HOXB5 Cooperates with NKX2-1 in the Transcription of Human RET

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Abstract

The enteric nervous system (ENS) regulates peristaltic movement of the gut, and abnormal ENS causes Hirschsprung’s disease (HSCR) in newborns. HSCR is a congenital complex genetic disorder characterised by a lack of enteric ganglia along a variable length of the intestine. The receptor tyrosine kinase gene (RET) is the major HSCR gene and its expression is crucial for ENS development. We have previously reported that (i) HOXB5 transcription factor mediates RET expression, and (ii) examine the interactions between HOXB5 and other transcription factors implicated in RET expression. We show that human HOXB5 binds to the promoter region 5’ upstream of the binding site of NKX2-1 and regulates RET expression. HOXB5 and NKX2-1 form a protein complex and mediate RET expression in a synergistic manner. HSCR associated SNPs at the NKX2-1 binding site (-5G>A rs10900296; -1A>C rs10900297), which reduce NKX2-1 binding, abolish the synergistic trans-activation of RET by HOXB5 and NKX2-1. In contrast to the synergistic activation of RET with NKX2-1, HOXB5 cooperates in an additive manner with SOX10, PAX3 and PHOX2B in trans-activation of RET promoter. Taken together, our data suggest that HOXB5 in coordination with other transcription factors mediates RET expression. Therefore, defects in cis- or trans-regulation of RET by HOXB5 could lead to reduction of RET expression and contribute to the manifestation of the HSCR phenotype.

Introduction

In mammalian embryos, neural crest cells (NCC) migrate from the neural tube, enter the foregut and colonize the gut, where they differentiate into neurons and glia of the enteric nervous system [1]. The enteric nervous system (ENS) comprises a network of neuronal ganglia and glia within the gut wall, which controls gut peristalsis. In human, abnormal ENS development results in absence of ganglia at the caudal-most gut in newborns with Hirschprung disease (HSCR), and patients develop a life-threatening condition of intestinal obstruction due to defective peristalsis. HSCR is a complex oligogenic disease and a major ENS developmental disorder affecting newborns with variable incidence in different races, and is most often found in Asians (28 per 100,000 live births) [2].

The receptor tyrosine kinase gene RET encodes a receptor for glial cell-line derived neurotrophic factor (GDNF) on NCC and is crucial for ENS development [3–6]. RET is the major HSCR gene, and loss-of-function mutations in RET account for up to 50% of familial and 7–33% of sporadic cases of HSCR [7–12]. Other genes implicated in HSCR that account for 7% of cases encode proteins involved in signaling pathways such as the endothelin 3/endothelin receptor B, and transcription factors SOX10, PHOX2B, NKX2-1 (also known as TTF-1), which govern ENS development [1,13,14]. Some of these transcription factors, for example NKX2-1 and SOX10 bind to cis-regulatory elements of the RET gene and regulate RET expression [13,15,16]. Single nucleotide polymorphisms (SNPs) affecting the binding and regulatory activities of NKX2-1 and SOX10 on RET were found associated with a reduced expression level of RET in patients and increasing risk of HSCR development [17,18]. These data indicated that the manifestation of the HSCR phenotype may result from the interaction between altered genes of these signalling pathways, which may ultimately result in an altered expression of RET, and consequently in the HSCR phenotype.

In human and mouse embryonic guts, Homeobox (HOX) gene HOXB5 is expressed in both migrating NCC and the gut mesenchyme, and the expression pattern of HOXB5 is associated with the migration and differentiation of NCC, suggesting a unique role of HOXB5 in ENS and gut musculature development [19–22]. To investigate Hoxb5 function in ENS development and to circumvent the problem of functional redundancy, we generated transgenic mice that can be induced by Cre to express a chimeric protein (enb) in NCC. This chimeric enb5 repressor competes with wild-type Hoxb5 for binding to target genes and inhibits transcription, thereby blocking the developmental pathways that normally require Hoxb5. We showed that the Hoxb5 trans-activated Ret expression, and that perturbation of Hoxb5 function caused down-regulation of
Results

