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Short Communication

Interleukin-2 induces the proliferation of mouse primordial germ cells *in vitro*

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ABSTRACT Primordial germ cells (PGCs) are the stem cell precursors of the germ line. Several growth factors contribute to enlarging the PGC population by acting as mitogens, survival factors or both. Interleukin-2 (IL-2) has a growth-promoting activity for T and B-lymphocytes, but its role in PGCs had not yet been studied. Here, we show that PGCs isolated from 10.5, 11.5 and 12.5 day postcoitum (dpc) mouse embryos constitutively express the three subunits (α, β and γ) of the IL-2 receptor (IL-2R). In contrast, IL-2 mRNA was not detected in these cells. However, the addition of recombinant IL-2 to the culture medium increased the number of PGCs *in vitro* via a mitogenic effect, as indicated by bromodeoxyuridine incorporation assays. Neutralization of the IL-2 receptor using anti-IL-2R subunit antibodies inhibited this IL-2-mediated proliferative effect on PGCs from 11.5 dpc embryos. Together, these data are indicative of a paracrine effect of IL-2 on PGC proliferation. In this regard, we also compared the effect of IL-2 with other compounds such as basic fibroblast growth factor (bFGF), steel factor, leukemia inhibitory factor and forskolin, and found that the degree of proliferation induced by IL-2 was similar to that induced by bFGF and forskolin. These observations support the notion that similar patterns of molecular signaling may underlie the developmental pathways of hematopoietic and germ stem cell precursors.

KEY WORDS: *germinal cell differentiation, hematopoietic stem cell, PGC, IL-2, embryonic hematopoiesis*

Primordial germ cells (PGCs) are the stem cell progenitors of the germ line. They exhibit phenotypic differentiation without losing their pluripotency. In the mouse embryo, PGCs can be first detected around 7 days postcoitum (dpc) as a small cluster of alkaline phosphatase positive cells in the extraembryonic mesoderm, at the posterior region of the primitive streak and at the base of the allantois (McLaren, 2003). From this region, they move into the embryonic hindgut endoderm (8-9 dpc) and at 9.5 dpc start to migrate actively from the hindgut wall towards the growing urogenital ridges, where they finally colonize the developing gonads at 12.5 dpc (Molyneaux and Wylie, 2004). During their migratory phase, the PGC population expands from approximately 100 cells in 8.5 dpc mouse embryos, to 3,000 cells in 11.5 dpc embryos, reaching around 25,000 PGCs at 12.5 dpc (Tam and Snow, 1981). By 12.5 dpc, PGCs in the male genital ridge start entering into mitotic arrest, whereas female germ cells arrest at meiotic prophase I, which takes place later at 13.5/15.5 dpc (McLaren, 2003).

Somatic cells contribute to the increase in PGC numbers by means of soluble growth factors, which are known to stimulate the proliferation and/or survival of mouse PGCs in culture (De Felici *et al.,* 2004). For example, basic fibroblast growth factor (bFGF), tumor necrosis factor-α (TNF-α) and bone morphogenetic protein-4 (BMP-4) (Resnick *et al.,* 1992) are mitogens for PGCs. In contrast, other cytokines such as interleukin-4 (IL-4), interleukin-6 (IL-6), interleukin-11 (IL-11) and leukemia inhibitory factor (LIF) have been found to enhance the survival rather than the proliferation of PGCs in culture (De Felici and Dolci., 1991; Cooke *et al.,* 1996; Cheng *et al.,* 1994; Koshimizu *et al.,* 1996). Finally, Steel Factor (SF) is able to increase both PGC proliferation and survival

Abbreviations used in this paper: AGM, aorta-gonadomesonephros; AP, alkaline phosphotase; BMP, bone morphogenetic protein; BrdU, bromodeoxyuridine; dpc, days post coitum; F, forskolin; FGF, fibroblast growth factor; HSC, hematopoietic stem cell; IL, interleukin; LIF, leukemia inhibitory factor; PGC, primordial germ cell; SF, steel factor; TGF-β1, transforming growth factor beta1; TNF, tumor necrosis factor.

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(Godin *et al.,* 1991; Dolci *et al.,* 1991, Pesce *et al.,* 1993), whereas factors such as transforming growth factor-β1 (TGF-β1) and activin appear to negatively regulate PGC proliferation (Godin and Wylie, 1991).

