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The c-kit receptor is involved in the adhesion of mouse primordial germ cells to somatic cells in culture

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Abstract

The receptor encoded by the *W* (c-kit) locus is expressed on the membrane of mouse primordial germ cells, whereas its ligand termed stem cell factor (SCF), encoded by the *Sl* locus, is expressed on the membrane of somatic cells associated with both the primordial germ cell migratory pathways and homing sites. Using an in vitro short time assay which allows a quantitative measure of adhesion between cells, in the present paper we show that SCF/c-kit interaction can modulate primordial germ cell adhesion to somatic cells. We report that the adhesiveness of 11.5 dpc primordial germ cells to four types of somatic cells in culture (TM_4 cells, STO fibroblasts, bone marrow stromal cells and gonadal somatic cells) is significantly reduced by antibodies directed against c-kit receptor or SCF, as well by soluble SCF. This SCF/c-kit mediated adhesion seems independent of SCF-induced tyrosine autophosphorylation of c-kit receptor. Moreover, primordial germ cells showed a poor ability to adhere to a bone marrow stromal cell line carrying the Sl^d mutation (unable to synthesize membrane-bound SCF). This adhesiveness was not further impaired by anti-c-kit antibody. These results demonstrate that SCF/c-kit interaction contributes to the adhesion of primordial germ cells to somatic cells in culture and suggest that the role played by SCF in promoting survival, proliferation and migration of these cells in vitro and in vivo, demonstrated by several studies, might depend on the ability of the membrane-bound form of this cytokine to directly mediate primordial germ cell adhesion to the surrounding somatic cells. © 1997 Elsevier Science Ireland Ltd.

Keywords: C-kit receptor; Primordial germ cell; Stem cell factor; Cell adhesion

1. Introduction

In the mouse embryo, mutations in both the *Steel* locus (*Sl*) and the 'dominant white spotting' locus (*W*) result in a developmental defect of three stem cell populations, i.e. hematopoietic stem cells, melanoblasts and primordial germ cells. W mutation is allelic with the gene encoding the tyrosine kinase receptor c-kit, whereas *Sl* locus encodes the c-kit ligand, stem cell factor (SCF) (for a review see Fleishman, 1993). SCF, also referred to as steel factor (SF), kit ligand (KL) or mast cell growth factor (MGF), is normally expressed in two forms deriving from the translation of different splice-variants. Both forms are initially localized on the cell membrane. However, while the larger variant (45 kDa in mouse) is usually cleaved to produce a soluble form of SCF, the smaller form (32 kDa) remains preferentially associated with the cell membrane (Huang et al., 1992). The defects in *W* and *Sl* mutant embryos are consistent with a role for the SCF/c-kit system in promoting survival, proliferation and/or migration of the embryonic stem cell population reported above. Recent findings showed that migratory mouse primordial germ cells (PGCs) express the c-kit receptor, while SCF is expressed in cells present in the PGC migratory pathway and in the gonadal ridges (Matsui et al., 1990; Keshet et al., 1991). Moreover, in vitro culture studies show that soluble and/or membrane-bound SCF is essential for survival and/or proliferation of mouse PGCs and that soluble SCF is able to notably reduce apoptosis in such cells (for a review see De Felici and Pesce, 1994a).

In this paper we test the hypothesis that membrane-associated SCF may function as an adhesion molecule for PGCs by studying the ability of germ cells of various embryonic

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ages to adhere in culture to monolayers of cells expressing or lacking transmembrane SCF and the effect on this adhesion of antibodies against c-kit receptor or SCF and of soluble SCF.

