NOX5 is Expressed Aberrantly but Not a Critical Pathogenetic Gene in Hirschsprung Disease

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Abstract

Background:

Hirschsprung disease (HSCR) is a congenital disorder characterized by the absence of intramural ganglion cells in the distal gastrointestinal tract (GI), which results in tonic contraction of the aganglionic gut segment and functional intestinal obstruction. Recent studies have suggested NADPH oxidase 5 (NOX5) as a candidate risk gene for HSCR. In this study, we examined the function of NOX5 to verify its role in the development of enteric nervous system (ENS).

Methods:

HSCR tissue specimens (n = 10) were collected at the time of pull-through surgery and control specimens (n = 10) were obtained at the time of colostomy closure in patients. The NOX5 expression in aganglionic and ganglionic segments of HSCR colon and normal colon were analyzed by immunohistochemistry (IHC), western blot and RT-qPCR. The gene expression levels and spatiotemporal expression spectrum of NOX5 in different development stages of zebrafish embryo were investigated using real-time quantitative PCR (RT-qPCR) and in-situ hybridization (ISH). The enteric nervous system in NOX5 Morpholino (MO) knockdown and wild type (WT) zebrafish embryo was analyzed by whole-mount immunofluorescence (IF). Intestinal transit assay was performed to analyze the gastrointestinal motility in NOX5 knockdown and control larvae.

Results

NOX5 is strongly expressed in the ganglion cells in the proximal segment of HSCR colons and all segments of normal colons. Moreover, the expression of NOX5 is markedly decreased in the aganglionic segment of HSCR colon compared to the ganglionic segment. In zebrafish, NOX5 mRNA level is the highest in one cell stage embryos and it is decreased by the development of the embryos. Interestingly, the expression of NOX5 appears to be enriched in the nervous system. However, the number of neurons in the GI tract and the GI motility were not affected upon NOX5 knockdown.

Conclusions

NOX5 may play a role in the early development of zebrafish embryo, but not required for the ENS development and loss of NOX5 didn't affect the GI motility in zebrafish. Nevertheless, we demonstrated that NOX5 specifically expressed in the ganglionic cell in colon tissue and markedly decreased in the aganglionic segment.

Background

HSCR is caused by failures of the proliferation, differentiation or migration of enteric neural crest cells (ENCCs), resulting in aganglionosis of the distal part of the gastrointestinal tract (1). According to the length of the aganglionic segment, it can be further classified as short segment HSCR, long segment

HSCR, total colonic aganglionosis (TCA) and total intestinal aganglionosis (2). The aganglionosis then leads to severe intestinal obstruction, which requires surgical removal of the aganglionic segment to treat (3).

HSCR is a highly heritable disorder (OMIM 142623). Familial and syndromic HSCR show a Mendelian pattern of inheritance. However, the etiology of the sporadic HSCR seems to be intricate, presenting a non-Mendelian type inheritance and involving many genetic and environmental factors (4). So far, more than 20 HSCR susceptibility genes have been found to be associated with the development of ENS (5, 6). The reduced RET (encoding a tyrosine kinase) expression is the major risk of HSCR (6). The RET/GDNF/GFRA1 signaling pathway and the Endothelin 3-Endothelin Receptor B signaling pathway are the most common known pathways involved in HSCR development. Besides, transcription factors like SOX10, PHOX2B, ZEB2 etc. are also playing important roles in the development of ENS (4). However, only approximately 30% of all HSCR patients carry mutations in these genes, suggesting that there must be many other genes participating in the etiology of HSCR.

In the last few years, lots of novel susceptibility genes and variants for HSCR were identify by genome-wide association studies (GWAS) (7, 8). In a recent GWAS of HSCR, NOX5 was identified as a susceptibility gene (9). Furthermore, a follow-up association analysis between NOX5 polymorphisms and risk of HSCR indicated that the hereditary variants in NOX5 were significantly associated with HSCR susceptibility (10).

NOX5, which belongs to the NADPH oxidase family, is one of the major producers of reactive oxygen species (ROS) in mammalian cells (11). NADPH oxidases are membrane proteins that generate superoxides, particularly ROS, which have been shown to be participated in various signaling cascades and cellular processes including proliferation, apoptosis and migration. Dysfunction of the NOX enzyme could lead to abnormal levels of ROS that may cause disease (12). Previous studies have demonstrated that Nox5 is involved in various pathological conditions, including cancer, cardiovascular and atherosclerotic diseases (13-15). However, no in vivo or in vitro study has provided immediate evidence that NOX5 is required for the development or function of the ENS. In this study, we aimed to test whether loss of NOX5 would lead to the disruption of the biological processes of enteric neurons by using zebrafish.

Methods

Tissue collection

This study complied with the Declaration of Helsinki and was approved by the Review Board of Ethics Committee of Tongji Hospital. Consent forms were sent to patients and signed by patients or their legal custodians. Full-length resected bowel specimens obtained during pull-through operations for HSCR were collected from ten patients. Three of these patients had a history of preoperative HAEC. Resected tissues included aganglionic and ganglionic segments. Ganglionic segments were taken from the most proximal margin of the resected pull-through specimen while aganglionic segments were taken from the most

distal margin of the resected specimen. Control colonic specimens were obtained from the proximal colostomy limb at the time of stoma closure in patients with imperforate anus (n = 10). Tissue specimens were stored in three ways following collection. One portion of each specimen was fixed in formalin at room temperature, for paraffin embedding and immunochemistry. A second portion was snap frozen in a mold containing optimal cutting temperature medium and stored at - 80 °C for immunofluorescence. The remaining specimen was stored at ⬚80 °C for protein or total RNA extraction.

