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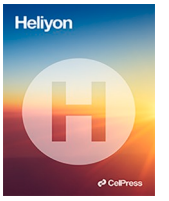

nkx2.3 is responsible for posterior pharyngeal cartilage formation by inhibiting Fgf signaling

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ABSTRACT

Nkx2.3, a transcription factor, plays important roles in various developmental processes. However, the mechanisms underlying *nkx2.3*'s regulation of pouch and pharyngeal arch development in zebrafish remain unclear. In this study, we demonstrated that knockdown or knockout of *nkx2.3* resulted in the absence of posterior ceratobranchial cartilages in zebrafish. The absence of posterior pharyngeal cartilages is a consequence of the compromised proliferation and differentiation and survival of cranial neural crest cells (CNCCs). Notably, we found that *nkx2.3* was not involved in endoderm pouch formation. Additionally, our findings suggested that *nkx2.3* negatively regulated Fibroblast growth factor (Fgf) signaling, as overexpression of *fgf8* could mimic the phenotype observed in *nkx2.3* morphants, suppressing CNCC differentiation. Moreover, inhibiting Fgf signaling restored the abnormalities in posterior cartilages induced by *nkx2.3* knockdown. These findings establish the essential role of *nkx2.3* in the development of posterior ceratobranchial cartilages through the inhibition of *fgf8*.

1. Introduction

The vertebrate skeleton develops from three embryonic stem cell lineages: the neural crest, somitic mesenchyme, and lateral plate. Among these lineages, neural crest-derived mesenchymal cells develop into the head skeleton [1]. The skull consists of the neurocranium (braincase) and viscerocranium (visceral skeleton, mainly composed of pharyngeal arches). The neurocranium provides protection for the brain, while the viscerocranium supports feeding and breathing functions [2]. Cranial neural crest cells (CNCCs) originate from the dorsal neural tube and undergo an epithelial-to-mesenchymal transition (EMT) before migrating from the rhombomeres into the pharyngeal arches and frontonasal plates, where they give rise to cartilages, bones, and other mesenchymal tissues [3]. The pharyngeal arches, which form on the lateral surface of the head in all animals, including humans, serve as the templates for the development of craniofacial structures in adulthood. Abnormal CNCCs development is known to contribute to various birth

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abnormalities affecting the craniofacial region in humans.

Each pharyngeal arch consists of a mesodermal core surrounded by CNCCs, with external ectodermal coverage and separation from other arches by an endodermal pouch [4,5]. Numerous studies have shown that proper endoderm morphogenesis is directly or indirectly necessary for craniofacial development in mice [6], chickens [7], and zebrafish [8,9]. Several signaling pathways, including Fibroblast growth factors (Fgf), retinoic acid (RA), bone morphogenetic proteins (BMP), and Hedgehog (Hh), have been previously identified as crucial for pharyngeal skeleton morphogenesis [8,10–14]. In zebrafish, impaired RA signaling or dysfunction of *pax1a/b* affects the development of the caudal pharyngeal pouches (PP)-2 and more posterior PPs [15]. The expression of *prdm1a*, regulated by Fgf andRA signaling pathways, plays a role in patterning the posterior craniofacial skeleton in zebrafish [16]. Although zebrafish craniofacial development has been extensively studied, the precise development of the more posterior arches remains relatively

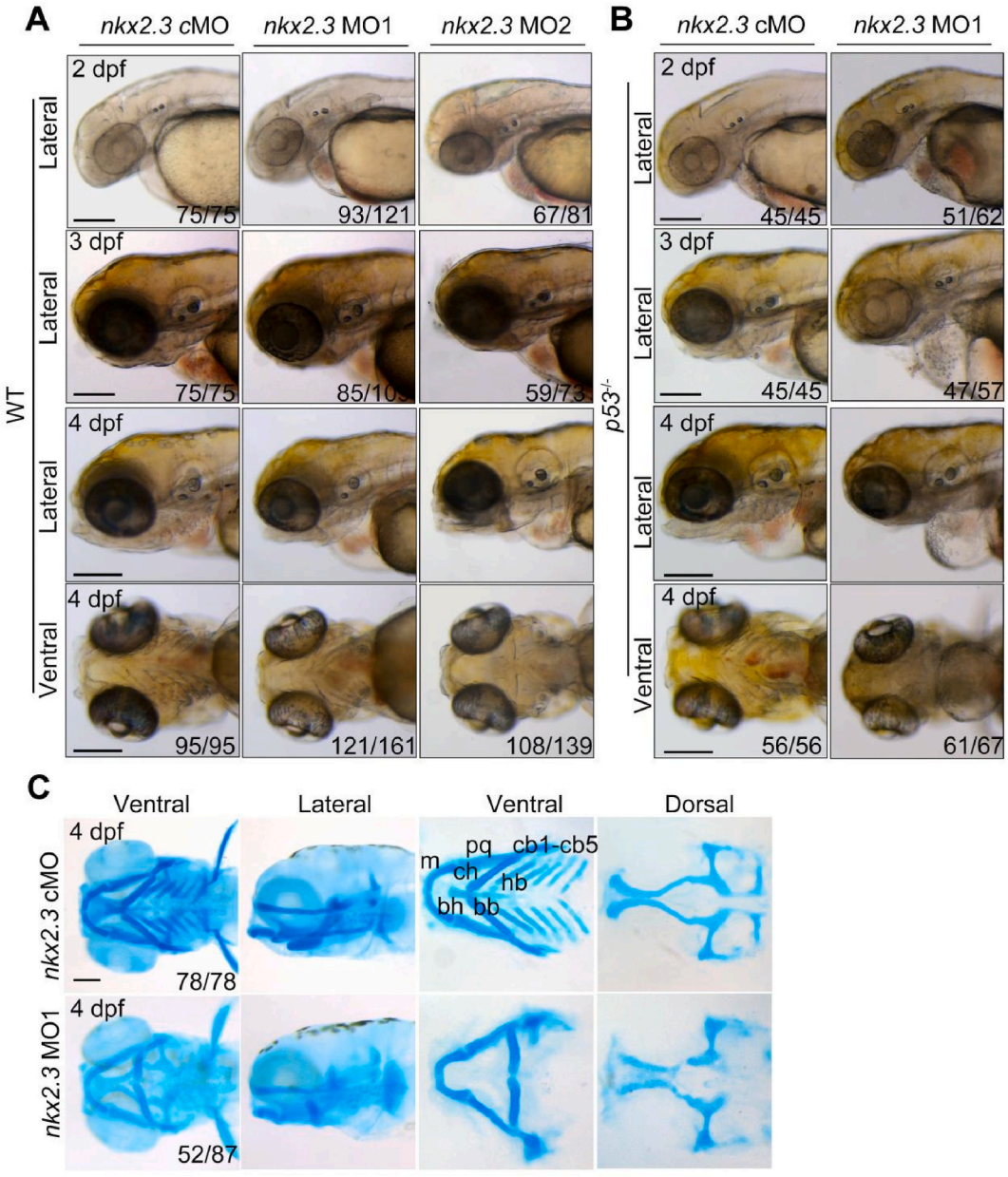

Fig. 1. Malformation of pharyngeal cartilages in *nkx2.3* morphants. (A) Morphology of WT embryos injected with *nkx2.3* MOs at indicated stages. Scale bars, 200 μm. **(B) Morphology of *p53* mutant embryos injected with *nkx2.3* MOs at 4 dpf.** Scale bars, 200 μm. **(C) Detection of craniofacial cartilages formation using Alcian blue staining.** The cartilages were shown anterior to the left and included the ceratobranchial (cb), ceratohyal (ch), Meckel's cartilage (m), basibranchial (bb), basihyal (bh), hypobranchial(hb), and palatoquadrate (pq) cartilages. Scale bars, 100 μm.

unknown.

