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Original Article

KL/KIT co-expression in mouse fetal oocytes

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ABSTRACT The tyrosine kinase receptor, KIT, and its ligand, KL are important regulators of germ cell development. The aim of this study was to examine in detail the expression of the genes encoding these proteins (*White* and *Steel*, respectively) during the fetal period (14.5-18.5 days *post coitum*, dpc) and the two weeks after birth in mouse ovaries using the highly sensitive *in situ* reverse-transcriptase polymerase chain reaction (*in situ* RT-PCR). *KL* and *KIT* mRNAs were not detected in 14.5-15.5 dpc ovaries but, between 16.5 and 17.5 dpc, most of the oocytes in the outer regions of the ovaries positively stained for both mRNAs. The majority of the co-expressing oocytes were identified at the zygotene/pachytene stage of meiotic prophase I. At 18.5 dpc, positive staining for *KL* mRNA was present only in the somatic cells in the outer regions of the ovaries. At birth, faint *KL* mRNA-labelled somatic cells were mainly found in the central region of the ovaries and, by P7-14, a higher level of expression was detected in the follicle cells of one- and two-layered growing follicles. Between 17.5 dpc and birth, most of the oocytes expressed *KIT* mRNA and, from P7 onward, there was a considerable accumulation of transcripts in the growing oocytes. The results of *in situ* RT-PCR were confirmed by RT-PCR on purified populations of oocytes, and at protein level by means of immunohistochemistry. The co-expression of *KL* and *KIT* in a fraction of fetal oocytes suggests that the KL/KIT system, besides the well known paracrine functions on germ cells, may exert a novel autocrine role during the mid-stage of the oocyte meiotic prophase. The possibility that this autocrine loop plays a role in sustaining the survival of fetal oocytes in this stage is supported by the finding that the addition to the culture medium of anti-KL or anti- KIT antibodies led to a significant increase in oocyte apoptosis in the absence of exogenous KL.

KEY WORDS: *Oocytes, KL, KIT, apoptosis, meiosis, in situ RT-PCR*

Introduction

The KIT tyrosine kinase receptor and its cognate ligand, KL which is also known as stem cell factor, steel factor or mast cell growth factor, are respectively encoded at the *White spotting* (*Kit* or *W*) and *Steel* (*Kitl* or *Sl*) loci (for a review, see Besmer, 1993). Mutations at both the *Kit* and *Kitl* loci cause deficiencies in germ cell development, as well as in the development of melanocytes, interstitial cells of Cajal, and cells at various stages of hematopoiesis (for a review, see Wagner *et al.*, 1991).

The expression of the gene encoding *KIT* or KL proteins has been previously studied during ovarian development in mice using a variety of methods, including *in situ* RNA hybridisation, immunohistochemistry, RNase protection and RT-PCR assays. mRNA encoding *Kit* and protein has been found in migrating and mitotic primordial germ cells, but not in oocytes undergoing the first stages of meiosis during fetal life (Manova and Bachvarova, 1991; De Felici *et al.*, 1996: Pesce *et al.*, 1997). Mouse oocytes in primordial follicles and throughout folliculogenesis have been found to contain mRNA encoding *Kit* and protein (Manova *et al.*, 1990; Horie *et al.*, 1991; Manova and Bachvarova, 1991). mRNA encoding *Kitl* and protein are expressed along the migration path of primordial germ cells and in the gonadal ridges (Matsui *et al.*, 1990; Keshet *et al.*, 1991; Motro *et al.*, 1991; De Felici *et al.*, 1996: Pesce *et al.*, 1997). In post-natal ovaries, *Kitl* is expressed in granulosa cells at all stages of follicle development, although its expression is low in primordial follicles and cumulus cells (Manova *et.*, 1993; Motro and Bernstein, 1993).

In normal mice, KL is produced from two alternatively spliced mRNAs as trans-membrane precursors, which may be proteolytically processed to produce soluble forms of KL (KL-1) or primarily found in the membrane-bound form (Kl-2) (Flanagan *et al.,* 1991; Huang *et al.,* 1992). Although little is known about the regulation of

Abbreviations used in this paper: dpc, day post coitum; RT-PCR, reverse transcription polymerase chain reaction.

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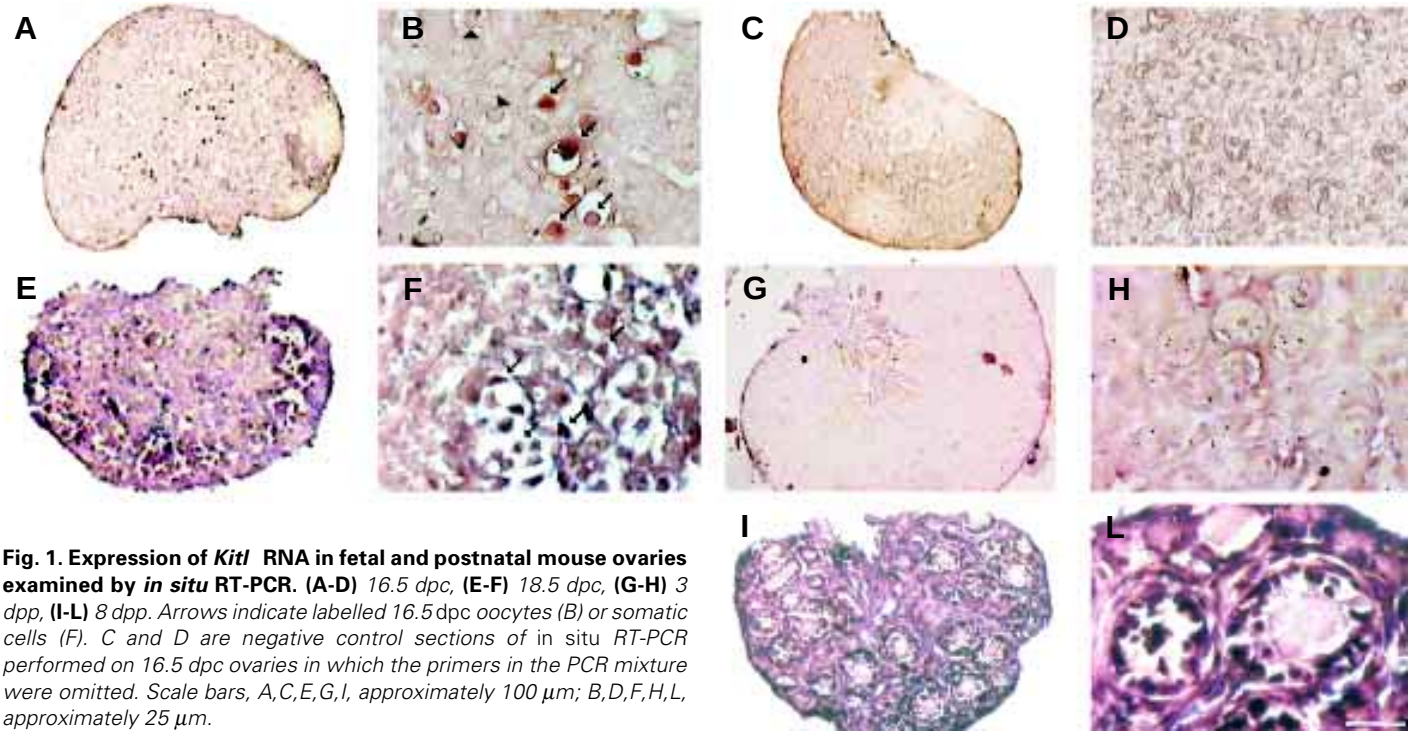

