NELL2 Modulates Cell Proliferation and Apoptosis via ERK Pathway in the Development of Benign Prostatic Hyperplasia

Jianmin Liu¹#, Daoquan Liu¹#, Xueneng Zhang²#, Yan Li¹#, Xun Fu¹#, Weixiang He¹, Mingzhou Li¹, Ping Chen¹, Guang Zeng¹, Michael E DiSanto³, Xinghuan Wang¹, Xinhua Zhang¹*

¹Department of Urology, Zhongnan Hospital of Wuhan University, Wuhan, China.
²Department of Urology, Yangxin County People’s Hospital, Yangxin, China.
³Department of Surgery and Biomedical Sciences, Cooper Medical School of Rowan University, Camden, NJ, USA.

#These authors contributed equally to this work.

*Corresponding Author Address
Dr. Xinhua Zhang MD, Ph. D
¹Department of Urology, Zhongnan Hospital of Wuhan University, 169 Donghu Road,
Wuhan 430071, P.R. China
Tel: 0086 27 67813043; Email: zhangxinhua@163.com

Keywords: benign prostatic hyperplasia, prostate, neural epidermal growth factor-like like 2, cell proliferation, apoptosis
Abstract

Benign prostatic hyperplasia (BPH) is a quite common illness but its etiology and mechanism remain unclear. Neural epidermal growth factor-like like 2 (NELL2) plays multifunctional roles in neural cell growth and is strongly linked to the urinary tract disease. Current study aims to determine the expression, functional activities and underlying mechanism of NELL2 in BPH. Human prostate cell lines and tissues from normal human and BPH patients were utilized. Immunohistochemical staining, immunofluorescent staining, RT-PCR and Western-blotting were performed. We further generated cell models with NELL2 silenced or overexpressed. Subsequently, proliferation, cycle, and apoptosis of prostate cells were determined by CCK-8 assay and flow cytometry analysis. The epithelial-mesenchymal transition (EMT) and fibrosis process were also analyzed. Our study revealed that NELL2 was upregulated in BPH samples and localized in the stroma and the epithelium compartments of human prostate tissues. NELL2 deficiency induced a mitochondrial-dependent cell apoptosis, and inhibited cell proliferation via phosphorylating ERK1/2 activation. Additionally, suppression of ERK1/2 with U0126 incubation could significantly reverse NELL2 deficiency triggered cell apoptosis. Consistently, overexpression of NELL2 promoted cell proliferation and inhibited cell apoptosis. However, NELL2 interference was observed no effect on EMT and fibrosis process. Our novel data demonstrated that upregulation of NELL2 in the enlarged prostate could contribute to the development of BPH through enhancing cell proliferation and inhibited a mitochondrial-dependent cell apoptosis via the ERK pathway. The NELL2-ERK system might represent an important target to facilitate the development of future therapeutic approaches in BPH.
(i) Benign prostatic hyperplasia (BPH) represents a bothersome burden negatively affecting aging males, but its etiology is still uncertain.

(ii) Neural epidermal growth factor-like like 2 (NELL2) shows a tight association with the urinary tract disease, including BPH. NELL2 was upregulated in human hyperplastic prostate while knockdown of this brain tissues-enrich gene resulted in an imbalance between prostate cells proliferation and apoptosis partly modulated by the extracellular signal-regulated kinase (ERK) pathway.

(iii) Alterations in the expression, functional activities of NELL2 may be a novel pathological mechanism of BPH. In addition, the NELL2-ERK system may represent a fundamental target to favor the progression of future therapeutic approaches in BPH.
Introduction

Benign prostatic hyperplasia (BPH), which causes lower urinary tract symptoms (LUTS), is a nonmalignant adenomatous overgrowth of the periurethral prostate gland commonly seen in aging men (1, 2). LUTS are a bothersome complaint in adult male population with a major impact on quality of life (QoL), and substantial economic burden (3, 4). The presence of histological BPH at autopsy is approximately 50% in those aged 51 to 60 years and is significantly increased with age (5). Histopathologically, the observed increase in cell number may be due to epithelial and stromal proliferation or to impaired programmed cell death leading to cellular accumulation. Androgens, estrogens, stromal-epithelial interactions, growth factors, and neurotransmitters may play a role, either singly or in combination, in the etiology of the hyperplastic process. Despite intense research efforts in the past five decades to elucidate the underlying etiology of prostatic growth in older men, cause-and-effect relationships have not been established. Thus, it is of great necessity to achieve a central knowledge of the molecular mechanisms involved in the etiology of BPH and discover new therapeutic strategies.

Neural epidermal growth factor-like (NEL) protein family was originally found in chickens (6), of which two related members NEL-like 1 (NELL2) and NEL-like 2 (NELL2) were subsequently identified in mammalian, including humans (7). NELL2, as a secreted multimeric glycoprotein containing several functional domains such as epidermal growth factor (EGF)-like domains, a thrombospondin-1-like domain, von Willebrand factor C domains and Ca\(^{2+}\) binding domains (8-10), was predominantly expressed in brain tissues (11). Furthermore, the expression of NELL2 had been previously investigated in tuberculous meningitis (12), in cancers of the kidney, bladder, central nervous system and prostate (13-16). Several studies suggested that this protein played a vital role in neural cell growth and differentiation as well as in oncogenesis (11, 17-19). Besides, NELL2 was involved in the regulation of female rat estrous cycle (20) and sterility appeared in NELL2 knocked out male mice (21). Estrogen and E2F1 may act as two upstream regulators of NELL2 (22, 23).

Among its multifunctional roles, a cell survival-promoting effect has been...
relatively well studied (23-25), which was mediated by an intracellular mitogen-activated protein kinases (MAPKs) pathway (26, 27). The MAPK pathway in mammals included c-Jun NH2-terminal kinase (JNK), p38 MAPK, and extracellular signal-regulated kinase (ERK), which played an important role in complex cellular programs like proliferation, differentiation, development, transformation, apoptosis and was involved in signal transduction from the cell membrane to nucleus in response to a wide range of stimuli (28, 29). Importantly, aberrant coordination of the MAPK cascades was observed in most cancers (30) and other human disease, including BPH (31, 32). Papatsoris et al (33) had reviewed that functional anomalies in the MAPK cascades might be involved in an imbalance between cell proliferation and programmed cell death, leading to increased growth, which facilitated the progression of BPH.

Recently, our group had performed mRNA expression profiling of three normal human prostates and five BPH tissues, and our microarray data (serial number: GSE119195) identified a total of 198 differentially expressed genes (DEGs) (34), including NELL2, which is one of the most significantly upregulated DEGs. Consistently, two publications had revealed that NELL2 was significantly upregulated in BPH tissues (16, 35). However, the in-depth functional activities and mechanisms research of this neuronal protein in the development of BPH still remained unclear. These observations suggested that NELL2 may act as a novel prostatic growth factor involved in development of BPH. In our current study, we investigated the expression of both NELL2 mRNA and protein in human hyperplastic prostate tissues. We further silenced and overexpressed the NELL2 gene in human prostatic cells to examine alteration of cell proliferation, cell apoptosis, epithelial-mesenchymal transition (EMT) and fibrosis process, as well as to identify those genes involved in the MAPK pathway.

