Alteration of cell adhesion and cell cycle properties of ES cells by an inducible dominant interfering Myb mutant

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The Myb transcription factors, c-Myb, A-Myb, and B-Myb, regulate cell differentiation and/or proliferation. To investigate the role of B-Myb in embryogenesis, we introduced an inducible dominant interfering Myb protein (MERT) into embryonic stem (ES) cells, which express B-Myb as an exclusive member of Myb family. Disruption of normal B-Myb function by the conditional activation of MERT caused a drastic morphological alteration of ES cells and G₁-S cell cycle arrest. The inhibition of B-Myb function by MERT dissociated tightly packed ES cell colonies into dispersed single cells that subsequently detached from the culture dish. Cell adhesion analyses revealed that suppression of B-Myb function reduced the adhesion with extracellular matrix proteins, such as laminin, collagen, and fibronectin. This reduction was presumably due to decreased cell surface expression of β 1 integrin. Embryoid body formation was also severely retarded by the activation of MERT. This impairment was attributed to reduced expression of E-cadherin, which functions as a homophilic intercellular adhesion molecule. Simultaneously, blocking B-Myb function did not alter the expression of differentiation markers. Our data indicate that B-Myb plays important roles in regulating cell adhesion and cell cycle progression. These results are well consistent with the recent report on the phenotype of B-Myb null mice and show that the regulation of cell adhesion is an important B-Myb function that has not yet been assumed. *Oncogene* (2001) 20, 1425–1434.

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Introduction

Three members of the Myb transcription factor family are found in mammals: c-Myb, A-Myb, and B-Myb (Lipsick, 1996; Ness, 1999; Weston, 1998). Gene targeting experiments have revealed that all three are involved in the control of cell differentiation and cell proliferation. These three members recognize the same target DNA sequence, but their expression patterns are

quite distinct (Mucenski *et al.*, 1991; Tanaka *et al.*, 1999; Toscani *et al.*, 1997). c-Myb is predominantly expressed in immature hematopoietic cells and is down-regulated with the differentiation and maturation of the cells (Westin *et al.*, 1982). The failure of definitive hematopoiesis in c-Myb targeting mice clearly demonstrated that c-Myb is a critical regulator of hematopoietic cell differentiation (Mucenski *et al.*, 1991). A-Myb is required for spermatogenesis and mammary gland development, as revealed by the phenotype of A-Myb-null mice (Toscani *et al.*, 1997). In contrast to the restricted expression of c-Myb and A-Myb, B-Myb expression is much broader in both adult and embryonic tissues (Nomura *et al.*, 1988; Sitzmann *et al.*, 1996). Recently, it was reported that homozygous B-Myb-deficient mutant mice died on embryonic day 4.5 to 6.5 (Tanaka *et al.*, 1999). Embryonic stem (ES) cells express B-Myb exclusively. In spite of much effort, researchers have failed to produce B-Myb^{-/-} ES cells, which suggests that B-Myb plays crucial roles in the maintenance of ES cells. At the same time, it is obviously difficult to investigate the role of B-Myb at the cellular level using a gene targeting strategy.

ES cells are derived from the inner cell mass of blastocysts (Bradley *et al.*, 1984; Martin, 1981). ES cells possess real totipotency, like the cells in the inner cell mass, since they can differentiate into any cell lineage once re-introduced into blastocysts. ES cells can be cultured as adherent cells on a layer of embryonic fibroblasts or in culture dishes coated with gelatin (Joyner, 1993). This suggests that some cell adhesion property of the cells is important for their maintenance. Numerous gene-targeting experiments have shown that ES cells are very useful for investigating gene function during mouse ontogeny. At the same time, analyses of the cell adhesion properties of null mutant ES cells have revealed that these cells are a powerful tool for analysing the molecular mechanisms of cell adhesion. For example, E-cadherin-deficient ES cells are defective in cell aggregation, presumably because of altered cell–cell adhesion (Larue *et al.*, 1996; Riethmacher *et al.*, 1995). β 1 integrin-deficient ES cells have altered cell morphology, migration, and adhesion to a number of substrates (Fassler *et al.*, 1995).

