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Original Article

Neuregulin 1 is required for the development of enteric neurons and vagus in zebrafish

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Abstract: Hirschsprung's disease (HSCR) is a congenital malformation of the colon characterised by the absence of enteric ganglion in the submucosal and myenteric plexuses. Recently genome-wide association studies have identified that neuregulin 1 (nrg1) gene might be a susceptibility locus for HSCR. This study aimed to investigate whether nrg1 is involved in the development of the enteric nervous system. By in situ hybridization, we revealed that nrg1 was mainly expressed in the mucous layer of the adult zebrafish gut. After been injected with nrg1 morpholino antisense oligonucleotides, zebrafish embryos showed delayed hatching, small head, pericardial edema, shortened-twisted trunk, and impaired motor ability. Some of these defects could be partially ameliorated by the injection of nrg1 mRNA. Further, we observed that knock down of nrg1 resulted in reduced number of enteric neurons (29.5 ± 5.8 vs 51.5 ± 8.3, P<0.01) and aganglionosis (an HSCR-like phenotype), and also affected the development of the vagal innervation of the gut. Taken together, our findings indicated that nrg1 played a critical role in the development of the enteric neurons as well as vagus nerve. Therefore, this work provides an evidence of nrg1 in the pathogenesis of HSCR.

Keywords: Neuregulin 1, Hirschsprung's disease, enteric neuron, vagus, zebrafish, morpholino

Introduction

Hirschsprung's disease (HSCR), also named aganglionic megacolon, is a congenital disorder characterized by the absence of the enteric ganglia along a variable length of the intestine, which can lead to tonic contraction of the affected segment, intestinal obstruction, and massive distension of the bowel [1]. HSCR can be contributed to a failure in the migration of enteric neural crest-derived cells (ENCCS) into the intestine during embryonic development. During normal prenatal development, cells originated from the neural crest migrate into the large intestine to form the enteric nervous system (ENS), which regulates the state of the wall and lumen of the gut and activates intrinsic reflexes that generate complicated and propulsive peristaltic movements [2]. However, in Hirschsprung's disease, this process is incomplete and part of the colon lacks these enteric neurons that regulate the activity of the colon. So the affected segment of the large intestine could not relax and pass stool through the bowel, leading to an obstruction. According to the extent of aganglionosis, HSCR can be classified into short segment (S-HSCR; 80% of the cases), long-segment (L-HSCR; 15%) and total colonic aganglionosis (TCA; 5%). The incidence of HSCR varies greatly by gender and racial, and is most prevalent among Asians (2.8 per 10,000 live births) [3].

Mounting evidence show that HSCR has a complex genetic etiology. A number of susceptible genes, such as RET, GDNF, SOX10, PHOX2B, SIP1, GFRA1, NRTN, NKX2.1, KIAA1279, EDNRB, NTRK3, EDN3, ECE-1, ZFXH1B, and L1CAM have been identified by candidate gene study [4-9]. Considerable literature shows that the RET gene, which encoding a tyrosine-kinase receptor, is the critical HSCR causative gene and its normal expression is crucial for the development of the ENS [10, 11]. Deletion of RET in the mouse or knockdown of RET in zebrafish resulted in complete intestinal agan-

glionosis [12, 13]. However, only 50% of the HSCR familial cases and 5-30% of sporadic cases can be attributed to mutations in the RET coding region, and mutations in other HSCR causative genes mentioned above account for about 7% of HSCR sporadic cases, indicating that there must be additional unidentified loci responsible for HSCR [14].

Recently genome-wide association studies (GWAS) have identified that neuregulin 1 (nrg1) may be a susceptibility locus for HSCR [15-17]. Nrg1 and its receptors, the ErbB family of tyrosine kinase receptors, are expressed in adult intestinal epithelia of both humans and mice, and the loss of ErbB2 is linked with postnatal colonic aganglionosis in mice [18]. However, whether loss-of-function of nrg1 can lead to HSCR has not been confirmed in an animal model. In view of there are many advantages of using zebrafish as a model organism, such as rapid life cycle, optical clarity, low cost to maintain, and rapid external embryonic development, zebrafish were selected in our research. What's more important is that zebrafish nrg1 shared 67% nucleotide identity with human nrg1, the high homology indicted that it might have the same function in zebrafish as it has in human.

