Smooth muscle expression of Cre recombinase and eGFP in transgenic mice

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Xin, H.-B., K.-Y. Deng, M. Rishniw, G. Ji, and M. I. Kotlikoff. Smooth muscle expression of Cre recombinase and eGFP in transgenic mice. Physiol Genomics 10: 211–215, 2002. First published July 2, 2002; 10.1152/physiolgenomics.00054.2002.—We report the generation of transgenic mice designed to facilitate the study of vascular and nonvascular smooth muscle biology in vivo. The smooth muscle myosin heavy chain (smMHC) promoter was used to direct expression of a bicistronic transgene consisting of Cre recombinase and enhanced green fluorescent protein (eGFP) coding sequences. Animals expressing the transgene display strong fluorescence confined to vascular and nonvascular smooth muscle. Enzymatic dissociation of smooth muscle yields viable, fluorescent cells that can be studied as single cells or sorted by FACS for gene expression studies. smMHC/Cre/eGFP mice were crossed with ROSA26/lacZ reporter mice to determine Cre recombinase activity; Cre recombinase was expressed in all smooth muscles in adult mice, and there was an excellent overlap between expression of the recombinase and eGFP. Initial smooth muscle-specific expression of fluorescence and Cre recombinase was detected on embryonic day 12.5. These mice will be useful to define smooth muscle gene function in vivo, for the study of gene function in single, live cells, and for the determination of gene expression in vascular and nonvascular smooth muscle.
lacZ reporter gene to smooth muscle tissues (8), was used to drive Cre recombinase and eGFP expression. An internal ribosome entry site (IRES) of the encephalomyocarditis virus was added upstream of the eGFP gene to permit the translation of two open reading frames for Cre and eGFP from one mRNA (6). The transgene was purified using a Qiagen kit and injected into fertilized mouse eggs by standard pronase for 20 min at 37°C overnight, embedded and sectioned, and sections digested with pronase C before immunostaining. Immunostaining with primary antibodies was followed by a biotinylated secondary antibody, labeling with streptavidin, and detection staining with primary antibodies was followed by a biotinylated secondary antibody, labeling with streptavidin, and detection.

Microscopy, cell dissociation, and sorting. eGFP fluorescence in cryosections and mouse embryos was visualized using epifluorescence microscopy. To determine Cre recombinase activity, male smMHC/Cre/eGFP mice from one founder line (SMCG2) were mated with female R26R mice (9) obtained from Jackson Laboratories. In this line a floxed stop element (primer 1, 5'-CCA ATT TAC TGA CCG TAC ACC-3'; primer 2, 5'-GTT TCA CTA TCC AGG TTA CGG-3'), or by Southern blotting EcoRI digested genomic DNA, using a probe from the coding region of Cre recombinase. Two founder lines were developed (SMCG2 and SMCG3).

Expression of eGFP in smMHC/Cre/eGFP mice. We bred two transgenic founder lines of smMHC/Cre/eGFP mice: SM2Cre/GFP and SM3Cre/GFP, which we refer

Results

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Fig. 1. Generation of smMHC<sup>CRe/EGFP</sup> transgenic mice. A: diagram of smMHC/Cre/eGFP transgene construct. B: detection of the transgene by PCR (left) and Southern blot analysis (right). The Southern blot shows DNA from wild-type (wt), SMCG2, and SMCG3 hemizygotes. Equal amounts of DNA were loaded for all three conditions, indicating higher transgene copy number in the SMCG2 line. eGFP, enhanced green fluorescent protein; smMHC, smooth muscle myosin heavy chain; IRES, internal ribosome entry site; TSH, thyroid stimulating hormone; SMCG2 and SMCG3, transgenic founder lines SM2<sup>Cre/GFP</sup> and SM3<sup>Cre/GFP</sup> mice, respectively.

Fig. 2. eGFP fluorescence restricted to smooth muscle in smMHC<sup>CRe/EGFP</sup> transgenic mice. A: composite light (left) and fluorescence (right) dissecting microscope images from 2-wk-old SMCG2 mouse. Note fluorescence confined to vascular and nonvascular (e.g., vas deferens) smooth muscle. B: ear notch from the same SMCG2 mouse; light and fluorescence of tracheobronchial smooth muscle (bottom). C: smooth muscle fluorescence in an embryo at 16.5 days post-coitum (dpc), showing light image of lung within chest (top) and fluorescence of tracheobronchial smooth muscle (middle), and cryosection through lung shows fluorescence confined to bronchial and vascular smooth muscle (bottom). Scale bars are 500 μm for top and middle and 100 μm for bottom.
to as lines SMCG2 and SMCG3. Southern blot analysis indicated a single integration site in both lines; SMCG2 mice displayed a higher transgene copy number, as indicated by comparison of the Southern blots under equal loading conditions (Fig. 1). SMCG2 hemizygotic mice were crossed to yield homozygotes, which could be distinguished from hemizygotes by the intensity of the hybridized band. SMCG2 transgenic mice were viable as hemizygotes and homozygotes; however, it was observed that homozygotes (SMCre/GFP,Cre/GFP) had smaller litter sizes and a higher incidence of perinatal mortality. As hemizygotes from both lines displayed intense eGFP fluorescence (see below), both lines were maintained as hemizygotes, and the SMCG2 line was chosen for rigorous analysis. As shown in Fig. 2, adult SMCG2 hemizygotic mice displayed intense GFP fluorescence restricted to vascular and nonvascular smooth muscle. Fluorescence was easily observed in newborn pups, and animals were rapidly genotyped by examination of a small section of ear or tail under a dissecting microscope for vascular fluorescence (Fig. 2B). Slight, diffuse fluorescence was observed in the atria at day 10.5, similar to previous reports for lacZ expression (14). More intense, localized eGFP fluorescence could be detected in the aorta and major airways at 12.5 days post-coitus (dpc), whereas smooth muscle fluorescence was prominent at 16.5 dpc in the intestine. An example of fluorescence in the lung of a 16.5 dpc embryo is shown in Fig. 2.

