ERK-MAPK signaling opposes Rho-kinase to promote endothelial cell survival and sprouting during angiogenesis

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Summary

Inhibition of ERK-MAPK signaling by expression of dominant-negative MEK1 in the tumor vasculature suppresses angiogenesis and tumor growth. In an organotypic tissue culture angiogenesis assay, ERK-MAPK inhibition during the migratory phase results in loss of bipolarity, detachment, and cell death of isolated endothelial cells and retraction of sprouting tubules. These effects are the consequence of upregulated Rho-kinase signaling. Transient inhibition of Rho-kinase rescues the effects of ERK-MAPK inhibition in vitro and in vivo, promotes sprouting, and increases vessel length in tumors. We propose a regulatory role of Rho-kinase by ERK-MAPK during angiogenesis that acts through the control of actomyosin contractility. Our data delineate a mechanism by which ERK-MAPK promotes endothelial cell survival and sprouting by downregulating Rho-kinase signaling.

Introduction

The tumor vasculature is an important target in cancer therapy, as the growth and metastasis of tumors depends on their vascular network (Folkman, 1990; Zetter, 1998). Understanding the signaling pathways that control angiogenesis will aid identification of therapeutic targets and the design of effective antiangiogenic therapies. Tumors employ diverse mechanisms for vessel formation, including sprouting from existing blood vessels (Ergun et al., 2001), de novo recruitment of endothelial precursors (Lyden et al., 2001), vessel remodeling (Patan et al., 1996), and cooption (Holash et al., 1999). Endothelial cell behavior during these processes is regulated by diverse signals from growth factors, cytokines, and the extracellular matrix (Ferrara, 2002). Studies show that such signals converge on common signaling pathways that control cell proliferation, survival, migration, and morphogenesis (Liotta and Kohn, 2001). The components of these signaling pathways therefore constitute potential targets for antiangiogenic therapy.

It has been suggested that the RAS-RAF-MEK-ERK signaling module is involved in tumor angiogenesis (Mukhopadhyay et al., 1998), and there is evidence in endothelial cells that MAPK-ERK signaling plays a central role during angiogenesis. *Mek1* knockout mice have defective angiogenesis in the placenta and are embryonic lethal (Giroux et al., 1999). Dominant-negative Raf targeted to the neovasculature of tumors induces endothelial apoptosis and inhibits tumor growth (Hood et al., 2002). Inhibition of ERK-MAPK signaling suppresses the formation of new blood vessels in response to growth factor stimulation in the chorioallantoic membrane in vivo (Eliceiri et al., 1998). ERK-MAPK signaling has been implicated in endothelial cell proliferation (Meadows et al., 2001), VEGF-mediated survival (Berra et al., 2000; Gupta et al., 1999), and protection against receptor-mediated apoptosis (Alavi et al., 2003). Another way ERK-MAPK signaling is thought to stimulate angiogenesis is by promoting endothelial cell motility (Eliceiri et al., 1998). Although the mechanism of activation of MAPK-ERK in endothelial cells by growth factors and integrins (Eliceiri et al., 1998) and the differential regulation of Raf-1 by different stimuli (Hood et al., 2003) have been characterized, it is not clear how ERK-MAPK signaling mediates its effects during tumor angiogenesis in vivo. Moreover, the mechanism by which ERK-MAPK promotes endothelial cell survival and influences motility are not understood.

In addition to ERK-MAPK signaling, there is evidence that Rho family GTPase signaling is involved in angiogenesis. Members of this family are activated in response to growth factors and cell-matrix adhesion and are key regulators of the actin cytoskeleton (Etienne-Manneville and Hall, 2002). Inhibition of Rho family GTPases disrupts tube formation in three-dimensional

SIGNIFICANCE

Delineating signaling pathways in endothelial cells in their physiological context is likely to generate more effective antiangiogenic therapies for cancer. In this study, we investigate the role of ERK-MAPK signaling in angiogenesis in tumors in vivo and in an organotypic angiogenesis assay. We show that ERK-MAPK opposes Rho-kinase-dependent actomyosin contractility to promote endothelial cell survival and vessel sprouting. While inhibition of ERK-MAPK in the tumor vasculature inhibits angiogenesis, inhibition of Rho-kinase promotes sprouting and increases vessel length. Our data delineate a role for ERK-MAPK signaling during angiogenesis and demonstrate that transiently inhibiting Rho-kinase may have a positive effect on tumor angiogenesis.

matrices in vitro (Cascone et al., 2003; Hoang et al., 2004). The activity of the prototypical member of the family, Rho, has been shown to be necessary for VEGF-driven angiogenesis in the chorioallantoic membrane, and the VEGF-mediated organization of endothelial cells into vessels in a skin angiogenesis model in vivo (Hoang et al., 2004). The serine/threonine kinases ROCKI/II (also referred to as ROKβ/a or Rho-kinase) are effectors of RhoA and RhoC. They control actomyosin contractility by leading to the phosphorylation of the regulatory myosin light chain II (MLC2) either directly or via inhibiting the myosin binding subunit (MYPT1) of myosin light chain phosphatase (MLCP) (Riento and Ridley, 2003). They also regulate actin assembly by activating LIM kinase (Riento and Ridley, 2003). The control of actomyosin contractility and actin assembly are thought to be key to the way Rho-kinase regulates cell migration, with a net positive or negative effect of Rho-kinase inhibition on migration depending upon the cell type and conditions (Riento and Ridley, 2003). In endothelial cells, Rho-kinase may have multiple functions during angiogenesis. Inhibiting Rho-kinase inhibits endothelial permeability, and it has been suggested that this is by disrupting endothelial cell tight junctions (Wojciak-Stothard and Ridley, 2002). In different assays of endothelial cell function in culture, Rho-kinase inhibition has been shown to disrupt vacuole formation and increase cellular protrusions, resulting in disruption of cord formation (Somlyo et al., 2003). These studies suggest that the regulation of Rho signaling plays an important role during angiogenesis.

Previously, it has been shown that pharmacological inhibition of ERK-MAPK activation compromises tumor growth in experimental animals without leading to toxicity (Sebolt-Leopold et al., 1999). Given the observations that ERK-MAPK signaling plays a role in angiogenesis (Eliceiri et al., 1998; Giroux et al., 1999; Gupta et al., 1999; Berra et al., 2000; Meadows et al., 2001; Hood et al., 2002), we sought to investigate whether selective inhibition of ERK-MAPK activation in the vascular compartment of tumors would inhibit tumor growth. We show that selective expression of dominant-negative MEK1 in the tumor vasculature results in reduced vascularization and tumor growth. To delineate the mechanism, we used an organotypic angiogenesis assay, in which vessel formation results from interaction of endothelial cells with fibroblasts (Bishop et al., 1999). Recently, it has been shown that such cocultures generate stable and long-lasting vessels when implanted in mice (Koike et al., 2004). By following endothelial cell migration and sprouting in real time by time-lapse microscopy, we show that ERK-MAPK signaling promotes endothelial cell survival and vessel sprouting by downregulating Rho-kinase activity. Rescue of the effects of ERK-MAPK inhibition by transiently inhibiting Rho-kinase in the tumor angiogenesis model confirms that this mechanism operates in tumors. Our data suggest that the balance between ERK-MAPK and Rho-kinase signaling is a key determinant of vascular morphogenesis in vivo and demonstrate that inhibition of ERK-MAPK in the vascular compartment of tumors alone can compromise tumor growth.

Results

ERK-MAPK signaling in the vascular compartment of tumors is necessary for angiogenesis and tumor growth

To block activation of the MAPKs ERK1/2 in blood vessels, we selectively expressed dominant-negative MEK1 (MEK1A; Cowley et al., 1994) in the vascular compartment of human tumor cell lines growing as xenografts in nude mice. Selective transgene expression in the vascular compartment of tumors is achieved by coinjection of tumor cells with irradiated ecotropic retrovirus producers (Mavria and Porter, 2001). In this experimental setting, the ecotropic retrovirus infects murine endothelial cells and some tumor-associated fibroblasts in the host-derived stroma but not the human cancer cells (Mavria and Porter, 2001 and Figure S1A in the Supplemental Data available with this article online). The irradiated virus producers disappear from the tumor mass within 2 weeks of tumor growth (Mavria and Porter, 2001). The BE colorectal cancer cell line was chosen because it forms well-vascularized tumors in mice (G.M., unpublished data). BE tumors with MEK1A transduction in the vascular compartment resulting from the coinjection of tumor cells with MEK1A virus producers grew at a slower rate compared to tumors with control EGFP transduction for 5 weeks after the tumors appeared (Figure 1A), whereas at later time as expected tumor growth resumed (data not shown). Coinjection of tumor cells with producer cells making an ecotropic retrovirus encoding a constitutively activated version of MEK1 (MEK1EE; Cowley et al., 1994) had little effect on the growth of these fast-growing tumors. In contrast, when the slow-growing breast cancer cell line MDA-MB-361 was coinjected with producer cells making the ecotropic MEK1EE virus, tumor growth was considerably enhanced compared with controls (Figure 1A). Therefore, manipulation of ERK1/2 signaling in the vascular compartment modifies tumor growth.

