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# Lentiviral tracking of vascular differentiation in bone marrow progenitor cells

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ABSTRACT

Lentiviral vectors encoding for identifiable marker genes controlled by lineage-specific promoters can be used to track differentiation of bone marrow progenitors into endothelial cells and/or smooth muscle cells. Human VE-Cadherin and Smoothelin-B promoters were cloned into a self-inactivating lentiviral vector (HR-VECad and HR-SMTHB) and used to drive expression of green fluorescent protein (eGFP). These constructs demonstrated specific promoter activity in mature endothelial and smooth muscle cells respectively *in vitro*. Lin⁻ bone marrow progenitor cells (Lin⁻ BMCs) in culture were used to test vector ability to track vascular differentiation. HR-VECad transduced Lin⁻ BMCs were plated on collagen-coated slides and grown in endothelial media, while HR-SMTHB transduced Lin⁻ BMCs were cultured on fibronectin-coated slides and grown in smooth muscle media. For *in vivo* differentiation assessment, lentiviral transduced Lin⁻ BMCs resuspended in Matrigel were injected subcutaneously into C57BL/6J mice. Explants were evaluated for eGFP expression. Lin⁻ BMCs grown in endothelial differentiation media produced groups of polygonal endothelial-like cells by days 16–21. When transduced with HR-VECad vector, these expressed eGFP in distinct cells within the colony by days 18–21, and coexpressed VE-Cadherin and eNOS. Lin⁻ BMCs grown in smooth muscle differentiation media produced spindle-shaped cells between days 10–14 in culture. When transduced with the HR-SMTHB vector, these cells showed eGFP expression at ~12 days, which increased over time and coexpressed αSMA, calponin and myosin heavy chain. Within Matrigel plugs containing HR-VECad transduced cells, eGFP⁺ constituted 0.4 ± 0.2% of total cells. In contrast, within Matrigel plugs containing HR-SMTHB transduced cells, eGFP⁺ cells constituted 0.2 ± 0.1% of total cells. These data demonstrate the feasibility of selectively marking BMC populations for cell fate determination.


## 1. Introduction

Bone marrow-derived progenitor cells have been shown to contribute to physiological and pathological changes in the vasculature (Hill et al., 2003; Heiss et al., 2005). These progenitor populations consist of a wide spectrum of cells with different differentiation capacities and functions (Herzog et al., 2003). The subset(s) of progenitor cells that participate in repair or contribute to vascular disease have yet to be fully identified and the mechanism by which these cells contribute to functional alterations in preclinical models is still not well understood (Taylor, 2004; Ott et al., 2005). Traditional tools to examine vascular differentiation events in progenitor cell populations have relied heavily on indirect immunofluorescence and immunohistochemical techniques. These methods have been exploited to detect diverse events *in vivo* such as bone marrow cell homing to sites of vascular injury, engraftment (incorporation) into the vessel wall and differentiation down a specific vascular lineage. However, it is increasingly evident that artefactual and methodological concerns surrounding immunodetection methods raise questions about the reliability of these techniques across different investigators and centers of study. Alternative, more biologically robust methods are therefore required to complement current techniques establishing cell differentiation and fate determination.

Lentiviral vectors encoding for identifiable marker genes controlled by lineage-specific promoters are an elegant tool that can be used to track differentiation of bone marrow progenitors into endothelial cells and/or smooth muscle cells. Indeed early studies in experimental models of cancer support use of these vectors in tracking cells injected into the whole animal (Hoffman, 2002; Hadjantonakis and Papaioannou, 2004). Bone marrow transplantation studies of donor cells that ubiquitously express a marker gene have shown that marrow cells can home to sites of vascular injury and engraft (Asahara et al., 1999; Shimizu et al., 2001; Sata et al., 2002; Foteinos et al., 2008). Using

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lineage-specific promoters, it is possible to estimate the rate and extent of cell-specific promoter activation, giving an insight into endothelial or smooth muscle cell differentiation.

Multiple candidate promoters have been identified, which are able to drive expression of transgenic tags in endothelial and smooth muscle cells. The Flk-1 and Tie-2 (Schlaeger et al., 1995) promoters have both been used in previous studies to identify bone marrow cell trans-differentiation into endothelial-like cells *in vivo* (Asahara et al., 1999; Kappel et al., 1999; De Palma et al., 2003; Xu et al., 2003; Duda et al., 2006; Foteinos et al., 2008). Unfortunately, the specificity of these promoter constructs has recently been questioned with not only detection in non-endothelial vascular cells (Yamashita et al., 2000) but also expression in monocyte/macrophage populations (Yang et al., 2004). αSMA and SM22α promoters have similarly been employed to track smooth muscle cells in remodeled vasculature (Wang et al., 1997; Hu et al., 2004; Osawa et al., 2006). However, it is clear that these constructs also have limitations in terms of lineage specificity. Therefore, for a more accurate estimation of the rate and the extent of vascular differentiation newer promoters with more lineage specificity need to be employed.

In the present study, lentiviral tracking using either the human VE-Cadherin or Smoothelin-B promoter to drive expression of green fluorescent protein (eGFP) was evaluated. These experiments demonstrate the vector's ability to selectively mark progenitor cell populations and transcriptional activate fluorescent marker genes to verify differentiation in specific cell phenotypes. This may allow for real-time tracking of bone marrow progenitor cell (BMC) differentiation *in vivo* without resorting to indirect analysis with methods such as immunohistochemistry.

