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Using a Histone Yellow Fluorescent Protein Fusion for Tagging and Tracking Endothelial Cells in ES Cells and Mice

Stuart T. Fraser¹, Anna-Katerina Hadjantonakis², Kenneth E. Sahr¹, Stephen Willey¹, Olivia G. Kelly^{3,4}, Elizabeth A.V. Jones⁴, Mary E. Dickinson^{3,4}, and Margaret H. Baron^{1,5,6,7,*}

¹Department of Medicine, Mount Sinai School of Medicine, New York, New York

²Developmental Biology Program, Memorial Sloan-Kettering Cancer Center, New York, New York

³Department of Molecular Physiology and Biophysics, Baylor College of Medicine, Houston, Texas

⁴Biological Imaging Center, California Institute of Technology, Pasadena, California

⁵Department of Molecular, Cellular and Developmental Biology, Mount Sinai School of Medicine, New York, New York

⁶Department of Oncological Sciences, Mount Sinai School of Medicine, New York, New York

⁷Department of Cell and Gene Medicine, Mount Sinai School of Medicine, New York, New York

Summary

We report the first endothelial lineage-specific transgenic mouse allowing live imaging at subcellular resolution. We generated an H2B-EYFP fusion protein which can be used for fluorescent labeling of nucleosomes and used it to specifically label endothelial cells in mice and in differentiating embryonic stem (ES) cells. A fusion cDNA encoding a human histone H2B tagged at its C-terminus with enhanced yellow fluorescent protein (EYFP) was expressed under the control of an Flk1 promoter and intronic enhancer. The Flk1::H2B-EYFP transgenic mice are viable and high levels of chromatin-localized reporter expression are maintained in endothelial cells of developing embryos and in adult animals upon breeding. The onset of fluorescence in differentiating ES cells and in embryos corresponds with the beginning of endothelial cell specification. These transgenic lines permit real-time imaging in normal and pathological vasculogenesis and angiogenesis to track individual cells and mitotic events at a level of detail that is unprecedented in the mouse.

Keywords

Flk1::H2B-EYFP; endothelial cells; fluorescent protein; EYFP; vasculogenesis; angiogenesis; imaging; transgenic mice; histone fusion

INTRODUCTION

Vascular development is a highly orchestrated series of events that initiates with the commitment and differentiation of mesodermal progenitors to the endothelial lineage. Endothelial proliferation, migration, and reorganization results in the formation of a primitive

* Correspondence to: Margaret H. Baron, Mount Sinai School of Medicine, Box 1079, Departments of Medicine and Molecular, Cell & Developmental Biology, 1425 Madison Ave. 11-70B, New York, NY 10029. E-mail: margaret.baron@mssm.edu. The first two authors contributed equally to this work.

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vascular network that undergoes remodeling and morphogenesis to mature vessels (Hirschi *et al.*, 2002; Yancopoulos *et al.*, 2000). Blood vessels form by two processes: vasculogenesis and angiogenesis. Vasculogenesis involves the recruitment of mesodermal cells (angioblasts) to the endothelial lineage and their assembly *de novo* into blood vessels. Angiogenesis is the generation of new blood vessels from the endothelial cells of existing blood vessels. Although long thought to be restricted to embryonic development, vasculogenesis has more recently been appreciated as one aspect of normal physiology and as a contributing factor in human disease. For example, neovascularization is a critical early event in the development of cancers (for reviews, see Folkman *et al.*, 2001; Tang and Conti, 2004).

