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Role of leukemia inhibitory factor and its receptor in mouse primordial germ cell growth

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SUMMARY

The pleiotropic cytokine leukemia inhibitory factor (LIF) is able to promote the growth of mouse primordial germ cells (PGCs) in culture. It is unclear whether LIF acts directly on PGCs or indirectly via feeder cells or embryonic somatic cells. To understand the role of LIF in PGC growth, we have carried out molecular and cell culture analyses to investigate the role of both the LIF ligand and its receptor in PGC development. LIF is able to stimulate PGC growth independently of the presence of feeder cells supporting the hypothesis that LIF acts directly on PGCs to promote their growth. We show here that transcripts for the low-affinity LIF receptor (LIFR), an integral component of the functional LIF receptor complex, are expressed in the developing gonad. Fluorescence-activated cell sorter (FACS) analysis, using an anti-LIFR antiserum, demonstrates that LIFR is present on the surface of PGCs, suggesting that PGCs are likely to be a direct target of LIF action in culture. Signalling via LIFR is essential for PGC growth in culture since the anti-LIFR antiserum, which blocks LIF binding to its receptor, abolishes PGC survival in culture. Two LIF-related cytokines, namely oncostatin M and ciliary neurotrophic factor, can also promote PGC growth in culture in addition to LIF. Thus one or more of these LIFR-dependent cytokines may play an important role in PGC development in mice.

Key words: primordial germ cells (PGCs), leukemia inhibitory factor (LIF), oncostatin M (OSM), ciliary neurotrophic factor (CNTF), interleukin-6 (IL-6), basic fibroblast growth factor (bFGF), steel factor (SLF), c-kit receptor tyrosine kinase, LIF receptor (LIFR), embryonic stem (ES) cells, embryonic germ (EG) cells

INTRODUCTION

Primordial germ cells (PGCs) are the embryonic precursors of the sperm and ova of adult animals (recently reviewed by McLaren, 1992; Wylie, 1993). Mouse PGCs are first identified in the embryo at 7.0 days post coitus (dpc) as a small population of cells expressing alkaline phosphatase (AP) (EC.3.1.3.1) (Ginsburg et al., 1990). During the subsequent 5 to 6 days of development, they migrate to the gonad anlagen and proliferate rapidly to establish a population of approximately 25,000 germ cells (Tam and Snow, 1981). Shortly after arrival in the gonad, at around 13.5 dpc, PGCs stop mitotic cell division and enter mitotic arrest in the developing testis or meiosis in the ovary. Despite the importance of PGCs to the survival of animal species, until recently very little was known about the growth factors regulating PGC proliferation and differentiation. A major advance in our understanding of PGC growth control came with the characterization of two sterile mouse mutants, *Dominant White Spotting* (*W*) and *Steel* (*Sl*). These two loci encode a tyrosine kinase receptor (c-kit) and its cognate ligand, Steel factor (SLF), respectively (reviewed by Witte, 1990). The c-kit receptor is expressed by PGCs while SLF is expressed by surrounding somatic cells and by the feeder cells used for culturing PGCs in vitro (Matsui et al., 1990; Dolci et al., 1991; Godin et al., 1991). We and others have demonstrated that SLF is essential for PGC survival in vitro as it is in vivo (Dolci et al., 1991; Godin et al., 1991; Matsui et al., 1991).

Although SLF is required for PGC survival in culture, PGC proliferation is not tightly correlated with the amount of SLF produced by feeder cells. Certain feeder cells such as STO embryonic fibroblasts produce additional diffusible factors that, together with SLF, stimulate PGC proliferation (Dolci et al., 1991). One such factor is leukemia inhibitory factor (LIF) (Matsui et al., 1991). Even though STO cells produce LIF, addition of exogenous, recombinant LIF is able to promote further the growth of PGCs cultured on STO feeder cells (Resnick et al., 1992; Matsui et al., 1992). In the presence of a third factor, basic fibroblast growth factor (bFGF), SLF and

LIF promote long-term proliferation of PGCs in culture. The resultant cell lines, which we have termed embryonic germ cells, or EG cells, resemble undifferentiated embryonic stem (ES) cells (Resnick et al., 1992; Matsui et al., 1992; Stewart et al., 1994).

LIF is a cytokine consisting of about 200 amino acids that can act in different ways on a variety of different cell types (Gearing et al., 1987; Gough et al., 1988). LIF was first discovered by virtue of its ability to inhibit proliferation of M1 mouse monocytic leukemic cells and to induce them to differentiate into macrophages (Hilton et al., 1988a,b; Metcalf et al., 1988). LIF was also identified as a differentiation inhibitory activity (DIA) that is able to prevent ES cells from differentiating and therefore to maintain them in an undifferentiated state (Smith et al., 1988; Williams et al., 1988). In addition, LIF stimulates proliferation of cultured DA-1a mouse myeloid cells (Moreau et al., 1988). In synergy with interleukin 3, LIF can augment the proliferation of human hematopoietic progenitors and potentiate megakaryocyte production in culture (Leary et al., 1990; Metcalf et al., 1991). Based on analyses of protein secondary structure and genomic organization, LIF has been placed in an emerging family of cytokines which includes interleukin-6 (IL-6), oncostatin M (OSM) and ciliary neurotrophic factor (CNTF) and with which LIF shares some overlapping biological activity (Rose and Bruce, 1991; Bazan, 1991). Our understanding of the pleiotropic functions of LIF was advanced by the molecular characterization of the cell surface receptor for LIF. The LIF receptor comprises a low-affinity, LIF-binding subunit (referred to here as LIFR) and a second transmembrane molecule, gp130, which was originally identified as the IL-6 signal transducer (Gearing et al., 1991, 1992). Activation of the LIF receptor complex requires heterodimerization of LIFR and gp130, and is accompanied by tyrosine phosphorylation of both subunits (Ip et al., 1992; Taga and Kishimoto, 1992; Davis et al., 1993). As members of the emerging cytokine receptor family, these two related transmembrane proteins serve as components of bipartite or tripartite receptors for multiple cytokines. For example, the LIFR/gp130 complex also functions as a high affinity receptor for OSM (Gearing et al., 1992). Addition of a third component, the low affinity receptor for CNTF (CNTFRα), converts the bipartite LIFR/gp130 complex into a tripartite, high-affinity receptor for CNTF (Ip et al., 1992; Davis et al., 1993; Baumann et al., 1993). Thus, both LIFR and gp130 are required for the signal transduction of multiple cytokines including LIF, OSM and CNTF. That the same heterodimeric receptor complex is used in the signal transduction for LIF, OSM and CNTF may in part explain the pleiotropic and overlapping functions of these related cytokines.

