Exp. Anim. 59(5), 615–622, 2010

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Flk1-GFP BAC Tg Mice: An Animal Model for the Study of Blood Vessel Development

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Abstracts: The mouse *Flk1* (also called *Kdr* or *Vegf-r2*) gene encodes a receptor for VEGF-A. *Flk1* is expressed in endothelial cells of the developing embryo. Recent studies have shown that *Flk1* is expressed by multi-potent mesodermal progenitors, which give rise to various hematopoietic and cardiovascular cell lineages during development, and in differentiating ES cells, which may be used for cell transplantation therapy to treat cardiovascular diseases. Given its developmental and clinical importance in cardiovascular tissues, an animal model of *Flk1* activity would be very useful. Here, we report the generation of *Flk1-GFP* BAC transgenic mice for monitoring *Flk1* gene expression during development. We show that GFP expression in these mice serves as a surrogate marker for developing endothelial cells. Immunohistochemical analysis showed that the regions of expression of GFP and endogenous FLK1 largely overlap. Uniform GFP expression was observed in most endothelial cells at 8.5 dpc and thereafter. *Flk1-GFP* BAC transgenic mice should be useful for the study of both vascular development and pathological angiogenesis.

Key words: angiogenesis, cardiogenesis, Flk1, vasculogenesis, VEGF-A

Introduction

The mouse *Flk1* gene encodes a receptor for vascular endothelial growth factor A (VEGF-A) [5]. During the later stages of embryonic development, *Flk1* is expressed abundantly in endothelial cells [17]. Gene targeting experiments have shown that *Flk1*-deficient mice die between 8.5 and 9.5 days postcoitum (dpc) due to the absence of blood vessels and blood islands [15]. Recent studies have shown that *Flk1*-positive progenitor cells are multi-potential, and can differentiate into smooth muscle cells and cardiomyocytes in addition to hematopoietic and endothelial lineages during mouse development and ES cell differentiation [2, 3, 9, 12, 13, 16, 18]. Importantly, these properties have been observed in both mouse and human ES cells in *in vitro* differentiation models [19].

In the adult, *Flk1* is expressed at a low level in normal

(Received 5 June 2010 / Accepted 28 June 2010)

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vascular beds, but is expressed more strongly in the neovasculature associated with tumor formation [5]. VEGF-A signaling is an important regulatory pathway controlling tumor angiogenesis. Although our understanding of the process of angiogenesis has grown, the precise molecular and cellular mechanisms of vascular development are still poorly understood. Therefore, the generation of an animal model of *Flk1* gene activity should lead to a better understanding of these processes, and should also be useful for the development of more effective approaches for treating conditions such as cancer that depend on angiogenesis.

Kappel and coworkers identified an enhancer activity in the first intron of the *Flk1* gene, which directs *Flk1* expression in endothelial cells of the yolk sac region at 7.5 dpc [7, 8]. This enhancer was successfully used to drive mCherry expression in endothelial cells by other researchers [11, 14]. However, deletion of this enhancer has no significant effect upon *Flk1* expression in mesodermal progenitors at 7.5 dpc or in endothelial cells at 8.5 dpc [3]. These results suggest that an as-yet unidentified enhancer directs *Flk1* expression in endothelial cells and early mesodermal cells.

Here, we report the generation of *Flk1-GFP* BAC (bacterial artificial chromosome) transgenic mice to monitor *Flk1* gene expression during development. Immunohistochemical analysis showed that the distribution of expression of GFP (green fluorescent protein) and endogenous FLK1 significantly overlapped. Uniform GFP expression was observed in most endothelial cells at 8.5 dpc and thereafter. Thus, the *Flk1-GFP* BAC transgenic mice generated in this study should be useful for studying vascular development and pathological angiogenesis.

Materials and Methods

Construction of the Flk1-GFP transgene

BAC clones covering the *Flk1* locus were obtained from Invitrogen. One clone (RP24-125B24) was modified using the RED/ET recombination technique (Gene Bridges, Heidelberg, Germany). A DNA fragment encoding GFP-SV40 polyA (Clontech, Mountainview, CA, USA) was ligated to a DNA fragment encoding an FRT PGK-gb2-neo expression cassette comprised of a prokaryotic promoter and neomycin-resistance gene flanked by FRT sequences (Gene Bridges). Homology arms for the first exon of *Flk1* were ligated to both ends of the reporter cassette by PCR amplification. *Escherichia coli* carrying the BAC were transformed with a RED/ET expression plasmid, pSC101-BAD-gbaA. Recombinants were identified by screening for kanamycin resistance followed by PCR analysis. The Kan/Neo cassette was excised by inducing FLPe expression after introduction of the FLPe expression plasmid (Gene Bridges).