To determine whether the observed reduction in tumor growth was associated with defective angiogenesis, tumors were dissected out for analysis of their blood vessels by staining for the endothelial cell marker CD31. Representative pictures of BE tumors with dominant-negative MEK1 transduction in the vascular compartment are shown in Figure 1B. We quantitated the overall CD31-stained area as a total measure of vasculature. Vessel length, the number of branching points, and vessels with open lumens were used as measures of vessel morphogenesis. Quantitation revealed 25% reduction in vessel area, 71% reduction in the number of vessels with open lumens, and 42% reduction in the number of branching points of MEK1A tumors compared to control (Figures 1C–1E). In addition, there was a 49% decrease in the number of longer vessels (Table 1). Consistently, MDA-MB-361 tumors expressing constitutively active MEK1 (MEK1EE) in the vascular compartment had larger vessels with open lumens compared to controls (Figure S1C). Altogether, these results show that ERK-MAPK signaling is necessary for tumor angiogenesis and that inhibition of ERK-MAPK signaling in the vascular compartment alone can compromise tumor growth.

Distinct stages during tube formation in endothelial-fibroblast cocultures

In order to investigate the mechanism of inhibition of angiogenesis in response to inhibition of ERK-MAPK signaling, we chose a tissue culture organotypic angiogenesis assay (TCS Angiokit) in which tubule formation results from the interaction between human umbilical vein endothelial cells (HUVECs) and human dermal fibroblasts (HDFs). Coculture of these cell types gives rise to tubules with lumens embedded in naturally produced extracellular matrix (Bishop et al., 1999) that show a better resemblance to capillaries in vivo than tubes formed by monocultures of endothelial cells in matrigel (Donovan et al., 2001).

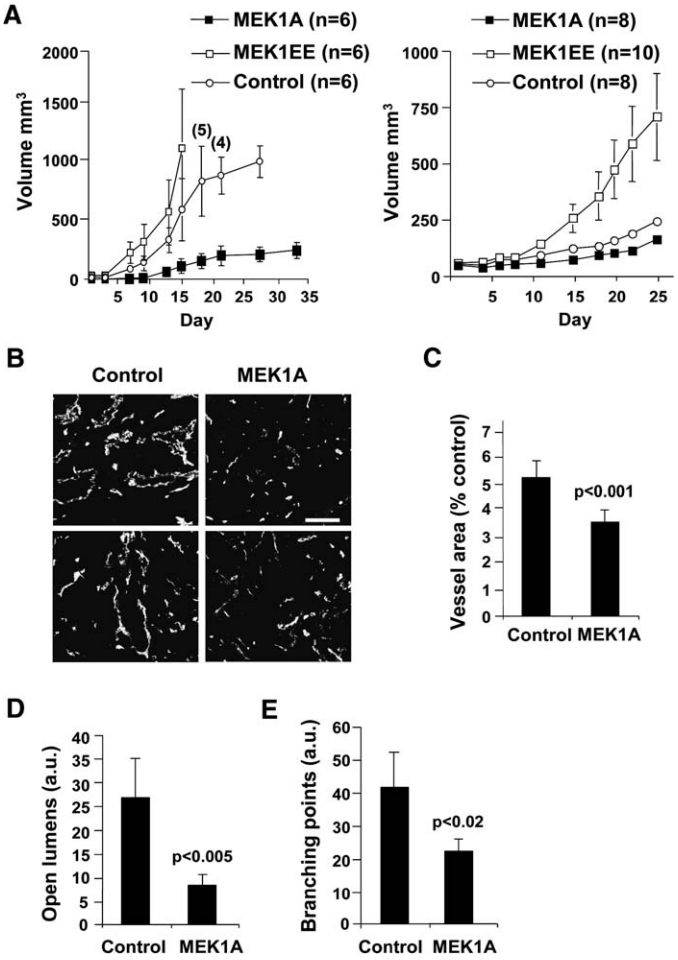

Figure 1. Expression of dominant-negative MEK1 in the vascular compartment of tumors suppresses angiogenesis and tumor growth

A: Tumor growth analysis. Tumors with MEK1A (dominant-negative MEK1), MEK1EE (constitutively active MEK1), or control (EGFP) retroviral transduction in the vascular compartment were set up as described in Experimental Procedures (left panel, BE colon carcinoma; right panel, MDA-MB-361 breast carcinoma). Graphs indicate the mean tumor growth rates ± SD. x axis: days after the appearance of tumors. Shown on the BE control growth curve are the numbers of animals with tumor size < 1500 mm^3. All tumors with MEK1EE expression in the vasculature reached maximum diameter by day 15. **B:** Tumor vessel appearance. BE tumors were analyzed when they reached approximately 0.6 cm diameter; cryosections were examined by immunofluorescence for CD31 expression. Two sections typical of the difference between tumors with MEK1A and control retroviral transduction are shown. Scale bar, 100 μm. The fractional area covered by $CD31^+$ features (**C**), the number of vessels with open lumens (**D**), and the number of branching points (**E**) are represented as mean ± SD (n = 4 tumors). Counts for each tumor were from eight sections, representing four different levels. For each section, the microscopic field (×20) showing the greatest level of CD31 staining was counted.

Angiogenesis in this system depends on the two major angiogenic factors bFGF and VEGF (Ferrara, 2002). FGF is supplied in the culture medium (TCS CellWorks), whereas VEGF is produced by the coculture system (see Table S1). Both antibodies to VEGF and VEGF antisense reagents have been shown to inhibit tube formation in this system (Table S2). The coculture assay is sensitive to a large number of well-established inhibitors of angiogenesis, including thrombospondin derivatives, endostatin, RGD peptide, and MMP inhibitors (Table S2). In this study, we modified the coculture assay by using HUVECs stably expressing EGFP to permit monitoring tubule formation by time-lapse microscopy. Real-time tracking after initiation of the coculture revealed three distinct stages during the assay. In the early stage of the coculture, endothelial cells appeared flattened, had multiple protrusions, and exhibited random cell motility (Figure 2 and data not shown). During this stage, endothelial cells often formed small islands, and there was high endothelial cell proliferation (data not shown). This early stage was followed by a migratory phase approximately 1 week after initiation of the coculture (Figure 2). During this phase, endothelial cells became bipolar, migrated in a directional manner, and assembled into tubules (Figure 2 and Movie S1). In addition, there was sprouting from islands of endothelial cells that had formed during the proliferative phase and from early tubules, and there was little or no proliferation (Movie S1). After 2 weeks in coculture, the tubules were established, there was further thickening, and some elongation could be observed (Figure 2 and data not shown). At this stage, there was expression of VE-cadherin at endothelial cell junctions (data not shown).

Table 1. Vessel length analysis

	Vessel length per field (a.u.)	Frequency (%) of vessel length (a.u.)			Median (a.u.)
		0–3	3–5	>5	
Control	141.6 (±9.3)	72.6	14.5	12.8	1.8
MEK1A	104.1 (±11.1)*	80.8	12.4	6.5	1.5
MEK1A + Y27632	134.7 (±11.9)**	76.6	11.5	11.9	1.6
Y27632	132.1 (±11.9)	66.5	15.5	17.9	2.0

Vessel length is the average value of the sum of individual vessel lengths in each of 32 fields representing the area of highest vessel density of a section. Sections were from four tumors per group, with eight sections representing four different levels in a tumor. *$p < 0.002$ with respect to control (n = 4); **$p < 0.02$ with respect to DN MEK (n = 4). a.u., arbitrary units.

