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STAT3 activation is sufficient to maintain an undifferentiated state of mouse embryonic stem cells

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Embryonic stem (ES) cells can be maintained in an undifferentiated state in the presence of leukemia inhibitory factor (LIF). LIF acts through a receptor complex composed of a low affinity LIF receptor (LIFRβ) and gp130. We reported that the intracellular domain of gp130 plays an important role in self-renewal of ES cells. In the present study, we examined the signaling pathway through which gp130 contributes to the self-renewal of ES cells. Mutational analysis of the cytoplasmic domain of gp130 revealed that the tyrosine residue of gp130 responsible for STAT3 activation is necessary for self-renewal of ES cells, while that required for SHP2 and MAP kinase activation was dispensable. Next, we constructed a fusion protein composed of the entire coding region of STAT3 and the ligand binding domain of the estrogen receptor. This construction (STAT3ER) induced expression of junB (one of the targets of STAT3) in ES cells in the presence of the synthetic ligand 4-hydroxytamoxifen (4HT), thereby indicating that STAT3ER is a conditionally active form. ES cells transfected with STAT3ER cultured in the presence of 4HT maintained an undifferentiated state. Taken together, these results strongly suggest that STAT3 activation is required and sufficient to maintain the undifferentiated state of ES cells.

Keywords: ES cells/estrogen receptor/gp130/LIF/STAT3

Introduction

Mouse embryonic stem (ES) cells are pluripotent cell lines derived from the inner cell mass of the pre-implantation mouse embryo (Evans and Kaufman, 1981; Martin, 1981). This pluripotency can be maintained in the presence of leukemia inhibitory factor (LIF) (Smith *et al.*, 1988; Williams *et al.*, 1988), a factor which belongs to the interleukin (IL)-6 cytokine family, which includes IL-6, IL-11, ciliary neurotropic factor, oncostatin M and cardiotropin-1. The effect of LIF is mediated through a cell surface receptor complex composed of the low affinity LIF receptor (LIFRβ) and gp130, a common receptor subunit of the IL-6 cytokine family (Hibi *et al.*, 1990; Gearing *et al.*, 1991). LIF binds with LIFRβ, which then forms a high affinity heterodimer complex with gp130.

Both LIFRβ and gp130 apparently have no intrinsic protein kinase domains, but do constitutively associate with the Jak family of non-receptor cytoplasmic protein tyrosine kinases (Stahl *et al.*, 1994). Binding of LIF with the LIFRβ–gp130 heterodimer results in the rapid activation of Jaks and subsequent phosphorylation of LIFRβ and gp130 on their tyrosine residues (Boulton *et al.*, 1994; Stahl *et al.*, 1994; Nakamura *et al.*, 1998). In ES cells, three Jak family kinases, Jak1, Jak2 and Tyk2, are activated upon LIF-stimulation (Ernst *et al.*, 1996). After tyrosine phosphorylation of LIFRβ and gp130, SH2 domain-containing signaling molecules such as STATs and SHP2 are recruited to the receptors and are then phosphorylated by Jaks on their tyrosine residues (Boulton *et al.*, 1994; Boeuf *et al.*, 1997; Nakamura *et al.*, 1998; Niwa *et al.*, 1998). STATs are transcription factors which form SH2-mediated dimers after tyrosine phosphorylation (Becker *et al.*, 1998; Chen *et al.*, 1998). Dimerized STATs are then translocated to the nucleus where they bind to DNA followed by direct specific transcriptional initiation (Darnell, 1997). In ES cells, STAT1 and STAT3 were found to be activated (Boeuf *et al.*, 1997; Starr *et al.*, 1997; Nakamura *et al.*, 1998; Niwa *et al.*, 1998). On the other hand, SHP2 is a protein tyrosine phosphatase containing two SH2 domains, and functions upstream of the Ras/MAP kinase pathway (Fukada *et al.*, 1996). In addition to the molecules described above, activation of non-receptor tyrosine kinases such as Hck, and phosphorylation of PLCγ, phosphatidylinositol 3-kinase etc. were also induced by the stimulation of LIF or other IL-6 family cytokines (Boulton *et al.*, 1994; Ernst *et al.*, 1994). Thus, signaling pathways through the LIFRβ–gp130 heterodimer are difficult to elucidate.

To examine the self-renewal signaling through cytoplasmic domains of LIFRβ and gp130 in ES cells, we had constructed previously chimeric receptors containing the extracellular domain of human granulocyte macrophage-colony stimulating factor (hGM-CSF) receptor (hGMR) α- or β-chain and the intracellular domain of mouse LIFRβ or gp130 (Nakamura *et al.*, 1998). Since hGM-CSF binds with human GM-CSF receptors but not with mouse receptors (Lee *et al.*, 1985), this chimeric receptor system is useful for evaluating effects of homo- or heterodimerization of cytoplasmic domains of cytokine receptors (Nakamura *et al.*, 1998; Tomida *et al.*, 1999). Using this system, we found that either LIFRβ–gp130 heterodimerization or gp130 homodimerization could transmit self-renewal signal in ES cells, while LIFRβ homodimerization could not dose (Nakamura *et al.*, 1998). These findings mean that the certain signals through the cytoplasmic domain of gp130 play an important role in the self-renewal of ES cells.

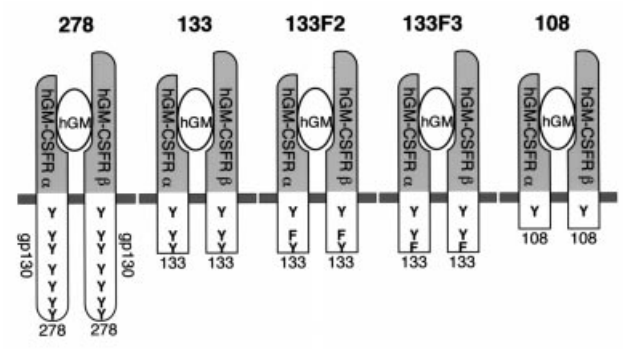

Fig. 1. Structures of mutant chimeric receptors. Chimeric receptors are composed of the extracellular domain of hGM-CSF receptor α- or β-chain (gray boxes) and the transmembrane and cytoplasmic domains of mouse gp130 (open boxes). Chimeric receptors 278, 133 and 108 have 278 (full-length), 133 and 108 amino acid residues in their cytoplasmic domains, respectively. 133F2 and 133F3 receptors are the truncation mutants in which the second and the third tyrosines (Y) from the membrane are replaced by phenylalanines (F).

