Mini Review

Genetic dissection of cardiac progenitor migration

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The formation of heart is regulated by coordinated complex processes: cardiac progenitors migrate from both sides of the anterior lateral plate mesoderm toward the midline and differentiate into the heart tube including atrium and ventricle. Forward genetic analysis using zebrafish has identified several mutants defective in myocardial migration, resulting in two hearts known as cardia bifida. Identification of genes responsible for the cardia bifida mutations has revealed key players that regulate the migration and assembly of cardiac progenitors. Both anterior endoderm and the extraembryonic tissue yolk syncytial layer (YSL) provide for this process, thereby controlling the coordinated movement of cardiac progenitors. Further, the signaling pathway mediated by sphingosine 1-phosphate (S1P) is essential for the migration of cardiac progenitors in zebrafish.

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The heart is one of the earliest organs to develop and function during vertebrate embryogenesis[1]. Zebrafish is an ideal model organism to study cardiac morphogenesis[2], because the processes of heart formation are visible in optically transparent embryo. In vertebrates, cardiac progenitors including presumptive myocardial and endocardial cells originate from both sides of the anterior lateral plate mesoderm (ALPM). During the segmentation period characterized by the formation of somites, bilateral cardiac progenitors migrate to the midline to form the heart cone (Fig.1)[3]. Subsequently, the heart cone extends to form the heart tube including two chambers (atrium and ventricle). Since molecular structures and developmental expression patterns of cardiac genes are well conserved between zebrafish and mammals, shared genetic pathways should be present in cardiac morphogenesis. However, the molecular mechanism of cardiac progenitor migration is not fully understood in vertebrates. Therefore, this review focuses on key regulators in myocardial migration, presented by the functional analysis of zebrafish cardia bifida mutants that fail to migrate and subsequently differentiate into two chambers at abnormal lateral positions. Zebrafish mutant screening has identified several cardia bifida mutations as shown in Table 1. Identification of genes responsible for these mutants allows genetic dissection of the coordinated movement of cardiac progenitors.

Contribution of genes involved in endoderm formation to cardiac morphogenesis

It is noteworthy that cardiac progenitors migrate on the sur-

Table 1

Mutant name	Gene	Cardiac defects	Other defects
1. molecules involved in endoderm specification			
bonnie and clyde (*bon*)	*mixer* [6]	cardia bifida	reduced endoderm tissues
casanova (*cas*)	*sox32* [7,8]	cardia bifida	absence of endoderm
2. molecules involved in endoderm and/or mesoderm specification			
one eye pinhead (*oep*)	*oep* (*EGF-CFC family*) [4]	cardia bifida, reduced myocardial precursors	reduced endoderm and mesoderm tissues
faust (*fau*)	*gata5* [5]	cardia bifida, reduced myocardial precursors	reduced endoderm tissues
hands off (*han*)	*hand2* [12]	cardia bifida, reduced myocardial precursors	reduced pectoral fin tissue
3. S1P signaling molecules and an adhesion molecule			
miles apart (*mil*)	*s1p2* [13]	cardia bifida	tail blisters
two of hearts (*toh*)	*spns2* (*spinster-like 2*) [17,18]	cardia bifida	tail blisters
natter (*nat*)	*fibronectin* [20]	cardia bifida	absence of anterior somite boundaries

face of anterior endoderm that may provide a supporting role for myocardial migration. Functional analysis of zebrafish mutants defective in endoderm specification suggests that anterior endoderm plays a pivotal role in the regulation of cardiac progenitor migration. Impairment of the nodal co-receptor *one eye pinhead* (*oep*; EGF-CFC family)[4] and transcription factors *faust* (*fau*)/*gata5*[5], *bonnie and clyde* (*bon*)/*mixer*[6] and *casanova* (*cas*)/*sox32*[7,8] disrupts endoderm specification, resulting in diminished numbers and defective movement of cardiac progenitors. Consistent with these findings, mice defective in endoderm specification exhibit cardia bifida[9]. Since transplantation of the endoderm-committed cells from wild-type embryos into endoderm-deficient *cas/sox32* mutants can restore the migration of cardiac progenitors[10], the anterior endoderm is indispensable for the regulation of cardiac progenitor migration. Consistent with this observation, surgical removal of anterior endoderm in chick embryos results in severe defects in myocardial migration[11]. However, the molecular mechanism through which anterior endoderm contributes to the appropriate differentiation and coordinated movement of cardiac progenitors remains unknown.

