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Effect of ghrelin receptor ligands on proliferation of prostate stromal cells and on smooth muscle contraction in the human prostate

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1. Introduction

1.1 Definition of LUTS

Lower urinary tract symptoms (LUTS) are a group of symptoms related to the prostate, urethra and bladder. LUTS have three components: voiding or obstructive symptoms, storage or irritative symptoms and post-micturition symptoms [1]. The voiding symptoms include hesitancy, which means a longer wait time for the stream of urine to start, weak stream, straining during micturition, dripping after urination is over or intermittent stream. The storage symptoms include urgency (feeling an urgent need to urinate), frequency (a short interval between requirement to urinate), nocturia (have to pass urine more than two times during the night and waking from micturition), and urge incontinence (a sudden, intense urge to micturition after an uncontrolled loss of urine) [2]. The post-micturition symptoms, which have received little attention, involve a feeling of incomplete emptying [3]. All three components have a potential burden on daily quality of life [4].

Chronic conditions such as obesity, diabetes or hyperglycaemia, vascular hypertension or OSA (obstructive sleep apnoea) may be related with LUTS [5, 6]. Especially based on epidemiologic studies, a relationship between metabolic syndrome and LUTS has been discussed in recent years, and is now widely accepted [7]. Lifestyle is also an important factor; heavy smoking, water intake during the night time, alcohol abuse or caffeinated drinks (tea, coffee, cola drinks), and even lack of physical activity can worsen storage symptoms [8, 9].

Voiding symptoms are most commonly caused by an obstruction at the outlet of the bladder base, which impedes the passage of urine [10]. Causes of this obstruction include an enlarged prostate gland and an increased smooth muscle tone in the prostate. In fact, benign prostatic hyperplasia (BPH) is one of the

most common causes of LUTS. However, there is no evidence of linkage between prostate volume and LUTS occurrence. Other causes of LUTS include neurological disorders (especially, in overactive bladder, neurological factors induce storage symptoms), stroke, taking medicine, drug abuse and mental illness [11].

Recently, the relationship between LUTS and metabolic syndrome (MetS) has become a popular topic of epidemiologic studies [5, 6, 12]. Before exploring this relationship, it is necessary to understand the definition of MetS. MetS is a term encompassing several parts that reflect abundant nutrition, sedentary lifestyle, and superfluous adiposity; these include obesity, insulin resistance, increased blood pressure and pro-inflammatory state [13]. MetS is regarded more as a cluster of different conditions than as a simplex disease, which means that the pathophysiological mechanism, treatment and prevalence are complex and numerous hormone levels are different from those of healthy people [14]. In other words, the risk of several diseases will increase under this disordered hormone level. MetS is related to an approximate doubling of cardiovascular system disorder risk and 5-fold increased risk for incidence of type 2 diabetes mellitus (T2DM) [15, 16]. In addition, current literature points out the important role of MetS-induced metabolic derangements in the development of benign prostate enlargement (BPE), which means an increased size of gland secondary to BPH. Patients who are overweighed have a higher risk of having MetS as a determinant of their prostate enlargement [17].

1.2 Epidemiology, etiology and nature history of LUTS

An epidemiological study from 2010 predicted that an estimated number of 2.3 billion individuals will be influenced by any types of LUTS [18]. Focusing on the LUTS patients' distribution in 2018, Asia has the greatest absolute population of LUTS, followed by Europe, Africa, North America, and South America [18]. For Europe, the increasing LUTS population is mainly due to the enlargement of the overall ageing group [4]. Although these results are dynamic,

they still shown that the worldwide burden of LUTS is increasing. In the next decade, the global expenditure of LUTS will keep rising [19]. Until now, epidemiologic studies have noted that a group of ageing-related diseases [20], cardiovascular disease and a combination of these disorder conditions, such as MetS, have a significant effect on LUTS [21].

Furthermore, emerging clinical studies suggest that MetS may have a close relationship with BPE, which may be considered as the possible cause of LUTS [21]. Recently, strong evidence indicated that patients with MetS exhibited greater prostate sizes in the European population, suggesting that MetS participates in the development of BPH/LUTS [22]. Other etiologic factors, i.e. diet and lifestyle, which may lead to MetS, may also contribute to the process of BPH/LUTS [3]. Based on a multicentre and prospective study of a cohort database on US men, researchers suggested that the effect of MetS on BPH/LUTS pathogenesis may start in the early stage of adulthood, especially increasing body mass index (BMI), higher prostate volume and inflammation status of the prostate [23].

The prostate gland has two growth periods. The first occurs in early puberty, when the prostate doubles in the size; the second is continuing slow growth for many years when the man is approximately aged 25 years [24]. This slow growth generally does not lead to problems until late in life; then, it can result in clinically relevant BPH [25]. Normally, BPH symptoms are rare in patients younger than 40 years old, but more than 50 % of men after retirement (60 years old) and 90 % of men in their 70s and 80s have at least any LUTS [26]. The burden of LUTS also needs full consideration. Recently, a novel study evaluated the burden of LUTS using severity and bother of the symptoms and suggested that for younger male patients, post-micturition dribble was the greatest burden, while in middle-aged and elderly men, urgency and nocturia were the most burdensome symptoms. In conclusion, the burden of LUTS increases with increasing age [27].

1.3 Pathogenesis of LUTS suggestive to BPH

LUTS occur in male and female patients, and increase with age. In male patients, LUTS commonly occurs in parallel with BPH. Based on the anatomy of the lower urinary tract, the prostate is one of the accessory organs of the male reproductive system along with urethral glands, which is divided into three zones (peripheral, transitional and periurethral zone) [28]. BPH commonly occurs in the transitional and periurethral zone of the prostate. Enlargement of the prostate induces LUTS by two approaches: 1) as the so called "static component", where the enlargement of prostate tissue induces compression of the urethra and finally bladder outlet obstruction (BOO); 2) the so called "dynamic component" describes an increased prostate smooth muscle tone, which contributes to urethral obstruction in parallel to the enlargement [29] (Figure 1). A clinical study suggests that as the age increases, the size of the prostate also increases and the prevalence of LUTS raised significantly with the age [30].

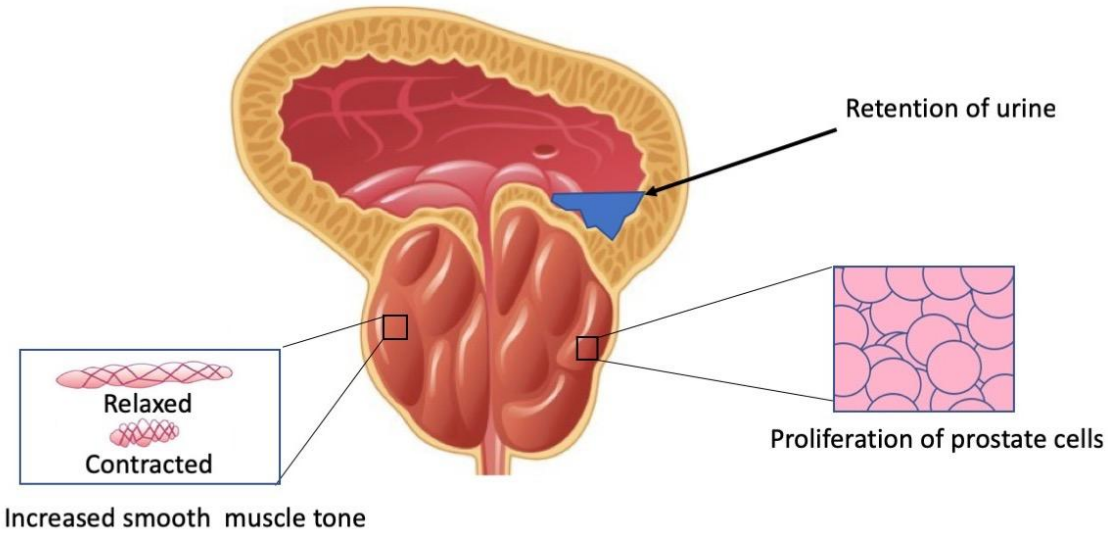

Figure 1. Smooth muscle contraction and proliferation of prostate cells are the two key processes causing urethral obstruction and LUTS suggestive to BPH. Enlargement of the prostate induces bladder outlet obstruction, resulting voiding symptoms or even retention of urine. In advanced BPH, the risk of acute urinary retention (AUR) will be increased and medications are required in moderate-to-severe LUTS patients. Meanwhile, the alteration of

prostate smooth muscle tone is another key component of LUTS suggestive to BPH (All diagram materials are from https://www.123rf.com and https://www.vectorstock.com/).

While the mechanisms, how BPH causes urethral obstruction and BOO are clear, the precise aetiology of BPH is still not completely clear. Certainly, there is an increased number of stromal and epithelial cells in the median lobe of the prostate, which may be due to proliferation or reduced programmed cell death [31-33]. Both of these mechanisms can lead to cellular accumulation and prostate enlargement [34, 35]. Furthermore, it has been suggested that androgens are vital to the process of BPH [36]. Nevertheless, dihydrotestosterone (DHT) is the active metabolite, which leads to prostatic growth. Testosterone is processed to DHT by the enzyme 5-alpha reductase (5-AR). However, the pathogenesis of BPH is not only a DHT-related mechanism [37].

Androgen receptors located in the prostate contribute to the development of BPH [38, 39]. Recent in vivo data suggested that estrogens take part in androgen-related enlargement of the prostate, by sensitization for androgen [40]. It is generally accepted that BPH is mainly a stromal disease, but the foci of initiating events is still unclear [41]. As mentioned by several reports, chronic inflammation may contribute to the genesis of BPH [42]. Cytokines (IFN-α, IL-2, IL-6, IL-8 and IL-15) were identified and confirmed in the process of fibro-muscular prostatic growth [43], however, the precise role of these cytokines in the development of BPH remains unclear.

It is widely accepted that a multifactorial aetiology contributes to LUTS suggestive of BPH [44]. Ageing is one of the most critical risk factors for development of LUTS [39]. The detailed molecular pathogenesis of BPH/LUTS has not been well established. However, in addition to age, inflammation, sex hormones, and metabolic factors have all been implicated [45].

1.3.1 Age

In younger male patients, LUTS can be caused by chronic prostatitis, which can lead to persistent dysuria and other symptoms that could cause men great concern. In clinical practice, younger men worry that dysuria could be caused by sexually transmitted diseases (STD) or cancer in genitourinary system [46]. Among elderly men, the most bothersome symptoms were urgency and nocturia. According to reports and patients' surveys, middle-aged men who were near retirement had even patterns of experienced burden for each symptom, what makes sense as age has an important role in LUTS [27, 47]. Even though urgency was the most bothersome symptom in elderly men, patients have higher risks for serious complications from other diseases (hypertension, heart disease and diabetes) than from the urinary system. Urologists can predict the prostate volume via free prostate-specific antigen, total PSA and patient age [48]. There is an acclaim in considering BPH as a part of the process of ageing [49]. From the above, it is suggested that the global economic costs of LUTS/BPH treatment will continue to increase in the following decades [18, 50]. Because the options for treatment are still limited, the exploration of putative new targets and novel medications is necessary.

1.3.2 Inflammation

Chronic inflammation is a risk factor for fibrosis in many organs, such as liver and kidney [51]. High presence of chronic inflammation infiltrates in prostate specimens, including radical prostatectomy specimens and transurethral resection prostate strips [52]. Chronic inflammation may also be linked to BPH in pathological diagnosis [52]. Recently, a prospective study including 167 autopsied prostates indicated that the presence and location of chronic inflammation were significantly associated with development of BPH; however, there was no association with prostate cancer [53]. Evidence from epidemiological studies implies that prostatic inflammation is related to the development of BPH, which tends to stimulate the continuous response to

metabolic stress signals [54]. A modest overall increased prevalence of BPH/LUTS in overweighed individuals is probably explained by the prostatic inflammation hypothesis. Currently, studies seek to estimate whether urinary cytokines reflect clinical attributes of LUTS suggestive of BPH [55]. The cytokines, including chemokine (C-X-C motif) ligand 1 (CXCL-1), CXCL-8, CXCL-10, C-C motif chemokine ligand 2 (CCL2), CCL3, and CCL5 in the recruitment of cluster of differentiation 3 T cell co-receptor (CD3+) and CD20+ , appear to take part in the process of LUTS by means of enhancing the chronic inflammatory response in prostate parenchyma [56, 57].

1.3.3 Sex hormones

5α-reductase regulates the transformation of testosterone to DHT. Higher DHT expression parallels prostate enlargement. Treatment with 5α- reductase inhibitors (5ARI) has been reported to decrease prostate the volume in BPH, indicating the irreplaceability of DHT in the development of prostate growth [58]. Even though androgens are necessary for morphology and the basic function of the prostate, oestrogen also maintains vital biological functions. Estrogen plays a critical role in BPH development, and the prostate is widely considered as an estrogen target tissue [59, 60]. Furthermore, more details in the literature concluded that estrogens and selective estrogens receptor modulations directly and indirectly have effects on not only growth but also differentiation of the prostate [40]. Therefore, hormone research on therapeutic effects for BPH will be promising for the ageing male patients [61].

1.3.4. Metabolic factors

Recent literature reported that patients with metabolic syndrome and related diseases, including obesity, hyperinsulinaemia, and insulin resistance, have a high risk to develop BPH/LUTS. The assumption of Vignozzi and colleagues suggested a plausible hypothesis, implying the importance of metabolic factors in the development of BPH. Their hypothesis drew attention to this linkage and

may be useful in exploring the relationship between metabolic syndrome and BPH [17, 61]. According to this theory, the step-stone of this process is chronic subclinical inflammation, which depends on the fat tissue of the patient and may be overlapped by metabolic factors, and indeed alters the sex hormone levels. The combined effect of these three hits reflect on disordered growth factors, which lead to the enlargement of prostate gland. In basic studies, non-genomic animal models of MetS, generated by feeding rabbits with high-fat diet for 12 weeks, exhibited severe prostatitis-like syndrome, tissue remodelling and bladder dysfunction [17]. In addition, several inflammatory cytokines and chemokines secreted by prostate cells increased in animal models of MetS [17]. A study via metabolomics analysis in several male patients with LUTS suggested that the profiles of amino acids in the plasma may contribute to metabolic syndrome [62]. Meanwhile, increasing glutamate and decreasing arginine, asparagine, citrulline and glutamine are associated with LUTS in males [62].

More recent evidence highlighted that obesity significantly raised the occurrence of BPH, and a relationship exists between diabetes and BPH [63]. Furthermore, MetS is thought to be associated with BPH/LUTS, likely through chronic inflammation. Cross-sectional and longitudinal studies tend to focus on vascular risk factors that appear to be related to the occurrence and degree of LUTS [64]. Similarly, moderate physical activity may reduce the risk of BPH [65]. Various mechanisms for this linkage have been discussed, such as reduced oxidative damage to the prostate cells and decreased sympathetic tone of prostate smooth muscle [65]. Because obesity appears to reduce the effect of dutasteride, these observations suggested that the development of new prevention strategies and treatment focus on weight loss and lifestyle, adiposity, and a individualized treatment of BPH according to patient comorbidities [66]. The mechanism of this effect might be related to the normalization of the various hormone levels in the serum of patients suffering with metabolic syndrome.

1.3.5 Other urologic diseases associated with LUTS

In clinical practice, it is observed that other diseases also produce LUTS, and the symptoms of several diseases are similar to LUTS. For example, LUTS associated with ketamine abuse has recently been a topic at least in Asia [67]. This syndrome has a clear cause and will cease if the ketamine use is suspended. However, urologists tend to ignore the true cause of this syndrome because the patients conceal their history [68]. Therefore, having a clear understanding of other diseases that produce LUTS is necessary and important for the following treatment.

1.3.5.1 Overactive bladder (OAB)

Patients with LUTS due to OAB likely suffer for urinary incontinence (UI) [69]. OAB causes a subset of storage LUTS defined by the International Continence Society as urgency, usually with frequency and nocturia [70]. Notably, male patients who are suffering with LUTS associated with OAB are more likely to respond to antimuscarinic therapy than to α_1-blockers [71]. This points to the divergent mechanisms underlying the voiding symptoms caused by BPH, and the storage symptoms caused by OAB.

1.3.5.2 Bladder tumour

In a recent, retrospective cohort study, the investigators demonstrated the linkage between LUTS and occurrence of initial bladder tumour [72]. Tumours in the lower urinary tract may also cause LUTS [19]. For instance, a case report showed that soft tissue chondroma located in the bladder and caused LUTS and nocturia [73]. Tumours in the lower urinary tract may cause compression in urethra or impair the bladder outlet, which will finally result in obstructive LUTS resembling those caused by bladder outlet obstruction in BPH [4]. However, in such patients, the cancer and tumour burden will be most bothersome, and therapy will focus on cancer treatment [74].

1.3.5.3 Spinal cord injury

Neurogenic lower urinary tract dysfunction is a very common and under-assessed disease, which affects the quality of life of patients and is a consequence of many diseases, such as stroke, Parkinson disease and spinal cord injury (SCI) [75-77]. SCI damages the descending motor and ascending sensory pathways, preventing normal bladder function and urethral control [77]. All of these lead to LUTS.

1.3.5.4 Prostate cancer

The linkage between prostate cancer and LUTS still needs more support from clinical data. The debate about this relationship is currently focused on PSA testing. The PSA test is frequently used for confirming prostate cancer [78-80]. However, the test's limitation may cause false results and over diagnosis. In a cohort study based on a large population, development of LUTS was strongly related to localized prostate cancer, but there was no association with fatal prostate cancer (defined as who suffering from regional or distant metastases). In other words, monitoring of LUTS in the early stage of cancer is not helpful because the urinary symptoms are not only induced by prostate cancer [81].

