Rspo1/Wnt signaling promotes angiogenesis via Vegfc/Vegfr3

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SUMMARY
Here, we show that a novel Rspo1-Wnt-Vegfc-Vegfr3 signaling pathway plays an essential role in developmental angiogenesis. A mutation in R-spondin1 (rspo1), a Wnt signaling regulator, was uncovered during a forward-genetic screen for angiogenesis-deficient mutants in the zebrafish. Embryos lacking rspo1 or the proposed rspo1 receptor kremen form primary vessels by vasculogenesis, but are defective in subsequent angiogenesis. Endothelial cell-autonomous inhibition of canonical Wnt signaling also blocks angiogenesis in vivo. The pro-angiogenic effects of Rspo1/Wnt signaling are mediated by Vegfc/Vegfr3(Flt4) signaling. Vegfc expression is dependent on Rspo1 and Wnt, and Vegfc and Vegfr3 are necessary to promote angiogenesis downstream from Rspo1-Wnt. As all of these molecules are expressed by the endothelium during sprouting stages, these results suggest that Rspo1-Wnt-Vegfc-Vegfr3 signaling plays a crucial role as an endothelial-autonomous permissive cue for developmental angiogenesis.

KEY WORDS: Angiogenesis, Vegfc, Zebrafish

INTRODUCTION
The Wnt and vascular endothelial growth factor (VEGF) signaling pathways are highly conserved mediators of cell fate specification and patterning (Rossant and Howard, 2002). VEGF signaling plays a well-documented central role in the vasculature, regulating endothelial specification, differentiation, migration and survival during angiogenesis and lymphangiogenesis (Olsson et al., 2006). Wnt signaling has also been implicated in endothelial function. Many Wnt signaling molecules are expressed in the developing vessels, and studies carried out in vitro using cultured endothelial cells have documented effects of Wnt signaling on endothelial proliferation, survival and migration (Franco et al., 2009; Liebner et al., 2008; Zerlin et al., 2008). Several in vivo studies on Wnt/β-catenin pathway signaling have also reported that this signaling pathway regulates various aspects of cardiovascular development, including artery/vein specification, blood-brain barrier formation and vascular smooth muscle integrity (Cohen et al., 2008; Corada et al., 2010; Hurlstone et al., 2003; Shu et al., 2002; Stenman et al., 2008). However, targeted disruption of Wnt signaling genes in mice has not resulted in widespread dramatic defects in developmental angiogenesis, perhaps owing to extensive functional overlap between these genes, and the in vivo role of Wnt signaling in the vasculature and the identity of crucial downstream pathways remain unclear (Franco et al., 2009; Ishikawa et al., 2001; Monkley et al., 1996; Xu et al., 2004; Ye et al., 2009).

A variety of genes have been shown to influence the Wnt signaling pathway or its output by modulating activities of Wnt pathway members (Chen et al., 2007; Cohen et al., 2008; Junge et al., 2009). Among these, R-spondins are a family of secreted ligands (Rspo-1–4) that have been shown to enhance Wnt signaling (Kim et al., 2008). Frizzled, LRP6 and a specific receptor called kremen are believed to act as a receptor complex for Rspo signaling (Binnerts et al., 2007; Kazanskaya et al., 2004; Kim et al., 2008; Nam et al., 2007; Wei et al., 2007), although their mechanism of action is not well understood (Binnerts et al., 2007; Kazinski et al., 1991; Nam et al., 2006; Wei et al., 2007). Different R-spondin family members have been shown to modulate a wide variety of functions in different tissues (Hendrickx and Leyns, 2008; Kim et al., 2006), including in the vasculature. R-spondin 3 has been shown to promote expression of VEGFA in Xenopus and mouse embryos and to regulate endothelial specification and differentiation (Kazanskaya et al., 2008). Wnt signaling is also reported to promote expressions of VEGFA and VEGFC in cultured human endothelial and tumor cells (Skurk et al., 2005; Zhang et al., 2001). A recent study also reported that transgenic expression of an activated form of β-catenin affects vascular remodeling and arterial-venous specification by activating Dll4 expression and thus increasing Notch signaling (Corada et al., 2010). Dll4/Notch signaling regulates angiogenesis and tip cell specification in mouse and zebrafish embryos (Elkholm et al., 2007; Lobov et al., 2007; Roca and Adams, 2007; Siekmann and Lawson, 2007). Despite these and other reports implicating Wnt signaling in various aspects of endothelial function, little is yet known about how its modulators affect blood vessel formation and vascular development in vivo.