2. Results

The TM_4 cell line was initially selected as an adhesion substrate for studying adhesion molecules involved in PGC/somatic cell interactions because of the ability of these cells to support PGC survival and proliferation through direct cell-to-cell contacts (De Felici et al., 1992, 1994a). Using a short term adhesion assay (45–60 min), we found that the adhesiveness of 11.5 dpc PGCs to TM_4 cells varied between 44 and 89% (mean ± standard error 61 ± 3.5) while that of 14.5 dpc oocytes was generally higher (50–90%; 74 ± 4.2) and that of 14.5 dpc prespermatogonia was lower (10–45%; 23 ± 5.5) (Table 1). To verify whether the SCF/c-kit interaction, which is crucial for PGC survival and proliferation, is also involved in mediating germ cell adhesion, we studied the effect of ACK-2 monoclonal antibody on their adhesiveness to TM_4 cells. ACK-2 recognizes the extracellular domain of the c-kit receptor and antagonistically blocks its receptor function (Ogawa et al., 1990, 1993; Nishikawa et al., 1991). The results reported in Fig. 1A show that the preincubation of 11.5 dpc PGCs in the presence of ACK-2 notably reduced the number of PGCs adhering to TM_4 cell monolayers. Similarly, PGC adhesiveness was significantly reduced when TM_4 cells were pretreated with an antibody that is able to neutralize the survival activity of soluble SCF on PGCs in culture (our unpublished results) or when the soluble form of SCF was added to the medium during the adhesion assay (Fig. 1B,C). These treatments, however, did not affect the adhesiveness to TM_4 cells of 14.5 dpc oocytes and prespermatogonia (data not shown).

Studies on the expression pattern of SCF performed with immunoblot (Fig. 2) and immunohistochemistry (Fig. 3A,B) indicated that TM_4 cells express transmembrane SCF. In addition, PCR analysis and immunohistochemistry showed that high levels of c-kit were expressed by 11.5 dpc

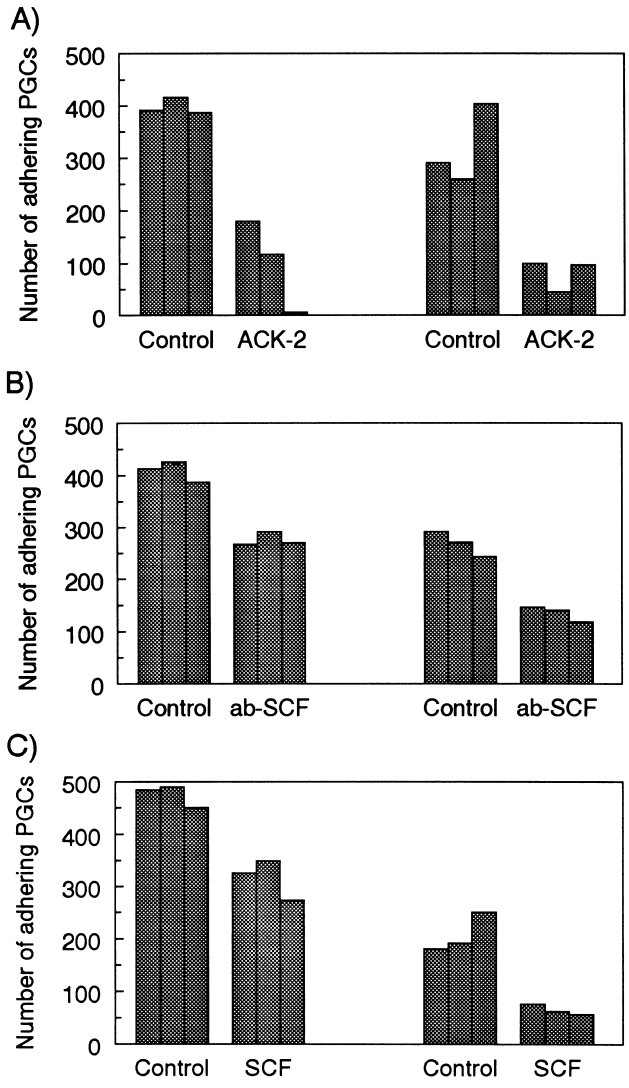

Fig. 1. Effect of 10 μg/ml anti-c-kit antibody (ACK-2) (A), 10 μg/ml anti-SCF antibody (ab-SCF) (B) and 500 ng/ml SCF (C) on the adhesion of 11.5 dpc PGCs to TM_4 cells. Each column represents the number of adhering PGCs in a single well; for each treatment, two representative experiments are shown. For all experiments (at least three for each treatment) differences are statistically significant ($P < 0.05$ determined by Student's *t*-test).

