Normal Function of Myf5 During Gastrulation Is Required for Pharyngeal Arch Cartilage Development in Zebrafish Embryos

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Abstract

Myf5, a myogenic regulatory factor, plays a key role in regulating muscle differentiation. However, it is not known if Myf5 has a regulatory role during early embryogenesis. Here, we used *myf5*-morpholino oligonucleotides [MO] to knock down *myf5* expression and demonstrated a series of results pointing to the functional roles of Myf5 during early embryogenesis: (1) reduced head size resulting from abnormal morphology in the cranial skeleton; (2) decreased expressions of the cranial neural crest (CNC) markers *foxd3*, *sox9a*, *dlx2*, and *col2a1*; (3) defect in the chondrogenic neural crest similar to that of *fgf3* morphants; (4) reduced *fgf3/fgf8* transcripts in the cephalic mesoderm rescued by co-injection of *myf5* wobble-mismatched mRNA together with *myf5*-MO1 during 12 h postfertilization; (5) abnormal patterns of axial and non-axial mesoderm causing expansion of the dorsal organizer, and (6) increased *bmp4* gradient, but reduced *fgf3/fgf8* marginal gradient, during gastrulation. Interestingly, overexpression of *fgf3* could rescue the cranial cartilage defects caused by *myf5*-MO1, suggesting that Myf5 modulates craniofacial cartilage development through the *fgf3* signaling pathway. Together, the loss of Myf5 function results in a cascade effect that begins with abnormal formation of the dorsal organizer during gastrulation, causing, in turn, defects in the CNC and cranial cartilage of *myf5*-knockdown embryos.

Introduction

VERTEBRATE EMBRYO MUST undergo multiple developmental stages to form a multicellular organism, including gastrulation, neurulation, and organogenesis. During gastrulation, the nodal and Wnt pathways are responsible for inducing two initial mesodermal domains: the axial mesoderm that constitutes the dorsal organizer and the remaining non-axial mesoderm.^{1,2} The non-axial mesoderm can, in turn, be divided into three regions: paraxial, intermediate, and lateral mesoderm. The mechanisms that control axial and non-axial mesoderm identity during gastrulation are well defined.^{3,4} Moreover, it is well known that the determination and differentiation of skeletal muscles from paraxial mesoderm are controlled by myogenic regulatory factors (MRFs), including Myf5, Myod, Myogenin, and MRF4.⁵ The roles these four MRFs play in myogenesis are well studied. However, we noticed that zebrafish *myf5* is expressed in the paraxial mesoderm during 75% epiboly to bud stage when myogenesis is still not processed in embryos,^{6,7} whereas *myod* is only expressed in the adaxial cells during 50% epiboly to

bud stage.⁸ Thus, it is unclear whether MRFs play roles during early gastrulation.

It has been demonstrated that most vertebrate cranial and pharyngeal (viscerocranial) bones, and all pharyngeal cartilages, are derived from the neural crest.^{9,10} Cranial neural crest (CNC) cells originate adjacent to the neural ectoderm and migrate toward the mandibular, hyoid, and branchial arches to form seven pharyngeal arches. Segmentation of the CNC cells into distinct streams is coupled with the segmentation of the hindbrain into rhombomeres (r1–7).^{10–12} Craniofacial development requires an orchestrated communication between progenitor cells derived from the cranial paraxial mesoderm and CNC cells derived from the neural ectoderm.¹³ In frog and chick embryos, CNC cells can reorganize skeletal and muscle patterns when they are grafted to ectopic anterior–posterior levels.¹⁴ Rinon *et al.*¹⁵ proposed that CNC cells control craniofacial development by regulating positional interactions with mesoderm-derived muscle progenitors. TGF- β -mediated fibroblast growth factor 10 (FGF10) signaling in CNC cells can control the development of myogenic progenitor cells during tongue morphogenesis.¹⁶ In

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Pax3^{-/-} (splotch-delayed [Spd]) or *Myf5*^{-/-}:*Myod*^{-/-} double mutant mice, the morphology of the cranial cartilage, ribs, and joint was abnormal.¹⁷⁻²³ In contrast to zebrafish *Myod*^{-/-} single mutants, *Myf5*^{-/-}:*Myod*^{-/-} double mutants displayed more severe defects in cranial cartilage development where almost all ventral pharyngeal skeleton is lost.²⁴ Taken together, this evidence strongly suggested that (1) communication between CNC cells and mesoderm is required for normal development and (2) *myf5* and *myod* might also play roles beyond their functions during myogenesis.²⁴ We previously reported that inhibition of *myf5* transcription dramatically reduced *krox20* and *pax2.1* expression in hindbrain. Moreover, *myf5* morphants have a severely reduced head size,²⁵ indicating that *Myf5* is a crucial factor involved in head formation and hindbrain development. However, it is still unknown whether abnormal head size is caused by abnormal development of CNC cells when *Myf5* is lost. Therefore, the molecular mechanism that underlies *myf5* in the context of craniofacial development needs to be investigated in more detail.

To address these questions, we used *myf5*-morpholino oligonucleotides (MO) to knock down *myf5* expression and demonstrated a cascade effect in which the absence of *Myf5* leads to the loss of dorsal mesoderm organization, that is, axial and non-axial patterning, resulting in the failure of cell fate determination, followed by a series of developmental defects, including reduced head size resulting from abnormal morphology in the cranial skeleton and decreased expressions of *foxd3*, *sox9a*, *dlx2*, and *col2a1*, all markers of CNC cells. Additionally, expression of *fgf3/fgf8* was greatly reduced in the cephalic mesoderm of *myf5* morphants at 12 hours post-fertilization (hpf), eventually affecting patterning of pharyngeal pouches and resulting in the defective development of cranial cartilage.

Materials and Methods

Ethics statement

The animal use protocol listed below has been reviewed and approved by the National Taiwan University Institutional Animal Care and Use Committee (IACUC) with ethics approval number NTU-99-72. No specific ethics approval was required for this project, as all zebrafish (*Danio rerio*) used in this study were between 0 to 5 days old. Given the age of the embryos, pain perception has not yet developed at these earlier stages and so this is not considered as a painful procedure.

