Implications of Endocrine Gland–Derived Vascular Endothelial Growth Factor/Prokineticin-1 Signaling in Human Neuroblastoma Progression

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Abstract Purpose: Neuroblastoma is a common pediatric tumor that is derived from improperly differentiated neural crest cells (NCC). We recently revealed that endocrine gland–derived vascular endothelial growth factor/prokineticin-1 (EG-VEGF/Prok-1) is a key factor mediating the growth and differentiation of enteric NCCs during development. In this report, we further elucidate its role in neuroblastoma progression.

Experimental Design: We studied the expression and copy number of EG-VEGF/Prok-1 receptors (PK-R1 and PK-R2) in 26 neuroblastoma tumors by real-time reverse transcription-PCR and immunohistochemical analysis. Implication of EG-VEGF/Prok-1 signaling in neuroblastoma progression was further shown in a neuroblastoma cell line (SK-N-SH).

Results: We found that all neuroblastoma samples from stages II to IV expressed both PK-R1 and PK-R2. Kruskall-Wallis signed rank tests revealed that the expression level of PK-R1 transcript is associated with the stages and metastasis of the neuroblastoma (P < 0.05), and PK-R2 is persistently higher in advanced-stage neuroblastoma samples. About 38% of the neuroblastoma tumors (10:26) possessed MYCN amplification, whereas no PK-R1 and PK-R2 amplifications were detected, suggesting that the overexpression of the receptors was not due to gene amplification. Subsequent functional studies showed that EG-VEGF/Prok-1 activates the Akt pathway to induce the proliferation of neuroblastoma cells. Targeted down-regulation studies revealed that EG-VEGF/Prok-1–mediated proliferation requires the presence of these two receptors, and that PK-R2 is essential for inhibiting apoptosis. In vitro migration and invasion assays also indicated that EG-VEGF/Prok-1 significantly enhances the cell migration/invasion of SK-N-SH.

Conclusions: Our study has shown for the first time that aberrant EG-VEGF/Prok-1 signaling favors neuroblastoma progression and could be a potential target for future neuroblastoma treatment.
related and belong to a newly identified AVIT protein family (12). These proteins are distributed widely in mammalian tissues and are known to bind to two closely related G protein–coupled receptors, PK-R1 and PK-R2. Receptor activation leads to the mobilization of calcium, the stimulation of phosphoinositide-3-kinase turnover, and the activation of the mitogen-activated protein kinase (MAPK) signaling pathways (13). It is known that these two prokinetins subserve similar functions and are involved in a variety of activities in various tissues. Originally identified as potent agents in contracting the smooth muscle of the gastrointestinal tract (14), they were later shown to act also as angiogenic mitogens, promoting angiogenesis in the ovary (11, 15) and testis (16) and inducing the proliferation, migration, and fenestration of endothelial cells derived from the adrenal gland (11). These molecules have also been shown to act as the survival factor modulating the growth, survival, and function of cells of the innate and adaptive immune systems, including hematopoietic stem cells and lymphocytes (17). In the central nervous system, Prok-2 not only supports neuronal survival (18), but also controls pain sensation (19) and behavioral circadian rhythms (10). More recently, we have shown that EG-VEGF/Prok-1 also mediates the development of the enteric nervous system from neural crest–derived emigrants. EG-VEGF/Prok-1 acts as a gut mucosa-derived factor and promotes the survival/proliferation and differentiation of enteric NCCs. It also works coordinately with the glial cell line–derived neurotrophic factor and provides an additional layer of signaling refinement for the glial cell line–derived neurotrophic factor pathway during enteric nervous system development.

This study was undertaken to determine whether aberrant prokineticin signaling promotes neural crest–derived neuroblastoma progression and, if so, the underlying mechanisms by which these effects are mediated. We show here that a high expression level of PK-R1 is associated with malignant clinical characteristics of the tumor, and that EG-VEGF/Prok-1 directly influences neuroblastoma progression by promoting the proliferation and migration/invasion of neuroblastoma cells.

