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The role of stem cell factor and of alternative *c-kit* gene products in the establishment, maintenance and function of germ cells

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ABSTRACT The *c-kit* gene plays a fundamental role during the establishment, the maintenance and the function of germ cells. In the embryonal gonad the c-kit tyrosine kinase receptor and its ligand Stem Cell Factor (SCF) are required for the survival and proliferation of primordial germ cells. In the postnatal animal, c-kit/SCF are required for the production of the mature gametes in response to gonadotropic hormones, i.e. for the survival and/or proliferation of the only proliferating germ cells of the testis, the spermatogonia, and for the growth and maturation of the oocytes. Finally, a truncated *c-kit* product, tr-kit, specifically expressed in post-meiotic stages of spermatogenesis and present in mature spermatozoa, causes parthenogenetic activation when microinjected into mouse eggs, suggesting that it might play a role in the final function of the gametes, fertilization.

KEY WORDS: *c-kit, stem cell factor, spermatogenesis, oogenesis, fertilization.*

The effect on gametogenesis of mutations at the *w* and *Sl* locus in mice

Mutations in the *W* (dominant White spotting) and *Sl* (Steel) loci in the mouse cause defects in pigmentation, anemia and sterility (Russell, 1979). These defects are due to the lack of stem cells belonging to three lineages: melanocytes, hematopoietic cells and germ cells. Although *W* and *Sl* mice show the same phenotype, some fundamental differences had led to hypothesize the existence of genes with different functions in the two loci (Russell, 1979). Bone marrow transplants from a wild type into a *W* recipient is able to rescue its anemic phenotype, while it is completely ineffective in a *Sl* mouse. On the other hand, hematopoietic stem cells from a *Sl* mouse are functional when transplanted in a wild type recipient, whereas hematopoietic stem cells from a *W* mouse are not (Russell, 1979). These observations suggested that the defect carried by the *W* mutations was intrinsic to the stem cells, whereas the defects carried by the *Sl* mutations derived from the environment surrounding the stem cells, which was supposed to play a fundamental function in their development and differentiation. Thus, the phenotype observed in the *W* and *Sl* mice was considered the result of the lack of proliferation and migration of stem cells originating the three lineages (Russell, 1979).

Molecular cloning of the genes responsible for the *W* and *Sl* mutations has confirmed the initial hypothesis. The proto-oncogene *c-kit* is located on mouse chromosome 5 and encodes a transmembrane tyrosine-kinase receptor that belongs to the family of the PDGF receptor (Besmer *et al.*, 1986; Yarden *et al.*, 1987; Qiu *et al.*, 1988). A series of studies led to its identification as the product of the *W* locus (Chabot, *et al.*, 1988; Geissler *et al.*, 1988). The parallelism between the *W* and *Sl* phenotype led to the hypothesis the *Sl* locus contained the gene encoding the growth factor specifically recognized by the c-kit receptor. This hypothesis was indeed confirmed with the cloning of the cDNA of a new pleiotropic growth factor in the *Sl* locus which was able to bind and activate c-kit. The multiple names given to this growth factor (MGF=Mast Cell Growth Factor; SLF=Steel Factor; KL=Kit ligand; SCF=Stem Cell Factor) reflect its many functions (Witte *et al.*, 1990; Besmer, 1991; Williams *et al.*, 1992).

The proto-oncogene *c-kit*

The proto-oncogene *c-kit* was initially identified as the cellular homologue of the viral oncogene *v-kit* (Besmer *et al.*, 1986). The product of the *c-kit* gene is a ~150 kDa tyrosine-kinase receptor that belongs to the same family as the α and β PDGF receptors and c-fms, the receptor for the Colony Stimulating Factor 1 (Ullrich and Schlessinger, 1990). The extracellular region of this family of

Abbreviations used in this paper: SCF, Stem Cell Factor; SH2, SRC Homology 2; SH3, SRC Homology 3; PGCs, Primordial Germ Cells; tr-kit, truncated c-kit; PLC, Phospholipase C; IP3, Inositol 1,4,5 Triphosphate.

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receptors is organized in 5 immunoglobulin-like domains and is devoted to ligand recognition and binding. The transmembrane region, a short hydrophobic stretch of amino acids, anchors the receptor to the plasma membrane. The intracellular region contains the kinase domain of the receptors required for transduction of the signal conveyed by the cognate ligands. The kinase domain of this class of receptors is characteristically split in two regions by an interkinase sequence which separates the ATP binding pocket from the phosphotransferase catalytic site (Yarden *et al.*, 1987; Qiu *et al.*, 1988). The juxtamembrane region, the interkinase region and the short carboxy terminal tail that follows the phosphotransferase catalytic site play an important role in the c-kit-mediated signaling. Indeed, upon ligand binding, dimerization of the receptor and activation of the intrinsic tyrosine kinase activity of the receptor occur. The consequent autophosphorylation of several tyrosine residues in the non-catalytical regions of the intracellular domain creates docking sites for specific src-homology 2 (SH2) containing proteins (Fig. 1). These proteins are either phosphorylated by c-kit or serve as adaptor molecules that bring other substrates in close proximity (Lev *et al.*, 1991; Herbst *et al.*, 1991; Rottapel *et al.*, 1991). Several signal transducers which interact directly with the autophosphorylated c-kit receptor have been identified, such as the p85 subunit of phosphatidylinositol 3-kinase (PI3K; Serve *et al.*, 1994), SH2-containing tyrosine phosphatases (Kozlowski *et al.*, 1998), src-related kinases (Price *et al.*, 1997), JAK2 (Brizzi *et al.*, 1994), STAT1 (Deberry *et al.*, 1997), and adaptor proteins such as SOCS1 (De Sepulveda *et al.*, 1999), SHC (Lennartsson, 1999), GRB2 and GRB7 (Thommes *et al.*, 1999). Less well defined is the interaction with other transducers such as the Ras-GTPase activating protein and phospholipase C γ–1 (PLCγ1; Herbst *et al.*, 1995) or the Tec kinase (Tang *et al.*, 1994). These transducers regulate different cell-specific responses such as proliferation, survival and motility, either by acting directly or after activation of downstream pathways culminating in the activation of protein kinase C (PKC; Blume-Jensen, 1994), AKT (Blume-Jensen *et al.*, 1998), and mitogen-activated protein kinases (MAPKs) such as extracellular-signal-regulated kinases 1/2 (ERK1/2; Hemesath *et al.*, 1998), or Jun-kinase (JNK; Timokhina *et al.*, 1998).