Transgenic zebrafish lines

The zebrafish embryos and larvae of AB strain, and transgenic lines *Tg(α-actin:RFP)*,²⁶ *Tg(fli1a:EGFP)*²⁷, and *Tg(-80Kmyf5:GFP)*,²⁸ were obtained and raised according to standard procedures.²⁹ Fluorescence was visualized with a fluorescent stereomicroscope (MZ FLIII; Leica) or a confocal spectral microscope (TCS SP5; Leica).

Fish embryos and whole mount in situ hybridization

The procedures for whole mount *in situ* hybridization were described previously.³⁰ The designations of zebrafish head muscle and cartilage follow the scheme of Schilling and Kimmel.³¹

Knockdown microinjection of zebrafish embryos

All MOs (Gene-Tools) were prepared at a stock concentration of 1 mM and were diluted to the desired concentrations, including 4, 2, or 1 ng, right before injection into each one- to two-cell stage embryos. MO sequences were as follows: *myf5*-MO1, TACGTCCATGATTGGTTTGGTGTG³²; *myf5*-MO2, GATCTGGGATGTGGAGAATACGTCC²⁴; *myod*-MO, ATATCCGACAACCTCCATCTTTT²⁶; *fgf3*-MO, CATTTGTGGCATGGCGGGATGTCGGC³³; and *p53*-MO, GCGCATTGCTTTGCAAGAATTG³⁴.

Detection of apoptotic cell death

Apoptosis assay was performed using the DeadEnd™ Colorimetric TUNEL System (Promega). Embryos were fixed in 4% paraformaldehyde (PFA) overnight at 4°C, and chorions were then removed and transferred into 100% methanol overnight at -20°C. The embryos were rehydrated thrice for 5 min with phosphate-buffered saline (PBS)+0.1% Tween 20 solution and covered with 100 μL of equilibration buffer for 10 min. Embryos were then incubated in 98 μL of equilibration buffer mixed with 1 μL of biotinylated nucleotide and 1 μL of TdT enzyme for 1 h at 37°C. These embryos were then washed thrice with 2× standard saline citrate solution for 5 min each and then with 2× PBS solution for 5 min each. The endogenous peroxidase was blocked by immersing the embryos in 0.3% hydrogen peroxide for 5 min. Then, the embryos were incubated in 100 μL of streptavidin horseradish peroxidase solution for 30 min. The embryos were washed thrice in PBS for 5 min each and then mixed with diaminobenzidine components in 100 μL of staining solution just before observation.

RNA injection

Capped mRNAs of *fgf3*, *myf5*, *myod*, and *gfp* were synthesized according to the protocol of the manufacturer (Epicentre). The *myf5* mRNA used to carry out rescue experiments was designed to mutate at the wobble position of *myf5* mRNA, in which seven nucleotides were mismatched to the corresponding nucleotides of *myf5*-MO1 without altering the amino acid residues. These mutated nucleotides within *myf5* mRNA caused no effect of translational inhibition by *myf5*-MO1. The synthesized mRNAs were diluted to 0.44 ng/μL *fgf3* mRNA, 44 ng/μL *myf5* mRNA, 11 ng/μL *myod* mRNA, and 44 ng/μL *gfp* mRNA with distilled water. About 2.3 nL of mRNA was injected into each one-cell stage embryo.

Plasmid constructs

Heat-shock inducible expression plasmid pHsp-*fgf3* was constructed, in which the production of *fgf3* mRNA was controlled by heat-shock promoter (Hsp70). After microinjection of plasmid pHsp-*fgf3* into the one-cell stage embryos, *fgf3* mRNA was conditionally overexpressed starting at 6 hpf when embryos were treated at 37°C for 10 min. Heat-shock induction was processed every 12 h until embryos developed at 36 or 120 hpf.

Cartilage staining

The Alcian blue staining protocol was modified from Neuhauss *et al.*³⁵ Embryos at 5 days postfertilization (dpf) were anesthetized using 0.02% buffered tricaine (Sigma) and

fixed overnight in 4% PFA at 4°C. After washing with PBS, embryos were stained overnight in 0.1% Alcian blue, which was dissolved in acidic ethanol (70% ethanol and 5% concentrated hydrochloric acid), washed extensively in acidic ethanol, dehydrated, and then stored in 80% glycerol. For better exposure of cartilage elements, embryos were digested with 0.02% trypsin.

Results

Effect of myf5 knockdown on head size, cranial cartilage formation, and cranial muscle development

The zebrafish transgenic line *Tg(α-actin:RFP)* is ideal for studying the dynamic changes of cranial muscle in embryos since all muscle of this line is tagged with RFP. Head muscle defects in *myf5* and *myod* morphants were observed with RFP signal (Fig. 1A–C). In *myf5* morphants, almost all head muscles were lost at 72 hpf (Fig. 1B). However, some of the cranial muscles were still present in the *myod* morphants (Fig. 1C), including extraocular muscles, such as superior oblique and inferior oblique muscles; dorsal pharyngeal muscles, such as levator arcus palatini, dilator operculi, adductor hyoideus, and adductor operculi; and trunk migratory head sternohyoideus muscle. By comparing the heads of MO1-treated embryos with those of wild-type (WT) embryos (Fig. 1D), we noticed that the *myf5* and *myod* morphants had abnormal craniofacial structures (Fig. 1E, F). The head size of the *Myf5* knockdown morphants was greatly reduced. Further, we used Alcian blue staining to detect the cranial cartilage for the WT (Fig. 1G, J), *myf5* morphants (Fig. 1H, K), and *myod* morphants (Fig. 1I, L) at 5 dpf. We found that *myf5* morphants had severe chondrodysplasia in craniofacial cartilage. Specifically, the derivatives of the branchial arches, such as basibranchial, ceratobranchial, and hypobranchial, were completely lost in *myf5* morphants (Fig. 1H, K). Morphologically abnormal cartilage, such as Meckel's cartilage, and palatoquadrate, basihyal, and ceratohyal cartilage, were also observed (Fig. 1H, K). However, cranial cartilage development of the *Myod* knockdown embryos was only mildly affected. For example, Meckel's and palatoquadrate cartilages were developed abnormally, but they were not lost since all branchial arch-derived cartilages could be detected (Fig. 1I, L). Such results suggest that *myf5* plays a more substantial role in cranial cartilage development than *myod*. We also examined the expression of *col2a1*, another chondrocyte marker, and found that it was correctly transcribed in the neurocranium of *myf5* morphants; however, compared with WT (Fig. 1M, O), *col2a1* was greatly reduced in the branchial arches, especially in ceratobranchial cartilages, at 72 and at 120 hpf (Fig. 1N, P). Thus, we concluded that the reduced head size in *myf5* morphants was caused by the disruption of cranial cartilage development.