Table 1

Adhesion of germ cells isolated from mouse embryonic gonadal ridges (PGCs) and fetal gonads (oocytes and prespermatogonia) to various cell monolayers (means and standard errors of at least three experiments are resorted)

	Adhesion (%) (mean ± SE)				
	TM_4	SCs	STO	Wt	Sl^d-1
PGCs	61 ± 3.5	48 ± 6.5	44 ± 4.3	40 ± 5.1	25.5 ± 3.2
Oocytes	74 ± 4.2	–	–	–	–
Prespermatogonia	23 ± 5.5	–	–	–	–

SCs, somatic cells from 11.5 dpc gonadal ridges; Wt, wildtype bone marrow stromal cells; Sl^d-1, bone marrow stromal cells from *Sl*^{d} mice; –, not determined.

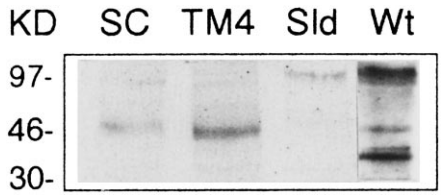

Fig. 2. Immunoblot analysis of the expression of SCF by gonadal somatic cells (SC), TM_4, Sl/Sl^d-1 (Sld) and wildtype (Wt) bone marrow cells. A band with an apparent molecular weight of approximately 45 kDa corresponding to potentially soluble SCF (Flanagan and Leder, 1990; Huang et al., 1992) is detected in protein extracts taken from gonadal somatic cells, TM_4 and Wt cells, but not from Sl/Sl^d-1 cells. In Wt cells a band of about 35 kDa corresponding to the transmembrane SCF form is also visible.