Interleukin-2 (IL-2) is a potent, multifunctional cytokine that plays a central role in the growth-stimulatory activity of T lymphocytes. It also participates in multiple biological processes, including the growth and differentiation of B lymphocytes, the liberation of lymphokine-activated killer lymphocytes and the proliferation and maturation of oligodendroglial cells (Gaffen and Liu, 2004). The biological effects of IL-2 are mediated by a specific cell surface receptor (IL-2R) which consists of three subunits (IL-2Rα, IL-2Rβ and IL-2Rγ), also known as p55, p75 and p64, respectively (Wang *et al.,* 2005). The expression of different combinations of these three components gives rise to the generation of various subtypes of the IL-2 receptor, each of which exhibits different binding affinities for IL-2 (Kim *et al.,* 2006). The IL-2Rα subunit is responsible for specific binding of IL-2, whereas both the β and γ subunits increase the strength of ligand receptor binding and enable the receptor to transduce its signal to the cytoplasm (Wang *et al.,* 2005). Recent studies have shown that the γ chain is present as a functional subunit in many cytokine receptors, such as those for IL-4, IL-7, IL-9, IL-13, IL-15 and IL-21, (Noguchi *et al.,* 1993; Giri *et al.,* 1994; He and Malek, 1995; Sugamura *et al.,* 1996; Asao *et al.,* 2001). Consequently, it has been designated as the common γ chain.

IL-2 is expressed in hematopoietic stem cells (HSC) in 7.5 dpc embryos (Yoder, 2001, 2004). At this stage, HSCs are found in extraembryonic sites such as the yolk sac and the trophoblast, but at 10.5 to 12.5 dpc they are also located in embryonic organs and regions such as the liver, thymus and the aorta-gonadomesonephros region (AGM) (Godin *et al.,* 1995; Cairns *et al.,* 2003), thus coinciding spatially with PGCs. The fact that the lineage specification of both cell types occurs within the same embryonic territory, at similar developmental stages and may be regulated by common genomic *loci* has led to the proposal that PGCs might be the precursors of HSCs or that they could have a common embryonic stem cell precursor. Indeed, EG-PGC-derived cells can be easily induced to differentiate into hematopoietic cells when cultured in methylcellulose in the presence of IL-3 (Rich, 1995; Othaka, *et al.,* 1999). Similarities between HSCs and PGCs led us to wonder if PGCs share also the mitogenic response to IL-2 previously observed in hematopoietic cells. In the present work, we show that the three subunits of IL-2R (α, β and γ), but not IL-2, are constitutively expressed on the surface of PGCs isolated from 10.5, 11.5 and 12.5 dpc embryos and that this receptor mediates a mitogenic effect of IL-2 on PGCs cultured *in vitro*.

Results and Discussion

Expression of components of the IL-2/IL-2R system

The IL-2/IL2R system is normally expressed in T, B and NK lymphocytes to stimulate their proliferation via a community effect (Leonard *et al.,* 1984). Other kinds of cells, such as dendritic cells (Granucci *et al.,* 2001), macrophages (Gaffen and Liu, 2004) and

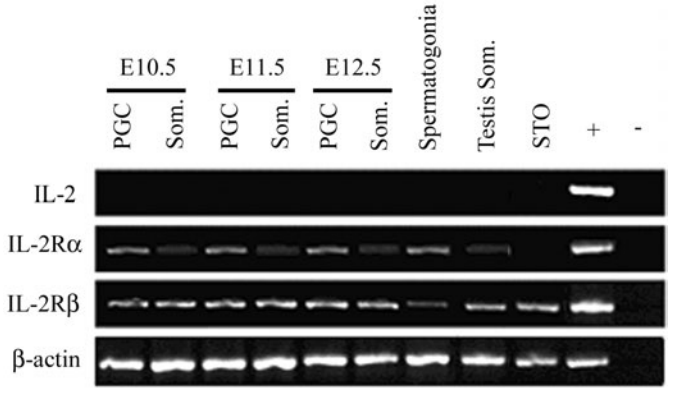

Fig. 1 (Left). Expression of IL-2, IL-2Rα and IL-2Rβ genes in primordial germ cells (PGCs) and somatic cells. *The expression of IL-2, IL-2Rα, IL-2Rβ and β-actin was analyzed by RT-PCR in PGCs and gonadal somatic cells from E10.5, E11.5 and E12.5 embryos, spermatogonia, isolated testis somatic cells and the STO mouse embryonic fibroblast cell line. RNA from murine splenic mononuclear cells activated with phytohemagglutinin-M was used as a positive control for IL-2, IL-2Rα and IL-2Rβ. Negative controls (-) consisted of PCR reaction performed without cDNA. The sizes of the amplified products are shown in Table 1. The α and β subunits of the IL-2R are expressed in PGCs and somatic cell samples at 10.5, 11.5 and 12.5 dpc, but IL-2 mRNA was not detected in any of them. Only the IL-2Rβ subunit is present in the STO cell line.*