Protein extraction and Western blot

Each protein sample of zebrafish was extracted from 30 embryos. The western blot was then performed as previously described (16). The rabbit anti-NOX5 antibody (Abcam, Cambridge, UK, ab191010) was used at a concentration of 1:1000. The rabbit anti-beta III Tubulin (Tuj1) antibody (Abcam, Cambridge, UK, ab18207). The HRP linked goat anti-rabbit secondary antibody (Abcam, Cambridge, UK) was used at 1:10,000 dilution. Beta actin (dilution 1:1000, Abcam, Cambridge, UK) was used as the loading control. The relative level of protein was determined by the normalized density of each band in the western blot using the ImageJ software.

Immunohistochemistry

Sections (4 μm) on silane-coated slides (Muto Pure Chemicals Co., Ltd., Tokyo, Japan) were deparaffinized in xylene and dehydrated in solutions with decreasing concentrations of ethanol. After rehydration and blocking of endogenous peroxidase activity with 3% of hydrogen peroxide for 10 min, heat-induced epitope retrieval was performed for 20min in 0.01M citrate buffer (pH 6.0) in a pressure cooker. Primary antibody for NOX5 was used at 1:100 and incubated for 30 min. After washing and incubation with EnVision™ for 30 min, color products were developed using the Liquid DAB+ as chromogen. The sections were counterstained with hematoxylin before dehydration and coverslipping. Slides processed without primary antibody were prepared as negative controls.

Zebrafish housing/breeding

The zebrafish protocols were approved by the Institutional Animal Care and Use Committee at Tongji Hospital. The AB strain zebrafish were maintained according to standard procedures(17). Embryos were raised in E3 medium at 28.5°C and staged as previously described(18). 0.003% N-phenylthiourea was added to E3 medium to inhibit melanization.

Real-time quantitative PCR

Total RNA was isolated from 0.2, 6, 12, 24, 48, and 72hpf embryos and colon tissues with TRIzol reagent (Life Technologies, Carlsbad, CA). RT-qPCR and data analysis were performed using LightCycler96 (Roche Diagnostics). Relative expression levels were calculated using β-actin as internal reference. The experiments were repeated three times with biological replicates. Zebrafish primers (NOX5 primer: Forward, 5'- ATT CACGGCACT GAAACGGA-3', Reverse, 5'-GGAGCTCCGCATGATT TACCT A-3'; b-actin primer: Forward, 5'-CGAGCTGTCTTCCCATCCA-3', Reverse, 5'-TCACC AACGTAGCTG TCTTTCTG-3').

Human primers (Tuj1 primer: Forward, 5'- GGA AGAGGGCGAGATGTACG-3', Reverse, 5'-GGGTTTAGACACTGCTGGCT-3'; b-actin primer: Forward, 5'- CCTTCCTGGGCATGGAGTC-3', Reverse, 5'-TGA TCTTCATTGTGCTGGGTG-3'. NOX5 primer: Forward, 5'-CCAGAAAGTGGCTG CTGAGA-3', Reverse, 5'-AGCTTGGAGAGGTGAGGCTA-3)

Whole-mount in situ hybridization

The 0.2, 6, 12, 48hpf Embryos (n=15 for each phase) were collected and processed for whole-mount ISH as previously described(19). Embryos

A 735-bp fragment of the NOX5 cDNA was amplified using the following primers: Forward: 5'-CGGAGGTCTCTGGATCATGC-3', Reverse: 5'-ATGTGCAGCCACAA CGTTTC-3'. A T7 promoter was added to the reverse primer. The ISH probe was then generated by in vitro transcription using T7 RNA polymerase.

Microinjection of morpholino antisense oligonucleotides

NOX5 knockdown experiments using MO were carried out as previously described (20). ATG morpholino antisense oligonucleotides targeting NOX5 were designed and synthesized as follows: NOX5-MO 5'-CGGGTGTCATCATCCAGACTCAT-3', a 5-nucleotide-mismatch morpholino was used as control: 5'-CGGcTGaCtTgATCCAcAC TCAT -3'. Zebrafish embryos were injected with 5ng of the MO at the one cell stage. The knockdown efficiency was validated using western blot.

Whole-mount Immunofluorescent Staining

Whole-mount Immunofluorescent staining with the anti-HuC/D antibody (A-21271, Life Technologies) was performed to examine the enteric neurons along the GI tract. The 5dpf embryos (n=20) were collected and fixed with 4% PFA overnight. The embryos were washed with PBS. After incubation in blocking solution (2% goat serum, 2mg/ml BSA in 1 x PBS) for 1 hour at room temperature, embryos were incubated with the anti-HuC/D antibody (1:500) in blocking solution overnight at 4 °C. After two washes in PBS for 10 min each time, embryos were incubated in the secondary antibody solution, 1: 1,000 Alexa Fluor rabbit anti-mouse IgG (A11001, Life Technologies) in PBS, for 1 hour at room temperature. Finally, the images were acquired using LSM 800 confocal microscope (Zeiss, Germany). The number of HuC/D-positive cells in the gut was then quantified using ImageJ. All of the experiments were repeated for three times.

