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Neuregulin 1 is involved in enteric nervous system development in zebrafish

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ABSTRACT

Background: Hirschsprung's disease (HD, also known as congenital colon aganglionosis) is a congenital disorder characterized by the absence of intramural ganglion cells in the distal gastrointestinal tract, which results in tonic contraction of the aganglionic gut segment and functional intestinal obstruction. Recent studies have indicated neuregulin 1 (NRG1) as a new candidate gene involved in the development of the enteric nervous system (ENS) in humans.

Methods: In our study, we investigated the role of NRG1 in zebrafish ENS development by assessing NRG1 expression patterns during ENS development. Knockdown, overexpression and rescue zebrafish models of NRG1 were created to evaluate differences in phenotype, numbers of enteric neurons, ENS-related factors and nerve fiber arrangements.

Results: NRG1 was expressed in zebrafish intestine at both the larval and adult stage. We also found that decreased expression of NRG1 resulted in reductions in enteric neuron number and decreased expression of ENS development markers. Moreover, NRG1-knockdown zebrafish exhibited a disordered arrangement of nerve fibers.

Conclusions: Collectively, these results demonstrated that NRG1 expression might play a role in zebrafish ENS development. In addition, by modulating NRG1 expression, we created a model that may be useful for investigating the mechanism underlying HD pathogenesis.

Aganglionosis, also known as Hirschsprung's disease (HD), is a congenital disorder characterized by the absence of ganglion cells along variable lengths of the distal gastrointestinal tract [1,2]. Aganglionosis is attributed to a failure of neural crest cell (NCC) migration, proliferation, and/or differentiation during enteric nervous system (ENS) development during embryogenesis. Regions of the gastrointestinal tract that lack neurons are known as "aganglionic" areas [3]. During development, NCCs separate from the neural tube and migrate throughout the body before differentiating into neurons, glial cells, cartilage cells and melanocytes [4]. The ENS comprises both neurons and glial cells within the gut walls and regulates intestinal motility, immune function, luminal secretion and blood flow. ENS development in the zebrafish is a conserved process that is simpler than the equivalent process in amniotes [5]. The development process can be divided into two stages: First, NCCs derived from the vagal neural crest migrate into the anterior region of the gut primordium. Second, NCCs migrate from the foregut to the hindgut and then differentiate into enteric neurons and glial cells [6].

Aganglionosis is a multigenic disorder. In recent years, a series of studies has revealed that many transcription factors and signaling molecules/receptors are related to ENS development. The RET/GDNF family receptor alpha 1 (GFRα1)/glial cell derived neurotrophic factor (GDNF) and endothelin receptor-B/endothelin-3 signaling pathways play major roles in the pathogenesis of aganglionosis. The RET gene, encoding a tyrosine kinase receptor, is required for normal ENS development in different species, including zebrafish. Mutations in the coding sequence of RET can explain 50% of the familial cases and approximately 20% of the sporadic cases of HD. More susceptibility loci, such as paired-like homeobox 2B (Phox2B), sex determining region Y-box 10 (Sox10), and Sonic hedgehog (Shh), have been subsequently identified. However, experimental models are needed to investigate the roles of these genes in the pathogenesis of aganglionosis [7].

A genome-wide association study (GWAS) [8] identified that variations in neuregulin 1 (NRG1) increased the risk of RET-mediated disease, and common and rare variants of NRG1 were shown to contribute to aganglionosis [9]. This study also indicated that NRG1 is expressed in intestinal mucosa and ganglions in both humans and

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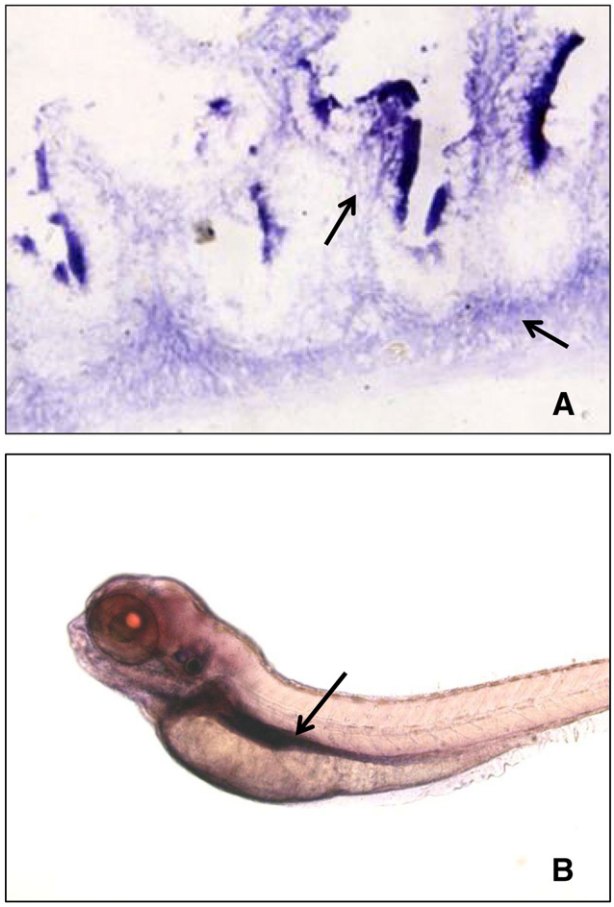

Fig. 1. NRG1 expression in the zebrafish gut. NRG1 expression in an adult zebrafish gut, mainly localized to the epithelium (the left arrow) and the muscularis externa layer (the right arrow) (A). Whole-mount *in situ* embryos hybridized with NRG1 antisense riboprobes showing NRG1 expression in the gut tube at 60 hpf (B).

