

Suppression of SHP-2 and ERK Signalling Promotes Self-Renewal of Mouse Embryonic Stem Cells

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The propagation of pluripotent mouse embryonic stem (ES) cells depends on signals transduced through the cytokine receptor subunit gp130. Signalling molecules activated downstream of gp130 in ES cells include STAT3, the protein tyrosine phosphatase SHP-2, and the mitogen-activated protein kinases, ERK1 and ERK2. A chimaeric receptor in which tyrosine 118 in the gp130 cytoplasmic domain was mutated did not engage SHP-2 and failed to activate ERKs. However, this receptor did support ES cell self-renewal. In fact, stem cell colonies formed at 100-fold lower concentrations of cytokine than the unmodified receptor. Moreover, altered ES cell morphology and growth were observed at high cytokine concentrations. These indications of deregulated signalling in the absence of tyrosine 118 were substantiated by sustained activation of STAT3. Confirmation that ERK activation is not required for self-renewal was obtained by propagation of pluripotent ES cells in the presence of the MEK inhibitor PD098059. In fact, the growth of undifferentiated ES cells was enhanced by culture in PD098059. Thus activation of ERKs appears actively to impair self-renewal. These data imply that the self-renewal signal from gp130 is a finely tuned balance of positive and negative effectors. © 1999 Academic Press

Key Words: ES cells; self-renewal; gp130; SHP-2; STAT3; ERK.

INTRODUCTION

The mammalian embryo develops from a founder population of pluripotent stem cells, the epiblast. These rapidly dividing stem cells persist only transiently *in vivo*, differentiating to form extraembryonic mesoderm and the three germ layers of the embryo proper during gastrulation. However, epiblast cells are intrinsically immortal and can give rise to stem cell tumours, teratocarcinomas, when grafted to ectopic sites (Solter *et al.*, 1970). Furthermore, when the epiblast of a mouse blastocyst is explanted in culture, immortal embryonic stem (ES) cell lines can be isolated (Brook and Gardner, 1997; Evans and Kaufman, 1981; Martin, 1981).

Propagation of ES cells is dependent on the presence of the cytokine LIF (Smith *et al.*, 1988; Smith and Hooper, 1987; Stewart *et al.*, 1992; Williams *et al.*, 1988). LIF promotes the proliferation of undifferentiated stem cells through the activation of a heteromeric complex containing two related cytokine receptor subunits, gp130 and the low-affinity LIF receptor, LIF-R (Davis *et al.*, 1993; Gearing and Bruce, 1992; Gearing *et al.*, 1991). Homodimerisation of gp130 alone, in response to the cytokine interleukin-6 (IL-6)

and a soluble form of IL-6 receptor (sIL-6R), can also support the derivation and propagation of ES cell lines (Nichols *et al.*, 1994; Yoshida *et al.*, 1994). A functional analysis of signals transduced through gp130 therefore provides an opportunity to elucidate the molecular mechanisms that regulate the propagation of ES cells and underpin pluripotency.

Signal transduction via gp130 depends upon the activation of JAK kinases, a class of nonreceptor tyrosine kinases that associate with the membrane proximal box1/box2 region of cytokine receptors (Darnell *et al.*, 1994). Upon activation, JAKs phosphorylate tyrosines in the intracellular domain of gp130 creating binding sites for proteins containing Src-homology-2 (SH2) domains. These proteins can in turn be phosphorylated, resulting in the activation of a variety of signalling molecules, including STATs (signal transducer and activator of transcription) 1 and 3 (Stahl *et al.*, 1995), the tyrosine phosphatase SHP-2, the mitogen-activated protein kinases (MAPK) ERK1 and ERK2 (Fukada *et al.*, 1996), insulin receptor substrate-1 (IRS-1) (Argetsinger *et al.*, 1995), Grb2-associated docking protein (Gab1) and phosphatidylinositol (PI)-3 kinase (Takahashi-Tezuka *et al.*, 1998), and the nonreceptor tyrosine kinases Hck and Btk

(Ernst *et al.*, 1994; Matsuda *et al.*, 1995). Amongst these, the STAT and MAPK signalling pathways appear to be the principal mediators of the biological responses to ligands that activate gp130 (Fukada *et al.*, 1996; Minami *et al.*, 1996; Nakajima *et al.*, 1996; Sheng *et al.*, 1997; Shimosaki *et al.*, 1997).

STATs are a family of latent transcription factors that upon recruitment to a receptor become phosphorylated, dimerise, and then translocate to the nucleus where they regulate transcription of target genes (Darnell, 1997). We have recently shown that activation of STAT3 is required for maintaining the pluripotent phenotype of ES cells (Niwa *et al.*, 1998). Chimaeric gp130 receptors unable to engage STAT3 were incapable of signalling self-renewal, whilst overexpression of a STAT3 interfering mutant caused ES cells to differentiate. However, in the absence of constitutively active forms of STAT3, we have been unable to determine whether this regulator alone is sufficient or if other signals are also required for self-renewal.

gp130 can also associate with the protein tyrosine phosphatase SHP-2 (Fukada *et al.*, 1996). This widely expressed enzyme has been implicated in signal transduction from receptor tyrosine kinases and is regarded as a positive effector of the MAPK signalling cascade (reviewed in Van Vactor *et al.*, 1998). SHP-2 contains a C-terminal catalytic domain and two tandemly arranged SH2 domains at its N terminus. The most N-terminal SH2 domain binds to one of the phosphorylated tyrosines on gp130, situated 118 amino acid residues from the membrane (Symes *et al.*, 1997). This interaction both recruits SHP-2 to the receptor complex and causes a conformational change which increases its catalytic activity (Hof *et al.*, 1998). Genetic and biochemical studies in *Drosophila* have identified the Daughter of Sevenless (DOS) protein as a substrate for corkscrew, the *Drosophila* homologue of SHP-2, and have shown a requirement for DOS in receptor tyrosine kinase signalling (Herbst *et al.*, 1996). Interestingly, a mammalian homologue of DOS, Gab1, has been shown to interact with SHP-2 (Holgado-Madruga *et al.*, 1996; Takahashi-Tezuka *et al.*, 1998). Recent evidence suggests that engagement of the gp130 receptor subunit results in the formation of a complex containing SHP-2, Gab1, and PI-3 kinase, resulting in activation of PI-3 kinase and of ERKs (Takahashi-Tezuka *et al.*, 1998). In addition, tyrosine phosphorylation of SHP-2 following recruitment to gp130 can create binding sites for the adaptor protein Grb2, which may also couple gp130 to the ERK pathway through interactions with SOS and Ras (Fukada *et al.*, 1996; Li *et al.*, 1994).

Stimulation of ERK1 and ERK2 has been shown to play a key role in mediating mitogenic responses of cells to growth factors (Cowley *et al.*, 1994; Pages *et al.*, 1993; Vouret-Craviari *et al.*, 1993; Weber *et al.*, 1997). Activation of this pathway via gp130 has also been reported to promote the proliferation of BAF B03 cells and to block apoptosis in cardiomyocytes (Fukada *et al.*, 1996; Sheng *et al.*, 1997). Here we confirm that activation of gp130 does stimulate the phosphorylation of SHP-2 and activation of ERK1 and

ERK2 in ES cells. Surprisingly, however, these signals are not required for the efficient propagation of stem cells. On the contrary, elimination of the phosphotyrosine binding site for SHP-2 from gp130 or inhibition of ERK activation enhanced ES cell self-renewal.