Formation of correctly patterned vascular structures is in part genetically programmed but also requires environmental cues that organize the assembly of angioblasts/endothelial cells at specific locations within the embryo (Bautch and Ambler, 2004; Flamme *et al.*, 1997; Fouquet *et al.*, 1997; Yancopoulos *et al.*, 2000). Maturation of embryonic blood vessels may also be hemodynamically determined (Isogai *et al.*, 2003; Jones *et al.*, 2004). Therefore, to understand in detail the dynamic events leading to vascular development in the mouse, it will be essential to image individual endothelial cells in live embryos. Transgenic mouse lines carrying *lacZ* reporters have been used by a number of investigators to examine blood vessel formation (e.g., see Kappel *et al.*, 1999; Ronicke *et al.*, 1996; Schlaeger *et al.*, 1997). However, because identification of cells expressing the *lacZ* transgene requires tissue fixation, this analysis provides only a snapshot of a given developmental stage. Advances in microscopic imaging methods and improvements in genetically encoded fluorescent proteins have led to the development of powerful tools for studying cell behavior in live embryos. For example, endothelial-specific green fluorescent protein (GFP) reporters have proven useful for noninvasive observation of vascular development in zebrafish (Isogai *et al.*, 2003; Lawson and Weinstein, 2002; Motoike *et al.*, 2000). The uniform expression of GFP within the endothelial cells of these animals limits their utility in following cell movements: the position of a given cell can be tracked in three dimensions (3D) over time (i.e., in 4D) only if it is present among a group of nonexpressing cells, as in a mosaic or mixed-lineage population (e.g., see Anderson *et al.*, 2000; Bak and Fraser, 2003; Hadjantonakis *et al.*, 2001; Srinivas *et al.*, 2004; Tam and Rossant, 2003). Ultimately, it will be desirable to construct anatomical models for analysis of normal, mutant, and pathological vascular development. This goal can be achieved if each cell can be marked with a single, easily identifiable tag that is visible at subcellular resolution (Hadjantonakis and Papaioannou, 2004; Hadjantonakis *et al.*, 2003; Koster and Fraser, 2001; Megason and Fraser, 2003; Plusa *et al.*, 2005). The large size and uniform shape of the nucleus in most cell types makes it ideal for such high-resolution imaging, permitting the tracking of cell division and movement (Hadjantonakis and Papaioannou, 2004).

To develop a noninvasive marker that could be used to track individual endothelial cells, we took advantage of a histone H2B fusion protein that labels DNA during all phases of the cell cycle while preserving cell morphology and behavior (discussed in Hadjantonakis and Papaioannou, 2004). Fluorescent tagged histone fusions can incorporate into chromatin without any adverse effects on cell viability in culture (Kanda *et al.*, 1998). By comparison with reporters containing nuclear localization sequences (nls), histone fusions generate a superior signal-to-noise ratio. They also have the distinct advantage that the signal remains bound to the target even during cell division, when the nuclear envelope has broken down. In contrast, nls-tagged markers (both GFP- and *lacZ*-based) and native fluorescent proteins become dispersed throughout the cell during mitosis, so that tracking of that cell becomes much more difficult (discussed in more detail by Hadjantonakis and Papaioannou, 2004). GFP fusions to histones have been used to label nuclei in live transgenic or retrovirally transduced zebrafish, flies, and quail (Das *et al.*, 2003; Gong *et al.*, 2004; Koster and Fraser, 2001; LaRue *et al.*, 2003; Savoian and Rieder, 2002). Recently, we described a constitutively expressed fusion cDNA encoding a human histone H2B tagged with a C-terminal GFP that permits

imaging and cell tracking at nuclear resolution in vivo in mouse embryos (Hadjantonakis and Papaioannou, 2004). In this study, we generated and evaluated a human histone H2B fusion tagged with a spectrally distinct enhanced yellow fluorescent protein (EYFP) that is expressed under the control of an *Flk1* (also known as vascular endothelial growth factor (VEGF) receptor-2 or *Kdr*) promoter and intronic enhancer. Flk1 is a receptor tyrosine kinase and functions as the main signaling receptor for VEGF during embryogenesis and neovascularization in adult tissues (Millauer *et al.*, 1993, 1994; Yamaguchi *et al.*, 1993; Yancopoulos *et al.*, 2000). It is the earliest endothelial receptor known to be expressed in nascent mesoderm (Kabrun *et al.*, 1997; Kataoka *et al.*, 1997; Yamaguchi *et al.*, 1993). Homozygous Flk1::H2B-EYFP transgenic mice are viable and fertile and exhibit chromatin-localized reporter expression in endothelial cells. As this reporter permits visualization of individual endothelial cells within a population, it also facilitates the tracking of cell position over time. Thus, it can be applied to multidimensional studies of cell behavior and cell fate in vivo. The fluorescent H2B-EYFP fusion protein binds to nucleosomes and uniformly labels endothelial cell nuclei in differentiating embryonic stem (ES) cells, mouse embryos, and adult tissues. The H2B-EYFP fusion has the important advantage that it withstands fixation while still retaining both fluorescence and nuclear localization. These Flk1::H2B-EYFP mice and ES cells are the first endothelial lineage-specific transgenic lines for imaging of endothelial cells at subcellular resolution and they provide powerful new tools for studying vascular development. They also provide a foundation for the future evaluation of the effects of pharmacologic agents or genetic mutations on vasculogenesis and angiogenesis.

