Zp3–cre, a transgenic mouse line for the activation or inactivation of *loxP*-flanked target genes specifically in the female germ line

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The site-specific DNA recombinase Cre is being used to develop a new generation of tools for controlling gene expression in mice [1]. Cre mediates the recombination of two directly repeated target (*loxP*) sites to a single *loxP* site, with concomitant excision of the DNA segment flanked by the *loxP* sites (the 'floxed' DNA). Such recombination can function to activate a gene by excising a floxed DNA segment that blocks expression because it either separates the regulatory and coding sequences of the gene [2] or interrupts the gene's open reading frame. Conversely, DNA excision can inactivate a gene if an essential fragment of the gene is floxed [3]. Gene activation or inactivation *in vivo* can be achieved by mating two different animals, one carrying a 'target gene' with appropriately placed *loxP* sites and one carrying a *cre* transgene. In most cases, the specificity of the system is dependent upon stringent regulation of *cre* expression. We describe here a mouse line in which *cre* expression is controlled by regulatory sequences from the mouse *zona pellucida 3* (*Zp3*) gene, which is normally expressed exclusively in the growing oocyte prior to the completion of the first meiotic division [4]. We show that in target-bearing *Zp3–cre* mice, Cre-mediated recombination of the target gene apparently occurs in 100 % of oocytes. Moreover, Cre activity is not detected in the somatic tissues of most target-bearing *Zp3–cre* mice. Potential uses for this mouse line are discussed.

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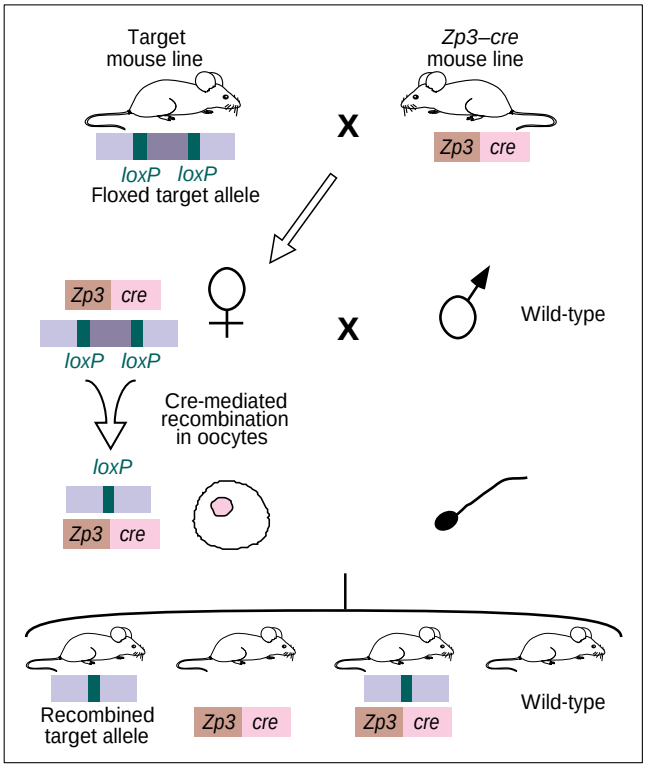
Results and discussion

To construct the pZp3–cre plasmid used to produce transgenic mice, we first modified the bacteriophage P1 *cre* gene [5] so that the ATG start codon and five bases immediately upstream of it were replaced by the sequence CCACCATGGGCCCAAAGAAGAAGGAGAAGGTT. These changes served to optimize translational efficiency [6] and to insert the sequence that encodes the nuclear localization signal from the T antigen of simian virus 40 (SV40) [7] into the 5′ region of the *cre* open reading frame. This modified *cre* DNA was then placed downstream of a 6.5 kilobase (kb) *Kpn*I restriction fragment that contains *Zp3* regulatory regions previously shown to drive expression of the firefly luciferase reporter gene in an oocyte-specific manner [8] and upstream of a 0.89 kb *Hin*dIII–*Kpn*I fragment that contains the polyadenylation signal from the human β-actin gene (K. Sturm and R. Pedersen, personal communication). The resulting pZp3–cre construct was injected into mouse zygotes (strain FVB/N) to produce

