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Main field of study in Molecular biology

Characterization of human STAMP2 promoter and 5' flanking sequence

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60 study points

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List of abbreviations

ABC	ATP-binding cassette
ActD	Actinomycin D
AIB1	Amplified in breast cancer-1
AR	Androgen receptor
ARA	Androgen receptor associated coregulator
ARE	Androgen response element
ARR	Androgen responsive region
ATP	Adenosine 5'-triphosphate
BAC	Bacterial artificial chromosome
bp	Base pairs
BRCA1	Breast cancer 1, early onset
CBP	CREB-binding protein
CDK	Cyclin dependent kinase
C/EBP- β	CCAT/Enhancer-Binding Protein-β
ChIP	Chromatin immunoprecipitation
CHX	Cycloheximide
CRE	cAMP response element
CREB	cAMP response element-binding
CTD	C-terminal domain
DBD	DNA-binding domain
DHT	Dihydrotestosterone
DNA	Deoxyribonucleic acid
DNAse I	Deoxyribonuclease I
EEA1	Early endosome antigen 1
Elk 1	E-26-like protein 1
EMSA	Electrophoretic mobility shift assay
ESE2	Ets factor 2
EtOH	Ethanol
Ets-2	Epithelium-specific factor 2
E2F	E2 (adenoviral protein) factor
FAD	Flavin adenine dinucleotide
FGF	Fibroblast growth factor
FKBP5	FK506- binding protein 5
FOX	Forkhead box
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GR	Glucocorticoid receptor
GRE	GR response element
GREF	Glucocorticoid responsive element matrix family
GRIP1	Glucocorticoid receptor interacting protein 1
GSK3β	Glycogen synthase kinase-3 β
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HSP	Heat shock protein
hSTAMP2	Human Six transmembrane protein of prostate 2
IL	Interleukin
JMJD2C	Jumonji domain containing 2C
Kb	Kilo base
KLK	Kallikrein
LBD	Ligand binding domain
LNCaP	Lymph node prostate cancer
LSD1	Lysine-specific histone demethylase 1
MAPK	Mitogen-activated protein kinase
MPSS	Massively parallel signature sequencing
mSTAMP2	Mouse Six transmembrane protein of prostate 2
NADPH	Nicotinamide adenine dinucleotide phosphate
NCoR	Nuclear receptor corepressor
NEP	Neutral endopeptidase
NFκB	Nuclear factor kappa B
Nox	NADPH oxidase
NR	Nuclear receptor
NTD	NH_2-terminal domain
NuRD	Nucleosome remodeling and deacetylating
PCa	Prostate cancer
P/CAF	p300/CBP-associated factor
PCR	Polymerase chain reaction
PDEF	Prostate-derived Ets factor
PIAS	Protein inhibitor of activated STAT
PIN	Prostatic intraepithelial neoplasia

PI3K	Phosphoinositide-3 kinase
PolII	Polymerase II
PSA	Prostate specific antigen
PTEN	Phosphatase and tensin homolog deleted on chromosome ten
qRT-PCR	Quantitative real time PCR
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SAGE	Serial analysis of gene expression
SHBG	Sex hormone-binding globulin
SMRT	Silencing mediator for retinoic acid receptor and thyroid-hormone receptor
SRA	Steroid receptor activator
SRC-1	Steroid receptor coactivator-1
STAMP	Six transmembrane protein of prostate
STAT	Signal transducer and activator of transcription
STEAP	Six-transmembrane epithelial antigen of the prostate
TBP	TATA box-binding protein
TCTP	Translationally controlled tumor protein
TF	Transcription factor
TFBS	Transcription factor binding site
TFIIF	Transcription factor IIF
TIARP	Tumor necrosis factor α-induced adipose-related protein
TIF2	Transcriptional intermediary factor 2
TNF-α	Tumor necrosis factor-alpha
TRAMP	Transgenic adenocarcinoma mouse prostate
TSAP6	Tumor suppressor-activated pathway-6
TSS	Transcription start site
TST	Testosterone
UTR	Untranslated region
6TM	Six-transmembrane

General introduction

Prostate cancer (PCa) is the most common cancer and a second cause of cancer death in American men [1]. Initially tumors are androgen dependent for growth and regress as a consequence of the androgen-ablation therapy. However, in spite of the good therapeutic response, the majority of these reoccur in a hormone refractory state [2]. Since no efficient cure has so far been found for the hormone-independent state of the PCa, it has been of high interest to identify potential PCa diagnostic biomarkers and therapeutic targets. For this purpose the androgen receptor (AR) and the molecular mechanisms of its action are being studied in our laboratory. The second focus of this study is androgen target genes. One of these, the human six transmembrane protein of prostate 2 (hSTAMP2), is a novel androgen regulated gene originally identified by our group [3]. Our recent findings suggest its possible function in the prostate physiology and pathology. This work involved the molecular analysis of its promoter and 5'-flanking sequences.

1.1 Prostate gland and androgens

1.1.1 The prostate gland

The prostate is a walnut-size exocrine gland that surrounds the urethra at the neck of the urinary bladder in men (reviewed in [4]). Its main function is to store and supply the fluid that composes up to 30% of the volume of semen. It develops by the ninth week of embryonic life from several sets of tubules envaginating from the primitive posterior urethra. The prostate is composed of several lobes that are continuous in an adult. The lobes are composed of lined with secretory epithelium alveoli that drain into the prostatic urethra through a series of tubules. The secretory parenchyma is held together by smooth muscles and connective tissue.

1.1.2 Androgens

Androgens, the male sex steroid hormones, are, in addition to polypeptide growth factors [5], required for prostate differentiation and maintenance of its function. Testosterone (TST), of which 95% is produced by Leydig cells in testis, is the main androgen circulating in the bloodstream of men. In circulation its availability to the tissues is regulated by several proteins to which it is bound. These are: sex hormone-binding globulin (SHBG) and to a lesser extend albumin and corticosteroid-binding globulin [6]. In the prostate, free TST enters the cells, where it is converted by the enzyme 5α-reductase to its active form, 5α-dihydrotestosterone (DHT) (Figure 1). Both TST and DHT can bind to the AR, a member of the steroid receptor superfamily; however, DHT's affinity for the AR is five times higher than that of TST. DTH binding induces a change in AR conformation that is more resistant to degradation [2]. In the absence of androgens, AR resides in the cytoplasm in a complex with heat shock proteins (HSPs). Upon ligand binding, it undergoes conformational change, dissociates from the HSPs and becomes hyperphosphorylated. The HSP-release unmasks dimerization motifs and nuclear localization signal [7]. AR is thus ready to enter the nucleus, dimerize and bind to androgen response elements (AREs). AREs, located in promoters and/or enhancers of androgen regulated genes, are sequences from which AR, in concert with various co-factors, can up- or down-regulate gene transcription.

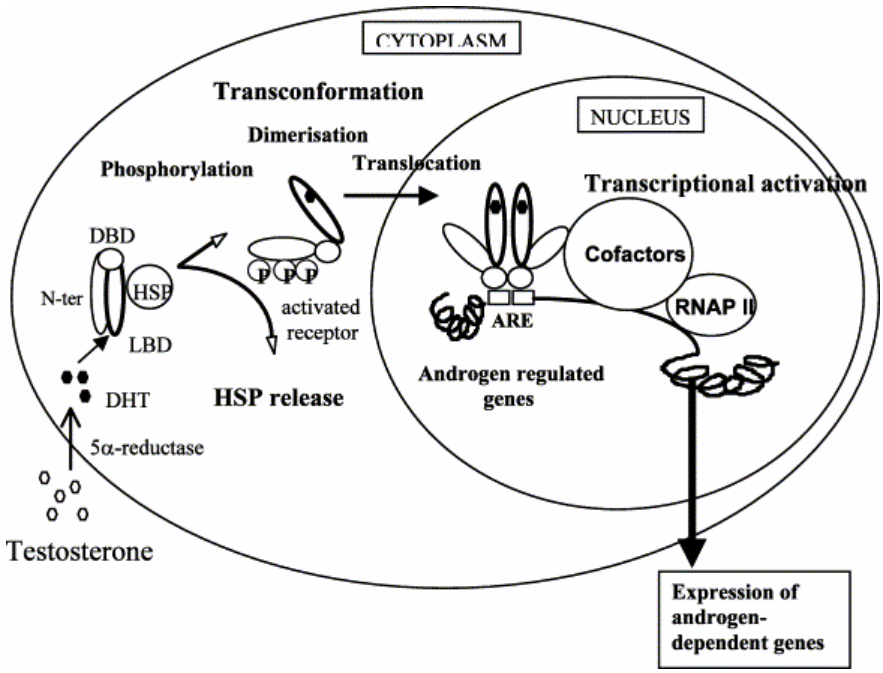

Figure 1. General mechanism of androgen mediated gene regulation
Free testosterone enters the cell through the plasma membrane, and after conversion to DHT by 5α-reductase binds to the ligand binding domain (LBD) of the AR. AR then dissociates from the complex with HSPs, becomes phosphorylated, dimerizes and enters the nucleus where it can bind to AREs and, in concert with cofactors, activate/repress gene expression. (Figure taken from [7])

1.2 AR and its role in gene transcription

1.2.1 The androgen receptor

The AR gene is localized on the X chromosome. It has eight exons and is approximately 90 kilo bases (kb) long. AR is expressed in the majority of tissues and its level depends on the tissue type, age and developmental stage [7]. Structural analyses have revealed that AR contains 4 distinct domains that are more or less conserved among the nuclear receptor family members: a highly conserved central deoxyribonucleic acid (DNA)-binding domain (DBD), hinge region, carboxy-terminal ligand-binding domain (LBD), and a less conserved NH_2-terminal domain (NTD) [8,9]. Further studies localized two regions responsible for the transcriptional activation, 1) a ligand-independent activation domain AF-1 placed in NTD and 2) AF-2 situated in the LBD [2]. Centrally localized DBD contains nine cysteines, of which eight are involved in formation of two zinc-finger domains. In addition to DNA

binding, DBD has been implicated in AR nucleo-cytoplasmic shuttling due to the presence of a non-classical nuclear export signal [10]. Interaction between NTD with LBD has been shown to mediate active conformation of the AR and reduce androgen dissociation [11,12]. A number of phosphorylation sites in the AR have been reported and it has been observed that the ligand-induced AR hyperphosphorylation correlates with its activation (reviewed in [7,13]). Recent findings indicate that the phosphorylation pattern is influenced by compartmentalization of the AR, where androgen-independent phosphorylation was observed for the cytoplasmic AR and androgen-dependent phosphorylation on different serine residues was detected on the nuclear AR [14]. Phosphorylation specificity and its exact function still remain largely unclear. It has been suggested that action of both AR and its cofactors are directly modulated by phosphorylation [15,16].

1.2.2 The androgen response elements

Although heterodimerization has been reported for the AR and the glucocorticoid receptor (GR) , it is has been shown that AR binds AREs mainly in a homodimeric manner [7]. The ARE consensus sequence, GG(A/T)ACAnnnTGTTCT [17], is very similar to that of GR, although non-conventional AREs have also been proven important for androgen specificity [7]. Co-localization of AREs with the GR response elements (GREs) suggests that AR, GR as well as the rest of nuclear receptors (NRs) interact with same sequences, leaving specificity of NR binding still not fully answered [7]. For the most highly androgen regulated genes, AREs can be found in the proximal promoter as well as in the distant enhancer sequences, either upstream or downstream of the first exon [18,19]. These multiple AREs are thought to cooperate in mediating transcription activation. A looping mechanism (Figure 2) has been proposed for the prostate specific antigen (PSA) gene, that involves interaction of AREs situated in the proximal promoter and the enhancer placed about 4 kb upstream of the transcription start site (TSS) [20]. Recently described ARE containing-enhancer of another androgen regulated gene, FK506- binding protein 5 (FKBP5), has been observed as far as 65 kb downstream, in the fifth intron, and a novel mechanism for indirect communication between enhancer and basal transcription machinery has been proposed [18]. To point out another example, neutral endopeptidase (NEP) gene was analyzed and in addition to previously identified ARE in exon 24 and androgen responsive region (ARR) in the NEP promoter, ARE1 and ARE2 in 3' untranslated region (UTR) and intron 17, respectively,

have been found [19]. These examples illustrate how the placement of AREs can vary greatly from one gene to another.

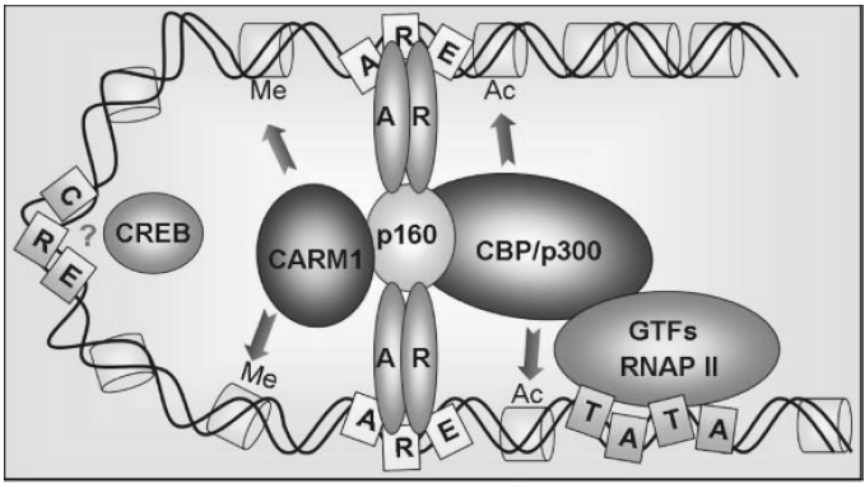

Figure 2. Proposed looping mechanism for the interaction between AREs in the proximal promoter and enhancer of the PSA gene
The contact between AREs located at both sites is bridged by coactivators that together with the transcription machinery form a complex with ARs. (Figure taken from [21])

1.2.3 The AR dynamics

Contrary to the widely acknowledged paradigm of stable NR interaction with DNA, a recent study of AR dynamics revealed a dramatically more transient character of this interaction [22]. AR has been shown to recycle between DNA-bound and unbound state with a half life of about 10 sec [22]. The character of cofactors recruited and the effect on the gene expression seems to be dependent on the rate of this recycling, which was shown to be slower for the agonist-bound AR, than for the antagonist-bound AR, resulting in gene activation [22].

The dynamics of AR has also been reported to be dependent upon the place in which it is bound to DNA. The AR loading and polymerase II (Pol II) recruitment onto the proximal promoters and enhancers was monitored by chromatin immunoprecipitation (ChIP) analysis in the PSA and kallikrein 2 (KLK2) genes and it was shown that AR residence time on the enhancer was more transient than on the promoter [23]. Quantification experiments undertaken in the same study showed twenty times more AR on the enhancer than on the promoter. However, the main Pol II containing complex was assembled on the

promoter. Both AREs in the enhancer and the promoter were needed for the maximum activity [23].

1.2.4 AR action and its cofactors

ARE-bound AR is able to recruit the transcription machinery and its N-terminus has been shown to interact with transcription factor IIF (TFIIF) and TATA box-binding protein (TBP) [24]. AR-mediated transcriptional activation or repression depends on the character of recruited cofactors, most of which are also utilized by other members of the steroid hormone receptors. Coactivators include the p160 family, p300/CBP-associated factor (P/CAF), cAMP response element-binding (CREB)-binding protein (CBP) and p300, all of which have the intrinsic histone acetyltransferase (HAT) activity. Other AR-associated coactivators are: androgen receptor associated coregulator (ARA) 70, ARA55 and ARA54 [25]. Still another coactivator, SWI/SNF, possesses adenosine 5'-triphosphate (ATP)-dependent chromatin remodeling activities, and TRAP/DRIP mediates recruitment of the core transcription factors [26]. Jumonji domain containing 2C (JMJD2C) and lysine-specific histone demethylase 1 (LSD1) are histone demethylating proteins that have been shown to associate with AR and cooperatively demethylate lysine 9 on histone 3 and thus aid androgen regulated gene activation [27,28]. Taken together, so far identified AR coactivators seem to contribute to gene transcriptional regulation in many different ways. This variety may account for the gene- and context-dependent specificity of AR's action.

