Journal of Histochemistry & Cytochemistry 61(7) 522–528

DOI: 10.1369/0022155413486159
jhc.sagepub.com
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Crlz-1 Is Prominently Expressed in Spermatogonia and Sertoli Cells during Early Testis Development and in Spermatids during Late Spermatogenesis

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Summary
The expression of the *Crlz-1* gene in mouse testis, where it was found to be expressed most highly among the tested mouse organs, was analyzed spatiotemporally by employing RT-PCR and in situ hybridization techniques with the aid of immunohistochemistry and/or immunofluorescence methods. In 1-week-old neonatal testis, *Crlz-1* was strongly expressed in the spermatogonia and Sertoli cells in its seminiferous cord. In 2- to 3-week-old prepubertal testis, where Sertoli cells cease to proliferate, *Crlz-1* expression dropped and remained weakly at the rim layer of seminiferous cords and/or tubules, where spermatogonia are present. In the adult testis at 12 weeks after birth, *Crlz-1* was expressed mainly in the spermatids near the lumen of seminiferous tubules. In a further in situ hybridization of *Crlz-1* in the 12-week-old adult testis with hematoxylin nuclear counterstaining, *Crlz-1* was mainly expressed at step 16 of spermatids between stages VII and VIII of seminiferous tubules as well as in their residual bodies at stage IX of seminiferous tubules. (J Histochem Cytochem 61:522–528, 2013)

Keywords
Crlz-1, Sertoli cell, spermatogonia, spermatid, seminiferous tubule

Crlz-1 (charged amino acid–rich leucine zipper-1), also named *UTP3* (U three protein 3) in a bioinformatic search, was originally cloned by its ability to bind to core binding factor β (CBFβ) (Sakuma et al. 2001), which is known to heterodimerize with Runx (runt-related transcription factor) to increase its DNA-binding affinity (Gu et al. 2000). Previously, we reported that *Crlz-1* is expressed specifically in the pre–B-cell stage of B-cell development (Lim et al. 2006) and mobilizes cytoplasmic CBFβ to the nucleus to allow nuclear Runx to heterodimerize with it (Park et al. 2009). The heterodimer of Runx/CBFβ has been known to be important in many developmental processes such as hematopoiesis (de Bruijn and Speck 2004), osteogenesis (Stein et al. 2004), and epithelial cell proliferation (Bae and Choi 2004). Recently, *Crlz-1* was also found to be a target gene of the Wnt (wingless-related MMTV integration site)/β-catenin signaling pathway as its promoter was bound by lymphoid enhancer factor-1 (LEF-1) (Park et al. 2011) together with β-catenin as a binding partner (unpublished result).

In a further attempt to obtain additional clues about the functional roles of the *Crlz-1* gene, we examined its expression levels in various mouse organs by Northern blot analysis. Among several tested mouse organs, testis was found to express *Crlz-1* most highly, verifying the performance of a

Received for publication November 19, 2012; accepted March 14, 2013.

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further temporal and spatial analysis of its gene expression pattern in testis.

In testis, there are three major types of cells—germ cells, Sertoli cells, and Leydig cells. Sertoli cells, which support the spermatogenic development of germ cells within the seminiferous tubules of testis, are known to begin to proliferate during fetal development and then cease to do so at about 2 weeks after birth in mice (Walker 2003). Coincidently, with the cessation of proliferation, Sertoli cells start to differentiate, with their morphological changes including the production of secreted proteins as well as the formation of tight junctions between them. On the other hand, spermatogonia germ cells continually divide not only to renew themselves but also to generate spermatozoa through the steps of spermatocytes and spermatids in the meiotic process of spermatogenesis (Ahmed and de Rooij 2009). Spermatids undergoing spermiogenesis, including spermiation, which is the final differentiation process of spermatogenesis, experience extensive morphological changes, including the formation of acrosome and tail, and their excess cytoplasm forming the residual bodies (Lee et al. 2011). Leydig cells, also known as interstitial cells of Leydig, are found between the seminiferous tubules in testis. They produce testosterone in the presence of luteinizing hormone.

