Immunoreactivity of *c-kit* Receptor Protein during the Prehibernation Period in the Oviduct of the Chinese Brown Frog, *Rana chensinensis*

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ABSTRACT. The objective of this study was to investigate immunoreactivity of the *c-kit* receptor in the oviduct of *Rana chensinensis* during the prehibernation period. Histological examination of oviducts was performed during the prehibernation period. The sections of oviduct were immunostained by the avidin-biotin-peroxidase complex method using rabbit polyclonal antisera raised against the rat *c-kit* receptor and PCNA. Total proteins were extracted from oviductal tissues and used for Western blotting analysis. Immunohistochemistry revealed the presence of the *c-kit* receptor and PCNA in the oviduct tissues during the prehibernation period. Also, positive signals for the *c-kit* receptor and PCNA by Western blotting were observed in oviduct tissues during the prehibernation period. These results suggested that the *c-kit* receptor might play a regulatory role in oviductal hypertrophy in the brown frog, *Rana chensinensis*.

KEY WORDS: *c-kit* receptor, oviduct, *Rana chensinensis*.

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The *c-kit* proto-oncogene encodes a transmembrane receptor and is associated with maturation of several cell types, including germ cells. The gene is widely expressed as a single 5-kb transcript localized to human chromosome 4 and to mouse chromosome 5 [5, 12, 19, 39]. The *c-kit* receptor whose ligand is a stem cell factor (SCF) belongs to the family of receptors for platelet-derived growth factor and colony-stimulating factor. It plays an important role in the signal transduction pathway that regulates cellular growth on repair [37]. The *c-kit* receptor is the gene product of the murine white spotting (W) locus [1, 26] mutation, which leads to defects in hematopoiesis, melanogenesis and gametogenesis [12]. The expression of *c-kit* receptor has been detected in various normal, fetal and adult tissues, including the gonads, brain, adrenal gland, skin, breast and bone marrow, and in several human malignancies like seminomas, including lung and brain cancers and glioblastomas [14, 16, 32]. The *c-kit* proto-oncogene encodes the receptor tyrosine kinase kit, which has also been shown to be important for normal mast cell survival, proliferation, differentiation and migration [6, 7, 27, 35, 40]. Evidence has accumulated that the *c-kit* receptor plays a critical role in the development of the mammalian ovary, oogenesis, folliculogenesis, blastocyst implantation and uterine growth and repair [8, 13, 22, 33]. Recently, the *c-kit* receptor has received increasing attention for its role in the reproductive function of lower vertebrates [18, 29, 41].

The Chinese brown frog (*Rana chensinensis*) is a special amphibian in northeastern China, as it has been used widely in traditional Oriental medicine [42]. The reproductive period for *R. chensinensis* is dependent on latitude and altitude and usually occurs from February to June following hibernation. However, one specific physiological phenomenon that occurs in *R. chensinensis* is that the oviduct abnormally expands prior to hibernation and not during the breeding period. Moreover, dessicated oviduct of the female *R. chensinensis*, Oviductus Ranae, is a valuable Chinese crude drug and is recorded in the Pharmacopoeia of the People's Republic of China (2005 edition) [36]. In the application of Oriental medicine, oviduct of *Rana chensinensis* is used to replenish the kidney essence, to nourish the yin and to moisten the lung [38]. The aim of present study was to investigate immunoreactivity of the *c-kit* receptor in the oviduct of *R. chensinensis* during the prehibernation period and to elucidate the relationship between the *c-kit* receptor and oviductal hypertrophy in *R. chensinensis*.

In October, 2010, ten adult female Chinese brown frogs (*Rana chensinensis*) were obtained during the prehibernation period from Jilin Baekdu Mountain Chinese Brown Frog Breeding Farm, Jilin Province (125°40'E–127°56'E, 42°31'N–44°40'N), China (Fig. 1). All the animals were treated in accordance with the National Animal Welfare Legislation. All experimental procedures were carried out in accordance with the guidelines established by the Beijing Forestry University. Both the left and right oviducts were collected from the frogs. One oviduct from the left side was immediately fixed for 12 hr in 4% paraformaldehyde (Sigma) in 0.05 M PBS, pH 7.4, for histological and immunohistochemical observations; another oviduct from the right side was immediately stored at -80°C for Western blot-

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Fig. 1. A female Chinese brown frog (*Rana chensinensis*).

ting detection.

Oviduct samples were dehydrated in an ethanol series and embedded in paraffin wax. Serial sections ($4\ \mu\text{m}$) were mounted on slides coated with poly-L-lysine (Sigma). Some sections were stained with hematoxylin-eosin (HE) for observations of general histology.

