Functional cooperation of spns2 and fibronectin in cardiac and lower jaw development

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Summary
The lipid mediator sphingosine-1-phosphate (S1P) is a regulator of cardiac development in zebrafish, as disruption of its receptor s1pr2 or transporter spns2 causes migration defects in cardiac progenitors. To examine the genetic interaction of S1P signaling and the cell adhesion molecule fibronectin, we have established a fn;spns2 double mutant. Cardiac migration defects in fn;spns2 mutants were more severe than those in fn or spns2 mutants. We further found that the lower jaw morphology was disorganized in the fn;spns2 mutant, while it had a slightly shortened anterior–posterior distance in the ventral pharyngeal arch in fn and spns2 mutants relative to wild type. Knockdown of fn in the s1pr2 mutant, but not in the s1pr1 mutant, resulted in severe defects in cardiac migration and ventral pharyngeal arch arrangement. Further, in the background of the fn mutant, knockdown of endothelin receptor A (ednra), which was downregulated in the spns2 mutant, caused pharyngeal defects resembling those in the fn;spns2 mutant. These results strongly suggest that Spns2-S1PR2 signaling and fibronectin cooperatively regulate both cardiac and lower jaw development in zebrafish.

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Key words: Sphingosine-1-phosphate, spns2, fibronectin, Jaw, Heart

Introduction
The bioactive lipid mediator sphingosine-1-phosphate (S1P) plays important roles in various types of biological processes including angiogenesis, inflammation and immunity (Skouara and Hla, 2009; Spiegel and Milstien, 2011; Hisano et al., 2012). In zebrafish, S1P is involved in cardiac development by regulating the myocardial migration, as evidenced by the migration defects of cardiac progenitors in an S1P receptor (S1PR) and an S1P transporter mutant, the s1pr2 and spns2 mutants (Kupperman et al., 2000; Osborne et al., 2008; Kawahara et al., 2009). S1PRs consist of at least five G protein-couple receptors (S1PR1–S1PR5) that show differential expression patterns during mouse embryogenesis (Ohuchi et al., 2008; Meng and Lee, 2009). Intercellular S1P signaling through S1PRs activates various downstream signaling pathways (Takwa et al., 2012), leading to diverse cellular responses including cell proliferation, differentiation and cell migration. However, the developmental function of S1P signaling through the S1P-S1PR axis remains largely unclear.

The cell adhesion molecule fibronectin is a major component of the extracellular matrix (ECM) and is involved in various cellular processes including cytoskeletal organization and cell migration (Wierzbiacka-Patynowski and Schwarzbauer, 2003). The fibronectin (fn) mutant, which shows a loss of fibronectin function in zebrafish, has defective boundary formation in its anterior somites (Koshida et al., 2005), suggesting fibronectin contributes to the epithelialization of somites. Additionally, treatment of anti-fibronectin antibody in chick embryos inhibits myocardial migration (Linask and Lash, 1986). In mice, myocardial specification is normally observed in fibronectin knockout mice, whereas myocardial migration is inhibited (George et al., 1997). It has also been shown that fibronectin is required for adherens junction formation between cardiac progenitors in zebrafish (Trinh and Stainier, 2004). These studies all suggest an important role for fibronectin in vertebrate cardiac development. Nevertheless, there is much uncertainty on how fibronectin cooperates with other signaling molecule(s) when regulating cardiac development and other organogenesis.

In this study, we established a double mutant, fn;spns2, to investigate the genetic interaction of fibronectin and S1P signaling in zebrafish. We found the two separated hearts phenotype in the fn;spns2 double mutant was more severe than that in either fn mutant or spns2 mutants. Further, the anterior–posterior distance of the lower jaw was shorter in the fn and spns2 mutants, while the ventral pharyngeal arch structure was significantly impaired in the fn;spns2 double mutant. Our results genetically reveal a functional cooperation between S1P signaling and fibronectin for the regulation of myocardial migration and lower jaw formation.