Because LIF can act on a wide variety of cells types, interpretation of its role in PGC culture assays is complicated. LIF could act directly on PGCs or it could stimulate feeder cells or embryonic somatic cells to produce growth-promoting factors for PGCs. It is also not clear how LIF acts to stimulate PGC growth, although several models have been postulated. LIF could act as a survival factor, a mitogen, or as a differentiation inhibiting activity (De Felici and Dolci, 1991; Matsui et al., 1991; Resnick et al., 1992; Pesce et al., 1993). To understand the role of LIF in PGC growth, we have carried out a series of experiments examining the action of both LIF and its receptor. We report here that the LIFR is present on the surface of PGCs and is essential for PGC growth in culture. The stimulation of PGC proliferation by LIF is not alleviated by the presence of SLF and bFGF, and is independent of feeder cells, indicating that LIF has a distinct and direct function in supporting PGC growth. Two LIF-related cytokines, OSM and CNTF can also promote PGC growth in culture. The potential functions of these LIFR-dependent cytokines in PGC growth and development will be discussed.

MATERIALS AND METHODS

PGC isolation and culture

PGC isolation and culture were performed as described previously. Briefly, single cell suspensions of dissected genital ridges from 11.5 dpc B6C3F1 mouse embryos were prepared by trypsin digestion and cultured in 96-well microtiter plates as previously described (Dolci et al., 1991). PGCs were maintained in 200 μl DMEM (high glucose) plus 15% fetal calf serum (FCS) for up to 2 days with the indicated factors or antibodies, either on gamma-irradiated STO feeder cells (5,000 rads) or on gelatin-coated plates (0.1% gelatin in H_2O). PGCs were identified by alkaline phosphatase histochemistry, as described previously (Donovan et al., 1986; Dolci et al., 1991).

Growth factors and neutralizing antibodies

Recombinant murine SLF (a soluble form of SLF) made in yeast was a generous gift of Dr D. E. Williams (Immunex). Recombinant human bFGF, murine LIF (ESGRO) and a neutralizing monoclonal IgG (ACK-2) against the c-kit receptor (Nishikawa et al., 1991) were purchased from GIBCO/BRL. Recombinant human OSM, rat CNTF, murine IL-6 and a purified rat monoclonal IgG against murine IL-6Rα (Vink et al., 1990) were purchased from Genzyme. An anti-serum against the murine LIFR was raised by immunizing rabbits with a purified, recombinant protein containing the murine LIFR extracellular domain. The anti-LIFR serum was selected by its ability to block LIF binding to both the low- and high-affinity LIF receptors and to block LIF-induced proliferation of DA-la myeloid cells (Gearing, unpublished data).

Biological assays for measuring the specific activity of cytokines

The specific activities of recombinant LIF, OSM, CNTF and IL-6 were assessed by their effects on M1 cell proliferation and D3 ES cell differentiation, and normalized to that of LIF. M1 murine monocytic leukemic cells were maintained in RPMI-1640 medium plus 10% FCS. Addition of either LIF, OSM or IL-6 inhibits proliferation of M1 cells monitored by [^{3}H]thymidine incorporation into chromosomal DNA (Metcalf et al., 1988; Rose and Bruce, 1991). The concentration of murine LIF required for achieving the half maximal activity (ED_{50} or 50 units/ml) of growth inhibition towards M1 cells was determined as 0.5 ng/ml, which is consistent with the specific activity (100 units/ng) determined by the supplier (GIBCO/BRL). Using the same M1 cell assay, the specific activities of recombinant human OSM and murine IL-6 were measured as 40 units/ng and 100 units/ng respectively. The activity of the monoclonal antibody (mAb) against murine IL-6Rα was also tested in the M1 cell assay. At 10 μg/ml the antibody completely blocked growth arrest and differentiation of M1 cells by IL-6 (250 units/ml) but had no effect on M1 cell growth inhibition induced by LIF or OSM.

CNTF by itself is inactive towards M1 cells (Davis et al., 1993), but it is able to substitute for LIF in maintaining D3 ES cells in an un-differentiated state (see below). D3 ES cells (obtained from the American Type Culture Collection) were maintained in an undifferentiated state on gelatin-coated dishes in DMEM (high glucose) supplemented with 15% FCS, L-glutamine, sodium pyruvate, non-

essential amino acids, β-mercaptoethanol and 1000 units/ml of LIF. Withdrawal of LIF results in ES cell differentiation accompanied by loss of alkaline phosphatase activity (Smith et al., 1988; Williams et al., 1988; Pease et al., 1990). The effects of OSM, CNTF and IL-6 were assayed by their ability to maintain AP activity of D3 ES cells. We have developed a quantitative assay to monitor the growth of undifferentiated ES cells based on their AP activity using a chromogenic substrate p-nitrophenyl phosphate (Cheng and Donovan, unpublished data). Using this assay, the ED_{50} of LIF (ESGRO) for D3 cell growth is measured as 1 ng/ml (or 100 units/ml). In addition to LIF, both OSM and CNTF are able to maintain the growth of undifferentiated D3 ES cells with specific activities of 40 units/ng and 20 units/ng respectively, based on a comparison with LIF. IL-6 is unable to inhibit D3 ES cell differentiation. Similar observations on the effects of OSM, CNTF and IL-6 on ES cell differentiation have been reported recently (Conover et al., 1993; Yoshida et al., 1994; Wolf et al., 1994). The amounts of recombinant cytokines used in PGC culture assays were, therefore, based on their specific activities rather than their total protein concentration.

Characterization of the anti-LIFR serum

Protein lysates from M1 cells were used for determining the specificity of the anti-LIFR antiserum. Following a published protocol used in the demonstration of LIF-induced LIFR phosphorylation (Ip et al., 1992), 5×10^6 M1 cells were treated with 50 ng/ml LIF or IL-6 at 37°C for 5 minutes and lysed in 0.5 ml of Tris-buffered saline plus 1% NP-40. The soluble proteins were then incubated either with the anti-LIFR rabbit serum (1:10 dilution) or with (5 μg of) an anti-phosphotyrosine mAb 4G10 (Upstate Biotechnology, Inc.). After precipitation with protein A-sepharose beads (Pharmacia), the precipitated proteins were separated by SDS-PAGE and then transferred onto a PVDF nylon membrane (Millipore) by electroblotting. The membrane was first probed with another anti-phosphotyrosine mAb (PY20, Signal Transduction Inc.) conjugated to horseradish peroxidase (HRP). After stripping, the filter was reprobed with the anti-LIFR serum at 1:2,000 dilution followed by a secondary anti-rabbit antibody conjugated to HRP (Boehringer Mannheim Biologicals). An ECL detection system (Amersham) was used to detect bound HRP immunoconjugates.

