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Endocardial cells are a distinct endothelial lineage derived from Flk1+ multipotent cardiovascular progenitors

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ABSTRACT

Identification of multipotent cardiac progenitors has provided important insights into the mechanisms of myocardial lineage specification, yet has done little to clarify the origin of the endocardium. Despite its essential role in heart development, characterization of the endocardial lineage has been limited by the lack of specific markers of this early vascular subpopulation. To distinguish endocardium from other vasculature, we generated an *NFATc1-nuc-LacZ* BAC transgenic mouse line capable of labeling this specific endothelial subpopulation at the earliest stages of cardiac development. To further characterize endocardiogenesis, embryonic stem cells (ESCs) derived from *NFATc1-nuc-LacZ* blastocysts were utilized to demonstrate that endocardial differentiation *in vitro* recapitulates the close temporal–spatial relationship observed between myocardium and endocardium seen *in vivo*. Endocardium is specified as a cardiac cell lineage, independent from other vascular populations, responding to BMP and Wnt signals that enhance cardiomyocyte differentiation. Furthermore, a population of Flk1+ cardiovascular progenitors, distinct from hemangioblast precursors, represents a mesodermal precursor of the endocardial endothelium, as well as other cardiovascular lineages. Taken together, these studies emphasize that the endocardium is a unique cardiac lineage and provides further evidence that endocardium and myocardium are derived from a common precursor.

Introduction

Precise coordination of several distinct cell lineages is required for heart development. The cardiac precursors of the first and second heart fields, having passed through the primitive streak during gastrulation, populate a region of anterior mesoderm known as the cardiac crescent (Buckingham et al., 2005; Kirby, 2002). With lateral folding, these precursors coalesce into a linear heart tube composed of an outer myocardium and inner endocardium. This tube serves as the structural basis for subsequent morphogenesis, as migrating neural crest and proepicardium contribute to vital structural features of the outflow tract and coronary vasculature (Brown and Baldwin, 2006; Mikawa and Gourdie, 1996). Genetic analyses and fate mapping have documented the origins and functions of these cell lineages in cardiogenesis with one notable exception. Despite being one of the earliest cardiac populations, little is known about the origin of the endocardium.

Endocardial cells first emerge from the ventral surface of the cardiac mesoderm into the space between myocardial cells and anterior visceral endoderm. With tubular heart formation, myogenic epithelium retains N-cadherin while endocardial precursors down-regulate expression (Linask, 1992). At this stage of development, endocardial cells are distinguished from myocardium by the expression of vascular makers, such as Flk1 (VEGFR-2), CD31/Pecam-1, and VE-cadherin (Baldwin, 1996; Drake and Fleming, 2000). The endocardium is unique from other endothelium both in terms of this developmental relationship with myocardium, but also in its critical role in valvulogenesis and trabeculation. Valve formation is initiated when endocardial cells delaminate into the underlying cardiac jelly, a process termed epithelial-to-mesenchymal transition (EMT) (Barnett and Desgrosellier, 2003). Failure to undergo EMT or disruption of signals from the endocardium to cushion cells results in a variety of valve defects. In myocardial trabeculation, loss of *Neuregulin* in ventricular endocardium or its myocardial receptors *ErbB2*/*ErbB4* results in an absence of trabeculation and decreased myocyte

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proliferation (Gassmann et al., 1995; Lee et al., 1995; Meyer and Birchmeier, 1995).

Whether endocardium and myocardium share a common progenitor in the cardiac mesoderm remains controversial, with existing models predicting either a common multipotent progenitor or two spatially sequestered subpopulations within the cardiac crescent. Recent evidence from zebrafish models suggests that endocardial cells are derived from the hematopoietic and vascular lineage, and then migrate to invest the developing myocardial tube (Bussmann et al., 2007). Retroviral tracing in the avian embryo indicates that these two populations are distinct prior to gastrulation (Cohen-Gould and Mikawa, 1996; Wei and Mikawa, 2000). In contrast, growing evidence suggests endocardium and myocardium are derived from a common multipotent mesodermal progenitor in the mouse. Fate mapping analyses in mice indicate mesodermal cells expressing *Mesp1* and *Flk1* contribute to both endocardium and myocardium (Motoike et al., 2003; Saga et al., 2000). Cre-mediated lineage tracing of *Nkx2.5*+ and *Isl1*+ cardiac populations suggest that as late as the cardiac crescent, subsets of myocardium, endocardium, vascular endothelium, and smooth muscle are derived from a common precursor (Cai et al., 2003; Stanley et al., 2002). These findings imply a close developmental relationship between the endocardium and myocardium, but detailed examination has proven difficult due to the limitations of *in vivo* mouse models.

Since its initial description, embryonic stem cell (ESC) differentiation has emerged as a robust *in vitro* model of lineage specification, as mesodermal derivatives such as endothelium, hematopoietic cells, and cardiomyocytes develop in a sequential pattern mirroring embryonic development (Keller, 2005). ESC differentiation has previously been utilized to define the origin and specification of the mesodermal hemangioblast, a common hematopoietic and endothelial precursor (Choi et al., 1998; Nishikawa et al., 1998). Recent studies of cardiac specification in ESC differentiation models have clarified the progression of lineage commitment required to establish cardiovascular cell populations. From ESCs expressing GFP under the mesodermal gene *Brachyury,* a Flk1+ multipotent cardiovascular progenitor capable of generating cardiomyocytes, smooth muscle cells, and endothelium were characterized (Kattman et al., 2006). Other approaches, using the early cardiac markers *Isl1* and *Nkx2.5*, have also isolated progenitor populations possessing various levels of cardiovascular cell potency (Christoforou et al., 2008; Moretti et al., 2006; Wu et al., 2006). However, the lack of a specific marker for the early endocardium prevents direct examination of the endocardium in these studies, and consequently, they failed to draw a distinction between the endocardium and other endothelium.

