Stat3-Efemp2a modulates the fibrillar matrix for cohesive movement of prechordal plate progenitors

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ABSTRACT

 Recently, emerging evidence has shown that Stat3 controls tumor cell migration and invasion. However, the molecular mechanisms by which Stat3 controls the cell movement remain largely unknown. Embryonic gastrula progenitors display coordinated and orientated migration, called collective cell migration. Collective cell migration is the simultaneous movement of multiple cells and is universally involved in physiological and pathological programs. Stat3 activity is required for the migration of gastrula progenitors, but it does not affect cell specification, thus suggesting that gastrula movements are an excellent model to provide insight into Stat3 control of cell migration in vivo. In this study, we reveal a novel mechanism by which Stat3 modulates extracellular matrix (ECM) assembly to control the coherence of collective migration of prechordal plate progenitors during zebrafish embryonic gastrulation. We show that Stat3 regulates the expression of Efemp2a in the prechordal plate progenitors that migrate anteriorly during gastrulation. Alteration of Stat3-Efemp2a signaling activity disrupted the configuration of fibronectin (FN) and laminin (LM) matrices, resulting in defective coherence of prechordal plate progenitor movements in zebrafish embryos. We demonstrate that Efemp2a acts as a downstream effector of Stat3 to promote ECM configuration for coherent collective cell migrations in vivo.

KEY WORDS: Stat3-Efemp2a signaling, Extracellular matrix, Collective cell migration

INTRODUCTION

 The Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway transfers signals derived from intrinsic and environmental stimuli to control the expression of target genes by the binding of STAT proteins to gene promoters (Aaronson and Horvath, 2002). In mammals, there are several STAT genes, including STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B and STAT6. The fact that disruption of the Stat3 gene leads to early embryonic lethality in mice indicates that Stat3 performs important and unique functions in vivo (Takeda et al., 1997). Constitutively active STAT3 has been identified in a number of human tumor types. Emerging evidence shows that STAT3 controls cell migration, motility and invasion in human tumor cells (Azare et al., 2007; Teng et al., 2009). STAT3 has been found to promote tumor invasion and metastasis in various cancers, including ovarian, bladder, pancreatic and prostate carcinomas and melanoma. Consistent with these pathological roles, conditional knockout of Stat3 in mouse keratinocytes blocks the wound-healing process in skin (Sano et al., 1999). Stat3 has also been found to control the cell migration of mouse embryonic fibroblasts and keloid-derived fibroblasts (Lim et al., 2006). These data suggest that regulation of cell migration is an essential function of Stat3. However, the molecular mechanism by which Stat3 controls cell migration remains largely unknown.

During gastrulation in zebrafish embryonic development, Stat3 is activated on the dorsal side of the embryo via the maternal Wnt/β-catenin pathway. Stat3 activity is required for the anterior migration of prechordal plate progenitors. Knockdown of Stat3 causes abnormal anterior migration, resulting in a mispositioned head and a shortened anterior-posterior (AP) axis, but produces no defects in early cell fate specification (Yamashita et al., 2002). As a transcription factor, Stat3 controls the expression of downstream genes to regulate essential functions in many biological progresses. For example, Liv1, a breast cancer-associated zinc-transporter protein, functions as a downstream target of Stat3 in the epithelial-mesenchymal transition (EMT) during zebrafish gastrulation (Yamashita et al., 2004). Stat3 has been shown to play crucial roles during gastrulation, and the study of gastrula movements might provide insight into the molecular mechanisms by which Stat3 controls cell migration in vivo.

Gastrulation during early embryonic development is an excellent model for the coordinated and orientated cell migration of groups of cells (McMahon et al., 2008; Arboleda-Estudillo et al., 2010; Dumortier et al., 2012). The coordinated and orientated migration of a group of cells, called collective cell migration, is essential for many developmental, physiological and pathological progresses, including embryonic morphogenesis, wound healing, tissue regeneration and invasion of cancers of epithelial origin (Friedl and Gilmour, 2009). A series of collective cell movements, including epiboly, internalization and convergent extension, shapes the basic body plan and forms the embryonic germ layers during embryonic gastrulation (Rohde and Heisenberg, 2007). The key movements of the gastrula cells are convergence and extension movements that narrow and lengthen the embryonic body, respectively. Several distinct cell populations undergo collective cell movements during convergence and extension, including the prechordal plate progenitors migrating toward the animal pole as a cohesive sheet of cells and the lateral mesendoderm cells moving toward the embryonic body axis (Montero et al., 2005; Arboleda-Estudillo et al., 2010).