RESULTS

Generation of an Flk1::H2B-EYFP Transgene to Label Nuclei

A pCX::H2B-EYFP vector was constructed from pCX::H2B-EGFP (Hadjantonakis and Papaioannou, 2004) by replacing the EGFP fragment with EYFP (ClonTech, Palo Alto, CA) and was used to test the function of the H2B-EYFP fusion in ES and COS cells (not shown). The H2B-EYFP fusion displayed strong chromatin-localized fluorescence throughout the culture period, without perturbing cell morphology, proliferation rate, or mitotic index, as compared to nontransfected controls, and mitotic figures were easily identified (see below). Having established the subcellular localization and neutrality of the H2B-EYFP fusion in vitro, we isolated *Flk1* BAC clones and generated a construct (Fig. 1) containing previously characterized *Flk1* promoter and intronic enhancer regions (Kappel *et al.*, 1999; Ronicke *et al.*, 1996). These sequences were demonstrated to drive expression of a linked reporter in endothelial cells, using immunostaining (Kappel *et al.*, 1999), fluorescence activated cell sorting (FACS), or uptake of DiI-labeled acetylated low-density lipoprotein (DiI-Ac-LDL) (Hirai *et al.*, 2003).

Expression of the Nuclear Endothelial Reporter During Differentiation of ES Cells

The Flk1::H2B-EYFP construct was introduced into ES cells by electroporation. Clones were isolated and tested for transgene expression after 3 days of differentiation as embryoid bodies (EBs). Dispersed cells from EBs were analyzed by FACS; the highest-expressing clone (4C5) was selected for the analysis shown in Figure 2. In all ES lines tested the transgene was expressed in undifferentiated ES cells cultured with LIF. Similar fluorescence was observed in ES cells expressing native GFP under the control of this Flk1 promoter/enhancer cassette (Hirai *et al.*, 2003). The ectopic expression in undifferentiated ES cells may reflect the absence of negative regulatory element(s) in the transgene. When ES cells were induced to differentiate in the absence of LIF, EYFP fluorescence was downregulated but was still easily detected through day 6 (Fig. 2). In contrast, in Flk1::GFP ES cells, native GFP fluorescence decreased rapidly to <5% at day 2 and was undetectable by day 3 (Hirai *et al.*, 2003). While these differences could be explained in part by the culture conditions used or perhaps to position

effects at the chromosomal site of integration, we propose that the nuclear EYFP reporter may offer greater sensitivity because of its high signal-to-noise ratio and its persistence through all phases of the cell cycle (see below). Thus, the strong nuclear expression enabled us to follow proliferating cells for several days in culture.

During embryoid body differentiation, virtually all cells that stained with an anti-Flk antibody were also EYFP-positive, as expected (not shown). When the EYFP-positive population was analyzed simultaneously for expression of endogenous Flk1, c-kit, or CD31 (PECAM-1, expressed on angioblast/endothelial cells), significant populations of Flk1+c-kit+ and Flk1+CD31+ cells were detected on day 4 (25% and 30%, respectively) and increased by day 6 in culture (88% and 55%, respectively, Fig. 2). These observations confirm that the EYFP-positive cells display surface properties of angioblastic/endothelial cells.

Expression of the H2B-EYFP Fusion Protein During Embryonic Vascular Development

From seven Flk1::H2B-EYFP transgenic mouse lines derived (see Materials and Methods), two were chosen for further analysis. The expression of the H2B-EYFP fusion was assessed in staged embryos ranging from late gastrulation/early headfold (~E7.5) to E17.5; the two transgenic lines gave indistinguishable patterns of expression. As anticipated from previous reports (Hirai *et al.*, 2003; Kappel *et al.*, 1999), the combination of *Flk1* regulatory elements used for the Flk1::H2B-EYFP construct (Fig. 1) was not active in the extraembryonic mesoderm (yolk sac) prior to endothelial lineage specification. Transgene fluorescence was not detected in whole embryos prior to the appearance of the first somites, but was observed in the yolk sac from the 2-somite stage onward and, by the 5-somite stage, in the heart and dorsal aortae (bilateral primordia of the future aorta) as well (Fig. 3). Fluorescent endothelial nuclei were found throughout the primitive vascular network of the distal yolk sac by E8.25. At later stages of yolk sac morphogenesis, major vessels as well as capillaries expressed the H2B-EYFP fusion protein (Fig. 4a–e). However, by late gestation large EYFP-negative vessels were detected within the yolk sac adjacent to brightly fluorescing large vessels (not shown), suggesting that downregulation of the transgene begins before birth. In embryos with intact extraembryonic membranes, counterstaining of the visceral endoderm revealed that fluorescent nuclei were confined to the mesodermal compartment of the yolk sac (Fig. 4f). Dividing EYFP-positive cells could be identified upon dynamic imaging of live embryos, as demonstrated in the time-lapse sequence shown in Figure 4g.

