Available online at www.sciencedirect.com
ScienceDirect
Biochimica et Biophysica Acta 1773 (2007) 536–545
Biochimica et Biophysica Acta
BBA
www.elsevier.com/locate/bbamcr

Prokineticin-1 modulates proliferation and differentiation of enteric neural crest cells

Elly S.W. Ngan [a,*], K.Y. Lee [a], Francesco Y.L. Sit [a], H.C. Poon [a], Jacqueline K.Y. Chan [a], M.H. Sham [b], Vincent C.H. Lui [a], Paul K.H. Tam [a,*]

[a] *Department of Surgery, University of Hong Kong, Pokfulam, Faculty of Medicine Building, 21 Sassoon Road, Hong Kong, SAR, China*
[b] *Department of Biochemistry, University of Hong Kong, Pokfulam, Hong Kong, China*

Received 5 October 2006; received in revised form 23 January 2007; accepted 24 January 2007
Available online 2 February 2007

Abstract

Prokineticins (Prok-1 and Prok-2) belong to a newly identified AVIT protein family. They are involved in variety of activities in various tissues, including smooth muscle contraction of the gastrointestinal tract and promoting proliferation of endothelial cells derived from adrenal gland. Importantly, they also act as the survival factors to modulate growth and survival of neurons and hematopoietic stem cells. In this study we demonstrated that Prok-1 (but not Prok-2) protein is expressed in the mucosa and mesenchyme of the mouse embryonic gut during enteric nervous system development. Its receptor, PK-R1 is expressed in the enteric neural crest cells (NCCs). To elucidate the physiological role(s) of Prok-1 in NCCs, we isolated the NCCs from the mouse embryonic gut (E11.5) and cultured them in the form of neurospheres. In an *in vitro* NCC culture, Prok-1 was able to activate both Akt and MAPK pathways and induce the proliferation and differentiation (but not migration) of NCCs via PK-R1. Knock-down of PK-R1 using siRNA resulted in a complete abolishment of Prok-1 induced proliferation. Taken together, it is the first report demonstrating that Prok-1 acts as a gut mucosa/mesenchyme-derived factor and maintains proliferation and differentiation of enteric NCCs.

Keywords: Prokineticin-1; Neural crest cell; Proliferation; Differentiation

1. Introduction

The enteric nervous system (ENS) in mammals is derived from the neural crest cells (NCC) which enter the foregut and colonize the entire wall of gastrointestinal tract [1]. Failure to completely colonize the gut results in the absence of enteric ganglia, as seen in Hirschsprung's (HSCR) disease in humans. Evidence increasingly indicates that neuron maturation/development is mediated by a combination of mechanism, involving both regional environmental factors as well as cell-intrinsic mediators encoded in NCCs. Glial-derived neurotrophic factor (GDNF) and its receptor, *Ret*, are the most critical players in ENS development [2,3]. Coding region mutations in *RET* account for approximately 50% of familial HSCR cases [4] and mice with null mutations in either *Ret* (Ret^{k-}) or *Gdnf* ($Gdnf^{-}$) have complete intestinal aganglionosis [5–10]. Other factors, such as endothelin 3 (EDN3) and its receptor (EDNRB) [11]; sonic hedgehog (Shh) and its receptor (Ptc) [12]; Sox 10 [13]; Phox2b [14], Mash-1 [15] are also known to be crucial for ENS development. However, coding region mutations in the HSCR genes identified so far cannot account for all HSCR patients. Incomplete penetrance of the mutations and variable expression of the phenotype indicate that additional genes may be implicated in the disease. This has led to the search for other susceptibility genes that could lead to HSCR.

* Corresponding authors. E.S.W. Ngan is to be contacted at Department of Surgery, University of Hong Kong, Pokfulam, Faculty of Medicine Building, 21 Sassoon Road, Hong Kong, SAR, China. Tel.: +852 2819 9631; fax: +852 2816 9621. P.K.H. Tam, Department of Surgery, University of Hong Kong, Pokfulam, K15, Queen Mary Hospital, Hong Kong, SAR, China. Tel.: +852 2855 4850; fax: +852 2817 3155.
E-mail addresses: engan@hkucc.hku.hk (E.S.W. Ngan), paultam@hkucc.hku.hk (P.K.H. Tam).

0167-4889/$ - see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamcr.2007.01.013

Prokineticin -1 and -2 (Prok-1 and Prok-2) are also referred to as endocrine gland derived vascular endothelial growth factor (EG-VEGF) and Bv8, respectively [16,17]. They belong to a newly- identified family of secreted proteins, the AVIT protein family [18]. These proteins were first identified in the gastrointestinal tract as potent agents mediating muscle contraction [17]. More recently, they have been found to be widely distributed in mammalian tissues. Prok-1 mRNA expression has been described in a variety of tissues, in steroidogenic glands such as the ovary, testis, adrenal gland and placenta [19–23], and also in the brain, colon, skeletal muscle, small intestine, spleen, thymus liver, bladder, prostate and uterus [19,24,25]. Prok-2, on the other hand, is most highly expressed in testis, colon, brain and peripheral blood leukocytes [16,26,27].

These two prokineticins bind not only to PK-R1, but also the other closely related G protein-coupled receptor, PK-R2. Receptor activation leads to mobilization of calcium, stimulation of phosphoinositide turnover, and activation of p44/p42 MAPK signaling pathways [28]. It is currently known that prokineticins possess diverse biological functions. Besides promoting the contraction of gastrointestinal smooth muscle, they also promote angiogenesis in ovary [23,29] and testis [26]; induce proliferation, migration and fenestration of endothelial cells derived from adrenal gland [17]; support neuronal survival [30]; cause hyperalgesia of skin; and control pain sensation [31] and behavioral circadian rhythms [18]. These two receptors have also been found recently in human and mouse hematopoietic stem cells and specific mature blood cells, including lymphocytes. Prokineticins can modulate growth, survival, and function of cells of the innate and adaptive immune systems, possibly through autocrine or paracrine signaling mechanisms [27]. Given the reported expression patterns and activities of the mammalian orthologs in the brain [16,30], reproductive tract [19,23,24,32], and cells of the immune system [27], the molecules seem to have subserved similar functions across evolution. Besides being angiogenic factors, prokineticins seem to be universal survival/mitogenic factors for various cells including endothelial cells, neuronal cells, lymphocytes and hematopoietic stem cells.

Interestingly, prokineticins and their receptors have been detected in mouse embryos as early as at E7, though their roles during embryonic development remain unclear. In this report we provide evidence that Prok-1 is involved in ENS development. First, we demonstrated that Prok-1 protein is expressed in the mucosa and mesenchyme of the mouse embryonic gut during ENS development (E11.5–E15.5). Secondly, its receptor PK-R1 (but not PK-R2) was shown to be present in enteric neural crest cells (NCCs). More importantly, functional studies using NCC culture demonstrated that Prok-1 activates Akt and MAPK pathways and induces the proliferation and differentiation of NCCs. The receptor PK-R1 activation was critical for Prok-1 induced proliferation and was completely abolished by knocking down PK-R1 using siRNA. Taken together, these findings provide evidence that Prok-1 is a new modulator for the proliferation and differentiation of enteric NCCs.

2. Methods and materials

2.1. Animals

For cultures of enteric NCCs, wild-type ICR mice were used. The day of a vaginal plug observed was considered E0.5.

2.2. Immunohistochemistry

Immunohistochemical study was performed on mouse embryos at different embryonic stages (E11.5–E17.5). Mouse embryonic samples were fixed and embedded in paraffin, subsequently sectioned and mounted on glass slides. Rabbit polyclonal antiserum to hProk-1 (1:200) was generated against a synthetic peptide of the C- terminus of hProk-1 (residues 87–105) (Zymed, San Francisco, CA) as described (27); hPK-R1 (1:150); hPK-R2 (1:150) (Lifespan Biosciences, Inc, Seattle, WA) were used for immunohistochemistry. For histological analysis, paraffin sections of mouse embryos were rehydrated using standard protocols and microwaved for 10 min in 10 mM sodium citrate pH 6.0. Sections were then incubated with the antibodies followed by incubation with secondary antibodies, anti-rabbit-IgG-Texas Red (1:200, Molecular Probe, Eugene, OR) and anti-mouse-IgG-FITC (1:200, Calbiochem, San Diego, CA) and mounted with aqueous mounting media (Vector, Burlingame, CA).