Fig. 1. Expression of *Kitl* RNA in fetal and postnatal mouse ovaries examined by *in situ* RT-PCR. (A-D) *16.5 dpc,* **(E-F)** *18.5 dpc,* **(G-H)** *3 dpp,* **(I-L)** *8 dpp. Arrows indicate labelled 16.5* dpc *oocytes (B) or somatic cells (F). C and D are negative control sections of* in situ *RT-PCR performed on 16.5 dpc ovaries in which the primers in the PCR mixture were omitted. Scale bars, A,C,E,G,I, approximately 100 μm; B,D,F,H,L, approximately 25 μm.*

these two alternative transcripts, the ratio of KL-1 and KL-2 mRNA differs between the ovaries of mice of different ages (Manova *et al.*, 1993).

Studies of mice with mutations at the *Kit* and *Kitl* loci, as well as *in vitro* results, have indicated that the KL/KIT system plays a pleiotropic role in the development of primordial germ cells (the precursors of adult gametes) by promoting their survival/proliferation and migration (for a review, see De Felici, 2000). Other important functions of the KIT receptor during mouse oogenesis are to favour the survival of oocytes as they reach the last stages of the meiotic prophase (Pesce *et al.*, 1997) and to promote oocyte growth (Packer *et al.*, 1994; Klinger and De Felici, 2002). Taken together, these data support the idea that the somatic components of the ovary produce KL during pre-natal and post-natal oogenesis, and that this growth factor has paracrine action on germ cells expressing the KIT receptor.

In different cell types, binding of KL causes KIT receptor dimerization and receptor autophosphorylation. The activated receptor in turn phosphorylates different substrates and thus activates distinct signalling pathways. These include the phosphatidylinositol 3-kinase (PI3-K)/Akt/mTOR/p70^{S6K}, Ras/mitogen activated protein linase (MAPK), the Janus kinase (JAK)/signal tranducer and activation of transcription (STAT), and the Src signalling pathways (for review, see Rameh *et al.,* 1999; Ueda *et al.,* 2002). In mouse primordial germ cells, De Miguel *et al.*, recently shown that KIT receptor phosphorylation leads to a PI3-K-independent Akt activation and a parallel MAPK stimulation (De Miguel *et al.*, 2002). However, none of these signals have been identified in mouse oocytes.

The apparent absence of functional KIT receptor during oogenesis in oocytes entering the meiotic prophase, and the lack of precise information concerning the expression of *Kitl* in fetal ovaries, prompted us to analyse in detail the expression of these genes in developing mouse ovaries between 14.5 dpc and two weeks postbirth using the highly sensitive technique of *in situ* RT-PCR.

It is known that a dramatic wave of oocyte apoptosis occurs during this period of mouse oogenesis (Borum, 1961; Bakken and McClanahan, 1978; Pepling and Spradling, 2001). Following these episodes, the number of germ cells decreases from approximately 20,000 at 13.5 dpc to about 6,000-10,000 at birth (Tam and Snow, 1981; Burgoyne and Baker, 1981; Pepling and Spradling, 2001). A number of lines of evidence indicate that apoptosis is responsible for the loss of germ cells in the developing ovary (Pesce *et al.*, 1993; Coucouvanis *et al.*, 1993; Pesce and De Felici, 1994; Pepling and

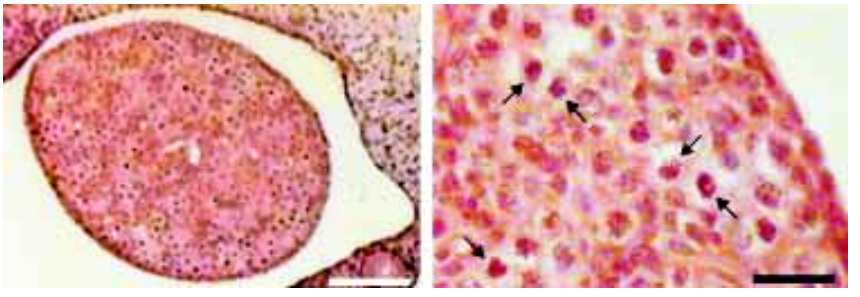

Fig. 2. Histological appearance of hematoxylin-eosin stained sections of 16-17 dpc mouse ovary. *Arrows indicate zygotene/pachytene oocytes. Scale bars: left panel, approximately 80 μm; right panel, approximately 25 μm.*