Here, we show that a novel Rspo1-Wnt-Vegfc-Vegfr3 signaling pathway is essential for developmental angiogenesis. During embryogenesis, mesodermally derived endothelial progenitor cells (angioblasts) first come together to form the primary vascular bed by a process called vasculogenesis. The primary vascular bed subsequently undergoes remodelling, as well as sprouting and growth of new vessels from the pre-existing vessels by a process...
called angiogenesis. Using a newly identified mutant in rspos1 and variety of other methods for functionally manipulating different members of this pathway, we show that canonical Wnt signaling is required downstream of rspos1 for endothelial sprouting angiogenesis. We also further show that Rspos1/Wnt signaling promotes sprouting angiogenesis by upregulating endothelial vegfc expression. Our results reveal a new signaling pathway required for developmental angiogenesis.

MATERIALS AND METHODS

Zebrafish strains and genetic mapping

Tg(fli-EGFP)y7 fish were maintained on the EK background and used for ENU mutagenesis. Bulked segregant analysis and fine genetic mapping were carried out as described previously (Lawson et al., 2003). The Tg(fli-ntEGFP)y7 is reported elsewhere (Roman et al., 2002). The Tg(flk:mCherry)206 line was generated using a transgene construct in which cytoplasmic mCherry is driven by the endothelial kdrl/flk1 promoter (assembled using Gateway technology). The construct was injected into EK embryos and founders were screened for endothelial mCherry expression. The brightest mCherry-expressing transgenic zebrafish was propagated to establish the line. Zebrafish strains were maintained and bred, and embryos were staged as described elsewhere (Kimmel et al., 1995; Westerfield, 2000).

Measuring endothelial cell proliferation, endothelial cell count and ISV phenotype

Endothelial cell numbers were counted using Tg(fli-ntEGFP)y7 transgenic zebrafish in which all endothelial cell nuclei are endogenously green fluorescent and can be easily counted. Mutant and wild-type embryos from the same clutch were mounted and trunk regions were imaged using 20× water immersion objective on an Olympus confocal microscope at the same clutch were mounted and trunk regions were imaged using 20× Tg(flk:mCherry)206 line was generated using a transgene construct in which cytoplasmic mCherry is driven by the endothelial kdrl/flk1 promoter (assembled using Gateway technology). The construct was injected into EK embryos and founders were screened for endothelial mCherry expression. The brightest mCherry-expressing transgenic zebrafish was propagated to establish the line. Zebrafish strains were maintained and bred, and embryos were staged as described elsewhere (Kimmel et al., 1995; Westerfield, 2000).

Plasmid DNA cloning

Endothelial specific Tole2(FLk:mCherry-2A-vegfc) and Tole2(FLk:mCherry constructs were assembled using Gateway technology (Kwan et al., 2007; Provost et al., 2007; Villefranc et al., 2007).

BIO drug treatment

A 100 mM stock solution of (2′,3′E)-6-bromoidirubin-3′-oxime (BIO; EMD biosciences, catalog number 361552) was prepared in DMSO and diluted in embryonic medium E3 to a final concentration of 0.5 mM. For early embryonic treatments, manually dechorionated embryos were placed in E3/BIO solution from 10 hpf until shield stage (6 hpf), then fixed and processed for staining. For late-stage treatments, dechorionated embryos were placed in E3/BIO solution from 16 hpf to 40 hpf and analyzed at different stages by live imaging or staining. Embryos in E3 solution containing DMSO carrier alone were used as control for all experiments.

Microinjections

Morpholinos were injected into one-to two-cell stage zebrafish embryos at the indicated doses, as described previously (Gore et al., 2008). Morpholinos used in the study are as follows: rspos1 translation blocking MO (translation start site underlined), 5′-CCAGCGCCAGCTCCCAAAGCT-3′: rspos1 exon 1 splice donor MO, 5′-AGAAGCATCAGCAGCTTCTCCGGTCT-3′; rspos1 exon 4 splice acceptor MO, 5′-CTTAGATCATCGACGCGCTGAAAAT-3′; wnt2b translation blocking MO (Ober et al., 2006), 5′-GTGTGCGATATAGGATGATCTCCGG-3′; krm1 translation blocking MO (translation start site underlined), 5′-AAGCTGCGACTTCCACAGGATCC-3′; krm1 5′-UTR MO, 5′-TGTAGTCTGACGTCTGAGCTTT-3′; krm1 exon 1 splice donor MO, 5′-GATTCCTGTGATGAAAGACAGAC-3′; vegfc translation blocking MO (translation start site underlined) (Ober et al., 2004), 5′-GAAATACCAATAAGTGACTTTAG-3′; flt4 splice donor MO (Covassin et al., 2006), 5′-TTAGGAAATGGTTCTCACTGAG-3′. Synthetic mRNA and plasmid DNA injections were carried out as described previously (Gore et al., 2008).

