VE-cadherin-CreER^{T2} Transgenic Mouse: A Model for Inducible Recombination in the Endothelium

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To introduce temporal control in genetic experiments targeting the endothelium, we established a mouse line expressing tamoxifen-inducible Cre-recombinase (Cre-ER^{T2}) under the regulation of the vascular endothelial cadherin promoter (VECad). Specificity and efficiency of Cre activity was documented by crossing VECad-Cre-ER^{T2} with the ROSA26R reporter mouse, in which a floxed-stop cassette has been placed upstream of the β-galactosidase gene. We found that tamoxifen specifically induced widespread recombination in the endothelium of embryonic, neonatal, and adult tissues. Recombination was also documented in tumor-associated vascular beds and in postnatal angiogenesis assays. Furthermore, injection of tamoxifen in adult animals resulted in negligible excision (lower than 0.4%) in the hematopoietic lineage. The VECad-Cre-ER^{T2} mouse is likely to be a valuable tool to study the function of genes involved in vascular development, homeostasis, and in complex processes involving neoangiogenesis, such as tumor growth. *Developmental Dynamics 235:3413–3422, 2006.* **© 2006 Wiley-Liss, Inc.**

Key words: endothelium; VE-cadherin; Cadherin-5; Cre-recombinase; tamoxifen; transgenic mice; tumor angiogenesis

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INTRODUCTION

Gene inactivation in the mouse germ line is a powerful technique to study gene function in a whole animal setting. However, ablation of a gene in all cells can result in complex phenotypes, or in lethality. To overcome such problems, several strategies have been developed that result in tissue-specific gene inactivation. Among these strategies is the Cre-loxP recombination system (Sauer, 1998; Metzger and Chambon, 2001). This approach takes advantage of the properties of P1 phage Cre-recombinase, a 38-kDa enzyme that recognizes a 34-bp sequence called loxP (for locus of X-over of P1), initially discovered in the bacteriophage P1 (Orban et al., 1992). Introduction of two loxP sites flanking a DNA target sequence enables binding by Cre-recombinase and either inversion or excision of this sequence, depending on the respective orientation of the loxP sites. Specificity of the resulting deletion is accomplished by the appropriate selection of a tissue-specific promoter to drive expression of the P1 Cre-recombinase.

ABBREVIATIONS CIVE C57Bl/J, Inducible, VE-cadherin Cre-recombinase mouse **VECad** Vascular Endothelial Cadherin **Cre-ER^{T2}** cre recombinase-tamoxifen receptor fusion protein **IP** intraperitoneal

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In the case of endothelial-specific gene targeting, several Cre lines have been developed with varying degrees of endothelial specificity (Gustafsson et al., 2001; Kisanuki et al., 2001; Motoike et al., 2003; Alva et al., 2006). Although extremely informative, the deletion of a wide array of genes using these lines has resulted in hemorrhage and eventual embryonic lethality. For these reasons, development of "inducible" Cre-recombinase models is now considered advantageous when the objective is to dissect the contribution of specific genes during pathological conditions and homeostasis.

Inducible control of Cre activity has been achieved by fusing sequences from the Cre gene with sequences encoding a mutant form (ER^{T2}) of the estrogen receptor ligand-binding domain (Danielian et al., 1993; Feil et al., 1997; Metzger and Chambon, 2001; Leone et al., 2003). ER^{T2} functions as a specific receptor for tamoxifen and is unresponsive to natural estrogens or other physiological steroids. Cre-ER^{T2} brings Cre activity under control of a synthetic ligand 4-hydroxy-tamoxifen (4-OHT). In the absence of 4-OHT, Cre-ER^{T2} protein remains sequestered in the cytoplasm by hsp90 (Mattioni et al., 1994; Picard, 1994), preventing Cre-mediated recombination events in the nucleus. Binding of 4-OHT to the mutated receptor disrupts interaction with hsp90, allowing the translocation of Cre protein to the nucleus where it can initiate loxP-specific recombination events.

In this report, we describe the generation and characterization of a transgenic mouse (CIVE23) (CIVE = C57Bl/J, Inducible, VE-cadherin Cre-recombinase, line #23) in which Cre-ER^{T2} expression is directed to endothelial cells by a VE-cadherin promoter cassette.

RESULTS AND DISCUSSION

We placed the Cre-ER^{T2} expression cassette developed previously (Feil et al., 1996) under regulatory control of the mouse VE-cadherin gene promoter region (−2486, +24). The construct also contains a synthetic intron (ivs) derived from the rabbit β-globin locus and a SV40 polyadenylation sequence (Fig. 1A). The VE-cadherin promoter element has been shown to direct LacZ expression in the endothelium during embryonic development and in adult organs as well (Gory et al., 1999; Alva et al., 2006). We tested the activity of VECad-Cre-ER^{T2} expression cassette by transient transfection of endothelial cell lines with or without a Cre-responsive LacZ reporter (Fig. 1B). Activity was only found in endothelial lines in the presence of 4-OHT and when both vectors were used (Fig. 1B).

Transgenic mice were generated by microinjection of the VECad-Cre-ER^{T2} transgene into one-cell mouse embryos. A total of eight lines were obtained, and four were selected for further characterization. Southern analysis indicated that each line harbored one to three copies of the transgene (data not shown). To test the efficiency and specificity of these lines, the VECad-Cre-ER^{T2} animals were intercrossed with the ROSA26R reporter mice (Soriano, 1999). Genotyping for both transgenes was performed by polymerase chain reaction (PCR; Fig. 1C). ROSA26R mice have a floxed stop-cassette upstream of the LacZ gene. In the presence of Cre-recombinase activity, the stop-cassette is excised, LacZ is expressed and β-galactosidase (β-gal) activity can be detected using the 5-bromo-4-chloro-3-indoyl β-D-galactopyranoside (X-Gal) staining procedure. Initial analysis showed that one of the four lines (CIVE23) demonstrated highly penetrant excision of the ROSA26R stop cassette. Here we present the full characterization of this line.

To examine the efficiency of recombination during embryonic development, embryos were harvested 2 days after maternal injection of tamoxifen. At embryonic day (E) 12.5, recombination was observed in the developing vasculature throughout the embryo (Fig. 2a). Evidence of Cre-ER^{T2} activity was noted by whole-mount staining in the vascular plexus of the head (Fig. 2b), blood vessels of the limb (Fig. 2d), and in the developing liver and heart (Fig. 2e and f, respectively). Recombination was also observed in blood vessels of the corresponding yolk sac (Fig. 2c). Analysis at E16.5 (also 2 days post-tamoxifen injection) showed a similar degree of specificity. Microscopic analysis of β-gal staining demonstrated that recombination occurred in all organs examined and with similar penetration. Positive endothelial cells were noted in the dermis (Fig. 2g), quadriceps (Fig. 2h), tongue (Fig. 2i), large vessels in the heart (Fig. 2j), kidney (Fig. 2k), lung (Fig. 2l), spleen (Fig. 2m), and bone (Fig. 2n). Similar evaluations have been also performed at E9.5, E11.5, E14.5, E15.5, E18.5, and E19.5 (data not shown). For the most part, Cre activity was specific to endothelial cells, except for the hematopoietic lineage. In particular, induction of tamoxifen before E11.5 resulted in labeling of hematopoietic cells in the liver (Fig. 2e). Cre-excision was more penetrant in capillaries, followed by arteries and veins (compare capillaries with the vein in Fig. 2g, and with the large arteries in Fig. 2j). We have also evaluated Cre-ER^{T2} activity in lymphatic endothelium. While mice harboring the constitutive VECad-Cre showed extensive deletion in skin lymphatics (Alva et al., 2006), this was not the case for Cre-ER^{T2} unless tamoxifen injection was done before E11.5 (data not shown).

