Published in final edited form as:
Genesis. 2008 December ; 46(12): 738–742. doi:10.1002/dvg.20437.

Cre Recombinase Activity Specific to Postnatal, Premeiotic Male Germ Cells in Transgenic Mice

Patricia I. Sadate-Ngatchou[1,*], **Christopher J. Payne**[1,2,*], **Andrea T. Dearth**[1,2], and **Robert E. Braun**[1,2,3]

[1]Department of Genome Sciences, University of Washington School of Medicine, Seattle, Washington

[2]The Jackson Laboratory, Bar Harbor, Maine

Summary

We have generated a transgenic mouse line, Tg(Stra8-cre)1Reb (Stra8-cre), which expresses improved Cre recombinase under the control of a 1.4 Kb promoter region of the germ cell-specific stimulated by retinoic acid gene 8 (*Stra8*). *cre* is expressed only in males beginning at postnatal day (P)3 in early-stage spermatogonia, and is detected through pre-leptotene-stage spermatocytes. To further define when *cre* becomes active, we crossed Stra8-cre males with Tg(ACTB-Bgeo/GFP)21Lbe (Z/EG) reporter females and compared the expression of Enhanced Green Fluorescent Protein (EGFP) with the protein encoded by the zinc finger and BTB domain containing 16 (*Zbtb16*) gene, PLZF – a marker for undifferentiated spermatogonia. Co-expression of EGFP is observed in the majority of PLZF+ cells. We also tested recombination efficiency by mating Stra8-cre;Z/EG males and females with wild-type mice and examining EGFP expression in the offspring. Recombination is detected in >95% of Z/EG+ pups born to Stra8-cre;Z/EG fathers but in none of the offspring born to transgenic mothers, a verification that *cre* is not functional in females. The postnatal, premeiotic, male germ cell-specific activity of Stra8-cre makes this mouse line a unique resource to study testicular germ cell development.

Keywords

Cre recombinase; Stra8 promoter; spermatogonia; spermatocytes; Z/EG

These experiments were initiated to produce a transgenic mouse line that expresses *cre* in undifferentiated spermatogonia. To date, the use of *cre*-mediated recombination to inactivate genes in developing germ cells at specific stages has been limited by the restricted expression patterns of available *cre* drivers. Recombination in primordial germ cells is possible using the alkaline phosphatase, liver/bone/kidney *Alpl*$^{tm1(cre)Nagy}$ mouse line (Lomeli *et al.*, 2000). However, significant developmental events occur between the onset of *cre* at embryonic day (E)9.5 and the appearance of spermatogonia in the postnatal testis. Additional *cre* activity is detected in the placenta, intestine, and neural tube. Excision of floxed DNA in primary spermatocytes and elongating spermatids is achievable using synaptonemal complex protein 1 Tg(Sycp1-cre)4Min (Vidal *et al.*, 1998) and protamine 1 Tg(Prm-cre)58Og (O'Gorman *et al.*, 1997), respectively, but the temporal expression of these transgenes is too late to be of use in undifferentiated spermatogonia. Meanwhile, the expression of *cre* driven by the growth differentiation factor 9 (*Gdf9*) and zona pellucida 3

[3]Correspondence to: Robert E. Braun, The Jackson Laboratory, 600 Main Street, Bar Harbor, Maine 04609, bob.braun@jax.org.
[*]These authors contributed equally to this work

(*Zp3*) promoters is restricted to developing oocytes (Lan *et al.*, 2004; Lewandoski *et al.*, 1997).

We therefore selected the promoter of the premeiotic male and female germ cell-specific gene *Stra8* to drive expression of improved Cre recombinase. Endogenous *Stra8* is first expressed in ovarian germ cells at E12.5 and continues until E16.5, while in males it is first transcribed in early-stage spermatogonia in the postnatal testis and persists in premeiotic germ cells throughout adulthood (Menke *et al.*, 2003; Oulad-Abdelghani *et al.*, 1996). A 1.4 Kb *Stra8* promoter fragment was recently fused to *EGFP* coding sequence, and the resulting transgene was shown to be a marker for spermatogonial stem cells (SSCs), a subpopulation of undifferentiated spermatogonia (Nayernia *et al.*, 2004). We have used this same 1.4 Kb promoter region to generate Stra8-cre by inserting it upstream of the improved *cre* coding sequence, shown to exhibit improved expression over the conventional prokaryotic *cre* (Shimshek *et al.*, 2002).