Fluoresence-activated cell sorting (FACS) analysis

A single-cell suspension from genital ridges of 12.5 dpc mice was incubated with a mouse mAb, TG-1, which recognizes a PGC surface marker, the SSEA-1 antigen (1:10 dilution of the hybridoma culture supernatant), or an isotype-matched mouse IgM (20 μg/ml). Each cell suspension was also labelled with either the anti-LIFR antiserum or a normal rabbit serum (NRS) control both at 1:10 dilution for 30 minutes on ice. After extensive washing with PBS, a mixture of two fluorochrome-conjugated goat IgGs (anti-mouse IgM-fluorescein isothiocyanate [FITC] and anti-rabbit IgG-phycoerythrin [PE], both from Sigma) was added to each cell suspension and incubated for 30 minutes on ice. The samples were then washed extensively in cold PBS. Two-color FACS analysis was performed using an EPICS profile analyzer instrument (Coulter corporation). The two control antibodies were used to set up arbitrary gates to separate positive cells from cells with background levels of fluorescence.

RNA protection assays for detecting LIFR transcripts

A genomic DNA fragment containing the cytoplasmic portion of the mouse LIFR was cloned by homology to the human LIFR (Gearing, unpublished data). A LIFR probe was constructed by cloning a 480 base pair (bp) segment from the mouse LIFR genomic DNA fragment (*Xba*I to *Bam*HI) into the corresponding sites in the Bluescript SK+vector (Stratagene). The anti-sense RNA probe (520 bp) for the LIFR was synthesized in the presence of [α-^{32}P]CTP by T7 RNA polymerase using the manufacturer's protocol (Stratagene). RNA was isolated from 11.5 dpc genital ridges, STO cells, D3 ES cells and EG cells derived from PGCs of 8.5 dpc embryos with RNAzol-B (Tel-Test, Inc.) by the method of Chomczynski and Sacchi (1987) using the manufacturer's protocol. After hybridizing overnight with 10 μg of total RNA or transfer RNA (Sigma), the un-hybridized probe was trimmed with RNases A and T1. A β-actin probe (139 bp) was also used in each hybridization reaction as an internal control. The RNase-resistant RNAs (radioactive) were separated by size in a 6% acrylamide-urea sequencing gel. The protected β-actin fragment is 114 bp long and the protected fragment for the mouse LIFR mRNA is 310 bp long. This portion of the LIFR transcript corresponds to the cytoplasmic region from amino acids 890 to 993 numbered according to the published human sequence (Gearing et al., 1991).

RESULTS

Effect of growth factors on 11.5 dpc PGC growth in culture

The stimulatory effects of SLF, LIF, and bFGF or combinations of these factors on the growth of PGCs at both premigration and postmigration stages were examined. This report is focused on the stimulation of postmigratory PGCs, since the stimulatory effects of these growth factors on premigratory PGCs have been published previously (Resnick et al., 1992; Matsui et al., 1992). Postmigratory PGCs were isolated from embryos of 11.5 dpc at which time approximately 70% of PGCs have reached the genital ridges (McCoshen and McCallion, 1975; Tam and Snow, 1981). In these short-term growth assays, PGCs were cultured on a monolayer of mitotically inactive STO fibroblast cells. After a fixed period of time in culture, either in the presence or absence of various growth factors, the numbers of PGCs were determined by virtue of their AP activity. Soluble SLF slightly increased the PGC number at day 1 and day 2 of the culture period (data not shown). Recombinant LIF moderately increased the number of 11.5 dpc PGCs cultured on STO feeder cells even though the STO feeder cells produce a certain amount of LIF (Fig. 1A). bFGF by itself is also able to stimulate PGC proliferation on STO cells to a greater extent than either exogenous LIF or SLF. The stimulation of PGC growth by LIF is also observed in the presence of bFGF and the stimulatory effects of LIF and bFGF are additive. The number of PGCs increased more than two fold in 2 days if both LIF and bFGF were added.

We further tested the effect of LIF in feeder-independent cultures established from 11.5 dpc genital ridges. In these cultures, PGCs together with embryonic somatic cells are plated onto gelatin-coated substrata (Godin et al., 1991; Matsui et al., 1991). In the presence of soluble SLF, PGC numbers increased during the first 24 hours of culture and then begun to decline (data not shown), consistent with previous reports (Matsui et al., 1991). Addition of LIF partially prevented the decline of PGC numbers over a 48 hour period (Fig. 1B). The stimulatory effect of LIF on PGC growth observed in these feeder-independent cultures was greater than that observed when PGCs were cultured on STO cells. The enhancement of PGC survival by LIF was further enhanced by the addition of bFGF, in a manner similar to its effect on PGC growth on STO cells (see Fig. 1A). Therefore, the action of LIF and bFGF on PGCs are not dependent on the presence of STO feeder cells. These data suggest that LIF (and bFGF) might act directly on PGCs to promote their survival and growth in culture. These results also illustrate that stimulation of PGC growth by LIF is

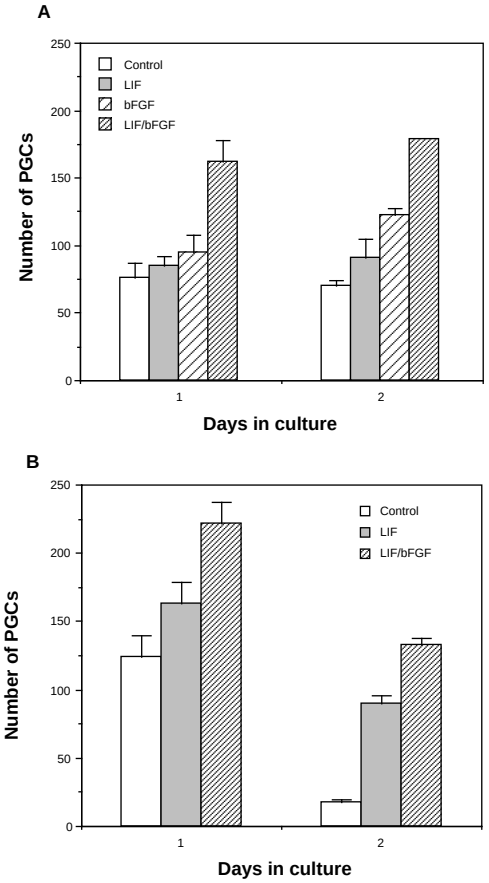

Fig. 1. Analysis of 11.5 dpc PGC growth in culture. PGCs in single cell suspensions were cultured in 96-well microtiter plates in the presence or absence of LIF (1000 units/ml) and bFGF (1 ng/ml). Cells from the equivalent of 0.2 embryos were plated on irradiated STO cell feeders (A), or 0.5 embryo-equivalent cells were seeded on gelatin-coated plastic in the presence of 100 ng/ml soluble SLF (B). After 1 or 2 days the cultures were fixed and PGCs were identified by histochemical staining for alkaline phosphatase. The mean and SD of PGC numbers were calculated from four (A) or three (B) duplicate wells.

not restricted to a specific stage of PGCs during development since it promoted the growth in culture of both premigratory and postmigratory PGCs (Resnick et al., 1992; Matsui et al., 1992; and this data). LIF has a distinct function in regulating PGC growth which is independent of SLF and bFGF.

