Developmental Biology ■ (■■■■) ■■■–■■■

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/locate/developmentalbiology

Patterning mechanisms of the sub-intestinal venous plexus in zebrafish

Michela Goi, Sarah J. Childs *

Department of Biochemistry and Molecular Biology and Alberta Children's Hospital Research Institute, University of Calgary, 3330 Hospital Drive NW, Calgary, AB, Canada T2N 4N1

ARTICLE INFO

Article history:
Received 23 February 2015
Received in revised form
5 October 2015
Accepted 12 October 2015

Keywords:
Zebrafish
Vein
Sub-intestinal venous plexus
Vegf
Bmp
PlexinD1

ABSTRACT

Despite considerable interest in angiogenesis, organ-specific angiogenesis remains less well characterized. The vessels that absorb nutrients from the yolk and later provide blood supply to the developing digestive system are primarily venous in origin. In zebrafish, these are the vessels of the Sub-intestinal venous plexus (SIVP) and they represent a new candidate model to gain an insight into the mechanisms of venous angiogenesis. Unlike other vessel beds in zebrafish, the SIVP is not stereotypically patterned and lacks obvious sources of patterning information. However, by examining the area of vessel coverage, number of compartments, proliferation and migration speed we have identified common developmental steps in SIVP formation. We applied our analysis of SIVP development to *obd* mutants that have a mutation in the guidance receptor PlexinD1. *obd* mutants show dysregulation of nearly all parameters of SIVP formation. We show that the SIVP responds to a unique combination of pathways that control both arterial and venous growth in other systems. Blocking Shh, Notch and Pdgf signaling has no effect on SIVP growth. However Vegf promotes sprouting of the predominantly venous plexus and Bmp promotes outgrowth of the structure. We propose that the SIVP is a unique model to understand novel mechanisms utilized in organ-specific angiogenesis.

1. Introduction

Organ-specific regulation of sprouting, migration, proliferation and vascular network establishment during angiogenesis is one of the less well understood aspects of vascular development (Geudens and Gerhardt, 2011). One important role of the vascular system is to deliver nutrients acquired from the mother, or from absorption from the digestive system, to tissues and organs. To do so, blood vessels need to be in close contact with embryonic nutrient sources. In the adult, digestive system vessels need to be organized in highly efficient vascular networks.

Here, we focus on the development of vessels in the sub-intestinal venous plexus (SIVP), a set of predominantly venous angiogenic vessels that initially obtain nutrients from the yolk and transfer them to the developing embryo body through the adjacent yolk syncytial layer (YSL), and that will later support the distribution of blood to the digestive system in the larva and adult fish (reviewed in Carvalho and Heisenberg, 2010; Donovan et al., 2000). The developing sub-intestinal venous plexus (SIVP) has been used as an easily visible vascular bed to screen for molecules that influence angiogenesis including pro- or anti-angiogenic factors (Chan et al., 2012; Kuo et al., 2011; Nicoli et al., 2009, 2007; Nicoli and Presta, 2007; Raghunath et al., 2009; Serbedzija et al., 1999). However, the study of the effects of these molecules is limited by poor knowledge of SIVP development, including whether or not this venous plexus is similar to other vascular beds in its development.

Little is known about early development of visceral vasculature in any animal model system but an anatomical atlas suggests that in zebrafish the supraintestinal artery (SIA) that delivers blood, and the bilateral sub-intestinal veins that collect the blood from the digestive system, start to develop around 2 dpf (Isogai et al., 2001). The SIVP is suggested to sprout from the duct of Cuvier (future common cardinal vein) and connect to the posterior cardinal vein (PCV) (Isogai et al., 2001; Nicoli and Presta, 2007). Around 3 dpf, the SIVP which has extended on the large surface of the yolk ball, appears as a vascular basket with compartments delimited by veins. The most anterior part of the right and left SIVPs drain into the hepatic sinusoids of the liver through the two hepatic portal veins. At 4 dpf, with the reduction of yolk size as the embryo feeds on it, the left SIVP starts regressing and empties into the right SIVP. Later, the blood from the posterior gut will only use the right SIVP to reach the liver (Isogai et al., 2001). Given that this plexus vascularizes essential visceral organs, gross defects in SIVP

* Corresponding author.
E-mail address: schilds@ucalgary.ca (S.J. Childs).

http://dx.doi.org/10.1016/j.ydbio.2015.10.017
0012-1606/

patterning are likely not compatible with life.