In this article, we report that *Crlz-1* was highly expressed in the proliferating spermatogonia and Sertoli cells in the seminiferous cords during early mouse testis development at neonatal ages and, thereafter, it was weakly expressed around at the rim layers of seminiferous cords and/or tubules, where spermatogonia are present. However, during late spermatogenesis at adult ages, *Crlz-1* was expressed mainly in the differentiating spermatids at the luminal side of the seminiferous tubules. Although the physiological significance of these expression patterns of the *Crlz-1* gene during testis development remains to be answered in the future, their potential roles have been discussed with regard to both the Wnt/β-catenin signaling pathway and Runx/CBFβ transcriptional activity.

Materials and Methods

Mice

Mice (FVB strain) were obtained from KOATECH (Pyeongtaek, Korea) and raised until use in the conventional animal care room of the university. All mice experiments were performed with the approval (KHUASP (SU)-13-D) of Kyung Hee University Institutional Animal Care and Use Committee and thereby according to its guidelines.

Northern Blot

Total RNA from the various mouse tissues were obtained using TRIzol reagent (Life Technologies; Carlsbad, CA) and electrophoresed on a 1% formaldehyde agarose gel. Northern blot was performed with the random primer-^{32}P-labeled *Crlz-1*, as well as *G3PDH* cDNA probes, following basically the same procedure as described in the molecular cloning: a laboratory manual (Green and Sambrook 2012).

Reverse Transcriptase–Coupled PCR

Testes were dissected, rinsed in cold PBS, and homogenized in TRIzol reagent (1 ml TRIzol per 100 mg tissue) using a pestle (CLS-5002-005; Chemglass Life Sciences, Vineland, NJ). The homogenate was cold-centrifuged at 12,000 × g for 10 min, and its supernatant was transferred into a new tube. Chloroform (0.2 ml of chloroform per 1 ml TRIzol reagent) was added to the tube, which was shaken vigorously for 15 sec and then incubated at room temperature for 2 min. The tubes were cold-centrifuged at 12,000 × g for 15 min, and the upper aqueous phase was transferred into a new tube. Isopropyl alcohol (0.5 ml isopropanol per 1 ml TRIzol) was added to the tubes, mixed well and then incubated at room temperature for 10 min. The tubes were cold-centrifuged at 12,000 × g for 10 min, and the precipitate was rinsed with 1 ml 75% ethanol. The precipitate was treated with RQ1 RNase-free DNase (M610A; Promega, Fitchburg, WI) at 37C for 30 min to exclude any DNA contamination. RNA was finally extracted by phenol/chloroform, precipitated by absolute ethanol, and rinsed with 75% ethanol. Then, 1 μg of this RNA precipitate was reverse-transcribed into cDNA and amplified using the selected primers in a PCR. The primers for *Crlz-1* were 5′-CTATTTTAGCAATCTGGTAGGTAATG (forward) and 5′-CATCCTGTTATAGAAAGGCTTGTTACC (reverse), and the primers for β-actin were 5′-TGAACCCTAAGGC CAACCGTG (forward) and 5′-GCAGCTCATAGCTCT TCTCCAGGG (reverse).

In Situ Hybridization and Immunohistochemistry

Testes were dissected from mice, rinsed in cold PBS, and then fixed in 4% paraformaldehyde in PBS on a rocker at 4C for 3 hr to overnight. The fixed testes were incubated in PBS containing 15% sucrose and then in PBS containing 30% sucrose at 4C until they sank to the bottom. The testes were then embedded with OCT compound (TissueTek 4583; Sakura, Torrance, CA) and stored frozen in a sealed container at −70C. Testis sections (5–10 μm) were prepared onto the RNase-free slides using a cryo-microtome, dried at room temperature for 1 hr, and stored at −20C. The prepared slides were fixed in 4% paraformaldehyde at room temperature for 10 min, washed twice with cold PBS for 5 min each time, and then treated with proteinase K (50 ng/ml) in a PK buffer (50 mM Tris-HCl [pH 7.5], 5 mM EDTA) at room temperature for 10 min.

