Snail modulates the assembly of fibronectin via α5 integrin for myocardial migration in zebrafish embryos

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The Snail family member snail encodes a zinc finger-containing transcriptional factor that is involved in heart formation. Yet, little is known about how Snail regulates heart development. Here, we identified that one of the duplicated snail genes, snai1b, was expressed in the heart region of zebrafish embryos. Depletion of Snai1b function dramatically reduced expression of α5 integrin, disrupted Fibronectin layer in the heart region, especially at the midline, and prevented migration of cardiac precursors, resulting in defects in cardiac morphology and function in zebrafish embryos. Injection of α5β1 protein rescued the Fibronectin layer and then the myocardial precursor migration in snai1b knockdown embryos. The results provide the molecular mechanism how Snail controls the morphogenesis of heart during embryonic development.

The Snail family member snail (also known as snail1) encodes a zinc finger-containing transcriptional factor. Disruption of snail in mice causes death at E7.5 before cardiac development. Mouse embryos with conditional disruption of snail after E8, die at E9.5, partially due to severe cardiovascular defects. In addition, Snail has been shown to play a direct role in endocardial cushion formation in a mouse model with a conditional snail knockdown. The data indicate that embryonic Snail deficiency is lethal in mice and Snail displays important function during the cardiac development. However, the mouse models are difficult to be examined and further to visualize how Snail controls heart development during embryonic development. Zebrafish, on the other hand, can survive without cardiovascular function during the first 7 days of development, which makes it a very good model for studying essential genes during heart development. Therefore, we used zebrafish embryos to address the mechanisms how Snail modulates cardiac development during embryonic development.

There are two duplicated snail1 genes in zebrafish, namely snai1a and snai1b. They exist in the paraxial and axial mesoderm and have been implicated in the anterior migration of axial mesendoderm. Here, we show that snai1b plays a key role in the migration of cardiac precursors by modulating the extracellular assembly of fibronectin (Fn) via the expression of α5 integrin. Our results provide the molecular mechanism that how Snail controls heart formation during zebrafish embryonic development.

Results
Knockdown of snai1b induces cardiac defects. In order to identify the location of snai1b expression in zebrafish embryos, we detected snai1b, myl7, and fn expression by whole-mount ISH. We found that snai1b was expressed in the anterior lateral plate mesoderm (LPM), where locates myocardial precursors, next to the neural crest and with myl7 expression (Supplementary Fig. S1A–D). These results suggest that Snai1b played a role during heart development.

We then injected snai1b antisense morpholino (MO) into embryos at the one-cell stage to determine the Snai1b functions during heart development. The results showed the same phenotypes during zebrafish embryonic development as described. In addition, we observed that more than 70% embryos injected with snai1b MO displayed a delay in cardiac fusion at 24 hours post fertilization (hpf). At 48 hpf, over 55% of embryos displayed gross cardiac defects, including large pericardial edemas, loss of cardiac looping (Figure 1A and B), and weakened heart throb. In order to confirm the snai1b MO specificity, we used a construct that expresses a Snai1b-GFP fusion protein contained snai1b 5'-UTR sequence to test the efficiency of snai1b MO. As expected, the snai1b MO efficiently depleted the expression of the Snai1b-GFP protein (Supplementary Fig. S2A–F), indicating the snai1b
MO specifically disrupts the function of Snail in zebrafish embryos. Then, we injected snailb MO at different concentrations into zebrafish embryos and identified that higher dose of snailb MO resulted in more abnormal embryos at 48 hpf (Supplementary Fig. S2G). Injection of snailb mRNA without 5′-UTR sequence, which the snailb MO binds to, reduced the defects to 41.6% at 24 hpf and 17.8% at 48 hpf (Figures 1G and 2G–J). In addition, snaila mRNA was unable to rescue the phenotype induced by snailb knockdown. Together, the results indicate that snailb displays essential function during the heart morphogenesis in zebrafish embryos.

Next, we injected snailb MO into the myl7::GFP transgenic zebrafish embryos, which expresses GFP in the developing heart, to visualize the heart development. The results showed that depletion of snailb induced abnormal cardiac looping in the myl7::GFP embryos. At 48 hpf, most snailb morphants developed band-like hearts with the atrium on the left or at the midline and had a small ventricle (Figure 1C and D). These phenotypes were even more pronounced at 72 hpf (Figure 1E and F). In contrast, the flk1::GFP transgenic zebrafish embryos exhibited normal endocardium formation in snailb morphants (Figure 1H and I). In addition, most band-like hearts formed in the morphants were able to contract and drive blood circulation, but not as efficiently as controls, indicated by the flow of the blood cells. These results further indicate that snailb is required for embryonic cardiac development in zebrafish embryos.

