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TECHNOLOGY REPORT

Postnatal Male Germ-Cell Expression of Cre Recombinase in Tex101-iCre Transgenic Mice

Zhenmin Lei,[1]* Jing Lin,[1] Xian Li,[1] Shengqiang Li,[1] Huaxin Zhou,[2] Yoshihiko Araki,[3] and Zi-Jian Lan[2]*

[1]Department of OB/GYN and Women's Health, University of Louisville Health Sciences Center, Louisville, Kentucky
[2]Department of Molecular, Cellular and Craniofacial Biology, Birth Defects Center, University of Louisville Health Sciences Center, Louisville, Kentucky
[3]Institute for Environmental and Gender-Specific Medicine, Juntendo University Graduate School of Medicine, Urayasu City, Chiba, Japan

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Summary: We have generated a transgenic mouse line that expresses improved Cre recombinase (*iCre*) under the control of the testis-expressed gene 101 (*Tex101*) promoter. This transgenic mouse line was named *Tex101-iCre*. Using the floxed *ROSA* reporter mice, we found that robust Cre recombinase activity was detected in postnatal testes with weak or no activity in other tissues. Within the testis, Cre recombinase was active in spermatogenic cells as early as the prospermatogonia stage at day 1 after birth. In 30- and 60-day-old mice, positive Cre recombinase activity was detected not only in prospermatogonia but also in spermatogenic cells at later stages of spermatogenesis. There was little or no Cre activity in interstitial cells. Breeding wild-type females with homozygous floxed fibroblast growth factor receptor 2 (*Fgfr2*) males carrying the *Tex101-iCre* transgene did not produce any progeny with the floxed *Fgfr2* allele. All the progeny inherited a recombined *Fgfr2* allele, indicating that complete deletion of the floxed *Fgfr2* allele by *Tex101-iCre* can be achieved in the male germline. Furthermore, FGFR2 protein was not detected in spermatocytes and spermatids of adult *Fgfr2*$^{fl/fl}$*;Tex101-iCre* mice. Taken together, our results suggest that the *Tex101-iCre* mouse line allows the inactivation of a floxed gene in spermatogenic cells in adult mice, which will facilitate the functional characterization of genes in normal spermatogenesis and male fertility. genesis 48:717–722, 2010. © 2010 Wiley-Liss, Inc.

Key words: testis; iCre recombinase; prospermatogonia; spermatogenesis; promoter

INTRODUCTION

Our goal is to characterize the gene regulatory network within spermatogenic cells (e.g., spermatocytes and round spermatids) during postnatal development. To achieve this goal, we want to adapt the *Cre/loxP* system (Gu et al., 1994; Sauer and Henderson, 1989) to uncover the physiological functions of spermatogenic cell-expressing genes, especially those genes (e.g., the germ-cell nuclear factor gene) that are critical for embryonic development (Chung *et al*., 2001; Fuhrmann *et al*., 2001). One critical factor in the generation of spermatogenic cell-specific knockouts using the Cre/loxP strategy is the activity of Cre recombinase expressed in *Cre* transgenic mice. Several lines of *Cre* transgenic mice such as *TNAP-Cre*, *Vasa-Cre*, *Stra8-iCre*, *TSPY-Cre*, *Pgk2-Cre*, *Sycp1Cre*, *c-kit-Cre*, *Enolase-Cre*, *Ngn3-Cre*, *Protamine-Cre*, and *SynCre* (Hammond and Matin, 2009; Lee *et al*., 2001; Rempe *et al*., 2006) have been successfully generated. These mice can express Cre recombinase either in primordial male germ cells in embryos or in spermatogonia, spermatocytes, round spermatids, and/or elongating spermatids in postnatal testes. In our previous effort to characterize the function of germ-cell nuclear receptor, we have used *TNAP-Cre*, *Sycp1Cre*, and *Enolase-Cre* mouse lines but failed to completely delete the floxed germ cell nuclear receptor gene in spermatocytes and round spermatids (Lan and Cooney, unpublished data). Incomplete deletion of floxed genes in male germ cells by *TNAP-Cre* and *Sycp1Cre* has also been observed in other laboratories (Kimura *et al*., 2003; Rasoulpour and Boekelheide, 2006; Rassoulzadegan *et al*., 2002). To date, successful use of the aforementioned *Cre* transgenic mice to completely and specifically delete-floxed genes in spermatocytes and round spermatids has not yet been reported. This information prompted us to generate a *Cre* transgenic mouse line that can express Cre in spermatocytes and round spermatids at a high level. In this report, we describe the generation and characterization of a *Cre* transgenic mouse line driven by the promoter of testis-expressed gene 101 (*Tex101*) (Kurita *et al*., 2001; Takayama *et al*., 2005; Tsukamoto *et al*.,

