Changes in the expression and functional activities of Myosin II isoforms in human hyperplastic prostate

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Abstract

Benign prostatic hyperplasia (BPH) is a common disease among aging males with the etiology remaining unclear. We recently found myosin II was abundantly expressed in rat and cultured human prostate cells with permissive roles in the dynamic and static components. This study aimed to explore the expression and functional activities of myosin II isoforms including smooth muscle myosin II (SMM II) and non-muscle myosin (NMM II) in the hyperplastic prostate. Human prostate cell lines and tissues from normal human and BPH patients were used. H&E, Masson’s trichrome, immunohistochemical staining, in vitro organ bath, RT-PCR and Western-blotting were performed. We further created cell models with NMM II isoforms silenced and proliferation, cycle, and apoptosis of prostate cells were determined by CCK-8 assay and flow cytometry. Hyperplastic prostate SM expressed more SM1 and LC17b isoforms compared to their alternatively spliced counterparts, favoring a slower more tonic-type contraction and greater force generation. For BPH group, blebbistatin (BLEB, a selective myosin II inhibitor), exhibited a stronger effect on relaxing phenylephrine (PE) pre-contracted prostate strips and inhibiting PE induced contraction. Additionally, NMMHC-A and NMMHC-B were upregulated in hyperplastic prostate with no change in NMMHC-C. Knockdown of NMMHC-A or NMMHC-B inhibited prostate cell proliferation and induced apoptosis, with no changes in cell cycle. Our novel data demonstrates that expression and functional activities of myosin II isoforms are altered in human hyperplastic prostate, suggesting a new pathological mechanism for BPH. Thus, the myosin II system may provide potential new therapeutic targets for BPH/lower urinary tract symptoms (LUTS).
Clinical Perspectives

(i) Benign prostatic hyperplasia (BPH) is one of the most common disease among aging males yet its etiology remains unclear.

(ii) Hyperplastic prostate SM expresses relatively more SM1 and LC17b isoforms, favoring a slower more tonic-type contraction and stronger force generation. NMMHC-A and NMMHC-B were upregulated in human hyperplastic prostate while knockdown of these isoforms resulted in prostate cells proliferation and apoptosis. Blebbistatin (BLEB) could relax prostatic strips and exhibited higher efficacy in BPH patients.

(iii) Alterations in the expression and functional activities of myosin II isoforms may be a novel pathological mechanism of BPH. In addition, BLEB may be a new therapeutic for BPH/lower urinary tract symptoms (LUTS).
Introduction

Benign prostatic hyperplasia (BPH) is one of the most common disease among aging males. Its incidence increases with age and reaches 90% at the age of 85\(^{(1)}\). The pathophysiology of BPH has two components, one is a static component that causes increased prostate volume, and the other is a dynamic component resulting in increased prostatic tone (active and passive tension) and bladder decompensation and overactivity\(^{(1)}\). Although aging and androgens play important roles in the development of BPH, its exact etiology remains unclear. Recently, we demonstrated that myosin II was abundantly expressed in the rat prostate where it could regulate prostatic tone and cell proliferation\(^{(2)}\). However, the myosin II in human prostate was less well studied. Exploring myosin II in human hyperplastic prostate may provide new insight into the pathophysiological mechanisms and therapeutic targets for BPH.

Myosin II, a motor protein in eukaryotic cells, induces a wide range of biological functions when coupled with actin\(^{(3)}\). Myosin II includes skeletal muscle myosin II, cardiac myosin II, smooth muscle (SM) myosin II (SMM II) and non-muscle myosin II (NMM II). The expression of the three muscle myosins is restricted largely to their respective muscle tissue, while NMM II is expressed in most mammalian cells including muscle cells. Our recent study demonstrated that SMM II is a contractile apparatus which mainly regulated prostatic SM tone while NMM II may regulate prostatic cell proliferation\(^{(2)}\).

SMM II is a hexameric molecule consisting a pair of myosin heavy chains (MHC) and two pairs of myosin light chains (MLC, both MLC\(_{17}\) and MLC\(_{20}\))\(^{(4)}\). After
alternatively splicing on smooth muscle myosin heavy chain (SMMHC) pre-mRNA, 5’ end isoforms (SM1 and SM2) and 3’ end isoforms (SM-A and SM-B) are produced, respectively(5, 6). Similarly, MLC_{17} also has 3’ end isoforms (LC_{17a} and LC_{17b})(7).

Indeed, previous studies have demonstrated that SMM II isoform composition may influence force generation and force maintenance(8, 9). The SM2, SM-B and LC_{17a} isoforms are associated with a faster more phasic-type contraction (e.g. urinary bladder), whereas the SM1, SM-A and LC_{17b} isoforms are associated with a slower more tonic force generation (e.g., aorta)(10-14). Recently, we demonstrated that the normal rat prostate expressed less SM2 (11.4%), more LC_{17a} (83.8%) and almost similar SM-B (58.8%) compared to their corresponding alternatively spliced isoform, exhibiting an intermediate contraction profile between phasic-type and tonic-type(2).

Meanwhile, we also demonstrated that SMM II isoform composition and contractility profile is altered in the castrated rat prostate, which expresses more SM2 and SM-B but less LC_{17a}, favoring a faster more phasic-type contraction(15). However, SMM II isoform composition and correlating contractility profiles for human prostate remain undefined. Moreover, whether the expression and functional activity of SMM II is altered in hyperplastic prostate is worth exploring.

