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Effects of ADP ribosylation factor 6 (ARF6) inhibition by NAV2729 or RNA interference in human prostate stromal cells

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Table of content

1. Introduction

1.1 Definitions

Lower urinary tract symptoms (LUTS) include a panel of symptoms involving the urethra, the bladder, and in men, the prostate.

Conventionally, LUTS consist of storage, voiding, and post-micturition symptoms (Table 1) (Abrams 2002, Gratzke, Bachmann et al. 2015).

Table1: Classification, symptoms and characteristics of LUTS.

LUTS		
Voiding (obstructive) symptoms	**Storage (irritative) symptoms**	**Post-micturition symptoms**
Weak stream	Increased daytime frequency	Feeling of incomplete emptying
Splitting or spraying	Nocturia	Post- micturition dribbling
Intermittent stream (Intermittency)	Urgency	
Hesitancy	Urinary incontinence	
Straining		
Terminal Dribbling		

Nearly half of the men suffering from benign prostatic hyperplasia (BPH) show enlarged prostate glands, known as benign prostatic enlargement (BPE), from which half could develop bladder outlet obstruction (BOO) (Hollingsworth and Wilt 2014). Changes in prostate smooth muscle contractility, and increased prostate size often accompany LUTS/BPH (McVary, Roehrborn et al. 2011).

Even though surgery, excessive fluid intake, urinary tract infections, or anticholinergic drugs can trigger acute urinary retention (AUR), AUR is most commonly caused by LUTS/BPH and represents a major complication that requires hospitalization. Other BPH complications are in parts related to complications of chronic urinary retention, including recurrent urinary tract infections, hematuria, male erectile dysfunction, formation of bladder stones, and damages in the bladder tissues or kidneys (Speakman and Cheng 2014).

The condition of LUTS is mostly quantified by quantitative symptom scores. The most commonly used indicator to evaluate symptom severity is the *International Prostate Symptom Score* (IPSS), which is an index assessed by internationally standardized questionnaires (Barry and O'Leary 1995). As a diagnostic tool, IPSS subjectively quantifies the degree of symptoms in LUTS. The application of IPSS is recommended for men with LUTS during their diagnosis and treatment evaluation (Gravas, Cornu et al. 2019).

Another diagnostic tool used in diagnosing BPH is uroflowmetry, a simple and non-invasive tool to assess impaired voiding in BOO. Uroflowmetry determines the volume of urine passed per unit of time. The most important result from the uroflowmetry is the maximal urinary flow rate (Q_{max}), which is relevant in the context of BOO (Ather and Memon 1998).

According to studies based on population, with the increase of patients' age, the prevalence of moderate-to-severe LUTS increases, and patients' Q_{max} decreases (Jacobsen, Girman et al. 2001).

1.2 Epidemiology

Epidemiologic data point to a high prevalence of LUTS, with no significant cultural variation (Litman and McKinlay 2007).

LUTS are among the most commonly seen clinical complaints in aging men (Martin, Haren et al. 2011). The prevalence increases with proceeding age, and severe LUTS are most common in older men (Boyle, Robertson et al. 2003). Around one-fifth of male adults report moderate-to-severe LUTS, which include urinary voiding and storage problems. (Maserejian, Chen et al. 2013).

LUTS prevalence has been shown to increase over time. An estimation extrapolated that 1.9 billion people in the world were affected by LUTS in 2008, and the number turns into nearly 2.3 billion until 2018, from which the number of patients suffering from voiding symptoms of LUTS in 2008 (917 million) is estimated to increase by 18.5% by 2018 (Irwin, Kopp et al. 2011). In another report, the number of male patients in the US alone having LUTS is estimated to reach 11 million by 2030 (Jacobsen, Girman et al. 1995), bringing huge economic costs of LUTS treatment.

However, it was found that the prevalence of routine prescription medication for patients with LUTS is low, nearly 90% of them had no medication usage (Kupelian, Wei et al. 2006), reflecting that the patients' access to medical care remains to be a problem.

1.3 Etiology

1.3.1 Voiding symptoms of BPH

In the strict sense, BPH is a histological diagnosis occurring with and without increases in prostate volume, but often includes an overgrowth in the transition

zone, resulting in the progressive formation of obstructive LUTS (Strand, Costa et al. 2017). The most reported reason associated with LUTS in men is BPH, which is closely related to age. LUTS due to BPH are related to BOO and benign prostatic obstruction (BPO), in which benign prostatic enlargement (BPE) resulting from BPH plays a role (Abrams 2002, Kupelian, Wei et al. 2006). It was reported that the risk of having moderate to severe LUTS and having significant BOO could increase by 5 times, and 3 times respectively, once the men's prostate volumes are higher than 50 cm^3 (Lepor 2004).

Changes in prostate smooth muscle contractility, as well as bladder outlet obstruction due to the enlargement of the prostate, could contribute to LUTS (Roehrborn 2008). Factors related to urethral compression might be involved in the pathophysiology of LUTS. Increased prostate smooth muscle contractility, enlargement of the prostate, which mainly associates with the increased number of epithelial and stromal cells due to increased cell proliferation and decreased cell death, are all involved (Roehrborn 2008). The etiology behind the hyperplasia include factors such as hormones, stem cells, growth factors, fibrosis, and autoimmunity (Strand, Costa et al. 2017). Consequently, both processes are targets in the medical management of LUTS/BPH (Oelke, Bachmann et al. 2013, Hennenberg, Stief et al. 2014). Thus, and as explained below in detail, α_1-adrenoceptor antagonists or phosphodiesterase-5 inhibitors may result in rapid relief of symptoms, by relaxing smooth muscle of the prostate (Oelke, Bachmann et al. 2013, Hennenberg, Stief et al. 2014). 5α-reductase inhibitors in turn, may inhibit prostate growth in BPH, resulting in reduction in prostate volume, and are used to prevent progression and complications (Oelke, Bachmann et al. 2013).

However, epidemiological data showed that women are also highly vulnerable to LUTS (Boyle, Robertson et al. 2003), as the prevalence of LUTS among women is as high as in men (Coyne, Sexton et al. 2009). Thus, the cause-effect relationship between prostatic overgrowth, BOO, and LUTS has been doubted due to the uncertainty of correlation between BPE and LUTS (Abrams, Cardozo et al. 2003). It is now believed that in addition to BPH and BPE, other elements are also related to LUTS (Abrams 1994). LUTS should be considered as symptoms with multifactorial etiology, in which disorders occur functionally or structurally in urological or non-urological systems (Bergdahl, Aus et al. 1998, Drake 2014, He, Wang et al. 2016).

1.3.2 Detrusor dysfunctions

Detrusor underactivity (DU) often causes symptoms like feeling of incomplete bladder emptying, sensations of residual urine after voiding, and hesitancy, which are very similar to BPH-related voiding symptoms. It is related to decreased strength and/or duration of the detrusor, leading to extended bladder emptying and/or incapable of completely emptying the bladder in a normal period of time (Abrams 2002). Lack or decreases of detrusor contractility, or of dysfunctions in the innervation of detrusor muscle could account for the etiology of these symptoms (Cucchi, Quaglini et al. 2007). Ageing, diabetes mellitus, BOO, pelvic surgery, and neurologic disabilities could all be potential risk factors of DU (Yu and Jeong 2017).

Detrusor overactivity (DO) is a urodynamic dysfunction with involuntary detrusor contractions during the filling phase which may be spontaneous or uncontrolled (Abrams 2002). Detrusor overactivity is involved in the pathological progression of storage LUTS. It could be either “myogenic” or

"neurogenic" (Peyronnet, Mironska et al. 2019). Non-neurogenic factors include chronic bladder irritation, aging of the bladder, and BOO. Neurogenic factors may include cortical inhibition dysfunctions, neurodegenerative disorders, and damage of the spinal cord (Hampel, Gillitzer et al. 2003). DO was traditionally regarded as the cause of overactive bladder (OAB), while after urodynamic analyses, no evidence of DO could be concluded in OAB patients, implying the possibility that other mechanisms, such as a role of urothelium or suburothelium and bladder afferent signaling, the interaction of urethra and OAB, some non-urological disorders like metabolic syndrome, sex hormone deficiency and subclinical autonomic nervous system dysfunctions (Peyronnet, Mironska et al. 2019) might be involved.

1.3.3 Inflammation

Increasing evidence shows that inflammation may play a role in the BPH pathophysiology as well as for the progression of LUTS. In prostate biopsy samples and tissue from prostatic resection of surgery for BPH, chronic and acute inflammation were commonly reported (Kohnen and Drach 1979, Gerstenbluth, Seftel et al. 2002, Di Silverio, Gentile et al. 2003, Anim, Kehinde et al. 2006). A study (Nickel, Roehrborn et al. 2007) found that prostatic inflammation and prostatitis symptoms are related. They found men whose biopsy specimens showed signs of acute inflammation have a higher chance of developing worsening of LUTS compared to those with no signs of acute inflammation. In the urothelium, chronic bacterial infection may also play a role in the etiology (Sorrentino, Cartwright et al. 2015). Symptoms like bladder pain, urgency, and nocturia were all bacteriuria-related, E. coli, Enterococcus, and other coliforms are the organisms most commonly detected (Sorrentino, Cartwright et al. 2015).

Other studies have found different levels of associations between having prostatic inflammation and AUR. In cross-sectional studies, after examining prostatic specimens, AUR patients have a higher chance of having evidence of inflammation compared to men with BPO (Tuncel, Uzun et al. 2005). This study found a relevance between inflammation in the prostate and AUR regardless of prostate volume (Kefi, Koseoglu et al. 2006). A study showed that prostatitis was a more vulnerable LUTS factor in men whose prostate volumes are less than 50 ml. The volume itself was a more significant risk factor than prostatitis when the prostate volume is more than 50 ml (Chung, Yu et al. 2012).

Other organs of the urinary tract, like the bladder, also show association to the inflammation process, which contributes to the LUTS both in the presence and in the absence of prostate enlargement (Sauver and Jacobsen 2008). Besides, the C-reactive protein, a non-specific biomarker of the presence of inflammation in the serum, is correlated with LUTS in men and women (Hsiao, Lin et al. 2012, Kim, Doo et al. 2015).

The mechanisms of how inflammation is leading to the growth of the prostate are still not very clear. However, the process of inflammation may contribute to prostatic enlargement by inducing tissue damage and chronic repairment, causing persistent stimulation of prostatic cells and the subsequent hyperplasia, or through decreasing prostatic apoptosis (Sauver and Jacobsen 2008, Morgia and Russo 2018).

These findings suggest that inflammation in both men and women might be involved in the presence and progression of LUTS.

1.3.4 Urinary tract stones

Urinary stones are hard masses formed inside the urinary tract and may lead to LUTS (Rao, Preminger et al. 2011). Hyperparathyroidism, dehydration, renal tubular acidosis, high animal-source protein or vitamin C consumption, and lack of water or calcium intake are all factors that could lead to urinary stones. As reported, 22% of all kinds of urinary stones are ureteric stones, and around 68% of the ureteric stones are distal ureteric stones (Jayant, Agrawal et al. 2014). Patients suffering from renal and ureteral stones, especially in the distal ureter, could potentially be accompanied by LUTS (Jiang and Kuo 2014).

1.3.5 Other factors of BOO

Other urinary tract problems related to urinary tract surrounding tissues are bladder cancer, prostate cancer, urethral stricture, and hypertrophy of the bladder neck. These dysfunctions may also cause LUTS (Lepor 1998, Ficarra, Rossanese et al. 2014, He, Wang et al. 2016).

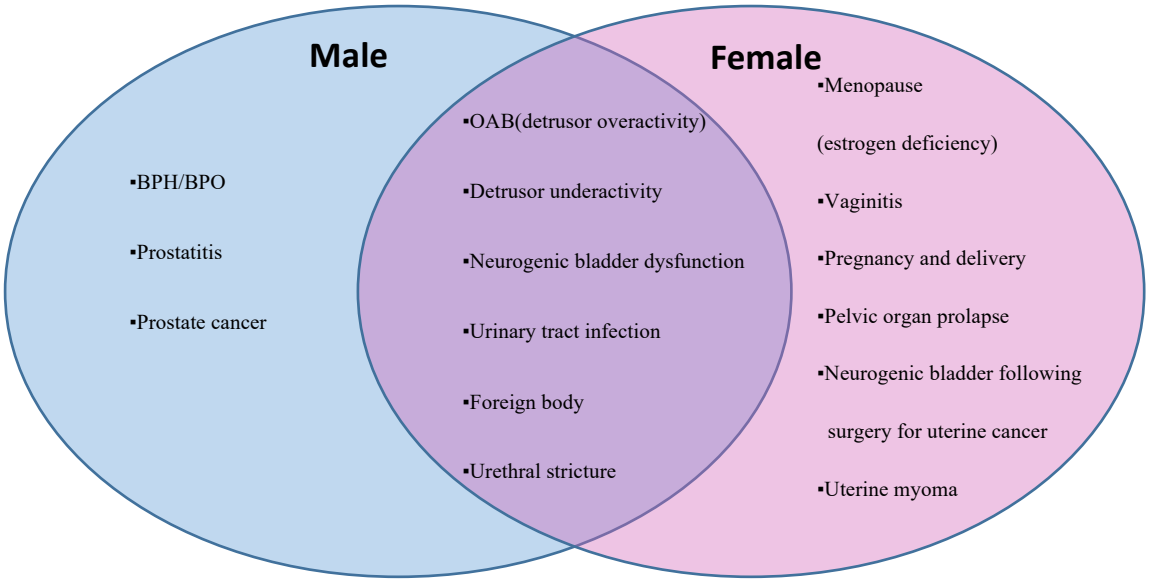


Figure 1: Causes of female and male LUTS (Gratzke, Bachmann et al. 2015, Takahashi, Takei et al. 2016).

1.4 Risk factors

1.4.1 Aging

Age is the most correlative factor that causes LUTS (Lepor 2004). In terms of prevalence, the precise estimates vary due to different definitions and cohorts, but the overall prevalence of LUTS increases clearly with age (not differed by sex or race/ethnicity). Symptoms such as weak stream, intermittency, urgency, nocturia, and incomplete emptying have the closest correlation with age (Chute, Panser et al. 1993).

No observations of histologic BPH have been reported in men under the age of 30 (Lepor 2004). However, almost half of the men exhibit histologic evidence of BPH by their 60s, and this chance increased up to 90% by their 90s (Lepor 2004). Pieces of evidence are showing that the prevalence of histologic BPH is similar around the world (Roehrborn and JD 2002).

For most men suffering from LUTS/BPH without treatment, symptoms will continue to progress and significantly worsen with increasing age (Lee, Garraway et al. 1998).

1.4.2 Metabolic Factors

Preclinical and clinical studies have found that metabolic aberrations such as metabolic syndrome, obesity, dyslipidemia, and diabetes together with their associated comorbidities like sex steroid alterations and inflammation are playing a role in the development and progression of LUTS/BPH (Sebastianelli and Gacci 2018).

The prostate volume was found to be associated with several metabolic factors in a prospective clinical study (Gacci, Sebastianelli et al. 2017). As reported, different metabolic features were linked to certain corresponding diameters of the prostate. For example, the craniocaudal diameter, the anteroposterior diameter, and the laterolateral diameter were found to be linked to high triglycerides, low high-density lipoprotein (HDL) cholesterol, and high systolic blood pressure, respectively. Obesity and prostate volume are also found to be related (Parsons, Carter et al. 2006), as reported, every 1 kg/m^2 increase in body mass index (BMI) is accompanied by a 0.4 ml increase in prostate volume. Compared to non-obese men, obese (BMI >35 kg/m^2) are 3.5 times more likely of having prostate enlargement compared to non-obese (BMI < 25 kg/m^2) ones.

Basically, the existing evidence of the relationship between metabolic aberrations and LUTS is related to the BPH and BPE. Besides, it also has been confirmed that increased annual BPE growth rate and sympathetic activity are related to metabolic syndromes (Oelke, Baard et al. 2008, Soler, Andersson et al. 2013).

The exact mechanisms behind these findings are not well understood, but it is believed that up-regulated sympathetic activity, inflammatory conditions, and pelvic ischemia, might be involved (Soler, Andersson et al. 2013, Sebastianelli and Gacci 2018).

1.4.3 Other risk factors

There are risk factors like genetic reasons or childhood nocturnal enuresis which might be related to LUTS (Coyne, Sexton et al. 2009, Parsons 2010). Illnesses like arthritis, asthma, chronic anxiety, depression, diabetes, heart disease, irritable bowel syndrome, neurological conditions, sleep disorders, and vitamin D deficiency are reported to cause LUTS as a comorbid syndrome (Coyne, Sexton et al. 2009). The multifactorial etiology of LUT suggests that LUTS are broadly related to other diseases and conditions, and can also act in a genetic-environment way.

1.5 The role of BPH in LUTS

LUTS can be caused by multifactorial reasons, but BPH remains one of the most crucial factors. The BOO due to prostatic enlargement has often been considered as the cause of pathophysiology of LUTS in men (Shapiro and Lepor 1995). Many voiding symptoms like intermitted stream, terminal dribbling, incomplete voiding, or even urinary retention could be attributed to BPH, which is responsible for BPO and BOO (Abrams 2002, Chapple 2011). Generally, the expansion of prostate size due to the increased growth and decreased apoptosis of prostate cells together with the increased prostate smooth muscle tone during aging could compress the urethra, and cause problems during bladder emptying

and urinary flow and lead to associated LUTS (Cioanta and Muschter 2000, Hennenberg, Stief et al. 2014, Oelke, Shinghal et al. 2015, Guneyli, Ward et al. 2017). In other words, the enlarged hyperplastic prostate is inducing BOO by dynamic and static mechanisms (Lepor 1989). The prostatic enlargement due to the up-regulated stromal proliferation is responsible for the static mechanism, whereas the prostate smooth muscle tone is involved in the dynamic mechanism. Both epithelial and stromal cell expansion are playing a role in the development of LUTS/BPH.