*Correspondence to: Zhenmin Lei or Zi-Jian Lan, Department of OB/GYN and Women's Health, University of Louisville Health Sciences Center, Louisville, Kentucky or Birth Defects Center, University of Louisville Health Sciences Center, Louisville, Kentucky.
E-mail: z0lei001@gwise.louisville.edu or z0lan001@gwise.louisville.edu
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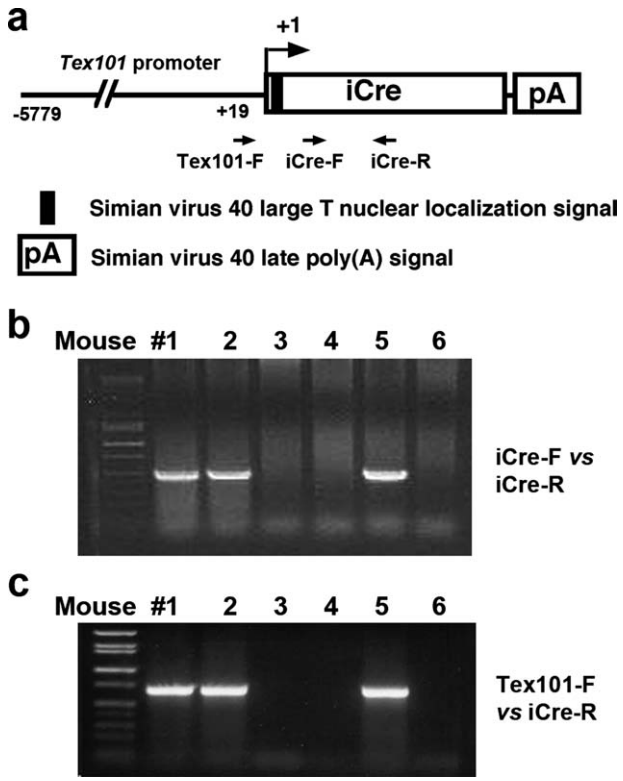

FIG. 1. Generation of *Tex101-iCre* mice. (**a**) Schematic representation of the *Tex101-iCre* transgene. The mouse *Tex101* promoter fragment (−5779 to +19) was inserted upstream of an improved Cre (*iCre*) expression cassette which contains a consensus Kozak sequence, simian virus 40 (SV40) large T nuclear localization signal, the iCre-coding sequences, and a SV40 poly(A) tail. (**b**) Genomic PCR using the iCre-F and iCre-R primers showing the presence of *iCre* in three mouse lines. (**c**) Allele-specific PCR using the Tex101-F and iCre-R primers showing the presence of the *Tex101-iCre* transgene in all three mouse lines.

2007). A codon-improved Cre recombinase (*iCre*) gene (Shimshek *et al*., 2002), instead of prokaryotic *Cre* (Gu *et al*., 1994), was used to enhance the expression level of Cre recombinase and improve the recombination efficiency of loxP sites.