The structure of the transgene is depicted in Figure 1a. After pronuclear injection of the transgenic construct and examination of resulting offspring (data not shown), one male was found to exhibit germline expression of Stra8-cre. This male was used to expand the line, and embryos were isolated from subsequent transgenic matings at two stages, E14.5 and E16.5, to assess transcriptional activity by RT-PCR (Fig. 1b). Surprisingly, none of the female embryos showed any cre mRNA expression during the window when endogenous *Stra8* is active in the female. Male embryos, as expected, did not express the transgene. When testes were examined for cre transcript at E18, P3, and P7 (Fig. 1c), expression was first detected at P3 and became much stronger by P7. In P7 pups, various organs were isolated to assess the specificity of Stra8-cre expression (Fig. 1d). Of the eight organs examined, only testis showed the cre transcript. Postnatal ovaries, as with embryonic females, exhibited no *cre* expression, suggesting that the transgene is non-functional in females. To examine Stra8-cre protein expression in P7 males, anti-cre antibody was used on testis cross-sections (Fig. 1e). At this early stage of postnatal development, cre protein was detected in the majority of spermatogonia within the seminiferous tubules.

Immunohistochemistry was also performed on adult testis (Fig. 2a). Cre was observed in spermatogonia and pre-leptotene spermatocytes residing along the basement membrane. As positive staining was seen in different stages of the seminiferous epithelial cycle (Russell, 1990), we conclude that Stra8-cre expression begins in Type A spermatogonia and ends in pre-leptotene spermatocytes, the last developmental stage before the onset of meiosis. To determine whether Cre recombinase functions in undifferentiated spermatogonia, a subset of Type A, we mated Stra8-cre males to Z/EG reporter females that constitutively express a floxed *Bgeo* until *cre*-mediated excision, after which EGFP becomes active (Novak *et al.*, 2000). Seminiferous tubules of Stra8-cre;Z/EG males were examined by whole-mount immunocytochemistry for the co-expression of EGFP and PLZF, a marker of undifferentiated spermatogonia (Buaas *et al.*, 2004;Costoya *et al.*, 2004). As shown in Figures 1b-c″, the majority of PLZF+ cells also expressed EGFP, indicating Stra8-cre activity. Additional, fainter EGFP+ cells that lack PLZF staining represent more differentiated germ cells (Figs. 1b″,c″). Interestingly, some PLZF+ spermatogonia did not exhibit EGFP, suggesting that *cre* becomes active in a subpopulation of undifferentiated spermatogonia.

Finally, we tested recombination efficiency by mating Stra8-cre;Z/EG males with wild-type females, and Stra8-cre;Z/EG females with wild-type males. Offspring produced from these matings were examined for constitutive EGFP expression that would result from the transmission of the recombined Z/EG allele through transgenic sperm or eggs. Table 1 reveals that of the pups born to Stra8-cre;Z/EG fathers, >95% receiving the Z/EG allele

exhibited recombination. In contrast, none of the pups born to Stra8-cre;Z/EG mothers receiving Z/EG showed recombination (Table 1). This finding verifies that *cre* is non-functional in females.

Exactly why Stra8-cre is male germ cell-specific is not clear. The promoter region used in this study contains both retinoic acid receptor-binding elements identified previously (Giuili *et al*., 2002); it is the same fragment that generated the *Stra8*-EGFP transgene, demonstrated to be a marker for SSCs (Nayernia *et al.*, 2004). It is likely that the transgene lacks key enhancers important for expression in females. Nonetheless, these experiments have yielded a *cre* driver that will facilitate the assessment of gene function in postnatal, premeiotic male germ cells.

Materials and Methods

Generation of Transgenic Stra8-cre Mice

First, the -1400/+7 fragment of *Stra8* genomic DNA (Nayernia *et al.*, 2004), where +1 is the transcription start site, was isolated from FVB/NJ mouse DNA and amplified using PCR primers designed with an AvrII recognition site at the 5′ end of the forward primer, gStra8F1avrII (5′-TCT CCT AGG AAC TTG CCT CCA AGG GGG TAG G-3′), and an XhoI recognition site at the 5′ end of the reverse primer, gStra8R1xhoI (5′-TGC TCG AGA CGA CTG CCC GTC GCA GAA TAA G-3′). The resulting product was T/A cloned into pGEMTeasy vector, followed by excision using AvrII and XhoI. This fragment was then substituted for the *GnRH* promoter in the plasmid pL29mGnRH.iCre (Shimshek *et al.*, 2002) via ligation following the excision of the *GnRH* fragment using AvrII and XhoI. Because pL29mGnRH.iCre contains two XhoI recognition sites, this ligation was performed in two steps. First, the *Stra8* promoter was ligated to the plasmid vector backbone to produce a 4.39 Kb product. Second, the sequence between the two XhoI sites in the plasmid vector comprising the *cre* coding region (~1.26 Kb) was amplified by PCR, T/A cloned into pGEMTeasy vector, digested with XhoI, and ligated to the XhoI-treated fragment that contained the *Stra8* promoter. This ligation product, called pL29Stra8.iCre (~5.65 Kb), was used to transform DH5α cells, and the plasmids from the resulting colonies were sequenced to verify proper insertion and orientation of fragments.

