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Inhibition of prostate smooth muscle contraction and prostate stromal cell growth by the WNK inhibitor WNK463 and WNK silencing

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Zusammenfassung (Deutsch):

Einleitung: α1-Adrenozeptor-Antagonisten stellen den Goldstandard in der medikamentösen Therapie von Symptomen des unteren Harntraktes bei einer benignen Prostatahyperplasie (BPH) dar. Ihre Effektivität ist jedoch begrenzt, auf Grund nicht-adrenerger Kontraktionen der glatten Muskulatur der Prostata. Parallel zu einem erhöhten glattmuskulären Tonus in der Prostata trägt auch das hyperplastische Wachstum zu den BPH-bedingten Symptomen bei, so dass Kombinationstherapien erforderlich sind, um beide Prozesse gleichzeitig medikamentös zu hemmen. Daraus ergibt sich ein Bedarf an neuen Medikamenten mit höherer Effektivität. Vor kurzem veröffentlichte Untersuchungen deuteten auf eine Kontraktions-fördernde Rolle der WNK Kinasen außerhalb der Prostata hin. In der vorliegenden Arbeit wurden die Effekte des WNK-Inhibitors WNK463 sowie eines Silencings der Expression der WNK-Isoformen WNK1-4 in humanen Prostatageweben und in kultivierten Stromazellen der Prostata untersucht.

Methoden: Die Effekte von WNK463 auf glattmuskuläre Kontraktionen wurden in Organbad-Experimenten untersucht. Die Effekte von WNK463 und des WNK-Silencings auf die Viabililtät, Proliferation, Apoptose, Kontraktion und Aktin-Organisation von WPMY-1-Zellen wurden in Zellkultur-Experimenten durch CCK-6-Assays, RT-PCR, EdU-Assays, Durchflusszytometrie, Zellkontraktions-Assays und Phalloidin-Färbungen untersucht.

Ergebnisse: WNK463 hemmte neurogene Kontraktionen humaner Prostatageweben, bei Konzentrationen von 0,5 µM und 10 µM. Kontraktionen durch die α1-adrenergen Agonisten Noradrenalin, Phenylephrin und Methoxamin wurden durch 10 µM WNK463 gehemmt, wohingegen 0,5 µM WNK463 zu keinen oder nur geringfügigen Hemmungen führte. Kontraktionen durch das Thromboxan A2-Analogon U46619 und durch Endothelin-1 wurden sowohl durch 0,5 µM als auch 10 µM WNK463 gehemmt. WNK463 senkte die Viabilität von Stromazellen, bei Konzentrationen von 5 µM und 10 µM, jedoch nicht bei 1 µM. Die Proliferation, erfasst durch EdU-Assays und als mRNA-Expression von Ki-67 wurde durch 10 µM WNK463 gehemmt. WNK463 induzierte bei Konzentrationen von 1 µM und 10 µM eine Apoptose. Sowohl die Kontraktion von WPMY-1-Zellen, als auch ihre Aktinorganisation wurden durch 10 µM WNK463 gehemmt. Ein Silencing jeder

Isoform für sich, also entweder WNK1, -2, -3 oder -4 führte zu einer Hemmung der Viabilität, Proliferation, Kontraktion und Aktin-Organisation, bzw. zur Induktion einer Apoptose.

Schlussfolgerungen: WNK463 hemmt adrenerge und nicht-adrenerge, sowie neurogene Kontraktionen humaner Prostatagewebe. In kultivierten Stromazellen der Prostata führten WNK463 bzw. Silencing aller vier WNK Isoformen zu Senkungen der Viabilität, Proliferation, Kontraktion und Aktin-Organisation, sowie zur Induktion einer Apoptose. WNK Kinasen könnten einen denkbaren Angriffspunkt für medikamentöse Interventionen bei Symptomen der BPH darstellen.

Abstract (English):

Background: α_1-Blockers still represent the gold standard for treating lower urinary tract symptoms (LUTS) suggestive of benign prostatic hyperplasia (BPH). However, the limited effectiveness of α_1-blockers may be associated with non-adrenergic contractions of prostate smooth muscle. In addition to smooth muscle contraction, prostate growth may contribute to LUTS suggestive of BPH as well, and combination therapies are still required to address both at once. Consequently, novel medications with improved efficacy are required. Recent studies suggested procontractile roles of WNK kinases, outside the prostate. Here, the effects of the WNK inhibitor WNK463 and WNK1-4 silencing were examined on human prostate tissues and human prostate stromal cells.

Methods: The effects of WNK463 on smooth muscle contractions were examined in an organ bath, and the effects of WNK463 and WNK1-4 knockdown on viability, proliferation, apoptosis, cell contraction and action organization of WPMY-1 cells were examined by CCK-8, RT-PCR, EdU, flow cytometry, cell contraction assay and phalloidin staining, respectively, in cell culture.

Results: WNK463 inhibited neurogenic contractions of human prostate tissues, using concentrations of 0.5 µM and 10 µM. Smooth muscle contractions induced by the α_1-adrenergic agonists noradrenaline, phenylephrine, and methoxamine were reduced by 10 µM WNK463, while 0.5 µM WNK463 showed a no or only small inhibitions of contractions. Contractions by the thromboxane A_2 analog U46619 and endothelin-1 were also reduced as well by 0.5 µM and by 10 µM WNK463. WNK463 reduced the viability of stromal cells at concentrations of 5 µM and 10 µM, but not at a concentration of 1 µM. Proliferation (in EdU assays and reflected by Ki-67 mRNA) was suppressed by 10 µM WNK463. Apoptosis was induced by 1 µM and 10 µM WNK463. Cell contraction was inhibited by 10 µM WNK463, as well as action organization. In addition, WNK expression was silenced by siRNAs for WNK isoforms 1-4, which showed similar inhibitory effects on viability, proliferation, apoptosis, cell contraction and actin organization.

Conclusions: WNK463 inhibited non-adrenergic and adrenergic, as well as neurogenic contractions in human prostate tissues. Substantial inhibitions were observed on viability, proliferation, reduction of apoptosis, cell contraction and actin

organization in human prostate stromal cells by WNK463 and silencing of WNK isoforms 1-4. All effects of WNK463 on stromal cells were mimicked by silencing of WNK expression. WNKs might be a promising target for medical improvement of LUTS.

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5-ARIs	5α-Reductase inhibitors
7-AAD	7-Aminoactinomycin
ANOVA	Analysis of variance
APC	Allophycocyanin
ARF6	ADP-ribosylation factor 6
ATP	Adenosine 5'-triphosphate
AUR	Acute urinary retention
BOO	Bladder outlet obstruction
BPE	Benign prostatic enlargement
BPH	Benign prostatic hyperplasia
BPO	Benign prostatic obstruction
BSA	Bovine Serum Albumin
$CaCl_2 \cdot 2H_2O$	Calcium chloride dihydrate
CCK-8	WST-8-based cell counting kit
cGMP	Cyclic guanosine-3‘,5‘-monophosphate
DAG	Diacylglycerol
DAPI	4’,6’-Diamidino-2-phenylindoledihydrochloride
DHT	Dihydrotestosterone
DMSO	Dimethyl sulfoxide
ED	Erectile dysfunction
EDTA	Ethylenediaminetetraacetic acid

EdU	Ethynyl-deoxyuridine
EFS	Electric field stimulation
ET-1	Endothelin-1
FACS	Fluorescence Activated Cell Sorter
FCS	Fetal calf serum
GEFs	Guanosine nucleotide exchange factors
GPCR	G protein-coupled receptor
GTPase	Guanosine triphosphate hydrolyzing protein
ILK	Integrins and integrin-linkes kinase
IP_3	Inositol-1,4,5-trisphosphate
IPSS	International Prostate Symptom Score
KCl	Potassium chloride
K-H	Krebs-Henseleit
KH_2PO_4	Potassium dihydrogen phosphate
LUTS	Lower urinary tract symptoms
MAPK	Mitogen-activated protein kinases
MD	Mean differences
$MgCl_2$	Magnesium chloride
$MgSO_4 \cdot 7H_2O$	Magnesium sulfate heptahydrate
MLC	Myosin light chain
MLCP	MLC phosphatase
MRAs	Muscarinic receptor antagonists
MYPT1	Myosin targeting subunit

NaCl	Sodium chloride
$NaHCO_3$	Sodium bicarbonate
NCC	Sodium chloride cotransporter
NKCC	Sodium-potassium chloride cotransporters
NO	Nitric oxide
OAB	Overactive bladder
OD	Optical density
OSR1	Oxidative stress responsive-1
PAKS	p21-activated kinases
PBS	Phosphate-buffered saline
PDE5Is	Phosphodiesterase 5 Inhibitors
PI3-K	Phosphatidylinositol 3-kinase
PIP_2	Phosphatidylinositol-4,5-bisphosphat
PKB	Protein kinase B
PKC	Protein kinase C
PLC	Phospholipase C
PLKs	Polo-like kinases
PSA	Prostate-specific antigen
PV	Prostate volume
PVR	Post-void residual
Q_{max}	Maximum urinary flow rate
QoL	Quality of life
RNase	Ribonuclease

rPX	Radical prostatectomy
RT-PCR	Real time polymerase chain reaction
SD	Standard deviation
SFKs	Src family kinases
siRNA	Small interfering RNA
SPAK	Ste20/SPS1-related proline/alanine-rich kinase
ß-ME	ß-Mercaptoethanol
STK16	The serine/threonine kinase 16
TURP	Transurethral resection of the prostate
TXA_2	Thromboxane A_2
WNK	With no lysine/K kinases
α_1-blockers	α_1-adrenoceptor antagonist
β_3-agonists	β_3-adrenoceptor agonists

1. Introduction

1.1 Definition

Benign prostatic hyperplasia (BPH) shows a high prevalence in older men worldwide. BPH includes histological changes in the prostate and often alterations in the prostate size, while the common symptoms caused by BPH are generally described as lower urinary tract symptoms (LUTS)[1]. Histologically, the proliferation of smooth muscle, glandular (epithelial) tissue and connective tissue in prostatic transition zone are responsible for the symptoms through mechanisms mainly involving testosterone and dihydrotestosterone (DHT)[2]. It is well-known that not all men with BPH will develop symptoms, but the symptoms are more common with an increment of age. However, the severity of symptoms is not related to the prostate volume on physical examination[3].

LUTS is a nonspecific term, which has been defined as urinary symptoms associated with storage (irritative), voiding (obstructive) or postmicturition[3-6]. Storage symptoms include incontinence, urgency, nocturia, daytime frequency and abnormal bladder sensation, and are related to overactive bladder (OAB) due to detrusor overactivity (DO) in the urinary bladder wall. Voiding symptoms include slow or weak stream, intermittency, hesitancy, straining, incomplete emptying and terminal dribbling, whereas post-micturition symptoms consist of incomplete emptying and post-micturition dribble, which are both attributed to the prostate and BPH[3,7,8]. Although LUTS do not usually cause severe sickness, they can impair men's quality of life (QoL), and lead to irreversible damage of the urogenital tract[7]. Furthermore, the International Prostate Symptom Score (IPSS) is used to stage the severity of LUTS in men, which is generally categorized as:

Points range from 0 to 7: free of symptom or mild;

Points range from 8 to 20: moderate;

Points range from 20 to 35: severe.

1.2 Epidemiology

LUTS are characterized by high and age-dependent prevalence, approaching 50% to 75% in men at an age of 50 years[9-11], reaching up to 80% in men at an age of 70, while prevalence increases to 88% to 90% in men at an age over 81[11-14]. Case numbers are continuously increasing with the demographic transition and the age-dependency of prevalence. Estimations for the year 2008 extrapolated the worldwide number of male patients with voiding symptoms to 515 million (plus 332 million cases of post-void symptoms), increasing to 612 million men with voiding symptoms for 2018 (plus 397 million cases of post-void symptoms)[15]. For most aging men, storage and voiding symptoms increased in frequency and severity with age without any medical treatment[8,16].

A prospective observational study from the United States reported that among 6909 men, nearly 30% suffered for mild LUTS; 50% suffered for moderate LUTS, and around 15% suffered for severe LUTS[17]. The average IPSS was 11.5 (range from 0 to 35)[17]. In a French study[18], based on the assessment of IPSS, 18.8% of the males in France scored 0 points (free of symptom), 67% scored 1-7 points (mild), 13% scored 8-19 points (moderate), and 1.2% scored ≥ 20 points (severe). These outcomes were also supported by cross-sectional analyzes among symptomatic men, where 75% of symptoms were mild, 21% were moderate, and 4% were severe[19].

1.3 Etiology and risk factors

1.3.1 Etiology

LUTS in men may be attributed to various situations, including benign prostatic enlargement (BPE) and benign prostatic obstruction (BPO). BPE is characterized by the proliferation of smooth muscle and/or glandular (epithelial) cells in the transition zone of the prostate, and includes an increased prostate volume. BPO is defined as urethral obstruction caused by the prostate gland at the position of the bladder neck and occurs with BPE, while obstruction by bladder outlet obstruction (BOO) may occur with and without BPE[20]. Male LUTS may be caused by the

enlarged prostate by two components: 1) static component: enlarged prostate volume results in BPO/BOO, due to compression of the urethra and/or of the bladder outlet region; and 2) dynamic component: increased prostate smooth muscle tone causes compression of the urethra and/or of the bladder outlet region[5,21]. OAB is often primary referred to an overactive detrusor, but may also occur secondary to obstruction caused by BPE and BPO, which may be a primary cause of LUTS in male patients[22-25].

1.3.2 Risk factors

Several studies[8,21,26] suggest that parallel to aging, changeable factors, such as age, lifestyle, smoking, alcohol, genetic predisposition, obesity, diabetes, hypertension, depression, hyperlipidemia and hypogonadism, are also partly responsible for the development of LUTS. Table 1 summarizes the main conditioning and worsening factors of LUTS.

Table 1. Risk factors of LUTS

Conditioning factors	Worsening factors
Age	Obesity
Lifestyle	Diabetes
Smoking	Hypertension
Alcohol	Depression
Genetic predisposition	Hyperlipidemia
-	Hypogonadism

1.4 The role of BPH in LUTS

It has been assumed that almost 1/4, or even 1/2 of men with BPH will develop LUTS, and almost half of men with LUTS are part of BOO due to BPH or bladder

dysfunction[27]. The pathophysiology of LUTS in men results from BOO or bladder dysfunction or a combination of these etiologies[8,28]. BPH accompanied by prostate gland enlargement is associated with BOO (static component); this is the most widely accepted basis for LUTS[21]. Obstruction may also be caused by an increased smooth muscle tone, where α_1-adrenoceptors and other receptors induce smooth muscle contraction and finally increase prostate smooth muscle tone, leading to BPO and LUTS[5,29]. Beyond that, detrusor contractions during normal voiding are mediated by muscarinic receptors (M2- and M3- type), while spontaneous and unvoluntary detrusor contractions causing symptoms in OAB are probably non-cholinergic and non-neurogenic[4,23].

Voiding symptoms due to BPH are believed to be caused by urethral obstruction. The obstruction is attributed to increased smooth muscle tone in the prostate and/or prostate growth in BPH, impairing bladder emptying, urinary flow and voiding[30]. Progression of BPH and LUTS leads to the formation of postvoid urine and to acute urinary retention (AUR), finally resulting in (recurrent) urinary system infections or renal function impairment, which may be life-threatening and requires immediate interventions and surgery. Impacts on the QoL due to LUTS suggestive of BPH are less harmful but very common, while further consequences include social isolation and depressions.

1.5 Mechanisms of targets for mediation in prostate smooth muscle contraction

It is well established that activation of α_1-adrenoceptors induces smooth muscle contraction through activating intracellular signaling pathways (protein kinase C (PKC), calcium and RhoA/Rho kinase)[31-33]. It is also clear that smooth muscle contraction arises from essential prerequisites containing myosin light chain (MLC) phosphorylation, actin organization, and attachment of filaments to membranes and anchoring cells to the extracellular matrix [34,35].

α_1-Adrenoceptors belong to the family of G protein-coupled receptors (GPCR), include three subtypes (classified as α_{1A}, α_{1B}, and α_{1D}) and are expressed in the prostate[31,33,36]. It is well recognized that G proteins dissociate from the receptors

after activation of the receptor, and that the G_α subunit ($G\alpha_{q/11}$, $G\alpha_{12}$, $G\alpha_{13}$), one subunit of the heterotrimeric G proteins, subsequently leads to activation of phospholipase C (PLC) and of the small monomeric GTPase RhoA[31,32,37-39]. After its activation, the second messengers (inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG)) are generated by the hydrolyzation of phosphatidylinositol-4,5-bisphosphat (PIP2) by PLC. IP_3 gives rise to elevations of cytosolic calcium concentrations by release from intracellular stores and by calcium influx from the extracellular space into the cell across the cell membrane. Subsequently, MLC kinase is activated by binding of Ca^{2+} and calmodulin, and induces MLC phosphorylation and smooth muscle contraction. In parallel, PKC is activated by DAG[31,32,37-39], which inhibits MLC phosphatase by phosphorylation of myosin targeting subunit (MYPT1) or by activation of the MLC phosphatase inhibiting factor CPI-17. Consequently, MLC phosphorylation and contraction are increased by MLC phosphatase inhibition.

In parallel to α_1-adrenoceptors, non-adrenergic agonists may also mediate prostate smooth muscle contractions, including endothelin-1(ET-1), or thromboxane A_2 (TXA_2)[30,40]. In addition, the panel of procontractile signaling pathways of prostate smooth muscle contraction is not confined to calcium-, PKC- or RhoA/Rho kinases-dependent mechanisms, but characterized by much higher complexity[41]. The endothelin-1-mediated smooth muscle contraction may be associated with the activation of three signaling pathways[42]: (i) Activation of PLC/DAG/IP_3 by ET-1, (ii) Activation of mitogen-activated protein kinases (MAPKs) by ET-1, (iii) Activation of phosphatidylinositol 3-kinase (PI3-K) and protein kinase B pathway (PKB) by ET-1. TXA_2 mediates smooth muscle contraction by activating of TXA_2 receptor, which is based on mechanisms involving at least Rho kinase and calcium[40]. Muscarinic receptors mediate smooth muscle contractions in the bladder wall and possibly in non-human prostates, but not in human prostates, while phosphodiesterase-5 promotes relaxation of smooth muscle and is involved in the pathogenesis of LUTS as well[4,43] (Figure 1).