The extent of the defective formation of cranial muscle and cartilage in the *myf5* morphants was dependent on the amount of the *myf5*-MO1 injected. As shown in Table 1, of the embryos injected with 4 ng of *myf5*-MO1, 83% of the surviving embryos lost cranial muscle, and 82% of the embryos lost ceratobranchial cartilage, while for embryos injected with 0.5 ng of *myf5*-MO1, the percentage of embryos losing cranial muscle and ceratobranchial cartilage was 3% and 52%, respectively. Similar phenotypes were observed for the embryos injected with *myf5*-MO2, which was still capable of

effectively knocking down *myf5* translation (data not shown). We also examined the target specificities of *myf5*-MO1 and *myf5*-MO2. In the control group, we observed the GFP signal at 28 hpf when *gfp* mRNA was co-injected with either *myf5*-MO1 or *myf5*-MO2 at the one-cell stage (Supplementary Fig. S1A, C, respectively; Supplementary Data are available online at www.liebertpub.com/zeb). However, the GFP signal was absent when *gfp* mRNA containing a complementary site for *myf5*-MO1 was co-injected with *myf5*-MO1 (Supplementary Fig. S1B). Similarly, the GFP signal was also absent when *gfp* mRNA containing a complementary site for *myf5*-MO2 was co-injected with *myf5*-MO2 (Supplementary Fig. S1D). Thus, the MOs we used were target-specific. Additionally, when embryos were injected with *myf5*-MO1, the phenotypes were observed at developmental stages as early as 12–14 hpf and as late as 120 hpf. However, we found that these head defects could be rescued by injection of *myf5* wobble-mismatched mRNA (Supplementary Figs. S2 and S3), suggesting that the defects during head development are indeed caused by the absence of *Myf5*. Importantly, cranial muscles and cartilages were still not observed as late as 8 dpf, indicating that the defects of *myf5* and *myod* morphants did not result from developmental delay. Thus, these results suggest that *Myf5*, previously known as a regulator in muscle development, is also involved in the development of cranial cartilage.

Myf5 is required for activation of genetic programs that define the chondrogenic neural crest cells

Neural crest cells are known to pattern the muscles of the pharyngeal region¹⁴. The pharyngeal cartilages are derived from migrating CNC cells. The appearance of pharyngeal arches is a characteristically morphogenic event during the pharyngeal period. To determine whether *Myf5* knockdown affects pharyngeal cartilage development, we used (1) *Tg(fli1a:EGFP)*, in which the pharyngeal arch neural crest expressed GFP and (2) Zn8 antibody to label the endoderm pouch³⁶. When compared with WT embryos at 32 hpf (Fig. 2A–C), only the first and second arches were formed in *myf5* morphants, while the third to fifth pharyngeal arches were lost (Fig. 2D–F). We then examined the neural crest marker *dlx2* in *myf5* morphants. In 24 hpf WT embryos, the expression of *dlx2* was observed in three distinct streams of CNCs that migrate to the mandibular (I; 1), hyoid (II; 2), and two branchial arches (III; 3, 4; Fig. 2G, I). In *myf5*-MO1-injected embryos, *dlx2* was normally expressed in the mandibular and the hyoid arches, but it was slightly reduced in the first and the second branchial arches (Fig. 2H, J). However, compared with WT (Fig. 2L, N), *dlx2* expression was greatly reduced in the branchial arches of the *myf5*-MO1-injected embryos at 36 hpf, resulting in the development of only four pairs of post-migratory crest (Fig. 2M, O), instead of six pairs of crest that had already developed in WT embryos at this stage of development.

To rule out the possibility that the craniofacial defects we observed were caused by nonspecific MO toxicity,³⁷ we co-injected *p53*-MO with *myf5*-MO1 and found that the reduced *dlx2* expression was not rescued in *myf5* morphants, either at 24 or at 36 hpf (Fig. 2K, P). These results indicate that the reduced *dlx2* signals in *myf5* morphants were not caused by nonspecific toxicity. Therefore, our results suggest that *Myf5* is essential for cartilage formation derived from migrating

FIG. 1. Inhibition of *myf5* translation leads to abnormal cranial muscle formation and cartilage differentiation and reduced head size. WT embryos, morphants (*myf5*-MO1 and *myod*-MO), and stages (hpf) are as indicated in each figure. (A–C) Viewed under fluorescent microscope; (D–P) viewed under a light microscope; (G–L) Alcian blue staining; and (M–P) using whole mount *in situ* hybridization to detect the transcripts of *col2a1*. (A) Tracing of head muscles at a late stage by using the zebrafish line Tg(α -actin:RFP). (B, C) Head muscles were dramatically reduced in both *myf5* and *myod* morphants. Head size (D–F) and cartilage differentiation (G–L) were reduced in *myf5*-MO1-injected embryos (E, H, and K), but only mildly affected in *myod* morphants (F, I, and L). Five pairs of ceratobranchial (cb) arches (J and L; black arrows) were completely absent in the *myf5* morphants (K). ah, adductor hyoideus; am, adductor mandibulae; ao, adductor operculi; cb, ceratobranchial; ch, ceratohyal; do, dilator operculi; dpw1–5, dorsal pharyngeal wall 1–5; hh, hyohyoideus; ih, interhyoideus; ima, intermandibularis anterior; imp, intermandibularis posterior; io, inferior oblique; ir, inferior rectus; lap, levator arcus palatini; m, Meckel's cartilage; mr, medial rectus; pq, palatoquadrate; sh, sternohyoideus; so, superior oblique; tv 1–5, transversus ventralis 1–5. hpf, hours postfertilization; WT, wild-type; MO, morpholino oligonucleotides.

