 Basement membrane assembly of the integrin α8β1 ligand nephroneectin requires Fraser syndrome–associated proteins

Daiji Kiyozumi,1 Makiko Takeichi,1 Itsuko Nakano,1 Yuya Sato,1 Tomohiko Fukuda,2 and Kiyotoshi Sekiguchi1

1Laboratory of Extracellular Matrix Biochemistry, Institute for Protein Research, Osaka University, Suita, Osaka 565-0871, Japan
2Sekiguchi Biomatrix Signaling Project, Japan Science and Technology Agency, Aichi Medical University, Nagakute, Aichi 480-1195, Japan

Introduction

Fraser syndrome (FS) is a rare autosomal recessive multiorgan disorder characterized by cryptophthalmos, syndactyly, renal agenesis, and a variety of morphogenetic defects (Slavotinek and Tifft, 2002). Approximately 45% of human cases are stillborn or die within the first year, primarily from pulmonary and/or renal complications (Boyd et al., 1988). Based on the phenotypic similarities, a group of mutant mice, called blebbing mice, are considered to be animal models of human FS (Darling and Gossler, 1994). Blebbing mice are characterized by epidermal blistering, classified as dystrophic epidermolysis bullosa, during the embryonic period and exhibit FS-like developmental defects, including cryptophthalmos, syndactyly, and renal agenesis (Darling and Gossler, 1994).

The molecular basis of FS had long been unknown until causative mutations were identified in FS patients and blebbing mice. Four genes encoding Fras1, Frem2, QBRICK (also named Frem1), and Grip1 have been found to be disrupted in FS patients and/or blebbing mice (Bladt et al., 2002; McGregor et al., 2003; Vrontou et al., 2003; Smyth et al., 2004; Takamiya et al., 2004; Jadeja et al., 2005; Timmer et al., 2005; Kiyozumi et al., 2006; Alazami et al., 2009). Among these gene products, QBRICK, Fras1, and Frem2 are members of a novel family of ECM proteins characterized by 12 consecutive CSPG repeats and a varying number of Calx-β domains. Fras1 and Frem2 are produced by epithelial cells as membrane proteins with a short C-terminal tail containing a PDZ domain-binding motif (McGregor et al., 2003; Vrontou et al., 2003; Jadeja et al., 2005; Timmer et al., 2005) and secreted after proteolytic processing near the juxtamembrane region (Kiyozumi et al., 2006; Carney et al., 2010). In contrast, QBRICK is produced by mesenchymal cells as a secreted protein (Smyth et al., 2004; Kiyozumi et al., 2006). These three proteins meet together at the basement membrane (BM) zone between the epithelial and mesenchymal tissues and reciprocally stabilize one another by forming a ternary complex. When any one of these proteins is lost or truncated, the other proteins fail to

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defects associated with FS may result from loss of QBRICK from BMs as an integrin \( \alpha_8\beta_1 \) ligand. In this study, we investigated the role of QBRICK in the pathogenesis of FS by addressing its potential role as an integrin \( \alpha_8\beta_1 \) ligand at embryonic BMs. Our data indicate that ablation of QBRICK expression significantly reduces the ability of BMs to interact with integrin \( \alpha_8\beta_1 \), despite the fact that QBRICK does not account per se for the reduced integrin \( \alpha_8\beta_1 \) binding. Instead, QBRICK is necessary for the BM assembly of nephronectin, another integrin \( \alpha_8\beta_1 \) ligand critical for renal development. Our findings shed light on the hitherto unknown mechanism that defines the ability of BMs to interact with integrin \( \alpha_8\beta_1 \) through the coordinated assembly of RGD-containing integrin ligands beneath the embryonic BMs.

**Results**

QBRICK is necessary for embryonic BMs to interact with integrin \( \alpha_8\beta_1 \)

To examine whether loss of QBRICK affects the ability of BMs to interact with integrin \( \alpha_8\beta_1 \), we performed in situ integrin binding assays in which recombinant soluble forms of integrins were incubated with frozen sections of mouse embryos to visualize the integrin binding activities in tissues. Recombinant integrin \( \alpha_8\beta_1 \) bound to various organs in E15.5 embryos, including the skin, lung, and choroid plexus, where the bound integrins stably localize at BMs (Kiyozumi et al., 2006). Grip1 is an intracellular adaptor protein that contains multiple PDZ domains, with which it binds to Fras1 and Frem2 (Takamiya et al., 2004). Dysfunction of Grip1 impairs the secretion of Fras1 (Takamiya et al., 2004) and results in the breakdown of the ternary complex between the three FS-associated proteins (Kiyozumi et al., 2006).

Although the reciprocal stabilization of QBRICK, Fras1, and Frem2 is apparently indispensable, it remains elusive how these molecules operate at BMs to regulate organ development. Little is known about the biochemical functions of QBRICK, Fras1, and Frem2, except that QBRICK has an Arg-Gly-Asp (RGD) integrin-binding motif and is capable of mediating cell adhesion that is dependent on RGD-binding integrins such as \( \alpha_8\beta_1 \) (Kiyozumi et al., 2005). It has been shown that integrin \( \alpha_8\beta_1 \) ligands are expressed at embryonic BMs and play important roles in organogenetic processes involving epithelial–mesenchymal interactions. For example, nephronectin, a BM protein that binds to integrin \( \alpha_8\beta_1 \), has been shown to play a pivotal role in kidney morphogenesis, as nephronectin-deficient mice frequently exhibit renal agenesis or hypoplasia (Linton et al., 2007). Mice deficient in integrin \( \alpha_8\beta_1 \) expression also display severe defects in kidney morphogenesis (Müller et al., 1997). Given that renal agenesis is one of the typical phenotypes of FS and its model animals and that QBRICK is an RGD-containing ligand for integrin \( \alpha_8\beta_1 \), some of the developmental defects associated with FS may result from loss of QBRICK from BMs as an integrin \( \alpha_8\beta_1 \) ligand.

In this study, we investigated the role of QBRICK in the pathogenesis of FS by addressing its potential role as an integrin \( \alpha_8\beta_1 \) ligand at embryonic BMs. Our data indicate that ablation of QBRICK expression significantly reduces the ability of BMs to interact with integrin \( \alpha_8\beta_1 \), despite the fact that QBRICK does not account per se for the reduced integrin \( \alpha_8\beta_1 \) binding. Instead, QBRICK is necessary for the BM assembly of nephronectin, another integrin \( \alpha_8\beta_1 \) ligand critical for renal development. Our findings shed light on the hitherto unknown mechanism that defines the ability of BMs to interact with integrin \( \alpha_8\beta_1 \) through the coordinated assembly of RGD-containing integrin ligands beneath the embryonic BMs.
were localized at BMs and colocalized with immunoreactivity for laminin-γ1 (Fig. 1, A–C). The binding of integrin α8β1 in situ was abolished by adding EDTA or GRGDSP peptide, but not by adding GRGESP peptide (unpublished data), confirming that the binding was divalent ion–dependent and therefore intrinsic to the ligand-binding activity of integrin α8β1. In Qbrick−/− embryos, however, the integrin α8β1 binding to BMs was greatly reduced compared with wild-type littermates (Fig. 1, A–C).