Inducible dominant interfering proteins are of immense benefit for investigating the function of

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intracellular proteins, such as transcription factors (Badiani *et al.*, 1994; Lyon and Watson, 1996; Saitou *et al.*, 1995; Taylor *et al.*, 1996; White and Weston, 2000). In order to analyse the function of B-Myb in ES cells, we made use of MERT (Myb-Engrailed-estrogen receptor-tag), an inducible dominant protein that interferes with Myb. MERT consists of the DNA binding domain of c-Myb, the *Drosophila* Engrailed repressor domain, and a modified estrogen receptor hormone-binding domain (ER-HBD) (Taylor *et al.*, 1996). The DNA binding domain allows the Engrailed repressor to bind specifically to the target gene sequences of Myb family members, thereby interfering with the expression of Myb-dependent genes. The modified ER-HBD can only be activated by an estrogen analog, 4-hydroxytamoxifen (4-OHT), which enables MERT to suppress Myb function in a 4-OHT-dependent fashion (Littlewood *et al.*, 1995). MERT is an inducible version of Myb dominant interfering proteins, and represses Myb activity conditionally. Consequently, MERT is a very powerful method for analysing the function of genes for which double negative mutant cells are difficult to obtain, like B-Myb (Tanaka *et al.*, 1999).

We expected that inhibition of B-Myb function would alter some aspect of differentiation, since c-Myb and A-Myb play important roles in cell differentiation in the tissues where they are expressed (Mucenski *et al.*, 1991; Toscani *et al.*, 1997). Although activation of MERT in undifferentiated ES cells gave rise to drastic morphological changes, no expression of differentiation markers for the three germ layers were detected. Instead, expression of cell adhesion molecules, such as $\beta 1$ integrin and E-cadherin, was significantly reduced by the activation of MERT. Our examination showed that B-Myb plays important roles in regulating cell adhesion and cell cycle progression in ES cells.

Results

Inhibition of B-Myb activity by the activated MERT

MERT represses the transcriptional activation of c-Myb in a 4-OHT dependent manner. ERT, which is a defective version of MERT lacking the DNA binding domain of c-Myb, was used as a control (Taylor *et al.*, 1996). It is reasonable to expect that MERT also inhibits the activity of B-Myb, since c-Myb and B-Myb have very homologous amino acid sequences and very similar consensus binding sequences (Nomura *et al.*, 1988). At first, we confirmed that the activated MERT could inhibit the transcriptional activation by B-Myb in the context of ES cells. ES cell clones which constitutively expressed ERT or MERT were transfected by lipofection with a Myb responsive reporter gene, 6MBS-Luc, consisting of six tandem repeats of Myb responsive elements and luciferase gene (Takahashi *et al.*, 2000). Then, the transfected ERT and MERT clones were cultured in the presence or absence of 4-

Figure 1 Inhibition of B-Myb induced transactivation by the activated MERT ES cells constitutively expressing ERT or MERT were transfected by lipofection with a Myb responsive reporter gene, 6MBS, consisting of six tandem repeats of Myb responsive elements, tk promoter and luciferase gene. The transfected ERT and MERT clones were cultured in the presence (closed bars) or absence (open bars) of 4-OHT. Data are the mean $\pm$ standard deviation of three samples. Statistically significant difference ($P < 0.05$ by Student's *t*-test) is marked with an asterisk

OHT. As shown in Figure 1, the transactivation of 6MBS was significantly reduced only in the MERT clone by the addition of 4-OHT. Since ES cells express B-Myb exclusively, it was felt that MERT should enable us to analyse the roles of B-Myb in these cells. The activation of MERT is caused by the translocation of MERT from cytoplasm to nucleus by the addition of 4-OHT (Mary Argent-Katwala and Kathy Weston, personal communication).

Inhibiting Myb activity caused cell-cycle arrest

A bicistronically EGFP-expressing plasmid (MERT-IE) and a control plasmid (ERT-IE) were constructed by ligating IRES-EGFP downstream from MERT and ERT, respectively, in order to use EGFP for *in vivo* identification of MERT or ERT expressing ES cells. MERT-IE and ERT-IE were electroporated into D3 ES cells and stable transformants were obtained. ES cell clones transfected with MERT-IE or ERT-IE plasmids were designated as MIE and EIE, respectively. In preliminary experiments, EGFP expression of the stable clones was examined by flow cytometry. The majority of the MIE (42 out of 45) and EIE (16 out of 21) clones showed a two-peak pattern of EGFP expression. Clones MIE7, MIE24, and EIE15, which all showed the two-peak pattern (Figure 2, left panels), were used for cell growth and cell cycle analyses, in order to use the EGFP-negative cell population as an internal negative control.

