

The Sycp1-Cre Transgenic Mouse and Male Germ Cell Inhibition of NF- κ B

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ABSTRACT: Successful spermatogenesis requires autocrine, paracrine, and endocrine signaling throughout the testes. The seminiferous tubules contain somatic Sertoli cells in tight association with numerous germ cell populations. To address the *in vivo* biologic roles of genes during spermatogenesis, spatial and temporal restriction of gene inhibition is a useful approach. To this end, Cre-LoxP technology can produce cell-specific knockdowns of genes, allowing dissection of the underlying processes that manifest as functional deficits in whole animals. Here we report the use of the synaptonemal complex protein 1-Cre (Sycp1-Cre) to create germ cell-specific nuclear factor κ B knockdown mice through floxed I κ B kinase β . We observed a LoxP gene recombination rate of approxi-

mately 43% using Sycp1-Cre, as determined by offspring genotype. In addition, we confirm that, with multiple generations, the LoxP sites fail to recombine due to epigenetic modification. This detailed examination of the meiotic Sycp1-Cre recombinase activity highlights the obstacles to germ cell-specific gene inhibition through Cre/LoxP technology in the testis. Taken together, these data demonstrate a need for early spermatogonial expression of Cre recombinase, as an alternative to meiotic Cre expression, for the creation of germ cell-specific knockout mice.

Key words: Testis, Cre/LoxP, spermatogenesis.

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Introduction

We sought to create germ cell-specific knockdowns of nuclear factor κ B (NF- κ B) activity using a Cre-LoxP murine system (Yu and Bradley, 2001). Mice lacking the NF- κ B subunit p65 (RelA) die during embryogenesis due to massive hepatocyte apoptosis (Beg et al, 1995). Restriction of gene deletion spatially or temporally through Cre-LoxP technology is one solution to the dilemma of embryo lethality associated with global knockout mice. Due to the large number of positive and negative regulating NF- κ B subunits, the upstream I κ B kinase (IKK) complex subunit IKK β was disrupted to inhibit NF- κ B activation (Karin, 1999; Li et al, 1999). We were provided with exon 3 floxed IKK β (*Ikk β ^{F/F}*) mice (Li et al, 2003; Egan et al, 2004). To create germ cell-specific NF- κ B knockdown mice, synaptonemal complex protein 1-Cre recombinase (Sycp1-Cre) was used (Vidal et al, 1998; Sage et al, 1999).

The *Sycp1* gene is expressed endogenously in both sexes, while promoter truncation restricts expression to

male meiosis (Sage et al, 1995; Vidal et al, 1998; Sage et al, 1999). Fusion of a truncated Sycp1 promoter to Cre recombinase resulted in a mouse useful for LoxP recombination in the testis during meiosis and as a method of obtaining LoxP recombined sperm for mating (Vidal et al, 1998; Sage et al, 1999; Chung et al, 2004). Expression of Cre mRNA was observed in leptotene/zygotene spermatocytes, while recombination occurred several days later in pachytene spermatocytes of stages V to VIII (Chung et al, 2004).

Recently it was discovered that expression of Sycp1-Cre during meiosis resulted in epigenetic methylation of LoxP site cytosines, blocking Cre/LoxP-mediated recombination (Rassoulzadegan et al, 2002). The authors hypothesized that presence of the Sycp1-Cre transgene with a floxed gene in spermatocytes disrupted normal imprinting, leading to a heritable silencing of LoxP sites. These results suggested that male offspring of Sycp1-Cre transgene-carrying LoxP male mice could lose their ability to perform Cre/LoxP recombination. This aberrant methylation of LoxP sites was also passed on to future generations (Rassoulzadegan et al, 2002).

Through mating of floxed IKK β (*Ikk β ^{F/F}*) and Sycp1-Cre (Cre) mice, we created heterozygous mice without (*Ikk β ^{F/A}*) and with (Cre-*Ikk β ^{F/A}*) recombinase. We sought to compare *Ikk β ^{F/F}*, Cre-*Ikk β ^{F/F}*, *Ikk β ^{F/A}*, and Cre-*Ikk β ^{F/A}* mice to study the biologic roles of NF- κ B in the *in vivo* murine testis; however, we discovered a progressive loss of LoxP recombination efficiency due to silencing of the LoxP sites in *Ikk β ^{F/F}* mice.

