Spermatogenesis arrest caused by conditional deletion of Hsp90α in adult mice

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Biology Open 1, 977–982
doi: 10.1242/bio.2012646
Received 4th January 2012
Accepted 11th July 2012

Summary

It is controversial whether a functional androgen receptor (AR) on germ cells, including spermatogonia, is essential for their development into sperm and, thus, initiation and maintenance of spermatogenesis. It was recently shown that many spermatocytes underwent apoptosis in the testes of Hsp90α KO mice. We had generated Hsp90α KO mice independently and confirmed this phenotype. However, the important question of whether Hsp90α is required to maintain spermatogenesis in adult mice in which testicular maturation is already completed could not be addressed using these conventional KO mice. To answer this question, we generated a tamoxifen-inducible deletion mutant of Hsp90α and found that conditional deletion of Hsp90α in adult mice caused even more severe apoptosis in germ cells beyond the pachytene stage, leading to complete arrest of spermatogenesis and testicular atrophy. Importantly, immunohistochemical analysis revealed that AR expression in WT testis was more evident in spermatogonia than in spermatocytes, whereas its expression was aberrant and ectopic in Hsp90α KO testis, raising the possibility that an AR abnormality in primordial germ cells is involved in spermatogenesis arrest in the Hsp90α KO mice. Our results suggest that the AR, specifically chaperoned by Hsp90α in spermatogonia, is critical for maintenance of established spermatogenesis and for survival of spermatocytes in adult testis, in addition to setting the first wave of spermatogenesis before puberty.

Key words: Hsp90α, Spermatogonia, Spermatogenesis, Androgen receptor

Introduction

Androgens are essential steroid hormones in initiation and maintenance of spermatogenesis as well as in the development of secondary male sex maturation. Nevertheless, it is still controversial whether the androgen receptor (AR) of germ cells is indispensable for spermatogenesis (Lyon et al., 1975; Johnston et al., 2001). Studies using germ cell-specific AR deficient mice showed almost normal spermatogenesis as well as fertility (Tsai et al., 2006). The AR gene in these mice is completely deleted in all germ cells beyond the pachytene stage; however, it remains unknown whether it is deleted in spermatogonia (Tsai et al., 2006; Wang et al., 2009).

Heat shock protein (Hsp) 90 is one of the most highly expressed cytosolic molecular chaperones, comprising 1% of the total cellular protein even in non-stressed conditions. It interacts with several hundred client proteins, simultaneously recruiting its own battery of co-chaperones, to facilitate their correct folding, transport, and degradation, thus maintaining normal cellular functions in ATP-dependent manner (Taipale et al., 2010; Echeverria and Picard, 2010). There are two distinct isoforms of Hsp90, Hsp90α and Hsp90β, whose homology is nearly 86% at the amino acid level. Both isoforms are thought to function in various tissues in a mostly redundant manner.

We previously generated Hsp90α-null mice to investigate the role of Hsp90α in antigen-cross presentation. During these studies we became aware that Hsp90α male mice were infertile and found that this resulted from the lack of sperm formation (Ichiyanagi et al., 2010; Imai et al., 2011). But before that, Grad et al. reported that Hsp90α gene trap mutant mice, in which Hsp90α is deleted throughout the entire life of the mouse, developed testicular atrophy caused by apoptosis of spermatocytes (Grad et al., 2010). In this report, there was a further intriguing observation that in one of the two distinct mutant lines the expression of Hsp90α was greatly reduced initially but resumed around day 45. Nonetheless, the mice still remained infertile (Grad et al., 2010). These results strongly imply a critical role of Hsp90α in establishing the first wave of spermatogenesis before puberty, and this in turn led us to the question of whether Hsp90α is required for the maintenance of normal spermatogenesis after testicular maturation is completed.

Based on the outcome of the study of Grad et al., we designed our mutant mice to be tamoxifen-inducible via the loxP-cre system, thus allowing Hsp90α-deletion in adult mice. We observed severe apoptosis occurring in germ cells beyond the pachytene stage, results that led us to conclude that Hsp90α is indispensable for the maintenance of germ cell development even in the mature adult testis. Moreover, we found that expression of AR is absent or reduced in spermatogonia of Hsp90α KO testes, whereas it is strongly expressed in WT testes. Our results indicate that the AR of spermatogonia, which is specifically chaperoned by Hsp90α, is critical for the initiation and maintenance of spermatogenesis.