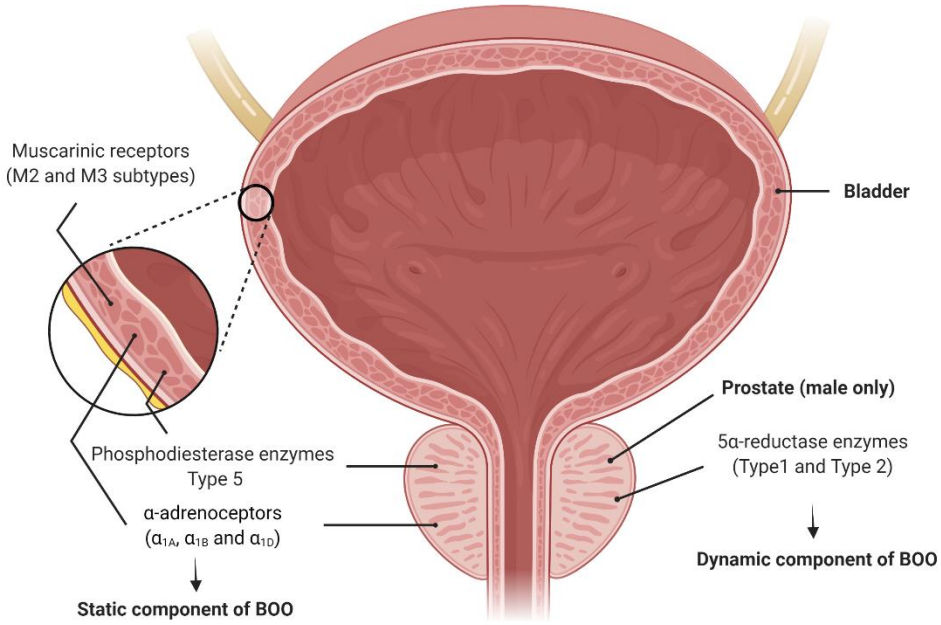


Figure 1. Mechanisms and targets in prostate smooth muscle contraction and lower urinary tract symptoms. The pathophysiology of LUTS in men results from bladder outlet obstruction (BOO) or bladder dysfunction or a mixture of these etiologies. The α_1-adrenoceptors and muscarinic receptors induce smooth muscle contraction in the prostate and bladder, respectively. Phosphodiesterases cause smooth muscle relaxation in the prostate and bladder, while 5α-reductase enzymes promote prostate growth and enlargement by conversion of testosterone to DHT. (Created with BioRender.com**)**

1.6 Pharmacological therapy for LUTS

1.6.1 Monotherapy

α_1-adrenoreceptor antagonist (α_1-blockers)

α_1-Adrenoceptors are highly expressed in the prostate and urethra[33] After their activation, strong contractions are induced in smooth muscle cells and subsequently, urethral resistance increases[33,44]. Therefore, α_1-blockers addressing α_1-

adrenoceptors on prostate tissues may relieve smooth muscle tone, and thus reduce urethral resistance and improve voiding symptoms[45]. Several α_1-blockers are available including tamsulosin, alfuzosin, silodosin, doxazosin, and terazosin[46]Clinical trials reported that α_1-blockers might relieve symptoms (assessed by IPSS) by maximally 30%-50% and raise the maximum urinary flow rate (Q_{max}) by maximally 40%[47,48]. Several studies also declared that α_1-blockers have high efficacy in short- and long-terms clinical trials[49,50]. However, α_1-blockers neither change the size of the prostate nor prevent the incidence of acute urinary retention (AUR) in follow-up studies[47,51].

5α-Reductase Inhibitors (5-ARIs)

It is widely accepted that DHT is produced from testosterone by the enzyme 5α-reductase, which can promote the development of BPH and LUTS[52,53]. 5-ARIs can suppress the transformation between testosterone and DHT, thus decreasing the prostate volume (PV) and serum levels of prostate-specific antigen (PSA)[54,55]. There are only two 5-ARIs (finasteride and dutasteride) available for clinical application. 5-ARIs are most suitable for moderate-to-severe LUTS patients or those with an enlarged prostate. 5-ARIs may decrease IPSS scores and prostate size by approximately 18%-28% and 15%-30%, respectively, and increase Q_{max} by around 1.5-2.0 ml/s in long-term treatment[56]. Moreover, it has been shown[57], that LUTS patients with PV ≥ 40 mL achieve better effects in reducing the risk of AUR and surgery than patients with a smaller PV after receiving dutasteride. Moreover, it has been reported[58]that finasteride may be a more value therapy for patients with a PV > 30 ml. However, these two clinical studies both showed little efficacy in patients with smaller prostate size.

Muscarinic Receptor Antagonists (MRAs)

M2- and M3- subtype receptors are highly expressed on the bladder smooth muscle cells[59,60]. MRAs affect detrusor smooth muscle by suppressing contractions by muscarinic cholinergic receptors[61]. The MRAs drugs including tolterodine, darifenacin, solifenacin, fesoterodine, propiverine, oxybutynin and trospium, which are the first-line OAB drug therapy[62]. According to the role of

muscarinic receptors in detrusor contractions (at least during normal voiding), MRAs are for treatment of storage symptoms of LUTS, such as the urgency of urination, frequent micturition and urinary incontinence[63]. Medications of this drug class could inhibit detrusor overactivity[4,64]. Interestingly, there was a more significant improvement in LUTS/BPH patients with a small-sized prostate or a low-leveled PSA after receiving MRAs drugs than those with a large-sized prostate or high-leveled PSA[65,66].

Phosphodiesterase 5 Inhibitors (PDE5Is)

PDE5Is are an available option for treatment of moderate-to-severe LUTS[67]. PDE5Is can relax the smooth muscle in prostate tissue and in the bladder detrusor, what has been supposed to account for improvements of LUTS via increasing the level of intracellular cyclic guanosine monophosphate (cGMP)[68-70]. Accordingly, smooth muscle relaxation by PDE5Is involves signaling by nitric oxide (NO)[71]. So far, only tadalafil has been approved for clinical application for male LUTS in Europe, with or without erectile dysfunction[46]. Previous clinical trials[72-74] demonstrated that patients with significant improvement in IPSS score were observed after receiving PDE5Is, but no considerable increase of Q_{max}.

β_3-adrenoceptor agonists (β_3-agonists)

β-Adrenoceptors (β_2 and β_3 subtype) are abundant in the urethra, bladder and prostate. In the bladder, the β_3 subtype shows higher levels than the β_1 and β_2 subtypes[33]. β_3-agonists can exert action on improving symptoms of LUTS by activating the β_3-adrenoceptors, resulting in smooth muscle relaxation in the detrusor and urethra[75-77]. Mirabegron is a selective β_3-agonist and available for treatment of storage symptoms, as it may raise clinically relevant improvement of OAB symptoms (including nocturia, frequency, urgency), although it shows no significant impact on Q_{max} or detrusor tone[78,79]. Moreover, a higher adherence rate and more obvious improvements in QoL were observed using mirabegron (50 mg) compared to antimuscarinics[80].

1.6.2 Combination therapies

α_1-blockers plus 5-ARIs

Currently, α_1-blockers combined with 5-ARIs are available and commonly used for patients with moderate-to-severe LUTS or patients with a high level of PSA, high PV, lower $Q_{max,}$ and higher post-void residual (PVR)[81]. Two clinical trials[82,83] showed that α_1-blockers plus 5-ARIs could achieve larger benefits in preventing the development of BPH, in reducing the likelihood of AUR and in decreasing the risk for surgery than single-drug treatment. In addition, combination treatment was considered in patient with long-term use (>12 months), and adherence seems to be significantly associated with the severity of symptoms, i. e. the stronger the symptoms, the longer the patients remained in treatment[81].

α_1-blockers plus MRAs

A combination of α_1-blockers and MRAs achieves higher satisfactory efficacy in relieving persistent storage symptoms compared to single α_1-blockers treatment[85]. A previous trial investigated alfuzosin plus darifenacin in LUTS patients with storage symptoms compared with single alfuzosin therapy[86]. The results showed better outcomes for daytimes frequency and urgency rates, when alfuzosin was combined with darifenacin. In a clinical trial investigating the long-term efficacy and safety of combination therapy (8 mg silodosin and 20 mg propiverine), participants under combination therapy achieved better effects on subjective symptoms and on storage function in patients with BOO and OAB symptoms[87]. The results also suggested that the adherence to this treatment did not show noticeable changes with increasing medical expenditure.

α_1-blockers plus β_3-agonists

In parallel to α_1-blockers and MRAs, α_1-blockers plus β_3-agonists may improve the storage symptoms better than α_1-blocker monotherapy according to a match study from Japan[89]. A placebo-controlled, double-blind study reported that participants with BOO and OAB receiving tamsulosin 0.2 mg plus mirabegron 50 mg reduces the risk of storge symptoms and relieves OAB symptoms better

compared to the tamsulosin-alone group[90]. Recently, another double-blind, randomized, multi-center study[91] was used to investigate the effectiveness and side effect of tamsulosin plus mirabegron. The results demonstrated the safety of this combination therapy, but showed no significant differences in PVR or Q_{max}.

MRAs plus β_3-agonists

Generally, and although the combination of MRAs and β_3-agonists does not belong to the first-line options of pharmacological therapy, this combination may achieve a higher treatment efficacy[92]. Mirabegron (25 or 50 mg) plus solifenacin (2.5 or 5 mg) may prevent the incidence of urinary frequency and urge urinary incontinence as well as adverse events, with better outcomes compared to monotherapy[93]. Also, according to a placebo-controlled, randomized study[94], mirabegron (25 or 50 mg) plus solifenacin (2.5, 5 or 10 mg) showed better improvements in mean volume voided per micturition and urgency than a single-drug therapy (solifenacin 5 mg). Mirabegron plus solifenacin has been shown to be well-tolerated, while no apparent extra adverse events were observed.

1.6.3 Limitations of current therapies

Although α_1-blockers are the first-line therapy for patients with BPH and moderate-to-severe LUTS, a certain ceiling of effectiveness became increasingly obvious for this class of drugs. To date, controlled studies show that α_1-blocker with the so far highest subtype selectivity of all available α_1-blockers (i. e., silodosin and tadalafil) do not provide greater improvements than the previously available medications. Thus, α_1-blockers improve symptoms (assessed by IPSS) by maximally 30%-50% and an increase the Q_{max} by maximally 40%[30,46,95-98]. These improvements are slightly better than placebos, which improve the IPSS by 10%-34%, and the Q_{max} up to 28%[30,46,95-99]. Consequently, approximately 69% of participants are not alleviated by α_1-blockers, and still 36%-45% of them even not by α_1-blockers plus 5-ARIs[100,101]. Along with the disproportional side effects, 65% of patients do not continue medication 12 months after the first prescription of α_1-blockers due to the dissatisfaction about the efficacy[102]. Low adherence to

medical treatment represents a high incidence rate of complications, hospitalization, and surgery due to BPH[102].

Although 5-ARIs can reduce PV in most moderate-to-severe LUTS patients, 25% of them show slight improvements and even get worse of their symptoms[103]. Particularly for patients with PV no more than 40 ml, 5-ARIs show less efficacy than the placebo[104]. Furthermore, 5-ARIs treatments are associated with several side effects in patients, including erectile dysfunction (ED), gynecomastia and decreased libido[105]. Some studies reported that dutasteride is associated with low-risk prostate cancer[106,107]. PDE5Is are also reported with a range of side effects (flushing, nausea, dyspepsia, headache, back pain and myalgia)[108]. In addition, the long-term (≥ 12 months) application for the persistence and safety of PDE5Is stay unknown, and their efficacy in decreasing PV and preventing disease progression still needs to be explored.

In face of these limitations, due to the age-dependency of prevalence, increasing case numbers and the demographic transition, novel medications are of high demand, requiring a) well-studied prostate smooth muscle contraction and b) discovery of potential candidate targets. For decades, basic and clinical research mainly focused on α_1-adrenoceptors and three intracellular signaling pathways (PKC, calcium and RhoA/Rho kinase) in prostate smooth muscle contraction and LUTS. It is known that the restricted effectiveness of α_1-blockers may be caused by non-adrenergic contractions of prostate smooth muscle, and that intracellular signaling leading to contraction is much more complex than previously assumed[109]. Non-adrenergic agonists may induce prostate smooth muscle contractions similar to α_1-adrenoceptors, which are opposed to α_1-blockers and may consequently keep obstruction and symptoms despite treatment with α_1-blockers[30,109]. In parallel, the panel of procontractile mechanisms involved in prostate smooth muscle contraction is not restricted to PKC-, calcium- or Rho kinase-dependent pathways, but is obviously characterized by a much higher diversity. Thus, several compounds were recently identified, which 1) inhibited non-adrenergic plus adrenergic contractions at once in human prostate tissues, 2) inhibited growth and growth and contraction of prostate cells simultaneously, what 3) pointed to previously unknown players of prostate smooth muscle contraction. Together, such findings appear promising, but demonstrate at the

same time, that molecular mechanisms of prostate smooth muscle contraction and growth are still unclear, despite their high clinical relevance.

1.6.4 Potential compounds for therapy of LUTS

Several new targets including Src family kinases (SFKs)[110], p21-activated kinases (PAKS)[111], polo-like kinases (PLKs)[112], integrins and integrin-like kinase (ILK)[113], the serine/threonine kinase 16 (STK16)[114], ADP-ribosylation factor 6 (ARF6)[115] and others have been further explored with regard to inhibiting human prostate smooth muscle contractions and growth of stromal cells. This pointed to connections between prostate smooth muscle tone and growth, and suggested that potential, unknown procontractile signaling pathways exist. Some of these targets and compounds were also examined in other organs, where such compounds inhibited vascular or airway smooth muscle, which initiated analog investigations for prostate smooth muscle. Therefore, compounds which inhibit smooth muscle contraction of any smooth muscle-rich tissue, or actin organization or MLC phosphorylation are possible candidates, especially in the prostate smooth muscle contraction.

Src family kinases (SFKs)

SFKs, a subfamily of non-receptor tyrosine kinases, are highly expressed in vascular smooth muscle cells and endothelial cells[116-120], which are involved in a range of cellular functions through the interaction with growth factors, receptors and cortactin[121]. Recent findings[122-126] strongly suggest that cell proliferation and smooth muscle contraction in different tissues and cell types are obviously connected with SFKs. In addition, SFK inhibitors could inhibit smooth muscle contractility in the uterus, alimentary tract, airways and blood vessels[127-133]. It has also been reported that SFK inhibitors have inhibitory effects on contraction and proliferation in prostate tissues and prostate stromal cells[110]. These findings showed that SFKs have a potential for suppressing prostate smooth muscle contraction and stromal cell growth.

P21-Activated kinases (PAKs)

PAKs, a family of serine/threonine kinases, play critical regulatory roles in many physiological processes, including regulation of contraction, neuronal function, cytoskeleton organization and cell cycle[134-136]. Previous studies also reported that PAKs might relax the smooth muscle in different organs (airways and blood vessels) [38,137-144] and significantly affect the airways and vascular smooth muscle by mediating the cell cycle[145-148]. Recently, a study[111] using PAK inhibitors demonstrated that it might be a potential target for inhibiting non-adrenergic (neurogenic- and endothelin-) induced prostate smooth muscle contractions, and stromal cell growth.

Polo-like kinases (PLKs)

PLKs, a subfamily of highly conserved serine/threonine kinases, are essential regulators in cell cycle, proliferation and cellular growth[149]. PLK1 is best known for involving cell cycle regulation and progression, as well as the regulation of the airway and vascular smooth muscle[150-155]. It has been reported that PLK inhibitors may inhibit α_1-adrenergic contraction in human prostate tissue, while showing a few changes in contractions induced by endothelin and TXA_2[112]. Consequently, the PLK-dependent pathway may offer novel approaches to inhibit prostate smooth muscle contraction, which has the potential for technological advancement and clinical application.

Integrins and integrin-linkes kinase (ILK)

Integrins are transmembrane protein and consist of a combination of α and β subunits[156]. Integrin-linked kinases (ILKs), a subfamily of serine/threonine-protein kinases, interact with integrin β1 and the β3 cytoplasmic domain and phosphorylates integrin β1[157,158]. Integrins and ILK have multiple cell functions, including linking the extracellular matrix with the actin organization, cell motility, adhesion, cell proliferation, apoptosis, and cancer development[159-168]. A recent study [113] reported that integrins and ILK might exert a significant effect on prostate

tissues. The results suggested that integrins and ILK inhibitors could inhibit non-adrenergic (neurogenic- and TXA_2-) induced prostate smooth muscle contraction.

The serine/threonine kinase 16 (STK16)

STK16, a new subfamily of serine/threonine kinases, is widely distributed and its homologs are conserved among all eukaryotes[169-173]. Several studies[174,175] revealed that STK16 might interfere with cell adhesion and actin organization. Recent evidence[114] showed that a STK16 inhibitor inhibits non-adrenergic smooth muscle contractions in the human prostate and shows little efficacy in the detrusor. Also, the STK16 inhibitor likely has a selective effect on adrenergic and non-adrenergic contractions, verifying multiple regulations of receptor-induced smooth muscle contractions in LUTS.

ADP-ribosylation factor 6 (ARF6)

ARF6, a small monomeric guanosine triphosphate hydrolyzing protein (GTPase), is a well-known protein related to various biological processes, including cell proliferation, migration and invasion, which is known for mainly regulating functions in membrane traffic and actin remodeling[176-178]. In the human prostate, the cytohesin inhibitor secinH3 inhibits ARF6 activity and smooth muscle contraction, suggesting a cytohesin-ARF6 pathway associated with smooth muscle contraction[179]. In addition, the ARF6 inhibitor, NAV2729, showed significant inhibition of α_1-adrenoceptor-induced contraction and stromal cell growth in the human prostate[115,180]. These findings suggested that ARF6 might be a putative target for the pharmacological therapy of LUTS.

1.7 With no lysine/K kinases (Lysine deficient protein kinases)

With no lysine/K kinases (WNK kinases, WNK1-4) are a family of serine/threonine kinases defined by the deficiency of a highly conserved lysine in subdomain II that involves binding adenosine 5’-triphosphate (ATP) and the catalyzing phosphoryl transfer in other kinases[181-184]. WNKs appear to have multiple roles

in the biological functions of different kinds of tissues[185-191]. The well-known functional role of WNKs is the phosphorylation of Ste20/SPS1-related proline/alanine-rich kinase (SPAK) and oxidative stress responsive-1 (OSR1)[192-196]. Activation of NCC (sodium chloride cotransporter) attributed to NCC phosphorylation by WNK-SPAK/OSR1 kinase cascade[197], results in reabsorption of NaCl and consequently in hypertension. Moreover, promoting vasocontraction in the vasculature is regulated by WNK-mediated SPAK/OSR1 signaling through NKCC (sodium-potassium chloride cotransporters)[187,198-200]. In addition, WNKs have been shown to function in the kidney, such as electrolyte homeostasis and regulation of ion transport[190], and may also play an essential role in vascular smooth muscle contractility[185]. Previous studies also revealed that WNK1 promoted cell proliferation in vascular smooth muscle cells[201] and human endometrial stromal cells[202]. In parallel, and considering similarities in smooth muscle contractions in the prostate and in blood vessels, it may be presumed that WNKs might have a similar procontractile function in the prostate. To date, the effects of WNK kinases or inhibitors on prostate smooth muscle have obviously not yet been investigated. Therefore, the functional roles of WNK kinases in the prostate tissue will be explored here.

2. Objective of this thesis

LUTS is a common non-malignant disease with high and age-dependent prevalence, with high impact on the QoL in large parts of the elderly population[203]. Current medical therapy of LUTS still shows encouraging results for improving symptoms and avoidance of surgery[81]. However, these options have an obvious therapeutic ceiling in terms of efficacy and adherence, and show side effects [204-207].

It is essential to explore potential targets and compounds with higher efficacy and adherence for future pharmacological therapy of LUTS patients. Thus, the objective of this thesis was first to evaluate the effects of the WNK inhibitor WNK463 on the contraction of human prostate tissues, and on the growth of prostate stromal cells. Secondly, silencing of WNK expression was applied to confirm the participation of WNKs in these processes and to identify involved WNK isoforms in prostate cells.

The thesis addressed the following topics:

- Effects of WNK463 on neurogenic, adrenergic and non-adrenergic contractions of human prostate tissues;
- Effects of WNK463 on contractility (cell contraction assay), proliferation and growth-related functions of prostate stromal cells;
- Effects of isoform-specific WNK silencing on contractility, proliferation, and inhibitor sensitivity in prostate stromal cells.