First, the EGFP expression patterns of these clones were examined in the presence or absence of 4-OHT. The percentages of EGFP-expressing cells in clones MIE7 and MIE24 started to decrease around 24 h after the addition of 4-OHT (Figure 2, bold lines in the middle panels). This decrease continued, and high-EGFP-expressing cells (R2 population in the middle

Figure 2 EGFP expression pattern of parental ES cells, clone EIE15, clone MIE7, and clone MIE24 in the presence or absence of 4-OHT. Parental ES cells, the EIE clone, and MIE clones were cultured in the absence (thin lines) or presence (bold lines) of 10^{-7} M 4-OHT. The numbers of high-EGFP-expressing MIE cells were drastically reduced in the presence of 4-OHT. Fractions R1 and R2 were used in the experiment shown in Figure 4

panels) disappeared almost completely after 48 h in the presence of 4-OHT (Figure 2, right panels). In contrast, the pattern of EGFP expression of clone EIE15 was not significantly different in the presence or absence of 4-OHT (Figure 2, middle and right panels). Therefore, the presence of 4-OHT inhibited the growth of MERT clones, but not that of parental or ERT cells (Figure 3). There was no significant increase in the dead cell number of MERT clones in the presence of 4-OHT (data not shown). In addition, no significant increase in apoptosis was detected by propidium iodide staining, Hoechst 33258 staining, or DNA ladder detection, in the presence of 4-OHT (data not shown). These results suggest that the 4-OHT-induced decrease in MERT-expressing cells was due to inhibition of cell proliferation rather than cell death.

Next, we investigated whether the activated MERT brought about the cell-cycle abnormality through the inhibition of B-Myb. Clones MIE7, MIE24, EIE15, and parental ES cells were cultured in the presence or absence of 4-OHT for 24 h. The cells of the MIE and EIE clones were sorted into EGFP⁻ and EGFP⁺ populations (R1 and R2 fractions in the middle panels of Figure 2). These sorted cells and non-sorted parental ES cells were stained with propidium iodide and their cell cycle compositions were analysed by flow cytometry. As shown in Figure 4, the 4-OHT-induced activation of MERT led to a 2–3-fold increase in the percentage of cells in G₀-G₁ phase in the EGFP⁺ fractions of clones MIE7 and MIE24 (from 18.1 to 44.2% and from 15.3 to 46.9%, respectively). However, no significant alteration was observed in the EGFP⁻

fractions. In contrast, 4-OHT had no significant effects on the cell cycle composition of parental ES cells or clone EIE15. These data indicate that only ES cells expressing the dominant interfering Myb protein were arrested in G₁ phase by activation of the protein. Thus, activation of MERT induced G₁-S cell cycle arrest and subsequent cell growth retardation. Taken together with circumstantial evidence concerning the possible roles of B-Myb in cell cycle progression as discussed below, this verifies that MERT is useful for examining B-Myb function in ES cells.

Matrix adhesion properties altered by interference with Myb activity

Unexpectedly, drastic morphological changes were observed in the 4-OHT-activated MIE clones, as shown in Figure 5. In the absence of 4-OHT, the MIE clones grew as tightly packed colonies, just like parental ES cells. However, adding 4-OHT resulted in single round cells that were detached or only loosely attached to the gelatin-coated dishes or neighboring cells (Figure 5f, h). Morphological changes in two representative MIE clones are shown: one is clone MIE7, which shows a two-peak pattern of EGFP expression (Figure 2), the other is clone MIE3, in which almost all ES cells show high expression of EGFP (data not shown). The addition of 4-OHT gave rise to similar morphological changes in these two clones, but the percentages of colonies showing these changes were significantly higher in clone MIE3 than in clone MIE7. This is probably attributable to the higher percentage of MERT-expressing ES cells in clone MIE3.

There are two explanations for this morphological change: some alteration of the state of cell differentiation or reduced cell adhesion activity. The former is unlikely, because no expression of differentiation marker genes, such as FGF-5, HNF-4, and Brachyury for the three germ layers, was detected in total cell population after the activation of MERT by 4-OHT (data not shown). Therefore, the latter possibility was examined in more depth, using clone MIE3, in which the vast majority of cells show high EGFP expression. In subsequent experiments, clone EIE12, which showed a single high peak expression of EGFP similar to clone MIE3 (data not shown), was used as a negative control.

