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p38 MAP kinase negatively regulates endothelial cell survival, proliferation, and differentiation in FGF-2–stimulated angiogenesis

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The p38 mitogen–activated protein kinase (p38) is activated in response to environmental stress and inflammatory cytokines. Although several growth factors, including fibroblast growth factor (FGF)-2, mediate activation of p38, the consequences for growth factor–dependent cellular functions have not been well defined. We investigated the role of p38 activation in FGF-2–induced angiogenesis. In collagen gel cultures, bovine capillary endothelial cells formed tubular growth-arrested structures in response to FGF-2. In these collagen gel cultures, p38 activation was induced more potently by FGF-2 treatment compared with that in proliferating cultures. Treatment with the p38 inhibitor SB202190 enhanced FGF-2–induced tubular morphogenesis by decreasing apoptosis, increasing DNA synthesis and cell proliferation, and enhancing the kinetics of cell differentiation including increased expression of the Notch ligand Jagged1. Overexpression of dominant negative mutants of the p38-activating kinases MKK3 and MKK6 also supported FGF-2–induced tubular morphogenesis. Sustained activation of p38 by FGF-2 was identified in vascular endothelial cells in vivo in the chick chorioallantoic membrane (CAM). SB202190 treatment enhanced FGF-2–induced neovascularization in the CAM, but the vessels displayed abnormal features indicative of hyperplasia of endothelial cells. These results implicate p38 in organization of new vessels and suggest that p38 is an essential regulator of FGF-2–driven angiogenesis.

Introduction

Angiogenesis, the formation of capillaries from preexisting vessels, plays a central role in a variety of physiological and pathological conditions (Folkman, 1995; Risau, 1997). Angiogenesis can be divided into a series of temporally regulated events involving proteolytic digestion of the extracellular matrix, proliferation and migration of endothelial cells, formation of functional capillaries, and remodeling of the vascular bed through apoptosis. Several growth factors, such as fibroblast growth factor (FGF)* and vascular endothelial growth factor (VEGF), initiate these events. Mitogen-activated protein kinases (MAPKs) are common participants in growth factor–induced signal transduction pathways from the membrane to the nucleus. Activated MAPKs are known to regulate the activities of transcription factors or kinases further downstream by phosphorylation and thereby control gene expression and cellular function. Prior to this study, three major classes of MAPKs have been identified in mammalian cells including extracellular signal–regulated kinase (ERK), c-jun NH_2-terminal kinase (JNK), and p38 mitogen-activated protein kinase (p38) (Waskiewicz and Cooper, 1995). It is well established that ERK is activated by most growth factors in various types of cells of consequence for cell proliferation and differentiation. In contrast, it is generally considered that JNK and p38 are activated by environmental stress, bacterial lipopolysaccharide, and inflammatory cytokines (Kyriakis and Avruch, 1996). Although many JNK and p38 activation stimuli are proapoptotic, the biological outcome of their activation is highly divergent and appears to be largely dependent on the cell type or cellular context.

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*Abbreviations used in this paper: ATF, activating transcription factor; BCE, bovine capillary endothelial; CAM, chick chorioallantoic membrane; ERK, extracellular signal–regulated kinase; FGF, fibroblast growth factor; HA, hemagglutinin; HUVE, human umbilical vein endothelial; JNK, c-jun NH_2-terminal kinase; MAPK, mitogen-activated protein kinase; MEF, monocyte enhance factor; NCS, newborn calf serum; p38, p38 mitogen–activated protein kinase; VEGF, vascular endothelial growth factor; vWF, von Willebrand factor.

Key words: p38 mitogen–activated protein kinase; differentiation; angiogenesis; fibroblast growth factor; vascular endothelial growth factor

Recent studies have demonstrated that p38 is also activated by several growth factors including FGF-2 (Ono and Han, 2000). In certain types of cells, FGF-2 activates p38 and its downstream target MAPK-activated protein kinase-2 via a Ras-dependent pathway, which ultimately leads to transcriptional activation of cyclic AMP response element-binding protein and activating transcription factor (ATF)-2 (Tan et al., 1996; Xing et al., 1996). MKK3 and MKK6 are relatively specific upstream regulators of p38 (Ono and Han, 2000). To date, four isoforms of the p38 family, namely p38α, p38β, p38γ, and p38δ, have been identified; these are encoded by distinct genetic loci but share a common Thr-Gly-Tyr motif in kinase subdomain VIII (Ono and Han, 2000).

In this report, we describe the role of p38 activation in FGF-2–induced angiogenesis. We present evidence that p38 activity is induced by FGF-2 in endothelial cells in vivo and that p38 negatively regulates endothelial cell differentiation by decreasing the expression of differentiation-specific proteins such as the Notch ligand Jagged1. Inhibition of p38 function leads to increased vessel formation, but a large proportion of the vessels appeared nonfunctional.

Results

FGF-2 induced tubular morphogenesis in bovine capillary endothelial cells

Tubular morphogenesis of primary endothelial cells in three-dimensional collagen gels involves differentiation of the cells to form vessel-like structures. Cells in the tubular structures are growth arrested (Folkman and Haudenschild, 1980; Kubota et al., 1988; Ingber and Folkman, 1989), which allows analysis of differentiation-specific events. In agreement with previous studies (Montesano et al., 1983; Folkman et al., 1989) FGF-2–treated bovine capillary endothelial (BCE) cells cultured in collagen gels underwent tubular morphogenesis with dramatic changes in cell morphology (Fig. 1, A–H) compared with cell cultured on gelatin-coated dishes (Fig. 1 I). FGF-2 is a well-established potent inducer of tubular morphogenesis (Montesano et al., 1986; Sato et al., 1991; Pepper et al., 1992). We examined the

