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Short Communication

Endothelium in the pharyngeal arches 3, 4 and 6 is derived from the second heart field

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ABSTRACT

Oxygenated blood from the heart is directed into the systemic circulation through the aortic arch arteries (AAAs). The AAAs arise by remodeling of three symmetrical pairs of pharyngeal arch arteries (PAAs), which connect the heart with the paired dorsal aortae at mid-gestation. Aberrant PAA formation results in defects frequently observed in patients with lethal congenital heart disease. How the PAAs form in mammals is not understood. The work presented in this manuscript shows that the second heart field (SHF) is the major source of progenitors giving rise to the endothelium of the pharyngeal arches 3 – 6, while the endothelium in the pharyngeal arches 1 and 2 is derived from a different source. During the formation of the PAAs 3 – 6, endothelial progenitors in the SHF extend cellular processes toward the pharyngeal endoderm, migrate from the SHF and assemble into a uniform vascular plexus. This plexus then undergoes remodeling, whereby plexus endothelial cells coalesce into a large PAA in each pharyngeal arch. Taken together, our studies establish a platform for investigating cellular and molecular mechanisms regulating PAA formation and alterations that lead to disease.

1. Introduction

During embryogenesis, paired pharyngeal arch arteries (PAAs) form symmetrically relative to the embryonic midline, connecting the heart with the dorsal aortae. In birds and mammals, the first and second PAAs regress, whereas the PAAs 3, 4 and 6 undergo asymmetric remodeling giving rise to the aortic arch arteries (AAAs) (Hutson and Kirby, 2007; Olson, 2002). Defects in PAA development are common features of congenital heart disease, warranting further detailed investigation into cellular and molecular mechanisms regulating their formation.

The argument of whether or not mammalian PAAs arise by angiogenesis or by vasculogenesis dates back to the early 1900s (Congdon, 1922). Investigations of human, rabbit, chicken and mouse embryos demonstrated the presence of endothelial plexus in the pharyngeal region prior to the appearance of PAA lumens, and it was hypothesized that this vascular plexus gave rise to the PAAs (Bremer, 1912; Congdon, 1922; DeRuiter et al., 1993a, 1993b, 1992; Li et al., 2012; Waldo et al., 1996). However, the origin of the endothelial cells in the plexus and subsequently, in the PAAs has remained unknown. Resin filling of embryonic vasculature demonstrated the presence of small vascular branches connected to the dorsal aortae and the aortic sac at early stages of PAA formation, suggesting that the PAA endothelium arose by branching off from these vessels (Hiruma et al., 2002). In contrast, recent studies using genetic labeling and the Tie2-Cre transgenic mice ruled out the dorsal aorta as the source of the PAA endothelium (Li et al., 2012). Understanding the origin of endothelial progenitors giving rise to the PAAs is imperative for elucidating the mechanisms regulating PAA formation and alterations that lead to congenital heart disease.

Broadly, the anterior mesoderm, which includes the PAA endothelium arises from *Mesp1*-expressing progenitors during early gastrulation (Liang et al., 2014; Papangeli and Scambler, 2013). It is also known that a small proportion of the PAA endothelium expresses

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Nkx2.5 or derives from Nkx2.5-expressing progenitors (Paffett-Lugassy et al., 2013). However, a precise embryonic tissue that gives rise to the PAA endothelium in mice is unknown. It is also not known how endothelial progenitors are recruited into the pharyngeal arches and the mechanisms regulating the formation of PAA lumens.

In the studies described below, we determined that the PAA endothelium in the pharyngeal arches 3 – 6 arises from a subset of the splanchnic mesoderm within the *Mesp1* lineage. This subset is defined by the expression of the *Isl1* transcription factor and the Mef2C-AHF-Cre transgene and is known as the second heart field (SHF) (Evans et al., 2010; Verzi et al., 2005; Vincent and Buckingham, 2010). Using carefully-staged mouse embryos, we demonstrate that cells within the SHF express *VEGFR2*, delaminate from the SHF, migrate into the pharyngeal mesenchyme, and form a plexus of small blood vessels, which then remodels into the PAA in each arch.

Mutations in *Tbx1*, *Gbx2*, *PlexinD1* and *Fgf8* interfere with PAA formation (Calmont et al., 2009; Gitler et al., 2004; Jerome and Papaioannou, 2001; Lindsay et al., 2001; Macatee et al., 2003; Merscher et al., 2001); However, which particular stages of PAA formation are regulated by these genes is unknown. Identification of PAA endothelial progenitors and cellular mechanisms regulating PAA formation are important steps toward understanding the etiology of congenital heart disease.

2. Materials and methods

2.1. Mouse strains

Cdh5(PAC)-CreERT2 transgenic mice were a gift from Dr. Ralph Adams (Wang et al., 2010). Rosa^{mTmG} mice, Gt(ROSA)26Sortm4(ACTB-tdTomato–EGFP) generated by (Muzumdar et al., 2007) were purchased from Jackson Labs (stock number 007676). Tg(Mef2c-cre)2Blk/Mmnc strain, also known as the Mef2C-AHF-Cre transgenic mice (Verzi et al., 2005) were obtained from the Mutant Mouse Resource and Research Center (stock number 030262-UNC). The *Isl1^{Cre}* strain was a gift from Sylvia Evans (Cai et al., 2003). All experimental procedures were approved by the Institutional Animal Care and Use Committee of Thomas Jefferson University and conducted in accordance with federal guidelines for humane care of animals.

