Impact of *TMPRSS2-ERG* fusion gene on prostate cancer cell response to chemotherapy, radiotherapy and androgen deprivation therapy

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by

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Slav Roumenov Ovtcharov, Balliol College, DPhil in Surgery, Trinity Term 2015

Abstract

Many aspects of the mechanisms by which prostate cancer (PCa) progresses from being a confined tumour to advanced metastatic and castration-resistant disease remain unclear. The aim of this study is to evaluate *in vitro* the potential role of the fusion gene *TMPRSS2-ERG* in the response of PCa cells to ionising radiation (IR) and androgen deprivation therapy (ADT). This research focused on assessing the presence of the *TMPRSS2-ERG* transcript across various PCa cell lines and identifying any correlation between the *TMPRSS2-ERG* transcript and other genes, particularly genes related to DNA damage repair pathways. Several genes involved in cell metabolism and development were found to correlate with *TMPRSS2-ERG* but not genes involved in DNA repair. In accordance with previous reports, this research confirmed a proliferative advantage for cells expressing ERG. However this project also tested the role of ERG-status in response to chemotherapy, radiation and ADT. The data showed that VCaP and DuCaP cells exposed to low-dose radiation demonstrated decreased viability irrespective of their ERG-status. Similarly ADT decreased the viability of VCaP cells and seemed to neutralise the proliferative advantage of *TMPRSS2-ERG* positive cells. Stimulation with dihydrotestosterone caused increased radioresistance of *TMPRSS2-ERG* positive cells. Treatment with taxanes showed stronger effect on cells with lower ERG expression. This work suggests that the proliferative advantage conferred by ERG overexpression in *in vitro* models can be neutralised by castration and IR.

Посвещавам този труд на засегнатите от рак

Сърцето и мислите ми са завинаги с вас

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List of Abbreviations

ADT androgen deprivation therapy

AR Androgen receptor

BPH Benign prostatic hyperplasia

CZ Central Zone

CRPC Castration-resistant prostate cancer

CSS Charcoal Stripped Serum

DBD DNA binding domain

DBS double-strand damage repair

DDR DNA damage response

DHT Dihydrotestosterone

ERG V-Ets Avian Erythroblastosis Virus E26 Oncogene Homolog

ETS E26 transformation-specific

ETV1 Ets Variant 1

hCG human Chorionic Gonadotropin

HGPIN high-grade prostatic intraepithelial neoplasia

HR Homologous recombination

IR Ionising radiation

LH Luteinizing Hormone

miRNAs MicroRNAs

NHEJ Non-homologous end joining

OS Overall survival

PAP Prostatic acid phosphatase

PCa Prostate Cancer

PZ Peripheral

PIN Prostate intraepithelial neoplasia

PSA Prostate specific antigen

SNPs single nucleotide polymorphisms

siERG siRNA targeting the expression of the ERG oncogene

siRNA Small interfering RNA

T testosterone

TMPRSS2 Transmembrane Protease, Serine 2

TZ transition zone

UGS Urogenital Sinus

UV Ultra Violet

γ-H2AX Phosphorylated chromatin protein histone H2AX

53BP1 Tumour suppressor p53-binding protein 1

Chapter 1. Introduction

1.1 The prostate gland

The prostate gland is a component of the male reproductive tract. The position of the organ is anterior to the rectum, inferior to the bladder, posterior to the symphysis pubis and superior to the perineal membrane. The prostate surrounds the urethra which carries urine from the bladder and semen from the two ejaculatory ducts (Figure 1.1.1-1).

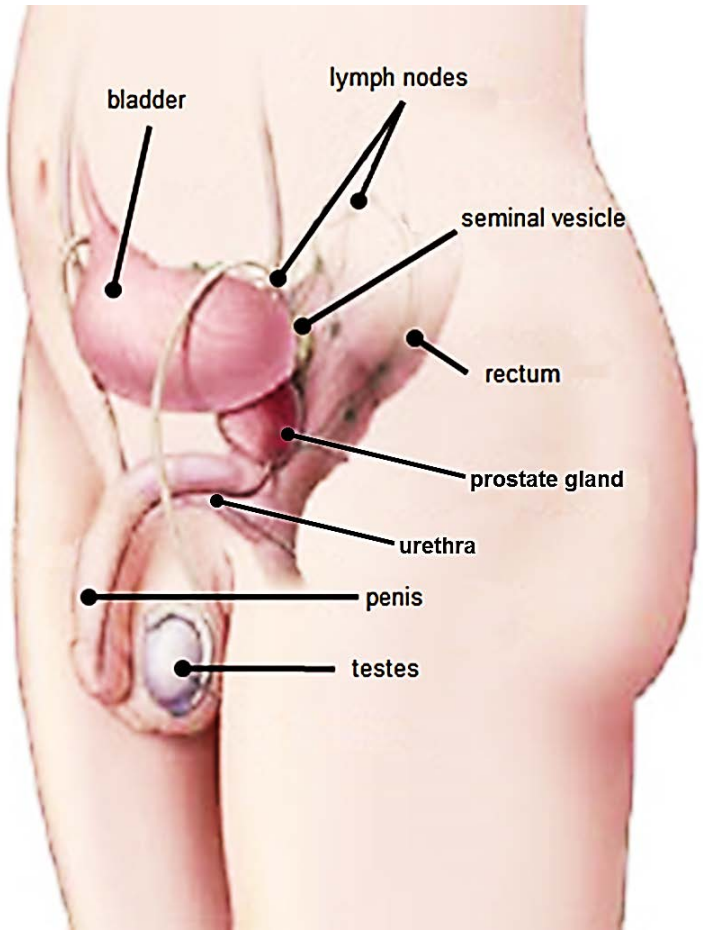


Figure 1.1.1-1 The prostate gland in men. The prostate gland sits below the bladder and in front of the rectum. Figure adapted from (National Institutes of Health 2014)

1.1.1 Prostate Development

Different transcription factors along with various hormonal, autocrine, and paracrine signalling pathways are involved in the development of the prostate gland (Prins & Putz 2008). The developmental process itself is continuous but can be divided into five different stages; determination, budding, branching morphogenesis, differentiation, and maturation during puberty. Embryologically most of the male accessory sex glands arise from the Wolffian ducts, which are of mesodermal origin, while the prostate gland is an endodermal structure that arises from the budding of tissue in the urogenital sinus (UGS). The development of the prostate gland is driven by androgens produced in the testes and begins at around 9 weeks of gestation. Initially this is controlled by human chorionic gonadotropin (hCG) produced by the placenta. As a result Leydig cells are stimulated to produce testosterone (T) (Sultan 2001; Byne 2006). After that, the foetus begins secreting its own luteinizing hormone (LH) that continues to stimulate the Leydig cells resulting in the completion of testicular descent and penile growth. Further molecular signals commit cells of the UGS to undergo prostatic differentiation. These UGS cells are epithelial and establish buds that spread into the surrounding UGS mesenchyme. The development of the prostate gland in the human male takes place during the second and third trimester and is complete at birth. The growth and proliferation of prostate epithelial cells are dependent on dihydrotestosterone (DHT). Surgical or chemical castration during key stages of prostate development leads to inhibition of prostate development (Lasnitzki & Mizuno 1977; Cunha 1973; Jost 1953; Price 1936). However, it has also been demonstrated that prostate determination can carry on to a significant

extent in the absence of androgens due to irreversible commitment of the cells (Lasnitzki & Mizuno 1977; Cunha 1973). At puberty, the structure of the prostate gland becomes more complex with tubuloalveolar glands organised in lobules. These are enclosed by stroma, which includes smooth muscle cells, fibroblasts, vasculature and nerves. Despite the exponential growth during puberty, the prostate gland is a slow-growing organ with doubling time of 2.76 years in adulthood (Berry et al. 1984). The pubertal prostate gland is controlled by androgens, which have increased levels during puberty. The androgens levels are sustained in adulthood and the size of the prostate gland stays about the same until the age of 40. At that stage the size of the prostate gland is enlarged as part of benign prostate hyperplasia. This is particularly evident in men over 50.

1.1.2 Anatomy

The prostate gland is enclosed by a thin fibrous capsule consisting of collagen and elastin with smooth muscle fibres. The main components of the prostatic tissue are glandular tissue and fibromuscular or stromal tissue (Deering et al. 1994). The glandular tissue contains acini and ducts. The periphery of the acini and the luminal layers in the acini comprise epithelial cells. The stromal layer consists of connective tissue, fibroblasts and smooth muscle fibres. Within the stroma there are many other cell types such as endothelial cells, lymphocytes, nerve cells and dendritic cells. The stromal layer also has blood vessels that supply nutrients and hormones to the epithelial cells (Figure 1.1.2-1). Histologically three different cell lineages in the pseudostratified columnar epithelium define the prostate gland. Differentiated non-proliferating luminal cells

are arranged as an uninterrupted layer of polarised columnar cells responsible for the secretion of proteins such as prostate specific antigen (PSA) and are characterised by cytokeratin 8 and 18 expression. These cells also express high levels of androgen receptor (AR). A second type of cell is the proliferating basal cell which is situated below the luminal layer, and is characterised by expression of cytokeratin 5 and 14, and p63. Basal cells demonstrate very low or undetectable expression of AR. Both basal and luminal cells have been thought to be the cells of origin for prostate cancer (PCa) (Goldstein et al. 2010; X. Wang et al. 2009). A third type of cell is the neuroendocrine cells, which are less frequent and have an unclear function. No AR expression has been reported in this cell type. Along with these three cell types, the prostate epithelium contains a small population of stem cells, which account for the morphogenesis and cellular turnover in the normal human prostate gland. Usually these cells are located within the basal layer of the epithelium and account for 0.5-1% of the cells (Isaacs & Coffey 1989). The intermediate type of cells are situated between basal and luminal cells. Their biology remains unknown although they were suggested as precursor cells of prostate cancer (van Leenders et al. 2003).

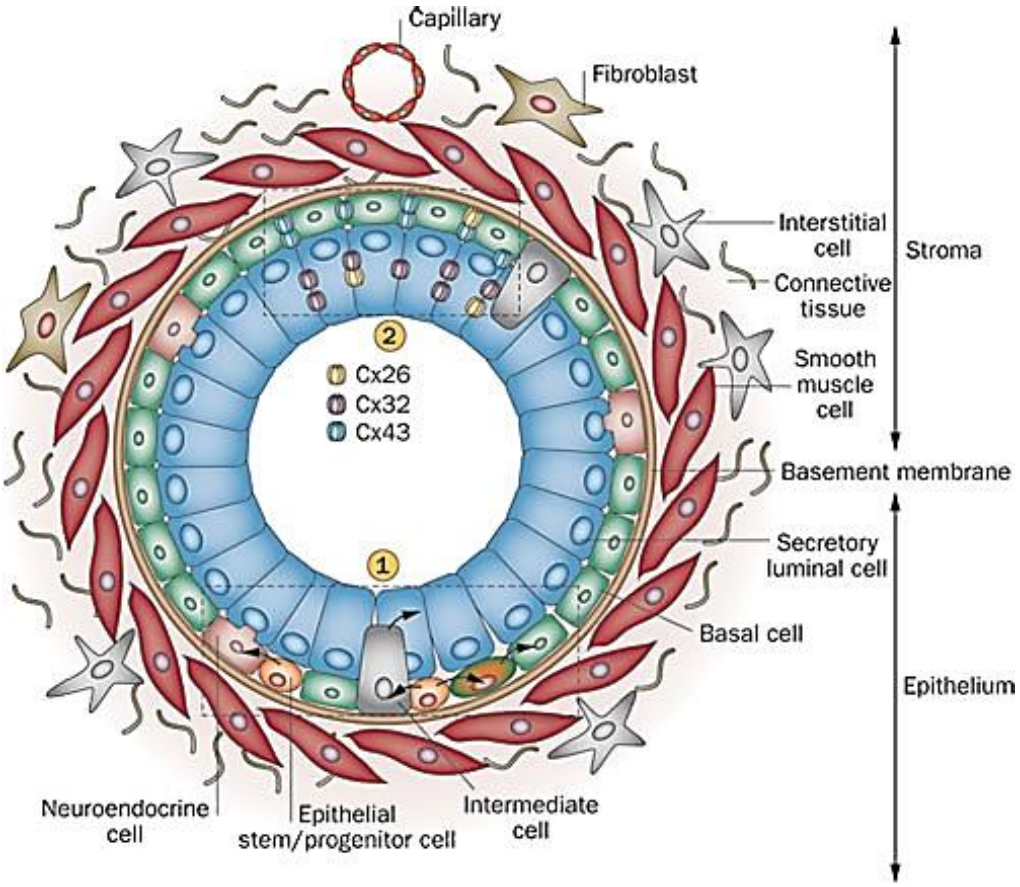


Figure 1.1.2-1 Structure of the prostate epithelial parenchyma and stromal matrix. The basal layer of the epithelium gives rise to basal and neuroendocrine cells. They also differentiate into luminal progenitors, which produce secretory cells. Elevated expression of connexins Cx32 and Cx43 is linked to alternative cell lineages. The role of Cx26 in this process is unidentified. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Urology (Czyż et al. 2012)

1.1.3 Prostate Zonal Anatomy

Although the adult prostate gland lacks an apparent lobular structure McNeal suggested a way to classify the prostate gland from a clinical point of view (McNeal 1988; McNeal 1981; McNeal 1969). This classification is based on regions or zones and divides the organ into three morphological zones: peripheral zone (PZ), central zone (CZ), transition zone (TZ), and anterior fibromuscular stroma (AFS, Figure 1.1.3-1). Up to 75% of prostate adenocarcinomas arise within the PZ (Hamm et al. 2009), whilst 25-40% of PCa arises within the TZ.

Several studies reported that cancers arising within the TZ are confined within the organ and have a more favourable outcome compared to cancers arising within the PZ. However a subclass of TZ cancers have a significant risk of metastatic disease (Shannon et al. 2003; Greene et al. 1991; McNeal et al. 1988; Noguchi et al. 2000). TZ cancers appear to arise within benign prostatic hyperplasia (BPH) nodules (Davis et al. 2010; Patel et al. 2011; Kabalin et al. 1989), whereas the CZ appears unaffected by either PCa or BPH.

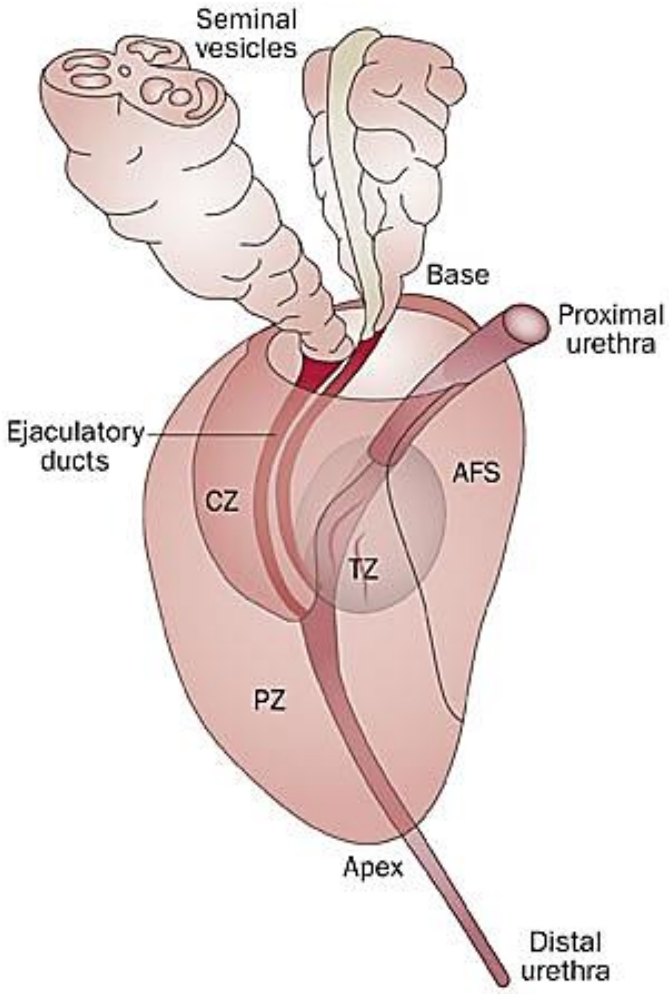


Figure 1.1.3-1 Zonal anatomy of the prostate gland. Three zones can be defined within the prostate including the central zone (CZ), the transition zone (TZ) and the peripheral zone (PZ). The PZ is deficient anteriorly and this area is filled by the fibromuscular stroma (AFS). Permission obtained from Elsevier Ltd © Wein, A. J. et al. Campbell-Walsh Urology 9th edition (Saunders Elsevier, Philadelphia, 2007)

1.1.4 Function

The main function of the prostate gland is to contribute to seminal fluid which makes up 20-30% of the total volume of fluid in the ejaculate. The secretion from the prostate gland is continuous but intensifies during sexual stimulation. The prostatic fluid is initially acidic but when mixed with alkaline secretions originating in the seminal vesicles results in seminal fluid with an alkaline pH of 7.2-8.0. Important components of the seminal fluid include proteolytic enzymes such as PSA and prostatic acid phosphatase (PAP) along with calcium, zinc, simple sugars and citric acid. The function of PSA is to liquefy coagulated semen and hence improve the chances of fertilisation. In addition, PSA is involved in the rapid cleavage of the seminal vesicle–secreted proteins semenogelin I and II (Lilja 1985). The main function of semenogelin I is to hinder the motility of intact spermatozoa and to capacitate the sperm by inhibiting mobility post-ejaculation. Therefore, PSA is linked to the production of progressively motile sperm (de Lamirande et al.). PSA is produced mainly in the acini and ducts of the prostate gland. The prostate gland also contains numerous valves organised in a way that supports the flow of semen into the urethra during ejaculation. Prostatic secretions also have a protective effect on the urinary tract by preventing bacteria from colonising the urethra.

1.2 Prostate cancer

1.2.1 Origin of cancer

Early insights into the origin of cancer appeared in 1914 when the German biologist Theodor Boveri presented his model of tumourigenesis (Hardy & Zacharias 2005). His work showed that individual chromosomes possess specific traits, and that only particular combinations can promote normal development. Boveri suggested that errors during cell division can affect the normal segregation of chromosomes leading to aberrations in the hereditary material such as chromosome aneuploidy, thus producing cell clones that can give rise to cancer. More recently these remarks were extended to include somatic mutations within single genes, known as tumour suppressor genes and oncogenes, that can inhibit or promote cancer progression respectively (Hardy & Zacharias 2005; Gibbs 2003; Hameroff 2004; Manchester 1997).

To date the general understanding is that cancer is a process in which somatically acquired genetic mutations cause normal cells to transform into malignant cells with uncontrolled cell division, abnormal differentiation, and inhibited apoptosis, resulting in imbalances in tissue homeostasis (Stratton et al. 2009). Malignant cells in turn can activate angiogenesis in the microenvironment, and are capable of invading into adjacent tissues or spread (metastasise) to other sites of the body. In 2000 Hanahan and Weinberg introduced a model that describes six hallmarks of cancer (Figure 1.2.1-1), and this has subsequently been extended to include two further hallmarks (Hanahan & Weinberg 2011). The first of these

hallmarks is the development of growth signal autonomy through the generation of growth signals by the cancer cell, thereby decreasing the cell's dependency on the signalling from the normal tissue microenvironment. Next is the capability of the cell to escape antiproliferative signalling, and the acquired ability to escape programmed cell death (apoptosis), which allows the tumour cell population to expand. Another important hallmark of cancer cells is their limitless replicative potential, termed "immortalisation" (Wright et al. 1989). Without oxygen and nutrients supplied by blood vessels, the cancer cells have a weaker ability to expand. Therefore they also need to be able to foster the formation of new blood vessels, a process known as "angiogenesis". One of the most significant traits of cancer cells is their capacity to spread and form metastases in other organs and this is the cause for 90% of cancer-related deaths (Mehlen & Puisieux 2006; Nguyen et al. 2009; Monteiro & Fodde 2010). The processes enabling the acquisition of these hallmarks include genomic instability and the development of premalignant or malignant lesions, which can cause an inflammatory state managed by innate immune cells. Additional novel hallmarks of cancer cells include their ability to re-programme the energy metabolism of cells, and to escape destruction by immune cells (Hanahan & Weinberg 2011).

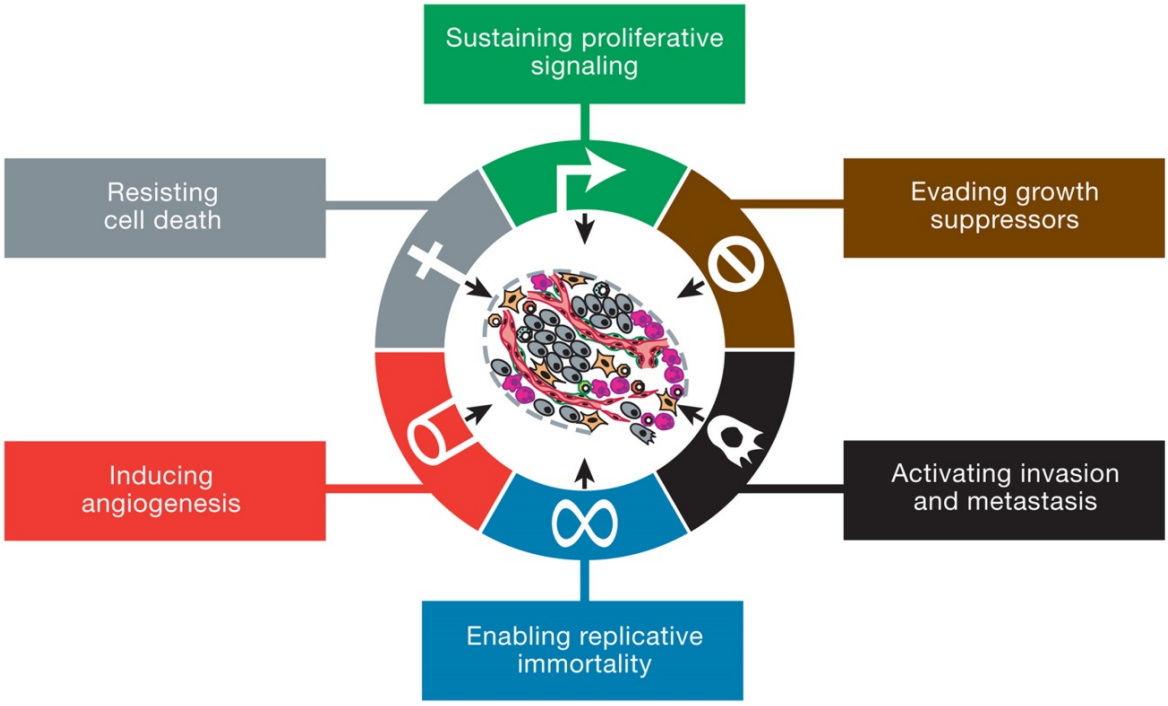


Figure 1.2.1-1 Hallmarks of cancer. The figure represents the six hallmark of cancer as suggested by Hanahan and Weinberg in 2000. Reprinted from Hallmarks of Cancer: The Next Generation (Hanahan & Weinberg 2011) with permission from Elsevier.

The extent of mutational signatures involved in tumourigenesis is very heterogeneous and complex. Somatic mutations can comprise various events related to the efficacy of DNA damage and DNA maintenance processes, such as base insertion, deletion or substitution, defective DNA repair, and copy number chromosomal variations and rearrangements (translocations). Similarly some mutations are likely to arise as a result of intrinsic errors of the cell machinery during DNA replication. Various epigenetic alterations can also be transmitted during mitotic cell division (Laird 2005). In addition, there are different exogenous or endogenous mutagen exposures or stimuli that can cause somatic mutations (Loeb & Harris 2008). For example, it has been demonstrated that smoking and

UV-light are accountable for lung cancer and melanoma of the skin respectively (Pfeifer 2010).

Different sets of mutated genes have been identified and relate to numerous cancer types. Cancer is therefore often perceived as being a combination of several diseases and not a single disease (Alexandrov et al. 2013; Seyfried et al. 2014; Seyfried 2012; Stratton 2011). The management of cancer and its different manifestations therefore call for a personalised approach that is tailored to each patient's unique tumour gene expression profile. It is now clear that almost every kind of genetic error can be detected in cancer cells. Interestingly, different sets of mutated genes can be found in different types of cancer (Ferlay et al. 2010; Hanahan & Weinberg 2011). However it remains doubtful whether some of these mutations are indeed related to tumourigenesis. In fact many of them do not confer selective growth advantage and are regarded as "passengers". On the other hand, "driver mutations" represent a subgroup of abnormalities that promote clonal expansion, invasion, and metastasis of cancer cells (Stratton 2011; Stratton et al. 2009).

New research data and clinical findings are emerging which suggest that the interaction between tumour and stromal cells plays a crucial role in tumourigenesis. This interaction is part of a complex system within a particular organ known as tissue homeostasis and involves the whole organism. The most common sites of origin of cancer are in epithelial tissues such as skin, prostate, breast, and colon, causing the deaths of millions of people across the globe each year. The leading cause of cancer death worldwide is lung cancer (Cancer Research UK 2014).

1.2.2 Incidence, mortality and risk factors

PCa is the most common male non-dermatological cancer in Europe with around 417,000 new cases in 2012 and 41,700 in the UK in 2011. The incidence of PCa in the UK is the 17th highest in Europe. PCa accounts for around 92,300 deaths in the European Union in 2012 and around 10,800 deaths in the UK alone (Cancer Research UK 2014). Because of its frequency the disease causes significant morbidity to patients and society and an economic burden to healthcare providers (Roehrborn & Black 2011). PCa originates in the prostate gland, which is dependent on a variety of different signalling pathways, transcription factors, and hormones (Prins & Putz 2008). The most prominent hormones involved in prostate regulation and development are testosterone and its more potent form DHT. The interplay of these androgens and androgen-driven signalling is also a part of PCa progression.

The pace of the disease can be unpredictable in individual men, varying from very slow growth to moderately fast progression (Freedland 2011). In most cases PCa can be slow-growing and may not cause any problems or symptoms for many years. In fact many men die with but not because of PCa. Some studies indicate that the lifetime risk of a man being diagnosed with PCa is 16%. At the same time the risk of an individual dying from PCa is 3% (Brawley 2012; Yin et al. 2008). Therefore, the more aggressive cases of PCa require adequate treatment in order to prevent dissemination outside of the prostate gland and development of metastases. Thus a key decision in PCa diagnostics is which patients have aggressive disease and require radical therapy, and which men have less aggressive PCa.

The causes of PCa are largely unknown but there are some well-established risk factors, such as age, race, and family history. As with cancers in other organ sites, PCa starts with alterations in one or more cells which results in uncontrolled increased cell proliferation and/or decreased cell death. Notably, these changes also reflect PCa resistance to hormone therapy and chemotherapy, which are key steps in tumour progression and metastasis (Adams & Cory 2007; Lowe et al. 2004; Johnstone et al. 2002). In the UK the rates of PCa have increased almost three fold during the last 30 years and currently there are more than 40,000 men being diagnosed with prostate cancer every year. The death rate is more than 10,000 a year, which means one man dies every hour because of PCa. This makes it the 4th most common cause of cancer death, representing approximately 7% of all cancer-related deaths, and the 2nd most common cause of cancer death in men in 2011 (Cancer Research UK 2014). In the UK in 2008-2010 the highest incidence rates (75%) were in men over 65 years of age, and only 1% in men under 50 years (Figure 1.2.2-1). Similarly the mortality rate is highest in older men, with an average of 73% of deaths occurring in men over 75 years of age (Cancer Research UK 2014).

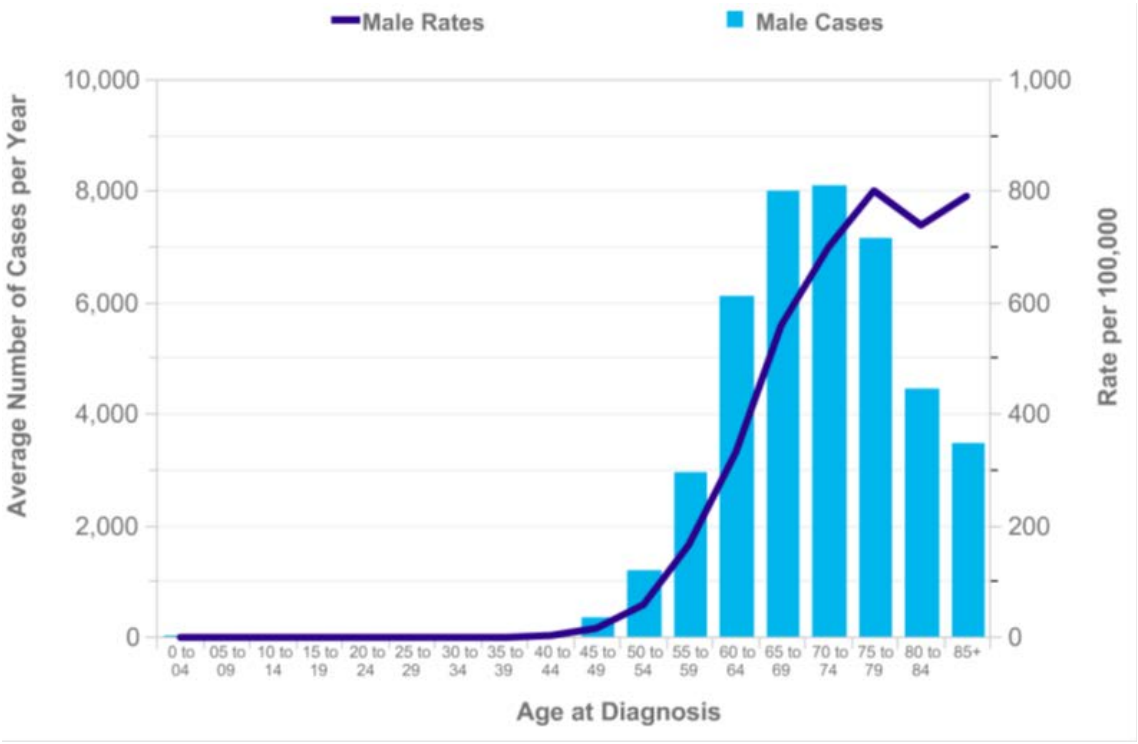


Figure 1.2.2-1 Incidence of PCa. Rates per 100,000 Population, Males, UK, 2009-2011. Prepared by Cancer Research UK - original data sources are available from http://www.cancerresearchuk.org/cancer-info/cancerstats

It has been demonstrated that family history plays an important role in PCa incidence. For example studies have indicated that men with an affected father have a 112-140% higher risk of developing PCa. Men with an affected brother have a 187-230% higher risk, and when men have a mother diagnosed with breast cancer the risk rises by 19-24%. The risk also increases when more than one first-degree relative, or a second-degree relative, is affected (Kiciński et al. 2011; Johns & Houlston 2003; Bruner et al. 2003). Regarding ethnicity, the cancer statistics for 2005 in the United States show that African Americans have a 62% higher risk of developing PCa compared to the Caucasian population. Furthermore, levels of incidence are much lower in Asian Americans and American Indians than in any other group investigated (Jemal et al. 2005). Other factors that affect the risk for PCa are diet, obesity, and sexual health.

1.2.3 Genetic predispositions

Genetic variations are very common in cancerogenesis. While germline mutations are inheritable, somatic mutations occur in cells such as those of the prostate epithelium. Many PCas have structural genetic lesions such as amplifications, deletions, or rearrangements. Different mutations associated with PCa are thought to have the potential to improve diagnosis and treatment of the disease. Different approaches for discovery of new mutations in PCa have now moved to large-scale techniques for genome sequencing and transcriptome analysis. Importantly, androgen signalling has been identified as a key mediator in the generation of some mutations. For instance, about half of all prostate tumours harbour a translocation of E26 transformation-specific (*ETS*) transcription factors, such as Ets Variant 1 (*ETV1*) or v-ets avian erythroblastosis virus E26 oncogene homolog (*ERG*), to the androgen responsive promoter of the transmembrane protease serine 2 (*TMPRSS2*) gene (Tomlins et al. 2005). However, a large number of PCas are ETS negative and little is known about the drivers of tumourigenesis in those cases. Point mutations are not very common in PCa, but where they occur, they result in a single substitution of a nucleotide base pair that may affect a single amino acid within a protein, or cause a truncated protein. On a nucleotide level, small insertion or deletion of nucleotides, termed “indels”, can cause frameshift mutations. As a result the reading frame of the gene is altered resulting in a change of the gene product.

One of the genes that is most-commonly mutated across *ETS*-negative cancers is the *SPOP* gene. This could suggest that *SPOP* is an alternative driver of cancer progression, which diverges from *ETS*-related translocations. Mutations in the

SPOP gene were found in about 6 to 13% of primary PCas, in 15% of metastatic tumours, and in 0% of benign samples. Moreover, it was observed that the presence of a SPOP mutation is mutually exclusive from ETS chromosomal aberration (Razzak 2012). PCas with a SPOP mutation had a higher rate of 5q21 and 6q21 deletions, which suggests they might comprise a separate group of cancers (Barbieri et al. 2012). The main function of SPOP is to regulate various signalling pathways such as steroid receptor signalling, c-Jun kinase and the hedgehog pathway (Liu et al. 2009; Li et al. 2011). Other ETS-negative PCas overexpress the serine peptidase inhibitor Kazal type 1 (SPINK1) which is also known as a tumour-associated trypsin inhibitor (Scott A Tomlins et al. 2008).