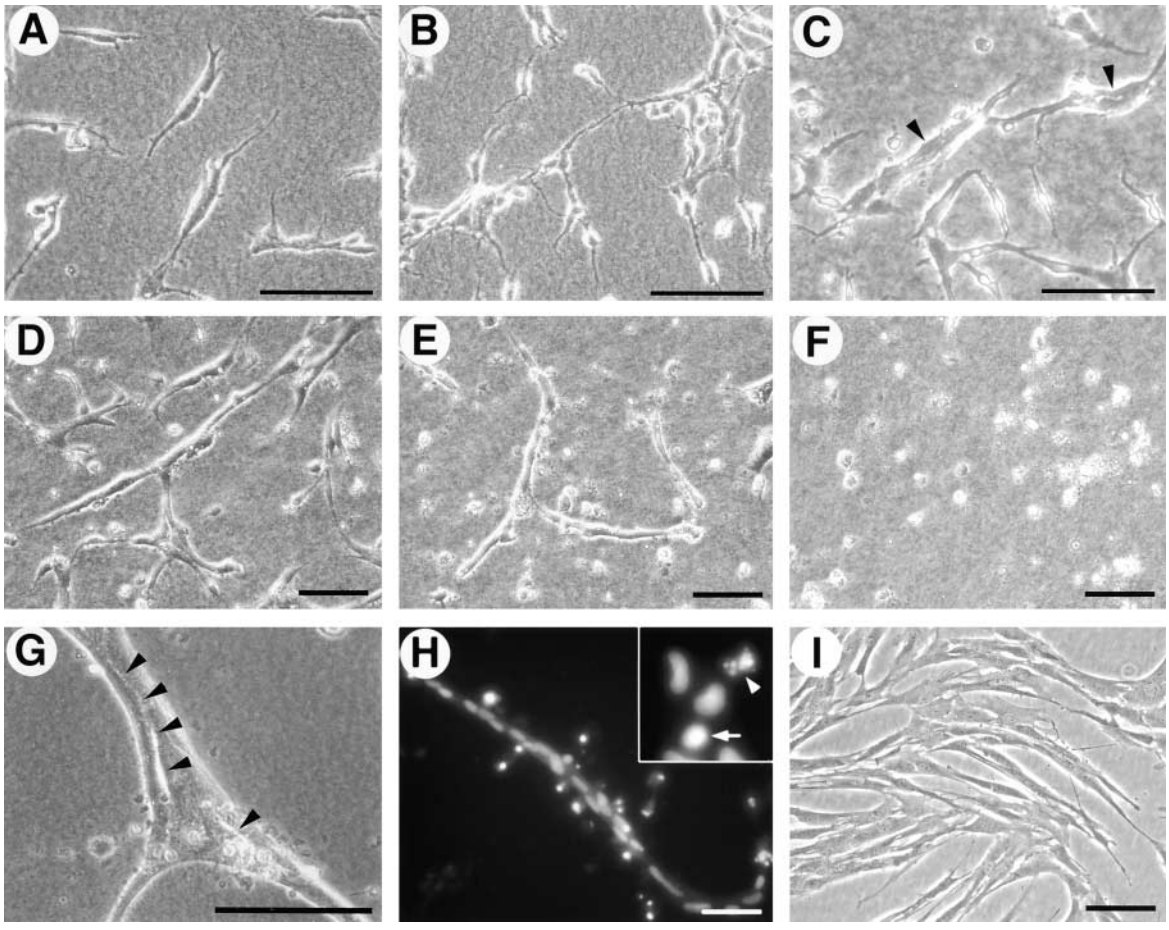

Figure 1. **FGF-2–dependent induction and regression of tubular morphogenesis in collagen gel cultures.** (A–G) Kinetics of FGF-2–induced tubular morphogenesis of BCE cells cultured in collagen gels. After 16 h of serum starvation, BCE cells were seeded between two layers of collagen gels and stimulated with 10 ng/ml FGF-2. Photographs were taken at 1 (A), 3 (B), 16 (C), 24 (D), 48 (E and G), and 72 h (F) after onset of stimulation. Arrowheads in C indicate fusion of endothelial cell bodies. Arrowheads in G indicate capillary lumen observed as a translucent slit within the tubules. (H) DNA staining of BCE cells undergoing tubular morphogenesis. FGF-2–stimulated BCE cell cultures in collagen were incubated for 48 h, fixed, and stained with Hoechst 33342. The arrow and the arrowhead indicate hypercondensed and fragmented nuclei, respectively. (I) Morphology of BCE cells cultured on a gelatin-coated dish for 24 h. Bars, 100 μm.

time course of tubular morphogenesis of BCE cells treated with 10 ng/ml FGF-2. Initially, cells aggregated and extended fine branches (Fig. 1 A), and by 3 h the branches started to connect (Fig. 1 B). Subsequently, by 16 h cells lined up along the branches and fused (Fig. 1 C, arrowheads) and became organized in tube-like structures by 24 h (Fig. 1 D). Between the 24- and 48-h time periods, capillary lumen were formed and observed as translucent slits within the tubules (Fig. 1 G, arrowheads) as described in previous reports (Ingber and Folkman, 1989; Montesano et al., 1991). By 48 h, cultures started to collapse and tubes eventually dissolved (Fig. 1 E). Cells released from dissolved tubes exhibited morphologic features characteristic of apoptosis, including cell rounding, membrane blebbing, and cell shrinkage, and displayed hypercondensed (Fig. 1 H, arrow) and fragmented (Fig. 1 H, arrowhead) nuclei when stained with Hoechst 33342. Tubes disappeared completely after an ~72-h incubation (Fig. 1 F). Addition of fresh growth factor did not prevent regression of the tubes. Tubular morphogenesis in the basal condition in the absence of FGF-2 was very rare. Thus, the tubular structures were regulated by a balance between FGF-induced differentiation and regression/apoptosis. The tubular structures were induced by FGF-2 at concentrations from 5 ng/ml, and maximal induction of tubular morphogenesis was seen at ~10–30 ng/ml FGF-2 in agreement with previous studies (Montesano et al., 1986; Pepper et al., 1992).

FGF-2 activates p38 in collagen gel cultures

We next addressed the question whether p38 was activated by FGF-2 and VEGF in BCE cells. FGF-2 stimulation increased p38 activity transiently in proliferating BCE cells cultured on gelatin as measured by phosphorylation of ATF-2 (Fig. 2 A). In the FGF-2–treated differentiating cells undergoing tubular morphogenesis on collagen gels, p38 activity was increased 10-fold (Fig. 2 B; quantified in Fig. 2 C). As expected, p38 was strongly activated by hyperosmolar stress (300 mM NaCl) in both types of culture (Fig. 2, A and B). Stimulation with VEGF at different concentrations maximally induced p38 activity 2.2-fold in the collagen gel culture (Fig. 2 B).

The pyridynyl imidazole derivatives SB202190 and SB203580 have been shown to be highly selective inhibitors of p38 activity (Lee et al., 1994; Cuenda et al., 1995; Jiang et al., 1996) with no effect on the activity of ERK, JNK, or other protein kinases. They inhibit the enzymatic activity of p38 by competing with ATP for binding to p38 (Young et al., 1997). Of the four described p38 isoforms, α, β, and γ appear to be sensitive to inhibition by SB202190 (Li et al., 1996; Jiang et al., 1997). Indeed, addition of 10 μM SB202190 to the in vitro kinase assay inhibited both FGF-2– and NaCl-induced activation of p38 in BCE cells (Fig. 2 D), whereas addition of SB202474, an inactive structural analogue of SB203580 (Lee et al., 1994), had no effect. We employed these compounds to examine how p38 activation affects FGF-2–induced tubular morphogenesis.

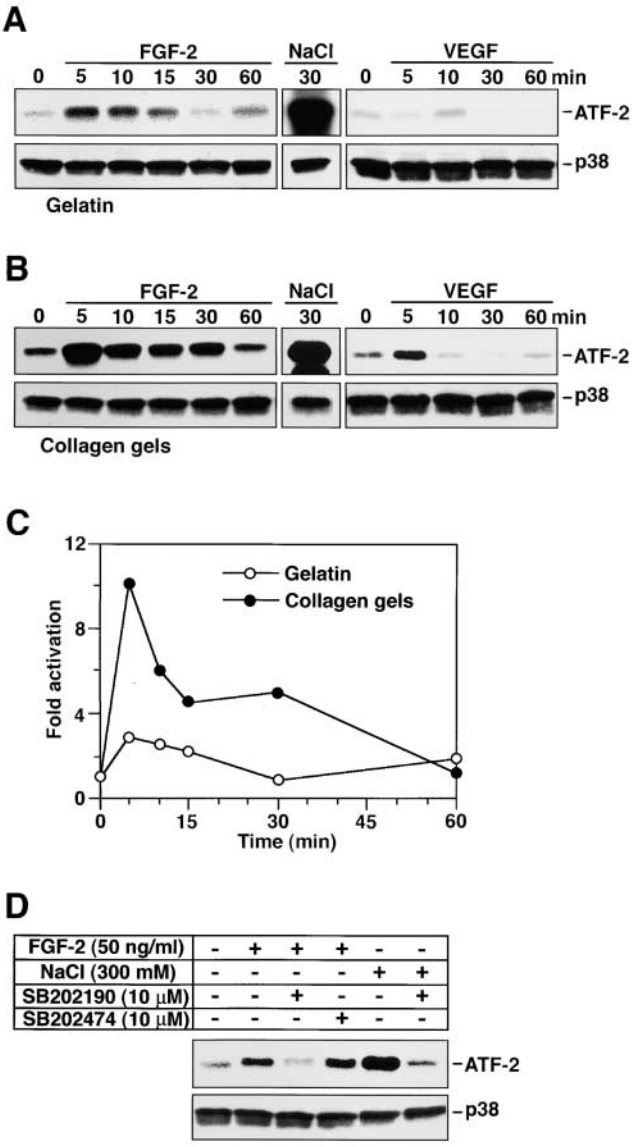

Figure 2. **FGF-2 effectively activates p38 in collagen gel cultures.** (A and B) BCE cells cultured on gelatin-coated dishes (A) or on collagen gels (B) were serum starved and treated or not with 50 ng/ml FGF-2 or 100 ng/ml VEGF for 5–60 min or with 300 mM NaCl for 30 min. After treatment, cells were lysed, immunoprecipitated using the anti-p38 antibody, and analyzed by ATF-2 phosphorylation in vitro (top). Aliquots of lysates were analyzed by immunoblotting with the anti-p38 antibody (bottom). (C) Quantification of the p38 activity induced by FGF-2 stimulation in A and B. (D) p38 activity was induced in BCE cells by treatment with FGF-2 for 5 min or with NaCl for 30 min. Cells were lysed, immunoprecipitated with the anti-p38 antibody, and the precipitates were preincubated with 10 μM SB202190, a p38 inhibitor, or 10 μM SB202474, an inactive compound, followed by ATF-2 phosphorylation in vitro (top). Aliquots of the lysates were analyzed for equal loading of p38 (bottom).