To further examine the degree of deletion, we performed a progressive evaluation of Cre-ER^{T2}–mediated excision. A single intraperitoneal (IP) dose of tamoxifen in pregnant females resulted in 35% excision (on average) by 1 day after injection (Fig. 3a). Over time, due to the genomic nature of the excision, the progeny of recombined cells retained the deletion and were able to express LacZ (Fig. 3b–d); however, the number of recombined cells increased only slightly to 42% (on average) by E18.5 (Fig. 3d).

The percentage of recombined cells was only increased with further exposure to tamoxifen (Fig. 3e–h). Multiple injections of tamoxifen starting at E10.5 resulted in 95% excision (on average) by E18.5 (Fig. 3g and h). As observed in previous reports, we found that activation by Cre-ER^{T2} occurs within the first 24 hr after tamoxifen injection, but the drug is quickly metabolized thereafter (Hayashi and McMahon, 2002). Based on macroscopic and histological evaluation, exposure to tamoxifen did not reveal any developmental defects or embryonic viability. However, we found that the drug (even a single dose by E9.5 or later) prevents delivery of the pups. Thus

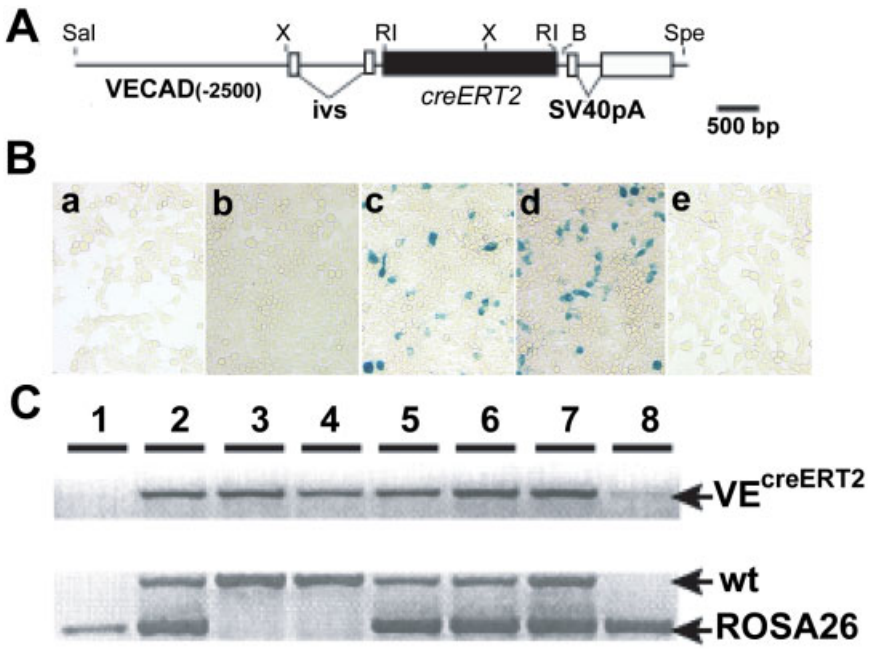

Fig. 1.

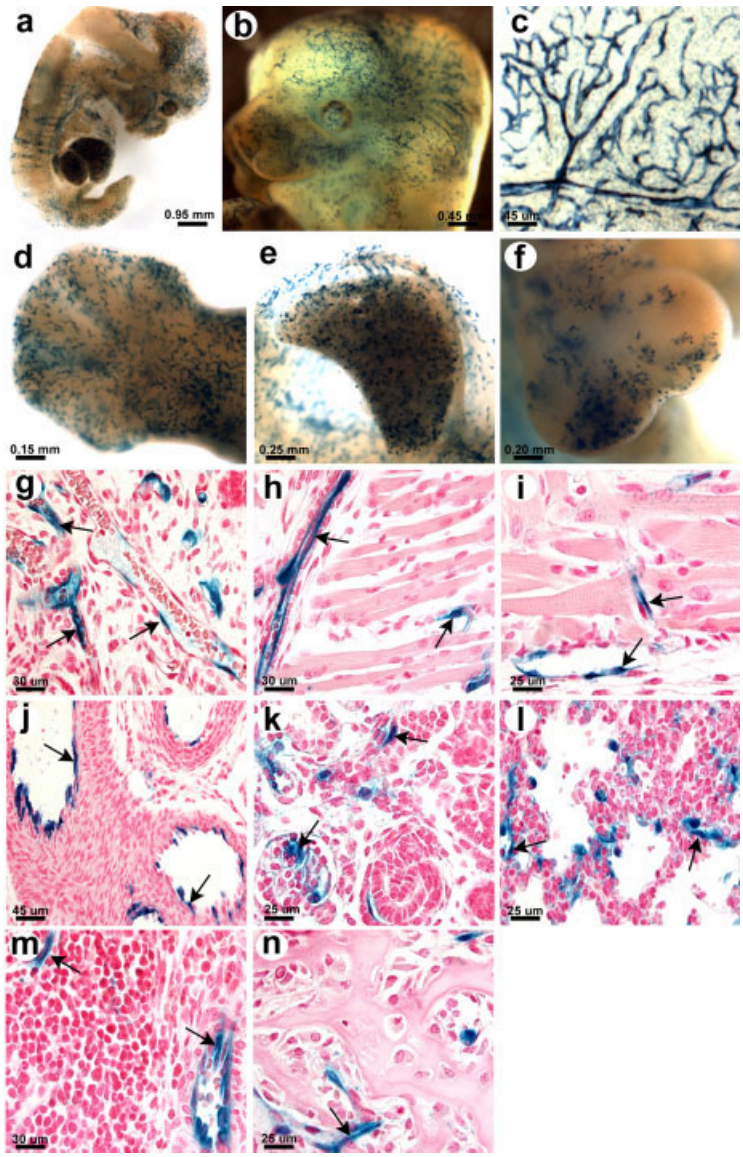

Fig. 2.

C-sections and surrogate mothers were necessary for analysis of later time-points.