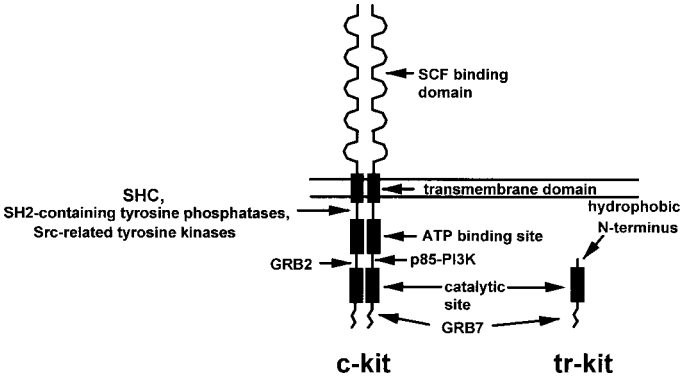

Fig. 1. Schematic representation of two distinct *c-kit* gene products *(modified from Rossi et al., 1999): the 150 kDa transmembrane tyrosine kinase SCF-responsive receptor, expressed in male and female germ cells at both mitotic and meiotic stages of differentiation (c-kit), and the truncated tyrosine kinase expressed only in post-meiotic male germ cells (tr-kit). Some of the SH2-containing signaling proteins which are known to interact with specific tyrosine residues in the cytoplasmic domain of c-kit are also shown.*

The *c-kit* gene encodes at least three different mRNAs, with three corresponding protein variants (Reith *et al.*, 1991; Rossi *et al.*, 1992). Two of these protein variants derive from a 5.5 kb mRNA and can be distinguished for the in-frame insertion of four amino acids (Gly-Asn-Asn-Lys) in the extracellular region of the receptor, due to alternative splicing. The only functional difference reported between these two isoforms is the increased basal level of autophosphorylation of the shorter form. However, it is not known whether these two isoforms show a different tissue specific expression pattern, or whether the shorter form, which displays a higher level of autophosphorylation and is constitutively more active, represents a signal for the cells expressing it (Reith *et al.*, 1991). The third protein variant is encoded by an alternative mRNA of 3.2 kb derived from alternative promoter usage and shows an extremely specific pattern of expression. Indeed, this shorter mRNA is only expressed in post-meiotic germ cells of the testis (Sorrentino *et al.*, 1991; Rossi *et al.*, 1992; Albanesi *et al.*, 1996) and it encodes a truncated form of the cytoplasmic region of the c-kit receptor (Rossi *et al.*, 1992). This truncated variant of c-kit has been named "tr-kit" (Sette *et al.*, 1997), and its possible function during male gamete development and function will be discussed later (Fig. 1).

Stem cell factor

The c-kit ligand, Stem Cell Factor (SCF), is produced in a soluble and a membrane bound form, resulting from alternative splicing around the exon 6 of the gene. The soluble form of SCF derives from proteolytic cleavage of a membrane bound precursor by a not yet identified protease (Williams *et al.*, 1992). The membrane bound form is originated by a splicing process that bypasses exon 6, which encodes for a sequence of 28 extracellular amino acids containing the proteolytic cleavage site. Both forms are expressed in a variety of tissues, and the ratio between the two can vary from tissue to tissue and in different developmental stages. Human SCF is translated with a 25 amino acids leader sequence, followed by a 185 amino acid extracellular sequence, a 27 amino acid transmembrane region and a 30 amino acid intracellular region. Murine SCF shows 83% homology with human SCF. However activation of c-kit by SCF appears to be highly species-specific (Fleischman, 1993; Williams *et al.*, 1992).

The molecular characterization of a *Sl* mutation led to the hypothesis that the soluble and membrane bound forms of SCF serve different functions. Brannan and colleagues (1991) showed that the *Steel* Dickie mutation results in a SCF protein which lacks the transmembrane and cytoplasmic regions. However, the purified recombinant SCF Dickie has the same biological activity as the wild type soluble SCF. In spite of this, homozygous *Sl*-Dickie mice lack pigmentation, are sterile and severely anemic, displaying all the hallmarks of a c-kit/SCF lack of function. This observation suggests that the transmembrane form of SCF plays a fundamental role during the establishment and the maintenance of the hematopoietic, melanocytic and germinal stem cells (Flanagan *et al.*, 1991; Brannan *et al.*, 1991). In support to this hypothesis are observations obtained with cell lines stably transfected with either the soluble or the transmembrane forms of SCF. Toksoz *et al.* (1992) have shown that transmembrane SCF is able to support