As seen in Figure 5, the endothelial cells of vessels in the head and upper trunk (shown for E9.5 embryos, Fig. 5b,d,e) as well as vessels in more caudal regions of the embryo (Fig. 5f–h) expressed the H2B-EYFP fusion protein. Although quantitative studies have yet to be conducted, it may be noteworthy that the vessels of the head initially appeared to exhibit a higher level of fluorescence intensity than more caudal vessels. At later stages, expression of the H2B-EYFP fusion protein was more uniform throughout the vasculature. The definitive endoderm (E8.25) contained no detectable fluorescent signal. Vascular structures outlined by EYFP-positive nuclei were easily seen in developing organs (Fig. 6). Fluorescent nuclei were identified in the vasculature of a variety of organs, including the heart and lungs, skin, ovary, kidney and adrenal gland, stomach and pancreas (Fig. 6), and dorsal aortae.

Expression of the H2B-EYFP fusion was restricted to the vascular endothelium at all stages examined. Endothelial-specific expression was confirmed by immunostaining of cryosections with anti-PECAM-1 (for this figure, anti-GFP, which cross-reacts with EYFP, was used but was found to be unnecessary, as strong fluorescence withstood fixation). The EYFP and PECAM-1 signals overlapped in structures with vascular morphology (shown for different tissues from E15.5 embryos in Fig. 7d,h,i). Erythroid cells were detected within these structures, as demonstrated by immunostaining with anti-Ter119 (Fig. 7m,n,p) or anti-CD71 (transferrin receptor, not shown).

Adult Expression of the Transgene

Fluorescent nuclei were not detected in the endothelium of the major blood vessels in the adult tissues examined, as expected from a previous study in which the same promoter and enhancer regions were used (Kappel *et al.*, 1999). However, bright endothelial expression was observed throughout the smaller vessels and capillaries of most adult tissues, including the spleen, epicardium, renal capillaries and glomeruli, lung, internal capillaries of the brain, peritoneal membrane, and testis (examples of which are shown in Fig. 8).

DISCUSSION

Here we report the first lineage-specific transgenic ES cell and mouse lines that can be imaged at subcellular resolution. As observed for the ubiquitously expressed H2B-EGFP fusion (Hadjantonakis and Papaioannou, 2004), high levels of transgene expression are maintained upon breeding, indicating that the Flk1::H2B-EYFP reporter is developmentally neutral and does not interfere with mitosis or meiosis. This reporter permits direct visualization of active chromatin in situ in endothelial cells: using stereomicroscopic and confocal laser scanning confocal microscopy, we were able to identify and track endothelial cells expressing the H2B-EYFP reporter in real time, in interphase nuclei, as well as nuclei of cells undergoing mitosis or cell death (discussed in Hadjantonakis and Papaioannou, 2004). The H2B-EYFP reporter withstands fixation, allowing processing and long-term storage of samples.

The bright signal obtained using the Flk1::H2B-EYFP nuclear reporter, even in the hemizygous state, offers a particular advantage for the direct isolation of EYFP-positive endothelial cells in a single step, using fluorescence-activated cell sorting (FACS) (Hadjantonakis and Nagy, 2000). FACS-sorted Flk1::H2B-EYFP cells could be used for transplantation experiments. The evaluation of engrafted tissues for cell fusion events (Terada *et al.*, 2002; Ying *et al.*, 2002) would be simplified because donor and recipient nuclei could be easily distinguished. Vascular endothelial cells display structural, functional, and molecular heterogeneity (discussed by Caprioli *et al.*, 2004). The ability to isolate endothelial cells from specific vascular beds of Flk1::H2B-EYFP animals for gene expression profiling may open up new areas of exploration in vascular biology.