ERK-MAPK signaling in endothelial cells during the migratory phase is necessary for tube formation in endothelial-fibroblast cocultures

To investigate the role of ERK1/2 signaling in endothelial cells during tube formation, we inhibited ERK1/2 activation during the migratory phase of the endothelial-fibroblast coculture 1 week after its initiation, using the pharmacological inhibitor PD184352 at concentrations that inhibit activation of ERK1/2 but not ERK5 (Mody et al., 2001). Treatment of the cocultures for 48 hr during this phase had a marked antiangiogenic effect (Figure 3A). EC proliferation at earlier stages was unaffected by inhibition of ERK-MAPK signaling, while treatment of cocultures once the tubes had already established had little effect (data not shown), arguing that ERK1/2 signaling is necessary during the migratory phase. To determine whether there was apoptosis in response to inhibition of ERK-MAPK signaling, we stained the cocultures with TUNEL (Figure 3B). We observed that it was single endothelial cells that were TUNEL positive after PD184352 treatment as opposed to endothelial cells that had assembled into tubules. However, cell rounding was noticeable at the tips of those tubules (Figure 3B). Using time-lapse microscopy, we found that cell death occurred over a period of 12–18 hr of inhibitor treatment (Movie S2). To find out whether this effect was on endothelial cell signaling, ERK1/2 was inhibited specifically in endothelial cells by means of expressing

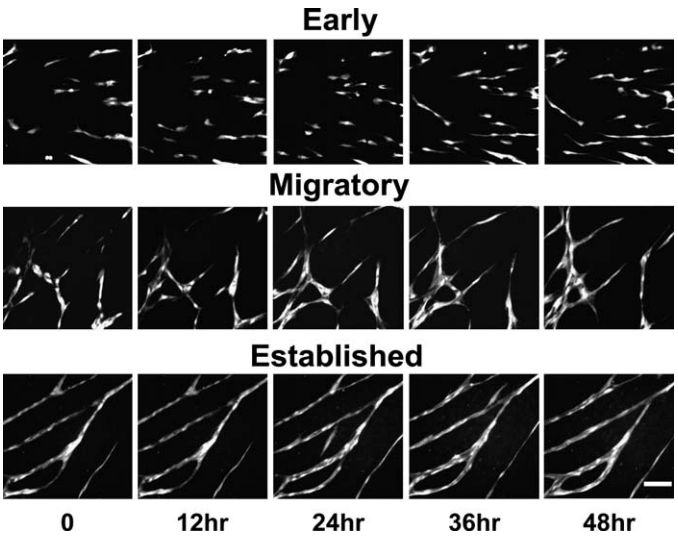

Figure 2. Three stages during angiogenesis in coculture of endothelial cells with fibroblasts

HUVEC-EGFP were cocultured with human dermal fibroblasts (HDFs), and tube formation was followed by time-lapse microscopy. Video stills are shown at 12 hr intervals over a 48 hr period for each stage. Three stages of endothelial behavior are distinguishable: ''early,'' where endothelial cells proliferate and have random motility (images are shown at days 4–6 after initiation of a coculture); ''migratory,'' where endothelial cells adopt a bipolar morphology, migrate in a directional manner, assemble into vessels, and sprout (images are from a day 7–9 coculture); and ''established,'' where vessels become thicker with some elongation (images are from days 14–16 of a coculture). Scale bar, 50 μm.

MEK1A in HUVECs before coculturing them with fibroblasts. Expression of MEK1A in HUVECs resulted in inhibition of angiogenesis in coculture (Figure 3C). Stable expression of MEK1A in HUVECs had no effect when the cells were cultured alone. In an alternative approach to inhibit ERK1/2 activation specifically in endothelial cells, HUVECs were transfected with siRNAs against *Raf-1* and *B-Raf* and seeded onto a confluent layer of fibroblasts. When seeded on confluent fibroblasts, HUVECs rapidly enter the migratory phase with little or no proliferation and form tubes (see Movie S4). Figure 3D shows that there was reduced tube formation when ERK1/2 activation was inhibited with Raf siRNAs. We found that it was necessary to silence expression of both *Raf-1* and *B-Raf* to downregulate ERK phosphorylation (Figure S2), arguing that both these kinases contribute to the activation of ERK1/2 in HUVECs (data not shown). These experiments show that ERK1/2 signaling in endothelial cells during the migratory phase is required for endothelial cell survival. Since both Raf-1 and B-Raf were necessary to control ERK-dependent survival during the migratory phase in this study, it is possible that the documented ERK-independent role of Raf-1 in endothelial cell survival (Alavi et al., 2003) is more relevant during the proliferative stage.

ERK-MAPK signaling is necessary for endothelial cell bipolarity, attachment, and survival during the migratory phase

During migration and vessel formation, endothelial cells adopt bipolar and elongated cell morphology (Figure 2). While following the survival of isolated endothelial cells in response to PD184352 treatment, time-lapse microscopy revealed distinct changes in endothelial cell morphology as shown by loss of bipolarity, preceding endothelial cell apoptosis (Movie S2). To demonstrate this better, we seeded HUVECs stably expressing MEK1A on confluent fibroblasts. There was loss of bipolarity of HUVEC-MEK1A cells within 18 hr of seeding (Figure 4A). Within 24 hr, HUVEC-MEK1A had detached, while control HUVECs had started aligning into tube-like structures. Movies of loss of bipolarity, detachment, and cell death can be seen in Movies S3 and S4. A loss of bipolarity that was similar to that seen in HUVEC-MEK1A was observed when HUVECs seeded on confluent fibroblasts were treated with PD184352 or Raf siRNAs (Figures 4B and 4C, respectively) that led to detachment from the fibroblast layer and cell death, as revealed by time-lapse microscopy (Figures 4B and 4D). These experiments suggest that endothelial apoptosis in response to inhibition of ERK-MAPK signaling results from changes in cell morphology leading to altered cell adhesion and detachment from the extracellular matrix.

ERK-MAPK signaling is necessary for sprouting by endothelial cells

The ablation of angiogenesis in response to inhibition of ERK1/2 signaling during the migratory phase in coculture (Figure 3A) suggested that, in addition to isolated endothelial cells, inhibition of ERK1/2 signaling affected the tubules that had already formed at this stage. In order to confirm this hypothesis and understand the mechanism underlying the response, we followed sprouting tubules during PD184352 treatment by time-lapse microscopy. The movies revealed that sprouting tubules retracted during PD184352 treatment, whereas control tubules continued remodeling (Figure 5A). The retraction induced by PD184352 treatment was irreversible, as there was no recovery when the inhibitor was washed out (Figure 3 and data not shown). To confirm that retraction was due to inhibition of ERK-MAPK signaling in endothelial cells, we measured the number of sprouts in tubules formed by HUVECs transfected with Raf siRNAs seeded on confluent fibroblasts. Figure 5B shows that there was reduced number of sprouts per vessel when ERK1/2 activation was inhibited in endothelial cells with the combination of Raf siRNAs.

ERK-MAPK opposes Rho-kinase signaling during vessel formation

The rounding of single cells and retraction of sprouting vessels observed in response to inhibition of ERK-MAPK signaling suggest that signaling pathways that control the morphology of endothelial cells are deregulated. Signaling through Rho and Rho-kinase has been implicated in cell retraction (Amano et al., 1998). Previous studies have shown that inhibition of ERK-MAPK signaling in cancer cells can lead to upregulation of Rho-Rho-kinase signaling (Vial et al., 2003). As cocultures contain a small proportion of endothelial cells (approximately 5% in the migratory phase), the activation status of Rho-kinase signaling in endothelial cells could not be determined by direct biochemical analysis. Therefore, we looked by immunofluorescence for changes in the phosphorylation state of myosin light chain 2, a target of Rho-kinase signaling involved in cell retraction (Amano et al., 1996, 1998). When control 1-week-old cocultures were stained, some pMLC was detected that often localized to the tips of vessels. Treatment of cocultures with PD184352 significantly increased pMLC staining in endothelial cells within 4 hr of treatment (Figure 6A, left panel). In these