LIFR is expressed in the genital ridge

To elucidate the function of LIF in PGC growth in vivo, we first need to know whether the bipartite, high-affinity LIF receptor, consisting of LIFR and gp130, is expressed in the developing gonad. Since gp130 is reported to be ubiquitously expressed in all murine tissues and cells examined (Saito et al., 1992), we decided to focus first on the expression of the LIFR, the LIF-binding subunit of the bipartite receptor for LIF. After the initial cloning of a murine LIFR cDNA encoding a secreted protein lacking transmembrane and cytoplasmic domains (Gearing et al., 1991), a genomic fragment containing the murine LIFR cytoplasmic domain was obtained (Gearing,

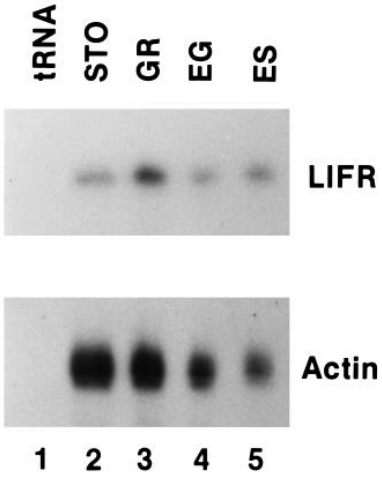

Fig. 2. RNase protection assay for detecting LIFR transcripts. Transfer RNA (tRNA, lane 1), or total RNAs made from either STO cells (STO, lane 2), 11.5 dpc genital ridges (GR, Lane 3), PGC-derived EG cells (EG, lane 4) or D3 ES cells (ES, lane 5) were hybridized with radioactive LIFR and β-actin probes. The top panel shows the protected fragments for LIFR and and the bottom panel for β-actin.

unpublished data). Using this DNA fragment as a probe, we examined the expression of the LIFR in the developing gonad by RNase protection analysis (Fig. 2). In RNA samples isolated from genital ridges and several cell lines, a protected fragment of the correct size was observed while transfer RNA did not yield any signal. As expected, mouse ES cells express the LIFR since LIF can act on ES cells to inhibit their differentiation. The LIFR transcript is detected in genital ridges of 11.5 dpc mouse embryos as well as in EG cells derived from PGCs. LIFR is also expressed in STO cells. At present, it is unclear whether the LIFR in STO cells is important for their own growth or for supporting PGCs cultured on STO feeder cells. Since the effects of LIF are greater in the absence of STO cells, this suggests that the potential action of LIF on STO cells is not crucial for PGC proliferation.

PGCs make LIFR proteins on their cell surface

To determine further whether PGCs per se in genital ridges express a LIFR, we made use of an antiserum raised in rabbits against a recombinant murine LIFR protein. This anti-LIFR serum was shown to block the binding of LIF to COS cells transfected with the *LIFR* gene, but not the binding of human OSM to cells transfected with the *gp130* gene which is a low-affinity receptor for human OSM (Gearing, unpublished data). We have further confirmed its specificity against the LIFR by using M1 mouse leukemic monocytic cells which express both LIFR and gp130, and terminally differentiate into macrophages when exposed to LIF, OSM or IL-6 (Metcalf et al., 1988; Rose and Bruce, 1991). It has previously been reported that in M1 cells LIFR and gp130 form a bipartite complex and are tyrosine-phosphorylated upon LIF activation, whereas only gp130 is tyrosine-phosphorylated in response to the IL-6 activation (Taga et al., 1992; Davis et al., 1993). Fig. 3A shows the phosphorylation of LIFR (approximately 190 kDa) and gp130 (approximately 145 kDa) in M1 cells treated with LIF.

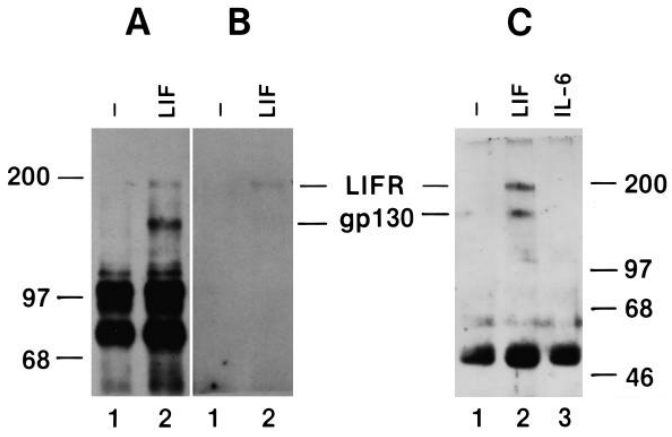

Fig. 3. Specificity of the anti-LIFR serum. Cellular lysates from untreated M1 cells (lane 1), or M1 cells treated with LIF (lane 2) or IL-6 (lane 3) were either precipitated with an anti-phosphotyrosine antibody (A,B) or with the anti-LIFR serum (C). The precipitated proteins were separated on 8% SDS-PAGE gels and then transferred onto nylon membranes. The positions of size-standard proteins (in KDa) are marked on the side for each gel. The LIFR (190 KDa) and gp130 (145 KDa) are denoted in the middle. (A) Phosphorylation of LIFR and gp130 in M1 cells following LIF activation. Tyrosine-phosphorylated proteins were identified with a second anti-phosphotyrosine antibody conjugated to horseradish peroxidase. (B) Specific recognition of LIFR by the anti-LIFR antiserum. The same protein blot used in A was stripped and reprobed with the anti-LIFR antiserum, followed by detection with an anti-rabbit IgG conjugated to peroxidase. (C) Phosphorylation of proteins precipitated by the anti-LIFR serum. Tyrosine-phosphorylated proteins were identified as described in A.

When the same blot was probed with the anti-LIFR serum instead of the anti-phosphotyrosine antibody used in Fig. 3A, only the LIFR protein was seen (Fig. 3B). In addition, the anti-LIFR serum is able to immunologically precipitate tyrosine-phosphorylated LIFR and gp130 in M1 cells treated with LIF in Fig. 3C, lanes 1 and 2), but not tyrosine-phosphorylated gp130 in M1 cells treated with IL-6 (Fig. 3C, lane 3). These data demonstrate that the anti-LIFR serum specifically recognizes the LIFR protein but not the related gp130 protein. However, we can not rule out the possibility that the anti-LIFR serum can recognize other unidentified receptors that are related LIFR.

We further used this anti-LIFR serum to examine whether the LIFR protein is present on the cell surface of PGCs (Fig. 4). In a two-color FACS analysis, disassociated PGCs were identified by expression of the SSEA-1 antigen and account for about 11% of the total cells isolated from genital ridges. Within the PGC population (SSEA-1^+ cells), approximately 50% of the cells stained positive with the LIFR antiserum by comparison with the control serum (Fig. 4). This result demonstrates that the LIFR protein is present on the surface of PGCs and suggests that PGCs are therefore likely to be a direct target of LIF action observed in culture. The LIFR antiserum also stains a population of SSEA-1-negative cells. Since the SSEA-1 antigen is down regulated on the PGC surface after they reach the gonad (Donovan et al., 1986), these cells could represent SSEA-1-negative PGCs. Alternatively these cells could represent gonadal somatic cells that express the LIFR. Macrophages have been identified in the developing urogenital ridge (De Felici et al., 1986) and represent a cell type known to bind I^{125}-LIF and therefore to express a LIF receptor (Hilton et al., 1988b).

