Journal of Pediatric Surgery (2009) **44**, 1904–1912

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Journal of Pediatric Surgery

www.elsevier.com/locate/jpedsurg

Transcriptional regulation of *RET* by Nkx2-1, Phox2b, Sox10, and Pax3

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Received 22 October 2008; accepted 25 November 2008

Key words:
RET;
Nkx2-1;
Phox2b;
Transcription

Abstract

Background: The rearranged during transfection (*RET*) gene encodes a single-pass receptor whose proper expression and function are essential for the development of enteric nervous system. Mutations in *RET* regulatory regions are also associated with Hirschsprung disease (HSCR) (aganglionosis of the colon). We previously showed that 2 polymorphisms in RET promoter are associated with the increased risk of HSCR. These single nucleotide polymorphisms overlap with the NK2 homeobox 1 (Nkx2-1) binding motif interrupting the physical interaction of NKX2-1 with the *RET* promoter and result in reduced *RET* transcription. In this study, we further delineated Nkx2-1–mediated *RET* Transcription.

Methods and results: First, we demonstrated that PHOX2B, like SOX10 and NKX2-1, is expressed in the mature enteric ganglions of human gut by immunohistochemistry. Second, subsequent dual-luciferase-reporter studies indicated that Nkx2-1 indeed works coordinately with Phox2b and Sox10, but not Pax3, to mediate *RET* transcription. In addition, identification of Phox2b responsive region in *RET* promoter further provides solid evidence of the potential functional interaction between Phox2b and *RET*.

Conclusion: In sum, Phox2b and Sox10 act together with Nkx2.1 to modify *RET* signaling and this interaction may also contribute to HSCR susceptibility.

Neural crest cells are multipotent cells that arise from the dorsal region of the fusing neural tube. During embryogenesis, neural crest cells migrate to their target regions and differentiate into different structures including enteric nervous system (ENS), which controls the blood flow, absorption, secretion, and motility of the gut.

The rearranged during transfection (*RET*) gene encodes a single-pass transmembrane receptor whose expression and proper function are crucial for the migration and differentiation of neural crest cell–derived enteric neuron progenitors, hence, the development of ENS [1]. Indeed, *RET* is the major gene implicated in Hirschsprung disease (HSCR), which is characterized by the absence of enteric ganglia in the gut region. Transcription of *RET* is a complex process that involves the dynamic interaction of transcription factors with the core promoter, other regulatory sequences, such as enhancer and repressor sequences. Any alteration in such a complicated mechanism may lead to defective *RET* expression that may be associated not only with the HSCR but also with the variability presented by the HSCR phenotype.

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doi:10.1016/j.jpedsurg.2008.11.055

NKX2-1, PHOX2B, SOX10, and PAX3 are crucial for the development of neural crest cells. Importantly, all these transcription factors are involved in *RET* transcription. There is no *Ret* expression in the homozygous *Phox2b*-knockout mice, which presents with a phenotype reminiscent of HSCR in humans [2]. Genetically, *Phox2b* polymorphisms also interact with *RET* polymorphisms to increase the risk of HSCR [3].

It has been demonstrated that Pax3 is required for enteric ganglia formation and that Pax3 and Sox10 regulate *c-ret* in a synergistic manner through activation of a conserved Sox10/Pax3-responsive enhancer [4]. In fact, mutations in *SOX10* are associated with syndromic HSCR cases (Waardenburg syndrome type IV), which are characterized by the auditory-pigmentary defects and HSCR phenotype.

NK2 homeobox 1 (NKX2-1), which was previously known as thyroid transcription factor 1, binds to the *RET* promoter and transactivates *RET* transcription [5]. Because all these transcription factors play crucial roles in regulating *RET* transcription in enteric neural crest cells, we set out to investigate whether Nkx2-1 cooperated with Phox2b, Sox10, or Pax3 in the regulation of *RET* transcription.

1. Materials and methods

1.1. Cell cultures

Human (SK-N-SH) and mouse (Neuro-2A) neuroblastoma cell lines were cultured in complete growth medium: Dubelcco modified Eagle medium (Gibco, Grand Island, NY) supplemented with heat-inactivated 10% fetal bovine serum, 2 mmol/L of L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (penicillin-streptomycin) (Gibco) at 37°C, 5% CO_2.

1.2. RNA extraction and reverse transcriptase polymerase chain reaction

Reverse transcriptase polymerase chain reaction (RT-PCR) was also performed to examine the expression of *Ret*, *Nkx2-1*, *Phox2b*, *Sox10*, and *Pax3* in the cell lines used. Total RNA was extracted from cell lines with TRIzol Reagent (Invitrogen, Carlsbad, Calif) according to manufacturer's instruction. Reverse transcriptase polymerase chain reaction was performed using 2 μg of total RNA with the SuperScript One-Step RT-PCR Systems (Invitrogen). Conditions of the PCR are summarized in Table 1.

1.3. Immunohistochemistry

Immunohistochemical study was performed on human gut. Samples were fixed and embedded in paraffin, subsequently sectioned, and mounted on glass slides. Anti-Phox2b (1:800, a gift from Stanisla Lyonnet, Necker Enfants Malades Hospital, France) and anti-Ret (1:100, Neuromics, Edina, Minn) antibodies were used for immunohistochemistry. For histologic analysis, paraffin sections of human gut were rehydrated using standard protocols and microwaved for 10 minutes in 10 mmol/L sodium citrate (pH 6.0). Sections were then incubated with the antibodies followed by incubation with secondary antibodies, anti-goat-IgG-Texas Red (1:200, Molecular Probe, Eugene, Ore) and anti-rabbit-IgG-FITC (1:200, Calbiochem, San Diego, Calif), and mounted with aqueous mounting media (Vector, Burlingame, Calif).

Table 1 Summary of primers used in this study

Gene/amplicon	Primers	Temperature (°C)
RET	Forward: 5′-ACA CCA AGG CCC TGC GGC G-3′ Reverse: 5′-GGA AGG TCA TCT CAG CTG AG-3′	54
Ret	Forward: 5′-GCT GCA TGA GAA TGA CTG GA-3′ Reverse: 5′-GAA GGA GTA GGC CCT GGG TA-3′	60
NKX2-1	Forward: 5′-ACG TGA GCA AGA ACA TGG C-3′ Reverse: 5′-GGT GGT TCT GGA ACC AGA TC-3′	56
Phox2b	Forward: 5′-AGT CCTGTA TGG CTG GGA TG-3′ Reverse: 5′-ACC ACC AGA GCA GTC CGT AC-3′	54
Sox10	Forward: 5′- ATG CAG CAC AAG AAA GAC CA-3′ Reverse: 5′- ATA GGG TCC TGA GGG CTG AT-3′	60
Pax3	Forward: 5′-GGA GGC GGA TCT AGA AAG GAA G-3′ Reverse: 5′-CCC CCG GAA TGA GAT GGT TGA A-3′	56
RET 372 bp	Forward: 5′-CCC GCA CTG AGC TCC TAC-3′ Reverse: 5′-CGC CCG TGC GCG-3′	56
RET 200 bp	Forward: 5′-GCC TAG CTT CAG TCC CGC-3′ Reverse: 5′-CGC CCG TGC GCG-3′	56
RET 100 bp	Forward: 5′-GGG CGG GGA TGG GGC GGC-3′ Reverse: 5′-CGC CCG TGC GCG-3′	56