MEKK3 is required for endothelium function but is not essential for tumor growth and angiogenesis

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Deng Y, Yang J, McCarty M, Su B. MEKK3 is required for endothelium function but is not essential for tumor growth and angiogenesis. Am J Physiol Cell Physiol 293: C1404–C1411, 2007. First published August 8, 2007; doi:10.1152/ajpcell.00058.2007.—Mitogen-activated protein kinase kinase kinase 3 (MEKK3) plays an essential role in embryonic angiogenesis, but its role in tumor growth and angiogenesis is unknown. In this study, we further investigated the role of MEKK3 in embryonic angiogenesis, tumor angiogenesis, and angiogenic factor production. We found that endothelial cells from Mekk3-deficient embryos showed defects in cell proliferation, apoptosis, and interactions with myocardium in the heart. We also found that MEKK3 is required for angiopoietin-1 (Ang1)-induced p38 and ERK5 activation. To study the role of MEKK3 in tumor growth and angiogenesis, we established both wild-type and Mekk3-deficient tumor-like embryonic stem cell lines and transplanted them subcutaneously into nude mice to assess their ability to grow and induce tumor angiogenesis. Mekk3-deficient tumors developed and grew similarly as control Mekk3 wild-type tumors and were also capable of inducing tumor angiogenesis. In addition, we found no differences in the production of VEGF in Mekk3-deficient tumors or embryos. Taken together, our results suggest that MEKK3 plays a critical role in Ang1/Tie2 signaling to control endothelial cell proliferation and survival and is required for endothelial cells to interact with the myocardium during early embryonic development. However, MEKK3 is not essential for tumor growth and angiogenesis.

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MITOGEN-ACTIVATED PROTEIN KINASE (MAPK) cascades form major intracellular signaling networks and are essential to transduce extracellular signals to the appropriate intracellular targets to initiate cellular responses leading to cell proliferation, differentiation, or apoptosis (4, 8, 9). Each MAPK is activated by a MAPK kinase (MAP2K), which, in turn, is activated by a MAP2K2 kinase (MAP2K3) (37, 40). MEKK3 is a member of the MEKK/STE11 subgroup of the MAP3K family (2, 10) and is capable of activating multiple downstream MAPKs, including ERK1/2, JNK, big MAPK/ERK5, and p38 (5, 22, 27, 46). In addition, MEKK3 has also been shown to activate the IκB kinase (IKK)/NF-κB pathway (22, 36, 47).

Previously, we have reported that MEKK3 plays an important role in early embryonic cardiovascular development, especially in angiogenesis and heart development (46), by activating myocyte-specific enhancer factor (MEF2)c, a transcription factor crucial for early embryonic development (1, 29), through the p38 MAPK cascade (46). MEF2c has also been shown to be a downstream target of ERK5, one of the major MAPks activated by MEKK3 and MEKK2 via MEK5 (3, 5, 21, 25). Disruption of ERK5 has been shown to cause multiple extraembryonic vascular and embryonic cardiovascular defects due to failure of endothelial cells (ECs) (21, 32, 44). Moreover, targeted deletion of ERK5 in adult mice led to lethality due to an EC failure (21). A study (31) on p38-α knockout mice showed that p38-α has an essential role in placenta angiogenesis but not embryonic cardiovascular development. However, since there are different isoforms of p38 in mammalian cells (19, 23, 28, 43), other p38 isoforms other than p38-α may function in embryonic cardiovascular development. In addition, MEKK3 has also been found to play a balancing role between pro- and antiapoptotic signals in ECs by regulating the cross-talk between the phosphatidylinositols 3-kinase (PI3K)/Akt and p38 signaling pathways (17). Both PI3K/Akt and p38 pathways are known to play important roles in ECs during vascular development (16, 18, 31). Thus, both biochemical and genetic studies have suggested that the two MAPK modules, MEKK3-ERK5-MEF2c and MEKK3-p38-MEF2c, may function in embryonic cardiovascular development.