Signaling via the LIFR is essential for PGC growth in culture

Since the anti-LIFR serum blocks LIF binding, we tested its effect on PGC growth in culture. Addition of the LIFR antiserum reduced the number of PGCs in culture in a dose-dependent manner, whereas the control rabbit serum had little effect on PGC growth (Fig. 5A). At higher concentrations the anti-LIFR serum reduced the number of PGCs by more than 75% and did not alter the morphology of feeder cells (data not shown). The blocking effect of the LIFR antiserum observed in these assays is comparable to that of a mAb recognizing the mouse c-kit receptor, ACK-2 (Matsui et al., 1991). Although the addition of exogenous LIF has only a moderate effect on PGC proliferation on STO feeder cells (which produce a sub-optimal amount of LIF) (see Fig. 1), the blocking experiment shown here illustrates that signaling via the LIFR protein (or a highly related receptor) is required for PGC growth in culture.

We further tested whether the presence of bFGF could overcome the requirement for LIF and SLF signaling. The addition of bFGF, LIF and SLF at their optimal concentration doubled the number of PGCs in 2 days, consistent with previous results (see Fig. 1). Addition of bFGF did not alleviate the blocking effect of neutralizing antibodies against either the

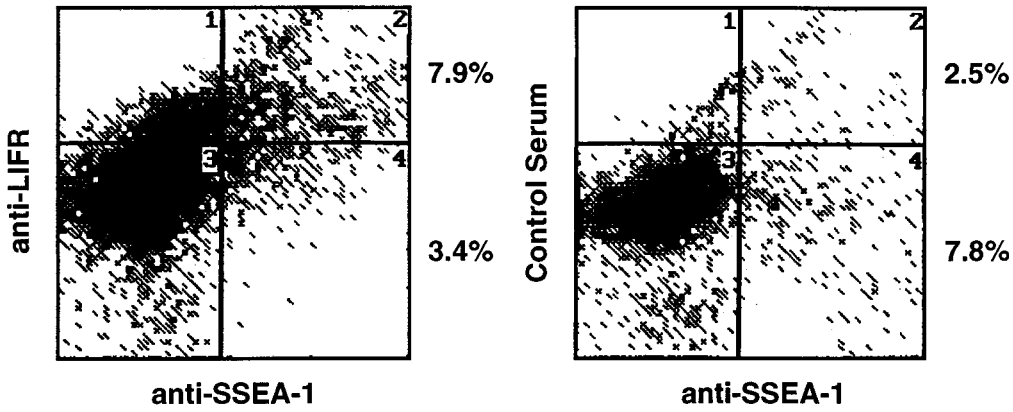

Fig. 4. FACS analysis of the LIFR expression in PGCs. Single cell suspensions from genital ridges were incubated with an antibody recognizing the SSEA-1 antigen, plus either the anti-LIFR antiserum (left panel) or normal rabbit serum (right panel). The intensity of the both fluorochromes associated with the two classes of antibodies from each intact cell was plotted in log scale in both X and Y axes, respectively. The percentile of the SSEA-1 positive population (PGCs) are shown on the right in each panel.

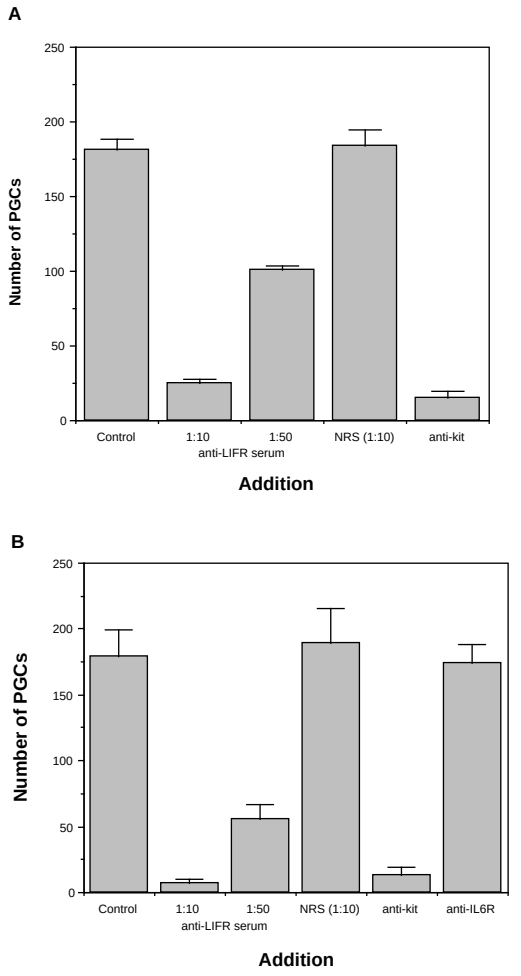

Fig. 5. Effect of neutralizing antibodies on PGC growth in culture. Cells from the equivalent of 0.4 embryos (A) or 0.2 embryos (B) were seeded on irradiated STO cell feeders in 96-well plates. PGCs were maintained for 2 days in the absence (A) or presence (B) of 1 ng/ml bFGF and 1000 units/ml LIF, and the indicated antibodies. The anti-serum against LIFR was used at either 1:10 or 1:50 dilution. A normal rabbit serum (NRS) was used at 1:10 dilution. The concentrations of the two rat monoclonal IgGs, ACK-2 (anti-kit) and anti-IL-6Rα, were 1 μg/ml and 10 μg/ml respectively. The mean and SD of PGC numbers from three duplicate wells were plotted.

LIFR or the c-kit receptor (Fig. 5B). The inability of bFGF to bypass the requirement for signaling via the c-kit receptor confirms the conclusion that the SLF/c-kit signal pathway is absolutely required for PGC growth both in vivo and in vitro. Similarly, the data presented here illustrate that signal transduction via the LIFR protein is also essential for PGC growth in vitro.