TABLE 1. THE PERCENTAGES OF DEFECTIVE PHENOTYPE OCCURRING IN CRANIAL MUSCLE AND CARTILAGE WHEN MYF5 WAS KNOCKED DOWN

<i>myf5</i> -MO1 injected concentration	Cranial muscle defect (%) ^a			Ceratobranchial cartilage defect (%)		
	Loss	Reduced	Normal	Loss	Reduced	Normal
Uninjected	0 (0/16)	0 (0/16)	100 (16/16)	0 (0/16)	0 (0/16)	100 (16/16)
0.1 ng	0 (0/35)	22 (8/37)	73 (29/37)	0 (0/37)	22 (8/37)	73 (29/37)
0.5 ng	3 (2/62)	76 (47/62)	21 (13/62)	52 (32/62)	34 (21/62)	14 (9/62)
2 ng	19 (8/43)	81 (35/43)	0 (0/43)	79 (34/43)	21 (9/43)	0 (0/43)
4 ng	83 (55/66)	17 (11/66)	0 (0/66)	86 (57/66)	14 (9/66)	0 (0/66)

^aCranial muscle defect was observed at 72 hpf, except medial rectus, inferior rectus, and superior rectus muscles, which was normal in *myf5*-MO1-injected embryos. Cartilage defect was observed after embryos were fixed and staining was performed.
hpf, hours postfertilization.

chondrogenic crest cells, especially the third CNC stream: branchial arches.

fgf3 knockdown results in defects similar to those of *myf5* knockdown

Fgfs belong to a family of extracellular signaling molecules involved in diverse facets of craniofacial development.^{33,36}

Interestingly, *myf5* morphants displayed defects similar to those of the *fgf3* morphants, such as lost branchial cartilage.³³ To determine whether *Myf5* is involved in *Fgf3* signaling during pharyngeal cartilage development, we compared the defects found in *myf5* morphants with those found in *fgf3* morphants. After Alcian blue staining of cartilage, we found that the defects of *myf5* and *fgf3* morphants were similar.

FIG. 2. *Myf5* knockdown embryos failed to develop pharyngeal arch structure and exhibited reduced prechondrogenic gene expression in cranial neural crest cells. *Tg(fli1a:EGFP)* indicates the neural crest (green) at the pharyngeal arch region, and *Zn8* indicates the endoderm pouch (red). In 32 hpf WT embryos, five pharyngeal arches can be observed (A–C). In *myf5*-morphants, only the first and second arches were detected. The third to fifth arches were lost in *myf5* morphants (D–F). At 24 and 36 hpf, the prechondrogenic gene *dlx2* was expressed at the third arch stream in WT embryos. However, *dlx2* was reduced significantly at the third arch stream in *myf5* morphants (G–J, L–O). Co-injection of *myf5*-MO1 and *p53*-MO did not rescue the reduced expression of *dlx2* at the third arch stream (K, P). I, mandibular arch; II, hyoid arch; III, branchial arch; 1–6, pharyngeal arch; arrows in (D) indicate the lost of neural crest; brackets in (G), (H), (L) and (M) indicate the branchial arch.

Specifically, compared with WT (Fig. 3A), the cartilage for pharyngeal arches 3–7 was lost at 120 hpf (Fig. 3B, C). We then detected expression of ERK-inducible transcription factors *pea3* and *erm*, which are completely Fgf-dependent during the early stages of zebrafish development.^{38,39} We found that (1) *pea3* transcripts were detected at the precursor cells of CNC located at pharyngeal arches from two to four at 24 hpf (Fig. 3D) and (2) *erm* transcripts were expressed at the precursor cells of CNC located at pharyngeal arches from one to four at 24 hpf (Fig. 3G). However, *pea3* and *erm* transcripts were hardly detectable in the posterior part of pharyngeal arch four in either *myf5* or *fgf3* morphants (Figs. 3E, F, H, I). Therefore, it is likely that Myf5 modulates craniofacial cartilage development via the *fgf3* signaling pathway.

Knockdown of *myf5* leads to the loss of *fgf3/8* expression at the cephalic mesoderm, but *fgf3* mRNA can rescue the craniofacial cartilage defects caused by *myf5* knockdown

Because the defects in *myf5* morphants are similar to those of *fgf3* morphants, we hypothesized that Myf5 and Fgf3 may function in the same signaling pathway. To test this hypothesis, we investigated the molecular interaction between Myf5 and Fgf3. Our results showed *fgf3/fgf8* expression in the cephalic mesoderm at 12 hpf (Fig. 4A, E), but it was lost in embryos either injected with *myf5*-MO1 alone or injected with *myf5*-MO1 plus *p53*-MO (Fig. 4B, C, F, G). However, the defective expression of *fgf3/fgf8* in the cephalic mesoderm of *myf5* morphants could be rescued by injection of wobble-

altered mRNA of *myf5* (Fig. 4D, H). These data suggest that the expression of *fgf3/fgf8* at the cephalic mesoderm is dependent on the normal expression of *myf5* during early embryogenesis. It has been shown that Fgf signaling from the cephalic mesoderm is required for the induction of pharyngeal pouches and the specification of epibranchial placodes.^{36,40} Thus, we further proposed that *myf5* deficiency could lead to craniofacial cartilage defect via reduced *fgf3/8* signaling from the cephalic mesoderm.

Next, we analyzed the patterns of such CNC markers as *foxd3* and *sox9a* during early embryogenesis. Compared with WT (Fig. 4I, N), the expressions of *foxd3* and *sox9a* at the premigratory neural crest and optic region were greatly reduced in the embryos injected either with *myf5*-MO1 alone or with *myf5*-MO1 plus *p53*-MO (Fig. 4J, K, O, P). However, when embryos were injected with either *myf5*-MO1 plus *myf5* wobble-mismatched mRNA or *myf5*-MO1 plus plasmid pHsp-*fgf3* and then heat-shocked to induce *fgf3* expression at 6 hpf, we found that the expressions of *foxd3* and *sox9a* were restored at 12 hpf (Fig. 4L, M, Q, R), indicating that the reduced expression of CNC markers in the *myf5* morphant is dependent on *fgf3* signaling.

Previous studies revealed that the migrating CNC cells undergo apoptosis in the absence of Fgf signaling.³³ Compared to untreated embryos, the apoptotic signals were increased throughout the head region in the *myf5*-MO1-injected embryos at 30 hpf (Fig. 5A vs. B and 5G vs. H). In contrast, when we co-injected *myf5* mRNA with *myf5*-MO1, the number of apoptotic signals decreased significantly compared with the occurrence of apoptosis in embryos injected with *myf5*-MO1 only (Fig. 5C, I).