**Materials and Methods**

**Human prostate tissues collection.**

Normal prostate tissue was obtained from eight young brain-dead men (mean age,
28.2±4.4 years old) undergoing organ donation at the Organ Transplant Center of Zhongnan Hospital, with pathological examination revealing no hyperplasia. A total of 8 specimens of BPH samples (mean age, 69.4±5.7 years old) were obtained from the patients who underwent transurethral resection prostate in the department of urology, Zhongnan Hospital of Wuhan University. Post-operative prostate pathology examination revealed BPH by two separate pathologists. Prostate tissues were divided into two strips and were, respectively, stored in liquid nitrogen for PCR and western blotting analysis and stored in 10% neutral buffered formalin for immunofluorescence microscopy. All human samples were obtained after the approval of the Hospital Committee for Investigation in Humans and after receiving written informed consent from all patients or their relatives. All human studies were conducted in accordance with the principles of the Declaration of Helsinki.

**Tissue immunofluorescent staining.**

Tissues were sectioned in 10 μm thick slices and thawed, mounted onto glass slides using a cryostat (Leica CM 1850, Wetzlar, Germany), air-dried, and fixed for 10 min in ice cold acetone. Slides were washed in PBS and incubated for 2 h in a mixture of PBS supplemented with 0.2% Triton X-100 and 0.1% bovine serum albumin, followed by incubation overnight with the primary antibodies (listed in Supplementary Table S1). The secondary antibodies (listed in Supplementary Table S2) employed to visualize the localization of NELL2 was Cy3-conjugated goat anti-rabbit IgG (1:1,000). DAPI was used for staining the nucleus. Visualization was done with a Laser Scanning Confocal Microscope (Olympus, Tokyo, Japan).

**Cell culture.**

Human benign prostatic enlargement epithelia cell line BPH-1 (Cat. #BNCC339850) was purchased from the Procell Co., Ltd. In Wuhan, China. Identification of the cell lines was performed at the China Center for Type Culture Collection in Wuhan, China. SV40 large-T antigen-immortalized stromal cell line WPMY-1 (Cat. #GNHu36) was purchased from the Stem Cell Bank, Chinese Academy of Sciences in Shanghai, China. The human normal prostate epithelial cell line RWPE-1 (Cat. #CRL-11609) was obtained from American Type Culture
Collection (ATCC). The BPH-1 cells were cultured in RPMI-1640 medium (Gibco, China) containing 10% fetal bovine serum (FBS) (Gibco, Australia). The WPMY-1 cells were cultured in DMEM medium (Gibco, China) containing 1% penicillin G sodium/streptomycin sulfate and 5% FBS. The RWPE-1 cells were maintained in prostate epithelial cell medium (PEpiCM, ScienCell Research Laboratories) with 1% prostate epithelial cell growth supplement (PEpiCGS, ScienCell Research Laboratories) and 1% penicillin/streptomycin solution (P/S, ScienCell Research Laboratories). All the cell lines were recently authenticated and were cultured in a humidified atmosphere consisting of 95% air and 5% CO₂ at 37°C.

**Knockdown and over-expression of NELL2 and MXRA5 in human prostate cells.**

NELL2-target specific small interfering RNA (siRNA) was synthesized by GenePharma Ltd. in Suzhou, China. The cells were transiently transfected with siRNA using Lipofectamine transfection reagent. When BPH-1, WPMY-1 and RWPE-1 cells were 30–50% confluent in six-well culture plates, the cell culture medium was replaced with fresh medium 30min before transfection. The transfection media were prepared according to the manufacturer’s instructions and incubated at room temperature for 10min. Subsequently, 200 μl of the lipofectamine complex solution was added to each well. After incubation for 6 h at 37°C in 5% CO₂, the cell culture medium was replaced with fresh medium and incubated for 48 h. The GFP fluorescence was evaluated as a reporter for the transfection efficiency. The knockdown of MXRA5 in the WPMY-1 cells followed previous protocol(34). The sequences of each siRNA are summarized in Supplementary Table S3.

NELL2 cDNA was polymerase chain reaction (PCR) amplified from a cDNA library of human prostate cell lines and then cloned into a 2 × FlagpcDNA3 empty vector performed with a one-step method to construct the homologous recombination vectors. After transfection by plasmid for 48 h, alterations of NELL2 at transcriptional and protein levels were evaluated by the qRT-PCR and western blotting analyses. The over-expression of MXRA5 in BPH-1 cells followed previous protocol(34).

**Pre-treatment using MKK inhibitor for rescue experiments.**

Before siRNA transfection, prostate cells were pretreated by MKK inhibitor
U0126 (MedChemExpress, China) at 10 μM for 2 h to deactivate ERK1/2(36, 37).

Prostate cells in the untreated group were pre-incubated with appropriate amount of vehicle (0.1% DMSO). Both groups were submitted for the RNA interference and alterations of cell viability and apoptosis were measured by cell counting kit-8 (CCK-8) assay and flow cytometry analysis, respectively.

**CCK-8 assay.**

The viability of cells was examined by CCK-8 (MedChemExpress, China) assay. Briefly, the neurons (approximately 5000 cells/well) were seeded in poly-L-lysine-coated 96-well plates and subjected to various treatments as described above. CCK-8 solution (10 μl/100 μl) was added to each culture well, and neurons were incubated for 2 h at 37 °C. Finally, the absorbance at 450 nm was measured by a microplate reader (cat. no. SpectraMaxM2; Molecular Devices, Sunnyvale, CA, USA) at the same time for each day.

**Flow cytometry analysis**

For cell cycle analysis, BPH-1, WPMY-1 and RWPE-1 cells (1 × 10⁶ cells) were harvested, washed with PBS, and then centrifuged. Pellets were resuspended with 1 ml DNA staining solution, which contained 50 μg/ml propidium iodide and 0.1 mg/ml RNaseA, and 10 μl permeabilization solution (Multisciences). The DNA content distribution was analyzed by flow cytometry analysis (Beckman, Cat. #FC500) after incubation in the dark at 37°C for 30 min. For cell apoptosis analysis, FITC Annexin V Apoptosis Detection Kit I (BD Biosciences, USA) was used. BPH-1, WPMY-1 and RWPE-1 cells (1 × 10⁶ cells) were harvested and then stained with FITC Annexin V Apoptosis Detection Kit I according to the manufacturer’s instruction.

**Cell immunofluorescence staining.**

For cell immunofluorescence microscopy, cells were cultured as aforementioned, followed by seeding on 12 mm coverslips and washing by ice cold phosphate-buffered saline (PBS, pH = 7.4). The coverslips were then fixed with 4% paraformaldehyde (PFA) for 30 min, followed by 0.1% Triton X-100 incubation and blocked in goat serum for 30 min at room temperature. Then incubated with primary antibody (listed in Supplementary Table S1) at room temperature for 2 h, washed with...
PBS and incubated with Cy3-labeled or FITC-labeled secondary antibody (listed in Supplementary Table S2) for 1 h. Nuclei were labeled with DAPI (2 μg/ml). Visualization was done with a Laser Scanning Confocal Microscope (Olympus, Tokyo, Japan).

**RNA isolation, reverse transcription, and quantitative real-time PCR (qRT-PCR) analysis.**

Total RNA was isolated from frozen tissues and cell lines using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions and quantitated at 260/280 nm using a NanoPhotometer spectrophotometer (IMPLEN, Westlake Village, CA, USA). 2 μg of total RNA was reverse-transcribed to complementary DNA (cDNA) via the SuperScript II First-Strand Synthesis System according to the manufacturer (Invitrogen). QRT-PCR was performed to determine the level of mRNA expression of a gene of interest based on SYBR green using a Bio-Rad (Hercules, CA, USA) CFX96 system. The expression levels of genes were normalized to the expression of GAPDH mRNA and compared by 2^ΔΔCT method. Primer sequences are listed in Supplementary Table S4. Values were normalized for amplified GAPDH alleles.