Similar to the structure of SMM II, NMM II is also a hexameric molecule consisting of three components: a pair of MHCs, a pair of regulatory MLCs that regulate NMM II activity and a pair of essential MLC that stabilize the MHC structure. There are three NMMHC isoforms (NMMHC-A, NMMHC-B and NMMHC-C), encoded by three different genes (MYH9, MYH10 and MYH14), respectively(16-18).
In contrast to the role of SMM II in mediating prostatic SM tension, NMM II was demonstrated to play important roles in cellular “housekeeping”-type processes, including cell proliferation, adhesion, migration, along with synthetic and secretory functions(3, 19). Since NMM II is highly expressed in embryonic tissues and downregulated in mature rats(20), NMM II is also referred to embryonic myosin II. Indeed, previous studies demonstrated that NMMHC-A and NMMHC-B play essential roles in tissue formation and organ development, including visceral endoderm(21), placenta(22), alveolar(23), coronary vasculature(24), kidney(25), etc. NMMHC-C was highly expressed in mouse cochlea and was crucial for auditory function(26). New glands, which can only be seen in the embryonic period, are often found in human hyperplastic prostate. It therefore has been proposed that the occurrence of BPH is the “reawakening” of the embryonic process involving prostate mesenchyme induced epithelial differentiation(27). Our recent study also identified these three NMM II isoforms in rat prostate tissues and cultured human prostate cells, and we showed that NMM II might play an important role in cell proliferation and BPH development(2). Again, these NMM II isoforms were less well studied in the human hyperplastic prostate.

Currently, with regard to the therapeutic treatment of BPH, 5α-reductase inhibitors (reducing prostate volume) and α-adrenergic blockers (decreasing prostatic SM tone) are the first line agents and are effective per se. However, side effects frequently occur such as dizziness, asthenia and sexual dysfunction. In addition, approximately 30% of BPH patients still require surgical treatment(28). Therefore, it
is necessary to identify new therapeutic targets for BPH.

Blebbistatin (BLEB), a small cell permeable selective myosin II inhibitor, was originally discovered as an inhibitor of NMM II(9). Our recent study found that BLEB significantly inhibits proliferation of human epithelial and stromal cells \textit{in vitro}, and that intra-prostatic injection of BLEB can reduce the volume of the rat prostate(29). In addition, BLEB has been suggested to inhibit SM contraction with near equipotency as for NMM II(30-32). We also demonstrated that BLEB could relax rat prostate strips in a dose-dependent manner(33). However, the effect of BLEB on human normal and hyperplastic prostate has been less well examined and whether its efficacy is altered in the hyperplastic prostate remains unknown.

Materials and Methods

Human tissue acquisition.

Human hyperplastic prostate tissues were obtained from ten male patients (mean age, 68.3±3.5 years) needing to undergo radical cystectomy for infiltrating bladder cancer. All samples showed BPH and no tumor infiltration, which were identified by two separate pathologists. Normal prostate tissue was obtained from ten brain-dead men (mean age, 31.7±2.5 years) undergoing donation at the Organ Transplant Center of Zhongnan Hospital, with pathological examination revealing no hyperplasia. Additionally, aorta, vena cava, corpus cavernosum (CC) and bladder tissues from normal humans were also obtained. 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.

**Human prostatic cell lines.**

Two prostatic cell lines BPH-1 and WPMY-1 were used for our current study. BPH-1, human benign prostatic enlargement epithelia cell line (Cat. #BNCC339850, purchased from the Procell Co., Ltd., Wuhan, China), was cultured in RPMI-1640 medium (Gibco, China) containing 10% fetal bovine serum (FBS) (Gibco, Australia). WPMY-1, SV40 large T antigen-immortalized stromal cell line (Cat. #GNHu36, purchased from the Stem Cell Bank, Chinese Academy of Sciences, Shanghai, China), was cultured in DMEM medium (Gibco, China) containing 1% penicillin G sodium/streptomycin sulfate and 5% FBS. All cells were cultured in a humidified atmosphere consisting of 95% air and 5% CO$_2$ at 37 ºC. Identification of the cell lines was performed at the China Center for Type Culture Collection in Wuhan, China.

**H&E Staining and Masson’s Trichrome Staining.**

Human prostate tissues fixed in 10% neutral buffered formalin for 48 h were processed routinely for paraffin embedding. The paraffin-embedded tissue sections (4 μm) were stained with hematoxylin and eosin using standard techniques. The paraffin sections were deparaffinized in xylene, followed by graded alcohols. Masson composite staining solution (Fuzhou Maxim Biotech Co., Ltd., Fuzhou, China) was added dropwise for 10 min. The sections were subsequently washed with distilled water, differentiated in phosphomolybdic-phosphotungstic acid solution for 10 min,
and incubated with blue staining solution for 5–10 min. Next, the sections were rinsed briefly in distilled water and differentiated in 1% acetic acid solution for 2 min. After being dehydrated quickly through 95% alcohol and then absolute alcohol, the sections were cemented using neutral gum for observation. Using this procedure, prostatic SM cells were stained red, collagen fibers were stained blue and epithelial cells were stained orange. In each sample, we analyzed three areas under magnification (×100). The choice of three fields was randomized without specific areas of a demarcated slide. The area percentage of epithelial, SM and collagen fibers were quantitated with Image Pro Plus 6.0 software, respectively.