First, the effect of activated MERT on the plating efficiency of these clones and parental ES cells was examined. One thousand ES cells were seeded onto non-coated or gelatin-coated dishes in the presence or absence of 4-OHT, and the numbers of colonies were counted 3 days later. As shown in Figure 6a, the plating efficiencies were drastically reduced in the MIE clones, in which B-Myb function was inhibited, while no effects were observed in the controls. Next, cell attachment to various extracellular matrix proteins, such as laminin, collagen, and fibronectin, was analysed. Clones MIE3 and EIE12 and parental ES cells were incubated in the presence or absence of 4-OHT for 15 h. Then, the cells were trypsinized and

Figure 3 Inhibition of B-Myb activity reduces cell numbers. Parental ES cells, the EIE clone, and MIE clones were cultured in the absence (open circles) or presence (closed circles) of 4-OHT. The numbers are shown as the mean $\pm$ standard deviation of four samples. At the indicated time points, samples were stained with Trypan blue and counted. The presence or absence of 10^{-7} M 4-OHT produced no significant differences in cell viability (data not shown)

plated onto tissue culture 96-well dishes coated with gelatin, laminin, collagen, or fibronectin. The adhesion of the cells was analysed 1 h later as described in the Materials and methods section. The MIE3 cells showed significantly reduced cell adhesion to all of the substrates in the presence of 4-OHT (Figure 6b). Essentially the same results were seen with two other MIE clones (data not shown). In contrast, the adhesion activity of parental ES cells and EIE12 cells was not decreased at all by the addition of 4-OHT.

The decreased attachment to extracellular matrix led us to investigate $\beta 1$ integrin expression in the MIE cells. When clones MIE3 and MIE7 were incubated in the presence of 4-OHT, single round cells were detached from the gelatin-coated dishes or neighboring cells after incubation for 48 h. This non-adherent fraction showed virtually no $\beta 1$ integrin expression on the cell surfaces, while the remaining adherent fraction showed $\beta 1$ integrin expression similar to cells cultured in the absence of 4-OHT (Figure 7a). These results suggest that down-regulation of $\beta 1$ integrin

expression caused by the suppression of B-Myb transactivation activity itself or subsequent reduced expression of B-Myb-regulated genes was responsible for the reduced cell adhesion activity, at least in part. However, RT-PCR analysis did not show any difference in $\beta 1$ integrin expression between the non-adherent and adherent fractions of the MIE clones (data not shown).

Activated MERT affects embryoid body formation

In order to analyse the developmental capacity of MERT cells, the embryoid body (EB) formation activity of clone MIE3 was investigated. For EB formation, 10^4 ES cells were cultured in methylcellulose semisolid media without LIF. 4-OHT was added 2 days after initiating the EB culture and the number of EB was counted 4 days later. Clone MIE3 produced remarkably fewer EB in the presence of 4-OHT (Figure 8a). However, some ES cell clusters that did not adhere to each other and did not produce EB remained

Figure 4 Inhibition of B-Myb activity causes G₁-S phase cell cycle block. Parental ES cells and clones EIE15, MIE7, and MIE24 were seeded in culture medium in the presence or absence of 4-OHT as indicated for 24 h. Fractions R1 and R2 from Figure 2 were sorted to give EGFP⁻ and EGFP⁺, respectively. These sorted cells and non-sorted parental ES cells were stained with propidium iodide and their cell cycle compositions were analysed by flow cytometry. The 4-OHT-induced activation of MERT led to a 2–3-fold increase in the percentage of cells in G₀-G₁ phase in the EGFP⁺ fractions of clones MIE7 and MIE24

Figure 5 Inhibition of B-Myb activity produces morphological changes. Parental ES cells (a,b) and clones EIE15 (c,d), MIE7 (e,f), and MIE3 (g,h) were cultured in the absence (a,c,e,g) and presence (b,d,f,h) of 10^{-7} M 4-OHT for 24 h. Detached or loosely attached single ES cells are seen only in 4-OHT(+) MIE clones

(Figure 8b,f). Since one of the most important cell adhesion molecules responsible for the initial phase of EB formation is E-cadherin, surface expression of E-cadherin on the cells detached from gelatin-coated

dishes by 4-OHT was examined. As shown in Figure 7b, the level of E-cadherin expression was significantly reduced in the non-adherent fraction of MIE cells. However, Northern blot hybridization revealed that the E-cadherin mRNA level was not decreased in these cells (data not shown).