A significant advantage and major rationale for the generation of smMHC/Cre/eGFP mice is the ability to identify individual myocytes in complex tissues for physiological or gene expression studies. Accordingly, the fluorescence of individual myocytes within the smooth muscle matrix and of dissociated myocytes was examined. Individual myocytes were easily observed in cryosections of smooth muscle tissue (not shown), and single myocytes were dissociated from vascular and nonvascular smooth muscle tissues to determine the effect on eGFP expression. As shown in Fig. 3, individual cells from these mice display intense cytosolic fluorescence after enzymatic digestion. In confocal images fluorescence was observed diffusely throughout the cytoplasm, and cells could be easily separated from non-
smooth muscle cells by FACS. To establish the feasibility of separation of myocytes from other cell types by FACS, two separate sorts were conducted of dissociated aortas from 3-wk-old mice. In both experiments fluorescent myocytes were readily separated from other cell types, yielding a homogeneous population of fluorescent cells. Sorted cells constituted 14% of the total cell suspension from whole aorta.

Expression of Cre recombinase. To determine the expression of Cre recombinase, SM2Cre/GFP mice were crossed with R26R mice, in which a stop element flanked by loxP sequences upstream of the lacZ coding sequence has been knocked into the ROSA26 locus (9). F1 offspring were genotyped for both alleles and double heterozygotes crossed to yield SM2Cre/GFP,R26R mice for analysis. SM2Cre/GFP,R26R displayed prominent lacZ staining in smooth muscle tissues, whereas no staining was observed in SM2Cre/GFP mice. LacZ expression was observed as early as 12.5 dpc, similar to previous findings (14). In some experiments, tissues were stained for lacZ expression, and fluorescence was visualized in the same tissues. As shown in Fig. 4, A and B, there was strong overlap between Cre-mediated β-galactosidase expression and eGFP fluorescence, verifying the use of fluorescence as a marker for conditional gene recombination. To further document the concordant expression of Cre recombinase and eGFP at a cellular level, tissues from wild-type and transgenic mice were sectioned, and immunostaining was performed using anti-GFP and anti-Cre recombinase antibodies. As shown in Fig. 4C, eGFP immunodetection demonstrated diffuse cytosolic staining confined to smooth muscle cells; Cre expression was similarly confined to smooth muscle cells, but showed the expected nuclear localization in myocytes. The smooth muscle-specific nature of expression was further confirmed by the observation of positive vascular myocytes within cardiac (Fig. 4C) and skeletal (not shown) muscle. Vascular smooth muscle expression of Cre and eGFP was observed in all tissues, including renal arteries and arterioles (Fig. 4C). Similarly, expression of the transgene was observed in adult mice in all nonvascular smooth muscle, including smooth muscle layers of the male and female reproductive tracts (not shown) and the circular and longitudinal muscle of the gastrointestinal tract (Cre recombinase expression shown in Fig. 4C).

DISCUSSION

Smooth muscle cells reside within a complex matrix that includes closely related mesenchymal cells such as fibroblasts, glia, neurons, and often closely adherent endothelial cells. The separation of myocytes from the surrounding cellular environment to isolate muscle-specific adaptations or disease responses, particularly in the vasculature of the mouse, is therefore quite challenging. The ability to accurately examine shifts in gene expression in smooth muscle such as are likely to occur in vascular disease would be substantially improved by the ability to separate myocytes by FACS or microdissection methods utilizing markers of cell lineage. To address this difficulty, we have engineered a transgenic mouse expressing eGFP and Cre recombinase under the control of the smMHC gene promoter. smMHC appears to be the most smooth muscle-restricted gene identified to date (10) and has been shown to direct expression of a reporter gene to smooth muscle (8). Although full promoter activity requires a large gene fragment including the first intron of the smMHC gene (~16 kb), the restriction of the transgene to smooth muscle tissue was deemed essential for the utility of this transgenic line. The specificity of this promoter has previously been exploited to develop a mouse expressing smooth muscle-restricted Cre recombinase (14). By combining eGFP with Cre recombinase on a bicistronic construct, smMHC/Cre/eGFP mice should be useful tools for conditional gene inactivation or activation, as well as the analysis of gene expression in myocytes by fluorescence-based cell separation techniques. The transgenic lines reported here express the bicistronic transgene beginning in late embryonic development in a tissue-restricted fashion, although early fluorescence was detected in the atrium between days 10.5 and 12.5 dpc, consistent with previous reports (14). Fluorescence expression also provides a rapid and convenient method of genotyping by observation of a small segment of tissue for vascular fluorescence. Finally, we found that eGFP fluorescence and Cre expression are well linked, providing a convenient in vivo marker for Cre-mediated genetic manipulations.

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