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Reciprocal localization of transcription factors YY1 and CP2c in spermatogonial stem cells and their putative roles during spermatogenesis

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ABSTRACT

Maintaining stemness and permitting differentiation mediated by combinations of transcription factors (TFs) are key aspects of mammalian spermatogenesis. It has been established that yin yang 1 (YY1), a target factor of mammalian polycomb repressive complex 2 (PRC2) and a regulator of stemness, is involved in the stable maintenance of prophase stage spermatocytes. Recently, we have demonstrated that the TF CP2c partners with YY1 in some cells to antagonistically regulate the other protein's function. To date, the functional roles of YY1 and CP2c in spermatogonial stem cells and their derived germ cells remain unclear. Here, we investigated the expression of YY1 and CP2c in mouse gonocytes and germ cells using tissue immunohistochemical and immunofluorescence analyses. At E14.5, both YY1 and CP2c were stained in gonocytes and Sertoli cells in testicular cords, showing different proportion and density of immunoreactivity. However, in adult testes, YY1 was localized in the nuclei of spermatogonial stem cells and spermatocytes, but not in spermatozoa. It was also detected in spermatogonia and spermatids in a stage–specific manner during spermatogenic cycle. CP2c could be detected mostly in the cytoplasm of spermatocytes but not at all in spermatogonial stem cells, indicating mutually exclusive expression of CP2c and YY1. Interestingly, however, CP2c was stained in the cytoplasm and nucleus of spermatogonia at elongation and release stages, and co-localized with YY1 in the nucleus at grouping, maturation, and releasing stages. Neither YY1 nor CP2c was expressed in spermatozoa. Our data indicate that YY1 strongly localizes in the spermatogonial stem cells and co-localizes heterogeneously with CP2c to permit spermatogenesis, and also suggest that YY1 is essential for stemness of spermatogonial stem cells (SCs) whereas CP2c is critical for the commitment of spermatogonia and during the progression of spermatogonia to spermatids. This evaluation expands our understanding of the molecular mechanism of spermatogonia formation as well as spermatogenesis in general.

1. Introduction

Polycomb-group protein complexes (PcGs) are key regulators of embryonic stem cell pluripotency and somatic differentiation. Polycomb repressive complex 1 (PRC1) is involved in the long-range intra- and inter-chromosomal interactions between polycomb target loci (Schoenfelder et al., 2015) and functions together with PRC2 as a master regulator of genome architecture, regulating stem cell (SC) self-renewal via a cell-autonomous mechanism in both embryonic and adult SCs (Chiacchiera et al., 2016; Schoenfelder et al., 2015; Simon and Kingston, 2013). However, in male germ cells PRC2 is a key regulator of proliferation and differentiation via gene silencing (Mu et al., 2014; Orlando, 2003). YY1 (also known as DELTA, NF-E1, UCRBP, INO80S, and YIN-YANG-1), a member of PRC2, is a zinc-finger transcription factor (TF) belonging to the GLI-Kruppel class and is involved in repression and activation of a number of diverse promoters (Shi et al., 1991). Constitutive knockout of the *YY1* gene in mice results in embryonic lethality around the peri-implantation stage (Donohoe et al., 1999). Moreover, YY1 also functions as a transcriptional activator, repressor, or

Abbreviations: E, elongation stage; G, grouping stage; IHC, immunohistochemistry; LTR, long terminal repeat; M, maturation stage; PcGs, polycomb-group protein complexes; PRC, polycomb repressive complex; R, release stage; SC, stem cell; TF, transcription factor; TRS, Tris-buffered saline containing 0.1% Tween 20; YY1, yin yang 1.

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co-regulator, either dependent or independent of its DNA binding affinity (Deng et al., 2010). Importantly, it has been suggested that YY1 is involved in the maintenance of spermatocytes by regulating the heterochromatic state and double-strand break formation during spermatogenesis (Wu et al., 2009). Moreover, although YY1 is a stemness factor (Perekatt et al., 2014), whether YY1 is involved in the maintenance of spermatogonial stemness in mammals remains unclear. In addition, its role in the spermatogonial SC-derived germ cells is not fully understood.

The TF CP2c (also known as TFCP2, LSF, (α)CP2, LBP-1c, UBP-1, and SEF-1) (Barnhart et al., 1988; Bing et al., 1999; Huang and Liao, 1994; Jones et al., 1988; Kang et al., 2005a; Kim et al., 1987, 1988; Wu et al., 1988; Yoon et al., 1994) is a YY1 interacting protein that binds sequences within the HIV-1 long terminal repeat (LTR) initiation region and recruits YY1 to the LTR. These factors then cooperatively recruit HDAC1 to the LTR, resulting in inhibition of transcription (Romerio et al., 1997; Coull et al., 2000). CP2c itself plays an important role in the control of cell proliferation, cell cycle, and differentiation, and also functions as an oncogene in multiple cancers (Santhekadur et al., 2012). Like YY1, CP2c functions either as a transcription activator or repressor. There are six CP2 isoforms in humans (LBP-1a, -1b, -1c, -1d, -9 and LBP-32) and four in mice (CP2a, CP2b, CP2c and CRTR-1) (Kang et al., 2005a; Yoon et al., 1994) that exhibit a wide range of DNA binding and transcriptional activities via interaction among themselves as well as with other proteins (Bruni et al., 2002; Casolaro et al., 2000; Kang et al., 2005a, 2010; Zhou et al., 2000). We recently found that CP2c and YY1 directly interact with each other and mutually suppress expression of their target genes, suggesting that their differential cellular distribution may be an important factor for regulating cell differentiation and fate decision (Kang et al., 2005b; Kim et al., manuscript in preparation). However, the functional interplay of YY1 and CP2c has not been investigated in spermatogonial SCs and their derived germ cells.

