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

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ABSTRACT

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 (ENS). Mutations in *RET* regulatory regions are also associated with Hirschsprung's disease (HSCR) (aganglionosis of the colon). We ~~have~~ previously showed that two polymorphisms in *RET* promoter are associated with the increased risk of HSCR. These SNPs 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. First, we demonstrated that PHOX2B, like SOX10 and NKX2-1, is expressed in the mature enteric ganglions of human gut. Second, subsequent dual-luciferase-reporter studies indicated that Nkx2-1 indeed works co-ordinately 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. In sum, Phox2b and Sox10 act together with Nkx2.1 to modify RET signalling and this interaction may also contribute to HSCR susceptibility.

KEYWORDS: *RET*; Nkx2-1; transcription

INTRODUCTION

Neural crest cells are multipotent cells which 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 (Schuchardt et al., 1994). Indeed, *RET* is the major gene implicated in Hirschsprung's 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 which may be associated not only with the HSCR but also with the variability presented by the HSCR phenotype.

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 (Pattyn et al., 1999). Genetically, *Phox2b* polymorphisms also interact with *RET* polymorphisms to increase the risk of HSCR (Miao et al., 2007).

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 (Lang and Epstein, 2003). 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 (TTF1), binds to the *RET* promoter and transactivates *RET* transcription (Garcia-Barcelo et al., 2005). Since all these transcription factors play crucial roles in regulating *RET* transcription in enteric neural crest cells, we set out to investigate whether Nkx2-1 co-operated with Phox2b, Sox10 or Pax3 in the regulation of *RET* transcription.

MATERIALS AND METHODS

Cell cultures

A human (SK-N-SH) and a mouse (Neuro-2A) neuroblastoma cell lines were cultured in complete growth medium: Dubelcco's Modified Eagle Medium (DMEM) (Gibco, USA) supplemented with heat inactivated 10% fetal bovine serum, 2mM of L-glutamine, 100U/ml penicillin and 100µg/ml streptomycin (Penicillin-Streptomycin) (Gibco, USA) at 37 ^{o}C, 5% CO_2.

RNA extraction and reverse transcriptase polymerase chain reaction (RT-PCR)

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, CA, USA) according to manufacturer's instruction. RT-PCR was performed using 2µg of total RNA with the SuperScript™ One-Step RT-PCR Systems (Invitrogen, Carlsbad, CA, USA). Conditions of the PCR are summarized in Table 1.

Gene/Amplicon	Primers	Tm (°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-372bp	**Forward: 5'**-CCC GCA CTG AGC TCC TAC-**3'** **Reverse: 5'**-CGC CCG TGC GCG-**3'**	56

RET-200bp	**Forward: 5'**-GCC TAG CTT CAG TCC CGC-**3'** **Reverse: 5'**-CGC CCG TGC GCG-**3'**	56
RET-100bp	**Forward: 5'**-GGG CGG GGA TGG GGC GGC-**3'** **Reverse: 5'**-CGC CCG TGC GCG-**3'**	56

Table 1. Summary of primers used in this study.

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, MN) antibodies were used for immunohistochemistry. For histological analysis, paraffin sections of human gut were rehydrated using standard protocols and microwaved for 10 min in 10mM sodium citrate pH6.0. Sections were then incubated with the antibodies followed by incubation with secondary antibodies , anti-goat-IgG-Texas Red (1:200, Molecular Probe, Eugene, OR) and anti-rabbit-IgG-FITC (1:200, Calbiochem, San Diego, CA) and mounted with aqueous mounting media (Vector, Burlingame, CA).

Construction of vectors

Luciferase-reporter plasmid

About 3.7kb of the human *RET* promoter (from the nucleotide 1545 to 5270 of the GenBank accession number AF032124) was cloned upstream of the luciferase reporter in the pXP1 vector (GenBank accession no. AF093683) as previously described (Garcia-Barcelo et al., 2005). In order to generate the sequential deletion reporter constructs, different lengths of the *RET* promoter (372bp, 200bp and 100bp DNA fragments upstream of the *RET* transcription start site) were amplified by PCR. The

PCR products were then subcloned into the pGL3-Basic vector (Promega, USA) upstream of the Firefly luciferase gene. Conditions of the PCR are summarized in Table 1.

Nkx2-1, Phox2b, Sox10 and Pax3 expression vectors

Nkx2-1-, and *Phox2b*- expression vectors were the gifts from Dr. Parviz Minoo (University of Southern California, Los Angeles, CA, USA) (Hamdan et al., 1998) and Dr. Diego Fornasari (University of Milan and CNR-Institute of Neuroscience, Milan, Italy), respectively. *Sox10* expression construct was purchased from OriGene Technologies, Inc. (Rockville, Maryland, USA). The mouse *Pax3* cDNA was obtained from Prof. Peter Gruss (Max Planck Institute of biophysical chemistry, Department of Molecualr Cell Biology, Göttingen, Germany) and then subcloned into pcDNA3.1 at *Hind*III and *Xho*I sites.

