocyte differentiation stage-dependent function of FOG-1. Unexpectedly, the number of megakaryocytes increased two-fold when FOG-1 was expressed after day 8 (Fig. 3D).

To elucidate the effect of FOG-1 on megakaryopoiesis, we carried out two additional experiments. The first experiment involved a simple suspension culture that contained TPO, in which the megakaryocytes were maintained in an exclusive manner. The induced cells were transferred to the culture medium that contained TPO in the absence of OP9 layers, and the megakaryocytes were counted on days 10 and 12 (Fig. 3E). Under this condition, FOG-1 enhanced cell proliferation.

The second experiment consisted of a colony formation assay. OP9 cell differentiation was induced until day 8. The induced cells were transferred to methylcellulose semisolid medium that contained TPO, and the numbers of small, medium, and large colonies were assessed. During the course of the experiment, FOG-1 was expressed for the indicated time periods (Table 1). Compared to the control culture, the numbers of small and middle

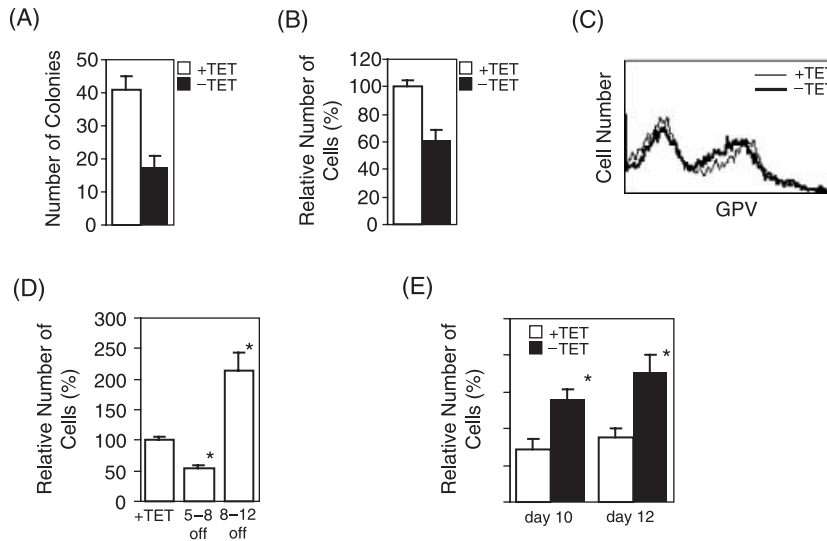


**Figure 2** Effect of FOG-1 induction on erythropoiesis. A total of  $10^5$  cells that were induced on day 5 were seeded on to the OP9 stroma cell layer, and the cells were cultured in the presence or absence of tetracycline. (A) Number of colonies on day 7 of induction of the Tet-regulated FOG-1 clone. Tetracycline was removed starting on day 5. Data are shown as the mean  $\pm$  SD of six samples, and are significantly different ( $P < 0.0001$  by the *t*-test). (B) Number of cells on day 8 of differentiation induction in the presence of EPO. Data are shown as the mean  $\pm$  SD of six samples, and are significantly different ( $P < 0.0001$  by the *t*-test). (C) Surface expression of the lineage marker TER-119 and CD71 (transferrin receptor) on day 8 in the presence of EPO. Tetracycline was removed starting on day 5. (D) Number of cells on day 12 in the presence of EPO. Tetracycline was removed at the time-points indicated in the figure. Data are shown as the mean  $\pm$  SD of six samples. \*Significantly different ( $P < 0.001$  by the *t*-test) from the control (+ TET). (E) Surface expression of the lineage marker TER-119 on day 12 in the presence of EPO. Tetracycline was removed starting on day 5. (F, G) Morphology of the cells on day 12 of induction in the presence of EPO and in the presence (F) or absence (G) of tetracycline. Tetracycline was removed starting on day 5.

sized colonies decreased significantly when FOG-1 was expressed between days 5 and 8, and between days 5 and 12. In contrast, these numbers were not affected, and the number of large colonies was significantly increased, when FOG-1 was expressed starting on day 8. Therefore, FOG-1 over-expression between days 5 and 8 represses the production of megakaryocyte progenitors from multipotential haematopoietic progenitors, whereas FOG-1 expression after day 8 enhances the proliferation of megakaryocytes.

**Characterization of the effects of FOG-1 on late-stage megakaryocyte differentiation**

The expression patterns of FOG-1, GATA-1, and GATA-2 were analysed after the cells were transferred to the suspension culture condition, starting at day 8. Western blot analysis was carried out to examine the FOG-1 expression levels (Fig. 4A). Endogenous FOG-1 expression decreased after day 8. Although FOG-1 expression was detected on days 10 and 12, even in the presence of



**Figure 3** Effect of FOG-1 on megakaryopoiesis. (A) Number of colonies on day 8 of induction in the presence of TPO. Data are shown as the mean  $\pm$  SD of six samples, and are significantly different ( $P < 0.0001$  by the *t*-test). (B) Number of cells on day 12 in the presence of TPO. Tetracycline deprivation started on day 5. Data are shown as the mean  $\pm$  SD of six samples, and are significantly different ( $P < 0.01$  by the *t*-test). (C) Surface expression of the lineage marker GPV on day 12 in the presence of TPO. Tetracycline deprivation started on day 5. (D) Number of cells on day 12 in the presence of TPO. The timing of tetracycline deprivation is shown in the figure. Data are shown as the mean  $\pm$  SD of six samples. \*These data are significantly different ( $P < 0.001$  by the *t*-test) from the control (+ TET). (E) Number of megakaryocytes in the suspension culture. A total of  $4 \times 10^4$  megakaryocytes that were induced on day 8 were seeded on to non-coated dishes, and cultured in the presence or absence of tetracycline. \*These data are significantly different ( $P < 0.001$  by the *t*-test) from the control (+ TET).