Snailb controls the migration of cardiac precursors. The morphological defects in the hearts of snailb disrupted animals led us to determine which stages during heart development were controlled by Snailb. Cells that contribute to the formation and development of the heart in the embryo undergo several phases of migration. The heart precursor cells migrate towards the anterior-lateral plate mesoderm after involuting during the early stages of gastrulation, subsequently, they fuse at the midline, where they form the linear heart tube12. The whole-mount ISH showed that the myocardial precursors with myl7 expression were located bilaterally in control embryos at 15 hpf (Figure 2A). In contrast, the myocardial precursors were not localized in bilateral groups and were farther away from the midline in the snailb morphants (n = 19/25; Figure 2B). At 18 hpf, the bilateral groups of myocardial precursors began to fuse together at the midline in controls, while they remained bilateral distribution in the snailb morphants (n = 20/27; Figure 2C and D). By 24 hpf, the precursors merged at the midline to form a single heart tube in control animals and started to migrate to the midline in the snailb morphants (n = 12/17; Figure 2E and F). These results indicate that snailb modulates the migration of cardiac precursors during heart embryonic development.

In addition, snailb morphants had decreased myl7 expression detected by ISH (Figure 1 and 2A–D), suggesting the defect of myocardial precursors on cell fate in the morphants. To examine the fate of myocardial precursors in the snailb morphants, we used TUNEL staining to detect apoptosis. We found that snailb morphants had high levels of apoptosis. Knocking down p53 reduced apoptosis levels in the heart region (Supplementary Fig. S3A–C), but did not alleviate the other snailb MO phenotypes, as judged by myl7 expression at 15 hpf (Supplementary Fig. S3D–F). Then myl7 expression by qRT-PCR showed that its expression was not altered in snailb morphants (Supplementary Fig. S3G), indicating that total cells with myl7 expression remained in snailb morphants. The results demonstrate that reduction of myl7 expression detected by ISH results from the spreading distribution of myl7 expressing cells on the mesodermal plate in snailb morphants, not the reduction of myl7 expressing cells, and further confirm that snailb controls the migration of cardiac precursors during heart embryonic development.

Figure 1 | Knockdown of Snailb induces cardiac morphology defects. (A, B): Phenotypes of snailb knockdown at 48 hpf, lateral view. Black arrows: heart valve. (C–F): Changes in heart shape in myl7::GFP embryos injected with control (Ctl) and snailb MO at 48 hpf and 72 hpf. A, atrium; V, ventricle. (G): Quantification of phenotypes produced by snailb morphants at 48 hpf, with or without snailb mRNA at different concentrations. (H, I): Heart endocardium in flk1::GFP embryos injected with Ctl and snailb MO at 72 hpf. White arrows: heart. Scale bars: 100 μm.
Snail1b maintains Fibronectin layer in the heart region. Several lines of evidence support the fact that Fibronectin (Fn) is required during myocardial precursor migration\textsuperscript{1,15}, and that snail modulates Fn deposition\textsuperscript{12}. Thus, we examined Fn deposition in zebrafish embryos at 17 hpf when myocardial precursors begin to fuse. The results showed that fine Fn layers were deposited in the extracellular matrix at the midline between the endoderm and cardiac precursors, and surrounded the myocardial precursors later on in control embryos. In contrast, the Fn deposition were dramatically reduced and displayed non-continuous patterns in the region surrounding the myocardial precursors in snail1b morphants, especially in the midline region (Figure 3A–D), indicating that snail1b is required for proper Fn deposition in the embryonic heart region of zebrafish. Since Fn plays important function in morphological segmentation boundary of somites in zebrafish embryos\textsuperscript{13} and knockdown of snail1b affects the shape of somite\textsuperscript{2}, we also measured the Fn pattern in the abnormal somite boundary regions and identified that Fn assembly was disrupted significantly in snail1b morphants (n = 16/22; Supplementary Fig. S7). The results are consistent with previous observations\textsuperscript{2} and indicate that the Fn assembly mediated by Snail1b also plays a critical role in morphogenesis of somite.

Next, we examined the expression of fn mRNA in snail1b morphants by in situ hybridization and found that fn expression was not altered after Snail1b knockdown in zebrafish embryos (Supplementary Fig. S4A and B). In fact, qRT-PCR results indicated that the levels of fn mRNA were higher in snail1b morphants at 17 hpf than those in control embryos (Supplementary Fig. S4C). The data indicate that snail1b does not directly regulate fn expression. Consistent with these observations, Fn protein co-injected with snail1b MO did not rescue the defects caused by Snail1b knockdown (Table 1). The results suggest that the defective generation and formation of cardiac precursors are caused by the abnormal Fn deposition.