Inhibition of p38 activation enhances FGF-2–induced tubular morphogenesis

We examined the consequence of inhibition of p38 activity for FGF-2–induced tubular morphogenesis in BCE cells cultured in collagen gels. After a 16-h serum starvation, BCE cells were cultured between collagen gels and treated or not with FGF-2 in the presence or absence of 10 μM SB202190. Tubular morphogenesis was enhanced by treatment with SB202190 in an FGF-2–dependent manner (Fig. 3, A and B). Thus, in cultures treated with both FGF-2 and SB202190 for 24 h an increased number of cells were engaged in tube formation, and there was

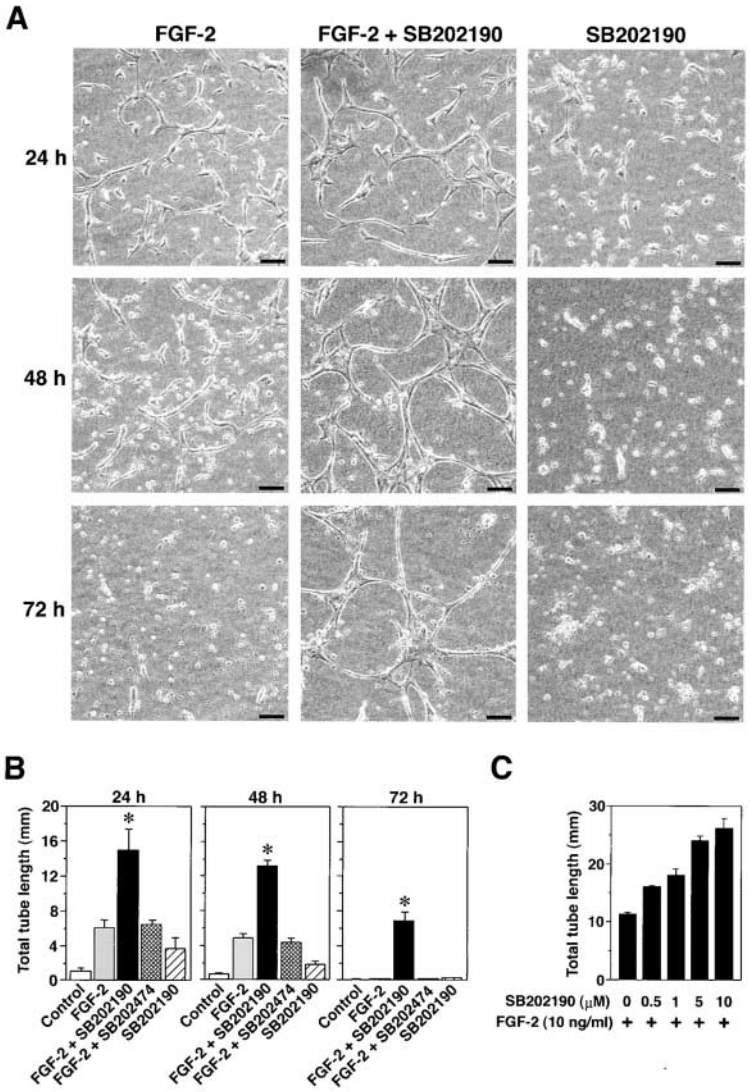

Figure 3. **SB202190 treatment enhances FGF-2–induced tubular morphogenesis.** (A and B) BCE cell collagen gel cultures were pretreated for 1 h with 10 μM SB202190 or 10 μM SB202474 and then treated or not with 10 ng/ml FGF-2. The cells were photographed after 24, 48, and 72 h of incubation (A). Total length of tubular structures from three fields (×10 objective) per well was measured using NIH image software (B). Treatment with FGF-2 and SB202190 demonstrated marked increases in the total tube length from 24 to 72 h. Similar results were obtained in five independent experiments. Asterisks indicate significantly different from FGF-2 or FGF-2 + SB202474 at $p < 0.05$ (unpaired Student's *t* test). (C) Dose response effect of SB202190 on FGF-2–induced tubular morphogenesis was measured at 24 h of incubation. Each bar shows the mean ± SD of duplicate wells. Bars, 100 μm.

an overall increase in tube length (for quantification see Fig. 3 B). By 72 h, the tubular structures had regressed in the FGF-2–treated cultures, whereas cultures treated with FGF-2 together with SB202190 still contained tubular structures of considerable length. The enhancing effect was dose dependent at concentrations from 0.5 to 10 μM of SB202190 (Fig. 3 C). Similar results were obtained when cells were treated with SB203580, another inhibitor of p38 (unpublished data). In contrast, the inactive compound SB202474 had no effect (Fig. 3 B). These findings indicate that inhibition of p38 activation enhances FGF-2–induced tubular morphogenesis.

To exclude the possibility of any unspecific effects of the SB202190 treatment on FGF-2–induced tubular morphogenesis, BCE cells were transiently cotransfected with cDNA constructs encoding MKK3 and MKK6, MAPK kinases which specifically phosphorylate p38. MKK3 and MKK6 are activated by dual phosphorylation on Ser-189/Thr-193 and Ser-207/Thr-211, respectively. Dominant negative versions of MKK3 and MKK6 were constructed by replacing these phosphorylation sites with alanine residues as described previously (Raingeaud et al., 1996). Expression of the dominant negative MKK3/6 caused a 1.9-fold increase in the total tube length at 24 and 48 h of incubation and an 18.3-fold increase at 72 h compared with the vector control (Fig. 4, A and B), effects similar to those seen in the SB202190-treated cultures. In contrast, expression of wild-type MKK3/6 resulted in a suppressed tubular morphogenesis with an increase in the number of apoptotic cells.

The level of p38 activity in the transfected cells was analyzed under basal and FGF-2–stimulated conditions. In the empty vector-transfected cells, increased activation of p38 was detected in the collagen gel cultures from 6 to 48 h incubation (Fig. 4 C), whereas no activation was detected in the gelatin cultures in spite of the continued high expression level of p38. Expression of wild-type MKK3/6 slightly (1.5-fold at 24 h incubation) enhanced the level of activated p38, whereas dominant negative MKK3/6 clearly inhibited p38 activation in the collagen gel culture (Fig. 4 C). Due to the regression of the tubes and apoptosis of the cells, the expression levels of p38 declined with time in these cultures.

FGF-2 treatment of BCE cells overexpressing wild-type MKK3/6 slightly (1.3-fold) increased the level of activated p38, whereas cells expressing dominant negative MKK3/6 failed to respond to FGF-2 with increased p38 activity (Fig.

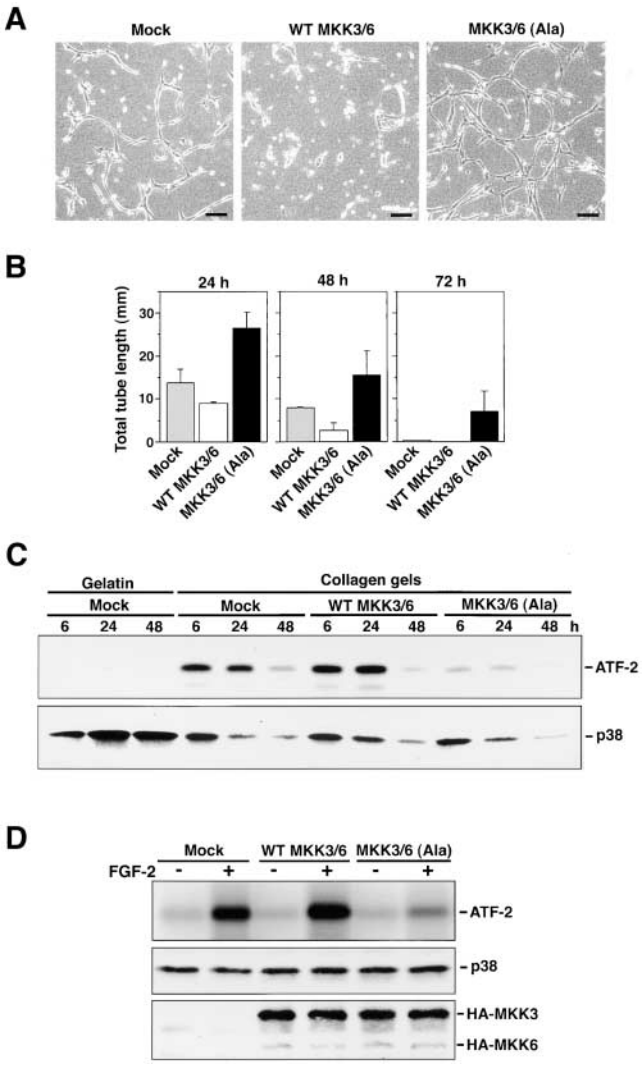

Figure 4. **Overexpression of dominant negative MKK3/6 enhances FGF-2–induced tubular morphogenesis.** BCE cells were transiently transfected with 3 μg wild-type HA-MKK3 and 3 μg wild-type HA-MKK6 (WT MKK3/6) or 3 μg dominant negative HA-MKK3 and 3 μg dominant negative HA-MKK6 (MKK3/6 [Ala]) or alternatively 6 μg empty vector (Mock). At 24 h after transfection, the cells were seeded between two layers of collagen gels and treated with 10 ng/ml FGF-2. (A) Morphology of the cells after 24 h of stimulation. (B) Total length of tubular structures was measured as described in the legend to Fig. 3 B. Each bar shows the mean ± SD of two independent experiments. (C) The transfected BCE cells cultured on gelatin-coated dishes or on collagen gels were lysed, and then p38 activity and protein levels of p38 were measured as described in the legend to Fig. 2. (D) At 24 h after transfection, the cells were serum starved for 16 h and treated or not with 50 ng/ml FGF-2 for 5 min. The cells were then lysed, and p38 activity was measured (top). One part of the cell lysates was immunoblotted with anti-p38 antibody (middle) or anti-HA mAb (bottom). Bars, 100 μm.

