microRNA-206 modulates an Rtn4a/Cxcr4a/Thbs3a axis in newly forming somites to maintain and stabilize the somite boundary formation of zebrafish embryos

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1. Introduction

MicroRNAs (miRNAs) are short (approx. 22 nt) endogenous non-coding RNAs that regulate gene expression at the post-transcriptional level by silencing target gene(s) through pairing between the seed sequence(s) of miRNA and the 3'-untranslated region (3'UTR) of target messenger RNAs (mRNAs). To promote dynamic equilibrium of expression among genes, miRNAs play an important role in cell differentiation, tissue identity [1] and normal development [2]. In particular, microRNA-206 (miR-206) has been reported as a regulator of muscle proliferation and differentiation, but its function in the mesoderm and somite cells of embryos remains unclear. Importantly, miR-206 can be detected at the one-cell stage of zebrafish embryos [3], and its expression increases in somites between 12 and 16 hpf [4,5]. Therefore, we
employed the labelled microRNA pull-down (LAMP) assay [6] of mRNAs extracted from 16-hpf zebrafish embryos and found that *reticulon 4a* (*rtn4a*) is a target gene for *miR-206* at this developmental stage. Zebrafish Rtn4a is essential for embryonic development and patterning of the nervous system [7,8]. However, the role Rtn4a plays at the early stage of somite boundary formation has not been elucidated.

Somite boundary formation of vertebrates is an example of developmental mesenchymal–epithelial transition (MET). More specifically, the presomatic mesoderm (PSM), an area of mesoderm in the neurulating embryo, consists primarily of mesenchymal cells. These mesenchymal cells surrounding the PSM become epithelial cells through MET and separate from the PSM to form somites [9–11]. Somites are transient structures that are present on both sides of the body axis from head to tail, first forming a repetitive and metameric configuration and later differentiating into skin, skeletal muscle and axial bone in late embryogenesis. As somites separate from each other, a morphological boundary is formed, termed the gap or cleft [9–11]. Therefore, we can define somite formation as the reiterated subdivision of paraxial mesoderm into paired, epithelial spheres of cells on either side of the midline [12]. Studies reveal a pre-patternning process in the anterior of the PSM before the morphological appearance of somite pairs. Cooke & Zeeman [13] proposed a clock and wavefront model to explain the pattern formation of PSM. They explain that a clock mechanism controls cell oscillations between anterior and posterior somitic identities in the PSM. During this process, the position of future somite boundaries is selected in the PSM. Both anterior and posterior somitic identities are responsible for boundary formation. Therefore, this boundary formation process makes the vertebrate a particularly good model with which to study MET [14].

Importantly, the boundary formation process may be considered the product of a two-step signalling cascade. The first step ensures normal development of somite and new boundary formation, and the second ensures proper maintenance of the boundary gap. In zebrafish, during the first developmental process, fluctuate expression patterns of two Hes-related genes, *her1* and *her7*, oscillate in PSM, known as the segmentation clock genes, controlling and coordinating the orderly process of oscillation [15,16]. Expression of Notch ligand DeltaC also oscillates during somitogenesis [17,18]. The oscillation phase of DeltaC expression is synchronized with that of *her1* and *her7*. The *tbx6* gene is an essential factor for the formation of the somite boundary [19], because the Tbx6 protein domain denotes the position of the succeeding somite boundary that will be formed during orderly somite segmentation. Furthermore, *ripply1* and *ripply2* restrict *tbx6* expression in the anterior edge of newly forming somites [20]. Particularly, in zebrafish, Mesp is not essential for Ripply-dependent boundary positioning, while it is required for the generation of morphological boundary and rostro-caudal polarity formation [21].

To maintain the boundary gap in the newly formed boundary, somite cells produce extracellular matrix (ECM) to form muscle plasticity and myotendinous junction (MTJ) [22]. Unlike amniotes, such as mouse and chicken, zebrafish undergoes simultaneous epithelialization at both anterior and posterior border cells [11]. EphA4 and Ephrinb2 signalling induces the MET of somite boundary formation and ECM assembly in zebrafish [23,24]. Rap1b, a GTPase, acts downstream of Ephrin reverse signalling and contributes to Integrin inside-out activation, resulting in fibronectin polymerization at somite boundaries [25]. Recently, Julich et al. [26] reported that Cadherin 2 (Cdh2) is also essential for the epithelialization of cells along the somite boundary. Cdh2 causes Integrin α5 inactivation within the paraxial mesoderm mesenchyme through cell–cell cohesion. When embryos start to form a new boundary, Cdh2 expression decreases along the nascent boundary, resulting in the accumulation of fibronectin. Thereafter, outside-in Integrin signalling begins to activate phosphorylated focal adhesion kinase (p-FAK) in boundary cells through the Integrin receptor [26].

Based on this foundation, we provide further insight into the molecular regulatory pathway that underlies the involvement of *miR-206* in the somite boundary formation of zebrafish embryos. Specifically, we confirm that *miR-206* plays a role in somite boundary formation at the early stage through silencing *rtn4a* expression. Furthermore, we found that C–X–C motif chemokine receptor 4a (*Cxcr4a*) represses the expression level of Rtn4a. Cxcr4a has been reported to be involved in somite rotation in zebrafish embryos [27] and somite morphogenesis in *Xenopus laevis* embryos [28]. Knockdown of *cxcr4a* in *Xenopus* resulted in defective formation of the somite boundary [28]. Additionally, we demonstrated that Cxcr4a is able to repress the expression of thrombospondin 3a (*Thbs3a*), an ECM protein. Finally, we proved that Thbs3a is involved in mediating the epithelialization of somite boundary cells that affect somite boundary formation. Thus, for the first time, we have demonstrated that a *miR-206/rtn4a/cxcr4a/thbs3a* axis is also importantly involved in controlling somite boundary formation during somitogenesis of zebrafish embryos.

2. Material and methods

2.1. Zebrafish husbandry and microscopy observation

Wild-type zebrafish (*Danio rerio*) AB strain (University of Oregon) and transgenic lines *Tg(myf5:GFP)* [29] and *Tg(a-actin:RFP)* [30] were used. Production and stage identification of embryos followed the description by Westerfield [31] and Kimmel et al. [32]. Microscopy observation was performed with a fluorescent stereomicroscope (Leica) and a confocal spectral microscope (Nikon).