Endothelial specificity of the transgene was also examined in adult mice. Adult VECad-Cre-ER^{T2}/ROSA26R mice were injected with 2 mg of tamoxifen for 5 consecutive days. Three weeks after the last injection, mice were sacrificed and recombination was assessed in different organs. In the abdominal wall, LacZ-positive cells were observed in capillaries next to skeletal muscle fibers with high penetration (Fig. 4a). The same was noted in the endothelium of the coronary network of the heart (Fig. 4b). β-gal staining was also found in the vasculature of the tongue (Fig. 4c). In the liver, recombination was seen in the endothelial cells of the central venule and sinusoids (Fig. 4d). Endothelial cells surrounding the pancreatic acini were also positive (Fig. 4e), as were the

Fig. 1. The VECad-Cre-ER^{T2} construct was tested in vitro and then introduced as a transgene in C57/Bl6 zygotes. **A:** cDNA encoding the tamoxifen-sensitive Cre-ER^{T2} was subcloned downstream of the murine VE-cadherin endothelial-specific promoter. **B:** The construct was tested in microvascular endothelial cell lines for specificity using a LacZ reporter floxed and driven by the CMV promoter. Cultures were exposed to 4-hydroxy-tamoxifen (4-OHT; 50 nM) for 16 hr (a–d only) and evaluated by β-gal staining (all panels): nontransfected cells (a), transfection with only the VECad-Cre-ER^{T2} construct (b), transfection with both vectors (c), transfection with both constructs at higher concentration than in c (d), transfection with both constructs and exposure to vehicle for 16 hr (e). β-gal activity is seen in blue. **C:** Polymerase chain reaction (PCR) analysis of genomic DNA isolated from the tail of 6-week-old VECad-Cre-ER^{T2} transgenic mice crossed to the ROSA26R mouse (129S-Gt(ROSA)26Sor^{tm1Sor}/J). Each lane (1–8) represents DNA from each mouse in one litter. The upper gel shows genotyping for VECad-Cre-ER^{T2}. The bottom gel shows genotyping for the presence of ROSA26 transgene (per Soriano, 1999).

Fig. 2. Tamoxifen-induced recombination in endothelial cells of VECad-Cre-ER^{T2} embryos. **a:** β-Gal staining (blue) of an embryonic day (E) 12.5 embryo (48 hr after maternal tamoxifen administration) after vibratome sectioning (150 μM). Note recombination throughout the vasculature. **b–f:** Whole-mount β-Gal staining of head (b), yolk sac (c), upper limb (d), liver (e), and heart (f). **g–n:** Microscopic assessment from E16.5, 48 hr after maternal tamoxifen injection: dermis (g), quadriceps (h), tongue (i), heart (j), kidney (k), lung (l), spleen (m), and bone (n). Arrows indicate the presence of β-gal staining, indicating recombination. β-gal, blue; nuclear counterstain, red.

capillaries of the intestinal villi and within the adipose tissue (Fig. 4f and k). The β-gal staining was observed in spiral arteries and capillaries of the endometrium (Fig. 4g). Positively stained endothelial cells were also noted in the seminal vesicle (Fig. 4h), bladder (Fig. 4i), and alveolar capillaries in the lung (Fig. 4j). The analysis revealed endothelial-specific recombination in all organs examined at a range of 71–92% deletion. We have also examined skin, brain, testis, prostate, and ovary with equivalent degrees of recombination (data not shown).

Tamoxifen treatment of neonatal animals is difficult because the peritoneal cavity is extremely small in very young animals. We evaluated three different modalities of tamoxifen delivery in postnatal day (P) 1–3 pups. These included (a) feeding, (b) topical skin exposure, (c) intragastric injection. Figure 5 presents a summary of the results from evaluations that compare daily doses of tamoxifen (at P1, P2, and P3) followed by examination 1 week after exposure to the drug. Feeding pups with tamoxifen (0.05–0.1 mg) resulted in specific, yet weak deletion (12–26%; Fig. 5a–c). In contrast, intragastric injection (0.05 mg) resulted in 65–89% recombination efficiency (Fig. 5g–i).

While we anticipated that rubbing 4-OHT onto the skin would lead to local deletion in dermal vessels, we found instead that this treatment resulted in systemic, but relatively weak, vascular deletion (Fig. 5d–f). There was no preferential recombination in the dermis under the area rubbed (data not shown). This method has been used particularly for epidermal-specific Cre-ER^{T2} models where local exposure to tamoxifen resulted inrecombination restricted to the treated region of skin (Vasioukhin et al., 1999). Nonetheless, it appears

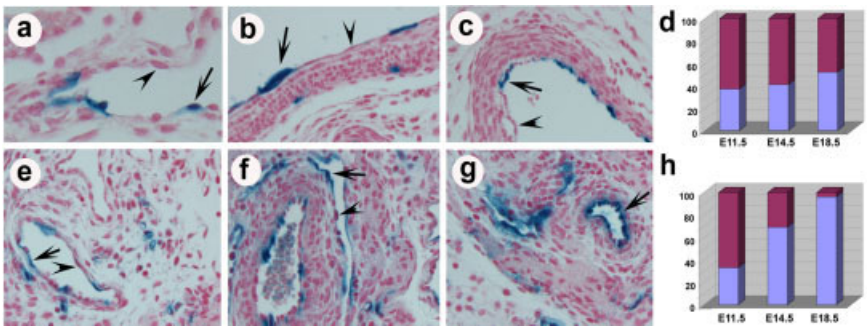

Fig. 3.

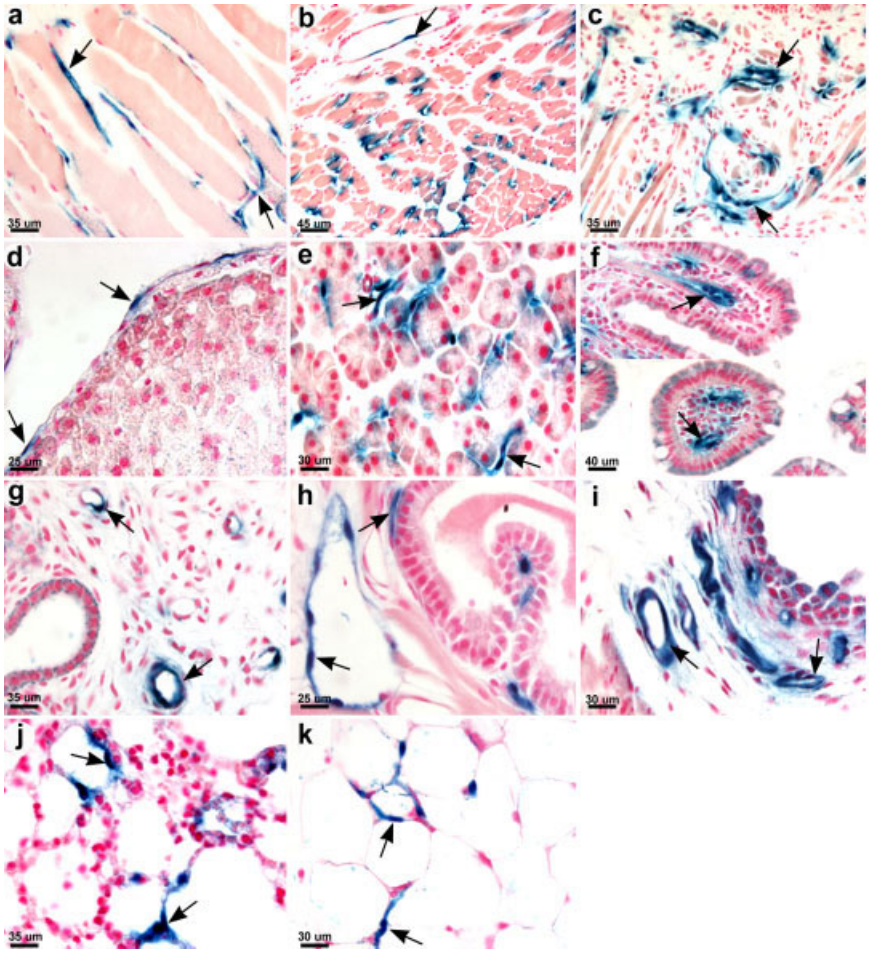

Fig. 4.