Results and Discussion
Cardiac progenitor migration regulated by Spns2 and fibronectin
The heart tube develops from bilateral cardiac progenitors in the anterior lateral plate mesoderm in all vertebrate (Miura and
Yelon, 2011) and laterally positioned cardiac progenitors coordinately move toward the midline and fuse to form the heart tube. In zebrafish, disrupting Slpr2 (S1P receptor) or spns2 (SIP transporter) results in defective migration of these cardiac progenitors and in cardiobifida (two separated hearts), indicating S1P signaling regulates myocardial migration (Kupperman et al., 2000; Osborne et al., 2008; Kawahara et al., 2009). It has been shown that the cell adhesion molecule fibronectin also contributes to the cardiac morphogenesis, as fn mutants partially penetrate the cardiobifida phenotype (Trinh and Stainier, 2004). Our established fn\(^{k\text{259}}\) mutant, a null mutant with a premature termination at codon 241, predominantly presented a straight heart tube phenotype (31/133 embryos obtained from the crossing of fn\(^{\text{ko157}}\) heterozygous fish) at 25 hours post-fertilization (hpf), but also had a minor population showing cardiobifida (3/133 embryos), a low penetration result that may be explained by the genetic background. To examine the genetic interactions between S1P signaling and fibronectin, we generated double mutant zebrafish fn\(^{k\text{259}},\text{spns2}\(^{\text{ko157}}\) by the crossing spns2\(^{\text{ko157}}\) and fn\(^{k\text{259}}\) mutants. As shown in Fig. 1, the fn;spns2 double mutant displayed two widely separated hearts (19/322 embryos obtained from the crossing of fn\(^{\text{ko157}},\text{spns2}\(^{\text{ko157}}\) heterozygous fish). The distances between hearts were much greater than those of the spns2 (cardiobifida; 30/145 embryos obtained from the crossing of fn\(^{8;5}\) n; spns2\(^{8;5}\) mutants) or fn mutants (straight heart tube; 31/133 embryos obtained from the crossing of fn\(^{\text{ko259}},\text{spns2}\(^{\text{ko157}}\) heterozygous fish) (supplementary material Table S1). The genotypes of individual mutants were confirmed by genomic sequencing after taking pictures, wt (A,E,I), fn mutant (B,F,J), spns2 mutant (C,G,K) and fn;spns2 double mutant (D,H,L). Scale bars: 200 \(\mu\text{m}\). (M) Average distances between two hearts from multiple experiments; error bars represent standard deviations.

**Lower jaw development is cooperatively regulated by Spns2 and fibronectin**

The facial skeleton is formed from mutual interactions between cranial neural crest cells and both the pharyngeal endoderm and ectoderm of zebrafish. Additionally, secreted proteins such as Endothelin1, BMPs and Fgfs are key regulators involved in the craniofacial development (Alexander et al., 2011; Yamauchi et al., 2011). It has been shown that the spns2 mutant displays a disorganized anterior pharyngeal endoderm (Osborne et al., 2008). We noticed that our fn;spns2 double mutant exhibits severe defects in ventral facial morphology. Therefore, we examined the pharyngeal arch structure of individual mutants by Alcian Blue staining at 4 days post-fertilization (dpf). The anterior–posterior distance of the ventral pharyngeal arches (Meckel’s, palatoquadrate and ceratohyal cartilages) in the spns2 or fn mutants was shorter than that of wild type (Fig. 2; supplementary material Table S2). On the other hand, the number and morphology of the ceratobranchial arch were relatively normal. The pharyngeal defects are consistent with a recent report that demonstrated morphological defects of the lower jaw in both slpr2 and spns2 mutants (Balczerski et al., 2012). We also found that the cell adhesion molecule
Fibronectin is also required for the proper lower jaw development (Fig. 2; supplementary material Table S2). In clear contrast, the structure of the ventral pharyngeal arch (Fig. 2D, asterisks) was disorganized in the fn;spns2 double mutant, whereas that of the dorsal pharyngeal structure (trabecular cartilage) appeared normal (Fig. 2D, cross). Jaw development is regulated by the cooperation of several transcriptional factors including the hand2, dlx and nkx family genes (Miller et al., 2003; Trinh et al., 2005; Talbot et al., 2010). Whole-mount in situ hybridization using the pharyngeal markers, hand2, dlx2 and nkx2.3 at 30 hpf revealed that their anteroventral expressions of hand2, dlx2 and nkx2.3 were reduced in fn;spns2 mutants (hand2, n = 7; dlx2, n = 7; nkx2.3, n = 7) compared to the their posterior expressions, whereas the expression patterns and intensities of these markers were normal in fn mutants (hand2, n = 7; dlx2, n = 5; nkx2.3, n = 7) and spns2 mutants (hand2, n = 7; dlx2, n = 8; nkx2.3, n = 8). The genotypes of individual mutants were confirmed by the direct sequencing of spns2 and fn genomic loci after taking pictures. Because the first pouch endoderm is required for the pharyngeal arch formation (Alexander et al., 2011), our results suggest that Spns2 and fibronectin contribute to the formation of the ventral pharyngeal arch by regulating the anteroventral expression of various pharyngeal markers.