We also tested the effect on PGC growth of an antibody which specifically recognizes IL-6Rα, the IL-6-binding subunit of the IL-6 receptor. This mAb has been shown to block IL-6 action on several cell lines in vivo and in vitro (Vink et al., 1990), and further tested by us for its effect on IL-6-induced M1 cell growth inhibition and differentiation (see materials and methods). At a concentration of 10 μg per ml, the mAb against IL-6Rα had little effect on PGC growth (Fig. 5B), while it fully blocked M1 cell

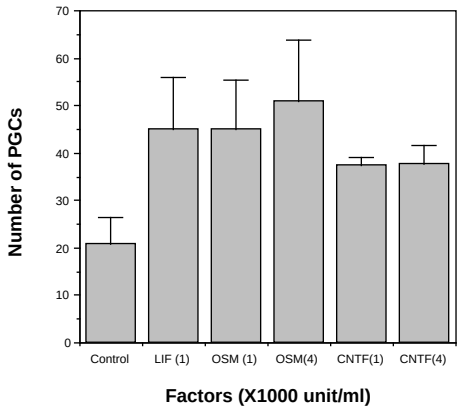

Fig. 6. Effect of LIF and related factors on PGC growth in culture. Cells from the equivalent of 0.5 embryos were seeded on gelatin-coated wells (in the absence of STO feeder layers). PGCs were maintained with 100 ng/ml SLF in addition to the indicated factors for 2 days. One thousand units per ml of LIF, OSM and CNTF, or 4000 units/ml of OSM and CNTF were used in the assay. PGCs were identified by AP histochemistry. The mean and SD of PGC numbers from three duplicate wells were plotted.

growth inhibition induced by IL-6 (see Materials and methods). These data suggest that, while signalling via c-kit and LIFR are essential for PGC growth in culture, signalling via the IL-6Rα is not required for their growth.

OSM and CNTF can also promote PGC growth in culture

The LIFR forms part of the receptor complex for other members of the LIF cytokine family such as OSM and CNTF. Since these cytokines share some overlapping functions with LIF in several culture systems, we also tested the ability of these factors to promote PGC growth in culture. Like LIF, recombinant OSM and CNTF slightly increased the numbers of the PGCs cultured on STO cells (data not shown). Because the stimulatory effect observed is weak and since STO feeder cells are known to produce LIF (and possibly other cytokines related to LIF), we further tested the stimulatory activity of these cytokines on PGC survival cultured in the absence of feeder cells. In this culture system, PGCs are highly dependent on LIF for their survival and the stimulatory effect of LIF on PGC growth was greater than that observed when PGCs were cultured on STO feeder cells (see Fig. 1B). In addition to LIF, recombinant OSM and CNTF also promoted PGC survival (Fig. 6). Combinations of two or three of these cytokines did not further increase PGC numbers (data not shown). These data suggest that either LIF, OSM or CNTF can function directly on PGCs as growth promoting factors. In the same assay, addition of IL-6, at concentrations between 100 and 10,000 units/ml, had little effect on PGC survival or proliferation (data not shown). This data is consistent with data demonstrating that signalling via the IL-6 receptor is not required for PGC growth in culture (see Fig. 5B).

DISCUSSION

Previous studies have demonstrated that LIF can stimulate the proliferation of PGCs cultured on feeder cells. Because LIF

can act on a wide variety of cell types, interpretation of its role in PGC growth is complicated. Here we have demonstrated that LIF can act to stimulate PGC proliferation in the absence of STO feeder cells, suggesting that LIF may act directly on PGCs (Fig. 1). Using a sensitive RNase protection assay, we have also shown that transcripts for the LIF-binding subunit (LIFR) of the high affinity LIF receptor complex are detectable in the developing gonad (Fig. 2). FACS analyses demonstrate that approximately 50% of PGCs (SSEA-1+ cells) can be labeled using a specific anti-LIFR antiserum (Fig. 4). Taken together these data demonstrate that PGCs express a LIF receptor and strongly suggest that PGCs are a direct target of LIF action in vitro. We have further shown that the anti-LIFR antiserum completely blocks PGC growth in culture in a manner similar to antibodies to the c-kit receptor tyrosine kinase (Fig. 5). These data suggest that signalling via the LIFR (or a highly related receptor) is required for PGC survival in vitro and may be required for PGC survival in vivo.

It has been proposed that all mammalian cells, excluding blastomeres, may require multiple peptide factors for survival (Raff, 1992). For example, oligodendrocytes require three classes of factors for survival in vitro: (1) insulin and insulin-like growth factors; (2) neurotrophins, particularly neurotrophin-3, and (3) CNTF, LIF or IL-6 (Barres et al., 1993). Whereas a single factor promotes short-term survival, at least one factor from each class is required to promote long-term oligodendrocyte survival in vitro. Some of these survival factors, such as neurotrophin-3, also function as mitogens when cytokines of the other classes are present (Barres et al., 1993). The data presented here suggest that LIF, like SLF, is required for PGC survival in vitro although it seems likely that LIF may also act as a co-mitogen in the presence of other growth factors (Matsui et al., 1991, 1992; Resnick et al., 1992). The notion that LIF is a PGC survival factor is consistent with data showing that it can suppress programmed cell death (apoptosis) of PGCs in culture (Pesce et al., 1993).

Although we have argued that signalling via the LIFR/gp130 complex is required for PGC survival, mice deficient in LIF are fertile (Stewart et al., 1992; Escary et al., 1993). Three explanations seem plausible. First, animals can have severely reduced numbers of PGCs and still be fertile. For example, *Steel*^{17H} animals, which have an abnormal SLF cytoplasmic tail, are fertile as females even though PGC numbers are reduced by 90% in comparison to wild-type littermates (Brannan et al., 1992). A direct examination of PGC numbers in LIF-deficient animals may allow this question to be addressed. Secondly, if LIF is the physiological ligand, another related cytokine may substitute for its function in PGC development in LIF-deficient animals. Data presented here clearly demonstrate that two LIF-related cytokines, OSM and CNTF can substitute for LIF in promoting PGC survival and proliferation in vitro and therefore could possibly substitute for LIF in vivo. Thirdly, LIF may not be the natural physiological ligand for PGCs. Since OSM and CNTF can stimulate PGC survival and proliferation in culture in a manner comparable to that of LIF they, or an as yet unidentified member of this cytokine family, may be the natural physiological ligand. Since CNTF can stimulate PGC survival and proliferation these data also suggest that PGCs may express CNTFRα. Recent studies demonstrated that ES cells express a CNTFRα and that CNTF can inhibit ES cell differentiation in a manner comparable to LIF (Conover et al., 1994; Yoshida et al., 1994; Wolf et al., 1994; see Materials and methods). The data shown here provide a further example of CNTF acting outside the nervous system and suggest a potential role for CNTF in PGC development. CNTF-deficient animals are also fertile (Masu et al., 1993), but the same arguments apply to these animals as apply to LIF-deficient animals. We are currently trying to determine if one or more these LIFR-dependent cytokines function as a physiological ligand to promote the growth of PGCs in vivo.

