The *c-kit* receptor protein in the testis of green frog *Rana esculenta*: seasonal changes in relationship to testosterone titres and spermatogonial proliferation

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Abstract

The green frog *Rana esculenta* is a seasonal breeder. The cyclic changes between almost arrested and highly activated spermatogenesis offer an ideal model to study basic mechanisms of spermatogenesis. In this study, we demonstrated, to our knowledge for the first time, *c-kit* receptor positive cells in the testis of this amphibian. The presence of *c-kit* receptor protein was confirmed by western blotting (Wb) analyses carried out in the testis during all the three main phases of the sexual cycle. The antibody recognized a band of about 150 kDa that was correlated with the positive staining in the germinal epithelium. The immunolabelling for *c-kit* receptor, evaluated by immunohistochemistry (IHC), was localized in I and II spermatogonia (SPG), in I and II spermatocytes, in both elongating spermatids and spermatozoa and in the Leydig cells. Furthermore, *c-kit* expression showed a seasonal pattern connected with both testicular and plasma profiles of testosterone during the reproductive cycle. The highest expression of *c-kit* receptor occurred during the reproductive period, when the testis exhibited the maximum concentration of testosterone. In this period, the mitotic activity of germ cell, assessed by both Wb and IHC analyses for proliferating cell nuclear antigen (PCNA), was intensive. Indeed, during the post-reproductive period, testosterone titres were the lowest and the expression of both PCNA and *c-kit* receptor protein in the testis, although present, is minor when compared with the reproductive phase. This evidence suggests that cell division can continue sufficiently to accumulate SPG for the next spring, when new germinal cells undergo multiplication. Finally, during the pre-reproductive period, testosterone levels begin to increase and mitotic activity of germinal epithelium is comparably enhanced. These events seem to precede the period of maximum stimulated spermatogonial proliferation, i.e. the reproductive period. These results suggest that the *c-kit* receptor may play a role in germ cell proliferation and provide a basis for future detailed investigation of regulatory factors of the proliferation of SPG.

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Introduction

The *c-kit* proto-oncogene encodes for a transmembrane tyrosine-kinase receptor (Besmer *et al.* 1983, Yarden *et al.* 1987) with homology to receptors for platelet-derived growth factor and colony-stimulating factor-1 (Yarden *et al.* 1987, Chabot *et al.* 1988, Qiu *et al.* 1988). *c-kit* is the gene product of the murine white spotting (W) locus (Besmer *et al.* 1986, Nocka *et al.* 1989), mutation which leads to defects in haematopoiesis, melanogenesis and gametogenesis (Geissler *et al.* 1988).

In rodent testis, the *c-kit* expression starts on post-natal day 5 and is restricted to differentiating type A spermatogonia (SPG), type B SPG, primary spermatocytes (SPC; Yoshinaga *et al.* 1991, Manova *et al.* 1993, Vincent *et al.* 1998, Sette *et al.* 2000) and Leydig cells (Manova *et al.* 1990, Motro *et al.* 1991, Rossi *et al.* 1991). For its exclusive expression, *c-kit* receptor could

be used as a specific marker for these germinal cell populations (Dym *et al.* 1995, Morena *et al.* 1996). The *c-kit* mRNA was found to be expressed at high levels in SPG, and at lower levels in meiotic pachytene SPC (Sorrentino *et al.* 1991). A truncated version of *c-kit* was detected in spermatids and spermatozoa (SPZ; Albanesi *et al.* 1996). Moreover, the immunohistochemical analysis demonstrated the presence of *c-kit* in acrosomal granules of round spermatids and the acrosomal region of SPZ (Sandlow *et al.* 1996a, 1996b).