The EGF-containing fibulin-like extracellular matrix protein 2a (Efemp2, also known as Fibulin 4/Fbln4) is an ECM protein and member of the fibulin family. The fibulin family proteins are characterized by a carboxy-terminal fibulin domain and several repeated calcium-binding epidermal growth factor domains
Targeted disruption of Efemp2 abolishes elastogenesis and causes severe lung and vascular defects (McLaughlin et al., 2006). Mutations in EFEMP2 in humans lead to diaphragmatic hernia, aortic aneurysm, tortuous pulmonary arteries and mild generalized lax skin (Dasouki et al., 2007). During elastogenesis, Efemp2 tethers lysyl oxidase (LOX) to tropoelastin to form intact elastic fibers (Horiguchi et al., 2009).

In this study, we show that Stat3 regulates the expression of efemp2a in prechordal plate progenitors. In a series of in vivo and in vitro experiments, we found that Efemp2a regulated the cell-driven remodeling of ECM structures and modulated the self-assembly of FN and LM proteins into fibrillar networks. Alteration of Stat3 or Efemp2a activity disorganized the FN and LM fibrillar matrix in zebrafish embryos, resulting in defective coherence in the collective movements of prechordal plate progenitors. Efemp2a was able to rescue the defective FN and LM fibrillar matrix and, subsequently, the abnormal coherent migration of gastrula progenitors in Stat3-deficient embryos during gastrulation. Our results indicate that Efemp2a acts as a downstream effector of Stat3 to modulate ECM assembly for coherent collective cell migrations in vivo.

**RESULTS**

**Efemp2a functions as a downstream target of Stat3 for the gastrula movements during zebrafish embryonic development**

Coherent cell movement is essential for gastrulation. To date, several guidance cues that determine the direction, motility and cohesion of gastrula cell migration have been identified. Among these, Stat3 activity is required for the anterior migration of prechordal plate progenitors, but it shows no effects on cell fate specification. Stat3 can serve as an excellent model for understanding the mechanisms by which the gastrula movements are regulated during embryonic development, and we therefore examined the function of Stat3 in gastrula migration in the zebrafish embryo. First, we screened the gene expression in Stat3-deficient embryos. Expression of the gene efemp2a was significantly reduced in the posterior axial mesendoderm (Fig. 1A-D). Consistent with these results, the expression of efemp2a was elevated in the anterior axial mesendoderm (Fig. 1E,F). The cells of the leading edge became spherical and separated, and the prospective prechordal plate undergoing coherent movement. The definitive endoderm expressing sox32 showed no trend toward convergence and the distribution of prechordal plate progenitors expressing gsc was disordered in Efemp2a-depleted embryos (Fig. 2E–F'). These data indicate that knockdown of Efemp2a induces severe impairment in anterior migration and convergent movements, resulting in mispositioning of anterior tissue and a shortened AP axis. However, we observed no significant changes of ntl, lefty (lft) and gata5 expression, which are related to mesendoderm specification, at the onset of gastrulation in Efemp2a morphants (Fig. 2G–J'). Our results indicate that Efemp2a function correlates with gastrula migration but not cell specification.

We tracked the prechordal plate progenitors by time-lapse imaging during mid-gastrulation and assessed the cell behaviors as described previously in Stat3- or Efemp2a-deficient conditions (Kai et al., 2008) (supplementary material Fig. S3A). These monitored cells appeared to move slowly and with reduced coherence and persistence in either Stat3-depleted or Efemp2a-depleted embryos (Fig. 3A,B). Efemp2a mRNA could restore the lower persistence (77.8%, n=9) and coherence (66.7%, n=9) caused by itself deficiently available, whereas coherence (66.7%, n=9) rather than persistence was restored by the addition of efemp2a mRNA to Stat3-depleted embryos, resulting in recovery of the migration distance along the AP axis (Fig. 3B). Next, we examined the morphology of the prospective prechordal plate undergoing coherent movement. The cells of the leading edge became spherical and separated, and they frequently crawled over one another (supplementary material Fig. S5B,C) in both Stat3- and Efemp2a-deficient embryos. In particular, there were more spaces between these cells, resulting in a wider region of gsc expression in each type of morphant (supplementary material Fig. S5E). In control embryos, the cells were spread and contacted one another (supplementary material Fig. S5A). These morphological changes were consistent with the lower coherence in Stat3- or Efemp2a-deficient embryos. We also examined the speed, persistence and coherence of lateral mesendoderm cells by differential interference contrast microscope (DIC) time-lapse (supplementary material Fig. S3B and Fig. S4). Neither Stat3 nor Efemp2a induced coherence defects (supplementary material Fig. S4B). These results indicate that Stat3-Efemp2a signaling mainly controls the coherent migration of
prechordal plate progenitors and not the persistence, which might be controlled by other Stat3 target genes.

