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 Fässler

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^{RGE/RGE} embryos) died as a result of vascular rupture and extensive bleeding. FN^{RGE/RGE} 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 Vas Biol. 2009;29:00-00.)

Key Words: fibronectin ▪ RGD ▪ integrin ▪ angiogenesis ▪ vascular smooth muscle cell ▪ vascular maturation ▫ vascular remodeling ▫ vascular rupture ▫ vascular invasion ▪ vascular remodeling ▪ vascular maturation

During vessel development in embryos, the endothelial precursor 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 EIIIA 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

Received November 24, 2008; revision accepted July 20, 2009.
From the Department of Molecular Medicine (S.T., M.M., E.M., R.F.), Max Planck Institute of Biochemistry, Martinsried, Germany; the Department of Biochemistry (T.N., M.S., T.K.) and the Department of Diabetes and Endocrinology (S.T., I.I., T.A., S.K.), Saitama Medical University, Japan; and University College Dublin, School of Biomolecular and Biomedical Science (S.B.), Ireland.
Correspondence to Seiichiro Takahashi, PhD, Department of Diabetes and Endocrinology, Saitama Medical University, 38 Morohongo, Moroyama, Iruma-gun, Saitama 350-0495, Japan. E-mail se_takah@saitama-med.ac.jp
© 2009 American Heart Association, Inc.

Arterioscler Thromb Vas Biol is available at http://atvb.ahajournals.org DOI: 10.1161/ATVBAHA.108.181164
cell interactions with FN are mediated largely by the integrin family, and endothelial cells have been shown to express a variety of integrins (ie, 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^{RGE/RGE} 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 iso-DGR 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^{RGE/RGE} 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 Medical Faculty, University of Tokyo (Japan) and Saitama Medical School (Japan).

#### Generation of Embryos

Generation of FN^{RGE/RGE} knockin mice has been described previously. FN^{RGE/RGE} embryos 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, 50 mmol/L Tris-HCl pH 8.0, 10 mmol/L EDTA, 100 mmol/L NaCl, and 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 seconds, 30 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 (Roche) 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, Germany), BrdU (Roche Diagnostics), Ki67 (DakoCytomation), αSMA (Sigma-Aldrich), ZO-1 (Invitrogen), 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 antianti-mouse, antiPECAM-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^{RGE/RGE} 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 defects in vascular circulation, we investigated whether the defect would be observed in FN^{RGE/RGE} 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 was obviously positive in wild-type embryos (supplemental Figure I). At E9.5, the BrdU and Ki67 staining patterns in FN^{RGE/RGE} 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^{RGE/RGE} 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.

Vascularogenesis 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^{RGE/RGE} 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 endodermal cell layers, and lined by endothelial cells in both strains (Figure 1B), indicating that vascularogenesis does occur in mutant embryos. However, in the FN^{RGE/RGE} yolk
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 vasculature of FNRGE/RGE yolk sacs was a primitive meshwork.10 FN deposition at the extraembryonic mesoderm; e, endoderm; de, extraembryonic mesoderm.

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,15 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 anastomosed 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 remod-
Figure 3. 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.

The Fibronectin RGD Motif Regulates Vascular Stability

Next, we closely examined the structure of vessels in mutant embryos. In the wild type, endothelial cells are closely surrounded by mesenchymal cells, at which large amount of FN deposition is evident (Figure 4A and 4C). In the mutant, endothelial sheets of dorsal aorta and umbilical vein are detached from the mesenchymal tissues with FN or without FN (arrowheads in Figure 4B and 4D). Endothelial cells are missing and the endothelial sheet has clearly collapsed, leading to leakage of primitive blood cells into the extraembryonic space (asterisks in Figure 4B and supplemental Figure IVAb). The overall level of CD31 expression (supplemental Figure VIII) did not appear to be decreased, suggesting that the number of endothelial cells in the mutant embryos was not decreased. In a previous study we had demonstrated that death of tail-bud–derived mesodermal cells attributable to apoptosis started from E9.512 and therefore this blood vessel detachment may result from the apoptosis of endothelial cells. However, TUNEL analysis showed no apoptosis in endothelial cells (supplemental Figure IVB), indicating that this blood vessel detachment in mutant embryos was not the result of endothelial cell death (arrowhead in supplemental Figure IVB).