Discussion

We used MERT, an inducible version of dominant interfering Myb proteins, to investigate the function of B-Myb in ES cells. Two phenomena were observed when Myb transactivation activity was interfered with in ES cells. One was cell-cycle arrest at G₁ phase, with a subsequent reduction in cell number. Cell-cycle arrest was as expected, since it occurs with the ectopic expression of B-Myb (Sala and Calabretta, 1992; Sala *et al.*, 1996) or the introduction of antisense B-Myb (Arsura *et al.*, 1992). Unlike hematopoietic cells (Frampton *et al.*, 1996; Taylor *et al.*, 1996), however, interfering with Myb function did not cause apoptotic cell death. The other noticeable, unexpected feature was the alteration of cell adhesion activity. This defective cell adhesion was caused by abnormalities of both cell-extracellular matrix adhesion and cell–cell adhesion, specifically via reduced cell surface expression of $\beta 1$ integrin and E-cadherin, respectively. However, the levels of $\beta 1$ integrin and E-cadherin mRNA expression were not altered by the inhibition of B-Myb.

Mutant mice lacking c-Myb show severe impairment of definitive hematopoiesis, probably due to some defects in hematopoietic stem or progenitor cells (Mucenski *et al.*, 1991). A-Myb-deficient males have a block in spermatogenesis and subsequent infertility (Toscani *et al.*, 1997). Moreover, in female mice, there is underdevelopment of the breast epithelial compart-

Figure 6 Inhibiting B-Myb influences plating efficiency and cell attachment activity. (a) The plating efficiency of parental ES cells (par) and clones EIE12 (E12), MIE3 (M3), and MIE7 (M7) was analysed in the presence (gray bars) or absence (open bars) of 4-OHT. The cells were cultured on non-coated and gelatin-coated dishes for 3 days. Data are shown as the ratio of colonies in the presence and absence of 10^{-7} M 4-OHT and are the mean $\pm$ standard deviation of four samples. Statistically significant differences ($P < 0.05$ by Student's *t*-test) are marked with an asterisk. (b) Cell attachment activity of parental ES cells and clones EIE12 and MIE3 was analysed after culturing for 15 h in the presence (gray bars) or absence (open bars) of 4-OHT. Extracellular matrix proteins are shown as the following: GL, gelatin; LM, laminin; CL, collagen type IV; FN, fibronectin. Data are shown as the ratio of attached cells in the presence and absence of 10^{-7} M 4-OHT, and are the mean $\pm$ standard deviation of four samples. Statistically significant differences ($P < 0.05$ by Student's *t*-test) are marked with an asterisk.

ment of the mammary gland following pregnancy. These findings suggest the attractive hypothesis that Myb family proteins play important roles during cell differentiation, especially in the maintenance or differentiation of immature progenitor or stem cells. Evidence from *myb*-related genes in plants reinforces this notion. Glabra 1 of *Arabidopsis thaliana* encodes a Myb-related protein found at the top of the trichome pathway (Oppenheimer *et al.*, 1991). Trichomes are branched, hair-like projections that arise from a single cell in the epidermal layer. Glabra 1 is required for both initiation and maintenance of cell fate. However, in ES cells, abrogation of B-Myb function did not alter the differentiation status of the cells at all (data not shown).

Another important role of Myb protein activity is the inhibition of apoptosis. Blocking c-Myb function causes apoptotic cell death in mouse T cells (Taylor *et al.*, 1996) and switching off a Myb-Ets fusion protein in chicken myeloblasts causes apoptosis (Frampton *et al.*, 1996). However, loss of c-Myb protein activity does not necessarily induce apoptosis in all hematopoietic cells. When a dominant negative Myb protein is switched on in mouse erythroleukemia cells, the cells

do not die, but arrest in G_1 phase instead and differentiate (Weston, 1998). Moreover, when a Myb-Ets fusion protein is turned off in chicken myeloid-erythroid progenitors, no death is observed, but cells differentiate into thrombocytes (Frampton *et al.*, 1995). Unlike all these cases, only G_1 arrest was observed when B-Myb function was repressed in ES cells, but neither apoptosis nor differentiation occurred. This dissociation of G_1 arrest and the other phenomena is attributable to differences in the functions of c-Myb and B-Myb or differences in the cell types used.