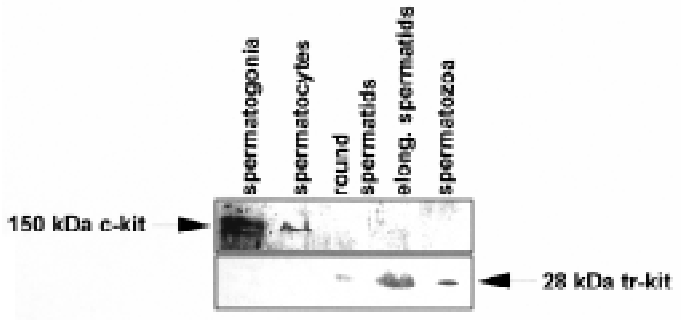

Fig. 2. *Western blot analysis of equal amounts of protein extracts from purified male germ cell populations obtained from mouse post-natal testis at different stages of differentiation, probed with a polyclonal antibody directed against the 13 carboxy-terminal amino acids encoded by the mouse c-kit open reading frame.*

long-term growth of human hematopoietic progenitor cells *in vitro*, whereas soluble SCF shows only a transient effect. Dolci *et al.* (1991) and Matsui *et al.* (1991) have also shown that transmembrane SCF is required for primordial germ cell survival *in vitro*. These observations suggest that the transmembrane form of SCF is absolutely required during embryonal development for the correct establishment of the three stem cell lineages that give rise to melanocytes, germ cells and hematopoietic cells.

The role of scf/c-kit in the establishment of the male and female germ cell line

In the mouse embryo, primordial germ cells (PGCs) originate from a limited number of cells that separate from the extra-embryonal mesoderm at the stage of 7-7.5 days post coitum (dpc). PGCs begin to migrate towards the gonadal ridges from the base of the allantoid and begin to actively proliferate in coincidence with the expression of c-kit (Manova *et al.*, 1991) to reach a number of approximately 25.000 at 13.5 dpc. (see for review: De Felici e Pesce, 1994). Once reached the gonadal ridges, PGCs continue to proliferate before entering a process of differentiation that will be completed at a later time with the formation of mature gametes. The end of their proliferative process coincides with the end of c-kit expression on these cells (Manova *et al.*, 1991). *W* and *Sl* mice have a drastically reduced number of PGCs in the gonadal ridges, which probably results from the reduced proliferation and survival of these cells as well as the failure to migrate from the base of the allantoid. Several lines of evidence from *in vitro* studies support the hypothesis that alterations of the SCF/c-kit system are responsible for these defects *in vivo*. It has been shown that PGCs are only able to survive and proliferate *in vitro* in co-culture with nurse cells that produce SCF, and that the transmembrane form of the growth factor is much more efficient than the soluble form (Godin *et al.*, 1991; Dolci *et al.*, 1991; Matsui *et al.*, 1991). More recent experiments have shown a synergy of effects between SCF and other growth factors such as the leukemia inhibitory factor (LIF), and molecules that induce a rise in intracellular cAMP levels, such as the pituitary adenylate cyclase activating polypeptides (PACAPs), to support PGCs survival *in vitro* (Dolci *et al.*, 1993; De Felici *et al.*, 1993; Pesce *et al.*, 1996). Furthermore, in the absence of nurse cells, PGCs undergo an apoptotic process *in vitro*, which can be synergistically prevented or attenuated by the presence of SCF and/or LIF in the culture medium (Pesce *et al.*, 1993). In this context, it is interesting to notice that, while c-kit is expressed by the PGCs during their migration toward the gonadal ridges, in situ hybridization and immunohistochemical experiments have shown that SCF is expressed by the cells present along the migratory path of PGCs (Matsui *et al.*, 1990). Finally, *in vitro* experiments have also shown a role for SCF/c-kit in the adhesion PGCs to layers of nurse cells (Matsui *et al.*, 1991; Pesce *et al.*, 1997).

Once they reach the gonadal ridges, PGCs begin a process of differentiation that will eventually lead to the production of mature gametes. The SCF/c-kit system plays a fundamental role also during the development of the male and female gonads, by supporting the production and survival of mature germ cells.

The role of scf/c-kit in the proliferation and/or survival of male mitotic germ cells

In the adult mouse testis, the c-kit receptor is mainly expressed by spermatogonia (Manova *et al.*, 1990; Sorrentino *et al.*, 1991) and by Leydig cells (Motro *et al.*, 1991). Type A spermatogonia are the cell type in which maximal levels of both c-kit RNA and protein are observed, followed by type B spermatogonia (Manova *et al.*, 1990; Sorrentino *et al.*, 1991; Yoshinaga *et al.*, 1991). Indeed, the full length c-kit receptor can be easily immunoprecipitated from rat spermatogonia (Dym *et al.*, 1995), it can be readily localized in mouse spermatogonia by either indirect immunofluorescence (Albanesi *et al.*, 1996) or by western blot analysis (Fig. 2), and it has been detected by immunohistochemical methods also in hamster and monkey spermatogonia (von Schonfeldt *et al.*, 1999). Moreover, the c-kit receptor mRNA and protein are expressed also in human spermatogonia (Natali *et al.*, 1992; Sandlow, 1996). The expression of the mRNA for the full length c-kit receptor has also been demonstrated in mouse spermatocytes, albeit at lower levels, but it ceases completely during meiosis and it is absent in post-meiotic cells (Sorrentino *et al.*, 1991; Rossi *et al.*, 1992). Indeed, in spermatocytes the c-kit protein either is not present (Manova *et al.*, 1990; Yoshinaga *et al.*, 1991; von Schonfeldt *et al.*, 1999) or, at least, is barely detectable (Fig. 2; Vincent *et al.*, 1998).