This report therefore provides the first evidence for the implication of EG-VEGF/Prok-1 signaling in the neuroblastoma progression, suggesting that EG-VEGF/Prok-1 signaling might be an attractive therapeutic target for neuroblastoma treatment.

Materials and Methods

Primary neuroblastoma tumor samples. A total of 26 neuroblastoma tumor specimens were included in this study. Fourteen samples were obtained from the Department of Surgery at the University of Hong Kong and 12 from the Second Affiliated Hospital of China Medical University, Shenyang. All subjects (5 females and 21 males) were unrelated Han Chinese and recruited between July 2001 and December 2005. Tumors were classified according to the International Neuroblastoma Staging System on the basis of postoperative histopathological examination. These included 3 tumors of stage I, 8 of stage II, 13 of stage IV, and 2 of missing data. Ages at diagnosis of the patients ranged from 4 months to 9 years (median, 28.5 months; mean, 29.3 ± 4.2 months). Ethical approval for this study was obtained from the Institutional Review Board of the University of Hong Kong/Hospital Authority Hong Kong West Cluster, and the Second Affiliated Hospital of China Medical University, Shenyang. Informed consent was received from each subject.

Cell culture. The human neuroblastoma SK-N-SH cell line from the American Type Culture Collection (Manassas, VA) was grown in DMEM/10% fetal bovine serum (Invitrogen, Rockville, MD). Reverse transcription-PCR. Total RNA was isolated from the SK-N-SH cell line 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 done using specific primers: PK-R1 (forward: 5′-GCGCCGATTGGAACCTC-3′; reverse: 5′-GCCGCCAGCTTCTGTGAGCTGC-3′); PK-R2 (forward: 5′-CCGGACAGCTCCTGGAGACATGG-3′; reverse: 5′-CGTGTGGAACCCCGGACTGTCG-3′) for 40 cycles; and actin (forward: 5′-GAATTCATTTTGGAGACCTTCA-3′; reverse: 5′-CCGGATCTCATCCTGGCCTGAAGTC-3′) for 25 cycles. The estimated sizes of reverse transcription (RT)-PCR products for hPK-R1, hPK-R2, and actin were 506, 432, and 306 bp, respectively. Results were normalized and expressed relative to the internal control, actin.

RNA interference. The PK-R1 short interfering (si) RNA (AAAGA-TAAGGTAAGACTCTAA), PK-R2 siRNA (AAGCGTCCTCTTTATGGCTAA), and a nonspecific siRNA control (AATTTCTCGAACTGTCATGC) were used. SK-N-SH cells numbers were used in triplicate for 24 h in the presence or absence of the phosphoinositide-3-kinase inhibitor (10 μmol/L, LY294002; Calbiochem, San Diego, CA). Bromodeoxyuridine (1:1,000) was added into the culture 4 h before the assays. The cultures were fixed, blocked, and incubated with anti-bromodeoxyuridine antibody. The cell proliferation rate was measured using Cell Proliferation ELISA kit, colorimetric (Roche, Indianapolis, IN). Three independent experiments were done, and each experiment was done thrice.

Apoptosis. Apoptosis was measured using the Annexin V-FITC apoptosis detection kit from BD PharMingen (San Jose, CA). Antibodies to 0.1 to 0.2 × 106 cells per well were grown for 24 h in DMEM containing 0.1% fetal bovine serum, alone or with EG-VEGF/Prok-1 (4 μg/mL) in a six-well plate. Both adherent and floating cells were collected and resuspended in 1× cold binding buffer (10 mmol/L, MgCl2, 0.4% bovine serum albumin) for analysis. Cells were also stained with propidium iodide to detect dead cells. Analysis was done on a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) using CellQuest software (Becton Dickinson). Unstained cells were classified as “live;” cells stained for Annexin V only were “early apoptotic;” cells stained for both Annexin V and propidium iodide were “late apoptotic;” and cells stained for propidium iodide only were “dead.”