In the present study, we examined the signaling pathway through which gp130 contributes to the self-renewal of ES cells, and for this, several mutants of hGMR–gp130 chimeric receptors were used. Our results strongly suggest that STAT3 activation through gp130 is required to maintain the undifferentiated state of ES cells. Using a conditionally active form of STAT3, we also found that STAT3 activation is sufficient for the self-renewal of ES cells.

Results

Construction and expression of mutant chimeric receptors in ES cells

Mouse gp130 has seven tyrosine residues in its 278 amino acid cytoplasmic domain (N640–Q917). Among them, the second tyrosine residue (Y757) from the membrane is responsible for activation of the MAP kinase cascade through SHP2 (Stahl *et al.*, 1995; Fukada *et al.*, 1996), and any one of four tyrosine residues [the third (Y765), fourth (Y812), sixth (Y904) and seventh (Y914)] containing the YXXQ motif is available for STAT3 activation (Stahl *et al.*, 1994; Yamanaka *et al.*, 1996). To determine whether SHP2/MAPK or STAT3 signaling pathway through gp130 is involved in self-renewal of ES cells, we introduced deletion and point mutations into the cytoplasmic domains of hGMRα–gp130 and hGMRβ–gp130 chimeric receptors, as shown in Figure 1. The chimeric receptors '278' (hGMRα–gp130, hGMRβ–gp130) have full-length cytoplasmic domains of gp130. '133' receptors are truncation mutants with cytoplasmic domains of 133 amino acid residues, and possess minimal tyrosine residues responsible for SHP2 (Y757) and STAT3 (Y765) activation. '133F2' receptors are mutants in which the second tyrosine (Y757) of 133 was replaced by phenylalanine. '133F3' receptors are mutants in which the third tyrosine (Y765) of 133 was replaced by phenylalanine. '108' receptors are truncation mutants with cytoplasmic domains of 108 amino acid residues, and have no tyrosine residues required for SHP2 or STAT3 activation. These mutant chimeric receptors were introduced into ES cells, and several stable transfectants were established.

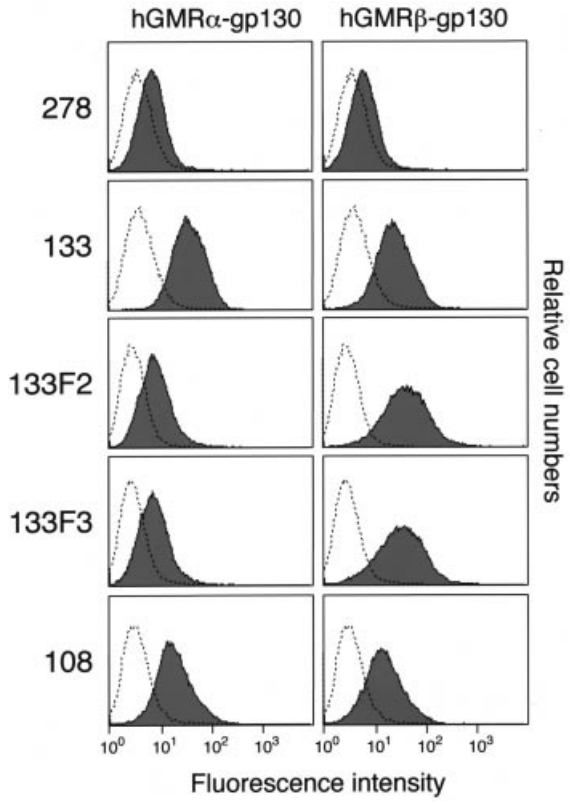

Fig. 2. Expression of mutant chimeric receptors in ES cells. ES cells were transfected with various chimeric receptors, and the stable transfectants were established. The expression of hGMRα–gp130 (left) or hGMRβ–gp130 chimeric receptors (right) was examined by flow cytometry, using an antibody against the extracellular domain of hGM-CSF receptor α- or β-chain, respectively. Dotted line histograms represent unstained cells and solid line histograms represent cells labeled with the anti-hGM-CSF receptor antibodies.

The expression of transfected receptors was confirmed by flow cytometry analysis, using an antibody against the extracellular domain of hGMR α- or β-chain (Figure 2).

Characterization of mutant chimeric receptors

To confirm the potential of mutant chimeric receptors to activate STAT3, SHP2 and MAP kinase, ES cells expressing chimeric receptors were stimulated with hGM-CSF and the cell extracts were subjected to Western blot analysis. When the extracts were immunoprecipitated with anti-STAT3 antibody and then probed with anti-phosphotyrosine antibody, the chimeric receptors 278, 133 and 133F2, but not 133F3 or 108, induced tyrosine phosphorylation of STAT3, in response to hGM-CSF (Figure 3A). On the other hand, when these extracts were immunoprecipitated with anti-SHP2 antibody and then probed with anti-phosphotyrosine antibody, the chimeric receptors 278, 133, and 133F3, but not 133F2 or 108, induced tyrosine phosphorylation of SHP2 in response to hGM-CSF (Figure 3B). Similar results were obtained when whole cell extracts were probed with anti-phospho (T202/Y204) MAP kinase (ERK1 and ERK2) antibody (Figure 3C). Therefore, 278 and 133 can activate both STAT3 and SHP2/MAP kinase, 133F2 can activate only STAT3, 133F3 can activate only SHP2/MAP kinase, and 108 cannot activate either STAT3 or SHP2/MAP kinase.