Transient transfection and dual-luciferase assay

In order to assess the transactivation activity of each transcription factor on the *RET* promoter, SK-N-SH ($2x10^4$) or Neuro-2A ($3x10^5$) cells were plated on tissue culture plates 24 hours prior to transfection. Cells were transiently co-transfected with 50ng pRL-SV40, 300ng reporter constructs and appropriate amount of *Nkx2-1*, *Phox2b*-, *Sox10*- or *Pax3*- expression vector as indicated using 1.25µl Lipofectamine™2000 (Invitrogen, USA).

Cells were then lysed and the luciferase activity was analyzed with the Dual-Luciferase Reporter Assay System (Promega) on a MicroLumatPlus LB 96V instrument (Berthold Technology). The activity of *RET* promoter was expressed as relative

luciferase unit (RLU) after normalized with the internal control (*Renilla* luciferase). The experiments were performed at least three times each in triplicate. The bar chart represents the mean value of RLU +/- standard derivation (SD).

Statistical analysis and in silico prediction of Phox2b binding site in the RET promoter

Statistical analysis of the luciferase data was performed with one-way ANOVA. The significance level was set at 5%. Tukey's test was used for *post hoc* pair-wise comparisons.

The transcription factor binding sites were predicted by the Matinspector program (http://www.genomatix.de/) with default parameters.

RESULTS

Differential expression of Nkx2-1, Phox2b, Sox10 and Pax3 in two neuroblastoma cell lines

Nkx2-1, Phox2b, Sox10 and Pax3 are four key transcription factors for *Ret* expression. Thus, we first evaluated their expression in one human (SK-N-SH) and one mouse (Neuro-2A) neuroblastoma cell lines by RT-PCR. As shown in Figure 1, *Ret*, *Nkx2-1* and *Phox2b* were expressed in these two neuroblastoma cell lines; while *Pax3* was only expressed in Neuro-2A and neither of these cell lines expresses *Sox10.* It is mention-worthy that *PHOX2B*, endogenously expressed in SK-N-SH, contains a 20bp deletion in the polyalanine tract that introduces a premature termination codon and results in a truncated protein (van Limpt et al., 2004). Since *Ret* is expressed in both SK-N-SH and Neuro-2A, these two neuroblastoma cell lines were used to delineate the transcriptional regulation of *Ret* expression in this study.

PHOX2B is expressed in the myenteric plexus of human adult gut

Previous study from our group have shown that *NKX2-1* is expressed in enteric ganglions of prenatal and postnatal guts (Garcia-Barcelo et al., 2005), and PHOX2B was also detected in the enteric ganglia of the developing ENS (Amiel et al., 2003). Given that *NKX2-1* and *PHOX2B* are co-expressed in these two neuroblastoma cell lines, we speculated that *PHOX2B* may be also persistently expressed even in the postnatal stage. To address this question, we have performed immunohistochemistrial study using sections from human postnatal gut. As shown in Figure 2, the ganglion cells in myenteric plexus are expressing RET (red) and also costained with PHOX2B (green),

indicating that PHOX2B are persistently coexpressed with RET in enteric ganglions in both embryonic and postnatal stages.

Activation of RET promoter by Nkx2-1, Sox10 and Pax3

The differential expression of *Nkx2-1*, *Sox10* and *Pax3* in SK-N-SH and Neuro-2A prompted us to examine their potential transactivation function on *RET* promoter in these cell lines. We used a reporter construct which contains ~3.7kb 5'flanking sequence of *RET* promoter spanning -3527 to +194 relative to the transcription start site. Within this region, we found binding motifs for Nkx2-1, Sox10 and Pax3, but not Phox2b, as summarized in Figure 3A. As indicated, Nkx2-1 binding motif is adjacent to transcription start site at -5. On the other hand, the binding sites for Sox10 and Pax3 are located further upstream at -3417 and -3398, respectively.

To examine the transactivation activity of these transcription factors, luciferase reporter assays were performed using this ~3.7kb *RET* promoter construct and the Neuro-2A cells. Overexpression of Nkx2-1 significantly activated the *RET* promoter by 6.5 folds when compared with the vector control (Figure 3B). Similarly, Sox10 (Figure 3C) and Pax3 (Figure 3D) also elevated the promoter activity by 2.7 and 2 folds, respectively. It is noteworthy that these transcription factors also upregulated the *RET* promoter with similar extent in SN-K-SH (data not shown). Taken together, all these three transcription factors could significantly upregulate the *RET* promoter activity in both neuroblastoma cell lines tested.