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Materials and Methods

Animals

Animals were housed at 30% to 70% humidity and 70°F ± 2°F on 12-hour light/dark cycles with ad libitum access to Purina Rodent Chow 5001 and water. All procedures involving animals were performed in accordance with the guidelines of Brown University's Institutional Animal Care and Use Committee in compliance with National Institute of Health guidelines. The Sycp1-Cre mice were obtained from the Jackson Laboratory (Bar Harbor, Me; strain B6;D2-Tg(Sycp1-cre)4Min/J). These Sycp1-Cre mice have been characterized in detail (Vidal et al, 1998; Sage et al, 1999; Rassoulzadegan et al, 2002; Chung et al, 2004). The laboratory of Michael Karin provided the *Ikkβ^{F/F}* mice (University of California San Diego). Characterization of the functionality of the *Ikkβ^{F/F}* mice has been performed (Li et al, 2003). Unless otherwise noted, all chemicals were purchased from Sigma Aldrich (St Louis, Mo).

Genotyping

Tail snips (4–6 mm long) were incubated in proteinase K and ATL buffer (DNeasy Kit, Qiagen Inc, Valencia, Calif) overnight at 55°C. DNA extraction and purification were performed using the DNeasy Kit. DNA yield ranged from 20 to 80 ng/μL, and 7.5 μL was used for the subsequent 50-μL polymerase chain reaction (PCR). Primers used were: IKKβ Up 5'-aagatgggcaactgtcatgtg-3' and Dp 5'-catcagggatcctgcagaca-3'; IKKβ-Lox 5'-gtcatttccacagccctgtga-3' and 5'-cctgtctatagaagcacaac-3'; IKKβ-Δ 5'-tagtccaactggcagcgaat-3' and 5'-cgctaggttaagatgctgtct-3'; Cre 5'-tgatggacatgttcagggatc-3' and 5'-cagccaccagcttgcata-3'; Y chromosome 5'-atgccgttctgcaccaagaa-3' and 5'-cgtggccttaaaactgag-3'; interleukin 2 (IL-2) 5'-ctagccacagaattgaagatct-3' and 5'-gtagtg-gaaattctagcatc-3'. Spermatozoa real-time PCR genotyping was performed on DNA extracted from caudal epididymides incubated at 37°C in 10 mL of phosphate-buffered saline for 30 minutes before centrifugation and proteinase K digestion with the addition of 10 mM DTT. Both IL-2 and total IKKβ were used as DNA housekeeping controls. The 4% Δ allele for *Ikkβ^{F/F}* mouse sperm is representative of the background of the system. Real-time PCR was performed on a Bio-Rad iCyclerIQ with SYBR green plus ROX, and quantification was performed using the Pfaffl method (Pfaffl, 2001).

Spermatid Head Count

Freshly isolated testes were detunicated, weighed, and homogenized in 1.5 mL of saline-merthiolate-thimerosol buffer as previously described (Rasoulpour et al, 2003). Samples were blinded, and spermatid heads were counted on a hemocytometer 4 independent times to ensure consistency.

Methylation Studies

Sodium bisulfite treatment of DNeasy-isolated genomic tail DNA from re-derived *Ikkβ^{F/F}* and F4–F5 generation *Ikkβ^{F/F}* mice was performed using the Chemicon CpG Fast Genome

Methylation Kit (Chemicon International, Inc, Temecula, Calif). Re-derived *Ikkβ^{F/F}* mice were never mated to the Sycp1-Cre transgene and therefore should be devoid of the epigenetic modification observed with expression of this transgene. PCR on sodium bisulfite-treated DNA was performed with methylation-specific primers (IKKβ-Up and IKKβ-Dp) to amplify DNA containing the LoxP sequence. PCR products were purified with the Qiagen PCR Purification Kit and shipped to the Yale Keck facility for DNA sequencing.

Statistics

The mean and standard error of the mean (SEM) were calculated for each data point and represented as mean ± SEM. One-way pairwise analysis of variance followed by the Bonferroni correction, Student's t-test, or χ^2 was used for all statistical analyses with significance at $P < .05$.

Results

Generation of Cre-Ikkβ^{F/F} Knockdown Mice

To create specific knockdowns of NF-κB, the well-characterized IKKβ floxed (*Ikkβ^{F/F}*) mouse was used as a global inhibitor of NF-κB activity, resulting in blockage of the classical NF-κB pathway. These *Ikkβ^{F/F}* mice have been well characterized and recombine efficiently (Li et al, 2003).