The most notable abnormality induced by switching on MERT was the change in cell adhesion properties that resulted in the detachment of ES cells and the failure of EB formation. The failure of cell adhesion could be accounted for by defects in cell-extracellular matrix attachment and cell-cell adhesion, which are presumably caused by the defective surface expression of $\beta 1$ integrin and E-cadherin, respectively. It is possible that this failure is a secondary event following cell cycle arrest in G_1 -S transition, since cell adhesion and cell proliferation events are closely related (Giancotti and Ruoslahti, 1999; Ginsberg *et al.*, 1992). Cell-cell interaction and integrin-mediated cell

Figure 7 Expression of β -1 integrin and E-cadherin on adherent and non-adherent cell fractions of MIE clones after inhibiting B-Myb. Surface expression of β -1 integrin (a) and E-cadherin (b) on adherent and non-adherent cells 48 h after adding 4-OHT. Bold and thin lines show the expression of the cells cultured with and without 10^{-7} M 4-OHT, respectively

adhesion to the extracellular matrix play regulatory roles in cell cycle progression (Levenberg *et al.*, 1999; Manabe *et al.*, 1999; Zhao *et al.*, 1998). To the contrary, certain cell-cycle-related molecules, such as cyclin C and cdk6, control cell adhesion (Fahraeus and Lane, 1999; Liu *et al.*, 1998). Although significant cell cycle arrest after switching on MERT was observed as early as 18 h after the addition of 4-OHT (data not shown), the reduction in cell adhesion activity was detected around 15 h. This time course suggests that the defects in the cell cycle and cell adhesion occurred almost simultaneously or that the latter began a little earlier, which implies that the defect in cell adhesion was not a secondary event of the defect in the cell cycle. To examine this interpretation further, we analysed ES cells that were arrested in G_1 phase by mimosine, a cell cycle-synchronization agent (Krude, 1999). Although mimosine caused $\sim 50\%$ of the ES cells to arrest as G_1 cells, which is 2–3-fold higher

than in untreated ES cells (data not shown), no defective cell adhesion was observed as following. The proportion of non-adherent ES cells per total ES cells was $0.32 \pm 0.07\%$ (mean $\pm$ standard deviation of triplicated samples) under normal culture condition. The proportion was not significantly altered by the addition of mimosine ($0.48 \pm 0.11\%$, P value > 0.2 by Student's t -test). Taken together, the defective cell adhesion is not a consequence of the cell-cycle arrest. Since the cell cycle block is primarily caused by the inhibition of B-Myb function as discussed above, the abnormalities in cell cycle and cell adhesion are independent events caused by the activated MERT.

As shown in Figure 7a, there was virtually no surface expression of β 1 integrin on the detached ES cells. The defective cell matrix attachment to collagen, fibronectin, and laminin can be explained by the defective β 1 integrin expression, because treatment with blocking antibodies against cell-surface β 1 integrin (kindly provided by Dr Yagita) similarly reduced adhesion of ES cells to these substrates (data not shown). β 1 integrin targeted ES cells show similar cell adhesion defects, but no detachment of those cells was observed (Fassler *et al.*, 1995). This discrepancy is probably due to the defective expression of E-cadherin in addition to absent β 1 integrin expression on the surface of MERT-activated ES cells. Similarly, the profound defects in the production of EB can be explained by the defective E-cadherin expression. The defects in β 1 integrin expression might contribute to the poor EB formation to some extent, but the effect of β 1 integrin should not be crucial, since β 1 integrin null embryos develop normally to the blastocyst stage (Fassler and Meyer, 1995; Fassler *et al.*, 1995) and cell aggregation for EB formation is only slightly retarded.