3. Materials and methods

3.1 Reagents and materials

Table 2. Reagents used in this study

Products	Manufacturer
Potassium chloride (KCl)	Roth, Germany
Sodium chloride (NaCl)	Roth, Germany
Sodium bicarbonate ($NaHCO_3$)	Roth, Germany
Magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$)	Roth, Germany
Potassium dihydrogen phosphate (KH_2PO_4)	Applichem, Germany
Calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$)	Sigma-Aldrich, USA
D(+)-Glucose	Roth, Germany
L-Norepinephrine hydrochloride	Sigma-Aldrich, USA
(R)-(−)-Phenylephrine hydrochloride	Sigma-Aldrich, USA
Methoxamine hydrochloride	Sigma-Aldrich, USA
Custodiol® HTK cardioplegia	Dr. Franz Köhler Chemie, Germany
Endothelin-1	Enzo, USA
U46619	Enzo, USA
Carbogen	Linde Gas, Germany
WNK463	Selleck Chemicals, USA
siRNA-WNK_{1-4}	Thermo Fisher Scientific, USA
siRNA-Negative Control	Thermo Fisher Scientific, USA
Opti-MEM	Life Technologies, UK

Human Prostate Stromal Cell (PrSC) Avalanche™ Transfection Reagent	EZ Biosystems
RPMI 1640	Gibco, USA
10 % fetal calf serum (FCS)	Gibco, USA
Phosphate-buffered saline (PBS)	Gibco, USA
4', 6'-diamidino-2-phenylindoledihydrochloride (DAPI)	Invitrogen, USA
Reverse transcription buffer 10×	Promega, USA
Magnesium chloride ($MgCl_2$)	Promega, USA
dNTP	Promega, USA
AMV reverse transcriptase	Promega, USA
Ribonuclease (RNase) inhibitor	Promega, USA
RNase-Free Water	Promega, USA
SYBR™ Green	Roche, USA
RT^2 qPCR Primer	QIAGEN, Germany
AllPrep DNA/RNA/Protein Mini Kit	QIAGEN, Germany
5-Ethynyl-deoxyuridine (5-EdU)	Thermo Fisher Scientific, USA
Triton X-100	Thermo Fisher Scientific, USA
Cell Counting Kit-8	Sigma, USA
Phalloidin	Sigma, USA
Cell Contraction Assay (Floating Matrix Model)	Cell Biolabs, USA
7-ADD	BD Science, UK
APC Annexin V	BD Science, UK
Bovine Serum Albumin (BSA)	Sigma, USA
Dimethyl sulfoxide (DMSO)	Roth, Germany
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich, Germany

Table 3. siRNAs used in this study

Target gene symbol	Sense strand (5'-3')	Anti-sense strand (5'-3')
WNK1	CAAUGAGUCAGAUAUCGAAtt	UUCGAUAUCUGACUCAUUGtc
WNK2	CUAUAAGUCUAGUAGCAAAtt	UUUGCUACUAGACUUAUAGgt
WNK3	GCCUCACGUUUGUCAGUAUtt	AUACUGACAAACGUGAGGCat
WNK4	GGCUUUUGCCCUAUCCAUUtt	AAUGGAUAGGGCAAAAGCCga

Table 4. Devices used in this study

Devices	Manufacturer
Tissue Bath System-720MO	DMT (Danish Myotechnology), Denmark
Electrical Field Stimulation generator (DMT)	DMT, Denmark
Lab pump	KNF- Neuberger, USA
Waterbath	Thermo Fisher, USA
Centrifuge	Thermo Fisher, USA
Nanodrop™	Thermo Fisher, USA
Cell culture incubator	Thermo Fisher, USA
Lab-Tek chamber slides	Thermo Fisher, USA
Fluorescence Activated Cell Sorter (FACS)	Becton-Dickinson,USA
Laser scanning microscope	Leica SP2, Germany
Light Cycler PCR system	Roche, Switzerland
Superfrost® microscope slides	Thermo Fisher, USA
Vibration shaker-Vortex 3	IKA, Staufen, Germany
Gel electrophoresis system	Bio-rad, Germany

3.2 Prostate tissue samples

Human prostate tissue samples were acquired from patients with prostate cancer undergoing radical prostatectomy (rPX) at the Department of Urology, Klinikum

Großhadern, Ludwig-Maximilians University of Munich (LMU). Tissue samples were taken from the periurethral zone, given that prostate cancers (70+%) mainly occur in the peripheral zone[208,209]. All prostates (stored in Custodiol® for transport) were conveyed to the Department of Pathology directly after the rPX surgery. Tissues from transurethral resection of the prostate (TURP) or under radiotherapy were excluded because these samples are traumatized, which may influence the results and are much smaller than the samples from rPX[205]. Tissues were completely anonymized and transported to the research lab.

3.3 Cell culture

Human prostate stromal cells (WPMY-1 cells) are an immortalized, commercially available cell line acquired from human prostate stromal without prostate cancer[210]. WPMY-1 cells used here were purchased from the American Type Culture Collection (ATCC Manassas, VA, USA). WPMY-1 cells were routinely cultured in RPMI 1640 (Gibco, USA) medium containing 10% fetal calf serum (FCS) (Gibco, USA) and 1% streptomycin (Gibco, USA)/penicillin (Gibco, USA) at 37°C in a 5% CO_2 humidified atmosphere.

3.4 Silencing of WNK expression

Cells were transfected with target-specific small interfering RNA (siRNA) duplexes (Ambion Silencer® Select library, Life Technologies, Carlsbad, CA, USA), diluted in Opti-MEM with a final concentration of 50 nM of siRNAs in the cell culture medium. Subsequently, Human Prostate Stromal Cell (PrSC) Avalanche™ Transfection Reagent (EZ Biosystems, College Park, MD, USA) was mixed with Opti-MEM (1:40). Cells were transfected after they reached 70%-80% confluence, and cultured without antibiotics 24 h prior to transfection. Following drop-wise addition of the transfection mixture, plates were centrifuged (300 g, 5 min), and incubated (37°C, 5% CO_2, 5 h) before replacement of the fresh medium containing the antibiotic. For real-time polymerase chain reaction (RT-PCR), cells were analyzed after 72 h. WST-8-based cell counting kit (CCK-8) and EdU assays were performed 72 h after transfection.

3.5 Real time polymerase chain reaction (RT-PCR)

3.5.1 RNA extraction and measurement

- Preparation: Buffer RLT with ß-ME (250 µl ß-mercaptoethanol (ß-ME) plus 25 ml RLT); Buffer RPE; 70% ethanol; Buffer RW1; PBS.
- Six well plate with cells washed with PBS twice times
- 350 µl buffer RLT and 350 µl 70% ethanol were added together to each well, and mixed adequately.
- 700 µl mixed sample was transferred to RNeasy Mini spin columns in a collection tube (2 ml), centrifuged (15 s, 4°C，8000 ×g), and the collection tube was thrown away.
- 700 µl buffer RW1 was added to the column, centrifuged (15 s, 4°C，8000 ×g), and the flow-through was discarded. This step was repeated.
- 500 µl buffer RPE was added to the column, centrifuged (15 s, 4°C，8000 ×g), and the collection tube was thrown away.
- 500 µl buffer RPE was added to the column and centrifuged (1 min, 4°C，8000 ×g).
- The RNeasy Mini spin column was placed in a new collection tube (1.5 ml).
- 30 µl RNase-Free water was added to each column, centrifuged (2 min, 4°C，8000 ×g), and the RNeasy Mini spin column was thrown away.
- The quantity of RNA concentration was measured by a Nanodrop spectrophotometer.

3.5.2 Reverse Transcription (RT)

Reverse Transcription Kit was used for in vitro transcription of the RNA samples (1 µg) to cDNA (Table 5).

Table 5. Reverse Transcription

Components	Volumes (µL)
$Mgcl_2$	4
dNTP	2
10×RT buffer	2
Random primers	1
rRNAsin	0.5
AMV reverse transcriptase	0.5

3.5.3 Real-time polymerase chain reaction (RT-PCR)

RT-PCR using FastStart Essential DNA Green Master and SYBR Green I Kit was performed to quantify the expression of target genes and GAPDH by a Light-Cycler® 96 Instrument (Roche, Switzerland). PCR reactions in a volume of 10 µl (Table 6) were carried out based on the protocol. The settings of the LightCycler® 96 Instrument were as follows:

Initialization	95°C	10 min	
Denaturation	95°C	10 s	40 cycles
Annealing	60°C	10 s	
Extension/elongation	72°C	15 s	
Melting Curve	95°C	15 s	
	60°C	60 s	
	95°C	15 s	

Table 6. Components of RT-PCR

Components	Volumes (µL)
Green Master 1	5
Green Master 2	3
cDNA	1
Primers	1
Total	**10**

3.6 Cell proliferation assay

WPMY-1 cells were cultured in 16-well chambered coverslips (Thermo Scientific, USA) with the desired density of 80% confluence. On the next day WNK463/DMSO (24 h) or Scramble/siWNK1-4 /Negative control transfection (72 h) were added to the cells. After a further 24 or 72 h, a working solution of EdU (2X) in an FCS-free medium was added. Cells were incubated for at least 24 h, followed by proceeding immediately with cell fixation and permeabilization.

EdU assays were performed in cultured cells, to examine the effects of WNK463 or silencing on proliferation. In this assay, proliferating cells are visualized by means of a fluorescing probe (5-TAMRA) combined with counterstaining with DAPI. EdU assay was carried out by the following steps:

- WPMY-1 cells were seeded on coverslips in fresh medium with 80% confluence for 24 hours.
- FCS-free medium containing 5% EdU was prepared and used to replace the previous medium.
- Cells were incubated with WNK463/DMSO for 24 hours or transfected by Wildtype/scramble siRNA/siWNK1-4 for 72 hours.
- Medium was removed and cells are washed twice with PBS.
- 100 µL of 3.7% formaldehyde was added to each well, and incubated at room temperature for 15 minutes.

- Formaldehyde solution was removed and cells were washed twice by 3% of BSA in PBS.
- BSA solution was removed and 100 µL 0.5% Triton X-100 in PBS was added to each well, incubated in a shaker at room temperature for 20 minutes.
- Triton solution was removed and cells were washed twice by 3% of BSA in PBS.
- Wash solution was removed and 50 µL reaction cocktail was added to each well, incubated in a shaker at room temperature (kept in a dark place) for 30 minutes.
- Reaction cocktail was removed and cells were washed three times with 3% of BSA in PBS.
- 50 µL DAPI was added to each well, and incubated in a shaker at room temperature for 15 minutes (kept in a dark place).
- DAPI was removed and cells were washed three times with 3% of BSA in PBS.
- Wash solution was removed and cells were covered by glass.
- Analysis by a laser scanning microscope (Leica SP2, Wtzlar, Germany)

3.7 Viability assay

Effects of WNK463 or of silencing of WNK1-4 on the viability of cultured cells was examined by a WST-8-based cell counting kit (CCK-8) (Sigma Aldrich, Munich, Germany). Effects of test compounds were assessed in three different concentrations, and compared to viability in controls without inhibitors. Viability assays were carried out by following steps:

- Seeding of WPMY-1 cells (5000 cells/well in inhibitor group, 500 cells/well in a knockdown group) in 96-well plates, incubating the plates at 5% CO_2, 37°C in the incubator for 24 hours.
- Replacing the medium with an FCS-free medium.
- Adding WNK463/DMSO to each well, incubation for further 24 hours or transfection with the wildtype/scramble siRNA/siWNK1-4 to each well, followed by incubation for further 72 hours.

- Incubating the treated cells for 24 hours, 48 hours, 72 hours.
- After incubation (24 hours, 48 hours, 72 hours), 10 µL CCK-8 was added to each well.
- Incubation of the cells for 2 hours, and measuring the plate by a microplate reader at 450 nM absorbance.
- Analysis of optical density (OD) values.

3.8 Phalloidin Staining

Visualization of actin organization was performed by fluorescence staining with FITC-coupled phalloidin. Phalloidin-based staining detects exclusively polymerized, filamentous actin, but not monomeric, non-filamentous actin. This allows the assessment of cytoskeleton organization. Phalloidin Staining was carried out by the following protocol:

- Seeding of WPMY-1 cells on coverslips in fresh medium with 80% confluence for 24 hours.
- Preparation of FCS-free medium containing 5% EdU and using it to replace the previous medium.
- Cells were incubated with WNK463/DMSO for 24 hours or transfected by Wildtype/scramble siRNA/siWNK1-4 for 72 hours.
- Medium was removed and cells were washed twice with PBS.
- 100 µL of 3.7% formaldehyde was added to each well, followed by incubation at room temperature for 15 minutes.
- Formaldehyde solution was removed and cells were washed twice by 3% of BSA in PBS.
- BSA solution was removed and 100 µL 0.5% Triton X-100 in PBS was added to each well, followed by incubation in a shakerat room temperature for 20 minutes.
- Triton solution was removed and cells were washed twice by 3% of BSA in PBS.
- Phalloidin (Sigma-Aldrich, Munich, Germany) was prepared in a fume cupboard, protect from light.

- 50 µL phalloidin was added to each well, and incubated in a table shaker at room temperature for 40 minutes (kept in a dark place).
- Phalloidin solution was removed and cells were washed twice by 3% of BSA in PBS.
- 50 µL DAPI was added to each well, followed by incubation in a shaker at room temperature for 15 minutes (kept in a dark place).
- Wash solution was removed and cells were covered by glass.
- Analysis by a laser scanning microscope (Leica SP2, Wetzlar, Germany)

3.9 Cell apoptosis assay

Early apoptosis and dead cells were detected by flow cytometry. These different stages of cells could be stained by allophycocyanin (APC) annexin V and 7-aminoactinomycin D (7-AAD) (BD Biosciences, Franklin Lakes, USA). Cell apoptosis assay was performed by the following steps:

- Cells were seeded in 6-wells plates and cultured for 24 hours.
- WNK463/DMSO was added to each well, and cells were incubated for further 24 hours or wildtype/scramble siRNA/siWNK1-4 were transfected to each well, and cells were incubated for further 72 hours.
- 1X annexin V binding buffer (1 mL 10X annexin V binding buffer mixed with 9 mL distilled water) was prepared.
- WPMY-1 cells were harvested.
- Cells were washed once with 1X PBS.
- Cells were resuspended with 1 mL annexin V binding buffer.
- 5 µL of APC annexin V and 5 µL of 7-AAD were added to each tube (100 µL).
- Incubation at room temperature for 15 minutes (kept in a dark place).
- 400 µL annexin V binding buffer was added to each tube.
- Analyzes by flow cytometry.

3.10 Cell contraction assay

The contraction of cultured stromal cells can be evaluated by collagen matrix contraction assays, where cells are seeded and grown in a matrix plug. Contraction and relaxation of cells result in changed diameters of the matrix plug, which can be quantified. Assays were performed using the Floating Matrix Model Cell Contraction Assays (Cell BioLabs), according to the following manufacturer's instructions:

Table 7. Preparation of Collagen gel working solution

Reagents	**24-Well**
Collagen Solution	9.54 mL
5X Medium or PBS	2.46 mL
Neutralization Solution	340 µL
Total	**12.34 mL**

- Collagen gel working solution was prepared (Table 7).
- WPMY-1 cells were harvested and resuspended in fresh medium at 5×10^6 cells/Ml.
- Cell suspension (2 parts) and cold collagen gel working solution (8 parts) were mixed. Formation of air bubbles were avoided.
- 500 µL of the mixture was added to each well of the 24-well Cell Contraction Plate
- Incubation of the plate at 5% CO_2, 37°C in the incubator for 1 hour.
- After the collagen polymerization, fresh medium (1 mL) was added to each well. Formation of air bubbles was avoided.
- Monitoring of wells for contraction at different time endpoints (1h, 2h, 3h, 6h, 12h, 24h, 48h)
- Measuring the collagen gel size (Area or Diameter) change by Image J.

3.11 Tension measurements

Organ bath experiments allow to induce, visualize, and quantify contractions of intact, fresh tissues. In each experiment, each of the four chambers of one organ bath was occupied with a tissue sample (around 6 x 3 x 3 mm), and all four tissues in a single experiment were derived from the same prostate. Subsequently, the inhibitor was applied to two chambers, while solvent (control for inhibitor) was applied to the two remaining chambers. Thus, each experiment was based on double determination. After pretension, KCl-induced contractions were assessed for each sample, as EFS- and agonist-induced tensions were later expressed as % of contraction induced by KCl. Following incubation (30-45 min), contractions were induced by application of electric field stimulation (EFS) or by application of exogenous agonists (concentration-response curves), including noradrenaline, phenylephrine, methoxamine, U46619, endothelin-1. Only one curve was constructed with each sample. EFS and all agonists were examined in separate experiments and using prostates from different patients. Experiments were carried out by the following steps:

- Preparation of Krebs-Henseleit (K-H) solution (as shown in Table. 8), remaining KH solution in the liquid thermostatic bath at 37°C and continuously carbogen (including 95% O_2 and 5% CO_2).
- Chambers were washed with K-H solution three times and refilled with 10 mL K-H solution.
- The prostate tissues were split into approximately 6 x 3 x 3 mm for each chamber.
- Each sample tissue was fixed on the suspended needle of the chambers.
- All the sample tissues need to proceed pretension (stretch to maximal 6.0 ± 0.1 mN), this process was repeated three times (each time took 15 minutes) until the tension remain 4.9 ± 0.1 mN.
- After the pretension, 400 µL KCl solution (2 M) was added to each chamber, inducing the maximal KCl-indued prostate smooth muscle contraction.
- All chambers were washed with K-H solution three times and replenished with 10 mL K-H solution.

- 10 µL WNK463 (dissolved in DMSO at a concentration of 0.5 µM and 10 mM) was added to two chambers as an experiment group, while 10 µL DMSO for another two chambers as a control group.
- Incubation for 30 minutes before the agonist-induced or EFS-induced contraction was started.
- After incubation, EFS-induced or agonist-induced contractions were performed. Frequency-response curves (including 2 Hz, 4 Hz, 8 Hz, 16 Hz, 32 Hz) for EFS or concentration-response curves for agonist (cumulative concentrations, as shown in Table.9-11) were recorded.
- Analysis: tensions at each frequency and each agonist concentration were calculated and expressed as % of contraction induced by KCl in the same sample.

Table 8. Preparation of Krebs-Henseleit (K-H) solution in Organ bath.

Solutions	Component	Volume/Quality
KH1	KCl	8.8 g
	NaCl	172.4 g
	$KH_2●PO_4$	4.1 g
	$CaCl_2●H_2O$	9.2 g
	$MgSO_4●7H_2O$	7.4 g
	Deionized Water	1000 mL
KH2	$NaHCO_3$	32.5 g
	Deionized Water	1000 mL
Krebs-Henseleit (K-H) solution	KH1	43.5 mL
	KH2	43.5 mL
	Glucose	1.62 g
	Deionized Water	1000 mL

Table 9. Concentration of Noradrenaline/Phenylephrine/Methoxamine in the organ bath.

Concentration (mM)	Volume (µL)	Final Concentration (µM)
0.1	10	0.1
0.1	20	0.3
1	7	1
1	20	3
10	7	10
10	20	30
10	70	100

Table 10. Concentration of U46619 in the organ bath.

Concentration (mM)	Volume (µL)	Final Concentration (µM)
0.1	10	0.1
0.1	20	0.3
1	7	1
1	20	3
10	7	10
10	20	30

Table 11. Concentration of Endothelin-1 in the organ bath.

Concentration (mM)	Volume (µL)	Final Concentration (µM)
0.04	2.5	0.01
0.04	5	0.03
0.4	1.75	0.1
0.4	5	0.3
0.4	17.5	1
0.4	50	3

3.12 Data and statistical analysis

Data from organ bath experiments are calculated and analyzed in the form of means, plus standard deviation (SD)of each experiment. The whole concentration-response curves were compared using two-way analysis of variance (ANOVA), and contractions at single concentrations were compared using multivariate analysis. A Student's t-test was used to compare paired groups, while a Dunnett's test was used to compare in series containing more than two groups. Tests and calculation of mean differences (MD) with 95% CI will be performed using the SPSS version 20 (IBM SPSS Statistics. Armonk, New York: IBM Corporation). P values <0.05 were considered significant. Cell apoptosis assay was measured by FlowJo version 10 (Tree Star Inc., Ashland, OR, USA). Viability assay, cell proliferation assay, phalloidin staining and cell contraction assay were quantified by ImageJ (NIH, Maryland, USA).

