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Epicardial GATA factors regulate early coronary vascular plexus formation

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ABSTRACT

During early development, GATA factors have been shown to be important for key events of coronary vasculogenesis, including formation of the epicardium. Myocardial GATA factors are required for coronary vascular (CV) formation; however, the role of epicardial localized GATAs in this process has not been addressed. The current study was conducted to investigate the molecular mechanisms by which the epicardium controls coronary vasculogenesis, focusing on the role of epicardial GATAs in establishing the endothelial plexus during early coronary vasculogenesis. To address the role of epicardial GATAs, we ablated GATA4 and GATA6 transcription factors specifically from the mouse epicardium and found that the number of endothelial cells in the sub-epicardium was drastically reduced, and concomitant coronary vascular plexus formation was significantly compromised. Here we present evidence for a novel role for epicardial GATA factors in controlling plexus formation by recruiting endothelial cells to the sub-epicardium.

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Introduction

The origin of coronary vascular (CV) endothelial cells has been a longstanding topic of interest and investigation. It was initially thought that coronary endothelial cells arose from the heart itself (Manasek, 1969). Based on cell lineage tracing studies in avian systems, an extra-cardiac structure derived from the septum transversum termed the proepicardium (PE) was subsequently proposed as the origin of CV endothelial cells (Mikawa and Gourdie, 1996). These findings led to a model in which endothelial precursor cells, along with other CV precursor cells, migrate from the PE to the myocardium where they form the epicardium. A subset of epicardial cells then invades the sub-epicardial space to differentiate into the coronary endothelial cells, coronary smooth muscle cells, and cardiac fibroblasts that form the coronary vasculature (Dettman et al., 1998; Merki et al., 2005; Mikawa and Gourdie, 1996). The observation that blocking PE attachment to the myocardium halted CV development supported the idea that all CV cells are derived from the PE, including vascular endothelial cells, or at least, that the PE is required for differentiation of endothelial cells that make up the coronary vasculature (Manner, 1993; Olivey et al., 2004). Recent murine studies indicate that coronary endothelial cells have multiple sources of origin and derive largely from the sinus venosus, the PE, and the endocardium (Katz et al., 2012; Red-Horse et al., 2010).

The coronary vascular system consists of a network of coronary arteries and coronary veins. The coronary arteries are generally located within the myocardium, while the coronary veins are typically located in the sub-epicardial space between the epicardium and myocardium (Lavine and Ornitz, 2008). It was thought that endothelial cells that contribute to the coronary arteries and veins derived from separate sources within the developing heart (Wu et al., 2012). However, recent work indicates that endothelial cells, which contribute to both the coronary veins and the coronary arteries, derive from the sub-epicardial endothelial cells (SEECs) (Red-Horse et al., 2010; Tian et al., 2013). These findings place the appearance of endothelial cells in the sub-epicardium as a critical early step in coronary vascular formation.

Multiple studies indicate that formation of the endothelial CV plexus involves complex reciprocal signaling between cells in the epicardium and myocardial cells of the sub-epicardium (Lavine and Ornitz, 2008; Olivey and Svensson, 2010; Tomanek et al., 2010). Plexus formation has typically been used as the benchmark in many of these studies; therefore, less has been done to distinguish regulatory control mechanisms that are operating prior to plexus formation. Formation of the coronary vascular plexus can be envisaged as having at least three steps: 1-migration and appearance of endothelial cells or precursors in the sub-epicardium; 2-coalescence

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of vascular endothelial cells; and 3-assembly of the initial plexus. Overall, plexus formation has been found to be dependent on epicardial–myocardial reciprocal signaling that centers on an angiogenic FGF-SHH-VEGF/Ang-2 signaling axis (Lavine and Ornitz, 2009). The role of this axis in recruiting endothelial cells, mobilizing resident hemangioblasts, or assembly of the initial plexus from endothelial precursors is the topic of significant investigation. In the studies here, we present evidence that addresses GATA-dependent regulatory control of these earliest steps and suggests a model in which the epicardium is the source of signals that regulate the appearance of SEECs.

The GATA family of transcription factors are important regulators of vascular development (Lepore et al., 2005; Watt et al., 2004; Xin et al., 2006; Zhao et al., 2005). The six members of the GATA family are divided into two sub-groups based on their expression profiles and roles in various developmental processes. GATA1/2/3 are involved in hematopoietic development, while GATA4/5/6 regulate cardiac and endodermal formation (Molkentin, 2000). Global knockout of either *Gata4* or *Gata6* results in failure of extra-embryonic tissues leading to embryonic lethality during gastrulation, while *Gata5* knockouts were found to be viable (Koutsourakis et al., 1999; Molkentin et al., 1997, 2000). Rescue of extra-embryonic tissue failure by tetraploid complementation revealed that *Gata4* knockouts are unable to form a PE, demonstrating a central role for GATA4 in CV development (Watt et al., 2004). Little is known concerning the role of GATA6 in CV development; however, compound heterozygous $Gata4^{+/-}$ $Gata6^{+/-}$ mice die at embryonic day 13.5 (E13.5) due to vascular defects (Xin et al., 2006). This finding raises the possibility that GATA4 and GATA6 are required for CV development. Consistent with this idea, in vitro work has shown that GATA6 can promote angiogenesis via suppression of anti-angiogenic, autocrine signaling (Froese et al., 2011). Moreover, friend of GATA-2 (FOG-2) a well-known GATA cofactor, has been found to be crucial for the formation of the vascular plexus (Tevosian et al., 2000). These findings suggest roles for GATA factors in coronary vascular development and a more specific role for epicardial GATAs.