Endothelial cell culture and treatments

Murine endothelial cell line MSS31 was kindly provided by Dr Yanai (Tohoku University, Japan) (Yanai et al., 1991) and cultured in α-minimal essential medium (MEM, GIBCO) containing 10% FBS. For activation of Wnt signaling, cultured MSS31 endothelial cells were treated with 16 ng/ml Wnt3a (R&D) or with 6 μM (2′,3′E)-6-bromoidirubin-3′-oxime (BIO; EMD biosciences) for 2 days.

RT-PCR analysis

Total cellular RNA from morpholino injected embryos and cultured MSS31 endothelial cells was isolated using Trizol reagent and treated with DNase I. Total cDNA was synthesized using the Thermoscript or SuperScript III first-strand synthesis supermix kit (Invitrogen) and PCR analysis was carried out using Qiagen Top Taq PCR kit or by Qiagen One-Step RT-PCR kit. Primers used in this study can be found in Table S2 (supplementary material).

RESULTS

dtvt135 mutants have a specific defect in angiogenesis

We identified down the tubes (dtvt135), a zebrafish mutant with a specific defect in angiogenesis, in a Tg(fli-EGFP)y7 transgenic-based forward-genetic screen for vascular mutations. Initial vasculogenesis takes place normally in dtvt135 mutants. Primary vessels in the head (e.g. primordial hindbrain channels) and trunk (e.g. dorsal aorta, DA, posterior cardinal vein, PCV) are properly
formed and carry normal circulatory flow (data not shown). By contrast, the subsequent growth of angiogenic vessels is strongly inhibited. Trunk intersegmental vessels (ISV) are either absent or greatly reduced in number and length in 26 hpf dtty135 mutants compared with their wild-type siblings (Fig. 1A-C; see also Fig. 2L). There is some limited recovery of ISV later in development.

Fig. 1. dtty135 mutants have defects in angiogenesis, but not in endothelial specification or in vasculogenesis. (A) Diagram of a zebrafish embryo with the red box highlighting the region shown in B and C, and a blue box highlighting the region shown in D-G. (B,C) Confocal images of trunk vessels in 26 hpf Tg(fli-EGFP)y1 wild-type sibling (B) and dtty135 mutant (C) zebrafish, showing normal formation of the vasculogenic dorsal aorta and posterior cardinal vein but failure to form the angiogenic intersegmental vessels (arrows in B, asterisks show absence in C) in dtty135 mutants (lateral views, anterior towards the left). (D,E) The hindbrain vessels imaged in F,G. Primordial hindbrain channels (PHBC) are in blue, basilar artery (BA) is in red and the central arteries (CA) are in black. (F,G) Confocal images of hindbrain vessels in 48 hpf Tg(fli-EGFP)y1 wild-type sibling (F) and dtty135 mutant (G) zebrafish, showing normal formation of vasculogenic primary vessels, including the PHBC and BA, but failure to form the angiogenic CA that penetrate the hindbrain (dorsal views, anterior towards the left). (H,I) In situ hybridization of endothelium in the trunk of 26 hpf wild-type sibling (H) and dtty135 mutant (I) zebrafish with a probe for vecdn, showing normal expression levels in dtty135 mutants (lateral views, anterior towards the left). ISV are seen in wild-type siblings (arrows in H) but not in dtty135 mutants (asterisks in I). (J,K) Transmitted light images of 26 hpf Tg(fli-EGFP)y1 wild-type sibling (J) and dtty135 mutant (K) zebrafish, showing reduced width of yolk extension but otherwise normal morphology of dtty135 mutants (J) compared with their wild-type siblings (K). Images are lateral views, anterior towards the left. Endothelial proliferation is reduced in dtty135 mutants. (L) Total number of endothelial cells present in the three posteriormost trunk segments, measured in Tg(fli-nEGFP)y7 transgenic dtty135 mutant or wild-type sibling animals, at 26 hpf and 40 hpf. (M) Total number of phosphohistone H3-positive endothelial cells present in the three posteriormost trunk segments, measured in phosphohistone and GFP antibody-probed Tg(fli-nEGFP)y7 transgenic dtty135 mutant or wild-type sibling animals, at 26 hpf and 40 hpf. Data are mean±s.e.m. (N,O) Higher-magnification confocal images of ISV sprouts in wild-type sibling (N) and dtty135 mutant (O) embryos at 20 hpf, showing reduced filopodial protrusions in mutants. (P) Total number of filopodia formed by control and mutant sprouts. Scale bars: 50 μm in B,C,F,G; 100 μm in H-K; 20 μm in N,O.
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A) LG16