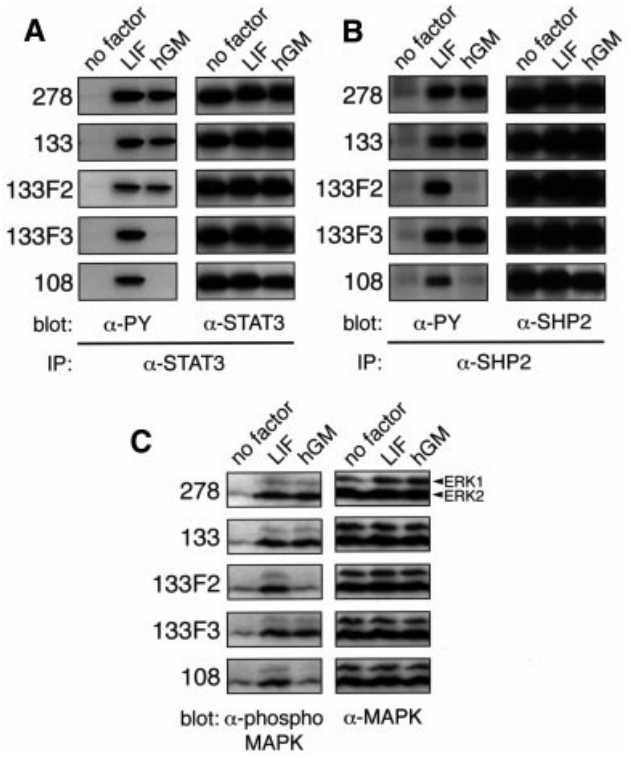

Fig. 3. Phosphorylation of STAT3, SHP2 and MAP kinase. (**A**) After the depletion of LIF and FCS for 12 h, ES cell lines expressing chimeric receptors were either left unstimulated or were stimulated with hLIF (100 ng/ml) or hGM-CSF (100 ng/ml) for 10 min, and then lysed in NP-40. Lysates containing 500 μg of protein were precipitated using anti-STAT3 antibody (2 μg), subjected to SDS–PAGE, and transferred to PVDF membrane. The membrane was first probed with anti-phosphotyrosine antibody (left), then stripped and reprobed with anti-STAT3 antibody (right). (**B**) ES cell lysates (500 μg of protein) were precipitated using anti-SHP2 antibody (1 μg), subjected to SDS–PAGE, and transferred to PVDF membrane. The membrane was first probed with anti-phosphotyrosine antibody (left), then reprobed with anti-SHP2 antibody (right). (**C**) ES cell lysates (100 μg of protein) were subjected to SDS–PAGE, and transferred to PVDF membrane. The membrane was first probed with anti-phospho(T202/Y204)-MAP kinase antibody (left), then reprobed with anti-MAP kinase antibody (right). Arrowheads indicate the positions of ERK1 and ERK2.

STAT3 activation correlates with self-renewal of ES cells

To determine whether mutant chimeric receptors can transmit self-renewal signals in response to hGM-CSF, ES cell lines expressing chimeric receptors (Figure 1) were cultured in the presence of hGM-CSF. In the presence of LIF (Figure 4, center), all the cell lines formed compact colonies, a characteristic phenotype of undifferentiated ES cells. On the other hand, in the absence of factors (Figure 4, left), all the cells became large and flattened, a phenotype of differentiated ES cells. In the presence of hGM-CSF without LIF (Figure 4, right), ES cell lines expressing receptors 278, 133 or 133F2 formed compact colonies, while those expressing the receptors 133F3 and 108 became large, flattened cells. These results strongly suggest that STAT3 activation is indispensable for self-renewal of ES cells while SHP2/MAP kinase activation through gp130 is dispensable.

Construction of a conditionally active form of STAT3

The results described above suggest that STAT3 activation is required for the self-renewal of ES cells. Consistent with this notion, other investigators found that the dominant-negative forms of STAT3 lead to differentiation of ES cells (Boeuf *et al.*, 1997; Niwa *et al.*, 1998). However, it has remained unclear whether or not STAT3 activation is indeed sufficient for the self-renewal of ES cells. To elucidate this further, we constructed a fusion protein composed of the entire coding region of mouse STAT3 and the modified ligand binding domain (G525R) of mouse estrogen receptor (Littlewood *et al.*, 1995) (Figure 5). The modified ligand binding domain binds with the synthetic steroid ligand 4-hydroxytamoxifen (4HT) but not with 17β-estradiol. Moreover, this mutant has no transactivation activity (Littlewood *et al.*, 1995).

To determine whether this fusion protein (STAT3ER) can function as a conditionally active form in response to 4HT, we transfected BOSC23 cells, a 293-derived cell line, with STAT3ER construct and a luciferase reporter containing four acute phase responsive elements (STAT3 binding sequence) (Nakajima *et al.*, 1996). When BOSC23 cells transfected with the empty expression vector (mock) were stimulated with LIF, luciferase activity was remarkably increased (Figure 6A). And this induction of luciferase activity by LIF was enhanced when BOSC23 cells were transfected with the STAT3ER construct, suggesting that STAT3ER retains the potential to transmit signals through LIF receptors. On the other hand, 4HT stimulation resulted in a significant induction of luciferase activity, but only when cells were transfected with STAT3ER. The ability of STAT3ER to induce luciferase activity was abolished by a mutation in its DNA-binding domain (E434A, E435A). This mutation also inhibited the induction of luciferase activity by LIF, probably because it acted as a dominant-negative STAT3 (Nakajima *et al.*, 1996). STAT3ER did not induce a luciferase reporter containing β-casein promoter, which has a STAT5 responsive element (Wakao *et al.*, 1994), although STAT5aER did induce the luciferase reporter in response to 4HT (Figure 6B). STAT3ER also did not induce a luciferase reporter containing an estrogen responsive element (data not shown). These results indicate that STAT3ER can mimic activated STAT3 in the presence of 4HT even without cytokine stimulation.

Characterization of STAT3ER

Next, STAT3ER was introduced into ES cells and stable transfectants were established. The expression level of STAT3ER in the ES clone used in this study is shown in Figure 7D. When ES cells expressing STAT3ER were stimulated with LIF, Northern blot analysis showed that junB and egr1 were induced (Figure 6C). JunB is one of the known targets of STAT3 (Fujitani *et al.*, 1994), whereas egr1 is thought to be located at the downstream of MAP kinase (Fukada *et al.*, 1996). In contrast, 4HT stimulation induced junB but not egr1 (Figure 6C). Since 4HT stimulation did not induce junB expression in parental ES cells, or ES cells expressing STAT5aER, these results suggest that 4HT specifically activates the STAT3 pathway in ES cells expressing STAT3ER.

Phosphorylation of STAT3ER induced by 4HT stimulation

Upon cytokine stimulation, STAT3 is phosphorylated on the tyrosine residue (Y705), dimerizes and can bind DNA