**Western blotting analysis.**

Tissues and cell lines were lysed and ultrasonicated in RIPA reagent containing protease inhibitor and phosphatase inhibitor (Sigma-Aldrich) on ice for 30 min. The supernatant was collected after centrifugation at 14,000 × g for 10 min at 4 °C. Then, the protein concentration was measured by bicinchoninic acid (BCA) assay. Protein extracts were isolated by sodium dodecyl sulfate-polyacrylamide (SDS–PAGE) gel and then transferred to a polyvinylidene fluoride membrane (PVDF, Millipore, Billerica, MA, USA) using a Bio-Rad wet transfer system. The membrane was then blocked in tris-buffered saline with 0.05% tween 20 (TBST) buffer containing 5% skim milk, and incubated sequentially with primary and secondary antibodies (listed in Supplementary Table S1, S2). An enhanced chemiluminescence kit (Thermo Scientific Fisher, Waltham, MA, USA) was used to expose the bands.

**Statistical analyses.**
All analyses were performed at least three times and represented data from three individual experiments. The data values were expressed as the means ± standard deviation (SD). Statistical analysis was performed using either the Student’s t test (two groups compared) or one way ANOVA and Tukey post-tests with SPSS 20.0 (multiple means compared). Statistical significance was considered as a p value < 0.05.

Results

The expression and localization of NELL2 in human prostate tissues and cell lines.

Normal and hyperplastic prostate were harvested (8 control samples and 8 BPH samples) from our institute. In hyperplastic prostate, both the mRNA and protein level of NELL2 were significantly increased over 2-fold (Fig. 1A, B). Meanwhile, the immunofluorescence staining showed NELL2 was localized in both the stromal and the epithelium compartments of human prostate (Fig. 1C, D). Negative controls omitting the primary antibody failed to stain (Fig. 1E) and positive controls using rat brain tissue showed a strong immune positivity (Fig. 1F). Consistently, immunohistology demonstrated NELL2 was present both in cultured human epithelium cell BPH-1 (Fig. 1G) and stromal cell WPMY-1 (Fig. 1H). Hence, epithelial cells and stromal cells were used in our subsequent studies.

Downregulation of NELL2 inhibited cell survival in prostate cell lines.

To investigate the functional activities of NELL2, a cell model of NELL2 deficiency was established by 3 distinct NELL2-target-specific-siRNAs (si-NELL2s) transfection. The knockdown efficiency of siRNA was validated by qRT-PCR and the mRNA level of NELL2 was remarkably downregulated (Fig 2A). Si-NELL2-2 was selected for use in further experiment due to its highest inhibitory effect. Consistently, NELL2 was significantly silenced at protein level when si-NELL2-2 was transfected both BPH-1 and WPMY-1 cells (Fig. 2B). Cell proliferation was further analyzed by CCK-8 assay and ki-67 immunofluorescence staining for these transfected BPH-1 and WPMY-1 cells, which revealed that knockdown of NELL2 significantly inhibited cell
proliferation at both 48 and 72h (Fig. 2C). Similarly, the relative number of ki-67 positive cells was significantly reduced by NELL2 deficiency (Fig. 2D).

**Downregulation of NELL2 resulted in cell apoptosis and ERK pathway activation.**

To better understand the underlying mechanism of this cell survival-inhibiting effect, cell cycle and cell apoptosis was further detected via flow cytometry analysis. Compared with the control cells, downregulation of NELL2 had no effect on cell cycle promotion (Fig 3A) but triggered a significant increase of the apoptotic BPH-1 and WPMY-1 cells (Fig. 3C). The alteration of proteins involved in cell cycle and cell apoptosis were analyzed by western blotting, exhibiting no change of CDK2/4 (Fig 3B), an upregulation of BAX and downregulation of Bcl-2 (Fig 3D, E). Furthermore, cleaved caspase 3, as a downstream protein of BAX and Bcl-2 in the apoptosis cascade, was also significantly increased (Fig 3D, E). Moreover, we found that Cytochrome c was also upregulated, suggesting that NELL2 deficiency could trigger prostatic cell apoptosis through a mitochondrial-dependent manner (Fig 3D, E). The MAPK family (including ERK1/2, JNK and P38), as one of the most fundamental signal pathways for cell proliferation and apoptosis, was also altered after suppression of NELL2 with phosphorylated ERK1/2 (p-ERK1/2) significantly enhanced in BPH-1 and WPMY-1 cells but a slight effect on p-JNK and p-p38 (Fig. 3F).

**Downregulation of NELL2 induced cell apoptosis through ERK1/2 activation.**

To verify this remarkable change of p-ERK1/2, the BPH-1 and WPMY-1 cells were pre-treated with U0126 (A MKK inhibitor, MKK is an upstream activator of ERK) and then transfected with si-NELL2 and controls. The CCK-8 assay indicated that the impact of cell growth delay induced by si-NELL2 could be significantly recovered by U0126 (Fig. 4A). Importantly, suppression of ERK1/2 with U0126 incubation could significantly reverse NELL2 deficiency triggered cell apoptosis exhibited by flow cytometry analysis (Fig. 4B, statistically analyzed in Fig. 4C). We further determined relevant proteins by western blotting analysis, revealing a reduction of p-ERK1/2 and a reversal of BAX/Bcl-2 ratio, as well as a decrease of cleaved caspase 3 and Cytochrome c (Fig. 4D, statistically analyzed in Fig. 4E).
Downregulation of NELL2 had no effect on EMT and fibrosis process.

It was reported that NELL2 promoted neural differentiation through increasing N-cadherin expression (19). As a calcium-dependent glycoprotein, N-cadherin was critical for a variety of pathophysiological mechanisms, including EMT in BPH (38). Therefore, we analyzed the mRNA level of several EMT markers after si-NELL2 transfection in BPH-1 cells, observing an upregulation of the epithelial marker (E-cadherin) and a downregulation of mesenchymal markers (N-cadherin, vimentin and snail). However, this mild change did not reach a statistical difference (Supplementary Fig. S1A). Furthermore, as two fibrosis markers, the mRNA level of α-SMA and collagen I did not change significantly after downregulation of NELL2 in WPMY-1 cells (Supplementary Fig. S1B).

Overexpression of NELL2 promoted cell proliferation and decreased cell apoptosis.

The plasmid was transfected into BPH-1 and WPMY-1 cells to over-expressed the NELL2 protein. Compared to vectors, both the mRNA and protein levels of NELL2 were significantly upregulated determined by qRT-PCR (Fig. 5A) and western blotting analysis (Fig. 5B). The CCK-8 assay indicated that NELL2 promoted cell growth at 48 and 72h (Fig. 5C). The flow cytometry analysis also revealed that NELL2 inhibited the apoptotic BPH-1 and WPMY-1 cells (Fig. 5D). Furthermore, western blot analysis validated an upregulation of Bcl-2 and a downregulation of BAX, cleaved caspase 3, Cytochrome c and p-ERK1/2, which were the direct opposite of what we observed after NELL2 deficiency (Fig 5E).

NELL2 could promote cell proliferation and suppress cell apoptosis validated by another cell line RWPE-1 and another NELL2 siRNA.

Cell proliferation was analyzed by CCK-8 assay for these transfected BPH-1, WPMY-1 and RWPE-1 cells, which revealed that knockdown of NELL2 by two specific siRNAs in RWPE-1 cells and knockdown of NELL2 by another siRNA in BPH-1 and WPMY-1 cells significantly inhibited cell proliferation at both 48 and 72h (Fig. 6A). Cell apoptosis was further detected via flow cytometry analysis. Compared with the control cells, downregulation of NELL2 by two specific siRNAs in RWPE-1...
cells and knockdown of NELL2 by another siRNA in BPH-1 and WPMY-1 cells triggered a significant increase of the apoptotic cells (Fig. 6B). Furthermore, NELL2 overexpression promoted cell growth (Fig 6C) and inhibit cell apoptosis (Fig 6D) in RWPE-1 cells.