Generation of transgenic animals

Recombinant BAC DNAs were purified using a BAC100 column (Nippon Genetics, Tokyo, Japan) and linearized by Pl-Scel digestion. Fertilized eggs collected from BDF_1 females were used for pronuclear injection, and the injected eggs were transplanted into pseudopregnant ICR females (SLC Inc., Shizuoka, Japan). The mice were maintained on an ICR genetic background. *Flk1-GFP* BAC Tg animals were genotyped and discriminated from *Flk1-GFP* knock-in animals by a pair of primers: 5'-GTTCCTATACTTTCTAGAGAAATGG-3', 5'-GAGTTATTTAGTTTAATACACC-3'. Fluorescent images of the whole body were taken by a VB7010 microscope (Keyence). This study was approved and conducted in accordance with the Regulations for Animal Experimentation of the University of Tsukuba.

Immunohistochemistry

Embryos were dissected, staged according to the method of Downs and Davies [1], and fixed with 4% paraformaldehyde (PFA) overnight. After washing with PBS, the embryos were soaked in 30% sucrose, mounted on OCT compound, and then 4 mm cryosections were prepared. Immunohistochemistry was performed with anti-FLK1 (clone AVAS12; BD) and anti-GFP (rabbit polyclonal; MBL), as previously described [4]. In brief, after blocking in PBSMT (PBS + 2% skim milk + 0.1% tween20) for 1 h, the sections were incubated with the first antibodies at 4°C overnight. The sections were washed twice in PBST for 5 min and then incubated with fluorochrome-conjugated secondary antibodies at room temperature for 1 h. Nuclei were visualized by incubation with 2 mg/ml Hoechst 33342 for 10 min at room

temperature and the sections were mounted in Perma-Fluor Mounting medium (Thermo Electron Corp., Pittsburgh, PA, USA). The images were captured by a Leica DC500 digital camera system. Whole mount immunohistochemistry was performed and Cy3-conjugated anti-rat antibody (Jackson Immunological, West Grove, PA, USA) was used as the 2nd antibody. Leica confocal microscopy SP5 or BioRevo (Keyence, Osaka, Japan) was used for imaging.

Mouse aortic ring assay

Thoracic aortas were removed from *Flk1-GFP* BAC Tg mice and immediately transferred to a culture dish containing serum-free Dulbecco's Modified Eagle Medium (Invitrogen, Carlsbad, CA, USA). The peri-aortic fibroadipose tissue was carefully removed with fine microdissecting forceps and iridectomy scissors. One-millimeter-long aortic rings (approximately 15 per aorta) were sectioned and extensively rinsed in DMEM. Ring-shaped explants of mouse aorta were then embedded in collagen gels prepared by mixing eight volumes of Cellmatrix type 1-A (Nitta Gelatin), one volume of 10 × M199 medium, and one volume of 2.2% $NaHCO_3$. The collagen gels containing the aortic rings were polymerized by incubation at 37°C for 30 min. Each dish contained 300 μl of DMEM containing 50 ng/ml human VEGF-A (R&D systems, Minneapolis, MN, USA). The cultures were kept at 37°C in a humidified environment for one week and examined using a microscope (BioRevo, Keyence).

Results

Generation of Flk1-GFP BAC transgenic mice

We previously generated *Flk1-GFP* knock-in animals that carried a gene encoding *GFP* in the first exon of *Flk1*[3]. GFP expression recapitulated that of endogenous FLK1 protein expression from the time point corresponding to the onset of FLK1 expression and thereafter. This knock-in mouse is a useful mouse model for monitoring *Flk1* gene activity during development and in the adult [3, 10]. However, the level of FLK1 expression is only half of that observed in wild-type animals because one of the two alleles is replaced by the cDNA encoding *GFP* [3]. Haplo-insufficiency of *Flk1* could theoretically have phenotypic consequences, although we note that the *Flk1-GFP* knock-in mice are fertile and do not show any apparent vascular anomalies [3]. Kappel and coworkers showed that the *Flk1* promoter region and the enhancer within the first intron can be exploited to monitor the distribution of *Flk1* expression [7]. The transgenic animals exhibited fluorescence in endothelial cells during development and in the adult [7]. However, the transgene used contains only part of the *Flk1* regulatory sequence and its enhancer activity is moderate (M. E., unpublished observation). Therefore, in this study we aimed to generate a new animal model of *Flk1* gene expression. We used a BAC clone that carries the whole *Flk1* genomic region as well as the upstream and downstream regions (Fig. 1). *GFP* cDNA was introduced into the first exon of the *Flk1* gene in the BAC clone using the RED/ET recombination technique (Fig. 1). The resulting GFP-carrying BAC was injected into pronuclei so as to create *Flk1-GFP* BAC Tg animals. Five out of nine lines showed clear GFP fluorescence within newborn skin and at the tip of the adult tails (Fig. 1).

