Development 135, 3655-3664 (2008) doi:10.1242/dev.027284

The Wnt signaling regulator R-spondin 3 promotes angioblast and vascular development

Olga Kazanskaya[1,*], Bisei Ohkawara[1,*], Melanie Heroult[2], Wei Wu[1,3], Nicole Maltry, Hellmut G. Augustin[2] and Christof Niehrs[1,†]

The vertebrate embryonic vasculature develops from angioblasts, which are specified from mesodermal precursors and develop in close association with blood cells. The signals that regulate embryonic vasculogenesis and angiogenesis are incompletely understood. Here, we show that R-spondin 3 (Rspo3), a member of a novel family of secreted proteins in vertebrates that activate Wnt/β-catenin signaling, plays a key role in these processes. In *Xenopus* embryos, morpholino antisense knockdown of Rspo3 induces vascular defects because Rspo3 is essential for regulating the balance between angioblast and blood cell specification. In mice, targeted disruption of *Rspo3* leads to embryonic lethality caused by vascular defects. Specifically in the placenta, remodeling of the vascular plexus is impaired. In human endothelial cells, R-spondin signaling promotes proliferation and sprouting angiogenesis in vitro, indicating that Rspo3 can regulate endothelial cells directly. We show that vascular endothelial growth factor is an immediate early response gene and a mediator of R-spondin signaling. The results identify Rspo3 as a novel, evolutionarily conserved angiogenic factor in embryogenesis.

KEY WORDS: R-spondin, VEGF, Placenta, Vasculogenesis, Wnt, *Xenopus*, Mouse

INTRODUCTION

In vertebrate embryos, blood and vascular endothelial cells are formed during gastrulation from the mesoderm, and both lineages develop in close association. The vasculature forms from vascular endothelial precursor cells, or angioblasts, which proliferate and build a tubular network by a process of coalescence and fusion. Following vasculogenesis, growth and arborization of this network lead to branching angiogenesis, which continues in the adult organism (Risau and Flamme, 1995). Vasculogenesis proceeds in close association with hematopoiesis, and in mouse these processes occur both extra-embryonically, in the yolk sac, the allantois and the placenta, as well as intra-embryonically, in the aortic primordia (Moore and Metcalf, 1970; Gekas et al., 2005; Ottersbach and Dzierzak, 2005; Zeigler et al., 2006). The homologous compartments in the *Xenopus* embryo are the mesoderm of the dorsal lateral plate near the pronephros and pronephric ducts (intra-embryonic equivalent) and the ventral blood islands (extra-embryonic equivalent) (Cleaver et al., 1997; Cleaver and Krieg, 1998; Ciau-Uitz et al., 2000; Walmsley et al., 2002).

The close association of hematopoietic and angioblastic cell development is probably due to the fact that both cell types derive from a mesodermal hemangioblast precursor, for which there is good evidence in zebrafish, *Xenopus*, chick, mouse and humans (Eichmann et al., 1997; Kennedy et al., 1997; Choi et al., 1998; Jaffredo et al., 1998; Nishikawa et al., 1998; Jaffredo et al., 2000; Walmsley et al., 2002; Huber et al., 2004; Ferguson et al., 2005; Vogeli et al., 2006; Kennedy et al., 2007) (reviewed by Red-Horse et al., 2007; Xiong, 2008) [but see Ueno and Weissman (Ueno and Weissman, 2006)].

Little is known about the growth factors that control the cell fate decision between hematopoietic cells and angioblasts, with exception of vascular endothelial growth factor (VEGF). VEGF plays a central role in many aspects of vascular development and embryonic angiogenesis (Carmeliet et al., 1996; Ferrara et al., 1996; Coultas et al., 2005) (reviewed by Ferrara, 1999; Tammela et al., 2005; Olsson et al., 2006). In particular, VEGF signaling is necessary and sufficient for promoting early endothelial differentiation in vertebrates (Carmeliet et al., 1996; Ferrara et al., 1996; Eichmann et al., 1997; Shalaby et al., 1997; Koibuchi et al., 2006).

Unlike for angioblast specification, a plethora of growth factors are known to regulate angiogenesis, including (but not limited to) VEGF, angiopoietin, PDGF, ephrin, Delta-Notch, BMPs, FGF and EGF (reviewed by Rossant and Howard, 2002). More recently, Wnt/Frizzled signaling has been added to this list. Wnts are glycoproteins that can activate multiple receptors and pathways, the best characterized of which is the Wnt/β-catenin pathway, which involves Frizzled (Fz) and LRP5/6 receptors (Logan and Nusse, 2004). Gain- and loss-of-function experiments have implicated Wnt signaling in promoting growth and differentiation of endothelial cells in vitro as well as angiogenesis in vivo (reviewed by Zerlin et al., 2008). However, the role of Wnt/β-catenin signaling in angioblast specification and vasculogenesis is unclear. R-spondins (Rspo1-Rspo4) encode a novel family of secreted proteins in vertebrates, which activate Wnt/β-catenin signaling and interact with LRP6 (Kazanskaya et al., 2004; Nam et al., 2006; Wei et al., 2007; Kim et al., 2008). R-spondins are involved in embryonic patterning and differentiation in frogs, mice and humans (Kazanskaya et al., 2004; Kim et al., 2005; Aoki et al., 2006; Blaydon et al., 2006; Parma et al., 2006; Aoki et al., 2008; Bell et al., 2008).

Here, we have focused on *Rspo3*, which we found to be prominently expressed in hematopoietic organs. Analysis of *Rspo3* by gain- and loss-of-function experiments in both mouse and *Xenopus*

[1]Division of Molecular Embryology, German Cancer Research Center, Im Neuenheimer Feld 581, D-69120 Heidelberg, Germany. [2]Division of Vascular Oncology and Metastasis, Medical Faculty Mannheim, University of Heidelberg, and German Cancer Research Center, Im Neuenheimer Feld 581, D-69120 Heidelberg, Germany. [3]Department of Biological Sciences and Biotechnology, Tsinghua University, 100084 Beijing, P. R. China.

*These authors contributed equally to this work
†Author for correspondence (e-mail: niehrs@dkfz-heidelberg.de)

Accepted 8 September 2008

reveals that this novel growth factor plays an essential role during vertebrate vasculogenesis and angiogenesis. In *Xenopus* embryos, Rspo3 regulates the balance between hematopoietic and endothelial differentiation by promoting angioblast specification and inhibiting blood cell specification. We go on to show that *Rspo3* mouse mutants die of angiogenesis defects in yolk sac and placenta. To determine whether these effects represent a direct action on endothelial cells, we demonstrate that recombinant R-spondin promotes proliferation and angiogenesis in endothelial cell lines in vitro. Finally, we show that Rspo3 triggers Wnt/β-catenin signaling to activate the immediate early target gene *Vegf*, which mediates the effects of Rspo3. The results shed light on the poorly understood growth factor regulation of early angioblast specification and vascular development.

MATERIALS AND METHODS

Embryos, explants, in situ hybridization and mRNA synthesis

In vitro fertilization, embryo and explant culture, whole-mount staining, microinjection and culture of *Xenopus* embryo explants were carried out essentially as described (Gawantka et al., 1995). Prospective ventral blood islands were dissected from stage 13, 16 or 20 embryos by cutting the ventral of the embryo from the posterior limit of the cement gland to the anus. In situ hybridization on cryosections was performed according to Yang et al. (Yang et al., 1999). O-dianisidine staining was carried out without fixation as described (Huber et al., 1998). VEGFR inhibitors were used at 1 μM KRN633 [N-(2-Chloro-4-((6,7-dimethoxy-4-quinazolinyl)oxy) phenyl)-N′-propylurea, Calbiochem] and 2 μM MAZ51 [3-(4-Dimethylamino-naphthalen-1-ylmethylene)-1,3-dihydroindol-2-one, Calbiochem], respectively. For bead implantations, Affigel beads (BioRad) were rinsed in PBS and incubated with affinity-purified recombinant xRspo2 or VEGF (Promocell) at 1 μg/μl overnight at 4°C. Beads soaked in BSA were used as controls. BSA-, Rspo2- or VEGF-soaked beads were transplanted into the prospective ventral blood islands of neurulae (stage 15) and embryos fixed for analysis at tadpole stage (stage 25). The antisense morpholino oligonucleotide targeting *X. tropicalis Rspo3* (gi: 114149217) and *X. tropicalis* VEGFA (gi: 77626411) were 5′atgcaattgcgactgctttctctgt and 5′cggtgaggctacagtgtaacagtgt.

RT-PCR

RT-PCR assays were carried out with primers described previously (see Dosch et al., 1997; Glinka et al,. 1997); additional primers were *XSCL* (forward, actcaccctccagacaagaa; reverse, atttaatcaccgctgcccac), *Xα-globin* (forward, tccctcagaccaaaacctac; reverse, cccctcaattttatgctggac), *Xmsr* (forward, aacttcgctctcgctcctccatac; reverse, gccagcagatagcaaacaccac), *XVEGF A* (forward, aggcgagggagaccataaac; reverse, tctgctgcattcacactgac) and mα-globin (forward, actttgatgtaagccacggc; reverse, tagccaag-gtcaccagcag).

qPCR assays were performed using an LC480 light cycler (Roche). Details of PCR primers used for amplification of mouse transcripts can be provided on request.

Targeted disruption and analysis of mouse embryos

Mice carrying a targeted disruption of *Rspo3* (gi:94388197) were obtained commercially from Artemis (Cologne). In brief, targeted mutagenesis of murine *Rspo3* was carried out in mouse embryonic stem cells following standard procedures, using the targeting vector shown in Fig. S3A in the supplementary material. Transgenic mice were generated on a C57Bl/6 background via standard diploid injection. Homozygous mutant embryos were generated by heterozygote intercrosses. C57Bl/6 heterozygotes were then backcrossed to CD1 females for at least six generations. No significant phenotypic differences were detected between homozygous embryos in C57BL/6 and CD1 background. Mouse tail tips or portions of yolk sacs or embryos were used for genotyping by PCR. Genotyping was performed by PCR analysis using three primers: 5′ATGCTTTGAGGCTTGTGACC; 5′TGCACCGACTCCAGTACTGG; and 5′TACATTCTGGTTTCTCAT-CTGG.

