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The LIM-homeodomain transcription factor Islet2a promotes angioblast migration

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ABSTRACT

Angioblasts of the developing vascular system require many signaling inputs to initiate their migration, proliferation and differentiation into endothelial cells. What is less studied is which intrinsic cell factors interpret these extrinsic signals. Here, we show the Lim homeodomain transcription factor *islet2a (isl2a)* is expressed in the lateral posterior mesoderm prior to angioblast migration. *isl2a* deficient angioblasts show disorganized migration to the midline to form axial vessels and fail to spread around the tailbud of the embryo. *Isl2a* morphants have fewer vein cells and decreased vein marker expression. We demonstrate that *isl2a* is required cell autonomously in angioblasts to promote their incorporation into the vein, and is permissive for vein identity. Knockout of *isl2a* results in decreased migration and proliferation of angioblasts during intersegmental artery growth. Since Notch signaling controls both artery-vein identity and tip-stalk cell formation, we explored the interaction of *isl2a* and Notch. We find that *isl2a* expression is negatively regulated by Notch activity, and that *isl2a* positively regulates *flt4,* a VEGF-C receptor repressed by Notch during angiogenesis. Thus Isl2a may act as an intermediate between Notch signaling and genetic programs controlling angioblast number and migration, placing it as a novel transcriptional regulator of early angiogenesis.

1. Introduction

During development of the vascular system, angioblasts, the precursors of endothelial cells, transform from a group of individual cells into a branched tree of arterial and venous networks through specification, migration and tubular morphogenesis. The mechanisms by which angioblasts migrate and are assigned to arterial and venous identities have been the subject of intense investigation.

Angioblasts are specified in both the anterior and posterior lateral posterior mesoderm (lpm) forming the head and trunk vessels respectively. Both populations migrate medially to form the first great vessels of the embryo by vasculogenesis. How the artery and vein organize into separate vessels is not completely clear. Lineage studies in zebrafish suggest that artery and vein cells may be specified in the lpm prior to migration (Zhong et al., 2001), while other studies suggest that an artery forms first, followed by transfer of cells to form a vein (Herbert et al., 2009). Transfer of artery and vein cells is also observed in the mouse embryo (Kim et al., 2008). While vessels that form later by angiogenesis express distinct molecular markers (Wang et al., 1998), artery and vein specific markers are often not expressed or co-expressed in both vessels in the early embryo. Recently Kohli et al., observed two stripes of migrating angioblasts in the early embryo with the inner stripe more likely to become artery, and the outer more slowly migrating stripe more likely to become vein (Kohli et al., 2013).

Recent genetic studies have demonstrated that artery-vein identity is genetically determined (Lamont and Childs, 2006; Swift and Weinstein, 2009). Notch signaling is a major contributor to arterial identity as loss of Notch leads to a decrease in the number of cells expressing arterial markers and an increase in cells expressing venous markers (Siekmann and Lawson, 2007). While a number of transcription factors and signaling molecules have been identified to promote arterial identity in addition to Notch (Diez et al., 2007; Fischer et al., 2004; Kim et al., 2008; Krebs et al., 2004; Siekmann and Lawson, 2007; Zhong et al., 2001), only one transcription factor has been identified that promotes a venous identity, the orphan nuclear receptor CoupTFII (Nr2f2). CoupTFII is expressed by venous endothelial cells in mice (Pereira et al., 1999;

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Yamazaki et al., 2009; You et al., 2005) and CoupTFII null mice have a severe reduction or complete loss of the bilateral trunk cardinal veins (You et al., 2005) indicating that it plays a key role in their development.

Notch signaling is also used again during later vascular development to decide which cell of a developing sprout will take the lead position. During angiogenesis of the zebrafish intersegmental arteries (ISAs), angioblasts sprouting from the dorsal aorta either take on highly motile tip cell that senses attractive and repulsive cues in the extracellular environment through the extension of filopodia, or a stalk cell that lumenizes to form a patent vessel (Gerhardt et al., 2003). Angioblasts actively compete for the tip cell position through VEGFR control of Dll4 expression (Jakobsson et al., 2010). In turn, antagonistic regulation by Notch-Dll4 of the vascular endothelial growth factor c (Vegfc) – Fms-related tyrosine kinase 4 (Flt4) signaling cascades regulates the number of angioblasts contributing to each ISA. Knockdown of Notch signaling results in a significant increase in the number of angioblasts per ISA and increase in Flt4 expression (Siekmann and Lawson, 2007). Conversely, loss of Vegfc or Flt4 leads to stalled ISA growth and a decreased number of angioblasts in each ISA (Covassin et al., 2006; Siekmann and Lawson, 2007). Transient activation of Notch signaling also results in stalled ISA growth mid-somite, suggesting that Notch represses the Vegfc-Flt4 signaling cascade (Leslie et al., 2007).