FIG. 3. The defects in the chondrogenic neural crest cells of Myf5 knockdown embryos were similar to those of *fgf3* morphants. WT embryos, morphants (*myf5*-MO1 and *fgf3*-MO), embryonic stages (hpf), and *in situ* hybridization probes are as indicated in each figure. (A–C) Ventral view of the cranial cartilage after embryos at 120 hpf were stained with Alcian blue. Compared with WT (A), the branchial cartilage did not develop normally in *myf5* and *fgf3* morphants, and abnormal morphology can be observed in ceratohyal cartilage (ch), Meckel's cartilage (m), and palatoquadrate cartilage (pq) (B, C). At 24 hpf, pharyngeal arch 4 of *pea3*- and *erm*-positive neural crest cells was reduced in *myf5* and *fgf3* morphants (D–F, G–I). Pharyngeal arches 1–4 are labeled in (D–I). FGF, fibroblast growth factor.

FIG. 4. The defective expressions of *fgf3*, *fgf8*, *foxd3*, and *sox9a* in *myf5*-knockdown embryos could be rescued by injection of *myf5*-MO1 plus either *myf5* mRNA or *fgf3* mRNA. Dorsal view of the expression patterns of *fgf3* (A–D), *fgf8* (E–H), *foxd3* (I–M), and *sox9a* (N–R) in head region of embryos at 12 hpf. In WT embryos, *fgf3* (A) and *fgf8* (E) were used to label the cephalic mesoderm (brackets); *foxd3* was used to label the premigratory neural crest (I); and *sox9a* was used to label the premigratory neural crest and optic region (N, brackets). In *myf5* morphants, *fgf3* and *fgf8* in the cephalic mesoderm were absent (B, F); *foxd3* (J) and *sox9a* (O) were greatly reduced. Co-injection of *p53*-MO could not rescue the absent expressions of *fgf3* (C) and *fgf8* (G) at the cephalic mesoderm or the reduced expressions of *foxd3* (K) and *sox9a* (P) in the neural crest caused by *myf5*-knockdown. However, injection of wobble-mismatched mRNA of *myf5* could restore the expressions of *fgf3* (D), *fgf8* (H), *foxd3* (L), and *sox9a* (Q) in *myf5*-morphants. Embryos were injected with *myf5*-MO1 plus plasmid pHsp-*fgf3* and then treated with heat shock to induce *fgf3* expression at 6 hpf, followed by analysis of *foxd3* and *sox9a* expressions at 12 hpf. Similar to WT embryos, the expressions of *foxd3* (M) and *sox9a* (R) in the neural crest and optic region were normal. The numbers shown in the lower-right corner of panels (A–R) indicate the number of phenotypes out of the total number of embryos examined. Brackets in (A–H) indicate the cephalic mesoderm; brackets in (N–R) indicate the otic vesicle.

To understand whether the apoptotic cells in the head of *myf5*-morphants were CNC cells, we employed embryos derived from transgenic line *Tg(-4.9 sox10:EGFP)*, in which CNC cells were labeled in green. After *myf5*-MO1 was injected into embryos, the TUNEL assay was performed to detect the amount of apoptotic signals labeled in red. We found a high frequency of yellow signals detected in the *myf5*-MO1-injected embryos, indicating that CNC cells undergo apoptosis in the absence of *myf5* (Supplementary Fig. S4). These findings suggest that apoptosis plays a significant role in the reduction of head size and that *myf5* inactivation results in the loss of pharyngeal cartilage formation.

Next, to understand whether reduced Fgf signaling in *myf5*-knockdown embryos causes CNC cells to undergo apoptosis, we injected *myf5*-MO1 plus *fgf3* mRNA. Results showed that the apoptotic signaling was reduced (Fig. 5D, J). However, the number of apoptotic cells remained unchanged in the embryos injected with *myf5*-MO1 plus either *gfp* mRNA (Fig. 5E, K) or *myod* mRNA (Fig. 5F, L). Additionally, even though overexpression of *fgf3* mRNA in *myf5* morphants resulted in reduced apoptotic signals, it also rescued the expression of *dlx2* (Fig. 5M vs. P). Similar to WT embryos, the six pharyngeal arches were observed in the embryos injected with *myf5*-MO1 plus plasmid pHsp-*fgf3* (Fig. 5M, P). However, only four *dlx2*-positive pharyngeal arches were observed in embryos injected

with *myf5*-MO1 plus *p53*-MO (Fig. 5N). Nevertheless, this defective expression of *dlx2* could be restored by co-injection of *myf5*-mRNA (Fig. 5O). At late stage, the cranial cartilage defects caused by *myf5*-knockdown were rescued by overexpression of *myf5* and *fgf3*, while in embryos injected with *myf5*-MO1 plus *p53*-MO, these cartilage defects remained unchanged (Fig. 5Q–T). The percentage of each group of embryos with rescued ceratobranchial cartilage was shown in Figure 5U. We noted that the occurrence of ceratobranchial cartilage defects caused by injection of 4 ng *myf5*-MO1 was decreased when embryos were co-injected with 0.1 to 2.5 pg of *fgf3* mRNA, 100 pg *myf5* mRNA, or 23 to 58 pg of pHsp-*fgf3*. On the basis of these findings, we concluded that *Myf5* modulates craniofacial development and that the *fgf3* signaling pathway is involved.

Myf5 is required for Spemann organizer patterning during gastrulation

While the above data clearly indicate that *Myf5* specifically modulates craniofacial development, *myf5* transcripts were not detected in the head during the early segmentation to Prim-5 stage. To determine whether *myf5* exists prior to segmentation, we analyzed the expression of *myf5* during early to late gastrulation. Our results demonstrated that *myf5* was expressed in the non-axial mesoderm at 50–60% epiboly, the