In embryos at various stages of development, uniform nuclear expression was detected in the endothelial cells of the aorta, small vascular structures, and in the microvasculature. The *Flk1* regulatory elements included in this transgene are active largely in developing endothelial cells, including those proliferating within the vasculature of tumors (Heidenreich *et al.*, 2000; Licht *et al.*, 2004). We anticipate that the Flk1::H2B-EYFP mouse will permit analysis of vascular defects associated with genetic mutations at a level of detail that has not previously been possible in the mouse, as it can be crossed to existing mutant lines and may be useful for screening of mutations affecting vascular development. For the first time, it will be possible to address fundamental questions such as the extent to which vascular remodeling occurs through the birth, rearrangement, death, or orientated division of endothelial cells. These lines should facilitate the identification in adult tissues of areas of abnormal vascularization. Therefore, they may constitute a useful preclinical model for testing angiogenesis inhibitors for treatment of cancers, macular degeneration, diabetic retinopathy, and chronic inflammatory diseases involving neovascularization. In combination with intravital microscopy, which can provide high resolution in three dimensions in deeper tissues of the live animal (Brown *et al.*, 2001; Jain *et al.*, 2002), the Flk1::H2B-EYFP mouse could provide a readout in real time in a number of preclinical models of human cancer. These transgenic mice will, therefore, create new possibilities for the study of normal and aberrant vascular development from embryo to adult and in models for human disease.

MATERIALS AND METHODS

Isolation of Flk1 Regulatory Sequences

Two Flk1 BAC clones were isolated by screening a murine 129/SvJ library (Incyte Genomics, Wilmington, DE) using PCR. An ~0.9 kb Flk1 fragment containing 623 base pairs (bp) of upstream regulatory sequence and the 290 bp 5' UTR (see fig. 2 of Ronicke *et al.*, 1996) and a 511 bp intron 1 enhancer region (Accession no. AF061804 and fig. 3 of Kappel *et al.*, 1999) were PCR-amplified from Flk1 BAC1, subcloned into pCR2.1 (Invitrogen, Carlsbad, CA), and sequenced. The promoter and intron 1 enhancer (enh) regions were then subcloned into pBluescript SK(-) (Stratagene, La Jolla, CA) as KpnI/EcoRI and EcoRI/BamHI fragments, respectively, to produce a subclone, pFlk1enh, containing the *Flk1* promoter and enhancer regions separated by an EcoRI site. Primer sequences are available upon request.

Generation of H2B-EYFP Fusion Gene

The coding sequence for the human histone H2B gene (X57127) was amplified from genomic DNA by PCR using *Pfx* Polymerase (Invitrogen) and cloned into pCR4 TOPO (Invitrogen) to generate pH2B. The H2B fragment from pH2B was then cloned into plasmid pEYFP-N1 (BD Biosciences, San Jose, CA) to generate pH2B-EYFP. The resulting fusion was reamplified by PCR and cloned into the XhoI site of pCAGGS (Hadjantonakis and Papaioannou, 2004) to generate pCX::H2B-EYFP. This vector was used to test function of the H2B-EYFP fusion following transient transfection of R1 ES and Cos-7 cells using Eugene6 Transfection Reagent as per the manufacturer's recommendations (Roche Diagnostics, Basel, Switzerland). The H2B-EYFP fragment plus an SV40 poly(A) sequence was then reamplified by PCR and cloned into the EcoRI site of pFlk1enh (see above) to yield pFlk1-H2B-EYFP. All primer sequences are available upon request.

Generation of Flk1::H2B-EYFP Transgenic Mice

The entire insert from pFlk1::H2B-EYFP, containing Flk1 regulatory regions and the Flk1::H2B-EYFP-pA fragment, was excised from pFlk1enh by digestion with SalI and XbaI and gel-purified for pronuclear injection into C57BL/6 × C3H (B6C3) F1 hybrid embryos, using standard methods (Nagy *et al.*, 2003). Injections were performed by the Mount Sinai Mouse Genetics Shared Resource Facility. Several transgenic founder animals were obtained, genotyped by PCR, and screened for EYFP expression based on detection of a fluorescent signal from an ear-punch biopsy using a stereo dissecting microscope. The two lines showing highest levels of expression were chosen for further analysis. They were maintained as hemizygotes on a C57BL/6 background. For embryo studies, transgenic males were crossed with ICR outbred or C57BL/6 females.