**Efemp2a deficiency disrupts focal adhesions, thereby affecting the generation of actin stress fibers and cellular protrusions**

As described previously, the oriented protrusions toward the animal pole are required for the anterior migration of prechordal plate progenitors (Dumontier et al., 2012). Three types of cellular protrusions (lamellipodia, filopodia and blebs) have been observed in these progenitors (Diz-Muñoz et al., 2010) (Fig. 4A-C).

We examined the frequency of protrusions from prechordal plate progenitors during mid-gastrulation. When Efemp2a was depleted, the frequency of blebs was significantly increased, accompanied by the generation of fewer filopodia and lamellipodia (Fig. 4D). Lamellipodia and filopodia depend on actin filaments (Diz-Muñoz et al., 2010). Therefore, we injected Lifeact-GFP cDNA into embryos to visualize the changes to actin filaments in actin-rich protrusions. Long protrusions containing actin were detected in control migrating progenitors (Fig. 4Ea-Ee). By contrast, actin formed foci and did not generate lamellipodia or filopodia in Efemp2a-deficient progenitors (Fig. 4Ec-Ef).
Previous work has shown that cell migration requires cellular protrusions to couple to the ECM through the plasma membrane via focal adhesions (Gupton and Waterman-Storer, 2006). Focal adhesions that are formed by adhesion complexes perform crucial scaffolding and signaling roles in the interaction of cells with the ECM (Carragher and Frame, 2004). Therefore, we detected the focal adhesion components Fak (Protein tyrosine kinase 2ab/Ptk2ab – Zebrafish International Resource Center) and Vinculin in the prechordal plate region. Focal adhesions were dramatically diminished or disrupted in Efemp2a-deficient embryos (Fig. 4F). This phenotype was also observed in cultured HUVEC cells infected with an adenovirus expressing Efemp2a full-length protein or its mutant C350 on FN or LM substrate. Both Efemp2a and C350 were able to disrupt the formation of focal adhesions and actin fibers in cells (Fig. 4H,I). Together, these results suggest that the decrease in focal adhesions disrupts the formation of actin-based cellular protrusions in Efemp2a-deficient embryos, and there is probably a correlation between the reduction in focal adhesions and the ECM.

**Efemp2a interacts with soluble FN and LM to shape their matrices**

To understand the character of Efemp2a, we transplanted prechordal mesendodermal (PCME) cells from the shield of Efemp2a-MO-injected or Cll-MO-injected donors into the shield of wild-type or Efemp2a-depleted host embryos and monitored their migration (supplementary material Fig. S6A,C). We also co-transplanted lateral mesendodermal (LME) cells from Efemp2a-MO or Cll-MO donors into the lateral blastoderm margin of wild-type or Efemp2a-depleted host embryos (supplementary material Fig. S6B,D). These results demonstrate that Efemp2a plays a non-cell-autonomous role in controlling the migration of gastrula cells. Combined with immunoblots of human cells ectopically expressing Efemp2a, these results confirm that Efemp2a is an extracellular protein (supplementary material Fig. S6E).

