Tie-1-directed expression of Cre recombinase in endothelial cells of embryoid bodies and transgenic mice

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SUMMARY

Tissue-specific gene inactivation using the Cre-loxP system has become an important tool to unravel functions of genes when the conventional null mutation is lethal. We report here the generation of a transgenic mouse line expressing Cre recombinase in endothelial cells. In order to avoid the production and screening of multiple transgenic lines we used embryonic stem cell and embryoid body technology to identify recombinant embryonic stem cell clones with high, endothelial-specific Cre activity. One embryonic stem cell clone that showed high Cre activity in endothelial cells was used to generate germline mice. The in vivo efficiency and specificity of the transgenic Cre was analysed by intercrossing the tie-1-Cre line with the ROSA26R reporter mice. At initial stages of vascular formation (E8-9), LacZ staining was detected in almost all cells of the forming vasculature. Between E10 and birth, LacZ activity was detected in most endothelial cells within the embryo and of extra-embryonic tissues such as yolk sac and chorioallantoic placenta. Ectopic expression of Cre was observed in approximately 12-20% of the adult erythroid, myeloid and lymphoid cells and in subregions of the adult brain. These results show that the tie-1-Cre transgenic strain can efficiently direct deletion of floxed genes in endothelial cells in vivo.

Key words: Cre recombinase (Cre), Cre-loxP, β-Galactosidase (LacZ), Endothelial cell, Transgenic mouse, tie-1 promoter

INTRODUCTION

The vascular system is the first organ that forms during embryogenesis. Common progenitors of blood and endothelial cells form at around E8 by differentiation of mesenchymal precursor cells and are called hemangioblasts (Risau, 1995). In the blood islands of the yolk sac, endothelial cell differentiation occurs in close association with hematopoietic precursor cells, while within the embryo endothelial cells mainly differentiate from mesoderm as solitary angioblasts (Risau, 1995). Endothelial cells of various organs have many common morphological and functional properties, but also characteristic differences (Garlanda and Dejana, 1997). A few examples are the fenestrated endothelium in the kidney glomeruli, the sinusoidal endothelium in the liver, and the highly specialised endothelium in the brain microvasculature providing the blood-brain barrier. Even within the same organ, heterogeneity exists between large and small vessels, and between arteries and veins. Endothelial cells play crucial roles in a wide variety of specific functions, including tissue homeostasis, fibrinolysis and coagulation, blood-tissue exchange, vasotonyus regulation, and in the activation and extravasation of blood cells in physiological and pathological conditions (Risau, 1995). The exact molecular mechanisms underlying these various functions, however, are often unclear. Targeted inactivation of genes expressed in endothelial cells is a powerful tool to tackle these questions. If the gene of interest, however, is expressed in many cell types, gene ablation might result in early embryonic lethality preventing the analysis of its function in endothelial cells. In such cases, an endothelial cell-restricted gene inactivation would be desired. Tissue-specific gene inactivation can be achieved using either the Cre-loxP or the FRT/flip recombination system. Up to now, no mouse line expressing Cre recombinase in endothelial cells was available for such experiments.

Here we describe the generation and characterisation of a transgenic mouse strain in which the Cre recombinase expression is directed to endothelial cells by the mouse tie-1 promoter. The tie gene encodes a receptor tyrosine kinase expressed at early stages of vascular development. It is first detected at E8 in angioblasts of the head mesenchyme, in endothelial cells of the dorsal aorta and in the blood islands of the yolk sac (Korhonen et al., 1994). The same promoter fragment has previously been used to direct expression of reporter gene in endothelial cells undergoing vasculogenesis and angiogenesis in vivo (Iljin et al., 1999; Korhonen et al., 1995).
One ES cell clone that showed high Cre activity in endothelial cells was used to generate transgenic mice. They efficiently delete loxP flanked DNA sequences in endothelial cells in vivo. Therefore, the *tie-1-Cre* strain is an excellent tool for analysis of gene functions in endothelial cells using the Cre-loxP recombination system.