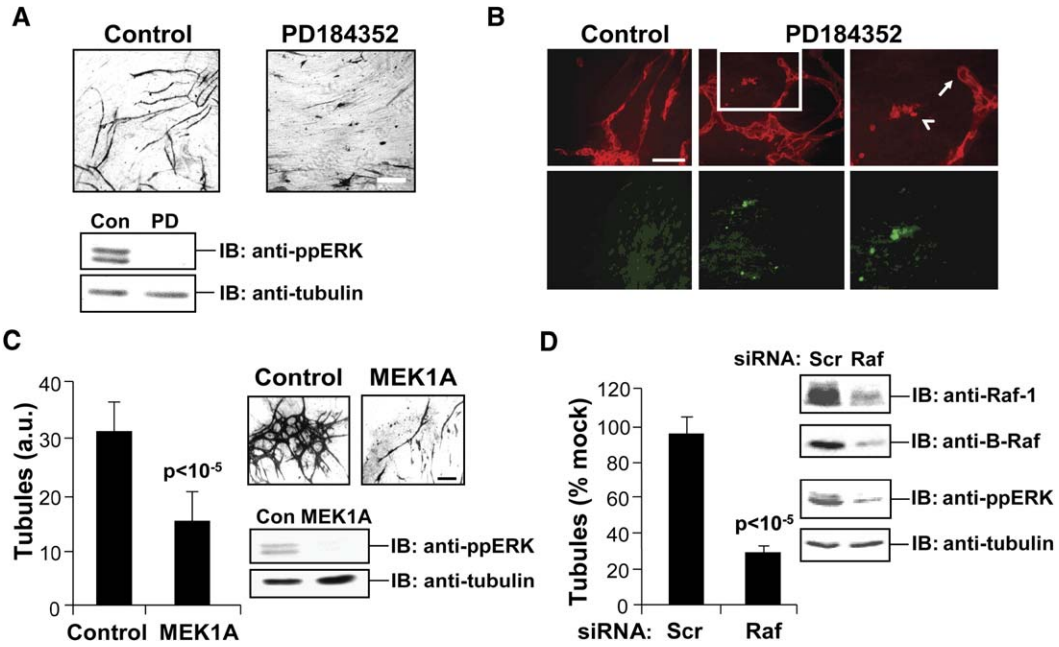

Figure 3. Inhibition of ERK-MAPK signaling in endothelial cells during the migratory phase results in abrogation of tube formation

A: HUVECs were cocultured with HDFs, and after 7 days the MEK inhibitor PD184352 (2 μM) was applied for 48 hr; the inhibitor was then removed and replaced with fresh medium. Vessel formation was assessed by CD31 staining after a further 5 days. Inset: Western blot shows ERK1/2 phosphorylation in HUVECs treated with PD184352 compared to control. Scale bar, 100 μm.

B: HUVECs were cocultured with HDFs, and after 7 days the MEK inhibitor PD184352 was applied for 18 hr. The cocultures were then fixed, HUVECs were visualized by CD31 immunostaining, and apoptotic cells were detected by TUNEL. Scale bar, 50 μm. The rightmost panels are ×2 magnifications of the inset shown in the middle panel. Open arrow shows apoptotic endothelial cells; closed arrow shows cell rounding at tip of vessel.

C: Vessel formation of HUVECs expressing dominant-negative MEK1 (MEK1A) compared to control HUVEC-EGFP was assessed by CD31 staining after 2 weeks coculture with HDFs. Results shown are representative of three independent experiments using three different pools of HUVEC-MEK1A carried out in triplicates. Tubule count is represented as ±SD (n = 9 microscopic fields at ×4 magnification). Images show representative vessel formation after 2 weeks coculture with HDFs of HUVECs expressing MEK1A compared to control. Scale bar, 100 μm. Western blot shows ERK1/2 phosphorylation in HUVECs expressing MEK1A compared to control.

D: HUVECs transfected with Raf siRNAs or scrambled control oligonucleotides were seeded on confluent HDFs. Tubule count was after 5 days. Results are average of three independent experiments (±SD). Upper inset shows Western blots for expression of Raf-1 and B-Raf, and lower inset shows ERK1/2 phosphorylation in HUVECs transfected with Raf siRNAs compared to control.

experiments, retraction was seen at 8 hr. This increase in MLC2 phosphorylation was reversed by treatment with the pharmacological inhibitor of the kinase activity of Rho-kinase Y27632 (Uehata et al., 1997), demonstrating that increased MLC2 phosphorylation was the consequence of Rho-kinase activity. A similar increase in pMLC staining was observed in isolated endothelial cells transfected with Raf siRNAs that had been seeded on confluent fibroblasts (Figure 6A, right panel). This increase in pMLC staining was also reversed by treatment with Y27632 (data not shown). These experiments show that inhibition of ERK1/2 signaling in endothelial cells leads to increased Rho-kinase activity, suggesting that ERK1/2 act to modulate signaling through Rho-kinase and thereby regulate actomyosin contractility via MLC2 phosphorylation.

Transient inhibition of Rho-kinase and actomyosin contractility rescues the effects of inhibition of ERK-MAPK signaling on tube formation in coculture

To test the hypothesis that ERK1/2 act to inhibit Rho-kinase signaling in endothelial cells, we examined whether the effects of inhibition of ERK-MAPK signaling could be rescued by inhibition of Rho-kinase. Rho-kinase signaling was blocked using Y27632 (Uehata et al., 1997) or H1152 (Ikenoya et al., 2002) inhibitors. Inhibitor was added prior to the point of loss of bipolarity and retraction, as determined by time-lapse microscopy. Y27632 treatment reversed the vessel retraction response caused by treatment with PD184352 (Figure 6B and Movies S5 and S6). Additionally, Y27632 treatment rescued the loss of bipolarity of endothelial cells in response to inhibition of ERK1/2 by Raf siRNAs (Figure 6C) and PD184352 (data not shown). Importantly, transient inhibition of Rho-kinase during the migratory phase rescued tube formation in cocultures treated with PD184352 (Figure 6D) but had no effect on its own (data not shown). When Rho-kinase alone was inhibited with Y27632 for a longer period of time (48 hr), there was increased tube formation (Figure 6D), suggesting that angiogenesis can be negatively regulated by Rho-kinase signaling. Similarly, treatment with H1152 led to significant rescue of the effects of inhibiting ERK-MAPK signaling on tube formation (Figure 6E). Because we have used two structurally unrelated inhibitors (Uehata et al., 1997; Ikenoya et al., 2002), these results strongly argue that ERK-MAPK is acting on Rho-kinase (Ikenoya et al., 2002). To confirm that these effects result from inhibition of signaling in the endothelial cells, we used an alternative model, where HUVECs alone are embedded in thin matrigel containing VEGF and form loops. PD184352 treatment resulted in cell rounding and retraction of a subpopulation of HUVECs and reduced formation of loops. This reduction in loop formation

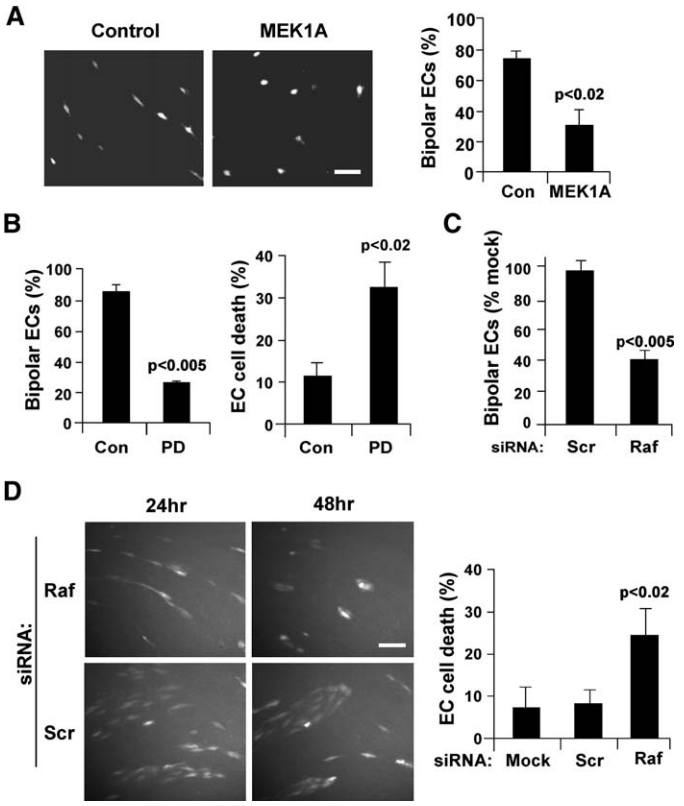

Figure 4. Inhibition of ERK-MAPK signaling in endothelial cells results in loss of bipolarity, detachment, and cell death

A: HUVECs expressing dominant-negative MEK (MEK1A) or control HUVEC-EGFP were marked with Oregon green and seeded on confluent HDFs. Bipolarity (length/width > 3) was measured after 18 hr. Results are average of three independent experiments (±SD) using three different pools of HUVEC-MEK1A or control cells. Scale bar, 50 μm.