In this study, we identified the expression of nrg1 in zebrafish colon and, based on it, we analyzed its function in the development of the ENS by knocking down it with antisense morpholino in zebrafish embryos. Deformities, disturbed growth and development as well as death can be observed by interfering nrg1 expression of zebrafish embryos. In addition, nrg1 can affect the survival, proliferation, migration of enteric neurons. Further study showed that nrg1 is not only involved in the development of enteric neurons, but also in the development of vagal innervation of the gut.

Materials and methods

Animals

Wild-type zebrafish (AB type) were maintained, and embryos were raised according to the *Zebrafish Book*. Zebrafish embryos were kept in a 28.5°C embryo medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, and 0.33 mM $MgSO_4$) and were staged by hours post-fertilisation (hpf) or days postfertilisation (dpf) as described [19]. All experimental procedures were approved by the Animal Care and Use Committee of Huazhong University of science and technology.

Whole mount in situ hybridization

We performed the whole-mount in situ hybridization and immunohistochemistry according to standard protocols. The cDNA clones of neurogenin 1 were used to prepare riboprobes. Primers for amplification of the template cDNA were designed by using the software of Primer Premier 5.0 and NCBI-Primer BLAST. Digoxigenin-labeled RNA probes were synthesized in vitro using a DIG RNA labeling kit (Roche). Embryos were fixed in 4% paraformaldehyde (containing 1‰ DEPC), then the embryos were digested with proteinase K, embryos were prehybridized and incubated in HYB+ containing RNA probe (Promega), then blocked with BSA and incubated with anti-DIG antibody and visualized with NBT/BCIP. The chromogenic embryos were preserved in PBST with 30% glycerol.

Morpholino injections

Nrg1 morpholino antisense oligonucleotides (nrg1-MO) were synthesized by Gene Tools (Philomath, USA). Morpholino oligonucleotides (MO) were diluted in 1× Danieau buffer (58 mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4$, 0.6 mM $Ca(NO_3)_2$, 5.0 mM HEPES pH 7.6) for injection at concentrations shown below. Approximately 1 nl of MO solutions was injected into one- to two-cell stage embryos. For comparison, the same amount of control-MO was injected. The sequences and the concentrations for injections are shown below: nrg1-MO (5'-CTTTGCCTGCTTTCACCTCAGCCAT-3') at 0.5 ng/μl, control-MO (5'-CCTCTTACCTCAGTTACAATTTATA-3') at 0.5 ng/μl. Control/nrg1-morpholino and EGFP-nrg1 lineared vector were co-injected into zebrafish embryos; the strength of green fluorescence was used to evaluate knockdown efficiency.

Assessment of enteric neurons

Immunohistochemistry of the neuronal marker HuC/D was conducted as described to assess the enteric neurons in the distal intestine [20]. Embryos were fixed in 4% paraformaldehyde (containing 1‰ DEPC), then the embryos were digested and washed, anti-Hu MAb 16A11 antibody (Molecular Probes) was added, at last the embryos were incubated by fluorescent sec-

