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Original Article

Delta/notch-like epidermal growth factor-related receptor (DNER) orchestrates stemness and cancer progression in prostate cancer

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Abstract: DNER, Delta/Notch-like epidermal growth factor (EGF)-related receptor, is a neuron-specific transmembrane protein carrying extracellular EGF-like repeats. The function of DNER in prostate cancer has not been evaluated. Here, we showed that the upregulation of DNER is observed in various cancers, including prostate cancer. Knockdown of DNER in PC-3 cells inhibited cell proliferation, migration and invasion as well as tumorigenesis in PC-3 xenografts. DNER knockdown specifically inhibited cell growth in spheroids. RT-PCR and western blot analysis were performed, and CD44, HES1 and GLI1 expression was significantly decreased in DNER knockdown cells. Thus, DNER promotes prostate cancer progression and the growth of PC-3 cells by modulating the primary genes of cancer stem cells.

Kaywords: DNER, prostate cancer, stemness, proliferation, metastasis

Introduction

Prostate cancer is a significant health problem and the second leading cause of cancer-related death in men, resulting in 913,000 new cases and over 261,000 deaths worldwide each year [1]. During the past decade, cancer stem cells (CSCs), defined as small populations of self-renewal and multipotential differentiation, have been an increasing concept in many malignancies [2]. One or more highly conserved signal transduction pathways involved in development and tissue homeostasis, including the Notch, Sonic hedgehog, and Wnt pathways, are persistently activated in CSCs [2]. In addition, the ability of CSCs contributes to the growth, metastasis, recurrence and drug resistance of cancer [3]. Therefore, targeting these pathways to control stem-cell replication, survival and differentiation will be useful for the future development of new treatment strategies.

Notch signaling plays an indispensable role in regulating cell-fate decisions during prostate cancer progression [4, 5]. Constitutive Notch proteins are large transmembrane proteins that can be activated by the direct interaction of Notch ligands, such as DSL (Delta, Serrate and Lag) family members, expressed on apposing cells. There are share repeated EGF-like motifs in their extracellular regions between Notch and DSL family members for physiological interactions. Delta/Notch-like epidermal growth factor (EGF)-related receptor (DNER, also named HE60 and BET) is a transmembrane protein carrying extracellular EGF-like repeats and specifically expressed in somatodendritic regions of the cerebellum [6, 7]. However, DNER lacks the DSL binding domain, which is considered essential for Notch ligands [8, 9]. Although there are some structural differences, DNER has been identified as a Notch ligand that mediates cell-cell interactions [8]. In fact, DNER has also been reported to exert pathological functions as both an oncogenic and anti-oncogenic factor [10, 11].

The role of DNER in other cancers, such as glioblastoma, prompted us to analyze the effects of this protein on prostate cancer proliferation,

invasion and metastasis. Taken together, the results suggest that DNER could be of potential interest for promoting cancer progression and the growth of prostate cancer cells by modulating the primary genes of cancer stem cells.

Materials and methods

Cell lines and antibodies

Human prostate cancer cell line, PC-3 cells were from ATCC (Manassas, VA) and cultured according to their protocols. Tubulin was purchased from Cell Signaling Technology and DNER (orb156622) antibody was purchased from Biorbyt company. HES1 (sc-166410), HEY1 (sc-134362), GLI-1 (sc-515751) and CD44 (sc-65265) antibody were purchased from Santa Cruz Biotechnology.

Cell viability assay

The thiazolyl blue tetrazolium bromide (MTT) (Amresco, Solon, OH, USA) was dissolved in phosphate buffered saline (PBS) at a concentration of 5 mg/ml, filtered, and stored at 4°C. PC-3 cells were seeded into a 96-well plate at 4000 cells per well in the DMEM containing 10% FBS. After 12 hours, the medium was replaced with serum-free, glucose-free DMEM overnight. Cells were treated with control siRNA lentiviruses and DNER siRNA lentiviruses for 3 days. For the viability assay, 20 μl MTT was added into each well. The absorbance at 570 nm was measured using an ELISA reader (Biotek, Winooski, Vermont, USA) and used to determine relative cell numbers in each well. The viability of control cells was 100%.