MATERIALS AND METHODS

ES Cell Culture and Transfection

ES cells were maintained without feeder cells in Glasgow modification of Eagle's medium containing 10% foetal calf serum, 0.1 mM 2-mercaptoethanol, and LIF. D027 cells have both copies of the *lif* gene inactivated by homologous recombination and an IRES- β geo reporter gene inserted within the *Oct4* gene locus (Dani *et al.*, 1998). ZIN40 cells carry a nuclear-localised β -galactosidase marker gene that is widely expressed in differentiated cell types (Mountford, 1995). For transfections of D027 cells, 2×10^7 cells were electroporated with 100 μ g of linearised plasmid DNA at 0.8 kV and 3 μ F in a 0.4-cm cuvette using a Bio-Rad gene pulser. Stably transfected clones were selected in medium containing 20 μ g/ml zeocin (Invitrogen). For introduction of the episomal constructs into MG1.19 cells (Gassmann *et al.*, 1995), 5×10^6 cells were electroporated with 20 μ g of supercoiled plasmid DNA at 0.2 kV and 960 μ F. Stably transfected colonies were selected in medium containing 100 μ g/ml hygromycin B for 8 days.

Plasmid Construction

The GRgp130 chimaeric receptors were generated by fusing the extracellular domain of the human granulocyte colony stimulating factor receptor (G-CSFR) (Baumann *et al.*, 1994) to an *EcoRI* fragment of mouse gp130 containing the transmembrane domain and the entire cytoplasmic region (Hibi *et al.*, 1990). The phenylalanine substitution of tyrosine 118 was introduced into the intracellular domain of gp130 by PCR overlap mutagenesis (Higuchi *et al.*, 1988). The PCR product was substituted into the GRgp(278) chimaera and sequenced. The receptor cDNAs were inserted within expression vector pCAGIZ. This vector contains a bicistronic expression cassette consisting of cytomegalovirus enhancer-human β -actin promoter, a site for insertion of the receptor cDNA, an internal ribosome entry site, and the zeocin-resistance gene (Niwa *et al.*, 1998). Full-length cDNAs encoding human SHP-2 (Bennett *et al.*, 1996) were inserted into both pCAGIZ and an otherwise identical plasmid, pCAGIH, in which the zeocin-resistance gene was substituted by the hygromycin-resistance gene.

Self-Renewal Assay

Expression of β -galactosidase from the *Oct4* locus in D027 cells was quantitated in an ONPG assay. ES cells were plated at 5000 cells per well in 24-well dishes and cultured for 6 days in the presence or absence of cytokine. When cells were treated with PD098059, they were plated at 2500 per well and cultured overnight in normal growth medium prior to addition of the inhibitor. On day 6, cells were washed once with PBS and lysed in 0.4 ml of 0.25 M Tris, pH 7.5, 0.5 mM DTT, 0.5% NP-40. Lysate (40 μ l) was mixed with 100 μ l of ONPG buffer (60 mM Na_2HPO_4 , 40 mM NaH_2PO_4 , 10 mM KCl, 1 mM MgCl_2 , 50 mM 2-mercaptoethanol, 1.2 mM ONPG) in a microtitre plate and incubated at 37°C for 2–4

h and the absorbance was read at 420 nm. All assays were performed in triplicate.

Immunoprecipitation and Immunoblotting

One day after plating ($2-3 \times 10^6$ cells per 100-mm dish), ES cells were refed with medium containing 1% foetal calf serum and lacking cytokines. The following day, cells were transferred to serum-free medium for 4 h prior to stimulation with IL-6 (100 ng/ml plus soluble receptor) or G-CSF (30 ng/ml) for 15 min. Cells were then washed once with ice-cold PBS and scraped off in 1 ml of ice-cold lysis buffer (150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 0.5% NP-40, 1 mM NaVO₃, 1 mM EDTA, 0.5 mM PMSF). Cleared lysates were incubated with 1 μ g anti-SHP-2 antibody (Santa Cruz) at 4°C for 1 h and then protein A-Sepharose was added and the incubation continued overnight. Immunoprecipitates were solubilised in 2 \times SDS sample buffer, electrophoresed on a 10% SDS-polyacrylamide gel, and electroblotted onto nitrocellulose. After overnight treatment with blocking buffer (25 mM Tris-HCl, pH 7.4, 2.7 mM KCl, 140 mM NaCl, 0.1% Tween 20, 1% BSA) the membranes were probed sequentially with anti-phosphotyrosine 4G10 (Transduction Laboratories), anti-Gab1 (Takahashi-Tezuka *et al.*, 1998), and anti-SHP-2 antibodies (Santa Cruz). Blots were incubated with horseradish peroxidase-coupled anti-mouse or anti-rabbit IgG and developed using ECL reagents (Amersham). Antibodies were stripped from the membranes between probeds by incubation at 50°C for 30 min in 62.5 mM Tris-HCl, pH 6.8, 2% SDS, 100 mM 2-mercaptoethanol.

For analysis of STAT3 and ERK phosphorylation, 1×10^6 ES cells were plated per well of 6-well dishes. Cells were serum starved and treated with cytokines as described above and then lysed in 100 μ l SDS sample buffer. Ten-microlitre aliquots were fractionated on a 10% SDS-polyacrylamide gel, electroblotted onto nitrocellulose, and probed with anti-ERK and anti-STAT3 antibodies according to the directions provided by the supplier (New England Biolabs).

Chimaera Analysis

ZIN40 ES cells (Mountford, 1995) were injected into C57BL/6 blastocysts and transferred into pseudopregnant mice. Mice were sacrificed at day 9.5 of pregnancy and the embryos were stained with X-gal.

Formation of Embryoid Bodies

Trypsinised IOUD2 ES cells (Dani *et al.*, 1998) were diluted to 1.5×10^4 cells/ml and allowed to aggregate in 10- μ l hanging drop cultures for 2 days in the absence of LIF. They were then transferred to bacterial dishes containing medium with or without PD098059. After 5 days of culture, the embryoid bodies were collected and plated overnight in gelatine-coated wells of a 6-well tissue culture dish. Embryoid bodies were either stained with X-gal on the following day or cultured for a further 5 days and then stained.

RESULTS

Tyrosine 118 Is Required for gp130-Dependent Phosphorylation of SHP-2 in ES Cells

Previous studies in BAF pro-B cell lines have shown that gp130-dependent activation of SHP-2 and the MAPKs,

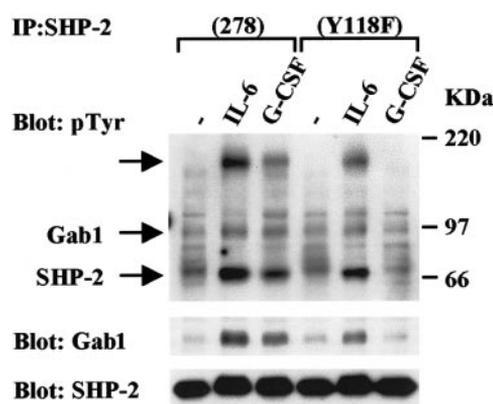


FIG. 1. gp130-dependent phosphorylation of SHP-2 in ES cells. ES cells expressing either the GRgp(278) or the GRgp(Y118F) chimaeric receptors were induced with IL-6 (100 ng/ml plus sIL-6R) or G-CSF (30 ng/ml) for 15 min. SHP-2 protein was immunoprecipitated from lysates of unstimulated or stimulated cells, fractionated on a SDS-polyacrylamide gel, and transferred to a nitrocellulose membrane. The filter was probed sequentially with anti-phosphotyrosine antibody (top), anti-Gab1 antibody (middle), and anti-SHP-2 antibody (bottom). The positions of tyrosine-phosphorylated forms of SHP-2, Gab1, and the higher molecular weight tyrosine phosphoprotein are indicated by arrows.

ERK1 and ERK2, is mediated via a tyrosine located 118 amino acid residues from the membrane within the cytoplasmic region of gp130 (Fukada *et al.*, 1996). To examine the functional role of tyrosine 118 in ES cells, we constructed cDNAs encoding chimaeric receptors consisting of the extracellular domain of the G-CSFR fused to the trans-membrane and cytoplasmic region of gp130. Since ES cells do not normally express the G-CSFR and show no self-renewal response to G-CSF (data not shown), these chimaeric receptors can be used to examine signalling independent of endogenous cytokine receptors (Niwa *et al.*, 1998). cDNAs encoding either the unmodified chimaeric receptor, GRgp(278), or a mutated receptor in which phenylalanine had been substituted for tyrosine 118, GRgp(Y118F), were cloned into the pCAGIZ expression vector and stably introduced into D027 ES cells by electroporation.