2.3. In situ hybridization

The Prok-1 and PK-R1 containing plasmids [24] were linearized with the appropriate restriction enzymes. The DIG-labelled antisense and sense transcripts were generated using T3 and T7 RNA polymerase separately with DIG RNA labeling kit (Roche, Basel Switzerland). Slides were dewaxed, rehydrated, and hybridized overnight in a humid box (containing 50% formamide, 5× SSC) with a sense or antisense DIG-labelled probe in hybridization solution (50% deionized formamide, 0.3 M NaCl, 5 mM EDTA, 20 mM Tris–HCl, 10% dextran sulphate, 2× Denhardt's buffer, 0.5 mg/ml yeast tRNA, 10 mM DTT) at 60 °C. Following a series of washings under stringent conditions (2× SSC/50% formamide and 0.1× SSC/30% formamide at 60 °C), the hybridization signal was amplified with Tyramide Signal Amplification system and Streptavidin-FITC conjugate in accordance with the manufacturer's protocol (TSA Biotin System; PerkinElmer, Wellesley, MA). The sections were then stained with the secondary antibody conjugated with Texas Red (anti-rabbit-IgG-Texas Red, 1:200, Molecular Probe). Finally, sections were counter-stained with DAPI and mounted with aqueous mounting media (Vector).

2.4. Neurosphere 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), washed with L15, 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 [12]. Mesenchyme cells grow as a monolayer, and NCCs form clusters. Cultures were replated (8×10^4 cells per each 35-mm wells) once every 3–5 days, and very few mesenchyme cells remained in culture after three passages.

2.5. Immunofluorescence

Immunofluorescence analyses were performed to localize NCCs and their derivatives using antibodies that recognized antigens of NCCs, neurons, glia, myofibroblast, proliferative cells, and apoptotic cells in accordance with a previously-described protocol [12]. Immunocytochemical analysis for NCCs was performed either using coated slides and the cytospin system (Thermo Shandon, Pittsbrugh, PA) or cells plated on chamber slides (Nalge Nunc). NCCs were fixed and blocked, were then incubated with primary antibodies: Ret (1:50,

BIL, Japan); β-tubulin, Tuj1 (1:200, Covance, Denver, PA); glial fibrillary acidic protein, GFAP (1:200, DAKO, Copenhagen, Denmark); Neurofilament M (1:500, Chemicon); Smooth muscle actin, SMA (1:400, DAKO); Tyrosine hydroxylase, TH (1:200, Chemicon); S100β (1:1000, Sigma) and nNOS (1:200, Cell signaling Tech., Beverly, MA, USA). The secondary antibodies used were Texas Red conjugated anti-rabbit (1:200) and FITC conjugated anti-mouse (1:200) from Molecular Probes and Zymed, respectively. Ret expressing cells were visualized with Tyramide Signal Amplification system and Streptavidin-Texas Red conjugate, in accordance with the manufacturer's protocol (TSA Biotin System).

2.6. BrdU incorporation and immunocytochemistry

BrdU incorporation was performed using In situ Cell Proliferation kit Fluos (Roche, Indianapolis, IN). The cells were treated with recombinant mouse Prok-1 (ED50 is 2–4 μg/ml, R&D, Minneapolis, MN) with concentrations of 2–4 μg/ml, recombinant human GDNF (50 ng/ml, PeproTech, Rocky Hill, NJ) or in combination for 24 h. BrdU was added 16 h before the termination of culture. Culture was fixed, blocked and incubated with anti-Ret antibody (1:50) (IBL). The immunosignal was amplified with Tyramide Signal Amplification system and Streptavidin- Texas Red conjugate. Genomic DNA was denatured in 4N HCl (5 min at RT). After PBS washing, the cultures were incubated with fluorescein-conjugated anti-BrdU antibody (Roche). Slides were washed in PBS and mounted with aqueous mounting media.

2.7. Cell proliferation assay

NCCs (Passage 4) were seeded at 1×10^4 cells /well in 96-well culture plates in NCC medium 20 h before treatment. The cells were treated with recombinant mouse Prok-1 with concentrations of 2–4 μg/ml in triplicate for 24 h in presence or absence of the PI-3-K inhibitor (10 μM, LY294002) and MAPK inhibitor (20 μM, PD98059) (Calbiochem), respectively. BrdU (1:1000) was added into culture 16 h before the assay. The culture was fixed, blocked, and incubated with anti-BrdU antibody. The cell proliferation rate was measured using Cell Proliferation ELISA kit, colorimetric (Roche). Three independent experiments were preformed and each in triplicate.

2.8. TUNEL

Apoptotic cells in neurosphere were analyzed on cytospin preparation of neurospheres using in situ Cell Death Detection kit, Fluorescein (Roche), following the manufacturer's protocol.

2.9. Gut explant culture

E11.5 midguts were isolated from ICR mice and both ends of the gut were placed on the filter membrane (0.45 μm, black gridded HABG, Millipore) as described [12]. The explants were then cultured in DMEM supplemented with 10% FBS in the absence (vehicle) or presence of Prok-1 (4 μg/ml), GDNF (100 ng/ml) or in combination for 48 h. Then, $Tuj1^+$ NCCs migrated onto the membrane were then visualized by immunostaining with anti-Tuj1 antibody.

2.10. RT-PCR

Total RNA was isolated from NCCs by TRIzol reagent (Life technologies Inc.) and reverse transcribed in 20 μl reaction system using SuperScript™ RNA Amplification System, in accordance with the manufacturer's instructions. PCR reactions were performed using specific primers: PK-R1 (Forward: 5′ GCGGCATTGGAAACTTCA3′; Reverse: 5′GGCCCACGAATTCTATGCC 3′) for 35 cycles; PK-R2 (Forward: 5′CGGCAGCTCTCCTGGGAG-CATGGC3′; Reverse: 5′CGTCTGGAACCCAGGGACTGCC3′) for 40 cycles; NOS (Forward: 5′GATCCAGTGGTCCAAGCTGCAG3′; Reverse: 5′CAG-CAGCATGTTGGACACAGCG3′); ChAT (Forward: 5′GACCAGC-TAAGGTTTGCAGCCAG3′; Reverse: 5′CTCAACTGAGTGAACA-GATCA3′); VIP (Forward: GTAGGCTGGATGACAGGATG3′; Reverse: 5′ GTAGTTATCTGTGAAGACGGC3′; NLF (Forward: 5′CCAGGTA GCCGCCATCAGCCAG3′; Reverse:5′GCGAGCCTGCAGGTTGCG-CAGA3′) for 30 cycles; actin (Forward: 5′ GAATTCATTTTTGAGACCTT-CAA 3′; Reverse: 5′ CCGGATCCATCTCTGCCTCGAAGTCCA 3′) for 25 cycles. The estimated sizes of RT-PCR products for PKR1, PK-R2, NOS, ChAT, VIP, NLF and actin were 506, 432 bp, 462 bp, 314 bp, 298 bp, 320 bp and 306 bp, respectively.