2.2. Tracing endothelial lineage in pharyngeal arches

Tamoxifen solution (20 mg/ml) was prepared by dissolving Tamoxifen (Sigma, #T5648) in corn oil. The solution was heated at 40 °C and rocked at 1000 rpm using Eppendorf Thermomixer® for 3 h; insoluble particles were removed by centrifugation. To label endothelial cells with GFP prior to the formation of the PAAs 3 – 6, we crossed ROSA^{mTmG} females with Cdh5(PAC)-CreERT2 transgenic males, and injected 4 mg of tamoxifen per 25 g of body weight, into pregnant females in the morning (10–11 am) of day E7.5. Embryos were dissected in the morning of day E9.5, and were subjected to whole mount staining with the antibodies against GFP (1:500, Aves labs, #GFP-1020) and VEGFR2 (1:200, R & D, #AF644). Nuclei were stained with DRAQ5. Stained embryos were then imaged using Olympus FV500 confocal microscope. Three-dimensional (3D) reconstructions and surface renderings were performed using Imaris (Bitplane, USA).

2.3. Whole-mount immunofluorescence staining and confocal image acquisition

Embryos at E9.5 or E10.5 were dissected and fixed at 4 °C in 4% paraformaldehyde overnight for ~ 15 h with gentle rocking. Embryos were then rinsed and washed with PBS every 30 min for 1–2 h. The number of somites (s) was counted in each embryo analyzed. Prior to staining, the head, forelimb buds and trunk posterior to the forelimb buds in E10.5 embryos were trimmed off. E9.5 embryos and younger were used without trimming. During all incubations, embryos were kept in 2 ml eppendorf tubes (Fisherbrand, #02-681-258), with 1 embryo per tube. Embryos were first incubated in 500 µl of blocking buffer (PBS with 0.1% Triton X100 and 10% Donkey serum) overnight at 4 °C with gentle rocking, then with 450 µl of blocking buffer containing 1° antibodies for 72 h at 4 °C, with gentle rocking. The following 1° antibodies were used: anti-PECAM1 (BD Pharmingen, #553370, 1:200), anti-VEGFR2 (R & D, AF644, 1:200), anti-ERG (Abcam, ab110639, 1:100), and anti-GFP (Aves lab, #GFP1020, 1:500). After the incubation with 1° antibodies, embryos were rinsed and washed every hour with PBST (PBS with 0.1% Triton X100) for at least 5 h. Embryos were then incubated with 450 µl of 2° antibodies diluted 1:300 in blocking buffer for 48 h at 4 °C. Alexa-labeled 2° antibodies were purchased from Invitrogen. FITC conjugated anti-chicken IgY antibodies were from Jackson Immunoresearch, #703-546-155. After staining with 2° antibodies, embryos were washed as above, and incubated with DRAQ5 (1:500 dilution in PBST, Cell signaling technology, #4048) for 48 h to stain nuclei. This extra incubation step was necessary because the presence of serum in the blocking buffer interferes with DRAQ5 staining. Prior to imaging, embryos were dehydrated in 50% methanol in PBS for 5 min, followed by 100% methanol two times for 5 min each, and cleared in BABB generated by mixing benzyl alcohol (Sigma, #B1042) and benzyl benzoate (Sigma, #B6630) at 1:2 (v/v). This procedure eliminates the native GFP and tdTomato fluorescence from ROSA^{mTmG} mice, allowing the use of any combination of the fluorophores described above. Images of entire pharyngeal arches were acquired with Olympus FV500 confocal microscope, collecting optical sections every 0.62 µm through the entire thickness of the embryo.

2.4. 3D quantitative analyses of confocal images

Overall, we analyzed 6 pharyngeal arches from embryos having 30–31 somites (s), 16 pharyngeal arches from embryos with 33–34 s, and 10 pharyngeal arches from embryos with 36–39 s 3D image reconstruction and analyses of cell numbers and distributions were performed using Imaris, and the methodology is illustrated in Sup. Figs. 1–2. To label and quantify the number of endothelial cells in the PAA and plexus of the 4th arch, we used the surface function in Imaris to create a segment encompassing the 4th pharyngeal arch, excluding all other regions (Sup. Fig. 1**A – C**). We then generated a new channel encompassing all of the endothelial cells within this segment (Sup. Fig. 1**D**, region in red). To separate the PAA endothelium from the plexus, we segmented the pharyngeal arch artery endothelium as shown in Sup. Fig. 1**F – G** and generated a new surface containing Pecam1$^+$ cells within the PAA (Sup. Fig. 1**H**). We then generated a new channel containing endothelial cells in the PAA (Sup. Fig. 1**I, PAA**). The channel with plexus endothelial cells was generated by excluding endothelial cells in the PAA (Sup. Fig. 1**J**). To quantify the number of endothelial cells, ERG$^+$ nuclei within these channels were counted using the Spots function in Imaris, as illustrated in Sup. Fig. 2. This number was confirmed by manual counting in several samples.

The number of SHF-derived endothelial cells in each PAA was quantified in 4 E10.5 embryos, ranging from 33 s to 35 s, 2 of these embryos were from the *Isl1^{Cre}; Rosa^{mTmG}* strain and the other 2 embryos were from the Mef2C-AHF-Cre; *Rosa^{mTmG}* strain. All embryos were stained with antibodies to GFP, VEGFR2 and ERG, and pharyngeal arches were imaged using confocal microscopy, collecting optical sections through the entire thickness of the embryo, sampling every 0.62 µm. The number of GFP$^+$ and GFP$^-$ endothelial cells in each pharyngeal arch was determined in optical sections spaced 15 – 20 µm, using the Imaris software. A total of 707 endothelial cells were examined in the 3rd PAAs, 589 endothelial cells were analyzed in the 4th PAAs, and 370 endothelial cells were analyzed in the 6th PAAs.