The Sycp1-Cre transgenic mouse was utilized to create germ cell-specific NF-κB knockdown mice. Therefore, *Ikkβ^{F/F}* mice were mated to Sycp1-Cre and subsequently backcrossed to *Ikkβ^{F/F}* to produce *Ikkβ^{F/F}* mice containing the Sycp1-Cre transgene (Cre-*Ikkβ^{F/F}*). To maximize NF-κB inhibition, male Cre-*Ikkβ^{F/+}* mice were mated to female *Ikkβ^{F/F}* mice to produce some *Ikkβ^{F/Δ}* pups with 1 IKKβ allele floxed and the other globally recombined (Figure 1).

By using various mating schemes, *Ikkβ^{F/F}*, Cre-*Ikkβ^{F/F}*, *Ikkβ^{F/+}*, Cre-*Ikkβ^{F/+}*, *Ikkβ^{F/Δ}*, and Cre-*Ikkβ^{F/Δ}* male mice were created. Body weights, testis weights, and histopathologic evaluation of testis sections from these mice were all unremarkable (data not shown). These data indicated that the male mice with different IKKβ genotypes had similar baseline spermatogenesis.

Evaluation of Fertility and LoxP Recombination in Mating Schemes

A detailed analysis of the prevalence of floxed or deleted alleles in offspring from parents in the mouse colony is presented in Figures 2 and 3. Mating scheme #1 shows data collected from mating a male *Ikkβ^{F/F}* and female *Ikkβ^{F/Δ}*; mating scheme #2 was the inverse, showing data from mating a male *Ikkβ^{F/Δ}* to female *Ikkβ^{F/F}*. The purpose of evaluating the genotype of the offspring from these parents was to assess putative embryonic lethal

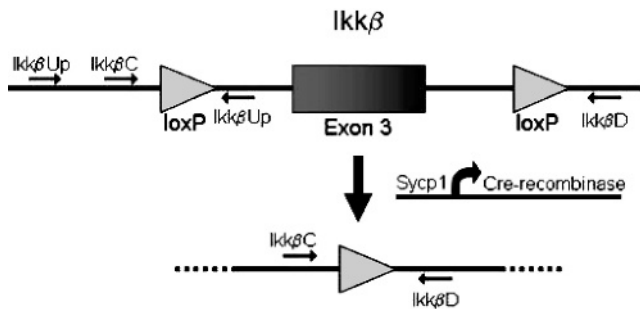


Figure 1. Exon 3 of the $I\kappa B$ kinase β gene is floxed by LoxP sites. Recombination of the LoxP by Cre recombinase results in deletion of exon 3 and subsequent inactivation of kinase activity. The Cre enzyme is under the control of the synaptonemal complex protein 1 promoter, normally expressed only during meiosis, that has been truncated to restrict expression to male meiosis.

phenotypes and any benefit/hindrance that $Ikk\beta^{\Delta}$ (Δ) sperm would have in fertilization. The 278 offspring resulting from mating scheme #1 (male $Ikk\beta^{F/F}$ and female $Ikk\beta^{F/\Delta}$) showed a nonsignificant trend (by χ^2 test) favoring the $Ikk\beta^{F/F}$ genotype by 55.8%. In contrast, in mating scheme #2 (inverse of #1; female $Ikk\beta^{F/F}$ and male $Ikk\beta^{F/\Delta}$), there was a nonsignificant trend in the opposite direction with only 43.5% $Ikk\beta^{F/F}$ pups ($n = 106$). Neither of these pup genotype frequencies diverged significantly from the expected Mendelian ratio of 50:50.