Cell migration and invasion assays. Cell migration/motility was measured using the Falcon cell culture polyethyleneterphthalate inserts with a pore size of 8 μm in a 24-well format (Falcon, Le Pont de Claira, France). Cells were trypsinized and washed twice with serum-free medium (DMEM). Cells (5 × 104) were then seeded in 350 μL DMEM medium alone or with EG-VEGF/Prok-1 (4 μg/mL) in the top chamber of the cell culture inserts, whereas the bottom chamber was filled with 900 μL DMEM supplemented with 10% fetal bovine serum as chemoattractant. Cells were then incubated for 16 h under normal growth conditions. For cell invasion, cells were prepared as above but were seeded on extracellular matrix–coated, 8-mm, pore-sized Matrigel invasion chambers (BD Biosciences, San Jose, CA) and cultured for up to 2 h.

To determine the number of cells migrating (uncoated membrane) or invading (extracellular matrix–coated membrane) through the
membrane, membranes were fixed and stained with 0.5% crystal violet solution. After washing with water, nonmigrating or noninvading cells were removed by wiping the top of the membrane with a cotton-wool tip. Cells migrating or invading through the membrane were manually counted using magnified (×200) digital pictures of insert/membranes (eight fields for each membrane). Experiments were done at least twice, with samples run in triplicate. An invasion index was calculated as the number of cells invading through the membrane in the treated cells divided by the number of cells migrating through the membrane in the control.

**Immunoblotting.** To analyze the levels of phosphorylation of Akt and MAPK proteins, cells were plated at 0.5 × 10⁵ cells per well in 100-mm dishes 2 days before treatment. The attached cells were then starved in phenol red-free medium in the absence of fetal bovine serum for 24 h before the treatment. The cells were then treated with EG-FGFProk-1 (4 μg/mL) in the presence or absence of the phosphoinositide-3-kinase inhibitor (LY294002, 10 μmol/L) and MAPK inhibitor (PD98059, 20 μmol/L) 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 1:1,000 dilution of antibodies against phospho-Akt (Ser473), phospho-MAPK (p42/p44), total Akt, or total MAPK (p44 and p42; Cell Signaling Technology, Beverly, MA). The same membranes were analyzed with a 1:1,000 dilution of anti-β-actin monoclonal antibody (Chemicon International, Inc., Temecula, CA) as a protein-loading control. All blots were incubated with 1:5,000 dilution of secondary horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibody (Amersham Pharmacia Biotech, Piscataway, NJ).

**Immunohistochemistry.** An immunohistochemical study was done on human neuroblastoma tissue samples. Neuroblastoma samples were fixed and embedded in paraffin, subsequently sectioned, and mounted on glass slides. Rabbit polyclonal antisera to PK-R1 (1:150; Lifespan Biosciences, Inc., Seattle, WA), anti-p21 (1:150), and anti-Ki-67 (ab14314; Abcam) were used for the immunohistochemistry. For histologic analysis, paraffin sections of mouse embryos were rehydrated using standard protocols and microwaved for 10 min in 10 mmol/L sodium citrate (pH 6.0). Sections were then incubated with the antibodies and subsequently incubated with secondary antibodies (Calbiochem) and mounted with aqueous mounting media (Vector, Burlingame, CA).

**Quantitative reverse transcription-PCR.** RNA for RT-PCR was extracted from neuroblastoma samples using TRIzol reagent (Invitrogen) and reverse transcribed in 20 μL as above. Quantitative PCR was done in the reaction mix, which consisted of 1× mastermix, actin forward and reverse primers, actin probe, forward and reverse primers for hPK-R1 (accession no. Hs367765) and hPK-R2 (accession no. Hs375029), and their probes (Applied Biosystems, Foster City, CA). The reaction mix (18 μL) was aliquoted into tubes, and 2 μL cDNA was added. Duplicated 20-μL samples plus positive and negative controls were placed in a PCR plate, and wells were sealed with optical caps. The PCRs were carried out using an ABI Prism 7700 (Applied Biosystems). All primers and probes were designed by Applied Biosystems. Data were analyzed and processed using Sequence Detector version 1.6.3 (Applied Biosystems) in accordance with the manufacturer's instructions. Primers and probes were optimized, and the linearity of the results validated in serial dilution of a cDNA pool. Results were expressed relative to an internal positive standard (cDNA obtained from a single sample of neuroblastoma) included in all reactions.