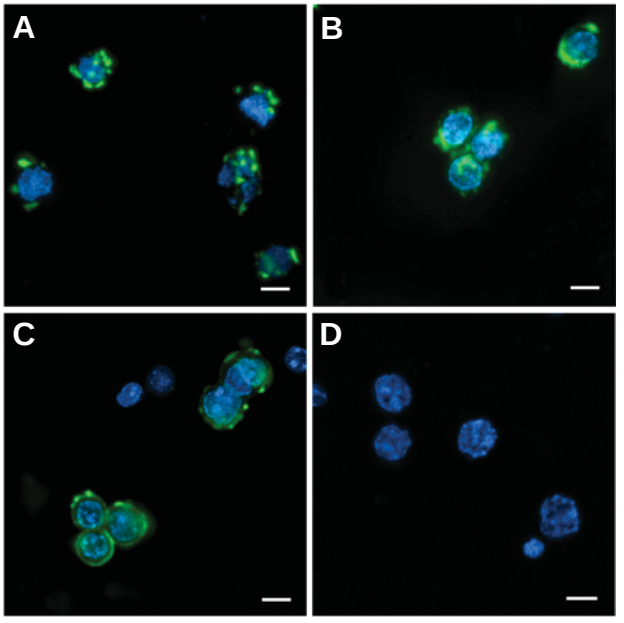

Fig. 2 (Right). Immunolocalization of the α, β and γ subunits of IL-2R. *Immunofluorescent confocal microscope images of PGCs from 11.5 dpc embryos, using anti-IL-2Rα* **(A)**, *anti-IL-2Rβ* **(B)** *and anti- IL-2Rγ antibodies* **(C)**. *Cells were fixed with 4% paraformaldehyde and incubated with the corresponding anti-mouse IL-2R α, β and γ antibody, followed by FITC conjugated rat anti-mouse IgG.* **(D)** *As a negative control, PGCs were only incubated with the secondary antibody. All subunits were found to be located on the surface of PGCs. The objective used was x 60. The image format was 1024 x 1024 pixels. Bar, 10 μm.*