B) y135

C) rspo1

D) rspo1 MO, 5 ng

E) krm1 MO, 0.5 ng

F) rspo1 MO, 5 ng

G) krm1 MO, 0.5 ng

H) rspo1 MO, 2.5 ng

I) krm1 MO, 0.25 ng

J) ctrl MO

K) rspo1 MO + krm1 MO

L) rspo1 and krm1 morpholino knockdown

M) dtt5 p55

N) Murine Rspo1 protein rescue

Fig. 2. R-spondin 1 and its receptor kremen 1 are required together for angiogenesis. (A) dtt5 p55 genotype interval on linkage group 16, with the number of recombinants per meiosis, position of BAC clones and position of the rspl locus indicated. (B) Rspo1 protein domain structure, with two furin domains (FU) and a thrombospondin domain (TSP). The dtt5 p55 mutation changes serine 193 to leucine in the TSP domain. (C) A zebrafish embryo with the red box highlighting the region imaged in (D)-(K). (D,E) In situ hybridization of the trunk of 24 hpf wild-type zebrafish embryos probed for rspl (D) or krm1 (E), with expression observed in the axial vasculature (arrowheads). (F-K) Confocal images of trunk vessels in 26 hpf Tg(fli-EGFP)y1 wild-type zebrafish injected with either 5 ng rspl MO (F), 0.5 ng krm1 MO (G), 2.5 ng rspl MO (H), 0.25 ng krm1 MO (I), 5 ng control MO (J) or 2.5 ng rspl MO + 0.25 ng krm1 MO (K). (L) Quantitation of the intersegmental vessel (ISV) phenotypes of 26 hpf Tg(fli-EGFP)y1 dtt5 p55 mutant or morpholino-injected embryos. (M,N) Confocal images of ISV in 26 hpf Tg(fli-EGFP)y1 dtt5 p55 mutant embryos that were either not injected (M) or were injected intramuscularly in the trunk with murine R-spondin (N). (O) Quantitation of the intersegmental vessel (ISV) phenotypes of uninjected or mouse R-spondin-injected dtt5 p55 zebrafish at 28 hpf. In L and O the bars show the percentages of ISV that have failed to sprout (blue), ISV that have grown only up to the horizontal myoseptum halfway up the trunk (red) and ISV that have grown all the way to the dorsal trunk to form the DLAV (yellow). The number of segments counted is shown above each bar on the graphs. Scale bars: 100 μm in D; 50 μm in E; 50 μm in F-K,M,N.

(after 2 dpf) but the vessels remain stunted and unable to form a complete trunk vascular network. The lymphatic network also fails to form in dtt5 p55 mutants, although this probably reflects the lack of a properly formed primary vasculature (data not shown). Cranial central arteries (Isogai et al., 2001) are also absent in 26 hpf dtt5 p55 mutants (Fig. 1D-G). Analysis of a variety of endothelial- or hematopoietic-specific markers reveals no defects in specification or differentiation of the endothelial or hematopoietic lineages (Fig. 1H-I; supplementary material Fig. S1), and no gross morphological defects, circulation defects or overall developmental delay are observed in dtt5 p55 mutants (Fig. 1J,K and data not shown). In addition to a defect in angiogenic sprouting and endothelial cell migration, dtt5 p55 mutants are also defective in angiogenic endothelial cell proliferation. dtt5 p55 mutants initially have a normal number of endothelial cells in the DA and CV, but, unlike wild-type siblings, the number of endothelial cells does not increase at later (40 hpf) stages of development (Fig. 1L). The number of phosphohistone H3-positive endothelial cells is also reduced in mutant DA and CV compared with wild-type siblings (Fig. 1M). High-resolution imaging reveals that migration and protrusive activity of the angiogenic sprouts that do eventually form in dtt5 p55 mutants is substantially reduced (supplementary material Movies 1 and 2), with fewer filopodial structures (Fig. 1N-P). In addition to endothelial effects, dtt5 p55 mutants also show reduced skeletal musculature at later stages of trunk development. However, initial specification and patterning of muscle is normal (supplementary material Fig. S1).