Results and Discussion

Production of Hsp90αKO and conditional KO mice

The EIIa-cre mouse carries a *cre* transgene under the control of the adenovirus EIIa promoter that targets expression of Cre recombinase to the early mouse embryo (JAX Mice Database – 003724 B6.FVB-Tg(EIIa-cre)C5379Lmgd/J). Breeding the mice to *loxP*-flanked Hsp90α mice (*Hsp90aa 1*^{neo}) (Fig. 1A) produced mice of three genotypes – *Hsp90aa* Exon 9,10-floxed mice (*Hsp90aa 1*^{flox}), mice with a deletion of *neo* and Exons 9 and10 on chromosome (*Hsp90aa 1*$^{-}$) (Fig. 1A) and *neo*-floxed mice (not shown). Complete Hsp90αKO (*Hsp90aa 1*$^{-/-}$) mice were obtained by interbreeding the *Hsp90aa 1*$^{-/+}$ mice (Imai et al., 2011). We obtained a total of 375 F1 mice and the genotypic ratio of the offspring was *Hsp90aa 1*$^{+/+}$:*Hsp90aa 1*$^{-/+}$: *Hsp90aa 1*$^{-/-}$=113:194:68. The number of homozygous *Hsp90aa 1*$^{-/-}$ offspring was significantly lower than the expected Mendelian ratios, suggesting that *Hsp90aa 1*$^{-/-}$ embryos survive by chance, not in every case, possibly through compensatory contribution of the other isotype of Hsp90, Hsp90β.

By breeding the Hsp90α^{flox} (*Hsp90aa 1*^{flox}) to B6CreER^{T2} mice, we obtained inducible mutant mice, thus, Hsp90α$^{flox/flox}$/CreER^{T2} and Hsp90α$^{flox/+}$/CreER^{T2}. CreER^{T2} mice were generated by knock-in of a fusion gene composed of *Cre* recombinase and the *ER*^{T2} domain of human estrogen receptor gene with three mutations (G400V/M542A/L544A) into the *Rosa 26* region (Seibler et al., 2003). Binding of 4-hydroxy tamoxifen to CreER^{T2} on cell surface causes relocation of the fusion protein