The data presented here show that PGCs express a functional LIF receptor composed of LIFR (or a highly related molecule) presumably in association with gp130. These data also argue that activation of this receptor is required for PGC survival. To understand the role of the LIF receptor in PGC development, the temporal and spatial patterns of LIFR and gp130 expression during different stages of PGC development need to be determined. It will be of particular interest to examine LIFR and gp130 expression around 13.5 dpc when PGCs cease mitotic division and undergo differentiation. Activation of the LIF/gp130 complex has been shown to activate DNA binding of acute-phase response factor (APRF), a transcription factor related to interferon-stimulated gene factor-3α (ISGF-3α). LIF, OSM and CNTF (as well as IL-6 and IL-11) cause tyrosine phosphorylation of APRF and ISGF-3α as well as inducing kinase activity in the Jak-Tyk family of cytoplasmic tyrosine kinases (Bonni et al., 1993; Lutticken et al., 1994; Stahl et al., 1994). The demonstration that LIF can act directly on PGCs suggests that these proteins may be expressed and activated in PGCs. Understanding the role of this signalling pathway in PGC development may help elucidate both the function and mechanism of action of LIF and related cytokines in PGC development.

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REFERENCES

Barres, B. A., Schmid, R., Sendnter, M. and Raff, M. C. (1993). Mutiple extracellular signals are required for long-term oligodendrocyte survival. *Development* **118**, 283-295.

Baumann, H., Ziegler, S. F., Mosley, B., Morella, K. K., Pajovic, S. and Gearing, D. P. (1993). Reconstitution of the response to leukemia inhibitory factor, oncostatin M, and ciliary neurotrophic factor in hepatoma cells. *J. Biol. Chem.* **268**, 8414-8417.

Bazan, J. F. (1991). Neuropoietic cytokines in the hematopoietic fold. *Neuron* **7**, 197-208.

Bonni, A., Frank, D. A., Schindler, C. and Greenberg, M. E. (1993). Characterization of a pathway for ciliary neurotrophic factor signaling to the nucleus. *Science* **262**, 1575-1579.

Brannan, C. I., Bedell, M. A., Resnick, J. L., Eppig, J. J., Handel, M. A., Williams, D. E., Lyman, S. D., Donovan, P. J., Jenkins, N. A. and Copeland, N. G. (1992). Developmental abnormalities in Steel17H mice result from a splicing defect in the steel factor cytoplasmic tail. *Genes. Dev.* **6**, 1832-1842.

Chomczynski, P. and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**, 156-159.

Conover, J. C., Ip, N. Y., Poueymirou, W. T., Bates, B., Goldfarb, M. P., DeChiara, T. M. and Yancopoulous, G. D. (1993). Ciliary neurotrophic factor maintains the pluripotentiality of embryonic stem cells. *Development* **119**, 559-565.

Davis, S., Aldrich, T. H., Stahl, N., Pan, L., Taga, T., Kishimoto, T., Ip, N. Y. and Yancopoulos, G. D. (1993). LIFR beta and gp130 as heterodimerizing signal transducers of the tripartite CNTF receptor. *Science* **260**, 1805-1808.

De Felici, M., Heasman, J., Wylie, C. C. and McLaren, A. (1986). Macrophages in the urogenital ridge of the mid-gestation mouse fetus. *Cell Differ.* **18**, 119-129.

De Felici, M. and Dolci, S. (1991). Leukemia inhibitory factor sustains the survival of mouse primordial germ cells cultured on TM4 feeder cells. *Dev. Biol.* **147**, 281-284.

Dolci, S., Williams, D. E., Ernst, M. K., Resnick, J. L., Brannan, C. I., Lock, L. F., Lyman, S. D., Boswell, H. S. and Donovan, P. J. (1991). Requirement for mast cell growth factor for primordial germ cell survival in culture. *Nature* **352**, 809-811.

Donovan, P. J., Stott, D., Cairns, L. A., Heasman, J. and Wylie, C. C. (1986). Migratory and postmigratory mouse primordial germ cells behave differently in culture. *Cell* **44**, 831-838.

Escary, J. L., Perreau, J., Dumenil, D., Ezine, S. and Brulet, P. (1993). Leukaemia inhibitory factor is necessary for maintenance of haematopoietic stem cells and thymocyte stimulation. *Nature* **363**, 361-364.

Gearing, D. P., Gough, N. M., King, J. A., Hilton, D. J., Nicola, N. A., Simpson, R. J., Nice, E. C., Kelso, A. and Metcalf, D. (1987). Molecular cloning and expression of cDNA encoding a murine myeloid leukaemia inhibitory factor (LIF). *EMBO J.* **6**, 3995-4002.

Gearing, D. P., Thut, C. J., Van de Bos, T., Gimpel, S. D., Delaney, P. B., King, J., Price, V., Cosman, D. and Beckmann, M. P. (1991). Leukemia inhibitory factor receptor is structurally related to the IL-6 signal transducer, gp130. *EMBO J.* **10**, 2839-2848.

Gearing, D. P., Comeau, M. R., Friend, D. J., Gimpel, S. D., Thut, C. J., McGourty, J., Brasher, K. K., King, J. A., Gillis, S., Mosley, B., Ziegler, S. F. and Cosman, D. (1992). The IL-6 signal transducer, gp130: an oncostatin M receptor and affinity converter for the LIF receptor. *Science* **255**, 1434-1437.

Ginsburg, M., Snow, M.H.L. and McLaren, A. (1990). Primordial germ cells in the mouse embryo during gastrulation. *Development* **110**, 521-528.

Godin, I., Deed, R., Cooke, J., Zsebo, K., Dexter, M. and Wylie, C. C. (1991). Effects of the steel gene product on mouse primordial germ cells in culture. *Nature* **352**, 807-809.

Gough, N. M., Gearing, D. P., King, J. A., Willson, T. A., Hilton, D. J., Nicola, N. A. and Metcalf, D. (1988). Molecular cloning and expression of the human homologue of the murine gene encoding myeloid leukemia-inhibitory factor. *Proc. Natl. Acad. Sci. USA* **85**, 2623-2627.

Hilton, D. J., Nicola, N. A. and Metcalf, D. (1988a). Purification of a murine leukemia inhibitory factor from Krebs ascites cells. *Anal. Biochem.* **173**, 359-367.

Hilton, D. J., Nicola, N. A. and Metcalf, D. (1988b). Specific binding of murine leukemia inhibitory factor to normal and leukemic monocytic cells. *Proc. Natl. Acad. Sci. USA* **85**, 5971-5975.

Ip, N. Y., Nye, S. H., Boulton, T. G., Davis, S., Taga, T., Li, Y., Birren, S. J., Yasukawa, K., Kishimoto, T., Anderson, D. J., Stahl, N. and Yancopoulos, G. D. (1992). CNTF and LIF act on neuronal cells via shared signaling pathways that involve the IL-6 signal transducing receptor component gp130. *Cell* **69**, 1121-1132.

Leary, A. G., Wong, G. G., Clark, S. C., Smith, A. G. and Ogawa, M. (1990). Leukemia inhibitory factor/differentiation-inhibiting activity/human interleukin for DA cells augments proliferation of human hematopoietic stem cells. *Blood* **75**, 1960-1969.

Lutticken, C., Wegenka, U. M., Yuan, J., Buschmann, J., Schidler, C., Ziemiecki, A., Harpur, A. G., Wilks, A., Yasukawa, K., Taga, T., Kishimoto, T., Barbieri, G., Pellegrini, S., Sendner, M., Heinrich, P. C. and Horn, F. (1994). Association of transcription factor APRF and protein kinase Jak1 with the interleukin-6 signal transducer gp130. *Science* **263**, 89-92.