**Table 1** Number of megakaryocyte colonies after conditional expression of FOG-1

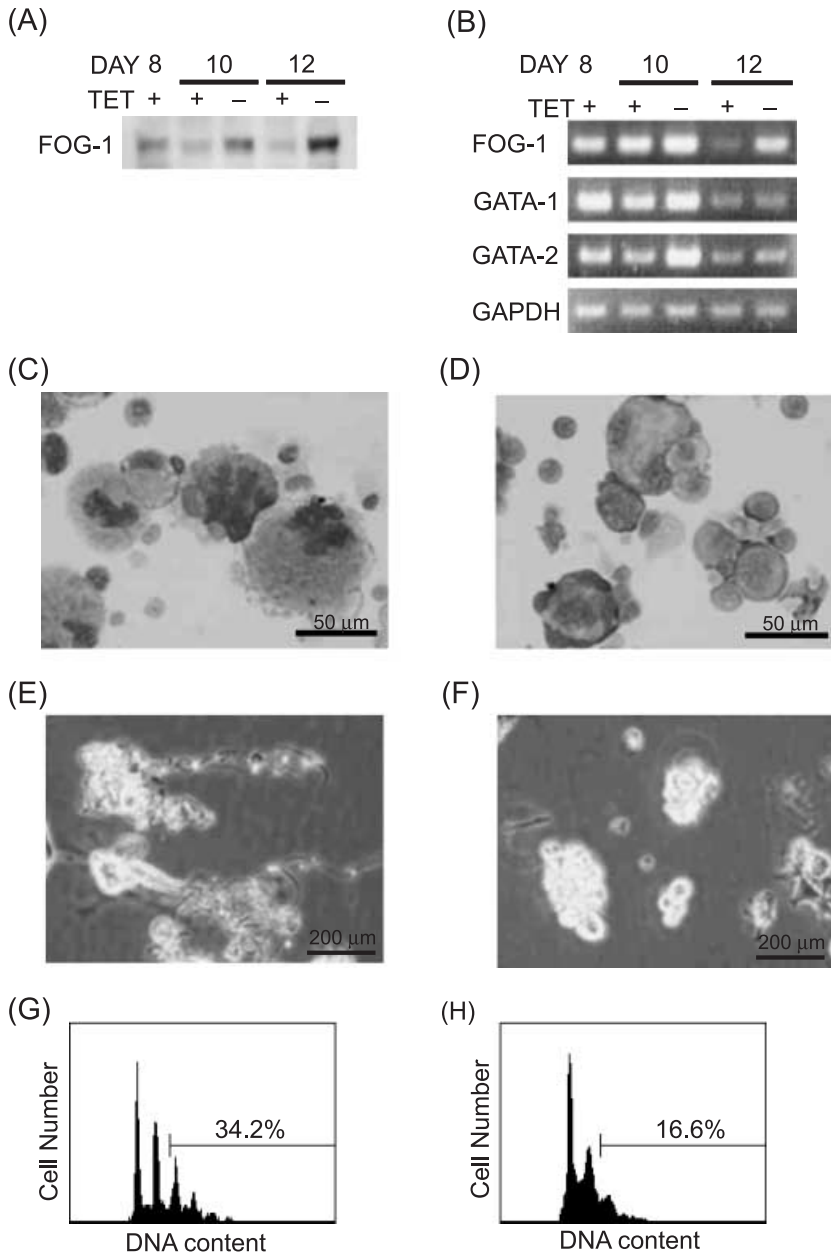
Conditional expression of FOG-1		Number of colonies			
Day 5–8	Day 8–12	Small	Middle	Large	Total
–	–	116 $\pm$ 9	38 $\pm$ 7	3 $\pm$ 1	157 $\pm$ 15
+	–	33 $\pm$ 2*	9 $\pm$ 2†	1 $\pm$ 0	42 $\pm$ 2*
+	+	36 $\pm$ 3*	12 $\pm$ 1†	2 $\pm$ 0	50 $\pm$ 3*
–	+	109 $\pm$ 10	46 $\pm$ 7	20 $\pm$ 4‡	175 $\pm$ 18

$1 \times 10^4$  day 5 cells were plated on to OP9 stromal layer and passed on to methylcellulose medium at day 8. After day 5, 10 ng/mL TPO was added into medium. Tetracycline was deprived as shown in the table. The number of colonies was counted after another 7 days. Small colonies and large colonies consisted of less than 20 cells and more than 100 cells, respectively. Data are shown as the mean  $\pm$  SD of five samples. \* and †The data are significantly different from those of '–' ( $P < 0.0001$  and  $P < 0.001$  by Student's *t*-test, respectively); ‡ The data is significantly different from those of the other conditions ( $P < 0.001$  by Student's *t*-test).

tetracycline, expression was higher in the absence of tetracycline. The results of the RT-PCR analysis of FOG-1 correlated well with the Western blot findings (Fig. 4B). The expression of GATA-1 was not affected significantly by FOG-1, while GATA-2 expression was higher in the cells that over-expressed FOG-1.

The effects of FOG-1 on late-stage megakaryocyte maturation were analysed; FOG-1 was over-expressed in

the suspension culture starting on day 8. The cell size and cytoplasm were smaller and darker, respectively, than in the control on day 12 (Fig. 4C,D). Phase contrast microscopic examination showed that control megakaryocytes promoted proplatelet formation, but that FOG-1-over-expressing megakaryocytes did not (Fig. 4E,F). The level of polyploidization of the FOG-1-over-expressing megakaryocytes was significantly lower than that of the