Expression of GFP recapitulated by Flk1-GFP BAC Tg

We observed strong and uniform fluorescence in endothelial cells at 8.5 dpc in three transgenic animals when we examined three transgenic lines which showed fluorescence within newborn skin and at the tip of the adult tails, indicating efficient recapitulation of the pattern of endogenous *Flk1* expression in endothelial cells at that stage (Fig. 2). Thereafter, we characterized two *Flk1-GFP* BAC Tg mice, lines 244 and 270. Immunohistochemical analysis revealed that the distribution of GFP expression corresponded almost exactly with that of endogenous FLK1 within the endothelial cells including those of the dorsal aorta (Fig. 2). Analysis of the two independent transgenic lines (lines 244 and 270) at 8.5 dpc revealed that most if not all GFP expression overlapped with that of the endogenous FLK1 protein (Fig. 2). These Tg embryos also showed strong GFP expression whose distribution was similar to that of endogenous FLK1 protein at later stages, such as 9.5 dpc (Fig. 3). Confocal microscopy was used to investigate whether the distribution of GFP expression overlapped with that of endogenous FLK1 in the yolk sac region of 9.5 dpc embryos. Analysis of the two independent trans-

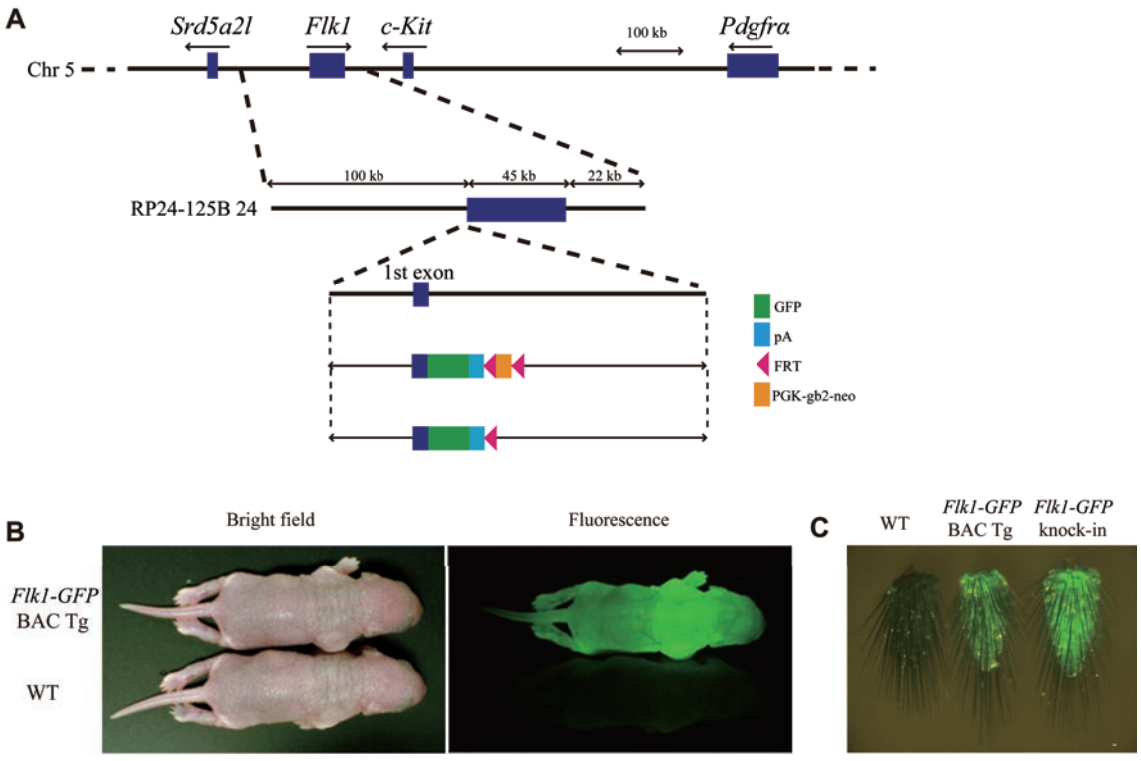

Fig. 1. Generation of *Flk1-GFP* BAC Tg mice. (A) Schematic representation of the *Flk1-GFP* BAC transgene used in this study. The *Flk1* BAC clone (RP24-125B24) carries 22 kb and 100-kb upstream and downstream genomic regions, respectively. The BAC transgene without PGK-gb2-neo cassette was used to generate transgenic animals. (B) Gross appearance of *Flk1-GFP* BAC Tg mice. Bright field (Left column) and GFP fluorescence images (Right column). Note that the GFP signal is seen throughout the entire bodies of newborn mice (P0). (C) Genotyping of *Flk1-GFP* BAC Tg mice. The BAC Tg mice were generally genotyped based on fluorescence of the tail.