**MATERIALS AND METHODS**

**Generation of the tie-1-Cre expression construct**

The *tie-1*-Cre expression construct was generated in two steps. First, a floxed neomycin cassette was introduced into a plasmid containing a 1.15 kb *HindIII*-ApaI fragment of the mouse *tie-1* promoter (Iljin et al., 1999). In the second step, an *EcoRI*-XhoI fragment of pMC1-Cre (Gu et al., 1993) containing a nuclear localisation signal, Cre cDNA and a pA+ signal and a neomycin resistance cassette flanked by two loxP sites was introduced into the construct.

**ES cell culture, generation and analysis of embryoid bodies and generation of transgenic mice**

The expression construct was electroporated into R1 ES cells. ES cell culture was performed as previously described (Fässler and Meyer, 1995). G418 resistant ES clones were screened by Southern blot analysis of ES cell DNA. Genomic DNAs were digested with *EcoRI* and *HindIII*, fractionated on 0.7% agarose gels, transferred to Hybond-N+ filters (Amersham) and probed with a [32P]dCTP labeled 1.15kb *HindIII*-ApaI fragment of the *tie-1* gene and a [32P]dCTP labeled 1.7 kb *EcoRI*-XhoI fragment of the Cre gene to analyse the integration of the transgene.

Wild-type R1 and *tie-1*-Cre ES cells were used to generate embryoid bodies. For in vitro differentiation in embryoid bodies, ES cells were cultured in hanging drops as previously described (Bloch et al., 1997). Briefly, 800 cells were cultured in 20 μl of DMEM, supplemented with 20% fetal calf serum, non-essential amino acids, and 0.1 mM β-mercaptoethanol, hanging from the lid of a Petri dish for 2 days allowing the formation of embryoid bodies and then for 3 days in bacteriological dishes. Subsequently, the aggregates were plated on Tissue Tek chambers precoated with 0.1% gelatine and incubated for 9 days. The embryoid bodies were generated either in the presence or absence of G418 throughout the procedure. Immunofluorescent staining of embryoid bodies was performed as previously described (Bloch et al., 1997) using rat anti-PECAM-1 as a primary (Pharmingen) and Cy3-conjugated goat anti-rat secondary antibody (Jackson).

The transgenic ES cell clone 9 was used to generate germline chimeras according to standard procedures (Fässler and Meyer, 1995). Mice heterozygous for the *tie-1*-Cre transgene were subsequently crossed with the ROSA26R reporter mouse strain (Soriano, 1999).

**LacZ staining**

For whole-mount staining, dissected embryos and yolk sacs between E8-E10.5 were fixed for 30 minutes at room temperature in fixation buffer containing 0.2% glutaraldehyde, 5 mM EGTA, 2 mM MgCl2, and 0.1 M sodium phosphate, pH 7.3. Fixed embryos were then washed three times with 0.1 M sodium phosphate buffer containing 2 mM MgCl2, 0.2% NP-40 and 0.1% Na-deoxycholate. The staining was carried out in washing buffer supplemented with 2.1 μg/ml potassium ferrocyanide, 1.6 μg/ml ferricyanide, and 1 mg/ml X-gal. Staining reactions were performed overnight at 37°C. Embryos were subsequently embedded in paraffin, sectioned and counter-stained with Chromotrop2R. Older embryos (E11.5-E17.5) and dissected adult organs were fixed overnight in fixation buffer at 4°C, incubated in 20% sucrose in 0.1 M phosphate buffer for 1 day and embedded in Tissue Tek. Frozen sections (8-10 μm) were stained for LacZ activity as previously described. In all experiments, embryos lacking the *tie-1*-Cre transgene served as negative controls and demonstrated that the staining was a specific indicator of Cre-mediated activation of the reporter gene.

**Flow cytometry and antibodies**

Single cell suspensions from adult double-transgenic thymus, spleen and bone marrow were investigated by flow cytometry. The cells were loaded with fluorescein di-β-D-galactopyranoside (FDG) to detect LacZ-positive cells (Nolan et al., 1998) and immunostained with markers for the erythroid, myeloid and lymphoid lineages. The following biotinylated antibodies were used as lineage markers: anti-CD3 (2C11), anti-CD45R/B220 (RA3-6B2), Ter119, Gr-1 (RB6-8C59) and anti-CD11b (Mac-1, M1/70) (all Pharmingen). Biotinylated antibodies were visualised with streptavidin-PE (Southern Biotechnologies). Dead cells were excluded from analysis on a FACScalibur (Becton Dickinson) by propidium iodide (1 μg/ml, Sigma) counter-staining.

