Molecular analysis of anti-androgenic effects of atraric acid on human prostate cancer growth *in vivo* and androgen receptor-mediated negative regulation of the TERT gene

Dissertation

zur Erlangung des akademischen Grades

doctor rerum naturalium

(Dr. rer. nat)

vorgelegt dem Rat der Medizinischen Fakultät

der Friedrich-Schiller-Universität Jena

von Dipl. Biol. Sophie Bartsch

geboren am 06.08.1986 in Weißenfels

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Tag der öffentlichen Verteidigung: 01.07.2014

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Abbreviations

AA	atraric acid
ADT	androgen deprivation therapy
AF	activation function
AR	androgen receptor
ARE	androgen response element
as	anti-sense
bFGF	basic fibroblast growth factor
bp	base pairs
BPH	benign prostatic hyperplasia
°C	degree Celsius
CCS	charcoal stripped serum
Cdk	Cyclin-dependent kinase
ChIP	chromatin immunoprecipitation
cm	centi meter
CoA	coactivator
CoR	corepressor
CRPC	castration-resistant prostate cancer
DBD	DNA-binding domain
DHT	dihydrotestosterone
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
ECM	extracellular matrix
EGFR	epidermal growth factor receptor
ER	estrogen receptor
FCS	fetal calf serum
g	gram

h	hour
HDAC	histone deacetylase
HSP	heat shock protein
IHC	immunohistochemical staining
IQR	interquartile range
i.p.	intra peritoneal
kb	kilo bases
l	liter
lacZ	β-galactosidase
LBD	ligand binding-domain
LBP	ligand-binding pocket
luc	luciferase
min	minutes
µ	micro
m	milli
M	molar
NGS	normal goat serum
NHR	nuclear hormone receptor
NLS	nuclear localization signal
n	nano
NTD	N-terminal domain
qRT-PCR	quantitative real-time polymerase chain reaction
p	pico
PCa	prostate cancer
P-pRB	phosphorylated RB protein
pRB	retinoblastoma protein
PSA	prostate specific antigen
R1881	methyltrienolone

RNA	ribonucleic acid
ROI	region of interest
rpm	rounds per minute
RPMI1640	Roswell Park Memorial Institute
s	sense
S	subline
s.c.	subcutaneous
sec	seconds
SEM	standard error of the mean
TERT	human telomerase reverse transcriptase
TLS	translation start site
UAS	upstream activating sequence
VEGF	vascular endothelial growth factor
WB	Western blot
wt	wild-type

Zusammenfassung

Prostatakrebs (PCa) ist eine der häufigsten Krebserkrankungen bei Männern der westlichen Welt. Es handelt sich um eine heterogene Erkrankung, die häufig mit genomischen Veränderungen einhergeht.

Im Frühstadium wächst der Prostatatumor androgenabhängig. Der Androgenentzug stellt daher eine wichtige Therapieform dar. Nach dem anfänglichen Erfolg der Behandlung, kommt es jedoch zum erneuten Tumorwachstum. Dieses fortgeschrittene und aggressivere Tumorstadium ist auch als kastrationsresistenter PCa (CRPC) bekannt. Das Versagen der Therapie kann hierbei nicht nur dem Verlust der Androgenrezeptor (AR)-Funktion zugeschrieben werden, da der Rezeptor vom CRPC weiterhin exprimiert wird und nicht nur wachstumsfördernd sondern auch tumorsuppressiv wirkt.

Zur Optimierung der gezielten PCa-Wachstumsinhibierung ist ein tiefgreifendes Verständnis molekularer Mechanismen, die an dem beschriebenen Entwicklungsprozess beteiligt sind, notwendig. In diesem Zusammenhang sind die Identifizierung neuer AR-Antagonisten und deren Wirkungsweise von besonderer Bedeutung.

Eines der AR-Zielgene ist die katalytische Untereinheit der humanen Telomerase, TERT. In der vorliegenden Studie konnte gezeigt werden, dass der AR, abhängig vom zellulären Kontext, sowohl die Aktivierung des TERT-Gens als auch dessen Repression vermittelt. TERT, als einzelnes AR-Zielgen, spiegelt daher die entgegengesetzten Effekte der Androgen-Signalgebung wider. Die AR-vermittelte Regulation der TERT-Expression wurde in der vorliegenden Arbeit funktionell analysiert. Dabei konnte eine kleine Region, die AR-Liganden-abhängig reprimiert wird, im proximalen TERT-Promotor identifiziert werden. AR-Antagonisten wie die natürliche Substanz Atrarsäure, die das Wachstum von CRPC-Zellen inhibiert, wurden in diesem Kontext ebenfalls untersucht. Es konnte nachgewiesen werden, dass Atrarsäure zwar die AR-abhängige Transaktivierung hemmt, nicht aber die AR-vermittelte Genrepression des TERT-Gens.

Des Weiteren konnte anhand von immundefizienten Mäusen, die Xenotransplantate menschlicher CRPC-Zellen trugen, gezeigt werden, dass Atrarsäure das Prostatatumorwachstum *in vivo* hemmt. Die Analyse der humanen Tumore deutet auf eine Aktivierung der pRB-Signalkaskade hin, welche im Zusammenhang mit der Behandlung steht. Signalmoleküle, die durch diesen Signalweg angesprochen werden, sind interessanterweise häufig in fortgeschrittenen Tumorstadien beeinträchtigt. Diese Daten bestärken die Wirksamkeit von Atrarsäure als neuartigen AR-Antagonist *in vivo*.

Summary

Prostate cancer (PCa) is a major health concern of men in Western countries, and represents a heterogeneous disease with a high frequency of genomic alterations.

At initial phases the tumor depends on androgens therefore, androgen ablation became an essential therapy of PCa. After first being successful, the treatment eventually fails and tumors progress to the more aggressive form of the disease called castration-resistant prostate cancer (CRPC). Since the androgen receptor (AR) was reported to have growth promoting as well as tumor suppressive activities, and remains to be expressed in CRPC, the failure of therapy is not simply attributed to a loss of AR function.

A deeper understanding of molecular mechanisms of processes involved and the identification of the mode of action of novel AR antagonists may help to optimize targeted PCa growth inhibition.

One of the AR target genes is the catalytic subunit of the human telomerase, TERT. In the present study it could be demonstrated that the AR mediates TERT gene activation as well as repression depending on cellular context. Hence, TERT as a single AR target reflects the contrasting effects of androgen signaling. The AR-mediated regulation of TERT expression was functionally analyzed. Thereby, a small AR-responsive region was identified within the proximal TERT promoter, which is repressed in a ligand-dependent manner. It was demonstrated that AR antagonists, like the natural compound atraric acid, which inhibits the growth of CRPC cells, do not block AR-mediated gene repression of the TERT gene while inhibiting AR-dependent transactivation.

Furthermore, a murine xenograft model of CRPC revealed that atraric acid inhibits the growth of human prostate tumors *in vivo*. Analyzing the tumors suggests that a molecular signaling pathway, which is targeted by the treatment, is the pRB signaling axis. Interestingly, signaling molecules involved in this pathway are frequently affected in advanced PCa specimens strengthening the potency of atraric acid as a novel AR antagonist.

1 Introduction

1.1 Effects of androgens in prostate cancer

Prostate cancer (PCa) is the most common malignancy among males and the second-leading cause of cancer-related deaths of men in developed countries (Siegel *et al.,* 2012). Huggins and Hodges (1941) discovered that PCa depends on androgens at initial phases. Therefore, androgen ablation became a major form of PCa therapy. The tumor responds with growth regression to androgen deprivation therapy (ADT). However, after first being successful the cancer eventually recovers and castration-resistant PCa (CRPC) develops. The response duration ranges from a few months to many years and one cannot predict how the patient will respond (Gelmann, 2002). Since PCa is a multifactorial and heterogeneous disease, research focused on the detection of putative biomarkers to optimize PCa therapy and may provide a molecularly targeted therapy (Beltran *et al,* 2013; Huang *et al.,* 2013; Yap *et al.,* 2012). In this context, combinatorial treatments have been suggested as well as targeting certain cell signaling pathways and AR signaling (Floc'h *et al.,* 2012; Huang *et al.,* 2013). At the stage of CRPC the tumor still relies on androgen receptor (AR) signaling (Decker *et al.,* 2012). Thereby, modifications of the AR pathway occur including AR gene amplification and mutation, increased receptor expression and androgen synthesis, as well as changes in coregulatory proteins and ligand-independent activation (Beltran *et al.,* 2013; Scher & Sawyers, 2005).

A frequently occurring mutation is the AR-T877A, which is a point mutation within the ligand binding domain (LBD) of the receptor. Amongst others, this mutation still allows AR-mediated transactivation upon the binding of the clinically used AR antagonist hydroxyflutamide (Veldscholte *et al.,* 1990). This seems to be caused by a conformational change of the receptor, which is usually obtained upon agonist binding (Zhou *et al.,* 2010). Therefore, the mutation provides a growth advantage of the respective cells overcoming PCa therapy. Hence, new AR antagonists (also referred to as anti-androgen) have been developed in order to treat resistant tumors. One of those is MDV3100 (enzalutamide) (Tran *et al.,* 2010). However, as for other anti-androgens a resistant AR mutation has already been identified, AR-F876L (Balbas *et al.,* 2013). AR mutations are part of the evolving cancer adapting to a certain therapy. An

alternating therapy may be applied to overcome these effects. For this purpose differently acting anti-androgens may be needed to avoid and treat resistant PCa tumors.

Although it is clear that androgens are essentially involved in the development of PCa a correlation of androgen levels with PCa risk could not be clarified (Brawer 2003; Harman 2001; Morgentaler & Rhoden 2006; Roddam *et al.,* 2008). Interestingly, the risk to develop PCa increases with age. The average age at the time of diagnosis is 70 years coinciding with a decrease of androgen levels during aging (Hankey *et al.,* 1999; Ferrini & Barrett-Connor, 1998; Purifoy *et al.,* 1981). Androgen signaling affects a great variety of cellular processes. Thereby, in the healthy organism AR signaling maintains a balance between proliferation and apoptosis. However, in PCa an imbalance towards a more proliferative state occurs (Isaacs *et al.,* 1994). The age-dependent decline of androgen levels may disrupt the molecular balance of prostate cells, and leads to a deregulation of AR signaling resulting in tumorigenesis (Algarté-Génin *et al.,* 2004). ADT may further disrupt androgen and AR signaling modifying the expression of target genes and activating alternative growth pathways (Marques *et al.,* 2010). Hence, a further approach treating CRPC involves the dual targeting of cell cycle and AR signaling pathways (Floc'h *et al.,* 2012; Lin *et al.,* 2013a;b).

1.2 AR signaling

The AR performs distinct physiological functions throughout life by regulating unique gene sets. Thereby, it plays a crucial role in male development, fertility and behavioral manifestations. The prostate gland depends on androgens for development and the maintenance of integrity. However, they are also crucial in benign prostatic hyperplasia (BPH) and PCa (Gelmann, 2002).

1.2.1 AR structure

The AR is part of the steroid hormone receptor family of transcription factors, which share a modular structure. The receptor consists of an N-terminal domain (NTD), which represents the primary effector region, and is largely responsible for the receptor-mediated transactivation. Additionally, the NTD is the primary site of coactivator (CoA)

binding and includes the activation function 1 (AF-1) (**Figure 1**). The central DNA-binding domain (DBD) is highly conserved among the steroid hormone receptors. It includes two zinc fingers, which form the DNA-binding structure, and recognize specific responsive promoter regions of target genes. The DBD, as well as the adjacent ligand-binding domain (LBD), are essential for receptor dimerization (Gelmann, 2002). The DBD and the LBD are separated from one another by the hinge region (**Figure 1**). The hinge region is a short sequence, which contains a part of the ligand-dependent nuclear localization signal (NLS). Its second part is located in the DBD (Lonergan & Tindall, 2011). There are two further NLS one in the NTD and another in the LBD. These different NLS are linked to specific pathways for nuclear import (Kaku *et al.,* 2008). The C-terminal LBD consists of 12 helices, which form a ligand-binding pocket (LBP). Agonist binding to the LBP induces a conformational change upon which helix 12 folds over the pocket and encloses the ligand. Thereby, the formation of AF-2 is induced triggering the recruitment of CoAs (Zhou *et al.,* 2010). Ligand-biding also induces an N-/C-terminal interaction, which primes receptor dimerization and is essential for the activity of the AR (Lonergan & Tindall, 2011).

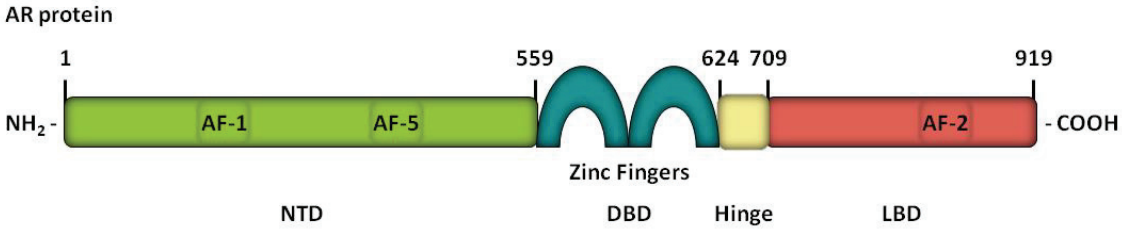


Figure 1: AR protein structure. The AR has a modular structure. The receptor consists of an N-terminal domain (NTD), a central DNA-binding domain (DBD), including two zinc finger motifs, a hinge region and a C-terminal ligand-binding domain (LBD). The localization of the activation functions 1, 2 and 5 (AF-1, -2, -5) is indicated. The numbers represent amino acid positions within the AR protein. The schematic diagram is adapted in part from Galani *et al.* (2008).

In general, anti-androgens, which have been described so far, target the LBP of the receptor. In the presence of antagonists helix 12 is positioned in a way favoring the binding of corepressors (CoR) suppressing AR action and inhibiting transcription initiation (Dotzlaw *et al.,* 2002). Herein, the receptor has different affinities to different ligands. The structure of a ligand determines the number of interactions made. Thereby, even minor changes in ligand structure have a great impact on the strength

of the interaction, hence different ligands exert different actions (Pereira de Jésus-Tran *et al.,* 2006).

Additionally, Caboni *et al.,* (2012) suggested a further site, which is accessible to antagonist interaction apart from the LBP. The binding of antagonists to this site induces the displacement of CoAs.

1.2.2 AR function

In a classical mode of action the AR is localized in the cytoplasm before ligand binding. It is bound to heat shock proteins (HSP) and other chaperones. The binding of a ligand to the receptor induces conformational changes releasing HSPs. Subsequently, the receptor can translocate into the nucleus, dimerize and interact with responsive DNA regions (Gelmann, 2002). The classical androgen response element (ARE) consists of two palindromic half-sites separated by a three nucleotide spacer. The consensus sequence with high AR-binding affinity is: GGA/TACAnnnTGTTCT (Roche *et al.,* 1992). The interaction of the ligand-bound AR with specific DNA regions triggers the formation of protein complexes at promoter regions modifying target gene transcription (Dehm & Tindall, 2006). AR action is mediated by a variety of coregulators, which amplify its signal including Sp1, p160 and CREB-binding protein, Ets1, NF1, GATA and FOXA1 (Jia *et al.,* 2008; Louie *et al.,* 2003; Lu *et al.,* 2000; Massie *et al.,* 2007).

However, there are substantial variations between AREs in different promoter regions. Even single nucleotide differences affect AR affinity and mediate specific regulatory effects (Gelmann, 2002). Alternative consensus sequences have already been described (Lin *et al.,* 2009). The transcriptional activation potential of a certain ARE is not only affected by the nucleotide divergence but also by the spacer and flanking regions (Nelson *et al.,* 1999). Additionally, the androgen and gene-specific response seems to be regulated by the orientation of the repeats regarding a direct or inverted repetition of a half-site. A varying composition and context of AREs induce different receptor conformations. Herein, AREs confer multiple regulatory functions, even within one nucleus (Bolton *et al.,* 2007; Claessens *et al.,* 2001; Denayer *et al.,* 2010). In a natural promoter context of target genes multiple AREs are frequently found, whose

correct spatial order is essential for transcription outcome (Gelmann, 2002). Furthermore, they are commonly adjacent to other transcription factor binding motifs (Bolton *et al.,* 2007). AREs are able to cooperate with each other and enhance their activities. These cooperative elements can induce dramatic alterations of the DNA structure and are part of a cell type-specific regulation (Cleutjens *et al.,* 1996; Cleutjens 1997; Reid *et al.,* 2001). Many sites of AR recruitment have been identified within the human genome. Herein, the set of available transcription factors and certain chromatin states are essential for the differential regulation of gene expression mediated by androgens (Jia *et al.,* 2008; Lin *et al.,* 2009).

There are further variations of the 'classical mode of action'. It has been demonstrated that the unbound receptor may also partly remains in the nucleus. Herein, its subcellular localization was reported to be cell type-specific (Gerdes *et al.,* 1998). Additionally, the AR not only exerts genomic effects. It also is involved in protein degradation and post-transcriptional modifications of other signaling molecules. In this context, it has been reported that the AR signaling influences the cell cycle on multiple levels including changes in Cyclin protein levels, Cyclin-dependent kinase (Cdk) activities and Retinoblastoma protein (pRB) phosphorylation (Knudsen *et al.,* 2008; Migliaccio *et al.,* 2000; Ye *et al.,* 1999; Xu *et al.,* 2006).

The AR is not only responsible for gene and protein activation but also their repression. Expression profiling in PC3-AR cells suggests that there might be even more genes down- than up-regulated in an AR-dependent manner (Lin *et al.,* 2009). Amongst others, androgen-mediated repression plays a role in the regulation of cell growth, differentiation, survival, migration and metabolic pathways (Grosse *et al.,* 2012; Prescott *et al.,* 2007). As indicated above, AR-mediated repression is primarily reported upon antagonist binding. This event triggers the recruitment of CoRs, which cause modifications and remodeling of the chromatin status. However, there are further modes of AR-mediated repression, which may involve negative AREs within promoter regions of target genes (Grosse *et al.,* 2012).

It has been shown that gene repression is mediated through diverse mechanisms. The AR may be recruited to responsive regions within promoter or enhancer regions of responsive target genes and either interacts with other proteins or interferes with protein binding of activating factors (Grosse *et al.,* 2012). Thereby, an interaction with

histone deacetylases (HDACs) or polycomb group proteins is possible to induce chromatin remodeling (Lanzino *et al.,* 2010; Wu *et al.,* 2013). The AR can be recruited to enhancer and promoter regions of one target gene not only for gene activation but also for gene repression. Consequently, an AR-occupied enhancer can communicate with the promoter inducing DNA looping, and the recruitment of chromatin modifying proteins (Shang *et al*, 2002; Wu *et al*, 2013). Furthermore, as described for gene activation AR-mediated gene repression is involved in tumor suppressive and growth promoting activities. Herein, the AR-mediated gene regulation seems to depend on the availability of other regulative proteins and chromatin state (Cai *et al.,* 2013b; Grosse *et al.,* 2012).

1.3 Telomerase and TERT expression in PCa

One gene, which has been demonstrated to be negatively regulated in an androgen- and AR-dependent manner, is the human telomerase reverse transcriptase (TERT) (Moehren *et al.,* 2008). TERT represents the catalytic subunit of the telomerase holoenzyme (Nakamura *et al.,* 1997). Furthermore, it consist of a templating RNA subunit (hTR) and telomere-associated proteins providing further regulative function and telomere stability (**Figure 2**) (Feng *et al.,* 1995; Liu *et al.,* 2004). The enzyme was first described in 1985 by Greider and Blackburn and is essential for the elongation and integrity of chromosomal ends, known as telomeres. Telomeres consist of hundreds to thousands of G/C-rich hexameric repeats (Greider & Blackburn, 1985). Amongst others, they are essential for chromosomal integrity. In the absence of telomerase activity, telomeres gradually shorten while cells undergo successive cell division (Greider, 1996). This is due to the inability of the DNA polymerase to replicate linear chromosomal ends also referred to as end-replication problem (Levy *et al.,* 1992). DNA damage response pathways are induced if telomeres reach a critical length. Consequently, cells enter a permanent cell cycle arrest or undergo apoptosis.

However, TERT expression has also been linked to telomerase-independent activities. It can be involved in the regulation of growth factor-related gene expression. Zhou *et al.,* (2009) could show that TERT induces the expression of vascular endothelial growth factor (VEGF). Jin *et al.,* (2010) suggested a TERT-basic fibroblast growth factor (bFGF)

axis, which is correlated with p53 inactivation increasing the resistance to DNA damage response pathways. Additionally, TERT may promote a stemness gene signature in cancer through the induction of epidermal growth factor receptor (EGFR) expression (Beck *et al.,* 2011). Furthermore, Tang *et al.,* (2008) reported a correlation of TERT and VEGF expression in PCa but not BPH specimens.

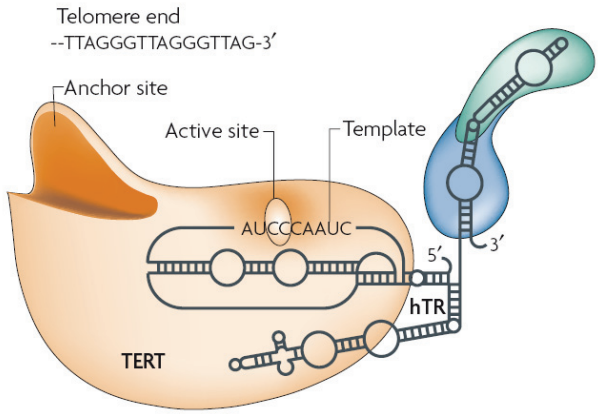


Figure 2: The telomerase holoenzyme. The human telomerase consists of a templating RNA subunit (hTR), the telomerase reverse transcriptase (TERT) (large protein structure) and associated proteins (small protein structure). TERT provides the catalytic subunit of the telomerase. Associated proteins are essential for complete elongation and telomere stability. The shaded region of the TERT protein represents the telomere substrate anchor site, which is bound during telomere synthesis. The elongation of telomeres occurs at the active site, at which hTR is localized. The figure was adapted from Harley (2008).