genic lines at 9.5 dpc revealed that most if not all GFP expression overlapped with that of the endogenous FLK1 protein (Fig. 4). GFP expression was also observed within the blood vessels of adult skin (Fig.5), indicating that GFP fluorescence in *Flk1-GFP* BAC Tg animals recapitulate endothelial expression of FLK1 from the embryonic stages to the adult.

Expression of GFP in the aortic ring assay

We next subjected the Tg mice to the aortic ring assay, which measures angiogenic activity, in particular the sprouting process. A piece of aorta from an adult was cultured in type IV collagen matrix and endothelial sprouts were observed in the presence of VEGF-A. GFP positive endothelial sprouts from the aortic ring were observed in real time (Fig. 6, data not shown). Immunohistochemical analysis using anti-FLK1 antibody showed that GFP protein expression completely overlapped that of the endogenous FLK1 protein (Fig. 6).

Taken together, these results suggest that the genomic region on the BAC clone is capable of recapitulating the distribution of the transgene expression, mirroring that of endogenous *Flk1* expression, and that the *Flk1-GFP* BAC transgenic mice generated in this study should be useful for studying vascular development and pathological angiogenesis.

Discussion

Flk1 plays essential roles in vasculogenesis and angiogenesis during development, as well as in the pathological angiogenesis that occurs during tumor growth [5]. Therefore, there is a need for animal models that can faithfully monitor *Flk1* gene activity. Although two genetically engineered mouse strains have previously been described that can monitor *Flk1* gene activity, there