Tie2 is an EC-specific receptor tyrosine kinase that plays an important role in embryonic vascular development (34). Disruption of Tie2 in mice resulted in embryonic lethality at approximately embryonic day 10.5 (E10.5) due to cardiac failure, hemorrhage, and other vascular defects (34). Tie2 is dimerized, autophosphorylated, and activated by binding to its ligand, angiopoietin-1 (Ang1), in ECs (30, 35, 41). Mice deficient for Ang1 have a similar phenotype as Tie2-deficient mice (38). Ang1 is an EC-specific mitogenic factor and is able to activate the MAPKs p38, ERK1/2, and JNK in cultured ECs (20, 24). However, the mechanism of MAPK activation by Ang1/Tie2 is still unknown. Since the phenotype of Mekk3-deficient mice resembles that of Tie2- and Ang1-deficient mice, it is likely that MEKK3 may be responsible for transducing Ang1/Tie2 signals to downstream MAPKs.

In this study, we further characterized the development of the vascular system in Mekk3-deficient embryos and determined the role of MEKK3 in ECs. We found that the proliferation and survival of Mekk3-deficient ECs were impaired. Interactions of Mekk3-deficient ECs with the myocardium were also affected in Mekk3-deficient embryos. Consistently, the dorsal aorta in mutant embryos was dilated or disrupted, suggesting a lack of support of ECs by smooth
muscle cells (SMCs). At the biochemical level, we found that MEKK3 is required for Ang1-induced p38 and ERK5 activation.

Because angiogenesis plays a critical role in tumor development and growth (13), we also investigated if MEKK3 is involved in these processes. We found that Mekk3-deficient tumors grew at the same incidence and rate as Mekk3 wild-type tumors. Mekk3-deficient tumors produced similar levels of VEGF and induced angiogenesis in a manner indistinguishable from control wild-type tumors. Collectively, these results suggest that while MEKK3 is a key intracellular signaling molecule for ECs, it is not essential for tumor angiogenesis or growth of tumors.

MATERIALS AND METHODS

Preparation of E9.5–10.5 wild-type and Mekk3−/− embryos. To obtain Mekk3−/− embryos, Mekk3+/− female mice were set up for time mating with Mekk3−/− male mice as previously described (46). Virginal plugged female mice were killed on days 9.5–10.5, and embryos and yolk sacs were harvested. Embryos and yolk sacs were fixed overnight in 4% paraformaldehyde for immunohistochemical staining.

All procedures in this study were performed in accordance with National Institutes of Health Guidelines for the Care and Use of Laboratory Animals (NIH Pub. No. 85-23, Revised 1996), and the protocol was approved by the Institutional Animal Care and Use Committee of the University of Texas and M. D. Anderson Cancer Center.

Immunohistochemistry. Embryos and yolk sacs for immunohistochemical staining were embedded in paraffin and cut into 6- to 7-μm-thick sections. Tissues for frozen sections were embedded in OCT (Tissue-Tek) and cut into 8-μm-thick sections. Frozen sections were fixed in acetone and acetone-chloroform. The hematoxylin and eosin staining procedure followed a standard protocol. Immunohistochemical staining with anti-smooth muscle actin (SMA; 1A4, Sigma), anti-PECAM (MEC13.3, Pharmingen), anti-VEGF (A20, Santa Cruz Biotechnology), and anti-proliferating cell nuclear antigen (PCNA) antibodies (BD Biosciences) was performed by basic immunoperoxidase procedures.

Bromodeoxyuridine incorporation assay. Mice carrying E10 embryos were injected with 250 μl of 250 μg/ml bromodeoxyuridine (BrdU; Sigma) through their tail veins. After 1.5–2 h, mice were killed, and embryos were collected. Embryos were fixed in 70% ethanol in PBS, embedded in paraffin, and cut into 6- to 7-μm sections. After deparaffinization, tissues were treated with 1% Triton X-100 in PBS, 2 M HCl in PBS, and 0.1 M Tris-HCl (pH 7.6) successively, followed by basic immunoperoxidase procedures with anti-BrdU antibody (B44, Becton Dickinson). TUNEL assay. E9.5 embryos were fixed in 4% paraformaldehyde overnight and embedded in paraffin. Apoptotic cells were detected by TUNEL assay using the In Situ Cell Death Detection Kit (Roche) according to the manufacturer’s instructions.

