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Human disease-associated extracellular matrix orthologs ECM3 and QBRICK regulate primary mesenchymal cell migration in sea urchin embryos

Daiji KIYOZUMI[1)], Shunsuke YAGUCHI[2,3)], Junko YAGUCHI[2)], Atsuko YAMAZAKI[2)] and Kiyotoshi SEKIGUCHI[1)]

[1)]*Laboratory of Extracellular Matrix Biochemistry, Institute for Protein Research, Osaka University, 3-2 Yamadaoka, Suita, Osaka 565-0871, Japan*
[2)]*Shimoda Marine Research Center, University of Tsukuba, 5-10-1 Shimoda, Shizuoka 415–0025, Japan*
[3)]*PRESTO, JST, 4-1-8 Honcho, Kawaguchi, Saitama 332-0012, Japan*

Abstract: Sea urchin embryos have been one of model organisms to investigate cellular behaviors because of their simple cell composition and transparent body. They also give us an opportunity to investigate molecular functions of human proteins of interest that are conserved in sea urchin. Here we report that human disease-associated extracellular matrix orthologues ECM3 and QBRICK are necessary for mesenchymal cell migration during sea urchin embryogenesis. Immunofluorescence has visualized the colocalization of QBRICK and ECM3 on both apical and basal surface of ectoderm. On the basal surface, QBRICK and ECM3 constitute together a mesh-like fibrillar structure along the blastocoel wall. When the expression of ECM3 was knocked down by antisense-morpholino oligonucleotides, the ECM3-QBRICK fibrillar structure completely disappeared. When QBRICK was knocked down, the ECM3 was still present, but the basally localized fibers became fragmented. The ingression and migration of primary mesenchymal cells were not critically affected, but their migration at later stages was severely affected in both knock-down embryos. As a consequence of impaired primary mesenchymal cell migration, improper spicule formation was observed. These results indicate that ECM3 and QBRICK are components of extracellular matrix, which play important role in primary mesenchymal cell migration, and that sea urchin is a useful experimental animal model to investigate human disease-associated extracellular matrix proteins.
Key words: Fraser syndrome, FREM1, FREM2, QBRICK, sea urchin

Introduction

Extracellular matrices (ECMs) play crucial roles in transmitting extracellular signals to cells. Various cellular processes including adhesion, migration, survival, proliferation, and differentiation are supported by cellular interactions with ECMs [1]. Dysfunction of these ECM proteins causes a spectrum of severe hereditary developmental disorders in human, including Fraser syndrome (FS), Manitoba oculotrichoanal syndrome, and bifid nose with or without anorectal and renal anomalies [2–5]. In these disorders, morphogenetic processes of multiple organs such as kidney, eye, digit, and skin are deficient. By using animal models, dysfunctions of ECM proteins FREM2, QBRICK (also named as FREM1) and FRAS1 have been identified to be associated with Fraser syndrome-like developmental defects [2–9]. FREM2, QBRICK, and FRAS1 constitute a protein family characterized by the presence of 12 chondroitin sulfate proteoglycan (CSPG) repeats and single

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Corresponding authors: D. Kiyozumi. e-mail: kiyozumi@biken.osaka-u.ac.jp
S. Yaguchi. e-mail: yag@shimoda.tsukuba.ac.jp
K. Sekiguchi. e-mail: sekiguch@protein.osaka-u.ac.jp
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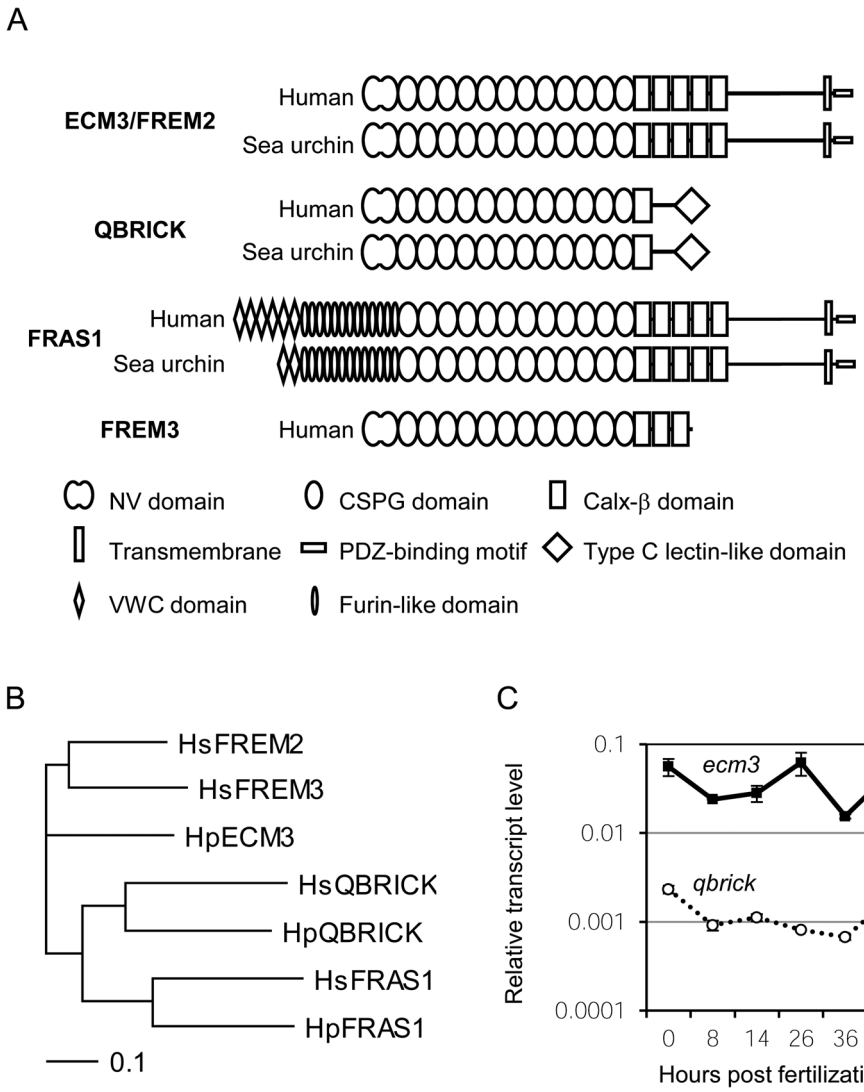