Fig. 3. Percentage of deletion during development depends on the induction protocol. **a–c:** A single dose of tamoxifen (1 mg, intraperitoneally) was administered to pregnant females at embryonic day (E) 10.5 and deletion was evaluated at E11.5 (a), E14.5 (b), and E18.5 (c). **d:** Quantification of the deletion was performed in four embryos by counting a total of 500 endothelial cells per specimen and assessing the percentage of positive (blue, β-gal positive) and negative (red, nuclear counterstain) cells. **e–h:** Percentage of deletion increased with prolonged exposure to tamoxifen. **e,f:** A single dose of a pregnant female at E10.5 resulted in 37% deletion by E11.5 (e), dual dose of a pregnant female at E10.5 and E12.5 resulted in increased percentage of recombination to 62% by E14.5 (f). **g:** Three injections at E10.5, E12.5, and E14.5 resulted in 95% excision by E18.5. **h:** Quantification of the degree of deletion is shown. Arrows point to positive cells and arrowheads to negative endothelial cells. In the histogram, β-galactosidase–positive cells are shown in blue and negative cells are shown in red.

Fig. 4. Tamoxifen-induced recombination in endothelial cells of VECad-Cre-ER^{T2} adult organs. A cohort of five young adult animals (8–12 weeks) were evaluated following a regimen of tamoxifen 2 mg/day for 5 consecutive days. **a–k:** Representative microscopic images showing β-Gal staining (blue) and nuclear counterstain (red) from quadriceps (a), heart (b), tongue (c), liver (d), pancreas (e), intestine (f), uterus (g), seminal vesicle (h), bladder (i), lung (j), and adipose tissue (k). Arrows indicate positive recombined endothelial cells.

that the absorption of 4-OHT by dermal capillaries leads to systemic delivery and excision in endothelial cells in multiple organs. From these results, we determined that intragastric injection of neonatal pups was the most efficient method for induction of recombination in young postnatal animals.

We also evaluated various 4-OHT exposure regimens in adult mice (Fig. 6). In the first series of experiments, 6- to 8-week-old VECad-Cre-ER^{T2}/ROSA26R mice were exposed to IP injections of 0.05, 0.5, or 2 mg of 4-OHT or vehicle for 5 consecutive days, according to published methods (Metzger et al., 1995; Imai et al., 2001; Metzger and Chambon, 2001). One week after the last injection, mice were killed and recombination was analyzed by evaluation of the β-gal activity in the abdominal wall muscle. As shown by macroscopic analysis (Fig. 6Aa,c,e) recombination was induced in a dose-dependent manner. Microscopic analysis revealed an increasing number of β-gal–positive cells in the endothelium of muscle capillaries (Fig. 6Ab,d,f). Colocalization of the β-gal–positive cells with CD31 immunohistochemistry confirmed that the recombination occurred specifically in endothelial cells (Fig. 6Ag). Double transgenic animals treated with the vehicle alone showed no recombination (Fig. 6Ah). These results document the specificity and penetration of this model. In addition, they demonstrate that an appropriate dosage is critical for deletion efficiency. Quantification of deletion was performed with a cohort of mice that received intraperitoneal injections with 0.5 mg of tamoxifen on 5 consecutive days (Fig. 6Ba–c). The data show deletion of 36% by 48 hr and 65% at 1 week after injection (Fig. 6Bd). However, this percentage does not change thereafter (Fig. 6Bc,d). During the course of these studies, we did not observe any deleterious effects of tamoxifen treatment.

Subsequently, we were interested in examining whether age in adult mice affected the degree of recombination. Thus, we exposed cohorts of mice at 3 weeks, 6 weeks, 8 weeks, and 24 weeks to tamoxifen (1 mg daily IP for 3 days), and evaluation was done 1 week after injection. As can be concluded from Figure 7A, the degree of excision correlated inversely with age, with higher rates of excision observed in younger animals (compare 3 weeks Fig. 7Aa with 24 weeks 7Ad). This finding could, at least in part, relate to the continued expansion (proliferation) of endothelial cells in young animals, that is, penetration of the excision is increased by the proliferation of already recombined cells. Nonetheless, the significant differences noted between 8 weeks (Fig. 7Ac) and 24 weeks (Fig. 7Ad) are not as easily explained by differential proliferative capacities, as by 8 weeks most of the vasculature has already completed expansion.

To examine the potential for varying expression of Cre-ER^{T2}, we examined expression of Cre-ER^{T2} protein in these mice using antisera against Cre-recombinase. To our surprise, we noted that Cre-ER^{T2} protein was highly expressed in endothelial cells of adult mice, including robust expression in cells where recombination had not yet taken place. Thus, in older adult mice, Cre-ER^{T2} expression did not directly result in a β-gal signal at the time points analyzed (Fig. 7B). The specific mechanism for this finding is unclear; however, three possibilities could explain these results: (1) the ROSA promoter used to drive the β-galactosidase (LacZ) reporter may not be efficiently used in certain endothelial cells of older mice; (2) alterations in the chromatin status of older endothelial cells may prevent recombination in some cells; or (3) activity of Cre or the Cre-ER^{T2} fusion is suppressed in some endothelial cells in older animals. Additional experiments are needed to discriminate between these possibilities. Nonetheless, these analyses highlighted the relevance of age in experiments involving genomic recombination events.

A common problem with so-called "endothelial-specific" promoters has been "leaky" expression in hematopoietic lineages. One motivation to develop a VE-cadherin Cre-recombinase mouse (Alva et al., 2006) was to bypass this problem. Nonetheless, the constitutive VE-Cadherin-Cre mouse model shows a 50–60% penetration in the hematopoietic lineages (Alva et al., 2006). In contrast, the inducible VE-Cad-Cre-ER^{T2} model induced with 2 mg of tamoxifen daily for 5 days and evaluated 3 weeks postinduction showed by histological evaluation (Fig. 8a) and by fluorescence-activated cell sorting (FACS) analysis (Fig. 8b) a relative minor (0.3% ± 0.1% over control) subpopulation of bone marrow cells that were positive for β-gal. These findings indicate that the labeled cells in the constitutive VE-Cad-Cre line are progeny of cells that expressed VE-cadherin at earlier developmental times. In contrast, the VECad-Cre-ER^{T2} bone marrow exhibits adult cells that have activated the VE-cadherin promoter. These cells when transplanted are found in the recipient and morphologically appear to be hematopoietic in nature (instead of endothelial or stromal cells; Fig. 8c). Whether these cells are also capable of contributing to the vasculature remains to be tested.