**RESULTS AND DISCUSSION**

The *tie-1*-Cre expression construct (Fig. 1A) was electroporated into R1 ES cells. G418-resistant ES clones were screened by Southern blot using probes specific for the *tie-1* promoter and the Cre cDNA to verify the integration of the transgene (Fig. 1B). Clones expressing a functional *tie-1*-Cre transgene in endothelial cells were identified using an in vitro assay. ES cells were differentiated in embryoid bodies to obtain endothelial cells. In clones where the transgene is expressed, endothelial cells should lose their floxed neomycin resistance cassette due to the Cre activity. As a result, these cells become neo-sensitive and die in the presence of G418. Since expression of PECAM-1 precedes that of *tie-1* in embryoid bodies (Vittet et al., 1996), the differentiated endothelial cells were visualised by PECAM-1 staining. An extensive vascular network was evident by PECAM-1 staining in embryoid bodies derived from wild-type ES cells as well as cells from clone 9 (Fig. 1C). On the contrary, when embryoid bodies from clone 9 were cultured in the presence of G418, only very poorly developed capillary-like structures and scattered PECAM-positive cells were present suggesting that the endothelial cells die soon after differentiation and activation of the *tie-1*-Cre transgene (Fig. 1C). Out of five clones tested, two showed a behaviour similar to clone 9, i.e. few PECAM-positive cells and no visible endothelial network in embryoid bodies cultured in the presence of G418. This in vitro screening procedure has significant advantages in terms of time, effort and expense when compared to the generation and analysis of several conventional transgenic mouse strains. The assay is easy to perform and is applicable to investigate transgenic expression in any cell type easily obtained in embryoid bodies such as endothelia, muscle, nerve, and epithelia.

ES clone 9 carried one copy of the integrated transgene (Fig. 1B) as judged by comparing hybridisation signals obtained from the transgene to that obtained for the endogenous *tie-1* promoter. This clone was used to generate germline chimeras according to standard procedures (Fässler and Meyer, 1995). Analysis of the progeny of founder animals showed that the transgene was transmitted at a frequency of 50%. Moreover, mice homozygous for the transgene were viable, fertile and showed no overt abnormalities indicating that the random insertion of the transgene did not disrupt an essential gene.
To determine the expression of a functional Cre in vivo, tie-1-Cre transgenic mice were intercrossed with the ROSA26R indicator strain in which the LacZ reporter gene is activated after Cre-mediated excision of a loxP flanked neomycin cassette (Soriano, 1999). Embryos resulting from such matings were dissected at E8-E17.5 during development and at adult

Fig. 1. Tie-1-Cre transgene, Southern blot analysis and in vitro differentiation analysis of transfected ES cells. (A) The expression construct is comprised of a 1.15 kb fragment of the mouse tie-1 promoter linked to a nuclear localisation signal, the coding sequence of Cre recombinase followed by a polyadenylation signal. In addition the construct contains a floxed neomycin resistance cassette. (B) After transfection, DNA from G418-resistant ES colonies was genotyped by Southern blot using tie-1- and Cre-specific probes. (C) Functional in vitro analysis of endothelial cell Cre expression. PECAM-1 staining of wild-type and ES cells from clone no 9 differentiated in embryoid bodies for 5 days in suspension culture and 9 days in tissue culture chambers. A prominent capillary network is present in embryoid bodies derived from wild-type ES cells. An equally well developed vascular network is evident in embryoid bodies derived from clone 9 grown in normal media. When embryoid bodies derived from clone 9 are cultured in the presence of G418, only very poorly developed vessel-tubes and scattered PECAM-positive cells are present due to activation of Cre in endothelial cells leading to excision of the neomycin resistance cassette and loss of G418-resistance. Bar, 250 μm.