Discussion

Our novel data demonstrated that NELL2 was localized in the stroma and the epithelium compartments of human prostate tissues and upregulated in hyperplastic prostate tissues. We also showed that NELL2 deficiency could induce a mitochondrial-dependent cell apoptosis, and inhibit cell proliferation via phosphorylating ERK1/2 activation in vitro (Fig 7). Our study suggested that NELL2 might play a vital role in the development of BPH.

NELL2, one of two human NEL genes, is also known as NEL-related protein 2 (NRP2), and was founded in the cytoplasm. It is expressed abundantly in the adult neurons with the highest expression in the hippocampus and the cerebellum(9, 17, 39). Furthermore, NELL2 is not only a key regulator of normal neural differentiation(18, 24), but also plays a fundamental role in metabolism regulation(40), male fertility(21) and neurological disorders, i.e., neuroblastoma(15). Interestingly, several publications indicated that this neuronal protein was very closely related to human urinary tract disease, including cancers of kidney, bladder and prostate(13, 14, 41). With regarding to BPH, NELL2 was firstly reported to be expressed in human hyperplastic prostate specimens in 2001(35). In our current study, NELL2 was strongly expressed in human prostate tissues and cultured human prostate cell lines. There are adequate experimental evidences demonstrating that prostatic stromal and epithelial cells maintain a sophisticated autocrine/paracrine type of communication(42, 43). Therefore, it is of great necessity for our subsequent study to determine that NELL2 proteins can either be expressed solely in the epithelia or in both epithelia and stroma.

We demonstrated that NELL2 protein was localized in both epithelia and stroma of human hyperplastic prostate, which was not entirely consistent with a previous report from DiLella et al(35), who had investigated that NELL2 mRNA was predominantly localized in the epithelial basal cell layer in transurethral resection of prostate (TURP).
This discrepancy in localization could be attributed to different detection methods: i.e., mRNA detected by in situ hybridization (ISH) analysis was different from protein detected by immunofluorescence staining. It was reported that prostate-associated gene 4 (PAGE4), a prostate-specific gene, its mRNA appeared to be highly expressed in prostatic epithelial cells of normal prostate(44) while its protein was not detectable in the same tissues(45). Moreover, its mRNA was specifically expressed in prostatic epithelial cells of prostate cancer (PCa) tissues(44) but its protein was not strongly stained in the epithelial cells of the same tissues(45).

The rich expression of NELL2 could contribute to BPH. Indeed, current study showed the mRNA and protein levels of NELL2 were upregulated in human hyperplastic prostates when compared with normal controls. Similarly, Shah and colleagues(16) performed gene expression analysis by a DNA microarray and found numerous genes were upregulated in both BPH and PCa, including NELL2. However, the potential roles of this highly altered gene in BPH were still uncertain over past two decades.

To explore the possible functional activities and underlying mechanisms of NELL2 involved in the progression of BPH. We selected three common human prostate cell lines BPH-1, WPMY-1 and RWPE-1 to generate NELL2 deficiency cell model utilizing NELL2-target specific siRNA treatment. Indeed, the knockdown efficiency was confirmed at mRNA and protein levels. Furthermore, downregulation of NELL2 led to cell growth delayed and apoptosis but had no effect on cell cycle in human prostate cells. Meanwhile, proteins associated with apoptosis were strongly altered, especially upregulation of BAX (a pro-apoptotic molecule) and downregulation of Bcl-2 (an anti-apoptotic molecule), which strictly controlled the intrinsic apoptotic pathway(46). Cytochrome c, as a well conserved electron-transport protein and a part of the respiratory chain localized to mitochondrial intermembrane space(47), was also increased. Upon apoptotic stimulation, cytochrome c released from mitochondria, which further triggered caspase 3 (an effector caspase) activation with its active form cleaved caspase 3 increased(48, 49), and eventually leads to apoptosis(50), suggesting that NELL2 deficiency could induce a
mitochondrial-mediated apoptosis. On the other hand, overexpression of NELL2 induced a cell survival-promoting effect and inhibited cell apoptosis. As many of the prostatic stromal-epithelial interactions observed during the development of BPH may be mediated by soluble growth factors, we further investigated the mRNA alteration of EMT markers after downregulation of this prostatic growth factor but no significant difference of EMT markers was observed. Furthermore, we analyzed the mRNA level of fibrosis markers after NELL2 silenced, as fibrosis may act as a risk factor contributing to BPH/LUTS etiology (51-54). Either, α-SMA and collagen I, which were triggered to express during prostate stromal fibroblasts differentiating into myofibroblasts (55), were found no change.

All above observations suggested that upregulated NELL2 mainly affected proliferative-apoptotic imbalance rather than EMT and fibrosis processes in the progression of BPH. Several lines of evidence demonstrated that a cell growth-promoting effect induced by NELL2 was observed in human breast cancer cells (22), retinal ganglion cells (25) and cultured rat embryonic hippocampal neurons (24). Indeed, NELL2 could modulate both prostate stromal cell and epithelial cell proliferation.

The ERK cascades, a well characterized MAPK signal pathway, were activated by MKK1/2 (also known as mitogen-activated protein kinase kinase, MAPKK). MKK1/2, modulated by Raf-Ras GTPase, whose activation was induced by tyrosine kinase receptors (RTKs) such as the epidermal growth factor receptor (EGFR) (56). For JNK and p38 MAPK, they are activated by various types of stress-induced responses (29). There is indirect evidence that all these three MAPK cascades might be implicated in several pathological alterations by modulating the local prostatic environment to favor the progression of BPH. Androgens, a generally acknowledged contributor of BPH, might induce the activation of the ERK cascades directly (57). Besides, it is putative that downregulation of apoptosis in BPH might be implicated in inactivation of the JNK/p38 MAPK apoptotic pathways (58-60). Interestingly, NELL2 deficiency could predominantly upregulated p-ERK1/2 in BPH-1 and WPMY-1 cells, with only a mild effect on p-JNK and p-p38, suggesting downregulation of NELL2 appeared to trigger
cell apoptosis via ERK activation rather than p38 and JNK. These outcomes of our experiments were not entirely consistent with a previous publication from Aihara et al (26), who reported that recombinant rat NELL2 enhanced survival of primary cultured neurons, which was partly modulated by activation of JNK rather than ERK. This discrepancy between the two studies seems to be result from differences of cells and treatments.

