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Epicardial HIF signaling regulates vascular precursor cell invasion into the myocardium

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ABSTRACT

During cardiogenesis, a subset of epicardial cells undergoes epithelial–mesenchymal-transition (EMT) and the resulting epicardial-derived cells (EPDCs) contribute to the formation of coronary vessels. Our previous data showed hypoxia inducible factor-1α (HIF-1α) expression at specific sites within the epicardium and support a link between hypoxia inducible factors (HIFs) and the patterning of coronary vasculogenesis. To better understand the autocrine role of HIFs in the epicardium, we transduced adenovirus mediated expression of constitutively active HIF-1α (AdcaHIF1α) into the embryonic avian epicardium where the vascular precursors reside. We found that introducing caHIF1α into the epicardial mesothelium prevented EPDCs from proper migration into the myocardium. *In vitro* collagen gel assays and *ex vivo* organ culture data further confirmed that infection with AdcaHIF1α impaired the ability of EPDCs to invade. However, the proficiency of epicardial cells to undergo EMT was enhanced while the movement of EPDCs within the sub-epicardium and their differentiation into smooth muscle cells were not disrupted by caHIF1α. We also showed that the transcript level of Flt-1 (VEGFR1), which can act as a VEGF signaling inhibitor, increased several fold after introducing caHIF1α into epicardial cells. Blocking the activation of the VEGF pathway in epicardial cells recapitulated the inhibition of EPDC invasion. These results suggest that caHIF1α mediated up-regulation of Flt-1, which blocks the activation of the VEGF pathway, is responsible for the inhibition of EPDC myocardial migration. In conclusion, our studies demonstrate that HIF signaling potentially regulates the degree of epicardial EMT and the extent of EPDC migration into the myocardium, both of which are likely critical in patterning the coronary vasculature during early cardiac vasculogenesis. These signals could explain why the larger coronaries appear and remain on the epicardial surface.

Introduction

The epicardium is the last cardiac layer to emerge, arising from the pro-epicardial serosa to form the outermost layer of the heart. This tissue plays crucial roles during embryonic heart development, especially in coronary vasculogenesis (Hiruma and Hirakow, 1989; Mikawa and Fischman, 1992; Poelmann et al., 1993; Viragh and Challice, 1981; Winter and Gittenberger-de Groot, 2007). Concurrent with the formation of the epicardium, a subset of epicardial cells undergoes epithelial–mesenchymal transformation (EMT) and starts migration into the sub-epicardial matrix. Some of these epicardial-derived cells (EPDCs) remain within the sub-epicardium whereas a subpopulation migrates farther into the compact zone of the myocardium. EPDCs have the ability to differentiate and give rise to a variety of cell types, including cellular elements of the coronary vasculature (Mikawa and Fischman, 1992; Olivey et al., 2004; Poelmann et al., 1993; Wessels and Perez-Pomares, 2004). *In vivo* and *in vitro* assays reveal the functions of some important signals directing coronary vessel formation, involving adhesion molecules, transcription factors and several growth factors (Lee et al., 2006; Morabito et al., 2001; Olivey and Svensson, 2010). These factors, which are important for epicardial EMT and further steps in coronary vessel formation, could inherently originate from the epicardium and EPDCs as well as from the myocardium (Olivey and Svensson, 2010). Although some elements involved in EMT and subsequent steps in epicardial cell differentiation and migration have been identified, the mechanisms that drive the specific spatial and temporal pattern of coronary vasculogenesis are largely unknown.

It has been proposed that hypoxia triggers coronary vascular development. A range of cellular responses to hypoxia is mediated

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by hypoxia-inducible factors (HIFs), a heterodimer composed of a constitutively expressed HIFβ subunit and an oxygen sensitive HIFα subunit (Wenger, 2002). To date, three HIFα genes and over 100 HIF regulated genes have been identified (Wenger et al., 2005). Components of the HIF complex have been described to be required for normal development and patterning of the cardiovascular system (Dunwoodie, 2009; Ramirez-Bergeron and Simon, 2001). Loss of hypoxia inducible factor-1α (HIF-1α) in the mouse severely disrupts myocardial and vascular endothelial development and embryos die around E10 (Iyer et al., 1998; Ryan et al., 1998). It was also reported that the absence of HIF-1β (ARNT) in mice results in abnormal cardiac morphogenesis and embryonic lethality by E10 (Adelman et al., 2000; Maltepe et al., 1997). Using the avian model, it was shown that environmental oxygen influences embryonic angiogenesis and hypoxia treatment causes myocardial and coronary artery anomalies and an increase of capillary density in hypoxic regions (Nanka et al., 2008). Our previous studies utilizing the hypoxia marker EF5 [2-(2-nitro-1H-imidazol-1-yl)-N-(2,2,3,3,3 pentafluoropropyl)acetamide] revealed the atrioventricular junction (AVJ), ventricular apex, and interventricular septum (IVS) are relatively hypoxic in embryonic hearts. Many of these regions corresponded to the sites where major coronary vessels develop. Furthermore, hypoxia indicators and nuclear labeling of HIF-1α were co-localized in these hypoxic regions (Wikenheiser et al., 2006). Altered HIF-1α expression levels in the myocardium disrupted normal patterning of coronary vessels, suggesting that differential levels of hypoxia within the embryonic myocardium modulate coronary vessel development through transcriptional regulation by HIF-1 (Wikenheiser et al., 2009, in press).