FIG. 5. Overexpression of Fgf3 can rescue the craniofacial defects caused by *myf5* knockdown. Dorsal (A–F) and lateral (G–L) views of WT embryos and embryos injected either with *myf5*-MO1 alone or with *myf5*-MO1 plus *myf5* mRNA, *fgf3* mRNA, *gfp* mRNA, or *myod* mRNA. The TUNEL assay was evaluated at 30 hpf. Compared with WT embryos (A, G), apoptotic signals significantly increased in *myf5* morphants (B, H). However, the apoptotic signals were significantly reduced in the embryos injected with *myf5*-MO1 plus either *myf5* mRNA or *fgf3* mRNA, compared with apoptotic signaling in the *myf5*-MO1-injected embryos (C, D, I, J). Embryos injected with *myf5*-MO1 plus either *gfp* mRNA or *myod* mRNA did not exhibit any reduction of apoptotic signaling (E, F, K, L). Additionally, compared with WT (M), the expression domain of *dlx2* in the embryos injected *myf5*-MO1 plus *p53*-MO was reduced (N), but this defect could be rescued by injection of *myf5*-MO1 plus either *myf5* mRNA or plasmid *pHsp-fgf3* (O, P). Similarly, compared with WT (Q), ceratobranchial cartilage in the embryos injected with *myf5*-MO1 plus *p53*-MO was absent (R). This defect could be rescued by injection of *myf5*-MO1 plus either *myf5* mRNA or *pHsp-fgf3* (S, T). (U) The diagram illustrates the percentages of embryos with defective ceratobranchial cartilage among all the surviving embryos injected with *myf5*-MO1 (4 ng) alone or injected with *p53*-MO (8 ng), *fgf3* mRNA (0.1-, 0.5-, 1.0-, and 2.5-pg), *myf5* mRNA (100 pg), or *pHsp-fgf3* (23- and 58-pg) at 120 hpf. ■ Loss: ceratobranchial cartilage was all lost; ■ Reduced: at least one of five pairs, but fewer than five pairs of ceratobranchial cartilage were present; □ WT-like: all five pairs of ceratobranchial cartilage were present. The numbers shown at upper-right corner of panels (A–T) indicate the number of phenotypes out of the total number of embryos examined. Pharyngeal arches 1–6 are labeled in (M–P). Arrows in (Q), (S) and (T) indicate the presence of ceratobranchial cartilages.

shield stage when involution of the first prospective mesendodermal cells leads to the formation of axial and non-axial mesendodermal tissue (Fig. 6A, B). Interestingly, *myf5* was only expressed on both sides of the Spemann organizer. Double *in situ* hybridization of *myf5* and *floating head* (*flh*) revealed that the strip pattern of *myf5* bordered on the Spemann organizer (Fig. 6F). At 7–9 hpf, *myf5* was expressed in

the paraxial mesoderm segmental plate and adaxial mesoderm (Fig. 6C, D). Nevertheless, at shield stage, *myod* was only expressed in a few cells on either side of the Spemann organizer (Fig. 6G). At 7–9 hpf, *myod* was expressed in the adaxial cells, but not in the lateral plate mesoderm (Fig. 6H, I). At 13 hpf, both *myf5* and *myod* were strongly expressed in the somites and adaxial cells (Fig. 6E, J).

FIG. 6. Non-axial mesoderm cells that express *myf5* during early gastrulation contributed to cranial mesodermal cells at later developmental stages. *Myf5* (A–E) and *myod* transcripts (G–J), as evaluated by whole mount *in situ* hybridization. The stages of development (hpf) are as indicated in the upper right of each figure. Images are vegetal pole view (A) or dorsal view (B–D, F–I) and posterior view (E, J). *Myf5* expression was first observed in the non-axial mesoderm at the shield stage (A, B), followed by the paraxial mesoderm segmental plate and adaxial mesoderm at 7–9 hpf (C, D) and then in the somites, adaxial cells, and presomitic mesoderm at 13 hpf (E). Double *in situ* hybridization of *myf5* and *flh* transcripts was also performed (F), revealing that the strip pattern of *myf5* bordered on the Spemann organizer. Meanwhile, *myod* was first expressed in a few cells on both sides of the organizer at the shield stage (G), followed by the adaxial cells at 7–9 hpf (H, I) and then in the somites and adaxial cells at 13 hpf (J).

Next, we investigated whether knockdown of *myf5* would cause abnormal gene expression during early gastrulation. *Tbx1*, which is required for interactions between the cranial mesoderm and the neural crest cells that form the pharyngeal cartilages,⁴¹ was expressed at the paraxial mesoderm in WT during shield stage (Fig. 7A, B). When *myf5* was knocked down, *tbx1* transcripts at the paraxial mesoderm were reduced (Fig. 7C). Interestingly, with the knockdown of *tbx1*, the *myf5* transcript was also reduced (Fig. 7D, E), suggesting that the interaction between *myf5* and *tbx1* is likely a positive feedback mechanism. Additionally, knockdown of *myf5* resulted in an ectopic expansion of *flh*-positive cells in the notochord (Fig. 7F–I). This expansion of axial mesoderm was also demonstrated by the expansion of *gsc* expression, which marks the prechordal plate mesoderm (Fig. 7K, L). In addition, the broadened dorsal clearing of *her1* expression was also observed (Fig. 7M, N). Knockdown of *myod* did not affect the patterning of the dorsal organizer (Fig. 7J). Further, co-injection of *myf5* mRNA led to the rescue of the phenotype caused by *myf5*-MO1 injection, suggesting that the *myf5*-MO1-induced phenotype was specific (Fig. 7O).

It is well known that the neural crest is induced by a combination of secreted signals and that neural crest induction is achieved by combining the same classes of molecules as those required for neural plate patterning: Bmp, Wnts, and Fgfs.^{42–45} Therefore, we analyzed whether the signaling of Bmp, Wnts, and Fgfs was affected in the absence of *myf5* during early embryogenesis. Compared to WT embryos (Fig. 8A), the expression domain of *bmp4* was expanded from the ventral domain to the dorsal domain when *myf5* was knocked down (Fig. 8F). While *fgf3* was reduced at the dorsal domain (Fig. 8B vs. G), the *fgf3* marginal gradient was lost at the ventral region in *myf5* morphants (Fig. 8C vs. H). The expanded expression of *fgf8* at the dorsal organizer might

have resulted from a change in the border of the dorsal organizer, and apparent decrease in the *fgf8* marginal gradient (Fig. 8D vs. I and 8E vs. J). Since the Fgf signals antagonize ventralizing Bmp signals, our data showed that *myf5* function may be involved in balancing the gradient of Fgf and Bmp signals during gastrulation. Taken together, our data revealed that *Myf5* plays an important role in mesoderm patterning and that normal expression of *myf5* from non-axial mesoderm is required for Spemann organizer patterning during gastrulation.