The expression of c-kit in the only proliferating cells during spermatogenesis, the spermatogonia, has led to the hypothesis that the SCF/c-kit interaction is required for the proliferation and/or survival of these cells. Further support to this hypothesis comes from the observation that, in mice, both the soluble and membrane-bound form of SCF are expressed by the nurse cells of spermatogonia, the Sertoli cells (Rossi *et al.*, 1991, 1993; Manova *et al.*, 1993; Tajima *et al.*, 1991a; Marziali *et al.*, 1993), whose intimate contact with the germ cells is necessary for their survival and differentiation. The selective site of testicular SCF expression in the Sertoli cell has also been confirmed in the rat (Munsie *et al.*, 1997) and in humans (Sandlow *et al.,* 1996). The expression of the mRNA for SCF is induced by the pituitary hormone FSH (follicle stimulating hormone) in pre-puberal mouse Sertoli cells cultured *in vitro*, through an increase in cAMP levels (Rossi *et al.*, 1991, 1993). Stage-dependent induction of SCF mRNA expression by FSH has also been observed in the adult rat testis (Yan *et al.*, 1999), and the maximal levels of SCF mRNA induction are observed in stages of the seminiferous epithelium which show the maximal sensitivity to FSH stimulation, and in which type A spermatogonia are actively

dividing. Interestingly, the soluble and membrane forms of SCF are differentially expressed during testis development. Sertoli cells from prepuberal mice mainly express the mRNA encoding for the transmembrane form, while the mRNA encoding for the soluble form is expressed at higher levels later, in coincidence with the beginning of the spermatogenic process, and the two transcripts are expressed at equivalent levels in the adult testis (Rossi *et al*., 1993). Moreover, FSH and/or cAMP analogs, beside increasing SCF mRNA levels, also modify the splicing pattern of the two isoforms in cultured mouse Sertoli cells in favour of the mRNA encoding for the soluble form (Rossi *et al*., 1993). In agreement with these observations is the finding that the highest levels of the transmembrane form of SCF are detected immunohistochemically in stages VII-VIII of the mouse seminiferous epithelium, and that, at these stages, a peculiar pattern of expression over the whole Sertoli cell membrane, rather than just in proximity of the basal layer of germ cells, is observed (Vincent *et al.*, 1998). The equivalent stages in rat testis are the less sensitive to FSH stimulation in the adult testis (Parvinen, 1982). This pattern of expression could be physiologically relevant, since it has been reported that the transmembrane form of SCF, but not the soluble form, is required for mouse germ cells adhesion to Sertoli cells in culture (Marziali *et al.*, 1993): it is conceivable that the transmembrane form of SCF might be involved in the progression through the blood-testis barrier of mitotic germ cells entering the first meiotic prophase at stages VII-VIII. Recently, it has been reported that the splicing pattern of SCF mRNA in Sertoli cells might also be influenced by local changes of pH in the seminiferous epithelium (Mauduit *et al*., 1999). FSH and/or cAMP regulation of SCF expression in rat Sertoli cells is due, at least in part, to transcriptional activation (Yan *et al*., 1999), and cAMP-responsive regions of the SCF promoter have been identified in the 5' flanking regions of the human (Taylor *et al*., 1996), rat (Jiang *et al*., 1997) and mouse (P. Grimaldi *et al*., manuscript in preparation) SCF genes.

Early studies performed in *W* mutant mice suggested that c-kit plays a critical role in the differentiation from type A to type B spermatogonia (Koshimizu *et al*., 1991). Moreover, a peculiar *Steel* mutation, Sl^{17H}, resulting in a splicing defect in the SCF cytoplasmic tail, in the homozygous condition induces sterility in males but not females, due to loss of spermatogonia during postnatal development (Brannan *et al*., 1992).

The first evidence that c-kit plays an important role in mitotically dividing male germ cells came from the observation that addition of the soluble form of SCF to *in vitro* cultured male germ cells from 7-8 day old mice at mitotic stages of differentiation stimulates DNA synthesis selectively in type A, but not in type B spermatogonia (Rossi *et al*., 1993). SCF-induced thymidine incorporation in cultured spermatogonia reflects DNA replication, rather than DNA repair, since it is completely abolished by aphidicolin, which specifically inhibits DNA polymerase α and arrests cells in early S-phase (Geremia *et al*., 1994). Selective induction of DNA synthesis in type A spermatogonia by soluble SCF has been confirmed using an *in vitro* tissue culture system for stage-defined seminiferous tubules of the adult rat testis (Hakovirta *et al*., 1999). The important role of the SCF/c-kit system in the maintenance of the germ cell line in the mouse post-natal testis is also suggested by *in vivo* and *in vitro* experiments in which the interaction between SCF and c-kit was blocked by an antibody directed against the extracellular region of the receptor. Under these conditions, c-kit expressing type A spermatogonia, but not c-kit negative spermatogonial stem cells, are depleted (Yoshinaga *et al*., 1991) and unable to proliferate (Tajima *et al*., 1994), and differentiating spermatogonia show increased levels of apoptosis (Packer *et al*., 1995). The role of soluble SCF in promoting survival and/or proliferation of rat and porcine spermatogonia has also been reported (Allard *et al*., 1996; Dirami *et al*., 1999).

SCF can trigger both mitogenic and anti-apoptotic responses

It has been argued that stimulation of DNA synthesis *per se* does not necessarily mean that SCF acts as a bona fide mitogenic factor, i.e. that SCF stimulates entry into the mitotic cell cycle of dividing type A spermatogonia. According to this view, the stimulation of spermatogonial DNA synthesis by SCF could simply reflect SCF action as a survival factor that prevents programmed cell death in cells which are intrinsically committed to proliferate (Packer *et al*., 1995; Hakovirta *et al*., 1999). However, appearance of actively proliferating type A_1-A_4 spermatogonia coincides with re-expression of the c-kit receptor, which is not expressed in post-natal spermatogonial stem cells, i.e. type A_0 spermatogonia (Manova *et al*., 1990; Tajima *et al*., 1994; Dym, 1994). Moreover, we have recently found that SCF does prevent apoptosis induced by growth factor deprival in cultured mouse spermatogonia, but this effect is more evident in type B spermatogonia (S. Dolci *et al*., manuscript in preparation), rather than in type A_{1-4} spermatogonia, the cell type in which DNA synthesis is selectively stimulated (Rossi *et al*., 1993; Yan *et al*., 1999). This might imply that the soluble form of SCF sustains the proliferation of differentiating type A spermatogonia, and, at the same time, it does not stimulate proliferation of type B spermatogonia, but rather it prevents their cell death before they enter the meiotic stages of differentiation, when c-kit expression is down-regulated. The basis for the different cellular response in type B with respect to type A spermatogonia, might depend on their lower levels of c-kit expression (Sorrentino *et al*., 1991). Indeed, in cultured c-kit expressing mast cells, higher doses of soluble SCF are required to trigger G1/S transition and promoting cell cycle progression than those required for suppressing apoptosis (Yee *et al*., 1994). Moreover, it is known that transient versus sustained stimulation of intracellu-