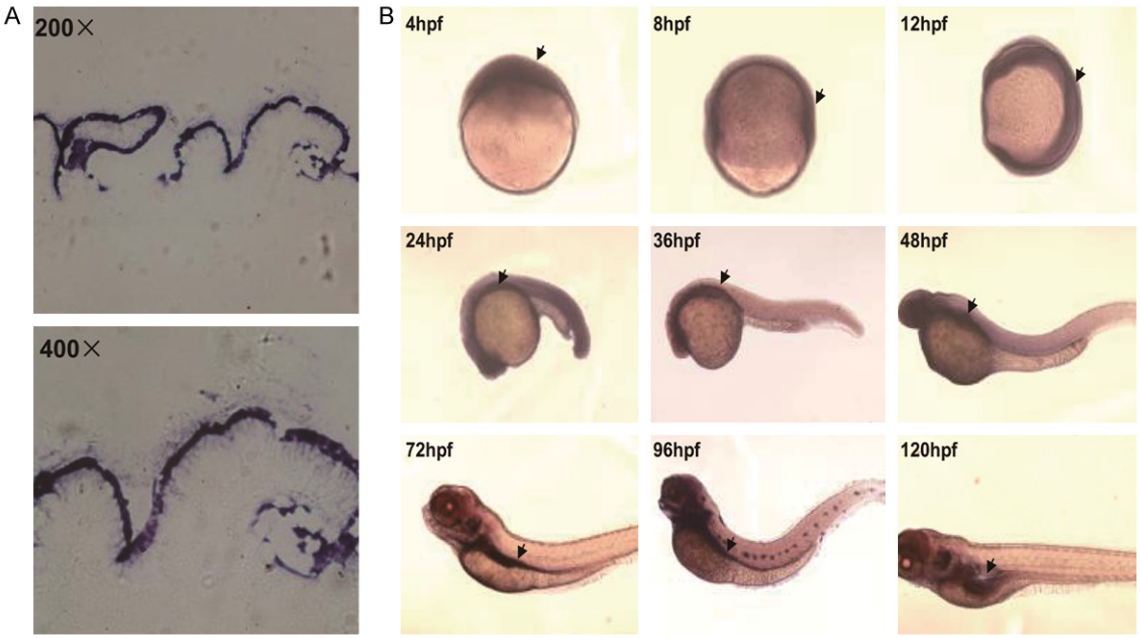

Figure 1. Expression of nrg1 in zebrafish gut. A. Nrg1 expressed in mucous layer, no expression was detected in muscularis and serosa. B. Nrg1 started to express in blastocyst stage (4 hpf). Strong expression was detected in gastrulation stage (8 hpf) and somite stage (12 hpf), mainly located in the animal pole. In pharyngula period (24 hpf, 36 hpf, 48 hpf), nrg1 expression was weak; the nrg1 expression level increased from hatching period (72 hpf, 96 hpf), and started to decrease from 120 hpf. Scale bars = 200 μm.

ondary antibody. Images were captured using a confocal microscope (Nikon). Finally HuC/D positive cells were counted in the 30 μm length of distal-most intestine.

Staining of intestinal vagal nerve fibers

Acetylated tubulin (AcT) was used as a marker of the vagal nerve fibers as described [21]. Embryos were fixed in 4% paraformaldehyde (containing 1‰ DEPC), then the embryos were digested with proteinase K, after washed by PTD (PBS, triton X, DMSO) , acetylated α-tubulin antibody (Sigma) was added, 4°C overnight, at last the embryos were incubated by fluorescent secondary antibody.

Statistical analysis

Data (presented as mean ± SD) were evaluated by Student's t-test and one-way ANOVA with Tukey's post hoc test, respectively. $P<0.05$ was considered statistically significant.

Results

Expression of nrg1 in zebrafish gut

Expression of nrg1 was detected in the mucous layer of adult zebrafish intestine, but absent in muscularis and serosa (**Figure 1A**). As **Figure 1B** shows, nrg1 started to express in blastocyst stage. Strong expression was detected in gastrulation stage and somite stage, which mainly located in the animal pole. In pharyngeal period, the expression of nrg1 was weak, after that the nrg1 expression level increased from hatching period, and started to decrease from 120 hpf.

Function of nrg1 in the development of zebrafish embryo

In this study, control/nrg1-morpholino and EGFP-nrg1 lineared vector were co-injected into zebrafish embryos, the strength of green fluorescence was used to evaluate the knockdown efficiency. Then nrg1-MO or/and nrg1-RNA was/were injected into embryos to observe phenotypes resulted from nrg1 expression changes. As is shown, strong fluorescence expression was detected in 12 hpf embryos injected by control-MO and nrg1-EGFP linearized vector, while embryos injected by nrg1-MO and nrg1-EGFP linearized vector manifested weakened green fluorescence (**Figure 2A**). It demonstrated that nrg1 can be knocked down by nrg1-MO efficiently. Compared with control morpholino-