Zebrafish intestinal transit assay

The tracer was prepared by mixing 100 mg of egg yolk, 150 μL of yellow-green fluorescent 2.0-um polystyrene microspheres (Invitrogen, Carlsbad, CA, USA) and 50 μL of deionized water as previously described (21). For 7dpf zebrafish larvae (n=65 for Control, n=75 for NOX5-Mo), approximately 2 mg of tracer powder was administered per Petri dish in the morning. After 3, 6, and 9 hours, the larvae were anaesthetized by 0.2%. tricaine (Sigma, St Louis, MO, USA) and imaged using a fluorescent dissecting microscope (Axio Zoom.V16, Zeiss, Germany). For scoring the transit efficiency, the zebrafish intestine

was artificially divided into four zones according to anatomical landmarks and the larvae was grouped based on the anterior extent of the tracer.

Statistical analysis

The embryos were selected by Simple random sampling. Data were analyzed using the GraphPad Prism software package (version 5; GraphPad Software Inc., La Jolla, CA, USA) and are presented as the mean ± standard error of the mean. Differences between two groups were analyzed using an unpaired t-test with Welch's correction. Analysis of variance (ANOVA) was used to compare data of more than two groups. Pearson's chi-square tests were used to assess the difference between 7 dpf wild-type and NOX5-MO group transit profiles at different time points. The experiments and data analyzing were finished by different researchers. The analyst didn't know the grouping scheme in advance.

Results

NOX5 is located in the enteric neuron membrane.

We first performed western blots and RT-qPCR of NOX5 in the aganglionic segments and the ganglionic segments in 10 patients with HSCR and 10 normal colon samples. The results shown that NOX5 is indeed expressed in the colon. Interestingly, NOX5 levels in the aganglionic segment of HSCR colon are markedly decreased compared to ganglionic segment and controls, whereas its expression levels in the ganglionic segments are not significantly altered (Fig 1-2). Besides, the expression of NOX5 is similar to Tuj1, which is the specific neuron marker in ENS. Next, we performed IHC and HE staining to examine the expression profile of NOX5 in colon. NOX5 can be observed in the ganglion cells in the myenteric and submucosa plexus of both the HSCR and control colons. However, NOX5 expressed hardly in aganglionic segments (Fig 3). Collectively, these data show that NOX5 is highly expressed in the ganglionic neurons of the colon, suggesting that it may play certain roles in these cells.

Expression pattern of NOX5 in zebrafish embryos

To analyze the function of NOX5 in vivo, we chose to use zebrafish as a model system. We performed ISH to determine NOX5 expression pattern in different stage (0.2, 6, 12, 24, 48, 72hpf) of zebrafish embryos. NOX5 is a maternal factor which exhibits a uniform expression pattern during the early stages of embryonic development (Fig 4 A-C). After 24hpf, it is mainly expressed in the central nervous system, the origin of enteric neurons (Fig 4 D-E). In addition, to qualify the relative level of NOX5 in different stages, we performed Rt-qPCR analyses and revealed that NOX5 expression is the highest in the one-cell stage and is decreased in the following stages until reaching plateau at around 48 hpf to 72 hpf (Fig 5). Since the zebrafish enteric neurons are differentiated and migrated to their final location within the first 5 days of embryonic development, our data indicate that NOX5 is indeed expressed during the period of enteric neuron development.

NOX5 is not required for zebrafish ENS development

To test whether zebrafish ENS development require NOX5, we designed a MO to knockdown NOX5 expression level. Upon injection of 5 ng NOX5-MO at one cell stage, the protein levels of NOX5 were significantly reduced until 5 dpf (Fig SF1). We then used HuC/D immunofluorescence staining to detect the enteric neurons in 5 dpf embryos. The results showed that the number of enteric neurons in NOX5 morphants are not significantly altered compare with controls (Fig 6A-C). Moreover, the gut morphology was indistinguishable between the morphants and the controls. Thus, our data indicate that NOX5 is dispensable for enteric neuron system development in zebrafish.

GI transit function is not affected by NOX5 knockdown

We further tested whether NOX5 is required for GI transit function by feeding larvae fluorescent microspheres in an emulsion of egg yolk and examining the microsphere transit through the GI at various time points. Upon feeding, both the control and NOX5 knockdown group larvae were anaesthetized and photographed at 3, 6 and 9 h. For quantification, we divided the whole GI into four zones and calculated the number of larvae with the tracer in each zones (Fig 7A). Representative images of the tracer location in control and morphant at different time points are displayed in Fig 7B. There is no significant difference of the tracer patterns between the two groups of embryos (Fig 7C, 3 h, $p = 0.32$, 6 h, $p = 0.49$, 9 h, $P = 0.59$). These data suggested that NOX5 is not required for ENS function or GI transit in zebrafish embryos.

Discussion

HSCR is a highly heritable disorder (22). Previously, a genome-wide association study (GWAS) with 123 sporadic HSCR patients and 432 unaffected controls identified NOX5 as a new susceptibility gene for HSCR (9). Furthermore, an association analysis between NOX5 polymorphisms and risk of HSCR in 187 patients and 283 unaffected controls showed that the genetic variants in NOX5 were significantly associated with HSCR susceptibility, particularly for the L-HSCR and TCA subtypes (10). Encouraged by these findings, we embarked on elucidating NOX5 function in ENS.