Fig. 1. Fraser syndrome-associated proteins are conserved in sea urchin. (A) Schematic representation of human 12-CSPG proteins ECM3/FREM2, QBRICK, and FRAS1 and their sea urchin orthologues. Human FREM3 is also shown. (B) A phylogenetic representation of human (Hs) and sea urchin (Hp) 12-CSPG proteins. (C) Quantitative RT-PCR analyses for *ecm3* and *qbrick* expression during embryonic development. The transcript level of *mitCO1* at any timing of development is set to 1 and relative expression levels are shown.

or multiple Calx-β motifs (Fig. 1A) [3, 6–8]. The molecular functions of 12-CSPG ECM proteins have been partially clarified in vertebrate animal models such as mice [3, 6–10]. Genetic and biochemical studies unveiled that FREM2, QBRICK, and FRAS1 stabilize reciprocally for their stable deposition in the embryonic ECM and this reciprocal stabilization is crucial for proper morphogenesis [8, 11]. Despite their apparent indispensability, it is largely uncertain how these 12-CSPG proteins regulate cell behaviors during morphogenetic processes. This is mainly because of technical difficulty to observe on-going morphogenetic processes in mammalian embryos in utero.

Sea urchins are echinoderms, and together with hemichordates are the closest relatives of chordates. The sea urchin embryos and larvae are transparent, which let us observe the dynamic behaviors of mesodermal tissues associating with ECMs from the outside of the body directly. In sea urchin embryos, the inner surface of the epithelial wall is covered by a thin basal lamina associated with a sparse meshwork of ECMs. The blastocoelar ECM fibers have been morphologically examined by light and electron microscopy [12–15]. It has been especially emphasized that a variety of cues from the blastocoelar ECMs contributes to the regulation of spatial organization of primary mesenchyme cells (PMCs) during larval spicule morphogenesis. Interestingly, ECM3, an ortholog of mammalian 12-CSPG protein FREM2 was reported to exist in sea urchin embryos [16, 17]. This gives us an opportunity to further characterize the

molecular function of Fraser syndrome-associated 12-CSPG proteins by using sea urchin.

By using sea urchin *Hemicentrotus pulcherrimus*, a model organism popular in the field of biology in Japan, we show that the orthologs of human disease-associated 12-CSPG ECM protein family are involved in sea urchin PMC migration. The findings will give us a new insight to understand how the dysfunction of 12-CSPG ECM proteins provoke FS-like developmental defects.

Materials and Methods

Animals and embryo culture

Embryos of *Hemicentrotus pulcherrimus* collected around Shimoda Marine Research Center, University of Tsukuba, and around Marine and Coastal Research Center, Ochanomizu University were used. The gene information is based on *Hemicentrotus pulcherrimus* genome and transcriptome database, HpBase [18]. The gametes were collected by intra-coelomic injection of 0.5 M KCl and the embryos were cultured by standard methods with filtered natural seawater at 15°C.

Phylogenetic analysis

Phylogenetic trees were generated using the neighbor joining method in ClustalW2.1 [19] on website (http://clustalw.ddbj.nig.ac.jp/). Multiple sequence alignment parameters were as follows: protein weight matrix: gonnet; gap open: 10; gap extension: 0.20; gap distances: 5; no end gaps: no; iteration: none; numiter: 1; clustering: NJ.