Cell culture. Wild-type and Mekk3-deficient mouse embryonic fibroblasts were prepared and cultured in 10% FBS in DMEM as previously described (47). Brain ECs (BECs) were maintained as previously described (14).

Plasmid construction. For construction of pHA-Fv-Tie2K plasmid, the intracellular kinase domain of Tie2 (amino acids 771–1122) was amplified by PCR and inserted into the vector pSH1/SN-E-FV-Fvls-E (6, 15) at the Sall site to allow an in-frame fusion of the intracellular kinase domain of Tie2 (Tie2K) with the FKBP12V36 (Fv) domain from the vector.

Lentiviral infection and transient transfection. Production of the Mekk3 short interfering (si)RNA-producing lentivirus was performed as previously described (26). For lentiviral infection, 3 × 10^5 BECs were plated in a six-well plate and cultured at 37°C in 5% CO2 overnight. The overnight-cultured BECs were then centrifuged with lentivirus at a multiplicity of infection of 10 at 2,300 rpm for 90 min in the presence of 8 μg/ml polybrene. Viral supernatants were removed and replaced with 3 ml fresh DMEM plus 10% FBS containing antibiotics 6 h after centrifugation. Seventy-two hours later, MEKK3 protein levels in infected cells were determined by immunoblot analysis.

For transient transfection of mouse embryonic fibroblasts, 1 μg of pCMV-flag-p38 was cotransfected with 2 μg of pHA-Fv-Tie2K or empty vector pSH1/SN-E-FV-Fvls-E using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Transfected cells were analyzed 36 h later.

Chemically induced dimerization. Synthetic dimerizer AP20187-mediated HA-Fv-Tie2K dimerization was carried out as previously described (6, 12).

Immunoblot analysis. Mouse embryonic fibroblasts cultured in a six-well plate were starved for 12 h before being stimulated with 100 ng/ml AP20187 or vehicle control. BECs were also starved for 12 h before being stimulated with 100 ng/ml recombinant human Ang1 (R&D) for the indicated time periods. Cell lysates were prepared and analyzed by immunoblot analysis as previously described using antibodies against phosphorylated (p)-p38, p-JNK, and ERK5 (Cell Signaling Technology, Boston, MA); against p38 and JNK1 (Santa Cruz Biotechnology), against MEKK3 (BD Biosciences); and against pTyr (PY20; Transduction Laboratories). Anti-p-MEKK3 was as described in our previous study (48).

Growth of wild-type and Mekk3−/− tumors. Tumor-like wild-type and Mekk3−/− embryonic stem (ES) cells were prepared and characterized as described in Ref. 46. Tumor-like ES cells (1 × 10^6) were injected subcutaneously into nude mice, and tumor growth was monitored 3 times/wk using calipers. Tumor volume was calculated by the following formula: volume = W^2 × L/2, where W is the shortest diameter and L is the longest diameter. For each experiment, 3 mice/group were used, and statistical analysis was performed.

Microvessel density of wild-type and Mekk3−/− tumors. To determine the extent of vascularization in ES cell-derived tumors, blood vessels were stained with anti-PECAM antibody. The microvessel density was counted in multiple sections of at least three subcutaneous tumors from each cell line, and SEs were calculated.

RESULTS

Mekk3 deficiency causes blood vessel dilation and defects in endothelium-myocardium interactions. Normal angiogenesis requires not only ECs but also other cell types, such as SMCs. Proper interactions between ECs and SMCs play an important role in angiogenesis (33, 45). Since our previous study (46) suggested that MEKK3 is not involved in angiogenic growth factor production but is essential for embryonic angiogenesis, it is possible that MEKK3 may control the signaling pathways that are essential for the integrity of the vasculature and for an appropriate EC-SCM interaction. To investigate this possibility, we analyzed the cross sections of E10 wild-type and Mekk3−/− embryos by hematoxylin and eosin staining. As shown in Fig. 1, we found dilation and rupture of the dorsal aorta in E10 Mekk3−/− embryos, which were associated with local hemorrhage. In addition, the dorsal aorta c usual to the heart was also found dilated in mutant embryos (Fig. 1A, c and d).

