SRF selectively controls tip cell invasive behavior in angiogenesis

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SUMMARY
Efficient angiogenic sprouting is essential for embryonic, postnatal and tumor development. Serum response factor (SRF) is known to be important for embryonic vascular development. Here, we studied the effect of inducible endothelial-specific deletion of Srf in postnatal and adult mice. We find that endothelial SRF activity is vital for postnatal growth and survival, and is equally required for developmental and pathological angiogenesis, including during tumor growth. Our results demonstrate that SRF is selectively required for endothelial filopodia formation and cell contractility during sprouting angiogenesis, but seems dispensable for vascular remodeling. At the molecular level, we observe that vascular endothelial growth factor A induces nuclear accumulation of myocardin-related transcription factors (MRTFs) and regulates MRTF/SRF-dependent target genes including My9, which is important for endothelial cell migration in vitro. We conclude that SRF has a unique function in regulating migratory tip cell behavior during sprouting angiogenesis. We hypothesize that targeting the SRF pathway could provide an opportunity to selectively target tip cell filopodia-driven angiogenesis to restrict tumor growth.

KEY WORDS: Filopodia, SRF, Sprouting angiogenesis, Actin, Myosin, Mouse

INTRODUCTION
Blood vessel network formation by sprouting angiogenesis involves a sequence of morphogenic events including the initial formation, guidance and extension of sprouts, anastomosis and lumen formation, allowing the formation of new vascular loops (Potente et al., 2011). Recent studies illustrate that individual endothelial cells (ECs) adopt distinct phenotypes and functions within the sprout, and the coordination of their dynamic behavior in response to tissue signals and cell-cell communication is instrumental for pattern formation (Potente et al., 2011). This is best understood for VEGF-VEGFR-mediated activation of ECs and DLL4/Notch-mediated selection of leading tip and trailing stalk cells in the vascular sprout (Hellström et al., 2007; Leslie et al., 2007; Sickmann and Lawson, 2007; Suchting et al., 2007; Benedito et al., 2012). Key features of tip cells are the extension of long, thin, actin-based filopodia and the ability to invade into avascular tissues (Dorrell et al., 2002; Gerhardt et al., 2003). The directional extension, the presence of various guidance receptors on filopodia, and the observation of tip-tip contact via filopodia all suggest that these structures are crucial mediators of tip cell function, analogous to filopodia functions in axonal growth cones during nervous system development and in Drosophila tracheal tip cells during airway morphogenesis (Ghabrial et al., 2003; Adams and Eichmann, 2010). VEGFA has been shown to promote endothelial migratory behavior and filopodia formation (Gerhardt et al., 2003; Lamalice et al., 2004), and VEGFA gradients modulate tip cell filopodia activity (Ruhrberg et al., 2002). Filopodia formation involves complex and dynamic rearrangement of actin cytoskeleton networks, enabling directed cell migration (Mattila and Lappalainen, 2008). However, we currently lack a good mechanistic understanding of how VEGFA regulates endothelial tip cell filopodia formation and tip cell invasion (Lamalice et al., 2007; De Smet et al., 2009).

In Drosophila trachea development, pruned mutants show defective tip cell filopodia formation and consequently impaired tracheal tip cell elongation and branching (Guillemin et al., 1996). The pruned gene (blistered) in Drosophila encodes the serum response factor (SRF), an evolutionarily conserved transcription factor that regulates the expression of genes through binding to a CArG box [CC(A/T)6GG] (Miano et al., 2007). SRF function appears to be particularly important in processes involving cell migration, including Drosophila tracheal morphogenesis, dorsal root ganglion terminal innervation and angiogenesis (Franco and Li, 2009; Miano, 2010). SRF transcriptional output is regulated mainly by its cooperation with two divergent co-factor families: the ternary complex factors ELK1, ELK3 (NET) and ELK4 (SAP1), which are under control of the MAPK signaling cascade (Buchwalter et al., 2004); and the myocardin-related transcription factors (MRTFs) MRTF-A (MKL1, MAL) and MRTF-B (MKL2), which are regulated by the actin polymerization state (Miralles et al., 2003; Vartiani et al., 2007).

Inactivation of the Srf gene in embryonic ECs leads to decreased vascular density, alteration in tip cell morphology and disruption of EC junctions. These defects result in aneurysm-like structure formation and hemorrhage, ultimately causing embryonic death at E14.5 (Franco et al., 2008; Holtz and Misra, 2008). In order to gain deeper insight into the specific role of SRF in vascular morphogenesis, we studied SRF function in postnatal angiogenesis. Induced endothelial Srf deletion at selected time points in postnatal mice led to dramatic growth retardation and decreased viability. Srf deletion induced systemic hypovascularization and severe retinal

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angiopathy. Genetic mosaic analysis illustrated that cell-autonomous filopodia defects and reduced actin contractility are the primary causes of the angiogenic defects. We also find evidence for VEGFA-mediated MRTF nuclear accumulation. In this context, MRTF-SRF activity controls the expression of contractility genes such as MyI9, Myh9 and Myh10, and decreased expression of MyI9 is sufficient to impair EC migration. We further find that SRF expression is increased in human tumor endothelium, and Srf deletion in mouse tumor models impairs tumor angiogenesis. Together, these results demonstrate the strict requirement of MRTF-SRF pathway activity in sprouting angiogenesis, and provide a basis for selectively targeting tip cell behavior as a potential mechanism to curb pathological angiogenesis.