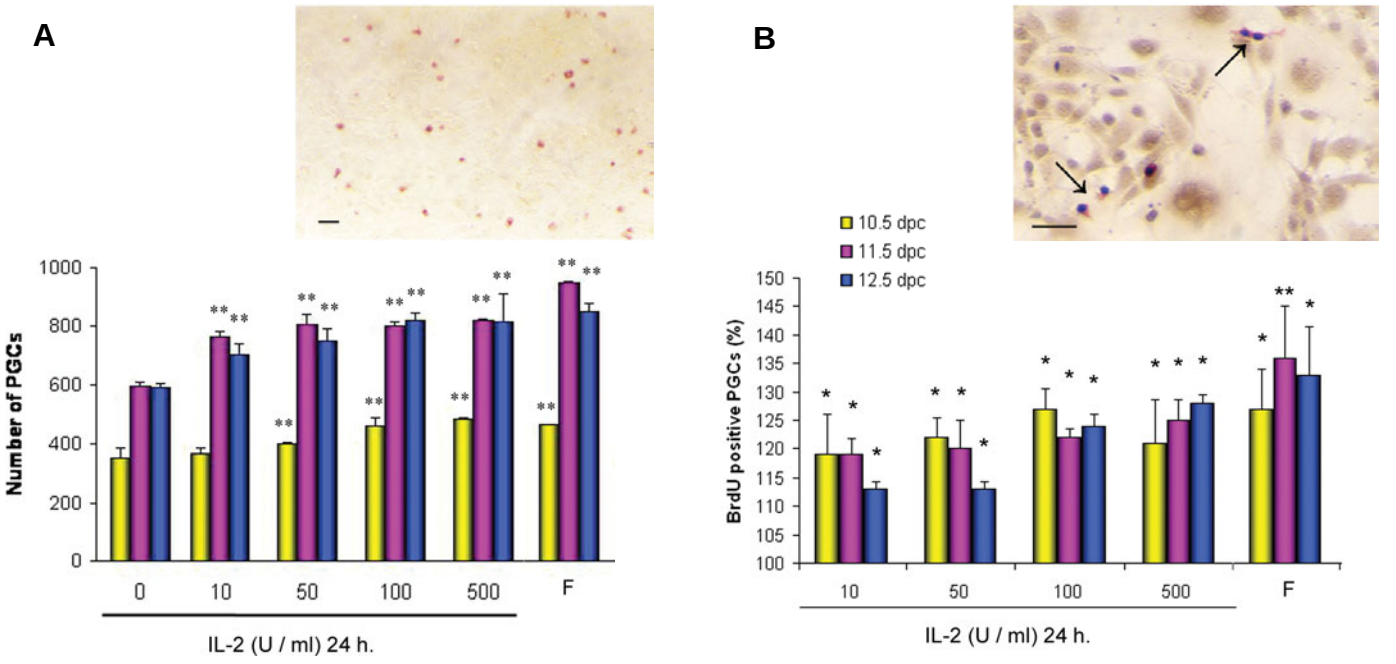

Fig. 3. Effect of IL-2 on PGC proliferation. (A) *The effect of various doses of mouse recombinant IL-2 on the number of PGCs, measured as alkaline phosphatase (AP) positive cells, isolated from 10.5, 11.5 and 12.5 dpc embryos, cultured on STO feeder cells and treated for 24 h. Bars represent the mean number of PGCs per well plus standard deviation of three replicate wells. After 24 hours, IL-2 at 10 U/ml showed a significant proliferative effect in 11.5 and 12.5 dpc PGCs, but not in 10.5 dpc (p<0.01). Nevertheless, at doses of 50, 100 and 500 U/ml, IL-2 did significantly increase the number of PGCs from 10.5, 11.5 and 12.5 dpc embryos (p<0.01). The illustration shows a culture of 10.5 dpc PGCs treated for 24 h with IL-2 (500 U/ml) on STO feeder cells and stained for AP (Bar, 100 μm).* **(B)** *Proportion (%) of BrdU-labeled cells with respect to the total number of AP positive cells, taking as control (100%) the number of double-stained cells obtained in non-treated cultures. PGCs isolated from 10.5, 11.5 and 12.5 dpc embryos were cultured for 1 day as above and treated with the indicated doses of IL-2 for 24 h. Subsequently, they were labeled with BrdU and histochemically double-stained for BrdU and AP. The increase in proliferation was around 12-25% in all the PGC cultures. The forskolin (F) control produced a proliferative effect between 25-35% in PGCs from 10.5, 11.5 and 12.5 dpc embryos. (*) Significant at P<0.05; (**) significant at P< 0.01. The illustration to the right shows a culture of PGCs after BrdU incorporation assay plus IL-2 (500 U/ml) treatment. Black arrows point to proliferating PGCs, which are double stained for BrdU and AP (Bar, 50 μm).*

melanoma cells (Boyano *et al.,* 1998; García Vazquez *et al.,* 2000) also express this cytokine and its receptor. In the present study, expression of the IL-2, IL-2Rα and IL-2Rβ genes was analyzed by RT-PCR using total RNA purified from PGC and somatic cell samples with a purity higher than 90% (Fig. 1). The PCR products were then subjected to electrophoresis and IL-2Rα and IL-2Rβ subunit bands were detected in the lanes corresponding to PGCs and somatic cells from 10.5, 11.5 and 12.5 dpc embryos, spermatogonia stem cells and testicular somatic cells. IL-2Rβ mRNA was also detected in the STO mouse embryonic fibroblast cell line. However, IL-2 expression was not observed in any of the analyzed samples. Expression of IL-2Rγ was not examined by PCR since its presence in PGCs has been previously reported (Cooke *et al.,* 1996).

To verify the expression of IL-2R subunit peptides (α, β and γ) on the surface of PGCs, indirect immunofluorescence assays were performed using confocal microscopy. IL-2Rα (Fig. 2A), IL-2Rβ (Fig. 2B) and IL-2Rγ (Fig. 2C) peptides were found to be expressed on the surface of PGCs at 11.5 dpc. As a negative control, we omitted the primary antibody during the immunocytochemical procedure, which led to the absence of immunofluorescence (Fig. 2D). Thus, we show that PGCs might be a target of the IL-2 cytokine since α, β and γ chains of the IL-2R were found to be expressed in isolated PGCs from 10.5, 11.5 and 12.5 dpc embryos, at mRNA and protein levels. We also show that the α and β subunits of the IL-2R are still expressed in postnatal spermatogonia (Fig. 1).

Effects of IL-2 and IL-2R blockade on PGC proliferation

PGCs at 10.5, 11.5 and 12.5 dpc were cultured on STO fibroblast feeder layers in the presence of 15% FCS and treated with IL-2 at 0, 10, 50, 100 and 500 U/ml for 24 hours. Each treatment was performed in triplicate and alkaline phosphatase quantification experiments were repeated three times. The mean values associated with the three experiments are illustrated in Fig. 3A. IL-2 did not exhibit any effect on 10.5 dpc PGCs at 10 U/ml after 24 hours, but a significant proliferative effect was observed in 11.5 and 12.5 dpc PGCs (p<0.01). Nevertheless, at doses of 50, 100 and 500 U/ml, IL-2 did significantly increase the number of PGCs from 10.5, 11.5 and 12.5 dpc embryos (p<0.01). As a positive control, PGCs from the same experiments were cultured in the presence of forskolin at 10 μM for 24 hours and the proliferative effect was found to be quite similar to that of IL-2. This result indicates that IL-2 may act as a paracrine or endocrine growth factor for mouse PGCs.