Fgfs are a type of extracellular signaling molecule that have been implicated in various aspects of pharyngeal arch development [10,17]. Studies on Fgf8 function in chick and mouse, as well as *fgf3* function in zebrafish, have demonstrated the roles of Fgfs in the differentiation and survival of postmigratory CNCCs [10]. The endoderm-derived Fgf3 and Fgf8 are crucial for pharyngeal cartilage formation. Specifically, simultaneous deletion of *fgf3* and *fgf8* in zebrafish results in a near-complete absence of pharyngeal and neurocranial cartilage [18]. Inhibition of Fgf8 signaling can lead to slight deviations in anterior nasal cartilage and loss of nasal bone in chicken embryos [19]. Fgf18 is involved in coordinating chondrogenesis, and its absence results in defective cartilage formation in mice [20]. Furthermore, abnormalities in specific Fgf signaling pathways can contribute to a range of craniofacial malformations in humans [21]. Despite previous studies on Fgf and cartilage development, the exact mechanism by which Fgf regulates cartilage development remains unclear.

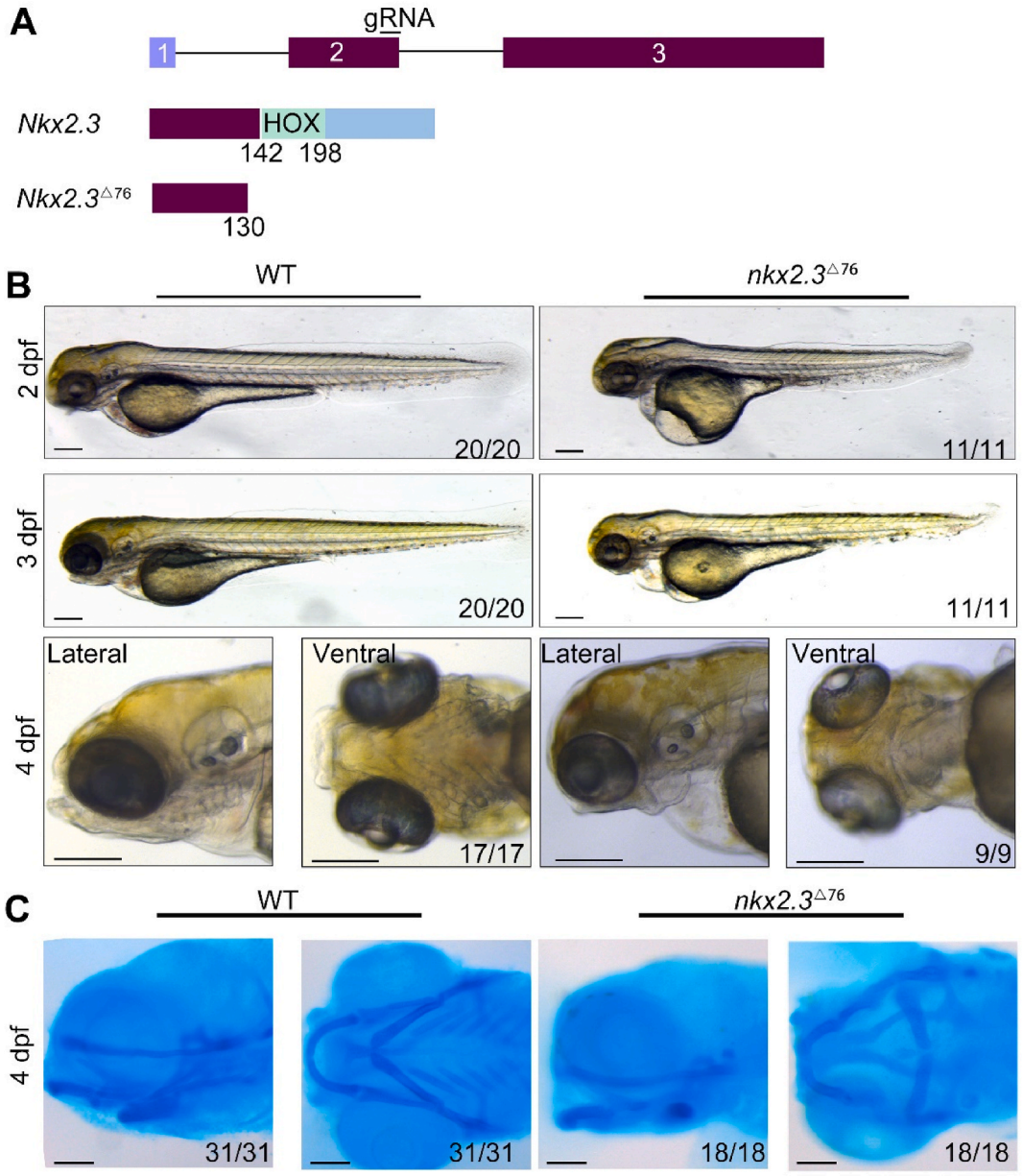

Fig. 2. Malformation of pharyngeal cartilages in *nkx2.3* mutants. (A) Knockout of *nkx2.3* by CRISPR-Cas9 system. The *nkx2.3*$^{\triangle 76}$ allele was identified by the targeting of the second exon of the *nkx2.3* locus (top panel), which carried a 76-bp deletion resulting in a premature stop codon. The truncated protein in *nkx2.3*$^{\triangle 76}$ mutants only has 130 amino acids (bottom panel). **(B) Morphology of *nkx2.3* mutant embryos at indicated stages.** Scale bars, 200 μm. **(C) Photomicrographs of the cartilages stained with Alcian blue.** The cartilages were shown anterior to the left and included the ceratobranchial (cb), ceratohyal (ch), Meckel's cartilage (m), and palatoquadrate (pq) cartilages. Scale bars, 100 μm.

The NK-2 class homeobox genes play crucial roles in vertebrate development. *Nkx2.3* is involved in the development of various structures, including the thyroid, sublingual salivary glands, lingual epithelium, and mandibular odontogenic epithelium. In mice, *Nkx2.3* is widely expressed in the embryonic pharyngeal floor endoderm [22]. It is important for tooth cusp formation and regulates the fate of enamel junctional cells. Knockdown of *Nkx2.3* leads to defects in the maturation and cellular organization of salivary glands [23]. Additionally, *nkx2.3* participates in the development of other components. In mice, chick, Xenopus and zebrafish, *nkx2.3* is known to be involved in the commitment and/or differentiation of cardiomyocytes [24]. Targeted inactivation of the *Nkx2.3* gene in mice results in severe morphological alterations in the intestine and spleen, affecting normal development and function [25]. *Nkx2.3* is also essential for the formation of visceral mesoderm [26].

Our previous study has shown that BMP signaling is necessary for specifying the differentiation of *nkx2.3*$^{+}$ cells into the pouch epithelium in zebrafish. During early somitogenesis, *nkx2.3*$^{+}$ cells located in the lateral region of the pharyngeal endoderm give rise to the pouch epithelium [27]. BMP2b activates Smad effectors in endodermal cells and induces the specification and morphogenesis of *nkx2.3*$^{+}$ progenitor cells, thereby promoting pharyngeal pouch development. Fgf, along with BMP and other signaling molecules, is expressed in the endoderm and is involved in the formation of the pharyngeal skeleton [27]. Therefore, we hypothesized that *nkx2.3* may engage in pharyngeal cartilage formation by activating the expression of the Fgf signaling pathway.