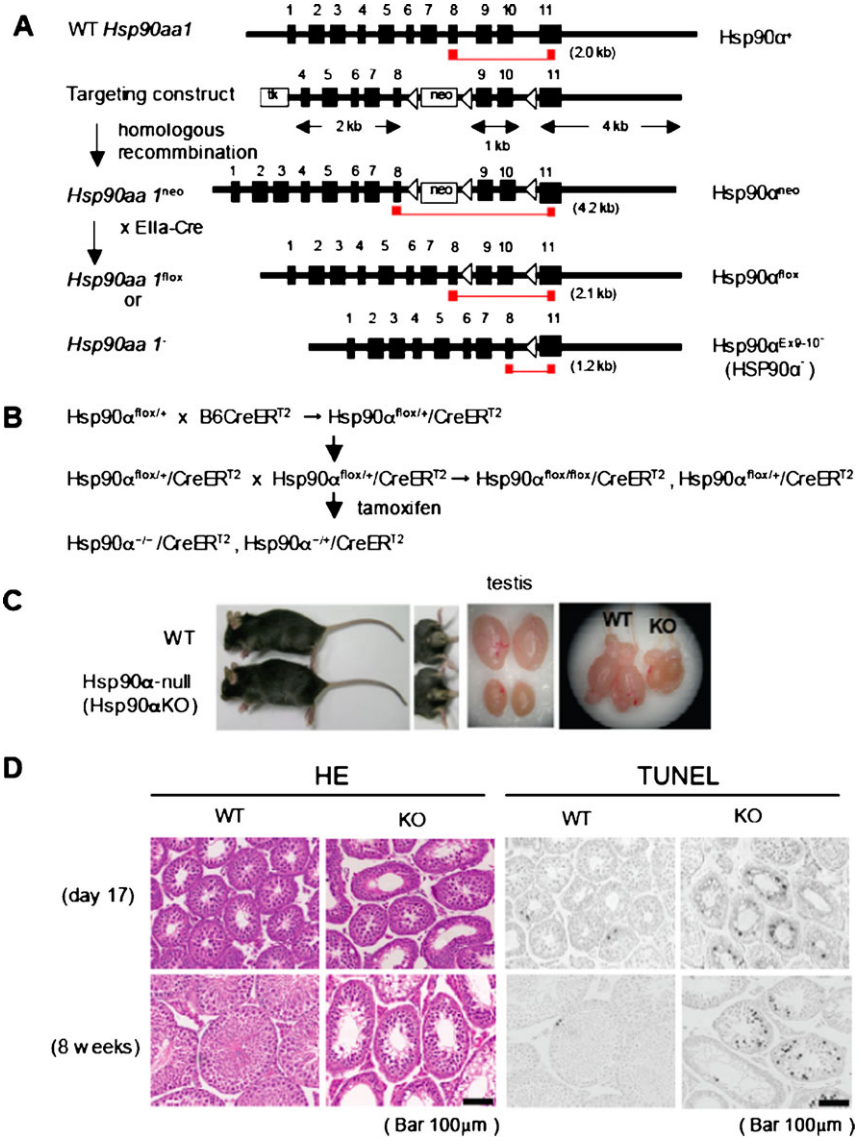

Fig. 1. Strategy for production of *Hsp90α* KO mice. (**A**) Generation of Hsp90α floxed mice and Hsp90α KO mice. Homologous recombination of the targeting construct into the *Hsp90aa1* gene resulted in production of Hsp90α^{neo} mice. By mating with EIIa-Cre mice, Hsp90α^{flox} and Hsp90α$^{Ex9-10-}$ mice were obtained. The primers (red boxes) used for PCR detection of the recombination status were on exon 8 and 11, respectively. (**B**) Generation of Hsp90α conditional KO mice. Hsp90α$^{flox/flox}$/CreER^{T2} and Hsp90α$^{flox/+}$/CreER^{T2} were generated by the indicated breeding procedures. Administration of tamoxifen resulted in production of Hsp90α$^{-/-}$ and Hsp90α$^{-/+}$ mice, respectively. (**C**) Hsp90α KO adult mice (8 weeks) underwent progressive testicular atrophy. (**D**) H&E staining and a TUNEL assay of the testes of day 17 and 8-week-old WT and Hsp90α KO mice. Scale bars=100 μm.

to the nucleus, where it deletes floxed genes. Oral administration of tamoxifen (5 mg) to the mice resulted in conditional deletion of exon 9 and 10 of *Hsp90aa 1*^{flox}, thus producing Hsp90α$^{-/-}$/CreER^{T2} and Hsp90α$^{-/+}$/CreER^{T2} mice (Fig. 1B).

Testicular atrophy in Hsp90αKO adult mice

Hsp90αKO mice grew normally, nearly comparably to wild type (WT) controls (Fig. 1C). However, we found that male 8-week-old Hsp90αKO mice were infertile and had small scrotums compared to the WT mice. Indeed, their testes were significantly smaller, close to one third the normal size, although the seminal vesicles were not significantly altered (Fig. 1C). Microscopic sperm counts revealed that no spermatozoa could be recovered from the caudal epididymis of 8 to 10 week Hsp90αKO mice. Notably, the size of the prepubescent testes (day 17 after birth) was nearly comparable between Hsp90αKO and WT mice (data not shown).

Decreased spermatogenesis in HSP90αKO mice

To determine which cell type in the testes is affected in Hsp90αKO mice, histological analysis was performed. By H&E staining, there was only a marginal reduction of germ cell development in Hsp90αKO day 17 testes; however, developmental arrest became apparent with progressive age (8 weeks) (Fig. 1D, left panel). TUNEL analysis revealed apoptosis occurring in germ cells after the pachytene stage in day 17 testes and there was increased severity at 8 weeks (Fig. 1D, right panel). The results clearly indicate that reduced numbers of developmental germ cells is caused by apoptosis induction in Hsp90αKO testes with increasing age. The summary of statistics for numbers of TUNEL positive cells is shown in Table 1. Overall, our results obtained from the analysis of Hsp90αKO testes are consistent with those of Grad et al., who used *Hsp90aa1* mutant mice produced by the gene trap method (Grad et al., 2010).