In several differentiated cell types, SHP-2 becomes tyrosine phosphorylated following its recruitment to a tyrosine phosphorylated gp130 receptor subunit (Fukada *et al.*, 1996; Nakashima *et al.*, 1997; Stahl *et al.*, 1995). To examine whether SHP-2 undergoes this modification in ES cells, SHP-2 immunoprecipitates were prepared from GRgp(278) and GRgp(Y118F) transfectants following stimulation with either IL-6 (plus sIL-6R) or G-CSF and probed for phosphotyrosine by Western blotting (Fig. 1). An increase in phosphorylated SHP-2 was detected in cells stimulated through either the endogenous gp130 or the GRgp(278) receptor. Two additional tyrosine phosphoproteins coprecipitated with the phosphorylated SHP-2. The protein migrating at approximately 100 kDa represents Gab1, an

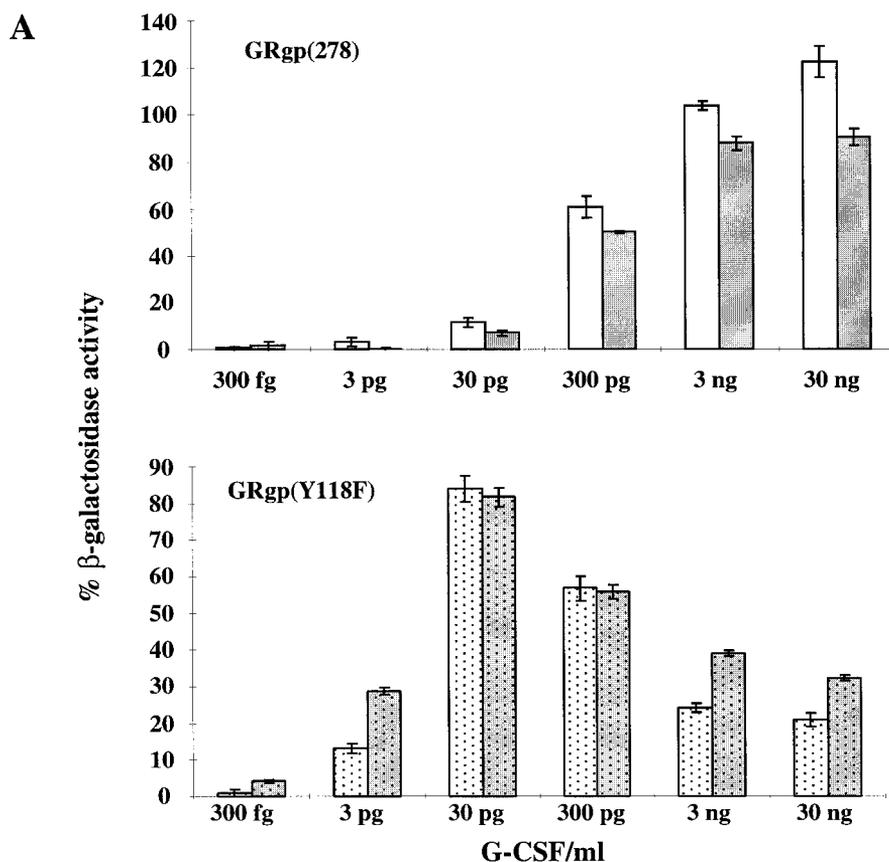


FIG. 2. Effect of mutating tyrosine 118 on gp130-dependent self-renewal and growth of ES cells. (A) Stem cell renewal mediated by GRgp(278) and GRgp(Y118F) chimaeric receptors in response to G-CSF. Self-renewal, as measured by β -galactosidase expression from the *Oct4-LacZ* locus was assayed after 6 days in culture with G-CSF (300 fg/ml to 30 ng/ml). Data for two independent clones are represented as means \pm SEM for duplicate determinations of triplicate samples normalised relative to the response with IL-6 (100 ng/ml plus sIL-6R). (B) Photomicrographs of X-gal-stained, representative colonies formed by GRgp(278) and GRgp(Y118F) transfectants after 6 days culture with 300 fg, 30 pg, and 30 ng/ml of G-CSF. (C) Photomicrographs of representative colonies formed by GRgp(278) and GRgp(Y118F) transfectants after 6 days culture with no cytokine, IL-6 (100 ng/ml + sIL-6R), G-CSF(30 ng/ml), or IL-6 (100 ng/ml + sIL-6R) and G-CSF(30 ng/ml).

IRS-1-related adaptor protein previously reported to associate with phosphorylated SHP-2 (Holgado-Madruga *et al.*, 1996; Takahashi-Tezuka *et al.*, 1998; Weidner *et al.*, 1996), while the higher molecular weight phosphoprotein likely represents phosphorylated receptor. Phosphorylation of SHP-2 did not increase following stimulation of the GRgp(Y118F) receptor, indicating that tyrosine 118 is necessary for recruitment of the phosphatase to gp130 in ES cells.

SHP-2 Activation Is Not Required for ES Cell Self-Renewal

In order to determine whether activation of SHP-2 is necessary for the propagation of ES cells, the response of GRgp130 transfectants to G-CSF was measured in a self-

renewal assay. D027 cells have a *LacZ* gene inserted within the stem-cell-specific gene, *Oct4* (Dani *et al.*, 1998). As a consequence, expression of this integrated reporter gene is restricted to undifferentiated ES cells and the resulting β -galactosidase activity provides a measure of stem cell self-renewal. In addition, both copies of the *lif* gene have been inactivated through gene targeting, thus reducing autocrine stimulation of ES cell growth (Dani *et al.*, 1998). β -Galactosidase activity from two independently isolated clones for each receptor construct was measured in medium-density cultures after 6 days of treatment with 300 fg/ml to 30 ng/ml G-CSF.

The data presented in Fig. 2A show that self-renewal of GRgp(278) transfectants increased in a dose-dependent manner, reaching a plateau at 3–30 ng/ml G-CSF. In contrast, the maximal self-renewal response of GRgp(Y118F) ES