MXRA5 was also one of the most markedly changed DEGs in our microarray dataset reported previously (34). This two DEGs both have some effect on ERK signaling. However, MXRA5 knockdown reduced while NELL2 knockdown enhanced p-ERK1/2 but suppressed proliferation of human prostatic cells. We further observed neither knockdown nor overexpression of NELL2 had any obvious effect on the expression of MXRA5 (Supplementary Fig S2A, B). Also, neither knockdown nor overexpression of MXRA5 had any obvious effect on NELL2 expression (Supplementary Fig S2C, D). These observations revealed that there was no interaction between NELL2 and MXRA5 at transcriptional and translational levels, which suggested that the same cell proliferation-delayed effect induced by these two different molecules might due to two distinct and independent mechanisms. We did, indeed, observe increased levels of mitochondrial-dependent apoptosis rather than cell cycle arrest associating with ERK activation induced by NELL2 deficiency. However, knockdown of MXRA5 induced G0/G1 arrest instead of inhibiting apoptosis partially dependent on ERK deactivation. Generally, activation of ERK1/2 promotes cell survival but it also showed pro-apoptotic effect under certain conditions. Accumulated evidences indicated that ERK1/2 signaling can block apoptosis at levels upstream, downstream, or unrelated to change of mitochondrial transmembrane potential and cytochrome c release (61-63), which might via suppressing the functions of pro-apoptotic proteins (64) or enhancing the activity of anti-apoptotic molecules (65, 66) in the mammalian. It is known that MAPK family members participate in regulating the cell cycle in various ways and ERK is mainly involved in regulating the progression of G0/G1 to the S phase (67). Actually, MXRA5 knockdown induced cell cycle arrest at G0/G1 phase associated with ERK1/2 reduction but had no effect on
apoptosis, subsequently led to cell growth delayed. On the other hand, ERK1/2 could function in a pro-apoptotic manner under some circumstances with not completely understood mechanisms. Previous studies reported that inhibition of ERK1/2 activity, for instance, could promote cell growth and suppress apoptosis induced by DNA damage stimuli (e.g., etoposide, UV) or antitumor compounds (e.g., resveratrol, shikonin)(68-72). In addition, ERK1/2 activation was found enhancing cisplatin-induced cell death in neuroblastoma cell lines(73) and mediated apoptosis in a p53-independent manner(74). Thus, NELL2 knockdown might suppress cell proliferation and trigger a mitochondrial dependent apoptosis via ERK1/2 in prostatic cells, which could be activated by phosphorylation on specific sites in particular subcellular compartments and promote either intrinsic or extrinsic apoptotic pathways by induction of mitochondrial Cytochrome c release, caspase 8 activation or autophagic vacuolization(75)

U0126 is a potent, non-ATP competitive and selective MEK1/2 inhibitor(76). As ERK1/2 was directly activated by MEK1/2, we suppressed ERK1/2 by pre-treating U0126 in the BPH-1 and WPMY-1 cells, observing a reduced protein level of p-ERK1/2. Furthermore, the delayed cell growth induced by NELL2 deficiency was significantly reversed, and the apoptotic cell rates were significantly reduced by ERK1/2 inhibition, as well as the proteins involved in cell apoptosis were recovered. All these observations indicated that NELL2 could promote cell proliferation and inhibit cell apoptosis via ERK pathway activation in the development of BPH.

Indeed, the difference in age between groups may influence the outcome with normal prostate samples being obtained from young donors and BPH samples obtained from aging males. Thus, the differential expression of NELL2 identified in the present study may be attributed to different age, instead of BPH. However, Luo et al(77) harvested the normal prostate samples from histologically normal regions within the radical prostatectomy specimens. They compared gene expression profile from normal samples with age ≥59 ys and BPH samples with age ≤59 ys and showed similar alterations as in their analysis with the presence of age difference (52.5 vs. 70.6, normal vs. BPH). Consistently, our current analysis identified the same
Given that upregulation of NELL2 in the enlarged prostate could contribute to the development of BPH, the relationship of NELL2 expression with prostate size was further analyzed by our group in a cohort of 104 BPH patients. However, no significantly positive relationship of NELL2 expression with prostate size was found (data not shown). It will be interesting to investigate in the future.

Collectively, our current study reveals that NELL2 protein is localized in both the stroma and the epithelium compartments of human prostate tissues and both NELL2 gene and protein expression increased in hyperplastic prostate. Knockdown of NELL2 inhibited prostatic cell proliferation and induced a mitochondrial-dependent cell apoptosis while overexpression of NELL2 observed a opposite but expected outcome. Moreover, this process is completely dependent on activation of the ERK pathway.

Our data suggests that the NELL2-ERK system could play vital roles in the development of BPH and it might be rediscovered as new therapeutic targets for BPH. In the future, genetic mice with conditional knockout NELL2 gene in prostate could be an intriguing model to explore the in vivo function of NELL2.

Data availability statement
The data used to support the findings of this study are available from the corresponding author upon reasonable request.

Acknowledgements
We thank the staff at Zhongnan Hospital of Wuhan University for their help in completing the study.

Funding details
This study was supported in part by National Natural Science Foundation of China (N.82070780 and N.81770757). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.
Author contributions

J.M.L., D.Q.L, X.N.Z., Y.L. and X.F. contributed equally to this work. J.M.L., X.N.Z. and Y.L. designed the experiment and J.M.L. wrote the first draft. J.M.L., D.Q.L. and X.F. conducted most of the experiments, W.X.H., M.Z.L., P.C., X.H.W. and G.Z. helped to analyze the results and collected the specimens. M.E.D and X.H.Z. critically revised drafts of the manuscript, provided important intellectual input and approved the final version for publication. J.M.L. and X.H.Z contributed to the writing of the manuscript. All authors reviewed the manuscript.

Disclosure of interest

No potential conflict of interest was reported by the authors.
Figure legends

Fig. 1 The localization and expression of NELL2 in human prostate tissues and cultured human prostate cells.

A, B: The relative expression level of NELL2 in BPH tissues versus normal prostate tissues at the mRNA and protein levels (i, ii). C, D: Immunolocalization of NELL2 for normal human prostate and BPH prostate. E: Negative controls omitting the primary antibody failed to stain. F: Positive control using rat brain tissue showed a strong immune positivity for NELL2 protein. G, H: Immunofluorescence of NELL2 in human epithelial cells (BPH-1) and stromal cells (WPMY-1). DAPI (blue) indicates nuclear staining and Cy3-immunofluorescence (red) indicates NELL2 protein staining. Sections of all sample were used for immunofluorescence experiments and representative graphs were selected into figure. *p < 0.05. Student’s t test. The scale bars are 100 μm.

Fig. 2 Knockdown of NELL2 inhibits cell proliferation in BPH-1 and WPMY-1 cells.

A: Knockdown efficiency of NELL2 at the mRNA levels with three different siRNA sequences (si-NELL2 1, 2, and 3) in BPH-1 and WPMY-1 cells. B: Representative western blot band (i) and relative densitometric quantification of NELL2 (ii) treated with si-NELL2 of the highest inhibitory efficiency (si-NELL2 2) and si-con in BPH-1 and WPMY-1 cells. C: The cell viability of BPH-1 and WPMY-1 after knockdown of NELL2 at different time points by CCK8 assay. D: The Ki-67 staining of BPH-1 and WPMY-1 after knockdown of NELL2 (i). Statistical analysis of Ki-67 positive cells per field in BPH-1 and WPMY-1 after knockdown of NELL2 (ii). NS means no significant difference, *p < 0.05, **p < 0.01, ***p < 0.001. ANOVA or Student’s t test. The scale bars are 100 μm.

Fig. 3 Knockdown of NELL2 has no effect on cell cycle but induces cell apoptosis and ERK1/2 pathway activation in BPH-1 and WPMY-1 cells.

A: Flow cytometry analysis for BPH-1 and WPMY-1 cells treated with
si-NELL2-2 for 48h compared with control cells (i). Percentages (%) of cell populations at different stages of cell cycles were listed within the panels (ii). B: Immunoblot assay of proteins in relation to cell cycle in BPH-1 and WPMY-1 after knockdown of NELL2. C: Flow cytometry analysis of the cell apoptosis in BPH-1 and WPMY-1 after knockdown of NELL2 for 48h. PI PE-A in y-axis stands for the fluorescence intensity of propidine iodide (PI) and FITC-A in x-axis stands for the fluorescence intensity of Fluorescein isothiocyanate (FITC) labelled Annexin V (i). Calculation area of the apoptosis rate was percentage of Annexin V+/PI+ cells. B: Statistical analysis reveals the apoptotic rate (%) of BPH-1 and WPMY-1 after knockdown of NELL2 (ii). D, E: Immunoblot assay and relative densitometric quantification of apoptosis-related proteins in BPH-1 and WPMY-1 after knockdown of NELL2. F: Immunoblot assay (i) and relative densitometric quantification of MAPK proteins (ii) in BPH-1 and WPMY-1 after knockdown of NELL2. NS means no significant difference, *p < 0.05, **p < 0.01; Student’s t test.