2.2. Searching for the putative target genes of *miR-206*

To search for the putative target genes of *miR-206*, we performed LAMP assay [6] with some modifications. The pre-*miR-206* was labelled with biotin and then mixed with cell extracts. The putative target genes were precipitated by anti-biotin agarose beads (Sigma) and transformed into cDNA by reverse transcriptase-polymerase chain reaction (RT-PCR). Finally, these putative cDNAs for *miR-206*-targeting were further combined with Zebrafish Whole Genome Microarray (Agilent).

2.3. Plasmid constructs

We designed primers to perform PCR from the cDNA library of zebrafish embryos at 20 hpf to clone the complete 3’UTR segment of each cDNA of *cited3* (*NM200078, +942 to +1638*), *gadd45ab* (*NM001002216, +587 to +1217*), *zwt42*...
co-injected 5 ng mRNA which was normalized as 100%. In zebrafish embryos, we activity obtained from phRG-TK alone was the control group, which was normalized as (20 embryos per group). The injection for 60 embryos which were randomly collected from ¼ fold change of microinjection into one embryo as follows: pre-miR-206

gene [33].

The dissociation procedure of zebrafish embryonic cells was modified from Lee et al. [35]. Briefly, the mir-206- MO-injected and control-mRNA-injected embryos from Tg(mfat5:GFP) at 20 hpf were incubated with trypsin (Sigma; 59427C) for 20 min at room temperature. Embryos were shattered by pipetting to completely separate cells from the tissue. Then, the GFP(+) cells were sorted by a cell sorter (BD FACS Aria III). The GFP(+) cells were collected in TRIZOL solution (Thermo Fisher Scientific) for RNA extraction.

2.8. Whole-mount in situ hybridization

Whole-mount in situ hybridization (WISH) followed the method described previously by Lin et al. [30] with exceptions. The 22-nt antisense sequences of mir-206 (EXIQON) [5] and the cDNA coding for rtndal (NM001079912), ccrx4a (NM131882), thbs3a (NM173225), gga8 (NM131281), deltad (NM130955), her1 (NM130788), tbt6 (NM153666), mesp1 (NM131551), mesp2 (NM131552), dgc8 (NM001122749), pom1 (NM001048067), nikras2 (NM001003433), zgc56251 (BC046025), sald (NM001080609) or sdc4 (NM001048149) were used as probes.

2.9. Immunohistochemistry

Immunohistochemistry was performed according to the protocol described previously by Koshida et al. [36] with some modifications. In this study, antibodies such as anti-fibronectin (Sigma; 1:200), anti-β-tubulin (Sigma; 1:1000), anti-laminin (Sigma; 1:100) and anti-phosphor FAK [pY397] (Thermo; 1:200), were used. Alexa 488 goat anti-rabbit IgG (Rockland) and Alexa 488 goat anti-mouse IgG (Life Technologies) served as secondary antibodies at a 1:1000 dilution in blocking solution. Rhodamine–phalloidin (Thermo; 1:200) was added in the blocking solution to detect F-actin.

2.10. Quantitative RT-PCR

For each experiment, we collected 100 embryos in 500 μl of Trizol reagent (Invitrogen) and stored them at −80°C. Total RNA was isolated according to the manufacturer’s instructions. For quantitative RT-PCR (qPCR), first-strand cDNA was generated using 1 μg of total RNA. Both cDNA concentrations were adjusted to 200 ng ml⁻¹, and q-PCR was performed using the 7900HT Fast Real-Time PCR System (Applied Biosystems, USA) according to the manufacturer’s instructions. Forward and reverse primers designed for cloning each gene by PCR were as follows: GCATCGGCA CAAAATTGACC and TIGAATTCCTGTTGTCACACGT for rtndal, CTGCTGTGTTGCGATTGC and GGAATCACCTCC AGCAGCA for ccrx4a, GAGAAATCATTTTGTCCCAATC and ACCTGTTACGTTGTAACCTG for thbs3a, and CTCC TCTTGGTGTTGCTG and CCGATTTCCTCTCAACGC TCT for ef1a. Expression levels of transcripts were determined by comparison with a standard curve from total RNA isolated from wild-type (WT) embryos.

2.4. Validation of mir-206-targeting genes by luc activity assay

Dual luc reporter assay (Promega) was carried out in cell lines HEK-293T and C2C12 and zebrafish embryos by following the method described previously [5] with some modifications. We co-transfected 40 ng of plasmid pGL3-TK, which served as an internal control, 200 ng of each examined plasmid, including phRG-TK, phRG-TK-cited3-3’UTR, phRG-TK-gadd45ab-3’UTR and phRG-TK-rtn4a-3’UTR containing cited3-, gadd45ab-, and rtn4a-3’UTR sequences, respectively, were driven by the upstream regulatory elements of zebrafish Myogenic Factor 5 (Myf5) gene [33].

2.5. Antisense morpholino oligonucleotides used to perform knockdown experiments

All morpholino oligonucleotides (MOs) were purchased from Gene Tools (USA) and prepared according to the protocol published by Gene Tools. The sequence and injected amount of each MO were as follows: mir-1-MO (AATACA TACTTCTTATTCCTCA, 8 ng) [5], mir206-MO (GATCTCA CTGAAACACACACTTCC, 8 ng) [5], mir206-5-mis-MO (GAgATCACATGAAACACACACTTCC, 8 ng) (the mismatched nucleotides are underlined) [5], rtndal-MO (GAAAA CAAAACACACTGTGACC, 2 ng), ccrx4a-MO (AGAA GTTTCCTTATAGTGCCTAT, 8 ng) [34], and thbs3a-MO (AGATAAAGGCAAGATTTTGCTGCT, 1 ng).

2.6. RNA preparation and mRNA overexpression

RNA and capped mRNAs were synthesized according to the manufacturer’s protocol (Epigen). The resultant RNAs were diluted with distilled water for final molecular mass of microinjection into one embryo as follows: pre-miR-206 RNA, 200 pg; pre-miR-1 RNA, 200 pg; cited3 mRNA, 200 pg; rtndal mRNA, 200 pg; rtndal mRNA, 200 pg; and thbs3a mRNA, 400 pg.

2.7. Fluorescence-activated cell sorting

Whole-mount in situ hybridization (WISH) followed the method described previously by Lin et al. [30] with exceptions. The 22-nt antisense sequences of mir-206 (EXIQON) [5] and the cDNA coding for rtndal (NM001079912), ccrx4a (NM131882), thbs3a (NM173225), gga8 (NM131281), deltad (NM130955), her1 (NM130788), tbt6 (NM153666), mesp1 (NM131551), mesp2 (NM131552), dgc8 (NM001122749), pom1 (NM001048067), nikras2 (NM001003433), zgc56251 (BC046025), sald (NM001080609) or sdc4 (NM001048149) were used as probes.