To clarify whether the impaired integrin binding was specific to the α8β1 isoform, we performed in situ binding assays with integrin α3β1, which binds to laminin-511 and laminin-332, the major laminin isoforms of epidermal BMs (Nishiuichi et al., 2006). Integrin α3β1 bound to the BMs of both wild-type and Qbrick−/− embryos, and showed no significant difference between them (Fig. 1 D). Similarly, as shown in Fig. 1 (E and F), the BMs in Qbrick−/− embryos remained unaffected with respect to their abilities to bind integrins α6β4 and α7X2β1, other laminin-binding integrins with distinct ligand specificities (Nishiuichi et al., 2006). These findings indicate that the integrin α8β1 binding ability is specifically deprived from BMs by ablation of QBRICK.

Next, we investigated whether the impaired binding of integrin α8β1 coincides with the attenuated expression of QBRICK in other FS model animals. We investigated Grip1 mutant Grip1−/− and Frem2 mutant Frem2−/− mice, both of which exhibit attenuated expression of QBRICK at BMs (Kiyozumi et al., 2006). In both types of mutant embryos, the binding of integrin α8β1 to BMs was significantly reduced in the epidermis (Fig. 1, G and H), which is consistent with the diminished expression of QBRICK at BMs (Fig. 1, G and H). These findings indicate that the impaired ability of BMs to bind integrin α8β1 coincides with the attenuated expression of QBRICK irrespective of the causative mutation.

Although integrin α8β1 is broadly expressed, it has a critical function in kidney development (Müller et al., 1997). Because renal dysmorphogenesis is one of the developmental defects observed in FS animals (Fig. 2 A; Darling and Gossler, 1994), we reexamined the occurrence of renal dysmorphogenesis in Qbrick−/− mice, and found that >90% of Qbrick−/− mice exhibited renal defects; i.e., 10 unilateral renal agenesis and two unilateral or bilateral renal dysgenesis among 13 E15.5 embryos, demonstrating a close association of renal agenesis with QBRICK deficiency. Therefore, we examined the integrin α8β1 binding activities of the BMs in the developing kidneys of Qbrick−/− embryos. Metanephric development starts by budding of a ureteric bud from the mesonephric duct at around embryonic day 11.5 (E11.5). The metanephric mesenchyme interacts with the ureteric bud BM through integrin α8β1 (Müller et al., 1997). In wild-type embryos, integrin α8β1 bound to the BMs of the mesonephric duct and ureteric bud (Fig. 2, B and C), where QBRICK was also expressed (Fig. 2, D and E). In the kidney of Qbrick−/− littermates, however, the integrin binding was greatly diminished (Fig. 2, B and C).

Glial cell line–derived neurotrophic factor (GDNF), a critical nephrogenic factor (Moore et al., 1996; Pichelin et al., 1996; Sánchez et al., 1996), has been shown to require integrin α8β1–dependent interactions of the metanephric mesenchyme with

**Figure 2. Impaired binding of integrin α8β1 in the developing kidney of Qbrick−/− mice.** (A) Unilateral renal agenesis observed in Qbrick−/− mice. Bar, 5 mm. (B and C) The left panels show integrin α8β1 (green) bound to the BMs of the E10.5 mesonephric duct (B) and E11.5 ureteric bud (C) of wild-type (top) and Qbrick−/− (bottom) embryos. The right panels show the BMs counterstained with an anti–laminin-γ1 chain antibody (magenta). (D and E) QBRICK immunofluorescence (green) in the developing kidney of wild-type (top) and Qbrick−/− (bottom) embryos at E10.5 (D) and E11.5 (E). The BMs were counterstained with an anti–laminin-γ1 chain antibody (magenta). The open and closed arrowheads indicate the mesonephric duct and ureteric bud, respectively. The asterisks indicate nonspecific binding of the anti-QBRICK antibody to the mesenchyme. Bars, 50 µm. (F) The expression levels of Gdnf and Pax2 transcripts in the E11.5 metanephros of Qbrick−/− control (open bars) and Qbrick−/− (shaded bars) littermates were determined by quantitative RT-PCR and normalized by the expression level of Gapdh. The expression level in control mice was set at 1. Each bar represents the mean ± SD (error bars; n = 3). ***, P < 0.01, significant difference by Student’s t test.
To address this possibility, we generated knock-in animals, QbrickRGE mice, in which the RGD integrin-binding motif in QBRICK was substituted with Arg-Gly-Glu (RGE) to inactivate the integrin \( \alpha_8\beta_1 \) binding activity (Fig. 3, A–C).

Immunofluorescence staining showed that QbrickRGE/RGE mice expressed the mutant QBRICK protein at comparable levels to those of QBRICK in wild-type mice at the BMs of the embryonic skin (Fig. 3 D), ureteric bud (Fig. 3 E), lung, and choroid plexus (Fig. S1, A and B). The expression levels of Fras1 and Frem2 also remained unaltered in QbrickRGE/RGE embryos (Fig. S1, C–H). QbrickRGE/RGE mice were born at the expected Mendelian ratios (wild-type/heterozygous/homozygous = 34:80:36) and phenocopied neither the renal defects observed in integrin \( \alpha_8\beta_1 \)-null mice and FS model mice (Fig. 3 F) nor the other developmental defects associated with FS, namely cryptophthalmos, syn-dactyly, and dystrophic epidermolysis bullosa (Fig. 3, G and H; diminished in the metanephros at E11.5 (Fig. 2 F), the stage when Gdnf reduction is observed in integrin \( \alpha_8\beta_1 \)-deficient mice (Linton et al., 2007). The expression of Pax2, a transcription factor critical for metanephric development, remained unaffected in the QbrickRGE/RGE metanephros (Fig. 2 F), similar to the case for integrin \( \alpha_8\beta_1 \)-deficient mice (Linton et al., 2007). These findings are consistent with the view that the loss of QBRICK impairs integrin \( \alpha_8\beta_1 \)-mediated signaling, which eventually results in renal dysgenesis.