In the presence of an antagonist, AR can recruit corepressors, as it has been shown for the bicalutamide-bound AR [29]. Corepressor complexes include the nuclear receptor corepressor (NCoR) and silencing mediator for retinoic acid receptor and thyroid-hormone receptor (SMRT) that in turn recruit histone deacetylase (HDAC) activity containing the corepressor complexes Sin3/Rpd3 and nucleosome remodeling and deacetylating (NuRD) complex [30]. In addition it has been reported that NCoR and SMRT also can be recruited by agonist-bound AR and thus, in the presence of DHT, the outcome of AR's action depends on the relative levels of coactivators versus corepressors, both competing for AR binding [31]. Conversely it has been shown that AR antagonists can act as agonists in cells with increased AR level and this antagonist-agonist conversion is associated with alterations in the recruitment of cofactors to the promoters of AR target genes [32]. The fact that AR's

action outcome not only depends on the ligand, but also on the context (specific cofactors present) at the transcription site, may further explain complexity and specificity of AR action. It is important to mention that in addition to the proteins which cooperate with AR at the gene locus, there are other factors that can modulate AR mediated gene activation by modulating AR activity, before it enters the nucleus. For instance, caspase 8 has recently been shown to repress AR-dependent gene expression by disrupting AR amino- and carboxy-terminal interactions and thus preventing nuclear translocation of AR [33].

In addition to directly affecting transcription, AR has also been shown to indirectly act on gene expression in an androgen-independent manner. Some of this evidence comes from the studies of the AR's occupancy of DNA at the PSA locus. In the absence of androgen, AR was shown to be absent from any site in the PSA locus in the prostate cancer cells; however, ribonucleic acid (RNA) interference mediated AR knockdown further reduced already low PSA expression. This suggests that the low androgen-independent PSA expression still requires functional AR [34]. In another study by the same group, the PSA expression in the absence of DHT was shown to be significantly higher for the androgen-independent cell line, than for the androgen sensitive cell line [35]. It was shown that this was a result of ligand-independent AR binding to the PSA promoter, even though the level of the steady-state AR occupancy was relatively low. This study suggests a link between the transcription activating chromatin modifications and sustained AR signaling [35]. Studies of histone modifications at the androgen target gene loci as well as knock down of HDAC-inhibitors further confirmed the importance of histone modification-state in AR-dependent gene activation/repression [23,36].

Another mechanism for AR-dependent transcription activation, that is independent on DNA binding, so called "triggering" of coactivators have been suggested [37]. In this study it was observed that the AR could activate transcription through binding to coactivators without itself being tethered to DNA. These AR-coactivator interactions were shown to involve distinct AR and coactivator regions than in case when AR itself was bound to DNA [37]. Another nonclassical, independent of AR-DNA interaction, actions of androgens has been described in Steroli cells where androgen was capable of regulating CREB-mediated gene expression via the mitogen-activated protein kinase (MAPK) pathway [38].

1.3 AR target genes

High throughput gene expression analysis techniques, like serial analysis of gene expression (SAGE), massively parallel signature sequencing (MPSS), and microarray-based techniques, have allowed for identification of potential androgen regulated genes in cells of prostate origin [25]. As mentioned before, the best understood action of AR is thought to involve DNA binding and requires the presence of the AREs in the gene locus. Prostate specific antigen (PSA) is probably the best characterized androgen regulated gene. It is situated in the kallikrein locus on the chromosome 19q and codes for secreted glycoprotein that belongs to the kallikrein family of serine proteases [39]. Requirement of the AR for PSA expression has been demonstrated in the androgen-negative and PSA-negative prostate cancer cells, PC-3, in which transfection of the AR and androgen treatment induced PSA expression in a AR dependent manner [40]. Transcriptional regulation of PSA involves synergetic interaction between multiple AREs, of which two are situated in the proximal promoter (AREI and AREII) and one cluster of AREs is placed in upstream enhancer (AREIII) about four kilobases away (Figure 3) [21]. Acetylation events and chromatin remodeling have been reported around all three AREs. Mutational analysis showed 99% reduction in PSA promoter activity, when the AREIII was mutated, with 80% and 50% for AREI and AREII respectively [29,41,42]. Based on ChIP analysis and insulator studies of the entire region spanning from the enhancer to the promoter, a combined model for PSA enhancer-promoter interaction has been proposed [20]. It involves both physical enhancer-promoter contact mediated by DNA looping and mediator complex, and tracking of the polymerase through the whole 4kb from the enhancer to the promoter. This Pol II tracking is dependent on Pol II C-terminal domain (CTD) phosphorylation [20]. Other non-consensus and weak AREs, present in the PSA locus, may contribute to its transcriptional activation, together with the putative cAMP response element (CRE) site found in the enhancer [43]. In addition, some other factors were indicated to enhance PSA expression, including two other steroid receptors (glucocorticoid receptor and progesterone receptor) [44-46], as well as GAGATA binding protein, Ets factor 2 (ESE2), and prostate-derived Ets factor (PDEF) [47-49].

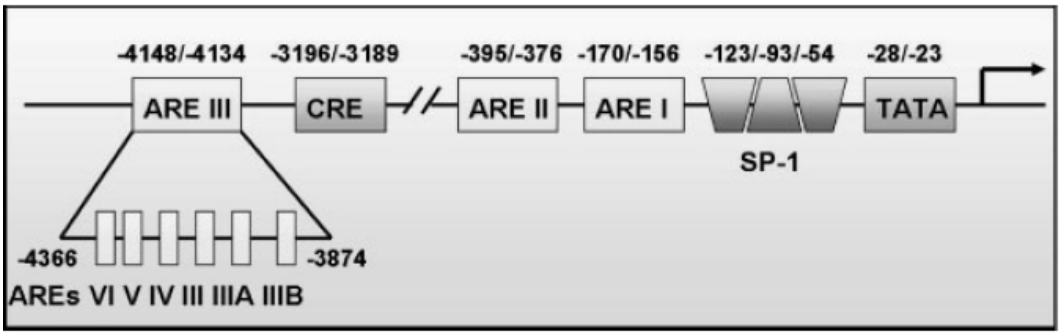

Figure 3. The PSA 5' regulatory region
The regulatory region consists of the promoter with two AREs, SP-1 site and TATA box and the enhancer with the multiple AREs and the single CRE. (Figure taken from [21])

To name another example of AR-target genes, NKX3.1, is a human homeobox gene, that maps to 8p21 region, of which loss of heterozygosity has been reported to be associated with tissue differentiation and loss of androgen-responsiveness [50]. NKX3.1 has been found to be specifically expressed in the prostate epithelium and its expression in the LNCaP cell line has been shown to be upregulated in a time- and androgen concentration- dependent manner [50]. Prescott and coworkers observed NKX3.1 gene regulation by androgens at the transcriptional level, with no need of *de novo* protein synthesis [51]. Other studies showed its high expression in an androgen-independent PCa xenograft model in the absence of androgens, suggesting NKX3.1 deregulation in advanced PCa [52]. Reported nuclear localization of the NKX3.1 protein coincides with its proposed function as homeobox TF [52]. Still another androgen-regulated gene, Kallikrein 4 (KLK4), has been identified and cloned as the fourth member if the kallikrein protease family [53,54]. Different splice-variants of the KLK4 mRNA and predominantly nuclear localization of the KLK4 truncated variants have been reported [55]. KLK4 has also been shown to be regulated by multiple hormones in the PCa cells and deregulated in the androgen-independent PCa [55]. Additionally KLK4 protein has been reported to be highly upregulated in malignant versus normal prostate [56]. Its overexpression has been shown to induce proliferation of PCa cell lines and knockdown of endogenous KLK4 in human lymph node prostate cancer (LNCaP) cells inhibited cell growth, suggesting possible role of KLK4 as a poliferative factor in the PCa development [56].

1.4 Prostate cancer and AR

1.4.1 Prostate cancer

Androgen-dependent epithelial and stromal growth and differentiation is important for both developing prostate and prostatic carcinoma. In this section the events leading to PCa will be reviewed, focusing on the AR and its action. According to the statistical analysis in United States in 2007, prostate cancer, together with colorectal cancer, is a third cause of cancer deaths in men at the age of 40 and above and it accounts for the 29% new cancer cases and 31% cancer deaths among men [1]. This makes it the most common non skin cancer and a third cause of cancer death in men.

After puberty age, the prostate size is regulated by androgens that can stimulate cell proliferation and inhibit apoptosis. Most PCas arise from the peripheral zone of the prostate. Prostatic intraepithelial neoplasia (PIN) is the first morphologically detectable stage of the PCa and can occur as early as in men's twenties. Further the disease progresses slowly, until cancer development around the age of 60 [57]. Around one third of local prostate tumors can become locally invasive and metastatic, spreading to other tissues; bone, liver and lung [57]. Initially tumors are androgen dependent for growth and regress as a consequence of the androgen-ablation therapy treatment. However the majority of these reoccur in a hormone refractory state [2]. This reoccurrence characteristic for PCa is believed to be the consequence of the clonal selection and epigenetic/adaptive mechanisms. After androgen depletion of the multifocal tumors and PIN, the preexisting androgen-independent cells (~1 in 10^5 -10^6) can expand leading to formation of an androgen-independent tumor. Alternatively, same ratio can reflect the frequency of the mutations responsible for "advantageous" genetic or epigenetic events, such as mutations in the AR gene that render it responsive to other hormones, changes in signaling pathways that affect AR activity, changes in the level of AR cofactors, etc. (see below) [58].

1.4.2 AR's role in the prostate cancer development

Because no effective therapy has yet been found for the reoccurrence of PCa, the main focus has been directed on studying the mechanisms that cause progression of PCa from

the androgen naive to the hormone refractory state. It has been observed that the androgen-responsive genes, initially downregulated upon androgen-ablation therapy, become restored in the androgen independent tumors [59]. This raises a question whether the AR can act in a ligand-independent manner, and if so, what are the mechanisms behind it. A number of such mechanisms have been proposed, pointing out the AR as a promising potential therapeutic target. First, overexpression of the steroid hormone coactivators and of the AR itself has been suggested [58]. AR gene amplification (leading to both mRNA and the protein level increases), as well as its increased stability, activity and nuclear localization has been observed in recurrent tumors [58]. Moreover, a point mutation in AR LBD has been proposed as one of the events that can make the AR sensitive to very low concentration of androgens, as well as responsive to other non-androgenic ligands [58]. Activation of such hypersensitive AR can thus occur in the prostate, where some extracellular androgen is still present even after androgen castration. Additionally, the prostate cells have the potential ability to provide intracellular source of androgens by *de novo* synthesis from cholesterol [58]. Both hypersensivity of AR to low testosterone level as well as AR ligand-independent activation have been proposed to be caused by growth factors [60]. Phosphoinositide-3 kinase (PI3K)/Akt, or Ras/MAPK signaling pathways, stimulated by auto- and paracrine growth factors can cause activation of AR, or its partners, by phosphorylation [13]. Thus both overexpression of the growth factors and mutations of the components of these signaling cascades can lead to elevated activation of AR in the absence of the ligand [13]. Finally, there is a range of other events that are common for cancer development in general. Some of these are loss of tumor suppressor genes, upregulation of oncogenes and overexpression of antiapoptotic genes, such as Bcl-2 and Bcl-xL, leading to upregulation of survival pathways [61,58].

1.4.3 Investigation of AR target genes

It has been of high interest to identify potential PCa diagnostic biomarkers or therapeutic targets. Appreciating AR's role in development and progression of PCa, extended studies of AR target genes has been conducted and a number of genes have thus been proposed as promising therapeutic targets. The previously mentioned homeobox NKX3.1 gene is one of these. Its loss by deletion of the chromosome 8p region is associated with hormone-refractory and malignant stage of PCa, which suggests its possible function as a tumor

suppressor gene [50]. PSA, described in the previous section, has been widely used as an indicator of PCa [62]. There is a relatively high rate of false-positive results of PSA screening, because elevated level of PSA may also indicate prostatitis or benign prostatic hyperplasia (reviewed in [63]). In spite of that some believe that PSA screening tests, leading to early diagnosis and treatment, is one of the factors that contribute to declining mortality rate of the patients diagnosed with early stages of PCa (reviewed in [64]). Researchers have thus been focused on detecting novel genes, differentially expressed in healthy versus cancerous prostate, and studying their expression regulation including the role of the AR and other factors as well as epigenetic mechanisms. As far as epigenetics is concerned, locus specific chromatin alterations of the PSA and KLK2 genes have been shown to contribute to elevated gene expression both in presence and absence of DHT in the androgen-independent PCa cells [35]. The microarray technology has made the wide scale gene expression analysis possible.

It is important to mention the model systems that have been used to both identify and study newly discovered androgen regulated genes. The LNCaP cell line is the main cell-based system used for large-scale gene expression studies and one of the few that retains some of the PCa's features. It is epithelial in origin, expresses AR and AR-regulated genes and is androgen sensitive for growth and survival in culture and xenografts [25]. It is also essential to mention the importance of androgen-independent AR-negative PCa cell lines used in PCa studies, with DU 145 and PC-3 being the most widely used lines [65,66]. One has to keep in mind that each of these cell lines, although all representing relapsed androgen independent PCa, arose independently and thus has its own unique characteristics [66]. Finally, rat prostate [25], and transgenic mice [67,68] have been used to study the PCa in more physiological context. However the genetically-engineered mouse models do not simultaneously reflect all the changes leading to PCa development in humans. Fundamental anatomic differences between human and murine prostate is another caveat of these models (reviewed in [4]).

1.5 STAMP/STEAP family members

1.5.1 STAMP/STEAP family

In the search for potential targets for prostate cancer diagnostics and treatment, the six-transmembrane protein of the prostate (STAMP/STEAP (six-transmembrane (6TM) epithelial antigen of the prostate)) family has been identified [3,69-75]. To date, the identified family members include: STEAP, STAMP1(STEAP2), STAMP2(STEAP4)/TIARP (tumor necrosis factor α-induced adipose-related protein) (mouse homologue) and STAMP3(STEAP3) also called TSAP6 (tumor suppressor-activated pathway-6) and its rat homologue pHyde.

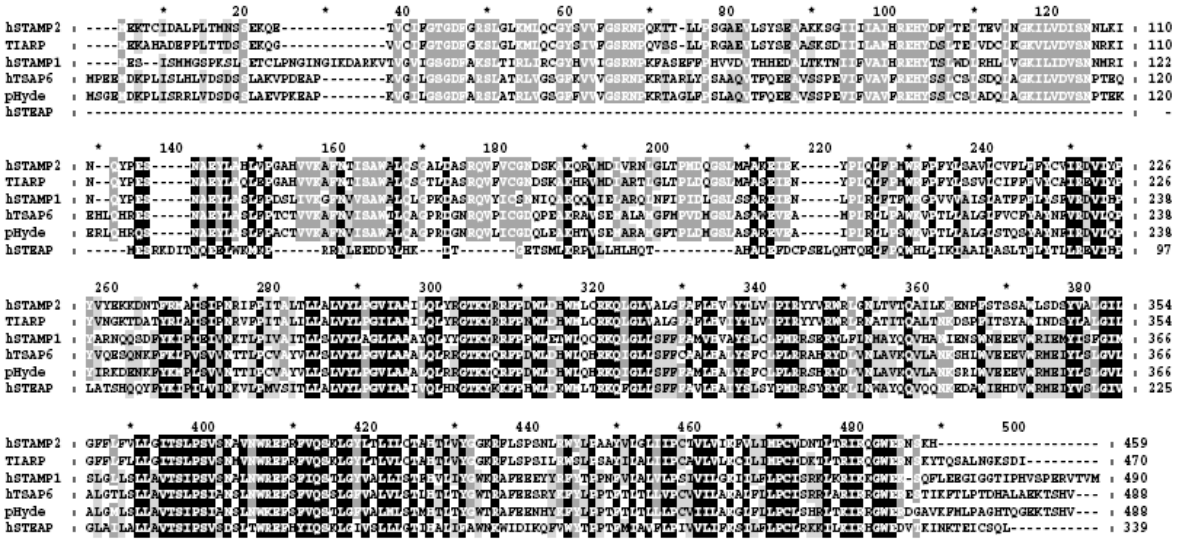

Figure 4. Alignment of the STEAP family members
Clustal and GenDoc alignment of the STAMP/STEAP family members (hSTAMP2, TIARP, hSTAMP1, hTSAP6, pHyde and hSTEAP) is shown. The black shaded residues indicate completely conserved residues. Rresidues conserved in 5 or 4 sequences are shaded dark and light gray respectively. (Figure taken from [3])

1.5.2 STEAP

STEAP was originally identified as highly expressed in human prostate tissue[70]. Also known as prostate-specific cell-surface antigen, it was shown to be located at the cell-cell junction in the secretory prostatic epithelium. As well as being upregulated in the prostate, it has also been shown to be highly expressed in bladder, colon, ovarian and Ewing

carcinoma cancer cell lines [70]. The mouse homolog, mSTAMP, which has 80% sequence homology to STEAP, was cloned from cDNA extracted from a prostate tumor cell line of the transgenic adenocarcinoma mouse prostate (TRAMP) mouse[71]. Mouse STEAP mRNA was shown to be largely prostate specific and strongly detected in primary tumor and metastasis of the TRAMP mice [71]. STEAP, unlike other family members, lacks part of the N-terminal domain (Figure 4).