For in situ hybridization (ISH), the treated slides were washed three times with PBS, each for 10 min, fixed again

in 4% paraformaldehyde at room temperature for 5 min, and washed three times with PBS, each for 10 min. For the hybridization reaction, 20 µl of hybridization solution (50% formamide, 5× Denhardt's solution, 5× saline sodium citrate [SSC], 250 µg/ml yeast tRNA, 500 µg/ml sonicated salmon sperm DNA) was dropped onto the slides and the slides were preincubated in a humidified box at 50C for 2 hr to overnight. The preincubated hybridization solution was removed and replaced with fresh hybridization solution containing the digoxygenin-labeled *Crlz-1* RNA probe, and the hybridization reaction was continued at 50C overnight. The hybridized slides were sequentially washed twice in 1× SSC at 60C for 20 min each, and twice in 2× SSC at 37C for 10 min each. The slides were then treated with 20 µg/ml RNase A in NTE buffer (500 mM NaCl, 10 mM Tris-HCl [pH 8.0], 1 mM EDTA) at 37C for 30 min, sequentially washed once in 2× SSC at room temperature for 10 min and twice in 0.2× SSC at 60C for 30 min each, and finally rinsed in PBST (PBS with 0.1% Triton X-100) at room temperature for 10 min. The rinsed slides were blocked with 5% sheep serum in PBST at 4C for 1 hr to overnight. The blocking solution was removed and replaced with fresh blocking solution containing alkaline phosphatase (AP)–conjugated anti-digoxygenin Fab fragment antibody (11-093-274-910; Roche, Basel, Switzerland). These slides were incubated in a humidified box at 4C overnight, washed three times in PBST at room temperature for 30 min each, and rinsed in the AP buffer (100 mM Tris-HCl [pH 9.5], 50 mM $MgCl_2$, 100 mM NaCl, 0.1% Tween 20) containing 5 mM fresh levamisole at room temperature for 5 min. The AP reaction was performed using BCIP/INT (BB0072/0280; BioBasic, Markham, Canada) in the AP buffer containing 5 mM fresh levamisole at room temperature. The AP reaction was stopped in an appropriate time with a solution consisting of 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA, and the samples were mounted using ImmunohistoMount (sc-45086; Santa Cruz Biotechnology, Santa Cruz, CA) for microscopy.

For immunohistochemistry, the frozen tissue sections were rinsed with PBST and blocked with PBST containing 5% goat serum (sc-2043; Santa Cruz Biotechnology) at room temperature for 1 hr. The blocked tissue sections were incubated with rabbit anti-VASA antibody (ab13840; Abcam, Cambridge, UK) at room temperature for 2 hr, washed three times with PBST at room temperature for 5 min each, and then incubated with AP-conjugated goat anti–rabbit IgG (sc-2007; Santa Cruz Biotechnology) at room temperature for 1 hr. Tissue sections were washed three times with PBST at room temperature for 5 min each, and reacted with AP substrates following the same procedure as for the ISH above.

To perform both the ISH for *Crlz-1* and immunofluorescence (IF) for *VASA* or *MIS* on the same tissue sections, after the above hybridization step of ISH for *Crlz-1* without proteinase K treatment, the tissue sections were blocked with 5% donkey serum in PBST at 4C for 1 hr. The blocked tissues were incubated with sheep anti-digoxigenin (11-093-274-910; Roche) and rabbit anti-VASA (ab13840; Abcam) or goat anti-MIS antibodies (sc-6886; Santa Cruz Biotechnology) at 4C overnight. The tissue sections were washed three times with PBST at room temperature and incubated with Alexa Fluor 633 donkey anti–sheep IgG (A-21100; Life Technologies, Carlsbad, CA) and Alexa Fluor 488 donkey anti–rabbit IgG (A-21206; Life Technologies) or Alexa Fluor 488 donkey anti–goat IgG antibodies (A-11055; Life Technologies) at room temperature for 1 hr. Finally, the tissue sections were washed three times with PBST at room temperature for 5 min each and mounted using DAKO fluorescent mounting medium (S3023; DAKO, Glostrup, Denmark) for fluorescence microscopy (DMI 6000B; Leica, Wetzlar, Germany).