In normal prostate tissue and BPH specimens telomerase is inactive. However, in early stages of prostate malignancies TERT expression is detectable and telomerase activity was verified in more than 80 % of PCa specimens (Sommerfeld *et al.,* 1996; Zhang *et al.,* 1998). Telomerase activation has also been linked to metastatic behavior since it is clearly detectable in circulating tumor cells, which are required during the progression to an invasive state (Fizazi *et al.,* 2007). There is no other tumor-associated gene, which is as widely expressed in different cancer types. As indicated above, the activation of the telomerase stops the shortening of telomeres. Hence, it blocks the induction of massive genomic instability resulting in cell death, which might limit tumor growth (Harley, 2008). Nevertheless, genomic instability also contributes to tumor progression. In this context, Sommerfeld *et al.* (1996) showed that telomeres in PCa cells are rather short compared to normal prostate tissue and BPH.

Telomerase repression represents a tumor suppressive mechanism, which is tightly regulated (Harley, 2008). Telomerase activity is primarily regulated by the expression of its catalytic subunit TERT (Meyerson *et al.,* 1997; Nakamura *et al.,* 1997; Ulaner *et al.,* 1998). However, TERT expression is not necessarily correlated to telomerase activity (Kyo *et al.,* 2003). TERT is regulated by a variety of transcription factors. Thereby, the role of the AR in this process seems ambiguous. In a non-human primate and in a rat model it has been shown that castration induces telomerase activation *in vivo*. These studies suggest a negative regulation of telomerase activity by androgens (Meeker *et al.,* 1996; Ravindranath *et al.,* 2001). In contrast, Iczkowski *et al.* (2004) demonstrated that in patients who underwent pre-operative complete androgen ablation TERT expression was significantly inhibited. These patients were treated with Casodex, a clinically used anti-androgen, and Goserelin acetate, a gonadotropin releasing hormone super-agonist. Interestingly, Goserelin acetate suppresses both the production of androgens and estrogens (Murphy *et al.,* 1987). The estrogen receptor (ER) is a well-known activator of TERT expression (Kyo *et al.,* 1999). Furthermore, Guo *et al.* (2003) employed androgen-dependent CWR22 PCa cells in xenograft experiments. Upon castration of the mice TERT expression decreased but was regained if mice were treated with androgen (Guo *et al.,* 2003). Hence, androgens seem to affect TERT expression positively. Soda *et al.* (2000) suggested a more complicated relation of androgens and TERT expression, since they saw no effect of androgens on telomerase activity in two androgen-independent PCa cell lines and in normal prostate cells but an induction in androgen-dependent LNCaP cells. They supposed that the regulatory mechanism of telomerase varies during PCa progression. The induction of TERT gene expression by androgens in LNCaP cells was also described by other groups (Bouchal *et al.,* 2002; Guo *et al.,* 2003; Moehren *et al.,* 2008). Moehren *et al.,* (2008) demonstrated that ARwt is able to mediate TERT promoter repression in an androgen-dependent manner. They suggested that in the normal prostate AR signaling is balanced. Herein, it may be involved in the maintenance of low TERT expression status; hence telomerase activity is not detectable. However, during PCa development and progression cells escape this tumor suppressive mechanism, e.g. by AR mutation, driving androgen-induced TERT promoter activation (Moehren *et al.,* 2008).

Calado *et al.* (2009) suggested that the stimulatory effect of androgens may be due to the conversion of androgens into estrogens by the aromatase CYP19. This is followed by the activation of the ER inducing TERT gene expression and telomerase activity. This process can be mediated by both ERα and ERβ (Kondoh *et al.,* 2007; Kyo *et al.,* 1999). Indeed LNCaP cells do express ERβ (Lau *et al.,* 2000).

However, hormone receptors are not the only regulators of TERT expression. Transcriptional regulation of TERT seems to involve complex molecular mechanisms. Two E-boxes have been identified within the TERT promoter. These are bound by c-Myc driving gene transcription (Kyo *et al.,* 2008; Poole *et al.,* 2001). Additionally, there are at least five Sp1-binding sites within the proximal TERT promoter (Kyo *et al.,* 2000). Furthermore, AP-1, AP-2, TGFβ, NF1, HER2/Neu and Ets proteins have been reported to modulate TERT promoter activity (Deng *et al.,* 2007; Goueli & Janknecht, 2004; Kyo *et al.,* 2008; Poole *et al.,* 2001; Takakura *et al.,* 2005). These factors represent a small selection of TERT-regulating factors, however, all of them also interact with the AR (Chipuk *et al.,* 2002; Darne *et al.,* 1997; Massie *et al.,* 2007; Sato *et al.,* 1997; Ye *et al.,* 1999; Yuan *et al.,* 2005). Since TERT expression is highly specific to cancer cells, whereas key modifying transcription factors are not, chromatin state as well as the specific subset of available transcription factors may determine actual TERT promoter activity (Kyo *et al.,* 2008). In this context, it has been shown that e.g. Sp1 does not act alone: On the one hand Sp1 has been shown to interact with c-Myc or ER in order to activate TERT expression (Kyo *et al.,* 1999; Kyo *et al.,* 2000). On the other hand Sp1 can form a complex with Sp3 inducing the recruitment of HDACs, which results in TERT gene repression (Won *et al.,* 2002). These studies indicate the complexity of TERT gene regulation.

CpG islands within the proximal TERT promoter may serve as further regulatory mechanism. However, their exact role in TERT promoter activity is not quite clear, yet (Dessain *et al.,* 2000). Zhu *et al.* (2010) reported that a repressive chromatin environment and the nucleosomal conformation appear to be a major mechanism tightly suppressing TERT gene expression in the majority of human somatic cells. Whereas, during tumorigenesis modifications in chromatin state and/or alterations in the expression level of transcription factors, cofactors and HDACs seems to induce a switch from promoter repression to activation (Kyo *et al.,* 2008; Zhu *et al.,* 2010).

1.4 Effects of atraric acid on AR signaling

There is a variety of anti-androgens available, which repress PCa growth. However, tumors usually progress to therapy resistance. Additionally, some of those substances may even induce unwanted effects. Recently it has been demonstrated that Casodex and MDV3100 may promote PCa metastasis (Lin *et al.,* 2013a;b). Especially under Pten-deficient conditions MDV3100 was reported to induce tumor cell invasion (Jia *et al.,* 2013). Pten-loss occurs in approximately 44 % of PCa specimens (Beltran *et al.,* 2013). The identification and characterization of new anti-androgens inhibiting PCa growth despite resistance or receptor mutation, as well as clear clinical diagnostics may be essential for PCa therapy. In the end, this may provide a better suited treatment with regard to a personalized PCa therapy (Beltran *et al.,* 2013; Huang *et al.,* 2013; Yap *et al.,* 2012).

Amongst others phytotherapy has been suggested as a treatment of BPH and prevention of PCa. A registered phytotherapeutic agent is Tadenan®. Tadenan® is an extract of the bark of the African plum tree *Pygeum africanum* (Dreikorn, 2002). From this bark extract the natural compound atraric acid (AA) was isolated, since it shows anti-androgenic effects (Schleich *et al.,* 2006). AA has been shown to repress AR-mediated target gene expression *in vitro* and *ex vivo* (PhD thesis Hessenkemper, 2013; Papaioannou *et al.,* 2009). Compared to the IC_{50} of other clinically used anti-androgens AA (IC_{50}=3 µM) is fairly efficient. It is more potent than flutamide (IC_{50}=116 µM), slightly weaker than Casodex (IC_{50}=1 µM), and less effective than MDV3100 (IC_{50}=36 nM) (Roell & Baniahmad, 2011; Tran *et al.,* 2010).

However, AA not only inhibits PCa cell growth of androgen-dependent and -independent growing cells but also suppresses cell invasion through an extracellular matrix (Papaioannou *et al.*, 2009). The latter effect is promising compared to MDV3100, which may induce PCa metastasis as indicated above. The growth-inhibitory effect of AA seems to be partially due to the induction of cellular senescence *in vitro* and *ex vivo* (PhD thesis Hessenkemper, 2013; PhD thesis Roediger, 2012). In general, effects induced upon AA treatment seem to be AR-dependent since PCa cells which lack AR expression (PC3 cells) do not show growth inhibitory effects and AA shows a high specificity for AR compared to other hormone receptors (Bachelor thesis

Dittmann, 2013; Papaioannou *et al.,* 2009). Computational analysis demonstrated that AA acts as a competitive inhibitor, hence it binds to the LBD of the AR. Thereby, three potential binding sites of AA within the LBD of the receptor were identified (Papaioannou *et al.,* 2009). The observed AA-mediated reduction of PCa cell growth seems to rely on the ability of AA: (I) to decelerate agonist-induced nuclear translocation, (II) to increase receptor mobility coinciding with reduced foci formation, and (III) to attenuate N-/C-terminal interaction of the AR. All of which interfere with AR function. In this context, it has been suggested that the higher AR mobility may destabilize DNA-binding of the AA-bound AR. Thereby, reduced chromatin recruitment of the receptor was verified by ChIP (PhD thesis Hessenkemper, 2013). In contrast, to other clinically used anti-androgens AA binding does not induce the recruitment of common CoRs to the AR (Papaioannou *et al.,* 2009). However, the reduced N-/C-terminal interaction may obstructs CoA binding attenuating target gene expression (PhD thesis Hessenkemper, 2013).

Therefore, AA was suggested as a novel chemical platform molecule inhibiting AR-mediated target gene expression and PCa growth (Roell & Baniahmad, 2011).

2 Objective

The AR plays an essential role in the development and progression of PCa. Therefore, AR signaling is a major target of PCa therapy, and androgen ablation is a preferred treatment. After first being responsive, tumors eventually recover and CRPC develops. During PCa progression changes in signaling pathways and AR-mediated gene regulation occur. Understanding mechanisms involved helps to identify new and effective therapies (Debes & Tindall, 2002). One promising target of cancer therapy is the TERT gene expression, which was reported to be regulated by the AR (Moehren *et al.,* 2008). However, AR-mediated TERT gene regulation is ambiguous. The androgen-stimulated receptor seems to be involved in both TERT repression and activation (Diploma thesis Bartsch, 2011; Bachelor thesis Schreiber, 2011). Sites of AR-mediated promoter activation have been identified, whereas regions mediating TERT repression still have to be characterized. For this purpose functional analysis of the proximal TERT promoter were conducted. Additionally, it should be examined how the two anti-androgens AA and MDV3100 influence AR-dependent TERT gene regulation. This represents a first approach trying to characterize the impact of anti-androgens on AR-mediated gene repression.

The natural occurring anti-androgen AA previously has been shown to inhibit PCa cell growth *in vitro* (Papaioannou *et al.,* 2009). In the present study, the effect of AA treatment on PCa growth *in vivo* should be verified via prostate carcinoma xenografts. Furthermore, cell signaling pathways addressed by the treatment, as well as effects on AR target gene expression were examined.

The identification and mechanistic characterization of new anti-androgens helps to improve PCa therapy, thereby presenting potency and weakness of new drugs to provide an optimal treatment of the heterogeneous appearance of PCa.

3 Material and methods

3.1 Hormones and chemicals

Atraric acid (AA) and dihydrotestosterone (DHT) were obtained from Sigma-Aldrich (Taufkirchen, Germany), Methyltrienolone (R1881) from Perkin Elmer (Waltham, MA, USA), Enzalutamide (MDV3100) from Selleck chemicals LLC (Houston, Texas, USA).
For *in vitro* experiments all compounds were dissolved in dimethylsulfoxide (DMSO). The final concentration of DMSO in the culturing medium never exceeded 0.2 %. Control incubations (without test compounds) were performed with the appropriate volume of DMSO. For *in vivo* experiments AA was injected as an homogenous suspension in 0.5 % (w/v) polysorbat 80 (Tween 80) in deionized sterile water. An aqueous solution of 0.5 % (w/v) Tween 80 in deionized water was applied as vehicle control.

3.2 Plasmids

The androgen-responsive pMMTV-luc plasmid, which contains a luciferase (luc) reporter driven by the mouse mammary tumor virus long terminal repeats is described by Gast *et al.*(1998). The pCMV-lacZ plasmid expressing β-galactosidase under control of the cytomegalovirus promoter, which was employed as internal control in reporter gene assays, the pSG5 vector, which was used as empty vector control, the ARwt and the AR-T877A expression vectors as well as the pGL3-TERTΔ3996 plasmid are described by Moehren *et al.* (2008). The latter one expresses the luc gene driven by the TERT promoter. The promoter includes 3996 bp upstream of the TLS (translation start) of the TERT gene thereafter referred to as full-length TERT promoter. The p4xUAS-TATA-luc reporter plasmid is described by Moehren *et al.* (2004). It contains a TATA box, which is localized upstream of the luc gene driving its expression. Upstream of the TATA box are four upstream activating sequence (UAS) sites. The p4xUAS-TERT-3988/-3276-TATA-luc was generated by C. Reeb, AG Baniahmad, Jena University Hospital, Germany. Additional TERT deletion constructs were cloned as follows: The backbone of all constructs used in the present work is the p4xUAS-TATA-luc vector, which was digested with *Sal*I and treated with FastAP (Thermo Scientific, Hamburg,

Germany). The linearized vector was either ligated with the appropriate TERT deletion or treated with Klenow (Thermo Scientific, Hamburg, Germany) to gain blunt ends, and ligated subsequently (Diploma thesis Bartsch, 2011). p4xUAS-TERT-3988/-3752 s: pGL3-TERTΔ3996 was treated with *Eco*105I, *Eco*72I and Klenow and ligated to the linearized vector. p4xUAS-TERT-1301/-238 s: pGL3-TERTΔ3996 was treated with *Nhe*I, *Eco*72I and Klenow and ligated with vector (Diploma thesis Bartsch, 2011). p4xUAS-TERT-440/-89 s: pGL3-TERTΔ3996 was treated with *Bst*EII/*Cfr*42I and Klenow and ligated with vector. p4xUAS-TERT-240/-1; p4xUAS-TERT-180/-1; p4xUAS-TERT-120/-1 s and as: pGL3-TERTΔ3996 was used as template for a PCR reaction (primer: TERT-240/-1 fw: GGGGGGTCGACCGTGGCGGAGGGACTGGGGACC; TERT-180/-1 fw: GGGGGGTCGACTCCTCCGCGCGGACCCCG; TERT-120/-1 fw: GGGGGGTCGACGGGCCCTCCCAGCCCCTCC; for all 3 constructs rev: GGGGGGTCGACCGCGGGGGTGGCCGG). PCR product was digested with *Sal*I and inserted into the *Sal*I site of the linearized vector. p4xUAS-TERT-58/-1; p4xUAS-TERT-120/-58 s and as: The following oligos were annealed, phosphorylated and inserted into the *Sal*I site of the linearized p4xUAS-TATA-luc vector: TERT-58/-1 s: 5'-TCGACCAGGCAGCGCTGCGTCCTGCTGCGCACGTGGGAAGCCCTGGCCCCGGCCACCCCCGCG-3'; TERT-58/-1 as: 5'-TCGACGCGGGGGTGGCCGGGGCCAGGGCTTCCCACGTGCGCAGCAGGACGCAGCGCTGCCTGG-3'; TERT-120/-58 s: 5'-TCGACGGGCCCTCCCAGCCCCTCCCCTTCCTTTCCGCGGCCCCGCCCTCTCCTCGCGGCGCGAGTTT-3' TERT-120/-58 as: 5'-TCGAAAACTCGCGCCGCGAGGAGAGGGCGGGGCCGCGGAAAGGAAGGGGAGGGGCTGGGAGGGCCCG. The successful cloning was verified by restriction analysis and sequencing.

3.3 Cell culture

African green monkey kidney CV1 cells, which lack endogenous expression of steroid hormone receptors, were cultured in Dulbecco's modified Eagle's medium (DMEM) (41966-029, Life Technologies, Darmstadt, Germany) supplemented with 5 % heat inactivated fetal calf serum (FCS; Invitrogen, Darmstadt, Germany), penicillin (100 U/ml), streptomycin (100 µg/ml) and 25 mM HEPES (pH 7.5-7.8). The androgen-dependent human PCa cell lines LNCaP S1 (Protopopov *et al.*, 2002) and S2, the latter ones were obtained from Dr. J. Klug, IMT University Marburg, were cultured in Roswell

Park Memorial Institute (RPMI1640) (11835-063, Life Technologies, Darmstadt, Germany) supplemented with 10 % FCS, penicillin (100 U/ml), streptomycin (100 µg/ml), 25 mM HEPES (pH 7.5-7.8) and 1 % sodium pyruvate. The androgen-independent growing C4-2 cells, which are derived from the LNCaP cell line (Thalmann *et al.*, 1994), were cultured in DMEM supplemented with 20 % F12 medium, 10 % FCS, 5 µg/ml insulin, 5 µg/ml apotransferin, 0.25 µg/ml Biotin, 25 µg/ml Adenin, penicillin (100 U/ml), streptomycin (100 µg/ml) and 25 mM HEPES (pH 7.5-7.8). Cells were incubated at 37 °C in a humidified atmosphere with 5 % CO_2. Prior to hormone treatment and during the experiments cells were cultured in hormone-depleted medium containing charcoal stripped serum (CCS).

3.4 Prostate tissue

Human PCa tissue samples were obtained from four patients who underwent prostatectomy. Specimens were kindly provided by the Institute of Urology, Jena University Hospital, Germany, prior to ethical approval (3286-11/11). Immediately after surgery, tissue samples were sliced (with a physical dimension of approximately 2x2 mm), and cultured at 5 % CO_2 and 37 °C for 48 h. RPMI1640 cell culture medium, supplemented with 10 % CCS, penicillin (100 U/ml), streptomycin (100 µg/ml), 25 mM HEPES (pH 7.5-7.8) and 1 % sodium pyruvate, was applied. The medium contained the indicated compounds (PhD thesis Hessenkemper, 2013; PhD thesis Roediger, 2012). Tissue sections were used subsequently for mRNA extraction and qRT-PCR.

3.5 Xenograft C4-2 tumors in immunodeficient mice

Experiments were approved by the Thüringer Landesamt für Lebensmittelsicherheit und Verbraucherschutz, Germany (02-006/11). Five-week-old male athymic nude mice (Harlan Laboratories, Rossdorf, Germany) were employed in xenograft experiments. The animals were housed in a pathogen free facility. C4-2 PCa cells should be grafted into the intact (non-castrated) mice. Prior to injection C4-2 cells were expanded *in vitro*. Cells were harvested and the cell pellet was resuspended in 50 µl 1x PBS (130 mM NaCl, 7.79 mM Na_2HPO_4, 3.077 mM NaH_2PO_4, pH 7.0) per 10^6 cells. The cell suspension was mixed 1:1 with BD Matrigel™ (BD Biosciences, Heidelberg, Germany).

100 µl cell suspension containing 10^6 cells were injected subcutaneous (s.c.) into both flanks of each nude mouse with a 24 G needle. After the tumors reached a size of approximately 80 mm^3 mice were treated with vehicle or AA (100 mg/kg). Mice were treated daily by intra peritoneal (i.p.) injection. Tumor size was measured every third day using a caliper (tumor volume = (length x $width^2$) x 0.56). Mice were weighted once a week. Mice were sacrificed if weight loss exceeded 20 % of initial weight or after 26 days of treatment. At the end of the experiment, subcutaneous tumors, prostate, seminal vesicles, testis, liver, kidney and levator ani were removed. The weight of the levator ani was determined. Tissues were snap frozen in liquid nitrogen and sections of PCa tumor tissues were stored in 1x PBS supplemented with 0.02 % NaN_3 at 4 °C for immunofluorescence staining.

3.6 Reporter gene assays

Transfection of CV1 cells was done using a modified $CaPO_4$ method (Wigler *et al.,* 1978). For transfection 10^5 cells per well were seeded on 6-well cell culture dishes using DMEM supplemented with 5 % CCS (Dotzlaw *et al.,* 2003). Experiments were prepared as doublet. Four to six hours later cells were transfected with 1 µg reporter construct, 1.6 µg AR expression plasmid or empty control vector (pSG5) and 0.2 µg pCMV-lacZ for internal normalization (Moehren *et al.,* 2008). Media was removed after 16 to 20 h, and cells were washed with 1x PBS (see 3.5). Fresh medium was added containing the respective substances. Cells were stimulated with R1881, DHT, AA, MDV3100 or combinations of those at the indicated concentrations. DMSO was used as solvent control. Cells were harvested 72 h after hormone induction. Luc and β-galactosidase activity were assayed. The latter one being used for normalization of the luc activity (Gerlach *et al.,* 2011).

3.7 Cell growth assays

LNCaP and C4-2 cells, respectively, were seeded in the appropriate medium supplemented with 5 %FCS on 12-well cell culture plates. 10^4 cells were seeded per well. After three days medium was replaced by fresh medium containing DMSO or R1881 in the appropriate concentration. Concurrently, first group of cells was

trypsinized and counted using a Neubauer counting chamber representing day 0. Three days later remaining cells were counted. Four wells were prepared per treatment.

3.8 Quantitative reverse transcription PCR (qRT-PCR)

LNCaP and C4-2 cells, respectively, were seeded on 6-well cell culture dishes (2×10^5 cells per well) in the appropriate medium supplemented with 10 % CCS. After two days (LNCaP S1 and S2 cells) or one day (C4-2 cells) of cultivation cells were treated with the indicated substances for 24 h. This time schedule was adopted to the experimental setup described by Moehren *et al.* (2008). PCa tissues were taken from mice (see 3.5) and prepared as follows.