4 D, top). Expression of exogenous MKK3/6 was confirmed by immunoblotting using anti-hemagglutinin (HA) monoclonal antibody; the expression of MKK3 clearly exceeded that of MKK6 (Fig. 4 D, bottom). Taken together, these data strongly support the notion that p38 activation exerts a negative regulatory role in tubular morphogenesis.

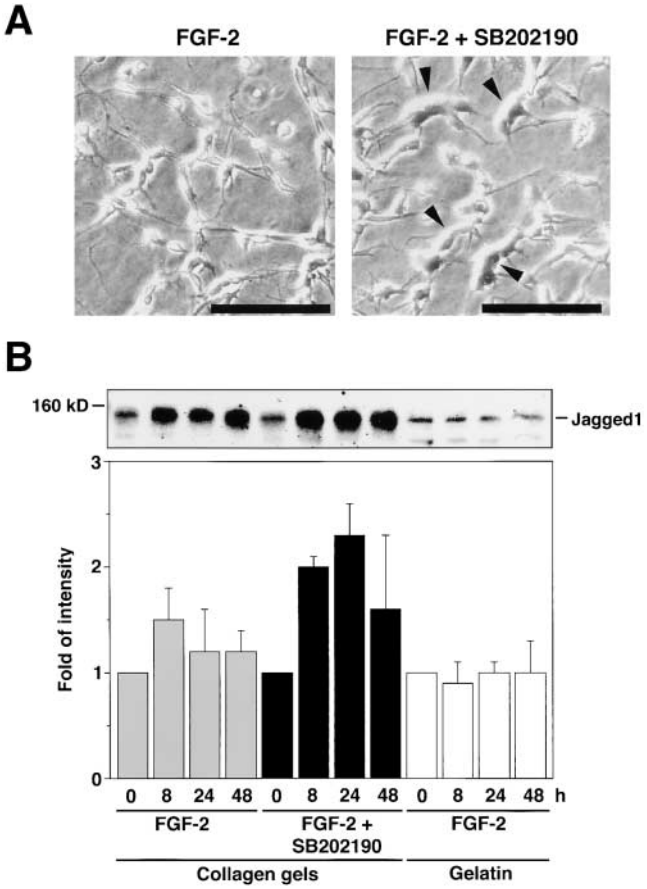

Figure 5. **SB202190 treatment increases the kinetics of FGF-2–induced cell differentiation.** (A) BCE cell collagen cultures were preincubated for 1 h with 10 μM SB202190 (right) or 0.1% DMSO (left) followed by treatment with 10 ng/ml FGF-2. The cells were photographed after 8 h of incubation. Arrowheads indicate fused cell bodies. (B) BCE cell cultures on collagen gels or on gelatin-coated dishes were pretreated for 1 h with 10 μM SB202190 or 0.1% DMSO followed by treatment with 10 ng/ml FGF-2 and extraction at various time points with SDS sample buffer. Samples were immunoblotted with anti-Jagged1 antibody. The degree of Jagged1 expression was quantified by NIH Image software. Each bar shows the mean ± SD of three independent experiments. Bars, 100 μm.

SB202190 treatment directly enhances FGF-2–induced cell differentiation as indicated by expression of Jagged1 during tubular morphogenesis

Recent studies have shown that p38 plays an essential role in cell differentiation in several different cell types (Engelman et al., 1998; Morooka and Nishida, 1998; Zetser et al., 1999). To examine the effect of p38 inhibition on endothelial cell differentiation, we first compared the cell morphology after 8 h of incubation in collagen gel cultures treated with FGF-2 alone and those treated with a combination of FGF-2 and SB202190. At that stage, cells treated with FGF-2 alone were connected by thin extensions (Fig. 5 A, left). In cultures treated with both FGF-2 and SB202190, cell bodies had fused in a majority of cells in the culture (Fig. 5 A, right, arrowheads). As shown in Fig. 1 C, fusion of endothelial cell bodies was induced by FGF-2 alone only after 16 h.

Several studies have demonstrated that Notch signaling is required for angiogenic vascular remodeling (Xue et al.,

1999; Krebs et al., 2000; Uyttendaele et al., 2001) and Jagged1, a ligand for its transmembrane receptor Notch, has been implicated in the regulation of endothelial cell differentiation in vitro (Zimrin et al., 1996; Uyttendaele et al., 2000). We next tested the possibility that the FGF-2–induced p38 pathway regulates endothelial cell differentiation via modulating expression of Jagged1. Expression of Jagged1 increased in collagen gel cultures between the 8- and 48-h time points (Fig. 5 B). The induction was specific for the full-length 150-kD form of Jagged1. In contrast, expression of Jagged1 was low and did not increase in the FGF-2–treated proliferating gelatin-seeded culture. Treatment with SB202190 in the presence of FGF-2 enhanced the expression of Jagged1 in the collagen gel cultures (a 2.3-fold increase from basal expression at the 24-h time point) compared with that with FGF-2 alone (a 1.2-fold increase from basal expression at the 24-h time point). These results support the notion that p38 negatively regulates differentiation of the endothelial cells. Moreover, it is possible that Jagged1/Notch signaling is involved in this regulatory pathway.

SB202190 treatment affects apoptosis, DNA synthesis, and proliferation during tubular morphogenesis

It is known that p38 regulates apoptotic cell death in response to cellular stress or certain inflammatory cytokines (Kyriakis and Avruch, 1996). We determined the extent of apoptosis in FGF-2–treated BCE cell cultures in collagen gels in the presence or absence of SB202190 by staining with Hoechst 33342. Cells released from dissolved tubes displayed hypercondensed and fragmented nuclei, hallmarks of apoptosis (Fig. 1 H). Quantitation of apoptotic cells showed that endothelial cell cultures underwent massive apoptosis in the absence of growth factors during the first 24 h of culture (Fig. 6 A); in the presence of FGF-2, 20% of the cells were apoptotic. There was no effect of the FGF-2 plus SB202190 treatment compared with FGF-2 alone at 24 h, but at 48 h cells treated with FGF-2 in combination with SB202190 were protected from apoptosis. Treatment with SB202190 alone had a marginal anti-apoptotic effect, probably depending on the endogenous production of growth factors. The exposure of phosphatidylserine on the outer leaflet of the plasma membrane serves as a sensitive marker for early stages of apoptosis (Martin et al., 1995). To determine whether decreased apoptosis contributed to the early effect of SB202190 in promotion of tubular morphogenesis, exposed phosphatidylserine was detected by use of fluorescein-conjugated annexin V. As shown in Fig. 6 B, the percentage of annexin V–positive cells was not affected by FGF-2 plus SB202190 treatment compared with FGF-2 alone or FGF-2 plus SB202474 treatment at 8 and 24 h when FGF-2–induced tubular morphogenesis was modulated by SB202190 treatment. In contrast, treatment with FGF-2 and SB202190 significantly inhibited an increase in annexin V–positive cells at 48 h. These results indicate that FGF-2 regulates cell survival through activation of p38 during the late phase of tubular morphogenesis but that early effects of SB202190 treatment leading to increased tube length are independent of inhibition of apoptosis.

