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by Gratiana E. Wijayanti

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The Exression Of The C-Kit Protein In The Germline Of A Marsupial

Wijayanti, G.E.[1], G. Shaw[2] and M. B. Renfree[2]
[1]Faculty of Biology Jenderal Soedirman University, Purwokerto, Indonesia
[2]Department of Zoology, the University of Melbourne, Parkville, VIC, Australia
e-mail: gratiana.wijayanti@unsoed.ac.id or gatiana_wij@lycos.com

Introduction

The *Kit* gene encodes a receptor (c-kit receptor) belonging to the family of type III transmembrane tyrosine kinase receptors and its transcript encodes a 975 amino acid protein which may be glycosylated. The mature protein has a molecular mass ranging from 125 to 160 kDa (Dubreuil *et al*., 1990; Fleischman, 1993). The kit ligand, Mast cell growth factor (also known as Stem cell factor), cDNA encodes a protein of 248 amino acids (Williams *et al*., 1992). The c-kit receptor and its ligand play a role in the transduction of extracellular signals and are known to control cell proliferation, cell survival, motility and differentiation (Besmer *et al*., 1993).

Mutations in the murine *white spotting* and *steel* loci (corresponding to *c-kit* and *Steel* respectively) result in deficiencies of three cell lineages, including the pigmentary system, haematopoetic system and germ cells during embryogenesis and in post natal animals (Russell, 1979; Silvers, 1979).

In the normal mouse, there are *c-kit* transcripts in the primordial germ cells as early as E7.5 (Manova and Bachvarova, 1991). Expression of *c-kit* is high during the proliferative phase, E7.5 to E13. As germ cells enter mitotic arrest or meiotic prophase, *c-kit* receptor expression is no longer detected (Manova and Bachvarova, 1991; Bachvarova *et al*., 1993). *C-kit* receptor is re-expressed in oocytes at a high level at all stages of postnatal development starting in the diplotene stage (Manova *et al*., 1990). In the postnatal testis, *c-kit* transcripts are detected in Leydig cells, spermatogonia, primary spermatocytes and round spermatids (Manova *et al*., 1990, 1993; Rossi *et al*., 1992). Round spermatids contain mRNA encoding a truncated protein consisting of only part of the intracellular domain (Albanesi *et al*., 1996). The c-kit protein has been detected on both Leydig cells and spermatogonia (Manova *et al*., 1990, 1993; Yoshinaga *et al*., 1991) and synthesis of the truncated protein in post meiotic cells has also been reported (Albanesi *et al*., 1996).

In sheep, Tisdall *et al*. (1999a) reported *c-kit* expression during the early stages of oogenesis and showed that its messenger RNA is expressed as early as embryonic day 24. Expression stays high until embryonic day 55 and stops when meiosis starts. These results are in a good agreement with an earlier report that used *in situ* hybridization to show that meiotic germ cells do not express *c-kit*, while oocytes that have reached the diplotene stage of meiosis and are enclosed in primordial follicles re-initiate *c-kit* expression (Clark *et al*., 1996).

In the brushtail possum (*Trichosurus vulpecula*), *c-kit* gene expression is localized in the germ cells and somatic cells during the first 15 days of life and later at day 30 and into adult life, *c-kit* expression is restricted to the germ cells (Eckery *et al*., 2002). It is not clear whether *c-kit* is also expressed during prenatal life in the brush-tail possum.

The c-kit gene has not been characterized in the tammar wallaby. It would be interesting to know whether the c-kit receptor expressed in a similar pattern to those in the brushtail possum. The aim of this study was therefore to examine the presence of c-kit protein in tammar germ cell in pre and postnatal development.

Materials and Methods

Animals

Tammar wallaby tissues ranging from fetuses (n=7), pouch young (25) to adult (3) gonads were used in this study. For fetuses, only sections through the gonadal region were stained. For post-natal gonads, sections through the mid region of the gonad were used. The age groups reflect the various stages of ovarian development in the tammar as described by Alcorn and Robinson (1983). Briefly, in the female tammar, during the first 22 days of pouch life the ovary differentiates and develops a cortex and medulla. From days 23-50 post-partum is the proliferative phase and the onset of meiosis. Days 51-110 encompass the degenerative stage, where there is large scale degeneration of oocytes. Folliculogenesis also begins at this time, and is essentially complete by day 110. Males were also examined at similar stages.

