OPEN

SUBJECT AREAS:
CELL MIGRATION
ORGANOGENESIS

Received
21 October 2013

Accepted
7 March 2014

Published
26 March 2014

Correspondence and
requests for materials
should be addressed to
H.X. (xuhongm@
yahoo.com)

* These authors
contributed equally to
this work.

Snail modulates the assembly of fibronectin via $\alpha 5$ integrin for myocardial migration in zebrafish embryos

Liangjun Qiao*, Hongwei Gao*, Ting Zhang, Lulu Jing, Chun Xiao, Yue Xiao, Ning Luo, Hongyan Zhu, Wentong Meng, Hong Xu & Xianming Mo

Laboratory of Stem Cell Biology, Center for Medical Stem Cell Biology, State Key Laboratory of Biotherapy, West China Hospital, Sichuan University, Chengdu, 610041, China.

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, *snailb*, was expressed in the heart region of zebrafish embryos. Depletion of Snailb function dramatically reduced expression of $\alpha 5$ 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 $\alpha 5\beta 1$ protein rescued the Fibronectin layer and then the myocardial precursor migration in *snailb* 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 development¹. Mouse embryos with conditional disruption of *snail* after E8, die at E9.5, partially due to severe cardiovascular defects². In addition, Snail has been shown to play a direct role in endocardial cushion formation in a mouse model with a conditional *snail* knockdown³. 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 development⁴. Therefore, we used zebrafish embryos to address the mechanisms how Snail modulates cardiac development during embryonic development.

There are two duplicated *snail* genes in zebrafish, namely *snaila* and *snailb*⁵. They exist in the paraxial and axial mesoderm^{6,7} and have been implicated in the anterior migration of axial mesendoderm^{5,8}. Here, we show that *snailb* plays a key role in the migration of cardiac precursors by modulating the extracellular assembly of fibronectin (Fn) via the expression of $\alpha 5$ integrin. Our results provide the molecular mechanism that how Snail controls heart formation during zebrafish embryonic development.

Results

Knockdown of *snailb* induces cardiac defects. In order to identify the location of *snailb* expression in zebrafish embryos, we detected *snailb*, *myl7*, and *fn* expression by whole-mount ISH. We found that *snailb* was expressed in the anterior lateral plate mesoderm (LPM), where locates myocardial precursors⁹, next to the neural crest and with *myl7* expression (Supplementary Fig. S1A–D). These results suggest that Snailb played a role during heart development.

We then injected *snailb* antisense morpholino (MO) into embryos at the one-cell stage to determine the Snailb 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 *snailb* 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 *snailb* MO specificity, we used a construct that expresses a Snailb-GFP fusion protein contained *snailb* 5'-UTR sequence to test the efficiency of *snailb* MO. As expected, the *snailb* MO efficiently depleted the expression of the Snailb-GFP protein (Supplementary Fig. S2A–F), indicating the *snailb*

Figure 1 | Knockdown of *Snail1b* induces cardiac morphology defects. (A, B): Phenotypes of *snail1b* 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 *snail1b* MO at 48 hpf and 72 hpf. A, atrium; V, ventricle. (G): Quantification of phenotypes produced by *snail1b* morphants at 48 hpf, with or without *snail1b* mRNA at different concentrations. (H, I): Heart endocardium in *flk1::GFP* embryos injected with Ctl and *snail1b* MO at 72 hpf. White arrows: heart. Scale bars: 100 μ m.

MO specifically disrupts the function of Snail in zebrafish embryos. Then, we injected *snail1b* MO at different concentrations into zebrafish embryos and identified that higher dose of *snail1b* MO resulted in more abnormal embryos at 48 hpf (Supplementary Fig. S2G). Injection of *snail1b* mRNA without 5'-UTR sequence, which the *snail1b* 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, *snail1a* mRNA was unable to rescue the phenotype induced by *snail1b* knockdown. Together, the results indicate that *snail1b* displays essential function during the heart morphogenesis in zebrafish embryos.