Cell-cell interactions play a crucial role in blood vessel development, especially in angiogenesis. Thus, we examined interactions between the endocardium and myocardium in Mekk3-deficient hearts. Indeed, a dissociation of the endocardium from myocardium was found in hearts of Mekk3−/− mice...
at E10 (Fig. 1B). These results suggest that the dilation and rupture of blood vessels caused by Mekk3 disruption may due to the disability of recruitment of SMCs by ECs.

**Mekk3 deficiency causes decreases in cell proliferation.** Because MEKK3 is a major intracellular signaling molecule involved in transducing many receptor-mediated signals, it is possible that in Mekk3-deficient embryos, the upstream receptor-mediated signals were blocked, which may lead to defective cell function. A previous study (11) in cell lines suggested that MEKK3 might be involved in regulating cell proliferation. To address this question, we determined cell proliferation in wild-type and Mekk3-deficient E9.5 yolk sacs by immunohistochemical staining (using anti-PCNA) and BrdU incorporation and staining assays. As shown in Fig. 2A, cell proliferation in Mekk3−/− yolk sacs was significantly decreased compared with that in wild-type yolk sacs. To determine the percentage of proliferating ECs, multiple sections (>10 blood islands each) of wild-type and Mekk3−/− yolk sacs were counted. The percentage of BrdU-positive cells in Mekk3−/− yolk sacs was 7% compared with 51% in wild-type yolk sacs (Fig. 2B).

Mekk3 deficiency causes increased apoptosis. Blocking angiogenic or mitogenic signals, such as by endothelial growth factors withdrawal or serum starvation, induces apoptosis. Since MEKK3 is implicated in transducing angiogenic signals in ECs, it is possible that disruption of Mekk3 may render ECs more susceptible to apoptosis. Indeed, a role of MEKK3 in regulating EC apoptosis by regulating the cross-talk between PI3K/Akt and p38 signaling pathways has been suggested (17). To investigate if MEKK3 signals are required for EC survival, we analyzed EC apoptosis in wild-type and Mekk3−/− embryos. As shown in Fig. 3, we observed a significant increase in the numbers of apoptotic ECs in Mekk3−/− embryos.

Mekk3 deficiency causes defects in Ang1/Tie2 signaling. Ang1/Tie2 signaling plays a vital role in embryonic vascular development. Since deficiency of Ang1/Tie2 signaling causes similar defects as those in Mekk3-deficient mice, we decided to examine the role of MEKK3 in Ang1/Tie2 signaling in ECs. We found that Ang1 was able to induce MEKK3 phosphorylation in Mekk3−/− embryos, suggesting that MEKK3 might be involved in regulating cell proliferation. To address this question, we determined cell proliferation in wild-type and Mekk3−/− yolk sacs by immunohistochemical staining (using anti-PCNA) and BrdU incorporation and staining assays. As shown in Fig. 2A, cell proliferation in Mekk3−/− yolk sacs was significantly decreased compared with that in wild-type yolk sacs. To determine the percentage of proliferating ECs, multiple sections (>10 blood islands each) of wild-type and Mekk3−/− yolk sacs were counted. The percentage of BrdU-positive cells in Mekk3−/− yolk sacs was 7% compared with 51% in wild-type yolk sacs (Fig. 2B).

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lation at S526 (Fig. 4A), an active phosphorylation site for MEKK3 (48). This result suggested that MEKK3 is a downstream target of Ang1/Tie2. We then knocked down MEKK3 expression in BECs by siRNA and determined how it would affect Ang1-induced MAPK activation (Fig. 4, B and C). Our data showed that knockdown of MEKK3 impaired Ang1-induced p38 and ERK5 but not JNK activation (Fig. 4C).