In the testis, the ligand of *c-kit* receptor stem cell factor (SCF) is uniquely expressed by Sertoli cells (Motro *et al.* 1991, Rossi *et al.* 1991, Tajima *et al.* 1991, Yan *et al.* 1999). The signalling cascade involving SCF and *c-kit* receptor system has been demonstrated to be indispensable for spermatogenesis and spermatogonial proliferation/differentiation/survival (Vincent *et al.*

1998, Blume-Jensen *et al.* 2000, Sette *et al.* 2000). Binding of SCF to *c-kit* receptor activates the tyrosine kinase receptor, followed by phosphoinositide 3-kinase and p70 S6 kinase activation to promote spermatogonial cell proliferation (Feng *et al.* 2000). In addition, i.v. injection of blocking anti-*c-kit* MAB activated Cdc42-associated tyrosine-kinase 2 (ACK2) caused a depletion of differentiating type A SPG (Yoshinaga *et al.* 1991) with an increased rate of apoptotic death in SPG and in dividing SPC (Packer *et al.* 1995). In mutant mice, abnormalities of the SCF/*c-kit* gene expression, such as gene deletions, point mutations, alternative splicing defects, lead to different changes in spermatogenesis (e.g. decreased primordial germ cell migration, decreased in spermatogonial proliferation; for reviews, see Morrison & Takahashi 1993, Loveland & Schlatt 1997).

However, the literature has mainly been focused on the localization and on the role of SCF/*c-kit* system in mice and rodents, species characterized by continuous spermatogenesis. Only recently were *c-kit* receptor positive SPG and SPC found in the testis of a seasonal breeder, roe deer, *Capreolus capreolus* and the changes of *c-kit* expression correlated to spermatogenic activity and the cyclic fluctuation of steroid hormones (Roleans *et al.* 2002). The release of testosterone is known to be a prerequisite for normal sperm production (see, for reviews, Zirkin 1993, Sharpe 1994). On the other hand, SCF is expressed in the testis by Sertoli cells under testosterone stimulation (Mi *et al.* 2004) and Leydig cells are a target for SCF (Rothschild *et al.* 2003). The SCF stimulates DNA synthesis in type A SPG cultured *in vitro* (Rossi *et al.* 1993, Hakovirta *et al.* 1999) and the injection of *c-kit* antibodies blocks their proliferation *in vivo* (Yoshinaga *et al.* 1991, Packer *et al.* 1995). Furthermore, there are several lines of *in vitro* data that indicate FSH regulates SCF expression in isolated rat Sertoli cells (McLean *et al.* 2002). In addition, the protein proliferating cell nuclear antigen (PCNA) is essential for the proliferation of SPG. It is also utilized in cell-cycle control through direct interaction with cyclin and cdk complex, where it allows progression through the G1/s boundary (Zhang *et al.* 1993). For these considerations, PCNA is utilized as an endogenous and molecular marker for mitotic and testicular epithelium proliferation (Chieffi *et al.* 2000).

Excluding the data reported for mammalian species, there are no studies yet available on *c-kit* receptor and on its putative involvement in the spermatogenesis of lower vertebrates. Regarding gonadal activity, seasonal breeders offer a good model to study basic mechanisms on the regulation of spermatogenesis without any manipulations. The purpose of this study was to investigate the expression of *c-kit* receptor in the testis of the amphibian *Rana esculenta*, which exhibits a well-known seasonality of reproduction (Rastogi *et al.* 1976, 2005, Di Fiore *et al.* 2005). The selective labelling of SPG to *c-kit* receptor protein and its expression during the seasonally activated and reduced proliferation could provide information on the

time and the role of interrelationships with other factors and cellular activities regulating spermatogenesis. Therefore, to better understand the control mechanisms regulating spermatogenesis, we evaluated the seasonal fluctuations of *c-kit* expression with both testicular and plasma profiles of testosterone during the reproductive cycle. Finally, the expression of *c-kit* receptor is assessed and related to mitotic activity of the testis of *R. esculenta*, tested with both immunolocalization and western blot analyses for PCNA.