Tex101, a germ cell-specific gene, is expressed in prospermatogonia before the initiation of spermatogenesis (Takayama *et al*., 2005). During the first wave of spermatogenesis, *Tex101* is highly expressed in spermatocytes and spermatids as well as spermatozoa, but not in spermatogonia, Sertoli cells, or interstitial cells (Takayama *et al*., 2005). The *Tex101* promoter (−5779 to +19) fragment (Tsukamoto *et al*., 2007) was inserted into the promoterless mini-iCre expression cassette, pGL3B-iCre (Lan *et al*., 2004), to generate the transgene vector, pGL3B-Tex101-iCre. Microinjection of the *Tex101-iCre* transgene (Fig. 1a), which contains the 5.8-kb *Tex101* promoter, the 1.1-kb iCre-coding sequence (Shimshek *et al*., 2002), and a SV40 late poly(A) signal, into the pronuclei of fertilized eggs (from the intercross of C57-B6/SJL mice) followed by transfer of the microinjected eggs into pseudo-pregnant mothers, produced one male (mouse #1) and five females (mouse #2 to #6) pups (Fig. 1b,c). Genomic PCR analysis showed that three mice (#1, #2, and #5) carried the *Tex101-iCre* transgene (Fig. 1b,c). After breeding with wild-type mice, these three mice successfully transmitted the transgene to their offspring.

R26R reporter mice (Soriano, 1999) were used to determine the tissue and cell specificity of Cre recombinase in three germline-transmitted *Tex101-iCre* mouse lines. Breeding of these three lines of *Tex101-iCre* mice with *R26R* mice produced *R26R* and *Tex101-iCre;R26R* mice. A variety of tissues (e.g., heart, kidney, liver, lung, spleen, adrenal gland, pancreas, thymus, testis, and ovary) were collected from 60-day-old *R26R* and *Tex101-iCre;R26R* mice and subjected to β-galactosidase staining. Positive blue staining in tissues indicates that Cre recombinase is active. As expected, no β-galactosidase staining was observed in the testis and other tissues of *R26R* mice (Fig. 2a, data not shown). In *Tex101-iCre;R26R* mice generated from the *Tex101-iCre* mouse lines #1 and #5, positive blue staining was observed in all tested tissues (data not shown), indicating ectopic expression of Cre recombinase in these two mouse lines. However, in *Tex101-iCre;R26R* mice generated from the *Tex101-iCre* mouse line #2, robust positive β-galactosidase staining was observed in the testis (Fig. 2b). Spotty blue staining was also observed on the surface of the kidney but not other tested tissues including the ovary (Fig. 2c, data not shown). Negative staining in the ovary was also observed in *Tex101-iCre;R26R* mice (derived from the *Tex101-iCre* mouse line #2) at 17.5 days postcoitum (dpc) (Fig. 2d). These results suggest that *Tex101-iCre* line #2 expresses Cre recombinase predominantly in the testis, not the ovary.

To determine the ontogeny of the expression of Cre recombinase, we performed β-galactosidase staining of testes from *Tex101-iCre;R26R* mice (derived from the *Tex101-iCre* mouse line #2) and *R26R* littermates at 17.5 dpc and at days 1, 8, 30, and 60 after birth. As anticipated, there was no blue staining in testicular sections from *R26R* mice (data not shown). In *Tex101-iCre;R26R* mice at 17.5 dpc, there was no positive blue staining in the testis (Fig. 2e). However, spotty positive blue staining was observed in some prospermatogonia in *Tex101-iCre;R26R* mice at day 1 after birth (Fig. 2f). At postnatal day 8, positive blue staining in prospermatogonia was more evident in the testis (Fig. 2g). At postnatal day 30, robust blue staining was observed in all seminiferous tubules, but not in interstitial cells (Fig. 2h). Within the seminiferous tubules, positive staining was observed in prospermatogonia, spermatogonia, spermatocytes, and round spermatids (Fig. 2i). Similarly, robust blue staining was observed in all seminiferous tubules, more specifically in spermatogenic cells including some prospermatogonia in 60-day-old *Tex101-iCre;R26R* testes (Fig. 2j,k). These results are at least partly consistent with endogenous *Tex101* expression in the testis (Takayama *et al*., 2005). Our results suggest that Cre recombinase in the *Tex101-iCre* mouse line #2 is active in prospermatogonia as early as at postnatal day 1 and in spermatogenic cells