S1PR2, but not S1PR1, cooperates with fibronectin in cardiac and lower jaw development

Spns2 functions as an S1P transporter (Kawahara et al., 2009), suggesting that some S1PRs contribute to cardiac and jaw development. The s1pr2 mutant has been found to present the cardia bifida phenotype in zebrafish (Kupperman et al., 2000), whereas three independent groups recently reported that the knockdown of s1pr1 causes severe defects in cardiac and vascular development (Gaengel et al., 2012; Tobia et al., 2012; Mendelson et al., 2013), with the circulation of blood cells being particularly impaired in s1pr1-depleted embryos. We therefore examined the functional interaction of S1PR1/2 and fibronectin. Using TALEN (transcription activator-like effector nuclease) technology, we recently established s1pr1 and s1pr2 knockout fish (Hisano et al., 2013; Ota et al., 2013). We confirmed that s1pr2 knockout mutants show cardia bifida phenotype (Fig. 3E). However, no obvious cardiac or vascular defects in s1pr1 knockout mutants were seen during early embryogenesis (Fig. 3C; supplementary material Movies 3, 4). Thus, we conclude that zygotic s1pr1 mutants showed normal blood circulation and intersegmental vessel angiogenesis, which disagrees with the aforementioned reports. Those studies all used identical S1PR1-morpholino. It is possible that the S1PR1-morpholino caused off-target effects or also affected the maternal
message of s1pr1. To explain the different conclusions, it would be best to study a maternal-zygotic s1pr1 mutant, which will be available in a future study. When Fn-MO (10 ng) was injected into the s1pr2 mutant, a more severe cardia bifida phenotype was induced compared to the s1pr2 mutant (Fig. 3E–G). Consistent with these results, severe defects in cardiac migration and lower jaw morphology were observed when S1PR2-MO (10 ng) was injected into fn mutants (supplemental material Fig. S1), which agrees with previous knockdown analysis that used S1PR2-MO and Fn-MO (Matsui et al., 2007). Such a cooperative cardiac defect was not observed in s1pr1 embryos injected with Fn-MO (supplementary material Table S1), which instead showed a straight heart tube phenotype similar to that in fn mutants. Fn-MO-injected s1pr1 mutants embryos had a slightly shorter anterior–posterior distance in their ventral pharyngeal arch structure, quite unlike the disorganized ventral pharyngeal arch structure observed in Fn-MO-injected s1pr2 mutants (Fig. 3H–J; supplementary material Table S2). Additionally, severe defects in cardiac migration and lower jaw morphology were observed when S1PR2-MO (10 ng) was injected into fn mutants (supplemental material Fig. S1). These results suggest that S1PR2, but not S1PR1, cooperates with fibronectin in both cardiac and lower jaw development. It has been reported that both s1pr2 and spns2 mutants lack the anterior endoderm (Balczerski et al., 2012). Because signaling pathways through the Spns2-S1PR2 axis regulate cell proliferation of the anterior endoderm tissue and therefore affect the positioning of the ventral pharyngeal arch, further analysis will be required to clarify how S1P signaling and fibronectin together control the movement and adhesion of anterior pharyngeal endodermal cells.