The degree of hyperplasia varies on a large scale. Stromal and epithelial cells contribute to around 80% and 20% of the hyperplastic volume of the prostate, respectively (Bartsch, Müller et al. 1979). Smooth-muscle cells make up a big portion of the stromal hyperplasia tissue (Shapiro, Becich et al. 1992), which is essential for increasing prostate smooth muscle tone and the following urethral compression or obstruction.

A study examined complete prostates from autopsy in 281 men at different ages found that on average, not only hyperplastic but also normal prostate volume increase with age, and the stromal volume increase played a more critical role (Arenas, Romo et al. 2001). Other studies also reported an increase of proliferation and decrease of apoptosis in the hyperplastic prostate epithelial cells compared to the normal prostate cells, implying that the increased epithelial cell densities in the prostate also account for the increased volume during aging (Berges, Vukanovic et al. 1995, Diez 1998).

So far, the mechanisms behind the up-regulated proliferation in stromal and epithelial cells of enlarged prostates remain not well understood. As a

multicellular organ, epithelia, stroma, and leukocytes may all get involved in BPH progression, making it harder to clarify the cellular and molecular mechanisms behind prostatic enlargement (Strand, Costa et al. 2017).

1.6 Burden of LUTS

With prolonged life expectancy and increasing health awareness, LUTS is causing more and more society and individual burdens (Taub and Wei 2006).

1.6.1 Burden for society

The high prevalence of LUTS requires an extensive health-care system and raises enormous socioeconomic costs (Speakman, Kirby et al. 2015). Expenses consist of two parts, including the costs for diagnosis and treatment, and the indirect costs related to lost working time and intangible costs under the influence of quality of life. Only the worldwide annual medical costs for LUTS/BPH alone were estimated to range from USD 3 to 10 billion, and perhaps higher in the future due to the increasing aged male population (Ventura, Oliver et al. 2011). Apart from the direct health care costs, daily productivity and activities are also seriously impaired. BPH affects around 50% of men in their working age (Wei, Calhoun et al. 2005). LUTS have a large economic relevance considering that the population is aging, and the prevalence of LUTS increases during aging. Therefore it is expected that the burden and costs of LUTS increase in the future (Kirby, Kirby et al. 2010).

1.6.2 Burden of individuals

In terms of individuals, LUTS negatively impacted the quality of life and health conditions, and the conditions of symptoms were related to bad health-related quality of life (Welch, Weinger et al. 2002).

Physical and mental well being

LUTS/BPH usually include a reduction in Q_{max}, and may lead to bothersome symptoms like inability to void, poor force of urine stream, and incomplete emptying (Roehrborn 2008).

In a cohort study with 5872 people involved, the risks of falling down and osteoporotic fractures were evaluated. The moderate to severe urinary symptoms increased the annual risk of at least one fall compared with mild symptoms, mainly due to urgency, urination initiation difficulties, and nocturia (Parsons, Mougey et al. 2009).

Sexual function is another aspect affected by LUTS. Aging can cause decreased sexual function, whereas, among the aged, sexuality is still highly demanded. Impaired sexual activity has been reported among men with LUTS, according to an Italian study (Mirone, Carone et al. 2017). Symptoms related to male sexual function like erectile dysfunction (Kaminetsky 2006), reduced quantity of ejaculate, and uncomfortable ejaculation (Schou, Holm et al. 1996, Höfner, Claes et al. 1999) are highly reported in LUTS patients.

Psychological factors related to LUTS have been addressed in several studies, among which several cross-sectional studies revealed the link between depression and LUTS (Asplund, Henriksson et al. 2004, Fitzgerald, Link et al.

2007, Coyne, Sexton et al. 2009, Wong, Woo et al. 2010). The molecular mechanism of causing depression by urological problems has also been studied. Pathogeneses involved between LUTS and depression include defect of serotonin synthesis, increased adrenergic tone, and the hypothalamic-pituitary axis (Laumann, Kang et al. 2008, Steers, Litman et al. 2008). Besides, inflammation was also found to be associated with the pathogenesis of major depression and LUTS (Miller, Maletic et al. 2009, Johnson, Abbasi et al. 2010).

Quality of sleep

The definition of nocturia by the International Continence Society (ICS) is explained as "the complaint that the individual has to wake at night one or more times to void." (Van Kerrebroeck, Abrams et al. 2002). Evidence showed that nocturia is one of the symptoms that have the strongest correlation with IPSS (Van Dijk, Wijkstra et al. 2010). Meanwhile, the prevalence of nocturia remains high among men with LUTS or BPH, and a cross-sectional study showed it could reach 83% who receive no treatment (Hernández, Estivill et al. 2008). In a questionnaire investigation, men suffering from nocturia declared a significantly lower nocturia-specific Quality of Life score than the non-nocturia group (Abraham, Hareendran et al. 2004). Insomnia prevalence is found to be higher in LUTS individuals when comparing with common people. A correlation between IPSS score and sleep quality was found (Marklund, Spångberg et al. 2010). Thus, poor sleep could be induced from all the above, mainly because of the sleep fragmentation and the subsequent lack of sleep (Middelkoop, Smilde-van den Doel et al. 1996).

Daily activities

To deal with their symptoms, people who have LUTS have to change their lifestyles and daily activities for avoiding symptoms and inconveniences, for

example, a large portion of the patients refuse to drink before going to sleep, and some also try not to go to places without bathrooms or to avoid long-distance traveling. Moreover, sometimes they escape relationships due to having LUTS (Scarpa 2001).

Impact on partners

LUTS are not only bringing discomfort to the patients themselves; partners are also bothered by men's symptoms. Though LUTS is not life-threatening, the situation would not leave the partners unaffected. Partner's main complaints including sleep disturbance, reduced sexual activity, and social life impairment, which caused bothersome problems in their partnerships (Mitropoulos, Anastasiou et al. 2002).

1.7 Treatment of LUTS

Drugs for LUTS treatment are among the most frequently prescribed medications for the elderly. A large variety of drugs and herbal preparations are available for treating LUTS.

α_1-Adrenoceptor antagonists ("α_1-blockers") and 5α-reductase inhibitors are the most commonly used medications for voiding symptoms (Caine, Pfau et al. 1976). Anticholinergic agents or the β_3-adrenoceptor agonist mirabegron are usually selected alone or together with α_1-blockers as a combination therapy to treat storage symptoms consistent with OAB (Roehrborn 2005, Tyagi, Tyagi et al. 2011). The detailed classifications of LUTS treatment are as follows.

1.7.1 α_1-Adrenergic blockers

Early reports have found the existence of a large amount of α_1-adrenergic receptors in the prostate stroma (Michel and Vrydag 2006). Evidence also showed α_1-adrenergic receptors in prostatic adenoma and variable results in the bladder neck region (Caine, Raz et al. 1975). Thus, it was suggested that stimulation of these receptors could cause increased smooth muscle tone in the prostate as well as bladder neck, leading to obstructive symptoms. In 1976, the nonselective α_1-antagonist phenoxybenzamine was first examined to improve symptoms of LUTS/BPH (Caine, Pfau et al. 1976).

Now it is clear that the α_1-adrenergic receptors are playing a major role in mediating the prostate smooth muscle tone. The up-regulated tone of the prostate smooth muscle could lead to an impairment in the urinary flow rate (i.e., obstruction) and aggravation of LUTS. The inhibition of these receptors has confirmed their benefits in improving urinary flow rates and LUTS (Caine, Perlberg et al. 1978). α_1A, α_1B, and α_1D are three subtypes of α_1-adrenergic receptors, α_1A is regarded as the most important one in the prostate, constituting approximately 80% according to ligand binding assays (Roehrborn 2005). Notably, not all the LUTS/BPH patients receiving α_1-blocker treatment have a positive response. Responder rates vary according to the standard that defines responses and the sample's eligibility. Considering tamsulosin, as an example, single doses of 0.4 mg were found to lead responder rates to 67% (placebo: 44%) in one study (Abrams, Schulman et al. 1995) and 66% (placebo: 49%) in another (Chapple, Wyndaele et al. 1996). Besides, by far, there is still no α_1-adrenergic receptor blocker which has a proven effect on reducing prostate volume or serum PSA.

Appropriate candidates treating LUTS/BPH include tamsulosin, terazosin, doxazosin, alfuzosin, and silodosin (Oelke, Bachmann et al. 2013). Effects of different α_1-blockers may vary in different patients. It is worth selecting an alternative α_1-blocker once the original one does not lead to a satisfying response.

Side effects of α_1-adrenergic blockers

When phenoxybenzamine was firstly introduced in treating LUTS/BPH, it showed side effects such as tiredness, dizziness, impaired ejaculation, and nasal congestion (Lepor 2007). The currently available α_1-adrenergic blockers, namely terazosin, doxazosin, tamsulosin and silodosin, presented similar adverse event spectrums, but among these four α_1-adrenergic blockers, dizziness, fatigue, and asthenia are more commonly seen in terazosin and doxazosin. However, tamsulosin and silodosin induce more ejaculatory dysfunctions like retrograde ejaculation (RE) (Roehrborn 2005). A study showed that 90% and 35% of patients had decreased ejaculate volume and anejaculation, respectively, after taking tamsulosin 0.8 mg /day (Hellstrom and Sikka 2006). Decreased blood pressure is the least common with silodosin and the most with doxazosin and terazosin. Thus, doxazosin and terazosin are usually taken at bedtime, and the dose should be adjusted according to efficacy and tolerance (Mobley, Feibus et al. 2015).

1.7.2 5α-reductase inhibitors

Testosterone can be converted to dihydrotestosterone (DHT) by 5α-reductase isoenzymes. Compared to testosterone, DHT is considerably far more potent in activating androgen receptor (AR). The functions of DHT include alterations of

the deoxyribonucleic acid, leading ultimately to such metabolic effects as protein synthesis, secretion, and growth of the prostate (Roehrborn 2005).

Finasteride and dutasteride are two 5α-reductase inhibitors that have been introduced in the early 1990th. Finasteride inhibits 5α-reductase type II, whereas dutasteride inhibits both type I and type II. However, clinical evidence did not show that inhibiting both subtypes is more beneficial than only type II inhibiting (Nickel 2004). After a duration of treatment within 6-12 months, finasteride and dutasteride could reduce prostate volume by 18-28% and serum PSA by around 50% (Naslund and Miner 2007). With long-term treatment, serum DHT concentration could decrease by nearly 70% with finasteride and 95% with dutasteride. Both 5-ARIs reduced the prostate DHT level by 85-90% (Gratzke, Bachmann et al. 2015).

5α-Reductase inhibitors (5ARIs) are recommended by the European Association of Urology (EAU), to be suitable for men with moderate-to-severe LUTS and an increased prostate volume (>40 ml), or to prevent progression or complications such as AUR, and the need for surgery (Oelke, Bachmann et al. 2013). They could lead to remission of symptoms after a treatment duration of 3-6 months.

Side effects of 5ARIs

It was reported that semen production is widely impaired among patients during 5ARI treatment (Cai, Fratianni et al. 1994, McConnell, Roehrborn et al. 2003). Reports about other side effects of treating LUTS/BPH patients with 5ARIs are rare. There is a slight chance (less than 5%) of patients who will suffer from side effects such as gynecomastia, tenderness, impotence, loss of libido, ejaculatory dysfunction (Tarter and Vaughan Jr 2006). In terms of gynecomastia or

tenderness, it is reported that they are more common in patients under dutasteride (3.5%) treatment compared to the finasteride (1.2%) (Carlin, Seftel et al. 1997).

Of note, in patients treated with 5ARIs, it was found that there is a slight increase in chance to result in more aggressive prostate cancer, which is with higher Gleason scores (>7), though both 5ARIs were found to decrease the overall incidence of prostate cancer (Thompson, Goodman et al. 2003). However, this potential risk of developing high-grade prostate cancer is still under considerable debate (Tarter and Vaughan Jr 2006, Schmitz-Dräger, Fischer et al. 2007).

1.7.3 Phosphodiesterase type-5 inhibitor therapy

Phosphodiesterase-5 (PDE5) inhibitors can prevent the cyclic guanosine monophosphate (cGMP) from being degraded by PDE5 (Hatzimouratidis and Hatzichristou 2007), thus increasing the intracellular cGMP concentration, and finally relaxing smooth muscle cells. During the past decade, PDE5 inhibitors were used as drugs for the treatment of erectile dysfunction. The PDE5 inhibitor-related relaxation of smooth muscle could occur in the urethra and prostate, bladder neck, or even the corpus cavernosum. Three trials in uncontrolled design revealed that when treating erectile dysfunction patients with sildenafil, a positive effect on LUTS/BPH also shows up, which proved its clinical benefit in LUTS/BPH (Sairam, Kulinskaya et al. 2002, Ying, Yao et al. 2004, Mulhall, Guhring et al. 2006).

There are randomized and placebo-controlled trials using sildenafil, vardenafil, and tadalafil. Their findings showed that compared to placebo, PDE5 inhibitors significantly improved IPSS (McVary, Monnig et al. 2007, McVary, Roehrborn et al. 2007, Roehrborn, McVary et al. 2008, Stief, Porst et al. 2008).

In vitro, organ-bath experiments confirmed the concentration-dependent smooth muscle relaxation effect of PDE5 inhibitors by measuring the contractility of the bladder, urethral, and prostate smooth muscle tissues (Tinel, Stelte-Ludwig et al. 2006).

Among all PDE5 inhibitors, tadalafil has the longest half-life of 17.5 h, with a drug efficacy duration of up to 36 h, allowing a once-a-day application for patients (Dong, Hao et al. 2013). Large scale clinical trials using tadalafil alone or together with α_1-blockers treating LUTS/BPH confirmed its effect on reduction of IPSS, improvement on the quality of life (QoL), which led to its approval for the treatment of voiding symptoms (Porst, Oelke et al. 2013, Mónica and De Nucci 2019).

1.7.4 Anticholinergic (Antimuscarinics) agents

Tolterodine, oxybutynin, fesoterodine, darifenacin, solifenacin and trospium are common anticholinergic drugs (Oelke, Bachmann et al. 2013). They act by a mechanism that blocks acetylcholine action on detrusor muscle which inhibits the contractility of smooth muscle. It has been shown that muscarinic receptors are expressed on bladder smooth muscle cells (Chess-Williams 2002). Anticholinergics are recommended as a primary choice for OAB storage symptoms, particularly urgency and frequency, when conservative options fail (Burkhard, Bosch et al. 2017).

Anticholinergic agents could achieve LUTS improvements such as increased QoL and reduced micturition frequency, urgency, and incontinence. Nocturia and

IPSS were also reduced though statistical significance was not reached (Abrams, Kaplan et al. 2002).

Regarding the different anticholinergic drugs, studies showed that their efficacy varies, indicating that solifenacin and fesoterodine may have advantages over tolterodine (Ginsberg, Schneider et al. 2013). However, EAU guidelines do not recommend a first-line antimuscarinic (Oelke, Bachmann et al. 2013).

Discontinuation rates could reach 90% among patients during anticholinergic agents treatment (Füllhase, Chapple et al. 2013). The most accepted reasons for abandoning this therapy could be either the lack of efficacy or the possibility of acute and chronic adverse events, such as xerostomia, constipation, and blurred vision (Chancellor, Migliaccio-Walle et al. 2013). Of note, several medications from the anticholinergic agent class can lead to side events related to central nervous system, due to their possibility of crossing the blood-brain barrier.

1.7.5 β_3-adrenoceptor agonists

Recently, β_3-adrenoceptor agonists opened a new horizon in treating storage symptoms caused by OAB, and they may emerge as a first-line medication option for these patients (Burkhard, Bosch et al. 2017).

β_3-adrenoceptor agonists can directly relax detrusor smooth muscle (Turner and Brading 1997). Mirabegron, a β_3-agonist, can lead to the detrusor smooth muscle

relaxation, and therefore improve bladder filling, and reduce storage symptoms including urgency, frequency, and nocturia (Mobley, Feibus et al. 2015).

The beneficial effects of mirabegron in the improvement of LUTS were confirmed in phase II and phase III clinical trials, when taking 25 mg or 50 mg daily (Herschorn, Barkin et al. 2013, Khullar, Amarenco et al. 2013). Systematic reviews and meta-analyses also concluded similar results (Kelleher, Hakimi et al. 2018).

Other advantages of mirabegron, especially over antimuscarinics, include reducing micturition frequency and urinary incontinence with just mild side effects, and no hypertension or cognitive changes were detected. Besides, there is no evidence showing that the use of mirabegron causes cardiovascular complications or diseases (Wagg, Staskin et al. 2018, White, Siddiqui et al. 2018, Chapple, Cruz et al. 2020).

Other β_3-adrenoceptor agonists like solabegron and vibegron are being developed and researched, or under trials studying their efficacy and safety.

1.7.6 Serotonin and norepinephrine reuptake inhibitors (SNRIs)

Other than relieving depression symptoms or anxiety disorders and nerve pain, one compound of the serotonin and norepinephrine reuptake inhibitor (SNRIs) class, duloxetine, was reported in some studies to be able to act as a pharmacologic treatment for stress urinary incontinence or mixed urinary

incontinence in women (Ghoniem, Van Leeuwen et al. 2005, Shamliyan, Kane et al. 2008). The most commonly seen side effects are nausea and vomiting, constipation, insomnia, dry mouth, dizziness, somnolence, and fatigue (Bump, Voss et al. 2008, Alan, Eren et al. 2014).

In terms of men, duloxetine was found to release incontinence in patients after prostatectomy. Meanwhile, duloxetine was found to shorten the time to release incontinence, but the final continent rate of the patients remains unchanged. (Alan, Eren et al. 2014).

In animal models, SNRIs demonstrated a substantial effect on the modulation of urination, revealing possibilities that SNRIs may effect through the central nervous system, acting with brain areas related to social and behavioral control of micturition (Redaelli, Ricatti et al. 2015).

1.7.7 Plant extracts

A great diversity of medications based on plant extracts, including single extract or combinations which come from more than one plant are available for LUTS treatment. Widely used plants products include *Cucurbita pepo* (pumpkin seeds), *Pygeum africanum* (bark of the African plum tree), *Hypoxis rooperi* (south African star grass), *Serenoa repens* (syn. sabal serrulata; saw palmetto), *Secale cereale* (rye pollen), and *Urtica dioica* (roots of the stinging nettle) (Dedhia and McVary 2008).