In this study, we investigated the developmental roles of *nkx2.3* in the formation of posterior pharyngeal cartilages using knockdown and knockout methods. Our findings demonstrate that depletion of *nkx2.3* led to defective formation of posterior ceratobranchial cartilages, resulting from defects in both CNCC proliferation, survival and differentiation. We further elucidated the mechanism behind this observation, revealing that *nkx2.3* deficiency stimulated Fgf signaling. Importantly, inhibiting Fgf signaling

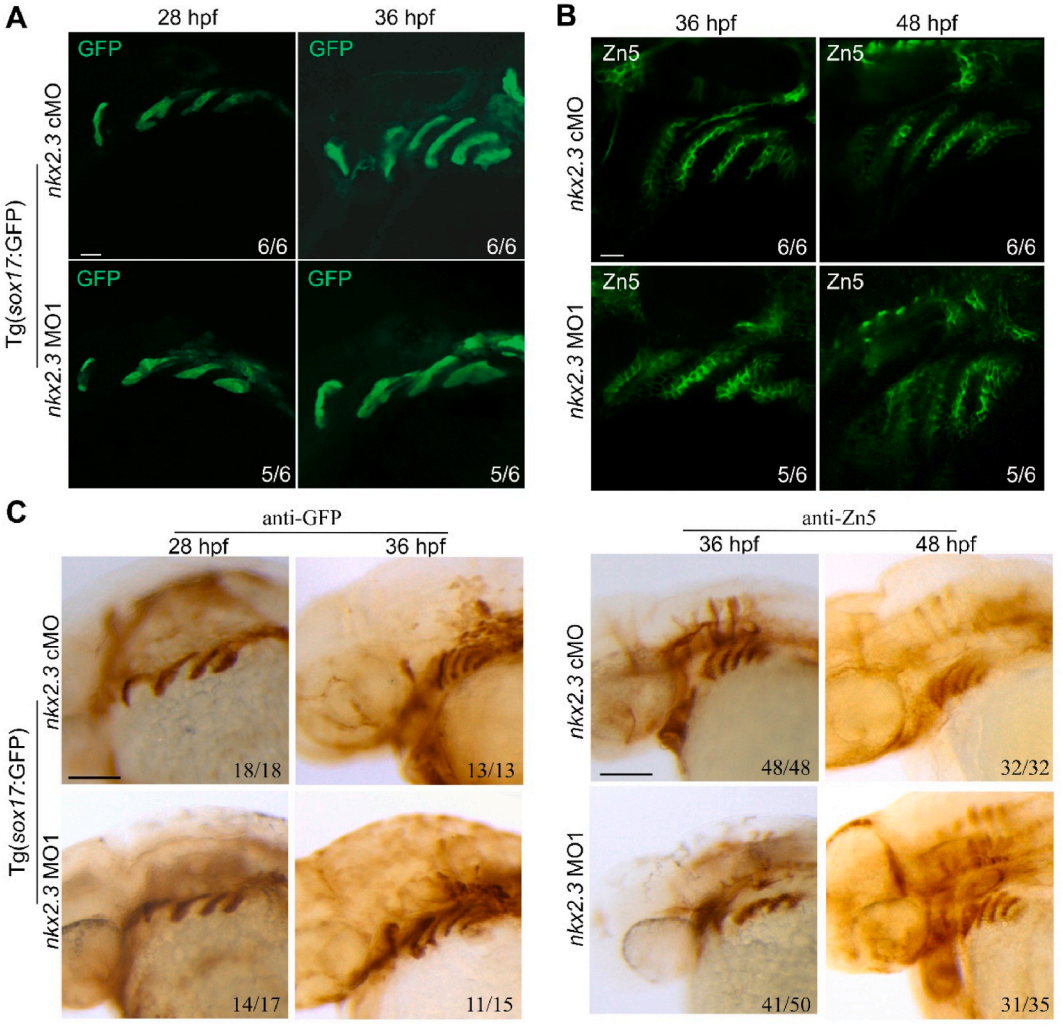

Fig. 3. Pharyngeal pouches are normally developed in *nkx2.3* morphants. (A) Embryos were immunostained with the anti-GFP antibody at the indicated stages. Scale bars, 20 μm. **(B) Embryos were immunostained with the anti-Zn5 antibody at the indicated stages.** Scale bars, 20 μm. **(C) Embryos were immunostained with the anti-Zn5 antibody at the indicated stages.** Scale bars, 100 μm. Images are shown with a lateral view and with the anterior to the left.

could restore the loss of posterior cartilages in *nkx2.3* morphants. Our results suggest that *nkx2.3* acts as a regulator of the Fgf signaling pathway and plays a critical role for the formation of posterior cartilages.

2. Results

2.1. *nkx2.3 is essential for the formation of the posterior pharyngeal cartilages*

Our previous studies, as well as those of conducted by others, have demonstrated significant expression of *nkx2.3* in the endoderm

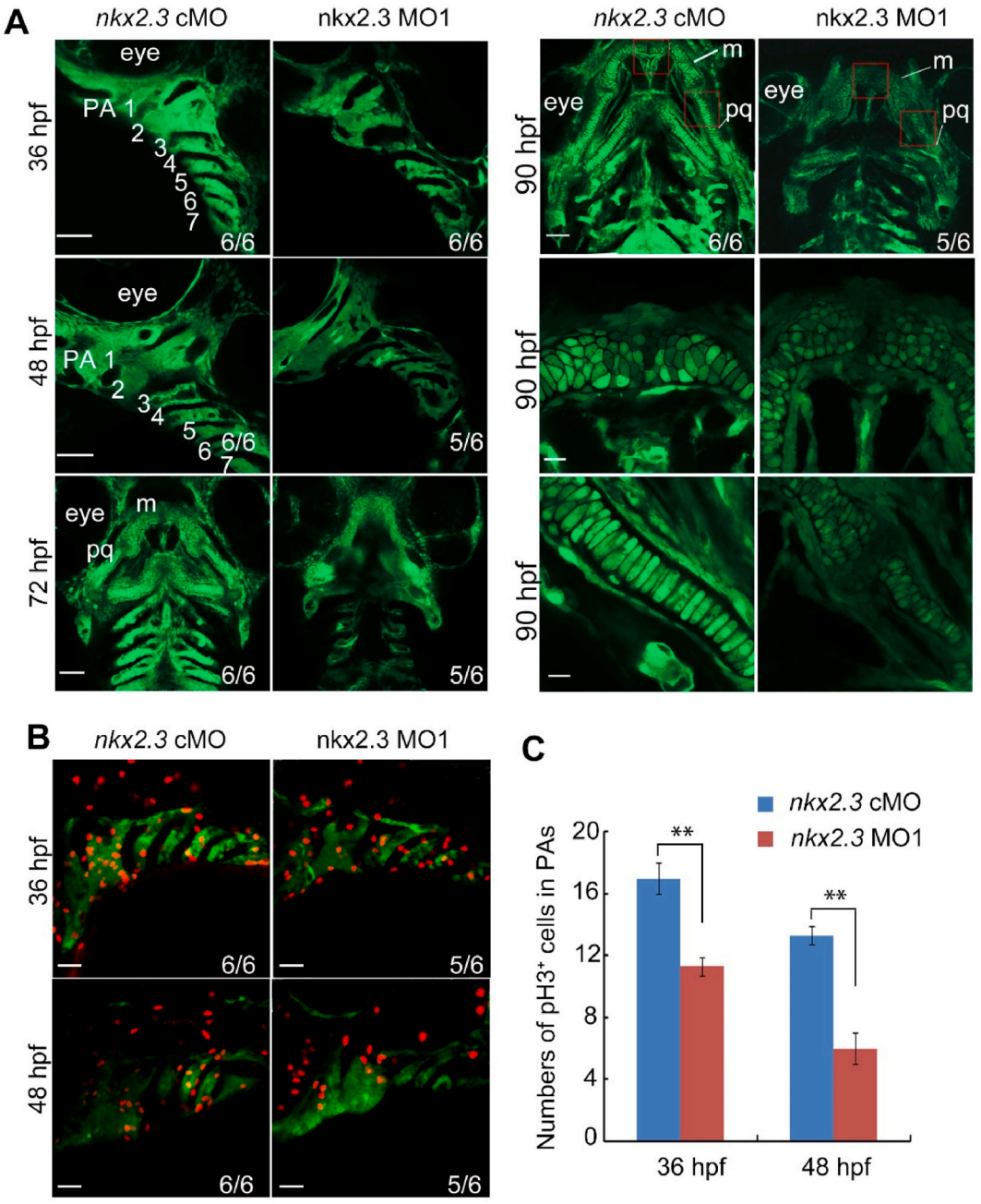

Fig. 4. Proliferation of CNCCs is compromised in nkx2.3 morphants. (A) Time-lapse imaging of *nkx2.3* morphants and control embryos in Tg(*fli1*-GFP) from 36 to 90hpf. The bottom right is an enlarged view of the red box in the top right figure at 90 hpf. Scale bars: 20 μm. **(B) Immunostaining of embryos in the background of Tg(*fli1*:GFP) with** pH3 **at indicated stages.** Scale bars: 20 μm. **(C) The number of** pH3 **and GFP double-positive cells in the pharyngeal arches.** Data are represented as mean ± SD. **P < 0.01.

