Regulation of the prostate-specific hTGP (*TGM4*) gene and its potential use for prostate cancer gene therapy.

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Abstract

Prostate cancer is a very important health problem in the UK and worldwide. Available treatments such as radiotherapy, surgery and androgen ablation have failed to significantly increase the life expectancy of prostate cancer patients. Therefore researching new treatments is paramount. A promising approach for prostate cancer is suicide gene therapy. In this work, the use of the NTR/CB1954 enzyme/prodrug system delivered by a baculovirus vector was investigated for its application in prostate cancer cell lines and prostate primary epithelial cultures. The results suggest that the NTR/CB1954 system is highly efficient in causing cell death in prostate cell lines and prostate primary epithelial cultures. The use of a baculovirus vector to deliver the NTR gene resulted in increased transduction of prostate cancer cell lines in comparison to non-malignant prostate and non-prostate cell lines. To target NTR expression to prostate cells the regulation of the hTGP promoter was dissected. hTGP expression was confirmed to be highly prostate specific and mainly regulated by retinoic acid, androgens, retinoic acid receptor gamma and the androgen receptor. This work presents the first evidence of an interaction between these nuclear receptors and challenges the current model for prostate specific expression. Finally a baculovirus encoding the NTR gene under the control of the hTGP promoter was tested in prostate cancer cell lines resulting in moderate cell death. These findings are very encouraging but in order to use the hTGP promoter for gene therapy there needs to be further manipulation of the sequence to optimise its potency while maintaining its prostate specificity The use of NTR and baculovirus coupled to the targeting controlled provided by the hTGP promoter could develop into a potent and specific approach to treat prostate cancer.

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Author's declaration

I state that this thesis represents my own unaided work, except where acknowledged in the text, and has not been submitted previously in consideration for a degree at this, or any other university.

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1. INTRODUCTION

1.1 Prostate anatomy and physiology

The human prostate is a small glandular organ located close to the base of the bladder and surrounding the urethra. The main function of this gland is the production of fluids that are part of the semen composition (Vo and Goodman 2001).

The prostate can be divided into four anatomical zones; the **central** is the second largest zone, forms the majority of the prostate's base and surrounds the ejaculatory ducts. The **peripheral** is the largest of the zones; it surrounds the central zone and a portion of the urethra. The **transition** zone surrounds the proximal urethra and grows throughout the lifespan of men. Finally there is the **anterior** zone, which is devoid of glandular activity and composed of muscular and fibrous tissue (figure 1).

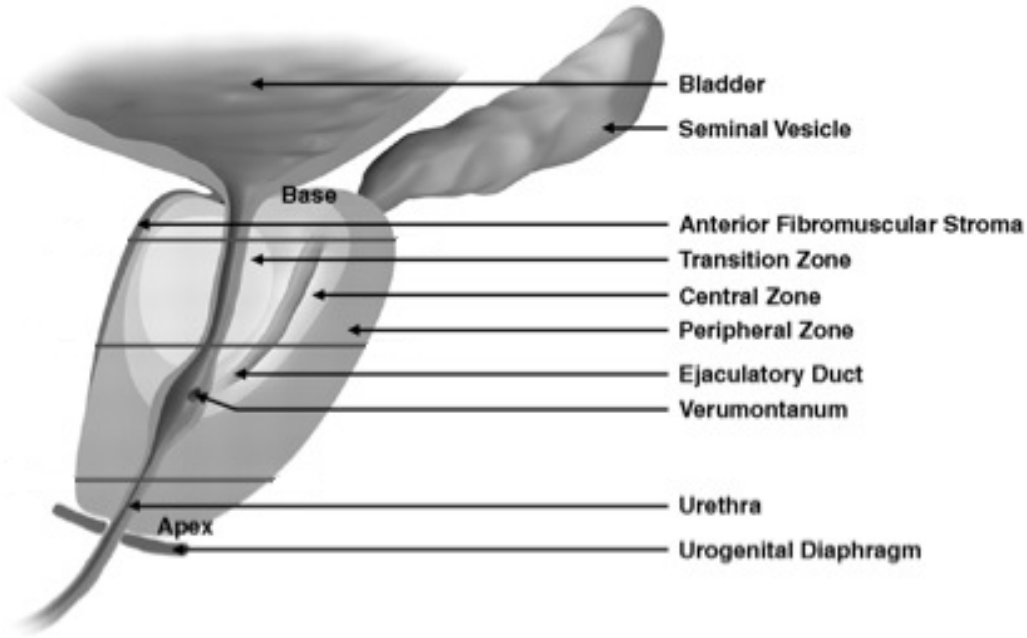


Figure 1 Human prostate anatomy, sagittal view (Kundra et al. 2007).

The prostate is a highly organised organ. The epithelial compartment is formed by a cellular bilayer with phenotypically distinct cells (figure 2). The luminal stratum is the more abundant cellular phenotype in prostatic epithelium; it is comprised of terminally differentiated cells that produce high amounts of secreted proteins such as the prostate specific antigen and prostatic acid phosphatase. Luminal cells are characterized by their high expression of androgen receptor (AR) and cytokeratins 8 and 18 (Brawer et al. 1985; Sherwood et al. 1990; Bonkhoff and Remberger 1993). Basal cells are less differentiated in comparison to luminal cells; they do not produce secreted products and are not directly dependent on androgens as luminal cells are and therefore express very low levels of AR (De Marzo et al. 1998). Basal cells express CD44, cytokeratin 5, cytokeratin 14 and p63, a p53-homologue important in prostate for cell lineage commitment and development (Signoretti et al. 2005; Grisanzio and Signoretti 2008). Neuroendocrine cells, a scarce third phenotype in the prostate epithelium, are differentiated cells that are androgen independent and express various neuropeptides such as chromogranin A and serotonin. Neuroendocrine cells are thought to provide signals to support the growth of luminal cells (Abrahamsson et al. 1998; Bonkhoff 1998; di Sant'Agnese 1998).

Further analysis of the prostate epithelium has uncovered an additional degree of complexity. The discovery of cells with intermediate characteristics between luminal and basal phenotypes has prompted the proposition of a hierarchy model that constantly renews the prostate epithelium. The discovery of a subpopulation of basal cells with a high proliferation rate that expresses both basal and luminal-associated cytokeratins (Hudson et al. 2001; Lang et al.

2001), the isolation of prostate epithelial stem cells (Richardson et al. 2004) and prostate stem cell lineage tracking using lentiviruses encoding fluorescent protein genes under the control of promoters active in late stages of differentiation (Frame et al. 2010), support the hierarchical model for the prostate epithelium.

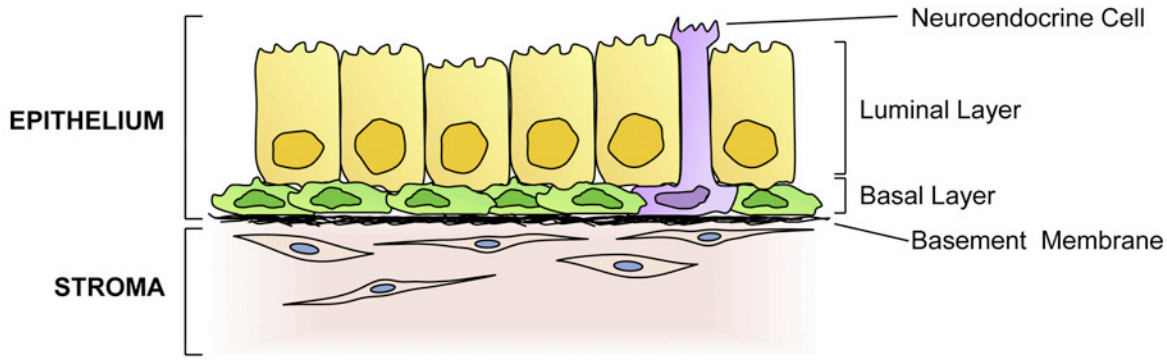


Figure 2. Human prostate epithelium, modified from (Oldridge et al. 2011)

1.1.1 Prostate development

The prostate gland develops from the urogenital sinus (UGS), which has an endodermal origin (Prins and Putz 2008). Prostate development commences with the commitment of UGS cells to prostatic cell fate. This is followed by the formation of UGS epithelial buds that penetrate into the surrounding UGS mesenchyme (Prins and Putz 2008). Branching morphogenesis of the prostate buds, which occurs when the elongating UGS epithelial buds contact the prostate mesenchyme, is co-ordinated with epithelial and mesenchymal differentiation (Prins and Birch 1995). Epithelial cells differentiate into basal and luminal cells. This differentiation is characterized by fluctuating patterns of cytokeratin and AR expression. Mesenchymal cells differentiate into periductal smooth muscle and fibroblasts (Hayward et al. 1996).

1.1.2 Regulation of prostate development by hormones

The onset of prostate development is mainly dependent on the presence of androgens (Cunha 1973). Despite the androgen requirement for prostate development, constant presence of these hormones is not needed to trigger differentiation. The study carried out by Cunha showed that UGM explants from male mice grown in the absence of androgen produced budded structures if the UGM explants were obtained after the mice started to produce testosterone. This means that androgen trigger an irreversible commitment that continues in the absence of this hormone (Cunha 1973). Interestingly, AR needs to be expressed in the UGS mesenchyme but not in the UGS epithelia, to promote prostatic morphogenesis. This was demonstrated through tissue recombinant studies. Grafted AR-deficient murine UGS epithelium combined with wild-type

murine UGS mesenchyme resulted in androgen dependent ductal morphogenesis. In a complementary study, wild-type murine UGS epithelium combined with AR-deficient murine UGS mesenchyme grafts resulted in vaginal-like differentiation (Cunha and Chung 1981; Cunha et al. 1987).

Despite the major role of androgens in prostate biology, other hormones can regulate prostate development. Oestrogen exposure during early development can modify prostate development by altering the expression of genes such as NKX3.1 and HOX13, which are closely involved in prostate development (Prins et al. 2001; Huang et al. 2004). Retinoic acid (RA) also plays an important role in prostate development. Retinoic acid, mainly through the retinoic acid receptors (RARs), controls the proliferation and differentiation of prostate epithelium (Peehl et al. 1993; Seo et al. 1997). Underlying the importance of this hormone in prostate development is the description of prostate squamous metaplasia in mice lacking RARG expression, which render the mice completely sterile (Lohnes et al. 1995).

1.2 Steroid receptors in the prostate

1.2.1 Androgen receptor

The AR is a nuclear receptor and transcription factor and it is a member of the steroid and nuclear receptor superfamily (Montgomery et al. 2001; Heinlein and Chang 2002). In the absence of ligand the AR is present in the cytoplasm of cells, interacting with heat shock proteins (HSPs) and cytoskeletal proteins that allow efficient ligand binding (Veldscholte et al. 1992; He et al. 1999; He et al. 2000; Cardozo et al. 2003). After ligand binding the AR undergoes conformational changes that affect its interaction with other proteins and DNA (Liao et al. 2003). One of the key results of these conformational changes is AR detachment from HSPs. This facilitates AR interaction with proteins such as ARA70, Filamin-A and importin-α, which bind to the nuclear localisation signal (NLS), enabling nuclear shuttling and dimerisation (Rahman et al. 2004; Schaufele et al. 2005; Cutress et al. 2008).

Once in the nucleus, active AR binds to consensus DNA sequences named androgen responsive elements (AREs). AR binding to AREs triggers the recruitment of histone acetyltransferase (HAT) enzymes, co-regulators and transcription machinery that activates transcription from target genes (figure 3) (Heinlein and Chang 2002; Powell et al. 2004).

The AR regulates genes involved in a variety of biological processes. Genes that encode prostate secreted products such as PSA, kallikrein 2 and prostatic acid phosphatase have been characterised as androgen regulated (Nelson et al. 1998). Genes related to cell survival are also androgen regulated. Androgens decrease the levels of p53 in a time and dose dependent manner (Rokhlin et al. 2005). In the rat ventral prostate the same effect is observed probably induced

by the increased expression of the negative p53 regulator Mdm2 and down regulation of the positive p53 regulators Hoxa5 and Egr1 (Nantermet et al. 2004). Interestingly, the apoptotic regulator caspase-2 has been shown to be regulated by the AR in prostate cells. Upon androgen treatment, capase-2 expression decreases in a dose-dependent manner. The AR directed regulation of the caspase-2 gene was confirmed by demonstrating AR binding to an ARE located in the intron 8 of this gene (Rokhlin et al. 2005).

Cell cycle-related proteins are also regulated by the AR. The cyclin-dependent kinase inhibitor p21, induced after androgen stimulation, has been described as a direct AR target (Lu et al. 1999). In a recent study, it was found that genes such as E2F1, CDC25, CDK6, CDC14 and CDC2, all related to cell cycle progression and regulation, were up regulated following androgen treatment (Massie et al. 2011). In the same report it was noted that genes related to glucose, lipid, nucleotide and amino-acid metabolism are also positively regulated after androgen treatment, which emphasises the importance of the AR as a key regulator of prostate biology.

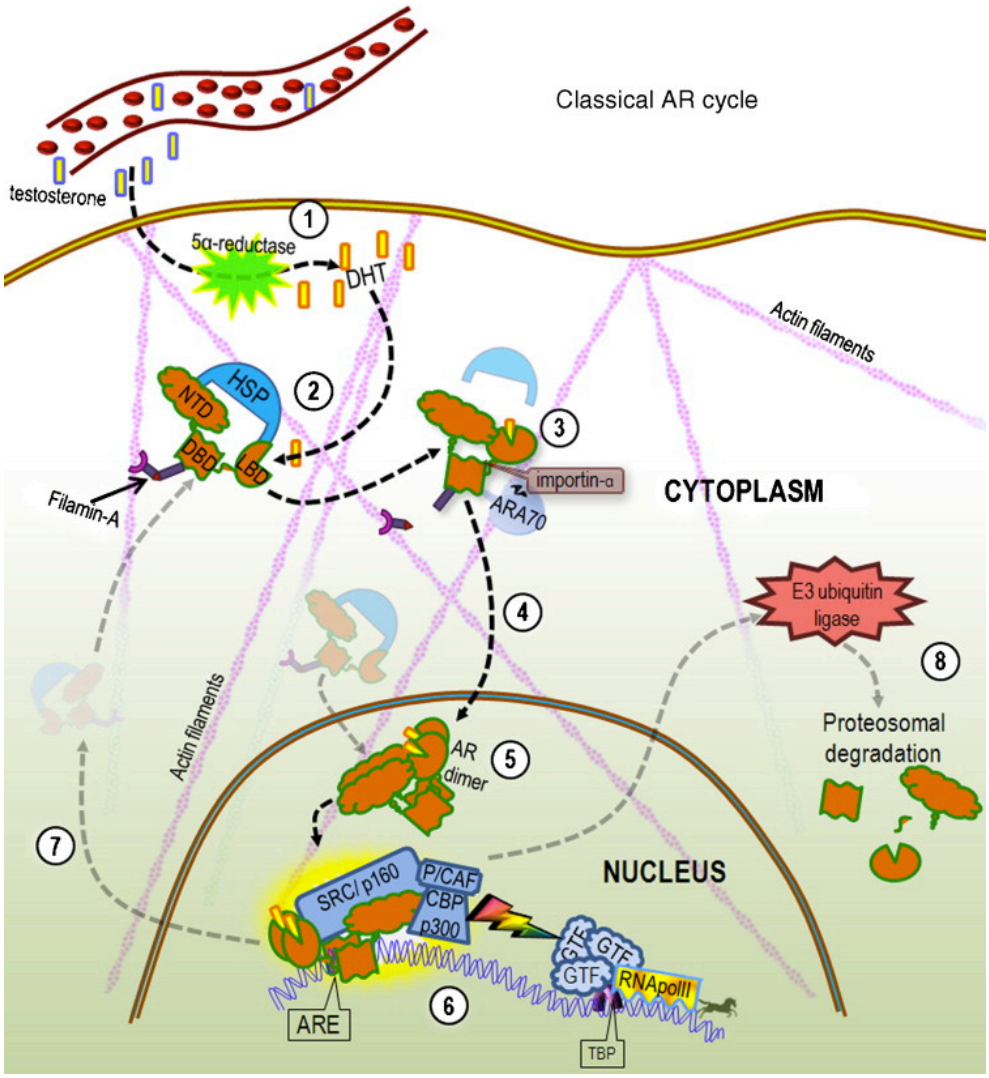


Figure 3. Androgen receptor signalling pathway. Testosterone is transformed to DHT by the enzyme 5α-reductase (1). DHT binds to the AR promoting its dissociation from HSPs chaperone complexes and allowing it to interact with importin-α and ARA70 which stabilises the receptor (2 and 3) and promote translocation to the nucleus (4). Once in the cell's nucleus AR dimerises (5) and binds to AREs in target genes promoting transcription by recruiting co-activators such as P/CAF and CBP/p300 (6). Non-ligand-bound AR is then shuttled back to the cytoplasm in preparation for more ligand binding (7) or degraded by the proteasome (8). Taken from (Bennett et al. 2010)

1.2.2 Retinoic acid receptors

The RARs, like the AR, belong to the superfamily of nuclear receptors. There are three different isotypes, RARα, RARβ and RARγ, encoded by three different genes (Chambon 1996). The role of the RARs is emphasised by the effects of vitamin A deficiency (VAD) syndrome both during development (congenital malformation affecting ocular cardiac, respiratory and urogenital systems) and after birth (growth retardation, widespread squamous metaplasia of glandular and transitional epithelia and degeneration of testis, retina and motoneurons) (Wilson et al. 1953; Chambon 1996; Morriss-Kay and Ward 1999; Mark et al. 2006). Vitamin A is the precursor of RA, which is the main ligand that triggers the RARs activity. In the absence of ligand, the RARs are found in the nucleus. They form heterodimers with the retinoid X receptors and are bound to specific sequences in the DNA termed retinoic acid responsive elements or RAREs, composed of direct repeats of a core hexameric motif (Leid et al. 1992; Mangelsdorf and Evans 1995). When RA binds to the RARs it causes the dissociation of co-repressors such as NCoR, SMRT and HDACs, from the RAR-RXR complex due to protein conformational changes (Egea et al. 2001). These changes in protein structure uncover surfaces for the interaction of the RAR-RXR complex with co-activators including the SRC/p160 family and p300/CBP (Glass and Rosenfeld 2000; McKenna and O'Malley 2002). The main role of these co-activators is the relaxation of the surrounding chromatin through histone modifications (Rosenfeld et al. 2006) which allows the posterior recruitment of the transcription machinery to the promoter (Dilworth and Chambon 2001; Woychik and Hampsey 2002) that in turn activates gene transcription (figure 4).

The RARs, being the main RA receptors, participate in a variety of biological processes, which are essential for the development and homeostasis of many organs and systems. The RARs can control the expression of HOX genes, thus have a direct role in the early development of many organs and systems including heart, urogenital system, eyes, pancreas and lungs (Duester 2008). Apoptosis is another important process regulated by RARs. Caspase 7 and 9 are up-regulated by RA. Caspase 9 is a direct target of RARs due to the presence of a functional RARE in its second intron (Donato and Noy 2005), which explains the increase in apoptosis following RA treatment. Also, RARB has been shown to regulate HOXA5, a potent inducer of cell death in breast cells, through a RARE site located at the 3' end of the gene. Down-regulation of RARB abolishes the apoptotic effects of RA treatment due to low expression of HOXA5 (Chen et al. 2007). Differentiation is another key process regulated by RARs. RARs increase the expression of proteins that modify chromatin, transcription factors and signalling effectors that enhance differentiation (Gudas and Wagner 2011). RARs are known to be involved in bone (Karakida et al. 2011), neuron (Ito et al. 2011), prostate (Peehl et al. 1993), liver (Huang et al. 2009) and stem cell (Purton et al. 2006; Chatzi et al. 2010) differentiation among other cell types and tissues.

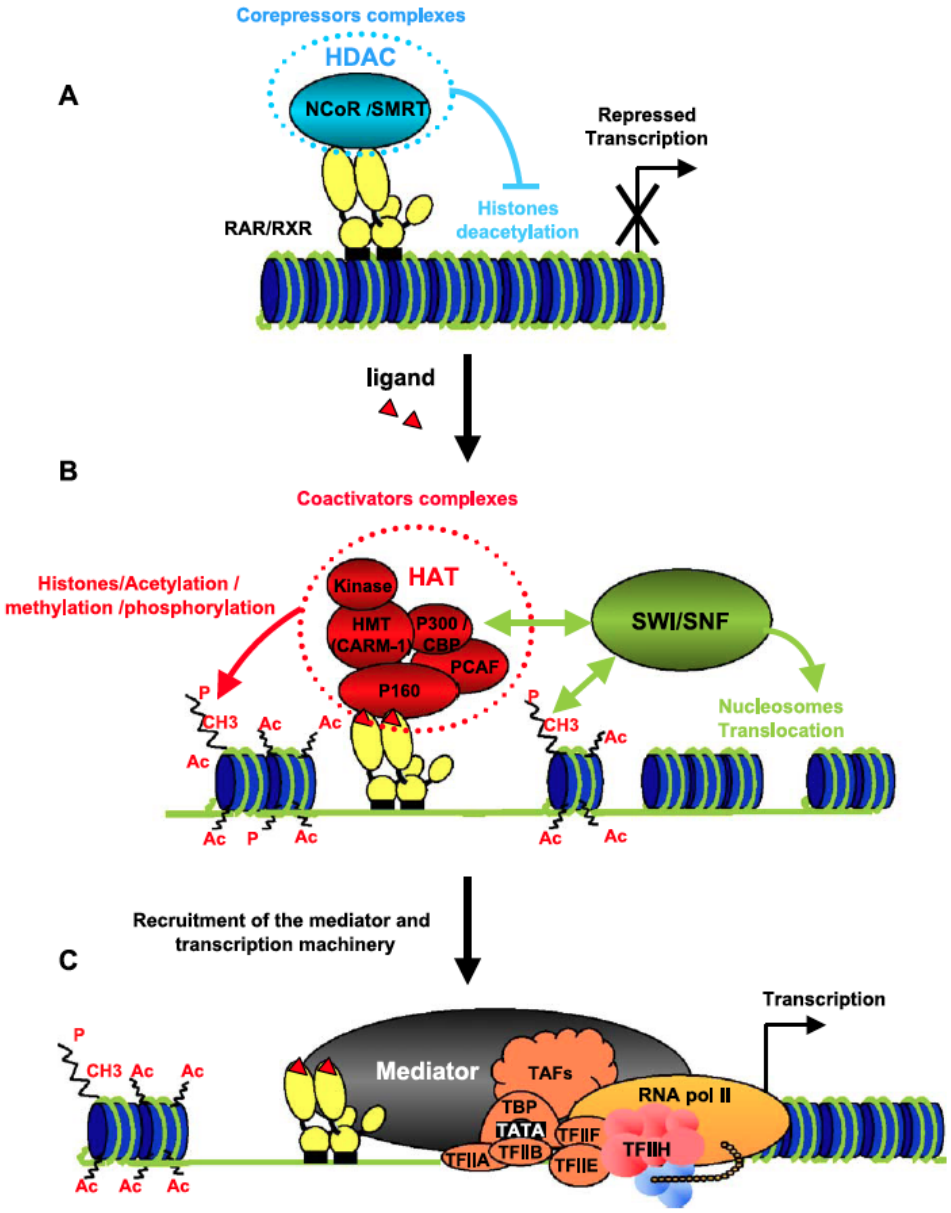


Figure 4. Retinoic acid-mediated gene expression. A. In the absence of ligand the RAR/RXR heterodimers are bound to the DNA and interact with co-repressors such as HDAC and NCoR to actively repress transcription. B. Following ligand binding, the RAR/RXR dimers undergo a conformational change that triggers interaction with co-activators such as PCAF, p300/CBP and other proteins with HAT activity to promote chromatin remodelling. C. Once the chromatin is in an open state, ligand bound RAR/RXR recruits the transcription machinery including TBP, the TFs, the mediator complex and RNA pol II to the transcription start site to begin transcription. Figure taken from (Bastien and Rochette-Egly 2004).

1.3 Prostate cancer

Prostate cancer is a predominant health problem in the UK, as one in every four new cases of diagnosed cancer in men is prostate cancer. This disease has an age-related component, with the majority of prostate cancer cases detected in men aged over 60 years (Cancer Res UK Prostate Cancer 2010). Despite the role of aging in prostate cancer there are incidence differences between populations that suggest prostate cancer is more than a by-product of age.

1.3.1 Risk factors for prostate cancer

-Inflammation

Inflammation seems to be connected to the onset of prostate cancer. Administration of PhIP (2-amino-1methyl-6phenyl-imidazo[4,5-b]pyridine), a potent inductor of inflammation, in rodents results in prostate hyperplasia and prostate intraepithelial neoplasia (PIN) which represents a precursor lesion to prostate cancer (Borowsky et al. 2006). Interestingly, regions of focal atrophic epithelium in the prostate can be associated with an inflammatory process. These regions, termed proliferative inflammatory atrophy or PIA, display increased proliferation and are often adjacent to PIN and adenocarcinoma (De Marzo et al. 1999). Possible causes for inflammation in the prostate could include physical trauma, bacterial or viral infection, altered hormone levels or diet (De Marzo et al. 2007). It has been shown that induced bacterial prostatitis in mice results in lesions resembling PIA and down regulation of Nkx3.1 expression, a key tumour suppressor gene often down regulated in prostate cancer (Khalili et al. 2010). These findings are supported by the known

susceptibility of the prostate gland to infections and the identification of multiple bacteria in prostatectomy samples (Sfanos et al. 2008).

-Oxidative stress and DNA damage

One of the major factors contributing to prostate cancer might be oxidative stress and subsequent DNA damage. Damage to DNA can occur when reactive oxygen species (ROS) accumulate inside cells and the detoxifying enzymes fail to cope with the challenge (Minelli et al. 2009). The prostate seems to be especially vulnerable to oxidative stress with inflammation, hormonal deregulation and diet as the main factors contributing to the accumulation of ROS. The observation that enzymes involved in ROS detoxification have low expression in pre-malignant lesions and prostate cancer, and the role of the tumour suppressor gene Nkx3.1 in regulating the expression of genes that respond to oxidative damage implies a role for oxidative stress in the development of malignancies (Bostwick et al. 2000; Ouyang et al. 2005).

-Genomic alterations

Genomic analysis of prostate cancer has revealed diverse chromosomal alterations and rearrangements associated with carcinogenesis. Gains at 8q and losses at 3p, 8p, 10q, 13q and 17p are examples of well-documented chromosomal alterations (Lapointe et al. 2007; Taylor et al. 2010). The identification of some of these changes in PIN and PIA lesions suggest the possibility that these genomic changes contribute to the carcinogenesis process. The mechanisms by which these lesions could induce cancer include copy number alterations of genes like *NKX3.1*, *PTEN* and *MYC*. How deregulation of

these genes leads to prostate cancer will be discussed further in following sections.

-Loss of senescence

Senescence is a cell cycle arrest in which cells remain viable but do not proliferate even when challenged with mitogen signals (d'Adda di Fagagna 2008). Senescence has been proposed to act as a mechanism of tumour suppression following oncogenic insults. Senescence has been shown as a frequent process in benign prostate hyperplasia (BPH) (Choi et al. 2000), with $p14^{arf}$ and $p16^{ink4a}$, genes associated with senescence, increasing with aging, particularly in non-malignant tumours (Zhang et al. 2006). In genetically engineered mice that lack *Pten* expression, PIN lesions arise with a senescent phenotype. Loss of senescent phenotype is achieved by inactivating p53 and Skp2, which suggests that senescence could be a barrier that prevents transformed cells from progressing to advanced disease stages (Chen et al. 2005; Narita et al. 2010).

1.3.2 Genes involved in prostate cancer

-PTEN

Phosphatase and tensin homologue (PTEN) is a 403 amino-acid protein localized in the plasma membrane and nucleus of the cells. Its main function is to down-regulate the intracellular levels of phosphatidylinositol-(3,4,5)-triphosphate (PIP3) (Maehama and Dixon 1998), which is a major product of the PI3K enzyme. PIP3 promotes phosphorylation of Akt by recruiting it to the cell membrane and activating phosphoinositide dependent kinases (PDKs)

(Kandel and Hay 1999). Phosphorylated Akt plays an important role in cell survival, promoting phosphorylation and inactivation of Bad and caspase 9, active components of the cell's death machinery (Datta et al. 1997 and Cardone et al. 1998). The importance of PTEN in prostate cancer development is underlined by the fact that PTEN is localized in the 10q23 region which is often deleted in prostate cancer (Wang et al. 1998b). PTEN inactivation is sustained in different prostate cancer cell lines and xenografts. In PC3 cells homozygous deletion of PTEN was detected, while in LNCaP cells the exon1 bears a frame-shift mutation that prevents PTEN translation (Vlietstra et al. 1998).

PTEN has also been associated with the onset of castration-resistant prostate cancer. Loss or reduction of PTEN expression paves the way for castration-resistance as demonstrated in mouse models where conditional PTEN deletion stimulates prostate cancer metastasis and androgen-independent proliferation (Wang et al. 2003).

-NKX3.1

NKX3.1 gene encodes a transcription factor homeodomain protein that is essential for prostate function and morphogenesis (Bhatia-Gaur et al. 1999). It belongs to the NK subfamily of homebox genes and is highly expressed in the prostate. This important gene is located in a chromosomal region, 8p21, where loss of heterozygosity often occurs in PIN lesions and prostate cancer tumours (Asatiani et al. 2005). Lack of alterations in the remaining allele raise the possibility of NKX3.1 haploinsufficiency as a mechanism to abolish NKX3.1 activity (Abdulkadir et al. 2002). Around 50% of PIN lesions and primary prostate tumours and as high as 80% of metastatic tumours show decreased

NKX3.1 expression, suggesting a possible role for NKX3.1 in prostate carcinogenesis and tumour aggressiveness (Bowen et al. 2000). One of the proposed mechanisms by which NKX3.1 protects prostate cells is its role in DNA damage response. Inactivation of this gene in mice results in a poor response to oxidative damage, while NKX3.1 expression in human prostate cancer cell lines protects against DNA damage (Ouyang et al. 2005; Bowen and Gelmann 2010). NKX3.1 has also been shown to suppress tumour growth in mice and cell proliferation and androgen independent growth *in vitro* (Kim et al. 2002).

-MYC

Several studies have demonstrated that MYC mRNA levels are up-regulated in PIN lesions and prostate cancer in comparison to BPH and normal tissue (Fleming et al. 1986; Buttyan et al. 1987). While MYC overexpression at the mRNA level seems consistent, MYC protein levels and localisation in prostate cancer is still unclear. Presence of MYC in the cytoplasm of cancer cells has been reported (Jenkins et al. 1997; Yang et al. 2005). However, MYC presence was localised in the nuclei of the cells, with little difference between benign and cancer samples (Fox et al. 1993). A more recent article describing the use of a rabbit monoclonal antibody against MYC, validated for immunocytochemistry, shows a strong nuclear localisation and a higher expression in malignant versus benign samples (Gurel et al. 2008).

A chromosomal region commonly amplified in advanced and recurrent prostate cancer is 8q24.21. This region contains the gene MYC, and its amplification is thought to be related to the high mRNA and protein expression found in

prostate cancer (Gurel et al. 2008). While gain of the chromosome 8 is relatively frequent, MYC locus amplification appears to be less common (Nupponen et al. 1998), and when it does occur is in the order of a few-fold increase, a modest up-regulation when compared to expression of NMYC in other cancers such as neuroblastoma. Evidence so far shows a poor correlation between 8q24 amplification and MYC expression, mostly because 8q24 gain is infrequent in PIN lesions, while MYC in the same lesions is higher than in benign tissues (Gurel et al. 2008). MYC overexpression promotes prostate cancer development through differential regulation of genes and proteins. Nkx3.1 expression is low in high MYC expressing cells (Ellwood-Yen et al. 2003), while hTERT expression is increased (Wang et al. 1998a) and EZH2 is up-regulated by MYC due to down-regulation of mir26a that represses EZH2 expression (Koh et al. 2010),

While current evidence suggests a role for MYC in prostate cancer, more studies are necessary to consolidate current evidence and provide a mechanism by which MYC promotes the development of prostate cancer.

1.4 AR and castration-resistant prostate cancer

The role of AR in prostate homeostasis has been discussed previously, however it also plays a major role in prostate cancer. Survival of prostate tissue is closely linked to androgen (figure 5A). Androgen deprivation causes high rates of apoptosis in prostate cells that interact with the stromal component (figure 5B), but not in those cultured without stroma where androgen depletion only causes low rates of proliferation (Gao et al. 2006). The importance of AR for prostate cells survival and its role in the development of prostate cancer resulted in the treatment of this disease by means of limiting the concentration of androgens available to the tumour. Although tumour shrinkage and prostate cancer biomarker PSA levels decrease after androgen deprivation, almost invariably, prostate cancer relapses with an acquired resistance to castration/castration-like treatments (Feldman and Feldman 2001). Castration-resistant prostate cancer (CRPC) retains AR expression, suggesting that this receptor is still playing an important role (Han et al. 2005). Experiments with xenografts, selected by their castration-resistance, show that these cells have a higher AR expression (figure 5C) and that treatment of these cells with AR antagonists had an agonist effect, stimulating AR activity (Chen et al. 2004). Other mechanisms to retain AR activity include gain-of-function mutations that increase protein stability, a broader response to other steroid hormones, increased sensitivity to androgens (figure 5D) and ligand independent activity (figure 5E) (Zhao et al. 2000; Robzyk et al. 2007; Steinkamp et al. 2009). Despite the assumption that AR performs the same activities in CRPC, a new study suggests that actually, AR activity in CRPC cells activates a different pathway to promote growth and survival. In CRPC, in contrast to castration-

sensitive prostate cancer, the AR up-regulates M-phase cell-cycle genes including UBE2C, a gene that disables the M-phase checkpoint (Wang et al. 2009).

Whether castration-resistant prostate cancer is an inevitable development of prostate cancer is still debatable. There are currently two main hypotheses to explain the onset of CRPC. The adaptation model proposes that androgen deprivation forces androgen-dependent cells to adapt in order to survive the new conditions. The clonal selection model suggests that the proliferation of CRPC cells is the result of the expansion of a rare set of previously quiescent cells that were castration-resistant preceding androgen deprivation (Isaacs and Coffey 1981).

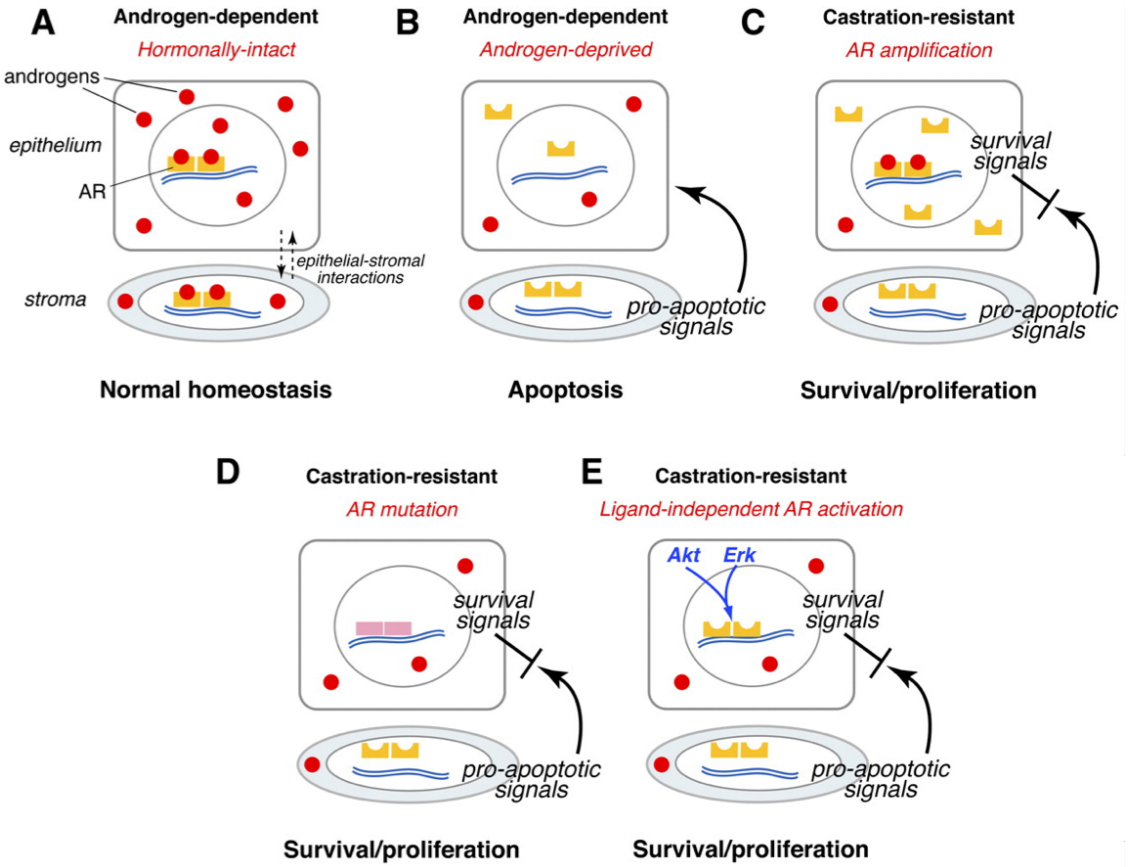


Figure 5. Castration-resistance molecular mechanisms. A. Normal response to androgen in epithelial cells: high concentrations of androgen trigger AR activity. B. Low androgen concentrations stimulate pro-apoptotic signal production by the stroma component to induce cell death in the epithelium. C. AR amplification results in AR activity in a low androgen environment and overcomes the pro-apoptotic signals coming from the stroma. D. AR mutations can stabilise the AR, promoting its activity in a low androgen environment. E. Activation of other signalling pathways can induce AR activity and oppose the pro-apoptotic signals caused by the low androgen concentration. Modified from (Shen and Abate-Shen 2010).

1.5 Prostate cancer stem cells

There are currently two main models that attempt to explain the cellular origin of prostate cancer and other solid tumours. The first hypothesis, known as the stochastic model, proposes that within the prostate tumour the majority of the cells possess high tumorigenic potential. Tumour heterogeneity develops from the expansion of clones with growth/resistance advantages and are decisive for the evolution of the tumour (Shackleton et al. 2009). An alternative model proposes the existence of cancer stem cells (CSC) to explain the heterogeneity found in prostate tumours and acquired resistance to treatments, particularly to androgen withdrawal (figure 6). According to this model, CSC accumulate mutations and alterations during their extended lifespan, that result in the generation of malignant progeny following an aberrant differentiation program (Rosen and Jordan 2009). Some of the proposed characteristics of prostate CSC are androgen independence, due to lack of expression of AR, indefinite self-renewal potential, asymmetric division and a high rate of tumour initiation ability.

Several observations support the CSC theory; one of the most interesting being that androgen deprivation causes regression and apoptosis in prostatic epithelium, but once androgens are restored, the prostate undergoes full recovery both structurally and functionally (English et al. 1987; Evans and Chandler 1987). This implies that within the prostate epithelium there must be a subset of cells with the ability to give rise to the prostate hierarchy that are castration resistant. A similar observation occurs when a prostate cancer patient undergoes chemical castration treatment. The tumour shrinks and PSA levels decrease during the early stages of the treatment. Unfortunately, in almost all

cases the tumour relapses and becomes castration-resistant and further treatments are limited to palliative actions (Denmeade and Isaacs 2002). This suggests the presence of cells with stem-like properties in prostate cancer tumours that are resistant to castration and generate a progeny with the same characteristics. Moreover, the same cell surface markers used to identify prostate epithelial stem cells (Richardson et al. 2004) can be used to isolate cells with increased clonogenicity, proliferation and self-renewal from prostate cancer biopsies (Collins et al. 2005), suggesting that normal epithelial prostate stem cells are the cell of origin for prostate cancer.