B: HUVEC-EGFP were seeded on confluent HDFs, treated with PD184352 (2 μm), and followed by time-lapse microscopy. The percentage of cells with bipolar morphology was determined after 10 hr treatment, and cell death was scored from time-lapse movies over 24 hr. Results are average of three independent experiments (±SD).

C: HUVEC-EGFP were transfected with Raf siRNAs or scrambled control oligonucleotide duplexes and seeded on confluent HDFs. The percentage of cells with bipolar morphology was determined 48 hr after transfection. Results are average of three independent experiments (±SD).

D: HUVEC-EGFP were transfected with Raf siRNA or scrambled control oligonucleotide duplexes, seeded on confluent HDFs, and followed by time-lapse microscopy. Cell death was scored from the movies over 3.5 days after siRNA transfection and is shown as average of three independent experiments (±SD). At least 40 cells from each group were followed in each experiment by time-lapse microscopy. Scale bar, 50 μm.

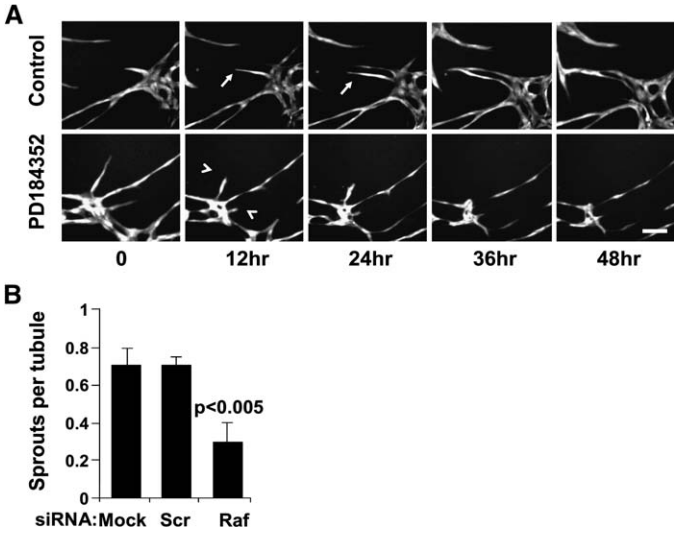

Figure 5. Inhibition of ERK-MAPK signaling results in tube retraction

A: Vessel retraction in response to inhibition of ERK1/2 activation. HUVEC-EGFP cocultured with HDFs were treated with PD184352 (2 μM) 1 week after initiation of the coculture. Vessel sprouting was followed by time-lapse microscopy. Still images are shown at 12 hr intervals from start of inhibitor treatment. Closed arrows show sprouting points in control vessels. Open arrows indicate retraction points in vessels treated with PD184352. Scale bar, 50 μm.

B: HUVEC-EGFP transfected with Raf siRNA or scrambled control oligonucleotides were seeded on confluent HDFs. Sprouting points on vessels were counted after 5 days. Results are average of three independent experiments (±SD).

was rescued with treatment with either Y27632 or H1152 (Figure S4), confirming that the rescue by Rho-kinase inhibition was via a direct effect on endothelial cells (Figure S4). In parallel with the observation that inhibition of Rho-kinase alone increased tube formation in the coculture system (Figure 6D) inhibition of Rho-kinase alone increased loop formation in the thin matrigel assay (Figure S4).

To extend the link between ERK-MAPK signaling, Rho-kinase, MLC2 phosphorylation, and actomyosin contractility, we examined whether inhibiting actomyosin contractility would overcome the effects of inhibiting ERK-MAPK signaling on tube formation. Transient treatment of cocultures with blebbistatin, an inhibitor of myosin II ATPase activity (Straight et al., 2003), rescued tube formation in cocultures treated with PD184352 but had no effect on its own (Figure 6F). These findings confirm that cell retraction and death following inhibition of ERK-MAPK signaling is the consequence of increased actomyosin contractility resulting from increased Rho-kinase signaling and MLC2 phosphorylation in endothelial cells.

Abrogation of angiogenesis in vivo through inhibition of ERK-MAPK signaling is overcome by Rho-kinase inhibition

These observations in tissue culture suggest that ERK1/2 signaling is required in endothelial cells to oppose the effects of Rho-kinase signaling on cell morphology during angiogenesis. To find out if this regulatory pathway operates during tumor angiogenesis in vivo, we investigated whether treatment with the Rho-kinase inhibitor Y27632 would rescue the effects of expression of dominant-negative MEK1 in the vascular compartment of tumors. Since cells get infected with the MEK1A virus before the tumors establish in the in vivo model, we administered the inhibitor early, when tumors had just started appearing, and continued treatment for 5 days. Tumors were analyzed when they had reached 0.6–0.7 cm diameter, and blood vessel formation was studied by staining for CD31 and scoring for blood vessels with open lumens and branching points. Representative pictures of tumors with MEK1A transduction in the vascular compartment and control, treated with Y27632 or control vehicle, are shown in Figure 7A. The reduction in angiogenesis in tumors with expression of dominant-negative MEK1 in the vascular compartment was abrogated when Rho-kinase was inhibited by treatment with Y27632. There was rescue of the overall

amount of vasculature as shown by the increase in the CD31 stained area (Figure 7B), the number of vessels with open lumens (Figure 7C), and total vessel length (Table 1). Although we found no significant increase in the overall amount of vasculature in response to Rho-kinase inhibition alone in tumors, there was marked increase in the amount of branching points (Figure 7D). These results suggest that the inhibition of angiogenesis resulting from blockade of ERK1/2 signaling in the tumor vasculature is a consequence of aberrant Rho-kinase activity.

To confirm that the reduction in angiogenesis in tumors in response to inhibition of ERK-MAPK signaling and the rescue by Rho-kinase inhibition were via a direct effect on endothelial cells, we employed an alternative in vivo angiogenesis model in which endothelial cells are attracted into VEGF-containing matrigel plugs and form vessels (Eliceiri et al., 1999). Introduction of retrovirus producer cells into these plugs results in infection and transgene expression in the vessels (Eliceiri et al., 1999; Figure S1B). In agreement with the results from the tumor model, there was reduced vessel formation in VEGF-matrigel plugs containing dominant-negative MEK1 retrovirus producers compared to control (Figures 7E and 7F). The reduction in vessel formation was again rescued by treatment with the Rho-kinase inhibitor Y27632 (Figures 7E and 7F). In similar experiments using PD184352 treatment rather than expression of dominant-negative MEK1 to block ERK-MAPK signaling in the VEGF-matrigel plugs, there was reduced angiogenesis in response to PD184352 that was rescued by treatment with Y27632 (data not shown).

In agreement with the observation in coculture that inhibition of ERK1/2 upregulated Rho-kinase-dependent MLC2 phosphorylation, there was increased pMLC in tumors treated with the MEK1 inhibitor PD184352 (Figure 7G). Altogether, these results demonstrate that the interplay between ERK1/2 and Rho-kinase signaling that we have identified in the endothelial-fibroblast coculture assay operates in vivo.

Discussion

To investigate the role of ERK-MAPK signaling in tumor angiogenesis, we have selectively inhibited ERK-MAPK by expression of dominant-negative MEK1 in the vascular compartment of tumors. A role of ERK-MAPK in tumor angiogenesis has been suggested (Mukhopadhyay et al., 1998) but not investigated mechanistically. Inhibition of ERK1/2 signaling in the tumor vasculature resulted in vessel reduction, defective vascular morphogenesis, and reduced tumor growth. These results raise the possibility that drugs to inhibit activation of ERK-MAPK signaling (Sebolt-Leopold and Herrera, 2004) may act both on tumor cells and endothelial cells.