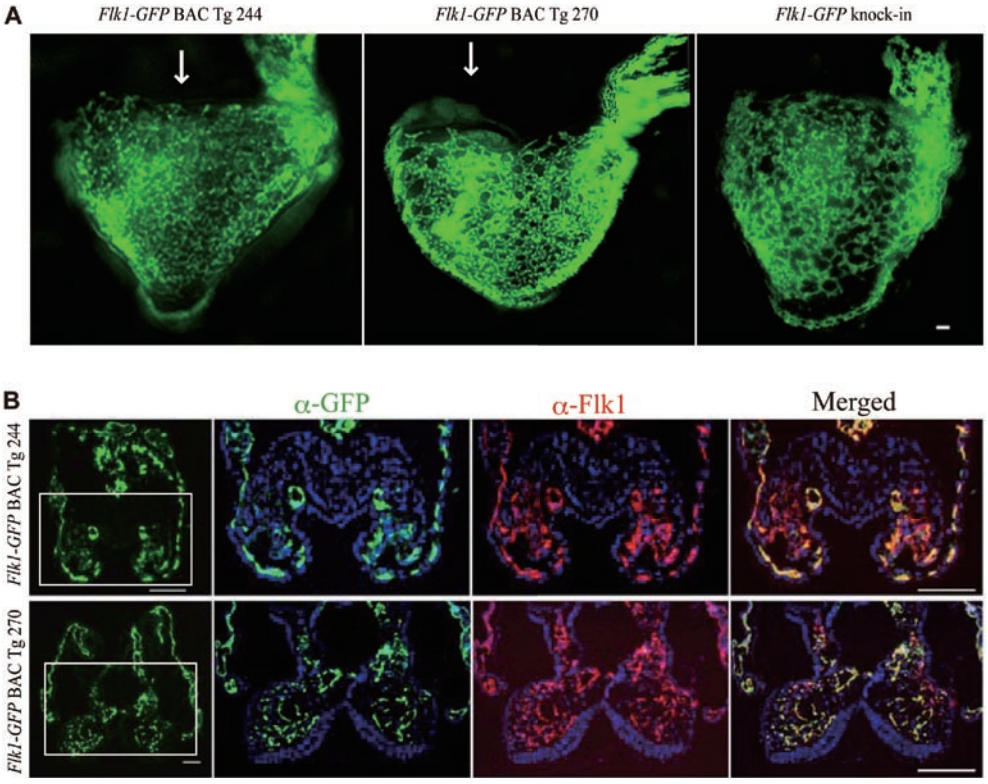

Fig. 2. GFP expression in *Flk1-GFP* BAC Tg embryos at 8.5 dpc. Fluorescent images of two independent *Flk1-GFP* BAC Tg and *Flk1-GFP* knock-in embryos at 8.5 dpc (A). Overlapping distribution of the expression of GFP and endogenous FLK1 protein (B). Section indicated in (A) was subjected to immunohistochemical analysis with anti-GFP and anti-FLK1 antibodies to further compare the distribution of their expression.

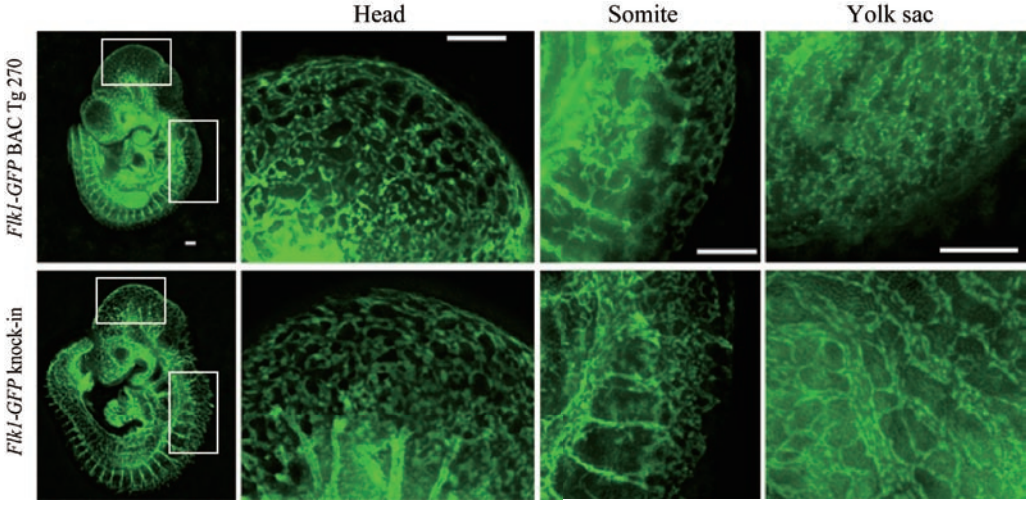

Fig. 3. Comparison of GFP expression in *Flk1-GFP* BAC Tg and *Flk1-GFP* knock-in embryos at 9.5 dpc. Higher magnification of the head region, intersomitic vessels and the yolk sac.