Male PGCs start to undergo mitotic arrest normally at 12.5 dpc

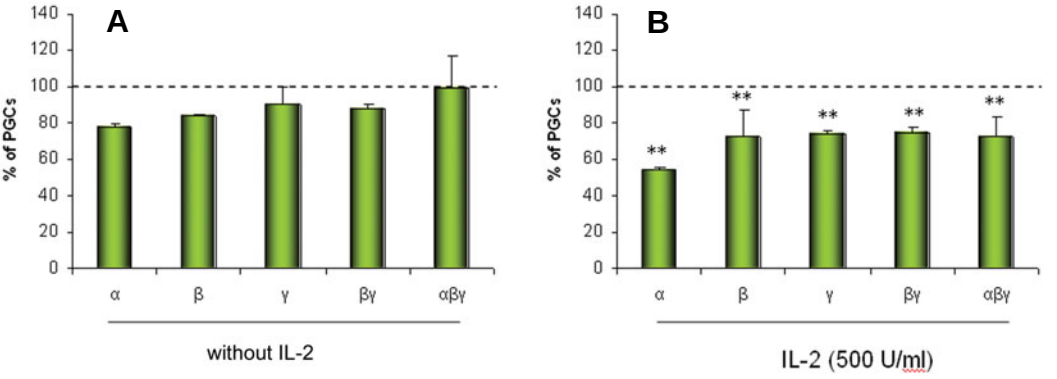

Fig. 4. Inhibition of IL-2 mediated proliferation of PGCs using anti-IL-2Rα, anti-IL-2Rβ and anti-IL-2Rγ blocking antibodies, either alone or in combination. *11.5 dpc PGCs were incubated with blocking antibodies for 2 h and subsequently with 500 U/ml of recombinant IL-2 for 24 hours. The number of PGCs was determined by counting AP positive cells. Blockade of the different subunits had no significant effect on the PGC numbers in untreated cultures* **(A)** *while in IL-2 treated cultures the blocking antibodies reduced very significantly (up to 45%; ** = p<0.01) the number of PGCs compared with IL-2 treated cultures without blocking antibodies (dotted line)* **(B)**. *The data represent the mean ± SD of triplicate cultures from three separate experiments.*

but, curiously enough, 12.5 dpc and 11.5 dpc PGCs show a very similar response to IL-2. Moreover, these cells are the only ones that significantly increase their proliferation rate at the lower IL-2 concentration (10 U/ml), suggesting that they express more IL-2 receptor than 10.5 dpc cells, or that their IL-2 receptor is more sensitive or more efficiently coupled to second messenger cascades. This finding opens up the possibility that IL-2 could delay or even revert the mitotic arrest which occurs in 12.5 dpc male PGCs. Indeed, IL-2 mediated reversion of mitotic arrest may constitute one component of the pathological progression of embryonal carcinoma of the gonads (Diez-Torre *et al.,* 2004).

Bromodeoxyuridine (BrdU) incorporation assays were performed in order to examine if IL-2 was acting as a mitogen or a survival factor in this cell population. In these experiments PGCs from 10.5, 11.5 and 12.5 dpc embryos were double-stained for BrdU incorporation and alkaline phosphatase activity (Fig. 3B). We found that there were changes in the proportion of BrdU positive cells in 10.5, 11.5 and 12.5 dpc PGCs treated with IL-2 at all doses. Increase in proliferation was around 12- 25% in PGCs from 10.5 to 12.5 dpc embryos with respect to the BrdU positive PGCs in untreated cultures (control, considered as 100%). The forskolin control (10 μM) produced a proliferative effect of around 25-35% in PGCs from 10.5, 11.5 and 12.5 dpc embryos. These assays reveal that the increase in number of PGCs in culture after treatment with IL-2 was due to a mitogenic rather than a survival effect. This IL-2 effect is different to that of other interleukins, such as IL-4, IL-6 and IL-11, which only exert a survival effect (De Felici and Dolci., 1991; Cooke *et al.,* 1996; Cheng *et al.,* 1994; Koshimizu *et al.,* 1996). However, it coincides with the proliferative effect of IL-2 on T lymphocytes (Cornish *et al.,* 2006).

We neutralized IL-2 receptor subunits with anti-IL-2Rα, β and γ blocking antibodies, independently or as a combination of subunit blockers, for 2 hours and then the cells were incubated in the presence or absence of 500 U/ml IL-2 for 24 hours (Fig. 4). We found that blocking the IL-2Rα, β and γ subunits independently or as combinations of βγ or αβγ subunit blockers did not have a significant effect on PGC proliferation rate (Fig. 4A). However, under the same conditions but in the presence of IL-2, a significant inhibition of PGC proliferation was observed (Fig. 4B). The percentage of inhibition was 45% when the α subunit was neutralized, while other blockades led to an inhibition of about 30% compared with those cultures treated with 500 U/ml IL-2 in the absence of blocking antibodies. These results demonstrate that this effect is specifically mediated by the IL-2R, since blockade of any of its subunits with subunit-specific antibodies abrogated the IL-2 mitogenic effect on PGC cultures.