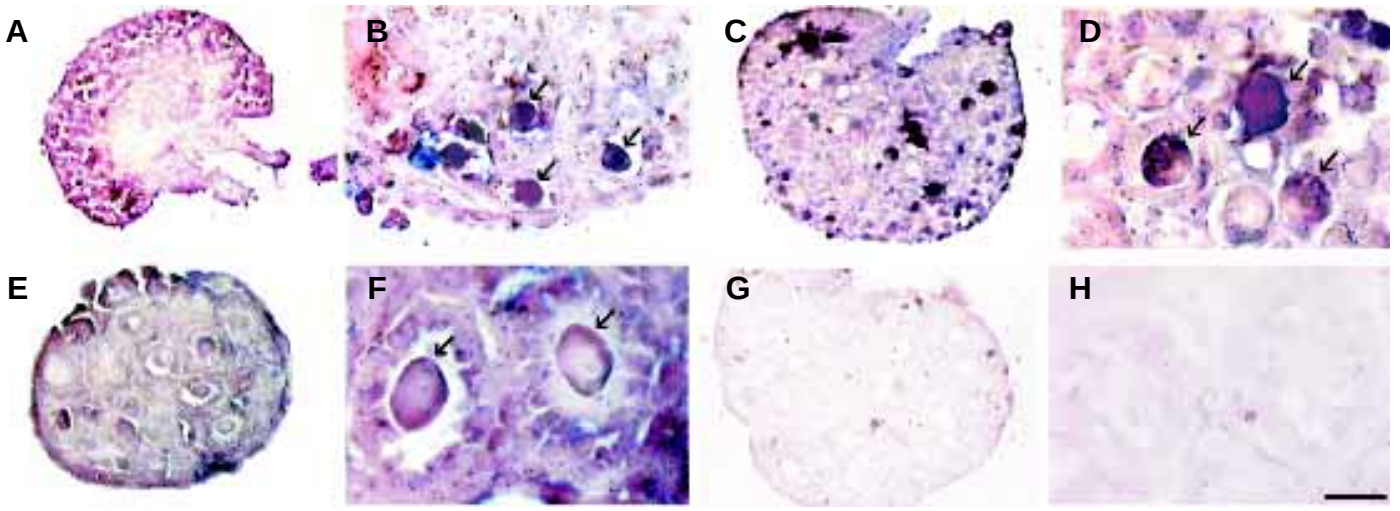

Fig. 3. Expression of *Kit* RNA in fetal and postnatal mouse ovaries examined by *in situ* RT-PCR. (A,B) *16-17* dpc, **(C,D)** *3* dpp, **(E,F)** *8* dpp, **(G,H)** *control sections from an 8 dpp ovary (see Materials and Methods). Arrows indicate labelled oocytes. Scale bars: A,C,E,G, approximately 100 μm, B,D,F,H, approximately 20 μm.*

Spradling, 2001). Since the KL/KIT system is necessary to prevent apoptosis in a number of different cell types, including primordial germ cells and male germ cells (for a review, see Datta *et al.,* 1999), one aim of our work was to verify whether oocyte apoptosis in the mid- and late stages of the meiotic prophase might be due to the absence of KIT receptor and/or the limited availability of KL. Similarly, oocytes in the primordial follicle undergo a higher rate of apoptosis than growing oocytes (Faddy *et al.,* 1983). In this stage, both apoptosis and the lack of growth might be due to limited KL availability. To examine this latter possibility, we extended our analysis to ovaries of the first two weeks after birth when the pool of primordial follicles is established and the first wave of oocyte growth begins.

Results

Localisation of mRNAs encoding KL and KIT in Fetal and P7-14 Ovary

No above-background expression of either alternatively spliced transcript of *Kitl* was observed in 14.5-15.5 dpc ovaries (not shown). Figure 1 shows the results of *in situ* RT-PCR using *Kitl* primer set II in 16.5-18.5 dpc (Fig. 1 A-F) and 3-8 *dpp* ovaries (Fig. 1 G,L). A significant expression for KL mRNAs was seen in some of oocytes in the outer region of the 16.5 dpc ovaries (Fig. 1 A,B). Hematoxylin-eosin staining of consequent sections of the same ovaries made it possible to determine that the *in situ* RT-PCR positive oocytes correspond to oocytes at the zygotene/pachytene stage of meiotic prophase I (Fig. 2). By 18.5 dpc, *Kitl* expression was detected in the somatic cells in the outer region of the ovary (Fig. 1 E,F). At birth and 3 *dpp*, faintly KL mRNA labelled somatic cells were found primarily in the central region of the ovary, with minimal expression in the primordial follicle cells (Fig. 1 G,H). From P8 onward (Fig. 1 I,L), a progressively higher level of expression were detected in the follicle cells of one- and two-layered growing follicles. Similar results were obtained using *Kitl* primer set I and III (not shown).

The expression of mRNA for KIT was not detectable in ovaries before 16.5 dpc (not shown). Between 16.5 dpc and birth, oocytes expressed variable but progressively increasing amounts of *Kit* mRNA (Fig. 3) and, from P7 onward, there was a considerable strong accumulation of transcripts in the growing oocytes (Fig. 3 E,F).

RT-PCR performed on fetal ovaries and isolated oocytes from 16.5-18.5 dpc embryos confirmed that oocytes express both *Kit* and *Kitl*. In such RT-PCR analyses, however, oocytes seemed preferentially to express the membrane bound KL (Fig. 4).

Expression of KL and KIT Protein in Fetal Ovaries

As *in situ* RT-PCR revealed the stage-dependent expression of both *Kitl* and *Kit* in fetal oocytes at the mid-stages of the meiotic prophase that has not been reported in previous studies (Manova and Bachvarova, 1991; Manova *et al.,* 1993; Horie *et al.,* 1991), we concentrated on verifying the presence of KL and KIT proteins in these oocytes. Immunohistochemistry experiments performed with an anti-KL antibody cross reacting with both membrane bound and soluble KL isoforms (see, Materials and Methods), revealed in general co-expression of both proteins in zygotene/pachytene oocytes of 16.5-17.5 dpc ovaries; KIT staining was more concentrated on the oocyte surface whereas KL was cytoplasmic widespread (Fig. 5).

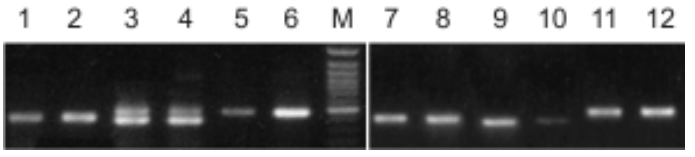

Fig. 4. RT-PCR analysis of *Kit* and *Kitl* expression in fetal ovaries and oocytes from 16.5 and 18.5 dpc mouse embryos. *Lanes are as follows (1) Kit (385 bp)16.5 dpc ovary; (2) Kit 18.5 dpc ovary; (3) Kitl 16.5 dpc ovary; (4) Kitl 18.5 dpc ovary; (5) actin 16.5 dpc ovary; (6) actin 18.5 dpc ovary; (7) Kit 16.5 dpc oocytes; (8) Kit 18.5 dpc oocytes; (9) Kitl 16.5 dpc oocytes; (10) Kitl 18.5 dpc oocytes; (11) actin 16.5 dpc oocytes; (12) actin 18.5 dpc oocytes. Note that both membrane-bound KL (375 bp) and soluble KL (459 bp) are expressed in the ovary, while oocytes express preferentially membrane bound KL.*

Subsequent experiments using the same antibodies and postnatal ovaries confirmed the expression patterns of *Kitl* and *Kit* mRNAs revealed by *in situ* RT-PCR also at protein level (not shown).