The decreased apoptosis in the SB202190-treated cultures was accompanied by increased DNA synthesis and cell proliferation. Fig. 7, A and B, shows that the relative number of BrdU-positive cells, indicative of S-phase entry, was 1.2% in the FGF-2–treated collagen gel cultures. This proportion increased to 13.2% in cultures treated with both FGF-2 and SB202190 for 24 h. Control cultures receiving FGF-2 together with SB202474, or SB202190 alone, did not show an increased number of BrdU-positive cells (Fig. 7 B). The effect of SB202190 treatment on endothelial cell proliferation during 3 d in collagen culture was assessed. As shown in Fig. 7 C, the number of cells decreased in control cultures and in cultures treated individually with FGF-2 or SB202190. This is in agreement with previous reports that cell proliferation is arrested during tubular morphogenesis (Folkman and Haudenschild, 1980; Kubota et al., 1988; Ingber and Folkman, 1989). Cultures treated with a combination of FGF-2 and SB202190 showed a slight increase in cell number on day 1 and 2. The

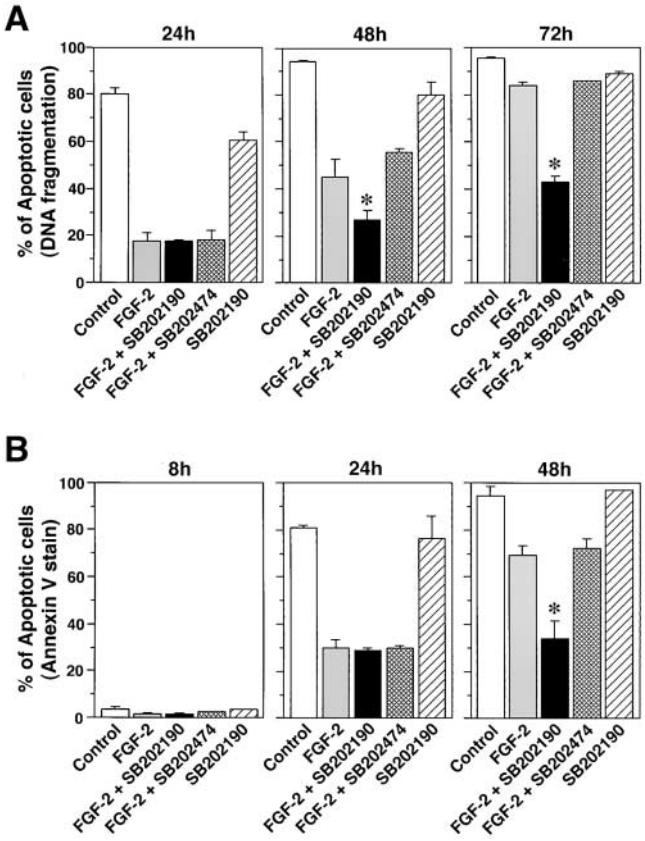

Figure 6. **Apoptosis in differentiating endothelial cells treated with FGF-2 and SB202190.** (A) BCE cell collagen cultures were pretreated or not with 10 μM SB202190 or 10 μM SB202474 for 1 h followed by stimulation or not with 10 ng/ml FGF-2. The cells were fixed and stained with Hoechst 33342 at 24, 48, and 72 h after incubation. Apoptotic nuclei were quantified by analysis of nuclear chromatin morphology from five fields (×20 objective) per well. (B) Cells were stained with fluorescein-conjugated annexin V and with propidium iodide and counterstained with Hoechst 33342 at 8, 24, and 48 h after incubation. Apoptotic cells stained annexin V positive, but propidium iodide negative were quantified from three fields (×20 objective) per well. In the presence of FGF-2, treatment with SB202190 reduced the frequency of apoptotic cells at 48 and 72 h but not at 8 and 24 h of incubation. Each bar shows the mean ± SD of duplicate wells. Asterisk indicates significantly different from FGF-2 or FGF-2 + SB202474 at $p < 0.03$ (unpaired Student's *t* test).

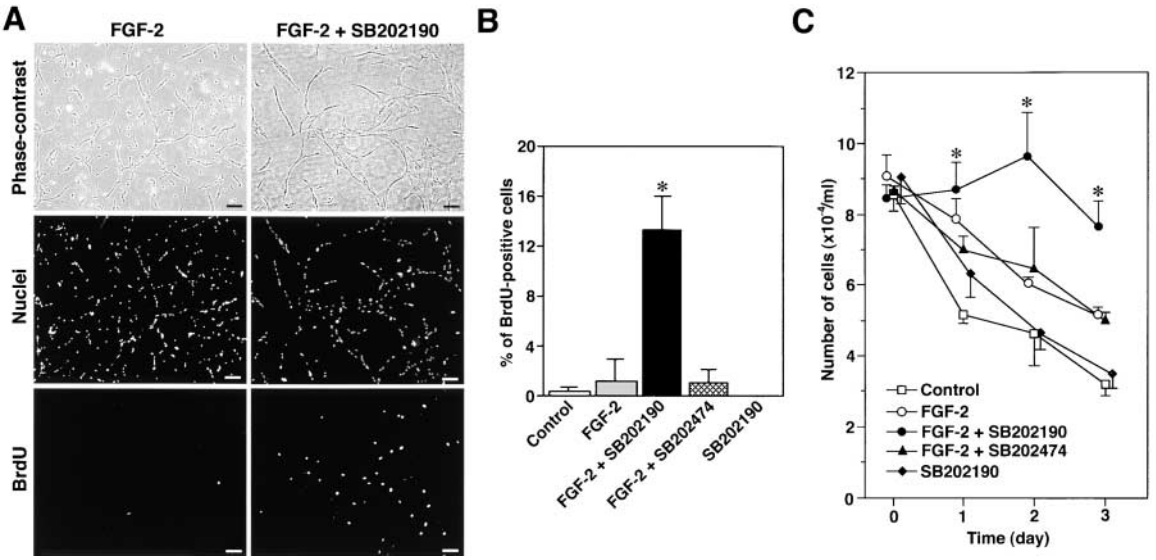

Figure 7. **SB202190 treatment leads to DNA synthesis and cell proliferation in collagen gel cultures in an FGF-2–dependent manner.** (A and B) BCE cell collagen cultures were pretreated or not with 10 μM SB202190 or 10 μM SB202474 for 1 h after which time FGF-2 was added to 10 ng/ml. After 24 h of incubation, BrdU (10 μM) was added, and after an additional 4 h of incubation the cells were fixed and stained with FITC-labeled anti-BrdU mAb (A, bottom). Nuclei were counterstained with Hoechst 33342 (A, middle). For comparison, cells in the same field were photographed in phase–contrast (A, top). BrdU-positive nuclei from five fields (×20 objective) per well were counted, and the percentage of BrdU-positive nuclei among the total nuclei was calculated (B). Each bar shows the mean ± SD of at least triplicate wells from two individual experiments. Asterisk indicates significantly different from FGF-2 or FGF-2 + SB202474 at $p < 0.01$ (unpaired Student's *t* test). (C) BCE cell collagen cultures in 24-well dishes were pretreated or not with 10 μM SB202190 or 10 μM SB202474 followed by treatment with FGF-2 as described above. At 0, 1, 2, and 3 d of incubation, the collagen gel layers were digested with collagenase, cells were trypsinized, and the number of cells was determined using a Beckman Coulter counter. Similar results were obtained in two independent experiments. Each value is the mean ± SD of triplicate wells. Asterisk indicates significantly different from FGF-2 or FGF-2 + SB202474 at $p < 0.04$ (unpaired Student's *t* test). Bars, 100 μm.

control compound SB202474 did not support FGF-2–dependent cell proliferation, indicating that the effect of SB202190 resulted from inhibition of p38. These data show that p38 inhibition in combination with FGF-2 treatment led to increased DNA synthesis at 24 h treatment and a stabilization of cell numbers during the 3 d of treatment with SB202190.

FGF-2 but not VEGF activates p38 in vascular endothelial cells in the chick embryos

FGF-2 treatment of the chick embryos is known to induce angiogenesis in the chick chorioallantoic membrane (CAM). To examine whether p38 activity was associated with growth factor–induced angiogenesis, we measured the kinetics of p38 activation in the CAMs that were stimulated with FGF-2. Increased activation of p38 was detected in the CAMs 10–60 min after their exposure to FGF-2 (Fig. 8 A). Furthermore, phosphorylated p38 was detected by anti–phospho-p38 immunostaining of endothelial cells in the CAM tissue at 2 h after treatment with FGF-2 (Fig. 8 B). In contrast, the immunoreactivity was not detected when the CAM was treated with VEGF, although VEGF also induces neovascularization of the CAM. These results demonstrate that FGF-2 activates p38 in endothelial cells during angiogenesis in vivo.