Tamoxifen-inducible deletion of the Hsp90α gene in a wide variety of tissues

To determine the effect of conditional deletion of the *Hsp90aa1* gene in adult mice, Hsp90α floxed mice were treated with oral tamoxifen. Genomic DNA was extracted from freshly isolated tissues (Fig. 2A) and the region between Exon 8 and Exon 11 of the *Hsp90aa1*gene was amplified by PCR, as indicated in Fig. 1A. Administration of tamoxifen resulted in deletion of the floxed Exons 9 and 10, which was demonstrated by identification of a 1.2 kb band (Fig. 2B). The Hsp90α^{+} and Hsp90α^{flox} alleles could be identified as 2.0 kb and 2.1 kb bands, respectively (Fig. 2B). Although Exon 9 and Exon 10 were deleted, levels of Hsp90α were not reduced at day 6 except in liver (Fig. 2C, day 6). Accordingly, both Hsp90α$^{flox/flox}$/CreER^{T2} and Hsp90α$^{flox/+}$/CreER^{T2} mice had normal sized testes (Fig. 2D, day 6).

Inducible deletion of Hsp90α results in testicular atrophy

We next recovered tissues at day 30 after administration of tamoxifen (Fig. 2A). Unlike the case on day 6, Hsp90α was markedly downregulated in all tissues tested (Fig. 2C, day 30). On day 30, testes of Hsp90α$^{flox/flox}$/CreER^{T2} but not Hsp90α$^{flox/+}$/CreER^{T2} mice showed atrophy, diminishing to a size similar to that of Hsp90α KO testes (Fig. 2D, day 30). Indeed, the weight of the testes in Hsp90α$^{flox/flox}$/CreER^{T2} mice were around one-third that of Hsp90α$^{flox/+}$/CreER^{T2} and WT mice (Fig. 2E).

Inducible deletion of Hsp90α in adult mice results in complete arrest of spermatogenesis due to severe apoptosis of germ cells beyond the pachytene stage

Histological analysis revealed pronounced, severe apoptosis and deletion of spermatocytes in testes with conditionally deleted Hsp90α, similar to what was observed in the conventional Hsp90α KO mice. No germ cell development, including the pachytene stage, remained (Fig. 3, upper panel). A TUNEL assay demonstrated that germ cells after pachytene stage underwent apoptosis, leading to a complete arrest of spermatogenesis and cell death of meiotic spermatocytes (Fig. 3, lower left panel). By contrast, Leydig and Sertoli cells seem to be not much impaired, rather closely resembling normal cells. Hsp90β does not compensate for Hsp90α during germ cell development in the testes of the conditional knockout mice. Relevant to this point, Hsp90β appears to be mainly expressed in Sertoli cells, whereas Hsp90α is expressed specifically in primordial and mature germ cells (Lee, 1990; Vanmuylder et al., 2002). Therefore, the limited expression of Hsp90β in germ cells might explain why Hsp90β could not rescue germ cell development in Hsp90α KO testes.

Aberrant expression of androgen receptor (AR) in Hsp90α-deficient testes

Androgen-AR interaction plays an important role in spermatogenesis since AR functions as testosterone-dependent transcription factor, initiating expression of an array of androgen-responsive genes (Chang et al., 1988a; Chang et al., 1988b; Lubahn et al., 1988; Heinlein and Chang, 2002). Based on

Table 1. Statistical analysis of TUNEL positive cells. Number of TUNEL positive cells per 100 tubules were counted. The testes were derived from WT and Hsp90αKO mice at age of 17 days and 8 weeks.

Groups	Age	No.	Number of TUNEL positive cells	Number of tubules	TUNEL positive cells per 100 tubules	Mean ± SD
WT	17 days	1	182	339	54	55±1
		2	43	80	54	
		3	128	227	56	
	8 weeks	1	71	311	23	24±2
		2	60	232	26	
		3	53	236	22	
Hsp90α KO	17 days	1	763	287	266	262±3
		2	543	209	260	
		3	922	352	262	
	8 weeks	1	192	83	231	234±3
		2	352	149	236	
		3	338	144	235	