pouches of zebrafish from 24 to 72 hpf [10,12,27]. To investigate the role of *nkx2.3* in the development of the pharyngeal arches, we used specific antisense morpholinos (MO) targeting the ATG site (referred to as *nkx2.3*-MO1) and a splice-acceptor site (referred to as *nkx2.3*-MO2). The effectiveness of *nkx2.3*-MO1 was confirmed by its ability to inhibit the expression of green fluorescent protein (GFP) fused with the 5'UTR of *nkx2.3*, containing the target sequence of *nkx2.3*-MO1 (Fig. S1). At 4 days post-fertilization (dpf), embryos microinjected with 10 ng of *nkx2.3*-MO1 showed morphological abnormalities, such as smaller heads and diminished jaw structures, compared to the groups injected with a control MO (referred to as *nkx2.3*-cMO) containing a five-base mismatch (Fig. 1A). Similar phenotypes were observed in embryos treated with *nkx2.3*-MO2 (Fig. 1A). We ruled out a general off-target effect of morpholinos by injecting 10 ng of *nkx2.3*-MO1 into *p53* mutant embryos, which displayed the same phenotype as *nkx2.3*-MO1 in wild-type (WT) embryos (Fig. 1B). To further validate the specificity of these phenotypes, a rescue experiment was performed. Coinjection of 300 pg of *nkx2.3* mRNA and 10 ng of *nkx2.3*-MO1 effectively alleviated the head and jaw malformations (Fig. S2), indicating the involvement of *nkx2.3* in head development. Alcian blue staining revealed that cartilages derived from the first arch, Meckel's (m) and palatoquadrate (pq), developed normally in *nkx2.3* MO1-injected embryos (Fig. 1C), as did second arch cartilages such as ceratohyal (ch) and hyosymplectic (hs) (Fig. 1C). However, all cartilage structures derived from the posterior arches, specifically the ceratobranchial (cb) cartilages, were noticeably absent (Fig. 1C). Additionally, the hypobranchials (hb) and epibranchials (eb) were absent as well.

Subsequently, we generated *nkx2.3* mutants by CRISPR-Cas9 technology. Using CRISPRscan, two sgRNAs were designed to target exon 2 of the *nkx2.3* gene. As a result, we obtained the *nkx2.3*$^{\Delta 76}$ mutant, which carries a 76-bp deletion and is predicted to produce a truncated *Nkx2.3* protein with 130 residues, lacking the HOX domain (Fig. 2A). Embryos from *nkx2.3*$^{\Delta 76\ +/-}$ incrosses exhibited normal morphology, except for a slightly shorter body length at 48 hpf (Fig. 2B). However, from 72 hpf onwards, the embryos showed heart edema and flattened jaws (Fig. 2B). Alcian blue staining of *nkx2.3*$^{\Delta 76}$ embryos revealed a lack of ceratobranchial cartilage formation compared to wild-type (WT) embryos at 4 dpf (Fig. 2C). These findings suggest that *nkx2.3* is necessary for the formation of posterior cartilages in zebrafish.

2.2. *nkx2.3 is not essential for endoderm pouch segmentation*

Endoderm-derived pharyngeal pouches play essential roles in the development of all pharyngeal cartilages [6,8,28]. Given *nkx2.3*'s strong expression in the endoderm pouches, we aimed to investigate its involvement in the development of pharyngeal pouches in zebrafish. To block *nkx2.3* function, we injected *nkx2.3* MO1 into Tg(*sox17*:GFP) transgenic embryos at the one-cell stage. In control embryos, endodermal pouch development occurs from anterior to posterior, with all pouches formed by 38 hpf [10]. Fig. 3A demonstrated that the distribution of GFP in the endodermal pouches of *nkx2.3* morphants was not significantly different from that in *nkx2.3* cMO-injected embryos at 28 and 36 hpf (Fig. 3A). Additionally, we used Zn5 antibody to label endoderm pouches in zebrafish [29] and observed normal pouch formation in *nkx2.3* morphants at 36 and 48 hpf (Fig. 3B). Furthermore, immunochemistry staining with GFP and Zn5 antibodies detected in Tg(*sox17*:GFP) and WT embryos confirmed the normal formation of endoderm pouches in *nkx2.3* knockdown embryos at various stages (Fig. 3C). Considering the requirement of endoderm pouches for thymus formation, we investigated whether *nkx2.3* also played a role in thymus development. At 4 dpf, we performed ISH to detect the expression of several thymus marker genes, including *ccl25a*, *rag1*, and *foxn1*. As expected, knocking down *nkx2.3* had no effect on the expression pattern of these marker genes (Fig. S3). These findings show that *nkx2.3* is not responsible for endoderm pouch formation.

2.3. *Disrupting nkx2.3 results in a reduced proliferation of CNCCs in zebrafish larva*

To better understand the development of pharyngeal arches, we performed time-lapse imaging of CNCCs in transgenic zebrafish, Tg (fli1:EGFP), where EGFP expression was specifically controlled by the fli1 promoter in CNCCs [21,22]. In WT embryos, at 36 hpf, GFP-positive cells migrated into seven pharyngeal segments, and the chondrification of the first pharyngeal cartilages initiated around 48 hpf [30]. We discovered that the morphology of pharyngeal cartilages in *nkx2.3* morphants at 36 and 48 hpf appeared normal, except for the posterior arches, which exhibited a thinner structure compared to embryos injected with *nkx2.3* cMO (Fig. 4A). As development progresses, this phenotype became more pronounced (Fig. 4A). At 72 hpf, early signs of cell arrangements that contribute to cartilage formation are evident [31]. WT chondrocytes in ceratohyal maintained their elongated and stacked arrangement at later time points (72–96 hpf). Notably, *nkx2.3* morphants had almost no chondrocytes in the posterior arches compared to control MO-injected embryos (Fig. 4A). These findings indicate that *nkx2.3* is essential for chondrocyte development.

Subsequently, we examined cell death and proliferation in developing *nkx2.3* morphants and control embryos. The decreased number of CNCCs observed in our live imaging experiments could be attributed to diminished cell proliferation, increased cell death, or a combination of both factors.

To test this hypothesis, we knocked down *nkx2.3* in Tg(*fli1*:GFP) transgenic fish embryos, followed by immunofluorescence experiments using pH3 and GFP antibodies at 36 and 48 hpf. The results showed that *nkx2.3* morphants displayed fewer proliferating pH3^{+} CNCCs compared to cMO-injected embryos (Fig. 4B and C), indicating a reduction in CNCC proliferation. Moreover, we used the TUNEL assay to investigate apoptosis in CNCCs of *nkx2.3* morphants. The results showed that in *nkx2.3* knockdown embryos, apoptotic signaling was increased in CNCCs at 72 hpf and 90 hpf compared to controls; whereas apoptotic signaling was not significantly different at 36 hpf and 48 hpf (Fig. S4), indicating that *nkx2.3* is necessary for CNCC survival.