ES Cell Transfection, Differentiation, and Flow Cytometry

pFlk1::H2B-EYFP was linearized by digestion with NotI and co-electroporated with pSV2Neo into E14 ES cells using a Gene Pulser apparatus (Bio-Rad Laboratories, Hercules, CA; 125 µF, 400 V). Following selection in medium containing G418, drug-resistant clones were isolated. To assess expression of the EYFP reporter, ES cells were allowed to differentiate into embryoid bodies (EBs) as described previously (Kennedy and Keller, 2003). At different times in culture (see legend to Fig. 3), EBs were dispersed using 0.05% trypsin/EDTA (Gibco Invitrogen, Grand Island, NY). Single-cell suspensions were incubated with biotinylated anti-Flk1 (subclone M218) and phycoerythrin (PE)-conjugated anti-CD31 (clone MEC 13.3; BD-Pharmingen, San Diego, CA) or PE-conjugated anti-c-kit (clone 2B8; BD-Pharmingen) antibodies. Flk1 monoclonal antibody was affinity-purified using Protein G-Sepharose and biotinylated using the Fluoreporter Biotin-XX Protein Labeling Kit (Molecular Probes, Eugene, OR; Cat. no. F2610). Flk1 expression was detected using allophycocyanin (APC)-

conjugated streptavidin (Pharmingen). Dead cells were excluded according to propidium iodide uptake. Flow cytometry was performed using a FACScalibur instrument (Becton Dickinson, San Jose, CA) in the Mount Sinai Flow Cytometry Core Facility.

Dissection and Preparation of Embryos and Adult Tissues

Embryos (0.5 dpc on the day of plug) and organs were dissected in either phosphate-buffered saline (PBS) or N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffered Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum, then cultured for live imaging as described previously (Jones *et al.*, 2002; Hadjantonakis and Papaioannou, 2004) or fixed for further processing prior to imaging. For time-lapse imaging, samples were cultured in a standard tissue culture incubator or on a microscope stage under conditions promoting the culture of mouse embryos (50% rat serum: 50% DMEM buffered with bicarbonate as described by Jones *et al.*, 2002) and maintained under physiological conditions in a closed temperature-controlled, humidified, and oxygenated (95% air, 5% CO₂) chamber (Solent Sci, Portsmouth, UK, or custom made). For cytoplasmic counterstaining, samples were incubated for 10 min in Cell Tracker Orange (Molecular Probes, C-2927) at a dilution of 1:500 in dissecting or culture medium. Nuclei were stained with Draq5 (Alexis, Ames, IA) according to the manufacturer's instructions. Embryos were kept in a tissue culture incubator at 37°C or at room temperature during staining. Samples were then washed twice in dissecting or culture medium prior to imaging. Whole embryos were kept in PBS in MatTek (Ashland, MA) glass-bottom dishes (Cat. no. P35g-1.5-14C) during imaging. For further processing, specimens were fixed in paraformaldehyde (PFA, 4% in PBS) at 4°C for 4 h (embryos) or 6–8 h (adult tissues). They were then washed in PBS and stored in PBS at 4°C. Samples were then imaged directly or prepared for vibratome or cryosectioning as follows. Following fixation the samples were kept in 4% sucrose/PBS (w/v) at 4°C overnight. The following day the concentration of sucrose was increased to 20% (in PBS) and the samples kept again overnight at 4°C. They were then transferred to a 1:1 (v/v) solution of sucrose (30%):vibratome embedding solution (prepared by combining 450 ml PBS, 2.2 g gelatin, 70 g bovine serum albumin, 90g sucrose) and equilibrated overnight at 4°C, then embedded in a mixture of vibratome embedding solution (3 mL) and glutaraldehyde (300 µL of a 25% stock). Vibratome sections (200 µm for embryos, 100–150 µm for adult tissues) were stored immersed in PBS for up to several days at 4°C. For cryosectioning, embryos were fixed in 4% PFA in PBS for 6 h, washed with PBS, and equilibrated in 20% sucrose in PBS overnight at 4°C. They were then transferred to Tissue-Tek OCT embedding medium (Sakura Finetek, Torrance, CA) for 3 h, placed into embedding molds, and snap-frozen in liquid nitrogen. Sections (20 µm) were cut using a Leica CM3050 cryostat, allowed to adhere to Fisherbrand Superfrost/Plus glass slides, and air-dried overnight. For immunostaining, sections were fixed for 10 min in 4% PFA in PBS, washed three times in PBS, and incubated with 5% normal goat serum for 20 min at room temperature. Next, the sections were incubated with primary antibody for 2 h at room temperature, washed three times in PBS, and incubated with secondary antibody for 1 h at room temperature. Following three final washes in PBS the sections were mounted in Vectashield Mounting Medium with DAPI (Cat. no. H-1200, Vector Labs, Burlingame, CA). The primary antibodies used were: rabbit anti-jellyfish GFP (Molecular Probes, 1:500 dilution); rat antimouse PECAM-1 (clone MEC 13.3), or rat antimouse Ter119 monoclonal antisera, both purchased from BD-Pharmingen (dilutions of 1:250 and 1:400, respectively). Secondary antibodies were used at a dilution of 1:500 and were: fluorescein isothiocyanate (FITC)-conjugated antirabbit IgG (BD-Pharmingen) and Alexa Fluor 568-conjugated goat antirat Ig (H+L) (Molecular Probes).