HOXB5 trans-activates RET promoter

Our group has previously reported that Hoxb5 induces transcription of Ret in mice [23]. To study if HOXB5 protein trans-activates human RET promoter, we examined the transcriptional activity of HOXB5 on a luciferase reporter construct consisting of 3.7kb human RET promoter sequence 5′ upstream of luciferase gene. SK-N-SH neuroblastoma cell was shown to express RET, NKX2-1, PHOX2B, but not SOX10, PAX3 [25]. In addition, SK-N-SH cell also did not express HOXB5 (our unpublished data). Therefore, SK-N-SH cell was chosen to examine the regulatory role of HOXB5 on RET gene. HOXB5 displayed a 7-fold induction of transcription from human RET promoter, as compared to empty vector control pRC/CMV (Figure 2A). In situ analysis identified HOX consensus binding motif in the proximal promoter region of human RET gene, suggesting that HOX may regulate RET expression by binding to the promoter (Figure 2A, B). To delineate the cis-element in the promoter responsible for HOXB5 induction, 5′ deletion reporter constructs spanning −1205 to +196 (DeL1D), and −177 to +196 (minimal promoter construct) were generated and tested for HOXB5 induction. HOXB5 induced luciferase activity from DeL1D comparable to that from the full-length promoter. In contrast, luciferase activity from the minimal promoter construct was significantly lower than those from the full-length and DeL1D reporter constructs (Figure 2C), which suggested that the region spanning from −1205 and −177 was important for HOXB5 induction.

Overlapping PCR fragments spanning the promoter region (−1205 to −177) were tested for HOXB5 trans-activation to demarcate the HOXB5-responsive element. It was found that HOXB5 induced luciferase activity from D2 fragment (spanning −932 to −666) with heterologous SV40 promoter (Figure 2D; D2-Luc). To directly confirm that HOXB5 enhances luciferase expression by physical association with D2, EMSA and ChIP analysis were performed in HOXB5 transfected SK-N-SH cells. D2 fragment was shown to bind to HOXB5 as revealed by EMSA. A Protein-DNA complex was observed when nuclear extract or HOXB5-GST fusion protein was incubated with biotin labeled D2 fragment probe (D2 probe), and this binding was abolished when 100-fold molar excess of cold probe was added (Figure 3A). ChIP analysis was performed with an antibody to HOXB5; binding to D2 was detected by real-time quantitative PCR. Our result showed that binding of HOXB5 to D2 was enriched compared to the non-specific IgG control (Figure 3B). Furthermore, EMSA showed that HOXB5 bind to the oligonucleotide D2A that contain the HOX binding sequence (Figure 3C). HOXB5 was found neither associate with nor trans-activate from the other PCR fragments (Figure S1).

HOXB5 synergizes NKX2-1 in RET transcription

Our group previously reported that NKX2-1 trans-activates the RET promoter and is important for the expression of RET [13]. HOX binding site is located 874 nucleotides upstream of NKX2-1 promoter site in the RET promoter, suggesting that these transcription factors may cooperate in the regulation of RET transcription. HOXB5 was co-transfected with NKX2-1 into SK-N-SH cells to examine the cooperation between these two transcription factors in the trans-activation of RET promoter. The inductions were calculated as fold activations relative to the empty vector pRC/CMV. HOXB5 showed a 7.3-fold induction, and NKX2-1 displayed a 4.5-fold induction from the RET promoter. In contrast, 14.5-fold induction was observed when HOXB5 and NKX2-1 were co-transfected, which was significantly higher (p<0.05) than the sum of trans-activation of HOXB5 and NKX2-1 alone (Figure 4A). Binding of NKX2-1 to RET promoter is sensitive to the promoter SNPs, which overlap the NKX2-1 binding site [13]. To examine if the promoter SNPs affects the synergistic cooperation between HOXB5 and NKX2-1, mutated full-length reporter constructs with the two SNPs (-5G>A and -1A>C) and one SNP (-1A>C) were examined for trans-activation by HOXB5 and NKX2-1. In line with our previous findings, SNPs (-5G>A and -1A>C) and SNP (-1A>C) reduced the trans-activation of RET promoter by NKX2-1 (Figure 4A). However, these SNPs did not affect the HOXB5 trans-activation. The synergistic cooperation of HOXB5 and NKX2-1 in the trans-activation from the RET promoter was reduced drastically by the SNPs. We further knockdown the endogenous NKX2-1 expression in SK-N-SH by siRNA. Reduction of NKX2-1 expression by 70±2% (mean±SD; p=0.005; n=3) (Figure 4B) resulted in a significant reduction of HOXB5 trans-activation of RET promoter by 68.7±7% (mean±SD; p=0.00003; n=3) (Figure 4C). Synergistic interaction was not observed between HOXB5 and other transcription factors PHOX2B, SOX10 and PAX3, that have been shown capable of trans-activating RET promoter. Instead, only additive effects were observed between HOXB5 and SOX10 in the trans-activation of RET promoter (Figure S2).