Materials and Methods

Reproductive aspects of sexual cycle in Rana esculenta male

The green frog *R. esculenta* is a seasonal breeder living in the Mediterranean area. The animals have a body length of about 6.5–7.0 cm and a body weight of about 8.5–10.5 g. The reproductive cycle of this amphibian begins at the end of 2nd year of age and includes the transition between high and low activities of the testis. In fact, the processes of reproduction resurge at the beginning of spring, proceed actively through the summer and decline gradually in the winter, decreasing to an almost total quiescence for a short time (Rastogi *et al.* 1976, 2005, Di Fiore *et al.* 2005). Briefly, the sexually quiescent frogs emerge in January–February (pre-reproductive period); soon, gonads and secondary sexual characteristics (SSC) start their development and in little time become reproduction functional (March–June, reproductive period). In this period, the frogs mate and eggs are deposited. Then from July to December (post-reproductive period), despite the still favourable temperature and photoperiod, testicular activity gradually declines and SSC regresses. In this period, some SPZ are produced, but there is no spermiation.

Seasonal changes in the weight of the testes reflect the state of spermatogenesis. In fact, the seasonal maximum of testicular weight occurs when the testes are in an advanced stage of spermatogenesis and therefore in reproductive period (0.30–0.35 g). In post-reproductive period, the testicular weight decreases until 0.21–0.25 g and the seasonal minimum corresponds to the stage of pre-reproductive period (0.14–0.19 g).

Animals

Taking into account the reproductive characteristics, adult males of *R. esculenta* were captured in the countryside of Caserta (Italy) during the pre-reproductive (January–February), reproductive (March–June) and post-reproductive (October–December) phases. To evaluate a possible stressing cause that can impair the reproductive processes of male frogs, some animals were killed in the field and the others were transferred to a laboratory terrarium with a photothermal regimen consistent with the period of the year. They were fed on mealworms and *Drosophila* fruit flies *ad libitum*. Mortality rates were low (<5%).

The technical procedures illustrated below have been carried out either on group of field animals or on group of animals captured and transferred in laboratory terrarium. No differences have been registered between two groups.

Collection of samples

Soon after capture, animals for each phase of the reproductive cycle were anaesthetized by immersion in a ratio of 1:1000 tap water solution of MS-222 (tricaine methylsulphonate; Sigma). Blood samples were centrifuged at 800 *g* for 15 min and the resulting plasma was stored at -80°C for testosterone assay as reported below. The testes were rapidly dissected out. From each animal, one testis was frozen in liquid nitrogen for testosterone assay and western blot analyses, while the other testis was fixed by immersion in Bouin's fluid and processed for histology and immunohistochemistry (IHC).

The methods of capture and dissection and the captive rearing conditions were in accordance with Italian law (D. L.vo 116/92) and authorized by the appropriate Italian government administrative office (Servizio Veterinario della A.S.L. 44, Prot. Vet. 22/95).

Protein extract preparation

A pool of three frozen frog testes coming from three different animals were homogenized directly into lysis buffer containing 5 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 1 mM phenylmethylsulphonyl fluoride, 1 μg leupeptin, 0.5 mM sodium orthovanadate, 20 mM sodium pyrophosphate and clarified by centrifugation at 14 000 r.p.m. for 10 min. Protein concentrations were estimated using a modified Bradford assay (Bio-Rad). This procedure has been carried out for all the three phases of the sexual cycle.

Western blot analysis

Forty micrograms of proteins were boiled in Laemmli buffer for 5 min before electrophoresis. The sample was subjected to SDS-PAGE (10% polyacrylamide) under reducing conditions. After electrophoresis, proteins were transferred to nitrocellulose membrane (Immobilion Millipore Corporation, Bedford, MA, USA); completeness of transfer was assessed using prestained protein standards (Bio-Rad). The membrane was treated for 2 h with blocking solution (TBS/T: 5% nonfat powdered milk in 25 mM Tris (pH 7.4); 200 mM NaCl; 0.5% Triton X-100) and was incubated overnight with rabbit-anti *c-kit* receptor antibody (Santa Cruz, CA, USA) diluted in the ratio of 1:500. After washing with the TBS/T and TBS, membrane was incubated with a horseradish peroxidase-conjugated secondary antibody (1:2000; Chemicon, Temecula, CA, USA) for 60 min and washed again. Indeed, for PCNA detection, a mouse MAB against recombinant PCNA

(Dako Corp., Hamburg, Germany) diluted in the ratio of 1:2000 was used. Then the membranes were incubated for 60 min with the anti-mouse peroxidase conjugated (1:5000; Chemicon). Reactions were detected using the ECL system (Amersham Life Science).

