Maintenance of Vascular Integrity in the Embryo Requires Signaling through the Fibroblast Growth Factor Receptor*

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Basic fibroblast growth factor (FGF)-2 is important for vessel formation and/or maintenance of vascular integrity in the embryo. FGF signaling may be mediated through transmembrane tyrosine kinase receptors or directly through intracellular pathways that do not involve receptor activation. To determine the role of receptor-mediated signaling in endothelial cells, an adenovirus encoding truncated FGF receptor (FGFR)-1, under the control of the cytomegalovirus promoter, was expressed in endothelial cells. FGF signaling was impaired, as indicated by inhibition of MAPK phosphorylation. Functional consequences included inhibition of endothelial cell migration and induction of apoptosis. To address the role of endothelial FGFR signaling in vascular development, recombinant adenovirus encoding a dominant-negative FGF was injected into the sinus venosus of embryonic day 9.0 cultured mouse embryos. Previous studies demonstrated that transgenes delivered via adenovirus, under the control of the cytomegalovirus promoter, are expressed selectively in the developing vasculature. Embryos expressing a control adenovirus developed normally, whereas those expressing the FGFR-1 mutant exhibited abnormal embryonic and extra-embryonic vascular development. These data demonstrate that FGF, by signaling through the FGFR, plays a pivotal role in the development and maintenance of a mature vascular network in the embryo.

Vasculogenesis is the differentiation of vascular endothelial precursors (angioblasts) from mesoderm, followed by the formation of large vessels in the embryo and of the primitive vascular plexus in the yolk sac (1–4). Basic fibroblast growth factor (bFGF)† is important for inducing mesoderm to form angioblasts and hematopoietic cells (1, 5–8). Although direct in vivo evidence that FGFs are important for vasculogenesis in vivo has previously been lacking, re-evidence that FGFs are important for vasculogenesis in vivo and/or vascular maintenance had previously been lacking, recent work by our group demonstrated that endogenously produced FGFs are essential for the development and/or maintenance of vascular integrity during embryogenesis (9).

One mechanism by which the FGF family of growth factors mediates their biological functions is by signaling through cell-surface receptors (10–16). Evidence also exists that FGF-2 can trigger a biological effect through direct nuclear interactions (12, 17, 18). Multiple isoforms of FGF-2, generated by alternative initiation of translation, are differentially distributed within the cell. The low molecular mass isoform of FGF-2 (18 kDa) is primarily localized to the cytoplasm and acts through cell-surface receptors, whereas the high molecular mass isoforms of FGF-2 (22, 22.5, and 24 kDa) are targeted to the nucleus and may signal independently of transmembrane receptor pathways (12, 17, 19–22). Our previous study using an antisense RNA approach to inhibit the expression of one or more members of the FGF family could not determine whether the effects of FGF on vasculogenesis were mediated through intracellular signals or through interaction with FGFRs on the cell surface (9).

Strategies directed at decreasing FGF availability can assess the importance of cell-surface receptor versus nuclear signaling in regulating vascular development. Expression of a truncated FGFR lacking a cytoplasmic tyrosine kinase domain allows for the formation of nonfunctional heterodimers with wild-type endogenous receptors, thereby inhibiting receptor-mediated signal transduction (23–31). In the present study, expression of a dominant-negative FGFR in human umbilical vein endothelial cells resulted in inhibition of FGF signaling as evidenced by impairment of MAPK activation. Disruption of FGFR signaling induced endothelial cell apoptosis and inhibition of endothelial cell migration in response to FGF, but not in response to VEGF. To test the hypothesis that signaling through the FGFR is essential for normal vascular development, a mouse embryo culture model was employed (9, 32). A dominant-negative mutant of FGFR-1 was expressed in the developing endothelium of an embryonic day 9.0 mouse embryo to inhibit FGFR signaling. Overexpression of a dominant-negative FGFR in the mouse embryo disrupted embryonic and extra-embryonic vascular development and prevented the formation of a mature vascular network. Thus, signaling by FGF through its transmembrane receptor is essential for normal endothelial cell function in vitro and the development and maintenance of the embryonic vasculature in vivo.