**Fig. 4** Recovering siNELL2-induced prostate cell apoptosis by U0126 to deactivate ERK1/2.

A: BPH-1 and WPMY-1 cells were pre-treated by U0126 at 10 μM for 2 h and treated by si-NELL2 for 48 h, comparing with control cells. Cell proliferation of the BPH-1 and WPMY-1 cells was analyzed by CCK-8 assay. B: Apoptotic cells staining with Annexin V and PI were revealed by flow cytometry analysis and the apoptotic rates were statistically analyzed (C). D, E: Immunoblot assay and relative densitometric quantification for phosphorylated and total ERK1/2 as well as apoptotic proteins (BAX, Bcl-2, and cleaved-caspase 3). Cell types, treatment of siRNA and protein masses were indicated. *p < 0.05, **p < 0.01. ANOVA.

**Fig. 5** Overexpression of NELL2 promotes cell proliferation and inhibits cell apoptosis.

A: qRT-PCR validated the efficiency of NELL2 overexpression at transcriptional level in BPH-1 cells. B: Immunoblot assay (i) and relative densitometric
quantification of NELL2 proteins (ii) in BPH-1 and WPMY-1 after overexpression of NELL2. C: The cell viability of BPH-1 and WPMY-1 after overexpression of NELL2 at different time points by CCK8 assay. D: Flow cytometry analysis of the cell apoptosis in BPH-1 and WPMY-1 after overexpression of NELL2 for 48h (i). Statistical analysis reveals the apoptotic rate (%) of BPH-1 and WPMY-1 after overexpression of NELL2 (ii). E: Immunoblot assay (i) and relative densitometric quantification for phosphorylated and total ERK1/2 as well as apoptotic proteins (BAX, Bcl-2, Cytochrome c and cleaved caspase 3) (ii). For compared with vector, *p < 0.05, **p < 0.01; Student’s t test.

Fig 6 NELL2 could promote cell proliferation and suppress cell apoptosis validated by another cell line RWPE-1 and another NELL2 siRNA.

A: The cell viability of RWPE-1 after knockdown of NELL2 using two specific siRNAs at different time points by CCK8 assay (i). The cell viability of BPH-1 and WPMY-1 after knockdown of NELL2 using additional si-NELL2-1 at different time points by CCK8 assay (ii, iii). B: Flow cytometry analysis of the cell apoptosis in RWPE-1, BPH-1 and WPMY-1 after knockdown of NELL2 for 48h (i). Calculation area of the apoptosis rate was percentage of Annexin V+/PI+ cells. The apoptotic rates were statistically analyzed (ii). C: The cell viability of RWPE-1 after overexpression of NELL2 at different time points by CCK8 assay. D: Flow cytometry analysis of the cell apoptosis in RWPE-1 after overexpression of NELL2 for 48h (i). The apoptotic rates were statistically analyzed (ii). *p < 0.05, **p < 0.01. ANOVA or Student’s t test.

Fig 7 Schematic model proposed for the central roles of NELL2-ERK system in the development of BPH.

The rich expression of NELL2 could suppress ERK pathway activation, then inhibit an apoptotic inducer BAX expression, Cytochrome c release and caspase 3 activation, and subsequently promote cell proliferation and reduce cell apoptosis. Consequently, the imbalance between cell proliferation and cell apoptosis induced by NELL2-ERK cascade contributed to the development of BPH.
Reference

1. Gratzke C, Bachmann A, Descazaud A, Drake MJ, Madersbacher S, Mamoulakis C, et al. EAU Guidelines on the Assessment of Non-neurogenic Male Lower Urinary Tract Symptoms including Benign Prostatic Obstruction. Eur Urol. 2015;67(6):1099-109.

2. Kim EH, Larson JA, Andriole GL. Management of Benign Prostatic Hyperplasia. Annual review of medicine. 2016;67:137-51.

3. Taub DA, Wei JT. The economics of benign prostatic hyperplasia and lower urinary tract symptoms in the United States. Current urology reports. 2006;7(4):272-81.

4. Speakman M, Kirby R, Doyle S, Ioannou C. Burden of male lower urinary tract symptoms (LUTS) suggestive of benign prostatic hyperplasia (BPH) - focus on the UK. BJU Int. 2015;115(4):508-19.

5. Berry SJ, Coffey DS, Walsh PC, Ewing LL. The development of human benign prostatic hyperplasia with age. J Urol. 1984;132(3):474-9.

6. Matsuhashi S, Noji S, Koyama E, Myokai F, Ohuchi H, Taniguchi S, et al. New gene, nel, encoding a Mr 93 K protein with EGF-like repeats is strongly expressed in neural tissues of early stage chick embryos. Dev Dyn. 1995;203(2):212-22.

7. Watanabe TK, Katagiri T, Suzuki M, Shimizu F, Fujiwara T, Kanemoto N, et al. Cloning and characterization of two novel human cDNAs (NELL1 and NELL2) encoding proteins with six EGF-like repeats. Genomics. 1996;38(3):273-6.

8. Kuroda S, Oyasu M, Kawakami M, Kanayama N, Tanizawa K, Saito N, et al. Biochemical characterization and expression analysis of neural thrombospondin-1-like proteins NELL1 and
NELL2. Biochem Biophys Res Commun. 1999;265(1):79-86.

9. Watanabe TK, Katagiri T, Suzuki M, Shimizu F, Fujiwara T, Kanemoto N, et al. Cloning and characterization of two novel human cDNAs (NELL1 and NELL2) encoding proteins with six EGF-like repeats. Genomics. 1996;38(3):273-6.

10. Rao Z, Handford P, Mayhew M, Knott V, Brownlee GG, Stuart D. The structure of a Ca(2+)-binding epidermal growth factor-like domain: its role in protein-protein interactions. Cell. 1995;82(1):131-41.

11. Kim H, Ha CM, Choi J, Choi EJ, Jeon J, Kim C, et al. Ontogeny and the possible function of a novel epidermal growth factor-like repeat domain-containing protein, NELL2, in the rat brain. Journal of neurochemistry. 2002;83(6):1389-400.

12. Yang Y, Mu J, Chen G, Zhan Y, Zhong J, Wei Y, et al. iTRAQ-based quantitative proteomic analysis of cerebrospinal fluid reveals NELL2 as a potential diagnostic biomarker of tuberculous meningitis. International journal of molecular medicine. 2015;35(5):1323-32.

13. Nakamura R, Oyama T, Tajiri R, Mizokami A, Namiki M, Nakamoto M, et al. Expression and regulatory effects on cancer cell behavior of NELL1 and NELL2 in human renal cell carcinoma. Cancer Sci. 2015;106(5):656-64.

14. Osman I, Bajorin DF, Sun TT, Zhong H, Douglas D, Scattergood J, et al. Novel blood biomarkers of human urinary bladder cancer. Clinical cancer research : an official journal of the American Association for Cancer Research. 2006;12(11 Pt 1):3374-80.

15. Maeda K, Matsuhashi S, Tabuchi K, Watanabe T, Katagiri T, Oyasu M, et al. Brain specific human genes, NELL1 and NELL2, are predominantly expressed in neuroblastoma and other embryonal neuroepithelial tumors. Neurologia medico-chirurgica. 2001;41(12):582-8;
16. Shah US, Getzenberg RH. Fingerprinting the diseased prostate: associations between BPH and prostate cancer. J Cell Biochem. 2004;91(1):161-9.