Mice were mated overnight and the morning of vaginal plug detection was defined as embryonic day (E) 0.5. For routine histological analysis, tissues were fixed in 4% paraformaldehyde overnight and embedded in paraffin wax for sectioning. Sections (4 μm) were stained with Hematoxylin/Eosin. For whole-mount in situ hybridization, the embryos were fixed and processed as described previously (del Barco Barrantes et al., 2003).

BrdU-incorporation assay

BrdU labeling of embryos was performed in vitro to avoid the effects of placental-embryo deficiency. Whole concepti (E8.5 and E9.5) were dissected from the decidua and incubated at 37°C with 100 μM BrdU for 4 hours. Embryos and extra-embryonic tissues were fixed in paraformaldehyde and embedded in paraffin. Immunochemistry for BrdU was performed as previously described (Schorpp-Kistner et al., 1999). The percentage of BrdU incorporating cells was counted for three independent groups of siblings, using three wild-type and three mutant samples (allantois and chorionic plate) each.

Analysis of β-galactosidase and β-catenin in mouse embryos

BATGAL$^{+/-}$/*Rspo3*$^{-/-}$ and *BATGAL*$^{+/-}$/*Rspo3*$^{+/+}$ mice were fixed at different stages and stained for β-galactosidase activity using a standard protocol. Then, allantois and chorionic plates were removed, embedded in Mowiol, photographed and the number of stained cells within a fixed area was counted. The number of β-galactosidase positive cells was counted for three independent groups of siblings, using three wild-type and three mutant samples (allantois and chorionic plate) each.

For quantification of nuclear β-catenin staining, embryos were photographed in a Zeiss confocal microscope (LSM510). The average intensity of Hoechst staining and background β-catenin staining in the cytoplasm of cells was determined using ImageJ (http://rsb.info.nih.gov/ij) in brightness-normalized images. The intensity of β-catenin staining in the nuclei was measured, background staining was subtracted, and the staining ratio between nuclear β-catenin and nuclear Hoechst was calculated for each nucleus.

Preparation of Rspo2 conditioned medium

Transfection of HEK293T cells with *X. laevis Rspo2* (gi:54145367) and harvest of conditioned medium were as described (Kazanskaya et al., 2004). Anti-FLAG M2-Agarose beads (Sigma) were incubated overnight with *X. laevis* Rspo2-conditioned medium or control medium from untransfected HEK 293T cells. After washing, xRspo2FLAG recombinant protein was eluted from beads using FLAG peptide (Sigma).

Endothelial proliferation assay

Human umbilical vein endothelial cells (HUVEC; PromoCell) were cultured in endothelial cell growth medium (Promocell) supplemented with 10% fetal bovine serum (FBS). For proliferation studies, cells were plated at 50% confluence in a 96-well plate; the next day they were supplemented with VEGF and *X. laevis* Rspo2 proteins for 48 hours, after which BrdU (10 μM) was added to each well for 4 hours. BrdU analysis of cell proliferation was carried out using Cell Proliferation ELISA BrdU chemiluminescent from Roche Applied Science.

Chorioallantoic membrane (CAM) assay

For chicken chorioallantoic membrane (CAM) assay, chicken eggs were incubated at 37°C in a humidified chamber. On day 3 of development, a window was made in the outer shell and on 6 day of development a 20 ml of xRspo2-FLAG or control beads or filter disk carrying recombinant VEGF (Sigma-Aldrich,100 ng/filter) was placed onto the surface of the CAM. The beads (anti-FLAG M2-Agarose, Sigma) were incubated overnight with *X. laevis* Rspo2 conditioned medium or control medium from untransfected HEK 293T cells and washed three times in PBS. After 5 days of incubation, the filter disks and the attached CAM were excised, washed with PBS and processed for histology using Hematoxylin/Eosin staining.

Spheroid-based angiogenesis assay

Endothelial cell spheroids of defined cell number were generated as described previously (Korff and Augustin, 1998). In brief, 12 hours after transfection, HUVEC were suspended in culture medium containing 0.2% (w/v) carboxymethylcellulose (Sigma) and seeded in nonadherent round-bottom 96-well plates (Greiner). Under these conditions, all suspended

cells contribute to the formation of a single spheroid per well of defined size and cell number (400 cells/spheroid). Spheroids were generated overnight, after which they were embedded into collagen gels. The spheroid containing gel was rapidly transferred into prewarmed 24-well plates and allowed to polymerize (30 minutes), then 100 µl endothelial basal medium with or without the indicated growth factors was added on top of the gel. After 24 hours, in vitro capillary sprouting was quantified by measuring the cumulative length using a digital imaging software (Axioplan, Zeiss). In order to obtain a measure of the cumulative sprout length per spheroid, every sprout from 10-15 spheroids was assessed, and from these data the mean cumulative sprout length per spheroid was calculated.

siRNA transfections

HUVECs were seeded in six-well dishes at a density of 1.2×10^5 cells per well 24 hours prior to experimental use. Predesigned annealed small interfering RNA (siRNA) (400 pmoles) control and directed to human VEGFR2 (Control siRNA and KDR ID#220 Ambion; http://www.ambion.com/) were transfected using oligofectamine (Invitrogen; http://www. invitrogen.com/) according to the manufacturer's instructions. The transfection was carried out in 1 ml OptiMEM (Invitrogen) for 4 hours. The medium was changed to ml ECGM. HUVEC spheroids were generated 24 hours after transfection. Downregulation of VEGF-R2 mRNA was analyzed using reverse-transcription polymerase chain reaction (RT-PCR) after 48 hours.

RESULTS

Rspo3 is expressed in blood forming organs in both *Xenopus* and mouse

Following our finding that R-spondins are novel Wnt regulators and that *Rspo2* is involved in early frog development (Kazanskaya et al., 2004), we wished to extend our analysis to other *Rspo* family members. We specifically focused on *Rspo3*, because it showed an early embryonic expression, suggesting an essential role during early patterning. The expression pattern of mouse *Rspo3* was meanwhile reported (Aoki et al., 2006) and we confirm that expression of the gene shows a complex pattern: the brain, neural tube, tail, heart, somites and limbs (see Fig. S1A-C in the supplementary material; data not shown). In addition, we found that mouse *Rspo3* was prominently expressed in sites of vasculogenesis and angiogenesis. Already in E8.0 embryos, *Rspo3* is expressed in the posterior primitive streak (Huber et al., 2004) and the allantois, which are prominent sources of hematopoietic cells (Fig. 1A). *Rspo3* expression persists in the allantois at later stages (Fig. 1B) and also becomes expressed in the chorionic plate of the placenta at E9.0 (Fig. 1B,C; see Fig. S1D,F in the supplementary material), and in heart and umbilical cord starting from E9.5 (see Fig. S1A-C in the supplementary material). *Rspo3* is also expressed in the blood vessels of the embryo proper (Fig. 1D).

Similar to mouse, *Xenopus Rspo3* is expressed in the CNS, and in a variety of precursors of the vasculature, such as dorsal lateral plate and ventral blood islands (Fig. 1E-K), which give rise to adult and embryonic blood, respectively. In particular, *Rspo3* shows prominent co-expression with or nearby the angioblast marker *Msr* (Devic et al., 1996) in the vitelline vein precursors in the periphery of the vbi, surrounding and partially overlapping the more centrally expressed *Scl*, an early marker of blood forming cells (Mead et al., 1998) (Fig. 1I-K; see Fig. S2 in the supplementary material). *Rspo3* is also co-expressed with *Vegf* in the hypochord (Fig. 1G,H), a tissue that is important for formation of the dorsal aorta (Cleaver et al., 2000). We conclude that *Rspo3* is prominently expressed in or close by endothelial cells, and their precursors in *Xenopus* and mouse.

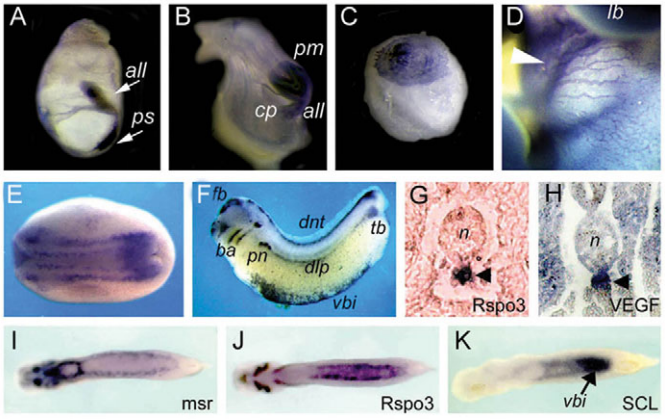

Fig. 1. *Rspo3* is expressed in endothelial cells and their precursors. (A-K) *Rspo3* expression by in situ hybridization during mouse and *Xenopus* embryogenesis. (A-D) Mouse embryo. (E-H) *X. laevis*. (I-K) *X. tropicalis*. (**A**) E8.0 embryo; anterior towards the left. *Rspo3* expression is in the primitive streak and in the allantois. (**B**) E8.5 embryo; anterior towards the top. *Rspo3* expression is prominent in the posterior mesoderm and in the allantois. (**C**) *Rspo3* expression in vasculature of the chorionic plate (E8.5). (**D**) Expression in the umbilical vein (arrowhead) (E12.5). (**E**) Stage 17; dorsal view, anterior leftwards, showing expression in the neural plate. (**F**) Stage 28; lateral view with anterior leftwards, showing expression in organs labeled. (**G**,**H**) Transverse section at tailbud stage showing expression of *Rspo3* (G) and *Vegf* (H) in the hypochord (arrowheads). (**I-K**) Expression of indicated genes in stage 24; ventral view, anterior leftwards, showing expression of *Msr* and *Rspo3* in the periphery of the ventral blood islands. Abbreviations: all, allantois; ba, branchial arches; cp, chorionic plate; fb, forebrain; dnt, dorsal neural tube; dlp, dorsal lateral plate; lb, limb bud; n, notochord; pm, posterior mesoderm; pn, pronephric sinus; ps, primitive streak; tb, tailbud mesoderm; vbi, ventral blood island.