Finally, a 3.1 Kb fragment excised by AvrII and BsiWI from pL29Stra8.iCre was purified and microinjected into the pronuclei of fertilized eggs of FVB mice. The resulting transgenic mouse line was named FVB/N-Tg(Stra8-cre)1Reb (Stra8-cre). This line of Stra8-cre has been given to The Jackson Laboratory and JAX® Mice for distribution, with JAX Stock # 008208. For some experiments, Stra8-cre mice were crossed with STOCK Tg(ACTB-Bgeo/GFP)21Lbe/J (Z/EG) reporter mice obtained from The Jackson Laboratory. Wild-type FVB/NJ mice were also used in specific matings.

RT-PCR for cre mRNA Expression

Embryos were dissected from female mice at E14.5, E16.5, and E18 following timed matings of Stra8-cre intercrosses. The heads of the embryos were removed and the remaining caudal regions were prepared for total RNA isolation in dounce tissue homogenizers using TRIzol® Reagent (Invitrogen, Carlsbad, CA). Genotyping for the *Sry* gene was performed to confirm the sex of each embryo. Male and female pups were euthanized at P3 and P7, and the following organs were removed for total RNA isolation: brain (B), heart (H), kidney (K), lung (L), spleen (S) and testes (T) from males; ovaries (O) from females. Total RNA was reverse transcribed into cDNA using random hexamer primers (Invitrogen). To detect *cre* expression, a 179-bp fragment was amplified by PCR using primers ic202F (5′-GTG CAA GCT GAA CAA CAG GA-3′) and ic381R (5′-AGG

GAC ACA GCA TTG GAG TC -3′) under the following conditions: 95°C for 5 min, followed by 40 cycles of 95°C for 30 sec. and 60°C for 1 min., with a final extension at 72°C for 7 min. A 207-bp fragment of βActin was amplified as a control transcript using primers Actb-F70 (5′-CCA GTT CGC CAT GGA TGA CGA TAT-3′) and Actb-R277 (5′-GTC AGG ATA CCT CTC TTG CTC TG-3′) under the same thermocycling conditions. Products were separated on 3% (w/v) agarose gels.

Immunohistochemistry on Cross-Sections and Whole-Mount Tubules

For analysis of cross-sections, testes were isolated from postnatal Stra8-cre mice at various ages and fixed in neutral-buffered formalin [3.7% (v/v) formaldehyde in phosphate-buffered solution (PBS)] overnight at 4°C. Fixed testes were then dehydrated, embedded in paraffin wax, and cut into 5μm-thick sections. Following rehydration, antigen-retrieval was performed by boiling the cross-sections in 10 mM sodium citrate, pH 6.0, for 10 min. After rinsing in dH_20, some cross-sections were incubated in 0.3% (v/v) hydrogen peroxide in MeOH for 20 min. to inhibit endogenous peroxidase activity. All samples were blocked in 3% (v/v) normal goat serum in PBS for 1 h at room temperature, followed by an overnight incubation at 4°C with anti-cre rabbit polyclonal antibody at 1:500 dilution (Novagen/EMD Biosciences, Madison, WI). After 2×10 min. washes in PBS, some cross-sections were incubated for 1h at room temperature with biotin-conjugated goat anti-rabbit antibody diluted 1:500 (Zymed/Invitrogen). For these samples, tertiary antibody incubation was performed by the addition of streptavidin-HRP antibody at 1:100 dilution for 20 min. Peroxidase activity was then visualized by using a DAB substrate kit following the manufacturer's instructions (Zymed/Invitrogen). Other cross-sections were incubated with AlexaFluor 488-conjugated goat anti-rabbit secondary antibody at 1:1000 dilution (Molecular Probes/Invitrogen). All samples were then rinsed in dH_20. Coverslips were affixed with glycerol/vinyl alcohol medium and cross-sections were examined on a Nikon E600 microscope using brightfield optics.