**In vitro organ bath studies.**

After procurement from patients (or brain-dead men), fresh prostate specimens were immediately stored in ice-cold Krebs-Hensleit (Krebs) buffer with continuous bubbling of 95% O₂ and 5% CO₂ and then transported on ice to laboratory. Prostate specimens were cut into vertical strips in the same direction, and the dimensions of the prostate strips were approximately 1 × 0.5 × 0.5 cm. As previously described(33), human prostate strips with identical lengths were mounted longitudinally in a 10 ml organ bath (Multi-Myograph Model 810MS; Danish Myo Technology; Aarhus, Denmark). The myograph was connected in line to a PowerLab 4/30 Data Acquisition System (ADInstruments; Colorado Springs, CO, USA) and in turn to a Dual-Core processor Pentium computer for real-time monitoring of physiological force. The prostate strips were equilibrated at least 1 h in Krebs buffer at 37°C with continuous
bubbling of 95% O₂ and 5% CO₂. The buffer was composed of (in mM): NaCl 110, KCl 4.8, CaCl₂ 2.5, MgSO₄ 1.2, KH₂PO₄ 1.2, NaHCO₃ 25 and dextrose 11) and during the organ bath experiments, the Krebs buffer was changed every 15 minutes. During the process of equilibration, the tension of the strips was continuously adjusted to 1,000 mg. After equilibration, prostate strips were contracted with 60 mM KCl and allowed to reach the maximum force. Then the strips were washed several times with buffer and tension was brought to baseline, subsequently, cumulative concentrations (10⁻⁷~10⁻³ M) of phenylephrine (PE) were administered. The maximum force of KCl depolarization was taken as 100% and the force generated by PE was normalized to a percentage of this value. After the contraction experiment was completed, the strips were washed several times using buffer and the tension was reduced to the baseline. Next, strips were pre-contracted with 10⁻⁵ M PE (a dose that induce submaximal contraction) and allowed to reach stable tension and then the relaxant effects of increasing doses of blebbistatin (BLEB) were evaluated. Additionally, after pre-incubation with 15 mM BLEB for 30 min, its inhibitory effect on PE (10⁻⁵ M) contractility was also tested.

**Knockdown of NMMHC-A and NMMHC-B in prostatic cells.**

MYH9- and MYH10-target specific small interfering RNAs (siRNAs) were synthesized by GenePharma Ltd. in Suzhou, China. BPH-1 and WPMY-1 cell lines were transfected with MYH9-siRNAs (si-NMMHC-As) or MYH10-siRNAs (si-NMMHC-Bs) using lipofectamine 2000 (Invitrogen, USA), according to the
manufacturer’s instruction. The sequences of si-NMMHC-As and si-NMMHC-Bs are listed in Supplementary Table 1. After transfection by si-NMMHC-A or si-NMMHC-B for 48 h, alterations of NMMHC-A and NMMHC-B at transcriptional and protein levels were evaluated by the qRT-PCR, Western-blot.

**CCK-8 (cell counting kit-8) assay.**

After transfection with siRNAs for 48 h, 2500 BPH-1 or WPMY-1 cells /100 μl medium were seeded in 96-well plates and allowed to grow for another four days. Then 10 μl CCK-8 solution was added into each well and incubated at 37°C for 1 h. The absorbance at 450 nm was measured by a microplate reader (cat. no. SpectraMax M2; Molecular Devices, Sunnyvale, CA, USA) at the same time for each day.

**Flow cytometry analysis for cell cycle and apoptosis.**

1 × 10^6 cells were harvested for cell cycle and apoptosis analyses. For cell cycle analysis, cells were centrifuged and then pellets were resuspended with PBS containing 50 μg/ml propidium iodide (Multi Sciences, Hangzhou, China) and 0.1 mg/ml RNaseA (20 μg/ml in PBS) in the dark. After incubation at 37°C for 30 min, the DNA content distribution was analyzed by flow cytometry analysis (Beckman, Cat. #FC500). Cell apoptosis was analyzed by flow cytometry using the Annexin V-FITC apoptosis analysis kit (Sungene Biotech., Tianjin, China), according to the manufacturer’s instructions.
Total RNA extraction and cDNA synthesis.

As described in a previous study from our group (33), total RNA was isolated from tissues and cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocol. One μg of total RNA was reverse-transcribed to cDNA via the SuperScript II First-Strand Synthesis System according to the instructions (Invitrogen).

Competitive reverse transcriptase polymerase chain reaction (competitive RT-PCR).

A total of 20 μL reaction volumes including 2 μL of the RT product cDNA (50 ng/μL), 10 μL blue TSINGKE Master Mix (TSE004, TSINGKE, Wuhan, China), 1μL each of upstream and downstream primer (50 μM) and 6 μL ddH2O were used to perform polymerase chain reaction (PCR). SM-A/SM-B, SM1/SM2 and LC17a/LC17b alternatively splice isoforms were amplified with competitive PCR using a GeneAmp 9700 thermal cycler (Applied Biosystems, Foster City, CA, USA). The primer sequences are shown in Supplementary Table 2. The cycling conditions were an initial 5 min at 94°C followed by 35 cycles (30 sec at 94°C, 30 sec at 55°C and 120 sec at 72°C), ending with a final one-time 7-min incubation at 72°C to ensure extension of all products. These three pairs of myosin isoforms are generated via alternative splicing, which allowed us the opportunity to perform competitive PCR for each pair of isoforms by designing primers that flanked the insert regions.