Imaging

Stereomicroscopic images were acquired in AxioVision (Carl Zeiss Microsystems, Thornwood, NY) using an AxioCam MRC camera mounted on a Leica MZFLIII or MZ16FA

stereodissecting microscope equipped with epifluorescent illumination and appropriate filter sets (Chroma Technology, Rockingham, VT). Laser scanning confocal data were acquired using a Zeiss LSM510 META mounted on an Axiovert 200M or using an LSM 5 PASCAL mounted on an Axiovert 100. Objective lenses used include a Zeiss Achroplan 63x/NA1.4, Zeiss C-Apochromat 40x/NA1.2, Zeiss Plan-Apochromat 20x/0.75NA and a Zeiss Fluar 5x/0.25NA. EGFP/EYFP was excited using a 488 nm argon laser (Lassos) at 488 nm; Cell Tracker Orange was excited using a 543 nm HeNe laser; and Draq 5 was excited using a 633 nm laser. Images were acquired as sequential optical x-y sections taken at 0.1–2 μ m z intervals. Raw data were processed using Zeiss AIM software (Carl Zeiss Microsystems). Time-lapse movies and movies of 3D projections from z-stacks were assembled in QuickTime Player (Apple Computer). The EYFP signal in the confocal images was pseudocolored yellow, while Draq5 and Cell Tracker Orange stains were pseudocolored purple or blue, respectively. Stereomicroscopic images were acquired on a color CCD camera and are depicted in green, the color observed with the epi-fluorescence filter cube used for acquiring the data.

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FIG. 1.

Construction of a *Flk1::H2B-EYFP* transgene. **a:** Structure of BAC clone containing *Flk1* locus. Positions of 5' upstream regulatory region (UPR) and intronic enhancer (enh) are shown. The *Flk1* gene comprises 28 exons (Ex), of which the first two are indicated. Blue boxes represent the upstream regulatory region (UPR) and intronic enhancer (enh). **b:** Cartoon of *Flk1::H2B-EYFP* construct used for generation of transgenic ES cells and mice. The SV40 polyadenylation (pA) signal originated from pCX::H2B-EYFP (see Materials and Methods). The figure is drawn approximately to scale. In1; intron 1. B, BamHI; K, KpnI; N, NotI; P, PstI; R, EcoRI; S, SalI; X, XhoI; Xb, XbaI.

FIG. 2.

Expression of Flk1::H2B-EYFP transgene during ES cell differentiation. **a:** Expression of Flk1::H2B-EYFP during ES cell differentiation was examined by flow cytometry. The blue line in the histogram represents the Flk1::H2B-EYFP cells while the red line represents nontransgenic ES cells used as a control. The EYFP reporter was detected in undifferentiated ES cells. Strong expression of the H2B-EYFP fusion was maintained through day 2 and declined gradually over the next 4 days. **b,c:** The expression of hematopoietic/vascular markers on Flk1::H2B-EYFP⁺ cells was examined at day 4 (**b**) or day 6 (**c**). Cells from Flk1::H2B-EYFP EBs were stained with antibodies against Flk1, c-kit, and CD31 (see Materials and Methods). The EYFP-positive population was analyzed simultaneously for expression of endogenous Flk1, c-kit, or CD31 (PECAM-1). Significant populations of Flk1⁺c-kit⁺ and Flk1⁺CD31⁺ cells were detected on day 4 and increased by day 6 in culture, confirming that the EYFP-positive cells display surface properties of angioblastic/endothelial cells.