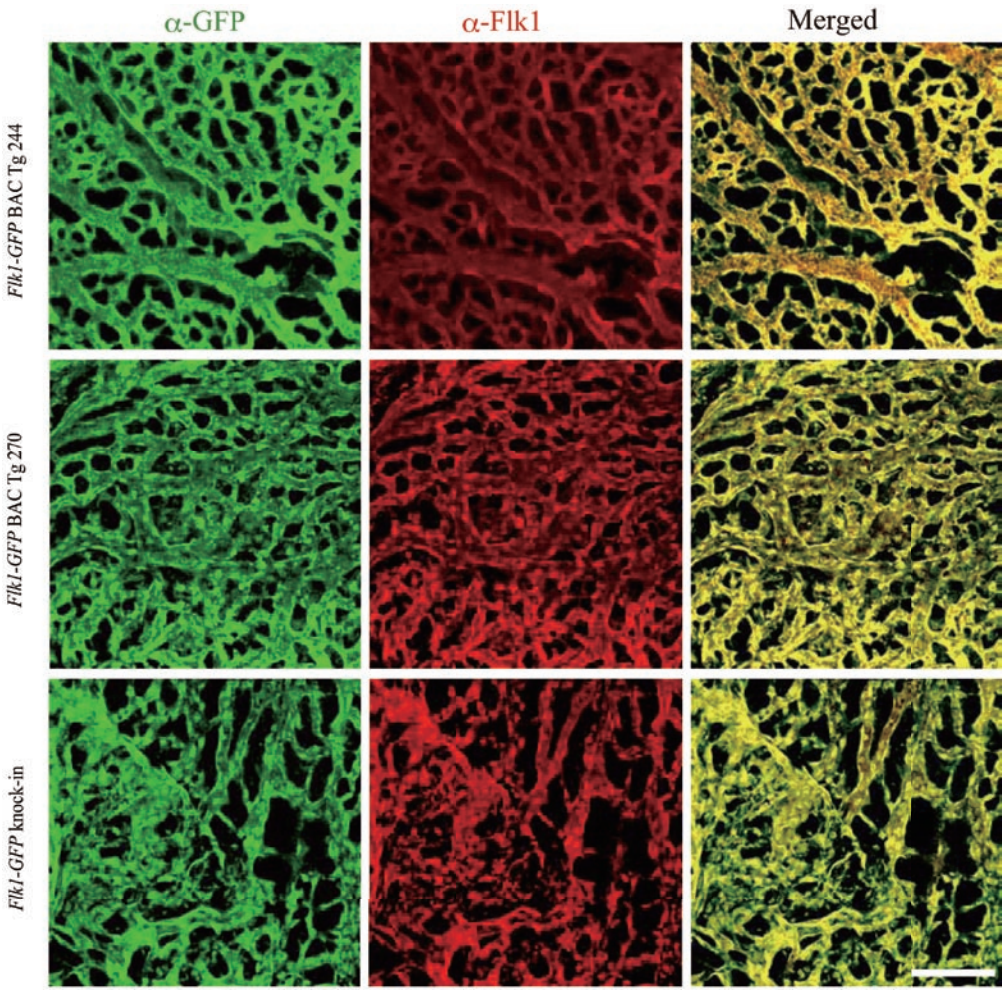

Fig. 4. Overlapping distribution of expressions of GFP and endogenous FLK1 protein in the yolk sac of *Flk1-GFP* BAC Tg embryos at 9.5 dpc. Confocal microscopy was used to observe GFP and FLK1 expression.

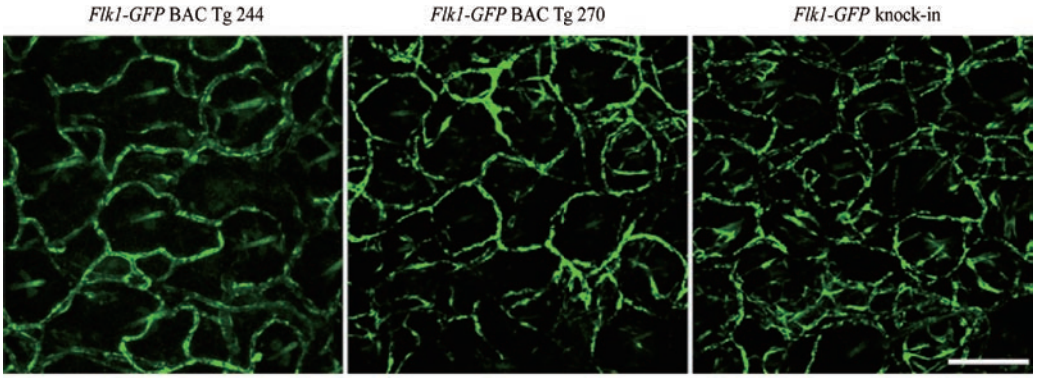

Fig. 5. Expression of GFP in the skin of adult *Flk1-GFP* BAC Tg mice. Note that two independent *Flk1-GFP* BAC Tg mice showed comparable GFP expression with that of *Flk1-GFP* knock-in mice.