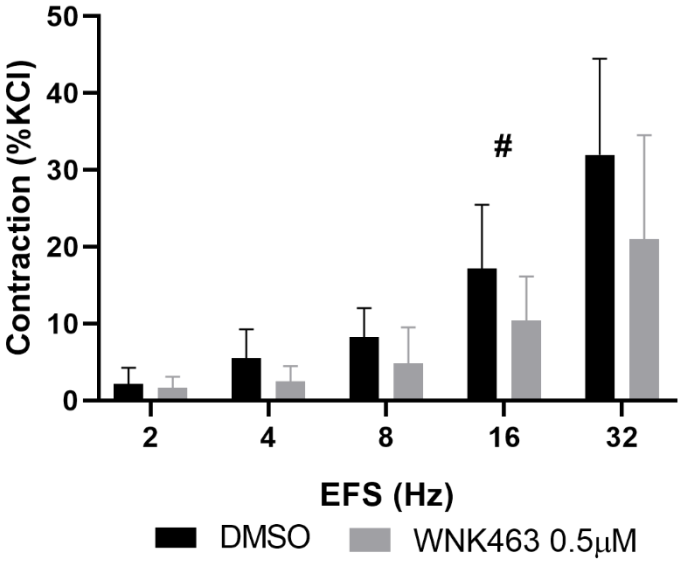
4. Results

4.1 Effect of WNK463 on prostate smooth muscle contractions

4.1.1 Effects of WNK463 on EFS-induced contractions

EFS (different frequencies from 2 to 32 Hz) induced prostate smooth muscle contraction in a frequency-dependent way, which were reduced by 0.5 µM WNK463 and by 10 µM WNK463 (Figure 2). Analysis of variance was performed to compare DMSO and WNK463 groups. According to the results, a concentration of 0.5 µM (P=0.003, between DMSO and WNK463) and 10 µM WNK463 (P=0.023, between DMSO and WNK463) significantly inhibited EFS-induced contractions. Subsequent multivariate analysis pointed to inhibition of EFS-induced contractions at stimulation frequency of 32 Hz (p=0.001) by 10 µM WNK463 (Figure 2).

A



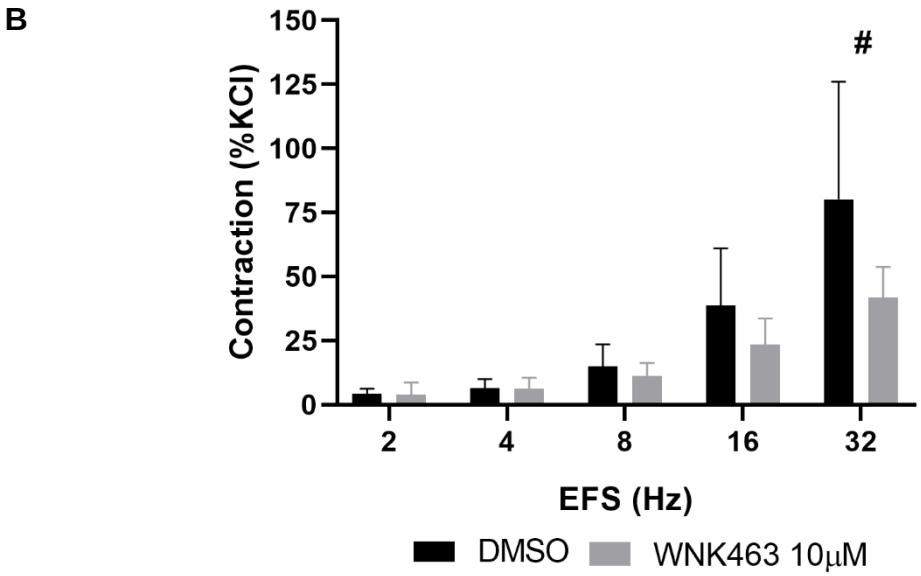


Figure 2. Effects of WNK463 on EFS-induced contractions of human prostate tissues. EFS (different frequencies from 2 to 32 Hz) induced contractions of human prostate tissue. The effects of WNK463 on EFS-induced contraction were compared between the control groups (n=5) and the WNK463 groups (n=5). Tensions were calculated and expressed as % of contractions induced by KCl (80 mM). Data are calculated as means ± SD on each experiment ($^{\#}$ P<0.05 for DMSO group vs. WNK463 group).

4.1.2 Effects of WNK463 on noradrenaline-induced contractions

Noradrenaline (cumulative concentrations from 0.1 to 100 µM) induced prostate smooth muscle contraction in a concentration-dependent way, which were reduced by 10 µM WNK463, but not by 0.5 µM WNK463 (Figure 3). Analysis of variance was performed to compare DMSO and WNK463 groups, indicating that a concentration of 10 µM WNK463 significantly inhibited noradrenaline-induced contractions (P<0.001, between DMSO and WNK463). Multivariate analysis pointed to significant inhibition of noradrenaline-induced contractions at concentrations of 30 µM (p<0.001) and 100 µM (p<0.001) (Figure 3).

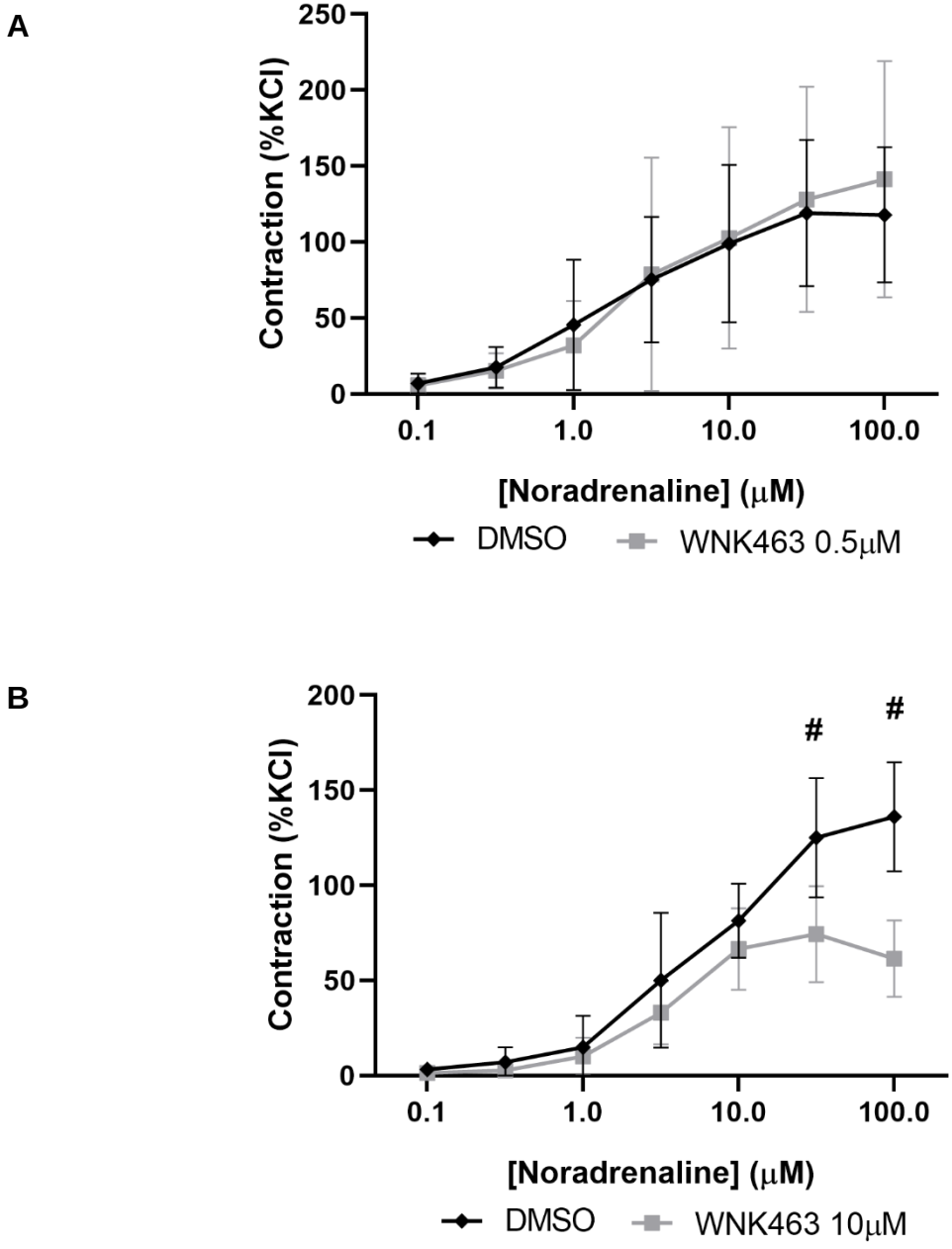
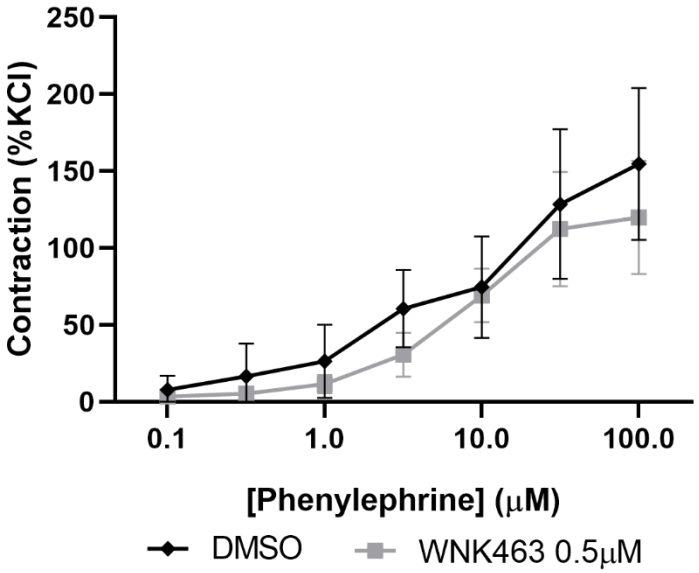


Figure 3. Effects of WNK463 on noradrenaline-induced contractions of human prostate tissues. Noradrenaline (cumulative concentrations from 0.1 to 100 µM) induced contractions of human prostate tissue. The effects of WNK463 on noradrenaline-induced contractions were compared between the control groups (n=5) and the WNK463 groups (n=5). Tensions were calculated and expressed as % of contractions induced by KCl (80 mM). Data are calculated as means ±SD on each experiment ($^{\#}$ P<0.05 for DMSO group vs. WNK463 group).

4.1.3 Effects of WNK463 on phenylephrine-induced contractions

Phenylephrine (cumulative concentrations from 0.1 to 100 µM) induced prostate smooth muscle contractions in a concentration-dependent way, which were reduced by 10 µM WNK463, but not 0.5 µM WNK463 (Figure 4). Analysis of variance was performed to compare the DMSO and WNK463 groups, indicating that a concentration of 10 µM WNK463 significantly inhibited phenylephrine-induced contractions (P=0.001, between DMSO and WNK463), while subsequent multivariate analysis pointed to significant inhibition on phenylephrine-induced contractions at concentrations of 30 µM (p=0.009) and 100 µM (p<0.001) (Figure 4).

A



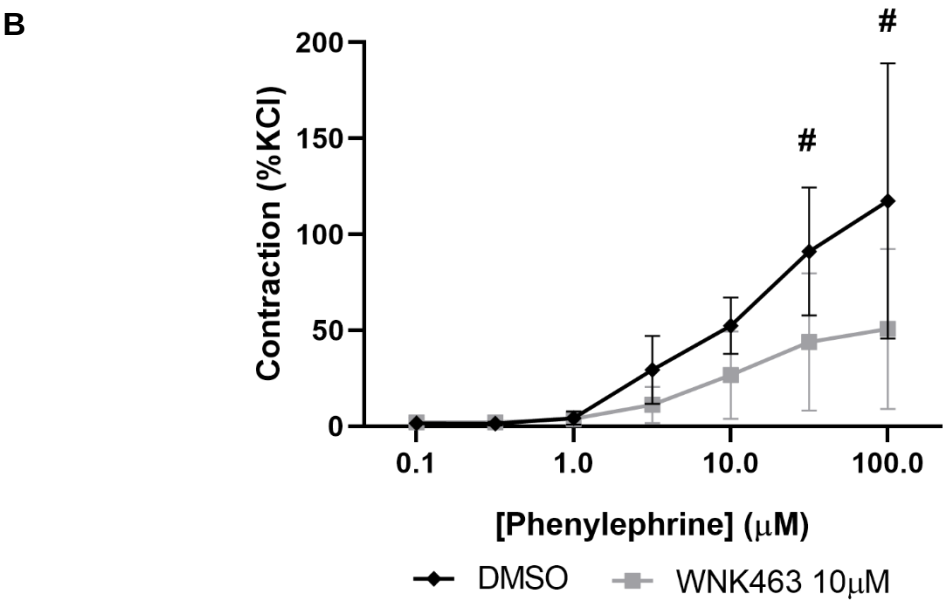


Figure 4. Effects of WNK463 on phenylephrine-induced contractions of human prostate tissues. Phenylephrine (cumulative concentrations from 0.1 to 100 µM) induced contractions of human prostate tissue. The effects of WNK463 on phenylephrine-induced contractions were compared between the control groups (n=5) and the WNK463 groups (n=5). Tensions were calculated and expressed as % of contractions induced by KCl (80 mM). Data are calculated as means ±SD on each experiment ($^{\#}$ P<0.05 for DMSO group vs. WNK463 group).

4.1.4 Effects of WNK463 on methoxamine-induced contractions

Methoxamine (cumulative concentrations from 0.1 to 100 µM) induced prostate smooth muscle contraction in a concentration-dependent way, which were reduced by 10 µM WNK463, but not by 0.5 µM WNK463 (Figure 5). Analysis of variance was performed to compare the DMSO and WNK463 groups, indicating that a concentration of 10 µM WNK463 significantly inhibited methoxamine-induced contractions (P=0.001, between DMSO and WNK463), while multivariate analysis pointed to significant inhibition of methoxamine-induced-induced contractions at concentrations of 10 µM (p=0.048) and 30 µM (p=0.041) (Figure 5).

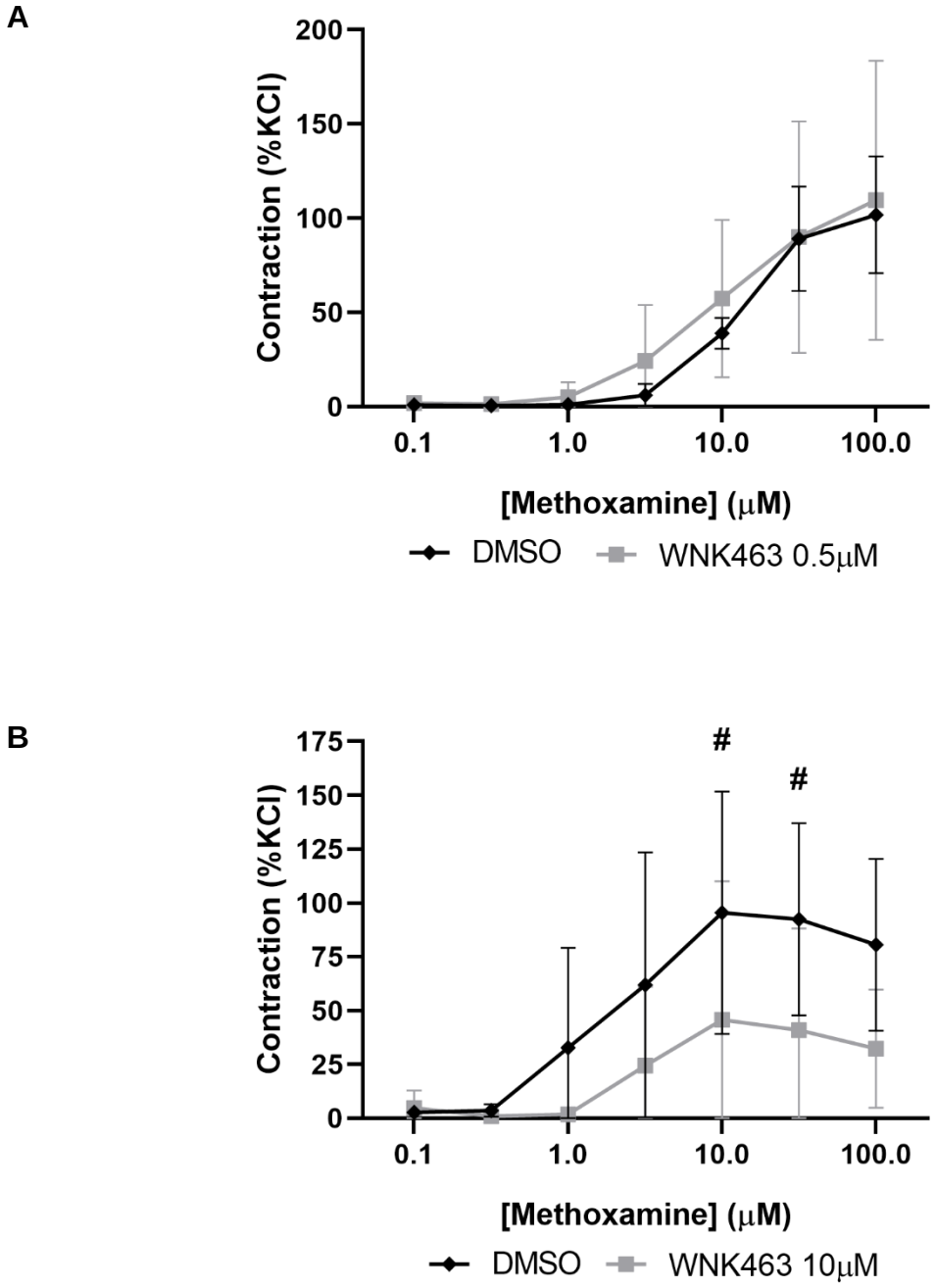
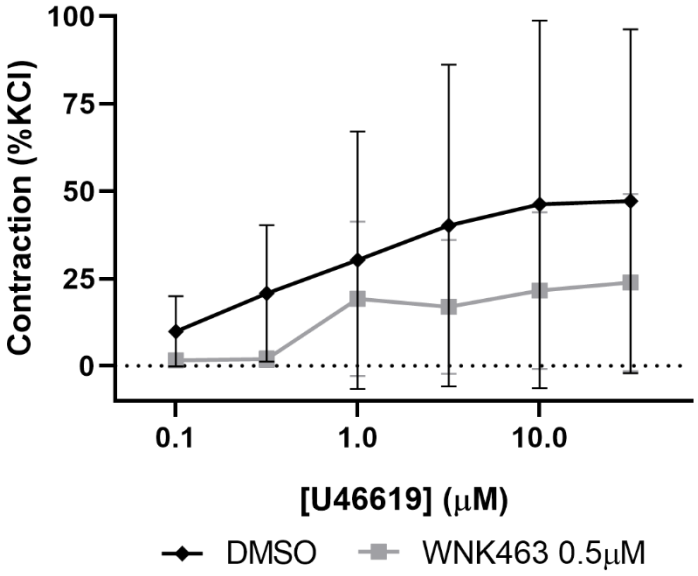


Figure 5. Effects of WNK463 on methoxamine-induced contractions of human prostate tissues. Methoxamine (cumulative concentrations from 0.1 to 100 µM) induced contractions of human prostate tissue. The effects of WNK463 on methoxamine-induced contractions were compared between the control groups (n=5) and the WNK463 groups (n=5). Tensions were calculated and expressed as % of contractions induced by KCl (80 mM). Data are calculated as means ±SD on each experiment ($^{\#}$ P<0.05 for DMSO group vs. WNK463 group).