-178/-6 of RET promoter is crucial for Phox2B mediated transcription

In Figure 4A, we found that Phox2b also increases the promoter activity of *RET* (~1.8 folds, $p<0.05$) in Neuro-2A, it is consistent with the previous finding (Bachetti et al., 2005). Bachetti *et al*. also showed that the putative binding site(s) for Phox2b may reside at the region between -307 and +53 of the *RET* promoter relative to the transcription start site. To further identify the putative Phox2b binding site, three additional 5' deletion reporter constructs were generated which contain the regions spanning -178 to +194 (RET-372bp), -6 to +194 (RET-200bp) and +94 to +194 (RET-100bp) of the *RET* promoter relative to the transcription start site. Consistently, Phox2B increased the luciferase activity by 1.8 folds with the proximal 372bp of *RET* promoter (RET-372bp) and it was comparable to that of the full length promoter (Figure 3A & B). Deletion of the additional 172bp (RET-200bp) from the 5' end resulted in a ~80% reduction in the transcriptional activity, suggesting the presence of a putative Phox2B binding site(s) in this 172bp (Figure 4B).

Subsequent bioinformatic analysis revealed that this 172bp DNA fragment contains multiple putative binding motifs for numerous transcription factors including GC-Box factor SP1, Kruppel-like factor (KLF) and vertebrate homologues of enhancer of split complex (HES1) etc as indicated in Figure 4C. Nevertheless, no putative Phox2b binding site ('ATTA' or 'TAAT') was found in this region (Figure 4C).

Sox10, Phox2b and Nkx2-1 work coordinately to mediate RET transcription

Given that 1) the above transcription factors are important for the expression of *RET* and they upregulate *RET* promoter activity; 2) Sox10, Pax3 and Nkx2-1 physically bind to the *RET* promoter (Garcia-Barcelo et al., 2005;Lang and Epstein, 2003); and 3) Nkx2-1 only transactivate *RET* transcription in the presence of Sox10 and Pax3 binding

sites (Garcia-Barcelo et al., 2005), we then examined whether Phox2b, Sox10 or Pax3 work coordinately with Nkx2-1 in stimulating *RET* transcription. In order to evaluate the functional interaction between these transcription factors on *RET* transcription, similar luciferase assays were performed using the Neuro-2A cells in which Nkx2-1 was co-transfected with Phox2b, Sox10 or Pax3.

Co-expression of Phox2b with Nkx2-1 showed significantly higher luciferase activity (4.2-fold) when compared with either Nkx2-1 (2.9-fold) or Phox2b (1.8-fold) alone (Figure 5A), suggesting that Phox2b can enhance Nkx2-1 induced *RET* promoter activity. Similar coordination was also found when Nkx2-1 was co-transfected with Sox10 in which 3.9-fold increment in promoter activity was seen, while Nkx2-1 or Sox10 alone only elevated luciferase activity by 1.8-fold and 1.9-fold, respectively (Figure 5B). Unlike Phox2b or Sox10, over-expression of *Pax3* could not further enhance Nkx2-1-activated *RET* transcription. Only 1.8-fold increment of the *RET* promoter activity was detected when *Pax3* was coexpressed with *Nkx2-1* (Figure 5C).

DISCUSSION

Proper gene expression is tightly regulated by dynamic assembly of transcription complex in the regulatory sequence of the gene which involves the binding of transcription factors specifically to their consensus DNA sequences, protein-protein interaction among enhancers, repressors as well as other cofactors. Aberrant gene expression may lead to disease. In this study, we demonstrated that 1) PHOX2B, like NKX2-1, is persistently expressed not only in the prenatal, but also in the postnatal enteric ganglions; 2) Nkx2-1, Phox2b, Sox10 and Pax3 stimulate *RET* transcription individually in neuroblastoma cells; 3) the -6/-178 region of the *RET* promoter is essential for Phox2b-activated *RET* transcription; 4) Phox2b or Sox10 interact with Nkx2-1 to mediate *RET* transcription; 5) Nkx2-1 does not co-operate with Pax3 in *RET* transcription.

NKX2-1, PHOX2B, SOX10 and PAX3 are dispensable for *RET* expression (Garcia-Barcelo et al., 2005;Pattyn et al., 1999;Lang and Epstein, 2003) and have been associated with the defective ENS development (HSCR). Here, we found that NKX2-1 (Garcia-Barcelo et al., 2005), SOX10 (Fu et al., 2003) and PHOX2B are co-expressed with *RET* in enteric ganglions of the postnatal gut. We believed that these factors may not only contribute to ENS development, but also the maintenance of *RET* expression in the mature enteric ganglions.