The observation that HOXB5 and NKX2-1 bound to the RET promoter, and HOXB5 synergized NKX2-1 in the RET transcription, prompted us to investigate if HOXB5 physically associates with NKX2-1. Using antiserum against HOXB5 for pull-down and anti-NKX2-1 for the detection of NKX2-1 protein
Figure 2. Localization of cis-regulatory element in RET promoter responsible for HOXB5 trans-activation. A, Schematic diagram of the human RET promoter. “+1” denoted transcription start of RET gene, and the first ATG of RET gene was indicated. The 5’ ends of sequential deletion constructs of RET promoter were indicated with nucleotide positions. Location of the D2 element was indicated. B, Nucleotide sequence of the human RET gene promoter was shown with the locations of various primers used for the generation of deletion constructs. Primers pair used for the generation of D2 PCR fragment for EMSA, and for the PCR amplification in ChIP assays were indicated. The PCR primers (UD1 forward and reverse primer) used for real-time quantitative PCR for the binding of D2 to HOXB5 were indicated with «. The D2A oligonucleotide probe (bold and italic) which was specifically bound by HOXB5 as shown by EMSA was underlined with dotted arrow, and the putative HOXB5 binding site in D2 was indicated by filled boxed. NKX2-1 binding site was boxed, and the two SNPs affecting NKX2-1 binding were indicated with downwards arrows. Putative binding sites of other transcription factors were boxed and labeled accordingly. Abbreviations: TFIIIB, transcription factor II B; SREBP, sterol regulatory element-binding protein; MTE, human motif ten element. C, Trans-activation of HOXB5 on full-length and 5’ deletion RET promoter was
in the protein complex, NKX2-1 was co-precipitated with HOXB5 in the Co-IP experiment (Figure 5A). As revealed by ChIP analysis, the chromatin region containing D2 was precipitated with anti-HOXB5 and anti-NKX2.1 (Figure 5B). In addition, binding of D2 to HOXB5 and NKX2-1 was further confirmed by ChIP and quantitative PCR (Figure 5B). Taken together all these suggested that HOXB5 and NKX2-1 formed a protein complex and bound to the RET promoter.

Discussion

Expression and proper function of RET are crucial for the development of ENS [3–6]. Loss-of-function mutations in RET have been identified in both familial and sporadic cases of HSCR [7,8,10]. Genetic variants in the cis-regulatory elements of RET also play a role in mediating individual susceptibility to HSCR, by compromising the RET expression [17,18]. Therefore, to unravel the molecular mechanisms of the regulation of RET expression is critical in understanding the molecular etiology of HSCR.

The minimal promoter (−177 to +196) of the human RET gene contains regulatory elements for the binding of basal transcription factors essential for basal RET expression [26–28]. On the other hand, tissue-specific expression of RET is regulated by tissue-specific transcription factors, working cooperatively via interactions with their respective cis-regulatory elements in the RET promoter [15,16,25].