4.1.5 Effects of WNK463 on U46619-induced contractions

U46619 (cumulative concentrations from 0.1 to 30 µM) induced prostate smooth muscle contraction in a concentration-dependent way, which were inhibited by 0.5 µM WNK463 and 10 µM WNK463 (Figure 6). Analysis of variance was performed to compare the DMSO and WNK463 groups, and demonstrate that a concentration of 0.5 µM (P=0.025, between DMSO and WNK463) and 10 µM WNK463 (P<0.001, between DMSO and WNK463) significantly inhibited U46619-induced contractions. Subsequent multivariate analysis pointed to significant inhibition of U46619-induced contractions at concentrations of 0.3 µM (p=0.024), 1 µM (p=0.003), 3 µM (p=0.003) and 10 µM (p=0.006) by 10 µM WNK463 10 µM group, (Figure 6).

A



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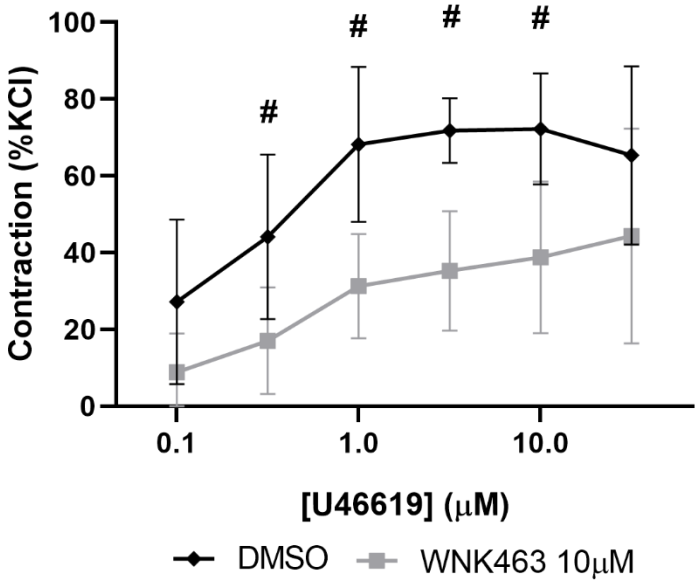


Figure 6. Effects of WNK463 on U46619-induced contractions of human prostate tissues. U46619 (cumulative concentrations from 0.1 to 30 µM) induced contractions of human prostate tissue. The effect of WNK463 on U46619-induced contractions were compared between the control groups (n=5) and the WNK463 groups (n=5). Tensions were calculated and expressed as % of contractions induced by KCl (80 mM). Data are calculated as means ±SD on each experiment ($^{\#}$ P<0.05 for DMSO group vs. WNK463 group).

4.1.6 Effects of WNK463 on endothelin-1-induced contractions

Endothelin-1 (cumulative concentrations from 0.1 to 3 µM) induced prostate smooth muscle contraction in a concentration-dependent way, which were reduced by 0.5 µM WNK463 and 10 µM WNK463 (Figure 7). Analysis of variance was performed to compare the DMSO and WNK463 groups, and demonstrate that concentrations of 0.5 µM (P<0.05, between DMSO and WNK463) and 10 µM WNK463 (P<0.05, between DMSO and WNK463) significantly inhibited endothelin-1-induced contractions. Subsequent multivariate analysis pointed to significant inhibition of endothelin-1-induced contractions at concentrations of 1 µM (p=0.031) by 0.5 µM WNK463 and at a concentration of 3 µM (p=0.043) by 10 µM WNK463 (Figure 7).

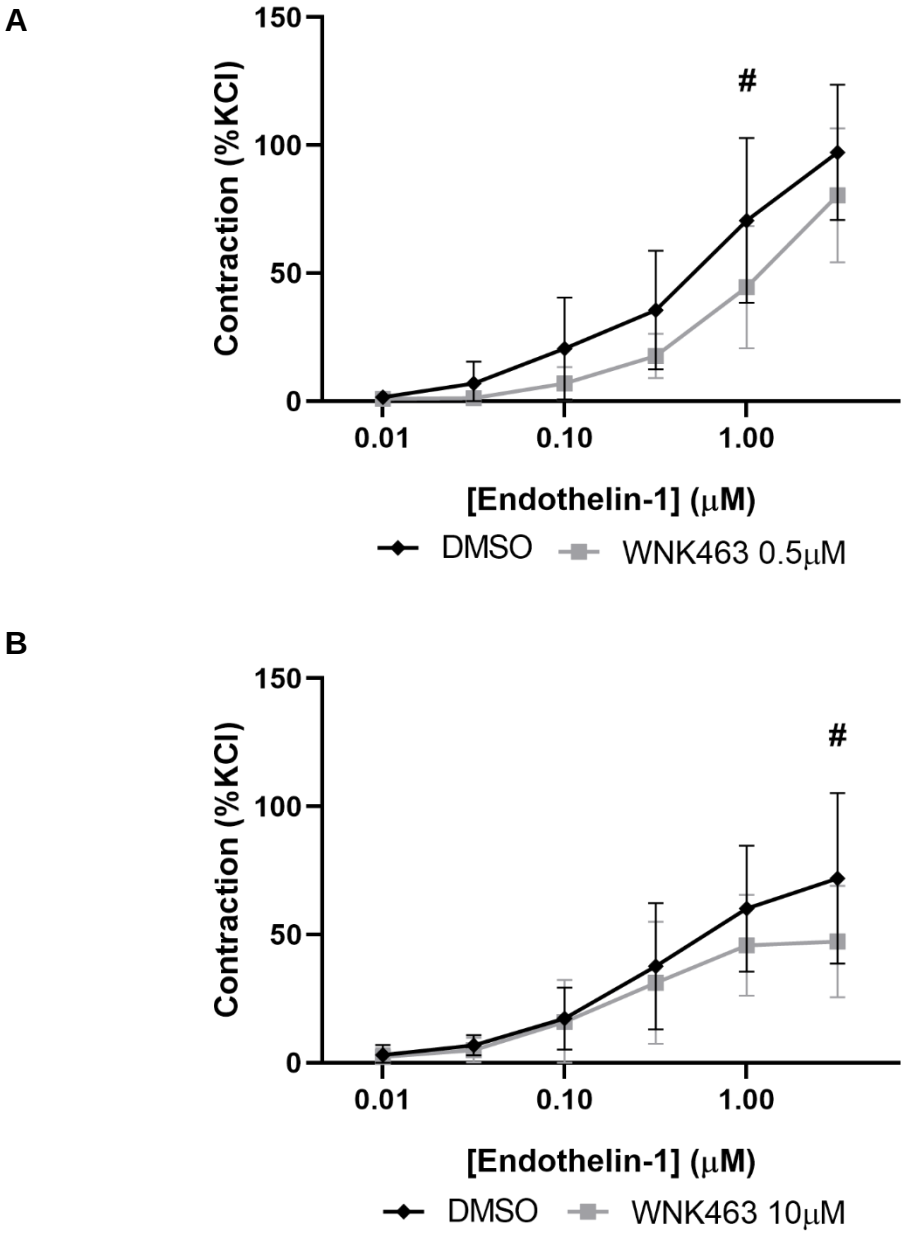


Figure 7. Effects of WNK463 on endothelin-1-induced contractions of human prostate tissues. Endothelin-1 (cumulative concentrations from 0.1 to 3 µM) induced contractions of human prostate tissue. The effects of WNK463 on endothelin-1-induced contractions were compared between the control groups (n=5) and the WNK463 groups (n=5). Tensions were calculated and expressed as % of contractions induced by KCl (80 mM). Data are calculated as means ±SD on each experiment ($^{\#}$ P<0.05 for DMSO group vs. WNK463 group).

4.2 Detection of WNK mRNAs by RT-PCR in human prostate tissues and prostate stromal cells (WPMY-1 cells)

WNK1-4 mRNA expressions were detected by RT-PCR in human prostate tissues and WMPY-1 cells. Figure 8 shows semiquantitative comparisons of expression levels from human prostate tissue samples (n=5) and from independent experiments of WPMY-1 cells (n=5), for each of the four WNK isoforms. Higher mRNA expression of WNK1-4 were observed in human prostate tissues than WPMY-1 cells.

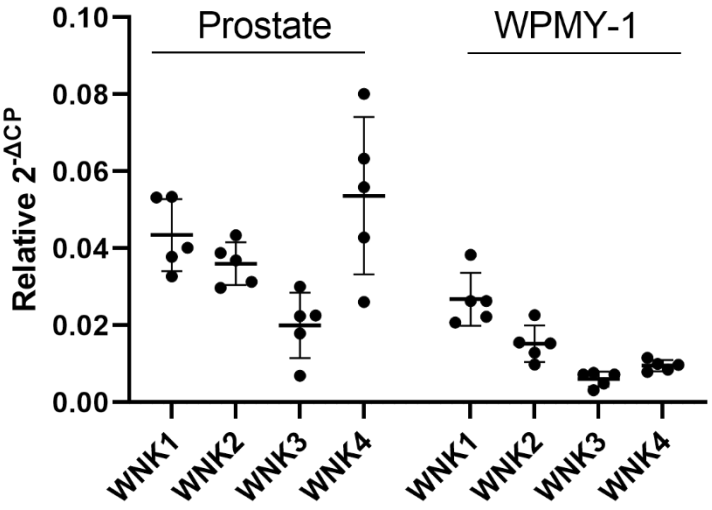


Figure 8. Detection of WNK mRNA expression in human prostate tissues and WPMY-1 cells. WNK1-4 mRNA expression from prostate tissue samples (n=5) and WPMY-1 cells (n=5 independent experiments) was detected by RT-PCR. Shown are all individual values from n=5 independent experiments.

4.3 Effects of WNK463 on cell culture

4.3.1 Effects of WNK463 on viability of human prostate stromal cells (WPMY-1 cells)

CCK-8 assay was performed to examine the effects of WNK463 on viability of WPMY-1 cells. WNK463 reduced the viability of stromal cells at concentrations of 5 µM and 10 µM, but not at a concentration of 1 µM (Figure 9). After different

periods (24–72 h) of exposure, concentration-dependent declines of viability were observed by WNK463 (concentration of 1 µM, 5 µM and 10 µM) in WPMY-1 cells, while there was no time-dependent effect on viability within these periods.

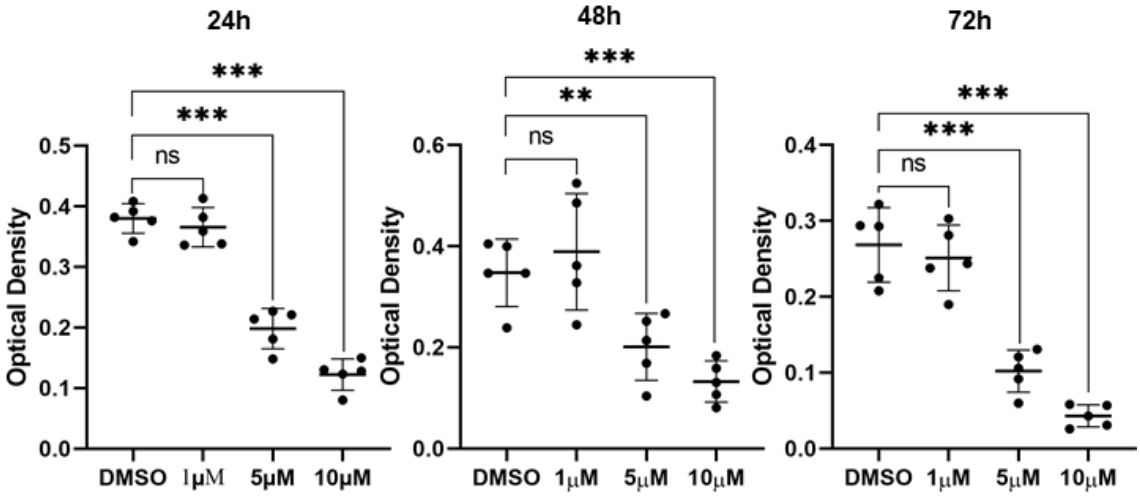


Figure 9. Effects of WNK463 on viability of WPMY-1 cells. Viability was examined by CCK-8 assay, after cells were incubated with DMSO or WNK463 (concentration of 1 µM, 5 µM and 10 µM). Shown are all individual values from n=5 independent experiments as well as the mean and P values (ns, no significance; **, P<0.01; ***, P<0.001).

4.3.2 Effects of WNK463 on cell proliferation of human prostate stromal cells (WPMY-1 cells)

4.3.2.1 Ki-67 mRNA expression

RT-PCR was used to examine the cell proliferation as different expression of Ki-67 mRNA between the inhibitor group (WNK463 10 µM) and control group (DMSO). Compared to control group, lower level of Ki-67 mRNA was detected in WNK463 treated cells (Figure 10, P<0.05).

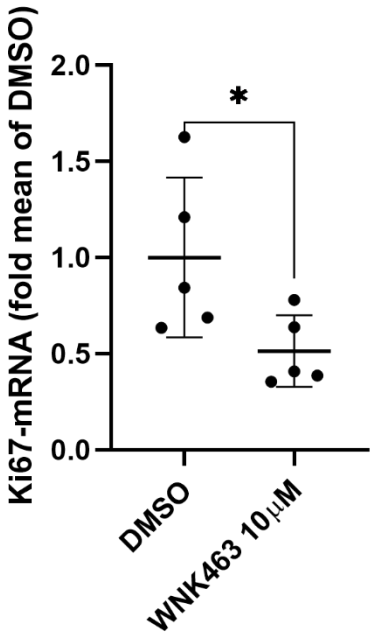


Figure 10. Effects of WNK463 on proliferation of WPMY-1 cells assessed by detection of Ki-67 mRNA expression. Cell proliferation was examined by assessment of Ki-67 mRNA content by RT-PCR, after cells were exposed to DMSO or WNK463. Shown are all individual values from n=5 independent experiments (fold of mean of control) or percentage of proliferating cells. (*, P<0.05; **, P<0.01; ***, P<0.001).

4.3.2.2 Cell proliferation by EdU assay

EdU assay was used to examine the cell proliferation rate after WPMY-1 cells were exposed to 1 µM WNK463, 10 µM WNK10 µM or DMSO. Compared to the DMSO group, the cell proliferation rate decreased in cells treated with 1 µM WNK463 (P<0.01) and with 10 µM WNK463 (P<0.001) (Figure 11).

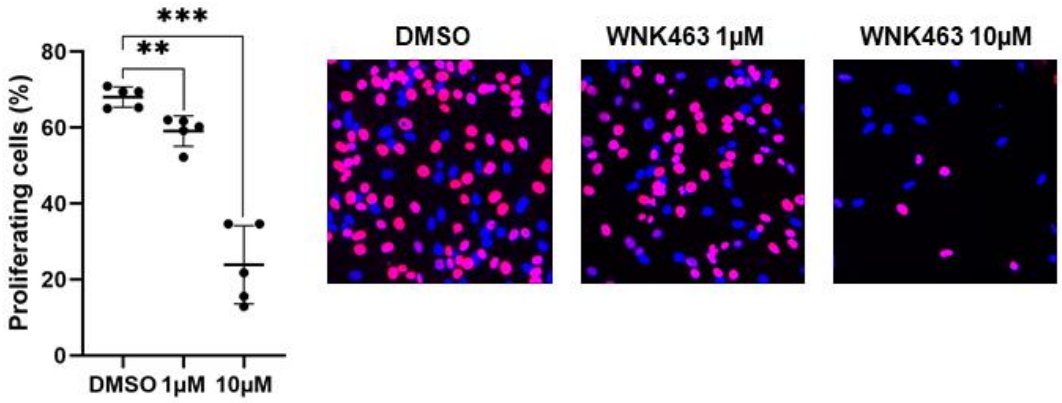


Figure 11. Effects of WNK463 on proliferation of WPMY-1 cells in EdU assay. Cell proliferation was examined by EdU assays, after cells were exposed to DMSO or WNK463 (n=5 independent experiments). Shown are all individual values from n=5 independent experiments (fold of mean of control) for the percentage of proliferating cells. (**, P<0.01; ***, P<0.001), together with images from representative experiments.

4.3.3 Effects of WNK463 on apoptosis of human prostate stromal cells (WPMY-1 cells)

Flow cytometry was used to detect the percentages of cells in early apoptosis and of dead cells by labeling for 7-AAD and annexin V. The percentage of early apoptosis (7-AAD: negative, annexin V: positive) was increased after cells were exposed to 1 µM WNK463 and 10 µM WNK463 (Figure 12). The average percentage of early apoptotic cells ranged about 1.64 % [1.01 to 2.62] after being exposed to solvent, but raised to 3.99 % [3.3-4.53] after being exposed to 1 µM WNK463, and to 8.02 % [6.2 to 10.2] after being exposed to 10 µM WNK463 (Figure 12). The percentage of dead cells (7-AAD: positive, annexin V: positive) was increased by 1 µM and by 10 µM WNK463 as well (Figure 12). The average percentage of dead cells amounted to 1.80 % [0.97 to 2.62] after being exposed to solvent, but raised to 8.78 % [6.46 to 12.4] after being exposed to 1 µM WNK463, and to 16.62 % [3.3-4.53] after being exposed to 10 µM WNK463 (Figure 11).

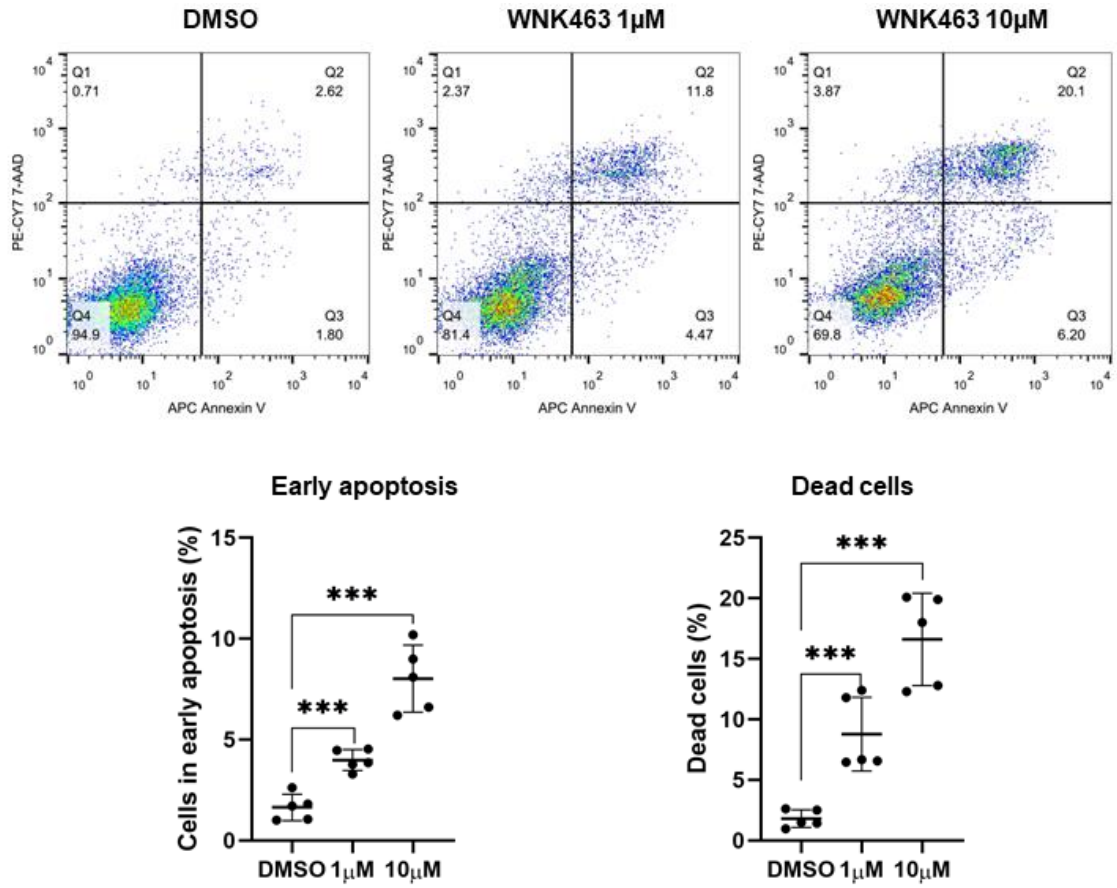
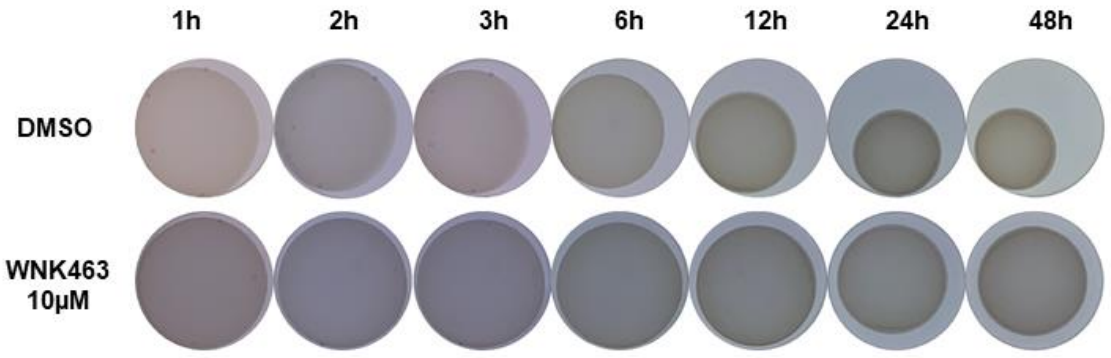


Figure 12. Effects of WNK463 on apoptosis of WPMY-1 cells. Cells in early apoptosis and dead cells were detected by flow cytometry, after cells were exposed to DMSO or WNK463. Shown are all individual values from n=5 independent experiments (***, P<0.001), together with means, SD and images from representative experiments.