Serial sections of oviducts were incubated with 10% normal goat serum to reduce background staining caused by the second antibody. The sections were then incubated with rabbit anti-rat *c-kit* receptor antiserum (1:500) (Santa Cruz Biotechnology) and rabbit anti-rat PCNA antiserum (1:500) (Santa Cruz Biotechnology) for 12 hr at room temperature. The sections were then incubated with a second antibody, goat anti-rabbit IgG conjugated with biotin and peroxidase with avidin, using a rabbit ExtrAvidin staining kit (Sigma) and then visualized with 30 mg 3,3-diaminobenzidine solution in 150 ml of 0.05 M Tris-HCl I^{-1} buffer, pH 7.6, plus $30\ \mu\text{l}$ H_2O_2 . The reacted sections were then counterstained with hematoxylin solution (Merck, Tokyo, Japan). Preabsorption of the antiserum was performed with an excess of antigen peptides (Sigma) for the control experiments.

The oviducts were weighed and diced into small pieces using a clean razor blade. The tissue was then homogenized in a homogenizer containing $300\ \mu\text{l}$ of 10 mg/ml PMSF stock and incubated on ice for 30 min at 4°C throughout all procedures. Homogenates were centrifuged at $12,000 \times g$ for 10 min at 4°C . Protein extracts ($25\ \mu\text{g}$) were mixed with an equal volume of $2\times$ Laemmli sample buffer. Equal amounts of each sample were loaded and run on a 12% SDS-PAGE gel at 18 V/cm and transferred to nitrocellulose membranes using a wet transblotting apparatus (Bio-Rad, Richmond, CA, U.S.A.). The concentration of SDS used for protein gel and protein sample buffer was 10%. The membranes were blocked in 3% BSA in TBS solution for 1 hr at room temperature. Primary incubation of the membranes was carried out using a 1:500 dilution of both rabbit anti-rat *c-kit* receptor antiserum and rabbit anti-rat PCNA antiserum for 1 hr. Secondary incubation of the membrane was then carried out using a 1:1,000 dilution of goat anti-rabbit IgG tagged with horseradish peroxidase for 1 hr. Finally, the membrane was developed using 25 mg 3,3-diaminobenzi-

Fig. 2. Anatomic localization and morphology of an oviduct from *Rana chensinensis*. (a) Black ovaries in the anterior of the cavum abdominis and one pair of white oviducts in the posterior position of the cavity. (b) A dissected oviduct obtained during the prehibernation period. The scale bars represent 1 cm.

Fig. 3. Histological appearance of *Rana chensinensis* oviducts obtained during the prehibernation period and stained with hematoxylin-eosin (HE). During prehibernation, the tubule lumen (a), epithelial cells (b) and lobules consisting of stroma cells (c) were observed in oviduct tissues. EC: epithelial cell. ST: stroma tissue. TL: tubule lumen. The scale bars represent $200\ \mu\text{m}$ (a) and $50\ \mu\text{m}$ (b, c).

dine solution in 25 ml TBS-T buffer (0.02 M Tris, 0.137 M NaCl and 0.1% Tween-20, pH 7.6) plus $3\ \mu\text{l}$ H_2O_2 . Preabsorption of the antiserum was performed with an excess of antigen peptides (Sigma) for the negative control.

Anatomic and morphologic observations of oviducts of *R. chensinensis* obtained during the prehibernation period are shown in Fig. 2. Black ovaries in the anterior section of the cavum abdominis and one pair of white oviducts in the posterior section of the cavity were observed in the abdominal cavity (Fig. 2a). An enlarged view of an oviduct during the prehibernation period is shown in Fig. 2b.

The histological appearances of oviduct tissues from the prehibernation period in October are shown in Fig. 3. The oviduct tissues included the tubule lumen, epithelium and lobules (Fig. 3a). The lobules consisted of stroma cells (Fig. 3c).

Immunoreactivity for the *c-kit* receptor and PCNA was observed in the oviduct tissues during the prehibernation

Fig. 4. Immunoreactivity of the *c-kit* receptor and PCNA in expanded oviduct tissues expanded of *Rana chensinensis* during the prehibernation period. Immunostaining for the *c-kit* receptor was observed in the cytoplasm of stroma cells (a) and epithelial cells (b) during the prehibernation period. The immunolocalization of PCNA was in the nuclei of stroma cells (c) and epithelial cells (d) during the prehibernation period. In order to clearly show the immunolocalization of *c-kit* and PCNA in the oviduct, high magnification images for *c-kit* receptor (Fig. 4, a) and PCNA (Fig. 4, c), indicated by a hollow triangle, are shown in the bottom left areas of figures. No immunostaining was detected in control sections in which the primary antibody was absorbed with an excess of antigen peptides (e, f). EC: epithelial cell. ST: stroma tissue. The scale bars represent 50 μ m.

period (Fig. 4). The positive staining for *c-kit* receptor was also observed in the cytoplasm of stroma cells (Fig. 4a) and epithelial cells (Fig. 4b). The immunostaining of PCNA was also present in the nuclei of stroma cells (Fig. 4c) and epithelial cells (Fig. 4d). No immunoreactivity was detected in control sections in which the antiserum was preabsorbed with an excess of antigen peptides (Fig. 4e, f).

