The Fibronectin RGD Motif Is Required for Multiple Angiogenic Events During Early Embryonic Development

Seiichiro Takahashi, Markus Moser, Eloi Montanez, Takanari Nakano, Makoto Seo, Steffen Backert, Ikuo Inoue, Takuya Awata, Sigehiro Katayama, Tsugikazu Komoda, Reinhard Fassler

Objective—Fibronectin (FN) is widely expressed during embryonic development, most prominently around developing vasculature. Studies of mice bearing an FN-null mutation have demonstrated that FN plays a role in vascular development, but the functions of the RGD motif of FN in vascular development remain unknown.

Methods and Results—Here we report that mouse embryos in which the RGD motif of FN had been replaced with an inactive RGE motif (FN<sub>RGE/RGE</sub> embryos) died as a result of vascular rupture and extensive bleeding. FN<sub>RGE/RGE</sub> embryos displayed multiple defects of angiogenesis, including failure of embryonic and yolk sac vasculature remodeling, defective placental blood vessel invasion, and defective heart development, although initial vacuolization occurred. Detailed histological examination of the embryos revealed endothelial cell sheet detachment from the underlying mesenchyme, and delayed differentiation or recruitment of vascular smooth muscle cells (VSMCs) around the heart and dorsal aorta.

Conclusions—These findings demonstrate that although FN is essential for both vasculogenesis and angiogenesis, the RGD motif plays specific roles in angiogenesis, including vascular invasion, remodeling, stabilization, and maturation. (Arterioscler Thromb Vasc Biol. 2009;29:00-00.)

Key Words: fibronectin  RGD  integrin  angiogenesis  vascular smooth muscle cells  vascular maturation  vascular stabilization

Early formation of a functioning vascular system during embryonic development is required to support the increasing metabolic demands of the growing embryo. The first components of the vasculature that undergo morphogenesis are the endothelial cells. The vasculature forms via two distinct processes: vasculogenesis and angiogenesis. Vasculogenesis involves the in situ differentiation of endothelial cells from mesoderm-derived precursors and assembly of the differentiating endothelial cells into discrete blood vessels. Angiogenesis results in formation of the primordia of the heart and large vessels as well as the rather homogeneous primary capillary network found in both the embryo and extraembryonic structures, such as the yolk sac. Angiogenesis involves the proliferation and migration of endothelial cells found in the primary vascular structures and leads both to sprouting into previously avascular organs and remodeling of the initially homogeneous capillary network to form small and large vessels. Whereas vasculogenesis occurs only during early embryogenesis, angiogenesis accompanies and is required for normal growth of both embryonic and postnatal tissues, for continuous remodeling of the adult female reproductive system, and in pathological situations such as wound healing and tumor growth.8–7

During vessel development in embryos, the endothelial cells and differentiated cells are regulated by a number of environmental cues, including growth factors such as fibroblast growth factors and vascular endothelial growth factors, cytokines, proteoglycans, extracellular adhesive glycoproteins, and also through interactions with the extracellular matrix (ECM).

Fibronectin (FN) is an element of the ECM that is prominently deposited around the developing vasculature. Experiments involving knockout of FN have shown that it is essential for vascular development, and that it is more important for vasculogenesis than for angiogenesis.8,9 In the absence of FN, no blood vessels form in the vitelline yolk sac, and no aortic endothelial cell tubes form in the embryo. On the other hand, FN-knockin embryos whose FN EIIA and EIIIB (extra fibronectin type III domain A and B, respectively) have been deleted display angiogenic defects, including vascular hemorrhage, failure of remodeling of the embryo and yolk sac vasculature, defective placental vessel invasion, and vascular smooth muscle cell (VSMC) differentiation or migration defect(s).10 These findings suggest that endothelial–FN interactions play essential roles in vascular development in vivo. Endothelial...
cell interactions with FN are mediated largely by the integrin family, and endothelial cells have been shown to express a variety of integrins (i.e., α5β1, αvβ1, αvβ3, and αvβ5), all of which are receptors for the RGD motif in FN. In a previous study, we replaced the RGD motif of FN with an inactive RGE motif in mice (FN^RGERGE embryos). The FN mutation caused 100% lethality with shortening of the posterior trunk, absence of tail bud–derived somites, and severe vascular defects. Surprisingly, however, mice with RGD inactivation showed FN assembly in the same way as wild-type mice. Studies of this knockin mouse model and cells have indicated the presence of a novel RGD-independent FN assembly pathway. In addition, we and another investigator have identified and characterized the isoDGR motif in FN-I5 as a functional novel αvβ3 integrin binding site that is capable of initiating FN matrix assembly.