Finally, we examined whether recombination was induced by tamoxifen in newly formed vessels during tumor neovascularization. Lewis Lung carcinoma cells and c-neu transformed mammary epithelial cells were delivered subcutaneously into adult VECad-Cre-ER^{T2}/ROSA26R mice. Tamoxifen was injected 1 week postinoculation of the tumor cells. Five days later, tumor tissue was harvested, sectioned, and stained for evaluation of reporter expression. The β-gal activity in blood vessel-like networks was clearly observed on the surface of the tumor (Fig. 9Aa). Microscopic analysis revealed that recombination occurred in cells forming blood vessels among the tumor cells (Fig. 9Ab,c). CD31 immunohistochemistry of previously X-Gal–stained sections demonstrated that recombination occurred in tumor endothelium (Fig. 9Ad). In the inducible CreER^{T2}mouse model (VECad-Cre-ER^{T2}), we did not detect β-gal reactivity in inflammatory cells that colonized the tumor (Fig. 9Ac). Interestingly, the degree of excision in the tumor endothelium was more significant than in multiple normal tissues. We also performed matrigel angiogenesis assays and noted multiple positive cells in networks and tube-like structures (Fig. 9B). These outcomes highlight the potential utility of the VECad-Cre-ER^{T2} model for studies involving neovascularization events in the adult.

Recently, two inducible endothelial Cre-lines have been reported: Tie2-

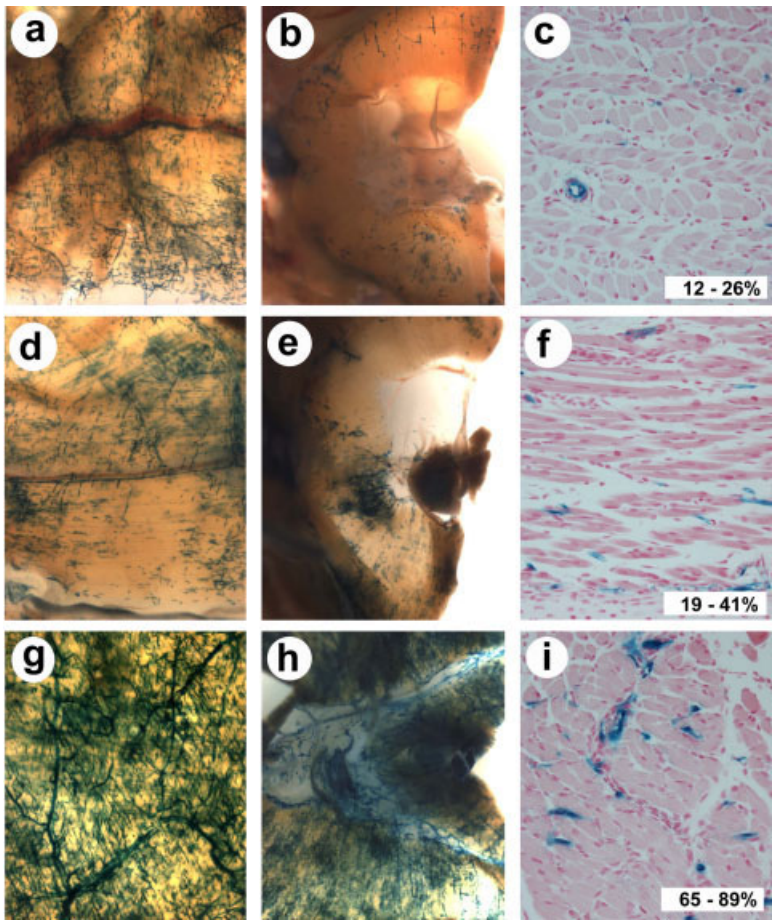

Fig. 5.

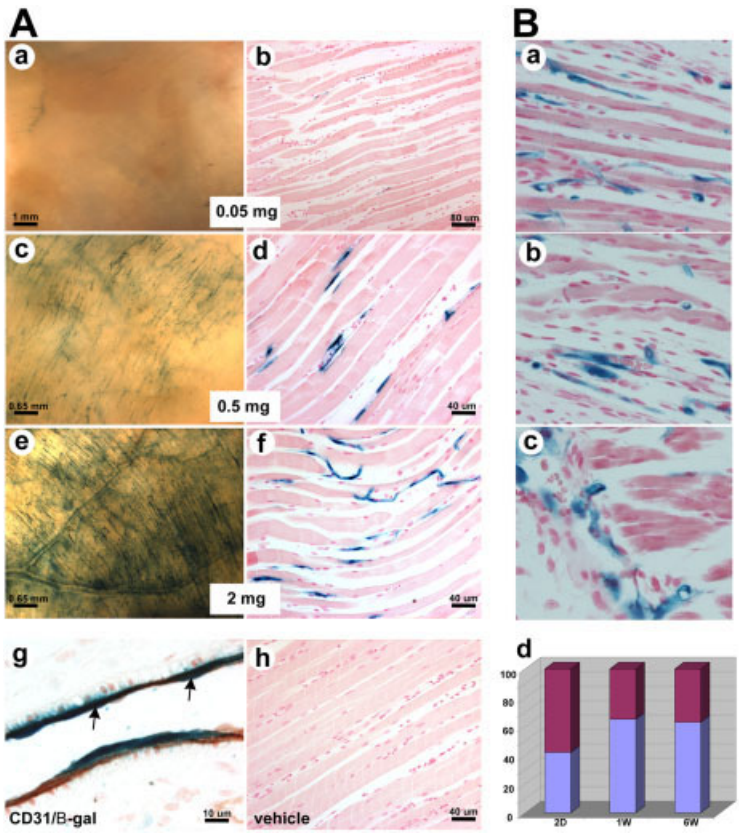

Fig. 6.

Cre-ER^{T2} (Forde et al., 2002) and SCL-Cre-ER ^{T2} (Gothert et al., 2005). With the first line, recombination in the endothelium was observed after a 5-week induction period with a high oral tamoxifen dose. In both lines, penetration in the hematopoietic lineage was also reduced in comparison with their constitutive counterparts. Because the characterization of those mice was not performed in a similar manner, it is difficult to make direct comparisons among all three inducible models. However, they each offer an opportunity to explore temporal deletions in the endothelium.

Since interpretation of specific gene deletion phenotypes requires a precise understanding of the population of

Fig. 5. Efficiency of Cre-recombinase in VECad-Cre-ER^{T2} in neonatal mice. Tamoxifen was given orally to neonates at postnatal day (P) 1, P2, and P3. a–i: LacZ expression was then assessed 1 week postdose. **a,b:** Whole-mount β-gal staining of abdominal muscle (a) and diaphragm (b). **c:** Histological evaluation of the abdominal muscle: β-gal, blue; nuclear counterstain, red. In a cohort of three mice, the degree of recombination after oral treatment was 12–26% as indicated in the figure. d–f: Topical application of 4-hydroxy-tamoxifen (4-OHT) for 3 consecutive days in neonatal mice evaluated 1 week postdose. **d,e:** Whole-mount β-gal staining of abdominal muscle (d) and diaphragm (e). **f:** Histological evaluation of the abdominal muscle. In a cohort of two mice, the degree of recombination range from 19–41%, as indicated in the figure. g–i: Intragastric injection of tamoxifen (0.05 mg) in neonatal mice for 3 days was evaluated 1 week postdose. **g,h:** Whole-mount β-gal staining of abdominal muscle (g) and diaphragm (h). **i:** Histological evaluation of the abdominal muscle. Evaluation of three mice revealed a percentage of recombination of 65–89% after three doses.