Soft agar growth assay

The soft agar growth assay was performed according to a standard method, as described previously [12]. Cells were digested with trypsinization and plated in six parallel sets. After 18 days colonies were fixed (50% methanol in 0.9% saline solution for 15 min followed by methanol for another 15 min), stained (0.5% crystal violet) and counted as a survivor if containing more than 50 cells. Surviving fraction (SF) was calculated as: number of colonies/(number of cells plated × plating efficiency), where plating efficiency (PE) was defined as: number of control colonies obtained/number of control cells plated.

Cell cycle assay

The analysis of cell cycle was used by Cell Cycle Analysis Kit (BD Biosciences, San Jose, CA) and measured according to their protocols. PC-3 cells ($5.0*10^5$ cells per well) were cultured in 6 well/plates and allowed to adhere to the well walls for 12 hours. After starvation overnight, the cells were treated with control siRNA lentiviruses and DNER siRNA lentiviruses for 72 hours. Subsequently, the cells were collected, washed in PBS and fixed in 70% ethanol at 4°C overnight. Then, the cells were centrifuged at 2000 rpm for 5 minutes at 4°C temperature. The supernatant was removed and the cells were resuspended in 4 mL of PBS and centrifuged again. The supernatant was removed and the cell pellet was 100 μl RNase A incubating for 30 min at 37°C. Finally, 400 μl PI (propidium iodide) was added and the cells were incubated for an additional 30 minutes in the dark at 37°C. The cell cycle distribution was determined by flow cytometry for DNA content (FACScan, Becton Dickinson, Germany). Results were presented as percentage of PC-3 cells in different phases of cell cycle (G0-G1, S and G2-M) in relation to total number of cells counted.

Cell migration assay

PC-3 cells were grown to confluent monolayers on 6-well plates with parallel streaks in bottom. And vertical wounds were created by a pipette tip. Mitomycin C (Amresco) was used to inhibit cell viability. 1% FBS also was used in the assay. Progression of migration was observed and photographed at various times, four fixed fields were analyzed for each well. The percentage of inhibition was expressed using control wells at 100%. Wound images were taken with a digital camera mounted on light microscope. The wound gap widths were measured using Image J software.

Cell invasion assay

The cell invasion assay was carried out using a Transwell assay. The upper chamber of each 8.0 μm pore size Transwell apparatus (Corning, NY, USA) was coated with Matrigel (BD Biosciences, San Jose, CA). PC-3 cells were added to the upper chamber at a density of $2*10^6$ cells/ml (100 μl per chamber) after starved overnight and incubated with different

conditions and normal medium in the lower compartment. For 24 hours of incubation, the cells on the upper surface were removed by a cotton swab. The invaded cells were fixed with 4% paraformaldehyde solution, stained with 0.1% crystal violet, and quantified by manual counting and ten randomly chosen fields were analyzed for each group.

Western blots

Cells were collected with lysis buffer after being washed three times with ice-cold PBS. Lysates were boiled in SDS loading buffer for 10 min then cleared by centrifugation (14, 000 rpm, 10 min, 4°C). The proteins were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and detected with specific antibodies.

RNA isolation and real-time PCR

According to manufacturer instructions, total RNA was extracted using TRIzol (Invitrogen) and cDNA was performed by using the Reverse Transcript Kit (QIAGEN, Duesseldorf, Germany). Real-time PCR was performed in triplicate using miScript SYBR Green qRT-PCR kit (QIAGEN) on the CFX-96 Real-time PCR Detection System (Bio-Rad). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was chose as an endogenous normalization control. The cycle threshold (Ct) value was used for quantification using the $2^{-\Delta\Delta Ct}$ method. Sequences of the primers are: DNER, forward: 5'-ACCACGAAGCCGTCAGA-3'; reverse: 5'-AACCAGGAAGGCAAACAC-3'; GAPDH, forward: 5'-TGACTTCAACAGCGACACCCA-3'; reverse: 5'-CACCCTGTTGCTGTAGCCAAA-3'.

Animal studies

The six-week-old male nude mice were purchased from Vital River, Beijing. The animals were handled according to the protocol approved by the Institutional Animal Care and Use Committee of Renmin Hospital of Wuhan University. The PC-3 cells treated with control siRNA lentiviruses and DNER siRNA lentiviruses at limiting dilution (1×10^7) were subcutaneously injected with Matrigel into on the back of the mice. Tumors were allowed to grow for 16 days (approximately 5 mm in diameter). Tumor size was measured using a Vernier caliper every day until 28 days.