Figure 1



Mating scheme to assess Cre activity in the female germ line of *Zp3–cre* transgenic mice. By crossing *Zp3–cre* mice with a line of mice carrying the floxed target gene, female *cre*$^+$/target$^+$ progeny were obtained. The *cre* and target genes segregate independently in these females, and thus when they are mated to wild-type males, progeny of four different genotypes are obtained. If *cre* has been expressed in the oocyte, the target gene is inherited in the recombined form, regardless of whether the *cre* transgene is also inherited. Thus, Cre activity in the female germ line can be assayed by determining the frequency at which *cre*$^+$/target$^+$ females transmit the target gene in recombined form.

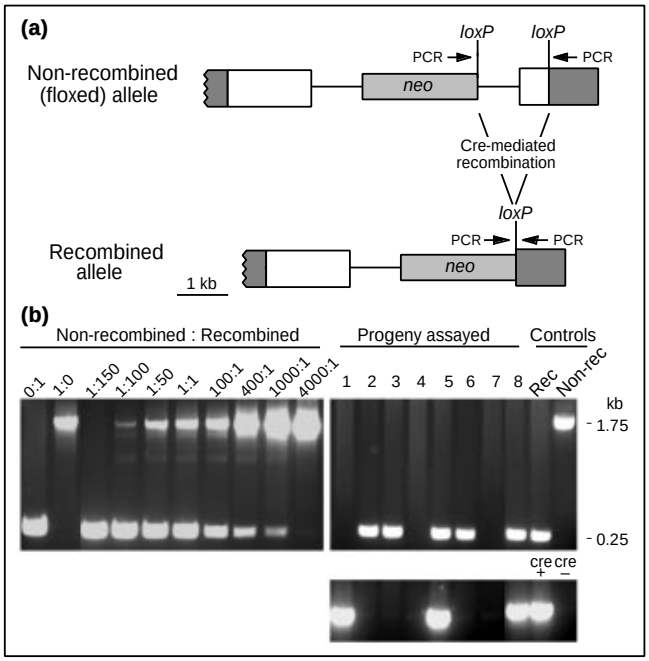
transgenic founder mice, and the *Zp3–cre* mouse line described here was established.

Cre activity in the female germ line was assessed using the mating scheme illustrated in Figure 1. *Zp3-cre* mice were mated to mice carrying a floxed target gene, and female *cre*$^+$/target$^+$ progeny were selected for further study. As a target gene we used an endogenous gene, *Gbx2*, modified by homologous recombination so that it contains two *loxP* sites (Fig. 2a and our unpublished work). Mice heterozygous for either the floxed (non-recombined) *Gbx2* allele or its recombined derivative are phenotypically normal. A single polymerase chain reaction (PCR) assay, using primers that hybridize to sequences flanking the *loxP* sites in the target gene, distinguishes between non-recombined and recombined *Gbx2* alleles (Fig. 2a). When the substrate for the PCR is the non-recombined allele, the amplification product is a 1.75 kb fragment, whereas when the substrate is the recombined allele it is a 0.25 kb fragment. When the substrate is wild-type mouse DNA, no amplification product is detected, because one of the PCR primers hybridizes to sequences within the *neo* expression cassette that are not present in wild-type DNA. Because we expected that the smaller fragment representing the recombined allele would be preferentially amplified, we were concerned that in DNA from animals carrying both alleles, the non-recombined allele would be difficult to detect. However, control experiments showed that the 1.75 kb amplification product could still be detected when the PCR substrate was DNA containing the non-recombined allele diluted 100-fold with DNA containing the recombined allele. Conversely, the 0.25 kb amplification product could still be detected when the ratio of non-recombined:recombined allele in the DNA was 4000:1 (see Fig. 2b; the band was clearly detectable in the gel).