Efemp2a has been reported previously to promote the assembly of Elastin, an ECM component, and we therefore examined the expression of elastin during gastrulation. FN and LM, two other ECM components (supplementary material Fig. S7B,C), but not Elastin (supplementary material Fig. S7A), were expressed during gastrulation in zebrafish embryos. Therefore, we hypothesized that Efemp2a acts on the expression or function of FN or LM. The mRNA expression levels of FN and LM were not altered in Efemp2a-depleted embryos (supplementary material Fig. S8). Subsequently, we examined the interactions between Efemp2a and FN or LM via co-immunoprecipitation. The collected culture media containing abundant GFP-tagged Efemp2a or C350, and little FN and LM (Fig. 5B,G,M-O), were incubated with or without soluble FN or LM. After overnight incubation, the supernatant and deposition were separated from each mixture by centrifugation. The supernatants were subjected to immunoprecipitation with anti-GFP, anti-FN or anti-LM antibody, and the GFP-tagged proteins were observed to actively associate with FN or LM (Fig. 5D-F,I-L). By contrast, few GFP fusion proteins were detected in depositions containing abundant FN or LM (Fig. 5P-T), and it was likely that a fraction of Efemp2a, C350 and GFP non-specifically followed insoluble polymers during centrifugation (Fig. 5U). These immunoprecipitation assays indicate that Efemp2a and its mutant C350 are able to interact with soluble FN and LM. However, these interactions cannot maintain binding with insoluble polymers of FN and LM, suggesting that Efemp2a is involved in the function of FN and LM.

Next, we examined FN and LM self-assembly with and without Efemp2a or the C350 mutant in vitro. FN and LM proteins were able to self-assemble into fine fibers with branches in the presence of fetal bovine serum (FBS) or only bovine serum albumin (BSA) in Hanks’ Balanced Salt Solution (HBSS) (Fig. 6A,B,E). In the presence of purified GFP-tagged Efemp2a, the FN and LM fibres became more branched and thicker, respectively (Fig. 6A,B,E), and then the depositions of FN and LM became larger and denser than the controls (Fig. 6C,6D,6F). Altogether, Efemp2a displays a modulatory function on the assembly of FN and LM into insoluble fibrillar meshwork. Given
the multi-cellular microenvironment *in vivo*, we tested the effect of Efemp2a on ECM formation in cultured MDCK or HeLa cells. These cells, which were infected with or without C350 adenovirus (Ad-C350), were cultured with different concentrations of recombinant EFEMP2 in combination with FN or LM. Excess EFEMP2 protein could promote FN and LM deposition compared with control, and the stimulatory effects depended on the dose of EFEMP2 (supplementary material Fig. S9Aa-Ad,Ba-Bd). By contrast, in cells overexpressing the C350 mutant, the assembly of FN and LM was perturbed, resulting in failure to generate fine FN or LM matrices (supplementary material Fig. S9Af,Bf). Notably, the effect of EFEMP2 could be disrupted by the Efemp2a mutant C350 protein (supplementary material Fig. S9Ae,Be). Thus, Efemp2a can, indeed, further FN and LM assembly.

**Stat3-Efemp2a controls the configuration of the ECM for the gastrula migration in zebrafish embryos**

We next assessed whether knockdown of Efemp2a or Stat3 disrupted ECM assembly *in vivo*. Immunostaining in whole embryos and 10-μm sections of zebrafish embryos was performed to examine the structure of the ECM. In the control embryos and sections, FN and LM ECM assembled continuous fibrillar matrices in the anterior regions (Fig. 7Aa,Af,Ba,Bg). By contrast, the fibrillar mesheswork of FN and LM were disrupted in Efemp2a- or Stat3-deficient embryos, and the deposition of FN and LM was scattered (Fig. 7Ah,Ac,Ag,Ah,Bb,Bc,Bh,Bi). The ECM defects in Efemp2a- or Stat3-depleted embryos were rescued by an appropriate level of supplementation with zebrafish *efemp2a* mRNA (Fig. 7Ad,Ac,Ai,Aj,Bd,Be,Bj,Bk). However, overexpression of *efemp2a* mRNA also disrupted the continuous FN and LM ECM fibrillar matrix and induced the formation of dense foci within the gastrulae (Fig. 7Bf,Bi). Examination of the embryo ultrastructure also showed defective ECM in the Stat3- or Efemp2a-deficient embryos (Fig. 7C). It has been reported that the assembly and three-dimensional fibrillar organization of FN regulate cell adhesion, spreading and migration in *Xenopus* embryos (Darribère and Schwarzbauer, 2000; Rozario et al., 2009). The 70-kD N-terminal fragment of FN (70 kD-FN) was shown to obstruct FN matrix formation (Rozario et al., 2009). We confirmed that 70 kD-FN perturbed the formation of the FN fibrils *in vitro* and in zebrafish embryos (supplementary material Fig. S10A-F). Tracking the migrating prechordal plate progenitors in the embryos injected with 70 kD-FN (70 kD-FN) was shown to obstruct FN matrix formation (Rozario et al., 2009). We confirmed that 70 kD-FN perturbed the formation of the FN fibrils *in vitro* and in zebrafish embryos (supplementary material Fig. S10A-F). 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These phenotypes are highly similar to those caused by the disruption of Stat3-Efemp2a signaling. Collectively, these results indicate that Stat3-Efemp2a signaling modulates ECM assembly for the cohesive migration of prechordal plate progenitors in embryos during gastrulation. Previous studies have shown that disruption of the ECM or Stat3 can cause an epithelial-mesenchymal transition (EMT) (Yamashita et al., 2004; Radisky, 2005). However, ISH showed that the expression of EMT marker genes, including e-cadherin (cdh1 – Zebrafish International Resource Center), tjp1a, n-cadherin (cdh2 – Zebrafish International Resource Center) and snai1b, were not coincident changes following alteration of efemp2a activity in vivo (supplementary material Fig. S11A). In vitro, the protein expression level of EMT markers, including E-cadherin, ZO-1, N-cadherin and Zeb1, were also not significantly changed in cultured HeLa cells overexpressing Efemp2a or C350 (supplementary material Fig. S11B). These results exclude the possibility that a shift of the activity in Efemp2a induces EMT to affect the migration of gastrula cells. Moreover, we detected the expression of focal adhesion receptors, including itgav, itgb1 and sdc4, in Efemp2a-depleted embryos. These results showed that itgav expression was reduced, but itgb1 and sdc4 expression levels were increased in the prechordal plate region (supplementary material Fig. S12). Thus, the data do not indicate that Efemp2a controls ECM assembly through the integrin or syndecan pathways.

