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Prokineticin-1 (Prok-1) works coordinately with glial cell line-derived neurotrophic factor (GDNF) to mediate proliferation and differentiation of enteric neural crest cells

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Abstract

Enteric neural crest cells (NCC) are multipotent progenitors which give rise to neurons and glia of the enteric nervous system (ENS) during fetal development. Glial cell line-derived neurotrophic factor (GDNF)/RET receptor tyrosine kinase (Ret) signaling is indispensable for their survival, migration and differentiation. Using microarray analysis and isolated NCCs, we found that 45 genes were differentially expressed after GDNF treatment (16 h), 29 of them were up-regulated including 8 previously undescribed genes. Prokineticin receptor 1 (PK-R1), a receptor for Prokineticins (Prok), was identified in our screen and shown to be consistently up-regulated by GDNF in enteric NCCs. Further, PK-R1 was persistently expressed at a lower level in the enteric ganglions of the *c-Ret* deficient mice when compared to that of the wild-type littermates. Subsequent functional analysis showed that GDNF potentiated the proliferative and differentiation effects of Prok-1 by up-regulating PK-R1 expression in enteric NCCs. In addition, expression analysis and gene knock-down experiments indicated that Prok-1 and GDNF signalings shared some common downstream targets. More importantly, Prok-1 could induce both proliferation and expression of differentiation markers of *c-Ret* deficient NCCs, suggesting that Prok-1 may also provide a complementary pathway to GDNF signaling. Taken together, these findings provide evidence that Prok-1 crosstalks with GDNF/Ret signaling and probably provides an additional layer of signaling refinement to maintain proliferation and differentiation of enteric NCCs.

Keywords: GDNF; Prok-1; Neural crest cell

1. Introduction

Neural crest cells (NCC) arise from the junction between the presumptive epidermis and the neural plate during neurulation. They are similar to embryonic stem cells in that they have a high degree of plasticity and are able to give rise to neurons and glia of the peripheral nervous system (PNS), skeleton and smooth muscle of aortic arch, chondrocytes, osteocytes, melanocytes, chromaffin cells, supporting cells and hormone producing cells in various organs.

Vagal and sacral NCCs contribute to neurons and glia of the enteric nervous system (ENS) during fetal development. Once they reach the gut, NCCs proliferate, differentiate and colonize the developing bowel. ENS development is then completed by establishing synaptic networks among neurons and glia, which regulate the peristalsis and secretory activity of the bowel. Appropriate pool size of NCCs is crucial for the normal and

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complete colonization of the gut [1]. In humans, defects in the NCCs may result in congenital colonic aganglionosis, known as Hirschsprung disease (HSCR) or megacolon [2].

Gial cell line-derived neurotophic factor (GDNF) is one of the most critical factors mediating the enteric NCC pool for the proper ENS development. GDNF binds on the RET tyrosine kinase and a glycosylphosphatidylinositol (GPI)-anchored ligand binding subunit (GFRα) to confer its survival, differentiation and migration signalings for enteric NCCs. In humans, mutations in *RET* account for approximately 50% of familial HSCR cases [3,4], but mutations of the *Gdnf* and *GFRα-1* genes are not a common aetiological event in HSCR [5]. Conversely, mice deficient in either *Ret* (*Ret*$^{k-}$), *Gdnf* (*Gdnf*$^{-}$) or *Gfrα1* (*Gfrα1*$^{-}$) have complete intestinal aganglionosis [6–13], suggesting GDNF/RET signaling is indispensable for ENS development.

Recent advance in microarray technology facilitates the identification of genes implicated in ENS development. Molecular characterization using microarray analysis to compare gut NCCs to whole-fetus RNA identified a panel of genes uniquely or highly expressed in the NCCs [14]. It includes numerous genes linked to HSCR, including Ret, Gfra1, EDNRB and Sox10, further confirming the biological significances of these pathways in ENS development. More recently, expression profiling studies on developing guts of *c-Ret* mutant mice (*Ret*$^{k-}$/ *Ret*^{k}) have been performed by two different groups [15,16]. *c-Ret* mutant mice display a complete intestinal aganglionosis [6], therefore, it is a useful model for identification of genes specifically expressed in ENS. In their screens, they have identified a large cohort of candidate ENS marker genes within the mammalian gut, and several of them have been proven to have biological implications. In particular, *Arhgef3* and *Ctnnal1* located in HSCR susceptibility loci, 3p21 and 9q31, respectively [15]; and a synaptosomal associated protein called Snap 25 mediates the NCC migration and neurite extension [16]. After careful comparisons of these reports, we found that only nineteen genes are common in these two studies. The deviations may be due to stage difference of the mutant mice used in their studies and therefore may represent the stage-specific ENS markers.