1.5.3 STAMP1/STEAP2

The six transmembrane protein of prostate 1 (STAMP1) [69], also known as STEAP2 [72] , has been shown to be up to ten fold higher expressed in normal prostate (epithelial cells) compared to other tissues at mRNA level [72]. It is also strongly upreguated in prostate tumors, both primary and hormone-refractory, with higher expression observed in the latter ones. STAMP1 is exclusively expressed in the androgen dependent, AR-positive LNCaP cells, with almost undetectable levels in the AR-negative DU145 and PC-3 cell lines [69]. Its colocalisation to plasma membrane, trans-Golgi network, vesicular tubular structures, as well as colocalisation with the early endosome antigen 1 (EEA1) suggests that STAMP1 may play role in the secretory and endocytic pathways [69,72].

1.5.4 STAMP2/STEAP4

The six transmembrane protein of prostate 2 (STAMP2) [3] also known as STEAP4 or tumor necrosis factor-alpha-induced adipose-related protein (TIARP) [73], is expressed (both at mRNA and protein level) at the plasma membrane upon adipocyte differentiation, in response to tumor necrosis factor-alpha (TNF-α) [73]. TIARP can be stimulated by interleukin (IL)-6, another adipocytokine implicated in pathogenesis of insulin-resistance [76]. Thus in addition to a proposed role in adipocyte development, STAMP2 is thought to regulate homeostasis by modulating the interplay between the inflammatory and metabolic responses in mice [77]. The human STAMP2 will further be discussed in one of the sections below.

1.5.5 STAMP3/STEAP3/TSAP6

The rat homologue of STAMP3 /STEAP3/TSAP6 pHyde, was originally cloned from the Dunning rat PCa cells [74]. pHyde has been shown to inhibit growth of the DU145 and LNCaP cells *in vitro* and contribute to reduction of the DU145-mouse tumors *in vivo*, possibly through stimulation of p53 and induction of apoptosis [74]. Its ectopic expression has been shown to induce apoptosis in DU145 through a caspase-3 dependent pathway [78]. STAMP3/TSAP6 is a p53 inducible protein (with p53-response elements found in its promoter) that regulates apoptosis and cell cycle by directly interacting with NIX (proapoptotic Bcl-2 related protein) and Myt1 kinase (negative regulator of G2-M transition) [79]. Another study has found that STAMP3/TSAP6 interacts with translationally controlled tumor protein (TCTP), a protein promoting histamine release, whose secretion was induced by the STAMP3/TSAP6 ovexpression [80].

1.5.6 Structural analysis and possible function

The 6TM domain, shared by all STAMP/STEAP proteins, is flanked by a large N-terminal and a short C-terminal domain (Figure 4). Its structure displays similarities to channel and transporter proteins [3]. Studies undertaken utilizing interactive profile sequence similarity search have indicated that the 6TM of the STAMP/STEAP proteins have high sequence similarity to the respective domains in nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Nox) and YedZ protein family members [81]. The eukaryotic Nox proteins are involved in production of reactive oxygen species (ROS), that contain N-terminal heme binding transmembrane domain and two C-terminal domains binding flavin adenine dinucleotide (FAD) and NADPH [82]. In addition to being involved in bacterial defense they have been implicated in signal transduction, cancer, mitogenic signaling, growth, angiogenesis, and modification of the cellular matrix proteins [81-83]. A Yed2 oxidoreductase family member found in *E. coli,* has been shown to bind heme and is suggested to be involved in electron transfer. Based on these findings it has been hypothesized that STAMP/STEAP proteins might be involved in oxidation/reduction events that may influence cell signaling, cancer and apoptosis [81].

Additionally, the 6TM proteins have been previously implicated in connection with disease states. For instance, an ATP-binding cassette (ABC) transporter gene mutation was reported to be responsible for Tangier disease [84]. In addition the Wilson disease is a result of malfunction of the putative copper-transporting P-type ATPase, ATP7B, leading to toxic accumulation of copper in the liver and brain [85]. This latter protein, ATP7B, in addition to the structural similarities seems to share similar function, namely copper transport. Recently it was shown that STAMP3/STEAP3, mammalian ferrireductase was critical for erythroid iron homeostasis [86]. The same group argues that tissue-expression pattern of the four murine members of STAMP/STEAP family, STEAP, STAMP1, STAMP3 and STAMP2, is relevant to metal homeostasis [87]. It was shown that the proteins co-localize with transferrin and transferrin receptor, which indicate possible function in iron uptake. The same study suggests that STAMP1, STAMP2 and STAMP3 can indeed reduce both iron and copper and simulate their cellular uptake *in vitro* [87]. These interesting findings as well as the ones described above, establish the basis for further functional studies of the STAMP/STEAP family.

1.6 Human STAMP2

Earlier work conducted in our laboratory led to the identification and cloning of the STAMP2 gene while searching for homologues of the previously identified STAMP1 gene [3]. STAMP2 gene is around 26 kb long, consists of five exons and four introns (Figure 5a) and is located on chromosome 7 close to two other members of this family, STEAP and STAMP1 (Figure 5b). Its amino acid sequence is 64% conserved and 44% identical to that of STAMP1. Additionally it has 78% amino acid sequence identity to TIARP (mouse homologue) as well as significant similarity to pHyde (rat homologue of STAMP3)(Figure 4). While its C-terminal contains the 6TM domain, three protein motifs have been identified in its N-terminal domain. These are: dinucleotide-binding domain, F420-dependent NADP oxidoreductase motif and a motif resembling the pyrroline 5-carboxylate reductase involved in amino acid metabolism and transport [3]. STAMP2 mRNA has been shown to be strongly expressed in placenta, lung, heart and prostate tissues (Figure 6a). Its expression in the LNCaP cells was increased upon androgen stimulation (Figure 6b), in contrast to other STEAP protein family members. Northern blot analysis showed that no

STAMP2 expression could be detected in the AR-negative cell lines, DU 145 and PC-3, or other tissue cancer cell types tested. The subcellular distribution of the GFP-tagged STAMP2 protein included: Golgi complex, *trans*-Golgi network, plasma membrane, vesicular-tubular structures and endoplasmic reticulum. Moreover, the rapid trafficking of the protein localized to vesicular-tubular structures in the cytosol and colocalisation with the EEA1 might suggest the potential function in endocytic and secretory pathways [3]. Further experiments showed that STAMP2 mRNA expression was significantly upregulated in prostate tumors, compared to normal glands. Overexpression of STAMP2 in the STAMP2 negative PC-3 cell line resulted in increased number and size of cell colonies, while overexpression of STAMP2 in COS7 and DU 145 increased cell growth [3]. These results indicate that one function of STAMP2 may be involved in the development of the prostate carcinoma, which makes it a potential therapeutic target and biomarker for this disease [3]. The murine homolog of the same protein, TIARP, has been implicated in the regulation of metabolic homeostasis, as well as in the pathology of insulin-resistance [73,76,77]. Another report suggests that the TIARP functions as a metaloreductase [87]. With respect to studying the genesis and physiology of the PCa, the fact that STAMP2 expression is regulated by androgens in the human prostate is particularly relevant. However, more studies are necessary in order to further elucidate and understand the function of STAMP2.

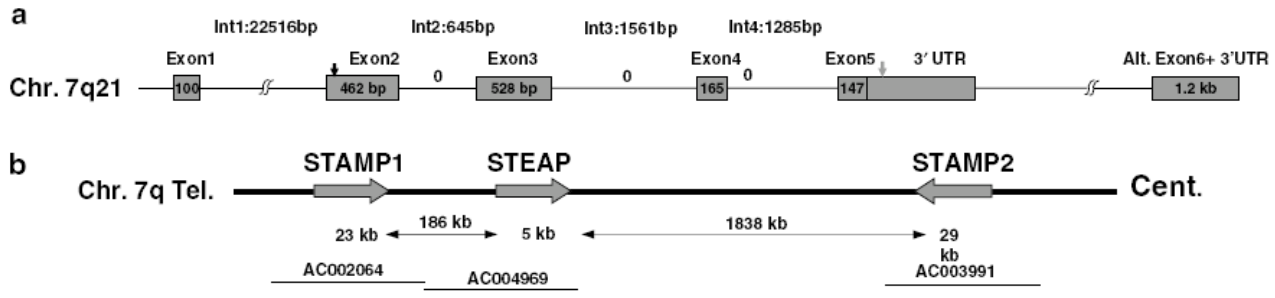

Figure 5. Schematic structure and location of the STAMP2 gene
a) Sites of the exons (boxes) and introns are shown and the position of predicted start and stop codon are indicated by arrows (black and gray respectively). b) The relative location of the three STAMP/STEAP family members on chromosome 7 are shown. The telomeric and centrometic ends of the chromosome, and location of the bacterial artificial chromosome (BAC) clones and the distance between the genes are indicated. (Figure taken from [3])

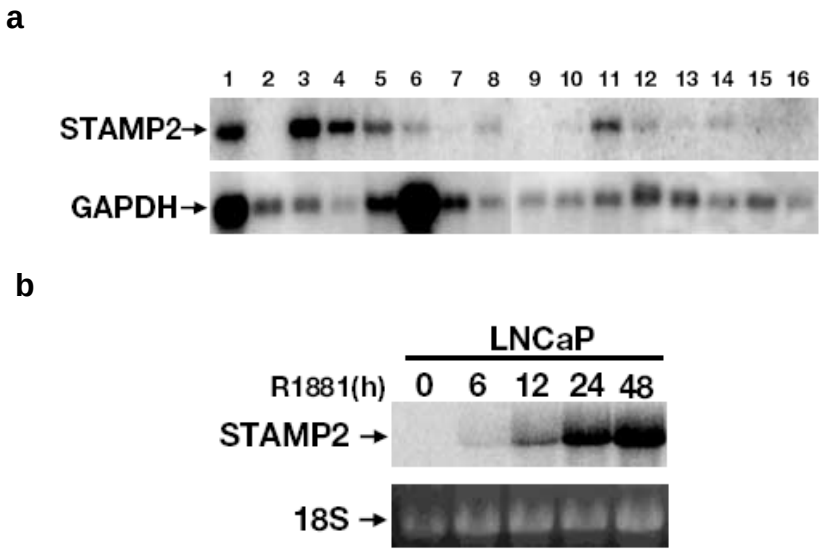

Figure 6. Characterisation of the STAMP2 gene expression
a) Northern blot was probed with STAMP2 or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA. The lanes represent mRNA from: 1. heart, 2. brain, 3.placenta, 4. lung, 5.liver, 6. skeletal muscle, 7.kidney, 8. pancreas, 9. spleen, 10. thymus, 11. prostate, 12 testis, 13. ovary, 14. small intestine, 15. colon, 16. peripheral blood leukocyte. The stronger hybridization observed for GAPDH in lanes 1. and 6. its due to its higher expression in these tissues. b) Northern analysis of the total RNA represents the time course of STAMP2 mRNA accumulation in LNCaP cells treated with the R1881 (synthetic androgen). (Figure taken from [3])

1.7 Transcription studies: computational and experimental

1.7.1 Computational analysis

Each of tens of thousands of genes in the eukaryotic cell has a unique expression pattern. Most of the information about a particular gene's expression program is encoded in its DNA sequence. Thus, in order to understand the mechanism that guides transcription of each gene, it is essential to identify and characterize its promoter, enhancer/silencer elements (cis-acting elements) and the transcription factor (trans-acting factor) binding sites that they contain [88]. Easily accessible bioinformatics tools, developed within the last three decades, allow for the quick wide-scale analysis of the eukaryotic genes. This involves, for example, detection of putative transcription factor (TF) binding sites (TFBS) in the assumed promoter region. Moreover, a genome-wide prediction of TFBPs is carried

out by software on a query sequence that can span over many kilo bases. For these analysis, in addition to complicated algorithms, it was necessary to create electronic databases with consensus sequences for promoter elements and TFBSs [89]. Such databases are made based on the collection of experimentally defined TFBPs and promoter elements in a set of co-regulated genes. The Gene2Promoter (i) program found at the Genomatix webpage (Table 1), is an example of an online software tool that has been designed to identify putative promoters in query sequences. This is carried out by identifying the locus in a query sequence and subsequently listing all alternative transcripts and promoters for that locus. It can also analyze the localized genes and promoters using ElDorado (ii), Genomatix genome annotation. MatInspector (iii) is another Genomatix software tool utilizing a large matrix, and matrix family (iv), library, for the TFBS in order to locate matches in the query DNA sequence [90,91].

1.7.2 Experimental analysis

Bioinformatics has now become an essential and integral area of molecular, cell biological, and biochemical research. Nevertheless, it should be recognized that bioinformatics provides predictions which need to be experimentally supported as false positive and false negative results are possible. Thus laboratory experimentation need to be utilized in order to gain empirical evidence that would supplement the hypothetical computational analysis provided by bioinformatics. For example, to validate promoter localization of a gene, primer extension analysis can be used to reveal the actual transcription start site and help to localize the core promoter region (reviewed in [89]). DNA-pull down strategies, including chromatography and electrophoretic mobility shift assay (EMSA), are effective methods for identification and isolation of the sequence-specific DNA binding factors from nuclear extracts (reviewed in [89]). EMSA is based on the fact that a protein-bound labeled DNA fragment will migrate slower on the gel compared to the control unbound labeled DNA fragment. Deoxyribonuclease I (DNase I) footprinting assay is another method for detecting TFBSs at a single base pair resolution (reviewed in [89]). It utilizes the DNase I to cleave radioactively labeled DNA. Followed by electrophoresis, the exact fragments protected from digestion by bound proteins, can be revealed. Another experimental procedure, ChIP assay, examines the protein-chromatin interactions in vivo (reviewed in [89]). In this method one first cross-links the proteins attached to DNA with formaldehyde.

After shearing the DNA, specific antibodies are used to pull down the TFs of interest and following reversal of crosslinking the immunoprecipitated DNA fragment can be amplified. These methods are able to detect specific TFs that physically interact with a particular DNA sequence. In order to examine the functional relevance of these interactions and to detect promoter and/or enhancer activity in a chosen DNA fragment, reporter gene assays can be applied (reviewed in [92]). As a general rule, the DNA fragment containing the putative promoter is cloned upstream of a reporter gene, of which expression can easily be detected and quantified. Such analysis has been automated and a range of different reporter gene vectors are available. Some of these are designed to detect and study promoters, others to work with enhancers. For additional evidence, site directed mutagenesis can be utilized. By mutating the potentially important cis- and trans-acting elements, in combination with above described laboratory techniques, one can verify their importance for gene regulation.

1.8 Aim of study

The STAMP/STEAP protein family includes novel genes, whose function is not fully known. As detaile above, it has been previously shown that expression of the human STAMP2 mRNA is significantly upregulated in prostate tumors compared to normal glands and that STAMP2 overexpression can induce cell proliferation [3]. In addition, STAMP2 expression is stimulated by androgen treatment and it is detectable only in AR positive LNCaP cells. To get a better understanding of STAMP2 function, it is essential to study its transcriptional regulation. The work described in this thesis is aimed at identifying and cloning the STAMP2 promoter and 5'-flanking sequences and the factors that are involved in STAMP2 transcription. Furthermore, work is presented which aimed to determine how direct the androgen regulation of STAMP2 expression is.

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(iii) http://www.genomatix.de/online_help/help_matinspector/matinspector_help.html

(iv) http://www.genomatix.de/online_help/help_matinspector/matrices.html

Manuscript

Characterization of human STAMP2 promoter and 5' flanking sequence

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Summary

Prostate cancer is the most common non-skin cancer and the third leading cause of cancer death in men in developed countries. No effective cure has yet been found to treat the hormone refractory state of PCa. In addition to study the actions of the AR, a focus has been directed towards identifying androgen-regulated genes as potential biomarkers and therapeutic targets for the disease. hSTAMP2 is one of these genes identified in our laboratory when searching for genes differentially expressed in the early stages of prostate cancer. Our previous studies have shown that its mRNA is highly expressed in the prostate tissue and upregulated in prostate tumor samples. Moreover, its overexpression increased colony formation and cell growth of PCa cell lines, suggesting a possible function in prostate pathology. Furthermore, its mRNA expression appeared to be androgen stimulated. These results suggest a possible androgen –dependent *cis*-regulatory elements located in the hSTAMP2 5'flanking sequence. Computational analyses reveal a number of transcription factor binding sites and androgen response elements (AREs) that may be of importance for the hSTAMP2 gene regulation. Reporter gene assays show the different effect that androgen has on the hSTAMP2 putative promoter activity in two different PCa cell lines. The complexity of the androgen regulation of hSTAMP2 expression is supported in experiments where LNCaP cells were treated with CHX, suggesting the requirement of *de novo* protein synthesis for the AR mediated hSTAMP2 gene regulation. Additional studies are needed to identify potential AREs located in the sequences further upstream and downstream of the hSTAMP2 gene.