The results of Western blotting analysis for the *c-kit* receptor and PCNA in the oviduct tissues are shown in Fig. 5. Positive immunodetection of *c-kit* receptor was identified in proteins extracted from oviducts. A positive signal of PCNA was also detected in proteins extracted from ovi-

Fig. 5. Western blotting of the *c-kit* receptor and PCNA in the oviduct tissues of *Rana chensinensis* during the prehibernation period. The left side shows the 10% range prestained SDS-PAGE standards (lane M). Total protein was extracted from whole oviduct tissue obtained during prehibernation. (lane Oct.). Preabsorption of the antiserum with an excess of antigen peptides was used for the negative control on the right side (lane N.C.). The positive bands of *c-kit* receptor and PCNA were observed in the positions of approximately 150 and 37 kDa, respectively.

ducts. No immunostaining was detected for the *c-kit* receptor and PCNA when using preabsorption of the antiserum with an excess of antigen peptides (Fig. 5, lane N.C.). The *c-kit* receptor and PCNA antibodies detected proteins in oviduct tissues that migrated to a position of approximately 150 and 37 kDa.

The present results clearly demonstrated that immunoreactivity for the *c-kit* receptor was present in oviducal epithelial cells and stroma cells of *R. chensinensis* during the prehibernation period. The presence of the *c-kit* receptor protein was confirmed by Western blotting analyses carried out in the enlarged oviduct tissues. These findings suggested that the *c-kit* receptor may play a regulatory role in oviducal hypertrophy. The present results provided a basis for future detailed investigation of regulatory factors in oviducal hypertrophy.

Ovarian estrogens control development of sex accessory structures such as the hypertrophy of oviduct prior to sexual maturation and during each season prior to ovulation. The oviducts regress when estrogen synthesis declines after breeding [9]. In this study, a special physiological phenomenon, the hypertrophy of *R. chensinensis* oviducts, occurred during the prehibernation period. Histological observation indicated that epithelial cells and a large number of stromal cells were observed in oviducal tissues expanded during the prehibernation period. These findings implied that the

hypertrophy of the oviduct was independent of ovarian steroid hormones, which could mean that locally produced intrinsic regulators might play a role in the oviducal hypertrophy of *R. chensinensis*. Cell growth and differentiation are controlled by a number of regulatory factors, including steroid hormones and peptide growth factors [30]. It is becoming increasingly evident that nuclear proto-oncogenes play a key role in coordinating steroid hormone actions in different tissues [30]. It is well documented that the *c-kit* receptor has important roles in the reproductive system because it is involved in almost all aspects of development and growth, especially cell survival, proliferation, differentiation and migration [2, 4, 11, 20, 21, 23, 25]. Evidence from humans also indicated that *c-kit* receptor was expressed in interstitial cells in the human fallopian tube [34]. The present study was the first demonstration of the expression of the *c-kit* receptor in the oviduct of vertebrates besides humans. Aberrant ligand-independent activation of the *c-kit* receptor has been implicated in the development and progression of tumors, in particular those of stroma origin [10, 17]. Although we do not understand the importance of the *c-kit* receptor in reproduction of *R. chensinensis*, the results of this study showed that the *c-kit* receptor was present in oviduct tissues expanded during the prehibernation period. This suggested that as the intrinsic regulator, the *c-kit* receptor might play a regulatory role in oviducal hypertrophy.

The *c-kit* receptor is a higher conservative molecule, and its cDNA has been isolated in other vertebrates, such as the chicken [31], *Xenopus* [3, 15] and zebrafish [28]. In this study, we carried out immunohistochemistry for *c-kit* receptor protein in oviduct tissues using a rabbit polyclonal anti-*c-kit* protein antibody, and the results showed that the *c-kit* receptor protein was predominantly localized in epithelial cells and stroma cells during the prehibernation period. To test our immunohistochemical results, we also performed Western blot analysis of the *c-kit* protein in an oviducal extract of *R. chensinensis*, and the results indicated that the polyclonal antibody recognized a single protein of about 150 kDa in the oviduct tissue of this species. Similar evidence has also been reported in *Rana esculenta*; that is, the *c-kit* receptor was present in testicular tissues, and the *c-kit* antibody recognized a protein band of a similar size, approximately 150 kDa, in the germinal epithelium [29]. The most commonly used method to evaluate cellular proliferation is PCNA immunostaining. PCNA is the auxiliary subunit of DNA polymerase delta and is also involved in several additional processes in the nucleus, most notably DNA repair [24]. In the present study, to identify further oviducal epithelial cell and stroma cell proliferation and differentiation in *R. chensinensis* during the prehibernation period, oviducal tissues were also evaluated by both Western blotting and immunohistochemical detection of PCNA. The positive expression of PCNA suggested that mitotic activity of oviducal epithelial and stroma cells of *R. chensinensis* occurs during the prehibernation period. Taken together, these results indicated that the intrinsic regulators including the *c-*

kit receptor might play a regulatory role in oviducal cell proliferation and differentiation in *R. chensinensis*. Future studies will be necessary to investigate steroid hormone concentrations and differential expression of the *c-kit* receptor during the pre- and posthibernation periods.

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