R-spondin 1 and kremen 1 are required together for angiogenesis

To examine the molecular nature of the dtt5 p55 phenotype, we positionally cloned the defective locus. The mutation maps to a sequenced interval on linkage group 16 containing the R-spondin 1 (rspl) gene (Fig. 2A). R-spondins are a family of secreted ligands (Rspo1-4) that have been shown to enhance Wnt signaling. Frizzled, LRP6 and a specific receptor called kremen are believed to act as a
Canonical Wnt signaling is required endothelial cell-autonomously for angiogenesis

As noted above, previous data has suggested that Rspo/kremen signaling functions upstream from or together with the Wnt signaling pathway. During early embryogenesis, a large number of Wnt ligands are expressed in and around the developing vasculature. None of these Wnt ligands have been reported to show a strong vascular phenotype, however, suggesting that multiple Wnt ligands may contribute together to endothelial signaling in conjunction with Rspo1 in the developing trunk. We reasoned that if Rspo1 signaling is acting via the Wnt pathway then reduction of some of these Wnt ligands might show synergistic effects in combination with Rspo1 knockdown. We chose the Wnt ligand wnt2bb to test this because a previous report (Ober et al., 2006) showed that wnt2bb is expressed in endothelial cells during early stages of zebrafish embryogenesis, although its function is required for liver specification at later stages (see also Fig. 3A,B). We found that in addition to these later liver defects, wnt2bb knockdown also causes defects in ISV sprouting and growth, although these defects are not as severe as those found in dtr-135 mutants or rspo1 morphants (Fig. 3C-G). However, co-injection of lower doses of wnt2bb and rspo1 morpholinos that fail to elicit a phenotype on their own results in a strong ISV phenotype (Fig. 3F,G; P<0.0049; supplementary material Table S1), suggesting that rspo1 and wnt2bb are also required together for angiogenesis. To examine whether Wnt signaling is required cell-autonomously in
the endothelium, we inhibited Wnt/β-catenin signaling in vivo by transgenic endothelial overexpression of Axin. Axin is a negative regulator of Wnt signaling that forms a complex with GSK3β and APC, marking β-catenin for destruction in the absence of active Wnt signaling (reviewed by Angers and Moon, 2009). We expressed Axin-EGFP fusion protein using the endothelial-specific flk promoter. Unlike endothelial cells expressing the control flk:EGFP transgene (Fig. 3H,K), flk:axin-EGFP transgene-expressing endothelial cells either failed to form ISV sprouts or formed stunted ISVs (Fig. 3I,K), as observed upon depletion of Wnt2b, kremen or Rspo1 proteins. These data indicate that endothelial cell-autonomous Wnt/β-catenin signaling is required for in vivo angiogenesis.

To confirm that activation of Wnt/β-catenin signaling is required downstream from Rspo1 function during angiogenesis, we examined zebrafish homozygous for apcmcr/+, a mutation previously shown to promote maintenance of active Wnt/β-catenin signaling by reducing degradation of β-catenin (Haramis et al., 2006; Hurlstone et al., 2003). We injected rspo1 morpholinos into embryos derived from apcmcr/+ incrosses (Fig. 4A-C). Rspo1 MO-injected wild type (+/+) or heterozygous (apcmcr/+) embryos showed reduced ISV formation (Fig. 4A,C), but rspo1 MO-injected homozygous (apcmcr/apcmcr) mutants developed relatively normal ISVs (Fig. 4B,C), showing that downstream activation of β-catenin signaling can rescue the angiogenic defects caused by loss of Rspo1. This was further confirmed using treatment with BIO [(2’Z,3’E)-6-bromoindirubin-3’-oxime], a small cell-permeable drug that upregulates β-catenin signaling by inhibiting GSK3β (Meijer et al., 2003). Zebrafish embryos treated with BIO during blastula stages (2.5-6.0 hpf) ectopically express the dorsal marker chordin, as expected for early upregulation of Wnt signaling, whereas treatment during early somitogenesis increases vascular expression of known canonical Wnt/β-catenin target gene cyclin D1 (ccnd1) (Shutman et al., 1999) (Fig. 4D-G). BIO-treated rspo1 MO-injected embryos show improved sprouting and elongation of cranial central arteries compared with DMSO control-treated animals (Fig. 4H,I), whereas BIO-treated dtty135 mutant embryos show improved sprouting and elongation of trunk ISVs compared with DMSO control-treated dtty135 mutants (Fig. 4J-L). Activation of Wnt/β-catenin signaling in apcmcr mutants or BIO treatment of wild-type zebrafish embryos also promotes increased proangiogenic activity and branching of both trunk ISVs and cranial central arteries (supplementary material Fig. S4).