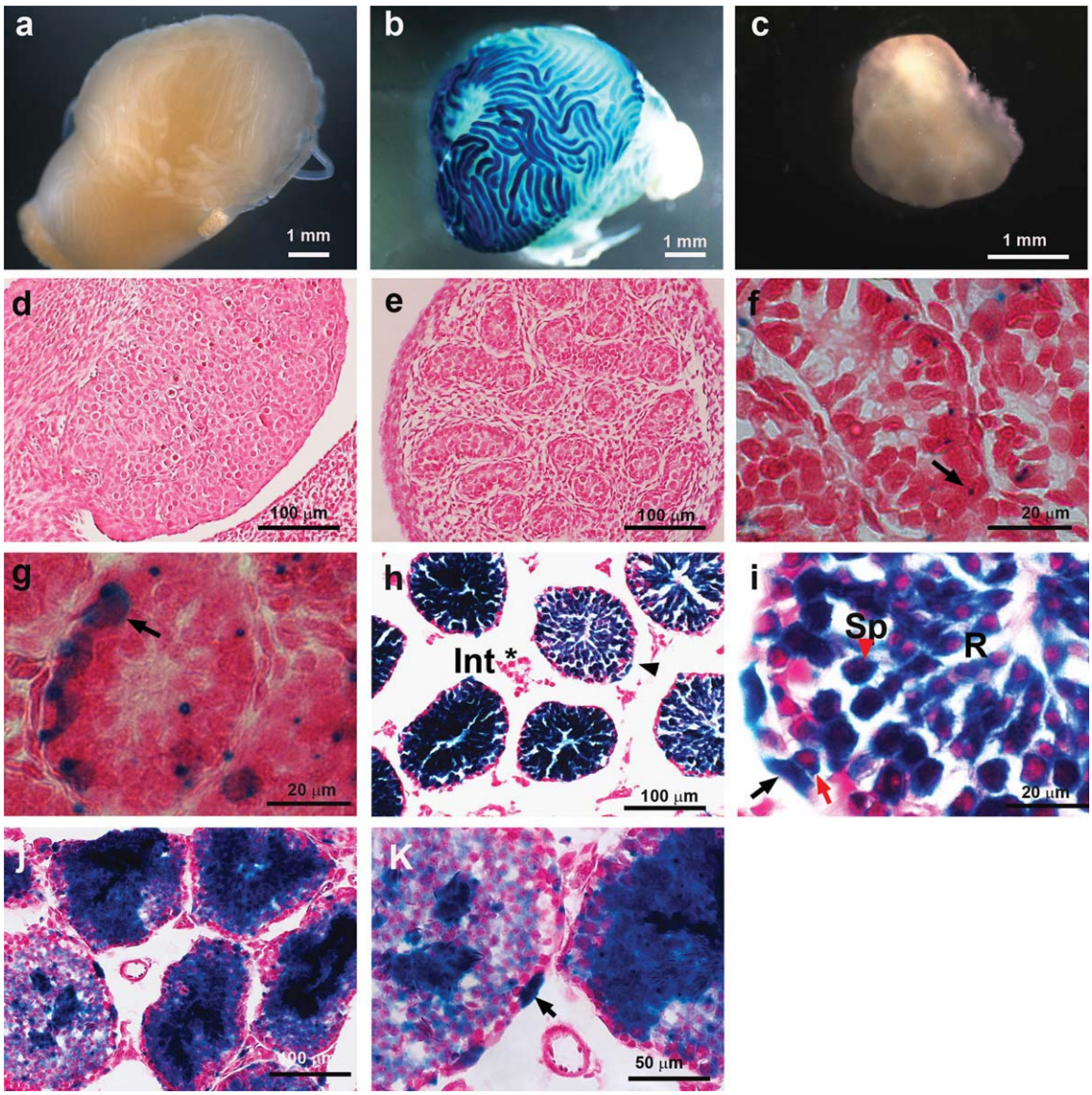

FIG. 2. β-Galactosidase staining showing Cre recombinase activity in spermatogenic cells of *Tex101-iCre;R26R* mice. (**a**) Whole-mount staining of a 60-day-old *R26R* testis. (**b–k**) β-Galactosidase staining of gonads from *Tex101-iCre;R26R* mice. (b) Whole-mount staining of a 60-day-old testis. (c) Whole-mount staining of a 60-day-old ovary. (d) Negative staining in the ovary at 17.5 dpc. (e) Negative staining in the testis at 17.5 dpc. (f) Positive and spotty staining in prospermatogonia (black arrow) at postnatal day 1. (g) Positive staining in prospermatogonia (black arrow) at postnatal day 8. (h–i) Positive staining in spermatogenic cells, but not interstitial cells (Int, indicated by the star symbol) at postnatal day 30. Panel (i) is a higher magnification of the tubule (black arrowhead) in panel (h). In panel (i), the black arrow indicates prospermatogonia; red arrow, spermatogonia; red arrowhead, spermatocyte (Sp); R, round spermatids. (j, k) Positive staining in spermatogenic cells in the testis at postnatal day 60. Panel (k) is a higher magnification of panel (j). Black arrow, prospermatogonia.