For whole-mount immunocytochemistry, seminiferous tubules were dissected from testes isolated from postnatal Stra8-cre;Z/EG mice at various ages and processed as previously described (Payne and Braun, 2006). Anti-EGFP mouse monoclonal antibody (JL-8; Clontech, Mountain View, CA) and anti-PLZF rabbit polyclonal antibody (H-300, Santa Cruz Biotechnology) were used in these studies at 1:50 dilution. AlexaFluor-conjugated secondary antibodies were diluted 1:1000 (Molecular Probes/Invitrogen). Tubules were examined by epifluorescence microscopy with FITC and TRITC filter sets.

Assessment of Stra8-cre Recombination Efficiency

Matings were established between Stra8-cre;Z/EG males and wild-type FVB/NJ females, and between Stra8-cre;Z/EG females and wild-type FVB/NJ males. Offspring produced from these matings were euthanized at 3-5 d of age and genotyped for the presence of *EGFP*. Heads were removed from the euthanized pups, and the remaining torsos were rinsed in PBS and sectioned in half. One half was examined for EGFP expression by visualizing tissues using epifluorescence microscopy with an FITC filter. The other half was fixed in 2% (v/v) paraformaldehyde in PBS on ice for 1.5 h, then washed 3×15 min. in PBS containing 2mM $MgCl_2$, 0.01% (w/v) sodium deoxycholate, and 0.02% (v/v) NP-40. Torsos were stained overnight at room temperature with 0.5 mg/ml X-Gal, 5mM potassium ferrocyanide, and 5mM potassium ferricyanide. Following 3×10 min. washes in PBS, torsos were examined for lacZ staining using brightfield microscopy. Mice positive for EGFP expression and negative for lacZ staining were scored as successfully exhibiting recombination at the Z/EG allele, transmitted through either sperm or egg from the transgenic parent.

Acknowledgments

The authors thank Dr. Rolf Sprengel for the kind gift of plasmid pL29mGnRH.iCre. Correspondence and requests for materials should be addressed to R.E. Braun at bob.braun@jax.org.

Literature Cited

Buaas FW, Kirsh AL, Sharma M, McLean DJ, Morris JL, Griswold MD, de Rooij DG, Braun RE. Plzf is required in adult male germ cells for stem cell self-renewal. Nat Genet. 2004; 36:647–652. [PubMed: 15156142]

Costoya JA, Hobbs RM, Barna M, Cattoretti G, Manova K, Sukhwani M, Orwig KE, Wolgemuth DJ, Pandolfi PP. Essential role of Plzf in maintenance of spermatogonial stem cells. Nat Genet. 2004; 36:653–659. [PubMed: 15156143]

Giuili G, Tomljenovic A, Labrecque N, Oulad-Abdelghani M, Rassoulzadegan M, Cuzin F. Murine spermatogonial stem cells: targeted transgene expression and purification in an active state. EMBO Rep. 2002; 3:753–759. [PubMed: 12151334]

Lan ZJ, Xu X, Cooney AJ. Differential oocyte-specific expression of Cre recombinase activity in GDF-9-iCre, Zp3cre, and Msx2Cre transgenic mice. Biol Reprod. 2004; 71:1469–1474. [PubMed: 15215191]

Lewandoski M, Wassarman KM, Martin GR. Zp3-cre, a transgenic mouse line for the activation or inactivation of loxP-flanked target genes specifically in the female germ line. Curr Biol. 1997; 7:148–151. [PubMed: 9016703]

Lomeli H, Ramos-Mejia V, Gertsenstein M, Lobe CG, Nagy A. Targeted insertion of Cre recombinase into the TNAP gene: excision in primordial germ cells. Genesis. 2000; 26:116–117. [PubMed: 10686602]

Menke DB, Koubova J, Page DC. Sexual differentiation of germ cells in XX mouse gonads occurs in an anterior-to-posterior wave. Dev Biol. 2003; 262:303–312. [PubMed: 14550793]

Nayernia K, Li M, Jaroszynski L, Khusainov R, Wulf G, Schwandt I, Korabiowska M, Michelmann HW, Meinhardt A, Engel W. Stem cell based therapeutical approach of male infertility by teratocarcinoma derived germ cells. Hum Mol Genet. 2004; 13:1451–1460. [PubMed: 15163638]

Novak A, Guo C, Yang W, Nagy A, Lobe CG. Z/EG, a double reporter mouse line that expresses enhanced green fluorescent protein upon Cre-mediated excision. Genesis. 2000; 28:147–155. [PubMed: 11105057]

O'Gorman S, Dagenais NA, Qian M, Marchuk Y. Protamine-Cre recombinase transgenes efficiently recombine target sequences in the male germ line of mice, but not in embryonic stem cells. Proc Natl Acad Sci U S A. 1997; 94:14602–14607. [PubMed: 9405659]