In summary, we show that Stat3 controls efemp2a expression in the prechordal plate progenitor cells (supplementary material Fig. S13). Efemp2a protein is secreted to promote FN and LM assembly into a matrix. The proper FN and LM fibrillar matrices maintain collective migration for correct embryonic development at early stages. Because ECM assembly and collective cell migration are involved in cell proliferation, differentiation, migration and survival in many biological processes, such as immune response, hematopoiesis, neurogenesis and tumorigenesis, our study provides new insights into the molecular mechanisms underlying how ECM assembly and collective cell migration may function together to perform these biological processes in vivo.
DISCUSSION

Efemp2a controls the proper spatiotemporal formation of FN and LM fibrillar matrix in vivo

In the present study, we found that Efemp2a was expressed earlier than tropoelastin during embryonic development, indicating that Efemp2a performs unknown functions during early embryonic development. Indeed, the results presented here demonstrate that Efemp2a performs an essential function in the modulation of FN and LM assembly in vitro or in vivo. We show that FN and LM undergo self-assembly in vitro. Under conditions of HBSS with serum or BSA, FN and LM are able to form fine fibrils with branches. Full-length Efemp2a increases the branching of FN and the average diameter of LM fibers. These FN or LM fibrils form larger and denser depositions compared with controls. The Efemp2a mutant, C350, reduces the ability of both FN and LM to form fine fibrils with branches, and these short fibrils do not readily generate a fine meshwork. In vivo, knockdown of efemp2a expression in zebrafish embryos disrupts the assembly of FN and LM into fibrillar meshwork. In addition, overexpression of...
efemp2a mRNA induces the formation of FN and LM foci. These results are consistent with previous observations that FN and LM assembled into fibers and fibrillar matrices without any additional enzymatic proteins and/or energy (Ulmer et al., 2008). Here, we show that Efemp2a can interact with soluble FN and LM. Increased or decreased efemp2a expression, or overexpression of its mutant, is able to disrupt the fine FN and LM fibrillar matrix, but it does not disturb the assembly of FN and LM in vitro and in vivo. These findings indicate that the function of Efemp2a in the formation of FN and LM fibrillar matrix is different from its role in the formation of elastic fibers. These data also indicate that Efemp2a directly promotes the assembly of LM and FN to form a matrix without the assistance of other enzymatic proteins. Additional in vitro biochemical experiments are required to address the biochemical mechanism by which Efemp2a molds the LM and FN matrix.