The RGD-dependent integrin-binding activity of QBRICK is dispensable for BMs to interact with integrin \( \alpha_8\beta_1 \)

Next, we investigated whether the diminished integrin \( \alpha_8\beta_1 \) binding in QbrickRGE/RGE embryos could be a direct consequence of the absence of the integrin binding ability of QBRICK.
and not depicted). Furthermore, the integrin α8β1 binding to the BMs of the epidermis (Fig. 3 I), ureteric bud (Fig. 3 J), lung, and choroid plexus (Fig. S1, I and J) appeared unaffected in Qbrick<sup>RGE/RGE</sup> embryos, indicating that QBRICK accounts for only a small fraction of the ability of BMs to bind integrin α8β1. These findings indicate that QBRICK regulates integrin α8β1 binding and renal development independently of its own integrin-binding activity.

**QBRICK regulates the expression of the other integrin α8β1 ligands nephronectin and MAEG**

Given the impaired BM binding of integrin α8β1 in Qbrick<sup>−/−</sup> mice, we examined the alternative possibility that QBRICK regulates the expression of other integrin α8β1 ligands in BMs. We focused on nephronectin and its homologue MAEG, both of which are localized at BMs and serve as ligands for integrin α8β1 (Brandenberger et al., 2001; Osada et al., 2005). In Qbrick<sup>−/−</sup> littermates, nephronectin was detected at the epidermal BM, but its immunoreactivity was greatly diminished compared with wild-type littermates (Fig. 4 A). Similarly, the expression of MAEG was reduced in the Qbrick<sup>−/−</sup> epidermal BM (Fig. 4 B). Diminished expression of nephronectin and MAEG was also apparent in the BMs of the developing lungs and choroid plexus in Qbrick<sup>−/−</sup> embryos (Fig. S2). Reduced expression of nephronectin at embryonic BMs was also observed in other F5 animal models, i.e., Frem<sup>2<sub>bino</sub></sup> and Grip<sup>1<sub>phob</sub></sup> mice (Fig. 4, C–F), where the BM deposition of QBRICK was greatly diminished (Fig. 1).

Nephronectin has been shown to play a critical role in renal development (Linton et al., 2007). In the developing kidney, nephronectin was detected at the BM of the mesonephric duct at E10.5 and that of the ureteric bud at E11.5 (Fig. 4, G and H), which is consistent with a previous study (Brandenberger et al., 2001). MAEG was undetectable at these renal BMs (Fig. 4, I and J). In Qbrick<sup>−/−</sup> embryos, the expression of nephronectin was greatly diminished at the BMs of the developing metanephros (Fig. 4 B). Diminished expression of nephronectin and MAEG in BMs of the epidermis and other organs. The diminished expression of nephronectin and MAEG in Qbrick<sup>−/−</sup> mice was further confirmed by Western blot analyses of tissue extracts from embryonic kidneys and skin (Fig. 4, K–M). No differences in the expression levels of laminin and integrin α8 were detected between wild-type and Qbrick<sup>−/−</sup> mice by the same analyses. Similar results were obtained when tissue extracts from Frem<sup>2<sub>bino</sub></sup> and Grip<sup>1<sub>phob</sub></sup> embryos were analyzed by Western blotting (Fig. 4, N and O). In contrast, diminished expression of nephronectin and MAEG was not observed in Qbrick<sup>RGE/RGE</sup> embryos (Fig. S3), indicating that the BM assembly of nephronectin and MAEG is independent of the ability of QBRICK to interact with integrin α8β1.

Although QBRICK and MAEG have been shown to possess an RGD motif and mediate integrin α8β1–dependent cell adhesion (Kiyozumi et al., 2005; Osada et al., 2005), their direct interactions with integrin α8β1 have not been demonstrated. Solid-phase binding assays using recombinant integrin α8β1 demonstrated that QBRICK and MAEG were capable of binding to integrin α8β1 with only weak affinities that were more than two orders of magnitude less than that of nephronectin (Fig. 4 P). These results indicate that nephronectin is the major determinant for the ability of BMs to bind integrin α8β1, which is consistent with the observation that the ability of BMs to bind integrin α8β1 remained uncompromised in Qbrick<sup>RGE/RGE</sup> embryos. These findings raise the possibility that QBRICK defines the ability of BMs to interact with integrin α8β1 by regulating the assembly of nephronectin, a high-affinity ligand for integrin α8β1, into BMs.

**Integrin α8β1 binding by MAEG is dispensable for embryonic development**

The weak binding of MAEG to integrin α8β1 makes it unlikely that the decreased integrin α8β1 binding to the BMs of Qbrick<sup>−/−</sup> mice is caused by reduced expression of MAEG. To further exclude this possibility, we generated mutant mice in which the RGD motif was deleted from MAEG. Because the RGD sequence of MAEG is located within a central linker segment, we generated mutant mice in which exons 9 and 10 of the Maeg gene encoding the linker segment were deleted (Fig. 5, A–D). The resulting mutant mice (Maeg<sup>ARGD</sup>) were apparently normal and did not phenocopy any FS-like developmental defects (unpublished data). In Maeg<sup>ARGD</sup> mice, the mutant MAEG protein was still localized at the embryonic epidermal BM (Fig. 5, E and F). The expression levels of QBRICK and nephronectin at the epidermal BMs were comparable between wild-type and Maeg<sup>ARGD</sup> embryos at E15.5 (Fig. 5, G and H). Western blotting analyses further showed that the expression levels of nephronectin and integrin α8 in the embryonic skin and kidney were comparable between Maeg<sup>ARGD</sup> and wild-type littermates (Fig. 5, I and J). The binding of integrin α8β1 was also comparable between the mutant mice and wild-type littermates (Fig. 5 K), which indicates that the contribution of MAEG to the integrin α8β1 binding activity of BMs is only marginal.