A key group of enzymes, termed Poly (ADP-ribose) polymerases (PARPs), regulate the repair of DNA single-strand breaks (SSBs) through base excision repair (BER). Thus BER of radiation-induced SSBs can be affected by inhibiting PARP activity. Furthermore, inhibition of DNA repair may cause aggravation of damage. For example PARP1 participates in the BER and SSB repair following irradiation. Inhibition of PARP1 leads to the formation of further DSBs because of the conversion of SSBs, which were not repaired by replication (Begg et al. 2011; Chalmers et al. 2010). Supposedly this leads to increased radiosensitivity *in vitro* (Noël et al. 2006; Dungey et al. 2008). In addition, PARPs are involved in controlling the transcription of the *AR* and other genes such as *ERG*, *FLI1*, and *ETV1*. Studies with PARP inhibitors suggest a tumour suppressing function for PARPs in preclinical models of PCas with *TMPRSS2-ERG* chromosomal aberrations, and in *EWS-FLI1* positive Ewing's sarcomas (Brenner et al. 2011; Sebastian de Bono et al. 2011; Brenner et al. 2012). Ongoing clinical trials are currently testing a role for PARP inhibitors in the treatment of ERG positive

tumours. PARP inhibitors can target cells with malfunctioning homologous recombination (HR), for example in tumours with dysfunctional BRCA1 or BRCA2 proteins, which are key players in HR. In PCa germline mutations in the *BRCA1* and *BRCA2* genes are associated with more aggressive disease and higher metastatic potential (Kote-Jarai, Leongamornlert, et al. 2011).

Increased signalling of the phosphoinositide-3-kinase pathway (PI3K) is very common in the vast majority of advanced PCas, occurring in between 25% and 70% of cases. A very frequent event in PCa is deletion of the phosphatase and tensin homolog gene (*PTEN*), which is a key tumour suppressor gene in the PI3K pathway. PTEN dephosphorylates intermediate products of lipid signalling, which results in deactivation of PI3K. Studies of its effects in mouse models, xenografts, and *in vitro* highlight the importance of *PTEN* as a tumour suppressor gene in PCa (Trotman et al. 2003; Chen et al. 2011; Carver et al. 2009). Another well-known tumour suppressor gene in PCa is *p53*, which is frequently mutated in this malignancy. p53 activates DNA repair, cell-cycle arrest and programmed cell death. Almost 40% of castration-resistant prostate cancers (CRPCs) express mutated p53 (Beltran et al. 2013).

Neuroendocrine differentiated PCa is predominantly a lethal subset of PCa that can arise in the late stages of this malignancy. This tends to metastasise to visceral organs, and recently it was shown that Aurora Kinase A (*AURKA*) is amplified in 65% of treated and hormone naïve cases, and in 86% of metastatic cases (Mosquera et al. 2013).

A post-mortem examination of metastatic lesions revealed numerous mutations in genes related to the AR signalling axis, including the *AR* itself, forkhead box A1 (*FOXA1*) which directly interacts with AR, CYP11B1, NKX3-1, FASN, GATA2,

TNK2, and PXN. Interestingly, variations were also observed in some ETS genes such as *ETS2*, which has a similar DNA binding domain to ERG, (Wei et al. 2010), *ETV3*, which does not have a defined role in PCa, *ELF1* and *ELK3*. Previous research has suggested a tumour suppressor function for *ETS2* (Demichelis et al. 2009; Perner et al. 2006; Yoshimoto et al. 2006). Additionally, frequent mutations have been observed in genes encoding histone-modifying proteins such as MYST4, MLL2, MLL3, MLL5, CHD1, CHD3, and NCO1, and DNA repair proteins such as BRCA2, ATM, XRCC4, and RAD 50. Some mutated genes, such as *OR5L1* and *CDK12*, are not yet known to have a defined function in PCa. However, previous data suggested that *CDK12* is also mutated in ovarian cancer (Cancer Genome Atlas Research Network 2011). Furthermore, well-known oncogenes such as *KRAS*, *SPOP*, *ROS1*, *JAK2*, *RET* and *MET*, or tumour-suppressors such as *PTEN*, *TP53*, *RB1*, *ZFHX3* and *APC*, were also found to be mutated in PCa. This data may shed light on the numerous processes involved in resistance to treatment (Grasso et al. 2012).

1.2.4 Candidate epigenetic biomarkers in prostate cancer.

Epigenetics defines a group of heritable controls capable of changing genome function without causing any changes to the DNA sequence. Epigenetic changes involve precisely orchestrated chemical reactions that activate or deactivate specific regions of the genome at specific times. Some of these reactions can participate in carcinogenesis and cellular transformation (Feinberg & Tycko 2004; Schulz & Hatina 2006; Garcia-Manero & Gore 2005; Hake et al. 2004). To date in addition to genetic mutations and chromosomal aberrations, epigenetic

modifications are increasingly being studied in order to establish their pathological effect and mechanisms of disrupting gene function. The three most studied mechanisms in remodelling of chromatin structure are DNA methylation, histone modifications and microRNAs (miRNAs).

1.2.4.1 DNA methylation

DNA methylation is a process which is dependent on the activity of DNA methyltransferases (Goldberg et al. 2007; Lopez-Serra & Esteller 2008; Sharma et al. 2010). Over 90% of PCas demonstrate altered promoter methylation of the glutathione-S-transferase P1 (*GSTP1*) gene making this an interesting candidate for a novel biomarker. The *GSTP1* gene expresses an enzyme involved in protection of the DNA molecule from oxidizing agents and electrophilic substances (Henrique & Jerónimo 2004). *GSTP1* methylation demonstrates superior specificity when compared to PSA testing for PCa diagnosis (>90% specificity for *GSTP1*,~20% for PSA) but its sensitivity is modest and similar to PSA (Wu et al. 2011). The DNA methylation levels of GSTP1 may potentially be utilised to distinguish PCa from other conditions of the prostate including benign prostate hyperplasia (BPH) and high-grade prostatic intraepithelial neoplasia (HGPIN) (Lee et al. 1994; Nakayama et al. 2003). Furthermore, the combination of this biomarker with the *APC* gene can increase specificity and sensitivity even further (Jerónimo et al. 2004). Other advantages of GSTP1 are its association with particular stages of PCa, and the possibility of detecting this epigenetic change in body fluids (Jerónimo et al. 2004; Enokida et al. 2005; Li et al. 2004). *APC* is another candidate biomarker that is hypermethylated in all stages of PCa.

The *APC* gene is a tumour suppressor and participates in cell migration, adhesion, and Wnt signalling. The protein can also be detected in body fluids and is often studied along with *GSTP1*.

1.2.4.2 Histone modifications and chromatin remodelling

Particular modifications of histones can be used as a prognostic marker in PCa. For example modifications of H_3K_9Ac, $H_3K_{27}Me_3$, $H_3K_4Me_2$, $H_4R_3Me_2$, $H_4K_{12}Ac$, and $H_3K_{18}Ac$ have been related to PCa stage. The combined use of $H_3K_{18}Ac$ and $H_3K_4Me_2$ improves detection of relapsing PCa in men with low grade disease (Seligson et al. 2005). Total levels of $H_3K_{18}Ac$ and $H_3K_4Me_2$ have also been found to be predictive of advanced PCa (Bianco-Miotto et al. 2010). One of the most studied histone-modifying enzymes is the histone methyltransferase Enhancer of Zeste Homologue 2 (EZH2), which catalyses trimethylation of lysine 27 of histone H3 producing $H_3K_{27}Me_3$. EZH2 was found to be the most significantly upregulated gene in a study of progressing PCa (Varambally et al. 2002). Changes in $H_3K_{27}Me_3$ levels have been reported in PCa and other cancer types including breast, ovarian and pancreatic cancer. A total loss of $H_3K_{27}Me_3$ has been linked to adverse prognosis (Rogenhofer et al. 2012; Wei et al. 2008), whilst the total level of $H_3K_{27}Me_3$ has been found to be higher in metastatic PCa cases (Ellinger et al. 2012).

1.2.4.3 MicroRNAs

MicroRNAs (miRNAs) comprise a group of small (18–25 nucleotide sequence), non-coding, single-stranded RNAs. They can bind their complementary target

mRNAs at the 3′ untranslated region (UTR) after transcription and thus down-regulate the expression of particular genes through the prevention of protein translation. The expression of miRNAs in PCa is a developing area of biomarker research. Potential advantages of miRNAs are that they are tissue specific, highly-stable, and can be extracted from blood, urine, and other body fluids (Lu et al. 2005; Mitchell et al. 2008; Bianco-Miotto et al. 2010). Abnormal expression of miRNAs has been related to prognosis, invasiveness and metastasis of different cancer types including PCa. However, out of approximately 50 miRNAs only a small group has been studied in more detail and connected to the development of PCa. Expression of miR-31, miR-96 and miR-205 have been linked to Gleason score in PCa. miR-125b, miR-205, and miR-222 have been linked to stage (Martens-Uzunova et al. 2012). miR-96 has been found to be prognostic for biochemical recurrence, and higher expression of the molecule was indicative of an adverse prognosis. Another study identified miR-9, miR-141, miR-200b, miR-375 and miR-516-3p as being the most overexpressed miRNAs among 667 miRNAs in the serum of 21 men with PCa. Further validation of this group in another cohort of 45 men with PCa identified miR-141, miR-200b and miR-375 to be predictive of high-risk cancer and higher Gleason score (Brase et al. 2011).

1.2.4.4 Single nucleotide polymorphisms

To date around 70 independent susceptibility loci termed single nucleotide polymorphisms (SNPs) have been linked with PCa. These have been identified through genome-wide association studies (GWAS) in Europeans, Asians and

African Americans, and are associated with increased PCa risk (Amundadottir et al. 2006; Yeager et al. 2007; Gudmundsson et al. 2007; Al Olama et al. 2009; Schumacher et al. 2011; Kote-Jarai, Amin Al Olama, et al. 2011; Xu et al. 2012). The molecular mechanism underlying their biological role in PCa however remains largely unknown, but the main concept revolves around impact on gene regulation by influencing the recruitment between transcription factors and transcriptional enhancer elements (Schödel et al. 2012; Cowper-Sal lari et al. 2012; Ward & Kellis 2012). Most of the SNPs occupy noncoding regions of the human genome and may have regulatory roles (Freedman et al. 2011; Hindorff et al. 2009).

Many SNPs are associated with PCa and act upon the androgen biosynthesis pathway. For instance, the *CYP11A1* gene encodes the cytochrome 450 side chain cleavage enzyme, which catalyses the initial phase of androgen synthesis. The $(tttta)_n$ repeat is the most studied SNP in this gene and was linked to an increased risk of aggressive PCa. Remarkably, lack of the $(tttta)_4$ allele in some patients was correlated with a higher risk of developing metastases compared to individuals with the $(tttta)_4$ (Kumazawa et al. 2004).

The HSD3B family is another class of important enzymes involved in the catalysis of active DHT into inactive metabolites in steroid-regulated tissues including the prostate. Two SNP variants, namely rs1047303 and rs1819698, have been found to correlate with a higher risk of PCa in Caucasian populations (Chang et al. 2002). However subsequent reports have failed to confirm the statistical significance of this data (Cunningham et al. 2007).

Various other SNPs act along the androgen metabolic pathway. Prostate growth is dependent on DHT signalling and the function of the steroid 5α-reductase type

II enzyme (SRD5A2) which irreversibly metabolises testosterone into DHT. In 1999 a study of PCa risk in African Americans and Latino Americans identified a substitution variant of SRD5A2 termed rs928285, and this was found to be associated with a higher risk of aggressive PCa (Makridakis et al. 1999). However a subsequent study reported that the potential effect of this variant in Caucasians is less than 1% (Ntais et al. 2003). Furthermore, a more recent analysis reported that SRD5A2 has no effect on PCa risk (Beuten et al. 2009).

Many studies have focused on establishing the potential role of two SNPs in the transactivation domain of AR which are termed (CAG)n and (GGN)n and which are trinucleotide repeat segments. An increased risk of PCa was observed in men with less than 21 CAG repeats (Zeegers et al. 2004). However a subsequent replication study identified a significant but reverse pattern (Lindström et al. 2006).

Rs2735839 occupies a region, adjacent to the *KLK3* gene encoding PSA. Previous data has linked this SNP to serum PSA expression. Furthermore, it has been suggested that there is a link between this SNP and PCa, which is not necessarily related to PSA screening since the samples used were not from screened individuals (Ahn et al. 2008).

In general the vast majority of SNPs studied in PCa have low penetrance. It remains unclear which variants play an important role in PCa risk and which are a result of other genetic variants of a particular gene. In addition, a number of polymorphisms display individual patterns of variation in different ethnic groups, geographical regions and races. This may also be due to differences in diagnostic procedures, sample sizes or the relative gene milieu. Nevertheless the question

as to whether SNPs associated with PCa can improve the overall strategy of assessing genetic risk remains unanswered.

1.2.5 Gene fusions

Oncogenes and gene fusions participate in cancer pathogenesis. Gene fusions usually arise as a result of somatic genomic rearrangements. Many gene fusions have been found in leukaemias, sarcomas and lymphomas where they lead to the expression of functional fusion proteins (Rowley 2001). Gene fusions are present in practically all cases of chronic myeloid leukaemias (CML), 15% - 30% of B cell and T cell tumours, and in around 20% of acute myeloid leukaemias (AML). In the case of soft tissue and bone cancers the percentage of gene fusions is between 15% - 20% (Mitelman et al. 2007). Gene fusions were previously thought to be rare in malignant epithelial cancers. Whilst they were common in papillary thyroid cancers, occurring in around 40% of cases, this type of cancer represents only 1% of all cancer cases and is therefore uncommon. In more commonly occurring tumour types, for example lung cancer, breast cancer and cancers of the digestive system, the percentage of malignancies with gene fusions is less than one. However, in 2004 this interpretation was revised as it was shown that the proportion of genomic rearrangements across various tumour types including haematological, epithelial, and mesenchymal tumours was similar (Mitelman et al. 2004). In 2005 recurrent mutations were identified in PCa cases by Tomlins et al. using new bioinformatics techniques termed “Cancer Outlier Profile Analysis” (Tomlins et al. 2005). It was shown that chromosomal rearrangements in PCa included members of the ETS family and lead to the

formation of fusion proteins. As a consequence of these rearrangements ETS proteins become controlled by androgen-responsive promoter elements (Tomlins et al. 2005; Tomlins et al. 2006; Tomlins et al. 2007; Helgeson et al. 2008). These oncogenic fusions lead to elevated levels of ETS factors driven by androgens and AR signalling. This may in turn promote PCa growth and progression (Hermans et al. 2006; Mehra et al. 2007; Perner et al. 2007).

1.2.6 The ETS family

The E26 transformation-specific (ETS) superfamily of proteins comprises evolutionarily conserved oncogenic transcription factors that consist of 27 members in humans. They share a DNA-binding motif, or ETS domain, which is approximately 80 to 90 amino acids long. The ETS proteins bind purine-rich DNA regions and can act as activators or repressors of transcription. The core motif 5'-GGAA/T-3' is flanked by other nucleotides (Graves & Petersen 1998). ETS proteins are linked with diverse functions such as cell proliferation, differentiation, apoptosis, lymphoid cell development, angiogenesis, and cancer cell invasion (Sementchenko & Watson 2000). Many of these transcription factors are involved in tumour progression and may contribute to a coordinated programme resulting in tumour metastasis and invasion (Wasylyk et al. 1993). Although their exact function remains unclear, ETS family members have been involved in chromosomal rearrangements in leukaemias, lymphomas and Ewing's sarcomas (Oikawa & Yamada 2003; Yagasaki et al. 2001). The recurrent gene rearrangements in prostate cancer usually include one 5' partner, such as *TMPRSS2* or *SLC45A3*, and a 3' partner, such as *ERG, ETV1*, *ETV4* or *ETV5*.

Emerging data suggests that the ETS fusions play crucial roles in PCa development and thus offer potential new targets for treatment.

1.2.7 The ERG gene

The avian acute leukaemia virus E26 is replication-defective and was identified as a cause of mixed erythroid-myeloid leukaemia in chickens (Moscovici et al. 1981; Radke et al. 1982). Its genome contains two oncogenes, *v-myb* and *v-ets*. The ERG protein is a member of the ETS family of oncogenes and is located on chromosome 21q22.2. Emerging data suggests that different diseases such as leukaemia, Down's syndrome and Alzheimer's disease are each associated with aberrations in genes on chromosome 21. A recent study showed that ERG expression status demarcates the total proteome for tumours of the prostate. ERG-expressing tumours demonstrated enhanced activity for cell growth and survival pathways, while tumours lacking ERG had enhanced proteasome and redox function pathways (Tan et al. 2014). A new physiological role of ERG in the inhibition of cytokine-driven vascular inflammation has also been identified. It was shown that ERG-driven pathways repress the nuclear factor NF-κB–dependent transcription and TNF-α–induced inflammation (Sperone et al. 2011). Another study reports on the prognostic impact of ERG in patients with cytogenetically normal acute myeloid leukaemia (CN-AML). The data demonstrated that in patients aged over 60 years there is a correlation between low ERG expression and better disease-free survival and overall survival, as well as a higher complete remission rate (Schwind et al. 2010). This data corresponds with previous discoveries in younger patients with CN-AML showing that lower ERG levels are

associated with higher rates of complete remission and longer event-free survival (Marcucci et al. 2007).

1.2.8 TMPRSS2 protease

The serine protease gene family includes protein-cleaving enzymes with numerous functions such as blood coagulation, tissue remodelling, digestion, invasiveness and apoptosis. Three serine proteases (PSA, hK2, and PRSS18) are prostate-specific and are regulated by androgen hormones in the process of liquefaction of semen (Rittenhouse et al. 1998; Nelson 1999). Furthermore, ectopic expression of these proteases may promote tumour growth and invasion by interacting with the extracellular matrix. PSA in particular is capable of cleaving glycoproteins in the extracellular matrix and thereby promotes cancer cell invasion (Webber et al. 1995). The biological role of another transmembrane protease serine gene, *TMPRSS2*, is however unclear. *TMPRSS2* encodes a 492 amino acid protein which is regulated by androgens (Lin et al. 1999), but *in vivo* studies suggest that it normally has functional redundancy or plays a nonvital role. In fact, *TMPRSS2* deficient mice do not develop abnormally compared with wild-type controls, and no alterations in prostatic secretions or in the normal function and histology of the prostate was observed. Furthermore, no defect was detected in terms of fertility. Also, a lack of *TMPRSS2* was not related to cell abnormalities, PCa development or reduced survival (Kim et al. 2006). TMPRSS2 contains five distinct regions including a type II transmembrane protease domain (suggesting that TMPRSS2 is expressed on the cell surface), a scavenger-receptor cysteine-rich domain which is probably associated with binding of cell

surface molecules, an LDL receptor class A domain, and intracellular regions (Paoloni-Giacobino et al. 1997). A previous study described an autocatalytic cleavage of the protease domain in mice compared with other human tissues. TMPRSS2 has high levels of expression in normal and cancer prostate epithelium. Lower levels of expression are observed in colon, lung, kidney and pancreatic epithelium. The *TMPRSS2* gene has been reported as being a fusion partner of ETV1 and ETV4 (Tomlins et al. 2005; Tomlins et al. 2006; Hermans et al. 2006).

1.2.9 TMPRSS2-ERG fusion

The most common recurrent chromosomal rearrangement in PCa results in the formation of the *TMPRSS2-ERG* fusion gene, which is found in nearly 50% of PCas (Tomlins et al. 2009; Mosquera et al. 2009). It has been suggested that the presence of *TMPRSS2-ERG* in a single focus of PCa is mutually exclusive to the presence of chromosomal abnormalities involving one of the other ETS family members (Tomlins et al. 2005; Tomlins et al. 2006; Svensson et al. 2010). It has been demonstrated that in around 90% of samples with higher levels of ERG expression there is a gene fusion between the 5' untranslated region of androgen-regulated TMPRSS2 and the transcription factor ERG, thus supporting the idea that the primary mode of ERG overexpression is through a *TMPRSS2-ERG* rearrangement (Tomlins et al. 2005; Perner et al. 2007). The *TMPRSS2* and *ERG* genes are normally situated in close proximity to each other, being only 2.85MB apart, and they have the same orientation on chromosome 21q22.2. In around 60% of *ERG* fusion positive PCas the main mechanism of translocation of *ERG*

and *TMPRSS2* is through deletion of the intervening sequence (Edel) (Hermans et al. 2006; Perner et al. 2006; Iljin et al. 2006). Less commonly, the intervening sequence is inserted into another chromosome (Esplit) (Tomlins et al. 2009). On Figure 1.2.9-1 is shown a common model that describes the formation of the fusion gene *TMPRSS2-ERG*. In a clinical setting, the most common way of detecting the fusion between TMPRSS2 and ERG involves fluorescence in situ hybridization (FISH) or polymerase chain reaction (RT-PCR). Nevertheless, these two methods are time consuming and require substantial expertise. Interestingly, in 2010 Park et al identified a reliable ERG-targeting antibody that demonstrated strong correlation between ERG expression and *TMPRSS2-ERG* presence detected using FISH analysis (Park, Tomlins, Mudaliar, et al. 2010). The ERG antibody was found to have high specificity and sensitivity for ERG immunohistochemistry in determining the status of *TMPRSS2-ERG*.

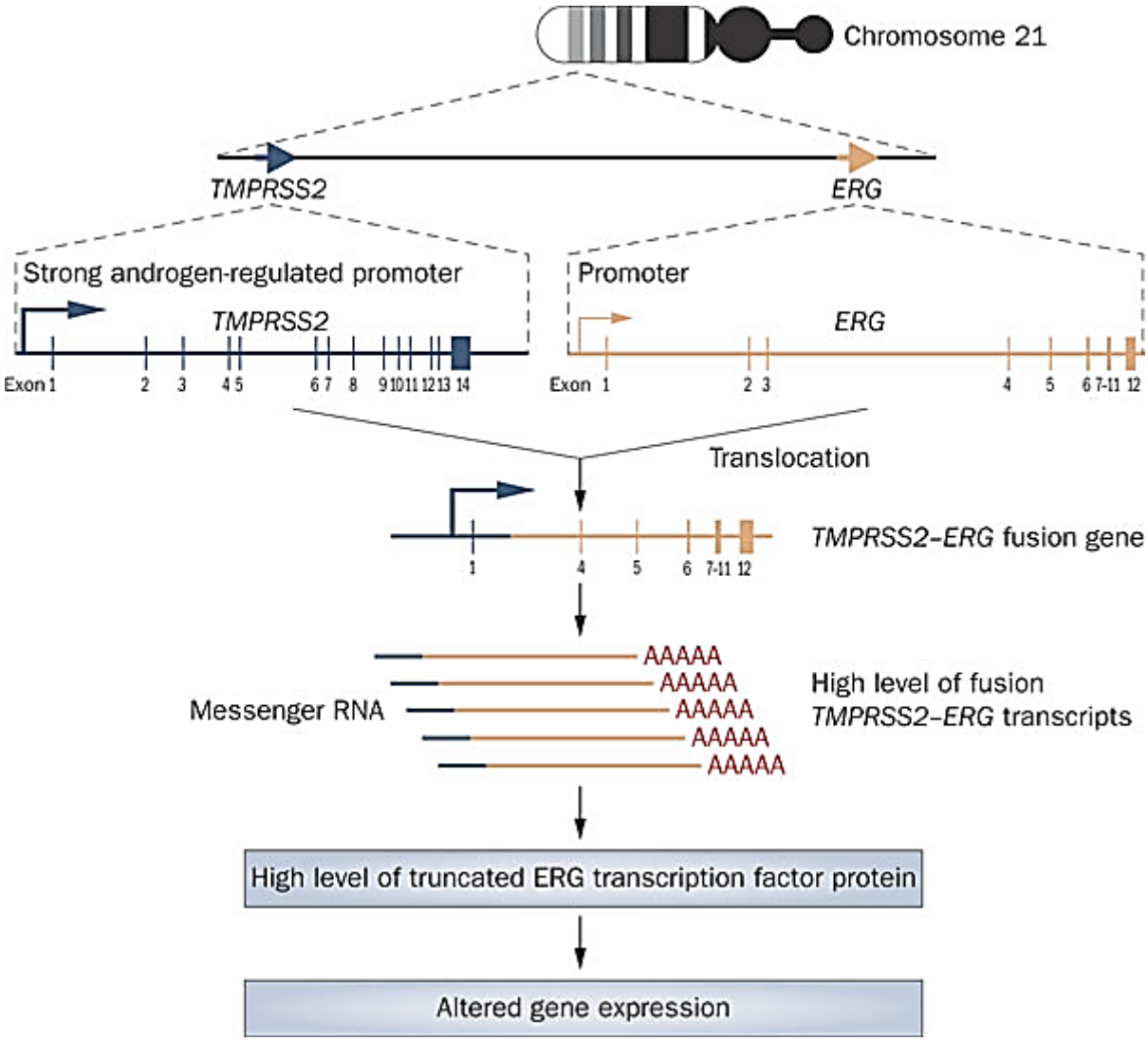


Figure 1.2.9-1 A model of formation of *TMPRSS2-ERG* transcript. The TMPRSS2 contains an androgen-regulated promoter, which becomes fused to the ERG transcription factor. This results in the formation of the *TMPRSS2-ERG* transcript (middle), which produces a truncated ERG involved in the expression of various genes. Reprinted by permission from Macmillan Publishers Ltd: [Nature Reviews Urology] (Clark & Cooper 2009), copyright (2009).

Previous data indicated that androgen signalling causes chromatin alteration and promotes co-localisation of *TMPRSS2* and *ERG* in prostate cells (Rickman et al. 2012). As a consequence, double strand-breaks are more likely to result in fusion genes (Lin et al. 2009). The mechanism of AR-promoted juxtaposition could be an explanation for the exclusivity of *TMPRSS2-ERG* to prostate cells (Mani et al.

2009). Moreover, the AR signalling axis induces co-localisation of AR and topoisomerase II beta (TOP2B) at genomic breakpoints of *TMPRSS2-ERG*. This initiates recombinogenic double-strand break via TOP2B. AR can in turn mediate the generation of *de novo TMPRSS2-ERG* transcripts (Haffner et al. 2010). The fusion of these two genes can result in higher levels of mRNA fusion product expression in response to androgen- and oestrogen-stimulation, and can encode a truncated ERG protein. This data provides potential new insights into the molecular biology of androgen-dependent development of prostate tumours. Previous studies suggested that an ERG rearrangement may be an initiating molecular event playing a key role in the development of PCa. However, the experimental data suggests that although ERG can initiate local lesions and prostatic intraepithelial neoplasia (PIN), it is not sufficient alone to initiate carcinoma (Perner et al. 2007; Zong et al. 2009; Carver et al. 2009). It is noteworthy that the combination of ERG overexpression and either PTEN loss or AKT up-regulation is associated with invasive carcinoma or neoplastic transformation as shown on Figure 1.2.9-2.

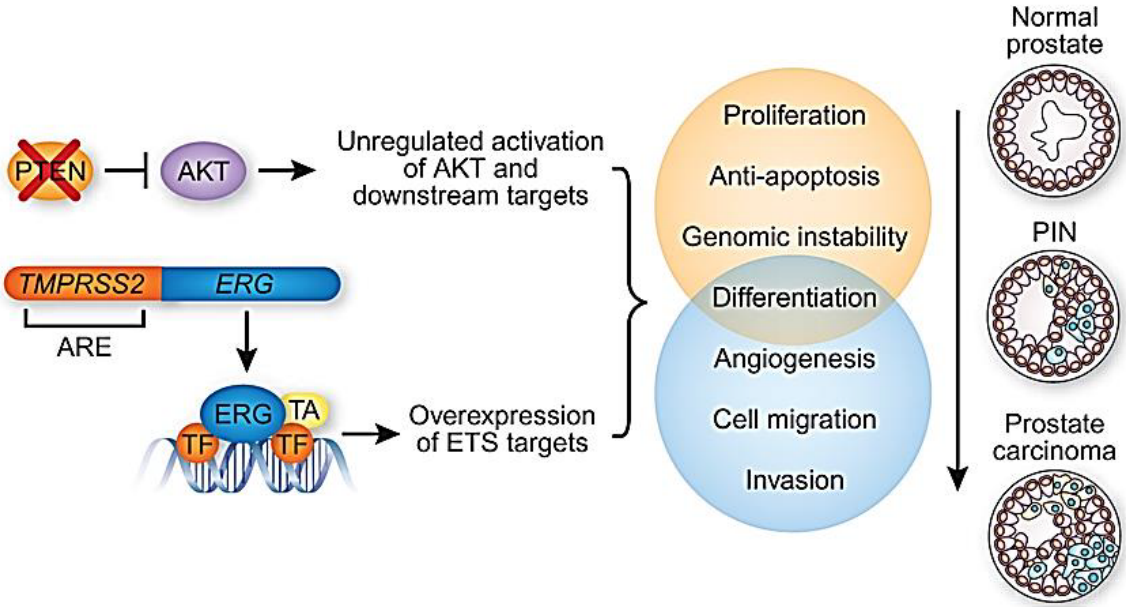


Figure 1.2.9-2 A model of PCa progression involving TMPRSS2-ERG. The illustration describes a potential cooperation between TMPRSS2-ERG and PTEN loss resulting in AKT upregulation. This in turn mediates the advancement to PCa by activating downstream signalling pathways. As a result the selective advantage of premalignant prostatic intraepithelial neoplasia (PIN) cells escalates. Abbreviations: ARE, androgen response elements; TA, transcriptional activator; TF, transcription factor. Reprinted by permission from Macmillan Publishers Ltd: [Nature Genetics] (Squire 2009), copyright (2009)

Other prostate diseases such as BPH and proliferative inflammatory atrophy (PIA) are *TMPRSS2-ERG* fusion negative (Cerveira et al. 2006; Mosquera et al. 2008; Tomlins et al. 2005). However, 19% of HGPIN and 19% of clinically staged T1 PCas expressed *TMPRSS2-ERG*, whilst 30% of hormone naïve metastases and 33% of CRPC cases harbour the fusion gene. The mean age of the patients was 63 years (ranging from 48 to 76) (Attard, Clark, et al. 2008; Demichelis et al. 2007; Perner et al. 2007). The presence of the fusion gene has also been shown to be greater in moderate to poorly differentiated PCas (41%) in comparison to well differentiated tumours (7%) (Rajput et al. 2007).

Different research groups have studied the potential connection between *ERG* gene expression and cancer aggressiveness in the context of heterogeneity amongst *TMPRSS2-ERG* mRNA variants. The results are conflicting, and some

studies relate *TMPRSS2-ERG* to aggressive tumours, whereas others relate it to indolent cancers. Possible explanation for this may be the heterogeneous, multifocal nature of PCa.

1.2.10 Diagnosis

Presently there is no single test for the detection of PCa, and early stage PCa is usually symptomless. Diagnosis of PCa initially involves a prostate-specific antigen (PSA) blood test and digital rectal examination of the prostate and if either of these are abnormal a trans-rectal ultrasound (TRUS)-guided prostate needle biopsy is taken for diagnosis. Other investigations which may be undertaken include computerised tomography (CT), magnetic resonance imaging (MRI), and radionuclide bone scan to identify metastatic disease if suspected.

Since the 1980s the introduction of PSA testing has significantly improved the diagnosis of PCa. In addition, the introduction of PSA testing coincides with a fall in PCa mortality rates in some countries. However, it is unclear whether this fall is purely because of PSA testing or because of improved treatment options. Ideally the use of PSA testing should reduce mortality rates, but PSA lacks specificity for PCa, and an uptake in PSA testing has also led to the problem of overdiagnosis and overtreatment of indolent prostate tumours (Walter et al. 2006). In 2009 a large randomised screening trial in the US concluded that there was no significant difference in mortality between men who received annual screening and a control group (Andriole et al. 2009). However this study had a substantial rate of prostate screening among men in the control arm. Nevertheless in 2012 the United States Preventive Services Task Force issued

a recommendation against PSA-based screening practices for PCa (Moyer 2012). Interestingly, a screening trial in Europe reported a 20% relative reduction in PCa mortality in men who received PSA screening (Schröder, Hugosson, et al. 2009). Despite the problems associated with a lack of specificity, potential overdiagnosis and overtreatment, PSA remains the single major biomarker for men at increased risk of PCa (Fleshner & Lawrentschuk 2009; Schröder, Roobol, et al. 2009). At present the vast majority of newly-diagnosed PCas are either confined within the prostate or locally advanced, without spread beyond the pelvis. Several methods can be used to treat PCa including active surveillance, radical prostatectomy, radical radiotherapy, and androgen deprivation therapy, with treatment options depending on various aspects of the malignancy including tumour grade and tumour stage.

One of the main challenges in PCa diagnosis is the heterogeneous and multifocal nature of the disease which can result in variable clinical outcomes (Arora et al. 2004; Ruijter et al. 1996). The presence of multiple independent foci of adenocarcinoma in a prostate needle biopsy also raises various diagnostic and clinical questions. For example a single small cancer focus may be missed or diagnosed incorrectly.

There are several prostate cell types that are abnormal but not necessarily cancerous. Prostatic intraepithelial neoplasia (PIN) refers to prostate cells that look premalignant under the microscope but have not yet become invasive. Low-grade PIN does not involve any specific treatment and its relation to PCa remains unclear. High-grade PIN on the other hand indicates an increased risk of PCa and therefore it may require repeat biopsies to be taken from the prostate depending on local diagnostic protocols. Atypical small acinar proliferation

(ASAP) refers to cells that look malignant but their number is insufficient to confirm the presence of a tumour microscopically. Since ASAP may be associated with PCa elsewhere in the gland it is usually followed by a repeat biopsy. Proliferative inflammatory atrophy (PIA) refers to abnormal cells that are associated with areas of inflammation and this may evolve into PIN or PCa.

The need for novel biomarkers that can be utilised for screening, prognostication, and detection in body fluids such as blood, serum, or urine is of ongoing interest. Promising biomarkers need to be further validated and evaluated as to whether they can improve clinical outcome. Some of the biomarkers researched in the past may be useful clinically but to date none of them have shown sufficient superiority to PSA testing to replace this test.