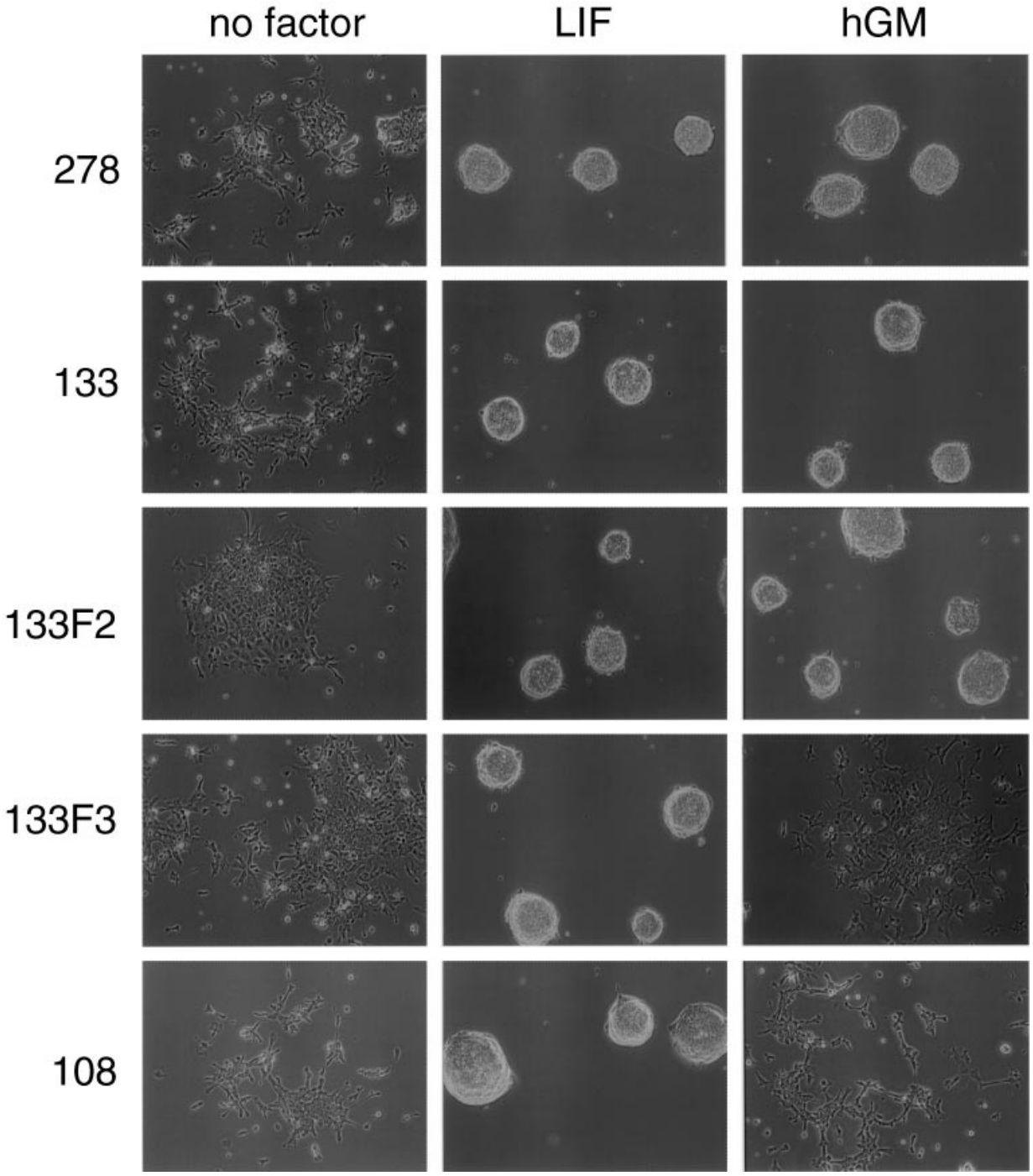

Fig. 4. Morphology of ES cells expressing chimeric receptors. ES cell lines expressing mutant chimeric receptors were cultured for 5 days in the presence of hLIF (10 ng/ml) or hGM-CSF (20 ng/ml), or without factors. On day 3, ES cells were trypsinized and replated. The undifferentiated state was determined by the maintenance of compact colony formation.

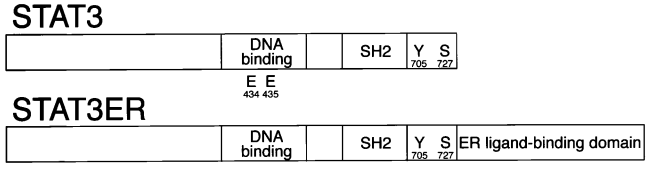

Fig. 5. Structure of a conditionally active form of STAT3. A conditionally active form of mouse STAT3 was constructed by fusing the entire coding region of STAT3 to the modified ligand binding domain (G525R) of mouse estrogen receptor.

(Akira *et al.*, 1994; Becker *et al.*, 1998). Moreover, it was reported that serine phosphorylation (S727) enhances the transcriptional activity of STAT3 (Wen *et al.*, 1995). We then asked if STAT3ER is phosphorylated by 4HT stimulation at tyrosine or serine residues. In this experiment, ES cells expressing STAT3ER were stimulated with either LIF or 4HT for 10 min up to 24 h, then the cell extracts were immunoprecipitated with anti-STAT3 antibody and subjected to Western blot analysis using anti-phosphotyrosine, anti-phospho (Y705) STAT3 and anti-phospho (S727) STAT3 antibodies. As shown in

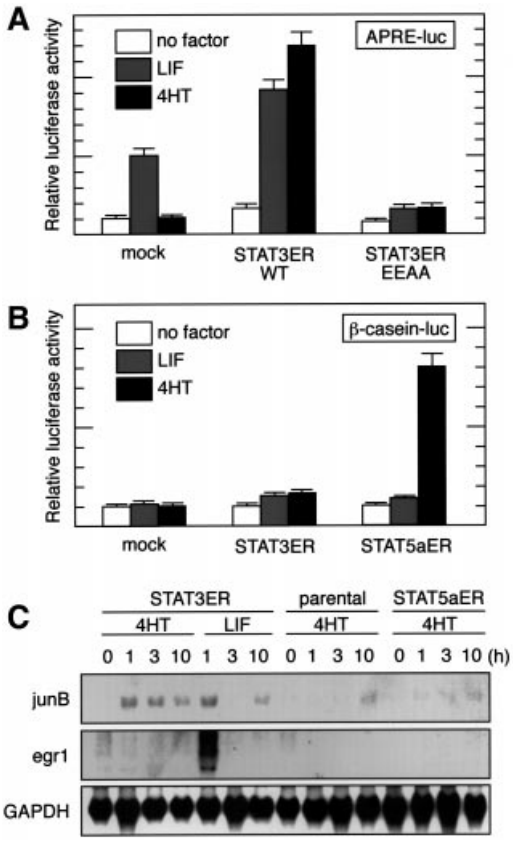

Fig. 6. Function of a conditionally active form of STAT3. (**A**) Luciferase assay. BOSC23 cells were transfected with 2 μg of (4×) APRE-junB minimal promoter–luciferase reporter construct together with 2 μg of STAT3ER or STAT3ER mutant (E434A, E435A) in the pCAGGS vector or an empty expression vector (mock). Twenty-four hours after transfection, cells were either left unstimulated or were stimulated with hLIF (100 ng/ml) or 4HT (1 μM) for 24 h, and cell extracts were prepared and measured for luciferase activity. Data represent mean ±SD of three independent experiments. (**B**) Luciferase assay. BOSC23 cells were transfected with 2 μg of β-caseinen–luciferase reporter construct together with 2 μg of STAT3ER or STAT5aER in the pCAGGS vector or an empty expression vector (mock). Twenty-four hours after transfection, cells were either left unstimulated or were stimulated with hLIF (100 ng/ml) or 4HT (1 μM) for 24 h, and cell extracts were prepared and measured for luciferase activity. Data represent mean ±SD of two independent experiments. (**C**) Northern blot analysis of ES cells. After factor starvation for 48 h, parental ES cells or ES cells expressing STAT3ER or STAT5aER were stimulated with hLIF (100 ng/ml) or 4HT (1 μM) for the indicated time periods, then harvested and RNAs were prepared. Total RNAs (30 μg) were separated by agarose gel, transferred to nylon membrane and hybridized with junB, egr1 or GAPDH cDNA probes.