**MATERIALS AND METHODS**

**Mice**

We used the following mouse lines: Srf floxed (SRFfl/fl) (Parlakian et al., 2004); PDGFB-c-ICreER (Claxton et al., 2008); the Cre reporter Rosa26-mTmG (mTmG) (Muzumdar et al., 2007); and LifeAct-GFP (Riedl et al., 2004); PDGFb-iCreER (Claxton et al., 2008); the Cre reporter Rosa26-recombination experiments, tamoxifen was injected (20 μl/g body weight of 1 mg/ml solution) at postnatal day (P) 2 before eyes were collected at P5 onwards. In mosaic recombination experiments, tamoxifen was injected (20 μg/g of 0.04 mg/ml solution) at P3 before eyes were collected at P6. N-[N-(3,5-difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyI ester (DAPT; 100 μg/g; Sigma) was injected subcutaneously. This study conformed to institutional guidelines for the use of animals in research.

**Mouse chimera generation**

At 3.5 days post-coitus (dpc), embryos of SRFfl/fl/PDGFB-cICreER/mTmG and SRFfl/fl/PDGFB-cICreER/mTmG mice were used to isolate ESCs, which were cultured in standard ESC media with the MEK inhibitor PD0325901 (Stemgent). ESCs were characterized and injected into 3.5-dpc staged Balb/cOlaHsd wild-type embryos and re-implanted into pseudopregnant foster females using standard protocols (Bryja et al., 2006).

**Cell culture and VEGFA stimulation**

HUVECs (PromoCell) were routinely cultured in EGM2-Bulletonk (Lonza). HUVECs at passage four were plated at 50,000 cells/cm² on gelatin precoated coverslips. At 80% confluence, medium was replaced with EGM2-growth factor-free/0.2% FBS overnight, and cells were then left untrated or stimulated with VEGFA165 (100 ng/ml; Peprotech). The wound-healing assay was performed as described previously (Franco et al., 2008).

**siRNA experiments**

The ON-TARGET set of four siRNAs against human SRF, MKL1, MKL2 and MYL9 was purchased from Dharmacon. HUVECs were transfected with 25 nM siRNA using the DharmaFECT 1 transfection reagent.

**Gene expression assays**

RNA extraction was performed using the RNeasy Micro Kit (Qiagen) and reverse transcription of mRNA with the First-Strand cDNA Synthesis Kit (Roche). Semi-quantitative real-time PCR was performed using a 7900HT Fast Real-Time PCR System and TaqMan gene expression probes (Applied Biosystems).

**Histology and immunofluorescence**

Retinas were collected from P5 onwards and fixed with 2% paraformaldehyde (PFA) in PBS for 5 hours at 4°C. Paraaffin embedding, cryosectioning and immunohistochemistry of tumor and mouse tissues were as described previously (Parlakian et al., 2004). Paraaffin human tumor tissue arrays were purchased from Biochain and processed as normal paraaffin sections. Immunohistochemistry was performed using the following antibodies: anti-CD31 (PECAM1, 1:400), anti-ICAM2 (1:100), anti-Ve-cadherin (cadherin 5, 1:50) (BD Bioscience); anti-active caspase 3 (1:400), anti-SMA-Cy3 (1:1000) (Sigma); anti-phosphohistone H3 (1:400), anti-MYH10 (1:100), anti-MYH9 (1:200) (Cell Signaling); anti-MRTF-A (1:50), anti-ERG1/2/3 (KCNH2/6/7, 1:100), anti-SRF (H300, 1:100 and G20, 1:200), anti-Ki67 (1:400) (Santa Cruz); anti-NG2 (CSPG4, 1:200) (Chemicon); anti-ZO1 (TJP1, 1:100) (Invitrogen); anti-DLL4 (1:100) (R&D Systems); anti-MYL9 (1:100), anti-p-MYL9 (1:400) (Abcam); and anti-collagen IV (1:400) (AbD Serotec). Primary antibodies were followed by incubation with an anti-rat IgG Cy3 (1:400) (Jackson Lab) and/or anti-rabbit IgG Alexa 488 or Alexa 647 (1:400), anti-goat IgG Alexa 488 (1:400), Alexa 488-conjugated phallolidin (1:100), and Alexa 647-conjugated isoeclin B4 (1:1000) (Invitrogen). DAPI (Sigma) was used to label nuclei. For confocal microscopy we used a Carl Zeiss LSM780. Super-resolution microscopy was performed using a DeltaVision OMX 3D structured illumination (3D-SIM) microscope (Applied Precision). Images were analyzed with ImageJ (NIH), Adobe Photoshop CS4 (Adobe Systems) and Imaris 7.4.0 (Bitplane) software.

**Western blot analysis**

Nuclear/cytosol fractioning and western blot analysis were performed as described previously (Parlakian et al., 2004) using the following antibodies: anti-MRTF-A (1:500), anti-MRTF-B (1:500), anti-SRF (1:500), anti-GAPDH (1:10,000) (Santa Cruz); anti-pMYL9 (1:2000), anti-MYL9 (1:2000), anti-β-tubulin (1:10,000), anti-lamin B1 (1:5000) (Abcam); anti-RHOA (1:2000), anti-CDC42 (1:2000), anti-pMYL9 (1:5000), anti-MYH10 (1:2000), anti-MYH9 (1:2000) (Cell Signaling). Corresponding HRP-conjugated secondary antibodies (1:5000; GE Healthcare) were used for chemiluminescence detection.