HOXB5 binds to the HOX consensus sequence at 875bp 5’ upstream of transcription start of RET gene, and deletion of the binding sequence abolishes HOXB5 trans-activation. HOXB5 and NKX2-1 form protein complex binding to the RET promoter, and work in a synergistic manner in mediating RET expression. Down-regulation of NKX2-1 by siRNA suppresses HOXB5 trans-activation of RET promoter, indicating that association with NKX2-1 is essential for HOXB5 in the regulation of RET expression. NKX2-1 binds at the region overlapping with the transcription start site, and mediates RET transcription [13]. HSCR associated SNPs at the NKX2-1 binding site (-5G>A rs10900296; -1A>C rs10900297) reduce NKX2-1 binding and trans-activation of RET [13,14,18,29–31]. These promoter SNPs have no direct effect on HOXB5 trans-activation activity, but abolish the synergistic interaction between HOXB5 and NKX2-1 on RET transcription, indicating that binding of NKX2-1 to the promoter is required for HOXB5 and NKX2-1 to work synergistically in mediating RET expression. NKX2-1 is a developmentally regulated homeodomain transcription factor [32], and possesses two tran-

![Figure 3. HOXB5 physically associates with human RET promoter.](image-url)
scriptional activation domains that interacts with other proteins to regulate target gene transcription [33]. We have previously indentified a Gly→Ser mutation in the NKX2-1 transcriptional activation domain in one HSCR patient. This mutation (Gly322-Ser) reduces the NKX2-1 induction of RET promoter without affecting its binding onto the RET promoter, suggesting that the Gly→Ser mutation may interfere with the protein-protein interactions of NKX2-1 with other transcription factors [14]. Further experiments are needed to ascertain if HOXB5 interacts with NKX2-1 via these transcriptional activator domains.

Our data show only an additive effect between HOXB5 and SOX10 on the trans-activation from the full-length RET promoter containing the SOX10/PAX3-responsive element. In line with a lack of synergistic interactions between HOXB5 with SOX10 and PAX3, HOXB5 exhibits comparable trans-activation from the full-length promoter and truncated promoter (Del.D construct) in which the SOX10/PAX3-responsive element is deleted. These data indicate that HOXB5 mediates transcription of RET independent of SOX10 or PAX3.

Although NCC express SOX10, PAX3, HOXB5, NKX2-1 and RET, co-expression of these transcription factors and RET is not always found in all SOX10-, PAX3- or HOXB5-expressing tissues [15,19,34]. Furthermore, SK-N-SH cells express RET and NKX2-1, but not SOX10, PAX3 and HOXB5 [25] (Figure S3). Using dual

**Figure 4.** HOXB5 synergizes NKX2-1 in trans-activation of human RET gene. A, Wild-type or mutated (SNPs -5G→A and -1A→C) or (-1A→C) full-length RET promoter was transfected with HOXB5 or NKX2-1 alone or in combination into SK-N-SH cells. Relative luciferase units for each combinations were determined, and fold increase (mean±SD) was calculated as compared to luciferase unit of empty vector (pXP1 Basic), which was arbitrarily regarded as 1. B, Expression levels of NKX2-1 mRNA were determined by real-time RT-PCR quantitation in non-transfected SH-N-SH cell and SH-N-SH cell transfected with NKX2-1 siRNA or negative control siRNA (Ctrl siRNA). Relative expressions of NKX2-1 were calculated with reference to non-transfected cell, which was arbitrarily regarded as 100%. C, full-length RET promoter was transfected with HOXB5 alone or in combination with NKX2-1 siRNA or negative control siRNA (Ctrl siRNA). Relative luciferase units for each combination were determined, and fold increase (mean±SD) was calculated as compared to luciferase unit of empty vector (pRC/CMV), which was arbitrarily regarded as 1.

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luciferase-reporter assays, we have previously shown that NKX2-1 indeed works coordinately with PHOX2B and SOX10, but not PAX3, to mediate RET transcription [25]. It would appear that the expression of RET is regulated by a number of transcription factors including HOXB5, NKX2-1, SOX10, PAX3 and PHOX2B, and that none of these transcription factors is necessary or sufficient for RET transcription. Instead, these transcription factors work either in a synergistic and/or additive manner to mediate RET expression.