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Immunohistochemistry was performed according to the protocol described previously by Koshida et al. [36] with some modifications. In this study, antibodies such as anti-fibronectin (Sigma; 1:200), anti-β-tubulin (Sigma; 1:1000), anti-laminin (Sigma; 1:100) and anti-phosphor FAK [pY397] (Thermo; 1:200), were used. Alexa 488 goat anti-rabbit IgG (Rockland) and Alexa 488 goat anti-mouse IgG (Life Technologies) served as secondary antibodies at a 1:1000 dilution in blocking solution. Rhodamine–phalloidin (Thermo; 1:200) was added in the blocking solution to detect F-actin.
2.11. Western blot analysis

Total proteins extracted from embryos were analysed on a 10% SDS-PAGE followed by western blot analysis according to the procedures described by Lin et al. [5], except that the yolk was removed and the antibodies against Rtn4a (Abk; 1:1000), FAK (Cell Signaling; 1:1000), phosphor FAK [pY397] (Thermo; 1:1000), cell division control protein 42 homologue (cdc42) (New East; 1:500), active cdc42 (New East), α-tubulin (Sigma-Aldrich; 1:5000), GADPH (Santa Cruz; 1:1000), mouse-HRP (Santa Cruz; 1:5000) and rabbit-HRP (Santa Cruz; 1:5000) were used.

2.12. Defective formation of somite boundary

When embryos were injected with miR-206-MO, rtnda4 mRNA, cxxr4a-MO and thbl3a mRNA, the somite boundary formation from the sixth to 20th somite of the embryos was examined at 20 and 48 hpf. We calculated the number of embryos exhibiting defective somite boundary formation, as indicated by at least one incompletely formed boundary at either side of the trunk.

3. Results

3.1. Screening of the target genes for miR-206

Although miR-206 could be detected in the one-cell stage of zebrafish embryos [3], it was significantly increased in somites during developmental stages between 12 and 16 hpf [4,5]. To understand the functions of miR-206 at that particular stage, we searched for the target genes of miR-206 at 16 hpf through LAMP assay. In total, 117 putative target genes for miR-206 were screened (electronic supplementary material, table S1). Four of them, including celp3p0-interacting transactivator with Glu/Asp-rich carboxy-terminal domain 3 (cited3), growth arrest and DNA-damage-inducible alpha b (gadd45ab), zinc finger protein 142 (znf142) and reticulon 4a (rtnda4), were selected for further study because they were specifically expressed in somites.

To further confirm whether cited3, gadd45ab, znf142 and rtnda4 were the target gene(s) of miR-206, we cloned their 3’ UTRs and fused them downstream of reporter cDNA encoding Renilla luc and driven by herpes simplex virus thymidine kinase promoter (TK). Thus, four expression plasmids were constructed: phRG-TK-cited3-3’UTR, phRG-TK-gadd45ab-3’UTR, phRG-TK-znf142-3’UTR and phRG-TK-rtnda4-3’UTR (figure 1a). These constructs were separately co-injected with pcS2-miR-206 into cell lines HEK-293T and C212 (figure 1b). Compared with the luc activity from the phRG-TK group (control) which was normalized as 1, the luc activities of HEK-293T cells transfected with phRG-TK-cited3-3’UTR, gadd45ab-3’UTR, znf142-3’UTR and rtnda4-3’UTR were 0.42 ± 0.05, 0.76 ± 0.16, 1.04 ± 0.17 and 0.44 ± 0.07, respectively, while in the C212 cells they were 0.69 ± 0.07, 1.10 ± 0.09, 0.88 ± 0.11 and 0.57 ± 0.07, respectively (figure 1b). Since luc activity was greatly inhibited by miR-206 in both non-muscle and muscle cell lines transfected with phRG-TK-cited3-3’UTR and phRG-TK-rtnda4-3’UTR, we chose only cited3 and rtnda4 for further in vivo experiments.

For the in vivo assay, we constructed phRL-Myf5-cited3-3’UTR, phRL-Myf5-gadd45ab-3’UTR, PhRL-Myf5-znf142-3’UTR and phRL-Myf5-rtnda4-3’UTR (figure 1c), in which the luc reporter was driven by zebrafish myf5 promoter, a somite-specific promoter [33]. These constructs were co-injected with either pre-miR-1 RNA or pre-miR-206 RNA into one-cell zebrafish embryos. Compared with the luc activity of control embryos injected with phRL-Myf5 alone, which was normalized as 1, the luc activities of embryos injected with pre-miR-206 RNA combined with plasmids phRL-Myf5-cited3-3’UTR, phRL-Myf5-gadd45ab-3’UTR, phRL-Myf5-znf142-3’UTR and phRL-Myf5-rtnda4-3’UTR were 0.49 ± 0.09, 1.05 ± 0.06, 0.72 ± 0.02 and 0.26 ± 0.03, respectively (figure 1c). On the other hand, the luc activities of embryos injected with pre-miR-1 RNA combined with plasmids phRL-Myf5-cited3-3’UTR, phRL-Myf5-gadd45ab-3’UTR, PhRL-Myf5-znf142-3’UTR and phRL-Myf5-rtnda4-3’UTR were 0.96 ± 0.07, 1.08 ± 0.07, 0.75 ± 0.04 and 1.05 ± 0.07, respectively (figure 1c). This evidence indicated that miR-206 can only specifically silence the reporter gene through cited3 and rtnda4-3’UTR, even though miR-1 and miR-206 have identical seed sequences. However, because rtnda4-3’UTR showed more obvious inhibition by miR-206, we focused on target gene rtnda4 for further study.

3.2. miR-206 was unable to silence reporter gene expression driven by mutated 3’UTR of rtnda4

The FindTar, RNA22 and RNAHybrid software programs were used to analyse the 3’UTR of zebrafish rtnda4, and three putative binding sequences for miR-206 in rtnda4-3’UTR were found. We therefore mutated the nucleotides at these positions and constructed plasmids phRL-Myf5-rtnda4-3’UTR-mnt1, -mnt2 and -m3, in which 1353 ~ 1379 nt, 1422 ~ 1444 nt and 1554 ~ 1576 nt were mutated, respectively (figure 1d). Compared with the luc activity of embryos injected with pre-miR-206 plus phRL-Myf5, which was normalized as 1, the luc activities of embryos injected with pre-miR-206 RNA combined with phRL-Myf5-rtnda4-3’UTR, -rtnda4-3’UTR-mnt1, -rtnda4-3’UTR-mnt2 and -rtnda4-3’UTR-mnt3 were 0.31 ± 0.02, 0.40 ± 0.08, 0.97 ± 0.16 and 0.36 ± 0.09, respectively (figure 1d). This evidence indicated that injection of phRL-Myf5-rtnda4-3’UTR-mnt2 abolishes the silencing effect of miR-206. Taken together, it was plausible to conclude that miR-206 silences the translation of reporter gene through binding rtnda4-3’UTR at 1422 ~ 1444 nt.

