Stem Cell Research (2014) 12, 222–232

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SHORT REPORT

Smooth muscle cells largely develop independently of functional hemogenic endothelium

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Received 24 May 2013; received in revised form 24 October 2013; accepted 26 October 2013
Available online 5 November 2013

Abstract Vascular smooth muscle cells represent a major component of the cardiovascular system. *In vitro* studies have shown that FLK1$^+$ cells derived from embryonic stem (ES) cells can differentiate into both endothelial and smooth muscle cells. These FLK1$^+$ cells also contain a mesodermal precursor, the hemangioblast, able to produce endothelial, blood and smooth muscle cells. The generation of blood precursors from the hemangioblast was recently shown to occur through a transient cell population of specialised endothelium, a hemogenic endothelium. To date, the lineage relationship between this cell population and smooth muscle cell progenitors has not been investigated. In this study, we generated a reporter ES cell line in which expression of the fluorescent protein H2B-VENUS is driven by the α-smooth muscle actin (α-SMA) regulatory sequences. We demonstrated that this reporter cell line efficiently trace smooth muscle development during ES cell differentiation. Although some smooth muscle cells are associated with broad endothelial development, we established that smooth muscle cells are mostly generated independently from a specialised functional hemogenic endothelium. This study provides new and important insights into hematopoietic and vascular development, which may help in driving further progress towards the development of bioengineered vascular grafts for regenerative medicine.

Introduction

The blood vessel and hematopoietic systems are the first functional organs formed in the developing embryo. Endothelial cells are initially generated through vasculogenesis to produce a primitive vascular plexus. Subsequent angiogenesis lead to the expansion and remodelling of this initial endothelial network (Patan, 2004). The newly formed endothelial vessels become rapidly associated with mural cells of the smooth muscle cell lineage. These cells are either referred to as

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1873-5061
http://dx.doi.org/10.1016/j.scr.2013.10.009

vascular smooth muscle cells if they encircle larger vessels or as pericytes if they reside within the wall of small vessels such as capillaries and post-capillary venules. These cells regulate blood flow through contraction and have also been proposed to control endothelial cell proliferation and differentiation by direct signalling and deposition of extracellular matrix (Betsholtz et al., 2005). Hampered smooth muscle cell differentiation or function results in severe vascular defects such as abnormal vessel morphology and increased permeability leading to a number of cardiovascular disorders including congenital heart diseases, aortic aneurysm, atherosclerosis, hypertension, and restenosis (Carvalho et al., 2004; Guo et al., 2007; Hellstrom et al., 2001, 1999; Lindahl et al., 1997; Milewicz et al., 2008; Zhu et al., 2006). In Hutchinson–Gilford progeria syndrome, loss of smooth muscle cells has been suggested to lead to progressive arterial occlusion that causes death from myocardial infarction or from stroke at an average age of thirteen years (Varga et al., 2006).