**Tumor studies**

Three-month-old SRFfl/fl and SRFfl/wt mice were treated by tamoxifen injection every second day over 10 days prior to tumor cell line injection. Xenograft tumors were generated by injecting 1×10⁶ Lewis lung carcinoma cells (3LL) subcutaneously into control and SRFiEC-KO mice. Tumor volume was measured every 4 days starting from the eighth day after tumor implantation. Volume was calculated using volume=width² × length × 0.52, as described (Galaup et al., 2006). At a given time point, mice were sacrificed and tumors were either fixed and embedded in paraffin or frozen in OCT solution (Miles Laboratories, Elkhart, IN, USA).

**Intraocular injections**

Intraocular injections were performed as described previously (Gerhardt et al., 2003). Right eye intraocular injection of 0.5 μl VEGFA165 (660 ng/ml in PBS; Peprotech) was performed using a Hamilton microsyringe. Four hours later pups were sacrificed and eyes collected and processed for immunohistochemistry.

**Hematopoietic cell counts**

Blood samples were analyzed using an MS-9 automated hematology analyzer (Melet Schloesing Technologies).

**Flow cytometric analysis**

After disruption and homogenization of hematopoietic tissues, cells were stained with antibodies raised against specific markers of hematopoietic lineages as reported (Ragu et al., 2010a; Ragu et al., 2010b). The cytometric detection of dsTomato Red and eGFP from bone marrow cell suspension lineage as reported (Ragu et al., 2010a; Ragu et al., 2010b). Flow cytometric data were analyzed using FlowJo Software (Tree Star).

**Quantification and statistical analysis**

For quantification of tip cells and branching points we scored tip cells as previously described (Gerhardt et al., 2003; Franco et al., 2008). All quantitative data were analyzed using unpaired Student’s t-tests for comparison with control mice at specific times using Prism 5.0 (GraphPad) and Excel (Microsoft) software. The data shown are mean ± s.e.m.

**RESULTS**

Postnatal endothelial-specific deletion of Srf causes systemic hypovascularization and impairs growth and survival

To inactivate SRF in ECs at specific stages, we crossed the Srf floxed mouse line (SRFfl/β) with mice carrying an inducible version
of Cre recombinase under the control of the Pdgfb promoter (PDGFb-iCreER) (Parlakian et al., 2004; Claxton et al., 2008). Tamoxifen-induced Cre recombinase activation excises Srf exon 2, which encodes the MADS box DNA-binding domain, and therefore creates a non-functional truncated SRF protein (Parlakian et al., 2004) (Fig. 1A).

SRFfl/fl and SRFfl/fl/PDGFb-iCreER mice, hereafter referred to as SRFwt or SRFiEC-KO, were given a single tamoxifen injection at P2 and analyzed on the following days. Efficient and specific Cre recombination in ECs was confirmed with the Cre recombinase reporter mouse line mTmG (Muzumdar et al., 2007) (supplementary material Fig. S1). Srf exon 2 deletion was confirmed by PCR on tail DNA and by western blot on lung extracts (Fig. 1B,C). A single tamoxifen injection at P2 led to a significant reduction in size and weight at P12 and to the reduced survival of SRFiEC-KO mice, as compared with SRFwt (Fig. 1D-F).

SRFiEC-KO mice showed an overall decrease in organ size, even when normalized to body weight (supplementary material Table S1) and died prematurely, averaging at P50. Heterozygous deletion of Srf in ECs did not result in any apparent defects and SRFfl/wt PDGFb-iCreER mice were indistinguishable from wild-type mice (data not shown). Analysis of blood vessel density in SRFiEC-KO mice identified a general hypovascularization in all investigated tissues, including muscle, kidney, lung and brain (Fig. 1G-I; data not shown). FACS and hematological analysis excluded hematopoietic deficiencies as a potential cause of the decreased viability (supplementary material Fig. S2). Thus, endothelial SRF is indispensable for postnatal blood vessel formation, and the reduced viability of mutant mice is likely to be associated with the insufficient vascularization of vital organs.

Deletion of Srf in postnatal ECs leads to decreased retinal sprouting angiogenesis and to severe persistent retinal angiopathy

To gain a mechanistic understanding of the underlying vascular defects, we investigated the postnatal retina of SRFiEC-KO mice. Expression analysis demonstrated that SRF is strongly expressed in a subset of ECs in active vascular sprouting regions, with the highest level observed in endothelial tip cells (Fig. 2A,E). In comparison, ECs in arteries, veins and capillaries of more central and established vessels show lower expression (Fig. 2B,C,E). However, ECs in other vascular beds showed variable SRF expression levels, with high levels observed in brain (supplementary material Fig. S3) and very low levels in peritoneal vasculature (Fig. 2D).

Given that VEGFA signaling regulates SRF in vitro (Chai et al., 2004; Franco et al., 2008) and that the SRF protein expression
pattern follows the proposed VEGFA gradients in the mouse retina (Gerhardt et al., 2003), we asked whether VEGFA affects SRF in vivo. Indeed, acute intraocular VEGFA injection significantly increased SRF protein levels in retinal ECs (supplementary material Fig. S4). Deletion of Srf in postnatal ECs at P2 led to a significant delay in radial expansion of the vascular bed and to reduced vascular density at subsequent stages of retinal development (Fig. 2F-I). Vascular sprouts in SRF^EC-KO mice displayed a blunted morphology, with clusters of cells that gave the sprout a ballooning appearance (Fig. 2J,K). However, consistent with previous reports on the embryonic vasculature (Franco et al., 2008), the number of sprouts at the vascular front was not significantly affected in the SRF^EC-KO retina (Fig. 2L). SRF-deficient tip cells also expressed similar levels of DLL4 protein compared with SRF^wt controls (supplementary material Fig. S5). However, the vascular front in SRF-deficient retinas extended conspicuously few and shorter filopodia (Fig. 2M,N). Measuring the distance from the base of filopodia projections to the corresponding tip cell nuclei in mutant mice (n=8); *P<0.05, **P<0.01, ***P<0.001 (unpaired Student’s t-test); error bars indicate s.e.m. Scale bars: 40 μm in A-D; 500 μm in FG; 20 μm in JK.