Discussion

Distinct functions of Myf5 and Myod in craniofacial development in zebrafish

Myf5 and *Myod* have been intensively studied in various species and are known to play different roles during trunk myogenesis. The regulation of *Myf5* is more complex and is markedly different from that of *Myod*.^{46–49} Recently, we demonstrated that *Myf5* and *Myod* exhibit distinct functions during craniofacial myogenesis in zebrafish.^{26,32} Other than myogenesis, however, the roles that *myf5* and *myod* play during embryogenesis have thus far been unclear. In this study, we demonstrated that *Myf5* is necessary for cranial cartilage development in zebrafish. Specifically, severe chondrodysplasia in craniofacial cartilage and greatly expanded Spemann organizer at the shield stage were observed in *myf5* morphants, but not in *myod* morphants. However, overexpression of *fgf3* could rescue the cranial cartilage defects caused by *myf5*-MO1, suggesting that *Myf5* modulates craniofacial cartilage development through the *fgf3* signaling pathway. Thus, we propose that *Myf5* plays roles in craniofacial development during zebrafish embryogenesis in a manner distinct from that of *Myod*.

FIG. 7. *Myf5* knockdown affected the expression domain of axial and non-axial mesoderm during gastrulation. Whole mount *in situ* hybridization of *tbx1* (A–C), *myf5* (D, E), *flh* (F–J, O), *gsc* (K, L), and *her1* (M, N) was performed in WT, *tbx1* morphant, *myf5* morphant (*myf5*-MO1), and the *myod* morphant (*myod*-MO) at shield stage (A–C, F–G, K–O) and 8 hpf (D, E, H–J), as seen in these dorsal views. *Tbx1* expression in the paraxial mesoderm was decreased in *myf5* morphants (A–C); *myf5* expression was also decreased in the paraxial mesoderm when *tbx1* was knocked down (D, E). Noticeable expansion of *flh*-expressing (G, I; arrow) and *gsc*-expressing (L; arrow) cells was observed in *myf5* morphants. The expression of *her1* displayed a wider dorsal clearing (N; arrow) when compared with the WT embryos (M vs. N). In contrast, the expression of *flh* appeared normally in the *myod* morphant (J). The expression of *flh* became normal in embryos injected with *myf5* mRNA plus *myf5*-MO1 (O).

FIG. 8. Signaling of both *fgf* and *bmp* was affected in the *myf5*-knockdown embryos during gastrulation. Using whole mount *in situ* hybridization to examine the expression patterns of *bmp4* (A, F), *fgf3* (B, C, G, H), and *fgf8* (D, E, I, J) in WT embryos (A–E) and *myf5* morphants (*myf5*-MO1; F–J) at shield stage. Compared to WT embryos (A), the expression of the ventral marker *bmp4* was upregulated at the ventral region (v) and extended to the dorsal part in *myf5*-knockdown embryos at shield stage (F). In contrast, compared with WT embryos (B, C), the expression of *fgf3* in the dorsal organizer (do) was reduced (G, H), and the expression of *fgf8* in the dorsal region (do) was also reduced (D, E vs. I, J). The borders of *fgf3* and *fgf8* at the dorsal organizer were significantly extended (arrows mark the size of dorsal organizer).

Previous studies revealed that the lack of myogenesis in head muscles could lead to skeletal defects in mouse and chick.^{17–22} However, our present data demonstrated that almost all the cranial muscles are lost in *myod* morphants, while cranial cartilage is still formed, although they displayed abnormal shapes. This evidence is consistent with the result obtained from *myod* mutants.²⁴ It is also true that defective cranial skeletogenesis, which is observed in *myod* and *myf5* double mutants,²⁴ is very similar to the *myf5*-knockdown phenotype reported in this study. Therefore, our data indicate that Myf5, not Myod, is the key factor mediating cranial cartilage development. Additionally, we found when Myf5 is lost during gastrulation, the cranial mesoderm cells fail to secrete the Fgf3/8, resulting in abnormal craniofacial development. Similar mechanism is also observed in mouse and chick tendon formation.^{50,51} Taken together, we highlight an alternative mechanism that musculature is not the key factor for cranial skeletogenesis. Instead, Myf5 is the key factor for cranial skeletogenesis.

Adequate expression of myf5 during gastrulation determines precisely distributed patterning of axial and non-axial mesoderm

Zebrafish gastrulation starts with the involution/ingression of prospective mesendodermal cells.⁵² At the shield stage, involution of the first prospective mesendodermal cells leads to the formation of axial and non-axial mesendodermal tissue. In this study, we detected *myf5* transcripts in the prospective non-axial mesoderm (Fig. 6) at the shield stage. Knockdown of Myf5 resulted in a significant expansion of *flh* and *gsc* expression domains in the dorsal organizer (Fig. 7). This evidence strongly supports the hypothesis that Myf5 plays important roles during gastrulation.

In zebrafish, the paraxial mesoderm is derived from two symmetrical territories of the marginal zone located on either side of the Spemann organizer. Kodjabachian *et al.*⁵³ proposed a model representing the interaction network between axial and paraxial cells. In this model, *ntl* blocks *spt* expression in notochord precursors by inducing *flh*, which consequently represses *spt*. Dal-Pra *et al.*⁷ demonstrated that the knockdown of FoxA2 and FoxA3 results in dorsal expansion of *myf5* and that the expression area of *myf5* could even be fused along the axis. In the present study, we found that Myf5 knockdown resulted in the expansion of *flh* expression in axial mesoderm (Fig. 7). We also noticed that the expression of *ntl* increased in the *myf5*-MO1-injected embryos (data not shown). The expansion of *flh* and the widened dorsal clearing of *her1* expression indicate that the non-axial mesoderm was pushed away by axial mesoderm, suggesting that the non-axial mesoderm might have lost its ability to restrict the distribution of the axial mesoderm, resulting in expansion of the Spemann organizer. Therefore, we concluded that an adequate spatio-temporal expression of *myf5* in the *myf5*-positive non-axial mesoderm is required to maintain normal patterns of the axial and non-axial mesoderms during gastrulation.

Additionally, it is known that Fgf signals may antagonize ventralizing Bmp signaling during gastrulation.⁵⁴ While our data also showed increased *bmp4* gradient from the ventral site to the dorsal site, we also found a reduced *fgf3/fgf8* marginal gradient during gastrulation in the *myf5*-knockdown embryos. These results suggest that Myf5 in the non-axial

mesoderm is important for mediating the balance between the gradient distribution of Fgf and Bmp signaling during gastrulation. Thus, in non-axial mesoderm during gastrulation, these observations, when taken together, suggest that *myf5* functions to both restrict the expansion of axial mesoderm and mediate the balance of Fgf and Bmp gradients.