RNA was isolated from cells or tissues using peqGOLD TriFast™ (Peqlab, Erlangen, Germany) according to manufacturer's instructions. A 2-step protocol was employed in qRT-PCR: 2 µg of RNA were applied in cDNA synthesis, which was performed with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Darmstadt, Germany) following manufacturer's instructions. After the cDNA synthesis reaction samples were diluted 1:1 with DEPC-treated water. The quantitative real time PCR was performed using the *Sso* Fast™EvaGreen® Supermix (Bio-Rad, München, Germany) following manufacturer's protocol carried out with the aid of the CFX96™ Real Time PCR detection system (Bio-Rad, München, Germany). Primer were applied at 10 µM stocks. The annealing temperatures were adjusted as indicated (**Table 1**). qRT-PCR results were analyzed via the $\Delta\Delta C_t$ method (Pfaffl, 2001) using the CFX Manager™ software from Bio-Rad (München, Germany).

Table 1: Primer sequences for indicated genes employed in qRT-PCR

Gene	Primer	Sequence 5' → 3'
Actin	fw	CACCACACCTTCTACAATGAGC
	rev	CACAGCCTGGATAGCAACG
AR	fw	TCAGCATTATTCCAGTGGATGGGC
	rev	TGGTAGAAGCGTCTTGAGCAGGAT
CCL2	fw	ATCACCAGCAGCAAGTGTCCC
	rev	ATGGAATCCTGAACCCACTTCTGC

Cyclin D1	fw	TCAACCTAAGTTCGGTTCCGATG
	rev	GTCAGCCTCCACACTCTTGC
E2F1	fw	GCAGAGCAGATGGTTATGG
	rev	GATCTGAAAGTTCTCCGAAGAG
FKBP5	fw	GAGGAAACGCCGATGATTGGAGAC
	rev	CATGCCTTGATGACTTGGCCTTTG
Maspin	fw	CAGACACCAAACCAGTGC
	rev	ACTCATCCTCCACATCCTTG
Med1	fw	GAGAATCCTGTGAGCTGTCCG
	rev	CTTCAGTTTGTTGTCCCCTGGA
MMP9	fw	ACGACGTCTTCCAGTACCGA
	rev	TTGGTCCACCTGGTTCAACT
Nov/CCN3	fw	ACCGTCAATGTGAGATGCTG
	rev	TGGATGGCTTTGAGTGACTTC
p16^{INK4a}	fw	CTTGCCTGGAAAGATACCG
	rev	CCCTCCTCTTTCTTCCTCC
p21$^{CIP1/WAF1}$	fw	TCGACTTTGTCACCGAGACACCAC
	rev	CAGGTCCACATGGTCTTCCTCTG
PSA	fw	GAGGCTGGGAGTGCGAGAAG
	rev	TTGTTCCTGATGCAGTGGGC
RPL13a	fw	GTATGCTGCCCCACAAAACC
	rev	TGTAGGCTTCAGACGCACGAC
Sox2	fw	GAATGCCTTCATGGTGTGGTC
	rev	TTAGCCTCGTCGATGAACGG
Sox9	fw	TTTCCAAGACACAAACATGACCTATCC
	rev	TCCTCCACGCTTGCTCTGAAG
TERT	fw	CGGAAGAGTGTCTGGAGCAA
	rev	GGATGAAGCGGAGTCTGGA
TMPRSS	fw	CCTGCAAGGACATGGGCTATA
	rev	CCGGCACTTGTGTTCAGTTTC

Primer were employed as 10 µM stock solutions. Annealing temperature is 60 °C, except of FKBP5 primer, here an annealing temperature of 65 °C was applied. TERT primer were adopted from Meeran *et al.* (2010).

3.9 Western blotting

C4-2 cells were seeded at a confluence of 25 % on 10 cm cell culture plates in the appropriate medium supplemented with 10 % CCS. One day after seeding, hormone induction with the indicated substances followed. Since DHT is metabolized, medium containing the respective compounds was refreshed daily. Cells were washed with ice cold 1x PBS after three days of treatment (see 3.5), harvested with 1 ml 1x PBS and centrifuged (600 g, 5 min, 4 °C). The cell pellet was resuspended in five-times the volume of the pellet NETN buffer (100 mM NaCl, 20 mM Tris/HCL pH 8.0,1 mM EDTA, 0.5 %NP-40) supplemented with phosphatase inhibitors (50 mM NaF, 100 µM Na_3VO_4, 10 mM β-Glycerophosphate). For cell lysis the reaction was incubated 10 min on ice. Cells were broken open by three cycles of freezing (in liquid nitrogen) and thawing (in 37 °C water bath). The suspension was centrifuged (21,500 g, 15 min, 4 °C). The supernatant was collected and represents whole cell extracts used for Western blot.

For PCa tissues, sections (≤ 50 mg) were taken and pestled in liquid nitrogen. 400 µl of ice cold lysis buffer [50 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 % TritonX-100, 1 mM Na_3VO_4, 1 mM NaF, 2.5 mM NaP-P (NaP-P: 77.4 mM Na_2HPO_4, 22.6 mM NaH_2PO_4, pH 7.4)] freshly supplemented with 1x complete protease inhibitor tablet (Roche, Penzberg, Germany) and 1 mM PMSF were added and mashed. The tissue was ultrasonified in 4 °C water bath for 30 min. Samples were incubated on ice for 10 min and collected by centrifugation (21,500 g, 2 min, 4 °C). Supernatants were aliquoted. Tissue and cell extracts were diluted in sterile deionized water as required.

The extracts were separated on SDS-PAGE and blotted onto a PVDF membrane (Roche, Penzberg, Germany). Blotted PVDF membranes were stored in and washed with TBS-T buffer (50 mM Tris pH 7.5, 150 mM NaCl; 0.1 % Tween 20) Western blot analysis was performed by using the appropriate antibodies (**Table 2**) and the enhanced chemiluminescence detection method (GE Healthcare, München, Germany). Primary antibodies were incubated over night at 4 °C and secondary antibodies (**Table 2**) at room temperature for 30 min. Each incubation was followed by three washing steps with TBS-T at 5 min. The protein signals were detected by ImageQuant™ LAS 4000 (GE Healthcare, München, Germany). Band intensities were quantified via LabImage 1D software (Kapelan Bio Imaging solutions, Leipzig, Germany). Hereby, specific bands

were defined as region of interest (ROI), background reduction was adjusted and band intensities were calculated by the program. The band intensity of the protein of interest was set relative to the loading control (β-Actin or α-Tubulin).

Table 2: Antibodies applied in immunoblotting.

Antibody	Company, Ref. no.	Dilution	Protein band size
mouse anti-β-Actin	Abcam, ab6276	1:10,000	42 kDa
rabbit anti-α-Tubulin	Abcam, ab15246	1:2000	50 kDa
mouse anti-Cyclin D1	Cell Signaling, 2926	1:2000	36 kDa
rabbit anti-E2F1	Santa Cruz, sc-193	1:1000	60 kDa
mouse anti-AR	Biogenex, 256M	1:2000	110 kDa
rabbit anti-PSA	Cell Signaling, 2475	1:2000	29 kDa
mouse anti-p21$^{WAF1/Cip1}$	Cell Signaling, 2946	1:2000	21 kDa
rabbit anti-pRB	Abcam, ab6075	1:1000	105 kDa
rabbit anti-P-pRB(S807/811)	Cell Signaling, 9308	1:1000	110 kDa
rabbit anti-P-pRB(S780)	Cell Signaling, 9307	1:1000	110 kDa
mouse anti-cleaved PARP	Cell Signaling, 9546	1:2000	PARP 116 kDa cleaved PARP 89 kDa
bovine anti-rabbit IgG HRP *	Santa Cruz, sc-2370	1:10,000	
goat anti-mouse IgG HRP *	Santa Cruz, sc-2005	1:10,000	

All antibodies were diluted in TBS-T (see 3.9). Secondary antibodies (*) are conjugated to horseradish peroxidase (HRP).

3.10 Immunofluorescence tissue staining

PCa tumor tissue sections stored in 1x PBS + 0.02 % NaN_3 (see 3.5) were incubated in 30 % saccharose in 1x PBS + 0.02 % NaN_3 over night for dehydration. Sections were submerged in Tissue-Tek® O.C.T. Compound (Sakura, Staufen, Germany) and slowly frozen on dry ice, if needed, tissues were stored at -20 °C prior to sectioning. 20 µm tissue sections were cut and placed on glass slides for immunofluorescence analysis.

Slides were washed in 1x PBS for 5-10 min and placed in Shandon Sequenza immunostaining racks (Thermo Scientific, Hamburg, Germany) providing an humidified chamber. Slides were blocked in blocking solution [5 % normal goat serum (NGS), 1x PBS-T (1x PBS, 0.25 % Triton-X100)] for one hour and rinsed once with 1x PBS for 5 min. Primary antibodies (**Table 3**) were applied to the sections over night at 4 °C. Simultaneously, slides were incubated without Ki67 primary antibody to determine background of secondary antibody (goat anti-rabbit Alexa Fluor 488) for future quantification. Slides were washed three times with 1x PBS at 5 min. Secondary antibodies (**Table 3**) were applied to the sections for 90 min in the dark at room temperature. Slides were washed once with 1x PBS for 5 min and nuclei were stained with Hoechst33258 (Molecular Probes, Darmstadt, Germany; dilution: 1:10,000in 1x PBS) for 15 min at room temperature. Slides were washed three times with 1x PBS for 5 min. Tissue sections were covered with Fluoromount-G™ (Southern Biotech, Eching, Germany) and sealed with nail polish. Images were captured using a Leica TCS SP5 confocal Scanner Fluorescence Microscope (Solms, Germany) and a 20x objective.

Fluorescence signals of Ki67 were quantified using ImageJ software as described previously (Arqués *et al.,* 2012). Ki67 signals were quantified per nucleus analyzed subtracting background signals (corresponding to the signals verified for slides, which were not incubated with Ki67 primary antibody but goat anti-rabbit Alexa Fluor 488). Relative quantification represents the level of expression of Ki67 protein per nucleus analyzed (Arqués *et al.,* 2012).

Table 3: Antibodies applied in immunofluorescence staining.

Antibody	Company, Ref. no.	Dilution
rabbit anti-Ki67	Vector Laboratories, VP-RM04	1:100
rat anti-CD31	BioLegend, MEC13.3	1:500
goat anti-rabbit Alexa Fluor 488 *	Invitrogen, A-11008	1:1000
goat anti-rat Alexa Fluor 555 *	Invitrogen, A-21434	1:1000

All antibodies were diluted in 1x PBS-T + 3 % NGS (see 3.5). Secondary antibodies (*) are conjugated to Alexa Fluor dyes.

3.11 Statistical analyses

Statistical analyses of the data were performed by Mann-Whitney *U* test or Wilcoxon signed-rank test using GraphPad Prism software. Outliers were identified by Grubb's test. The significance level was set at $p<0.05$.

4 Results

The AR plays a pivotal role in the development and progression of PCa (Gelmann, 2002). Since the cancer depends on androgens at initial phases ADT became a major form of PCa therapy. Even though, first successful the tumor eventually recovers and CRPC develops. However, the tumor still partly relies on AR signaling (Parray *et al.,* 2012). For the treatment of this more advanced stage new therapeutic approaches need to be developed. Hence, the identification of new anti-androgens and an understanding of the mechanisms involved in tumor growth regression as well as a deep insight into AR-mediated gene regulation are essential.

The human TERT gene, which is essential for telomerase activity, and stimulates growth factor signaling in an telomerase-independent manner, represents one gene regulated by the AR, however, in an ambiguous manner. Furthermore, TERT gene expression is induced in PCa specimens relative to BPH (Beck *et al.,* 2011; Tang *et al.,* 2008).

4.1 Both wild-type and mutant AR mediate TERT repression in an androgen concentration-dependent manner

Moehren *et al.* (2008) could show that the ARwt represses the full-length TERT promoter in an androgen-dependent manner. Herein, the first 3996 bp upstream of the TLS (translation start) of the TERT gene were referred to as full-length promoter. However, to analyze whether this effect is androgen concentration-dependent CV1 cells, which lack endogenous steroid receptors, were employed in reporter gene analyses. For this purpose, cells were co-transfected with a full-length TERT promoter construct (pGL3-TERTΔ3996) and an ARwt expression plasmid or the empty vector (**Figure 3 A,B**). Subsequently, they were treated with R1881, a synthetic androgen, which is more stable than the natural occurring DHT. In the absence of the human AR, R1881 is not able to mediate the repression of the TERT promoter construct (**Figure 3 A**). However, in the presence of ARwt increasing concentrations of the androgen mediate an increasing repressive activity. The strongest repression was seen for 0.1 up to 1 nM of the hormone, higher concentrations slightly released the repressive effect (**Figure 3 B**). Furthermore, a suppressive effect was already observed

independent of androgen treatment comparing solvent controls of vector-transfected (**Figure 3 A**) and ARwt expression plasmid-transfected cells (**Figure 3 B**).

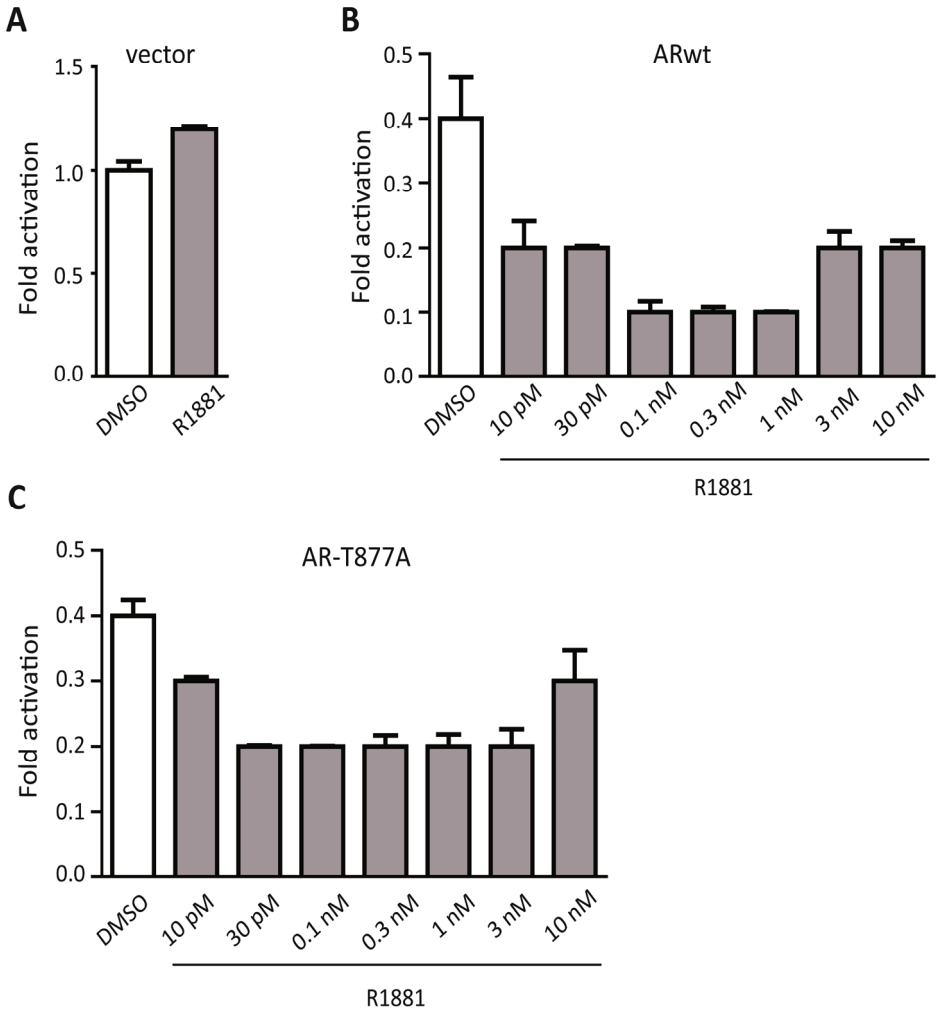


Figure 3: TERT promoter repression is AR- and androgen concentration-dependent. Reporter gene assays were conducted to analyze TERT promoter activity in the presence of ARwt and AR-T877A, respectively. CV1 cells were co-transfected with (**A**) pSG5, (**B**) pSG5-ARwt or (**C**) pSG5-AR-T877A (1.6 µg), pGL3-TERTΔ3996 (1 µg), and pCMV-lacZ (0.2 µg). The latter one represents the expression vector for β-galactosidase, which was used for normalization. Cells were treated with solvent (DMSO) or increasing R1881 concentrations as indicated and incubated for 72 h. (**A**) Vector control was treated with 10 nM R1881, solely. Normalized luciferase units are shown as fold activation. All values were calculated relative to values derived from (**A**) DMSO-treated vector-transfected cells, which were arbitrarily set as 1. Error bars indicate SEM.

Since, these differences of effectiveness of androgen-mediated repression were observed for ARwt the same experimental setup was applied for the mutant AR-T877A. AR-T877A represents a point mutation within the LBD of the steroid receptor (Veldscholte *et al.,* 1990) and was described not to be able to mediate androgen-

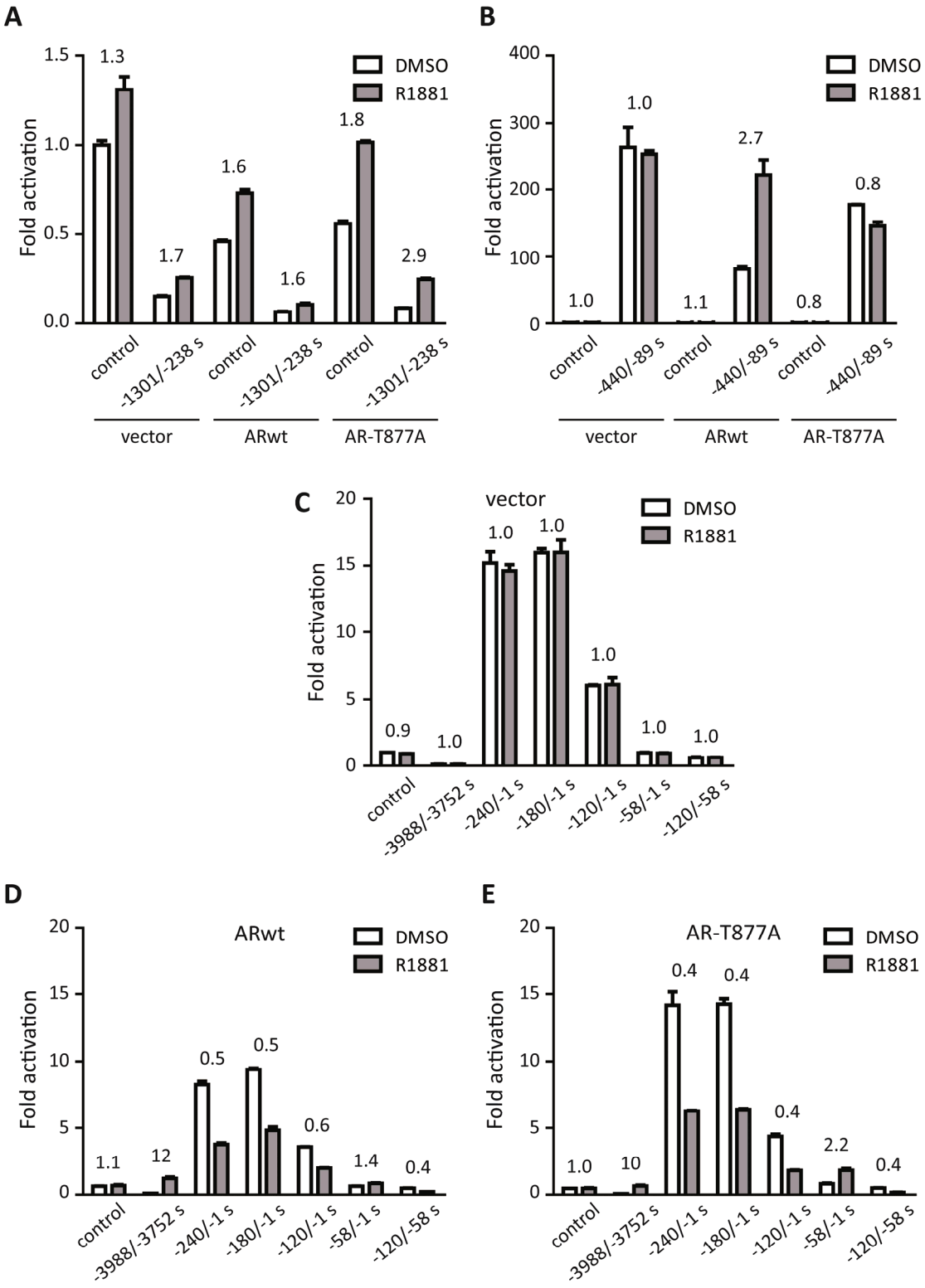
dependent repression of TERT expression at an R1881 concentration of 10 nM (Moehren *et al.,* 2008). The data herein indicate that AR-T877A represses TERT promoter activity even in the absence of hormone to a similar rate as ARwt. This repression is enhanced with increasing androgen concentrations (**Figure 3 C**). However, in contrast to ARwt, in the presence of the mutant AR the lowest R1881 concentration applied (10 pM) and the highest concentration (10 nM) did not cause a further repression of TERT promoter activity compared to DMSO-treated AR-T877A expressing CV1 cells, this is in accordance to the data published by Moehren *et al.* (2008).