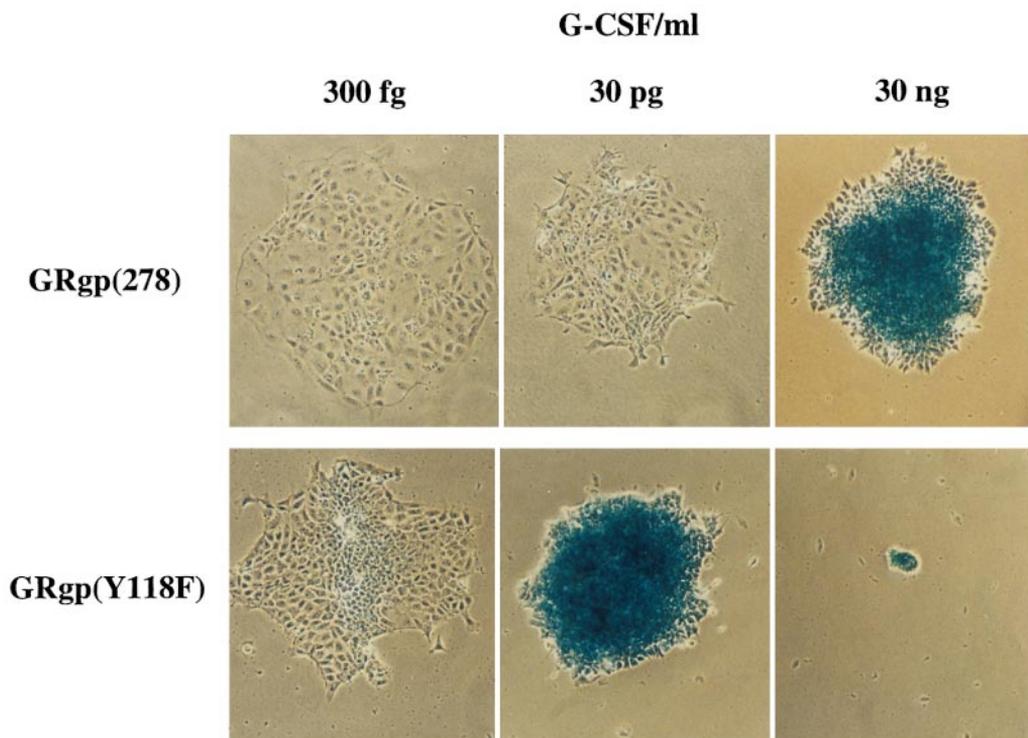
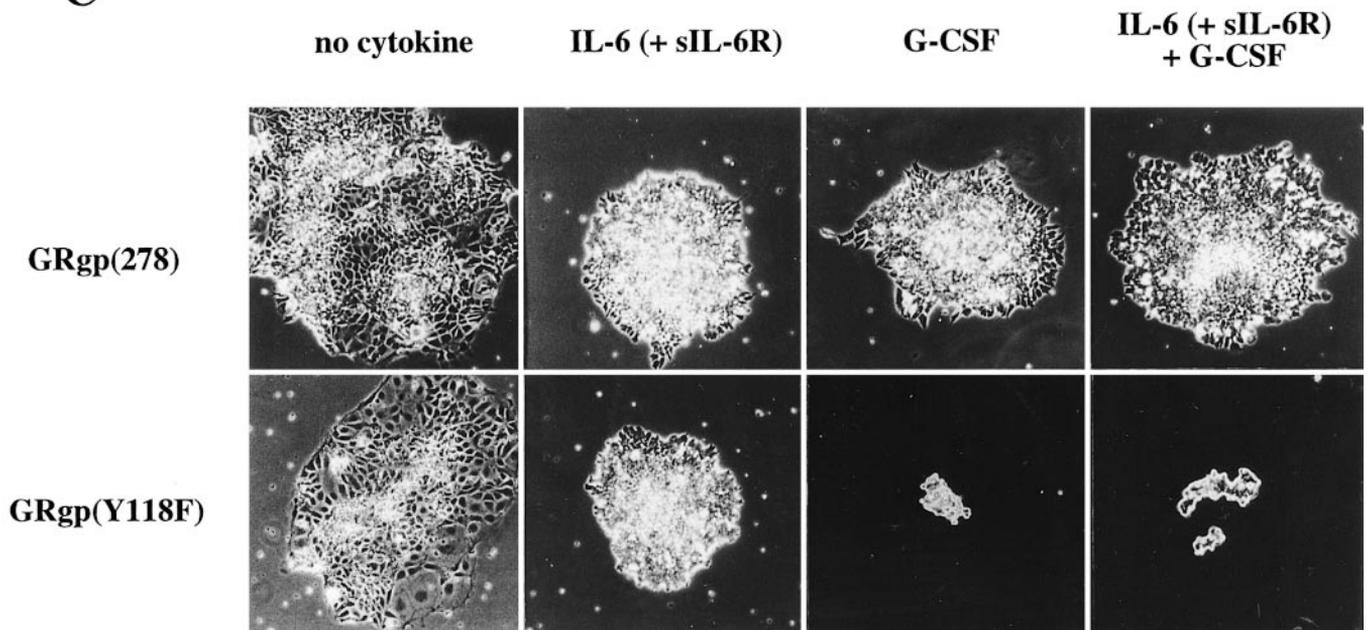
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FIG. 2—Continued

cells was achieved at just 30 pg/ml G-CSF. The morphology of the GRgp(Y118F) colonies maintained in 30 pg/ml G-CSF was typical of undifferentiated ES cells (Fig. 2B). This result establishes that activation of SHP-2 through tyrosine 118 is not required to direct ES cell self-renewal. Equivalent levels of both receptor chimaeras were expressed at the cell surface of ES cell transfectants, as judged by binding studies with ¹²⁵I-labelled G-CSF (data not shown). Therefore the shift in dose response suggests that substitution of Y118 directly alters signalling activity.

Interestingly, at higher concentrations of G-CSF, GRgp(Y118F) transfectants formed small aggregates of cells rather than the more flattened colony morphology normally associated with undifferentiated ES cells (Fig. 2B). These colonies expressed β -galactosidase and stained positive for the stem cell marker alkaline phosphatase (Fig. 2B and data not shown), indicating that the ES cells remained undifferentiated. This was confirmed by the resumption of typical ES cell growth and colony morphology when, following treatment with G-CSF, these cultures were refed with medium containing IL-6 plus sIL-6R (data not shown).

The unusual appearance of GRgp(Y118F) cells in high concentrations of G-CSF is unlikely to be simply due to an increase in affinity of the Y118F receptor for G-CSF because this response is not observed in wild-type cells treated with high levels of LIF or in GRgp(278) transfectants treated with saturating levels of IL-6 (plus sIL-6R), G-CSF, or IL-6 (plus sIL-6R) plus G-CSF (Fig. 2C). Furthermore the phenotype of GRgp(Y118F) cells in high levels of G-CSF was maintained when cells were simultaneously stimulated with G-CSF and IL-6 (plus sIL-6R). This observation excludes the explanation that the unusual ES cell morphology is due to a partial loss of self-renewal signals and suggests that the phenotype arises from hyperactivation of signals downstream of gp130. Collectively these data point to a key role for tyrosine 118 in downregulating gp130 signalling in ES cells.

Attenuation of the STAT3 Signal Is Mediated via Tyrosine 118

We have previously established that activation of STAT3 is essential for gp130-dependent self-renewal of ES cells (Niwa *et al.*, 1998). To determine whether mutating tyrosine 118 affects this key regulator, activation of STAT3 was compared in GRgp(278) and (Y118F) transfectants. Stimulation of cells for 25 min with 30 fg/ml to 300 ng/ml of G-CSF did not reveal a significant difference between the levels of tyrosine phosphorylation of STAT3 induced by the chimaeric receptors (data not shown). However, signalling through the receptors was distinguished when the duration of the STAT3 signal was examined (Fig. 3). Cells were stimulated with either G-CSF or IL-6 (plus sIL-6R) for 25 min and refed with cytokine-free medium and then samples were collected at 40-min intervals. A similar time course for the decay of phosphorylated STAT3 was obtained following stimulation through either the endogenous gp130 receptor or GRgp(278), with the signal being undetectable by 120 min. In contrast, the activation of STAT3

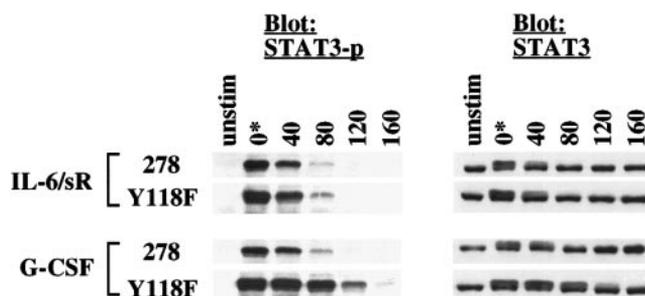


FIG. 3. Decay of activated STAT3 following stimulation of gp130 and the chimaeric GRgp130 receptors. GRgp(278) and GRgp(Y118F) ES cell transfectants were stimulated with IL-6 (100 ng/ml plus sIL-6R) or G-CSF (30 ng/ml) for 25 min (0*) and refed with cytokine-free medium and samples were collected at 40-min intervals. Immunoblots of cell lysates were probed sequentially with an antibody specific for the active phosphorylated form of STAT3 and then with an antibody that recognises both phosphorylated and unphosphorylated STAT3. Note that activation of STAT3 is associated with the appearance of a slower migrating STAT3 species, presumed to be the serine phosphorylated form of STAT3.

was sustained in G-CSF-treated GRgp(Y118F) cells and could still be detected at 160 min. This result indicates that tyrosine 118 mediates a signal that normally attenuates the activation of STAT3.