## 2. Methods and materials

### 2.1. Lentiviral vector construction and generation

Using high fidelity PCR (Roche Applied Science, West Sussex, UK), 1350 bp human VE-Cadherin (−1130/+120) (Prandini et al., 2005) and 3561 bp human Smoothelin-B (−3372/+120) (Rensen et al., 2002) promoter regions were amplified and were cloned into the EcoRI–BamHI sites of pHR-CSGW vector, a second-generation lentiviral transfer construct (kindly provided by Adrian Thrasher, Institute of Child Health, London, UK). Lentiviral vectors were generated by a three-plasmid calcium phosphate transfection procedure. Briefly, 293T cells were transfected with the transfer plasmid vector together with a packaging plasmid (pCMV-ΔR8.91 encoding the packaging proteins Gag-Pol, Rev, Tat) and an envelope expression plasmid (pMD.G encoding G protein of the vesicular stomatitis virus (VSV)). The expression of GFP was driven by one of three different internal promoters, SFFV, hVE-Cadherin, and hSMTH-B. In addition, the transfer vector backbone contained the woodchuck hepatitis virus posttranscriptional regulatory element (Zufferey et al., 1999), and the central polypurine tract to enhance levels of transcription and gene expression. Viral supernatants were collected starting 48 h and continuing for 4 consecutive days after transfection, and then filtered through a 0.45-μm filter. Viral supernatants were concentrated ~100-fold by ultracentrifugation in a Sorval centrifuge, for 90 min at 72,000*g*. Using these protocols, titers of $\sim 5 \times 10^7$–$3 \times 10^8$ TU/mL were achieved.

### 2.2. Transduction of cell lines

HEK-293T and HeLa cells were obtained from the American Type Culture Collection (Middlesex, UK), and HUVEC and AoSMC obtained from Clonetics (BD Biosciences, Franklin Lakes, NJ). All cultures were maintained in media recommended by suppliers. In total, $5 \times 10^4$ cells were plated on a 12-well plate 24 h before transduction. For BMC transduction, whole bone marrow was collected from mice by flushing femurs and tibias with RPMI 1640 (Sigma, St Louis, MO) supplemented with 100 U penicillin/streptomycin (Sigma, St Louis, MO). $Lin^-$ cells were isolated using magnetic bead separation with a mouse Lineage cell Depletion kit (Miltenyi Biotec GmbH, Germany). Cells were resuspended in X-Vivo 15 (Lonza, Basal, Switzerland) supplemented with 50 ng/mL mSCF (Peprotech, London, UK) at a density of $2 \times 10^6\ mL^{-1}$. Vectors were diluted in X-Vivo 15 supplemented with 50 ng/mL mSCF and 5 μg/mL Polybrene (Millipore, Billerica, MA). Transduction was performed in round bottom 96-well plates for 24 h in a 200 μl reaction volume.

### 2.3. Methocult assay

Methocult M3334 media (StemCell Technologies, Canada) was used according to manufacture's instructions. Briefly, after $Lin^-$ BMC transduction with HR-CSGW, cells were washed 3 times with pre-warmed PBS and resuspended to $1 \times 10^6$ cells/mL in X-Vivo 15. Cells were diluted 1:10 in Methocult and plated onto 35 mm dishes in triplicate. Cells were cultured for 7 days at 37 °C, 5% $CO_2$ and ~95% humidity. Colony-forming units were counted and reported per $10^5$ $Lin^-$ cells.

### 2.4. Fluorescent activated cell sorting

Three to five days after transduction, $Lin^-$ BMCs were lifted using PBS and washed twice with 0.5% PBSA. Cells were stained with anti-mouse cKit-APC or -PE (BD Biosciences 1:500) for 30 min on ice. Live cells were gated on forward and side scatters using BD FACSCaliber (BD Biosciences, Franklin Lakes, NJ), and evaluated for eGFP and cKit expression by 2D dot plots using WinMDI 9.2 software.

### 2.5. Lentiviral integration assay

Genomic DNA was isolated from transduced cells using DNeasy Isolation Kit (Qiagen, West Sussex, UK). PCR for eGFP-WPRE region was performed using Platium Taq Polymerase (Invitrogen, Carlsbad, CA) with the following primers: eGFP-F (5′- CTGACCTACGGCGTGCAGTGC-3′) and WPRE-R (5′- CCTCGATGTTGTGGCGGATC-3′) annealing at 58 °C. Amplicons were visualized on 1.5% agarose gel as a 327 bp fragment.

### 2.6. Bone marrow in vitro differentiation

Twenty-four hours after transduction, $Lin^-$ bone marrow cells were washed 3 times in pre-warmed PBS. Cells were resuspended at $5 \times 10^5\ mL^{-1}$ in appropriate differentiation media and plated on collagen or fibronectin (Sigma, St Louis, MO) coated 8-well chamber slides (Lab-Tek™ II Chamber Slide™ System, Nunc GmbH & Co. GK). Smooth muscle outgrowth cells were grown for three weeks in SMGM-2 (Lonza, Basal, Switzerland) supplemented with 25 ng/mL PDGF-$_{BB}$ and 2.5 ng/mL TGF-$_{\beta 1}$. Endothelial outgrowth cells were grown for three weeks in EGM-2 (Lonza, Basal, Switzerland) supplemented with 50 ng/mL VEGF and 25 ng/mL bFGF. All cytokines were purchased from Peprotech (London, UK).

### 2.7. In vivo Matrigel Angiogenesis Assay

Twenty-four hours after transduction, $Lin^-$ bone marrow cells were washed 3 times in pre-warmed PBS. Cells were resuspended