Taken together, both ARwt as well as AR-T877A mediate TERT promoter repression even in the absence of hormone, thereby, the ARwt is more effective in mediating androgen-dependent repression

4.2 AR-mediated TERT repression requires a region within the proximal TERT promoter

Previous studies could show that the AR is recruited to two regions within the TERT promoter, the -4 kb and the -0.1 kb region (Moehren *et al.,* 2008). Thereby, the distal site of the TERT promoter seems to be directly bound by the AR (Diploma thesis Jatzkowski, 2010) and can mediate androgen-dependent promoter activation (Diploma thesis Bartsch, 2011; Bachelor thesis Schreiber, 2011). In order to identify the TERT promoter region, which is responsible for AR-mediated TERT repression, promoter deletion constructs were generated and functionally analyzed. Thereby, the responsiveness of ARwt and AR-T877A were compared in the presence of R1881 at a concentration of 10 nM. A construct spanning -1301 to -238 bp upstream of the TLS of the TERT gene showed a repressive activity in the absence of AR relative to control. This region excludes most of the reported activating Sp1 sites (Poole *et al.,* 2001). Since there was a weak induction of promoter activity upon androgen treatment in the absence of AR expression (vector), an AR-dependent regulation of TERT-1301/-238 s could not be verified (**Figure 4 A**), which is consistent with previous data (Diploma thesis Bartsch, 2011). In contrast, the TERT-440/-89 s construct, which includes most of the activating Sp1 sites (Poole *et al.,* 2001), potently induces luc expression compared to control vector, independent of AR and androgens (**Figure 4 B**). This induction was in part repressed by the unliganded ARwt but not its mutant counterpart. The repressive

activity of ARwt was released upon androgen treatment (**Figure 4 B**). A further deletion of the TERT promoter spanning the first 240 bp upstream of the TLS (TERT-240/-1 s) also induced promoter activity relative to control vector (**Figure 4 C**). However, the construct was repressed in the presence of ARwt and this effect was enhanced upon androgen treatment (**Figure 4 C,D**).



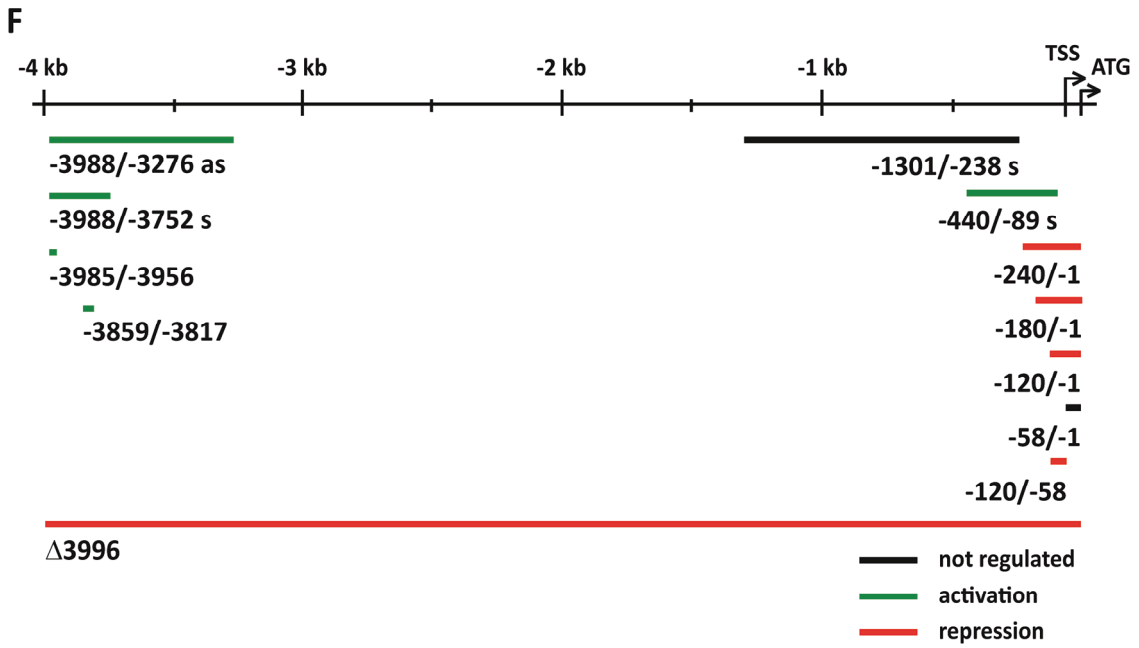


Figure 4: AR-dependent TERT repression is mediated through a proximal promoter region. Reporter gene assays were conducted to identify the AR-responsive element within the proximal TERT promoter. (**A**) CV1 cells were co-transfected with either pSG5 or one of the AR expression vectors (pSG5-ARwt, pSG5-AR-T877A) (1.6 µg), p4xUAS-TATA-luc (control) or the TERT-1301/-238 promoter construct (1 µg) and pCMV-lacZ (0.2 µg). Cells were treated with DMSO or R1881 (10 nM) for 72 h. All values were calculated relative to DMSO-treated vector-transfected cells, which were arbitrarily set as 1. (**B**) CV1 cells were treated as described above, and co-transfected with a reporter construct containing promoter DNA from -440 to -89 bp relative to the translation start site (TLS) of the TERT gene. Values were calculated relative to DMSO-treated vector-transfected control. (**C-E**) Cells were transfected with one of the indicated TERT promoter deletion constructs and (**C**) pSG5, (**D**) pSG5-ARwt or (**E**) pSG5-AR-T877A, all values were calculated relative to DMSO-treated vector-transfected control, which was set as 1. Numbers above the bars indicate fold hormone induction. For further explanations see **Figure 3**. (**F**) Schematic overview of TERT constructs including their response to androgens. All TERT constructs were cloned into the p4xUAS-TATA-luc vector except of TERTΔ3996 whose backbone is the pGL3 basic vector. Numbers are relative to the TLS (ATG) of the TERT gene. The transcription start site (TSS) at position -58 is indicated(NM_198253.2).

This finding is similar to the one observed for the full-length TERT promoter construct (**Figure 3 A**). In the presence of R1881 AR-T877A represses the promoter construct to a similar degree as ARwt. A further shortening of the promoter spanning the first 180 bp mimicked these effects (**Figure 4 C-E**). For the deletion construct TERT-120/-1 s the androgen-independent activity of the receptor was almost abolished but the androgen-dependent repression was maintained for both wt and mutant receptor. A reduction of the TERT promoter to the first 58 bp caused the loss of the androgen-

mediated repression. Interestingly, a slight up-regulation was verified for activated AR-T877A but not for ARwt.

Thus, the down-sizing of the TERT promoter from -120 to -58 bp permits the androgen-dependent repression by both ARwt and AR-T877A (**Figure 4 C-E**). The distal TERT promoter construct, TERT-3988/-3752 s seems to include a strong repressive element comparing it to control vector (**Figure 4 C**), however, this repression was abrogated by both ligand activated wt and mutant AR (**Figure 4D,E**).

Therefore, the TERT promoter is ambiguously regulated by androgen-activated AR. The distal promoter around -4 kb bears at least two positive AREs (**Figure 4 F**) whereas the proximal promoter mediates TERT repression in an androgen-dependent manner. The minimal AR-dependent repressive element seems to span a region from -120 to -58 upstream of the TLS of the TERT gene (**Figure 4 F**).

4.3 AR-mediated repression of the TERT promoter is orientation-dependent

A region spanning 60 bp of the proximal TERT promoter, which is responsive to the androgen-stimulated AR was identified. Subsequently, TERT constructs were employed in antisense orientation for reporter gene analyses and the responsiveness of ARwt and AR-T877A was determined. This approach should help to receive a further understanding of the role of the location of the AR responsive region in relation to the TSS (-58 bp) and other regulative sites within the TERT promoter.

The results indicate that the higher basal activity of the -240/-1 TERT promoter construct relative to the control vector in the absence of androgen and AR is independent of its orientation (**Figure 5 A**). Similar observations were made for the other promoter deletion constructs (**Figure 5 B**). However, the AR-mediated androgen-dependent repression of the proximal TERT promoter depends on the sense orientation of the constructs. An anti-sense orientation of TERT-240/-1 caused an induction of the promoter activity in the presence of androgen-stimulated ARwt, and interestingly no change of promoter activity if the activated AR-T877A was present (**Figure 5 A**).

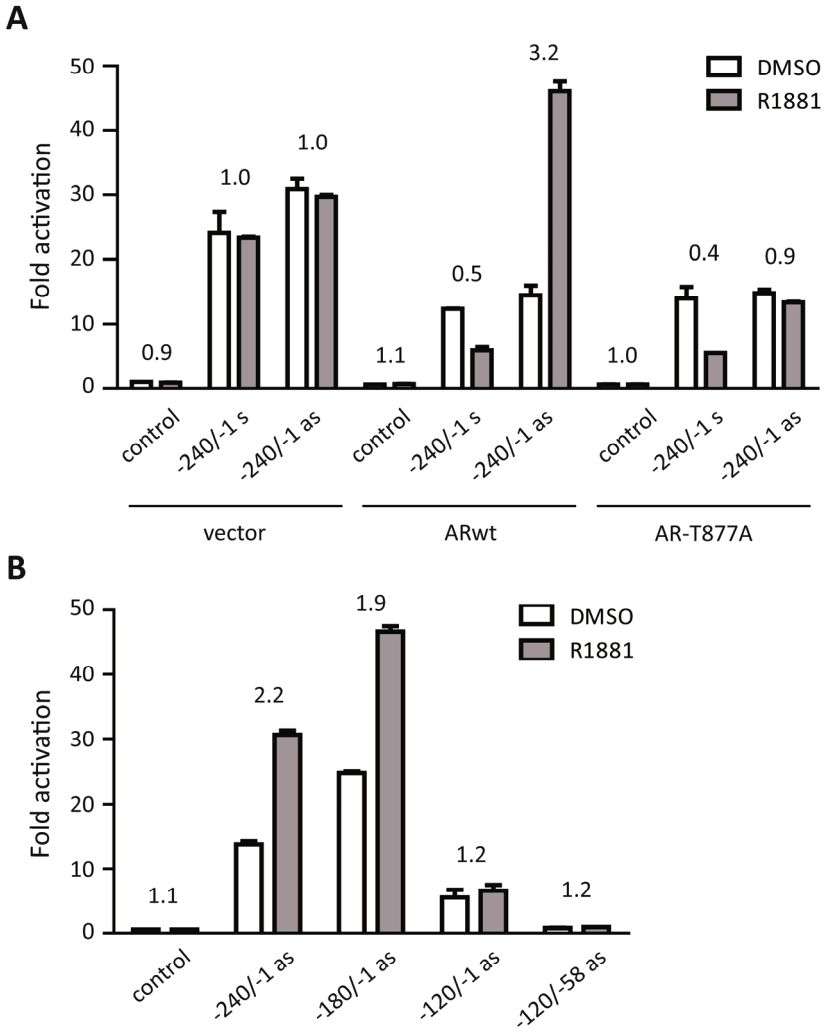


Figure 5: AR-mediated repression of the proximal TERT promoter depends on its orientation. The orientation dependence was determined through analyses of promoter constructs cloned in sense (s) and antisense (as) orientation into a reporter plasmid. (**A**) Cells were transfected with either control vector (p4xUAS-TATA-luc) or a -240/-1 TERT promoter construct in s or as orientation and pSG5, -ARwt or -AR-T877A. (**B**) Cells were transfected with an ARwt expression plasmid and one of the indicated antisense promoter constructs. DMSO or 10 nM R1881 were applied. Numbers indicate fold hormone induction. For further explanations see **Figure 3**.

The TERT-180/-1 as construct is weakly activated by the R1881-activated ARwt, as well. For the TERT-120/-1 deletion and the minimal repressive element TERT-120/-58 neither an activation nor a repression was detectable in anti-sense orientation (**Figure 5 B**).

Hence, the androgen-dependent repression of the proximal TERT promoter by the AR relies on the orientation or positioning of the androgen responsive region.

4.4 Androgen-induced repression is maintained upon anti-androgen treatment

Anti-androgens alter the AR pathways since they compete with androgens for binding to the receptor and block the receptor's transactivating potential. Nevertheless, it is not known how anti-androgens change the repressive activity of the receptor on TERT expression. For that purpose the natural occurring compound AA and the synthetic MDV3100, which is in clinical trials (Semenas *et al.,* 2013), were applied in reporter gene assays. For these analyses an R1881 concentration of 0.1 nM, instead of 10 nM (see 4.2 and 4.3), and a DHT concentration of 10 nM was applied to use androgen-anti-androgen ratios, which were published to be most effective (Papaioannou *et al.,* 2009; Tran *et al.,* 2010) and owing to the solubility of the substances. The MMTV promoter as a well-characterized AR-responsive promoter was used as control of the anti-androgenic activity of the substances. As expected, neither AA nor MDV3100 influenced MMTV promoter activity in the absence of androgens, and both AA and MDV3100 strongly reduced androgen-induced activation of the MMTV promoter. In contrast to AA, which still enabled a weak promoter activation, the co-treatment of androgen with MDV3100 completely blocked promoter activation (**Figure 6 A,C**). These results verify that both compounds exhibit an anti-androgenic activity at the concentrations employed.

To demonstrate if AR-mediated TERT repression is influenced by anti-androgen treatment, the full-length TERT promoter construct was employed in reporter gene assays and cells were treated with the indicated substances. Both DHT and R1881 induced a repression of the promoter activity, which was hold if AA was present simultaneously, whereas AA as single agent had no influence on its activity compared to solvent control (**Figure 6 B**). The anti-androgenic activity of both substances was then tested via the TERT-3988/-3752 s construct, which includes at least two functional positive AREs (Diploma thesis Bartsch, 2011; Bachelor thesis Schreiber, 2011). Interestingly, in this context AA and MDV3100 showed a similar potential of inhibiting AR-mediated transactivation. Both compounds almost completely blocked R1881-induced promoter activation (**Figure 6 D**). Whereas, DHT-induced promoter activation was less efficiently repressed (**Figure 6 D**) bearing in mind that this promoter region appears in a natural context and shows an approximately 15-fold weaker hormone

induction than the MMTV promoter (**Figure 6**). Surprisingly, the minimal repressive element TERT-120/-58 s was not significantly repressed at these lower androgen concentrations (**Figure 6 E**).

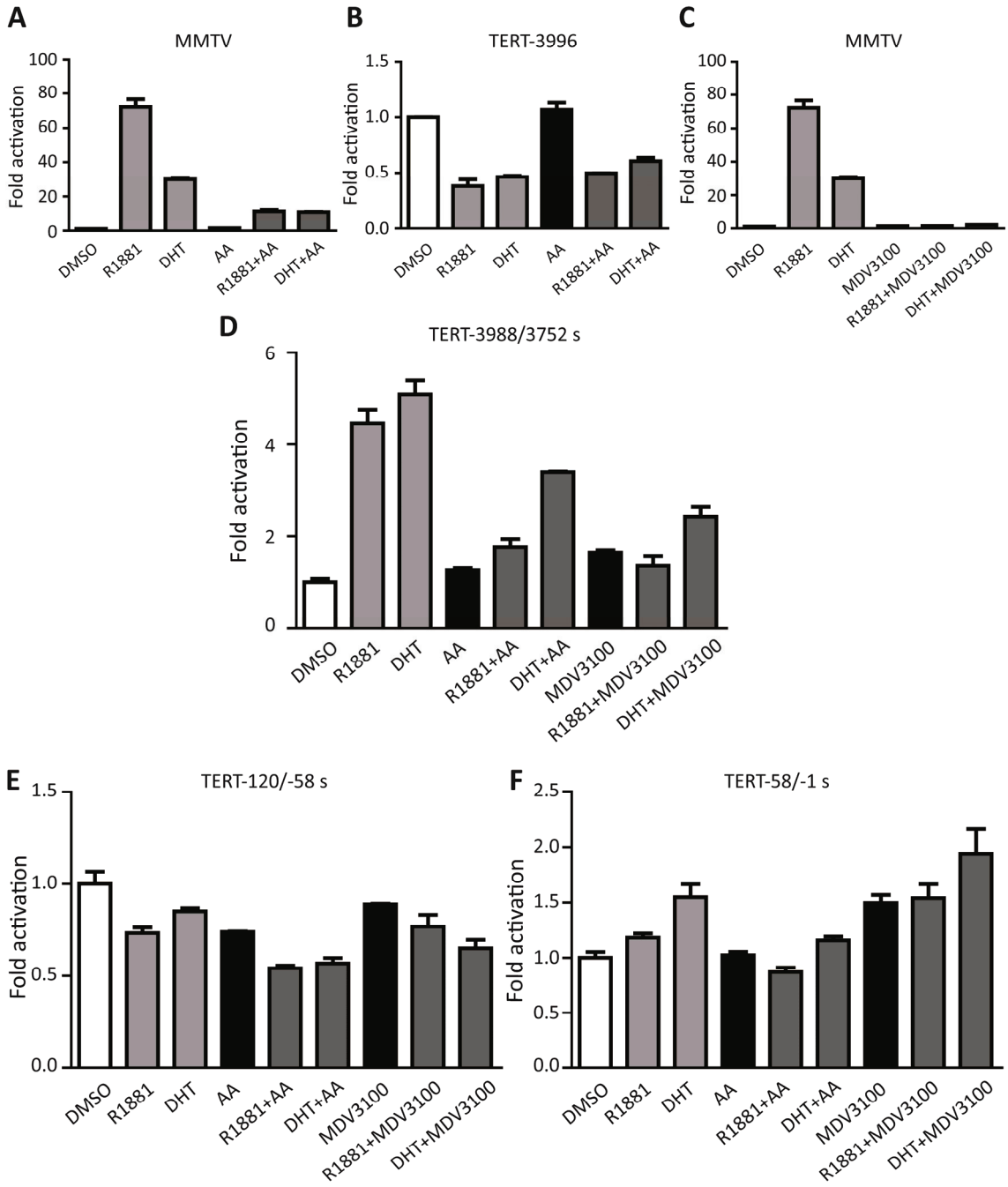


Figure 6: TERT promoter response upon anti-androgen treatment in the presence of ARwt. Reporter gene assays were employed to analyze TERT promoter activity in the presence of androgens and/or anti-androgens. CV1 cells were co-transfected with pSG5-ARwt, pCMV-lacZ and (**A, C**) pMMTV-luc, (**B**) pGL3-TERTΔ3996, (**D**) p4xUAS-TERT-3988/3752 s, (**E**) p4xUAS-TERT-120/-58 s, (**F**) p4xUAS-TERT-58/-1 s (1 µg). Cells were treated with DMSO, R1881 0.1 nM, DHT 10 nM, AA 30 µM, MDV3100 10 µM or combinations of those and incubated for 72 h. Normalized luciferase units are shown as fold activation. Values were calculated relative to DMSO controls, which were arbitrarily set as 1. Error bars indicate SEM.

Anyhow, these concentrations were sufficient to repress full-length TERT promoter 3-fold (**Figure 6 B**) indicating that this region may depend on its surrounding sequences or chromatin. There was no clear difference between androgen and anti-androgens treatment concerning promoter activity. A 2-fold repression was gained only when androgen and AA were present simultaneously (**Figure 6 E**). In contrast, the TERT-58/-1 s construct, which seemed not to be responsive to R1881-stimulated ARwt, showed a weak activation in the presence of DHT and MDV3100. In this context there seemed to be a synergistic effect of the two substances reaching a 2-fold induction if cells were co-treated with DHT and MDV3100 but not with DHT and AA (**Figure 6 F**).
Taken together, anti-androgens inhibit the transactivating potential of the AR, however, in a certain promoter context they may still allow for AR-mediated repression indicating that AR-dependent activation mechanistically differs from repression.

4.5 Cell environment matters: TERT expression is differentially regulated by androgens depending on cellular context

Different groups published that TERT expression is not repressed but induced upon androgen treatment in the androgen-dependent growing LNCaP cells (Guo *et al.,* 2003; Moehren *et al.,* 2008; Soda *et al.,* 2000). However, in PC3-AR cells Moehren *et al.* (2008) reported a repression of TERT expression if stimulated ARwt is present. In this work it could be shown via reporter gene assays that the AR-T877A in general is able to mediate TERT repression, hence TERT repression does not specifically rely on ARwt. This effect was further analyzed in different PCa cell lines, which were employed in TERT mRNA expression analyses. Three cell lines were chosen, which are closely related to each other: androgen-dependent growing LNCaP cells, which were present as two sublines (S1, S2), and androgen-independent growing C4-2 cells, which are derived from LNCaP cells; all of which express the AR-T877A mutant. First, to get an idea of the growth differences, all cell lines were treated with increasing concentrations of R1881 and employed in growth assays. C4-2, in contrast to the two LNCaP cell lines, showed no detectable response to the hormone. Their growth was stable independent of R1881 (**Figure 7 A**). Both LNCaP S1 and S2 cells grew biphasically in the presence of androgens with a growth promoting effect at low androgen

concentrations and growth inhibition at high androgen concentrations. Thereby, LNCaP S2 cells were less sensitive to the growth inhibitory effects compared to LNCaP S1 cells, at least after three days of treatment (**Figure 7 A**).