1.3.5.5 Chronic pelvic pain syndrome

Different groups of symptoms have been regarded as chronic pelvic pain syndrome, including chronic, abacterial prostatitis, interstitial cystitis, and painful bladder syndrome [82]. Some studies suggested that the prostate factors may be important in the pathogenesis of chronic pelvic pain syndrome, which also plays a role in LUTS suggestive of BPH [83]. For instance, approximately 30 % of men who diagnosed with chronic prostatitis, also report symptoms relative to pelvic pain syndrome [84].

1.3.6 Genetic determinants

Aetiologic studies on BPH/LUTS appear difficult and complex because various processes participate in prostate enlargement and are suggestive of LUTS, such as prostatic stromal cell growth and altered smooth muscle tone. A series of genetic alterations may contribute to BPH/LUTS [18]; however, there is a pressing need for improved understanding of the relationships between the symptoms and target genes [85]. Nevertheless, patients with large prostates are susceptible to serious LUTS; however, some patients with the same prostate volume may not experience bothersome LUTS and do not require any medication [86]. Therefore, genetic determinants appear possible, and a genome-wide association study (GWAS) is necessary for LUTS research. Recently, a GWAS study based on 5000 subjects reported that GATA3 could be a promising candidate gene for BPH/LUTS research. In BPH/LUTS populations, the expression of GATA3 is marginally increasing, which means that this gene may contribute to the development of BPH/LUTS [8]. Although this result is still preliminary and does not reach a significant level for GWAS, it could be regarded as a new vision for exploring the aetiological mechanism of BPH/LUTS in the future.

1.4 The role of MetS in LUTS

The importance of metabolic syndrome such as diabetes and obesity in clinical work increased. The morbidity and mortality associated with metabolic syndrome are expensive and represent a serious burden to numerous patients [87]. Recent studies reported that metabolic syndrome probably plays a role in the development of LUTS [23, 88, 89]. MetS is a group of diseases and states, including obesity and pro-inflammatory state. Behind these various patterns of disease, the main pathophysiology is insulin resistance. There are several factors surrounding insulin resistance that affect the development of metabolic syndrome and related target organs such as liver, kidney and prostate.

Furthermore, MetS may induce the metabolic stress to prostate cells and also play an important role in LUTS suggestive to BPH.

1.4.1 Obesity and LUTS

A case-control study showed that compared with normal BMI subjects, men who were obese had medium higher odds of suffering LUTS [90]. Another clinical population-based cohort trial suggested that the risk of developing LUTS increased with waist circumference and BMI in women [91]. Interestingly, physical activity was a protective factor for obese patients. The researchers used daily walking time as an evaluation index and found that the men who walked daily had decreased chances of developing LUTS [22]. Currently, the mainstream hypothesis is that obesity has a linkage with LUTS in ageing men, but the mechanism of this association is still unclear [92-95].

1.4.2 Insulin resistance in smooth muscle

Various related pathways may explain the association of insulin resistance (IR) and BPH/LUTS. The first could be that the state of sympathetic nerve activity is upregulated [23]. Furthermore, disordered insulin levels lead to increased endothelin levels in vascular smooth muscle cells [96]. The increased nerve activity and overexpressed endothelin may lead to higher prostate smooth muscle tone [97]. At the same time, higher insulin levels may induce a chaotic trophic hormone state, which probably increases the prostate volume. Abnormal regulation of the insulin-like growth factor (IGF) axis has been reported in the development of BPH and the onset of prostate cancer [98, 99].

The pathogenesis of BPH includes a disruption of the balance of hormonal and other growth factors, such as IGF, leading to cellular proliferation of prostate stromal cells and to decreased extent in the epithelial regions of the prostate [99]. IGF and its binding proteins (IGFBPs) may contribute to the aetiology of BPH/LUTS [100]. Not only endogenous and exogenous hormones but also

genetic profiles affect expression level of circulating IGFs and IGFBPs. Furthermore, IGFBP3 has unique growth-inhibitory and pro-apoptotic actions. IGFBP3 is widely reported to inhibit angiogenesis by means of reducing vascular endothelial growth factor (VEGF) [99]. In a case-control study of BPH patients, which recruited 1454 individuals, researchers suggested that the risk of clinical BPH showed a positive relationship with the IGFI/IGFBP3 ratio, while clinical BPH risk was also related to IGFBP3 [101]. Moreover, this study depicted that modest associations between BMI and IGF contribute to the risk of development of BPH. Obese men in the upper 25 % of IGFI/IGFBP3 ratio had almost twice the risk of clinical BPH compared to those in the lower 25 % [102].

Insulin resistance or insulin sensitivity are very common in individuals with abnormal metabolic activity, and these alter not just the β-cells of pancreas but also the target organs [103]. In practice, obesity could be the impetus of insulin resistance, suggesting that ageing male patients suffering from diabetes and obesity at the same time will be the susceptible group of BPH/LUTS [104].

1.4.3 Pro-inflammatory molecules in LUTS

Several inflammatory factors are elevated in the metabolically abnormal individuals, such as C- reaction protein (CRP), tumour necrosis factor (TNF), IL-6 and IL-8. The main reason is that obesity induces cytokine release in parallel to macrophage infiltration [105]. This cytokine and inflammatory cell increase could maintain a pro-inflammatory state in the prostate [106]. Inflammatory infiltrates were commonly observed in prostate tissue. Cytokines IL-6 and IL-8, for example, are raised in MetS and may lead to inflammation in the development of BPH/LUTS; they can also be secreted by prostate stromal cells with cytokine stimulation or recruitment by inflammatory cells such as macrophages, and both result in enlargement of prostatic tissues. Furthermore, IL-8 could be a promising biomarker for BPH/LUTS [107].

1.4.4 Environmental factors that contribute to LUTS

In many patients, MetS becomes manifest in diseases such as obesity, hypertension, dyslipidaemia and diabetes. Individuals with MetS may be exposed to environmental stressors such as diet [108]. A twin study suggested that environmental factors, including diet, are associated with health in urinary system [109]. For instance, low-fat diets, grilled meat, vitamins, protein and vegetables are important factors in the development of BPH/LUTS [9]. Considering the substantial environmental effects, future researches tend to focus on identifying specific environmental risk factors that cause LUTS [9].

1.5 Pharmacological treatment for LUTS

For many years, surgery has been regarded as the standard therapy for relieving BPH/LUTS. In recent years, the new medical therapy has changed the guidelines of BPH/LUTS management, and moved surgery, mainly in the form of transurethral resection of the prostate, or laser procedures to the second line. In the cases of non-bothersome LUTS, watchful waiting is an option for the patients who have few risks of acute urinary retention (AUR) and other complications [4].

For individuals with moderate-to-severe LUTS, α_1-adrenoceptor antagonists (α_1-blockers) represent the mainstream medical therapy for BPH/LUTS [19]. Furthermore, patients suffering from moderate-to-severe LUTS and an enlarged prostate (prostate volume more than 40 mL) can obtain 5ARIs for supplement [110]. They can slow down or prevent the disease progression with regard to AUR and requiring for further surgery treatment. BPH/LUTS is accepted as a unique condition that influences the quality of life (QoL) [111]. However, non-selective α_1-blockers should be carefully applied in elderly patients in case of the occurrence of orthostatic hypotension [112]. For instance, the first non-selective α_1-blockers phenoxybenzamine was reported to be effective in BPH, but has several side effects, which include tiredness, dizziness, nasal

stuffiness, and hypotension [113]. After that, long-acting selective α_1-blockers are recommended by the American Urological Association (AUA) and European Association of Urology (EAU), which are effective and show better tolerability than non-selective α_1-blockers [114]. However, long-acting selective α_1-blockers may still damage sexual ability [4]. Moreover, LUTS patients without valid and stable treatment may have higher risk of renal insufficiency and chronic kidney disease (CKD), which may threat life [115]. Therefore, it is important to keep a high patient compliance of pharmacology treatment. The adherence to drug therapy in BPH/LUTS is based on the balance between efficacy and side effects. Several drug classes are available for medical treatment of LUTS, which were approved based on clinical trials and preceding preclinical research [19].

1.5.1 Monotherapy

1.5.1.1 α_1-blockers

1.5.1.1.1 Mechanism of action

Generally, the effect of endogenously released noradrenaline on smooth muscle cells in the prostate is inhibited by α_1-blockers. α_1-blockers have been suggested to decrease prostate smooth muscle tone and BOO [4]. Smooth muscle is a major cellular constituent of the prostatic stroma in BPH [116]. According to the high expression of α_1–adrenergic receptors in the human prostate smooth muscle, the adrenergic innervation in prostatic function is important for development of LUTS suggestive to BPH [117]. In controlled phase III clinical studies, obvious decreases in International Prostate Symptom Score (IPSS) were observed, parallel with improvements in maximum flow rate (Qmax) of approximately 3 points relative to placebo [112]. α_1-blockers improve the situation of many patients, as evidenced by numerous clinical studies. Consequently, they emerged to the most important medication for treatment of LUTS suggestive of BPH. Nevertheless, α_1-blockers have almost no effect on bladder outlet resistance, and

obstruction is barely improved by α_1-blockers. Thus, other mechanisms of action than smooth muscle relaxation in the prostate may also be relevant. Outside the prostate (e.g., urinary bladder and/or spinal cord), α_1-adrenoceptors and α_1-adrenoceptor subtypes (α_1b- or α_1d-adrenoceptors) may play a role as mediators of various effects [117] (Figure 2). In blood vessels, other non-prostatic smooth muscle cells, and even the central nervous system, α_1-adrenoceptors may regulate adverse events [118].

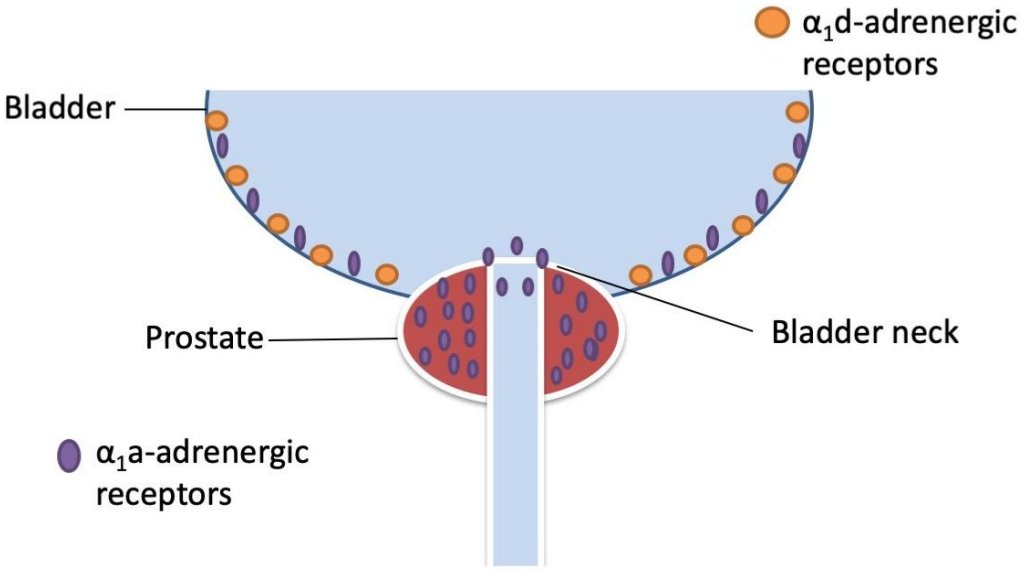

Figure 2. According to the receptor distribution in the lower urinary tract, the α_1a-adrenergic receptor subtype is primarily located in the prostate and bladder neck, with a few others within the bladder. Therefore, the α_1a-adrenergic receptors subtype predominates in prostate tissue and regulates smooth muscle tone.

1.5.1.1.2 Pharmacology of α_1-blockers

Various pharmacologic therapies are the first choice for treatment of BPH/LUTS. Normally, α_1-blockers are the preference for the patients. The usage of α_1-adrenergic blockers is based on targeting prostate α_1a-adrenergic receptors. Physicians pick up and prescribe several alpha-blockers in real-life clinical practice [119]. Three different subtypes of α_1-adrenergic receptors have been characterized: α_1a-adrenergic receptors, α_1b-adrenergic receptors, and α_1d-adrenergic receptors, which differ in amino acid composition [119]. The

α_1a-adrenergic receptors subtypes predominate in prostate tissue and regulate smooth muscle tone. Additionally, expression of the α_1a-adrenergic receptors subtype is raised in BPH prostatic tissue compared to non-BPH prostatic tissue. In non-BPH prostatic tissue, the proportion of α_1a to α_1b to α_1d receptors was 63:31:6; in BPH tissue, the ratio was 85:14:1 [118, 120]. Pharmacologic urologic selectivity of α_1-blockers relies on the binding affinities to these subtypes. Tamsulosin is 3 times more selective for the α_1a-adrenergic receptors subtype than for the α_1b- and α_1d-adrenergic receptors [121]. Naftopidil has a three times greater affinity for the α_1d- than for the α_1a-adrenergic receptors [122]. Silodosin is highly selective for α_1a-adrenergic receptors, with a 162 times greater affinity than for α_1b-adrenergic receptors and an approximately 50 times greater affinity than for α_1d-adrenergic receptors [123]. Alfuzosin is also a selective α_1-blocker, which provides quick and continuing relief of LUTS [124].

1.5.1.2 5ARIs

1.5.1.2.1 Mechanism of action

5-Alpha-reductases are enzymes that regulate the conversion of testosterone into dihydrotestosterone (DHT). In the 1970s, the critical role of the 5AR-dependent testosterone to DHT transformation in the development of BPH was claimed [125] (Figure 3). Following animal experiments and phase III clinical studies, finasteride (Proscar) and dutasteride (Avodart) were used in the therapy of BPH with LUTS. In the clinical studies, 5ARIs decreased prostate volume by 20 % to 30 % and also reduced scores of LUTS, while the risk of AUR and surgical treatment within 3 to 6 months was also reduced [126]. These highly selective inhibitors do not directly block the activity of testosterone and do not affect the circulating testosterone concentration. The effects of these 5ARIs are limited to the target organ, including prostate and scrotal skin, which lead to local production of DHT [127, 128].

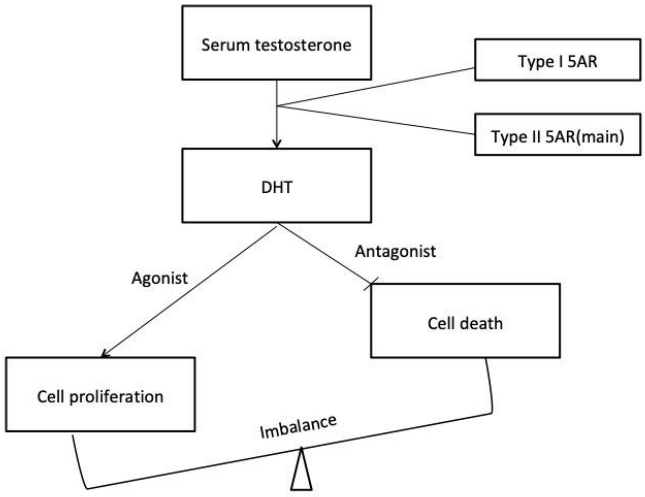

Figure 3. In the prostate cells, serum testosterone is converted to dihydrotestosterone, which relies on the 5ARs. There are two main types within the prostate cell (type 1 and type 2) [126]. Type 2 is the predominant form in prostate cells, but type 1 is still expressed. DHT enhances prostatic cell proliferation and suppresses apoptosis, i. e. blocks the process of programmed cell death.

1.5.1.2.2 Pharmacology of 5ARIs

According to available clinical evidence, finasteride monotherapy is regarded as an option in patients with medium to severe LUTS and a high prostate volume (>40 mL) [129]. Furthermore, FIN is also used to treat patients who do not have prostate enlargement [130]. Unlike finasteride, which inhibits 5AR isoform type II, dutasteride inhibits not only 5AR isoform type II but also type I [126]. Dutasteride has been called a dual inhibitor, and unlike finasteride, dutasteride has effects in patients with prostate volume ranging from 30 mL to 40 mL. Side effects such as erectile dysfunction (ED), decreased libido and ejaculatory disorders (EjDs) appear to be the most common complaints in clinical work, which result from 5ARIs [4]. Therefore, open discussion of the sexual side effects with patients who need this therapy is necessary and important [126].

1.5.1.3 Muscarinic receptor antagonists

Muscarinic receptor antagonists are not exclusive treatments for LUTS suggestive of BPH. In fact, their use in BPH is even argued by theoretical risks

that post-void residual urine may increase by such drugs, and detrusor inhibition may cause voiding difficulties and life-threatening acute urinary retention [131]. Muscarinic receptor antagonists are approved for treatment of OAB and storage symptoms [131]. In the past, muscarinic receptor antagonists were mostly examined in women because it was accepted, that male LUTS are mainly related to prostate disorder; therefore, physicians believe that male patients should be treated with prostate-specific medications [132]. Nevertheless, usage of muscarinic receptor antagonists in male patients suffering from LUTS suggestive of BPH and with symptoms of OAB has become increasingly relevant in the last decade. However, because of the absence of studies on the long-term effects of muscarinic receptor antagonists on LUTS, the prescription of muscarinic receptor antagonists should be cautioned [133]. Available medicines are fesoterodine, darifenacin, propiverine, oxybutynin, tolterodine, solifenacin, and trospium chloride.