Masu, Y., Wolf, E., Holtmann, B., Sendtner, M., Brem, G. and Thoenen, H. (1993). Disruption of the CNTF gene results in motor neuron degeneration. *Nature* **365**, 27-32.

Matsui, Y., Zsebo, K. M. and Hogan, B. L. (1990). Embryonic expression of a haematopoietic growth factor encoded by the Sl locus and the ligand for c-kit. *Nature* **347**, 667-669.

Matsui, Y., Toksoz, D., Nishikawa, S., Nishikawa, S., Williams, D., Zsebo, K. and Hogan, B. L. (1991). Effect of Steel factor and leukaemia inhibitory factor on murine primordial germ cells in culture. *Nature* **353**, 750-752.

Matsui, Y., Zsebo, K. and Hogan, B. L. (1992). Derivation of pluripotential embryonic stem cells from murine primordial germ cells in culture. *Cell* **70**, 841-847.

McCoshen, J. A. and McCallion, D. J. (1975). A study of primordial germ cells during their migratory phase in steel mutant mice. *Experientia* **31**, 589-590.

McLaren, A. (1992). Development of primordial germ cells in the mouse. *Andrologia* **24**, 243-247.

Metcalf, D., Hilton, D. J. and Nicola, N. A. (1988). Clonal analysis of the actions of the murine leukemia inhibitory factor on leukemic and normal murine hemopoietic cells. *Leukemia* **2**, 216-221.

Metcalf, D., Hilton, D. J. and Nicola, N. A. (1991). Leukemia inhibitory factor can pontentiate murine megakaryocyte production in vitro. *Blood* **76**, 50-56.

Moreau, J.-F., Donaldson, D., Bennett, F., Witek-Giannotti, J., Clark, S. C. and Wong, G. G. (1988). Leukaemia Inhibitory factor is identical to the myeloid growth factor human interlukin for DA cells. *Nature* **336**, 690.

Nishikawa, S., Kusakabe, M., Yoshinaga, K., Ogawa, M., Hayashi, S., Kunisada, T., Era, T., Sakakura, T. and Nishikawa, S. (1991). In utero manipulation of coat color formation by a monoclonal anti-c-kit antibody: two distinct waves of c-kit-dependency during melanocyte development. *EMBO J.* **10**, 2111-2118.

Pease, S., Braghetta, P., Gearing, D., Grail, D. and Williams, R. L. (1990). Isolation of embryonic stem (ES) cells in media supplemented with recombinant leukemia inhibitory factor (LIF). *Dev Biol* **141**, 344-352.

Pesce, M., Farrace, M. G., Piacentini, M., Dolci, S. and De, F. M. (1993). Stem cell factor and leukemia inhibitory factor promote primordial germ cell survival by suppressing programmed cell death (apoptosis). *Development* **118**, 1089-1094.

Raff, M. C. (1992). Social controls on cell survival and death: an extreme view. *Nature* **303**, 390-396.

Resnick, J. L., Bixler, L. S., Cheng, L. and Donovan, P. J. (1992). Long-term proliferation of mouse primordial germ cells in culture. *Nature* **359**, 550-551.

Rose, T. M. and Bruce, A. G. (1991). Oncostatin M is a member of a cytokine family that includes lekeumia inhibitory factor, granulocyte colony-stimulating factor and interleukin 6. *Proc. Natl. Acad. Sci. USA* **88**, 8641-8645.

Saito, M., Yoshida, K., Hibi, M. and Kishimoto, T. (1992). Molecular cloning of a murine IL-6 receptor-associated signal transducer, gp130, and its regulated expression in vivo. *J. Immunol.* **148**, 4066-4071.

Smith, A. G., Heath, J. K., Donaldson, D. D., Wong, G. G., Moreau, J., Stahl, M. and Rogers, D. (1988). Inhibition of pluripotential embryonic stem cell differentiation by purified polypeptides. *Nature* **336**, 688-690.

Stahl, N., Boulton, T. G., Farruggella, T., Ip, N. Y., Davis, S., Witthuhn, B. A., Quelle, F. W., Silvennoinen, O., Barbieri, G., Pellegrini, S., Ihle, J. N. and Yancopoulos, G. D. (1994). Association and activation of Jak-Tyk kinases by CNTF-LIF-OSM-IL-6 β receptor components. *Science* **263**, 92-95.

Stewart, C. L., Kaspar, P., Brunet, L. J., Bhatt, H., Gadi, I., Kontgen, F. and Abbondanzo, S. J. (1992). Blastocyst implantation depends on maternal expression of leukaemia inhibitory factor. *Nature* **359**, 76-79.

Stewart, C. L., Gadi, I. and Bhatt, H. (1994). Stem cells from primordial germ cells reenter the germ line. *Dev. Biol.* **161**, 626-8.

Taga, T. and Kishimoto, T. (1992). Functional inhibition of hematopoietic and neurotrphic cytokines by blocking the interlukin 6 signal transducer gp130. *Proc. Natl. Acad. Sci. USA* **89**, 10998-11001.

Tam, P. P. L. and Snow, M. H. L. (1981). Proliferation and migration of primordial germ cells during compensatory growth in mouse embryos *J. Embryol. Exp. Morph.* **64**, 133-147.

Vink, A., Coulie, P., Warnier, G., Renauld, J.-C., Stevens, M., Donckers, D. and Van Snick, J. (1990). Mouse plasmacytoma growth in vivo: enhancerment by interleukin 6 (IL-6) and inhibition by antibodies directed against IL-6 or its receptor. *J. Exp. Med.* **172**, 997-1000.

Williams, R. L., Hilton, D. J., Pease, S., Willson, T. A., Stewart, C. L.,

Gearing, D. P., Wagner, E. F., Metcalf, D., Nicola, N. A. and Gough, N. M. (1988). Myeloid leukaemia inhibitory factor maintains the developmental potential of embryonic stem cells. *Nature* **336**, 684-687.

Witte, O. N. (1990). Steel locus defines a new multiplepotent growth factor. *Cell* **63**, 5-6.

Wolf, E., Kramer, R., Polezhaewa, I., Thoenen, H. and Brem, G. (1994). Efficient generation of cherimic mice using embryonic stem cells after long-term culture in the presence of ciliary neurotrophic factor. *Transgenic Research* (in press).

Wylie, C. C. (1993). The biology of primordial germ cells. *European Urology* **23**, 62-66.

Yoshida, K., Chambers, I., Nichols, J., Smith, A., Saito, M., Yasukawa, K., Shoyab, M., Taga, T. and Kishimoto, T. (1994). Maintaince of the pluripotential phenotype embryonic stem cells through direct activation of gp130 signalling pathways. *Mech. Dev.* **45**, 163-171.

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