When treating LUTS with plants extracts, ß-sitosterol, fatty acids, phytosterols, and lectins are compounds that might play a role (Madersbacher, Berger et al. 2008). Anti-inflammatory, anti-androgenic, and oestrogenic effects were observed in vitro, but the exact mechanisms supporting the usage of plant extracts treating LUTS remain unclear (Levin and Das 2000, Buck 2004, Madersbacher, Berger et al. 2008).

Studies show the plants extracts are bringing benefits to LUTS, mainly by reducing IPSS (Chatelain, Autet et al. 1999, Fornara, Madersbacher et al. 2020).

1.7.8 Combination therapy

To reduce symptoms and prevent progression, and to deal with voiding plus storage symptoms together, it is reasonable to combine different medical agents in LUTS treatment since it is possible to take advantage of different mechanisms and to compensate for drawbacks of each drug.

α_1-blocker and 5α-reductase inhibitor

Since α_1-blockers and 5α-reductase inhibitors, the two main drug classes for the treatment of LUTS/BPH act by completely different mechanisms, and no contraindication of using both drugs together has been reported, and finally, considering the delay of remission of symptoms after a 5α-reductase inhibitor treatment could be 3-6 months, combination therapy of both drugs appears possible as they are complementary to each other in the treatment of LUTS. This combination therapy is advised by most (if not all) urological guidelines, including the American Urological Association (AUA) (Foster, Barry et al. 2018) and EAU (Gravas, Cornu et al. 2019). Of note, this combination therapy is

suitable for moderate-to-severe LUTS patients with an increased risk of disease progression (Gravas, Cornu et al. 2019).

α_1-blocker plus antimuscarinic

Due to the commonly seen coexistence of both voiding and storage symptoms (referred to as mixed LUTS) in men, combination therapy of α_1-blockers with antimuscarinic agents appears possible. There are two strategies of this combination therapy, the fixed combination and the add-on combination. In the fixed-dose combination therapy, fixed-dose, single-capsule combination of α_1-blockers and antimuscarinics are used, while in add-on therapy, antimuscarinics are added to α_1-blockers in patients with persistent storage symptoms. Several studies have reported the efficacy of both combination therapies for the treatment of OAB as well as voiding/BPH symptoms in men (MacDiarmid 2008, Nishizawa, Yamaguchi et al. 2011, Takeda, Nishizawa et al. 2013, van Kerrebroeck, Chapple et al. 2013, Drake, Sokol et al. 2016, Moss, Rezan et al. 2017).

As recommended by EAU guidelines, using both α_1-blockers and antimuscarinics simultaneously could be a choice for moderate-to-severe LUTS patients if either of the single medication is insufficient (Gravas, Cornu et al. 2019).

α_1-blocker and β_3-adrenoceptor agonist

Due to the limited effectiveness of α_1-blocker in monotherapy, men suffering from LUTS under α_1-blocker treatment may also receive additional β_3-adrenoceptor agonist treatment similar to antimuscarinics. Supported by data

showing that the β_3-adrenoceptor agonist mirabegron is effective and well-tolerated, it can be an option as an alternative treatment choice to replace antimuscarinics in combination therapy (Ichihara, Masumori et al. 2015, Wada, Iuchi et al. 2016).

However, these studies were not double-blinded or placebo-controlled. In fact, this combination is still poorly examined, but subject of current ongoing trials. Although there is evidence indicating that combination therapies increase efficacy, generally, combination therapies tend to be second or third-line pharmacological treatment. In addition, except for the side effects due to the properties of these two kinds of drugs, the disadvantages of combination therapy also include increased costs.

α_1-Blocker and PDE5 inhibitors (PDE5Is)

Recently, PDE5I in combination with α-blocker has been shown to be more effective than PDE5I alone treating erectile dysfunction with LUTS/BPH (Barragán-Arteaga and Reyes-Vallejo 2016). It was reported that 43.0% to 82.5% of the male LUTS patients also have erectile dysfunction, which made this combination promising (Rosen, Altwein et al. 2003). The PDE5I/α_1-blocker therapy showed its effect on the improvement of Q_{max}, it also brought benefits in reducing postvoid residual volume and detrusor pressure at maximum flow. Compared with PDE5I or α_1-blocker used alone, this combination therapy reduced IPSS significantly (Barragán-Arteaga and Reyes-Vallejo 2016). The mechanisms behind the better outcome with this combination therapy remain not yet fully defined. There are pieces of evidence that PDE5 inhibitors may act by increasing the effects of α_1-blockers on neurogenic contractions of the human prostate and bladder neck (Angulo, Cuevas et al. 2012).

1.7.9 Limitations and drawbacks of current therapies

Though α_1-blockers improve symptoms (IPSS) by 30-50%, and improve the Q_{max} by 20-40% (Oelke, Bachmann et al. 2013), generally, full improvements cannot be reached, and effects of placebo were also remarkable when treating male LUTS, showing a symptom (IPSS) improvement by 10–34%, and a Q_{max} increase up to 27% (Hennenberg, Stief et al. 2014). In addition, 30%-35% of the patients responding to α_1-blocker treatment only have a reduction in IPSS of less than 25%. As much as 69% of patients feel disappointed with α_1-blockers, and even with α_1-blocker and 5-ARI combination therapy, still 36-45% of the patients are unsatisfied (Füllhase, Chapple et al. 2013, Hennenberg 2021). As a result, high discontinuation rates can be seen, as reported, only 35% of patients insisted on their medication one year after the first α_1-blocker usage (Cindolo, Pirozzi et al. 2015, Cindolo, Pirozzi et al. 2015), leading to high BPH-related hospitalization and surgery rate (Cindolo, Pirozzi et al. 2015). In terms of 5-ARIs, 5-ARIs could not bring more improvements than placebo in patients with prostates volume < 40 ml (Boyle, Gould et al. 1996). Besides, a long onset time is needed till the 5-ARIs are bringing benefits of symptoms (usually requires three months) (Naslund and Miner 2007). Taken together, current therapies still have limitations, alternative options with higher efficacy and fewer limitations are of high demand.

1.7.10 Novel compounds for the pharmacotherapy of LUTS

To date, the efficacy and safety of available medical options have been thoroughly evaluated, especially the market-dominating α_1-blockers and 5α-reductase inhibitors. However, for numerous patients with severe symptoms, invasive and unpleasant surgeries are still essential (Ventura, Oliver et al. 2011).

In addition to the established options such as tamsulosin, finasteride, tadalafil, solifenacin, mirabegron, and their combination therapies, and as the ongoing basic and clinical research in the field of urology develops, there are new candidates of pharmacologic treatment emerging.

Novel α_1-blockers

Established α_1-blockers are mainly used for male LUTS, regardless of the existence of BPE. What draws major concerns are adverse effects on blood pressure, which may be related to the antagonism of α_1B-adrenoceptors in arterial vascular smooth muscles (Roehrborn and Schwinn 2004, Schwinn and Roehrborn 2008). Consequently, the latest introduced α_1-blockers show higher subtype-selectivity for α_1A- versus α_1B- or α_1D-adrenoceptors, which are especially preferable for elderly patients who are more vulnerable to cardiovascular risks and require higher tolerability (de Mey 1998).

RS-17053, an agent developed by Roche Bioscience (Palo Alto, CA, USA), demonstrated very high specificity to α_1A-adrenoceptor (Ford, Arredondo et al. 1996). However, in organ bath, norepinephrine-induced tissue contractions can only be inhibited by high concentrations of RS-17053, and it was indicated that different variants of the α_1A-adrenoceptor might exist in human lower urinary tract smooth muscle that are discriminated by RS-17053 (Marshall, Burt et al. 1996). Consequently, the compound did not proceed to applications.

Another α_1-blocker, L-771,688 (or SNAP 6383, Merck Research Laboratories), which has an even higher specificity over other adrenoceptors, showed inhibition of phenylephrine-induced human or animal prostate smooth muscle contractions. *In vivo* experiments suggested that L-771,688 effectively reduced the urethral pressure with only a slight influence on blood pressure (Chang, Chen et al. 2000). This evidence suggested that L-771,688 might be a good option for treating LUTS/BPH considering its high specificity and less cardiovascular-related effects. In addition, it dose-dependently improved peak urine flow rates and showed good tolerance in BPH patients (Marks, Curtis et al. 2000). Thus this selective α_1A-adrenoceptor antagonist appears to be a potential option in the treatment of BPH, but further studies are required.

Cetrorelix (luteinizing hormone-releasing hormone antagonist)

Previously presented evidence showed the luteinizing hormone-releasing hormone (LHRH) is related to the proliferation of prostate stromal cells and other hormone-dependent events. The existence of LHRH receptors in the human epithelial cell line BPH-1 and in human prostate tissue was also confirmed (Siejka, Schally et al. 2010, Rozsa, Nadji et al. 2011). Therefore, a role of LHRH in regulating prostate growth and causing voiding symptoms may be assumed.

The antagonist of LHRH, cetrorelix (SB-75), was found to function in multiple ways that may improve the management of BPH-related LUTS. On the one hand, in cultured cells, cetrorelix reduced the proliferation of glandular epithelial cells in response to growth factors. On the other hand, it lowers the circulating testosterone levels in vivo (Rick, Schally et al. 2011).

Based on the findings that inflammation, growth factors, and hormones could also possibly get involved in the progression of LUTS/BPH (Debruyne, Tzvetkov et al. 2010), it was suggested that the inhibition of LHRH receptors directly in the prostate or suppression of pro-inflammatory cytokines and growth factors in a transcriptional level could be part of the mechanisms (Rick, Schally et al. 2011). LHRH antagonists alone, or in combination with other agents could be an option in the context of treating LUTS/BPH.

Selective cannabinoid (CB) receptor agonists

The endocannabinoid system (ECS), which consists of the cannabinoid (CB) receptors, their ligands, and enzymes controlling the formation and turnover of endocannabinoids, have been proven to play a role in regulating the functions of the lower urinary tract. Besides, in human lower urinary tract tissues, the existence of ECS components (CB1, CB2, fatty acid amide hydrolase (FAAH)) has been proven by Western blot and immunohistochemistry (Bakali, Elliott et al. 2013, Castiglione, Benigni et al. 2014, Hedlund and Gratzke 2016).

It was reported that CP 55940, an agonist of CB1 and CB2 receptors, decreased nerve-induced contractions in human prostatic strips (Gratzke, Weinhold et al. 2010). Similarly, in rat models, the agonist WIN 55,212-2 induced inhibitory effects of neurogenic contractions of the smooth muscle through epithelial CB1 receptors (Tokanovic, Malone et al. 2007).

The CB agonists also showed effects in inhibiting neurogenic contractions of bladder tissues from patients or animals (Pertwee and Fernando 1996, Gratzke, Streng et al. 2009), indicating that CB receptor agonists could be potential

candidates for management of LUTS in both voiding and storage symptoms, but none of the selective CB receptor agonists have been studied in clinical settings so far.

GTPase-related agents

Monomeric GTPases are GTP-binding proteins involved in actin remodeling, gene expression, cell polarity, cell cycle, cell proliferation, and smooth muscle contraction. Since the small monomeric GTPase RhoA was found to promote vascular smooth muscle contraction in a manner that increases calcium sensitivity (Hirata, Kikuchi et al. 1992) and a similar role of RhoA was demonstrated in smooth muscles from the bladder (Rajasekaran, Wilkes et al. 2005) and prostate (Takahashi, Nishimura et al. 2007), several studies described the role of Rho-kinase activation in lower urinary tract smooth muscle contraction (Morelli, Vignozzi et al. 2007, Morelli, Filippi et al. 2009).

Numerous newly emerged findings suggest that monomeric non RhoA GTPases, such as Rac GTPases, cell division control protein 42 homologs (CDC42), Ras, Rap1b, and Rab GTPases, may play an essential role in smooth muscle contraction (Li, Wang et al. 2020).

1.8 Mechanisms of prostate smooth muscle contraction

Prostate smooth muscle contractions, as well as smooth muscle contractions in other organs, are all associated with the interaction of myosin with actin filaments. Three key factors are involved (Hennenberg, Stief et al. 2014).

(1) the phosphorylation of myosin light chains (MLC);

(2) polymerization of monomeric actin and formation to filaments;

(3) anchoring of actin filaments on the cell membrane.

The cytosolic free calcium concentration in the smooth muscle plays a crucial role in regulating smooth muscle contractility (Breemen and Saida 1989). Generally, increased Ca^{2+} concentration in the cytosol contributes to smooth muscle cell contraction. Once voltage-gated Ca^{2+} channels open, an influx of Ca^{2+} occurs, Ca^{2+} will bind to calmodulin, and finally, myosin light chain kinase (MLCK) becomes activated. Ca^{2+}/calmodulin-dependent MLCK induces MLC phosphorylation, and subsequent phosphorylation of MLC enables the myosin to bind to the actin filament, thus initiating the smooth muscle contraction (Hennenberg, Stief et al. 2014).

Some mediators can induce the contraction of prostate smooth muscle (Hennenberg, Stief et al. 2014). For example, α_1-adrenoceptors are transmembrane receptors, which are members of the G protein-coupled receptors family, they mediate physiological responses to noradrenaline. Among α_1-adrenoceptors, it was assumed that the α_1A subtype plays a major role in prostate smooth muscle contraction (Andersson, Lepor et al. 1997).

A conformational transformation of the receptor occurs once the ligand binds to it, and this process allows the receptor's interaction with the G protein

(Hennenberg, Stief et al. 2014). Thereafter, the interaction of the G protein and intracellular effector systems occurs (Somlyo and Somlyo 2000). In the inositol-1,4,5-triphosphate (IP_3)-activated, calcium-dependent pathway, phospholipase C (PLC) is activated by the G protein and hydrolyzes phosphatidylinositol 4,5-biphosphate (PIP_2) into DAG and IP_3. IP_3 binds to calcium channels on the endoplasmic reticulum and allows calcium release from the endoplasmic reticulum. This results in depolarization, and subsequent opening of voltage-gated Ca^{2+} channels on the membrane, and capacitative Ca^{2+} influx. The binding of Ca^{2+} and calmodulin will then activate MLCK and induce smooth muscle contraction as described above. Meanwhile, DAG activates PKC and causes cellular responses, including triggering contraction by inhibition of MLC phosphatase (Somlyo and Somlyo 2000).

In another pathway, calcium sensitivity is increased after the activation of RhoA/Rho-kinase and, in parallel and independently, of protein kinase C (Hennenberg, Stief et al. 2014). Ca^{2+} sensitization of smooth muscle contraction is the principal mechanism of smooth muscle contraction by inhibiting the smooth muscle myosin phosphatase (MLCP) that dephosphorylates the MLC in smooth muscle. The active form of the small GTPase RhoA activates the Rho-kinase and phosphorylates the regulatory subunit of MLCP, and inhibits its phosphatase activity, thus maintained the contractile state (Somlyo and Somlyo 2000).

Other than adrenoceptors, the non-adrenergic mediators endothelin-1 (ET-1) and thromboxane A_2 (TXA_2) are also involved in triggering prostate smooth muscle contraction (Strittmatter, Gratzke et al. 2011, Hennenberg, Miljak et al. 2013, Hennenberg, Acevedo et al. 2017, Yu, Gratzke et al. 2019). ET-1 or the TXA_2

analog U46619 both could induce concentration-dependent contractions of human prostate tissues (Yu, Gratzke et al. 2018).

Like α_1-adrenoceptor activation, ET-1 receptor stimulation also activates PLC and converts PIP_2 to IP_3 and DAG, thus elevating the concentration of intracellular calcium and participating in muscle contraction (Abdel-Latif and Zhang 1991).

Thromboxane A_2 (TXA_2) is an inflammatory mediator synthesized from prostaglandin H_2 (PGH_2) with the help of TXA_2 synthase (TXS). It functions by activating the TXA_2 receptor (TXA_2-R) (Nakahata 2008). A study performed in nonmalignant human prostate tissue confirmed the expression of TXA_2-R and TXS in the prostate stroma by Western blot and immunohistochemistry (Strittmatter, Gratzke et al. 2011). In organ bath experiments, the TXA_2 analog U46619 caused prostate smooth muscle contraction concentration-dependently, and it was thought that calcium- and Rho kinase-dependent signaling pathways, or other mechanisms might be involved (Strittmatter, Gratzke et al. 2011).

1.9 Possible role of ARF6

ADP-ribosylation factor (ARF) GTPases belong to the Ras superfamily of small GTPases. The ARF family consists of six ARF isoforms, ARF1-6. According to their structural properties, ARF isoforms consist of three classes, which are class I (ARF1, ARF2, and ARF3), class II (ARF4 and ARF5), and class III (ARF6). The class I ARFs mainly regulate plasma membrane transport (Donaldson 2003, D'Souza-Schorey and Chavrier 2006). So far, the precise functions of class II proteins remain unknown, but it is believed that class I together with II of ARFs

are involved in the secretion process pathway in the ER-Golgi system (Volpicelli-Daley, Li et al. 2005).

The main functions of ARF6 include regulation of vesicular trafficking, membrane lipids remodeling, and actin organization-related pathways (Van Acker, Tavernier et al. 2019). Guanosine diphosphate (GDP)-bound ARF6 is existing as an inactive form, whereas GTP-bound as an active form. GTPase-activating proteins (GAPs) like GIT1, ARAP2, and ACAP1, could promote the deactivation of ARF6 by accelerating hydrolyzation of GTPase-bound GTP (Yamauchi, Miura et al. 2017, Vitali, Girald-Berlingeri et al. 2019). Activation of ARF6 requires guanine nucleotide exchange factors (GEFs), which mediate the exchange of ARF6-bound GDP into GTP.