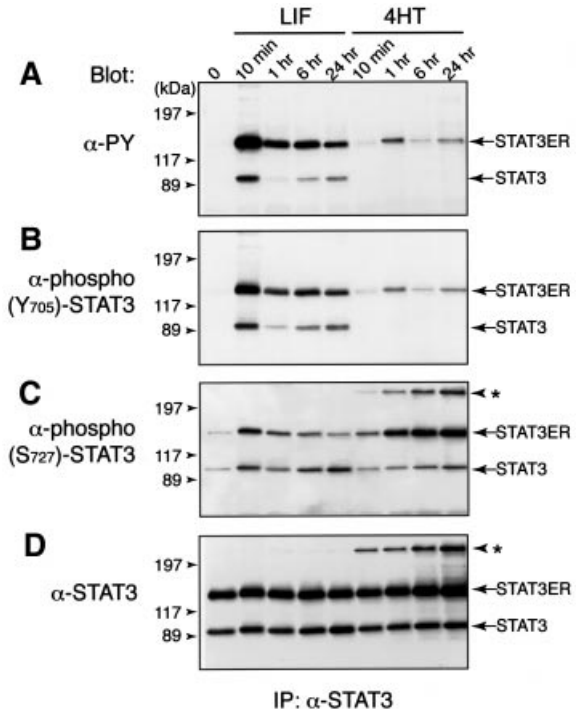

Fig. 7. Tyrosine and serine phosphorylation of STAT3 and STAT3ER. After the depletion of LIF for 12 h and subsequent LIF and FCS starvation for 12 h, ES cells expressing STAT3ER were stimulated with hLIF (100 ng/ml) or 4HT (1 μM) at the indicated periods, and then lysed in NP-40. Lysates containing 500 μg of protein were precipitated using anti-STAT3 antibody (2 μg), subjected to SDS–PAGE, and transferred to PVDF membrane. The membrane was probed with anti-phosphotyrosine antibody (**A**), anti-phospho (Y705) STAT3 (**B**), anti-phospho (S727) STAT3 (**C**) or anti-STAT3 (**D**) antibody. The bands (indicated by an asterisk) which appeared after 4HT stimulation in (C) and (D) are likely to be the dimer of STAT3ER.

Figure 7A (a blot with anti-phosphotyrosine antibody) and Figure 7B [a blot with anti-phospho (Y705) STAT3 antibody], LIF-stimulation induced tyrosine phosphorylation of both endogenous STAT3 and STAT3ER. Interestingly, endogenous STAT3 was dephosphorylated rapidly whereas dephosphorylation kinetics of STAT3ER was slow. In contrast, 4HT-stimulation resulted in tyrosine phosphorylation of STAT3ER but not of endogenous STAT3.

On the other hand, as shown in Figure 7C [a blot with anti-phospho (S727) STAT3 antibody], both LIF- and 4HT-stimulation induced serine phosphorylation of endogenous STAT3 and STAT3ER. In the case of LIF stimulation, the kinetics of serine phosphorylation (Figure 7C) was roughly similar to that of tyrosine phosphorylation (Figure 7B). But in the case of 4HT-stimulation, the kinetics of serine phosphorylation (Figure 7C) differed from that of tyrosine phosphorylation (Figure 7B). The significant dephosphorylation at the serine residue was not observed in either endogenous STAT3 or STAT3ER within 24 h of 4HT stimulation.

STAT3 activation is sufficient for self-renewal of ES cells

To determine whether STAT3 activation is sufficient for the self-renewal of ES cells, parental ES cells or ES cells expressing STAT3ER, STAT5aER or STAT6ER (Kamogawa *et al.*, 1998) were cultured in the presence of LIF or 4HT (Figure 8). In the presence of LIF, all the cell lines formed compact colonies. On the other hand, in the presence of 4HT, only ES cells expressing STAT3ER formed compact colonies. STAT3ER mutated at the DNA-binding domain (E434A, E435A) also did not contribute to the compact colony formation in response to 4HT (data not shown). The morphologically undifferentiated state of ES cells expressing STAT3ER was maintained for at least 30 days (15 passages) in the presence of 4HT without LIF (data not shown). The undifferentiated state of ES cells was further confirmed by the alkaline phosphatase activity and the expression of stage-specific embryonic antigen-1 (SSEA-1), a specific marker of the undifferenti-