However, the precise phenotype of FN^RGERGE embryos has not been reported. In the present study we demonstrated that inactivation of the RGD motif in mice results in a severe circulation defect that differs from the circulation defect seen in mice lacking FN. Our findings indicate that (1) the interaction between FN RGD and α5 integrin plays critical roles in angiogenic vascular invasion, remodeling, and stabilization, and (2) nonligation of integrins to the FN-RGD motif reduces the expression of α smooth muscle (αSMA) around the aorta and heart; (3) Moreover, the RGD motif appears to function in the differentiation or maintenance of Pax3-expressing cells that are precursors of vascular smooth muscle cells.

Materials and Methods

Ethics Statement

All experiments were approved by the Animal Research Committees of the Max Planck Institute of Biochemistry (Germany) and Saitama Medical University (Japan).

Generation of Embryos

Generation of FN^RGERGE knockin mice has been described previously. Inbred embryos and yolk sacs were dissected in PBS, embedded in paraffin, and used for immunohistochemical analysis or directly for whole-mount immunolabeling. The yolk sac and a small piece of each embryo were removed and lysed in buffer containing 50 mmol/L Tris-HCl pH 8.0, 10 mmol/L EDTA, 100 mmol/L NaCl, 0.1% SDS, and 1.2 mg/mL proteinase K. DNA was subjected to PCR with FN-specific primers under the following conditions: 94°C, 1 minute; 60°C, 1 minute 30 s; 72°C, 1 minute 30 s, for 33 cycles.

Immunohistochemistry

Samples were fixed in 4% PFA in PBS, pH 7.2, overnight, then dehydrated in a graded ethanol series, and embedded in paraffin (Paraplast X-tra, Sigma-Aldrich). Immunohistochemical staining of the sections was performed as described previously. To assess BrdU incorporation, mice were injected with BrdU (100 μg/g body weight) 1.5 hour before killing. Antibodies against the following were used for immunohistochemical staining: fibronectin (Chemicon), laminin α1 chain (provided by T. Sasaki, Max Planck Institute of Biochemistry, Martinsried, Germany), BrdU (Roche Diagnostics), Ki67 (DakoCytomation), αSMA (Sigma-Aldrich), ZO-1 (In vitrogen), CD31 (PharMingen), and endomucin. Fluorescence-conjugated secondary antibodies (goat anti-rat Cy3, goat anti-mouse Cy3, goat anti-rabbit FITC, and donkey anti-rabbit Cy3) were purchased from Jackson ImmunoResearch Laboratories, and goat anti-rabbit Alexa 488 was obtained from Sigma-Aldrich. Antibodies were diluted in accordance with the manufacturers’ recommendations.

Whole-Embryo Immunohistochemistry

Yolk sacs and embryos were fixed overnight in Dent fixative (80% methanol: 20% DMSO). The samples were treated with 6% H2O2 in methanol for 1 hour at room temperature to quench endogenous peroxidase. They were then rehydrated in PBS containing 0.1% Tween-20, incubated in antibody buffer (5% BSA in PBS) 2×1 hour, and exposed to primary antibody overnight at 4°C. After a 5- to 7-hour wash in 0.1% Tween-20 in PBS, the samples were incubated with secondary antibodies overnight at 4°C, and then washed in 0.1% Tween-20 in PBS 3×1 hour. Rat antiendothelin, anti-PECAM-1 (PharMingen), and αSMA (Sigma-Aldrich) monoclonal antibodies were used for whole-embryo immunohistochemistry.

Whole-Mount RNA In Situ Hybridization

Whole-mount in situ hybridization was performed as described previously.