Next, the PCR products were electrophoresed on a 2% agarose gel to separate
them. Then the PCR products were visualized using GelStar staining and ultraviolet illumination. Band density was quantified by reflectance scanning of gel photographs obtained with a BioDoc-It camera set-up (UVP; Upland, CA, USA) using a Bio-Rad (Hercules, CA, USA) GS-700 imaging densitometer. Finally, the Bio-Rad Molecular Analyst 1D program was used for analyzing and quantitating the relative SMM II isoforms expression for each pair of isoforms. Relative expression of SM-A was calculated by the ratio of SM-A isoform expression to the total expression of SM-A and SM-B. In a similar manner, relative expressions of LC17b and SM1 isoforms were also determined.

**Real-Time reverse transcriptase polymerase chain reaction (Real-Time RT-PCR)**

As previously described(34), RT products were amplified in a 96-well plate in a 20 μL reaction volume with all samples run in triplicate, using the CFX Connect Real-Time PCR System™ (Bio-Rad). The following experimental protocol was utilized: denaturation (95°C for 10 min to activate the polymerase) followed by an amplification program repeated for 40 cycles (95°C for 15 sec, then 60°C for 60 sec) using a single fluorescence measurement. Primer sequences are shown in Supplementary Table 2. Target genes were amplified using SYBR Green for amplicon detection. For relative quantification, gene expression was normalized to expression of the RPL19 ribosomal housekeeping gene as an internal control and compared by $2^{-ΔΔCT}$ method.
SDS-PAGE and Western-Blotting Analysis.

As previously described (34), proteins were extracted from tissues and cells using RIPA (Radio-Immunoprecipitation Assay) lysis buffer (Sigma-Aldrich, St Louis, Mo) (containing 1 × PBS, 1% IGEPAL CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 10 mM EDTA) with freshly added phenylmethanesulphonylfluoride (PMSF; Sigma Aldrich, St. Louis, MO, USA) and sodium orthovanadate (Sigma Aldrich). An aliquot of 100μg of each sample was electrophoresed on a 7.5% or 12.5% sodium dodecyl sulfate–polyacrylamide (SDS-PAGE) gel (Wuhan Boster Biological Technology Ltd, Wuhan, China) and transferred to polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA) using a Bio-Rad wet transfer system. The membrane was blocked for 2h at room temperature with Tris–buffered saline with 0.1% [v/v] Tween (TBST) containing 5% [w/v] non-fat dry milk. The membranes were incubated overnight with corresponding protein primary antibodies (Information of primary antibodies was listed in Supplementary Table 3). After washing several times, the membranes were incubated with secondary antibody at room temperature for 2h. Detection of reaction antigen was performed with an enhanced chemiluminescence (ECL) kit (Thermo Scientific Fisher, Waltham, MA, USA). The bands were quantified by reflectance scanning of gel photographs obtained with a BioDoc XRS+ camera using Bio-Rad Molecular Imager® ChemiDoc™ XRS+ System and Quantity One® SW 1-D Analysis Software (Bio-Rad).
Immunohistochemistry (IHC)

As previously described (33), human prostate tissues fixed in 10% neutral buffered formalin for 48 hours were routinely processed for paraffin embedding. Samples were sectioned at 5 μm and deparaffinized in xylene followed by descending grades of ethanol (100%, 95%, 70%, 30%). Antigen retrieval was performed in 10 mM sodium citrate buffer at pH 6.0, heated to 96°C, for 30 min., followed by proteinase K treatment for 10 min. Endogenous peroxidase activity was quenched using 3% hydrogen peroxide in PBS for 15 min. Blocking was performed by incubating sections in 5% normal donkey serum with 2% BSA for 1 hr. The sections were stained by routine IHC methods, using horse radish peroxidase polymer conjugate (Invitrogen), to localize the antibody bound to antigen, with diaminobenzidine as the final chromogen. All immunostained sections were lightly counterstained with haematoxylin. The primary antibodies (information listed in Supplementary Table 3) to target proteins were incubated for 1 hour at room temperature. Slides were evaluated for immunostaining by light microscopy.

For each slide, twenty area fields under 100 × magnification were selected randomly from each specimen (ten from stroma and another ten from epithelial compartment). For each field, IOD (integrated optical density) was calculated using Image Pro Plus 6.0 software. The mean density was calculated by IOD/area and the average values were used for target protein expression quantitative analyses.

Statistical analysis.

Results are expressed as mean ± SD for n experiments. Statistical analysis used
the Student’s t-test with Excel software (two-sample treatments compared). $p < 0.05$ was considered significant.

**Results**

**Histological examination for human prostate.**

Human hyperplastic prostate showed obviously stroma hyperplasia accompanied with epithelial hyperplasia including epithelial layer thickening, twisted and folded, and papillary fronds protruding into the glandular cavities (Fig. 1A). In sections of Masson’s trichrome staining, compared to control group, hyperplastic prostate showed increased component of epithelia ($p < 0.05$), SM ($p < 0.05$) and collagen fibers ($p < 0.05$) (Fig. 1B).

**In vitro contractility of human hyperplastic prostate strips.**

In response to adrenergic stimulation (PE), human prostate strips produced significant force in a dose-dependent manner. Notably, hyperplastic prostate strips generated more force. As shown in Fig. 2A, isolated prostatic strips from both normal human and BPH patients reached maximal contraction at $10^{-4}$ M PE, but maximal contraction forces were around 125% and 175% of KCl depolarization induced tension, respectively.