Like Nkx2-1, Sox10 and Pax3, Phox2b alone was also able to up-regulate *RET* transcription. Nevertheless, there is no known Phox2b binding site identified in the *RET* promoter so far. Indeed, a previous finding from Bachetti *et al* showed that Phox2b confers its full transactivation function with the proximal 360bp *RET* promoter (Bachetti et al., 2005), and it is believed that the putative binding motif(s) is residing

between +53 and -307. In this study, we used a series of 5' deletion mutants to further localize the Phox2b responsive region at -6/-178 of the *RET* promoter. Again, no putative Phox2b binding site ('ATTA' or 'TAAT') could be identified in this fragment, implying that Phox2b may not bind onto the *RET* promoter directly. This observation, on the other hand, reinforces the hypothesis that Phox2b may act as a bridge to recruit other transcriptional regulators to form a transcription complex, in turn, mediates the *RET* transcription. Sequence analysis showed that there are three potential binding sites for SP1 protein (SP1) and two for Kruppel-like factor (BKLF), and one for Hes1 within this 172bp. It suggests that Hes1, SP1 or KLF may interact with Phox2b to mediate *RET* transcription. Noteworthy, Hes1 and KLF4 are expressed in neural crest progenitor (unpublished data) and implicated in neural stem cell maintenance and neurogenesis (Cau et al., 2000;Li et al., 2005). Therefore, interaction between Phox2b and these two factors may contribute to the neural crest specific expression of *RET* and ENS development. Future study on the functional interaction between these factors may further delineate the underlying mechanism by which Phox2b mediates the neural crest specific expression of *RET*.

Several models have been proposed to explain the co-operation mechanism among transcription factors. The 'kinetics model' suggests that different transcription factors may accelerate different steps, such as the assembly of transcription complex and elongation of the transcript (Herschlag and Johnson, 1993). If more than one rate-determining step is accelerated, the transcription efficiency will be increased exponentially. However, if only one rate-determining step was accelerated, the transcription efficiency would increase modestly. The co-operative DNA binding model suggests that the synergism could be achieved by the DNA binding of a transcription

factor which may increase the DNA binding affinity of another transcription factor.

In our system, the additive effect between Nkx2-1 and Sox10 or Phox2b in *RET* transcription regulation is best explained under the kinetics model whereby Nkx2-1 may only contribute to the Sox10 or Phox2b-regulated transcriptional step. If these transcription factors were implicated in the same rate-determining step, the transcription efficiency would only be increased additively (Herschlag and Johnson, 1993). Alternatively, Phox2b and Sox10 may not have direct interaction with Nkx2-1. They may contribute to the Nkx2-1 regulated *RET* transcription through other coactivators or tissue specific transcriptional factors.

Evidently, Nkx2 and Pax family members have been shown to have functional cross-talk in various systems. For instance, NKX2-1 directly interacts with PAX8 to regulate thyroid-specific expression of thyroglobulin (*Tg*) (Espinoza et al., 2001). In the developing pancreata, Nkx2-2 and Pax4, despite of working through two independent pathways, they both are involved in mediating the expression of same set of pancreatic beta-cell early differentiation markers such as *Hlxb9*, *Pdx1* as well as insulin (Sosa-Pineda, 2004). Therefore, Nkx and Pax can work co-ordinately either by direct interaction or via parallel pathways. Here, we have shown that Nkx2-1 does not have direct interaction with Pax3. However, we cannot rule out the possibility that they would work in parallel to provide signal refinement for the *RET* expression during ENS development.

In sum, this is the first integrated study showing the potential functional interaction of multiple transcription factors with Nkx2-1 to mediate the *RET* transcription. A delicate balance between these factors will therefore ensure the proper expression of *RET* during different developmental windows. Identification of promoter

region responsible for Phox2b transactivation further confirms its biological significance on *RET* expression. It also supports the notion that both functional and genetic interactions between *PHOX2B* and *RET* contribute to the development of HSCR disease (Miao et al., 2007).

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FIGURE LEGENDS

Figure 1. Expression of *RET, NKX2-1, Phox2b, Sox10* and *Pax3* in SK-N-SH and Neuro-2A cells. RT-PCR analysis indicates that *RET*, *NKX2-1*, *Phox2b*, *Sox10* and *Pax3* are differentially expressed in two neuroblastoma cell lines (SK-N-SH and Neuro-2A). +ve: positive control; -ve: negative control.