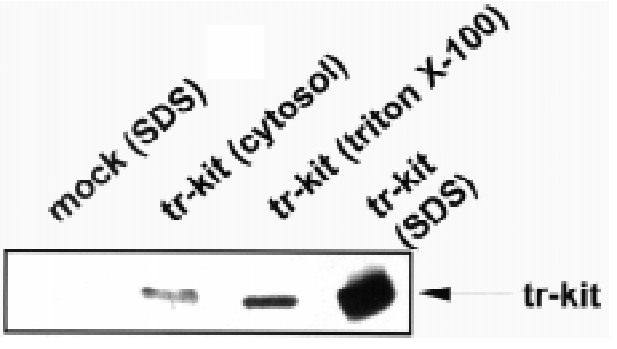

Fig. 3. *Western blot analysis of equal amounts of protein from soluble (cytosol), detergent solubilized (Triton X-100) and detergent-insoluble (SDS) extracts from control COS cells (mock) or from COS cells producing recombinant tr-kit after transfection with a eukaryotic expression plasmid (tr-kit).*

lar transducers by activated tyrosine kinase receptors causes differential responses (proliferation versus growth arrest, or induction of apoptosis versus promotion of survival and differentiation) and that the specific cellular response depends on the cell cycle situation of the responding cell, i.e. whether it is in a quiescent state or actively proliferating (Marshall, 1995; Blagosklonny, 1999). It is possible that *in vivo* these role are played differentially by the soluble and the transmembrane form of SCF, which could release signals of different strength to mitotic germ cells (transient versus sustained stimulation), as they do in hematopoietic precursors (Toksoz *et al*., 1992). In support to this hypothesis are studies performed in cryptorchid testes of mice heterozygous for the *Steel* Dickie mutation, which only express a soluble, but not a transmembrane form of SCF: proliferation of type A_{1-4} spermatogonia was apparently unaffected in surgically reversed cryptorchid testes of these animals, suggesting that soluble SCF is sufficient for their divisions, but a dramatic reduction in type B spermatogonia was observed, with consequent impairment of spermatogenesis (Tajima *et al*., 1991b).

The fact that SCF can be both a mitogenic and an anti-apoptotic stimulus in the same cell type has been clearly demonstrated in c-kit expressing cells of the hematopoietic lineage. In mast cells, activation of the PI3K/Rac/JNK pathway is required for SCF-induced proliferative response, whereas inhibition of JNK accompanies SCF-mediated protection from radiation-induced apoptosis (Timokhina *et al*., 1998). A key role is probably played by PI3K activation in both situations, since PI3K also activates AKT, which is required to block the apoptotic pathway (Franke *et al*., 1997). Indeed c-kit-mediated inhibition of apoptosis in a hematopoietic cell line is coupled to PI3K-dependent activation of AKT (Blume-Jensen *et al*., 1998), and selective PI3K inhibitors block the anti-apoptotic effects of SCF in germ cells during fetal oogenesis (Morita *et al*., 1999). Moreover, SCF stimulation of mast cells is accompanied by a proliferative response, but also by simultaneous induction of SOCS1 expression; SOCS1, in turn, binds to the autophosphorylated c-kit receptor, to the GRB2 adaptor protein, and to the VAV GEF protein, with consequent block of both the Ras/Raf/MEK/ERK and Rac/JNK pathways, which are required for the mitogenic response (De Sepulveda *et al*., 1999). It has been proposed that c-kit-mediated induction of SOCS1 in mast cells suppresses the mitogenic potential of c-kit while maintaining SCF-dependent anti-apoptotic signals, and that SOCS1 is an inducible switch which modulates the SCF-mediated proliferative response in favour of a cell survival response (De Sepulveda *et al*., 1999).

More detailed studies on signal transduction pathways stimulated by c-kit activation in proliferating spermatogonial cells and their following effects on progression of the cell cycle and prevention of apoptosis are needed to establish whether SCF acts on these cells as a mitogenic or survival factor, or both, and whether it might also influence their differentiation into meiotic spermatocytes.