In addition, human hyperplastic prostate exhibited a decreased shortening velocity compared to control tissue. This was reflected by a longer time to 50% PE induced maximum contraction for BPH tissue that was significantly slower than that
of controls (Fig. 2B), being 80.03±18.35 S and 48.67±10.63 S (BPH vs. control, \( p < 0.05 \)), respectively. As shown in Fig. 2, the representative tracings of contraction in the response to \( 10^{-5} \) M PE, hyperplastic prostate (Panel D) exhibited a slower more tonic-type contraction compared to normal prostate tissue (Panel C).

**Composition of SMM II isoforms in human hyperplastic prostate is altered.**

The composition of SMM II isoforms in human normal tissue (prostate, aorta, vena cava, corpus cavernosum (CC) and bladder) were detected by competitive RT-PCR (Fig. 3A). Among these tissues, aorta expressed the highest ratio of these three isoforms (90.3% SM-A, 78.3% LC\(_{17b}\), 64.6% SM1), while bladder expressed the lowest (74.6% SM-A, 11.2% LC\(_{17b}\), 55.3% SM1). Of note, these three isoforms in human normal prostate tissue were all between that of bladder and aorta, which was 79.9% for SM-A, 30.5% for LC\(_{17b}\) and 61.4% for SM1, respectively.

As shown in Fig. 3B & 3C, when compared to controls, human hyperplastic prostate expressed higher relative ratio of LC\(_{17b}\) (47.7% vs. 30.5%, \( p < 0.01 \)) and SM1 (69.3% vs. 61.4%, \( p < 0.05 \)), with no statistical difference in relative ratio of SM-A between groups (82.8% vs. 79.9%).

**Blebbistatin strongly relaxes human prostate strips.**

As a selective myosin II inhibitor, Blebbistatin (BLEB) relaxed PE pre-contracted human prostate SM strips in a dose-dependent manner (Fig. 4A, B). At 10 \( \mu \)M, BLEB could almost decrease the tension of the prostate strips to baseline. In addition, BLEB exhibited more effective relaxation on human hyperplastic prostate strips (Fig. 4C). Consistently, pre-incubation of prostate strips with 15 \( \mu \)M BLEB
effectively inhibited $10^{-5}$ M PE-induced tension increase for both control (Fig. 4D) and BPH patients (Fig. 4E), with inhibition percentage increased by approximately 15% for prostate strips from BPH patients (Fig. 4F).

**Immunohistochemistry analysis of SM MHC and NMM II in human prostate.**

As shown in Figure 5, SM MHC was abundant only in the stroma component of human prostate tissue (Fig. 5A), while NMMHC-A (Fig. 5B), NMMHC-B (Fig. 5C) and NMMHC-C (Fig. 5D) were localized both in stroma and epithelial compartments. With regard to quantitative analysis (Fig. 5E), MHC protein expression was elevated in stroma of hyperplastic prostate. Meanwhile, NMMHC-A and NMMHC-B proteins expression were also increased in both stroma and epithelial compartments of hyperplastic prostate. However, NMMHC-C protein expression showed no change between BPH and control group in either the stroma or epithelial compartments.

**Expression of SM MHC and NMM II isoforms in human prostate.**

The expression of SM MHC and NMM II isoforms (NMMHC-A, NMMHC-B, NMMHC-C) of human prostate were further determined by Western blotting (Fig. 6A, B) and RT-PCR (Fig. 6C). SM MHC, NMMHC-A and NMMHC-B were upregulated in hyperplastic prostate tissue at both mRNA and protein levels. As for NMMHC-C, no alteration of expression was observed between control and BPH groups.

**Knockdown of NMMHC-A or NMMHC-B protein expression in BPH-1 and WPMY-1 cells inhibited prostate cell proliferation and promoted apoptosis.**

To create a cell model of NMMHC-A deficiency, three distinct MYH9-target-specific-siRNAs (si-NMMHC-As) were transfected into BPH-1 and
WPMY-1 cells. After 48 h, the knockdown efficiency was validated by RT-PCR and Western blot analysis (Fig. 7A). For both cell lines, the inhibitory efficiency of si-NMMHC-A-2 was more than 80%, therefore this siRNA was chosen for further experiment. Similarly, the efficiencies of three distinct MYH10-target-specific-siRNAs (si-NMMHC-Bs) were determined (Fig. 7B) and si-NMMHC-B-1 was chosen for further experiment.

Then proliferation, apoptosis and cell cycle stage of transfected cells were analyzed. CCK-8 assay showed that knockdown of NMMHC-A or NMMHC-B significantly inhibited both BPH-1 and WPMY-1 cells proliferation (Fig. 7C). In addition, as revealed by flow cytometry analysis, knockdown of NMMHC-A or NMMHC-B promoted prostate cells apoptosis (Fig. 7D). For NMMHC-A knockdown, the apoptosis rate increased by 13.5% for BPH-1 cells, ($p < 0.01$) and 6.8% for WPMY-1 cells, ($p < 0.01$), respectively, while for NMMHC-B knockdown, the apoptosis rate increased by 4.4% for BPH-1 cells ($p < 0.01$) and 6.3% for WPMY-1 cells, ($p < 0.01$), respectively. However, as determined by flow cytometry analysis, there were no changes in cell cycle stage after knocking down NMMHC-A or NMMHC-B either in BPH-1 or WPMY-1 cells (Fig. 7E).