Antibodies

For the construction of expression plasmids encoding glutathione-S-transferase (GST) fusion proteins, the cDNA fragment encoding the sea urchin ECM3 antigen (a.a. 20-271) and sea urchin QBRICK antigen (a.a. 23-241) was amplified by PCR and cloned into pGEX 4T-1 (GE healthcare, Chicago, IL, USA). For bacterial expression of GST-fusion proteins, the *E. coli* BL21 strain was transformed with individual expression plasmids and the expressed proteins were affinity-purified using glutathione sepharose 4 FF (GE healthcare). Rats and guinea pigs were immunized with GST-fused QBRICK and ECM3 proteins, respectively. Specific antibodies were purified by affinity purification using antigen-conjugated beads.

Whole-mount *in situ* hybridization and immunofluorescence

Whole-mount *in situ* hybridization was performed as described previously [20, 21]. *sm50* RNA-probe was used for detecting PMC [22]. Immunohistochemistry for detecting ECM3 and QBRICK was performed as follows. Embryos were fixed with 3.7% paraformaldehyde/sea water at room temperature for 10 min then treated with 0.05 M HCl, 137 mM NaCl at 4°C for 5 min. Embryos were then incubated with 10 μg/ml of anti-ECM3 and anti-QBRICK antibody diluted in 3% BSA in 0.1% Tween20/PBS at 4°C overnight. The primary antibodies were detected with secondary antibodies conjugated with Alexa-568 and Alexa-488 (Thermo Fisher, Waltham, MA, USA). The specimens were observed with a Zeiss Axio Imager.Z1 equipped with Apotome system, and optical sections were stacked and analyzed with ImageJ and Adobe Photoshop. Panels and drawings for figures were made with Microsoft PowerPoint.

Microinjection of morpholino antisense oligonucleotides (MO)

Microinjection into fertilized eggs was performed as described previously [23]. We used the following morpholinos (Gene Tools, Philomath, OR, USA) at the indicated concentrations in 24% glycerol in injection needles; Two different morpholinos blocking translation of ECM3 (*ecm3*-MO1 and *ecm3*-MO2) and QBRICK (*qbrick*-MO1 and *qbrick*-MO2) were used to confirm the specificity of ECM3 and QBRICK function. The efficacy of morpholinos was also confirmed by immunofluorescence using their specific antibodies (Fig. 2 and Supplementary Fig. 1). The morpholino sequences were the following:
ecm3-MO1: 5'-ATGGTGAAGAAAACTGCCGCTAAAC-3', *ecm3*-MO2: 5'-AAAGCTCTTGATGCAACACTCAAAC-3', *qbrick*-MO1: 5'-GTGATGCCTTTTACAGCATTCATCC-3', *qbrick*-MO2: 5'-GTCCAAGCCCAGAAGATGTGGTTAT-3, and *qbrick*-MO2inv: 5'-TATTGGTGTAGAAGACCCGAACCTG-3.

Dose effect of each MOs on gross developmental events such as gastrulation was checked and no non-specific toxic effect was observed under the concentration range employed in this study (Supplementary Fig. 2). No toxicity of high dose of MO on embryonic development was confirmed with 4.5 mM of *qbrick*-MO2inv, a control MO with an inverted sequence of *qbrick*-MO2 (Supplementary Fig. 2). Dose effect of MOs was summarized in Supplementary Fig. 3.

Quantitative RT-PCR analysis

Quantitative RT-PCR was performed as previously described [8]. The primer sequences were the following: *ecm3*: 5'-ACCCCAGTATGGCTACATCATGAAC-3' and 5'-GCCACCTGCATCTCTGACTGATACA-3',