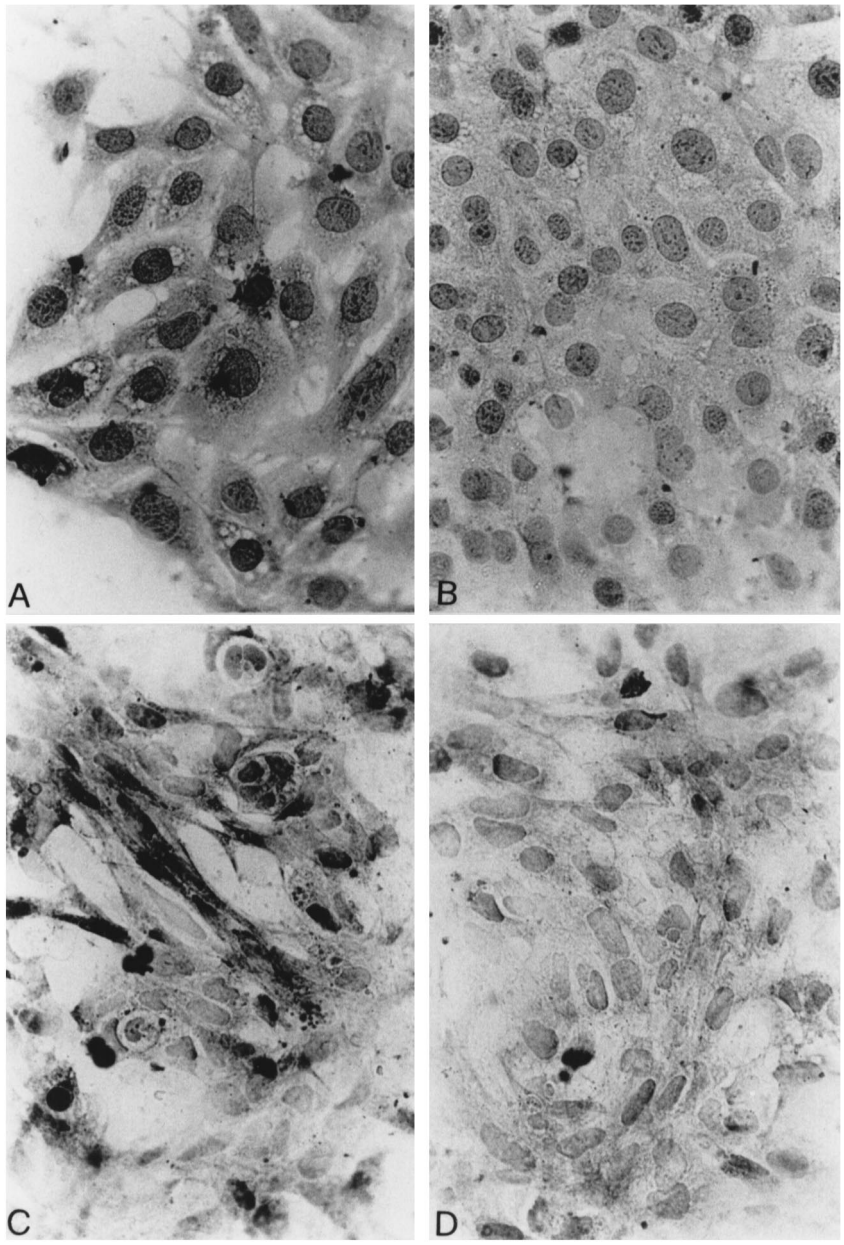

Fig. 3. Immunohistochemical demonstration of the presence of SCF in TM_4 (A) and gonadal somatic cells (C) in culture. (B,D) Control samples in which non-immune rabbit serum was used instead of primary anti-SCF antibody. The immunolocalization of SCF protein shows specific staining on the surface/cytoplasm and for an unknown reason also in the nucleus.

PGCs, while the receptor was not detectable or expressed at very low levels by germ cells at later stages of development (Figs. 4 and 5).

We also studied the adhesion of 11.5 dpc PGCs to cell monolayers obtained from somatic cells of gonads of the same embryonic age, to STO fibroblasts and to bone marrow stromal cells derived from normal mice or from mice carrying the Sl^d mutation. While gonadal somatic cells, STO fibroblasts and bone marrow stromal cells express membrane-bound SCF (Matsui et al., 1990; Huang et al., 1992; this paper, see below), Sl/Sl^d-1 bone marrow stromal cells can synthesize soluble SCF only (Flanagan and Leder, 1990). This was confirmed by immunoblot (Fig. 2), immunohistochemistry (Fig. 3C) and PCR analysis (Fig. 6). Adhesion assays showed that although 11.5 dpc PGC adhesiveness to gonadal somatic cell monolayers was quite variable ranging from 35 to 70% (48 ± 6.5) (Table 1), it was always significantly reduced by ACK-2 treatment (Fig. 7). Similarly, the adhesion of PGCs to STO fibroblasts and bone marrow stromal cells ranged from 35 to 61% (44 ± 4.3 and 40.5 ± 5.1, respectively) (Table 1) and was markedly reduced by 30–40% by ACK-2 antibody. On the other hand, PGC adhesion to Sl^d stromal cells was low (15–30%, 25 ± 3.2) (Table 1) and was not affected by ACK-2 treatment (data not shown).

Since the c-kit receptor has been shown to exhibit intrin-

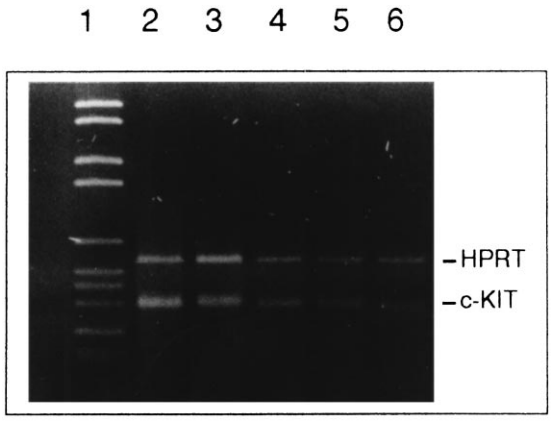

Fig. 4. RT-PCR analysis of c-kit mRNA expression (amplified product 385 bp) in germ cells of various postcoital ages; molecular markers (lane 1), 11.5 dpc (lane 2), 12.5 dpc female (lane 3), 12.5 dpc male (lane 4), 14.5 dpc female (lane 5), 14.5 dpc male (lane 6) The amplified product of the housekeeping gene HPRT (580 bp) is also shown.

sic tyrosine kinase activity and this activity is postulated to play a role in its signaling function, we used the tyrosine kinase inhibitor genistein to verify if this signal is important for SCF/c-kit mediated PGC adhesion. The results showed that treatment with genistein in the range of 10–100 μM does not reduce PGC adhesion to TM_4 and gonadal somatic cells.