To address the Cre/LoxP recombination rate throughout spermatogenesis, the Sycp1-Cre transgene was carried in sires (Figure 3). Genotyping the offspring from these sires gave an indication of the LoxP recombination frequency that occurs upon the completion of spermatogenesis. Mating scheme #3 assessed the LoxP recombination frequency in early generations of the mouse colony. In the F1 generation of the colony, male $Cre-Ikk\beta^{F/+}$ mice were mated to $Ikk\beta^{F/+}$ females to assess germline transmission of a Δ allele. The percentage of offspring with a Δ allele ($Ikk\beta^{F/\Delta, +/\Delta}$) versus everything else ($Ikk\beta^{F/F, F/+, +/+}$) revealed that 21.7% of the 106 pups received a Δ sperm. Due to chromatin condensation into protamines during spermiogenesis, no Cre/LoxP recombination should occur after spermatogenesis. Therefore, these data indicated that by the end of spermatogenesis, 21.7% of the elongate spermatids carried a single $Ikk\beta^{\Delta}$ allele. Since the sire genotype was $Cre-Ikk\beta^{F/+}$, 100% recombination of available LoxP sites would mean 50% of the sperm would have a Δ and the remaining 50% would be IKK wildtype (+) sperm ($Ikk\beta^{+}$). Since 50% Δ in the offspring is the maximum, the 21.7% Δ we observed suggested a Cre/LoxP recombination rate of approximately 43.4%.

When $Cre-Ikk\beta^{F/\Delta}$ mice were first generated, their fertility was evaluated. Mating scheme #4 (Figure 3)

Mating Scheme #1

Parents	M $Ikk\beta^{F/F}$	F $Ikk\beta^{F/\Delta}$
Offspring (n=278)	56.5% $Ikk\beta^{F/F}$	43.5% $Ikk\beta^{F/\Delta}$

Mating Scheme #2

Parents	M $Ikk\beta^{F/\Delta}$	F $Ikk\beta^{F/F}$
Offspring (n=106)	42.5% $Ikk\beta^{F/F}$	57.5% $Ikk\beta^{F/\Delta}$

Figure 2. Offspring genotype segregated by mating schemes highlighting parental genotype and observed genotypes from these matings without Cre recombinase. Mating schemes #1 and #2 consist of a doubly floxed parent ($Ikk\beta^{F/F}$) mated to an NF- κ B knockdown ($Ikk\beta^{F/\Delta}$). Mendelian ratios would be 50:50 for the offspring. Observed numbers do not significantly deviate from the expected 50:50. (Significance $P < .05$ by χ^2 test).

displays results from 3 independent matings of $Cre-Ikk\beta^{F/\Delta}$ males with 2 female C57BL/6J wildtype ($Ikk\beta^{+/+}$) mice each. For clarity, the expected offspring genotypes based on 100%, 0%, and 43.4% (based on mating scheme #3) were calculated. If 100% of the LoxP alleles in the sperm recombined, one would expect 100% of the pups to carry a gene for IKK β . On the other hand, if 0% of the LoxP alleles recombined, then the expectation would be that 50% of the pups carry a Δ gene (since the sires are $Cre-Ikk\beta^{F/\Delta}$). Finally, if the recombination rate established by mating scheme #3 was observed here (in which approximately 43.4% of the LoxP alleles underwent Cre/LoxP recombination), then 71.7% of the offspring would carry the Δ allele.

Surprisingly, of the 119 pups from these 3 mating pairs, only 57.9% had a Δ allele. This was not significantly different from the baseline 50% and far less than the 71.7% recombination rate expected based on mating scheme #3. Since the baseline 50% of the Δ alleles in the offspring were from the Δ allele globally expressed in the sires, the 57.9% of $Ikk\beta^{F/\Delta}$ pups suggested a recombination rate of 15.8%. Therefore, we observed 43.4% Cre/LoxP recombination of LoxP sites during spermatogenesis in $Cre-Ikk\beta^{F/+}$ males, yet only 15.8% recombination in $Cre-Ikk\beta^{F/\Delta}$ testes. To address this conundrum and corroborate these results, epididymal spermatozoa were genotyped.

Real-time PCR evaluations of epididymal sperm from $Ikk\beta^{F/F}$, $Ikk\beta^{F/\Delta}$, $Cre-Ikk\beta^{F/F}$, and $Cre-Ikk\beta^{F/\Delta}$ mice (generations F4–F5) were performed (Figure 4). Primers