**Discussion**

Our novel data demonstrated the alteration in expression and functional activity of SMM II and NMM II isoforms in human hyperplastic prostate tissue. Human hyperplastic prostate tissue expressed more SM1 and LC$_{17b}$ isoforms than normal
prostate, correlating with a switch to a slower more tonic SM contraction phenotype at the functional level. In addition, the myosin II inhibitor BLEB could potently relax human prostatic SM, and it exhibited higher inhibitory efficacy for hyperplastic prostate tissue compared to normal prostate control. We also determined that NMMHC-A and NMMHC-B were upregulated in hyperplastic prostate, while knockdown of NMMHC-A or NMMHC-B could inhibit proliferation and induce apoptosis of prostatic cells.

Our recent study showed that rat prostatic SM could generate significant force in response to KCl depolarization or α1-adrenoceptor stimulus(2), which suggested this active force might play an important role in the pathophysiology of BPH. Indeed, our current study found that human prostatic SM exclusively distributed in the stroma component, which also generated significant force like rat. Moreover, we found that human hyperplastic prostate strips generated more force than normal prostate in response to PE (Fig. 2). This increase in active tension, which was mediated by the adrenergic nervous system, may contribute to the development of BPH/LUTS. Our Masson’s trichrome staining revealed that the expected increase in SM content for human hyperplastic prostate (Fig. 1), which may lead to the stronger force. Consistently, expression of SMMHC (a strong marker of the SM phenotype) was also elevated at both the mRNA and protein level in our current study (Fig. 5 & 6). In contrast, a previous study from Lin and his colleagues(35) found that SMMHC was downregulated in human hyperplastic prostate when compared with normal prostate. However, they only detected the expression of SMMHC at the mRNA level.
Moreover, previous studies demonstrated that $\alpha_{1a}$-adrenoceptors were upregulated during BPH(36). In human prostate, $\alpha_{1a}$-adrenoceptor is the most abundant subtype among three $\alpha_1$-adrenoceptor subtypes ($\alpha_{1a}$, $\alpha_{1b}$ and $\alpha_{1d}$), and it is expressed particularly in the prostatic stroma and mainly mediates active tension in human prostatic smooth muscle. Therefore, upregulation of this $\alpha_1$-adrenoceptor subtype may also contribute to the increase in force generation.

Subsequently, we thoroughly determined the composition of the SMM II isoforms and found the prostate expressed relatively more SM-A (79.9%), less LC$_{17b}$ (30.5%) and equal SM1 (61.4%) compared to their alternatively spliced isoform counterpart. However, the composition of SMM II isoforms in human prostate was different from that in rat normal prostate(2, 15), which expressed more SM1 (88.6%), less LC$_{17b}$ (16.2%) and almost similar SM-A (41.2%). Interestingly, both human and rat prostatic strips exhibit an intermediate phenotype between tonic- and phasic-contraction.

In addition, human hyperplasia prostate tissue expressed more LC$_{17b}$ and SM1 isoforms than normal prostate (Fig. 3), with no change in expression ratio of SM-A, which favored a more tonic-type contraction. Previous studies had demonstrated that the relative higher ratio of LC$_{17b}$ to LC$_{17a}$ isoform in SM was associated with slower shortening velocity and a more tonic contraction(37-39). Indeed, we found that the time to 50% maximum contraction of prostate strips from BPH patients was longer than that of controls (in Fig. 2). Similar to the LC$_{17b}$ isoform, it was demonstrated that the SM-B isoform was associated with a more phasic contraction, faster shortening
velocity and higher ATPase activity, whereas the SM-A isoform was found to be expressed more in slower and more tonic type SM with lower ATPase activity (10-12). However, the relative ratio of SM-A to SM-B was not alterations between BPH and normal groups. With regard to SM1/2 isoforms, selective knock out of the SM2 isoform (all SM1 isoform remaining) in mice was shown to lead to increases in KCl and carbachol-induced contractions (40). In addition, transgene overexpression of SM1 in mice enhanced SM contraction while transgene overexpression of SM2 attenuated SM contraction (41). Therefore, based on above two SM1/2 genetic manipulation studies, the increased expression ratio of SM1 in human hyperplastic prostatic SM (in Fig. 3) may contribute to the increased force generation which was demonstrated in our current study.

Besides the alterations in expression and functional activity of SMM II, we also observed changes in NMM II expression in hyperplastic prostate. Our recent study demonstrated that all three NMM II isoforms were richly expressed in rat prostate tissue and also expressed in human prostate cell lines (2). Consistently, our current study also identified these isoforms in human prostate tissue and additionally found NMMHC-A and NMMHC-B are upregulated in both the stroma and epithelial compartments of hyperplastic prostate (Fig. 5 & 6). Considering that NMM IIs are known to play important roles in “housekeeping” processes and are essential for tissue formation and organ development, these two upregulated isoforms (NMMHC-A and NMMHC-B) may be mechanistically associated with the occurrence and development of BPH. Indeed, our recent study found BLEB (a selective myosin ATPase inhibiting
agent) could downregulate NMMHC-A and NMMHC-B protein expression, and induce prostate cell apoptosis (29). Similarly, we found knockdown of NMMHC-A inhibited prostatic cells proliferation and induced apoptosis. Previous studies demonstrated that NMMHC-A could promote cancer cell growth via the p53 (42) and MAPK/AKT signaling pathways (43), and regulate the epithelial-mesenchymal transition (EMT) process (44-46). It is also well known that p53 is a crucial mediator of cell cycle arrest and cell apoptosis, and evidences demonstrated that accumulation of mesenchymal-like cells derived from the prostatic epithelial cells via EMT process were associated with BPH development (47-49). Interestingly, our recent microarray study also found that the MAPK pathway, which regulates cell proliferation and cell cycle, was linked to BPH (34). Therefore, these findings suggest that NMMHC-A may be involved in the development of BPH through the p53 pathway, MAPK signaling and the EMT process. In contrast, a previous study (35) showed no change in NMMHC-A expression between hyperplastic and normal prostate, which was not consistent with our current findings. However, they only examined expression at the mRNA level, while our current study performed more comprehensive studies such as RT-PCR, Western-blotting and IHC. In summary, the NMMHC-A in prostate remains less studied, and the in-depth exploration to elucidate the mechanisms involved will be intriguing.