The quantification of the bands was obtained by SCAN densitometry. Data are expressed as percentage relative to the maximum value obtained for reproductive period (taken as 100%). The protein levels were determined and quantified in three separate determinations.

Immunohistochemical localization of c-KIT receptor

To investigate the presence of *c-kit* receptor protein in the germinal epithelium of testis, *c-kit* immunolabelling was carried out. Five randomly chosen sections of the testis, for each frog from each phase of the sexual cycle (three frogs for phase), were examined. Testicular sections fixed in Bouin's solution and embedded in paraffin wax were used. The slides were washed in PBS 0.1 M (pH 7.4) for 15 min, treated with 3% H_2O_2 for 45 min to inhibit endogenous peroxidase and rinsed in PBS. The slides were then incubated in the blocking solution containing 20% normal goat serum (Sigma) with 10% BSA in PBS for 1 h at room temperature. After blocking, the slides were rinsed in PBS-BSA 10% and incubated overnight with polyclonal rabbit anti-*c-kit* antibody (1:200; Santa Cruz). After three rinses in PBS-BSA 10%, the slides were incubated sequentially with biotinylated goat anti-rabbit antibody (1:500; Sigma) diluted in PBS-BSA 10% and streptavidin (1:200, Pierce, IL, Rockford, USA). Bound antibody was visualized using 3,3'-diaminobenzidine tetrahydrochloride solution (Dako Corp.) according to the manufacturer's instructions. Sections were counterstained with haematoxylin, dehydrated, mounted and visualized by light microscope.

The scoring of immunoreaction was performed on five randomly chosen sections from each animal testis on the total of five different fields for each slide.

Immunohistochemical localization of PCNA protein

To evaluate germ cells, the PCNA IHC was performed according to a recent study reported by [Raucci et al. \(2004\)](#). Serial deparaffinized sections of frog testes were sequentially incubated in normal goat serum (1:20; Sigma) for 45 min to reduce undesired background staining, primary antiserum (monoclonal mouse anti-PCNA diluted in the ratio of 1:400 with 10% BSA; Dako Corp.) overnight at 4°C in a dark moist chamber, biotinylated secondary antibody (goat anti-mouse IgG diluted in the ratio of 1:500; Sigma), and streptavidin (1:200; Pierce). The immunopositivity was visualized by exposure to 3,3'-diaminobenzidine tetrahydrochloride in Tris buffer (Dako Corp.). Sections were mounted with a synthetic medium.

Controls

Specificity of immunostaining for both *c-kit* receptor and PCNA was determined by (a) omission of primary antiserum, (b) substitution of the primary antiserum with 0.1 M PBS or no immune serum (1:200) and (c) primary antiserum preabsorbed overnight with synthetic antigen (only for *c-kit* receptor). These controls completely eliminated immunostaining.

Histology

After dissection, frog testes were rapidly removed and fixed in Bouin's fluid for 48 h at room temperature. The fixative was then extracted with 70% ethanol and the tissues were embedded in paraffin wax. The histological exam of the testes was carried out in deparaffinized sections (7 μ m), rehydrated and stained with haematoxylin and eosin reaction, as described in Mazzi (1977).

Testosterone assays in plasma and testis

Testosterone determinations in the plasma were conducted utilizing the Enzyme Immunoassay Kits (Adaltis, Bologna, Italy). The following limits of detection were sensitivity 50 pg/ml (intra-assay variability 4.0%, inter-assay variability 9.0%). Plasma samples (100 μ l) were vortexed with ethyl ether (1:10, v/v) for 5 min and centrifuged at 3000 g for 10 min. The upper phase (ethyl ether) was transferred to a glass tube. Two extractions were performed. The pooled ether phases were left to evaporate on a hot plate at 40–50 °C under a hood. The residue was dissolved in a 0.5 ml sodium phosphate buffer 0.05 M (pH 7.5), containing BSA at a concentration of 10 mg/ml, and then utilized for the assay. Tissue samples (testis) were homogenized in the ratio of 1:10 (w/v) with distilled water. The homogenate was then mixed vigorously with ethyl ether (1:10, v/v) and the ether phase was withdrawn after centrifugation at 3000 g for 10 min. Three extractions were performed. Pooled ether extracts were dried and then utilized for testosterone immunoassay as previously reported. Testosterone recovery was 85% from plasma and 80% from tissues.