Instead of ENS markers, we screened for the GDNF positively regulated genes. To achieve this, we have performed microarray analysis using the purified enteric NCCs. We found that 45 genes are differentially expressed and 29 of them are up-regulated upon the GDNF treatment (16 h). Among these 29 up-regulated genes, 72% of them (21/29) have also been found to be reduced in *Ret* mutant mice (*Ret*$^{k-}$/ *Ret*^{k}) [15,16]. In addition, we have also identified eight genes, including a G-protein coupled receptor called GPR73 or Prokineticin receptor -1 (PK-R1), which have not been described in the previous screens with *Ret* mutant mice [15,16]. Here, we focused, in particular, on a cross-talk between GDNF/Ret and Prokineticin-1 (Prok-1) which has been recently demonstrated via PK-R1 to mediate differentiation and proliferation of enteric NCCs [17]. Therefore, in this study we have revealed not only a panel of new candidate genes implicated in GDNF/Ret signaling, but also a new pathway in which Prok-1 may provide an additional layer of signaling refinement to maintain proliferation and differentiation of enteric NCCs.

2. Materials and methods

2.1. Animals

Mice carrying the *Ret.k*$^{-}$ mutation were crossed randomly with a mixed genetic background [11]. The genotype of embryos and animals was determined by PCR, as has been described previously [11]. The day of a vaginal plug observed was considered E0.5. For cultures of enteric NCCs, wild-type ICR, *Ret.k*$^{-}$ mutant mice and their wild-type littermates were used.

2.2. NCC culture

E11.5 ICR mouse guts (from stomach to hindgut) were dissected in L15 medium (Invitrogen, Rockville, MD). Guts were washed with Ca^{2+} and Mg^{2+} free PBS and digested with collagenase/dispase (0.2 μg/ml each; 37 °C for 10 min). Digested guts were triturated into single cells and filtered through cell strainers (100 μm and then 40 μm). NCCs were then isolated from the mesenchymal cells using MACS® separator (Miltenyi Biotec, Bergisch Gladbach, Germany) and anti-p75^{NTR} antibody (Abcam, MA, USA) according to the manufacturer's protocol. Isolated cells were resuspended in NCC medium, DMEM containing 15% Chick embryo extract (SIL, Sussex, UK), FGF (20 ng/ml, Sigma, St. Louis, MO), EGF (20 ng/ml, Sigma), Retinoic acid (35 ng/ml, Sigma), N2 (1%), B27 (2%, Invitrogen), β-mercaptoethanol (50 mM, Sigma), and plated onto poly-D-lysine- and fibronectin (20 μg/ml each, Life Technologies Inc.)-coated wells according to a previously-described protocol [17,18]. The NCCs were seeded at clonal density (1×10^5 cells per each 35-mm wells and 5×10^3 cells per each 20-mm well). Subsequent immunostaining analysis using anti Ret antibody (Neuromics, MN, USA) showed that the purity of isolated NCC is approximately 80%. The differentiation capability of isolated cells has also been tested in presence of GDNF (50 ng/ml) (supplement 1).

Ret.k^{-} mutant mice: Esophagus and stomach of the homozygous *Ret.k*$^{-}$ mice and their wild-type littermates were dissected out and digested as stated above. Due to the limited amount of mutant embryos, the NCCs were enriched by multiple replating as described [17] instead of being isolated with cell sorting system used in the wild-type ICR mice. The digested cells from one individual gut were first grown in a 5-mm well (48 well plate) for 3 days with the NCC medium. After genotyping, the NCCs of the same genotype (approximate from 6 to 8 mutant embryos) were pooled together and grew at the clonal density. The third passage of the NCCs was used for the subsequent experiments. NCCs were stained with p75^{NTR} antibody to assess the purity of culture. The purity of the third passage of NCC culture was higher than 80%.

2.3. Microarray analysis

NCCs were cultured in presence or absence of GDNF (50 ng/ml) for 8 and 16 h. RNA was then extracted from treated and untreated NCCs using TRIzol reagent (Invitrogen) and purified by RNeasy mini kit (Qiagen, Hilden, Germany). Three independent experiments have been performed. Three 5 μg total RNA samples from treated and untreated NCCs were used to prepare material for hybridization of six GeneChip Mouse Expression Set Genome 430 2.0 arrays (Affymetrix) according to the manufacturer's protocol. Hybridization, scanning and generation of raw expression data were performed by a core facility at the Genome Research Center, University of Hong Kong. Data analysis was performed by using GENE SPRING 7 software (Agilent Technologies, Palo Alto, CA).

2.4. Reverse Transcription-PCR (RT-PCR)

Total RNA was isolated from NCCs by TRIzol reagent (Invitrogen) and reverse transcribed in 20 μl reaction system using SuperScript™ RNA Amplification System (Invitrogen), in accordance with the manufacturer's instructions. PCR reactions were performed using specific primers (Table 1). All the PCRs were performed separately and in the linear range of amplification (25–35 cycles). The product bands were then scanned for relative expression by comparing with that of the standard, β-actin.

2.5. Quantitative RT-PCR

RNA for RT-PCR was extracted from NCCs using TRIzol Reagent (Invitrogen) and reverse transcribed in 20 μl as above. Quantitative PCR was