Preparation of Digoxigenin-Labeled Crlz-1 *RNA Probe for ISH*

The 201-bp *Stu*I DNA fragment, which is positioned between +505 nt and +705 nt from the transcription start site of the *Crlz-1* gene (Park et al. 2011), was cloned into the klenow-blunted *Dra*II site of the pBluescript II KS(+) plasmid (Stratagene; La Jolla, CA). Two plasmid clones of oppositely oriented inserts were obtained. The two plasmids were then linearized by *Xho*I and used to make the sense and antisense RNA probes by T3 RNA polymerase (P208C; Promega) with digoxigenin-11–UTP RNA labeling mix (11277073910; Roche). Both the sense and antisense RNA probes were purified by spin columns (17072; Intron, Seongnam, Korea) and verified by gel electrophoresis and dot blot hybridization.

Results

Crlz-1 was expressed most highly in the testis among the tested mouse organs (Fig. 1). To analyze further this highest expression of *Crlz-1* in testis, we traced both temporally and spatially the expression of *Crlz-1* within the dissected testis organ.

In a temporal RT-PCR analysis of whole testis after birth, *Crlz-1* was expressed highly at 1 week, whereas its expression dropped to remain weak from 2 weeks after birth (Fig. 2).

For a further temporal and spatial analysis of *Crlz-1* gene expression in testis, the ISH technique was employed to localize *Crlz-1* expression within the testis; this was because a suitable antibody was not available for immunohistochemistry. However, additional immunohistochemical experiments were also performed using the same or adjacent tissue sections of testis to localize the expression of *VASA* or *MIS* as a marker for germ cells or Sertoli cells, respectively. A strong expression of *Crlz-1* was localized in the proliferating spermatogonia and Sertoli cells in the seminiferous cord of 1-week-old neonatal testis, where spermatogonia were the