Cancer stem cell spheroid assay

PC-3 cells were dissociated to single cells by incubation in 0.05% trypsin-EDTA solution (Invitrogen), and re-suspended in serum-free medium containing 20 ng/ml EGF (R&D Systems), 0.4% bovine serum albumin (BSA, Sigma), B27 Supplement (1:50, Invitrogen) and 4 mg/ml insulin (Invitrogen). For quantification of the sphere forming capacity, between 1000 and 9000 cells were seeded in serum-free medium containing 1% methylcellulose (Sigma) into individual wells of poly (2-hydroxyethyl metacrylate) (Sigma) coated 96-well plates. After 7 to 10 days all spheres containing 4 or more cells were counted.

Lentivirus preparation and transfection

DNER siRNA lentiviruses were obtained from GeneChem Biotechnology (Shanghai, China), target sequences of human DNER (GenBank accession no. NM_139072.3). PC-3 cells cultured at a density of 5×10^5 cells per 6 well plate. After incubated 24 hours, the cells were transfected with DNER siRNA lentiviruses and control sequences using CON077 (GeneChem Biotechnology, China) following the manufacturer's instructions. Cell lysates were collected and RT-PCR were performed to detect mRNA expression.

Statistical analysis

All experiments were done independently at least three times. Results are represented as the Mean ± SD. The quantification of the relative increase in protein expression was performed using Image J software and was normalized with the control protein expression in each experiment. Significant differences between groups were analyzed by using the paired t-test. A *P*-value of < 0.05 was considered statistically significant.

Results

Expression levels of DNER are different between normal and cancer tissues

To determine whether differences in the expression levels of DNER exist among normal and cancer tissues from different sources, the expression of this gene was further analyzed using quantitative PCR in various normal and cancer tissues. As shown in **Figure 1A-C**, the upregulation of DNER was observed in lung and pancreatic cancers using RT-PCR, while no significant difference in DNER expression was detected between astrocytoma and normal brain tissues. Simultaneously, positive DNER expression was indicated by brown staining in