NADPH oxidases (Nox), comprising seven family members (Nox1-Nox5 and dual oxidase 1 and 2), are the major producers of reactive oxygen species in mammalian cells. NOX5 was first reported in 2001 based on a blast search using the C-terminus of gp91[phox] as bait to identify novel transcripts (23). The exact pathophysiological significance of NOX5 remains unclear, but it seems to be important in the physiological regulation of sperm motility, vascular contraction and lymphocyte differentiation, and NOX5 hyper activation has been implicated in cardiovascular disease, kidney injury and cancer (24). One of the distinguishing features about NOX5 is its dependence on Ca^{2+} for its regulation (23). Activation of NOX5 in response to elevated Ca^{2+} is a multi-phased process (25). The amount of Ca^{2+} necessary to activate NOX5 fully is high, and accordingly, additional systems involving regulatory proteins are operational that increase sensitivity to Ca^{2+}, thereby facilitating ROS generation. Hence, NOX5 can be activated directly by Ca^{2+} or indirectly by interacting with other proteins and kinases, such as Ca^{2+}-bound calmodulin or PKC (26). Intriguingly, NOX5 is the first and only NADPH oxidase to be crystallized,

providing opportunities to design specific NOX5 inhibitors and activators, which is crucial for biomedical research and potentially for therapeutic utility (27).

We used zebrafish as the animal model to explore the function of NOX5. The result of spatiotemporal expression spectrum of NOX5 in zebrafish embryo indicated that NOX5 might play a role in the early development of zebrafish, which is similar to the previous study about the expression of NOX family in zebrafish (28). However, after completely knockdown the NOX5 protein, our results showed that there no difference in HuC/D positive neurons number of GI between the normal group and the NOX5 knockdown group, indicated that the development of ENS in zebrafish did not require the NOX5. Besides, the results of GI transit assay suggested that the GI motility also did not affected by the absence of NOX5 protein. Generally, GI motility is affected by enteric neural crest cells that form the ENS and undergo extensive migration from the caudal hindbrain to colonize the total GI tract (29). The results of the in vivo study in zebrafish showed that the loss function of NOX5 could not cause the absence of enteric neuron in zebrafish, which is the most important characteristic of HSCR.

However, our data of IHC showed that the expression of NOX5 located on the enteric neuron membrane. RT-qPCR, western blot showed that NOX5 strongly expressed in the myenteric ganglionic cell in the proximal and normal segment of HSCR colon and hardly expressed in the distal segment of colon with the absence of ganglionic cell. On the one hand, abnormal distribution of ICCs had been observed in the aganglionic segment colon of HSCR patients (1). On the other hand, cell migration, contraction and proliferation cannot complete without $Ca2^+$-dependent processes, and this is particularly pertinent to NOX5, because NOX5 itself is regulated by $Ca2^+$ (23). Moreover, in bowel motility, $Ca2^+$ is regulated by ICCs, which are essential to generate and propagate the electrical cyclical activity (slow waves) in the intestines. Therefore, we hypothesize that the aberrant expression of NOX5 in aganglionic segment may occur secondary to abnormal release of $Ca2^+$. More importantly, our study demonstrated for the first time that NOX5 expresses in the ganglionic cells specifically in colon tissue in this current study. NOX5 may serve as a typical neuron marker for enteric ganglionic cell to determine the occurrence and development of HSCR. It should be noted that the balance between $Ca2^+$ and NOX5 was destroyed, which may lead to further deterioration of spasm in distal segment of HSCR patients. In other words, abnormal release of $Ca2^+$ results in the decreased expression of NOX5 through an unknown positive feedback mechanism, which persistently cause anomaly concentration of $Ca2^+$. In other words, abnormal release of $Ca2^+$ result in the decreased expression of NOX5 through an unknown positive feedback mechanism, which persistently cause anomaly concentration of $Ca2^+$.

However, the variation regularity of NOX5 have not been found in ENS. The association between NOX5 and $Ca2^+$ remain unclear and further studies are required to elucidate the decreased level of NOX5 in the aganglionic segment colon of HSCR patients.

Conclusions

Our findings show that NOX5 may play a role in the early development of zebrafish embryo, but not required for the ENS development and loss of NOX5 did not affect the GI motility in zebrafish. Nevertheless, we demonstrated that NOX5 specifically expressed in the ganglionic cell in the colon tissue and markedly decreased in the aganglionic segment.

Abbreviations

HSCR: Hirschsprung Disease

GI: gastrointestinal

NOX5: NADPH oxidase 5

ENS: enteric nervous system

MO: Morpholino

Declaration

Ethics approval and consent to participate

Consent for publication

Not applicable.

Availability of data and materials

Data sharing is not applicable to this article as no datasets were generated or analysed during the current study.

Competing interests

The authors declare that they have no conflict of interest.

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"Jiexiong Feng" provided the experiment funds of this research and interpreted the data and revised the manuscript.

Authors' contributions

JW and JX carried out all experiments, acquired and interpreted the data, and wrote the manuscript. XFC and XYM breeding the zebrafish. DDZS and YJC collected the human colon samples. JXF and BX conceived of the study, acquired and interpreted the data, and obtained funding for the studies presented. All authors read and approved the final manuscript.