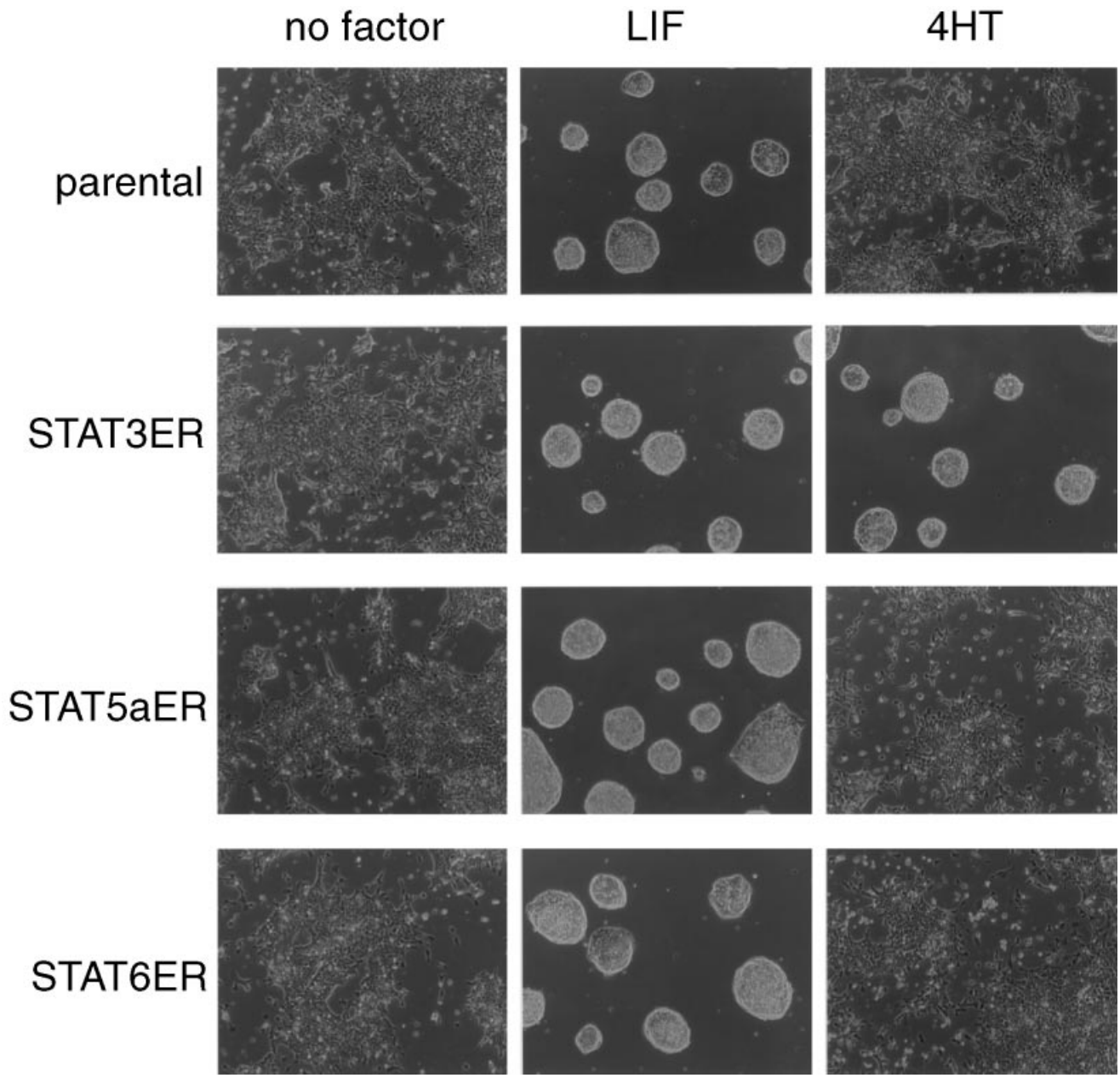

Fig. 8. Morphology of ES cells expressing STAT3ER, STAT5aER or STAT6ER. Parental ES cells, or ES cells expressing either STAT3ER, STAT5aER or STAT6ER were cultured for 4 days in the presence of hLIF (10 ng/ml) or 4HT (1 μM), or without factors. The undifferentiated state was determined by the formation of compact colonies.

ated state of ES cells (Solter and Knowles, 1978). As shown in Figure 9A and B, ES cells maintained by STAT3ER and 4HT were alkaline phosphatase positive and expressed SSEA-1.

To demonstrate the pluripotency of ES cells maintained by STAT3ER and 4HT, ES cells expressing STAT3ER maintained for 7–10 passages in the presence of either LIF or 4HT were injected into C57BL/6 blastocysts, which normally form black mice. The ES cells used in this experiment have albino coat color markers. As judged by coat color contribution, both ES cells lines produced chimeric mice, and there was no obvious difference between the effect of LIF and 4HT (Figure 9C). These results indicate that STAT3 activation is sufficient and specific to maintain the undifferentiated state of ES cells.

Discussion

STAT3 is activated by a variety of factors, including the IL-6 family of cytokines, granulocyte-colony stimulating factor (G-CSF), epidermal growth factor and leptin, and plays a critical role in various cell responses (Akira, 1997). For example, STAT3 is necessary for differentiation of M1 leukemia cells into macrophages (Nakajima *et al.*, 1996; Yamanaka *et al.*, 1996), differentiation of cerebral cortical precursor cells into astrocytes (Bonni *et al.*, 1997), anti-apoptosis in pro-B cell lines (Fukada *et al.*, 1996), proliferation of T cells (Takeda *et al.*, 1998) and early development of mouse embryos (Takeda *et al.*, 1997). In these studies, a series of chimeric cytokine receptors, dominant-negative forms of STAT3 and STAT3-knockout strategies were used to elucidate the significance of STAT3.

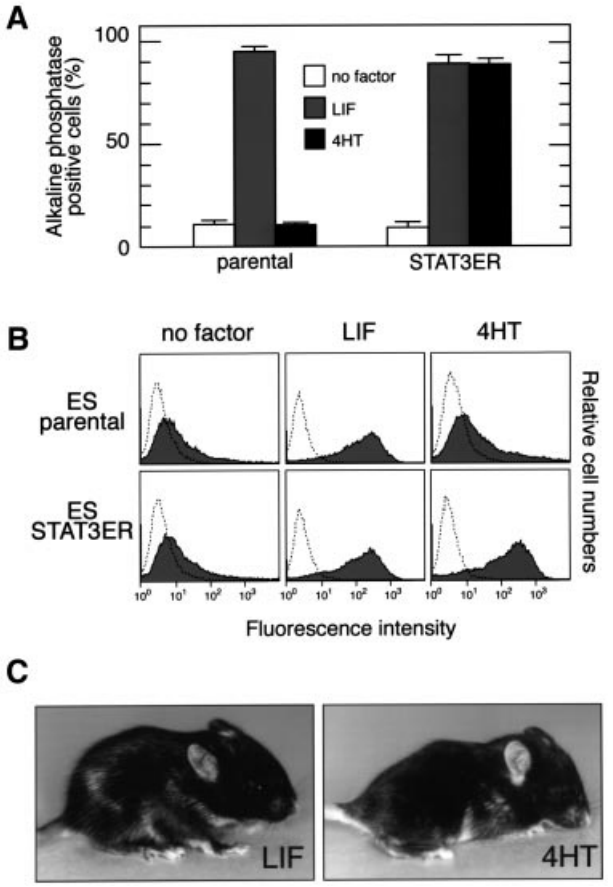

Fig. 9. Maintenance of undifferentiated state of ES cells expressing STAT3ER by 4HT. (**A**) Parental ES cells or ES cells expressing STAT3ER were cultured for 6 days in the presence of hLIF (10 ng/ml) or 4HT (1 μM), or without factors, and were stained for alkaline phosphatase activity using Diagnostic Kit (Sigma). The ratio of alkaline phosphatase-positive cells was scored from the total of randomly chosen 300 cells. Data represent mean ±SD of three independent experiments. (**B**) Parental ES cells (upper) or ES cells expressing STAT3ER (lower) were cultured for 6 days in the presence of the indicated factor. The expression of SSEA-1 was examined by flow cytometry. Dotted line histograms represent unstained cells and solid line histograms represent cells labeled with anti-SSEA1 antibody. (**C**) A3-1 ES cells expressing STAT3ER were cultured for 7–10 passages in the presence of LIF or 4HT without feeder cells, and injected into C57BL/6 blastocysts. The injected embryos were then transferred into pseudopregnant females. Chimerism was determined by the coat color. Chimeric mice derived from ES cells expressing STAT3ER maintained by either LIF (left) or 4HT (right) are shown.