Anti-KL and anti-KIT Antibodies increase Oocyte Apoptosis in Culture

With the aim of verifying whether the KL/KIT system plays an autocrine role in preventing apoptosis in a fraction of oocytes at the zygotene/pachytene stage, we used a culture system that allows the *in vitro* culturing of fetal oocytes with minimal (less than 10%) somatic cell contamination (De Felici e*at al.*, 1999; Klinger and De Felici, 2002). The oocytes were isolated from 16.5 dpc ovaries and cultured for 1-2 days in serum-free DMEM + BSA in the absence or presence of anti-KL antibodies reported to neutralize the biological activity of KL (MAB455, R&D System) or ACK-2 antibody known to specific inhibit the action of KL by binding to its KIT receptor (Ogawa *et al.*, 1991; Nishikawa *et al.*, 1991). Analysis of oocyte apoptosis by means of the *in situ* detection nick translation end-labelling method (TUNEL) indicated that a 1-day or a 2-day culture of oocytes in continuous presence of 10 μg/ml anti-KL or 5 μg/ml ACK-2, respectively, significantly increased apoptosis to above the threshold control values (Tukey's test $P < 0.01$, Fig. 6). The same concentration of rat IgG used as control did not cause any increase in oocyte apoptosis (not shown). On the other hand, as expected (De Felici *et al.*, 1999), the addition of soluble 100 ng/ml KL did not significantly affect the percentage of oocytes undergoing apoptosis during two days of culture (Fig. 6).

Discussion

The present study investigated the expression of *Kit* and *Kitl* in mouse ovaries during fetal and early postnatal life using the highly sensitive *in situ* RT-PCR technique, which allows low abundant mRNAs to be detected and assigned to specific cell types in heterogeneous tissues. In addition to confirming previous results concerning the expression in the postnatal ovary of *Kit* and *Kitl* in growing oocytes and granulosa cells, respectively, we detected a stage-specific expression of both genes in oocytes located in the outer region of 16.5-17.5 dpc fetal ovaries that has not been revealed by previous studies. The finding that oocytes at the zyotene/pachytene stage of the meiotic prophase I co-expressed both *Kit* and *Kitl* was confirmed by the presence of KIT and KL proteins. Taken together, these results suggest that the KL/KIT system plays an autocrine role in oocytes reaching the mid-stage of meiotic prophase I. In line with this view, we found that the addition to the culture medium of a blocking antibody against KL or the KIT receptor caused a significant increase in apoptosis in 16.5 dpc oocytes cultured in virtually absence of somatic cells. While the effect of the anti-KL antibody was already evident after 1 day of culture, ACK-2 significantly increased oocytes apoptosis only after 2 days. This difference could be due to different efficency of the blocking antibodies employed or to another signalling pathway involved in oocyte survival and that could be activated by KL cross-reacting to an additional receptor. On the other hand, these last results indicate that a KL-dependent KIT receptor tyrosine kinase activity is necessary for the survival of such oocytes and suggests a novel KL-mediated autocrine loop at this meiotic stage when KL is not available from the somatic cells. The findings that the addition of exogenous soluble KL is little effective in preventing oocyte apoptosis during the first days of culture (this paper and De Felici *et al.*, 1999) while it significantly reduces apoptosis at later culture times (De Felici *et al.*, 1999), suggest that KL autocrine likely weak signaling although necessary during the first stages of meiosis becomes insufficient at later stages when KL paracine signals turn out to be more relevant.

In addition to oocyte survival, both KL autocrine and/or paracrine signals may be necessary for an efficient activity of meiotic checkpoint proteins and/or to favour meiotic progression in a stage-dependent fashion. A meiotic checkpoint that prevents meiotic progression when defects in recombination and synapsis occur has been described at the pachytene stage in a number of species, including mammals (for a review, see Roeder and Bailis, 2000). Although many components of the meiotic checkpoint pathways in mammal oocytes remain to be identified, it is likely that they act by triggering apoptosis at distinct meiotic stages in order to eliminate the oocytes that have undergone recombination and synapsis errors. It is possible to hypothesise that the defective oocytes may be eliminated as a result of apoptosis activation by checkpoint proteins or because they are unable to express a sufficient number of receptors for the growth factors necessary for their survival. In addition, apoptosis may serve to eliminate supernumerary oocytes that have escaped meiotic checkpoints and are competing for the limited amounts of trophic factors produced by the surrounding somatic cells. A large number of proteins are probably involved in the meiotic checkpoint. The ATM and Rad3/Meih1p/mei-41/Atr proteins are probable candidates as detectors of DNA damage or abnormal DNA replication in both mitotic and meiotic cells (Keegan *et al.*, 1996; Barlow *et al.*, 1998), but their precise role and regulation in oocyte

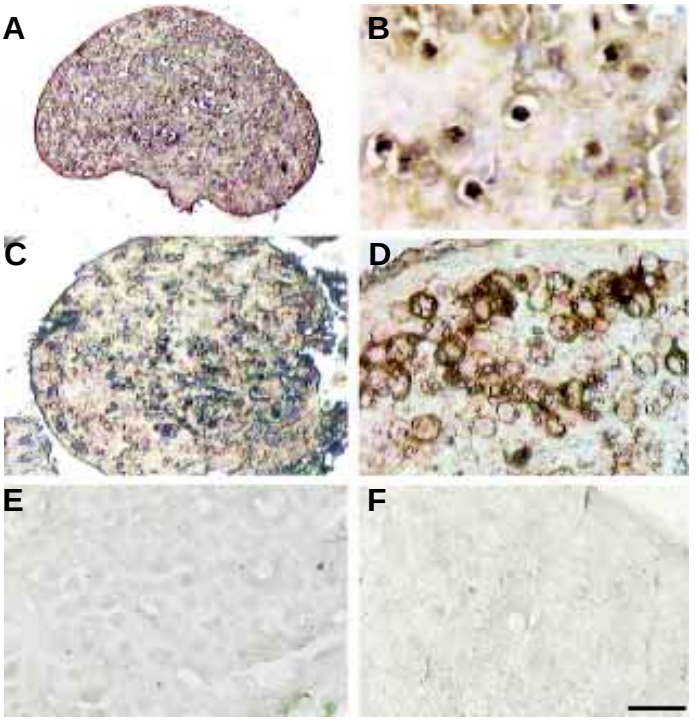

Fig. 5. Immunohistochemical analysis of KL (A,B) and KIT (C,D) protein expression in 16.5 dpc mouse ovaries. **(E,F)** *Control negative sections for KL and KIT antibodies, respectively (see Materials and Methods). Scale bars: A,C, 100 μm; B-F, approximately 25 μm.*

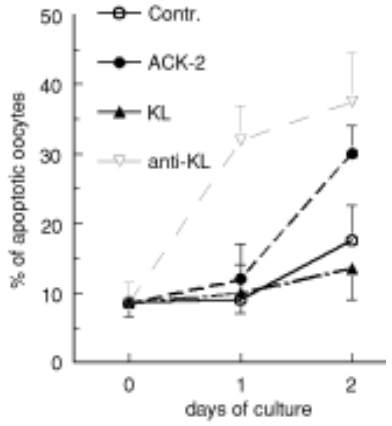

Fig. 6. Effects of anti-KL, ACK-2 antibody or soluble KL on oocyte apoptosis *in vitro*. *The oocytes were obtained from 16.5 dpc ovaries and cultured in serum-free DMEM+ BSA for 1-2 days with or without 10 μg/ml anti-KL or 5 μg/ml ACK-2 antibody or 100 ng/ml KL under the conditions described in Materials and Methods. The TUNEL histochemistry analysis revealed a significant increase in apoptosis in the oocytes cultured in the presence of anti-KL (1 and 2 days of culture) or ACK-2 antibodies (2 days of culture).*

apoptosis needs to be determined. The recent findings that the kinase activity of ATM can be stimulated by growth factor signalling (insulin: Yang and Kastan, 2000) support the idea that growth factors may activate molecular pathways by directly modulating DNA checkpoint proteins. Furthermore, interactions involving the KIT receptor might be critical for the oocyte meiotic progression as has been suggested in the case of pachytene spermatocytes in adult mice testis (Vincent *et al.*, 1998).