The involvement of ARF6 in increased proliferation in both malignant or nonmalignant cells has been proven (Hongu and Kanaho 2014, Bourmoum, Charles et al. 2016, Yamauchi, Miura et al. 2017). In addition, ARF6 is known to promote processes related to actin fiber formation and cytoskeletal organization, like cell migration, cell adhesion, and platelet activation (Urban, Quick et al. 2016, Duan, Zhang et al. 2018, Greig and Bulgakova 2020). Consequently, a role of ARF6 in a) proliferation of prostate cells, and b) actin-dependent prostate smooth muscle contraction appears possible.

2. Objective of this thesis

It is believed that both the increased prostate smooth muscle contractility and the prostate enlargement due to increased prostate cell proliferation could cause compression in the urinary tract, impairing urinary flow and leading to LUTS/BPH (Hennenberg, Stief et al. 2014).

α_1-Blockers are widely used for LUTS/BPH treatment by relaxing prostate smooth muscle tone together with subsequent improvement of bladder emptying, because activated α_1-adrenoceptors lead to contraction of prostate smooth muscle cells (Caine, Raz et al. 1975). In turn, 5α-reductase inhibitors inhibit prostate growth by reducing serum and intraprostatic concentrations of DHT, leading to an involution of prostate tissue (Steers 2001, Kim, Brockman et al. 2018).

These established medications have insufficient overall efficacy, which may cause high discontinuation rates of patients. In addition, the current medications intend to target inhibition of prostate smooth muscle contraction or prostate growth separately. In fact, connections between both processes have been poorly understood to date. Moreover, growing evidence is showing that non-adrenergic factors are involved in prostate contractions in parallel to α_1-adrenoceptors. As a result, these non-adrenergic contractions could keep a compressed status of the urethra regardless of the usage of α_1-blockers, and may explain the limitations and the existence of non-responders to the treatment of α_1-blockers (Hennenberg, Acevedo et al. 2017).

These pieces of evidence suggest that it is of imperative demand to develop novel strategies to aim at prostate contractions and prostate growth at the same time, to attain drugs with increased efficacy and fewer non-responders.

The possibility that ARF6 might enhance the human prostate smooth muscle contraction has been proposed recently, based on evidence that the cytohesin inhibitor secinH3 inhibited ARF6 activity and contraction of human prostate tissues, and impaired actin organization in cultured prostate stromal cells (Herlemann, Keller et al. 2018).

Together with the proven function that ARF6 is promoting cell proliferation in several cell lines, ARF6 inhibition in reducing prostate smooth muscle contraction and prostate growth at the same time appears possible, but remains to be proven. Recently, a small molecule inhibitor NAV2729 which was assumed to be specific for ARF6, has become available (Yoo, Shi et al. 2016, Benabdi, Peurois et al. 2017, Yamauchi, Miura et al. 2017).

Consequently, the effects of NAV2729 were here examined in a cultured prostate stromal cell line. Furthermore, to assess the effects of ARF6 silencing in mRNA level, ARF6-specific siRNAs were used. To understand the possible limited efficacy regarding the specificity of NAV2729, the effects of NAV2729 were also assessed in prostate stromal cells with silenced ARF6 expression.

The purpose of this thesis was to examine a possible role of ARF6 in prostate smooth muscle contraction and stroma proliferation. Detailed questions were as follows:

● Does the ARF6 inhibitor NAV2729 inhibit proliferation in prostate stromal cells?

● Does the ARF6 inhibitor NAV2729 impair the actin organization in prostate stromal cells?

● Does the ARF6 inhibitor NAV2729 increase apoptosis of prostate stromal cells?

● Does the ARF6 inhibitor NAV2729 inhibit the contraction of prostate stromal cells?

● Does the ARF6 inhibitor NAV2729 reduce the activity of ARF6 protein in prostate stromal cells?

● Can ARF6 silencing mimic the effect of ARF6 inhibitor NAV2729 in prostate stromal cells?

● Is NAV2729 specific for ARF6?

3. Materials and methods

3.1 Reagents and devices

Table 2: Reagents used in this thesis.

Products	Manufacturer
RPMI 1640	Gibco, USA
penicillin/streptomycin	Gibco, USA
10% fetal calf serum (FCS)	Gibco, USA
Phosphate-buffered saline (PBS)	Gibco, USA
Trypsin-EDTA	Thermo Fisher Scientific, USA
NAV2729	Tocris, UK
Dimethyl sulfoxide (DMSO)	Roth, Germany
Cell counting kit 8 (CCK8)	Sigma-Aldrich, Germany
Silencer Select ARF6 siRNA	Thermo Fisher Scientific, USA
Silencer Select scramble control	Thermo Fisher Scientific, USA
Human Prostate Stromal Cell (PrSC) Avalanche™ Transfection Reagent	EZ biosystems
Roti®-Histofix	Roth, Germany
Triton™ X-100	Sigma-Aldrich, Germany
BSA	Gibco, USA
EdU-Click 555 cell proliferation assay	Baseclick, Germany
Phalloidin	Sigma-Aldrich, Germany
4',6'-diamidino-2-phenylindole-dihydrochloride (DAPI)	Invitrogen, USA
Mounting solution	Thermo Fisher Scientific, USA
RNeasy Mini kit	QIAGEN, Germany
Reverse Transcription buffer 10X	Promega, USA
dNTP Mix	Promega, USA
Random Primers	Promega, USA
AMV Reverse Transcriptase	Promega, USA
RNase Inhibitor	Promega, USA
RNase Free Water	Promega, USA
SYBR™ Green	Roche, USA
GAPDH (NM_002046)	QIAGEN, Germany
ARF6 Primer (NM_001663)	QIAGEN, Germany
mouse monoclonal anti ARF6 antibody (sc-7971)	Santa Cruz Biotechnology, USA
mouse monoclonal anti β-actin antibody (sc-47778)	Santa Cruz Biotechnology, USA
rabbit anti phospho-myosin light chain 2	Cell Signaling, USA
rabbit anti-myosin light chain 2	Cell Signaling, USA
rabbit anti-phospho-MYPT1	Cell Signaling, USA

rabbit anti-MYPT1	Cell Signaling, USA
secondary biotinylated horse anti-mouse IgG	Vector Laboratories, CA
ARF6 pull-down assay kit BK033	Cytoskeleton, USA
ARF1 pull-down assay kit BK032	Cytoskeleton, USA
RAC1 pull-down assay kit BK035	Cytoskeleton, USA
RhoA pull-down assay kit BK036	Cytoskeleton, USA
Protease inhibitor	Thermo Fisher Scientific, USA
radioimmunoprecipitation assay (RIPA) buffer	Sigma-Aldrich, Germany
Dc-Assay protein concentration kit	Bio-Rad, Germany
SDS sample buffer	Roth, Karlsruhe, Germany
Milk powder	Roth, Germany
Dithiothreitol (DTT)	Sigma-Aldrich, Germany
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich, Germany
Tris base	Sigma-Aldrich, Germany
Polyvinylidene-Fluoride (PVDF)	Sigma-Aldrich, Germany
CytoSelect™ 24-Well Cell Contraction Assay Kit (Floating Matrix Model)	Cell Biolabs, USA
Trichloroacetic acid (TCA) solution	Sigma-Aldrich, Germany
Sulforhodamine B sodium salt (SRB)	Sigma-Aldrich, Germany
Acetic acid	Roth, Germany

Table 3: Devices and software used in this thesis.

Products	Manufacturer
Cell culture incubator	Thermo Fisher Scientific, USA
Waterbath	Thermo Fisher Scientific, USA
Lab-Tek chamber slides	Thermo Fisher Scientific, USA
Laser scanning confocal microscope	Leica SP2, Germany
Light Cycler PRC system	Roche, Switzerland
Gel electrophoresis system	Bio-rad, Germany
Protran® nitrocellulose membranes	Schleicher&Schuell, Germany
Chemiluminescence (ECL) Hyperfilm	GE Healthcare, Germany
Film developers	Kodak, USA
ImageJ software	National Institutes of Health, USA
SPSS version 20	IBM SPSS Statistics, USA

3.2 Cell culture

3.2.1 Cell line

Experiments were performed using WPMY-1 cells (ATCC Manassas, VA, USA), a non-malignant cell line derived from prostate stromal tissue from a 54-year male (Webber, Trakul et al. 1999). Smooth muscle cells are the predominant cell type in the typical prostate stroma composition, and it has been reported that WPMY-1 cells represented characteristics of prostate smooth muscle cells, as they express vimentin, α-smooth muscle actin (α-SMA), calponin (Webber, Trakul et al. 1999, Li, Xu et al. 2019) and α_1A-adrenoceptors (Wang, Gratzke et al. 2016).

3.2.2 Cell thawing

Steps for cell thawing were performed according to the following protocol.

1. The cells from liquid nitrogen were quickly thawed at 37 °C using a water bath.

2. Thawed cells were diluted with pre-warmed Roswell Park Memorial Institute (RPMI) 1640 (Gibco, Carlsbad, CA, USA) culture medium with 10% FCS (Gibco, Carlsbad, CA, USA) and 1% penicillin/streptomycin (Gibco, Carlsbad, CA, USA) in T75 flasks.

3. Flasks were put into an incubator (Thermo Fisher Scientific, USA) in a 37 °C, 5% CO_2 environment.

3.2.3 Cell passaging and cryopreservation

Steps for cell passaging and cryopreservation were performed according to the following protocol.

1. Removal of the medium in the flasks by aspiration.

2. Cells inside the flasks were washed with 37 °C prewarmed PBS (Gibco, USA) two times.

3. 1-2 ml Trypsin-EDTA (Thermo Fisher Scientific, USA) was dispensed into each flask to cover the cells completely.

4. After incubation at 37 °C for 2 min and subsequent detaching of the cells from the surface, 10 ml RPMI 1640 with 10% FCS was added to the flasks followed by resuspension for blocking the digestion.

5. Centrifugation was applied to this medium to harvest cell pellets, followed by resuspension of the pellets with new culture medium. Finally, the cells were transferred to new flasks.

6. For cell cryopreservation, the resuspended cells were mixed with freezing medium to reach a final concentration of 10% DMSO, 40% FCS, and 50% cell suspension, then the cells were stored in cryotubes in -80 °C for short-term or liquid nitrogen for long-term storage.

3.3 siRNA Transfection

Prostate stromal cell (PrSC) AvalancheTM transfection reagent (EZ Biosystems, College Park, MD) was used to transfect WPMY-1 cells with the siRNA. Cells were cultured in antibiotic-free medium 24 h prior to transfection, and 70% confluence of the cells should be reached on the day of transfection. RT-PCR, Western blot or any other experiments were all performed at least 72 h after transfection. To examine the effects of NAV2729 on ARF6-silenced cells, NAV2729 was applied 48 h following transfection. Cell counting kit 8 (CCK8) and 5-Ethynyl-deoxyuridine (EdU) were conducted after an additional 24 h.

Specific steps for transfection were performed according to the following protocol.

1. Dilution of the ARF6 siRNA (Silencer Select ARF6 siRNA, sequence AGACGGUGACUUACAAAAAtt), or Silencer Select scramble control siRNA (sequence not published) into OptiMEM® to a final concentration of 50 nM, followed by vortexing.

2. For the creation of a transfection master mix, transfection reagent was added to the OptiMEM®-diluted siRNA in a volume ratio of 1:40, and immediately vortexed for 10 seconds.

3. Incubation at room temperature for 15 min.

4. The transfection mixture was added into each corresponding well or flask. In every 1 ml medium, 100 µl transfection master mix should be added.

5. Plates or flasks were gently shaken, followed by centrifugation for 5 min at 300 g.

6. Plates or flasks were put back to the incubator at 37 °C, 5% CO_2 for 5 h.

7. Removal of the medium containing the transfection reagent, 37 °C pre-warmed fresh medium was then added. Another 72 h incubation was performed before further analyses.

3.4 Real time polymerase chain reaction (RT-PCR)

3.4.1 RNA extraction and quantification

Steps for RNA extraction and quantification were conducted using the following protocol.

1. Cells were prepared in 6 well plates for RNA extraction. When extracting the RNA, there should be around 1 x 10^6 cells in each well.

2. The medium was discarded, the wells were washed with PBS once, followed by addition of buffer RLT (600 µl) in each well to disrupt the cells.

3. Cells were resuspended in the buffer, and the lysate was added into corresponding microcentrifuge tubes.

4. The lysate was transferred into QIAshredder spin columns placed in 2 ml collection tubes, the tubes were centrifuged for 2 min at 12000 g. The flow-through was discarded.

5. 700 µl of the buffer RW1 was added to the spin columns placed in new 2 ml collection tubes, centrifugation was applied at 12000 g for 15 s, the flow-through was discarded.

6. Step 5 was repeated once again.

7. 500 µl Buffer RPE was added to the spin columns, followed by centrifugation for 15s at 12000 g, the flow-through was discarded.

8. Step 7 was repeated once again, but centrifugation was applied for 2 min at 12000 g instead of 15 s at 12000 g.

9. Collection tubes together with the liquid inside were discarded, spin columns were placed in new 2 ml collection tubes. Finally, 30 µl nuclease-free water was added right in the middle of the membrane inside the spin column, followed by centrifugation for 1 min at 12000 g, the spin columns were discarded and the isolated RNA was inside the collection tubes.

10. Quantification was performed by using a nanodrop spectrophotometer.

3.4.2 Reverse Transcription

Steps for the reverse transcription were performed according to the following protocol.

1. 1 µg total RNA was diluted in 10 µl nuclease-free water in a microcentrifuge tube from each sample, the microcentrifuge tube was placed at 70 °C for 10 min.

2. The tube was centrifuged briefly to get rid of the bubbles, and then placed on ice.

3. For each RNA sample, the reverse transcription mix using a kit (Promega, Madison, WI, USA) was prepared (Table 4).

Table 4: Composition of reverse transcription mix

Component	Amount
$MgCl_2$, 25 mM	4 µl
Reverse Transcription 10X Buffer	2 µl
dNTP Mixture,10 mM	2 µl
Recombinant RNasin® Ribonuclease Inhibitor	0.5 µl
AMV Reverse Transcriptase (23 U/) µl	0.65 µl
Random primers	0.5 µl
Total RNA	10 µl (1 µg)
Nuclease-Free Water	To a final volume of 20 µl

4. The mixture was incubated for 10 min at room temperature, then it was incubated at 42 °C for 1 h for reverse transcription.

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

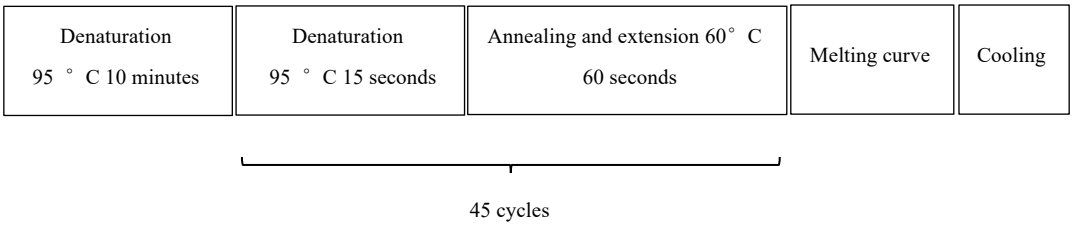
The primers provided by Qiagen (Hilden, Germany) are based on the RefSeq accession numbers, NM_001663 for ARF6, NM_002417 for Ki-67, and NM_002046 for GAPDH. The reactions were performed with Roche Light Cycler (Roche, Basel, Switzerland) using LightCycler FastStart DNA MasterPlus SYBR Green (Roche, Basel, Switzerland). Each reaction was conducted in a reaction mix (10 µl system, Table 5).

Table 5: Composition of RT-PCR mix.

Components	Amount
LightCycler SYBR Green	5µl
ready-to-use primer	1µl
Sample	1µl
RNase-free water	3µl

The settings on the Light Cycler were as Figure 2.

Figure 2: Light Cycler settings



The data were analyzed, using the crossing point (CP) value, which stands for the cycles needed to meet a set threshold fluorescence signal strength (Livak and Schmittgen 2001, Wong and Medrano 2005). ΔCP values were calculated by subtracting the CP of GAPDH from the target CP in each sample ($\Delta CP = CP_{measured\ gene} - CP_{GAPDH}$). Values were represented as $2^{-\Delta CP}$ and normalized to the mean values of corresponding controls (resulting in relative $2^{-\Delta CP}$).

3.5 CCK8 Viability assay

Steps for the viability assay were performed according to the following protocol.

1. WPMY-1 cell suspension was prepared and counted under a microscope.

2. 100 µl cell suspension containing 5000 cells was seeded into each well of the 96-well plates, followed by incubation at 37 °C, 5% CO_2 for 24 h.

3. After 24 h, medium containing 1 μM, 2.5 μM, 5 μM NAV2729, or DMSO was prepared. The old medium in each well of the 96-well plates was discarded and replaced by 100 μl fresh medium containing NAV2729 or DMSO.

4. At the time point as indicated, 10 μl of CCK8 reagent was added to each well of the 96-well plates, then the plates were incubated for 2 h in the incubator before measuring.

5. Absorbance was measured at 450 nm using a microplate reader.

3.6 EdU assay

EdU assay in WPMY-1 cells was performed with an "EdU-Click 555" proliferation assay kit (Baseclick, Tutzing, Germany).

Steps for the EdU assay were performed according to the following protocol.

1. WPMY-1 cells were digested and added into 16 well Lab-Tek chamber slides (Thermo Fisher, Waltham, MA, USA) and incubated at 37 °C, 5% CO_2 atmosphere. Intervention was induced when the cells reach 70% confluence.

2. Treatment with NAV2729 in the indicated concentrations was performed, or siRNA transfection (using scramble control and wildtype WPMY-1 cells as control groups) was applied.

3. EdU was added 12 h before fixation. The final concentration of EdU in each well should be 10 μM.

4. Right before fixation, residual EdU-containing medium was discarded, the cells were washed with PBS two times.

5. Residual PBS was discarded, and 90 µl Roti®-Histofix was added to each well and incubated for 15 min at room temperature for fixation.

6. Histofix solution was discarded, and cells were washed with 3% bovine serum albumin (BSA, diluted with PBS) 2 times.

7. Supernatant was discarded, and 90 µl 0.5% Triton™ X-100 solution was applied in each well and incubated at room temperature for 20 min.