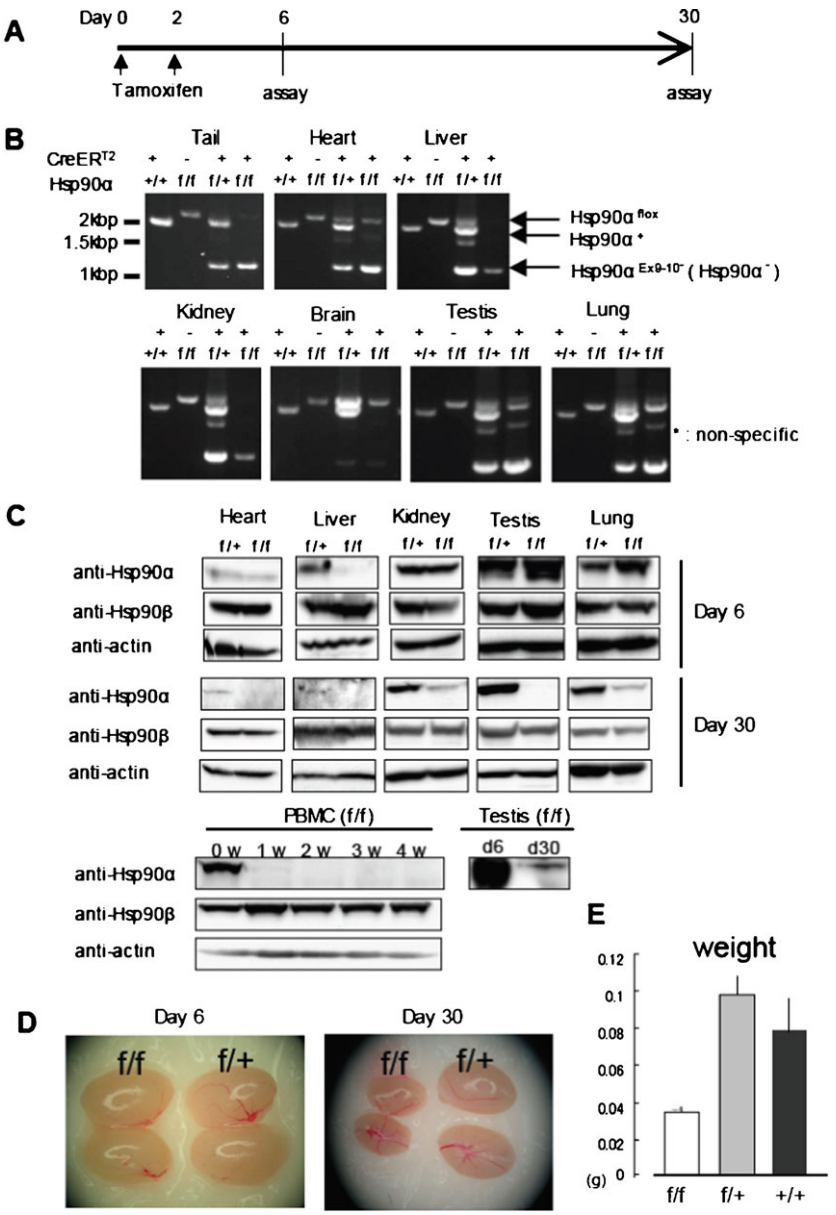

Fig. 2. Tamoxifen-induced conditional deletion of *Hsp90α* results in testicular atrophy. (**A**) The scheme of oral administration of tamoxifen and removal of the testes for assay. (**B**) Genome analysis after administration of tamoxifen. There were some non-specific bands in lanes 3 and 4 in each panel. (**C**) Expression levels of Hsp90α and Hsp90β in the indicated tissues tested at day 6 and day 30 after administration of tamoxifen. Hsp90α but not Hsp90β is largely downregulated at day 30. (**D**) Testes obtained at day 6 and 30 after treatment with tamoxifen are shown. The testis recovered from f/f but not f/+ mice underwent atrophy at day 30. (**E**) Weight (g) of the testes recovered from f/f, f/+, and +/+ mice at day 30 are shown as bar graphs. (n=6 from 3 mice of each genotype; error bars indicate SD).

the fundamental importance of AR, we wondered whether AR expression was normal in individual cells (germ cells) of the testis in the absence of Hsp90α. We used specific antibodies and performed immunohistochemical analysis for estrogen receptor (ER)α, ERβ and AR of both WT and Hsp90α KO testes (both 8-week-old). On germ cells, we found dominant expression of ERα and AR in spermatogonia and to a lesser extent in spermatocytes of WT testis, whereas the pattern was disrupted and/or even reversed in Hsp90α KO testis (Fig. 4). In order to discriminate between spermatogonia and spermatocytes, we further performed immunohistochemistry in mirror sections of 8 week and day 17 testes with antibodies specific to synaptonemal complex protein 3 (SCP3) that is expressed in spermatocyte but not in spermatogonia. Consistent with the results in Fig. 4, AR was expressed in germ cells containing both spermatocytes (SCP3 positive) and spermatogonia (SCP3 negative) in WT testes, whereas it was positive in spermatocytes but not in spermatogonia in Hsp90α KO testis (Fig. 5A). In prepuberal testes, on the other hand, AR was positive in only spermatocytes in both WT and Hsp90α KO testes (Fig. 5B). Thus, AR is weakly or not expressed in spermatogonia even in WT testis before puberty, suggesting that AR in spermatoginia is critical in initiation of germ cell development. In other words, most seminiferous tubules showed strong expression of AR (and/or ERβ) in spermatocytes, but little or none in spermatogonia in Hsp90α KO mice, implying that Hsp90α KO testis lacks the androgen-AR signaling in spermatogonia required for subsequent germ cell development.