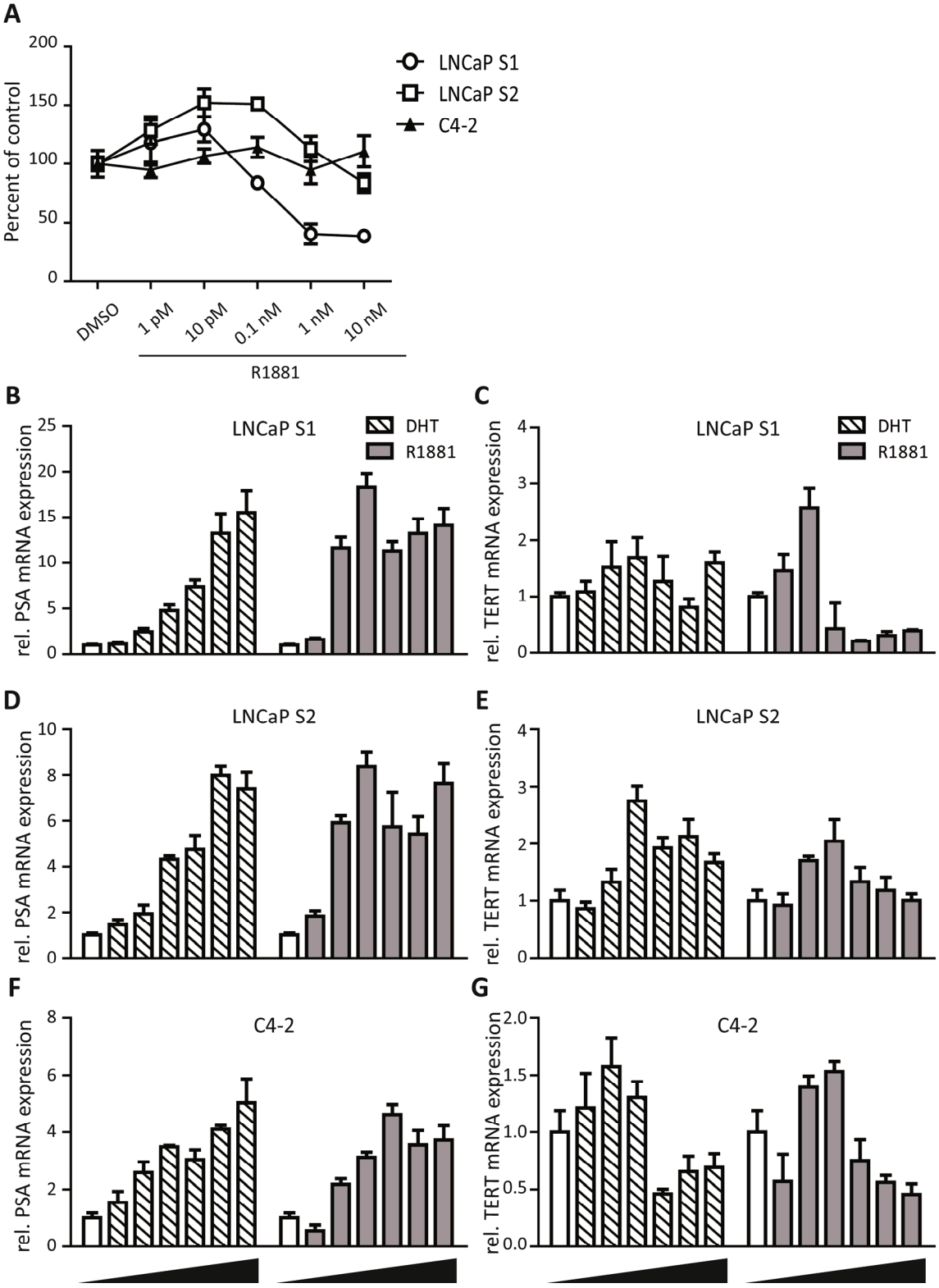


Figure 7: TERT mRNA expression is differentially regulated upon androgen treatment in related PCa cell lines. The androgen-dependent growing LNCaP cells (present as two sublines - S1, S2) and the androgen-independent growing C4-2 cells were employed to analyze androgen concentration-dependent TERT mRNA expression by qRT-PCR. (**A**) Equal amounts of LNCaP S1, S2 and C4-2 cells were seeded and treated with DMSO or increasing concentrations of R1881. The number of cells was counted after 3 days of treatment. Cell numbers were calculated relative to DMSO, which was set as 100 % of cell growth. (**B,C**) The androgen-dependent LNCaP S1 cells and (**D,E**) LNCaP S2 cells, and (**F,G**) the androgen-independent C4-2 cells were treated with solvent (DMSO - white bars) or increasing concentrations of DHT or R1881 indicated by black triangles (30 pM, 0.1 nM, 0.3 nM, 1 nM, 3 nM, 10 nM). 24 h later cells were harvested and mRNA extracts were employed in qRT-PCR. qRT-PCR was performed for (**B,D,F**) PSA mRNA, (**C,E,G**) TERT mRNA and β-Actin. The latter one was used for normalization. Values were calculated relative to DMSO, which was set as 1. Error bars indicate SEM.

Independent of the growth, all three cell lines showed an up-regulation of PSA mRNA expression in a concentration-dependent manner after 24 h of treatment reaching a plateau at higher androgen concentrations (**Figure 7 B,D,F**). The androgen response of TERT mRNA expression, however, was quite different. There was hardly any change of TERT expression in LNCaP S1 cells upon DHT treatment. At low R1881 concentration LNCaP S1 cells showed a growth induction, simultaneously, TERT expression was induced. At higher R1881 concentrations TERT expression was strongly repressed in LNCaP S1 cells correlating with growth inhibition by androgens (**Figure 7 C**). In LNCaP S2 cells, which were less sensitive to growth inhibition, TERT expression was induced treating cells with DHT at a concentration of 0.3 nM, at higher DHT concentrations induction was maintained but weaker. In contrast, R1881 induced TERT expression at a concentration of 0.1 and 0.3 nM this effect was abolished at higher R1881 concentrations (**Figure 7 E**). TERT expression was reduced 2-fold upon DHT treatment (1 nM) in C4-2 cells. Additionally, the inhibitory effect remained but was slightly reduced at higher DHT concentrations. The cells responded similar if R1881 was applied (**Figure 7 G**).

These results indicate that androgen-mediated induction of AR target gene expression (e.g. PSA) can be quite stable amongst different PCa cell lines, whereas androgen-dependent TERT regulation depends on cellular context and ligand most likely involving the availability of other regulative factors and chromatin status.

4.6 TERT expression is repressed by androgen and AA in human PCa tissue *ex vivo*

In the present work it was verified that AR stimulated by androgens or anti-androgens can mediate TERT down-regulation *in vitro*. Next, it was analyzed whether TERT mRNA expression is repressed in primary malignant PCa tissue. For this purpose, *ex vivo* studies with human prostate tumor tissue were conducted. The tissue was derived from prostatectomy. Small tissue specimens of 2x2 mm were employed and treated with R1881 (1 µM, 10 nM), DHT (1 µM) or AA (0.1 mM) post-operative. The high concentrations were chosen to ensure that the substances penetrate the tissue blocks. After two days of treatment RNA was extracted and employed in qRT-PCR (**Figure 8**). The relative TERT mRNA expression was highly variable in the untreated control samples. Despite this, there was a weak, but not statistically significant ($p<0.05$), down-regulation of TERT in presence of R1881 (1 µM) and AA. However, there was hardly any regulation mediated by DHT, which can be metabolized (**Figure 8**). Unfortunately, there were not sufficient tumor tissue samples available to statistically analyze a second R1881 concentration (10 nM) hence data is only shown for R1881 1 µM.

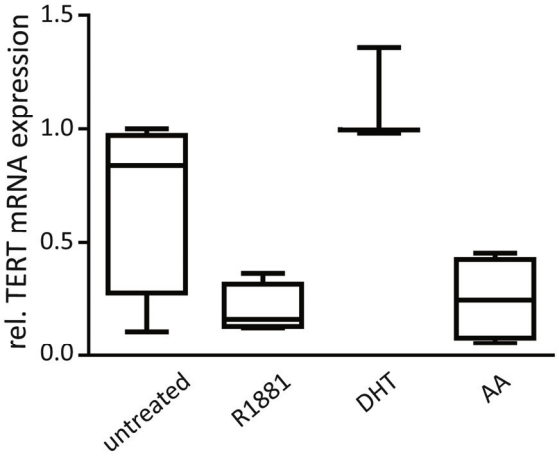


Figure 8: TERT mRNA expression is repressed by R1881 and AA in human PCa tissue *ex vivo*. Prostate tumor specimens derived from prostatectomy were treated post-operative with androgens and AA to analyze TERT mRNA expression. The PCa specimens were treated daily with R1881 (1 µM), DHT (1 µM) or AA (0.1 mM) for 2 days. qRT-PCR was performed for TERT, β-Actin and RPL13a. Tukey box plot of relative TERT mRNA expression normalized to both β-Actin and RPL13a was used to compare the different treatment groups (n = 4). Boxes show the lower and upper inter-quartile. Horizontal lines indicate the median and whiskers show a maximum of 1.5 IQR. Outliers were excluded.

Nevertheless, the two samples, which were available for the R1881 10 nM treatment, showed a similar trend in TERT regulation as the higher concentration (data not shown). Therefore, TERT mRNA expression seems to be repressed upon R1881 or AA treatment *ex vivo* strengthening the data obtained *in vitro*.

4.7 AA treatment influences the p16-pRB signaling axis in C4-2 cells

The repression of TERT mRNA by the AR is preserved in the presence of AA, whereas the transactivation of the PSA gene is reduced representing an advantageous effect of the anti-androgen. Additionally, AA prevents cell invasion through an ECM as well as the AR N/C-terminal interaction and decelerates AR nuclear translocation (Papaioannou *et al.,* 2009; PhD thesis Hessenkemper, 2013). AA inhibits PCa cell growth involving the induction of cellular senescence (Papaioannou *et al.,* 2009; PhD thesis Roediger, 2012). Thereby, TERT repression may be a preferable by-product of AA-treatment strengthening other more basal growth inhibitory mechanism. The signaling underlying AA-induced growth arrest is not completely understood, yet. In this context, the androgen-independent C4-2 cells, as a model of the more challenging CRPC, were used for gene and protein expression analyses upon AA treatment to gain further insight into the pathways, which may be targeted to reduce cell growth.

C4-2 cells were treated with R1881 (0.1 nM), DHT (10 nM), AA (30 µM) or combinations of those and gene and protein expression was analyzed. Changes in protein expression were determined after 72 h of treatment to get a hint of long-term treatment, whereas mRNA expression was verified after 24 h of treatment to analyze direct effects of AR on target gene expression. A possible mechanism how anti-androgens mediate the inhibition of AR signaling is by destabilization of AR protein. Papaioannou *et al.* (2009) reported that AR protein expression is not changed upon AA treatment in androgen-dependent LNCaP cells. In the present work it could be shown that in androgen-independent C4-2 cells AA treatment alone does not change AR protein expression. However, both R1881 and DHT induce a reduction of AR protein, which was preserved if cells were co-treated with DHT and AA but was reversed if cells were co-treated with R1881 and AA (**Figure 9 A**). These results indicate that AR stability may be differentially regulated depending on the ligand and cell line.

Even though AR protein is reduced upon androgen treatment PSA mRNA expression is induced. Hence, either AR degradation is not apparent after 24 h of treatment or there still is enough functional AR present to mediate transactivation. The R1881-mediated PSA mRNA expression is repressed if cells were co-treated with AA. In contrast, the DHT-induced PSA mRNA expression was not suppressed by AA. AA alone did not change PSA expression (**Figure 9 B**).

As expected, TERT expression was weakly induced by R1881 (0.1 nM) and repressed 2-fold by DHT treatment (10 nM) in C4-2 cells. The R1881-mediated induction was reversed by co-treatment with AA and the inhibitory effect of DHT was weakened (**Figure 9 B**). The mRNA expression of an important cell cycle regulator and AR target gene, p21 (Lu *et al.,* 2000), was not changed upon either treatment. Modifications in Cyclin D1 expression, another AR target and cell cycle modulator (Lanzino *et al.,* 2010; Xu *et al.,* 2006), were minor (**Figure 9 B**). Nevertheless, both signaling molecules were changed on protein level depending on the treatment. p21 and Cyclin D1 were reduced on protein level by either androgen and p21 protein level was marginally changed in the presence of AA. The reduction of p21 was preserved if cells were co-treated with androgen and AA. However, strongest effects on p21 protein expression were linked to DHT treatment (**Figure 9 A**). The impact of AA treatment on Cyclin D1 expression was slightly different. The simultaneous treatment with DHT and AA caused a reduction of expression compared to DHT treatment alone and Cyclin D1 protein was stabilized if R1881 and AA were present (**Figure 9 A**). Since Cyclin D1 protein expression was in part strongly influenced related signaling molecules were analyzed.

The p16-pRB protein axis, which also influences Cyclin D1 activity, is a tumor suppressive mechanism, and has been linked to the induction of cellular senescence by R1881 and AA in the androgen-dependent LNCaP cells (PhD thesis Roediger, 2012). Hence, p16 and pRB have been analyzed in more detail. The expression of the tumor suppressor p16 was induced upon androgen and AA treatment reaching highest expression if cells were co-treated with DHT and AA. In contrast, the treatment of cells with R1881 and AA did not result in induction of p16 mRNA (**Figure 9 B**). Phosphorylation of pRB was also induced by androgen treatment this effect was reversed upon co-treatment with AA, especially if AA treatment occurred in the presence of DHT (**Figure 9 A**).

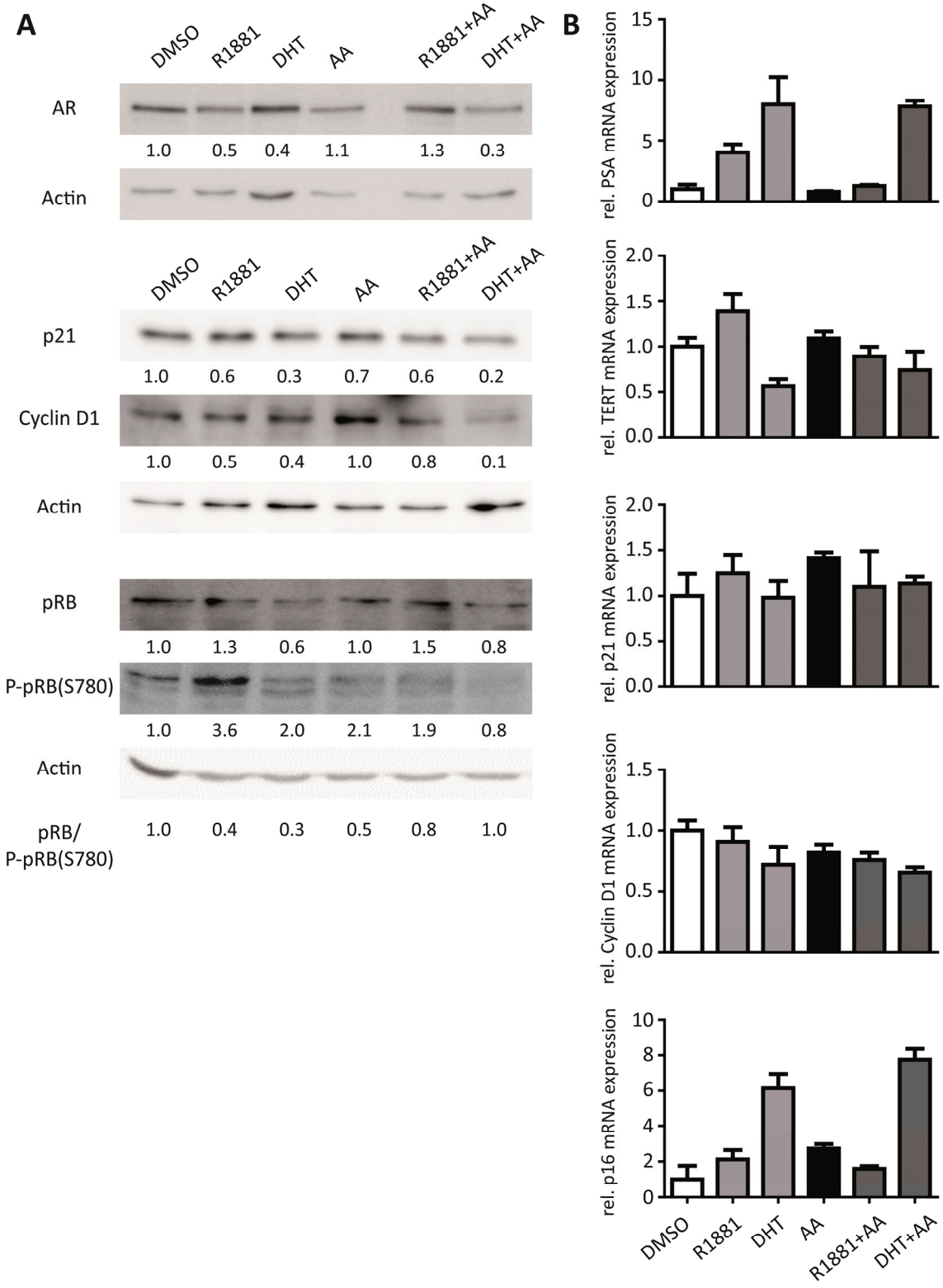


Figure 9: AA treatment modulates the expression of signaling molecules in C4-2 cells. Alterations of androgen-induced (**A**) protein and (**B**) gene expression in the presence of AA were analyzed in C4-2 cells. Cells were seeded and treated with DMSO, R1881 (0.1 nM), DHT (10 nM), AA (30 µM) or combinations of those. (**A**) 72 h later cells were harvested and protein extracts were employed in WB. Protein bands were quantified by LabImage D1 - the expression of the protein of interest was normalized to β-Actin expression. For phosphorylated RB protein (P-pRB) the upper most band was quantified. Relative intensities of protein bands

are presented as numbers below the blot. DMSO-treated control was set as 1. For protein band size see **Table 2**. (**B**) After 24 h of hormone treatment cells were harvested and qRT-PCR was performed with mRNA extracts. qRT-PCR was performed for PSA, TERT, p21, Cyclin D1, p16, β-Actin and RPL13a. Both β-Actin and RPL13a were used for normalization. Values were calculated relative to DMSO, which was set as 1. Error bars indicate SEM.

Taken together, these results indicate that AA treatment modulates the p16-pRB signaling axis involving the reduction of Cyclin D1 protein, the induction of p16 mRNA and the dephosphorylation of pRB, exerting strongest effects if AR is stimulated by DHT. This pathway may be involved in the growth inhibitory effect of the anti-androgen. Additionally, the data suggest that R1881 and DHT exert differential effects in modulating signaling molecule expression.

4.8 AA treatment inhibits prostate tumor growth *in vivo*

Papaioannou *et al*. (2009) demonstrated that AA inhibits the growth of different PCa cell lines including the androgen-independent C4-2 cells. To assess whether AA inhibits PCa growth *in vivo* male immunodeficient nude mice harboring C4-2 xenograft tumors, as a model of androgen-independent growing human PCa, were treated i.p. with AA. Tumor and murine tissues were prepared and further analyzed. The tumor growth was reduced upon AA treatment compared to vehicle (**Figure 10 A**). Thereby, AA did not cause any detectable side effects concerning parameters verified herein. The body weight of vehicle- and AA-treated mice did not differ from each other (**Figure 10 B**). Levator ani weight, as an androgen-responsive tissue (Johansen *et al.,* 2007), was not altered (**Figure 10 E**). Additionally, the proliferation rate of murine intact prostate, determined by Ki67 immunostaining, was independent of treatment (**Figure 10 C,F**) as well as the expression of different AR target genes in diverse murine tissues (Bachelor thesis Dittmann, 2013, data not shown). However, the proliferation marker Ki67 was decreased upon AA treatment in xenografted PCa tumor tissue (**Figure 10** D,F). CD31 was used as endothelial marker to stain vessels within the C4-2 tumors. Both vehicle- and AA-treated tumors became vascularized (**Figure 10 F**).

Hence, AA seems to inhibit tumor growth *in vivo* without affecting other host tissues suggesting a tissue specificity of AA action.