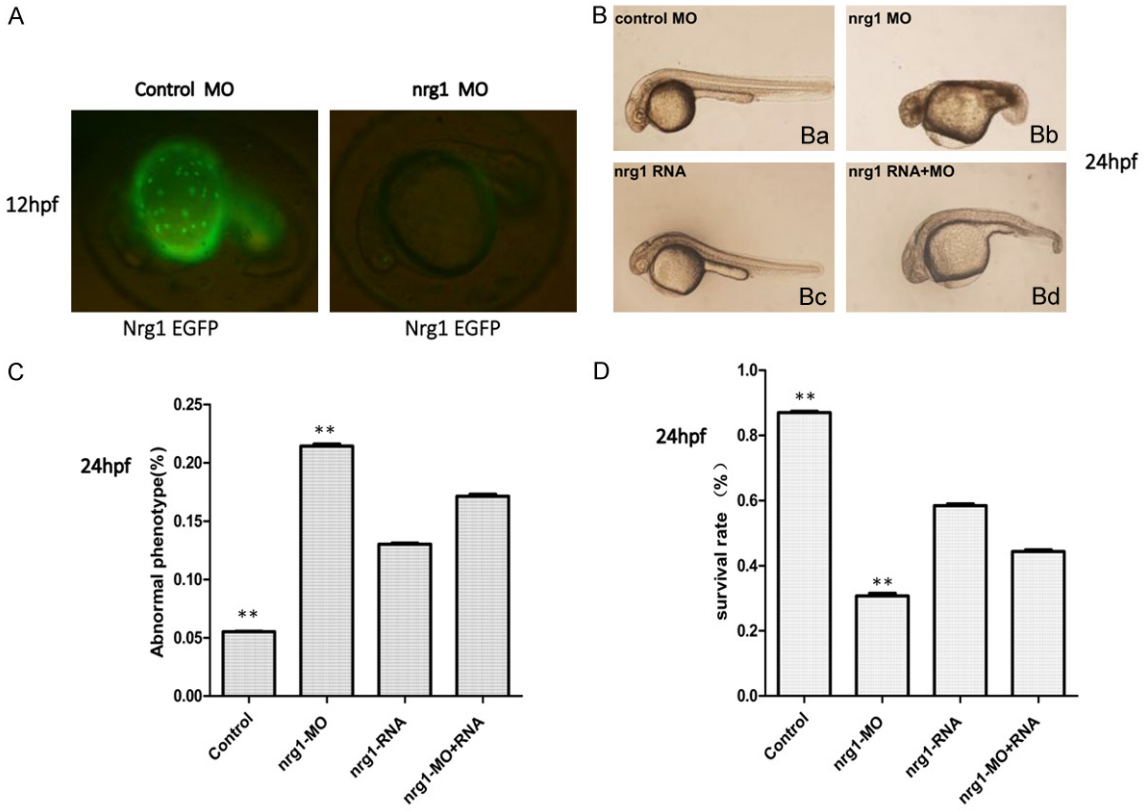

Figure 2. Knockdown of Neuregulin 1 in zebrafish embryo. A. EGFP fluorescence showed that nrg1-MO blocked translation of the Neuregulin 1-EGFP fusion protein at 12 hpf. B. Compared with control MO-injected group (Ba), nrg1 MO-injected embryos (Bb) had smaller head, shorter-twisted trunk and tail. The phenotype could be partially rescued when the embryos were co-injected with nrg1 RNA (Bc). There was no significant phenotypic variation in the nrg1 mRNA group (Bd). Scale bars = 200 μm. C and D. Statistical analysis of abnormal phenotype and survival rate at 24 hpf. All results were represented as the mean ± SD of four separate experiments (40-60 embryos in each experiment). Error bars represent SD. (*P<0.05, **P<0.01).

injected group (**Figure 2Ba**), nrg1-MO-injected embryos had smaller head, shorter-twisted trunk and tail (**Figure 2Bb**). The nrg1-MO phenotype above could be partially rescued when the embryos were co-injected with nrg1 mRNA (**Figure 2Bc**). There was no significant phenotypic variation in the nrg1 mRNA group (**Figure 2Bd**). After been knocked down by nrg1-MO, the lack of neuregulin-1 protein can lead to lower survival rate and higher malformation rate (P<0.01) compared with control group (**Figure 2C** and **2D**).