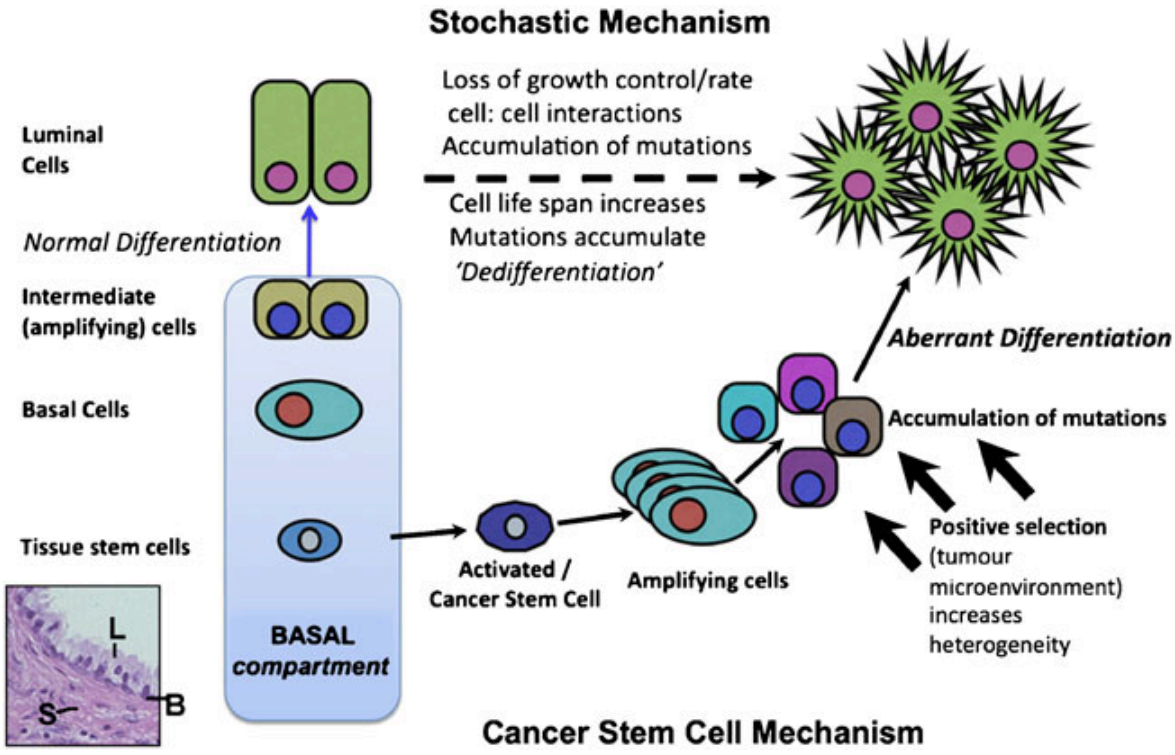


Figure 6. Stochastic and cancer stem cell mechanisms that could lead to prostate cancer development. The stochastic mechanism proposes that luminal cells suffer a loss of growth control and cell:cell interactions that when combined with a cell life-span increase, produces dedifferentiated cancer cells. The cancer stem cell mechanism proposes that normal stem cells suffer mutations that activate malignancy and that these cancers stem cells follow an aberrant differentiation program. Figure adapted from (Maitland et al. 2011)

1.6 Current treatments for prostate cancer.

Current treatments available for prostate cancer are often offered to patients depending on the stage of the disease. Gleason grade (Gleason 1966) and PSA score (Placer and Morote 2011) are the most used tools in prostate cancer diagnosis. Gleason grade scores prostate cancer using 5 grades depending on the differentiation status of the tissue and although it was established in 1966 it remains the most useful prognostic tool for prostate cancer. PSA levels are correlated to the presence of prostate cancer. The higher the PSA levels the more likely to detect prostate cancer, however this test is not 100% reliable and cannot distinguish between aggressive and non-aggressive cancers.

1.6.1 Radical prostatectomy

Radical prostatectomy is the most successful treatment for localized prostate cancer when compared to other treatments (Bill-Axelson et al. 2005). This approach is intended to completely remove all the cancerous tissue that is confined within the prostatic capsule and therefore could be curative. There are two main downsides to this approach; first, for this treatment to be effective, the cancer needs to be detected at a very early stage without clear signs of spreading outside the prostate, something that even with the current screening methods is still very unlikely. Secondly, there are a number of possible side effects such as sexual dysfunction and urinary incontinence (Catalona et al. 1999).

1.6.2 Radiotherapy

Another treatment for organ-confined prostate cancer is radiation therapy. It consists of the use of ionizing radiation over a portion of tissue to control its growth through DNA damage and the cascade response generated by it. Radiation therapy for prostate cancer has two variants; external beam radiotherapy or brachytherapy (Duchesne 2011).

External beam radiotherapy refers to the use of an external source of ionising radiation targeting a particular part of the body where the tumour is localised, in this case, the prostate. Brachytherapy is the internal use of radioactive seeds located next to the tumour. While the principle of both treatments is the same, there are certain differences between both approaches. Whereas external beam radiation therapy is less invasive, brachytherapy reduces the amount of healthy tissue that is irradiated and therefore some of the undesired side-effects caused by radiation (Jani and Hellman 2003).

1.6.3 Androgen deprivation therapy

Another treatment that can be used as a monotherapy or in combination with surgery or radiotherapy is androgen deprivation therapy. This treatment is often used to treat advanced or metastatic prostate cancer, and relies on the luminal cells' dependence on androgens for survival. Since cells with a luminal phenotype comprise the majority of the prostate tumour, androgen deprivation results in a rapid decrease in tumour size. However, in time, the majority of the tumours treated with androgen deprivation therapy relapse, becoming more aggressive and insensitive to androgen deprivation therapy and other common treatments (Yagoda and Petrylak 1993; Rashid and Chaudhary 2004). Other

well-documented side effects that prevent the wider use of androgen deprivation therapy are osteoporosis, skeletal complications, arterial stiffness, cognitive decline and fatigue (Isbarn et al. 2009).

1.6.4 Chemotherapy

Chemotherapy is often considered the last line of defence against CRPC. Commonly used drugs include docetaxel, paclitaxel and vinblastine. These drugs target dividing cells by inhibiting the mitotic spindle assembly therefore preventing cells from completing mitosis (Yvon et al. 1999). However, CRPC can become resistant to these agents by decreasing proliferation rates and increasing resistance to apoptosis (Berges et al. 1995). Combination of chemotherapeutic agents and androgen deprivation therapies prolong the mean time life expectancy in a range of months only (Seruga and Tannock 2011).

Current treatments against prostate cancer have failed to take into account the heterogeneous nature of prostate cancer. Their outcome is the selection of prostate cancer cells which are resistant to these therapies, making the cancer more aggressive. Therefore, new therapies specifically designed to consider the many characteristics of prostate cancer are needed to provide a successful treatment with the ultimate outcome of improving patient's life expectancy and quality.

1.7 Gene therapy as an alternative treatment for prostate cancer

Gene therapy is by definition the transplantation of normal genes into cells in place of missing or defective ones in order to correct inherited genetic disorders. However, the term gene therapy has also been applied to the delivery of therapeutic genes to treat a given non-genetic disease. The main aim of gene therapy is the successful delivery of therapeutic genetic material to a specific tissue or cell (El-Aneed 2004). To achieve this goal, two types of vectors capable of delivering genetic material can be used, viral and non-viral vectors. Among the most studied non-viral vectors we find cationic lipids (Felgner et al. 1994), cationic polymers such as polyethylenimines (Breunig et al. 2005) and poly (L-lysine) (Ward et al. 2001). These non-viral vectors are considered safer vehicles for gene transfer, since they are not modified pathogens and do not trigger a host immune response, but their ability to transduce cells *in vivo* and *in vitro* is significantly lower when compared to the efficiency of viral vectors (Breunig et al. 2005). Conversely, viral-vectors, with the advantage of a more efficient gene delivery, need to be engineered to suppress their unregulated reproduction, immunogenicity and need to be used in limiting doses due to their potential toxic effect. At present, several viruses have been selected as promising vectors for gene delivery but in order to understand the advantages and disadvantages in the use of these viruses, a basic knowledge of their biology is necessary.

1.7.1 Viral vectors for gene therapy

1.7.1.1 Adenovirus

Human adenoviruses are non-enveloped icosahedral particles with a double stranded DNA genome. The adenoviral capsid contains minor and major capsid proteins, encoded in the adenovirus late genes. Hexon proteins are the major component of the adenoviral capsid. They possess a β-barrel structural motif found in the icosahedral capsids of many DNA viruses. At the capsid vertices a penton capsomere, a covalent complex of two proteins, attaches to a fiber protein containing a globular knob domain in the distal tip. While hexon proteins play a major role in the structure of the capsid itself, pentons and fibers are involved in virus-cell interactions and viral tropism (Glasgow et al. 2006).

-Adenovirus Infection

The virus entry to the cell is mediated by the coxsackie/adenovirus receptor (CAR), which interacts with the globular knob domain in the fiber protein. This interaction results in a clathrin-mediated endocytosis of viral particles. As the viral particles travel through the endosome pathway within the cell cytoplasm, the virus-encoded proteases release the core components from the capsid; these components are then directed to the nucleus passing through the nuclear pore to initiate transcription of viral genes. The first genes to be transcribed are termed *E1, E2, E3* and *E4* (E stands for early transcribed gene). These genes change the cell regulation, inhibit apoptosis, shut down the cell protein synthesis, provide viral replication machinery, and facilitate the process of viral particle exit from the host cell. The DNA replication starts from both termini of the viral genome, and from then on, late transcription events initiate. There are

five late transcripts named L1, L2, L3, L4 and L5; the products of these transcripts are the structural components of the virus. The presence of these components leads to virus assembly, maturation and eventual viral egress from the cell (Russell 2000).

-Adenovirus Relevant Features for Gene Therapy

Adenovirus can infect dividing and non-diving cells (Quantin et al. 1992), which is a very valuable attribute when trying to target differentiated or slow growing cells. They can also be modified in order to prevent uncontrolled replication and potential oncogenicity and to increase their transgene carrying capacity. Deletion of the E1 gene region causes the virus to become replication-deficient and dependent on a helper cell line for viral propagation; by deleting the *E3* gene the carrying capacity can be augmented, because E3 is a non-essential replication region, even though its presence is desirable in oncolytic adenoviruses, give its capacity to modulate the immune response (Sharma and Andersson 2009). The need for larger cloning capacity has led to the creation of high capacity or “gutless” vectors containing only inverted terminal repeats and packaging signals (for replication and packaging of viral DNA) and therefore they can enclose inserts up to 37 kb (Young et al. 2006).

1.7.1.2 Retrovirus

Retroviruses are wide-spread among the animal kingdom. Most retroviral virions are spherical particles of around 80-100 nm in diameter. They are encircled by a lipid bilayer derived from the host cell plasma membrane and contain viral envelope proteins. Inside the envelope is the viral capsid, mainly comprised of

the product of the viral *gag* gene. The retrovirus' genome consists of two copies of an RNA molecule along with a tRNA primer for reverse transcription and small amounts of reverse transcriptase protein. Complex retroviruses encode several other proteins involved in viral replication or cell response to the virus (Anson 2004).

-Retrovirus Infection

The first step of retroviral infection is the interaction of viral particles with the cell surface. There is not enough data to conclude if this first interaction occurs through specific molecules, but it is likely that the implicated proteins are dissimilar from the viral receptor responsible for the entry process. After this first interaction, viral particles use cell surface proteins as specific receptors. In order to gain access into the host cell the viral envelope proteins interact with these cell surface proteins. This interaction leads to a fusion between both cellular and viral membranes, and releases the viral core into the cytoplasm. Once the viral core is released, two processes are triggered: the reverse transcription of the viral RNA genome and a partial and progressive disassembly or uncoating of the viral particle. Subsequently, the viral DNA needs to reach the nucleus and it is likely that the cytoskeleton is being used to gain access to this cellular organelle. As part of their life cycle, retroviruses need to integrate the reverse transcribed DNA into the host genome. A good number of retroviruses are incapable of accessing the intact nuclei and must wait for nuclear breakdown during mitosis (even though, some retrovirus can replicate in non-dividing cells which might mean that they are capable of entering an intact nucleus). Once in the nucleus the integration process is

directed by the viral protein integrase. The position in the cell genome where integration occurs seems to be chromatin-status and viral-type dependent. After integration, the provirus is transcribed as another normal cell gene, which leads to the generation of viral proteins and viral RNA genome. These transcripts are transported to the cytoplasm where translation of viral capsid proteins occurs; subsequently viral particles are assembled and coated by the cell plasma membrane (which already has the viral envelope proteins) when budding out of the cell (Nisole and Saib 2004).

-Retrovirus Relevant Features for Gene Therapy

The retrovirus life cycle includes an integration step into the host genome and stable transmission to daughter cells, an important characteristic when trying to achieve long-term transgene expression. As the virus itself encodes all the genes needed for viral replication, it is easy to remove all viral genes and maintain only long terminal repeats required for RNA genome packaging. Retrovirus vectors were initially based on Moloney murine leukaemia virus (MoMuLV) that is incapable of crossing the nuclear membrane, and therefore can only infect dividing cells. This distinctive attribute is convenient because cancer cells are in constant proliferation, hence the retrovirus vector can only infect the dividing cancer cells and is harmless to the healthy tissue. Unfortunately, a tumor consists of dividing and non-dividing cells, which would result in partial infection and poor therapeutic impact. This problem stimulated the interest in lentivirus vectors, that can infect dividing and non-dividing cells with high efficiency. Lentiviruses are engineered in the same way as retroviruses, deleting all virulence genes and using helper cell lines for

replication and packaging. Still, random insertion into the DNA genome and the activation of cellular oncogenes are problems that require further research (Young et al. 2006).

1.7.1.3 Herpes Simplex Virus

Herpes Simplex Virus (HSV) is a double-stranded DNA virus with a lipid envelope that is embedded with glycoproteins responsible for receptor-ligand interactions. Between the envelope and the capsid there is a protein layer called tegument that has functions related to host protein synthesis down-regulation, viral gene induction and virion assembly. Finally, the icosadeltahedral capsid contains 152 kb of dsDNA (Burton et al. 2001).

-Herpes Simplex Virus Infection

The entry of HSV into host cells initiates with the attachment of HSV glycoproteins to heparan sulphate molecules in the cell surface. This binding promotes the virus union with the viral entry receptor, which can be heparan sulfate, herpes virus entry mediator (HVEM) or members of the nectin family. The recognition of the entry receptor results in the fusion between the viral envelope and the cell membrane, releasing the nucleocapsid into the cytoplasm. The nucleocapsid is then transported to the nucleus by the cytoskeleton. If the virus follows the lytic pathway, a regulated sequential expression of genes termed immediate early (IE), early (E) and late (L) initiate. The expression of the IE genes relies on the protein VP16, a tegument component, that in association with cellular factors activates their transcription. The successful IE gene expression leads to transcription of the E genes, which then change the

intracellular environment to favour viral replication. The structural proteins encoded by the L genes are produced after the replication of the viral genome; the assembly of viral particles always leads to cell death. If the DNA enters a latent state, then it persists as an episomal element with almost no transcriptional activity (Lachmann 2004).

-HSV Relevant Features for Gene Therapy

HSV based vectors have a large transgene cloning capacity as almost half of their 152 kb genome is dispensable for replication purposes. In the same way as most viral vectors, HSV vectors contain gene deletions to abolish viral gene expression or enclose several conditional mutations that suppress viral gene expression, because the expression of even a few viral genes could lead to cytotoxicity. HSV vectors have a useful quality, in that they can persist in a latent state when they reach sensory neurons. This is a way to achieve long-term expression, but since a large part of the population has already been in contact with wild-type replicative efficient HSV, and as the infection is latent, the probability of recombination and thus cytotoxic effects is larger than desired (Lachmann 2004).

1.7.1.4 Adeno-Associated Virus

Adeno-Associated virus (AAV) belongs to the family *Parvoviridae*, genus *Dependovirus*, because productive infection can only occur in the presence of a helper virus. AAV are small (22-25 nm) non-enveloped viruses with linear single-stranded DNA contained in an icosahedral capsid. It is the only

mammalian DNA virus known to be capable of integrating at a relatively specific site of the human genome (Daya and Berns 2008).

-AAV Infection

AAV first attaches to heparan sulfate proteoglycans, and the binding to $\alpha_V\beta_5$ integrin heterodimers, fibroblast growth factor receptor type 1 and the hepatocyte growth factor receptor c-Met triggers the internalization process. The virus pathway leading to the nucleus has not been clearly identified, but it seems that this pathway initiates with receptor-mediated endocytosis and then liberation to the cytoplasm due to a pH dependent process (Bartlett et al. 2000). It is not clear if the virus accesses the nucleus through the nuclear pore complex, but the pathway that follows (nuclear pore-dependent or independent) is likely to depend on the presence of a helper virus. Once the virus is in the nucleus it starts expressing regulatory proteins that, depending on the presence of a helper virus, up- or down-regulates DNA replication. When viral replication is promoted, viral proteins interact with cellular factors and promote DNA synthesis, transcription and translation of structural genes, which then lead to virion assembly, and the egress of viral particles out of the cell. If there is no helper virus inside the cell, then the AAV genome integrates into the human chromosome 19 (a region designated as AAVS1), still being able to initiate a productive infection when a helper virus infects the cell (Goncalves 2005).

-AAV Relevant Features for Gene Therapy

The apparent non-pathogenic nature of AAV and its capability of site directed integration make this virus a good candidate for gene therapy purposes. The

most common way of generating recombinant AAV particles is transfecting cells with a plasmid containing the gene of interest flanked by AAV inverted terminal repeats (ITRs) and with another construct containing the viral r*ep* and *cap* genes (for replication and capsid formation, respectively). In the presence of Ad helper functions (either by infection or by transfection) the gene of interest is rescued from the plasmid backbone and packaged into AAV capsids (Goncalves 2005). Despite the multiple advantages of the AAV system, the lack of large cloning capacity is a significant weakness.

1.7.1.5 Baculovirus

The Baculovirus family comprises a wide range of invertebrate and insect viruses. The most used species for gene therapy purposes is the Autographa californica nucleopolyhedrovirus or AcMNPV. Its genome is comprised of double-stranded and covalently closed circular DNA. AcMNPV have rod-shaped capsids which are covered by an envelope containing peplomers made of gp64 protein. AcMNPV can also be found in structures named occlusion bodies. These are formed in the nucleus and are enveloped nucleocapsids embedded in a protein matrix (O´Reilly DR 1992). Since in this work AcMNPV was the only baculovirus species used, hereafter will refer to AcMNPV as baculovirus for ease of reference.

-Baculovirus Infection

Infection in cell culture comprises three basic phases: early, late and very late. In the early phase, the viral particles gain entry into the cell through interaction with the cell membrane and adsorptive endocytosis. The nucleocapsid is then

transported to the nucleus, following interaction with the nuclear pore complex. After reaching the nucleus, viral RNA is rapidly transcribed and the cells undergo several changes, including cytoskeleton and chromatin rearrangements to support the viral cycle. In the late phase, DNA replication, late gene expression and baculovirus production all take place. In the very late phase the nucleocapsids are enveloped in the nucleus and covered by a polyhedrin protein matrix. It is important to note that baculovirus is incapable of productively infecting mammalian cells, even though its DNA can be found in the nuclei of these cells (O´Reilly DR 1992).

-Baculovirus Relevant Features for Gene Therapy

Baculoviruses have been largely studied as vectors for expression of human proteins in an insect cell-based system. The advantages of the baculovirus protein expression system are correct protein folding, diverse post-translational modifications and high safety when using these viral agents, as they are considered non-pathogenic to humans. In the 1980s it was found that baculovirus DNA can reach the nucleus of mammalian cells without any sign of transcription of viral DNA (Tjia et al. 1983). The list of permissive cells to baculovirus transduction has expanded; it comprises not only a broad number of human cell lines (table 1), but also other vertebrate cell lines. It has become evident that the molecule responsible for viral attachment and entry to mammalian cells is the viral protein gp64. After attachment, the virus enters the cell via endocytosis and follows the endosomal pathway, escaping to the cytoplasm (a process dependent on gp64) and then being transported to the nucleus using the cytoskeleton (Stanbridge et al. 2003; Hu 2006) (figure 7). The

facts that baculovirus can transduce a mammalian cell without expressing most viral genes, that most humans have not been in contact with the virus and hence do not show previous immunity against it, and the highly scalable nature of baculovirus production, make this vector a very interesting and promising option for human gene therapy.

Cell type	Reference
HeLa	(Condreay et al. 1999)
Huh-7	(Condreay et al. 1999)
HepG2	(Boyce and Bucher 1996)
HEK293	(Sollerbrant et al. 2001)
WI38	(Condreay et al. 1999)
MRC5	(Palombo et al. 1998)
MG63	(Condreay et al. 1999)
ECV-304	(Airenne et al. 2000)
HUVEC	(Kronschnabl et al. 2002)
PC3	(Stanbridge et al. 2003)
KATO-III	(Shoji et al. 1997)
SAOS-2	(Condreay et al. 1999)
Pancreatic β cells	(Ma et al. 2000)
Keratinocytes	(Condreay et al. 1999)
Bone marrow fibroblast	(Condreay et al. 1999)
Primary foreskin fibroblast	(Dwarakanath et al. 2001)
Primary neural cells	(Sarkis et al. 2000)
Primary hepatocytes	(Boyce and Bucher 1996)
Mesenchymal stem cells	(Ho et al. 2005)

Table 1. Human cell types permissive to baculovirus transduction. Table adapted from (Hu 2006).

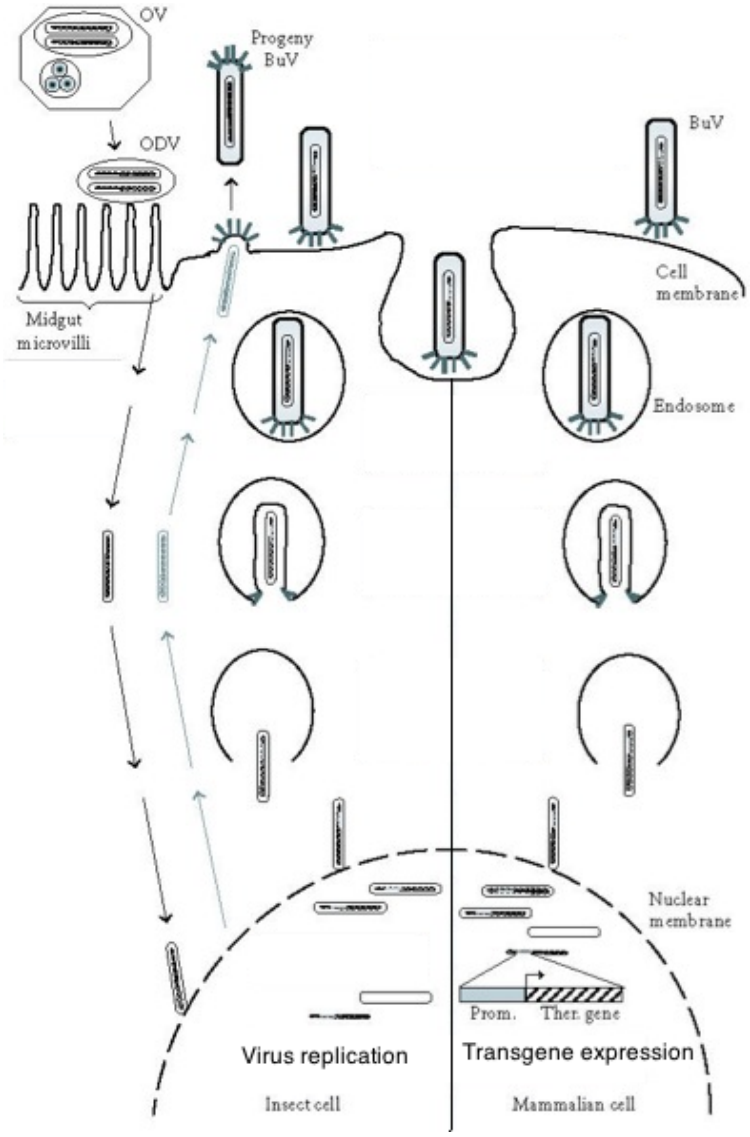


Figure 7. Baculovirus infection of insect cells (left) and transduction of mammalian cells (right). In insects there are two forms of infection, the first one occurs when occluded virus (OV) is released by the alkaline environment of the midgut and taken into the cell's cytoplasm, where the protein matrix is degraded, liberating the viral core. The second one occurs when a progeny baculovirus (BuV) enters in contact with an insect cell through the gp64 proteins on the membrane of the virus, which triggers endocytosis and membranes fusion between the viral membrane and the endosome, releasing the viral core into the cytoplasm. In both cases, the viral core is taken to the nucleus where virus replication begins. In mammalian cells the baculovirus gains entry into the cell using the gp64 protein present in its envelope. The baculovirus core is released into the cytoplasm and then taken into the cell's nucleus where the transgene can be expressed from a mammalian promoter. Figure adapted from (Stanbridge et al. 2003).

1.8 Gene Therapy Strategies for Prostate Cancer

The persistent study of prostate cancer biology has led to the development of potential gene therapeutic strategies that, depending on its foundation, aim for apoptosis, suicide therapy, immune system activation, oncolysis and correction of defective genes. All these strategies take advantage of the cancer cell's molecular modifications, which allow it to proliferate at a great rate, evade the immune system, and change to an aberrant expression pattern.

1.8.1 Corrective Gene therapy

This strategy attempts to use tumour suppressor genes to restore the proper regulation of the cell cycle or use antisense sequences to down-regulate an oncogene that contributes to the abnormal cell cycle in prostate cancer. An important therapeutic gene used for this purpose is the tumour suppressor gene *p16*; due to the finding that *p16* inactivation is common in an elevated number of prostate cancer cases. Experiments expressing the *p16* gene in several prostate cancer cell lines have shown growth inhibition and/or senescence, depending on the genetic background of the cell line (Steiner et al. 2000). Another suitable candidate is the tumour suppressor gene *p53*, as abnormal p53 function is commonly associated with an advanced stage and metastasis in prostate cancer. Adenovirus containing p53 gene have been used to treat prostate cancer in a mouse model with good results (Eastham et al. 1995). The over expression of the *myc* oncogene in prostate cancer has prompted the use of antisense mRNA to decrease the protein levels of myc, a strategy that is able to suppress and in some cases eradicate human tumours growing in nude mice (Lu 2001). In a similar way the transduction of pro-apoptotic genes to cancer

cells (*caspase-7* and *Bax*) have a positive effect on the tumour mass reduction (Mazhar and Waxman 2004). As these methodologies require almost 100% of transduction efficiency, it is more likely that they are used as a paired approach rather than as single therapies.

1.8.3 Oncolytic Gene Therapy

Oncolytic gene therapy refers to the use of cancer, tumour or tissue-selective replication competent viruses to kill malignant cells. These viruses show an increased ability to replicate in cancer/tissue-specific cells in comparison to non-malignant/unspecific cells. Conditional replication in cancer cells can be achieved by the use of cancer related or tissue specific promoters regulating the expression of key viral replication genes. One of the most used regulatory regions to control adenovirus replication is the hTERT promoter. In these replication-competent viruses, hTERT has been used to control the expression of E1A, therefore limiting viral replication to hTERT expressing cells (Onimaru et al. 2010; Doloff et al. 2011). Another exploitable approach is to use tissue specific approaches to regulate viral replication. The ARR_2PB promoter has been used to restrict the replication of the HSV virus to prostate cells, resulting in enhanced tumour specificity and lysis (Lee et al. 2010).This therapy has the advantage of needing relatively low amounts of virus to induce a therapeutic effect, since the therapeutic particles amplify within the cancer cells. However, the host immune response is still a challenge to be addressed by the oncolytic approach, since circulating antibodies and Cytotoxic T-cell response limit the therapeutic effect of these viruses (Davis and Fang 2005).

1.8.4 Suicide Gene Therapy

This approach is also known as gene-dependent enzyme prodrug therapy (GDEPT), and consists of the introduction of a metabolizing enzyme into target cells followed by the addition of a prodrug in a systemic way. The enzyme then will transform the non-toxic prodrug into a cytotoxic form, which in turn will kill the enzyme expressing cells (figure 8). The advantage of this approach is that high transduction efficiency is not required because of the so called “bystander effect”, which is thought to occur by the toxic agent diffusing to neighboring cells. The most used systems for GDEPT are the HSV gene for thymidine kinase in combination with the gancyclovir prodrug and the cytosine deaminase with the 5-fluorocytosine prodrug (MacRae et al. 2006).

Thymidine kinase is an enzyme encoded by the herpes simplex virus 1 (HSV-1) and it participates in the reactivation of the viral life cycle after a period of latency. This enzyme can be used to phosphorylate the prodrug gancyclovir (GCV), which is the rate limiting step in the conversion of GCV to its cytotoxic form (Portsmouth et al. 2007). Following phosphorylation by viral thymidine kinase the monophosphorylated GCV is then transformed to a triphosphate form by cellular enzymes. This compound is capable of inhibiting cellular DNA polymerases, and it can be incorporated into nascent DNA molecules leading to single-strand breaks and cellular death by means of apoptotic and non-apoptotic pathways depending on the cell model (Portsmouth et al. 2007). The main disadvantage when using HSVtk/GCV is that the active prodrug is unable to diffuse to neighbouring cells and the bystander effect has less impact on cell killing.

Cytosine deaminase is another enzyme used for suicide gene therapy. A specific feature is that the gene is only found in bacteria and fungi, but not in humans. Yeast cytosine deaminase is the preferred type because of its higher processive ability effect for the prodrug 5-fluorocytosine (5-FC), which then is converted to 5-fluorouracil (5-FU), a chemotherapeutic agent used to treat carcinomas in humans. 5-FU can then be converted into other compounds that interfere with the activity of the thymidylate synthase and can be incorporated into RNA and DNA, interfering with nuclear processing of rRNA and mRNA and causing DNA damage. These effects are augmented by the bystander effect of the active prodrug, that occurs without the need for cell-cell contact or facilitated diffusion (Portsmouth et al. 2007). These effects lead to cell growth inhibition and apoptosis-mediated cell death in a variety of cancers including prostate cancer (Freytag et al. 2003).

Nitroreductase enzyme is another suitable candidate for GDEPT, as it can process the CB1954 prodrug from a low cytotoxicity compound to a fully functional alkylating agent that causes extensive DNA cross-linking (Searle et al. 2004). It has been shown in a mouse fibroblast cell line that the nitroreductase/CB1954 system is functional even when only 10% of the cells were expressing nitroreductase. In the same study, transgenic mice expressing nitroreductase in T cells but not in other tissues showed a decrease in T cell population after CB1954 treatment, while other tissues studied remained unaffected (Drabek et al. 1997). The principal advantages of the nitroreductase/CB1954 system is the low transduction efficiency required for cell killing; CB1954 does not display cross-resistance with any cytotoxic agent

currently in use, and is effective under hypoxic conditions (Portsmouth et al. 2007). Moreover, a mutant bearing an amino acid change, has proved to be more efficient at activating CB1954 (Grove et al. 2003), and recently a nitroreductase double-mutant has been generated with a higher specificity for CB1954 than for other substrates (Race et al. 2007). The use of the NTR/CB1954 prodrug system could be particularly successful in prostate cancer given the low proliferation rate and heterogeneity of the disease and the ability of the activated prodrug to kill both proliferating and non-proliferating cells (Jaberipour et al. 2010).

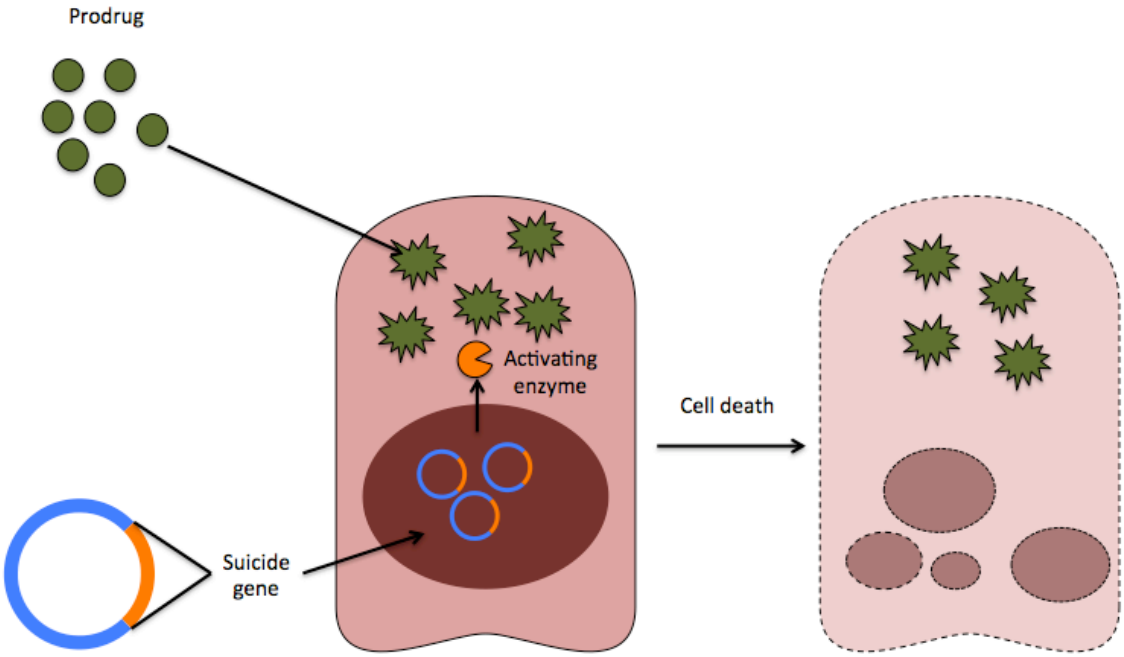


Figure 8. Suicide gene therapy or GDEPT**.** This approach relies on an enzyme encoding or suicide gene in combination with a non-cytotoxic prodrug. When the gene is delivered, target cells begin to produce the activating enzyme. Following prodrug treatment the enzyme transforms the prodrug into a potent cytotoxic agent that accumulates in the cell leading to apoptosis/cell death.

1.9 Targeting Viral Tropism

Another strategy that can be used to direct the expression of therapeutic genes is to change or modulate viral tropism. Viruses have a natural “preference” for certain molecules on the cell surface that allows them to gain entry into the cell. In some cases, this natural tropism might be desirable, but in most cases an attenuation of natural viral tropism is desirable.

1.9.1 Pseudotyping

Pseudotyping is “changing the tropism of a virus by replacing the viral attachment protein with that of a related virus” (Waehler et al. 2007) (figure 9A). Among the vectors that can be subject to pseudotyping are adenovirus, AAV, retrovirus and lentivirus. A common technique is to co-transfect plasmids, one with the coding sequence of the desired attachment protein and another with the rest of the necessary elements to give rise to a functional viral vector (Waehler et al. 2007). Pseudotyping has been performed in enveloped (Schnierle et al. 1997) and non-enveloped viruses (Mercier et al. 2004). It is more difficult to achieve good results in substituting proteins in non-enveloped viruses due to the high similarity needed between the native and the pseudotyping molecule, in order to preserve the capsid structure and functionality which can be easily disrupted.

1.9.2 Adaptor Proteins

Another approach is to use adaptor proteins to link the target molecule in the cell and the viral attachment protein in the virus surface (figure 9B). This can be achieved using receptor-ligand complexes, where the viral receptor is

genetically fused to the ligand of a receptor expressed in the target cell or tissue (Waehler et al. 2007). A further method, called chemical conjugation, covalently links the targeting ligand to the vector using Polyethylene glycol (PEG) (Waehler et al. 2007).

1.9.3 Genetic Incorporation

A more promising method is genetic incorporation (figure 9C). This approach aims to fuse a recognition sequence in the capsid or surface proteins of virions in a way that it can mediate the attachment of the viral particle to the target cell. The expression of a single-chain antibody on the viral surface has been tested in adenovirus, AAV, retrovirus and herpes simplex virus (Menotti et al. 2006; Waehler et al. 2007), showing good results. Moreover, as the introduction of large peptides to the viral structure may lead to incorrect folding and affect the way the virion is assembled, a less risky approach is the use of small peptide motifs. These are less likely to affect the tertiary structure of the protein, where they might be inserted, and yet they can keep high specificity (Waehler et al. 2007).

Despite the high potential for changing the natural tropism of viral vectors, this methodology is not being exploited in the prostate cancer area. One of the few published studies of this approach uses a viral vector resulting from the combination of an M13 derived filamentous phage and AAV. The resultant chimeric vector, which displays an RGD-4C peptide, is able to bind the αV integrins that are regularly over-expressed in tumour and endothelial cells. This chimeric vector was first tested in cell cultures showing high specificity for αV

integrin-positive cells. The chimeric vector was also tested in a mouse model of human prostate cancer, and showed high transgene expression in the prostate. Even when the liver showed non-specific clearance of the phage, there were no signs of infection in that organ (Hajitou et al. 2006).

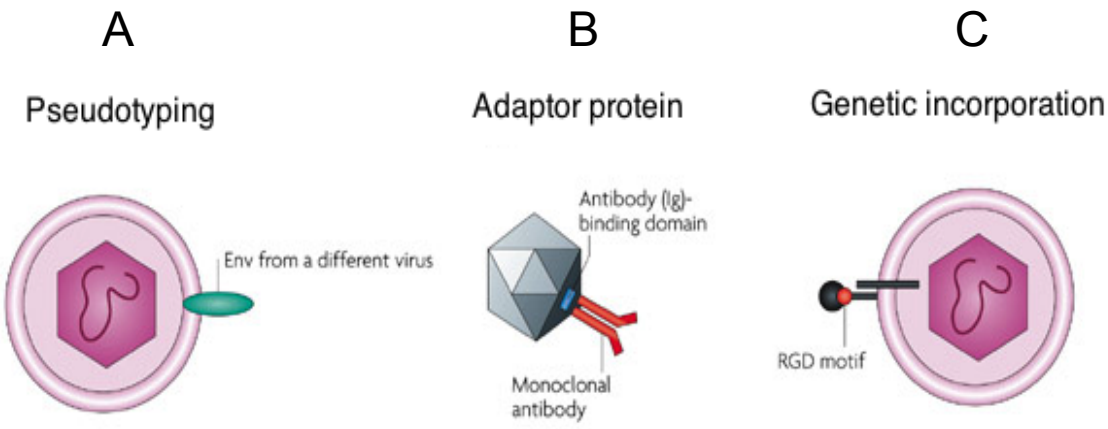


Figure 9. Different approaches for virus re-targeting. A. Pesudotyping consists of substituting a native virus envelope protein for that of a different strain or virus. B. Adaptor proteins facilitate integration of the viral attachment protein and the cell receptor on the surface of the virus. This technique also enables targeting of specific cell receptors, using specific antibodies bound to the virus through an IgG binding domain. C. Genetic incorporation is a term often used to describe genetic modifications to viral proteins in order to add a new binding domain. This can be obtained by fusing the viral protein to a single chain antibody or by including a motif for a different receptor.

1.9.4 Transcriptional targeting

If gene therapy strategies for cancer were used systematically, without aiming to target cancer cells, undesired expression of potentially toxic genes would be likely to occur in healthy tissue. This problem may be overcome by transcriptional targeting. This approach intends to limit the expression of therapeutic genes to a specific tissue or cell population, taking advantage of the distinctive cellular transcription factors in each tissue or by exploiting the over expression of some of these in cancer cells. Thus, using a promoter that can be activated only by a certain transcription factor(s), can limit the expression of the desired gene(s) to a tissue or cell population. Many promoters have been used to target the expression of therapeutic genes. These promoters can be classified as promoters based on tumour biology (telomerase and VEGF promoters), which are reported to have a higher activity in tumour cells; tissue specific promoters (prostate specific antigen or PSA and probasin promoters in the case of prostate) and microenvironment responsive promoters (table 2) (Haviv and Curiel 2001; Robson and Hirst 2003).

Tumour biology specific	**Reference**
hTERT	(Yu et al. 2011)
MUC1	(Doloff et al. 2011)
AFP	(Ma et al. 2010)
CEA	(Fong et al. 2010)
Prostate specific promoters	
PSA	(Kraaij et al. 2007)
PSMA	(Coulter et al. 2010)
Probasin	(Trujillo et al. 2010)
PPT	(Danielsson et al. 2011)
Microenvironment-specific promoters	
Flt-1	(Kaliberova et al. 2009)
HRE elements	(Kwon et al. 2010)
GRP78	(Azatian et al. 2009)

Table 2. Tumour, tissue and microenviroment-specific promoters used for gene therapy purposes.