8. Triton™ X-100 solution was discarded, and cells were washed with 3% BSA two times, followed by staining of the fixed cells with the EdU assay cocktail (30 µl/well) for 30 min in the dark (Table 6).

Table 6: EdU cocktail (for staining of 8 wells).

Reaction buffer (10X)	100 µl
Catalyst-solution	40 µl
Dye Azide (10mM)	2 µl
Buffer additive (10x)	100 µl

9. EdU cocktail solution was discarded, and cells were washed with 3% BSA 2 times and stained with 1 µg/ml DAPI solution in the dark at room temperature for 15 min.

10. Cells were washed with 3% BSA, then the chambers from the glass slides were removed carefully, glass slides were finally gelled.

11. The observation time for the cells using a laser scanning microscope (Leica SP2, Wetzlar, Germany) was within one week after staining due to the limited lifetime of fluorescence.

3.7 Phalloidin assay

Steps for the phalloidin staining were performed according to the following protocol.

1. WPMY-1 cells were digested and seeded in 16 well Lab-Tek chamber slides (Thermo Fisher, Waltham, MA, USA) and incubated at 37 °C, 5% CO_2 atmosphere.

2. Treatment with NAV2729 in the indicated concentrations was performed, or siRNA transfection (using scramble control and wildtype WPMY-1 cells as control groups) was applied.

3. Right before fixation, the residual medium was discarded, and the cells were washed with PBS two times.

4. Residual PBS was discarded, and 90 µl Roti®-Histofix was added to each well and incubated for 15 min at room temperature for fixation.

5. Histofix solution was discarded, cells were washed with 3% BSA (diluted with PBS) 2 times.

6. Supernatant was discarded, and 90 µl 0.5% Triton™ X-100 solution was applied in each well and incubated at room temperature for 20 min.

7. Triton™ X-100 solution was discarded, and cells were washed with 3% BSA two times and stained with fluorescein isothiocyanate- (FITC-) labeled phalloidin (100 µM), 40 µl in each well for 40 min in the dark.

8. Phalloidin solution was discarded, cells were washed with 3% BSA 2 times and stained with 1 µg/ml DAPI solution in the dark at room temperature for 15 min.

9. Cells were washed with 3% BSA subsequently, then the chambers were carefully removed from the glass slides, and glass slides were finally gelled.

10. The observation time for the cells using a laser scanning microscope (Leica SP2, Wetzlar, Germany) was within one week after staining due to the limited lifetime of fluorescence.

3.8 Sulforhodamine B (SRB) colony formation assay

The SRB method has been widely applied since its introduction in 1990 (Skehan, Storeng et al. 1990). It is designed to evaluate cytotoxicity and proliferation in studies based on cells. (Vichai and Kirtikara 2006). Here, SRB colony assay was performed to further exam the influence of NAV2729 on cytotoxicity and proliferation of the WPMY-1 cells.

Steps for the colony formation assay were performed according to the following protocol.

1. WPMY-1 cells were digested and diluted in the medium. In each well of the 6-well plates, 100 cells were seeded.

2. Cells were incubated at 37 °C, 5% CO_2. After 48 h, NAV2729 was applied in the indicated concentrations.

3. After 14 days of incubation, the cells were washed twice with PBS and fixed by trichloroacetic acid (TCA) solution overnight (2 ml each well of the 6-well plate, 4 ℃), and then the plates were washed with water five times followed by 0.4% SRB solution staining for 30 min at room temperature.

4. Every well was washed five times with 1% acetic acid, the colonies were then counted.

3.9 Contraction assay

The contraction ability of cells was assayed using a CytoSelect™ 24-Well Cell Contraction Assay Kit (floating matrix model version, Cell Biolabs, USA). The contraction inhibitor 2, 3-butanedione monoxime (BDM) included in the kit was used as a standard control (final concentration 10 mM) as suggested by the manufacturer. The contraction of the cells was indicated by the shrink of the floating collagen. Pictures of the collagen were taken at the indicated time points.

Steps for the contraction assay were performed according to the following protocol.

1. Cells were digested and resuspended in fresh RPMI medium to reach 5×10^6 cells/ml.

2. Cell contraction matrix was created using one part of cell suspension and four parts of pre-cold collagen matrix solution.

3. 500 µl of the cell contraction matrix which contains cells was added to each well of the 24-well plate provided in the kit.

4. The plate was incubated for 1 h at 37 °C, 5% CO_2.

5. After collagen polymerization, 1 ml of 1640 RPMI medium (with NAV2729, contraction inhibitor, or DMSO) was added from the top of each well.

6. The collagen contraction was monitored in the indicated time points by taking pictures with a camera. The data was shown in the percentage of the contracted (reduced) collagen area (measured with Image J software) compared to the corresponding well area at each time point.

3.10 Western blot

Steps for the Western blot were performed according to the following protocol.

1. Wildtype, ARF6 silenced, and scramble control WPMY-1 cells were prepared.

2. The medium in the flasks was discarded, the cells were washed with ice-cold PBS two times.

3. All of the residual PBS was aspirated, followed by the addition of 600 µl ice-cold radioimmunoprecipitation assay (RIPA) buffer (Sigma-Aldrich, Germany) inside each flask.

4. Flasks were placed horizontally on ice for 25 min for lysis.

5. After lysis, the cells were scraped by plastic cell scrapers, and then the lysed solution was collected in microfuge tubes.

6. Centrifugation of the tubes at 16000 g for 20 min to pellet the cell debris. The supernatant which contains the protein was split into two parts, which were stored at -20 ℃, or directly used for protein quantification.

7. Protein concentration was quantified with Dc-Assay protein concentration kit (Bio-Rad, Germany) following the instructions provided by the manufacturer.

8. Protein samples were boiled with sodium dodecyl sulfate (SDS) sample buffer for 10 min.

9. Samples (20 μg/lane) were loaded to SDS-polyacrylamide gel, followed by electrophoresis at 180 V for 30 min to separate the proteins.

10. Membrane transfer was performed by electroblotting with Protran® nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) at 19 V for 30 min.

11. PBS with 5% milk powder (Roth, Karlsruhe, Germany) was used to block the membranes for unspecific binding sites at 4 °C overnight.

12. Membranes were incubated with the primary antibodies at 4 °C overnight. Primary antibodies were prepared in PBS containing 0.1 % Tween 20 (PBS-T) and 5 % milk powder.

13. Membranes were washed with PBS-T solution three times (every five min).

14. Membranes were washed with PBS one time.

15. Chemiluminescence (ECL) Hyperfilm (GE Healthcare, Freiburg, Germany) was used to develop the blots. Densitometric quantification of the blots was carried out by ImageJ (National Institutes of Health, Bethesda, MD).

3.11 Phosphorylation assessment

WPMY-1 cells were incubated in T75 flasks. After reaching the confluence of around 70%, phosphorylation assessment was performed.

Steps for the phosphorylation assessments were performed according to the following protocol.

1. NAV2729 (to reach a final concentration of 5 μM), or solvent DMSO was added to the corresponding flasks, followed by incubation for 2 h.

2. Proteins were isolated and quantified, using the same method as mentioned in the Western blot part.

3. Phospho-MYPT1, MYPT1, phospho-MLC, MLC, and β-actin Western blot analyses were performed.

4. Densitometric quantifications were conducted using the method mentioned in the Western blot part.

3.12 Pull-down assay

Pull-down assays were performed to separate and differentiate active GTPases from their inactive, and here, effects of NAV2729 on GTPase activities in WPMY-1 cells were measured with a pull-down assay kit. After being treated with NAV2729 or solvent, protein of cells was isolated and subjected to pull-down assays and Western blot analyses.

Steps for the pull-down assay were performed according to the following protocol.

1. WPMY-1 cells were prepared in T75 flasks. After reaching a confluence of 70%, pull-down assay was performed.

2. NAV2729 (to reach a final concentration of 5 μM), or solvent DMSO was applied to the corresponding flasks and incubated for 2 h.

3. Proteins were isolated and quantified, using the same method as mentioned in the Western blot part.

4. For each measurement, 300 μg protein sample was prepared on ice, ARF1, ARF6, RAC1, and RhoA activities were measured using the pull-down assay kits BK032, BK033, BK035, BK036 (Cytoskeleton, USA) respectively, following the instructions provided by the manufacturer.

5. Western blot analyses and the following densitometric quantification were applied as mentioned in the Western blot part.

3.13 Statistical analysis

Data are shown as means ± standard error. SPSS version 20 (IBM SPSS Statistics, IBM Corp., Armonk, NY) was used for all statistical analyses. Concerning unpaired observations, multivariate analysis of variance and two-way analysis of

variance was used. P values < 0.05 will be regarded as statistically significant. All experiments being subjected to statistical analyses were based on at least five independent experiments.

4. Results

4.1 Effects of NAV2729 on the viability of WPMY-1 cells

CCK8 was used to assess responses in viability to NAV2729. Three concentrations (1, 2.5, and 5 µM) and five time-points (1 h, 2 h, 24 h, 48 h, 72 h) were tested (Figure 3). Short incubation periods showed a concentration-dependent trend in viability decrease, reaching 19 ± 1.1% from 5 µM after 1 h and 21 ± 1.3% after 2 h. However, the decreases were not significant. A clear concentration and time-dependent effect on the viability of WPMY-1 cells was seen after 24 h, showing a significant decrease of viability by all concentrations compared with the solvent treated group, ranging between 44 ± 2.7% from 1 µM and 89 ± 1.6% from 5 µM after 24 h to nearly complete reduction of viability for all three concentrations after 72 h.

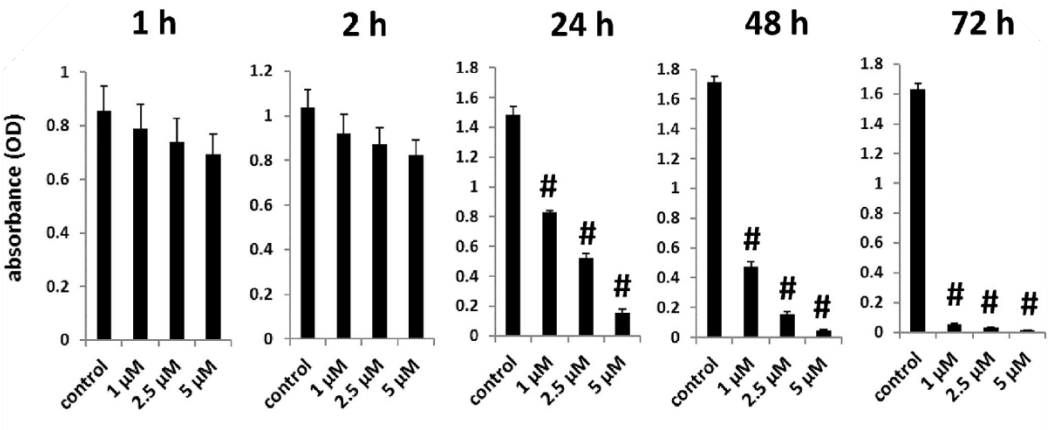


Figure 3: CCK8 assays, to assess effects of NAV2729 on the viability of WPMY-1 cells. WPMY-1 cells were treated with NAV2729 in concentrations and time periods as indicated. DMSO-treated cells with the same environment were used as controls. Data are shown as means ± S.E. from n=5 independent experiments for every setting (#, $p<0.05$ versus corresponding control).

4.2 EdU proliferation assay

EdU assay was applied to assess the influence of NAV2729 on the proliferation ability of WPMY-1 cells (Figure 4). After staining, the numbers of total nuclei and the EdU-stained nuclei were counted, and the percentage of proliferating cells was calculated in each group. NAV2729 led to a concentration-dependent decrease in proliferation. 2.5 µM NAV2729 reduced the proliferation rate by 20 ± 2.5%. 5 µM NAV2729 reduced the proliferation rate by 40 ± 1.9%.

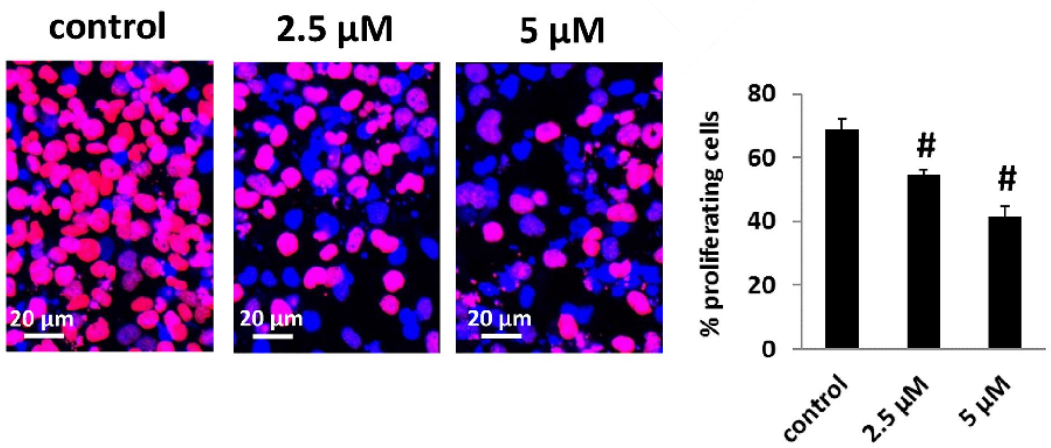


Figure 4: EdU assays, to assess effects of NAV2729 on the proliferation of WPMY-1 cells. NAV2729 was applied to WPMY-1 cells in concentrations indicated. The proliferation rate was evaluated by EdU assay. DMSO-treated cells under the same conditions were used as controls. Shown are representative experiments and means ± S.E. (error bars) from n=5 independent experiments (#, $p<0.05$ versus control).

4.3 Assessment of Ki-67 content

The proliferation indicator Ki-67 was here semi-quantitatively evaluated by RT-PCR. Ki-67 mRNA expression levels were reduced concentration-dependently

by NAV2729 treatment (24 h), reaching 76 ± 9.1% reduction by 5 µM NAV2729 (Figure 5).

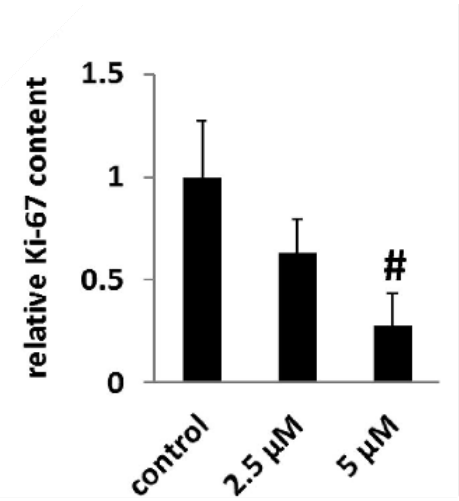


Figure 5: NAV2729 was applied to WPMY-1 cells with indicated concentrations. mRNA content of the proliferation marker Ki-67 was quantified by RT-PCR. DMSO-treated cells under the same conditions were used as controls. Shown are means ± S.E. (error bars) from n=5 independent experiments (#, $p<0.05$ versus control).

4.4 Effects of NAV2729 on colony formation in WPMY-1 cells

Colony formation ability was evaluated by counting the colonies of WPMY-1 cells from the wells of 6-well plates after 14 days of incubation with solvent or NAV2729. Concentration-dependent inhibition of cell colony formation was

found in NAV2729 treated groups (Figure 6), amounting to 58 ± 11.5% reduction of colony formation under 5 µM NAV2729 treatment.

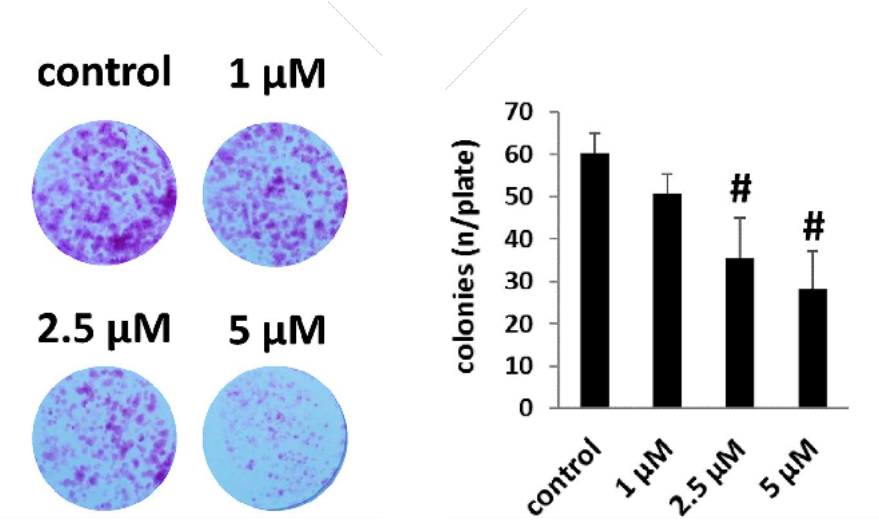


Figure 6: Colony formation assays to assess effects of NAV2729 on the growth of WPMY-1 cells. NAV2729 was applied to WPMY-1 cells in indicated concentrations during the colony formation. DMSO-treated cells under the same conditions were used as controls. Shown are representative experiments and means ± S.E. (error bars) from n=5 independent experiments (#, $p<0.05$ versus control).

4.5 Effects of NAV2729 on actin organization of WPMY-1 cells

WPMY-1 cells were treated with NAV2729 (5 µM, 24 h) as an experimental group or with DMSO as a control group under the same conditions. Pictures taken by a fluorescence microscope showed polymerized, filamentous actin in the control cells after phalloidin staining. In the DMSO-treated control group,

the structure of the actin was fusiform-shaped, stretched filaments are partially crossing each other. Treatment with NAV2729 led to actin deformation, breakdown, showing shortening, regression of actin bundles, and loss of phalloidin-stained actin. Quantification confirmed that treatment with NAV2729 in WPMY-1 cells induced a significant decrease of actin formation compared with the solvent control group (Figure 7).