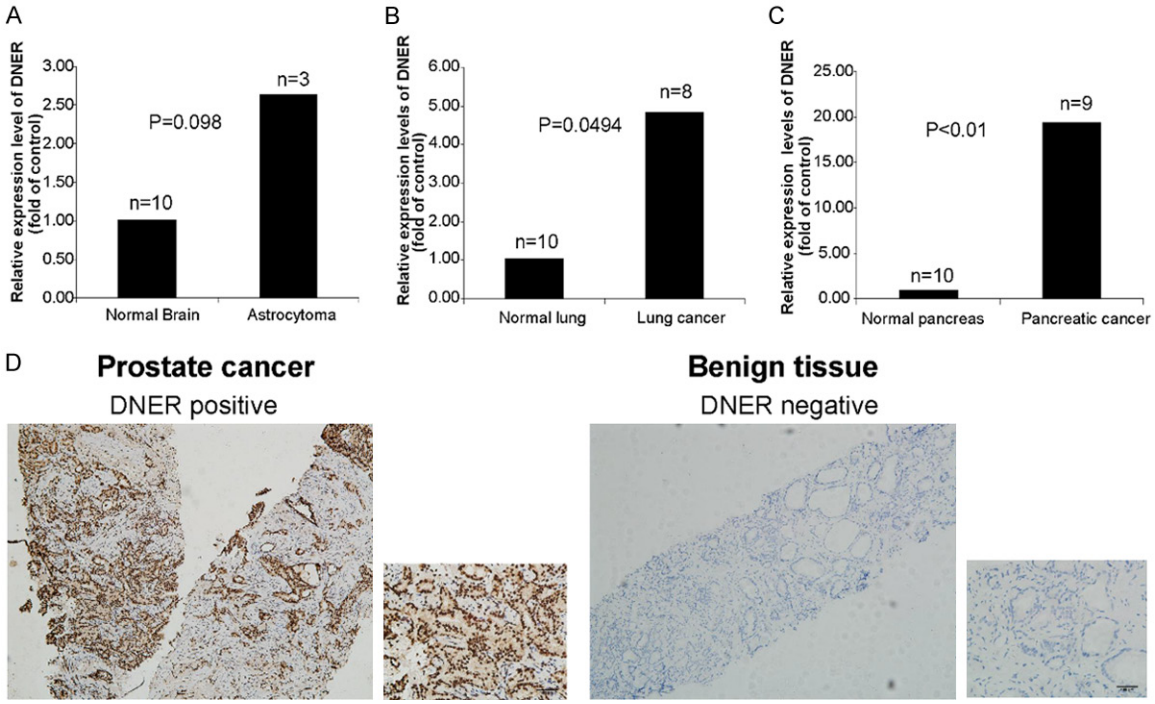

Figure 1. Expression levels of DNER between normal and cancer tissues. Expression analysis of DNER by RT-PCR in different between normal and cancer tissues from brain (A), lung (B), and pancreas (C). * $P < 0.05$, ** $P < 0.01$ versus control values. (D). Representative findings of tumors stained strongly (left) or benign tissues weakly (right). Paraffin-embedded human tissues samples were analyzed for DNER expression by IHC using anti-DNER antibody.

the plasma membrane and/or cytoplasm of prostate cancer tissues compared with benign disease via IHC staining assays (**Figure 1D**).

Depletion of DNER abolishes prostate cancer cell growth, migration and invasion

To investigate the effects of DNER on prostate cancer progression, DNER was knocked down in prostate cancer PC-3 cells (**Figure 2A**). The results demonstrated that DNER knockdown attenuated the growth of PC-3 cells, which was evident in MTT and soft agar growth assays (**Figure 2B** and **2C**). Consistent with this observation, the decreased expression of DNER increased the subpopulation in the G1 phase and decreased the proportions of cells in the S and G2/M phase (**Figure 2D**), while the knockdown of DNER inhibited both cell migration and invasion (**Figure 2E** and **2F**). These observations suggested that DNER significantly promotes prostate cancer growth, migration and invasion.

Depletion of DNER abolishes prostate cancer cell tumorigenesis

The role of DNER in the tumorigenesis of prostate cancer cells was confirmed by injecting PC-3 cells or PC-3 cells depleted of DNER in the backs of nude mice. As shown in **Figure 3**, 16 days after injection, DNER knockdown significantly suppressed the tumor formation of PC-3 cells. These observations suggested that DNER significantly promotes prostate cancer tumorigenesis.

Depletion of DNER suppresses prostate cancer cell stemness

The spheroid assay was established to analyze whether DNER enhances tumor stemness. As shown in **Figure 4A**, DNER knockdown significantly inhibited cell growth in spheroids. We examined the mRNA levels of potential targets in cells expressing DNER or DNER knockdown to explore the potential mechanism of the DNER-mediated promotion of prostate cancer cell stemness. As shown in **Figure 4B** and **4D**, DNER knockdown specifically impaired CD44 expression, and the PC-3 cells treated in spheroid assay were considered as positive controls. As shown in **Figure 4C** and **4D**, DNER knockdown reduced the expression of HES1 and GLI1 at both the gene and protein levels but had no impacted on HEY1 expression, indicating that DNER was essential for tumor stemness.