Finally, we compared the proliferative effect of IL-2 with other cytokines which are known mitogen and/or survival factors such as bFGF (mitogen), LIF (survival factor) and SF (mitogen + survival factor). We cultured PGCs at 11.5 dpc on STO feeder layers in the presence of 15% FCS, supplemented with IL-2 (500 U/ml), bFGF (10 ng/ml), SF (20 ng/ml) or LIF (10 ng/ml) for 24 hours. We found that the proliferative effect of IL-2 was very similar to that of bFGF, SF and LIF in PGCs from 11.5 dpc embryos ($p<0.05$) (Fig. 5), supporting the idea that IL-2 plays a physiologically relevant role in the regulation of the PGC population at this developmental stage. Since a variety of growth factors and cytokines increase PGC numbers *in vitro*, as mentioned before, the lack of one of them would not necessarily have a significant effect on germ line development, because its function could be replaced by other factors (factor redundancy). In fact, transgenic mice with deletion of the γ subunit of the IL-2R (necessary for IL-2 signal transduction) do not show any significant alteration of their gonads (DiSanto *et al.,* 1995).

Common role of the IL-2/IL-2R system in PGC and HSC development

A possible physiological role for IL-2 in PGCs *in vivo* is supported by the presence of this cytokine in the embryo in places where PGC migration and proliferation take place. Expression of IL-2 *in vivo* has been shown in several tissues such as the decidua, placenta (von Rango *et al.,* 2003) and embryonic liver, thymus, pharyngeal blood vessels and omentum (Godin *et al.,* 1995; Reya *et al.,* 1996). The expression of IL-2 and its receptors in various tissues of the postimplanted embryo was found to be associated with the location of HSCs and their differentiation into extrathymic T cells during development (Murray *et al.,* 1998). IL-2 is also expressed by HSCs which spatially coincide with PGCs in early embryos (Zeigler *et al.,* 2006). In addition, it is already well known that the embryo receives maternal growth factors through the placenta (McLennan and Koishi, 2004), raising the possibility that maternal IL-2 could also have a role in PGC development.

Our findings of a functional IL2/IL2R system for PGC proliferation show another similarity between HSCs and PGCs, supporting

the hypothesis of a common stem cell precursor or, at least, a close developmental pathway for these two cell lineages. Genetic studies have shown that the development of hematopoietic cells parallels that of germ cells. Natural mutations at the *W* and *Steel* loci, encoding the tyrosine kinase receptor c-Kit and its SF ligand (also known as SCF, MGF or KL), respectively, determine a phenotype causing embryonic death at 14-15 dpc due to anemia. *W/W* and *Sl/Sl* embryos also lack germ cells and melanoblasts (for a review see Besmer, 1991). SF exerts pleiotropic effects on PGC and hematopoietic cell proliferation and survival (Matsui *et al.,* 1991, Godin *et al.,* 1991; Dolci *et al.,* 1991, Carson *et al.,* 1994) and both PGCs and HSCs adhere to somatic cells by expressing the membrane-bound variant of SF (Pesce *et al.,* 1997; Bendall *et al.,* 1998). The enhanced SF-mediated survival of germ and hematopoietic cells *in vitro* is probably due to the suppression of similar apoptotic pathways, which involve the control of expression of Bcl-2 and Bax (Pesce *et al.,* 1993; Carson *et al.,* 1994; De Felici *et al.,* 1999). During mouse embryogenesis, restriction of hematopoietic potential to cells of the posterior part of the epiblast is established. Before gastrulation, all epiblastic cells are equally able to enter the hematopoietic lineage while, as gastrulation proceeds, this ability is confined to the extraembryonic mesodermal compartment, where progenitors of HSCs and PGCs are allocated (Kanatsu and Nishikawa, 1996; Lawson and Hage, 1994). Interestingly, stimulation of anterior epiblastic cells of gastrula stage embryos with secreted Bmp-4 inhibits their differentiation pathway as neuroectodermal cells and restores their ability to form hematopoietic colonies (Kanatsu and Nishikawa, 1996). Moreover, ablation of Bmp-4 by homologous recombination impairs the formation of the extraembryonic mesodermal compartment (Winnier *et al.,* 1995) and affects, dose-dependently, the segregation of the PGC founder population and hematopoietic precursors (Lawson *et al.,* 1999; McLaren, 1999; Fujiwara *et al.,* 2001). Mutation of the β1-integrin gene causes a hematopoiesis defect in fetal liver but not in yolk sac (Fassler and Meyer, 1995). This suggests that mechanisms of cell adhesion/migration are involved in the allocation of a precursor of HSCs in the aorta-gonadomesonephros (AGM) before they enter into the dorsal aorta. Similar integrin-mediated adhesion mechanisms have been also suggested for PGCs (De Felici and Dolci, 1989; French-Constant *et al.,* 1991). In line with these arguments, it has been shown that mice defective for β1-integrin also exhibit an impaired migration of PGCs towards the AGM region and gonadal ridges (Anderson *et al.,* 1999). Moreover, HSCs and PGCs appear to share homing mechanisms mediated by SDF-1 and its receptor CXCR4. In fact, mice lacking either SDF-1 or CXCR4 have impaired colonization of bone marrow and gonads (Ma *et al.,* 1998; Ara *et al.,* 2003).