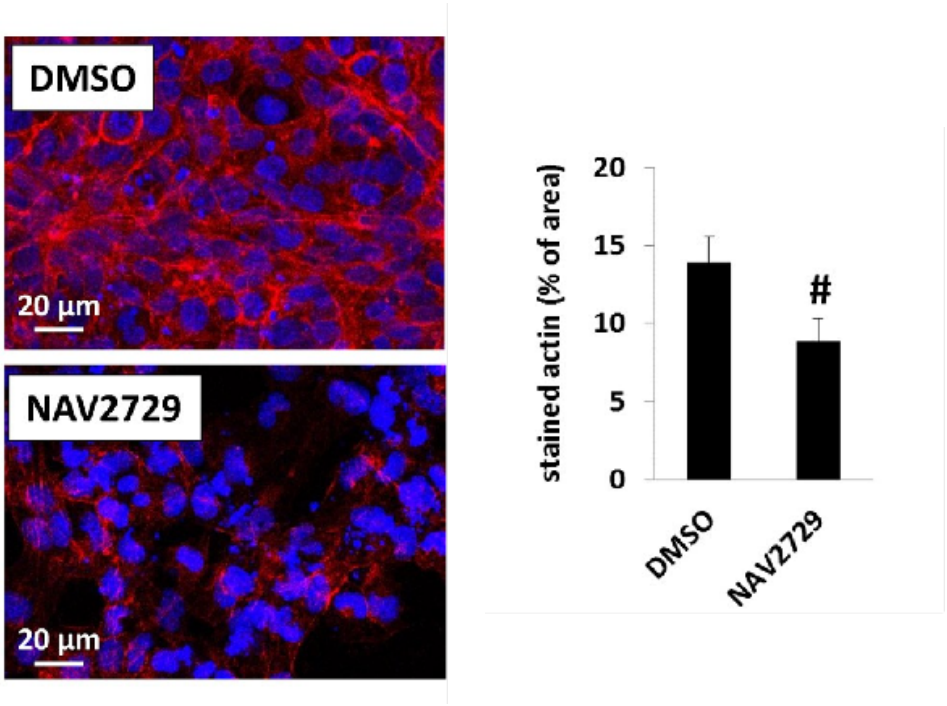


Figure 7: Phalloidin staining to assess effects of NAV2729 on actin organization in WPMY-1 cells. NAV2729 (5 µM) was applied to WPMY-1 cells for 24 h followed by phalloidin staining to visualize actin filaments. Shown are pictures from representative experiments and means ± S.E. (error bars) from n=5 independent experiments in both series (#, $p<0.05$ versus control).

4.6 Effects of NAV2729 on WPMY-1 cell contraction

A collagen matrix contraction assay was performed to measure the contraction of cells under different conditions. After collagen polymerization followed by the addition of NAV2729 or DMSO, the 24-well plate was visualized and pictures were taken in all groups from 3 h to 24 h. NAV2729 induced concentration-dependent contraction inhibition, as reflected by reduced changes in the collagen size (Figure 8). The contraction developed within the first 3 h and then continue to contract slowly. Within the first 3 h, 2.5 µM NAV2729 did not reduce contraction significantly comparing to the control (DMSO). In contrast, 5 µM and 10 µM NAV2729 reduced contraction by 6.8 ± 2.5% and 16.1 ± 3.6%, respectively. Within 12 h, 2.5 µM, 5 µM, and 10 µM NAV2729 reduced contraction by 5.1 ± 1.9%, 7.4 ± 2.0%, 18 ± 5.7% respectively. 24 h after application of NAV2729 or DMSO, 2.5 µM, 5 µM, and 10 µM NAV2729 reduced contraction by 4.4 ± 1.1%, 7.4 ± 0.8%, 21 ± 5.7% respectively.

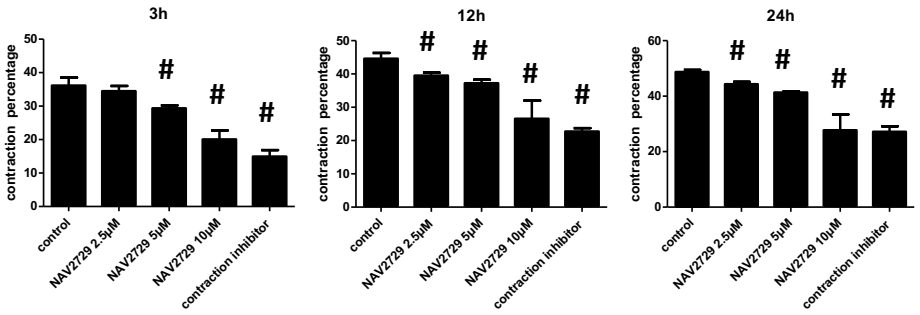


Figure 8: Contraction of WPMY-1 cells under control (DMSO) and different concentrations of NAV2729. Contraction was recorded and quantified in all concentrations within 24 h after collagen polymerization. Contraction was compared among control and all NAV2729-treated groups. As cells contract, the collagen shrinks in its size and thus higher contraction shows smaller collagen size, indicated by a higher area contraction percentage. Shown are diagrams from

5 independent experiments, with means ± S.E. (error bars) as quantified and calculated by these experiments (#, $p<0.05$ versus corresponding control).

4.7 Effects of ARF6 silencing by siRNA in WPMY-1 cells

ARF6 siRNA transfection led to down-regulation of ARF6 expression in WPMY-1 cells. Western blot was applied for assessing the protein expression. Reduced ARF6 protein expression was shown compared with scramble control and wildtype WPMY-1 cells (Figure 9A). RT-PCR was applied for measuring mRNA expression and showed reduced levels of ARF6 mRNA compared with the scramble control and wildtype groups (Figure 9B). These readouts confirmed that the transfection was successful, i.e. that ARF6 expression was successfully silenced at protein and mRNA levels.

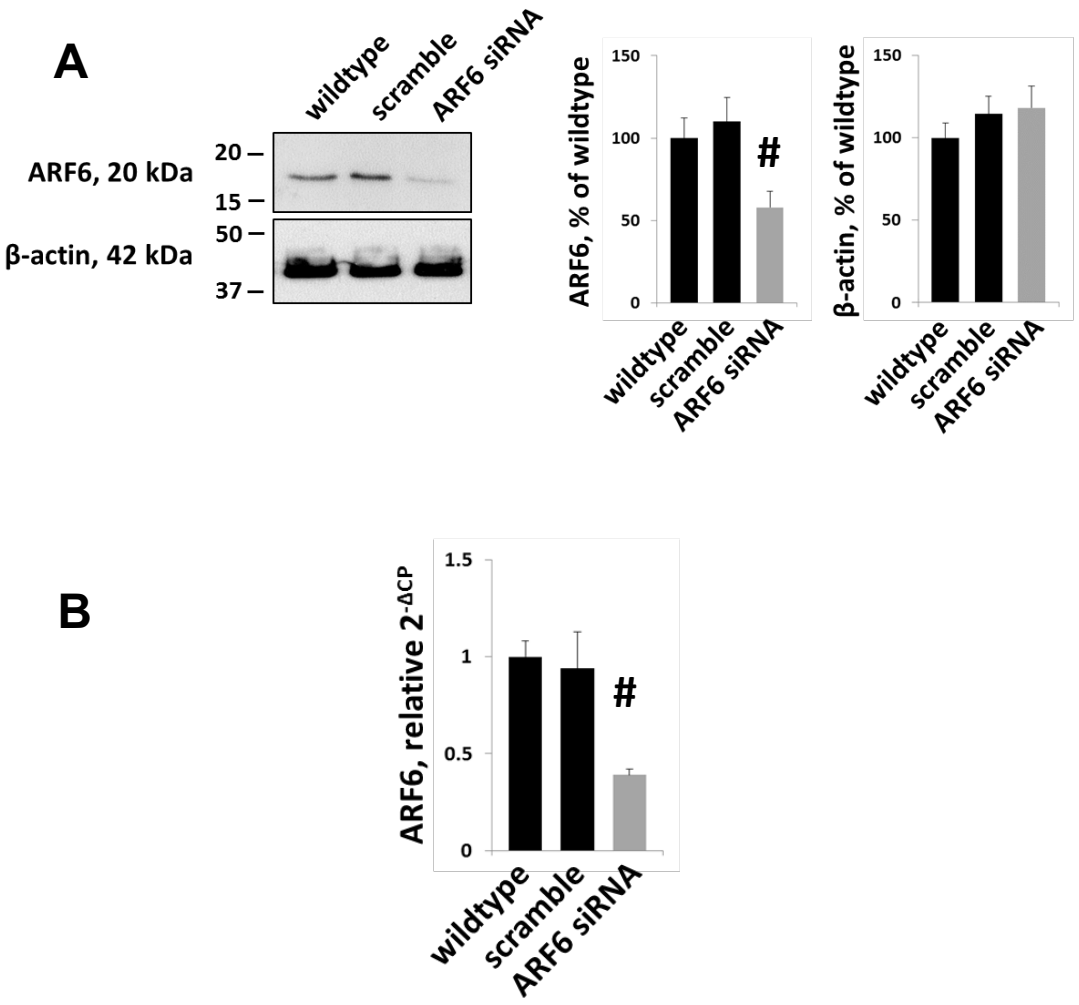


Figure 9: WPMY-1 cells were transfected with scramble or ARF6 siRNA, the ARF6 expression level was compared between each group by A) Western blotting and B) RT-PCR. Shown are representative experiments and means ± S.E. (error bars) from n=5 independent experiments in all series (#, $p < 0.05$ versus wildtype and scramble). Positions of marker bands next to the bands of interest are indicated at the left of each blot (sizes in kDa).

4.8 Effects of ARF6 silencing by siRNA on the viability of WPMY1 cells

72 h after Silencer Select ARF6 siRNA or scramble control siRNA transfection, viability was evaluated by CCK8. Knockdown of ARF6 expression mimicked the effects of NAV2729 on viability. In the ARF6 siRNA-transfected cells, viability decreased by 32 ± 7.1 and 22 ± 9.0% compared to the wildtype WPMY-1 cells and the scramble control siRNA transfected group.

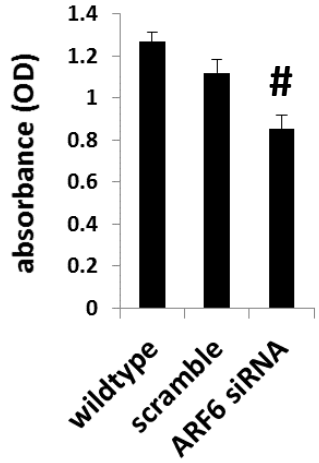


Figure 10: Viability was compared by CCK8 assay. Shown are means ± S.E. (error bars) from n=5 independent experiments between wildtype WPMY-1 cells and WPMY-1 cells transfected with scramble siRNA or ARF6-specific siRNA (#, $p<0.05$ versus wildtype and scramble).

4.9 Effects of ARF6 knockdown by siRNA on the proliferation of WPMY-1 cells

EdU assay was conducted 72 h after Silencer Select ARF6 siRNA and scramble control siRNA transfection. Three groups were assayed (ARF6 siRNA, scramble control, wildtype). The rate of proliferation in WPMY-1 cells with ARF6 knockdown decreased by 34 ± 2.3% compared to wildtype cells and by 30 ± 1.8% compared to the scramble control siRNA transfected cells.

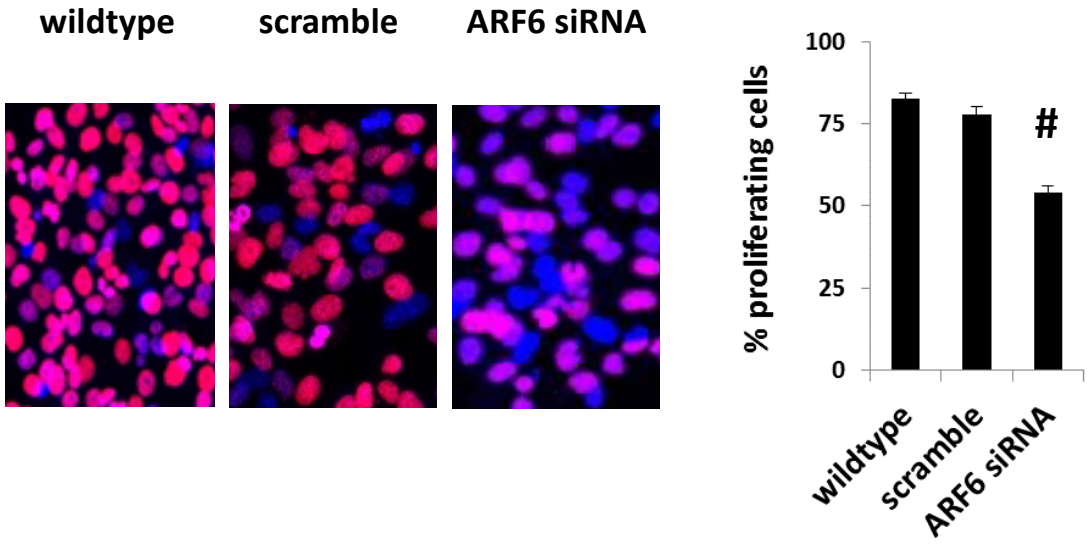


Figure 11: The proliferation of WPMY-1 cells in wildtype, scramble control, and ARF6-silenced groups was evaluated with EdU assay. Shown are representative EdU pictures and a diagram with means ± S.E. (error bars) from n=5 independent experiments (#, $p<0.05$ versus wildtype and scramble).

4.10 Effects of ARF6 knockdown by siRNA on actin organization of WPMY-1 cells

The Silencer Select ARF6 siRNA and scramble control siRNA were transfected into WPMY-1 cells. Actin staining in cells transfected with scramble control resembled the staining in untreated wildtype cells. The knockdown of ARF6 by siRNA mimicked the effects of NAV2729 on the deformation and breakdown of actin organization.

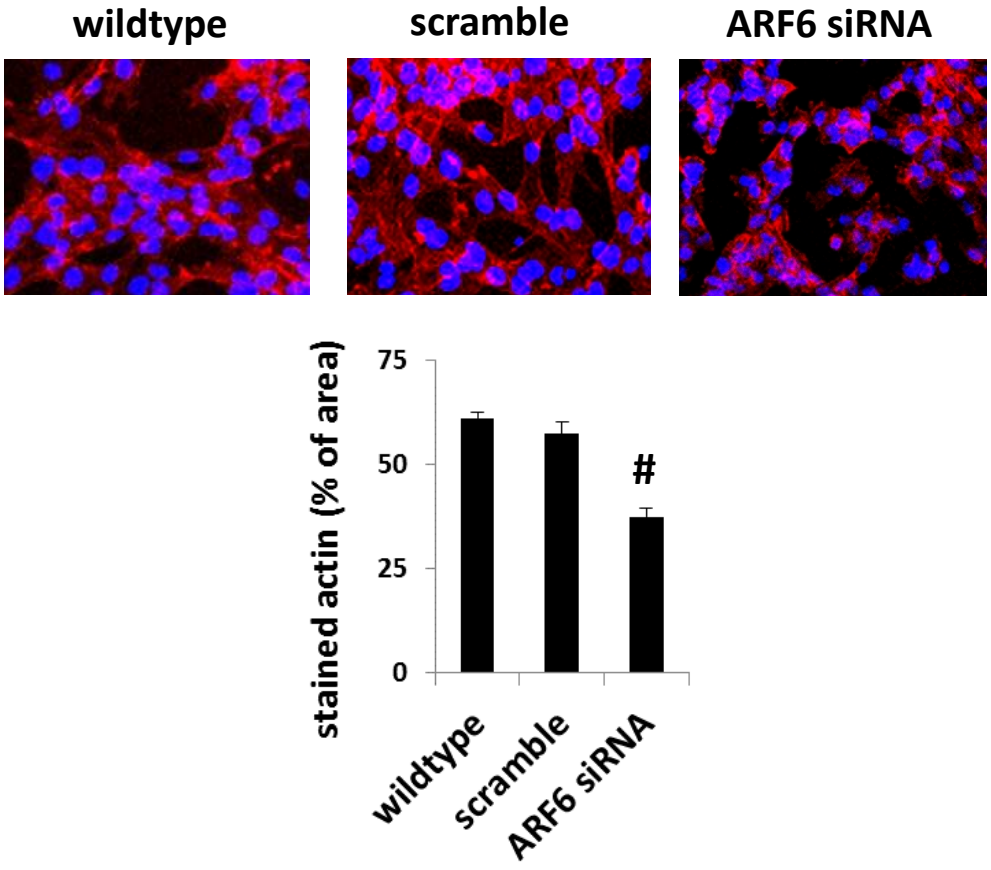


Figure 12: WPMY-1 cells from wildtype group, scramble control group, and ARF6-silenced group were subjected to phalloidin staining to visualize polymerized actin. Shown are representative phalloidin pictures and a diagram with means ± S.E. (error bars) from n=5 independent experiments (#, $p<0.05$ versus wildtype and scramble).

4.11 Effects of ARF6 silencing by siRNA on WPMY-1 cell contraction

ARF6-silenced WPMY-1 cells showed reduced contraction, compared to the wildtype or the scramble control settings. 3 h after transfection, ARF6 silenced cells showed a reduction in contraction of 16.6 ± 2.5% compared to the wildtype, and of 12.7 ± 1.2% compared to the scramble control. 12 h after transfection, ARF6-silenced cells showed a reduction in contraction of 16.4 ± 2.3% compared to the wildtype, and of 11 ± 3.6% compared to the scramble control. 24 h after transfection, ARF6 silenced cells showed a reduction in contraction of 9.7 ± 1.8% compared to the wildtype but no significant difference compared to the scramble control.