Fig. 2. Temporal and spatial expression of LacZ in tie-1-Cre and ROSA26R double transgenic embryos and yolk sacs detected by whole-mount LacZ-staining. (A) At E8.5, LacZ-positive cells are located in the developing heart tube, dorsal aorta and the head mesenchyme. (B) Ventral view of the same embryo, showing strong LacZ staining in the paired dorsal aorta (arrowheads). (C) In the E8.5 yolk sacs LacZ-positive cells mark the forming vasculature. (D) By E10.5, LacZ staining is more pronounced and nearly continuous throughout the embryonic vascular system. (E) A higher magnification of an E10.5 embryo showing the LacZ-positive capillary plexus of the head. (F) At E10.5, the continuous vascular network of the yolk sac is positive for LacZ staining. At all stages analysed, ROSA embryos lacking the tie-1-Cre transgene were negative for LacZ staining (not shown).
stages and examined by LacZ staining. At all stages analysed, no LacZ activity was detected in ROSA26R control embryos lacking the *tie-1-Cre* transgene.

In the *tie-1-Cre* and ROSA26R double transgenic embryos LacZ activity was detectable already at E8-E8.5 (Fig. 2A). Most cells in the developing heart tube and in the dorsal aorta were LacZ-positive (Fig. 2A and B). In the yolk sac, cells of the forming vessels were stained (Fig. 2C). By E9.5, LacZ-positive cells were observed in the developing heart, dorsal aorta, head mesenchyme and in the yolk sac (not shown). At E10, LacZ staining was detected in most cells of the embryonic vascular system (Fig. 2D and E). Positive cells were found in the developing heart, developing plexus of the head (Fig. 2E), intersomitic vessels and the carotid arteries. In yolk sac, the vascular tree showed a distinct LacZ staining (Fig. 2F). On histological sections, strong LacZ signals were visible in the heart endocardium and in the endocardial cushions (Fig. 3A) that forms via transdifferentiation of endothelium into mesenchyme (Sinning et al., 1992). Since the Cre-mediated activation of the reporter gene occurs before the transdifferentiation, the resulting mesenchymal cells express the activated reporter gene. The endothelial lining of the dorsal aorta was strongly stained, and a few positive hematopoietic cells were visible in the lumen (Fig. 3B). At E14.5, the prominent capillary network of the lung was strongly stained whereas the bronchioli were negative (Fig. 3C). In liver, LacZ staining was detected in the large veins, in the nuclei of the sinusoidal endothelial cells and in a few scattered cells possibly of hematopoietic origin (arrow in Fig. 3D). The vessels of the developing kidney glomeruli and those between the tubuli were LacZ-positive (Fig. 3E) and a strong LacZ staining was present in the intersomitic vessels (Fig. 3G). The capillaries within the brain were LacZ-positive while the brain tissue is devoid of staining. (H) Capillaries in the spinal cord and the skin (arrows) are LacZ-positive. Abbreviations: a, atrium; v, ventricle; ect, endocardial cushion tissue; da, dorsal aorta. Bars: 500 μm (A,B); 250 μm (C,E-H); 125 μm (D).
Taken together, between E10 and until birth, most endothelial cells showed a positive LacZ staining. These data are mainly in agreement with previously published data on the expression of the endogenous tie-1 gene (Dumont et al., 1995; Korhonen et al., 1994). The tie-1 promoter is not active in endothelial cells of the liver sinusoids (Korhonen et al., 1995), while we detected LacZ staining in these cells. This is most likely due to Cre-mediated activation of the LacZ gene in endothelial cells before the sinusoidal spaces form. The tie-1-Cre induced LacZ expression follows that of the endogenous tie-1 gene expression (Dumont et al., 1995; Korhonen et al., 1994) also at the initial stages of vascular formation, indicating that Cre quickly deleted the floxed DNA sequence and activated the LacZ reporter gene.

To locate LacZ-positive cells in adult tie-1-Cre and ROSA26R double transgenic mice, kidney, lung, heart, liver and brain were investigated by LacZ staining. In kidney, capillaries of the glomeruli and vessels surrounding the tubuli were LacZ-positive (Fig. 4A). Strong LacZ staining was also detected in capillaries of the alveolar network of the lung while the epithelium of the bronchioli were devoid of staining (Fig. 4B). Small vessels and capillaries between the myocardial muscle fibers showed LacZ staining (Fig. 4C), and the same was observed for the thin endocardial lining. In liver, the central venule and the nuclei of the endothelial cells lining the sinusoidal spaces were LacZ-positive. (F) The capillaries of the brain are LacZ positive. (G) Several areas of the brain show a strong LacZ signal. (H) Higher magnification of the cerebral cortex showing that neurons of all layers are LacZ positive. (I) Higher magnification of the hippocampus showing LacZ positive neurons of the dentate gyrus and CA3, and positive staining of a proportion of cells in CA2, while cells of CA1 are devoid of staining. Abbreviations: m, myocardial muscle layer; e, endocardial lining; cv, central vein; s, sinusoidal spaces; dg, dentate gyrus. Bars: 250 μm (A-C, F); 125 μm (D,E); 2 mm (D); 500 μm (H,I).