3. Discussion

SCF is produced as either a 248 amino acid transmembrane polypeptide or a soluble form of 164 residues. The two types of RNA produced by alternative splicing of the primary transcript encode transmembrane forms, but only one of them can be efficiently cleaved at the cell surface (Flanagan and Leder, 1990). Recent results suggest that soluble and cell-bound SCF might have distinct functions. For example, the soluble form induces melanocyte dispersal and mast cell proliferation, while the cell surface form promotes melanocyte survival and hematopoietic and mast cell adhesion (Kaneko et al., 1991; Adachi et al., 1992; Avraham et al., 1992; Kodama et al., 1994; Wehrle-Haller and Weston, 1995). Furthermore, interaction between the c-kit receptor and membrane-bound SCF appears to be responsible for the adhesion of spermatogonia to Sertoli cells (Marziali et al., 1993).

Previous reports demonstrated that SCF is essential for PGC survival but is unable to directly stimulate their proliferation (Dolci et al., 1991, 1993). Most interesting, while soluble SCF is able to prevent apoptosis in PGCs for a short time only (Pesce et al., 1993), feeder cells expressing the transmembrane form of SCF permit long lasting PGC survival (Dolci et al., 1991). In this paper, we present the first evidence that SCF/c-kit interaction plays a role in mediating PGC adhesion to somatic cells, probably in synergism with other molecules. We demonstrate that in vitro PGC adhesion to cell monolayers expressing membrane-bound SCF (TM_4, gonadal somatic cells, STO fibroblasts and bone marrow stromal cells) is markedly reduced by antibodies against c-kit receptor or SCF, or by soluble SCF (Fig. 1). However, since PGC adhesion was not completely inhibited by these treatments, it appears that additional mechanisms are involved in PGC adhesion in these systems. The existence in embryonic germ cells of SCF/c-kit-dependent and -independent adhesion mechanisms is further supported by the finding that PGCs showed a consistent though poor ability (15–30%) to adhere to cells lacking transmembrane SCF (Sl/Sl^d-1 bone marrow stromal cells) and by the evidence that other epitopes are likely to play a role in adhesive interactions between PGCs and somatic cells (De Felici and Pesce, 1994b). Indeed, 14.5 dpc oocytes which express very low amounts of c-kit receptor (Manova et al., 1990; Manova and Bachvarova, 1991; this paper) appear to adhere to TM_4 cells with efficient SCF/c-kit-independent mechanisms (Table 1), thus confirming that different and developmentally regulated mechanisms can modulate germ cell adhesiveness to somatic cells.

In recent years it has become apparent that there is considerable interdependence, overlap and similarity between the cytokine and adhesion molecule systems. Some cytokines found in association with cell membranes and with the extracellular matrix might act to directly mediate cell adhesion. The results reported in this paper indicate that SCF may be considered a member of this new emerging cytokine family. On the basis of our results and other studies reported above, we suggest that SCF/c-kit adhesion is crucial for PGC survival and consequently, for their ability to respond to mitotic signals (i.e. increase of intracellular cAMP; De Felici et al., 1992; Dolci et al., 1993; Pesce et al., 1996). Moreover, SCF/c-kit-mediated adhesion might be involved in PGC migration. Clumping of PGCs characteristic of W^e/W^e mice (Buehr et al., 1993) suggests that in this mutation the ability of PGCs to adhere normally to cellular substrates can be impaired, this affecting their migratory ability. Keshet et al. (1991) describe a gradient of SCF expression along the path of migratory PGCs and suggest that this may provide a mechanism for the control of 'cell homing' through chemotaxis. On the other hand, Godin et al. (1991) showed that although SCF did not exert a chemotropic effect on mouse PGCs in vitro, it was able to stimulate their motility. In addition, soluble SCF promotes dispersion of melanoblasts in the mouse embryo (Wehrle-Haller and Weston, 1995) and is a chemoattractant for mast cells (Meininger et al., 1992).

While tyrosine kinase activity appears to be essential for c-kit-mediated survival and proliferation signals (Ogawa et al., 1990; Ogawa et al., 1993; Blume-Jensen et al., 1991; Matsui et al., 1991; Nishikawa et al., 1991), it seems unnecessary for PGC adhesion to somatic cells. In fact, pretreatment of PGCs with genistein does not reduce their adhesion to TM_4 or gonadal somatic cells. Similarly, the inhibition by PKC activation of tyrosine autophosphorylation of c-kit