17. Jeong JK, Kim HR, Hwang SM, Park JW, Lee BJ. Region- and neuronal phenotype-specific expression of NELL2 in the adult rat brain. Mol Cells. 2008;26(2):186-92.

18. Nelson BR, Claes K, Todd V, Chaverra M, Lefcort F. NELL2 promotes motor and sensory neuron differentiation and stimulates mitogenesis in DRG in vivo. Dev Biol. 2004;270(2):322-35.

19. Kim DH, Kim HR, Choi EJ, Kim DY, Kim KK, Kim BS, et al. Neural epidermal growth factor-like like protein 2 (NELL2) promotes aggregation of embryonic carcinoma P19 cells by inducing N-cadherin expression. PLoS One. 2014;9(1):e85898.

20. Ryu BJ, Kim HR, Jeong JK, Lee BJ. Regulation of the female rat estrous cycle by a neural cell-specific epidermal growth factor-like repeat domain containing protein, NELL2. Mol Cells. 2011;32(2):203-7.

21. Kiyozumi D, Noda T, Yamaguchi R, Tobita T, Matsumura T, Shimada K, et al. NELL2-mediated lumicrine signaling through OVCH2 is required for male fertility. Science (New York, NY). 2020;368(6495):1132-5.

22. Kim DH, Roh YG, Lee HH, Lee SY, Kim SI, Lee BJ, et al. The E2F1 oncogene transcriptionally regulates NELL2 in cancer cells. DNA Cell Biol. 2013;32(9):517-23.

23. Choi EJ, Kim DH, Kim JG, Kim DY, Kim JD, Seol OJ, et al. Estrogen-dependent transcription of the NEL-like 2 (NELL2) gene and its role in protection from cell death. J Biol Chem. 2010;285(32):25074-84.
24. Kim HR, Kim DH, An JY, Kang D, Park JW, Hwang EM, et al. NELL2 Function in Axon Development of Hippocampal Neurons. Mol Cells. 2020;43(6):581-9.

25. Munemasa Y, Chang CS, Kwong JM, Kyung H, Kitaoka Y, Caprioli J, et al. The neuronal EGF-related gene Nell2 interacts with Macf1 and supports survival of retinal ganglion cells after optic nerve injury. PLoS One. 2012;7(4):e34810.

26. Aihara K, Kuroda Si, Kanayama N, Matsuyama S, Tanizawa K, Horie M. A neuron-specific EGF family protein, NELL2, promotes survival of neurons through mitogen-activated protein kinases. Molecular Brain Research. 2003;116(1-2):86-93.

27. Choi EJ, Kim DH, Kim JG, Kim DY, Kim JD, Seol OJ, et al. Estrogen-dependent Transcription of the NEL-like 2(NELL2) Gene and Its Role in Protection from Cell Death. Journal of Biological Chemistry. 2010;285(32):25074-84.

28. Kim EK, Choi EJ. Compromised MAPK signaling in human diseases: an update. Archives of toxicology. 2015;89(6):867-82.

29. Zhang W, Liu HT. MAPK signal pathways in the regulation of cell proliferation in mammalian cells. Cell research. 2002;12(1):9-18.

30. Maik-Rachline G, Hacohen-Lev-Ran A, Seger R. Nuclear ERK: Mechanism of Translocation, Substrates, and Role in Cancer. Int J Mol Sci. 2019;20(5).

31. Xu H, Fu S, Chen Y, Chen Q, Gu M, Liu C, et al. Oxytocin: its role in benign prostatic hyperplasia via the ERK pathway. Clin Sci (Lond). 2017;131(7):595-607.

32. Wang K, Pascal LE, Li F, Chen W, Dhir R, Balasubramani GK, et al. Tight junction protein claudin-1 is downregulated by TGF-β1 via MEK signaling in benign prostatic epithelial cells. Prostate. 2020;80(14):1203-15.
33. Papatsoris AG, Papavassiliou AG. Molecular 'palpation' of BPH: a tale of MAPK signalling? Trends in molecular medicine. 2001;7(7):288-92.

34. Xiao H, Jiang Y, He W, Xu D, Chen P, Liu D, et al. Identification and functional activity of matrix-remodeling associated 5 (MXRA5) in benign hyperplastic prostate. Aging. 2020;12(9):8605-21.

35. DiLella AG, Toner TJ, Austin CP, Connolly BM. Identification of genes differentially expressed in benign prostatic hyperplasia. The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society. 2001;49(5):669-70.

36. Wang Q, Zhang Y, Zhu J, Zheng H, Chen S, Chen L, et al. IGF-1R inhibition induces MEK phosphorylation to promote survival in colon carcinomas. Signal transduction and targeted therapy. 2020;5(1):153.

37. Wang Y, Tan X, Tang Y, Zhang C, Xu J, Zhou J, et al. Dysregulated Tgfbr2/ERK-Smad4/SOX2 Signaling Promotes Lung Squamous Cell Carcinoma Formation. Cancer research. 2019;79(17):4466-79.

38. Xu D, Chen P, Xiao H, Wang X, DiSanto ME, Zhang X. Upregulated Interleukin 21 Receptor Enhances Proliferation and Epithelial-Mesenchymal Transition Process in Benign Prostatic Hyperplasia. Frontiers in endocrinology. 2019;10:4.

39. Oyasu M, Kuroda S, Nakashita M, Fujimiya M, Kikkawa U, Saito N. Immunocytochemical localization of a neuron-specific thrombospondin-1-like protein, NELL2: light and electron microscopic studies in the rat brain. Brain research Molecular brain research. 2000;76(1):151-60.

40. Jeong JK, Kim JG, Kim HR, Lee TH, Park JW, Lee BJ. A Role of Central NELL2 in the
Regulation of Feeding Behavior in Rats. Mol Cells. 2017;40(3):186-94.

41. Luo J, Dunn TA, Ewing CM, Walsh PC, Isaaacs WB. Decreased gene expression of steroid 5 alpha-reductase 2 in human prostate cancer: implications for finasteride therapy of prostate carcinoma. Prostate. 2003;57(2):134-9.

42. La Vignera S, Condorelli RA, Russo GI, Morgia G, Calogero AE. Endocrine control of benign prostatic hyperplasia. Andrology. 2016;4(3):404-11.

43. Wang N, Dong BJ, Quan Y, Chen Q, Chu M, Xu J, et al. Regulation of Prostate Development and Benign Prostatic Hyperplasia by Autocrine Cholinergic Signaling via Maintaining the Epithelial Progenitor Cells in Proliferating Status. Stem cell reports. 2016;6(5):668-78.

44. Iavarone C, Wolfgang C, Kumar V, Duray P, Willingham M, Pastan I, et al. PAGE4 is a cytoplasmic protein that is expressed in normal prostate and in prostate cancers. Molecular cancer therapeutics. 2002;1(5):329-35.

45. Fu S, Liu T, Lv C, Fu C, Zeng R, Kakehi Y, et al. Stromal-epithelial interactions in prostate cancer: Overexpression of PAGE4 in stromal cells inhibits the invasive ability of epithelial cells. J Cell Biochem. 2020;121(11):4406-18.

46. Hu G, Zhang J, Xu F, Deng H, Zhang W, Kang S, et al. Stomatin-like protein 2 inhibits cisplatin-induced apoptosis through MEK/ERK signaling and the mitochondrial apoptosis pathway in cervical cancer cells. Cancer Sci. 2018;109(5):1357-68.

47. Schägger H. Respiratory chain supercomplexes of mitochondria and bacteria. Biochim Biophys Acta. 2002;1555(1-3):154-9.