By following tubule formation in endothelial cell-fibroblast cocultures by time-lapse microscopy, we demonstrated that inhibition of ERK-MAPK signaling results in upregulation of Rho-kinase signaling and cell contractility, leading to detachment of isolated endothelial cells and retraction of sprouting vessels. Significantly, we observed these effects only under conditions where migration and tubule formation occurs and not with endothelial cells cultured on tissue culture plastic (data not shown). The interplay between ERK-MAPK and Rho-kinase signaling appears to operate in vivo, as inhibition of ERK-MAPK signaling led to increased MLC phosphorylation in tumor vessels, and the defects in vascularization were rescued by inhibition of Rho-kinase in vivo. Our results suggest that the level of Rho-kinase activation must be accurately regulated in angiogenic vessels. We hypothesize that reduced actomyosin contractility is necessary to allow endothelial cell migration and sprouting. This is consistent with the finding that actomyosin contractility has been shown to oppose protrusive activity driven by actin assembly (Totsukawa et al., 2004) (see Figure 8). Furthermore, reduced actomyosin contractility appears to be necessary to allow cell spreading and survival during endothelial cell migration, when there are looser cell-cell and cell-matrix attachments.

The retraction of tubules in response to increased Rho-kinase activity is consistent with reports that activation of Rho signaling by the microtubule depolymerizing agent vinblastin results in collapse of tubes in three-dimensional matrix, and vessel lumens in the chorioallantoic membrane (Bayless and Davis, 2004). The report by Bayless and Davies demonstrates that aberrant activation of Rho signaling can mediate cytoskeletal disruption of angiogenic vessels, although it does not address whether these effects are mediated by Rho-kinase. The retraction of tubules observed in tissue culture in response to inhibition of ERK-MAPK signaling and activation of Rho-kinase is consistent with the reduction in vessel length and number of open lumens seen in tumors. Both in tissue culture and in vivo, these effects were rescued by inhibiting Rho-kinase. Surprisingly, inhibition of Rho-kinase alone in vivo increased sprouting and vessel length. These data therefore suggest that inhibition of Rho-kinase promotes vascular remodeling during angiogenesis. This suggests that studies using inhibition of Rho-kinase as a strategy for cancer treatment should include monitoring tumor vascularization to determine whether Rho-kinase inhibition is promoting angiogenesis.

We have shown that inhibition of ERK1/2 signaling resulted in apoptosis of isolated endothelial cells. Although ERK-MAPK signaling has been implicated in the survival of other cell types by acting on the apoptotic machinery (reviewed in Chang et al., 2003; Sebolt-Leopold and Herrera, 2004), our data suggest that endothelial cell death in response to inhibition of ERK1/2 activation resulted from changes in cell shape and detachment from the extracellular matrix as a consequence of increased Rho-kinase activity during the migratory phase. Our conclusions are supported by the following: the predominant response to inhibition of ERK1/2 signaling during endothelial cell migration was loss of bipolarity. Following cell rounding, apoptosis took place approximately 10–12 hr later as revealed by time-lapse microscopy. Cell rounding and retraction were rescued by Rho-kinase inhibition but were not inhibited by ZVAD (see Movie S7), demonstrating that cell rounding and retraction are not the result of the induction of apoptosis. It is well established that endothelial cells depend on adhesion to the extracellular matrix to receive survival signals through the engagement of integrins (Frisch and Ruoslahti, 1997; Meredith et al., 1993). In addition to cell adhesion, cell spreading is also necessary for endothelial cell survival (Chen et al., 1997; Re et al., 1994). As a direct demonstration of the importance of spreading, seeding endothelial cells on substrates of small surface area leads to apoptosis even in the presence of integrin engagement (Chen et al., 1997). Therefore, although cell contractility is necessary for both cell adhesion and spreading (Balaban et al., 2001), its levels must be tightly regulated in endothelial cells during angiogenesis to support cell survival and allow migration. The finding that ERK1/2 signaling promotes cell survival through regulating

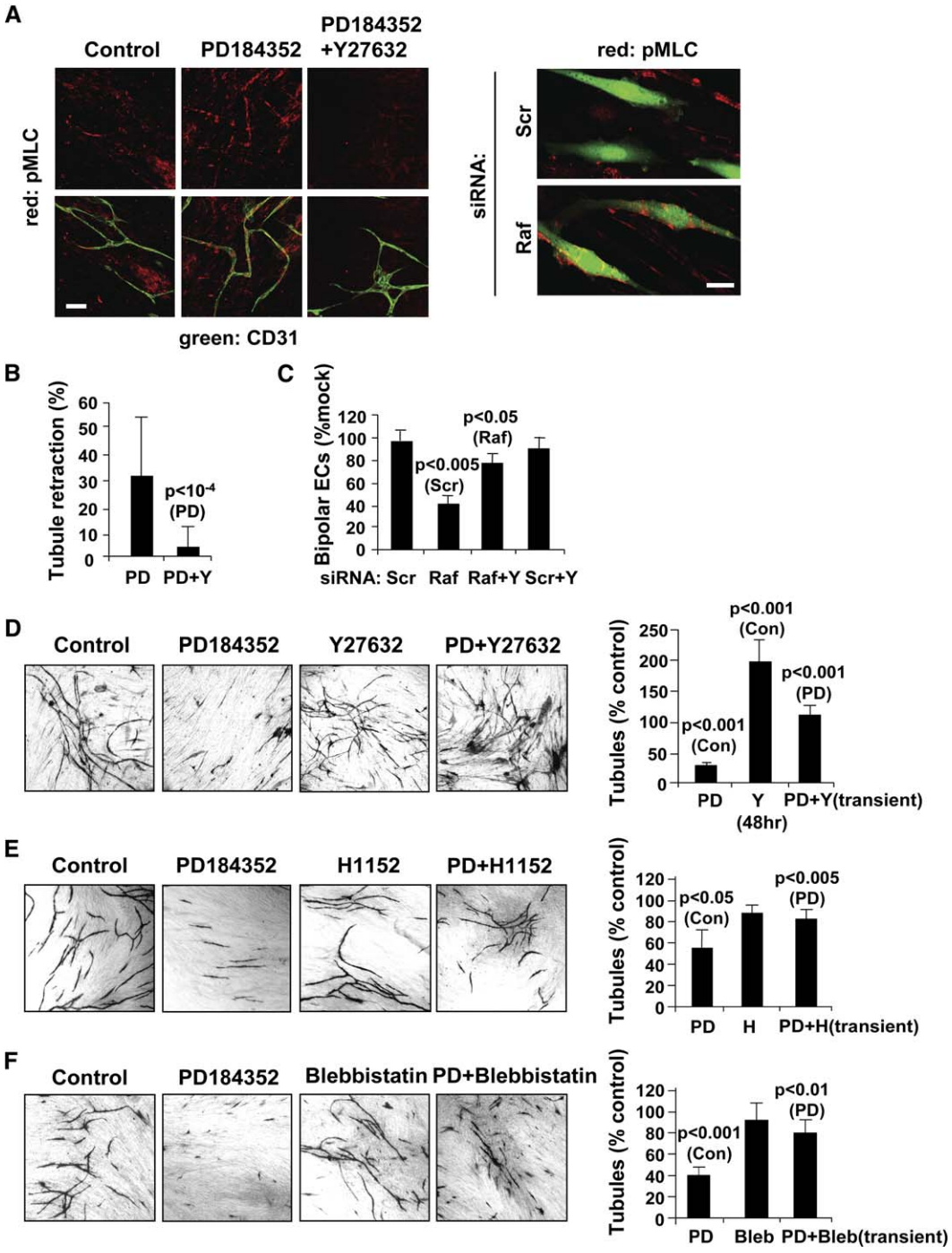

Figure 6. Endothelial cell bipolarity and retraction are rescued by transient inhibition of Rho-kinase and actomyosin contractility in coculture

A: Inhibition of ERK-MAPK signaling leads to elevated Rho-kinase activity in coculture. Left panel: 1 week after initiation, HUVEC-HDF cocultures were treated with PD184352 (2 μM) for 4 hr and analyzed by immunofluorescence for phosphorylated MLC2 (Ser-19). Where indicated, Y27632 (10 μM) was applied 2 hr before the cocultures were stained. Scale bar, 50 μm. Right panel: HUVEC-EGFP transfected with scrambled (scr) or Raf siRNAs were seeded on confluent HDFs. Scale bar, 10 μm.

B: Sprouting of HUVEC-EGFP cocultured with HDFs was followed by time-lapse microscopy 1 week after initiation of coculture. The percentage retraction with respect to initial tubule length is shown for tubules treated with PD184352 compared to combination with Y27632 after 12 hr treatment. Results are averages of three independent experiments (±SD). At least six tubules from each group were followed in each experiment by time-lapse microscopy.