Genes involved in myocardial cell differentiation are also required for myocardial migration

Proper population numbers and myocardial differentiation of cardiac progenitors appear to be important for myocardial migration, because mutations in *hand2* and *gata5* lead to severe defects in myocardial differentiation and migration, resulting in cardia bifida[5,12]. *hand2*, which is a basic helix-loop-helix transcription factor, is expressed in a broad domain of the ALPM and subsequently in myocardial cells. Disruption of *hand2* leads to a significant reduction of myocardial cells and a substantial suppression of myocardial migration[12]. Because the initial expression of the NK-type homeobox transcription factor, *nkx2.5*, which is one of the earliest known markers for cardiac progenitors, is not affected in *hand2* mutants[12], it is still unclear how *hand2* regulates the differentiation of myocardial progenitors.

gata5, which is essential for endoderm formation, is also expressed in myocardial cells and therefore may regulate both endodermal and myocardial cell differentiation. Interestingly, both *oep* (a nodal co-receptor) and *gata5* are required for initial cardiac expression of *nkx2.5* and myocardial differentiation[4]. *gata5* expression in cardiac progenitors is significantly inhibited in embryos lacking zygotic *oep* (*Zoep* mutants), but forced expression of *gata5* can restore the *nkx2.5* expression in myocardial cells of these mutants[4]. These results suggest that the nodal signaling involving Oep is required for the induction and maintenance of *gata5* expression in cardiac progenitors and Gata5 subsequently regulates the expression of *nkx2.5* in myocardial differentiation. Further study is required to clarify how *hand2* and *gata5* contribute to cardiac differentiation and coordinated movement of myocardial progenitors.

Importance of S1P signaling regulators in cardiac progenitor migration

It is an important question which signaling pathway is involved

in the regulation of myocardial migration. Since mutation of the *miles apart* (*mil*) gene, which encodes *sphingosine 1-phosphate* (*S1P*) *receptor-2/s1p2*, exhibits cardia bifida without disrupting myocardial specification[13], the S1P signaling pathway mediated by the S1P receptor $S1P_2$ plays a vital role in myocardial migration in zebrafish (Fig.2E). S1P is a secreted sphingolipid mediator that activates the G protein-coupled S1P receptors ($S1P_1$-$S1P_5$), leading to cellular responses including cell proliferation, cell death, migration and differentiation[14-16]. Although *mil/s1p2* is broadly expressed during gastrulation and segmentation periods, the sheet of anterior endoderm is disorganized in the *mil/s1p2* mutants[17]. Cardiac defects in the *mil/s1p2* mutants are rescued by anterior endoderm replacement with wild-type cells[17], suggesting that the *mil/slp2* function in anterior endoderm is required for myocardial migration.

Recent reports demonstrate that disruption of a twelve-pass transmembrane-domain protein Spns2/Two of hearts (Toh)/ Spinster-like 2 (Spinl2) leads to cardia bifida[17,18] with tail blisters (Table 1), resembling the phenotypes of *mil/s1p2* mutant (Fig.1D-1F). Spns/Spinster family proteins include Spns1/ Spinl1, Spns2/Spinl2/Toh and Spns3/Spinl3 in the zebrafish and human genomes. Cardia bifida in the *toh/spns2* mutants is restored by mRNA injection of *spns2/spinl2*, but not *spns1/spinl1* or *spns3/spinl3*, suggesting that Spns/Spinster family proteins possess divergent functions. One *toh* allele (*ko157*) contains a missense mutation in the *spns2* gene with a substitution of arginine to serine at amino acid position 153, Spns2(R153S). Introduction of Spns2-EGFP, but not Spns2(R153S)-EGFP or Spns1-EGFP, enhances S1P export from the cells (Fig.2E), indicating that Spns2 functions as a S1P transporter[18]. *spns2* is strongly expressed in the yolk syncytial layer (YSL) located below the developing myocardial progenitors during the segmentation period (Fig.2A,B)[18]. Interestingly, knockdown of *spns2* but not of *mil/s1p2* in the YSL resulted in cardia bifida. On the other hand, injection of *spns2* mRNA in the YSL could rescue cardia bifida in *toh/spns2* mutants, but injection of *spns2*(*R153S*) did not (Fig.2C,D)[18]. These results indicate that Spns2 function in the YSL is indispensable for myocardial migration. Thus, the Spns2-$S1P_2$ signaling pathway is essential to control the migration of myocardial progenitors.