Next, we injected *snail1b* 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 *snail1b* induced abnormal cardiac looping in the *myl7::GFP* embryos. At 48 hpf, most *snail1b* 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 *snail1b* 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 *snail1b* is required for embryonic cardiac development in zebrafish embryos.

Snail1b controls the migration of cardiac precursors. The morphological defects in the hearts of *snail1b* disrupted animals led us to determine which stages during heart development were controlled by *Snail1b*. 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 tube¹⁰. 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 *snail1b* 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 *snail1b* 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 *snail1b* morphants ($n = 12/17$; Figure 2E and F). These results indicate that *snail1b* modulates the migration of cardiac precursors during heart embryonic development.

In addition, *snail1b* 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 *snail1b* morphants, we used TUNEL staining to detect apoptosis. We found that *snail1b* 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 *snail1b* 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 *snail1b* morphants (Supplementary Fig. S3G), indicating that total cells with *myl7* expression remained in *snail1b* 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 *snail1b* morphants, not the reduction of *myl7* expressing cells, and further confirm that *snail1b* controls the migration of cardiac precursors during heart embryonic development.

Figure 2 | Defects in cardiac development detected by myocardial precursor. (A–J): *myl7* expression marking myocardial precursor cells in zebrafish embryos. (A, C, E, H): embryos injected with Ctl MO develop normally at 15, 18, 24, and 48 hpf. (B, D, F, I): embryos injected with *snailb* MO display cardiac fusion delays (B, D, F) and band-like heart shape at 48 hpf (I). *Myl7* expression (black arrows) is reduced at 15 and 18 hpf (B, D). (G, J): embryos injected with both *snailb* MO and mRNA display partial rescue of heart defects. (K–M): rescue of heart defects (arrowhead) in embryo injected with *snailb* MO and mRNA at 48 hpf.

The examination of endoderm formation by *foxa2* expression in *snailb* morphants at 26 hpf showed that there was not different in both control and morphant embryos (Supplementary Fig. S3H and I). Since endoderm formation appeared to be normal in the *snailb* morphants, we excluded the possibility that defects in endoderm formation were the reason for the delay or inhibition of myocardial fusion in these mutants. Taken together, the results indicate that *Snailb* is not necessary for the specification of myocardial precursors and the formation of endoderm during embryonic heart development.

***Snailb* maintains Fibronectin layer in the heart region.** Several lines of evidence support the fact that Fibronectin (Fn) is required during myocardial precursor migration^{11,12}, and that *snail* modulates Fn deposition¹³. 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 *snailb* morphants, especially in the midline region (Figure 3A–D), indicating that *snailb* 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 embryos¹⁴ and knockdown of *snailb* affects the shape of somite⁵, we also measured the Fn pattern in the abnormal somite boundary regions and identified that Fn assembly was disrupted significantly in *snailb* morphants ($n = 16/22$; Supplementary Fig. S7). The results are consistent with previous observations⁵ and indicate that the Fn assembly mediated by *Snailb* also plays a critical role in morphogenesis of somite.

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

$\alpha 5$ integrin acts downstream of *Snailb* for Fn deposition in the embryonic heart. Since integrins are essential for Fn fibril formation¹⁵, we tested the expression of several integrins in zebrafish embryos during heart formation (Figures 4A–C and Supplementary Fig. S5). The results showed that $\alpha 5$ integrin was markedly decreased in *snailb* morphants at 15 hpf compared to that in control embryos ($n = 11/16$; Figure 4A, B). The examination of expressing patterns and *snailb* mRNA induction showed that $\alpha 5$ integrin was co-expressed with *snailb* mRNA and was up-regulated in the LPM by *snailb* mRNA ($n = 14/21$; Figure 4C, Supplementary Fig. S1E, Supplementary Fig. S5J).