4.3.4 Effects of WNK463 on cell contraction of human prostate stromal cells (WPMY-1 cells)

Collagen matrix contraction assay was performed to assess the cell contractions of WPMY-1 cells by comparison of matrix diameter sizes and areas. After adding fresh medium with DMSO or WNK463 (10 µM) and assessment after different periods (1 h, 2 h, 3 h, 6 h, 12 h, 24 h and 48 h), the matrix diameter sizes as well as gel area changes were higher in the 10 µM WNK463 group compared to the DMSO group, indicating that WNK463 inhibited cell contractions of WPMY-1 cells (Figure 13). After 1 h, average sizes of matrix diameter amounted to 1.39 mm [1.12 to 1.81] in the DMSO group, but to 0.95 mm [0.67 to 1.18] in the WNK463

group. In addition, average ratios of gel-area/well-area amounted to 0.82 [0.77 to 0.86] in the DMSO group, and to 0.88 [0.85 to 0.91] in the WNK463 group. After 2 h, average sizes of matrix diameter amounted to 1.50 mm [1.12 to 1.97] in the DMSO group, but to 1.05 mm [0.91 to 1.18] in the WNK463 group. In addition, average ratios of gel-area/well-area amounted to 0.81 [0.75 to 0.86] in the DMSO group, and to 0.86 [0.85 to 0.88] in the WNK463 group. After 3 h, average sizes of matrix diameter amounted to 1.78 mm [1.30 to 2.26] in the DMSO group, and to 1.21 mm [1.00 to 1.39] in the WNK463 group. In addition, average ratios of gel-area/well-area amounted to 0.78 [0.72 to 0.83] in the DMSO group, and to 0.85 [0.82 to 0.87] in the WNK463 group. After 6 h, average sizes of matrix diameter amounted to 2.21 mm [1.39 to 2.90] in the DMSO group, and to 1.41 mm [1.33 to 1.48] in the WNK463 group. In addition, average ratios of gel-area/well-area amounted to 0.72 [0.65 to 0.80] in the DMSO group, and to 0.81 [0.75 to 0.83] in the WNK463 group. After 12 h, average sizes of matrix diameter amounted to 3.97 mm [3.27 to 4.39] in the DMSO group, but to 2.27 mm [1.94 to 3.21] in the WNK463 group. In addition, average ratios of gel-area/well-area amounted to 0.54 [0.50 to 0.61] in the DMSO group, and to 0.72 [0.62 to 0.76] in the WNK463 group. After 24 h, average sizes of matrix diameter amounted to 5.52 mm [4.64 to 5.89] in the DMSO group, but to 3.48 mm [3.02 to 4.45] in the WNK463 group. In addition, average ratios of gel-area/well-area amounted to 0.40 [0.37 to 0.48] in the DMSO group, and to 0.60 [0.54 to 0.64] in the WNK463 group. After 48 h, average sizes of matrix diameter amounted to 6.13 mm [5.69 to 6.43] in the DMSO group, but to 3.41 mm [3.10 to 3.57] in the WNK463 group. In addition, average ratios of gel-area/well-area amounted to 0.35 [0.33 to 0.39] in the DMSO group, and to 0.60 [0.58 to 0.60] in the WNK463 group.



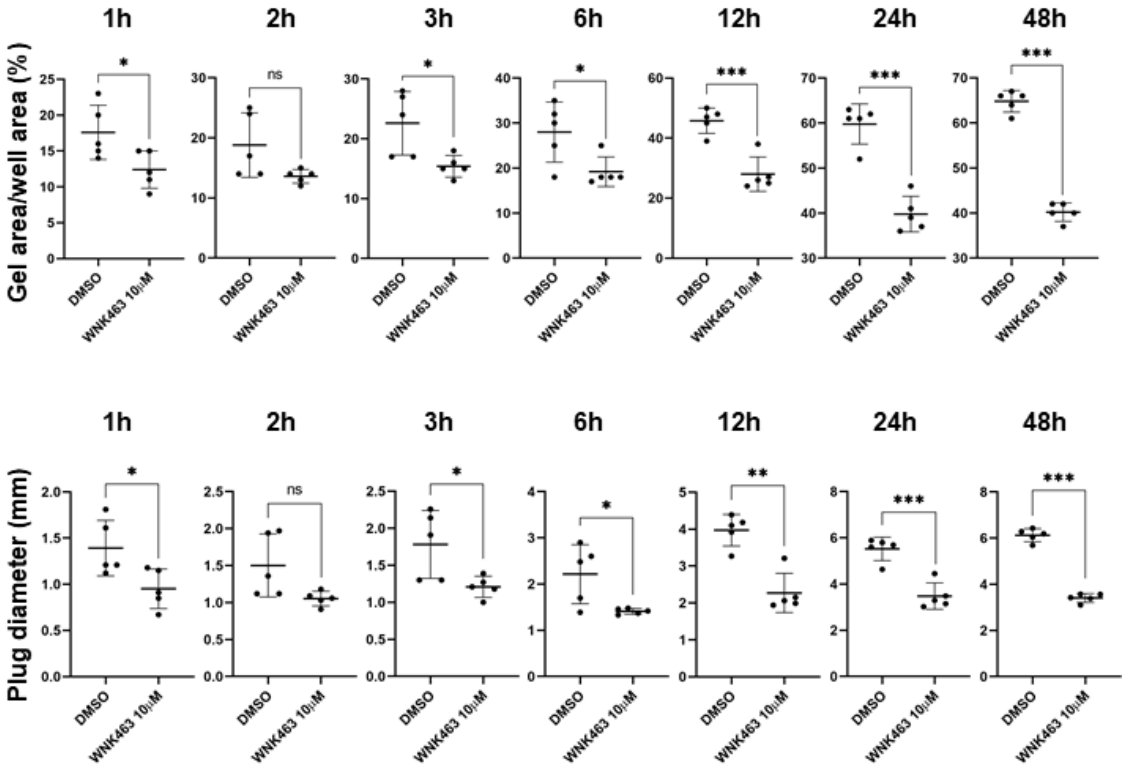


Figure 13. Effects of WNK463 on cell contractions of WPMY-1 cells. Cell contractions were compared between cells treated with DMSO or WNK463 for 1 h, 2 h, 3 h, 6 h, 12 h, 24 h, 48 h. Collagen matrix contraction assay was performed to assess the cell contractions after different period after adding a fresh medium. Changes were expressed as changes in plug diameters (delta), and as ratios of gel-area/well-area. Shown are all individual values from n=5 independent experiments. (ns, no significance; *, P<0.05; **, P<0.01 ***, P<0.001), together with images from representative experiments.

4.3.5 Effects of WNK463 on actin organization of human prostate stromal cells (WPMY-1 cells)

Phalloidin staining was used to examine the polymerized actin in WPMY-1 cells. In control (DMSO) groups, actin was well organized with bundles and long filaments, as well as parallel arrangement and filaments criss-crossed from different cells (Figure 14). Concentration-dependent effects on actin organization were observed while cells were exposed to WNK463 1 μM, 10 μM for 24 h. After treatment with 1 μM WNK463, phalloidin-stained filaments were invisible in most areas, and the phalloidin-stained area covered less than the control group (Figure

13). Actin organization was significantly broken down after treatment with 10 µM WNK463, and only very short and thin filaments remained in these cells (Figure 13).

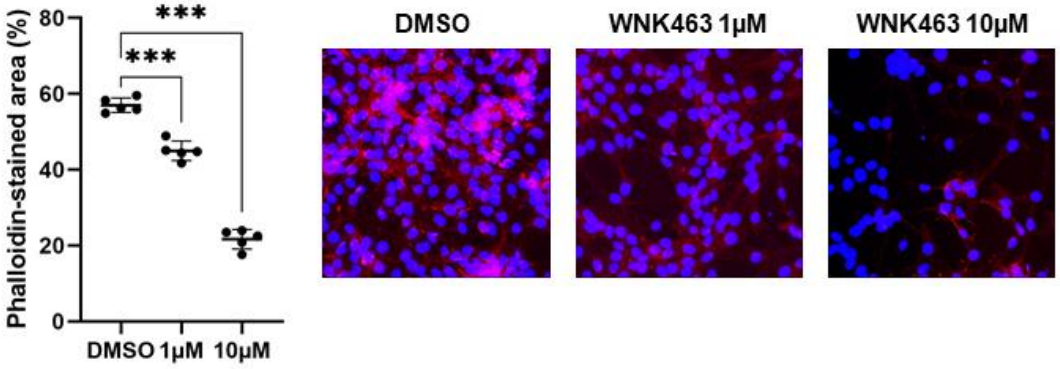


Figure 14. Effects of WNK463 on actin organization of WPMY-1 cells. WPMY-1 cells were exposed to DMSO or WNK463 (1 µM or 10 µM) for 24 h. Actin filaments were dyed by phalloidin, resulting in red staining, while nuclei were dyed with DAPI, resulting in blue staining. Concentration-dependent effects were observed on actin organization of WPMY-1 cells. Shown are all individual values from n=5 independent experiments (*p<0.05 vs. DMSO), together with images from representative experiments.

4.4 Effect of WNK1-4 silencing on cell culture

4.4.1 Silencing of WNK1-4 expressions in human prostate stromal cells (WPMY-1 cells)

Expressions of WNK isoforms 1-4 were downregulated by isoform-specific WNK siRNAs in WPMY-1 cells. After transfection with siRNAs for WNK isoforms 1-4, WNK1-4 mRNA levels decreased compared to wildtype cells and scramble siRNA by RT-PCR (Figure 15).

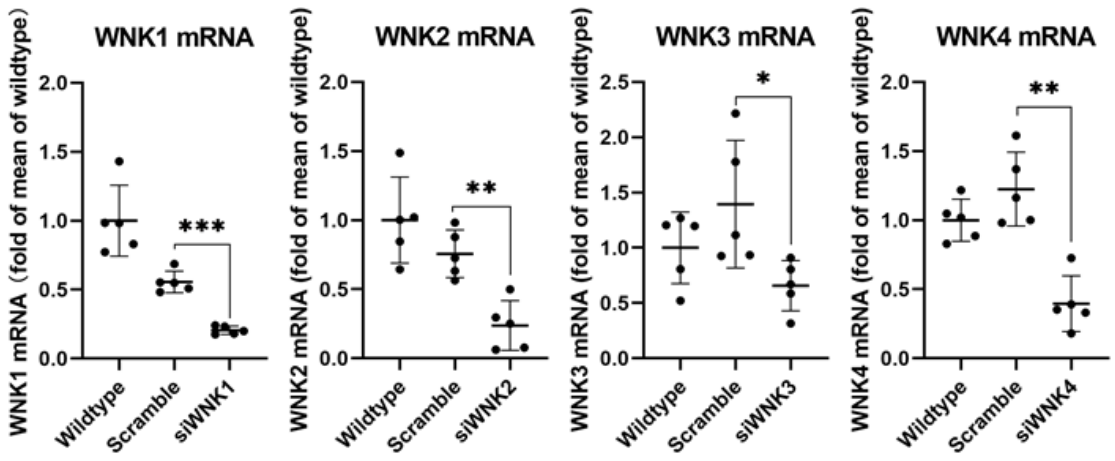


Figure 15. Silencing of WNK1-4 mRNA expressions in WPMY-1 cells. Expression of WNK1-4 mRNAs was semiquantitatively compared by RT-PCR in WPMY-1 cells, after transfection with scramble siRNA or with siRNAs for WNK isoforms 1-4, and in non-transfected WPMY-1 cells ("wildtype"). Shown are all individual values from n=5 independent experiments (fold of mean of wildtype) for RT-PCR.

4.4.2 Effect of WNK1-4 silencing on viability of human prostate stromal cells (WPMY-1 cells)

After transfection of WPMY-1 cells with scramble siRNA and WNK1-4 siRNAs for 72 h and in non-transfected WPMY-1 cells kept under the same conditions, viability was assessed by CCK-8 assay. Compared to non-transfected cells and to cells transfected with scramble siRNA, transfection with WNK1-4 siRNAs reduced the viability of WPMY-1 cells, assessed 24 h after transfection. Thus, significant reduction of viability was observed after transfection with WNK1 siRNA ($P<0.01$), WNK2 siRNA ($P<0.01$), WNK3 siRNA ($P<0.01$), and WNK4 siRNA ($P<0.01$) (Figure 16).

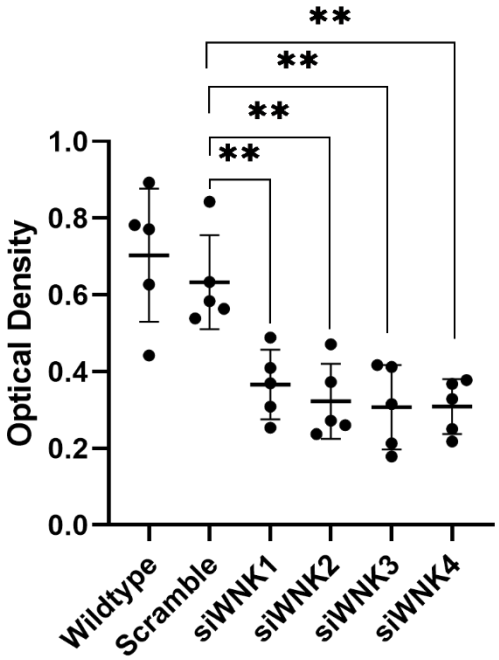


Figure 16. Effects of WNK1-4 silencing on viability of WPMY-1 cells. Viability was assessed by CCK-8 assay after transfection with scramble siRNA or with siRNAs for WNK isoforms 1-4, and in non-transfected WPMY-1 cells ("wildtype"). Shown are all the individual values from n=5 independent experiments (**, P<0.01).

4.4.3 Effects of WNK1-4 silencing on cell proliferation of human prostate stromal cells (WPMY-1 cells)

4.4.3.1 Ki-67 mRNA expression

After transfection with scramble siRNA and WNK1-4 siRNAs for 72 h, Ki-67 mRNA expression was assessed by RT-PCR as a readout for cell proliferation. All four WNK1-4 siRNAs reduced the expression of Ki-67 mRNA. Compared to scrambled siRNA-transfected cells (Figure 17), significantly lower expression of Ki-67 mRNA was detected after transfection with WNK1 siRNA (P<0.001), WNK2 siRNA (P<0.001), WNK3 siRNA (P<0.001), and WNK4 siRNA (P<0.001).

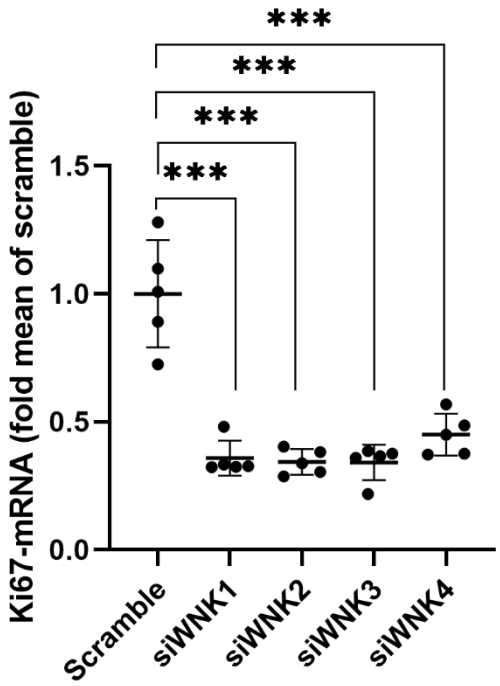


Figure 17. Effects of WNK1-4 silencing on Ki-67 mRNA expression of WPMY-1 cells. Ki-67 mRNA content in WPMY-1 cells was semiquantitatively assessed by RT-PCR, after cells were transfected with scramble siRNA, or with isoform-specific WNK siRNAs. Shown are all individual values from n=5 independent experiments (fold of mean of control).

4.4.3.2 Cell proliferation by EdU assay

After transfection of WPMY-1 cells with scramble siRNA and WNK1-4 siRNAs for 72 h and in non-transfected WPMY-1 cells kept under the same conditions, the rate of cell proliferation was assessed by EdU assay. After transfection with isoform-specific WNK siRNAs, lower proliferation rates were observed, compared to non-transfected WPMY-1 cells and to WPMY-1 cells transfected with scramble siRNA. Compared to scramble siRNA, the proliferation rate was decreased after transfection with WNK1 siRNA ($P<0.01$), WNK2 siRNA ($P<0.001$), WNK3 siRNA ($P<0.001$), and WNK4 siRNA ($P<0.001$) (Figure 18).