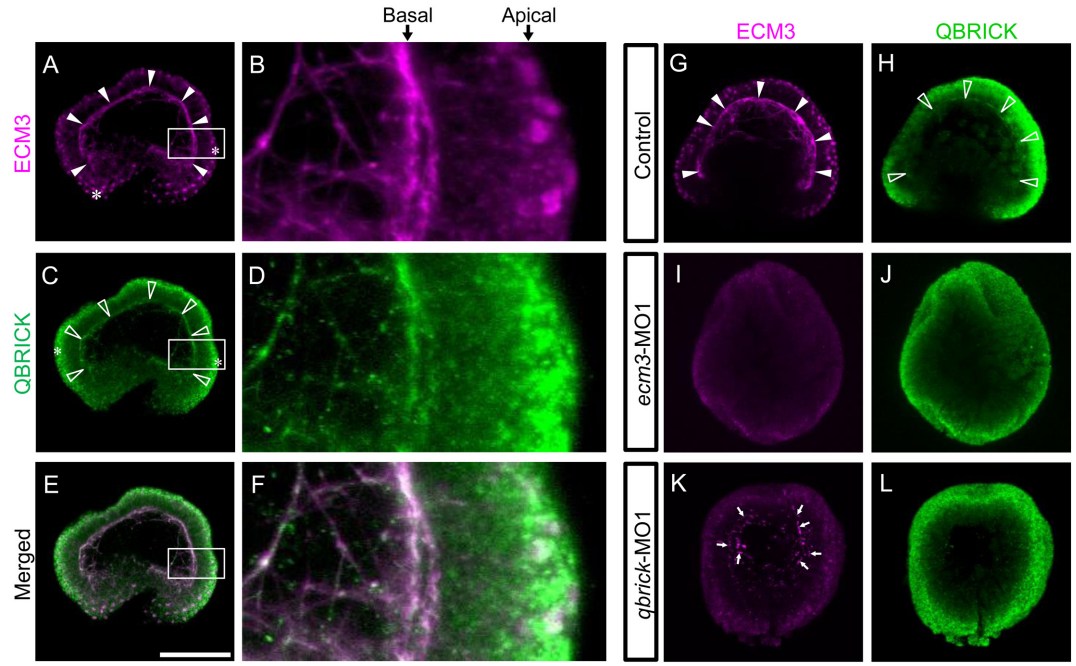

Fig. 2. Protein localization of 12-CSPG proteins ECM3 and QBRICK. (A–F) Co-immunofluorescence of embryos for ECM3 (magenta) and QBRICK (green) at 23 hpf. ECM3 (A, B), QBRICK (C, D), their merged view (E, F). Panels B, D, and F are magnified views of boxed area in panel A, C, and E. Closed and open arrowheads in (A) and (C) indicate blastocoel wall localization of ECM3 and QBRICK, respectively. Asterisks in (A) and (C) indicate apically localized immunoreactivities. Bar, 50 μm. (G–L) Immunofluorescence images of ECM3 and QBRICK in MO-injected embryos. Glycerol-injected (G, H) *ecm3*-MO1-injected (I, J), and *qbrick*-MO1-injected (K, L) embryos. Immunofluorescent signals of ECM3 (magenta in G, I, K), QBRICK (green in H, J, L) are shown. Closed and open arrowheads indicate ECM3 and QBRICK localizations in control embryos, respectively. Arrows indicate fragmented ECM3 localization in QBRICK morphants.

qbrick: 5'-TGCAAGGTGGAGGTGGCGCTCGAT-3' and 5'-TCGTAACTTTACCACATCATGGTC-3', and *mitCO1*: 5'-CCGCATTCTTGCTCCTTCTT-3' and 5'-TGCTGGGTCGAAGAAAGTTG-3'.

Results

FS-associated 12-CSPG ECM protein family in sea urchin *H. pulcherrimus*

Among human FS-associated ECM proteins, i.e., FRAS1, FREM2, and QBRICK, only ECM3 was reported as a ortholog of human FREM2 [16, 17]. Sea urchin proteins orthologous to human FRAS1 and QBRICK were therefore surveyed based on protein sequence homology search. By analyzing genomic and RNA sequences (http://cell-innovation.nig.ac.jp/Hpul/) [18], a full-length sequence encoding sea urchin QBRICK (HPU_07299) was identified (Fig. 1A). A mRNA sequence encoding sea urchin FRAS1 (HPU_12374) was also identified (Fig. 1A), but it lacks 5' region.

In mammals, there is another 12-CSPG family protein, named FREM3 [11, 24]; Mammalian FREM3 also possesses domain composition similar to other FS-associated ECM proteins, but *Frem3* mutant mice do not show FS-like defects [25]. Sea urchin FREM3 ortholog was not identified by the survey employed in this study (Figs. 1A and B).

Expression and localization of ECM3 and QBRICK in the developing sea urchin embryos

Among sea urchin *ecm3*, *qbrick*, and *fras1*, the expression of *ecm3* and *qbrick* was further investigated as their full-length mRNAs were identified. The expression of *ecm3* and *qbrick* transcripts was examined by quantitative RT-PCR, and both were expressed during early stages of embryonic development (Fig. 1C). The relative amount of *ecm3* transcript was 23–77 times more than that of *qbrick* throughout development.

To see protein distribution, antibodies specific to ECM3 and QBRICK were raised and immunofluorescence was performed. In 23 h post fertilization (hpf) embryos, ECM3 was present as fibrillar structures on the basal surface blastocoel wall (Figs. 2A and B). In addition to basal distribution, ECM3 was also present at the apical surface of the ectoderm as spotted pattern (Figs. 2A and G). QBRICK was present on the basal surface of ectoderm (Figs. 2C and D), where ECM3 and QBRICK

constitute together a two-dimensional mesh-like fibrillar structure (Figs. 2E and F). In addition to basal localization, QBRICK was also observed at the apical region of ectoderm as spotted pattern (Fig. 2C).