2.11. Immunoblotting

To analyze levels of phosphorylation of AKT and MAPK proteins, cells were plated at 0.2×10^6 cells/well in 6-well dish 2 days prior to treatment. The attached cells were then starved in phenol-red free medium in absence of CEE for 16 h before the treatments. The cells were then treated with Prok-1 (4 μg/ml) in the presence or absence of PI-3-K inhibitor (LY294002, 10 μM) and MAPK inhibitor (PD98059, 20 μM) for 24 h before harvesting. In total, 20 μg of total protein from cell lysates was separated on 10% SDS-polyacrylamide gels and blotted with a 1:1000 dilution of antibodies against phospho-AKT (Ser 473), phospho-MAPK (p44/p42), total AKT, or total MAPK (p44 and p42) (Cell Signaling). The same membranes were analyzed with a 1: 2500 dilution of anti-β-actin monoclonal antibody (Chemicon International, Inc., Temecula, CA, USA) as a protein loading control. All blots were incubated with 1:5000 dilution of secondary HRP-conjugated anti-mouse or anti-rabbit antibody (Amersham Pharmacia Biotech., Piscataway, NJ, USA).

2.12. Statistical analysis

Differences among multiple treatment groups were analyzed by one-way analysis of variance ANOVA followed by the Tukey posttest. A p value less than 0.05 represented a statistically-significant difference.

3. Results

3.1. Prok-1 is a mucosa/mesenchymal-derived factor in mouse embryonic gut

In mice, NCCs reach the foregut at approximately embryonic day 10.5 (E10.5) and complete their colonization by ~E14.5. To substantiate the potential role(s) of these peptides in enteric nervous system development, spatial–temporal expression patterns of Prok-1 and Prok-2 in the guts of mouse embryos were analyzed by immunohistochemistry and *in situ* hybridization. At E11.5 upwards, NCCs coalesced into presumptive myenteric region between developing longitudinal and circular muscles at the outer circumference underneath the serosa as a layer of cells and express NCC marker, $p75^{NTR}$ and the neuronal precursor marker, Tuj1. Immunohistochemistry using antibody against Prok-1 showed that Prok-1 protein is expressed in both the mucosa and mesenchyme. At E15.5, relatively higher expression of Prok-1 was observed in mucosa (Fig. 1C). The primary-minus control or staining with peptide pre-absorbed antibody (data not shown) showed a weak non-specific signal (Fig. 1, Ctrl, D). This observation was further confirmed by localizing Prok 1 transcripts detected in mucosa/mesenchyme region using *in situ* hybridization (Fig. 1E–G). Prok-1 expression was not detected in the enteric ganglia at the stages examined (Fig. 1). On the contrary, the structurally related family member Prok-2 was not detected in the embryonic guts by *in situ* hybridization (data not shown).

3.2. PK-R1, but not PK-R2, is expressed in enteric NCCs

The existence of receptors is required for Prok-1 to exert its function(s), so the expressions of the receptors of prokineticins,

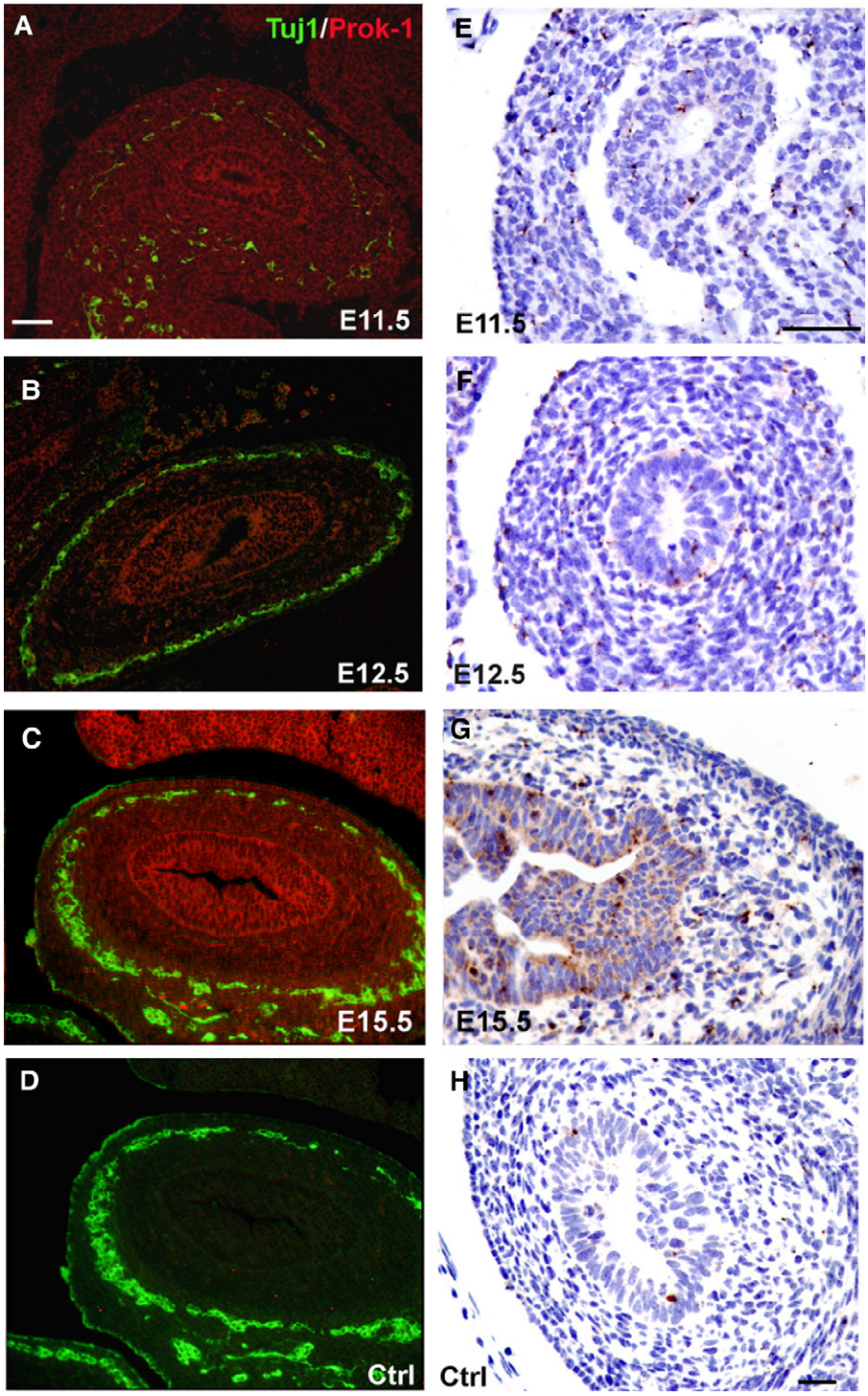

Fig. 1. Spatial and temporal expression of Prok-1 in mouse embryonic guts. (A–C) Immunohistochemistry using antibody specific to Prok-1. Prok-1 protein was localized at the mucosa and mesenchyme of the embryonic guts (E11.5–15.5) (Red). (D) The immunosignals were specific because no signal was detected in the primary-minus control (Ctrl). NCCs were localized at the gut mesenchyme using anti-Tuj1 antibody (Green). (E–H) *In situ* hybridization analysis of Prok-1 expression in mouse embryonic guts (E11.5–15.5, E–G) using DIG-labeled antisense and sense (Ctrl, H) probes. Consistently, Prok-1 transcripts were located in both the mucosa and mesenchyme. Signal was specific because no signal was detected when sense probe was used (Ctrl, H). Bars 100 μm.