48. Salakou S, Kardamakis D, Tsamandas AC, Zolota V, Apostolakis E, Tzelepi V, et al.
Increased Bax/Bcl-2 ratio up-regulates caspase-3 and increases apoptosis in the thymus of patients with myasthenia gravis. In vivo (Athens, Greece). 2007;21(1):123-32.

49. Ma Y, Zhu B, Yong L, Song C, Liu X, Yu H, et al. Regulation of Intrinsic and Extrinsic Apoptotic Pathways in Osteosarcoma Cells Following Oleandrin Treatment. Int J Mol Sci. 2016;17(11).

50. Liu X, Kim CN, Yang J, Jemmerson R, Wang X. Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. Cell. 1996;86(1):147-57.

51. Ma J, Gharaee-Kermani M, Kunju L, Hollingsworth JM, Adler J, Arruda EM, et al. Prostatic fibrosis is associated with lower urinary tract symptoms. J Urol. 2012;188(4):1375-81.

52. Djavan B, Lin V, Seitz C, Kramer G, Kaplan P, Richier J, et al. Elastin gene expression in benign prostatic hyperplasia. Prostate. 1999;40(4):242-7.

53. Cantiello F, Cicione A, Salonia A, Autorino R, Tucci L, Madeo I, et al. Periurethral fibrosis secondary to prostatic inflammation causing lower urinary tract symptoms: a prospective cohort study. Urology. 2013;81(5):1018-23.

54. Bauman TM, Nicholson TM, Abler LL, Eliceiri KW, Huang W, Vezina CM, et al. Characterization of fibrillar collagens and extracellular matrix of glandular benign prostatic hyperplasia nodules. PLoS One. 2014;9(10):e109102.

55. Rodriguez-Nieves JA, Macoska JA. Prostatic fibrosis, lower urinary tract symptoms, and BPH. Nature reviews Urology. 2013;10(9):546-50.

56. Pierce KL, Luttrell LM, Lefkowitz RJ. New mechanisms in heptahelical receptor signaling to mitogen activated protein kinase cascades. Oncogene. 2001;20(13):1532-9.

57. Peterziel H, Mink S, Schonert A, Becker M, Klocker H, Cato AC. Rapid signalling by
816 androgen receptor in prostate cancer cells. Oncogene. 1999;18(46):6322-9.
817 58. Ip YT, Davis RJ. Signal transduction by the c-Jun N-terminal kinase (JNK)--from inflammation to development. Current opinion in cell biology. 1998;10(2):205-19.
818 59. Theyer G, Kramer G, Assmann I, Sherwood E, Preinfalk W, Marberger M, et al. Phenotypic characterization of infiltrating leukocytes in benign prostatic hyperplasia. Laboratory investigation; a journal of technical methods and pathology. 1992;66(1):96-107.
819 60. Li C, Hu WL, Lu MX, Xiao GF. Resveratrol induces apoptosis of benign prostatic hyperplasia epithelial cell line (BPH-1) through p38 MAPK-FOXO3a pathway. BMC complementary and alternative medicine. 2019;19(1):233.
820 61. Tran SE, Holmstrom TH, Ahonen M, Kahari VM, Eriksson JE. MAPK/ERK overrides the apoptotic signaling from Fas, TNF, and TRAIL receptors. J Biol Chem. 2001;276(19):16484-90.
821 62. Qiao L, Studer E, Leach K, McKinstry R, Gupta S, Decker R, et al. Deoxycholic acid (DCA) causes ligand-independent activation of epidermal growth factor receptor (EGFR) and FAS receptor in primary hepatocytes: inhibition of EGFR/mitogen-activated protein kinase-signaling module enhances DCA-induced apoptosis. Molecular biology of the cell. 2001;12(9):2629-45.
822 63. Shonai T, Adachi M, Sakata K, Takekawa M, Endo T, Imai K, et al. MEK/ERK pathway protects ionizing radiation-induced loss of mitochondrial membrane potential and cell death in lymphocytic leukemia cells. Cell death and differentiation. 2002;9(9):963-71.
823 64. Biswas SC, Greene LA. Nerve growth factor (NGF) down-regulates the Bcl-2 homology 3 (BH3) domain-only protein Bim and suppresses its proapoptotic activity by phosphorylation. J Biol Chem. 2002;277(51):49511-6.
65. Domina AM, Vrana JA, Gregory MA, Hann SR, Craig RW. MCL1 is phosphorylated in the PEST region and stabilized upon ERK activation in viable cells, and at additional sites with cytotoxic okadaic acid or taxol. Oncogene. 2004;23(31):5301-15.

66. Garcia J, Ye Y, Arranz V, Letourneux C, Pezeron G, Porteu F. IEX-1: a new ERK substrate involved in both ERK survival activity and ERK activation. The EMBO journal. 2002;21(19):5151-63.

67. Meloche S, Pouysségur J. The ERK1/2 mitogen-activated protein kinase pathway as a master regulator of the G1- to S-phase transition. Oncogene. 2007;26(22):3227-39.

68. Xu C, Shen G, Yuan X, Kim JH, Gopalkrishnan A, Keum YS, et al. ERK and JNK signaling pathways are involved in the regulation of activator protein 1 and cell death elicited by three isothiocyanates in human prostate cancer PC-3 cells. Carcinogenesis. 2006;27(3):437-45.

69. Tang D, Wu D, Hirao A, Lahti JM, Liu L, Mazza B, et al. ERK activation mediates cell cycle arrest and apoptosis after DNA damage independently of p53. J Biol Chem. 2002;277(15):12710-7.

70. Saito R, Krauze MT, Noble CO, Drummond DC, Kirpotin DB, Berger MS, et al. Convection-enhanced delivery of Ls-TPT enables an effective, continuous, low-dose chemotherapy against malignant glioma xenograft model. Neuro-oncology. 2006;8(3):205-14.

71. Shih A, Davis FB, Lin HY, Davis PJ. Resveratrol induces apoptosis in thyroid cancer cell lines via a MAPK- and p53-dependent mechanism. The Journal of clinical endocrinology and metabolism. 2002;87(3):1223-32.

72. Wu Z, Wu LJ, Tashiro S, Onodera S, Ikejima T. Phosphorylated extracellular signal-regulated kinase up-regulated p53 expression in shikonin-induced HeLa cell apoptosis.
73. Woessmann W, Chen X, Borkhardt A. Ras-mediated activation of ERK by cisplatin induces cell death independently of p53 in osteosarcoma and neuroblastoma cell lines. Cancer chemotherapy and pharmacology. 2002;50(5):397-404.

74. Tang D, Wu D, Hirao A, Lahti JM, Liu L, Mazza B, et al. ERK activation mediates cell cycle arrest and apoptosis after DNA damage independently of p53. J Biol Chem. 2002;277(15):12710-7.

75. Cagnol S, Chambard JC. ERK and cell death: mechanisms of ERK-induced cell death–apoptosis, autophagy and senescence. The FEBS journal. 2010;277(1):2-21.

76. Favata MF, Horiuchi KY, Manos EJ, Daulerio AJ, Stradley DA, Feeser WS, et al. Identification of a novel inhibitor of mitogen-activated protein kinase kinase. J Biol Chem. 1998;273(29):18623-32.

77. Luo J, Dunn T, Ewing C, Sauvageot J, Chen Y, Trent J, et al. Gene expression signature of benign prostatic hyperplasia revealed by cDNA microarray analysis. Prostate. 2002;51(3):189-200.
NELL2 

ERK1/2 

BAX expression 
Cytochrome c release 
Caspase 3 activation 

Cell apoptosis 

Cell proliferation 

BPH