Endothelin receptor A, a possible mediator downstream of Spns2-S1PR2 signaling
Both Spns2 and S1PR2 are involved in cardiac and lower jaw development. To identify the genes regulated by the Spns2-S1PR2 axis, we performed microarray analysis. Total RNA was
isolated from uninjected, S1PR2-MO (10 ng)-injected and Spns2-MO (10 ng)-injected embryos at 25 hpf, and their gene expression profiles were compared. We found that the expression of *endothelin receptor A* (*ednra*) was downregulated in both Spns2-depleted and S1PR2-depleted embryos (see Materials and Methods). Consistent with this result, the expression of *ednra* in the pharyngeal arches of the *spns2* mutant (*n* = 10) was reduced compared to that of wild type (*n* = 7) (Fig. 4A,B). In both zebrafish and mouse, disruption of *endothelin1* (*edn1*) causes a loss or transformation of the lower jaw (Nair et al., 2007; Tavares et al., 2012). Because Edn1 functions through its cognate type-A receptor Ednra, Ednra can be considered a key regulator in pharyngeal development (Nair et al., 2007). However, it is not clear how Ednra cooperates with other molecule(s) during jaw development. Therefore, we investigated the functional interaction between Ednra and fibronectin. Heart morphology seemed normal when Ednra-MO (10 ng) was injected into wild-type embryos (*n* = 13), while cardiac defects in Ednra-MO injected fn embryos (*n* = 11) were slightly more severe than those of fn embryos (Fig. 4C,D; supplementary material Table S1). Further, a disorganization of the ventral pharyngeal arch arrangement in Ednra-MO injected fn embryos (*n* = 10) was observed relative to that of Ednra-MO injected wild-type embryo (*n* = 7) (Fig. 4E,F; supplementary material Table S2). The genotypes of individual mutants were confirmed by the direct sequencing of *spns2* and *fn* genomic loci after taking pictures. Although in a cell culture system Edn1 increases the adhesion of amelanotic melanocytes to fibronectin (Ma et al., 2006), how Edn1 signaling affects fibronectin function remains unclear. Further analysis will be required to determine whether Edn1 regulates the fibronectin-mediated cellular interaction during the ventral pharyngeal arch arrangement. One clue comes from both  

S1P-S1PR2 and End1-Ednra being critical in the patterning of the ventral pharyngeal arch, which agrees with our demonstrating that Ednra, a possible downstream target of the Spns2-S1PR2 axis, synergizes with fibronectin to promote the lower jaw development.

**Conclusion**

In this study, using zebrafish genetic mutants (*fn, spns2* and *fn;spns2*), we demonstrated that Spns2-S1PR2 signaling and the cell adhesion molecule fibronectin cooperatively regulate the migration of cardiac progenitors. Further, Spns2-S1PR2 and fibronectin synergize to promote ventral pharyngeal cartilage formation. Because the expression of *ednra* in the pharyngeal arches of *spns2* mutants is reduced, we propose that Ednra contributes to the lower jaw arrangement by cooperating with fibronectin.

**Materials and Methods**

**Zebrafish mutants**

Mutant alleles of *fibronectin* (*fn*) and *spns2* were used (Koshida et al., 2005; Kawahara et al., 2009). To obtain double mutants, the *spns2* allele was crossed into the *fn* allele. Embryos of *fn* and *spns2* were obtained from *fn* and *spns2* heterogeneous fish. Embryos of *spns2* and *fn* were obtained from *spns2* and *fn* *S1P-S1PR2* and *End1-Ednra* being critical in the patterning of the ventral pharyngeal arch, which agrees with our demonstrating that Ednra, a possible downstream target of the Spns2-S1PR2 axis, synergizes with fibronectin to promote the lower jaw development.