1.2.11 Grading

PCa is graded using the Gleason score system, which assigns a grade from one to five and reflects the similarity between cells in the cancerous tissue and normal cells in the prostate. Grade 1 indicates high similarity with normal cells while Grade 5 indicates very abnormal cell morphology and growth patterns. Two areas that make up most of the cancer are assessed and added together to produce the Gleason sum score. The Gleason sum score can vary between two and ten. Cancers with Gleason sum scores of six or less are called well-differentiated or low-grade cancers. Cancers with Gleason sum score seven are called moderately differentiated or intermediate-grade. Cancers with Gleason sum score between eight and ten are called poorly-differentiated or high-grade. In

conclusion higher Gleason sum scores are associated with more aggressive PCa.

1.2.12 Staging

In order to predict clinical outcome it is necessary to assess the correct stage of a tumour growth by analysing the findings of PSA measurement and DRE, sometimes supplemented by CT or MRI. Staging is generally required if there is a clinical suspicion of metastases. The staging of PCa is based on the TNM system which evaluates tumour (T), lymph nodes (N), and metastases (M).

1.2.13 Treatment

Once PCa has been diagnosed and staged it is necessary to develop an appropriate treatment plan for each individual patient based around tumour factors and patient factors such as age and comorbidity.

Low risk localised PCas may be managed by “active surveillance” which describes careful monitoring of the cancer using regular PSA testing, DRE, and intermittent biopsies in order to detect any potential tumour progression, at which point an active treatment option such as surgery or radiotherapy is offered. Active surveillance can save many men from overtreatment when they do not have life-threatening PCa, and the regular tests ensure that any symptoms and signs of cancer progression are identified in a timely manner.

“Watchful waiting” describes a follow-up with less frequent tests and more focus on changes in symptoms, and is a potential option for men with greater comorbidity. The emphasis here is more about symptom control than cure.

Radical prostatectomy is a surgical treatment strategy aimed at removing the prostate gland and the tumour tissue with curative effect. Side effects can include urinary incontinence and erectile dysfunction.

Radical radiotherapy is another curative option which uses high-energy x-rays or additional types of radiation to treat cancer by destroying malignant cells. It is used for localised or locally advanced PCa, although palliative radiotherapy can also be used to slow down the growth of metastases. Short-term side effects of radiotherapy involve discomfort in the treated area, loss of pubic hair, diarrhoea and cystitis. Long-term side effects can involve urinary incontinence, erectile dysfunction, inflammation of the rectum, diarrhoea, bleeding and discomfort.

Androgen deprivation therapy (ADT) is often utilised before, during and after radiotherapy in order to increase the effect of radiation and reduce the chances of cancer recurrence. This treatment can include the use of antiandrogens in order to hinder androgen signalling. Hormone therapy may have various side effects such as erectile dysfunction, hot flushes, sweating and swelling of the male breasts. In the long-term the patient may experience weight gain, bone thinning (osteoporosis), depression and an increased risk of myocardial infarction. A surgical alternative to hormone therapy is the removal of the testicles, known as orchiectomy.

Chemotherapy is mainly used for metastatic tumours that do not respond well or at all to hormone therapy. The use of different drugs aims to destroy cancerous

cells by interfering with cell growth. Possible side effects of chemotherapy include the killing of immune cells, infections, hair loss, infertility and vomiting.

Usually radical treatment of PCa is aimed at the whole prostate gland, but focal therapy is a developing concept currently undergoing research.

1.3 Radiotherapy

Radiotherapy is considered one of the most effective curative options for the treatment of localised PCa. Radiation is measured in an SI unit named the Gray (Gy) where 1 Gy is equivalent to the energy of 1 J per kg of mass. The term Gray mainly refers to absorbed radiation dose. Radiotherapy is not always effective with the standard radiation doses (74 Gy in 2 Gy fractions) and there are different ways of improving its efficacy such as raising the radiation dose, improving the distribution of the dose, and integrating image-guidance into daily treatment delivery. Different studies of intermediate and high-risk PCas have suggested improved outcomes from radiotherapy when it is combined with neoadjuvant, concurrent, and adjuvant hormone therapy.

1.3.1 Physiological implications of radiotherapy

The irradiation of any biological system triggers a cascade of processes divided into physical, chemical, and biological phases. During the physical phase interactions occur between the charged particles and atoms of the tissue. As a result a high-speed electron passes across a mammalian cell and ejects orbital electrons from atoms (termed “ionisation”) or raises other electrons to higher

energy levels in the same atom (termed “excitation”). This can lead to a cascade of ionisation events. During the chemical phase the damaged atoms and molecules react with other components of the cell and this can result in breakage of chemical bonds and the production of broken molecules known as free radicals. These are very reactive and aim to reach equilibrium with regards to their electronic charge. In this fashion changes can occur in biologically important molecules. All subsequent processes, including various enzymatic reactions and DNA damage repair, occur during the biological phase. Failure to repair some lesions can cause cell death. Cells do not die instantly and sometimes they can undergo several mitotic divisions beforehand. It can take several weeks before the first effects of radiation may be observed. These can include breakdown of the skin or mucosa, haemopoietic cell damage, and other effects. Secondary effects may be caused by the cell death and can result in compensatory cell proliferation in normal tissue or in tumours. At a later stage additional effects can be observed, such as fibrosis and telangiectasia of the skin, or blood vessel damage. Also the appearance of secondary malignancies can be a long-term side-effect of radiation damage (Joiner & Kogel 2009).

In everyday practice radiotherapy is often delivered to patients in 2 Gy fractions 5 times per week, so a 7-8 week period of treatment will total 66 to 78 Gy. This practice is based on the idea that most cancer cells are rapidly dividing, but slowly growing cells have a longer proliferation cycle and therefore more time for lesions to repair between sequential fractions. In the case of PCa it may be beneficial to fractionate with fewer, but larger, fractions.

1.3.2 DNA damage and repair pathways

Ionising radiation (IR) has the ability to ionise or eject electrons randomly from molecules within the cell. The strongest biological damage is produced by ejected electrons that can subsequently cause additional ionisations. Ionised molecules can trigger a cascade of reactions resulting in the breaking of chemical bonds. Most molecules such as mRNA or proteins have multiple copies and can undergo rapid turnover, thus the effects of IR can be minimised. However, DNA has only two copies in the cell, therefore disruptions in the DNA molecule can cause significantly greater damage to the cell, and can often cause cell death. It has been suggested that DNA is the main target for cell death caused by radiation, therefore cells and organisms have developed specialised repair mechanisms involving various protein interactions to maintain the correct structure of the DNA molecule. For example, double-strand DNA breaks are an extremely dangerous form of DNA damage, and in order to avoid propagation of mutations it is essential to detect and repair DNA damage before DNA replication and cell division.

Ionising radiation can cause lesions that, depending on the scale, can trigger different repair mechanisms. Some of these detect and repair bases, such as base excision repair (BER) or single-strand breaks (SSBR). Double strand breaks can trigger homologous recombination (HR) or non-homologous end-joining (NHEJ). It has been shown that these repair mechanisms are very effective. For example, 1 Gy will cause cell death in only 30 % of irradiated mammalian cells although the damage will be to more than 1000 nucleotide bases, with approximately 1000 single-strand DNA breaks and 20 to 40 double-strand breaks.

In general within seconds of cells being exposed to ionising radiation specialised proteins detect damaged regions. Next, the chromatin structure undergoes remodelling so that it becomes more accessible to the repair proteins. The whole repair process is constantly monitored in order to ensure that the chromatin is reset to its original shape. The DNA damage response (DDR) process is therefore a very complex and synchronised system involving various signalling pathways which can be divided into two groups of events; termed “sensors” and “effectors” of DNA damage. As their name suggests, the “sensors” actively monitor DNA and recognise damaged regions. These proteins signal to many other proteins which in turn activate three effector signalling pathways involving cell death, checkpoints, and DNA repair. The sensors of DNA damage determine the initial response of cells to DSB. The initial steps of this process involve the recruitment of large numbers of proteins to the site of DNA lesion. Usually they cluster in small regions or speckles called “ionising radiation induced foci” (IRIF), and these can be observed microscopically. It has been suggested that every focus represents a point from which DNA repair and further recruitment of effectors of DDR takes place.

1.3.3 Phosphorylated H2AX

Within minutes of DSB occurring, one of the very early stress responses in the DDR is the phosphorylation of a histone protein called H2AX. This protein takes part in the formation of the core nucleosome structure as DNA is wrapped around it. H2AX is a variant of the H2A histone protein, is located throughout the whole nucleus, and accounts for up to 15% of the H2A protein in the cell. The

phosphorylated form of H2AX is also known as γ-H2AX and this participates in the recruitment of different downstream proteins implicated in the DDR. It has been demonstrated that cells lacking the expression of this protein are radiosensitive. The presence of phosphorylated H2AX can be viewed with a microscope and has been utilised in the research of DSB repair mechanisms in irradiated cells. The presence of γ-H2AX is a precise method for identifying the presence and repair of individual DSBs in the nucleus after exposure of the cell to IR (Joiner & Kogel 2009).

1.3.4 53BP1

p53-binding protein 1 is another important surrogate marker for DNA damage response in irradiated cells. The protein is a mediator/adaptor protein of the DDR (Figure 1.3.4-1) and is a member of the BRCA1 carboxyl terminus (BRCT) repeat family (Bork et al. 1997; Abraham 2002). The BRCT domains are found in proteins involved in DDR pathways, such as the breast cancer protein BRCA1. The first evidence of 53BP1 function in DDR was visualised in irradiated cells stained with an anti-53BP1 antibody showing copious foci in the nuclei of cells treated with IR (Anderson et al. 2001; Rappold et al. 2001; Wang et al. 2002). Interestingly, the focal relocalisation of 53BP1 in response to DNA damage overlapped with nuclear foci of γ-H2AX, thereby indicating authentic sites of DNA lesions.

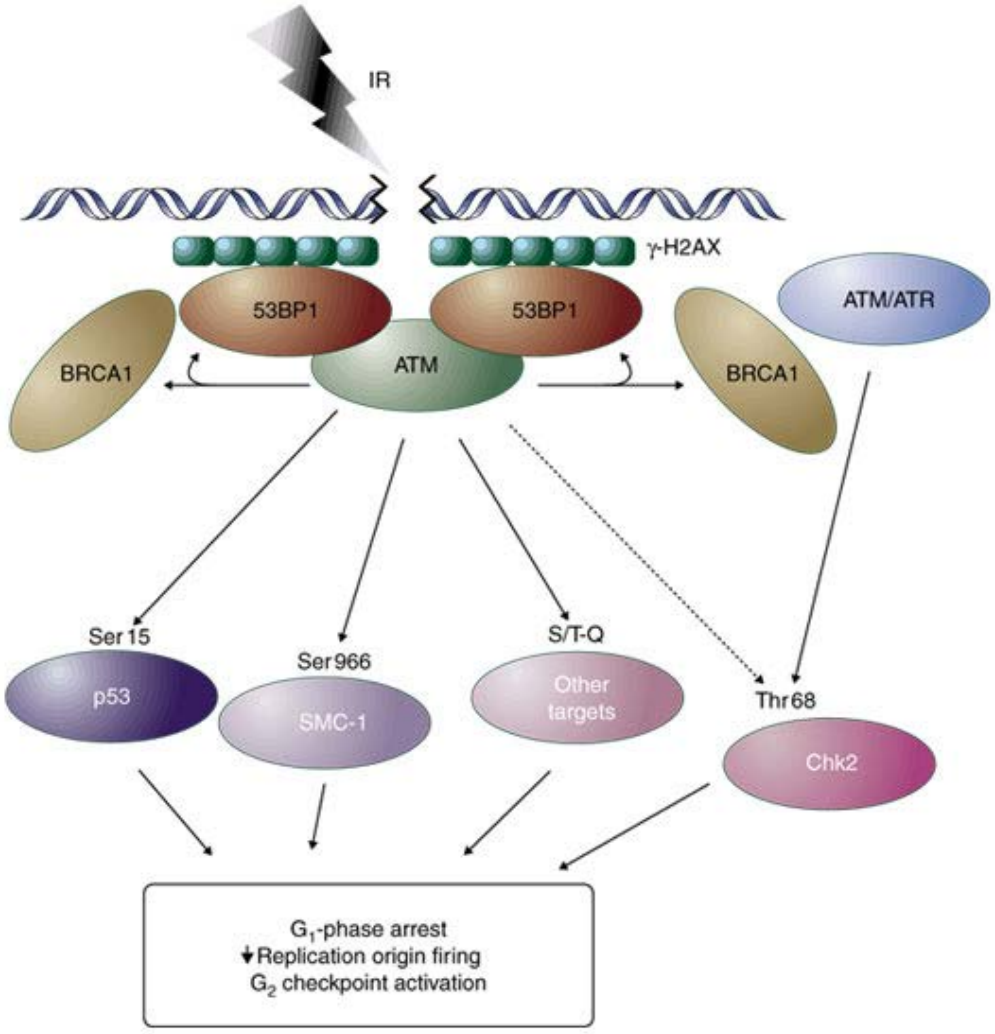


Figure 1.3.4-1 Early events in response to IR. IR and various genotoxic events cause DSBs. An early event in response to this is the phosphorylation of histone H2AX by mostly ataxia telangiectasia mutated (ATM) protein at the site of DSB or close to it. This interaction is required for further recruitment and phosphorylation of 53BP1 by ATM. The function of 53BP1 is to mediate the binding of ATM to numerous downstream targets such as p53 and SMC-1. The activation of Chk2 kinase may involve a different protein of the BRCT repeat family of proteins, which circumvents 53BP1. Reprinted by permission from Macmillan Publishers Ltd: Nature Cell Biology (Abraham 2002) Copyright 2002.

53BP1 is a mediator of the DDR and as such facilitates ATM-dependent phosphorylation events. Independent studies have demonstrated the importance of 53BP1 in checkpoint activation and DNA repair. Abrogation of 53BP1 results in intact or partial G2/M checkpoint cell cycle arrest. Furthermore, loss of 53BP1 function leads to genome instability. Following IR 53BP1 undergoes nuclear

relocalisation and is recruited to sites of nuclear foci in which upstream partners include phosphorylated H2AX (Celeste et al. 2003).

1.4 Hormonal therapy

1.4.1 Androgen Receptor

Although the exact mechanisms driving PCa remain to be fully elucidated, the Nobel-laureate Charles Huggins and his colleagues demonstrated the essential relationship between androgens and PCa progression (Huggins 1941). This led to the utilisation of hormonal therapy as a standard practice for advanced PCa. Androgen signalling is dependent upon the interaction of androgens with the nuclear AR. Signalling via the AR axis plays a pivotal role in both normal prostate development and prostate carcinogenesis.

1.4.2 Structure and function of androgen receptor

The AR is a 110 kDa protein which belongs to the superfamily of nuclear receptor transcription factors. The AR is encoded by the *AR* gene which is located on chromosome Xq11-12 (Lubahn et al. 1988; Brown et al. 1989; Committee 1999). Since the *AR* gene is situated on the X chromosome only a single-copy exists in men, and this consequently facilitates the phenotypic manifestation of mutations. The structure of the AR exhibits distinct functional regions and is similar to the structure of other steroid hormone receptors. The N-terminal domain has

transcriptional regulatory function and is encoded by a large first exon. This domain contains the ligand independent activation domain AF-1 which defines co-regulator binding and affects transcriptional activation. The central DNA-binding domain (DBD) is highly conserved and is encoded by exons two and three. The C-terminal ligand-binding domain (LBD) is encoded by exons four to eight and contains the ligand-dependent transactivation function AF-2. The LBD prevents the action of the receptor in the absence of ligand. The DBD and the LBD are separated by a hinge region comprising a nuclear localisation signal (Claessens et al. 2008; Lamont & Tindall 2010).

Several structurally diverse AR isoforms have been described in the literature. Intriguingly some of these show an association with aggressive prostate tumour growth (Watson et al. 2012; Zhang et al. 2011). For example the splice variant AR-V7 (or AR3) is missing the LBD and therefore is ligand-independent. As a result, the AR-V7 is constitutively active and triggers the expression of AR dependent genes. Furthermore, the expression of some AR isoforms increases after androgen depletion and decreases in the presence of testosterone (T). In some cases the expression of AR isoforms is also associated with the expression of a full-length AR. However, the increased cell growth caused by AR isoforms lacking LBD can be inhibited by LBD-targeting anti-androgen drugs such as MDV3100. Another effective method of inhibiting PCa cell growth is to use siRNA targeting the full-length AR mRNA, which indicates the importance of expression of the full-length protein in castration-resistant tumours (Watson et al. 2010).

AR is activated by the binding of androgen ligands. Androgens belong to a family of steroid hormones including the main androgen testosterone (T) and its more potent analogue DHT. T is produced by Leydig cells in the testes as a result of

stimulation by luteinising hormone (LH). In the prostate epithelial cells T is converted to DHT by steroid 5α-reductase (Russell & Wilson 1994; Fang et al. 1969). DHT has a 10-fold higher binding affinity for the AR than is the case for T, and therefore DHT plays a greater role in prostate development (Wilson & Gloyna 1970). Furthermore, DHT acts in a paracrine fashion to stimulate the secretory function of the epithelial AR. Studies of 5alpha-reductase deficiency in human males identified a complete lack of prostate morphogenesis but normal formation of the ductus deferens and seminal vesicles (Siiteri & Wilson 1974). Other androgens involved in the androgen signalling pathway are dehydroepiandrosterone and androstenedione which are both produced by the adrenal cortex and have a weaker effect.

In its “poised state” the ligand-free AR is sequestered in the cytoplasm and is bound by a heat shock protein (HSP) heterocomplex which protects the receptor from degradation. In the presence of a hormone ligand the AR undergoes conformational change and the HSPs dissociate. Next, the AR forms a homo-dimer with the ligand followed by nuclear translocation (Edwards & Bartlett 2005). In the nucleus the AR-ligand complex acts as a transcription factor and binds to specific androgen responsive element sites in the promoter regions of target genes. This in turn recruits coactivators which alter the chromatin structure. Eventually the complex is joined by RNA polymerase and transcription is initiated (Heinlein & Chang 2004). Further studies describe the function of AR as a coactivator which triggers of downstream kinase actions such as MAPK and PI3K, or as a repressor of certain genes (Baniwal et al. 2009; Unni et al. 2004; Castoria et al. 2004; Lu 2000).

The levels of expression of AR in the urogenital sinus (UGS) mesenchyme are elevated before and during morphogenesis of the prostate gland. At the same time, AR expression in the epithelium is limited. AR expression in the epithelium is initiated after budding and branching has started (Cunha et al. 1992; Cunha & Chung 1981). In the normal prostate the AR is mainly responsible for driving the differentiation of luminal epithelial cells and the expression of genes associated with the normal function of the prostate gland such as *PSA*. In PCa, however, the function of the AR is less clear but probably relates to activation of cell proliferation, cell cycle regulation and cell survival (Wang et al. 2009; Wang et al. 2007; Xu et al. 2006; Knudsen 1998).

1.4.3 Androgen deprivation therapy in prostate cancer

As PCa arises from prostate epithelium, PCa cells usually maintain their AR expression, and this has successfully been used in androgen deprivation therapy (ADT) as a standard therapy. Over 90% of men with PCa respond to ADT alone, but eventually castration-resistant tumours will evolve leading to aggressive carcinoma and poor outcome (Gittes 1991; Palmberg et al. 1999). Moreover, within two years of ADT 50% of cancers progress to castration-resistant PCa (CRPC) (Sharifi et al. 2005; Ramon & Denis 2007). Thus it remains unclear which patients will benefit from the therapy and how long this benefit will last. Several clinical trials have compared the effect of radiotherapy in combination with ADT versus radiotherapy alone. The trials focused on high-risk or intermediate-risk patients, and identified significant increases in disease-free and overall survival after combined use of radiotherapy and ADT (Bolla et al. 1997; Jones et al. 2011).

In addition, when ADT is combined with radiotherapy, post-treatment prostate biopsy series show better local control (Zelefsky et al. 2008). Another more recent study demonstrated a positive correlation between AR signalling and DNA repair genes. The results suggest that increased AR signalling promotes resistance to radiotherapy by initiating more rapid DNA damage repair in irradiated cells. This supports the concept that ADT synergises with radiotherapy (Polkinghorn et al. 2013).

1.4.4 Androgen signalling in prostate cancer

During PCa progression several changes occur in the AR signalling axis. One of the first changes is seen in the different pathways which incorporate AR function in normal and cancer cells. While in normal cells androgen-driven cell growth requires paracrine stimuli from the stromal cells, in cancer cells the signalling pathway of AR switches to an autocrine mode where no stromal interaction is required (Gao et al. 2001).

1.4.5 Mechanisms of castration resistance in prostate cancer

There are several models explaining the mechanisms by which tumours can escape ADT. These include amplification of the AR locus itself resulting in higher levels of AR mRNA and protein (Ford et al. 2003; Edwards et al. 2003; Brown et al. 2002; Visakorpi et al. 1995; Koivisto et al. 1997). Another model describes various AR alterations that can widen the range of AR ligands or bypass the activation by ligand completely. This results in AR activation by other steroid

hormones (Zhao et al. 2000; Taplin 2007; Brooke et al. 2009; Yuan & Balk 2009; Culig 1993; Tan 1997). Interestingly, some drugs such as bicalutamide and flutamide have been shown to switch from being an AR antagonist to being a strong agonist. In this case the drugs effectively select for mutated cancer cell clones that are then stimulated by the drug (Steinkamp et al. 2009). A third model describes several AR isoforms that can completely bypass ligand activation. Their shared feature is the lack of regions within the C-terminal LBD which leads to continuous AR activation (Hörnberg et al. 2011; Hu et al. 2009; Guo et al. 2009; Dehm et al. 2008; G. Jenster, H.A. van der Korput, J. Trapman 1995; Sun et al. 2010). ADT can also be evaded by dysregulated function of various cis-acting coactivators and corepressors that affect the normal AR function as a transcription factor, and thereby promotes PCa progression (Gregory, He, et al. 2001; Xu et al. 2009; Chmelar et al. 2007; Agoulnik et al. 2005; Halkidou et al. 2003; Johnson R.T., et al. 2001). Finally, ligand-independent AR activation through cross-talk with signal transduction pathways can occur. Usually these signalling pathways activate the AR indirectly by facilitating the phosphorylation of AR coactivator molecules. For example, EGF, IGF, IL-6, Wnt, PI3K and Ras-Raf-MAP kinase pathways all mediate the AR response to low levels of androgens (Weber & Gioeli 2004; Gioeli et al. 1999; Wu et al. 2006; Schweizer et al. 2008; Hernes et al. 2004; Krueckl et al. 2004). An outline of the different pathways by which PCa becomes androgen independent is shown on Figure 1.4.5-1.

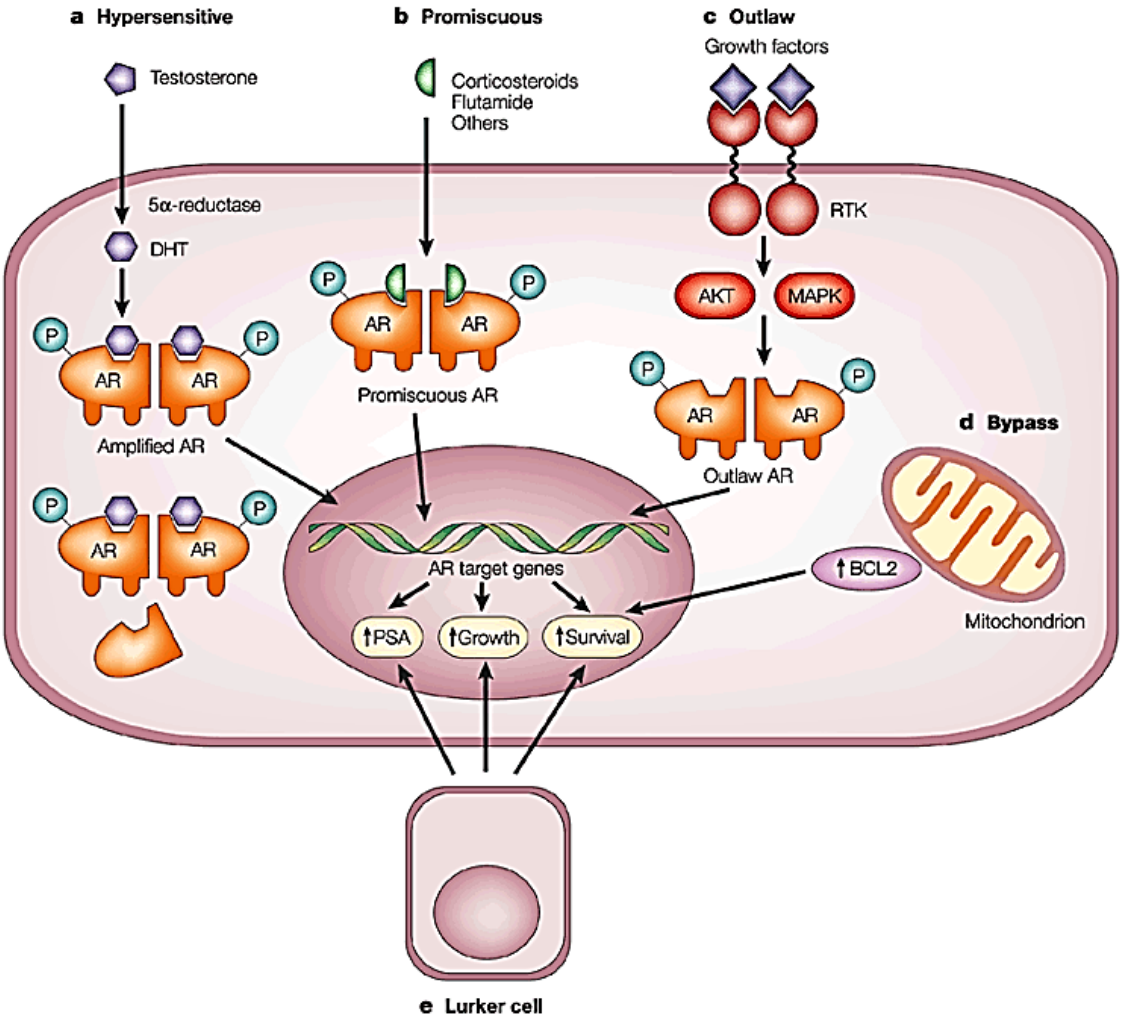


Figure 1.4.5-1 Five different models describe the process of developing androgen independence. a. illustrates the hypersensitive pathway where as a result of gene amplification more AR is expressed. This pathway can also include increased sensitivity of AR. **b.** The promiscuous pathway describes wider AR specificity, which includes non-androgenic molecules. **c.** The outlaw pathway includes receptor tyrosine kinases (RTKs) that mediate phosphorylation of AR by AKT or MAPK pathways leading to release of AR. **d.** The bypass pathway includes parallel survival pathways including the anti-apoptosis pathway and protein BCL2, which avoids the need for AR or a ligand. **e.** The lurker cell pathway involves androgen independent cancer cells that might be selected for through different treatments. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Cancer (Feldman & Feldman 2001), copyright (2001)

Numerous androgen-dependent genes become overexpressed during the progression to CRPC (Mousses et al. 2001; Amler et al. 2000). In fact, most CRPCs express AR (Hobisch et al. 1995; Visakorpi et al. 1995; Ruizeveld de Winter et al. 1994) although the expression levels of AR do not always correlate with cancer cell response to ADT. Also, the AR expression levels do not appear

to correlate with any particular tumour phenotype when evaluating the histological grade or clinical stage. Furthermore, the levels of AR are actually increased in CRPC in contrast to untreated tumours (Koivisto et al. 1997; Linja et al. 2001; Latil et al. 2001). It is rather uncommon to have the expression of AR abolished in CRPC but this can occur in some cases through hypermethylation of the AR promoter (Kinoshita et al. 2000). The *TMPRSS2-ERG* fusion gene has an androgen responsive element and therefore is under the control of AR resulting in the overexpression of the ERG transcription factor which thereby promotes PCa progression (Tomlins et al. 2005).

1.4.6 Strategies to target Androgen Receptor axis

There are several strategies that target the function of AR at different levels (Figure 1.4.6-1). The core strategy targets the signalling from the hypothalamus to the pituitary gland in order to supress the levels of circulating T. This includes the use of LHRH analogous such as leuprorelin (Prostap®) or goserelin (Zoladex®) for treatment of locally advanced tumours. The main advantage of these drugs is that orchiectomy can be avoided. Common side effects include low libido, hot flushes and sweats, tiredness, bone pain, and skin problems.

The function of AR can also be targeted directly. The traditional use of antiandrogens such as flutamide (Drogenil®) and its derivative bicalutamide (Casodex®) has proved less effective because of continuous stimulation of the AR by co-activators, or in some cases because of the agonistic effects of these drugs (Chen et al. 2009). As a result, occasionally treatment withdrawal in advanced PCa can benefit the patient (Small et al. 1997). In 2012 the US Food

and Drug Administration (FDA) approved the use of enzalutamide (Xtandi®, previously known as MDV3100), which is an oral second-generation anti-androgen drug used to treat metastatic CRPC. Enzalutamide is a potent drug which prevents the nuclear localisation of the AR, and hence prevents its binding to DNA. Moreover, it can inhibit the recruitment of transcriptional coactivators (U.S. Food and Drug Administration 2012; Tran et al. 2009). The randomised Phase III “AFFIRM” trial revealed significantly prolonged overall survival with enzalutamide compared to placebo in men who have failed prior docetaxel treatment (Nadal et al. 2014; Sternberg et al. 2014). In terms of safety and tolerability, enzalutamide was reported to cause seizure events in about 1% of the patients, but in general, it appears to have a promising side effect profile. In another Phase III trial, namely “Prevail”, the use of enzalutamide reduced the risk of death by 29% compared to the placebo group. The most common side effects included hypertension, fatigue and hot flush. The occurrence of seizure was reported in one patient in each treatment group (Tomasz et al. 2014). Abiraterone acetate is an emerging agent for treatment of advanced PCa. Abiraterone indirectly inhibits the AR axis by blocking a critical enzyme, CYP17, which is involved in T synthesis. This causes impaired synthesis of intracrine T, early adrenal androgen and peripheral circulation of T (Attard, Reid, et al. 2008; de Bono et al. 2011; Reid et al. 2010; Attard et al. 2009; Yap et al. 2008). The activity and safety of abiraterone was confirmed in initial phase II/III clinical trials with overall range of response between 25% and 60% (Attard, Reid, et al. 2008). Overall abiraterone causes a decline in circulating levels of androgens but may not be effective in carcinomas with different splice variants. Furthermore abiraterone may indirectly cause an increase in the levels of corticosteroids,

which is referred to as the toxicity of abiraterone (Sartor et al. 2011). The consequences of increased corticosteroids include hypertension, hypokalaemia, oedema and fluid retention.

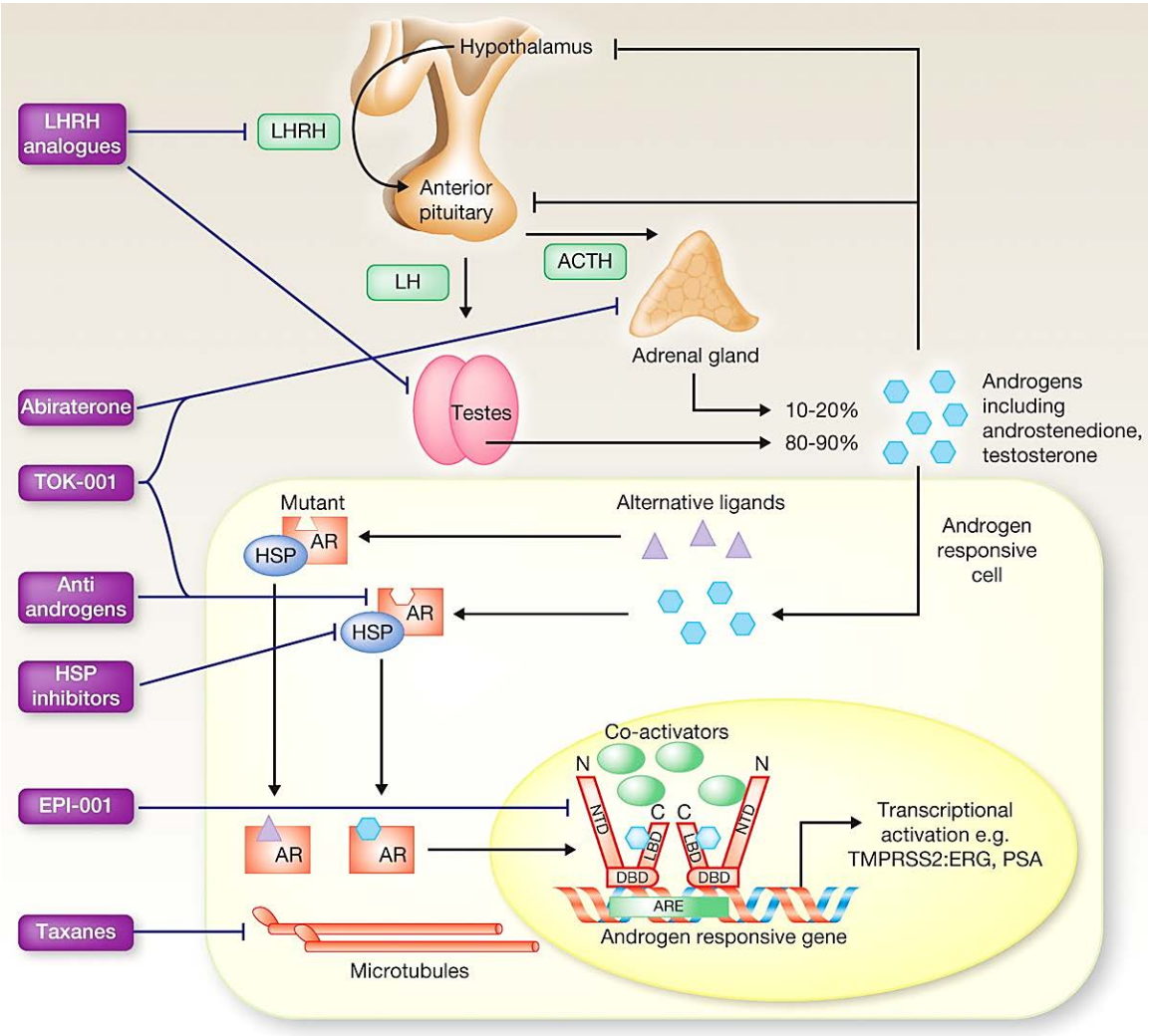


Figure 1.4.6-1 Different therapeutic strategies for targeting AR signalling. A negative feedback loop from the hypothalamus-pituitary-gonadal axis regulates the production of androgens. The luteinising hormone releasing hormone (LHRH) promotes the release of luteinising hormone (LH) from the pituitary. The LH then activates androgen synthesis in the testes. ACTH is produced by the anterior pituitary and promotes adrenal synthesis. AR binds to androgens or alternative ligands and translocates to the nucleus, where it binds the androgen responsive elements of *TMPRSS2-ERG*, *PSA* and other genes. Numerous drugs target the LBD or androgens, but more novel strategies involve NTD or chaperone proteins targeting. The use of taxanes disrupts the microtubules. NTD, N-terminal domain; DBD, DNA binding domain; LBD, ligand binding domain. Adapted from New Strategies in Metastatic Prostate Cancer: Targeting the Androgen Receptor Signaling Pathway, Attard et al. 2011, with permission from AACR

1.5 Chemotherapy for Prostate Cancer

Chemotherapy is an important treatment option for men with metastatic CRPC and several new therapeutic agents have recently been approved by the US Food and Drug Administration (FDA). At diagnosis the vast majority of PCas are sensitive to initial androgen deprivation therapy (ADT) but the effect is temporary and after an average of one to three years the cancer cells become resistant to ADT and continue to proliferate. Eventually the disease will progress to CRPC and some men with end-stage disease may benefit from chemotherapy (Pendleton et al. 2007).