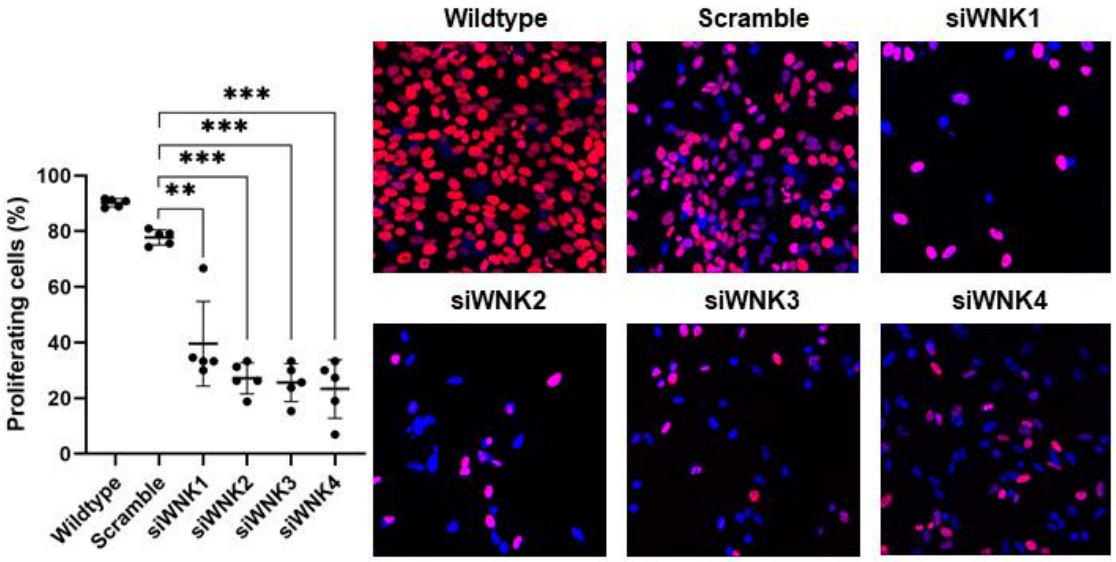
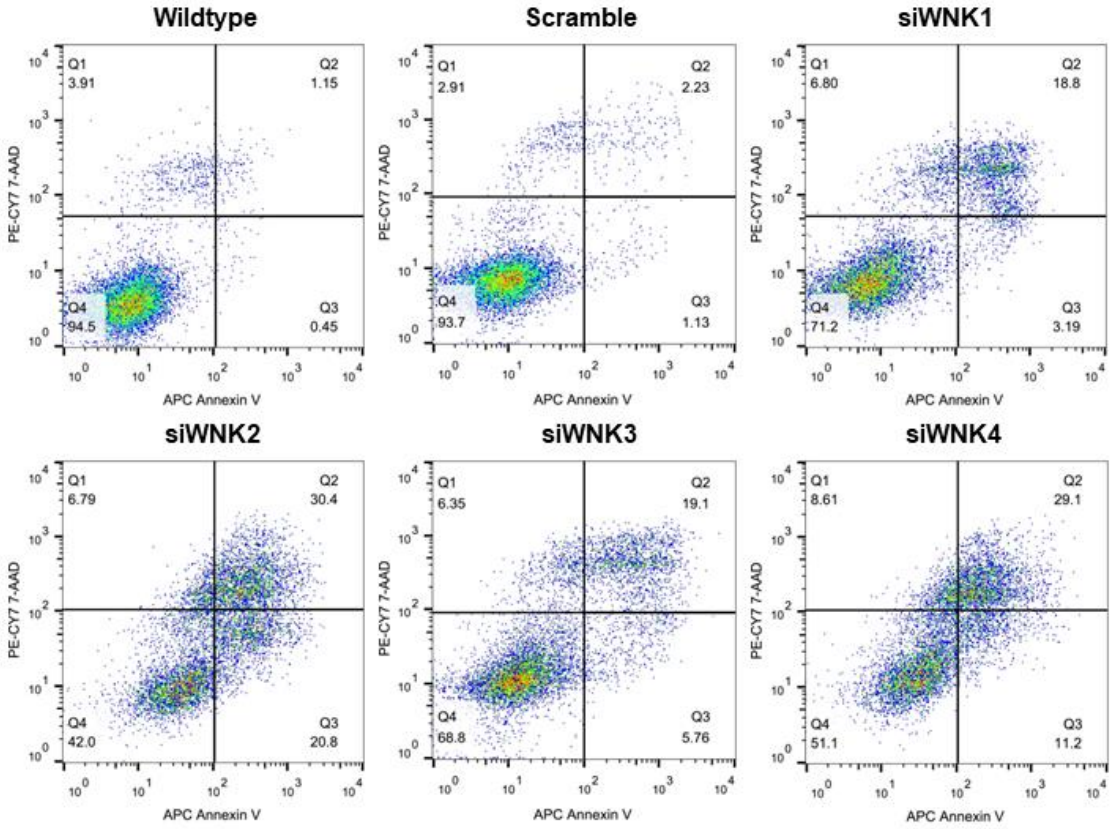


Figure 18. Effects of WNK1-4 silencing on proliferation rate of WPMY-1 cells. Cell proliferation was examined by EdU assay, after cells were transfected with scramble siRNA and WNK1-4 siRNAs, and in non-transfected cells kept under identical conditions ("wildtype"). Shown are all individual values from n=5 independent experiments (fold of mean of control) for percentage of proliferating cells (**P<0.01, ***P<0.001), together with images from representative experiments.

4.4.4 Effects of WNK1-4 silencing on apoptosis and cell death in human prostate stromal cells (WPMY-1 cells)

After transfection of WPMY-1 cells with scramble siRNA and WNK1-4 siRNAs for 72 h and in non-transfected WPMY-1 cells kept under the same conditions, the percentage of cells showing apoptosis and cell death was assessed by flowcytometry. Transfection with WNK1-4 siRNA raised the percentage of cells in early apoptosis and the percentage of dead cells, compared to non-transfected cells and to cells transfected with scramble siRNA (Figure 19). The average percentage of early apoptotic cells amounted to 1.01 % [0.45 to 1.43] in non-transfected cells and to 1.74 % [0.60 to 3.58] in cells transfected with scramble siRNA, but raised to 5.73 % [3.19 to 8.22] after transfection with WNK1 siRNA, 6.94 % [2.56 to 20.80] after transfection with WNK2 siRNA, 5.53 % [2.10 to 12.60] after transfection with WNK3 siRNA, and to 4.98 % [1.80 to 11.20] after transfection with WNK4 siRNA. The average percentage of dead cells amounted to 2.36 % [1.12 to 5.85] in non-transfected cells and to 4.45 % [1.13 to 7.88] after transfection with scramble siRNA, but raised to 23.90 % [16.70 to 30.90] after transfection with WNK1, 19.26 % [14.30 to 30.40] after transfection with WNK2 siRNA, 17.86 %

[14.10 to 19.10] after transfection with WNK3 siRNA, and to 24.54 % [14.70 to 31.80] after transfection with WNK4 siRNA.



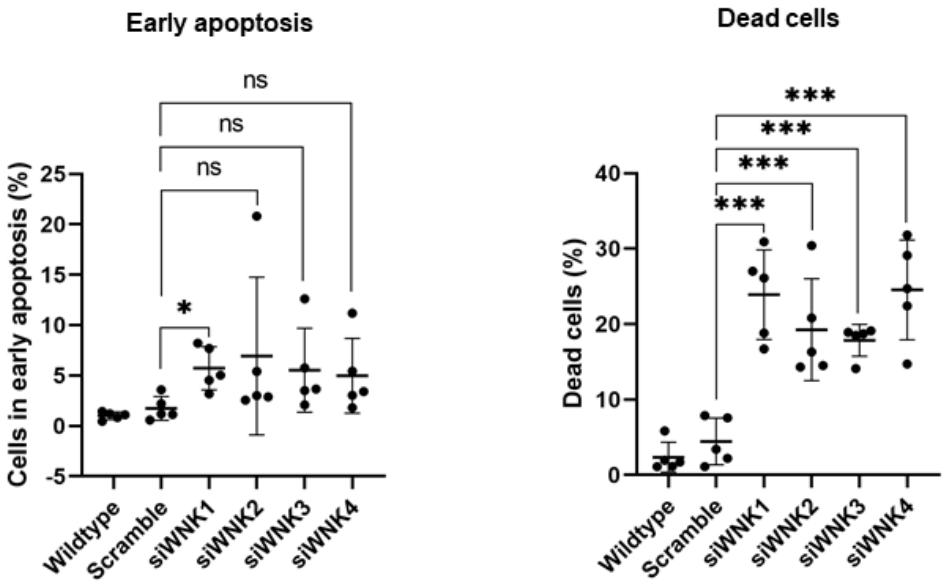


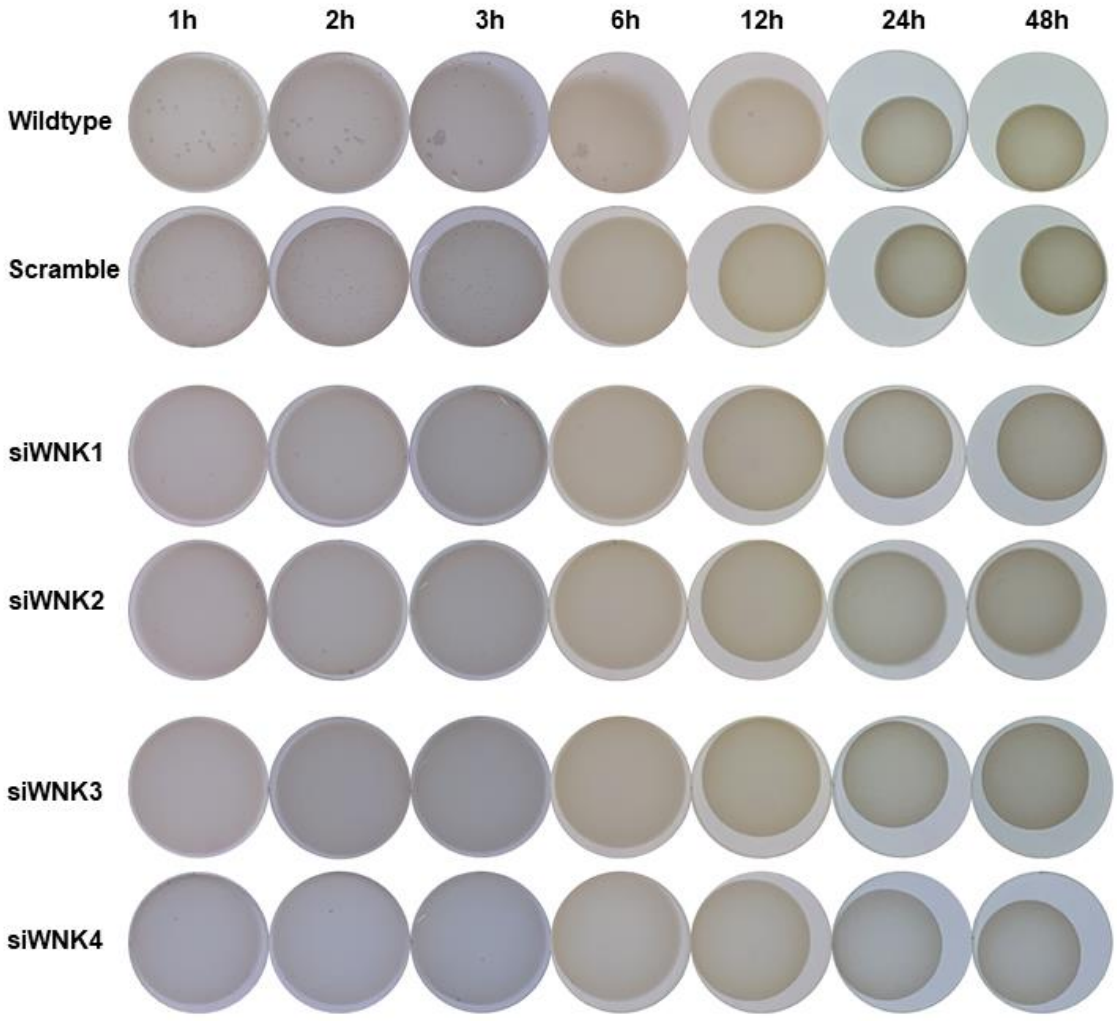
Figure 19. Effects of WNK1-4 silencing on apoptosis and cell death in WPMY-1 cells. Early apoptosis and cell death were detected by flow cytometry, after cells were transfected with scramble siRNA and WNK1-4 siRNAs, and in non-transfected cells kept under the same conditions ("wildtype"). Shown are all individual values from n=5 independent experiments (ns, no significance; *, P<0.05; ***, P<0.001), together with images from representative experiments.

4.4.5 Effects of WNK1-4 knockdown on cell contraction of human prostate stromal cells (WPMY-1 cells)

Cell contraction was assessed after transfection of WPMY-1 cells with scramble siRNA or WNK1-4 siRNAs, and in non-transfected cells by collagen matrix contraction assays. Compared to cells transfected with scramble siRNA, matrix diameter sizes as well as gel area changes were higher in cells transfected with WNK1-4 siRNA at different time points (assessed 1-48 h after adding of fresh medium), suggesting that WNK1-4 silencing reduced cell contraction of WPMY-1 cells (Figure 20). After 1 h, average sizes of matrix diameter amounted to 1.34 mm [0.67 to 2.12] and 1.63 mm [0.78 to 2.72] in non-transfected cells and cells transfected with scramble siRNA, but to 1.17 mm [0.75 to 1.66], 0.82 mm [0.61 to 1.40], 1.23 mm [0.73 to 1.48] and 1.08 mm [0.43 to 1.51] in cells transfected with WNK1, WNK2, WNK3 or WNK4 siRNA. In addition, average ratios of gel-area/well-area amounted to 0.84 [0.77 to 0.91] and 0.79 [0.71 to 0.89] in non-transfected cells and cells transfected with scramble siRNA, but to 0.89 [0.85 to 0.92], 0.90 [0.87 to 0.93], 0.85 [0.81 to 0.91] and 0.86 [0.81 to 0.94] after transfection with WNK1, WNK2, WNK3 or WNK4 siRNA. After 2 h, average sizes of matrix diameter amounted to 1.70 mm [1.30 to 2.24] and 1.53 mm [1.00 to 1.94] in non-transfected cells and cells transfected with scramble siRNA, but to 1.07 mm [0.75 to 1.62], 0.84 mm [0.46 to 1.13], 1.12 mm [0.82 to 1.69] and 1.17 mm [0.81 to 1.87] after transfection with WNK1, WNK2, WNK3 or WNK4 siRNA. In addition, average ratios of gel-area/well-area amounted to 0.80 [0.77 to 0.84] and 0.81 [0.77 to 0.84] in non-transfected cells and cells transfected with scramble siRNA, but to 0.87 [0.86 to 0.91], 0.89 [0.86 to 0.94], 0.88 [0.89 to 0.91] and 0.86 [0.81 to 0.89] after transfection with WNK1, WNK2, WNK3 or WNK4 siRNA. After 3 h, average sizes of matrix diameter amounted to 1.78 mm [0.73 to 2.29] and

2.05 mm [1.34 to 2.80] in non-transfected cells and cells transfected with scramble siRNA, but to 1.55 mm [1.14 to 1.97], 1.11 mm [0.95 to 1.35], 1.45 mm [0.80 to 1.93] and 1.37 mm [0.97 to 1.94] after transfection with WNK1, WNK2, WNK3 or WNK4 siRNA. In addition, average ratios of gel-area/well-area amounted to 0.76 [0.71 to 0.78] and 0.76 [0.72 to 0.82] in non-transfected cells and cells transfected with scramble siRNA, but to 0.81 [0.77 to 0.85], 0.86 [0.84 to 0.89], 0.83 [0.78 to 0.87] and 0.85 [0.80 to 0.88] after transfection with WNK1, WNK2, WNK3 or WNK4 siRNA. After 6 h, average sizes of matrix diameter amounted to 2.73 mm [2.07 to 3.54] and 2.35 mm [1.30 to 2.94] in non-transfected cells and cells transfected with scramble siRNA, but to 1.90 mm [1.33 to 2.55], 1.62 mm [1.32 to 2.31], 1.91 mm [1.33 to 2.15] and 1.81 mm [1.36 to 2.36] after transfection with WNK1, WNK2, WNK3 or WNK4 siRNA. In addition, average ratios of gel-area/well-area amounted to 0.69 [0.62 to 0.78] and 0.73 [0.68 to 0.83] in non-transfected cells and cells transfected with scramble siRNA, but to 0.77 [0.72 to 0.83], 0.79 [0.73 to 0.81], 0.78 [0.74 to 0.83] and 0.78 [0.73 to 0.82] after transfection with WNK1, WNK2, WNK3 or WNK4 siRNA. After 12 h, average sizes of matrix diameter amounted to 4.63 mm [2.95 to 5.91] and 4.09 mm [3.67 to 4.55] in non-transfected cells and cells transfected with scramble siRNA, but to 2.56 mm [1.88 to 2.99], 2.18 mm [1.45 to 3.02], 2.43 mm [2.06 to 2.88] and 2.62 mm [2.20 to 2.89] after transfection with WNK1, WNK2, WNK3 or WNK4 siRNA. In addition, average ratios of gel-area/well-area amounted to 0.48 [0.39 to 0.65] and 0.54 [0.50 to 0.58] in non-transfected cells and cells transfected with scramble siRNA, but to 0.70 [0.65 to 0.77], 0.74 [0.64 to 0.78], 0.72 [0.64 to 0.78] and 0.68 [0.63 to 0.73] after transfection with WNK1, WNK2, WNK3 or WNK4 siRNA. After 24 h, average sizes of matrix diameter amounted to 6.09 mm [5.36 to 6.68] and 5.38 mm [4.92 to 5.82] in non-transfected cells and cells transfected with scramble siRNA, but to 4.79 mm [3.31 to 5.84], 2.93 mm [1.87 to 3.77], 3.71 mm [3.13 to 4.46] and 3.45 mm [2.80 to 4.15] after transfection with WNK1, WNK2, WNK3 or WNK4 siRNA. In addition, average ratios of gel-area/well-area amounted to 0.36 [0.31 to 0.41] and 0.42 [0.39 to 0.46] in non-transfected cells and cells transfected with scramble siRNA, but to 0.48 [0.40 to 0.61], 0.68 [0.58 to 0.80], 0.57 [0.51 to 0.63] and 0.61 [0.53 to 0.71] after transfection with WNK1, WNK2, WNK3 or WNK4 siRNA. After 48 h, average sizes of matrix diameter amounted to 6.41

mm [5.78 to 6.89] and 5.96 mm [5.74 to 6.16] in non-transfected cells and cells transfected with scramble siRNA, but to 4.57 mm [3.52 to 5.34], 4.72 mm [3.63 to 5.09], 4.71 mm [3.75 to 5.30] and 4.29 mm [3.83 to 4.76] after transfection with WNK1, WNK2, WNK3 or WNK4 siRNA. In addition, average ratios of gel-area/well-area amounted to 0.34 [0.30 to 0.38] and 0.37 [0.35 to 0.39] in non-transfected cells and cells transfected with scramble siRNA, but to 0.50 [0.44 to 0.59], 0.49 [0.45 to 0.57], 0.49 [0.43 to 0.56] and 0.53 [0.51 to 0.58] after transfection with WNK1, WNK2, WNK3 or WNK4 siRNA.