MATERIALS AND METHODS

Construction of Recombinant Adenovirus—Replication-deficient human type 5 adenovirus containing the Escherichia coli β-galactosidase gene under the control of the cytomegalovirus immediate-early enhancer/promoter (AdCMV/βlacZ) was provided by the Institute for Human Gene Therapy of the University of Pennsylvania. A recombinant adenovirus containing a 1.1-kilobase pair fragment of the murine FGF-2 cDNA in the antisense orientation (AdCMV/antisense FGF-2) was generated as described (9). An adenoviral vector containing a dominant-negative mutant of murine FGFR-1 (AdCMV/DNFGFR) was con-
essential growth medium (Clonetics, Walkersville, MD). Cells were isolated as described previously (34) and maintained in high titer stocks were prepared by infecting human embryonic kidney VECs infected with either AdCMV/lacZ or AdCMV/DNFGFR were plated in the upper chamber (5\times10^4 cells/well) and harvested using cold lysis buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS in PBS) containing a protease inhibitor mixture (Roche Molecular Biochemicals). Cell lysates were centrifuged at 14,000 \times g for 4 °C. Samples were separated by SDS-polyacrylamide gel electrophoresis on 10% Tris/glycine gels (Novex, San Diego, CA) and electrophoretically transferred to nitrocellulose membranes. Membranes were blocked in PBS containing 5% nonfat dry milk and 0.2% Tween 20 at room temperature. Transgene expression was detected by incubating the membrane with a monoclonal antibody to c-Myc (clone 9E10, Oncogene Products) at 2 °C and blocked with 3% goat serum in PBS for 2 ha t room temperature. The sections were then counterstained with methyl green or nuclear fast red (Vector Labs, Inc.). Protein bands were quantified using a gel documentation system (Gel Doc 2000, Bio-Rad, Hercules, CA). Protein samples were subjected to SDS/PAGE, transferred onto nitrocellulose membranes, and probed with appropriate antibodies. Immunoreactive bands were visualized with chemiluminescence detection reagents (ECL, Amersham Pharmacia Biotech). To assess the presence of endogenous FGF-R1 protein, membranes were stripped and reprobed using a polyclonal antibody against a peptide sequence to the carboxyl terminus of human FGF-R1 (Santa Cruz Biotechnology, Santa Cruz, CA). To control for loading and transfer efficiency, blots were probed with a monoclonal antibody against β-actin (Sigma). Cell Proliferation—HUVECs were infected with 5\times10^3 particles/cell of either AdCMV/lacZ or AdCMV/DNFGFR for 12 h and then replated at 1.7 \times 10^5 cells/well in 6-well dishes. The cells were incubated in either essential growth medium or minimal EBM containing 2% fetal bovine serum and 25 ng/ml bFGF (Life Technologies, Inc.) was used as a control. The primers used to detect the dominant-negative and endogenous FGFRs were 5'-GTGGAGAATGAGTATGGG-3' and 5'-AGTCTTCTGAGTG-3'. PCR was performed using a forward primer (5'-TCTTCACTTGCCTTACCTGTCTGCG-3') by injection of phenol red dye into the vitelline arteries of embryos injected with AdCMV/lacZ (control), AdCMV/DNFGFR, or AdCMV/antisense PFG-2.