at later stages of spermatogenesis. Cre recombinase activity displayed in *Tex101-iCre* mice is earlier than in *PGK-Cre*, *c-Kit-Cre*, *Enolase-Cre*, *Sycp1Cre*, and *Protamine-Cre* mice. Thus, *Tex101-iCre* transgenic mice can be used to delete genes of interest in spermatogenic cells as early as the prospermatogonia stage in postnatal testis.

Having determined that Cre recombinase in the *Tex101-iCre* mouse line #2 can efficiently induce excision of the artificial DNA fragment in the *ROSA* locus, we investigated whether Cre recombinase in *Tex101-iCre* mouse line #2 can delete a floxed endogenous gene fragment in male germ cells. The floxed *Fgfr2* mice (Yu *et al.*, 2003) were used to test the efficiency of Cre recombinase in *Tex101-iCre* mice. Breeding of *Tex101-iCre* females with $Fgfr2^{fl/fl}$ males produced *Tex101-iCre;*$Fgfr2^{fl/+}$ mice (Fig. 3a). Female *Tex101-iCre;*$Fgfr2^{fl/+}$ mice were then crossed with male $Fgfr2^{fl/fl}$ mice to generate *Tex101-iCre;*$Fgfr2^{fl/fl}$ males. To determine whether the floxed *Fgfr2* allele was completely deleted by *Tex101-iCre* in the male germline, we performed genotyping analysis of the progeny from the breeding of