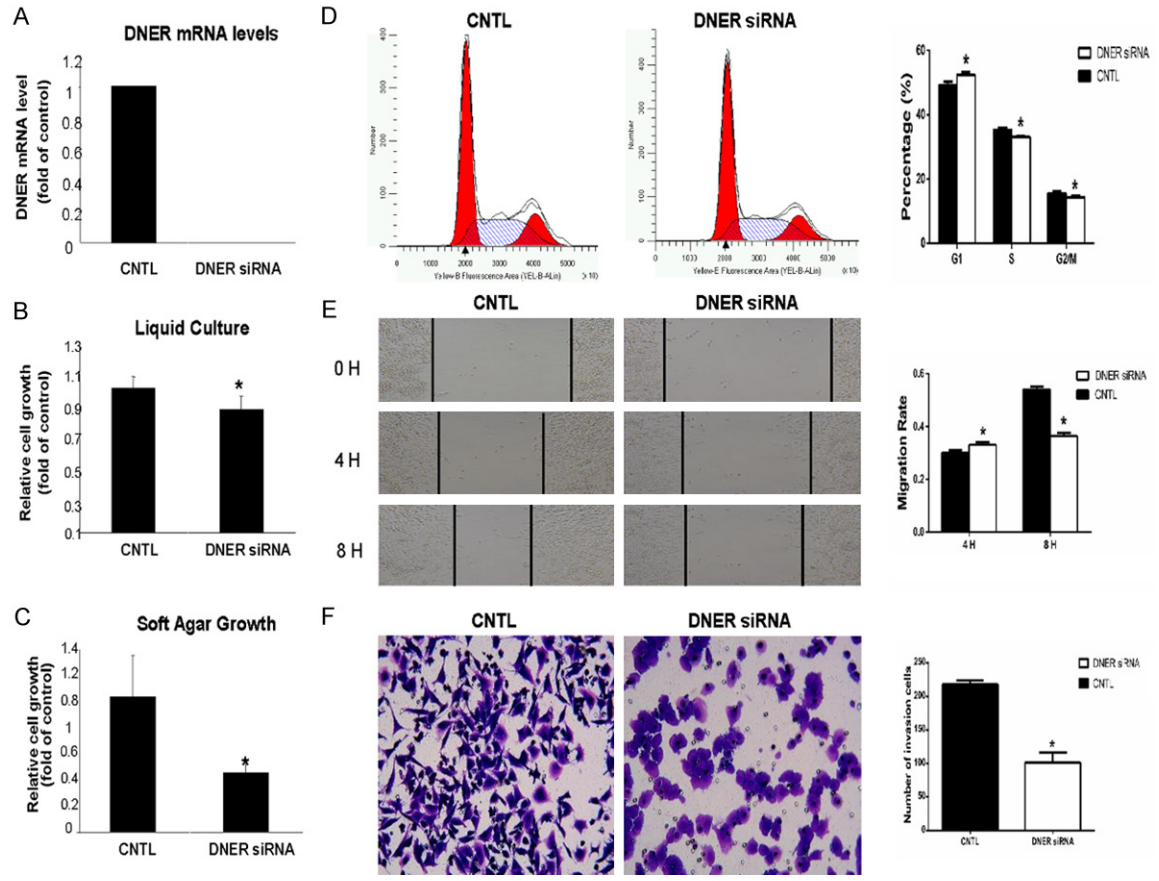

Figure 2. Depletion of DNER abolishes prostate cancer cell growth, migration, invasion. DNER siRNA was transfected into PC3 cells and the stable cell lines were generated by selection using puromycin and the expression of DNER was detected by RT-PCR (A). After selection the cells were plated in either anchorage dependent (liquid culture) or anchorage independent (soft agar) culture conditions. Cell growth was measured by MTT assay after 3 days (B) or alamar blue staining after one week for soft agar assay (C). (D) Cell cycle assay was used to examine the effects of DNER knockdown on cells cycle by flow cytometry analysis, and cell population (%) in each phase was quantified. (E) We transfected PC3 cells with DNER siRNA or control siRNA and detected changes in their motility using wound healing assay. The migration rate was correlated to cell migration ability. (F) Transwell assay was used to detect the effects of DNER knockdown on cells invasion. Changes in the number of cells penetrating the membrane in Transwell invasion assay. The bars represent the mean values ± SD triplicate (n = 3). * $P < 0.05$, ** $P < 0.01$ versus control values. CNTL: control group.