Results

Vascular Remodeling Defects in FN^RGERGE Yolk Sacs and Placentas

FN-RGE homozygous embryos are characterized by shortening of the posterior trunk and absence of tail bud–derived somites, resembling the phenotype of α5 integrin–deficient mice, and begin to die on embryonic day 10.5 (E10.5). Because α5 integrin binds strongly to the RGD motif in FN, and α5 integrin–deficient mice exhibit defective circulation, we investigated whether the defect could be observed in FN^RGERGE embryos. At E10.5, pregnant female mice were injected with BrdU, and their entire litters were dissected 5 hours later. BrdU incorporation was detected by immunohistochemical staining, and in parallel a series of sections was stained for the nuclear antigen Ki67. BrdU staining was clearly positive in wild-type embryos at E10.5 (supplemental Figure I, available online at http://atvb.ahajournals.org), but there was no significant incorporation of BrdU into FN^RGERGE embryos at E10.5, suggesting either a defect in cellular proliferation or defects in placentation or yolk sac circulation, resulting in greatly reduced availability of BrdU to the embryo. Ki67 staining, however, was not significantly different from that in the wild-type embryos (supplemental Figure I). At E9.5, the BrdU and Ki67 staining patterns in FN^RGERGE embryos were basically the same as those in the wild-type mice (data not shown). These findings suggest that the defect in the circulatory system of the mutant embryos started after E9.5. We concentrated our analysis on FN^RGERGE embryos mainly at embryonic days 8.5 and 9.5, because the defects they show at this stage are not yet as severe as those at later stages, thus reducing the possibility that the circulatory defects are attributable to secondary effects of the FN mutation.

Vasculogenesis is first observed in the yolk sac. Yolk sacs from E9.5 wild-type embryos were found to contain large vitelline blood vessels, whereas FN^RGERGE embryos had pale yolk sacs that lacked clearly observable blood vessels (Figure 1A). The findings in FN-null embryos demonstrated that FN is necessary for endothelial cell attachment to the yolk sac endoderm and for blood-island formation. We examined the blood islands using CD31 antibody staining, which showed that they were composed of mesodermal and endocardial cell layers, and lined by endothelial cells in both strains (Figure 1B), indicating that vasculogenesis occurs in mutant embryos. However, in the FN^RGERGE yolks

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sac, blood islands were enlarged and less FN was deposited/retained at the endoderm–endothelial basement membrane interface (Figure 1B and supplemental Figure II). Next, we visualized the vascular network by staining for CD31 at E8.5, E9.0, and E9.5. As shown in Figure 1C, at E8.5 the organization of the endothelial cells appeared normal in the mutant, indicating that the formation of a primitive vascular plexus is unaffected. Initial angiogenesis had evidently taken place in the wild-type yolk sac at E9.0, as the primitive vascular plexus had undergone modification to form rudimentary vessels, and both small capillaries and large vitelline collecting vessels had differentiated by E9.5, whereas the vascular system of FNRGE/RGE yolk sacs remained a primitive meshwork. Bar: 200 μm. e indicates extraembryonic mesoderm; e, endoderm; m, mesoderm; m indicates extraembryonic mesoderm; m

Figure 1. Yolk sac defects in FNRGE/RGE embryos. A, Morphology of the yolk sacs at E9.5. Large vitelline blood vessels (arrowhead) are evident in the wild-type yolk sac, but not in the FNRGE/RGE mutant. B, Staining for FN and CD31 in the yolk sacs at E9.5. Arrowheads indicate the endoderm–endothelial basement membrane interface. C, Whole-mount CD31 staining of wild-type and FNRGE/RGE yolk sacs at E8.5, E9.0, and E9.5. In the wild-type yolk sacs, blood vessels have remodelled progressively to form large (arrow) and small (arrowhead) vessels, whereas the vascular system of FNRGE/RGE yolk sacs is a primitive meshwork. Bar: 10 μm. e indicates extraembryonic mesoderm; e, endoderm; m, mesoderm; m indicates extraembryonic mesoderm; m