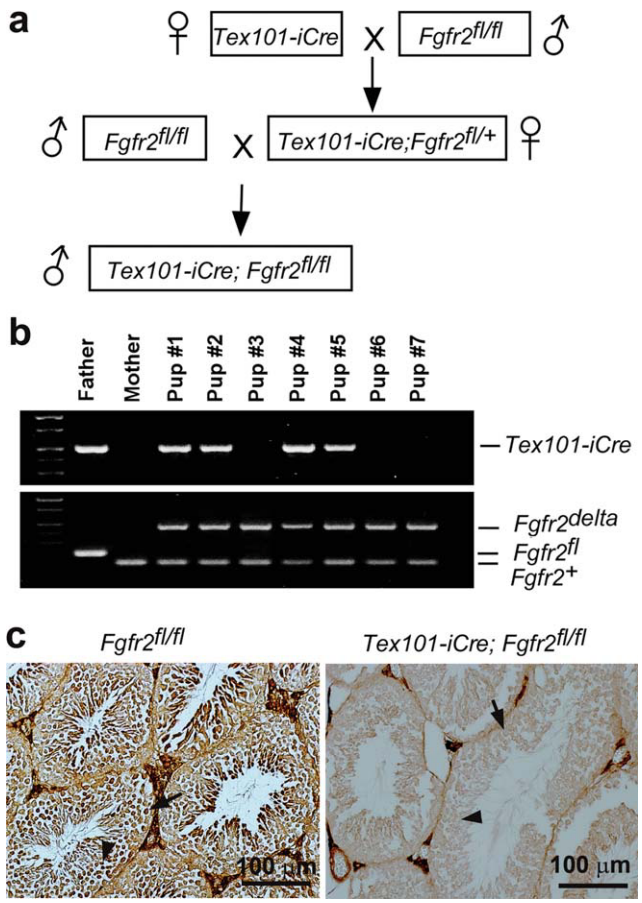

FIG. 3. Deletion of the floxed *Fgfr2* allele by *Tex101-iCre* in the male germline and spermatogenic cells. **(a)** A breeding scheme for generating *Tex101-iCre*;*Fgfr2*$^{fl/fl}$ male mice. **(b)** A representative PCR genotyping result of a litter of pups produced from breeding a *Tex101-iCre*;*Fgfr2*$^{fl/fl}$ male with a *Fgfr2*$^{+/+}$ female. Note the lack of the *Fgfr2*^{fl} allele and the presence of the *Fgfr2*^{delta} allele in all pups, indicating complete deletion of the floxed *Fgfr2* allele in the male germline, regardless of the presence of the *iCre* transgene in the progeny. **(c)** Immunohistochemical studies showing the expression of the FGFR2 protein in adult *Fgfr2*$^{fl/fl}$ (left panel) and *Tex101-iCre*;*Fgfr2*$^{fl/fl}$ (right panel) testes. Note the loss of the FGFR2 protein in spermatocytes (arrows) and spermatids (arrowheads), but not in interstitial cells of *Tex101-iCre*;*Fgfr2*$^{fl/fl}$ testes.

Tex101-iCre;Fgfr2$^{fl/fl}$ males with *Fgfr2*$^{+/+}$ females. If Cre recombinase is active in the spermatogenic cells, the floxed *Fgfr2* allele (*Fgfr2*^{fl}) will be converted to the recombined *Fgfr2*^{delta} allele regardless of the presence or absence of *Tex101-iCre* transgene in the progeny. Indeed, no floxed *Fgfr2* allele was detected in 40 pups generated from three *Tex101-iCre;Fgfr2*$^{fl/fl}$ male mice, and a recombined *Fgfr2*^{delta} allele was observed in all progeny. A representative genotyping result is shown in Figure 3b. These results show that Cre recombinase expressed in adult *Tex101-iCre* males is sufficient to completely delete the floxed *Fgfr2* allele in the male germline.

It has been shown that FGFR2 is expressed in spermatocytes and spermatids as well as interstitial cells within adult rat testis (Cancilla and Risbridger, 1998). To validate the deletion of *Fgfr2* in spermatogenic cells within mouse testis, we performed immunohistochemical studies to examine FGFR2 expression in adult *Tex101-iCre;Fgfr2*$^{fl/fl}$ testes. As expected, FGFR2 protein was detected in spermatocytes, spermatids, and interstitial cells of control *Fgfr2*$^{fl/fl}$ mice (Fig. 3c, left panel). In *Tex101-iCre;Fgfr2*$^{fl/fl}$ testes (Fig. 3c, right panel), the FGFR2 protein was also detected in interstitial cells. However, FGFR2 expression in spermatocytes and spermatids was not observed in *Tex101-iCre;Fgfr2*$^{fl/fl}$ littermates (Fig. 3c, right panel). These results suggest the complete deletion of the floxed *Fgfr2* in spermatogenic cells but not in interstitial cells by Tex101-iCre, resulting in complete loss of the FGFR2 protein in spermatocytes and spermatids. However, no obvious defects in spermatogenesis and male fertility were observed in *Tex101-iCre;Fgfr2*$^{fl/fl}$ male mice (data not shown), indicating that FGFR2 in spermatocytes and spermatids is dispensable for normal spermatogenesis and male fertility. Regardless, our results suggest that *Tex101-iCre* transgenic mice (Line #2) can be used for conditional deletion of genes in spermatogenic cells to uncover their functions in spermatogenesis and male fertility.

In conclusion, we generated and characterized a *Tex101-iCre* mouse line that can express Cre recombinase in spermatogenic cells as early as the prospermatogonia stage. More importantly, we found complete deletion of the floxed *Fgfr2* allele in spermatogenic cells in the adult testis. We believe that these *Tex101-iCre* transgenic mice will be useful to determine the physiological functions of genes during spermatogenesis. In addition, this transgenic mouse line can be used for conditional expression of transgenes specifically in spermatogenic cells as early as the prospermatogonia stage.