1.5.1 Taxanes

The taxanes are a group of drugs which block cell division by hindering cell cycle progression through centrosomal impairment, induction of abnormal spindles and suppression of spindle microtubule dynamics. Taxanes are extracted from plants of the genus Taxus (yews). In the 1970s the first taxane, paclitaxel, was approved by the FDA. Later another more potent semisynthetic drug named docetaxel was developed and used in the treatment of refractory ovarian and breast cancers. The taxanes possess a unique ability to bind various sites of the beta-tubulin subunit of microtubules and thereby facilitate microtubule bundling. As a result, these microtubule bundles interfere with the natural dynamics of other microtubules in the cell. This leads to mitotic block and apoptosis of the affected cells. However, cancer cells can develop resistance to these agents as a result of the function of a gene called *multidrug resistance (MDR) 1*. The *MDR 1* gene

overexpresses the P-glycoprotein in cancer cells. The biological role of this protein is an ATP-dependent efflux pump that controls drug accumulation levels in multidrug-resistant cells (Chen et al. 1986). Newer drugs have lower affinity for P-glycoprotein and therefore are more efficient for docetaxel-resistant tumours (Kartner et al. 1983).

In 2004 two very important randomised phase III trials, SWOG 9916 and TAX-327, reported an improvement in overall survival of patients treated with docetaxel-based therapy (Tannock et al. 2004; Petrylak et al. 2004). As a result of these trials for a number of years docetaxel was the standard drug used for patients with CRPC. However, there was no effective treatment for patients whose tumours became refractory to docetaxel. This changed in 2010 when another mitotic spindle inhibitor, cabazitaxel (Jevtana®), was approved by the FDA. Further novel drugs have been approved recently for advanced metastatic PCa including above mentioned abiraterone and enzalutamide.

1.6 Aims and objectives of the study

The aim of this study is to evaluate *in vitro* the potential role of *TMPRSS2-ERG* in the response of PCa cells to ADT, IR and chemotherapy, and assess potential advantages for cells harbouring *TMPRSS2-ERG*.

- Initial investigations focused on the general characterisation of cell models overexpressing ERG. These cells were stimulated with androgens and the changes in their ERG levels and DNA damage response proteins were analysed.
- Secondly, the physiological effects in cells subjected to siRNA-mediated *ERG* knockdown were studied. In particular cell growth and viability were assessed after radiation treatment and ADT. In addition response to taxanes was tested.
- Finally *TMPRSS2-ERG* harbouring cell models were subjected to radiation treatment in order to evaluate molecular changes in their DNA damage response protein levels. In particular the surrogate markers of IR-induced damage (53BP1 and γ-H2AX) were investigated in order to trace the molecular changes caused by IR.

In summary this work aimed to investigate the potential role of the *TMPRSS2-ERG* fusion gene in the events underlying the response of PCa to IR. A key objective was to interpret our observations and ERG expression in relation to the clinical setting, and with regards to the dilemma whereby some patients respond better than others to radiotherapy.

Chapter 2. Materials and methods

2.1 In Vitro Cultures

2.1.1 Validation and sources of cell models used in this work

All cell lines used in this work are summarised in Table 2.1-1. They were obtained either from our in-house cell library in which case they were subjected to a microsatellite genotyping method of validation conducted by HPA Culture Collections, or they were purchased from ATCC.

Table 2.1-1 List of cell lines used in this work

Cell line	Site of origin	Species	Immortalisation	Reference
VCaP	Spinal cord metastasis	Human	xenograft	(Rubin et al. 2000; Korenchuk et al. 2001)
DuCaP	Dura metastasis	Human	xenograft	(Lee et al.; Rubin et al. 2000)
LnCaP	Lymph node metastasis	Human	xenograft	(Gibas et al. 1984; Horoszewicz et al. 1983)
DU-145	Brain metastasis	Human	-	(Mickey et al. 1977; Stone et al. 1978)

M2182	Prostate epithelial	Human	SV40	(Jackson-Cook et al. 1996; Bae et al. 1998)
PC3	Bone metastasis	Human	-	(Chen 1993; Kaighn et al. 1979)
BPH1	Benign hyperplastic prostatic epithelia	Human	SV40	(Hayward et al. 2001; Hayward et al. 1995)
P4E6	Early stage PCa	Human	HPV	(Maitland et al. 2001)
22RV1	Primary, (CWR22R-2152)	Human	xenograft	(Sramkoski et al. 1999)
PNT1A	Normal prostate epithelium	Human	SV40	(Berthon et al. 1997; Cussenot et al. 1991)
PNT2C2	A subline of PNT2 cells	Human	SV40	(Berthon et al. 1995)
RWPE1	Normal prostate epithelium	Human	HPV	(Bello et al. 1997; Webber et al. 1996)
ST-2	Bone marrow	Mouse	-	(Tong et al. 1999; Ogawa et al. 1988)
Jurkat	T lymphocyte cells cell leukemia	Human	-	(Schneider et al. 1977; Gillis & Watson 1980)

DuCaP and RWPE1 (WPE1-NB14, ATCC® CRL-2850) cell lines were authenticated by Health Protection Agency Culture Collections, UK (Table 2.1-2

and Table 2.1-3**)**. Short tandem repeat (STR) DNA typing methodology was used. This methodology is based on microsatellites in the human genome which contain STR markers allowing for validation of individual cell lines at DNA level. A set of STR loci including D5S818, D13S317, D7S820, D16S539, vWA, TH01, TPOX, CSF1PO and amelogenin (AMEL) for gender determination are used in the process. PCR primers specific for these STR loci produce fragments of a different size which can then be easily visualised using gel electrophoresis technique. Alleles that may fall in the same size range require the use of fluorescent labelling of PCS primers. The obtained STR cell line profiles were interrogated using an online authentication search engine available on http://www.dsmz.de/STRanalysis. Certain mismatches were observed and the expression of certain cell-specific proteins was validated using Western Blot.

Table 2.1-2 STR genotyping of DuCaP cells

	Locus names								
Locus	**D5S818**	**D13S317**	**D7S820**	**D16S539**	**VWA**	**TH01**	**AM**	**TPOX**	**CSF1PO**
Query	*12* *12*	*11* *12*	*9* *12*	*9* *9*	*18* *19*	*9.3* *9.3*	*XY*	*11* *11*	*10* *12*
DuCaP	12 12	11 12	9 12	9 9	18 19	9.3 9.3	XY	8 11	10 12

Table 2.1-3 STR genotyping of RWPE1 cells

	Locus names								
Locus	**D5S818**	**D13S317**	**D7S820**	**D16S539**	**VWA**	**TH01**	**AM**	**TPOX**	**CSF1PO**
Query	*12* *15*	*8* *14*	*10* *11*	*9* *11*	*14,1* *8*	*8,* *9.3*	*X* *X*	*8* *11*	*13* *13*
RWPE1	12 15	14 14	10 11	9 11	18 18	8 9.3	XX	8 11	13 13

2.1.2 Growth conditions

Cells were maintained in optimal growth conditions according to the supplier's protocol. The VCaP cell line was obtained from ATCC (#CRL-2876) and the DuCaP cell line was available at the Nuffield Department of Surgical Sciences (NDS). They were grown in DMEM medium containing 4.5 g/L glucose (Invitrogen) and supplemented with 10% foetal bovine serum (FBS) (Invitrogen). In accordance with the provider's protocol, no antibiotics were used for these cell lines. LnCaP and Du145 cells were purchased from ATCC; PNT1A, PNT2C2 and P4E6 were gifted by Professor Norman Maitland (University of York); Jurkat and PC3 from the Radiobiology Research Institute (RRI); ST-2 were obtained from ATCC. These cell lines were all maintained in RPMI-1640 medium supplemented with 10% foetal bovine serum and penicillin (100 units/mL)/streptomycin (100 mg/mL; P/S). RWPE-1 cells were obtained from NDS and maintained in Keratinocyte Serum-Free Media supplemented with EGF (2.5 µg), Bovine Pituitary Extract (25 mg) (GIBCO) and P/S. All cell lines were maintained in 5% CO_2 incubator at 37 °C. All cell lines were routinely screened for mycoplasma every month using MycoAlert® Assay kit (Lonza) according to the manufacturer's protocol.

2.1.3 Freezing and defrosting of cell cultures

VCaP and DuCaP cells were placed in long-term storage by suspending in 5% dimethyl sulfoxide (DMSO) (Sigma) pre-mixed with standard growth medium and 20% FCS. For all other cell lines 10% DMSO was used. Cryogenic vials were

placed at -80°C overnight and stored in liquid nitrogen thereafter. Defrosting of cells was done by gently thawing at 37°C and immediate removal of the DMSO rests by re-suspending in PBS and centrifugation and 240 x g for 5 minutes. Thereafter cell pellets were re-suspended in standard medium.

2.1.4 Cell counting

For growth curve analysis, dead cells and total cell number were counted using The NucleoCounter® NC-100 (ChemoMetec, Denmark) according to the manufacturer's protocol.

2.2 siRNA transfection

Control siRNA and siRNA targeting the *ERG* gene were ordered from Dharmacon (USA) and handled as required by manufacturer. The concentration of the stock solution was 20 µM and the concentration used for VCaP and DuCaP cells was 40 nM and 100 nM, respectively. The siRNA used were purchased from ThermoScientific including ON-TARGETplus Human ERG (2078) siRNA-SMARTpool and control ON-TARGETplus Non-targeting Pool. The sequence of the target sequence for ERG are GAUCCUACGCUAUGGAGUA, GUGAAUGGCUCAAGGAACU, GCGCUACGCCUACAAGUUC and GGACAGACUUCCAAGAUGA. Briefly, the required amount of siRNA was mixed with Hank's Balanced Salt Solution (HBSS, Life Technologies) whilst the transfection reagent, DharmaFECT1 (Dharmacon) was incubated in Opti MEM® (Life Technologies) for 5 minutes. The amount of transfection reagent diluted in

50 µL (96-well plates) was 0.13 µL and 0.26 µL for VCaP and DuCaP cells respectively. Next, the two solutions were combined for further 20 minutes. After that, the required amount of cells was resuspended in growth medium without antibiotics and mixed with the siRNA-transfection complexes. Cells were plated at 500,000 cells/well in a 6-well plate, at 100,000 cell/well in a 24-well plate, and at 16,000 cells/well in a 96-well plate. After 24 hours, the medium was replaced with fresh medium in order to avoid the toxic effects of the transfection reagent.

2.3 Western Blot

For Western Blot experiments VCaP and DuCaP cells were cultured in T-75 cm^3 or T-125 cm^3 flasks. At 90% confluence cells were passaged. Cells were washed with phosphate buffered saline (PBS) followed by incubation with 0.05 % Trypsin-EDTA (Sigma) at 37% for 5-10 minutes. Cells were split 1:3 and the passages used for all experiments were between 6 and 20. For siRNA treatment, 500,000 cells were reverse transfected and seeded in 6-well plates for treatment. The day after transfection medium was replaced with fresh medium or medium containing drugs. After further 24 hours the cells were irradiated. When collecting the cell lysates, cells were firstly washed with PBS and then scraped in lysis buffer made up of 50 mM HEPES pH 7.4, 0.1% NP40, 250 mM NaCl and protease inhibitor cocktail (Invitrogen).The samples were collected in 1.5 mL Eppendorf tube, then frozen on dry ice and placed at -80°C for long-term storage.

2.3.1 Protein extraction

Samples were defrosted on ice for 20 minutes. This was followed by a centrifugation step at 12,000 x g for 15 minutes at 4°C. The supernatant was then carefully moved to a new Eppendorf tube and either stored for further use at -80°C or subjected to protein concentration measurement.

2.3.2 Bradford assay

Protein concentration was determined using Coomassie Plus Bradford assay (Thermo Scientific). The protein solution was firstly diluted 1:10 and then 5 µL of the diluted material was mixed with 250 µL of Bradford assay in duplicate in a 96-well plate. Known dilutions of BSA (Sigma) were used to calculate a standard curve. Absorbance was measure at 595 ηm using Infinite® 200 PRO (Tecan) and Magellan software.

2.3.3 Membrane blotting

Lysates containing 10-20 µg of protein were mixed with NuPage® LDS sample buffer and NuPage® reducing agent. Samples were then heated to 70°C and separated on a NuPage® 4-12% BisTris precast gels using Novex tank (Invitrogen) at 150 V for 1 hour. NuPage® MOPS SDS running buffer was used for electrophoresis, and following this step proteins from the gels were transferred onto a nitrocellulose membrane (GE Amersham) in 20% methanol NuPage® transfer buffer for 2 hours at 20 V or overnight at 5 V. After protein transfer, the membrane was washed in tris-buffered saline containing 0.1% Tween (TBST).

This step was followed by the addition of blocking solution containing 5% milk (Sigma) diluted in TBST or 5% BSA (Sigma) diluted in TBST for 1 hour at room temperature. Primary antibodies (Table 2.3-1) were diluted in blocking solution overnight at 4˚C. This was followed by three washes of the membrane in TBST and 1 hour incubation with secondary antibody conjugated to Horseradish Peroxidase (HRP) (Thermo Scientific) diluted 1:5000. Next, three wash cycles of the membrane were performed and detection of the antibodies was conducted using chemiluminescnece reagent (Pierce ECL Western Blotting Substrate, Thermo Scientific).

Table 2.3-1 List of Western Blot antibodies used in this work

Protein	Size (kDa)	Host	Dilution	Company	Cat. №	Reference	Antigen
ERG	55	Rabbit	1:10000	Epitomics	5115-1	(Park, Tomlins, Brenner, et al. 2010)	aa 450 to the C-terminus
PSA (C-19)	34	Goat	1:1000	Santa Cruz	sc-7638	(Thomas et al. 2011)	C-terminus
AR (N-20)	110	Rabbit	1:10000	Santa Cruz	sc-816	(Vija et al. 2014)	N-terminus
alpha Tubulin	50	Mouse	1:10000	eBioscience	14-4502	(Breitling & Little 1986)	aa 426-430 C-terminus
Anti-γ-H2AX (JBW301)	17	Mouse	1:1000	Millipore	05-636	(Okamoto et al. 2013)	phosphorylated Ser139
HSP73	66-78	Rat	1:10000	Enzo	ADI-SPA-815	(Heimdal et al. 2014)	native HSP73
p21 (F-5)	21	Mouse	1:500	Santa Cruz	sc-6246	(Shi et al. 2013)	aa 1-159 native p21
p53(DO-1)	53	Mouse	1:10000	Santa Cruz	sc-126	(Sane et al. 2014)	aa 11-25 N-terminus

2.3.4 Quantification

Exposed films were digitised and ImageJ software was used to quantify the intensity of each band. Figures were assembled using Microsoft Office.

2.4 Ionising radiation

All irradiation experiments were conducted using IBL 637 caesium irradiator (CIS Bio International). Doses of 0 to 8 Gy were delivered to cell samples with a dose rate of 0.98 Gy/min. Control samples were mock irradiated under the same conditions.

2.5 anti-γ-H2AX and 53BP1 foci formation assay

VCaP and DuCaP cells were pre-treated with ERG-targeting siRNA or non-targeting control. Cells were seeded in 96 well plates at 10,000 cells per well and irradiated after 48 hours. After fixation in 4% paraformaldehyde (PFA) the cells were permeabilised and blocked with PBS solution containing 0.1% Triton (Sigma), 1 % bovine serum albumin (BSA, Thermofisher Scientific) and 1% goat serum (Invitrogen) for 1 hour at room temperature. Next cells were incubated with a primary antibody overnight at 4C° and after three washing steps, with secondary antibody for 1 hour at room temperature. Finally, cells were washed 3 times with PBS followed by addition of 0.5 µg/mL 4’,6’-diamidino-2-phenylindole (DAPI).

Table 2.5-1 List of antibodies for immunofluorescence and fluorophores

Antibody	Dilution	Company	Cat. №	Reference	Antigen
53BP1	1:1500	Cell Signalling	#4937	(Park et al. 2014)	central region
anti-γ-H2AX	1:1500	Millipore	05-636	(Okamoto et al. 2013)	phosphorylated Ser139
Alexa Fluor 546	1:1200	Invitrogen	A-21085		
Alexa Fluor 488	1:1200	Invitrogen	A-11017		

2.6 Microscopy

Foci detection was performed using IN Cell Analyser 1000 automated epifluorescence microscopy (GE Healthcare) or laser confocal microscopy (Zeiss). Images acquired using In Cell Analysed 1000 where quantified using Workstation software (v3.5). The laser confocal microscope was used to acquire ten images per well with at least 100 cells. The average number of foci per nuclei in this images was analysed using FociCounter software.

2.7 Drugs and compounds

DHT (Sigma) was dissolved in ethanol and added to cells 24 hours before irradiation at concentrations between 1 nM – 10 nM. Therapeutic agents used in the experiments include paclitaxel (New England Biolabs) and docetaxel, both of which were dissolved in DMSO. All drug stock solutions were stored at -20C°. For drug treatment experiments 500,000 cells were seeded in 6-well plates and cultured for 24 hours in growth media supplemented with 10% charcoal stripped serum (CSS) (Invitrogen) and P/S.

2.8 MTS Tetrazolium Viability Assay

Cell viability was assessed using CellTiter 96 AQueous Non-Radioactive assay (Promega) following the manufacturer's protocol. The kit is based on the principle that a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] is reduced by viable cells in order to generate a formazan product which is directly solubilised in the culture medium. The measured quantity of formazan during the phase of exponential growth is proportional to the number of viable cells *in vitro* (Goodwin et al. 1995; Cory et al. 1991). Depending on the cell growth a measurement was performed once every day or once every other day for each multi-well plate. The measurement was conducted using Tecan Infinite 200 multiplate reader. The absorbance of formazan product was measured at 490 nm and a reference absorbance was measured at 630 nm. The reference absorbance was used to remove background signal by subtracting this value from the 630 nm absorbance reading. Cells were seeded at a density of 10,000 cells per well in 96 well plates. Half of the medium was changed every third day. Four independent experiments were performed in quadruplicates. The optical density values were used to calculate relative cell number normalised to day zero.

2.9 Quantitative Real-Time Polymerase Chain Reaction

2.9.1 RNA isolation

RNA isolation was performed in DNA- and RNAse-free environment. Samples were carefully treated following the TRIzol® (Invitrogen) manufacturer's protocol. 1 mL of TRIzol® reagent was added to each well and the cell lysate was transferred to an Eppendorf tube and incubated for 10 minutes at room temperature. At this stage on certain occasions samples were moved to -80°C for long-term storage. Each sample was then thoroughly mixed with 200 µL chloroform and subsequently centrifuged at 10,000 x g for 15 minutes at 4 ˚C. After this step, the aqueous phase (top) was carefully transferred into a new DNA- and RNAse-free Eppendorf tube (Sigma). In the next step, RNA was precipitated by mixing with 500 µL Isopropanol and centrifugation at 10,000 x g at 4°C for 10 minutes. The supernatant was removed and the remaining pellet was washed with 1 mL of 75% ethanol. Next, the pellet was centrifuged at 7,500 x g at 4°C for 5 minutes and air dried. After removal of all ethanol residues, the RNA pellet was resuspended in 40 µL DNA and RNAse- free water. The RNA pellet was completely dissolved by incubating at 60°C for 10 minutes. Each sample was treated with 1 µL (2 units) of DNase (Ambion) at 37°C for 30 minutes followed by inactivation of DNAse at 75°C for 10 minutes. RNA concentration was measured using Nanodrop ND-1000 Spectrophotometer (Thermo Scientific). Samples were transferred into a -80°C freezer for long-term storage.

Reverse transcription was conducted using PCR ProtoScript M Mulv kit (New England Biolabs). 1 µg of RNA was used for cDNA synthesis. The required amount was mixed with 2 µL of oligo dT and incubated at 70°C for 5 minutes. Next, samples were kept on ice, and mixed with premade mix of 10 µL M-Mulv and 2 µL reverse transcriptase. The reverse transcription reaction was performed at 42°C for 1 hour followed by enzyme inactivation at 80°C for 5 minutes.

2.9.2 Quantitative Polymerase Chain Reaction

Predesigned and preoptimised TaqMan primers (Life Technologies) were used for the qPCR analysis. All the primers are proprietary and can be obtained from the company using their catalogue number and assay ID (Table 2.9-1). For each qPCR reaction mixture a 2 µL of TaqMan assay, 10 µL TaqMan Gene Expression MasterMix, 1ηg cDNA and water were mixed. The final volume of the reaction mixture was 20 µL. qPCR analysis was conducted using Stratagene 4700 Thermocycler (Agilent). The thermal protocol starts at 50°C for 2 minutes, followed by 10 minutes at 95°C and 40 polymerisation cycles of 15 seconds at 95°C and 1 minute at 60°C. Fold change was calculated by using the comparative threshold cycles method. All samples were normalised to an endogenous control and the ratio was used to calculate differences in expression levels between the sample and control reactions.

Table 2.9-1 List of Taq polymerase primer probes

Gene	Probe Exons	Dye	Cat #	Assay ID
ERG	6-7, 7-8, 8-9, 9-10, 10-11	FAM	4331182	Hs01554634_m1
TMPRSS2-ERG fusion	-	FAM	4331182	Hs03063375_ft
AR	4-5	FAM	4331182	Hs00171172_m1
KLK3 (PSA)	1-2	FAM	4331182	Hs02576345_m1
Beta Actin	1	VIC	4326315E	-

2.10 Microarray and microRNA analysis

2.10.1 Sample preparation and Quality Control

VCaP and DuCaP cells were treated with ERG-targeting siRNA and after 48 hours RNA was isolated as described above. At least 500 ηg RNA from two independent experiments was sent for Microarray and microRNA analysis conducted by Toray Industries Inc, Tokyo, Japan. The company conducted quality control analysis using Agilent 2100 Bioanalyzer, the samples were labelled using miRCURY LNA microRNA Hy5 Power labelling kit (Exiqon, Denmark).

2.10.2 Hybridisation

Labelled samples were loaded onto 3D-Gene Human miRNA Oligo chip - 2 arrays for microRNA analysis. Samples were loaded for microarray analysis onto 3D-Gene Human Oligo chip 25k version 2.1. Hybridised samples were then scanned and normalised using 3D-Gene Scanner 3000 (Toray) at 635 ηm.

2.10.3 Signal detection

Images were evaluated using 3-D-Gene Extraction (Toray, Japan). The intensity of the background signal was determined as the mean signal intensity of the blank spots where those with the 5% highest and lowest signal intensities were dismissed. The detected spots were determined as having signal intensity over 2 SD plus mean of background signal intensity. The signal intensities of detected spots were subtracted with the background signal and the mean of the background-subtracted signal intensity of duplicate spots was used for the further analysis. Highlighted data was removed from the analysis.

2.11 Statistical Analysis

GraphPad Prism 4.0 software (GraphPad, CA) was used to perform all statistical tests. Growth curve and cell viability analysis were evaluated using 2-way ANOVA followed by Bonferroni's multiple comparison test. The Bonferroni post-test was conducted by the software in order to compare replicate means by columns. For the remaining experiments unpaired two-tailed Student's t-test was used. Data is expressed as the mean value ± standard error of the mean (SEM) and was derived from triplicates within each of at least three independent experiments. P values ≤ 0.05 were deemed statistically significant. The following nomenclature was applied *$p < 0.05$, **$p < 0.01$.

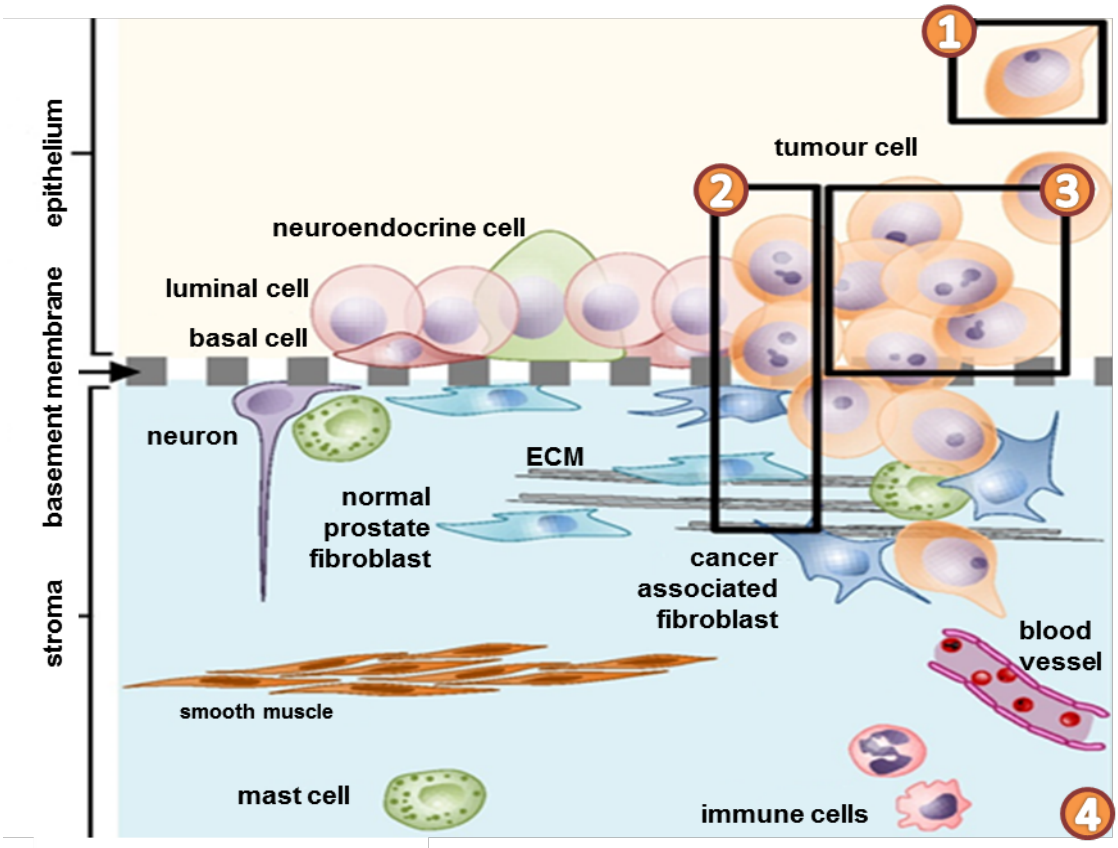
Chapter 3. Identification of appropriate cell models for TMPRSS2-ERG studies and their characterisation before radiotherapy

3.1 Introduction

Model systems are a fundamental element of PCa research and these include *in vitro* models, such as culture of established PCa cell lines in plastic dishes and *in vivo* models, such as xenografts of human PCa cell lines in immunodeficient animal models. The selection of a particular cell line for a study plays a decisive role in the interpretation of the results and comes with limitations related to the use of a particular model. PCa research has strongly been disadvantaged by the lack of reliable cell models capable of accurately recapitulating the biology underlying PCa initiation and progression. This has been a significant barrier to the improvement of our knowledge of the prostate disease, and the utilisation of different strategies for the testing of new therapeutic agents and treatments. Furthermore, PCa research in model organisms has mainly been centred on animal models that have significant limitations and only partially resemble the human malignancy. It has been demonstrated that only nonhuman primates and dogs can spontaneously develop PCa (Mubiru et al. 2008; Leroy & Northrup 2009). Mice and other rodent models, on the other hand, have widely been used to reveal the principal mechanisms of prostate carcinogenesis. Some of the most common animal models include the TRAMP and LADY viral oncogene mouse

models, which are pathologically similar and develop progressive forms of PCa (Ishii et al. 2005; Chiaverotti et al. 2008; Greenberg et al. 1995; Gingrich et al. 1997; Kasper et al. 1998). One important characteristic of these models is that the tumours exhibit neuroendocrine phenotype and neuroendocrine PCas account for only about 10% of cases (Masumori et al. 2001). In addition the rapid rate of disease progression in these models is in dissonance with the rate of PCa in men. The variability in androgen dependence of the tumours in TRAMP mice was also found to be significant with about 20% of the rodents losing genitourinary volume after castration (Gingrich et al. 1997). More recently transgenic mouse models which harbour the *TMPRSS2-ERG* fusion gene, and thereby overexpress ERG, were also developed (Klezovitch et al. 2008; Scott A. Tomlins et al. 2008; Carver et al. 2009). However, each of these models is ultimately limited by their nonhuman nature, which drastically narrows their application to human PCa disease initiation, progression and metastasis. Some *in vivo* PCa models overcome the nonhuman nature and other weak points but they are expensive and do not address experimental and cancer latency. These models include primary cells, tissue slice grafts and cancer tissue xenografts. However the characteristics of xenografted cell lines also deviate from human PCa due to the host milieu which is usually that of an immunodeficient murine model subjected to subcutaneous injection. Various *in vitro* PCa models seem to be the only models that address issues such as the nonhuman origin of the cells, financial research expenses and cancer latency. Yet these models have typically lost their ability to recapitulate the natural progression of PCa and metastases, which *in vivo* models retain. In addition, they grow in an artificial milieu. *In vitro*

models usually include immortalised cell lines growing in monocultures that do not simulate multicellular parameters and are not representative of the high prostatic heterogeneity and the complex *in vivo* microenvironment. Significant advances have been made thanks to the development of 3D model systems such as spheroids. These provide a better understanding of prostate pathology but require further optimisation (Figure 2.10.3-1).



	Advantages	Disadvantages
1 2D monocultures	Simple and available	Reductionist approach
	Easy to reproduce	Abnormal milieu
		Aberrant morphology
		No structural integrity
2 Biomatrix co-cultures (2.5D)	Multicellular	Limited structure & cell composition
	Epithelial-stromal interaction	Atypical surface and culture milieu
	Suitable for ECM tests	
3 Spheroid aggregates (3D)	3D cell interactions	Limited composition
	Simple and cheap	Variability in spheroid formation, size & shape
	High-throughput (hanging drop)	No control of cell aggregation & structure
		Typically homogenous
4 Bioengineered & *ex vivo* (3D)	Retains original tissue morphology & features	Technically complex
	Heterogenous 3D frame	Limited culture duration (ex vivo), dependent on patient tissue availability
	Control & definition of scaffold	Expensive

Figure 2.10.3-1 Various in vitro PCa model systems bear certain limitations in recapitulating the natural disease progression. Figure adapted from (Ellem et al. 2014).

Up until recently most PCa research involving cell lines has been focused on the three mainstays or “classic” cell lines, namely PC3, DU145 and LNCaP, each of which originated from a metastatic tumour. LNCaP cells bear mutated AR, whilst PC3 and Du145 can grow independently from AR signalling. These cell models therefore do not recapitulate the primary PCa disease where AR signalling is fundamental and the ADT is the front-line therapy. Indeed, AR-dependent genes, including *PSA* and *TMPRSS2*, are frequently overexpressed in CRPC (Cai & Balk 2011). In the case of *TMPRSS2-ERG* the signalling from the AR is transmitted through an androgen responsive element within the first exon of *TMPRRSS2* and thus the fusion gene results in overexpression of ERG (>100 fold) under the control of AR. Therefore a lot of work has now been devoted to the development of AR-positive and AR-responsive cell lines due to the nature of CRPC which usually retains AR expression. For example, the VCaP cell model was isolated from a vertebral PCa metastasis and it is a model that can recapitulate some of the main features of clinical CRPC. This cell line is both AR-sensitive and tumorigenic, whilst it also expresses mutated p53, Rb, AR and PSA, and it harbours the *TMPRSS-ERG* fusion gene (Korenchuk et al. 2001; Tomlins et al. 2005). Furthermore, the subtype of *TMPRSS2-ERG* translocation present in this cell line is the most frequent in clinical PCa, i.e. type III, which includes the non-coding *TMPRSS2* exon 1 fused to *ERG* exon 4 (Sun et al. 2008; Wang et al. 2008).