Nrg1 for the development of enteric neurons

Total missing of enteric neurons were detected in 11.1% (5 in 45) of the nrg1-MO group that mimicked HSCR. For the rest, the knockdown of nrg1 resulted in a decrease in the number of enteric neurons at 120 hpf (29.5 ± 5.8 vs 51.5 ± 8.3, P<0.01) (**Figure 3A**). At 66 hpf, the differentiated enteric neurons were first observed with HuC/D antibody at the anterior region in control and nrg1 knockdown group. Hereafter, in control group, the HuC/D positive cells extended to the posterior region, and the number of the differentiated enteric neurons increased. Knockdown of nrg1 blocked the migration of enteric neurons, especially in the stage the precursors migrating along the intestine (**Figure 3B**).

Nrg1 for the development of vagus in gut

As is shown in (**Figure 4A**), at 120 hpf, intestinal vagal nerve bundles were thick, short and chaotic in nrg1 knockdown zebrafish. At 7 dpf, vagal nerve bundles in control group arranged reticularly and extended into intestinal tract. On the contrary, there was no nerve branch extend-

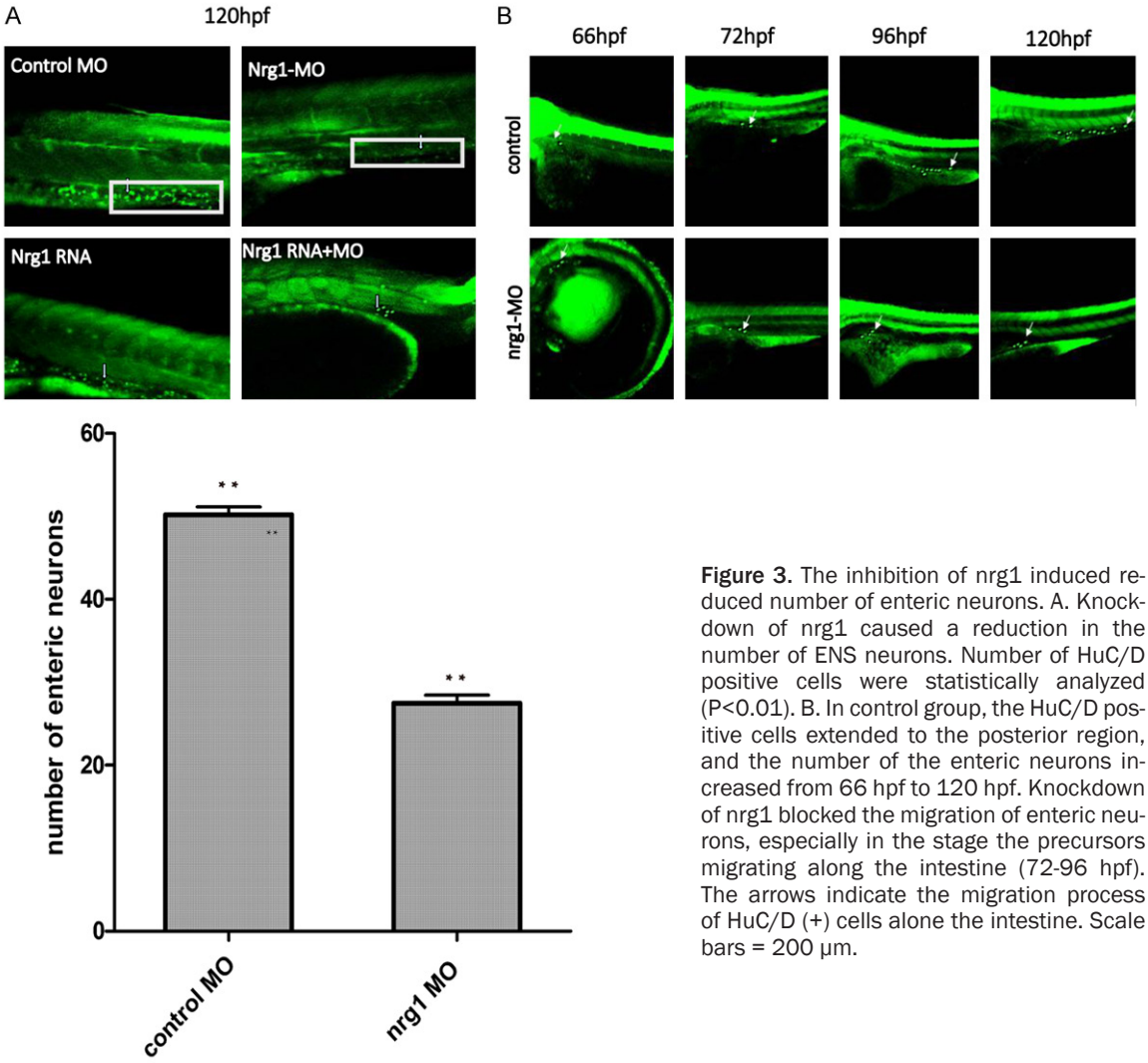

Figure 3. The inhibition of nrg1 induced reduced number of enteric neurons. A. Knockdown of nrg1 caused a reduction in the number of ENS neurons. Number of HuC/D positive cells were statistically analyzed ($P<0.01$). B. In control group, the HuC/D positive cells extended to the posterior region, and the number of the enteric neurons increased from 66 hpf to 120 hpf. Knockdown of nrg1 blocked the migration of enteric neurons, especially in the stage the precursors migrating along the intestine (72-96 hpf). The arrows indicate the migration process of HuC/D (+) cells alone the intestine. Scale bars = 200 μm.