SB202190 treatment enhances FGF-2–induced neovascularization but impairs neovascular morphogenesis in the chick embryos

To examine the role of p38 activation during angiogenesis, the CAMs were treated with the p38 inhibitor SB202190 and stimulated or not with FGF-2. FGF-2 induced neovascularization in the CAM (Fig. 9 A), the extent of which was estimated by counting number of vessel branches (Fig. 9 B). Cotreatment with FGF-2 and SB202190 but not with FGF-2 and the inactive compound SB20247, led to a significant increase in the number of vessel branches. Treatment with SB202190 alone had a small stimulatory effect, most likely due to endogenous production of growth factors in the chick embryo (Fig. 9, A and B). We next analyzed the morphology of the vessels in the CAMs by immunostaining with an anti–von Willebrand factor (vWF) antibody as a marker for endothelial cells. Blood vessels of different diameters consisting of monolayer of endothelial cells were visible in the CAMs treated with FGF-2 alone (Fig. 10, left) and in the CAMs treated with FGF-2 together with SB202474 (unpublished data). In contrast, the CAMs treated with FGF-2 and SB202190 displayed polymorphic microvessels with hyperplasia of endothelial cells (Fig. 10, right, arrowheads). The abnormal vascular structures were frequently observed for vessels <100 μm in diameter. Although the abnormal vessel lumina appeared partially or entirely closed, some of them still contained red blood cells (Fig. 10, right, arrows), suggesting that abnormal vessel structures were included in the estimation of apparently functional vessels in CAMs treated with FGF-2 and SB202190 shown in Fig. 9. About 50–70% of all blood vessels stained by the anti-vWF antibody displayed partial or complete occlusion of the lumina in four individual CAMs treated with FGF-2 and SB202190. These findings indicate that activation of p38 by FGF-2 stimula-

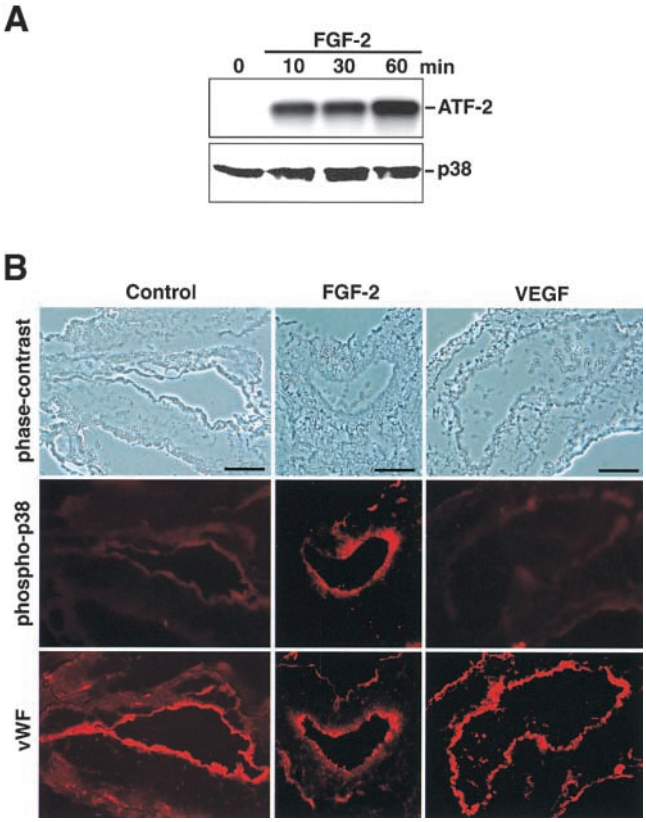

Figure 8. **Localization of phosphorylated p38 in FGF-2–treated chick embryos.** (A) Activation of p38 in the CAM was analyzed after treatment with or without 0.5 μg FGF-2 for 10–60 min and the CAM tissue was lysed, and p38 activity (top) and protein levels of p38 (bottom) were measured as described in the legend to Fig. 2. (B) Cryosections of CAMs that had been treated with 1 μg FGF-2 or 1 μg VEGF for 2 h or untreated (Control) were examined for phosphorylated p38 using indirect immunofluorescence and photographed in phase–contrast (top) and in fluorescence view (middle). Series of the sections were processed for indirect immunofluorescence using anti–human vWF antibody to detect endothelia of vessels (bottom). Bars, 50 μm.

tion is critical for formation of functional vessels in vivo by regulation of several aspects of endothelial cell function, such as differentiation, apoptosis, and proliferation.

Discussion

We show in this study that activation of p38 is induced by FGF-2 in endothelial cells undergoing tubular morphogenesis in collagen cultures. The dramatic morphological changes that the cell undergo accompanied by growth arrest and formation of lumen are indicative of a differentiation process, which is characterized by distinct signal transduction (Klint and Claesson-Welsh, 1999) and gene regulatory events (Kahn et al., 2000). Treatment with pharmacological p38 inhibitors and transient expression of dominant negative upstream regulators of p38 enhanced tubular morphogenesis in an FGF-2–dependent manner by regulating differentiation, apoptosis, and proliferation of the endothelial cells. We could also demonstrate FGF-2–induced activation of p38 in vivo in the chicken CAM. Inhibition of p38 augmented FGF-2–induced neovascularization in the CAM, but close inspection revealed that ∼60% of the vessels displayed partial or complete occlusion of the lumen due to hyperplasia of the endothelial cell pool.

The mechanistic explanation for the induction of p38 activation in the differentiating but not the proliferating endothelial cells could be based on specific gene regulation, since differentiation of endothelial cells is accompanied by induction of specific gene transcription (Kahn et al., 2000). On the other hand, we cannot exclude that p38 activation was dependent on the collagen matrix, since integrin signaling pathways are known to synergize with growth factor receptor pathways to regulate cell proliferation, adhesion, and migration (Clark and Brugge, 1995). The α2β1 integrin, which binds type 1 collagen, has been shown to mediate p38 activation in the osteosarcoma cell line Saos-2, and the signal transduction molecules Cdc42, MKK3, and MKK4 were implicated in this pathway (Ivaska et al., 1999). Activation of p38 by collagenous matrix has also been shown in human dermal fibroblasts (Ravanti et al., 1999). These results suggest that collagen matrix–endothelial cell interaction via integrins such as α2β1 may modulate FGF-2–induced p38 activation.

Recent studies have demonstrated that p38 activation is required for differentiation in several different cell types including neuronal cells (Morooka and Nishida, 1998), adipocytes (Engelman et al., 1998), and myoblasts (Zetser et al., 1999). We provide experimental evidence that activation of p38 directly modulates endothelial cell differentiation during tubular morphogenesis based on the following observations. First, inhibition of p38 in FGF-2–stimulated BCE cell collagen gel cultures enhanced FGF-2–induced tubular morphogenesis but did not affect apoptosis in the 8- and 24-h cultures (Fig. 3 B and Fig. 6). Second, treatment with SB212190 enhanced fusion of endothelial cells, which is an early step in the organization of the cells into tube-like structures (Fig. 5 A). Third, we found that expression of Jagged1, a ligand for Notch family transmembrane receptors, was increased during the early stage of tubular morphogenesis and that this expression was enhanced by treatment with a p38 inhibitor (Fig. 5 B). Recent studies have shown that Notch signaling is essential for endothelial cell differentiation during tubular morphogenesis. Rat brain microvascular endothelial cells, overexpressing Jagged1 or Notch4/int-3, a constitutive active form of Notch4, form tubular structures spontaneously (Uyttendaele et al., 2000). Targeted gene inactivation of Jagged1 and Notch1 and double inactivation of Notch1/Notch4 results in embryonic lethality with severe defects in vascular remodeling (Xue et al., 1999; Krebs et al., 2000). Expression of an activated form of Notch4 in the mouse embryonic endothelium causes disorganization of the vasculature and embryonic lethality (Uyttendaele et al., 2001). Notch4 is expressed specifically in endothelial cells during embryonic and adult life (Uyttendaele et al., 1996). Although the specificity of Jagged1 for binding to the various Notch receptors is still unclear, these findings imply that the interaction of endothelial Jagged1 with Notch4 regulates vascular morphogenesis. Our data support that induction of Jagged1 is coupled to tubular morphogenesis and that p38 may be a potent regulator of Jagged1 expression.

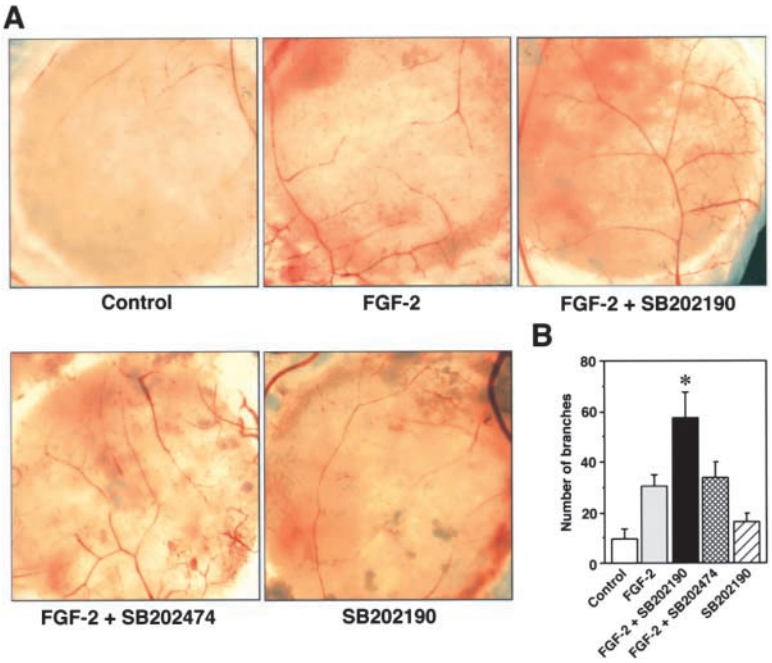

Figure 9. **SB202190 treatment enhances neovascularization in FGF-2–treated chick embryos.** (A) Angiogenesis was examined in 10-d-old chick CAMs treated with PBS (Control) or 0.1 μg FGF-2 in the presence or absence of 10 μM SB202190 or 10 μM SB202474. (B) The number of vessel branches was estimated as described previously (Brooks et al., 1994). Treatment with FGF-2 induced neovascularization in the CAMs. The addition of SB202190 enhanced FGF-2–induced neovascularization, whereas the addition of SB202474 had no effect. Similar results were obtained in three independent experiments. Each bar shows the mean ± SD from five to seven embryos. Asterisk indicates significantly different from FGF-2 at $p < 0.04$ (Mann-Whitney's U test).