2.4. *Disrupting nkx2.3 inhibits the differentiation of CNCCs in zebrafish larva*

To investigate the precise role of *nkx2.3* in pharyngeal cartilage development, we initially examined the expression of several

markers associated with CNCC specification and migration in *nkx2.3* morphants using WISH. Compared to the control group, *nkx2.3* morphants exhibited indistinguishable expression patterns of the early neural crest specification marker *foxd3* at 11 hpf (Fig. S5), indicating normal CNCC induction. Moreover, *nkx2.3* depletion had no discernible effect on CNCC migration, as the expression of postmigratory markers *dlx2* and *crestin* in *nkx2.3* morphants was comparable to that in control animals at 26 hpf (Fig. S5).

Furthermore, we performed Col2a antibody immunostaining to assess CNCC differentiation. In control embryos at 72 hpf and 100 hpf, clear Col2 signals were observed in the Meckel's (m) and palatoquadrate (pq) cartilages derived from the first pharyngeal arch, as well as in the ceratohyal (ch) and hyosymplectic (hs) cartilages derived from the second arch (Fig. 5A). However, in *nkx2.3* knockdown embryos at 72 hpf, the Col2a signals in cartilage m, pq, and ch were reduced. As development progressed, positive signals in the ceratobranchial cartilages (cb) nearly vanished (Fig. 5A), which is consistent with the findings from Alcian blue staining. These observations suggest a failure in chondrogenic differentiation in the posterior arches. Additionally, *nkx2.3* morphants exhibited decreased expression of chondrogenic marker genes such as *gsc*, *sox9a*, and *col2a* at 72 hpf, suggesting the crucial role of *nkx2.3* in chondrocyte development (Fig. 5B). These results demonstrate that while *nkx2.3* is not essential for CNCC induction or migration, it plays a vital role in CNCC differentiation.

2.5. *nkx2.3 regulates the development of posterior cartilages through modulating Fgf signaling*

Fgfs are essential for the differentiation and survival of post-migratory CNCCs and the morphogenesis of pharyngeal pouches [10]. To investigate the role of *nkx2.3* in regulating the formation of posterior cartilages through Fgf signaling, we performed ISH to detect the expression of *fgf3* and *fgf8* in *nkx2.3* morphants. The results showed higher levels of *fgf3* and *fgf8* expression in the pharyngeal region after *nkx2.3* knockdown compared to cMO-injected embryos (Fig. 6A), implying that *nkx2.3* may regulate the formation of posterior cartilages via Fgf signaling. Hans et al. [32] created a transgenic zebrafish line, named Tg(*hsp70:fgf8*), with the intention of inducing misexpression of *fgf8* under the control of the zebrafish temperature-inducible *hsp70* promoter. We sought to investigate whether misexpression of Fgf signaling using this transgenic line could lead to malformation of the posterior arches. As shown in Fig. 6B, we observed that heat shock-induced misexpression of *fgf8* led to a reduction in the size of the jaw structures. Alcian blue staining of the pharyngeal arches revealed that overexpression of *fgf8* in embryos resulted in normal formation of cartilages derived from the first and second arches, but there was a reduction or loss of posterior cartilages (cb1-cb5s) (Fig. 6C). To better understand how *fgf8* overexpression affected the lack of posterior cartilages, we examined CNCC differentiation using Col2a immunostaining. We found

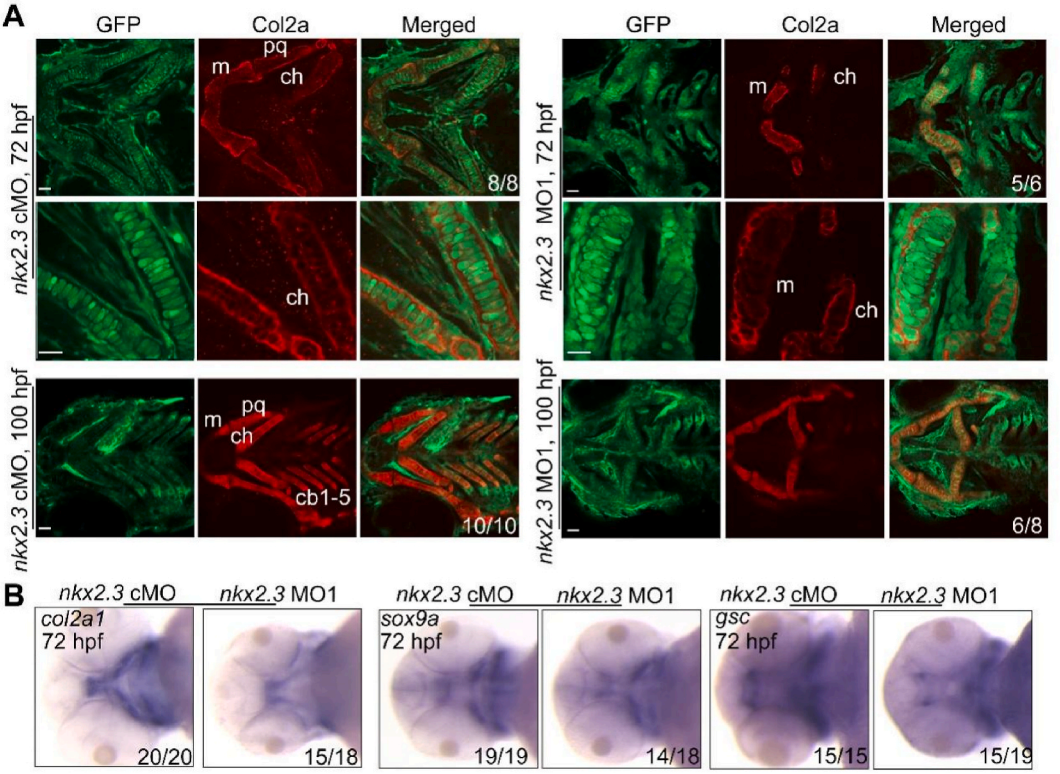

Fig. 5. Posterior arch skeletogenic CNCCs do not differentiate in nkx2.3 morphants. (A) Detection of Col2a proteins in the pharyngeal arches at indicated stages. Embryos of Tg*(fli1:*EGFP*)* injected with *nkx2.3* MOs were co-immunostained with anti-GFP (green) and anti-Col2a (red) antibodies. The palatoquadrate (pq), ethmoid plate (ep) and ceratohyal (ch) cartilages were shown with anterior to the left. Scale bars, 20 μm. **(B) The expression level of marker genes of CNCCs' differentiation was detected by WISH at 72 hpf.**