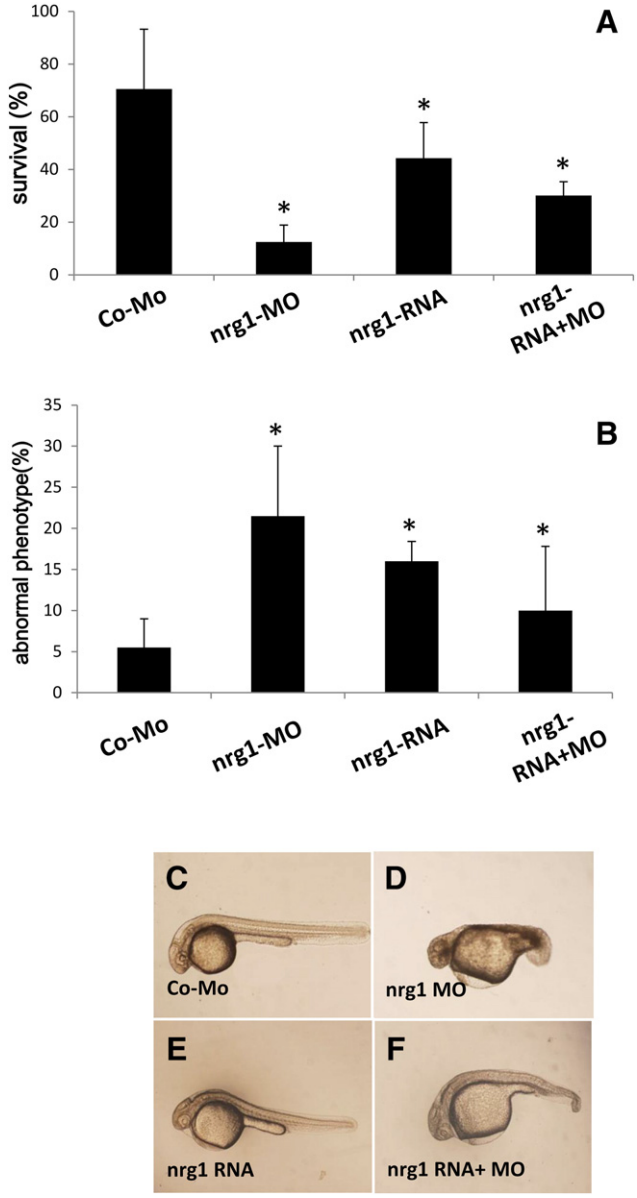

Fig. 2. Lateral views of phenotypes induced by altering NRG1 expression in zebrafish embryos. (A, B) Knockdown of NRG1 resulted in higher mortality and abnormal phenotype rate than in the control group and the rescue group (coinjected with NRG1 morpholino and RNA). Phenotypes were observed in different groups (C, D, E, F). The NRG1 mRNA group (E) and the rescue group (F) exhibited mild abnormal phenotypes compared with the NRG1-knockdown group (D). *$P<0.05$.

mice. The NRG1 ligand ErbB2/ErbB3 is expressed in NCCs and mature intestinal epithelium in mice; and perturbation of ErbB2 function results in ENS defects in mice [10]. These findings implied that a potential relationship between NRG1 and -normal ENS development; however, to date, no *in vivo* study has provided direct evidence of this relationship. In the current study, we demonstrated for the first time that NRG1 is expressed in adult zebrafish intestinal epithelium as well as in related regions of ENS development in embryos. In addition, the expression of ENS-related factors, and nerve fiber arrangement were all affected upon perturbing NRG1 expression, suggesting the critical role of NRG1 in ENS development in zebrafish.

1. Materials and methods

1.1. Zebrafish

Wild-type zebrafish (AB strain) were raised under standard laboratory conditions at 28.5 °C. On the day of fertilization (0 dpf), eggs were collected. Embryos were staged and fixed at specific hours post fertilization (hpf) as previously described [8].

1.2. Plasmids construction and RNA synthesis in vitro

The zebrafish homologue of NRG1 was amplified from adult zebrafish tissue by RT-PCR and subcloned into the pCS2 + plasmid. The constructs were linearized with the restriction enzyme *Xba* I and purified, and the purified NRG1 mRNA transcripts were prepared using a Riboprobe *In Vitro* Transcription System. Zebrafish gene fragments for crestin, ret., gdnf, phox2B, shh and sox10 were amplified from adult tissue with specific primer sets as previously described. The amplified genes were then subcloned into a pGM-T vector and linearized with the restriction enzyme *Spe* I. Digoxigenin-labeled probes were prepared from the linearized constructs using a Riboprobe *In Vitro* Transcription System.

1.3. Microinjection of morpholino antisense oligonucleotides

ATG morpholino antisense oligonucleotides targeting NRG1 were designed and synthesized as follows: NRG1-MO 5′-CTTTGCCTGCT TTCACCTCA GCCAT-3′, Standard Control-Mo (Co-Mo) from GeneTools was used as a non-specific control. Embryonic microinjection was performed at the 1- and 2-cell stage. For morpholino rescue experiments, 1 nl of NRG1-MO morpholino was coinjected into the yolk with NRG1 mRNA (50 ng/μl). Embryos were collected at different stages.