AR is chaperoned by Hsp90 before its association with testosterone and Hsp90 inhibitors caused AR-Hsp90 complex to dissociate, which might cause degradation of AR by the proteasome (Whitesell and Cook, 1996; Zhang et al., 2006). Although the precise reason why AR is missing in spermatogonia of the Hsp90α KO testis is unknown, it is plausible that AR undergoes degradation following deletion of *Hsp90α* gene since it is usually chaperoned by Hsp90α and not by Hsp90β. Although absent in spermatogonia, AR seemed to reappear in germ cells of

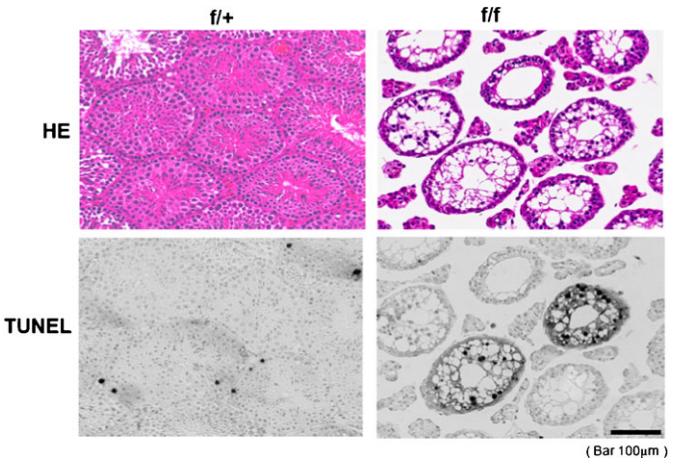

Fig. 3. Immunohistochemical analysis of testes recovered from tamoxifen-treated f/+ and f/f mice at day 30. The testes derived from f/f but not f/+ mice showed severe apoptosis of germ cells beyond the pachytene stage and spermatogenesis arrest, as indicated by H&E staining (upper panel) and the TUNEL assay (lower panel). Scale bar=100 μm.