In the case of prostate cancer gene therapy, the most common approach, given the characteristics of the prostate (particular gene expression profile and non-vital function), is the use of tissue specific promoters. PSA, human kallikrein-2 (hKLK2), probasin and prostate specific membrane antigen (PSMA) are some promoters currently under study to assess their therapeutic value in gene therapy. Nevertheless, the majority of these promoters show certain basal activity in other tissues or cell lines and weak expression when compared to strong promoters such as CMV (Latham et al. 2000; Yu et al. 2004). As a consequence, the engineering of prostate promoters through the addition or deletion of regulatory sequences has been the focus of many studies aiming to enhance the specificity and strength of prostate promoters. Recently a very elegant system for prostate specificity has been design by Woraratanadharm *et al*, in which a probasin-based promoter combined with a system regulated by tetracycline drives the transcription of EGFP (Woraratanadharm et al. 2007). This construction has been evaluated in two prostate cancer cell and two non-prostate cancer cell lines with good results, but *in vivo* evidence is clearly needed. Another approach is to fuse different promoter regions and enhancers from two different prostate specific promoters. By fusing different sections of probasin and PSA promoters, Kraaij *et al* were able to diminish the length of a chimeric promoter and still maintain the tissue specificity, while improving the promoter activity when compared to a PSA parental promoter (Kraaij et al. 2007). Another prostate specific promoter (PSMA), which is up-regulated by androgen deprivation, has been tested in two prostate cell lines and in induced tumours in mice, showing high specificity and low *in vivo* cytotoxicity (Zeng et al. 2007).

1.9.5 Human prostate-specific transglutaminase

The expression of the human prostate transglutaminase (hTGP) gene was found to be highly enriched in prostate tissue and indirectly regulated by androgens (Dubbink et al. 1998), and the minimal promoter region was mapped in the region -1 to -500 bp when tested in PC346C cells (Dubbink et al. 1998). In a later study it was found that a Sp1 binding-site in the proximal region of the promoter was necessary to induce transcription, and that the distal region of the 2.1 kb promoter exerted a negative regulation on transcription. The tissue specificity region was mapped outside the 2.1 kb length promoter due to expression in several non-prostate cell lines (Dubbink et al. 1999a). Similar to other prostate specific genes, hTGP expression was found to be up-regulated in the more differentiated prostate epithelial cells (Dubbink et al. 1999b). Given the high specificity of this protein in prostate tissue, further characterization of the distal promoter could shed light on the regulatory mechanisms of prostate restricted expression.

2. AIMS OF RESEARCH

There is a need for novel therapeutic strategies to tackle prostate cancer, since traditional treatments such as radiotherapy, surgery and androgen deprivation therapy fail. A promising approach for prostate cancer is gene therapy, given that the prostate is a non-essential organ with a specific gene expression profile. In order to design effective gene therapy strategies, there are several things to consider. Primarily, there is the vector of gene delivery. Baculovirus is an excellent candidate and was chosen in this study because it transduces several mammalian and human cells, does not induce a memory immune response and can hold large amounts of genetic material. Secondly, there is the mechanism of therapy. In this case we chose to study the NTR/CB1954 system because of its capacity to induce cell death in proliferating and non-proliferating cells even at low transduction efficiencies. Thirdly, specificity and control of gene expression is highly important. The hTGP promoter was chosen because it is one of the most highly prostate-specific genes and its regulation does not depend directly on androgens. The main objective of this project was to construct a baculovirus gene therapy vector encoding the NTR suicide gene under the regulation of the hTGP promoter.

Thus the main aims of this study were to:

1. Determine the effectiveness of the NTR/CB1954 system in inducing cell death in prostate cancer cells.

2. Assess the efficiency of baculovirus transduction of prostate cell lines and primary prostate epithelial cells.
3. Elucidate the factors and mechanisms regulating the hTGP gene expression, with a view to designing a prostate-specific promoter for use in a gene therapy vector.

To achieve these goals, prostate cancer cell lines were transfected with NTR and treated with CB1954 to evaluate the cell death rate caused by this system. Prostate cell lines (malignant and non-malignant) and prostate primary epithelial cultures were transduced with baculovirus to analyse the transduction efficiency and the baculovirus' ability to deliver the NTR gene and promote its expression.

To study the hTGP promoter, a bioinformatic analysis was carried out to find putative responsive elements that could regulate hTGP expression. Prostate cell lines were treated with androgens and retinoic acid and tested for hTGP expression. To dissect the role of the AR and RAR in hTGP regulation, receptor specific siRNA was used to knockdown AR and RAR levels, and the effect on hTGP expression was evaluated. To confirm AR and RAR binding and activity the hTGP promoter sequence was cloned and receptor binding was assessed by chromatin immunoprecipitation. Finally a baculovirus vector encoding the NTR gene under the control of the hTGP promoter was constructed and was tested in prostate cancer cell lines for NTR expression and cell death following CB1954 treatment.

3. MATERIALS AND METHODS

3.1 Cell Culture

3.1.1 Insect cell culture

Sf9 cells were obtained from Invitrogen and culture as monolayers in Grace's medium (Invitrogen), supplemented with 2mM L-Glutamine (Invitrogen) and 10% FCS (PAA) at 27°C. Cells were subcultured at 70% confluence and media was replaced every 3 days.

3.1.2 Human cell culture

-Cell lines

Human cell lines were purchased from either the American Type Culture Collection (ATCC, USA) or the European Collection of Animal cell culture (ECACC, UK) excluding PNT1A, PNT2C2 and P4E6 cells, which were established in our laboratory (Berthon et al. 1995; Maitland et al. 2001). PC346C cells were obtained from Dr. Robert Kraaij (Erasmus Medical Centre, The Netherlands). Tissue plasticware was purchased from Corning, and cells were routinely cultured in T25 flasks at 37°C with 5% CO_2, unless high amounts of cells were required in which case cells were grown in T150 flasks under the same conditions.

LNCaP, PNT1A and PNT2C2 were grown in Roswell Park Memorial Institute-1640 medium (RPMI, Invitrogen) supplemented with 10% Foetal Calf Serum

(FCS, PAA) and 2mM L-Glutamine (Invitrogen). PC346C were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen)/Ham's F12 (Lonza) (1:1 volume) supplemented with 2% FCS, 100U/ml penicillin, 100µg/ml streptomycin, 0.01% (w/v) BSA (Sigma), 10ng/ml EGF (Sigma), 1% (v/v) ITS-G (GIBCO), 0.1nM R1881 (*DuPont*-New England Nuclear), 1.4µM hydrocortisone (Sigma), 1nM triiodothyronine (Sigma), 0.1nM phosphoethanolamine (Sigma), 50ng/ml cholera toxin (Sigma), 0.1µg/ml fibronectin (Sigma) and 20µg/ml fetuin (Sigma). MCF7, T47D and HeLa cells were maintained in DMEM (Invitrogen) supplemented with 10% FCS and 2mM L-Glutamine.

-Primary Cultures

Patient samples were collected with ethical permission from York District Hospital (York) and Castle Hill Hospital (Cottingham, Hull). Prostate tissue was obtained only from patients who had given informed consent. Use of patient tissue was approved by the Local Research Ethics Committees. All patient samples were anonymised. Cells in culture were obtained from Dr Lindsay Georgopolus, Dr Fiona Frame, Dr Davide Pellacani, Dr April Frazer, Paula Kroon and Emma Oldridge. Cells were co-cultured on Collagen-I plasticware with irradiated STO murine feeder cells until growth was established in KSFM supplemented with 5ng/ml EGF, 50µg/ml bovine pituitary extract and 2mM L-Glutamine.

3.2 Foetal calf serum hormone depletion

To remove steroid compounds and other lipid-based hormones, 2g of Norvid A charcoal (Sigma) were mixed with 100ml of FCS (PAA) and refrigerated at 4°C overnight. The mixture was centrifuged at 5000RPM for 10 minutes to

precipitate the charcoal. Supernatant was repeatedly centrifuged at 5000RPM for 10 minutes until a clearer supernatant was visible. FCS was filtered using a 0.2μm filter and stored at 4°C until used.

3.3 SDS-PAGE and Western Blot

Cells were lysed with Cytobuster (Novagen) for whole cell lysates, or with NE-PER Nuclear and Cytoplasmic Extraction Reagent kit (Pierce) supplemented with 1X protease inhibitors (ROCHE) to obtain nuclear protein extracts. Before loading between 10-40μg of protein in each lane, samples were heated at 100°C for 10 minutes then placed on ice for 2 minutes. 10% Tris-SDS acrylamide gel was used to resolve the proteins. Precision Plus Kaleidoscope standards ladder (Biorad) was used for sizing and visualization of gel running pattern and protein transfer.

Resolved proteins were transfered into Immobilon-P membranes at 100V for 2h. Membranes were air-dried, wet with methanol, washed with TBS and blocked for 1h at room temperature (RT) in 1% (w/v) non-fat skimmed milk (Marvel) / TBS. Primary antibodies were diluted in 1% non-fat skimmed milk (Marvel) / TBS and incubated with the membranes for 1h at RT on a rocking table. Membranes were washed twice in TBS-Tween-20 0.1% (v/v) for 10 min followed by a wash in TBS and an incubation step in 0.5% non-fat skimmed milk (Marvel) / TBS for 15 minutes. Secondary antibodies were diluted in 0.5% non-fat skimmed milk (Marvel) / TBS and incubated for 1h at RT. After secondary antibody incubation, membranes were washed twice in TBS-Tween-20 0.1% (v/v) for 10 minutes followed by a washing step in TBS for 15 minutes.

HRP substrate (ROCHE) was added to the membranes followed by film (Hyperfilm ECL, Amersham) exposition. Films were manually processed using developer and fixer solutions (GBX, Kodak).

3.4 RNA isolation and cDNA synthesis

For RNA isolation the RNeasy Mini kit (Qiagen) was used according to manufacturer's instructions. Briefly, cells were harvested, resuspended in PBS and centrifuged in 1.5ml eppendorf tubes at 5000RPM for 4 minutes. Supernatant was discarded and the cell pellet lysed and resuspended in RLT buffer, which contains guanidine thiocyanate supplemented with 1% β-mercaptoethanol. Cell lysates were homogenised using QIAshredder columns. 1 volume of 100% ethanol was added to the homogenised lysates. The mixture was placed in RNeasy spin columns and centrifuged at 10000RPM for 30s to allow RNA binding to the column. After 2 washing steps the RNA was solubilised by adding H_2O to the column followed by a 1 minute spin at 10000RPM. For cDNA synthesis, 0.5-1μg total RNA was mixed with 50ng of random hexamer primers (Invitrogen) and 1μl of 2.5mM dNTPs mix (Invitrogen). The mixture was incubated at 65°C for 5 minutes then transferred to ice for 2 minutes. To complete the cDNA synthesis reaction 5X First strand buffer (Invitrogen), DTT (0.1M), RNase inhibitor (RNaseOUT, Invitrogen, 40U/reaction) and reverse transcriptase (Superscript II, Invitrogen, 200U/reaction) were added. cDNA synthesis reactions were incubated at 25°C for 10 minutes followed by a 42°C incubation for 50 minutes. To terminate the reaction samples were incubated at 70°C for 15 minutes. cDNA was purified using Qiagen's QIAquick

PCR Purification Kit. RNA and cDNA concentration was measured using a nanodrop ND1000 spectrophotometer (Thermo Scientific).

3.5 Generation of recombinant baculovirus

Recombinant baculoviruses, where the EGFP and nitroreductase (NTR) enzyme are under the control of the cytomegalovirus early promoter (CMV), were constructed by Dr Stephanie Swift. Recombinant baculovirus bearing the hTGP promoter controlling the expression of the NTR enzyme was produced using the Bac-to-Bac Baculovirus Expression System (Invitrogen). NTR was amplified using the Expand High Fidelity PCR system (Roche) using previously described conditions and specific primers (Appendix B) that added XhoI and XbaI sites at the 5' and 3' end of the PCR product respectively. The hTGP4.5-pGL3 plasmid was linearized using XhoI and XbaI enzymes (New England Biolabs) to create sticky ends.

Linearized plasmid was separated by gel electrophoresis; the right size band cut and purified using the QIAquick Spin kit (Qiagen) following manufacturer's protocol. Ligation reaction was set up using the Quick Ligation kit (New England Biolabs) combining 50ng of vector, 3-fold molar excess of insert Quick T4 DNA Ligase and incubating for 5 minutes at RT. DH5α bacteria (Invitrogen) were transformed with the ligation reaction and positive colonies screened by PCR using primers spanning the 5' hTGP promoter and the 3' NTR gene. PCR positive colonies were prepared and sent for sequencing to verify the integrity of the sequence. In order to clone the hTGP promoter-NTR enzyme sequences into the transfer vector pFASTBac1 (Invitrogen), SacI and XbaI enzymes (New England Biolabs) were used to excise this segment and linearized the vector. The removed fragment and the linearized pFASTBac1 vector were further

separated by gel electrophoresis and purified using the QIAquick Spin kit (QIAGEN).

Ligation was set up using the Quick ligation kit (New England Biolabs) using previously described conditions. DH5α bacteria (Invitrogen) were transformed with the ligation reaction and positive colonies screened by PCR using primers spanning the 5' hTGP promoter and the 3' NTR gene and by transfecting LNCaP cells and screening for NTR expression by WB. Positive colonies were grown and plasmid purified and transformed into DH10Bac *E.coli* (Invitrogen). The DH10Bac strain contains a baculovirus shuttle vector and a helper plasmid and allows site-specific recombination into the baculovirus genome when the cells are transformed with the pFASTBac1 vector. DH10Bac colonies were screened by PCR using primers adjacent to the hTGP promoter-NTR sequences and primers within those sequences. Positive colonies were grown and plasmid isolated and purified for further transfection into sf9 insect cells. 4 days after transfection, growing media containing recombinant baculovirus was centrifuged at 1300RPM, transfered to a 15ml Falcon tube and stored at 4°C.

3.6 Virus titration

Sf9 cells ($1x10^6$) were seeded in 6 well plates in duplicates 2 hours before viral infection. Serial dilutions of virus were prepared ranging from 10^{-2} to 10^{-6} for P1 titres and from 10^{-4} to 10^{-8} for P2 titres in 1X Grace's media supplemented with 5% FCS, 12.5µg Fungizone, 500U penicillin and 5000U streptomycin. Medium was removed from insect cells and overlaid with 200µl of virus dilution and left for 1h at RT on a rocking table. Cells were overlaid with a 1:1 mix of 2% (w/v) agarose and growing media and left at RT for 30 min to allow the agarose to

solidify. Wells were covered with 2ml of growing media and placed in a humidified chamber at 28°C for 4-5 days to allow infection to proceed. Plaques were stained for 2h with 0.025% neutral red (Sigma) and left to dry overnight at 28°C. A light box was used to identify and count lysis plaques. Titres were calculated on the basis of the average lysis plaques (in duplicate) and the dilution factor.

3.7 Baculovirus amplification

Sf9 cells ($2x10^7$) were seeded in T175 flasks (Corning) a day before the virus amplification. Cultures were infected for 1h with 0.1 pfu/cell diluted in 5ml of growing media on a rocking table. Infection was allowed to proceed for 5 days, then budded baculovirus in the supernatant was collected and centrifuged at 1300RPM for 10 minutes to remove contaminating insect cells and debris. Supernatant was stored in the dark at 4°C.

3.8 Viral DNA extraction

Baculovirus DNA was extracted by mixing 10µl of concentrated baculovirus with 89.4µl of virus lysis buffer (10mM Tris-HCl pH 8.3, 50mM KCl, 100µg/ml gelatine, 0.45% (v/v) Tween-20) and 0.6µl of proteinase K (10mg/ml). Mixture was incubated at 60°C for 1h, followed by an incubation step at 95°C for 10 minutes, then allowed to cool to RT. A PCR reaction was set up using 2µl of viral lysate as template and specific primers spanning the hTGP promoter or the NTR gene to analyse the presence of the desired sequences.

3.9 Virus concentration

Collected supernatant was ultracentrifuged at 24000RPM for 1h at 4°C using thinwall polyallomer tubes (Beckman) matched to a weight difference of 0.05g or less. Tubes were centrifuged in a LS-65 ultracentrifuge (Beckman) using the swing-out rotor SW28 (Beckman). After centrifugation supernatant was carefully removed leaving a white pellet. Pellet was overlaid with 1ml of PBS and left at 4°C overnight for the pellet to dissipate. Concentrated virus was titred as previously described.

3.10 Baculovirus transduction of human cells

LNCaP, PC346C, PNT1A, PNT2C2, P4E6 and PC3 cells were seeded in 96 well plates ($1x10^5$ cells/well) for MTS assay or in 48 well plates $2.2x10^5$ for FACS analysis. Growing media was replaced with serum-free media containing 500 pfu/cell and left incubating for 2h at 37°C unless otherwise specified. After incubation with the specific baculovirus, cells were added growing media and left for 24-72h at 37°C to allow transduction to proceed. Successful transduction was evaluated by western blot, MTS assay and/or FACS analysis depending on the recombinant baculovirus used.

3.11 RT-PCR and RT-qPCR

RT-PCR and RT-qPCR experiments were carried out using 10 and 50ng of cDNA/reaction as template, respectively. For qPCR experiments standard curves and primer efficiencies were evaluated to confirm the amplification of a single product and that the amplification efficiency was higher than 85%. Reactions for qPCR experiments were prepared in MicroAmp® Optical 96-Well

Reaction Plates (Applied Biosystems) using 10µl of Power SYBR Green 2X mix, 50ng of cDNA, 1.5µl 10µM forward primer, 1.5µl 10µM reverse primer and H_2O up to a total volume of 20µl. qPCR amplification experiments were run in triplicate on an ABI 7000 real-time PCR instrument and expression levels normalized to HPRT, which was used as a housekeeping gene. RT-PCR reactions were prepared in 0.2ml PCR tubes (Axygen) using the Platinum *Taq* DNA polymerase kit (Invitrogen). Reactions were prepared using 2.5µl 10X PCR Buffer, 0.5µl 10mM dNTP mixture, 0.75µl 10mM $MgCl_2$, 0.5µl forward primer, 0.5µl reverse primer, 0.1µl Platinum *Taq* DNA polymerase, 1µl of cDNA (10/ng/µl) and 19.15 µl H_2O. Reactions were run in the thermal block cycler GeneAmp PCR system 9700 (Applied Biosystems) and PCR products separated in 1-1.4% (w/v) agarose (Invitrogen) gels prepared with 1X TAE buffer (40mM Tris base, 1mM EDTA, 20mM Glacial Acetic Acid) and with GelRed (Biotium) at 1µl/ml to label DNA. Gels were visualized using the Gene Genious system (Syngene).

3.12 Flow cytometry

LNCaP, PC346C, PNT1A, PNT2C2, P4E6 and PC3 cells transfected with EGFP plasmid or transduced with Bv-EGFP were analysed by FACS to measure the number of EGFP positive cells. Cells were trypsinized, washed and resuspended in 500µl PBS and taken to the CyAn ADP for FACS analysis. At least 10,000 singlet events were recorded for each sample and each experiment was performed in duplicates. EGFP positive cells were analysed by plotting the FITC Log and the PE Log channels.

3.13 Gene expression profile in human tissues

TissueScan Human Normal Tissue qPCR Arrays (OriGene Technologies, Rockville MD) were used to screen for hTGP, PSA and TMPRSS2 expression in 48 different tissues using Taqman gene expression assays Hs00162710_m1, Hs02576345_m1 and Hs01120965_m1 and following manufacturer's instructions.

3.14 Bacterial transformation

Vials containing DH5α, Stbl3 or DH10Bac bacteria were thawed on ice for 30 minutes before transformation. DH10Bac bacteria were aliquoted into separate tubes (100µl/tube). 1-5ng of plasmid DNA was added to each vial and left on ice for 30 minutes. Cells were heat-shocked for 45s at 42°C without shaking. Cells were then placed on ice for 2 minutes, followed by the addition of 250µl (DH5α and Stbl3) or 900µl (DH10Bac) of RT SOC medium (Invitrogen). Vials were incubated at 37°C for 1h (DH5α and Stbl3) or 4h (DH10Bac) in a shaking incubator. DH5α and Stbl3 cells were plated in LB agar plates containing either 30µg/ml Kanamycin or 50µg/ml Penicillin and incubated for 24h before colony screening. DH10Bac cells were plated in LB agar plates containing 50µg/ml Kanamycin, 7µg/ml gentamycin, 10µg/ml Tetracyclin, 100µg/ml X-gal and 40µg/ml IPTG and left at 37°C for at least 48h to allow white/blue colouring of the colonies.

3.15 Bacterial cultures, plasmid isolation and purification

E. coli strains DH5α (Invitrogen), stbl3 (Invitrogen) and DH10Bac (Invitrogen) containing plasmids of interest were grown in LB liquid media (tryptone, yeast

extract and NaCl) overnight in the presence of specific antibiotics; ampicillin, kanamycin, gentamicin. Bacterial cultures were centrifuged at 4,500RPM and supernatant discarded. Cell pellets were resuspended using Qiagen's buffer P1 (50mM Tris-Cl pH 8.0, 10mM EDTA, 100µg/ml RNase A). Buffer P2 (200nM NaOH, 1% SDS (w/v)) was added to the mixture and mixed thoroughly by gently inverting the containing tube and left at RT for 5 minutes. Buffer P3 (3.0M potassium acetate, pH 5.5) was added and mixed by gently inverting the tube until a clear phase and a precipitate could be visible. The mixture was incubated in ice for 15 minutes, then centrifuged at 14000RPM for 30 minutes at 4°C. Supernatant was applied to the QIAGEN-tip to promote plasmid binding to the tip's resin, followed by a series of washing steps with buffer QC (1.0M NaCl, 50mM MOPS pH7.0, 15% isopropanol (v/v), 0.15% Triton X-100 (v/v)). Plasmid DNA was eluted using QF buffer (1.25M NaCl, 50mM Tris-Cl pH 8.5, 15% isopropanol (v/v)) and precipitated by adding 0.7 volumes of isopropanol and centrifuging at 14000RPM for 1h at 4°C. DNA pellet was washed with 70% ethanol (v/v) at RT, then resuspended in H_2O.

3.16 Generation of hTGP promoter constructs

The hTGP promoter sequence (4.5 kb) was amplified using the Expand High Fidelity PCR system (Roche), using specific primers (see appendix B) and the manufacturer's protocol. Briefly, template DNA (2ng) were mixed with specific primers (300nM), dNTPs (200µM), 10X Polymerase buffer and Expand High Fidelity enzyme mix (2.6U/reaction) in a total volume of 50µl. Samples were placed in the thermal block cycler GeneAmp PCR system 9700 (Applied Biosystems) using the following thermal profile: 1 cycle at 94°C for 2 minutes,

30 cycles of 15s at 94°C, 30s at 55°C and 4 minutes at 68°C and a final elongation step of 7 minutes at 68°C. Amplified hTGP promoter was cloned into the pEGFP-1 plasmid (Clontech) using the restriction enzymes *XhoI* and *SacII* (New England Biolabs). To clone the hTGp (4.5 kb) and the several deletion mutants into the pGL3 basic vector (Promega), the In Fusion cloning system (Clontech) was used following manufacturer's protocol (see appendix for primers sequence). Briefly, primers spanning the hTGP promoter were design to amplify the 4.5kb section, previously cloned into the pEGFP-1 plasmid and shorter versions (3.5, 3, 2.5, 2 and 1.5 kb, respectively). Amplification was carried out using the Expand High Fidelity PCR system (Roche) using the same conditions previously described. PCR products were ligated into the pGL3-basic plasmid using the In-Fusion kit enzymes and buffers. 1µl of ligation reaction was used to transform STBL3 (Invitrogen) chemically competent *E. coli*. All sections of the promoter that involved PCR amplification were subject to DNA sequencing to confirm the fidelity of the amplification.

3.17 Immunofluorescence

PC346C or LNCaP cells were seeded in Poly-D-lysine 8-well CultureSlides (BD) in charcoal stripped media for 48h. Before fixation, cells were briefly washed with PBS, fixed by adding cold methanol (-20°C) for 5 min, and then air-dried. Incubation in 10% goat serum (Sigma) for 1h was used to block non-specific antibody binding. Cells were incubated with AR antibody (sc-816) or IgG rabbit isotype (Sigma) as negative control in 1% BSA/PBS for 1h at room temperature. Secondary antibody goat anti-rabbit labelled with Alexa Fluor 488 (Invitrogen)

was incubated for 30 minutes in 1% BSA/PBS at room temperature. Slides were mounted with DAPI-containing VECTASHIELD (Vector Laboratories).

3.18 Plasmid transfection into human cell lines

Cells were seeded in 96- or 6-well plates and grown either in complete media (for NTR transfection) or charcoal stripped media (for hTGP promoter or retinoic acid sensitivity evaluation) for 24h previous to transfection. To measure retinoic acid responsiveness, Cignal RARE reporter (luc) kit plasmids (CCS-016L) from SABiosciences were transfected into LNCaP, PC346C, PNT1A and PNT2C2 cells using TransIT-LT1 for PC346C and PNT1A, PNT2C2 and TransIT-2020 for LNCaP cells as transfection reagents. Cells were transfected using a DNA:Transfection reagent ratio of 1:3 (µg:µl). For cells growing in 96 wells a total of 250ng of plasmid/well produced the best transfection efficiency, while for cells growing in 6-well plates 2.5µg were required to achieve the best percentage of transfected cells. Cells were treated with either vehicle (DMSO) or atRA (500nM) 18h after transfection and luciferase activity measured after a further 24h. For the functional analysis of the hTGp promoter, plasmid mixtures containing the different versions of the hTGP promoter and the pRL-CMV Vector (Promega) (in a 1:1 copy number ratio) were co-transfected into LNCaP cells grown for 24h in charcoal stripped media, using TransIT-2020 as a transfection reagent. 12h after transfection cells were treated with vehicle (DMSO), atRA (500nM) or R1881 (10nM) for a further 24h.

3.19 Plasmid transfection into Sf9 insect cells

Sf9 cells ($8X10^5$) were seeded in 6 well plates in 1:1 (v/v) growing media without antibiotics and unsupplemented Grace's Insect medium (without serum) 30 minutes before transfection. Baculovirus DNA (1µg) and 8µl of Cellfectin II (Invitrogen) were mixed separately with 100µl of unsupplemented Grace's Insect Medium. Diluted DNA and Cellfectin II were mixed by pipetting and left at RT for 15-30 minutes. The DNA-lipid mixture was then added drop wise to the cells and left at 28°C for 3-5h. Transfection mixture was removed and replaced with complete growing media supplemented with antibiotics, and incubated for 72h or after signs of viral infection were clearly visible.

3.20 Luciferase assay

Luciferase expression was measured using the Dual-Glo system (Promega) following manufacturer's protocol and the Polarstar Optima micro-plate reader (BMG). Lysis buffer containing luciferase substrate (1:1 v/v) was added to cells growing in 96 well plates and mixed by pipetting. Luciferase activity was measured 10 minutes after cell lysis. Stop & Glo reagent was added to the wells to quench luciferase activity and provide substrate for *Renilla* luciferase. *Renilla* luciferase activity was measured 10 minutes after Stop & Glo reagent addition.

3.21 MTS assay

CellTiter 96 AQueous One Solution Reagent (Promega) was thawed at RT before use. For cells growing in 100µl medium in 96 well plates, 20µl of CellTiter reagent were added and cells incubated at 37°C for 2-4h. As background controls wells containing cells without CellTiter reagent were used.

Absorbance was recorded at 490nm using a BMG Labtech POLARstar OPTIMA microplate reader.

3.22 siRNA transfection

LNCaP cells were grown in charcoal-stripped media for 24h in 6-well plates coated with L-Poly-lysine (Sigma) before transfection. A 2.5μM siRNA solution was prepared in RNase-free H_2O. In separate tubes siRNA and DharamaFECT 2 transfection reagent (Dharmacon), 3μl of reagent for every $2X10^5$ cells, were diluted using serum-free medium. Diluted siRNA and transfection reagent were mixed and left at RT for 20 minutes. Adding growing medium until reaching the desired final volume completed the transfection mix. The siRNA final concentration was 12.5nM per experiment. The specific siRNAs used were Silencer select (Applied Biosystems) siRNAs targeting RARB (siRNA ID: s11804), RARG (siRNA ID: s11807), AR (siRNA ID: s1538) or Negative control ♯1. Cells were harvested every 24, 48 and 72h for RNA extraction or every 48, 72 and 96h for protein extraction.

3.23 NTR half-life

LNCaP cells were transfected with the hTGP-NTR plasmid while growing in T25 flasks following the same basic procedure as previously described, but increasing the quantity of plasmid DNA to 6.5μg. 24h after transfection the cells were trypsinized and plated in 6-well plates ($3X10^5$ cells/well, 2 wells per time point) in the presence of atRA 500nM to stimulate NTR expression. 48h following atRA addition, cells were treated with 120μg/ml cyclohexamide (Sigma) and harvested every 2h up to 10h. Proteins were extracted and SDS-

PAGE followed by western blot to measure the levels of NTR after cyclohexamide treatment. As a positive control a western blot evaluating α-clusterin expression was performed using the Anti-clusterin α chain antibody (clone 41D, Millipore). β-actin levels were measured as a loading control and semi-quantitative densitometry performed using GelEval software (FrogDance).

3.24 Chromatin Immunoprecipitation (ChIP)

Cells were grown in T175 flasks in charcoal stripped media for 24h, then treated with either atRA (500nM), R1881 (10nM) or vehicle (DMSO) for 10h. After treatments cells were trypsinized and resuspended in 5ml media and treated with fomaldehyde to a final concentration of 1% for 10 minutes at RT with gentle shaking. Glycine was added to stop fixation to a final concentration of 0.125 M for 5 minutes at RT. Cells were washed with cold PBS and resuspended in cold swelling buffer (5mM Pipes pH 8, 85mM KCl) supplemented with NP-40 (final concentration of 0.2%) and protease inhibitors (Roche). The cell suspension was incubated in ice with gentle shaking for 20 minutes. Suspension was centrifuged at 3,000 rpm and resuspended in IP buffer TSE150 (0.1% SDS, 1% Triton, 2mM EDTA, 20mM Tris-HCl pH 8, 150mM NaCl) supplemented with protease inhibitors (Roche) and sonicated using a Bandelin Sonopuls HD 2070, for 21 cycles of 30s on/30s off at full power. Chromatin was centrifuged at 14,000RPM for 30 minutes, aliquoted and stored at -80°C. A sample of the sonicated chromatin was purified using a phenol/chloroform extraction to corroborate correct chromatin disruption. Protein A-sepharose beads (Sigma) were blocked by incubating them in an IP buffer TSE150 solution containing yeast tRNA (Sigma) to a final concentration of 1μg/ml and BSA (Sigma) to a

final concentration of 250µg/ml while rotating at 4°C for 4h. Chromatin was cleaned up by incubating 50µl of 50% pre-blocked protein A-sepharose beads with 20µg/IP of chromatin in a total volume of 1 ml TSE 150 buffer for 1.5h at 4°C while rotating. The suspension was centrifuged for 1 minute at 3,000 rpm and supernatant kept in a separate tube. 20 µl of the supernatant were kept to be used as INPUT control, then the rest was divided and incubated with either anti-RAR (sc-773), AR (sc-816) (Santa Cruz Biotech) or purified rabbit IgG (PP64B Millipore) at 4°C overnight. Antibody-protein-DNA complexes were recovered by incubation with 50µl of 50% pre-blocked protein A sepharose beads for 1.5h at 4°C. Beads were retrieved by centrifugation at 3,000 rpm for 1 minute at RT and washed with IP buffer TSE150, IP buffer TSE500 (0.1% SDS, 1% Triton, 2mM EDTA, 20mM Tris-HCl pH 8, 500mM NaCl), washing buffer (10mM Tris-HCl pH 8, 0.25M LiCl, 0.5% NP-40) and TE buffer (10mM Tris-HCl pH 8, 1mM EDTA). DNA was eluted by adding 100µl of elution buffer (1% SDS, 10mM EDTA, 50mM Tris-HCl pH 8) and an incubation step at 65°C for 15 minutes. Beads were centrifuged at 15,000RPM for 1 minute and supernatant transfered to a separate tube. Beads were rinsed with 150µl TE/1% SDS, vortexed, centrifuged at 15,000RPM and the supernatant pooled with the previous one. Immunoprecipitated DNA was left at 65°C overnight, then treated with proteinase K (Invitrogen) together with glycogen (Roche) for 2 h. DNA was purified by phenol/chloroform extraction. The percentage of Immunoprecipitation (%IP) was calculated taking into account the dilution factor and the level of amplification obtained from unprecipitated chromatin.

4. RESULTS

4.1 Efficiency of the NTR/CB1954 system in prostate cells

4.1.1 Determining prostate cell lines' sensitivity to CB1954

In order to test the efficiency of the NTR/CB1954 system in prostate cell lines, it was necessary to determine the optimal concentration of CB1954 to treat each cell line. PNT1A, PNT2C2 (benign cell lines), P4E6 (early-stage cancer), PC3, LNCaP and PC346C (malignant cell lines) were treated with increasing concentrations of the prodrug CB1954 (from 5 to 40μM) for a period of 72h. Following the incubation period, cells were tested for viability using the MTS assay that measures the cells' metabolic activity, which is correlated to their survival. Figure 10 shows the relative survival of the different cell lines. P4E6 and PNT2C2 showed high sensitivity to the CB1954 drug, displaying a decrease of 26% and 31% in relative survival, respectively, at 40μM. PC3, PC346C, LNCaP and PNT1A showed less sensitivity to CB1954 with a drop in relative survival between 7%-18% at 40μM. Based on these results, a 10μM concentration for P4E6 and PNT2C2 cells and 20μM for LNCaP, PC3, PC346C and PNT1A cells were chosen. All further experiments using CB1954 were conducted using these selected concentrations for each cell line to prevent unspecific toxicity caused by the prodrug treatment in the absence of enzyme.

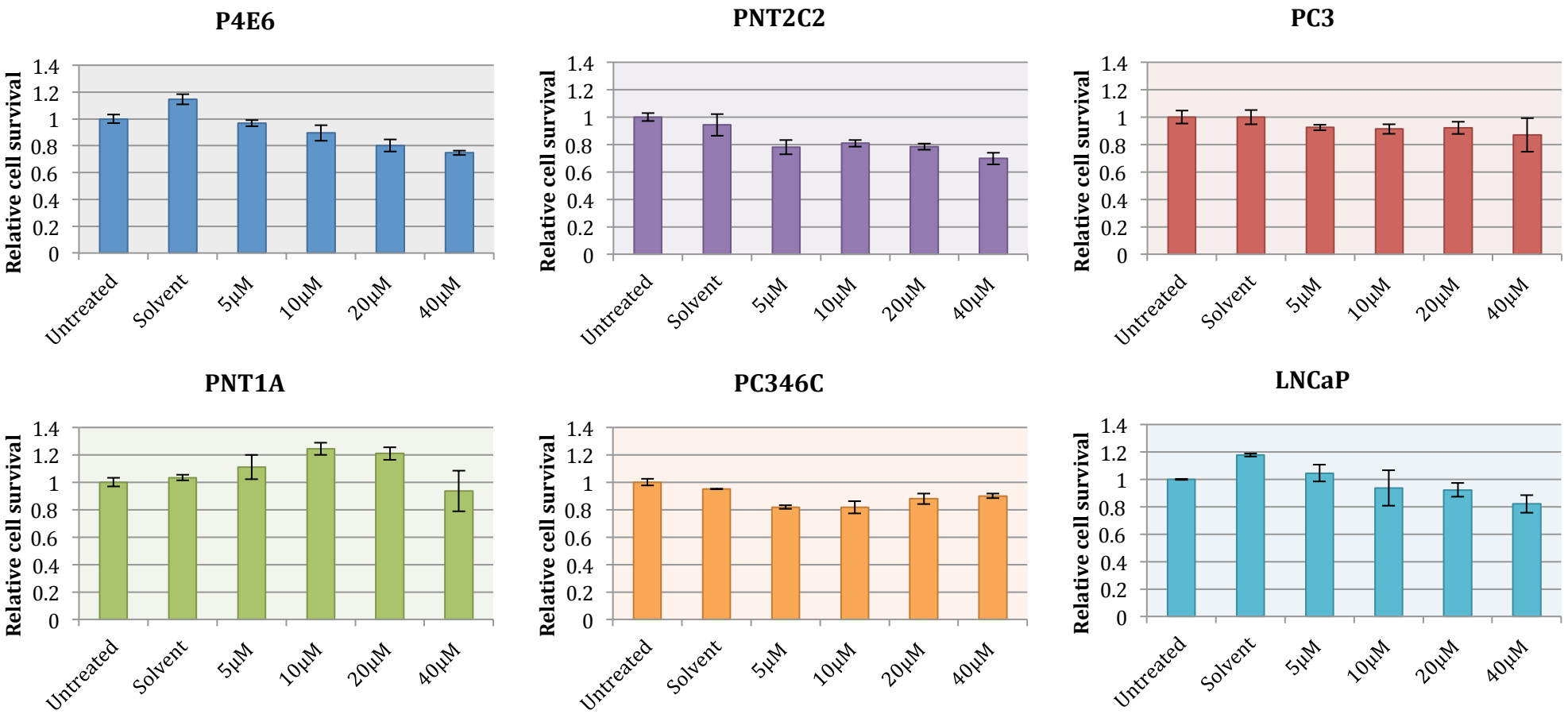


Figure 10. Determining prostate cell lines sensitivity to CB1954**.** Prostate cell lines P4E6, PNT2C2, PC3, PNT1A, LNCaP and PC346C were treated with CB1954 for 72h.

4.1.2 NTR expression in prostate cancer cells triggers cell death after CB1954 treatment.

To determine the susceptibility of prostate cancer cells to the NTR/CB1954 pro-drug system, PC3 and P4E6 cells were transiently transfected with two plasmids bearing either the wild-type NTR gene or a mutated version that confers higher enzymatic activity (NTR-mutant), under the control of the CMV promoter. Western blot analysis of NTR expression 24h after transfection (figure A) showed that both cell lines expressed high levels of NTR, either WT or mutant. To test whether the enzyme expressed in these cell lines was capable of catalysing the reduction of the CB1954 pro-drug, thus transforming it into a very powerful cytotoxic compound, transfected P4E6 cells were treated with CB1954 for 72h. P4E623A cells stably transfected with NTR-mutant were used as a positive control. Figure 11B shows that only cells that were transfected with either NTR WT or NTR-mutant and treated with CB1954 display decreased cell viability, which was comparable to the effect seen in P4E623A cells. Cells transfected with EGFP and treated with CB1954 showed a small decrease in cell viability most likely caused by the known EGFP toxicity (Liu et al. 1999).

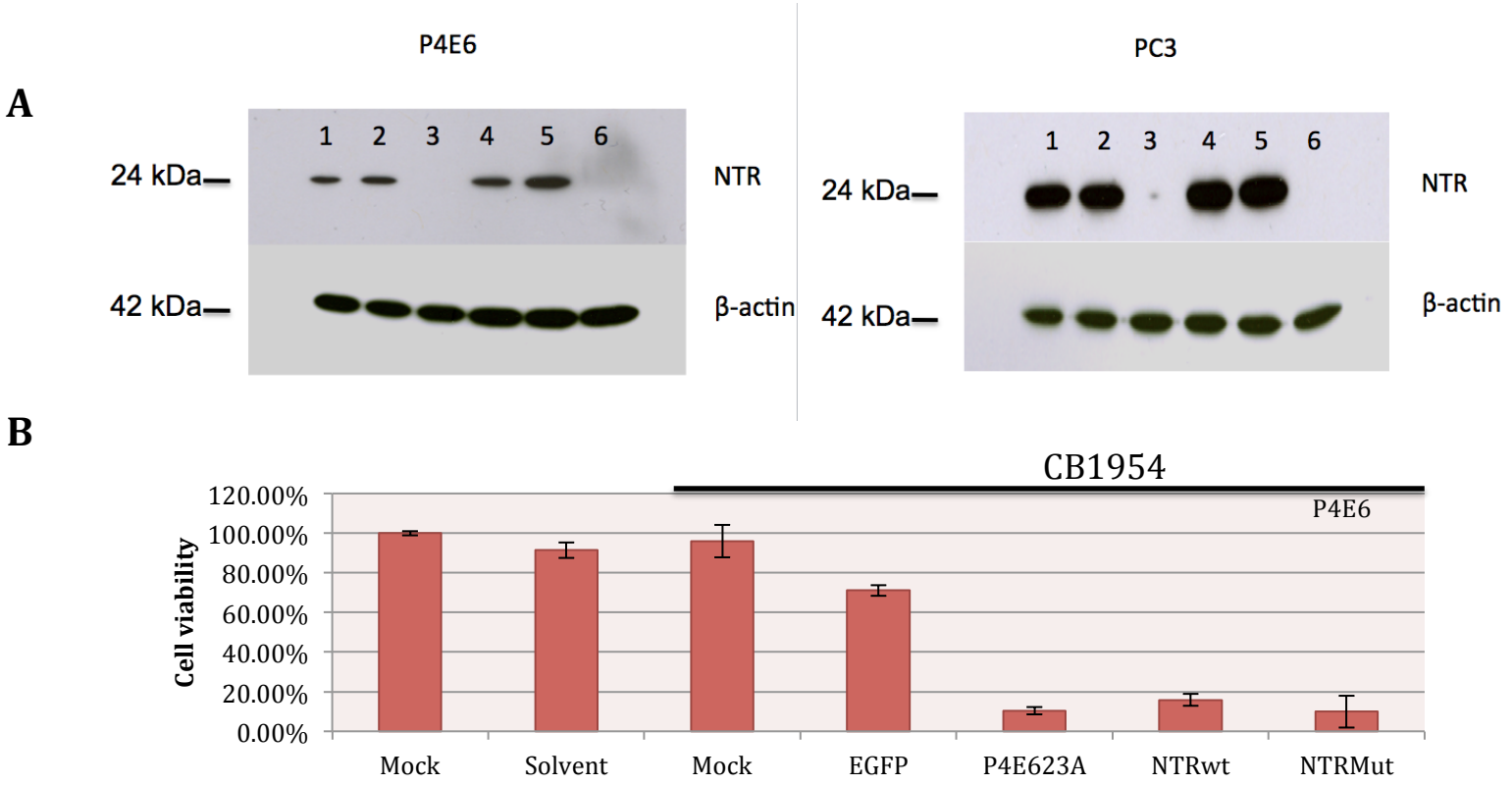


Figure 11. NTR enzyme expression in prostate cancer cell lines and induction of cell death after CB1954 treatment. A. Western Blot of NTR (WT and mutant) expression in transfected P4E6 and PC3 prostate cancer cell lines. β-actin was used as loading control. P4E623A are P4E6 cells stably transfected with a CMV-NTR plasmid. Lanes: **1**. NTR WT 24h **2**. NTR MUT 24h **3**. EGFP 24h **4**. NTR WT 48h **5**. NTR MUT 48h **6**. EGFP 48h. B. Cell viability, as measured by MTS assay, of P4E6 cells transfected with NTR (WT and mutant) 72h after CB1954 treatment (10μM).