Here, we analyzed the localization of YY1 in testis to evaluate the role of YY1 in maintaining the stemness of spermatogonia and spermatogenesis, and also examined CP2c expression profiles to determine the interplay between YY1 and CP2c in maintaining stemness and spermatogenesis.

2. Materials and methods

2.1. Preparation of tissue samples

CD1 mice were purchased from the Jackson Laboratory and maintained in a standard environment until use. The animals were handled according to NIH guidelines with approval from the institutional ethics committee of Hanyang University. To obtain E14.5 embryos, pregnant mice were sacrificed at day 14.5 of gestation. Mature testes were collected from 12-week-old mice. Testes samples were quickly fixed in 10% neutral buffered formalin, dehydrated in a graded series of ethanol, treated with xylene, paraffin embedded, and cut into 4-μm sections.

2.2. Immunohistochemistry (IHC)

Paraffin sections of samples were deparaffinized with xylene 3 times for 5 min each, rehydrated with an ethyl alcohol series and distilled water, and then washed with Tris-buffered saline (50 mM, pH 7.6) containing 0.1% Tween 20 (TRS). Antigen retrieval was performed by autoclaving the sections in sodium citrate buffer (10 mM, pH 6.0) for 30 min. After washing in TRS, the sections were incubated with Universal AP Red Enhancer (760-051, ultraView Universal Alkaline Phosphatase Red Detection Kit, VENTANA) or blocked with 3% hydrogen peroxidase solution for 10 min depending on visualization conditions. The sections were then incubated with the following primary antibodies in Dako antibody Diluent (S0809, DAKO) at 4 °C overnight: mouse anti-LSF antibody (CP2c specific antibody, 1:100 dilution, 610818, BD Biosciences) and rabbit anti-YY1 antibody (1:200 dilution, ab 109237, Abcam). After being washes in TRS, the sections were incubated with Universal HRP multimer (760-500, ultraView DAB Detection Kit, VENTANA) or Universal AP Red Multimer (ultraView Universal Alkaline Phosphatase Red Detection Kit) at room temperature for 30 min. After two more washes in TRS, the immunoreactivity of samples was visualized with a mixture of equal amount of Universal DAB H_2O_2 and Universal DAB Chromogen (ultraView DAB Detection Kit) or a mixture of equal amount of Universal AP Red Naphthol, Universal AP Red Fast Red A and Universal AP Red Fast Red B (ultraView Universal Alkaline Phosphatase Red Detection Kit) for 5–10 min. Samples were counterstained with Mayer's hematoxylin. Finally, the slides were washed with tap water and mounted with glycerol. Negative controls were obtained by substituting TRS for the primary antibody. For double staining, mouse anti-LSF antibody and rabbit anti-YY1 antibody were serially treated in the sections. After incubation with primary antibody, Dako Envision + System–HRP Labeled Polymer Anti-mouse (K4001, Dako) and ImmPRESS AP Anti-rabbit IgG (MP-5401, Vector Laboratories) were used as a secondary antibody, respectively. The immunoreactivity was visualized with a mixture of equal amount of Universal DAB H_2O_2 and Universal DAB Chromogen (ultraView DAB Detection Kit) or VECTOR Blue Alkaline Phosphatase substrate (SK-5300, Vector Laboratories). Samples were counterstained with Nuclear fast red solution (Sigma, N3020).

2.3. Confocal microscopy

Immunofluorescence double staining was performed using the same procedure as IHC until the primary antibody incubation step. After washing in TRS, the sections were incubated with Cy™3-conjugated AffiniPure goat anti-mouse IgG (H + L) (Cat. No 115-165-146, Jackson Immuno Research) for CP2c and Alexa Fluor 488-conjugated goat anti-rabbit IgG (H + L) secondary antibody (A-11008, ThermoFisher Scientific) for YY1. Counter staining was performed with DAPI (H-1200, Vector Laboratories). The immunofluorescence signals were examined using a Zeiss LSM 700 laser scanning microscope.

3. Results

3.1. Expression of YY1 and CP2c in E14.5 testis

In the testicular cord of E14.5 testes the gonocytes are surrounded by Sertoli cells (Fig. 1A & B). Nuclei of Sertoli cells were densely and strongly stained with hematoxylin (dark spots), whereas most nuclei of gonocytes had an even staining pattern (Fig. 1B). The expression of YY1 and CP2 was examined in the testicular cord of E14.5 testis by immunohistochemistry. The number of immunopositive cells within testicular cord was counted in 2 serial sections. YY1 was localized in the nuclei of about 86% of Sertoli cells and in about 38% of gonocytes with dark spots whose nuclei were a little bit densely stained with hematoxylin (Fig. 1C, C′ & E). CP2c was localized in about 18% of Sertoli cells and in about 30% of gonocytes, with only detectable level of staining (Fig. 1D, D′ & E). Mesenchymal cells surrounding the testicular cords were strongly stained in the nuclei with both YY1 and CP2c, and showed faint CP2c staining in the cytoplasm (Fig. 1C & D). This heterogeneous expression of YY1 in gonocytes, Sertoli cells, and mesenchymal cells suggests that YY1 is involved in germ cell-specific imprint-

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