Fig. 2. Srf deletion in postnatal endothelium disrupts retinal angiogenesis. (A-C) SRF expression pattern (green) in blood vessels (red, CD31) in P6 mouse retinas. SRF is strongly expressed in capillary tip (AA, white arrowheads) and stalk (AA, yellow arrowheads) cells, and weakly in arterial (B,B) or venous (C,C) ECs (yellow arrows), in contrast to the strong SRF expression in mural cells (cyan arrows). (D) In peritoneal blood vessels of 3-month-old adult mice, ECs (yellow arrows) express very low levels of SRF, in contrast to smooth muscle cells (cyan arrows). (E) Quantification of SRF fluorescence signal in ECs from P6 wild-type retinas. (F,G) Retinal blood vessels (red, isoclin B4) of P6 mice show decreased vessel sprouting in SRF^EC-KO compared with control (F). (H) Quantification of radial expansion of blood vessels at different ages (n=8). (I) Quantification of vascular branching points per unit area in mutant and control retinas (n=8). (J,K) Vascular sprouts (red, isoclin B4) in SRF^EC-KO present morphological deficiencies, with decreased and shorter filopodia projections, decreased cytoplasmic extension (blue line), ballooned morphology and EC nucleus clumping (cyan asterisk; green, Erg). (L) Quantification of filopodia bursts per sprouting front length (n=6). (M) Quantification of filopodia projections per filopodia burst in P6 retinas (n=6). (N) Quantification of cytoplasmic extension in P6 retinas, showing a decrease in the distance from the filopodia base to the corresponding tip cell nuclei in mutant mice (n=8); *P<0.05, **P<0.01, ***P<0.001 (unpaired Student’s t-test); error bars indicate s.e.m. Scale bars: 40 μm in A-D; 500 μm in FG; 20 μm in JK.
The mouse retinal vascular plexus becomes fully developed at ~P21 (Fruttiger, 2007). In order to understand whether retinal sprouting defects in SRFiEC-KO mice were transient or persistent, we studied the retinal vascular network at different developmental stages. At P12, almost all sprouts in SRFiEC-KO mice appeared ballooned, substantially enlarged and lacked filopodia projections (Fig. 3A,B). With evident lumens and perfusion (supplementary material Fig. S6), these structures resembled saccular or ‘berry-like’ aneurysms, despite originating from stalled sprouts. Vascularization of the deeper retinal layers was also severely affected, and very few vessels were formed at this stage in SRFiEC-KO compared with control retinas (Fig. 3A,H11032,B/11032). The defects persisted at later stages, with mutant retinas showing large saccular structures and an almost complete absence of deeper vascular plexuses (Fig. 3G-J; supplementary material Fig. S7).

To test whether SRF is specifically required at particular phases during retinal angiogenesis or might have additional functions in vessel maintenance, we gave a single tamoxifen injection at P10, after the onset of the secondary sprouting phase, and assessed the vasculature at P22. Of the three vascular layers in the mouse retina, the intermediate vascular layer is the last to be formed by sprouting angiogenesis (Fruttiger, 2007). Interestingly, only vessels forming after P10 (mostly the intermediate retinal plexus), but not established vessels, were affected and showed the same ballooned sprout phenotype (supplementary material Fig. S8).

These results indicate that SRF is selectively required for effective vascular sprouting, in particular for filopodia-driven tip cell invasion and anastomosis, rather than tip cell specification. Moreover, SRF appears to be dispensable for other EC functions, such as lumenization, artery and vein differentiation and vessel pruning (Fig. 2F; Fig. 3B; supplementary material Fig. S6). In addition, recruitment of mural cells was unaffected in SRFiEC-KO mice (supplementary material Fig. S9).

**Endothelial Srf deletion impairs tumor growth**

The selective role of SRF in tip cell filopodia formation and invasion presented us with a unique opportunity to investigate whether targeting tip cell function impacts tumor angiogenesis. In order to induce tumor formation, lung carcinoma cells were subcutaneously injected into 3-month-old SRFwt and SRFiEC-KO mice previously treated with tamoxifen. At day 12, tumors in SRFiEC-KO mice were already significantly smaller than in control mice, and at day 24 the tumors collected from control mice were almost triple the volume of those collected from SRFiEC-KO mice (supplementary material Fig. S9).
SRFτEC-KO mice were often hemorrhagic despite their reduced size. Histology highlighted a significant increase in necrotic regions within the tumor mass (Fig. 4C-E). In SRFτEC-KO mice, blood vessels were more confined to the periphery of the tumors (Fig. 4D) and vascular density was significantly decreased (Fig. 4F-H). Accordingly, we observed decreased tumor cell proliferation (Fig. 4I-K) and increased apoptosis (Fig. 4L-N) in the mutant mice. Tumors in SRFτEC-KO mice showed decreased recruitment of smooth muscle (SMA-positive) cells (supplementary material Fig. S10), but not of pericytes (NG2-positive cells). This decreased mural cell coverage in tumor vessels differs from the normal mural cell coverage observed during developmental angiogenesis in SRFτEC-KO mice (supplementary material Fig. S9; Fig. 1H). It is conceivable that the decrease in tumor angiogenesis, and thus reduced tumor oxygenation, in SRFτEC-KO mice could promote persistently high VEGF levels that further impair tumor vessel maturation.