Defects in Angiogenesis in the Mutant Embryos Themselves
Extensive defects in vascular morphogenesis were also observed in the FNRGE/RGE embryos themselves (Figure 3). Whole-mount staining for endomucin, a marker of endothelial cells, revealed a decrease in the complexity of the vascular network in the FNRGE/RGE embryos at E9.5 (Figure 3A and 3B). The cranial plexus in these embryos consisted mainly of large vessels that branched less frequently in comparison with the wild-type embryos (Figure 3C and 3D), and some large vessels appeared blunt-ended and discontinuous (arrowheads in Figure 3D). In the trunk of wild-type embryos, intersomitic blood vessels were apparent along the boundaries between adjacent somites (Figure 3E), and the intersomitic and dorsal-side vessels branched to form a highly anastomosing capillary network (arrows in Figure 3E). In contrast, in FNRGE/RGE embryos, intersomitic vessels were severely disorganized (Figure 3F), and discontinuous capillaries in somites and on the dorsal side were also observed (arrows in Figure 3F). These results suggest that initial vascularization also occurs normally in mutant embryos themselves, but that subsequent angiogenic vascular remodel-
Vascular Stability

The Fibronectin RGD Motif Regulates Vascular Stability

Defects of endothelial cell-mesenchymal contact in mutant embryos. Whole-mount endomucin immunostaining of endothelial cells at E9.5 (A through F). In the wild-type embryo (A) and at higher magnification (C and E), the blood vessels can be seen to have remodeled to form clearly branched vessels, but in the FNRGE/RGE embryo (B) and at higher magnification (D and F) abnormal wide irregular vessels are evident. Note the blunt-ended vessels in the mutant (arrowheads in D). Arrows in E and F point to small branching vessels that are less prevalent in the mutant embryo. Bar=200 μm.

Defects of blood vessel remodeling in FNRGE/RGE embryos. Whole-mount endomucin immunostaining of endothelial cells at E9.5 (A through F). In the wild-type embryo (A) and at higher magnification (C and E), the blood vessels can be seen to have remodeled to form clearly branched vessels, but in the FNRGE/RGE embryo (B) and at higher magnification (D and F) abnormal wide irregular vessels are evident. Note the blunt-ended vessels in the mutant (arrowheads in D). Arrows in E and F point to small branching vessels that are less prevalent in the mutant embryo. Bar=200 μm.

Because recruitment of pericytes and smooth muscle cells to the walls of nascent blood vessels affects vessel maturation and stabilization, we investigated the expression of α smooth muscle actin (αSMA) around the dorsal aorta at E9.5 and E10.5 (Figure 5). Most of the αSMA-expressing cells in the wild-type embryos started to surround the dorsal aorta on E9.5, and they had completely surrounded it by E10.5. However, the level of αSMA expression around the dorsal aorta in FNRGE/RGE embryos was clearly lower at E9.5 in comparison with wild-type embryos (Figure 5 and supplemental Figure VIII). Interestingly, αSMA staining in the myotome of somites was also reduced in the FNRGE/RGE embryos (arrows in Figure 5 and arrowheads in supplemental Figure VI). At E10.5 most of the dorsal aorta of the mutant embryo was surrounded by αSMA-expressing cells, but some of these cells had not reached the vessel. Abnormal αSMA staining was also observed in the heart. Examination of frontal heart sections from E9.5 wild-type and FNRGE/RGE embryos showed that FNRGE/RGE embryos at E9.5 had formed ventricles and atria and expressed αSMA with an apparently normal pattern similar to that in the wild-type embryos (supplemental Figures VI and VII). However, the myocardial layer appeared thinner and less complex in FNRGE/RGE embryos (supplemental Figure VII). These findings suggest a delay in recruitment or differentiation of these cells in the mutant embryos.

Because it has been reported that the αSMA-expressing cells of the dorsal aorta and myotome are derived from the same
paraxial mesodermal precursors that transiently express Pax3 and that these precursors migrate toward the dorsal aorta from the paraxial mesoderm.\(^{14}\) Pax3 is initially expressed in the presomitotic mesoderm (psm) just before somitogenesis and later in dermamyotomes and in the somites. Hence, Pax3 in FN\(^{RGE/RGE}\) embryos is expressed appropriately, as seen in wild type in Figure 6. However, the level of expression in the mutants was not coordinated along the midline and, consequently, was uncoordinated and asymmetrical across the midline, in comparison with the wild type. These results indicate that in FN\(^{RGE/RGE}\) embryos, differentiation or maintenance of differentiated Pax3-expressing cells is adversely affected, and that the defect causes a delay in the recruitment or differentiation of \(\alpha\)SMA-expressing cells around the heart and dorsal aorta.