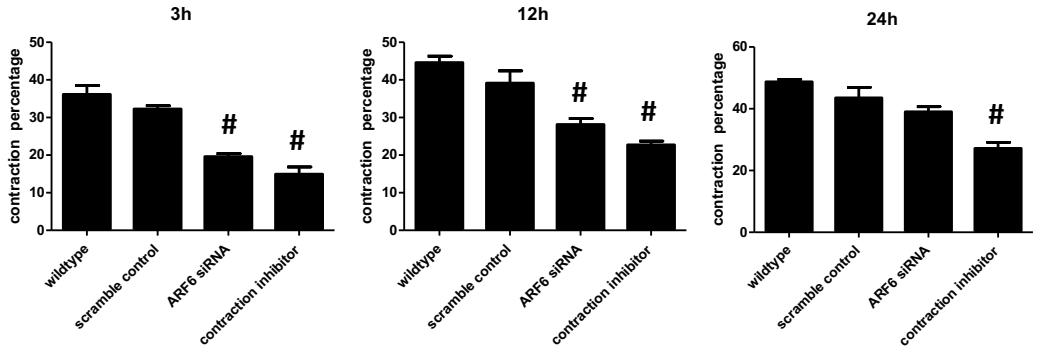


Figure 13: Contraction of WPMY-1 cells in wildtype, scramble control, and ARF6-silenced cells. Contraction was recorded and compared between groups at each time point. Shown are diagrams from 5 independent experiments, and with means ± S.E. (error bars) as quantified and calculated by these experiments (#, $p<0.05$ versus wildtype and scramble).

4.12 Effects of NAV2729 in ARF6-silenced WPMY-1 cells

For evaluating potential limitations of the specificity of NAV2729, the effects of NAV2729 on viability and proliferation in WPMY-1 cells with and without ARF6 silencing were assessed. Different from the results of wildtype WPMY-1 cells, low concentrations of NAV2729 (1 or 2.5 μM, 24 h) did not show effects on viability in ARF6-silenced WPMY-1 cells. 5 μM NAV2729 caused a reduction of viability by 58 ± 0.3% (Figure 14A) in ARF6-silenced WPMY-1 cells, this viability was still higher than that in the NAV2729-treated wildtype WPMY-1 cells which showed an almost complete reduction in viability by the same NAV2729 concentration (Figure 3). In EdU assays, treatment with NAV2729 (2.5 μM) in ARF6-silenced WPMY-1 cells showed no significant effect, while 5 μM NAV2729 still reduced the proliferation rate by 12 ± 2.8% (Figure 14B), and again, this effect of NAV2729 in ARF6 silenced cells was smaller than that in wildtype cells.

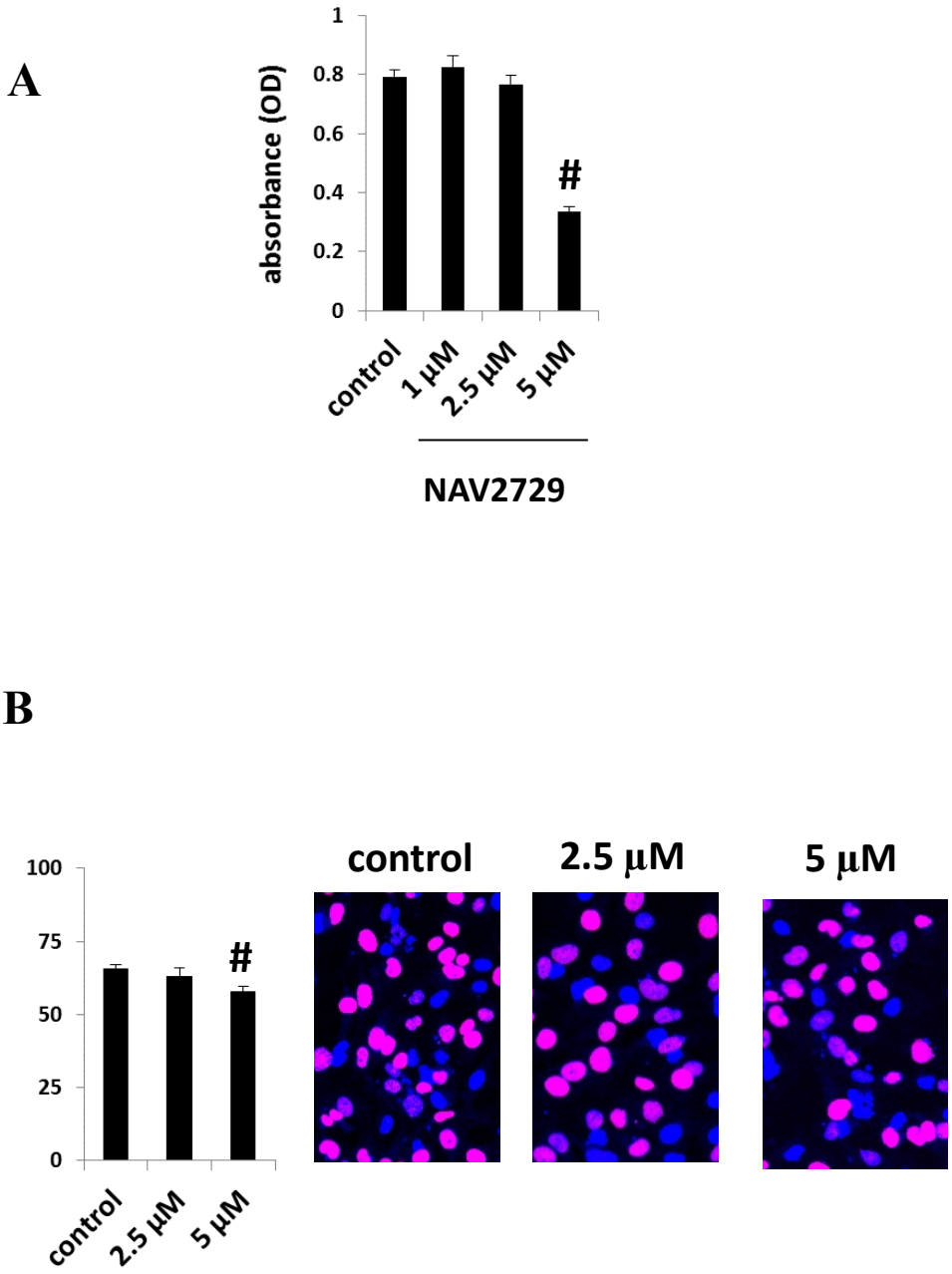
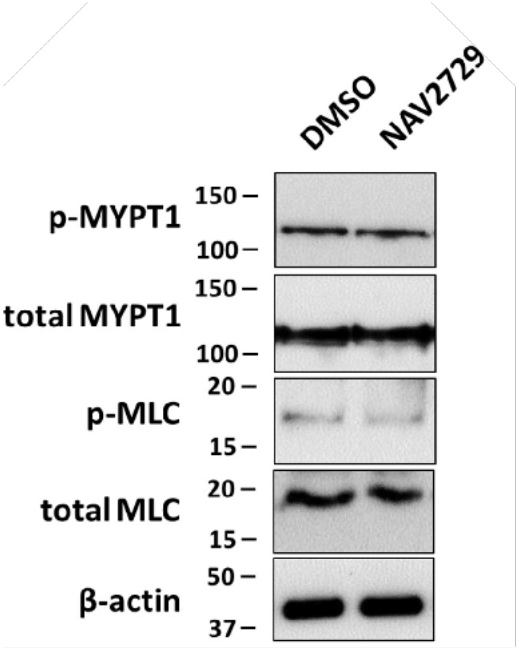


Figure 14: Impact of NAV2729 on viability and proliferation in ARF6-silenced cells. Here, the effects of NAV2729 on the indicated concentrations were assayed on viability and proliferation in ARF6-silenced cells, by CCK8 (A) and EdU assay (B). Shown are representative pictures and diagrams with means ± S.E. (error bars) from n = 5 independent experiments in both series (#, $p<0.05$ versus wildtype and scramble).

4.13 Effects of NAV2729 on myosin phosphatase-targeting subunit 1 (MYPT1) and MLC phosphorylation in WPMY-1 cells

Other than actin organization, phosphorylation of the MYPT1 causes increased MLC phosphorylation in procontractile signaling pathways, which plays a key role in smooth muscle contraction (Somlyo and Somlyo 2003). Here, expressions of phospho-MYPT1, total MYPT1, phospho-MLC, and total MLC were semiquantitatively compared by Western blotting between NAV2729 and DMSO-treated WPMY-1 cells. The content of all these proteins was not reduced by NAV2729, implying that the phosphorylation of MYPT1 or MLCs was not influenced by NAV2729 in WPMY-1 cells (Figure 15).



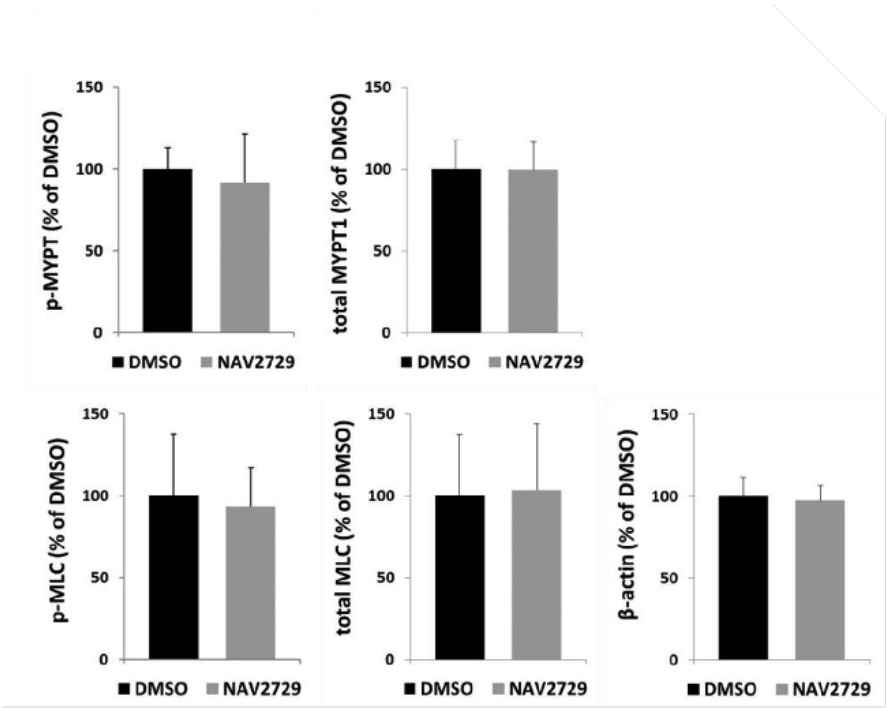


Figure 15: NAV2729 (5 µM, 2 h) or solvent (DMSO) were applied to WPMY-1 cells. The expressions of total and phosphorylated MYPT1 and MLC were compared semiquantitatively by Western blot between phospho-MYPT1 (threonine 696), total MYPT1, phospho-MLC (threonine 18/serine 19), and total MLC. Shown are representative experiments and quantification (n=4 independent experiments for p-MYPT1/MYPT1, and n=3 for p-MLC/MLC) (means ± S.E.). In each single experiment, samples from the same experiment were used for the control and inhibitor groups. Positions of marker bands next to the bands of interest are indicated at the left side of each blot (sizes in kDa).

4.14 Effects of NAV2729 on GTPase activities in WPMY-1 cells

NAV2729 significantly lead to the reduction of GTP-ARF6 content, but not total ARF6 content, indicated the inhibition of ARF6 activity in WPMY-1 cells. On the contrary, no evidence is showing activities of ARF1, RAC1, or RhoA are influenced by NAV2729 (Figure 16).

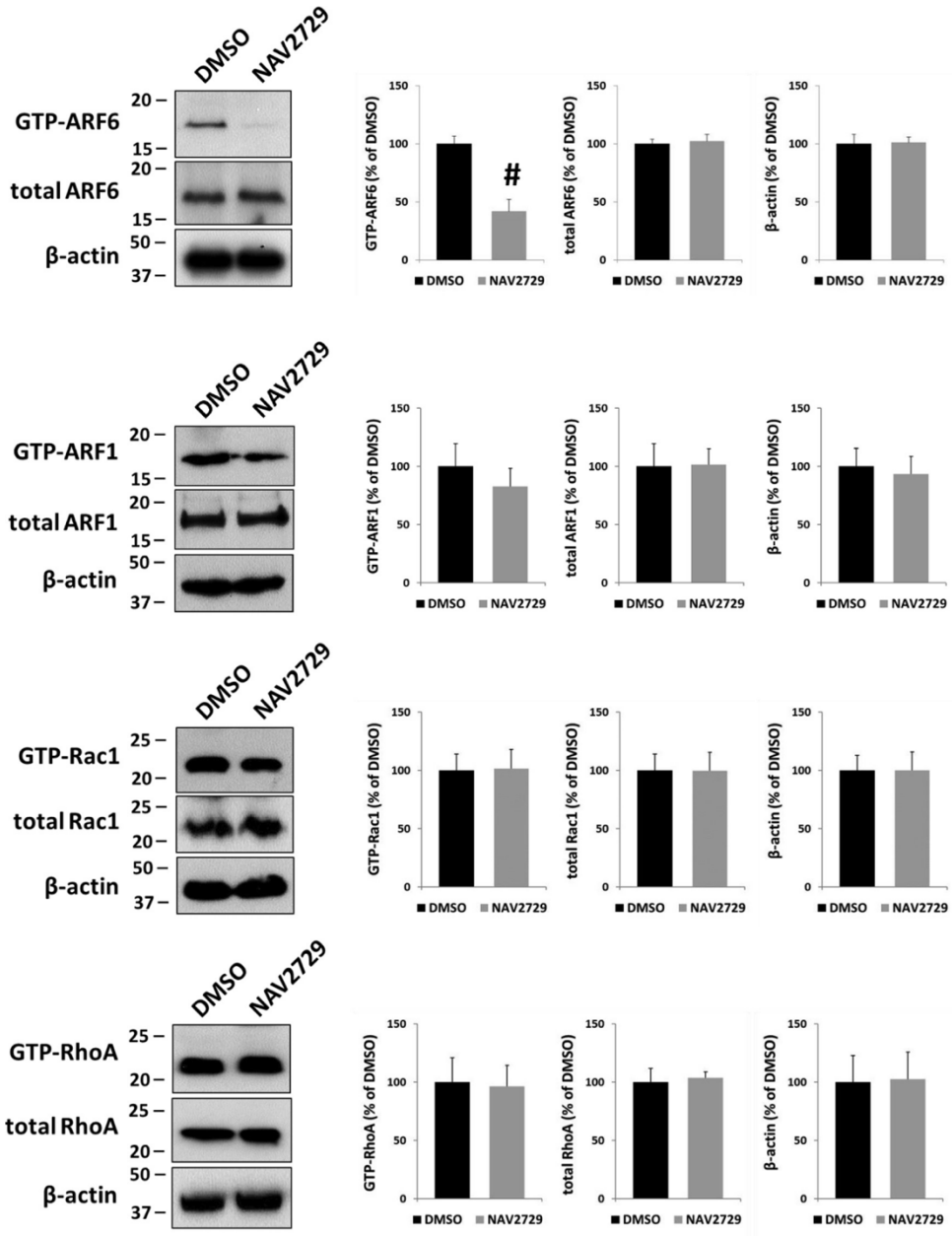


Figure 16: NAV2729 (5 μM, 2 h) or solvent (DMSO) were applied to WPMY-1 cells, and pull-down assays were subsequently performed to evaluate the content of active GTPases (GTP-ARF6, GTP-ARF1, GTP-RAC1, and GTP-RhoA). Western blot was performed to compare the total content of GTPases. Shown are representative experiments (left panels) and quantification from all experiments (n=7 for ARF6, n=6 for ARF1, n=5 for RAC1, n=4 for RhoA) (means ± S.E.(errorbars)) (#, $p<0.05$ for DMSO versus NAV2729). In each single experiment, samples from the same tissue were used for the control and inhibitor groups. Positions of marker bands next to the bands of interest are indicated at the left of each blot (sizes in kDa).

5. Discussion

The small monomeric GTPase ARF6 belongs to the large Ras superfamily that encompasses small GTPases located on chromosome 14q21.3. Established functions of ARF6 include intracellular vesicular trafficking, and regulation of endocytosis and endocytic recycling (Schweitzer, Sedgwick et al. 2011, Van Acker, Tavernier et al. 2019). Apart from the well-studied role of ARF6 in membrane trafficking, it is becoming increasingly clear that ARF6 associates with promoting proliferation in different cell types, including vascular smooth muscle cells (Bourmoum, Charles et al. 2016), endothelial cells (Ikeda, Ushio-Fukai et al. 2005), and many kinds of cancer cells (Li, Wang et al. 2009, Knizhnik, Kovaleva et al. 2012).

In addition, previous evidence pointed to a role of ARF6 in modulating the actin cytoskeleton (Donaldson 2002). The role of actin polymerization in smooth muscle force generation is widely accepted (Gunst and Zhang 2008). By inhibiting the actin polymerization followed by measuring the contractile responses to agonist stimulation, numerous reports based on smooth muscle tissues ranging from airway, vascular to uterine and intestinal showed strong inhibition of contraction and tension suppression (Gunst and Zhang 2008).

Since the proliferation and contraction property of prostate stromal cells is essential for the pathogenesis of LUTS suggestive of BPH, and as other studies confirmed the impact of small monomeric GTPases on BPH, such as RhoA, Rac, and others (Rajasekaran, Wilkes et al. 2005, Wang, Kunit et al. 2015), it was here speculated that ARF6, which is also a member of small monomeric GTPases, may be involved in control of prostate growth and smooth muscle contraction in

LUTS suggestive of BPH. Cytohesins are small guanine nucleotide exchange factors (GEFs) activating ARFs. The recent observation that SecinH3, a cytohesin inhibitor, inhibits ARF6 activity (Hafner, Schmitz et al. 2006, Grossmann, Yoo et al. 2013), and prostate smooth muscle contraction (Herlemann, Keller et al. 2018), suggested that a cytohesin/ARF6 pathway could be involved in the pathogenesis of LUTS suggestive of BPH.