MATERIALS AND METHODS

Animals

Healthy adult C57B6/SJL mice and the floxed ROSA, *R26R* (on the genetic background of 129S1/SvImJ) (Soriano, 1999), were purchased from The Jackson Laboratories (Bar Harbor, Maine). The floxed *Fgfr2* mice (on a mixed background of C57BL/6J × 129x1/SvJ) (Yu *et al.*, 2003) were generously provided by Dr. David Ornitz (University of Washington, St. Louis, MO). All mice were maintained on a 14-h light:10-h dark cycle, with free access to food and water in the vivarium of the University of Louisville. All experiments in this study were approved in advance by the Animal Welfare Committee of the University of Louisville.

Generation of *Tex101-iCre* Transgenic Mice

The *Tex101* promoter fragment (−5779 to +19) was isolated from the pGL-cPK plasmid (Tsukamoto *et al.*, 2007) by restriction digestion using *Pvu*II and *Kpn*I

enzymes followed by Klenow incubation (in the presence of dNTPs). Meanwhile, the promoter-less mini-iCre expression cassette, pGL3B-iCre (Lan *et al*., 2004), which contains a consensus Kozak sequence (Kozak, 1986), the nuclear localization signal peptide of a simian virus 40 (SV40) large T antigen, and coding sequences of the *iCre* gene (Shimshek *et al*., 2002), was digested with *Kpn*I and then incubated with the Klenow fragment to remove the 5′ overhang. The Klenow-treated *Tex101* promoter fragment (−5779 to +19) was cloned into the blunted *Kpn*I site of pGL3B-iCre, using the Stratagene T4 DNA ligation kit to generate the *Tex101-iCre* transgene expression vector pGL3B-Tex101-iCre. The nucleotide sequence of this transgene vector was confirmed by sequence analysis.

A 7.13-kb Sac I/Sal I DNA fragment from pGL3B-Tex101-iCre, which contains the 5.8-kb *Tex101* promoter, 1.1-kb iCre-coding sequence, and a SV40 late poly(A) signal (Fig. 1a), was purified from an agarose gel and then microinjected into the pronuclei of fertilized eggs (C57-B6/SJLF1 X C57-B6/SJLF1 zygotes). The microinjected eggs were then transferred to pseudo-pregnant mothers to generate *Tex101-iCre* mice.

Mouse Genotyping

Mouse tail genomic DNA was extracted as described previously (Lan *et al*., 2002). *Tex101-iCre* mice were genotyped by PCR analysis using iCre-F and iCre-R primers (Lan *et al*., 2004) or Tex101-F (5′-gttcaaccaatgagacctcg-3′) and iCre-R primers. PCR conditions for genotyping the $Fgfr2^{+}$, $Fgfr2^{fl}$, $Fgfr2^{delta}$, and *R26R* allele were reported previously (Lan *et al*., 2004; Poladia *et al*., 2006; Yu *et al*., 2003).

β-galactosidase Staining

Tex101-iCre female mice were crossed with *R26R* male mice to generate *Tex101-iCre;R26R* mice. Gonads at 17.5 dpc and tissues from 60-day-old mice were collected and then subjected to β-galactosidase staining according to the manufacturer's protocol (Specialty Media, NJ). Whole-mount stained gonads at 17.5 dpc were fixed in formalin, sectioned, and counter-stained with 0.1% nuclear fast red, as described previously (Lan *et al*., 2004). Testicular cryosections from mice at 1, 8, 30, and 60 days after birth were prepared and subjected to β-galactosidase staining, as described previously (Lan *et al*., 2009). Whole-mount tissues and testicular sections were examined under a Zeiss Axioskop 2 microscope (Carl Zeiss, Thornwood, NY).

Immunohistochemistry

Cryosections of testes from 3-month-old $Fgfr2^{fl/fl}$ and $Tex101\text{-}iCre;Fgfr2^{fl/fl}$ littermates were prepared as described previously (Lan *et al*., 2009). Immunohistochemical staining of these cryosections was performed using a rabbit polyclonal anti-FGFR2 antibody (sc-122, 1:250 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) and the Rabbit Vectastain ABC kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's protocol.

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