2.1 Introduction

PCa is the most common non-skin cancer and the third leading cause of cancer death in men in developed countries [1]. Development and growth of both the prostate and PCa depend on androgens that, after entering the cell, bind to and activate the AR. The ligand-bound AR then enters the nucleus where it interacts with AREs in the chromatin. In concert with recruited coregulators, it can either activate or repress expression of its target genes [2]. In the early stages, PCa relies on androgen for growth and can therefore regress in response to hormonal therapy. Androgen ablation by surgical castration and use of various inhibitors and antiandrogens, have been the critical therapeutic options for the PCa treatment so far [3]. However, in spite of the good initial therapeutic response, in most cases the tumors eventually recur as androgen-independent, adopting a lethally resistant phenotype. Since no efficient cure has been found to battle the hormone refractory state of PCa, much effort has been put into understanding the molecular mechanisms that govern androgens' effect on prostate physiology and pathology. It has been shown that upon progression of the PCa to hormone independence, AR signaling is still active and androgen-response genes are upregulated in a ligand-independent manner [4]. AR amplification, both at gene and protein level, is one of the cellular events that have been observed in prostate tumor progression. In addition, increased stability of the AR, as well as activation and nuclear localization has been proposed to account for maintained signaling in PCa. Furthermore, AR mutations, changes in the cellular level of AR coregulators, as well as overexpression of growth factors are thought to alter AR's ligand sensitivity and specificity (reviewed in refs. [5] and [6]). Thus all these proposed events, leading to amplification of the AR signaling under low testosterone level, point out that AR is a potential target in treating the androgen independent state of the PCa.

In quest for therapeutic targets and biomarkers for the disease, androgen target genes have been another important field of study. Development of methods for the wide scale gene expression analysis, both computational and laboratory, has contributed to identification of a large number of potential androgen regulated genes. Some of these are used as PCa markers. PSA, a member of the kallikrein protein family, is the best characterized androgen target gene and has been utilized in screening of patients as an indicator for the prostatic

disease [7]. Secreted PSA has been shown to be directly regulated by the androgen receptor that binds at multiple AREs located in the proximal promoter and distal enhancer of the PSA gene [8-10]. KLK2, a member of the same protein family, is another example of AR target genes of which regulatory elements have been well studied [11]. NKX.3.1 and KLK4 are two other proteins highly expressed in prostate that are also regulated by androgens [12-14]. In addition to the direct effect of DNA-bound AR on gene expression, DNA binding-independent action of AR, so called "triggering of coactivators" has been reported [15]. Another study suggests evidence for the ligand-independent and DNA-binding independent AR transcriptional activation of the PSA gene [16]. Alternatively, AR is able to affect expression of a gene indirectly by regulating mRNA synthesis of its transcriptional regulators. Moreover, nonclassical action of the AR, where the AR can regulate CREB-mediated gene expression via the MAPK pathway, has been reported in Sertoli cells [17]. Whether it binds directly to regulatory elements in DNA, or acts indirectly through other proteins, AR together with its target genes remain as leading potential factors in prostate pathophysiology.

While searching for highly expressed prostate tissue genes, a novel protein, STEAP, was identified. The STEAP protein was shown to be located at the cell-cell junctions of the prostate epithelial cells [18]. Later, another closely related protein family, STAMP, was identified [19]. So far identified family members include STAMP1/STEAP2, STAMP2/STEAP4/TIARP (mouse homologue) and STAMP3/TSAP6/pHyde (rat homologue) [18-25]. Structural computational analysis revealed that all these proteins (including STEAP) contain a 6TM domain, flanked by N- and large C-terminal domain and that their structure carries similarities to that of the channel and transporter proteins [20,26]. Based on sequence similarity searches it was shown that the 6TM of the STAMP proteins is a homologue to the respective domains in Nox and YedZ protein family members leading to the suggestion of the possible function in an electron transfer system [26]. Furthermore, studies of subcellular localization and biochemical assays have recently demonstrated that the murine STAMP1/STEAP2, STAMP2/STEAP4 and STAMP3/STEAP3 can reduce both iron and copper and mediate their uptake *in vitro* [27,28]. These findings and the fact that the 6TM proteins have previously been suggested to play a

role in disease states resulted in the hypothesis that STAMP proteins are important for both normal prostate and PCa development [29,30].

While searching for genes differentially expressed in early PCa, our group cloned a gene that had high sequence similarity to the previously identified STAMP1 and named it STAMP2 [20]. In addition to STAMP1, its amino acid sequence shows high similarity to the mouse homologue of STAMP2, TIARP, and the rat homologue of STAMP3, pHyde. There is evidence suggesting that this novel protein may be important for both normal prostate function and PCa progression. Firstly, STAMP2 mRNA was highly expressed in the prostate tissue. Secondly, while its expression could not be detected in a number of AR-negative cell lines, its expression was observed in the AR-positive LNCaP cells and was strongly stimulated by androgens. STAMP2 mRNA was also shown to be highly upregulated in prostate tumor samples versus normal prostate tissue. In addition, ectopic expression of STAMP2 in STAMP2-negative PCa cell lines increased colony formation and cell growth. Further experiments demonstrated that STAMP2 is localized to the plasma membrane and most of the intracellular membrane structures suggesting a possible function in endocytic and secretory pathways [20].

The prostate is a major secretory organ that is dependent on androgens for normal development as well as progression to carcinoma. The STAMP2 findings described above indicate that STAMP2 is a potential AR target gene which needs to be elucidated. It is also important to investigate whether the AR directly binds to regulatory sequences in the STAMP2 locus. In the study presented in this thesis the STAMP2 5'flanking sequence has been identified, characterized and analyzed.

2.2 Materials and methods

2.2.1 Materials

Agar and Luria-Bertani (LB) were purchased from Becton, Dickenson and Company. AquaPhenol™ was purchased from Q-BIOgene and ethanol and isopropanol were obtained from Arcus Kjemi AS. Ethidium bromide was purchased from Merck KGaA, agarose standard and 5'-bromo-4-chloro-3-indolyl-β-D-glactopyranoside (X-gal) were purchased from Saveen Warner AB. Glutaraldehyde was obtained from Electron Microscopy Sciences and hydroxymethyl aminomethane (TRIS), sodium chloride (NaCl) and sucrose were purchased from BDH. Activated charcoal, adenosine 5'-triphosphate (ATP), actinomycin D (ActD), ampicillin, chloramphenicol, bovine serum albumin (BSA), chloroform, cycloheximide (CHX), kanamycin, lysozyme, magnesium acetate, magnesium chloride ($MgCl_2$), octyl phenoxy polyoxy ethanol (Triton-X 100), potassium ferricyanide, potassium ferrocyanide, ribonuclease A (RNAse A) and sodium acetate (NaAc) were obtained from Sigma-Aldrich. The CTB-167B5 bacterial artificial chromosome (BAC) clone (AC003991), DH10B™ electro competent *Escherichia coli*, dithiothreitol (DTT), D-luciferin free acid with sodium salt, glycerol, Lipofectamine™ 2000, One Shot® MAX Efficiency® DH5α™ -T1^R chemically competent *E. coli*, OptiMEM® I, pCMV-β-gal, pCR®-Blunt II-TOPO® vector, pUC18, pUC 19, Superscript™ II Reverse Transcriptase and TRIzol® Reagent were purchased from Invitrogen. JETStar 2.0 Plasmid Midiprep Kit was obtained from Genomed, The E.Z.N.A.™ Plasmid Mini Kit II was purchased from Omega Bio-tek and QIAEX®II Gel Extraction Kit was obtained from Qiagen. pGL2-Basic Vector, rRNasin® RNase Inhibitor and RQ1 RNase-Free-DNase were purchased from Promega. Phusion™ High-Fidelity DNA Polymerase and DyNAzyme™ II DNA Polymerase were obtained from Finnzymes. Deoxyribonucleosine-5'-triphosphates (dNTPs), T4 DNA ligase, 2-Log DNA ladder and all restriction enzymes with their respective buffers were purchased from New England Biolabs. RPMI 1640, L-glutamine, trypsin/EDTA and penicillin/streptomycin were purchased from Bio-Whittaker-Cambrex, and fetal calf serum (FCS) were obtained from from PAA GmgH. The MycoAlert® Mycoplasma Detection Kit was purchased from Lonza. FuGENE 6 Transfection Reagent, LightCycler® 480 SYBR Green I Master and LightCycler® 480 Multiwell Plates 96 were

obtained from Roche Diagnostics. The Bio-Rad Protein Assay was purchased from BIO-RAD, and methyltrienolone (R1881) was purchased from Perkin Elmer™ life sciences. pSG5 was obtained from Stratagene and the pSG5-AR [31] and -285PB-LUC [32] plasmids have been described earlier. Oligo dTs and all primers were manufactured by Sigma-Genosys. The human prostate cell lines LNCaP, DU 145, and PC-3 were obtained from the American Type Culture Collection (Rockville, MD, USA).

2.2.2 Methods

2.2.2.1 Theoretical promoter analysis

The 5'-flanking sequence of the human (AF423422) and mouse (BC006651 and AJ319746) STAMP2 genes were scanned for putative promoters using the Gene2Promoter software (Genomatix). Transcription factor (TF) binding sites were predicted by the MatInspector (Genomatix) software [33]. As exclusion criterions, core sequence similarity was chosen at 1.00 and matrix similarity above 0.8. Other bioinformatics tools (Table 1) were then used for further promoter and TF binding sites analysis.

Table 1. List of the bioinformatics tools used in promoter and TF binding sites analysis

Software/Database	**Web page address**
Gene2Promoter and MatInspector (Genomatix)	http://www.genomatix.de
Promoter 2.0 Prediction Server	http://www.cbs.dtu.dk/services/promoter
Neutral Network Promoter Prediction	http://www.fruitfly.org/seq_tools/promoter.html
Softberry Promoter Prediction	http://www.softberry.com/berry.phtml
PROSCAN Version 1.7	http://www-bimas.cit.nih.gov/molbio/proscan
TFSEARCH	http://www.cbrc.jp/research/db/TFSEARCH.html
Transcription Element Search System	http://www.cbil.upenn.edu/cgi-bin/tess/tess
JASPAR database	http://jaspar.genereg.net

2.2.2.2 Reporter vector construction

2.2.2.2.1 Fragment amplification

The CTB-167B5 bacterial artificial chromosome (BAC) clone (AC003991) was amplified in *E.coli* bacteria and purified using the JETSTAR 2.0 Plasmid Miniprep Kit according to the manufacturer's instructions (Genomed). Primers (Table 2) were designed using the Primer3 program and blasted to known sequences (NCBI) to ensure specific binding. Fragments of the human STAMP2 (hSTAMP2) 5'flanking sequence were amplified by polymerase chain reaction (PCR) from the BAC clone described above. 0.02U Phusion™ High-Fidelity DNA Polymerase (Finnzymes), 200µM of dNTPs, 0.5mM of each primer and 140 ng template were used in 50µl reactions. The PCR program consisted of: 30 s at 98°C, 20 cycles (denaturation: 10 s at 98°C, annealing: 30 s at 55°C, and elongation: 30 s/kb at 72°C) followed by a 10 min final extension step at 72°C. The size and quantity of the products were examined on a 1% agarose gel. The following putative STAMP2 promoter fragments were generated using the primers indicated in brackets (referring to Table 2), position +1 refers to the first bp (basepair) of the 5' untranslated region (UTR): -5674 to +185 (primer pair 7 and 8), -4076 to +185 (primer pair 3 and 6), -2094 to +185 (primer pair 5 and 6), named 6kb, 4kb and 2kb respectively, and -5674 to -2061(primer pair 1 and 4), -4076 to -2061 (primer pair 3 and 4), -5674 to -4055 (primer pair 1 and 2), named F1-R2, F2-R2 and F1-R3 respectively. Fragments 6kb, 4kb and 2kb contained the predicted putative promoter (-499 to +103).

Table 2. Primers for the hSTAMP2 promoter region amplification

No.	Name	Sequence (5'→3')*	Position**	Annealing T (°C)
1	hStamp2F1	gacgcg|tcgacgcacaaattgcctggactggaatt *SalI*	-5674	55
2	hStamp2R1	cccgcg|gatcctgccttccttgcctgctctagtc *BamHI*	-4055	55
3	hStamp2F2	gacgcg|tcgacactagagcaggcaaggaaggca *SalI*	-4076	55
4	hStamp2R2	cccgcg|gatccggtttctttacaccagcatcacca *BamHI*	-2061	55
5	hStamp2F3	gacgcg|tcgactggtgatgctggtgtaaagaaacc *SalI*	-2094	55
6	hStamp2R3	cccgcg|gatccctgttcttcagtgcgatgggc *BamHI*	+185	55
7	hStamp2F1new	ggtac|cgcacaaattgcctggactggaatt *KpnI*	-5674	55
8	hStamp2R3new	c|tcgagctgttcttcagtgcgatgggc *XhoI*	+185	55
9	hStamp2F2_int	tttggtggtgcacaacatct	-597	55
10	hStamp2R3_int	gggcaaagcatccatacact	-3358	55
11	hStamp2F2_int2	gactcttagggacacgcagc	-2461	55
12	hStamp2F1_int	ttgcttcctggtctgtgttg	-4951	55
13	hStamp2R2_int	aggcctagagccagtgttca	-3480	55
14	pGL2b.F	tgtatcttatggtactgtaact		
15	pGL2b.R	ctttatgtttttggcgtcttcc		

* Sequences (1-13) designed using Primer3 software: http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi

** Relative position with respect to STAMP2 exon 1 first base (+1)

2.2.2.2.2 TOPO-cloning

Amplified fragments were cloned into the pCR®-Blunt II-TOPO® vector according to the manufacturer's instructions with some modifications (Invitrogen). 4µl of PCR product and

1µl of 1:5 diluted vector were used, the reactions were then incubated at room temperature over night. One Shot® MAX Efficiency® DH5α -T1^{R} chemically competent *E. coli* cells were transformed with 5µl TOPO reaction following the manufacturer's protocol and the transformation reaction was spread on LB plates containing 50µg/ml kanamycin. The transformation efficiency was determined by counting the number of colony forming units (CFU) using pUC19 as a positive control.