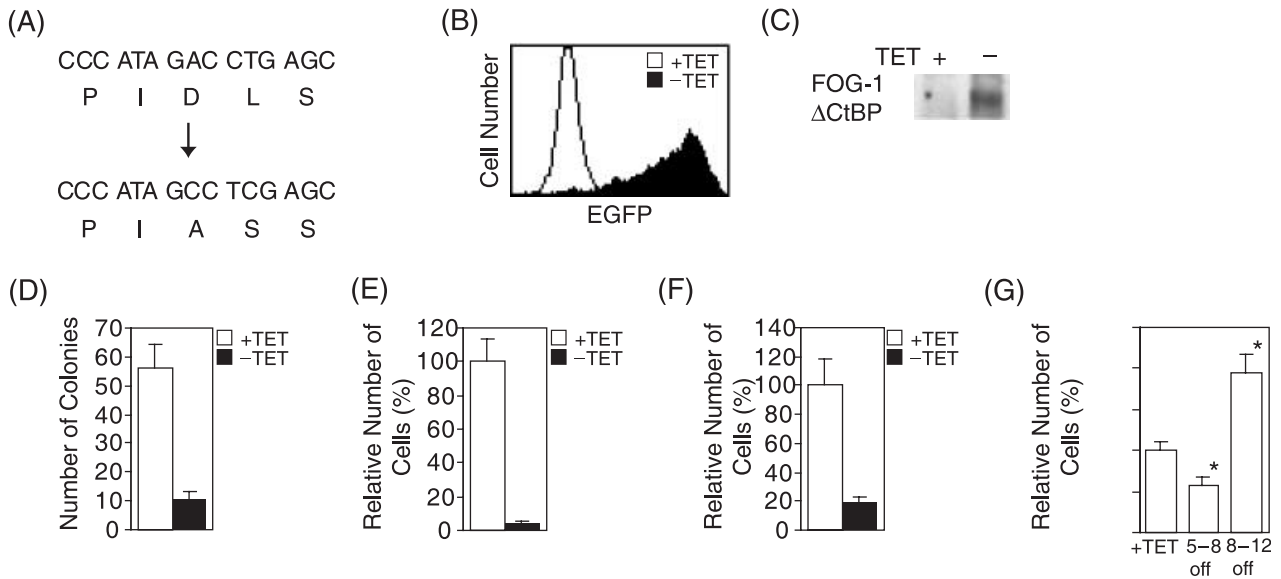
**Figure 4** Analysis of megakaryocytes following the over-expression of FOG-1 during terminal differentiation. (A) Western blot analysis of FOG-1 in megakaryocytes in the presence or absence of tetracycline. (B) RT-PCR analysis of expression of FOG-1, GATA-1, and GATA-2 in FOG-1-induced megakaryocytes. GATA-2 was induced by depriving the cells of tetracycline. (C, D) Morphologies of the cells on day 12 of induction in the presence (C) or absence (D) of tetracycline. (E, F) Proplatelet formation of megakaryocytes in suspension culture in the presence (E) or absence (F) of tetracycline. (G, H) Polyploidization of megakaryocytes in suspension culture in the presence (G) or absence (H) of tetracycline.

control (Fig. 4G,H). Taken together, our results demonstrate that late-phase over-expression of FOG-1 produces megakaryocytes of immature phenotype through the inhibition of differentiation.

**Effects of the C-terminal-binding protein on the function of FOG-1**

It has been reported that FOG-1 binds to a transcriptional corepressor, CtBP, and that this binding may influence FOG-1 function (Fox *et al.* 1999; Deconinck *et al.*

2000; Katz *et al.* 2002). A FOG-1 mutant that was unable to bind to CtBP (FOG-1ΔCtBP) was produced, and its effects on erythroid and megakaryocytic differentiation were analysed using the same experimental system described here. The FOG-1ΔCtBP mutation and its conditional expression pattern are shown in Fig. 5A–C. The data on FOG-1ΔCtBP over-expression are quite similar to those obtained for wild-type FOG-1 over-expression (Fig. 5D–G). FOG-1ΔCtBP repressed the production of haematopoietic progenitors and erythroid lineage cells. Although megakaryocyte production was



**Figure 5** Effects of FOG-1 $\Delta$ CtBP on erythropoiesis and megakaryopoiesis. (A) Mutation introduced in FOG-1 to eliminate binding with CtBP. (B, C) FACS analysis of the expression levels of EGFP (B) and FOG-1 (C) in a representative ES cell clone that conditionally expresses FOG-1 $\Delta$ CtBP, 24 h after tetracycline deprivation. (C) Western blot analyses of FOG-1 $\Delta$ CtBP expression in a representative ES cell clone 24 h after tetracycline deprivation. (D) Number of colonies on day 7 of induction of the Tet-regulated FOG-1 $\Delta$ CtBP clone. Data are shown as the mean  $\pm$  SD of six samples, and are significantly different ( $P < 0.0001$  by the  $t$ -test). Tetracycline was removed starting on day 5. (E, F) Number of cells on day 8 (E) and day 12 (F) in the presence of EPO. Data are shown as the mean  $\pm$  SD of six samples, and are significantly different ( $P < 0.0001$  by the  $t$ -test). Tetracycline was removed starting on day 5. (G) Number of cells on day 12 in the presence of TPO. Tetracycline was removed at the time-points shown in the figure. Data are shown as the mean  $\pm$  SD of six samples. \*These data are significantly different ( $P < 0.001$  by the  $t$ -test) from the control (+TET).