Rspo3 regulates hematopoietic cell fate in *Xenopus*

To investigate a possible role in *Xenopus* development, we inhibited *Rspo3* by injection of antisense morpholino oligonucleotides. The resulting tadpoles displayed ventral edema, as they are characteristically seen following vascular defects (Fig. 2A,B). Staining of erythrocytes with o-dianisidine confirmed that the experimental embryos develop fewer vessels and accumulate erythrocytes in the ventral side (Fig. 2C,D). Molecular marker analysis revealed an expansion of the hematopoietic markers α-globin and *Scl* in the ventral blood islands (vbi). By contrast, *Msr* expression was reduced, which was most pronounced in the lateral plate region, where vbi-derived angioblasts normally develop into the vitelline network (Fig. 2E-L). Rescue experiments carried out in ventral marginal zone explants (VMZ), which contain the vitelline vein and vbi precursors, validated the specificity of the morpholino: α-globin expression was expanded in *Rspo3* morpholino-injected VMZs, whereas expression was blocked by co-injection of morpholino and *Rspo2* mRNA (Fig. 2M-O). These results suggest a requirement of Rspo3 in promoting angioblast fate at the expense of the hematopoietic lineage in the vbi.

To confirm this conclusion, we misexpressed Rspo2 (which is better produced than Rspo3). To rule out the possibility that early embryonic misexpression of Rspo2 may act indirectly by affecting early patterning rather than by directly regulating hemangiogenic fates, we implanted beads soaked with recombinant Rspo2 or VEGF into the vbi precursors of late neurulae. We thereby avoided global

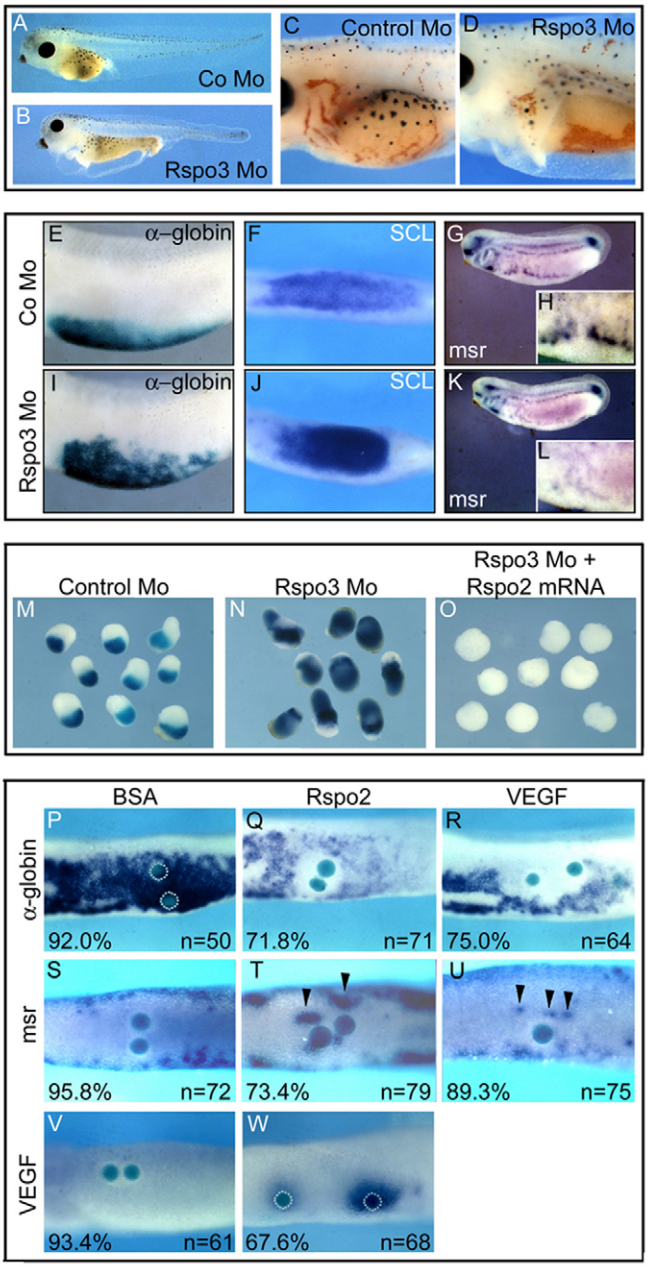

Fig. 2. Rspo3 regulates the balance between blood and endothelial differentiation. (A-D) *Rspo3* is required for blood vessel formation. *Xenopus tropicalis* embryos were injected equatorially into each blastomere at the four-cell stage with 2.5 ng control morpholino (Co Mo) or Rspo3 Mo as indicated. (**A**,**B**) Tadpole morphology; note edema in embryo injected with Rspo3 Mo (B; 69.0%, *n*=203) but not in control (A) (6.4%, n=173). (**C**,**D**) o-Dianisidine erythrocyte staining at tadpole stage. Poor blood vessel formation occurs in the head and erythroid cells accumulate in the ventral region with Rspo3 Mo (D). (**E-L**) *Rspo3* is required for angioblast formation. *Xenopus tropicalis* four-cell stage embryos were injected equatorially with 2.5 ng Co Mo or Rspo3 Mo, as indicated, into each blastomere and analyzed at tailbud stage by whole-mount in situ hybridization for the indicated genes. (H,L) Inset shows high magnification of the lateral region with *Msr*-expressing angioblasts. Control and Rspo3 Mo showed *Msr* staining at 93% (*n*=75) and 20% (*n*=87) frequency, respectively. (**M-O**) Rescue experiment of Rspo3 Mo-induced phenotype. *Xenopus tropicalis* four-cell stage embryos were injected with 2.5 ng of Co Mo or Rspo3 Mo with or without 50 pg *Xenopus Rspo2* mRNA, as indicated, into each ventral blastomere. At gastrula stage (st. 10.5), ventral marginal zones (VMZ) were explanted, cultured until stage 28 and processed for whole-mount in situ hybridization for α-globin. The upregulation of α-globin expression (23/24) in Rsop3 Mo-injected VMZ and the downregulation of α-globin expression (19/20) in Rspo3 Mo and *Rspo2* mRNA-injected VMZ was observed in two independent experiments. (**P-W**) BSA-, Rspo2- or VEGF-soaked beads (top label) were transplanted in the ventral blood island precursors in neurulae and processed for whole-mount in situ hybridization for the indicated markers (side label) at tadpole stage. Arrowheads indicate ectopic *Msr* induction. White circles indicate implanted beads.

overexpression, and targeted R-spondin signaling to the hematopoietic lineage. Rspo2 and VEGF beads both repressed α-globin, and instead induced patches of *Msr* expression (Fig. 2P-U). Furthermore, Rspo2 induced robust *Vegf* expression around the bead (Fig. 2V,W). This confirms that R-spondin signaling regulates the decision between blood and endothelial cell fates in the vbi and suggests that its effects are mediated by VEGF.

To further molecularly dissect R-spondin signaling in *Xenopus*, we used VMZ explants of microinjected embryos followed by RT-PCR analysis of marker genes. RT-PCR analysis showed that *Scl*, α-globin, *Msr* and *Vegf* are expressed in VMZ (Fig. 3A-D). *Rspo3* morpholino injection upregulated the expression of *Scl* and α-globin at the expense of *Msr* as well as of *Vegf* (Fig. 3A). Conversely, *Rspo2* and *Rspo3* mRNA injection, which generally behave indistinguishably in overexpression, induced *Msr* and *Vegf*, and blocked expression of *Scl* and α-globin (Fig. 3A,B). The effects of *Rspo3* gain- and loss-of-function were phenocopied by up- and downregulation of Wnt ligand, following microinjection of wild-type or dominant-negative *Wnt8* (Hoppler et al., 1996), respectively (Fig. 3A). Similar to *dnWnt8*, mRNA microinjection of *Dkk1*, a specific inhibitor of the Wnt receptor LRP5/6 (Glinka et al., 1998), upregulated *Scl* and downregulated angioblast markers (Fig. 3B). Furthermore *Dkk1* blocked the ability of *Rspo3* to upregulate *Msr* and *Vegf* (Fig. 3B, lanes 7-8). These results confirm that during vasculogenesis Rspo3 acts as promoting Wnt/β-catenin signaling.

As VEGF is known to control cell fate decision between hematopoietic cells and angioblast in chick (Eichmann et al., 1997), we tested two pharmacological VEGF receptor inhibitors (MAZ 51 and KRN 633) (Kirkin et al., 2001; Nakamura et al., 2004). Treatment of VMZs with both drugs at low μM concentration mildly phenocopied the effects of *Rspo3* inhibition (Fig. 3B, lanes 5-6) and partially inhibited the effect of *Rspo3* mRNA overexpression (Fig. 3B, lanes 10-11). As an alternative to VEGF receptor inhibitors, we used *Vegf* Morpholino injection, which, like Rspo3 Mo, upregulated *Scl* expression (Fig. 3C). These results indicate that VEGF promotes endothelial at the expense of blood cell differentiation in *Xenopus*. To corroborate the VMZ explant data, we used explanted prospective vbi from different embryonic stages for marker gene analysis (Fig. 3D). This confirmed *Vegf* expression in the vbi by qPCR, which was reduced by Rspo3 Mo. This was accompanied by downregulation of *Msr* and upregulation of *Scl*.