3.3. Overexpression of each isoform of Rtn4a causes abnormally transverse actin filaments in somites

Three rtnda4 isoforms are found in zebrafish, named rtnda4l, rtnda4am and rtnda4n [37,38]. They share identical C-terminal sequence and 3’UTR, including 1422 ~ 1444 nt, which was bound by miR-206 in the experiment described above (figure 2a). In order to exclude the off-target effect of MO injection, we knocked down endogenous miR-206 by injection of miR-206-MO which specifically inhibits both miR-206-1 and miR-206-2 in zebrafish embryos without affecting the production of miR-1 with the same seed sequence as that of miR-206 [5]. As control-MO, we used miR-206-3-mis-MO, as described previously [5]. When we injected mRNAs to individually overexpress rtnda4l, rtnda4am or rtnda4n in the zebrafish embryos, defective phenotypes exhibiting abnormal transverse actin filaments across the somite boundary could be observed at 48 hpf in embryos injected as noted above (figure 2b–g), except the control-MO injection group. By contrast, the A-band within

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actin filaments was arranged with no apparent change in order (figure 2b–g). Therefore, based on the data obtained from the rescue experiment, as shown in the electronic supplementary material, table S2, it was concluded that either knockdown of miR-206 or overexpression of each isoform of Rtn4a in embryos causes the observed defective phenotype.
These somite boundary formation defects can be rescued by overexpression of mature miR-206 RNA or knockdown of rtn4a. Thus, we hypothesized that either knockdown of miR-206 or overexpression of each isoform of Rtn4a caused a previously observed defective phenotype, but did not disturb the arrangement of actin or muscle fibre development.

Since overexpression of each isoform of Rtn4a caused defective actin filaments in somites, we focused on rtn4aI for further study. Using WISH, we found that miR-206 was detectable in somites and PSM as early as at 12 hpf, while rtn4aI was detected in somites at 16 hpf (electronic supplementary material, figure S1). Furthermore, using frozen sections, we observed that both miR-206 and rtn4aI were expressed in the fast muscle of trunk at 24 hpf (electronic supplementary material, figure S1), suggesting a tight, possibly regulatory, relationship between miR-206 and rtn4aI in the zebrafish somite.

3.4. Knockdown of miR-206 increases rtn4a mRNA and Rtn4aI protein in zebrafish embryos

Using qPCR, we quantified rtn4aI mRNA expression level in zebrafish embryos injected with miR-206-MO to specifically knock down endogenous miR-206. The amount of rtn4aI mRNA in the untreated WT embryos at 20 hpf was normalized as 1, and the amount of rtn4aI mRNA in embryos injected with miR-206-MO was 1.52 ± 0.31 (n = 3) (figure S2), which represents an approximately 52% increase. This qPCR result was consistent with WISH detection in embryonic somites, which demonstrated that the rtn4aI mRNA signal shown in miR-206-MO-injected embryos was stronger than that of WT (electronic supplementary material, figure S2).

Furthermore, compared with WT, we found that the protein level of Rtn4aI was increased in the miR-206-MO-injected embryos at 20 hpf (figure 2I). However, the protein level of Rtn4aI remained unchanged in the embryos injected with miR-1-MO (figure 2J, lane 2), which shares an identical seed sequences with miR-206 and whose antisense oligonucleotide sequence were previously described by Lin et al. [5], indicating that the amount of Rtn4aI protein is specifically regulated by miR-206, not miR-1, thus confirming our speculation.

3.5. Either knockdown of miR-206 or overexpression of rtn4aI causes defective somite boundary in embryos

To determine if any change of miR-206 and its target gene might cause a corresponding defect in embryos, we employed zebrafish transgenic line Tg(a-actin:RFP), in which muscle cells are tagged with red fluorescent protein (RFP) [30]. Embryos classified as donor groups received injection of the green fluorescent dye Dextran alone or injection of Dextran combined with either miR-206-MO or rtn4aI mRNA in Tg(a-actin:RFP). After Tg(a-actin:RFP) embryos were treated and developed at 4 hpf, 20–30 cells were taken from donor embryos and transplanted into the non-axial mesoderm of recipient (WT or MO/mRNA-injected) embryos at 4.7 hpf. The control group showed no somite boundary defect in recipient embryos (figure 3a–d) (n = 15). Additionally, when cells from WT embryos were transplanted into either miR-206-MO- (figure 3e–h) (n = 20) or rtn4aI-mRNA-injected recipients (figure 3i–l) (n = 18), no somite boundary defect was observed. However, somite boundary of recipient embryos transplanted with cells from embryos injected with either Dextran combined with miR-206-MO (figure 3m–p) (n = 21) or rtn4aI mRNA (figure 3q–t) (n = 24) appeared defective (ectopic or loss) by 23.8 and 33.3%, respectively, indicating that the number of somite boundary defects caused by knockdown of miR-206 and overexpression of Rtn4aI had increased. This quantitation experiment strongly suggests that either knockdown of miR-206 or overexpression of Rtn4aI results in defective formation of the somite boundary, indicating it is an example of a community effect.

Based on these findings, we asked if such defect resulted from abnormal regulatory factors involved in somite boundary formation, such as fgf8, deltal, her1, tbx6, mespa and mespb. As shown in the electronic supplementary material, figure S3, the expression patterns of these segmentation decision genes were not significantly altered in either miR-206-knockdown or Rtn4aI-overexpression embryos. These data suggested that the effect of the miR-206/rtn4aI axis on somite boundary formation is independent of the effect on somite formation, giving reason to hypothesize that it might be mediated through some unknown downstream effectors.