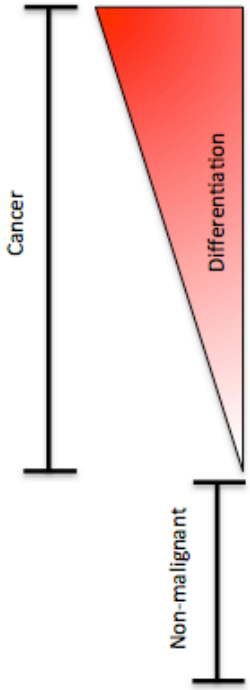
4.2 Baculovirus as a vector for prostate cancer gene therapy

4.2.1 Baculovirus effectively transduces prostate cancer cell lines

A panel of malignant and benign prostate cell lines were transduced with the recombinant baculovirus BV-EGFP which encodes the enhanced green-fluorescent protein (EGFP) under the control of the CMV promoter, to assess the transduction efficiency of the baculovirus in prostate cells. EGFP positive cells were counted 24h post-transduction, using FACS analysis and the results presented as a percentage of EGFP positive cells. Figure 12A shows that BV-EGFP effectively transduces prostate cancer cell lines LNCaP, PC346C and PC3, while early-stage cancer cell line P4E6 and benign cell lines PNT1A and PNT2C2 show low levels of EGFP expression. The same panel of cell lines were transduced with a recombinant baculovirus encoding the NTR-mutant gene under the control of the CMV promoter and 24h after transduction treated with CB1954 for a further 72h to evaluate cell viability. Prostate cancer cell lines LNCaP, PC346C and PC3 displayed massively decreased viability after CB1954 treatment (80% reduction, figure 12B). PNT1A and PNT2C2 cells showed high percentage of viable cells, with a reduction in cell viability of less than 20%, while P4E6 showed a decrease of almost 40% in cell viability. Cell viability after CB1954 treatment matched the transduction efficiency for each cell line, where the more efficiently transduced cell lines displayed higher cell death caused by the NTR enzymatic activity in the presence of CB1954. Interestingly, baculovirus seemed to be more effective at transducing malignant prostate cell lines than benign cell lines. This “preference” is displayed regardless of the differentiation status and proliferation rate of each cell line.

LNCaP and PC346C cells are more differentiated cells than PC3 (Table 3). They express AR, and PSA expression is stimulated by androgen treatment. The doubling time of P4E6, PC3, PNT1A and PNT2C2 is less than the doubling time for LNCaP and PC346C cells (Table 3). Therefore, the main difference between permissive and non-permissive cells could lie in the internal mechanisms that transport the baculovirus from the cytoplasm into the cell's nucleus. It has been reported that in HeLa cells transduced with baculovirus, the majority of the virus remains trapped in intracellular vesicles and is unable to reach the nucleus (Barsoum et al. 1997). This observation stresses the importance of effective endosomal escape in baculovirus transduction.

These results suggest that the baculovirus is able to deliver the NTR gene to prostate cancer cells and has the ability to transduce prostate cancer cell lines more efficiently than non-malignant cells.



Cell line	Differentiation markers	Doubling time	References
LNCaP	AR, PSA, CTK 8	60h	(Horoszewicz et al. 1983; Lang et al. 2002)
PC346C	AR, PSA, CTK 8	3 days	(Marques et al. 2005)
PC3	CTK 8	38.5h	(Hanigan et al. 1999; Lang et al. 2002)
P4E6	PSA, CTK 8	24h	(Lang et al. 2002; Lang et al. 2006)
PNT1A	PSA (low), CTK8	25h	(Degeorges et al. 1995; Lang et al. 2002)
PNT2C2	PSA, CTK 8	25h	(Lang et al. 2002)

Table 3. Differentiation markers and doubling times of prostate cell lines.

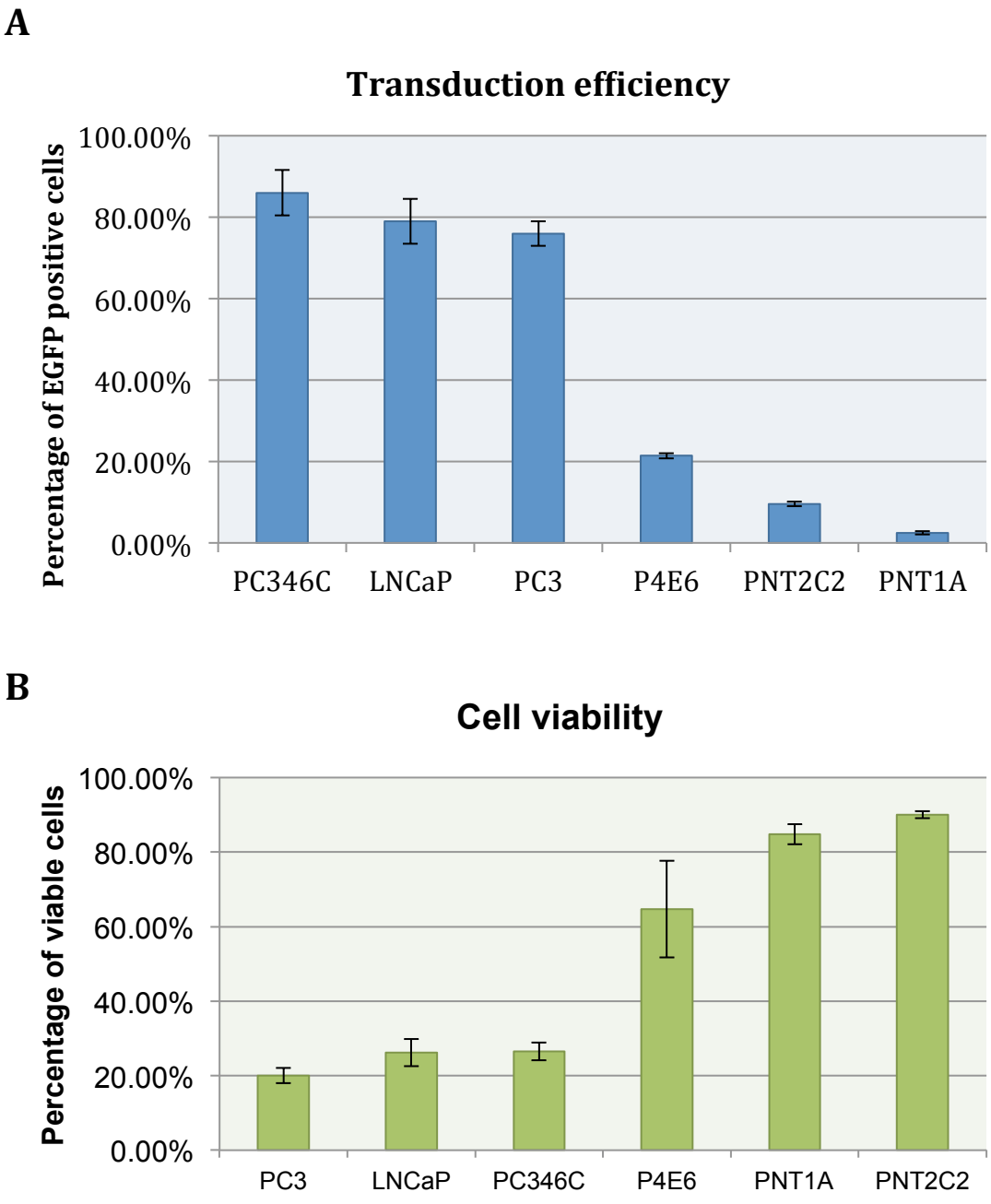


Figure 12. Baculovirus effectively transduces prostate cancer cell lines. A. Percentage of EGFP positive cells 24h after being transduced with a recombinant baculovirus encoding the EGFP gene (BV-EGFP) under the control of the CMV promoter as measured by FACS analysis. B. Cell viability, as measured by MTS assay, in cells transduced with a recombinant baculovirus encoding the NTR-mutant gene (BV-NTR), under the control of the CMV promoted and treated with CB1954 for 72h.

4.2.2 Baculovirus can efficiently transduce cultured cells derived from patients.

While the baculovirus showed a high transduction efficiency in human prostate cancer cell lines, it was also necessary to test its ability to transduce a more clinically relevant model such as cultured cells derived from patient tissue. Cells were seeded in collagen-coated wells and transduced with BV-NTR. As one of the controls, cells were transduced with the BV-EGFP and images of EGFP positive cells were taken 24h after transduction. CB1954 pro-drug was added 24h after transduction and the cells remained in culture for 48h before performing the MTS assay. Figure 13A shows data from three different primary samples transduced with the BV-EGFP. All samples showed the presence of EGFP positive cells indicating that the transduction was successful. Figure 13B shows the cell viability 48h after CB1954 addition in cancer and BPH samples. Noticeably there is no obvious difference between cancer and benign cultures, in contrast to the differences described in cell lines, suggesting that benign and cancerous prostate cells could be transduced equally.

Taken together, these encouraging results provide evidence of the potential for successful use in gene therapy of baculovirus in delivering the NTR enzyme into prostate epithelial cell lines and cultured cells derived from patients.

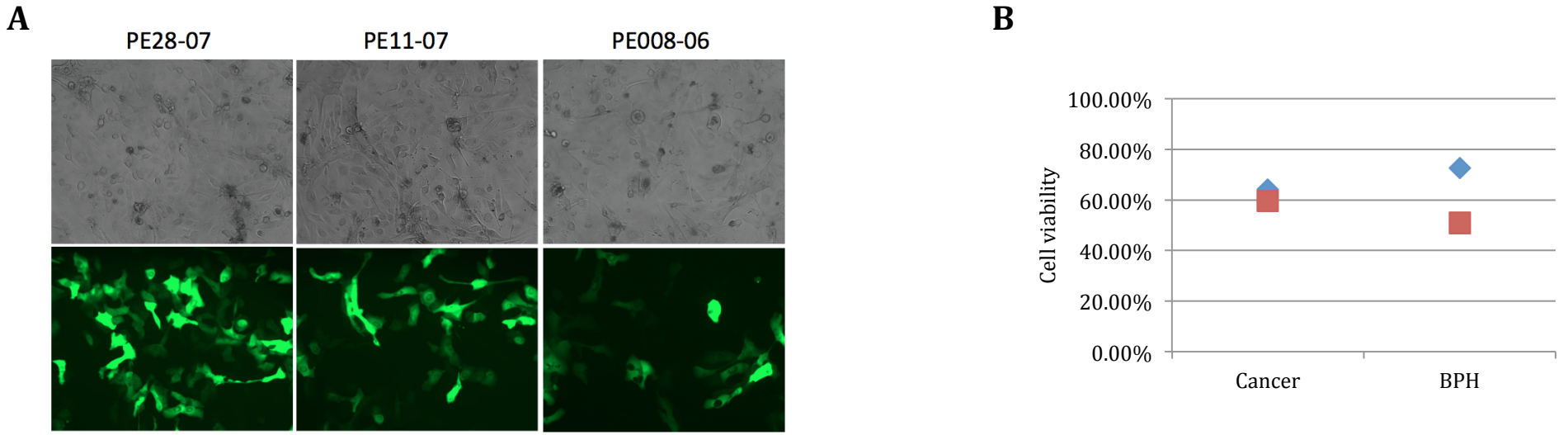


Figure 13. Patient-derived prostate cells grown *in vitro* are susceptible to baculovirus transduction. A. Patient-derived cells transduced with BV-EGFP showing fluorescent protein expression after 24h. PE28-07=cancer sample, PE11-07=castration resistant, PE008-06= cancer sample. B. Cell viability, as measured by MTS assay, in malignant and benign patient-derived prostate cells transduced with BV-NTR and treated with CB-1954 (20μM) for 48h.

4.3 hTGP promoter characterization

4.3.1 hTGP expression is highly prostate specific

In order to construct a successful gene therapy vector one of the most important characteristics is a tightly regulated tissue-specific promoter. For this the hTGP promoter was chosen as a potential prostate-specific promoter for use in our system.

Previous reports suggested that hTGP expression was restricted to the prostate using Northern blot analysis (Dubbink et al. 1998). To expand on this finding and quantitatively compare hTGP expression to that of other known prostate-specific genes such as PSA and TMPRSS2, a cDNA qPCR-array containing cDNA samples from 48 different healthy human tissues was carried out. Figure 14 shows the expression levels of hTGP, PSA and TMPRSS2 in a panel of different human tissues. hTGP expression is almost 200 times higher in the prostate than in the next highly expressing tissue, the testis. In total hTGP expression was detected in 21/48 tissues. While PSA expression was more than 400 times higher in the prostate than in the next highly expressing tissue (fat), PSA was detected in 32/48 tissues, suggesting that although hTGP could be less abundant in the prostate than PSA, it is crucially more prostate-specific. TMPRSS2 expression was highest in the prostate, but was surprisingly also detected in high levels in different tissues such as colon, pancreas, stomach and lungs (figure 14). TMPRSS2 mRNA expression could be detected in 39/48 tissues, casting serious doubts on what is considered to be a prostate-specific gene.

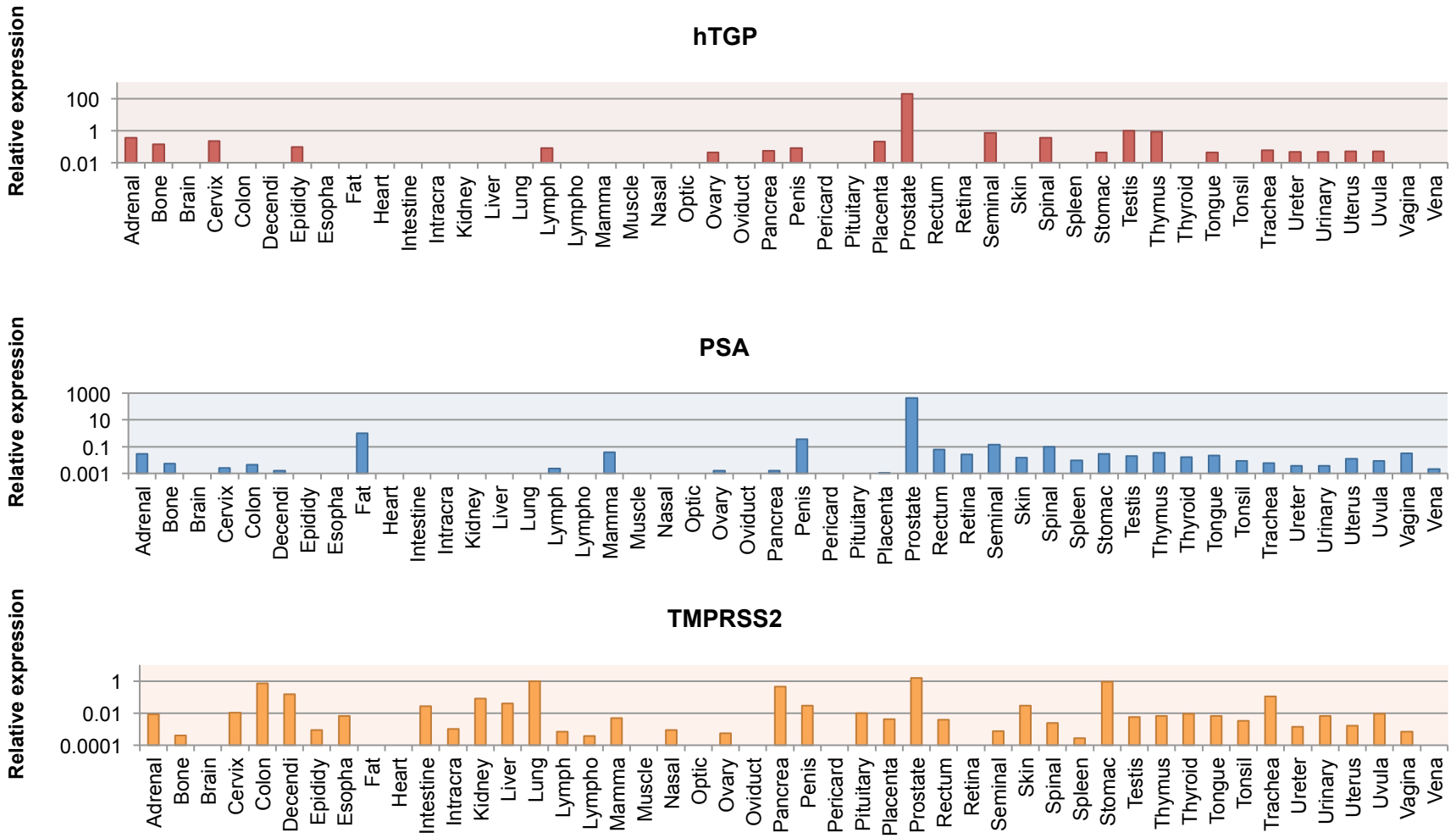


Figure 14. hTGP, PSA and TMPRSS2 mRNA expression profile in human tissues. Expression values were normalized to the second highest expressing tissue.

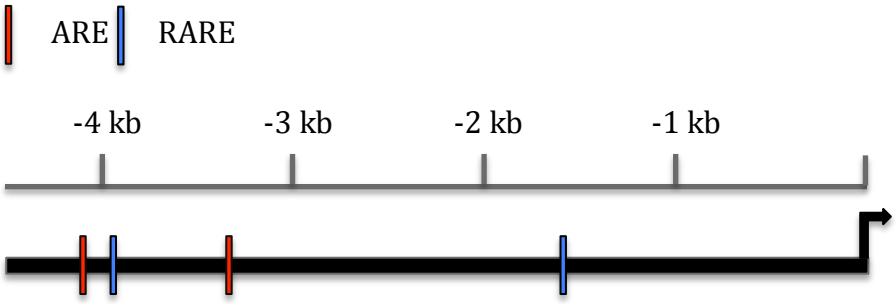
4.3.2 hTGP expression in prostate cell lines is controlled by retinoic acid

Previous reports suggested that androgens indirectly regulated hTGP expression in the prostate cancer cell line PC346C and that the elements responsible for this regulation were outside the 2.1kb proximal promoter characterized at this time (Dubbink et al. 1996; Dubbink et al. 1999a). It was therefore decided to carry out a bioinformatic analysis on a larger, 4.5kB portion of the hTGP in order to find putative binding sites for transcription factors important in prostate-specific expression. Figure 15A shows a schematic representation of AREs and RAREs found in the 4.5kb hTGP promoter and figure 15B shows the consensus sequences used to search for AREs and RAREs.

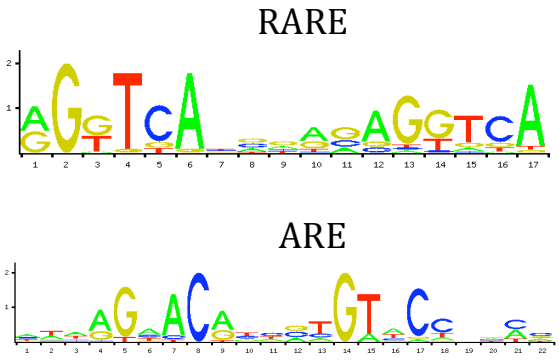
As mentioned in the introduction, AR and RARs play important roles in prostate development and homeostasis and thus it was decided to investigate the role of both receptors in the regulation of hTGP expression. A panel of prostate cell lines were treated with 500nM *all trans* retinoic acid (atRA) for 24h, and hTGP mRNA expression was assessed. Figure 15C shows that hTGP expression increased in LNCaP and PC346C in response to atRA treatment, while PNT1A and PNT2C2 cells showed a decrease in hTGP expression. To investigate the kinetics of the response seen in hTGP expression to atRA in LNCaP cells, RNA samples from LNCaP cells treated with 500nM atRA were taken after incubation time-points between 2-24h. hTGP expression significantly increased 4h after atRA treatment and continued increasing up to 24h after the start of the treatment (figure 15D). Such a rapid response suggested that the effect of atRA

on hTGP expression was caused directly by the activity of a receptor stimulated by atRA, most likely the RARs.

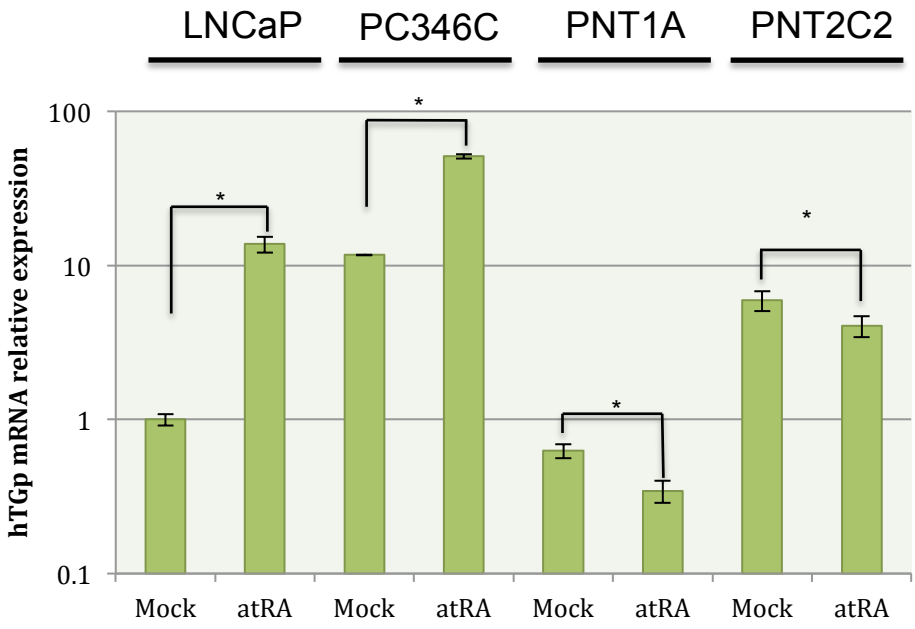
A


ARE
RARE
-4 kb
-3 kb
-2 kb
-1 kb

B


RARE
ARE

C


LNCaP
PC346C
PNT1A
PNT2C2
hTGp mRNA relative expression
100
10
1
0.1
*
*
*
*
Mock
atRA
Mock
atRA
Mock
atRA
Mock
atRA

D

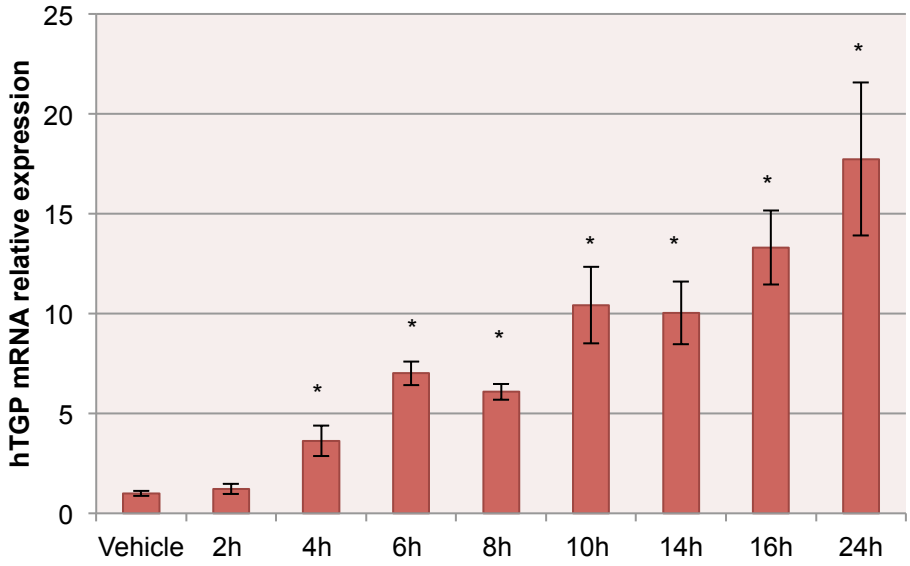


Figure 15. Retinoic acid regulates hTGP mRNA expression in prostate cell lines. A. Depiction of AREs and RAREs found in a 4.5kb portion of the hTGP promoter by bioinformatics analysis using the JASPAR database. B. Graphic representation of the consensus sequences used to determine AREs and RAREs in the hTGP promoter (taken from the JASPAR database website). C. hTGP mRNA expression in prostate cell lines LNCaP, PC346C, PNT1A and PNT2C2 treated with either 500nM atRA or vehicle for 24h as measured by qPCR. D. hTGP mRNA levels in LNCaP cells treated with 500nM atRA between 2-24h. The symbol * denotes statistical significance respect to control as measured by Student-T test ($p<0.05$).

4.3.3 Prostate cell lines have different abilities to activate transcription following atRA treatment

LNCaP and PC346C cells were shown to increase hTGP mRNA levels following atRA treatment, while PNT1A and PNT2C2 cells displayed decreased hTGP expression. To find out differences between the cell lines that could explain this differential regulation, total RAR protein levels and mRNA levels of RAR isoforms were evaluated. It was hypothesised that different RAR expression levels or expression of certain RAR isoform(s) could be responsible for the distinct hTGP regulation between cell lines. To test this hypothesis, western blot analysis evaluating RAR expression in LNCaP, PC346C, PNT1A and PNT2C2 was carried out. Figure 16A shows that total RAR protein levels were higher in PNT1A and PNT2C2 cells, meaning that the differential response of hTGP to atRA was not due to a lack of RAR expression driving.

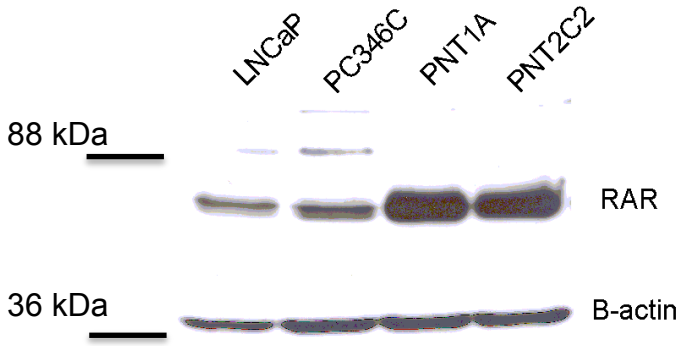
RARA, RARB and RARG mRNA levels across the panel of prostate cell lines did not produce a clear pattern, suggesting that preferential expression of one or more isoforms could be responsible for the differences between the cell lines' response to atRA treatment regarding hTGP expression (figure 16B).

To test the overall ability of each cell line to activate transcription following atRA treatment, prostate cell lines were transfected with a luciferase reporter plasmid where the active regulatory element is composed of a TATA box element and a tandem of RAREs, that upon ligand-bound RAR recognition and binding activate luciferase transcription (figure 16C). At 18h after transfection cells were treated with increasing concentrations of atRA, and luciferase activity measured

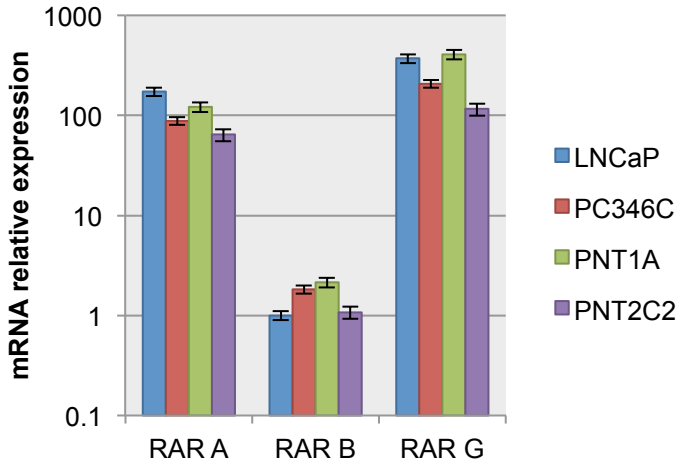
after a further 24h. LNCaP and PC346C were able to activate luciferase expression following atRA treatment reaching 10.6- and 15.7-fold induction, respectively when cells were treated with 1μM atRA (figure 16D upper panels). PNT1A and PNT2C2 cells also showed an increase in luciferase expression following atRA treatments, 2.8- and 4.5-fold increase, respectively (figure 16D bottom panels). However, the magnitude of this increase was modest when compared to that of LNCaP and PC346C after atRA induction.

These results suggested a correlation between the ability of the cell lines to induce transcription in response to atRA treatment and the induction of hTGP expression after atRA treatment. More interestingly was the observation that LNCaP and PC346C cells, which activate hTGP expression after atRA treatment, possess characteristics of a more differentiated cell type (AR and PSA expression), while PNT1A and PNT2C2 are less differentiated. This has more relevance since hTGP expression was only detected in the luminal compartment of the prostate (Dubbink et al. 1999b), meaning that it was expressed exclusively by more differentiated cells. Our results suggest that there is a differential regulation of hTGP expression depending on the differentiation status, which is supported by previous reports.

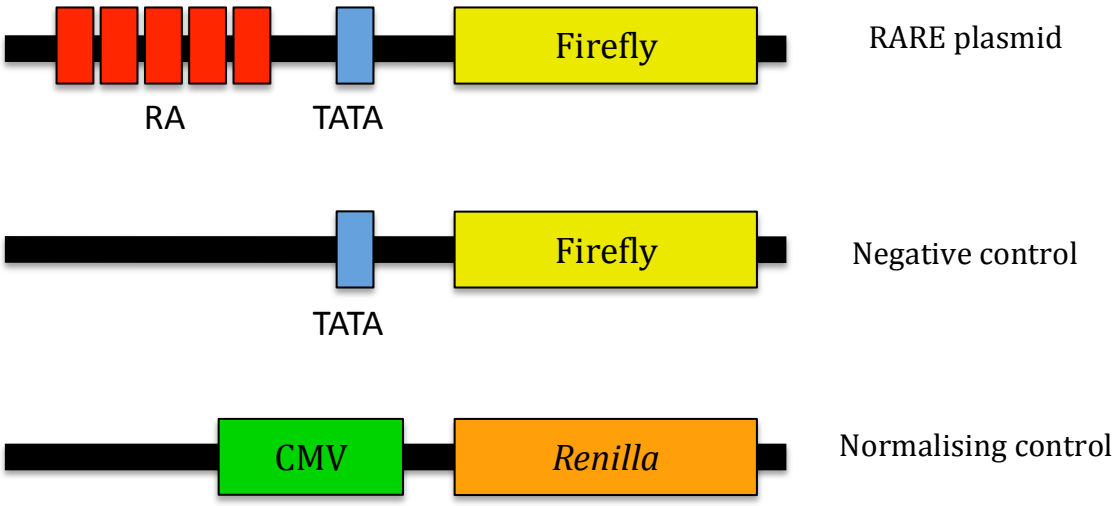
A


LNCaP
PC346C
PNT1A
PNT2C2
88 kDa
RAR
36 kDa
B-actin

B


1000
100
10
1
0.1
mRNA relative expression
RAR A
RAR B
RAR G
LNCaP
PC346C
PNT1A
PNT2C2

C


Firefly
RARE plasmid
RA
TATA
Firefly
Negative control
TATA
CMV
Renilla
Normalising control

D

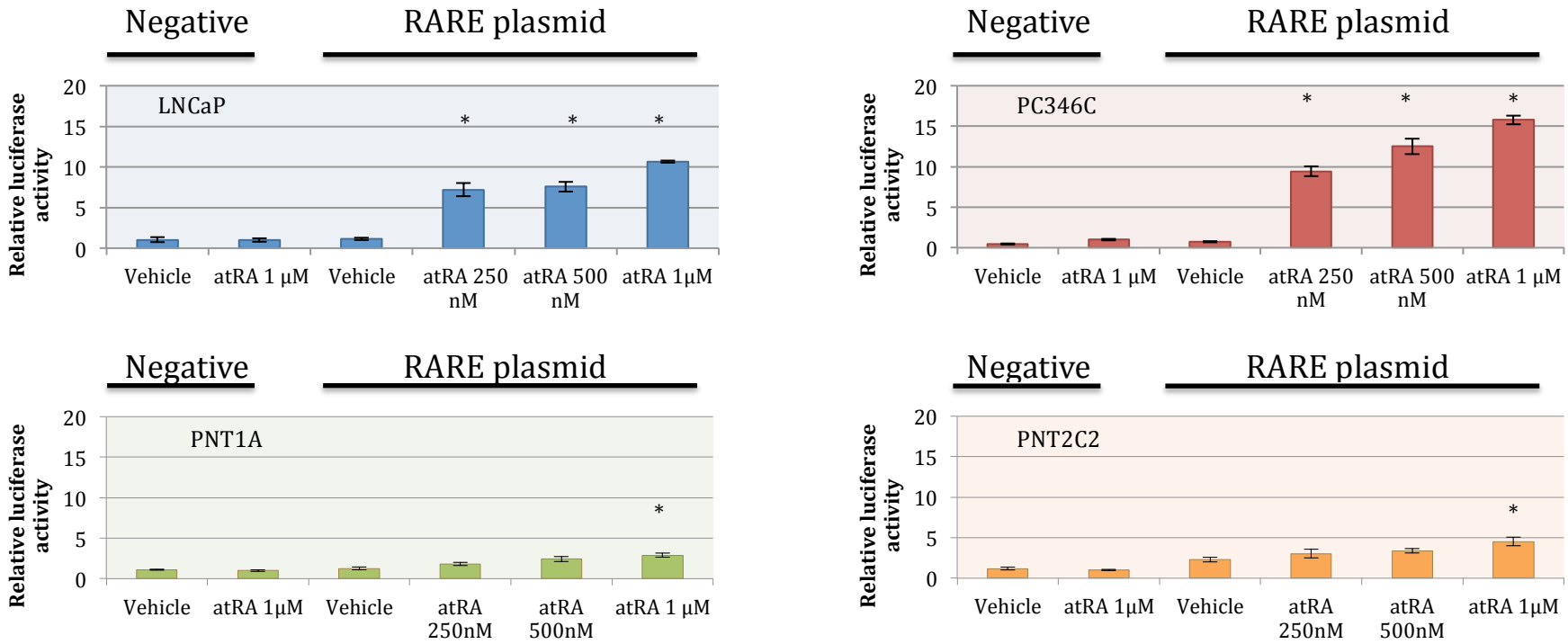
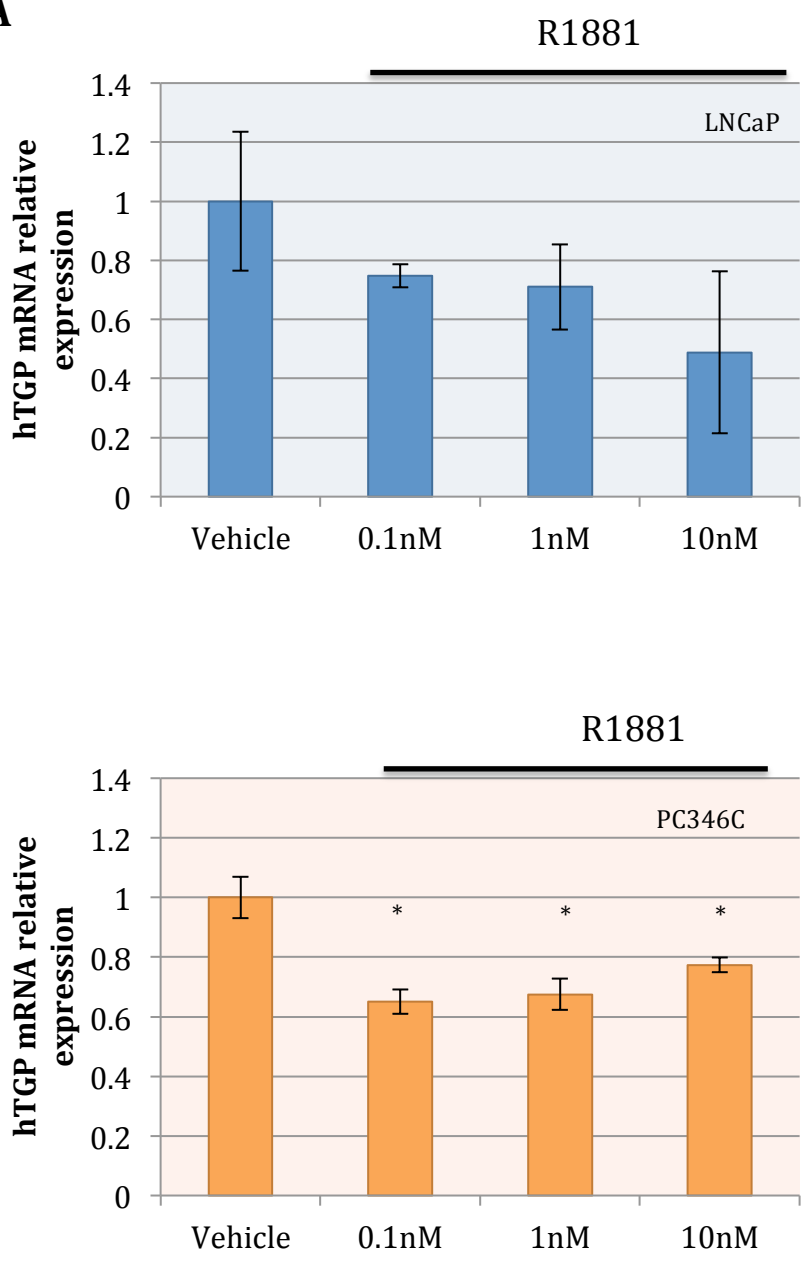


Figure 16. Differential regulation of hTGP expression could be caused by differential transcriptional activity in response to atRA treatment**.** A. Western blot analysis of RARs in LNCaP, PC346C, PNT1A and PNT2C2. β-actin was used as a loading control. B. RAR isoforms mRNA expression profile in LNCaP, PC346C, PNT1A and PNT2C2 cells measured by qPCR. C. RARE reporter and control plasmids depicting regulatory elements. D. Luciferase activity in transfected prostate cell lines in response to increasing concentrations of atRA. Luciferase activity was normalised to the values of the cells transfected with the negative control and treated with 1µM atRA. The symbol * denotes statistical significance with respect to control as measured by Student-T test ($p<0.05$).