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Figures
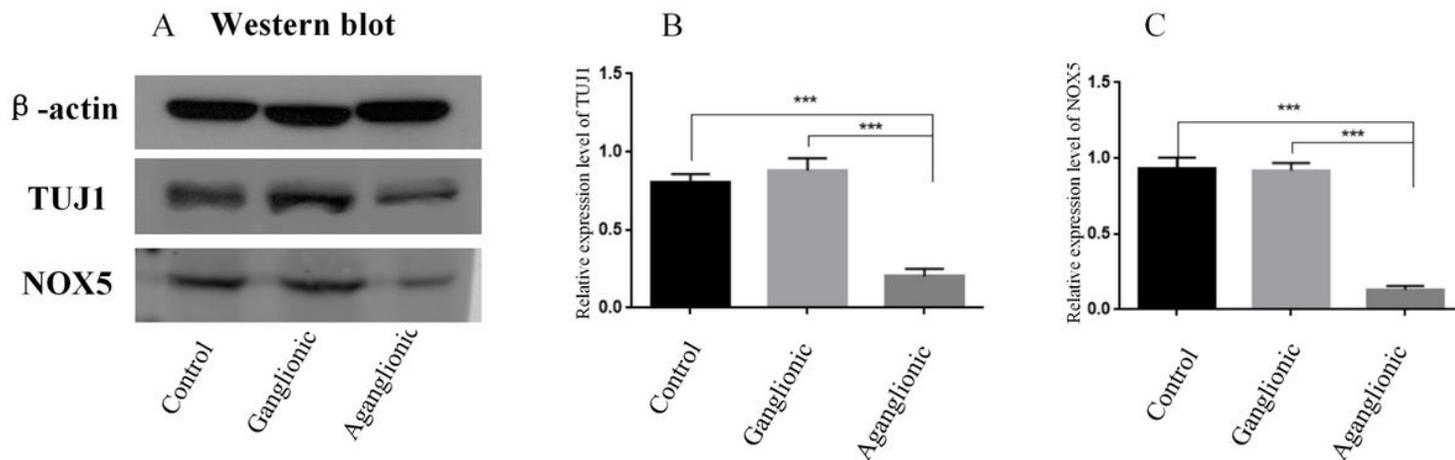
Figure 1