Localization of c-kit protein in the tammar testes and ovaries

Bouin's-fixed paraffin embedded sections were deparaffinised, rehydrated then subjected to antigen unmasking in 50mM Glycine pH 3.5 using a microwave oven for 10 minutes. After cooling to room temperature and rinsing in water, the sections were then incubated with 3% H_2O_2 in water to block any endogenous peroxidase activity. Following three times washing in TBS, the sections were incubated with 5% normal goat serum in TBS with 0.1% BSA for 40 min at room temperature. The sections were then incubated with polyclonal rabbit anti c-kit (C-19, SC-168, Santa Cruz Biotechnologies Inc.) at a 1:20 or 1:40 dilution in TBS with 0.1%BSA overnight at room temperature. After three washes in TBS, the sections were incubated with the secondary antibody biotinylated goat anti rabbit, at a 1:500 dilution for 40 min. Antigen-antibody binding was detected using Avidin-biotin Complex/HRP (Vectastain, Vector Laboratory) according to the manufacturers instruction and visualized using 0.6 mg/ml DAB with 0.03% H_2O_2. The sections were counterstained with Harris's haematoxylin, dehydrated, cleared and mounted in DPX. Negative control sections were processed simultaneously with the test sections and treated as the test sections but the primary antibody was replaced by normal goat serum.

Results

The earliest appearance of c-kit in the tammar was in the placenta of late fetuses. Germ cells from neonates were c-kit negative but from day one post partum they had pale cytoplasmic staining (Figure 1).

In the ovaries, by the end of the first week post partum germ cells had dark pericellular staining with paler cytoplasmic staining (Figure 1a-b). Reactivity in the germ cells was markedly reduced as they entered meiosis but increased again in oocytes in meiotic arrest. In the adult ovary there was pale to moderate cytoplasmic staining in oocytes in follicles of all developmental stages and pale cytoplasmic reactivity in luteal cells, thecal cells and granulosa cells adjacent to the theca. Pre-treatment of ovary sections with citrate buffer instead of glycine buffer intensified germ cell staining but did not alter the overall temporal or spatial distribution of c-kit within germ cells. It did, however, yield stromal staining not observed with antigen retrieval using glycine buffer. Such staining was observed in cells within the medulla of a day 19 post-partum ovary and in stromal cells surrounding germ cell nests in day 46 and 52 post-partum ovaries. In contrast to glycine-treated sections, granulosa cells in adult ovaries treated with citrate buffer were negative.