**Real-time quantitative PCR.** DNA was extracted from neuroblastoma tissues and blood (normal control) using QIAmp tissue kit (Qiagen) in accordance with the manufacturer's protocols. TaqMan PCR was then done using the ABI Prism 7700 (Applied Biosystems) with primers and probes for MYCN and TGN46 (accession no. BC02819) as described above. All primers and probes were designed by Applied Biosystems. A standard curve was constructed in each PCR run with 4-fold serial dilutions containing 20, 5, 1.25, 0.3125, and 0.078125 ng/mL of a healthy donor’s DNA, and the dosages of the target genes in each sample were interpolated using the standard curves. The gene copy number of a sample of DNA was determined by the ratio of the target gene dosage to the TGN46 dosage. Copy numbers were expressed as the average of two measurements.

**Statistical analysis.** Statistical analysis was done using the SPSS statistics software package (SPSS, Chicago, IL). Statistical comparisons for the PK-R1 and PK-R2 expression in patients were done with the Kruskall-Wallis signed rank test or Mann-Whitney U test as appropriate. Differences between stages (II-IV) were analyzed using the Kruskal-Wallis test, a nonparametric equivalent to one-way ANOVA. When the Kruskal-Wallis test was significant, the Mann-Whitney U test was used to analyze the observed differences between the three groups. Mann-Whitney U test was also used to analyze the differences between metastasis and nonmetastasis groups and neuroblastoma and non-tumor groups. The differences among multiple treatment groups were analyzed by one-way ANOVA, followed by Tukey’s test. A P value <0.05 represented a statistically significant difference.

**Results**

**Expression level of hPK-R1 correlates with the stages of neuroblastoma.** Total RNA was isolated from 26 neuroblastoma samples at various stages. Using quantitative RT-PCR,
expression levels of PK-R1 and PK-R2 in the neuroblastoma samples were examined. Consistently higher expressions of PK-R1 were found in the advanced-stage neuroblastoma samples. The expression levels of PK-R1 varied considerably, being 3.7 ± 1.9 at stage II, n = 3; 46.5 ± 70.6 at stage III, n = 8; and 110.9 ± 156.9 at stage IV, n = 13. An association was found between the expression of PK-R1 transscripts and the stages of the neuroblastoma (P < 0.05, Kruskall-Wallis signed rank and Mann-Whitney U tests; Fig. 1A), but not with the age (data not shown). Similarly, although the expression level of PK-R2 was relatively lower, higher expression was found in neuroblastoma samples from stage III (62.5%, five out of eight) and IV (61.5%, 8 out of 13; when compared with the mean value of its relative expression level in stage II; Fig. 1B). However, there was no significant association of hPK-R2 expression with the stage of neuroblastoma. It is noteworthy that the expression of PK-R1 was not associated to that of hPK-R2 in neuroblastoma (P > 0.05; data not shown).

To confirm the protein expression of PK-R1 and PK-R2 in neuroblastoma samples, immunohistochemical studies were done on sections of neuroblastoma samples from different stages (stages II, III, and IV). Immunosignals of PK-R1 were detected in the cytoplasm of all neuroblastoma cells (Fig. 1C), whereas PK-R2 was mainly expressed in a subset of cells (Fig. 1C). In addition, a high immunosignal for PK-R2 protein was also detected in some of the advanced neuroblastoma samples (stage III, Fig. 1C). All tumors displaying high mRNA levels also displayed high protein levels. Consistent with our real-time RT-PCR results, more intensive staining of PK-R1 was seen in the neuroblastoma samples from stage IV than in those from earlier stages (stages II and III).

Overexpression of PK-R1 and PK-R2 is not due to gene amplifications. Gene amplification is common in neuroblastoma and may result in overexpression of oncprotein. Using DNA-based real-time quantitative PCR, we simultaneously quantified hPK-R1 (2p13), hPK-R2 (2p12), and MYCN (2q24) and a reference gene, TGN46 (2q11), using a previously described protocol (20, 21). Copy number as hPK-R1/TGN46, hPK-R2/TGN46, and MYCN/TGN46 ratios of 26 neuroblastoma patients were shown in Fig. 2. MYCN amplification was detected in 10 patients (1 at stage II, 3 at stage III, and 6 at stage IV). None of them had hPK-R1 or hPK-R2 amplification. In addition, there was no correlation between the MYCN amplification and the overexpression of PK-R1 and PK-R2.