To determine whether $\alpha 5$ integrin was able to rescue the migration defect of myocardial precursors in *snailb* morphants, we co-injected *snailb* MO with $\alpha 5$ integrin mRNA at different concentrations. Accordingly, both co-injecting 50 pg and 100 pg $\alpha 5$ integrin mRNA rescued the myocardial defects in *snailb* morphants (Supplementary Fig. S6). Furthermore, we injected integrin proteins into the embryos to determine the function of $\alpha 5$ integrin in *snailb* morphants. The results showed that $\alpha 5\beta 1$ integrin protein was able to rescue the Fn fibril assembly at 17 hpf (Figure 3E and F) and the cardiac defects at 20 hpf (Figure 4D) in *snailb* morphants. Taken together, the results demonstrate that *Snailb* controls the expression $\alpha 5$ 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^{3,16}, and *snail* is additionally required for left-right asymmetry determination in the heart². Here, we show that *Snailb* 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 $\alpha 5$ mRNA or $\alpha 5\beta 1$ 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 migration¹⁷. In fact, mice mutant for *Snail* die at gastrulation, yet they can form mesoderm and express mesodermal markers¹. 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 *Snailb* controls the migration of myocardial precursors, rather

Figure 3 | Fn around myocardial precursors is modulated by Snail1b. Transverse sections of *myl7::GFP* (green) transgenic embryos immunostained for Fn (red). DAPI (blue) staining indicates nuclei. (A, B): Fn fibrils around myocardial precursors and across the midline in control embryos. (C, D): Fn levels decrease in *snail1b* MO injected embryos. (E, F): Fn levels are restored in embryos injected with $\alpha 5\beta 1$ integrin protein. (B, D, and F): Magnified view of dashed rectangle of A, C, and E, respectively. Scale bars: 50 μ m.

than modulating their generation. The data further demonstrate that Snail is required for cell movement, not fate determination.

Snail has been reported to play a role in endocardial development in mice³. However, the results presented here suggest that Snail1b does not involve in endocardial development in zebrafish. One possible reason might be that Snail1a can compensate for Snail1b function and maintains the generation of endocardial tissue. Another possibility is that Snail1b does not regulate the generation of endocardium,

which is derived from a distinct region in the anterior LPM where *snail1b* does not express¹⁸.

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 behaviors¹⁵, and is expressed early in embryonic development in the mesoderm as well as in between the embryonic germ layers^{19,20}. 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 E8.0²¹. Fn deficiency is in fact lethal within the first 10 embryonic days due to cardiovascular and vascular defects²². In zebrafish embryos, Fn deposition at the midline is required for the myocardial precursor migration and the formation of adhesion junctions among these cells^{11,12}. 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 Snail1b during cardiac development. Furthermore, in this study, the *snail1b* 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 mesoderm¹². The difference mainly results from the fact that Fn deposition was reduced, rather than completely inhibited in *snail1b* morphants. Indeed, cardiac precursors are partially surrounded by Fn deposition in these morphants.

Previous studies have shown that Fn accumulation is dependent on $\alpha 5$ integrin in zebrafish embryos^{14,23}. Overexpression of *snail* increases the expression of $\alpha 5\beta 1$ integrin²⁴. Our results display here that Snail1b regulates $\alpha 5$ integrin expression and controls Fn deposition, rather than expression, in the heart region of zebrafish embryos. Injection of $\alpha 5$ integrin mRNA or $\alpha 5\beta 1$ integrin protein is able to rescue the defect in Fn layer formation in Snail1b knock-down embryos, as well as to restore the migration of myocardial precursors. The results provide strong evidence that Snail1b regulates the expression of $\alpha 5$ 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 *snail1b* morphant⁵. 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 *snail1b* morphant (Supplementary Fig. S7). Since both $\alpha 5$ integrin and Fn play important roles in morphological segmentation boundary of somite in zebrafish embryos¹⁴, 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 embryo development. In present works, we observe that Snail1b is