**FS-associated BM proteins are capable of binding to nephronectin and MAEG**

Given the reduced BM deposition of nephronectin and MAEG in Qbrick<sup>−/−</sup> mice, we sought to further clarify how QBRICK modulates the assembly of nephronectin and MAEG into BMs. The levels of Npnt as well as Maeg transcripts in the E15.5 skin and whole embryos remained unaffected in Qbrick<sup>−/−</sup> mice (Fig. 6, A and B), indicating that QBRICK regulates the assembly of nephronectin and MAEG into BMs at a posttranscriptional level. Immunofluorescence examination of the embryonic skin at high-power magnification did not reveal any abnormal intracellular deposition of nephronectin or MAEG inside the epithelial and mesenchymal cells that faced each other across a BM (Fig. 6 C), making it unlikely that the secretion of nephronectin and MAEG was impaired by QBRICK deficiency. Furthermore, the expression levels of 18 different BM components were unaffected in Qbrick<sup>−/−</sup> embryos (Fig. S4). These findings indicate that the diminished assembly of nephronectin and MAEG occurs specifically and is not caused by a gross defect in the BM architecture.
Figure 4. **Impaired expression of nephronectin and MAEG in FS model mice.** (A–F) Immunofluorescence staining (green) for nephronectin (A, C, and E) and MAEG (B, D, and F) in the dorsal skin of Qbrick<sup>−/−</sup> (A and B), Frem2<sup>my/my</sup> (C and D), and Grip1<sup>eb/eb</sup> (E and F) mice, and their control wild-type or heterozygous littermates. (G–J) Immunofluorescence staining (green) for nephronectin (G and H) and MAEG (I and J) at the E10.5 mesonephric duct (G and I; open arrowheads) and E11.5 ureteric bud (H and J; closed arrowheads) in wild-type (top) and Qbrick<sup>−/−</sup> (bottom) littermates. The BMs were counterstained with an anti–laminin-γ1 chain antibody (magenta). Bars: (A–F) 20 µm; (G–J) 50 µm. (K and L) Immunoblot detection of laminin γ1 and β1 chains (LN), integrin α8 (Int8), nephronectin (Npnt), and MAEG in protein extracts from the kidney (K) and skin (L) of E15.5 Qbrick<sup>−/−</sup> mice and their wild-type littermates. (M–O) Immunoblot signal intensities of laminin, integrin α8, nephronectin, and MAEG in the kidney (left) and skin (right) of E15.5 wild-type (open bars) and Qbrick<sup>−/−</sup> (shaded bars; M), E15.5 Frem2<sup>my/my</sup> (open bars) and Frem2<sup>my/my</sup> (shaded bars; N), and E17.5 Grip1<sup>eb/eb</sup> (open bars) and Grip1<sup>eb/eb</sup> (shaded bars; O) mice. The signal levels in control mice were set at 1. Each bar represents the mean ± SD (error bars; n = 3–6). *, P < 0.05; **, P < 0.01; ***, P < 0.001, significant differences by Student’s t tests. (P) Titration curves of recombinant integrin α8β1 bound to the GST-fused NV domain of QBRICK (open triangles), GST-fused RGD linker segment of nephronectin (open diamonds), GST-fused RGD linker segment of MAEG (open squares), and GST (closed circles). Each point represents the mean ± SEM (n = 3).
Next, we addressed the possibility that QBRICK and/or other FS-associated proteins, i.e., Fras1 and Frem2, associate with nephronectin and MAEG, thereby anchoring these proteins at BMs. When nephronectin was immunoprecipitated from embryonic kidney extracts, QBRICK and Frem2 were also pulled down (Fig. 6 D). To further corroborate the association of nephronectin with QBRICK and other FS-associated proteins, we coexpressed FLAG-tagged nephronectin with QBRICK, Fras1, or Frem2 in 293F cells, and immunoprecipitated nephronectin from conditioned media with an anti-FLAG antibody. QBRICK, Fras1, and Frem2 were coprecipitated with nephronectin, but not with albumin cotransfected instead of nephronectin as a negative control (Fig. 6 F). We also performed similar experiments using nephronectin deletion mutants (Fig. 6 E). Deletion of the MAM domain, but not the EGF domain, significantly reduced the coprecipitation of QBRICK, Fras1, and Frem2. The RGD-containing linker segment pulled down none of the FS-associated proteins, suggesting that the segment is dispensable for the interactions of nephronectin with FS-associated proteins. Similarly, QBRICK, Fras1, and Frem2 were coimmunoprecipitated with MAEG, despite the finding that the EGF domain, rather than the MAM domain, was involved in the coimmunoprecipitation (Fig. 6 G). Immunoelectron microscopic observations revealed that nephronectin was localized at the sublamina densa region of the epidermal BM (Fig. 6 H), which is the region just beneath the lamina densa where QBRICK, Fras1, and Frem2 are also localized (Kiyozumi et al., 2006; Dalezios et al., 2007; Petrou et al., 2007a,b), in E15.5 embryos. Collectively, these findings indicate that QBRICK, together with Fras1 and Frem2, interacts with nephronectin (and MAEG) at the sublamina densa region and thereby regulate the assembly of these integrin ligands into BMs.
Figure 6. Nephronectin and MAEG associate with QBRICK, Fras1, and Frem2 in vitro. (A and B) The expression levels of Npnt and Maeg transcripts in E15.5 dorsal skin (A) and E15.5 whole embryos (B) of wild-type (shaded bars) and Qbrick−/− (open bars) littermates were determined by quantitative RT-PCR and normalized by the expression level of Gapdh. The expression level in wild-type mice was set at 1 for each transcript. Each bar represents the mean ± SD (in A, n = 6 for both wild-type and Qbrick−/−; in B, n = 5 for wild-type and n = 6 for Qbrick−/−). (C) Magnified views of epidermal basal cells (nephronectin producers) and dermal mesenchymal cells (MAEG producers) of E15.5 wild-type and Qbrick−/− mice stained with anti-nephronectin (top) and anti-MAEG (bottom) antibodies. The arrowheads indicate the position of the epidermal BMs. Bar, 5 µm. (D) E15.5 mouse kidney extracts were subjected to immunoprecipitation with an anti-nephronectin antibody or control rabbit IgG, and the resulting immunoprecipitates were analyzed by immunoblotting with anti-QBRICK, anti-Frem2, and anti-nephronectin antibodies. (E) Schematic views of FLAG-tagged nephronectin, MAEG, and their deletion mutants used for coimmunoprecipitation assays. (F) FLAG-tagged nephronectin or its deletion mutants were coexpressed with QBRICK, Fras1, or Frem2 and immunoprecipitated with an anti-FLAG monoclonal antibody. Proteins in the input samples or immunoprecipitates were specifically detected with anti-QBRICK, anti-HA (for Fras1), and anti-Myc (for Frem2) antibodies. (G) Coimmunoprecipitation experiments were performed for MAEG and its deletion mutants in a similar manner to those for nephronectin and its deletion mutants. (H) Immunoelectron microscopic analysis of embryonic skin. Ultrathin sections of ventral skin from E15.5 mouse embryos were labeled with an anti-nephronectin antibody, which was visualized by a 15-nm gold particle–conjugated secondary antibody. Nephronectin is located in the sublamina densa region of the epidermal BM (arrowheads). The arrows indicate the lamina densa. Bar, 200 nm.
Discussion

In the present study, we have demonstrated that the BMs of Qbrick−/− and other FS model animals show defects in binding to integrin α8β1, and that these defects arise from the diminished expression of nephronectin, but not QBRICK per se, at the BMs. Our data also showed that MAEG, another RGD-containing integrin α8β1 ligand, fails to stably assemble into BMs in these FS model animals, revealing a hitherto unknown mechanism that operates in the coordinated BM assembly of the three integrin α8β1 ligands, nephronectin, QBRICK, and MAEG. To the best of our knowledge, this is the first demonstration that QBRICK and other FS-associated BM proteins play a critical role in the BM assembly of integrin α8β1 ligands and thereby regulate the interactions of cells with BMs via integrin α8β1 and the signaling events thereafter.