Polymerease Chain Reaction—To detect the expression of the dominant-negative mutant of FGF-R1 in embryos injected with AdCMV/DNFGFR, PCR amplification was performed using specific target sequences. Total RNA from AdCMV/lacZ (control), AdCMV/antisense PFG-2, and AdCMV/DNFGFR-injected embryos was isolated using a total RNA isolation kit (Promega, Madison, WI) following the manufacturer's instructions. Reverse transcription using 2 μg of total RNA was performed with Superscript II reverse transcriptase (Life Technologies, Inc.). To reverse-transcribe dominant-negative FGF-R1, a reverse primer corresponding to the c-myc epitope (5’-TAGGTCTTCTCCTACT-TATGAGCTTCCTGTCTGCG-3’) was used. A random hexamer primer (Life Technologies, Inc.) was used as a control. The primers used to detect the dominant-negative and endogenous FGF-Rs were 5’-TGGGAGAATGAGTATGGG-GTAGTGGG-3’ (forward primer) and 5’-CAGTGGTACCTGTCTGGC-3’ (reverse primer). PCR was performed using a forward primer (5’-AGTCTTCTGAGTG-3’) and a reverse primer (5’-CATCCACAGTCTTCTAGTGG-3’) for detecting glyceraldehyde-3-phosphate dehydrogenase. Samples were separated by electrophoresis on a 2% agarose gel stained with ethidium bromide.

Immunohistochemistry—Cultured embryos were collected and dissected free of extra-embryonic membranes. For whole-mount immunohistochemistry, embryos (E6.5 to E7.5) were fixed in 4% paraformaldehyde/0.1 M phosphate buffer pH 7.4 containing 0.1% sodium dodecyl sulfate and 0.1% sodium periodate for 1 h at room temperature. Embryos were washed with 5% hydrogen peroxide in methanol, and blocked in PBS (50 ml) for 30 min at room temperature. Nonspecific staining was blocked by incubation with 3% normal goat serum/PBS for 60 min. Sections were incubated overnight at 4 °C with the primary antibody (1:5000; Sigma). Embryos were washed with PBS and PBS containing 0.1% Tween 20 before incubation with the secondary antibody (1:5000; Vector Labs, Inc.). The sections were then counterstained with methyl green or nuclear fast red (Vector Labs, Inc.).

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cleared twice in 100% xylene, rehydrated through a series of ethanol

phosphorylated p42/p44 MAPK (pMAPK) for 12 h and then stimulated with 25 ng/ml bFGF (6–8 h). HUVECs were infected with adenovirus encoding

ative FGFR.

B indicates that the endogenous FGFR was expressed at similar levels in both control- and AdCMV/DNFGFR-infected cells using a polyclonal anti-FGFR antibody (Santa Cruz Biotechnology). Membranes were also hybridized with a monoclonal antibody to β-actin to normalize for loading and transfer efficiency (C).

post-fixed in 4% paraformaldehyde, rinsed three times with PBS, and embedded in paraffin; and 6-μm sections were cut. Sections were cleared twice in 100% xylene, rehydrated through a series of ethanol washes (100 to 70%), washed with distilled water, and counterstained with hematoxylin.

RESULTS

Western Blot Analysis of the Truncated FGFR—To demonstrate the expression of the c-myc-tagged truncated FGFR protein, Western blot analysis was performed. The truncated FGFR protein was detected as a doublet between 50 and 67 kDa (Fig. 1A). The expression of the dominant-negative FGFR transgene was observed to be dose-dependent when increasing titers of AdCMV/DNFGFR were used for infection. To confirm these results, a monoclonal antibody to the ectodomain of FGFR-1 (Upstate Biotechnology, Inc., Lake Placid, NY) was used to reprobe the blot and demonstrated results identical to those using the anti-c-Myc antibody (data not shown).

To detect the endogenous FGFR, the blot was reprobed with a polyclonal antibody raised against a peptide sequence to the carboxyl terminus of human FGFR-1. As shown in Fig. 1B, endogenous FGFR-1 was expressed at similar levels in both AdCMV/lacZ- and AdCMV/DNFGFR-infected cells. To normalize for loading and transfer efficiency, a monoclonal anti-β-actin antibody was used to reprobe the blots (Fig. 1C).