Prokineticins mediate proliferation of human neuroblastoma cell line, SK-N-SH. The existence of receptors is required for EG-VEGF/Prok-1 to exert its function(s), so the expressions of the prokineticin receptors (PK-R1 and PK-R2) in a neuroblastoma cell line, SK-N-SH, was examined. RT-PCR analysis revealed that PK-R1 and PK-R2 were expressed in this neuroblastoma cell line (Fig. 3A).

To assess the mitogenic potential of EG-VEGF/Prok-1 in the neuroblastoma cell line, bromodeoxyxuridine incorporation assays were done in both the absence and presence of EG-VEGF/Prok-1. After 24-h treatments with vehicle (Ctrl), EG-VEGF/Prok-1 (2 and 4 μg/mL), quantitative cell proliferation ELISA assays were done. Increments of 22.4 ± 2.3% and 30.1 ± 2.3% (P < 0.05) on the proliferation rate were observed with 2 and 4 μg/mL EG-VEGF/Prok-1 treatments, respectively, relative to the vehicle-treated control (Fig. 3B). Interestingly, a similar proliferative effect was also observed with Prok-2 treatment (19.4 ± 2.2%, 2 μg/mL; and 30.9 ± 1.3%, 4 μg/mL; Fig. 3B; P < 0.05). In addition, prokineticins-treated SK-N-SH showed a 200% to 300% increase in cell number over the 4-day culture, compared with a 50% increase in the untreated control (Fig. 3C, P < 0.05).

EG-VEGF/Prok-1 induced proliferation is dependent on Akt phosphorylation. Signal transduction events initiated by EG-VEGF/Prok-1 in hematopoietic stem cells (17) and NCCs3 have recently been reported. Given that EG-VEGF/Prok-1 promotes mitogenesis, MAPK and Akt activation were evaluated by Western blot analysis of phosphorylated MAPK, p44 and p42, or phosphorylated Akt (a phosphoinositide-3-kinase target). In SK-N-SH cells, EG-VEGF/Prok-1 induced more marked Akt phosphorylation within 20 min of stimulation than in other cells. The activation was specific and inhibited by Akt inhibitor (10 μmol/L, LY294002; Fig. 3D). Surprisingly, unlike in NCCs, EG-VEGF/Prok-1 did not activate MAPK phosphorylation in SK-N-SH cells (Fig. 3E). Subsequent ELISA assay also showed that Akt inhibitor (10 μmol/L, LY294002) completely abolished the proliferative effect of prokineticin in SK-N-SH cells (Fig. 3F; EG-VEGF/Prok-1, 1.3 ± 0.18-fold; LY294002, 0.72 ± 0.07-fold relative to the vehicle-treated control; P < 0.05).
PK-R1 and PK-R2 are required for the prokineticin-induced proliferation. To directly evaluate whether PK-R1 or PK-R2 is crucial for EG-VEGF/Prok-1-mediated proliferation, we transfected PK-R1 siRNA, PK-R2 siRNA, or a control siRNA into the SK-N-SH cells and cultured for 24 h after EG-VEGF/Prok-1 (4 μg/mL) stimulation. According to RT-PCR analysis, PK-R1 and PK-R2 expression decreased significantly in cells transfected with PK-R1 and PK-R2 siRNA when compared with that of the cells transfected with the control siRNA (Fig. 4A and C). Furthermore, PK-R1 (Fig. 4B) and PK-R2 (Fig. 4D) knockdown completely abolished EG-VEGF/Prok-1–induced proliferation. Interestingly, the down-regulation of PK-R2 induces apoptosis. Annexin V staining revealed that PK-R2–down-regulated cells are less tolerant to serum starvation, and 20% more apoptotic cells were detected independent of EG-VEGF/Prok-1 treatment (Fig. 4E), whereas PK-R1 knockdown does not increase apoptotic cells. These results indicate that the expressions of both PK-R1 and PK-R2 are required for EG-VEGF/Prok-1–induced proliferation, and PK-R2 is essential for inhibiting apoptosis.