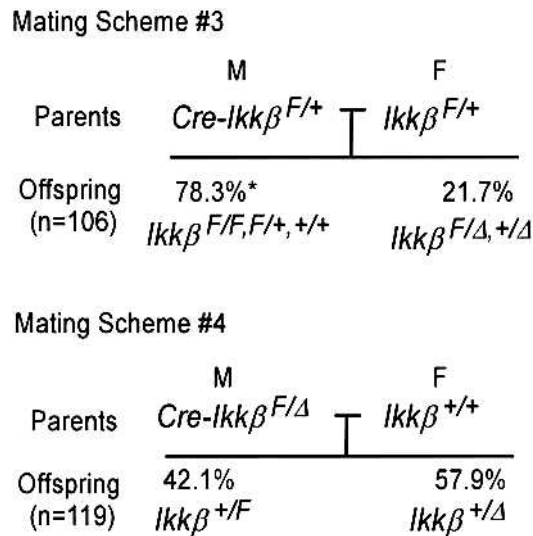


Figure 3. Mating schemes highlighting parental genotype and recombination rates with Cre recombinase-carrying sires. Mating scheme #3 (*Cre-Ikkβ^{F/+}* male mated to *Ikkβ^{F/+}* female) was performed to assess initial recombination rates in the mouse colony. With 100% recombination of the male floxed allele, the expected Mendelian ratio would be a 50:50 ratio of offspring with a floxed allele versus deleted (Δ). If there is 0% recombination of the male sperm, then 100% of the offspring should be in the left column. The 21.7% Δ observed is significant and indicates a recombination rate of 43.4% of available floxed alleles were recombined into Δ . Mating scheme #4 is the initial assessment of 1 group of NF- κ B knockdown mice. If there was 100% recombination then the ratio would be 0:100 with the male only donating Δ sperm. Note that the 57.9% Δ alleles observed is almost exactly the same as that of mating scheme #1b in which the male is *Ikkβ^{F/Δ}*. Similar to mating scheme #1, these results are not significantly different from 50:50. (*Significance $P < .05$ by χ^2 test).

for *Ikkβ^F*, *Ikkβ^Δ*, the Y chromosome, and IL-2 (housekeeping gene) were used in sperm genotyping. Spermatozoa from *Ikkβ^{F/F}* served as a negative control for the percentage of sperm with a Δ allele. Not surprisingly, 46% of the sperm from *Ikkβ^{F/Δ}* epididymides had a Δ allele. What was striking was the low percentage of Δ sperm in *Cre-Ikkβ^{F/F}* mice, indicating an apparent lack of recombination. In addition, the extent of recombination in *Cre-Ikkβ^{F/Δ}* mouse sperm was no different than *Ikkβ^{F/Δ}* sperm. These data suggested that recombination of the LoxP allele no longer occurred in these later generation mice. Also shown is the percentage of Y chromosome-positive sperm as an internal control.

To address epigenetic modification of LoxP sites from F4–F5 generation mice, sodium bisulfite treatment followed by sequencing was performed on 5 F4–F5 and 5 re-derived F1 *Ikkβ^{F/F}* mice. The re-derived F1 *Ikkβ^{F/F}* mice that had never been mated to the Sycp1-Cre had unmethylated LoxP site cytosines in their genomic DNA, as determined by sodium bisulfite treatment and subsequent DNA sequencing. In the

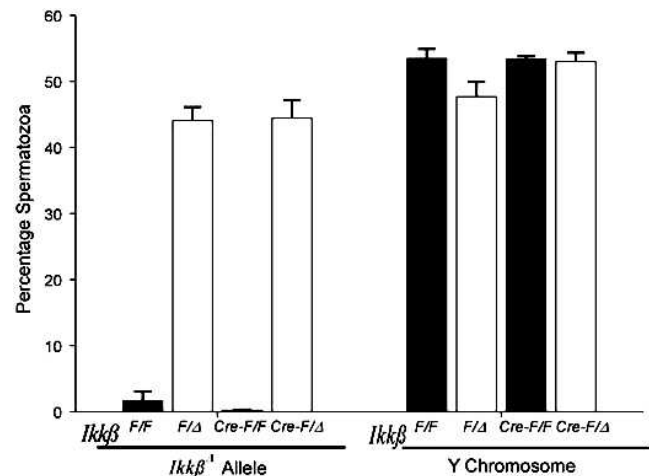


Figure 4. Epididymal spermatozoa real-time polymerase chain reaction genotyping for the deleted I κ B kinase β (*IKK β*) allele and Y chromosome. All 4 genotypes have approximately 50% Y sperm (right panel) as a control. The percentage of sperm with the Δ allele is at background levels for *Ikkβ^{F/F}* and *Cre-Ikkβ^{F/F}* in late-generation mice, while approximately 45% for *Ikkβ^{F/Δ}* and *Cre-Ikkβ^{F/Δ}* mice, indicating a lack of floxed recombination. Experiments were performed in triplicate with a housekeeping gene as an internal control. (n >3 for all genotypes).