1.5.1.4 β_3-adrenoreceptor agonists

In the urinary bladder, β_3-adrenoreceptor agonists are thought to cause relaxation of the bladder and prevention of urination [87], especially by relaxation of the detrusor [50]. Based on the effect on the detrusor, the application of β_3-adrenoceptor agonists on OAB has been established [134]. β_3-adrenoceptor mRNA is expressed in detrusor, and β_3-adrenoreceptor agonists were reported inhibited calcium oscillation in detrusor, which reduces detrusor contraction [135]. Functionally, the β_3-adrenoreceptor appears to be a promising target for pharmaceutical agents. According to preclinical studies, other evidence shows that the agonists of β_3-adrenoceptor is predominant over other β-adrenoreceptors and has the ability to reduce the detrusor contraction and good tolerability for OAB patients [136]. In clinical practice, mirabegron 50mg is the first licensed β_3-adrenoreceptor agonists for treating OAB patients and especially focus on the symptoms including micturition frequency and urgency [137].

1.5.1.5 PDE5

In mammalian species including human beings, the whole lower urinary tract expresses phosphodiesterase type 5 (PDE5) [138, 139]. Immunohistochemical (IHC) results depicted that PDE5 is localized in the lower urinary tract blood vessels, endothelial cells and smooth muscle cells. Furthermore, in in vitro studies, PDE5 inhibitors relaxed the contraction of isolated prostate and bladder neck strips. Based on this, PDE5 inhibitors were recommended by the European Association of Urology for treating LUTS suggestive to BPH [19]. Uniquely, PDE5 is expressed in human prostatic stromal cells, which are different from the lower urinary tract blood vessels. Several clinical evidences suggested that PDE5 inhibitors reduced the international prostate symptom score (IPSS) compared to the placebo. Based on a recent meta-analysis, monotherapy of PDE5 inhibitors was similar effective to α_1-blockers on reduction of postvoid residual urine (PVR) and IPSS [140]. According to guidelines for treatment of male LUTS, tadalafil is recommended and reaches an clinically objective improvement of LUTS, which is higher than that of placebos [141].

1.5.1.8 Herbal medicine

According to recent reports, in western countries, the investment and expenses in all types of BPH medications are rapidly increasing [19]. However, in eastern Asia, such as Japan and China , the expenditures of BPH therapeutics are still lower than in the western countries [142]. Several interactional studies suggest that plant extracts and herbal medicine occupy a considerable part of BPH pharmaceutical marketing [143, 144]. Serenoa repens (SeR), the most reported plant medicine for LUTS treatment, has been indicated by a multicentre study to significantly reduce the prostate volume in patients with BPH [145]. Gravas et al. suggested that SeR significantly reduced prostate inflammation in terms of histological parameters [145]. Morgia et al. suggested that SeR improved the slightly Qmax and IPSS in patients with moderate-severe LUTS suggestive to

BPH [146]. Nevertheless, they are not recommend by the guidelines of EAU or AUA [19, 147].

1.5.2 Combination therapies

1.5.2.1 Combination of α1-blockers with 5-ARIs

The most promising combination therapy is α_1-blockers with 5-ARIs, as demonstrated by several studies all over the world. According to Füllhase et al., some of these studies argued that the combination of α_1-blockers with 5-ARIs has been discussed thoroughly and scientifically for the groups in different ages [120, 130]. Generally, patients with large prostates (volume more than 40 mL), who have a high risk of complications, are the group with the largest profit from this combination therapy.

After considering the clinical index (IPSS, Q_{max}, and prostate volume), the effect of different treatment options was ranked by the physicians. The most recommended is combination therapy. A high-level clinical trial acclaimed that α_1-blockers and 5-ARI combination increased Q_{max} twice than α_1-blockers monotherapy [148]. In June 2010, a one set oral medication of dutasteride and tamsulsion (Jalyn, GSK, U.K.) was examined by the US Food and Drug Administration for use in BPH patients with enlarged prostate. The potential of combination therapy of α_1-block with 5-ARIs is apparent.

1.5.2.2 Combination of antimuscarinics and α_1-blockers

Male LUTS are often attributed to BOO suggestive of BPH and are treated with drugs targeting the prostate, including α_1-blockers and 5α-reductase inhibitors. However, these agents may not effect in many male patients with LUTS adequately, especially men with mixed LUTS, including voiding LUTS and storage LUTS. Antimuscarinics could be an alternative option for patients who are suffering with OAB symptoms despite treatment for prostatic enlargement. Therefore, Kaplan et al. suggested that antimuscarinics appear to be a powerful

and safe option for urgency, frequency, urgency urinary incontinence, and symptoms associated with OAB in male patients who are not suffering clinically significant bladder outflow obstruction, but antimuscarinics alone are not approved for the treatment of BPH. There is clinical evidence that antimuscarinics alone would increase the risk of AUR [149].

1.5.2.3 Combination of α_1-blockers and β_3-adrenergic receptor agonists

The β_3-adrenergic receptor plays a critical role in bladder smooth muscle relaxation [150]. Mirabegron, a β_3-adrenergic receptor agonist, is a first-line medication launched in 2011 that can be applied for the treatment of OAB symptoms. The side effects, including dry mouth and constipation, are lower with mirabegron than with anticholinergic drugs [4]. A randomized, controlled study reported that patients who have already received treatment of α_1-blocker can still benefit from the add-on treatment of mirabegron (50 mg per day) [151]. 50 % to 75 % male LUTS patients reported OAB symptoms, so that pharmacotherapy with an α_1-blocker alone could not reduce OAB symptoms [152]. Furthermore, the β_3-adrenergic receptor may increase bladder capacity and extend micturition interval [153]. These data suggest that combination of mirabegron with routine α_1-blocker management is an option to improve the QoL.

1.5.2.5 Combination of 5-ARIs and PDE5Is

According to clinical research, combination of different types of BPH treatment is superior to single management by α_1-blockers or 5-ARIs. Looking into the clinical practice patterns, 5-ARIs are commonly prescribed drugs for LUTS suggestive to BPH [119, 154], which decrease the development of BPH [155]. Combination of 5-ARIs with PDE5Is has been tested recently and the effect was estimated by several randomized controlled trials [126]. The combination therapy improves the effect in terms of clinical LUTS suggestive to BPH,

because PDE5Is principally inhibit contraction, and are applied for rapid symptom improvement [156]. Roehrborn et al. launched an international, randomized, double-blind, parallel study about the combination treatment of finasteride and tadalafil (both are 5 mg daily) [157]. Significant LUTS improvement was observed, and the effect lasted longer than finasteride 5 mg alone, which means that the combination effect could improve treatment outcomes more than monotherapy. Furthermore, decreased side effects and increased tolerance and safety were mentioned in this study. Therefore, for the treatment of LUTS secondary to the BPH, a combination strategy is worth applying to patients suffering medium and severe symptoms.

1.5.3 Limits of current therapies

First-line medical therapy for LUTS secondary to BPH in middle-aged male patients includes α_1-blockers and 5α-reductase inhibitors (5ARIs) [157]. α_1-blockers, as the mainstream option, decrease the IPSS and increase Q_{max} (a reduction of IPSS by 30-40 % and increase of Q_{max} about 20-25 %); they also improve QoL [4]. Six α_1-blockers have been reported for the medication therapy of LUTS secondary to BPH: terazosin, doxazosin, tamsulosin, naftopidil, alfuzosin, and silodosin. All successfully improve voiding LUTS vs. placebo [19, 120]. However, comparable effects of placebo were observed [158].Based on clinical studies, these α_1-blockers reduce IPSS and improve Q_{max} by not more than 50 %, meanwhile placebos may lead to improvements around 30 % [4]. Additionally, α_1-blockers present little effect on decreasing the prostate volume, or preventing the occurrence of AUR or the requirement for surgery [159]. The limitations of α_1-blockers may result from various causes, and raise the demand of new medications with higher efficacy on treating LUTS suggestive to BPH.

5-ARIs appear to be only effective in men with moderate to severe LUTS; for patients with prostate volume < 40 mL, 5-ARIs might not be more useful than placebo [155]. The short-term efficacy of 5-ARIs on symptom reduction is slow,

generally needing three months [126]. Furthermore, monotherapy is not sufficiently effective for patients with moderate-to-severe LUTS after the first round of therapy [160]. The risk of symptomatic progression, which is the primary aim of 5-ARI administration, can be reduced by not more that 35-40 % by monotherapy, and to maximally 66 % by combination therapies [161]. Moreover, based on a population-based cohort study, the discontinuation rate for 5-ARIs is high, peaking up to 70 % of patients who do not continue their medication after 1-year treatment [162]. Many patients stop taking medications because the effect of 5-ARIs is lower than expected or because they do not understand that the clinical improvement may need 3-6 months [163].

Sexual adverse effects, including decreased libido, are the most common complications resulting from the use of 5ARIs and have been documented to range from 0.9 to 38 % [164]. Some patients may stop taking medication because of anxiety of their sexual function. Studies of androgen receptors in animal models, which were based on cavernosal tissue of rats, suggested that reduction of DHT levels in the prostate may cause the effects of 5ARIs on sexual function [110]. Finasteride changes the levels of DHT in serum, which may lead to erectile dysfunction by decreasing levels of nitric oxide (NO) and reducing nitric oxide synthase (NOS) activity in the corpus cavernosum [165]. It is suggested that at comparative high doses, finasteride impaired erectile function by causing NOS activity disorder in the penis [149].

From the psychiatric side, low-dose finasteride (5-ARIs) may produce depression symptoms[119]. There is a demonstrated link between levels of androgen (low free testosterone) and depression, as depressive symptoms were found in men with prostate cancer treated with androgen deprivation, but the exact mechanism remains unknown [166]. Roemer et al. published research on the effects of finasteride on mice and found that 5ARI administration reduced neurogenesis of the hippocampal area, which was associated with depression [167].

The studies on PDE5 inhibitors are merging; however, in long-term application (more than 1 year) the tolerability and safety of PDE5 inhibitors remains unclear, and their effects on reducing prostate size and slowing disease progression still need to be researched [168]. The risk of adverse drug reactions (ADRs) resulting from PDE5 inhibitors is obviously dose-dependent. Additionally, the most common ADR is headache, reported in >10 % of patients who are managed with long-term PDE5 inhibitors [139]. Other ADRs, including dizziness, dyspepsia, flushing, rhinitis or nasal congestion, have also been reported from clinical studies[156].

In conclusion, to overcome these disadvantages and side effect of commonly used treatments for BPH, novel BPH therapies should be considered, and the deep mechanism should be understood. The efficacy of current medications is limited, even despite the widespread application of some drugs. Considering the disappointing continuation rate of current medications, other options for patients could be recognized in the future.

1.5.4 Novel compounds in pathophysiology and experimental therapy of LUTS

A number of studies introduced the concept that many more signaling pathways than previously known may promote prostate smooth muscle contraction and the development of prostate enlargement. Many attempts have been made with the purpose of providing novel solutions for LUTS suggestive of BPH, including approaches based on several GTPases and kinases [117, 169-172]. Taken together, it is becoming increasingly obvious that there is a much higher complexity than previously assumed which accounts for the mechanisms underlying prostate smooth muscle contraction. In recent years, an increasing number of new compounds have been identified as possible potential candidates for putative new therapy options for LUTS suggestive to BPH, and some are to be further explored for clinical applications.

1.5.4.1 G protein-coupled receptor kinases 2 and 3 (GRK2/3)

GRK2/3 are expressed in the human prostate [173, 174]. It has been proposed that GRK-mediated receptor phosphorylation may critically regulate activation of α_1-adrenoceptors in the human prostate. GRK2/3 are supposed to cause phosphorylation of β_2-adrenoceptors without involving protein kinase C (PKC) [169]. Furthermore, in the human prostate, GRK-mediated receptor phosphorylation may also important to connect α_1-adrenoceptors to β-arrestin-2 and other accessory binding partners [174]. α_1-adrenoceptors regulate prostate smooth muscle contraction, meanwhile, activation of intracellular signalling pathways by G-proteins is require also for other G-protein-coupled receptors; and receptor-mediated smooth muscle contraction appears to connect with GRK2/3-mediated receptor phosphorylation [173]. In human prostate smooth muscle, CMPD101, a selective GRK2/3 inhibitor, inhibits α_1-adrenergic, neurogenic, and non-adrenergic contractions. This reagent may be an interesting compound in this context, also regarding translational aspects of clinical studies [173].

1.5.4.2 Polo-like kinases (PLKs)

Polo-like kinases (PLKs), a group of serine-threonine kinases, have been widely associated with promotion of proliferation and also regulation of the cell cycle. In airway and vascular smooth muscle contraction, recent studies suggested that PLK1 plays a critical role [175]. It has been suggested that prostate smooth muscle contractions were decreased after treatment with several PLK inhibitors [176]. Endothelin-1 and thromboxaneA2 induced contractions were not sensitive to PLK inhibitors, which depicted a divergent regulation of adrenergic and non-adrenergic prostate smooth muscle contraction by PLKs [176]. It is possible that, in the hyperplastic human prostate, PLK1 promotes α_1-adrenergic smooth muscle contractions [176].

1.5.4.3 LIM kinases

Based on cardiovascular disease studies, a promising role appears possible for LIMKs, as it has been reported that α_1-adrenoceptors in the cardiovascular system lead to phosphorylation of cofilin via LIMKs activation [177]. Recently, a study using LIMK inhibitors demonstrated that LIMK inhibitors reduce contractions of human prostate tissues [178]. According to these results, in the prostate, LIMKs inhibitors inhibit smooth muscle contraction. In conclusion, LIMKs might be relative to bladder outlet obstruction and urethral obstruction in BPH [178].

1.5.4.4 Rac GTPase inhibitors

RhoA, a member of the superfamily of small monomeric GTPases plays an important role in contraction of smooth muscle [179]. According to recent studies, Rac (as another monomeric GTPase) regulates relaxation of airway smooth muscle, and also vascular smooth muscle [180]. In the human prostate, recent evidence suggested that all three isoforms of Rac are expressed in human prostate tissue, and inhibition of Rac GTPases decreased proliferation of prostate stromal cells and inhibited prostate smooth muscle contraction [181]. Taken together, Rac may be a promising new target for treatment of LUTS suggestive to BPH.

1.5.4.5 Src family kinases (SFKs)

SFKs, non-receptor tyrosine kinases, involved in the regulation of central cellular functions, such as regulation of the cell cycle and promotion of proliferation of different cell types [182]. Furthermore, based on studies about airways, gastrointestinal tract, and uterus SFKs inhibitors play a role in smooth muscle contraction in these organs [183]. Recent evidence showed that SFKs inhibitors affect the organization of actin filaments in prostate smooth muscle, which are required contraction. In line with this, SFKs inhibitors also inhibited smooth muscle contractions of human prostate tissues, and, in parallel, inhibited

the proliferation of prostate stromal cells [170]. These finding suggest that SFKs could be a possible target for inhibiting the smooth muscle contraction and proliferation at the same time.

1.5.4.6 Focal adhesion kinase (FAK)

Activation of FAK could be important for α_1-adrenoceptor-mediated contraction [184, 185]. In previous studies, a role of FAK for contraction of bladder smooth muscle has been reported [186]. Using a prostate cell culture model and human prostate tissues, it has been recently suggested that application of FAK inhibitors reduces prostate smooth muscle tone [187]. Importantly, FAK may be critical for the pathogenesis of LUTS suggestive to BPH.

1.6 Generally studies on development of Ghrelin system

1.6.1 History of Ghrelin

Kojima et al. proposed ghrelin as a new gastric hormone and clarified its function and structure [188]. The name of this hormone is a metaphor of growth, as abundant studies merged and suggested that this newly discovered hormone leads to the growth hormone (GH) release [189]. Ghrelin, a 28 amino acid acyl-peptide, is esterified with octanoic acid on Serine 3. GHSR-1a activation is necessary for acylation; however, it is widely accepted that des-acyl ghrelin is far more abundant than acyl-ghrelin [190]. Nevertheless, the main function of des-acyl ghrelin remains unknown. In 2002, Baldanzi et al. demonstrated the function of des-acyl ghrelin in cardiomyocytes and endothelial cells, suggesting that the des-acyl ghrelin could be a survival factor in the cardiovascular system [191]. After that, discussions around the ghrelin and des-acyl ghrelin are continuous, and they appear to be a promising pharmacologic topic.

1.6.2 Various forms

Generally, two forms of ghrelin have been reported in the human stomach. The first is the acylated ghrelin (AG), with the n-octanoylated serine at position 3, which is widely studied [192]. The second is the des-acyl ghrelin (DAG), produced by the other splicing of the ghrelin gene [193]. Additionally, as mentioned before, acylation is common and necessary for GH release in many processes involving the ghrelin system [190]; DAG is the usual form (plasma desacyl-ghrelin accounts for 75 % of total circulating ghrelin) in the ghrelin system [194, 195]. Furthermore, in human plasma and the stomach, some minor forms of ghrelin are expressed [196].

Acyl-transferase was recently identified as an enzyme that takes charge of ghrelin acylation, ghrelin *O*-acyltransferase (GOAT) [197]. Mouse genome results demonstrated that GOAT belongs to a family of 16 hydrophobic membrane-bound acyltransferases. This transferase includes porcupine (attaches to Wnt proteins) [198]. GOAT mRNA has a negative relationship with ghrelin in the major ghrelin-secreting tissues. Additionally, a novel 23-amino acid peptide was recently identified from rat stomach, called obestatin [199]. Obestatin is expressed from the mammalian preproghrelin gene. Several authors reported that the ghrelin gene products, such as acyl ghrelin, des-acyl ghrelin and obestatin, are all related to distinct feeding behaviours, and delicately coordinate digestive processes and regulation of energy balance [200-203].