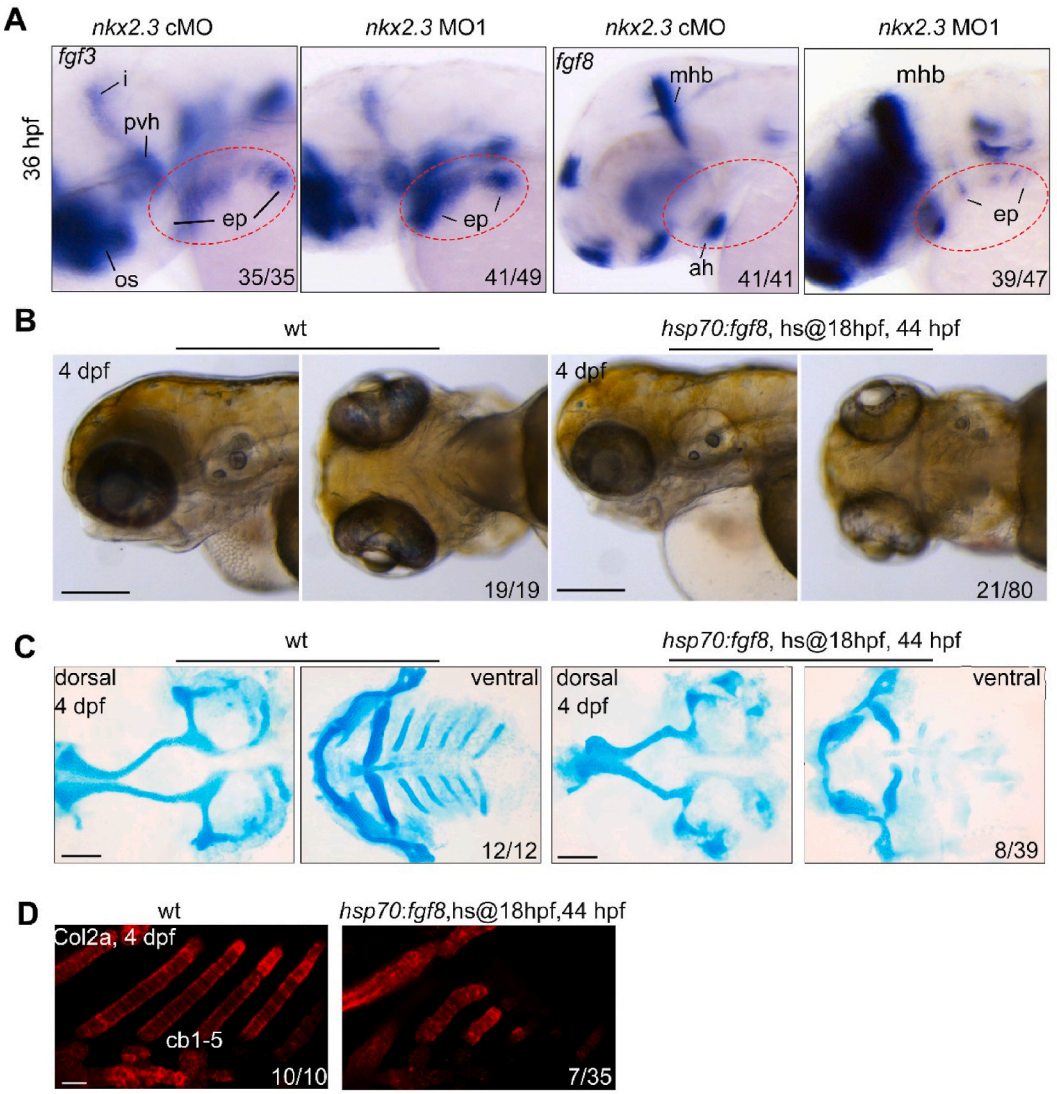

Fig. 6. Knockdown of *nkx2.3* increased Fgf signal and *fgf8* overexpression mimic the phenotype of *nkx2.3* morphants. (A) Expression pattern of *fgf3* and *fgf8* in *nkx2.3* morphants detected by ISH at 36 hpf. i, isthmus; os, optic stalk; pvh, posterior-ventral hypothalamus; ep, endodermal pouches; mhb, midbrain-hindbrain boundary; ah, adenohypophysis. **(B) Morphology of embryos with overexpressed fgf8.** Scale bars: 200 μm. **(C) Alcian blue staining of embryos in which *fgf8* was overexpressed.** Scale bars: 100 μm. **(D) Immunostaining of Col2a proteins in *fgf8* transgenic embryos at 4 dpf.** Scale bars: 10 μm.

reduced or absent Col2a expression in cb1-cb5s (Fig. 6D). These findings suggest that knockdown of *nkx2.3* activates Fgf signaling, while ectopic Fgf signaling represses CNCC differentiation, resulting in fewer or no posterior cartilages.

Further analysis revealed that the promoter of zebrafish *fgf8* contained 20 potential binding sites for *nkx2.3* (Table S1) according to JASPAR (http://jaspar.genereg.net), suggesting that *nkx2.3* might regulate the transcription of *fgf8*. Similarly, the promoter of human *FGF8* also contained 19 potential binding sites for Nkx2.3 (Table S2), suggesting a conserved regulatory role of *nkx2.3* in *fgf8*.

2.6. Inhibition of Fgf signaling partially corrects the malformation of posterior cartilages caused by nkx2.3 knockdown

Considering the activation of Fgf signaling in *nkx2.3* morphants, we investigated whether the inhibition of Fgf signaling could rescue the phenotype of posterior cartilages loss. To test this, we gave embryos injected with *nkx2.3* cMO and *nkx2.3* MO1 either SU5402, a selective inhibitor that binds to all Fgf receptors and inhibits tyrosine kinase activity [33], or DMSO at various dosages. We administered SU5402 at concentrations of 0.67 μM, 1.25 μM, 2.5 μM, 5 μM and 10 μM to *nkx2.3* cMO and *nkx2.3* MO1 injected embryos from 24 to 48 hpf, respectively. Our findings demonstrated that the decreased jaw structures brought on by *nkx2.3* knockdown were partially recovered in embryos treated with SU5402 at a dose of 2.5 μM from 24 to 48 hpf (Fig. 7A). However, when administered between 24 and 48 hpf, the inhibitor below 5 μM had no effect on the growth of *nkx2.3* cMO-injected embryos. In the

meanwhile, we discovered that SU5402 over 5 μM exacerbated the phenotype while SU5402 below 1.25 μM had no rescue impact on the smaller jaw structures caused by *nkx2.3* knockdown (data not shown). Using Alcian blue staining, we also evaluated the rescue impact of blocking Fgf signaling in *nkx2.3* morphants. In comparison to the DMSO-treated groups, the 2.5 μM SU5402 treatment had a rescue effect on the posterior cartilage defects (Fig. 7B). These results suggest that *nkx2.3* controls posterior arch development via Fgf signaling.

3. Discussion

We have demonstrated that *nkx2.3*$^{+}$ cells are present in the lateral pharyngeal endoderm during the early somite stages and give rise to the pouch epithelium [27]. Several other studies have also shown that *nkx2.3* plays an important roles in salivary gland and tooth morphogenesis in mice [22,23], as well as the development of small intestine and spleen in mice [25]. However, this is the first in-depth study focusing on the role of *nkx2.3* in pharyngeal arch morphogenesis. Here, by using the knockdown and knockout embryos,