In this chapter various cell lines were characterised to ensure their adequacy as experimental models for studying the impact of *TMPRSS2-ERG* fusion gene on cell response to androgen deprivation, IR and chemotherapy. The cell models were tested for expression of different proteins including ERG and AR. Suitable *in vitro* models were then characterised by assessing their response to AR stimulation. Androgen stimulated effects were measured and by targeting the expression of *ERG* we sought to determine gene expression changes focusing in particular on genes associated with DNA damage response. Finally, the effect of classical PCa chemotherapeutic drugs was assessed on cells with different endogenous expression of *TMPRSS2-ERG*.

3.2 Results

3.2.1 VCaP and DuCaP cell lines are the only in vitro models harbouring the TMPRSS2-ERG fusion gene

Here VCaP cells, which overexpress ERG, were used as a positive control to screen for further cell models that may overexpress the ERG protein. A panel of 14 prostate cancer cell lines, prostate non-malignant cell lines and non-prostate cell lines were selected for a Western Blot analysis of proteins related to androgen signalling, ERG, tumour suppressor protein p53 and the DNA-damage sensor protein γ-H2AX (Figure 3.2.1-1).

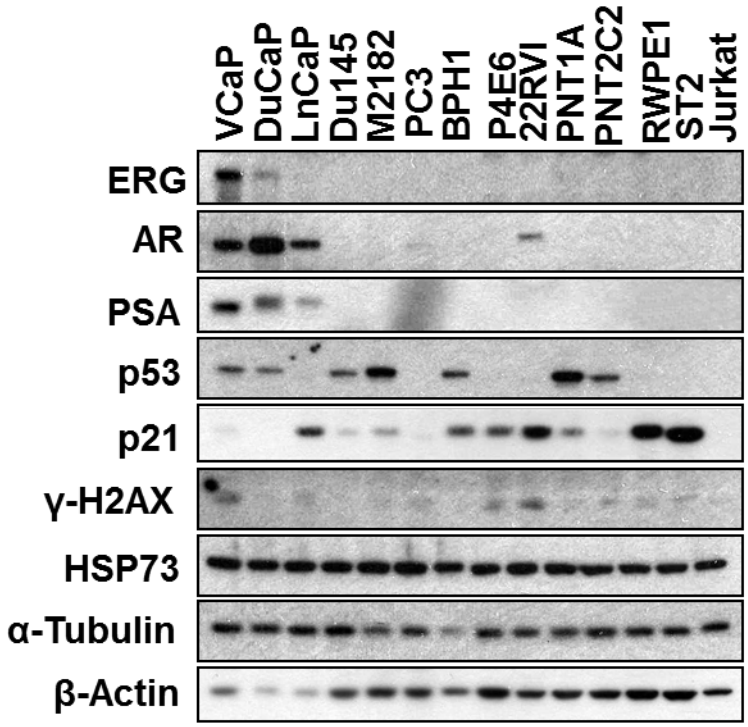


Figure 3.2.1-1 Expression of various proteins in PCa cell models used in this thesis. Various benign and cancer cell models were tested for the expression of proteins associated with AR signalling, response to IR, and the expression of tumour suppressor protein p53.

The Western Blot analysis showed that VCaP and DuCaP are the only cell lines expressing detectable levels of both ERG and AR proteins. AR was expressed more strongly in VCaP and DuCaP compared with LNCaP, 22RVI and PC3 cells. Interestingly, the expression of endogenous AR in DuCaP cells was significantly higher than in VCaP cells. Furthermore, the expression of PSA was clearly detectable in VCaP and DuCaP cells, which corresponds positively with their wild-type AR expression (van Bokhoven et al. 2003). Next, the expression of the tumour suppressor protein p53, which mediates the DNA damage-induced checkpoint, and the cyclin-dependent kinase inhibitor p21, which mediates p53-dependent G1 growth arrest, were investigated. A lack of functional p21 has been

linked to compromised radiation-induced cell cycle arrest (Brugarolas et al. 1995; Deng et al. 1995). p53 was found to be expressed in VCaP, DuCaP, Du145, M2182, BPH-1, PNT1A and PNT2C2 cells, whilst p21 was found in all but two of the cell lines (DuCaP and Jurkat) whereby the level of p21 in VCaP, PC3 and PNT2C2 was nearly undetectable. Coexpression of p53 and p21 was observed in VCaP, Du145, M2182, BPH-1, PNT1A and PNT2C2. It is worth noting that both VCaP and DuCaP have mutated *p53*, while in BPH-1 and PNT1A the function of p53 protein is compromised by the SV40T antigen used for immortalisation. The distribution of γ-H2AX suggests that the basal levels of expression were noticeably higher in VCaP and 22RVI cells. Taken together the data showed that out of 14 cell lines, VCaP and DuCaP cells were the ones that demonstrated overexpression of ERG. This observation correlated positively with expression of two key elements of the androgen signalling axis, AR and PSA. In accordance with previous research both VCaP and DuCaP cell lines were found to express p53. The expression of p21 was at marginally detectable levels in VCaP and undetectable in DuCaP cells. Interestingly, the two cell lines showed different steady-state levels of γ-H2AX expression with higher levels in VCaP.

Previous studies have described the difficulties in finding an endogenous protein that is consistently expressed across various PCa cell types. In the case of VCaP and DuCaP cells the preliminary results presented in this chapter indicate significant differences in the expression of some housekeeping genes. Therefore the expression of heat shock protein HSP73, α-Tubulin and β-Actin were compared in order to identify the most appropriate internal control. The

expression of HSP73 and α-Tubulin seemed to be the most consistent across all cell lines within the panel, and particularly in the case of VCaP and DuCaP cells.

3.2.2 Expression of TMPRSS2-ERG fusion gene correlates with wild-type ERG expression

Here the correlation between gene expression of wild-type *ERG* and *TMPRSS2-ERG* fusion gene was investigated by qPCR. The two genes were found to be co-expressed in VCaP and DuCaP cell lines (Figure 3.2.2 1 **A.** and **B.**) with their levels being highest in VCaP cells. The *TMPRSS2-ERG* fusion gene was not present in any of the remaining cell lines, whilst the wild-type *ERG* gene showed less than 0.5% expression in Jurkat cells relative to VCaP cells.

The gene expression of *AR* was disproportionally high in DuCaP cells compared to VCaP cells (Figure 3.2.2 1 **C.**) and this observation corresponds with the protein expression of AR seen in the immunoblots. Other cell lines such as LnCaP, PC3 and 22RVI were found to express low levels of *AR*.

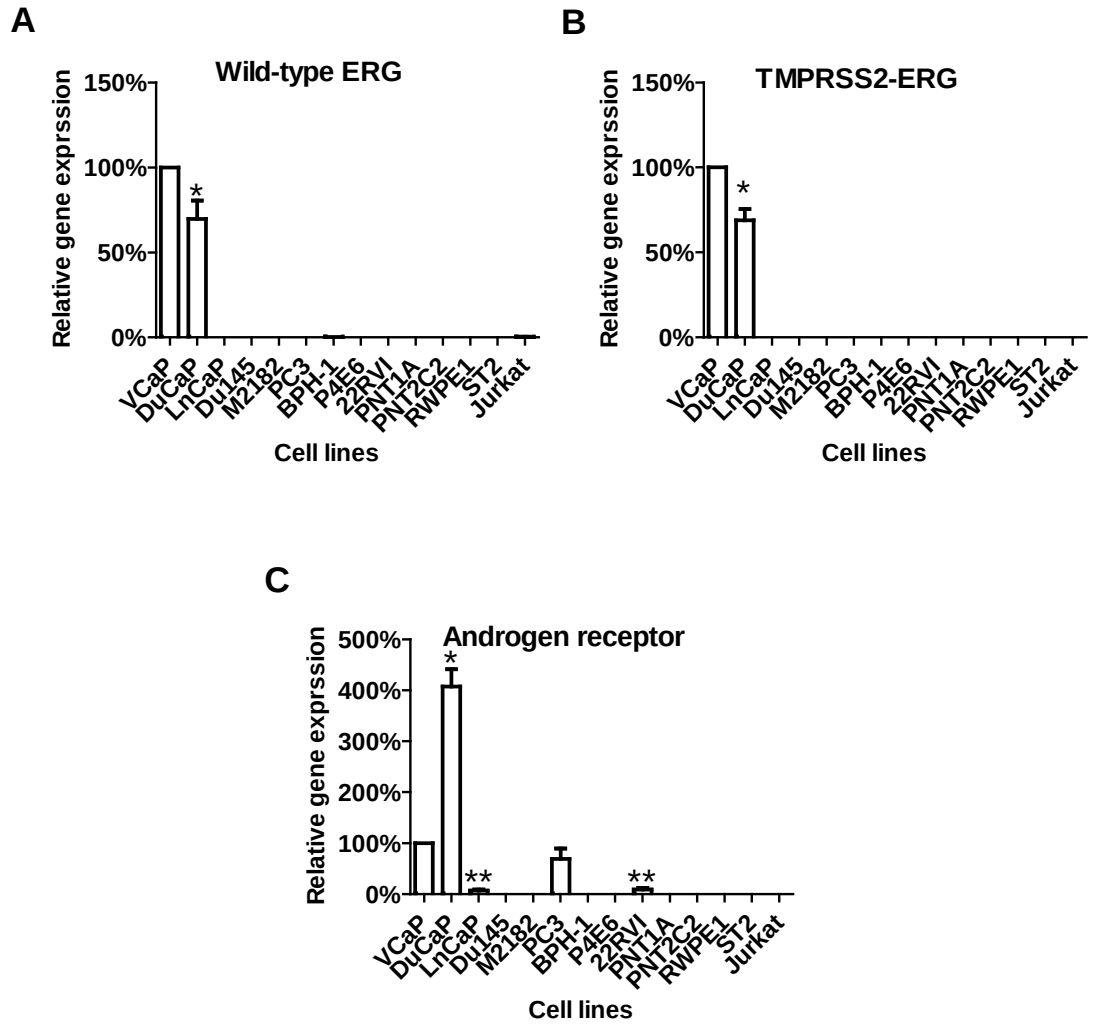


Figure 3.2.2-1 Gene expression analysis of *ERG*, *TMPRSS2-ERG* and *AR* in PCa cell lines, non-malignant prostate cells and non-prostate cell lines. Quantitative real-time PCR was used to evaluate the expression of different genes across a panel of cells and normalised to VCaP cells. Figure **A.** shows the expression of wild-type *ERG* **B.** shows the expression of the fusion gene *TMPRSS2-ERG* and **C.** shows expression of *AR*.

3.2.3 Dihydrotestosterone stimulation of VCaP and DuCaP cells

The expression of a functional AR is central to PCa cell growth and the levels of AR are often upregulated in CRPC. Previously it was believed that CRPC cells are strictly androgen independent but newer data indicated that CRPC normally remains hormone driven (Chen et al. 2004; Attard, Reid, et al. 2008; Waltering et

al. 2009). Here the aim was to identify the androgen response of VCaP and DuCaP cells by treating them with DHT, which has a slower dissociation rate and higher affinity for the AR than T (Mohler et al. 2011). Different concentrations of DHT were used for a qPCR analysis to investigate the expression of several genes implicated in the AR signalling axis and ERG transcription factor. In addition the levels of DNA damage proteins were also evaluated (Figure 3.2.3-1). The results showed that levels of ERG were marginally detectable in androgen free medium for both VCaP and DuCaP cells. In contrast, higher presence of AR was observed in androgen-free medium compared with DHT-supplemented medium. After DHT-stimulation the levels of AR decreased in both VCaP and DuCaP cells, where the relative decrease was around 50% for VCaP cells and up to 80% for DuCaP cells (Figure 3.2.3-1). Interestingly an inverse correlation was observed between the levels of AR and PSA in DHT-free medium. This was particularly evident in VCaP cells where the presence of higher levels of AR were associated with significantly lower levels of PSA. This changed after DHT addition when AR levels decreased and PSA levels increased. In DuCaP cells this phenomenon was not as distinct although a reduction in AR was also associated with a rise in PSA.

p53 was another gene that was affected by androgen signalling. A difference was observed in the response of VCaP and DuCaP cells to DHT stimulation although the overall pattern remained consistent. Absence of DHT caused a strong increase in p53 production, whereas the addition of DHT corresponded with a decrease in p53 which mirrored the production of AR.

When the effect of castration on γ-H2AX expression was evaluated a substantial rise in total γ-H2AX was observed in DHT-free medium. Addition of low DHT doses caused considerable reductions of this protein in both VCaP and DuCaP cells. Interestingly, 10 nM DHT seem to cause a subtle but detectable increase in γ-H2AX levels in both cell lines (Figure 3.2.3-1). Overall these results indicated that the expression of ERG in both VCaP and DuCaP models was greatly dependent on the presence of androgens in the culture medium, whereby significant downregulation was observed in androgen deprived milieu. The androgen deprived medium also resulted in increased AR expression, while PSA expression was reduced. Both VCaP and DuCaP cells demonstrated remarkable increase in p53 and γ-H2AX levels in androgen deprived medium. Nevertheless the addition of DHT to the medium caused a decrease in the expression of these proteins in both cell lines, although 10 nM DHT seemed to slightly increase their levels compared to 1 nM and 100 nM concentrations.

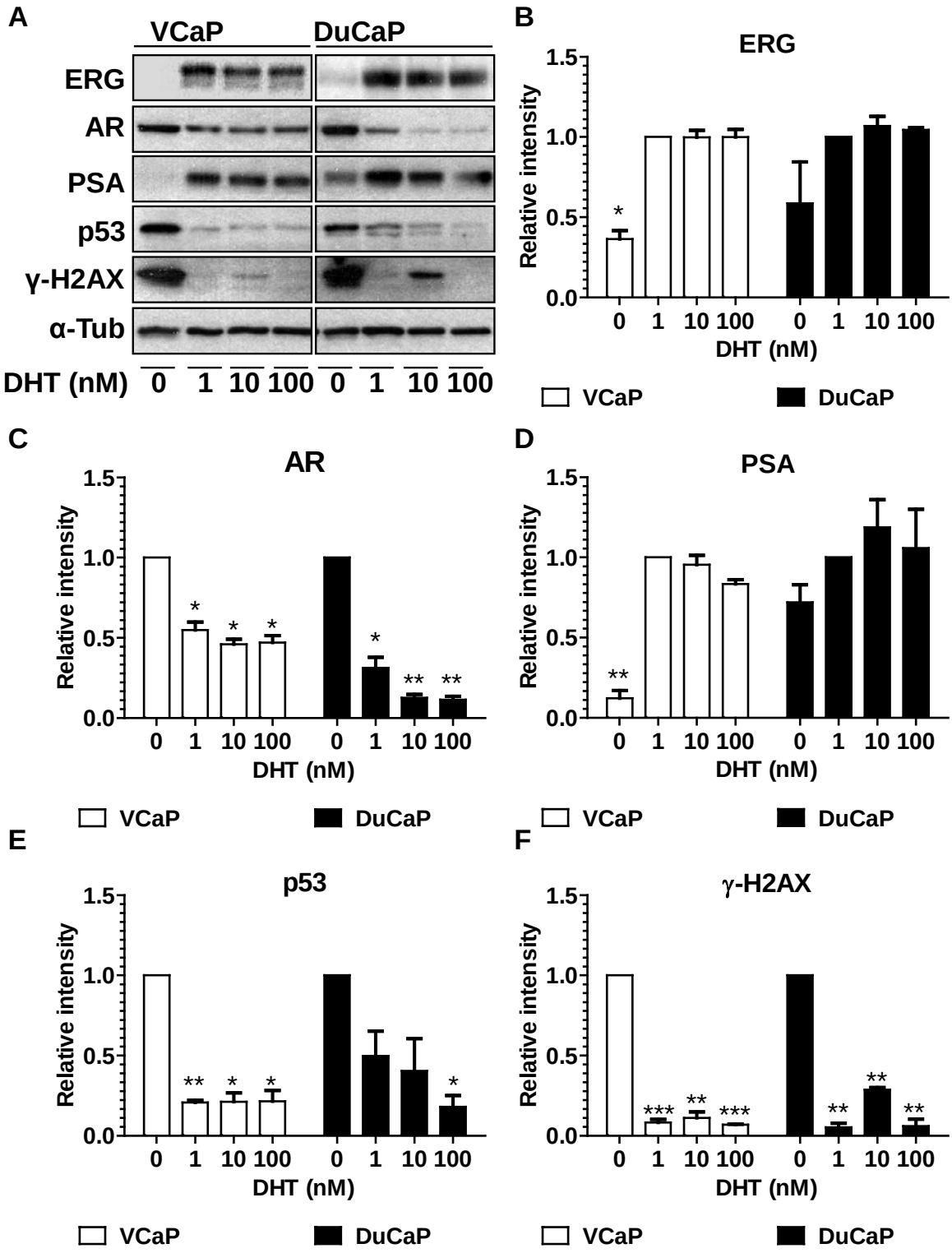


Figure 3.2.3-1 Effect of androgens on the expression of ERG, AR, PSA and DNA damage response proteins. A. VCaP and DuCaP cells were androgen-starved for 48 hours and then treated with different doses of DHT. Cell lysates were collected and the protein expression was determined using Western Blot analysis. **B-F.** The histograms display the relative intensity of protein expression quantified using ImageJ. The proteins analysed are shown on **B.** ERG, **C.** AR, **D.** PSA, **E.** p53 and **F.** γ-H2AX. Stars indicate significant difference between one normalised sample and the remaining treatments.

3.2.4 Difference in response to taxanes

Docetaxel (Taxotere®) and its historical predecessor paclitaxel (Taxol®) are both recommended for treatment of men with CRPC. Docetaxel is the only standard first-line chemotherapy for metastatic CRPC following ADT. In order to investigate a potential correlation between ERG levels and chemotherapy response, the cell viability of VCaP and DuCaP cells was analysed after administrating docetaxel or paclitaxel. Different doses of the respective drug were used and data was collected up to 72 hours in order to determine the most appropriate drug concentration for short-term effects. Overall, treatment of VCaP cells with docetaxel caused a marginal decrease in cell viability after 72 hours across the whole concentration range (Figure 3.2.4-1). The use of docetaxel between 1 nM and 40 nM induced a decrease in cell viability, but the use of higher concentrations did further reduced cell proliferation. In addition, longer exposure did not significantly affect the viability of VCaP cells after 72 hours. DuCaP cells on the other hand demonstrated unambiguous response to chemotherapy with docetaxel (Figure 3.2.4-1). After 24 hours, proliferation was already inhibited using docetaxel at a concentration of 10 nM. At concentration higher than 80 nM up to 3200 nM there was approximately two-fold decrease in cell viability after 24 hours and even higher reduction in cell viability was observed after 72 hours. In conclusion, the two cell lines, VCaP and DuCaP, responded differently to docetaxel treatment whereby DuCaP demonstrated remarkable decrease at concentrations as low as 10 nM, whilst VCaP appeared to remain almost unaffected by the treatment.

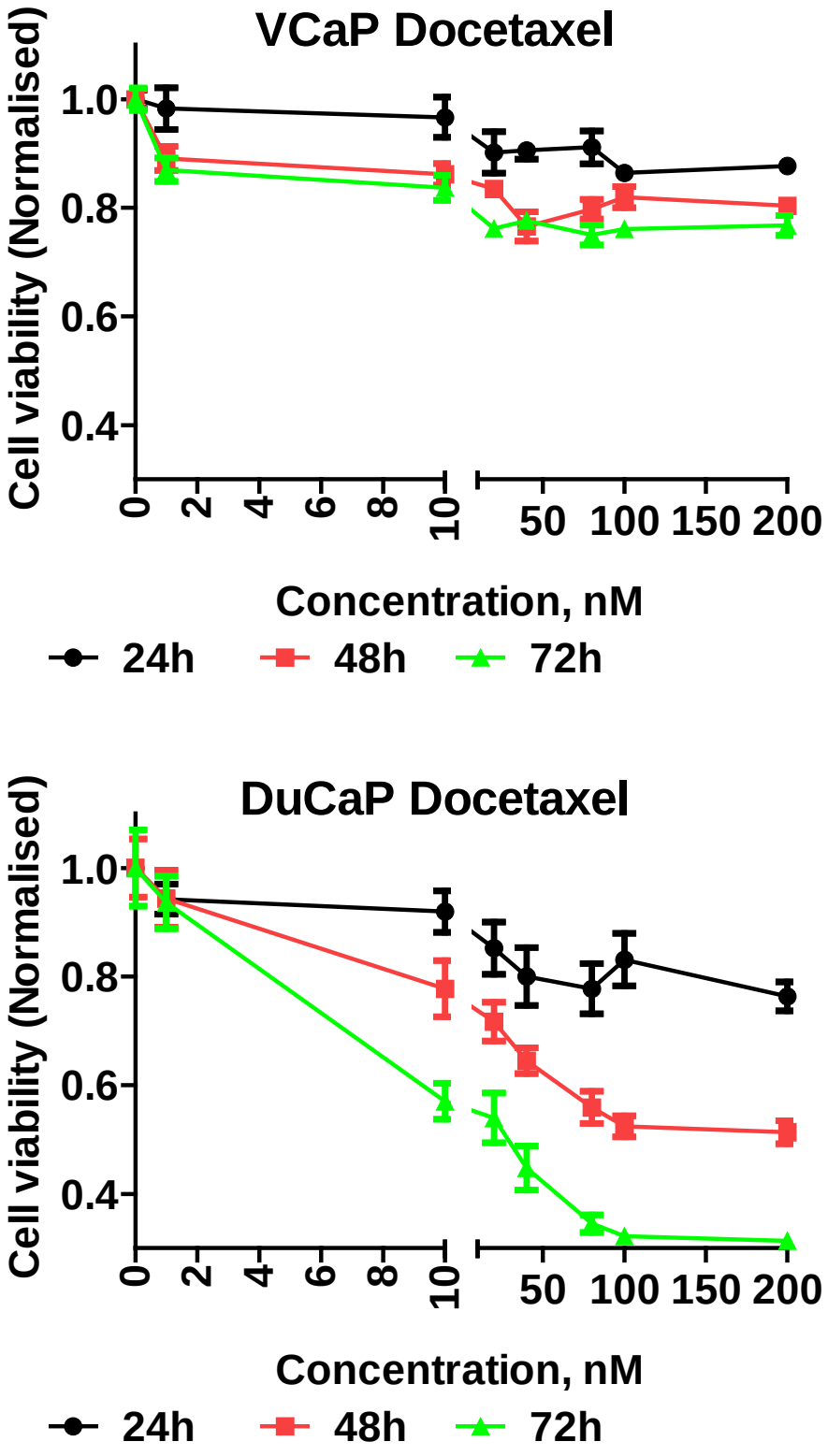


Figure 3.2.4-1 Difference in response to treatment with Docetaxel. VCaP (top figure) and DuCaP (bottom figure) cell were seeded in 96-well plates at 16,000 cells per well. The cells were treated with Docetaxel at the doses indicated. MTS readings of absorbance were performed 24, 48 and 72 hours after exposure to the drug.

In a similar way VCaP and DuCaP cell lines were treated with paclitaxel and the effect of the drug on cell viability was measured after 24, 48 and 72 hours using MTS assay (Figure 3.2.4-2). After 24 hours VCaP demonstrated reduced viability even at lowest concentrations. After 72 hours the reduction was even more prominent with 75% viability across the lowest 1 nM concentration. However it seemed that the reduction in cell viability levelled out at around 60% and was not influenced by further increase in the concentration. This might be linked to the slower doubling time in this cell line as mentioned in the discussion of this chapter. DuCaP cells on the other hand did not demonstrate substantial decrease in viability at 1 nM concentration even after 72 hours. Nevertheless a sudden decrease in viability to around 50% was observed at 5 nM concentration after 72 hours. Further increase in the concentration did further affected cell viability and at 10 nM concentration, cell viability was only 30%. Overall in comparison to docetaxel, paclitaxel seemed to have caused stronger reduction in cell viability of VCaP cells but the maximum effect caused cell viability to drop down to 60%. DuCaP cells on the other hand did not respond as rapidly to the lowest concentrations of paclitaxel but at 5 nM concentration and above viability was reduced to 30%. At certain doses of drug, no further decrease in cell viability was observed. This was probably due to precipitation of the corresponding drug and it was in the range 400 - 3200 nM for docetaxel and 20 - 160 nM for paclitaxel (data not shown).

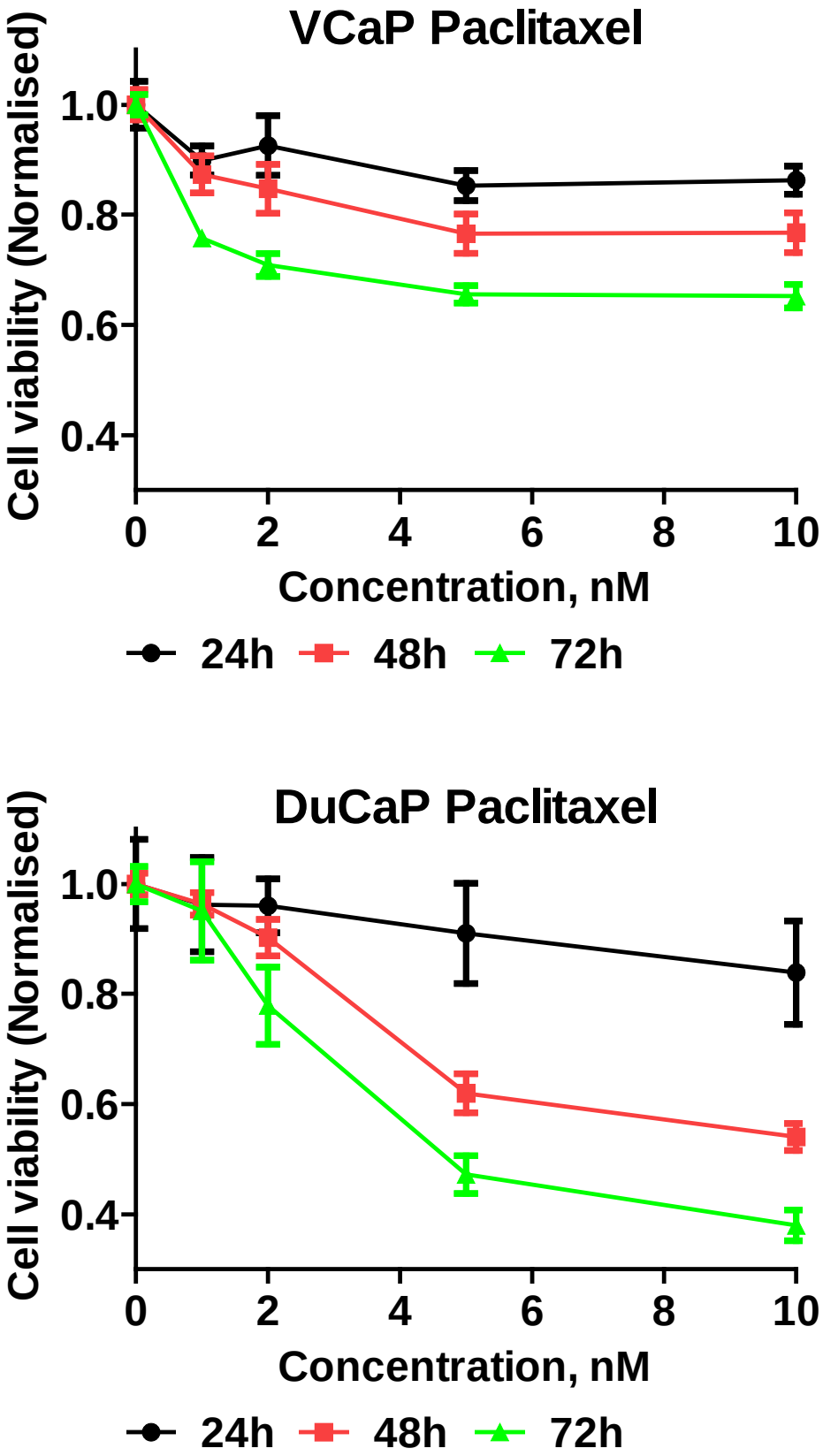


Figure 3.2.4-2 Difference in response to treatment with Paclitaxel. MTS assay was used to measure viability of VCaP (top) and DuCaP (bottom) cells after exposure to paclitaxel at the time points indicated.

3.2.5 Efficiency of ERG-knockdown and the effect of ERG silencing on gene expression in VCaP and DuCaP cells

Since the steady-state levels of ERG protein differ in VCaP and DuCaP cell lines, here it was sought to establish a reliable knockdown protocol for transfecting both cell lines with siRNA targeting the gene expression of ERG efficiently.

Figure 3.2.5-1 shows the use of ERG-targeting siRNA in VCaP cells and the corresponding levels of ERG oncoprotein. Cells were transfected with ERG siRNA, targeting all types of ERG isoforms, or non-targeting control siRNA oligos. The knockdown effect on *TMPRSS2-ERG* expression was assessed at the indicated time points. The efficiency of *TMPRSS2-ERG* knockdown was consistent throughout the assessed time points. At the gene expression level, the highest knockdown effect occurred after 24 hours. At the protein level, VCaP cells transfected with non-targeting control siRNA molecules showed a robust expression of ERG oncoprotein whereby ERG expression was successfully depleted in the ERG siRNA-transfected VCaP cells from 24 hours onwards, with the highest depletion after 48 hours. This data indicated that the strongest knockdown effect in VCaP cells occurs from 48 hours onwards.

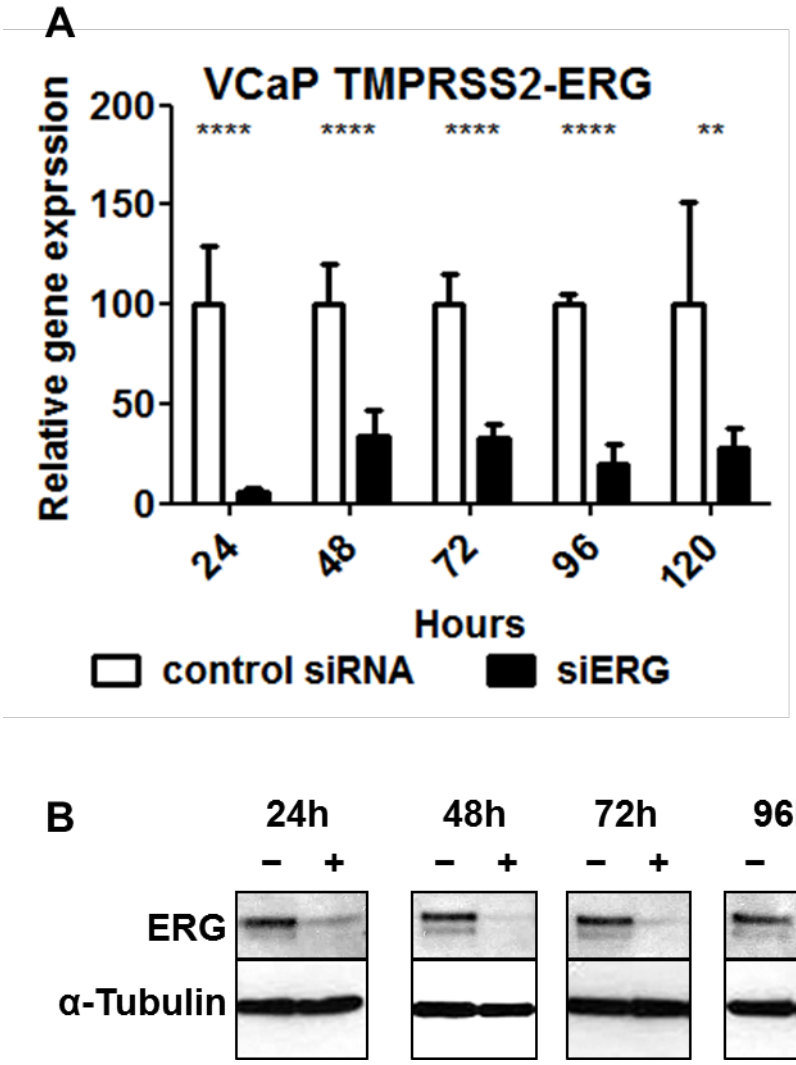


Figure 3.2.5-1 Efficiency of ERG knockdown in VCaP cells. A. qRT PCR analysis indicates gene levels of *TMPRSS2-ERG* after siRNA treatment. **B.** Corresponding protein samples were used for Western Blot analysis in order to confirm the efficiency of the ERG-targeting siRNA oligos used in this work.

After determining the ERG knockdown efficiency, a microarray analysis of VCaP cells was conducted by Toray Industries (Inc. Japan) to determine gene expression changes between ERG-positive and ERG-knockdown VCaP or DuCaP cells. For data analysis certain criteria were applied, which selected for genes that showed at least a two-fold change in expression and that were either significantly overexpressed or downregulated ($P < 0.05$) in VCaP and DuCaP

cells after ERG knockdown. The results are displayed in Figure 3.2.5-2 and a short summary of the genes' function and clinical relevance is listed in Table 3.2-1 and Table 3.2-2. Knockdown of ERG in VCaP cells was associated with a rise in the expression of genes that are involved in cell proliferation, cell cycle progression and regulation of the immune system. These genes include *GIMAP6*, *CDH17*, *CDH12*, *WDR62*, *ABHD2*, *HLA-E*, *FAM34A* and *GPR44*. Therefore VCaP cells that overexpress ERG have low levels of these genes.