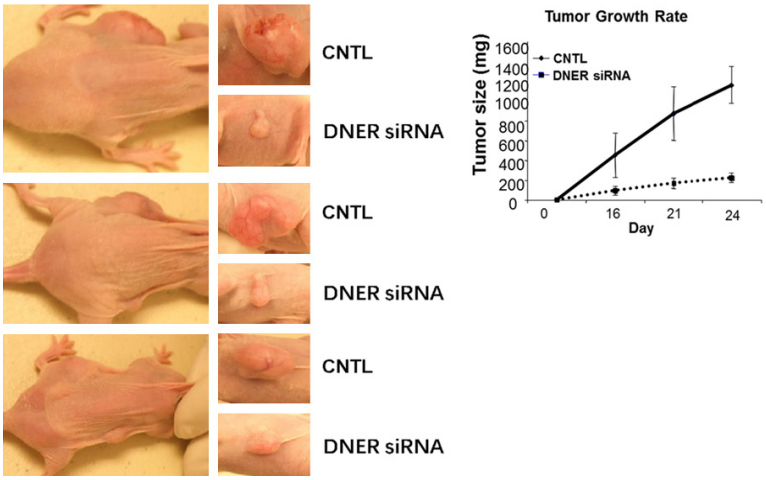

Figure 3. Depletion of DNER inhibits prostate cancer cell tumorigenesis. PC3 prostate cancer cells transduced with either lentiviral vectors DNER siRNA or empty vector were injected into nude mice. The ability of these cells to form tumors was monitored. The bars represent the mean values ± SD triplicate (n = 3). * $P < 0.05$, ** $P < 0.01$ versus control values. CNTL: control group.

Discussion

In the present study, the function of DNER on prostate can-

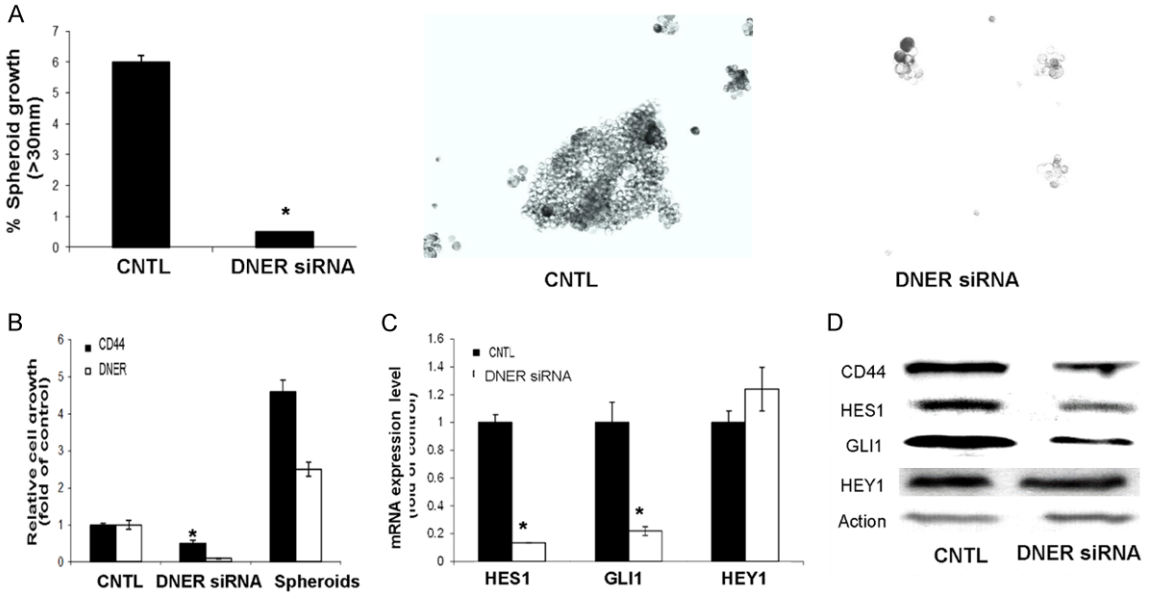

Figure 4. Depletion of DNER depresses prostate cancer cell stemness. A. Spheroid assay in PC3 cells transduced with DNER siRNA or empty vector (control) was applied to test the features of cancer stem cell. Spheroid forming capacity is depicted in % versus CNTL set to 100%. B. The expression of CD44 by RT-PCR after DNER knockdown. The expression in spheroid assay was use as a positive control. C. qPCR data of HES1, GLI1 and HEY1 expression in PC3 cells transduced with lentiviruses carrying vectors with the DNER siRNA or empty vector (control). Expression values were normalized to HES1, GLI1 and HEY1 expression in control cells was set to 100%. D. Representative Western blots of CD44, HES1, GLI1, HEYs and β-actin of PC3 cells transduced with lentivirus carrying vectors with the DNER siRNA or from control cells transduced with lentivirus carrying an empty vector. The bars represent the mean values ± SD triplicate (n = 3). * $P < 0.05$, ** $P < 0.01$ versus control values. CNTL: control group.

cer progression and stemness was evaluated. DNER, a neuron-specific EGF-like repeat-containing transmembrane protein, acts as a functional ligand of Notch during the morphogenesis of Bergmann glial cells and mediates functional communication via cell-cell interactions in the mouse cerebellum [8, 13]; however, the role of DNER in prostate cancer has not previously been studied. The results of the present study demonstrated both in vivo and in vitro that DNER may contribute to cancer progression and stemness in prostate cancer cells.