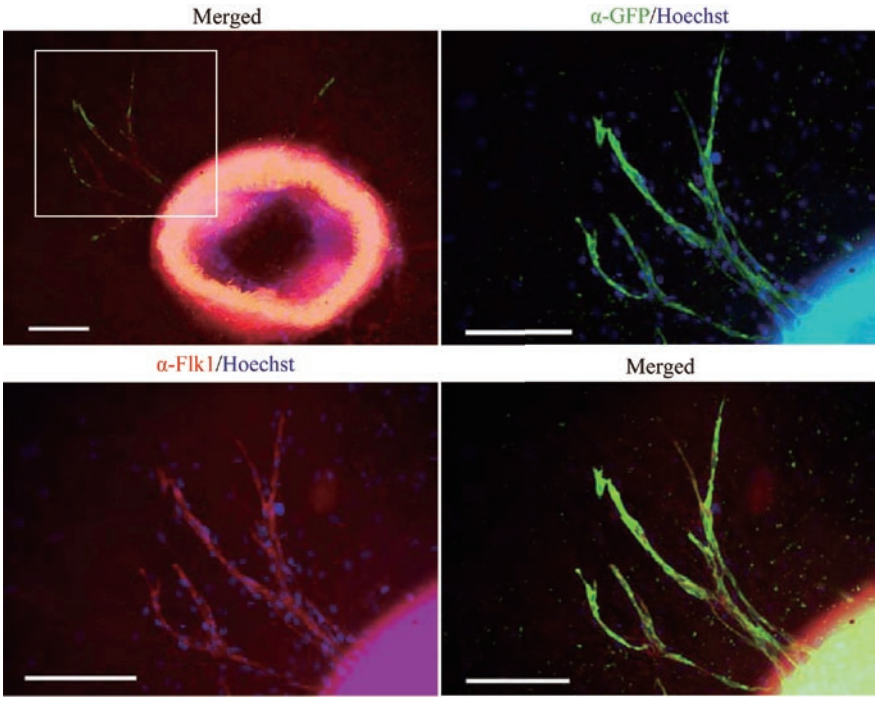

Fig. 6. GFP expression in aortic rings from *Flk1-GFP* BAC Tg (line 244) mice. Aortic rings were dissected from *Flk1-GFP* BAC Tg mice and were cultured in the presence of VEGF-A for 4 days. Endothelial sprouts were fixed and immunostained with anti-GFP antibody.

are advantages and disadvantages in the use of these strains. *Flk1-GFP* knock-in mice were generated by introducing a *GFP* cDNA into the first exon of the *Flk1* gene [3]. While endogenous *Flk1* expression was faithfully recapitulated in this strain, one of the two *Flk1* alleles was destroyed, raising a concern that haplo-insufficiency of the *Flk1* gene may create a phenotype [3]. On the other hand, another strain of *Flk1-lacZ* transgenic mice, carrying the promoter and first intron enhancer of the *Flk1* gene, also recapitulated endothelial expression of the FLK1 gene. Although its expression is relatively moderate and mosaic, raising a concern that all the FLK1-positive cells are not necessarily labeled with LacZ [7, M. E. unpublished]. Given that uniform and strong GFP expression was detected and that both *Flk1* alleles are intact in the *Flk1-GFP* BAC transgenic animals generated in this study, they should prove to be a useful animal model.

Angiogenesis during development is a complex process. The early blood vessels of the embryo and yolk sac in mammals develop by aggregation of angioblasts into a primitive vascular plexus, then undergo a complex remodeling process, whereby growth, migration, sprouting and pruning lead to the development of a functional circulatory system [5]. Tumor angiogenesis is also a complex process. A specialized subtype of endothelial cell known as the tip cell is believed to be involved in angiogenesis, but the molecular signaling pathways utilized by tip cells remain largely uncharacterized [6]. Since *Flk1-GFP* BAC transgenic animals can recapitulate strong and uniform GFP expressions in endothelial cells, they will be useful for noninvasively visualizing endothelial cells in the process of growth, migration, sprouting and pruning, and tip cell formation, at single cell resolution, during development and in *in vitro* angiogenesis models.

In conclusion, the *Flk1-GFP* BAC transgenic animals generated in this study should be useful in the study of molecular and cellular mechanisms of vascular development and pathological angiogenesis, and may provide opportunities to develop new anti-angiogenic agents.

Acknowledgments

We appreciate the technical assistance of Ms. Yuko Hamada the construction and pronuclear injection of *Flk1-GFP* BAC transgene. We also thank Dr. Yoshihiro Miwa for access to his microscope. We are also grateful to all members of the Takahashi Laboratory. This work was supported in part by Grant-in-Aid for Scientific Research (C, 20590273) (M. E.).