To confirm the role of MEKK3 in the Ang1/Tie2-p38 pathway, we utilized a well-characterized chemical-induced dimerization system to induce Tie2 dimerization and activation in wild-type and Mekk3−/− cells. We expressed a fusion protein that contains the intracellular kinase domain of Tie2 fused with a Phet36Val mutant (Fv) form of PKBP12 (7). The addition of a low AP20187 concentration to cells has been shown to activate various proteins fused with the Fv domain but to have no adverse effect on the cells (6, 12). We cotransfected a Flag-p38-expressing plasmid with either the HA-Fv-Tie2K plasmid or a control empty vector into either wild-type or Mekk3−/− MEFs and then determined p38 activation in the presence or absence of AP20187. As shown in Fig. 4D, the addition of AP20187 induced HA-Fv-Tie2K phosphorylation in 5 min in both wild-type and Mekk3−/− cells, indicating that AP20187 induced Tie2 dimerization and caused Tie2 autophosphorylation and activation. Without cotransfection of HA-Fv-Tie2K, there was no p38 phosphorylation either in wild-type or Mekk3−/− cells. In the presence of HA-Fv-Tie2K, p38 was phosphorylated in 5 min in wild-type cells but not in Mekk3−/− cells.

Fig. 3. Mekk3 deficiency causes increased apoptosis. Cross sections of E9.5 embryos were analyzed by a TUNEL assay for apoptosis. Apoptotic cells were stained green. ECs were stained red using anti-PECAM antibody, and nuclei were stained blue (by 4',6-diamidino-2-phenylindole). Arrows indicate endothelial cells (ECs) in either WT or Mekk3−/− embryos. Scale bars = 10 μm.

Fig. 4. Mekk3 is required for angiopoietin-1 (Ang1)/Tie2 signaling to p38 and ERK5. A: serum-starved brain ECs (BECs) were stimulated with 100 ng/ml Ang1 in serum-free DMEM for the indicated time points. Cell lysates were prepared, and MEKK3 was immunoprecipitated (IP) using anti-MEKK3 antibody. MEKK3 phosphorylation and expression levels were analyzed by immunoblot (IB) analysis. B: knockdown of Mekk3 was performed as described in MATERIALS AND METHODS. Cell lysates from BECs infected with Mekk3 short interfering (si)RNA and control virus were analyzed by IB analysis for MEKK3 expression. C: serum-starved BECs were stimulated with 100 ng/ml Ang1 in serum-free DMEM for the indicated time points. Cell lysates were analyzed by IB analysis with the indicated antibodies. D: wild-type and Mekk3−/− mouse embryonic fibroblasts were transfected with a Flag-p38 expression vector together with HA-Fv-Tie2K plasmid or control empty vector and then serum starved for an additional 12 h. Cells were then treated with 100 nM AP20187 for the indicated time points before being lysed. Flag-p38 and HA-Fv-Tie2K were immunoprecipitated using anti-Flag and anti-HA antibody, respectively. p38 and HA-Fv-Tie2K phosphorylation were analyzed by IB analysis using anti-phosphorylated (p-)p38 antibody and PY20 antibody, respectively.
Together, these results demonstrated that MEKK3 is critical for Tie2-induced p38 activation.

Mekk3 deficiency does not affect tumor-like cell growth. Since MEKK3 plays an essential role in embryonic angiogenesis, we then asked if MEKK3 is also required for tumor growth and tumor-induced angiogenesis. We established both wild-type and Mekk3−/− tumor-like cell lines from ES cells (46). ES cells are pluripotent cells derived from early mammalian embryos and possess tumor-like properties. If large numbers of ES cells are transplanted, they produce tumors (teratomas) (39).

Under normal growth condition, both wild-type and Mekk3−/− tumor-like cell lines grew similarly in vitro (data not shown). To further determine if MEKK3 is required for tumor growth in vivo, we implanted wild-type and Mekk3-deficient tumor-like ES cells subcutaneously into nude mice. Tumor growth was monitored from implanted mice initially at day 5 and continually through day 25, and tumor sizes were analyzed. As shown in Fig. 5A, the growth rates of wild-type and Mekk3-deficient tumors were almost identical, suggesting that Mekk3 is not required for tumor growth in vivo.