1.6.3 Mechanisms of action

Ghrelin directly connects to and activates growth hormone secretagogue receptor (GHSR) to enhance GH release. The post-translational modification of octanoylation appears to be critical for the progress of GH release [204]. In general terms, AG controls food intake, adiposity, and insulin secretion. By means of orexigenic neural circuits, ghrelin regulates systemic metabolism and stimulation of gut motility subsequently [192] (Figure 4). DAG has been less

reported, but also contributes to the stress response and may have an opposite role in regulation hypothalamic circuitry [205]. In animal models, the signals of serum AG related to food intake via the hypothalamus and then through the gastric vagal afferent pathway [203]. In vivo, signal transduction pathways should act via the nucleus of the solitary tract in the brainstem [206]. The nucleus of the solitary tract is connected to hunger signalling mediated by insulin [202]. Electro-physiologic studies indicate that AG suppresses firing of gastric afferent nerves [207]. Taken together, these findings are evidence for the concept that the ghrelin system is necessary for the central control of feeding behaviour via a ghrelin-associated orexigenic signalling cascade.

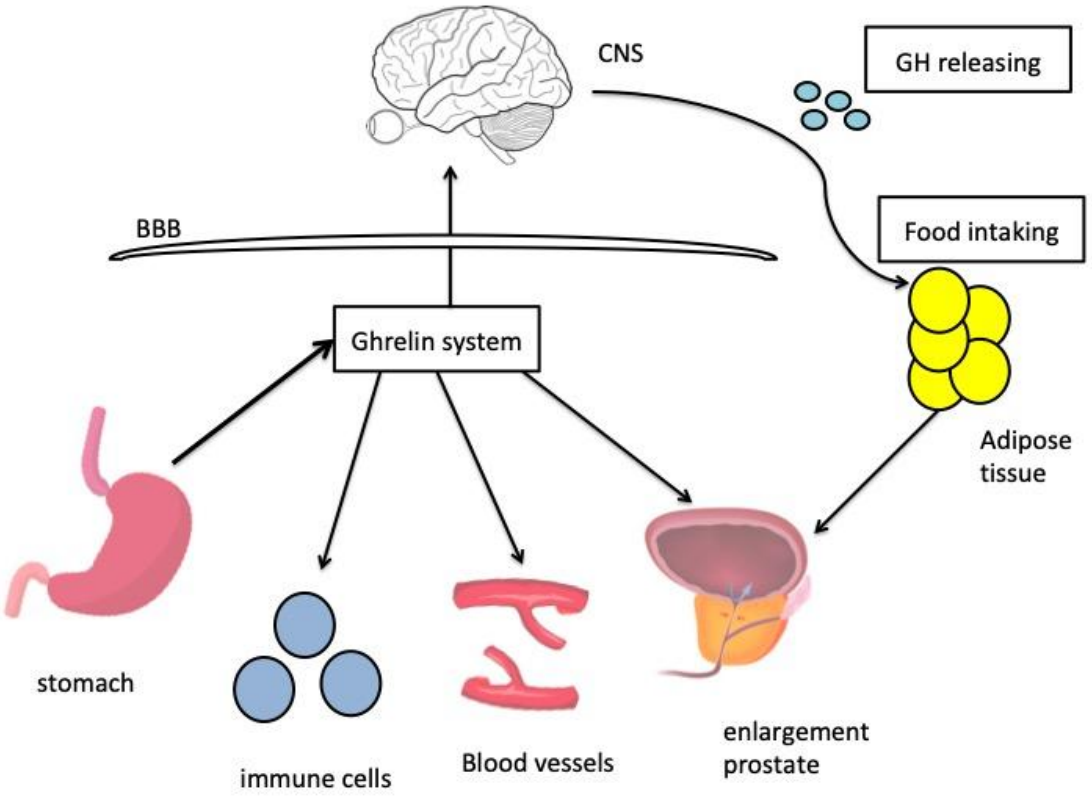

Figure 4. Ghrelin, which is mainly secreted by the stomach, affects various systems, including the central nervous system (ghrelin activates the GHSR and promotes growth hormone release), the immune system, and the cardiovascular system. AG controls food intake, increases pro-inflammatory factors, glucose and lipid metabolism, and promotes gastrointestinal motor function and cellular proliferation [208]. It may be speculated, that abnormal ghrelin-associated metabolism may contribute to the development of LUTS suggestive to BPH [94]. CNS: central nervous system, BBB: blood-brain barrier. (Diagram materials from https://www.vectorstock.com/)

1.6.5 Major functions

Of the functions of ghrelin, energy balance should be mentioned first. In the early 21th century, human studies have targeted the altered serum ghrelin expression in abnormally thin and obese subjects (based on BMI) [209]. For instance, AG level influence the endogenous circulating hormone levels in the process of insulin resistance and obesity [205]. Previous studies suggested that AG enhances proliferation of different cell types [210-212]. Interestingly, the role of DAG remained initially unclear, but it was recently reported that it inhibits the apoptosis of pancreatic beta-cells [213, 214]. The function of DAG on food intake is more complex and remains unclear. In fact, previous studies presented opposite results of the effect of DAG on proliferation in different types of cells [190, 215, 216]. Taken together, published data on functions of AG and DAG are conflicting. There are evidences suggested that AG and DAG have similar effects on food taken, meanwhile also opposite effects in other biological functions [217]. Recent findings suggested that the balance of the AG/DAG ratio may regulate insulin actions in the metabolic syndrome. The unbalanced AG/DAG ratio may contribute to the insulin resistance and increases body mass, and induces metabolic stress and abnormal hormone level in plasma subsequently [217].

Generally, serum ghrelin levels were much higher in patients with anorexia nervosa than in healthy subjects, and increasing weight reduced serum ghrelin to normal levels in these patients [216]. Researchers believe that the possible reason may be the loss of sensitivity or resistance to ghrelin expression in human serum. On the other hand, the ghrelin level, in cortical tissue, may be overexpressed in patients who are suffering with anorexia nervosa [218]. Ghrelin produces a significant rise in human plasma glucose levels, which are according to a decrease in insulin release [210, 219]. The explanation of this phenomenon has been debated for a long time. C. Gauna et al. suggested that the

administration of unacylated human ghrelin might lead to insulin sensitivity [220].

Ghrelin enriched T cells in elderly humans and animal models [221]. Meanwhile, the thymus involuted and T-cell production declined. Nevertheless, in an animal model, the long-term management of ghrelin increased GH/insulin-like growth factor I levels and stimulated growth and cell differentiation, meanwhile, increased T-cell proliferation [222]. Ghrelin also regulated the pro-inflammatory cytokines release [222]. In animal models, ghrelin decreased endotoxin-induced anorexia and cytokine production, while it improved mortality associated with lipopolysaccharide (LPS)-induced endotoxin shock [205]. Additionally, Collden et al. suggested that ghrelin and the GHSR are found in human T cells and monocytes [223]. Furthermore, it is specifically that ghrelin inhibited the chronic synthesis of pro-inflammatory anorectic cytokines (for instance, leptin, IL-6, interleukin 1 ß (IL-1 ß), and tumour necrosis factor-1).

Cardiovascular effects of ghrelin are also widely reported, particularly the effect of ghrelin on cardiovascular smooth muscle [224]. Various evidence supports a functional role for ghrelin in the proliferation of myocardial cells related to improved cardiac function [225]. Ghrelin inhibition of apoptosis in cardiomyocytes and in endothelial cells via the activation of extracellular signal-regulated kinase 1/2 and Akt serine kinases has been widely reported in experimental studies [226]. Another important point is that even though cardiomyocytes connect to ghrelin with high affinity, they do not express GHSR1a, which means that there are other subtypes of ghrelin receptors in the cardiovascular system [191].

Ghrelin, a gastrointestinal hormone, exerts its effects by cooperation with GHSRs [227]. Increasing evidence has suggested that ghrelin has a wide variety of biological functions, one of which is to promote motility of the gastrointestinal system [225]. For instance, administration of ghrelin in

peripheral organs increase the motility of the gastrointestinal tract. In vitro, ghrelin increases the contraction amplitude of the muscle strips, which is a dose-dependent process, even though the rhythm is not altered by ghrelin. In parallel, ghrelin receptors are widely found in gastrointestinal cell lines [228]. Ghrelin has a proliferative effect on Cajal cells, which produce slow waves of contraction and connect to adjacent smooth muscle cells to generate spontaneous rhythmic contractions [229]. Furthermore, based on histological studies, Cajal cells have been connected with adjacent smooth muscle cells of the gastrointestinal and urinary tract, which is also a target for ghrelin [230].

Taken together, the reported functions of ghrelin inspire researchers to explore the effect of ghrelin in other systems, such as the respiratory system [192]. The distribution of ghrelin receptors in tissues may indicate that they are the target tissues for ghrelin [231].

1.6.6 Role of ghrelin in disease

Ghrelin has been reported as the only known systemic orexigenic factor; in lean patients, serum ghrelin expression fluctuates according to energy intake. Alterations in ghrelin secretion appears to influence the fluctuations in serum ghrelin associated with different metabolic and disease states. Ghrelin has a strong growth hormone (GH)-releasing activity via stimulating of the GHSR1a [232]. Importantly, GHSRs are mainly concentrated in the unit of hypothalamus-pituitary. Two main functions should be mentioned: achieving appetite and regulating energy balance; also, managing pancreatic endocrine function and affecting glucose levels. Both play critical roles in the progress of obesity [233]. Surprisingly, various studies reported decreased ghrelin levels in obese patients [233, 234]. However, they did not distinguish between AG and DAG [192]. In fact, the balance of the AG/DAG level is altered in obese patients, so that the ghrelin system may contribute to obesity [235].

Additionally , it is potentially beneficial that ghrelin and its receptors reduce breakdown of proteins and weight loss in catabolic conditions, including cancer cachexia and age-related frailty [236, 237]. Clearly, ghrelin increases food intake and body weight in humans [233], and enhances cell proliferation and differentiation in different cell lines [223, 237-239]. Specially, in term of cardiovascular smooth muscle cells, ghrelin may increase the smooth muscle tone [240].

1.6.7 Summary

Ghrelin, a metabolism-related hormone, increases food intake in a dose-dependent manner, and increases appetite both by enhancing feeding by metabolic need and by rewarding feeding behaviour [241]. The latest studies on biological roles of ghrelin demonstrated that it could have various physiological functions. Ghrelin has potential GH-releasing, orexigenic activities, and critical effects on the cardiovascular and gastrointestinal systems [217].

Furthermore, ghrelin participates in inflammation and age-associated obesity [242]. The ghrelin signaling pathway is an important factor for prostate inflammation, which may induce the development of BPH and prostate cancer [243, 244]. Metabolic syndrome induces hormone alterations including unbalanced AG/DAG ratio, which increases metabolic stress [235]. Moreover, this metabolic stress enhances the development of BPH [245]. Together, a possible role of the ghrelin system as a molecular link connecting BPH with metabolic syndrome appears feasible. In fact, metabolic syndrome is a sustained disorder affecting levels of different metabolic hormones, which may involve or affect the ghrelin system [93].

1.7 Possible role of the ghrelin system in links between metabolic syndrome and LUTS suggestive to BPH

1.7.1 The relationship between the metabolic syndrome and BPH/LUTS

The relationship between metabolic syndrome and BPH/LUTS has been derived mostly from a series of cutting-edge epidemiologic studies [93]. Several components of metabolic syndrome, including obesity, glucose intolerance, dyslipidaemia, and hypertension, were identified as risk factors for BPH [246]. New studies on the development of BPH and BOO support the suggestion that metabolic syndrome can affect the natural course of these conditions [23]. Although this context has been unequivocally proven by clinical and epidemiological observations, the underlying molecular mechanisms are still widely unknown.

Adipokines, including ghrelin or other metabolic hormones, play a central role in the pathophysiology of metabolic syndrome [247]. However, they have not been considered as possible mediators linking BPH/LUTS with metabolic syndrome to date [248]. Previous studies addressing molecular mechanisms connecting BPH with metabolic syndrome focused on inflammatory mediators and lipoproteins [249]. Because ghrelin may increase smooth muscle contractility and promotes proliferation in several organs and cell types, a similar role in the prostate and for BPH/LUTS may be assumed, besides its role in metabolic syndrome.

1.7.2 Ghrelin system in regulation of smooth muscle tone

Considering the effect of enhancing smooth muscle contraction, ghrelin has a potential in different systems, including the cardiovascular and gastrointestinal systems [248]. In vascular smooth muscle, the modulation of contraction and arterial pressure control are related to the activities of ghrelin and receptors [250].

In vessels, saphenous vein, internal mammary artery and coronary artery, ghrelin receptors were widely detected [251]. Interestingly, ghrelin was widely represented on the surface of vascular smooth muscle [252].

Additionally, the ghrelin system was also been reported to influence the gastrointestinal system. Ghelardoni et al. found ghrelin mRNA and protein in the stomach and almost every section of the small intestine [253]. The mRNA level was maximal in the stomach; however, its protein level was quite below that measured in the lung. Thus, in the gut, ghrelin protein expression is dissociated from mRNA expression [192]. In isolated rat stomach smooth muscle strips, ghrelin enhanced the small cholinergic-mediated contraction, stimulated during electric field stimulation (EFS) [254].

In terms of the effect of ghrelin on smooth muscle contraction, Fang et al. suggested the ghrelin system may be a protective factor on vascular smooth muscle cells, and inhibits the increase in the calcium concentration of rat aorta vascular smooth muscle cells (VSMCs) [240]. Dimitrova et al. reported that, in human mesenteric arteries, the ghrelin system increased the force of contraction via Src kinase and mitogen-activated protein kinase (MEK) [255]. Using an animal model, Mladenov et al. suggested that ghrelin increased the tension of endothelin-1 induced contraction in pig femoral arteries [256]. According to previous studies, in the cardiovascular system, and also in the gastrointestinal system, ghrelin appears to promote smooth muscle contraction.

However, the effect of ghrelin and its receptors in human prostatic smooth muscle still need to be explored. A possible influence of the ghrelin system in LUTS suggestive of BPH appears possible and interesting, as prostate smooth muscle contraction is involved in LUTS and an important target for its medical therapy.

1.7.3 Ghrelin system plays a role in proliferation

Considering the cellular proliferation, a study on VSMCs, Liang et al. reported that the ghrelin system may inhibit the differentiation of VSMCs [257]. Moreover, Rossi et al. suggested that ghrelin inhibits angiotensin II-induced proliferation and contraction by means of the cAMP-protein kinase A pathway [257, 258]. Accordingly, they suggested that, in terms of vascular damage and remodelling, the ghrelin system is a potential therapeutic target.

In contrast, during cardiac remodelling, the effect of ghrelin remains unclear. Recently, a study suggested that ghrelin activates the Raf-1-MEK1/2-ERK1/2-BAD signalling pathway in the rat model due to its anti-apoptotic effect; at the same time, it inhibits cardiac fibrosis [225, 259]. Furthermore, in long-term ghrelin treatment, as reported by Yuan et al., vascular endothelial growth factor (VEGF) expression increased in the peri-infarct zone in myocardial infarction rats compared with a control group, suggesting that ghrelin could be a protective factor and improves angiogenesis [260].

However, to the best of my knowledge, there are no studies concerning ghrelin-induced proliferation in non-malignant prostate cells or in prostate smooth muscle contraction, but effects on prostate enlargement appear possible.

1.7.4. Ghrelin system in BPH?

Very limited studies are available on the role of ghrelin in BPH. However, a recently study suggested that significant positive correlation between serum PSA and total ghrelin in non-diabetic individuals with BPH, which provide an evidence that ghrelin may have a diagnostic role in BPH [261].

Because the ghrelin system may increase smooth muscle contractility and promotes proliferation in several organs and cell types, a similar role in the prostate and LUTS suggestive to BPH may be considered, within their role for

the metabolic syndrome. Taken together, studies about the effect of ghrelin system on LUTS suggestive to BPH are assumed to start from two aspects: the effect on contraction of prostate smooth muscle and the effect on the proliferation of prostate cells.

2. Aims

Current medical therapy of LUTS suggestive to BPH is still influenced by insufficient efficacy and low adherence to medications of patients [262]. Although pharmacotherapy in BPH has been reported as successful and essential, daily practice suggested that there are still inherent limitations. For instance, α_1-blockers improve the symptom scores no more than 50 % [181]. Recent study suggested that the drug adherence rate to α_1-blockers was acclaimed lower than 30 % after starting of the 12 months treatment [263].

Therefore, it is vital to find more effective options for treatment of LUTS suggestive of BPH. An all-round or single-compound medication for treatment of voiding symptoms would be optimal. In recent years, the possible linkage between contraction of smooth muscle and proliferation of prostatic cells has been researched, however, the one-pack solution of LUTS suggestive to BPH is still on the way.

Consequently, the aim of this study was to explore the possible role of the ghrelin system for proliferation of prostate stromal cells and for contraction of prostate smooth muscle.

Specifically, the following questions were subject of in this thesis:

1. Does a ghrelin agonist increase the viability of a prostatic stromal cell line?
2. Does a ghrelin agonist increase the proliferation of a prostatic stromal cell line?
3. Does a ghrelin agonist affect the cell cycle of a prostatic stromal cells?
4. Does a ghrelin agonist affect growth factor mRNA expression?
5. Does an inverse ghrelin agonist affect contractions of human prostate tissues?

3. Materials and methods

3.1 Reagents and devices

MK-0677 is a non-peptide, small molecular GHSR agonist, which has a higher affinity to the GHSR compared to ghrelin [264]. Stock solutions (10 mM) were prepared with dimethyl sulfoxide (DMSO), and kept at -20 ℃ until use. Aqueous stock solutions of noradrenaline (10 mM) were freshly prepared before each experiment.

PF-5190457, a member of a spiro-azetidino-piperidine series, was identified by Pfizer Pharmaceuticals by means of high-throughput screen method [265], which is potent and high selective GHSR inverse agonist that enhances glucose-stimulated insulin secretion (GSIS) [266]. Stock solutions (10 mM) were prepared with DMSO and kept at -20 ℃ until use.