Inhibition of SHP-2 Activity Promotes ES Cell Self-Renewal

To determine whether the inability to recruit and activate SHP-2 could account for the altered regulation of the GRgp(Y118F) receptor, we examined the effects of overexpressing mutated forms of SHP-2 on ES cell self-renewal. SHP-2 can potentially affect downstream signalling through two mechanisms; through its phosphatase activity or via direct phosphotyrosine-dependent interaction with the SH2 domain containing adaptor protein Grb2 (Bennett *et al.*, 1994; Milarski and Saltiel, 1994). cDNAs encoding wild-type SHP-2, a mutant lacking the two principal sites for tyrosine phosphorylation on SHP-2 (Y542/580F), or a catalytically inactive enzyme in which an essential cysteine is replaced with serine (C459S) were inserted into the pCAGIH expression vector. These plasmids contain the polyoma origin of replication and are maintained as episomes when introduced into MG1.19 cells, an ES cell line that has been engineered to constitutively express polyoma large T (Gassmann *et al.*, 1995). In addition to the relatively stable expression of the introduced cDNA, the transfection efficiency of these vectors is considerably higher than protocols dependent on stable integration, thus allowing rapid evaluation of the effects of introduced molecules in large numbers of independent transfectants (Niwa *et al.*, in preparation).

Plasmids were electroporated into MG1.19 cells and stable transfectants were isolated after 8 days selection in medium

containing hygromycin B and LIF. Similar numbers of hygromycin-resistant colonies were obtained for all the SHP-2 expression vectors. After staining with Leishman's reagent, a minimum of 300 randomly chosen colonies per construct were scored according to whether they contained predominantly stem cells, both stem and differentiated cells, or only differentiated cells (Fig. 4A). Compared with the empty vector control, expression of SHP-2 (WT) or (Y-F) had little effect on colony morphology other than a slight increase in the numbers of mixed colonies. In contrast, expression of the SHP-2 (C-S) mutant increased the proportion of colonies containing stem cells and reduced the number of those composed of differentiated cells. This suggests that inhibition of the phosphatase activity of SHP-2 enhances ES cell self-renewal.

To assess this in a more quantitative manner, D027 transfectants carrying stably integrated SHP-2 expression vectors were established and their self-renewal in response to LIF was measured via expression of the chromosomal *Oct4-LacZ* reporter gene. Immunoblotting confirmed that the introduced forms of SHP-2 were overexpressed by approximately 10- to 20-fold in the D027 transfectants (Fig. 4B). As shown in Fig. 4C, expression of the SHP-2 (C-S) mutant increased stem cell self-renewal compared with cells transfected with control vector. Both SHP-2 (WT) or (Y-F) slightly inhibited self-renewal. This result supports the notion that the phosphatase activity of SHP-2 can suppress ES cell self-renewal. There was, however, no indication of the unusual colony morphology observed with the GRgp(Y118F) receptor, indicating that another, as yet unidentified, mechanism may moderate gp130-dependent signalling through tyrosine 118.

Tyrosine 118 Is Necessary for Activation of ERK1 and ERK2

Since activation of SHP-2 can couple gp130 to the ERK pathway (Fukada et al., 1996), we examined whether tyrosine 118 was also required for activation of ERK1 and ERK2 in ES cells. Activation of ERKs in GRgp130 transfectants treated with G-CSF or IL-6 (plus sIL-6R) was assessed by immunoblotting with an antibody specific for the phosphorylated (activated) forms of ERK1 and ERK2 (Fig. 5). Basal levels of activated ERK were consistently detected in untreated cells following serum starvation. Increased ERK phosphorylation was observed in cells stimulated via the endogenous gp130 and GRgp(278) receptors. This was not evident on stimulation through the GRgp(Y118F) chimaera. Reprobing with an antibody specific for the tyrosine phosphorylated form of STAT3 confirmed that both chimaeric receptors were effective at activating STAT3. These results establish that tyrosine 118 mediates activation of the ERK pathway in ES cells.

Blockade of ERK Activation with PD098059 Does Not Impair ES Cell Propagation

The capacity of GRgp(Y118F) to signal self-renewal implies that ERK activation is not required for the propagation

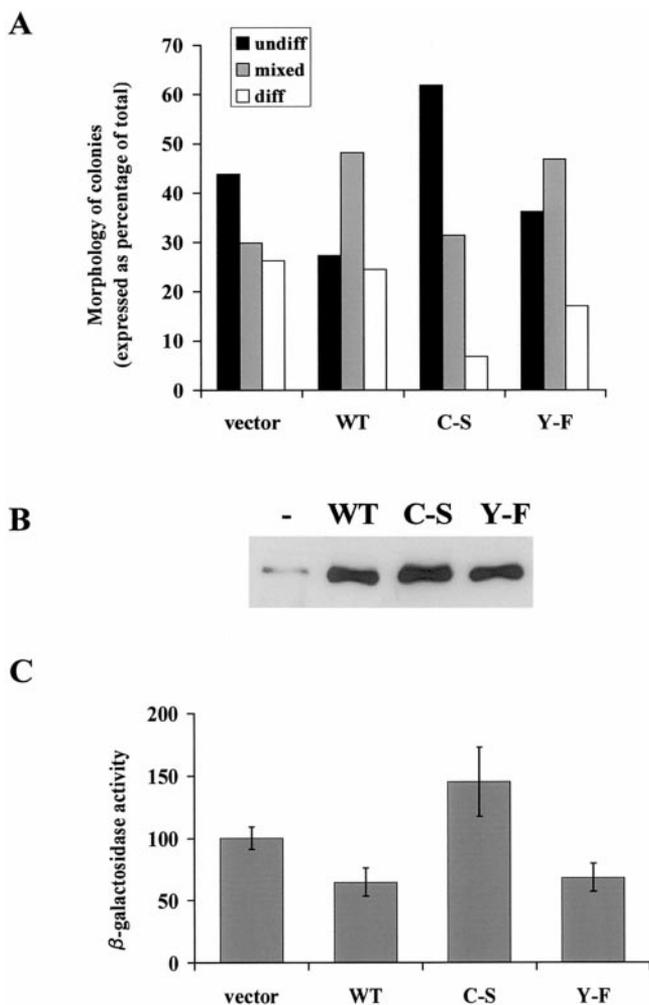


FIG. 4. Overexpression of catalytically inactive SHP-2 promotes ES cell self-renewal. (A) Self-renewal of MG1.19 cells expressing mutant forms of SHP-2. MG1.19 cells were electroporated with either the empty expression vector pCAGIH or constructs expressing WT, Y542/580F, or C459S forms of SHP-2. Hygromycin-resistant colonies (>300) were stained and scored on the basis of whether they contained predominantly stem cells (undiff) or differentiated cells (diff) or were composed of both cell types. (B) Overexpression of SHP-2 in D027 transfectants. Cell lysates prepared from representative clones were fractionated on a 10% SDS-acrylamide gel, electroblotted onto a nitrocellulose membrane, and probed with a SHP-2-specific antibody. (C) Self-renewal of D027 cells overexpressing SHP-2. Self-renewal of D027 cells stably transfected with either pCAGIZ (vector) or the pCAGIZ-based SHP-2 expression constructs was assayed by measuring β -galactosidase activity from the *Oct4-LacZ* locus. Cells were assayed after culture for 6 days in 100 units/ml LIF. Data for three independent clones are represented as means \pm SEM for duplicate determinations of triplicate samples and normalised relative to the mean value obtained for the pCAGIZ (vector) transfectants.