2.2.2.2.3 Screening of putative positive transformants

Putative positive colonies containing the transformed constructs were screened by whole-cell PCR using primers from Table 2. Bacterial clones were added to a master mix comprised of 200µM of dNTPs, 200µM of primers and 2U DyNAzyme^{TM} II DNA Polymerase (Finnzyme) in a total volume of 25µl. The PCR conditions were as follows: a 10 min cell lysis step at 95°C, followed by 35 cycles (30 s at 95°C, 35 s at 55°C and 1 min at 72°C) and a final extension step (6 min at 72°C). Positive clones were then grown in selective LB medium, containing 50µg/ml kanamycin, over night at 37°C. For diagnostic restriction enzyme digest, small scale plasmid DNA purification (STET miniprep) was used (Molecular Cloning, 3rd edition, Sambrook and Russel). Cells from 1.5ml overnight bacterial cultures were pelleted by centrifugation and resuspended in 400µl of STET (8% sucrose, 0.5% Triton X-100, 50mM EDTA, pH 8.0 and 10mM Tris-Cl, pH 8.0). 10µl of 1mg/ml RNAse and 30µl of 10mg/ml lysozyme were then added and mixture incubated on ice for 2-3 min. The tube was placed in boiling water for 60 s and then immediately put on ice. After 15 min centrifugation at +4°C (13000 rpm) the mucoid pellet was removed, the DNA precipitated with 400µl pre-chilled (-20°C) isopropanol and centrifuged as before. The DNA pellet was then washed twice with 70% ethanol and resuspended in MQ-water by a 15 min incubation step at 65°C. The size and orientation of the inserts were confirmed by diagnostic restriction enzyme digestion**.**

2.2.2.2.4 Generating pGL2-Basic reporter constructs

The 2kb forward and reverse putative STAMP2 promoter constructs, as well as the 4kb forward, the 6kb forward and 6kb reverse constructs were digested with restriction

enzymes from the respective TOPO vectors and cloned into the multiple cloning site (MCS) of the pGL2-Basic vector (Promega). The plasmids were amplified in *E.coli* and purified using the E.Z.N.A.^{TM} Plasmid Mini Kit II (Omega Bio-tek). 5µg of both inserts and pGL2-Basic vector digested with *KpnI* and *XhoI* were separated on a 1% agarose gel. The fragments were then excised and gel extracted using the QIAEX$^{®}$II Gel Extraction Kit according to the manufacturer's instructions (Qiagen). The concentration of purified DNA was then estimated by gel electrophoresis, comparing the intensity of the fragment to 1 µg of a 2-Log DNA ladder. The inserts were subsequently ligated into the pGL2-Basic vector using T4 DNA ligase according to the manufacturer's instruction (New England Biolabs). Between 200 and 600 ng insert was used for each reaction and the ligation was carried out at 16°C over night. The ratio of vector to insert used in the reaction was 1: 3. After transforming 2µl ligation reaction into 20µl DH10B electro-competent cells at 1.3 Volt and 200 Ω, the bacteria were shaken for 1 hour in antibiotic-free LB medium at 37°C and then spread out on 100µg/ml ampicillin containing LB plates. Putative positive colonies were screened by whole cell PCR and STET miniprep as described in section 2.2.2.2.3. For transfection experiments, the constructs were purified using the JETSTAR 2.0 Plasmid Midiprep Kit. Concentration and purity of the DNA were measured with a Lambda25 spectrophotometer (Perkin Elmer^{TM} life sciences) at 260 and 280nm. The three forward constructs were sequenced by ABI-lab, placed in the Department of Biology and Molecular Biosciences, University of Oslo, using primers from Table 2.

2.2.2.3 Cell culture

The human prostate cancer cell lines LNCaP, DU 145, and PC-3 were routinely maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FCS). All media used in the experiments were supplemented with 50U/ml penicillin, 50µg/ml streptomycin and 200 nM L-glutamine, if not indicated differently. The cells were incubated at 37°C in a humidified 5% CO_2, 95% air incubator. The culture medium was changed every 2-3 days. The passage number of LNCaP cells used in experiments was between 16 and 23. The MycoAlert® Mycoplasma Detection Kit (Lonza) was used to test cells for mycoplasma contamination.

2.2.2.4 Cell transfection and Reporter Experiments

2.2.2.4.1 DU 145 and PC-3 cells transfection

DU 145 and PC-3 cells were plated out in 6-well plates at density of 1.5×10^5 cells in 2ml 10% FCS- RPMI 1640 per well and grown until they reached 70% confluence. 2-3 hours prior to transfection, the medium was removed, cells rinsed with Phosphate buffered saline (PBS), pH 7.4 and 2ml 0.5% CT-FCS RPMI 1640 was added. The transfection was carried out according to the manufacturer's instructions (Roche Diagnostics). 3µl FuGene6 reagent diluted in 100 DMEM was used per well. The following constructs were used for transfections (amount of DNA given as ng per well). 150ng of -285PB-LUC vector was co-transfected with 15ng pSG5-AR vector. Similar molar ratios of empty pGL2-Basic (280ng), pGL2-Basic-6kbRev.(580ng), pGL2-Basic-2kbForw.(400ng), pGL2-Basic-4kbForw.(500ng) and pGL2-Basic-6kbForw (580ng) were each co-transfected with either pSG5-AR (75ng for DU 145, 30ng for PC-3 cells) or molar ratio of empty pSG5 (41ng for DU 145 cells, 16,5ng for PC-3 cells). pUC18 plasmid (carrier DNA) was added to each DNA mix to bring it up to a total concentration of 1µg DNA per well. After transfection, the cells were incubated at 37°C over night. The following morning, the medium was removed and replaced with 1ml 0.5% CT-FCS RPMI 1640 with either 10^{-8} M R1881 or vehicle (ethanol) and incubated for another 48 hours. All transfections were carried out in triplicates and each experiment was repeated at least twice. β-gal assay was used to assess transfection efficiency (Section 2.2.2.4.3).

2.2.2.4.2 LNCaP cells transfection

LNCaP cells were plated out in 2ml 10% FCS containing RPMI 1640 medium at a density of 2×10^5 cells per well in 6-well plates and grown until they reached 70 % confluency. Transfection of the same reporter constructs, as used for other cell lines, was carried out with FuGene6 and cells incubated as described above. No AR plasmid was co-transfected, since LNCaP is an AR-positive cell line. pUC18 was used as a carrier DNA to raise DNA amount to 1µg per well.

LnCaP cells were also transfected using Lipofectamine™ 2000, according to manufacturer's protocol (Invitrogen). Cells were transfected in 0.5% CT-FCS antibiotic free medium. The same amounts of constructs were used as described above and pUC18 was used to raise the DNA amount to 4µg per well. OptiMEM I was used to dilute both transfection reagent and DNA. All transfections were carried out in triplicates. β-gal assay was used to assess transfection efficiency (Section 2.2.2.4.3).

2.2.2.4.3 β-galactosidase (β-gal) assay

LNCaP cells were transfected with 1µg pCMV-βGAL vector per well in 6-well plates using FuGene6 or Lipofectamine 2000 and incubated, as described above. The media was removed; cells washed in PBS and then fixed for 5 minutes with 1.5ml 0.5% glutaraldehyde in PBS. After fixing the cells and washed with PBS, 1.5ml β-gal buffer (1× PBS, 10mM potassium ferricyanide, 10mM potassium ferrocyanide, 4.5M magnesium chloride, 1mg/ml X-GAL) was then added and the cells were incubated at 37°C over night. After wash with PBS, transfected cells (blue) were counted under a light microscope to determine the transfection efficiency (ratio of transfected cells / total number of cells).

2.2.2.4.4 Luciferase measurement

Transfected cells were rinsed with PBS and 150µl cell lysis buffer (25 mM Tris-HCL, pH 7.8, 2mM DTT, 10% glycerol, 1% Triton-X) was added to each well. The plates were then shaken for 5 min at room temperature. Cell lysates were transferred to eppendorf tubes and spun down for 2 min at 4°C (13000 rpm). 10µl supernatant and 50 µl of luciferase reaction mix (100mM Tris-O-acetate, pH 7.8, 10mM Magnessium-O-acetate, 1mM EDTA, 0.2mM D-luciferin, 2mM ATP) was used for measurement of luciferase activity. The luminescence of each sample was counted using a Victor^{2} ™ 1420 Multilabel Counter (Perkin Elmer™ life sciences) and recorded as counts per seconds (CPS). The Bio-Rad Protein Assay reagent was used to estimate total protein concentration in the cell lysates to normalize for reporter activity of each sample. A BSA dilution standard curve was used as external standard.

2.2.2.5 Inhibition of transcription and translation experiments

2.2.2.5.1 ActD / CHX treatment

LNCaP cells were plated out in 10-cm culture plates. After reaching 40% confluence, the following starvation protocol was applied: after removal of 10% FCS medium, cells were washed with PBS and 2% CT-FCS medium was added, after 48h the medium was changed to 0.5% CT-FCS medium and cells were starved for 24h. Cells were then pre-treated with either ActD (1µg/ml), CHX (1µg/ml), or vehicle (ethanol) for 60 min, followed by 24 h treatment with R1881 (10^{-8} M), or vehicle (ethanol). The cells were then washed with PBS and harvested by cell scraping. Two independent experiments in triplicate were carried out for each experiment.

2.2.2.5.2 RNA isolation

Total RNA was extracted using TRIzol® Reagent according to the manufacturer's instructions. The extracted RNA was treated with RQ1 RNase-Free-DNase (2U per 10 µg RNA) at 37°C for 60 min (Promega), followed by 3M sodium acetate (pH 5.2) and ethanol precipitation. RNA concentration and purity were measured with Lambda25 spectrophotometer (Perkin Elmer Life Sciences) at 260 and 280nm. To asses RNA quality, 2µl was run on 1 % agarose gel for 10 min at 200V. 4-5µg RNA was then reverse-transcribed by SuperScript™ II reverse transcriptase (Invirogen). Oligo-dTs were used as primers and rRNasin® RNase Inhibitor was added to inhibit potential RNAses. The quality of the cDNA and no RT negative controls were checked by PCR using hTCTP primers (Table 3).

2.2.2.5.3 qRT-PCR

Quantitative real time PCR (qRT-PCR) analysis were performed using LightCycler® 480 (Roche Diagnostics) in multiwell plates. Lightcycler® 480 SYBR Green I Master, 0.5µM of each primer and 2 µl of diluted cDNA template were used in 10µl PCR reactions (Table 3). The PCR program was comprised of a denaturation step (5 min at 95°C) and 45 cycles of: denaturation (5 s at 95°C), annealing (20 s at respective annealing temperature) and extension (30 s at 72°C). The PCR was carried out in duplicates for each sample and a template-free reaction was included for each primer pair as a negative control. For

normalization, the housekeeping gene GAPDH was amplified in parallel with each sample (Table 3) to compensate for quantity variation between the samples. To exclude non-specific PCR by-products and reveal presence of possible primer dimers, melting curve analysis was performed: 5 s at 95°C, 1 min at 65°C followed by continuous fluorescence measurement between 65°C and 97°C. Finally a cooling step (10 s at 40°C) was applied. The crossing point (CP) values were calculated by the software as the cycle number at which the increase of the product became logarithmic. Standard curves of each primer pair were used as standards. One negative and one positive (R1881 treated cells) reaction for each primer pair were run on a 1% agarose gel to verify product size.

Table 3. Primers and conditions used in qRT-PCR

Respective gene	**Sequence (5'→3')**	**Annealing T (°C)**	**Template dilution**	**Product size (base no.)**
GAPDH	F: gtcagtggtggacctgacct R: tcgctgttgaagtcagagga	60-66	1:5	150
PSA (KLK3)	F: ccctgagcacccctatcaac R: tgagtgtctggtgcgttgtg	66	1:5	398
NKX3.1	F: ggcctgggagtctcttgactccactac R: atgtggagcccaacccacagaaaatg	62	1:5	180
hSTAMP2	F: atgacagcaaagccaagcaa R: gcaaagcatccagtggtcaa	62	1:2	350
KLK4	F: atggaaaacgaattgttctgctcg R: atctggctccctggctctt	62	1:5	150
hTCTP	F: gaggggaagatggtcagtagg R: tgcttgatttgttctgcagc	60	1:5	278

2.2.2.6 Data analysis

The data was analyzed with Microsoft Office Excel 2003 software. Statistical analyses were performed using the Student's one-tailed t-test, where P values of $P<0.05$ and $P<0.01$ were considered statistically significant and highly significant respectively.

2.3 Results

2.3.1 Prediction of hStamp2 promoter and TF binding sites

To predict the location of the putative promoter of STAMP2, about 6 kilo base (kb) pairs of the hSTAMP2 gene (AF423422) 5'- flanking region was used as a query sequence in the Gene2Promoter program (Genomatix). The putative promoter region of STAMP2 was mapped to chromosome 7 (NC_000007), where the STAMP2 locus and three putative promoters were localized. The putative Promoter 1 (Figure 1) aligned with the hSTAMP2 first exon and was chosen for later analyses. The other two putative promoters identified were situated 22.1 and 27.6 kb downstream of the first exon. Previous experiments in our laboratory have identified the promoter of the STAMP2 mouse homologue, also called STEAP4 (BC006651) or TIARP (AJ319746). The putative human STAMP2 Promoter 1 has the same localization on the 5' gene region of STAMP2 as the mouse STAMP2 promoter identified. Since promoter location and elements are often evolutionary conserved between gene homologues, the putative human STAMP2 Promoter 1 was chosen for further analyses.

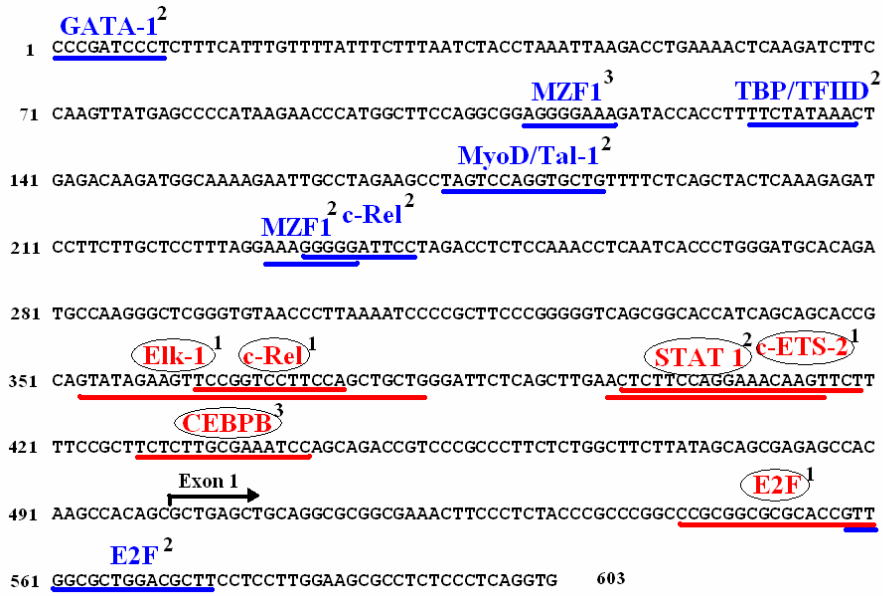


Figure 1. Sequence of hSTAMP2 putative promoter with predicted TF binding sites
The putative hSTAMP2 promoter region, identified by Gene2Promoter program, was used as query sequence in programs that identify TF binding sites. Sites predicted by more that one software (Table 1), are indicated in blue in the figure, each with the number of programs by which it was listed. The red, circled factors represent the sites identified by the MatInspector program that are common for both human and mouse STAMP2 putative promoter.

In order to predict TF binding sites, both the putative human and the mouse STAMP2 promoters, each about 600 base pairs (bp) long, were analyzed by the MatInspector software (Genomatix). When the exclusion criterion core similarity was set to 1 and matrix similarity was set to 0.8 or above, the program predicted 62 and 57 TF binding sites on both + and - strand for the human and the mouse promoters, respectively. Comparison of the hits revealed eight of the same, or belonging to the same family of TF binding sites that were situated in similar positions of both promoters. These are presented in Table 4 and indicated in Figure 1.

Table 4. TF binding sites common for the human and mouse STAMP2 putative promoters predicted by MatInspector

TF binding site Human	Position	Str.	Core sim.	Matrix sim.	Sequence *	TF binding site Mouse	Position	Str.	Core sim.	Matrix sim.	Sequence *
FOXK2	181 - 197	(-)	1.000	1.000	gctgagaaAACAgcacc	**FOXD1**	182 - 198	(-)	1.000	0.826	gctaagaaAACAgtaca
Elk-1	353 - 373	(-)	1.000	0.980	gaaggaccGGAActtctatac	**Elk-1**	359 - 379	(-)	1.000	0.839	gcacagctGGAAggaccagaa
Elk-1	362 - 382	(-)	1.000	0.839	cagcagctGGAAggaccggaa						
c-Rel	363 - 375	(+)	1.000	0.915	tccggtccTTCCa	**c-Rel**	360 - 372	(+)	1.000	0.915	tctggtccTTCCa
STAT1	396 - 414	(-)	1.000	0.951	ttgtttcctGGAAgagttc	**STAT1**	394 - 412	(-)	1.000	1.000	aagtttccgGGAAgagttc
STAT1	398 - 416	(+)	1.000	0.967	actcttccaGGAAacaagt	**STAT1**	396 - 414	(+)	1.000	0.962	actcttcccGGAAactttg
c-Ets-2	399 - 419	(+)	1.000	0.908	ctcttccAGGAaacaagttct	**Elk-1**	397 - 417	(+)	1.000	0.956	ctcttcccGGAAactttgctt
CEBP beta	428 - 442	(+)	1.000	0.943	tctcttgcGAAAtcc	**CEBP beta**	425 - 439	(+)	1.000	0.943	cctcttgcGAAAtca
E2F	544 - 560	(+)	1.000	0.900	ccgcgGCGCgcaccgtt	**E2F**	541 - 557	(-)	1.000	0.853	gtctgGCGCggctgcgg

* Capital letters mark the core sequence.