We noted the significance of STAT3 activation in LIF-dependent self-renewal of ES cells, determined using a series of chimeric receptor mutants. Along with recent reports showing that dominant-negative forms of STAT3 lead to differentiation of ES cells (Boeuf *et al.*, 1997; Niwa *et al.*, 1998), our findings indicate that STAT3 activation is required for self-renewal of ES cells. In addition to the requirement of STAT3 activation, we showed further that STAT3 activation is sufficient to maintain the undifferentiated state of ES cells [at least in the presence of fetal calf serum (FCS)], determined using a conditionally active form of STAT3. This is apparently the first report demonstrating the sufficiency of STAT3 activation in differentiation-suppression signaling.

Although STAT3 activation is sufficient for inhibiting the differentiation of ES cells, there is a threshold for the activity of STAT3 to completely maintain the undifferentiated state of ES cells. In our experiments, ES cell lines which express STAT3ER at a lower level were not fully maintained in the undifferentiated state by 4HT (data not shown). It was reported that the homodimerization of the cytoplasmic domain of G-CSF receptor, which can activate STAT3, partially but not fully prevents ES cell differentiation (Ernst *et al.*, 1996; T.Nakamura, T.Heike and T.Yokota, unpublished results). This may be explained by the weaker activation of STAT3 through the G-CSF receptor.

A plausible model explaining how STAT3ER is activated by 4HT is that STAT3ER is dimerized and activated through the 4HT-bound ligand binding domain of the estrogen receptor, without the tyrosine phosphorylation of STAT3. Actually, the STAT3ER dimer (>200 kDa) was observed when ES cells expressing STAT3ER were stimulated with 4HT (Figure 7D, indicated by an asterisk). Moreover, a conditionally active form of STAT6, a fusion protein between STAT6 and ER ligand binding domain, was reported and shown to be activated without tyrosine phosphorylation (Kamogawa *et al.*, 1998). However, our result showed that STAT3ER was weakly tyrosine phosphorylated on Y705 of STAT3, in response to 4HT in ES cells (Figure 7A and B). In addition, 4HT-stimulation also resulted in serine phosphorylation (Figure 7C). In this case, both STAT3ER and endogenous STAT3 functioned as substrates of 4HT-induced serine kinase. The 4HT-bound ER ligand binding domain may activates some kinases. Further analyses are needed to reveal the significance of the tyrosine/serine phosphorylation on STAT3ER.

One interesting point of tyrosine and serine phosphorylation of STAT3 is that dephosphorylation kinetics of STAT3ER differed from that of STAT3 (Figure 7). Wild-type STAT3 was rapidly dephosphorylated while STAT3ER was slowly dephosphorylated. Phosphatase(s) for STATs has yet to be well characterized, and the mechanism by which the phosphatase(s) recognizes and binds with STATs is largely unknown. But since phosphorylation sites on STAT3 are located at the C-terminus, it is possible that ER ligand binding domain that was fused to the C-terminus of STAT3 affected accessibility of the phosphatase(s).

Our findings also showed that SHP2 and MAP kinase activation through gp130 is dispensable for the self-renewal of ES cells. This finding is consistent with evidence that homozygous SHP2-deficient ES cells could be maintained in an undifferentiated state in the presence of LIF, although some abnormalities in differentiation were observed (Qu *et al.*, 1997). However, it is possible that the MAP kinase pathway is involved in proliferation or anti-apoptosis of ES cells, as we cultured ES cells in the presence of FCS. Although FCS apparently has no differentiation-suppression activity, it does contain several mitogens that induce the activation of MAP kinase cascade in ES cells (Ernst *et al.*, 1996). MAP kinase activation by FCS may compensate the SHP2/MAP kinase pathway, which is activated through gp130. Moreover, we observed that both LIF and FCS could induce the serine phosphorylation of STAT3 (our unpublished data). Thus, FCS may enhance the basal activity of STAT3 by serine phosphorylation.

Since STAT3 is a transcription factor, self-renewal of

ES cells would be maintained by the molecule(s) whose expression is regulated by STAT3, either directly or indirectly. One of the possible targets of STAT3 is a POU-domain transcription factor, Oct-3/4. Oct-3/4 is expressed in ES and embryonal carcinoma cells, and required to maintain the stem cell properties (Nichols *et al.*, 1998). However, induction of Oct-3/4 expression either by LIF- or 4HT-stimulation in ES cells expressing STAT3ER was never apparent (our unpublished data). Thus, Oct-3/4 seems not be a direct target of STAT3. Identification of targets of STAT3 is now under investigation.

Materials and methods

Cell culture and cytokines

The ES cell line A3-1 derived from embryos of 129/SvJ inbred strain (Azuma and Toyoda, 1991) was cultured on gelatin-coated dishes in the absence of feeder cells as described previously (Nakamura *et al.*, 1998). Recombinant human LIF (hLIF) and human GM-CSF (hGM-CSF) were kindly provided by Amgen. BOSC23, a HEK293-derived retroviral packaging cell line (Pear *et al.*, 1993), obtained through ATCC was maintained in Dulbecco's modified Eagle's medium containing 10% FCS. A synthetic (Z)4-hydroxytamoxifen (4HT), purchased from Research Biochemical International, was dissolved in ethanol (5 mM stock).

Plasmid construction

Chimeric cytokine receptors containing transmembrane and cytoplasmic domains of mouse gp130 and the extracellular domain of hGM-CSF receptor α (hGMRα–gp130) or β chain (hGMRβ–gp130) in pCAGGS expression vector (Niwa *et al.*, 1991) were described previously (Nakamura *et al.*, 1998). STAT3β was provided by Dr A.L.-F.Mui (DNAX Research Institute). The modified ligand binding domain (G525R) of mouse estrogen receptor (ER) in pBabe-puro vector was provided by Dr M.McMahon (DNAX Research Institute). STAT5a and a luciferase reporter plasmid containing β-casein promoter (Wakao *et al.*, 1994) was provided by Dr A.Miyajima (University of Tokyo). STAT6ER (Kamogawa *et al.*, 1998) was provided by Dr Y.Kamogawa (University of Tokyo). A reporter plasmid containing four copies of acute phase responsible element (APRE) in front of the minimal junB promoter linked to the luciferase gene (Nakajima *et al.*, 1996) was provided by Dr T.Hirano (Osaka University). A luciferase reporter plasmid containing estrogen-responsive element (Kato *et al.*, 1995) was provided by Dr S.Kato (University of Tokyo).