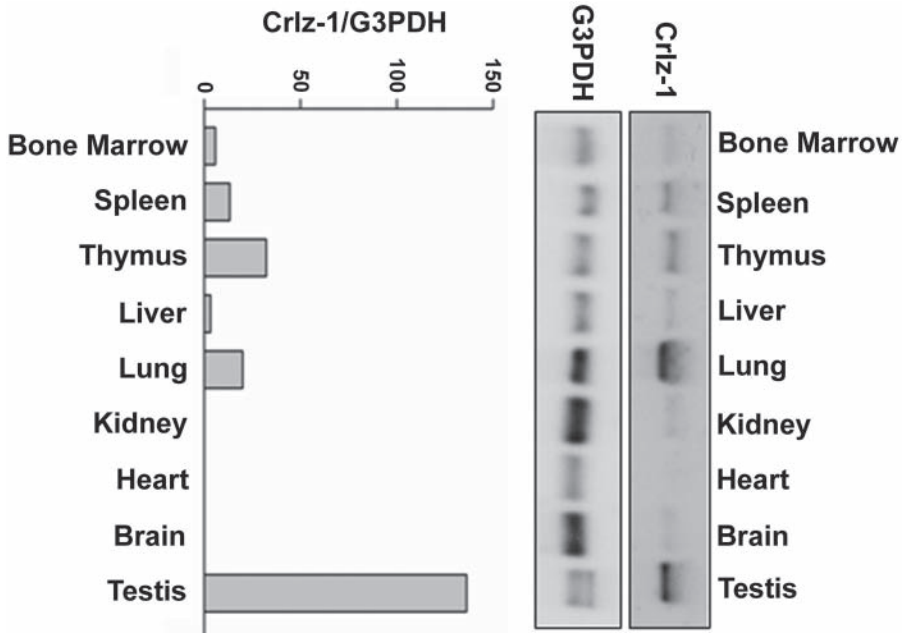


Figure 1. *Crlz-1* was expressed most highly in the testis among the tested mouse organs. Total RNA from various organs was separated by 1% formaldehyde agarose gel electrophoresis, transferred to nitrocellulose membrane, and probed with the radioactive ^{32}P-labeled *Crlz-1* or *G3PDH* cDNA. The expression of *Crlz-1* was normalized by the expression of *G3PDH* as a housekeeping gene control and graphed in terms of *Crlz-1*/*G3PDH*.

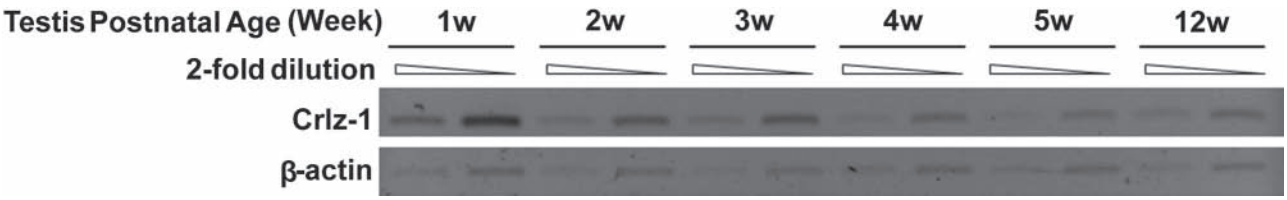
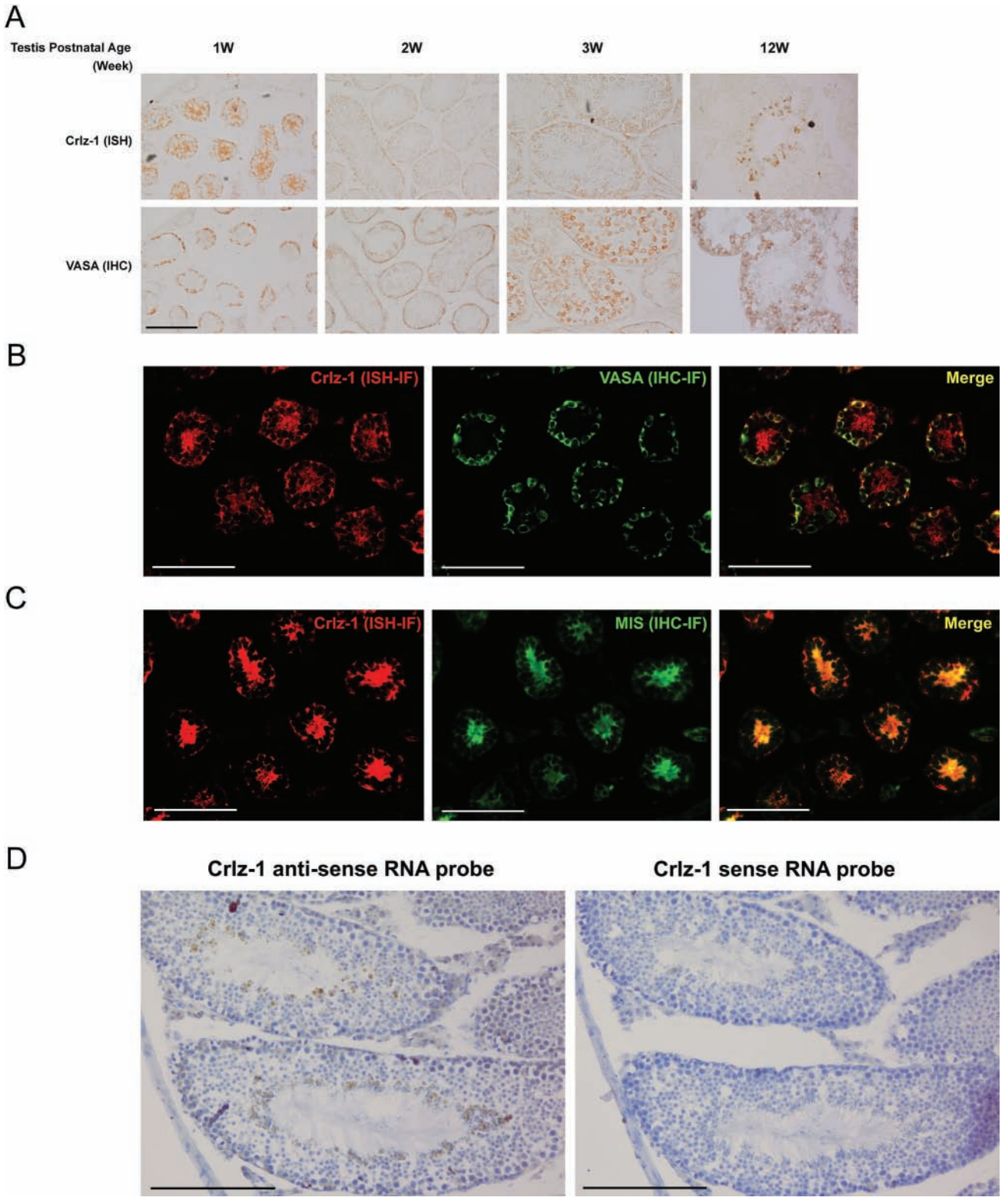