4.3.4 hTGP expression is repressed by androgens

Once it was established that hTGP expression could be regulated by atRA, it was decided to test the effect of androgens on the regulation of hTGP expression. For this purpose, AR expressing cells LNCaP and PC346C were treated with increasing concentrations of the synthetic androgen R1881 for 24h. hTGp expression decreased after R1881 treatments in both LNCaP and PC346C cells (figure 17A). Because R1881 and atRA had opposing effects on hTGP expression, it was decided to co-treat LNCaP and PC346C cells with R1881 and atRA together to evaluate whether the positive effect of atRA could antagonize the negative effect of R1881 on hTGP expression or vice versa. Figure 17B shows RT-PCR analysis of hTGP mRNA levels in LNCaP and PC346C cells either treated with atRA, R1881 or a combination of both compounds. As a positive control for R1881 treatments, PSA expression, known to be up-regulated by androgens, was monitored. As previously seen, hTGP mRNA levels in untreated cells were higher in PC346C than in LNCaP (figure 17B). After atRA treatment hTGP mRNA levels increased in both cell lines (compare lanes 5 and 6). However when the cells were co-treated with atRA and R1881, induction of hTGP mRNA was completely disrupted in LNCaP cells, while in PC346C hTGP mRNA levels decreased (figure 17C). These results indicated that androgen treatment was capable of cancelling the positive effect of atRA on hTGP expression.

A


R1881
LNCaP
hTGP mRNA relative expression
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Vehicle
0.1nM
1nM
10nM
R1881
PC346C
hTGP mRNA relative expression
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
*
*
Vehicle
0.1nM
1nM
10nM

B

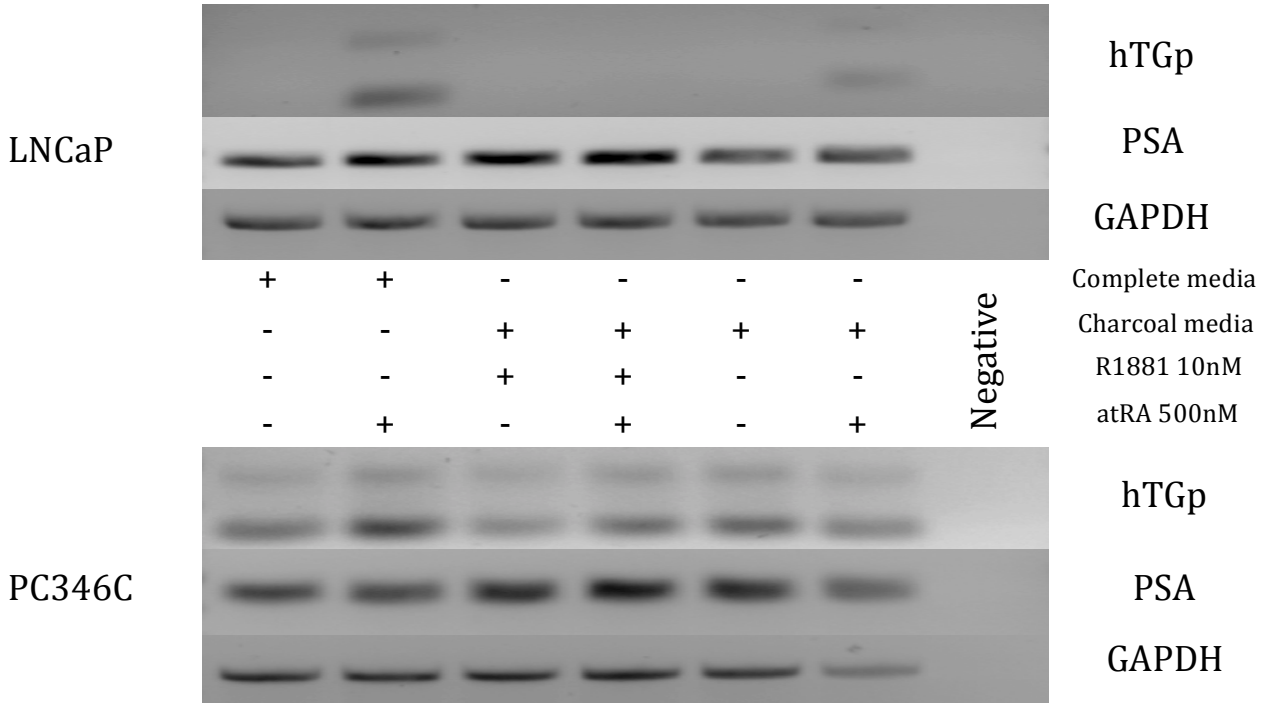


Figure 17. Androgens have a negative effect on hTGP mRNA expression**.** A. LNCaP and PC346C hTGP mRNA expression 24h after 10nM R1881 or vehicle treatments. B. hTGP, PSA and GAPDH mRNA expression in LNCaP and PC346C cells treated with 500nM atRA, 10nM R1881 or a combination of both hormones for 24h. The symbol * denotes statistical significance with respect to control as measured by Student-T test ($p<0.05$).

4.3.5 AR knockdown does not rescue hTGP expression after R1881 treatment in LNCaP cells

LNCaP cells were transfected with AR-specific siRNA to evaluate the effect of this protein knockdown in hTGP expression both in basal conditions and following R1881 treatment. AR mRNA was successfully knocked down (75% knockdown) following AR-specific siRNA transfection in LNCaP cells (figure 18A). AR protein also suffered a significant knockdown, as measured by western blot analysis, where AR protein could not be detected (figure 18B). AR knockdown in LNCaP cells was expected to rescue hTGP mRNA expression since androgen treatments decreased hTGP expression. However, AR knockdown did not result in hTGP expression rescue, but in a further repression of hTGP mRNA expression in basal conditions and following R1881 treatment (figure 18C).

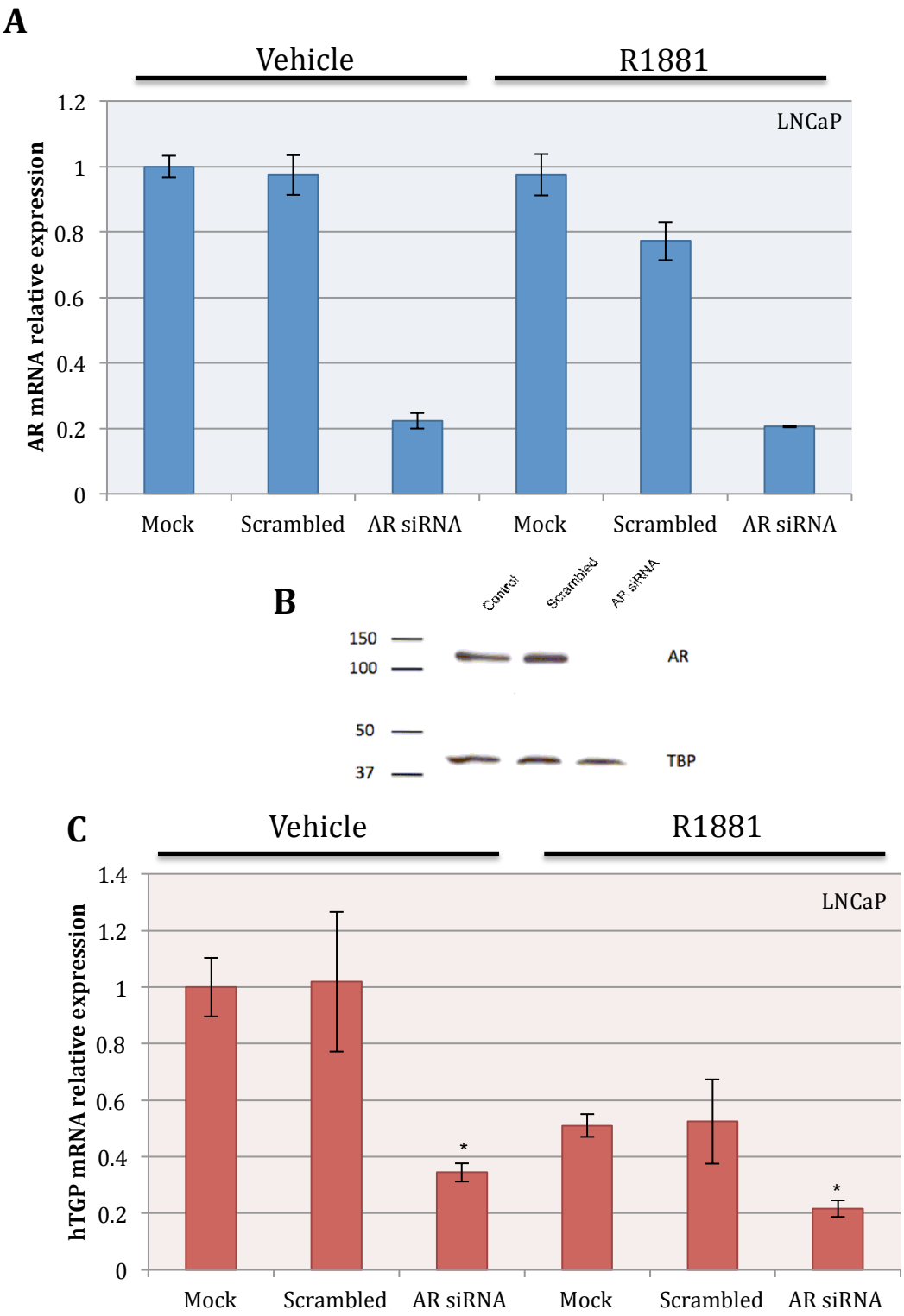


Figure 18. AR knockdown affects hTGP expression**.** A. AR mRNA expression in LNCaP cells transfected with AR-specific siRNA and treated with 10nM R1881 or vehicle as control. B. AR protein levels in LNCaP cells transfected with AR-specific siRNA 72h after transfection. TBP was used as a loading control. C. hTGP mRNA levels in AR knockdown LNCaP cells treated with 10nM R1881 or vehicle. The symbol * denotes statistical significance with respect to control as measured by Student-T test ($p<0.05$).

4.3.6 AR knockdown interferes with atRA-dependent hTGP expression

The observation that AR knockdown affected hTGP expression prompted the question of whether AR was necessary for atRA-dependent stimulation of hTGP mRNA levels. For this purpose, LNCaP cells were transfected with AR-specific siRNA and then treated with atRA for a further 24h. hTGP expression was negatively affected by AR knockdown in vehicle-treated cells as reported previously, but it also affected atRA-dependent hTGP mRNA expression, decreasing its expression by around 6.5-fold (figure 19A). To compare the behaviour of hTGP expression to that of a known AR-target gene, PSA levels in AR knockdown LNCaP cells were measured. Figure 19B demonstrates that PSA was down-regulated by AR knockdown in both vehicle-treated and R1881-treated cells. These results implied that AR was not regulating hTGP in the same way that it regulates PSA, or for that matter any other known prostate specific gene. To further investigate the role of the AR in hTGP expression, it was decided to evaluate how AR knockdown affected RAR expression. It was considered that if AR affected hTGP expression, it could do so indirectly by regulating the expression of any of the RAR, thus a down-regulation in AR would lead to a down-regulation in RAR expression and therefore low levels of hTGP expression. RAR mRNA levels in AR-knockdown LNCaP cells are shown in figure 19C. While RARB expression remained unaltered, RARG and RARA expression was increased in cells with low AR levels.

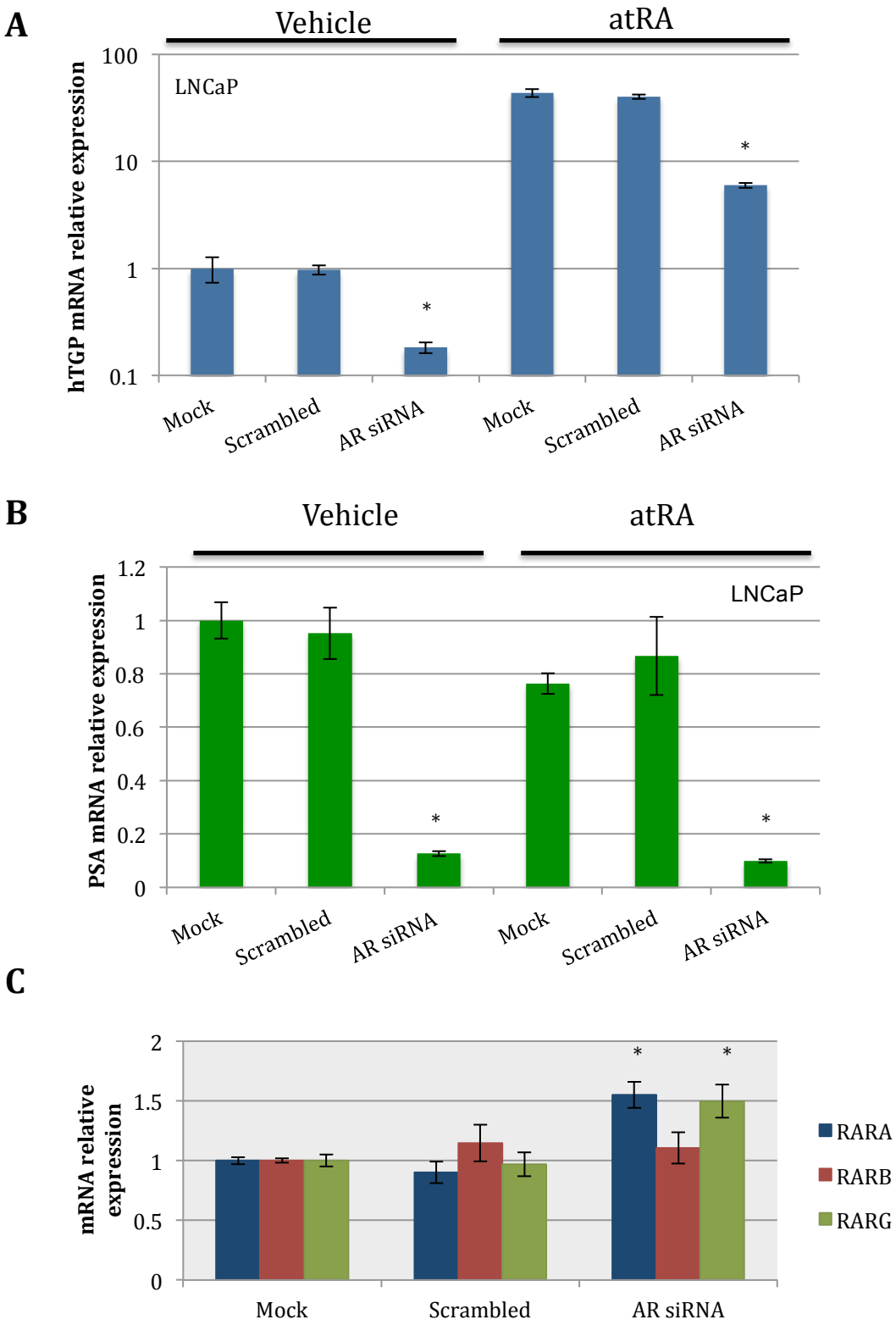


Figure 19. AR is necessary for atRA-dependent expression of hTGP. A. hTGP mRNA expression in LNCaP cells transfected with AR-specific siRNA and treated with 500nM atRA. B. PSA mRNA expression in LNCaP cells transfected with AR-specific siRNA and treated with 500nM atRA. C. RARA, RARB and RARG mRNA expression in AR-siRNA transfected LNCaP cells. The symbol * denotes statistical significance with respect to control as measured by Student-T test ($p<0.05$).

4.3.7 AR transcriptional activity is not necessary for atRA dependent hTGP up-regulation

When AR-specific siRNA was transfected into LNCaP cells, it induced a down-regulation in AR receptor protein levels. Low levels of AR caused hTGP expression to be down-regulated, but whether this down-regulation was caused by the lack of the AR transcriptional activity or the low protein levels could not be distinguished. In order to investigate if the participation of the AR transcriptional activity was necessary for the atRA-dependent hTGP up-regulation, LNCaP cells were treated with bicalutamide. Bicalutamide is an AR inhibitor that binds to the AR, allowing it to recognize and bind to AREs in the DNA but it prevents the recruitment of co-activators that promote transcription from target genes (Masiello et al. 2002). LNCaP cells were pre-treated with 5μM bicalutamide 12h before R1881 or atRA addition, and hTGP was evaluated after a further 24h. To investigate if the bicalutamide treatment was effective, PSA mRNA levels were measured in LNCaP cells treated with R1881 and/or 5μM bicalutamide. Figure 20A shows that PSA mRNA levels increased in response to R1881 treatment, while bicalutamide and R1881 co-treatment resulted in almost a 50% reduction in PSA mRNA expression when compared to R1881 treated cells. hTGP mRNA expression in cells co-treated with bicalutamide and atRA remained unaltered, implying that the AR transcriptional activity was not necessary for atRA-dependent up-regulation of hTGP mRNA levels (figure 20B).

Another hypothesis that could explain why AR was necessary for atRA-induced hTGP expression would be that atRA were directly or indirectly promoting AR

shuttling into the nucleus. It has been reported that the AR does not interact with atRA and therefore is not activated by this compound (Fong et al. 1993). However, whether atRA treatment indirectly alters AR cellular localization was still unknown. To investigate this, an immunofluorescence assay was performed in LNCaP and PC346C cells to determine the AR localization after R1881 and atRA treatments. LNCaP and PC346C cells were grown in charcoal-stripped media for 24h, then treated with R1881 or atRA for 2h before cell fixation. AR localization in cells grown in charcoal stripped media was both nuclear and cytoplasmic in both LNCaP and PC346C cells (figure 21 left panels). After R1881 treatment, most of the AR had shuttled to the nucleus and very little remained in the cytoplasm (figure 21 middle panels). After atRA treatment, AR was not visibly re-localized to the nuclei of the cells, suggesting that atRA did not indirectly alter AR subcellular localization (figure 21 right panels). Thus this mechanism is not important in the AR regulation of atRA-dependent hTGP expression.

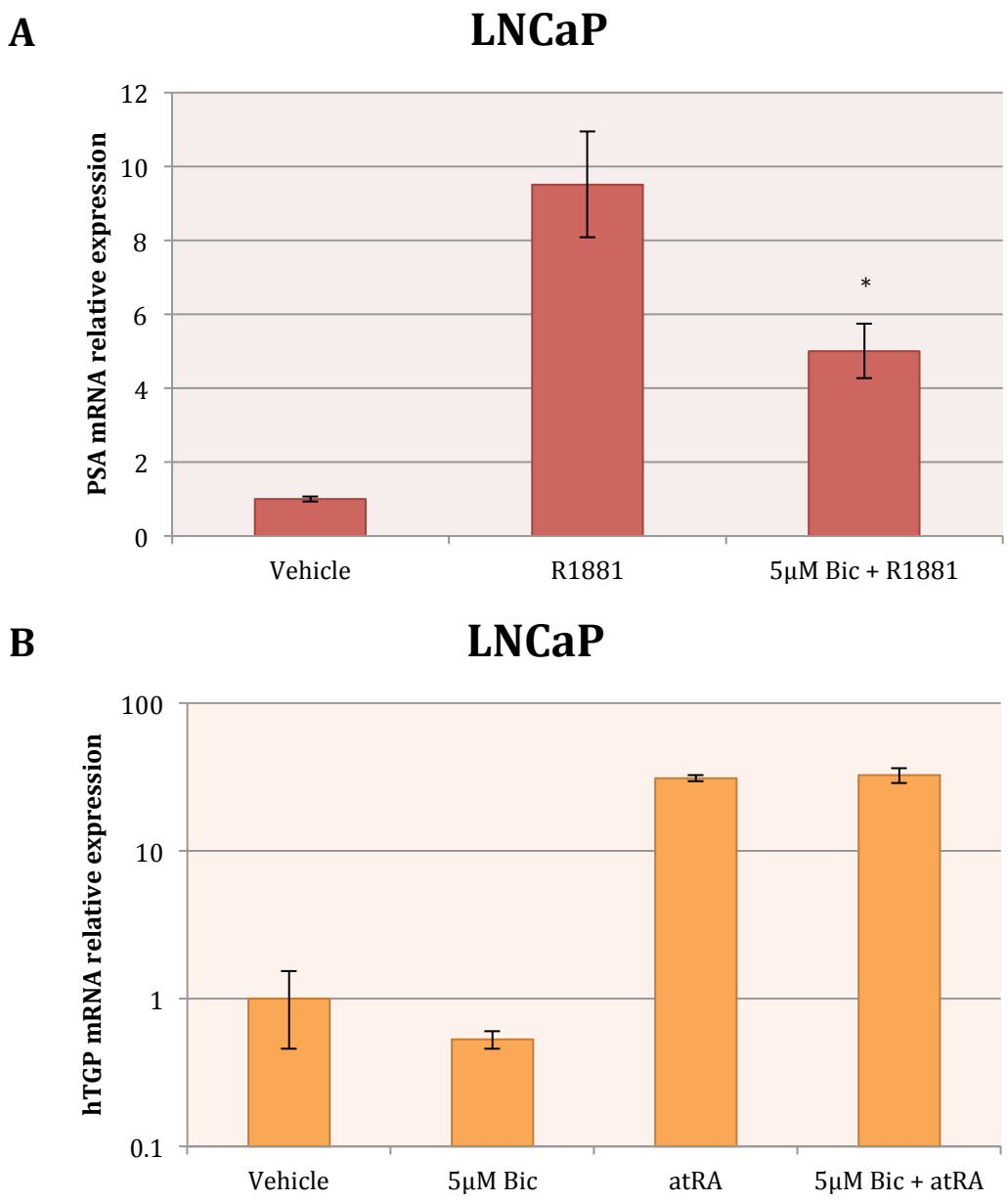


Figure 20. AR transcriptional activity is not necessary for atRA-induced hTGP mRNA expression**.** A. PSA mRNA expression in LNCaP cells treated with 10nM R1881, 5µM bicalutamide or a combination of both 24h after treatment. B. hTGP mRNA levels in LNCaP cells treated with 500nM atRA, 5µM bicalutamide or a combination of both for 24h. The symbol * denotes statistical significance with respect to R1881 treated cells as measured by Student-T test ($p<0.05$).

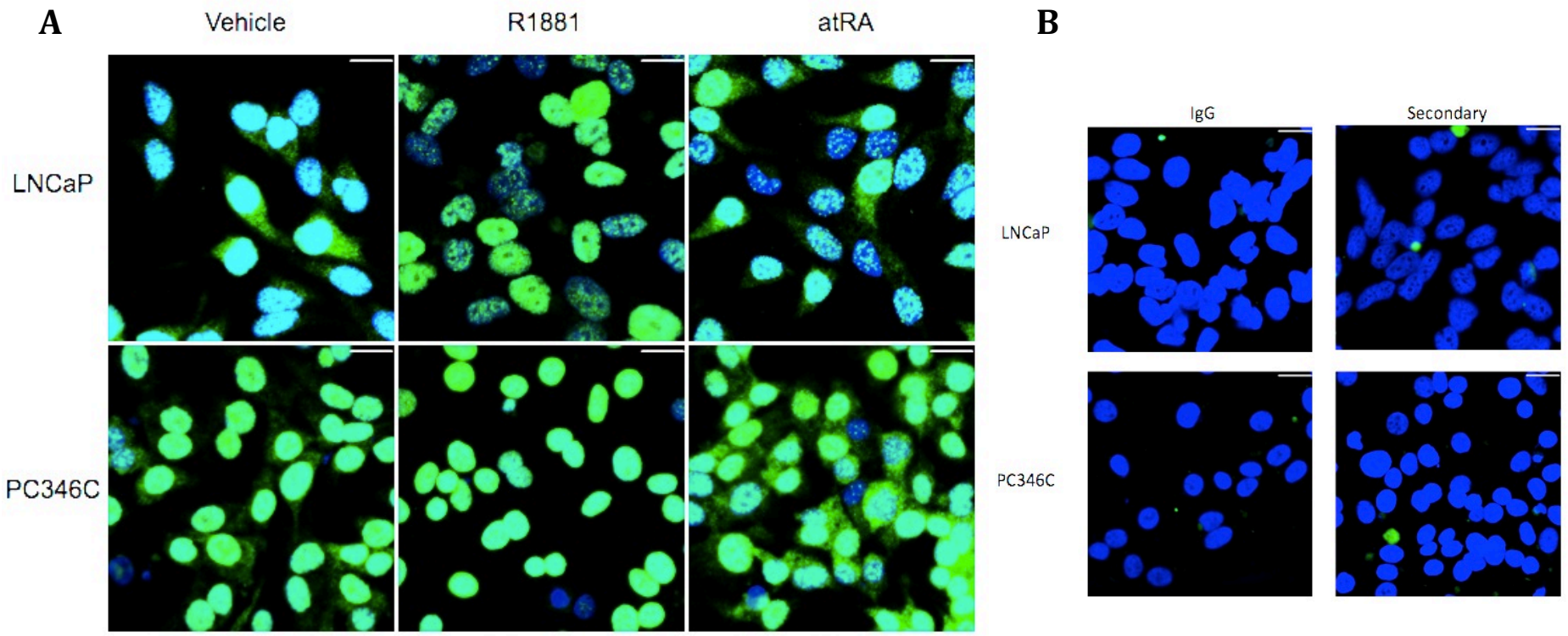


Figure 21. AR subcellular localization in LNCaP and PC346C cells. A. AR subcellular localization was detected by immunofluorescence in LNCaP and PC346C cells treated with either 10nM R1881 or 500nM atRA for 2h. B. Controls showing immunofluorescence procedure using IgG instead of primary antibody and secondary antibody only. The white bar in the pictures is equivalent to 20μm.

4.3.8 RARG plays a major role in atRA-dependent hTGP mRNA expression

Retinoic acid exerts most of its effects through the RARs. To confirm that atRA-dependent regulation of hTGP expression was mediated through the RARs, LNCaP and PC346C cells were treated with 250 and 500nM of the synthetic retinoid (4-[(E)-2-(5,6,7,8-Tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl]-benzoic acid) or TTNPB for 24h, and hTGP expression analysed by qPCR. This compound exclusively binds to the RARs and mediates transcription from their target genes (Astrom et al. 1990; Schug et al. 2007). Figure 22 shows that hTGP mRNA expression was up-regulated after TTNPB treatment in both LNCaP and PC346C cell lines. This result indicated that one or more of the RARs was capable of inducing hTGP upon ligand stimulation. Because of their importance in prostate biology, RARB and RARG were thought to be ideal candidates to regulate hTGP expression. RARB is expressed early in the rat prostate and is often found to be down-regulated in prostate cancer, while lack of RARG results in hyperplastic lesions in the prostate of knockout mice (Lohnes et al. 1995; Aboseif et al. 1997; Nakayama et al. 2001). To determine whether RARB and/or RARG were involved in hTGP regulation, LNCaP cells were transfected with RARB or RARG specific siRNAs to knockdown gene expression. In order to determine the best concentration of specific siRNA to knockdown RARB and RARG expression, LNCaP cells were transfected with 25 and 12.5nM of specific and scrambled siRNA. The 12.5nM concentration was chosen due to the effective gene knockdown and lack of significant alterations by the scrambled siRNA at this concentration (figure 23). Figure 24A and 24C show RARB and RARG knockdown at the mRNA and

protein level, confirming that the specific mRNAs and proteins were down-regulated in LNCaP cells.

hTGP levels in RARB knockdown cells suffered a small decrease in the absence of atRA treatment (figure 24B). However, when cells were treated with 500nM atRA for 24h, there was no difference in hTGP expression between the mock-transfected cells, cells transfected with the scrambled siRNA and cells transfected with the RARB siRNA. This result implied that while RARB participates in hTGP basal expression, its role in atRA-dependent hTGP expression was nil.

RARG knockdown also resulted in a decrease in basal hTGP expression (figure 24D), but when RARG was knocked down in LNCaP cells with 500nM atRA treatment there was more than a 50% decrease in hTGP expression compared to hTGP expression in mock and scrambled siRNA transfected cells. These results therefore elucidated the importance of RARG in the atRA-dependent regulation of hTGP expression.

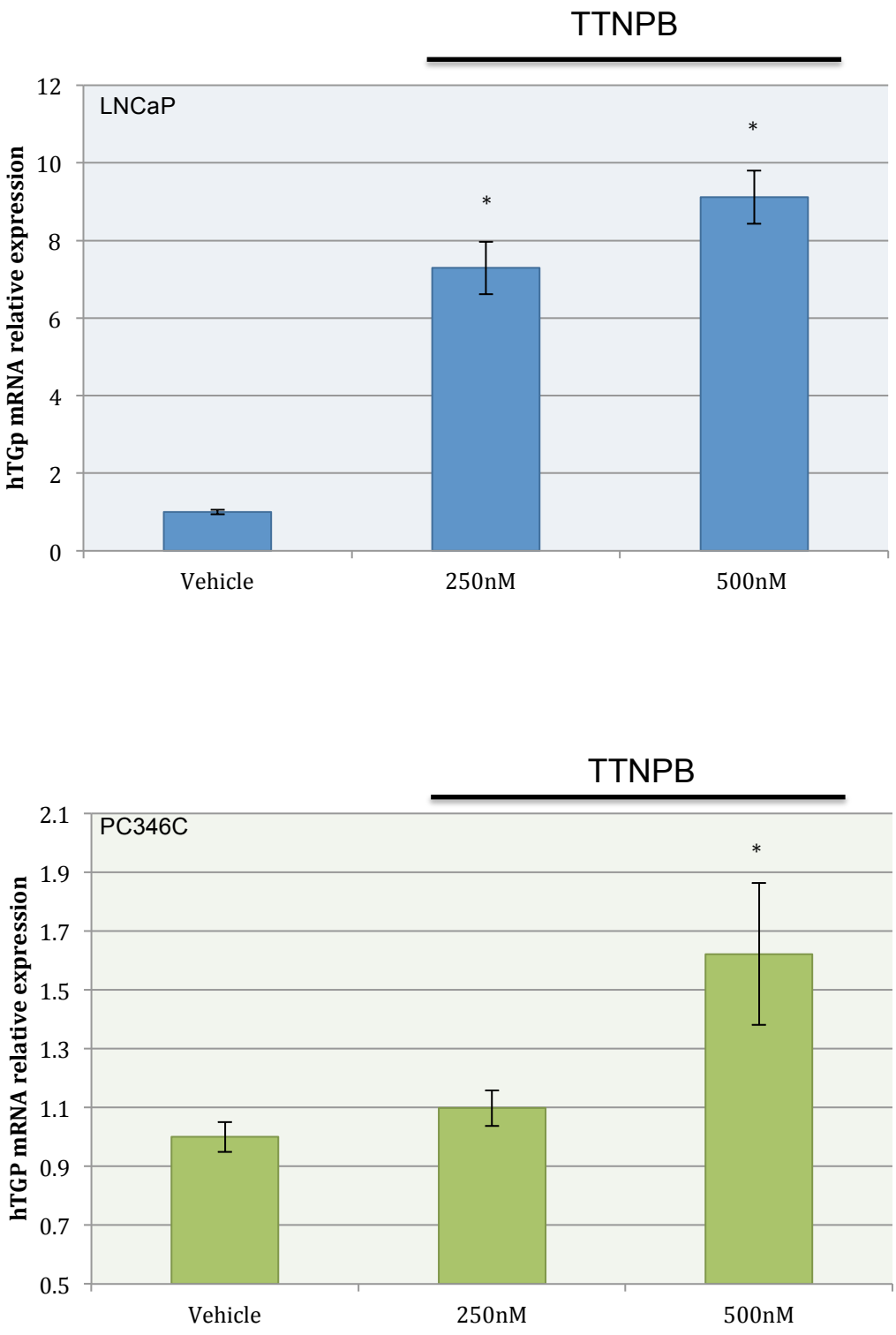


Figure 22. TTNPB activates hTGP expression in LNCaP and PC346C cells**.** The synthetic retinoid TTNPB, which exclusively binds to the RARs, is capable of promoting hTGP mRNA expression in LNCaP and PC346C cell lines 24h after treatment. The symbol * denotes statistical significance with respect to control as measured by Student-T test ($p<0.05$)**.**

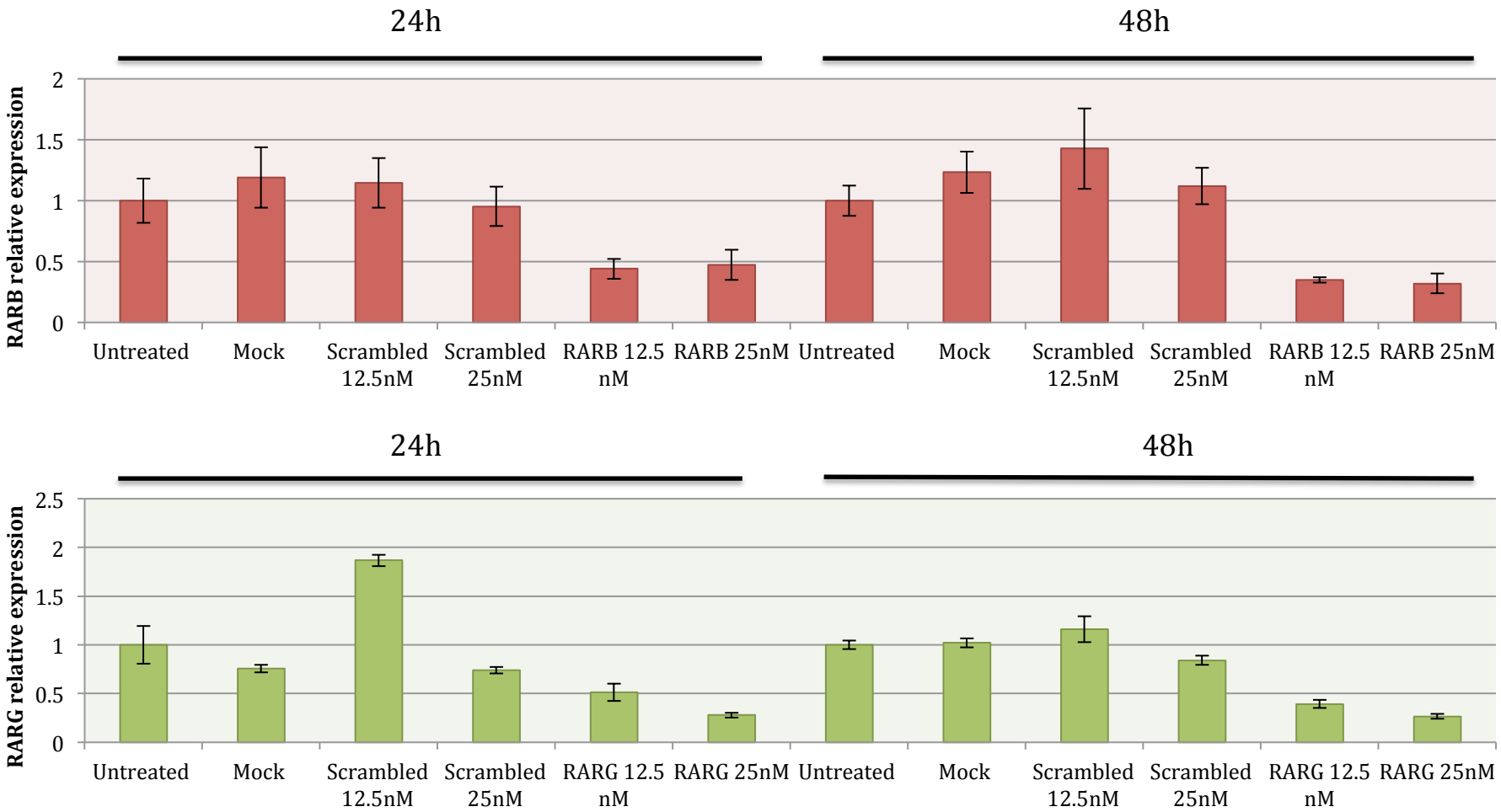
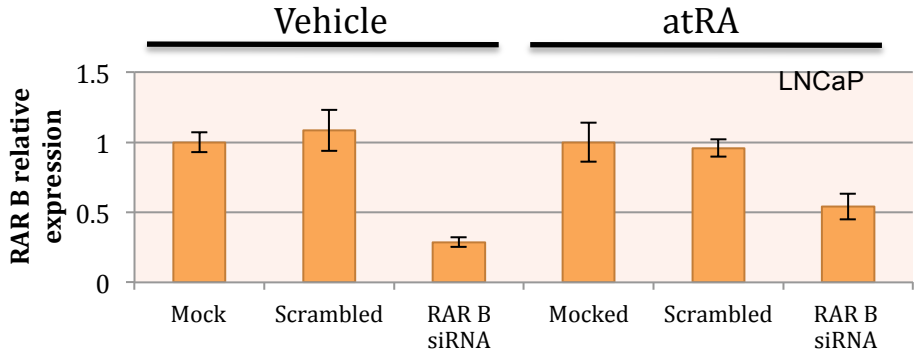
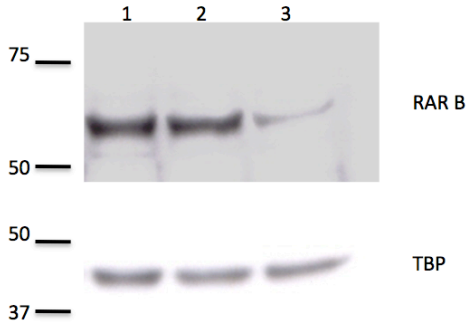


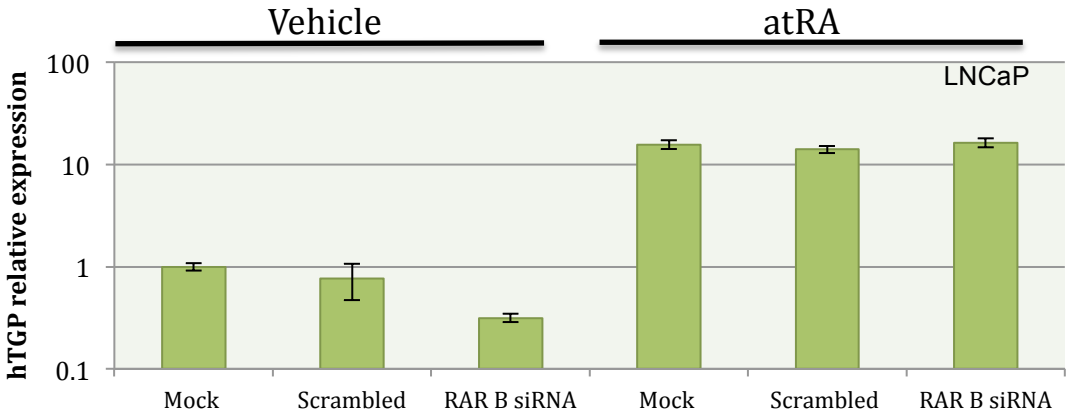
Figure 23. Selecting the best siRNA concentration to knockdown RARB and RARG expression. LNCaP cells were transfected with 25 or 12.5nM scrambled or specific siRNA and RARB and RARG mRNA expression was monitored at 24 and 48h.