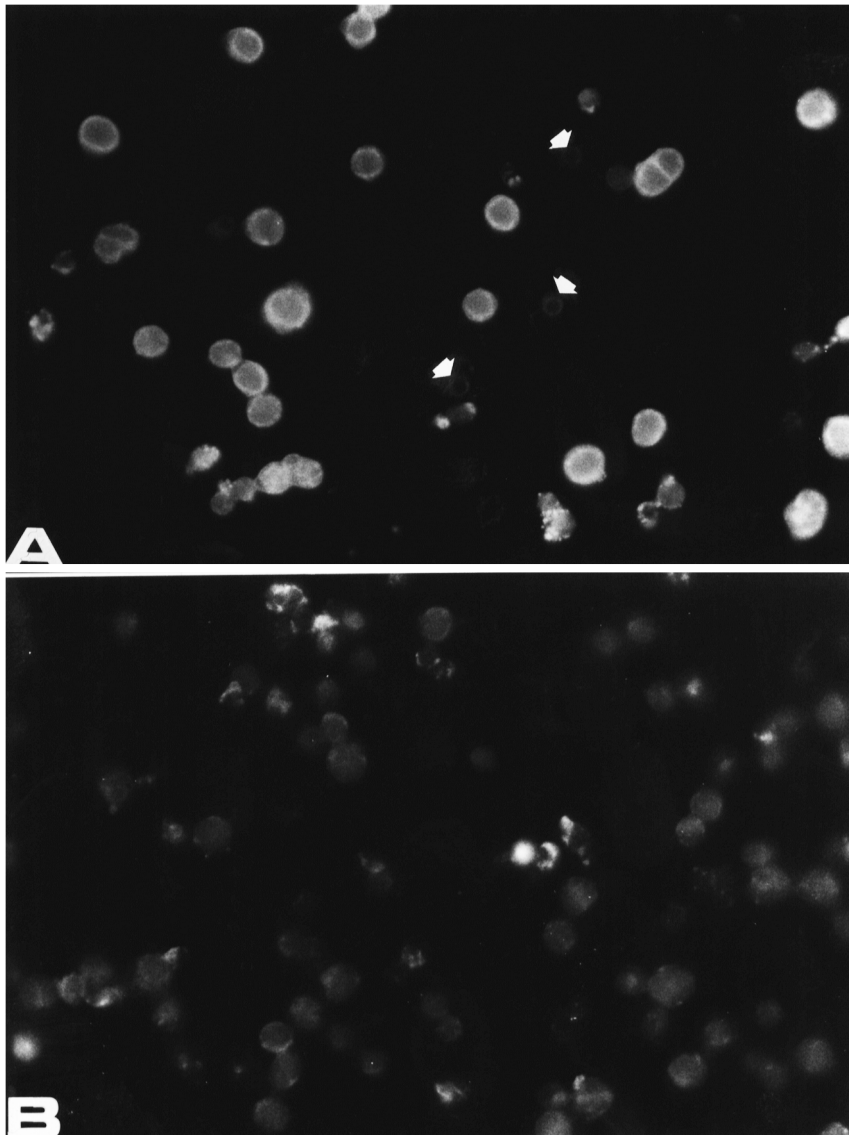

Fig. 5. Immunohistochemical demonstration of c-kit protein in isolated germ cells. While c-kit is strongly expressed in 12.5 dpc PGCs (A), it is barely visible in 14.5 dpc oocytes (B). Arrows indicate unlabeled contaminating somatic cells.

receptor in porcine aortic endothelial cells stably transfected with c-kit DNA (PAE/kit cells) does not affect its SCF binding affinity and stimulates the SCF-induced motility response consisting of circular actin reorganization as well as chemotaxis (Blume-Jensen et al., 1991; Blume-Jensen et al., 1993). These results suggest that the potential involvement of SCF/c-kit interaction in controlling cell motility and in particular PGC migration is independent of SCF-induced tyrosine autophosphorylation of c-kit receptor.

In conclusion, although distinct roles for soluble and membrane-bound forms of SCF in mouse PGCs development remain to be identified, our results indicate that transmembrane SCF may favor PGC adhesion to somatic cells. This adhesive interaction can be crucial for proper PGC response to other migration, survival and proliferation factors such as LIF and cAMP and when defective can lead to the defects which characterize the W and Sl mutations.