Figure 2. The expression of *Crlz-1* in testis was highest at 1 week after birth and remained weak thereafter. Total RNA was extracted from the whole mouse testis at various ages and analyzed for *Crlz-1* expression by reverse transcriptase–coupled PCR. The linearity of PCR was ensured by performing PCR using 2-fold diluted samples. β-actin was included as a loading control.

only germ cells at the rim layer of seminiferous cords, as verified by the expression of *VASA* (Fig. 3A).

In the additional immunofluorescence (IF) experiments using *VASA* and *MIS*, the cells where *Crlz-1* was expressed were shown indeed to be spermatogonia and Sertoli cells by colocalization with *VASA* and *MIS*, which were found at the rim layer and within the core of the seminiferous cord, respectively, in 1-week-old neonatal testis (Fig. 3B, C). In 2- to 3-week-old prepubertal testis, where Sertoli cells cease to proliferate, *Crlz-1* expression dropped only to remain weakly at the rim layer of seminiferous cords and/or tubules, where spermatogonia are present (Fig. 3A), correlating well with the RT-PCR results of Fig. 2. In the adult testis at 12 weeks after birth, *Crlz-1* was expressed mainly in the spermatids near the lumen of seminiferous tubules (Fig. 3A). Fig. 3D shows the negative control ISH image with the sense RNA probe of the *Crlz-1* gene and hematoxylin nuclear counterstaining in 12-week-old mouse testis; this verifies the validity of the antisense RNA probe for the *Crlz-1* gene in our ISH experiments.

We then performed ISH experiments with the hematoxylin nuclear counterstaining using the 12-week-old adult testis to define the *Crlz-1*–expressing steps of spermatids as well as stages of seminiferous tubule development. As judged by the size and shape of germ cells, their location and composition within each seminiferous tubule, and the architecture of each seminiferous tubule (Kotaja et al. 2004; Ahmed and de Rooij 2009), *Crlz-1* was mainly expressed at step 16 of spermatid formation between stages VII and VIII of seminiferous tubule development as well as in their residual bodies at stage IX of seminiferous tubule development (Fig. 4).