\textbf{α5 integrin acts downstream of Snail1b for Fn deposition in the embryonic heart.} Since integrins are essential for Fn fibril formation\textsuperscript{17}, we tested the expression of several integrins in zebrafish embryos during heart formation (Figures 4A–C and Supplementary Fig. S5). The results showed that α5 integrin was markedly decreased in snail1b morphants at 15 hpf compared to that in control embryos (n = 11/16; Figure 4A, B). The examination of expressing patterns and snail1b mRNA induction showed that α5 integrin was co-expressed with snail1b mRNA and was up-regulated in the LPM by snail1b mRNA (n = 14/21; Figure 4C, Supplementary Fig. S1E, Supplementary Fig. S5J).

To determine whether α5 integrin was able to rescue the migration defect of myocardial precursors in snail1b morphants, we co-injected snail1b MO with α5 integrin mRNA at different concentrations. Accordingly, both co-injecting 50 pg and 100 pg α5 integrin mRNA rescued the myocardial defects in snail1b morphants (Supplementary Fig. S6). Furthermore, we injected integrin proteins into the embryos to determine the function of α5 integrin in snail1b morphants. The results showed that α5β1 integrin protein was able to rescue the Fn fibril assembly at 17 hpf (Figure 4E and F) and the cardiac defects at 20 hpf (Figure 4D) in snail1b morphants. Taken together, the results demonstrate that Snail1b controls the expression α5 integrin which in turn controls Fn deposition necessary for myocardial precursor migration during heart morphogenesis in zebrafish embryos.

Discussion

Previous studies have shown that the combined expression of slug and snail is required for epithelial–mesenchymal transition (EMT) in cardiac cushion morphogenesis\textsuperscript{4,16}, and snail is additionally required for left-right asymmetry determination in the heart\textsuperscript{2}. Here, we show that Snail1b knockdown causes defects in myocardial precursor migration and delays cardiac fusion of these precursors in the zebrafish embryos. In addition, our results provide that the delayed fusion of myocardial precursors can be restored by α5 mRNA or α5β1 integrin protein, indicating that Snail-integrin signaling is essential for cardiac development in zebrafish.

Whether snail genes are mesodermal determinants has been long debated. There is increasing evidence indicating that the activity of Snail is related not only to cell fate, but also to cell migration\textsuperscript{18}. In fact, mice mutant for Snail die at gastrulation, yet they can form mesoderm and express mesodermal markers\textsuperscript{1}. The lethality of Snail mutants makes it difficult to investigate the role of Snail in organ formation in the mouse. Zebrafish, on the other hand, is an ideal model to investigate the role of Snail in organogenesis. Here, we show that Snail1b controls the migration of myocardial precursors, rather
Snail has been reported to play a role in endocardial development in mice. However, the results presented here suggest that Snailb does not involve in endocardial development in zebrafish. One possible reason might be that Snaila can compensate for Snailb function and maintains the generation of endocardial tissue. Another possibility is that Snailb does not regulate the generation of endocardium, which is derived from a distinct region in the anterior LPM where snailb does not express.

Our studies indicate that myocardial precursor migration defects are due to the disruption of the Fn layer. Previous studies have shown that Fn is a multi-domain ECM protein that mediates multiple cellular behaviors, and is expressed early in embryonic development in the mesoderm as well as in between the embryonic germ layers. Fn-deficient murine embryos do not undergo primitive heart tube fusion, but instead, form heart tubes with thickened myocardial tissue lacking cardiac jelly, and with abnormal endocardium at E8.0. Fn deficiency is in fact lethal within the first 10 embryonic days due to cardiovascular and vascular defects. In zebrafish embryos, Fn deposition at the midline is required for the myocardial precursor migration and the formation of adhesion junctions among these cells. Our results are consistent with these previous studies that support the idea that the fibrils of Fn is required for the migration of myocardial precursors and indicate that Fn deposition is indeed a downstream effector of Snailb during cardiac development. Furthermore, in this study, the snailb morphants show delayed fusion of the myocardial sheets and seemingly smaller hearts (Figure 2). However, the cardiac defects of natter/fn mutants are described as a lack of myocardial fusion at 24 hpf and single myocardial cells located in the anterior mesoderm. The difference mainly results from the fact that Fn deposition was reduced, rather than completely inhibited in snailb morphants. Indeed, cardiac precursors are partially surrounded by Fn deposition in these morphants.