C: HUVECs transfected with Raf siRNA or scrambled control oligonucleotides were seeded on confluent HDFs. The percentage of cells with bipolar morphology (length/width > 3) was determined 48 hr after transfection. Results are averages of three independent experiments (±SD).

D: Inhibition of Rho-kinase by Y27632 leads to rescue of tube formation when ERK1/2 activation is inhibited. Seven days after initiation, HUVEC-HDF cocultures were treated with PD184352 or Y27632 for 48 hr. In combination, Y27632 treatment was for 8 hr on day 1 and day 2 of 48 hr PD184352 treatment. After treatment, media containing inhibitors were removed and replaced with fresh tissue culture media. Tube formation was assessed by CD31 staining after a further 5 days. Tubule count is represented as ±SD (n = 9 microscopic fields at ×4 magnification from triplicate wells).

Rho-kinase activity does not exclude the possibility that ERK-MAPK regulates bona fide survival signals during endothelial cell proliferation, or to protect cells from receptor-mediated cell death (Alavi et al., 2003).

While inhibition of ERK-MAPK signaling had marked effects during the migratory phase, there was a much smaller effect in established tubes. This observation is in agreement with the findings of Eliceiri and coworkers, who showed that only the formation of new blood vessels in response to bFGF stimulation was compromised when ERK-MAPK was inhibited in the chorioallantoic membrane (Eliceiri et al., 1998). Therefore, there may be lower MAPK-ERK activity in established tubes in coculture. Alternatively, MAPK signaling may be uncoupled from Rho-kinase signaling in established tubes. In agreement with the proposed interplay between ERK1/2 and Rho-kinase signaling, we found elevated levels of phosphorylated MLC2 in established tubules compared to isolated endothelial cells and sprouting tubules, indicative of higher Rho-kinase activity. We would argue based on our results and those of others that during angiogenesis it is necessary to downregulate Rho-kinase to promote cell spreading and cell migration, whereas to form stable vessels upregulated Rho-kinase activity may be necessary to inhibit the generation of cell protrusions (Arthur and Burridge, 2001), maintain tight junctions (Wojciak-Stothard and Ridley, 2002), and stabilize vessels. This notion is supported by the finding that Rho signaling promotes the organization of confluent layers of endothelial cells into cords and the assembly of endothelial cells into vessels in vivo (Hoang et al., 2004). In mature vessels, survival factors and mechanical support from established cell-cell contacts and stable cell-matrix adhesions (Dejana, 2004) may protect the cells from the effects of elevated actomyosin contractility.

In summary, we have demonstrated that inhibiting MAPK-ERK in the vasculature of tumors inhibits angiogenesis and reduces tumor growth and revealed a role of ERK-MAPK signaling during angiogenesis, namely its role in downregulating Rho-kinase activity to allow endothelial cell spreading, survival, and sprouting of vessels. This mechanism has been elucidated by the use of an organotypic angiogenesis assay and confirmed by in vivo approaches. We propose that ERK-MAPK signaling plays a key role in controlling vascular morphogenesis through opposing the effects of Rho-kinase activation. As tumor vascularization may result from different mechanisms in different tumor types, detailed studies of human tumor material will be needed to determine in which tumor systems the vasculature can be compromised by targeting ERK-MAPK signaling and the induction of Rho-kinase-mediated retraction and cell death. Phospho-MLC2 and phospho-ERK may be useful markers in such studies. Potentially, some antiangiogenic agents that act on tumor blood vessels may act through downregulation of ERK-MAPK signaling and consequent upregulation of Rho-kinase signaling. Interestingly, combretastatin A-4-phosphate, a tumor vascular targeting agent, seems to work by upregulation of Rho-kinase and MLC activity, and its effects are abrogated by ERK1/2 activation (Kanthou and Tozer, 2002).

Experimental procedures

Cells, retroviral infection, and electroporation

Angiokit-validated HDFs and HUVECs were obtained from TCS CellWorks (Buckingham, UK) and cultured in DMEM 10% FCS and Large Vessel Endothelial Medium (TCS CellWorks), respectively. HUVEC-EGFP were generated by viral infection of HUVECs with an EGFP pBabe-puro (Morgenstern and Land, 1990) amphotropic, replication-defective retrovirus. EGFP-expressing cells were sorted by FACS. HUVEC-MEK1A were generated by infection with a MEK1A pBabe-puro amphotropic retrovirus, followed by selection in 0.5 μg/ml puromycin. All HUVECs were used up to passage 6, and their tube-forming ability was assessed in a 2 week coculture assay with HDFs (see below). Plasmid DNA and siRNAs were introduced into HUVECs by electroporation using the HUVEC (Vs.2) Nucleofector Kit (Amaxa GmbH, Germany).

Constructs and recombinant retroviruses

pEXV MEK1A (dominant-negative MEK1 with Ser-217 substitution to alanine) and retroviral vectors pBabe-puro MEK1A and MEK1EE (active MEK1 with Ser-217 and Ser-221 substitution to glutamic acid) have been previously described (Cowley et al., 1994). High-titer retrovirus producer lines were generated as previously described (Mavria and Porter, 2001).

Antibodies, siRNAs, and inhibitors

Antibody against phospho-MLC2 (Ser-19) was from Cell Signaling Technology, CD31 and Raf-1 were from BD Biosciences, B-Raf was from Santa Cruz Biotechnology, and phospho-ERK1/2 was from Sigma. siRNAs for *Raf-1* and *B-Raf* (Karasarides et al., 2004) were from Qiagen. PD184352 (Sebolt-Leopold et al., 1999), H1152 (Ikenoya et al., 2002), and blebbistatin (Straight et al., 2003) were from Calbiochem, and Y-27632 was from Tocris (Southampton, UK).

Immunofluorescence and time-lapse microscopy

For immunofluorescence, fixation was in 3.7% paraformaldehyde, and permeabilization was in 0.1% Triton X-100. Apoptotic cells were detected using the DeadEnd Fluorometric TUNEL System (Promega). Cells were imaged by confocal microscopy. Multisite microscopy was performed in a humidified, CO_2-equilibrated chamber using a Diaphot inverted microscope (Nikon, Kingston upon Thames, UK) equipped with a motorized stage (Prior Scientific, Oxford, UK) controlled by Simple PCI software (Compix, Cranberry Township, PA).

Coculture angiogenesis assays

Cocultures of HUVECs with HDFs (TCS Angiokit) were purchased from TCS CellWorks, Buckingham, UK. Pharmacological inhibitors were applied 7 days after initiation of the coculture with no change of media for 48 hr prior to application. Tube formation was assessed by immunohistochemistry using a mouse anti-human CD31 Tubule Staining Kit (TCS CellWorks). Anti-human CD31 was applied at 1:400 dilution and alkaline phosphatase-conjugated secondary at 1:500. The substrate was applied for 15 min and washed, and pictures of the cocultures were taken after 4–5 hr. Coculture assays of HUVEC-MEK1A and HUVEC-EGFP with HDFs were set up as previously described (Bishop et al., 1999). Short-term assays following electroporation of HUVECs were set up by seeding HUVECs on HDFs that had reached confluency after 7 days in culture. HUVECs marked with Oregon green (Molecular Probes) were cultured alone overnight before seeding on HDFs.

E: Inhibition of Rho-kinase by H1152 leads to rescue of tube formation when ERK1/2 activation is inhibited. Seven days after initiation, HUVEC-HDF cocultures were treated with PD184352 or H1152 (5 μM) for 24 hr. In combination with PD184352, H1152 was added 12 hr after PD184352. After treatment, media containing the inhibitors were removed and replaced with fresh tissue culture media. Tube formation was assessed by CD31 staining after a further 5 days. Tubule count is represented as ±SD (n = 9 microscopic fields at ×4 magnification from triplicate wells).

F: Inhibition of actomyosin contractility by blebbistatin leads to rescue of tube formation when ERK1/2 activation is inhibited. Cocultures were treated with PD184352 or blebbistatin (5 μM; Wilkinson et al., 2005) as in **E**. In combination, blebbistatin was added 12 hr after PD184352. Tubule count is represented as in **E**.