4. Experimental procedures

4.1. Isolation of germ cells and preparation of cell monolayers

For the adhesion assays, primordial germ cells, oocytes and prespermatogonia were isolated by EDTA-mechanical treatment of 11.5 dpc gonadal ridges and 15.5 dpc fetal gonads of CD-1 mouse embryos according to the method of De Felici and McLaren (1982). For RT-PCR analysis, purified PGCs and enriched populations of oocytes and prespermatogonia were obtained following the protocols described by De Felici and Pesce (1995) and De Felici and McLaren (1982).

The TM_4, STO and wildtype or Sl/Sl^d-1 bone marrow cell lines have been described along with their growth conditions by Mather (1980), Martin and Evans (1975) and Bos-

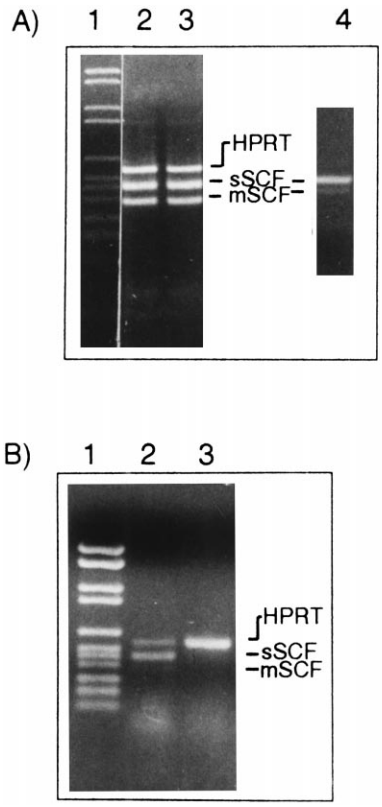

Fig. 6. RT-PCR analysis of SCF mRNA expression in (A) gonadal somatic cells of 11.5 dpc (lane 2), 12.5 dpc (lane 3) and STO fibroblasts (lane 4) and (B) wildtype (lane 2) and *Sl*/*Sl*^{d}-1 (lane 3) bone marrow cells. While cDNA-amplified products of 459 and 375 bp, respectively, for transcripts corresponding to the potentially soluble form and the transmembrane form of SCF were present in gonadal cells, STO and Wt cells, no amplified products were detectable in *Sl*/*Sl*^{d}-1 cells. The amplified product of the housekeeping gene HPRT (580 bp) is also shown. Lane 1, molecular weight markers.

well et al. (1990), respectively. Cell monolayers of gonadal cells were prepared by culturing the somatic cell fraction obtained following MiniMACS purification of 11.5 dpc PGCs (De Felici and Pesce, 1995) for 1 day. Cultures of TM_4 and gonadal somatic cells were carried out in a modified MEM supplemented with 5% horse serum and 2.5% heat-inactivated fetal calf serum (hFCS, Flow) in a 5% CO_2 incubator at 37°C (De Felici and Dolci, 1991). Sl/Sl^{d}-1 cells were cultured in DMEM supplemented with 10% hFCS on gelatin-coated dishes under the culture conditions reported above. All cultures were grown to subconfluency or confluency (see below) in the wells of a Heraeus Flexiperm-Micro 12 tissue chamber attached to a culture Falcon petri dish (3003).

4.2. Adhesion assay

Aliquots of germ cell suspensions were seeded on confluent cell monolayers and the Flexiperm tissue chamber was removed after 45–60 min of culture. The dish was then gently washed once with 5 ml of culture medium to remove unattached cells and cells were fixed with 5 ml of 4% paraformaldehyde for 5 min. The use of removable Flexiperm chambers allowed us to carry out multiple adhesion assays on the same dish using control and treated cells seeded at the same time on different cell monolayers and insured that all replicates were subjected to the same perturbation forces. The number of adhering germ cells was scored by two different investigators under an inverted microscope in at least two replicates for each experiment after identification by alkaline phosphatase staining (De Felici and Dolci, 1989). In some experiments, the number of germ cells in the cell suspensions was determined by allowing suspension samples to stick to poly-L-lysine coated coverslips (three replicates) and germ cell adhesion values were calculated as a percentage of this number. In the experiments in which the effect of antibodies or genistein on PGC adhesion was tested, PGCs or somatic cell monolayers (see Section 2) were preincubated for 40–60 min at 37°C in the presence of these compounds before the adhesion assay.