We also investigated endothelial SRF expression in human tumor samples. Immunostaining of tumor tissue arrays indicated that SRF was highly expressed in ECs in hepatocarcinomas (3/4) and astrocytomas (3/3), as compared with non-pathological control tissues (supplementary material Fig. S11).

We conclude that SRF is expressed and required during all stages of sprouting angiogenesis, including embryonic, postnatal and pathological angiogenesis. Based on our analysis we propose that SRF is a crucial regulator of tip cell filopodia formation and migration in sprouting angiogenesis.

**Impaired tip cell filopodia formation is a primary defect in SRFτEC-KO mice**

To directly test whether filopodia formation is truly dependent on SRF activity or whether tip cell specification is involved in the SRFτEC-KO phenotype, we blocked Notch signaling using a γ-secretase inhibitor (DAPT). Notch signaling negatively regulates tip cell formation, and DAPT dramatically increases tip cell formation in vivo, generating a highly dense vascular plexus (Hellström et al., 2007). Twenty-four hours post-DAPT injection, the vascular density appeared equally increased in SRFwt and SRFτEC-KO retinas (Fig. 5A-D), while radial expansion remained delayed in SRFτEC-KO retinas (Fig. 5E). Higher magnification of the vascular sprouting front showed that SRFτEC-KO ECs still possessed significantly fewer and shorter filopodia (Fig. 5C,D,F). Therefore, filopodia formation appears to be the primary defect of SRF- deficient ECs.

To assess the cell-autonomous function of SRF we lowered the tamoxifen concentration in order to induce mosaic inactivation of Srf in retinal ECs combined with the mTmG reporter as a marker for Cre activity (Fig. 5G,H). Mosaic SRF inactivation in ~20-30% of ECs caused no defects in radial expansion (Fig. 5O) and led to no other observable vascular phenotype. Given that lower doses of tamoxifen might not be sufficient for complete excision of the floxed Srf allele in all of the GFP-expressing cells, we generated embryonic stem cells (ESCs) from SRFτEC/PDGFb-iCreER/mTmG (Het ESCs) and SRFfl/fl/PDGFb-iCreER/mTmG (KO ESCs) mice.
Fig. 5. Filopodia defects are a primary cell-autonomous defect of SRF-deficient ECs. (A-D) Vasculature of P6 retinas (red, isolectin B4) from SRFwt (A,C) and SRFiEC-KO (B,D) mice after P2 tamoxifen injection and DAPT treatment for 24 hours. Both wild-type and mutant retinas show increased vascular density upon DAPT treatment. SRFiEC-KO tip cells (D) showed a decreased number and shorter filopodia (arrows) compared with SRFwt tip cells (C). (E) Quantification of radial expansion of DAPT-treated retinas (n=4). (F) Quantification of filopodia per filopodia burst in DAPT-treated retinas (n=4). (G,H) Confocal images of SRFfl/wt/PDGFb-iCreER/mTmG (G) and SRFfl/fl/PDGFb-iCreER/mTmG (H) retinas after low-dose tamoxifen injection at P3. Cells exposed to Cre recombinase activity express membranous eGFP signal (green). (I-N) Confocal images of chimeric retinas derived from injection of SRFfl/wt/PDGFb-iCreER/mTmG (I,J,L) and SRFfl/fl/PDGFb-iCreER/mTmG (K,M,N) ESCs into wild-type host embryos. SRFiEC-KO/mTmG tip cells show filopodia defects (green arrows in M,N), compared with control tip cells (green arrows in K,L). White arrows indicate host-derived wild-type filopodia bursts. Blue, isolectin B4; red, Erg. The boxed regions in I,J are magnified in K-N as indicated. (O) Quantification of radial expansion of DAPT-treated mosaic retinas (n=4). (P,Q) Srf knockout ECs have a smaller contribution to the tip position in the mosaic (P; 36±8.2%; n=4) or chimeric (Q; 27±8.3%; n=5) experiment, as compared with ~50% contribution of Srf heterozygous cells. (R) In mosaic experiments after 48 hours of DAPT treatment, SRFfl/fl/PDGFb-iCreER/mTmG ECs contribute normally to the tip cell position (52±17.4%; n=3). *P<0.05, **P<0.01, ***P<0.001 (unpaired Student’s t-test); error bars indicate s.e.m. Scale bars: 500 μm in A,B; 20 μm in C,D,K-N; 50 μm in G,H; 100 μm in I,J.
and injected them into wild-type blastocysts. Chimeric pups were thereafter treated with a full dose of tamoxifen at P2 to induce recombination in all Cre-expressing ECs, ensuring a more efficient method to produce Srf/knockout ECs in a mosaic vasculature. ECs derived from Het ECs contributed to the tip position and showed normal tip cell morphology with numerous filopodia (Fig. 5I,K,L). However, ECs derived from KO ECs showed a decreased capacity to be at the tip position at the vascular front (Fig. 5J), and, when occasionally found at the tip position, SRF-deficient ECs showed substantially fewer and shorter filopodia than control cells (Fig. 5M,N). In regions where the vasculature was mostly formed by KO ECs, the vascular front showed a similar vascular phenotype to that of SRF<sup>IEC-KO</sup> mice, but not in regions with a majority of Het ECs (supplementary material Fig. S12).