of ES cells. To test this hypothesis, D027 cells were cultured in the presence of the specific MEK inhibitor, PD098059 (Dudley et al., 1995). Treatment of ES cells with

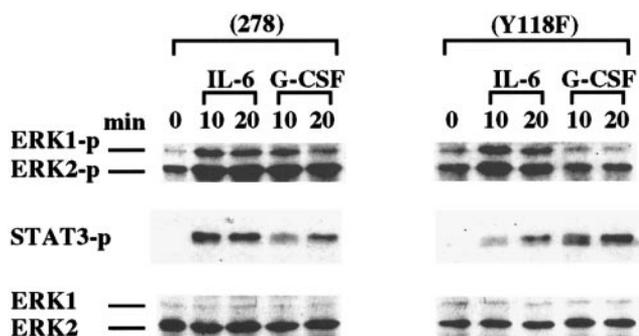


FIG. 5. gp130-dependent phosphorylation of ERK1 and ERK2 in ES cells. ES cells expressing either the GRgp(278) or the GRgp(Y118F) chimeric receptors were either untreated or stimulated with IL-6 (100 ng/ml plus sIL-6R) or G-CSF (30 ng/ml) for 10 or 20 min. Cell lysates were separated on a 10% SDS-acrylamide gel, electroblotted onto a nitrocellulose membrane, and probed sequentially with antibodies specific for the active phosphorylated forms of ERK and STAT3. Reprobing the stripped filter with an antibody that binds to both phosphorylated and dephosphorylated ERKs verified that equivalent amounts of protein were loaded in the samples.

3–25 μ M PD098059 did not inhibit self-renewal compared with cells cultured in vehicle alone (Fig. 6A). Surprisingly, in fact, the level of self-renewal increased in a dose dependent manner peaking at 12–25 μ M. At concentrations greater than 50 μ M PD098059 the growth of ES cells was impaired, possibly as a result of some nonspecific inhibitory effect of the drug, resulting in small colonies which were undifferentiated as shown by staining for β -galactosidase (data not shown).

To verify that ERK activation through gp130 was continuously suppressed by PD098059 in these long-term cultures, GRgp(278) cells were incubated for 48 h with the inhibitor plus LIF and then stimulated through the chimeric receptor with G-CSF. The immunoblot revealed that G-CSF-dependent phosphorylation of ERK1 and ERK2 was progressively reduced from 3 to 12 μ M and effectively blocked at 25 μ M PD098059 (Fig. 6B). The continued proliferation of undifferentiated ES cells at inhibitory concentrations of PD098059 confirms that gp130-dependent activation of ERK1 and ERK2 is not required for the propagation of ES cells.

The effect of PD098059 on self-renewal suggested that the inhibitor might alter the dose response of ES cells to LIF. Self-renewal of ES cells was assayed following treatment with 3–300 U/ml LIF in the presence of either 25 μ M PD098059 or vehicle (0.2% DMSO) (Fig. 6C). Treatment with PD098059 increased the level of β -galactosidase activity at all concentrations of LIF. This implies that the drug does not alter the cytokine dose dependency of ES cells but rather enhances their response to LIF. Significantly, PD098059 did not block the differentiation of ES cells in the absence of LIF.

ES Cells Propagated in PD098059 Remain Pluripotent

ES cell colony morphology and Oct4 expression are reliable indicators of the undifferentiated phenotype, but do not establish that the cells are pluripotent. We therefore determined whether ES cells propagated in the absence of gp130-dependent ERK signalling have the capacity to incorporate into the developing embryo and differentiate appropriately. Cells were cultured at low density (1000 cells/cm²) for 48 h in the presence of LIF plus 25 μ M PD098059 or in the absence of LIF. They were then refed with medium containing LIF but lacking the inhibitor for a further 24 h before microinjection into mouse blastocysts. ZIN40 cells were used in this experiment, since they carry a nuclear-localised β -galactosidase marker widely expressed in differentiated cell types (Mountford, 1995). Staining of midgestation embryos for β -galactosidase revealed that ES cells treated with PD098059 contributed to chimaeras (Fig. 7). In contrast, cells cultured in the absence of LIF for 48 h were incapable of colonising the embryo (data not shown). Significantly, live-born chimaeras have been obtained from ES cells cultured for 4 days in 25 μ M PD098059. Functional contribution of the cells to the germ line has been observed in two chimaeras, as evidenced by transmission of the ES cell-derived coat colour marker. This result confirms that gp130-dependent ERK activity is not required for maintaining the pluripotency of ES cells.

PD098059 Inhibits the Differentiation of ES Cells in Embryoid Bodies

The enhancement of ES cell self-renewal by PD098059 indicates that signals mediated through MEK may be involved in controlling ES cell differentiation. However, the physiological relevance of this process on a plastic substratum is unclear. To examine the effect of PD098059 in a more appropriate model of differentiation, ES cells were aggregated to form embryoid bodies and cultured in the presence of the MEK inhibitor. The IOUD2 ES cell line used in this experiment carries the *Oct4-LacZ* chromosomal reporter gene, thus allowing the identification of stem cells in embryoid bodies by staining for β -galactosidase activity (Dani *et al.*, 1998). ES cells were aggregated in hanging drops in the absence of LIF for 2 days and then maintained in suspension in bacterial dishes in the presence of 25–100 μ M PD098059 for a further 5 days. Embryoid bodies were then plated on gelatin-coated plates overnight and stained with X-gal. Results of a representative experiment are shown in Fig. 8. The proportion of stem cells increased in the presence of PD098059, in a dose-dependent manner, with the majority of cells in embryoid bodies in 75 μ M PD098059 staining positive for β -galactosidase. Importantly, the absolute number of stem cells also increased (compare 25 μ M with 75 μ M), indicating that over this range of concentrations of PD098059 the persistence of the stem cells was not due to an inhibition of proliferation. At 100 μ M PD098059, however, the growth of embryoid bodies was severely