The apical and basal localizations of ECM3 were disappeared when the translation of ECM3 was attenuated by specific morpholino antisense-oligonucleotides (MOs) (Fig. 2I, Supplementary Fig. 1A). Basally localized QBRICK disappeared in QBRICK morphants (Fig. 2L), while apically localized signals still persisted, raising a possibility that apical anti-QBRICK immunoreactivity includes some non-specific signals. When ECM3 expression was attenuated by MOs, basally localized QBRICK was also disappeared (Fig. 2J). When QBRICK expression was attenuated by MOs, apical ECM3 disappeared, while basal ECM3 signal decreased and residual ECM3 did no longer show as fibrils (Fig. 2K, Supplementary Fig. 1A).

The ECM3 fibers localized on the basal surface of ectoderm were further investigated by projecting confocal stacks two-dimensionally. In control embryos, ECM3 was observed as highly branched mesh-like structure (Figs. 3A and B). When ECM3 was knocked-down by MOs, ECM3 protein deposition disappeared completely (Figs. 3C and D). When QBRICK was knocked-down, the basally localized ECM3 was found as very poorly branched short fragments (Figs. 3E and F).

Impaired PMC migration in FREM2/ECM3 and QBRICK knockdown embryos

As a prominent event during early developmental stages of sea urchin embryogenesis is the migration of PMCs, the effect of ECM3 and QBRICK knockdown on PMC migration was studied. At 30 hpf in control embryos, PMCs align in a ring-like pattern and some of them migrate the wall of the blastocoel (Figs. 4A and B). In ECM3 morphants, however, the PMC distribution was severely impaired; after ingression of PMCs, they were recognized just as two clusters of cell-aggregation but not as cells migrating along blastocoel wall (Figs. 4C and D, Supplementary Fig. 2). In QBRICK morphants, the distribution of PMCs appeared similar to that in control, but PMCs migrating along blastocoelar wall were not observed (Figs. 4E and F, Supplementary Fig. 4). We performed more detailed time-course study on the PMC migration during early developmental stages, and found that ECM3 and QBRICK are dispensable for PMC ingression and early PMC migration (Supplementary Fig. 4). After ingression, PMCs dispersed and migrated slightly toward animal pole at 21 hpf, then localized on vegetal plate at 24 hpf in both *ecm3* and *qbrick*-MO-injected embryos. Once PMCs located on vegetal plate, then they migrated along blastocoel wall, and this step was impaired by ECM3 or QBRICK knockdown. The number of total PMCs in QBRICK morphants was comparable to that of control embryos, whereas that in ECM3 morphants was uncountable due to the severe

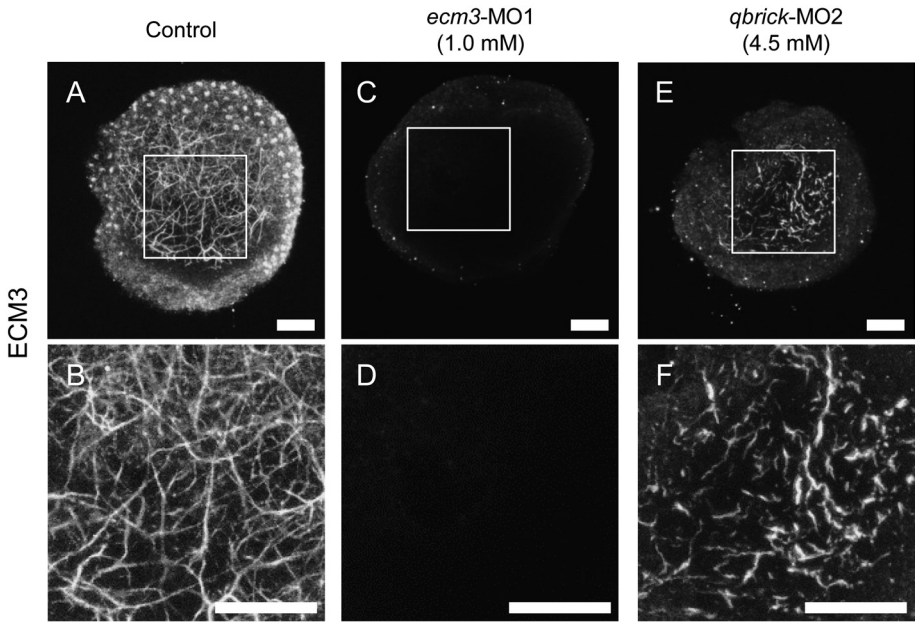

Fig. 3. Mesh-like fibrillar ECM3 on the basal surface of ectoderm. (A–F) Immunofluorescence of ECM3 in control (A, B), *ecm3*-MO (C, D), and *qbrick*-MO (E, F) injected embryos. B, D, and F are magnified views in the boxed areas in A, C, and E, respectively. Images are generated by projecting several z-stack images into a single plane. Bars, 20 μm.