Molecular mechanism of the BM assembly of nephronectin and MAEG

Because the expression levels of Npmt and Maeg transcripts remained unaffected in Qbrick−/− and other FS model mice, the diminished expression of nephronectin and MAEG at the BMs of these mutant animals probably results from defects in post-transcriptional events, namely impaired secretion or increased turnover of these proteins. Given that no intracellular inclusions of nephronectin and MAEG were detected in the epithelial and mesenchymal cells that faced each other across a BM in Qbrick−/− mice, the diminished BM deposition of these integrin α8β1 ligands does not seem to result from impaired secretion, but rather from increased turnover. Although the mechanisms for the turnover of nephronectin and MAEG in vivo remain to be elucidated, our data show that these RGD-containing integrin α8β1 ligands are associated with QBRICK and other FS-associated proteins in vivo, as indicated by the immunoprecipitation of nephronectin with QBRICK and Frem2 from embryonic tissue extracts. The interactions of nephronectin as well as MAEG with QBRICK and other FS-associated proteins were also confirmed by communoprecipitation of these proteins after recombinant expression in 293-F cells in vitro. Thus, it seems likely that the three FS-associated proteins forming a ternary complex at the sublamina densa region serve as a scaffold for the stable association of nephronectin and MAEG with BMs via direct or indirect interactions with these α8β1 ligands. In support of this possibility, nephronectin was found to be localized at the sublamina densa region by immunoelectron microscopy. The failure of the association of nephronectin and MAEG with the ternary complex of FS-associated proteins may well render these α8β1 ligands more susceptible to proteolytic degradation and lead to their increased turnover at BMs in Qbrick−/− and other FS model animals, irrespective of the causative mutation.

Pathogenesis of the renal dysmorphogenesis in FS

Among the developmental defects observed in FS animals, the renal defects overlap with those observed in integrin α8−/− mice. Initial outgrowth of the ureteric bud often fails or is delayed in both types of animals (Carter, 1959; Müller et al., 1997; McGregor et al., 2003; Pitera et al., 2008). To explain this phenotypic overlap, we obtained evidence that the integrin α8β1 binding by ureteric bud BMs was significantly impaired in the developing kidney of Qbrick−/− mice. The reduced integrin α8β1 binding in Qbrick−/− embryos was not caused by the lack of integrin α8β1 binding ability of QBRICK itself, but rather by the impaired expression of nephronectin, a high-affinity ligand for integrin α8β1. The expression of another ligand, MAEG, at BMs was also impaired in Qbrick−/− mice, but the contribution of MAEG as an integrin α8β1 ligand at BMs appears to be only marginal compared with that of nephronectin. Besides its weak activity for binding integrin α8β1, MAEG was hardly detected at the BM of the ureteric bud. Furthermore, MaegARGD mice developed normally and did not exhibit renal defects, making it unlikely that MAEG is involved in the renal defects in Qbrick−/− mice. In contrast, nephronectin-deficient mice exhibit clear defects in renal morphogenesis (Linton et al., 2007). Direct integrin binding assays revealed that nephronectin has an exceptionally high affinity for integrin α8β1 with a subnanomolar dissociation constant (Sato et al., 2009; this paper), whereas QBRICK and MAEG have affinities for integrin α8β1 that are more than two orders of magnitude less than that of nephronectin, thus emphasizing the importance of nephronectin as a physiological ligand for integrin α8β1. We also found that reduced Gdnf expression, to which the renal phenotypes of integrin α8−/− mice and nephronectin-deficient mice have been attributed (Linton et al., 2007), was recapitulated in the developing kidney of Qbrick−/− mice. Reduced Gdnf expression was also found in the metanephros of Fras1 mutant mice (Pitera et al., 2008). Given that nephronectin functions as a dominant ligand for integrin α8β1 at the ureteric bud BM and plays a central role in renal morphogenesis via the induction of Gdnf expression, we propose a model for the pathogenesis of the renal defects in FS, as schematically illustrated in Fig. 7. In this model, aberrant expression of QBRICK or other FS-associated BM proteins destabilizes the ternary complex of FS-associated proteins and results in the loss of FS-associated proteins from the ureteric bud BM (Kiyozumi et al., 2006; this paper). The absence of FS-associated proteins leads to failure of nephronectin assembly at the sublamina densa region of the BM, and therefore metanephric mesenchymal cells expressing integrin α8β1 cannot interact with the ureteric bud BM to induce the signaling events necessary for the induction of Gdnf in metanephric mesenchymal cells. As a consequence, the expression of Gdnf is attenuated and renal developmental defects occur. Our results showed that the reduction in Gdnf expression was only partial (~50%) in the Qbrick−/− metanephros. Nevertheless, this reduction may suffice to cause renal defects in Qbrick−/− mice, because renal agenesis sometimes occurs even in Gdnf−/− heterozygotes (Moore et al., 1996; Pichel et al., 1996; Sánchez et al., 1996).

It should be emphasized that ~90% of Qbrick−/− mice exhibited either bilateral or unilateral renal agenesis. This is in striking contrast to the renal phenotype of another Qbrick mutant strain, called bat mice, in which only 20% of the mice...
mice do not exhibit dystrophic epidermolysis bullosa, syndactyly, or cryptophthalmos (Linton et al., 2007). Because nephronectin and MAEG share the ability to associate with QBRICK, Fras1, and Frem2, it seems possible that MAEG compensates for the loss of nephronectin as a linker component, as has been shown for the attachment of the arrector pili muscle to the bulge BMs. It will therefore be interesting to examine whether ablation of both \textit{Npnt} and \textit{Maeg} recapitulates the defective dermal–epidermal interactions observed in FS model animals. It will also be interesting to investigate whether there are extracellular factors other than nephronectin and MAEG that can associate with FS-associated BM proteins. One such candidate is hemicentin-1, an ECM molecule critical for dermal–epidermal integrity in zebrafish (Carney et al., 2010). The hemicentin-1 nonsense mutation \textit{nagel} was shown to cause epidermal blistering in a synergistic manner with the Fras1 mutation \textit{pinfin} (Carney et al., 2010).