A Dominant-negative FGFR Interrupts MAPK Activation—To examine whether the expression of the truncated FGFR interferes with FGF signaling, cells were infected with either AdCMV/lacZ or AdCMV/DNFGFR and subsequently treated with bFGF or VEGF in the presence or absence of the MEK1 inhibitor PD98059 (Fig. 2). Treatment of control cells with bFGF or VEGF resulted in the stimulation of MAPK phosphorylation in the absence of the MEK1 inhibitor. Addition of PD98059 under the above conditions inhibited MAPK phosphorylation. In cells expressing the dominant-negative FGFR, bFGF-induced MAPK phosphorylation was effectively inhibited, whereas VEGF-induced MAPK phosphorylation was normal. These data demonstrate that the dominant-negative FGFR selectively disrupts FGFR signaling, as indicated by inhibition of MAPK activation.

A Dominant-negative FGFR Induces Cell Apoptosis and Inhibits Cell Migration—The AdCMV/lacZ-infected cells cultured in minimal EBM supplemented with FGF increased in number over time (Fig. 3). In contrast, cells expressing the mutant FGFR failed to increase in number when cultured under similar conditions and actually decreased in number with time, similar to AdCMV/lacZ-infected cells cultured in minimal medium without FGF. By microscopic examination, the cells expressing the dominant-negative FGFR were shrunken, disintegrated into small vesicles, and ultimately detached from the culture dishes. To determine whether the morphological
changes and decreased cell number were due to apoptosis, TUNEL staining was performed (Fig. 4). The proportion of TUNEL-positive cells expressing the dominant-negative FGFR was 37%, compared with 4% of control-infected cells supplemented with FGF. This was similar to β-galactosidase-expressing cells cultured without supplemented FGF. These results indicate that signaling through the FGFR is necessary for FGF-mediated cell survival.

Assays were performed to determine whether FGF signaling through cell-surface receptors induces cell migration (Fig. 5). Cell migration was stimulated by bFGF, VEGF, and serum in control cells. In contrast, expression of the dominant-negative FGFR significantly decreased bFGF-induced cell migration, whereas VEGF- or serum-induced migration was not inhibited in cells expressing the mutant FGFR.

Detection of the Dominant-negative FGFR Transgene in Vivo—To detect the expression of the transgene in embryos injected with AdCMV/DNFGFR, total RNA was isolated from embryos 22–26 h post-injection, and reverse transcription-PCR was performed (Fig. 6). AdCMV/DNFGFR viral DNA was used as a positive control. AdCMV/DNFGFR-injected embryos expressed the truncated FGFR (641 bp), whereas no transgene-specific bands were detected in the embryos injected with adenoviruses encoding lacZ or antisense FGF-2 (Fig. 6A). To detect endogenous FGFR-1, a random hexamer was used for reverse transcription. A 593-bp band corresponding to the endogenous FGFR was expressed in the samples from embryos injected with adenoviruses encoding lacZ, antisense FGF-2, and the dominant-negative FGFR (Fig. 6B). No bands were detected in samples from AdCMV/DNFGFR-injected embryos amplified in the absence of Taq polymerase. AdCMV/DNFGFR viral DNA as a positive control yielded a 593-bp band. As an internal control, reverse transcription-PCR using primers to glyceraldehyde-3-phosphate dehydrogenase was performed (Fig. 6C). These data demonstrate that injection of embryos with AdCMV/DNFGFR results in expression of the dominant-negative FGFR transgene.

Endothelial Cell-specific Expression of the Transgenes—Previous reports indicate that the expression of adenoviral vectors under the control of the cytomegalovirus enhancer and promoter is restricted to the endocardium of the heart and developing blood vessels (9, 32). Embryos injected with either AdCMV/lacZ or a mixture of AdCMV/DNFGFR plus AdCMV/lacZ (2:1 ratio) and cultured for an additional 24 h were stained for β-galactosidase-expressing cells cultured without supplemented FGF. This was similar to β-galactosidase-expressing cells cultured with supplemented FGF. These results indicate that signaling through the FGFR is necessary for FGF-mediated cell survival.