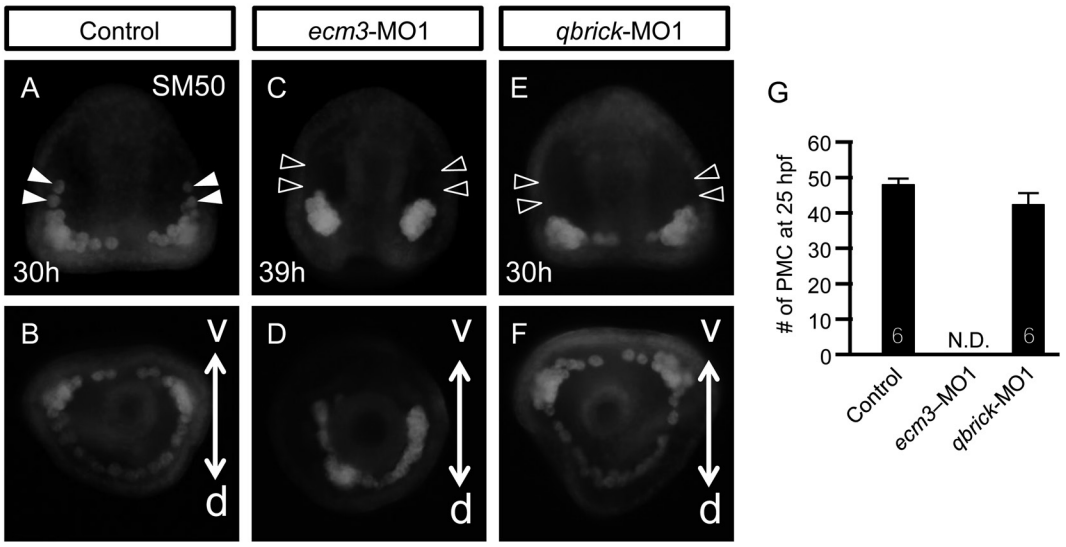

Fig. 4. PMC migration is abnormal in *ecm3* or *qbrick* knockdown embryos. PMC distribution are visualized by fluorescent *in situ* hybridization using *SM50* RNA-probe (green). (A, B) Glycerol-injected control embryos. (C, D) *ecm3*-MO-injected embryos. (E, F) *qbrick*-MO-injected embryos. The lateral (A, C, E) and vegetal (B, D, F) views of embryos are shown. Filled arrowheads in (A) indicate PMCs migrating along blastocoelar wall. Open arrowheads indicated the absence of migrating PMCs toward the anterior side in *ecm3* and *qbrick* morphants. (G) The number of PMCs in control or MO-injected embryos at 25 hpf. Each column represents mean number ± SD (bars). Numbers in bars represent *n*.

cell-aggregation (Fig. 4G).

Compared with PMC migration defects, key events of ectodermal development, including epithelial construction, oral-aboral polarity formation, opening mouth, and pigment cell localization on aboral side were not critically affected in ECM3 and QBRICK morphants (Supplementary Fig. 2).

Disordered spicule patterning by ECM3 and QBRICK knockdown

The spicule formation in the morphants was investigated further. At 72 hpf, compared with glycerol-injected control embryos (Figs. 5A and B), ECM3 morphants were abnormal; gastrulation occurred, but the aggregation of mesenchymal cells was still observed and no larval arms were formed (Fig. 5C). To examine the spicule formation quantitatively, embryos were smashed and the lengths of each part of spicules were measured (Fig. 5D). In ECM3 morphants, the spicules were severely affected and their length was too short to precisely measure (Figs. 5D, G and H). QBRICK morphants also possessed abnormal morphology with shorter arms (Fig. 5E), although such an abnormality appeared less severe than that observed in ECM3 morphants. The spicules were not fully but partially formed in QBRICK morphants (Fig. 5F). Preoral rods were often very short or missing in QBRICK morphants (Fig. 5F) and postoral rods were shortened, whereas body rods appeared normal (Figs. 5G and H). The overall features of spicule pattern observed in controls, ECM3 morphants, and QBRICK morphants were schematically summarized in Figs. 5I–K.

Discussion

Conserved 12-CSPG protein family in sea urchin

ECM3, a sea urchin ortholog of human FREM2 was previously reported [16, 17]. In addition to *ecm3*, sea urchin counterparts of human Fraser syndrome-associated genes *qbrick* and *fras1* were identified, although *fras1* mRNA information is still partial. These results suggest that all three genes encoding FS-associated ECM proteins are conserved between mammals and sea urchins. The expression of *qbrick* was confirmed at both transcript and protein levels. Although we did not focus on *fras1* in this study because of lacking a full-length information in the published mRNA database [18], it would be valuable to investigate FRAS1 protein expression and function by using specific antibody and MOs in the future study.