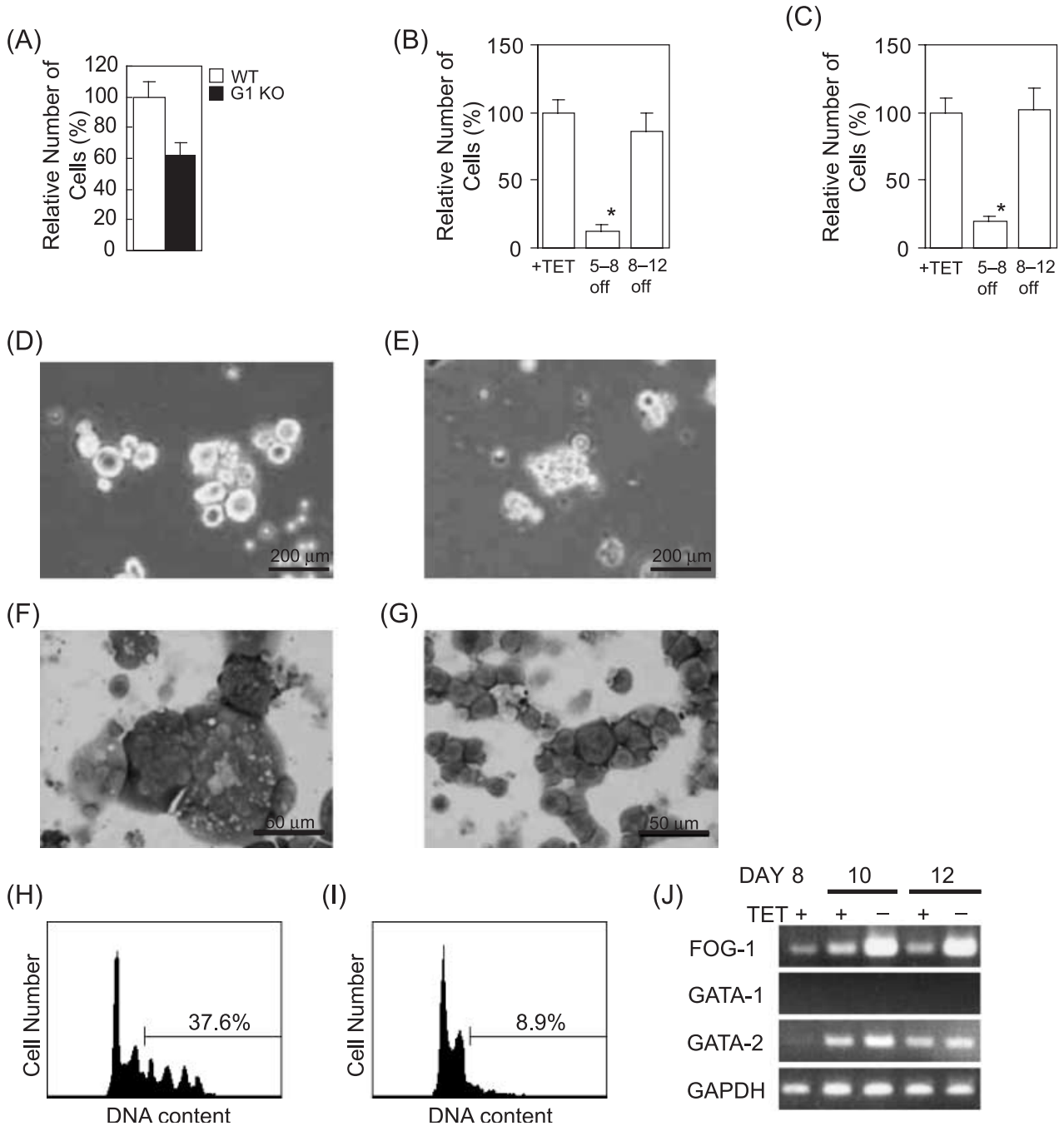
repressed by FOG-1 $\Delta$ CtBP over-expression from day 5 to day 8, it was enhanced by FOG-1 $\Delta$ CtBP over-expression from day 8. The phenotypes of the FOG-1 $\Delta$ CtBP-over-expressing megakaryocytes were identical to those of the FOG-1-over-expressing controls (data not shown).

#### Effects of FOG-1 and FOG-1 $\Delta$ CtBP on megakaryopoiesis in GATA-1-null cells

We produced GATA-1-null ES cells and generated Tet-off expression of FOG-1, to examine in detail the co-operative effects of GATA-1 and FOG-1. First, we examined the megakaryocyte development from GATA-1 null ES cells by OP9 system. The number of megakaryocytes was significantly reduced by the null mutation of GATA-1 (Fig. 6A). Differentiation of megakaryocytes was also impaired by the mutation (data not shown). Although megakaryocyte markers such as GPV, GPIIb and acetylcholine-esterase were normally expressed, proplatelet formation was hardly detected. Next, we examined the effects of FOG-1 and FOG-1 $\Delta$ CtBP under the GATA-1 null condition. When either FOG-1 or FOG-1 $\Delta$ CtBP was expressed between days 5 and 8,

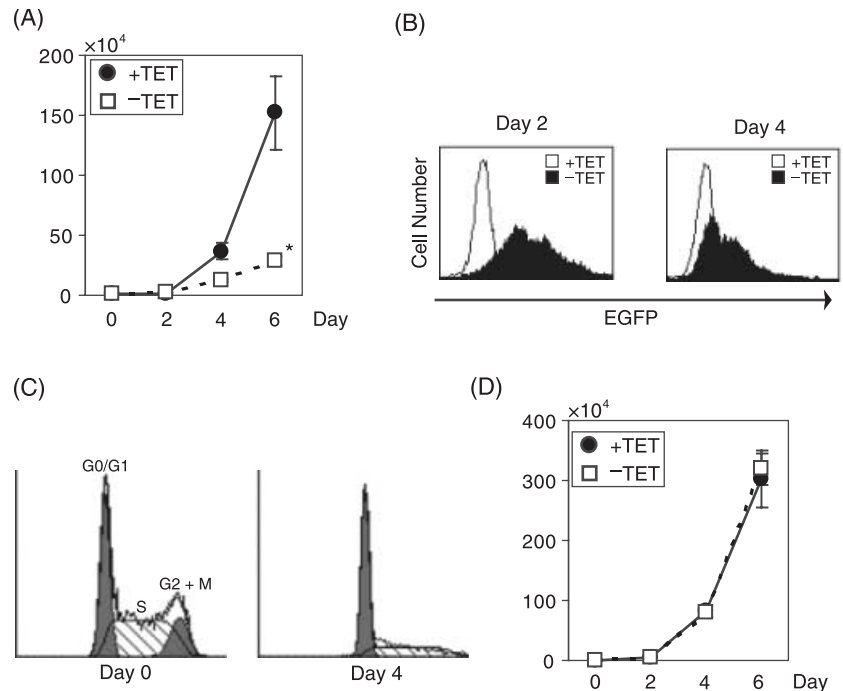
the number of megakaryocytes decreased (Fig. 6B,C). However, FOG-1 or FOG-1 $\Delta$ CtBP expression after day 8 did not alter the number of megakaryocytes. These data show that whereas the interaction with GATA-1 is indispensable for late-phase enhancement, it is dispensable for early phase inhibition by FOG-1. Although GATA-1 is dispensable for the early phase inhibition, GATA-1 null mutation brought about more profound inhibitory effect of FOG-1. This implies that simple co-operative effects of FOG-1 and GATA-1 cannot explain the inhibition by FOG-1.