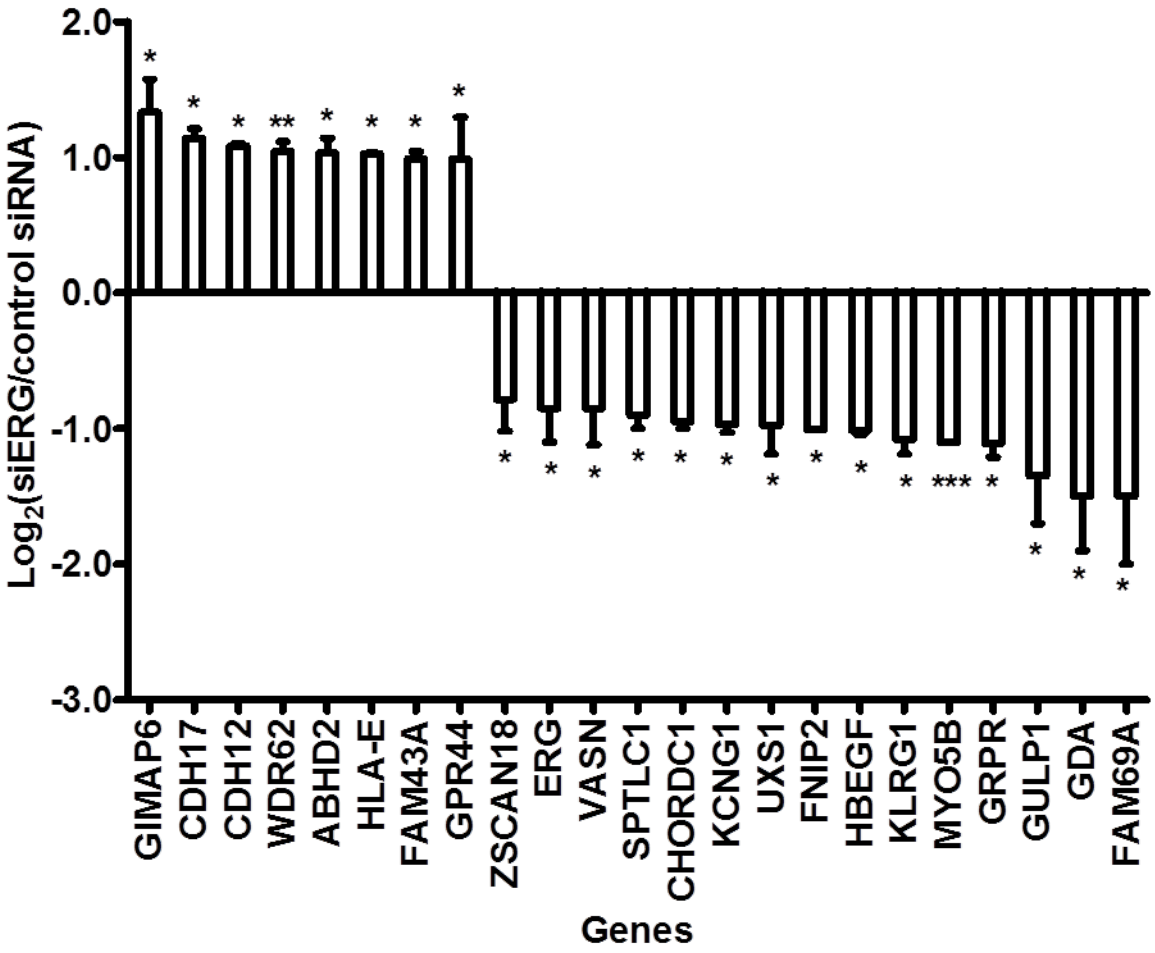


Figure 3.2.5-2 Gene expression analysis of VCaP cells. VCaP cells were treated with ERG-targeting siRNA and the change in gene expression was assessed using microarray analysis. The chart shows genes with more than one fold change in their expression and significant change to control. *P<0.05; **P<0.01; ***P<0.001, unpaired student t-test.

Table 3.2-1 List of genes that have been found to be significantly upregulated in VCaP cells following ERG knockdown

Gene	Function	Clinical relevance	VCaP status
GIMAP6	cell survival, lymphomyeloid cells	downregulated in non-small cell lung cancer (Shiao et al. 2008).	upregulated
CDH	cell adhesion and invasion	upregulated in various tumour cells	upregulated
WDR62	replication and cell cycle progression	upregulated in human gastric cancer tissues and cell lines (Zeng et al. 2013)	upregulated
ABHD2	suppresses cell migration	upregulated in atherosclerotic lesions (Miyata et al. 2008)	upregulated
HLA-E	inhibits natural killer cell recognition	better OS when absent (Zeestraten et al. 2014)	upregulated
FAM43A	unknown	unknown	upregulated
GPR44	unknown	unknown	upregulated

The genes that were downregulated in ERG knockdown VCaP cells and upregulated in the control VCaP cells were related to various metabolic pathways, immune response and cell division. These included *SCAN18*, *VASN*, *CHORDC1*, *KCNG1*, *UXS1*, *FNIP2*, *GDA*, *FAM69A*.

Table 3.2-2 List of genes that have been found to be significantly downregulated in VCaP cells following ERG knockdown

Gene	Function	Clinical relevance	VCaP status
ZSCAN18	transcriptional regulation	unknown	downregulated
VASN	inhibitor of TGF-beta signaling	unknown	downregulated
SPTLC1	metabolic pathways	mutations cause neuropathy and mitochondrial abnormalities	downregulated
CHORDC1	pegulates centrosome duplication, prevents tumorigenesis	unknown	downregulated
KCNG1	probable potassium channel subunit	unknown	downregulated
UXS1	biosynthesis	unknown	downregulated
FNIP2	unknown	unknown	downregulated
HBEGF	smooth muscle cell proliferation	upregulated in pancreatic, thyroid and lung cancers (Ota et al. 2013; Kuo et al. 2014; Ray et al. 2014)	downregulated
KLRG1	inhibits natural killer cells and T-cell	involved in arthritis, hepatitis C, and leukaemia (Melis et al. 2014; Shi et al. 2014; Göthert et al. 2013)	downregulated
MYO5B	intracellular transport and cell polarity	downregulated in gastric cancer, mutated in microvillus inclusion disease (Dong et al. 2012; Thoeni et al. 2014; Golachowska et al. 2012)	downregulated
GRPR	release of gastrointestinal hormones, smooth muscle contraction, epithelial proliferation	upregulated in several solid tumours including PCas (Mattei et al. 2014; Lee et al. 2013; Egloff et al. 2013; Nagasaki et al. 2012)	downregulated
GULP1	phagocytosis, glycosphingolipid and cholesterol transport	potential role in controlling Alzheimer's disease and Schizophrenia pathology	downregulated
GDA	microtubule assembly	unknown	downregulated
FAM69A	unknown	unknown	downregulated

As described for VCaP cells, Figure 3.2.5-3 shows the knockdown efficacy of the siRNA oligos used in DuCaP cells. In addition, it was also tested how ERG knockdown affected wild-type *ERG* versus *TMPRSS2-ERG* gene expression. Also DuCaP cells were investigated to identify changes that may occur in the expression of *AR* and *PSA* following *ERG* knockdown. The data from the qPCR analyses indicated a consistent and lasting silencing effect on both wild-type *ERG* and *TMPRSS2-ERG*. Even after 96 hours the levels of these genes were around 50% of those in the control cell population. The expression of ERG protein however remained downregulated even after 96 hours. The expression of *AR* and *PSA* did not present a clearly distinguishable pattern that may be associated with the knockdown of *ERG* oncogene. Nevertheless it appeared that the level of *AR* increased following ERG knockdown, with *AR* levels being inversely correlated with the levels of *ERG* (Figure 3.2.5-3). The presence of ERG protein was measured by Western Blot analysis and clear depletion of ERG was detected 24 hours after ERG-targeting siRNA transfection, with the highest downregulation seen after 48 hours onwards.

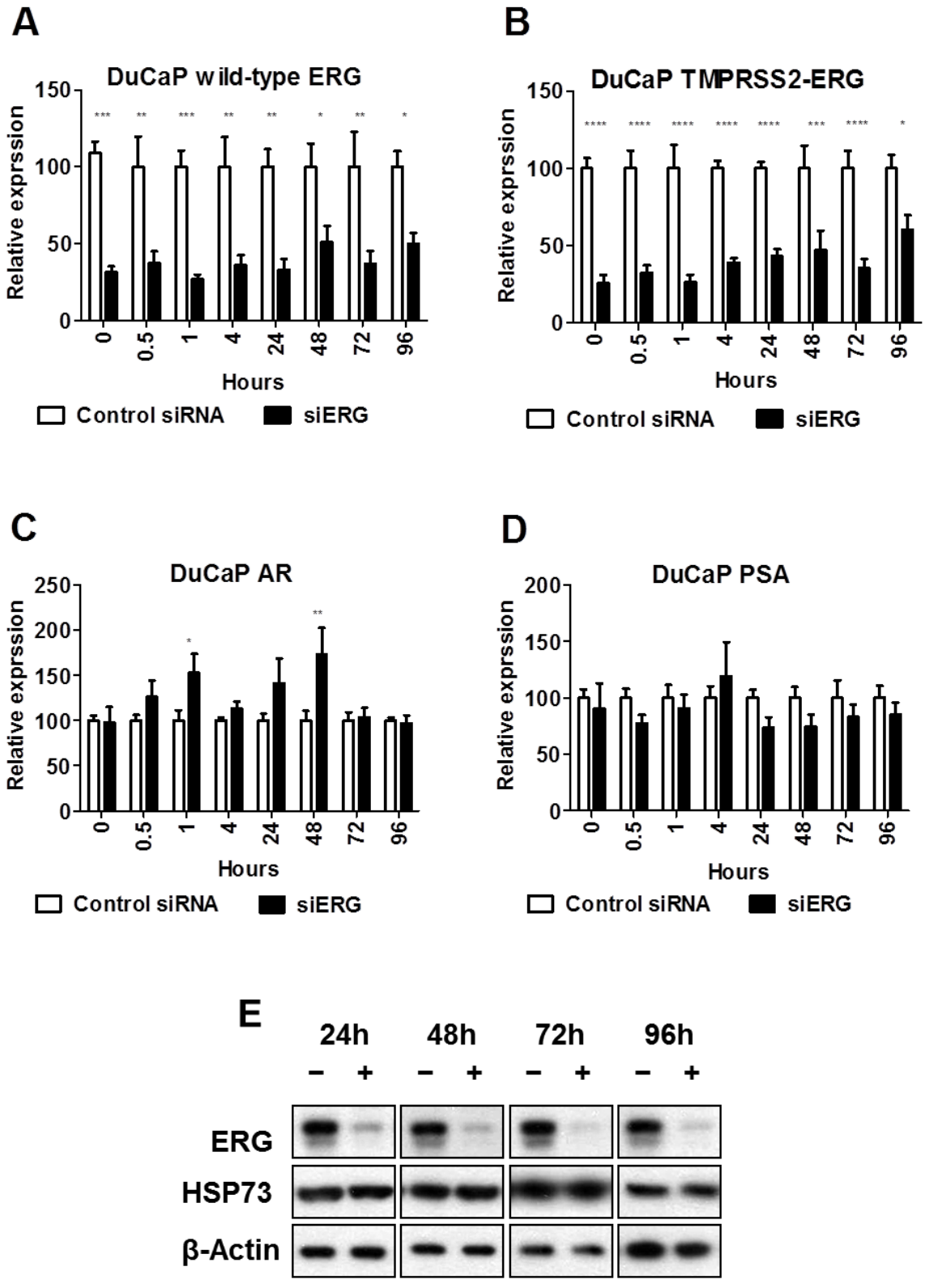


Figure 3.2.5-3 Effect of siRNA targeting ERG in DuCaP cells. DuCaP cells were transfected with siRNA targeting ERG (siERG). After 48 hours cells were 6 Gy irradiated, whereby time point zero indicates non-irradiated cells. The effect of siERG treatment was assessed at the indicated time points, which are relevant to the following experiments with IR. The figures show A. Wild-type ERG B. TMPRSS2-ERG C. AR and D. PSA. E. Western blot. 2-way ANOVA

Similar to VCaP cells, the optimisation of *ERG* knockdown in DuCaP cells was followed by a microarray analysis in which the effect of *ERG* silencing was compared to the endogenous overexpression of *ERG* in the control population of DuCaP cells. The same criteria were applied and only significant changes in gene expression levels of at least two-fold change were included. Many of the genes that showed elevated expression after knockdown such a *SLIT2*, *SLC27A2*, *SOX21*, *LHX3*, *LEPREL2* (Figure 3.2.5-4) were involved in development and metabolic pathways. For example slit homolog 2 (Drosophila) (*SLIT2*), which is a key element of axonal navigation during neural development and inhibits vascular smooth muscle cell migration, demonstrated more than four-fold increase in the *ERG*-knockdown DuCaP cells compared to the control.

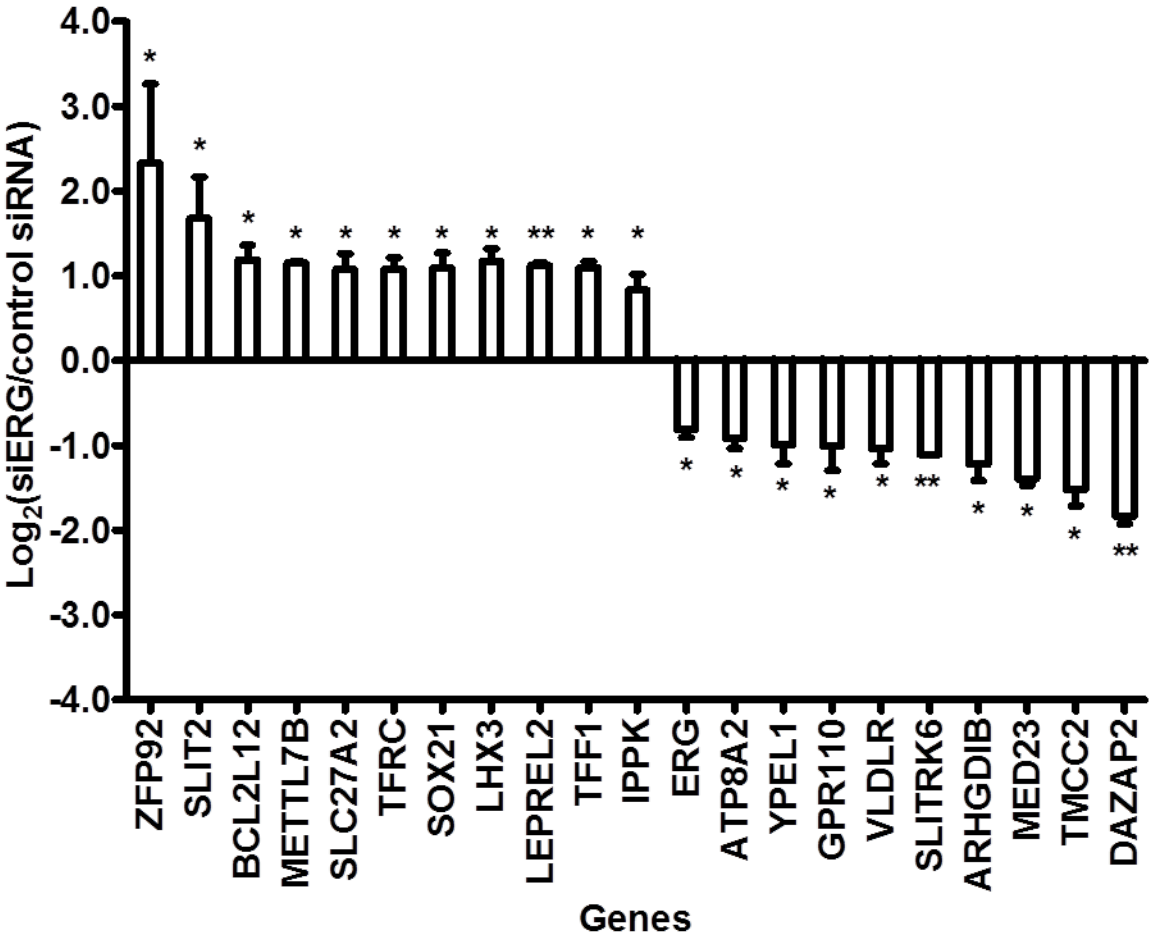


Figure 3.2.5-4 Microarray analysis of DuCaP cells. DuCaP cells were treated with ERG-targeting siRNA and the fold change expression was assessed using microarray analysis. As mentioned above, the criteria was to select genes that are significantly changed at least one fold change an these were included on the bar chart. *P<0.05; **P<0.01; unpaired student t-test.

Table 3.2-3 List of genes that are downregulated in DuCaP cells

Gene	Function	Clinical relevance	DuCaP status
ZFP92	unknown	unknown	Downregulated
SLIT2	controls angiogenic sprouting branching morphogenesis	Loss in breast, colon, and lung cancer (Dallol et al. 2003; Dallol et al. 2002). Downregulation linked to metastasis (Mehlen et al. 2011; Ballard & Hinck 2012; Göhrig et al. 2014)	Downregulated
BCL2L12	anti-apoptotic inhibitor of caspases 3/7, blocks p53	Upregulated in bladder (Foutadakis et al. 2014), favourable in breast (Thomadaki et al. 2007; Talieri et al. 2003), gastric (Florou et al. 2010) and colon cancers (Kontos et al. 2008). Unfavourable in glioblastoma (Stegh et al. 2007), nasopharyngeal carcinoma (Fendri et al. 2011), chronic lymphocytic leukaemia (Papageorgiou et al. 2011), acute myeloid leukaemia (Thomadaki et al. 2012)	Downregulated
METTL7B	unknown	unknown	Downregulated
SLC27A2	lipid biosynthesis	unknown	Downregulated
TFR1	iron uptake, development of erythrocytes	mediates hepatitis C virus entry (Martin & Uprichard 2013), upregulated in thyroid carcinoma (Magro et al. 2011)	Downregulated
SOX21	neurogenesis	Favourable expression in gliomas (Caglayan et al. 2013)	Downregulated
LHX3	pituitary development and motor neuron specification	Downregulation linked to pituitary hormone deficiency (Bechtold-Dalla Pozza et al. 2012; Sobrier et al. 2012)	Downregulated
LEPREL2	collagen biosynthesis	Downregulated in breast cancer (Shah et al. 2009)	Downregulated
TFF1	Stabilizes the mucous gel in gastrointestinal mucosa	Downregulated in gastric (J. Liu et al. 2014; Cobler et al. 2013) colorectal cancer (Huang et al. 2013). Enhances PCa cell invasiveness and metastasis (Bougen et al. 2013)	Downregulated
IPPK	DNA repair, endocytosis, and mRNA export	unknown	Downregulated

Genes that were downregulated in siERG-treated DuCaP cells included *ATP8A2*, *YPEL1*, *VLDLR*, *SLITRK6* relevant to brain development and metabolism.

Table 3.2-4 List of genes that are upregulated in DuCaP cells

Gene	Function	Clinical relevance	DuCaP status
ATP8A2	neural development	Expressed in brain. Suggested role in neuronal dysfunction upon mutation (Cacciagli et al. 2010; Onat et al. 2013)	Upregulated
YPEL1	development of the craniofacial complex	Suggested association with breast cancer risk (Olson et al. 2011)	Upregulated
GPR110	possibly EGFR signalling	Upregulated in prostate and lung cancer (Lum et al. 2010)	Upregulated
VLDLR	lipid metabolism, neural development	associated with severe CNS developmental disorders (Ozcelik et al. 2008; Moheb et al. 2008)	Upregulated
SLITRK6	neurite outgrowth and synaptic development	Mutations are linked to myopia, cochlear dysfunction and progressive auditory neuropathy (Morlet et al. 2014; Tekin et al. 2013). Suggested as molecular indicator of brain tumour properties and upregulated in medulloblastoma (Aruga et al. 2003)	Upregulated
ARHGDIB	regulator of Rho family GTPases	Upregulated in ovarian (Tapper et al. 2001) breast (Zhang & Zhang 2006) gastric (Cho et al. 2009) colorectal carcinoma (Li et al. 2012) and pancreatic cancer cells (Koide et al. 2006; Abiatari et al. 2009).	Upregulated
MED23	Required for transcriptional activation	Associated with mental retardation, conjunctivitis (Hashimoto et al. 2011). Low Med23 expression linked to better survival in Ras-active lung cancer (Yang et al. 2012)	Upregulated
TMCC2	unknown	unknown	Upregulated
DAZAP2	spermatogenesis, transcription regulation, RNA splicing	Downregulated in multiple myeloma (Luo et al. 2012; Shi et al. 2004)	Upregulated

Taken together the data indicated that ERG knockdown in VCaP cells drives the overexpression of genes linked to cell survival (*GIMAP6*), adhesion and invasion (*CDH17*, *CDH12*). Genes found to be downregulated together with ERG in VCaP cells, were linked to metabolic pathways (*SPTLC1*, *UXS1*), transcription (*ZSCAN18*), and intracellular transport (*MYO5B*). In DuCaP cells on the other hand, the knockdown of ERG caused overexpression of genes linked to angiogenic sprouting (*SLIT2*), and inhibitors of apoptosis (*BCL2L12*). The genes that were co-regulated with ERG were linked to neural development (*ATP8A2*, *YPEL1*, *VLDLR*, *SLITRK6*) and transcription (*MED23*, *DAZAP2*). Out of all genes that met the selection criteria only *GPR110*, *TFF1* and *GRPR* were previously linked to PCa.

3.2.6 Analysis of the basal expression levels of genes associated with DNA damage response and repair pathways

Changes in the DNA damage repair genes were not anticipated in the absence of IR. However it was of interest to analyse the obtained gene expression and confirm this. None of the key elements of the DSB repair pathways matched the initial selection criteria and therefore they were not included in the above figures and tables. In this subchapter a further analysis was conducted in order to evaluate the expression levels of major players in DNA repair in VCaP and DuCaP cells (Figure 3.2.6-1). Most of them were not statistically significant and showed less than a two-fold change in expression. Although in some cases it may appear that siERG treatment in VCaP cells caused slight increase in levels of

certain key DNA repair genes including *H2AX*, *RAD51*, *BRCA1* and *BRCA2* compared to control cells, these were not statistically significant (Figure 3.2.6-1 **A**). Similarly in DuCaP cells treatment with ERG-targeting siRNA seemed to have caused a decrease in some DNA repair genes and had no effect on the rest. The only genes that were altered significantly were *XRCC6* and *RAD50* genes which were both downregulated in the siERG-treated DuCaP population.

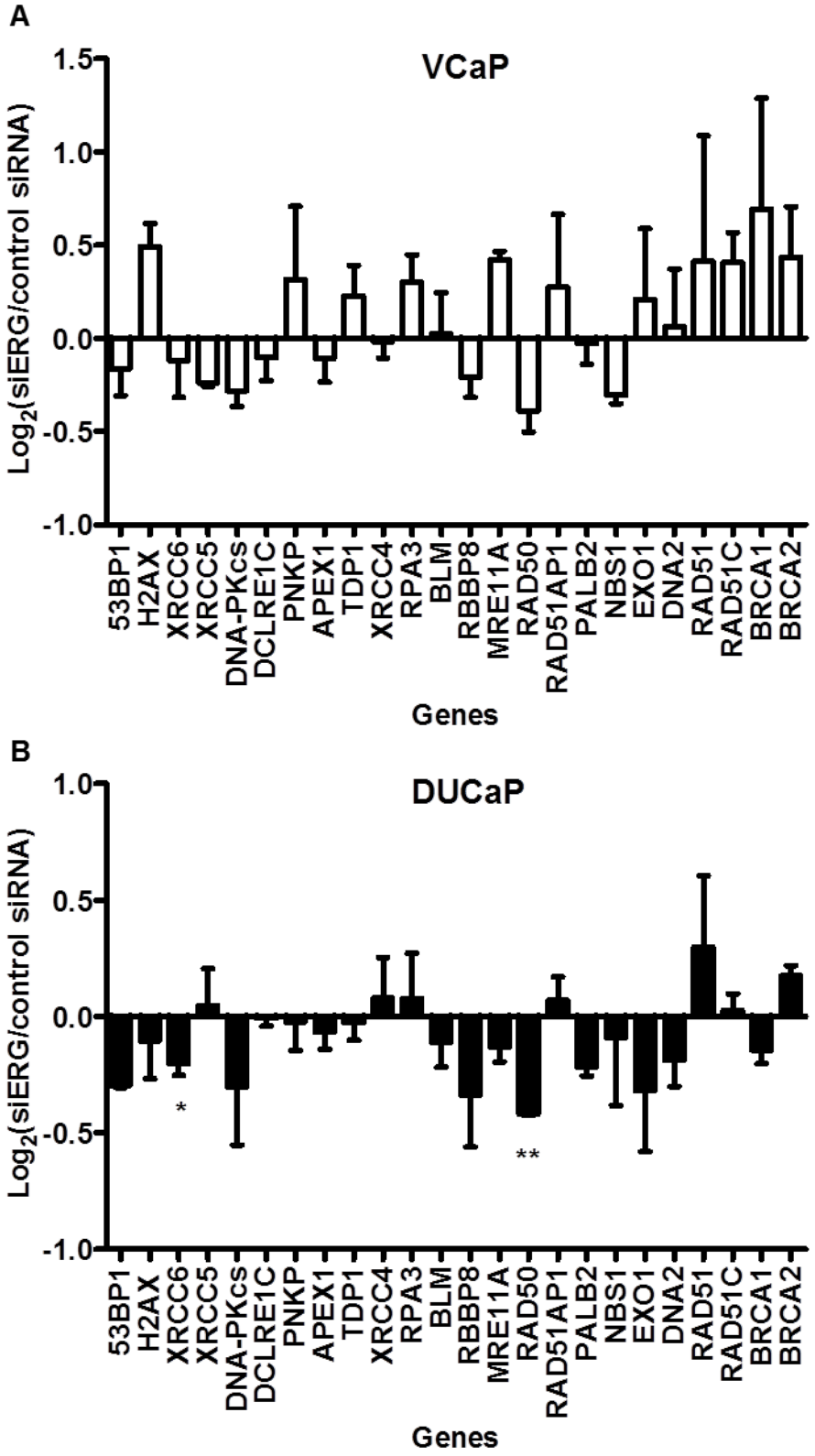


Figure 3.2.6-1 Changes in expression of DNA repair genes after siERG treatment. Only genes linked to DNA damage repair are shown in this figure. The data represents the expression of genes in A. VCaP and B. DuCaP cells treated with ERG-targeting siRNA. *P<0.05; **P<0.01; unpaired student t-test.

The table below sums up the expression of DNA repair genes in VCaP and DuCaP cells compared to their siERG-treated populations. Note that the Figure 3.2.6-1 A. and B. above show the gene levels in ERG-treated populations, whereas Table 3.2-5 describes gene levels in control VCaP and DuCaP cells. In our judgement as to whether a gene seems to be upregulated, downregulated or unchanged the *log* values from two independent experiments were matched and expressed as “Upregulated”, “Downregulated” or “Undefined” where the *log* values differed widely or were in contrast. The table also includes repair genes that are central to the DNA damage response for which the signal was below the threshold of detection and these were marked with “Unclear signal”. This data however is not conclusive and can only be used as a guideline for further studies on gene repair pathways.

Table 3.2-5 List of genes coding for proteins playing a role in DNA double-strand break repair pathways

Gene	Function	VCaP status	DuCaP status
53BP1 (TP53BP 1)	central element of chromatin-based DSB signalling; mediates the end-joining of distal DNA ends; promotes NHEJ	Upregulated	Downregulated
H2AX (H2AFX)	limits DNA accessibility to the cellular machineries; central in DNA repair, DNA replication, transcription and chromosomal stability	Downregulated	Undefined
ATM	sensor protein activated by DNA damage; phosphorylates H2AX and 53BP1; initiates DNA damage checkpoint upon DSBs and ionising UV A	Undefined	Unclear signal
XRCC6 (Ku70)	corrects defective DNA strand-break repair and sister chromatid exchange following treatment with ionising radiation and alkylating agents	Undefined	Upregulated
XRCC5 (Ku80)	involved in chromosome translocation, NHEJ, DSB repair	Upregulated	Undefined
DNA-PKcs (PRKDC)	keep broken DNA ends in close proximity and recruit end-processing factors	Upregulated	Downregulated
DCLRE1C	encodes Artemis required for recombination cell cycle control during DNA repair	Undefined	Undefined
PNKP	key role in DNA repair, functioning as part of both the NHEJ and BER pathways	Undefined	Undefined
APEX1	major apurinic/apyrimidinic endodeoxyribonuclease in the DNA BER pathway	Undefined	Undefined
TDP1	DNA repair enzyme that can remove a variety of covalent adducts from DNA through hydrolysis	Undefined	Undefined
XRCC4	involved in the DNA ends relegation complex	Undefined	Undefined
RPA3	part of a protein complex binding ssDNA and essential in DNA double-strand break repair and cell cycle checkpoint activation	Downregulated	Undefined
BLM	DNA replication and repair	Undefined	Undefined

RBBP8	endonuclease involved in a complex handling meiotic and mitotic DSBs; modulates BRCA1	Upregulated	Downregulated
MRE11A	part of the MRE11–RAD50–NBS1 (MRN) complex, central to DSB repair. MRE11A has nuclease activity.	Downregulated	Downregulated
RAD50	DNA double-strand break repair. Part of MRN complex.	Upregulated	Downregulated
RAD51AP1	linked to activation of HR and DSB repair; cooperates with PALB2 in promoting of D-loop formation by RAD51.	Undefined	Undefined
PALB2	important in HR; recruits BRCA2 and RAD51 to DNA breaks.	Undefined	Downregulated
NBS1 (NBN)	part of the MRN complex. Recruits PI3/PI4-kinase family members ATM, ATR, and probably DNA-PKcs.	Upregulated	Undefined
EXO1	5'->3' double strand exonuclease activity Functions in DNA mismatch repair.	Undefined	Undefined
DNA2	DNA replication and maintenance of nuclear and mitochondrial DNA	Undefined	Undefined
RAD51	key mediator of HR and DSB repair. Underwinds duplex DNA	Undefined	Undefined
BRCA1	nuclear phosphoprotein involved in transcription, DNA repair of DSBs and recombination; tumour suppressor.	Undefined	Downregulated
BRCA2	role in DSB repair and HR; tumour suppressor.	Downregulated	Upregulated

3.3 Discussion

The investigation in this chapter sought to characterise the cell line models appropriate to study the effect of *TMPRSS2-ERG* fusion gene in response to androgens, IR and chemotherapy. The fusion gene *TMPRSS2-ERG* was previously shown to be expressed in VCaP and DuCaP cells using qPCR

(Tomlins et al. 2005). The results in this chapter include an analysis of *TMPRSS2-ERG* expression in additional PCa cell lines, non-malignant prostate cells and other commonly known non-prostate cell models such as Jurkat cells. The expression of *TMPRSS2-ERG* gene accompanied by the expression of ERG protein were confirmed in VCaP and DuCaP cells (Figure 3.2.2-1). This was in concordance with the ERG protein levels detected by Western Blot (Figure 3.2.1-1). At the same time, *TMPRSS2-ERG*, wild-type *ERG* and ERG protein were not found to be expressed in the remaining cell lines. ERG expression in VCaP and DuCaP cells coincided with the expression of AR on a gene and protein level. Interestingly the protein and gene levels of *AR* in DuCaP cell were substantially higher than the one in VCaP. Given the fact that DuCaP cells have lower levels of *TMPRSS2-ERG* and wild-type *ERG* this may suggest a negative correlation between the expression of ERG and AR. In fact, a mechanism involving a negative feedback loop between AR and AR-regulated genes was suggested to regulate the levels of AR in VCaP cells, and this may involve *ERG* in *TMPRSS2-ERG* positive cancers (Cai et al. 2009; Cai et al. 2011). This chapter extends the research to DuCaP cells and confirms the possibility for such mechanism. This effect of androgens in the presence and absence of ERG are further explored in chapter 4. In addition, despite higher levels of AR in DuCaP cells, the presence of PSA protein was lower than in VCaP cells (Figure 3.2.1-1 and 3.2.2-1**)**. RNA levels of AR in DuCaP cells were also higher. Both VCaP and DuCaP cells express wild-type AR, but the difference in their metastatic sites may account for differences in the activity of their AR pathways. It is thus possible that the expression of *PSA* and *TMPRSS2-ERG* in DuCaP cells results in less protein

products compared to VCaP cells, where AR might be driving the expression of its target genes more efficiently. None of the remaining cell lines analysed in this study showed expression of *TMPRSS2-ERG* thus limiting significantly the number of *in vitro* models available for the study of the *TMPRSS2-ERG* fusion gene. However, wild-type expression of *ERG* was detected in Jurkat cells, albeit the expression level relative to VCaP cells was less than 0.5%. Expression of AR and PSA was observed in VCaP, DuCaP and LnCaP cells suggesting the use of these cell lines models for studying androgen signalling.

Furthermore, the expression of p53 and p53-related proteins was investigated in order to determine the steady-state levels. Expression of p53 did not correlate with the expression of p21, and this raised the question as to whether the p53/p21 pathway was in fact active in the cells investigated, and whether or not it can cause cell cycle arrest. In particular VCaP and DuCaP cells had relatively high baseline expression of p53, but borderline detectable expression of p21 in VCaP cells, and no detectable expression of p21 in DuCaP cells. This corresponds with the fact that *p53* is mutated in these cells (Korenchuk et al. 2001; van Bokhoven et al. 2003).