EG-VEGF/Prok-1, but not Prok-2, promotes migration and invasion of SK-N-SH cells. An association study revealed that the expression level of PK-R1 was significantly higher in the neuroblastoma patients with metastasis than in those without metastasis (Fig. 5A; \( P < 0.05 \), Mann-Whitney \( U \) test). This implies that prokineticin signaling may promote the metastasis of neuroblastoma. We therefore next aimed to determine whether the addition of EG-VEGF/Prok-1 and Prok-2 affects metastatic parameters, including migration and invasion.

Addition of EG-VEGF/Prok-1 to SK-N-SH cells significantly enhanced their migration/motility through the uncoated membrane (Fig. 5B; 150%, \( P < 0.05 \)), whereas the addition of Prok-2 had no such effect. Furthermore, PK-R1–down-regulated SK-N-SH cell was not responsive to EG-VEGF/Prok-1 stimulation, implying that PK-R1 is required for EG-VEGF/Prok-1–induced migration/motility of these cells. Similarly, down-regulation of PK-R2 also significantly reduced the migrating cells (Fig. 5C). A subsequent invasion assay also showed that EG-VEGF/Prok-1 dramatically increased SK-N-SH cell invasion throughout

*Fig. 3. EG-VEGF/Prok-1 induces the proliferation of human neuroblastoma cell line, SK-N-SH. A, RT-PCR analysis on the expression of PK-R1 and PK-R2 in the neuroblastoma cell line, SK-N-SH. B, cell proliferation ELISA assay. SK-N-SH cells were treated with vehicle, 2 and 4 μg/mL EG-VEGF/Prok-1 or Prok-2 for 24 h. Proliferating cells had been incorporated with bromodeoxyuridine for 16 h; then the relative proliferation rate was measured by ELISA using anti-bromodeoxyuridine antibody. Columns, mean of three independent assays, each in triplicate; bars, SE. Data were analyzed by one-way ANOVA followed by Tukey’s test. A \( P \) value <0.05 represents statistical significance. C, SK-N-SH cells were treated with vehicle, EG-VEGF/Prok-1 or Prok-2 for 4 d. Cell number was determined by a hemacytometer. Each point was measured in triplicate in each of the two independent experiments. D and E, Western blot analyses. SK-N-SH were cultured in the presence of vehicle, EG-VEGF/Prok-1 (4 μg/mL), EG-VEGF/Prok-1 + phosphoinositide-3-kinase inhibitor (LY294002, 10 μmol/L), and EG-VEGF/Prok-1 + MAPK inhibitor (PD98059, 20 μmol/L) for 20 min. Cell lysates were obtained, and Western blot was done. EG-VEGF/Prok-1 significantly increased the phosphorylation of Akt (D) but not MAPK (E) and compared with the vehicle. Activation of Akt signaling was inhibited by phosphoinositide-3-kinase inhibitor. F, cell proliferation ELISA assay. SK-N-SH cells were treated with vehicle, EG-VEGF/Prok-1 (4 μg/mL) in the presence or absence of phosphoinositide-3-kinase inhibitor (LY294002) for 24 h. Proliferating cells were incorporated with bromodeoxyuridine for 4 h; then the relative proliferation rate was measured by ELISA using the anti-bromodeoxyuridine antibody. Columns, mean of three independent assays, each in triplicate; bars, SE. Data were analyzed by one-way ANOVA followed by Tukey’s test. A \( P \) value <0.05 indicates statistical significance.*
extracellular matrix–coated membranes (by 2.5-fold) compared with untreated SK-N-SH cell controls (Fig. 5D; \( P < 0.05 \)). In sum, EG-VEGF/Prok-1 enhances neuroblastoma cell migration and invasion.