Table 1. Reagents used in this study.

Products	Provider
Dimethyl sulfoxide (DMSO)	Roth, Germany
RPMI 1640	Gibco, USA
10 % fetal calf serum (FCS)	Gibco, USA
Phosphate-buffered saline (PBS)	Gibco, USA
Sulforhodamine B (SRB)	Gibco, USA
4', 6'-diamidino-2-phenylindole-dihydrochloride (DAPI)	Invitrogen, USA
Magnesium chloride ($MgCl_2$)	Promega, USA
Reverse transcription buffer 10×	Promega, USA
dNTP Mix	Promega, USA
Random primers	Promega, USA

Custodiol	Köhler, Germany
Glucose	Sigma-Aldrich, Germany
Carbogen	Linde Gas, Germany
MK-0677	Tocris Bioscience, UK
PF-5190457	Tocris Bioscience, UK
Noradrenaline	Sigma-Aldrich, Germany
AMV reverse transcriptase	Promega, USA
Ribonuclease (RNase) inhibitor	Promega, USA
RNase Free Water	Promega, USA
SYBR™ Green	Roche, USA
RT^2 qPCR Primer	QIAGEN, Germany
RNase Away	Thermo Fisher Scientific, USA
AllPrep DNA/RNA/Protein Mini Kit	QIAGEN, Germany
5-Ethynyl-deoxyuridine (5-EdU)	Thermo Fisher Scientific, USA
Potassium chloride (KCl)	Roth, Germany
Sodium chloride (NaCl)	Roth, Germany
Calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$)	Roth, Germany
Magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$)	Roth, Germany
Sodium bicarbonate ($NaHCO_3$)	Roth, Germany
Potassium dihydrogen phosphate (KH_2PO_4)	Roth, Germany
10x Reaction Buffer with $MgCl_2$	Thermo Fisher Scientific, USA
Triton X-100	Thermo Fisher Scientific, USA
Mounting solution	Thermo Fisher Scientific, USA
Dithiothreitol (DTT)	Sigma-Aldrich, Germany
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich, Germany
Propidium iodide	Abcam, London, GB
RNase	Abcam, London, GB

Table 2: Devices used in this study.

Products	Provider
Tissue Bath System-720MO	DMT (Danish Myotechnology), Denmark
Electrical Field Stimulation	DMT, Denmark
Lab pump	KNF- Neuberger, USA
Thermostat	Memmert, Germany
Fluorescence Activated Cell Sorter (FACS)	Becton-Dickinson, USA
Light Cycler PCR system	Roche, Switzerland
Superfrost® microscope slides	Thermo Fisher, USA
Laser scanning microscope	Leica SP2, Germany
Lab-Tek chamber slides	Thermo Fisher, USA
Cell culture incubator	Thermo Fisher, USA
Waterbath	Thermo Fisher, USA
Centrifuge	Thermo Fisher, USA
Nanodrop™	Thermo Fisher, USA

3.2 Human prostate tissues

Human prostate tissues were obtained from patients undergoing radical prostatectomy for prostate cancer (n = 19). All prostates were transported in Custodiol® (Köhler, Germany) to the Department of Pathology immediately (less than 1 hour) after prostatectomy and then directly macroscopically examined by pathologists. Tissue samples were then obtained from the periurethral zone, where no tumors were observed. Patients were not included, if they were received transurethral resection of prostate (TURP) or brachytherapy

before radical prostatectomy. If any prostatic stones, or fibrotic parts were observed, these tissues were also excluded.

The research was carried out according to the Declaration of Helsinki of the World Medical Association, and has been approved by the ethics committee of the Ludwig-Maximillians University, Munich, Germany. Human prostate tissues were stored in Custodiol® at 4 ℃ for organ bath studies for a maximum of 90 min.

3.3 Cell culture

WPMY-1 cells (human prostate stromal cell line) used in this study were purchased from the American Type Culture Collection (ATCC Manassas, VA, USA). WPMY-1 is an immortalized cell line from human prostate stroma, without malignant transformation [267]. Cells were cultured in Roswell Park Memorial Institute (RPMI)1640 (Gibco, Carlsbad, CA, USA) supplemented with 10 % fetal bovine serum (FCS) (Gibco, Carlsbad, CA, USA) and 1 % penicillin (Gibco, Carlsbad, CA, USA) and streptomycin (Gibco, Carlsbad, CA, USA) at 37 ℃ in a humidified atmosphere with 5 % CO_2.

3.4 Real time polymerase chain reaction (RT-PCR)

RNA isolation from cells was performed using the RNeasy Mini kit (Qiagen, Hilden, Germany). RNA concentrations were measured spectrophotometrically. Reverse transcription to cDNA was performed with 1 µg of isolated RNA using the Reverse Transcription System (Promega, Madison, MI, USA). RT-PCR for Ki-67, IGF-1, EGF, FGF-2, TGF-β1, cyclin D1, CDK2, CDK4, E2F3 and actin was performed with a Roche Light Cycle (Roche, Basel, Switzerland) using primers provided by Qiagen (Qiagen, Hilden, Germany) as ready-to-use mixes. PCR reactions were performed in a volume of 25 µL. Denaturation was performed at specific temperature ranges. The specificity of primers and

amplification was demonstrated by subsequent analysis of melting points, which revealed single peaks for each target. Results were expressed using the ΔΔCP method, where number of cycles (Ct) at which the fluorescence signal exceeded a defined threshold for actin was subtracted from Ct values for targets, and values were calculated as ΔΔCP and normalized to each other.

3.4.1 Reverse Transcription (RT)

RNA samples were incubated at 70 ℃ for 10 min. Thereafter, 1 µg of RNA sample was reversing transcribed using Reverse Transcription System (Promega, Madison, WI, USA) (Table 3).

Table 3: Reverse Transcription

Components	Volumes (µL)
Random hexamer oligodeoxyribonucleotides	0.5
Ribonuclease inhibitor	0.5
Reverse-transcriptase	0.65
dNTP mix	2
10xRT buffer	2
$MgCl_2$	4
RNase-free water	10.35

3.4.2 Primers

All primers (Table 4) were provided by Qiagen (Hilden, Germany) as ready-to-use mixes (www.qiagen.com). All primers were stored at -20 ℃.

Table 4: Primers

Primer full name	RefSeq accession number
Marker of proliferation Ki-67 (Ki-67)	NC_000010.1

Cyclin-dependent kinase 2 (CDK2)	NM_001798
Cyclin-dependent kinase 4 (CDK4)	NM_000075.4
Insulin-like growth factor 1 (IGF-1)	NM_198541
Epidermal growth factor (EGF)	NM_00118130
Fibroblast growth factor 2 (FGF-2)	NM_031950
Tranforming growth factor β1 (TGF-β1)	NM_001142621
E2F transcriptin factor 3	NM_001949

RefSeq represent reference sequence from national center for biotechnology information (NCBI).

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

RT-PCR for proliferation markers, including Ki67, CDK2 and CDK4, growth factors, including IGF-1, EGF, TGF, FGF, and cell cycle regulating factor E2F3 and actin was performed with a Roche Light Cycler (Roche, Basel, Switzerland). PCR reactions were carried out in a volume of 25 µL, which contained 5 µL Light Cycler® FastStart DNA Master Plus SYBR Green I (Roche, Basel, Switzerland), 1 µL template, 1 µL primer, and 18 µL water. The settings run on the Light Cycler were used as followed.

Denaturation was performed for 10 min at 95 ℃, and amplification with 45 cycles of 15sec at 95 ℃ followed by 60 sec at 60 ℃. The specificity of primers and amplification was demonstrated by subsequent analysis of melting points, which revealed single peaks for each target. ΔΔCP crossing points (CP) method was used to express the results [268].

3.5 Cell proliferation assay

WPMY-1 cells were plated with a density of 50,000/well on a 16-well chambered coverslip (Thermo Scientific, Waltham, MA, USA). After 24 hours, cells were treated with MK-0677, or DMSO. After further 24 hours, the medium

was changed to a 10 mM 5-ethynyl-2'-deoxyuridine (EdU) solution in FCS-free medium containing agonist or solvent (DMSO). 20 hours later, cells were fixed with 3.7 % formaldehyde. EdU incorporation was determined using the "EdU-Click 555" cell proliferation assay (Baseclick, Tutzing, Germany) according to the manufacturer`s instructions. This method is a method for labeling DNA in vivo that allows to depict the replicate DNA in the context of well preserved cellular and chromatin ultrastructure. EdU is readily incorporated into cellular DNA during DNA replication.

In this assay, incorporation of EdU into DNA is assessed by detection with fluorescing 5-carboxytetramethylrhodamine (5-TAMRA). Counterstaining of all nuclei was performed with DAPI. Experiments were performed using the following protocol:

1. Cells were cultured on the glass coverslips in the RPMI medium with 10 % serum for 24 hours.
2. After the attachment of the WPMY-1 cell, the medium was replaced by serum free medium containing 5 % EdU.
3. Cells were exposed to MK-0677 or DMSO for 24 hours.
4. Medium was removed followed by washing the plates with PBS two times.
5. Cells on the plate were fixed with formaldehyde. Residual EdU medium mixture was removed and 90 µL paraffin was added in each well, followed by waiting for at least 15 min at room temperature before washing.
6. BSA/PBS solution was prepared in the plastic holder. Paraffin was removed from the plates and washed by 95 µL BSA/PBS solution two times.
7. Plates with cells were treated with 90 µL vortexed triton and incubated at room temperature for 20 min. Thereafter, 30 µL of EdU cocktail mixture was added in each well.
8. Residue was removed and covered with glass.
9. A laser scanning microscope (Leica SP2, Wetzlar, Germany) was applied to analysis the labeled cells.

3.6 Plate colony formation test

Cells were incubated at 37 ℃ for 14 days, then washed twice with phosphate-buffered saline, and fixed by 2 mL 10 % trichloroacetic acid (TCA) 4 ℃ overnight. After that, all plates were washed with cold water five times, and stained with 0.4 % Sulforhodamine B (SRB) solution (diluted in 1 % acetic acid) at room temperature for 30 minutes. Before analysis, all plates were labeled and washed by 1 % acetic acid five times. The number of colonies containing 50 cells or more was counted under a microscope. Experiments were performed using the following protocol:

1. 100 cells were seeded in each well in 6 well plates. Final confluence of 70 % growth area in one well of 6 well plate provides satisfactory results according to previous experimental results.
2. MK-0677 was applied to cells in different concentration. DMSO was applied to control group.
3. Cells were stained with SRB after 14 days.
4. Plates were washed by PBS two times.
5. 10 % TCA solution (250 μL to 1 mL per well) was added for fixation, which could totally cover the whole surface area of the plate.
6. Cells were incubated in 4 ℃ for at least 1 hour.
7. TCA solution was removed from the plates, and plates were dried in room temperature.
8. 1 mL SRB reagent (contain 1 % acetic acid) per well was added, followed by 30 min incubation at room temperature.
9. All plates were washed with 1 % acetic acid, and plates were dried in room temperature.
10. All plates were recorded and analyzed by ImageJ (NIH, Maryland, USA). Based on the equation as following, the formation efficiency could be obtained. The formation efficiency could represent the long-term proliferation ratio.

$$Plate\ colony\ formation\ efficiency = \frac{Number\ of\ colonies}{Number\ of\ cells\ seeded} \times 100\ \%$$

3.7 Cell cycle analysis

Cells were fixed with ethanol and centrifuged at 500 g for 5 min.The ethanol supernatant was discarded and cells were suspended in 1 mL of pre-cooled phosphate-buffered saline (PBS) for 1min, and then centrifuged at 500 g for 5 min. The cells were suspended in 200 μL propidium iodide (PI) and RNase staining mixture solution. After incubation in the dark at 37 ℃ for 30 min, cells were sorted on a fluorescence-activated cell sorting (FACS) flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). For setting the flow cytometer, the excitation should be regulated at 488 nm for generating DNA distribution histograms.

Based on setting, 10,000 events were analyzed for each sample using ModFit LT software (Verity Software House, Torpsham, ME, USA). Experiments were performed using the following protocol:

1. PI and RNase solution were prepared as follow:

1 x PBS	9.45 mL
20 x PI (1 mg/mL)	500 μL
200 x RNase (110,000 U/mL)	50 μL
PI and RNase Solution	10 mL

2. 200,000 cells each well in 6 well plates were seeded for this examination.
3. Medium was removed after the cell attachment, and cells were washed with PBS and trypsin in order to dispatch the cells from the flasks.
4. Once the cells were fully trypsinized, the cells were pelleted at 500 g for 5 min and the supernatant was discarded.
5. Cells were washed twice by PBS and centrifuged again, before fixation of all cells.

6. Cell pellet was gently suspended in 400 µL cold PBS. 800 µL ice cold 100 % ethanol was added into each well. Plates with cells were stored at 4 ℃ for at least 2 hours. After that, the prepared sample from 4 ℃ were transferred to the bench and equilibrate to room temperature.
7. Cells were treated with the PI and RNase solution on the cell samples, after two times cold PBS washing,
8. Samples were incubated at dark and 37 ℃ for around 20 min.
9. Samples were placed on ice and subjected to the FACS analysis.
10. Before the samples undergo the FACS analysis, the setting and tranquilization should be finished.
11. Appropriate (forward scatter) FSC vs. (side scatter) SSC gate were established to cell debris and cell aggregates.
12. Data were collected in FL2 (channel 2) via 488 nm laser illumination.
13. Markers were setted up on a histogram plot to discribe such like , < 2N, 2N, 2N-4N(DNA synthesis), 4N and > 4N intensity regions facilitates quantifying differences in DNA content between different samples. Expect to see a two-fold intensity different between 2N (G1 stage) and 4N (mitotic) PI peaks.

3.8 Viability assay

Viability of WPMY-1 cells was assessed using the cell counting kit (CCK-8) (Sigma Aldrich, Munich, Germany) according to the instruction provided by manufacturer. CCK-8 allows WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) to penetrate living cells, inducing formazan dye. This colorimetric assay reflects cell viability. Experiments were performed using the following protocol:

1. Cell suspension (20,000 cells per well) was seeded in 96-well plates and incubated in a humidified incubator (5 % CO_2, 37 ℃) for 24 hours.

2. Medium was replaced by serum free medium, which could diminish the influence of unknown concentration of serum, after cells attached in plates.
3. MK-0677 was added into each well (final concentrations were 100 nM to 300 nM). DMSO as control.
4. Cells were incubated for different time spans: 24 hours, 48 hours or 72 hours.
5. 10 μL of CCK-8 was added to each well of the plate at the end of each time point (24 hours, 48 hours or 72 hours). Separate control experiments were performed for each time point. Bubbles to the wells in this procedure were avoided.
6. Cells were incubated for 2 hours, and measured the absorbance at 450 nm in microplate reader.
7. OD-values were collected and analyzed.

3.9 Tension measurements

Smooth muscle contractions of human prostate tissue were assessed by myographic measurements using an organ bath of the model 720MO (Danish Myotechnology, Ahus, Denmark). DMSO for controls, and PF-05190457 were added 30 min before contractions were induced by electric field stimulation (EFS) or noradrenaline. In each experiment, tissue from the same prostate was allocated to all four channels of one organ bath, and DMSO and PF-5190457 were added to two of the four channels, respectively. For calculation, tensions were expressed as % of KCl-induced contraction. Experiments were performed using the following protocol:

1. Krebs Henseleit (KH) solution was prepared before organ bath experiments. KH was maintained at 37 ℃ and continuously bubbled with carbogen (95 % O_2, 5 % CO_2) in a thermostatic container.
2. Chambers were rinsed with KH solution and filled with 10 mL KH solution, before the experiments.
3. Prostate strips were carefully incised from fresh prostate tissues.

4. Once the prostate tissue was secured to the needles, the tissue was mounted in the organ bath.
5. All the tissues were stretched to 4.9 mN and equilibrated for 45 min. During this step, spontaneous decreases in tone were usually observed. Therefore, tension was adjusted three times during the equilibration period in order to obtain a stable resting tone of 4.9 mN.
6. High molar KCl (final concentration was 80mM) was added to each chamber to induce the maximal KCl-induced contraction of prostate strips. Subsequently, the chambers were washed with KH solution and refilled with KH solution.
7. PF-5190457 10 µL (10 mM stock solution, final concentration in the organ bath chamber was then 10 µM) dissolved in DMSO was added to two chambers. 10 µL DMSO was added to other two chambers as control. Subsequently, PF-5190457 and DMSO were applied to prostate strips at least 30 min before adding noradrenaline or applying EFS.
8. 30 min after adding PF-5190457 and DMSO, frequency-response curves for EFS and concentration-response curves for noradrenaline were obtained. Frequencies used for EFS in this study were 2 Hz, 4 Hz, 8 Hz, 16 Hz, and 32 Hz. The cumulative concentrations of noradrenaline were 0.1 µM, 0.3 µM, 1 µM, 3 µM, 10 µM, 30 µM and 100 µM.
9. For calculation, tensions were expressed as % of KCl-induced prostate smooth muscle contraction.

3.10 Statistical analysis

Data in this study are presented as means ± standard error of the mean (S.E.M) with the indicated number of each experiment. Both values were calculated using GraphPad Prism 6 (Statcon, Witzenhausen, Germany). Multivariate analysis of variance (ANOVA) used to calculate unpaired observation, and two-way ANOVA were used to calculate paired observations. Both of these

calculations were performed with SPSS® 20.0 (IBM SPSS Statistics, Armonk, New York: IBM Corporation). P values < 0.05 were considered to be statistically significant. Cell cycle analysis was measured by the Modfit LT system (Verity Software House, Topsham, USA). Cell colony assay was measured by the ImageJ (NIH, Maryland, USA). Modfit performs its automatic analysis of the sample. It scans the histogram for peaks. Based on the sizes and positions, the program attempts to determine the number of cell cycles in the sample.