Mekk3 deficiency in tumor cells does not affect tumor-induced angiogenesis. Since MEKK3 was found to be essential for early embryonic angiogenesis (46), its role in tumor angiogenesis is of particular interest due to the potential value in therapeutic control of tumor angiogenesis. To date, its role in tumor angiogenesis is still unclear. To investigate if MEKK3 is required for tumor angiogenesis, we analyzed the microvessel density in tumors derived from wild-type or Mekk3-deficient ES cells by immunohistochemical staining using an anti-PECAM antibody (Fig. 5B). Consistent with no growth retardation, we found no significant differences in the numbers of tumor blood vessels in wild-type and Mekk3-deficient tumors (Fig. 5C).

Mekk3 deficiency does not affect VEGF production. Tumor cells are capable of producing VEGF, one of the most important angiogenic growth factors functioning in tumor angiogenesis (13). Therefore, we determined if the production of this angiogenic factor was affected in Mekk3-deficient tumors. As shown in Fig. 6A, we found that the tumors developed in nude mice implanted with either wide-type or Mekk3-deficient ES cells expressed similar levels of VEGF, as determined by immunohistochemical staining. To further confirm that the protein level of VEGF was not affected by Mekk3 disruption, we also determined VEGF protein levels using immunohistochemical staining in embryos (Fig. 6B). Again, we found similar levels of VEGF in E9.5 wild-type and Mekk3-deficient embryos, consistent with our previous finding showing that Mekk3 deficiency did not affect mRNA levels of VEGF and bFGF in embryos (46). Taken together, these results suggested that MEKK3 is not involved in regulating VEGF expression and may be also not required for other blood vessel growth factor productions.

DISCUSSION

Our previous study (46) showed that MEKK3 plays an essential role in embryonic angiogenesis. In this study, we further characterized the defects in the cardiovascular system...
of Mekk3−/− embryos. We found that dorsal aortas in Mekk3−/− embryos were dilated and often leaky (Fig. 1A). These results partially explained why many of the Mekk3−/− embryos had hemorrhage. In addition, we found that the interaction between the endothelium and myocardium was disrupted in hearts of Mekk3−/− embryos (Fig. 1B), indicating a defect in the recruitment of SMCs by ECs.

Although early EC development through vasculogenesis was not affected in the absence of MEKK3, our previous study (46) indicated that there is an intrinsic defect in Mekk3−/− ECs that affects their ability to generate new vasculature even in the presence of normal levels of vascular and angiogenic factors. MEKK3 has been suggested to play a role in regulating cell proliferation (11) and apoptosis (17). In this study, we found decreased cell proliferation in Mekk3−/− ECs in vivo (Fig. 2). Interestingly, this defect seems to be EC specific, since we found Mekk3-deficient tumor cells grew at a similar rate as wild-type tumors both in vitro and in vivo (Fig. 5). Furthermore, we found increased apoptosis in Mekk3-deficient ECs (Fig. 3). These results suggest that MEKK3 may play an intrinsic role in EC proliferation and survival.

To begin understanding the molecular mechanism of MEKK3 signaling in ECs, we used siRNA to knock down MEKK3 expression in EC lines. These experiments showed that MEKK3 is required for both p38 and ERK5 MAPK activation by Ang1 (Fig. 4). In control siRNA-infected BECs, p38 activation was nicely induced by Ang1, but this induction was clearly ablated in MEKK3 knockdown BECs (Fig. 4C). Importantly, we found that activation of ERK5, a major downstream target of MEKK3 during embryonic cardiovascular development (5, 21, 32), was severely impaired in Mekk3 knockdown BECs (Fig. 4C). This result suggests that the Ang1/Tie2-MEKK3-ERK5 axis may be a major functional module in angiogenesis. Interestingly, the basal phosphorylation level of p38 was slightly augmented in Mekk3 knockdown BECs. This might be due to MEKK2 compensation, since MEKK2 shares almost identical catalytic domains with MEKK3 (6, 48). We did not observe a significant defect in JNK activation in this assay, suggesting that other MAP3Ks might be involved in JNK activation during angiogenesis. Alternatively, JNK activation by Ang1/Tie2 may not be the dominant MAPK cascade.