Similar to NMMHC-A, knockdown of NMMHC-B also inhibited prostatic cells proliferation and induced apoptosis. NMMHC-B has been reported to play an important role in extracellular matrix (ECM) remodeling (23) and regulation of the
EMT process (24, 50). The ECM is a complex network composed of a variety of molecules secreted by supporting cells. It not only provides an environment for cell survival and activities, but also regulates the shape, metabolism, function, migration, proliferation and differentiation of cells through signal transduction (51). Prostatic stromal-epithelial interactions are essential during development of the normal prostate (52) and associated with the etiology of BPH referred to as “reawakening” of the embryonic process (27), which suggests that NMMHC-B may be involved in the pathogenesis of BPH via regulating stromal-ECM-epithelial relationships.

NMMHC-C is a relatively newly identified NMM II isoform associated with deafness (53) and peripheral neuropathy (54). Recent studies also have demonstrated that NMMHC-C regulates cell polarity and invasion (55, 56) acting as an actomyosin cytoskeleton. Its role in the prostate is unclear, however, and no expression alteration was observed between the BPH and control groups in our present study.

Since our recent study demonstrated that BLEB could potently relax PE pre-contracted rat prostate SM (33), a similar effect on human prostate SM may be expected. Indeed, our current data revealed that BLEB also potently relaxed PE pre-contracted human prostate SM contraction and pre-incubation with BLEB attenuated PE induced prostate SM contraction, with sensitivity increased for BPH patients. Given that BLEB is a selective myosin ATPase inhibiting agent for both SMM II and NMM II, it may be that the upregulation of SMM II and NMM II isoforms in hyperplastic prostate (in Fig. 6) contribute to the increased efficacy of BLEB on the BPH group. Therefore, both passive and active forces in hyperplastic
prostate could be attenuated in the presence of BLEB, however, the factors that
determine passive tone in the prostate remain to be determined. The series elastic
elements in the stromal and epithelial cells and the ECM contribute to passive tissue
force, which is independent of active SM contraction. Meanwhile, α-blockade could
only reduce active tension, but not decrease passive tension in the prostate. BLEB
may reduce both active (through SMM II) and passive (possible through NMM II)
tension in the human prostate by inhibiting myosin II, which suggests that BLEB may
be a promising to effective agent with potential as a new therapeutic for BPH/LUTS.
However, BLEB has several unfavorable features including structural instability, high
hydrophobicity, cytotoxicity, phototoxicity and high fluorescence(57). Therefore, the
clinical application of BLEB may have some limitations, such as a wide range of
systemic side effects. In recent years, some scholars have modified the molecular
structure of BLEB to ameliorate its unfavorable physicochemical and biological
properties, while retaining its inhibitory properties to a large extent(58-62). The effect
of these novel BLEB derivatives on treating BPH/LUTS may be worth exploring in
future.

One limitation for our current study is that the protein levels of SMM II isoforms
were not determined because isoform-specific antibodies are not commercially
available at present. However, a previous study demonstrated that mRNA levels of
SMM II isoforms correlated well with protein expression(63).

In conclusion, we demonstrate alterations in the expression and functional
activities of myosin II isoforms between hyperplastic and normal prostate, which
suggests a new pathological mechanism for BPH. BLEB may be expected to be an effective potential new therapeutic for BPH/LUTS.

Data Availability Statement

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

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Author Contributions

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

Figure 1. Histological examination of human hyperplastic prostate.
(A) Hematoxylin and eosin (H&E) staining (upper) and Masson’s trichrome staining (bottom) of prostate tissue sections from human control and BPH, respectively. The scale bar is 100 μm. In Masson’s trichrome staining graphs, prostatic smooth muscle (SM) cells were stained red, collagen fibers were stained blue and epithelial cells were stained orange. (B) The bar graph for area percentage of different component (epithelia, SM and collagen fibers) between control and BPH group, respectively, from Masson’s trichrome staining (n = 10 different humans for each group). *p < 0.05 vs. control.

Figure 2. In vitro contractility of human prostate strips and analysis.
(A) Summary graph for in vitro contraction force of prostate strips in response to increasing doses (10^{-7}~10^{-3} M) of phenylephrine (PE) from normal humans and BPH patients (n = strips obtained from 10 different humans for each group, one strip was used for each human). The maximum response to 60 mM KCl was taken as 100%, while the contractile effect of PE was evaluated as a percentage of this response. Data was shown as mean ± SD. *p < 0.05 vs. control; **p < 0.01 vs. control. NS means no significant differences between groups. (B) Summary graph of time to 50% of PE induced maximum contraction for prostate strips from normal humans and BPH patients. Data was shown as mean ± SD. *p < 0.05 vs. control. The bottom panels are typical force tracings of human prostate strips in response to 10^{-5} M PE. (C) Control group; (D) BPH group. The x-axis represents time (min), while the y-axis represents force (mg).