No clear apoptosis was detected in the yolk sac or placental endothelial cells in mutant embryos (data not shown). Tight junctions were established between endothelial cells in mutant embryos (supplemental Figure VA), indicating normal contact between endothelial cells. Stains of laminin γ1 (supplemental Figure VII) and collagen IV staining (data not shown) suggested no abnormalities in the basement membrane. However, PCNA staining revealed normal expression of CD31, laminin γ1, and Zo1, thus the mutant embryo (supplemental Figure VIII). These results indicate that the RGD motif in FN is probably necessary for stable attachment of endothelial cells to the mesoderm, but not for endothelial cell proliferation and survival, organization of the endothelial sheet, or basement membrane formation.

Because recruitment of pericytes and smooth muscle cells to the walls of nascent blood vessels affects vessel maturation and stabilization,22 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 front heart sections from E9.5 wild-type and FNRGE/RGE embryos showed that FN in FNRGE/RGE embryos at E9.5 had formed thicker and more complex FN layer around the heart than in wild-type embryos (Figure VI). 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...
Figure 5. Defects in maturation of blood vessels. E9.5 and E10.5 embryos were stained for endomucin (green) and α-smooth muscle actin (αSMA) (red). SMA staining around the dorsal aorta and in the myotome of somites is reduced in the FN<sup>RGE/RGE</sup> embryos at E9.5. Bar=20 μm. da indicates dorsal aorta; m, myotome; s, somite.

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 presomitic mesoderm (psm) just before somite formation and later in dermamyotomes and in the presomitic mesoderm (psm) and somites. In normal embryos, αSMA expression around the midline and in the somites and midline, in comparison with the wild-type results, indicates that in FN<sup>RGE/RGE</sup> 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 αSMA-expressing cells around the heart and dorsal aorta.

Discussion

FN<sup>RGE/RGE</sup> 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 cells into the extraembryonic space, as a result of the unstable attachment of endothelial cell sheets to the underlying mesenchyme. The level of αSMA 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 αSMA expression (supplemental Figure VIII). On the basis of all the above findings, we concluded that FN RGD is required for multiple angiogenic processes, 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 α5β1 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 α5β1 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, in angiogenesis during early embryonic development,17,18 including vascular invasion, remodeling, stabilization, and maturation during early embryonic development.

Figure 6. Whole-mount RNA in situ expression of Pax3. (A and B) are whole-mount in situ expression of Pax3 (A and B) at E9.0. Expression of Pax3 in the heart (arrowheads in A and B indicate somite pairs with Pax3 expression) is reduced in the FN<sup>5-null</sup> embryos (C and D) of wild-type and FN<sup>RGE/RGE</sup> embryos at E9.0. Expression of Pax3 in the heart was evident in the presomitic mesoderm, dermamyotome, and dorsal neural tube, as in the wild-type, but in the uncoordinated and asymmetrical (Arrowheads in A and B indicate somite pairs with Pax3 expression) E9.0; r=100 μm. dm indicates dermamyotome; n, neural tube; psm, presomitic mesoderm.

Because of the high level of expression of FN around blood vessels and the role of α5β1 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 α5β1 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 α5β1, α5β1, α8β1, α8β1, αβ3, αβ3, αβ6, and αIIbβ3.11 Knockout studies of the α chain, except for the α5 chain, have shown that each of the null mutations results in a much less severe phenotype than the FN RGD<sup>+</sup> mutation dose (supplemental Figure IX). Ablation of integrin α5, on the other hand, results in both extensive vascular defects and early embryonic lethality.17 In embryos lacking integrin α5, greatly distended blood vessels are seen in the yolk sac and in the embryo itself. Additionally, overall blood vessel pattern complexity is reduced in α5-null tissues.18 This phenotype is strikingly similar to that observed in the FN<sup>RGE/RGE</sup> embryos. α5-null mutations in mice also showed that the absence of α5 affects somite genesis.23 The somite in the α5-null mutations showed misexpression of Pax3, as was the case in FN<sup>RGE/RGE</sup> embryos. The reduced smooth muscle cell coverage of the dorsal aorta, which was seen in FN<sup>RGE/RGE</sup> embryos, was also evident in α5-null embryos, although this was not examined in detail. α5β1 integrin binds to ADAM15,17,19 (a disintegrin and metalloprotease) and collagen XVIII as well as the RGD motif of FN.24 Analysis of each of the null mutations

Discussion

FN<sup>RGE/RGE</sup> 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 cells into the extraembryonic space, as a result of the unstable attachment of endothelial cell sheets to the underlying mesenchyme. The level of αSMA expression around the midline and in the somites and midline, in comparison with the wild-type results, indicates that in FN<sup>RGE/RGE</sup> embryos, differentiation or maintenance of differentiated Pax3-

Discussion

FN<sup>RGE/RGE</sup> 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 cells into the extraembryonic space, as a result of the unstable attachment of endothelial cell sheets to the underlying mesenchyme. The level of αSMA expression around the midline and in the somites and midline, in comparison with the wild-type results, indicates that in FN<sup>RGE/RGE</sup> 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 αSMA-expressing cells around the heart and dorsal aorta.