Extrinsic cues and intrinsic receptors guide the morphogenesis of the vascular system. Vascular endothelial growth factor A (VegfA) induces endothelial cell proliferation and migration while inhibiting apoptosis (Carmeliet et al., 1996; Liang et al., 2001; Shalaby et al., 1995). During intersegmental vessel (ISV) angiogenesis, *vegfA* is expressed mid-somite around the notochord in a gradient to attract the sprouting vessels while its receptor (*vascular endothelial growth factor receptor 2*, *vegfr2*) is expressed by the angioblasts (Fouquet et al., 1997). In the zebrafish, *vegfA* expression is induced by *sonic hedgehog* (*shh*) expression at the midline (Lawson et al., 2002). Together with Vegf, Notch signaling is necessary for arterial specification in the trunk, and for the decision to take on a tip (migratory, proliferative) or stalk (non-migratory, non-proliferative) identity in the developing intersegmental arteries of the zebrafish embryo (Siekmann and Lawson, 2007). Platelet-derived growth factor (Pdgf) signaling has also been reported to induce ISV sprouting (Wiens et al., 2010).

Venous sprouting can be easily studied in the fish as it is clearly visible in real time. Venous ISVs form through cellular emigration from the PCV (Isogai et al., 2003; Yaniv et al., 2006) to connect with the arterial ISVs and require VegfC/Flt4 signaling. *vegfC* ligand is expressed in the dorsal aorta (DA) and its receptor *flt4* (*vegfr3*) in the sprouting cells of the vein (Covassin et al., 2006; Hogan et al., 2009a, 2009b). Bone morphogenetic protein (Bmp) is an important cue for venous migration ventrally during formation of the caudal venous plexus (CVP). Interestingly, VegfA is not required for CVP sprouting suggesting a difference between arterial and venous sprouting in the formation of this bed (Wiley et al., 2011). Venous sprouting of the CVP is also sensitive to perturbation in prenylation (Choi et al., 2011) and Sphingosine-1-phosphate signaling. The S1P1 receptor is expressed in endothelial cells and inhibits filopodia formation to stabilize the vascular network. Absence or reduction of S1P1 in the CVP causes excessive filopodial extensions resulting in a fused plexus instead of the wild-type honeycomb-like structure (Ben Shoham et al., 2012; Mendelson et al., 2013). A third example of venous sprouting can be found in formation of the common cardinal veins which occurs by lumen ensheathment and is sensitive to *vegfC* levels (Helker et al., 2013). These examples highlight diverse mechanisms and cues for venous sprouting in different organs. Here we characterize the sprouting of a fourth venous bed the SIVP and find significant differences in the cues and morphology of its development to other venous beds.

Only a few mutants show growth defects in the SIVP. *out of bounds* (*obd*) mutants have a mutation in the angiogenic guidance receptor *plexinD1* and an overgrown SIVP (Childs et al., 2002). *obd* ISVs also show disrupted control of timing and direction of angioblast migration from the dorsal aorta and an altered and overgrown caudal vein plexus (Childs et al., 2002; Torres-Vazquez et al., 2004). Plexins are transmembrane semaphorin (Sema) receptors that provide guidance for migrating angioblasts, axonal guidance and pruning, sensory-motor circuit connectivity and immune system development (Gay et al., 2011). A model for Sema-PlexinD1 signaling suggests that integrin based adhesion is lost when PlexinD1 receptor is activated by ligand causing retraction of filopodia and cellular detachment from the extracellular matrix, thus restricting migration (Sakurai et al., 2010). In the trunk, *sema3* ligands are expressed in the somites and *plexinD1* in the endothelium. *plexinD1* expressing angioblasts receive a repulsive signal when they contact the somite thus limiting their pathway to the space between somites (Torres-Vazquez et al., 2004; Zygmunt et al., 2011). Semaphorin-PlexinD1 signaling also has a second function in promoting *delta-like 4* (*dll4*) expression in tip cells downstream of VegfA signaling and thereby altering the tip-stalk cell balance to limit angiogenesis (Kim et al., 2011).