As reported previously, proplatelet formation was scarcely detected in the GATA-1-null megakaryocytes (Fig. 6D) (Shivdasani *et al.* 1997). FOG-1 over-expression from day 8 reduced the size of the megakaryocytes (Fig. 6E) and promoted an immature phenotype compared with control (Fig. 6F,G). In addition, the percentage of polyploid megakaryocytes was significantly reduced by FOG-1 (Fig. 6H,I). FOG-1 $\Delta$ CtBP gave essentially similar results (data not shown). In the GATA-1 KO megakaryocytes, the GATA-2 expression level was similar to that of the control megakaryocytes (Figs 4B and 6J). Therefore, expression level of GATA-2 does not



**Figure 6** Effects of FOG-1 and FOG-1ΔCtBP on megakaryocytic differentiation in the GATA-1-null condition. (A) Number of megakaryocytes developed from control and GATA-1 null ES cells. These data are significantly different ( $P < 0.01$  by the *t*-test). (B, C) Number of cells in FOG-1- (B) and FOG-1ΔCtBP- (C) expressing clones on day 12 in the presence of TPO. Tetracycline was removed at the indicated time-points. Data are shown as the mean  $\pm$  SD of six samples. \*These data are significantly different ( $P < 0.0001$  by the *t*-test) from the control (+TET). (D, E) Phase contrast microscopy of the FOG-1-induced cells in suspension culture in the presence (D) or absence (E) of tetracycline. Tetracycline was removed starting on day 8. (F, G) May-Giemsa staining of the FOG-1-expressing cells on day 12 of induction in the presence (F) or absence (G) of tetracycline. Tetracycline was removed starting on day 8. (H, I) Polyploidization of FOG-1-expressing megakaryocytes in the presence (H) or absence (I) of tetracycline. Tetracycline was removed starting on day 8. (J) Expression of FOG-1, GATA-1, and GATA-2 in FOG-1-induced megakaryocytes.

**Figure 7** Effects of FOG-1 and FOG-1 $\Delta$ CtBP induction on the proliferation of ES cells. (A) Number of Tet-regulated FOG-1 clones in the presence or absence of tetracycline.  $10^4$  ES cells were plated at day 0, and the number of cells was counted at day 2, 4 and 6. Data are shown as the mean  $\pm$  SD of five sample, and the differences between +TET and -TET are statistically significant on day 4 ( $P < 0.01$ ) and day 6 ( $P < 0.0001$ ). (B) FACS analyses of the EGFP expression of FOG-1 two or four days after tetracycline deprivation. (C) Cell cycle analyses of ES cells zero or four days after the tetracycline deprivation. At day 0, the peak on the left side and on the right side show G0/G1 phase and G2 +M phase, respectively. The oblique line in the middle shows S phase. At day 4, S phase and G2 +M phase were decreased. (D) Number of Tet-regulated FOG-1 $\Delta$ CtBP clones in the presence or absence of tetracycline. Data are shown as the mean  $\pm$  SD of five samples.



seem to give significant influence on the megakaryocyte proliferation and differentiation. Since GATA-2 can collaborate with FOG-1, we cannot exclude the possibility that FOG-1 would play some role in megakaryocyte differentiation by synergizing with GATA-2. FOG-1 increased the expression of GATA-2 in the GATA-1-null context, but this increase was smaller than that in the control. Another haematopoietic GATA factor, GATA-3, was not expressed in the megakaryocytes under any condition (data not shown). These data show that FOG-1 inhibits megakaryocyte maturation in a GATA-1-independent manner.

#### Effects of FOG-1 and FOG-1 $\Delta$ CtBP on ES cell proliferation

We also analysed the effects of FOG-1 on ES cells. ES cell growth was impaired significantly by FOG-1 over-expression. After day 6, the number of cells was reduced to about 25% of the control number (Fig. 7A). The decrease in EGFP suggests poor proliferation of the FOG-1-expressing cells (Fig. 7B). Cell cycle analysis showed that the cells that expressed FOG-1 were arrested at the G0/G1 phase (Fig. 7C). This inhibitory effect of FOG-1 was similarly observed when FOG-1 was over-expressed in the GATA-1 null ES cells (data not shown). The over-expression of FOG-1 $\Delta$ CtBP produced completely different results. As shown in Fig. 7D, FOG-1 $\Delta$ CtBP did not affect the ES cell proliferation. Morphological ana-