ECM3 and QBRICK protein distribution in *H. pulcherrimus* embryos

In *H. pulcherrimus* embryos used in this study, ECM3 was detected on the basal and apical surface of ectoderm; ECM3 constituted mesh-like fibrillar structure on the basal surface, while they localized in a punctate manner on the apical surface. It is reported that ECM3 protein

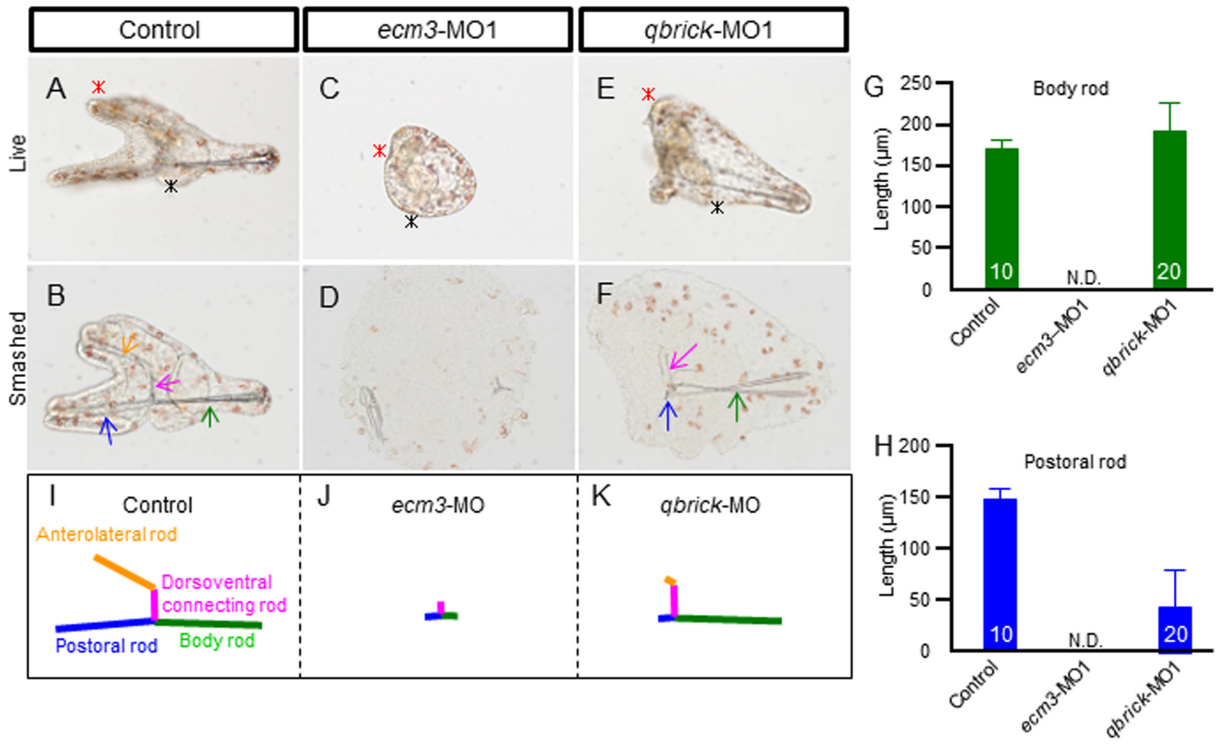

Fig. 5. Antisense morpholino knockdown of *ecm3* and *qbrick* causes skeletal patterning defects. (A–F) Glycerol or MO-injected embryos at 72 hpf. A, B, glycerol-injected control. C, D, *ecm3*-MO1 injected embryos. E, F, *qbrick*-MO1 injected embryos. Red asterisks, animal pole. Black asterisks, vegetal pole. In addition to side views (A, C, E), smashed views (B, D, F) are also shown to visualize endoskeletons. Arrows indicate each skeletal rod and arrow colors correspond to rod component indicated in (I). (G, H) The length of body rod (G) and postoral rod (H) in glycerol or MO-injected embryos. Each column represents average ± SD (bars). Numbers in bars represent *n*. (I–K) Schematic representation of skeletal rod pattern in control (I), *ecm3*-MO (J), and *qbrick*-MO (K) embryos.

is localized on the basal but not apical surface of ectoderm in green sea urchin *Lytechinus variegatus* [17]. The apical localization of ECM3 and QBRICK in addition to basal localization observed in *H. pulcherrimus* embryos in this study is probably species-specific variation. In Mediterranean sea urchin *Paracentrotus lividus*, apical localization of ECM protein Pl-nectin is reported [26], supporting the possibility of apical ECM protein localization in the embryonic ectoderm of *H. pulcherrimus*. Immunoreactivity against QBRICK was also detected on apical and basal surface. However, as apical anti-QBRICK immunoreactivity remained in embryos treated with *qbrick*-specific MOs, it cannot be concluded with present data that QBRICK localizes apically.