PK-R1 and PK-R2, were examined. Immunohistochemistry using PK-R1 specific antibody showed that PK-R1 protein is localized in both the gut mesenchyme and presumptive myenteric region underneath the serosa (Fig. 2). Tuj1 and PK-R1 double-staining in enteric NCCs confirms that PK-R1 is expressed by enteric neurons (Fig. 2C, G). The signal was specific because only weak signal was seen in the mucosa region when IgG isotype control was used (Fig. 2D). Expression

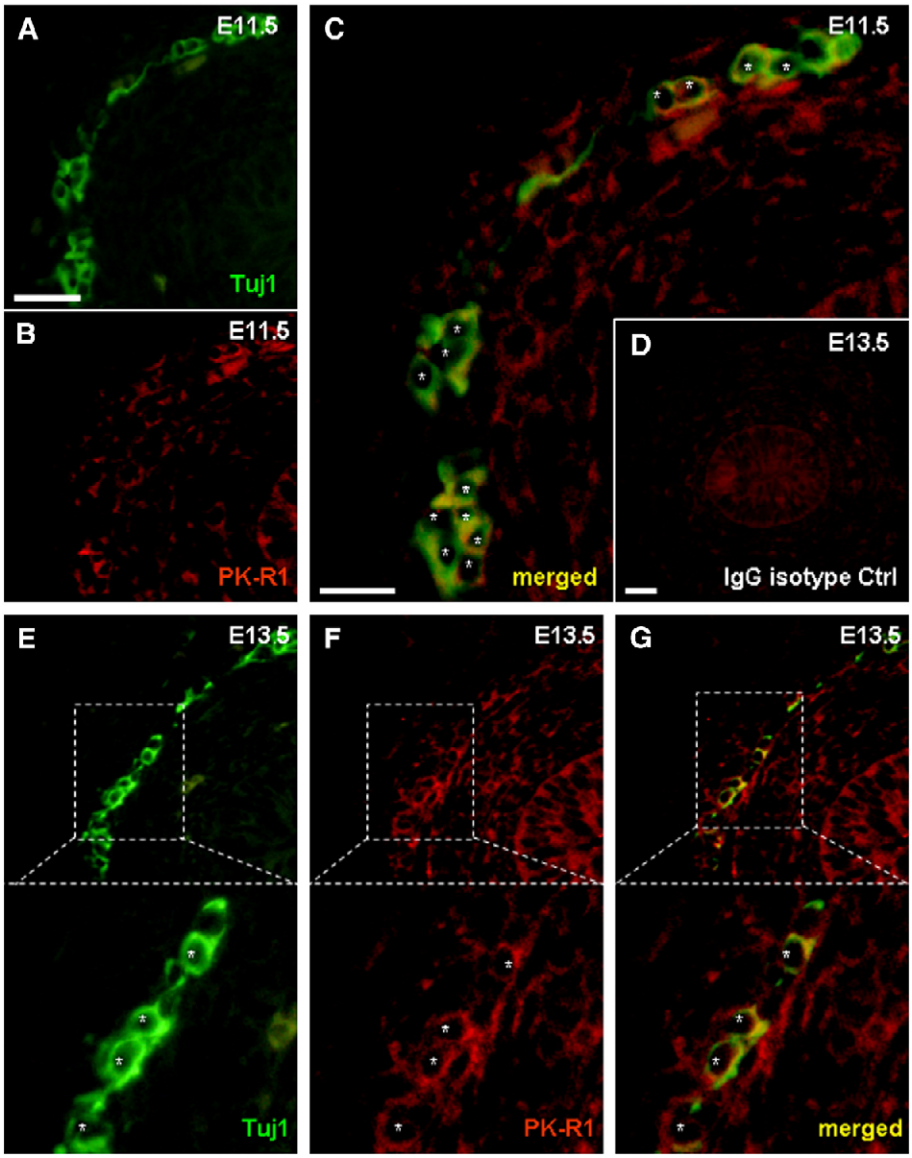

Fig. 2. Localization of PK-R1 in mouse embryonic guts. Representative figures from immunohistochemical analysis using antibody specific to PK-R1 on mouse embryonic gut (A–C: E11.5 and E–G: E13.5). NCCs were located at the gut mesenchyme with Tuj1 antibody (A and E, green). Immunosignal specific to PK-R1 protein was detected at both NCCs and mesenchyme of guts (B and F). (D) Staining with IgG isotype control showed a weak nonspecific signal. Photos A–C and E–G were taken at the same magnification (400×). Photos D were taken at 200×. Bars 100 μm.

of PK-R1 transcript in NCCs was further confirmed by *in situ* hybridization and using another NCC marker, $p75^{NTR+}$ (data not shown). Noteworthy, although PK-R1 was found in the embryonic gut, PK-R2 was not (data not shown).

3.3. Prok-1 promotes proliferation of NCCs

In an attempt to further demonstrate that PK-R1 is expressed in "primitive" NCCs, we isolated the NCCs from E11.5 embryonic guts and enriched in culture as described [12] for this study. The isolated NCCs are "primitive" ($p75^{NTR+}$/Ret^{+}/$Tuj1^{-}$) and expressed various transcription factor genes characteristic of embryonic NCCs, including slug, snail, twist, Pax3 and Sox10 (data not shown). Using semi-quantitative RT-PCR, we confirmed the expression of PK-R1 in NCCs, where human endometrium was used as positive control (Fig. 3A). To assess the mitogenic potential of Prok-1 on NCCs, BrdU incorporation assays were performed in the absence or presence of Prok-1. Based on the information provided by the supplier (R&D), the ED50 of the recombinant mouse EG-VEGF (Prok-1) is 1–4 μg/ml (85 nM–340 nM), which is determined by its ability to stimulate ^{3}H-thymidine incorporation by EJG cells (endothelial cell line derived from adrenal tissue). Therefore, the concentration we used in the experiments is the ED50 of this recombinant peptide recommended by the product provider (2 and 4 μg/ml). After 24-h treatments with vehicle (Ctrl) and Prok-1, NCCs were stained with NCC marker (Ret) and BrdU antibodies. Consistently, 26.6±2.7% of NCCs were proliferating (Ret^{+}/$BrdU^{+}$) in vehicle control, whereas Prok-1 increased proliferating cells (Ret^{+}/$BrdU^{+}$) to 43.5±3.7% ($p<0.05$) (Fig.