A
Testis Postnatal Age (Week)
1W
2W
3W
12W
Crlz-1 (ISH)
VASA (IHC)
B
Crlz-1 (ISH-IF)
VASA (IHC-IF)
Merge
C
Crlz-1 (ISH-IF)
MIS (IHC-IF)
Merge
D
Crlz-1 anti-sense RNA probe
Crlz-1 sense RNA probe

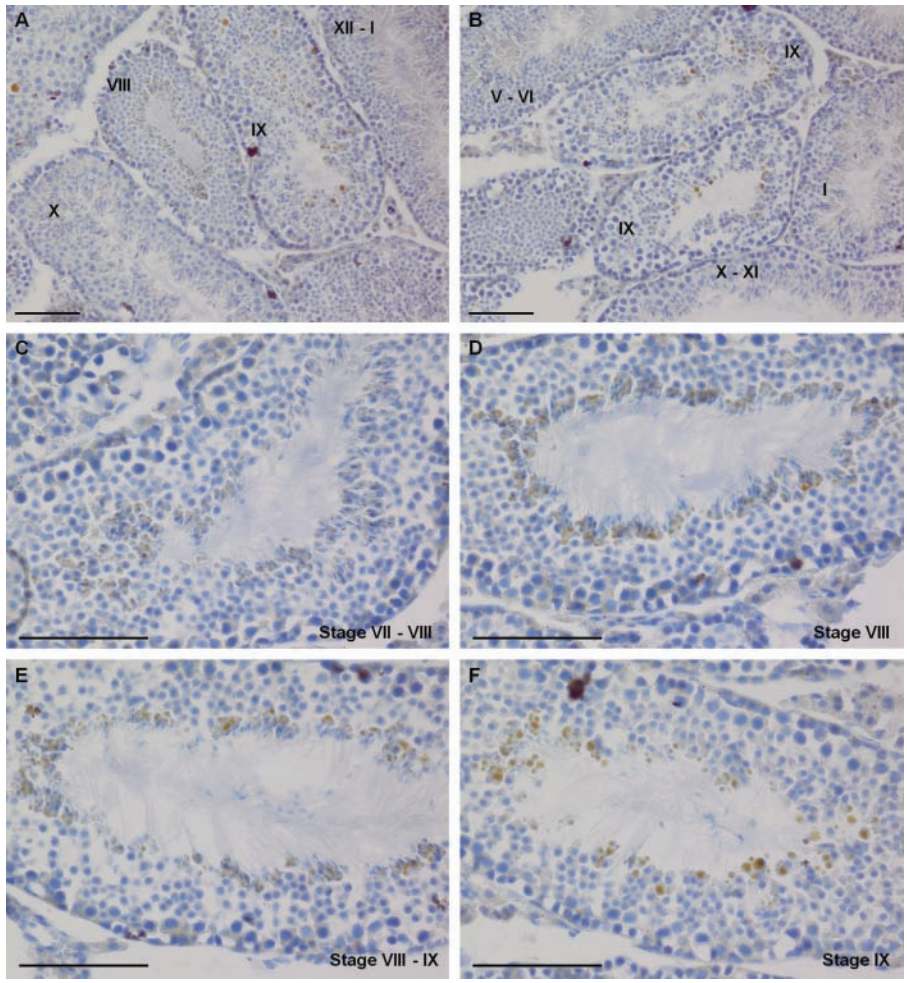


Figure 4. *Crlz-1* was mainly expressed at step 16 of spermatids between stages VII and VIII of seminiferous tubules as well as in their residual bodies at stage IX of seminiferous tubules in the adult testis. *Crlz-1* in a tissue section of 12-week-old adult testis was detected by the in situ hybridization technique as in Fig. 3. The steps of spermatid formation as well as the stages of seminiferous tubule development were defined with the hematoxylin nuclear counterstaining, which is blue, and represented by Arabic and Roman numerals, respectively (Ahmed and de Rooij 2009). Panels A and B show two different tissue sections of 12-week-old mouse testis consisting of various stages of seminiferous tubules, whereas panels C to F show the seminiferous tubules at stages VII to IX, the images of which were captured from other tissue sections of 12-week-old mouse testis. Scale bars: 100 μm.