Statistical analysis

Data were compared by ANOVA followed by Duncan's test for multi-group comparison and the Student's *t*-test for between-group comparison. All data were expressed as mean $\pm$ s.d. The level of significance was taken at $P < 0.01$ and $P < 0.05$.

Results

The results presented below refer to the animals captured and transferred in laboratory terrarium.

Expression of *c-kit* receptor protein during the seasonal cycle

The *c-kit* receptor protein was characterized by western blot and the specific band of about 150 kDa was detected during the annual cycle with a different expression status (Fig. 1). Strong expression of *c-kit* receptor was present in the reproductive period (Fig. 1, lane 2). By contrast, during the pre- and post-reproductive periods, the testis showed a lower expression of *c-kit* (Fig. 1, lanes 1 and 3 respectively).

Figure 1 Western blot detection of *c-kit* receptor protein in testicular extract of *Rana esculenta* during the sexual cycle. Protein (40 μ g/lane) was resolved by SDS-PAGE, transferred to nitrocellulose membrane and then incubated with antibody raised against *c-kit* receptor protein. A specific band of about 150 kDa was observed by comparison with co-migrating size markers (Bio-Rad). The amount of *c-kit* receptor protein was quantified by densitometry using the SCAN program. The quantification of the data is shown in the histogram. Data are expressed as percentage relative to the maximum value obtained for reproductive period (taken as 100%). The values shown represent the mean of three separate determinations. Each determination came from a pool of three testes of three different frogs. Error bars represent mean $\pm$ s.d. 1, pre-reproductive; 2, reproductive; 3, post-reproductive.

C-KIT receptor immunohistochemistry

The immunohistochemical detection of *c-kit* was carried out on serial slides of testis of green frog *R. esculenta* and revealed the presence of this receptor throughout the annual cycle (Fig. 2). The cytological elements of germinal epithelium show a positive reaction localized in both plasma membrane and cytoplasm. They were mainly I and II SPG, I and II SPC, elongating spermatids and SPZ. Among the somatic cells, Leydig cells also reacted with the *c-kit* receptor antibody (Fig. 2). The intensity of immunolabelling for *c-kit* receptor protein varied depending on the phase of the reproductive cycle. In the reproductive phase (Fig. 2C), the positivity was higher than the pre- (Fig. 2B) and post-reproductive (Fig. 2D) phases. The antibody used for *c-kit* detection was subjected to immunopreabsorption, revealing that the labelling was totally blocked by preincubation with the cognate peptide (Fig. 2A).

Figure 3 shows the immunopositivity of different cytological elements of the testis during the sexual cycle. In the reproductive period, strong *c-kit* positive staining was detected in I SPG (Fig. 3B₁); the immunopositivity continued to be seen on I SPG at the pre- (Fig. 3A₁) and post-reproductive (Fig. 3C₁) periods, but its intensity was less than the reproductive phase. In the pre-reproductive period, II SPG were slightly reacted with the *c-kit* receptor antibody (Fig. 3A₂). In the reproductive and post-reproductive periods, II SPG were intensely positive (Fig. 3B₂ and C₂ respectively). Moreover, the staining of I and II SPC of pre-reproductive testis (Fig. 3A₃ and A₄ respectively) was less intense than the reproductive (Fig. 3B₃ and B₄ respectively) and post-reproductive periods (Fig. 3C₃ and C₄ respectively). Strong immunopositivity can be seen also in both elongating spermatids and SPZ during reproductive period (Fig. 3B₅), although it decreases in post-reproductive period (Fig. 3C₅). In the pre-reproductive period, the reaction in SPZ is the lowest observed during all the phases of the sexual cycle (Fig. 3A₅).