Fig. 6. Specific and inducible VECad-Cre-ER^{T2}–mediated recombination in adult mice is dose-dependent. Tissues shown are the abdominal skeletal muscle. **A:** Adult mice (6–8 weeks) were injected intraperitoneally (IP) with either 0.05 mg (a,b); 0.5 mg (c,d), or 2 mg (e,f) of tamoxifen for 5 consecutive days. Macroscopic (a,c,e) and histological with β-gal (blue) and nuclear counterstain (red, b,d,f) evaluation was performed 7 days postinjection. Colocalization (arrow) of β-gal (blue) and PECAM/CD31 (red–brown), an endothelial-specific marker (g). Microscopic analysis of the abdominal wall muscle of VECad-Cre-ER^{T2} mice injected with vehicle alone (control mice; h). **B:** The percentage of deletion was quantified over time in mice that were injected with 0.5 mg of tamoxifen IP for 5 consecutive days. Percentage of deletion was evaluated 2 days (a), 1 week (b), and 6 weeks (c) after the last injection. Quantification is shown in d, each bar of the histogram shows positive cells in blue and negative cells in red.

cells affected, we have made an effort to quantify the specificity and penetration of the VECad-Cre-ER^{T2}. However, it should stress that interanimal variability was noted, particularly as age increased. In addition, factors such as background strain, weight (fat content), and pathological conditions that affect liver function were found to contribute to this variability. Therefore, the quantifications presented should be used as a guide for the relative (not absolute) degree of deletion in relation to a particular modality of induction (frequency and route).

An interesting point uncovered by our study was that induction by tamoxifen in "old" adults did not lead to significant levels of recombination, even in the presence of Cre. Although the mechanism behind this finding requires further investigation, it emphasizes the extreme relevance of determining the percentage of excision in the target population. In contrast, tamoxifen induction in neonates and young adults resulted in fairly significant penetration.

In summary, this study reports the generation and characterization of a tamoxifen-inducible endothelial-Cre (Cre-ER^{T2}) line for studies of lineage tracing and genomic recombination in vascular endothelium. Analysis of different modes and timing of recombination presented here should be of use to investigators interested in inducible genomic alterations in the endothelial lineage.

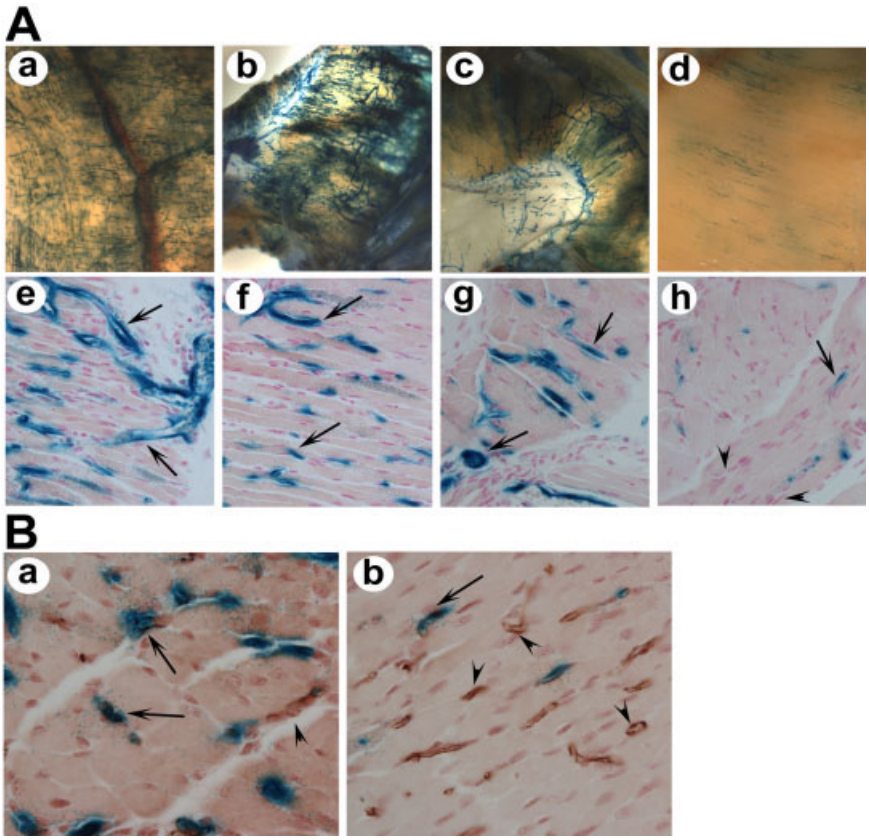

Fig. 7. Recombination in VE-Cad-Cre ERT2 mice varies inversely with age. Tissues shown are skeletal muscle. **A:** a–d: Whole-mount view. e–h: Histological sections. Mice at 3 weeks (a and e), 6 weeks (b and f), 8 weeks (c and g), and 24 weeks (d and h) were exposed to tamoxifen for 3 consecutive days (1 mg × 3 days, IP). **B:** Double staining of β-gal and Cre-recombinase shows frequent correlation (arrows) between recombination (blue) and Cre expression (brown) in mice at 3 weeks of age (a). In contrast, low correlation was noted in mice at 24 weeks (b), yet multiple vessels were expressing Cre-recombinase (arrowheads).

EXPERIMENTAL PROCEDURES

VECad-Cre-ER^{T2} Construct

A 3.2-kb *Avr*II/*Xba*I fragment of the pGS-Cre-ER^{T2} vector (Feil et al., 1996, 1997) containing the Cre-ER^{T2} cDNA was ligated into the murine 2.5-kb *VE-Cadherin* promoter (Gory et al., 1999). The resulting VE-Cad-Cre-ER^{T2} transgene is composed of the VE-cadherin promoter, a synthetic intron composed of sequences from the rabbit β-globin intron II, the Cre-ER^{T2} cDNA sequence and the polyadenylation site (poly A) from the SV40 early region (Fig. 1).

Transgenic and Reporter Mice

The VECad-Cre-ER^{T2} construct was tested in vitro and then introduced as a transgene into C57Bl6 and FVB/N backgrounds. Injections were performed into the male pronucleus of fertilized embryos by the transgenic mouse core facility of the University of California, Irvine.