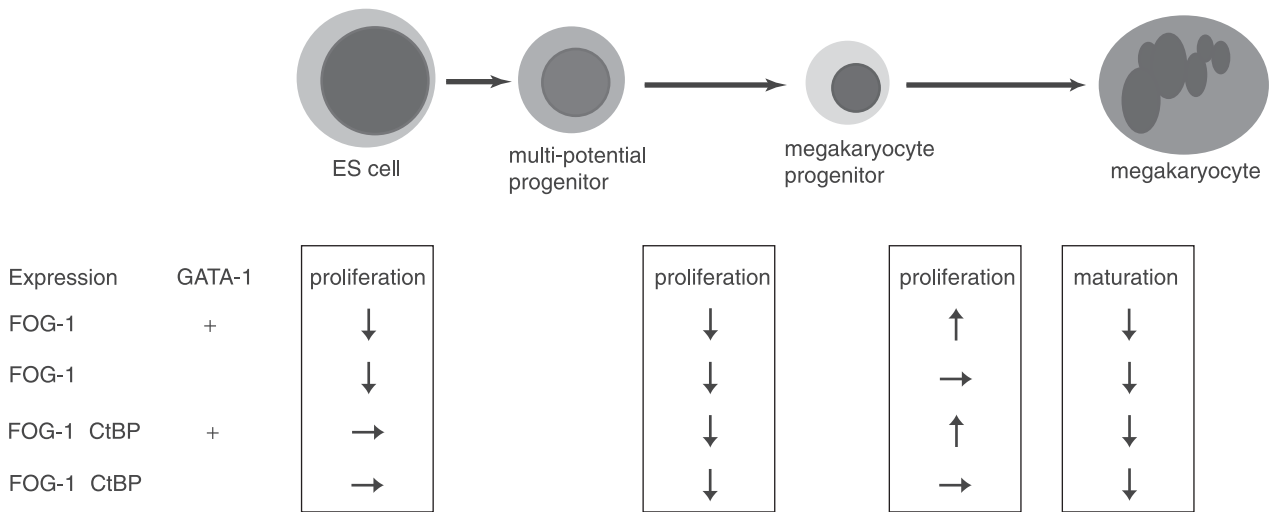
lysis showed no particular difference between the control and FOG-1-over-expressing ES cells (data not shown). Furthermore, the differentiation markers of the three germ layers were not altered by FOG-1 over-expression (data not shown). These data suggest that ES cell differentiation is not induced by FOG-1 over-expression. RT-PCR analysis revealed that none of the GATA factors were expressed in the ES cells (data not shown). Therefore, FOG-1 inhibits the proliferation of ES cells in a CtBP-dependent, GATA-independent manner.

#### Discussion

In this paper, we demonstrate that FOG-1 influences cell differentiation and proliferation in a differentiation context-dependent manner. Compared to the simple growth inhibition of erythroid cells and ES cells, the effects of FOG-1 on megakaryopoiesis are more complicated and are differentiation stage-dependent (Fig. 8). The potential molecular mechanisms involved in FOG-1 function were revealed using mutant cell lines and genetic analyses.

#### Differentiation stage-specific function of FOG-1

When FOG-1 was expressed between days 5 and 8, i.e. from the stage of multipotential or erythro-megakaryocytic progenitors to megakaryocytic progenitors, the proliferation of megakaryocytic cells was impaired. However,



**Figure 8** Schematic diagram of the effects of FOG-1 and FOG-1 $\Delta$ CtBP in the wild-type and GATA-1-null conditions.

during terminal differentiation of the megakaryocytes, FOG-1 enhanced the proliferation and inhibited the maturation of megakaryocytes. An elegant series of experiments and the analysis of GATA-1-mutant patients have revealed that FOG-1 is essential for megakaryopoiesis, and that this effect is dependent on co-operation with GATA-1 (Nichols *et al.* 2000; Freson *et al.* 2001, 2002; Mehaffey *et al.* 2001). Interaction between FOG-1 and GATA-1 would explain the enhanced proliferation of megakaryocytes, since in the GATA-1-null condition, proliferation was not enhanced by FOG-1 over-expression. However, the decreased proliferation of early megakaryocyte progenitors by FOG-1 was GATA-1-independent, since the effect was observed even in the GATA-1-null condition. This inhibitory effect might be explained by the co-operation of FOG-1 with GATA-2.

Considering the effect of GATA-2 on the enhancement of multipotential haematopoietic progenitors (Kitajima *et al.* 2002) and the phenotype of GATA-2-null mice (Tsai *et al.* 1994; Shivdasani & Orkin 1996), the reduced proliferation seen during the multipotential progenitor phase may be explained by FOG-1 interference with the transcription of GATA-2. Meanwhile, the increase in GATA-2 might enhance the proliferation of megakaryocytes in the late stage of differentiation, since GATA-2 expression on day 10 was increased by the expression of FOG-1 in the presence of TPO (Fig. 4B). However, since the simple over-expression of GATA-2 from day 8 of differentiation did not induce the proliferation or differentiation block of megakaryocytes (unpublished data), the increased expression of GATA-2 alone cannot account for the proliferative effect. Therefore, the concomitant expression and subsequent co-operative functions of

GATA-1 and FOG-1 may play crucial roles in the enhanced proliferation of megakaryocytes. In contrast, impaired terminal differentiation was observed even in the GATA-1-null condition. There are two possible explanations for this effect: firstly, FOG-1 alone plays important roles in this impairment or secondly, a redundant function of GATA-2 compensates for the function of GATA-1. In either case, it is conceivable that the molecular functions of FOG-1 in proliferation and differentiation can be distinguished with respect to the binding partner.

Both the GATA-1-deficient megakaryocytes and FOG-1-expressing wild-type megakaryocytes displayed poor proplatelet formation (Figs 6 and 4, respectively). These data show that GATA-1 is necessary for proplatelet formation and that FOG-1 represses proplatelet formation. It has been reported that FOG-1 activates or inhibits the transcriptional activity of GATA-1 in various gene expression systems (Tsang *et al.* 1997; Fox *et al.* 1999; Gaines *et al.* 2000; Kawabata *et al.* 2001; Wang *et al.* 2002). The transcriptional interference of FOG-1 with GATA-1 may account for the reduced proplatelet formation. Taken together, our results show that FOG-1 changes its biological activity according to the differentiation status during megakaryopoiesis, and that different co-operative partners are required for FOG-1 function at different cell stages.