Western blotting revealed significantly increased protein expression levels of NOX5 in the ganglionic HSCR specimens (n = 10) compared to aganglionic and normal control tissue (n = 10). Equal loading of electrophoresis gels was confirmed by ACTIN staining (p < 0.01 by one-way ANOVA).
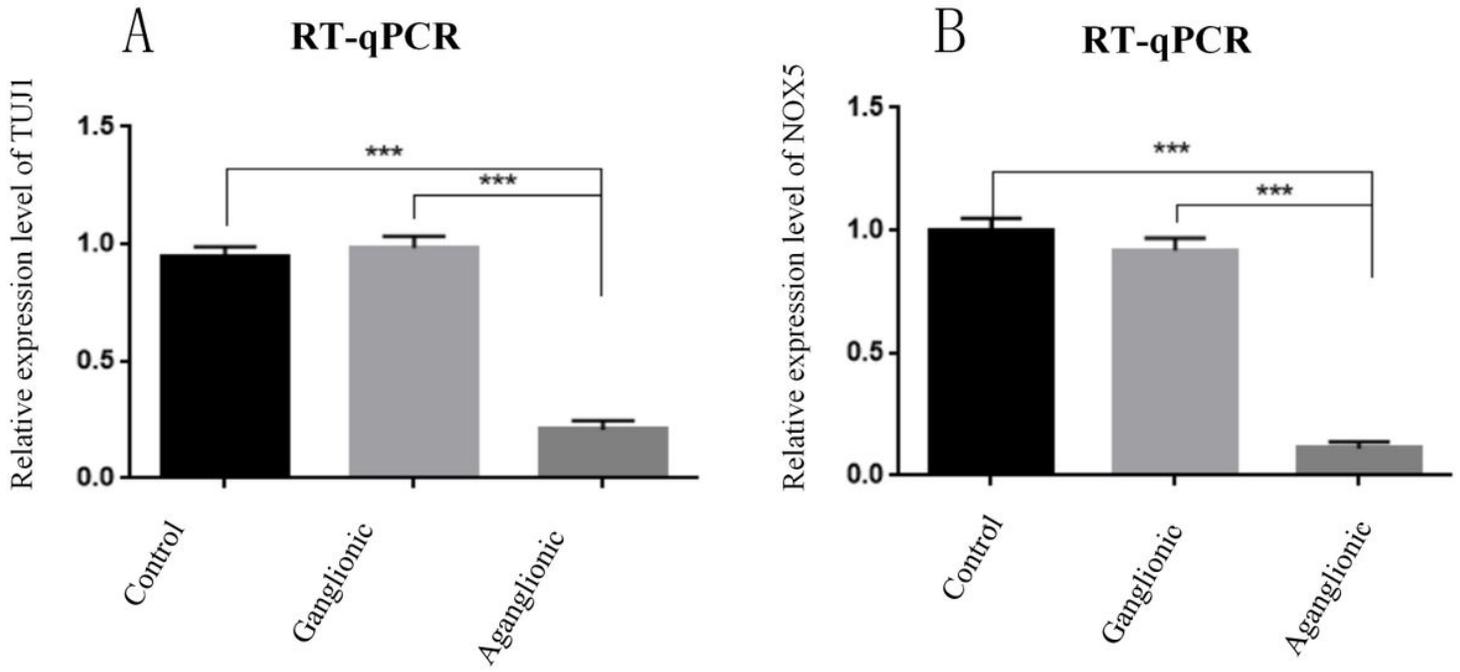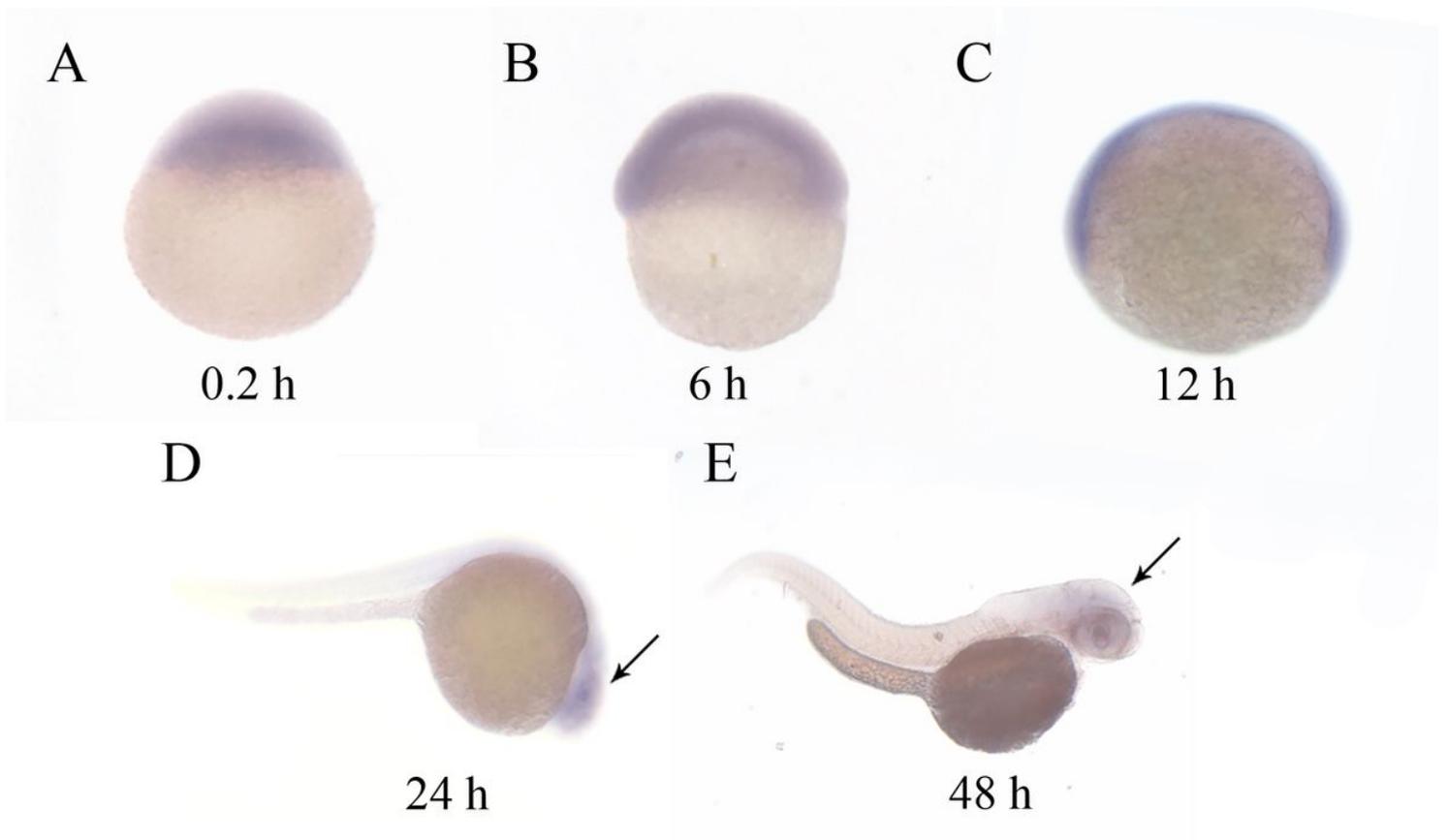
Figure 2