A wide range of biological activities have been attributed to the KL/KIT system, including proliferation and differentiation signals, a homing mechanism for migration, and anti-apoptotic action. Under normal conditions, *Kit* is generally expressed by precursors of cell lineages and KL by cells found in their environment. In both developing embryos and adults, there is a peculiar expression of KL/KIT by contiguous cell types. (Motro *et al.*, 1991). In particular, during mouse oogenesis, mRNA encoding *Kit* and protein has been found in mitotic primordial germ cells but not in oocytes undergoing the first stages of meiosis during fetal life (Manova and Bachvarova, 1991; De Felici *et al.*, 1996: Pesce *et al.*, 1997). Mouse oocytes in primordial follicles and throughout folliculogenesis have been found to contain abundant mRNA encoding *Kit* and protein (Manova *et al.*, 1990; Horie *et al.*, 1991; Manova and Bachvarova, 1991). mRNA encoding KITL and protein is expressed by cells along the migration path of primordial germ cells and in the gonadal ridges (Matsui *et al.*, 1990; Keshet *et al.*, 1991; Motro *et al.*, 1991; De Felici *et al.*, 1996: Pesce *et al.,* 1997); in post-natal ovaries, KITL is expressed in granulosa cells at all stages of follicle development, (Manova *et al.*, 1993; Motro and Bernstein, 1993). In normal tissues, a limited number of cell types co-expressing *Kit* and *Kitl* have been described, including mast cells in connective tissues (de Paulis *et al.*, 1999; Zhang *et al.*, 1998; Welker *et al.*, 1999), aortic endothelial and smooth muscle cells (Miyamoto *et al.*, 1997), ovarian surface epithelial cells (Parrot *et al.*, 2000), and neural crest cells (Guo *et al.*, 1997). The same applies to blastocytes during embryo development (Mitsunary *et al.*, 1999).

On the other hand, the co-expression of both genes and the presence of proteins have been observed in many human tumours such as lung cancer (Hibi *et al.*, 1991), breast carcinomas (Hines *et al.,* 1995), colorectal carcinomas (Lahm *et al.*, 1995), gynecological and testicular malignancies (Inoue *et al.*, 1994), myeloid leukemia (Pietsch, 1993), glioma (Stanulla *et al.*, 1995; Cohen *et al.*, 1994) and melanoma (Di Paola *et al.*, 1997). This suggests that, although an autocrine KL/KIT loop is involved in some normal biological activity, it is more often associated with growth deregulation.

In conclusion, the results reported in the present paper indicate that fetal oocytes may be one of the few normal cell types containing in addition to a paracrine mode of KL/KIT signalling an autocrine KL/KIT system. This latter may play an important role as an autocrine loop for maintaining survival and priming the meiotic progression of fetal oocytes as they reach the zygotene/pachytene stage of meiotic prophase I.

Materials and Methods

Collection and Culture of Oocytes

The oocytes were isolated from the ovaries of 16.5-18.5 days *post coitum* (dpc) embryos of CD-1 mice (Charles River, Italy) by means of EDTA-mechanical ovary disaggregation, and collected using a mouth-operated glass micropipette under a stereomicroscope, as they are easily identified by their morphology (De Felici and McLaren, 1983). They were then immediately frozen in liquid nitrogen for RT-PCR (see below). In some experiments, 16.5 dpc oocytes were cultured as follows. Briefly, oocytes were transferred to a Falcon tube containing 0.5 ml of DMEM (about $2x10^4$/ml), supplemented with antibiotics (75 mg/litre penicillin-G, 50 mg/litre streptomycin sulphate), 0.25 mM pyruvate, non-essential aminoacids, 5% horse serum (HS) and 2.5% fetal calf serum (FCS) (GIBCO, BRL). When indicated, oocytes were cultured in seum-free DMEM additioned with 20 mg/ml BSA (Fraction V, Sigma) (DMEM+BSA) in the presence of anti-KL (MAB456, R&D System) or ACK-2 (GIBCO, BRL) monoclonal antibodies or rat IgG (SIGMA) or soluble mouse recombinant KL (R&D System). The cultures were carried out for two days at 37°C in a humidified atmosphere of 5% CO_2 in air.

Apoptosis

The apoptosis of cultured fetal oocytes was evaluated by mixing 50 μl of the oocyte suspension with an equal volume of 3% agarose in PBS. Aliquots (50 μl) were smeared onto glass slides and allowed to solidify at room temperature. The slides were fixed with 4% paraformaldehyde for 10-15 min and washed with PBS. Finally, the cells were treated to identify those that were TUNEL-positive in accordance with the protocol of the *in situ* cell death detection kit (Roche Diagnostics, Italy). Fluorescence was detected under a Zeiss Axioplan-2 microscope, and the TUNEL-positive oocytes scored in a minimum of three fields (100 cells per field) under a 40x objective.

Primers for In Situ *RT-PCR*

The following sets of oligonucleotide primers were used to amplify cDNA:
Kit: 5'- AGTGCGGGAAGCCATCAAGG and
5'-CACTGCTGGTGCTCGGGTTT (441bp) (annealing temperature 64°C);
Kitl primer-set 1: 5'-CTACGAGATATGGTAATACAA and
ATCGCTACTGCTGTCATTC (annealing temperature 56°C),
Kitl primer -set II: 5'-TATAAAAGAATCTCCGAAGAG G and
5'-TACCAGCCACTG TGC GAAG (annealing temperature 62°C),
Kitl primer -set III: 5'- TAGTCATTGTTGGCTACGAG and
TTGCGGCTTTCCCTTTCTC (annealing temperature 54°C). The expected fragment length was 393 bp for *Kitl* primer set I, 500 bp or 416 bp for *Kitl*

primer set II, depending on the presence absence of exon 6, and 337 bp for *Kitl* primer set III. The reverse primer set I is specific for soluble KL, set III is specific for membrane-bound KL since it crosses the exon 5/7 boundaries (7 bp within exon 5 and 12 bp within exon 7) and set II recognizes both KL mRNA forms. In control RT-PCR all the above primers do not generate PCR products from genomic DNA during the extension cycle used because the expected fragments are exceedingly large, namely 10130 bp for K*it* and 4147, 9262 and 10598 bp for K*itl* set 1, set II and set III, respectively.