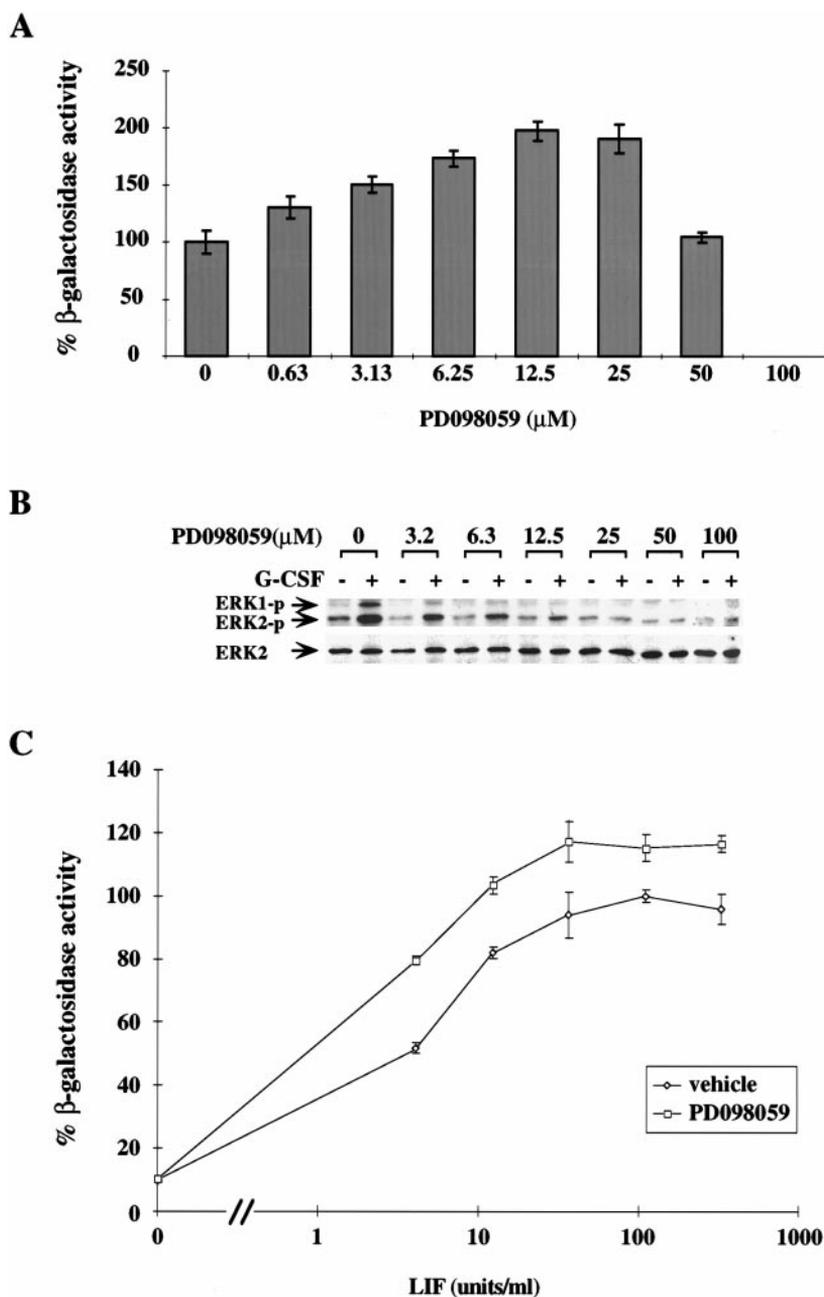


FIG. 6. Effect of the MEK inhibitor, PD098059, on ES cell self-renewal and ERK activation. (A) Self-renewal of ES cells treated with the indicated concentrations of PD098059. D027 ES cells grown at a subsaturating level of LIF (5 U/ml), to increase the sensitivity of the assay to changes in self-renewal signalling, were treated with PD098059 for 5 days and assayed for β -galactosidase expression from the *Oct4* locus. Data are means \pm SEM for duplicate determinations of triplicate samples normalised relative to the response in the absence of PD098059. (B) PD098059-dependent inhibition of ERK activation. GRgp(278)-transfected D027 cells were cultured with a subsaturating level of LIF (5 U/ml) and PD098059 for 48 h. Cells were then stimulated with G-CSF (30 ng/ml) for 10 min, lysed in sample buffer, and analysed for ERK activation by immunoblotting with phosphospecific anti-ERK antibodies. Subsequent probing of the filter with an antibody that binds to both phosphorylated and dephosphorylated ERKs confirmed that equivalent amounts of protein were loaded in all samples. (C) Effect of PD098059 on the dose response of ES cells to LIF. The dose response of D027 ES cells to LIF in 25 μ M PD098059 or vehicle (0.05% DMSO) was measured by β -galactosidase expression from the *Oct4* locus. Data are means \pm SEM for duplicate determinations of triplicate samples normalised relative to the maximum response of cells to treatment with LIF (100 U/ml) plus vehicle. No significant difference in ES self-renewal was observed between untreated and DMSO-treated cells.

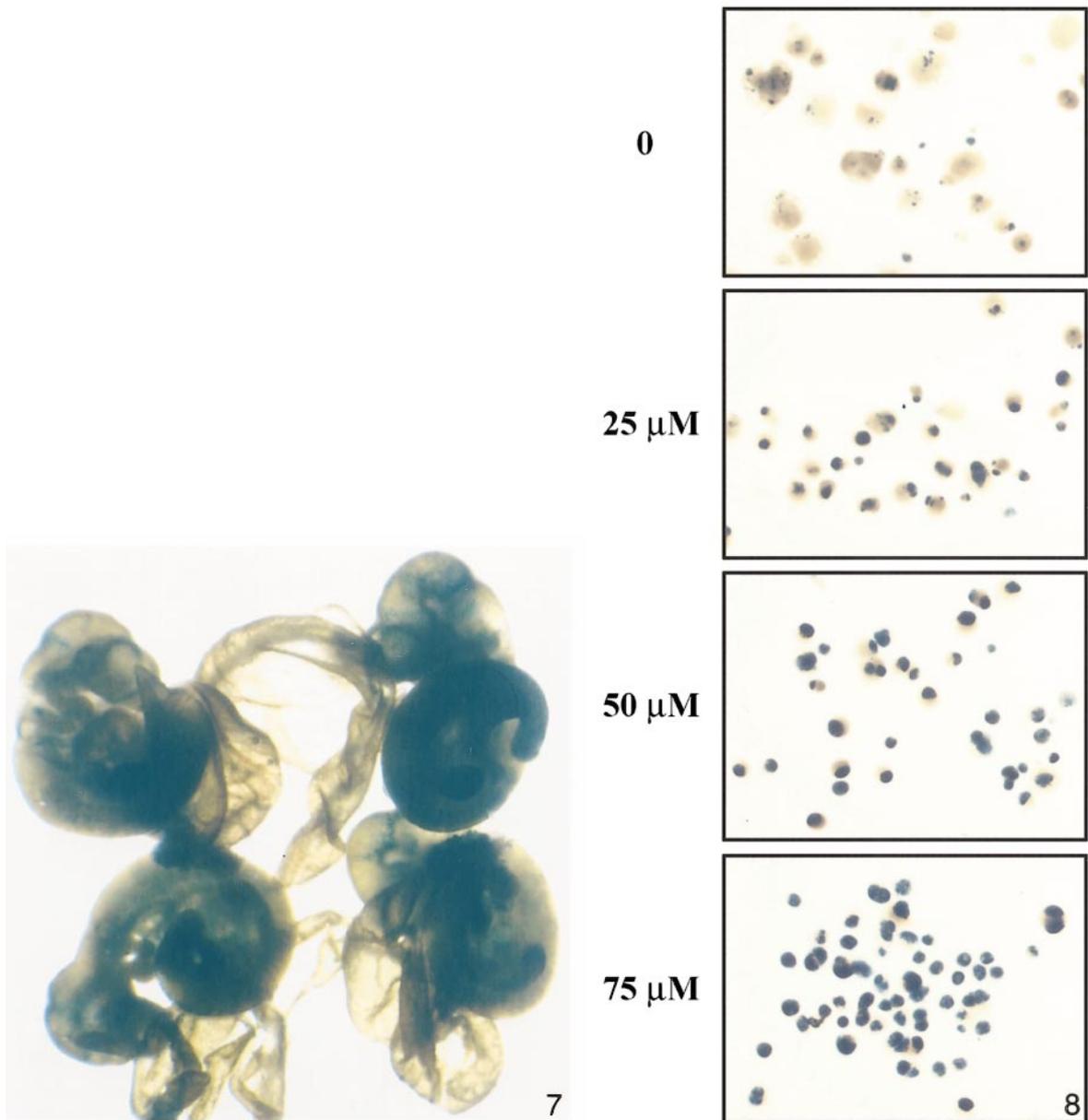


FIG. 7. Effect of PD098059 on ES cell pluripotency. ZIN40 ES cells were treated with 25 μM PD098059 plus 5 U/ml LIF for 48 h, refed with medium containing LIF for a further 24 h, and then microinjected into C57BL/6 blastocysts. Embryos were collected at day 9.5 of pregnancy and stained for β -galactosidase activity. Representative embryos are shown.

FIG. 8. Effect of PD098059 on ES cell differentiation in embryoid bodies. Aggregates of IOUD2 ES cells were formed in hanging drops without LIF for 2 days. They were then transferred to bacterial dishes and cultured for 5 days in medium containing either no inhibitor or 25, 50, or 75 μM PD098059. After attachment overnight on gelatinised plates, the embryoid bodies were stained for β -galactosidase activity.

inhibited, although most of the surviving cells did still express the *Oct4-LacZ* reporter gene (data not shown).