Figure 3. The spatiotemporal expression patterns of the *Crlz-1* gene in the developing mouse testis were examined by in situ hybridization. (A) *Crlz-1* was expressed highly in spermatogonia and Sertoli cells of 1-week-old neonatal testis as well as in spermatids of 12-week-old adult testis, whereas a weak expression of *Crlz-1* was found at the rim layer of seminiferous cords and/or tubules of 2- to 3-week-old prepubertal testis, where spermatogonia are present. *Crlz-1* (ISH) indicates the use of in situ hybridization to detect the expression of *Crlz-1*. *VASA* (IHC) indicates the use of immunohistochemistry to detect the expression of *VASA*, which is known to be expressed specifically in the germ cells. The signals of *Crlz1* (ISH) and *VASA* (IHC), which were performed using adjacent tissue sections, were obtained using alkaline phosphatase (AP) and BCIP/INT as a substrate, which gave a brick-red product. (B) *Crlz-1* (ISH-IF) and *VASA* (IHC-IF) were performed on the same tissue section of 1-week-old neonatal testis. Instead of using the AP-conjugated antibodies, fluorescence-labeled antibodies for immunofluorescence (IF) were employed to show the co-localization (yellowish-orange in merge) of *Crlz-1* (red) with *VASA* (green). (C) *Crlz-1* (ISH-IF) and *MIS* (IHC-IF) were performed on the same tissue section of 1-week-old neonatal testis as in panel B. Fluorescence-labeled antibodies for IF were again employed to show the co-localization (yellowish-orange in merge) of *Crlz-1* (red) with *MIS* (green). (D) The negative control experiments of in situ hybridization were also performed with the *Crlz-1* sense RNA probe on the adjacent tissue sections of testis to show the validity of the *Crlz-1* antisense RNA probe for our in situ hybridization experiments. One of those control experiments is shown here, in this case using 12-week-old mouse testis with the blue-colored hematoxylin nuclear counterstaining. The *Crlz-1* antisense RNA probe only gave a brick-red colored signal in an AP reaction but not its sense RNA probe. Scale bars: 100 μm (A–C); 200 μm (D).

Discussion

The Wnt/β-catenin signaling pathway as well as Runx/CBFβ transcriptional activity are well known for their roles in cellular proliferation and/or differentiation (Pencovich et al. 2011; Gough 2012; Niehrs and Acebron 2012; Nusse and Varmus 2012; Taniuchi et al. 2012; Valenta et al. 2012). The physiological significance of *Crlz-1* expression in testis might be viewed in terms of both the Wnt/β-catenin signaling pathway, as *Crlz-1* was found to be its target gene (Park et al. 2011) and Runx/CBFβ transcriptional activity, as *Crlz-1* was found to mobilize CBFβ to the nucleus to allow its heterodimerization with Runx in the nucleus (Park et al. 2009). Supporting these connections, β-catenin expression was strongly detected in a Western blot as well as in fluorescent immunohistochemical analyses, and LEF-1 was found to bind to the *Crlz-1* promoter in a chromatin immunoprecipitation (ChIP) analysis in a 1-week-old neonatal testis (data not shown).

With these potential connections of *Crlz-1* to the Wnt/β-catenin signaling pathway and Runx/CBFβ transcriptional activity, a strong expression of *Crlz-1* might be necessary for the intense proliferation of spermatogonia and Sertoli cells during early testis development, whereas weak expression of *Crlz-1* observed later at the rim layer of seminiferous cords or tubules, where spermatogonia are present, might be necessary for their homeostatic maintenance. However, the expression of *Crlz-1* in spermatids in late spermatogenesis may be necessary only for their differentiation because they do not proliferate. All these speculations remain to be tested in the future.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This work was supported by a grant (20110475) from the Kyung Hee University in 2011.

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