3.6. Genes that were predominantly impacted by both miR-206-knockdown and rtn4aI-overexpression in the somite of embryos

To discover potential regulatory factors mediating the effect of miR-206/rtn4aI axis in somites, we employed another transgenic line, Tg(myf5: GFP), in which the GFP reporter is driven by an upstream 80 kb of zebrafish myf5 such that GFP is expressed in the developing PSM and somite [29]. After we injected miR-206-MO and rtn4aI mRNAs separately into
Figure 2. Knockdown of endogenous miR-206 increases the level of Rtn4al protein, causing abnormal transverse actin filaments across the somite boundary. (a) The genomic structure of three isoforms of rtn4a genes. Locations of the rtn4a-MO binding site (red line), miR-206 binding site at the 3’UTR of rtn4a mRNA (yellow box), and recognized region of antibody against Rtn4a protein (green line) were indicated. (b) WT, (c) miR-206-MO-injected, (d) miR-206-5-mis-MO-injected, (e) rtn4al-mRNA-injected, (f) rtn4am-mRNA-injected, and (g) rtn4an-mRNA-injected embryos developed at 48 hpf were immunostained with fluorescent phalloidin to label F-actin. Panels (b’–g’) were the enlarged views of corresponding panels (b–g). The area between the two arrowheads indicates the abnormal transverse actin filaments across the somite boundary. (h) The relative amounts of rtn4al mRNA between WT deheaded embryos and miR-206-MO-injected deheaded embryos at 20 hpf were quantified by qPCR when that of WT was normalized as 1. One hundred embryos were used each time, and experiments were performed in triplicate (n = 3). (i) Western blot analysis of Rtn4al (35 kD) among proteins extracted from deheaded embryos at 20 hpf: in WT (lane 1), rtn4al-mRNA-injected embryos (lane 2), rtn4a-MO-injected embryos (lane 3) and miR-206-MO-injected embryos (lane 4). (j) Western blot analysis of Rtn4al among proteins extracted from deheaded embryos at 20 hpf: in WT (lane 1), miR-1-MO-injected embryos (lane 2), and miR-206-MO-injected embryos (lane 3). Student’s t-test was used for statistical analysis. Asterisk indicates significant difference at *p < 0.05. RD: Relative density. GAPDH and tubulin served as internal controls.
Tg(myf5:GFP) embryos, cells were dissociated from embryos at 20 hpf. We collected the GFP-expressing cells through fluorescence-activated cell sorting (FACS) and then performed microarray. We found 33 and 60 genes greatly increased and decreased, respectively, in both miR-206-knockdown embryos and the rtn4al-overexpression embryos compared with the untreated embryos (electronic supplementary material, figure S4). From these genes, we selected four upregulated genes, including DiGeorge syndrome chromosomal region 8 (dgcr8), protein O-mannosyl-transferase 1 (pomt1), NFKB inhibitor interacting Ras-like 2 (nkras2) and thbs3a, and four downregulated genes, including zgc 56251, Sal-like protein 4 (sall4), cxcr4a and syndecan 4 (sdc4), and used WISH to determine their proportional expression in somites relative to the results from microarray (electronic supplementary material, figure S5). On the basis of WISH results, we found that thbs3a and cxcr4a were highly expressed in the somite boundary region, and these two genes were chosen for further study.

### 3.7. Somite boundary formation defect caused by abnormal expression of cxcr4a and thbs3a is similar to that caused by abnormal expression of miR-206 and rtn4al

The somite boundary was normally developed in WT embryos at 20 hpf (figure 4a–c), while was incompletely formed in embryos injected with miR-206-MO (figure 4d–f), miR-206-5-mis-MO (figure 4g–i), rtn4al mRNA (figure 4j–l), cxcr4a-MO (figure 4m–o) and thbs3a mRNA (figure 4p–r).
The percentages of embryos exhibiting defective formation of the somite boundary among examined embryos were 0% ($n = 46$) in the WT group, 52.1% ($n = 48$) in the miR-206-MO-injection group, 0% ($n = 30$) in the miR-206-5-miR-MO-injection group, 56.4% ($n = 55$) in the rtr4al-mRNA-injection group, 59.2% ($n = 49$) in the cxc4a-MO-injected group, and 54.1% ($n = 49$) in the thbs3a-mRNA-injection group. We observed that defective somite boundary was randomly distributed along the trunk, even occurring on both sides of the trunk in zebrafish embryos. Therefore, to determine the

Figure 4. Somite boundary defect occurred in either cxc4a-knockdown or thbs3a-overexpression zebrafish embryos. (a–c) None treated control WT embryos, (d–f) miR-206-knockdown embryos, (g–i) miR-206-5-miR-MO-injected embryos (served as control), (j–l) rtr4al-mRNA-overexpressed embryos, (m–o) cxc4a-knockdown embryos, and (p–r) thbs3a-mRNA-overexpressed embryos were examined. Immunofluorescent staining was performed on the embryos at 20 hpf. (a,d,g,i,m,p) Fibronectin was labelled with green fluorescent signal to detect somite boundary. (b,e,h,k,n,q) Phalloidin was labelled with red fluorescent signal to detect F-actin. (c,f,i,l,o,r) Merge of the two signals. Places where the somite boundary was absent were marked by white arrowheads. The numbers shown on the lower-right corner are the percentages of defective somite boundary occurrence averaged from three independent experiments. (s) Quantification of the number of defective boundaries per embryo at the 6th to 20th pairs of somites on both sides of the trunk at 20 hpf.
disrupted boundary located at the 6th to 20th pairs of somites on both sides of the trunk in 20 hpf embryos, we quantified the number of embryos having this defective boundary among examined embryos. Additionally, we calculated the number of defective somites per embryo. As shown in figure 4s, we demonstrated that defective formation of somite boundary caused by abnormal expression of cxcr4a and thbs3a was completely congruent with that caused by abnormal expression of miR-206 and rtn4al. Additionally, we found that this somite boundary defect did not result from developmental delay because this defect could still be observed in the injected embryo up to 48 hpf (electronic supplementary material, figure S6 and table S2). Although somite patterning and somite boundary formation genes, such as fgf8, deltad, her1, tbx6, mespa, and mespb, were not significantly different from those of the control group (electronic supplementary material, figure S3), we observed that the ECM, consisting of fibronectin and laminin, was not correctly organized in the somite boundary of embryos at 20 hpf (figure 4) and 48 hpf (electronic supplementary material, figure S6; table S2) when embryos were injected with either miR-206-OMO or rtn4al mRNA. These results were similar to those reported by Goody et al. [39] for mkr2b-MO-injected embryos. Although somites were able to form normally starting at 20 hpf, we noticed that somite boundary formation could be disrupted and fail to form completely by 48 hpf by the failed epithelialization of somite boundary cells, in turn resulting in discontinuous MTJ [39,40]. Thus, some muscle fibres were abnormally transverse across places where the somite boundary had incompletely formed. This evidence supports that this somite boundary defect did not result from development of p-FAK to confirm its concentration at the intersomitic boundary. Therefore, we detected the distribution pattern from qPCR (figure 5j). Furthermore, we also employed qPCR to quantify the level of thbs3a mRNA expression in embryos injected either cxcr4a-MO alone or cxcr4a-MO combined with miR-206-MO, miR-206 RNA, rtn4al-MO or rtn4al mRNA. As shown in figure 5k, when cxcr4a was knocked down in embryos, thbs3a expression was increased, irrespective of whether miR-206 or rtn4al was increased or decreased. This line of evidence suggested that miR-206 and rtn4al do not regulate thbs3a expression in cxcr4a morphants. However, cxcr4a is an upstream negative regulator of the thbs3a gene. Therefore, Cxcr4a has a direct and significant effect on Thbs3a. Additionally, injection of miR-206-MO, rtn4al mRNA or cxcr4a-MO combined with thbs3a-MO in embryos resulted in the reduction of thbs3a expression. Interestingly, we demonstrated that the somite boundary formation defect caused by miR-206-knockdown, rtn4al-overexpression and cxcr4a-knockdown could be rescued by reduction of Thbs3a (figure 5f).