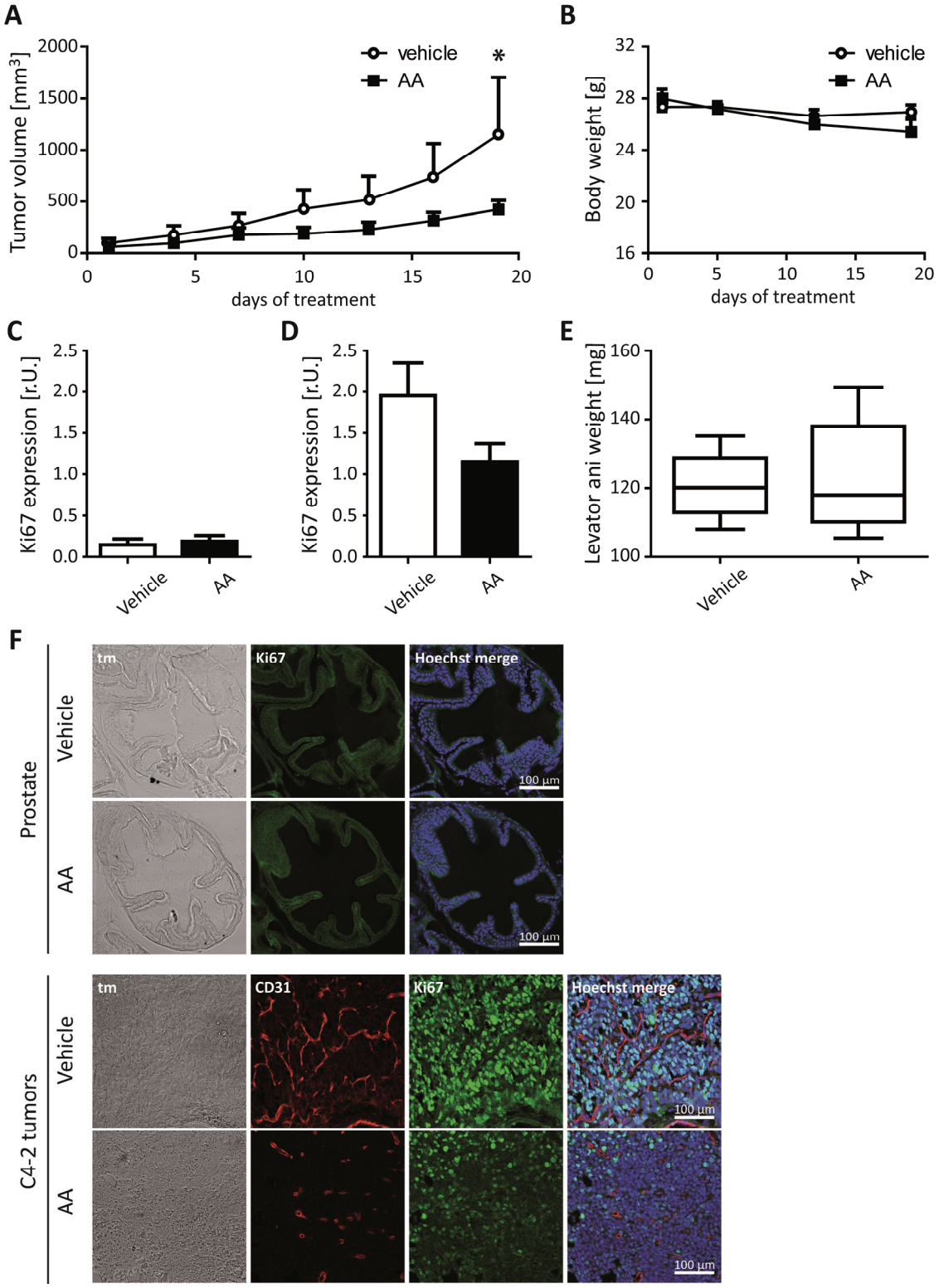


Figure 10: AA is active in a model of androgen-independent growing PCa. Male nude mice (vehicle: n=6, AA: n=5) received s.c. inoculation with C4-2 cells in each flank. On tumor formation mice were grouped and injected daily with vehicle or AA (100 mg/kg). Tumor measurements began at~80 mm^3. Tumor volume was measured every third day using a caliper. (**A**) Tumor volume was plotted against days of treatment. Tumors derived from animals treated with AA (n=7) had statistically different growth rates compared to vehicle (n=6) after 19 days of treatment (* $p<0.05$). (**B**) Body weight was measured once a week and plotted

against days of treatment. (**C-F**) Mice were sacrificed; murine tissues and tumor tissue were isolated. (**E**) Levator ani weight was compared between the vehicle and AA treatment groups and is illustrated as Tukey box plot. Boxes show the lower and upper inter-quartile. Horizontal lines indicate the median and whiskers show a maximum of 1.5 IQR. (**F**) IHC staining of Ki67 was performed in murine prostate and of Ki67 and CD31 in C4-2 tumor tissue. Representative images are shown. Ki67 staining of (**C**) murine prostate and (**D**) tumor tissue was quantified by Image J software. Obtained values were plotted representing the relative level of Ki67 expression per cell nucleus analyzed, noted as relative units (r.u.). (20 images were analyzed per tissue and treatment; murine prostate: p=0.80, tumor tissue p=0.17). Error bars indicate SEM.

4.9 AA treatment decreases PSA expression *in vivo*

The effects of AA treatment on AR signaling in human C4-2 tumors were further assessed by qRT-PCR and WB. As indicated before an anti-androgenic activity of a certain substance can be achieved by destabilization of AR protein. The treatment of C4-2 cells with AA did not cause further degradation of AR protein (**Figure 9**). In accordance with the *in vitro* data, AA treatment did not influence the AR protein stability *in vivo* (**Figure 11 D**) even though a reduced mRNA expression was detected in the presence of the anti-androgen (**Figure 11 A**). In contrast, the expression of the positively regulated AR target gene PSA was reduced on protein level (**Figure 11 D**) but not on mRNA level (**Figure 11 B**). Additionally, upon AA treatment TERT mRNA expression was marginally reduced (**Figure 11 C**). Bearing in mind that these mice were intact, hence produce endogenous androgens, which may already induce low levels of TERT expression. These results indicate that AA in part induces different functional effects *in vivo* and *in vitro*, which may reflect the interplay with the whole organism and the diverging duration of the treatment period, which is maximal three days *in vitro* but almost four weeks *in vivo*. Furthermore, the AA treatment induces changes on mRNA and protein level of AR targets, which do not necessarily have to be linked with each other.

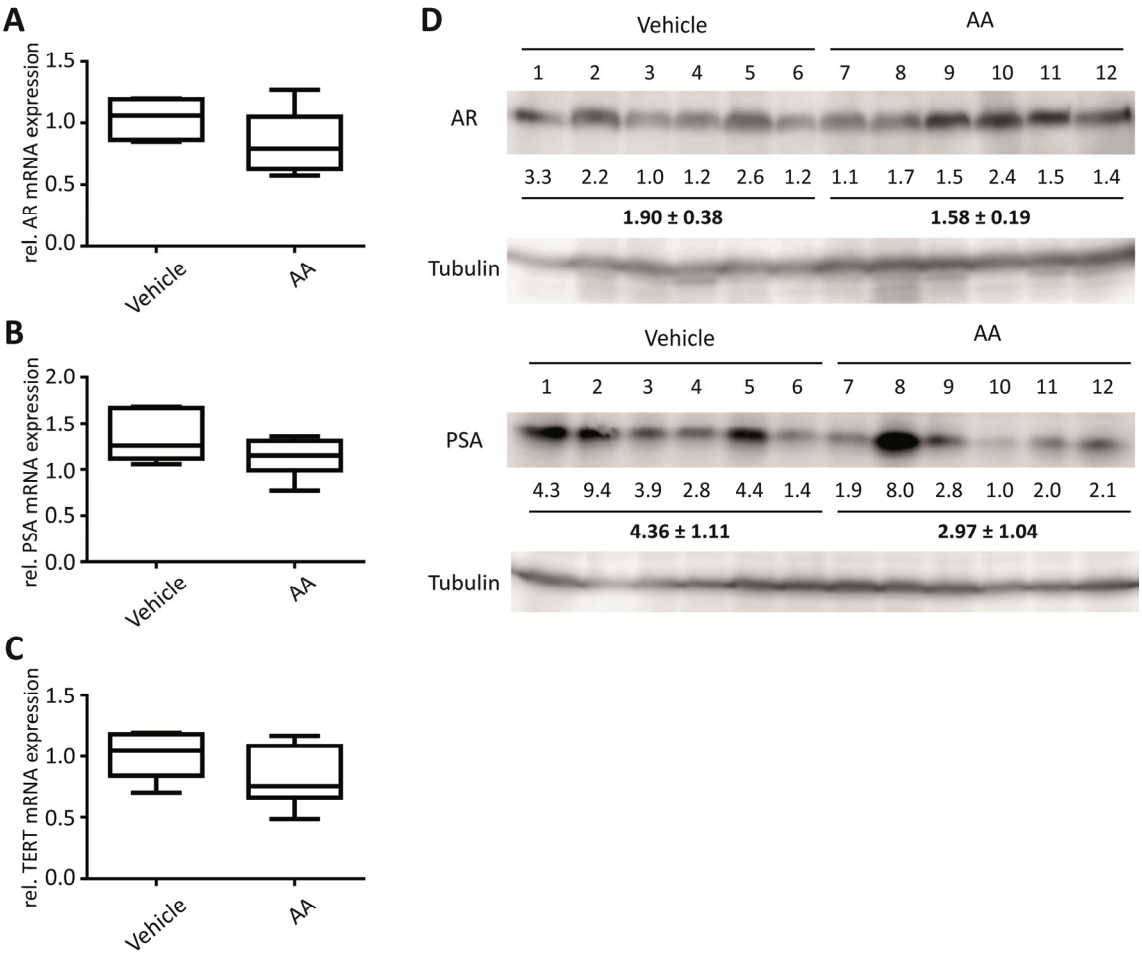


Figure 11: Effects of AA treatment on AR, PSA and TERT expression in C4-2 tumors. Male nude mice were treated as indicated (see **Figure 10**). After mice were sacrificed C4-2 PCa tumors were isolated and tissues were employed for mRNA and protein extraction. qRT-PCR was performed for (**A**) AR, (**B**) PSA, (**C**) TERT, β-Actin and RPL13a. The latter ones were used for normalization. The two treatment groups (vehicle: n=6, AA: n=8) were plotted as Tukey box plots whereat outliers were excluded (for further information see **Figure 10**, p-values see **Table 5**). (**D**) WB was conducted for AR and PSA protein. Protein bands were quantified by LabImage D1 - protein expression was normalized to β-tubulin. (For protein band size see **Table 2**.) Relative intensities of protein bands are presented as numbers below the blot. The sample with the weakest protein expression was arbitrarily set as 1. Each group (vehicle and AA, n=6) was averaged. Mean ± SEM are indicated. (AR protein: p=0.94; PSA protein: p=0.18)

4.10 AA treatment modifies the p16-pRB axis *in vivo*

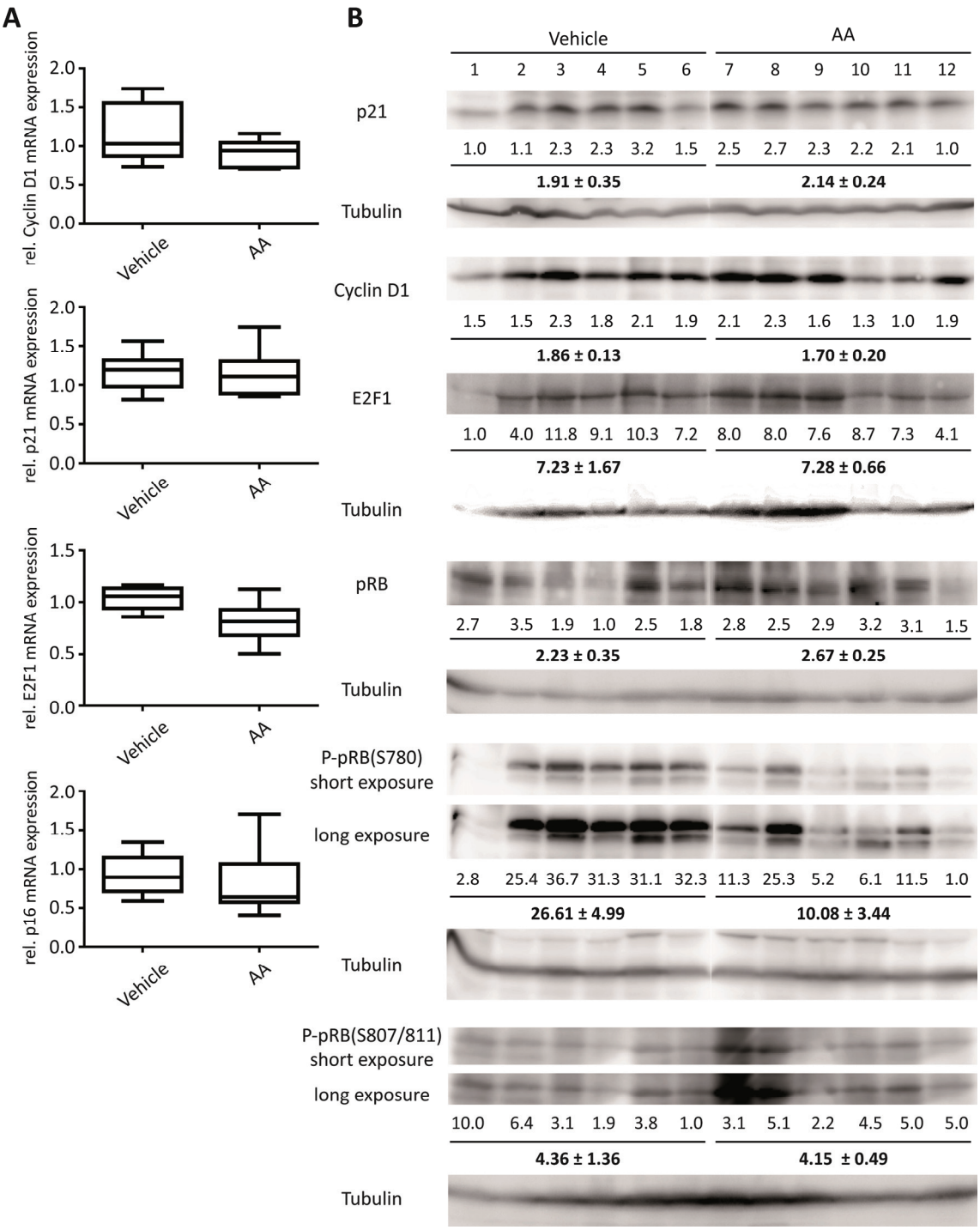
The treatment of xenografted nude mice with AA caused tumor growth regression. However, which pathways mediate this effect needs to be clarified, therefore, cell cycle factors were analyzed. Cyclin D1 expression as an important cell cycle regulator was reduced upon AA treatment in the presence of DHT in C4-2 cells in cell culture (**Figure 9**). Hence, Cyclin D1 expression was analyzed in the PCa tissue derived from

xenografted intact male nude mice. The expression of Cyclin D1 mRNA was neither changed on mRNA (**Figure 12 A**) nor on protein level *in vivo* (**Figure 12 B**). p21 expression, as an additional cell cycle modulator, whose expression was not changed by AA treatment in C4-2 cells *in vitro*, was unaffected by AA treatment *in vivo*, as well (**Figure 12 A,B**). Furthermore, the *in vitro* data indicated that in the presence of AA the p16-pRB pathway is modulated, hence E2F1, p16 and pRB expression were analyzed. E2F1 protein, which acts as a transcription factor, is bound and inactivated by pRB in a cell cycle-dependent manner (Nevins, 1998). Its expression was significantly reduced on mRNA level in AA-treated C4-2 PCa tumors compared to vehicle-treated animals (**Figure 12 A**). This effect was not passed on protein level (**Figure 12 B**). p16 mRNA expression, which was induced by DHT and AA *in vitro*, was not changed *in vivo* (**Figure 12 A**). pRB whole protein expression was also not modified by AA treatment, however, its phosphorylation was (**Figure 12 B**). S780 is among the phosphorylation sites of pRB which are initially phosphorylated by Cyclin D1-CDK4, and inhibit the binding to E2F1 (Kitagawa *et al.,* 1996). Indeed, P-pRB (S780) was significantly reduced in the presence of AA. In contrast, the phosphorylation at S807/811, which may regulate phosphorylation of other sites but is not directly involved in inhibition of E2F1 binding (Rubin, 2013), is not modulated comparing vehicle and AA group (**Figure 12 B, Table 4**).

Therefore, these results indicate that, as shown *in vitro*, the expression of cell cycle modulators is changed *in vivo*. Thereby, the most intriguing effect is the reduction of pRB phosphorylation at S780 upon AA treatment.

Another pathway how AA treatment may reduce tumor growth could involve the induction of apoptosis. Papaioannou*et al.* (2009) reported that there is no detectable induction of apoptosis upon AA treatment *in vitro*. A marker of cells undergoing apoptosis is PARP cleavage, which primarily is mediated by caspases (Kaufmann *et al.,* 1993). Furthermore, PARP plays a role in DNA repair and its cleavage has been implicated in the inactivation of this activity coinciding with the induction of apoptosis (D'Amours *et al.,* 2001). PARP expression is marginally induced in C4-2 tumor tissues derived from AA-treated mice, whereas the level of cleaved PARP is similar in the two treatment groups (**Figure 12 C**). However, there is no change of the PARP to cleaved PARP ratio detected in the AA group compared to control (**Table 4**). These data suggest that there is no change of the rate of PARP cleavage-mediated apoptosis due to AA

treatment. These data are in accordance with the *in vitro* data published by Papaioannou *et al.* (2009).



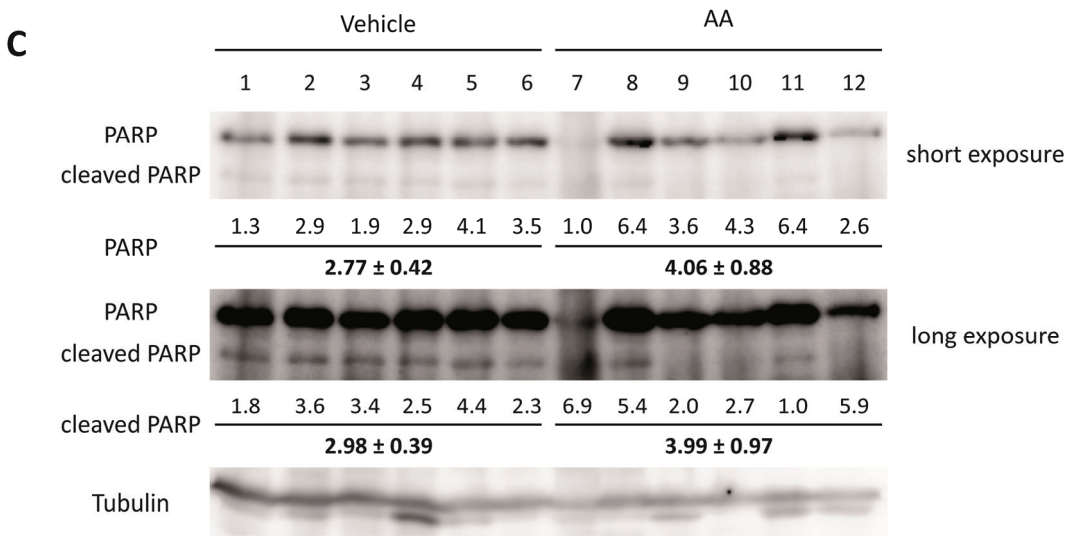


Figure 12: Effects of AA treatment on the expression of cell cycle modulators and PARP in C4-2 tumors. Male nude mice were treated as indicated (see **Figure 10**). After mice were sacrificed C4-2 PCa tumors were isolated and tissues were employed for mRNA and protein extraction. (**A**) qRT-PCR was performed for Cyclin D1, p21, E2F1, p16, β-Actin and RPL13a. The latter ones were used for normalization. The two treatment groups (vehicle: n=6, AA: n=8) were plotted as Tukey box plots whereat outliers were excluded (for further information see **Figure 10**, p-values see **Table 5**). (**B**) WB was conducted for p21, Cyclin D1, E2F1, whole (RB) and phosphorylated pRB (P-pRB) and (**C**) PARP and cleaved PARP. P-pRB(S780), P-pRB(S807/811) and PARP-cleaved PARP signals are shown in short and long exposure (n=6). Protein bands were quantified by LabImage D1 - protein expression was normalized to β-tubulin. For P-pRB the upper most band was quantified. Relative intensities of protein bands are presented as numbers below the blot. The sample with the weakest protein expression was arbitrarily set as 1. Each group (vehicle and AA) was averaged. Mean ± SEM are indicated. (p-values for protein expression: p21: p=0.7; Cyclin D1: p=0.7; E2F1: p=0.94, pRB: p=0.31; P-pRB(S780): p=0.04; P-pRB(S807/811): p=0.82; PARP: p=0.21; cleaved PARP: p=0.36).

Table 4: Ratio of protein expression of pRB and PARP.

Protein ratio	vehicle	AA	p-value
pRB/P-pRB(S780)	0.2198 ± 0.1489	0.5336 ± 0.2082	0.0649
pRB/P-pRB(S807/811)	0.7356 ± 0.2161	0.7246 ± 0.1434	0.8182
PARP/cleaved PARP	0.9524 ± 0.1360	1.926 ± 0.9385	0.4848

Protein ratios of pRB to phosphorylated RB protein (P-pRB) and PARP to cleaved PARP are indicated. Mean ± SEM of n=6 is shown.

4.11 AA affects the expression of different AR target genes

The AR as a transcription factor regulates the expression of a great variety of genes. Some of those are regulated directly by the AR others indirectly, some are activated others are repressed. Also, the processes AR target genes are involved in are of great variety. All together the AR has a wide spectrum of targets. In order to get a hint of the effects of AA treatment *in vivo* a selection of AR targets was chosen and analyzed by qRT-PCR, thereby, primary focus was on genes, which are mediators of cell cycle progression, and have been shown to influence metastatic behavior of PCa cells.

Table 5: Summary of data analyses of changes in mRNA expression of selected AR target genes upon AA treatment *in vivo*.