RET is repressed in most tissues and cells, and its expression is associated with a transition from a repressive chromatin to an open, permissive chromatin [35,36]. It is plausible to speculate that association of HOXB5 with HOX binding sequence and interaction with NKX2-1 at the transcription start site, may induce conformational change of the RET promoter bringing transcription factors in close proximity of minimal promoter facilitating the recruitment of basal transcription factors mediating RET transcription.

In conclusion, we showed that i) HOXB5 binds to crucial RET regulatory region and affects RET expression; ii) HOXB5 synergizes with NKX2-1 transcription factor, and iii) HOXB5 regulatory activity is compromised by sequence variants within RET regulatory regions. Taken altogether, our data suggests that HOXB5, in coordination with NKX2-1, plays an essential role in RET expression. Future studies shall focus in delineating the interplay of molecular components implicated in the regulation of RET expression, which will provide important mechanistic insights into the pathogenesis of HSCR.

Materials and Methods

Cell culture

Human neuroblastoma cell line SK-N-SH (#HTB-11) (ATCC, Manassas, USA) was cultured in Eagle’s minimal essential medium (EMEM) (ATCC, Manassas, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, Invitrogen, Carlsbad, USA) without antibiotics at 37°C and 5% CO2. Trypsin-EDTA solution (0.25%; w/v) was used for trypsinization of cells. Cells were stored in 5% (v/v) DMSO in liquid nitrogen.

Luciferase-reporter constructs

The 3.7 kb RET full-length promoter (1545 to 5270, AF032124, Genbank), the 372 bp minimal promoter and the mutated RET full-length promoter containing -5G>A and -1A>C or -1A>C alleles, were generated by PCR and cloned upstream of the luciferase reporter in the pXP1 vector (AF093683, GenBank) [13]. All of these constructs contain a 195 bp 5’UTR of RET gene. A 1.4 kb 5’ deletion construct (DeD), was generated by cloning of the PCR fragment (-1205 to -195) of RET gene upstream of the luciferase reporter in the pXP1 vector. Furthermore, D2A-Lac construct was generated by cloning of the PCR fragment (-932 to -666) of RET gene upstream of the luciferase reporter in the pGL3 vector (Promega, Madison, USA).

Transient transfection and dual-luciferase reporter assay

SK-N-SH cells were seeded on 24-well 18 hours prior to transfection. Cells were co-transfected with pRL internal control, reporter constructs and appropriate amount HOXB5 and/or NKX2-1 expression vectors with Lipofectamine 2000 (Invitrogen, Carlsbad, USA). Cell lysate was analyzed 24 hours by Dual-Luciferase Reporter Assay System (Promega, Madison, USA) on MicroLumatPlus LB 96V instrument. At least two independent triplicate or quintupled experiments were performed, and the luciferase activity was presented as relative luciferase unit (RLU) normalized with the Renilla luciferase internal control.
Table 1. Primers for PCR amplification.

| Amplification | Primers | Annealing Temperature |
|---------------|---------|-----------------------|
| Del.D (-1205 to 195) | Forward: 5'-TCTGCTCGTGGAGGTGAC-3' | 61°C |
|               | Reverse: 5' - AGTTGCTCTCCAGCGGTTC-3' | |
| D1 (-1205 to -912) | Forward: 5'-TCCTGCTGTGAGGTGAC-3' | 62°C |
|               | Reverse: 5'-AGGGTAGGTGGATGGG-3' | |
| D2 (-932 to -666) | Forward: 5'-GGCCACATCCACCTGAC-3' | 62°C |
|               | Reverse: 5'-CTCCAGACTGTGAGGGTGAC-3' | |
| D3 (-700 to -363) | Forward: 5'-TTTCCGGTTCCAAGGTTG-3' | 62°C |
|               | Reverse: 5'-CTGGCTTTTGGCCCTTCC-3' | |
| D4 (-387 to -115) | Forward: 5'-TGACAGCCGGAAGGGAACAG-3' | 62°C |
|               | Reverse: 5'-CGCTTTGAGCGCTAGTG-3' | |
| D2A (-932 to -666) | Forward: 5'-AGGCCACATCCACCTGAC-3' | 61°C |
|               | Reverse: 5'-CTCCAGACTGTGAGGGTGAC-3' | |