Requirement for an adhesion molecule for myocardial migration

The disruption of the adhesion molecule Fibronectin in mice and zebrafish results in defective myocardial migration, leading to cardia bifida[19,20]. The use of anti-Fibronectin blocking antibody in chick embryos induces to cardia bifida[21]. Further, functional suppression of Fibronectin by local injection of an RGD peptide in zebrafish embryos leads to migration defects in myocardial cells[22]. In zebrafish, Fibronectin is detected at the midline between endoderm and cardiac progenitors during segmentation periods (Fig.3). These results suggest that the epithelial organization of myocardial cells is important for coordinated myocardial migration. The expression of *fibronectin* in the medial domain is suppressed in endoderm-defective *cas/sox32* mutants[20], indicating that anterior endoderm is required for the localization of Fibronectin in the basal substratum surrounding the cardiac progenitors. The *natter/fibronectin* mutant exhibits the disruption of apicobasal polarity. Interestingly, S1P stimulation increased the interaction between primary cultured zebrafish cells and a Fibronectin substratum[22], whereas the cell-Fibronectin interaction was substantially inhibited by knockdown of *mil/s1p2*. Further, local application of Fibronectin around the anterior midline area rescues the cardia bifida phenotype of *mil/s1p2* mutant embryos[22], suggesting that the functional regulation of Fibronectin is one of the downstream events of S1P-mediated signaling (Fig.3). Consistent with these results, a disorganized endodermal sheet and diminished Fibronectin deposition around the migrating cardiac progenitors are observed in both *toh/spns2* and *mil/s1p2* mutants [17]. Thus, deposition of Fibronection may contribute to the establishment of positional clues essential for the migration of cardiac progenitors.

Conclusions

Functional analysis of endoderm-defective mutants (*oep*, *fau/gata5*, *bon/mixer*, *cas/sox32*) indicates that the anterior endoderm plays an important role in the regulation of myocardial migration. Further, appropriate myocardial differentiation, which is defective in *fau/gata5* and *has/hand2* mutants, appears to be essential for myocardial migration. Functional analysis of *toh/ spns2* and *mil/s1p2* mutants demonstrates that S1P functions as a signal required for myocardial migration. Furthermore, functional regulation of Fibronectin, which is one of the downstream events of S1P signaling, is required for the organization of the myocardial epithelia that may provide positional clues for myocardial migration. Thus, complex molecular interactions take place among different tissues to control myocardial migration. Gene expression profiling analysis with microarray chip in these cardia bifida mutants would be useful for the identification of novel regulators involved in cardiac progenitor migration. Further isolation and characterization of zebrafish cardia bifida mutants will provide a more precise genetic pathway of the co-

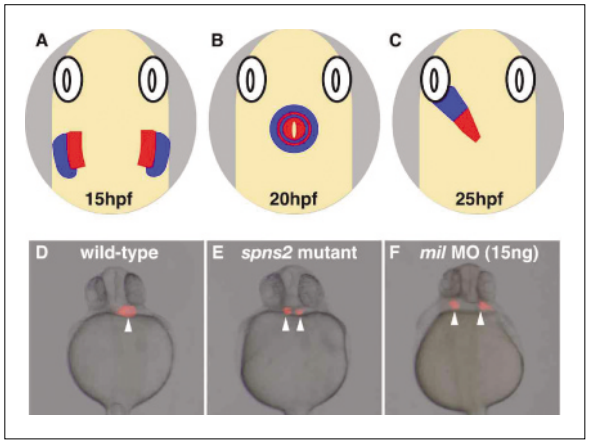

Fig.1 Zebrafish heart formation and cardia bifida mutations

(A-C) Schematic representation of heart development (dorsal view, anterior up). Bilateral cardiac progenitors (A) are composed of atrial precursors (blue) and ventricular precursors (red) around 15 hours post-fertilization (hpf). Subsequently, they migrate to the midline and fuse to form the cardiac cone (B) around 20 hpf. The cardiac cone extends to form the heart tube (C) including the atrium and ventricle around 25 hpf. Gray; yolk, yellow; embryonic body. (D-F) Cardia bifida is observed either in *spns2* mutant embryo or in *mil/s1p2* morphant (*mil* MO-injected embryo; 15 ng). Developing hearts (arrowheads) are visualized by monomeric red fluorescent protein (mRFP) expression driven by the promoter of the cardiac-specific gene, *cardiac myosin light chain 2* (*cmlc2*), in the transgenic line *Tg* (*cmlc2:mRFP*).