A


Vehicle
atRA
LNCaP
RAR B relative expression
1.5
1
0.5
0
Mock
Scrambled
RAR B siRNA
Mocked
Scrambled
RAR B siRNA


1
2
3
75
50
50
37
RAR B
TBP

B


Vehicle
atRA
LNCaP
hTGP relative expression
100
10
1
0.1
Mock
Scrambled
RAR B siRNA
Mock
Scrambled
RAR B siRNA

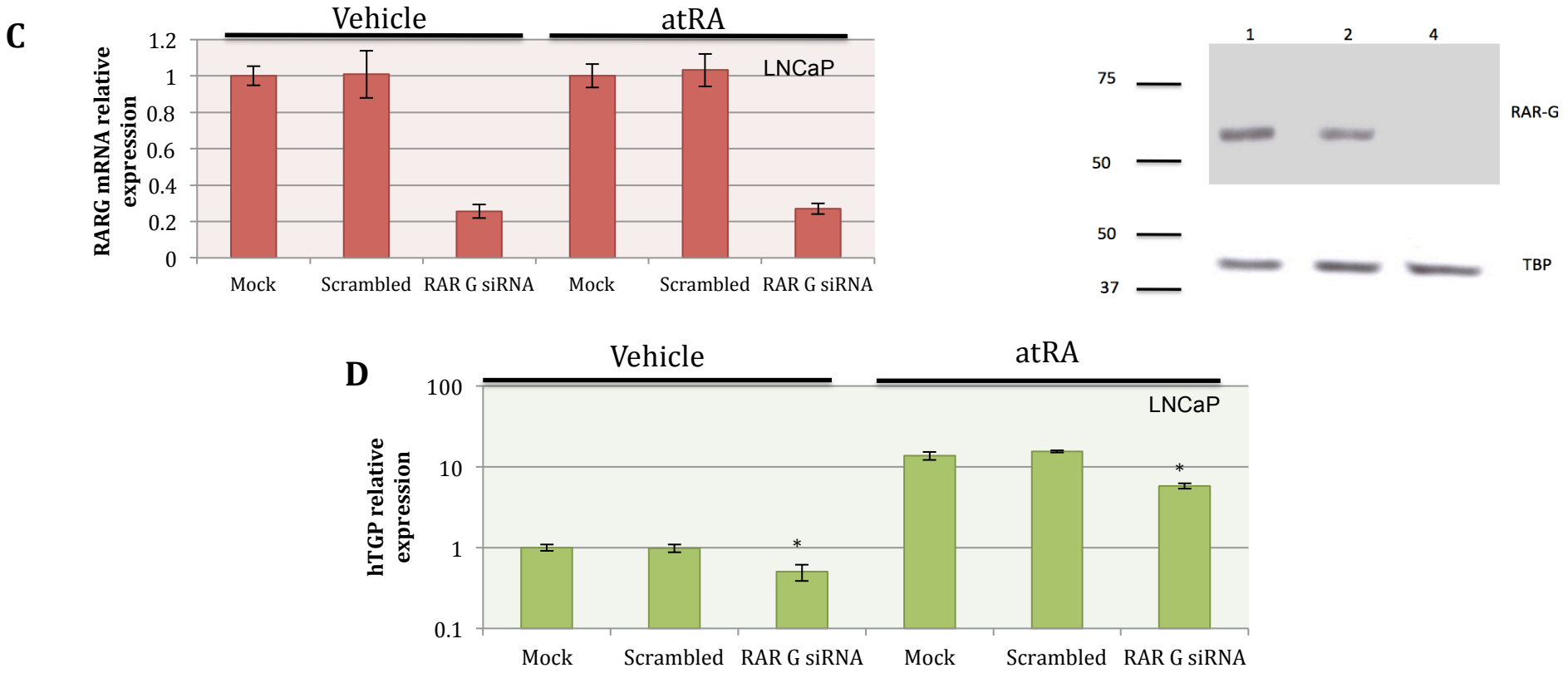


Figure 24. The role of RARB and RARG in hTGP regulation**.** A. RARB mRNA (left) and protein (right) levels in siRNA transfected LNCaP cells 24h after 500nM atRA or vehicle treatment for mRNA and 72h after siRNA transfection for western blot analysis. B. hTGP mRNA expression in RARB knockdown LNCaP cells treated with 500nM atRA or vehicle for 24h. C. RARG mRNA (left) and protein (right) levels in siRNA transfected LNCaP cells 24h after 500nM atRA or vehicle treatment for mRNA and 72h after siRNA transfection for western blot analysis. D. hTGP mRNA expression in RARG knockdown LNCaP cells treated with 500nM atRA or vehicle for 24h. The symbol * denotes statistical significance with respect to control as measured by Student-T test ($p<0.05$).

4.3.9 hTGP promoter characterization

A previous bioinformatics analysis suggested the presence of AREs and RAREs in a 4.5kb section of the hTGP promoter. To test whether functional AREs and/or RAREs were present in the hTGP promoter, a 4.5kb section of this regulatory element was cloned into the luciferase reporter plasmid pGL3 basic. As well as the 4.5kb hTGP promoter, truncated versions of the hTGP promoter lacking 5' DNA regions were cloned to investigate if loss of these regions resulted in lack of promoter activity (figure 25A). LNCaP cells were seeded in charcoal-stripped medium in 96 well-plates 24h before being co-transfected with the different hTGp-pGL3 promoter-reporter constructs with a normalising plasmid encoding the *renilla* luciferase gene under the control of the CMV promoter. 24h after transfection, cells were treated with either 500nM atRA, 10nM R1881 or vehicle, and luciferase activity was measured after a further 24h. Luciferase activity was normalised to the readings from LNCaP cells transfected with the empty vector (pGL3). Luciferase activity in cells transfected with the different version of the hTGP promoter showed increased activity when compared to the empty vector, demonstrating that all regions contained promoter activity. hTGP promoters between 1.5 and 3.5 kb showed the same luciferase activity in the absence of treatment, while the 4.5kb promoter showed increased luciferase expression, around 4-fold higher. This observation indicated the presence of a positive regulatory region located in the 5' region of the hTGP 4.5kb promoter that was active in cells without stimulation (figure 25B).

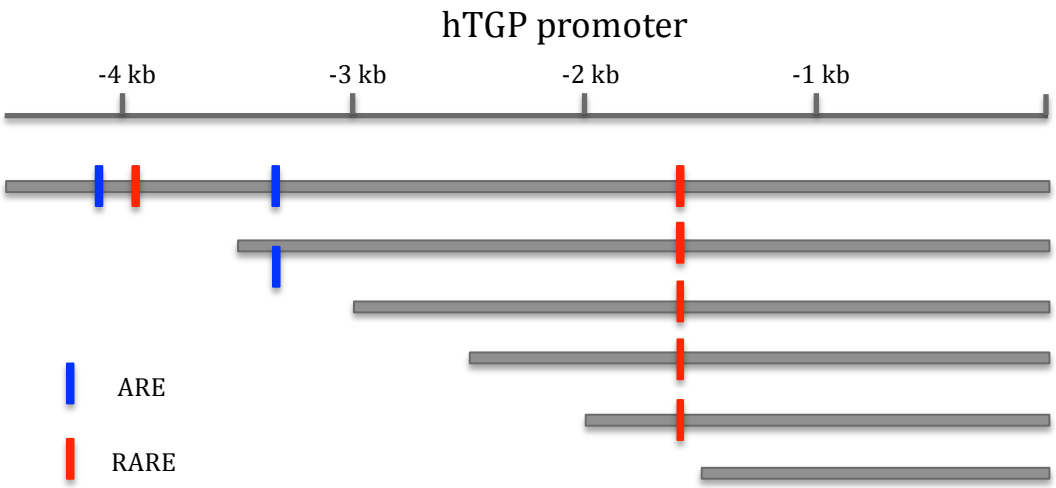
Transfected cells treated with 500nM atRA showed a significant decrease in luciferase activity, except for cells transfected with the 4.5kb hTGP promoter,

which displayed increased luciferase activity (more than 9-fold higher than vehicle treated cells), implying that within the region -4500 to -3550 of the hTGP there is at least one active RARE (figure 25C). This result is in accordance with the bioinformatics analysis suggesting the presence of a RARE located at -3962. The fact that further deletion of the hTGP promoter had no effect on luciferase activity conveyed that the other RARE found in the bioinformatics analysis was either not active or needed the presence of the 5' RARE or another regulatory sequence contained in the 5' region of the 4.5kb hTGP promoter for hTGP regulation.

R1881 treatment resulted in a decrease in luciferase activity only in those cells transfected with the 4.5kb hTGP promoter (around 40% reduction in luciferase activity), while no significant changes were seen in cells transfected with other constructs (as shown in figure 25D).

Taken together, these results indicated the presence of an active RARE and ARE in a 1kb region at the 5' of the 4.5kb hTGP promoter. Given that this region is not directly adjacent to the transcription start site of the gene and that there appeared to be no further important elements between this region and the minimal hTGP promoter, it is proposed that this region contains an enhancer regulated by both retinoic acid and androgen.

A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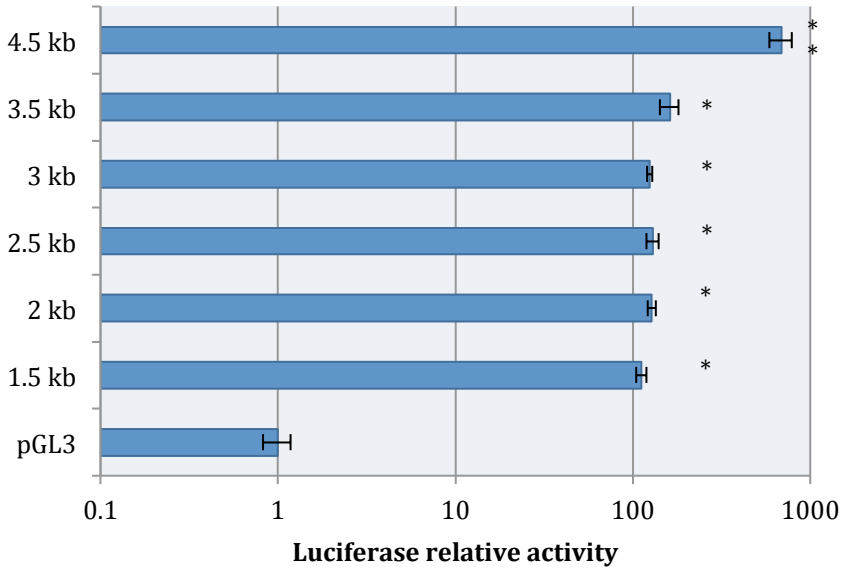
-3 kb
-2 kb
-1 kb
ARE
RARE

B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3.5 kb
3 kb
2.5 kb
2 kb
1.5 kb
pGL3
0.1
1
10
100
1000
Luciferase relative activity

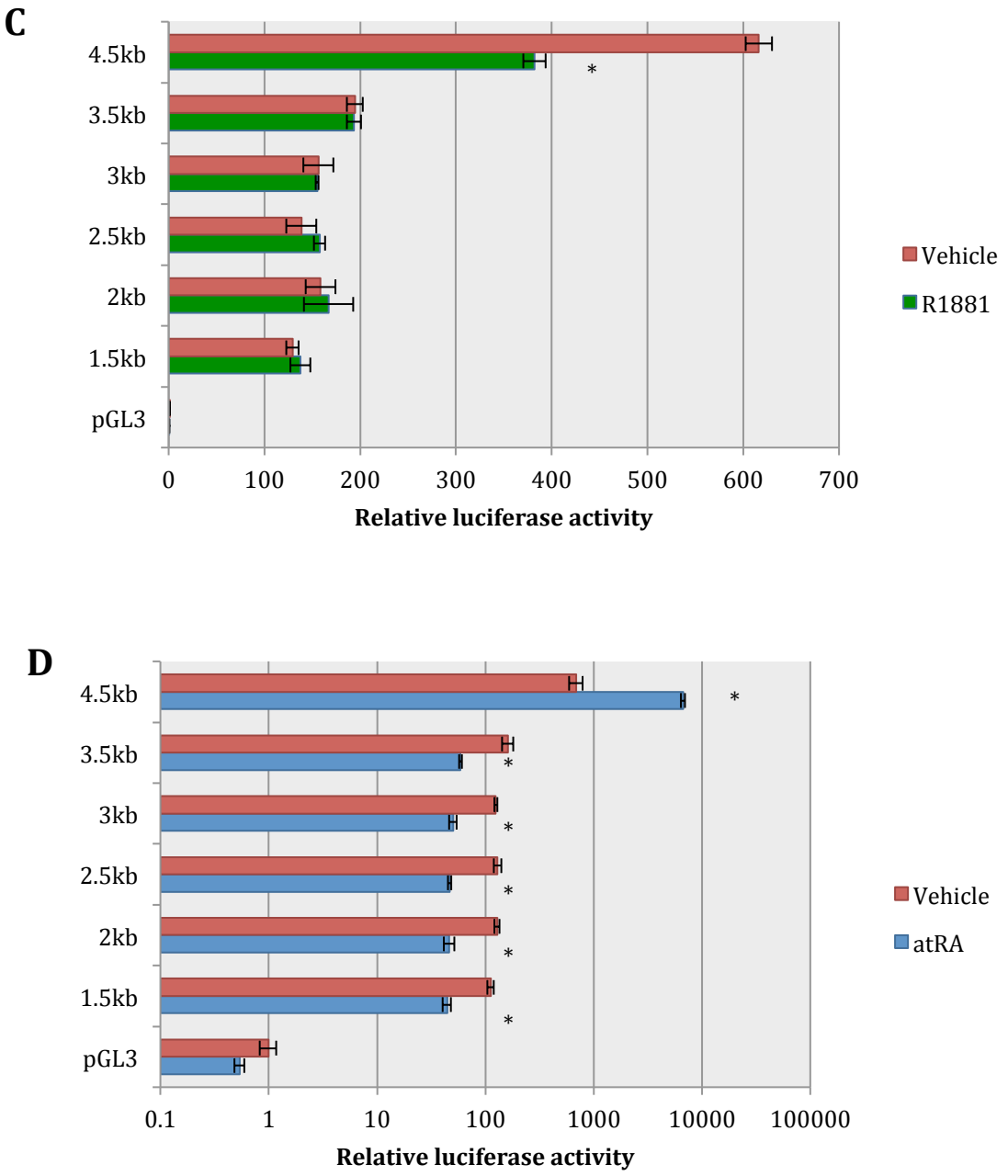


Figure 25. hTGP promoter analysis. A. Schematic representation of the hTGP regions, containing responsive elements cloned into the luciferase vector pGL3 basic. B, C and D. Relative luciferase activity in LNCaP cells co-transfected with different constructs containing the 4.5kb hTGP and several deletion mutant versions and the normalising plasmid CMV-pRL. Luciferase activity was measured 24h after transfection (B), 24h after 500nM atRA treatment, 48h after transfection (C), or 24h after 10nM R1881 treatment, 48h after transfection (D). The symbol * denotes statistical significance with respect to control as measured by Student-T test ($p<0.05$).

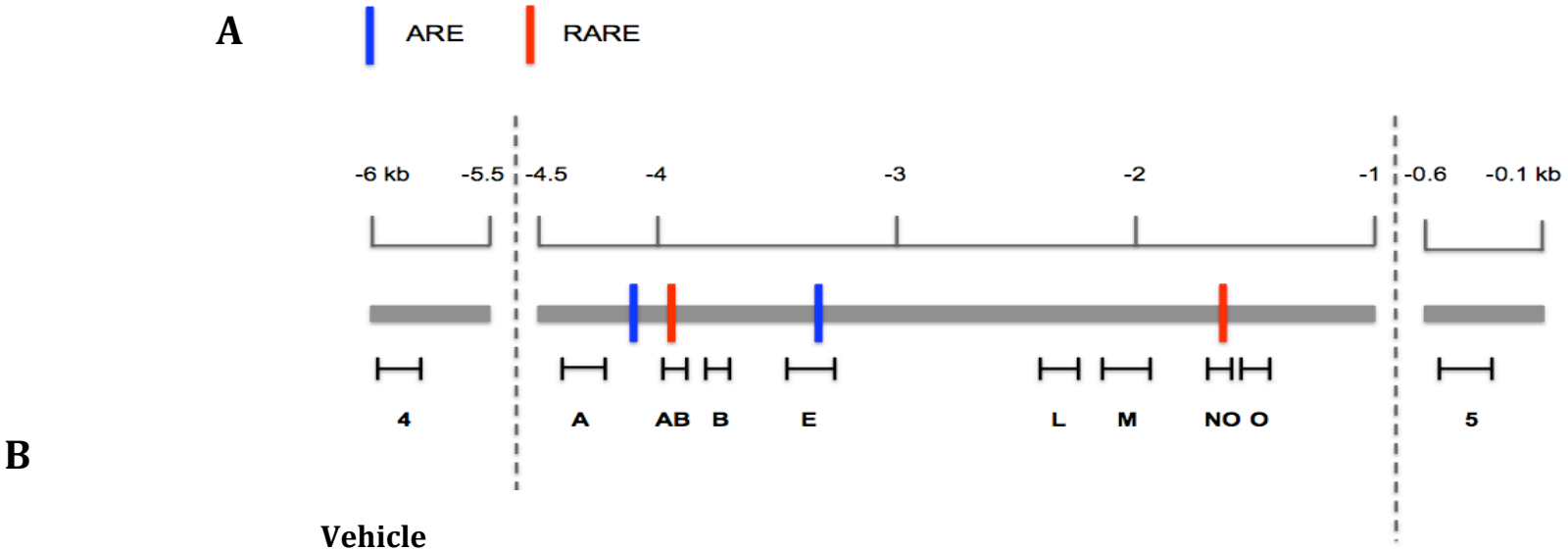
4.3.10 Mapping the direct binding of AR and RAR to the hTGP promoter

To complete the analysis of the hTGP promoter, it was decided to map the binding sites of the AR and RAR to this regulatory region. In order to do so, vehicle, atRA and R1881 treated LNCaP cells were subjected to chromatin immunoprecipitation (ChIP). LNCaP cells were grown in T150 flasks in charcoal stripped media for 24h, then treated with vehicle, 500nM atRA or 10nM R1881 for 10h. Following treatment, cells were fixed using paraformaldehyde, to promote the cross-linking of proteins and DNA, chromatin was extracted and sonicated to produce short-length DNA fragments. DNA-bound AR and RAR was immunoprecipitated using specific antibodies, and DNA purified by phenol-chloroform extraction. DNA fragments were amplified by qPCR using specific primers spanning the hTGP promoter in regions where either AR/RAR binding was expected (regions AB, B and NO), and in regions where receptor binding was not expected (regions 4, 5, N and M) as shown in figure 26A. RAR binding to the hTGP promoter in vehicle-treated cells was higher at regions -3745, -1647 and -1504, which are close to or within RAREs located at -3962 and -1647 (figure 26A and B). It was expected to find RAR binding to the DNA in the absence of ligand since the current model (Chambon 1996) suggests that RARs are able to bind to the DNA in the absence of ligand, while ligand binding promotes the recruitment of co-activators to the regulatory region to promote gene transcription. AR binding to the hTGP promoter in vehicle-treated cells matched the binding sites of the RAR in the same region, but tended to be lower than RAR binding across the hTGP promoter (figure 26C).

RAR binding to the hTGP promoter in atRA-treated LNCaP cells remained very similar to the pattern shown in vehicle treated cells. Interestingly, AR binding

across the hTGP promoter in atRA-treated LNCaP cells showed a uniform decrease showing similar levels as for the IgG control, except at the -3962 region where it remained constant (figure 26C). Since AR is necessary for complete hTGP mRNA expression following atRA treatment, it is noteworthy that the only region where AR binding remained unaltered is adjacent to the RAR binding site. This implied that AR binding in the -3962 region was important in the atRA-dependent hTGP expression, opening the possibility that the AR and the RAR might interact in the regulation of hTGP.

R1881 treatment in LNCaP cells caused a decrease in RAR binding to the hTGP promoter (figure 26D) but also a significant increase in AR binding to the -3962 region. Since R1881 treatment stimulated AR binding to the hTGP promoter, it is tempting to hypothesize that androgen-bound AR actively represses hTGP either by recruiting co-repressors or by impeding the RAR activity. Whether RAR discharge off the hTGP was caused by steric impediment or any other mechanism remains to be investigated. A proposed model for hTGP regulation, summarising the findings of this work is depicted in figure 27.


A
ARE
RARE
-6 kb
-5.5
-4.5
-4
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4
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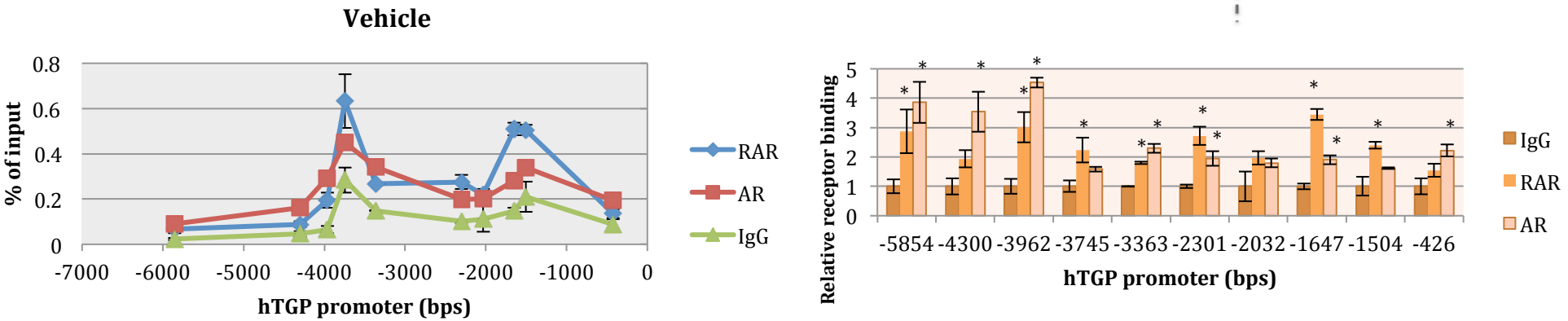

B
Vehicle
% of input
0.8
0.6
0.4
0.2
0
-7000
-6000
-5000
-4000
-3000
-2000
-1000
0
hTGP promoter (bps)
RAR
AR
IgG
Relative receptor binding
5
4
3
2
1
0
-5854
-4300
-3962
-3745
-3363
-2301
-2032
-1647
-1504
-426
hTGP promoter (bps)
IgG
RAR
AR

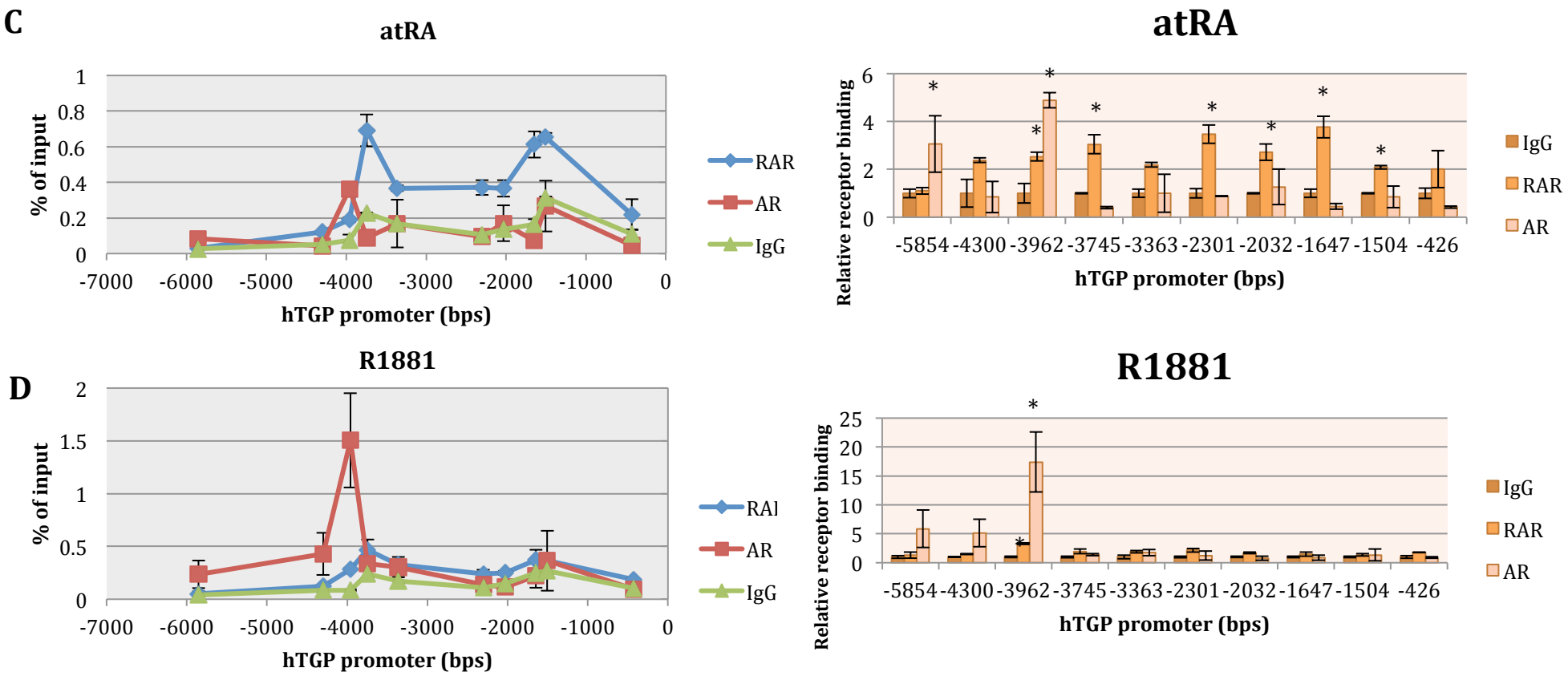


Figure 26. AR and RAR binding to the hTGP promoter**.** A. AREs, RAREs and primer amplifying regions for ChIP-qPCR analysis of the hTGP promoter. B. AR and RAR binding to the hTGP plotted as percentage of input (left) and relative binding (right) LNCaP cells treated with vehicle for 10h. C. AR and RAR binding to the hTGP plotted as percentage of input (left) and relative binding (right) LNCaP cells treated with 500nM atRA for 10h. D. AR and RAR binding to the hTGP plotted as percentage of input (left) and relative binding (right) LNCaP cells treated with 10nM R1881 for 10h. The symbol * denotes statistical significance with respect to IgG control as measured by Student-T test ($p<0.05$).

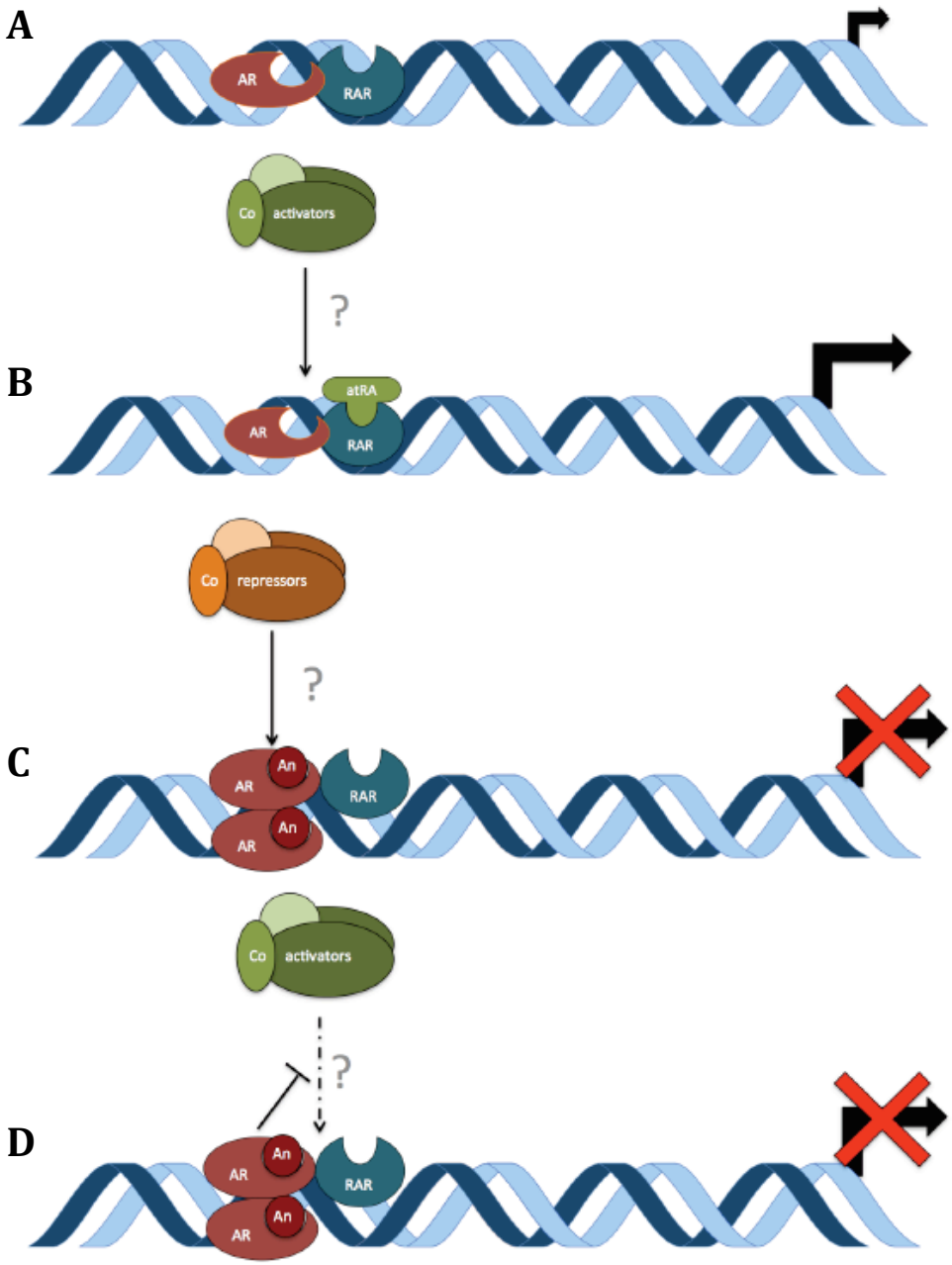


Figure 27. Proposed mechanism of hTGP regulation by retinoic acid and androgen. A. In an environment where retinoic acid and androgen concentrations are low, the AR and RAR are bound to the hTGP promoter allowing basal transcription. B. Retinoic acid-bound RAR activates transcription, with the help of the AR, most likely by recruiting co-activators to the hTGP promoter. C and D. Androgen-bound AR increases its binding to the hTGP promoter, actively repressing hTGP expression either by the recruitment of co-repressors or impeding the recruitment of co-activators and/or factors that allow hTGP expression.

4.4 Testing the baculovirus-hTGP-NTR in prostate cancer cell lines

4.4.1 Utility of the hTGP promoter in prostate cancer gene therapy

In order to test whether the NTR enzyme used to cause targeted cell death following CB1954 treatment could be expressed using the hTGP promoter as a regulatory sequence, NTR was cloned in place of luciferase in the hTGP 4.5kb-pGL3 plasmid, now re-named hTGP4.5-NTR.

LNCaP cells were grown in charcoal-stripped medium for 24h, then transfected with hTGP4.5-NTR plasmid. Cells were then treated with vehicle or 500nM atRA 24h after transfection, and NTR expression was measured by western blot analysis, 24h and 48h after vehicle or atRA treatment. Figure 28 shows that NTR expression could be detected only in cells transfected with the hTGP4.5-NTR plasmid that had also been treated with atRA. NTR expression was sustained at 24h and 48h following atRA treatment. When the NTR levels of the positive control, LNCaP cells transfected with the CMV-NTR plasmid, were compared to those of cells transfected with the hTGP4.5-NTR plasmid and treated with atRA using semi-quantitative densitometry, a 10-fold difference was observed between samples, indicating that while the hTGP promoter could be helpful for prostate targeting, it was also less strong than CMV in producing NTR enzyme (figure 28B).

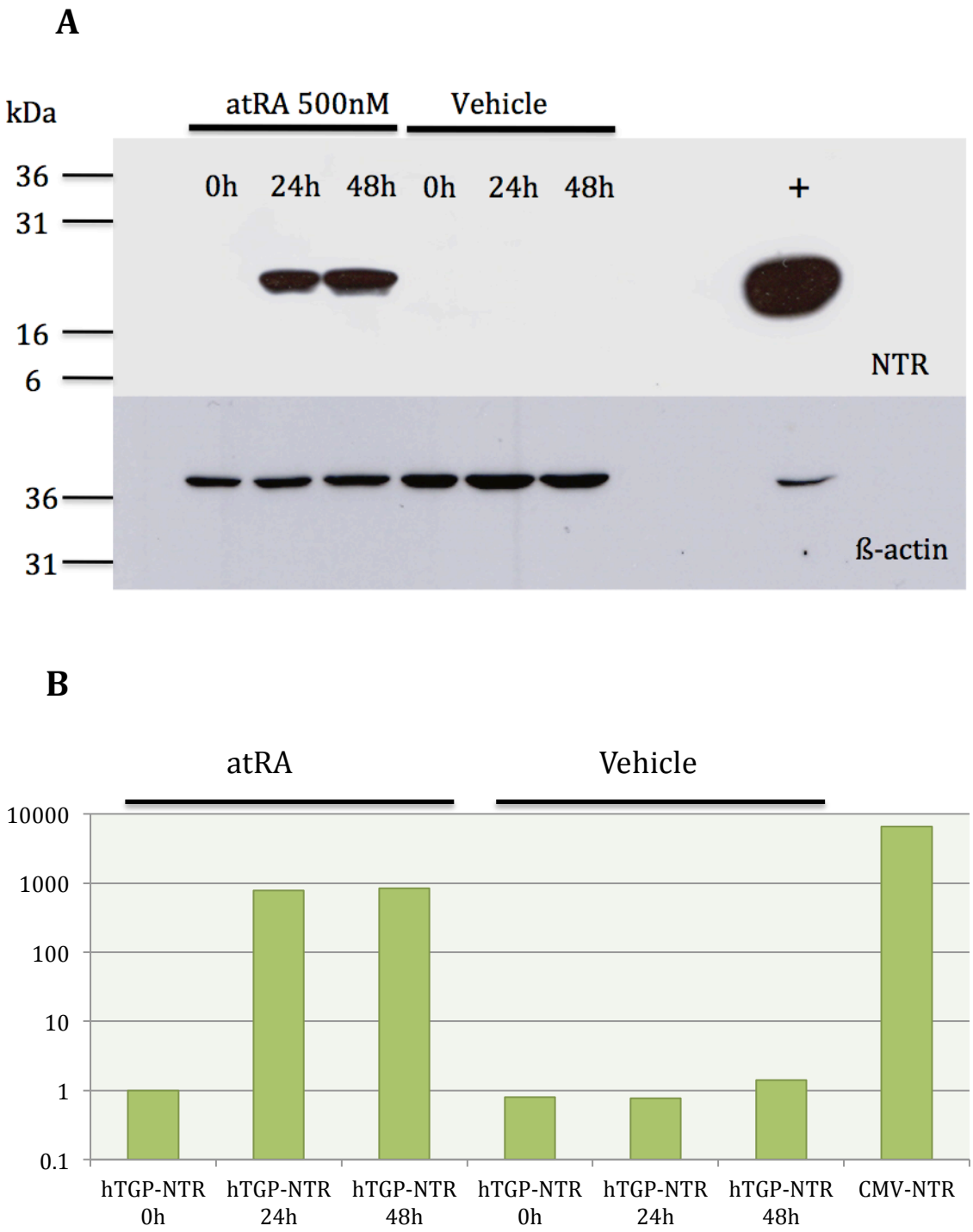


Figure 28. The hTGP promoter activates NTR transcription following atRA treatment**.** A. Western blot analysis showing NTR expression in transfected LNCaP cells, transfected with hTGP4.5-NTR and treated with either 500nM atRA or vehicle for 24h or 48h. β-actin was used as loading control. B. NTR expression as analysed by semi-quantitative densitometry. NTR expression was normalised to β-actin expression. The positive control consisted in whole protein extracts from LNCaP cells transiently transfected with a construct containing the NTR gene under the control of the CMV promoter.

4.4.2 hTGP promoter activity in prostate and non-prostate cell lines

LNCaP, PC3, PNT1A (prostate cell lines) and HeLa, MCF7 and T47D (non-prostate cell lines) were grown in complete medium and transfected with hTGP4.5-Luc plasmid and treated with 500nM atRA to assess the activity of the hTGP promoter in prostate and non-prostate cell lines. Figure 29A illustrates the firefly and *Renilla* luciferase activities in each cell treated either with vehicle or atRA. Firefly and *Renilla* luciferase readings are a reflection of promoter activity and transfection efficiency. Transfection efficiency was measured by *Renilla* luciferase readings. Since the early CMV promoter controls the expression of *Renilla* luciferase, it was expected to obtain similar *Renilla* luciferase activities in the different prostate and non-prostate cell lines. However *Renilla* expression varied from cell line to cell line by orders of 10- to more than 100-fold (figure 29A). This phenomenon could be explained by the differential ability of the transfection reagent to deliver plasmid DNA into different cell lines and the dissimilar activity of the CMV promoter in diverse cellular contexts (Cheng et al. 1993). Therefore, to compare the hTGP promoter activity in different cell lines, it would be necessary to either use a transfection method that can deliver plasmid DNA into all the different cell lines with exactly the same efficiency and/or a normalizing plasmid where the expression of *Renilla* luciferase was controlled by a promoter equally strong in all cell lines. To compare the hTGP promoter between different cell lines, the activity of each cell line transfected and treated with atRA was normalised to the activity of the same cell line transfected and treated with vehicle (figure 29B). Luciferase expression was enhanced by atRA treatment only in LNCaP cells. Not even PC3 or PNT1A cells showed an increase in luciferase expression following atRA treatment. This could be

related to the differentiation status of the prostate cell lines. While LNCaP represent a more differentiated cell type, PC3 and PNT1A have phenotypical characteristics of less differentiated cells. hTGP expression has been found to be restricted to the highly differentiated luminal cells in prostate (Dubbink et al. 1999b), suggesting that expression in less differentiated cell types is suppressed. Part of the suppression mechanism could rely on the promoter sequence and therefore PC3 and PNT1A cells were unable to up-regulate luciferase expression in response to atRA treatment. HeLa, MCF7 and T47D cells also failed to up-regulate luciferase expression under the control of the hTGP, following atRA treatment. Interestingly, although MCF7 cells are known to express RARs and AR (Ross-Innes et al. 2010; Subik et al. 2010), key transcription factors in hTGP regulation, no significant change was observed after atRA treatment. Whether the 4.5kb hTGP promoter contains enough information to express genes in a prostate specific manner requires further investigation.

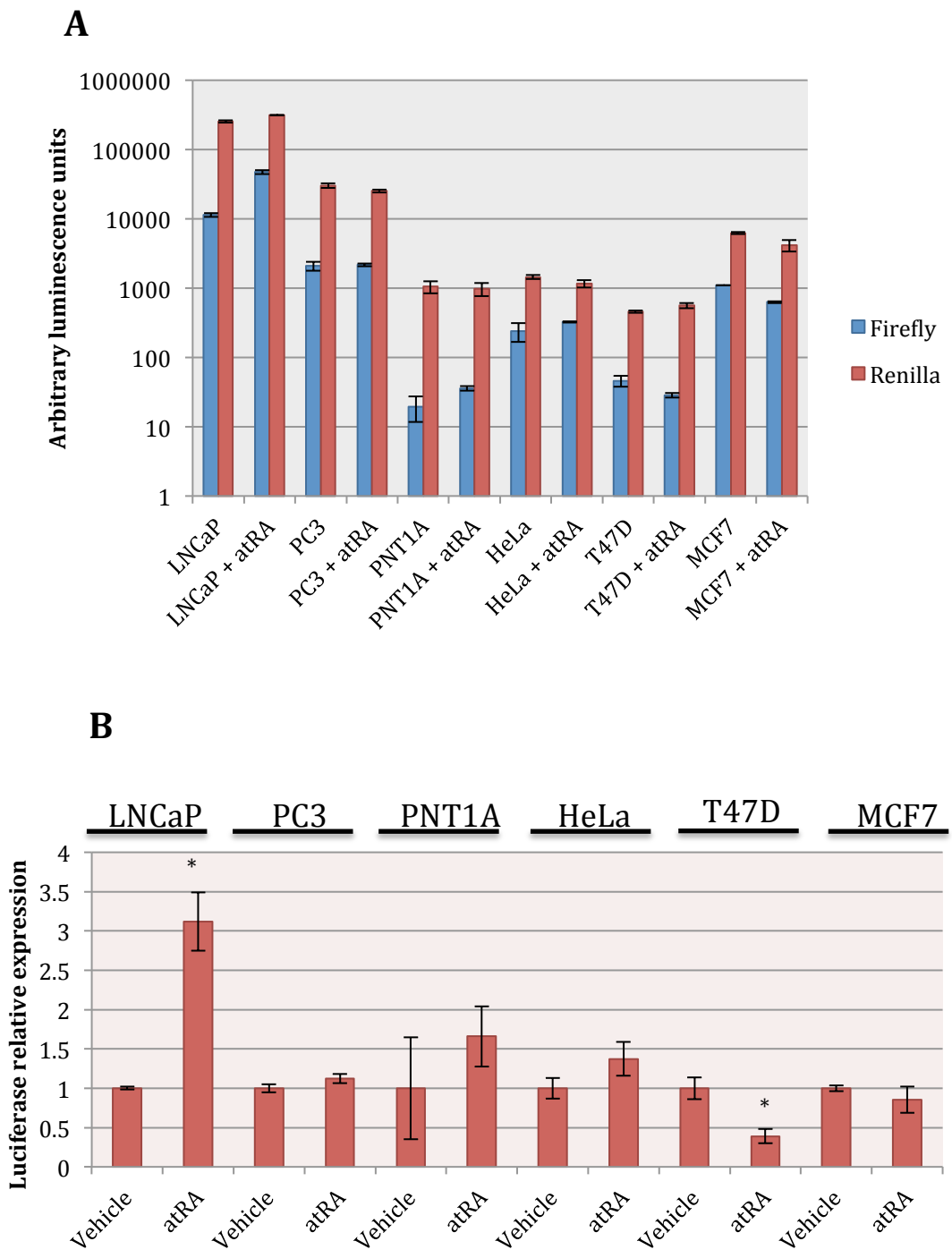


Figure 29. hTGP promoter activity in prostate and non-prostate cell lines. A. Luciferase activity of hTGP4.5-Luc transfected and atRA treated cells measured in arbitrary luminescence units. The CMV promoter controlled Renilla luciferase expression. B. Relative luciferase expression of hTGP4.5-Luc transfected and atRA treated cell lines normalised to vehicle treated samples. The symbol * denotes statistical significance with respect to vehicle control as measured by Student-T test ($p<0.05$).