On the basal surface of ectoderm, ECM3 and QBRICK constituted together mesh-like fibrillar ECM structure. In mice, both FREM2, a mammalian ECM3 ortholog, and QBRICK are localized at basal surface of epithelium [6, 8] and they form a molecular complex together with FRAS1 *in vitro* [8]. These mammalian FREM2 and QBRICK are known to constitute fibrillar structure at the basal surface of epithelium, although such a fibrillar structure is barely visible at an electron microscopic level [11]. Collectively, the molecular property of ECM3 and QBRICK to form fibrillar ECM structure on the basal surface of epithelium is conserved beyond phylum.

Reciprocally stabilized expression of ECM3 and QBRICK

When QBRICK was knocked down, the apical and basal deposition of ECM3 diminished as shown in Figs. 2E, 3E and 3F. Also, when ECM3 was knocked down, at least the basal deposition of QBRICK disappeared (Fig. 2J). As the transcript levels of *ecm3* and *qbrick* were not critically affected by MO injection (Supplementary Fig. 1), these results suggest that ECM3 and QBRICK stabilize their deposition in the extracellular space each other. This relationship is similar to that observed in mammals, in which reciprocal stabilization among ECM3 ortholog FREM2, QBRICK, and FRAS1 has been reported [8, 11]. Therefore, our data strongly suggests that the ability of FS-associated ECM proteins to stabilize in a reciprocal manner is conserved between echinoderm and mammals. It seems possible that reciprocal protein stabilization between ECM3 and QBRICK occurs through fiber formation, as ECM3 became short

and poorly branched fragments when QBRICK was knocked-down. Since *ecm3* transcript is more abundantly expressed compared with *qbrick*, and ECM3 was diminished but still persistently expressed in QBRICK MO embryos, the major component of fibers is ECM3, whereas QBRICK may support and stabilize fiber formation and branching by associating with ECM3 fiber.

Contribution of ECM3 and QBRICK to embryonic development

In mammals, it has been suggested that 12-CSPG proteins regulate mesenchymal cell behaviors during morphogenesis from several experimental facts; the mesenchyme was dissociated from the basement membrane [2, 3, 6–8] and mesenchymal gene expression was altered [10, 27] in Fraser syndrome model mice. In sea urchin, the contribution of ECM3 to PMC migration was previously suggested [17] but has not been fully experimentally resolved yet. In this study with the genetic modification by MO knock-down, it was clearly shown that ECM3 and QBRICK participate in PMC migration and the following skeletogenesis during sea urchin embryogenesis.

Since basally localized ECM3 and QBRICK can directly interact with PMCs, it seems possible that the basally localized ECM3/QBRICK fibers function as an adhesive substrate for PMC migration along blastocoel wall, and fragmented ECM3-positive fibers in QBRICK knock-down embryos is less active compared with mesh-like fibrillar ECM3 (Fig. 3). Alternative interpretation is that ECM3 and QBRICK act on epidermis, then the PMC migration was secondary affected, as both proteins are abundantly localized around epidermis (Fig. 2). To conclude how ECM3 and QBRICK act on mesenchyme, further investigation including identifying adhesive receptors for ECM3-containing fibers would be necessary.

Sea urchin as a model experimental animal to study human diseases

Easy access by light and fluorescent microscopy to developing sea urchin embryos has unveiled that 12-CSPG protein family is indispensable in wide range of deuterostomes for proper embryonic development. In mice, the deposition of FREM2 and QBRICK at basement membrane zone is interdependent [8]. This interdependency probably reflects the formation of supramolecular complex [8], although how they form supramolecular structure has largely been unknown. In sea urchin embryos, we found that mesh-like fibrillar ECM formation is prerequisite for stable ECM3 and QBRICK deposition. This stabilization model appears to apply to mammals, as in mice 12-CSPG proteins were also observed to associate with thin fibrillar structures, although such a structure is barely visible at immunoelectron microscopic level [11]. To investigate stabilization mechanism of 12-CSPG proteins, sea urchins would be advantageous over mice as model animals, because thick fiber formation is easily visible at conventional confocal microscopy level. In addition to MO as employed in this study, successful CRISPR/Cas9-mediated genome editing in shorter breeding cycle species [28] has expanded the usefulness of sea urchin as an experimental animal model for human diseases. Further investigation of 12-CSPG proteins using sea urchin would not only clarify on the molecular mechanism of sea urchin development but also be beneficial for better understanding of 12-CSPG protein-related genetic disorders in human.

Conflicts of Interests

The authors declare no competing or financial interests.

Author Contributions

Conceptualization: D.K., S.Y., K.S.; Methodology: D.K., S.Y., J.Y., A.Y.; Investigation: D.K., S.Y., J.Y., A.Y.; Data curation: D.K., S.Y.; Writing − original draft: D.K., S.Y.; Writing − review & editing: D.K., S.Y., K.S.; Visualization: D.K., S.Y.; Supervision: D.K., S.Y., K.S.; Project administration: D.K., S.Y., K.S.; Funding acquisition: D.K., S.Y., K.S.

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