Figure 2. Expression of *PHOX2B* and *RET* in myenteric plexus of human gut. Human postnatal gut section was stained with anti-RET and anti-PHOX2B antibodies. RET (red) and PHOX2B (green) are colocalized in neuroblast of the myenteric plexus. lm, longitudinal muscle; cm, circular muscle; mp, myenteric plexus. Scale bars, 50 μm.

Figure 3. Transactivation activities of Nkx2-1, Sox10 or Pax3 on *RET* promoter. (A) DNA sequence of the *RET* promoter used in this study. The binding sites of Sox10, Pax3 and Nkx2-1 were highlighted. Luciferase assays shows that (B) Nkx2-1, (C) Sox10 and (D) Pax3 activate *RET* promoter significantly. Luciferase activity was normalized with *Renilla* luciferase to obtain relative luciferase unit (RLU). Data are shown as the fold increase in relative promoter activities compared with that in the control (empty vector). Bars bearing "*" are statistically different from the control ($p<0.05$).

Figure 4. Identification of Phox2b responsive promoter region in *RET* gene. (A) Luciferase assay shows that Phox2b significantly activates *RET* transcription. (B) Deletion mutation analysis was performed with three sequential 5' deletion mutants of

the *RET* promoter. RET-372bp, RET-200bp and RET-100bp reporter constructs contain the proximal 372bp, 200bp and 100bp of *RET* promoter. Deletion of -6/-178 of *RET* promoter results in loss of Phox2b-mediated *RET* transcription. Luciferase activity was normalized with *Renilla* luciferase to obtain relative luciferase unit (RLU). Data are shown as the percentage change in relative promoter activities compared with that in the control (empty vector). Bars bearing "*" are statistically different from the control ($p<0.05$). (C) *In silico* prediction of putative transcription factor binding sites residing on the region -6/-178 of *RET* promoter. The putative binding motifs are underlined and labeled accordingly.

Figure 5. Functional interaction of Nkx2-1 with Phox2b, Sox10 and Pax3 on *RET* transcription. Luciferase assays were performed in Neuro2A cells to examine the potential interaction between Nkx2-1 with (A) Phox2b, (B) Sox10 and (C) Pax3. Luciferase activity was normalized with *Renilla* luciferase to obtain relative luciferase unit (RLU). Data are shown as the fold increase in relative promoter activities compared with that in the control (empty vector). Bars bearing "*" are statistically different from the control ($p<0.05$).

Figure 1

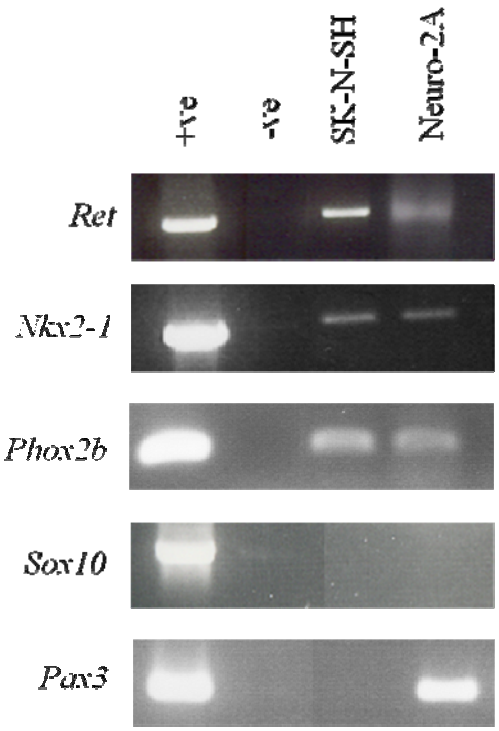


Figure 2

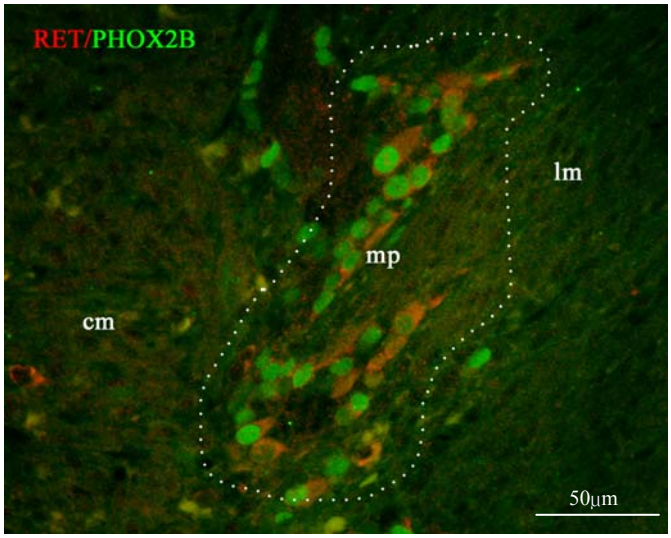
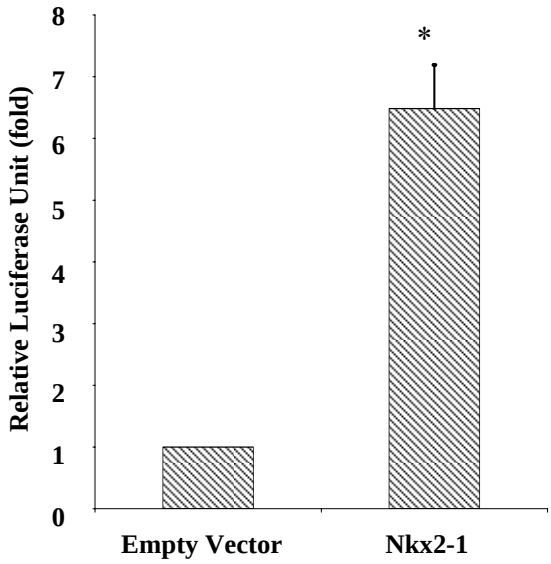


