Germ line activation of the Tie2 and SMMHC promoters causes noncell-specific deletion of floxed alleles

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de Lange WJ, Halabi CM, Beyer AM, Sigmund CD. Germ line activation of the Tie2 and SMMHC promoters causes noncell-specific deletion of floxed alleles. Physiol Genomics 35: 1–4, 2008. First published July 8, 2008; doi:10.1152/physiolgenomics.90284.2008.—Tissue-specific knockouts generated through Cre-loxP recombination have become an important tool to manipulate the mouse genome. Normally, two successive rounds of breeding are performed to generate mice carrying two floxed target-gene alleles and a transgene expressing Cre-recombinase tissue-specifically. We show herein that two promoters commonly used to generate endothelium-specific (Tie2) and smooth muscle-specific [smooth muscle myosin heavy chain (Smmhc)] knockout mice exhibit activity in the female and male germ lines, respectively. This can result in the inheritance of a null allele in the second generation that is not tissue specific. Careful experimental design is required therefore to ensure that tissue-specific knockouts are indeed tissue specific and that appropriate controls are used to compare strains.

RESULTS

Endothelial Cell-Targeted Mice

Tie2-Cre, herein termed eCre+ [Jackson Laboratory no. 004128, B6.Cg-Tg(Tek-cre)12Fiv/J], male mice were crossed to homozygous PPARGF/F (13) females to generate 162 F1 offspring, all of which were heterozygous for the floxed allele, and nearly 50% were eCre+ (PPARGF/F eCre+).1 Even in F1 mice, a PCR product with lesser intensity than either the floxed or wild-type alleles and confirmed as the null allele by DNA sequencing was evident (Fig. 1B, lanes 1 and 2). The null allele was detected in genomic DNA of 76% of the mice, all of which were eCre+. In the second generation, PPARGF/F eCre+ mice were backcrossed to PPARGF/F mice, generating 172 offspring. The PPARG null allele was detected in genomic DNA from 42% of the offspring, 31% of which were eCre+. The intensity of the null allele was low in eCre+ mice inheriting the transgene from the male, consistent with eCre activity in endothelial cells in the tail sample (Fig. 1B, lanes 3, 5, 6, and 11). On the contrary, the intensity of the null allele was equal to the floxed allele when it was transmitted through the female even in eCre+ mice (Fig. 1B, lane 15), indicating a 1:1 ratio of floxed and null alleles.

To investigate this “eCre-independent” gene deletion further, F2 offspring were retrospectively separated into two groups based on the sex of the eCre+ transmitting parent (Fig. 1C). The null allele was never detected in genomic DNA from eCre− F2 offspring when it was transmitted through the male germ line (Fig. 1B, lanes 4, 7–10), indicating that the Tie2 promoter is not active in the male germ line. However, when the eCre+ allele was transmitted through the female germ line, the null allele was detected in genomic DNA from 100% of PPARGF/F (Fig. 1B, lane 15) and 40.0% of PPARGF/F F2 offspring even if they genotyped as eCre−.

We next designed an RT-PCR assay to distinguish between the full-length Pparg transcript (originating from either the wild-type or floxed allele) and the null transcript (originating from conversion of a Pparg floxed allele to a null allele) (Fig. 2A). Aortic RNA from F2 mice in which the eCre transgene was transmitted through the male or female germ line were assayed by RT-PCR (Fig. 2B). A null transcript was not detected in Cre− offspring if the male parent was eCre+.

1 Care of the mice used in the experiments met the standards set forth by the National Institutes of Health in its guidelines for the care and use of experimental animals, and all procedures were approved by the University Animal Care and Use Committee at the University of Iowa.

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Fig. 1. Genotyping of Tie2-cre and peroxisome proliferator-activated receptor-γ (PPARG) mice. A: schematic showing the 3 alleles of Pparg. The positions of PCR primers, location of loxP sites (filled triangles), and the expected PCR product sizes are indicated. Genotyping of the Pparg floxed allele and the eCre or sCre transgenes was performed as previously described (13, 17). WT, wild type. B: PCR genotyping for Pparg and Tie2-Cre is shown. Lanes 1–2 are offspring of a 1st cross (♂ eCre × ♀ PPARGF/F); lanes 3–11 are offspring of a 2nd cross where eCre was passed through the male (♂ PPARGF/F × eCre × ♀ PPARGF/F); lanes 12–15 are offspring of a 2nd cross where eCre was passed through the female (♂ PPARGF/F × ♀ PPARGF/F × eCre). C: table shows the number of mice distributed by genotype. The expected number of animals is indicated in brackets. F2male/c and F2female/c represent the eCre transgene transmitted to F2 generation through the male and female germ line, respectively.

Fig. 2. RT-PCR assay. A: schematic of the mRNA species generated from the 3 alleles shown in Fig. 1A. The primers used for RT-PCR are shown along with their expected size. B: aortic RT-PCR products generated from F2 endothelial-null offspring. Lanes 1–4 are RT-PCR products generated from samples where eCre was passed through the male; lanes 5–8 are RT-PCR products generated from samples where eCre was passed through the female. The animal genotype as assessed by tail genomic DNA is indicated below the figure. Total RNA was isolated from thoracic aorta using TriReagent (Molecular Research Center), DNase I treated and repurified using RNeasy spin columns (Qiagen). cDNA was generated by reverse transcription PCR using Superscript III (Invitrogen); 10 ng of reverse transcribed RNA was PCR amplified using primers 1-forward (F): AGACCACTCGCATTCCTTTGACAT and 3-reverse (R): TCCGGCAGTTAAGATCACACC.