Taking advantage of our previous observation that Nuclear Factor of Activated T-cells (NFATc1/NFAT2) is an endocardial-specific transcription factor during early cardiac development, we investigated the existence of a common endocardial–myocardial progenitor by generating a bacterial artificial chromosome (BAC) transgene containing the *NFATc1* genomic locus (de la Pompa et al., 1998; Ranger et al., 1998). Possessing the regulatory elements necessary to distinguish endocardium from other endothelium, this *NFATc1-nuc-LacZ* BAC transgenic mouse faithfully recapitulated endogenous endocardial *NFATc1*. Using ES cells derived from *NFATc1-nuc-LacZ* BAC transgenic embryos, we demonstrate endocardiogenesis in ESC differentiation and document the close temporal–spatial relationship seen between endocardium and myocardium *in vivo* is reproduced *in vitro*. We show endocardium is derived from a specific mesodermal population as a cardiac cell lineage distinct from hematopoietic and vascular lineages. Furthermore, clonal analysis established this population contains cardiovascular progenitors with cardiomyocyte, endocardial, endothelial, and smooth muscle potential. Collectively, these studies provide evidence that endocardium is a unique cardiac lineage distinct from other endothelium, and that endocardium and myocardium are derived from a common cardiovascular progenitor.

Materials and methods

BAC targeting and transgenesis

To generate the *NFATc1-nuc-LacZ* BAC transgene, a nuclear-localized *LacZ* reporter (*nuc-LacZ*) and a *frt* flanked tetracycline resistance cassette (Tet^R) were inserted between homology arms corresponding to the 500 bp 5′ of the translational start site and 400 bp 3′ to the splice site of Exon1. Bacterial homologous recombination was performed as previously described (Lee et al., 2001). To establish transgenic mouse lines, the *NFATc1-nuc-LacZ* BAC transgene was prepared by cesium chloride gradient purification, and dialyzed into microinjection buffer (10 mM Tris, 15 μM EDTA pH 7.4)). BAC DNA was diluted to 2 ng/μl for injection into pronuclei of 0.5-day-old fertilized FVB zygotes and transferred into oviducts of pseudo-pregnant ICR females (Boyle et al., 2008). Animals were maintained in accordance with protocols approved by the Vanderbilt University Institutional Animal Care and Use Committee (IACUC).

Embryonic stem cell culture and differentiation

ESCs were cultured on irradiated mouse embryonic fibroblasts in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 15% FBS (HyClone), 2 mM L-glutamine, 100 U/ml Pen/Strep, 1 mM sodium pyruvate, 100 μM non-essential amino acids, 1000 U/ml recombinant leukemia inhibitory factor (ESGRO, Millipore) and 100 μM β-mercaptoethanol. ESCs were transferred to gelatinized plates 1–2 days prior to the initiation of differentiation. Embryoid bodies (EBs) were formed by either hanging drop or in suspension at a density of 2.5×10^{-4} in ESC differentiation media (DM) consisting of Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 15% FBS, 2 mM L-glutamine, 100 U/ml Pen/Strep, 200 μg/m transferrin (Roche), 0.5 mM L-ascorbic acid (Sigma), and 4.5×10^{-4} M monothioglycerol. Noggin/Fc, Wnt3A, Dkk-1 were obtained from R&D Systems. ESC lines from *NFATc1-nuc-LacZ* BAC transgenics were derived from E3.5 blastocyst-stage embryos plated on irradiated STO feeders in RESGRO complete ES medium (Millipore). After 5–6 days in culture, the inner cell mass outgrowth was selectively removed from the trophectoderm, trypsinized, and then replated (Liu and Labosky, 2008). Once colonies were visible, ESCs were transitioned to ES medium.

β-galactosidase and immunostaining

For immunofluorescence, embryo cryosections (10 μm) were fixed in 10% neutral buffered formalin (NBF); cells in culture were fixed with ice cold methanol:acetone (1:1). Tissues were blocked in 10% goat serum (Jackson Labs) prior to incubation with primary antibodies overnight at 4 °C. Antibodies used are listed in Supplemental experimental procedures. Samples were mounted with Vectashield containing DAPI (Vector Labs). Images were acquired using a Nikon Eclipse E800 epifluorescence microscope or Zeiss Upright LSM510 Confocal Microscope. MetaMorph v6.1 (Molecular Devices) was used to calculate MHC+ area per EB. For β-Galactosidase staining, whole-mount embryos, cryosections, and cells in culture were fixed with 0.2% glutaraldehyde, 2 mM $MgCl_2$, and 5 mM EGTA in PBS, or 10% NBF for dual β-Gal/immunoperoxidase staining, and stained with 1 mg/ml X-Gal and 5 mM potassium ferro/ferricyanate, 2 mM $MgCl_2$, 0.02% NP40, 0.01% sodium deoxycholate in PBS.

Flow cytometry

D3.5 EBs were dissociated using cell disassociation buffer (Gibco) and resuspended in Hanks Buffered Salt Solution (HBSS) with 2% FBS, and 25 mM HEPES. Samples were then incubated with CD16/CD32 (mouse, Ebioscence, 1 μg/ml) to block nonspecific binding prior to