Given that cells compete for the tip position and exchange positions dynamically (Jakobsson et al., 2010), we hypothesize that in a mosaic context SRF-deficient tip cells will be overtaken by wild-type cells, thus restoring sprouting. Studying the distribution of recombined cells among the SRF<sup>IEC</sup>-<sup>B</sup> versus SRF<sup>IEC</sup>-<sup>het</sup> cells (Fig. 5P), we observed a reduced contribution of SRF-deficient cells to the tip position upon using the low-dose tamoxifen approach (36±8.2%) or the chimeric approach (27±8.3%), as compared with the contribution of Srf heterozygous cells (~50%). Interestingly, DAPT treatment in mosaic SRF experiments rescued the contribution to the tip position, and we found that 52±17.4% of the tip cells originated from SRF-deficient cells (Fig. 5R; supplementary material Fig. S12).

Together, these results suggest that in mosaic situations SRF-deficient cells show a decreased competitive capacity to reach the tip position compared with control cells. Interestingly, this effect appears to depend on DLL4/Notch signaling, despite the fact that DLL4 levels are unaffected in homogenous populations of SRF-deficient ECs (supplementary material Fig. S5). Also, the increase in vascular density and the increase in the number of filopodia bursts upon Notch inhibition suggest that tip cell specification per se does not require SRF. Our results instead provide the first experimental evidence for a crucial role of filopodia formation in the process of tip cell competition. Using computational modeling of tip cell selection, Bentley and colleagues previously predicted that filopodia act as amplifiers of DLL4/Notch-mediated lateral inhibition, as a cell that extends more and longer filopodia will be able to reach more VEGF and thus receive more VEGFR2 (KDR) signaling input driving DLL4 expression (Bentley et al., 2008; Bentley et al., 2009).

**Endothelial actin filament organization defects in SRF<sup>IEC-KO</sup> mice**

Given the well-described role of SRF in transcriptional regulation of actin regulatory proteins, we analyzed actin polymerization and organization in the retinal ECs of SRF<sup>IEC-KO</sup> mice by intercrossing the SRF<sup>IEC-KO</sup> and LifeAct-GFP mouse lines. The LifeAct-GFP transgene expresses an actin-binding protein fused to GFP that labels filamentous actin specifically (Riedl et al., 2010). Importantly, the LifeAct-GFP transgene is strongly expressed by the retinal endothelium, allowing visualization of the actin cytoskeleton specifically in the vasculature (Fraccioli et al., 2012). By confocal microscopy, we observed a marked decrease in endothelial actin polymerization in SRF<sup>IEC-KO</sup>/LifeAct-GFP retinas in filopodia and at the filopodium base (Fig. 6A-D). In control retinas, tip cells showed a large number of fine actin filaments protruding from the cell body, which appeared interconnected with cortical actin (Fig. 6A-D). By contrast, SRF<sup>IEC-KO</sup> tip cells showed almost no organized actin filament-containing protrusions. We also observed decreased EC cortical actin, including at EC junctions, compared with control LifeAct-GFP littersmates (Fig. 6E,F). Phalloidin staining and super-resolution microscopy confirmed the presence of a disrupted actin network in the SRF-deficient ECs (supplementary material Fig. S13).

Using VE-cadherin and ZO1 as EC junction markers we observed that, at the sprouting front of SRF-deficient retinas, junction profiles appeared straighter, with less serrated profiles, compared with control retinas (Fig. 6G,H). These serrated cadherin profiles have being linked to myosin-dependent retrograde cadherin flow (Kametani and Takeichi, 2007), suggesting that, in addition to filopodia formation defects, SRF-deficient ECs might also have an impaired acto-myoosin network (Fig. 6G,H). In cancer cells and megakaryocytes, it has recently been shown that SRF controls expression of myosin heavy chains and Myl9 (Gilles et al., 2009; Medjkane et al., 2009), genes known to be important for acto-myoosin filament contraction (Vicente-Manzanares et al., 2009).

Immunostaining showed that Srf deletion in ECs led to a decrease in the expression of myosin heavy chain 10 (MYH10) (Fig. 6J) and decreased the level of phosphorylated regulatory myosin light chain Myl9 (Fig. 6K,L), suggesting an overall decrease in the contractility of SRF<sup>IEC-KO</sup> ECs. Thus, SRF is required for a structured actin cytoskeleton network in retinal ECs, and reduced actin polymerization and actin cytoskeleton contractility could underlie the filopodia formation defects and clumping of ECs observed in the SRF-deficient vascular sprout.

**MRTFs regulate the expression of genes linked to the contractility apparatus through SRF**

We investigated whether SRF transcriptional activity regulates the expression of genes involved in the acto-myoosin contractile apparatus. Knocking down SRF protein levels in human umbilical vein endothelial cells (HUVECs) using specific siRNAs led to a significant downregulation of MYH9, MYH10, MYL9 and beta-actin (ACTB) mRNA expression levels (Fig. 7A), indicating that SRF is important for the expression of these genes in ECs in vitro. SRF knockdown in HUVECs further showed a significant reduction in MYL9, MYH9 and MYH10 protein levels as measured by immunofluorescence intensity (Fig. 7B-F) or western blot (Fig. 7G). However, no significant difference in the ratio of phosphorylated (p) MYL9 to total MYL9 was observed (Fig. 7G), and no changes in RHOD or CDC42 protein levels were seen (supplementary material Fig. S14).