Gene product	Role in PCa linked to AR signaling	Reference	rel. mRNA expression± SEM (p-value)
AR	growth promoting	Debes & Tindall, 2002; Yuan *et al.,* 2013	vehicle: 1.040± 0.067 AA: 0.841 ± 0.085 (0.059)
CCL2	recruitment of monocytes, T- and dendritic cells, metastasis	Lin *et al.,* 2013a	vehicle: 0.303±0.058 AA: 0.415±0.195 (0.573)
Cyclin D1	cell cycle progression	Xu *et al.,* 2006	vehicle:1.158±0.155 AA: 0.913±0.059 (0.282)
E2F1	cell cycle progression	Davis *et al.,* 2006	vehicle: 1.037±0.047 AA: 0.802±0.066 (0.020) *
FKBP5	increases AR transcriptional activity	Febbo *et al.,* 2005	vehicle: 1.180±0.039 AA: 1.019 ±0.074 (0.081)
Maspin	inhibition of tumor cell invasion	He *et al.,* 2005	vehicle: 0.650±0.097 AA: 0.662 ±0.171 (0.662)
MED1	coactivator	Jin *et al.,* 2013	vehicle: 1.278±0.083 AA: 1.130±0.065 (0.181)
MMP9	PCa cell invasion	Lin *et al.,* 2013b	vehicle:0.937 ±0.074 AA: 0.988 ±0.159 (1.000)

Gene product	Role in PCa linked to AR signaling	Reference	rel. mRNA expression± SEM (p-value)
Nov/CCN3	regulator of cell differentiation	Wu *et al.,* 2013	vehicle: 0.720±0.215 AA: 0.967 ±0.097 (0.142)
p16^{INK4a}	inhibits cell cycle progression	Lu *et al.,* 1997	vehicle:0.926±0.107 AA: 0.830 ±0.148 (0.491)
p21$^{CIP1/WAF1}$	progression to CRPC	Fizazi *et al.,* 2002	vehicle:1.171±0.102 AA: 1.152±0.106 (1.000)
PSA	level elevated in PCa, proteolytic activity	Kim & Coetzee 2004	vehicle: 1.342±0.112 AA: 1.123±0.0702 (0.228)
Sox2	maintaining pluripotency, preventing differentiation	Kregel *et al.,* 2013	vehicle:0.738 ±0.236 AA: 0.665±0.173 (0.755)
Sox9	PCa development	Cai *et al.,* 2013a	vehicle: 1.083±0.147 AA: 1.043±0.090 (0.852)
TERT	regulates telomerase activity, induction of growth factor signaling	Guo *et al.,* 2003; Moehren *et al.,* 2008; Soda *et al.,* 2000; Tang *et al.,* 2008	vehicle: 1.009±0.082 AA: 0.829±0.084 (0.181)
TMPRSS	TMPRSS:ERG gene fusion, PCa progression	Cai *et al.,* 2009	vehicle: 1.133±0.128 AA: 1.124±0.135 (0.852)

Experiments were conducted as described (see **Figure 10**).The expression of various known AR target genes was analyzed by qRT-PCR. Genes were listed alphabetically. Relative gene expression of AR-regulated genes is indicated per treatment group (vehicle: n=6, AA: n=8). p-values were obtained upon Mann-Whitney-U test, which was done for mRNA expression of the normalized gene of interest comparing vehicle and AA group. (* $p<0.05$ statistically significant)

The cell cycle modulators have already been discussed (see 4.10) as well as PSA, TERT and AR itself (see 4.9). FKBP5 represents another positively regulated AR target gene, which seems to be the most stably induced one in different PCa cell lines. This protein was reported to increase AR-mediated transactivation (Febbo *et al.,* 2005). Interestingly, the treatment of xenografted nude mice with AA decreased the expression of FKBP5 mRNA in C4-2 tumors (**Table 5**). Similarly, MED1, which acts as an

AR CoA and plays an oncogenic role (Jin *et al.,* 2013), was marginally repressed upon AA treatment *in vivo* (**Table 5**). Another gene, which tends to be regulated comparing vehicle and AA treatment group, is Nov/CCN3. Nov is a critical regulator of cell differentiation (McCallum & Irvine, 2009) and there was an up-regulation of Nov/CCN3 in the presence of AA (**Table 5**). Other genes analyzed in the present study do not show differences in mRNA expression level comparing vehicle and AA group (**Table 5**). Changes in MMP9 and CCL2 expression have been linked to cell invasion both of which were reported to be induced by MDV3100 or Casodex (Lin *et al.,* 2013a;b). Interestingly, there was no induction on mRNA level by AA maybe pointing to an advantageous effect of AA compared to clinically used anti-androgens.

Taken together these results indicate that AA treatment modifies the mRNA expression of distinct AR target genes in CRPC xenografts *in vivo*, thereby may providing an environment which reduces tumor growth.

5 Discussion

A primary target of PCa therapy is the AR. However, therapeutic approaches currently used fail after approximately two years of treatment, hence it is essential to find new therapies providing beneficial effects on survival of PCa patients.

5.1 The ambiguity of AR-mediated TERT gene regulation

TERT represents the catalytic subunit of the telomerase holoenzyme (Nakamura *et al.,* 1997) and its expression correlates with growth factor-induced tumor progression (Jin *et al.,* 2010; Tang *et al.,* 2008). Growth factor pathways have been implicated as a therapeutic approach of PCa therapy as well as AR signaling has (Bellmunt & Oh, 2010). Hence, understanding AR-mediated TERT regulation may be a promising therapeutic approach to treat PCa. However, the regulative role of AR and androgens on TERT gene expression is controversially discussed. Furthermore, TERT is inconsistently regulated in LNCaP and C4-2 cells depending on cell context, ligand and ligand concentration as shown herein.

It has been shown that the AR is recruited to two sites of the TERT promoter, the distal -4 kb region and the proximal -0.1 kb region (Moehren *et al.,* 2008). These regions provide differential AR-mediated TERT promoter regulation.

It has been shown that the DBD of the AR directly interacts with, and positively regulates the distal TERT promoter (Diploma thesis Bartsch, 2011; Diploma thesis Jatzkowski, 2010; Bachelor thesis Schreiber, 2011). Thereby, the distal TERT promoter itself (TERT-3988/-3752) possesses a strong repressive activity independent of AR protein, which is released in the presence of androgen-bound AR. In contrast, as shown herein, the proximal TERT promoter holds a strong activating potential, which can be repressed by the androgen-stimulated AR. Additionally, the mutation of the AR protein does not necessarily influence the course of androgen-mediated TERT gene regulation, as it has been described previously (Moehren *et al.,* 2008). In the present study it was demonstrated that both ARwt and AR-T877A are able to mediate TERT gene repression. Thereby, ARwt is more efficient than its mutant counterpart. However, TERT gene repression by AR strongly depends on androgen concentration, promoter and cellular context.

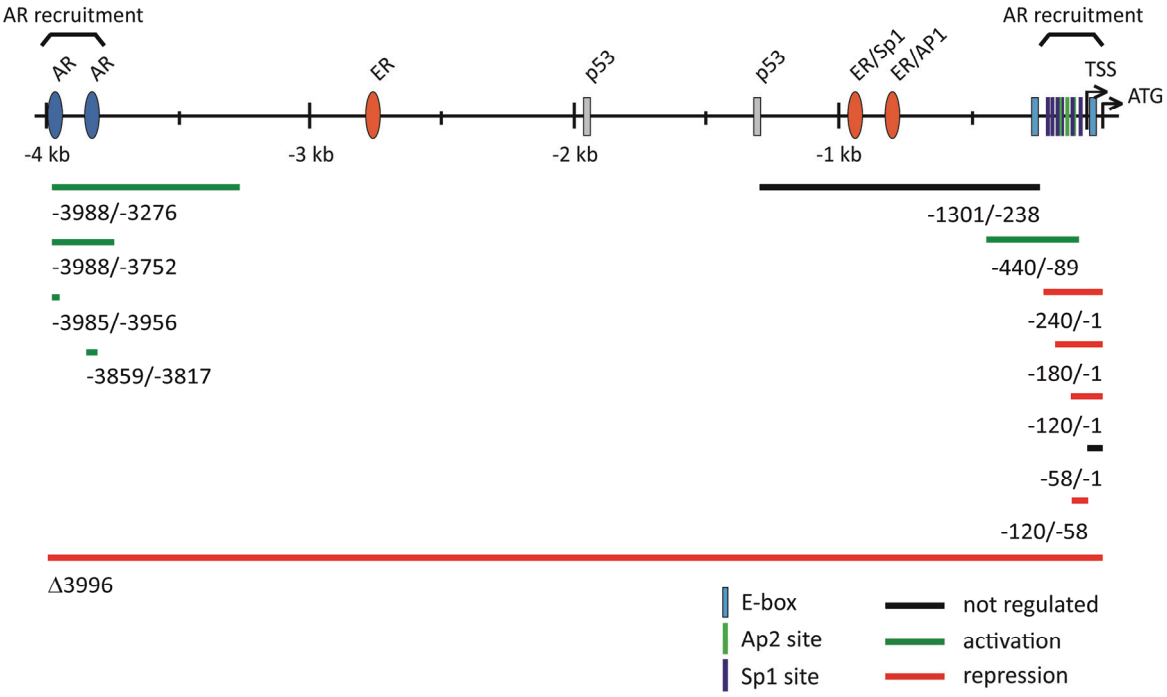


Figure 13: Schematic diagram of the TERT gene promoter and promoter constructs. The transcription start site (TSS) and the translation start site (ATG) are shown. In line with previous publications the adenine of the initiating ATG is denoted as position +1. Transcription factor binding sites are indicated as geometrical figures (reviewed by Poole *et al.,* 2001). AREs (green ellipse) were identified by A. Jatzkowzki (Diploma thesis, 2010). Sites of AR chromatin recruitment are shown (identified by Moehren *et al.,* 2008). Promoter fragments, which were employed in functional analysis, are represented below the TERT promoter. The course of AR-dependent androgen-induced promoter regulation is indicated by the color scheme. The activity of the promoter fragments refers to the natural sense orientation. The labeling of the promoter deletions denotes the position of the fragment within the promoter relative to the ATG. Androgen responsiveness of TERTΔ3996 was first documented by U. Moehren (PhD thesis, 2004). Androgen responsiveness of TERT-3988/-3276, TERT-3988/-3752 and TERT-3985/-3956 were first documented by S. Bartsch (Diploma thesis, 2011); of TERT-3859/-3817 was first documented by M. Schreiber (Bachelor thesis, 2011). The figure was adapted from S. Bartsch (Diploma thesis, 2011).

AR mediates TERT promoter repression through the proximal promoter (TERT-240/-1), which was observed even in the absence of hormone, and is enhanced upon androgen binding. The minimal repressive sequence of AR-mediated TERT repression could be narrowed down to a region spanning the first -120 to -58 bp upstream of the TLS of the TERT gene (**Figure 13**). This minimal sequence is repressed to a similar degree as full-length TERT promoter in the presence of AR and R1881 10 nM, however, at lower androgen concentrations (0.1 nM) the repression is weakened, which is not observed using the full-length promoter. These results indicate that surrounding DNA sequences seem to influence AR-mediated repression.

Interestingly, the repression is orientation-dependent since the TERT-240/-1 construct in anti-sense orientation resulted in an activation of reporter gene expression in the presence of androgen-stimulated ARwt, and no change of promoter activity if AR-T877A was present. This effect was maintained if the TERT-180/-1 construct was employed in anti-sense orientation for reporter gene assay, but was abolished if further shortened TERT promoter deletions were used. These results strengthen the stronger impact of ARwt on TERT promoter activity compared to the mutant AR and a determinant role of promoter context. Furthermore, ARwt repressed the TERT-440/-89 sense promoter construct in the absence of hormone. This repression was released if R1881 was added. In this context there was no change of promoter activity for AR-T877A independent of hormone. Thus, there seems to be an AR-responsive site located between -120 and -89 bp upstream of the TLS mediating androgen-dependent TERT repression.

As indicated, the experimental setup used herein allowed to functionally analyze an orientation-dependent regulation. Indeed the androgen response varied depending on promoter construct orientation. In general, the promoter is tightly packed with a variety of transcription factor binding sites (Kyo *et al.,* 2008; Poole *et al.,* 2001). Furthermore, in anti-sense orientation the minimal AR-responsive site was set into closer proximity of the TLS of the reporter gene. Activating transcription factors, which have been described to interact with the TERT promoter at position -120 to -89, include AP2, Sp1 and MAZ (Poole *et al.,* 2001). Previously, it has been published that Sp1 and MAZ play a role in AR-mediated gene regulation (Jiao *et al.,* 2013; Lu *et al.,* 2000). An AR-Sp1 interaction has been linked to gene repression in PCa cells by He *et al.* (2005). MAZ was reported to be up-regulated in PCa specimens being involved in cell proliferation and migration (Jiao *et al.,* 2013). Hence, an interaction of these transcription factors with the AR may account for the modulation of TERT promoter activity. Thereby, the positioning of the factors relative to the TLS is essential in determining absolute promoter activity as reported elsewhere (Ramirez-carrozzi & Kerppola, 2003). Therefore, AR-mediated TERT regulation likely depends on available co-factors as well as accessible binding sites, since specific regions can be blocked by histone modifications or DNA methylation. This complex interplay seems to provide information of androgen-mediated TERT activation or repression.

Interestingly, a similar promoter structure has been described for the PSA gene. PSA is one of the best studied positively regulated AR target genes. It has been published that PSA regulation by the AR is mediated by proximal promoter regions and an enhancer, which is located approximately 4 kb upstream. Thereby, the strong enhancer was described to be required for the high androgen-regulated expression of the target gene (Cleutjens, 1997). The -4 kb region of the TERT promoter may provide a similar function as the AR-responsive enhancer of the PSA gene. Binding of the AR to the distal androgen-responsive region may cause local modifications of chromatin by recruitment of HATs and CoAs inducing an interaction of the -4 kb region with the proximal promoter and driving gene transcription as reported for the PSA gene (Shang *et al.,* 2002; Wang *et al.,* 2005). AR recruitment to specific promoter sites can be related to histone modifications as discussed by Kim & Coetzee (2004). However, as indicated in the present study PSA gene expression is fairly stable in three related PCa cell lines expressing AR-T877A. Thereby, increasing androgen concentrations induce increasing PSA expression independent of the androgen applied. In contrast, TERT expression is induced by DHT at moderate concentrations, and marginally repressed at higher concentrations depending on the cellular context. Similar results were obtained for R1881, however, R1881 at high concentrations induced a more pronounced repression at least in LNCaP S1 cells. It has been published that the LBP is composed of hydrophobic and polar amino acids, which adapt to variable positions specific for the distinct agonist present. Thereby, the ligand structure determines the number of contacts made with the LBD and even minor alterations have a great impact on the strength of the interactions and the activity of the receptor (Pereira de Jésus-Tran *et al.,* 2006). In this context, it has been published that the two androgens induce slightly different receptor dynamics (Klokk *et al.,* 2007). Receptor dynamics correlate with the residence on chromatin, which may cause the differences observed herein.

As indicated in the present and previous studies increasing androgen concentrations impact differentially on cell growth and target gene expression (Culig *et al.,* 2002). A possible explanation may be the involvement of rapid signaling. Rapid signaling pathways are triggered by AR signaling and modify AR-mediated gene regulation. Their induction depends on androgen concentration (Lyng *et al.,* 2000). Varying androgen concentrations may also affect AR protein modifications. The binding of androgens

affects AR phosphorylation and acetylation, which in turn modify receptor activity (Fu *et al.,* 2003; Gioeli *et al.,* 2006; Yang *et al.,* 2007).

Interestingly, the androgen concentration-dependent induction of TERT expression observed in LNCaP and C4-2 cell lines was not found in reporter gene assays regarding TERT promoter activity. These effects indicate that the cell environment including the natural promoter context predicts the outcome of AR-mediated TERT regulation.

Taken together, the androgen-regulated expression of the TERT promoter depends on the characteristics of the cell line. This may include the level of AR expression and the presence of AR mutations as well as the level of co-factors, other transcription factors interacting with the promoter, DNA methylation and histone modifications.

Noteworthy, R1881 and DHT in part induce different effects, which may be also attributed to the metabolization of DHT. Metabolites of natural androgens can stimulate ERβ, which in turn is an activator of TERT gene expression (Calado *et al.,* 2009; Doering & Gladue, 1982; Kondoh *et al.,* 2007). The ERβ is expressed by LNCaP cells (Lau *et al.,* 2000) hence overlaying effects can occur. This effect may also account for the marginal up-regulation of TERT expression seen in PCa tissues *ex vivo*. Anyhow, the synthetic androgen R1881 and the antagonist AA tend to repress TERT expression in this context.

As indicated above, AA represses AR transactivation of the MMTV promoter. This repressive activity is even stronger if MDV3100 is applied. MDV3100, in contrast to AA, completely blocks androgen-induced transactivation of the MMTV promoter. However, in the context of the natural occurring promoter section TERT-3988/-3752 the effect of AA and MDV3100 treatment on AR-mediated promoter activation was similar. Furthermore, as indicated by the reporter gene assays conducted herein, AA represses AR-mediated transactivation but permits promoter repression.

TERT promoter activity was also analyzed under combinatorial treatment. Thereby, ratios of androgens to anti-androgens were applied, which were published to be most effective (Papaioannou *et al.,* 2009; Tran *et al.,* 2010). Since the solubility of the antagonists is restricted, the androgen concentrations had to be reduced. Therefore, androgen concentrations close to physiological levels were applied (Taieb *et al.,* 2003). Interestingly, at these androgen concentrations full-length TERT promoter was still repressed in reporter gene assays. In contrast, for the minimal repressive region

TERT-120/-58 repression was almost completely lost at an R1881 concentration of 0.1 nM, further suggesting an involvement of surrounding promoter regions. The AR may need stabilizing factors at the proximal promoter region to induce target gene repression, since the recruitment of the AR to the -0.1 kb region is already weaker than to the -4 kb region (Moehren *et al.,* 2008).

The co-treatment of either androgen plus AA induced a two-fold repression of the minimal repressive region. At the indicated concentrations there was an induction of the TERT-58/-1 promoter region in the presence of DHT but not R1881, which was sustained by co-treatment with MDV3100 but not AA. Hence, AA and MDV3100 seem to exert different effects on TERT promoter regulation.

It has been shown previously that different AR ligands induce differential conformations of the receptor (Kuil *et al.,* 1995). Ligand binding specifically alters the receptor conformation, chaperone composition and protein interactions (Bohl *et al.,* 2005; Osguthorpe & Hagler, 2011; Zhou *et al.,* 2010). These changes also influence AR acetylation and phosphorylation, which in turn results in differential regulation by different antagonists (Fu *et al.,* 2003; Gioeli *et al.,* 2006; Yang *et al.,* 2007).

Taken together, there are two AR-responsive regions within the TERT promoter. The proximal promoter region bears a site, which is repressed upon androgen and/or anti-androgen treatment, whereas the distal site is stimulated by androgen-bound AR. Both effects can be mediated by ARwt and AR-T877A in an androgen concentration-dependent manner. Thereby, it is likely that other factors influence the outcome of AR-mediated TERT regulation e.g. the availability of co-factors and chromatin status. Furthermore, the presence of an insulating region between both sites of AR recruitment may be possible.

The data suggest a hypothetical model of differential AR-mediated TERT gene regulation: In the normal prostate the AR may be recruited to the proximal TERT promoter upon androgen-binding. This event may be primed by or associated with the binding of other factors. The binding of AR induces the recruitment of further factors, which cause a repression of TERT gene expression (**Figure 14**). However, during PCa development AR signaling is disturbed and the cell environment changes including the availability of co-factors and the chromatin status. Hence, during PCa progression the DNA at the distal TERT promoter may be de-methylated or histones acetylated as a

licensing step of AR-binding. The androgen-stimulated AR binds to the AREs within the -4 kb region and to the proximal AR-responsive sites of the TERT promoter. CoAs and other factors are recruited to both sites inducing a chromosomal loop driving gene expression (**Figure 14**). Thereby, imbalances in AR signaling, e.g. by receptor amplification or mutation, may further support TERT gene expression by activated AR.

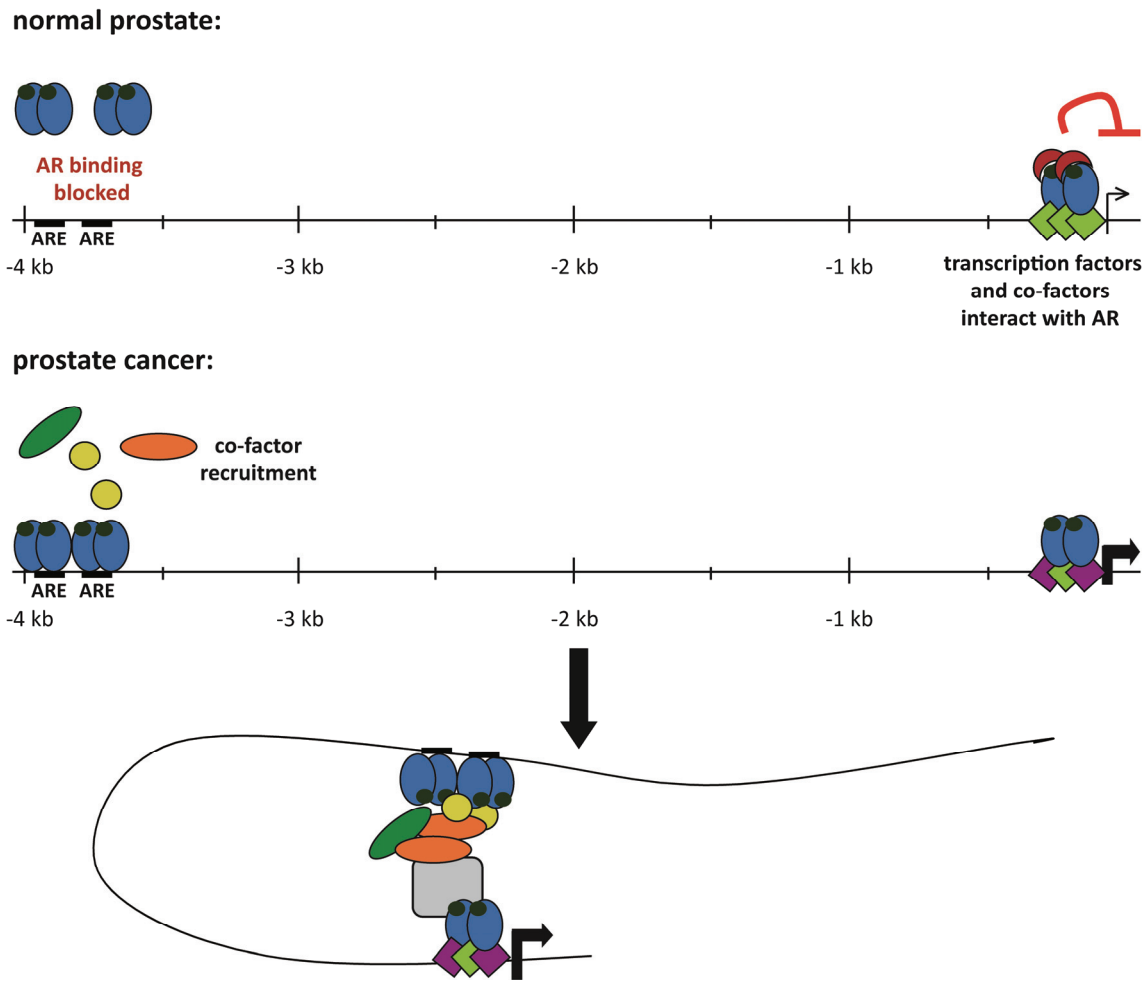


Figure 14: Model of AR-mediated TERT gene regulation. In the normal prostate agonist-bound AR (blue ellipse) is recruited to the proximal TERT promoter. Thereby, the receptor directly or indirectly interacts with the DNA. This event may be primed and/or followed by the recruitment of further factors (geometrical figures) contributing to a repressive complex. The binding of AR to the distal TERT promoter is blocked, e.g. by chromatin structure or DNA methylation. During PCa development chromatin structure and AR signaling are changed. The receptor is recruited to both the proximal and the distal TERT promoter region upon androgen binding. This is followed by a coordinated and ordered recruitment of further factors inducing the interaction of both TERT promoter sites allowing TERT gene transcription.