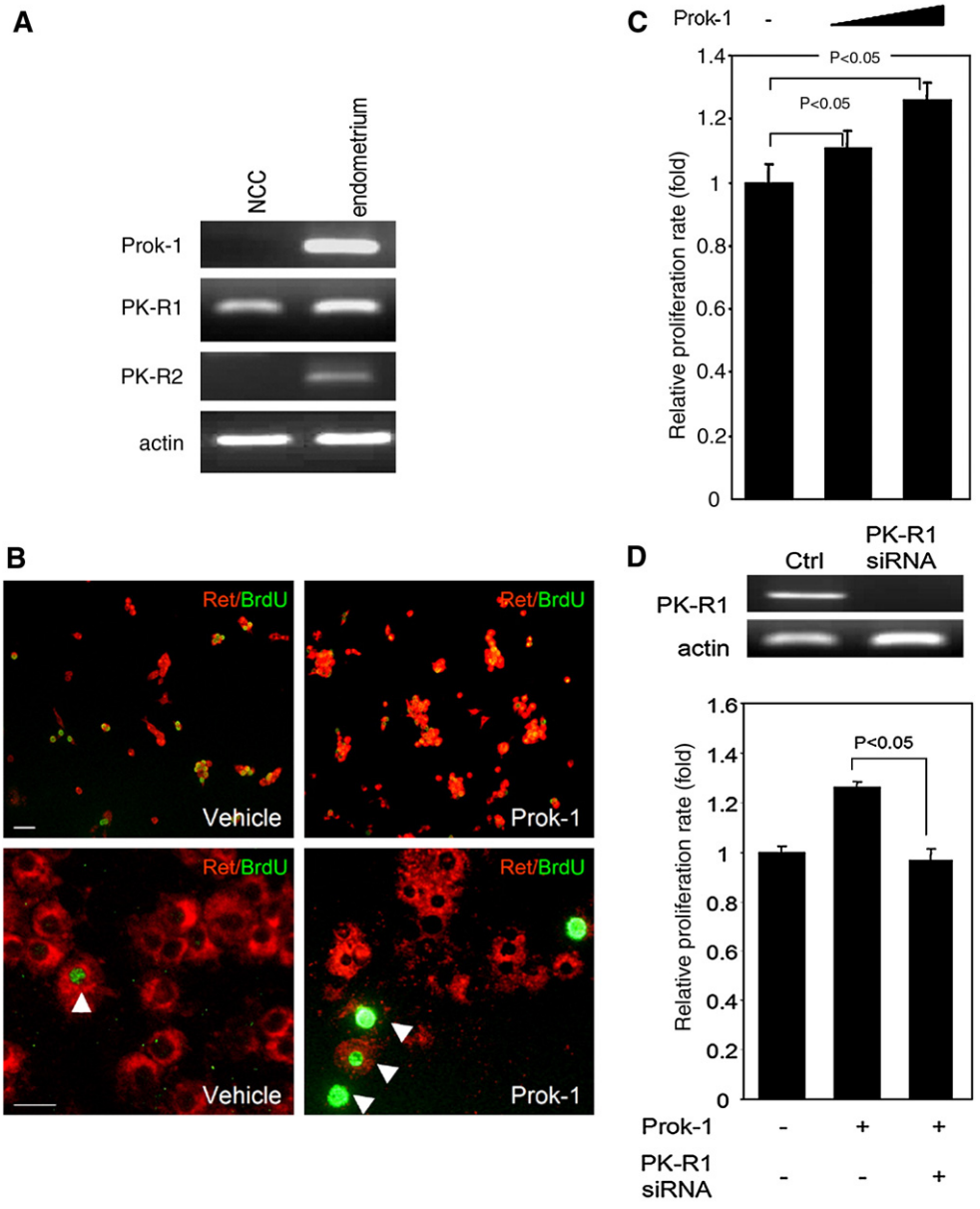

Fig. 3. Prok-1 promotes the proliferation of NCCs. (A) PK-1, but not Prok-1 or PK-R2, transcript was detected in isolated NCCs by RT-PCR. cDNA of human endometrium were used as the positive control. Actin was used as the internal control. (B) BrdU incorporation and immunocytochemistry. NCCs were cultured in the absence (Vehicle) or presence of Prok-1 for 24 h. NCCs were then stained with anti-Ret antibody (Red) and proliferative cells were detected with anti-BrdU antibody (Green). Pictures at upper panel and lower panel were taken at 200× and 400×, respectively. Bars 100 μm. The percentages of proliferating NCCs ($Ret^{+}/BrdU^{+}$) in Vehicle control and Prok-1 treated culture were summarized in the table below. (At least three randomly chosen fields of total 200 cells from each culture were counted) (C) Cell proliferation ELISA assay. NCCs were treated with vehicle, increasing concentration Prok-1 for 24 h. Proliferating cells had been incorporated with BrdU; then the relative proliferation rate was measured by ELISA using anti-BrdU antibody. (D) RT-PCR analysis on the expression of PK-R1 in NCCs 24 h after transfection with PK-R1 siRNA and the non-silencing control (Ctrl). Cell proliferation ELISA assays were performed in siRNA transfected NCCs treated with or without Prok-1. The value reported in bar charts represent the mean±SEM of 3 independent assays, each in triplicate. Data were analyzed by one-way ANOVA followed by the Tukey posttest. A p value less than 0.05 is representing statistical significance.

3B). Similarly, when a more quantitative cell proliferation ELISA assay was used, a dose dependent increment (2 μg/ml: 12.5±2.4% 4 μg/ml: 23.7±2.8% $p<0.05$) on the proliferation rate was observed with Prok-1 treatment, relative to the vehicle treated control (Fig. 3C). TUNEL assays were also performed to examine the apoptotic cells in the culture and only a very small proportion of apoptotic cells were found in the culture either in the presence or the absence of Prok-1 (data not shown).

To directly evaluate whether PK-R1 is crucial for Prok-1 mediated proliferation, we transfected PK-R1 siRNA or a control siRNA into the NCCs and cultured for 24 h after Prok-1 stimulation. According to RT-PCR analysis, PK-R1 expression decreased significantly in cells transfected with PK-R1 siRNA when compared to that of the cells transfected with the control siRNA (Fig. 3D). Consistently, PK-R1 knockdown completely abolished Prok-1 induced proliferation (Fig. 3D).

3.4. Prok-1 induces the differentiation, but not migration of NCCs

Besides promoting proliferation, Prokineticins have also been shown to be able to stimulate the differentiation of hematopoietic cells [27]. To further assess the ability of Prok-1 to induce differentiation of NCCs, Prok-1 was administrated to NCC culture for 48 h. Like GDNF, Prok-1 induced the expression of neuron- (Tuj1$^+$/TH$^+$/NFL$^+$) and glial- (GFAP$^+$/S100$^+$) specific markers (Fig. 4A). In addition, the long-term treatment with Prok-1 (6 days) also up-regulated the expression of neuronal nitric oxide synthase (nNOS) and vasoactive intestinal polypeptide (VIP), but not choline acetyltransferase (ChAT), substance P and iNOS (data not shown) (Fig. 4B and C). It indicated that Prok-1 is able to promote the differentiation of NCCs, at least, to neural/glial lineages.

Prokineticins have been implicated in the mediation of hematopoietic cell mobilization [27]. Therefore, the effect of