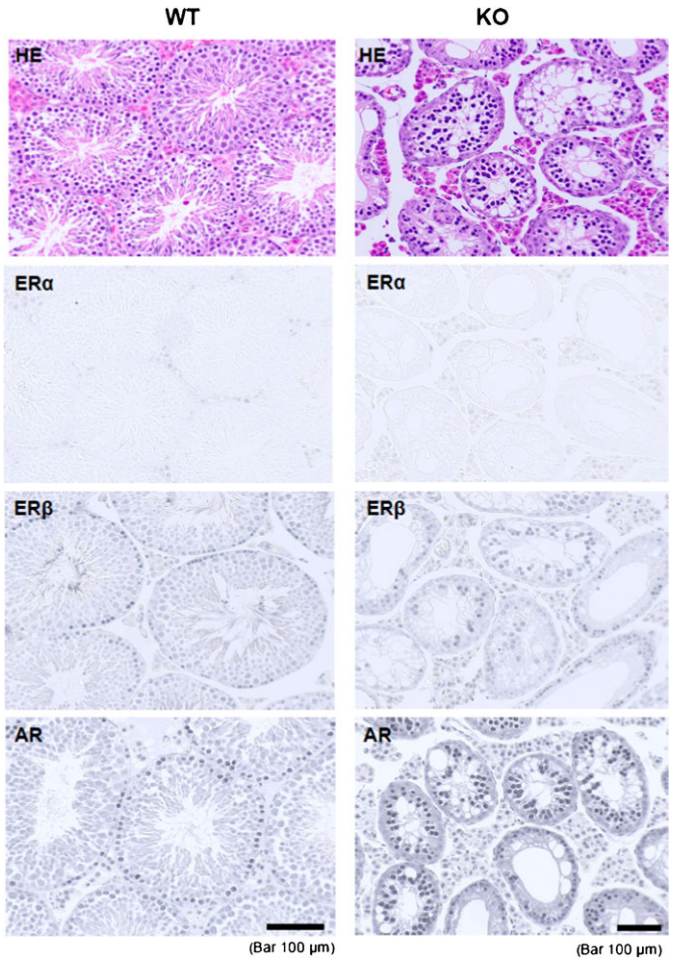

Fig. 4. Aberrant AR expression in Hsp90α KO testes. H&E staining of WT and knockout testes (Top row). Expression of ERα, (2nd row), ERβ (3rd row) and AR (4th row) in testes derived from WT and Hsp90α KO mice (8 weeks) was tested with specific antibodies. ERβ and AR mostly stained spermatogonia of WT testis. On the other hand, spermatogonia of Hsp90α KO testis were mostly negative and many spermatocytes stained positive, in marked contrast to the pattern in WT testes. Scale bars=100 μm.

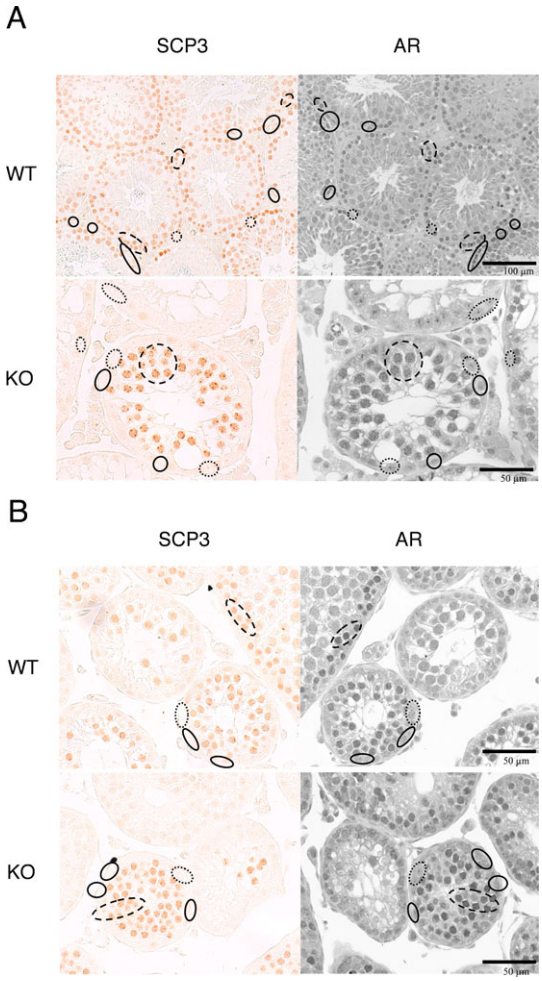

Fig. 5. SCP3 and AR staining in mirror sections of testes. Expression of SCP3 and AR in testes derived from WT and Hsp90α KO mice ((**A**) 8 weeks after birth, (**B**) 17 days after birth) was tested with specific antibodies. SCP3 stained spermatocytes with meiosis, not spermatogonia, not Sertoli cells. AR stained spermatogonia and spermatocytes of 8 weeks WT testis (A). On the other hand, spermatogonia of Hsp90α KO testis were mostly negative and many spermatocytes stained positive, in marked contrast to the pattern in WT testes (A). AR stained only spermatocytes in both WT and Hsp90α KO testis (17 days) (B). The marked cells are indicated as representative spermatogonia (solid lines), spermatocyte (dashed lines), and Sertoli cells (dotted lines). Scale bars=50 μm, except top row of A where scale bar=100 μm.

Hsp90α KO testis. This might be because the proteasome activity in germ cells (beyond the pachytene stage) already undergoing apoptosis is downregulated, which eventually causes accumulation of AR within the cells. The balance between Hsp90α-mediated chaperone activity and proteasomal activity determines the expression level of AR in germ cells. Hsp90α deficiency might influence this balance, giving rise to the observed aberrant expression pattern of AR in germ cells including spermatogonia. It is also possible that Hsp90α simply regulates (facilitates) transcriptional activation of AR gene in spermatogonia.