**Expression of Vegfc is specifically affected in dtty135 mutant embryos**

Wnt signaling has been reported to promote expression of VEGFA (Kazanskaya et al., 2008) or VEGFA and VEGFC (Skurk et al., 2005) in cultured human endothelial cells, so we examined the expression of zebrafish VEGFs, and VEGF receptors and other
vascular marker genes, in \textit{dtty135} mutants and their wild-type siblings. We found no change in the expression of \textit{vegfaa}, \textit{vegfab} or any of the VEGF receptors, including \textit{vegfr3/flt4} (Fig. 5A-F; supplementary material Fig. S1), but noticed a strong decrease in expression of \textit{vegfc} (Fig. 5G,H).

\textit{vegfc} has a dynamic expression pattern during early zebrafish development (Covassin et al., 2006; Ober et al., 2004). It is initially expressed in the hypochord, a trunk midline signaling structure located between the notochord and developing dorsal aorta (Covassin et al., 2006) (data not shown), but by 24 hpf \textit{vegfc} becomes expressed exclusively in the adjacent dorsal aorta (Covassin et al., 2006) and, importantly, also in the growing ISV (Fig. 5G,I,K). Although \textit{vegfc} is expressed in the hypochord at 18 hpf in \textit{dtty135} mutants, which is indistinguishable from wild-type animals (data not shown), by 24 hpf its expression in the dorsal aorta is absent or strongly reduced (Fig. 5H). \textit{vegfr3/flt4}, the presumptive zebrafish receptor for \textit{vegfc}, is also expressed in the vasculature, first in the dorsal aorta and posterior cardinal vein (Fig. 5E), which also express \textit{rsopo1}. Later during development, \textit{vegfr3/flt4} is expressed in posterior cardinal vein and growing ISV (Covassin et al., 2006; Hogan et al., 2009; Siekmann and Lawson, 2007) (Fig. 5K,L). We confirmed the tip cell expression of \textit{vegfr3/flt4} by performing double in situ hybridization (Fig. 5M). Similar \textit{vegfc} expression by endothelial cells of the developing dorsal aorta and intersegmental vessels during mouse embryonic development was reported previously using a \textit{lacZ} knock-in line (Tammela et al., 2008).

Expression of \textit{vegfc} is regulated by Wnt/\beta-catenin signaling

We examined whether \textit{vegfc} expression is reduced in mutants other than \textit{dtty135} with similar defects in ISV formation. \textit{etsrp331} and \textit{flk157} mutants, which are also defective in ISV formation, do not show reduced \textit{vegfc} (Fig. 6A-D). To examine directly whether Wnt/\beta-catenin signaling regulates \textit{vegfc} expression in endothelial cells in vitro, we treated mouse cultured endothelial cells with either Wnt/\beta-catenin signaling activator BIO or Wnt3a. Unlike control treatments, treatment with either BIO (Fig. 6E) or Wnt3a (Fig. 6F) activates \textit{vegfc} expression in cultured mouse endothelial cells. Similarly, in zebrafish embryos, control DMSO-treated \textit{rsopo1} morphants failed to express \textit{vegfc} in the dorsal aorta (Fig. 6G), but BIO-treated morphants regained dorsal aorta expression of \textit{vegfc} (Fig. 6H). Importantly, we also noted that higher level \textit{vegfc}...
Vegfc signaling is required downstream of Rspo/Wnt signaling

As our results indicated Rspo1/Wnt signaling promotes vegfc expression in the endothelium, we carried out additional experiments to test whether Vegfc/Vegfr3 signaling is required for angiogenesis downstream from Rspo1/Wnt. Injection of synthetic vegfc mRNA rescued ISV sprouting defects in dttr135 mutant embryos, whereas similar injection of vegfa mRNA failed to rescue (Fig. 7A-E), showing that exogenously supplied Vegfc, but not Vegfa, is sufficient to promote ISV sprouting in the absence of Rspo1. To determine whether endothelial-specific expression of vegfc can similarly rescue angiogenesis, we expressed either vegfc and mCherry together, or mCherry alone as a control, in dttr135 mutants using an endothelial-specific flk promoter (Fig. 7F-L). Endothelial cells expressing Vegfc and mCherry incorporated into normally sprouting and extending ISV (Fig. 7F-H,L), whereas endothelial cells expressing mCherry alone did not (Fig. 7I-L). These studies showed that vegfc is an essential component of Rspo1/Wnt downstream signaling required for sprouting angiogenesis.