In gain-of-function, *Vegf* mRNA injection upregulated *Msr* and blocked *Scl* expression (Fig. 4A, lane 3), similar to Rspo2/3 mRNAs. Importantly, *Vegf* overexpression rescued *Msr* expression in *Rspo3*-depleted embryos (Fig. 4A, lane 5). Conversely, *Vegf* morpholino injection blocked the ability of Rspo2-soaked beads to

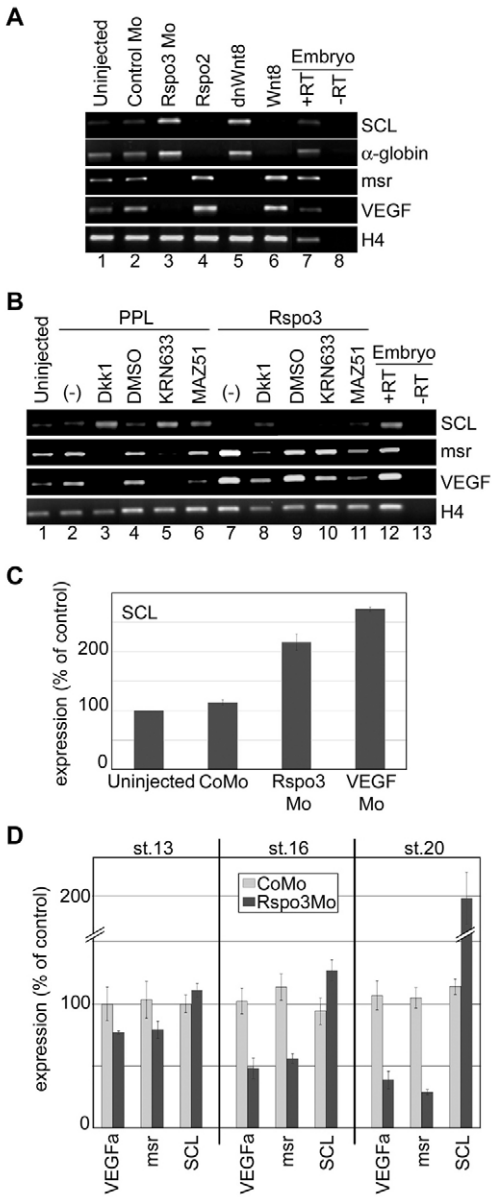

Fig. 3. Rspo3 acts via Wnt/β-catenin signaling to induce *Vegf*. (**A-C**) RT-PCR analysis or quantitative PCR analysis (qPCR) of indicated marker genes in ventral marginal zones (VMZ). Four-cell stage *Xenopus* embryos were injected with 2.5 ng of Control Mo or Rspo3 Mo, 10 ng of VEGF Mo, 50 pg *Xenopus Rspo2* mRNA, 250 pg dominant-negative *Wnt8* (dnWnt8), 50 pg *pCSKA-Wnt8* DNA, 150 pg *Xenopus Dkk1* DNA, 50 pg *Xenopus Rspo3* mRNA or 250 pg *Xenopus* VEGF-A_{170} mRNA, as indicated, into each ventral blastomere. VMZs were explanted at stage 10.5, cultured until stage 28 with or without treatment of VEGF receptor inhibitors (KRN or MAZ), and processed for RT-PCR (A,B) or qPCR (C). –RT, minus reverse transcription control. (**D**) Four-cell stage embryos were injected with 2.5 ng of Control Mo or Rspo3 Mo, as indicated, into both ventral blastomeres. Embryos were cultured until the indicated stage and prospective vbi regions were dissected for qPCR analysis. Gene expression in uninjected vbi explants was set to 100% (control).

inhibit α-globin expression (Fig. 4B), confirming the effects seen with VEGF receptor inhibitors. This indicates that R-spondin signaling is mediated by VEGF.

To test whether *Vegf* is an immediate early R-spondin target gene, we carried out induction experiments in the presence of cycloheximide. *Xenopus* animal caps (responders) were transiently conjugated with Wnt3a- or Rspo2-expressing animal caps (inducers) and then separated again to analyze the responders in isolation (see Fig. 4C for experimental set up). In these conjugates, responder caps showed *Vegf* induction, even when they were cycloheximide treated (Fig. 4D). This immediate early *Vegf* induction by Rspo2 and Wnt3a is consistent with the fact that *Vegf* is a direct β-catenin target gene, which harbors seven Tcf/Lef-binding sites (Easwaran et al., 2003).

We conclude that: (1) Rspo3 regulates the balance between hematopoietic and angioblast cell fate in the vbi; (2) it does so by promoting Wnt/β-catenin signaling, which (3) is required for expression of *Vegf*, that (4) acts as distal regulator of both cell fates in this cascade, promoting angioblast and inhibiting hematopoietic cell fate.

Rspo3 is required for mouse vascular development

To extend the analysis to mammals, we generated *Rspo3* mutant mice by targeted gene disruption (see Fig. S3 in the supplementary material). Homozygous *Rspo3* mutant embryos have been previously described (Aoki et al., 2006). The mice were reported to arrest development around E10 because of placental defects, the nature of which remained unknown. We confirmed that *Rspo3* mutant embryos arrest development at around day 10 and show placental defects (see below). In addition, we found that mutant mice show hemorrhages and have pale yolk sacs and placentas (Fig. 5A-C). These are characteristic features of mice that display embryonic vascular deficiencies. PECAM staining and histological analysis confirmed that the yolk sac of mutant embryos had an underdeveloped vasculature (Fig. 5D,E), indicative of an angiogenesis defect. Similar defects were seen in the vasculature of the embryo proper (Fig. 5F). Histological inspection of the placenta at E10.5 showed that embryonic blood vessels failed to invade the labyrinth layer in *Rspo3* mutants (Fig. 5G). Vascular staining for PECAM indicated that the primary capillary plexus was formed and vessels appeared normal at E9.5 (not shown). However, these vessels failed to undergo proliferation and remodeling, as is characteristic for later angiogenesis (Fig. 5H). These results indicate that Rspo3 signaling in the placenta is required to promote endothelial cell growth and remodeling, rather than initial differentiation and formation of the primary plexus. Consistent with this interpretation, BrdU labeling experiments showed a marked decrease in proliferating endothelial cells in the mutants by E9.5 (see below, Fig. 7E).

Rspo3 is required for Wnt/β-catenin-mediated induction of VEGF in the placenta

We next asked whether as in *Xenopus*, mouse *Vegf* may be regulated by Rspo3. Similar to Rspo3, VEGF is required for placental development (Carmeliet et al., 1996; Ferrara et al., 1996; Coultas et al., 2005) and *Rspo3* is co-expressed with *Vegf* and the endothelial marker *Vegfr2* (*Flk1*) in the placenta (see Fig. S1D-F in the supplementary material). Whereas *Vegfr2* expression in endothelia was mostly unaffected (Fig. 6A), expression of *Vegf* was strongly reduced when analyzed by both in situ hybridization (Fig. 6B) and RT-PCR (see Fig. S4 in the supplementary material). By contrast, expression of *Tpbpa* and *Csh1*, markers of giant trophoblast and spongiotrophoblast cells that produce VEGF (Coultas et al., 2005), was mostly unaffected (Fig. 6C,D). We conclude that *Rspo3* is required for *Vegf* expression and for endothelial cell proliferation to promote proper vascularization of the mouse placenta.

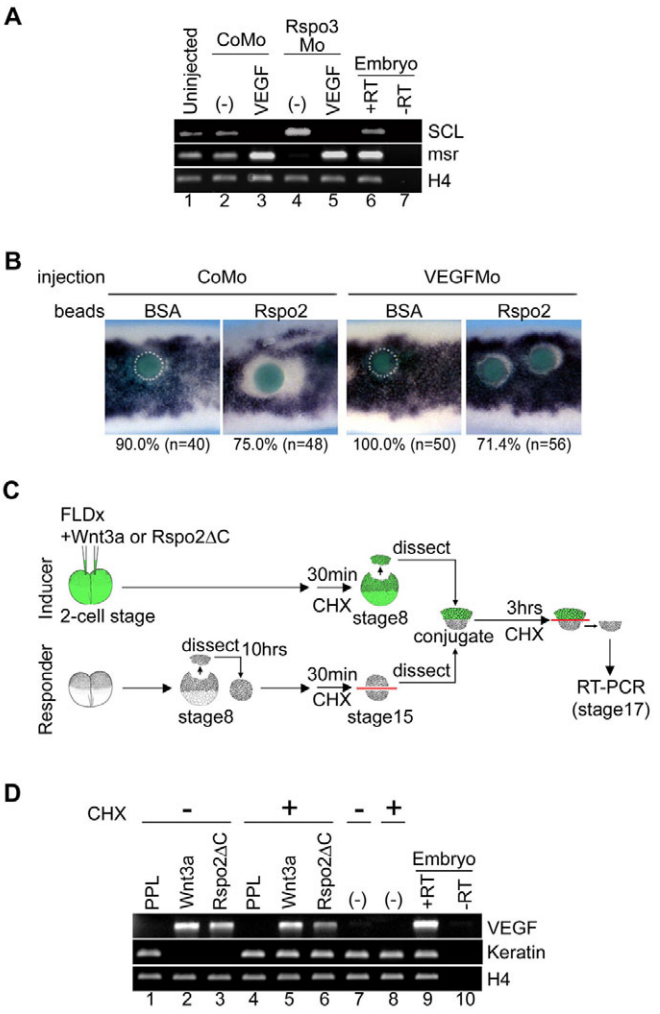

Fig. 4. *Vegf* mediates R-spondin/Wnt signaling during hematopoiesis. (**A**) RT-PCR analysis of ventral marginal zones (VMZ), experimental set up as in Fig. 3A-C. (**B**) Four-cell stage embryos were injected with 10 ng Co Mo or VEGF Mo, as indicated, into both ventral blastomeres. Beads soaked in BSA or Rspo2 were implanted at neurula stage (white circles), and processed for whole-mount in situ hybridization for α-globin at tadpole stage. (**C**) Experimental design for experiment in D. Top: 'inducer' embryos were injected with 100 pg *Wnt3a* or 500 pg Rspo2ΔC (well-secreted variant) mRNA plus lineage tracer FLDx (fluorescence lysinated dextran), cultured until stage 8 and treated with cycloheximide (CHX, 40 μg/ml) for 30 minutes. Lower panel: 'responder' uninjected animal caps were explanted at stage 8, cultured until control siblings reached stage 15, and treated with or without CHX (10 μg/ml) for 30 minutes. Responder animal caps were bissected, sandwiched with or without animal cap of inducer embryos and cultivated for another 3 hours to permit induction in the presence of CHX (10 μg/ml). Responder animal tissues were harvested and analyzed by RT-PCR shown in D. (**D**) *Vegf* induction is an immediate early response to Wnt/R-spondin signaling. Uninjected animal caps (responders) were co-cultured with fluorescently labeled animal caps injected with *Wnt3a* and *Rspo2* mRNA (inducers) as shown in C. Responder animal caps were cultured until control siblings reached stage 15 and treated with or without cycloheximide (CHX) for 3 hours. Responder animal caps were harvested separately and analyzed by RT-PCR for the markers indicated. As a control for the efficacy of CHX treatment, the (indirect) inhibition of keratin expression by *Wnt3a* and *Rspo2* (lanes 2-3) is blocked by CHX (lanes 4-9).