later generation mice, 3 out of the 5 had methylation of cytosines in the LoxP sequence of *Ikkβ^{F/F}*. The methylated LoxP sites on these mice all originated from a paternal *IKK β* -LoxP allele in a *Cre-Ikkβ^{F/F}* sire, while the unmethylated sequence had a maternally derived LoxP. These observations were consistent with the mechanism of epigenetic silencing previously reported with the Sycp1-Cre (Rassoulzadegan et al, 2002).

Discussion

Cre-LoxP technology can be useful to inhibit gene expression in a spatially or temporally restricted manner. This can be particularly valuable to the study of spermatogenesis due to the multiplicity of cell types within the testis. Here, the Sycp1-Cre mouse was used to study the roles of NF- κ B during spermatogenesis. We used *Ikkβ^{F/F}* mice mated with Sycp1-Cre transgenic mice to create *Ikkβ^{F/Δ}* and *Cre-Ikkβ^{F/Δ}* NF- κ B knockdown animals. During the creation and characterization of these mice, we observed low recombination rates and eventually a failure of recombination.

Initially surprising was the lack of a testicular phenotype in our NF- κ B knockdown (*Ikkβ^{F/Δ}* and *Cre-Ikkβ^{F/Δ}*) mice (data not shown). There was no reduction of fertility or fecundity. Having a deleted exon 3 (kinase domain) of *IKK β* was also not deleterious to developing offspring, as determined by the pup frequency from mating scheme #1 (Figure 2). Assessment of

the recombination rate of the Sycp1-Cre recombinase in initial male Cre-*Ikkβ^{F/+}* mice showed a 43.4% rate as measured by offspring genotyping. This modest LoxP recombination was of concern and far lower than the expected rate of 100% recombination based on previous characterization of Sycp1-Cre (Vidal et al, 1998). Given that Cre recombinase is a stable enzyme (Buchholz et al, 1996) and that expression of the Sycp1-Cre mRNA occurs in early meiosis (leptotene/zygotene spermatocytes) (Chung et al, 2004) (14 days before spermatid release from the seminiferous epithelium; Russell, 1990), the percentage of germ cells lacking a functional allele of IKKβ was unexpectedly low.

Assessment of the offspring of NF-κB knockdown Cre-*Ikkβ^{F/A}* mice indicated that these mice likely were subjected to epigenetic silencing due to methylated cytosines within the IKKβ LoxP sites. The Sycp1-Cre has been reported to cause a transvection phenomenon wherein once the Cre is present in a male, future offspring have increasingly greater amounts of methylated cytosines on their LoxP sites. This site-specific methylation increases progressively throughout generations. Importantly, a LoxP site with methylated cytosines is not recognized by the Cre-recombinase (Rassoulzadegan et al, 2002). Real-time PCR of epididymal sperm from these mice supported these observations. Sodium bisulfite treatment of genomic DNA from male F1 and F4–F5 *Ikkβ^{F/F}* mice revealed LoxP site cytosine methylation on (3 of 5) older generation *Ikkβ^{F/F}* mice, correlating with previous results (Rassoulzadegan et al, 2002).

An initial characterization of the Sycp1-Cre was performed by the developers of this transgenic mouse (Chung et al, 2004). They reported that Sycp1-Cre/Rosa26 mice displayed strong β-galactosidase positivity in pachytene spermatocytes. It is important to note, however, that primary spermatocytes are tetraploid. Therefore, the pachytene spermatocyte positivity reported in these studies could result from 1 to 4 LoxP recombinations per cell. In addition, germ cells exist in syncytia, and the β-galactosidase protein could possibly diffuse through cytoplasmic bridges between cells.

Clearly, Cre/LoxP technology has significant limitations in dynamic tissues such as seminiferous tubules. Complexities arising from tetraploidy and syncytia in male germ cells magnify the difficulty of cell type-specific gene knockout in cells with such high turnover.

These issues could, in part, be alleviated by using early spermatogonial-specific Cre transgenic mice; however, proper interpretation of Cre/LoxP technology effects in male germ cells can be complex.

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