Physiological roles of nephronectin and MAEG at BMs

Among the developmental defects observed in FS, dystrophic epidermolysis bullosa, syndactyly, and cryptophthalmos arise from defects in dermal–epidermal interactions. Both nephronectin and MAEG are highly expressed at the epidermal BM, implying their physiological roles in dermal–epidermal interactions. Because the dermal–epidermal integrity appears unaffected in mice deficient in integrin \( \alpha 8 \) (Müller et al., 1997), their functions as integrin \( \alpha 8\beta 1 \) ligands seem to be dispensable for dermal–epidermal interactions. Besides their roles as integrin \( \alpha 8\beta 1 \) ligands, they may function as linker components that anchor the epidermal BM to the mesenchymal ECM via associations with QBRICK, Fras1, and Frem2. Recently, nephronectin and MAEG were shown to function as BM components that anchor the arrector pili muscle to the BMs at the bulge of hair follicles (Fujiwara et al., 2011). These findings support the possibility that nephronectin and MAEG function as attachment sites that physically connect epidermal BMs with mesenchymal cells and/or ECMs. Apparently, nephronectin-null mice do not exhibit dystrophic epidermolysis bullosa, syndactyly, or cryptophthalmos (Linton et al., 2007). Because nephronectin and MAEG share the ability to associate with QBRICK, Fras1, and Frem2, it seems possible that MAEG compensates for the loss of nephronectin as a linker component, as has been shown for the attachment of the arrector pili muscle to the bulge BMs. It will therefore be interesting to examine whether ablation of both \textit{Npnt} and \textit{Maeg} recapitulates the defective dermal–epidermal interactions observed in FS model animals. It will also be interesting to investigate whether there are extracellular factors other than nephronectin and MAEG that can associate with FS-associated BM proteins. One such candidate is hemicentin-1, an ECM molecule critical for dermal–epidermal integrity in zebrafish (Carney et al., 2010). The hemicentin-1 nonsense mutation \textit{nagel} was shown to cause epidermal blistering in a synergistic manner with the Fras1 mutation \textit{pinfin} (Carney et al., 2010).

Possible role of the FS-associated proteins in epithelial-mesenchymal interactions

The stable expression of the three FS-associated proteins is strongly dependent on the concerted expression of all three proteins. QBRICK is secreted by mesenchymal cells, whereas Fras1 and Frem2 are secreted by epithelial cells. Thus, the BM assembly of these proteins requires appropriate cooperation of the epithelium and mesenchyme. Furthermore, the degree of the reciprocal stabilization of these proteins reflects how the interacting epithelium and mesenchyme fit with one another. Our data show that the expression of nephronectin and MAEG is regulated by the integrity of the three FS-associated BM proteins, and is therefore dependent on the combination between the epithelium and mesenchyme. Such regulated expression of

Figure 7. A model for renal defects in FS. In the developing metanephros, QBRICK and other FS-associated proteins associate with nephronectin in the sublamina densa region and secure its localization at BMs. Metanephric mesenchymal cells interact with nephronectin through integrin \( \alpha 8\beta 1 \). Stimulation of integrin \( \alpha 8\beta 1 \) induces Gdnf, which is necessary for further development of the metanephros (left). In FS, nephronectin is not stably deposited at BMs, owing to the loss of FS-associated proteins, and is eventually degraded. The loss of nephronectin leads to failure of Gdnf induction and subsequent metanephric development (right).
RGD-containing integrin ligands at BMs should be important for their function as signal transducers in epithelial–mesenchymal interactions because their expression needs to be fine-tuned spatially and temporally as organs develop. Nephronectin, which is expressed in the ureteric bud epithelium and recognized by integrin α8β1 expressed on the surface of the metanephric mesenchyme, is a typical “epithelium-to-mesenchyme” signal transducer. Regulated BM assembly of nephronectin by the FS-associated proteins would therefore facilitate the spatiotemporally regulated crosstalk between the epithelium and mesenchyme during renal development.

In summary, we have presented evidence that QBRICK, together with Fra1 and Frem2, defines the ability of BMs to bind integrin α8β1 through modulating the BM assembly of nephronectin, thereby regulating the integrin α8β1–mediated signals transmitted from the ureteric buds to the mesanephric mesenchyme. Further investigations of these FS-associated BM proteins should provide insights into the pathogenesis of FS as well as the molecular basis of epithelial–mesenchymal interactions during development.

Materials and methods

Animals
Qbrick<sup>−/−</sup> mice were produced by a homologous recombination that substituted the second exon of the Qbrick gene, which includes an initiation codon, with a neomycin-resistance gene as described previously (Kiyozumi et al., 2006), and maintained in a C57BL/6 background. The frem2<sup>My/Hu</sup>eb and frem1<sup>My/Hu</sup>eb mouse strains were obtained from Jackson Immunoresearch Laboratories, Inc. Male MY/Hu<sub>eb</sub> males were outcrossed with C57BL/6 female mice to obtain frem2<sup>My/my</sup>eb and frem2<sup>My/My</sup>eb litters. Male ATEB/Le<sub>eb</sub> males were outcrossed with female C57BL/6 mice, and the resulting frem<sup>2M2/2</sup> females were backcrossed to MY/Hu<sub>eb</sub> males to obtain frem<sup>2M2/2</sup> and frem<sup>2M2/2</sup> litters. Nephronectin, which binds integrin α5β1 heterodimers, was the protein used for histological analyses. The B6.Cg-Tg(CAG-cre):C2M020sb mouse strain expressing Cre recombinase under the control of the CAG promoter (BRC No. 01828) was provided by Institute of Physical and Chemical Research (RIKEN) Bio-Resource Center with the support of the National BioResource Project of the Ministry of Education, Culture, Sports, Science, and Technology, Japan.

To generate Qbrick<sup>−/−</sup> knock-in mice, a knock-in vector was constructed as follows: the vector included the 2-kb upstream Qbrick genomic sequence, a neomycin resistance gene sandwiched by loxP sequences, and a thymidine kinase gene (Fig. 3A). To introduce the RGE mutation, the 5-kb genomic sequence was digested with AscI and BamHI restriction sites. The resulting plasmids each contained an open reading frame expressing a polypeptide consisting of an N-terminal FLAG sequence, a linker region including the RGD sequence, a neomycin resistance gene flanked a neomycin resistance gene (Fig. 5B). To substitute exons 9 and 10, which encode the linker region including the RGD sequence, with a neomycin resistance gene, the targeting vector was introduced into strain 129 mouse embryonic stem cells. The targeted clones were identified by PCR and Southern blotting, and injected into C57BL/6 blastocysts to obtain chimeric mice. The resulting chimeric male offspring that transmitted the mutated Qbrick gene through the germline were crossed with C57BL/6 female mice to obtain Qbrick<sup>−/−</sup> males and Qbrick<sup>−/−</sup> females. Qbrick<sup>−/−</sup> male and Qbrick<sup>−/−</sup> female mice were maintained in a C57BL/6 background and crossed to obtain embryos lacking wild-type MAEG (Qbrick<sup>1−/−</sup>) and their control (Qbrick<sup>+/−</sup>) littermates. All the mouse experiments were performed in compliance with the institutional guidelines and were approved by the Animal Care Committee of Osaka University.