NOX5 antibodies positively stained ganglion cells (arrows) in the submucosal and myenteric plexus of the ganglionic (A, D, ×100) and normal colon (C, F, ×100) but not aganglionic colon (B, E, ×100). Each ISH section have a correspondent HE staining slide.
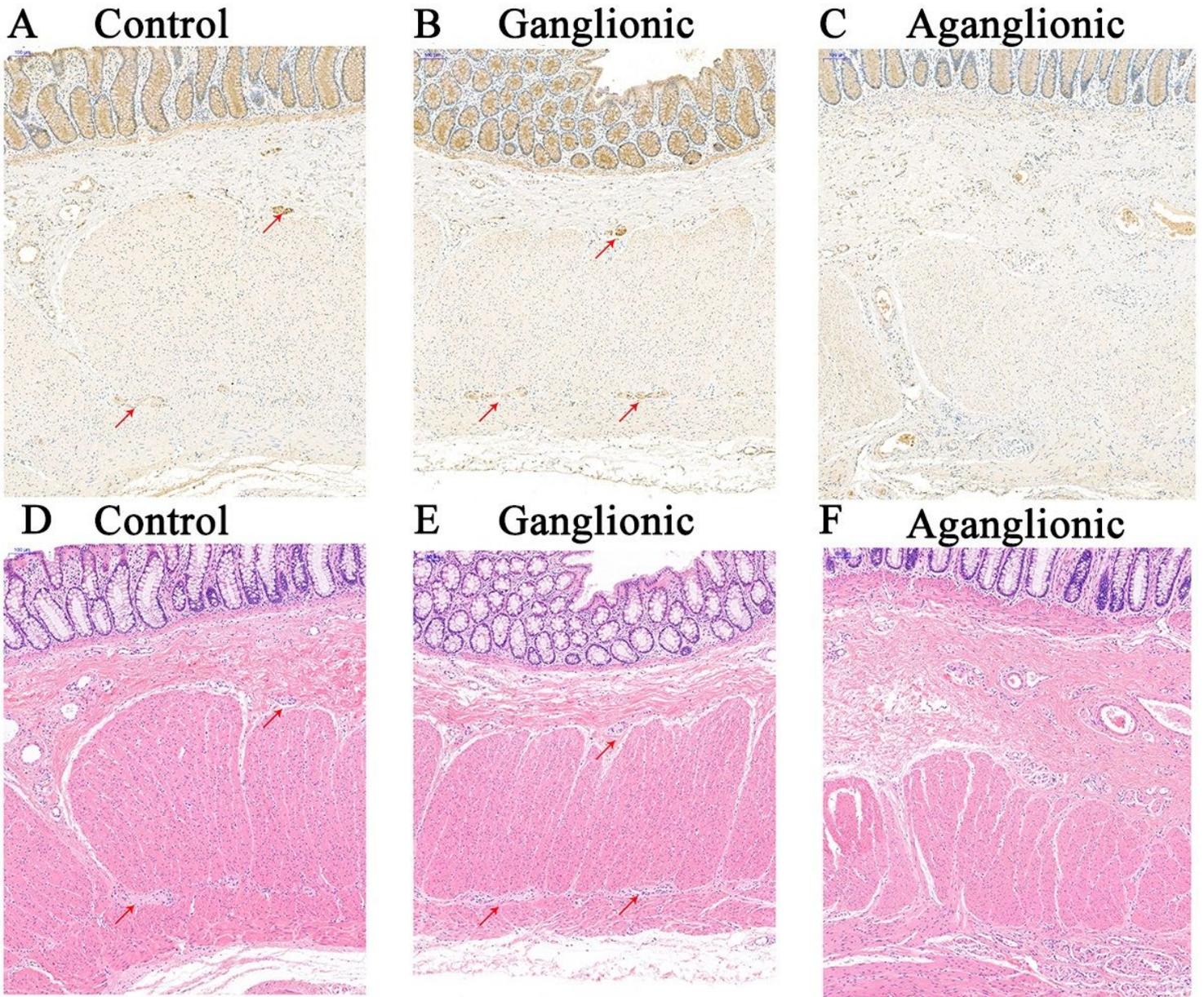
Figure 3

Broad NOX5 expression through the first 2 days of development. A-E: 0.2, 6, 12, 24, and 48 hpf. D-E: Lateral views of whole-mount ISH embryos probed with NOX5 antisense show a relative specific positive expression in central nervous system.