In Situ *RT-PCR*

Paraffin embedded tissue sections of fetal and post-natal ovaries were sectioned into 6 μm slices and analysed as previously described with minor modifications (Doneda *et al.*, 1997). Briefly, the sections were digested with proteinase K (SIGMA 5 μg/ml) at 37°C for 10 min and incubated overnight at 37°C with 10 units of an RNAse-free DNAse solution (Roche Diagnostics, Italy). The specimens were then incubated directly on the glass slide at 42°C for 60 min with 30 μl of a solution containing the antisense primer (1 μmole/L) and 200 units of avian mieloblastosis virus (AMV- Roche Diagnostics, Italy). The solution for cDNA amplification contained 1 μM of each sense and antisense primer, 100 mM Tris HCl, 25 mM $MgCl_2$, 50 mM KCl, 100 μM digoxigenin-dUTP (Roche Diagnostics, Italy) and 1 unit of Taq polymerase (BIOLINE). Then PCR cycles were then performed using an Omnislide thermal cycler (HYBAID), and the digoxigenin-labelled PCR product was detected in accordance with the recommendations of the supplier (Roche Diagnostics, Italy) with the only addition of 240 μg/mL levamisole (SIGMA). Tissue sections were not counterstained. Negative controls included: i) DNA amplification controls: omission of the reverse transcription step followed by normal cDNA amplification procedure. ii) non-specific amplification controls: omission of the primers in the PCR mixture will reveal non-specific staining due to endogenous priming, namely DNA fragments produced by the exonuclease activity of DNA polymerase, apoptosis and other artefacts as intrinsic alkaline phosphatase activity.

The control slides were processed in parallel to the test slides. In some experiments, hematoxylin-eosin staining of consequent sections of the same ovaries analysed for *in situ* RT-PCR was performed.

RT-PCR on Fetal Ovaries and Oocytes

For RT-PCR, RNA from whole ovaries or samples of 100-200 virtually pure oocytes individually collected from 16.5 and 18.5 dpc ovaries (see above) was extracted using the RNAzol kit as described by Pesce *et al.* (1998). RT-PCR was performed on 1 μg (where possible) or an aliquot of RNA extracted from oocytes containing an excess of yeast tRNA as a carrier. Briefly, the RNA was reverse-transcribed using the superscript one-step RT-PCR (Invitrogen) with primers specific for mouse *Kit* and *Kitl* and for cDNA amplification as described by Rossi *et al.* (1993a,b), and β-actin (Nichols *et al.*, 1998) as an internal control. Negative controls included samples in which the reverse transcriptional and *Taq* DNA polymerase or primers were omitted (not shown).

Immunostaining

The immunostaining procedure was performed on paraffin embedded sections of fetal and post-natal ovaries using the avidin-biotin peroxidase (ABC) method following the manufacture instructions (goat ABC staining system, Santa Crusz Biotechnology, Inc.). KIT protein was detected using goat polyclonal IgG antibody raised against a peptide mapping at the carboxy terminus of *KIT* of mouse origin (Santa Cruz Biotechnology: sc-1494). KL protein was detected using goat polyclonal IgG antibody raised against a peptide mapping at the amino terminus of KL of mouse origin and cross reacting with both membrane bound and soluble KL isoforms (Santa Cruz Biotechnology: sc-1303). After being dewaxed, the tissue sections were treated with 0.1% Triton-X for 10 min and incubated for 2 hrs with the primary antibody diluted 1:100. The immunoreaction was performed according to Hsu *et al.* (1981) and by developing peroxydase with 3, 3' diaminobenzidine (DAB, Sigma). The slides were then dehydrated in ethanol, cleared in xylene and mounted in eukitt (BDH). Negative control experiments were performed using isotype-matched goat IgG-(Sigma) instead of the primary antibody.

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References

BARLOW, C., LIYANAGE, M., MOENS, P.B., TARSOUNAS, M., NAGASHIMA, K., BROWN, K., ROTTINGHAUS, S., JACKSON, S.P., TAGLE, D., RIED, T., and WYNSHAW-BORIS, A. (1998). Atm deficiency results in severe meiotic disruption as early as leptonema of prophase I. *Development* 125: 4007-4017.

BAKKEN A.H.-and McCLANAHAN M. (1978). Patterns of RNA synthesis in early meiotic prophase oocytes from fetal mouse ovaries. *Chromosoma* 67: 21-40.

BESMER, P., MANOVA, K., DUTTLINGER, R., HUANG, E.J., PACKER, A., GYSSLER, C.-and BACHVAROVA, R.F. (1993). The kit-ligand (steel factor) and its receptor c-kit/W: Pleiotropic roles in gametogenesis and melanogenesis. *Development Supl.* 125-137.

BORUM, K. (1961). Oogenesis in the mouse. A study of the meiotic prophase. *Exp. Cell. Res.* 24: 495-507.

BURGOYNE, P.S.-and BAKER, T.G. (1981). Oocyte depletion in XO mice and their XX sibs from 12 to 200 days post partum. *J. Reprod. Fertil.* 61: 207-212.

COHEN, P.S., CHAN, J.P., LIPKUNSKAYA, M., BIEDLER, J.L.-and SEEGER, R.C. (1994). Expression of stem cell factor and c-kit in human neuroblastoma. The Children's Cancer Group. *Blood* 84: 3465-3472.

COUCOUVANIS, E.C., SHERWOOD, S.,W., CARSWELL-CRUMPTON, C., SPACK, E.G.-and JONES, P.P. (1993). Evidence that the mechanism of prenatal germ cell death in the mouse is apoptosis. *Exp Cell Res.* 209: 238-247.

DATTA, S.R., BRUNET, A.-and GREENBERG, M.E. (1999). Cellular survival: a play in three Akts. *Genes. Dev.* 13: 2905-2927.

DE FELICI, M. and MCLAREN, A. (1983). *In vitro* culture of mouse primordial germ cells. *Exp. Cell Res.* 144: 417-427.

DE FELICI, M., DI CARLO, A.-and PESCE, M. (1996). Role of stem cell factor in somatic-germ cell interactions during prenatal oogenesis. *Zygote* 4: 349-351.