Figure 4 | $\alpha 5\beta 1$ integrin protein rescues cardiac defects induced by Snail1b knockdown. *In situ* hybridization for indicated genes. (A–C): Expression pattern of $\alpha 5$ integrin in different embryos at 15 hpf, dorsal view. (D): different types of phenotypes displayed in injected embryos at 20 hpf. Graph shows phenotypic ratios of injected embryos at the same stage. Percentage of embryos of type 3 between embryos with *snail1b* MO and those with both *snail1b* MO and 25 pg $\alpha 5\beta 1$ integrin protein was statistically significant ($P = 0.023$, t -test). Ctl means Ctl MO, MO means *snail1b* MO.

involved in the movements during embryonic gastrulation as described previously⁵. 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-cadherin⁵, 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 procedure²⁵. Wild-type zebrafish belonged to the AB strain. The transgenic lines used were *Tg(myl7::GFP)*²⁶ and *Tg(flk1::GFP)*²⁷.

Antisense morpholino and mRNA or protein injections. Antisense morpholino oligonucleotides (MO) were purchased from Gene Tools (Philomath, OR, USA) and used as previously described²⁸. An MO with the sequence 5'-CCTCTTACCTCAGTTACAATTATA-3' was used as a control (Ctl MO). The *snail1b* MO, which blocks *snail1b* translation by hybridizing to bases -28 to -4, was used as previously described⁵. 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' CCACTAGAGCGCCGGACAGC 3'

Sense primer (ligate into pcDNA3.1(+) vector):

5' CCGGAATTCATGCCACGCTCATTTCTTGT 3'

Anti-sense primer (ligate into pcDNA3.1(+) vector):

5' CCGCTCGAGGAGCGCCGGACAGCAGCC 3'

Sense primer (ligate into pEGFP-N1 vector):

5' CCGGAATTCGCTGAAGTTTCGAGGGGATATTACA 3'

Anti-sense primer (ligate into pEGFP-N1 vector):

5' CCGGATCCCGGAGCGCCGGACAGCAGCC 3'

The integrin $\alpha 5$ mRNA was as previously described¹⁴. The *snail1b* and integrin $\alpha 5$ mRNA was obtained using the mMessage mMachine T7 Kit (Invitrogen). Embryos were injected at the one-cell stage with 3 ng *snail1b* MO or 10–200 pg mRNA per embryo. The integrin $\alpha 5\beta 1$ protein was bought from R&D systems (3230-A5-050).

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^{29,30}. The antisense RNA probe was synthesized from the relative cDNA with a digoxigenin (DIG) or fluorescein (FLU) RNA labeling kit (Roche). In brief, embryos were permeabilized with Proteinase K (10 μ g/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 : 200; Sigma, F3648), mouse anti-GFP antibody (1 : 500, Novus Biologicals, NBP1-47583). All Alexa-fluor-labeled secondary antibodies were purchased from Invitrogen and used at a 1 : 800 dilution. Embryos were fixed overnight at 4°C in 4% PFA in PBS, and then cut into 10- μ m sections. Immunohistochemistry was performed using these sections as previously described¹². 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 acetone for 5 min. After 3 washes with PBS, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) assay was performed using the Millipore *In Situ* Apoptosis Detection Kit as described³¹.

The quantitative PCR and Statistical analysis. The quantitative PCR was performed as previously described³². Statistical significance was evaluated with independent samples *t*-test. Differences were considered significant for $p < 0.05$.

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Acknowledgments

The authors thank the members of Dr. Mo's laboratory for their critical discussion and review of the manuscript. The English was edited by Christopher Brooks, Ph. D from Bioscience Editing Solutions. This work was supported by the National Basic Research Program of China (to X.M., 2009CB941200), the Nature Science Foundation of China (to H.X., 31171384), and the Key Project of Chinese Ministry of Education (to X.M., No. 109136).

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

Supplementary information accompanies this paper at <http://www.nature.com/scientificreports>

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Qiao, L.J. *et al.* Snail modulates the assembly of fibronectin via $\alpha 5$ integrin for myocardial migration in zebrafish embryos. *Sci. Rep.* **4**, 4470; DOI:10.1038/srep04470 (2014).

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