Stat3-Efemp2a shapes the matrix path for coherent collective migration

We analyzed the behavior of gastrula progenitors via time-lapse movies of living zebrafish embryos during gastrulation and observed that the prechordal plate progenitors migrate directionally as a group with cell-cell connections, consistent with previous findings (Dumortier et al., 2012; Tada and Heisenberg, 2012). During migration, the leader cells of the group extend forward by cell protrusions. As described previously, we also noticed that, during the progression of migration, the gastrula progenitors continuously express E-Cadherin along the interface between the epiblast and hypoblast throughout gastrulation (Montero et al., 2005).

On close review of the video data, we noticed that the collective migration of the prechordal plate progenitors is highly similar to the multicellular broad and flat rim of epithelial sheet migration (Khalil and Friedl, 2010). The leading edge cells in the migrating sheet of gastrula progenitors explore the environment, identify the migration path and form traction. As described previously, during the process of collective migration, the migrating cells make physical contact with the adjacent matrix (Montell, 2008). The contacts and subsequent crosstalk between the cells and matrix lead to the generation of a fibrillar matrix that acts as a track and supports the migration processes (Winklbauer and Nagel, 1991; Wang et al., 2008). The matrix track has structural and molecular properties that can perform additional functions in addition to guiding the direction of cell migration. For example, the newly formed matrix promotes the maintenance of collective front-rear polarity during collective cell migration. Indeed, we find that proper FN and LM assembly into a matrix beneath deep mesendoderm cells coincides with the migration of prechordal plate progenitors, suggesting that the FN and LM fibrillar matrix supports and directs the collective cell migration of gastrula progenitors. Under conditions in which knockdown of Efemp2a expression disrupts the structures of FN and LM matrix, the gastrula progenitors maintain their mobility and randomize migrating direction as individual cells, rather than as a coherent group of cells. The randomized migration of the
progenitors causes a mispositioned head and a shortened AP axis during gastrulation. These results indicate that the proper FN and LM fibrillar meshwork beneath deep mesendodermal cells is required for maintaining collective migration and for ensuring the appropriate direction of migration of the prechordal plate progenitors for correct embryonic development.

During embryonic gastrulation, the ECM components LM and FN are highly expressed in the anterior regions and form a continuous fibrillar meshwork. The ECM assembly is controlled by Stat3-Efemp2a signaling. Ectopic expression of Efemp2a can rescue the ECM defects and the lower coherence of migrating prechordal plate progenitors caused by Stat3 deficiency. In conclusion, our data demonstrate that Efemp2a is an effector of Stat3 signaling and plays a non-cell-autonomous role to control the coherence of cellular migration in gastrulation in zebrafish embryos.

MATERIALS AND METHODS

Zebrafish strains
Zebrafish were raised and maintained following standard procedure. Wild-type zebrafish belonged to the AB strain. All experiments involving the use of animals were conducted in compliance with the approved guidelines. The animal protocols were approved by the Animal Care and Use Committee of West China Hospital, Sichuan University, China.

Efemp2a cloning
Efemp2a cDNA was amplified from zebrafish embryos using the primers 5′-ATCCACTGGGCCGTTCCTCCTC-3′ and 5′-GACATGCAGAAATTGCCTCGT-3′ and subcloned into the pGEM-T easy vector to synthetize antisense or sense probes for in situ hybridization. The coding region of Efemp2a was subcloned into the pcDNA3.1 vector for mRNA synthesis.

In situ hybridization
The efemp2a antisense RNA probe was synthesized using a digoxigenin RNA labeling kit (Roche). Whole-mount in situ hybridization was performed as previously described (Thiss and Thiss, 2008).

Morpholino knockdown
Efemp2a tbMO (5′-CCGCATCTCTACACACCCTCTCATAC-3′), the primary MO used, targets the 5′ sequence spanning the start codon, and Efemp2a spMO (5′-AGGTTTCGCTCTTACCGTGA-3′) blocks the splicing of efemp2a mRNA. Stat3 MO (5′-GCCATGTGTGCCCCTTAATGATGTCCG-3′) and Stat5.1 MO (5′-GTTGACTTACACGAGTTGTTCC-3′) were reported in previous studies (Yamashita et al., 2002; Liu et al., 2009). A standard control MO was used as the control.