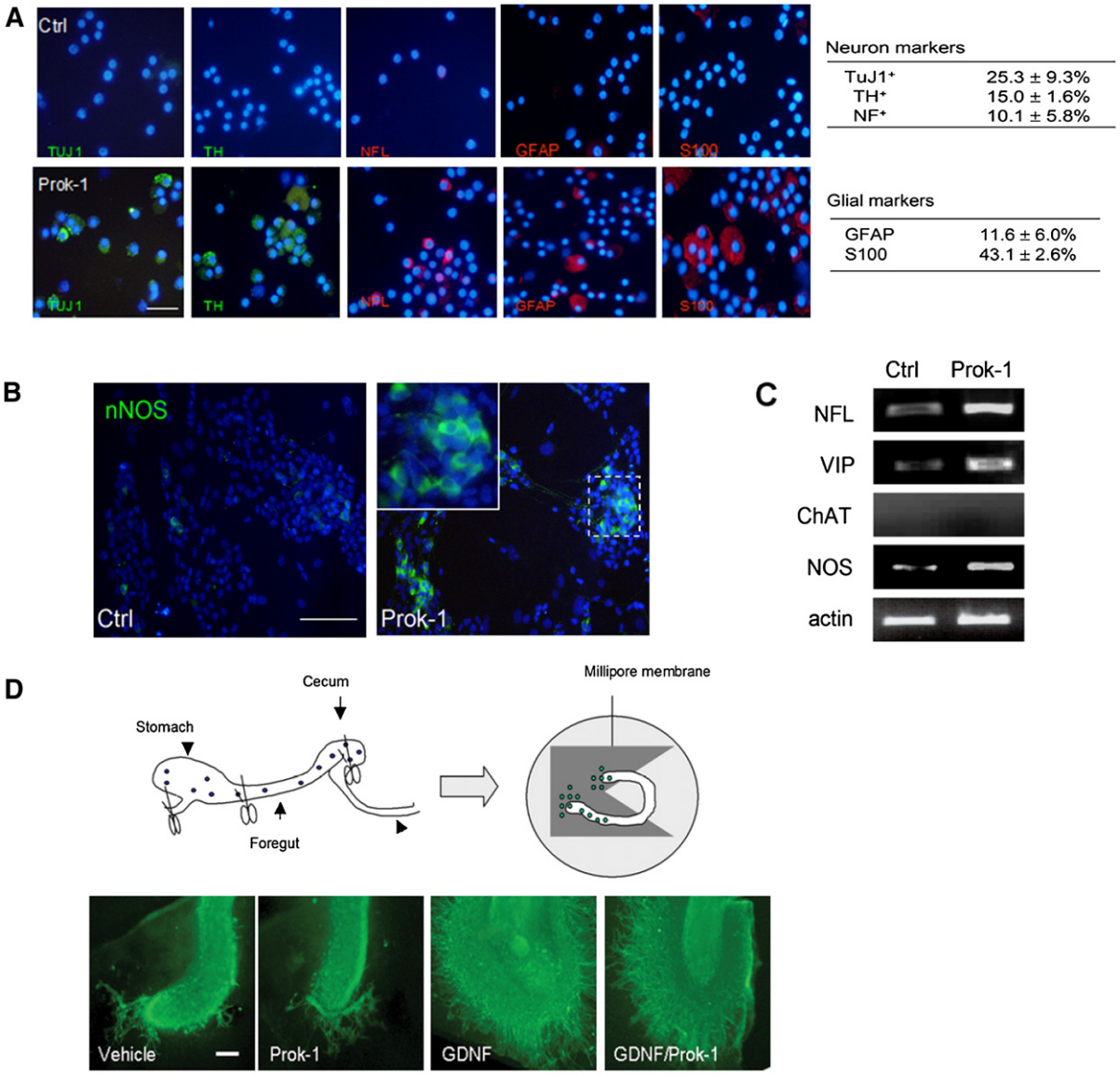

Fig. 4. Prok-1 promotes differentiation but not migration of NCCs. (A) Prok-1 induces expression of neuronal and glial markers in NCCs. Representative pictures for immunocytochemical analysis of Prok-1 treated NCCs. NCCs were cultured in the presence of vehicle or Prok-1 for 48 h. Then, the cells were cytospun onto the coated slides and subjected to immunocytochemical analysis using antibodies specific to neuronal- (Tuj1, TH and neurofilament) and glial- (GFAP and S100) lineages. In the vehicle control, majority of cells remained "primitive" and express neither neuronal nor glial markers. On the other hand, Tuj1$^+$, TH$^+$, Neurofilament$^+$. GFAP$^+$ or S100$^+$ NCCs were found in the culture containing Prok-1. The percentages of cells expressing neuronal and glial markers in Prok-1 treated culture were summarized in the table. At least three randomly chosen fields of total 200 cells from each culture were counted in two independent experiments. Pictures were taken at 400×. Bars 50 μm (B) Immunocytochemical analysis of Prok-1 treated NCCs with nNOS antibody. NCCs were cultured in the presence of vehicle or Prok-1 for 6 consecutive days. Pictures were taken at 200×. Bars 100 μm. (C) Expression of neurofilament L (NFL), vasoactive intestinal polypeptide, Choline acetyltransferase, nitric oxide synthase (NOS) in Prok-1 treated NCCs (6 days) were analyzed by RT-PCR. Actin was used as the internal control. (D) E11.5 mid- guts were isolated from ICR mice and placed on the filter membrane. The explants were then cultured in the absence (vehicle) or presence of Prok-1, GDNF or in combination for 48 h. Tuj1$^+$ NCCs migrated onto the membrane were then visualized by immunostaining with anti-Tuj1 antibody. Bar 200 μm.

Prok-1 on NCC migration was examined in an *ex vivo* gut explant culture in accordance with a previously described protocol [12]. E11.5 midguts were isolated from ICR mice and placed on the filter membrane. The explants were then cultured in the absence (vehicle) or presence of Prok-1, GDNF or in combination for 48 h. Tuj1$^+$ NCCs migrated onto the membrane were then visualized by immunostaining with anti-Tuj1 antibody. Unlike GDNF, Prok-1 was unable to induce the migration of NCCs of E11.5 gut in the *ex vivo* culture (Fig. 4D).

3.5. Prok-1 mediated proliferation depends on both MAPK and Akt phosphorylation

Recently, the signal transduction events initiated by Prok-1 in hematopoietic stem cells were reported [27]. Given that Prok-1 promotes mitogenesis and differentiation of NCCs, MAPK and Akt activation were evaluated by using MAPK and Akt inhibitors (PD98059 and LY294002) as well as phosphorylation-specific antibodies. Consistently, by ELISA assay, Prok-1 enhanced the proliferation of NCCs by 1.2±0.03 fold relative to untreated control. In the presence of MAPK inhibitor (PD98059), Prok-1 induced proliferation was completely abolished (1.0±0.05 fold relative to the vehicle treated control, $p<0.05$). Further, Akt inhibitor (LY294002) ablates not only the Prok-1 induced, but also the intrinsic proliferation of NCCs (0.76±0.05 fold) (Fig. 5A). Subsequent Western blot analysis of phosphorylated MAPK, p44 and p42, or phosphorylated Akt (a PI-3-K target) further confirmed the activations of the MAPK and Akt pathways by Prok-1 in NCCs. Comparable to other cells, Prok-1 markedly induced phosphorylation of MAPK within 20 min of stimulation. It is also noteworthy that unlike in other cells, Prok-1 mainly increased phosphorylated p44, but not p42 in NCCs (Fig. 5B). The activation was specific and inhibited by MAPK inhibitor (PD98059). Similarly, Akt phosphorylation was markedly increased above control culture at 20 min of incubation with Prok-1 and was abolished by Akt inhibitor (LY294002) (Fig. 5C).

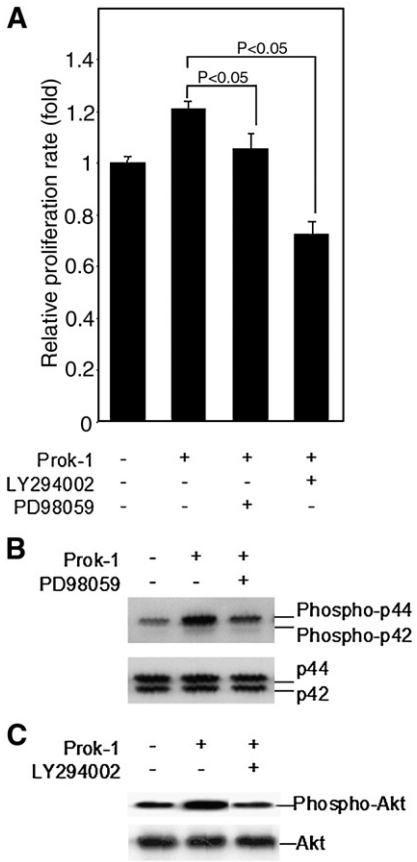

Fig. 5. Proliferative effect of Prok-1 is mediated by PI-3-K and MAPK pathways. (A) Cell proliferation ELISA assay. NCCs were treated with vehicle, Prok-1 in the presence or absence of PI-3-K inhibitor (LY294002) and MAPK inhibitor (PD98059) for 24 h. Proliferating cells were incorporated with BrdU and the relative proliferation rate was measured by ELISA using anti-BrdU antibody. The value reported in bar charts represent the mean±SEM of 3 independent assays, each in triplicate. Data were analyzed by one-way ANOVA followed by the Tukey posttest. A p value less than 0.05 indicates statistical significance. (B, C) Western blot analysis. NCCs were cultured in the presence of vehicle, Prok-1, Prok-1+MAPK inhibitor (PD98059) and Prok-1+PI-3-K inhibitor (LY294002) for 20 min. Cell lysates were obtained and western blot was performed. Prok-1 significantly increased the phosphorylation of both MAPK (B) and Akt (C) compared to vehicle. Prok-1-induced NCC Proliferation (A) and substrate phosphorylation (B, C) was inhibited by MAPK and PI-3-K inhibitors.