Vegfc signaling is believed to function primarily through Vegfr3 expressed by the tip cells during angiogenic sprouting. Blocking Vegfr3-mediated Vegfc signaling in mice leads to reduced angiogenic sprouting and vascular density (Tammela et al., 2008). To test whether Vegfc and Vegfr3 are required downstream of Rspo1/Wnt signaling, we injected MOs targeting the vegfc or Vegfr3/flk4 transcripts (Fig. 7M-S). Injections of full doses of these MOs resulted in absent or reduced ISV sprouting in wild-type embryos (Fig. 7M,N,S). Co-injection of low doses of either vegfc or vegfr3/flk4 MOs, which, on their own, failed to elicit significant phenotype (Fig. 7O,P,S), together with sub-phenotypic doses of rspo1 MO (Fig. 7H,L, Fig. 7S) yielded a strong effect on ISV sprouting and growth (P<0.0001; supplementary material Table S1), similar to that in dttr135 mutants (Fig. 7Q,S). Previous studies have reported a role for Notch signaling in angiogenesis and selection of endothelial tip cells (Hogan et al., 2009; Tammela et al., 2008). To examine whether Rspo1 signaling functions upstream of Notch, we injected rspo1 or control MO into a notch reporter transgenic line (Parsons et al., 2009). Vascular expression of the notch reporter was equivalent in rspo1 and control morphants (supplementary material Fig. S5). Furthermore, vascular expression of Notch pathway genes such as dll4, grl (supplementary material Fig. S1) and notch5 (data not shown) was unchanged in dttr135 mutants compared with their wild-type siblings. To determine whether Vegfc functions downstream from Notch, we examined vegfc expression following transgenic heat shock-inducible ubiquitous activation of Notch signaling, as we have reported previously (Lawson et al., 2001). The expression of vegfc was equivalent in Notch-activated and control embryos (supplementary material Fig. S5). These results suggest that Rspo-Wnt-Vegfc signaling functions independently from Notch signaling during embryonic angiogenesis.

DISCUSSION

Rspo1/Wnt signaling regulates angiogenesis

In this manuscript, we identify a novel signaling pathway essential for developmental angiogenesis. We provide multiple lines of evidence establishing the crucial role of Rspo1/Wnt signaling during developmental angiogenesis. Loss of Rspo1 function by means of genetic mutation or by morpholino mediated knockdown leads to suppression of angiogenic proliferation and to defects in both trunk and cranial vessel angiogenesis. Similarly, inhibiting functions of the presumptive receptor for R-spondin, kremen 1 (Binnerts et al., 2007) or the Wnt2bb ligand (both of which are expressed in the vasculature) also leads to defects in angiogenesis. Furthermore, inhibiting canonical Wnt/β-catenin pathway by transgenic overexpression of an Axin-GFP fusion protein under the control of the kdrl/flk1 promoter leads to sprouting defects similar to those resulting from loss of Rspo1 function, indicating that Wnt signaling is required for angiogenesis in an endothelial-autonomous fashion. We also demonstrate that genetic (via apcmcr mutation) or chemically induced (via BIO treatment) activation of Wnt signaling rescues Rspo1 angiogenesis defects and promotes increased vascular branching and sprouting. As noted above, multiple studies have reported roles for canonical Wnt signaling in endothelial cells during development and disease (Dejana, 2010; Franco et al., 2009; Zerlin et al., 2008). Canonical Wnt signaling appears to be active in the endothelium during development (Maretto et al., 2003), and a variety of studies have reported important roles for this signaling pathway during distinct stages of vascular development, including endothelial cell fate specification and endothelial cell proliferation, and vascular growth, integrity and regression (Goodwin et al., 2007; Liebner et al., 2008; Liu and Nathans, 2008; Stenman et al., 2008; Wang et al., 2007). In addition to canonical Wnt signaling, a recent study also reported an important role for non-canonical Wnt-Fli1 signaling in myeloid cells regulating retinal angiogenesis.
These studies underline the importance of this signaling pathway during vascular development. Our analysis of dtt<sup>y135</sup> mutants and downstream Wnt signaling shows that Rspo1/Wnt signaling is essential for all developmental angiogenesis, although it is not required for vasculogenesis.

Distinct roles of R-spondin family proteins during vascular development

R-spondin family proteins have been implicated in a diverse variety of developmental processes. Rspo2 regulates Xenopus muscle formation and tracheal and limb morphogenesis in mouse embryos (Stefater et al., 2011). These studies underline the importance of this signaling pathway during vascular development. Our analysis of dtt<sup>y135</sup> mutants and downstream Wnt signaling shows that Rspo1/Wnt signaling is essential for all developmental angiogenesis, although it is not required for vasculogenesis.