In summary, we provide evidence that PGCs express functional IL-2 receptors during their migration to and colonization of gonadal ridges and that the IL-2 cytokine increases the *in vitro* proliferation of PGCs through a mitogenic, rather than survival, effect. Thus, these results support the hypothesis that the IL-2/IL-2R system may be involved in the proliferation and differentiation of PGCs in mouse embryos, in a manner similar to that which occurs in embryonic hematopoiesis.

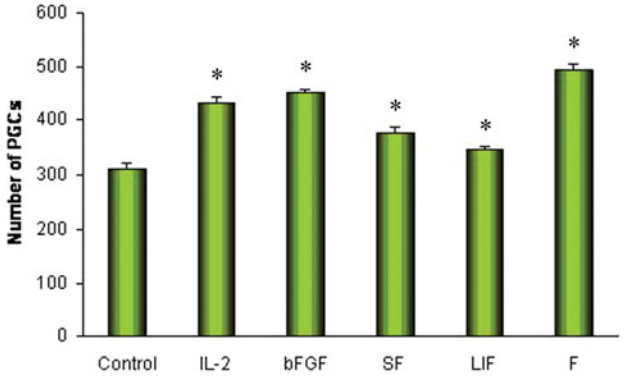

Fig. 5. Comparison of the effects of IL-2 and other growth factors on PGC proliferation. *11.5 dpc PGCs were cultured on STO feeder layers in the presence of 15% FCS, supplemented with IL-2 (500 U/ml), bFGF (10 ng/ml), SF (20 ng/ml), LIF (10 ng/ml) or forskolin (F, 10 μM) for 48 hours. The number of PGCs was determined by counting AP positive cells. The graph shows that the proliferative effect of IL-2 was very similar to that of bFGF and forskolin in PGCs from 11.5 dpc embryos ($p<0.05$). The effect of SF and LIF was always proliferative with respect to the control ($p<0.05$), but the effect of these cytokines was smaller than that of IL-2. The data represent the mean ± SD of triplicate cultures from three separate experiments. (*) Significant difference at $p<0.05$.*

Experimental Procedures

Isolation and culture of mouse PGCs

PGCs were isolated and purified from the gonadal ridges of mouse embryos at 10.5, 11.5 and 12.5 dpc using the Mini-Macs system (Pesce and De Felici, 1995). They were then cultured on mitomycin C inactivated STO fibroblast monolayers in DMEM. The medium was supplemented with 15% FCS, N-acetyl-cysteine (Sigma, St. Louis, MO, USA), sodium pyruvate (Sigma, St. Louis, MO, USA) and cells were seeded (about 1 gonadal ridge equivalent/well) in 24-well culture plates. The purity of the obtained PGC samples was higher than 90% as determined by PGC-specific alkaline phosphatase staining.

Isolation of spermatogonia cells

Spermatogonia were obtained from 6-7 day-old mice testes. Decapsulated testes were digested with collagenase-II (Sigma, St. Louis, MO, USA). Seminiferous tubuli were isolated after centrifugation at 85 g for 7 minutes and subsequently digested with a trypsin-EDTA salt solution (Invitrogen Life Technologies, Carlsbad, CA) and DNase-I (Sigma, St. Louis, MO, USA). The obtained cell suspension was filtered using a nylon net (Falcon; BD Biosciences, Heidelberg, Germany) to remove cell aggregates and was centrifuged at 390 g for 10 min. In order to separate spermatogonia from somatic cells, cell suspensions were plated into culture flasks and maintained at 32°C overnight in DMEM supplemented with 10% FCS, 32 mM NaHCO3, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma, St. Louis, MO, USA). Spermatogonia (which remained in suspension) and somatic cells (which adhered to the substrate) were collected separately and washed before experiments. Cell suspensions were incubated with anti-vimentin-FITC in order to analyze spermatogonia and somatic cell sample purity by flow cytometry analysis. Spermatogonia were identified as vimentin-negative cells, while testicular somatic cells were vimentin positive (data not shown). Only those samples with a purity over 90% were used for experiments.

Other Cells

The STO mouse embryonic fibroblast cell line (European Collection of Cell Cultures, Salisbury, UK) was cultured with DMEM supplemented with 10% FCS, 32 mM NaHCO3, 2 mM glutamine, 100 U/ml penicillin and 100

μg/ml streptomycin (Sigma, St. Louis, MO, USA). Murine splenic mononuclear cells were used as positive controls for IL-2/IL-2R expression. These cells were isolated, activated and cultured as previously described (Boyano *et al.,* 1998).

Reverse-transcription and PCR

Total RNA from PGCs, gonadal ridges and testicular somatic cells, spermatogonia, mouse embryonic fibroblasts STO and activated mononuclear cells was purified using the RNeasy Mini Kit (Qiagen, Hilden, Germany). Reverse-transcription was performed using the RETROscript kit (Ambion, Austin, TX, USA). PCR was achieved with the Platinum PCR Supermix (Invitrogen Life Technologies, Carlsbad, CA), according with the manufacturer's instructions. Mouse specific primers for PCR amplification were purchased from Invitrogen. Their sequences and annealing temperatures are summarized in Table 1. Annealing temperature ranged between 55 and 61°C for all primers. PCR products were subjected to electrophoresis in 2% agarose gels containing ethidium bromide (Merck, Darmstadt, Germany) in TBE 1X buffer and subsequently visualized under UV illumination (LKB Bromma 2011 macrovue).