In order to establish the relative steady-state levels of expression of proteins involved in the DNA-damage repair pathways, the cell panel was tested for expression of γ-H2AX. This was detected at elevated levels in VCaP, P4E6 and 22RVI cells. The presence of H2AX phosphorylation in non-irradiated cells has previously been linked to chromatin instability (Yoshikawa et al. 2009). Baseline expression of γ-H2AX was not found in non-irradiated DuCaP cells. Since VCaP and DuCaP cells are from the same patient, it is noteworthy that the extent of

histone phosphorylation varies between these two cell lines and this may suggest that they have different radiosensitivities. The expression of γ-H2AX in VCaP cells was associated with p53/p21 expression. The significance of γ-H2AX has previously been described in γ-H2AX-null cells which were unable to initiate cell cycle arrest at the G_2-M after IR (Fernandez-Capetillo et al. 2002). Usually cells subjected to IR can undergo mitotic catastrophe, which is the main mechanism of cell death (Swift & Golsteyn 2014). The effect of IR can also cause cell cycle arrest, and *p53* is essential for this process (B. Liu et al. 2014). Previous studies have suggested an important role for γ-H2AX in the p53/p21 pathway and initiation of cell cycle arrest (Fragkos et al. 2009). In addition, radiosensitive tumour cells usually retain γ-H2AX expression for a longer period of time than is the case for radioresistant cells, therefore this may suggest a difference in the response of VCaP and DuCaP cells to DNA damage.

After identifying the steady-state expression levels of AR-dependent and DNA damage response proteins, the effect of androgen deprivation was analysed by Western Blot. A comparison of VCaP and DuCaP cells grown in androgen-free medium versus androgen-supplemented medium revealed an interesting correlation between AR-dependent genes and DNA-damage response genes (Figure 3.2.3-1). DHT-supplemented medium caused an increase in ERG levels and a decrease in AR expression. Increased ERG levels also coincided with increased PSA levels. The effect of androgen deprivation on the expression of DNA-damage response proteins including γ-H2AX and p53 showed a direct correlation and a rapid reduction in their expression was observed following the addition of DHT. This may indicate that androgen deprivation *per se* may be a

major factor that needs to be taken into consideration when testing the effect of IR on PCa biology.

In order to evaluate the effect of chemotherapy on VCaP and DuCaP cells, the two cell lines were treated with two classes of taxanes, docetaxel and paclitaxel.

The treatment with docetaxel caused about 25% decrease in cell viability in VCaP cells and around 75% in DuCaP. Paclitaxel on the other hand resulted in about 30% decrease in cell viability in VCaP cells, whereas the decrease observed in DuCaP cells was similar to the one of docetaxel, around 75%. Previous studies on the effect of DTX in PC3, LnCaP and Du145 cells reported an effect after 24 hours post-treatment (Mediavilla-Varela et al. 2009; Pinski et al. 2001; Axiak-Bechtel et al. 2013; Liu et al. 2013). Use of 7.5 nM caused significant effect on cells expressing wild-type p53 and weaker effect on cells with mutant or null p53 (Liu et al. 2013). As mentioned above, VCaP and DuCaP cells harbour mutated p53 and this may explain the response of VCaP cells but also shows that the use of high doses of DTX may be effective, as demonstrated in DuCaP cells. In addition previous data reported that the use of up to 10 nM paclitaxel caused 80 % cell death in LnCaP cells after 24 hours (Zheng 2006). The clinically relevant doses of paclitaxel and docetaxel are ≤ 100 nM (Urien et al. 1996; McAuliffe et al. 2002; Andersen et al. 2006). A limitation of this experiment was the period of three days. This did not take into account differences in the doubling time of DuCaP and VCaP cells, 2 and 3 days respectively. Therefore a longer exposure to taxanes may be required in order to see effect on VCaP cells.

Next, a protocol for an efficient knockdown of ERG protein in both VCaP and DuCaP cell lines was established and the changes were analysed using qPCR, Western Blot and microarray analyses.

In VCaP cells the knockdown of *TMPRSS2-ERG* was determined at the protein and gene levels followed by a gene expression analysis comparing the gene levels in the ERG-knockdown VCaP versus the control-treated VCaP cells. Several of the downregulated genes were found to be linked to cancer. Downregulation of the GTPase immunity associated family member 6 (*GIMAP6*) gene was previously suggested to participate in the pathogenesis of non-small cell lung cancer (NSCLC) and to influence immune response to the tumours (Shiao et al. 2008). Cadherin-17 (*CDH17*) is another gene that was downregulated in the control VCaP cells. Interestingly, studies on *CDH17* determined increased expression of this gene in liver metastasis from colorectal cancer (Bartolomé et al. 2014) and suggested an important role in late metastatic events (Luque-García et al. 2010). Knockdown studies of *CDH17* also showed inhibition of cell proliferation, adhesion, migration and colony formation, as well as cell cycle arrest and apoptosis in human gastric cancer cells (Lin et al. 2014). Similarly Cadherin 12 (*CDH12*) was reported to promote proliferation, adhesion, migration and angiogenesis in colorectal cancer (Zhao et al. 2013). WD40 repeat-containing 62 (*WDR62*) is another gene that was downregulated in control VCaP cells but was found upregulated in gastric cancer and was correlated with poor prognosis (Zeng et al. 2013). Abhydrolase Domain Containing 2 (*ABHD2*) is a candidate for colorectal cancer-related gene (Yoshida et al. 2010). The absence of Major Histocompatibility Complex, Class I, E (*HLA-E*) gene, which was

downregulated in control VCaP cells, was previously associated with better outcome in colon cancer as the tumour cells are easier target for elimination by the natural killer cell (Zeestraten et al. 2014). The knockdown of ERG was linked to co-regulated downregulation of number of genes. The clinical relevance of many of these genes however including *ZSCAN18*, *VASN*, *CHORDC1*, *KCNG1*, *UXS1*, *FNIP2*, *GDA*, *FAM69A* was unknown and to our best knowledge the function of *FNIP2* and *FAM69A* has not yet been identified. The gene serine palmitoyltransferase long chain base subunit 1 (*SPTLC1*) is upregulated in VCaP cells and recent studies related mutations in this gene to neuropathy and mitochondrial abnormalities (Fridman et al. 2014; Myers et al. 2014; Marshall et al. 2014; Suh et al. 2014). Another gene that is upregulated in control VCaP cells is the Heparin-Binding EGF-Like Growth Factor (*HBEGF*), which is involved in bronchial proliferation and remodelling. Recent studies found this gene to promote breast cancer metastasis (Zhou et al. 2014) and to be upregulated in patients with hypospadias, a congenital hypoplasia of the penis in which there is a displacement of the urethral opening (Karabulut et al. 2013). Upregulation of the Killer Cell Lectin-Like Receptor Subfamily G, Member 1 (*KLRG1*) has been linked to chronically inflamed joints and arthritis (Melis et al. 2014). Myosin VB (*MYO5B*) is epigenetically silenced in human gastric cancer (W. Dong et al. 2012; Dong et al. 2013) which contrasts to our observation in the control VCaP cells. Furthermore the upregulation of Gastrin-Releasing Peptide Receptor (GRPR) in control VCaP cells was in concordance with its ectopical overexpression in 60% of colon cancers (Radulovic et al. 1991; Frucht et al. 1992) but also with its expression in small-cell lung cancer (Mattei et al. 2014), breast and PCa (Jensen

et al. 2008; Mansi et al. 2013; Cai et al. 2013). The last gene that was upregulated in the control VCaP population was the engulfment adaptor phosphotyrosine-binding-domain-containing 1 (*GULP1*), which mediates glycosphingolipid and cholesterol transport but also is involved in the phagocytosis of apoptotic cells. Due to the role of *GULP1* in cellular transport and its interaction with the amyloid-β precursor protein it was proposed a potential relevance in Alzheimer's disease (Beyer et al. 2012; Hao et al. 2011). In addition the role of *GULP1* in phagocytosis related this gene to schizophrenia (X. Chen et al. 2009). The precise role of these genes in PCa disease progression remains to be elucidated.

In DuCaP cells the knockdown of *TMPRSS2-ERG* was confirmed by qPCR along with evaluation of the levels of *AR* and *PSA*. The data suggested a possible inverse correlation between *ERG* gene expression and *AR* expression (Figure 3.2.5-3). Previous articles related DNA methylation of *SLIT2* to pancreatic adenocarcinoma and metastases in pancreatic cancer (Nones et al. 2014; Göhrig et al. 2014), hereditary breast cancer (Alvarez et al. 2013), non-small cell lung cancer (Suzuki et al. 2013) and ovarian cancer (R. Dong et al. 2012). Furthermore knockdown of *SLIT2* was shown to activate gastric cancer growth and metastasis (R. Shi et al. 2014) and downregulation of this gene was related to renal cell carcinoma (Ma et al. 2014). It was suggested that this gene acts as a tumour suppressor gene in various cancer types including colorectal cancer (Chen et al. 2013). BCL2-Like 12 (*BCL2L12*) is another gene found to be upregulated after ERG-knockdown. The fact that control DuCaP cells had lower levels of *BCL2L12* was in contrast with previous data showing an increase of this gene and unfavourable outcome in bladder tumours (Foutadakis et al. 2014) and

glioblastoma (Stegh & DePinho 2011; Jensen et al. 2013; Stegh et al. 2010). Increased *BCL2L12* was also observed in chronic lymphocytic leukaemia (Karan-Djurasevic et al. 2013; Papageorgiou et al. 2011) and acute myeloid leukaemia (Thomadaki et al. 2007) with poor prognostic value in the latter. However expression of *BCL2L12* was also linked to favourable outcome in breast cancer (M.-T. Lee et al. 2013), gastric cancer (Florou et al. 2010) and colon cancer (Kontos et al. 2008). Downregulation of *BCL2L12* was reported in advanced laryngeal tumours, malignant tongue neoplasms (Geomela et al. 2013). Next, the gene coding for Transferrin Receptor (*TRF1*) acts in the iron uptake and the development of erythrocytes, and is widely present in the central nervous system. We observed downregulation of *TRF1* in control DuCaP cells and reduced levels of *TRF1* were previously connected to inhibition of proliferation in pancreatic cancer cells (Jeong et al. 2014) and in patients with Parkinson disease (Yu et al. 2013) In contrast *TFR1* gene was found to be significantly increased in alcoholic liver disease (Dostalikova-Cimburova et al. 2014) and thyroid carcinoma (Magro et al. 2011). SRY (Sex Determining Region Y)-Box 21 (*SOX21*) was reported to be methylated with high frequency in colorectal cancer (Mitchell et al. 2014). On the other hand overexpression of *SOX21* was linked to decreased stem-like cell proliferation of gliomas cells in vivo (Caglayan et al. 2013). In fact *SOX21* was identified as a regulator of stem cell fate during early mouse development (Mallanna et al. 2010). LIM Homeobox 3 (*LHX3*) is involved in the development of cells of the pituitary gland and the nervous system. This gene was linked to medical conditions affecting the pituitary glands and motor neurons (Mullen et al. 2012; Cohen 2012; Bechtold-Dalla Pozza et al. 2012).The leprecan-like 2

(*LEPREL2*) gene was downregulated in control DuCaP cells and previously downregulation of this gene was linked to breast cancer (Shah et al. 2009). Further gene that was downregulated in the control DuCaP was the Trefoil Factor 1 (*TFF1*), which was found to be lost, downregulated or methylated in gastric cancer (Soutto et al. 2014; Feng et al. 2014; Tanaka et al. 2013) but overexpressed in salivary gland tumours (Chaiyarit et al. 2014) and breast cancer (Pelden et al. 2013). More importantly *TFF1* was found to promote PCa invasiveness and metastasis (Bougen et al. 2013). For some of the remaining genes including *ZFP92*, *METTL7B*, *SLC27A2*, there was not available data regarding their function and clinical relevance.

In DuCaP cells the ATPase, Aminophospholipid Transporter, Class I, Type 8A, Member 2 (*ATP8A2*) was upregulated and previous studies linked this gene to cerebellar ataxia, mental retardation, and dysequilibrium syndrome, cerebellar atrophy (Cacciagli et al. 2010; Onat et al. 2013). Additionally, a previous study reported an upregulation of *ATP8A2* in VCaP cells (Paulo et al. 2012). Another gene Yippee-Like 1 (Drosophila) (*YPEL1*) was shown to induce cell senescence and was linked to breast cancer risk (Olson et al. 2011). The next gene was the G Protein-Coupled Receptor 110 (*GPR110*), which has unknown function but recent report suggested that it may be critical for the transactivation of the epidermal growth factor receptor (*EGFR*) (Cho-Clark et al. 2014). Previous studies found it upregulated in lung and PCa (Lum et al. 2010) and this is in concordance with our observation. The Very Low Density Lipoprotein Receptor (*VLDLR*) is involved with the trafficking of metabolites required in the neuronal migration in the cerebral cortex and cerebellum. The clinical significance of

VLDLR is mainly connected to developmental disorders (Ozcelik et al. 2008; Moheb et al. 2008) and Alzheimer's disease (Xing et al. 2013). SLIT And NTRK-Like Family, Member 6 (*SLITRK6*) was another gene involved in neural development that was downregulated in the DuCaP cells treated with ERG-targeting siRNA. As a result mutations in this gene cause myopia and deafness in humans (Morlet et al. 2014; Tekin et al. 2013). In addition *SLITRK6* was linked to specific properties in brain tumours (Aruga et al. 2003). The next gene was the Rho GDP Dissociation Inhibitor (GDI) Beta (*ARHGDIB*). Recent report suggested that *ARHGDIB* was negatively regulated in lung cancer (Song et al. 2014). Nevertheless additional reports demonstrated in several cancers including gastric (Cho et al. 2009) and breast cancer (Zhang & Zhang 2006). The remaining two genes Mediator Complex Subunit 23 (*MED23*) and DAZ Associated Protein 2 (*DAZAP2*) are both involved in transcriptional regulation *MED23* was associated with better survival in lung cancer (Yang et al. 2012) whereas *DAZAP2* was found to be downregulated in myeloma (Luo et al. 2012; Shi et al. 2004). *XRCC6* and *RAD50* were the only DNA damage-related proteins found to be significantly upregulated in DuCaP cells. *XRCC6* encodes the Ku70 protein which can facilitate binding of defective DNA strands and is linked to DNA NHEJ pathway during DSB repair and recombination. *RAD50* is a component of the MRN complex, which is a key element in DSB repair, DNA recombination, preservation of telomere integrity and meiosis.

Taken together, the results in this chapter indicated the most appropriate cell models, VCaP and DuCaP, for studies on *TMPRSS2-ERG*. The steady-state protein levels of ERG in these models were linked to AR and PSA expression. In

addition ERG expression in VCaP cells coincided with higher γ-H2AX levels compared to DuCaP cells. Androgen deprivation caused substantial decrease in ERG and PSA in both VCaP and DuCaP cells. At the same time, androgen-deprivation resulted in increased levels of AR, p53 and γ-H2AX. The use of taxane in VCaP and DuCaP cells had stronger negative effect on DuCaP cells' viability. Downregulation of ERG in both cells lines caused significant changes in the levels of genes linked to metabolism, transcription or neural development. *XRCC6* and *Rad50* were the only DNA damage response genes found to be significantly upregulated in ERG-knockdown DuCaP cells and this observation needs to be validated in further studies involving irradiated and non-irradiated samples. In total, the preliminary findings from the microarray analysis did not show overlap between the gene changes in VCaP and DuCaP cells. In addition the data needs to be validated by Western Blot and/or qPCR analysis. Also it will be desirable to reproduce the experiment by including samples from at least five independent experiments and compare the effect from various combinations of siRNA sequences in order to exclude off-target effect.

Chapter 4. Physiological effects of ionising radiation and androgen deprivation

4.1 Introduction

Radiotherapy is a useful alternative to surgery in PCa treatment and imaging technologies along with dose-escalating radiation have substantially improved clinical outcomes. However selecting patients that may benefit from radiotherapy is not a straightforward process. These are usually individuals with non-indolent intermediate- and high-risk localised or locally advanced PCa with around 5% absolute risk decrease of mortality between 10 and 15 years. Different radiobiological models imply that radiotherapy is more efficient if doses are delivered in larger fraction sizes than in smaller more frequent doses, causing increased sensitivity in PCa cells (Fowler et al. 2001). Several randomised clinical trials reported that dose escalation leads to elevated risk for late gastrointestinal toxicity in 26% to 35%, which is in contrast to low doses of radiotherapy (Pollack et al. 2002; Dearnaley et al. 2007; Al-Mamgani et al. 2008). Late rectal toxicity results in rectal pain, rectal stricture and fistula, rectal inflammation causing incontinence and peri-rectal bleeding. Although many state-of-the-art techniques allow for delivery of high dose IR directly to the tumour rather than to the entire organ, thereby causing minimal effects on the healthy surrounding tissue, radioresistance remains a significant problem. To date some principal mechanisms of radioresistance have been identified and targeted using different drugs. Nevertheless monotherapy with radiation has limited effectiveness for high-risk patients. In randomised trials of monotherapy with low dose irradiation (65-70 Gy) versus long-

term ADT and radiation, the 10-year rate of PSA failure was higher than 75% (Pilepich et al. 2005; Horwitz et al. 2008; Pilepich et al. 2001; Bolla et al. 2010). Previous research has also found an association between IR and an increased risk of early and late toxicities. One of the major risks associated with radiotherapy is the high proportion of patients who encounter tumour regrowth after treatment with IR. The most concerning risk related to radiation remains the phenomenon of secondary malignancy induced by initial treatment, as reported by several studies (Preston et al. 2008; Raicu et al. 1993; Zelefsky et al. 2006; Peeters et al. 2005).

In clinical practice the combination of IR and ADT has been the standard for treatment of high-risk PCa for approximately 20 years. Multiple clinical trials have reported that the combination of radiotherapy and ADT gives better response than radiotherapy alone. Particularly for high and intermediate risk PCa the combination of IR and ADT proved to significantly increase the chances for disease-free survival (Jones et al. 2011; Bolla et al. 1997). Further studies focused on the mechanisms by which AR inhibition benefits radiotherapy and leads to increased PCa cell death, by using *in vitro* and *in vivo* models (Wo & Zietman 2008). Different mechanisms that may account for this trend include a decline in tumour cell hypoxia (Jain et al. 1998), DNA damage repair (Al-Ubaidi et al. 2013) and decline in AR-mediated growth without direct synergy (Pollack et al. 2001). In addition it was proposed that AR may assist the *de novo* formation of tumour translocations involving *TMPRSS2*, *ERG*, and *ETV1* in response to IR and that these are non-random events (Lin et al. 2009). This notion is in contrast with the popular theory that tumour translocations result from random DNA double-strand lesions and that during a process of selection, only DNA lesions conferring a proliferative advantage are retained. In this context, a study focussing on oestrogen receptor function discovered that there might be a system for coordinated regulation

of certain genes and their co-expression where an interaction between loci of different chromosomes is induced. The main player in this process of chromosomal movements was an isoform of an enzyme, topoisomerase II isoform B (TOP2B), that catalyses a temporary DSB to resolve DNA topological constraints. In summary the oestrogen signalling was linked to recruitment of this enzyme to oestrogen receptor DNA binding sites causing DSBs (Hu et al. 2008). In a similar fashion androgen signalling was found to recruit TOP2B and AR to DNA sites of *TMPRSS2-ERG* breakpoints. This resulted in the *de novo* formation of a *TMPRSS2-ERG* fusion gene (Haffner et al. 2010). Furthermore, DHT stimulation of AR sensitive PCa cells induced proximity between *TMPRSS2* and *ERG* loci, and consequently IR promoted the production of a *TMPRSS2-ERG* fusion transcript (Mani et al. 2009; Lin et al. 2009; Yu et al. 2010).

As mentioned in the beginning of this work the role of *TMPRSS2-ERG* in the pathology of PCa has long been disputed. No clear association between *TMPRSS2-ERG* status and outcome has been agreed (Demichelis et al. 2007; Robert K Nam et al. 2007; Attard, Clark, et al. 2008; Spencer et al. 2013; Pettersson et al. 2012) and no association with Gleason score has been determined (Chaux et al. 2011; R K Nam et al. 2007; Xu et al. 2014; Pettersson et al. 2012). Interestingly, a recent study reported an association between ERG expression and both advanced progression stage and better outcome. The study proposed that this paradox may be due to differences in the cellular pathways that underline ERG-positive and ERG-negative tumours (Taris et al. 2014). *In vitro* and *in vivo* studies showed that knockdown of *TMPRSS2-ERG* is linked to decreased cell proliferation, invasiveness and mobility (Sun et al. 2008; Wang et al. 2008; King et al. 2009). To date however there are no reported *in vitro* studies that have investigated the role of *TMPRSS2-ERG* status on cell response to IR in cells overexpressing ERG endogenously.

In this chapter the growth and proliferation of VCaP and DuCaP cells was tested after treatment with ERG-targeting siRNA or control siRNA in standard culture medium. The effect of exposure to IR was determined by counting cell number. In addition the proliferative activity of irradiated cells was measured using MTS assay after IR treatment. This experimental setup aimed to reveal whether the presence of *TMPRSS2-ERG* confers a growth advantage to irradiated cells. In the subsequent series of experiments it was investigated whether the elimination or addition of androgens alters this interplay. Since ADT is associated with various adverse effects including bone loss, muscle loss, hot flashes, metabolic changes, gynecomastia, and possibly increased cardiovascular events, it is of clinical interest and importance to determine whether IR *per se* is sufficient to diminish growth in cancer cells, and whether the expression of ERG modifies this response and to what extent this interaction depends on AR signalling.

4.2 Results

4.2.1 Morphological changes of VCaP cells after ionising irradiation treatment

As demonstrated in chapter 3 VCaP cells are AR dependent and the depletion or addition of DHT has a rapid effect on their molecular biology. Therefore their morphological response to castration and IR was investigated. VCaP cells were cultured in CSS in order to simulate ADT, while a second population of VCaP cells was maintained in 1 nM DHT-supplemented medium or standard growth medium before eventually IR was introduced (Figure 4.2.1-1). The physiologically relevant

concentration of DHT is between 1 and 10 nM (Castoria et al. 2014; Castoria et al. 2011; Cheng et al. 2010; Overcash et al. 2013). As demonstrated in Chapter 3, there was no significant difference between cells stimulated with 1 nM, 10 nM or 100 nM DHT (Figure 3.2.3-1). Therefore 1 nM DHT concentration was selected as the standard androgen treatment for VCaP cells. The morphology of the cells was observed three days post-irradiation. Upon IR, it was apparent that cells grown in CSS were proliferating less actively, without the formation of organ-like structures and clumps of cells. On the other hand, cells grown in DHT-supplemented medium did not present morphological differences to cells grown in standard culture medium, however they appeared to be less confluent. They formed clumps and structure-like patterns, which are characteristic for VCaP cells. It appeared that within the three days time-frame the effect of IR did not cause visible effects on cell morphology and a longer observation period may be required to evaluate potential differences.

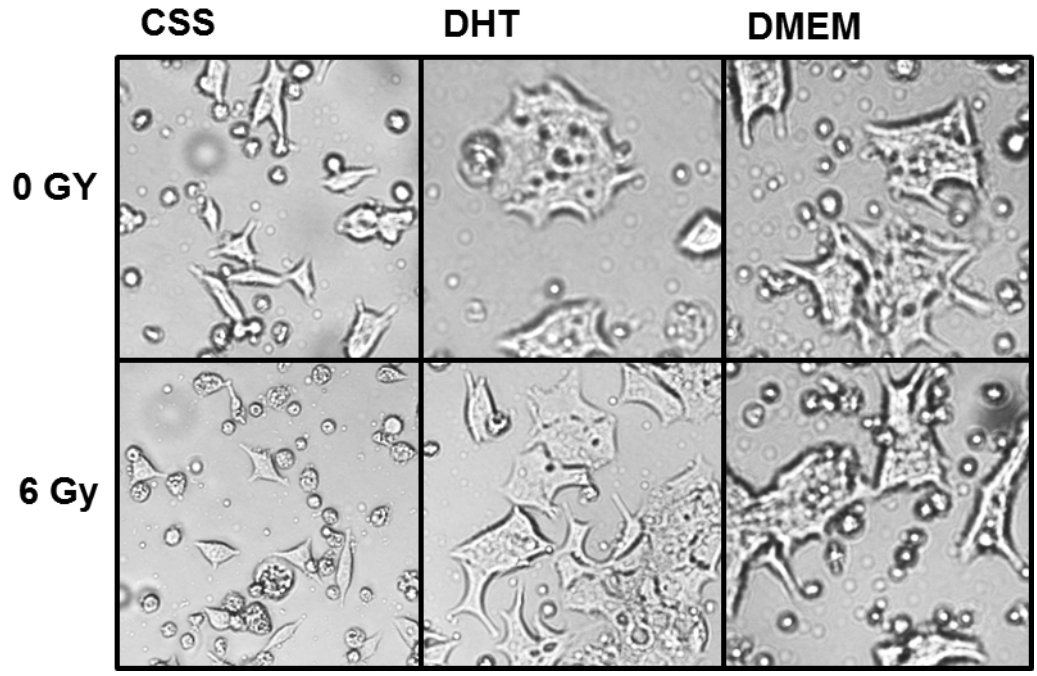


Figure 4.2.1-1 Short-term morphological differences in VCaP cells after combining castration and IR. VCaP cells were grown for 3 days in medium supplemented with either CSS only, CSS supplemented with 1 nM DHT, or standard DMEM supplemented with 10% FCS. The top row shows cells treated with sham irradiation. Bottom row shows cells irradiated at 6 Gy. Magnification: 10X.

Next VCaP cells were grown for an extended period of time (two weeks) in order to evaluate the effect of IR on cell morphology more extensively. However, VCaP cells were grown in standard culture medium only and were either treated with ERG-targeting siRNA or control siRNA for 48 hours, which was followed by 0 Gy or 6 Gy IR-treatment. The morphological changes were recorded every other day by taking bright-field microscopy images. As shown on Figure 4.2.1-2 during the first week no major differences were observed between ERG knockdown and control cells both in 0 Gy or 6 Gy treated cell populations. Although the control cell population appeared to be growing more actively an overall similarity in morphology was retained. During the second week, the 0 Gy-treated cells demonstrated active growth and achieved almost 90% cell confluence, whereby it appeared that the siERG-treated cells were less

confluent and had floating cells, likely dead cells, in the medium compared to the control siRNA-treated cells. Cells exposed to 6 Gy IR demonstrated aberrant growth pattern (Figure 4.2.1-3). In fact, across the 6 Gy treated populations, the siERG-treated cells resembled the characteristics of dead or necrotic cells. They had rounded structure and undefined borders of their cellular compartments. The morphology of 6 Gy treated control siRNA cell population was also similarly affected by IR. However more cells appeared to be alive and adhered to the bottom of the plate in comparison to 6 Gy treated siERG cells

Overall, the 14-days morphological analysis indicated that 0 Gy treated cells tend to form clumps of cells and achieved 90% confluency in the presence of ERG compared to 50% confluency in ERG-knockdown cells. In the case of 6 Gy treated cells, the formation of clumps was less apparent and cell confluency was less than 50%. 6 Gy treated ERG-knockdown cells formed very few cell clumps and the cells showed rounded shape.

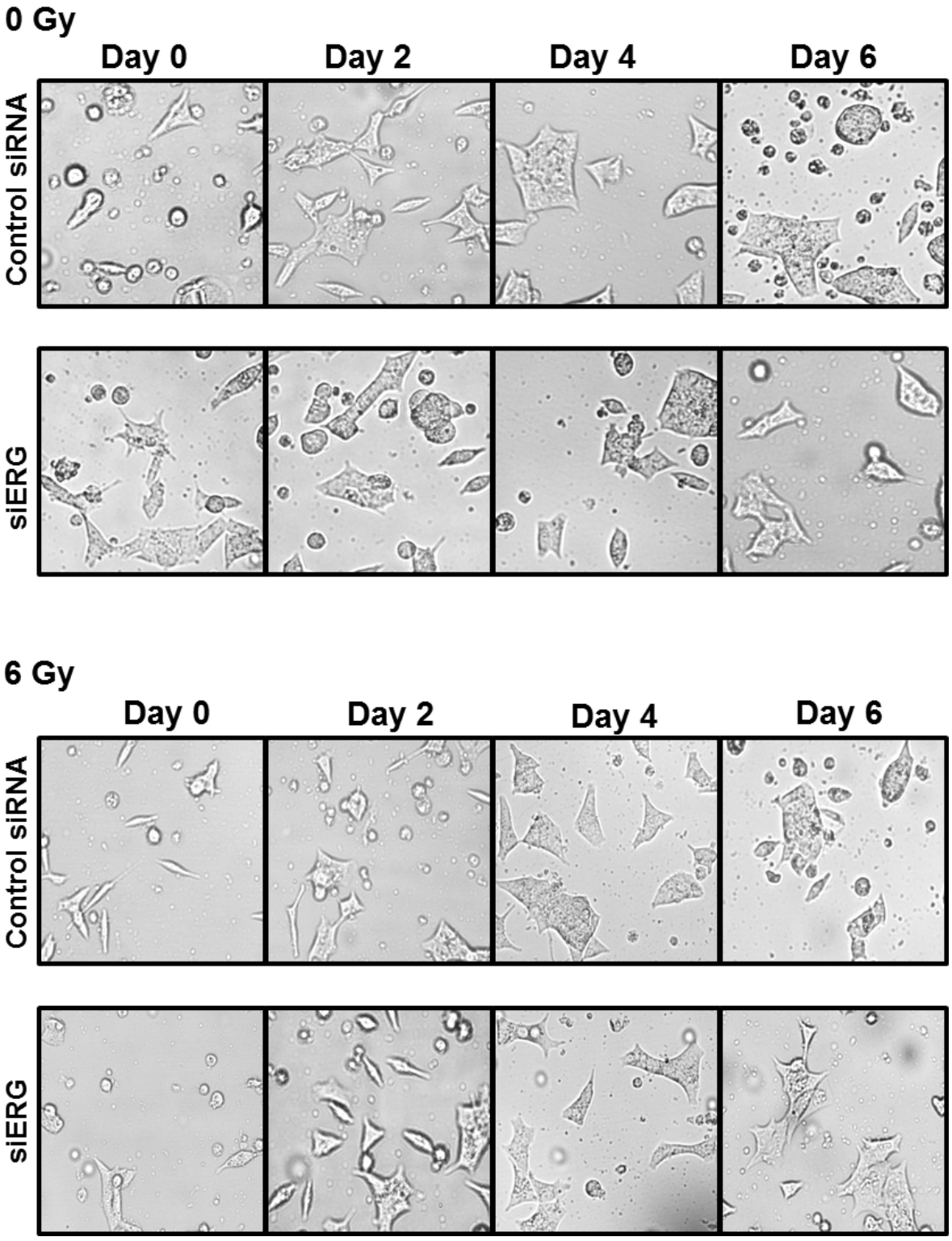


Figure 4.2.1-2 Long-term morphological differences between siERG treated and control population of VCaP cells. VCaP cells were reverse transfected and seeded in normal medium for 48 hours. Cells were subsequently irradiated at 6 Gy or 0 Gy and their morphology was observed microscopically. Top images represent 0 Gy irradiated cells and bottom images represent 6 Gy irradiated cells. The days are shown above each couple of images of siERG-treated and control siRNA treated VCaP cells. Magnification: 10X.

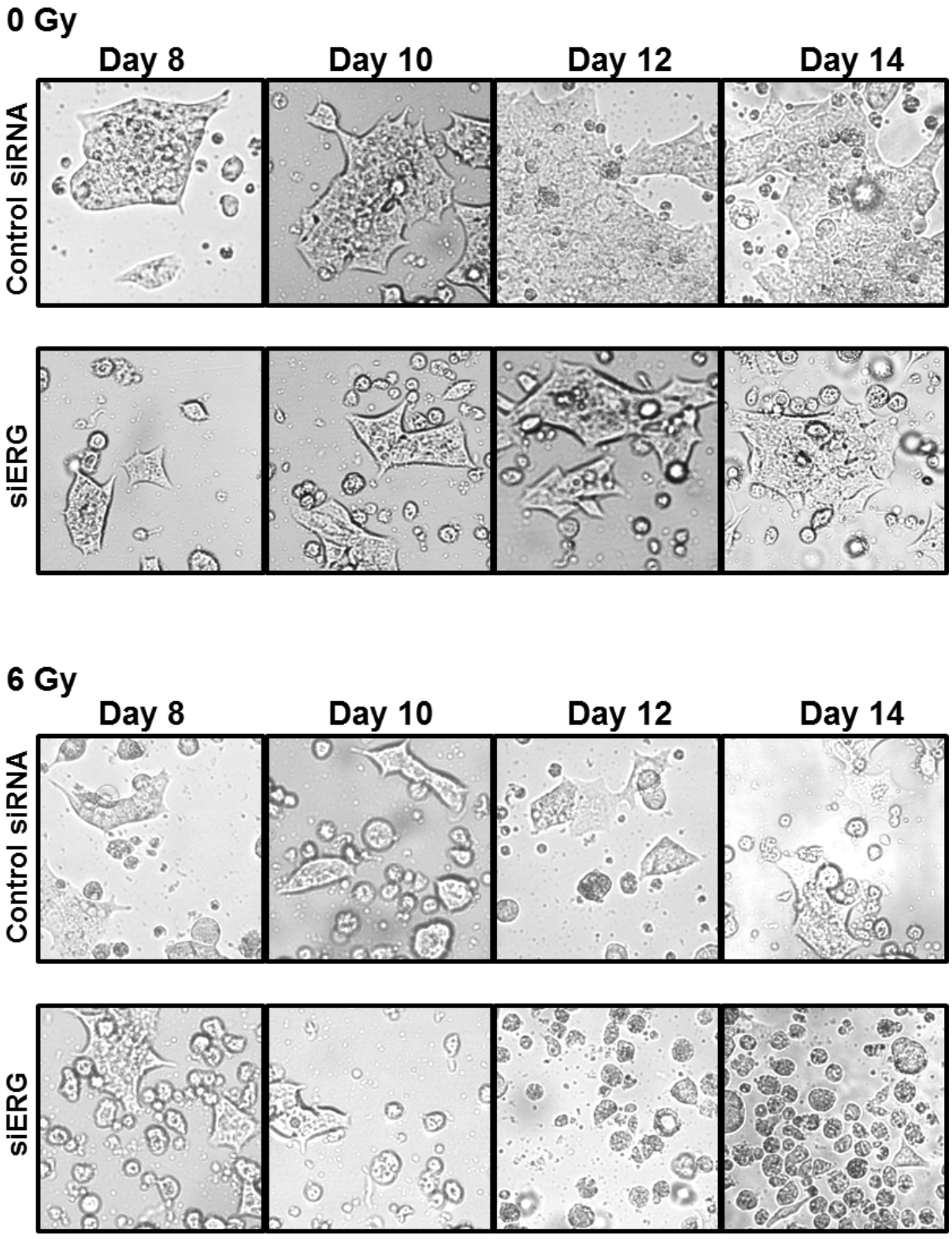


Figure 4.2.1-3 Morphological differences between siERG treated and control population of VCaP cells. As described in the previous figure here are shown the images representing VCaP cell morphology during the second week of the experiment, day 8 to day 14. The cells were grown in standard culture medium. Magnification: 10X.