Figure 4

NOX5 expressed during early zebrafish development. Relative mRNA levels of NOX5 derived via quantitative PCR normalized to ACTIN. N=6 for each data point.
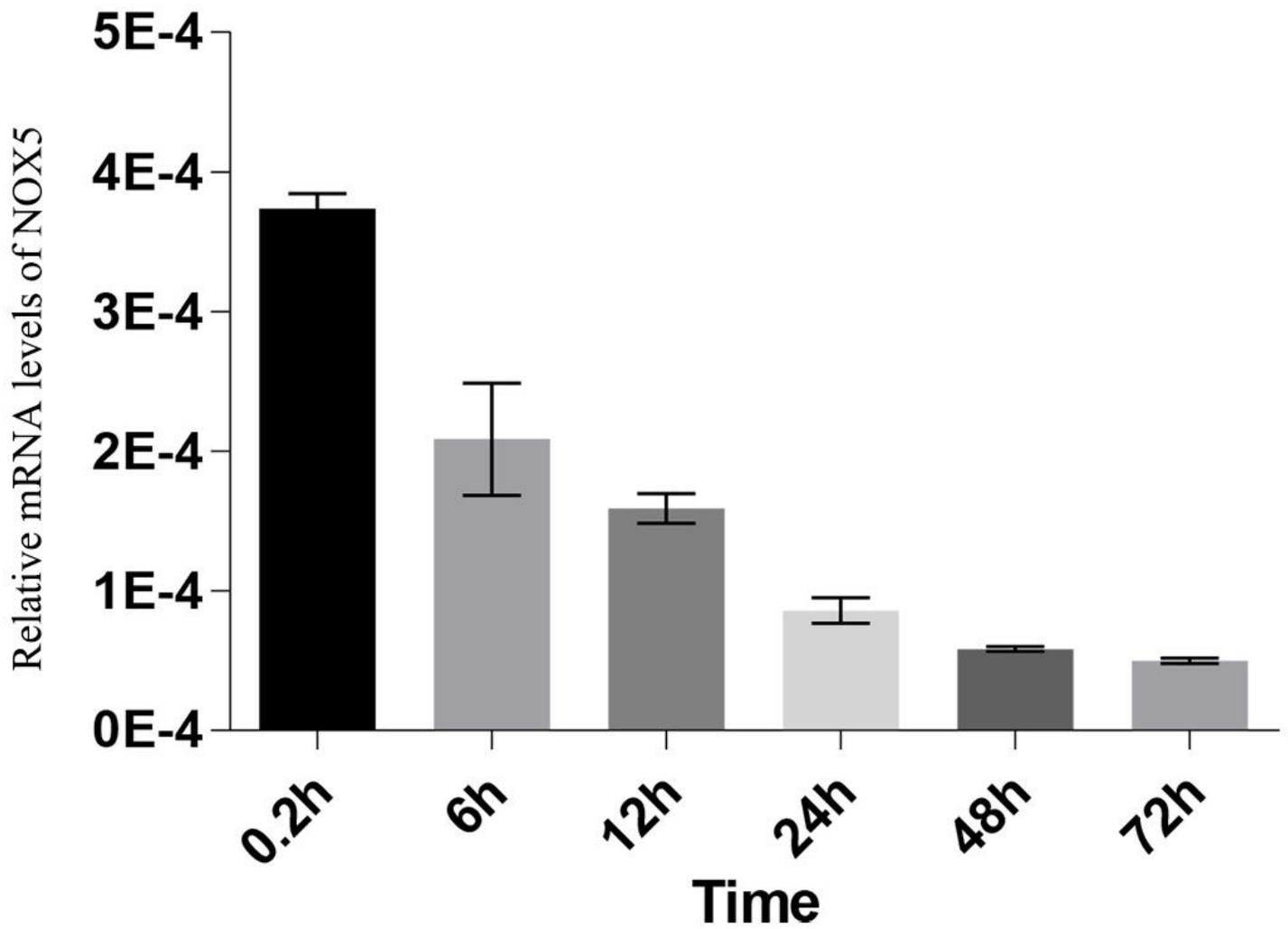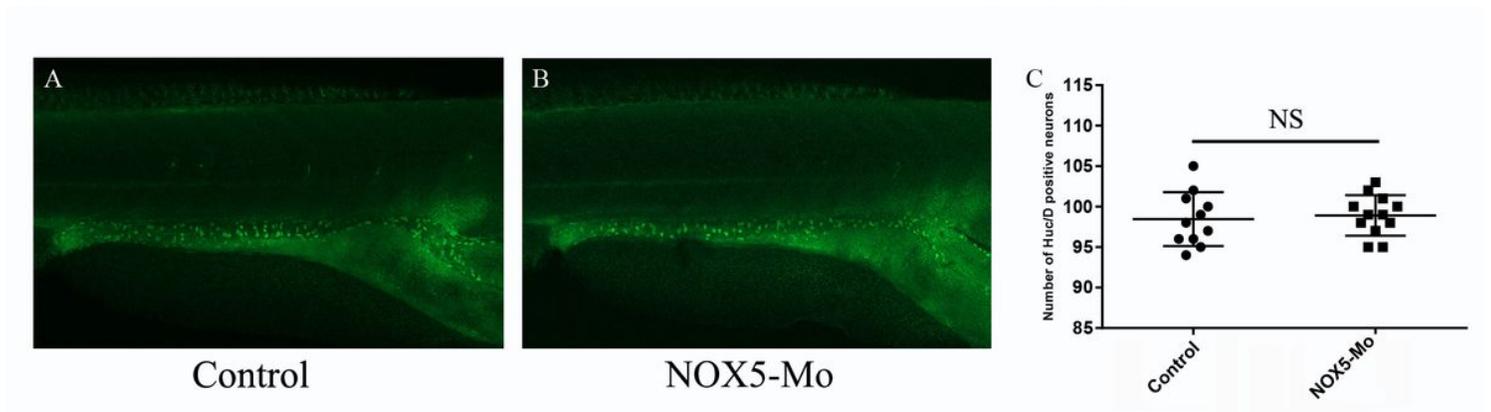
Figure 5

Immunostaining with HuC/D at 5dpf show no difference between control group (A) and NOX5-MO group (B). No statistical difference in HuC/D+ neuron numbers between two groups (C).

Figure 6

To evaluate intestinal transit at the population level, we categorized individuals within a population based on the location of tracer in the gastrointestinal tract. (A) Regions of the gut are divided to 4 zones based on anatomical landmarks. Zone 1 is the region of the intestinal bulb rostral to the swim bladder. Zone 2 is the region of the intestinal bulb ventral to the swim bladder. Zone 3 is the junction between the intestinal bulb and the mid intestine. Zone 4 consists of the mid- and posterior intestine. e, eye; sb, swim bladder. (B) Larvae were scored as zone 1, zone 2, zone 3, zone 4 and empty based on the anterior extent of tracer in the gastrointestinal tract (arrow). (C) Bar graphs representing the transit profile of a population of 7 dpf larvae at 3 h, 6h, 9h of transit time. Data are represented as the number of larvae classified into each zone category based on visual analysis of live larvae at the time specified. The difference of transit profiles of all time points between two groups show no statistically significant ($P > 0.05$).

Supplementary Files

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