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Detection of the Dominant-negative FGFR Transgene in Vivo—To detect the expression of the transgene in embryos injected with AdCMV/DNFGFR, total RNA was isolated from embryos 22–26 h post-injection, and reverse transcription-PCR was performed (Fig. 6). AdCMV/DNFGFR viral DNA was used as a positive control. AdCMV/DNFGFR-injected embryos expressed the truncated FGFR (641 bp), whereas no transgene-specific bands were detected in the embryos injected with adenoviruses encoding lacZ or antisense FGF-2 (Fig. 6A). To
Expression of the Dominant-negative FGFR Results in Abnormal Development of the Embryo Proper—The effect of dominant-negative mutant receptor expression on development of the embryo proper was examined. AdCMV/DNFGRF or AdCMV/αcZ was injected into the sinus venosus of cultured embryonic day 9.0 mouse embryos. At the time of injection, the embryos contained 10–12 somites and exhibited open neural folds. The hearts of the embryos continued to beat, but were not completely septated. Few circulating blood cells were visible in the yolk sac vasculature, and branching of the vasculature was incomplete.

Of the AdCMV/αcZ-injected control embryos, 54 of 59 developed normally, with complete turning of the embryos observed (Table I). Microscopic observation and microangiography demonstrated normal yolk sac vascular development. In contrast, of the 78 embryos injected with AdCMV/DNFGRF, 74 showed delayed embryonic development and did not complete turn within the yolk sac. Additionally, the same 74 embryos demonstrated abnormal formation of the yolk sac vasculature. Since the previous report from our laboratory demonstrated that expression of an antisense RNA to FGF-2 inhibits vascular development (9), we compared the phenotype of embryos expressing AdCMV/antisense FGF-2 with that of embryos expressing AdCMV/DNFGRF. The abnormal phenotype observed in the AdCMV/DNFGRF-expressing embryos was similar to that of embryos injected with AdCMV/antisense FGF-2. These results indicate that expression of a dominant-negative mutant FGFR during early embryonic stages disrupts vascular development in the embryo and yolk sac and delays the proper development of the embryos.

Expression of a Dominant-negative FGFR Results in Defec-
To examine the effects of the dominant-negative FGFR on developing embryonic vasculature, whole-mount PECAM staining was performed. The control-injected embryos showed a highly branched and organized vascular network within the developing head (Fig. 9A). In the trunk region, a well formed and organized capillary network between somites was observed (Fig. 9C), and an intact endocardium (Fig. 9E) was present.

In contrast, AdCMV/DNFGFR-injected embryos exhibited a poorly developed primitive intracranial vasculature (Fig. 9B). In 50% (n = 15) of the embryos, the primary capillary plexus surrounding the forebrain was absent. The endocardium (Fig. 9D) and intersomatic vessels (Fig. 9F) in embryos expressing a dominant-negative FGFR were incompletely formed. Thus, expression of the dominant-negative FGFR disrupts vascular integrity in the developing murine embryo.

Expression of a Dominant-negative FGFR Inhibits Vascular Network Formation in the Yolk Sac—To examine the vascular organization of the developing yolk sac, microangiography was performed by injecting phenol red dye into the vitelline arteries of viable embryos. As shown in Fig. 10A, dye injected into AdCMV/lacZ-injected control embryos circulated throughout the extensive yolk sac vasculature. Furthermore, dye was observed to circulate freely between the embryo proper and the yolk sac. In contrast, the majority of AdCMV/DNFGFR-injected embryos (90%) exhibited disorganized yolk sac vascular development (Fig. 10B). Phenol red dye injected into the vitelline arteries of these embryos remained localized to the site of injection and did not circulate through either the yolk sac or the embryo proper.