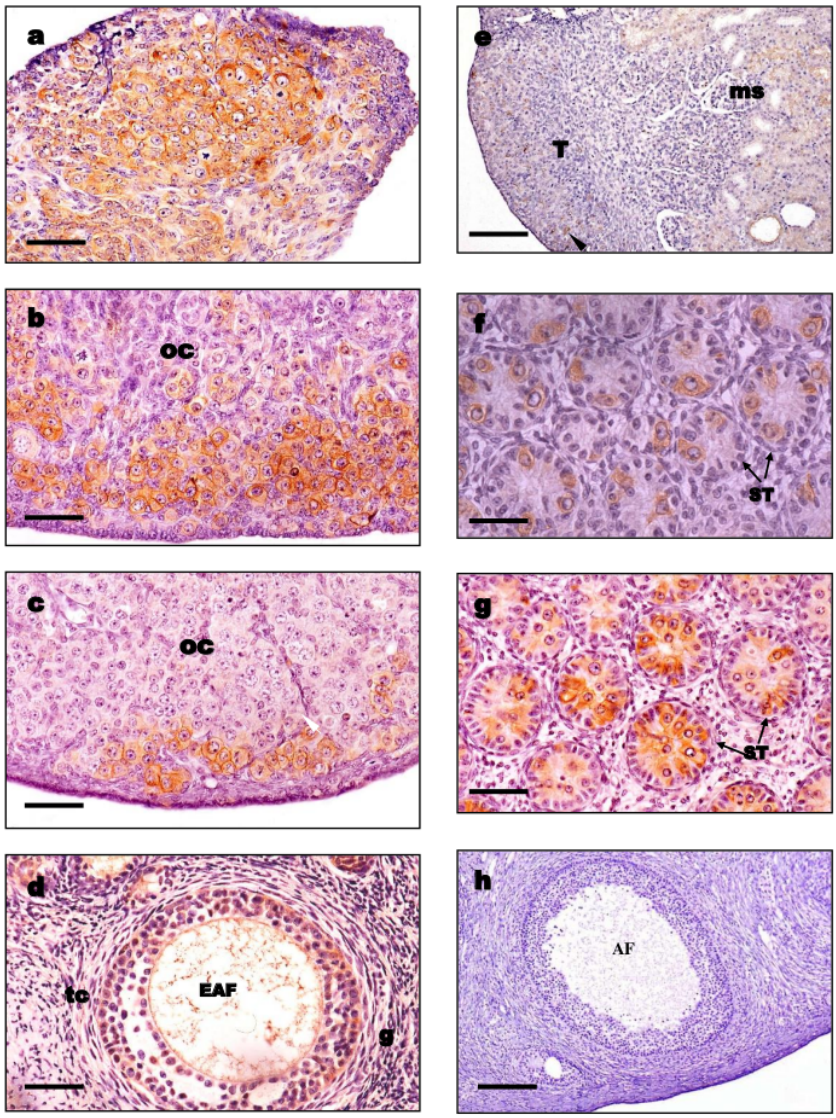

Figure 1. c-kit immunostaining in ovaries and testes of the tammar wallaby. (a) day 10 pp ovary. All oogonia were stained with the c-kit antibody as indicated by the brown staining; (b) day 21 pp ovary, in which most of the oogonia were strongly stained; (c) day 31pp ovary. Only a few oogonia at the periphery of the ovarian cortex were positive; (d) adult ovary. Oocytes of early antral follicles were stained with the c-kit antibody. The granulosa and thecal cells were also stained lightly; (e) day1 pp testis showing a few immunoreactive germ cells (arrow); (f,g) day 11 and 26 pp testes showing all of the gonocytes were immunoreactive but none of the Sertoli cells were positive. (h) The negative control ovary shows no staining. Abbreviations: oc= ovarian cortex; tc= theca; EAF= early antral follicle; T= testis; ms= mesonephros; ST=seminiferous tubules. Scale bar represents: 20 μm in (a-d, f,g), 5 μm in (e,h).

In the testes the number of positive germ cells and the staining intensity increased during the first four weeks of pouch life (Figure 1e-g) but then decreased until

by the ninth week there was little staining. Some testes from a variety of ages exhibited very pale, ubiquitous staining in almost all cells of the seminiferous tubules and often staining in the germ cells was not above this. In the adult testis small numbers of spermatogonia against the basement membrane had dark cytoplasmic staining and in some seminiferous tubules, round and elongating spermatids had pale indistinct cytoplasmic staining. Leydig cells were very positive. Pre-treatment of tissues with citrate buffer (pH 6.0) instead of glycine buffer (pH 3.5) reduced the background staining in the seminiferous tubules and accentuated staining in the germ cells.

Discussion

The timing of c-kit immunoreactivity in tammar germ cells was comparable to the timing of another c-kit antibody from Santa Cruz (M-14) which was reported to cross react with oogonia and oocytes at 14 –21 weeks of pregnancy and gonocytes of 13-19 weeks of pregnancy in humans (Robinson *et al*., 2001). The immunoreactivity of c-kit (M-14) with human germ cells before 13 weeks of gestation has not been examined. In the mouse, the presence of c-kit during the migratory phase was based on c-kit mRNA expression (Manova and Bachvarova, 1991). The protein is absent from migratory PGCs and first appears in oocytes on the day of birth (Manova *et al*., 1990; Horie *et al*., 1991; Yoshinaga *et al*., 1991), similar to the tammar. In the sheep, the c-kit protein could be detected in the ovary even when its mRNA transcript was not detected (Tisdall *et al*., 1999).

The present of c-kit receptor protein during the proliferative stage of tammar germ cell, suggested that the c-kit receptor may play a role in germ cell proliferation. The importance of c-kit and its ligand, SCF, on germline development was first observed in mice with mutations on White spotting (*W*) or Steel (*Sl*) genes. These mutants are germ cell deficient (Mintz and Russell, 1957). Since then, much research has been conducted to examine the exact function of these two genes. *In vitro* and *in vivo* experiments showed that c-kit and SCF interaction is important for germ cell proliferation (Yoshinaga *et al*., 1991; Tajima *et al*., 1994), germ cell survival (De Felici *et al*., 1992; Dolci *et al*., 1993; Pesce *et al*., 1996), and germ cell adhesion to somatic cells (Marziali *et al*., 1993; Pesce *et al*., 1997).

Functional assays to evaluate the function of c-kit (SCF signal transduction pathway) have been conducted by introducing an antibody that blocks binding of SCF to the c-kit receptor *in vitro*. The exposure of cultured ovaries and testes to 5 µg/ml to 10 µg/ml of anti c-kit antibody for 4 or 8 days significantly decreased mitotic activity in the ovaries (Wijayanti, 2003). This is consistent with a physiological role for c-kit in stimulating mitoses in the gonad. The mechanism for how c-kit/SCF interaction promotes mitosis in germ cells is not yet clear. Reynoud *et al* (2000) proposed that c-kit/SCF interaction produces mitotic effects on the granulosa cells of adult ovaries by inducing formation of proliferating factors such as GDF-9. Further studies need tobe conducted to elucidate the mechanism on c-kit receptor protein in controlling germ cell proliferation in the tammar wallaby.

Conclusion

The anti- c-kit antibody SC-168 only cross-reacted with post-natal tammar germ cells. The strongest immunoreaction was detected after the first day of pouch young life until the onset of first meiosis, which is coincident with the active proliferative stage of germ cell development in the tammar.

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