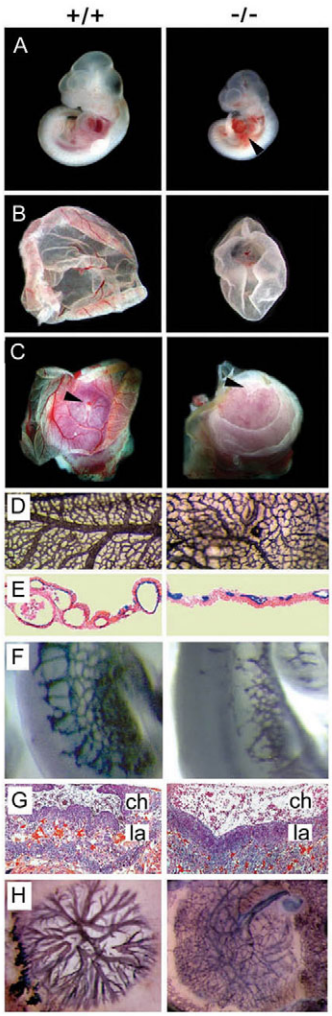

Fig. 5. *Rspo3* is required for mouse vascular development. (**A-C**) External morphology of wild-type and *Rspo3*$^{-/-}$ embryos (A), yolk sacs (B) and placentas (C) (E10.5). Enlarged pericardial sac with hemorrhage is marked by arrowhead in A. Note pale *Rspo3*$^{-/-}$ yolk sac in B and pale *Rspo3*$^{-/-}$ chorionic plate in C (arrowheads). (**D**,**E**) Yolk sac morphology in *Rspo3*$^{-/-}$ embryos (E9.5). (D) PECAM antibody staining of yolk sac vasculature at E9.5. (E) Histological section of the PECAM stained yolk sacs shown in D, counterstaining with Eosin. (**F**) PECAM staining of the trunk region of wild-type and *Rspo3*$^{-/-}$ embryos E9.5. (**G**,**H**) Placental defects in *Rspo3*$^{-/-}$ embryos. (G) Hematoxylin and Eosin staining of histological section. Note reduced embryonic vessels in *Rspo3*$^{-/-}$ labyrinth. (H) PECAM staining of placentas shown in C. Representative samples from 20 wild-type and 20 knockout embryos are shown.

Interestingly, two Wnt/β-catenin pathway components, *Wnt2* and frizzled5, are also required for the vascularization of the mouse placenta (Monkley et al., 1996; Ishikawa et al., 2001) and evidence for a general role of Wnt/β-catenin signaling in angiogenesis is accumulating (Zerlin et al., 2008). To corroborate therefore that the vascular defects in *Rspo3* mutants were also due to reduced Wnt/β-catenin signaling, we carried out immunofluorescence staining to monitor nuclear β-catenin. A marked reduction of nuclear β-catenin staining was observed in the mutant allantois (Fig. 6E,G). To confirm this downregulation of Wnt signaling, we bred *Rspo3* heterozygotes with the Wnt-reporter mouse line *BATGAL* (Maretto

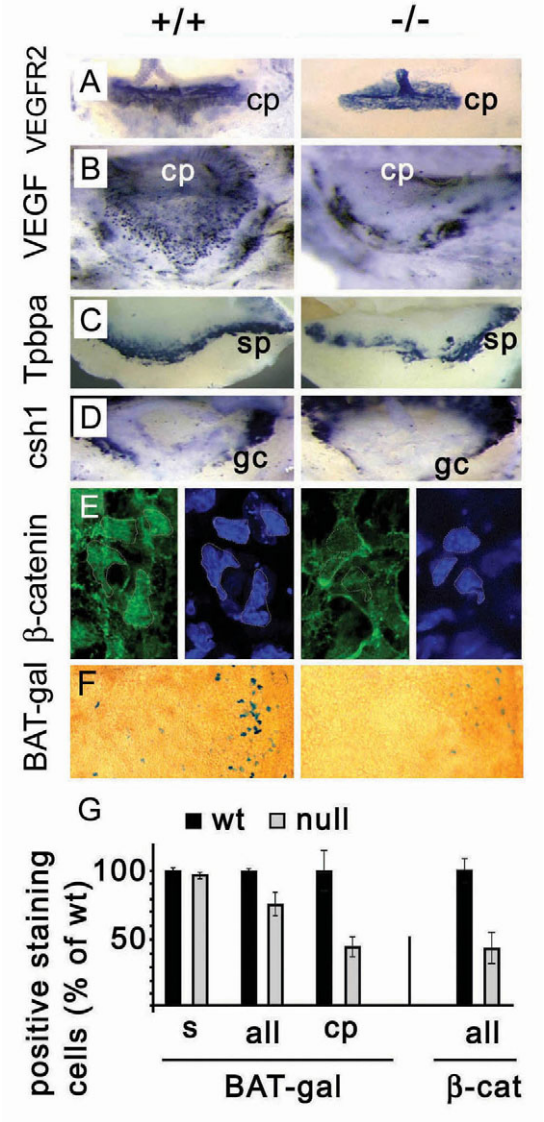

Fig. 6. *Rspo3* is required for Wnt/β-catenin mediated induction of *Vegf*. (**A**) *Vegfr2* expression. (**B**) *Vegfa* expression. (**C**) *Tpbpa* expression in spongiotrophoblast layer. (**D**) *Csh1* (placental lactogen 1) expression in giant cell border. (A-D) Representative samples from 20 wild-type and 20 knockout embryos are shown as indicated. (**E**) Immunofluorescence staining of β-catenin (green) and nuclei (Hoechst, blue) in the allantois (E8.5). Reduced β-catenin staining occurs in *Rspo3*$^{-/-}$ allantois. (**F**) *BATGAL* reporter activity in wild-type and *Rspo3*$^{-/-}$ chorionic plates. Representative samples from 20 wild-type and 20 knockout embryos are shown. (**G**) Quantification of *lacZ* staining in the allantois (all) (E8.5), somites (s) (E8.5) and chorionic plate (cp) (E9.5) and β-catenin staining in the allantois (all) (E8.5). The number of positive cells in wild-type embryos was set to 100% (see Materials and methods). Abbreviations: all, allantois; ch, chorion; gc, giant cells; sp, spongiotrophoblast; la, labyrinth; cp, chorionic plate.

et al., 2003), which drives *lacZ* expression at sites of Wnt signaling. Staining for β-galactosidase showed a *lacZ* signal in the chorionic plate of wild-type *BATGAL* mice, which was reduced in *Rspo3*$^{-/-}$/*BATGAL* allantoises and placentas (Fig. 6F,G). Taken together, these results suggest that *Rspo3* functions in placental vascular remodeling by promoting Wnt/β-catenin signaling to activate *Vegf* expression.

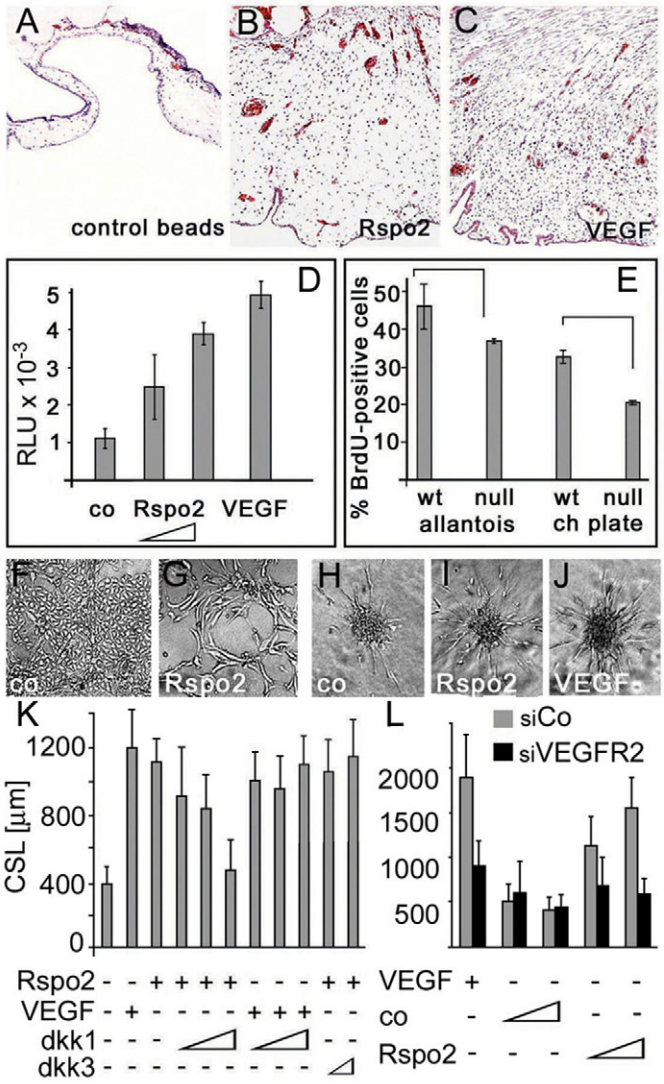

Fig. 7. R-spondin signaling promotes angiogenesis in vitro. (**A-C**) Chicken chorioallantoic membrane assay using beads soaked with Rspo2 or VEGF or control. (**D**) Cell proliferation assay using BrdU incorporation in human umbilical vein endothelial cells (HUVEC). RLU, relative light units. (**E**) Cell proliferation assay in control (wt) and Rspo3$^{-/-}$ (null) E9.5 embryos using 4 hours BrdU incorporation in vitro (n=9 embryos each). (**F**,**G**) Induction of capillary-like morphology of human dermal microvascular endothelial cells after treatment with recombinant Rspo2. (**H-J**) Three-dimensional spheroid capillary sprouting assays using HUVEC. (**K**,**L**) Quantification of spheroid assays. CSL, cumulative sprout length.