Vascular homeostasis, which prevails, for example, in the healthy male is thought to depend on a balance between progression and regression, regulated by the microenvironment (Folkman and Haudenschild, 1980). Recent studies indicate that apoptosis of the endothelium plays a central role in the regression of the vasculature (Dimmeler and Zeiher, 2000). Apoptosis-mediated cell deletion during tubular morphogenesis has been demonstrated in human umbilical vein endothelial (HUVE) cells (Pollman et al., 1999) and bovine aortic endothelial cells (Kuzuya et al., 1999). Activation of p38 is likely to be important in the induction of apoptosis and regression of angiogenesis in accordance with the well-established notion that apoptosis induced by several stress stimuli involves the action of p38 (Kyriakis and Avruch, 1996). In line with this view, our data showed that p38 inhibition reduced the degree of apoptosis in the differentiating primary endothelial cells (Fig. 6). Regulation of growth may be achieved in part by induction of apoptosis/regression but also partly by direct inhibition of cell cycle progression. The p38 pathway has been shown to play a negative regulatory role in cell cycle progression (Molnar et al., 1997; Takenaka et al., 1998; Yu and Sato, 1999). Yu and Sato (Yu and Sato, 1999) reported that inhibition of p38 correlated with decreased expression of cdk inhibitor $p27^{Kip1}$ and hyperphos-

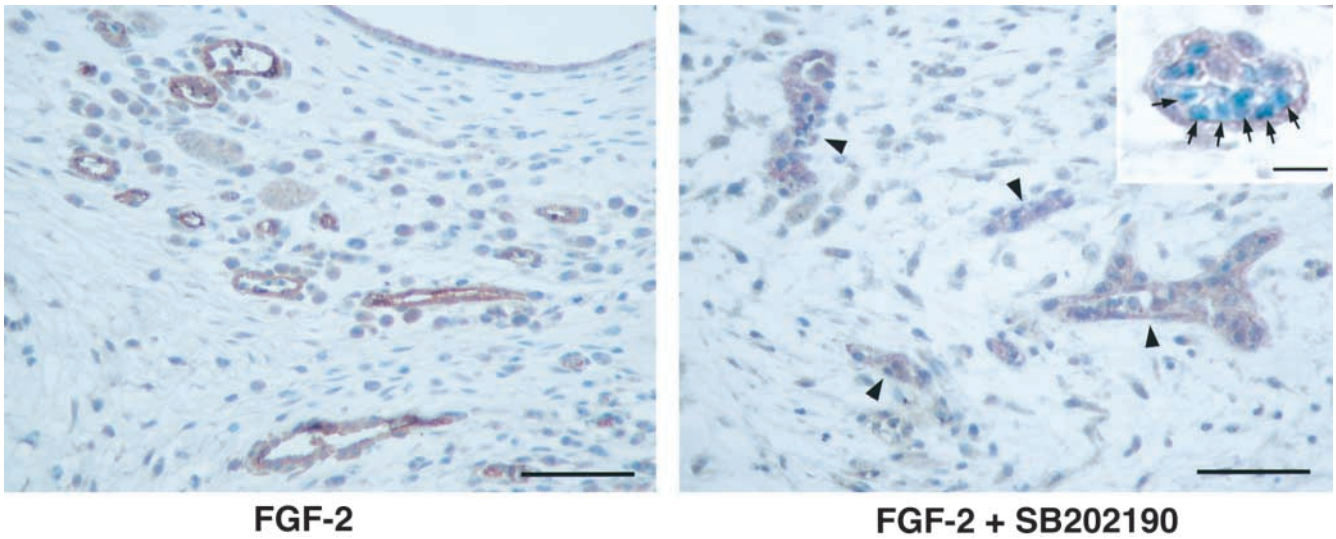

Figure 10. **SB202190 treatment impairs vascular morphogenesis in FGF-2–treated chick embryos.** Sections of CAMs that had been treated with 0.1 μg FGF-2 in the presence or absence of 10 μM SB202190 were examined immunohistochemically using an anti–human vWF antibody to detect endothelial cells. Cotreatment with FGF-2 and SB202190 induced hyperplasia of endothelial cells and abnormal vascular morphologies (arrowheads). Erythrocytes (arrows) were visible in a vessel with partially occluded lumen. Bars: (FGF-2 and FGF-2 + SB202190) 50 μm; (inset) 10 μm.

phorylation of the Rb tumor suppressor protein in HUVE cells. Therefore, growth arrest of endothelial cells during tubular morphogenesis may be mediated via a p38-mediated block in cell cycle progression (Fig. 7, A and B).

Gene inactivation of upstream regulators of p38 or individual p38 isoforms has demonstrated a role for the p38 pathway in vascular development and regulation of angiogenesis. Disruption of the p38α gene results in embryonic lethality around E12.5 due to severe defects in placental development (Adams et al., 2000; Mudgett et al., 2000; Tamura et al., 2000). The p38α-null placentas display a severe reduction in the labyrinthine layer and lack of intermingling embryonic and maternal blood vessels, consistent with a defect in vascularization. The p38α-null mice also display defects in cardiovascularization. However, this defect is likely to be secondary to the impairment in placental function (Adams et al., 2000). A similar phenotype with vascular defects in the placenta was noted in mice lacking expression of MEKK3, a p38 MAPK kinase kinase (Yang et al., 2000). On the other hand, inactivation of MKK3, a p38 MAPK kinase, did not reveal vascular abnormalities (Lu et al., 1999), indicating the complexity of the p38 pathway. Several different downstream targets of p38 have been identified, including protein kinases such as MAPK-activated protein kinase-2/3 and transcription factors such as ATF-2, Elk-1, CHOP, and myocyte enhance factor (MEF)2C (Ono and Han, 2000). For example, MEF2C is expressed in endothelial cells in the embryo and plays a crucial role in cardiovascular development (Lin et al., 1997, 1998). In embryos lacking expression of MEF2C, endothelial cells fail to organize properly and form vascular anomalies characterized by extreme variability in lumen size and defects in remodeling. Thus, MEF2C may be involved downstream of the p38-dependent signaling pathway for regulating vascular morphogenesis.

In the present study, treatment with the p38 inhibitor–enhanced FGF-2 induced the CAM neovascularization, but ~60% of the vessels displayed features indicative of hyperplasia of the endothelial cells with partially or completely closed lumen. These in vivo results correlate with the in vitro data, since the p38 inhibitor treatment induced cell proliferation and survival in BCE cells collagen gel culture. The morphological features of the vascular defect in p38α-null placentas are different from that in the CAMs treated with the p38 inhibitor. The cellular response to p38 activation may be dependent on the expression pattern of the p38 isoforms and the stimulating agent. Placentas express high level of p38α and p38δ but not p38β and p38γ (Wang et al., 1997), whereas vascular endothelial cells coexpress p38α, p38β, and p38δ (Hale et al., 1999). VEGF and placenta growth factor, which are implicated as major regulators of placenta angiogenesis, also activate p38 (Rousseau et al., 1997; Desai et al., 1999). Interestingly, VEGF-induced p38 activity has been shown to positively regulate migration and actin reorganization in HUVE cells (Rousseau et al., 1997). In this study, activation of p38 by VEGF was weaker and more transient in the differentiating primary microcapillary endothelial cell compared with treatment with FGF-2.

In conclusion, our data indicate that FGF-2–stimulated p38 signaling pathway has a critical role during angiogenesis in negative regulation of different aspects of endothelial cell function. This regulation may be required for the normal assembly of endothelial cells during vascular morphogenesis and the maintenance of established vessels.