**Discussion**

More than 90% of neuroblastoma tumors arise in the adrenal gland, suggesting a link between perinatal tumors and adrenal development (22). The adrenal medulla develops from NCCs in conjunction with the formation of the sympathetic nervous system. Initially, NCCs migrate from the neural tube, proliferate, and form distinct neuroblastic nodules within the adrenal gland. These neuroblastic nodules gradually decrease in number, and their outer cells eventually differentiate into pheochromoblasts that will ultimately become chromaffin cells of the adult adrenal medulla. There is compelling evidence that perturbation of the involution program of these neuroblastic nodules may result in perinatal neuroblastoma.

The adrenal gland is one of the tissues expressing a high level of EG-VEGF/Prok-1 (11) in which EG-VEGF/Prok-1 induces proliferation, angiogenesis, and migration of the adrenal cortex capillary endothelial cells (23). More recently, we have shown that enteric NCCs also express PK-R1, and that the EG-VEGF/Prok-1 signal from the gut endoderm mediates the growth and differentiation of NCCs via the paracrine pathway during the development of the enteric nervous system.

In this study, our results show for the first time that the overexpression of PK-R1 in neuroblastoma is associated with tumor progression. In considering a role for prokineticin signaling in neuroblastoma progression, we have done functional analysis using a neuroblastoma cell line. We showed that EG-VEGF/Prok-1 promotes the proliferation, growth, and migration of neuroblastoma cells. Furthermore, the expressions of both PK-R1 and PK-R2 are crucial for EG-VEGF/Prok-1–induced proliferation and migration. Target-specific knockdown of either PK-R1 or PK-R2 results in a complete abolition and reduction of EG-VEGF/Prok-1–induced proliferation and migration, respectively, in SK-N-SH. Additionally, PK-R2 is able to confer resistance to apoptosis of neuroblastoma cells, as seen in corpus luteum–derived endothelial cells (24). Downregulation of PK-R2 in SK-N-SH abolished its responsiveness to EG-VEGF/Prok-1 by inducing the apoptosis. In the neuroblastoma samples, expression levels of PK-R2 were persistently higher in advanced-stage tumors, suggesting that PK-R2 may also play a role in protecting the tumor cells from apoptosis, in turn promoting tumor progression. Noteworthy, the proliferative effects of prokineticins are relatively modest, implying that

**Fig. 4.** PK-R1 and PK-R2 are essential for EG-VEGF/Prok-1–mediated proliferation. A, RT-PCR analysis on the expression of PK-R1 and PK-R2 in SK-N-SH 24 h after transfection with PK-R1 siRNA and the nonsilencing control (Ctrl). B, cell proliferation ELISA assays were done in siRNA-transfected SK-N-SH cells treated with or without EG-VEGF/Prok-1 (4 \( \mu \)g/mL). C, RT-PCR analysis on the expression of PK-R1 and PK-R2 in SK-N-SH 24 h after transfection with PK-R2 siRNA and the nonsilencing control. D, cell proliferation ELISA assays were done in siRNA-transfected SK-N-SH cells treated with or without EG-VEGF/Prok-1 (4 \( \mu \)g/mL). E, PK-R1 or PK-R2 knockdown SK-N-SH cells subjected to serum starvation. Percentages of apoptotic cells with or without EG-VEGF/Prok-1 treatment were measured by Annexin V staining as described in Materials and Methods. Columns, mean of three independent assays, each in triplicate; bars, SE. Data were analyzed by one-way ANOVA followed by Tukey’s test. **, \( P \) value < 0.05 and indicates statistical significance.
other factors must be at play that determine the bulk of the proliferative drive in the neuroblastoma cells. Rather, Prok-1 has a major impact on the migration. EG-VEGF/Prok-1 is also a potent angiogenic factor, and it may therefore also promote vascularization in the development and metastasis of neuroblastoma (3, 25). Other than the receptors, elevated EG-VEGF/Prok-1 expressions have also been implicated in colorectal (26) and prostate cancers (27). EG-VEGF/Prok-1 induces angiogenesis and promotes cell proliferation and liver metastasis in colorectal cancers. Antisense EG-VEGF/Prok-1, on the other hand, successfully inhibited EG-VEGF/Prok-1–induced angiogenesis and tumor growth in mice (26). Together, these results suggest that EG-VEGF/Prok-1 promotes neuroblastoma progression not only by inducing proliferation, migration/invasion, and vascularization, but also by inhibiting the apoptosis of neuroblastoma cells.