Possible roles of scf/c-kit in female and male meiotic germ cells

Expression of the c-kit receptor ceases in fetal oocytes around the time they enter the prophase of the first meiotic division, but it resumes in post-natal oocytes, and is present at all the stages of their development (Manova *et al*., 1990; Horie *et al.,* 1991; Yoshinaga *et al*., 1991). Similarly to what has been described for Sertoli cells in the testis, granulosa cells surrounding the oocytes produce SCF (Manova *et al*., 1993). SCF expression in the female gonad is also under the control of gonadotropins (Motro and Bernstein, 1993; Ismail *et al*., 1996) and it has been proposed as a growth factor required for growth and maturation of the oocytes (Manova *et al*., 1993; Packer *et al*., 1994). The importance of SCF in post-natal oogenesis is also suggested by studies in *Steel-panda* and *Steel-contrasted* mutant mice, carrying DNA rearrangements located > 100 kb 5' of SCF-coding sequences, which lead to tissue-specific effects on SCF mRNA expression (Bedell *et al*., 1995). In both these mutants, SCF expression and spermatogenesis in the post-natal mutant testis is normal, but decreased SCF mRNA expression causes sterility in females only, by affecting the initiation and maintenance of ovarian follicle development. SCF has also been recently proposed to play a role in the maintenance of meiotic arrest in the oocyte. *In vitro* experiments indicate that soluble SCF delays spontaneous meiotic resumption in mouse oocytes, observed as germinal vesicle breakdown (GVBD), and this effect is blocked by anti-c-kit antibodies (Ismail *et al*., 1996). Moreover, the same authors have shown that microinjection of anti-sense c-kit RNA lowers the expression levels of c-kit in mouse oocytes and increases their ability to reenter meiotic progression (Ismail *et al*., 1997). The c-kit receptor is also expressed in ovulated oocytes (Manova *et al*., 1990; Horie *et al.,* 1991; Yoshinaga *et al*., 1991), that are arrested at metaphase of the second meiotic division under the action of proto-oncogene *c-mos* (Colledge *et al*., 1994; Hashimoto *et al*., 1994), but the function of c-kit and/or SCF at this stage is unknown.

The possibility that SCF plays a role during male meiosis as well was originally raised by the observation that intraperitoneal injection of antibodies directed against the extracellular portion of the c-kit receptor, together with inducing apoptosis of spermatogonial cells, also induced a striking increase in apoptosis of dividing spermatocytes at stage XII of the mouse seminiferous epithelium (Packer *et al*., 1995). Since the injected antibody cannot pass through the blood-testis barrier, clearly this observation cannot be ascribed to a direct effect of the antibody on spermatocytes, but it probably reflects an alteration of complex local paracrine networks which regulate meiotic progression of germ cells, as a consequence of death of spermatogonia in the basal layer. Similarly, alteration of local paracrine mechanisms could explain the dramatic impairment observed in meiotic divisions of late pachytene spermatocytes in mice heterozygous for the *Steel* Dickie mutation, since these mice also show a drastic reduction in the number of type B spermatogonia (Tajima *et al*., 1991b). However, it cannot be excluded that the c-kit receptor is re-expressed in late pachytene spermatocytes just before the meiotic divisions, similarly to what observed in post-natal maturing oocytes. Recently, it has been reported that the transmembrane form of SCF might play a role in transmeiotic progression in culture of mouse pachytene spermatocytes cocultured with the SCF-expressing 15-P1 cell line (Vincent *et al*., 1998). This has been argued on the basis of the observation that transmeiotic progression in this *in vitro* system was inhibited by the soluble form of SCF or by antibodies directed against the extracellular portion of the c-kit receptor. However, the c-kit protein has never been identified immunohistochemically in mouse spermatocytes using several anti-c-kit antibodies (Manova *et al*., 1990; Yoshinaga *et al*., 1991; Albanesi *et al*., 1996; Vincent

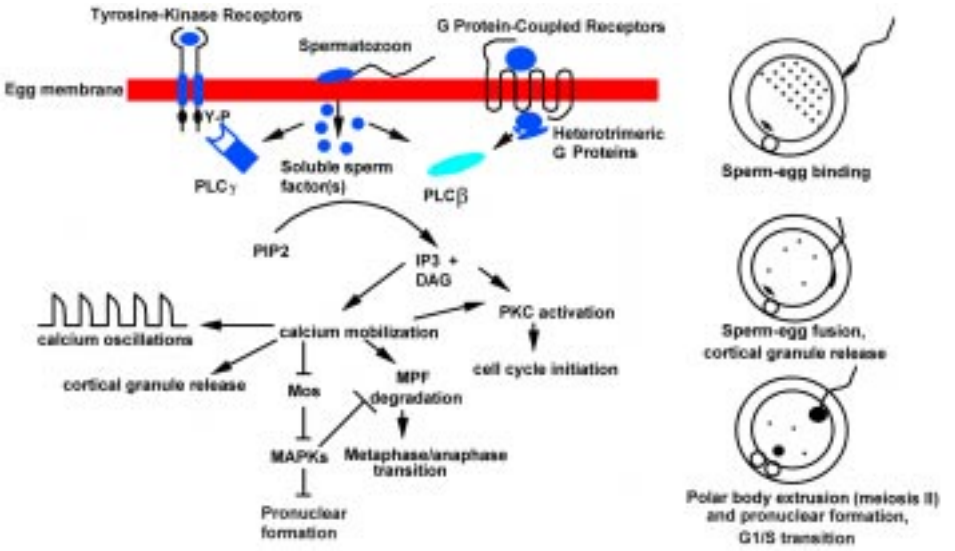

Fig. 4. Schematic representation of possible mechanisms through which spermatozoa can trigger egg activation at fertilization. *After egg-sperm binding, a tyrosine-kinase receptor or a G protein-coupled receptor on the egg plasma membrane is stimulated and activates either the γ or the β isoform of PLC, respectively. Alternatively, after sperm-egg fusion, a sperm factor (such as tr-kit) is released into the egg cytoplasm and triggers PLC activation. The consequent activation of PIP_2 hydrolysis generates the second messengers IP_3 and DAG which are responsible for the following events of egg activation: the calcium mobilization from IP_3-sensitive intracellular stores is followed by a peculiar pattern of calcium oscillations. The calcium mobilization is responsible for cortical granule release (which is, in turn, responsible for the block of polyspermy) and for the inactivation of the cytostatic Mos-MAPK-MPF circuitry, with consequent release of the oocytes from metaphase-II arrest, exit from the second meiotic division and extrusion of the second polar body. MAPK inactivation also allows chromosome decondensation and the reconstitution of the nuclear envelope, with the consequent formation of the female pronucleus. Calcium and DAG-dependent PKC activation might be responsible for progression into the first mitotic cell cycle.*