Figure 3

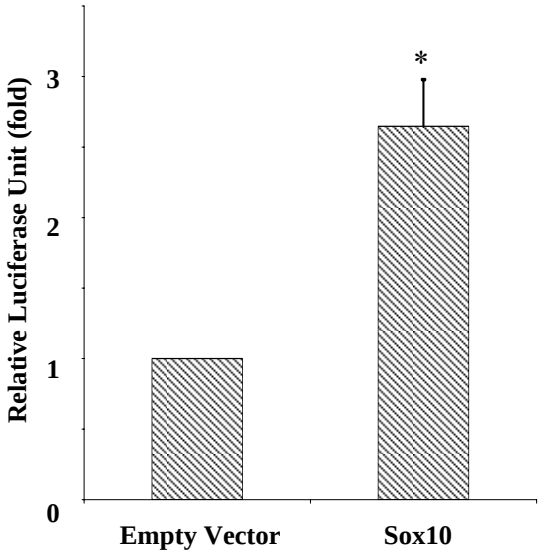
A)

```
-3516 acctcaatag cccgatgggg gagcagaggg cagtggcaga cagatgggaa actgaggcac
                                                    SOX10
-3456 cgaccacttt gctaacagtg cacacagcca gcctcagagt ctgtgcttcc aaccaccatg
      PAX3
-3396 tcacactgcc catgggaggg acctggaccc ctgcccacgc ccactctgag aggcctcggt
-3336 cagcagttcc aggaatgcca cccagccttc cagtatcacc aggaccgagc tgcgctcttg
      ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
 -96 accccacccg cctccggccc cgcctggccc acccctggac cgcccccgcc ccgccccgcc
                                                NKX2-1
 -36 cctacccgct cctcggcgca gccggcgctt gcctagcttc agtcccgcga ccgaagcagg
                                    +1
 +26 gcgcgcagca gcgctgagtg ccccggaacg tgcgtcgcgc ccccagtgtc cgtcgcgtcc
 +86 gccgcgcccc gggcggggat ggggcggcca gactgagcgc cgcacccgcc atccagaccc
+146 gccggcccta gccgcagtcc ctccagccgt ggccccagcg cgcacgggcg atggcgaagg
```

B)



C)



D)