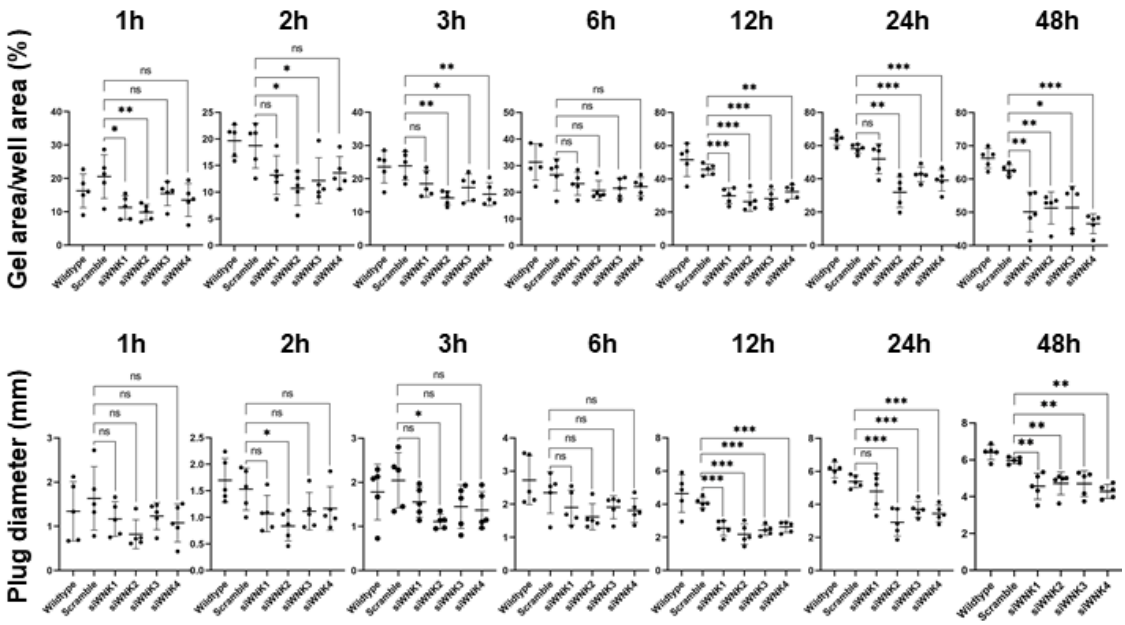


Figure 20. Effects of WNK1-4 silencing on cell contractions of WPMY-1 cells. Cell contractions were compared between cells transfected with scramble siRNA or WNK1-4 siRNA, and in non-transfected cells kept under identical conditions, 1 h, 2 h, 3 h, 6 h, 12 h, 24 h and 48 h after seeding to matrix plugs in collagen matrix contraction assays. Changes were expressed as changes in plug diameters (delta), and as ratios of gel-area/well-area. Shown are all individual values from n=5 independent experiments. (ns, no significance; *, P<0.05; **, P<0.01 ***, P<0.001), together with images from representative experiments.

4.4.6 Effects of WNK1-4 knockdown on actin organization of human prostate stromal cells (WPMY-1 cells)

Phalloidin staining was performed after transfection of WPMY-1 cells with scramble siRNA or with WNK1-4 siRNA, and in non-transfected cells kept under identical conditions. In non-transfected and scramble siRNA-transfected cells, actin was well organized with bundles and long filaments, as well as parallel arrangement and filaments criss-crossed from different cells (Figure 21), After transfected with WNK1-4 siRNAs and compared to cells transfected with scramble siRNA, actin organization was broken down, appartently completely. Only shorter and thinner filaments remained in these cells, compared to cells transfected with scramble siRNA (Figure 21).

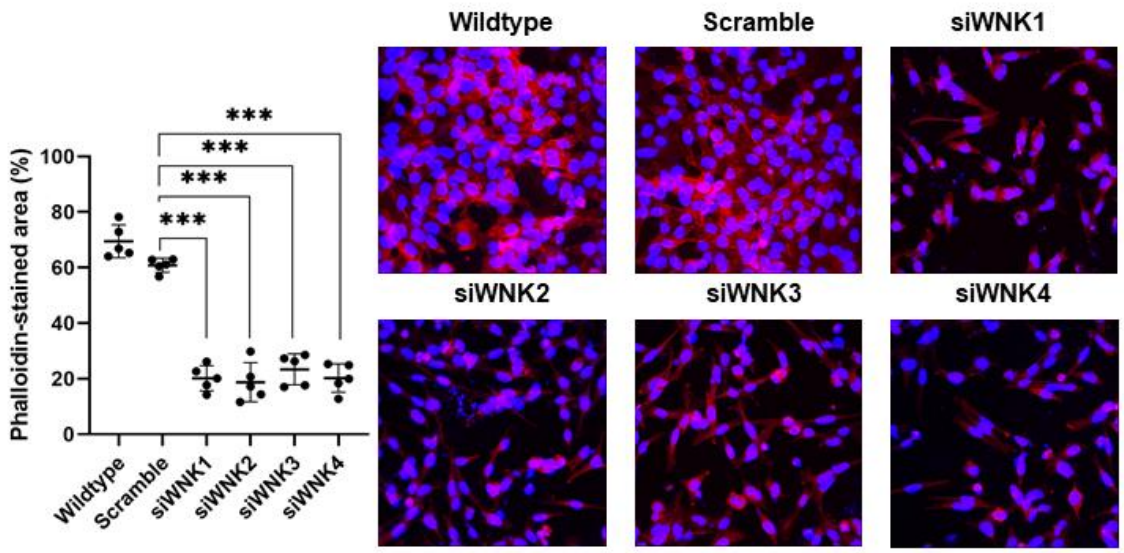


Figure 21. Effects of WNK1-4 silencing on actin organization of WPMY-1 cells. Phalloidin staining was performed in WPMY-1 cells transfected with scramble siRNA or WNK1-4 siRNAs for 72 h, or in non-transfected cells kept under identical conditions ("wildtype"). Shown are all individual values from n = 5 independent experiments (***p<0.001, siRNAs vs. scramble siRNA), together with images from representative experiments.

5. Discussion

Although α_1-adrenoceptors have long been considered as the main factor for prostate smooth muscle contraction and LUTS in BPH, an increasing number of studies suggested that non-adrenergic mediators (TXA_2, endothelin-1) may induce prostate smooth muscle contractions as well and in parallel to α_1-adrenoceptors[40,109,211]. Meanwhile, the limited effectiveness of α_1-blockers has been attributed to non-adrenergic mediators, which may keep prostate smooth muscle tone elevated despite treatment with α_1-blockers, and may account for medication refractory components in LUTS[30,109]. Similarly, the effectiveness of 5-ARIs in reducing prostate size is limited as well, and combination therapies are still required and often applied, although they are least tolerated and affected by high discontinuation rates. Consequently, novel medications to inhibit non-adrenergic and adrenergic smooth muscle contraction, plus growth in prostate cells are required. Ideal drug candidates would be single compounds, addressing all these factors at once. The results of this thesis suggest inhibition of smooth muscle contraction in isolated, human prostate tissues and inhibition of growth-related functions in stromal cells by the pan-WNK inhibitor WNK463, and suggest a contraction- and growth-promoting roles of WNKs in prostate stromal cells.

WNK kinases are serine/threonine kinases occurring in four isoforms, WNK1-4[184,212,213]. WNK1, WNK2, WNK3 and WNK4 are encoded by four genes located to chromosomes 12p13.33, chromosome 9q22.31, chromosome Xp11.22 and chromosome 17q21.31, respectively[183,212]. The WNK kinases are ubiquitously expressed. WNK1 highly distributed in the kidney, heart, testis, and skeletal muscle[214], WNK2 is mainly distributed in the brain, colon and heart[183], WNK3 is widely distributed in lung, brain, liver and kidney[183,215] and WNK4 was detected in the bile duct, colon, pancreas, kidney, skin and brain[183,216]. In the present thesis, expression of all four WNK isoforms was confirmed in human prostate tissues and WPMY-1 cells by RT-PCR. However, detection by Western blot analyses, which was originally also intended to be included in this thesis, failed. This was attributed to the high molecular weights of WNKs (251 kDa for WNK1, 243 kDa for

WNK2, 198 kDa for WNK3, 135 kDa for WNK4), which did not provide conclusive results. In fact, this was not unexpected, as electrophoresis is in fact unsuitable for large proteins (>200 kDa).

The findings regarding effects of WNK463 on prostate smooth muscle contraction are in line with previous reports, suggesting a role of WNKs for promotion of vascular smooth muscle. In fact, vascular and prostate smooth muscle show similarities, including the fact that contractions are induced by α_1-adrenoceptors, thromboxane A_2 and endothelin-1 in both types and reflected by cardiovascular side effects as the most common adverse events during therapy of LUTS suggestive of BPH with α_1-blockers. Thus, preceding reports suggesting WNK-driven vascular smooth muscle contractions were the basis to assume a similar role in prostate smooth muscle and initiated the examinations performed in this thesis. Mutations in WNK1 or WNK4, leading to upregulation and increased activity of RhoA/Rho kinase, can cause arterial hypertension in humans[212,213]. The critical role of WNKs for regulating blood pressure has been attributed to the regulation of electrolyte and body fluid homeostasis by the kidney, and to vascular function, including modulation of vascular smooth muscle contractility[185,190]. Thus, genetic modifications in mice leading to reduced WNK activity impaired α_1-adrenergic contractions of isolated vessels[187,199]. Vice versa, genetic modifications in mice leading to increased WNK activity resulted in increased α_1-adrenergic contractions of isolated vessels [185]. Accordingly, angiotensin-II, which induces contraction of vascular smooth muscle, increases phosphorylation of the WNK substrate NCC (WNK-OSR1/SAPK-NaCl cotransporter)[217]. In addition to NCC, SAPK (STE20/SPS1-related proline/alanine-rich kinase) and NKCC1 (Na^+-K^+-$2Cl^-$ cotransporter 1) are other major downstream substrates phosphorylated by WNKs[187,190]. Finally, increased WNK activity was reported to induce α_1-adrenergic contractions of renal and vascular vessels by activating RhoA[185], and another study suggested that WNK1 is essential for activation of PLCβ by $G\alpha_q$-coupled receptors[186]. RhoA and PLCβ are parts of important intracellular signaling pathways mediating smooth muscle contraction not only in vascular smooth muscle, but in prostate smoot muscle as well[30].

Here, the pan-WNK inhibitor WNK463 was applied to human prostate tissues in organ bath studies addressing smooth muscle contractions of these tissues, and inhibited neurogenic contractions induced by EFS, contractions induced by the three α_1-adrenergic agonists noradrenaline, phenylephrine and methoxamine, by the TXA_2 analog U46619 and by endothelin-1. These inhibitions were of divergent extent, and differed with WNK463 concentrations and contractile stimuli. Thus, inhibitions were large (roughly ranging around 50%) and occurred using 0.5 µM and 10 µM for EFS- and U46619-induced contractions, but were low or lacking using 0.5 µM but observed using 10 µM for α_1-adrenergic and endothelin-1-induced contractions. EFS-induced contractions of prostate tissues are neurogenic, at least most parts of these contractions, as previously demonstrated using the neurotransmission inhibitor, tetrodotoxin[218-221]. Thus, EFS-induced, neurogenic prostate smooth muscle contraction is driven by the release of endogenous neurotransmitters and subsequent activation of postsynaptic α_1-adrenoceptors on prostate smooth muscle cells[222-224]. The inhibition of α_1-adrenergic contractions by WNK463, observed in this thesis is in line with previous studies reporting relationships between WNKs and α_1-adrenergic vasocontration[185,187,199]. However, inhibition of α_1-adrenergic (and endothelin-1-induced) contractions required higher concentrations of WNK463 and were here observed using 10 µM, but not using 0.5 µM. Consequently, different mechanisms of inhibition may be assumed to account for α_1-adrenergic/endothelin-1-induced, and for EFS-/U46619-induced contractions, as the latter were sensitive to 0.5 µM WNK463.

The concentration of 0.5 µM may be (most probably) largely specific for WNKs, and inhibitory effects at this concentration may be largely caused by inhibition of WNKs. In contrast, for effects limited to 10 µM but not observed using 0.5 µM, off-target effects cannot be excluded. WNK463 is a pan-WNK-kinase inhibitor, which potently inhibited the activity of all four family members (WNK1, WNK2, WNK3, WNK4), with IC_{50} values of 5 nM,1 nM, 6 nM, and 9 nM, respectively.

WNK463 also showed significant suppression on WNK kinases in human embryonic kidney 293 cells, with an IC_{50} of 106 nM[225], which showed both low nanomolar affinity and high kinase selectivity[226].

In addition to functions in blood pressure regulation and vasocontraction, WNKs may promote the proliferation of different cell types, including vascular smooth muscle cells[226,227]. Consequently, an analog role may be presumed for smooth muscle cells outside the cardiovascular system, and was here examined using WPMY-1 cells. WPMY-1 cells (immortalized myofibroblast stromal cell line), were originally acquired from the stroma in the peripheral zone of a non-malignant human prostate[228]. WPMY-1 cells are similar to prostate smooth muscle cells and have been previously considered as such, with homogeneous characteristics and properties, which also expressed smooth muscle marker α-SMA [111] calponin and α_1-adrenoceptors [228]. Therefore, WPMY-1 cells were used here to examine the pharmacological effects of WNK463 and to study the impact of WNK1-4 silencing on cell growth and smooth muscle cell contraction. EdU assays cells suggested that WNK463 inhibited the proliferation of WPMY-1 cells. In doing so, the concentration of 10 µM achieved a larger inhibition than 1 µM, which was in line with the previous findings from other cell types[201,227]. The effect on proliferation rate was supported by results from RT-PCR, where a decline in the expression of the proliferation maker Ki-67 was noted. These findings were confirmed by concentration-dependent decreases in viability in CCK-8 assays. In parallel to inhibition of cell proliferation and as another function being critical for growth, apoptosis and cell death of WPMY-1 cells were increased at a concentration of 1 µM and 10 µM in flow cytometry. Together, these results show a significant inhibitory effect of WNK463 on the growth of WPMY-1 cells. Similar to prostate tissues in the organ bath, WNK463 inhibited contractions of WPMY-1 cells in cell contractions assays, which was paralleled by breakdown of actin organization, as seen after phalloidin staining. In fact, correct actin organization, including filament organization, is a requirement for smooth muscle contraction[30,41].

In order to confirm the role of WNKs in these functions, the impact of WNK1-4 silencing was examined. Silencing of all four WNK isoforms was confirmed by RT-PCR, and mimicked all effects of WNK463. Thus, compared to wildtype and scramble siRNA-transfected cells, WNK1-4 silencing showed similar effects on viability, proliferation, apoptosis, cell contraction and actin organization in WPMY-1 cells. Silencing of different isoforms of WNKs showed the same results, suggesting equal functions of all four isoforms in prostate stromal cells. Interestingly, WNK1-4 has been reported to exert action on proliferation or migration, apoptosis by MAPK signaling pathways or the SPAK/NKCC1 pathway[212,227,229-236]. Therefore, the effects of WNK463 on WPMY-1 cells may be associated with MAPK signaling pathways or SPAK/NKCC1 pathway as well.

Phalloidin staining on actin organization of WPMY-1 cells showed a significant breakdown of actin organization with short and thin filaments after exposure to WNK463 in a concentration-dependent way. In a collagen matrix contraction assay, contraction of WPMY-1 cells was significantly inhibited after adding WNK463. Since actin polymerization and correct organization to filaments is an ultimate prerequisite for smooth muscle contraction[34,35,237], actin disorganization might be the reason for inhibiting smooth muscle contraction by WNK463. It is commonly accepted that RhoA regulates actin organization centrally and actomyosin contractility in intracellular processes[238-242]. A previous study demonstrated that genetic modifications in mice leading to increased WNK activity resulted in increased α_1-adrenergic contractions of isolated vessels, assumed to be imparted by upregulation of the contraction-mediating GTPase RhoA[185]. Based on these evidences, it may be hypothesized that WNK is involved in cell contraction by regulating actin polymerization and cytoskeleton reorganization by the RhoA signaling pathway.

According to this thesis and the presented data, WNKs might be potential targets to address cell growth and smooth muscle contraction of prostate cells by pharmacologic treatment. WNK463 is orally available, and can be applied at least in

animal models. In order to explore the effect of WNK463, Yamada et al.[226] designed a rat model with spontaneously hypertension, and found that WNK463 could exert effect on cardiovascular and renal system, by impairing blood pressure and the phosphorylation of SPAK and OSR1. Lee al.[243] also found that WNK kinases inhibited by WNK463 will intensify K^+/Cl^- cotransporter 2 activity, raise the efficacy of γ-aminobutyric acid inhibition, and thereby inhibit seizure activity in rat models. Therefore, these proof-of-biology studies suggested an essential role for WNKs for important physiological processes and confirmed the prediction made for pharmacological WNK inhibition by human and rodent genetics in vivo. Together, available evidence from other organs and other cell types suggested an analog role for promoting smooth muscle contraction and growth in the prostate, which has been confirmed by the findings of this thesis. Accordingly, it would be feasible to examine effects of WNK463 on BPH and LUTS in animal models. Studies may include to explore the effects of WNK463 on prostate growth in animals with experimentally-induced BPH, where prostate growth and enlargement is induced by chronic testosterone application. Effects on LUTS may be investigated in rodents with partial urethral obstruction, mimicking bladder outlet obstruction in BOO. However, as the obstruction in these models are not caused by the bladder, any improvements of voiding in these models is typically referred to effects on the bladder. Voiding symptoms, purely caused by prostate enlargement in animal models can only be examined in dogs and monkeys, where the prostate circularly surrounds the urethra, similar to humans. In rodents, however, the prostate forms two lobes, without surrounding the prostate, so that BPH in rodents may not cause urethral obstruction and voiding symptoms[244]

6. Conclusion

The pan-WNK inhibitor WNK463 inhibits neurogenic, α_1-adrenergic and non-adrenergic smooth muscle contractions of human prostate tissues, but with divergent patterns regarding required inhibitor concentrations. Neurogenic and TXA_2-induced contractions were most susceptible to inhibition by WNK463, i.e. occurring at nanomolar concentrations. In parallel, WNK463 affected growth-related functions of cultured prostate stromal cells, including inhibition of proliferation and viability, and induction of apoptosis and cell death. All effects on growth-related functions, as well as contraction of WPMY-1 cells were mimicked by silencing of WNK isoforms 1-4, pointing to a certain role of all four isoforms in these functions. Considering that smooth muscle contraction and growth in the prostate are critical for pathogenesis of LUTS in BPH, WNKs may be putative targets of future drug treatment and WNK463 may be a promising candidate to be tested in animal models of BPH and LUTS.

7. References

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Affidavit

Affidavit

Liu, Yuhan

Surname, first name

I hereby declare, that the submitted thesis entitled:

Inhibition of prostate smooth muscle contraction and prostate stromal cell growth by the WNK inhibitor WNK463 and WNK silencing

…….

is my own work. I have only used the sources indicated and have not made unauthorised use of services of a third party. Where the work of others has been quoted or reproduced, the source is always given.

I further declare that the submitted thesis or parts thereof have not been presented as part of an examination degree to any other university.

Planegg,14.09.2022
place, date

Yuhan Liu
Signature doctoral candidate

List of publications

Publication related to thesis:

1. Y. Liu, R. Huang, A. Ciotkowska, A. Tamalunas, R. Waidelich, F. Strittmatter, C.G. Stief, M. Hennenberg, Inhibition of prostate smooth muscle contraction and prostate stromal cell growth by the WNK inhibitor WNK463 and WNK silencing. (Writing)

Other publications:

1. R. Huang, **Y. Liu**, A. Ciotkowska, A. Tamalunas, R. Waidelich, F. Strittmatter, C.G. Stief, M. Hennenberg, Concentration-dependent alpha1-Adrenoceptor Antagonism and Inhibition of Neurogenic Smooth Muscle Contraction by Mirabegron in the Human Prostate, Frontiers in pharmacology, 12 (2021) 666047.

2. R. Huang, **Y. Liu**, B. Li, R. Wang, A. Tamalunas, R. Waidelich, F. Strittmatter, C.G. Stief, M. Hennenberg, Inhibition of human prostate smooth muscle contraction by the inhibitors of protein kinase C, GF109203X, and Go6983, The Prostate, (2021).

3. B. Li, R. Huang, R. Wang, **Y. Liu**, C.G. Stief, M. Hennenberg, Picotamide inhibits a wide spectrum of agonist-induced smooth muscle contractions in porcine renal interlobar and coronary arteries, Pharmacol Res Perspect, 9 (2021) e00771.

4. A. Spek, B. Li, B. Rutz, A. Ciotkowska, R. Huang, **Y. Liu**, R. Wang, F. Strittmatter, R. Waidelich, C.G. Stief, M. Hennenberg, Purinergic smooth muscle contractions in the human prostate: estimation of relevance and characterization of different agonists, Naunyn-Schmiedeberg's archives of pharmacology, 394 (2021) 1113-1131.