Annual profile of testosterone level and histology

Figure 4 reports the seasonal fluctuation of testosterone levels detected in testis and plasma of green frog *R. esculenta* during the reproductive cycle. In all the major phases of the sexual cycle, substantial amounts of testosterone were found in both testicular extracts and plasma. The androgen level showed significant variation depending on the phase of reproductive cycle and reaching its higher content during the reproductive period (115 ± 8.0 ng/ml testis and 17.4 ± 1.5 ng/g plasma respectively). In the pre- and, particularly, post-reproductive periods, the testosterone content detected was significantly lower (48.6 ± 2.5 ng/g testis, 7.5 ± 0.7 ng/ml plasma and 25.3 ± 1.4 ng/g testis, 2.5 ± 0.4 ng/ml plasma respectively) than the reproductive period ($P < 0.01$). Figure 4 also reports the modification of germinal epithelium of testis through the sexual cycle. The testis of green frog

Figure 2 Immunohistochemical detection of *c-kit* receptor positive cells in seminiferous ampoules of *Rana esculenta* during pre-reproductive (B), reproductive (C) and post-reproductive (D) periods. Negative control, made by primary antiserum pre-absorbed overnight with synthetic antigen (A). ($\times 500$).

Figure 3 Immunohistochemistry for *c-kit* receptor protein in the testis of *Rana esculenta* during the sexual cycle. A₁–A₅=pre-reproductive period; B₁–B₅=reproductive period; C₁–C₅=post-reproductive period. Arrowhead, I SPG; arrow, II SPG; ▲, I SPC; △, II SPC; asterisk, spermatozoa (×1000).

R. esculenta shows a cystic pattern, i.e. the germ cells proliferate in coordinated clusters enclosed in membranous cyst walls (ampoules). In the pre-reproductive period, seminiferous ampoules of testis contained predominantly I and II SPG. Although I and II SPC and SPT were present, their number was low because they underwent degeneration. Some SPZ were present in the lumen. In the reproductive period, testicular epithelium contained the maximal number of I SPG and appeared highly differentiated. The highest number of II SPG was present in the cysts and their massive transformation into I SPC occurred; II SPC was also evident and the lumen was full of SPZ. In the post-reproductive period, high numbers of cysts still contained I and II SPC although their decrease was closely associated with the formation of SPT. Not many SPZ were present in the lumen.

Expression of PCNA protein during the seasonal cycle

Western blot analyses used for PCNA determination showed that testis contained a specific band of 36 about

kDa found during all the phases of the sexual cycle (Fig. 5). The expression of PCNA underwent fluctuation depending on the phase of the cycle. A higher expression of PCNA was seen in the reproductive period (Fig. 5, lane 2) than pre- and post-reproductive periods (Fig. 5, lanes 1 and 3 respectively).

PCNA immunohistochemistry

The presence of PCNA was examined in frog testes during the sexual cycle. IHC analyses were performed on serial sections and revealed the presence of PCNA protein in both cytoplasm and nucleus of I and II SPG and in the nuclei of SPC, while II SPC and spermatids remained negative.

In the pre-reproductive period, seminiferous ampoules of the testis showed fair immunoreactive material in I SPG multiplying. Moreover, II SPG and I SPC were moderately positive to PCNA (Fig. 6A). In the reproductive period, testicular epithelium showed the highest intensity of immunoreaction for PCNA. The positivity

Figure 4 Seasonal changes in testosterone content detected in both testis (■) and plasma (□) of green frog *Rana esculenta*, during the reproductive cycle. Results are expressed as mean $\pm$ s.d. obtained from five individual animals. Histological sections of testis of *Rana esculenta* during pre-reproductive, reproductive and post-reproductive periods. Arrowhead, I SPG; arrow, II SPG; ▲, I SPC; △, II SPC; L, lumen. haematoxylin/eosin (×500).

was localized in I and II SPG and in I SPC (Fig. 6B). In the post-reproductive period, the immunopositivity for PCNA was contained in identical cell types observed in the other two periods; but in this phase, the intensity of the immunolabelling was visibly lower than the pre- and post-reproductive phases (Fig. 6C).