After NAV2729, a small molecule compound that directly inhibits ARF6 has become available, addressing this assumption became possible. NAV2729 directly inhibits ARF6, involving a non-nucleotide competitive mechanism (Yoo, Shi et al. 2016). Hence, a series of experiments based on NAV2729 was carried out in this thesis, to evaluate its effects on the growth and contraction of prostate stromal cells, which are essential factors for the pathogenesis of LUTS suggestive of BPH.

WPMY-1 is an immortalized prostate stroma-originated adherent cell line, which is an appropriate cell line for mimicking the stromal components of the prostate in vitro. WPMY-1 cells show similarities to smooth muscle cells, as both of them have the capability of contraction and are close in morphology, described as elongated, spindle-shaped cells. Of note, they show co-expression of the smooth muscle marker α-SMA (Webber, Trakul et al. 1999), calponin, and α_1A-adrenoceptors (Wang, Gratzke et al. 2016).

These characteristics qualified the WPMY-1 cell line to be a suitable model in studying the role of ARF6 in smooth muscle contraction and stromal proliferation in LUTS suggestive of BPH in vitro. Several series of experiments

were carried out using the ARF6 inhibitor NAV2729 or by creating ARF6 knockdown by RNA silencing.

When evaluating proliferation under the impairment of ARF6 by NAV2729 in EdU assays, a significant reduction of proliferating cells became obvious. This was also supported by RT-PCR results, showing reduced content of the proliferation marker Ki-67. Colony formation assay further confirmed this by showing decreased colony-forming ability. These findings may be related to reduced viability in CCK8 assays. Together, this suggests a high impact of NAV2729 on different growth-related functions of prostate stromal cells.

Actin organization of WPMY-1 cells was examined by phalloidin staining. Results indicated a deformation of actin organization, reduced content of polymerized actin at a quantitative level by NAV2729，and in contraction assays, significant contraction inhibition was observed. Together, ARF6 may play a role in promoting the stroma growth, actin organization, and smooth muscle contraction in LUTS suggestive of BPH.

Further experiments based on ARF6 knockdown using siRNA mimicked the effects of NAV2729 on cell proliferation, actin organization, and cell contraction, confirming the involvement of ARF6 in the growth and contraction of WPMY-1 cells, and suggesting that the effects of NAV2729 were probably caused (at least mostly) by inhibition of ARF6.

MYPT1 and MLC phosphorylations are involved in contractions related to calcium, protein kinase C, and Rho kinase (Somlyo and Somlyo 2000, Somlyo and Somlyo 2003). According to the findings of this thesis, NAV2729 did not inhibit prostate stromal cell proliferation and contraction by inhibiting MYPT1 or MLC phosphorylation.

In our experiments on WPMY-1 cells, concentrations at 1 μM, 2.5 μM, 5 μM were applied, close to the concentration in a previous study which showed IC_{50} values of 1 and 3.4 μM in fluorometric and orthogonal radiometric nucleotide exchange assays, respectively (Yoo, Shi et al. 2016). To prove the specificity of NAV2729 in this thesis, pull-down assays were carried out in WPMY-1 cells, showing inhibition of ARF6 activity by NAV2729. In contrast, ARF1, RAC1, RhoA inhibition were not detected. This was in line with previous reports (Yoo, Shi et al. 2016), suggesting specific ARF6 inhibition by NAV2729. Our study confirmed that the effects are caused by targeting ARF6 instead of other GTPases. Specificity was also examined by applying NAV2729 to ARF6-silenced WPMY-1 cells. Compared to wildtype WPMY-1 cells, NAV2729 showed attenuated effects on proliferation and viability in ARF6-silenced cells. The remaining effects in ARF6-silenced WPMY-1 cells could be caused by residual ARF6 expression, or by slight off-target effects. Even though it is unclear which reason it might be, any non-specific effects of NAV2729 are insufficient to explain the strong inhibition of proliferation in wildtype WPMY-1 cells. Based on the experimental data presented here, a role of ARF6 in promoting prostate growth and prostate smooth muscle contraction appears possible. In addition, a high specificity without off-target effects of NAV2729, at least below a concentration of 5 μM in targeting of ARF6, could be concluded.

BPH occurs with progressive hyperplasia of both the glandular epithelial cells and the stromal cells (Strand, Costa et al. 2017). Complex cellular alterations can be involved in this process including changes in proliferation, differentiation, and apoptosis, which could result in the enlargement of the prostate gland (McNeal 1990, Price, McNeal et al. 1990, Lee and Peehl 2004). Both the smooth muscle contraction and the enlargement of the prostate as a result of stromal growth in BPH patients can promote urethral obstruction, impaired bladder outlet and consequent voiding symptoms (Oelke, Bachmann et al. 2013, Hennenberg, Stief et al. 2014). Thus, a relationship between contraction and stromal growth in LUTS suggestive of BPH may be assumed. In line with the experimental findings presented here, a dual role of ARF6 in the LUTS suggestive of BPH could be assumed. On the one hand, ARF6 activation leads to hyperproliferative prostate stromal cells. On the other hand, ARF6 may be crucial for regulating smooth muscle contraction. The proliferation of prostate stromal cells and the prostate smooth muscle contraction should not be regarded separately from each other in the light of the findings of this thesis. Instead, they should be considered as connected factors. The reason may be that both the proliferation and contraction depend on the correct cytoskeleton organization. During the mitosis process, the spindle apparatus is mainly formed by cytoskeletal components and is involved in the correct segregation of chromosomes (Wittmann, Hyman et al. 2001). This provides an explanation of the finding presented here that the disorganization of actin formation in WPMY-1 cells after NAV2729 treatment or ARF6 silencing is paralleled by reduced proliferation. Similar dual roles of further GTPases have also been reported, as inhibitors for RAC GTPases or Rho-kinase are believed to inhibit prostate smooth muscle contraction and stroma proliferation (Rees, Foxwell et al. 2003, Wang, Kunit et al. 2015).

Each type of the most used established drugs for LUTS, which are α_1-adrenoceptor blockers and 5α-reductase inhibitors, has clear drawbacks. α_1-Adrenoceptor blockers have a low responder rate among patients (Abrams, Schulman et al. 1995, Chapple, Wyndaele et al. 1996). 5α-reductase inhibitors do not inhibit prostate smooth muscle contraction, but function in a way to reduce prostate cell proliferation, thus reducing prostate size. Thus, three months or more may be required to achieve improvement of symptoms. Considering that NAV2729 inhibits prostate smooth muscle contraction, as shown in parallel investigations in the laboratory where this thesis was performed (Yu, Gratzke et al. 2019), and inhibits growth of stromal cells, it appears possible that NAV2729 may represent a compound targeting both at once, if applied *in vivo*. Thus, at least in experimental models *in vitro*, smooth muscle contraction and growth in the prostate can obviously both be inhibited by single drugs.

It could be speculated that a procontractile role of ARF6 similar to the role of ARF6 in promoting cell proliferation and smooth muscle contraction may also exist in other smooth muscle organs such as airways, gastrointestinal tract, or bladder, as similar roles of RhoA are also reported in other organs (Christ and Andersson 2007, Puetz, Lubomirov et al. 2009, Chiba, Matsusue et al. 2010, Rattan, Phillips et al. 2010, Loirand and Pacaud 2014). This would be important from a clinical view, considering that smooth muscle-based diseases include hypertension, complications of diabetes, obstructive lung diseases, and other diseases.

In our study, NAV2729 proved its effects on stroma growth and stroma contractions in vitro. Further investigations are needed to understand the underlying molecular mechanisms of ARF6 functions seen here. By MYPT1 and

MLC phosphorylation analyses, it can be at least concluded that the inhibition of contraction may not result from inhibition of MLC phosphorylation, as NAV2729 did not reduce the content of phospho-MYPT1, total MYPT1, phospho-MLC or total MLC.

NAV2729 could be a promising compound in the context of LUTS suggestive of BPH. Certainly, it is still unclear whether NAV2729 leads to clinically relevant improvements like urodynamic effects, remission of LUTS, or reduction of BPH. In addition, side effects or toxicity are not examined yet in vivo. Even though with the lack of further confirmation on animal models or clinical trials, our data raised evidence of applying a GTPase inhibitor NAV2729, which is targeting ARF6, to create a new therapy that could lead to a better outcome in LUTS management.

6. Conclusions

ARF6 is involved in promoting prostate stromal proliferation and prostate smooth muscle contraction. These functions can be inhibited by NAV2729. This inhibition shows specificity without off-target effects, at least below a concentration of 5 μM NAV2729. The effects of NAV2729 observed here do not result from inhibition of MLC phosphorylation. A role of ARF6 in targeting prostate stromal proliferation and smooth muscle contraction together in the management of LUTS suggestive of BPH might be possible. A similar role in other smooth muscle organs (blood vessels, airways, etc.) appears possible and could be of high clinical relevance.

7. Zusammenfassung

Symptome des unteren Harntraktes (*lower urinary tract symptoms*, LUTS) aufgrund einer benignen Prostatahyperplasie (BPH) sind durch eine überaus hohe Prävalenz gekennzeichnet. Im Gegensatz dazu sind die Möglichkeiten der medikamentösen Behandlung stark begrenzt. Den BPH-bedingten Beschwerden beim Wasserlassen liegt meist eine Verengung der Harnröhre zu Grunde. Diese ist zum einen durch einen erhöhten glattmuskulären Tonus in der Prostata, und zum anderen durch die Vergrößerung des Organs bedingt, und führt durch Beeinträchtiung der Blasenentleerung zu Symptomen. Folgerichtig stellen beide Vorgänge Angriffspunkte für medikamentöse Therapien dar. Option der ersten Wahl sind α_1-Adrenozeptor-Antagonisten („α_1-Blocker“), welche über eine Hemmung α_1-adrenerger Kontraktionen in der Prostata zu einer Erweiterung der Harnröhre und schnellen, jedoch nur teilweisen Verbesserung von Harnstrahl und Symptomen führen. Allgemein können maximal 50%ige Verbesserungen durch α_1-Blocker erwartet werden, während Plazebos zu Verbesserungen von Harnstrahl und Symptomen um 27% bzw. 40% führen. Darüberhinaus werden 5α-Reduktase-Inhibitoren eingesetzt, um das Wachstum der hyperplastischen Prostata hemmen und so die Progression sowie das Auftreten von Komplikationen zu verzögern. Kombinationstherapien beider Wirkstoffklassen kommen häufig zum Einsatz. Leider sind hierbei ausschließlich die Nebenwirkungen additiv, jedoch nicht die positiven Effekte. Folgen sind Abbruchraten bis zu 90% innerhalb des ersten Jahres nach Erstverschreibung, was zu Progression, Veschlechterung, Hospitalisierung und Operationen führt.

Glattmuskuläre Kontraktion und Wachstum in der hyperplastischen Prostata wurden jahrzehntelang überwiegend separat voneinander betrachtet, und kaum miteinander in Verbindung gebracht. Die hier präsentierten Ergebnisse weisen daraufhin, dass beide Vorgänge jedoch über die monomere GTPase miteinander

verknüpft sind. Arbeiten im Vorfeld der hier durchgeführten Untersuchungen führten zu der Vermutung, dass ARF6 einen treibenden molekularen Faktor der glattmuskulären Kontraktion in der Prostata, sowie des Zellwachstums sein könnten. Belege für eine prokontraktile Rolle von ARF6, sowie für eine ARF6-vermittele Proliferation in Prostatazellen standen jedoch bislang aus. Im Rahmen der vorliegenden Promotion konnte nun in einem Zellkulturmodell gezeigt werden, dass ARF6 die Kontraktion von prostatischen Stromazellen, sowie das Wachstum dieser Zellen antreibt, und dass sich beide Prozesse durch den mutmaßlichen ARF6-Inhibitor NAV2729 hemmen lassen.

Für die Untersuchungen wurde eine immortalisierte Zelllinie aus dem Stroma einer humanen Prostata verwendet (WPMY-1). Durch Transfektion mit einer siRNA wurde in diesen Zellen ein silencing der ARF6-Expression etabliert. Im Vergleich zu Zellen, die als Kontrolle mit unspezifischer siRNA transfiziert wurden, zeigten diese eine verminderte Viabilität (CCK8-Assays), eine verminderte Proliferation (EdU-Assays), eine (z. T. vollständig) gehemmte Kontraktilität (*matrix contraction*-Assays), sowie einen Zusammenbruch der Aktin-Organisation (Phalloidin-Färbungen). Dieselben Effekte, sowie ein vermindertes Wachstum in Zellkolonie-Bildungs-Assays war zu beobachten, wenn WPMY-1 Zellen mit NAV2729 behandelt wurden. Die Kontraktilitäts-Hemmung beruhte vermutlich auf dem Zusammenbruch des Aktinzytoskelets. Grundsätzlich ist neben einer korrekten Aktin-Organisation auch eine Phosphorylierung der leichten Myosinketten erforderlich. Diese wurde jedoch durch NAV2729 nicht herabgesetzt, worauf Western-Blot-Analysen mit phospho-spezifischen Antikörpern hinweisen. Die Effekte von NAV2729 waren in Zellen mit ARF6-silencing deutlich eingeschränkt, was auf eine hohe Spezifität des Inhibitors für ARF6 schließen lässt. Pull-down-Assays zur Untersuchung verschiedener GTPase-Aktiviäten bestätigten, dass NAV2729 in

WPMY-1 Zellen ARF6 hemmte, jedoch nicht die weiteren, z.T. Kontraktions-relevanten GTPasen RhoA, RAC1 oder ARF1.

Die Ergebnisse werden auf mehreren Ebenen diskutiert. Kontraktion und Wachstum in der Prostata werden zwar häufig gleichzeitig medikamentös gehemmt, was Kombinationstherapien erfordert. Eine behandlungsbedürftige Symptomausprägung, einhergehend mit einem hohen Progressionsrisiko ist v. a. in fortgeschrittenem Alter zu verzeichnen. Diese Altersgruppe ist stark von Polypharmazie betroffen, so dass neue Wirkstoffe, mit denen sich Prostata-bedingte Probleme durch eine einzige Substanz behandeln lassen könnten, aus klinischer Sicht potentiell von großem Interesse sind. Im grundlagenwissenschaftlichen Kontekt zeigen die vorliegenden Ergebnisse, dass Kontraktion und Wachstum in der Prostata miteinander verknüpft sind, und nicht unabhängig voneinander agieren. Für diese Verknüpfung scheint ARF6 eine essentielle Rolle zu spielen. Außerhalb des urologischen Zusammenhangs wiederum sind diese Ergebnisse die vermutlich ersten, die eine ARF6-vermittelte Kontraktion belegen, und könnten daher für andere glattmuskuläre Organe, Krankheiten und Fachgebiete relevant werden (arterielle Hypertension, obstruktive Lungenkrankheiten), sofern sich eine analoge Rolle von ARF6 in diesen Geweben bewahrheiten sollte.

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9. Abbreviations

5-AR	5α-reductases
5-ARIs	5a-reductase inhibitors
5-EdU	5-Ethynyl-deoxyuridine
α-SMA	Vimentin α-smooth muscle actin
AR	Androgen receptor
ARF	ADP-ribosylation factor
ATCC	American Type Culture Collection
AUA	American urological association
AUR	Acute urinary retention
BDM	2, 3-Butanedione Monoxime
BMI	Body mass index
BOO	Bladder outlet obstruction
BPE	Benign prostatic enlargement
BPH	Benign prostatic hyperplasia
BPO	Benign prostatic obstruction
BSA	Bovine serum albumin
CB	Cannabinoid
CCK	Cell Counting Kit
CDC42	Cell division cycle 42
cGMP	Guanosine monophosphate
CP	Crossing points

DAG	Diacylglycerol
DAPI	4’,6’-diamidino-2-phenylindole-dihydrochloride
DHT	Dihydrotestosterone
DMSO	Dimethyl sulfoxide
DO	Detrusor overactivity
DTT	Dithiothreitol
DU	Detrusor underactivity
EAU	European association of urology
ECL	Enhanced chemiluminescence
ECS	Endocannabinoid system
EDTA	Ethylenediaminetetraacetic acid
EdU	5-Ethynyl-deoxyuridine
ET-1	Endothelin-1
FAAH	Fatty acid amide hydrolase
FCS	Fetal calf serum
FDA	US Food and Drug Administration
FITC	Fluorescein isothiocyanate
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GAPs	GTPase-activating proteins
GDP	Guanosine diphosphate
GEFs	Guanine nucleotide exchange factors
GTP	Guanosine triphosphate
GTPase	Guanosine triphosphate kinase

HDL	High-density lipoprotein
IC_{50}	Half maximal inhibitory concentration
ICS	International Continence Society
IP_3	Inositol 1,4,5-trisphosphate
IPSS	International Prostate Symptom Score
LHRH	Luteinizing hormone-releasing hormone
LUTS	Lower urinary tract symptoms
MAPK	Mitogen-activated protein kinase
MLC	Myosin light chain
MLCK	Myosin light chain kinase
MYPT1	Myosin phosphatase-targeting subunit 1
NE	Norepinephrine
OAB	Overactive bladder
PBS	Phosphate-buffered saline
PBS-T	Phosphate-buffered saline-Tween
PDE	Phosphodiesterase
PGH_2	Prostaglandin H2
PI3K	Phosphoinositide 3-kinase
PIP_2	Phosphatidylinositol 4,5-biphosphate
PKC	Protein kinase C
PLC	phospholipase C
PSA	Prostate-specific antigen
PVDF	Polyvinylidene-Fluoride

QoL	Quality of life
Q_{max}	Maximum flow rate
RAC	Ras-related C3 botulinum toxin substrate
RE	Retrograde ejaculation
RhoA	Ras Homolog Family Member A
RIPA	Radioimmunoprecipitation assay
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
RT	Reverse Transcription
RT-PCR	Real time polymerase chain reaction
SDS	Sodium dodecyl sulfate
S.E.	Standard error
SNRIs	Serotonin and norepinephrine reuptake inhibitors
SRB	Sulforhodamine B sodium salt
TCA	Trichloroacetic acid
TXA_2	Thromboxane A2
UR	Urinary retention
UTIs	Urinary tract infections

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Affidavit

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I hereby declare, that the submitted thesis entitled:

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