Tail DNA from 58 progeny of a cross between *cre*$^+$/target$^+$ females and wild-type males was subjected to PCR analysis to determine the genotype of each animal. All possible categories of progeny were obtained in the expected Mendelian ratios (18 *cre*$^+$/target$^-$, 15 *cre*$^-$/target$^+$, 11 *cre*$^+$/target$^+$, and 14 *cre*$^-$/target$^-$). This analysis also revealed the recombination status of the target gene in the 26 target$^+$ offspring (Fig. 2b and data not shown). In each sample, only the 0.25 kb fragment was detected, whether or not the animal also carried *Zp3–cre*. This indicates that recombination occurred at 100 % efficiency as a consequence of Cre produced in oocytes. Similar results were obtained when tail DNA from the progeny of females carrying *Zp3–cre* and a different target transgene (a floxed allele of the *Fgf8* gene) was assayed for target recombination (E. Meyers, M.L. and G.R.M., unpublished observations), indicating that highly efficient Cre-mediated recombination in the female germ line is characteristic of our *Zp3–cre* mouse line and is not specific to a particular target gene. Preliminary studies in which the sexes were reversed, and the progeny of *cre*$^+$/target$^+$ males were assayed for recombination, indicated that the *Zp3–cre* transgene is not active in the male germ line (data not shown).

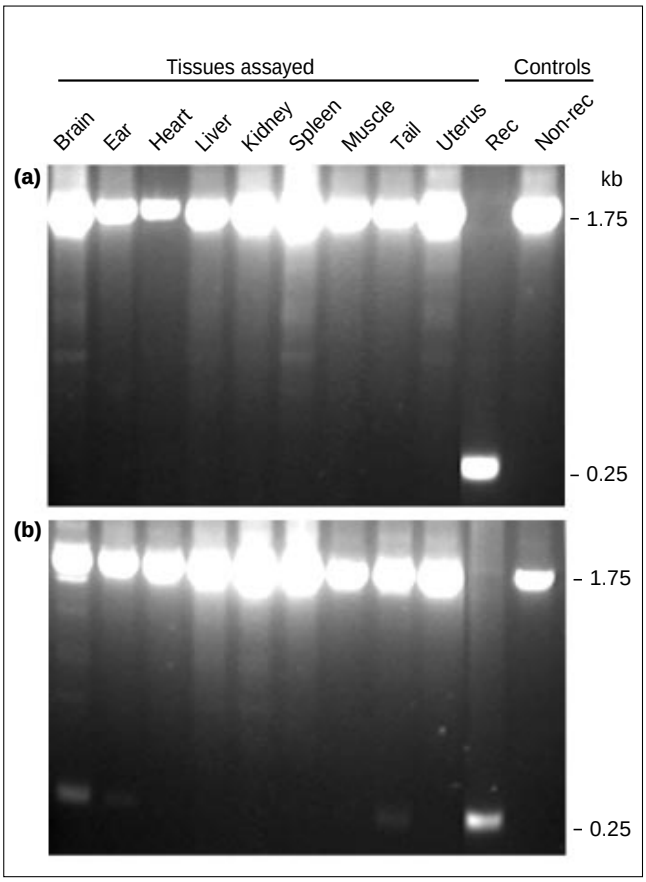
These data illustrate one use for which *Zp3–cre* mice are ideally suited: the generation at very high efficiency of progeny that carry the recombined (null) allele of a floxed target gene (such as *Gbx2* or *Fgf8*). The mouse lines carrying the non-recombined target gene remain available for future use in gene inactivation experiments employing mice that express *cre* in specific tissues. Two other *cre* transgenic

Figure 2



Assay for Cre-mediated DNA recombination in the female germ line. (a) Diagram of the target gene. The larger boxes represent exons of the *Gbx2* gene and the horizontal line intron DNA. White and grey shading represent coding and non-coding regions, respectively; the *neo* selection cassette was inserted into the intron. The floxed *Gbx2* gene (top) contains *loxP* sites flanking parts of the intron and the 3′ end of the *Gbx2* coding region; after Cre-mediated recombination (bottom), the DNA flanked by *loxP* sites is deleted and a single *loxP* site remains. The horizontal arrows represent primers used in the target gene PCR assay (5′-CTTGTGTAGCGCCAAGTG-3′ and 5′-GGTGCCTCTGAGTTCTGC-3′). (b) Assay for recombination status of the *Gbx2* target gene in progeny of a cross between a *cre*$^+$/target$^+$ female and a wild-type male. Control DNAs from mice carrying either the non-recombined or recombined target allele were mixed in the ratios indicated, and used as a substrate for the target gene PCR assay (left). Right panel, top: tail DNA from individual progeny (1–8) and control DNAs were used as substrates for the target gene PCR assay; right panel, bottom: the same progeny DNAs were assayed for the presence of absence of the *cre* transgene using primers that hybridize to the *cre* gene (5′-TGATGAGGTTCGCAAGAACC-3′ and 5′-CCATGAGTGAACGAACCTGG-3′). Control DNAs were derived from mice carrying the *cre* transgene or wild-type mice. In all target$^+$ progeny, the target was inherited in the recombined form, irrespective of whether the *cre* transgene was also inherited.