Immunofluorescence
Embryos or newborn mice were embedded in OCT for cryosectioning. 10-µm sections were fixed in absolute ethanol for 20 min at −20°C, then rehydrated with PBS. The antibodies used and their concentrations are shown in Table S1. After incubation with primary antibodies at 4°C overnight, the sections were incubated with 1:3,000-diluted Alexa Fluor 488–conjugated goat anti-rabbit IgG (Invitrogen) and 1:4,000-diluted Cy3-conjugated anti-rat IgG (Jackson Immunoresearch Laboratories) as secondary antibodies at 4°C for 2 h. To detect intracellular protein inclusions, frozen sections were fixed with 4% paraformaldehyde in PBS at 4°C for 15 min and permeabilized with 0.1% Triton X-100 in PBS at 4°C for 10 min before incubation with primary antibodies. The sections were mounted in Perma-Fluor (Thermo Fisher Scientific) and visualized using a laser scanning microscope (LSM5 PASCAL; Carl Zeiss) equipped with LD-Achromplan (20×, NA 0.4) and Plan-Neofluor (10×, NA 0.3; 40×, NA 0.75; 63×, NA 1.25) objective lenses at room temperature. The imaging medium was air for the 10×, 20×, and 40× objective lenses, and Immersol 518 F (Carl Zeiss) for the 63× objective lens. The LSM5 PASCAL software (Carl Zeiss) was used during image collection. Each set of stained sections was processed under identical gain and laser power settings. Each set of obtained images was processed under identical brightness and contrast settings, which were adjusted by the LSM image browser (Carl Zeiss) for clear visualization of BMs. At least four pairs of sections of mutant mice and their control littermates were examined, and similar results were obtained.

Immunoelectron microscopy
E15.5 mouse embryos were sequentially fixed in 0.5% glutaraldehyde and 4% paraformaldehyde in 30 mM Hapes, pH 7.4, 100 mM NaCl, and 2 mM CaCl<sub>2</sub>. After dehydration, the samples were embedded in LR white resin. Ultra-thin sections (70 nm) were labeled with a rabbit anti-nephronectin polyclonal antibody (Manabe et al., 2008), which was visualized using 15-nm gold particle-conjugated anti-rabbit IgG. The sections were post-stained with uranyl acetate and lead citrate. Images were acquired using a 1200EX electron microscope (JEOL Ltd.) operated at 80 kV.

Construction of plasmids for recombinant protein expression
A cDNA encoding the Ig κ-chain signal sequence followed by three tandem HA epitope tag sequences and Frem1 (amino acids 26–4,210) or a cDNA encoding the Ig κ-chain signal sequence followed by three tandem Myc epitope tag sequences and Frem2 (amino acids 40–3,160) were cloned into pcDNA3.1(+) (+) (Invitrogen) for expression of FLAG-tagged Frem1 or 3× Myc-tagged Frem2, respectively, as described previously (Kiyozumi et al., 2006). A full-length Qbrick cDNA was subcloned into pcDNA3.1(+) (+) (Invitrogen) for expression of FLAG-tagged albumin. A cDNA encoding full-length nephronectin was subcloned into pFLAG-CMV-5a (Sigma-Aldrich) for expression of FLAG-tagged albumin. The cDNA encoding full-length nephronectin was cloned into pFLAG-CMV [Invitrogen] for expression of FLAG-tagged nephronectin, as described previously [Sato et al., 2009]. For expression of nephronectin deletion mutant proteins, cDNAs encoding ΔEGF (amino acids 1–23; 257–361), ΔMAM (amino acids 1–4,19), or the linker segment alone (amino acids 1–23; 257–414) were individually subcloned into pFLAG-CMV, as described previously [Sato et al., 2009]. Mouse MAEG cDNAs encoding amino acids 19–550 (full length), 19–393 (ΔMAM), 254–550 (ΔEGF), and 254–393 (linker) were amplified by PCR with the following primer pairs: full length, 5′-GGGCGGGCGCGCTTAGACAGTTTGAGAGTTC-3′ and 5′-GCGCGCTTCTCAGTCAAACATCT-3′; ΔMAM, 5′-GGGCGGGCGCGCGGAGCTTACGGAAC-3′ and 5′-CTGAAAGCTTACGAGAATAAGGTA-3′; linker, 5′-CTCGGGGCGCGGCTTAGACAGTTTGAGAGTTC-3′ and 5′-CTGAAAGCTTACGAGAATAAGGTA-3′.

The cDNA fragments were subcloned into pSecFLAG, a modified vector of pSecTag2A [Invitrogen]. The pSecFLAG vector was generated by subcloning a double-stranded DNA encoding the FLAG sequence followed by a stop codon (sense strand sequence: 5′-GGATCCGGGTACCACTTTCCTTTCCTTCTTCTTCT-3′ and 5′-CTGAAAGCTTACGAGAATAAGGTA-3′) using the BamH1 and Xho1 sites. The cDNA fragments were digested with AscI and BglII and subcloned into the pSecFLAG expression vector using the Ascl and BamH1 restriction sites. The resulting plasmids each contained an open reading frame expressing a polypeptide consisting of an N-terminal signal sequence, a MAEG protein fragment, and a C-terminal FLAG tag.
Plasmids expressing the human integrin α3, α6, α7/2, and α8 extracellular domains fused to the ACID peptide with a FLAG tag, and the β1 and β4 extracellular domains fused to the BASE peptide with a 5X His tag were prepared as previously described (Nishii et al., 2006; Ido et al., 2007; Sato et al., 2009). cDNAs encoding an NV domain of QBRICK (amino acids 23–280), a linker segment of nephrocutinin (amino acids 256–414), or a linker segment of MAEG (amino acids 260–392) were individually electrotransfected into 293-F cells (Invitrogen), and the recombinant integrins were purified from the conditioned medium using Ni-NTA affinity columns, followed by anti-FLAG mAb affinity chromatography.

Recombinant GST fusion proteins were induced in Escherichia coli by overnight incubation with 0.1 mM IPTG at 25°C. The cells were then lysed by sonication, and the supernatants were passed through a glutathione-Sepharose 4B column (GE Healthcare). The bound proteins were eluted with 50 mM Tris-HCl, pH 8.0, containing 10 mM glutathione.