Discussion

This study ascertained the presence of the *c-kit* receptor in the testis of the green frog *R. esculenta* during the reproductive cycle. It also shed light on unmanipulated basic mechanisms regulating testicular activity in this model of amphibian seasonal breeders.

Results of western blot analysis demonstrated the expression of *c-kit* receptor in the testis of this species and indicated the polyclonal antibody recognized a single protein of ~ 150 kDa. Two forms of *c-kit* receptor with an apparent molecular size of 124 and 160 kDa have been detected in the rat testis (Dym *et al.* 1995) and

Figure 5 Western blot analyses of PCNA protein in testicular extract of *Rana esculenta* during the sexual cycle. Protein (40 μ g/lane) was resolved by SDS-PAGE, transferred to nitrocellulose membrane and incubated with antibody raised against PCNA protein. A specific band of about 36 kDa was observed by comparison with co-migrating size markers (Bio-Rad). The amount of PCNA was quantified by densitometry using the SCAN program. The quantification of the data is shown in the histogram. Data are expressed as percentage relative to the maximum value obtained for reproductive period (taken as 100%). The values shown represent the mean of three separate determinations. Each determination came from a pool of three testes of three different frogs. Error bars represent mean $\pm$ s.d. 1, pre-reproductive; 2, reproductive; 3, post-reproductive.

results indicate that the 124 kDa protein could be an immature protein, while the 160 kDa protein is the mature fully glycosylated form of *c-kit* (Nocka *et al.* 1989). On the other hand, there may be three isoforms of *c-kit* receptor proteins in mouse sperm, one isomeric isoform (33 kDa) that is expressed in the sperm tail and two testis-specific isoforms (150 and 48 kDa) that are expressed in the acrosomal region (Feng *et al.* 1998). It is also possible that the 33 kDa protein is generated by

Figure 6 Immunohistochemistry for PCNA in the testis of *Rana esculenta* showing a positive reaction in the cytoplasm and the nucleus of I (arrowheads) and II (arrow) SPG and I SPC (open arrow) during the pre-reproductive (A), reproductive (B) and post-reproductive (C) periods ($\times 500$).

proteolytic cleavage of 150 kDa c-kit receptor protein during sample preparation or represents a cross-reaction with a somatic protein. In addition, more recent reports propose that the binding of c-kit ligand to receptor induces c-kit dimerization autophosphorylation so that the c-kit receptor is present as both membrane-associated and soluble (Kierszenbaum 2006).

Using a rabbit polyclonal anti-c-kit, we also carried out the IHC for c-kit receptor protein in the germinal epithelium of testis. The finding showed that the c-kit receptor protein was predominantly localized on the membrane of I and II SPG, I and II SPC, elongated spermatids and SPZ in all the phases of the yearly cycle. Particularly, strong c-kit positivity staining was observed in the testis during the reproductive period, when the spermatogenesis was strongly activated (Rastogi *et al.* 1976, 2005, Di Fiore *et al.* 2005). Since the antibody was raised against the c-terminal domain of the c-kit receptor, which is known to be the intracellular domain of the receptor (Yarden *et al.* 1987, Qiu *et al.* 1988), it may be assumed that the signal was membrane associated (possibly intercellular). Previously, Yoshinaga *et al.* (1991) showed high expression of c-kit receptor in mouse testis in type A SPG, low expression in intermediate and type B SPG and in the earliest preleptotene SPC. Later, it was demonstrated that the c-kit receptor also is expressed in the acrosomal granules of round spermatids and the acrosomic region of SPZ (Albanesi *et al.* 1996, Sandlow *et al.* 1996a, 1996b, 1997, Feng *et al.* 1998), suggesting the receptor's involvement in some crucial step(s) for capacitation and/or the acrosome reaction (Feng *et al.* 2005).