Materials and methods

Tissue culture

BCE cells were isolated from calf adrenal cortex as described (Qi et al., 1999) and used at passage 10–14. For routine culture, the cells were cultured in DME (Life Technologies), 10% newborn calf serum (NCS), 2 ng/ml FGF-2 (Boehringer) on gelatinized dishes. For collagen gel cultures, collagen type I (Vitrogen; Cohesion Technologies, Inc.) was mixed with 10× Ham's F12 medium and 0.1 M NaOH (8:1:1) distributed in dishes or onto glass coverslips in 6-well plates and allowed to gel at 37°C overnight. When indicated, cells were starved in DME, 1% NCS for 16 h.

p38 assays

Serum-starved BCE cells were untreated or treated with 50 ng/ml FGF-2 or 100 ng/ml VEGF (Peprotech) for 5–60 min or with 300 mM NaCl for 30 min. Alternatively, BCE cells were seeded on collagen gels, incubated for 8 h, and then serum starved and stimulated. Cells were lysed in a Triton X-100 containing phosphatase and protease inhibitors, and immunoprecipitation was performed using an anti-p38 antibody (Gerwins et al., 1997). Samples were washed in kinase buffer (20 mM Hepes, pH 8.0, 20 mM $MgCl_2$, 2 mM $MnCl_2$, 1 mM DTT) and incubated for 30 min at 30°C in kinase buffer, 5 μCi of [γ-^{32}P]ATP (Amersham Pharmacia Biotech), and 5 μg recombinant NH_2-terminal fragment of ATF-2. Samples were resolved by SDS-PAGE in 12% gels and analyzed using a Bio-Imager BAS-1800II (Fuji).

For in vitro kinase inhibition assays, p38 was immunoprecipitated and preincubated or not with 10 μM SB202190 (Calbiochem-Novabiochem), a specific inhibitor of p38, or 10 μM SB202474 (Calbiochem-Novabiochem), an inactive compound, before the kinase assay. The p38 assays in the CAM was performed essentially according to Eliceiri et al. (1998). Briefly, 0.8 × 0.8 cm filter disks (Whatman) were saturated with 3 mg/ml cortisone acetate (Sigma-Aldrich) and soaked in PBS (40 μl for each filter) with or without FGF-2 (0.5 μg for each filter) on the CAMs. CAM tissue was harvested, snap frozen, and homogenized in a modified RIPA buffer containing 1% deoxycholic acid, 1% Triton X-100, and 0.1% SDS. Equivalent amounts (700 μg) of protein were analyzed as described above.

Tube formation assay

Serum-starved BCE cells were inoculated onto the collagen gels in 6-well dishes at 5×10^4 cells/cm^2 and incubated for 3 h at 37°C, and then a second layer of collagen was added. After gelling, cultures were treated with or without 10 ng/ml FGF-2 in DME, 1% NCS. In indicated experiments, SB202190, SB202474, or 0.1% DMSO was added to cells 1 h before addition of FGF-2. The cultures were incubated for 72 h without readdition of growth factors or inhibitors. The cells were examined using a phase–contrast microscope (Nikon Eclipse TE 300) at indicated time points. To quantify the length of tubular structures, three random phase–contrast photomicrographs (×10 objective) per well were taken, and the tubule length was measured using NIH image software (version 1.56). Tubes shorter than 100 μm were excluded from the measurements.

Detection of apoptotic cells

BCE cells treated or not with 10 ng/ml FGF-2 and 10 μM SB202190 or 10 μM SB202474 were fixed in 3% paraformaldehyde (Sigma-Aldrich) in PBS and stained with 5 μg/ml Hoechst 33342 (Molecular Probes) for 30 min. Nuclei with condensed chromatin and fragmented nuclei from five fields (×20 objective) per well were counted. For early detection of apoptosis, BCE cells were stained with fluorescein-conjugated annexin V using Annexin-V-FLUOS staining kit (Roche Molecular Biochemicals). Nuclei were counterstained with 5 μg/ml Hoechst 33342, and the number of annexin V–positive cells was counted from three fields (×20 objective) per well. Necrotic cells identified through propidium iodide were excluded from the estimation.

Plasmid constructions and transient transfections

Human cDNAs encoding MKK3 (sequence data available from GenBank/EMBL/DDBJ under accession no. L36719) and MKK6 (sequence data available from GenBank/EMBL/DDBJ under accession no. U39657) were generated by PCR from HL60 cells using primers where the 5′ primer contained the HA tag sequence YPYDVPDYA. The dominant negative versions of MKK3 and MKK6 (Raingeaud et al., 1996) were created by site-directed

mutagenesis where S189/T193 in MKK3 and S207/T211 in MKK6 were changed to alanines. All constructs were verified by DNA sequencing. Transient transfections of BCE cells with the different constructs were performed using LipofectAMINE™ (Life Technologies).

Cell proliferation assay

Serum-starved BCE cells were seeded at 9×10^4 cells/well on collagen gel–containing 24-well plates. After 3 h, cells were stimulated with 10 ng/ml FGF-2. In indicated experiments, 10 μM SB202190, 10 μM SB202474, or 0.1% DMSO was added to cells 1 h before addition of FGF-2. At indicated time points, the gel was solubilized with 20 μl collagenase (2.5 U/μl in PBS; Sigma-Aldrich) for 2 h at 37°C, after which time 230 μl trypsin/EDTA per well was added. Changes in the cell count were determined with a Beckman Coulter counter.

Detection of S-phase cells

Serum-starved BCE cells were inoculated onto collagen gel–containing coverslips at a density of 10^5 cells/cm^2 and incubated at 37°C for 2 h. Cultures were treated with 10 μM SB202190, 10 μM SB202474, or 0.1% DMSO for 1 h followed by addition of 10 ng/ml FGF-2. After 24 h incubation at 37°C, 10 μM BrdU (Sigma-Aldrich) was added, and incubation continued for 4 h. Cells were fixed in 70% ethanol for 30 min, washed with PBS, and incubated in 0.02 M NaOH for 2 min followed by five washes with PBS. FITC-conjugated anti-BrdU mAb (2:5 dilution; Becton Dickinson) was added, and after 1 h the cells were stained with 5 μg/ml Hoechst 33342 for counter staining of whole nuclei. The labeling index was expressed as the percentage of labeled nuclei/total nuclei from five fields (×20 objective) per well.

Immunoblotting

Serum-starved BCE cells were pretreated for 1 h with 10 μM SB202190 or 0.1% DMSO and then treated with 10 ng/ml FGF-2. The cells were lysed in 500 μl boiling SDS sample buffer at the indicated time points, and samples were analyzed by SDS-PAGE and immunoblotting using nitrocellulose filter (Hybond-ECL; Amersham Pharmacia Biotech). The blots were incubated with anti-Jagged1 antibody (1:200 dilution; Santa Cruz Biotechnology, Inc.) overnight at 4°C, washed, and then incubated with HRP-conjugated anti–rabbit Ig antibody (1:2,500 dilution; Amersham Pharmacia Biotech) followed by ECL detection (Amersham Pharmacia Biotech). For immunoblotting of p38 and HA-tagged constructs, one part of the cell lysates (80 μl) was separated by SDS-PAGE, transferred, and probed with anti-p38 antibody (1:1,000 dilution) or anti-HA mAb (1:1,000 dilution; Boehringer).

Angiogenesis assay in chicken embryos

The angiogenesis assay in the CAM was performed as described (Sasaki et al., 1999) using fertilized 10-d chick eggs in which the CAM was exposed by removing a 1 × 1 cm piece of the shell to allow application of Whatman filter disks saturated with 3 mg/ml cortisone acetate soaked in PBS (50 μl for each filter) with or without FGF-2 (0.1 μg for each filter) and 10 μM SB202190 or 10 μM SB202474. After 3 d of incubation, the CAM was cut around the filter and inspected in a double-blind procedure to determine the number of vessel branches in the area of the filter disk as described previously (Brooks et al., 1994).

Immunohistochemistry and immunofluorescence

For immunohistochemical analysis of endothelia in the CAM, treated CAM fragments were fixed with formalin and embedded in paraffin. Paraffin-embedded sections were deparaffinized and incubated with anti–human vWF antibody (1:100 dilution; Dako) followed by biotinylated anti–rabbit Ig (1:100 dilution; Dako). A positive reaction was detected by the ABC method (Dako) and visualized by the DAB reaction. Sections were counterstained with hematoxylin.

Immunofluorescence analysis of phosphorylated p38 in the CAM was performed essentially as described previously (Eliceiri et al., 1998). Treated CAM fragments were embedded in Tissue-Tek OCT compound (Sakura Fintek), frozen, and 4-μm cryostat sections were cut, fixed in acetone for 15 min, blocked in 1% BSA in PBS, incubated with anti–phospho-p38 antibody (1:50 dilution) or anti–human vWF antibody (1:100 dilution) overnight, washed, and incubated with TRITC-conjugated goat anti–rabbit IgG (1:50 dilution; Zymed Laboratories) for 30 min.

Statistical analysis

Results are expressed as mean ± SD. Statistical analyses were performed using StatView software (Version 4.5; SAS Institute, Inc.). Student's *t* test and Mann-Whitney's U test were used for intergroup comparisons.

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