Germ cell-specific AR KO mice did not show testicular atrophy, nor did they show spermatogenesis arrest, indicating that the androgen-AR interaction in germ cells is dispensable for their development (Tsai et al., 2006). These observations conflict with our hypothesis that Hsp90α-dependent AR expression in spermatogonia is essential in subsequent germ cell

development. However, the germ cell-specific AR KO mice were generated by mating the floxed AR mice with synaptonemal complex protein 1 promoter (*Sycp1*-cre) transgenic mice (Tsai et al., 2006). Sycp1 is expressed at an early stage of male meiosis, leptene to zygotene, therefore, the floxed AR allele might not be deleted in spermatogonia of these mice.

Sertoli cell- and Leydig cell-specific AR KO mice have a testicular phenotype very similar to the Hsp90α KO, i.e., no sperm formation, disrupted germ cell development and testicular atrophy (Chang et al., 2004; De Gendt et al., 2004; Holdcraft and Braun, 2004; Xu et al., 2007). However, in our Hsp90α KO mice, both Sertoli cells and Leydig cells appeared normal. It is possible that the AR-mediated transcriptional cascade in Sertoli- and Leydig-cells of our mice is not impaired due to the compensatory effect of Hsp90β, since this isotype rather than Hsp90α is mainly expressed in these cell types (Lee, 1990; Vanmuylder et al., 2002).

In summary, we have established *Hsp90α* conditional deletion mutant mice and found that spermatogenesis in adult testes was completely abrogated through apoptosis caused by inducible ablation of the *Hsp90α* gene. In addition, AR expression in spermatogonia was downregulated in Hsp90αKO testis. Although our results raised the possibility that AR-dependent signaling is essential in the subsequent germ cell development, further studies will be required to test this hypothesis.

Materials and Methods

Generation of *Hsp90α* conditional KO mice

Conditional HSP90α-null mice were generated by deleting exons 9 and 10, which encode the C-terminal region of the protein, in a cre-recombinase-dependent manner (Imai et al., 2011). Tamoxifen-dependent, inducible deletion of the floxed exons was performed according to a previous report (Hikida et al., 2009). Briefly, mice were orally administrated 100 µl 4-hydroxy tamoxifen (Sigma T5648) dissolved in corn oil (Sigma C8267) at a dose of 50 mg/kg, twice in a two-day interval. Mice were analyzed 4 d and 28 d after the last injection. Deletion efficiency of exons 9 and 10 was assessed by genomic PCR. The samples were subjected to Western blotting analysis using antibodies specific for Hsp90α (mouse mAb EMD-17D7, Calbiochem.), Hsp90β (rabbit polyclonal Abs, Lab Vision Corporation, Fremont, CA) and actin (Sigma).

Genomic-PCR

The primers used for genotyping of mice after oral administration of tamoxifen were forward (within exon 8): ttctctgaaggactactgtaccagaatgaa, and reverse (downstream of exon 11): gtgtgggggtgcgcaaagaaaagacacatt. The primers for detection of the *cre*-recombinase gene were forward: caccctgttacgtatagcc, and reverse: ctctgaccagagtcatcct.

Immunohistochemistry

Enzyme immunohistochemistry was performed on paraffin sections (5–6 µm) of the mouse testis, as described previously (Koji et al., 2001; Song et al., 2011). The antibody dilutions were as follows: rabbit anti-AR antiserum (Millipore AB561) was at 1:800, mouse monoclonal anti-ERα (BioGenex ER88) was at 1:100, mouse monoclonal anti-ERβ (BioGenex ERβ88) was at 1:200, and rabbit anti-SCP3 antibody (Novus Biologicals NB300-231) was at 1:750 (1.33 µg/ml). After the reaction with horseradish peroxidase (HRP)-conjugated second antibody, the sites of HRP deposition were visualized with 3,3′-diaminobenzidine-4HCl (DAB) and H_2O_2 in the presence or absence of nickel and cobalt ions (Shukuwa et al., 2006). In every experimental run, negative control samples were prepared by reacting the sections with normal rabbit serum (for AR) or normal mouse IgG (ERα and ERβ) instead of the specific first antibody. To identify apoptotic cells, Terminal deoxynucleotidyl transferase (TdT)-ated dUTP nick end-labeling (TUNEL) staining was performed according to the method (Gavrieli et al., 1992) with a slight modification (Koji et al., 2001).

Competing Interests

The authors have no competing interests to declare.

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