4.4.3 NTR half-life in LNCaP cells

NTR half-life in human cells is unknown. Since in this study it was intended to induce NTR expression using the hTGP promoter, that is weaker in comparison to the CMV promoter, it was necessary to measure NTR half-life to assess whether this would be a potential issue. For this purpose, LNCaP cells were transfected with the hTGP4.5-NTR plasmid and treated with 500nM atRA for 24h. Following atRA treatment, cells were treated with 5μM cycloheximide, a drug that halts protein synthesis, and cells were harvested at various time points ranging from 2-10h. Samples were analysed by western blot, using as a comparison, a protein with a well-characterized half-life in prostate cells, clusterin (Rizzi et al. 2009). Figure 30A shows that protein levels of clusterin, decreased over time, and were practically undetectable 10h after cycloheximide treatment. NTR appeared to be more stable, showing detectable levels 10h following cycloheximide treatment. Semi-quantitative densitometry measuring clusterin and NTR decay over time indicated a half-life of 2h for clusterin and 10h for NTR (figure 30B). Clusterin is considered as an unstable protein, given its rapid degradation. However, NTR was around 5 times more stable, indicating that NTR half-life would not be an issue in NTR/CB1954 treatments. This also implies that a very strong promoter is not necessarily needed and a weak promoter could also be of use to express NTR.

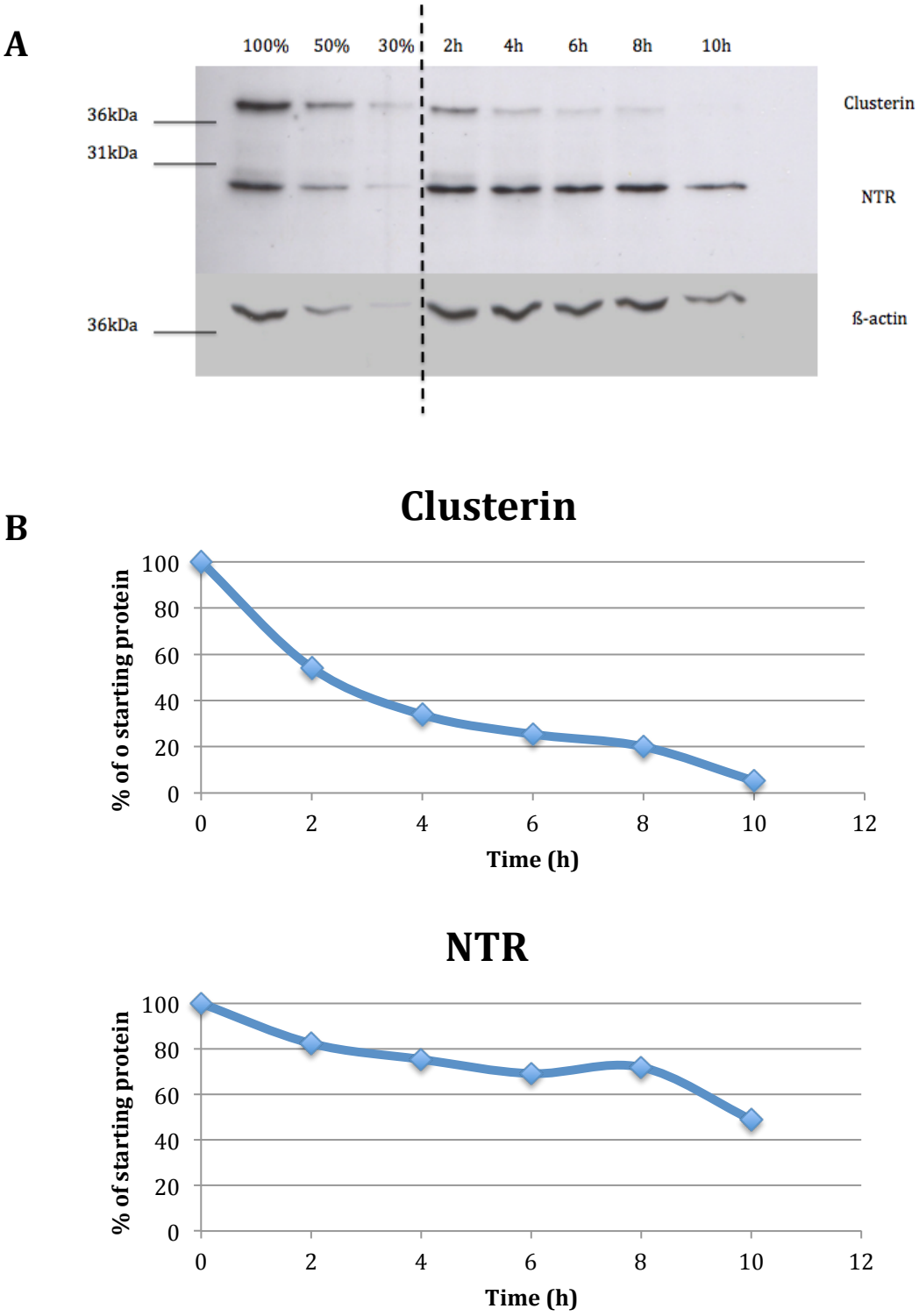


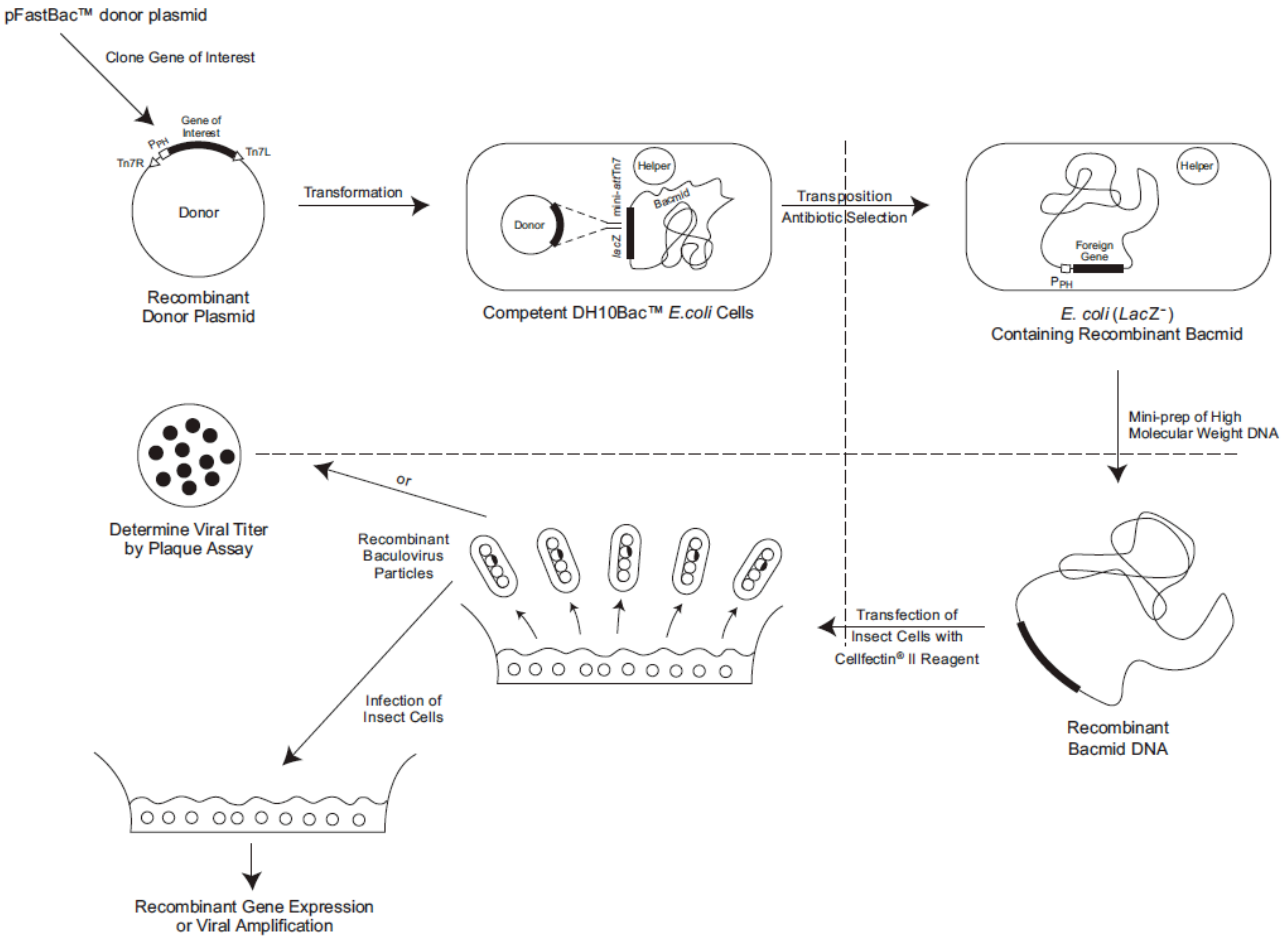
Figure 30. NTR half-life in human prostate cells. A. Western blot analysis of clusterin, NTR and β-actin of LNCaP cells transfected with hTGP4.5-NTR plasmid, treated with 500nM atRA to induce NTR expression, followed by a cyclohexamide treatment to inhibit protein synthesis and harvested between 2-10h. B. Semi-quantitative densitometry evaluating clusterin and NTR decay over time.

4.4.4 Building the hTGP4.5-NTR baculovirus

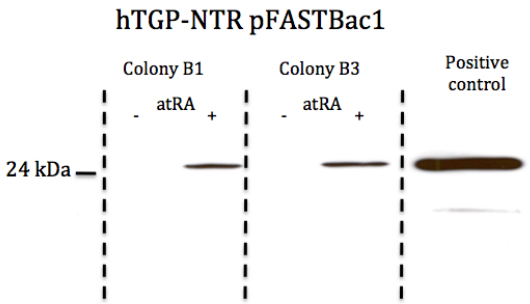
A recombinant baculovirus encoding the NTR gene under the control of the hTGP promoter was engineered using the Bac-toBac baculovirus expression system. The hTGP promoter and NTR sequences were cloned into the recombinant donor plasmid pFASTBac1. Cloning into this plasmid results in the cloned sequences being flanked by the donor Tn7L and Tn7R sequences to allow site-specific recombination. To confirm the correct cloning and functionality of the hTGP and NTR genes, plasmids were sequenced and two of those that contained the correct sequence were transfected into LNCaP cells that were further treated with 500nM atRA. Cells were lysed and total protein extracted and analysed by western blot. Figure 31B shows that both plasmids produce NTR expression following atRA treatment, while untreated cells displayed no visible levels of NTR in accordance with previous results. The hTGP4.5-NTR pFASTbac1 plasmid was then transformed into competent DH10Bac *E. coli* cells. These cells contain a recombinant baculovirus genome where a *LacZ* gene contained, within its sequence, the acceptor Tn7L and Tn7R sites. Successful site-directed recombination resulted in *LacZ* disruption and failure to produce blue colonies when bacteria were grown in the presence of IPTG and X-gal. Ten white colonies were tested for the presence of the hTGP promoter and NTR sequences by isolating the baculovirus genome and amplifying a section of it by PCR, using specific primers that flanked both human DNA sequences and should produce a 9kb product. Two baculovirus genomes from different colonies that showed presence of hTGP and NTR sequences were transfected into sf9 insect cells to initiate production of baculovirus particles. 96h after infection, supernatant was collected and stored

for further amplification. Before amplification of a baculovirus, its viral DNA was tested by PCR to verify whether it contained the hTGP promoter and NTR sequences using two sets of primers. The first one flanked both hTGP and NTR sequences, yielding a 9kb amplification product and was used to identify baculovirus genomes that contained the insert. The second set of primers was localized within the hTGP promoter and the NTR gene sequences and amplified a region of around 4.5 kb. Both baculovirus genomes isolated from viral particles showed the presence of hTGP and NTR sequences assuring the correct construction of the virus (figure 31C and 31D).

A


pFastBac™ donor plasmid
Clone Gene of Interest
Gene of Interest
Tn7R
Tn7L
Donor
Recombinant Donor Plasmid
Transformation
Donor
mini-attTn7
lacZ
Helper
Bacmid
Competent DH10Bac™ E.coli Cells
Transposition Antibiotic Selection
Helper
Foreign Gene
E. coli (LacZ⁻) Containing Recombinant Bacmid
Mini-prep of High Molecular Weight DNA
Recombinant Bacmid DNA
Transfection of Insect Cells with Cellfectin® II Reagent
Recombinant Baculovirus Particles
or
Determine Viral Titer by Plaque Assay
Infection of Insect Cells
Recombinant Gene Expression or Viral Amplification

B


hTGP-NTR pFASTBac1
Colony B1
Colony B3
Positive control
- atRA +
- atRA +
24 kDa

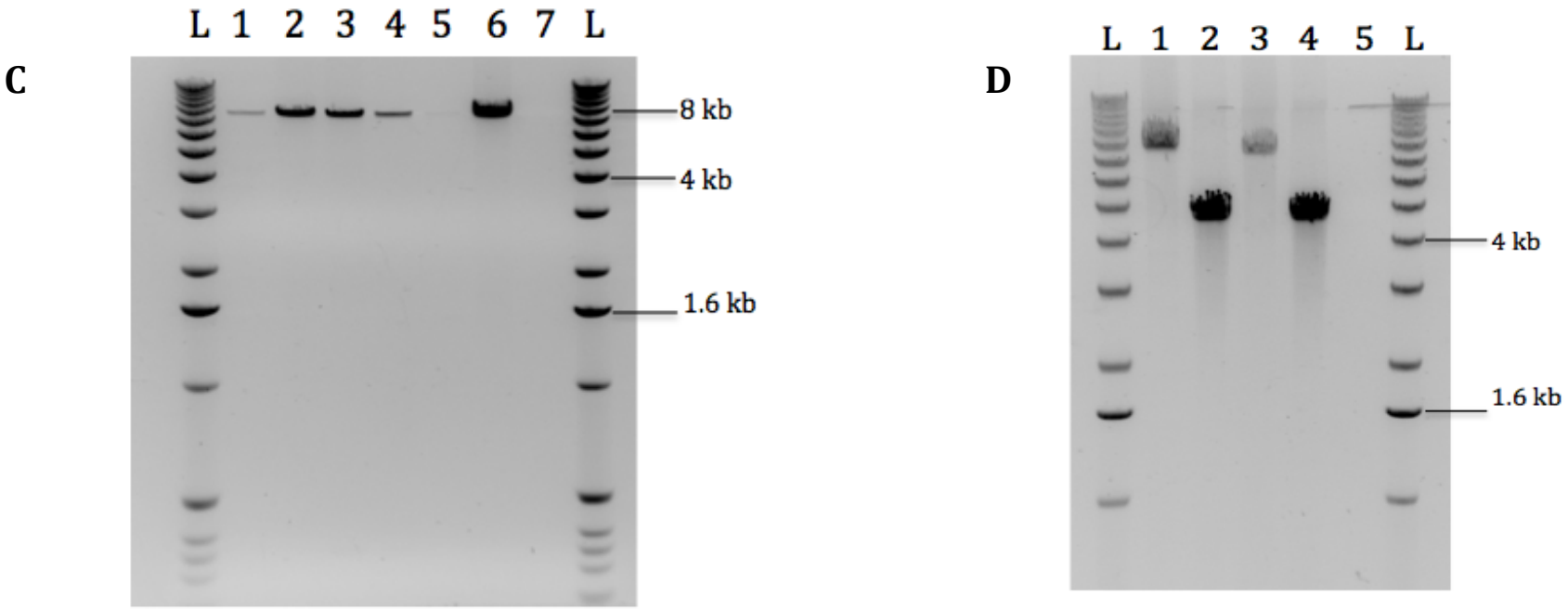


Figure 31. Engineering a prostate targeted baculovirus for gene therapy**.** A. Overview of the procedure to build the hTGP4.5-NTR baculovirus, taken from the Bac to Bac manual (4th September 2010, Invitrogen. The hTGP-NTR sequences were cloned into the pFASTBac1 vector. hTGP4.5-NTR pFASTBac1 was transformed into DH10Bac cells to promote site-specific recombination into the baculovirus genome. Baculovirus genome was isolated and transfected into sf9 insect cells to produce baculovirus particles. B. Western blot analysis of NTR expression in LNCaP cells transfected with the plasmid hTGP4.5-NTR pFASTBac1 and treated with 500nM atRA or vehicle for 24h. C. PCR analysis of the presence of hTGP promoter and NTR gene sequences in purified baculovirus genomes from bacterial colonies. Lanes 1-6 are baculovirus genomes from different colonies; lane 7 is the negative control. D. PCR analysis of the baculovirus genome extracted from viral particles generated by two different parental genomes. Lanes 1 and 3 were amplified using specific primers that flank the hTGP and NTR sequences in the Baculovirus genome. Lanes 2 and 4 were amplified using primers that amplified a sequence within the hTGP promoter and the NTR gene. Lane 5 is the negative control.

4.4.5 Testing the ability of the baculovirus to infect non-prostate cell lines

Before testing the ability of the hTGP4.5-NTR baculovirus to induce cell death, it was decided to test the baculovirus' ability to transduce non-prostate cell lines. It was of particular importance to perform this experiment in order to be able to discern the cell specificity of the hTGP promoter and the overall efficiency of the cells to be transduced by the virus. MCF7, T47D and HeLa cells were transduced with BV-EGFP using LNCaP cells as a positive control to test the susceptibility of each non-prostate cell line to be transduced by baculovirus. Figure 32 shows that non-prostate cell lines are very poorly susceptible for baculovirus transduction, when compared to LNCaP cells. Non-prostate cell lines showed less than 2% EGFP-expressing cells; in comparison to 68% EGFP expressing LNCaP cells. These results should be interpreted carefully, since the baculovirus used for this experiment used the CMV promoter as the regulatory element to control EGFP expression. As mentioned before, CMV has been shown to have different activity depending on the cell type context. Therefore, to measure to which degree EGFP expression was a measure of successful transduction without being affected by promoter activity, more experiments featuring baculoviruses expressing EGFP from different promoters are needed.

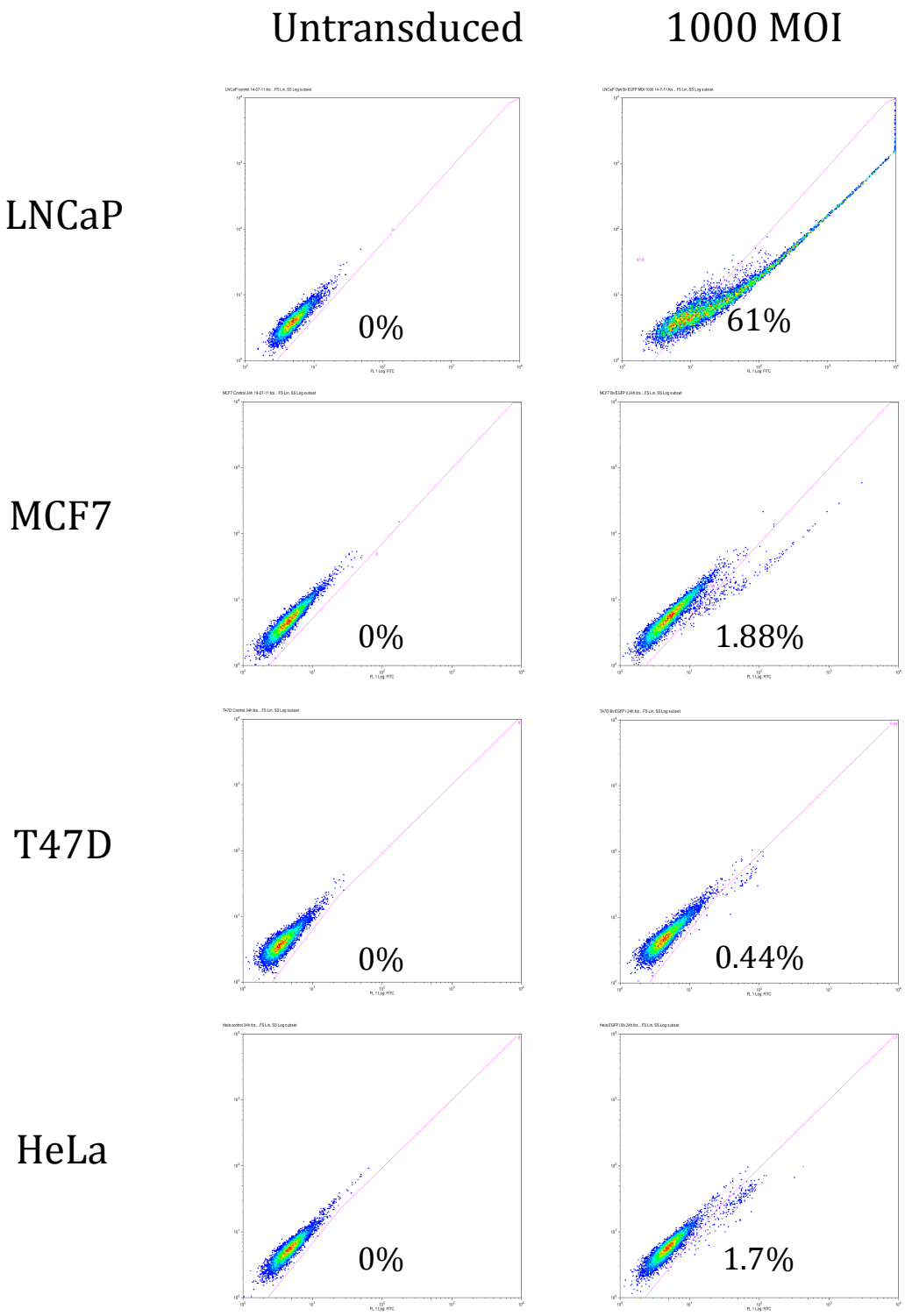


Figure 32. Non-prostate cell lines susceptibility to baculovirus transduction**.** MCF7, T47D and HeLa cells were transduced with the recombinant baculovirus BV-EGFP for 2h. 24h following transduction cells were harvested, washed and re-suspended in PBS. Percentage of EGFP positive cells was measured by FACS analysis. LNCaP cells were used as a positive control.

4.4.6 hTGP4.5-NTR baculovirus ability to cause cell death in LNCaP cells

LNCaP cells were transduced with the hTGP4.5-NTR baculovirus to test whether treating them with CB1954 could kill transduced cells. Cells were incubated with recombinant baculovirus for 4h at RT, then at 37°C for 24h. Transduced cells were treated with 500nM atRA (to stimulate NTR expression) or vehicle for 24h before CB1954 addition. As controls, untransduced cells were treated with vehicle, atRA, CB1954 or a combination. 72h after CB1954 treatment, cell viability was measured by MTS assay. Cells transduced with the hTGP4.5-NTR baculovirus and treated with CB1954 displayed a reduction in cell viability of 37%, around the reduction observed when cells were pre-treated with atRA (figure 33A), meaning that atRA treatment did not enhance cell death. Interestingly, cells transduced with the hTGP4.5-NTR baculovirus, but not treated with CB1954, also showed a decrease of 28.7%, suggesting that the virus on its own causes some degree of cell cytotoxicity.

To investigate why atRA pre-treatment did not enhance cell death following CB1954 exposure, LNCaP cells were transduced and treated with atRA or vehicle. Cells were harvested 24h following atRA treatment and protein was extracted to analyse NTR expression by western blot analysis. Figure 33B demonstrates that NTR expression in transduced LNCaP cells was undetectable, even after atRA treatment. This is most likely caused by the sequences surrounding the hTGP promoter, as it has been inserted in the baculovirus genome, since the virus used to transduce LNCaP cells was proved to have the hTGP promoter and NTR sequences intact. Therefore, gene expression was altered in the context of the viral genome in contrast to plasmid

alone. While further experiments, including the evaluation of NTR mRNA expression in baculovirus transduced cells and baculovirus genome transfection into mammalian cells, are needed to clarify at which step NTR expression is prevented, the findings of this work have taken baculovirus-based prostate cancer gene therapy a step forward. We have proved the value of the NTR/CB1954 system and baculovirus for its use not only in prostate cell lines but also in prostate epithelial primary cultures. By investigating the regulation of the hTGP promoter we have discovered a new interaction between AR and RAR that is actively regulating a highly prostate specific gene. These findings provide encouraging evidence of the potential use of our system for prostate cancer gene therapy.

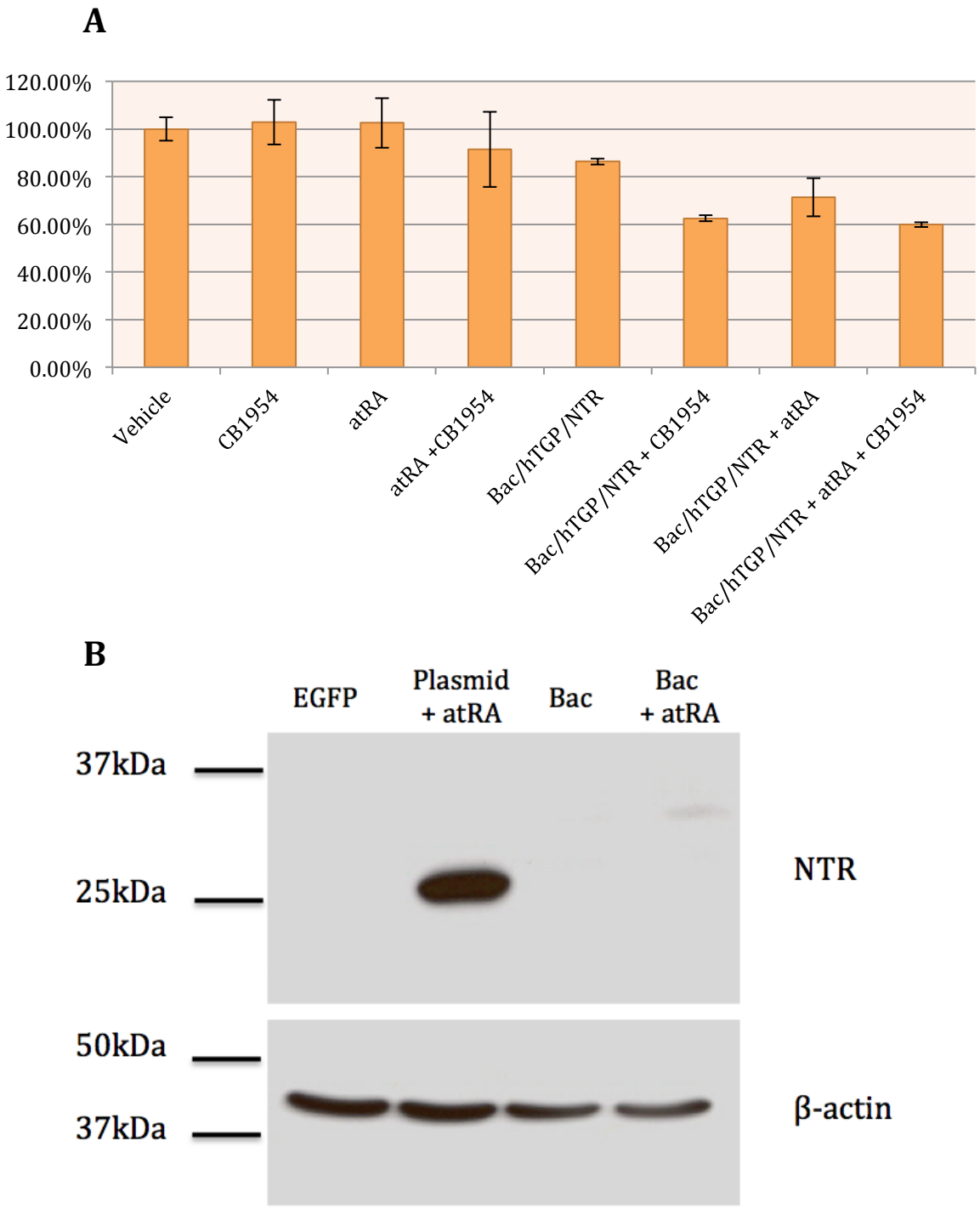


Figure 33. Activity of the hTGP4.5-NTR baculovirus in LNCaP cells**.** A. Percentage of viable cells transduced with the hTGP4.5-NTR baculovirus, treated with either 500nM atRA or vehicle for 24h, then treated with 20μM CB1954 or vehicle for 72h. Cell viability was measured by MTS assay. B. NTR protein expression in LNCaP cells transduced with hTGP4.5-NTR baculovirus and treated with 500nM atRA or vehicle for 24h. As a positive control cells were transfected with the hTGP4.5-NTR plasmid and treated with 500nM atRA (lane 2).

5. DISCUSSION

5.1 The role of RARs and AR in the prostate

The amount of research in the role of RARs and AR in prostate function and homeostasis is highly dissimilar. Although previous studies suggest a major role for retinoic acid and RARs in prostate homeostasis, a detailed examination of the mechanism of action remains understudied. It has been shown that the retinoic acid pathway and RARs expression are both enhanced in primitive murine prostate epithelial cells. Aldehyde dehydrogenase genes, which catalyse the transformation of retinol into retinoic acid, were shown to be up-regulated in the urogenital epithelial sinus, and in both foetal and adult prostate stem cell populations. *Rxra*, *Rarb* and *Rarg* have also been shown to be highly expressed in adult and foetal stem cells (Blum et al. 2009). This data suggested that retinoic acid signalling is important during prostate development, but also in adult prostate, and could play a role in the regulation of prostate stem cells.

Perhaps the most studied role of retinoic acid and RARs in prostate is its link to cancer development. RARB hypermetylation seems to be a common feature of malignant prostate cells in the majority of the patients (Vasiljevic et al. 2011). Retinoic acid treatment can cause apoptosis in prostate cancer cell lines PC3 and DU145, through up-regulation of the *TNFRSF12A, TNFRSF1A* and *TNFRSF10B* genes (Karabulut et al. 2011). Retinoic acid has also been used to enhance cell death in castration-resistant prostate cells subject to suicide gene therapy (Chen et al. 2008). While the means of protection against prostate cancer development conferred by retinoic acid remains unknown, research in

other cancer types reveals important mechanisms that could play a role in *prostate cancer. Retinoic acid induces the expression of* G0S2*,* TNFAIP2*,* SMAD3*, and* NRIP1 by RAR binding to the promoter of these genes in endometrial cells. Up-regulation of these genes could result in cell cycle arrest, apoptosis and reduced motility, features that are also increased when the cells were treated with retinoic acid *(Cheng et al. 2011).*

On the other hand, the role of AR in prostate homeostasis and prostate cancer has been investigated in some detail. Among the most exciting recent findings is the AR interaction with the transcription factor FOXA1. This regulatory mechanism has uncovered a new layer of complexity in AR activity. FOXA1 is able to both induce and prevent AR binding to a subset of AREs in the genome. Lack of FOXA1 expression results in an aberrant gene expression profile, that could lead to, or be a further step towards, prostate cancer development (Sahu et al. 2011). Interestingly, AR's role in prostate homeostasis is not restricted to growth and survival. AR targets include genes involved in metabolic processes. *GLUT1*, *HK1/2* and *PFKFB2*, genes involved in glucose uptake and glycolysis, and *FASN*/*ACACA*, genes playing a role in biosynthetic processes, are all genes up-regulated by androgen exposure, and are likely to contribute to prostate cancer by providing the raw materials and means for the cancer cells to maintain their growth (Massie et al. 2011).

These recent studies emphasise two key findings regarding AR activity. First, they stress the importance of the interaction of AR with other transcription factors and second, they highlight the notion that the activity of the AR is not

just to provide survival signals, but to help the cells grow by stimulating metabolic machinery.

The findings in the present work demonstrate that retinoic acid and RARs have an opposite effect on the regulation of the hTGP gene in comparison to treatment with androgens. It was also found that AR knockdown resulted in RARA and RARG mRNA up-regulation, and that retinoic acid treatment yielded low AR binding to the hTGP promoter. All these observations, of opposing effects between androgen and retinoic acid, suggest that one of the roles of retinoic acid in prostate homeostasis could be to counteract or moderate the effects of androgen.

Similar observations of the opposing effect of androgen and retinoic acid had been made in the past. In the AR-expressing breast cancer cell line T47D, retinoic acid treatment results in AR down-regulation of expression and activity (Hall et al. 1992). Moreover, retinoic acid treatment of LNCaP cells resulted in reduced AR binding activity, and repression of KLK3 and KLK2 expression, both prostate-specific and androgen regulated genes, in a dose dependent manner (Young et al. 1994). The effect of retinoic acid on LNCaP cells was however dependent on the presence of androgens. While retinoic acid treatment alone stimulated growth and differentiation, a combined treatment with androgens yielded growth inhibition (Esquenet et al. 1996), suggesting that the combined treatment promotes an interaction of the stimulated mechanisms triggered by these hormones. It is known that retinoic acid can have contrasting effects on proliferation and cell survival depending on the cell type. The effect of

retinoic acid on survival and growth appears to be determined by the expression of the retinoic acid binding proteins CRABP-II and FABP5. Retinoic acid induces cell death and proliferation arrest in cells that express high levels of CRABP-II and low levels of FABP5, mainly through the activity of the RARs. Induction of proliferation and survival is triggered by retinoic acid in cells with high FABP5 levels by inducing the activation of the PPARB/D (peroxisome proliferator-activated receptor beta/delta) (Schug et al. 2007). Therefore it would be interesting to investigate whether androgens stimulate or repress the expression of either CRABP-II or FABP5, which should result in the modulation of the response to retinoic acid, and explain the differential response to retinoic acid in the presence/absence of androgen.

When retinoic acid treatment induces cell death, Rb activation and down-regulation of AR protein expression precedes the activation of apoptosis in LNCaP cells (Gao et al. 1999), suggesting that retinoic acid could also be able to modulate the AR activity.

Another nuclear steroid receptor with an opposing role to the RAR, in breast cancer cells, is the oestrogen receptor (ER). Similarly to its effect on prostate cells, retinoic acid induces apoptosis and proliferation arrest in breast cancer cells, while oestrogen induces cell proliferation and survival. In MCF7 cells, RAR and ER binding sites have a high co-localization rate of 39.3% (within 1kb distance between binding sites) and the shared number of genes that they bind to is 59.8%. RAR and ER therefore compete to bind to overlapping sites, and the activation of one down-regulates the activity of the other (Hua et al. 2009).

However RAR and ER can also interact and cooperate to induce transcription of genes. It has been shown that the RARA can bind to oestrogen responsive elements (EREs) and that this binding depends on the presence of the ER and oestrogen (Ross-Innes et al. 2010). RARA interaction with the ER control the expression of about a third of all ER regulated genes. The mechanism of this interaction seems to rely on the ability of the RARA to facilitate ER/co-activator interactions (Ross-Innes et al. 2010). Presence of the RARA ligand, retinoic acid disrupts the cooperation between the nuclear receptors and favours the transcription or RARA regulated genes (Ross-Innes et al. 2010).

These studies clearly suggest an antagonistic role of retinoic acid to that of androgen regarding cell survival and growth in prostate cells. They also suggest that RARs can oppose the proliferative and survival effects of another steroid hormone, oestrogen, in breast cells. The findings in this work are not only in line with these previous reports, but provide a putative mechanism for the regulation of this antagonistic relationship. It was found that AR played a dual role in the regulation of hTGP expression. In the presence of atRA, AR cooperated with the RARG to induce hTGP expression, while the presence of androgen caused increased AR binding to the hTGP promoter that resulted in transcriptional repression.

It will be interesting to research if the regulatory circuit controlling hTGP expression can be found in more genes, a very likely event, but it may be more important to identify the roles of such genes, and to evaluate how their differential expression impacts on prostate function and homeostasis.

5.2 Prostate-specific expression

The role of AR in prostate-specific gene expression is widely acknowledged. Tissue-specific expression is regulated mainly through enhancers and the prostate is no exception. It has been recently described how FOXA1, a transcription factor highly expressed in the prostate, interacts with the AR to facilitate and restrict its binding to active enhancer elements, consequently regulating the expression of target genes (Wang et al. 2011).

The discovery of transcription factors, apart from AR, modulating the expression of prostate-specific genes, questions the assumed solitary role of AR in prostate-specific gene expression. KLK3 (or PSA) is probably the most studied prostate specific gene. It is widely used as a cancer biomarker and is known to be regulated by the action of androgens through the androgen receptor (Andreu-Vieyra et al. 2011). However, PSA is not solely regulated by the action of androgens. It was recently demonstrated that treating LNCaP cells with the cytokine IL-6 could induce PSA expression. The IL-6-dependent increase in PSA expression was mediated by the signal transducer and activator of the transcription 3 (STAT3), the heat-schock protein 90 (HSP90) and a previously described androgen enhancer region (Tsui et al. 2011).

FOLH1 is the best example of a prostate specific gene whose expression is down-regulated by the effects of androgen. As with most prostate specific genes, FOLH1 expression is regulated by an enhancer located in the third intron of the gene. This enhancer mediates, alongside the FOLH1 promoter, the androgen-dependent repression observed in prostate cells (Noss et al. 2002). One of the key transcription factors that bind to the FOLH1 enhancer is AP-3.

Site directed mutagenesis which caused a DNA sequence change in the AP-3 binding site, triggered the repression of the enhancer's activity. Also bound to this enhancer was the transcription factor NFATc1, suggesting a possible cooperation with AP-3 to induce FOLH1 expression (Lee et al. 2003). Recently described is the involvement of ERG and the TMPRSS2-ERG fusion in the regulation of FOLH1 expression. It was found that expression of the TMPRSS2-ERG fusion could inhibit FOLH1 expression. Since in this fusion the regulatory region, and some of the first exons of the TMPRSS2 gene, are fused to a section of the ERG gene, it is probable that androgen induction of this fusion is responsible for the down-regulation in FOLH1 expression. Interestingly, ERG siRNA knockdown resulted in increased FOLH1 expression, suggesting that it is ERG which is the factor controlling FOLH1 expression, since this effect could be observed in the presence of androgen (Yin et al. 2011).

NKX3.1 is another prostate-specific gene, which is regulated by multiple transcription factors. AR regulates NKX3.1 expression by binding to an enhancer at the 3' UTR of the gene that contains AREs (Thomas et al. 2010). The transcription factor ETS1 is also able to modulate NKX3.1 by binding to the gene's proximal promoter (Preece et al. 2011). ERG and ESE3, another ETS transcription factor, have been shown to regulate the expression of NKX3.1 through the induction of EZH2 (Kunderfranco et al. 2010). More interestingly perhaps is the observation that retinoic acid is also able to directly up-regulate NKX3.1 protein expression and transcription in LNCaP prostate cancer cells (Thomas et al. 2006).

In the present study it is described how retinoic acid drives the expression of the highly prostate specific gene hTGP, through the activation of the RARG. This is, up to date, only the second example of a prostate specific gene regulated by retinoic acid. It becomes clearer that the idea of AR and androgen as solitary regulators of prostate-specific expression needs to change to favour a more complex and realistic interplay between a plethora of different transcription factors. This will allow investigators to gain an insight into the complex control of tissue-specific expression that could lead to breakthroughs in many areas of science and medicine including gene and stem cell-based regeneration therapies.

5.3 Baculoviruses in gene therapy

Most of the vectors used for gene therapy purposes are viruses that naturally infect human cells and tissues. One of the advantages of using these viruses is that they possess specific means to infect human cells and therefore are highly efficient at delivering desired transgenes. However, since these are natural pathogens, the human body is prepared to sustain a rapid and efficient immune response upon detection of these parasites. This immune response poses the major obstacle in using human-infecting viruses for gene therapy, since the total amount of virus that can be applied to a subject is limited in quantity and in number of doses.

Baculovirus offers an alternative to these problems, featuring high transduction efficiency in vertebrate and mammalian cells (Airenne et al. 2011), while being unable to induce memory immune responses from human hosts. Research using baculovirus as a vector for gene therapy has increased recently. Neural

stem cells (NSC) with tropism for tumours were infected with a recombinant baculovirus encoding the herpes simplex virus thymidine kinase gene. Baculovirus-transduced NSC showed thymidine kinase expression for as long as three weeks, and injection of transduced NSC followed by gancyclovir treatment into mice growing human glioma xenografts resulted in inhibition of growth and prolonged survival (Zhao et al. 2011).