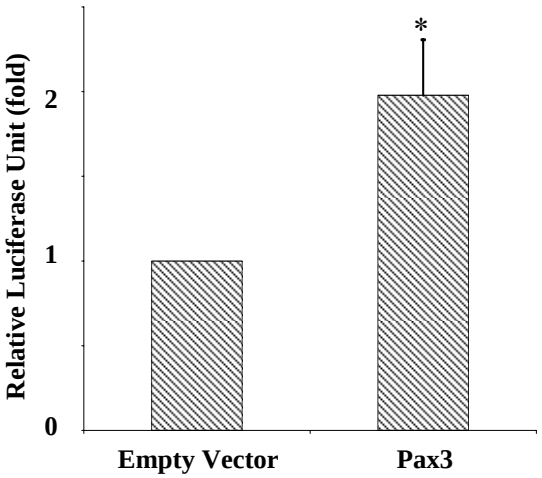
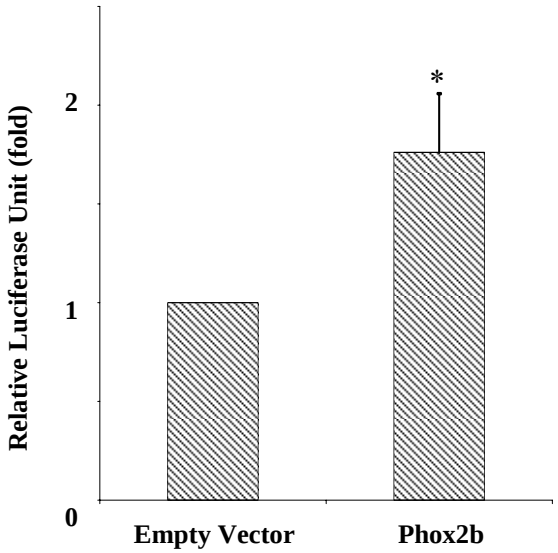
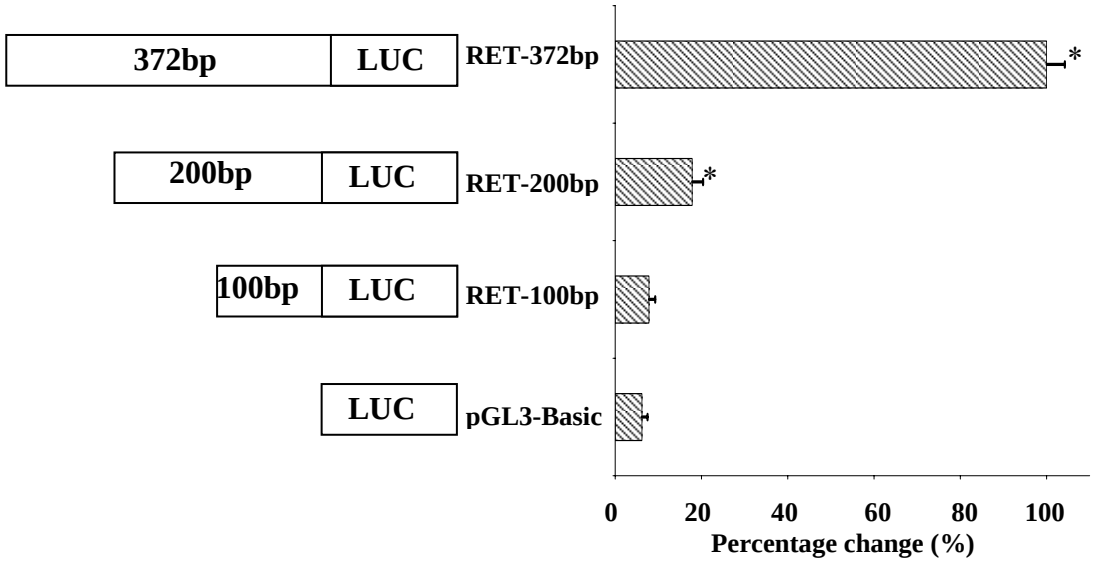


Figure 4

A)



B)

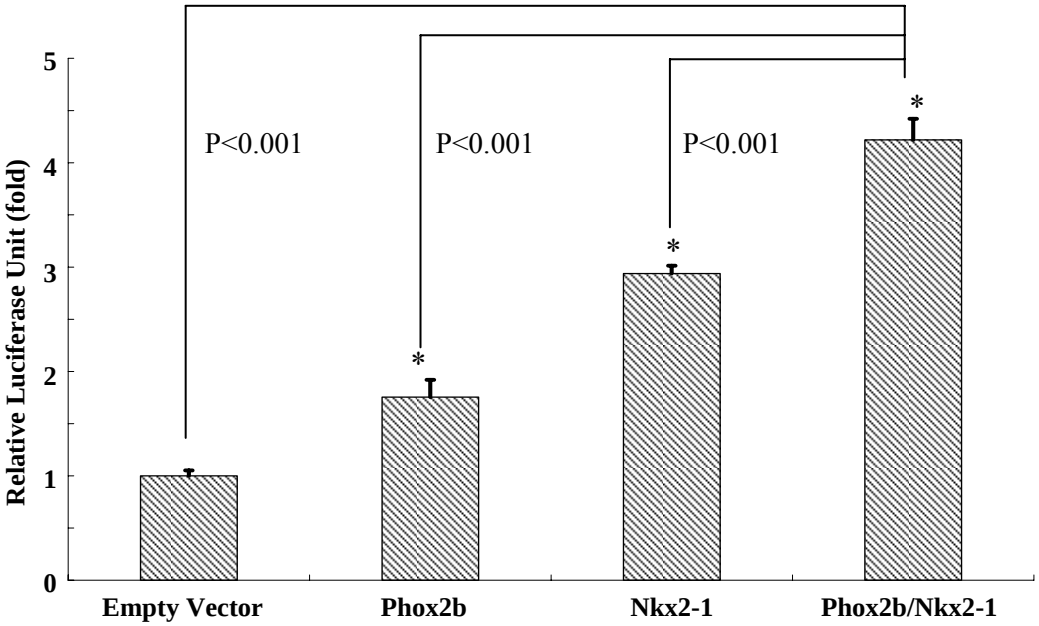


C)