Previous studies have shown that Fn accumulation is dependent on integrin in zebrafish embryos. Overexpression of snail increases the expression of integrin. Our results display here that Snailb regulates integrin expression and controls Fn deposition, rather than expression, in the heart region of zebrafish embryos. Injection of integrin mRNA or integrin protein is able to rescue the defect in Fn layer formation in Snailb knockdown embryos, as well as to restore the migration of myocardial precursors. The results provide strong evidence that Snailb regulates the expression of integrin to modulate Fn assembly required for myocardial precursor migration during zebrafish embryonic development.

Previous data show that the somites still formed but had an abnormal shape in the snailb morphant. In consistent with the data, we also identified that morphological segmentation boundary of somite is disturbed and also Fn assembly was disrupted significantly in the snailb morphant. Since both integrin and Fn play important roles in morphological segmentation boundary of somite in zebrafish embryos, it is reasonable to propose that snail-integrin signaling exists wider than the heart field to control embryonic morphogenesis in other tissues and organs during embryonic development. In present works, we observe that Snailb is
involved in the movements during embryonic gastrulation as described previously. Their data show that snail1b plays a role in anterior migration of the cell in the axial mesendoderm during gastrulation, via controlling the cell-cell contact mediated by E-cadherin, suggesting there are other signals controlled by snail1b to mediate anterior migration of the cell during gastrulation and the Snail1b involved signaling is required to be investigated in the future work.

Methods

Zebrafish strains. Zebrafish were raised and maintained following standard procedure. Wild-type zebrafish belonged to the AB strain. The transgenic lines used were Tg(my7::GFP)5 and Tg(fkh1::GFP)26.

Antisense morpholino and mRNA or protein injections. Antisense morpholino oligonucleotides (MO) were purchased from Gene Tools (Philomath, OR, USA) and used as previously described. An MO with the sequence 5′-CCTCTTACCCATGTTACATTTATA-3′ was used as a control (Cil MO). The snail1b MO, which blocks snail1b translation by hybridizing to bases -28 to -4, was used as previously described. The zebrafish snail1b coding region was cloned using PCR and was ligated into the pcdNA3.1(+) vector or pEGFP-N1 vector. The origin PCR primers for snail1b full sequence were as follows: Sense primer: 5′-GCTGAAGTTTCGAGGGGATATT-3′ Anti-sense primer: 5′-CCACTAGAGCGCCGGACAGCAGCC-3′

In situ hybridization and histology. Whole-mount in situ hybridization (ISH) or the double ISH with DAB and NBT/BCIP was carried out as previously described. The antisense RNA probe was synthesized from the relative cDNA with a digoxygenin (DIG) or fluorescein (FLU) RNA labeling kit (Roche). In brief, embryos were permeabilized with Proteinase K (10 μg/ml, Promega) and hybridized overnight at 65°C with the DIG-labeled and FLU-labeled antisense probes. After several washes at 65°C and room temperature, DAB staining followed NBT/BCIP (Roche) staining was performed according to the manufacturer’s instructions.

Immunohistochemistry. Primary antibodies used in this study included: rabbit polyclonal anti-fibronectin antibody (1:500; Sigma, F3648), mouse anti-GFP antibody (1:200; Sigma, F1399), mouse anti-GFP antibody (1:500, Novus Biologicals, NBP1-47583). All Alexa-fluor-labeled secondary antibodies were purchased from Invitrogen and used at 1:800 dilution. Embryos were fixed overnight at 4°C in 4% PFA in PBS, and then cut into 10-μm sections. Immunohistochemistry was performed using these sections as previously described. After washing with PBS, the sections were imaged using a Zeiss fluorescence microscope.

TUNEL assay. Embryos were fixed overnight at 4°C in 4% PFA in PBS, washed 3 times with PBS, and then permeabilized with acetic for 5 min. After 3 washes with PBS, terminal deoxynucleotidyl transferase-mediated UTP-biotin nick end labeling (TUNEL) assay was performed using the Millipore In Situ Apoptosis Detection Kit as described.

The quantitative PCR and Statistical analysis. The quantitative PCR was performed as previously described. Statistical significance was evaluated with independent samples t-test. Differences were considered significant for p < 0.05.

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Author contributions
L.Q. and H.G. designed and performed experiments and wrote the manuscript. T.Z. and L.J. performed the molecular, histological experiments. C.X. and N.L. performed the microinjection. Y.X. performed ISH. H.Z. and W.M. performed the tissue section. H.X. and X.M. contributed to interpretation of the experiments and completed the manuscript.

Additional information
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