Offspring were genotyped for Cre-ER^{T2} and R26R alleles using PCR. Briefly, DNA was generated from tail biopsies and PCR amplified for 39 cycles: denaturation at 94°C for 20 sec, annealing at 58.5°C for 20 sec, and elongation at 70°C for 45 sec. The primers used for the Cre PCR were Cre1 and Cre2, described by Feil and colleagues (Feil et al., 1996). The expected Cre PCR product size is 970 bp. Primers for the R26R allele have also been described previously (Soriano, 1999) and produce products of 600 bp for the wild-type (wt, +) allele and 320 bp for the LacZ reporter allele (−).

Analysis of Cre-ER^{T2} Activity in ROSAR26R Reporter Mice

To analyze Cre-recombinase activity, VECad-Cre-ER^{T2} transgenic males were crossed to females that carried the ROSA reporter allele R26R (Soriano, 1999). Tamoxifen (ICN) was dissolved in a sunflower seed oil/ethanol (10:1) mixture at 10 mg/ml. For initial screening, the double transgenic VECad-Cre-ER^{T2}/R26R animals were injected intraperitoneally with 2 mg of tamoxifen for 5 consecutive days. One week after the last injection, animals were anesthetized with Avertin (2-2-2-tribromoethanol, 0.5 mg/g in mice) and perfused with a fixative under conditions known to preserve β-gal activity (0.2% glutar-

aldehyde, 2 mM $MgCl_2$, 5 mM ethylenediaminetetraacetic acid [EDTA] in phosphate buffer saline [PBS]). The abdominal wall muscle was dissected and stained with X-Gal as described below.

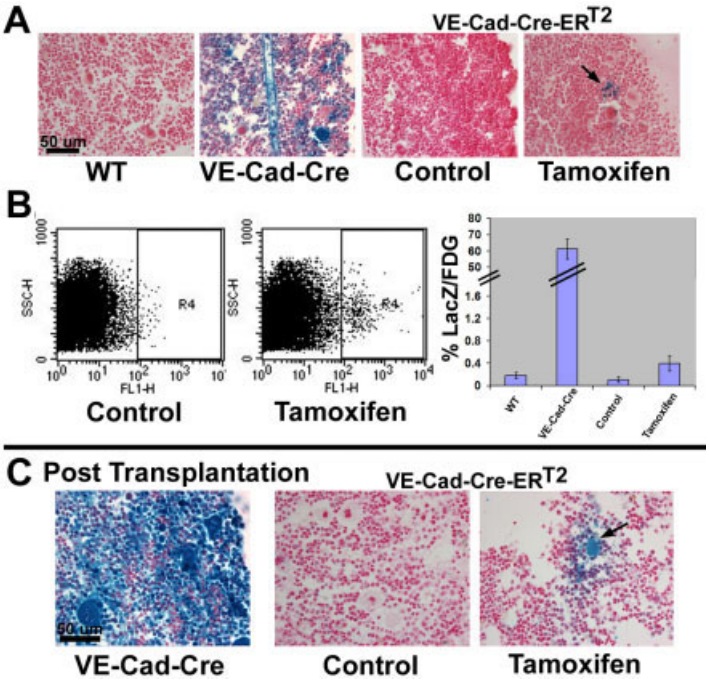

Fig. 8.

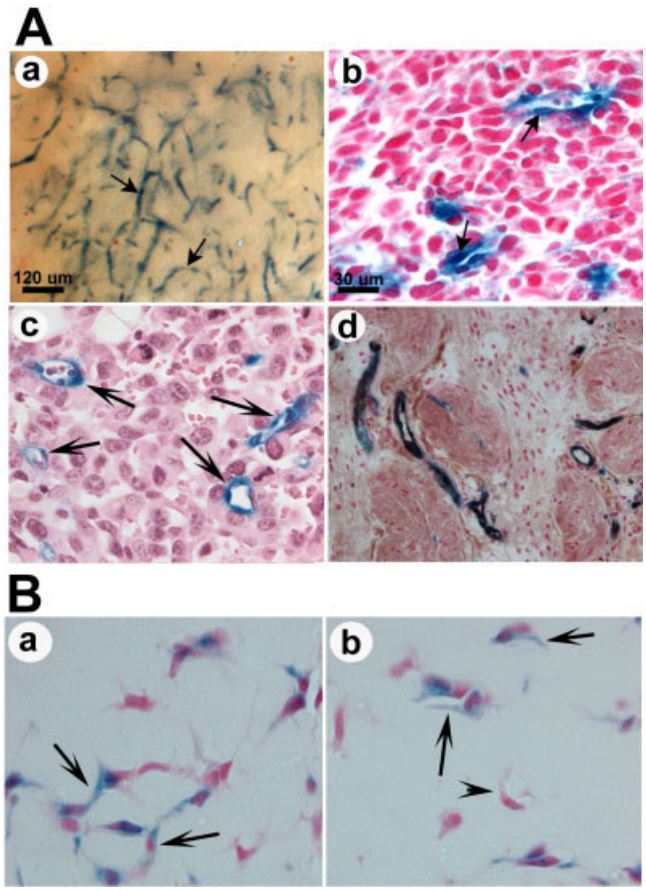

Fig. 9.

For dose–response experiments, 0.05, 0.5, and 2 mg of tamoxifen were injected IP into VECad-Cre-ER^{T2}/R26R animals for 5 consecutive days. One week later, animals were killed and the abdominal wall muscle was processed as described above. The evaluations of the recombination in the organs and in the hematopoietic compartments (bone marrow, spleen, thymus) were performed 3 and 12 weeks, respectively, after the last 2-mg injection of tamoxifen.

Fig. 8. Recombination in the adult hematopoietic lineage. Bone marrow from VECad-Cre (constitutive line; Alva et al., 2006) and inducible VECad-Cre-ER^{T2} was compared with wild-type animals (WT). VECad-Cre-ER^{T2} mice were injected with either vehicle (Control) or tamoxifen. Bone marrow was evaluated 3 weeks after tamoxifen induction (2 mg intraperitoneally for 5 consecutive days). **A:** β-gal stained (blue) specimens representative of each treatment and animal group with red nuclear counterstain. The arrow in the VECad-Cre-ER^{T2} tamoxifen-treated panel shows a small population of β-gal positive bone marrow cells induced in adult animals. **B:** Fluorescence-activated cell sorting (FACS) analysis of LacZ expression conducted to quantify the positive population. The first two dot plots depict Side Scatter (SSC) versus LacZ (FL1-H), with the positive β-gal population gated by R4. The chart depicts the percentage of β-gal–positive bone marrow cells as demonstrated by FACS, bars indicate standard error (n = 5 WT, n = 4 VECad-Cre, n = 3 each for VECad-Cre-ER^{T2} control and tamoxifen group). **C:** Histological examination of β-gal–stained marrow from recipient mice 4 months after whole bone marrow transplantation with the cells depicted in A and B. Note that bone marrow in the tamoxifen-induced animals resulted in reconstitution and exhibited a small subset of β-gal–positive bone marrow (arrow).