SIVP development is affected by lipoprotein levels. Mutation in the microsomal triglyceride transfer protein (*mtp*) causes excessive angiogenesis in the SIVP resulting in defective yolk absorption. *mtp* is expressed in the zebrafish yolk syncytial layer (YSL) and in the larval/adult gut and is important for the proper production of ApoB-containing lipoproteins (such as LDLs) that deliver lipids (Avraham-Davidi et al., 2012; Hussain et al., 2008; Marza et al., 2005). Low concentrations of lipoproteins decrease levels of *sflt1*, the soluble Vegfr1 (sFlt1) receptor. Since sFlt1 sequesters VegfA and therefore decreases signaling through Vegfr2 the end result is to enhance angiogenic sprouting (Avraham-Davidi et al., 2012; Kendall and Thomas, 1993; Roberts et al., 2004; Zygmunt et al., 2011). Interestingly, the intestinal lymphatics also grow in close relationship with the SIVP, may also have a role in lipid transportation, and may share similar signaling control (Okuda et al., 2012).

Using live imaging we trace SIVP development in real time. We find that the features of the developing SIVP are not hard-wired as they are in some other vascular beds of the zebrafish. The SIVP shows variable patterning among embryos, although we find common developmental morphologies. We identify commonalities in wild-type SIVP development in order to describe SIVP morphogenesis and apply it to the genetic *obd* mutants. Small molecule inhibition of the Vegf, Bmp and Mek/Erk pathways, but not other signaling pathways used in arterial growth, inhibit the proper formation of the SIVP. Our results suggest the developing gut vasculature responds to a unique set of growth factors, and is a model to shed insight into mechanisms of visceral organ angiogenesis.

2. Materials and methods

2.1. Zebrafish embryos

Embryos were collected and dechorionated through a brief treatment with pronase (Sigma-Aldrich, St. Louis, MO), incubated at 28.5 °C in E3 embryo medium and staged in hours post-fertilization (hpf) or days post fertilization (dpf). Endogenous pigmentation was inhibited from 24 hpf by the addition of 0.003% 1-phenyl-2-thiourea (PTU, Sigma-Aldrich) in E3 embryo medium. The fluorescent transgenic lines *Tg(fli:EGFP)^{y1}* (Lawson and Weinstein, 2002), *Tg(fli:EGFP)^{y7}* (Roman et al., 2002) were used to visualize cells and nuclei of endothelial cells respectively. *out of bounds* homozygous embryos *obd^{fov01b}* were used in all experiments using mutants (Childs et al., 2002). Morpholino knockdown (Gene Tools LLC, Corvallis, OR) used the following sequences: *bmp4* (5′-GTCTCGACAGAAAATAAAGCATGGG-3′) (Zeng and Childs, 2012), *vegfaa* (5′-GTATCAAATAAACAACCAAGTTCAT-3′) (Childs et al., 2002), *vegfab* (5′-GGAGCACGCGAACAGCAAAGTTCAT-3′) (Bahary et al., 2007) and *plexinD1* (5′-TGAGGGTATTTA-CAGTCGCTCCGC-3′) (Torres-Vazquez et al., 2004), at doses of 7, 2, 2 and 15.5 ng/embryo respectively.

2.2. Inhibitor treatments

Drug stocks were heated for 20 min at 65 °C and then diluted in E3 embryo medium and added to embryos from 4 or from 24 hpf. DMSO (D8418, Sigma) was used as a vehicle and control. Embryos were grown at 28.5 °C in the dark until imaging. Doses and sources are listed in Table S1.

2.3. Confocal imaging and measurements

Up to 10 embryos were mounted in 1% low melt agarose (Invitrogen) on glass bottom dishes (MatTek, Ashland MA) and imaged using a Zeiss LSM700 microscope using ZEN Black 2012

Please cite this article as: Goi, M., Childs, S.J., Patterning mechanisms of the sub-intestinal venous plexus in zebrafish. Dev. Biol. (2015), http://dx.doi.org/10.1016/j.ydbio.2015.10.017