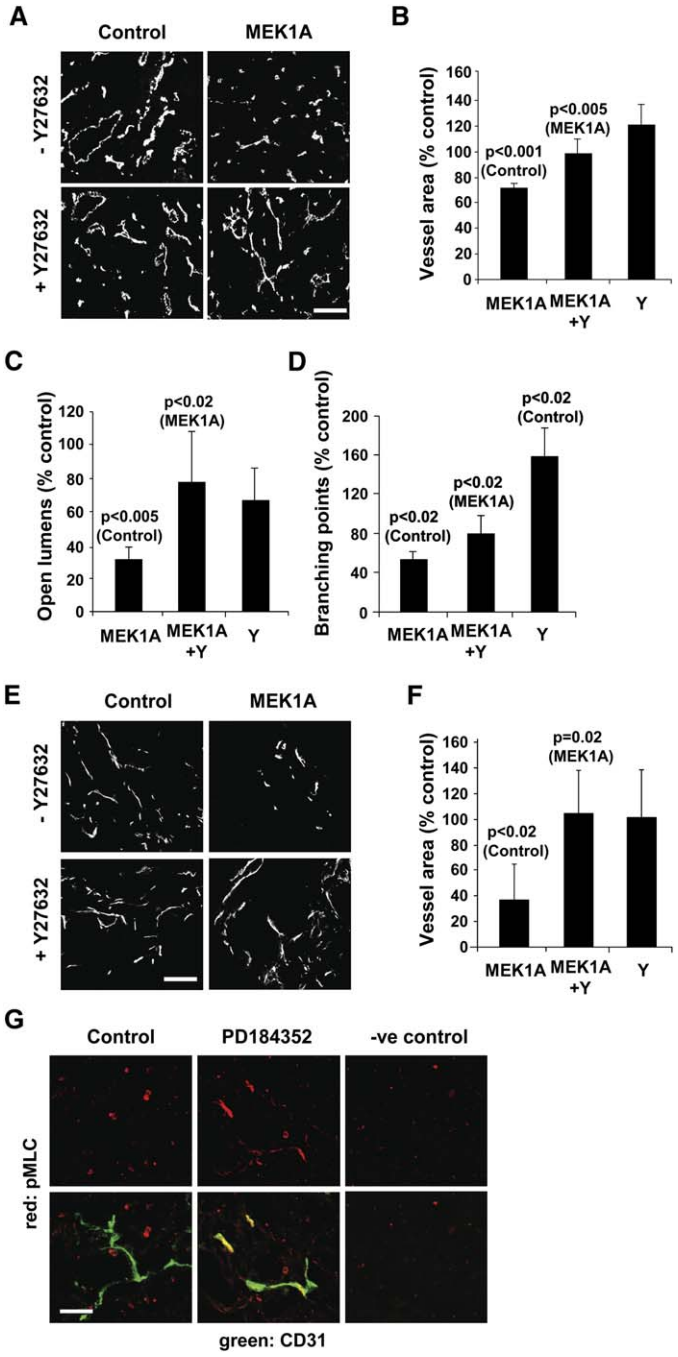

Figure 7. The block to angiogenesis in vivo resulting from inhibition of ERK-MAPK signaling is overcome by Rho-kinase inhibition

A–D: Inhibition of Rho-kinase leads to rescue of angiogenesis in tumors when ERK-MAPK signaling is inhibited in the tumor vasculature. BE tumors with MEK1A (dominant-negative MEK1) or control (EGFP) retroviral transduction in the vascular compartment were set up as described in Experimental Procedures. Y27632 was administered for 5 days after tumors appeared. Tumors were analyzed when they reached approximately 0.6 cm diameter; cryosections were examined by immunofluorescence for CD31 expression. **A:** Typical sections are shown of tumors with MEK1A or control transduction treated with Y27632 or vehicle. Scale bar, 100 μm. The fractional area covered by $CD31^+$ features (**B**), the number of vessels with open lumens (**C**), and the number of branching points (**D**) are represented as mean ± SD (n = 4 tumors). Counts for each tumor were from eight sections, representing four different levels. For each section, the microscopic field (×20) showing the greatest level of CD31 staining was counted.

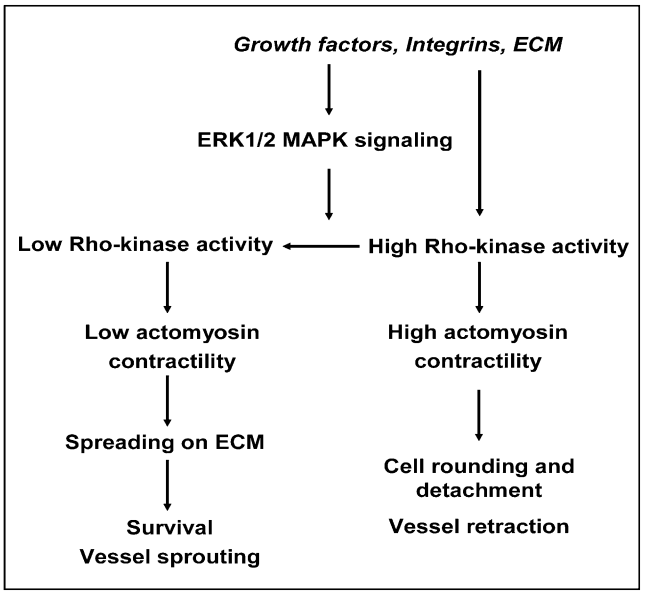

Figure 8. Model for interaction between ERK-MAPK signaling and Rho-kinase signaling during angiogenesis

In vivo angiogenesis assays, inhibitor treatment, and vessel measurement

All in vivo experimentation was performed according to United Kingdom Home Office Regulations and the United Kingdom CCCR guidelines. For tumor growth assays, BE or MDA-MB-361 tumor cells (1.5×10^6) were mixed with irradiated (20 Gy) ecotropic virus producers at 1:10 ratio and inoculated subcutaneously into the right flank of 5- to 6-week-old male MF1 nu/nu mice (Harlan). Tumor volumes were calculated according to the formula $V(mm^3) = (L \times W^2) \times 1/2$ (Ovejera et al., 1978). Where tumors reached 1500 mm^3 in the control group, animals were removed from the study according to UK Home Office guidelines. Treatment with Y27632 was intraperitoneal at 50 mg/kg body weight (Erik Sahai, personal communication), twice daily starting at 10 days after inoculation of tumor cells. Treatment with PD184352 was intraperitoneal at 200 mg/kg body weight (Sebolt-Leopold et al., 1999), daily starting after the tumors appeared. Matrigel angiogenesis assays were carried out essentially as previously described (Eliceiri et al., 1999) using growth factor-depleted Matrigel (Becton Dickinson) supplemented with VEGF (400 ng/ml). The plugs were excised 6 days after inoculation.

Cryosections (10 μm) for immunofluorescence were cut from dissected tumors and matrigel plugs frozen in OCT compound (Sakura) and analyzed by confocal microscopy using a ×20 objective. Automated analysis of the proportion of the image represented by CD31-positive cells and measurement of vessel length were performed using the Scion Image software. DAPI staining

E and **F:** Inhibition of ERK-MAPK signaling blocks angiogenesis in a VEGF-driven in vivo model and is overcome by inhibition of Rho-kinase. VEGF-matrigel plugs containing MEK1A or EGFP retrovirus producers were set up as described in Experimental Procedures. Y27632 was administered for 3 days starting 3 days after inoculation. Matrigel plugs were analyzed at the end of treatment; cryosections were examined by immunofluorescence for CD31 expression. **E:** Sections typical of the difference in vascular density between plugs with MEK1A virus in the presence or absence of Y27632 are shown. Scale bar, 100 μm. **F:** The fractional area covered by $CD31^+$ features is represented as mean ± SD (n = 4 tumors). Counts for each tumor were from sections from three different levels. For each section, the microscopic field (×20) showing the greatest level of CD31 staining was counted.

G: Inhibition of ERK-MAPK signaling leads to elevated levels of phosphorylated MLC2 in tumors. BE tumors were established in nude mice, and PD184352 was administered for 4 days after the tumors appeared. Tumors were analyzed at the end of treatment; cryosections were examined by immunofluorescence for expression of CD31 and phosphorylated MLC2 (Ser-19). Scale bar, 50 μm.

was used to confirm the lack of nucleated cells in vessels when scoring open lumens, and to distinguish the tumor area from the surrounding stroma (looser cell appearance). Vessels within the surrounding stroma were excluded from the analysis, as were arterial vessels identified by their autofluorescence in the green channel.

Supplemental data

The Supplemental Data include four supplemental figures, two supplemental tables, and seven movies and can be found with this article online at http://www.cancercell.org/cgi/content/full/9/1/33/DC1/.

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