et al., 1998; von Schonfeldt *et al.*, 1999), even though trace amounts might be present on their membrane, as indicated by western blot analysis with an antibody recognizing the intracellular domain (Fig. 2) and by the observation that they can be enriched by FACS separation using antibodies directed against the extracellular domain (Vincent *et al.*, 1998). The transmeiotic progression observed *in vitro* might reflect one of the two above mentioned possibilities: either the c-kit receptor is re-expressed in late pachytene spermatocytes just before the meiotic divisions, or a more complex interaction occurs, involving distinct c-kit positive cells (such as c-kit positive spermatogonia) present in the starting germ cell population used in these studies. Both possibilities agree with the observation that artificial activation of ERK1 in *in vitro* cultured pachytene spermatocytes leads to G2/M transition of the first meiotic division, suggesting that an unidentified extracellular signal from the seminiferous tubule environment actually regulates male meiotic progression (Sette *et al.*, 1999).

An alternative form of c-kit (tr-kit) is specifically expressed during spermiogenesis

The 5.5 kb c-kit mRNA is not expressed during the haploid phase of spermatogenesis (Sorrentino *et al.*, 1991; Rossi *et al.*, 1992). Nevertheless, the full length c-kit receptor has been claimed to be present in isolated mouse, rat and human spermatozoa, based on immunofluorescence and/or western blot analysis using an anti-c-kit antibody directed against the extracellular region of the receptor (Sandlow *et al.*, 1996; Feng *et al.*, 1997). The same authors also reported the localization of c-kit in the acrosomal region of spermatids and spermatozoa, and its possible involvement in the acrosome reaction of the sperm *in vitro* (Feng *et al.*, 1998). These data are in conflict with previous observations that the c-kit receptor protein is not detectable in haploid germ cells by immunocytochemistry on testis sections using the same anti-c-kit antibody (Yoshinaga *et al.*, 1991). Using a different antiserum, directed against the intracellular portion of the c-kit receptor, immunoreactivity was shown in both spermatogonia and spermatids in permeabilized sections of mouse, hamster and monkey testis (von Schonfeldt *et al.*, 1999). Notwithstanding that this antiserum is directed against the cytoplasmic domain of the c-kit receptor, spermatogonia were purified by magnetic cell sorting using such antibodies, probably because partial damage of the spermatogonial membrane allows their specific adsorption. However, spermatids were not enriched by this fractionation method, suggesting that, whereas the c-kit receptor is present in the spermatogonial membrane, a cytoplasmic c-kit immunoreactive protein, rather than a SCF-responsive membrane receptor, must be present in spermatids of all those species (von Schonfeldt *et al.*, 1999).

Indeed, the expression of the mRNA for the c-kit receptor is drastically reduced in premeiotic cells (spermatocytes) and is virtually absent in the postmeiotic spermatids (Sorrentino *et al.*, 1991). However, Northern blot analysis of germ cells at different developmental stages has shown the presence of two alternative mRNA, of 3.2 and 2.3 kb, encoded by the *c-kit* gene in the haploid cells of the mouse testis (Sorrentino *et al.*, 1991). Similar alternative transcripts have also been detected in the adult rat testis (Orth *et al.*, 1996). The two alternative spermatid-specific c-kit transcripts originate in 16th intron of the mouse *c-kit* gene, and contain all the exons downstream of it (Rossi *et al.*, 1992). These alternative c-kit mRNA encode for a truncated form of the receptor, called tr-kit (Sette *et al.*, 1997), with an ORF that starts in the intron 16 and encodes for 12 hydrophobic amino acids followed by the last 190 carboxy terminal residues of the c-kit receptor (Fig. 1). Thus, tr-kit lacks the extracellular portion of the receptor, the transmembrane region, the ATP binding site and the interkinase sequence of the full-length c-kit. Tr-kit contains the phosphotransferase domain and the cytoplasmic tail of c-kit, where several molecular interactions with signaling proteins take place (Herbst *et al.*, 1995; Sette *et al.*, 1997; Thommes *et al.*, 1999). *In vitro* transcription experiments with nuclear extracts from round spermatids have suggested the presence of an alternative promoter in the 16th intron of the *c-kit* gene (Albanesi *et al.*, 1996). Indeed, transgenic mice in

which the expression of the reporter gene LacZ is under the control of this intronic promoter have demonstrated the specific expression of β-galactosidase in the testis (Albanesi *et al.*, 1996). A more detailed analysis of the testes from these transgenic mice has revealed specific expression of the reporter gene uniquely in haploid germ cells. Indeed, β-galactosidase was expressed in a stage-specific manner, starting from step 8 of spermiogenesis, i.e. the haploid phase of spermatogenesis (Albanesi *et al.*, 1996). Thus, it appears that this intronic promoter of the *c-kit* gene is only active in the late stages of spermatogenesis, suggesting a role for this truncated c-kit protein variant either during spermatid differentiation or for the function of mature sperm.

tr-kit is expressed in spermatids and spermatozoa and induces the activation of metaphase II arrested oocytes