We also observed that DNER was highly expressed in various cancer tissues except for the brain. Based on these results, we examined whether DNER was associated with prostate cancer progression. Notably, DNER expression was upregulated in prostate cancers. In terms of structural sequence, DNER is closely related to the Notch receptor and its ligand Delta. Additionally, Notch ligand Delta-like 4 (DLL4) acts as a positive driver for tumor growth in prostate cancer [14]. Moreover, DLL4 expression is correlated with VEGFR-2 expression [15], and DLL4 functions as a negative regulator of tumor angiogenesis by activating Notch1 in endothelial cells [14, 16]. These results suggested that DNER might function by Notch signaling in prostate cancer progression.

As we observed cessation of cell growth by DNER knockdown, we confirmed that DNER played a key role in increasing proliferation, tumorigenicity, and metastasis in prostate cancer. However, DNER inhibited glioblastoma growth in vitro and in engraftments such as tumor xenografts [11]. It was initially speculated that the expression of DNER, as a specific gene inducing glioblastoma differentiation, was increased by histone deacetylase (HDAC) inhibitors, which may represent a protective regulation in cells to adjust to the toxicity of HDAC inhibitors. In addition, the capacity of Notch as a tumor suppressor in particular tissues is, in part, a consequence of its cell-intrinsic role in promoting cell cycle exit and differentiation [17]. Therefore, the functional mechanisms of DNER were distinguished between glioblastoma and other cancers. For glioblastoma, the differentiating effects of DNER in brain cancer

stem-like cells implicated this protein as a non-canonical Notch ligand [11], while DNER in the adipogenesis of human adipose tissue-derived mesenchymal stem cells (hAMSC) was regulated independently from Notch signaling [18]. Thus, further elucidation of the physiological function of DNER in diverse cancers is an important area of investigation.

The results of the present study indicated that PC-3 cells ceased to stemness and changed morphology via DNER knockdown. Additionally, the inhibition of DNER using siRNA induced the downregulation of CD44, which displayed stem cell characteristics and reduced the expression of HES1 and GLI1 as crucial mediator genes in Notch and Sonic hedgehog signaling, respectively. A number of studies have shown that CD44 is a widely used CSCs marker in human cancers [19-21], while HES1 and GLI1 are similarly involved in the self-renewal and tumorigenicity of stem-like cancer cells in various cancers [22-25]. Moreover, this interaction could account for the potential mechanisms among CD44, Hedgehog and Notch signaling. As shown in previous studies, HES1 and GLI1 inhibit the expression of CD44 [26, 27], and HES1 also mediates expression of GLI1 transcription [28]. However, we observed that DNER knockdown had no impact on the expression of HEY1. DNER specifically binds to Notch1-expressing cells to induce the intracellular cleavage of Notch1 at the S3 site and activate downstream target genes by means of different transcription factors. However, in contrast to classic Notch ligands, DNER lacks the so-called DSL Notch binding motif and instead binds to Notch1 by the first and second EGF-like repeats in its extracellular domain. The lower transactivation activity of DNER compared to Delta might reflect lower Notch receptor affinity to DNER. Alternatively, DNER might preferentially bind to other Notch family members, such as Notch2 or Notch3, which are also expressed in prostate cancer [29, 30]. In contrast, a previous study suggested that DNER was not a Notch ligand, and its true function remains unknown [31].

Taken together, the present study examined the effects of DNER in prostate cancer progression and stemness. Their expression indicates that DNER is likely to mediate various genes in cancer stem cells (CSCs). Further studies are needed to understand the specific mechanisms of DNER in the formation and maintenance of the stem cells of prostate cancer.

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Disclosure of conflict of interest

None.

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