4. Discussion

The hypothesis was tested that Prok-1 signaling is not limited to mediating the muscle contraction of gastrointestinal tract in mature gut but also regulates the development of the ENS from crest-derived emigrants. Transcripts encoding Prok-1, and its receptor PK-R1 were detected in the bowel as early as at E11.5 which is a critical time point for colonization. Both Prok-1 and PK-R1 expressions were persistent throughout development. However, NCCs express only PK-R1, and not the closely related receptor PK-R2. Together with the subsequent PK-R1 knock down experiment further confirmed that Prok-1 exhibits its functions only via PK-R1 during ENS development. As a matter of fact, the distinct expression patterns of Prokineticin receptors were observed in various tissues. In the adrenal gland, only PK-R1 was detected [33]. In the ovary, follicular cells predominantly express PK-R1, whereas corpus luteum-derived cells express high levels of both PK-R1 and PK-R2 [21]. Similarly, these two receptors also co-exist in the endometrium, but at different expression level [19,24]. Therefore, it is believed that prokineticins may have different angiogenic as well as non-angiogenic functions in different tissues via acting on either PK-R1 or PK-R2. Recently, *PK-R1*$^{-/-}$ and *PK-R2*$^{-/-}$ mice were generated [34]. *PK-R2*$^{-/-}$ mice exhibited hypoplasia of olfactory bulb and abnormal reproductive system, whereas *PK-R1*$^{-/-}$ showed no phenotypic defect in these two systems. It suggests that these two receptors are playing differential roles in various systems. *PK-R1*$^{-/-}$ mice are vital, indicating that a functional enteric nervous system can develop without PK-R1 expression. While subtle morphological or physiological deficits may exist, but not as severe as the aganglionosis associated with Ret$^{-/-}$ /Gdnf$^{-/-}$. *PK-R1*$^{-/-}$ mice would be a good model for the future study on the *in vivo* function of Prok-1 on ENS development.

In this report, we elucidated function of Prok-1 in an *in vitro* NCC culture system, which was established using E11.5 embryonic guts. Immunohistochemical studies demonstrated that, if not all, at least a population of NCCs at E11.5 embryonic guts have already committed to neural fate and expressed neuronal marker, Tuj1 (Fig. 1). After the NCCs were isolated and grew in the NCC specific growth medium, the majority of NCCs were shown to be "primitive" and did not express Tuj1 (Fig. 4). This is perhaps because the NCC growth medium only favors the growth of "primitive" cells and the committed or differentiated cells were removed after multiple re-plating. Alternatively, we cannot exclude the possibility that those committed cells are able to dedifferentiate. More importantly, the "primitive" ($p75^{NTR+}$/RET^{+}/$Tuj1^{+}$) NCCs express functional receptors for Prok-1 and are multipotent. In sum, this provided a firm foundation for us to assess the role of Prok-1 on NCC proliferation and differentiation.

Using our *in vitro* NCC culture system, we showed that Prok-1, like GDNF, is able to induce the proliferation and differentiation of NCCs. Downstream, both GDNF and Prok-1 share common elements, most prominently the MAPK and PI3K pathways, which provide multiple points of intersections between these two factors. As reported recently by Srinivasan *et. al*., the PI3K/Akt/FOXO signaling pathway (but not MAPK, pathway) is pivotal for GDNF stimulated ENS precursor survival and neurite extension [35]. In our study, we found that both Akt and MAPK signalings are essential for the Prok-1 induced proliferation of NCCs, and Akt and MAPK inhibitors completely abolish Prok-1 induced proliferation. Further, in some aspects, GDNF and Prok-1 also work quite differently. GDNF is a potent chemoattractant for NCCs [36], which mediates the rostro-caudal migration of NCCs for gut colonization. However, Prok-1 was not able to induce migration of NCCs in the *ex vivo* gut explant cultures. Taken together, GDNF and Prok-1 may share some but not all the downstream elements. Importantly, they exhibit similar biological functions. The functional redundancy of Prok-1 and GNDF may support the idea that Prok-1 provides a compensatory pathway to ensure the proper development of ENS. To demonstrate this idea, a double mutant (*c-Ret*$^{+/-}$ and *PK-R1*$^{-/-}$) mice may be required for the future studies.

Recently, Micci et al. successfully used neural stem cell isolated from the central nervous system (CNS) to perform transplantation in the stomach and rescued gastric function in neuronal nitric oxide synthase-deficient mice [37]. However, neurons of the CNS, although resembling enteric neurons in many ways, are derived from a distinct pool of precursor cells in early embryonic life. Enteric neurons are the end product of migratory stem cells originating in the neural crest. Therefore, based on the assumption that cells are more "naturally poised" to generate a particular tissue and require little or no manipulation either *in vivo* or *in vitro*, enteric neural crest cells are more obvious candidates for use in the cell replacement therapy for neuroenteric disorders. It is noteworthy that Prokineticins is a universal survival factor/mitogen and subserves similar functions in both CNS and peripheral nervous system (PNS). In CNS, mainly Prok-2, mediates neural protection or survival [16,30] and Prok-1 is a mediator for the proliferation of neuronal precursor cells in ENS. The data from this study, therefore, may not only enhance our understanding of the biology of ENS development, but also the future optimization of NCC or neural stem cell culture condition for the therapeutic use.

Acknowledgments

The authors are grateful to Prof. Sophia Tsai and Dr. MM Garcia-Barcelo for their invaluable comments and to Dr. David Wilmshurst who proofread the text. This work was supported by Seed Funding grant for Basic Research from the University of Hong Kong to ESW Ngan.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbamcr.2007.01.013.

References

[1] D. Newgreen, H.M. Young, Enteric nervous system: development and developmental disturbances—Part 2, Pediatr. Dev. Pathol. 5 (2002) 329–349.
[2] R.H. Baloh, H. Enomoto, E.M. Johnson Jr., J. Milbrandt, The GDNF family ligands and receptors—Implications for neural development, Curr. Opin. Neurobiol. 10 (2000) 103–110.
[3] M. Saarma, GDNF—A stranger in the TGF-beta superfamily? Eur. J. Biochem. 267 (2000) 6968–6971.
[4] M. Angrist, S. Bolk, B. Thiel, E.G. Puffenberger, R.M. Hofstra, C.H. Buys, D.T. Cass, A. Chakravarti, Mutation analysis of the RET receptor tyrosine kinase in Hirschsprung disease, Hum. Mol. Genet. 4 (1995) 821–830.
[5] P. Durbec, C.V. Marcos-Gutierrez, C. Kilkenny, M. Grigoriou, K. Wartiowaara, P. Suvanto, D. Smith, B. Ponder, F. Costantini, M. Saarma, GDNF signalling through the Ret receptor tyrosine kinase, Nature 381 (1996) 789–793.
[6] H. Enomoto, P.A. Crawford, A. Gorodinsky, R.O. Heuckeroth, E.M. Johnson Jr., J. Milbrandt, RET signaling is essential for migration, axonal growth and axon guidance of developing sympathetic neurons, Development 128 (2001) 3963–3974.
[7] M.W. Moore, R.D. Klein, I. Farinas, H. Sauer, M. Armanini, H. Phillips, L.F. Reichardt, A.M. Ryan, K. Carver-Moore, A. Rosenthal, Renal and neuronal abnormalities in mice lacking GDNF, Nature 382 (1996) 76–79.
[8] J.G. Pichel, L. Shen, H.Z. Sheng, A.C. Granholm, J. Drago, A. Grinberg, E.J. Lee, S.P. Huang, M. Saarma, B.J. Hoffer, H. Sariola, H. Westphal, GDNF is required for kidney development and enteric innervation, Cold Spring Harb. Symp. Quant. Biol. 61 (1996) 445–457.
[9] M.P. Sanchez, I. Silos-Santiago, J. Frisen, B. He, S.A. Lira, M. Barbacid, Renal agenesis and the absence of enteric neurons in mice lacking GDNF, Nature 382 (1996) 70–73.
[10] A. Schuchardt, V. D'Agati, L. Larsson-Blomberg, F. Costantini, V. Pachnis, Defects in the kidney and enteric nervous system of mice lacking the tyrosine kinase receptor Ret, Nature 367 (1994) 380–383.
[11] R.P. Kapur, Early death of neural crest cells is responsible for total enteric aganglionosis in Sox10(Dom)/Sox10(Dom) mouse embryos, Pediatr. Dev. Pathol. 2 (1999) 559–569.
[12] M. Fu, V.C. Lui, M.H. Sham, V. Pachnis, P.K. Tam, Sonic hedgehog regulates the proliferation, differentiation, and migration of enteric neural crest cells in gut, J. Cell Biol. 166 (2004) 673–684.
[13] E.M. Southard-Smith, L. Kos, W.J. Pavan, Sox10 mutation disrupts neural crest development in Dom Hirschsprung mouse model, Nat. Genet. 18 (1998) 60–64.