We thought that the expression of both β 1 integrin and E-cadherin mRNA might be reduced in the detached ES cells and examined this possibility. However, no significant reduction was observed by RT-PCR or Northern blot analysis. General reduction of cell surface molecules by switching on MERT is unlikely, since significant percentages of the cells show high expression of E-cadherin, even the non-adherent fraction in Figure 7b. This finding suggests that interference with Myb function might compromise translation or subsequent processing of the β 1 integrin and E-cadherin proteins, perhaps affecting vesicular transport towards the cell membrane or post-translational modifications, such as glycosylation (Bellis *et al.*, 1999), localization, and stabilization at focal adhesions. The identification of the B-Myb target gene(s) involved in the β 1 integrin and E-cadherin processing pathways remains a subject of future investigation. There was a tendency that the EGFP expression in non-adherent fraction was higher than that in adherent fraction, however, it was not in an 'all-or-none' fashion. Other cell adhesion related molecules might be affected post-transcriptionally by some indirect functions of activated MERT and those molecules would play some roles on the cell adhesion.

Figure 8 Embryoid body formation and its morphology. (a) The numbers of embryoid bodies produced from parental ES cells (par) and clones EIE12 (E12) and MIE3 (M3) are shown as the mean $\pm$ standard deviation of four samples. The numbers of EB in the absence and presence of 4-OHT are shown as open and gray bars, respectively. A drastic reduction of EB formation was observed by inhibiting B-Myb. Statistically significant difference ($P < 0.05$ by Student's *t*-test) is marked with an asterisk. (b) Photographs of cell clusters under EB-forming conditions. Parental ES cells (a,b), clone EIE12 (c,d), and clone MIE3 (e,f) were cultured under EB-forming conditions in the absence or presence of 4-OHT. Significant numbers of cell clusters that did not form EB were observed in MIE3 clones in the presence of 4-OHT (f)

Recently, B-Myb-deficient mice were produced by gene targeting and the phenotype was reported. The heterozygous mutants were healthy, but the homozygous mutants died at an early stage of development, around embryonic day 4.5~6.5. Although B-Myb^{-/-} blastocysts were indistinguishable from B-Myb^{+/-} or B-Myb^{+/+} ones at E3.5, B-Myb^{-/-} blastocysts cultured *in vitro* for 5 days showed severely impaired proliferation of the inner cell mass. In addition, B-Myb^{-/-} ES cell lines could not be obtained. In B-Myb^{-/-} mice, trophoctoderm cell development and trophoblast differentiation were observed in spite of the subsequent rapid abnormal growth of the blastocysts, which is very similar to the phenotype of $\beta 1$ integrin null mice. As shown in Figure 7, ES cells with activated MERT showed virtually no $\beta 1$ integrin expression, but significant E-cadherin expression. The impaired G₁/S transition of B-Myb^{-/-} embryos is surely one explanation for the early lethality of B-Myb^{-/-} embryos. Taken together, however, we believe that abnormalities in cell adhesion are another, and probably more important, cause of the defects in B-Myb^{-/-} embryos.

Concerning cell adhesion molecules regulated by Myb proteins, the $\alpha 4$ integrin gene is transcriptionally regulated by c-Myb in hematopoietic cells. A heterodimeric $\alpha 4\beta 1$ integrin (VLA4) is an essential cell adhesion molecule on hematopoietic cells. Our results show that B-Myb, another Myb family member, regulates cell adhesion molecules, including $\beta 1$ integrin and E-cadherin, although the regulating mechanisms remain to be elucidated. In conclusion, it is certain that Myb family genes play important roles in cell adhesion, as well as in cell proliferation and cell differentiation.

Materials and methods

Plasmid construction

pMCEF-MERT-IE and pMCEF-ERT-IE were constructed as follows. pCMV-IRES-EGFP (a kind gift of Dr A Kume, Jichi Medical School, Tochigi, Japan) (Kume *et al.*, 1999), which contains an *Xho*I site and a *Bam*HI site at the 5' and 3' ends of the IRES-EGFP fragment, respectively, was digested with *Xho*I, and blunt-ended with Klenow polymerase. This fragment was ligated with *Bam*HI linkers, digested with *Bam*HI, and cloned into the downstream *Bam*HI site of pMCEF-MERT and pMCEF-ERT (a kind gift of Dr K Weston (Taylor *et al.*, 1996)) to give rise to pMCEF-MERT-IE and pMCEF-ERT-IE, respectively. All ligated portions were confirmed by DNA sequencing.