Fig. 7. Efemp2a activity modulates the assembly of FN and LM in zebrafish embryos. (A) Whole-mount immunofluorescence staining for FN (a-e) and LM (f-j) in embryos injected with Ctl-MO (a,f), Stat3-MO (b,g), Efemp2a-MO (c,h), Stat3-MO combined with efemp2a mRNA (d,i), or Efemp2a-MO combined with efemp2a mRNA (e,j). Dashed lines indicate the leading edge of the prechordal plate in DAPI staining images. Animal-pole views show dorsal on the top. Scale bars: 100 µm. (B) Immunostaining analysis of the FN and LM matrix in sagitally sectioned embryos, injected with Ctl-MO (a,g), Stat3-MO (b,h), Efemp2a-MO (c,i), Stat3-MO combined with efemp2a mRNA (d,j), Efemp2a-MO combined with efemp2a mRNA (e,k) or efemp2a mRNA (f,l) at the end of gastrulation, using FN (a-f) and LM (g-l) antibodies. The arrowheads indicate the FN or LM matrix. Scheme shows sagittal section. A, anterior; P, posterior; Nc, notochord (m). Lateral views show dorsal to the right and anterior on the top. Scale bar: 200 µm. (C) TEM of sagitally sectioned embryos injected with Ctl-MO (a), Stat3-MO (b) or Efemp2a-MO (c) at the end of gastrulation. The arrowheads indicate the ECM structure and asterisks highlight the cells. Embryos were at the tail-bud stage (A-C). Scale bar: 500 nm.
Construction of Efemp2a mutants

Six mutants of zebrafish Efemp2a were designed based on conserved domain analysis and generated by PCR. The C-terminal deletion mutants included C70, C140, C210, C280 and C350. The N-terminal deletion N10 mutant lacks the signal peptide. Capped efemp2a mutant mRNA and full-length mRNA that lacked the morpholino sequence were synthesized as previously described (Zhang et al., 2011).

Microinjection

Injection of mRNA constructs or morpholinos was performed at the one- to four-cell stages. In all experiments, 8 ng Efemp2a-tbMO, Efemp2a-spMO or Stat3-MO was injected with 80 pg C350 mRNA. For the overexpression experiment, 100 pg efemp2a mRNA was also injected. For recovery experiments, 60 pg or 30 pg efemp2a mRNA was also injected.

Immunofluorescence staining

Immunofluorescence staining was performed as previously described (Zhang et al., 2011) using the following antibodies: anti-Efemp (Abnova, H00030008-M01; 1:400); anti-Fibronectin (Sigma, F3684; 1:400); anti-Laminin-1 (Thermo Scientific, RB-082-A1; 1:400); anti-Fak (Santa Cruz, sc-558; 1:500); and anti-Vinculin (Millipore, MAB3574; 1:500).

Time-lapse imaging and cell movement analysis

Time-lapse imaging of zebrafish embryos during gastrulation was performed as previously described (Arboleda-Estudillo et al., 2010). Embryos were flat-mounted and imaged under a Leica DM6000B fluorescence microscope or SP5 confocal microscope with 20° or 40° objectives. Images were taken at one frame per 30 s for 80 cycles during tracking of prechordal plate progenitors or at one frame per 20 s for 60 cycles during monitoring of lateral mesendoderm. ImageJ (NIH) software was used to track randomly chosen cells in every frame and to calculate parameters including speed, persistence and coherence was performed as previously described (Kai et al., 2008; Arboleda-Estudillo et al., 2010). For analysis of the frequency of cellular protrusions, frames were captured at 12 s intervals for 10 min by DIC time-lapse using a Zeiss Imager Z1 microscope with a 20° objective. Three independent experiments were performed, and Student’s t-test was used to evaluate comparisons between two groups.

Cell transplantation

Embryos were injected with Ct1-MO combined with FITC-dextran (Invitrogen) or with Efemp2a-MO combined with Rhodamine-dextran (Invitrogen), and donor cells were harvested. Cells were transplanted into wild-type host embryos or Efemp2a-MO-injected host embryos as previously described (Yao et al., 2010).