Moreover, capillaries throughout the brain showed positive staining for LacZ (Fig. 4F). In a few mice, not all endothelial cells of adult organs were LacZ-positive (70-90% in kidney, liver and brain, and 90-95% in lung and heart). Surprisingly, in adult brain an intense LacZ staining was also evident in certain neuronal populations (Fig. 4G). LacZ positive cells mark most cortical areas (Fig. 4G and H) including ventral/lateral cortical structures. LacZ staining was also detected in some neuronal populations of the hippocampus (Fig. 4G and I). Neurons of the dentate gyrus and CA3 were strongly LacZ positive, and so was a proportion of cells in CA2, while cells of CA1 were almost completely devoid of staining (Fig. 4I). Ventral thalamus lacked LacZ staining while dorsal thalamus including medial and lateral geniculate were LacZ positive (not shown). In midbrain, a few scattered LacZ positive cells were present. In cerebellum, the LacZ positive cells had a patchy distribution and some Purkinje cells, granule cells and deep nuclei showed LacZ staining (not shown). During early embryogenesis, no LacZ positive cells were detected in the developing brain while a very weak and diffuse LacZ staining was first evident at E17.5 (not shown) suggesting that the Cre expression most likely is turned on in postmitotic neurons. The tie-1 promoter had not previously been reported to be active in neuronal cells and therefore, this expression of the tie-1-Cre transgene most likely is ectopic. This unexpected
expression pattern of the tie-1-Cre transgene can be used to detect floxed genes in certain neuronal subpopulations at postnatal stages.

Apart from the wide LacZ staining of endothelial cells, also a proportion of the hematopoietic cells were blue (Fig. 3C). Counting on tissue sections revealed that 93 out of 700 blood cells (13%) were LacZ–positive at E10. This is not very surprising considering that the endothelial and hematopoietic lineages are derived from a common precursor, the hemangioblast (Choi et al., 1998) and that tie-1 has been suggested to be expressed already at this stage (Korhonen et al., 1992; Korhonen et al., 1994). It is not known, however, if and where the tie-1 promoter is active at later stages of hematopoiesis. Tie-1 expression has been reported in hematopoietic stem cells (Iwama et al., 1993; Yano et al., 1997), in some B cells (Hashiyama et al., 1996), and in several leukemic cell lines (Armstrong et al., 1993; Kukk et al., 1997).

To further characterise Cre expression in the hematopoietic lineage, cell suspensions from adult double transgenic thymus, spleen and bone marrow were investigated by flow cytometry. The cells were loaded with fluorescein di-β-D-galactopyranoside (FDG) to detect LacZ-positive cells (Nolan et al., 1988) and immunostained with markers for the erythroid, myeloid and lymphoid lineages. These results are summarised in Table 1. In thymus, 12% of the CD3-positive T cells were LacZ–positive. In spleen, 13% of the B220-positive B cells were positive for LacZ. In bone marrow, the numbers of LacZ-positive cells were 17% of the B220-positive B cell population, 14% of the Ter119-positive erythrocytes, 20% of the Gr-1-positive granulocytes and 19% of the Mac-1-positive granulocyte and monocyte population. Taken together, between 12-20% of cells from these hematopoietic lineages were positive for LacZ indicating that the tie-1-Cre transgene is not preferentially expressed in a certain population at later stages of hematopoiesis but rather in a common precursor cell.

In summary, we describe the generation of a tie-1-Cre transgenic mouse strain that efficiently mediate excision of floxed genes in endothelial cells in vivo. The availability of this strain will be a valuable tool for assessing gene functions in vascular development and disease, as well as dissecting endothelial cell biology through the use of the Cre/loxP recombination system. This will be particularly important if constitutive gene ablation experiments result in early embryonic lethality or lead to complex phenotypes that include vascular defects as well as defects in other organ systems.