Mechanistically, downstream of growth factor stimulation, SRF cooperates with MRTFs to control the expression of actin filament-related genes (Miano et al., 2007). Similarly, we observe that SRF and MRTFs regulate the expression of genes involved in the acto-myoosin contractile apparatus in ECs. Immunofluorescence intensity analysis showed a significant increase in the nuclear accumulation of endogenous MRTF-A upon VEGFA stimulation (Fig. 7H-J). In addition, cytoplasmic-nuclear fractioning revealed enrichment of nuclear MRTF-B upon VEGFA treatment (Fig. 7K). Interestingly, combined knockdown of both MRTF-A and MRTF-B in HUVECs led to decreased expression of MYL9, MYH9 and beta-actin (Fig. 7L), similar to the results observed in SRF knockdown experiments. However, no significant change in MYH10 expression was observed. Western blot analysis confirmed the strong downregulation of MYL9 protein levels in HUVECs (supplementary material Fig. S14).

Given the strong regulation of MYL9 expression by SRF and MRTFs, we decided to investigate whether defective MYL9 expression could explain the migratory deficiencies of SRF-deficient ECs. Using siRNA to target Myl9 mRNA for degradation,
we analyzed EC migration using the *in vitro* wound-healing assay. Remarkably, MYL9 knockdown caused significant migratory deficiencies when compared with control siRNA experiments, which mirrored the migratory phenotype observed for SRF or MRTF-A/B knockdowns (Fig. 7M). It is important to note that knockdown of MYL9 does not affect the expression of SRF or MRTFs themselves, and has only a small impact on other SRF-regulated genes (supplementary material Fig. S14), suggesting that defective EC migration is attributable to MYL9 knockdown.

Phalloidin staining of the different knockdown HUVECs showed that SRF and MRTF function has a strong impact on actin cable formation and total actin protein levels, in contrast to the effect of MYL9 knockdown, which has a low impact on filamentous actin compared with control samples. Accordingly, siRNA knockdown of SRF or MRTFs, but not of MYL9, significantly decreased the number of filopodia seen at the edge of the HUVEC monolayer (supplementary material Fig. S15). We conclude that an intact contractile apparatus is essential for proper EC migration, and SRF-mediated regulation of the expression of proteins involved in this process, including MYL9, MYH9 and MYH10, could be essential during sprouting angiogenesis.

**DISCUSSION**

EC migration is crucial for all aspects of blood vessel formation. Whether EC progenitors assemble during early vasculogenesis, or vessels sprout and remodel during angiogenesis, ECs need to be...
Fig. 7. VEGF modulates SRF transcriptional activity in ECs via MRTFs. (A) mRNA expression levels of the indicated genes in SRF knockdown HUVECs, showing that SRF activity is important for sustained expression of MYL9, MYH9, MYH10 and beta-actin (ACTB). However, VEGFR2 (KDR) levels were unchanged (n=3). (B-E) Immunofluorescence staining of HUVECs showed that knockdown of SRF (C,E) leads to decreased MYH10 (green, B,C) and MYH9 (green, D,E) protein expression, and decreased actin polymerization (phalloidin (red), D,E) compared with control siRNA transfection (B,D). (F) Fluorescence quantification shows significantly lower phalloidin, MYH9 and MYH10 intensity in SRF knockdown HUVECs. (G) Western blot analysis confirms that SRF is important for sustained expression of MYL9, MYH9 and MYH10. Tubulin (Tub.) was used as a loading control. (H,I) MRTF-A immunostaining (green) in starved (H) or VEGFA-stimulated (I) HUVECs showed that VEGFA induces MRTF-A nuclear accumulation. DAPI, blue. (J) MRTF-A fluorescence intensity quantification (n=3). (K) Western blot analysis of MRTF-B protein in cytoplasmic and nuclear fractions of HUVECs starved for 16 hours or stimulated with VEGFA for 2 hours, which led to a significant enrichment of MRTF-B in nuclear extracts upon VEGFA stimulation. Tubulin and lamin B1 were used as loading controls. (L) Knockdown of both MRTF-A (MKL1) and MRTF-B (MKL2) in HUVECs, showing that MRTF activity is important for expression of SRF, MYL9, MYH9 and ACTB. However, VEGFR (KDR) and MYH10 levels were unchanged (n=3). (M) Wound-healing migration assay used to quantify the overall motility of ECs when depleted of SRF, MRTF-A (MKL1) and MRTF-B (MKL2), or MYL9. Specific knockdown of SRF, MRTF-A/B and MYL9 all led to decreased EC migration compared with the control siRNA transfection. *P<0.05, **P<0.01, ***P<0.001 (unpaired Student’s t-test); error bars indicate s.e.m. Scale bars: 20 μm in B,C,H,I; 40 μm in D,E.
motile and to translocate with respect to the surrounding tissue (invasion) and each other (intercalation, remodeling). Here, we identify for the first time a molecular component that is selectively required for endothelial invasion. Most of the molecular regulators of EC migration that have been identified so far affect both sprouting and remodeling, such as WAVE2 (WASP2), PI3K and SYX (PLEKHG5) (Yamazaki et al., 2003; Garnaas et al., 2008; Graupera et al., 2008). SRF, however, is highly expressed in ECs at the sprouting front, and is selectively involved in filopodia formation and tip cell invasion during both embryonic and postnatal angiogenesis. The loss of endothelial SRF leads to dramatic and progressive morphological changes in vascular sprouts. Decreased angiogenesis was observed in all organs of SRFΔEC-KO mice analyzed in this study, which correlated with decreased organ growth and reduced body weight gain. SRFΔEC-KO mice died ~50 days following a single tamoxifen injection for reasons that remain unknown; however, we are tempted to speculate that generalized hypovascularization leads to severe growth retardation resulting in the observed decrease in viability.