R-spondin signaling promotes angiogenesis in vitro

As the results indicated that Rspo3 is required for vascular development, we determined whether R-spondin signaling may directly promote angiogenesis by activating endothelial cells. As recombinant protein we used both Rspo2 and Rspo3, because the former is better produced, whereas in our experience both are qualitatively interchangeable. Characteristic properties of angiogenic factors are their ability to induce endothelial proliferation and capillary morphogenesis. In chicken chorionic membrane (CAM) assays (Fig. 7A-C), implantation of Rspo3-soaked beads

induced a strong proliferative response in the CAM, accompanied by intense angiogenesis. A similar induction was seen with VEGF-soaked beads. Likewise, in human umbilical vein endothelial cells (HUVEC), recombinant Rspo2 induced cell proliferation, as did VEGF (Fig. 7D). Correspondingly, the proliferation of endothelial cells labeled in situ with BrdU in pregnant mice was reduced in *Rspo3*-null mutants (Fig. 7E).

To test whether R-spondin signaling can also induce the morphogenesis that is characteristic of angiogenic factors, we employed the human dermal microvascular endothelial cells (HDMEC) capillary tube formation assay, where Rspo2 readily induced capillary-like morphology (Fig. 7F,G).

Another angiogenic morphogenesis test is the spheroidal sprouting assay, in which collagen gel-embedded endothelial cells give rise to a three-dimensional capillary-like network upon angiogenic stimulation (Korff and Augustin, 1998). Similar to VEGF, Rspo2 induced endothelial sprouting in a dose-dependent manner (Fig. 7H-L). The ability of Rspo2 to induce sprouting was blocked by addition of the Wnt inhibitor Dkk1 but not by the related Dkk3, which does not inhibit Wnt signaling (Mao et al., 2001) (Fig. 7K). In contrast to Rspo2, the sprouting effect of VEGF was not affected by Dkk1. Once again the effects were dependent on VEGF signaling, as siRNA knockdown of *Vegfr2* (>70% by RT-PCR, not shown) blocked the ability of Rspo2 to induce sprouting (Fig. 7L). We conclude that R-spondin signaling can act directly on endothelial cells to promote angiogenesis by activating Wnt/β-catenin and VEGF signaling.

DISCUSSION

Rspo3 regulates hematopoietic/angioblast fate in *Xenopus*

An important issue in developmental biology relates to the mechanisms whereby multipotent precursors give rise to distinct differentiated progeny. One such fate decision, which is poorly understood, concerns the choice between hematopoietic and blood cell differentiation in hematopoietic organs where these cells develop in close association. The co-specification of these lineages is supported by a large body of evidence in all vertebrates, including chick, mouse, zebrafish and *Xenopus* (reviewed by Ferguson et al., 2005; Red-Horse et al., 2007; Xiong, 2008). In the hematopoietic regions of *Xenopus* embryos, for example, a combination of lineage tracing and gene expression experiments showed that both lineages pass through a progenitor state in which they co-express blood and endothelial genes (Ciau-Uitz et al., 2000; Walmsley et al., 2002). The co-development of both lineages and their requirement for the same genes begs the question of which cues regulate their separate specification? Although a number of growth factor signaling pathways, such as VEGF, TGFβ, BMP4 and hedgehog, have been identified that are required directly or indirectly for formation of both lineages (Dickson et al., 1995; Winnier et al., 1995; Carmeliet et al., 1996; Ferrara et al., 1996; Shalaby et al., 1997; Dyer et al., 2001; Vokes et al., 2004; Gering and Patient, 2005), the extrinsic factors that regulate the fate separation between these lineages remain largely unknown. A main conclusion of our study is that Rspo3-Wnt/β-catenin signaling plays a key role in this process, by promoting angioblast and inhibiting hematopoietic cell fate in *Xenopus* ventral blood island (Fig. 8). The early embryonic expression of *Rspo3* in the gastrula ventrolateral mesoderm in *Xenopus* is consistent with this proposition.

In support of our model, constitutive activation of Wnt/β-catenin signaling has been shown to block hematopoietic cell differentiation in transgenic mice (Kirstetter et al., 2006; Scheller et al., 2006).

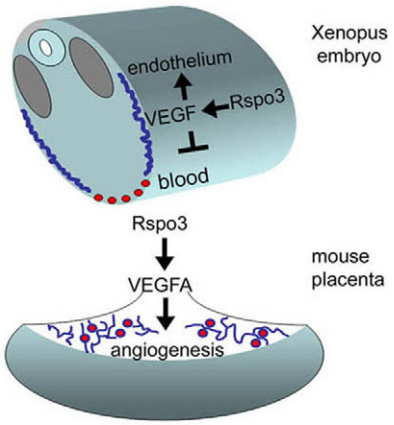

Fig. 8. Model of Rspo3 action in *Xenopus* and mouse vascular development. Blue lines indicate the embryonic vasculature in *Xenopus* embryos and mouse placental labyrinth; red spheres, blood cells.

Furthermore, experiments with Wnt2$^{-/-}$ embryoid bodies suggest that Wnt/β-catenin signaling is required for normal endothelial maturation and vascular plexus formation, and that Wnt2 suppresses hematopoietic differentiation in vitro (Wang et al., 2007).

The molecular analysis indicates that an essential mediator of Rspo3 is its immediate downstream target gene *Vegf*. In support of its crucial role, VEGF in chicken progenitor cells reduces hematopoietic and induces endothelial cell differentiation (Eichmann et al., 1997). A role of VEGF in regulating angioblastic versus hematopoietic cell fate decision is also corroborated by recent experiments in *Xenopus* (Koibuchi et al., 2006). As it acts upstream of the VEGF pathway, Rspo3 signaling may be the earliest step regulating this hematopoietic lineage bifurcation in the *Xenopus* vbi.

Our results indicating a negative role of VEGF in *Scl* expression and blood cell specification are in contrast to a body of studies specifically in mouse. For example, *VegfR2*-deficient mice lack both endothelial and hematopoietic cells, suggesting that VEGF signaling is essential in a common progenitor (Shalaby et al., 1997). Other mouse data also indicate a requirement for VEGF signaling for *Scl* expression and blood cell differentiation (reviewed by Ferrara, 1999; Tammela et al., 2005; Olsson et al., 2006). The reason for this discrepancy may be differences in the role of *Scl* and VEGF in regions of embryonic and definitive hematopoiesis, respectively. In mouse, *Scl* is a general hematopoietic marker expressed in common precursors of blood and endothelial cells, and required for the development of both lineages (Shivdasani et al., 1995; Visvader et al., 1998). Also in the *Xenopus* dorsal lateral plate and anterior vbi, which are responsible for definitive hematopoiesis, *Scl* and the endothelial marker *xfli* are co-expressed, suggestive of a common precursor (Walmsley et al., 2002). By contrast, in the *Xenopus* vbi, which give rise to embryonic blood and which is the focus of our study, *Scl* is a red blood lineage marker, the expression of which is mutually exclusive with the endothelial marker *xfli* (Walmsley et al., 2002) (compare Fig. 1I with Fig. 1K). As our study focuses on *Xenopus* primitive hematopoiesis, the role of *Vegf* and *Scl* may be different in organs of definitive hematopoiesis. We note, however, that VEGF signaling in *Scl* induction, primitive hematopoiesis and vasculogenesis is also not conserved between mouse and either zebrafish or chicken (Eichmann et al., 1997; Habeck et al., 2002; Patterson et al., 2005).

Rspo3, Wnt signaling and angiogenesis

The defects in mutant mice reveal that embryonic vasculature regulation is a conserved function of Rspo3-Wnt/β-catenin signaling, albeit at a different stage, namely in angiogenesis of the yolk sac and the placenta, where it is required for endothelial cell proliferation and remodeling. This is also supported by the pro-angiogenic effect of R-spondin signaling in adult endothelial cells. Taken together with its endothelial expression, this suggests an autocrine mode of Rspo3 signaling. Recently, it was shown that VEGF also functions in an autocrine fashion in endothelial cells during vascular development (Lee et al., 2007). Indeed, the rather broad effect of Rspo3 on early specification as well as later angiogenesis is reminiscent of VEGF, which likewise plays a role not only during early hematopoietic cell specification, but also during subsequent cell proliferation, vascular morphogenesis and remodeling, both in the embryo and in the adult (Ferrara 1999; Coultas et al., 2005).

There is large evidence that Wnt signaling plays an important role in angiogenesis. *Wnt2*, *Wnt4* and *Wnt7b* mutant mice have reduced embryonic or adult vasculature (Monkley et al., 1996; Shu et al., 2002; Jeays-Ward et al., 2003). Likewise, *Fz4* and *Fz5* mutant mice show defective angiogenesis (Ishikawa et al., 2001; Xu et al., 2004). The *Fz4* mutation is also linked to human familial exudative vitreoretinopathy (FEVR), which is caused by a failure of peripheral retinal vascularization (Robitaille et al., 2002). Mutations in *norrin*, which encoding a high affinity *Fz4* ligand, are also linked to FEVR (Xu et al., 2004). However, in most cases it remains unclear whether the angiogenic role revealed by these Wnt/Fz mutants involves Wnt/β-catenin signaling. At least in vitro it is the Wnt/β-catenin signaling pathway, which promotes endothelial cell proliferation (reviewed by Zerlin et al., 2008). Furthermore, Wnt/β-catenin signaling promotes expression of *Vegf* (Zhang et al., 2001), which is a direct β-catenin target gene (Easwaran et al., 2003).

Consistent with this, we find that R-spondins also promote endothelial cell proliferation and in vitro angiogenesis by activating Wnt/β-catenin signaling, as it is inhibited by Dkk1. This effect is blocked by siVEGFR2, suggesting that, as in *Xenopus* and mouse embryos, R-spondins impact endothelial cells ultimately through VEGF signaling. However, in HUVEC, we were unable to observe upregulation of *Vegf* by Rspo2 treatment (data not shown), whereas in *Xenopus* and mouse embryos, Rspo3 was required for *Vegf* expression. This suggests that there may be different mechanisms underlying the promotion of VEGF signaling, endothelial proliferation and morphogenesis by Rspo3.

Finally, from a medical perspective, the identification of Rspo3 as a secreted angiogenic factor may provide novel opportunities for pharmaceutical intervention in angiogenic and antiangiogenic therapies (reviewed by Ferrara, 1999; Dor et al., 2003).