To introduce mutations into the cytoplasmic domains of hGMRα–gp130 and hGMRβ–gp130, the entire coding regions were excised by *Xho*I, and cloned into the *Xho*I site of pSP72 vector (Promega). To construct 133, 133F2 or 133F3 mutants, plasmids was digested with *Bst*EII and *Mun*I, and the PCR-amplified fragment containing mutations was digested with *Bst*EII and *Mun*I, and ligated into the *Bst*EII–*Mun*I site of the plasmid. To construct the 108 mutant, the plasmid was digested with *Ppu*10I and *Mun*I and the annealed synthetic oligonucleotide containing mutations was then ligated into the *Ppu*10I–*Mun*I site of the plasmid. Mutant receptor constructs of hGMRα–gp130 and hGMRβ–gp130 were excised by *Xho*I, and cloned into the *Xho*I site of pCAGGS vector containing neomycin- and hygromycin-resistant genes, respectively.

To construct a full-length STAT3 (STAT3α), the entire coding region of STAT3β was excised by *Eco*RI and *Not*I, and ligated into the *Eco*RI–*Not*I site of pCR2.1 vector (Invitrogen). The C-terminal part (amino acids 581–755) of STAT3 was amplified by RT–PCR, using RNA prepared from ES cells, digested with *Sma*I and *Not*I, and ligated into the *Sma*I–*Not*I site of the plasmid. To construct a fusion protein composed of the entire coding region of STAT3 and the modified (G525R) ligand binding domain (amino acids 281–599) of mouse estrogen receptor, a *Bam*HI site was created by PCR at the 3′ end of the STAT3. Then the *Eco*RI–*Bam*HI fragment of STAT3 and the *Bam*HI–*Sal*I fragment of the ER ligand binding domain was ligated into the *Eco*RI–*Xho*I site of pCAGGS vector containing the puromycin acetyltransferase gene driven by the SV40 early promoter (Clontech). This fusion protein (STAT3ER) contains eight extra amino acids (DPGSSPNS), which are not present in either STAT3 or ER ligand binding domain, at the fusion point.

The entire coding region of mouse junB, egr1 and glyceraldehyde-6-phosphate dehydrogenase (GAPDH) was amplified by RT–PCR, using RNA prepared from ES cells, then subcloned into the pCR2.1 vector. Nucleotide sequences were confirmed using the 310 DNA sequencing system (Perkin-Elmer).

Establishment of stable transfectants

Stable transfectants of ES cells expressing chimeric receptors were obtained as described previously (Nakamura *et al.*, 1998), and expression of the chimeric receptors was confirmed by flow cytometry using a cell sorter (FACScan, Becton Dickinson) and monoclonal antibodies against the hGMRα (Santa Cruz Biotechnology, S-20) or hGMRβ (Santa Cruz Biotechnology, S-20). To establish ES cell lines expressing STAT3ER, 20 μg of expression plasmid of STAT3ER with the puromicin resistant gene was introduced into ES cells by electroporation, and transfectants were selected with 1 μg/ml puromicin (Sigma). Expression of STAT3ER was analyzed by Western blotting, using a monoclonal antibody against STAT3 (Transduction Laboratories).

Luciferase assay

BOSC23 cells (1×10^6 cells in 60 mm dish) were transiently transfected with 2 μg of the (4×) APRE-junB minimal promoter–luciferase reporter construct together with 2 μg of STAT3ER in the pCAGGS vector or an empty expression vector, using lipofectamine (Gibco-BRL) and OPTI MEM1 medium (Gibco-BRL). Twenty-four hours after the transfection, cells were scraped off using trypsin-EDTA solution, plated in three dishes and then were either left unstimulated or were stimulated with LIF (100 ng/ml) or 4HT (1 μM) for 24 h. Cells were harvested and lysed in 1 ml of buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 4 μg/ml aprotinin, 4 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4 and 1% NP-40). Luciferase activity was measured using a PicaGene luciferase assay kit (Toyo Inki, Japan) and a luminometer (Lumat LB9501).

Western blots

Anti-STAT3 antibody (C-20) and anti-SHP2 (C-18) antibodies were purchased from Santa Cruz Biotechnology, Inc. Anti-phospho (T202/Y204) MAP kinase, anti-phospho (Y705) STAT3 and anti-phospho (S727) STAT3 antibodies were from New England Biolabs, Inc. Anti-(ERK1+2) antibody was from Zymed Laboratories, Inc. Anti-phosphotyrosine (4G10) antibody was from Upstate Biotechnology, Inc. Immunoprecipitation and Western blotting were as described (Nakamura *et al.*, 1998). The bands were visualized using the ECL regent (Amersham).

Northern blots

After factor starvation for 48 h, 1×10^7 ES cells were stimulated with LIF (100 ng/ml) or 4HT (1 μM) for 1, 3 or 10 h, harvested, and RNAs were prepared using the TRIZOL Reagent (Gibco-BRL). Total RNAs (30 μg) were separated by 1% agarose formaldehyde gel and transferred to a nylon membrane (Hybond N^+, Amersham). Hybridization was performed at 68°C for 1–2 h, using QuickHyb hybridization solution (Stratagene) and the ^{32}P-labeled cDNA probe of entire coding region of junB, egr1 or GAPDH. The membrane was washed twice with 0.1× SSC containing 0.1% SDS at 60°C for 10 min, and radiolabeled bands were visualized, using a Image Analyzer (Fuji Film).

Stem cell assays and generation of chimeric mice

To determine the undifferentiated state of ES cells, based on their morphology, parental ES cells or ES transfectants were plated at 1×10^4 cells per well in gelatin-coated 6-well dishes then cultured for 4 or 5 days. Cells were either grown in the presence of 10 ng/ml hLIF, 20 ng/ml hGM-CSF or 1 μM 4HT, or without factors. To analyze the expression of stage-specific embryonic antigen-1 (SSEA-1) or the alkaline phosphatase activity, ES cells were plated at 1×10^4 cells per well in gelatin-coated 6-well dishes and cultured for 6 days in the presence of 10 ng/ml hLIF, 1 μM 4HT, or without factors. On day 6, cells were harvested and incubated with 1 μg/ml anti-SSEA-1 monoclonal antibody KM380 (Kyowa, Japan) and subsequently with R-phycoerythrin-conjugated goat anti-Ig M antibody (Caltac). The expression of SSEA-1 was measured by flow cytometry, using a cell sorter (FACScan). Alkaline phosphatase activity was determined using Diagnostic Kit (No. 86, Sigma) as described previously (Nakamura *et al.*, 1998). Microinjection of ES cells into C57BL/6J blastocysts was performed according to standard procedures (Robertson, 1987).

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