Notably, we show for the first time that SRF function is also conserved in pathological conditions. Tumors implanted in SRFΔEC-KO mice were poorly vascularized and had markedly reduced growth as a likely consequence of defective tip cell-driven angiogenesis. However, all other aspects of vessel morphogenesis, such as arterio-venous differentiation, lumen formation and vessel pruning were surprisingly unaffected, suggesting that EC motility during the sprouting and remodeling phases of angiogenesis rely on different molecular mechanisms. Curiously, no significant differences in VE-cadherin protein levels were observed in the retinal vessels of the SRFΔEC-KO mouse, which is in contrast to previous observations in the embryonic vasculature (Franco et al., 2008). This result might signify a specific role of SRF during embryonic vascular development.

The reasons for a specific requirement of SRF in tip cell invasion remain incompletely understood. Our results show that Srf knockout tip cells are unable to establish organized actin fibers and a contractile apparatus to drive tip cell filopodia extension and sprout elongation, suggesting that SRF deficiency disrupts essential cell mechanics that drive tip cell migration and cell rearrangements. In other organ systems and cellular contexts, it has been shown that migration depends on different actin-based structures at both the front and rear of the cell (Pollard and Cooper, 2009; Lecuit et al., 2011). De novo actin polymerization, via activity of the Arp2/3 complex and members of the formin family, promotes the oriented protrusive activity of cytoplasmic membranes. Myosin motor proteins use actin cables to create tension forces, promoting the contraction of cell structures (Pollard and Cooper, 2009; Lecuit et al., 2011). Although both modes use the same actin building blocks, different spatial arrangements and forces are applied on the actin networks (Lecuit et al., 2011). Impairing one or other mechanism will promote different phenotypes, as cells will be able to extend but not contract, or vice versa. In Drosophila trachea development, the excision of leading tip cells abrogates cell intercalation in the stalk, arguing that tracheal tip cells exert pulling forces on the invading sprout, promoting elongation (Causinus et al., 2008). Our present results would fit well with a scenario in which leading tip cell selectivity requires the de novo formation of actin filaments for filopodia formation and invasion, and requires acto-myosin contraction to exert pulling forces on the trailing stalk cells to elongate the vascular sprout. In this context, SRF seems to be important for both mechanisms because Srf null cells show defective filopodia, and the presence of Srf null ECs at the stalk position does not perturb normal sprouting.

Mechanistically, enhanced actin polymerization is known to stimulate gene transcription via a signaling pathway that is regulated by nuclear translocation of MRTFs, in which SRF functions as the main transcription factor (Vartiainen et al., 2007). Our results provide the first evidence for a functional MRTF-SRF axis in ECs. In the context of sprouting angiogenesis this raises the possibility that, in vivo, VEGF could control SRF activity by promoting actin polymerization and MRTF nuclear transport, which would contribute to the overall motility and behavior of endothelial tip cells (supplementary material Fig. S16). We propose that, during sprouting angiogenesis, ECs engage a VEGF-MRTF-SRF signaling pathway, the function of which is crucial for proper tip cell migration and invasion. Although Mrtfb (Mk2) knockout mice show a vascular phenotype, it is difficult to interpret because, like SRF, MRTF-B appears to have multiple functions, including roles in heart and smooth muscle cell development (Oh et al., 2005; Li et al., 2012). Therefore, endothelial-specific analysis of MRTF-A and MRTF-B function should be addressed in the future in order to elucidate the contribution of such a pathway in sprouting angiogenesis.

Recent results have shown that overexpression of Branchless, a Drosophila homolog of FGF, is enough to drive all the phenotypic features of tracheal tip cells in an Srf knockout background. The authors showed that SRF, in this context, is a non-essential regulator of tracheal tip cell function (Gervais and Casanova, 2011). Likewise, we found that SRFΔEC-KO retinas have similar numbers of sprouts to controls, and filopodia formation occurs at the right place and with the correct polarization, demonstrating that SRF is not essential for the specification and polarization of endothelial tip cells. However, despite these similarities between both systems, in ECs SRF does seem to have an essential role in regulating genes that support tip cell invasiveness and migration. Interestingly, Drosophila border cell migration, which provides a model for collective cell migration and invasion, is also controlled through activation of the MRTF-SRF signaling pathway downstream of PDGF/VEGF and EGF receptors (Friedl and Gilmour, 2009). The Drosophila MRTF-SRF pathway is needed to build a robust cytoskeleton necessary for the integrity and migration of border cells (Somogyi and Rørth, 2004), which strongly correlates with the conclusions of this study. Nevertheless, in the future it will be important to discriminate which genes under SRF regulation are crucial for filopodia formation and function of the acto-myosin contractile apparatus. In this study, we have identified Myl9 as being regulated by the MRTF-SRF pathway and that this could be important for EC motility in vivo. The link between MRTF-SRF, MYL9 and cell migration has previously been shown to be important for tumor and megakaryocyte migration in vivo (Gilles et al., 2009; Medjkane et al., 2009), and it is therefore likely that Myl9 inactivation could impair tip cell behavior.

Current strategies to curb tumor growth by targeting angiogenesis have reached the clinic, but are marred with difficulties as tumors switch to alternative pro-angiogenic pathways (De Bock et al., 2011). Strategies that combine anti-angiogenic and anti-tumor cell invasion, such as combinatorial inhibition of VEGF and c-MET (Sennino et al., 2012), appear highly promising, as they limit both primary tumor growth and its propensity to disseminate. Given that a growth factor–MRTF-A–SRF signaling pathway is important for acto-myosin mechanics driving tumor cell invasion (Medjkane et al., 2009) and, as we show here, is also selectively crucial for endothelial tip cell invasion, it would seem advantageous to target this axis simultaneously in tumors and ECs.
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Competing interests statement

The authors declare no competing financial interests.

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Supplementary material

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