Although the upstream pathways leading to arterio-venous specification are known, the effector molecules within each type of vessel are not completely characterized. Here, we sought to identify additional molecules that contribute to vessel growth and patterning. The Islet family of LIM homeodomain transcription factors is involved in the specification of different cell types including pancreatic and cardiovascular lineages (Ahlgren et al., 1997; Cai et al., 2003; Pfaff et al., 1996; Thaler et al., 2004) as well as the differentiation of several neuronal lineages (Elshatory et al., 2007; Elshatory and Gan, 2008; Pan et al., 2008). In the cardiovascular lineage, Isl1 plays a key role in the specification of all cell types of the heart including cardiomyocyte, endothelial and smooth muscle lineages (Bu et al., 2009; Cai et al., 2003; Kwon et al., 2009). In contrast, there is no reported cardiovascular phenotype after loss of Isl2 (Thaler et al., 2004). Here we demonstrate that zebrafish *isl2a* is expressed in the lateral posterior mesoderm that gives rise to angioblasts and show that it promotes angioblast migration, promoting assembly into the vein and ISA sprouting.

2. Materials and methods

2.1. Zebrafish strains

Wild type zebrafish Tupfel long fin (TL) embryos were raised and maintained according to Westerfield (Westerfield, 1995) with approval from the University of Calgary Animal Care Committee. Embryos were treated with 0.2 mM 1-Phenyl-2-Thiourea (PTU) (Sigma, St. Louis, MO) at four hours post fertilization (hpf) to prevent pigment formation. The transgenic lines: Tg(kdrl:GFP)^{la116}, Tg(fli1a:negfp)^{y7}, Tg(fli1a:egfp)^{y1} and Tg(kdrl: mCherry)^{ci5} have been described (Choi et al., 2007; Proulx et al., 2010; Roman et al., 2002).

2.2. In situ hybridization, staining and imaging

Templates for wholemount in situ hybridization (Lauter et al., 2011) for *isl2a* and *fli1a* were obtained by PCR using primers in Table S1 followed by in vitro transcription using T7 Polymerase (Promega, Madison, WI). The *flt4, mrc1, notch3* and *ephrinB2b* probes have been described (Lawson et al., 2001; Wong et al., 2009). Stained embryos were embedded in 3% methylcellulose and photographed with a Zeiss Axiocam HR camera (Carl Zeiss, Inc, Thornwood, NY) on a Zeiss SV11 stereomicroscope.

The number of cells in the vein and ISAs was counted from slices of confocal stacks. Confocal images were collected on either a Zeiss LSM510 or Zeiss LSM700 confocal microscope, and presented as maximal intensity projections generated in ImageJ or Zeiss Zen. For histology, embryos were sectioned at 5–7 μm in JB-4 plastic medium (Polysciences, Warrington, PA) and photographed with a Magnafire camera (Optronics, Galeta, CA).

2.3. Morpholino, mRNA and DNA injections

Morpholinos for each gene were obtained from Gene-Tools, LLC (Philomath, OR; sequences, doses and references are listed in Table S2) or Open Biosystems (Huntsville, AL) and resuspended in water. For enhanced knockdown, isl2a^{ATG} and isl2a^{e4i4} morpholinos were co-injected (*isl2a*^{MO}). Unless specified, all images show the phenotype with injection of the combination (isl2^{MO}).

Dominant negative *isl2a* (*isl2a*^{LIM}) or wild type *isl2a* coding region was amplified from cDNA as previously described (Segawa et al., 2001) using primers in Table S1. The Tol2kit was used to generate all constructs (Kwan et al., 2007). A Gateway compatible 0.8 kb *fli1a* promoter has been described (Christie et al., 2010). The p3E viral A2TagRFP construct was a kind gift from Chi-Bin Chien (Poulain and Chien, 2013). For transient expression, approximately 100 ng of plasmid DNA was co-injected with 25 pg of Tol2 mRNA into single-cell embryos.

2.4. Cell transplantation

Tg(kdrl:GFP)^{la116} donor embryos were injected with 10,000 MW rhodamine dextran (Invitrogen) and *isl2a*^{MO}. Some recipient embryos were also injected with *isl2a*^{MO}. At 30–50% epiboly, 50–100 cells were transplanted from donor embryos into the dorsal margin of non-GFP recipient embryos in 1 × Danieau solution. Recipient embryos developed in 0.3 × Danieau solution and 2.5 × Penicillin/Streptomycin (Invitrogen). At 30 hpf, recipient embryos were sorted for the presence of *kdrl:GFP* positive endothelial cells, mounted and photographed. Each experiment was performed at least twice to ensure reproducibility. Data was analyzed by the Chi Square statistic.

2.5. RNA extraction and quantitative RT-PCR (qPCR)

Total RNA was purified using the RNeasy Mini kit (Qiagen). Single strand cDNA was synthesized with Superscript III reverse transcriptase and oligo-dT primer (Invitrogen) according to the manufacturer's instructions. Quantitative RT-PCR was performed using the DNA Engine Opticon System (MJ Research Inc.) with iQ SYBR Green Supermix (BioRad) or using a StepOnePlus (Life Technologies) with SYBR Green PCR Master Mix (Life Technologies). Primers are listed in Table S1. Relative gene expression levels were analyzed by the $\Delta\Delta C_t$ method, with elongation factor 1α (EF1α) or beta actin 1 (actb1) as a reference gene. All reactions were performed as technical triplicates on biological triplicates.

2.6. DAPT treatment

A stock solution of 25 mM DAPT (Toronto Research Chemicals, North York, ON) dissolved in DMSO was diluted to 75 μM and added to embryos at 6 hpf. Control embryos were treated with an equivalent dose of DMSO alone.