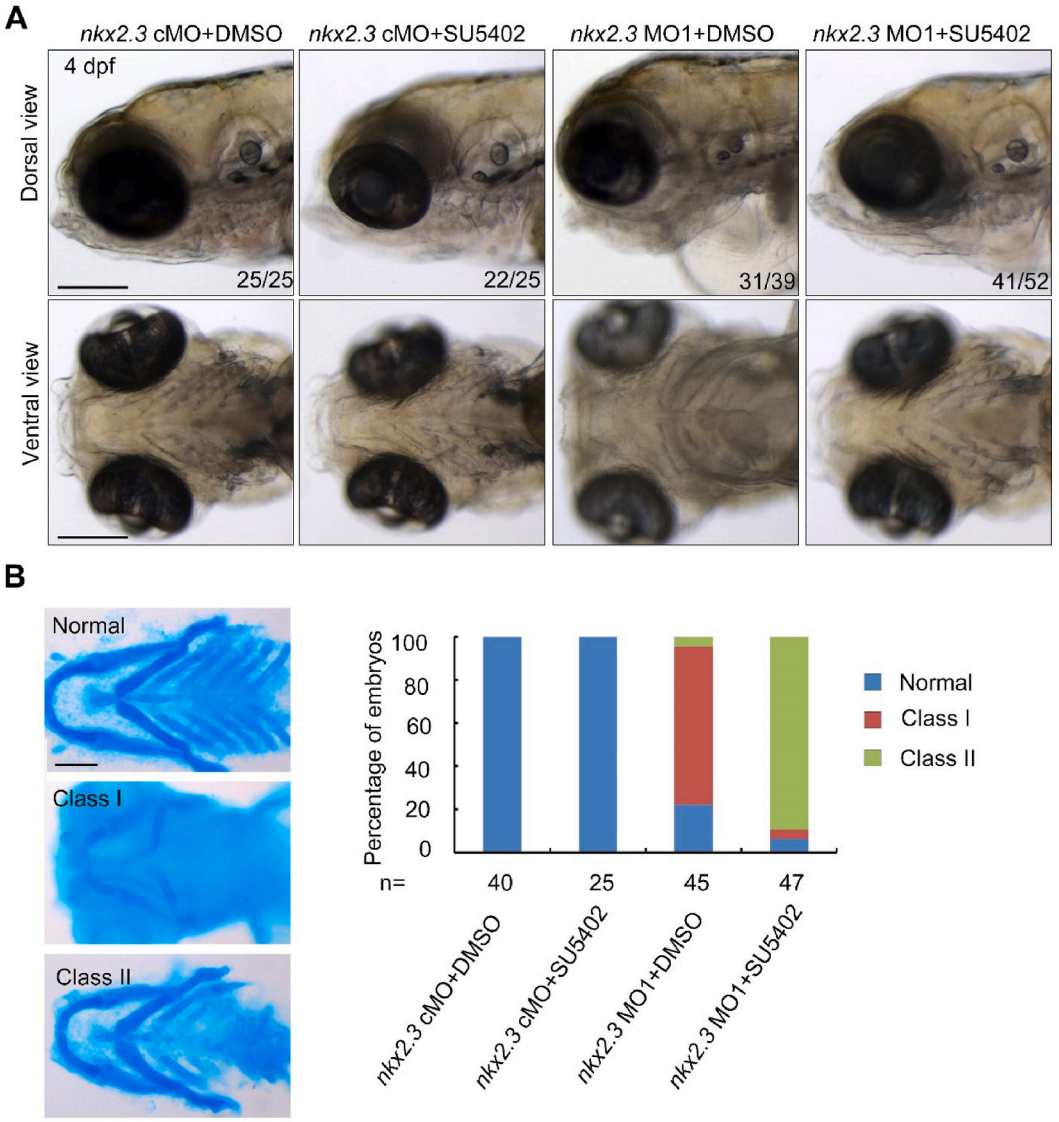

Fig. 7. Fgf signal inhibition can partially correct the malformation of the posterior cartilages in *nkx2.3* morphants. (A) Embryo morphology following SU5402 treatment on *nkx2.3* morphants. We tracked the embryos' growth and took pictures of them at 4 dpf. Embryos treated with 2.5 μM SU5402 between 24 and 48 hpf after receiving an injection of *nkx2.3* cMO or *nkx2.3* MO1 are shown in the representative images. Scale bars: 200 μm. **(B) Alcian blue staining of *nkx2.3* morphants treated with SU5402 and the statistic results.** At 4 dpf, the embryos of *nkx2.3* morphants and controls that had been treated with of 2.5 μM SU5402 were collected and stained with Alcian blue. Representative images of the various pharyngeal cartilage classes were shown in the left panel. Scale bars: 100 μm. The bar graph on the right displays the proportion of each type of embryo seen in the left panel in each treatment group.

we have demonstrated that *nkx2.3* is essential for the development of the posterior pharyngeal cartilage in zebrafish, independent of the development of the endoderm pouches. Knockdown of *nkx2.3* suppresses the proliferation and differentiation of CNCCs, with elevated expression levels of both *fgf3* and *fgf8* in the pharyngeal region. Overexpression of *fgf8* results in posterior cartilage loss, where the expression of *col2a* in these regions was reduced or absent. Inhibition of Fgf signaling partially rescued the posterior arch defects caused by the knockdown of *nkx2.3*.

Since the early 2000s, MOs have been used to knock down genes in various models, including zebrafish, sea urchins, and Xenopus [33]. However, concerns have been raised about the off-target consequences of MOs, such as neurotoxicity and developmental delay. Therefore, recommended guidelines for using MOs, including controls and rescue experiments, help researchers distinguish between specific phenotypes and off-target effects [33,34]. Guidelines for morpholino use in zebrafish request the creation of a mutant line to support phenotype specificity [34]. In our study, we created the *nkx2.3* mutant in zebrafish using the CRISPR-Cas9 approach. When we used F2 embryos for phenotypic research, we discovered that the posterior pharyngeal cartilages were also absent in *nkx2.3* mutant embryos, consistent with the defects in *nkx2.3* morphants. Unfortunately, the defects were recovered in the F3 generations. In 2019, Anne and colleagues reported that young *Dio2* zebrafish mutants exhibit hyperglycemia, whereas adult mutant zebrafish have normoglycemia [35]. These findings indicated a compensatory response in older *Dio2* knockout zebrafish, whereas younger mutants did not show the same response. However, Stainier et al. noted that the morphant can be used as an acceptable substitute for the mutant in subsequent experiments if the mutant's phenotype is compatible with that of the morphant [34]. Therefore, the mechanistic experiments in our study were performed in *nkx2.3* morphants.

Based on multiple studies using the zebrafish model, it has been observed that zebrafish mutants do not show the same severe phenotype as zebrafish morphants for the same genes [36]. This phenomenon in zebrafish mutants, but not in morphants, is due to the response of genetic compensation. In 2019, two outstanding works partially uncovered that genetic compensation can be triggered by the upregulation of compensating genes through controlling nonsense-mediated mRNA decay (NMD) and/or premature termination codons (PTC)-bearing mRNA, in conjunction with epigenetic machinery in mutant zebrafish [37,38].

Previous studies have identified the expression of *nkx2.3* in the pharyngeal endoderm [39], which is further supported by our previous data. We hypothesized that *nkx2.3* might be involved in endoderm development and performed a series of related experiments. At 4 dpf, we observed a loss of posterior cartilage cb1-cb5s in the *nkx2.3* morphants. However, *nkx2.3* knockdown embryos displayed normal formation of the endoderm pouch, indicating that *nkx2.3* is not associated with endoderm pouch development. Moreover, knockdown of *nkx2.3* did not result in any changes in the expression pattern of several thymic marker genes. However, *nkx2.3* is essential for the development of pharynx-derived cartilages. Analysis of *nkx2.3*-deficient mice revealed defects in maturation and cellular organization of the sublingual gland, absence of the cusps in the mandibular molars, and occasional absence of the third molar [22]. These findings suggest that *nkx2.3* plays a crucial role in the development of the zebrafish posterior pharyngeal cartilage, but is not involved in the formation of the endodermal pouch.

The formation of pharyngeal cartilages in zebrafish involves several steps, including CNCC specification, ventrolateral migration of CNCCs from rhombomeres along three distinct streams into the pharyngeal arches, prechondrogenic condensation, and chondrogenic differentiation [30,40,41]. Therefore, further insights are necessary to reveal the mechanisms underlying the behavior of CNCCs during pharyngeal cartilage development. Our observations indicate that CNCCs can be induced normally in the *nkx2.3* morphants, and the depletion of *nkx2.3* does not significantly affect CNCC migration. However, we did observe a failure of cartilage differentiation in the postembryonic arch, suggesting that *nkx2.3* is required for the chondrogenic differentiation of CNCCs, but not for the induction or migration.