Other programs, listed in Table 1, were then used for further screening of the hSTAMP2 putative promoter. The TF binding sites that were repeatedly predicted by two or three of the programs are shown in Figure 1. Since previous findings suggest that hSTAMP2 is an androgen target gene [20], the 5'flanking 6kb region of STAMP2 was screened for possible NR binding sites. Table 5 gives an overview of the possible NR target sequences identified.

Table 5. NR binding sites identified by MatInspector in the 6 kb 5'flanking region of hSTAMP2

NR binding site	Position *	Str.	Core sim.	Matrix sim.	Sequence **
Androgen receptor binding site, IR3 sites	-5640 to -5622	(+)	0.869	0.915	ataatacttccaGTACtaa
Progesterone receptor binding site, IR3 sites	-5505 to -5487	(-)	1.000	0.843	attaatagagaTGTTcttt
Androgene receptor binding site, IR3 sites	-5430 to -5412	(+)	1.000	0.961	tttgcacatcttGTTCttt
Glucocorticoid receptor, C2C2 zinc finger protein binds glucocorticoid dependent to GREs, IR3 sites	-4337 to -4319	(+)	0.833	0.862	tctgtaaagagtGTGCtgc
Progesterone receptor binding site, IR3 sites	-3367 to -3349	(-)	1.000	0.874	caccaccaaatTGTTctta
Androgen receptor binding site, IR3 sites	-3189 to -3171	(+)	0.956	0.909	tatgaactgtgtGTCCctc
Androgen receptor binding site, IR3 sites	-1405 to -1387	(+)	0.956	0.915	gagcaacttccaGTCCtgt
Glucocorticoid receptor, C2C2 zinc finger protein binds glucocorticoid dependent to GREs, IR3 sites	-968 to -950	(-)	0.893	0.860	tgtgtgtgtcttGTCCtgg

* With respect to the hSTAMP2 first exon base (+1)
** Capitals mark the core sequence

2.3.2 Generation of luciferase reporter constructs

In order to obtain experimental evidence for the gene regulatory potential of the 5'-flanking sequence, six different fragments upstream of the hSTAMP2 first exon were amplified by PCR using sequence specific primers (Table 2 and Figure 2) and inserted into the pGL2-Basic Vector, a promoter-less luciferase reporter gene construct.

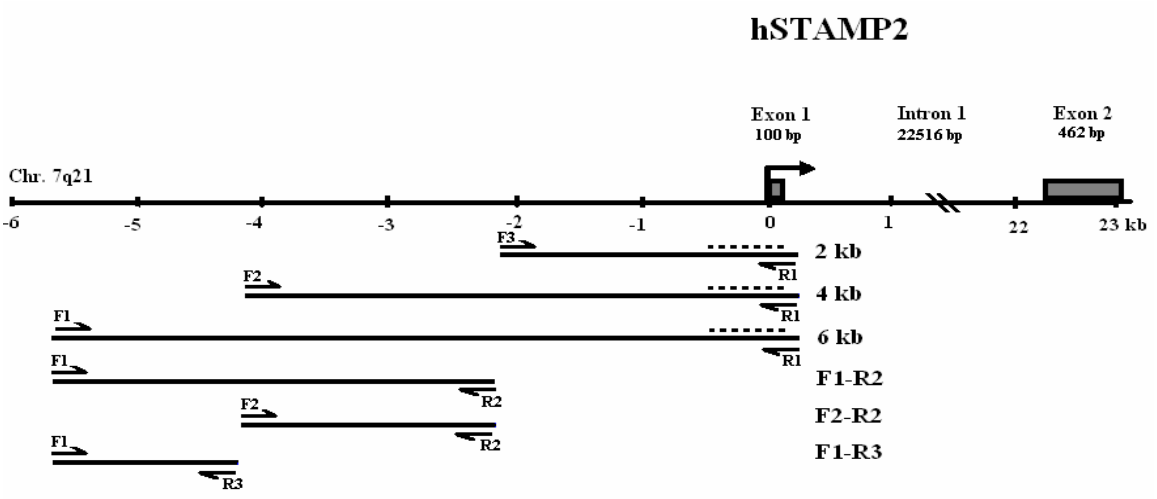
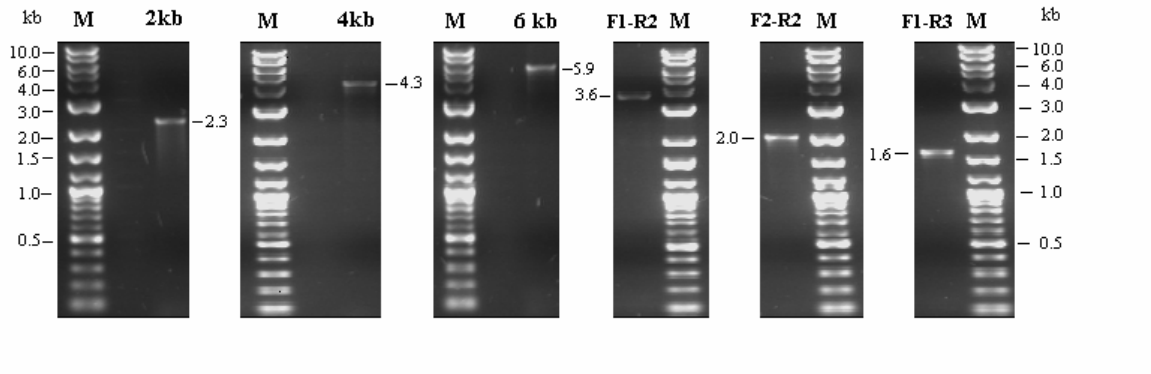


Figure 2. Amplification of the putative promoter fragments of the human STAMP2 gene
Six different length fragments were amplified from the hSTAMP2 5'flanking sequence the using primers indicated. The fragments' names are listed to the right of each fragment. Dashed lines show localization of the putative promoter. Localization of the hSTAMP2 first two exons (boxes) and first intron are shown.

The PCR-amplified fragments (Figure 2 and Figure 3A) were subjected to a two step cloning procedure (Figure 3B). The 2kb, 4kb and 6kb putative promoter fragments were cloned into the pCR®-Blunt II-TOPO® vector. The 5'-3' (forward) and the 3'-5' (reverse) of the 2kb fragment, the 4kb forward and 6kb forward and reverse fragments were then cloned into the *Kpn1* and *XhoI* site of the pGL2-Basic Vector. Sequencing of the 2kb and 4kb in the pGL2-basic vector confirmed their correct amplification and cloning. Sequencing of the 6kb in the pGL2-basic vector confirmed correct orientations and at least 93% correct sequence. The remaining ~ 400 bp of the 6kb fragment that could not be sequenced due to technical difficulties are currently being sequenced.

A



B

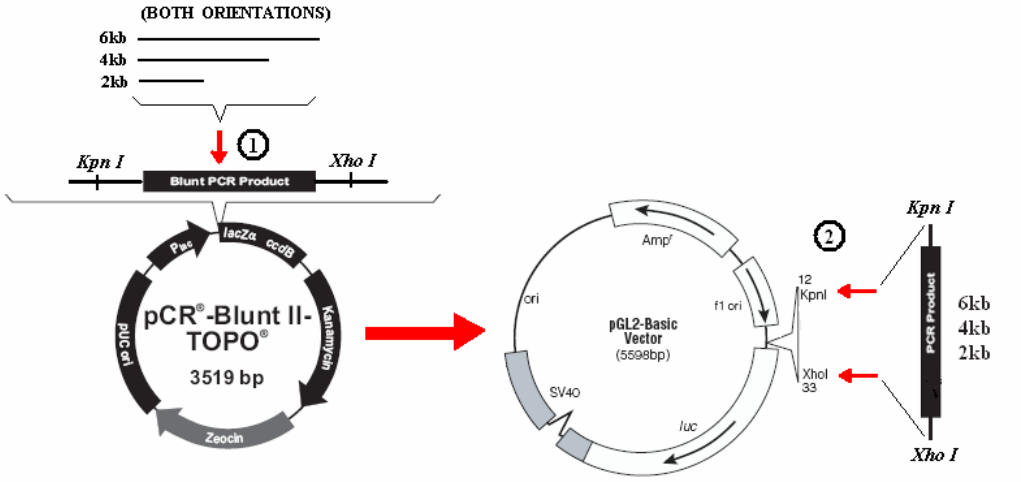


Figure 3. Construction and cloning of the luciferase reporter pGL2-Basic vector constructs
a) The correct size of each fragment was confirmed by running 5µl of the total 50µl PCR reaction by electrophoresis and comparing the size to the molecular marker (M). b) A two-step cloning strategy was used to generate the reporter gene constructs. (1) 2kb, 4kb and 6kb fragments were bi-directionally inserted into the pCR®-Blunt II-TOPO® vector. Next, 2kb forward and reverse, 4kb forward and 6kb forward and reverse clones were selected, inserts cut out with the *KpnI* and *XhoI* restriction enzymes and (2) inserted into the pGL2-Basic Vector cut with the same enzymes, upstream of the luciferase gene. The diagrams of both vectors are obtained form the manufacturers' manuals (Invitrogen, Promega).

The remaining amplified fragments, F1-R2, F2-R2 and F1-R3 (Figure 2 and 3A), none of which contained the predicted promoter, was designed for cloning into the pGL2-Promoter Vector in order to reveal possible enhancer elements located in more distant sequences upstream of the promoter. However due to time limitations these constructs have not been made.

2.3.3 Promoter analyses of hStamp2 5'-flanking sequences in prostate cancer cell lines

Since it was previously found that hSTAMP2 expression is prostate enriched and androgen dependent [20] we used human PCa cell lines to verify the expression potential of the three forward inserts. Using the cationic lipid method, the respective reporter constructs were co-transfected together with an AR expression vector (pSG5-AR) [31] into two AR-negative PCa cell lines, DU 145 and PC-3. After transfection, the cells were treated for 48 h with R1881, synthetic androgen. Vehicle (ethanol) was used for the control cells. The pSG5-AR amount used was optimized for both cell lines (data not shown). The -285-PB-LUC, described before as pPB(-285/+32)-LUC is a rat probasin promoter containing reporter plasmid [32] and served as a positive control, since its response is strongly androgen dependent. As shown in Figure 4a and 4b, the reporter expression of the positive control raised six to seven-fold upon androgen stimulation for both cell lines, indicating that both transfection and androgen treatment were successful. To examine the basal, androgen independent, activity of the putative promoter fragments, empty pSG5 was co-transfected with all three reporter constructs. In DU 145 cells co-transfected with empty pSG5 and the respective constructs (Figure 4A, pSG5) an increase in the reporter activity was observed that correlated with increasing insert length. Surprisingly, when the pSG5-AR construct were co-transfected with the promoter constructs a slight reduction (significant for the 2kb construct) of promoter activity upon androgen treatment was observed for all the constructs (Figure 4A, AR, black bar compared to gray bar). In addition AR expression appeared to have an androgen dependent inhibitory effect on the activity of the longest fragment.

In contrast, the same reporter activity experiment carried out in PC-3 cells, showed a significant induction of promoter activity upon androgen treatment when pSG5-AR was transfected for all the constructs (Figure 4B, AR, black bar versus gray bar). Additionally, expression of the reporter increased with the length of the insert upon androgen treatment. However, the fold induction was to a much less extent than to that of the positive control. In addition, the basal activity was stronger for the 6kb compared to the other two constructs (Figure 4B, pSG5). For both experiments empty pGL2-Basic vector, co-transfected with

pSG5-AR, served as a negative control and showed low promoter activity (data not shown). Co-transfection of pSG5-AR with pGL2-Basic Vector containing the 6kb reverse insert showed low promoter activity, indicating directional specificity of the expression potential of the promoter (data not shown). The transfection efficiency, examined by β-gal assay described in section 2.2.2.4.3, was ~70% and ~40% for the DU 145 and the PC-3 cells respectively (data not shown).

As described above, previous data from our laboratory have shown that STAMP2 is androgen regulated. This, together with the results of the transfection experiments in PC-3 cells (Figure 4B) suggests that the hSTAMP2 promoter requires the presence of the AR for activation. For this reason, the different constructs were transfected into the AR positive cell line, LNCaP for promoter analyses. However, despite numerous attempts, using two different cell passages and two different transfection reagents, the transfection efficiency of this cell line was too poor (under 5%) to obtain any reliable reporter activity. This certainly is a future area of investigation.

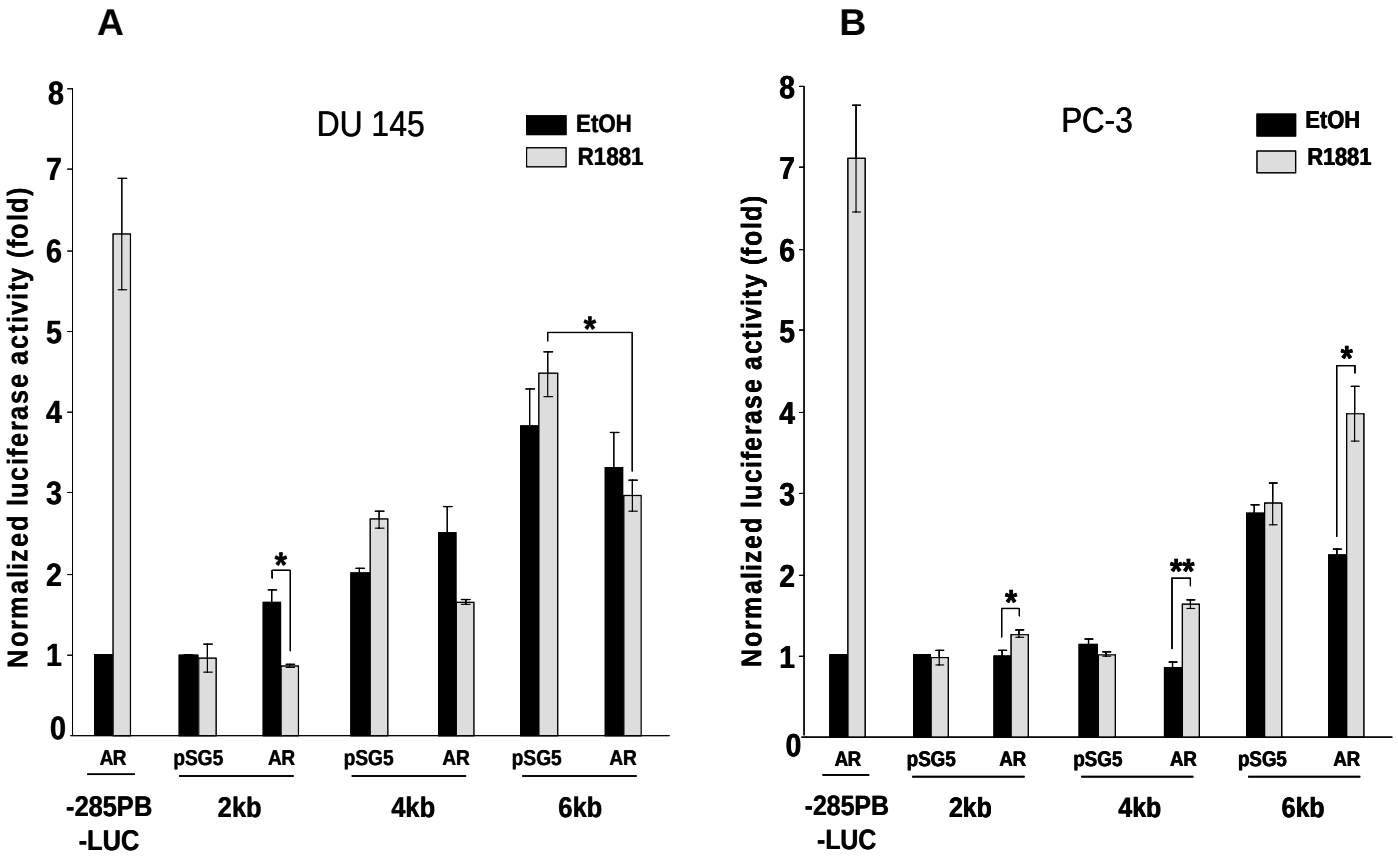


Figure 4. Normalized transcriptional activity of the hSTAMP2 5'flanking region in AR negative prostate cancer cell lines

2kb, 4kb, 6kb putative promoter luciferase reporter constructs were co-transfected with either empty vector (pSG5) or AR (pSG5-AR) expression plasmid into the (a) DU 145 or (b) PC-3 cells, as described in section 2.3.2.4.1. The cells were then treated for 48h with either vehicle (ethanol marked as EtOH) or R1881. -285PB-LUC, co-transfected with AR, served as a positive control for the androgen treatment. After harvesting, the cells were lysed and luminescence of each sample was measured to quantify reporter activity. Two experiments, each in triplicate, were carried out for each construct and the figure presents the data from the representative experiments. Values mark the mean of three measurements with the standard error (SE) bars. Values for the vehicle treated -285PB-LUC- and vehicle treated pSG5+2kb -transfected cells were set to one. P values were assessed by student's one-tailed t-test. Differences in reporter activity of vehicle treated versus androgen treated samples, that are statistically significant ($P<0.05$) or highly significant ($P<0.01$), are indicated by single and double star respectively. The significance has been calculated for the pSG5-AR-cotransfected 2kb, 4kb and 6kb and for the 6kb in DU 145 treated with androgen (pSG5 vs. AR) only.