Here we address the role of GATA4 and GATA6 in epicardial-mediated formation of the coronary plexus. Our results show that GATA4 and GATA6 are expressed in the epicardium and required for plexus formation. Using a Cre-lox system to conditionally knock out both *Gata4* and *Gata6* (dcKO) in an epicardial-specific manner, we found that the loss of epicardial GATAs resulted in a drastic loss of coronary plexus formation. These results suggest a model for formation of both coronary veins and arteries in which epicardial GATAs regulate the number of endothelial cells in the sub-epicardium.

Materials and methods

Animals

All animal experiments were approved by the Institutional Animal Care and Use committee (IACUC) at the Medical College of Wisconsin. The *WT1*(*RP23-8C14*)-*Cre* mouse line contains a BAC expression construct in which the *Cre* recombinase gene was inserted in the 5′ UTR of the first exon within the Wilms Tumor-1 gene. This construct was designed to target the epicardium and epicardial-derived cells. The *WT1*(*RP23-8C14*)-*Cre* line has been maintained on a C57B16/J background and was obtained as a generous gift from Dr. John Burch. The $Gata4^{f/f}Gata6^{f/f}$ mice were generated by crossing the previously described $Gata4^{f/f}$ and $Gata6^{f/f}$ mouse lines (Watt et al., 2004; Sodhi et al., 2006). The $Gata4^{f/f}$ line contains *loxP* sites flanking exons 3–5, which contain the nuclear localization and DNA binding domains. The $Gata6^{f/f}$ line contains *loxP* sites flanking exon 2, which contains most of the *GATA6* sequence. *Tie2-Cre, Rosa26R-eYFP, and Rosa26R-βgal* mice have been previously described (Kisanuki et al., 2001; Soriano, 1999; Srinivas et al., 2001).

Embryos were generated by timed matings designating E0.5 as noon on the day a vaginal plug was observed. Genotyping was performed with PCR by standard protocols using genomic DNA isolated from embryonic tail tissue.

Primers used are as follows:

Cre	F: GTT CGC AAG AAC CTG ATG GAC A
	R: CTA GAG CCT GTT TTG CAC GTT C
$Gata4^{f/f}$	F: CCC AGT AAA GAA GTC AGC ACA AGG AA
	R: AGA CTA TTG ATC CCG GAG TGA ACA TT
$Gata6^{f/f}$	F: GTG GTT GTA AGG CGG TTT GT
	R: ACG CGA GCT CCA GAA AAA GT

Histology and immunofluorescence

Embryos were harvested from timed pregnant females, photographed, and fixed overnight in zinc buffered formalin. Embryos were then embedded in paraffin wax, and sectioned at 5 μm according to standard protocols. Sections were then subject to antigen retrieval with 10 mmol/L citric acid buffer (pH 6.0) at 100 °C for 10 min. Sections were then blocked for 45 min and stained overnight with primary antibody in blocking solution. Sections were then washed three times with PBS and incubated with secondary antibody in blocking solution for 2 h. Finally, sections were washed three times with PBS and incubated in 100 mmol/L glycine with DAPI for 1 h.

Sections were immunofluorescently labeled according to standard protocols using anti-PECAM1 (1:100, BD Biosciences, clone MEC13.3), anti-ERG (1:500, Santa Cruz Biotechnology, clone C-20), anti-GFP (1:500, Invitrogen), anti-sarcomeric myosin (1:200, DSHB, clone MF-20), anti-smooth muscle myosin heavy chain (1:200, Biomedical Technologies BT-562), anti-GATA4 (1:100, Santa Cruz Biotechnology, sc-9053), anti-GATA6 (1:5000, a generous gift from Dr. Xiang-Xi Xu), anti-WT1 (1:100, DAKO, 6F-H2) and anti-SHH (1:100, DSHB, clone 5E-1) primary antibodies and corresponding fluorescent secondary antibodies from Invitrogen.

Images were captured using a Nikon Eclipse 80i microscope equipped with a Nikon Digital Sight DS-2MBW monochrome camera and NIS Elements-D imaging software. Photo processing was done with Photoshop.

Whole-mount immunohistochemistry

Whole-mount PECAM staining was conducted as previously described (Lavine et al., 2005). Isolated E12.5 hearts were fixed overnight in 4% paraformaldehyde. Following fixation, hearts were dehydrated with methanol and incubated in 6% hydrogen peroxide for 2 h. Hearts were then rehydrated, blocked in 5% goat serum/1% Triton X-100/PBS, and incubated overnight with PECAM primary antibody (1:100, BD Biosciences, clone MEC13.3). Following 5 × 1 h washes in blocking solution, hearts were incubated overnight with biotinylated goat anti-rat IgG secondary antibody (1:200, Vector). Following 5 × 1 h washes in blocking solution, hearts were incubated overnight in ABC reagent (Vectastain). After a final set of washes, the hearts were then exposed to DAB and imaged. PECAM stained hearts were then embedded, sectioned, lightly counterstained with hematoxylin, and imaged for quantification of vessels per section. Three sections from at least three wild type and three dcKO hearts were used for quantification.

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