Figure 3



Assay for Cre-mediated DNA recombination in somatic tissues of *cre*$^+$/target$^+$ mice. DNA from the tissues indicated was used as a substrate for the target gene PCR assay; (a) and (b) show samples from different individuals. In addition to the 1.75 kb PCR product, representing the non-recombined target allele, which is detected in all samples, a small amount of the 0.25 kb PCR product, representing the recombined allele, can be detected in the lanes containing brain, ear, and tail DNA from one individual (b). Control DNA samples as in Figure 2.

mouse lines have been described that can also be used to obtain offspring with a recombined target gene [9,10]. In the *cre*$^+$/target$^+$ animals obtained by mating those *cre* mice with target-bearing mice, Cre apparently functions in many cells at early stages of development, including germ cells or their precursors. Such *cre*$^+$/target$^+$ animals may thus be mosaic with respect to target gene recombination, but they are able to transmit a recombined target allele to their progeny.

Other potential uses of the *Zp3–cre* mice depend not only on the efficient function of Cre in the female germ line, but also on the absence of Cre activity in somatic tissues. To explore this issue, we mated *Zp3–cre* males with target$^+$ females and assayed for target gene recombination in various tissues from their *cre*$^+$/target$^+$ offspring. In preliminary studies, we found that the recombined allele was not detectable by Southern-blot analysis of genomic DNA from the *cre*$^+$/target$^+$ mice (data not shown). Thus we used the more sensitive PCR-based assay described above to determine the recombination status of the target gene in numerous tissues from four *cre*$^+$/target$^+$ mice. When DNA isolated from tissues of two individuals was used as the substrate for target gene amplification, only the 1.75 kb fragment representing the non-recombined allele was detected (Fig. 3a and data not shown). Even when the PCR amplification products from one of these individuals were assayed by Southern blot, the 0.25 kb fragment representing the recombined allele was not detected (data not shown). When DNA from the same tissues isolated from the two other individuals was assayed, similar results were obtained except that a small amount of the 0.25 kb PCR amplification product was detected in the tail, brain, and ear samples from both mice, and also in the uterus and muscle samples from one of the mice (Fig. 3b and data not shown). We assayed in this way for target gene recombination in tail DNA isolated from an additional 19 *cre*$^+$/target$^+$ mice. In 15/19 samples analyzed, only the 1.75 kb PCR amplification product representing the non-recombined allele was detected; in the remaining 4/19 samples both the 1.75 kb and the 0.25 kb fragments could be seen (data not shown). Because the control experiments indicate that the recombined allele can be detected when it constitutes as little as 0.025 % of the PCR substrate, these data indicate that in most individuals of this *Zp3–cre* line, expression of the *cre* gene is stringently regulated and expressed only in the female germ line. It should be noted, however, that in a second mouse line produced with our *Zp3–cre* construct, we found evidence of extensive recombination in somatic tissue from all *cre*$^+$/target$^+$ mice assayed (n = 13; data not shown).