DE FELICI, M., DI CARLO, A., PESCE, M., IONA, S., FARRACE, M.G.-and PIACENTINI, M. (1999). Bcl-2 and Bax regulation of apoptosis in germ cells during prenatal oogenesis in the mouse embryo. *Cell Death Diff.* 6: 908-915.

DE FELICI, M. (2000). Regulation of primordial germ cell development in the mouse. *Int J Dev Biol* 44: 575-80.

DE MIGUEL, P.M., CHENG, L., HOLLAND, E,C., FEDERSPIEL, M.J. and DONOVAN, P.J. (2002). Dissection of the c-Kit signaling pathway in mouse primordial germ cells by retroviral-mediated gene transfer. *Proc. Natl. Acad. Sci. USA.* 99, 10458-10463.

DE PAULIS, A., MINOPOLI, G., DAL PIAZ, F., PUCCI, P., RUSSO, T.-and MARONE, G. (1999). Novel autocrine and paracrine loops of the stem factor/-kinase network cell. *Int. Ach. Allergy Immunol.* 118: 422-425.

DI PAOLA, R.S., KUCZYNSKI, W.I., ONODERA, K., RATAJCZAK, M.Z., HIJIYA, N., MOORE, J.-and GEWIRITZ, A.M. (1997). Evidence for a functional kit receptor in melanoma, breast, and lung carcinoma cells. *Cancer Gene Ther.* 4: 176-182.

DONEDA, L., BULFAMANTE, G., GRIMOLDI, M.G., VOLPI, L.-and LARIZZA, L. (1997). Localization of fos, jun, kit and SCF mRNA in human placenta throughout gestation using *in situ* RT-PCR. *Early Pregnancy: Biology and Medicine* 3: 265-271.

FADDY, M.J., GOSDEN, R.G.-and EDWARDS, R.G. (1983). Ovarian follicle dynamics in mice: a comparative study of three inbred strains and an F1 hybrid. *J. Endocrinol* 96: 23-33.

FLANAGAN, J.G., CHAN, D.C.-and LEDER, P. (1991). Transmembrane form of the kit ligand growth factor is determined by alternative splicing and is missing in the Sld mutant. *Cell* 64: 1025-1035.

GUO, C.S., WEHRLE-HALLER, B., ROSSI, J.-and CIMENT, G. (1997). Autocrine regulation of neural crest cell development by steel factor. *Dev. Biol.* 184: 61-69.

HIBI, K., TAKAHASHI, T., SEKIDO, Y., UEDA, R., HIDA, T., ARIYOSHI, Y., TAKAGI, H.-and TAKAHASHI, T. (1991). Coexpression of the stem cell factor and the c-kit genes in small-cell lung cancer. *Oncogene* 6: 2291-2296.

HINES, S.J., ORGAN, C., KORNSTEIN, M.J. and KRYSTAL, G.W. (1995). Coexpression of the c-kit and stem cell factor genes in breast carcinomas. *Cell Growth Differ.* 6: 769-779.

HORIE, K., TAKAKURA, K., TAJI, S., NARIMOTO, K., NODA, Y., NISHIKAWA, S.I., NAKAYAMA, H., FUJITA, J. and MORI, T. (1991). The expression of c-kit protein during oogenesis and early embryonic development. *Biol. Reprod.* 45: 547-552.

HSU, S.M., RAINE, L. and FANGER, H. (1981). Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures. *J. Histochem. Cytochem.* 29: 577-580.

HUANG, E.J., NOCKA, K.H., BUCK, J. and BESMER, P. (1992). Differential expression and processing of two cell associated forms of the kit-ligand: KL-1 and KL-2. *Mol. Biol. Cell.* 3: 349-362.

INOUE, M., KYO, S., FUJITA, M., ENOMOTO, T. and KONDOH, G. (1994). Coexpression of the c-kit receptor and the stem cell factor in gynecological tumors. *Cancer Res.* 54: 3049-3053.

KEEGAN, K.S., HOLTZMAN, D.A., PLUG, A.W., CHRISTENSON, E.R., BRAINERD, E.E., FLAGGS, G., BENTLEY, N.J., TAYLOR, E.M., MEYN, M.S., MOSS, S.B., CARR, A.M., ASHLEY, T. and HOEKSTRA, M.F. (1996) The Atr and Atm protein kinases associate with different sites along meiotically pairing chromosomes. *Genes Dev.* 10: 2423-2437.

KESHET, E, LYMAN, S.D., WILLIAMS, D.E., ANDERSON, D.M., JENKINS, N.A., COPELAND, N.G. and PARADA, L.F. (1991). Embryonic RNA expression patterns of the c-kit receptor and its cognate ligand suggest multiple functional roles in mouse development. *EMBO J.* 10: 2425-2435.

KLINGER, F.G. and DE FELICI, M. (2002). *In vitro* development of mouse growing oocytes from fetal oocytes: stage-specific regulation by stem cell factor and granulosa cells. *Dev. Biol.* 244: 85-95.

LAHM, H., AMSTAD, P., YILMAX, A., BORBENYI, Z., WYNIGER, J., FISCHER, J.R., SUARDET, L., GIVEL, J.C. and ODARTCHENKO, N. (1995). Interleukin 4 down regulates expression of c-kit and autocrine stem cell factor in human colorectal carcinoma cells. *Cell Growth Differ.* 9: 111-118.

MANOVA, K., NOCKA, K., BESMER, P. and BACHVAROVA, R.F. (1990). Gonadal expression of c-kit encoded at the W locus of the mouse. *Development* 110: 1057-1069.

MANOVA, K. and BACHVAROVA, R. (1991). Expression of c-kit encoded at the W locus of mice in developing embryonic germ cells and presumptive melanoblasts. *Dev. Biol.* 146: 312-324.

MANOVA, K., HUANG, E.J., ANGELES, M., DELEON, V., SANCHEZ, S., PRONOVOST, S.M., BESMER, P., and BACHVAROVA, R.F. (1993). The expression pattern of the c-kit ligand in the gonads of mice supports a role for the c-kit receptor in oocyte growth and in proliferation of spermatogenesis. *Dev. Biol.* 157: 85-99.

MATSUI, Y., ZSEBO, M. and HOGAN, B. (1990). Embryonic expression of a haematopoietic growth factor encoded by Sl locus and the ligand for c-kit. *Nature* 347: 667-669.

MITSUNARY, M., HARADA, T., TANIKAWA, M., IWABE, T., TANIGUCHI, F. and TERAKAWA, N. (1999). The potential role of stem cell factor and its receptor c-kit in the mouse blastocyst implantation. *Mol. Hum. Reprod.* 5: 874-879.

MIYAMOTO, T., SASAGURI, Y., SASAGURI, T., AZAKAMI, S., YASUKAWA, H., KATO, S., ARIMA, N., SUGAMA, K. and MORIMATSU, M. (1997). Expression of stem cell factor in human aortic endothelial and smooth muscle cells. *Atherosclerosis* 129: 207-213.