Another baculovirus-based effort to treat glioma took advantage of the observation that sodium butyrate (NaBu) enhanced baculovirus transduction efficiency. The human glioma cell line U251 were transduced with a recombinant baculovirus armed with the WT p53 gene, to induce apoptosis, in combination with NaBu treatment. By combining NaBu with baculovirus transduction, p53 expression was improved, resulting in enhanced cell death rates. *In vivo* efficacy was corroborated by intratumoral injection of recombinant baculovirus alongside NaBu treatment of U251 tumours growing in nude mice. Baculovirus/NaBu treated tumours showed decreased proliferation (Guo et al. 2011).

The need for tumours to increase blood vessel formation to sustain their growth has also been targeted by gene therapy using baculovirus vectors. A recombinant baculovirus capable of expressing the fusion protein hEA, a fusion between human endostatin and angiostatin with anti-angiogenic activity has been shown to have anti-proliferative and anti-angiogenic potential. When injected intratumorally, in prostate cancer mouse xenografts, the recombinant baculovirus inhibited tumour growth and prolonged host survival, in comparison to the controls (Luo et al. 2011b).

One of the downsides of using baculoviruses is that gene expression is transient. Trying to confer stable expression a recombinant baculovirus expressing the sleeping beauty (SB) transposase and containing the hEA gene, flanked by inverted repeat/direct-repeat (IR/DR) elements recognized by the SB transposase, was built. This hybrid baculovirus showed increased duration of transgene expression when compared to traditional baculoviruses, affecting both tumour growth and prolonging survival in prostate and ovarian cancer allograft mice models (Luo et al. 2011a).

The use of baculovirus as a vector for prostate cancer gene therapy is investigated in prostate cancer cell lines in this work. The findings presented here suggest that, as observed by many other groups in the world, baculoviruses are capable of transducing human cancer cells with high efficiency. It was also found in this research that baculoviruses have a "preference" to transduce prostate cancer cell lines rather than normal prostate cell lines, cervical cancer or breast cancer cell lines. Expanding the research of which cell types can be transduced by baculoviruses will not only expand its use into permissive tissues but also will provide valuable information of which de-targeting strategies are required to prevent undesired gene expression in off-target tissues.

5.4 The NTR/CB1954 system

Suicide gene therapy is an attractive option to cause cell death among cancer cells. It confers the advantage of requiring to be expressed only by a fraction of a given population in order to induce widespread cell death by a mechanism denominated bystander effect. It is this particular characteristic that places the NTR/CB1954 system apart from the other suicide gene therapy methods. The NTR gene has been modified to adapt it to the mammalian codon usage. These changes resulted in enhanced expression, sensitivity to CB1954 and increased bystander effect (Grohmann et al. 2009). A clinical trial using a replication-defective adenovirus armed with the NTR gene was administered via intraprostatic injection and NTR expression could be found in the majority of the patients (Patel et al. 2009).

An interesting approach to induce NTR expression in cancer cells is to make use of hypoxia-responsive elements (HREs) to regulate the expression of NTR. By using a regulatory region comprised of the minimal CMV promoter linked to HREs from VEGF and erythropoietin, NTR expression could be targeted to tumour-hypoxic regions. This resulted in inhibition of tumour growth following prodrug administration (Harvey et al. 2011).

Another feature common to most tumours is the expression of human telomerase reverse transcriptase (hTERT). Using an hTERT regulatory region to control the expression of NTR and Herpes Simplex virus thymidine kinase (HSV-TK) coupled to gancyclovir and CB1954 treatment has been used as a combinatorial GDEPT treatment. A bicistronic adenovirus coding the HSV-TK and NTR genes separated by an IRES sequence and regulated by the hTERT

promoter showed higher anti-tumoural activity than single HSV-TK or NTR adenovirueses in breast cancer human cells (Yu et al. 2011).

In this study we have proved that NTR/CB1954 combination can efficiently kill prostate cancer cells. The use of the humanized NTR and a combination of different GDEPT enzymes is an exciting prospect for prostate cancer gene therapy.

5.5 Current gene therapy for prostate cancer

Gene therapy has shifted from being a futuristic approach, to a treatment that needs fine-tuning before reaching the bedside. One of the most used approaches for prostate cancer gene therapy is the utilization of oncolytic viruses. These viruses have the ability to replicate in cancer cells only due to the use of cancer-specific or in some cases tissue-specific promoters, regulating the expression of genes necessary for viral replication. One of the most exciting prospects for oncolytic gene therapy has been recently described. The approach uses the natural ability of macrophages to home to hypoxic areas to deliver the oncolytic virus to prostate tumours. The macrophages were co-transduced with an adenovirus where the E1A gene, necessary for proliferation, is under the control of the PPT promoter (prostate-specific) and a construct containing the E1A/B gene under the control of a promoter containing HREs. Using this system, adenovirus replication will begin only when the transduced macrophages reach hypoxic regions. Orthotopic LNCaP tumours growing in mice showed regression and absence of metastasis following injections of transduced macrophages (Muthana et al. 2011). Advantages of this system are the low requirement of viral particles to induce a therapeutic effect and the

delay of the immune response to the recombinant virus due to the fact that the virus is in a latent state while in the macrophage and therefore antigen expression should be minimal.

Although an exciting prospect, the use of oncolytic viruses and, the use of prostate-specific or cancer-specific regulatory regions to control the proliferation of the recombinant virus could yield other difficulties. Prostate-specific promoters are expressed in the majority of the cancer cells, since most of the prostate-specific promoters are active in more differentiated cells. However, prostate cancers do contain a small proportion of less differentiated cells in which these promoter would not be active, therefore selecting a population that is resistant to the new therapy. A possible solution for this problem is the combination of the oncolytic approach with suicide gene therapy. The use of a recombinant oncolytic vesicular stomatitis virus (VSV) encoding the cytosine deaminase/uracil phosphoribosyltransferase (CD::UPRT) suicide gene has proved to kill uninfected cells by means of the known bystander effect following prodrug treatment (Leveille et al. 2011).

Another alternative is the use of oncolytic viruses with more conventional treatments such as chemotherapy. Using a Reovirus with oncolytic activity alongside docetaxel synergistic cytotoxic effects could be observed. Combinatorial treatment resulted in increased apoptotic/necrotic cell populations, reduced growth and increased survival in mice bearing PC3 tumours in comparison to single treatments (Heinemann et al. 2011). Similar

results were obtained when using an oncolytic adenovirus and docetaxel in another prostate cancer xenograft model (Li et al. 2010).

A noteworthy approach is the sensitization of tumour cells to cell death/apoptosis by inhibiting anti-apoptotic genes and introducing pro-apoptotic ones. Adenoviruses coding the *REIC/Dkk-3* gene successfully induced cell death in PC3 prostate cancer cells. However, isolated resistant colonies showed overexpression of BiP/GRP78, an endoplasmic reticulum-residing chaperone protein. BiP/GRP78 siRNA knockdown rendered cells sensible to *REIC/Dkk-3* triggered apoptosis, suggesting that BiP/GRP78 plays a key role in resistance to *REIC/Dkk-3* mediated cell death (Tanimoto et al. 2010).

The same principle has been used to induce cell death by infecting cells with an adenovirus encoding the mda-7/IL-24. The product of this gene is a member of the IL-10 cytokine family and is known for its anticancer activities. However, its activity is inhibited by the action of the myeloid cell leukemia-1 (Mcl-1) protein, therefore its down-regulation is a pre-requisite for inducing cell death using this system. Instead of knocking down Mcl-1, cells were treated with BI-97C1, an Apogossypol derivative, to induce Mcl-1 pharmacological inhibition. Infection of prostate cancer cells growing in nude mice with the adenovirus encoding the mda-7/IL24 combined with BI97C1 resulted in growth inhibition, increased apoptosis and decreased Ki-67 expression. Similar results were observed in Hi-myc transgenic mice, which are prone to developing spontaneous prostate cancer (Dash et al. 2011).

In this work, the potential of a baculovirus system using a prostate-specific promoter and a GDEPT approach was evaluated for its use as a therapy for prostate cancer. It was confirmed that baculoviruses could deliver therapeutic genes not only into prostate cell lines but also prostate primary samples derived form patients. The NTR/CB1954 system also showed satisfactory results yielding high cell death in efficiently transduced prostate cells. While this work also uncovered a complex regulation governing hTGP expression, more research is needed to clarify the potential of this promoter region as a regulatory sequence driving the expression of the NTR enzyme. Genetic engineering to augment promoter strength while retaining tissue-specificity seems indeed necessary. Treatments that sensitize prostate cancer cells to NTR/CB1954 cell death or co-treatment with chemotherapy agents is also an exciting perspective.

Another use for the hTGP promoter would be to enable it for an oncolytic approach. Since hTGP expression is highly prostate specific, it could be used to restrict the proliferation of viruses to prostate cells. However, as mentioned before, the oncolytic approach should also take into account the existence of non-permissive cell types and therefore this method should be combined with other form of therapy, preferentially, suicide gene therapy.

5.6 Conclusion

This work has provided encouraging evidence supporting the use of baculovirus and suicide gene therapy as an alternative treatment for prostate cancer. The NTR/CB1954 system effectively killed prostate cancer cells. The baculovirus vector transduced not only prostate cancer cell lines but also prostate primary epithelial cell cultures. The dissection of the hTGP promoter revealed a layer of complexity regarding prostate specific-regulation. While many prostate-specific genes are mainly regulated by androgens through the AR, hTGP expression is regulated by an interaction between the AR and RARG. This new finding, will lead to a better understanding of how prostate-specific expression is achieved and will provide valuable information on the design and understanding of regulatory regions targeting prostate-specific expression.

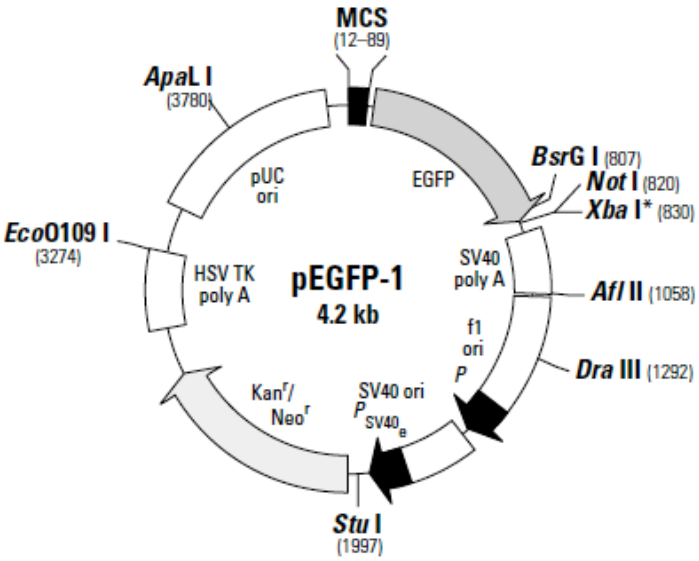
Appendices

A. Plasmid maps

- pEGFP1

pEGFP-1 Vector Information PT3026-5

GenBank Accession #: U55761 Catalog #6086-1

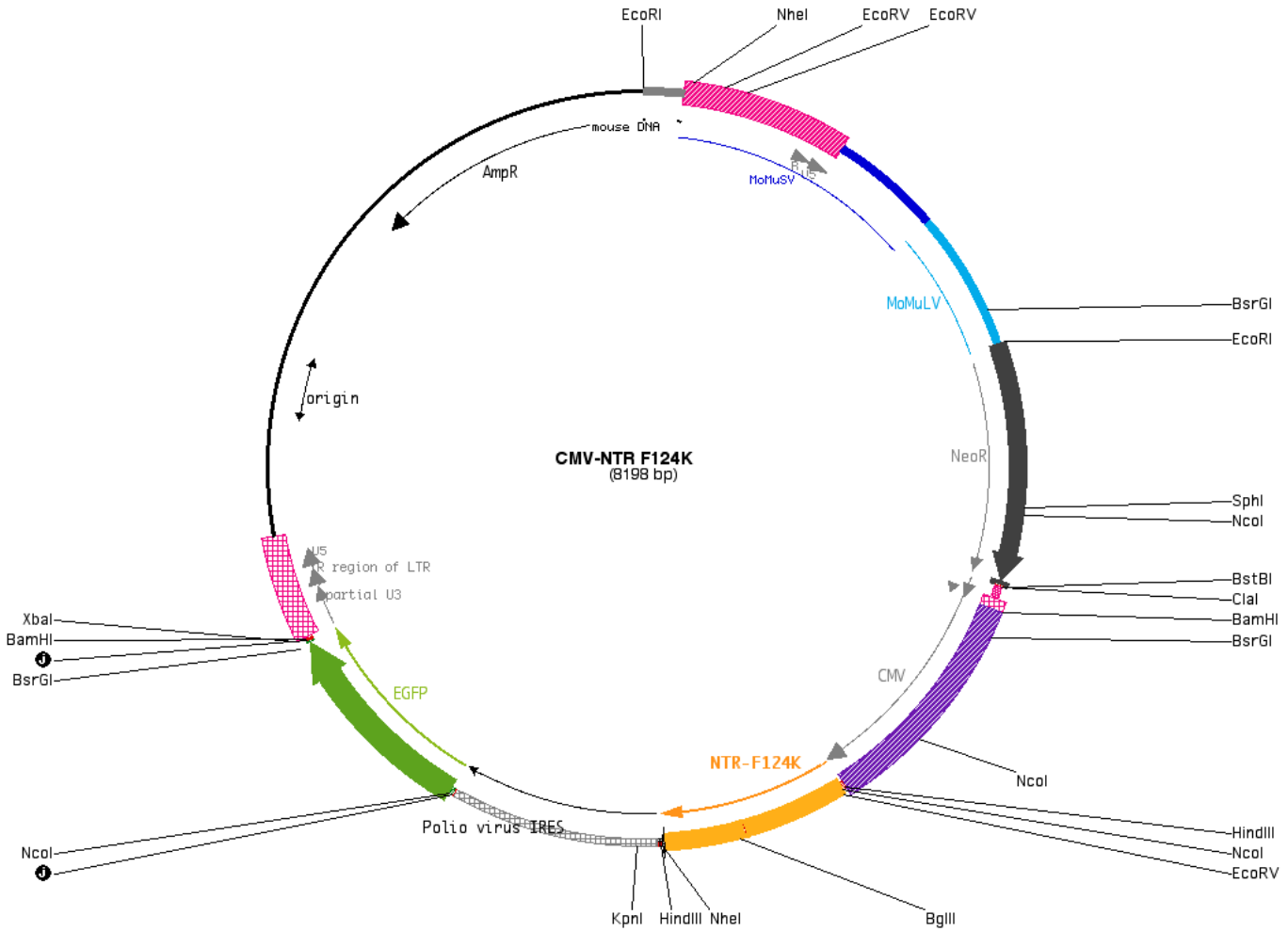


11 21 31 41 51 61 71 81 91 EGFP

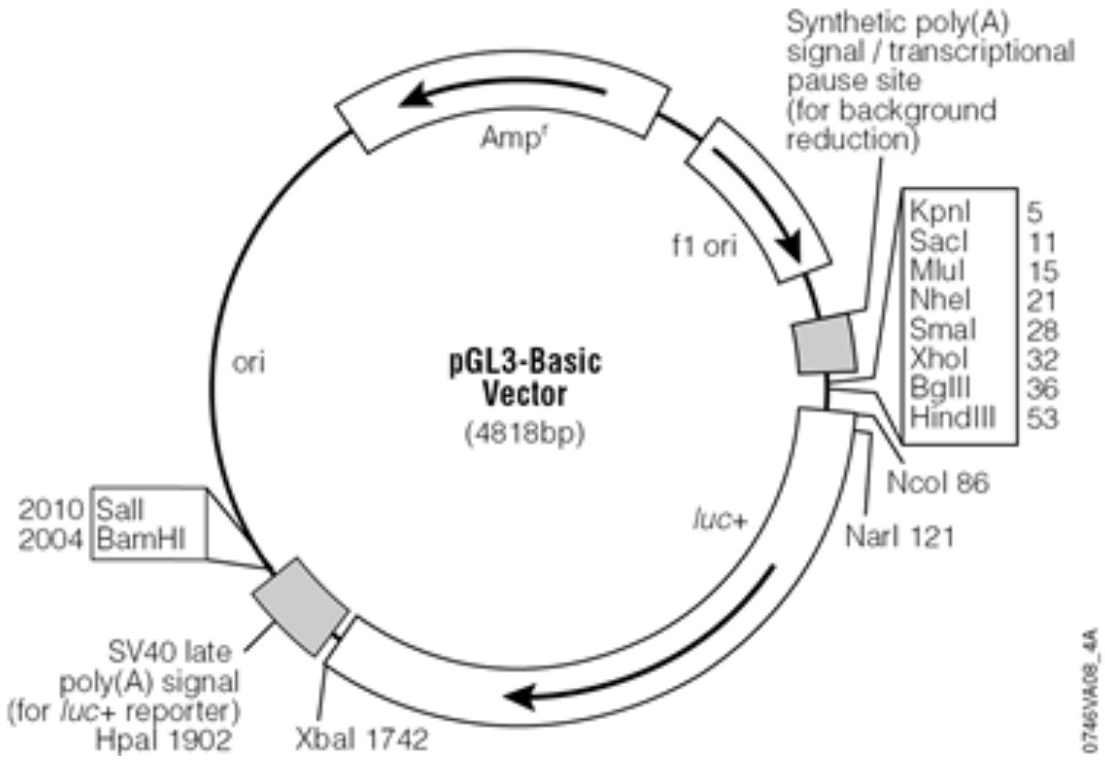
TA GCG CTA CCG GAC TCA GAT CTC GAG CTC AAG CTT CGA ATT CTG CAG TCG ACG GTA CCG CGG GCC CGG GAT CCA CCG GTC GCC ACC ATG GTG

Eco47 III Bgl II Xho I Sac I Ecl136 II Hind III EcoR I Pst I Sal I Acc I Kpn I Asp718 I Sac II Apa I Bsp120 I Xma I Sma I BamH I Age I

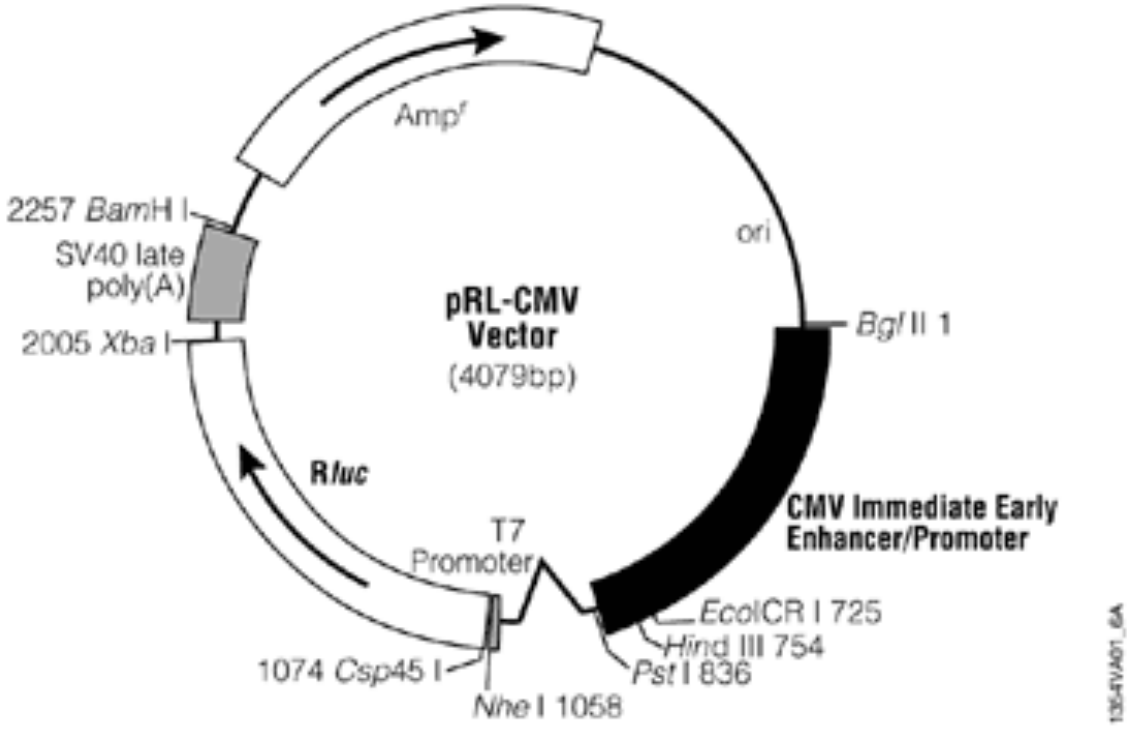
- CMV-NTR (F124K)



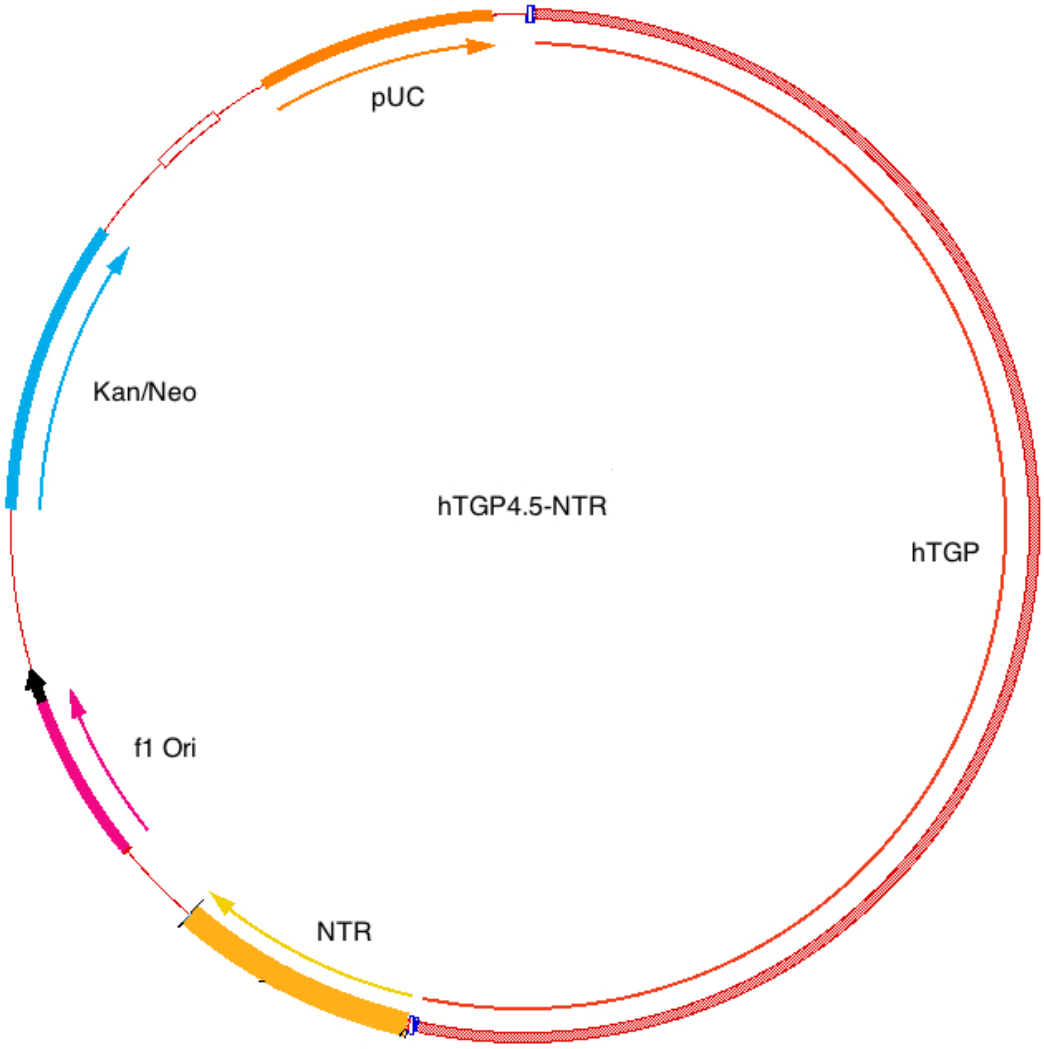
- pGL3-Basic


Synthetic poly(A) signal / transcriptional pause site (for background reduction)
Amp^r
f1 ori
ori
pGL3-Basic Vector
(4818bp)
luc+
KpnI 5
SacI 11
MluI 15
NheI 21
SmaI 28
XhoI 32
BglII 36
HindIII 53
NcoI 86
NarI 121
2010 SalI
2004 BamHI
SV40 late poly(A) signal (for luc+ reporter)
HpaI 1902
XbaI 1742
0746VA08_4A

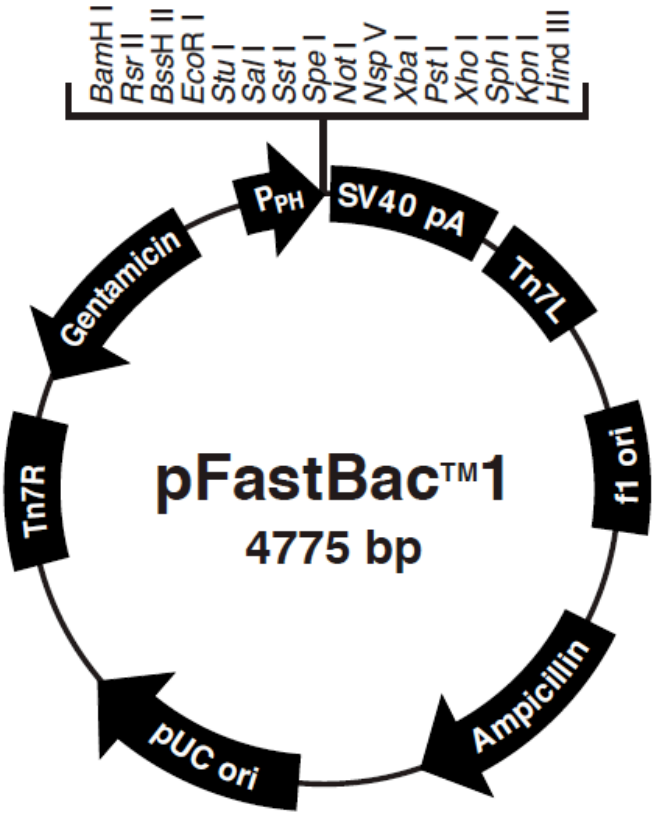
- pRL-CMV


Amp^r
ori
2257 BamH I
SV40 late
poly(A)
2005 Xba I
pRL-CMV
Vector
(4079bp)
Bgl II 1
Rluc
T7
Promoter
CMV Immediate Early
Enhancer/Promoter
EcoICR I 725
Hind III 754
Pst I 836
1074 Csp45 I
Nhe I 1058
135-4VA01_6A

- hTGP-NTR (EGFP1 backbone)



-pFASTBac1



Comments for pFastBac™1
4775 nucleotides

f1 origin: bases 2-457
Ampicillin resistance gene: bases 589-1449
pUC origin: bases 1594-2267
Tn7R: bases 2511-2735
Gentamicin resistance gene: bases 2802-3335 (complementary strand)
Polyhedrin promoter (P_{PH}): bases 3904-4032
Multiple cloning site: bases 4037-4142
SV40 polyadenylation signal: bases 4160-4400
Tn7L: bases 4429-4594

B. Primers

Primer name	Sequence 5'- 3'	Purpose
hTGP exon 1 for	GAGATAGAGTCTTCCCTGGCA	Amplify hTGP cDNA using RTPCR
hTGp exon2 rev	GGACTGCTCGTTTGGAACTCC	Amplify hTGP cDNA using RTPCR
GAPDH For	AAGGTGAAGGTCGGAGTCAA	Amplify GAPDH cDNA using RTPCR
GAPDH Rev	GGACACGGAAGGCCATGCCA	Amplify GAPDH cDNA using RTPCR
PSA fwd	ATGTGGGTCCCGGTTGTCTT	Amplify PSA cDNA using RT-PCR
PSA rev	TCAGGGGTTGGCCACGATGG	Amplify PSA cDNA using RT-PCR
PSAFor qPCR	TGTGCTTCAAGGTATCACGTCAT	Amplify PSA cDNA using qPCR
PSARev qPCR	TCAGGGGTTGGCCACGATGG	Amplify PSA cDNA using qPCR
5' qHPRT	GATGATGAACCAGGTTATGACC	Amplify HPRT cDNA using qPCR
3' qHPRT	CCAAATCCTCAGCATAATGATTAGG	Amplify HPRT cDNA using qPCR
hTGp mRNA A For	GGGGGCTGCCAGAAGTATCAAA	Amplify hTGP cDNA using qPCR
hTGp mRNA A Rev	CAGCACGGGGTCCCTCCTATC	Amplify hTGP cDNA using qPCR
RARG all-transcripts For	TGACCGGAACAAGAAGAAGAAAGAG	Amplify RARG cDNA using qPCR
RARG all-transcripts Rev	CTGGCAGAGCGAGGGGAAAGT	Amplify RARG cDNA using qPCR
RARB all-transcripts For	CCTGCCTTTGGAAATGGATGAC	Amplify RARB cDNA using qPCR
RARB all-transcripts Rev	TTGCTGGGTCGTCTTTTTCTGATA	Amplify RARB cDNA using qPCR
RARA all-transcripts For	GGCCCCCTCACCGACCTG	Amplify RARA cDNA using qPCR
RARA all-transcripts Rev	CGCTTCCGCACGTAGACCTTTAG	Amplify RARA cDNA using qPCR
AR1-2B qPCRFor	CATCTTGTCGTCTTCGGAAATGTTA	Amplify AR cDNA using qPCR
AR1-2BqPCRRev	GAAGCCTCTCCTTCCTCCTGTAGTT	Amplify AR cDNA using qPCR
Region4hTGpFor	ATGCAGTCTGTGGTATTTGTT ATGG	Amplify upstream

		region of the hTGP by qPCR
Region4hTGpRev	TTGGGTCTGGCTTCTTTCACTTAG	Amplify upstream region of the hTGP by qPCR
RegionAhTGpFor	TTGTCTGTACTGCTTCCGTGTTCC	Amplify region of the hTGP promoter by qPCR
RegionAhTGpRev	ATTTTCCCCCTGGTGTAGCATTAG	Amplify region of the hTGP promoter by qPCR
AB5’	CTGAAGTGCCAGGTTTGCTCCAT	Amplify region of the hTGP promoter by qPCR
AB3'	AAAAGAATCCAATAAACCCCGAAGTC	Amplify region of the hTGP promoter by qPCR
RegionBhTGpFor	ATTTACTAACTCCTCCCTGTCTCC	Amplify region of the hTGP promoter by qPCR
RegionBhTGpRev	GCTGCTGTTCATGGTGCTAAG	Amplify region of the hTGP promoter by qPCR
RegionEhTGpFor	CTTCCACCTGAGCACCCTGTCCT	Amplify region of the hTGP promoter by qPCR
RegionEhTGpRev	GCAAGAAGAGCCTGAAAACCAC	Amplify region of the hTGP promoter by qPCR
RegionLhTGpFor	AACTAAAACCCGGACCCTCTCA	Amplify region of the hTGP promoter by qPCR
RegionLhTGpRev	GATGCTTGCTTTTCTCTGTATTTC	Amplify region of the hTGP promoter by qPCR
RegionMhTGpFor	GTGCACTTCAGGGCTTGGTTTGT	Amplify region of the hTGP promoter by qPCR
RegionMhTGpRev	AGTGAGGGGGCTGAATAATGATGC	Amplify region of the hTGP promoter by qPCR
RegionNhTGpFor	ATCATTATTCAGCCCCCTCACTTT	Amplify region of the hTGP promoter by qPCR
RegionNhTGpRev	AATTTTAATGGCTATCTGCTCTGC	Amplify region of the hTGP promoter by qPCR
NO5'	CAACATTTCCACTTCAAGGCATTC	Amplify region of the hTGP promoter by qPCR
NO3'	TACAATCAGTGTTGCAAAGAATAAGGTT	Amplify region of the hTGP promoter by qPCR

RegionOhTGpFor	CACGCCTGGCAAGATGG	Amplify region of the hTGP promoter by qPCR
RegionOhTGpRev	CACGCCTGGCAAGATGG	Amplify region of the hTGP promoter by qPCR
Region5hTGpFor	TGCTTCTCAGTGCATACAACATCTC	Amplify region of the hTGP promoter by qPCR
Region5hTGpRev	TAAGTCTAGGAACCCAGGCTAACC	Amplify region of the hTGP promoter by qPCR
Infusion hTGp 4.5kb 5' region For	TATCGATAGGTACCGAGCTCATTCCAGAGCCATCCAGTTCCTTT	Amplify 4.5 kb hTGP promoter
Infusion hTGp 3.5kb 5' region For	TATCGATAGGTACCGAGCTCCCCCACAGGCACAGGCAT	Amplify 3.5 kb hTGP promoter
Infusion hTGp 3kb 5' region For	TATCGATAGGTACCGAGCTCGTGGGGGAGGGGGATGCT	Amplify 3 kb hTGP promoter
Infusion hTGp 2.5kb 5' region For	TATCGATAGGTACCGAGCTCACCCAGGATGTAGGCCCAGTTCT	Amplify 2.5 kb hTGP promoter
Infusion hTGp 2kb 5' region For	TATCGATAGGTACCGAGCTCCATCCCTGGGCTTTTGGTCTG	Amplify 2 kb hTGP promoter
Infusion hTGp 1.5kb 5' region For	TATCGATAGGTACCGAGCTCAATAGGAACAGCTCCATCTTGCCA	Amplify 1.5 kb hTGP promoter
Infusion hTGp 4.5kb 3' region Rev	GATCGCAGATCTCGAGAATGCCAGGGAAGACTCTATCTCTGAT	Amplify all deletion mutants
NitroR ApaI For	CCCTGGCATTCCGCGGGCCCAAGCTTCCACCATGGATATCATTTCT	Clone NTR into pGL3 basic plasmid instead of Luciferase
NitroR NotI Rev	TCTAGAGTCGCGGCCGCTCATTACACTTCGGTTAAGGTGATGTT	Clone NTR into pGL3 basic plasmid instead of Luciferase
XhoI-NTR For	CTAGCCCGGGCTCGAGATGGATATCATTTCTGTCGCCTTAAA	Sequence nitroreductase
XbaI-NTR Rev	CCGCCCCGACTCTAGAATTACACTTCGGTTAAGGTGATGTTTT	Sequence Nitroreductase
NTR seq For	AGCGTCATTCCACTAAGGCATTTG	pFastBac1 vector cloning site
NTR seq Rev	CGAGATTTCGGCAGCGTAGC	pFastBac1 vector cloning site
pFastBac1F	GCGTCACCCGGCAACCTT	Amplify pFASTBac1 plasmid
pFastBac1R	GGGAACTGGGTGTAGCGTCG	Amplify pFASTBac1 plasmid

pUC/M13 Forward	CCCAGTCACGACGTTGTAAAACG	Clone NTR into pGL3 basic plasmid instead of Luciferase
pUC/M13 Reverse	AGCGGATAACAATTTCACACAGG	Clone NTR into pGL3 basic plasmid instead of Luciferase

C. Antibodies

Target Protein	**Manufacturer**	**Isotype**	**Application**
Androgen Receptor	Santa Cruz Biotech SC-816	Rabbit Pab	WB, ChIP, IF
RAR all isoforms	Santa Cruz Biotech SC-773	Rabbit Pab	WB, ChIP
RAR-Beta	Santa Cruz Biotech SC-552	Rabbit Pab	WB
RAR-Gamma	Santa Cruz Biotech SC-550	Rabbit Pab	WB
Tata binding protein	Abcam Ab818	Mouse Mab	WB
Beta-Actin	Sigma A5316	Mab	WB
Nitroreductase	Prof Nicol Keith, University of Glasgow	Mouse Mab	WB
Clusterin 05-354	Millipore	Mouse Mab	WB

Abbreviations

5-FC= 5-fluorocytosine

AAV= Adeno-associated virus

Ad= Adenovirus

AR= Androgen receptor

ARE= Androgen responsive element

atRA= *all trans* retinoic acid

bp= base pairs

BPH= Benign prostate hyperplasia

BSA= Bovine serum albumin

Bv= Bavulovirus

CAR= Coxsackie/adenovirus receptor

CB1954= 5-(Aziridin-1-yl)-2,4-dinitrobenzamide

CD:UPRT= Cytosine deaminase/uracil phosphoribosiltransferase

cDNA= complementary deoxyribonucleic acid

ChIP= Chromatin immunoprecipitation

CMV= Citomegalovirus

CRPC= Castration-resistant prostate cancer

CSC= Cancer stem cell

CTK= Cytokeratin

DHT= Dihydrotestosterone

DMEM= Dulbecco's modified Eagle's medium

DMSO= Dimethyl sulfoxide

DNA= Deoxyribonucleic acid

dNTP= Deoxyribonucleotide triphosphate

dsDNA= Double stranded deoxyribonucleic acid

E= early

EDTA= Ethylenediaminetetraacetic acid

EGF= Epidermal growth factor

EGFP= Enhanced green fluorescent protein

ER= Oestrogen receptor

ERE= Oestrogen responsive element

FACS= Fluorescent activated cell sorting

FCS= Foetal calf serum

FITC= Fluorescein isothiocyanate

GAPDH= Glyceraldehyde 3-phosphate dehydrogenase

GCV= Gancyclovir

GDEPT= Gene-directed enzyme prodrug therapy

HAT= Histone acetyltransferase

HDAC= Histone deacetylase

hKLK2= Human kallikrein 2

HRE= Hypoxia responsive element

HRP= Horseradish peroxidase

HSP= Heat shock protein

HSV= Herpes simplex virus

HSVTK= Herpes simplex virus thymidine kinase

hTERT= Human telomerase reverse transcriptase

hTGP= Human prostate-specific transglutaminase

HVEM= Herpes virus entry mediator

IE= Immediate early

IgG= Immunoglobulin G

IL= Interleukin

IPTG= Isopropil-β-D-1-thiogalactopyranoside

IR/DR= Indirect repeat/direct repeat

ITR= Inverted terminal repeats

ITS-G= Insulin-transferrin-selenium

kb= kilobase

KSFM= Keratinocyte serum-free media

L= Late

LB= Luria Broth

mM= Milimolar

MOI= Multiplicity of infection

MoMuLV= Moloney murine leukemia virus

mRNA= Messenger ribonucleic acid

MTS= (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl-2H-tetrazolium)

NLS= Nuclear localization signal

nM= Nanomolar

nm= Nanometer

NSC= Neural stem cell

NTR= Nitroreductase

PAGE= Polyacrylamide gel electrophoresis

PBS= Phosphate buffered saline

PCR= Polymerase chain reaction

PDK= Phosphoinositide dependent kinase

PEG= Polyethylene glycol

pfu= Plaque forming unit

PhIP= 2-amino-1methyl-6phenylimidazo[4,5-b]pyridine

PIA= Proliferative inflammatory atrophy

PIN= Prostate intraepithelial neoplasia

PIP3= Phosphatidylinositol-(3,4,5)-triphosphate

PPAR= Peroxisome proliferator-activated receptor

PSA= Prostate specific antigen

PSMA= Prostate specific membrane antigen

PTEN= Phosphatase and tensin homologue

qPCR= Quantitative polymerase chain reaction

R1881= Methyltrienolone

RA= Retinoic acid

RAR= Retinoic acid receptor

RARE= Retinoic acid responsive element

Rb= Retinoblastoma

RNA= Ribonucleic acid

ROS= Reactive oxygen species

RPM= Revolutions per minute

RPMI= Roswell Park Memorial Institute

rRNA= Ribosomal ribonucleic acid

RT= Room temperature

RT-PCR= Reverse transcription polymerase chain reaction

SB= Sleeping beauty

SDS= Sodium dodecyl sulfate

siRNA= Small interfering ribonucleic acid

SOC= Super Optimal broth with Catabolic repressor

STAT= Signal transducer and activator of transcription

TBP= TATA binding protein

TBS= Tris-buffered saline

tRNA= Transfer ribonucleic acid

TTNPB= (E)-4-[2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthylenyl-1-propenyl]

benzoic acid

U= Unit

UGM= Urogenital sinus

UGS= Urogenital mesenchyme

v/v= Volume/volume

VEGF= Vascular endothelial growth factor

VSV= Vesicular stomatitis virus

w/v= Weight/volume

WT= Wild-type

X-Gal= 5-bromo-4chloro-3-indolyl-beta-D-galacto-pyranoside

µM= Micromolar

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