The oocyte specificity of Cre activity in our *Zp3–cre* mice makes them a valuable resource for studies aimed at determining the consequences of transgene expression when such expression causes lethality or some defect that precludes the establishment of a transgenic line. In such experiments, the target transgene (which will function only after being activated by recombination) could be one that causes ectopic expression of a normal gene, expression of a gain-of-function or dominant-negative mutation, or cell ablation (*via* toxin-gene expression) in a particular tissue. By mating *Zp3–cre* males to females carrying such a target transgene in the non-recombined (inactive) form, one would obtain *cre*$^+$/target$^+$ females that are phenotypically normal because the target transgene remains non-recombined (inactive) in somatic tissues. However, all the offspring of such females that inherit the target transgene will acquire it in the recombined (activated) form, and as they develop they will display the mutant phenotype. Thus, *Zp3–cre* mice provide a highly efficient means of producing experimental embryos carrying an activated target gene in all their cells. In principle, similar results could also be

obtained by mating target animals to mice in which *cre* is expressed at a very early stage of development [9,10], resulting in recombination (target activation) after fertilization in individual cells of *cre*$^+$/target$^+$ progeny. However, if recombinase activity were not sufficiently high to mediate target gene recombination in some cells, the embryos would be mosaic with respect to transgene activation, which would complicate the analysis of the mutant phenotype.

Zp3-cre mice are also potentially useful for inactivating endogenous genes specifically in the oocyte. By such inactivation, one can study the functions of genes that play a role in oocyte maturation, as well as those whose expression in the developing oocyte is necessary for development of the early embryo. In principle, oocyte-specific gene inactivation could be achieved by producing *Zp3–cre* females that carry both a null and a floxed allele of the gene of interest, or that are homozygous for the floxed allele. Recombination of the floxed allele(s) in the oocyte should result in eggs that are homozygous null for the gene of interest. This approach would be essential in cases in which females lacking the gene product in all their tissues (including oocytes) cannot be obtained because homozygosity for the null allele is lethal. This approach can only be used, however, if Cre-mediated recombination occurs in the oocyte before the gene of interest is expressed. It seems likely that Cre does indeed function early in oocyte maturation, prior to completion of the first meiotic division, because the target allele is always inherited in the recombined form by the progeny of *cre*$^+$/target$^+$ females irrespective of whether they also inherit the *cre* gene. Moreover, the Zp3 regulatory elements used to control *cre* gene expression in the *Zp3–cre* mouse line are known to drive gene expression in the growing oocyte. Nevertheless, it is conceivable that the target gene might be accessible for Cre-mediated recombination only after fertilization. If that were the case, then inheritance of the target in the recombined form might occur because Cre protein produced in the growing oocyte is present in sufficiently high amounts to mediate the recombination of the inherited target gene after fertilization.

This possibility can be explored by determining whether sufficient Cre activity remains in *Zp3–cre* oocytes to mediate the recombination of a target gene that has been brought in by the sperm at fertilization. We therefore mated *Zp3–cre* females with target$^+$ males, and assessed the recombination status of the target gene in tail DNA from their *cre*$^-$ progeny. Of the 44 progeny analyzed, 10 were found to be of the genotype of interest (*cre*$^-$/target$^+$): 4/10 animals carried only the recombined allele, whereas 6/10 carried the non-recombined allele (3/10 carried only the non-recombined allele, and 3/10 were mosaic for the two forms of the target gene). These results contrast sharply with our finding that 15/15 *cre*$^-$/target$^+$ progeny carried only the recombined form of the target gene when it was transmitted by *cre*$^+$/target$^+$ females, thereby providing strong evidence that, in the *Zp3–cre* mouse line, Cre-mediated recombination occurs very early in oocyte development. These results also indicate that sufficient Cre activity remains in mature oocytes to mediate the recombination of a paternally contributed target gene, at least in some cases. This observation is important because it indicates that to obtain *cre*$^+$/target$^+$ animals in which the target gene is not recombined in any cells (except female germ cells), the parental *Zp3–cre* gene must be contributed by the sperm and not by the oocyte. More importantly, taken together the data indicate that the *Zp3–cre* mouse line should be a valuable resource for carrying out oocyte-specific gene inactivation experiments. For example, two excellent candidates for this approach are the genes encoding β-catenin and E-cadherin. Both genes are expressed in the developing oocyte, and it has been proposed that this maternal expression is required for pre-implantation development [11], but this hypothesis cannot be tested using the standard gene-inactivation approach because embryos homozygous for null mutations are not viable [12,13].

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