[14] A. Pattyn, X. Morin, H. Cremer, C. Goridis, J.F. Brunet, The homeobox gene Phox2b is essential for the development of autonomic neural crest derivatives, Nature 399 (1999) 366–370.
[15] E. Blaugrund, T.D. Pham, V.M. Tennyson, L. Lo, L. Sommer, D.J. Anderson, M.D. Gershon, Distinct subpopulations of enteric neuronal progenitors defined by time of development, sympathoadrenal lineage markers and Mash-1-dependence, Development 122 (1996) 309–320.
[16] M.Y. Cheng, C.M. Bullock, C. Li, A.G. Lee, J.C. Bermak, J. Belluzzi, D.R. Weaver, F.M. Leslie, Q.Y. Zhou, Prokineticin 2 transmits the behavioural circadian rhythm of the suprachiasmatic nucleus, Nature 417 (2002) 405–410.
[17] M. Li, C.M. Bullock, D.J. Knauer, F.J. Ehlert, Q.Y. Zhou, Identification of two prokineticin cDNAs: recombinant proteins potently contract gastrointestinal smooth muscle, Mol. Pharmacol. 59 (2001) 692–698.
[18] A. Kaser, M. Winklmayr, G. Lepperdinger, G. Kreil, The AVIT protein family. Secreted cysteine-rich vertebrate proteins with diverse functions, EMBO Rep. 4 (2003) 469–473.
[19] S. Battersby, H.O. Critchley, K. Morgan, R.P. Millar, H.N. Jabbour, Expression and regulation of the prokineticins (endocrine gland-derived vascular endothelial growth factor and Bv8) and their receptors in the human endometrium across the menstrual cycle, J. Clin. Endocrinol. Metab. 89 (2004) 2463–2469.
[20] J.Y. Chung, Y. Song, Y. Wang, R.R. Magness, J. Zheng, Differential expression of vascular endothelial growth factor (VEGF), endocrine gland derived-VEGF, and VEGF receptors in human placentas from normal and preeclamptic pregnancies, J. Clin. Endocrinol. Metab. 89 (2004) 2484–2490.
[21] N. Ferrara, G. Frantz, J. LeCouter, L. llard-Telm, T. Pham, A. Draksharapu, T. Giordano, F. Peale, Differential expression of the angiogenic factor genes vascular endothelial growth factor (VEGF) and endocrine gland-derived VEGF in normal and polycystic human ovaries, Am. J. Pathol. 162 (2003) 1881–1893.
[22] N. Ferrara, J. LeCouter, R. Lin, Endocrine gland vascular endothelial growth factor (EG-VEGF) and the hypothesis of tissue-specific regulation of angiogenesis, Endocr. Res. 28 (2002) 763–764.
[23] J. LeCouter, J. Kowalski, J. Foster, P. Hass, Z.M. Zhang, L. Dillard-Telm, G. Frantz, L. Rangell, L. DeGuzman, G.A. Keller, F. Peale, A. Gurney, K.J. Hillan, N. Ferrara, Identification of an angiogenic mitogen selective for endocrine gland endothelium, Nature 412 (2001) 877–884.
[24] E.S. Ngan, K.Y. Lee, W.S. Yeung, H.Y. Ngan, E.H. Ng, P.C. Ho, Endocrine gland derived vascular endothelial growth factor is expressed in human peri-implantation endometrium, but not in endometrial carcinoma, Endocrinology 147 (1) (2005) 88–95.
[25] L. Zhang, N. Yang, J.R. Conejo-Garcia, D. Katsaros, A. Mohamed-Hadley, S. Fracchioli, K. Schlienger, A. Toll, B. Levine, S.C. Rubin, G. Coukos, Expression of endocrine gland-derived vascular endothelial growth factor in ovarian carcinoma, Clin. Cancer Res. 9 (2003) 264–272.
[26] J. LeCouter, R. Lin, M. Tejada, G. Frantz, F. Peale, K.J. Hillan, N. Ferrara, The endocrine-gland-derived VEGF homologue Bv8 promotes angiogenesis in the testis: localization of Bv8 receptors to endothelial cells, Proc. Natl. Acad. Sci. U. S. A. 100 (2003) 2685–2690.
[27] J. LeCouter, C. Zlot, M. Tejada, F. Peale, N. Ferrara, Bv8 and endocrine gland-derived vascular endothelial growth factor stimulate hematopoiesis and hematopoietic cell mobilization, Proc. Natl. Acad. Sci. U. S. A. 101 (2004) 16813–16818.
[28] T. Soga, S.I. Matsumoto, T. Oda, T. Saito, H. Hiyama, J. Takasaki, M. Kamohara, T. Ohishi, H. Matsushime, K. Furuichi, Molecular cloning and characterization of prokineticin receptors, Biochim. Biophys. Acta, Gene Struct. Expr. 1579 (2002) 173–179.
[29] T. Kisliouk, N. Levy, A. Hurwitz, R. Meidan, Presence and regulation of endocrine gland vascular endothelial growth factor/prokineticin-1 and its receptors in ovarian cells, J. Clin. Endocrinol. Metab. 88 (2003) 3700–3707.
[30] D. Melchiorri, V. Bruno, G. Besong, R.T. Ngomba, L. Cuomo, A. De Blasi, A. Copani, C. Moschella, M. Storto, F. Nicoletti, G. Lepperdinger, F. Passarelli, The mammalian homologue of the novel peptide Bv8 is expressed in the central nervous system and supports neuronal survival by activating the MAP kinase/PI-3-kinase pathways, Eur. J. Neurosci. 13 (2001) 1694–1702.
[31] C. Mollay, C. Wechselberger, G. Mignogna, L. Negri, P. Melchiorri, D. Barra, G. Kreil, Bv8, a small protein from frog skin and its homologue from snake venom induce hyperalgesia in rats, Eur. J. Pharmacol. 374 (1999) 189–196.
[32] C. Wechselberger, R. Puglisi, E. Engel, G. Lepperdinger, C. Boitani, G. Kreil, The mammalian homologues of frog Bv8 are mainly expressed in spermatocytes, FEBS Lett. 462 (1999) 177–181.
[33] R. Lin, J. LeCouter, J. Kowalski, N. Ferrara, Characterization of endocrine gland-derived vascular endothelial growth factor signaling in adrenal cortex capillary endothelial cells, J. Biol. Chem. 277 (2002) 8724–8729.
[34] S. Matsumoto, C. Yamazaki, K.H. Masumoto, M. Nagano, M. Naito, T. Soga, H. Hiyama, M. Matsumoto, J. Takasaki, M. Kamohara, A. Matsuo, H. Ishii, M. Kobori, M. Katoh, H. Matsushime, K. Furuichi, Y. Shigeyoshi, Abnormal development of the olfactory bulb and reproductive system in mice lacking prokineticin receptor PKR2, Proc. Natl. Acad. Sci. U. S. A. 103 (2006) 4140–4145.
[35] S. Srinivasan, M. Anitha, S. Mwangi, R.O. Heuckeroth, Enteric neuroblasts require the phosphatidylinositol 3-kinase/Akt/Forkhead pathway for GDNF-stimulated survival, Mol. Cell. Neurosci. 29 (2005) 107–119.
[36] H.M. Young, C.J. Hearn, P.G. Farlie, A.J. Canty, P.Q. Thomas, D.F. Newgreen, GDNF is a chemoattractant for enteric neural cells, Dev. Biol. 229 (2001) 503–516.
[37] M.A. Micci, K.M. Kahrig, R.S. Simmons, S.K. Sarna, M.R. Espejo-Navarro, P.J. Pasricha, Neural stem cell transplantation in the stomach rescues gastric function in neuronal nitric oxide synthase-deficient mice, Gastroenterology 129 (2005) 1817–1824.