2.3.4 *Effect of cycloheximide and actinomycin D on AR induction of hStamp2 expression and other androgen regulated genes*

Since the transfection results were conflicting in the two different cell lines described above, and did not indicate a direct effect of AR on the hSTAMP2 promoter activity in DU 145 cells, experiments were undertaken to test whether the AR directly or indirectly effects STAMP2 expression. In order to answer this question, LNCaP cells were pretreated with CHX, an inhibitor of protein synthesis, followed by R1881 treatment. A transcription inhibitor, ActD was used in parallel with the CHX. To monitor the effect of these two compounds on androgen induced gene expression, total RNA was extracted from the treated and untreated cells, reverse-transcribed and the cDNA was analyzed by qRT-PCR. Normalized mRNA expression of five different genes was assessed (Table 3). PSA and NKX3.1 were used as positive controls of the CHX treatment, as results of similar experiments have previously been reported [34-37] .

Figure 5 shows that the size of the different PCR products was as expected. None of the qRT-PCR controls were negative, indicating that the amplification is from cDNA only. Melting curve analysis confirmed the specificity of the PCR products.

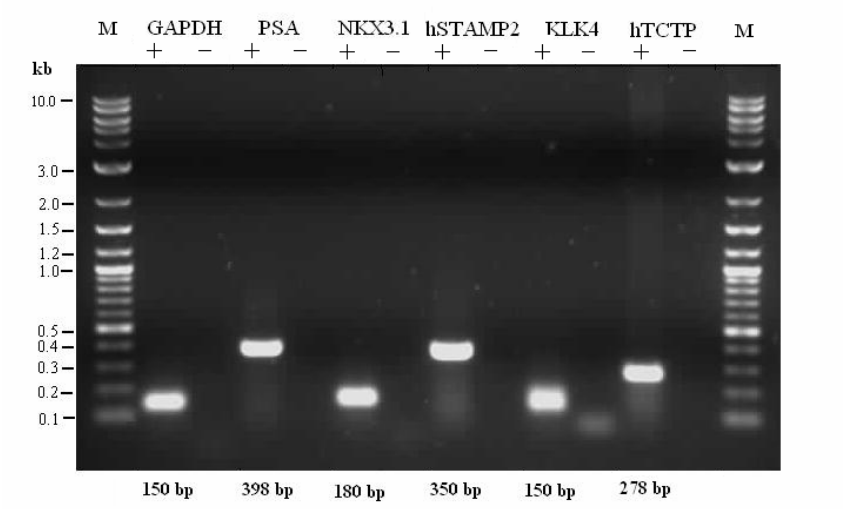
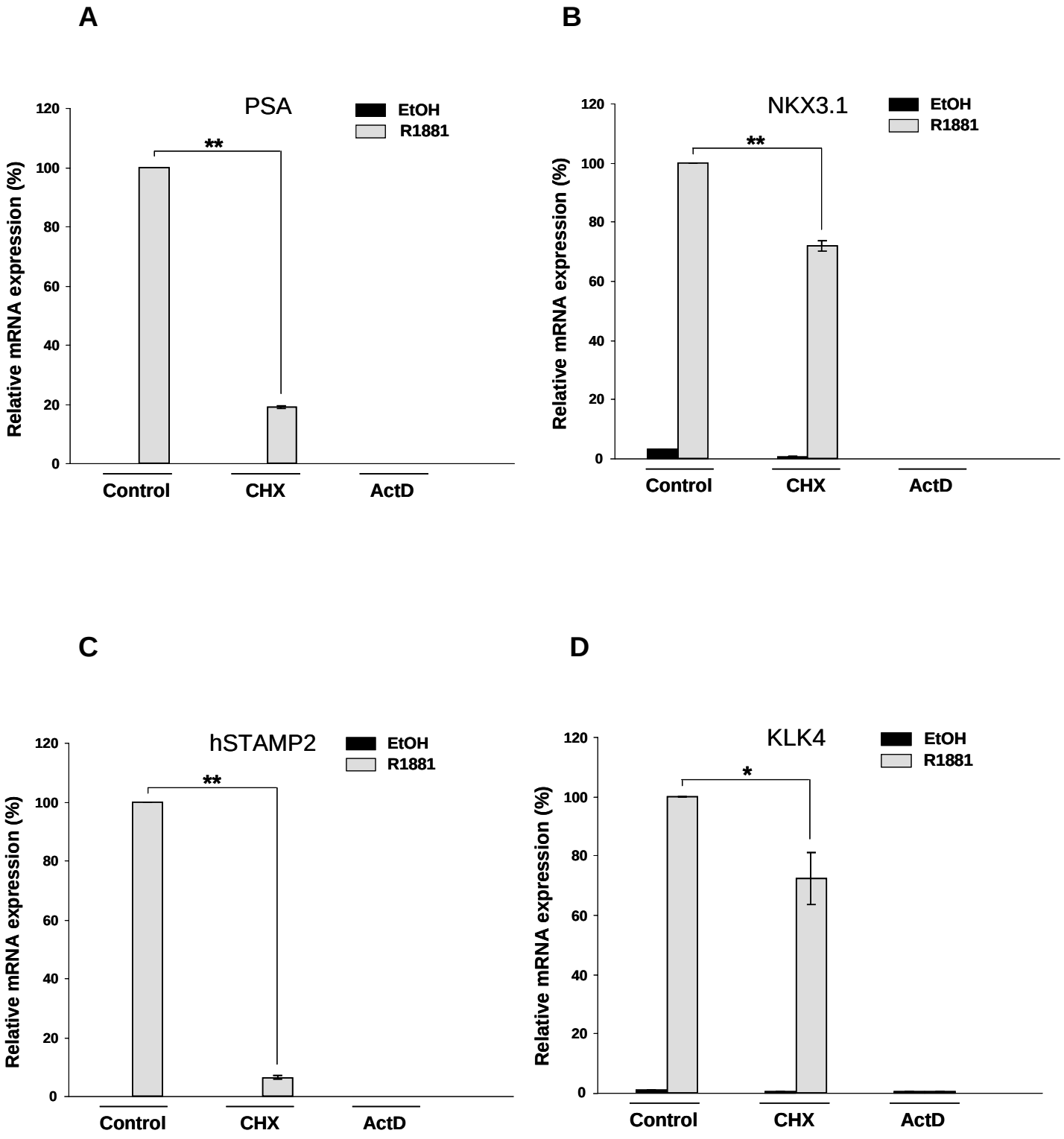


Figure 5. The size of the qRT-PCR products
Specificity of the PCR product was verified by running 5µl of the total 10µl PCR reaction on a 1% agarose gel. One positive and one negative sample for each primer pair is represented in the picture.

Figures 6A and Figure 6B show that CHX has an inhibitory effect on androgen induced PSA and NKX3.1 expression with 81% and 29% reduction respectively, when compared to samples not treated with CHX. As shown in Figure 6C, hSTAMP2 androgen induction was strongly CHX sensitive (reduced by 94% compared to control). Additionally, the mRNA expression level after treatment with CHX was examined of two other proteins that our group is working with, Kallikrein4 (KLK4) and human translationally controlled tumor protein (hTCTP). For KLK4, a CHX-mediated reduction (28%) of androgen induced KLK4 mRNA was observed (Figure 6D). hTCTP, of which expression was induced about 20% by androgen, revealed about four fold reduction in mRNA expression post CHX treatment (Figure 6E). Expression of the four out of five genes tested (PSA, NKX3.1, hSTAMP2 and KLK4) (Figure 6A-D) was almost totally inhibited by ActD pretreatment, in contrast a much smaller but significant reduction in hTCTP transcripts were observed (Figure 6E). In general, ActD treatment resulted in loss of about 50% cells due to cell death (data not shown). This however, should not influence the results, since the expression of each gene was normalized to the GAPDH expression.


A
PSA
EtOH
R1881
Relative mRNA expression (%)
120
100
80
60
40
20
0
**
Control
CHX
ActD
B
NKX3.1
EtOH
R1881
Relative mRNA expression (%)
120
100
80
60
40
20
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Control
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ActD
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hSTAMP2
EtOH
R1881
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120
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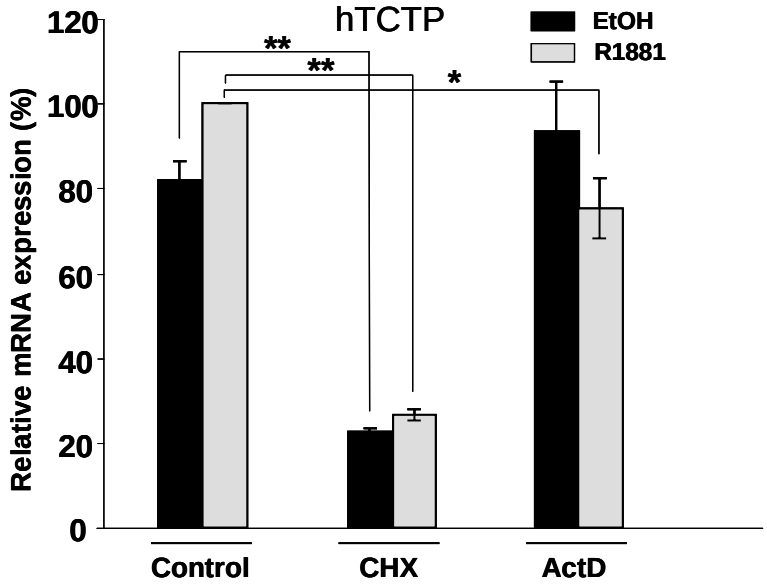

Figure 6. Normalized relative expression and effect of CHX and ActD on transcription of five androgen regulated genes

LNCaP cells were pretreated with CHX, ActD or vehicle (EtOH) for 60 min followed by 24h treatment with either R1881 or vehicle (EtOH). Total RNA, extracted from harvested cells was reverse transcribed and cDNA subjected to qRT-PCR analysis .Expression of five different genes were investigated: (a) PSA, (b) NKX3.1, (c) hSTAMP2, (d) KLK4 (e) hTCTP and measurements were normalized to GAPDH (housekeeping gene) and mRNA concentrations were deduced. Two independent experiments, each in triplicate, were carried out for each gene investigated and the figures sum up data from both experiments. Values mark the mean of the six measurements with the standard error (SE) bars. The value for R1881 treated control cells was set to 100%. P values were assessed by student's one-tailed t-test. Differences in mRNA concentration before and after treatment, that are statistically significant ($P<0.05$) or highly significant ($P<0.01$), are indicated by single and double star respectively. The values for some of the samples that was too low to appear in the figures are as follows: (a) Control, EtOH: 0.12, CHX, EtOH: 0.02, ActD, EtOH: 0.15, R1881: 0.13, (b) ActD, EtOH: 0.01, R1881: 8.9×10^{-3}, Control, EtOH: 0.02, CHC, EtOH: 0.05, ActD, EtOH: 3.7×10^{-5} , R1881: 3.9×10^{-5}.

2.4 Discussion

In this study the putative hSTAMP2 promoter has been identified, characterized and analyzed. As mentioned above, both the identified human and mouse STAMP2 putative promoters are situated at the same localization on the 5' gene region of the STAMP2 gene. Since homologues proteins between different species are likely to have promoters situated at similar sites in the gene locus, it is probable that the predicted human and mouse STAMP2 promoters are the true ones. Both promoters align with the first exon of the STAMP2 gene and share a number of TF binding sites located in same position in the promoter, as indicated in Table 4. Some of these and other TF binding sites, present only in the human promoter, were identified by more than one promoter program suggesting these putative TF sites are the true ones. The TF binding sites located only in the human promoter may reflect genuine evolutionary differences between two species. Some of the predicted TFs and their family members have been implicated in prostate and/or cancer development. E-26-like protein 1 (Elk-1) and epithelium-specific factor 2 (Ets-2) belong to the large family of Ets factors controlling cell proliferation, angiogenesis and metastasis during tumor progression [38]. Ets factors were observed to have distinct functions in epithelial cell gene regulation, while ESE-2 was shown to transactivate the PSA promoter [39]. In addition, reporter gene assays have shown that Forkhead Box (FOX) A1 and GATA2 are required for androgen-dependent PSA gene transcription [40,41]. c-Rel, a member of the nuclear factor kappa B (NFκB) family have been reported to be required for chromatin remodeling across the IL-2 gene promoter in T cells [42]. Furthermore, c-Rel was shown to activate antiapoptotic signaling, induce cell cycle progression and directly regulate transcription of the *e2f3a* promoter in B cells [43]. Another study identifies c-Rel as a negative regulator of the AR and shows that c-Rel, together with AR, is a part of the nucleoprotein complex that regulates the PSA promoter, and its overexpression downregulates PSA transcription in LNCaP cells [44]. Furthermore, simultaneous upregulation of NFκB and Ets have been observed in the abdominal aortic aneurysm [45]. NFκB and CCAT/Enhancer-Binding Protein-β (C/EBP- β), of which site is also identified in hSTAMP2 putative promoter, have been reported to cooperate in the regulation of the IL-6 promoter in PC-3 and DU 145 cells [46]. Signal transducer and activator of transcription

(STAT) 1 was observed to be overexpressed in DU 145 cells leading to drug resistance [47]. STAT pathways were also shown to be activated by fibroblast growth factors (FGFs), of which expression is increased and which act as auto- and paracrine growth factors in the PCa cells [48]. Moreover, STATs have been linked to IL-6 signaling, which has been implicated in PCa [49]. Since retinoblastoma tumor suppressor protein (pRB), inhibitor of the E2 (adenoviral protein) factor (E2F), is expressed in PC-3 and mutated in DU 145, it has been observed that E2F3 gene overexpression can enhance proliferation of DU 145, but not PC-3 cells [50]. Transcription of and transcription regulation mediated by both E2F1 and AR have been shown to be coregulated [51]. All the findings concerning these TFs may indicate their importance in hSTAMP2 regulation. However, the computational analyses are hypothetical. Thus in order to asses relevance of each factor, the bioinformatics result should be empirically confirmed by laboratory methods, like EMSA, ChIP, DNase I footprinting assay, and cotransfection of TFs with the hSTAMP2 promoter constructs in reporter assays.

The promoter analyses described above show that the 2kb fragment of the putative promoter of STAMP2 has promoter activity. In addition there was an increase in reporter activity that correlated with increasing insert length for both cell lines tested. These results suggest that the 2kb fragment contains the core promoter, while the upstream region encompasses the proximal promoter of STAMP2. An attempt has been made to identify elements of the core promoter. Three core promoter elements, TBP, E2F and C/EBP sites, have been identified by computational analysis (Figure 1) [52]. It was previously reported that promoter analysis of the STAMP1 gene did not identify any significant TATA or CAAT box consensus sequences, indicating that this gene is transcribed from a TATA-less promoter [19]. It has been proposed that many promoters do not contain consensus TATA boxes (reviewed in [53]). However the putative core promoter elements identified in this study suggest that the hSTAMP2 core promoter contains a TATA box. hSTAMP2 cDNA was blasted against the nonredundant (nr) expressed sequence tags (ETS) database in order to localize the TSS (data not shown). However the results were unclear and could not be linked to the results obtained by computational methods. Further laboratory experiments, including primer extension analysis, will need to be undertaken to identify the TSS and the core promoter elements.