4. Results

4.1 Effects of MK-0677 on WPMY-1 cells

4.1.1 Effects of MK-0677 on viability of WPMY-1 cells

After exposure to 100 nM and 300 nM of MK-0677 for 24 hours, relative OD values were 1.14 ± 0.11 and 1.12 ± 0.11, respectively ($p < 0.0001$ for control vs. MK-0677), compared to controls which were normalized to 1. After 48 hours of incubation with 100 nM and 300 nM of MK-0677, relative viability was 1.35 ± 0.23, and 1.24 ± 0.18, respectively ($p < 0.0001$ for control vs. MK-0677) compared to controls which were normalized to 1 (Figure 5).

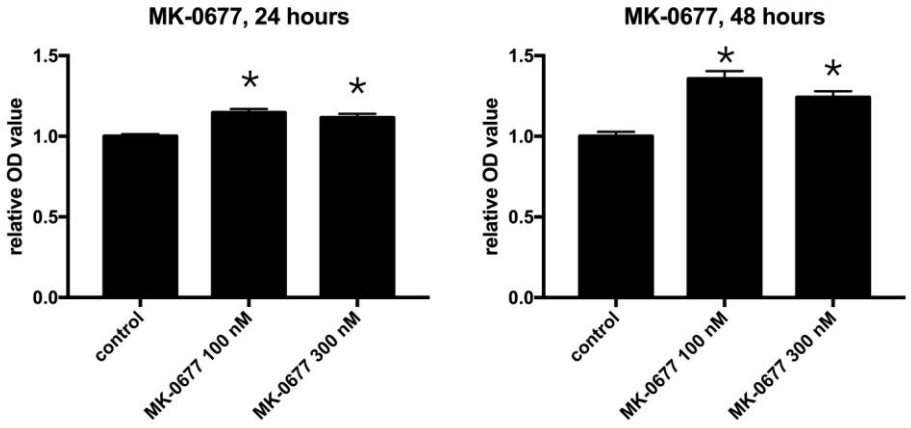

Figure 5: Effect of MK-0677 on viability of WPMY-1 cells was examined by CCK-8 assay. *p < 0.0001 for control vs. MK0-677. Data are shown as means ± S.E.M. from n = 5 independent experiments.

4.1.2 Effects of MK-0677 on proliferation of WPMY-1 cells in EdU assay

In EdU assay, nuclei of proliferating cells are shown in pink, while nuclei of non-proliferating cells are shown in blue. The relative number of proliferating cells (pink nuclei) was notably higher after treatment with 100 nM of MK-0677

(Figure 6), compared with solvent-treated controls. This increase of proliferation rate in response to 100 nM MK-0677 occurred after 24 hours of incubation.

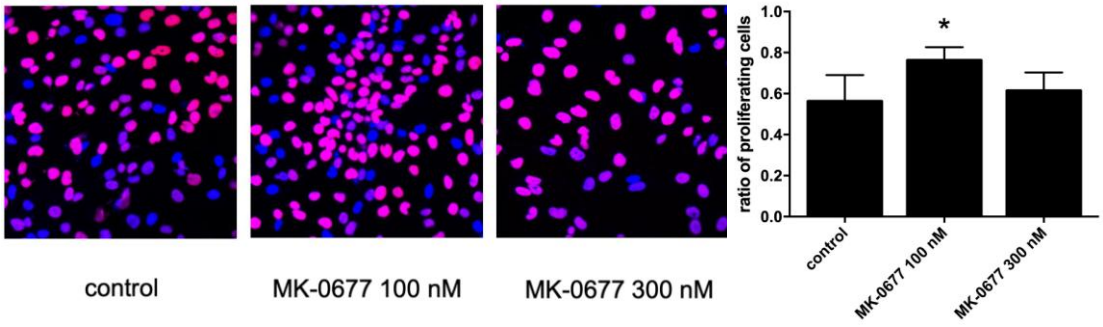

Figure 6: Proliferation rate assessed by EdU assay after incubation with solvent or MK-0677 (100 nM and 300 nM) for 24 hours. The number of cells showing proliferation (pink nuclei) was referred to the total number of cells (pink + blue nuclei). Shown are representative images and means ± S.E.M. from series with n = 5 independent experiments for each setting (* $p < 0.0001$ for corresponding control vs. MK-0677 100 nM).

4.1.3 Effects of MK-0677 on proliferation of WPMY-1 cells in colony plate assay

The initial cell number was 100 per well in the 6 well plates, which widely scattered in the whole growth area of every well. After 14 days incubation, formation of colony groups were observed, which are stained purple (Figure 7).

The number of stained colonies was counted. In plate colony assay, colony formation was increased by MK-0677 (for control vs. MK-0677 100 nM, numbers of colonies were 34.8 ± 5.6 and 49.6 ± 9.7, respectively). Colony formation was not significantly increased by 300 nM MK-0677, number of colonies were 43.8 ± 7.9.

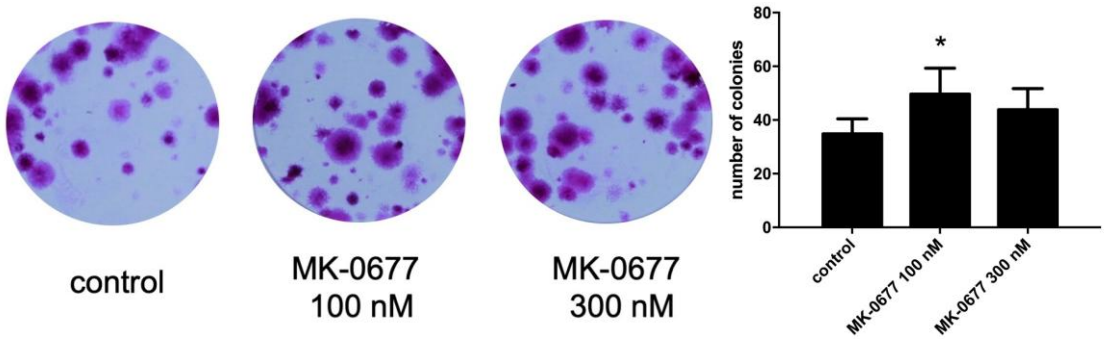

Figure 7: Effects of MK-0677 on proliferation of WPMY-1 cells in plate colony assay. WPMY-1 cells treated with 100 nM and 300 nM MK-0677 displayed much more and larger colonies (*p < 0.02 for MK-0677 100 nM vs. control). Shown are representative experiments (upper panel) and means ± S.E.M. from n = 5 independent experiments (lower panel).

4.1.4 Effects of MK-0677 on cell cycle of WPMY-1 cells

WPMY-1 cells were incubated with 100 nM MK-0677 for 24 hours (Figure 8 A), and cell populations in G1 phase, S phase and G2/M phase were assessed by means of flow cytometry. The percentage of cells in G1 phase was 63.4 % ± 2.44 % and 61.3 % ± 2.45 %, respectively (p = 0.0218, control vs. MK-0677 100 nM). Cell population in G2/M was 4.19 % ± 1.88 % and 7.21 % ± 1.02 %, respectively (p < 0.0001, control vs. MK-0677 100 nM). Histogram shown that MK-0677 significantly decreased the cell distribution in G1 phase and increased in G2/M phase after 24 hours.

After incubation with 100 nM MK-0677 for 48 hours (Figure 8B), the percentage of cell population in G1 was 63.0 % ± 2.97 % and 61.2 % ± 2.27 %, respectively p = 0.0654 for control vs. MK-0677 100 nM, meanwhile cell population in G2/M was 4.89 % ± 1.65 % and 7.18 % ± 0.95 %, respectively (p < 0.0001, control vs. MK-0677 100 nM). After 48 hours incubation of MK-0677, histogram shown that MK-0677 significantly increased in G2/M phase, but no effect on G1 phase.

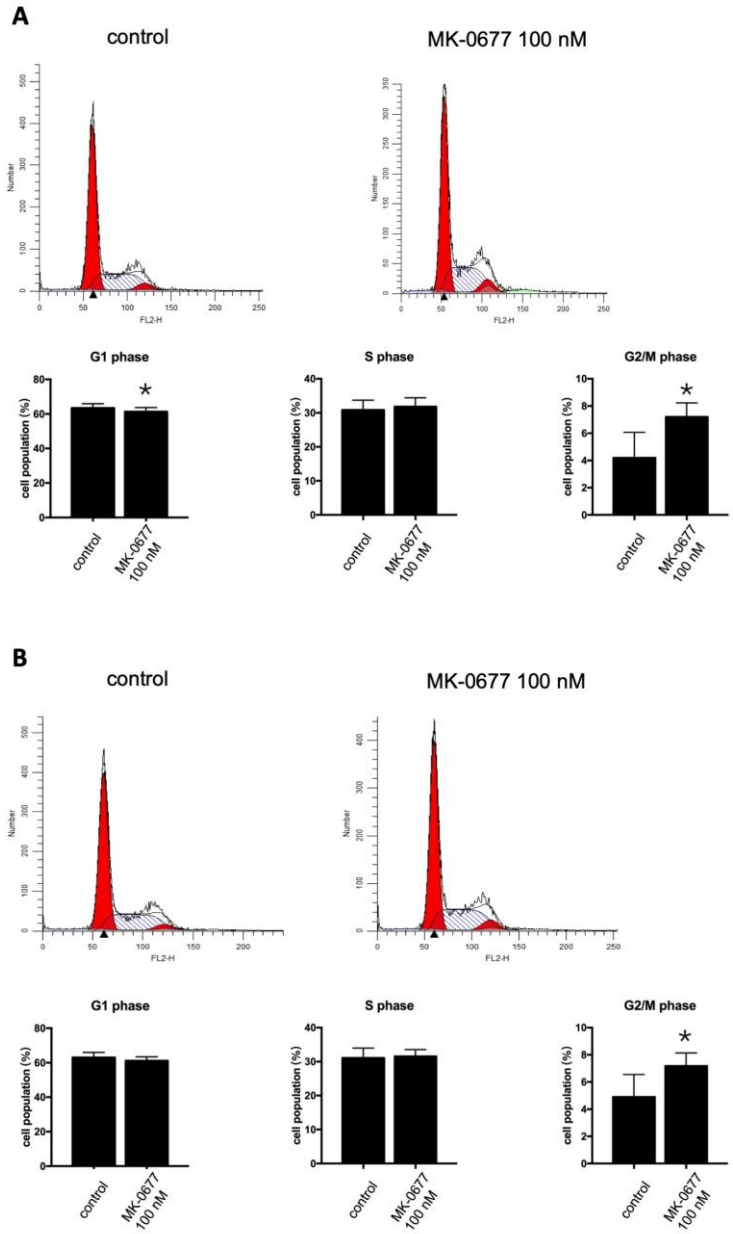

Figure 8: Effects of MK-0677 on cell cycle in WPMY-1 cells (A for 24 hours and B for 48 hours), assessed by flow cytometry analysis. Histograms show representative experiments, while diagrams show results from quantification of the cell population in the G1, S, and G2/M phase (After 24 hours, *$p < 0.03$ for G1 phase of control vs. MK-0677 100 nM, *$p < 0.0001$ for G2/M phase of control vs. MK-0677 100 nM; after 48 hours, *$p < 0.0001$ for G2/M phase of control vs. MK-0677 100 nM). Shown are means ± S.E.M. from n = 5 independent experiments.

4.2 Effect of MK-6077 on mRNA profiles of WPMY-1 cells

4.2.1 Effects of MK0-677 on proliferation markers of WPMY-1 cells via RT-PCR

Expression levels of Ki-67, cyclin D1, and CDK4 in WPMY-1 cells were assessed by RT-PCR. Significant higher expression levels of Ki-67, cyclin D1 and CDK4 were detected in MK-0677 treated cells, compared to solvent-treated cells (Figure 9).

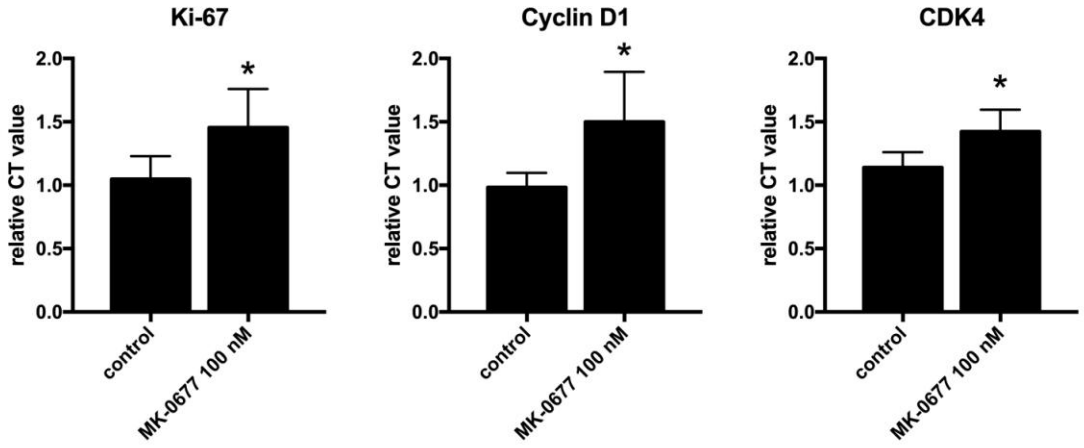

Figure 9: Effects of MK-0677 (100 nM, 24 h) on mRNA expression of different proliferation markers. Analyses were performed by RT-PCR for Ki-67, cyclin D1 and CDK4. Shown are means ± S.E.M. from n = 5 independent experiments for each primer (*p < 0.05 vs. control). Relative CT value are ΔΔCP values (2^-(Ct_{target}-Ct_{GAPDH}), which are normalized to the mean of the control group.

4.2.2 Effects of MK-0677 on cell cycle regulating factors mRNA of WPMY-1 cells

In WPMY-1 cells, mRNA of CDK2 and E2F3 were detected by RT-PCR. Significant lower expression levels of CKD2 were detected in MK-0677 treated cells, compared to solvent-treated cells. In contrast, expression levels of E2F3 were not changed (Figure 10).

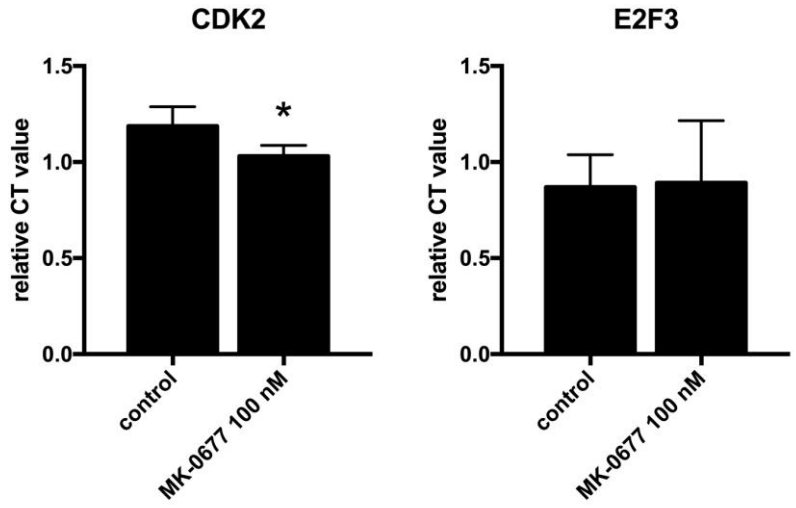

Figure 10: Effects of MK-0677 (100 nM, 24 h) on mRNA expression of cell cycle regulating factors, including CDK2 and E2F3. Shown are means ± SEM from series with 5 independent experiments for each primer (*p < 0.05 vs. control). Shown are means ± S.E.M. from series with n = 5 independent experiments for each primer (*p < 0.05 vs. control). Relative CT value are ΔΔCP values (2^-(Ct_{target}-Ct_{GAPDH}), which are normalized to the mean of the control group.

4.2.3 Effects of MK-0677 on growth factors of WPMY-1 cells

Expression levels of IGF-1, EGF-2, FGF-2 and TGF-β1 in WPMY-1 cells were assessed by RT-PCR. In WPMY-1 cells with incubated with 100 nM MK-0677 for 24 hours, significant higher expression levels of IGF-1 were observed compared to solvent-treated cells (Figure 11). However, there was no significant difference of expression levels of EGF-2, FGF-2 and TGF-β1 between the control and MK-0677 treated groups.