4.3. RT-PCR for SCF and c-kit mRNAs

For RT-PCR experiments, total RNA was obtained from cell monolayers after 1 day of culture and from freshly purified germ cells by the RNazol method (Cinna Biotecx) as described in Pesce et al. (1996). Total RNA (1 μg) was retrotranscribed by AMV RT (Promega) using oligo-d(T) as a primer. PCR amplification was performed using primers specific for *c-kit* and *Steel* specific for cDNA amplification as described in Rossi et al. (1993a), Rossi et al. (1993b). Primers for *Steel* allowed us to identify transcripts corresponding to the potentially soluble and the transmembrane forms of SCF (459 and 375 bp, respectively), but not the *Sl*^{d} transcript. The ubiquitous message HPRT was used to monitor RNA recovery (Rossi et al., 1993b).

4.4. Immunoblot for SCF

For immunoblot analysis, cells were lysed in a solution

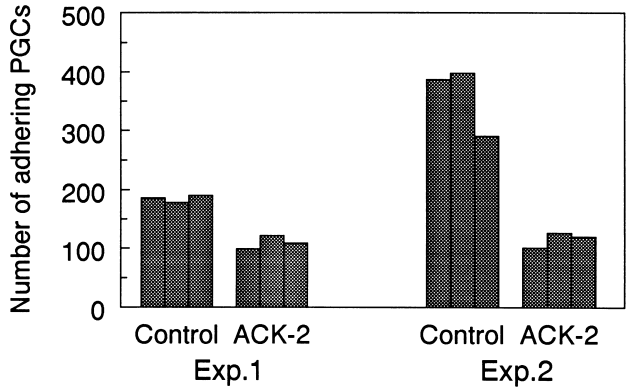

Fig. 7. Effect of anti-c-kit antibody (ACK-2) on the adhesion of 11.5 dpc PGCs to gonadal somatic cells. Because PGC adhesion on such cell monolayers was quite variable (see text), two representative experiments out of five performed are shown; each column represents the number of adhering PGCs in a single well. In all experiments ACK-2 caused a significant ($P < 0.05$) reduction of PGC adhesion.

containing 50 mM Tris, 150 mM NaCl, 20 mM EDTA, 1% deoxicholate, 0.1% SDS, 1 mM PMSF, 20 μg/ml leupeptin and 1 mM Na vanadate. Proteins (50 μg) were subjected to 4–20% gradient SDS/PAGE electrophoresis. After transfer to Hybond C nitrocellulose membranes, blots were treated according to standard procedures. Probing of the blots was performed using goat anti-mouse SCF (R&D Systems) (1:300) for 3 h at room temperature. After a second 1 h incubation period with anti-goat peroxidase-conjugated antibody (Zymed), blots were developed for the peroxidase reaction using a solution containing 500 μg/ml 3-3′-diaminobenzidine tetrahydrochloride and 0.03% H_2O_2.

4.5. Immunohistochemistry for SCF and c-kit

Immunohistochemistry for SCF was carried out on subconfluent monolayers of TM_4, Sl/Sl^d-1 and gonadal somatic cells. Cells were fixed with 4% paraformaldehyde in PBS for 15–30 min or methanol/acetone (1:1, v/v) for 2–3 min at room temperature and then thoroughly washed in PBS containing 3% BSA (PBS/BSA). Endogenous peroxidase was quenched by incubating the cells in 2% H_2O_2 in PBS for 10 min. The specimens were then exposed to goat anti-mouse SCF (R&D Systems) at a dilution of 1:100 in PBS/BSA for 3 h at room temperature or overnight at 4°C. After washing, the cells were incubated in peroxidase-conjugated rabbit anti-goat secondary antibody (Zymed) in PBS/BSA. Peroxidase was finally revealed as reported above.

Immunostaining of c-kit protein was performed on cryostat sections and on germ cells attached to poly-L-lysine coated slides. Samples were fixed with acetone for 5 min, rinsed with PBS/BSA and exposed to 1–5 μg/ml monoclonal rat anti-mouse c-kit (ACK-2, GIBCO) for 2 h. TRITC- or peroxidase-conjugated rabbit anti-rat IgG (Calbiochem) was used as secondary antibody.

For both antibodies controls consisted of samples treated in the same way except for omission of the primary antibody or its replacement with non-immune serum.

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