**Discussion**

FN\(^{RGE/RGE}\) embryos are characterized by multiple defects of angiogenesis in the yolk sac, placenta, and heart. The dorsal aorta of mutant embryos frequently ruptures, leading to leakage of primitive blood vessels into the extraembryonic space, as a result of the unstable attachment of endothelial cell sheets to the underlying mesenchyme. The level of \(\alpha\)SMA expression around the aorta in mutant was apparently less, thus also affecting vascular stability as well as maturation (Figure 5). These results clearly indicated that nonligation of integrins to the FN-RGD motif reduces the tolerance of blood vessels to mechanical stress. We also demonstrated that differentiation or maintenance of Pax3-expressing cells is affected in mutant embryos. This might be the reason for the downregulation of aortic \(\alpha\)SMA expression (supplemental Figure VIII). On the basis of all the above findings, we concluded that FN RGD is required for multiple angiogenic events, including vascular invasion, remodeling, stabilization, and maturation during early embryonic development.

Because of the high level of expression of FN around blood vessels and the role of \(\alpha 5\beta 1\) integrin, which strongly binds to the RGD motif in FN, in angiogenesis during early embryonic development,\(^{17,18}\) we expected that binding of the RGD motif to \(\alpha 5\beta 1\) would be essential for angiogenesis, and our detailed examination of the mutants provided the first evidence that this was indeed the case. At least 8 integrins have been reported to bind to the FN RGD motif, including \(\alpha\)V\(\beta\)1, \(\alpha\)V\(\beta\)3, \(\alpha\)V\(\beta\)5, \(\alpha\)V\(\beta\)6, and \(\alpha\)IIb\(\beta\)3.\(^{11}\) Knockout studies of the \(\alpha\) chain, except for the \(\alpha 5\) chain, have shown that each of the null mutations results in a much less severe phenotype than the FN RGD>\(\beta\)G mutation dose (supplemental Figure IX). Ablation of integrin \(\alpha 5\), on the other hand, results in both extensive vascular defects and early embryonic lethality.\(^{17}\) In embryos lacking integrin \(\alpha 5\), greatly distended blood vessels are seen in the yolk sac and in the embryo itself. Additionally, overall blood vessel pattern complexity is reduced in \(\alpha 5\)-null tissues.\(^{18}\) This phenotype is strikingly similar to that observed in the FN\(^{RGE/RGE}\) embryos. \(\alpha 5\)-null mutations in mice also showed that the absence of \(\alpha 5\) affects somitegenesis.\(^{23}\) The somite in the \(\alpha 5\)-null mutations showed misexpression of Pax3, as was the case in FN\(^{RGE/RGE}\) embryos. The reduced smooth muscle cell coverage of the dorsal aorta, which was seen in FN\(^{RGE/RGE}\) embryos, was also evident in \(\alpha 5\)-null embryos, although this was not examined in detail. \(\alpha 5\beta 1\) integrin binds to ADAM15,17,19 (a disintegrin and metalloproteinase) and collagen XVIII as well as the RGD motif of FN.\(^{24}\) Analysis of each of the null mutations
has shown that these proteins, except for the FN RGD>RGE mutation, are clearly not required for early embryonic development.25,26,27,28 Thus, among these proteins, interaction of integrin α5 with the FN RGD motif is uniquely essential for angiogenesis during early embryonic development (supplemental Figure IV).

We have previously reported that the FN fibers observed in mutant embryos are assembled via interaction between the isoDGR (isoAsp-Gly-Arg) motif in FN-I and αvβ3 integrin in vitro. These mutant FN fibers may function in vasculogenesis in vivo, because initial vasculogenesis occurs normally in FN RGER>GRG embryos, whereas the FN-null mutation results in severely defective vasculogenesis. It would be interesting to generate a mutant mouse strain in which the isoDGR motif in FN-I is knocked in.