Studies in transgenic mice and the characterization of human craniofacial disorders have shown that Fgf signaling is necessary for the development of membrane bone in the cranium [21,38]. Albertson et al. reported that attenuated *fgf8* signaling consistently results in biased LR asymmetric development of the pharyngeal arches and craniofacial skeleton in zebrafish [42], while *fgf8* haploinsufficiency leads to distinct craniofacial defects in adult zebrafish [43]. In our study, we found elevated expression levels of *fgf3* and *fgf8* in the pharyngeal region after the *nkx2.3* knockdown. Surprisingly, overexpression of *fgf8* resulted in a deficiency of posterior cartilage in cb1-cb5s with reduced or absent expression of Col2a. We hypothesized that *nkx2.3* knockdown inhibits CNCC differentiation through activating Fgf signaling, which subsequently resulted in the reduction or absence of posterior cartilages. In other words, inhibiting Fgf signaling could partially rescue the posterior cartilage malformation caused by *nkx2.3* knockdown, further validating this hypothesis. Additionally, we discovered that overexpressing *fgf8* in transgenic zebrafish lines Tg (*hsp70*:*fgf8*) caused a lack of the posterior pharyngeal cartilage. Based on these findings, we propose that fine maintenance of the balance of Fgf signaling activity is essential for the development of posterior pharyngeal arch cartilages in zebrafish. Next, we will explore the molecular mechanism by which *nkx2.3* regulates *fgf8*.

4. Materials and methods

4.1. Zebrafish strains and transgenesis

WT TU stocks of *Danio rerio*, along with the transgenic lines Tg(*sox17*:EGFP), Tg(*fli1*:EGFP), Tg(*hsp70*:*fgf8*), and *tp53* mutant line, were raised and maintained under standard laboratory conditions at 28.5 °C. Embryo stages were determined based on morphology as previously described [44]. All the methods were approved and performed in accordance with the relevant guidelines and regulations of the Institutional Ethics Committee of Hebei Medical University.

4.2. Morpholino (MO) and mRNA microinjection

nkx2.3-MO1 (5′-GAAGCATCATCAGGCGCTAAATGTG-3′) and *nkx2.3*-cMO (5′-GAAGCATCACTGGACGCTAAATGTG-3′) were designed to target the translational start region of zebrafish *nkx2.3* and were purchased from Gene Tools LLC. *nkx2.3*-MO2 (5′-CGCTGTTGGATTACATCTACAATGT-3′) was designed to target the splice junction target of intron 1 and exon 2. The linearized plasmids were used as templates to synthesize capped mRNAs *in vitro* using the mMessage mMachine kit (Ambion). Morpholinos or mRNAs were injected into the yolk of embryos at the 1-cell stage.

4.3. Generation of zebrafish nkx2.3 mutants lines using the CRISPR-Cas9 system

Guide RNA (gRNA) target sequences were selected using the CRISPRscan online tool (gRNA1: 5′-GGAACTGCAGGATAGATTTC-3′; gRNA2: 5′-GGCTGAGGTGCACTCGGACA-3′). Founder (F0) embryos co-injected with *nkx2.3* gRNAs and Cas9 protein were raised to sexual maturity and crossed with WT fish to generate F1 heterozygous carriers. F0 and F1 fish carrying mutations were identified by digesting with T7 endonuclease I, which recognizes and cleaves non-stringently matched DNA.

4.4. Whole-mount in situ hybridization and immunohistochemistry

Digoxigenin-UTP-labeled RNA probes for the genes *foxd3*, *dlx2*, *crestin*, *col2a*, *sox9a*, *gsc*, *fgf3*, *fgf8*, *ccl25a*, *foxn1* and *rag1* were synthesized *in vitro* from linearized DNA templates using the RNA Polymerase T7 system (Roche). As previously mentioned, Whole-mount in situ hybridization was performed using embryos at the indicated stages [41]. The fixed embryos were dehydrated in graded methanol, stored at −20 °C for at least 2 h, and then immunostained for Zn5, GFP, and pH3 and rehydrated three times for 5 min each in PBST (1 PBS, 0.1% TritonX-100). After three washes with PBST, the embryos were incubated at room temperature for 1 h in a blocking solution (5% fetal calf serum, 1% dimethylsulfoxide (DMSO), and 2% BSA in PBST). Monoclonal mouse anti-Zn5 (1:50, 111605, Zebrafish International Resource Center) or anti-pH3 antibody (1:1000; 3377, Cell Signaling Technology) and mouse anti-GFP (1:1000, A11120, Molecular Probes) antibodies were added and incubated at 4 °C overnight, followed by three 15-min washes with PBST. The embryos were then incubated overnight at 4 °C with fluorescence conjugated secondary antibody, including DyLight 488-conjugated goat anti-rabbit IgG (1:500; 711-545-152, Jackson), DyLight 594-conjugated goat anti-mouse IgG (1:500; 715-585-150, Jackson), DyLight 488-conjugated AffiniPure goat anti-mouse IgG (1:500; 715-545-150, Jackson), and DyLight 594-conjugated AffiniPure goat anti-rabbit IgG (1:500; 711-585-152, Jackson). Finally, the embryos were mounted in 1% low melting point agarose and imaged using a laser scanning confocal microscope (Zeiss LSM780). The protocols for Whole-Mount Immunohistochemistry of Zn5 and GFP in Zebrafish embryos were followed as previously described [45].

4.5. TUNEL assay

The ApopTag® Red In Situ Apoptosis Identification Kit (Millipore, S7165) was used with slight modifications, following the instructions provided by the manufacturer, to perform a TUNEL assay for identifying apoptotic cells in embryos [40]. The fluorescent microscope was used to study the embryos (Zeiss SteREO Discovery.V20).

4.6. Time lapse imaging

At 36 hpf, anaesthetized Tg(*fli1:EGFP*) embryos were embedded in 1% low-melt agarose. At specified time points, confocal stack images of the pharyngeal region were obtained using a META Zeiss 710 confocal microscope with a 20 × objective.

4.7. Embryonic treatments

Tg*(hsp70l:fgf8)* embryos were subjected to 40 °C heat shocks for 30 min at 18 hpf, 42 hpf, and 56 hpf to induce *fgf8* overexpression. The embryos were subsequently harvested at 96 hpf for phenotypic analysis and Alcian blue staining [46].

SU5402 (572630, Sigma) was dissolved in DMSO and used at various concentrations to inhibit Fgf activity in *nkx2.3* morphants. Embryos at 24 hpf were incubated with different doses of SU5402 under dark conditions, with fresh drug buffer being changed every 12 h. Subsequently, the embryos were washed and harvested at 96 hpf for further manipulations. Control embryos were treated with equivalent amounts of DMSO (0231, Amresco).

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Data availability statement

Data will be made available on request.

CRediT authorship contribution statement

Shuyan Yang: Writing – review & editing, Writing – original draft, Project administration, Formal analysis, Data curation, Conceptualization. **Xin Xu:** Writing – original draft, Project administration, Formal analysis, Data curation, Conceptualization. **Zheng Yin:** Methodology, Investigation, Formal analysis, Data curation. **Yuelin Liu:** Methodology, Investigation, Formal analysis, Data curation. **Handong Wang:** Methodology, Investigation, Formal analysis, Data curation. **Jin Guo:** Resources, Methodology, Investigation, Formal analysis, Data curation. **Fang Wang:** Resources, Methodology, Investigation, Formal analysis, Data curation. **Yihua Bao:** Resources, Methodology, Investigation, Formal analysis, Data curation. **Ting Zhang:** Software, Resources, Project administration, Funding acquisition, Formal analysis. **Shaoguang Sun:** Writing – review & editing, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Abbreviations

dpf	days post fertilization
hpf	hours post fertilization
GFP	green fluorescent protein
MO	morpholino
WT	wild-type
CNCCs	Cranial neural crest cells
PPs	pharyngeal pouches
PAs	pharyngeal arches
Ffg	Fibroblast growth factors
BMP	bone morphogenetic protein
RA	retinoic acid
Hh	Hedgehog
WISH	whole-mount RNA in situ hybridization
WB	western-blot
DMSO	dimethylsulfoxide;
m	Meckel's
pq	palatoquadrate
ch	ceratohyal
hs	hyosymplectic
cb	ceratobranchial
hb	hypobranchials
eb	epibranchials
NMD	nonsense-mediated mRNA decay
PTC	premature termination codons

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e21915.

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