We thank M. Schorpp-Kistner and P. Angel for advice and reagents. We thank Y. Audigier, M. Gessler, P. Gruss and S. Piccolo for providing materials, and K. Ellwanger, Y.-L. Huang and H. Weber for technical help. Supported by DFG (Ni 286/9-2 and Au83/9-1).

Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/135/22/3655/DC1

References

Aoki, M., Mieda, M., Ikeda, T., Hamada, Y., Nakamura, H. and Okamoto, H. (2006). R-spondin3 is required for mouse placental development. *Dev. Biol.* **301**, 218-226.

Aoki, M., Kiyonari, H., Nakamura, H. and Okamoto, H. (2008). R-spondin2 expression in the apical ectodermal ridge is essential for outgrowth and patterning in mouse limb development. *Dev. Growth Differ.* **50**, 85-95.

Bell, S. M., Schreiner, C. M., Wert, S. E., Mucenski, M. L., Scott, W. J. and Whitsett, J. A. (2008). R-spondin 2 is required for normal laryngeal-tracheal, lung and limb morphogenesis. *Development* **135**, 1049-1058.

Blaydon, D. C., Ishii, Y., O'Toole, E. A., Unsworth, H. C., Teh, M. T., Ruschendorf, F., Sinclair, C., Hopsu-Havu, V. K., Tidman, N., Moss, C. et al. (2006). The gene encoding R-spondin 4 (RSPO4), a secreted protein implicated in Wnt signaling, is mutated in inherited anonychia. *Nat. Genet.* **38**, 1245-1247.

Carmeliet, P., Ferreira, V., Breier, G., Pollefeyt, S., Kieckens, L., Gertsenstein, M., Fahrig, M., Vandenhoeck, A., Harpal, K., Eberhardt, C. et al. (1996). Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. *Nature* **380**, 435-439.

Choi, K., Kennedy, M., Kazarov, A., Papadimitriou, J. C. and Keller, G. (1998). A common precursor for hematopoietic and endothelial cells. *Development* **125**, 725-732.

Ciau-Uitz, A., Walmsley, M. and Patient, R. (2000). Distinct origins of adult and embryonic blood in Xenopus. *Cell* **102**, 787-796.

Cleaver, O. and Krieg, P. A. (1998). VEGF mediates angioblast migration during development of the dorsal aorta in Xenopus. *Development* **125**, 3905-3914.

Cleaver, O., Tonissen, K. F., Saha, M. S. and Krieg, P. A. (1997). Neovascularization of the Xenopus embryo. *Dev. Dyn.* **210**, 66-77.

Cleaver, O., Seufert, D. W. and Krieg, P. A. (2000). Endoderm patterning by the notochord: development of the hypochord in Xenopus. *Development* **127**, 869-879.

Coultas, L., Chawengsaksophak, K. and Rossant, J. (2005). Endothelial cells and VEGF in vascular development. *Nature* **438**, 937-945.

del Barco Barrantes, I., Davidson, G., Gröne, H. J., Westphal, H. and Niehrs, C. (2003). Dkk1 and noggin cooperate in mammalian head induction. *Genes Dev.* **17**, 2239-2244.

Devic, E., Paquereau, L., Vernier, P., Knibiehler, B. and Audigier, Y. (1996). Expression of a new G protein-coupled receptor X-msr is associated with an endothelial lineage in Xenopus laevis. *Mech. Dev.* **59**, 129-140.

Dickson, M. C., Martin, J. S., Cousins, F. M., Kulkarni, A. B., Karlsson, S. and Akhurst, R. J. (1995). Defective haematopoiesis and vasculogenesis in transforming growth factor-beta 1 knock out mice. *Development* **121**, 1845-1854.

Dor, Y., Djonov, V. and Keshet, E. (2003). Making vascular networks in the adult: branching morphogenesis without a roadmap. *Trends Cell Biol.* **13**, 131-136.

Dosch, R., Gawantka, V., Delius, H., Blumenstock, C. and Niehrs, C. (1997). Bmp-4 acts as a morphogen in dorsoventral mesoderm patterning in Xenopus. *Development* **124**, 2325-2334.

Dyer, M. A., Farrington, S. M., Mohn, D., Munday, J. R. and Baron, M. H. (2001). Indian hedgehog activates hematopoiesis and vasculogenesis and can respecify prospective neurectodermal cell fate in the mouse embryo. *Development* **128**, 1717-1730.

Easwaran, V., Lee, S. H., Inge, L., Guo, L., Goldbeck, C., Garrett, E., Wiesmann, M., Garcia, P. D., Fuller, J. H., Chan, V. et al. (2003). beta-Catenin regulates vascular endothelial growth factor expression in colon cancer. *Cancer Res.* **63**, 3145-3153.

Eichmann, A., Corbel, C., Nataf, V., Vaigot, P., Breant, C. and Le Douarin, N. M. (1997). Ligand-dependent development of the endothelial and hemopoietic lineages from embryonic mesodermal cells expressing vascular endothelial growth factor receptor 2. *Proc. Natl. Acad. Sci. USA* **94**, 5141-5146.

Ferguson, J. E., 3rd, Kelley, R. W. and Patterson, C. (2005). Mechanisms of endothelial differentiation in embryonic vasculogenesis. *Arterioscler. Thromb. Vasc. Biol.* **25**, 2246-2254.

Ferrara, N. (1999). Molecular and biological properties of vascular endothelial growth factor. *J. Mol. Med.* **77**, 527-543.

Ferrara, N., Carver-Moore, K., Chen, H., Dowd, M., Lu, L., O'Shea, K. S., Powell-Braxton, L., Hillan, K. J. and Moore, M. W. (1996). Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. *Nature* **380**, 439-442.

Gawantka, V., Delius, H., Hirschfeld, K., Blumenstock, C. and Niehrs, C. (1995). Antagonizing the Spemann organizer: role of the homeobox gene Xvent-1. *EMBO J.* **14**, 6268-6279.

Gekas, C., Dieterlen-Lievre, F., Orkin, S. H. and Mikkola, H. K. (2005). The placenta is a niche for hematopoietic stem cells. *Dev. Cell* **8**, 365-375.

Gering, M. and Patient, R. (2005). Hedgehog signaling is required for adult blood stem cell formation in zebrafish. *Dev. Cell* **8**, 389-400.

Glinka, A., Wu, W., Onichtchouk, D., Blumenstock, C. and Niehrs, C. (1997). Head induction by simultaneous repression of Bmp and Wnt signalling in Xenopus. *Nature* **389**, 517-519.

Glinka, A., Wu, W., Delius, H., Monaghan, A. P., Blumenstock, C. and Niehrs, C. (1998). Dickkopf-1 is a member of a new family of secreted proteins and functions in head induction. *Nature* **391**, 357-362.

Habeck, H., Odenthal, J., Walderich, B., Maischein, H. and Schulte-Merker, S. (2002). Analysis of a zebrafish VEGF receptor mutant reveals specific disruption of angiogenesis. *Curr. Biol.* **12**, 1405-1412.

Hoppler, S., Brown, J. D. and Moon, R. T. (1996). Expression of a dominant-negative Wnt blocks induction of MyoD in Xenopus embryos. *Genes Dev.* **10**, 2805-2817.