ed into intestinal tract in nrg1 knockdown zebrafish (**Figure 4B**).

Discussion

Neuregulins include a large family of EGF-like signaling proteins that have been implicated in cell-cell communication during development and disease. Nrg1 was the first member of the family to be identified due to diversified biological functions. The nrg1 proteins have been demonstrated to be critical for the development of the nervous system, heart, and mammary glands. Nrg1/Erbb signaling is involved in the survival, proliferation and migration of neural progenitor cells as well as synaptogenesis, myelination, and synaptic plasticity in the nervous systems [22-24]. In addition, nrg1 has also been suggested to be responsible for some mental diseases, such as schizophrenia and bipolar disorder [25, 26]. The dysregulation of nrg1 has been reported to be linked with breast cancer [27]. What's more, nrg1/ErbB signaling is required for cardiac morphogenesis, and it plays an essential role in maintaining the myocardial architecture during adulthood [28]. It is worth noting that nrg1 protein has various isoforms in the light of the N-terminal domains, it may explain the multiple roles of nrg1-ErbB signaling pathway. For example, nrg1 type III is required for normal sensory-motor gating as well as short-term memory [29], while normal synaptic transmissions and behaviors of mouse need proper expression level of nrg1 type I [30]. In zebrafish, nrg1-mediated signaling is critical for the formation of the dorsal root ganglion neurons, specification of oligodendrocytes, as well as the amputation-induced proliferation and migration of vertebrate regeneration [31-33]. In our study, morpholino-mediated