Histological examination of AdCMV/lacZ- and AdCMV/DNFGFR-injected embryos was used to demonstrate abnormalities in the yolk sac vasculature. Control embryos showed a normal endothelial cell lining between visceral endoderm and mesoderm as well as clearly formed vessels containing blood cells (Fig. 10C). In the yolk sac of embryos injected with AdCMV/DNFGFR, endothelial cells were apparent; however, no intact vessels were observed (Fig. 10D). Furthermore, blood islands containing red blood cells were fewer in number com-
FIG. 10. Expression of a dominant-negative mutant of the FGFR inhibits vascular network formation. Control-injected (A) and AdCMV/DNFGFR-injected (B) embryos were cultured for 22–26 h, and then phenol red dye was injected into the vitelline arteries to display the vasculature. Branch formation of vessels on the surface of the yolk sac was observed in the AdCMV/lacZ-injected embryo (A). The yolk sac in the AdCMV/DNFGFR-injected embryo (B) demonstrates disorganized vessels and lack of branching of vessels compared with the control embryo (C). A and D show hematoxylin staining of sections of paraffin-embedded yolk sac in AdCMV/lacZ-injected (C) and AdCMV/DNFGFR-injected (D) embryos. Numerous endothelial cells and blood cells were observed throughout the yolk sac in the control embryo (C), but not in the AdCMV/DNFGFR-injected embryo (D).

DISCUSSION

The FGF ligand and cell-surface receptor families have been demonstrated to be important for inducing mesoderm to form from vascular endothelial precursors (1, 5–8, 23, 58). In contrast to the direct function of FGFs in mesoderm induction, in vivo evidence suggests that FGFs do not play a direct role in vasculogenesis or in the maintenance of the vasculature. For example, knockout mice for FGF-2 (56), FGF-3 (57), and FGF-5 (38) are viable and show no vascular defects. Other data, such as studies showing that the homoygous mutants of FGF-4 (58) and FGFR-1 (39, 40) die during early embryogenesis, are difficult to interpret since any effect these molecules might have on later stages of embryogenesis, including vascular development, would not be detectable. There are, however, some data to indicate that FGFs play a role in vasculogenesis and/or maintenance of vascular integrity. Flamme and Risau (5) have demonstrated that avian epiblasts can be induced by FGFs to form blood islands in vitro. Previous work by our group using an antisense RNA approach provided evidence that endogenously synthesized FGFs can be induced by FGFs to form blood islands in vivo. These results indicate that the nucleus-localized isoforms of FGF-2 have specific biological function (17). These observations suggest that the effects of FGF on regulating vascular development during embryogenesis may be mediated either by signaling through transmembrane tyrosine kinase receptors or by direct nuclear interactions. Our previous study using an antisense approach to inhibit endogenous production of FGF ligand could not determine whether signaling through cell-surface receptors or through direct stimulation of intracellular pathways mediates the biological effects of FGF on murine vascular development (9).

This study was designed to determine if FGF signaling through FGFRs is required for endothelial cell survival, proliferation, and migration and for normal vascular development in the mouse embryo. Altering the availability of functional FGFRs by expressing a truncated receptor lacking the cytoplasmic tyrosine kinase domain effectively reduces functional receptor availability through a dominant-negative mechanism (15, 26, 46). It has been demonstrated that truncated receptors form heterodimers with endogenous FGFRs and thereby inhibit FGFR signaling. Inhibition of FGFR signaling in Xenopus embryos using a dominant-negative approach prevents mesoderm induction (23). Transgenic mice overexpressing a dominant-negative FGFR exhibit abnormal cardiac, lung, and keratinocyte development (27–29). Recent work by our group indicated that expression of truncated FGFR-1 induces differentiation of skeletal myocytes and results in apoptosis of vascular smooth
muscle cells (30, 31). Thus, the overexpression of a dominant-negative mutant receptor can be utilized to disrupt signaling through FGFRs, allowing the role of receptor-mediated signaling in regulating biological processes to be assessed.