In embryoid bodies maintained for a further 5 days after plating, the persistence of stem cells was significantly augmented by PD098059 (data not shown). Cardiomyocytes, identifiable as patches of beating cells, were found in

both untreated embryoid bodies and those cultured in the presence of 25 μM PD098059, thus demonstrating that the starting population of ES cells was capable of differentiating appropriately.

These results show that ES cell differentiation, under conditions that reflect events in the early embryo, can be

inhibited by blocking MEK activity. This suggests that ERK signalling may play an important role in the differentiation of pluripotent epiblast cells during gastrulation.

DISCUSSION

Engagement of the gp130 cytokine receptor subunit generates intracellular signals that block ES cell differentiation and promote self-renewal of these pluripotent stem cells (Yoshida *et al.*, 1994). Identification of the underlying mechanisms that regulate mouse ES cell propagation should allow the development of improved strategies for establishing pluripotent stem cell lines from other mammalian species including humans. In this regard, we have previously demonstrated that gp130-dependent propagation of ES cells does require STAT3 activation (Niwa *et al.*, 1998). Here we show that gp130-dependent recruitment of SHP-2 and the stimulation of the MAP kinases, ERK1 and ERK2, do not contribute directly to maintaining pluripotency. Rather they function to moderate the strength of gp130 signalling. In addition, we present evidence that ERK activation may directly promote the differentiation of ES cells.

Stimulation of ES cells through either endogenous gp130 or the chimaeric GRgp(278) receptor increased the tyrosine phosphorylation of SHP-2. This modification was blocked when tyrosine 118 of gp130 was mutated to phenylalanine, confirming that this single tyrosine is essential for recruitment of SHP-2 (Stahl *et al.*, 1995). Despite this deficit, the mutated receptor was fully capable of directing self-renewal of ES cells, proving effective even at low concentrations of activating ligand. This result establishes that SHP-2 activity is not required to maintain the pluripotent phenotype of ES cells and may in fact inhibit self-renewal signalling. The latter conclusion is supported by the enhanced self-renewal of ES cells overexpressing the catalytically inactive SHP-2 (C-S) mutant. Collectively these results suggest that SHP-2 activity suppresses or antagonises mechanisms that promote ES cell self-renewal. These findings provide an explanation for the phenotype of ES cells carrying a partial deletion of the endogenous *SHP-2* gene (Qu *et al.*, 1997). Although interpretation is complicated by the continued expression of a truncated SHP-2 protein, the mutant ES cells are viable, show a slight increase in their responsiveness to LIF, and exhibit an impaired capacity to differentiate *in vitro* (Qu and Feng, 1998).

Despite an established role for SHP-2 in coupling receptors to the Ras/MAPK signalling pathway, a negative regulatory function has also been implied through its homology to SHP-1, a suppresser of erythropoietin receptor function (Klingmuller *et al.*, 1995). Indeed, mutation of tyrosine 118 of gp130 has been shown to enhance STAT3 signalling in neuroblastoma and hepatoma cells (Kim *et al.*, 1998; Servi-dei *et al.*, 1998). Furthermore, transcription from STAT3-responsive promoter constructs was increased by overexpression of a catalytically inactive SHP-2 protein. This

pointed to the phosphatase as a likely mediator of this effect: a conclusion supported by sustained phosphorylation of both the Y118F receptor and its associated JAK kinases (Kim *et al.*, 1998).

Interestingly, signalling via the GRgp(Y118F) receptor was associated with restricted growth of ES cells in high concentrations of G-CSF. This intriguing observation indicates that excessive gp130 signalling can interfere with ES cell propagation. Hyperactivation of STAT3 is implicated in this phenotype because stimulation of the Y118F receptor results in prolonged tyrosine phosphorylation of STAT3 and sustained activation of endogenous STAT3 target genes, and is suppressed by combined mutagenesis of multiple STAT3 docking sites (T.B., C.S., I.C., A.S., unpublished). Significantly, overexpression of catalytically inactive SHP-2 enhanced self-renewal but did not recapitulate the effect obtained with the GRgp(Y118F) receptor, indicating that a loss of phosphatase activity of SHP-2 may not be solely responsible for the increased signalling activity.

Since ERKs are downstream of effectors of SHP-2 and have been proposed to play a role in self-renewal (Boeuf *et al.*, 1997; Ernst *et al.*, 1996), we examined the activation of these MAP kinases by the chimaeric receptors. Whereas stimulation of gp130 in ES cells triggered an increase in ERK1 and ERK2 phosphorylation, no response could be detected upon engagement of the Y118F chimaeric receptor. Furthermore, treatment with inhibitory concentrations of the MEK inhibitor PD098059 did not block but rather enhanced self-renewal of ES cells. Activation of ERK1 and ERK2, via either SHP-2 or another pathway such as Shc (Giordano *et al.*, 1997), therefore is not critical in maintaining the pluripotent phenotype of ES cells.

This lack of a requirement for gp130-dependent ERK activation may be related to the continuously proliferating nature of ES cells. An established function of ERKs in differentiated cells is to regulate the transition through G1/S, at least in part through the induction of cyclin D (Lavoie *et al.*, 1996; Weber *et al.*, 1997). However, ES cells have a relatively short G1 phase and appear to possess few of the G1-associated control mechanisms (Savatie *et al.*, 1994, 1996). Furthermore, this uncoupling from ERK signalling is consistent with the observation that ES cells continue to proliferate in the absence of serum, a powerful mitogen and inducer of ERK activity (Johansson and Wiles, 1995).

The increase in stem cell self-renewal on treatment with the MEK inhibitor PD098059 implies that ERK activity impairs the propagation of undifferentiated ES cells. Studies of gp130-dependent signalling in PC12 cells and in astrocytes have shown that inhibition of ERK activity resulted in enhanced STAT3-mediated transcription (Bonni *et al.*, 1997; Ihara *et al.*, 1997). This suggests that antagonism between STAT3 and ERK signalling could account for the effect of PD098059 in ES cells. Alternatively, signalling via ERKs may actively promote ES cell differentiation. Indeed, the persistence of stem cells in embryoid bodies treated

with PD098059 indicates that ERK activation could be a necessary component of the normal differentiation process.

Differentiation of ES cells is associated with induction of G1 cyclin expression, indicating the reestablishment of G1 cell cycle control mechanisms (Savatier *et al.*, 1996). This transition also occurs in the epiblast at gastrulation and there is increasing evidence that the Ras/MAPK signalling pathway plays a critical role in regulating these differentiation events. Inhibition of SHP-2 activity during embryonic development of either *Xenopus* or mice is associated with failure to gastrulate normally and defects in the formation of mesodermal cell lineages (Saxton *et al.*, 1997; Tang *et al.*, 1995). Downregulation of ERK activity in *Xenopus*, through overexpression of the MAP kinase phosphatase MKP-1, also inhibits mesoderm induction (LaBonne *et al.*, 1995). Furthermore, mutation of the gene encoding a transcription factor regulated by the Ras/MAPK signalling pathway, serum response factor (SRF), blocks gastrulation and formation of mesoderm in the mouse (Arsenian *et al.*, 1998). Significantly, however, loss of SRF activity is not reported to compromise the propagation of *srf*^{-/-} ES cells. The differentiation of epiblast cells may therefore represent a point at which embryonic cells become subservient to ERK-dependent growth control mechanisms. Interestingly, this is also the stage at which transplanted grafts of embryonic tissue lose the capacity to form malignant teratocarcinomas (Damajanov *et al.*, 1971).

The independence of ES cell self-renewal from ERK activation may have important practical applications. Our data suggest that inhibitors of the Ras/MAPK pathway should promote the propagation of undifferentiated ES cells. It is possible that by suppressing the growth and maturation of differentiated cell types, such inhibitors may facilitate the routine manipulation and *de novo* derivation of ES cells.

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