C-kit receptor is a conservative molecule and the cDNA has been isolated in lower vertebrates, such as chicken (Sasaki *et al.* 1993), *Xenopus* (Baker *et al.* 1995, Kao & Bernstein 1995) and zebrafish (Parichy *et al.* 1999). Addition of human recombinant SCF to newt testicular organ cultures stimulates the SPG proliferation and they progressed to the seventh generation, the penultimate stage before the primary spermatocyte stage. However, the SPG do not differentiate into SPC and do not die by apoptosis (Abè *et al.* 2002). These data are consistent with the previous results in mice where added SCF to cultured male germ cells stimulates DNA synthesis in type A SPG (Rossi *et al.* 1991, Hakovirta *et al.* 1999). Moreover, the i.v. injection of ACK-2, a MAB that blocks the binding of SCF to c-kit, causes a loss of differentiating type A SPG, but has no effect on the transition of intermediate SPG to type B SPG or primary SPC (Yoshinaga *et al.* 1991).

The expression of c-kit receptor protein in the testis of frog *R. esculenta* evaluated by western blot data parallels cyclic changes of germinal epithelium and is well correlated with both plasma and testicular levels of testosterone. In fact, the highest expression of c-kit receptor occurred during the reproductive period, when seminiferous ampoules were completely differentiated and both testicular and plasma content of T was at the maximum peak. Instead, during the pre- and post-reproductive periods, the expression of c-kit as well as testosterone levels was lower. The fluctuation of c-kit receptor protein was also correlated to mitotic activity of the testis, evaluated by both western blot and

immunohistochemical detection of PCNA during the sexual cycle. In the reproductive period, in fact, PCNA expression was the highest as well as the immunoreactivity of germinal epithelium to this mitotic factor. These findings suggest that *c-kit* receptor could be implied in the proliferation of the germinal epithelium of *R. esculenta*. On the another hand, Roleans *et al.* (2002) observed that in roe deer, *Capriolus capriolus*, the testis showed a specific pattern of spermatogonial *c-kit* receptor positive cell, in relation to testosterone levels.

In the amphibian *R. esculenta*, the highest level of testosterone during the reproductive phase is beneficial for spermatogenesis and the development and maturation of the androgen-dependent target organ (Rastogi *et al.* 1976, Delrio *et al.* 1980, Varriale & Serino 1994, Di Fiore *et al.* 2005). On the other hand, Raucci *et al.* (2004) demonstrated that the increase of testosterone titres causes and increase of seminiferous ampulae area and their spermatid and sperm contents. In addition, *in vitro* experiments carried out on cultured testicular germ cells of embryonic chickens demonstrated that the testosterone treatment induced stronger *c-kit* and PCNA expressions, indicating that this hormone promoted testicular germ cell proliferation probably through indirect effects on Sertoli cells (Mi *et al.* 2004). Furthermore, in cultured cells of mouse SPG, SCF enhanced *c-kit* expression of type A SPG by stimulating their progression into the mitotic cell cycle (Dolci *et al.* 2001).

In our study, light microscopy revealed that Leydig cells show a *c-kit* immunopositive reaction. *c-kit* localization in Leydig cells has already been known (Sandlow *et al.* 1996a, 1996b, 1997) and its expression is connected to a paracrine regulation of Leydig cell steroidogenetic function (Rothschild *et al.* 2003). However, soluble SCF might participate in the regulation of formation of Leydig cell population during testicular development. These data indicate that there is a reciprocal control between SCF production by Sertoli cells, testosterone production by Leydig cells and *c-kit* expression (Yan *et al.* 2000).

In conclusion, our study demonstrates for first time in non-mammalian vertebrate testis, the presence of *c-kit* receptor protein and suggests a direct subtle cooperation between the testosterone and the expression of *c-kit* during the seasonal cycle of *R. esculenta*. In addition, mitotic activity of germinal epithelium is well correlated with plasma and testicular levels of the androgen hormone.

As demonstrated in mammals, in *R. esculenta*, *c-kit* could play a pivotal role in the timing of the steps of the spermatogenesis. Future investigations should address the precise function of *c-kit* receptor in the processes of spermatogenesis. This will open further investigation on the involvement of regulatory factors in the proliferation of the SPG.

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