Figure 3. The composition of SMM II isoforms in human tissues.
(A) SMM II isoforms expression level in prostate, aorta, vena cava, corpus cavernosum (CC) and bladder tissue from normal humans (n = 10). Data was shown as mean ± SD. (B) Typical competitive RT-PCR bands of SMM II isoforms for prostate tissues from normal humans and BPH patients. (C) The bar graph for relative expressions of SM-A, LC_{17a} and SM1 between different groups (n = 10 different
humans for each group). Data was shown as mean ± SD. \( *p < 0.05 \) vs. control; \( **p < 0.01 \) vs. control. NS means no significant differences between groups.

**Figure 4. In vitro effect of Blebbistatin (BLEB) on human prostate strips.**

Upper panels of this figure showed the relaxant effect of BLEB on PE pre-contracted prostate strips. Representative force tracings of prostate strips from normal human (A) and BPH patients (B). Strips were pre-contracted with \( 10^{-5} \) M PE and then were allowed to reach a stable tension. The relaxant effect of increasing doses of BLEB (1, 5, 10 \( \mu \)M) was evaluated. The x-axis represents time (min), while the y-axis represents force (mg). (C) Summary graph of BLEB-induced relaxant effects on human prostate (\( n = 5 \) different humans for each group). The response to PE was taken as 100% while the relaxant effects of BLEB was evaluated as a percentage of this response. Data was shown as mean ± SD. \( *p < 0.05 \) vs. control. Bottom panels of this figure showed the BLEB-induced inhibitory effect on human prostate strips. Representative force tracings of prostate strips from normal human (D) and BPH patients (E). Strips were contracted with \( 10^{-5} \) M PE and then were washed to a baseline tension. After 30-min incubation with 15 \( \mu \)M BLEB, strips were contracted with \( 10^{-5} \) M PE again and the inhibition of BLEB was evaluated. The x-axis represents time (min), while the y-axis represents force (mg). (F) The summary graph of inhibition effect of BLEB on PE-mediated contraction of human prostate strips (\( n = 5 \) different humans for each group). The force for first response to PE was taken as 100% while the inhibition effect of BLEB was evaluated as a percentage of this response. Data was shown as mean ± SD. \( *p < 0.05 \) vs. control.

**Figure 5. Immunohistochemistry for SM MHC and NMM II isoforms expression in human prostate.**

Representative images of immunohistochemistry for MHC (A), NMMHC-A (B), NMMHC-B (C) and NMMHC-C (D) in prostate sections from human control and BPH groups. For each panel, the scale bars were 500 \( \mu \)m (upper) and 50 \( \mu \)m (bottom), respectively. (E) The quantitative analysis of MHC and NMM II isoforms expression...
in stroma and epithelial compartments (n = 10 different humans for each group). The mean density was calculated by IOD/area. Data was shown as mean ± SD. * means p < 0.05 vs. control; ** means p < 0.01 vs. control.

**Figure 6. Expression of SM MHC and NMM II isoforms in human prostate.**
(A) Representative Western blotting bands of target proteins and GAPDH in human prostate. Molecular weight (kDa) is indicated to the right of the blot. (B) The summary graph of relative protein expression in human prostate (n = 10 different humans for each group). Quantification of protein expression was calculated by the gray value ration of target protein/GAPDH. (C) Relative mRNA expression of target genes in human prostate (n = 10 different humans for each group). All values shown are mean ± SD. Experiments were repeated three times for each sample. *p < 0.05 vs. control; **p < 0.01 vs. control.

**Figure 7. Downregulation of NMMHC-A and NMMHC-B promotes prostate cell apoptosis and represses proliferation.**
(A) Verification of distinct NMMHC-A-siRNAs silencing efficacy at the mRNA (i) and protein (ii, iii) level in BPH-1 cells and WPMY-1 cells. (B) Verification of distinct NMMHC-B-siRNAs silencing efficacy at the mRNA (i) and protein (ii, iii) level in BPH-1 cells and WPMY-1 cells. ** means p < 0.01 vs. si-control. (C) CCK-8 assay was used to detect the viability of the BPH-1 (i) and WPMY-1 cells (ii) treated by si-control (black line), si-NMMHC-A (dotted line) and si-NMMHC-A (broken line). Data was shown as mean ± SD. ** means p < 0.01 vs. si-control. (D) Flow cytometry analysis of alterations of BPH-1 and WPMY-1 cells apoptosis rate by transfection of si-control, si-NMMHC-A and si-NMMHC-B. (i) 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. (ii) Summary graph of apoptosis rate among si-control, si-NMMHC-A and si-NMMHC-B groups. Each experiment was repeated three times with similar results and all values shown are mean ± SD. * means p < 0.05 vs. si-control (in same cell lines), ** means p < 0.01 vs. si-control (in same cell lines). (E) Flow cytometry
analysis for the BPH-1 and WPMY-1 cells cycle treated with si-NMMHC-A (or si-NMMHC-B) for 48h compared with si-control treated cells. Percentages (%) of cell populations at different stages of cell cycles were listed within the panels (i). The summary graph of percentage of cells in each phase from three independent experiments in BPH-1 (ii) and WPMY-1 cells (iii). Data was shown as mean ± SD.

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