Though the importance of myocardial HIF in regulating coronary vasculogenesis has long been accepted, the functions of epicardial-HIF during coronary vessel development have not been studied (Tomanek et al., 2003). To unravel the *in vivo* roles of epicardial-HIF signaling, we disturbed HIF-1α gene expression and analyzed the consequences *in ovo*, in *ex vivo* explant culture, and also in various *in vitro* systems. Here, we present evidence that HIF-1 has a complex regulatory role during specific steps of epicardial development. While EMT was stimulated by the forced expression of constitutively active HIF-1α (caHIF1α), EPDCs transduced with caHIF1α displayed profoundly impaired invasion into and migration within the myocardium. Our findings support the potential for micro-environmental hypoxia via HIFs and the VEGF pathway to regulate both EMT and their ability to migrate within the myocardium.

Materials and methods

Chicken and quail fertilized eggs

Fertile White Leghorn chicken (*Gallus gallus domesticus*) eggs were obtained from Case Western Reserve University's Squire Valleevue Farm (Cleveland, OH, USA) or from Charles River (MA, USA). Fertile quail (*Coturnix coturnix communis*) eggs were purchased from (Boyd's Bird Company, Inc. Pullman, WA, USA). Eggs were incubated in a humidified forced draft hatching incubator (G.Q.F. Manufacturing Co., Savannah, GA, USA) at 38 °C to the appropriate stages. The embryos were staged according to the method of Hamburger and Hamilton (HH, Hamburger and Hamilton, 1951).

In ovo injection of engineered adenoviruses into the pericardial space and assessment of EPDC migration

Using a modification of a previously described method (Fisher et al., 1997; Fisher and Watanabe, 1996), 0.5 μl adenovirus containing AdcaHIF1α or AdGFP (1×10^9 pfu/ml) was injected into the pericardial space of the avian heart at stage HH 24 (ED 4), when the surface of the heart is largely covered by the epicardium. The AdcaHIF1α virus bears a constitutively active form of human HIF-1α (caHIF1α) and green fluorescent protein (GFP) sequence which were driven by a dual CMV promoter (Kelly et al., 2003). The caHIF1α contains a deletion of the oxygen-dependent degradation domain (residues 392–520) and two missense mutations (Pro567Thr and Pro658Gln). AdGFP containing the CMV promoter that drives expression of GFP was used as a control. After injection, eggs were returned to the incubator and embryos were harvested at stage HH26 (ED 5) and HH28 (ED 5.5–6). GFP positive cells located in different regions and positions were scored as follows. Comparable areas were randomly chosen from non-serial sections for each group. The number of positive cells containing distinct DAPI stained nuclei within these areas were counted for each sample. At least 10 areas were scored for each different region and the average number was determined by the total number of positive cells divided by the total number of areas counted in each of six independent experiments for each stage (HH26 and HH28). DAPI positive cells at the AVJ were also counted using the same method as described before.

Collagen gel assay and quantification of migration

The collagen gel assay was performed as previously described with some modifications (Dokic and Dettman, 2006). Briefly, neutralized collagen I gel was added to 24 well plates (700 μl/well) and allowed to solidify at 37 °C in the incubator for 60 min. Stage HH25 (ED 4.5) avian hearts were harvested in sterile PBS and placed onto the surface of the gels which were covered by serum free M199 medium. After 24 h culturing at 37 °C, 5% CO_2, hearts were removed and epicardial monolayers were allowed to grow out on the surface of the gel in serum free medium for 24 h. For virus infection experiments, monolayers were incubated overnight with serum free M199 containing AdGFP or AdcaHIF1α (1×10^9 pfu/ml) and the medium was then replaced with M199 supplemented with 10% fetal bovine serum (FBS) or 10% FBS+50 ng/ml VEGFA to trigger migration. After 36 h incubation, gels were fixed in 4% paraformaldehyde (PFA) in 1 × PBS, washed and embedded in 1.5% agarose. Embedded samples were equilibrated in 30% sucrose (w/v) overnight at 4 °C and maintained at −80 °C. Cryosections (20 μm) were further stained with DAPI and imaged on a Leica DM2500 microscope. For inhibition experiments, epicardial monolayers were cultured in 10% FBS M199 medium containing DMSO (Fisher Scientific, MA, USA), DFO (150 μM, Sigma–Aldrich, MO, USA), SU5416 (15 μM, Calbiochem, CA, USA), MAZ51 (5 μM, EMD4 Biosciences, CA, USA), BSA (30 μg/ml, Fisher), Fc-sFlt1 (30 μg/ml, R&D systems, MN, USA) for 2 days before analysis.

To evaluate cell migration, the Image J software (NIH) was used to measure the distance for each GFP positive (for adenovirus experiments) or DAPI stained (for inhibition experiments) cell that migrated from the surface into the gel. At least 15 comparable non-serial sections were chosen and analyzed for each group per independent experiment. For each treatment group, the average distance of migration was calculated by the total distance of cell migration divided by the total number of cells counted. Three independent experiments were performed for each treatment.

Organ culture assay

For *ex ovo* migration assays, stage HH25 (ED 4.5) avian hearts were excised from embryos in sterile PBS and cultured overnight at 37 °C in serum free M199 medium added adenovirus (AdGFP or AdcaHIF1α, 1×10^9 pfu/ml). Then hearts were placed in fresh M199 medium supplemented with 10% FBS and cultured for