Discussion

FN<sup>RGE/RGE</sup> 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 cells into the extraembryonic space, as a result of the unstable attachment of endothelial cell sheets to the underlying mesenchyme. The level of αSMA expression around the midline and in the somites and midline, in comparison with the wild-type results, indicates that in FN<sup>RGE/RGE</sup> 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 αSMA-expressing cells around the heart and dorsal aorta.

Discussion

FN<sup>RGE/RGE</sup> 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 cells into the extraembryonic space, as a result of the unstable attachment of endothelial cell sheets to the underlying mesenchyme. The level of αSMA expression around the midline and in the somites and midline, in comparison with the wild-type results, indicates that in FN<sup>RGE/RGE</sup> 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 αSMA-expressing cells around the heart and dorsal aorta.

Discussion

FN<sup>RGE/RGE</sup> 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 cells into the extraembryonic space, as a result of the unstable attachment of endothelial cell sheets to the underlying mesenchyme. The level of αSMA expression around the midline and in the somites and midline, in comparison with the wild-type results, indicates that in FN<sup>RGE/RGE</sup> 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 αSMA-expressing cells around the heart and dorsal aorta.

Discussion

FN<sup>RGE/RGE</sup> 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 cells into the extraembryonic space, as a result of the unstable attachment of endothelial cell sheets to the underlying mesenchyme. The level of αSMA expression around the midline and in the somites and midline, in comparison with the wild-type results, indicates that in FN<sup>RGE/RGE</sup> 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 αSMA-expressing cells around the heart and dorsal aorta.

Discussion

FN<sup>RGE/RGE</sup> 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 cells into the extraembryonic space, as a result of the unstable attachment of endothelial cell sheets to the underlying mesenchyme. The level of αSMA expression around the midline and in the somites and midline, in comparison with the wild-type results, indicates that in FN<sup>RGE/RGE</sup> 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 αSMA-expressing cells around the heart and dorsal aorta.

Discussion

FN<sup>RGE/RGE</sup> 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 cells into the extraembryonic space, as a result of the unstable attachment of endothelial cell sheets to the underlying mesenchyme. The level of αSMA expression around the midline and in the somites and midline, in comparison with the wild-type results, indicates that in FN<sup>RGE/RGE</sup> 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 αSMA-expressing cells around the heart and dorsal aorta.

Discussion

FN<sup>RGE/RGE</sup> 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 cells into the extraembryonic space, as a result of the unstable attachment of endothelial cell sheets to the underlying mesenchyme. The level of αSMA expression around the midline and in the somites and midline, in comparison with the wild-type results, indicates that in FN<sup>RGE/RGE</sup> 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 αSMA-expressing cells around the heart and dorsal aorta.
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-I5 and αvβ3 integrin in vitro. These mutant FN fibers may function in vasculogenesis in vivo, because initial vasculogenesis occurs normally in FN\textsuperscript{RGE>GERG} 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-I5 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, 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, 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 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.

Acknowledgments

We thank Dr. Takahashi and Dr. Douglas for careful reading of the manuscript.

Sources of Funding

The work was supported by the Deutsche Forschungsgemeinschaft and the Max Planck Society.

Disclosures

None.

References

The Fibronectin RGD Motif Is Required for Multiple Angiogenic Events During Early Embryonic Development
Seiichiro Takahashi, Markus Moser, Éloi Montanez, Takanari Nakano, Makoto Seo, Steffen Backert, Ikuo Inoue, Takuya Awata, Sigehiro Katayama, Tsugikazu Komoda and Reinhard Fässler

Arterioscler Thromb Vasc Biol. published online August 27, 2009;
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2009 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/early/2009/08/27/ATVBAHA.108.181164.citation

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2009/09/03/ATVBAHA.108.181164.DC2
http://atvb.ahajournals.org/content/suppl/2009/09/11/ATVBAHA.108.181164.DC3

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
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.
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, Sigeiho 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, Sigeiho 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.