To further determine the role of MEKK3 in Ang1/Tie2 signaling, we asked if MEKK3 is required for Tie2, a receptor tyrosine kinase for Ang1, signaling to the downstream targets. Tie2 consists of an extracellular ligand binding domain for angiopoietins (Ang1–4) and an intracellular kinase domain for signaling (35). Following ligand Ang1 binding, Tie2 becomes dimerized and autophosphorylated on its kinase domain, which leads to the activation of downstream effectors, including MAPK cascades (30, 35). We used a chemical-inducible system to artificially activate Tie2 in either wild-type or Mekk3-deficient cells. Our results showed that MEKK3 is critical for Ang1/Tie2 signaling to the p38 MAPK pathway (Fig. 4D). Taken together, the above experiments showed that at least part of the MEKK3 signaling is mediated by p38 and ERK5 MAPKs in response to angiogenic factor Ang1 stimulation. Since both p38 (4) and ERK5 (42) MAPK pathways are involved in controlling cell proliferation and survival in response to growth factors and developmental signals, the above results also suggest that the activation of these two MAPK pathways by MEKK3 during early angiogenesis may play a critical role in EC proliferation and survival, thus partially explain the phenotype of Mekk3-deficient embryos.
Precisely how MEKK3 is involved in regulating the early cardiovasculogenesis remains largely unknown. Since MEKK3 is ubiquitously expressed, disruption of the Mekk3 gene in mice may indirectly affect blood vessel development. Future investigation with EC-specific conditional Mekk3 knockout mice is needed to formerly confirm the crucial intrinsic role of MEKK3 in ECs.

Understanding the function of MEKK3 may provide new strategies for developing drugs to control angiogenesis and perhaps tumor growth. Thus, we also investigated the role of MEKK3 in tumor growth and angiogenesis in this study. We established both wild-type and Mekk3−/− tumor-like ES cell lines and implanted them into nude mice, where tumors developed due to the tumor-like properties of ES cells. Although we found that Mekk3-deficient mouse embryonic fibroblast cells grew slower than wild-type cells under normal culture conditions (data not shown), surprisingly we did not find any difference in the growth of tumors derived from both kinds of ES cells (Fig. 5A).

Tumor angiogenesis, the formation of new blood vessels in tumor tissues, provides enough nutrition for tumor growth. Since Mekk3 deficiency in tumors did not affect tumor growth, we further examined tumor angiogenesis in wild-type and Mekk3−/− ES cell-derived tumors. Consistent with the results in tumor growth, no differences were found in tumor angiogenesis of Mekk3−/− tumors (Fig. 5, B and C). These results suggest that MEKK3 is dispensable for both tumor growth and angiogenesis. Our study also suggests that MEKK3 is not a good target in tumor cells for therapeutic treatment of cancer, although it is an important regulator of angiogenesis.

Angiogenesis requires coordinated action of a variety of growth factors, and tumor cells produce many of these factors, including VEGF, one of the most important growth factors involved in both tumor and embryonic angiogenesis (13). We found that the production of VEGF protein was similar in wild-type and Mekk3−/− tumors (Fig. 6A). We also did not find any differences in VEGF protein levels in wild-type and Mekk3−/− embryos (Fig. 6B), consistent with our previous finding at the mRNA level (46). Since the newly formed blood vessels in tumors are derived from host tissues that have the wild-type Mekk3 gene, these data suggest that both tumor types are able to produce sufficient angiogenic growth factors for new blood vessel formation.

Together, our study showed that MEKK3 is required for EC proliferation, survival, and interactions with the myocardium during early embryonic development, but is not essential for tumor growth and angiogenesis. This study also suggests that MEKK3 might be a better target for antiangiogenic therapy in ECs rather than in tumor cells.

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MEKK3 FUNCTION IN ENDOThelial CELLS

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