#### Functional association between FOG-1 and CtBP

CtBP is a broadly expressed corepressor protein that binds to the Pro-X-Asp-Leu-Ser (PXDL) motifs that are present in many transcription factors, including

Kruppel, Net, and the adenoviral E1A (Turner & Crossley 1998; Criqui-Filipe *et al.* 1999; Postigo & Dean 1999). FOG-1 has been reported to function as a repressor of GATA-1-activated transcription (Fox *et al.* 1999; Kawabata *et al.* 2001). This repression is mediated by a small region of FOG-1 (residues 724–834), within which the CtBP-binding motif is located. The binding of FOG-1 to CtBP appears to be essential for transcriptional repression, since FOG-1ΔCtBP does not efficiently repress GATA-1-activated transcription. These molecular data suggest the biological importance of the interaction between FOG-1 and CtBP.

Experiments using FOG-1ΔCtBP have provided some insights into the molecular functions of FOG-1. FOG-1ΔCtBP rescued erythropoiesis and megakaryopoiesis of a FOG-1-null cell line, and showed increased erythropoietic rescue, as compared to the wild-type FOG-1. In addition, mutant mice, into which the FOG-1ΔCtBP mutation had been introduced at the FOG-1 locus, did not show abnormal erythropoiesis (Katz *et al.* 2002). In *Xenopus*, erythropoiesis was inhibited by the over-expression of xFOG, mammalian FOG-1, and FOG-2 (Deconinck *et al.* 2000). Interestingly, the mutated FOG-2, which is unable to bind to CtBP, functioned as a better inducer of erythropoiesis.

We did not observe any enhancement of erythroid differentiation as a result of the over-expression of FOG-1. Instead, FOG-1 impaired significantly the proliferation of erythroid cells at all differentiation stages. This inhibitory effect was independent of the interaction of FOG-1 with CtBP, since the mutated FOG-1ΔCtBP showed similar reductions of erythroid cell proliferation. Although FOG has been reported to function as a repressor of red blood cells in both the mouse and *Xenopus*, the molecular mechanisms of repression seem to differ in terms of CtBP association. Although GATA-1 expression was reduced by FOG in *Xenopus*, it was not affected by FOG in our system. These results suggest that the molecular functions of FOG are different in mammals and amphibians. Alternatively, the data from *Xenopus* may reflect the effect of FOG-1 on the differentiation from mesoderm to haematopoietic cells, but not that of erythroid differentiation.

The inhibition of ES cell proliferation by FOG-1 was independent of GATA factors, since GATA factors 1–6 were not expressed in the ES cells. In addition, this inhibition was mediated by the binding of FOG-1 to CtBP, since FOG-1ΔCtBP had no effect on ES cell proliferation. These data show that FOG-1 functions as a cell proliferation repressor in combination with CtBP, and that FOG-1 function does not require GATA factors. Thus, FOG-1 induces impaired proliferation of haematopoietic cells

and ES cells, while the molecular function of FOG-1 varies among the different cell differentiation stages.

### Usefulness of ES cell differentiation for scrutinizing transcription factor function

In different cell differentiation contexts, FOG-1 either inhibits or enhances cell proliferation through different molecular mechanisms. This type of differentiation context-dependent function is not amenable to analysis with conventional loss- or gain-of-function experiments. In general, loss of function experiments gives more robust conclusion than over-expression experiments. Since conventional over-expression of genes by retroviral vectors reveals only limited aspects of gene function. First, it is difficult to control the switching on and off of gene expression by the retroviral vector. Thus, it is almost impossible to induce differentiation stage-specific or time-course-selected expression of the genes. Second, only the relatively long-term effects of the genes can be analysed, since the selection of infected cells takes a considerable amount of time. Third, we cannot exclude the possibility that target cells that are infected with retroviral vectors have altered functions and properties.

The experimental system described in this report overcomes these drawbacks. The time-course-dependent and various other functions of FOG-1 are clearly evident in this experimental system. Thus, conventional over-expression experiments are not adequate for elucidating the functions of transcription factors. We are currently analysing the functions of GATA-1, GATA-2, and Runx-1, all of which show cell differentiation context-dependent functions (data not shown). In some cases, conventional loss of function experiments is not sufficient to analyse the differentiation stage specific function of the genes. For example, recent conditional loss-of-function experiments with transcription factors, such as SCL and Runx-1, have shown that these genes are essential for early development of haematopoiesis but dispensable for adult haematopoiesis (Mikkola *et al.* 2003; Ichikawa *et al.* 2004). Our experimental system easily turns on and off the genes in a stage specific manner. We believe that conditional and stage restricted over-expression of various genes could provide reasonable results which are difficult to obtain by even conditional gene targeting analysis.

In *Xenopus*, xFOG repressed erythropoiesis but a study using a mouse erythroid cell line showed that erythroid differentiation (Deconinck *et al.* 2000). This discrepancy is attributable to species difference or different experimental system. Our present data strongly support the latter possibility. Because our data are well correlated with the data of xFOG but not consistent with that of mouse

erythroid cell line (Tsang *et al.* 1997; Crispino *et al.* 1999). Thus, the gene functions revealed by studies using cell lines do not necessarily reflect the authentic functions of these genes. In addition, the combination of gene targeting and conditional gene expression constitutes a powerful tool for examining the combinatorial functions of transcriptional activators.

## Experimental procedures

### Plasmid construction

The Tet-regulatable FOG-1 or FOG-1ΔCtBP construct was generated by inserting mouse FOG-1 or FOG-1ΔCtBP cDNA into the *EcoRI* site of pUHD10-3.IRES-EGFP (Era & Witte 2000).