Smooth muscle cells are characterised by the specific expression of several proteins such as α-smooth muscle actin (α-SMA), transgelin (SM22α), calponin and smooth muscle myosin heavy chain (MYH11) (Owens, 1995). α-SMA, encoded by the *Acta2* gene, is one of the six actin genes expressed in mammalian cells and is the first known marker of differentiated smooth muscle cells during vasculogenesis (Owens, 1995). During murine embryogenesis, vascular smooth muscle cells express high levels of α-SMA, (Mack and Owens, 1999; McHugh, 1995), initially within the embryonic heart rudiment around E8.5, then in the yolk sac and the aorta vasculature by E9.5–10.5 (Armstrong et al., 2010). The embryonic origin of smooth muscle remains poorly characterised and lineage-tracing studies suggest that, in distinct vessels, vascular smooth muscles or pericytes have different embryonic origins (Cheung et al., 2012; Majesky, 2007). The hemangioblast, or its *in vitro* equivalent — the blast colony forming cell (BL-CFC), is defined as a mesodermal progenitor cell with endothelial and hematopoietic potentials (Choi et al., 1998). A vascular smooth muscle cell developmental potential was also attributed to the hemangioblast, as both embryo-derived hemangioblast (Huber et al., 2004) and embryonic stem (ES) cell-derived BL-CFCs (Ema et al., 2003; Lu et al., 2009) were demonstrated to contain vascular smooth muscle potential. The generation of blood precursors from the hemangioblast was recently shown to occur through a transient cell population of specialised endothelium, a hemogenic endothelium (Lancrin et al., 2009). To date, the lineage relationship between this specific endothelial cell population and smooth muscle cell progenitors has not been investigated. Several previous studies suggest a close developmental relationship between endothelial and smooth muscle progenitors. For instance, mesodermal FLK1$^+$ cells generated from differentiated ES cells were shown to produce both smooth muscle and endothelial cells that organise into vessel-like structures (Yamashita et al., 2000). Furthermore, smooth muscle actin expression has been detected in CD34$^+$ cord blood endothelium and in endothelial cells at the luminal surface of adult aorta (Azuma et al., 2009; Lu et al., 2004). The generation of cardiomyocytes was also shown to be associated with common endothelial and smooth muscle development. In headfold stage murine embryos, or in differentiated ES cells, Kattman et al. identified a progenitor for cardiomyocytes with additional endothelial and vascular smooth muscle potential (Kattman et al., 2006; Yang et al., 2008).

In this study, we generated a mouse reporter ES cell line in which the expression of the fluorescent protein, H2B-VENUS, is driven from α-SMA regulatory sequences. We demonstrated that this reporter cell line allows to efficiently track smooth muscle development during murine ES cell differentiation. Although we observed the presence of rare H2B-VENUS$^+$ cells in enriched hemogenic endothelial cell populations, our findings established that smooth muscle cells were mostly generated independently from the specialised functional hemogenic endothelium.

Materials and methods

BAC recombineering

The bMQ116k23 BAC was purchased from the Sanger Institute. BAC carrying bacteria were transformed with pSC101-BAD-gbaA plasmid containing recombination proteins: *exo*, *bet* and *gam* under the control of arabinose-inducible promoter (gift from Francis Stewart laboratory, Dresden). The homology arms were cloned into a vector containing: *H2B-Venus* reporter gene, a floxed cassette PGK/EM7-TK-Neo and an ampicillin resistance gene. Recombineering was performed according to the method developed by Francis Stewart laboratory (Dresden, Germany). Details of oligonucleotides sequences used for various steps and screening of ES cells are available on request.

Gene expression analysis

Total RNA was extracted using RNeasy Plus Mini Kit (Qiagen). cDNA was generated using Omniscript RT Kit (Qiagen). Quantitative PCR was performed on ABI7900 (Applied Biosystems) using universal probe library (Roche), TaqMan Universal PCR Master Mix (Applied Biosystems) and gene specific primers (Supplemental Table 1). Gene expression was normalised to a reference gene (*β-Actin*) and represented as 2^-ΔCt.

ES cell culture

Murine ES cells were maintained and differentiated as described (Sroczynska et al., 2009b). Smooth muscle cells were grown in medium containing IMDM supplemented with 10% FCS, 2 mM L-glutamine, 180 μg/ml transferrin, 25 μg/ml ascorbic acid, 4.6×10^{-4} M MTG, 15% D4T conditioned medium (Choi et al., 1998), 10 ng/ml IL-6 (Peprotech) and 5 ng/ml VEGF (Peprotech). For contraction assays, cells were treated with carbachol (100 μM, Sigma) where indicated. Hemogenic endothelial cultures were performed as previously described (Lancrin et al., 2009). Single blast colonies were expanded in the medium previously described on a Ibidi μ-slide with Matrigel (Huber et al., 2004).

Fluorescence-activated cell sorting (FACS)

Flk1-bio, TIE2-bio, CD31-bio, c-KIT-APC, VE-CADHERIN-APC, CD41-PE and Streptavidin-PECy7 antibodies were purchased from eBioscience. α-SMA-Cy3 antibody was purchased from Sigma. All antibodies used in this study have been previously