RNA from 12.5 dpc embryo fetal liver was used as positive control for β-actin gene expression. RNA from murine splenic mononuclear cells, activated with phytohemagglutinin-M (Roche, Mannheim, Germany) was used as positive control for the presence of IL-2, IL-2Rα and IL-2Rβ mRNA. A negative control containing water instead of cDNA was included in PCR experiments.

TABLE 1

PRIMERS SEQUENCES USED FOR RT-PCR

Primer	Sequence	Amplicon size (bp)	T annealing: ºC
IL-2	F: 5´-ATGTACAGCATGCAGCTCGCATC -3´ R: 5´-GGCTTGTTGAGATGATGCTTTGACA -3´	502	55
IL-2Rα	F: 5´-ATGGAGCCACGCTTGCTGATGTTG -3´ R: 5´-CCATTGTGAGCACAAATGTCTCCG -3´	700	55
IL-2Rβ	F: 5´-CTCCGTGGACCTCCTTGACATAAATGTGG -3´ R: 5´- TGTTTCGTTGAGCTTTGACCCTCACCTGG -3´	348	58
β-actin	F: 5´-GTGGGCCGCTCTAGGCACCAA -3´ R: 5´-CTCTTTGATGTCACGCACGATTTC -3´	540	55

Immunofluorescence assays

PGCs were seeded onto chamber slides (Nunc, Naperville, USA) and fixed in 4% paraformaldehyde after 4 hours of culture. Cells were washed three times in phosphate buffered saline (PBS) and blocked with 10% FCS in PBS, before incubation with the primary antibody for 60 min at room temperature in 5% FCS in PBS. Specific rat antibodies were anti-mouse IL-2Rα (2 μg/ml) and anti-mouse IL-2Rβ (1 μg/ml), both from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA) and anti-mouse IL-2Rγ (1 μg/ml) from BD Biosciences, (Heidelberg, Germany). After three washes in PBS, cells were incubated with FITC-conjugated anti-rat IgG (30 μg/ml) (Sigma, St. Louis, MO, USA) in the same buffer as that used for the primary antibody for 60 min in the dark. Stained cells were washed with PBS containing 0.4 μg/ml 4',6-diamin-2-phenylindol-dihydrochloride (DAPI) and mounted onto Fluoromount G mounting medium (Electron Microscopy Sciences, Washington D.C., USA). Cells were examined with an Olympus Fluoview 500 confocal microscope.

Proliferation assays and neutralization

PGCs were seeded into 24-well microplates at an approximate concentration of one gonadal ridge equivalent/well on mitomycin-C inactivated STO cell monolayers in serum-containing medium with different concentrations of recombinant IL-2 (0, 10, 50, 100 and 500 U/ml) (Roche, Mannheim, Germany). bFGF (10 ng/ml, R&D systems), SF (20 ng/ml, R&D Systems), LIF (10 ng/ml) or forskolin (10 μM, Sigma) were used as a positive control in these experiments. LIF was isolated as described by Gearing *et al.,* 1989. Cells were cultured for 24 hours and their proliferation was determined by counting the number of alkaline phosphatase stained cells and expressing this as a percentage of control (cells incubated without IL-2). Proliferation was also quantified by counting alkaline phosphatase stained cells which exhibited nuclear incorporation of bromodeoxyuridine (BrdU) using the Amersham cell proliferation kit (RPN20) as previously described (De Felici and Dolci, 1989). Briefly, the cells were fixed after 2 hours of BrdU labeling and then PGCs were double-stained for alkaline phosphatase activity and BrdU incorporation. Each concentration of IL-2 was applied to three wells, and the experiment was repeated three times. For neutralization assays, PGCs were cultured on STO cell feeder layers for 2 hours without FCS in the presence of anti-mouse IL-2Rα (2 μg/ml), anti-mouse IL-2Rβ (1 μg/ml) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and anti-mouse IL-2Rγ (1 μg/ml) (BD Biosciences, Heidelberg, Germany). Then, they were incubated for 24 hours in the presence or absence of recombinant IL-2 (500 U/ml). Cell growth was determined by proliferation assays with alkaline phosphatase staining in microplates as described above. Proliferation was expressed as the percentage of PGCs with respect to the number of PGCs in similar conditions but in the absence of subunit antibodies (n=3 independent experiments).

Statistics

Values are expressed as means + standard deviation (n=3). Significance was defined as $p < 0.05$ (*) and $p < 0.01$ (**). Statistical analyses were performed using the *ANOVA* test with *SPSS 12.0* for *Windows*.

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