FOG-1ΔCtBP was generated as follows. The *SacI-EcoRI* fragment of murine FOG-1 cDNA was inserted into the *SacI* and *EcoRI* sites of pBluescript (Stratagene, La Jolla, CA, USA). The PIDLSK sequence motif was mutated by PCR to PIASSK (pBS-mutFOG), using the protocol of the Quikchange Site-Directed Mutagenesis Kit (Stratagene, CA, USA). This mutation has been previously described (Schaeper *et al.* 1995). Representative clones were subjected to automated fluorescent sequencing with the T7 and M13-reverse primers. This mutant fragment was integrated with the remainder of the *EcoRI-SacI* fragment of FOG-1 cDNA.

### Cell cultures and differentiation induction

E14tg2a ES cells and their clones were utilized in this study. These cells were maintained as previously described (Niwa *et al.* 1998). The culturing of OP9 stroma cells and the *in vitro* coculturing of ES cells with OP9 cells, to induce differentiation to haematopoietic cells, were performed as previously described (Nakano *et al.* 1994, 1996). Briefly, ES cells were transferred on to OP9 stromal cells in 6-well plates at a density of  $10^4$  cells per well. These induced cells were trypsinized at day 5, and  $10^5$  cells were seeded on to fresh OP9 cells. 2 U/mL EPO or 10 ng/mL TPO were added to support the differentiation and proliferation of erythroid cells and megakaryocytes, respectively.

Colony forming unit assays were performed as follows. First, the ES cells were induced into becoming haematopoietic cells by coculture with OP9 cells in a medium that contained 10 ng/mL TPO until day 8. On day 8, the induced cells were transferred into the methylcellulose culture medium Methocult M3232 (Stemcell Technology, Vancouver, BC, Canada) that was supplemented with 10 ng/mL TPO. The colonies were counted seven days after transfer to the methylcellulose medium.

### Establishment of Tet-regulated ES cell clones

The tetracycline regulatory system (Gossen & Bujard 1992) was used to obtain ES cell clones that conditionally expressed FOG-1. The expression of the gene was driven by a modified version of the Tet system, as developed by Era & Witte (2000). The production of Tet-regulated clones was carried out essentially as described

(Era & Witte 2000). Briefly, the E14tg2a ES cell clone ES2-1, which expresses constitutively the modified Tet-regulated transactivator under the control of the CAG promoter, was established. Then, the linearized plasmids of bicentric Tet-regulatable FOG-1 or FOG-1ΔCtBP genes (30 μg) were co-transfected with the neomycin plasmid pPGKneo (1 μg) into  $8 \times 10^6$  ES2-1 cells. For gene introduction, electroporation was performed at 0.23 kV and 500 μF using the Gene Pulser II (Bio-Rad, Hercules, CA, USA). The cells were selected using 200 μg/mL G418 (Sigma, St Louis, MO, USA) for 7–10 days in the presence of 1 μg/mL tetracycline (Sigma). The addition of tetracycline was essential, to avoid the expression of Tet-regulatable genes in ES cells. The clones whose EGFP expression was tightly regulated by tetracycline were selected. Among these clones, the expression of the desired genes was examined by Western blotting, as described below.

Tet-regulated GATA-1 null ES cells were established as followed. Targeting vector was designed as previously described (Fujiwara *et al.* 1996). The targeting construct was linearized and electroporated into the E14tg2a ES cell clone ES2-1. cDNA of FOG-1 or FOG-1ΔCtBP was introduced into the GATA-1 null ES cells as described above. Two clones were examined.

At least two independent clones, in which FOG-1 or FOG-1ΔCtBP expression was tightly regulated by Tet, were analysed and showed essentially similar results. The data on the representative clones are presented.

### Flow cytometry

The cells were harvested by mild pipetting, resuspended in Ca- and Mg-free phosphate-buffered saline (PBS) that contained 5% foetal calf serum, and stained with the PE-conjugated or biotinylated antibodies. The biotinylated anti-mouse GPV (1C2) antibody was purchased from Seikagaku Corporation (Tokyo). The biotinylated anti-TER-119 antibody was a kind gift from Dr T. Kina (Kyoto University, Kyoto, Japan). The biotinylated antibodies were visualized using PE-conjugated streptavidin (PharMingen, San Diego, CA, USA). The PE-conjugated anti-mouse CD71 antibody was purchased from eBioscience (San Diego, CA, USA). For the polyploidization analysis, the cells were suspended in 100 μL PBS that contained 0.1% Triton X-100, 0.2 mg/mL RNase A, and 50 ng/mL propidium iodide. The stained cells were analysed using the FACSCalibur (Becton Dickinson, Franklin Lakes, NJ, USA). For cell cycle analysis, the cells were suspended and analysed by the same method as for the polyploidization analysis. The cell cycle analysis was carried out using the ModFit™ LT software (Becton Dickinson).

### RT-PCR

Total RNA was recovered using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA), and 100 ng of total RNA was used for cDNA synthesis. Reverse transcription was performed using the ThermoScript RT-PCR system (Gibco BRL, Rockville, MD, USA). The primer sequences and PCR conditions for GATA-1 to GATA-6, FOG-1, and GPDH have been previously described (Suwabe *et al.* 1998; Tsang *et al.* 1998).

## Western blot analysis

The ES cells or the induced blood cells ( $2 \times 10^5$ ) were suspended in 15  $\mu$ L of a solution that contained 0.5% NP40, 100 mM NaCl, 1% glycerol, 1 mM ethylenediamine tetraacetic acid, 1 mM phenylmethylsulphonyl fluoride, and 20 mM Tris-HCl (pH 8.0). The protein samples were electrophoresed on a 7.5% SDS-Tris-glycine gel (BIO CRAFT, Tokyo), and transferred to an Immobilon PVDf membrane (Millipore, Bedford, MA, USA). The membranes were probed with the goat polyclonal anti-FOG-1 antibody (M-20; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The horseradish peroxidase-conjugated anti-goat IgG antibody (Zymed, San Francisco, CA, USA) was used as the secondary antibody. The membranes were stained with the enhanced chemiluminescence detection kit (Amersham Pharmacia, Buckinghamshire, UK).

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