In this study, MAPK phosphorylation was measured to assess the ability of the dominant-negative FGFR to inhibit FGF signal transduction. In cells expressing the dominant-negative FGFR, phosphorylation of MAPK mediated by bFGF is inhibited. However, VEGF, which signals through its own receptor, activates MAPK in cells expressing the dominant-negative FGFR. In addition, the MEK1 inhibitor PD98059 blocks the phosphorylation of MAPK by both VEGF and bFGF in AdCMV/ lacZ-infected control cells. Thus, the dominant-negative FGFR selectively inhibits FGF-induced signal transduction in human vascular endothelial cells.

This study examined the effect on endothelial cells of FGF signaling through the FGFR as opposed to direct nuclear signaling. Other growth factors, such as vascular endothelial growth factor and angioptin-1, have been shown to protect endothelial cells from apoptosis (47–49). The data shown here indicate that interruption of FGF signaling results in a reduction of cell number due to apoptotic cell death, which was confirmed by morphological changes as well as by TUNEL staining. Expression of the dominant-negative FGFR also inhibits endothelial cell migration in response to bFGF, but not to VEGF or 10% serum. Therefore, signaling mediated by bFGF through its cognate receptors and the subsequent activation of MAPK prevent apoptosis and stimulate cell migration. Since other signaling pathways utilize phosphatidylinositol 3'-kinase/Akt in inhibiting apoptosis (47–49), further studies are required to elucidate the detailed signaling pathways responsible for FGF anti-apoptotic signaling.

The data presented above demonstrate that signaling through the FGFR is essential for endothelial cell function in vitro. To determine the consequence of interrupting FGF signaling in vivo, an embryonic mouse model system was utilized to address the role of the FGFRs in regulating the development and maintenance of the vasculature. Previous studies reported that the delivery of transgenes to the early mouse embryo (embryonic day 9.0) by an adenovirus carrying the cytomegalovirus promoter/enhancer results in the expression of transgenes only in endothelial cells (9, 32). In the present studies, reverse transcription-PCR analysis indicated that the mutant FGFR transgene is detected only in the embryos infected with adenovirus expressing the mutant FGFR, and not in the embryos injected with control adenoviral vectors. Immunohistochemistry confirmed that the dominant-negative FGFR transgene is expressed in the endothelium of the developing embryos. Thus, this model allows selective expression of the developing FGFR in the developing vasculature.

Embryos expressing mutant FGFRs show disorganized yolk sac vascular development. This finding was confirmed by microangiography, performed by injecting phenol red dye into yolk sac vessels. Consistent with the microscopic observations, dye injected into an embryo expressing the mutant FGFR failed to circulate throughout the yolk sac. This was in contrast to the observed dye circulation in control embryos expressing lacZ. Histological examination indicated that the vascular network is disrupted in embryos expressing mutant FGFRs. In addition, several defects in the embryonic vasculature were observed with whole-mount PECAM staining. Therefore, the data presented here provide evidence that signaling through the FGFR is essential to the maintenance of the embryonic and extra-embryonic vascular network in the embryo. This study, however, does not rule out the possibility that intracellular signaling by nuclear forms of FGF-2 also plays a role in vascular development.

The intracellular pathways that are stimulated by cell-surface FGF signaling are not completely known. Embryos lacking Ras GTPase-activating protein, a negative regulator of Ras (50), and TEL, a member of the Ets transcription factor family (51), exhibit defects in yolk sac vascular organization similar to those in embryos expressing the mutant FGFR. Since Ets transcription factors have been shown to be downstream targets of Ras signaling (52, 53) and since Ras GTPase-activating protein is a substrate for receptor tyrosine kinase (54, 55), it is possible that these molecules participate in the intracellular signaling pathway that links FGFR activation to the regulation of vascular development in the embryo.

In summary, this study provides direct evidence that FGF plays a major role in the morphogenesis of the vascular system by signaling through cell-surface receptors. Further studies are required to address the specific signaling pathways involved in mediating vascular development during early embryogenesis.

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