References

1. Downs, K.M. and Davies, T. 1993. Staging of gastrulating mouse embryos by morphological landmarks in the dissecting microscope. *Development* 118: 1255–1266.
2. Ema, M., Faloon, P., Zhang, W.J., Hirashima, M., Reid, T., Stanford, W.L., Orkin, S., Choi, K., and Rossant, J. 2003. Combinatorial effects of Flk1 and Tal1 on vascular and hematopoietic development in the mouse. *Genes Dev.* 17: 380–393.
3. Ema, M., Takahashi, S., and Rossant, J. 2006. Deletion of the selection cassette, but not cis-acting elements, in taegeted Flk1-lacZ allele reveals Flk1 expression in multipotent mesodermal projenitors. *Blood* 107: 111–117.
4. Ema, M., Yokomizo, T., Wakamatsu, A., Terunuma, T., Yamamoto, M., and Takahashi, S. 2006. Primitive erythropoiesis from mesodermal precursors expressing VE-cadherin, PECAM-1, Tie2, endoglin, and CD34 in the mouse embryo. *Blood* 108: 4018–4024.
5. Ferrara, N. 2004. Vascular endothelial growth factor: basic science and clinical progress. *Endocr. Rev.* 25: 581–611.
6. Gerhardt, H., Golding, M., Fruttiger, M., Ruhrberg, C., Lundkvist, A., Abramsson, A., Jeltsch, M., Mitchell, C., Alitalo, K., Shima, D., and Betsholtz, C. 2003. VEGF guides angiogenic sprouting utilizing endothelial tip cell filopodia. *J. Cell Biol.* 161: 1163–1177.
7. Kappel, A., Rönicke, V., Damert, A., Flamme, I., Risau, W., and Breier, G. 1999. Identification of vascular endothelial growth factor (VEGF) receptor-2 (Flk-1) promoter/enhancer sequences sufficient for angioblast and endothelial cell-specific transcription in transgenic mice. *Blood* 93: 4284–4292.
8. Kappel, A., Schlaeger, T.M., Flamme, I., Orkin, S.H., Risau, W., and Breier, G. 2000. Role of SCL/Tal-1, GATA, and Ets transcription factor binding sites for the regulation of Flk-1 expression during murine vascular development. *Blood* 96: 3078–3085.
9. Kattman, S.J., Huber, T.L., and Keller, G.M. 2006. Multipotent flk-1+ cardiovascular progenitor cells give rise to the cardiomyocyte, endothelial, and vascular smooth muscle lineages. *Dev. Cell* 11: 723–732.
10. Kojima, T., Hirota, Y., Ema, M., Takahashi, S., Miyoshi, I., Okano, H., and Sawamoto, K. 2010. Subventricular zone-derived neural progenitor cells migrate along a blood vessel scaffold toward the post-stroke striatum. *Stem Cells* 28: 545–554.
11. Larina, I.V., Shen, W., Kelly, O.G., Hadjantonakis, A.K., Baron, M.H., and Dickinson, M.E. 2009. A membrane associated mCherry fluorescent reporter line for studying vascular remodeling and cardiac function during murine embryonic development. *Anat. Rec. (Hoboken)* 292: 333–341.
12. Moretti, A., Caron, L., Nakano, A., Lam, J.T., Bernshausen, A., Chen, Y., Qyang, Y., Bu, L., Sasaki, M., Martin-Puig, S., Sun, Y., Evans, S.M., Laugwitz, K.L., and Chien, K.R. 2006. Multipotent embryonic isl1+ progenitor cells lead to cardiac, smooth muscle, and endothelial cell diversification. *Cell* 127: 1151–1165.
13. Motoike, T., Markham, D.W., Rossant, J., and Sato, T.N. 2003, Evidence for novel fate of Flk1+ progenitor: contribution to muscle lineage. *Genesis* 35: 153–159.
14. Poché, R.A., Larina, I.V., Scott, M.L., Saik, J.E., West, J.L., and Dickinson, M.E. 2009. The Flk1-myr::mCherry mouse as a useful reporter to characterize multiple aspects of ocular blood vessel development and disease. *Dev. Dyn.* 238: 2318–2326.
15. Shalaby, F., Rossant, J., Yamaguchi, T.P., Gertsenstein, M., Wu, X.F., Breitman, M.L., and Schuh, A.C. 1995. Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. *Nature* 376: 62–66.
16. Wu, S.M., Fujiwara, Y., Cibulsky, S.M., Clapham, D.E., Lien, C.L., Schultheiss, T.M., and Orkin, S.H. 2006. Developmental origin of a bipotential myocardial and smooth muscle cell precursor in the mammalian heart. *Cell* 127: 1137–1150.
17. Yamaguchi, T.P., Dumont, D.J., Conlon, R.A., Breitman, M.L., and Rossant, J. 1993. flk-1, an flt-related receptor tyrosine kinase is an early marker for endothelial cell precursors. *Development* 118: 489–498.
18. Yamashita, J., Itoh, H., Hirashima, M., Ogawa, M., Nishikawa, S., Yurugi, T., Naito, M., Nakao, K., and Nishikawa, S. 2000. Flk1-positive cells derived from embryonic stem cells serve as vascular progenitors. *Nature* 408: 92–96.
19. Yang, L., Soonpaa, M.H., Adler, E.D., Roepke, T.K., Kattman, S.J., Kennedy, M., Henckaerts, E., Bonham, K., Abbott, G.W., Linden, R.M., Field, L.J., and Keller, G.M. 2008. Human cardiovascular progenitor cells develop from a KDR+ embryonic-stem-cell-derived population. *Nature* 453: 524–528.