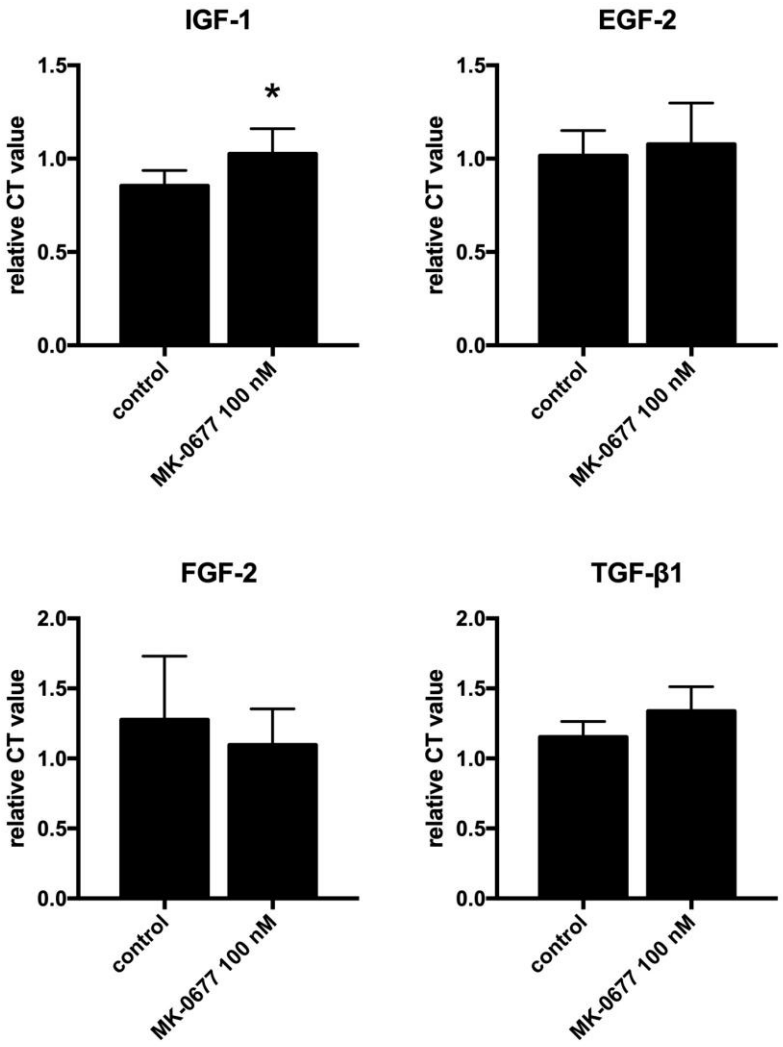

Figure 11: Effects of MK-0677 on mRNA expression of growth factors. Analyses were performed by RT-PCR for IGF-1, EGF, FGF-2 and TGF-β1 (growth markers). Shown are means ± S.E.M. from series with n = 5 independent experiments for each primer (*$p < 0.05$ vs. control. Relative CT value are ΔΔCP values (2^-(Ct_{target}-Ct_{GAPDH}), which are normalized to the mean of the control group.

4.3 Effects of PF-05190457 on human prostate smooth muscle contraction

4.3.1 Effects on EFS-induced contractions

Frequency-dependent contractions of prostate tissue were induced by means of EFS (2-32 Hz). PF-5190457 in a concentration of 10 µM decreased EFS-induced

contractions of prostate strips. Multivariate analysis was conducted to compare frequency-response curves at different stimulation frequencies (p = 0.008 compared to DMSO at 32 Hz). Two-way ANOWA was applied to compare the difference between DMSO and PF-5190457 groups (p = 0.07 vs. DMSO) (Figure 12).

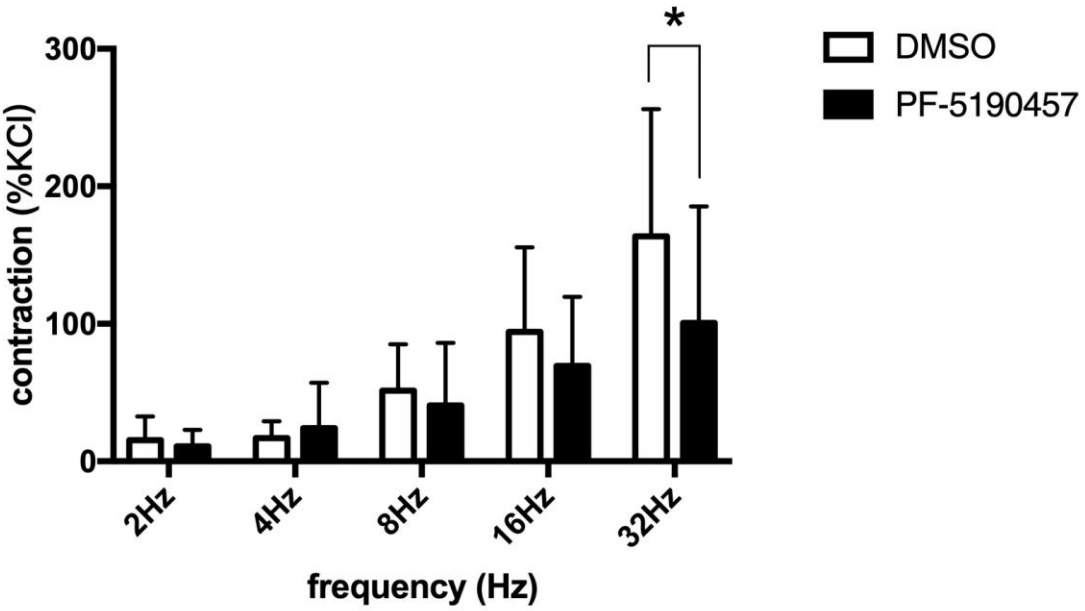

Figure 12: Effects of PF-5190457 on EFS-induced contractions of human prostate tissue. In an organ bath, contractions of prostate strips were induced by EFS. Shown are means ± S.E.M. from series using tissue from n = 10 patients, with tissues from each patient being allocated to the DMSO and the PF-5190457 group (*p < 0.05 for control vs. PF-5190457 in multivariate analysis). Contractions were referred to 2 M KCl-induced tension, to correct any heterogeneity of tissues and patients.

4.3.2 Noradrenaline-induced contractions

Concentration-dependent contractions of prostate tissue were induced by means of noradrenaline (0.1-100 µM). Multivariate analysis revealed that inhibition by PF-5190457 was significant at 10 and 30 µM of noradrenaline (p < 0.02 vs. DMSO). However, the inhibiting effect of PF-0519457 was inverted at 100 µM of noradrenaline (p < 0.02 vs. DMSO) (Figure 13). Two-way ANOWA was conducted to compare the difference between DMSO and PF-5190457 (p < 0.001 for PF-5190457 vs. DMSO).

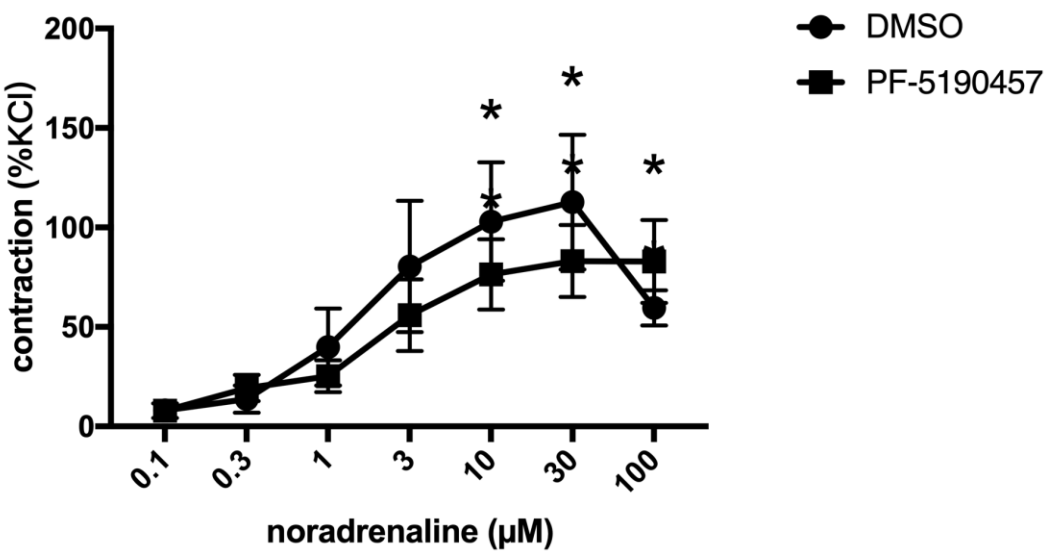

Figure 13: Effects of PF-5190457 on noradrenaline-induced contractions of human prostate tissue. Shown are means ± S.E.M. from a series using tissues from n = 9 patients, with tissues from each patient being allocated to a DMSO and a PF-5190457 group (*$p < 0.05$ for control vs. PF-5190457 in multivariate analysis). Contractions were normalized to 2M KCl-induced tension, to correct any heterogeneity of tissues and patients.

5. Discussion

Prostatic cell proliferation and prostate smooth muscle tone are essential for etiology and therapy of LUTS suggestive of BPH. Both are responsible for urethral obstruction and BOO in prostate enlargement. Currently, α_1-blockers to relax prostate smooth muscle are the first line treatment of male LUTS suggestive of BPH. However, it is widely accepted that the efficacy of α_1-blockers is limited [119]. In order to overcome the limitation of current treatments in BPH, novel therapies should be considered.

Metabolic factors should be taken into account in the treatment of LUTS suggestive to BPH. The present study may suggest that metabolic hormones promote growth in the hyperplastic prostate, which may initiate development of BPH and LUTS suggestive of BPH in patients with metabolic syndrome [21].

According to the results in another thesis of the lab, where this thesis was performed, ghrelin agonists increase prostate smooth muscle tone in α_1-adrenergic and neurogenic contraction. The present thesis suggests that ghrelin agonists may also increase the proliferation of prostate stromal cells. Here, an inverse GHSR agonist inhibited the α_1-adrenergic and neurogenic prostate smooth muscle contraction. Together, this supports the idea that the ghrelin system may take part in the development of LUTS suggestive of BPH. Inhibiting the activities of the ghrelin system could bring novel options for the treatment of LUTS suggestive to BPH. The ghrelin system may be a molecular mechanism connecting the development of BPH/LUTS with the metabolic syndrome. Such a relationship emerged clearly from previous epidemiologic studies, while underlying molecular mechanisms are still widely unclear.

5.1 Ghrelin and GHSR take part in proliferation and contraction in different systems

GHSR is a member of the G protein-coupled receptor family, and regulates growth hormone secretion. Expression of GHSR in adults is found in different regions, including the stomach, the central nervous system (CNS), pituitary gland, vascular endothelium and other peripheral tissues [269]. Ghrelin specifically binds to the GHSR with high affinity, and produces a complex compound of ghrelin-GHSR which is associated with internalization [188]. Based on neurological studies, activation of GHSR is critical for proliferation and migration of glioma cells [270]. Furthermore, emerging evidence suggested that GHSR activation enhances the proliferation of airway and gastrointestinal smooth muscle cells [271]. Ghrelin-enhanced proliferation and cell growth have been reported for different cell types [190, 222, 272, 273]. In a study using rat hippocampal neural stem cells (NSCs), ghrelin stimulated the cellular proliferation by means of accelerating progression of cell cycle phase from G1 to G2/M [274]. Wang et al. suggested that ghrelin improved proliferation of hepatocytes by regulating cell cycle via GSK3β/ catenin signaling pathway [200]. In detail, exposure of ghrelin leads to a shift from G1 phase to S phase in the case of hepatocytes, which means ghrelin could be a protective factor for patients who have liver injury. Thus, ghrelin is a mitotic signal from stomach, but effect on other target organs [275].

Additionally, ghrelin and its receptor were reported to effect on regulation of contraction, pressure control and cell proliferation in vascular smooth muscle [276]. For instance, ghrelin has an acute effect on relaxation of human artery via regulating vascular K^+ channels [277]. In terms of gastrointestinal smooth muscles, ghrelin has an important function on the prokinetic activities in the stomach, and increased the intestinal transit [278]. Based on animal experiments, ghrelin was reported to accelerate gastric emptying, and improve inter-digestive motility [279, 280]. However, there are conflicting reports on ghrelin effects on

cells. Rossi et al. suggested that ghrelin inhibits the proliferation of aortic smooth muscle cells [258]. Antiproliferative effects were reported on thyroid and breast cells [281, 282].

On the other hand, ghrelin and GHSR were reported to be involved in regulating smooth muscle contraction in different cell types. For instance, it has been suggested that ghrelin management maintains the spontaneous gastric contractions in rat model [283]. Using an in vitro experimental approach, the role of ghrelin and GHSR on airway smooth muscle contractions has been observed. Ghrelin and GHSR show a potential effect on airway smooth muscle contraction via NO signaling [284].

According to preliminary studies (preceding this thesis) on the human prostate tissue and prostatic stromal cells, GHSR mRNA is obviously expressed in both. The ghrelin system was assumed to play a role in the development of LUTS suggestive to BPH. In order to clarify whether ghrelin takes part in the proliferation of prostate cells, the ghrelin agonist MK-0677 was applied here in a prostate cell culture model. In terms of prostate smooth muscle contraction, the inverse agonist PF-5190457 was applied on prostate tissue.

5.2 MK-0677 enhances the proliferation of prostate cells and regulates their cell cycle.

MK-0677, which is a selective agonist of the ghrelin receptor, was developed as an orally available GHSR ligand [264, 285]. The compounds resulted from optimization of preceding lead compounds/precursors to induce growth hormone release in rat pituitary cell assays, where MK-0677 showed an EC_{50} of 1.3 nM [286]. Oral availability was first confirmed in dog models reference. 32 beagle dogs were treated with MK-0677 1.0 mg/kg oral administration for 14 days and the results suggested that MK-0677 can elevate serum IGF-1 levels [287]. MK-0677 may activate GHSR-coupled pathways and induces

GHSR-mediated effects with EC_{50} values in the nanomolar range (0.2-1.4 nM) [288]. MK-0677 was identified as a competitive receptor agonist [289]. In vitro, Lee et al. suggested that oral administration MK-0677 (4 mg/kg) in rats increased serum GH (1.8 times higher compared to basal levels), suggesting that oral administration of MK-0677 plays a role in the progression of GH-release [290].

Meanwhile, clinical trials about MK-0677 exist. In 1999, Murphy et al. published a clinical trial on MK-0677 in osteoblasts treatment in elderly patients [291]. The results suggested that GH and MK-677 have a stimulatory effect on bone remodeling. Adunsky et al. launched a phase II placebo-controlled clinical trial of MK-0677 provided novel solution to patients suffering from the hip fracture and going through the functional recovery therapy [292].

MK-0677-induced proliferation of WPMY-1 cells shown in this thesis suggests that the ghrelin system takes part in the growth of prostate cells, which was confirmed by different readouts, including EdU assay, CCK-8 and plate colony assay. Based on the data from flow cytometry, the connection between cell cycle regulation and ghrelin dependent proliferation has been demonstrated. By activating cyclin-CDK-dependent transcription, prostate stromal cells commit to enter a new cell cycle during G1, which might be a potential mechanism of ghrelin enhanced prostate cell proliferation.

The increased population of cells in the G2/M phase suggested that cell cycle has been accelerated by MK-0677 and over load fracture of G2/M cells were detected [293]. Combined with the other readouts including CCK-8 assay and EdU assay, the results depict that WPMY-1 cell number and viability are both increased after exposure to ghrelin agonists. According to the flow cytometry results, MK-0677 significantly increased the cell fracture in G2/M phase, but not in the G1 phase, so that the sensible explanation is that cell cycle has been accelerated, especially in G1 phase [294]. Taken together, ghrelin agonists may

promote the progression of cell cycle, and increase the proliferation of prostate cells.

5.3 MK-0677 alters the profile of cell cycle regulators and growth factors

After exposure to MK-0677, an increased expression of CDK4, and decreased expression of CDK2 were observed. Based on studies of separate animal models, CDK2/CDK4 play a role in cell cycle regulation and subsequently affect the proliferation in different types of cells [295]. For WMPY-1 cells, the current results may suggest that ghrelin arrests prostate stromal cells in the G2/M phase by regulating CDKs and cyclin [296]. Cyclins and CDKs are cooperating with each other in cell cycle. Additionally, CDKs has no function in the absence of a cyclin partner. Once activated by a cyclin partner, CDKs coordinate the entry into the next phase of the cell cycle [297]. This progression is controlled by means of a complicated signaling network. The CDKs, including CDK2 and CDK4 are major regulators of cell cycle progression [298] (Figure 14). Taken together, MK-0677 may regulate the cell cycle via CDK2/CDK4 in prostate cells.

Especially, CDK4 regulates the transfer of cells in the G1 phase into the S phase, and cooperates with other functional cytokines, immunoglobulin (IG) superfamily, and growth factors. Activity of CDK4 would regulate the function of E2F [299]. Subsequently, when the cell cycle into the S phase, E2F stimulates the following transcriptional activation of genes that control the rate of proliferation in prostate cells [296].

A main finding of this thesis is, that treatment with a ghrelin agonist increases the proliferation rate in prostate stromal cells. In BPH, the prostate enlargement may contribute to the symptoms associated to bladder emptying. Taken together,

ghrelin agonists may promote prostate cell proliferation by regulation of cell cycle and its regulators.

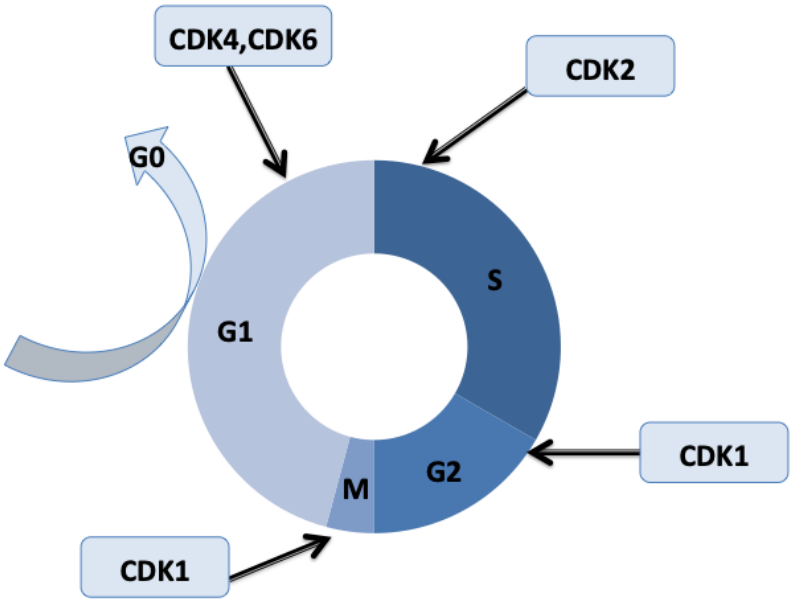

Figure 14: Typically, in a rapidly proliferating human cell line with a total cycle time of 24 hours, the G_1 phase (11 hours**)**, S phase **(**8 hours**)**, G_2 **(**4 hours**)**, and M **(**1 hour**)**, even though the duration of these phases are different in various cell lines [296]. In detail, G1 is the longest phase of the cell cycle, which represents the first step for cell duplicating. Altered expression of relative regulation factors like CKDs could influence the cell cycle phases, what can be analyzed by FACS.