5.2 AA influences the pRB-p16 signaling axis *in vitro*

The gold standard of PCa therapy is ADT. After first being successful, the tumor recurs and CRPC develops. New antiandrogens have been developed, e.g. MDV3100, to treat this more aggressive state of the cancer, however, the overall survival advantage is still modest (Yuan *et al.,* 2013). Hence, to build on this progress of PCa therapy it is essential to understand the mechanisms accounting for anti-androgenic activity and to develop strategies to overcome tumor growth progression. It has been shown previously that AA inhibits the agonist-induced AR transactivation coinciding with suppression of target gene expression. Furthermore, it inhibits proliferation of androgen-dependent and -independent growing PCa cells and reduces cell invasion (Papaioannou *et al.,* 2009). It decelerates agonist-induced AR nuclear translocation, prevents AR N/C interaction, inhibits DNA-binding of AR and induces cellular senescence (PhD theses Hessenkemper, 2013; PhD thesis Roediger, 2012). In the present study it could be demonstrated that the anti-androgen seems to mediate at least some of its growth repressing effects via the pRB pathway. The treatment of the androgen-independent growing C4-2 cells with AA resulted in a decreased protein expression of Cyclin D1 coinciding with an induced expression of p16 mRNA and a strong de-phosphorylation of pRB *in vitro*.

Cyclin D1 mRNA expression was hardly influenced by AA treatment, however, protein expression was. This effect may account for post-transcriptional regulation of Cyclin D1 by AR as published recently (Xu *et al.,* 2006). Another possible explanation could involve 4E-BP, which was described as key regulator of Cyclin D1. Thereby, 4E-BP if hypophosphorylated binds to eIF4E. eIF4E is a component of the mRNA capping complex promoting translation (Averous *et al.,* 2008). Different studies demonstrated that AR signaling can regulate 4E-BP phosphorylation, coinciding with its inactivation (Soifer *et al.,* 2012; Wu *et al.,* 2010). Interestingly, 4E-BP phosphorylation is frequently up-regulated in PCa specimens (Graff *et al.,* 2009).

AA exerted different effects depending on the androgen, which was used as co-treatment. It is suggested that the AR adapts to the structure of its specific agonist. R1881 was reported to built more contacts with the receptor than the endogenous androgen DHT (Pereira de Jésus-Tran *et al.*, 2006). Thereby, R1881 may has a higher

receptor affinity, is relative resistant to metabolism, and shows a low non-specific binding to proteins other than the AR (Brown *et al.*, 1981; Pereira de Jésus-Tran *et al.*, 2006). Since DHT binds weaker and is metabolized, higher concentrations were applied compared to R1881. The R1881-activated AR induces PSA expression, as well as the DHT-stimulated receptor. In the presence of R1881 and AA PSA expression is repressed. The reduced PSA expression may be caused by attenuated nuclear translocation of the receptor in the presence of AA. Furthermore, it is likely that less AR is recruited to the target gene promoter as suggested by ChIP-data of W. Hessenkemper (PhD thesis, 2013). However, if cells were co-treated with DHT and AA PSA expression was not reduced compared to DHT treatment alone. Brown *et al.* (1981) reported that the R1881-AR complex, despite the higher receptor affinity, is less stable than the DHT-AR complex, which may explain the effect observed herein.

Interestingly, PSA expression did not correlate with the decreased AR expression in C4-2 cells upon androgen treatment. This may be due to a delay of AR protein degradation (measured after 72 h) compared to PSA gene expression (measured after 24 h). Another explanation could be that there is still enough functional AR protein present for the induction of PSA expression.

An androgen-induced attenuation of AR expression was published previously. Two studies described that in LNCaP cells AR mRNA expression is reduced upon androgen treatment (Wolf *et al.,* 1993; Yeap *et al.,* 1999). Another group saw a marginal decrease of AR protein upon androgen treatment in LNCaP cells (Bouchal *et al.,* 2002). Nevertheless, post-transcriptional regulation of AR protein in C4-2 cells may be different to LNCaP cells since C4-2 cells adapted to androgen-independent growth conditions. Hence, their AR signaling might be active even at very low androgen concentrations. On the one hand, there could be a threshold level of activated AR protein, which is hold by receptor degradation. On the other hand, mutations of the NTD of the receptor may account for differences in androgen dissociation and AR degradation rates (Zhou *et al.,* 1995). The occurrence of further AR mutations in C4-2 cells cannot be excluded during cell cultivation.

AA did not influence the AR protein level in the presence of DHT. Nevertheless, a stabilization of AR was verified under co-treatment with AA and R1881, which, if the

previous assumption is correct, may imply a disadvantageous effect for the cell since signaling processes may be oversteered.

AA treatment had no detectable impact on p21 expression. Even though, p21 was reported to be positively regulated by androgens in LNCaP cells (Lu *et al.,* 2000). In C4-2 cells androgen treatment reduced p21 on protein but not mRNA level, further underlining differential processes in LNCaP and C4-2 cells. Additionally, TERT, as a growth promoting factor, stayed at low mRNA expression level in the presence of AA.

Taken together, AA treatment seems to modify the p16-pRB signaling axis, which in turn may be related to growth inhibition of androgen-independent C4-2 cells. Furthermore, AA treatment causes gene and protein specific alterations while no extensive reversal of all AR-mediated processes was observed. The overall data suggest that AA exerts its effects only in part on transcriptional level. Additionally, AA treatment seems to account for alterations of signaling cascades.

5.3 AA reduces PCa growth *in vivo*

The effect of AA was analyzed via human tumor xenografts. This model system allows to predict the drug response of a tumor in a human patient (Richmond & Su, 2008). Since an actual human tumor is observed molecular human PCa markers can be analyzed, which are not endogenously expressed by mice, e.g. PSA.

AA significantly reduced PCa growth of androgen-independent tumors *in vivo* coinciding with reduced Ki-67 expression. Thereby, no side effects were observed so far.

As for the cell culture data, target gene expression was not always consistent at protein and mRNA level. AR expression, for instance, was not changed at protein but it was decreased at mRNA level. Hence, there seem to be some post-transcriptional effects maintaining AR protein level. PSA protein was decreased upon AA treatment *in vivo* indicating that the AA concentration was suited to repress AR activity. TERT mRNA level was marginally reduced in the C4-2 tumor xenografts. *In vitro* AA had no effect on androgen-mediated TERT gene regulation. However, this may also be caused by differences in androgen concentration in the two experimental setups, as we already could see that TERT gene regulation is quite androgen concentration-dependent. TERT

down-regulation is favorable as it coincides with reduced telomerase activity and has an impact on growth factor-promoted tumor cell proliferation (Jin *et al.,* 2010; Tang *et al.,* 2008).

A further pathway, which may be involved in reduced cell proliferation of C4-2 tumors includes pRB signaling. As described for the C4-2 cells *in vitro*, a significant reduction of pRB phosphorylation at S780 was verified *in vivo* upon AA treatment. Thereby, the overall RB protein level was not changed neither was pRB S807/811 phosphorylation. The phosphorylation of the S780 site of pRB is mediated by the Cyclin D1-Cdk4/6 complex. This modification represses the binding of pRB to E2F1, and therefore allows E2F1 target gene expression (Kitagawa *et al.,* 1996). In contrast, S807/811 was reported to may prime the phosphorylation at other sites within the RB protein and does not interfere with pRB-E2F1 interaction (Rubin, 2013). E2F1 protein expression was not changed upon AA treatment. The dephosphorylation of pRB at S780 most likely enhances the interaction of pRB and E2F1. Since E2F proteins function as transcription factors primarily modulating gene expression of proteins involved in cell cycle progression, growth promoting factors may be reduced in AA-treated C4-2 tumors. This hypothesis may be encouraged by the significant E2F1 mRNA down-regulation upon AA treatment, reported herein, since one of the E2F1 target genes is E2F1 itself (Johnson *et al.,* 1994). Therefore, the process of AA-mediated reduction of cell proliferation involves the binding of AA to AR inducing the de-phosphorylation of pRB at S780. This in turn causes an interaction of pRB with E2F1, which cannot conduct its transactivating function. Hence, AA decreases the expression of cell cycle mediators. In this context, it has already been shown that androgens and the AR are able to induce the formation of the Cyclin D1-Cdk4 complex mediating the phosphorylation of RB protein. These processes may involve post-transcriptional or post-translational modifications of Cyclins and/or Cdks, which have been linked to AR signaling (Gregory *et al.,* 2001). The data suggest that AA interferes with these AR-mediated processes.

Another pathway could involve epidermal growth factors, which induce ERK1/2 resulting in pRB(S780) phosphorylation (Uchida, 2012). Interestingly, TERT has also been linked to VEGF expression, which is able to activate the ERK pathway (Tang *et*

*al.,*2008; Zhou *et al.,* 2009). However, if these pathways are linked to AA-induced growth reduction has to be clarified.

Additionally, the data suggest that p21 and PARP seem not to be involved in AA-induced growth reduction. PARP is specifically cleaved during apoptosis, however, it is not indispensable to this process (Wang *et al.,* 1997). Therefore, the data presented herein do not exclude apoptosis as a mechanism of AA-induced attenuation of proliferation, but make it unlikely.

Another important aspect of the study was to analyze the effect of AA treatment on gene expression of genes, which are related to metastatic behavior. Lin *et al.*(2013a;b) published that anti-androgen treatment as a form of ADT can induce cell invasion and PCa metastasis. Mechanisms involved include the up-regulation of CCL2 and MMP9*,* none of which is induced at mRNA level upon AA treatment. Med1 a CoA of AR, which was related to cell cycle progression (Jin *et al.,* 2013), was marginally decreased due to AA treatment *in vivo*. Interestingly, Med1 expression is induced by ERK signaling (Jin *et al.,* 2013) leaving room for speculation that ERK signaling may be reduced due to AA treatment, which in turn reduces Med1 expression and pRB phosphorylation. Two further, quite favorable effects are the slight up-regulation of Nov/CCN3, which induces cell differentiation and was reported to be negatively regulated by androgen-activated AR (Wu *et al.,* 2013). And the down-regulation of FKBP5, a positively regulated direct AR target gene, which increases the transcriptional activity of AR (Febbo *et al.,* 2005). These results indicate that AA treatment causes quite differential effects on AR-mediated processes. Furthermore, they suggest that there is no general inhibition of AR activity.

Taken together, these findings identify modulation of pRB phosphorylation status as a molecular mechanism of AA-induced growth reduction of human androgen-independent C4-2 PCa tumors. Hereby, most likely post-transcriptional processes mediated by AA-bound AR are involved. Furthermore, AA treatment neither increases the expression of genes related to metastasis nor induces p21 signaling or PARP-mediated apoptosis. However, the variability of responses amongst different individuals is high and links between pathways described herein are missing, hence further experiments are needed to gain a better understanding of processes involved in AA-induced growth regression.

6 Conclusion

CRPC is challenging for PCa therapy even though new compounds have been found, which reduce PCa progression their success is timely limited. Hence, understanding mechanisms involved in growth advantages and therapy resistance of CRPC are essential to generate new promising therapeutic approaches.

One gene, which is frequently up-regulated during cancer progression, is TERT. It could be shown that TERT is either activated or repressed by AR depending on cellular context. Two sites within the TERT promoter are targeted by the receptor providing the base for the contrasting regulation. In order to get a better understanding of mechanisms involved and to verify if the model of AR-mediated TERT regulation is true, it may be useful to perform chromosome conformation capture (3C) analysis. Hereby, cells showing androgen-induced repression should be compared to androgen-induced activation. Additionally, a screening of histone and/or DNA modifications of the TERT promoter comparing benign and malign tissues may be a goal of further research. These setups may also be used comparing none-treated with treated samples to verify if there are effects of anti-androgen treatment on TERT promoter regulation, which could be advantageous for therapy outcome. In this context, AA has been shown to allow TERT gene repression by AR.

Furthermore, AA treatment enables the de-phosphorylation of RB protein, which plays a role in diverse processes including cell cycle regulation, maintenance of chromosome structure and modification of histones (Rubin, 2013). Therefore, on the one hand upstream signaling should be analyzed to identify targets of AR signaling. The study of kinase activities, especially Cdks, upon AA treatment, as well as the Cyclin D1-Cdk4/6 complex formation should be the focus of future research. As shown herein, pRB de-phosphorylation coincides with a down-regulation of E2F1 mRNA expression but not the modulation of E2F1 protein level. Hence, the regulation of other E2F proteins, i.e. E2F2 and 3, should be verified relative to AA treatment, since they are also regulated by pRB (Tyagi *et al.,* 2002). Still, the effect of AA treatment on E2F1-pRB interaction may be determined by co-immunoprecipitation and the expression of E2F1 target genes by qRT-PCR and WB. Additionally, a knock-down or knock-out of pRB could confirm its role in AA-mediated growth regression.

On the other hand, continuative research has to focus on the effect of AA treatment on cell invasion, as it has been shown that other anti-androgens may induce metastasis (Lin *et al.,* 2013a;b). In this context, first results published by Papaioannou *et al.* (2009) and presented herein indicate that AA inhibits cell invasion through an extra cellular matrix, and does not induce expression of target genes involved in metastatic behavior. Furthermore, AR mutations, which are resistant to AA treatment, should be identified, as it has been done for MDV3100 and represents a crucial step to prevent therapy resistance (Balbas *et al.*, 2013).

To further clarify the potential of AA as a new anti-androgen it should be analyzed in a *Pten*-deficient context. *Pten*-loss is a frequently occurring event in prostate tumor progression, and *Pten*-deficient mouse models are available (Beltran *et al.*, 2013; Floc'h *et al.,* 2012; Jia *et al.,* 2013). The loss of *Pten* causes an increased Cyclin D expression and hyper-phosphorylation of pRB. Regarding the data of the present study, AA may be promising under these conditions.

Taken together, PCa is a heterogeneous disease and different biomarkers as well as a more rational drug design have been suggested (Beltran *et al.,* 2013; Huang *et al.,* 2013; Patel *et al.,* 2013; Yap *et al.,* 2012). A deep insight into the mechanism of how certain anti-androgens work and what causes therapy resistance may help to make a more personalized treatment available. Thereby, molecular pathways, which are involved in tumor progression, should determine the specific kind of therapy. Furthermore, alternating drug administration may be suggested in order to avoid resistance.

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8 Appendix

Curriculum vitae

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Berufserfahrung

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04/11-06/11	Institut für Humangenetik, Universitätsklinikum Jena ungeprüfte wissenschaftliche Hilfskraft, Planung und Durchführung molekularbiologischer Experimente
12/08-1/09	Lehrstuhl für Genetik, Friedrich-Schiller-Universität Jena studentische Hilfskraft, Planung und Durchführung molekular-biologischer Experimente
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Ausbildung

03/11	Abschluss als Diplom-Biologin an der Friedrich-Schiller-Universität Jena (Note: 1,2)
02/10-02/11	Praktisches Arbeiten an der Diplomarbeit am Institut für Humangenetik, Universitätsklinikum Jena. Thema: *Functional analysis of androgen response elements within the promoter of human telomerase reverse transcriptase*
03/09-11/09	Zwei Auslandssemester an der *University of Auckland*, Neuseeland Schwerpunktfächer: *Bioscience*, *Psychology*
10/07	Vordiplom (Note: 2,7) an der Friedrich-Schiller-Universität Jena
10/05-03/11	Biologie-Studium an der Friedrich-Schiller-Universität Jena Hauptfach: Genetik, Nebenfächer: Biochemie, Neurobiologie
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Publikationen

Roediger J, Hessenkemper W, Bartsch S, Huettner SS, Manvelyan M, Liehr T, Petersen I, Grimm M-O & Baniahmad A (in preparation) Supraphysiological androgen levels induce cellular senescence in human prostate cancer cells through the Src-AKTpathway

Thiele M, Rabe S, Hessenkemper W, Röll D, Bartsch S, Kraft F, Abraham T, Houtsmuller A, van Royen M, Giannis A & Baniahmad (2014) A Novel nor-homo-and spiro-oxetan-steroids target the human androgen receptor and inhibit both androgen-dependent and castration-resistant prostate cancer growth. *Current medicinal medicine*, Epub ahead of print

Grosse A*, Bartsch S*& Baniahmad A (2012) Androgen receptor-mediated gene repression. *Molecular and cellular endocrinology,*352(1-2), pp.46-56
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Präsentationen

3. Postgraduate Symposium on Cancer Research, Dornburg, Deutschland (Posterpräsentation) *Inhibition of telomerase expression by androgens: A novel role of androgen receptor mutations for prostate cancer development*

4. Postgraduate Symposium on Cancer Research, Dornburg, Deutschland (Posterpräsentation)*The androgen receptor inhibits human telomerase expression representing a protective role of AR signaling in prostate cancer*

Stipendien

12/13	Promotionsabschlussstipendium der Graduierten Akademie Jena

Sprachkenntnisse

Englisch – fließend in Wort und Schrift
Latein – Großes Latinum (Note: 2,0)
Französisch – Grundkenntnisse

DV-Kenntnisse

Office-Standardanwendungen: Word, Excel, PowerPoint;
routinierter Umgang mit GraphPad Prism 5, Adobe Illustrator
Kenntnisse im Umgang mit SPSS

Sonstige Tätigkeiten

05/14	Workshop: Projektmanagement für Postdocs
02/13	Workshop: *Understanding Statistics – The Basics*
09/12	Übungskurs zum Erlernen der Technik der offenen Kastration an Mäusen
03/12	Übungskurs zum Erlernen tierexperimenteller Methoden für Maus und Ratte
10/07-7/08	Fachsprachenzertifikat, Englisch an der Friedrich-Schiller-Universität Jena (Note: 2)

Jena, 16.07.2014 Sophie Bartsch

Ehrenwörtliche Erklärung

Hiermit erkläre ich, dass mir die Promotionsordnung der Medizinischen Fakultät der Friedrich-Schiller-Universität Jena bekannt ist,

ich die Dissertation selbst angefertigt habe und alle von mir benutzten Hilfsmittel, persönlichen Mitteilungen und Quellen in meiner Arbeit angegeben sind,

mich folgende Personen bei der Auswahl und Auswertung des Materials sowie bei der Herstellung des Manuskripts unterstützt haben: Prof. Dr. Aria Baniahmad,

die Hilfe eines Promotionsberaters nicht in Anspruch genommen wurde und dass Dritte weder unmittelbar noch mittelbar geldwerte Leistungen von mir für die Arbeit erhalten haben, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen,

dass ich die Dissertation noch nicht als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung eingereicht habe

und dass ich die gleiche, in wesentlichen Teilen ähnliche oder eine andere Abhandlung nicht bei einer anderen Hochschule als Dissertation eingereicht habe.

Jena, 16.07.2014 Sophie Bartsch

Danksagung

Am Ende dieser Arbeit möchte ich mich bei all den Menschen bedanken, die mich während meiner Promotionszeit unterstützt haben.

Bedanken möchte ich mich bei Dr. Aria Baniahmad für die Möglichkeit an diesem Thema zu arbeiten, für seine Unterstützung und humorvolle Art. Außerdem bedanke ich mich bei Dr. Christian Melle für die Erstellung des Zweitgutachtens und dass er trotz eines vollen Terminplans so einiges möglich macht. Auch meinem Drittgutachter möchte ich danken, dass er sich für die Begutachtung bereiterklärt hat.

Großer Dank gilt meinen Kollegen aus dem Institut für Humangenetik. Besonders Flo und Wiebke danke ich für das Korrekturlesen der Arbeit. Flo danke ich für seine Ideen und Unterstützung. und ich wünsche ihm einen Kettenhandschuh für die weitere Arbeit mit den Mäusen. Wiebke bin ich dankbar für die Zusammenarbeit am Projekt und die Problemdiskussionen auf vielerlei Hinsicht. Auch Dani möchte ich besonders danken, ohne ihr Organisationstalent und ihre motivierenden Worte wäre ich manchmal ganz schön verloren gewesen. Katrin und Christoph danke ich für die große Hilfe mit meinen 'Nudes', ohne euch würde ich wahrscheinlich immer noch Tumore messen. Auch Claudi, 'Moh' und Gabi danke ich für die schöne Zeit und das immer offene Ohr bei jeglichen Problemen. Auch bei allen ehemaligen bedanke ich mich für ihre Hilfe und viele gemeinsame lustige Stunden! Besonders sei hier Alek genannt, mein 'Kummerkasten', die mir immer Rückhalt gegeben hat und sich schon mal darauf einstellen kann, dass sie mich auch nach der Abgabe dieser Arbeit nicht los wird.
Vielen Dank auch an die übrig gebliebenen 'Bios' für die Bergfeste.
Christoph danke ich für die Unterstützung, das Auffangen, wenn man gerade mal wieder den Boden unter den Füßen verliert und die Geduld.

Der größte Dank gilt meiner Familie, ohne die ich heute nicht genau hier wäre. Danke für den Rückhalt, euer Verständnis und eure Motivation auch in den scheinbar schwierigsten Situationen des Lebens!!!