The gene reporter assays presented in this study (Figure 4) confirm the presence of a promoter in the 2kb 5' UTR fragment for both cell lines tested. The possibility that the pSG5 constructs possesses promoter activity itself has been excluded, as no decrease in activity was observed for the reporter constructs transfected alone (data not shown) comparing to when they were cotransfected with pSG5 empty. The promoter acts in directional manner, as no activity was observed for the empty pGL2-basic vector and pGL2 with the reverse 6kb putative hSTAMP2 fragment (data not shown). There is a tendency of increasing basal activity (when no AR is present) of all three constructs that is proportional to increasing length of the 5'flanking fragment in the DU 145 cells (Figure 4A, pSG5). This indicates that the promoter activity is androgen independent in this cell line. Interestingly, when AR is cotransfected into DU 145 cells and treated with androgen, an inhibitory effect is observed for the 2 kb fragment compared to when not treated with androgen (Figure 4A, black bars compared to gray bars). In addition AR expression appeared to have an androgen dependent inhibitory effect on the activity of the longest fragment (Figure 4A, 6kb, gray bar (pSG5) compared to gray bar (AR)). In conclusion, these results suggest that promoter activity in the DU 145 cell line is independent on the AR and that there are possible AR dependent inhibitory elements present in the 2kb 5'UTR as well as further upstream.

In contrast, the reporter activity assays carried out in PC-3 cells indicate that the promoter is AR dependent (Figure 4B). Although relatively high androgen-independent promoter activity was observed, a significant increase in reporter activity was seen for all three construct tested when AR was present and treated with androgen compared to when not treated (black bars compared to gray bars, AR, Figure 4). In addition, the androgen dependent promoter activity was proportional to increasing length of the 5'flanking fragment in the PC-3 cells. Interestingly, the same androgen independent increase in promoter activity that was observed for the DU 145 cells is observed between the four and six kb upstream in PC-3 cells, indicating possible presence of androgen independent enhancer elements between the four and six kb upstream of hSTAMP2 gene. However, these results indicate that there are cell line dependent differences for the activity of the putative hSTAMP2 promoter in DU 145 and PC-3 cells**.** Cell lines, although derived from

the same tumor, may express proteins at different levels or modification status, and these differences can, at least to some extent, explain different response to therapeutic treatment [54]. In addition, cell lines derived from different tumors might have different signaling pathway network.

Androgen depletion independent (ADI) PC can be divided into two types: type I, where the expression of the AR is lost, and type II, where AR acts as an oncogene [55]. PC-3 and DU 145 belong to the type I ADI PC cell lines. DU 145 is originally derived from a brain metastasis [56], while PC-3 is derived from a bone metastasis [57]. Both of these cell lines have the p53-signalling pathway disrupted. The PC-3 cell line does not express p53 due to a stop codon introduced by mutation, while DU145 express a mutated version of this protein [54]. In addition, the PC-3 cell line is phosphatase and tensin homolog deleted on chromosome ten (PTEN)-negative, resulting in a constitutively active PI3K/Akt pathway [58]. In contrast, the DU 145 cell line expresses PTEN while the pRB and bax-genes are mutated [59]. PC-3 cells do not express AR in spite of having no detectable changes in the gene itself compared cell lines expressing AR [60,61]. In DU 145 cells, the AR promoter is methylated and thus not expressed [61]. A number of studies have reported varying expression levels of signaling molecules between the DU 145 and PC-3 cell lines. One example is a study by Skjøth and Issinger where profiling analyses of factors involved in cell viability and apoptosis in PCa cell lines were carried out [54]. It was observed that DU 145 cells were more sensitive to cisplatin induced apoptosis, than the PC-3 and LNCaP cells [54]. Western analysis showed a number of signaling molecules being differently expressed in PC-3 and DU 145 cells. The same study indicated that the Akt activity was almost three fold higher in PC-3 cells compared to that of DU 145 cells. This result coincides with the previously reported mutation of the inactive PTEN gene in PC-3 cells [58]. In addition, Sharma and co workers have reported that PTEN negatively regulates the PI3K/Akt pathway, in which phosphorylation (inactivation) of glycogen synthase kinase-3 β (GSK3β) results in increased stability and nuclear level of β-catenin leading to elevated AR's activity [62]. β-catenin has previously been shown to augment AR transcriptional activity [63]. Thus inactive PTEN could also explain higher levels of phosphorylated GSK3β observed in PC-3 cells, as reported by Skjøth and Issinger [54]. These results suggest that the β-catenin/AR-mediated transactivation level might be higher in the AR-transfected PC-3 than the AR-transfected

DU 145 cells. Numerous studies have measured the expression level of the various AR cofactors in PCa cell lines. For instance, Comuzzi and collaborators reported that the CBP coactivator protein expression level was significantly higher in PC-3 and LNCaP cells compared to that in DU 145 cells [64]. Buchanan and coworkers have observed that another AR coactivator, glucocorticoid receptor interacting protein 1 (GRIP1), and an AR corepressor, SMRT, have more than two fold higher expression level in PC-3 cells than in DU 145 cells [65]. Furthermore, differences of constitutive activation of MAPK in the PC-3 cells versus DU 145 cells have been shown several times. To mention one example, Stangelberger and collaborates detected low levels of phosphorylated MAPK in PC-3 cells and PC-3 cell mice tumors, compared to DU 145 cells [66]. Fujimoto and coworkers presented a novel putative AR coactivator, ARA55 that, by yeast-two-hybrid and co-immunoprecipitation experiments, was found to bind AR [67]. Northern analysis showed that ARA55 mRNA was detected only in PC-3 cells but not in DU 145 and LNCaP cells [67]. In addition, another study reports that the steroid receptor activator (SRA), known to be a part of the steroid receptor coactivator-1 (SRC-1) complex [68], was detected at much higher levels in PC-3 cells, than in the DU145 and LNCaP cells [69]. Linja and collaborators measured the expression of 16 different AR coregulators in PCa cell lines, xenografts and clinical specimen [70]. It was reported that ARA24, amplified in breast cancer-1 (AIB1), and AIB3 expression was higher in the PC-3 cells compared to the DU 145 cells. Additionally, there was four-fold and over two-fold increase in expression of p300 and STAT1 respectively. On contrary SRC1, protein inhibitor of activated STAT (PIAS) 1, PIASx, breast cancer 1, early onset (BRCA1), transcriptional intermediary factor 2 (TIF2), β-catenin and NCoR1 were upregulated in the DU 145 cells [70]. The four fold upregulation of the β-catenin in DU 145 cells, compared to that of PC-3 cells, does not correlate with the early mentioned hypothesis concerning the constitutively active PI3/Akt pathway in PC-3 cells. From the studies described above, however, it is, possible that the NCoR corepressor upregulation in the DU 145 cells could account for the inhibitory effect of the AR upon androgen treatment observed in our luciferase assays. Moreover, the upregulation of the STAT1 in PC-3 cells is particularly interesting, since there is a STAT1 binding site identified in both human and mouse STAMP2 putative promoter (Table 4, Figure 1). In addition, Litvinov and coworkers claim that AR overexpression in the AR-negative cells itself is neither toxic nor advantageous and that the appropriate molecular mechanisms

must be present in the cell for the AR to act as either an oncogene or tumor suppressor. It was observed that lenti viral expression of AR in PC-3 cells resulted in upregulation of the cyclin dependent kinase (CDK) inhibitor, p21, and suppressed PC-3 cell growth (induced G1 arrest) both in culture and mouse xenografts. This was not observed for the DU 145 cells tested, leading to conclusion that of these two cell lines, only PC-3 cells retained the necessary coregulators for the AR to function as a tumor suppressor gene. In contrast to these findings, Nightingale and coworkers observed suppressed DU 145 cell growth due to stable AR expression and DHT treatment [71]. In summary, all these previous findings highlight significant differences between the DU 145 and PC-3 cells that may explain the different promoter activity of the hSTAMP2 promoter constructs with respect to androgen stimulation in our study.

Furthermore, it was previously reported by our group that hSTAMP2 expression did not correlate with AR level in PCa samples, suggesting involvement of other factors in the hSTAMP2 gene regulation [20]. In addition to the AR, AR cofactors are necessary for gene activation and their level and/or status may vary between these two cell lines. It has been reported before that NCoR and SMRT corepressors can be recruited by agonist-bound AR, leading to androgen dependent AR mediated gene repression [72]. The outcome of the action of ligand bound AR thus depends on the relative levels of coactivators versus corepressors at the transcription site. It is therefore possible that the slight inhibitory effect of the androgen treatment in the DU 145 cells could be due to nuclear levels of cofactors that would favor hSTAMP2 gene repression.

Alternatively, it is possible that both cell lines used in our experiments lack some of the cofactors for proper hSTAMP2 gene regulation. As illustrated in Figure 6C, androgen high induction of hSTAMP2 mRNA level in LNCaP cells was observed. Wild type AR and p53 expressed in the LNCaP cells are just two examples of potential factors that could account for differences in hSTAMP2 gene regulation between this cell line and the two other AR-negative PCa cell lines tested. LNCaP is an androgen-sensitive cell line obtained from lymph node metastasis [73,74]. The strength of the LNCaP system is that it retains several salient features of human PCa: epithelial origin, expression of the AR, as well as AR-regulated genes and androgen sensitivity for growth and survival in culture and xenografts

[75]. Over 90% of the PCa samples obtained from the patients are AR-positive [76]. It is therefore of high importance to repeat the reporter gene studies in the STAMP2 positive cell line LNCaP. In conclusion, our results thus provide the basis for more detailed studies of hSTAMP2 gene regulation, in which particular factors, including AR cofactors, should be addressed.

Table 5 lists the putative AREs identified in the putative hSTAMP2 promoter. As shown and discussed above, there was a significant increase in reporter activity by androgen for all three construct tested in PC-3 cells (Figure 4B). However, the fold difference of the androgen effect was to much less extent than the positive control. Even though the amount of the transfected AR was optimized (data not shown), the strongest induction obtained in the PC-3 cells was under two fold. As the constructs contain cis-regulatory elements taken out from the *in vivo* chromosomal context, it is possible that our 6kb fragment misses some regulatory sites, resulting in poor AR-dependent stimulation. In the recent study of Wang and coworkers it has been reported that only 38% AREs were located within 500 bp of TSS in androgen regulated genes in LNCaP cells [77]. Thus, functional AREs might be located further downstream or upstream of the gene, for instance in introns, as it has been shown for other androgen regulated genes [77-79]. To examine this, further analyses of hSTAMP2 genomic DNA are required. Since computational analysis of such long DNA fragments may give a high number of hits one could use GREF_GATA model for AR binding sites. The GREF_GATA model is a model used in computational analysis of DNA sequences that localizes the AREs by combining the glucocorticoid responsive element matrix family (GREF) matches with the GATA matrix family matches, based on previously observed distance correlation of the same two families [80]. Furthermore, additional mutational analysis of the putative AR binding sites would be useful to assess their importance for promoter activity.

In addition to the direct effect of AR through ARE binding, AR may act indirectly by regulating transcription of an intermediary gene that in turn affects transcription of a gene or the half life of its mRNA and/or protein. Since nongenomic actions of the AR has been documented [17] and no major effect of androgen stimulation of the hSTAMP2 promoter activity was observed in the reporter studies described above, we hypothesized that AR

may indirectly regulate hSTAMP2. In order to investigate this, LNCaP cells were pretreated with CHX, an inhibitor of protein synthesis, followed by R1881 treatment. A transcription inhibitor, ActD, was used in parallel with the CHX. Detection of the mRNA level of PSA and NKX3.1 were used as positive controls. The CHX effect on the PSA androgen induction, resulted in a significant decrease of PSA mRNA, as reported before [34-36] (Figure 6A). This suggests that, although AR directly binds to regulatory sites in PSA locus to activate transcription, proper androgen dependent expression of PSA requires *de novo* protein synthesis. On the other hand, as illustrated in Figure 6B and reported by Prescott and collaborators, a smaller, but still significant, decrease of NKX3.1 mRNA was detected upon CHX treatment compared to the non-treated samples suggesting that AR transcriptional activation is more direct for NKX3.1 expression and not as dependent of *de novo* synthesized protein as expression of PSA [37]. The results illustrated in Figure 6C confirm that expression of hSTAMP2 mRNA is AR dependent (black bars). When treated with androgen and CHX, there is a significant decrease in STAMP2 mRNA level compared to that treated with androgen only. This shows that hSTAMP2 mRNA expression is highly CHX sensitive and in similar fold to that of PSA, indicating that androgen induced transcription of STAMP2 involves action of labile proteins. This is in agreement with the suggestion that transcriptional regulation of hSTAMP2 regulation is a complex process, involving other factors than the AR alone. Androgen induction of the KLK4 gene was affected by CHX to a much lesser extent (Figure 6D), similarly to NKX3.1, suggesting that the androgen dependent transcriptional activation is more direct and less dependent on newly synthesized proteins that in case of the PSA and the hSTAMP2. The mRNA synthesis for the four gene described above was nearly blocked by the ActD treatment.

Experiments from our laboratory have shown that TCTP expression is androgen regulated (unpublished results). qRT-PCR and Western blot analyses indicate that TCTP is more regulated on protein level compared to mRNA level in response to androgen, indicating post-transcriptional regulation of TCTP by AR. In the experiments described above (Figure 6E) the effect of androgen stimulation on TCTP expression mRNA showed a 20% induction of TCTP mRNA upon androgen treatment. This low androgen induction might be due to the androgen treatment time (24 h), as the experiments carried out recently indicate that TCTP upregulation is higher after 48 h treatment. The significant decrease in

the amount of mRNA under CHX treatment could indicate that de novo protein synthesis is required for TCTP transcription, regardless of AR presence. Surprisingly, a small but significant reduction in hTCTP transcripts was observed after ActD treatment. This weak ActD effect may suggest long half life of TCTP mRNA, exceeding the 24 hour CHX treatment. Thus, the four-fold decrease in TCTP mRNA upon CHX treatment could also indicate that labile proteins are required for mRNA stability, for instance involved in mRNA sequestration in a cell. TCTP, also called histamine-releasing factor (HRF), has been reported as a secreted protein that participates in inflammatory responses [81]. Thus, storing of mRNA would allow for a quick synthesis of high number of proteins, with no need for gene transcription. Interestingly, a relatively long (~16 h) half life TCTP protein has been reported [82]. Such long protein half life allows for rapid mobilization upon activation by inflammatory signal. Further experiments are required to examine the TCTP mRNA half life in LNCaP cells. As it has been reported that TCTP secretion is regulated by TSAP6 (STAMP3), a STAMP family member [82], it would be interesting to examine possible interaction of the hSTAMP2 with the TCTP.

2.5 Conclusion and future perspectives

Our results indicate that the putative hSTAMP2 promoter was identified, containing a number of potential TF binding sites, of which some were also found in the mouse homologue. Some of these factors have been linked to prostate and cancer development. The reporter gene studies confirmed promoter activity in the 2kb fragment, in addition an increase in reporter activity correlated with increasing insert length. These results suggest that the 2kb fragment contains the core promoter, while the upstream region contains androgen dependent regulatory elements. No androgen effect was observed on the promoter activity in DU 145 cells. However, a significant increase in the promoter activity for all the constructs tested was observed upon androgen treatment in PC-3 cells. As discussed above, the differences in promoter activity of the STAMP2 promoter might reflect the heterogeneity of the two AR negative cell lines utilized. In addition, the androgen induced promoter activity observed in the PC-3 cell line was quite modest compared with the positive control. Taken together, these results may suggest indirect AR

involvement in the hSTAMP2 gene regulation that is cell line dependent. These data are also consistent with currently unidentified AREs in regions of the STAMP2 gene that are not analyzed in this study. These might be, located in further upstream and downstream enhancer regions, both canonical and noncanonical, with modified consensus sequence, AREs [77].

The complexity of the androgen regulation of hSTAMP2 expression was supported in experiments where LNCaP cells were treated with CHX, suggesting the requirement of *de novo* protein synthesis for AR mediated hSTAMP2 gene induction. It will be important to analyze the promoter activity of STAMP2 in LNCaP cells as factors important for STAMP2 expression might be present. In addition, other important future studies are co-transfections of the different TF that bind to the putative TF sites identified, as well as mutagenesis of the TF binding sites and AREs in the putative promoter, which might confirm the functional role of these factors. We could also utilize the ChIP-on-chip, a recently developed method that combines ChIP with tiled DNA oligonucleotide microarray analysis, and that has recently been used for mapping AR binding sites [77]. In addition, EMSA, ChIP and DNase I footprinting analyses could be used to reveal binding of the TFs to their respective sites in the cis-regulatory elements. The enhancer and/or silencer elements could be further examined by inserting the two to four and four to six kb 5'UTR fragments to the pGL2-Promoter Vector. In addition the primer extension analysis would define the transcription start site of the hSTAMP2 gene and by narrowing the 5'UTR 2kb fragment in reporter gene assays one could localize more exact position of the core promoter. These and other experiments may help to reveal the function of STAMP2, a protein that one day may prove to be a useful diagnostic and therapeutic target in battling the prostate cancer.

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