In WPMY-1 cells, up-regulated IGF-1 mRNA occurred here after exposure to MK-0677. This may suggest that a relationship between ghrelin system and IGF-1 exists in proliferation of prostate cells [300]. The relationship between the cellular proliferation and IGF-1 has been widely reported. For instance, in relation to growing children, activation of the ghrelin/ IGF-1 axis increases the body weights and stimulates longitudinal bone growth [301]. In prostate cell lines, IGF-1 signaling is indispensable to mediate estrogen-induced proliferation [302].

The results are in line with previous reports demonstrating a positive correlation between mRNA expression of IGF-1 and prostatic stromal cell growth. High

expression of IGF-1 may explain the alteration of cell cycle. Increased IGF-1 signaling has been reported to be involved in cell proliferation, cell survival and cell cycle.

A crosstalk of IGF-1 and CDK2/CDK4 was observed in different cell types [303, 304]. Growth factors, including IGF, EGF, TGF, FGF, have been reported to take part in the proliferation on different cell types [305]. Koshinaka et al. suggested that the GH-STAT5-IGF-1 axis existed in the plantaris muscle in a mice model and played an important role in preventing skeletal muscle atrophy [306]. Min et al. found that, by promoting nuclear localization of cyclin B/CDK1, IGF-I enhances activation of CDK1 kinase during G2/M in oligodendrocyte progenitor cells (OPCs) [307]. Furthermore, by means of stimulating of activation of PI3K/Akt/mTOR signaling, IGF-1 is essential for further progression of OPCs through G2/M.

In WMPY-1 cells, MK-0677-induced expression of IGF-1 may effect on CDK2 and CDK4, as suggested by RT-PCR. Subsequently, those CDKs, which are essential for promoting cell cycle progression, induce the proliferation of prostate stromal cells [305] (Figure 15). Thus, it appears obvious that the ghrelin system may be involved in the development of BPH/LUTS. IGF-1 signaling may be involved in ghrelin-induced proliferation in prostate cells.

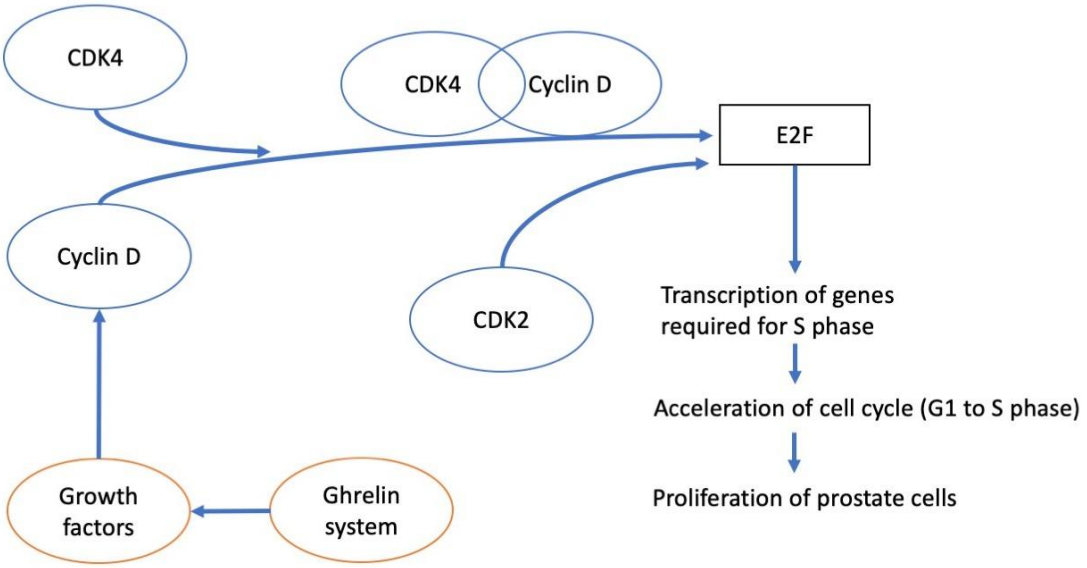

Figure 15: The ghrelin system plays a role in the metabolic syndrome and alters the expression of growth factors via regulating the activation of CDK4 [295]. Subsequently, CDK4 combined with cyclin D partly activate E2F-dependent transcription, and CDK2 may promote the E2F-dependent transcription. These two stimulating inputs induce the acceleration of cell cycle. Finally, the proliferation of prostate stromal cell was increased by this network.

5.4 PF-5190457 reduced the neurogenic and adrenergic contraction of prostate tissue

According to the findings of this thesis, it was observed that an inverse ghrelin receptor agonist, PF-5190457, reduced EFS-induced contractions. EFS-induced smooth muscle contractions of prostate tissue are caused by adrenergic neurotransmission and subsequent activation of postsynaptic α_1-adrenoceptors located on prostate smooth muscle cells [308]. Consequently, the effect of PF-5190457 on noradrenaline-induced contractions of prostate tissues was also examined. PF-5190457 reduced noradrenaline-induced contractions up to an noradrenaline-concentration of 30 µM, while an increased tone was observed at 100 µM noradrenaline. These changes were significant after multivariate analysis. Notably however, the overall effect of PF-5190457 on noradrenaline-induced contractions was an inhibition, as shown by the p-value resulting from comparison of the whole groups. Any explanation, why an increased tone was observed at 100 µM remains speculation at this stage and on the basis of the available data. The noradrenaline-curves with and without PF-5910457 may point to a competitive antagonism of α_1-adrenoceptors by PF-5910457, what would mean, that the whole effect is not caused by inverse agonism of GHSR, but antagonism of α_1-adrenoceptors.

Alternatively, these data may point to a cross-talk between GHSR and α_1-adrenoceptors, which remains to be understood in detail. Thus, downstream cross-talk appears possible to explain this phenomenon. Most important from a clinical and translational view appears, that the overall and predominant effect of

PF-5910457 on noradrenaline-induced contraction was an inhibition [309]. PF-5910457 was introduced as a potent, selective ghrelin receptor inverse agonist and the first inverse GHSR agonist that translated to clinical development [310]. Based on pharmacologic research, PF-5170457 has a superior effect on ghrelin receptor and less off-target selectivity than other GHSR inhibitors [201]. However, the information of PF-5910457 on clinical translation is limited. PF-5910457 were provide by Pfizer and is a high affinity and selective ghrelin receptor inverse agonist (K_d = 3 nM) [265]. A phase 1b human laboratory study suggest that heavy drinkers may benefit from administration of PF-5910457 [266]. This clinical study proved the safety and tolerability of PF-5190457.

The findings of this thesis suggested that PF-5910457 inhibits α_1-adrenergic contraction of prostate tissues, what is in line with the findings from a preceding thesis showing that ghrelin agonists enhance prostate contractions. Consequently, it may now be hypothesized that ghrelin management may improve LUTS suggestive to BPH. Nevertheless, the effect of PF-5910457 in vivo still needs to be confirmed in further studies, including animal models of BPH or with experimentally-induced LUTS.

5.5 Translational aspects

The findings suggest that ghrelin-induced proliferation of prostatic cells may take part in the development of BPH. However, we still need to explore the inverse agonists of ghrelin system in vivo. In order to benefit the patients who suffering with the LUTS suggestive to BPH, the effective medication or oral capsules are still needed.

In principal, studies based on antagonists may be the right way. But: GHSR shows high intrinsic activity, so that in vivo (e.g. in models of obesity) pure antagonists will block GHSR-effects by only 50 % [260]. Therefore, the latest developments of ghrelin research are inverse agonists, which show higher

efficacy than pure antagonists [266]. PF-5190457 is an inverse GHSR agonist that inhibits constitutive activities of GHSR and reduce the activates of acyl-ghrelin, which has stronger effect than GHSR antagonists, including BIM28163 and JMV2959 [310]. The experiments in this thesis are the first addressing the effects of an inverse GHSR agonist, i. e. PF-5190457, on human prostate tissue. The main finding is that PF-5190457 may inhibit prostate smooth muscle contraction. Therefore PF-5190457 represents a promising compound, which may proceed to a novel pharmacotherapy for patients with LUTS suggestive to BPH.

As another key finding of ghrelin agonist, that we observed MK-0677 improve the proliferation of prostate stromal cells. In prostate, ghrelin management may effect on the development of stromal cells growth. Prostate growth and smooth muscle contraction are main components for treatment of LUTS suggestive to BPH, and were regarded as two individual parts [311]. However, based on the current data suggesting that proliferation of prostate stromal cells and contraction of prostate smooth muscle are linked to metabolic hormone and its receptor. This may reveal the fact that treatment on disorder proliferation and contraction of prostate smooth muscle could rely on single compounds, which targets on the ghrelin system. Currently, based on the clinical studies, combination therapies are still required, which may be replaced by a single compound in the future [312].

5.6 Conclusions

The current results support the idea that the ghrelin system takes part in smooth muscle contraction and stromal growth in the hyperplastic human prostate. Considering low adherence to and low efficacy of medical LUTS therapies, other options with higher efficacy are necessary for the patients. The ghrelin system could be a promising target for the development of novel medications. Clinical trials using inverse ghrelin receptor inverse agonists appear possible and feasible.

Restricts of this thesis still exist. The thesis does not identify all the mechanisms by which ghrelin system effect on the proliferation of prostate cells and on contraction of prostate smooth muscle. It may be possible that ghrelin, through yet undescribed signaling pathway activates CDK2/CDK4 or IGF-1, leading to increased cyclin D1 expression on prostate stromal cells.

6. Conclusion

The ghrelin system may be active in the prostate, as activation of ghrelin receptors promotes growth of stromal cells and inverse agonists reduces prostate smooth muscle contraction. Ghrelin agonist-induced prostate cell proliferation may be related to regulation of IGF-1 and cell cycle. The results of this thesis suggest that a treatment based on the ghrelin system may have the potential to influence LUTS suggestive to BPH.

7. Summary

Lower urinary tract symptoms (LUTS) suggestive of benign prostatic hyperplasia (BPH) are one of the most unpleasant issues for elderly male, while metabolic syndrome is considered to be an essential clinical risk factor of BPH. However, the efficacy of available medications for their treatment is limited. Recently, epidemiologic studies proved relationships between metabolic syndrome and LUTS suggestive to BPH. Other preliminary studies suggested that ghrelin system may take part in this linkage.

By means of preclinical models, including human prostate tissue and cell culture, this thesis provides evidence that the metabolic hormone ghrelin increases the proliferation of prostate cells and, that a GHSR inverse agonist reduced prostate smooth muscle contraction. Results from different readouts confirmed a role of metabolic hormones in proliferation of prostate cells and regulation of cell cycle in BPH.

Both processes, i. e. growth of stromal cells and smooth muscle contraction in the prostate, both contribute to urethral obstruction and LUTS in BPH. Considering the current findings, which suggested stromal cell proliferation and smooth muscle contraction by ghrelin, it appears possible that patients with

LUTS suggestive to BPH may profit from ghrelin management. These findings may provide a basis for subsequent clinical trials of ghrelin inverse agonist.

8. Zusammenfassung

Symptome des unteren Harntraktes (lower urinary tract symptoms, LUTS) auf Grund einer gutartigen Prostatavergrößerung (benigne Prostatahyperplasie, BPH) stellen von ihrer Wahrnehmung her eine der stärksten gesundheitlichen Belastungen bei älteren Männern dar. Das metabolische Syndrom hingegen wird als weniger belastend wahrgenommen, bildet aber einen erheblichen Risikofaktor für kardiovaskuläre Erkrankungen und die BPH. Die Effektivität von Medikamenten zur Behandlung der BPH ist deutlich begrenzt und unzureichend. Epidemiologische Studien wiesen kürzlich Zusammenhänge zwischen dem metabolischen Syndrom und LUTS bzw. der BPH nach. Andere vorläufige Ergebnisse wiesen auf eine mögliche Rolle des metabolischen Hormons Ghrelin in dieser Verknüpfung hin.

In der vorliegenden Promotionsschrift werden Ergebnisse aus vorklinischen Untersuchungen präsentiert, welche eine Ghrelin-Rezeptor-induzierte Proliferation von Stromazellen der humanen Prostata (Zellkultur), sowie eine Abschwächung der glattmuskulären Kontraktion humaner Prostatagewebe (Organbad) durch einen inversen Ghrelin-Rezeptor Agonist belegen. Dabei bestätigen sich Ergebnisse aus verschiedenen Messmethoden (EdU- und Plate-Colony-Assay, mRNA-Expression verschiedener Marker, Durchflusszytometrische Messungen) gegenseitig, und weisen gemeinsam auf eine Ghrelin-induzierte Proliferation von Stromazellen der Prostata hin.

Beide Prozesse, also das Wachstum von Stromazellen und die glattmuskuläre Kontraktion in der Prostata tragen bei einer BPH zu einer Verengung der Harnröhre, und so zu Beeinträchtigungen von Harnstrahl und Blasenentleerung

bei, was schließlich Miktionsbeschwerden verursacht. Daher legen die hier präsentierten Beobachtungen nahe, dass Patienten mit LUTS auf Grund einer BPH von einem medikamentösen Ghrelin-Management profitieren könnten. Die hier gemachten Beobachtungen könnten die Grundlage für nachfolgende klinische Studien mit inversen Ghrelin-Rezeptor-Agonisten bilden.

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10. Abbreviation

5-ARIs	5α-reductase inhibitors
5-EdU	5-Ethynyl-deoxyuridine
AG	Acyl ghrelin
ANOVA	Analysis of variance
ATCC	American type culture collection
ATP	Adenosine 5’-triphosphate
AUR	Acute urinary retention
BOO	Bladder outlet obstruction
BMI	Body mass index
BPE	Benign prostate enlargement
BPH	Benign prostatic hyperplasia
$CaCl_2•2H_2O$	Calcium chloride dihydrate
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
CCK	Cell Counting Kit
CDK	Cyclin-dependent kinases
CKD	Chronic kidney disease
CP	Crossing points
Ct	Cycle threshold
DAG	Des-acyl ghrelin
DAPI	4’,6’-diamidino-2-phenylindole-dihydrochloride
DG	Diacylglycerol

DHT	Dihydrotestosterone
DMSO	Dimethyl sulfoxide
DO	Detrusor overactivity
DTT	Dithiothreitol
EC_{50}	Half maximal effective concentration
ED	Erectile dysfunction
EjD	Ejaculatory disorders
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
FACS	Fluorescence-activated cell sorting
FSC	Forward scatter
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GOAT	Ghrelin O acyl transferase
GPCR	G protein-coupled receptor
GHSR	Growth Hormone Secretagogue Receptor
IC_{50}	Half maximal inhibitory concentration
IG	Immunoglobulin superfamily
IP3	Inositol 1,4,5-trisphosphate
IPSS	International Prostate Symptom Score
KCl	Potassium chloride
KH	Krebs Henseleit solution
KH_2PO_4	Potassium hydrogen phosphate
LIMKs	LIM domain kinases
LUTS	Lower urinary tract symptoms
MAPK	Mitogen-activated protein kinase
MetS	Metabolic syndrome
$MgCl_2$	Magnesium chloride
$MgSO_4•7H_2O$	Magnesium sulfate heptahydrate
MLC	Myosin light chain
NaCl	Sodium chloride
$NaHCO_3$	Sodium bicarbonate

NSCs	Neural sten cells
OAB	Overactive bladder
OPCs	Oligodendrocyte progenitor cells
PBS	Phosphate-buffered saline
PDE	Phosphodiesterase
PDE5Is	Phosphodiesterase 5 inhibitors
PDEIs	Phosphodiesterase inhibitors
PI	Propidium iodide
PLC	Phospholipase C
PLK	Pole-like kinases
PK	Protein kinases
PKC	Protein kinase C
PSA	Prostate specific antigen
PVR	Post-void residual
Qmax	Maximum flow rate
RNA	Ribonucleic acid
RT-PCR	real time polymerase chain reaction
RP	Radical prostatectomy
RPMI 1640	Roswell Park Memorial Institute 1640
SEM	Standard error of the mean
SeR	Serenoa repens
SFK	Src family kinase
STD	Sexually transmitted diseases
SRB	Sulforhodamine B
SSC	Side scatter
T2DM	Type 2 diabetes mellitus
TCA	Trichloroacetic acid
TURP	Transurethral resection of prostate
UI	Urinary incontinence
UR	Urinary retention
VEGF	Vascular endothelial growth factor

VSMCs	Vascular smooth muscle cells
α_1-blockers	α_1-Adrenoceptor antagonists
β3-agonists	Beta3 adrenoceptor agonists

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12. Eidesstattliche Versicherung und Erklärung

Eidesstattliche Erklärung Hiermit erkläre ich, dass ich die vorliegende Arbeit eigenständig und ohne fremde Hilfe angefertigt habe. Textpassagen, die wörtlich oder dem Sinn nach auf Publikationen oder Vorträgen anderer Autoren beruhen, sind als solche kenntlich gemacht. Die Arbeit wurde bisher keiner anderen Prüfungsbehörde vorgelegt und auch noch nicht veröffentlicht.

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