In situ integrin binding

Frozen sections of mouse embryos were blocked with blocking buffer (3% BSA, 25 mM Tris-HCl, pH 7.4, 100 mM NaCl, and 1 mM MgCl₂) for 30 min at room temperature, then incubated with 1–10 µg/ml of recombinant integrins and a rat anti–laminin-1 monoclonal antibody (Millipore) in blocking buffer at 4°C overnight. After three washes with wash buffer (25 mM Tris-HCl, pH 7.4, 100 mM NaCl, and 1 mM MnCl₂) for 10 min at room temperature, the sections were incubated with 0.5 µg/ml of rabbit anti–Velcro (ACID/BASE coiled-coil) antibody (Takagi et al., 2001) in blocking buffer at room temperature for 2 h. The sections were then washed with wash buffer and incubated with 1:3,000-diluted Alexa Fluor 488-conjugated goat anti–rabbit IgG (Invitrogen) and 1:4,000-diluted Cy3-conjugated goat anti–rat IgG (Jackson ImmunoResearch Laboratories). After washing with wash buffer, the sections were mounted in PermaFluor (Thermo Fisher Scientific). The fluorescent probes were visualized with an LSM5 laser scanning microscope, as described for the immunofluorescence. At least four pairs of sections of mutant mice and their control littermates were examined, and similar results were obtained.

Solid-phase integrin binding assay

Solid-phase integrin binding assays were performed as described previously (Nishii et al., 2006). In brief, microtiter plates were coated with 80 µg/ml of recombinant integrins in 10 mM NaOH overnight at 4°C, and then blocked with 1% BSA. The plates were incubated with recombinant integrin α8β1 in the presence of 1 mM MnCl₂. The plates were washed with TBS containing 1 mM MnCl₂, 0.1% BSA, and 0.2% Tween 20, followed by quantification of bound integrin α8β1 by ELISA using a biotinylated rabbit anti-Velcro antibody and HRP-conjugated streptavidin.

Quantitative RT-PCR

Quantitative RT-PCR was performed as described previously (Kiyozumi et al., 2005). The primers used were as follows: Npnt, 5′-TGAGGAAA-CACGTTACCACGGAGG-3′ and 5′-AGGGGTTCTTGCAAGCACGGCA-3′; Maeg, 5′-ATAGGATGTAAGGTGGAAGAGGAGG-3′ and 5′-AGGACACAAGACTCAC-3′; Ndnt, 5′-GCTTCGTGAAGCCCGCCTGACACT-3′ and 5′-CTTCTTCTGTCTTAGATGGCCTTG-3′; GATAA-3′; Fox2, 5′-GAAGACACCACCCCTGTTAACC-3′ and 5′-ATTGAGACGTGCTCCTATGATT-3′; GATAA-3′; GATAA-3′; Gapdh, 5′-AGGGTGAAGGTGCGGTG-3′ and 5′-TGGCGGCGGACGATGAGA-3′. The expression levels of the individual transcripts were normalized by the expression level of Gapdh.

Western blotting

E15.5–E17.5 skin and kidneys were homogenized with SDS-PAGE sample buffer (2% SDS, 5% sucrose, and 63 mM Tris-HCl, pH 6.8), and the protein concentrations of the homogenates were determined using a BCA protein assay reagent kit (Thermo Fisher Scientific). The homogenates were supplemented with a 1/20 volume of 2-mercaptoethanol, boiled, and subjected to SDS-PAGE. The separated proteins were electrotransferred to a nitrocellulose membrane. The primary antibodies used were: rabbit anti–laminin (9393; Sigma-Aldrich), goat anti–mouse integrin α8 (R&D Systems), and rabbit anti–nephrocutinin (Manabe et al., 2008), and rabbit anti–MAEG (Osada et al., 2005). The bound primary antibodies were visualized with an Amersham ECL Prime Western blotting detection reagent kit (GE Healthcare). The intensities of the signals were quantified using ImageJ software (Abramoff et al., 2004).

Immunoprecipitation assays

Embryonic kidney extracts were prepared as described previously (Brandenberger et al., 2001), with slight modifications. In brief, E15.5 kidneys were solubilized in ice-cold TBS containing 1 mM PMSF, 0.7 µg/ml pepstatin A, and 10 µg/ml leupeptin using a Dounce homogenizer. After centrifugation at 18,000 g for 30 min, the pellet was resuspended in extraction buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM MgCl₂, 1.5% n-octylglycoside, 1 mM PMSF, 0.7 µg/ml pepstatin A, and 10 µg/ml leupeptin), sonicated, and centrifuged at 10,000 g for 20 min to remove debris. An anti-nephrocutinin polyclonal antibody or rabbit IgG, which was immobilized on Protein A Sepharose 4 Fast Flow Beads (GE Healthcare), was added to the kidney extract and incubated for 6 h at 4°C with gentle rotation. The beads were washed with extraction buffer three times, followed by treatment with SDS-PAGE sample buffer. The supernatant was recovered by centrifugation and subjected to Western blotting analysis. Immunoprecipitation was also performed using conditioned media containing recombinant proteins. Aliquots (5 ml) of Freestyle 293-F cells (Invitrogen) at 10⁶ cells/ml were transfected with 4 µg of plasmids encoding 3x HA-tagged Fasl, 3x Myc-tagged Fsm2, or QBRICK and 1 µg of plasmids encoding FLAG-tagged nephrocutinin, FLAG-tagged MAEG, or their deletion mutants using the 293Fectin transfection reagent (Invitrogen). After 3 d of culture, the conditioned media were recovered by centrifugation. The conditioned media were supplemented with a 1/20 volume of 1 M Tris-HCl, pH 7.4, a 1/10 volume of 10% Triton X-100, and a 1/38 volume of anti-FLAG M2 agarose beads (Sigma-Aldrich), and incubated at 4°C overnight with gentle rotation. The beads were washed three times with TBS containing 1 mM CaCl₂ and 1% Triton X-100. Proteins bound to the agarose beads were eluted with SDS-PAGE sample buffer and subjected to SDS-PAGE, followed by immunoblotting with anti-HA (Babco), anti-Myc (Santa Cruz Biotechnology, Inc.), anti-QBRICK (Sigma-Aldrich), and anti-QBRICK (Kiyozumi et al., 2005) antibodies.

Online supplemental material

Fig. S1 shows the immunofluorescence and in situ integrin α8β1 binding of QBRICK360/2005 mice. Fig. S2 shows the immunofluorescence of nephrocutinin and MAEG in QBRICK360/2005 mice. Fig. S3 shows the immunofluorescence and immunoblotting analyses of nephrocutinin and MAEG in QBRICK360/2005 mice. Fig. S4 shows the immunofluorescence of various BM proteins in QBRICK360/2005 mice. Table S1 summarizes the primary antibodies used for immunofluorescence. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201203065/DC1.

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