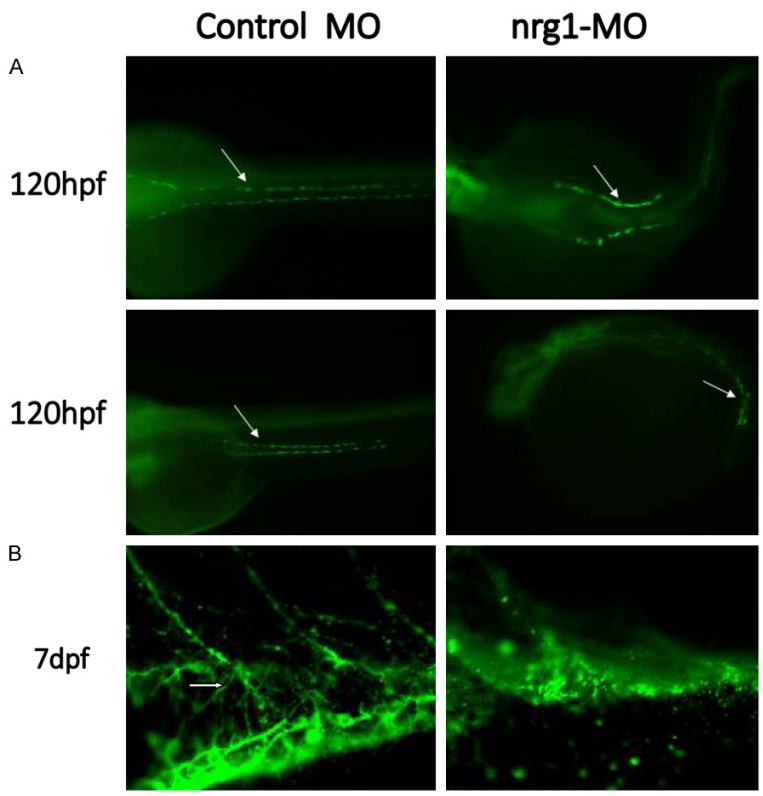

Figure 4. The role of nrg1 in the development of intestinal vagal nerve fibers. A. At 120 hpf, intestinal vagal nerve bundles were thick, short and chaotic in nrg1 knockdown zebrafish. B. At 7 dpf, vagal nerve bundles in control group arranged reticularly and extended into intestinal tract; on the contrary, there was no nerve branch extended into intestinal tract in nrg1 knockdown zebrafish. Scale bars = 200 μm.

knockdown of nrg1 embryos had small head and pericardial edema, indicating that nrg1 was involved in the development of nervous system and heart.

As is well known that HSCR is a congenital malformation of the colon characterised by the absence of parasympathetic intrinsic ganglion cells in the submucosal and myenteric plexuses [9]. Accumulated evidence has shown that nrg1 was implicated in the pathogenesis of HSCR [15, 17, 34]. So what we were most interested in was weather nrg1 is implicated in the development of the ENS. As shown in the current research, loss-of-function of nrg1 by nrg1-MO in zebrafish embryos could lead to aganglionosis, a phenocopy of HSCR in humans. The result demonstrated that nrg1 is involved in ENS development, and the knocking down of it results in a HSCR-like phenotype in zebrafish.

Previous research has suggested that high levels of nrg1 messenger RNA and protein expression were observed in gut tissues of patients with sporadic HSCR [35], by contrast, we found no significant phenotypic variation in the zebrafish embryos injected with nrg1 mRNA. But our findings are consistent with another research that HSCR patients were found to be associated with a significant reduction of the normal nrg1 protein levels [17]. It might be explained that there exist an "optimal" range of nrg1 protein level for the normal development of ENS, but more work should be done to clarify this hypothesis.

Following the finding that nrg1 was involved in the survival and proliferation of enteric neurons, we further explored whether it participated in the development of intestinal vagal nerve fibers. Different from most other vertebrates, the vagus innervates the whole intestine of the zebrafish, which is very important for the intestinal movement. In most cases we will see two major bundles of AcT (+) nerve fibers approaching the intestine at the mid or proximal level, one on either side. The bundles derived from the right and left nodose ganglion respectively, arranged reticularly and extended into intestinal tract, representing the branches of the vagus nerves of the intestine [21]. In our study, the intestinal vagal nerve bundles in nrg1-MO injected zebrafish embryos were thick, short and chaotic, and there was no nerve branch extended into intestinal tract. These results confirmed that nrg1 is critical for the development of the vagal innervation of the gut, maybe it's one of HSCR's pathogenesis. But the exact molecular mechanism remains to be elucidated.

Taken all together, the findings presented in our study reveals that nrg1 is vital for the development of the enteric neurons as well as vagus. The work is also expected to shed light on the HSCR's pathogenesis.

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Disclosure of conflict of interest

None.

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