Huber, T. L., Zhou, Y., Mead, P. E. and Zon, L. I. (1998). Cooperative effects of growth factors involved in the induction of hematopoietic mesoderm. *Blood* **92**, 4128-4137.
Huber, T. L., Kouskoff, V., Fehling, H. J., Palis, J. and Keller, G. (2004). Haemangioblast commitment is initiated in the primitive streak of the mouse embryo. *Nature* **432**, 625-630.
Ishikawa, T., Tamai, Y., Zorn, A. M., Yoshida, H., Seldin, M. F., Nishikawa, S. and Taketo, M. M. (2001). Mouse Wnt receptor gene Fzd5 is essential for yolk sac and placental angiogenesis. *Development* **128**, 25-33.
Jaffredo, T., Gautier, R., Eichmann, A. and Dieterlen-Lievre, F. (1998). Intraaortic hemopoietic cells are derived from endothelial cells during ontogeny. *Development* **125**, 4575-4583.
Jaffredo, T., Gautier, R., Brajeul, V. and Dieterlen-Lievre, F. (2000). Tracing the progeny of the aortic hemangioblast in the avian embryo. *Dev. Biol.* **224**, 204-214.
Jeays-Ward, K., Hoyle, C., Brennan, J., Dandonneau, M., Alldus, G., Capel, B. and Swain, A. (2003). Endothelial and steroidogenic cell migration are regulated by WNT4 in the developing mammalian gonad. *Development* **130**, 3663-3670.
Kazanskaya, O., Glinka, A., Del Barco Barrantes, I., Stannek, P., Niehrs, C. and Wu, W. (2004). R-Spondin2 is a secreted activator of Wnt/beta-Catenin signaling and is required for xenopus myogenesis. *Dev. Cell* **7**, 525-534.
Kennedy, M., Firpo, M., Choi, K., Wall, C., Robertson, S., Kabrun, N. and Keller, G. (1997). A common precursor for primitive erythropoiesis and definitive haematopoiesis. *Nature* **386**, 488-493.
Kennedy, M., D'Souza, S. L., Lynch-Kattman, M., Schwantz, S. and Keller, G. (2007). Development of the hemangioblast defines the onset of hematopoiesis in human ES cell differentiation cultures. *Blood* **109**, 2679-2687.
Kim, K. A., Kakitani, M., Zhao, J., Oshima, T., Tang, T., Binnerts, M., Liu, Y., Boyle, B., Park, E., Emtage, P. et al. (2005). Mitogenic influence of human R-spondin1 on the intestinal epithelium. *Science* **309**, 1256-1259.
Kim, K. A., Wagle, M., Tran, K., Zhan, X., Dixon, M. A., Liu, S., Gros, D., Korver, W., Yonkovich, S., Tomasevic, N. et al. (2008). R-Spondin family members regulate the Wnt pathway by a common mechanism. *Mol. Biol. Cell* **19**, 2588-2596.
Kirkin, V., Mazitschek, R., Krishnan, J., Steffen, A., Waltenberger, J., Pepper, M. S., Giannis, A. and Sleeman, J. P. (2001). Characterization of indolinones which preferentially inhibit VEGF-C- and VEGF-D-induced activation of VEGFR-3 rather than VEGFR-2. *Eur. J. Biochem.* **268**, 5530-5540.
Kirstetter, P., Anderson, K., Porse, B. T., Jacobsen, S. E. and Nerlov, C. (2006). Activation of the canonical Wnt pathway leads to loss of hematopoietic stem cell repopulation and multilineage differentiation block. *Nat. Immunol.* **7**, 1048-1056.
Koibuchi, N., Taniyama, Y., Nagao, K., Ogihara, T., Kaneda, Y. and Morishita, R. (2006). The effect of VEGF on blood vessels and blood cells during Xenopus development. *Biochem. Biophys. Res. Commun.* **344**, 339-345.
Korff, T. and Augustin, H. G. (1998). Integration of endothelial cells in multicellular spheroids prevents apoptosis and induces differentiation. *J. Cell Biol.* **143**, 1341-1352.
Lee, S., Chen, T. T., Barber, C. L., Jordan, M. C., Murdock, J., Desai, S., Ferrara, N., Nagy, A., Roos, K. P. and Iruela-Arispe, M. L. (2007). Autocrine VEGF signaling is required for vascular homeostasis. *Cell* **130**, 691-703.
Logan, C. Y. and Nusse, R. (2004). The Wnt signaling pathway in development and disease. *Annu. Rev. Cell Dev. Biol.* **20**, 781-810.
Mao, B., Wu, W., Li, Y., Hoppe, D., Stannek, P., Glinka, A. and Niehrs, C. (2001). LDL-receptor-related protein 6 is a receptor for Dickkopf proteins. *Nature* **411**, 321-325.
Maretto, S., Cordenonsi, M., Dupont, S., Braghetta, P., Broccoli, V., Hassan, A. B., Volpin, D., Bressan, G. M. and Piccolo, S. (2003). Mapping Wnt/beta-catenin signaling during mouse development and in colorectal tumors. *Proc. Natl. Acad. Sci. USA* **100**, 3299-3304.
Mead, P. E., Kelley, C. M., Hahn, P. S., Piedad, O. and Zon, L. I. (1998). SCL specifies hematopoietic mesoderm in Xenopus embryos. *Development* **125**, 2611-2620.
Monkley, S. J., Delaney, S. J., Pennisi, D. J., Christiansen, J. H. and Wainwright, B. J. (1996). Targeted disruption of the Wnt2 gene results in placentation defects. *Development* **122**, 3343-3353.
Moore, M. A. and Metcalf, D. (1970). Ontogeny of the haemopoietic system: yolk sac origin of *in vivo* and *in vitro* colony forming cells in the developing mouse embryo. *Br. J. Haematol.* **18**, 279-296.
Nakamura, K., Yamamoto, A., Kamishohara, M., Takahashi, K., Taguchi, E., Miura, T., Kubo, K., Shibuya, M. and Isoe, T. (2004). KRN633: a selective inhibitor of vascular endothelial growth factor receptor-2 tyrosine kinase that suppresses tumor angiogenesis and growth. *Mol. Cancer Ther.* **3**, 1639-1649.
Nam, J. S., Turcotte, T. J., Smith, P. F., Choi, S. and Yoon, J. K. (2006). Mouse cristin/R-spondin family proteins are novel ligands for the Frizzled 8 and LRP6 receptors and activate beta-catenin-dependent gene expression. *J. Biol. Chem.* **281**, 13247-13257.
Nishikawa, S. I., Nishikawa, S., Hirashima, M., Matsuyoshi, N. and Kodama, H. (1998). Progressive lineage analysis by cell sorting and culture identifies FLK1+VE-cadherin+ cells at a diverging point of endothelial and hemopoietic lineages. *Development* **125**, 1747-1757.
Olsson, A. K., Dimberg, A., Kreuger, J. and Claesson-Welsh, L. (2006). VEGF receptor signalling-in control of vascular function. *Nat. Rev.* **7**, 359-371.
Ottersbach, K. and Dzierzak, E. (2005). The murine placenta contains hematopoietic stem cells within the vascular labyrinth region. *Dev. Cell* **8**, 377-387.
Parma, P., Radi, O., Vidal, V., Chaboissier, M. C., Dellambra, E., Valentini, S., Guerra, L., Schedl, A. and Camerino, G. (2006). R-spondin1 is essential in sex determination, skin differentiation and malignancy. *Nat. Genet.* **38**, 1304-1309.
Patterson, L. J., Gering, M. and Patient, R. (2005). Scl is required for dorsal aorta as well as blood formation in zebrafish embryos. *Blood* **105**, 3502-3511.
Red-Horse, K., Crawford, Y., Shojaei, F. and Ferrara, N. (2007). Endothelium-microenvironment interactions in the developing embryo and in the adult. *Dev. Cell* **12**, 181-194.
Risau, W. and Flamme, I. (1995). Vasculogenesis. *Annu. Rev. Cell Dev. Biol.* **11**, 73-91.
Robitaille, J., MacDonald, M. L., Kaykas, A., Sheldahl, L. C., Zeisler, J., Dube, M. P., Zhang, L. H., Singaraja, R. R., Guernsey, D. L., Zheng, B. et al. (2002). Mutant frizzled-4 disrupts retinal angiogenesis in familial exudative vitreoretinopathy. *Nat. Genet.* **32**, 326-330.
Rossant, J. and Howard, L. (2002). Signaling pathways in vascular development. *Annu. Rev. Cell Dev. Biol.* **18**, 541-573.
Scheller, M., Huelsken, J., Rosenbauer, F., Taketo, M. M., Birchmeier, W., Tenen, D. G. and Leutz, A. (2006). Hematopoietic stem cell and multilineage defects generated by constitutive beta-catenin activation. *Nat. Immunol.* **7**, 1037-1047.
Schorpp-Kistner, M., Wang, Z. Q., Angel, P. and Wagner, E. F. (1999). JunB is essential for mammalian placentation. *EMBO J.* **18**, 934-948.
Shalaby, F., Ho, J., Stanford, W. L., Fischer, K. D., Schuh, A. C., Schwartz, L., Bernstein, A. and Rossant, J. (1997). A requirement for Flk1 in primitive and definitive hematopoiesis and vasculogenesis. *Cell* **89**, 981-990.
Shivdasani, R. A., Mayer, E. L. and Orkin, S. H. (1995). Absence of blood formation in mice lacking the T-cell leukaemia oncoprotein tal-1/SCL. *Nature* **373**, 432-434.
Shu, W., Jiang, Y. Q., Lu, M. M. and Morrisey, E. E. (2002). Wnt7b regulates mesenchymal proliferation and vascular development in the lung. *Development* **129**, 4831-4842.
Tammela, T., Enholm, B., Alitalo, K. and Paavonen, K. (2005). The biology of vascular endothelial growth factors. *Cardiovasc Res.* **65**, 550-563.
Ueno, H. and Weissman, I. L. (2006). Clonal analysis of mouse development reveals a polyclonal origin for yolk sac blood islands. *Dev. Cell* **11**, 519-533.
Visvader, J. E., Fujiwara, Y. and Orkin, S. H. (1998). Unsuspected role for the T-cell leukemia protein SCL/tal-1 in vascular development. *Genes Dev.* **12**, 473-479.
Vogeli, K. M., Jin, S. W., Martin, G. R. and Stainier, D. Y. (2006). A common progenitor for haematopoietic and endothelial lineages in the zebrafish gastrula. *Nature* **443**, 337-339.
Vokes, S. A., Yatskievych, T. A., Heimark, R. L., McMahon, J., McMahon, A. P., Antin, P. B. and Krieg, P. A. (2004). Hedgehog signaling is essential for endothelial tube formation during vasculogenesis. *Development* **131**, 4371-4380.
Walmsley, M., Ciau-Uitz, A. and Patient, R. (2002). Adult and embryonic blood and endothelium derive from distinct precursor populations which are differentially programmed by BMP in Xenopus. *Development* **129**, 5683-5695.
Wang, H., Gilner, J. B., Bautch, V. L., Wang, D. Z., Wainwright, B. J., Kirby, S. L. and Patterson, C. (2007). Wnt2 coordinates the commitment of mesoderm to hematopoietic, endothelial and cardiac lineages in embryoid bodies. *J. Biol. Chem.* **282**, 782-791.
Wei, Q., Yokota, C., Semenov, M. V., Doble, B., Woodgett, J. and He, X. (2007). R-spondin1 is a high affinity ligand for LRP6 and induces LRP6 phosphorylation and beta-catenin signaling. *J. Biol. Chem.* **282**, 15903-15911.
Winnier, G., Blessing, M., Labosky, P. A. and Hogan, B. L. M. (1995). Bone morphogenetic protein 4 is required for mesoderm formation and patterning in the mouse. *Genes Dev.* **9**, 2105-2116.
Xiong, J. W. (2008). Molecular and developmental biology of the hemangioblast. *Dev. Dyn.* **237**, 1218-1231.
Xu, Q., Wang, Y., Dabdoub, A., Samllwood, P. M., Williams, J., Woods, C., Kelley, M. W., Jiang, L., Tasman, W., Zhang, K. et al. (2004). Vascular development in the retina and inner ear: control by Norrin and Frizzled-4, a high affinity ligand-receptor pair. *Cell* **116**, 883-895.
Yang, H., Wanner, I. B., Roper, S. D. and Chaudhari, N. (1999). An optimized method for in situ hybridization with signal amplification that allows the detection of rare mRNAs. *J. Histochem. Cytochem.* **47**, 431-446.
Zeigler, B. M., Sugiyama, D., Chen, M., Guo, Y., Downs, K. M. and Speck, N. A. (2006). The allantois and chorion, when isolated before circulation or chorio-allantoic fusion, have hematopoietic potential. *Development* **133**, 4183-4192.
Zerlin, M., Julius, M. A. and Kitajewski, J. (2008). Wnt/Frizzled signaling in angiogenesis. *Angiogenesis* **11**, 63-69.
Zhang, X., Gaspard, J. P. and Chung, D. C. (2001). Regulation of vascular endothelial growth factor by the Wnt and K-ras pathways in colonic neoplasia. *Cancer Res.* **61**, 6050-6054.