FIG. 3.

Expression of Flk1::H2B-EYFP in the early somite stage embryo. Anterior view of a 5-somite stage embryo enclosed in the yolk sac membranes. Strong nuclear yellow fluorescence is evident in the yolk sac (ys), developing heart crescent (ht), and paired dorsal aortae (da). al, allantois; am; amnion. Scale bar = 50 μ m.

FIG. 4.

Expression of Flk1::H2B-EYFP in the yolk sac. **a,b:** Widefield images of an E9.5 yolk sac. Note that both the large vessel and capillaries are surrounded by EYFP-positive nuclei. A higher magnification view of capillaries is shown in **b**. **c,d:** Confocal images of E9.5 yolk sac. In **d** a confocal image was superimposed on a brightfield image; capillary edges can be seen. **e,f:** An E8.5 embryo with its extraembryonic membranes left intact was stained for 2 min in Draq5 then rinsed in PBS. Due to the brief period of staining, the dye penetrated only the outer visceral endoderm but not the mesodermal layer of the yolk sac. In **e** the yolk sac is shown en face along the plane of imaging. The 3D projection of z-stack was rotated 90° (**f**) to emphasize the specificity of the EYFP expression. **g:** 3D Time-lapse sequence showing the nucleus of a dividing cell in the yolk sac. Arrows indicate dividing cell (t = 0) or daughter cells (t = 4, 8, 12 min). Scale bars = 20 μm.

FIG. 5.

Expression of the H2B-EYFP fusion protein in E9.5 Flk1::H2B-EYFP transgenic embryos. Darkfield (**a,c**) and fluorescent (**b,d**) images of the same embryo. **e**: Magnified view of midbrain vasculature of E10.5 embryo, stereomicroscopic fluorescent image. **f**: 3D projection of confocal z-stack is shown for E9.5 midbrain vasculature. **g,h**: Stereomicroscopic fluorescent images of caudal end of E9.5 embryo. Intersomitic vessels (isv) are easily identified. **i**: 3D projections of z-stacks from confocal images of somitic vessels. Scale bars = 100 μ m. uv, umbilical vessel (uv); hindlimb bud (lb).

FIG. 6.

Flk1::H2B-EYFP expression in organs from an E15.5 stage embryo. The fluorescent stereomicroscopic images are paired with darkfield (**a,b,k**) or brightfield (**g,i**) images. **a,b**: Heart (h) and lungs (l). **c**: Heart with high-magnification view (**d**) of epicardial vessels (boxed area in **c**). **e,f**: Forelimb. **g,h**: Ovary (ov) and mesonephros (mn). **i,j**: Kidney (k) and adrenal gland (ad). **k,l**: Stomach (st) and pancreas (p). ll, left lung; rl, right lung.

FIG. 7.

Immunostained sections from E15.5 Flk1::H2B-EYFP transgenic embryos. Expression of the H2B-EYFP fusion protein (**b,f,j,n**, detected here using an antibody against GFP; see Materials and Methods) overlapped with that of PECAM-1 (**a,e,i**; merged images, **d,h,l**), an endothelial marker. Erythroid cells (detected using anti-Ter119, **m**) filled spaces lined by H2B-EYFP-expressing endothelial cells (merged images, **p**). **a–d**: Section through gut; scale bar = 20 μ m. **e–h**: Section through forelimb; scale bar = 20 μ m. **i–l**: Section just below epidermis; scale bar = 10 μ m. **m–p**: Section through dorsal trunk; scale bar = 50 μ m.

FIG. 8.

Flk1::H2B-EYFP expression in adult tissues. **a–c:** Epifluorescence images of epicardial vessels of adult heart. Boxed area in **a** is shown magnified in **b**. The large vessels of the epicardium, most of which do not express the transgene, are easily seen (blue arrowheads). A larger fluorescent vessel is indicated by the white arrow. **c:** 3D projections of z-stacks from confocal images of peritoneal blood vessels. 3D projection of confocal z-stack of testis overlaid on bright field image. **(d).** Cytoplasm stained with Cell Tracker Orange. Interstitial capillary (ic); blood vessel (bv); seminiferous tubule (s). Scale bars = 100 μ m.