Based on this line of evidence, we concluded that Cxcr4a is an upstream negative regulator controlling thbs3a expression in newly formed somites, while Rtn4al is an upstream negative regulator controlling cxcr4a expression. Meanwhile, miR-206 is present in PSM and somites (electronic supplementary material, figure S2), and it is able to repress Rtn4al, resulting in the higher expression of Cxcr4a, which, in turn, prevents excessive expression of Thbs3a in newly forming somites, leading to normal formation of somite boundaries during somitogenesis. These findings allowed us to propose a novel miR-206/rtn4al/cxcr4a/thbs3a regulatory cascade that mediates the formation of normal somite boundary.

3.8. Cxcr4a and Thbs3a are downstream effectors of the miR-206/rtn4al axis

To further confirm whether Cxcr4a and Thbs3a are downstream effectors of the miR-206/rtn4al axis, we individually injected miR-206-MO, rtn4al mRNA and thbs3a mRNA into one-cell zebrafish embryos, followed by detection of the expression level of cxcr4a mRNA at 20 hpf. In the WT embryos, we found that cxcr4a displayed a pronounced expression in PSM and boundaries in the newly forming somites, but only weak expression in mature somites (figure 5a). In the miR-206-knockdown and rtn4al-overexpression embryos, cxcr4a was reduced in newly forming somites (figure 5b,c), suggesting that cxcr4a is negatively regulated by rtn4a. However, cxcr4a remained unchanged in thbs3a-overexpression embryos (figure 5d), suggesting that cxcr4a is not regulated by thbs3a. These results observed from the WISH were consistent with the data obtained from q-PCR (figure 5i).

On the other hand, we individually injected miR-206-MO, rtn4al mRNA and cxcr4a-MO into one-cell zebrafish embryos, followed by detection of the expression level of thbs3a mRNA at 20 hpf. In the WT embryos, thbs3a was expressed at low level in the newly forming somites, but it was expressed at a relatively high level in mature somites (figure 5e). In the miR-206-knockdown, rtn4al-overexpression and cxcr4a-knockdown embryos, however, thbs3a was expressed at a relatively high level in the newly forming somites with pronounced expression in mature somites (figure 5f–h). Again, these WISH results were consistent with the data obtained from qPCR (figure 5j). During somite boundary formation, the epithelial cells of the somite boundary become columnar in shape, undergo MET, and exhibit cell polarity which makes centrosomes localize apically [23,36,41]. Using fluorescence-labelled γ-tubulin, we could trace the location of centrosomes in the epithelial cells of the somite boundary. In WT embryos, results showed that the epithelial cells of the boundary presented an oval or cylindrical shape and that centrosomes localize apically (figure 6a,b,b'). However, in the miR-206-MO-injected embryos (figure 6c',c), rtn4al-mRNA-injected embryos (figure 6d,d'), cxcr4a-MO-injected embryos (figure 6e,e') or thbs3a-mRNA-injected embryos (figure 6f,f'), the epithelial cells of the somite boundary presented a circular shape, and centrosomes did not localize apically (figure 6f'–f, arrowhead). Instead, centrosomes were localized randomly. This line of evidence suggested that the epithelial cells of miR-206-knockdown embryos, rtn4al-overexpression embryos, cxcr4a-knockdown embryos and thbs3a-overexpression embryos do not undergo epithelialization at somite boundaries. p-FAK is present in multiple receptor complexes and is located at the intersomitic boundary [42,43]. It is required for somite boundary formation during somitogenesis of zebrafish embryos [42]. Therefore, we detected the distribution pattern of p-FAK to confirm its concentration at the intersomitic
boundary of embryos injected with miR-206-MO and rtm4al mRNA, and resulted showed the absence of intracellular accumulation of p-FAK in boundary cells. Thus, we detected p-FAK signal in WT embryos and embryos injected with miR-206-MO, rtm4al-mRNA, ccr4a-MO and thbs3a-mRNA. In WT embryos, results showed that the p-FAK signal did not exhibit evenly in the entire boundary cells. Instead, p-FAK presented a high concentration toward the intersomitic position (figure 7a–c), as described previously by Crawford et al. [44]. However, unlike WT embryos, in the embryos injected with miR-206-MO (figure 7d–f), rtm4al mRNA (figure 7g–i), ccr4a-MO (figure 7j–l) and thbs3a mRNA (figure 7m–o), the p-FAK signal did not present a high accumulation pattern towards the intersomitic position (white arrows), indicating that these defective boundary cells were unable to process epithelialization by the absence of intracellular p-FAK accumulation in the somite boundaries.