However, the null transcript was detected in aorta even if the mice were eCre−, but only if the female parent was eCre+ transgene (compare lanes 2 and 4 with 5 and 6 in Fig. 2B).

Smooth Muscle Cell-Targeted Mice
We employed a similar strategy for the generation of PPARGF/F Smmh-Cre+ (sCre+). As it was known that the Smmh promoter is activated in the female germ line (M. Kotlikoff,
personal communication) we set up crosses only where the sCre\(^+\) allele was transmitted through the male germ line. All 193 F1 offspring were heterozygous for the floxed allele (PPARG\(^{FF}\)) and \(-50\%\) were sCre\(^+\) (Fig. 3). A breeding of PPARG\(^{FF}\) sCre\(^+\) F1 males with PPARG\(^{FF}\) females produced 362 F2 offspring with a near equal distribution among the expected genotypes. Strikingly, the null allele was detected in genomic DNA from all but one PPARG\(^{FF}\) mouse, irrespective of sCre genotype (Fig. 3, lanes 3, 8, and 9 vs. 6, 7, and 10). The null allele was not detected in any animals with a genotype of PPARG\(^{FF}\) sCre\(^-\) because they inherited the wild-type allele from the male sCre\(^+\) parent (Fig. 3, lanes 12, 15, and 16). The expression of the null allele was subsequently confirmed by RT-PCR in aorta from PPARG\(^{FF}\) mice irrespective of their sCre genotype (data not shown).

**DISCUSSION**

We generated two models in which we attempted to selectively ablate the Pparg gene in the endothelial and vascular muscle, respectively. Our data suggests that the Smmhc promoter drives Cre-recombinase expression in the male germ line prior to the second meiotic division, resulting in the global deletion of the paternally inherited floxed allele, even in sCre\(^-\) offspring. This hypothesis is supported by data showing that SMMHC protein is present in primary spermatocytes at the zygote to early diplotype stage (5). On the contrary, there was no evidence of male germ line expression of the eCre\(^+\) transgene. The eCre\(^+\) transgene, however, was apparently expressed in the female germ line. It is interesting to note that in many instances (40%), the null allele was detected at an intensity similar to that of the wild-type allele in F2 mice genotyped as PPARG\(^{FF}\) eCre\(^-\) when eCre\(^+\) (and the Pparg wild-type allele) was transmitted by the female. This efficient conversion of the paternal floxed allele suggests that there may be retention of Cre-recombinase activity in the early embryo after fertilization. Our data regarding activation of Cre-recombinase expression by the Tie2 and Smmhc promoters in the germ line are in agreement with other studies using other floxed genes (7, 9), strongly suggesting our observations are not an aberration caused by Pparg locus. Our data raise some concerns regarding study design and control selection that must be considered when interpreting the results of experiments where either the breeding scheme was not detailed or the sex of the Cre-transmitting parent considered. Unfortunately, it is difficult to assess if this has adversely affected published studies as often the details of the intercross between floxed and Cre mice are not very thorough and the amount of genotyping information provided is minimal. Consequently, can this problem be avoided? In terms of the Tie2-Cre model and other Cre-recombinase models with similar problems, complications arising from germ line Cre-recombinase activity can be avoided by transmitting the transgene through the male germ line. Recall that none of the Cre\(^-\) offspring from mice bred in this manner had the null allele and there was no expression of the null allele at the mRNA if the mice were Cre\(^+\). The situation is more complicated for the smooth muscle-specific promoter and other Cre-recombinase transgenes found to be expressed in both the male and female germ lines. In this case, mice expected to be Gene\(^E\)/null Cre\(^+\) or \(-\) in the F2 generation are effectively Gene\(^E\)/null Cre\(^+\) or \(-\). This would still allow the generation of a cell-specific null as long as the F2 mice are not continuously intercrossed. Further intercrossing could potentially generate mice that are Gene\(^null\)/null. Furthermore, care should be taken that only Gene\(^null\)/null Cre\(^-\) litters are selected as controls.

In conclusion, we have shown that activation of Cre expression by the Tie2 and Smmhc promoters occurs in the female and male germ lines, respectively. We believe that both these promoters can be used successfully to develop vascular-specific knockout mice providing that germ line expression of Cre is taken into account in design of the study. If investigators are uncertain whether their favorite Cre-recombinase transgene is expressed in germ cells, they should make an effort to ensure that their genotyping assay can detect and distinguish between the wild-type, floxed, and null allele and to keep accurate records of the genotypes of the offspring. Designing an RT-PCR assay that also detects an expressed null allele can also provide an important diagnostic tool to determine if faithful cell-specific knockout is occurring. If these issues are kept in mind, the
only factor that should influence the success of a cell-specific knockout project is the effectiveness of the promoter for driving Cre-recombinase in an appropriate spatial and temporal manner.

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REFERENCES