Finally, we will discuss the function of FN RGD-integrin interaction as a regulator of apoptosis. Several studies have shown that RGD-based peptides inhibit angiogenesis and induce endothelial cell apoptosis in vivo. There are 3 distinct models: One is the anoikis model,29,30 whereby cells start to undergo apoptosis when they lose their integrin-mediated adhesion. In the second model, RGD-based peptide induces apoptosis by directly activating caspases, without any involvement of integrins,31 and the third model suggests that unligated integrins promote apoptosis by directly recruiting caspase-8. Our present findings appear to support the second model, because inactivation of the FN RGD motif does not induce apoptosis in vivo. Another possibility is that the interaction between isoDGR in FN and αvβ3 integrin, which is inhibited by RGD-based peptide, functions to protect apoptosis.

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Disclosures

None.

References

16. Francis SE, Goh KL, Haldvall-Davidsen CL, Hiemstra P, M. Development DGR embryos, whereas the FN-null mutation results in severely defective vasculogenesis. It would be interesting to generate a mutant mouse strain in which the isoDGR motif in FN-I is knocked in.

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Editorial Expression of Concern

On August 27, 2009, we published ahead of print a study by Seiichiro Takahashi, Markus Moser, Eloi Montanez, Takanari Nakano, Makoto Seo, Steffen Backert, Ikuo Inoue, Takuya Awata, Sigehiro Katayama, Tsugikazu Komoda, and Reinhard Fässler (The fibronectin RGD motif is required for multiple angiogenic events during early embryonic development. Arterioscler Thromb Vasc Biol. 2009 August 27 Epub ahead of print; DOI: 10.1161/ATVBAHA.108.181164). It has come to our attention that there is an allegation of mismanagement of coauthorship. An investigation is under way, and appropriate action will be taken on completion.
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Seiichiro Takahashi, Markus Moser, Eloi Montanez, Takanari Nakano, Makoto Seo, Steffen Backert, Ikuo Inoue, Takuya Awata, Sigehiro Katayama, Tsugikazu Komoda, Reinhard Fässler

Retraction

For the paper by Seiichiro Takahashi, Markus Moser, Eloi Montanez, Takanari Nakano, Makoto Seo, Steffen Backert, Ikuo Inoue, Takuya Awata, Sigehiro Katayama, Tsugikazu Komoda, and Reinhard Fässler (The fibronectin RGD motif is required for multiple angiogenic events during early embryonic development. *Arterioscler Thromb Vasc Biol.* 2009 August 27 [Epub ahead of print]; DOI: 10.1161/ATVBAHA.108.181164), after an investigation by the Saitama Medical University Internal Investigation Committee, the Committee concluded that it was unethical for Dr. Takahashi to publish this paper for the following reasons:

1. Dr. Takahashi admitted that he forged the signatures of coauthors Markus Moser, Eloi Montanez, and Reinhard Fässler that appeared on the Authorship Responsibility and Copyright Transfer Agreement.
2. Signatures of the other coauthors were made by themselves, not by Dr. Takahashi.
3. None of the Japanese coauthors knew before publication that the signatures of Dr. Moser, Dr. Montanez, and Dr. Fässler were forged by Dr. Takahashi.
4. None of the coauthors advised Dr. Takahashi that the signatures of Dr. Moser, Dr. Montanez, and Dr. Fässler should be faked by Dr. Takahashi.
5. Neither Dr. Moser, Dr. Montanez, nor Dr. Fässler read the submitted version of original manuscript.
6. Some of the Japanese coauthors did not check the boxes that appear on 1-E of the Authorship Responsibility and Copyright Transfer Agreement. In such cases, Dr. Takahashi did that instead, without permission.
7. Dr. Takahashi sent an e-mail message to Dr. Moser, Dr. Montanez, and Dr. Fässler, using fake e-mail addresses, to pretend that the German coauthors knew about the paper. Some of the Japanese coauthors believed this message and signed the Authorship Responsibility and Copyright Transfer Agreement.

The editors, therefore, hereby retract the paper.