FN and LM self-assembly in vitro

Media were collected from cultured AD293 cells overexpressing GFP, Efemp2a-GFP or C350-GFP from adenovirus infection. The 10 ml of harvested culture medium was mixed with 20 µl Protein A/G agarose beads and 2 µg anti-GFP antibody (Novus, NB1-47584; 1:5000). The mixtures were rotated overnight at 4°C and then centrifuged at 1000 rpm for 1 min. The harvested beads absorbed GFP, Efemp2a-GFP or C350-GFP protein. These agarose beads were combined with 4 µmol soluble FN or LM and added into 1 ml HBSS with 10% FBS or 1% BSA, and these mixtures were rotated for 1 h at room temperature (RT). Next, the mixtures were centrifuged at 3000 rpm for 5 min, and immunofluorescence staining was performed for the precipitations, which included the insoluble production by FN or LM self-assembly and agarose beads, using anti-FN (Sigma, F3684; 1:500) or anti-LM (Thermo Scientific, RB-082-A1; 1:500) antibody. Finally, the precipitations were dropped onto glass slides, mounted and then observed using a fluorescence microscope. For measurements of mean density of the FN or LM matrix, Rhodamine-tagged FN or LM (Cytoskeleton) was used. The agarose beads absorbing GFP, Efemp2a-GFP or C350-GFP, together with 4 µg Rhodamine-tagged FN or LM, were added into 1 ml HBSS with 1% BSA and incubated for 1 h at RT. The mixtures were dropped onto glass slides, mounted and observed using a fluorescence microscope. The mean density (IOD sum/area sum) was measured by Image-Pro Plus 6.0 software.

Immunoprecipitation and immunoblotting

The media from cultured AD293 cells overexpressing GFP, Efemp2a-GFP or C350-GFP by adenovirus infection were collected. Soluble FN (Millipore) or LM (Sigma) was added into the medium and incubated for 1 h at RT. The supernatant and deposition were separated by centrifugation at 3000 rpm for 5 min. The supernatant, protein A/G agarose beads (Millipore) and antibody (anti-GFP, anti-FN or anti-LM antibody) were mixed and rotated overnight at 4°C. The agarose beads were harvested by centrifugation at 1000 rpm for 1 min and then washed three times. Samples were separated by SDS-PAGE, followed by electrophotic transfer onto a membrane. The membranes were blocked and immunoblotting was performed using the following antibodies: anti-Fibronectin (Sigma, F3684; 1:1000), anti-Laminin-1 (Thermo Scientific, RB-082-A1; 1:1000) and anti-GFP (Novus, NB1-47584; 1:1000). The GFP-tagged FN and LM in depositions were also detected by western blotting.

Chromosomal immunoprecipitation assay

ChiP was performed as described previously (Liu et al., 2012). Briefly, 150 embryos were injected with Stat3-Flag plasmid and harvested at the 20-somite stage. The embryos were placed in 1% formaldehyde, homogenized and then sonicated to generate 300-500 bp chromatin fragments. Fragmented chromatin was immunoprecipitated with Flag (Sigma, F1804; 1:1000) or IgG (Santa Cruz, sc-2025; 1:250) antibody. Nucleic acids were precipitated with 100% ethanol and used for PCR. Primer P1: 5′-CAATGGAGTACAAATATTGCA-3′ and 5′-TTAATCACACGGTGTCCTAAATA-3′; primer P2: 5′-ACAACACGGACCCGAGATT-3′ and 5′-TTTATGGCATTCCACCGT-3′.

Cell culture

The cell lines AD-293, MDCK and HUVEC were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal calf serum (FCS). HeLa cells were cultured in RPMI-1640 medium containing 10% FCS. All cultures were incubated at 37°C with 5% CO2. Medium was changed every 48 h. Soluble FN or LM and recombinant Efemp2 protein (Abnova) were added to the medium when required for the experiment.

Construction and infection of adenovirus

The recombinant adenovirus plasmids obtained by AdEasy technology were deployed with PacI and transfected into AD-293 cells using transfection reagents as described previously (Luo et al., 2007). After collection of the supernatants from primary viral transfection, AD-293 cells were infected by viral supernatants for the amplification and purification of high-titer recombinant adenoviruses. The cultured HeLa cells, HUVEC cells or MDCK cells were infected by viral supernatants containing high-titer recombinant adenoviruses. After 24 h, the infected cells were used for harvest in immunofluorescent staining.

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Competing interests

The authors declare no competing financial interests.

Author contributions

T.Z. and C.Y. designed, performed and analyzed experiments, and wrote this manuscript. L.Q. helped with the molecular and biochemical experiments. L.J., H.L., C.X. and N.L. helped with the molecular experiments; and S.L. helped with the transmission electron microscope experiment. W.M., H.Z. and J.L. helped with experimental analysis; and X.M. helped with experimental analysis and writing. H.X. conceived this study, designed the experiments, analyzed the data and wrote this manuscript.

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Supplementary material

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References