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in the tip cell to inhibit hyperbranching (Hogan et al., 2009; Roca and Adams, 2007; Tammela et al., 2008). Supporting stalk cells express soluble Flt1 through Vegfr2 to induce Vegfr3 and Dll4 in the tip cell (Lobov et al., 2007). Supporting stalk cells express soluble Flt1 (sVegfr1) and Notch. sVegfr1 helps sharpen the concentration gradient of available Vegfa to direct sprouting (Chappell et al., 2009), whereas Dll4/Notch signaling limits the number of tip cells specified (Hellstrom et al., 2007; Lobov et al., 2007; Siekmann and Lawson, 2007). Notch limits Veg receptor expression in stalk cells and Dll4 suppresses Vegfr3 in the tip cell to inhibit hyperbranching (Hogan et al., 2007; Roca and Adams, 2007; Tammela et al., 2008).

Vegfr3 signaling during angiogenesis

Vegfa is best-known as a pro-lymphangiogenic ligand for the Vegfr3 receptor, but recent evidence has shown that Vegfc-Vegfr3 signaling also has a role in angiogenic sprouting (Hogan et al., 2007; Tammela et al., 2008). Our data showing endothelial-specific expression strongly suggest that the Rspo1-Wnt-Vegfc-Vegfr3 pathway functions in an endothelial-specific fashion. The diagram as drawn shows autocrine Rspo1 and paracrine Vegfc endothelial signaling, but we are not able to determine from the data presented in this manuscript whether signaling is autocrine, juxtacrine or both. However, the pan-endothelial (not tip- or stalk-restricted) expression of the components of this pathway suggests that it provides a permissive cue for angiogenesis, rather than a selective cue for tip cells. Other published studies have shown that Vegfa produced by surrounding tissue signals through Vegfr2 to induce Vegfr3 and Dll4 in the tip cell (Lobov et al., 2007; Tammela et al., 2008). Supporting stalk cells express soluble Flt1 (sVegfr1) and Notch. sVegfr1 helps sharpen the concentration gradient of available Vegfa to direct sprouting (Chappell et al., 2009), whereas Dll4/Notch signaling limits the number of tip cells specified (Hellstrom et al., 2007; Lobov et al., 2007; Siekmann and Lawson, 2007). Notch limits Veg receptor expression in stalk cells and Dll4 suppresses Vegfr3 in the tip cell to inhibit hyperbranching (Hogan et al., 2007; Roca and Adams, 2007; Tammela et al., 2008).

A permissive endothelial cue for developmental angiogenesis

Together, our results provide genetic evidence for a novel Rspo1-Wnt-Vegfc signaling pathway required for developmental angiogenesis (Fig. 8). The fact that expression of rspo1, krm1, wnt2bb, vegfc and vegfr3 is restricted to the vasculature during early development, and that endothelial-specific suppression of Wnt signaling phenocopies loss of Rspo1, suggests that this pathway serves as an endothelial-autonomous modulator of angiogenesis during embryogenesis. It remains to be determined whether the entire pathway can function in an autocrine fashion at the level of a single endothelial cell. Autocrine Vegfa signaling has been shown to promote endothelial maintenance and vascular homeostasis (Lee et al., 2007). Similarly, a Vegfc/Vegfr3 autocrine loop enhances tumor associated lymphangiogenesis and tumor progression in a murine tumor model (Matsuura et al., 2009). Vegfc is initially produced as a pro-Vegfc dimmer that is proteolytically cleaved at its N- and C-terminus to generate multiple processed secreted forms. Indeed, reduced activity is associated with cleavage mutants that fail to properly process the pro-Vegfc dimmer (Joukov et al., 1997). However, cleavage of pro-Vegfc does not appear to be essential for its secretion, as unprocessed pro-Vegfc and cleavage mutant forms of Vegfc are both secreted efficiently, although they have different abilities to bind and/or activate the Vegfr3 receptor (Joukov et al., 1997; Siegfried et al., 2003). Therefore, it is not possible using these data to distinguish between possible paracrine and autocrine modes of signaling.

Using our in vivo model, we have shown a complete requirement of Vegfc signaling for developmental angiogenesis downstream of Rspo1/Wnt pathway. As abnormal Wnt and Vegfc signaling is frequently seen in different tumors and tumor cell lines (Laakkonen et al., 2007; Polakis, 2007; Stacke et al., 2002), the novel Rspo-Wnt-Vegfc-Vegfr3 pathway we have identified may play an important role during tumor angiogenesis and, potentially, other vascular pathologies.

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Competing interests statement
The authors declare no competing financial interests.

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DEVELOPMENT

choroidal neovascularization.

lipoprotein receptor, a negative regulator of the wnt signaling pathway and receptors.

Development
regulator of cardiovascular differentiation, morphogenesis and progenitor self-


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