Interestingly, it is known that Prok-1 and Prok-2 share the two PKRs (18, 28) and, to a certain extent, exhibit different biological functions. Like EG-VEGF/Prok-1, Prok-2 is able to induce the proliferation of neuroblastoma cells. However, only EG-VEGF/Prok-1 can mediate the migration and invasion of neuroblastoma cells. As regards the distinct expression patterns of EG-VEGF/Prok-1 and Prok-2 observed in various tissues, it is believed that EG-VEGF/Prok-1 (but not Prok-2) provides the survival signaling for neuroblastoma progression. Nevertheless, the exact roles of Prok-1 and Prok-2 remain unclear. It is possible that multiple ligands and homo-/hetero-oligomerization of receptors may occur, increasing the functional complexity of this system. A new appreciation of the intricacy of peptidergic G-protein-coupled receptors may help to further delineate the system.

MYCN gene amplification is known to occur in 25% to 30% of primary neuroblastoma (29, 30). However, this study found that 38% of patients were MYCN amplified. This overrepresentation perhaps reflects the fact that there were comparatively few early-stage tumor samples available for analysis. Given the lack of correlation between MYCN amplification and PK-R1 overexpression, the MYCN-nonamplified patients not included in the current study would probably not affect the association between high PK-R1 expression and aggressive neuroblastoma phenotype. To examine PK-R1 and PK-R2 amplifications in neuroblastoma, we used quantitative real-time PCR for quick screening on the relative copy numbers of the PK-R1 and PK-R2 genes in the normal control and the neuroblastoma patients. Consistently, no amplification was found, suggesting that the overexpressions of PK-R1 and PK-R2 in neuroblastoma are unlikely to be due to gene amplifications. On the other hand, given the HapMap data showing that the PK-R1 gene (2p13.1) is located at recombination hotspots, we believe that the overexpression of PK-R1 would be the result of the gene translocation. Alternatively, other survival signals may also mediate the

**Fig. 5.** EG-VEGF/Prok-1 promotes migration and invasion of neuroblastoma cells. A, expression level of PK-R1 is associated with the metastasis of neuroblastoma. Expression levels of PK-R1 in human neuroblastoma samples with and without metastasis were measured using real-time PCR. Mann-Whitney U test was done and showed significant association (P < 0.05) between the expression level of PK-R1 and the metastasis of neuroblastoma. B, cell migration assay on SK-N-SH with and without EG-VEGF/Prok-1 or Prok-2 (4 μg/mL). C, cell migration assay on PK-R1 or PK-R2–down-regulated SK-N-SH. D, invasion index for the EG-VEGF/Prok-1–treated SK-N-SH cells and the untreated control (Ctrl). Columns, mean of three independent assays, each in triplicate; bars, SE. Data were analyzed by one-way ANOVA followed by Tukey’s test. A P value < 0.05 indicates statistical significance.
expression of PK-R1. As evident in the in vitro NCC culture, expression of PK-R1 is mediated by the MAPK and Akt signaling pathways. Bioinformatics analysis also indicates that at least two putative activator protein-1 binding sites are found in the 5′ untranslated region of PK-R1. The activation of the activator protein-1 family of transcription factors, including c-Fos and c-Jun family members, was shown as one of the earliest nuclear events induced by growth factors that stimulate MAPK (31). Therefore, it is also possible that the aberrant survival signal(s) mediates PK-R1 expression by directly activating c-Fos and c-Jun via MAPK activation. Nevertheless, the underlying mechanism by which PK-R1 expression is regulated still remains unclear.

In conclusion, this study reveals the biological significance of prokineticin signaling in neuroblastoma and illustrates that expression of PK-R1 associated with the malignant disease outcome can in fact directly influence tumor progression. An understanding of the function and regulation of unfavorable neuroblastoma signaling, therefore, may help develop an effective therapeutic approach in the management of neuroblastoma in the future.

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