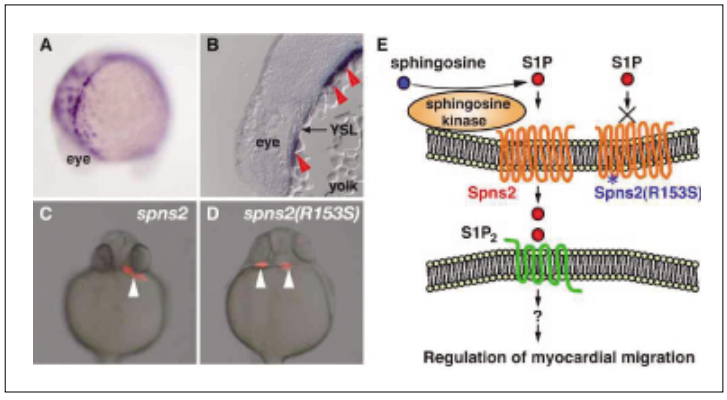

Fig.2 Spns2-S1P signaling is required for myocardial migration

(A and B) *spns2* expression at 15-somite stage. *spns2* is strongly expressed in the YSL around the anterior midline (red arrowheads). (C and D) Cardia bifida in the *spns2* mutant with *cmlc2:mRFP* is rescued by injection of *spns2* mRNA (250pg), but not the *spns2* mutant *spns2*(*R153S*) mRNA (250pg). Positions of hearts are indicated by white arrowheads. (E) Sphingosine kinase catalyses the formation of S1P from sphingosine. Introduction of Spns2, but not the Spns2 mutant Spin2(R153S), increases the export of S1P from the cells. S1P signaling mediated by Mil/S1P$_2$ regulates the migration of cardiac progenitors.

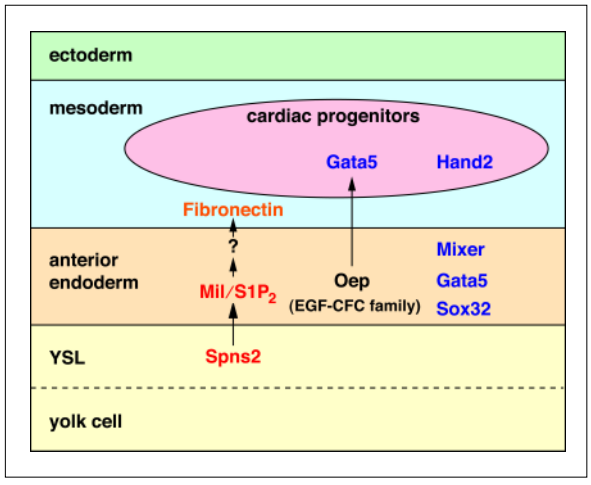

Fig.3 Genetic pathway of cardiac progenitor migration revealed by cardia bifida mutations

Four mutations (*oep*, *mixer*, *gata5* and *sox32*) affecting endoderm specification results in cardia bifida. Mutations in *hand2* and *gata5* prevent the myocardial differentiation. Nodal signaling, involving the co-receptor Oep, is required for *gata5* expression in myocardial cells. Cardia bifida is observed in *spns2* and *mil/s1p2* mutants that are defective in S1P signaling. The *fibronectin* mutant exhibiting cardia bifida presents a disorganization of epithelial integrity. Fibronectin deposition at the midline is inhibited in the *mil/s1p2* morphant, suggesting that the S1P signaling mediated by Mil/S1P$_2$ regulates the deposition or function of Fibronectin.

ordinated movement of cardiac progenitors. Since the molecular mechanism of organ formation during early embryogenesis is well conserved between teleosts and mammals, the findings generated from the genetic dissection of zebrafish myocardial migration would be directly applicable to the analysis of key regulators of mammalian cardiac morphogenesis.

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