A truncated c-kit protein of ~30 kDa has actually been detected in mouse testis cells using an antibody raised against the last 13 amino acids at the carboxy terminus of the c-kit receptor (Albanesi *et al.*, 1996). As shown in Fig. 2, this protein, named tr-kit, is first expressed in round spermatids, it accumulates in elongated spermatids and it is present in testicular and epididymal spermatozoa (Albanesi *et al.*, 1996; Sette *et al.*, 1997), whereas no trace of the ~150 kDa full-length c-kit receptor is detectable in haploid germ cells. A cytoplasmic c-kit immunoreactive protein has also been detected immunohistochemically in permeabilized hamster and monkey spermatids (von Schonfeldt *et al.*, 1999). We also found that a ~30 kDa c-kit immunoreactive protein is detectable by western blot in rat and human spermatozoa (unpublished observations). In the mature mouse sperm, tr-kit accumulates in the post-acrosomal region and in the midpiece (Sette *et al.*, 1997), which are proximal to the site of the sperm that fuses with the egg membrane at fertilization (Yanagimachi, 1994). Indirect immunofluorescence analysis on permeabilized spermatozoa localizes tr-kit in the residual sperm cytoplasm (Sette *et al.*, 1997). However, western blot analysis indicates that the majority of this protein is not present in the soluble fraction of spermatozoa, but it is found mostly in the Triton-X100 insoluble material, and the same subcellular distribution of tr-kit is observed in COS cells transfected with a tr-kit expression vector (Fig. 3). It is possible that the N-terminal hydrophobic residues present in tr-kit and encoded by *c-kit* intronic sequences (Fig. 1) are involved in its stable interaction with the particulate compartment. Interestingly, microinjection of a recombinant tr-kit expressed in the cytosolic fraction of tr-kit transfected COS cells is able to activate mouse oocyte arrested at metaphase II after ovulation (Sette *et al.*, 1997, 1998). The amount of recombinant tr-kit required for such activation is lower than the amount of tr-kit present in a single sperm (Sette *et al.*, 1998). Furthermore, oocyte activation follows the same pathway and timing described for the physiological activation at fertilization, with Ca^{2+}-mediated cortical granule exocytosis, completion of meiosis and extrusion of the second polar body, and decrease in MAPK activity, with the consequent formation of a parthenogenetic pronucleus (Sette *et al.*, 1997, 1998). These results have led to the hypothesis that tr-kit is a sperm factor released from the sperm into the oocyte at fertilization to trigger egg activation. Indeed, the molecular basis of egg activation at fertilization in the mouse are still unknown. Ovulated mouse oocytes are arrested at metaphase of the second meiotic division, with the chromosomes aligned at the equator of the meiotic spindle located near one pole of the cell. Sperm-egg fusion triggers a series of intracellular Ca^{2+} oscillations which are thought to cause the completion of meiotic division and the extrusion of the second polar body (Kline and Kline, 1992; Whitaker and Swann, 1993). Although the mechanism has not been elucidated, three hypotheses have been proposed to explain sperm-induced egg activation (Fig. 4):

1) a sperm-membrane ligand activates a tyrosine kinase receptor on the egg membrane;
2) a sperm-membrane ligand activates a G-protein-coupled receptor on the egg membrane;
3) the sperm releases cytosolic or particulate factors in the egg cytoplasm that trigger egg activation.

The first two hypotheses rely on experiments showing that microinjection of mRNA for receptors of either type allows activation of oocytes by the corresponding ligand (Yim *et al.*, 1994; Shilling *et al.*, 1994). However, the presence and functionality of such receptors in mouse oocytes has not been demonstrated. The third hypothesis is supported by experiments in which sperm cytosolic extracts from several species have been injected into ovulated oocytes and shown to induce Ca^{2+} oscillations and all the events characteristic of fertilization. (Swann, 1990; Homa e Swann, 1994; Dozortev *et al.*, 1995; Sette *et al.*, 1997; Wu *et al.*, 1997; Stricker, 1997). It is also known that sperm-egg fusion precedes the onset of Ca^{2+} oscillations in mouse eggs, supporting the hypothesis that a cytosolic sperm factor is released in the egg cytoplasm and triggers egg activation (Lawrence *et al.*, 1997). Mobilization of intracellular Ca^{2+} is probably achieved by activation of one or more PLC isoenzymes and the consequent rise in IP_3 intracellular levels (Miyazaki *et al.*, 1992, 1993; Dupont *et al.*, 1996). Interestingly, tr-kit-induced mouse oocyte activation is suppressed by co-injection of the SH3 domain of PLCγ1 and tr-kit is able to induce the tyrosine phosphorylation and activation of PLCγ1 when co-expressed in a heterologous system (Sette *et al.*, 1998). We could not demonstrate a direct physical interaction between tr-kit and PLCγ1 (Sette *et al.*, 1998). Moreover, the finding that the SH3, rather than the SH2 domain of PLCγ1 is required for tr-kit induced egg activation, led us to hypothesize that intermediate adaptor proteins are required to elicit tr-kit mediated stimulation of the enzyme. Since tr-kit lacks the ATP-binding site (Rossi *et al.*, 1992), it should lack intrinsic tyrosine kinase activity, and thus it should be unable to autophosphorylate or to phosphorylate tyrosine residues on downstream target proteins. The observation that microinjection of tr-kit mRNA is equally efficient in eliciting egg activation as the recombinant protein expressed in COS cells (Sette *et al.*, 1997), suggests that, if posttranslational modifications, such as phosphorylation, are required for tr-kit function, they can be achieved in the egg cytoplasm. Thus, it is possible that a distinct tyrosine kinase present in the egg cytoplasm phosphorylates tr-kit, thus allowing PLCγ1 stimulation and consequent egg activation.

Studies are underway to dissect the molecular mechanisms by which tr-kit elicits activation of PLCγ1 and to establish whether this truncated product of the *c-kit* gene is one of the sperm factors that plays a physiological role at fertilization in the mouse and in other mammalian species.

The crucial experiment to show whether tr-kit is important in sperm function during fertilization or whether it plays additional roles during spermiogenesis will come from studies conducted with

genetically modified mice. We are performing the genetic ablation through homologous recombination (Capecchi, 1989) of the intronic promoter sequences which drive tr-kit specific expression during spermiogenesis (Albanesi *et al.*, 1996). This was an obligate choice, since we know that normal protocols of replacement of exonic sequences shared with the full-length *c-kit* gene product would have just generated *W* mutant mice with no germ cells in their embryonal gonads (Russell, 1979).

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