MOTRO, B., VAN DER KOOY, D., ROSSANT, J., REITH, A. and BERNSTEIN, A. (1991). Contiguous patterns of c-kit and steel expression: analysis of mutations at the W and Sl loci. *Development* 113: 1207-21

MOTRO, B. and BERNSTEIN, A. (1993). Dynamic changes in ovarian *c-kit* and *steel* expression during the estrous reproductive cycle. *Dev. Dyn.* 197: 69-79.

NICHOLS, J., ZEVNICK, B., ANASTASSIADIS, K., NIWA, H., KLEWE-NEBENIUS, D., CHAMBERS, I., SHOLER, H. and SMITH A. (1998). Formation of pluripotrnt stem cells in the mammalian embryo depends on the POU transcription factor Oct-4. *Cell* 95: 379-391.

NISHIKAWA, S., KUSAKABE, M., YOSHINAGA, K., OGAWA, M., HAYASHI, S., KUNISADA, T., ERA, T., SAKAKURA, T. and NISHIKAWA, S. (1991). In utero manipulation of coat color formation by a monoclonal anti-c-kit antibody: two distinct waves of c-kit-dependency during melanocyte development. *EMBO J.* 10: 2111-2118.

OGAWA, M., MATSUZAKI, Y., NISHIKAWA, S., HAYASHI, S., KUNISADA, T., SUDO, T., KINA, T., NAKAUCHI, H. and NISHIKAWA, S. (1991) Expression and function of c-kit in hemopoietic progenitor cells. *J. Exp. Med.* 174: 63-71.

PACKER, A.I., HSU, Y.C., BESMER, P. and BACHVAROVA, R.F. (1994). The ligand of the c-kit receptor promotes oocyte growth. *Dev. Biol.* 161: 194-205.

PARROT, J.A., MOSHER, R., KIM, G. and SKINNER, M.K. (2000). Autocrine interaction of keratinocyte growth factor, hepatocyte growth actor, and kit-ligand in the regulation of normal ovarian surface epithelial cells. *Endocrinology* 141: 2532-2539.

PEPLING, M., E. and SPRADLING, A. C. (2001). Mouse ovarian germ cell cyst undergo programmed breakdown to form primordial follicles. *Dev. Biol.* 244: 339-351.

PESCE, M., FARRACE, M.G., PIACENTINI, M., DOLCI, S. and DE FELICI, M. (1993). Stem cell factor and leukemia inhibitory factor promote primordial germ cell survival by suppressing programmed cell death (apoptosis). *Development* 118 : 1089-1094.

PESCE, M. AND DE FELICI, M. (1994). Apoptosis in mouse primordial germ cells: a study by transmission and scanning electron microscope. *Anat. Embryol. (Berl)* 189: 435-440.

PESCE, M., DI CARLO, A. and DE FELICI, M. (1997). The c-kit receptor is involved in the adhesion of mouse primordial germ cells to somatic cells in culture. *Mech. Dev.* 68: 37-44.

PESCE, M., WANG, X., WOLGEMUTH, D.J. and SCHOLER, H.R. (1998). Differential expression of the Oct-4 transcription factor during mouse germ cell differentiation. *Mech. Dev.* 71: 89-98.

PIETSCH, T. (1993). Paracrine and autocrine growth mechanisms of human stem cell factor (c-kit ligand) in myeloid leukemia. *Nouv. Rev. Fr. Hematol.* 35: 285-286.

RAMEH, I. and CANTLEY, I.C. (1999). The role of phosphoinositide 3-kinase lipid products in cell function. *J. Biol. Chem.* 274: 8347-8350

ROEDER, G.S. and BAILIS, J.,M. (2000). The pachytene checkpoint. *Trends Genet.* 16: 395-403.

ROSSI, P., DOLCI, S., ALBANESI, C., GRIMALDI, P. and GEREMIA, R. (1993a). Direct evidence that the mouse sex-determining gene Sry is expressed in the somatic of male fetal gonads and in the germ cell line in the adult testis. *Mol. Reprod. Dev.* 34: 369-373.

ROSSI, P., DOLCI, S., ALBANESI, C., GRIMALDI, P., RICCA, R. and GEREMIA, R. (1993b). Follicle-stimulating hormone induction of Steel factor (SLF) mRNA in mouse Sertoli cells and stimulation of DNA synthesis in spermatogonia by soluble SLF. *Dev. Biol.* 155: 68-74.

STANULLA, M., WELTE, K., HADAM, M.R. AND PIETSCH, T. (1995). Coexpression of stem cell factor and its receptor c-Kit in human malignant glioma cell lines. *Acta Neuropathol.* 89: 158-165.

TAM P.P.L. AND SNOW M.H.L. (1981). Proliferation and migration of primordial germ cells during compensatory growth in mouse embryo. *J. Embryol. Exp. Morphol.* 64: 133-47.

UEDA, S., MIZUKI, M., IKEDA, H., TSUJIMURA, T., MATSUMURA, I., NAKANO, K., DAINO, H., HONDAZ, I. Z., SONOYAMA. I., SHIBAYAMA, H. *et al.* (2002). Critical roles of c-Kit tyrosine residues 567 and 719 in stem cell factor-induced chemotaxis: contribution of src family kinase and PI3-kinase on calcium mobilization and cell migration. *Blood*, 99: 3342-3349.

VINCENT, S., SEGRETAIN, D., NISHIKAWA, S., NISHIKAWA, S.I., SAGE, J., CUZIN, F. and RASSOULZADEGAN, M. (1998). Stage-specific expression of the Kit receptor and its kligand (KL) during male gametogenesis in the mouse: a Kit-KL interaction critical for meiosis. *Development* 125: 4585-4593.

WAGNER, E.F. and ALEXANDER, W.S. (1991). Of Kit and mouse and man. *Cell Differentiation* 6: 356-358.

WELKER, P., GRABBE, J., GIBBS, B., ZUBERBIER, T. and HENZ, B.M. (1999). Human mast cells produce and differentially express both soluble and membrane-bound stem cell factor. *Scan. J. Immunol.* 49: 495-500.

YANG, D.Q. and KASTAN, M.B. (2000). Participation of ATM in insulin signaling through phosphorylation of eIF-4E-binding protein 1. *Nature Cell. Biol.* 2: 893-896.

ZHANG, S., ANDERSON, D.F., BRADDING, P., COWARD, W.R., BADDELEY, S.M., MACLEOD, J.D., MCGILL, J.I., CHURCH, M.K., HOLGATE, S.T. and ROCHE, W.R. (1998). Human mast cells express stem cell factor. *J. Pathol.* 186: 59-63.

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