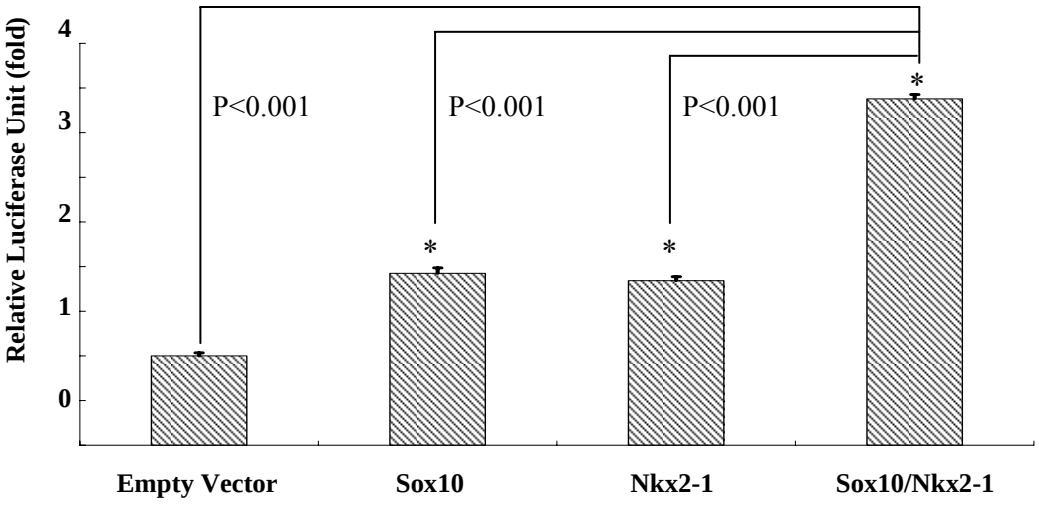
```
-178 cccgcactga gctcctacac gcgccggccc cggccgcacc ccgcgcacgc agagcaagca
                                                HES1
-118 ctggagcccc gccccttccc gcaccccacc cgcctccggc cccgcctggc ccacccctgg
          SP1                    BKLF                          BKLF
 -58 accgcccccg ccccgccccg cccctacccg ctcctcggcg cagccggcgc tt
     SP1              SP1
```

Figure 5

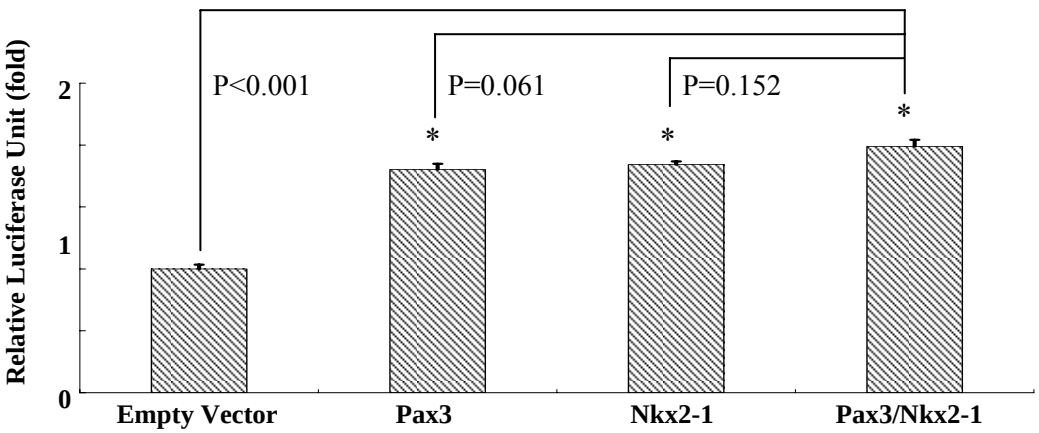
A)


Relative Luciferase Unit (fold)
0
1
2
3
4
5
P<0.001
P<0.001
P<0.001
*
*
*
*
Empty Vector
Phox2b
Nkx2-1
Phox2b/Nkx2-1

B)


Relative Luciferase Unit (fold)
0
1
2
3
4
P<0.001
P<0.001
P<0.001
*
*
*
*
Empty Vector
Sox10
Nkx2-1
Sox10/Nkx2-1

C)


Relative Luciferase Unit (fold)
0
1
2
P<0.001
P=0.061
P=0.152
*
*
*
*
Empty Vector
Pax3
Nkx2-1
Pax3/Nkx2-1