Fig. 9. Recombination of endothelial cells during neoangiogenesis in the adult. **A:** Tamoxifen-induced recombination in endothelial cells of the tumor vasculature. Whole-mount β-gal staining of a section (200 μm) of the Lewis Lung carcinoma (LLC) tumors in VE-Cad-Cre-ER^{T2} mice 1 week after injection of tamoxifen (a). Microscopic analysis of the β-gal stained LLC tumor, nuclear counterstain in red (b). Microscopic evaluation of β-gal stained c-neu tumors in VE-Cad-Cre-ER^{T2} mice (counterstained with hematoxylin; c). β-gal sections were also stained with PECAM (red–brown) (d). **B:** Tamoxifen-induced recombination in matrigel assays. Mice were either injected intraperitoneally with tamoxifen (1 mg × 1 dose) on the same day of matrigel implantation (a) or the matrigel plug contained OH-tamoxifen (50 nM; b). Matrigel plugs were extracted, stained for β-gal, fixed, and evaluated microscopically; nuclear is counterstain in red. Arrows show positive cells, arrowhead negative cells.

Recombination During Embryonic Development

Tamoxifen (1 mg) was injected IP into VECad-Cre-ER^{T2}/R26R pregnant female mice at the specified embryonic stages. Unless noted, 2 days later embryos were harvested after intracardial perfusion of pregnant females with 0.2% glutaraldehyde, 2 mM $MgCl_2$, 5 mM EDTA in PBS. Embryos were harvested, post-fixed for 10 min, and subjected to X-Gal staining as described below.

Tumor Experiments

Lewis Lung carcinoma (LLC) cells were routinely cultured in 10% fetal bovine serum (FBS) in Dulbecco's modified Eagle's medium (DMEM). For injections, confluent monolayers of LLC cells were washed and incubated 5 hr in serum-free DMEM. The cells were trypsinized, washed two times with serum-free DMEM and resuspended at a concentration of 10×10^6 cells/ml in serum-free DMEM. A total of 100 μl were injected intramuscularly into each flank of adult double-transgenic mice. One week after tumor cell injection, the mice received a single injection of 1 mg of tamoxifen (IP). Tumors were harvested 5 days after tamoxifen injection and subjected to X-Gal staining to reveal β-gal activity in LacZ-expressing cells, as described below.

Matrigel Assays

Mice (4–6 weeks old) were injected subcutaneously with 0.4 ml of matrigel (approximate protein concentration 9.9 mg/ml, Collaborative Research) premixed with vehicle (fatty acid-free bovine serum albumin), fibroblast growth factor-2 (1.3 μg/ml), vascular endothelial growth factor (1.4 μg/ml), and SPP (1 μg/ml), a protocol modified from Lee et al. (1999). Matrigel plugs were harvested and evaluated for β-galactosidase activity. Specimens were subsequently fixed with 4% paraformaldehyde in PBS, embedded in paraffin, dehydrated in ethanol and xylene, and stained with nuclear fast red.

Detection of β-Galactosidase Activity

For X-Gal staining, perfusion of fixed adult tissues (organs, tumors), or freshly isolated embryos or bone marrow, were post-fixed at 4°C in 0.2% glutaraldehyde, 2 mM $MgCl_2$, 5 mM EDTA in PBS, washed three times for 30 min at 37°C with detergent buffer (0.01% sodium deoxycholate, 0.02% Nonidet P-40, 2 mM $MgCl_2$ in PBS, pH 7.3), and then stained overnight in detergent buffer containing 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 1 mg/ml X-Gal. X-Gal was prepared at 25 mg/ml stock-solution in dimethylformamide. Whole-mount analysis was realized under a binocular magnifier.

For histological examination, stained samples were washed twice in PBS and post-fixed overnight in 1% paraformaldehyde at 4°C. After washes with PBS, samples were dehydrated and embedded in paraffin wax, and 7-μm sections were cut and counterstained with Nuclear fast red (Vector Labs). Bone marrow was embedded in Histogel (American Master Tech Scientific, Inc.) before dehydration and paraffin embedding.

Immunohistochemistry

Immunostaining for the endothelial marker PECAM1/CD31 was carried out with anti-PECAM-1/CD31 antibody (PECAM-1, platelet endothelial cell adhesion molecule-1; clone MEC 13.3; BD Pharmingen) after completion of X-Gal staining. Five-micrometer paraffin sections were clarified in xylene, rehydrated, and washed once in 1× PBS for 5 min. Endogenous peroxidase was inhibited with 0.9% H_2O_2 in methanol for 20 min. After one wash with 1× PBS, the slides were incubated 15 min at 37°C with proteinase K (0.1 mg/ml). After 2 washes with 1× PBS, sections were blocked with 1% rabbit serum, 0.05% Tween-20 in 1× PBS for 1 hr, and incubated overnight with anti-PECAM (1:100 in blocking solution) at 4°C. Sections were washed three times with 1× PBS, incubated 1 hr with biotinylated rabbit anti-rat antibody (1:100 in blocking solution) and immunoreactivity was visualized by the avidin–biotin complex method (Vectastain, Vector Laboratories). Sections were developed using the NovaRED substrate kit (Vector Laboratories). Immunohistochemistry for Cre-recombinase was performed in a similar manner using a polyclonal antibody (1:50 in blocking solution; Novagen), except, antigen retrieval was performed using boiling citrate buffer (pH. 6.8) for 10 min before blocking.

Quantification of Endothelial-Specific Deletion

Histological slides were prepared with two side-by-side 5-μm sections. One of those sections was exposed to PECAM antibodies, in addition to the X-Gal staining. The purpose of the PECAM was to identify the endothelial nature of the recombined cells. Because the frequent overlap of both colors resulted in a black and diffused staining, we used the nuclear red-stained sections to count nuclei of positive and negative cells and the serial PECAM-stained section to ensure the endothelial nature of the cell being counted. A total of 500 cells were counted per slide in three to four specimens and segregated in positive (blue, β-gal positive) and negative cells (red, nuclear counterstain).

Bone Marrow Analysis

Inducible VECad-Cre-ER^{T2} mice crossed with ROSA26 reporter lines were induced with 2-mg TXF IP injections daily for 5 days, vs. vehicle alone. In addition, VECad-Cre mice described previously (Alva et al., 2006) and wild-type FVB mice were analyzed as positive and negative histological and FACS controls. VECad-Cre-ER^{T2} bone marrow, 3 weeks after induction with TXF or vehicle, was removed from one femur and stained for LacZ as described above. Bone marrow from the paired femur was dissociated in sterile PBS and exposed to ammonium chloride solution (150 mM ammonium chloride, 1 M potassium bicarbonate, 10 mM EDTA) for 3 min at room temperature to mediate red cell lysis. Cells were then filtered (40 μm) and transplanted into lethally irradiated (1,000 cGy) C57Bl mice (5×10^6 cells by means of tail vein injection). A subset of the cells prepared for transplant also underwent FACS analysis as described (Nolan et al., 1988). In summary, bone marrow cells were hypotonically loaded with fluorescein di-β-D-galactopyranoside (Molecu-

lar Probes-Invitrogen) for LacZ expression and stained with 7AAD (BD Pharmingen) for viability. Cells were gated on cell size, viability, and negatively gated on FL-2 autofluorescence. Events analyzed totaled 50–100,000. Transplanted bone marrow underwent histological analysis of LacZ expression at approximately 4 months after transplant.

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