Cell culture

D3 mouse ES cells were maintained on embryonic fibroblast feeder cells in the presence of leukemia-inhibitory factor following standard methods (Joyner, 1993). To obtain stable transformants of ES cells, 8×10^6 cells were electroporated with 20 μ g of the linearized plasmid DNA at 230 V and 500 μ F using a Bio-Rad gene pulser and then selected in the presence of 250 μ g/ml G418 (Sigma, St Louis, MO, USA). To activate the MERT and ERT proteins, cells were cultured in the presence of 10^{-7} M 4-OHT (Sigma) in most experiments except luciferase assay. For luciferase analysis, 2 μ g of 6MBS-Luc plasmids (Takahashi *et al.*, 2000) was transfected with Lipofectamine 2000 (Gibco BRL, Rockville, MD, USA) in 6 well plates. 5×10^{-8} M 4-OHT was added 24 h after the transfection and luciferase activity was analysed 48 h after the transfection. In most experiments, feeder cells were removed by incubation on 0.1% gelatin-coated dishes for 30 min and then ES cells were harvested as non-adherent cells and analysed. For cell growth analysis, ES

cells were trypsinized and the numbers of viable cells were counted by Trypan blue exclusion.

FACS and cell cycle analyses

To analyse EGFP expression, single-cell suspensions obtained by trypsinization were washed twice with PBS, stained with 1 µg/ml propidium iodide (PI), and analysed using FACSCalibur (Becton Dickinson, Franklin Lakes, NJ, USA). PI-negative cells were examined for FL1 fluorescence intensity to detect EGFP. Single-cell suspensions were washed twice with PBS, and cells were sorted into EGFP-positive and EGFP-negative cells using FACS Vantage (Becton Dickinson). For cell cycle analysis, sorted cells were centrifuged and resuspended in PBS containing 0.1% Triton X-100, 0.5 mg/ml RNase, and 25 µg/ml PI. The samples were incubated for at least 15 min in the dark and analysed using FACSCalibur (Becton Dickinson). The cell cycle composition was analysed using the program ModFit LT.

Immunostaining

For immunostaining, cultured ES cells were harvested with cell dissociation buffer (Gibco BRL) and washed twice with PBS. Cells were incubated with 5 µg/ml rat anti-mouse CD29 (β1 integrin) monoclonal Ab (PharMingen, San Diego, CA, USA) or 10 µg/ml rat anti-mouse E-cadherin monoclonal Ab (Takara Biomedicals, Kusatsu, Japan). After washing, cells were incubated with 20 µg/ml mouse anti-rat phycoerythrin-conjugated monoclonal Ab (PharMingen, San Diego, CA, USA), washed, and analysed using FACSCalibur (Becton Dickinson).

Cell adhesion assay

The cell adhesion assay was carried out essentially as described previously (Priddle *et al.*, 1998). 96-well tissue culture plates (Nunc, Roskilde, Denmark) were coated with 0.1% gelatin, 50 µg/ml mouse laminin (Biomedical Technologies Inc., Stoughton, MA, USA), 10 µg/ml mouse type IV collagen (Gibco BRL), or 10 µg/ml human plasma fibronectin (Gibco BRL) overnight at 4°C, and then blocked with 2% BSA. ES cells were trypsinized. The trypsin was neutralized by the addition of FCS-containing medium, and the cells were resuspended (8×10^6 cells/ml) in serum-free medium. Then, 100 µl of cell suspension was added to each well and the cells were allowed to attach for 1 h at 37°C. Nonadherent cells were removed by washing three times with PBS, and the attached cells were fixed with 10% formaldehyde for 30 min, then stained with 1% toluidine blue for 1 h. The wells were washed extensively with water and dried in air. Then, the dye was extracted with 2% SDS. The absorbance at 620 nm was measured with a microtiter plate reader. The assays were carried out in four wells and the experiments were repeated more than twice.

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Embryoid body formation

Embryoid body formation was carried out essentially the same as described (Wiles, 1993). ES cells were trypsinized, suspended in LIF-free ES cell medium, and feeder cells were removed by incubating the cells on gelatin-coated dishes for 30 min. Non-adherent cells were harvested and re-suspended in α-MEM containing 1% methylcellulose, 15% fetal calf serum, and 1×10^{-4} M β-mercaptoethanol. Cells were plated on 35 mm suspension culture dishes (Corning, NY, NY, USA) at a density of 1×10^4 cells/dish. Two days after initiating the culture, 4-OHT was added at a final concentration of 1×10^{-7} M. Morphological examination and embryoid body counts were done 6 days after initiating the culture.

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