Oulad-Abdelghani M, Bouillet P, Decimo D, Gansmuller A, Heyberger S, Dolle P, Bronner S, Lutz Y, Chambon P. Characterization of a premeiotic germ cell-specific cytoplasmic protein encoded by Stra8, a novel retinoic acid-responsive gene. J Cell Biol. 1996; 135:469–477. [PubMed: 8896602]

Payne C, Braun RE. Histone lysine trimethylation exhibits a distinct perinuclear distribution in Plzf-expressing spermatogonia. Dev Biol. 2006; 293:461–472. [PubMed: 16549060]

Russell, LD.; Ettlin, RA.; Sinha Hikim, AP.; Clegg, ED. Histological and Histopathological Evaluation of the Testis. St. Louis: Cache River Press; 1990. p. 286

Shimshek DR, Kim J, Hubner MR, Spergel DJ, Buchholz F, Casanova E, Stewart AF, Seeburg PH, Sprengel R. Codon-improved Cre recombinase (iCre) expression in the mouse. Genesis. 2002; 32:19–26. [PubMed: 11835670]

Vidal F, Sage J, Cuzin F, Rassoulzadegan M. Cre expression in primary spermatocytes: a tool for genetic engineering of the germ line. Mol Reprod Dev. 1998; 51:274–280. [PubMed: 9771647]

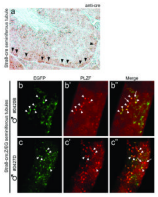

FIG. 1.

Testis-specific expression of Cre recombinase mRNA in postnatal Stra8-cre transgenic mice. (**a**) Schematic diagram of the transgene construct. A 1.4 Kb promoter fragment of *Stra8* is inserted upstream of the 1.26 Kb *cre* coding sequence, separated by the SV40 splice donor/ splice acceptor. -1400 and +7 denote the nucleotide positions of the *Stra8* fragment, and restriction endonuclease recognition sites utilized in the construction of the 3.1 Kb transgene are listed. (**b**) cDNA from male and female embryos isolated at E14.5 and E16.5 was analyzed by PCR using cre and βActin primers. No cre band was detected in any of the males or females (denoted by symbols) at either time point. (**c**) cDNA from testes isolated at E18, P3, and P7 was analyzed by PCR for cre and βActin. A faint cre band was detected at P3, with a much stronger band at P7; – RT, no reverse transcription control. (**d**) cDNA from different organs isolated from P7 transgenic offspring was analyzed by PCR for cre and βActin: B, brain; H, heart; K, kidney; L, lung; S, spleen; T, testis; O, ovary; -RT, no reverse transcription on testis RNA. Only the testis shows a specific cre band. Faint, non-specific product was amplified from lung cDNA. **(e)** Cross-section of P7 transgenic seminiferous tubules. Cre protein is detected by anti-cre antibody (green); DNA is labeled with DAPI (blue).

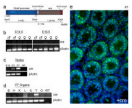

FIG. 2.

Initial Stra8-cre activity is detected in a subset of undifferentiated spermatogonia and its expression is observed through pre-leptotene stage spermatocytes. (**a**) Cross-section of a Stage VII seminiferous tubule isolated from a 6-week-old Stra8-cre mouse. Arrowheads point to pre-leptotene spermatocytes that express Stra8-cre, detected by anti-cre antibody. (**b-c″**) Whole-mount immunostaining of seminiferous tubules isolated from two different 2.5-week-old Stra8-cre;Z/EG mice, offspring resulting from a Stra8-cre male × Z/EG female cross. Arrowheads denote undifferentiated spermatogonia expressing the transcriptional repressor PLZF (red) that also express EGFP (green), a result of the excision of the *Bgeo* gene from the Z/EG allele by Stra8-cre (Merge, yellow). Arrows in **b″** and **c″** point to PLZF + spermatogonia that do not express EGFP.

Table 1

Assessment of Stra8-cre recombination efficiency on the Z/EG target allele

breeding pair		# of offspring $\left(\frac{Z/EG}{+}\right)$		% recombination in offspring $\left(\frac{Z/EG}{+}\right)$
$\frac{Stra8-cre}{+};\frac{Z/EG}{+} \times WT$		EGFP+	lacZ+	
♂ #2435	♀	66	2	97%
♂ #2562	♀	17	0	100%
♂ #2559	♀	25	1	96%
♀ #3113	♂	0	16	0%
♀ #2818	♂	0	12	0%

♂ = male; ♀ = female; Z/EG = Tg(ACTB-BGeo/GFP)21Lbe