**Conclusion**

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**Establishment of s1pr1 or s1pr2-knockout zebrafish**

TALEN constructs targeting s1pr1 or s1pr2 were described previously (Hisano et al., 2013; Ota et al., 2013). TALEN mRNAs (400 pg each) were injected into blastomeres at the 1–2 cell stage of zebrafish embryos. Identification of potential F0 founders and F1 embryos having mutant allele was performed by HMA (heteroduplex mobility assay) (Ota et al., 2013). The *s1pr1* and *s1pr2* alleles were isolated from +362 to +371 of the *s1pr1* coding region, while the *s1pr2* allele was deleted from +179 to +188 of the *s1pr2* coding region. To monitor the cardiac and
vessel development, the transgenic lines spns2-MO with a mosaic expression pattern of spns2-MO-injected (Log2 Ratio: 1.06).

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Alcian Blue staining

Embryos at 4 dpf were fixed overnight by 4% paraformaldehyde in PBST. Embryos were washed with acetic acid buffer (0.17% HCl and 70% ethanol) and incubated with 0.1% Alcian Blue (Sigma) in acetic acid buffer. After 3 times washing with acetic acid buffer, embryos were incubated for 10 min with maleic acid buffer (0.1 M maleic acid [pH 7.5]). Embryos were incubated with anti-DIG alkaline phosphatase (Roche) in blocking buffer (0.1 M NaCl, 0.1% Tween 20, 0.1% Vgatase) overnight at 4°C. Embryos were washed with phosphate buffer saline (PBS) containing 0.1% Tween 20 (PBST), color reactions were performed using BM purple (Roche) as the substrate for DIG labeling.

Acknowledgements

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Competing Interests

The authors have no competing interests to declare.

References


Supplementary Material
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Fig. S1. Knockdown phenotype of S1PR2 in fn mutant. (A,B) Cardiac morphology visualized by mRFP expression derived from Tg(cmlc2:mRFP).
Both images show ventral views at 28 hpf. (C,D) Lower jaw morphology at 4 dpf was visualized by Alcian Blue staining (ventral view). Genotyping was performed by genomic sequencing after taking pictures. wt (A,C) and fn mutant (B,D). Scale bars: 200 μm.

Table S1. Heart morphology.

<table>
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<tr>
<th>MO</th>
<th>Heart distance (μm)</th>
<th>sd</th>
<th>n</th>
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<tbody>
<tr>
<td>WT</td>
<td></td>
<td>0.00</td>
<td>10</td>
</tr>
<tr>
<td>Fn</td>
<td>13.20</td>
<td>27.84</td>
<td>10</td>
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<tr>
<td>S1PR1</td>
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</tr>
<tr>
<td>S1PR2</td>
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<td>145.56</td>
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<tr>
<td>spns2</td>
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<td>97.24</td>
<td>11</td>
</tr>
<tr>
<td>Fn-spns2</td>
<td></td>
<td>304.43</td>
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<tr>
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<tr>
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<tr>
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<tr>
<td>Fn</td>
<td>ednra</td>
<td>58.11</td>
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Table S2. Lower jaw morphology.

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<tr>
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<tr>
<td>S1PR2</td>
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<td>200.89</td>
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<tr>
<td>spns2</td>
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<tr>
<td>Fn-spns2</td>
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<td>84.00</td>
<td>8</td>
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<td>fn</td>
<td>247.90</td>
<td>10</td>
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<td>299.70</td>
<td>13</td>
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<tr>
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<td>fn</td>
<td>131.84</td>
<td>8</td>
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<td>WT</td>
<td>ednra</td>
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<tr>
<td>Fn</td>
<td>ednra</td>
<td>95.29</td>
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Movie 1. Beating heart in a wt embryo at 28 hpf.

Movie 2. Beating heart in a fn-spns2 double mutant embryo at 28 hpf.

Movie 4. Blood circulation in an s1pr1 mutant embryo at 28 hpf.