Small interference RNA knockdown of NKX2-1

Transfection of small interfering RNA (siRNA) was performed using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, USA) according to the siRNA-plasmid co-transfection protocol from Invitrogen. Human NKX2-1 siRNA (Stealth RNAi HSS144278) and negative control (Stealth RNAi 12933-200) were purchased from Invitrogen (Invitrogen, Carlsbad, USA).

Real-time PCR

Real-time PCR was performed on 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, USA) using Fast SYBR Green Master Mix (Applied Biosystems, Foster City, USA) according to the manufacturer’s protocol.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChiP) was performed using MAGnify™ ChiP System (Invitrogen, Carlsbad, USA) according to the manufacturer’s instruction. Briefly, 3 x 10^6 HOXB5/NKX2-1 transfected SK-N-SH cells were cross-linked in 1% formaldehyde and chromatin DNA was sheared by sonication (Vibra-cell VCX750 sonicator; 35% amplification, 20s ON plus 40s OFF, 25 cycles) to generate fragments of around 500 bp in length. Dynabead® were pre-coupled with anti-HOXB5 (133c3a, Santa Cruz), anti-NKX2-1 (H-190, Santa Cruz), mouse or rabbit IgG. Sheared chromatin DNA was incubated with corresponding antibody-coupled beads at 4°C with rotation for 3 hours. After pull-down and reversion of cross-linking, chromatin DNA was purified. Binding of D2 to HOXB5 and NKX2-1 was quantified by real-time quantitative PCR analysis.

Supporting Information

Figure S1 A, Schematic diagram of the human RET promoter. “+1” denoted transcription start of RET gene, and the first ATG of RET gene was indicated. B, Overlapping DNA fragments (D1 to D4) were generated by PCR and labeled with biotin for EMSA. Only D2 was able to bind to HOXB5 as shown by the retarded migration of the probe (arrow). C, Trans-activation of HOXB5 from D1 to D4 fragments were assayed by luciferase activity. Luciferase activity was normalized with Renilla luciferase to obtain relative luciferase unit. Fold increase (mean±SD) was determined relative to luciferase unit of empty vector (SV40-LUC) which was arbitrarily regarded as 1.

Figure S2 No synergistic interaction between HOXB5 and PHOX2B, SOX10 or PAX3 in the trans-activation of RET promoter. Wild-type full-length RET promoter was transfected with expression constructs of HOXB5, SOX10, PAX3 and PHOX2B either alone or in combination into SK-N-SH cells. Relative luciferase units for each combinations were determined, and fold increase (mean±SD) was calculated as...
compared to luciferase unit of empty vector (pXPI Basic), which was arbitrarily regarded as 1.

The expression of RET, NKX2-1, SOX10, PAX3, and HOXB5 was arbitrarily regarded as 1.

**Figure S3** SK-N-SH cells express RET and NKX2-1, but not SOX10, PAX3 and HOXB5. Total RNA was isolated from SK-N-SH cells for RT-PCR analysis to assay for the expression of RET, NKX2-1, SOX10, PAX3 and HOXB5. RT-PCR for β-actin was included as a positive control to test the integrity of the RNA and the RT-PCR reaction. PCR products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining.

**Author Contributions**
Conceived and designed the experiments: JZ M-MG-B PKHT VCHL. Performed the experiments: JZ VCHL. Analyzed the data: JZ M-MG-B PKHT VCHL. Contributed reagents/materials/analysis tools: JZ M-MG-B PKHT VCHL. Wrote the paper: VCHL.

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