To test the hypothesis that increased Thbs3a causes decreased expression of p-FAK within cells, we further employed mesodermal C2C12 cells which were cultured in undifferentiated condition. When C2C12 cells were treated with overexpressed mouse Thbs3a (mThbs3a), the expression of intracellular p-FAK[pY397] was decreased (electronic supplementary material, figure S7a), suggesting that the result observed in the mThbs3a-overexpressed cells, namely decreased expression of intracellular p-FAK[pY397], was essentially replicated in the boundary cells of thbs3a-mRNA-injected embryos and, hence, the absence of intracellular p-FAK accumulation in the somite boundaries, explaining, in turn, the inability to process epithelialization. Additionally, we found that active Cdc42 was increased in mThbs3a-overexpressed cells (electronic supplementary material, figure S7b), indicating that these cells trend toward epithelial–mesenchymal transition (EMT), which is unfavourable for epithelialization.
4. Discussion

4.1. Novel miR-206/rtn4a/cxcr4a/thbs3 pathway was found in newly forming somites to maintain and stabilize somite boundary formation

Skeletal muscle-specific miR-206 is known to regulate the differentiation of muscle cells [45]. Additionally, miR-206 represses the translation of mRNA encoding vascular endothelial growth factor Aa, resulting in inhibiting the angiogenesis of zebrafish embryos at 24–72 hpf [5,46]. Interestingly, in this study we reveal another biological function of miR-206 during embryogenesis, namely that miR-206 can repress the expression of rtn4a and thus play a role in the formation of the somite boundary; Phalloidin labelled with red fluorescent signal was used to mark F-actin, while γ-tubulin labelled with blue fluorescent signal was used to mark centrosomes. (b–f) Three fluorescent signals were merged; (b’–f’) were amplified from the corresponding panels (b–f). White arrowheads indicate centrosomes not localized apically in the epithelial cells of the defective somite boundary.

Figure 6. Change of expression levels of miR-206, Rtn4a, Cxcr4a or Thbs3a fails to epithelialize somites in zebrafish embryos. (a) A diagram depicts that centrosomes of epithelial cells at the somite boundary are localized apically when epithelial cells undergo MET. (b) WT embryos at 20 hpf; (c) knockdown of miR-206, (d) overexpression of rtn4a, (e) knockdown of cxcr4a and (f) overexpression of thbs3a. Fibronectin labelled with green fluorescent signal was used to mark the somite boundary; Phalloidin labelled with red fluorescent signal was used to mark F-actin, while γ-tubulin labelled with blue fluorescent signal was used to mark centrosomes. (b–f) Three fluorescent signals were merged; (b’–f’) were amplified from the corresponding panels (b–f). White arrowheads indicate centrosomes not localized apically in the epithelial cells of the defective somite boundary.

by miR-206, not miR-1. Based on this evidence, we concluded that the regulatory pathway related to somite boundary formation is miR-206-specific, which is strongly supported by the hypothesis proposed by Lin et al. [5], who demonstrated that miR-1 and miR-206 target different genes and play different roles during zebrafish embryogenesis.

The study of zebrafish Rtn4a has largely been confined to development of the nervous system [7,8]. Meanwhile, although rtn4a knockdown does not cause a significant defect of somite development, the effect of cxcr4a overexpression on somite development has not been reported. Pinzón-Olejua et al. [8] reported that the expression pattern of Rtn4a in zebrafish is noticeably present at the somite boundary, but the biological implication of Rtn4a in the somite boundary is still unknown. In this study, we demonstrated that Rtn4a plays a key role in somite boundary formation during somitogenesis. The reduced expression of Rtn4a, as mediated by miR-206, increased the expression of downstream cxcr4a, a gene well known to be involved in somite boundary formation [28], particularly in the newly forming somites of embryonic trunk. Our further study indicated that Cxcr4a represses the expression of downstream...
Thbs3a, an ECM protein. The reduced expression of Thbs3a favours epithelialization of boundary epithelium cells through MET to form boundaries in the newly forming somites. Therefore, we propose a novel regulatory pathway, *miR-206/cxcr4a/thbs3a*, which modulates somite boundary formation in zebrafish embryos.

We noticed that the expression levels among *rtn4a*, *cxcr4a* and *thbs3a* in the normal state are different between newly forming somites and mature somites during somitogenesis. As shown in figure 5, when we used WISH to examine the expression level of embryos at 20 hpf, we found that a relatively lower level of *rtn4a* was present in newly forming somites, which allowed a greater expression of *cxcr4a*, but a decreased persistence of *thbs3a*, finally allowing boundary cells to undergo MET and form normal boundaries in the newly forming somites of zebrafish embryos. Unlike the expression levels that occurred in newly forming somites, a relatively higher level of *rtn4a* was present in mature somites, which downregulated the *cxcr4a* expression, resulting in the upregulation of *thbs3a*. However, the biological implication of increased Thbs3a in mature somites during somitogenesis requires further investigation; it is well outside scope of the present study.

### 4.2. Structurally defective myotendinous junction might cause muscle fibres to cross somite boundary

When zebrafish embryos were treated with knockdown of *miR-206*, overexpression of *rtn4a*, knockdown of *cxcr4a*, or overexpression of *thbs3a*, defective somite boundaries were observed at early (20 hpf) and late (48 hpf) developmental stages. However, at late stage, some elongated myofibres were frequently observed to cross the somite boundary. It was speculative that this phenomenon may have resulted from incomplete formation of MTJ, as explained below. The somite boundary in zebrafish embryos forms in three separate stages before 24 hpf [40]. The first stage involves formation of the initial epithelial somite boundary when epithelial border cells surround an inner mass of mesenchymal cells [11]. During the second transitional stage, mesenchymal cells start to differentiate into muscle cells. Myotome boundary formation occurs during the third and final stage when fibronectin and p-FAK accumulate at somite border cells [40]. In zebrash muscle differentiation, the fibronectin-rich matrix concentrates adjacent to slow-twitch fibres, while the laminin-rich basement membrane concentrates adjacent to both slow-twitch and fast-twitch muscle fibres. Thereafter, MTJ forms in this ECM-rich area between muscle segments [22,50].

In *miR-206*-knockdown and *rtn4a*-overexpression embryos, we found that fibronectin and p-FAK did not accumulate correctly in the border cells of embryos as early as 20 hpf (figures 4 and 7). Additionally, their boundary cells did not properly process initial epithelial somite boundary formation at 20 hpf. Since laminin was then unable to accumulate correctly in the somite boundary at 48 hpf (electronic supplementary material, figure S6), MTJ was incompletely formed, as noted above. In this case, absence of any barrier between two somites, muscle cells differentiate and fuse into a long muscle fibre that crosses the compromised site of the somite boundary. However, whether there is any implication of the *miR-206/rtn4a/cxcr4a/thbs3a* axis...
reported in this study relative to MTJ formation at any particular stage in zebrafish embryos is an interesting issue for further study.