Normal patterns of axial and non-axial mesoderms determine cell fate of embryos

Multiple *nodal*-related genes are necessary for induction of mesoderm and endoderm.^{55–57} In zebrafish, two *nodal*-related genes, *sqt* and *cyclops* (*cyc*), have been defined.^{58–62} In *sqt*^{−/−} and *cyc*^{−/−} double mutants, almost all mesendodermal derivatives are lost, including notochord, trunk somites, pronephros, heart, blood, and gut.⁵⁹ In *sqt*^{−/−} and *cyc*^{+/−} embryos, widened dorsal clearing of *sqt* expression is observed. Fate map experiments revealed that the dorsal marginal cells in *sqt*^{−/−} and *cyc*^{+/−} mutants adopt neural fates, including spinal cord, hindbrain, and midbrain.⁶³ This evidence suggests that precisely distributed patterning of the axial and non-axial mesoderms during gastrulation is required for embryonic cell fate determination. Interestingly, in this study, we found that the absence of *myf5* resulted in abnormal development of both axial and non-axial mesoderms. Thus, the failure in proper patterning initiated a cascade that interferes with cell fate determination and causes a host of embryonic defects, as detailed above. We therefore propose that *myf5* is an important gene for maintaining correct mesoderm patterning in the non-axial mesoderm during early gastrulation.

Knockdown of Myf5 causes the reduced expression of fgf3 in cephalic mesoderm, resulting in defective development of craniofacial cartilage in embryos

The pharyngeal arches are unique transient structures that give rise to much of the head and neck skeleton.¹⁴ They are populated early in development by CNC cells originating from the posterior midbrain–hindbrain region in a precise rostral–caudal pattern.¹⁰ Considerable evidence indicates that Fgf signals from the cranial mesoderm are important for pharyngeal endoderm formation. Crump *et al.*³⁶ revealed that Fgf8 and Fgf3 are required in the neural keel and cranial mesoderm during early somite stages (10–14 somite stage) to promote first pouch formation. Nechiporuk *et al.*⁴⁰ also reported that mesoderm-derived Fgf3 and Fgf8 signals establish both the epibranchial placodes and development of the pharyngeal endoderm.

The zebrafish *fgf3* mutant *limabsent* (*lia*) shows reduction of almost all cartilage of the gill arches (pharyngeal arches 3–7) and *dlx2*-positive chondrogenic neural crest cells of arches 3–7.⁶⁴ Analysis of the *fgf3* knockdown demonstrated that the pharyngeal endodermal expression of *fgf3* is specifically required for survival of skeletogenic neural crest cells to form the posterior pharyngeal arches.^{65,66} In this study, we demonstrate that Myf5 is required for pharyngeal cartilage formation. Inhibition of Myf5 function results in the defective development of craniofacial cartilage, especially in the branchial arch derivatives. Interestingly, we found that *fgf3* and *fgf8* at the cephalic mesoderm were significantly reduced in *myf5* knockdown embryos during 12 hpf. Additionally, cranial defects in the *myf5* morphant were similar to those observed in the *fgf3* morphants. Also, similar to *fgf* knockdown

embryos, endoderm pouch formation in pharyngeal arches was lost in *myf5* morphants, as revealed by immunostaining.³⁶ Whole mount *in situ* hybridization also revealed that the expressions of *fgf3*, *erm*, and *pea3* are all reduced in *myf5* morphants. Further, although results from the TUNEL assay indicate that apoptotic signals are abundant in *myf5*, but not *myod*, morphants (Fig. 5), co-injection of *fgf3* mRNA with *myf5*-MO1 is sufficient to rescue the ceratobranchial cartilage defects and reduce the apoptosis. Taken together, we proposed that the defects in cranial cartilage formation in *myf5* morphants were caused by reduced Fgf signaling from the cephalic mesoderm.

Hinits *et al.*²⁴ reported that the cranial muscles of *myod*-null mutant zebrafish (*myod*^{th261}) were greatly reduced or entirely absent. Nonetheless, all major cranial cartilages were presented at 6 dpf in the *myod*^{th261} mutant, albeit in the absence of normal cranial cartilage morphogenesis. As shown in Figure 11 and L, these results are consistent with those presented in this study because we found that all branchial arch-derived cartilages could be detected in *myod*-knockdown embryos, although Meckel's and palatoquadrate cartilages were developed abnormally. However, it should be noted that Hinits *et al.*²⁴ also demonstrated that *myf5*^{hu2022} and *myod*^{th261} double mutant fish exhibited severe cranial skeletal defect, suggesting that Myf5 is necessary for cranial cartilage formation. Thus, the *myf5*-knockdown phenotypes, as presented here by injection of MO1 and MO2 into embryos, are plausible outcomes when Myf5 is absent in zebrafish embryos. While our MO-knockdown approach resulted in an observable defective phenotype, Hinits *et al.*⁶⁷ reported no phenotype in *myf5*-knockdown embryos. We might attribute the apparent differences to the effectiveness and concentration of MO they used. Additionally, although Hinits *et al.*⁶⁷ concluded that the *myf5*-null mutant also had no observed defects, these mutants were all lethal and could not grow to adulthood. Similarly, Giraldez *et al.*⁶⁸ previously reported that *Zdicer* mutants had no obvious defects at 7 to 10 dpf and that these mutants were lethal after 10 dpf. Interestingly, Giraldez *et al.*⁶⁸ also generated maternal-zygotic *dicer* (MZdicer) mutants that displayed morphogenesis defects, indicating that maternal mRNAs might play a role in development. Based on this evidence, reasoning arises that might explain why the *myf5*-null mutant had no observed defects in the Hinits *et al.*⁶⁷ model. Specifically, mutant embryos contain maternal *myf5* mRNA to sufficiently promote normal embryonic growth at least until 5 dpf. However, when we used the MO-knockdown approach, *myf5*-MO inhibited maternal *myf5* mRNA translation, resulting in an observable phenotype.

In summary, this article is the first report showing that Myf5, a well-known MRF involved in myogenesis, functions in gastrulation and plays roles in head mesoderm patterning, CNC survival, and cranial cartilage formation. This study opens up avenues to further investigate the possible regulatory network underlying the involvement of Myf5 in the cranial development of zebrafish.

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Disclosure Statement

No competing financial interests exist.

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