4.3. miR-206 affects somite boundary formation by regulating MET

As a therapeutic agent, miR-206 has been used to treat drug-resistant cells and cancer cells based on its ability to suppress EMT. For example, overexpression of miR-206 was used to treat breast cancer cells to inhibit the downstream genes of transforming growth factor (TGF)-β, such as NRPI and SMAD2, to reduce the migration and invasion of breast cancer cells [51]. Overexpression of miR-206 was also used to treat lung adenocarcinoma cisplatin-resistant cells to enhance MET protein level and, in turn, restrict the migration and invasion of lung cancer cells [52]. By contrast, knockdown of miR-206 favours cells undergoing EMT. This evidence indicates that miR-206 is involved in balancing the EMT/MET biological process. In this study, we observed that knockdown of miR-206 affects somite boundary formation in embryonic development through disturbance of somite boundary epithelial cells undergoing MET. Therefore, the use of miR-206 overexpression as a tumour suppressor through regulating EMT/MET supports our findings because miR-206 overexpression favours MET which results in epithelialization of boundary cells to form normal boundary, while miR-206 knockdown favours EMT which results in failure of epithelialization of boundary cells and forms a defective boundary.

4.4. miR-206 does not directly affect the expression changes of cxcr4a or thbs3a

We analysed two individual microarrays obtained from miR-206-knockdown embryos and rtn4a-overexpressed embryos. We found that the expressions of downstream cxcr4a and thbs3a were consistent in that cxcr4a was decreased and thbs3a was increased in both microarrays (electronic supplementary material, figure S5). However, neither cxcr4a nor thbs3a was included in the 117 putative target genes listed in the miR-206 LAMP assay, suggesting that neither gene was a direct target of miR-206. Then, using bioinformatics analysis, no corresponding sequences specific for miR-206 binding were located at the 3’UTRs of cxcr4a and thbs3a. Based on this line of evidence, it can be concluded that the expressions of cxcr4a and thbs3a in somites are not directly affected by miR-206. Instead, they are regulated by Rtn4a, which is mediated by miR-206, as determined in our results.

4.5. Rtn4a overexpression results in the loss of somite boundary formation

There are three types of Rtn4a, including Rtn4a1, Rtn4a3 and Rtn4a4n, which all share 188 amino acid residues at the C-terminal region. However, at the N-terminus, they contain 133, 23 and 7 amino acids, respectively [37]. Interestingly, we found that actin filaments were elongated across the somite boundary if all three Rtn4a subtypes were overexpressed in zebrafish embryos (figure 2), indicating that the

4.6. Cxcr4a expression affects somite boundary formation

Leal et al. [28] reported that inhibition of either SDF-1a or its ligand, Cxcr4, in Xenopus laevis embryos resulted in failed somite boundary formation. Since somite separation was not completely formed, myotome elongation and alignment were observed. Nakaya et al. [55] also reported that chick Cdc42, one of the Rho family members, is critical for MET processes of somite boundary cells. Unlike the active form of Cdc42 in mesenchymal cells, Cdc42 activity in boundary cells undergoing epithelialization is repressed, suggesting that Rho family activity is also involved in controlling the MET in boundary cells.

When Cxcr4a was inhibited in our zebrafish study, phenotypes such as defective somite boundary and muscle fibre crossover were observed. These phenotypes were similar to those of Xenopus embryos injected with cxcr4-MO [28]. Furthermore, we demonstrated that inhibition of Cxcr4a resulted in the increase of Thbs3a in zebrafish embryos. Interestingly, when mouse Thbs3a was overexpressed in C2C12 cells, we here also showed that intracellular Cdc42 was present in an active state (electronic supplementary material, figure S7b), which was unfavourable for MET. These lines of evidence suggest that boundary cells cannot undergo MET with absent downregulation of Thbs3a and inactivation of Cdc42.

4.7. Thbs3a expression affects somite boundary formation

Thbs3a, a secreted ECM protein, belongs to the thrombospondin family. Thrombospondin proteins mainly bind to receptors such as integrin, located on the cell membrane, resulting in the transduction of extracellular signals toward cells [56]. In Drosophila, Thbs (Tsp) has vital roles in integrin-dependent ECM organization at developing muscle/tendon attachment sites [57,58]. In zebrafish, Thbs4b is required for muscle attachment. In Thbs4b-deficient embryos, laminin is discontinuous at somite boundaries, suggesting that zebrafish Thbs4b plays a dual role: binding integrin and organizing the tendon ECM at MTJs to maintain muscle attachment [59].

In vertebrates, five thbs genes have been reported, including thbs3, thbs4 and thbs5 categorized as a subclass presenting as homo- and heteropentamers through a conserved coiled-coil structure [60,61]. Although in vitro assay demonstrated that Thbs interacts with other integrin ligands, such as laminin, collagen and fibronectin [62], it is still unclear if Thbs plays instructive or merely permissive roles in ECM organization and cell–ECM interactions.

Since Thbs3 and Thbs4 of zebrafish share a similar protein structure [63], it is plausible that Thbs4, together with Thbs3,
might form a heterodimeric structure to be functional. However, no evidence was forthcoming in the present study to suggest that zebrafish Thbs3a is in any way integrated with integrin-dependent ECM, leading to the speculation that zebrafish Thbs3a might be involved in maintaining the stability of integrin-dependent ECM because either overexpression or knockdown of Thbs3a expression level caused defective formation of the somite boundary.

Mouse Thbs1 and zebrafish Thbs3a are conserved in two domains, even though they contain different lengths of amino acid residues at the N-terminus [63]. However, they may have distinct functions in different cells. When mouse Thbs1 was added to vascular smooth muscle cells, the degree of intracellular p-FAK was upregulated [64]. By contrast, when zebrafish Thbs3a was overexpressed in somites, the degree of intracellular p-FAK was downregulated. When boundary cells undergo epithelialization, the level of intracellularly active Cdc42 is reduced [55], whereas overexpression of zebrafish Thbs3a results in an increased level of intracellular active Cdc42, which is unfavourable for epithelialization. Thus, our study is supported by Lynn et al. [64] and Osada-Oka et al. [65], who demonstrated that overexpression of Thbs1 favours EMT, resulting in enhanced migration of vascular smooth muscle cells in humans and leading to vascular intimal thickening. In our study, the overexpression of Thbs3a in zebrafish embryos favoured EMT, but normal boundary formation in newly forming somites was halted because it was also unfavourable for epithelialization of boundary cells.

**Ethics.** The National Taiwan University Institutional Animal Care and Use Committee (IACUC) reviewed and approved the protocol described below (NTU-103-EL-13). No specific ethics approval was required for this project, as all zebrafish used in this study were between 0 and 4 days old.

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