4.2.2 ERG presence correlates with increased cell number in non-irradiated VCaP and DuCaP cells but not in irradiated cells

The morphological evaluation of cell growth provided interesting clues as to how *TMPRSS2-ERG*-status may affect response to radiotherapy. Nevertheless, a more detailed quantitation of this effect was also necessary. Thus the cell growth of VCaP cells was quantitatively assessed by counting cell numbers. VCaP cells were transfected with ERG-specific siRNA, then plated in standard culture medium and after 48 hours cells were treated with 6 Gy or 0 Gy. The day of irradiation was defined as day zero and all subsequent measurements of cell number were normalised to the values determined on day zero. As shown on Figure 4.2.2-1 the initial growth of cells treated with *ERG*-targeting siRNA or control siRNA was comparable and no significant difference in the total cell number was observed in the cells exposed to 0 Gy IR. Interestingly towards the end of the second week of the experiment, it became evident that within the 0 Gy treated cells, the control siRNA-treated VCaP population proliferates significantly more than the siERG-treated VCaP population. This data corresponded with similar studies that have previously reported an elevated growth in cells overexpressing the ERG oncoprotein (Wang et al. 2008). The introduction of radiotherapy strongly affected this proliferative advantage for cells with ERG overexpression. VCaP cells exposed to 6 Gy IR showed similar percentage of viable cells in control siRNA-treated VCaP population and in siERG-treated VCaP population.

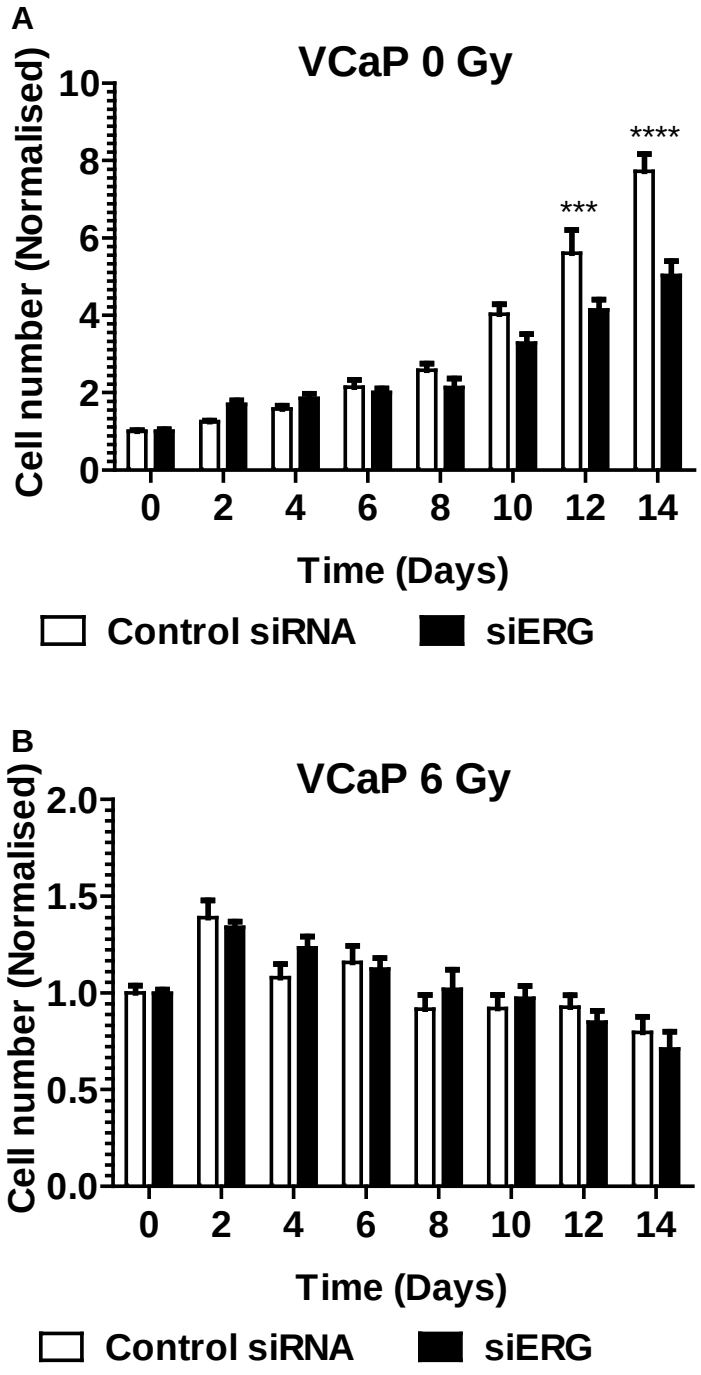


Figure 4.2.2-1 Expression of ERG does not affect response to treatment with 6 Gy irradiation. 100,000 cells were plated on 24 well plates and transfected with control siRNA or siERG. The plates were irradiated at 0 Gy or 6 Gy. Every other day the number of total cells were counted using NucleoCounter. **A.** Total cell number of 0 Gy irradiated cells. **B.** Total cell number of 6 Gy irradiated cells. **A-B.** The bar charts show normalised cell proliferation relative to day zero. Day zero indicates the day of irradiation. The data represents three independent experiments conducted in triplicates; *P<0.05; **P<0.01; ***P<0.001,****P<0.0001, Two-way ANOVA.

In a similar way DuCaP cells were also transfected with ERG-specific siRNA and subjected to radiotherapy. DuCaP cells were then grown in standard culture medium for seven days and their number was analysed (Figure 4.2.2-2). In accordance with the results from the VCaP cells, DuCaP cells treated with control siRNA demonstrated a growth advantage over the ERG-specific siRNA-treated cells when exposed to 0 Gy irradiation. This emphasises the importance of ERG overexpression for cell growth in non androgen-deprived and non-irradiated conditions. In addition the DuCaP cells proliferated quicker than the VCaP cells and they reached 90 % confluency within seven days. The introduction of 6 Gy IR caused continuous decline in the number of DuCaP cells after day two. It appeared that the cell number of both ERG-expressing and ERG-knockdown DuCaP cells declined.

In summary both VCaP and DuCaP cells had similar growth rates in non-androgen-deprived standard culture medium and in both cell lines the presence of ERG was linked to increased cell growth before radiotherapy. On the other hand the exposure to radiation had comparable damaging effects on both VCaP and DuCaP cells and this effect was irrespective of their ERG-status.

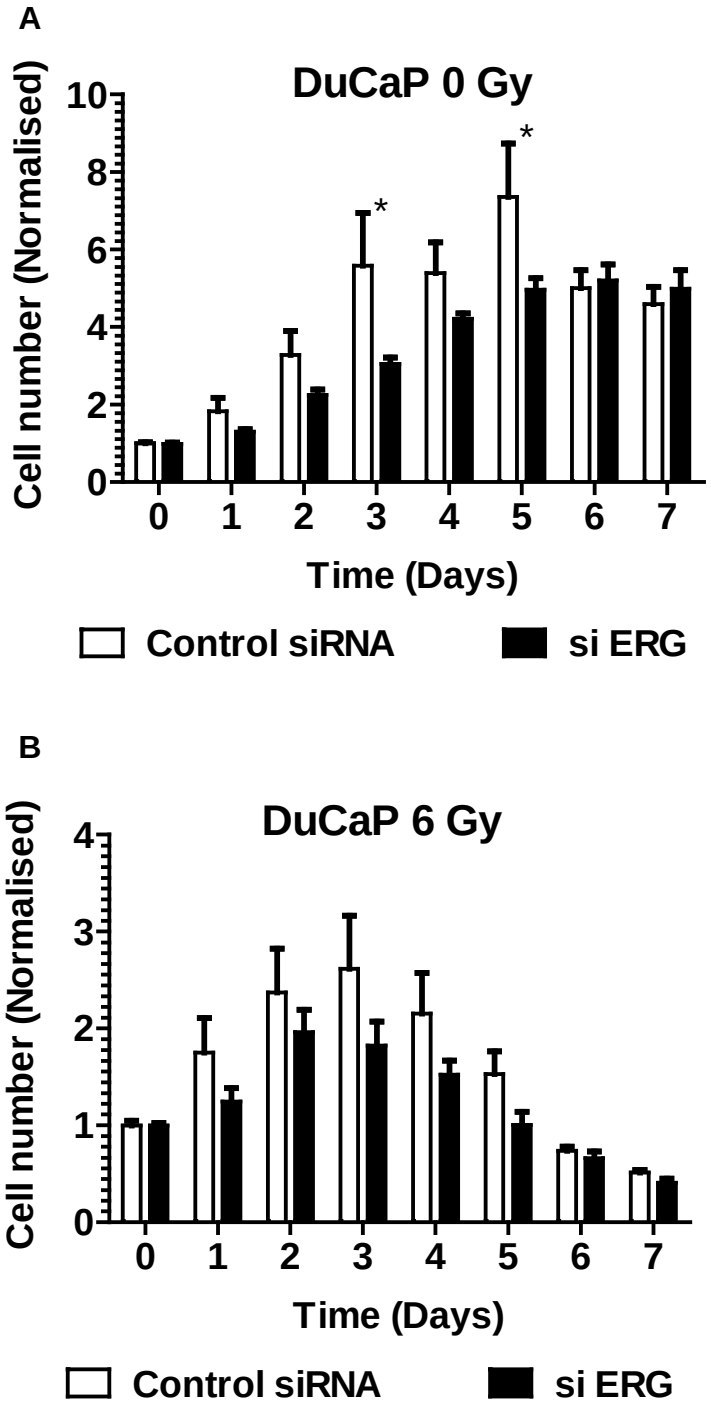


Figure 4.2.2-2 Growth curve, DuCaP cells. DuCaP cells were transfected with ERG targeting siRNA or control siRNA. Cells were irradiated after 48h at 6 Gy or with 0 Gy sham irradiation. The total number of cells was counted using NucleoCounter. Non-irradiated cells are shown on **A.** and irradiated cells on **B.** Cell number was normalised to the number of cells measured on day zero. The chart represents four independent experiments done in triplicates.

4.2.3 ERG presence promotes cell viability before, but not after, IR treatment

In order to evaluate the role of ERG on cell viability, VCaP and DuCaP cells were treated with ERG-targeting siRNA and control siRNA followed by dose-escalating radiation ranging including control 0 Gy, 2 Gy, 4 Gy, 6 Gy and 8 Gy IR, and viability was measured using MTS assay. Previously, it has been shown that prostate cells grown in vitro in 10% FCS are exposed to a T concentration that is not physiological. In fact the levels of T are more than 100 times lower than those in adult men. The use of CSS further reduces the levels of T by another 80% which results in levels that are well below those of castrated men (Sedelaar & Isaacs 2009). In this section, the viability of VCaP cells was determined in standard growth medium (Figure 4.2.3-1). VCaP cells (0 Gy) grew faster in the control siRNA treated cells compared to the ERG-specific siRNA-treated population. This was evident starting from the fourth day of culture (Figure 4.2.3 1 A.). Radiotherapy of control siRNA- and ERG-specific siRNA-treated VCaP cells with 2 Gy caused a decrease in viability that was similar in the two groups (Figure 4.2.3 1 B.). After 4 and 6 Gy IR, cells displayed increased proliferation up to day four in the two groups and then viability started to decrease from day four onwards (Figure 4.2.3 1 C. and D). After 8 Gy IR, cell viability remained close to the initial levels which suggested that there was no detectable increase in proliferation and there was no difference between the ERG-specific siRNA-treated and the control siRNA-treated VCaP cells. Overall, the exposure of VCaP cells grown in standard growth medium to various levels of IR was not associated with a proliferation advantage for cells expressing ERG, while in absence of IR, ERG overexpressing cells displayed proliferative advantage.

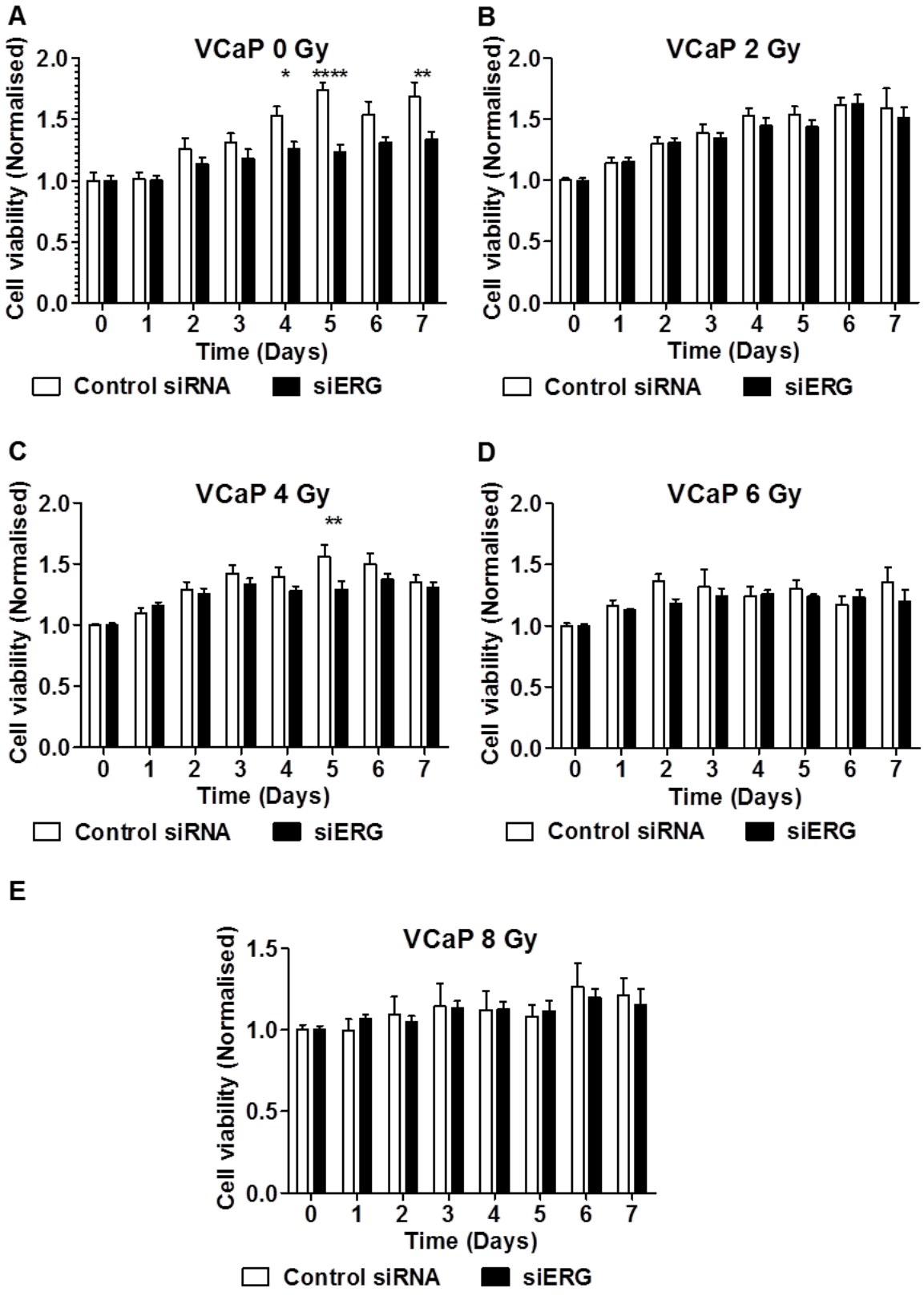


Figure 4.2.3-1 Cell viability assay of VCaP cells exposed to dose-escalating radiation. VCaP cells were grown in standard growth medium and irradiated at **A.** 0 Gy **B.** 2 Gy **C.** 4 Gy **D.** 6 Gy and **E.** 8 Gy IR. The data represents three independent experiments conducted in triplicates; *P<0.05; **P<0.01; ***P<0.001,****P<0.0001, Two-way ANOVA.

Similarly, viability of DuCaP cells was evaluated in response to radiotherapy in standard growth medium. DuCaP cells were transfected with either control siRNA or ERG-targeting siRNA for 48 hours before exposure to dose-escalating radiation. The results obtained confirmed the results obtained from the cell viability assay of VCaP cells. DuCaP cells overexpressing ERG demonstrated proliferative advantage in absence of IR from day three to day six (Figure 4.2.3-2). It is noteworthy that the doubling time of DuCaP cells was shorter than that of VCaP cells and this was reflected in a higher overall cell viability of DuCaP cells compared to VCaP cells. Nevertheless, the exposure to 2 Gy IR caused a decrease in cell viability and as shown on Figure 4.2.3-2, further escalation of the IR dose led to a higher decrease in cell viability. However, no difference was observed between ERG-specific siRNA- and control siRNA-treated DuCaP cells. This confirmed our previous observation that ERG overexpression conferred proliferation advantage only to cells that had not been exposed to radiation.

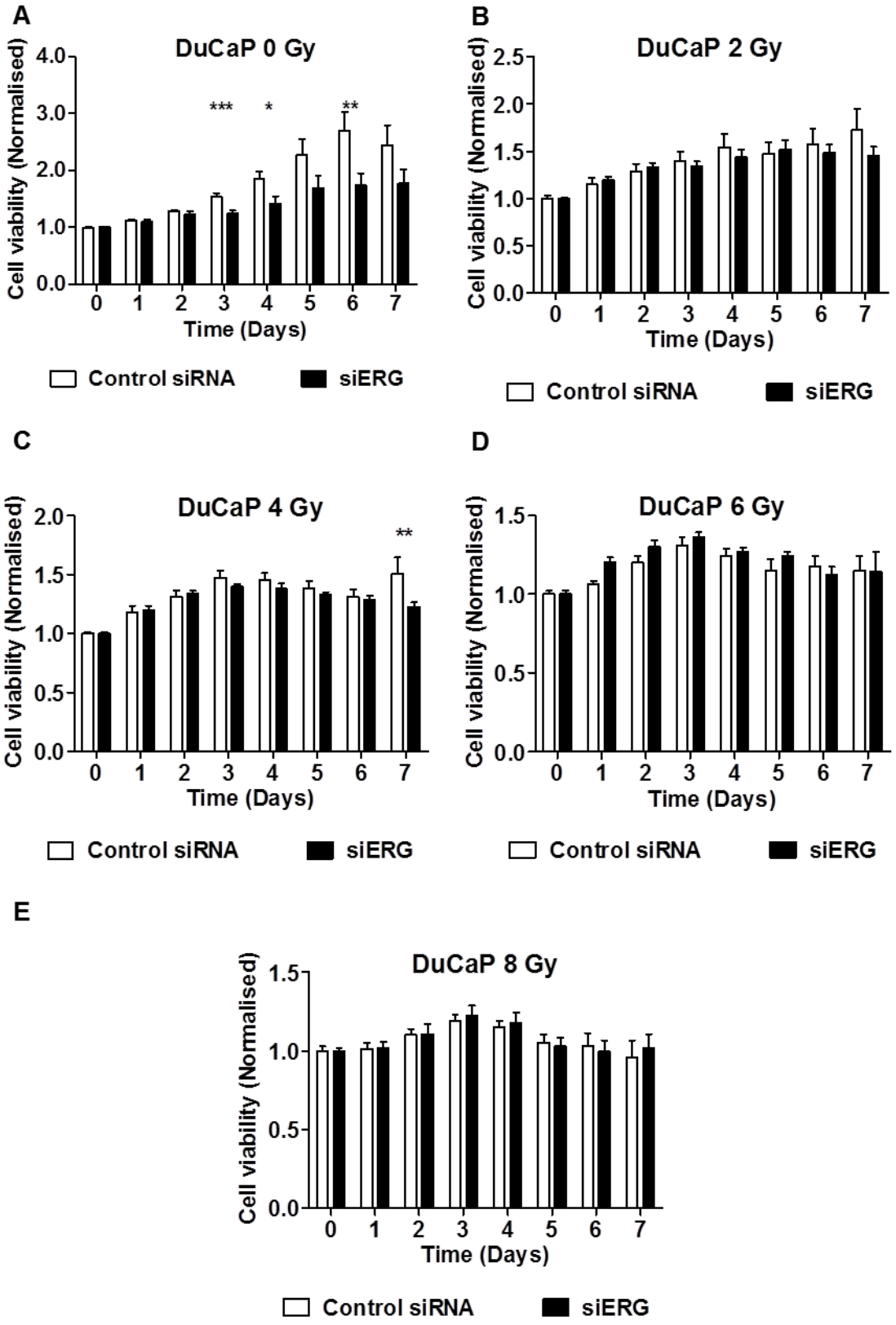


Figure 4.2.3-2 Cell viability assay of DuCaP cells exposed to dose-escalating radiation. DuCaP cells were reverse transfected and irradiated after 48 hours. The bar chart indicate normalised cell viability of cells irradiated at **A.** 0 Gy **B.** 2 Gy **C.** 4 Gy **D.** 6 Gy and **E.** 8 Gy IR. The data represents three independent experiments conducted in triplicates; *P<0.05; **P<0.01; ***P<0.001,****P<0.0001, Two-way ANOVA.

4.2.4 An androgen-deprived environment might neutralise the proliferative advantage of ERG overexpression

The AR signalling axis is a key factor in the development and progression of PCa, and tumour growth can be suppressed by ADT. In patients, chemical castration is the primary method of treatment for locally advanced or metastatic PCa tumours. Human PCa xenograft models of VCaP and DuCaP cells have been used to explore cellular and molecular changes in AR signalling underlying ADT. Both cell lines are unique models mimicking CRPC and expressing wild-type AR and *TMPRSS2-ERG* (van Bokhoven et al. 2003). Downstream targets of the AR include PSA which is also the most-studied and tested member of the androgen pathway. One of the most important and recent reports investigate the correlation between AR and its role in androgen-driven ERG expression (Tomlins et al. 2005). Furthermore, there is a significant overlap between AR and ERG binding sites. Interestingly it was reported that ERG functions as a AR repressor by binding at these sites or by directly inhibiting *AR* transcription (Yu et al. 2010).

Here the effect of different culture media on cell response to IR was compared. VCaP cells were transfected with control siRNA or ERG-targeting siRNA and after 24 hours, the medium of the cells was replaced with charcoal stripped serum (CSS) culture medium or CSS culture medium supplemented with DHT (CSS+DHT). Dose-escalating radiation ranging from control 0 Gy to 8 Gy was then used 48 hours after transfection Figure 4.2.4-1.

The effect of CSS on cell viability following 0 Gy IR led to almost unchanged levels of growth up to 15 days. The addition of DHT on the other hand resulted in increased VCaP cell viability, but this effect was strictly dependent on regular supplementation

with fresh DHT. The exposure of CSS-grown cells to 2 Gy, 4 Gy, 6 Gy or 8 Gy IR resulted in a subtle increase in cell viability towards the end of the second experimental week for the control siRNA-treated cells but not for ERG-specific siRNA-treated cells. This difference was statistically significant for the cells grown in DHT-supplemented medium. We found differences in cell viability of control siRNA treated cells and siERG treated VCaP cells exposed to 2 Gy, 4 Gy and 6 Gy IR. The data pointed towards proliferation advantage for cells overexpressing ERG when exposed to IR (Figure 4.2.4-1 and Figure 4.2.4-2)

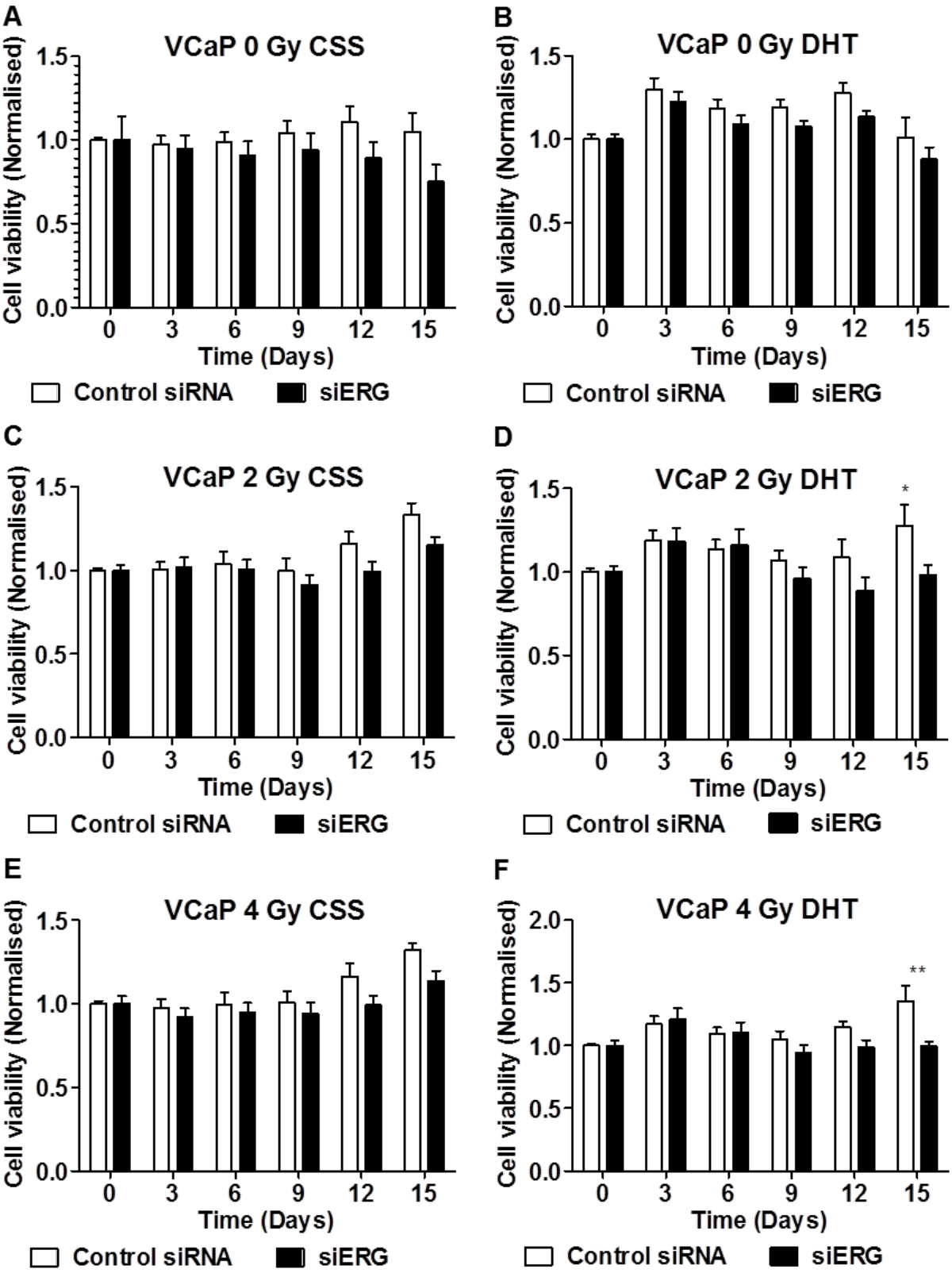


Figure 4.2.4-1 Cell viability of irradiated VCaP cells grown in androgen-free or androgen supplemented media. VCaP cells were reverse transfected with siERG or control siRNA and 48 hours later cells were subjected to dose-escalating radiation. A. Control irradiated cells grown in CSS. B. Control irradiated cells grown in 1 nM DHT supplemented medium. C. and D. show cells exposed to 2 Gy IR. E. and F. show cells exposed to 4 Gy IR.

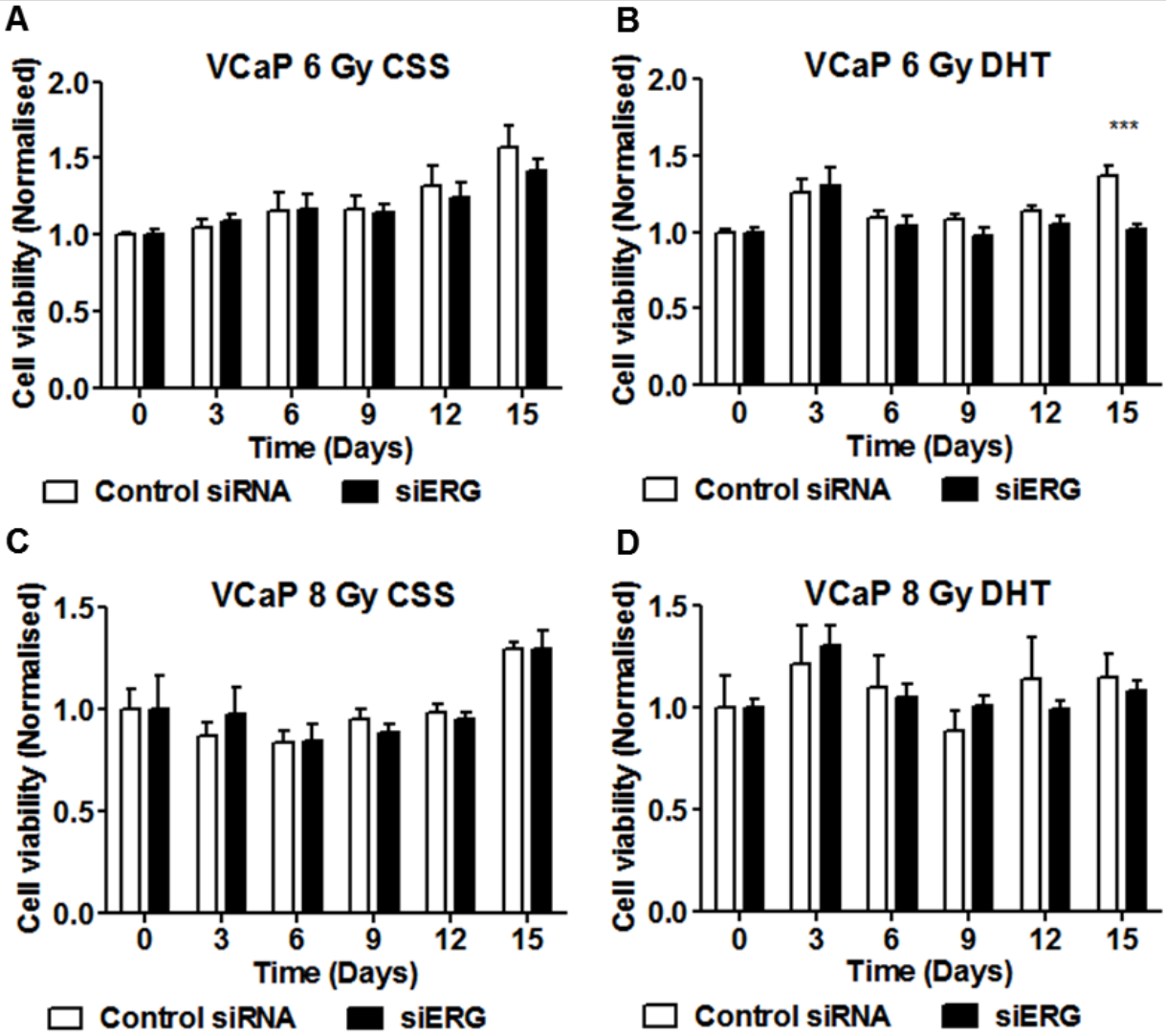


Figure 4.2.4-2 Cell viability of irradiated VCaP cells grown in androgen-free or androgen supplemented media. A. and **B.** show VCaP cells irradiated at 6 Gy and grown in CSS or DHT-supplemented medium, respectively. C. and D. show VCaP cells irradiated at 8 Gy.

4.3 Discussion

The optimal management of localised PCa is an important issue and several studies reported equivalence in outcome of surgery and radiotherapy. Currently there are no molecular markers that can help the decision making process to treat early stages of PCa, and the role of *TMPRSS2-ERG* in radioresistance remains unclear. A recent

study on intermediate-risk PCa patients reported no role for *TMPRSS2-ERG* in radioresponse (Dal Pra et al. 2013). The study investigated pre-treatment biopsies of individuals subjected to radiotherapy but the use of one biopsy per an index lesion did not reflect the multifocality and heterogeneity of PCa (Locke et al. 2012).

One *in vitro* study has reported rather conflicting results suggesting that, depending on the cell line and the fusion type, *TMPRSS2-ERG* may confer radiosensitivity or radioresistance (Swanson et al. 2011). This study however focused on PC3 and Du145 cell lines that are both androgen-independent and do not endogenously overexpress ERG. Moreover, PC3 cells are *PTEN* negative while Du145 cells are *PTEN*-positive, and loss of *PTEN* along with *TMPRSS2-ERG* expression has previously been related to increased carcinogenesis and tumour aggressiveness (Carver et al. 2009; Nagle et al. 2013; King et al. 2009).

The colony formation assay is the gold standard for evaluating the effect of IR. The assay tests the ability of a single cell to form a colony of 50 or more cells. The assay has widely been used to assess radiation sensitivity of various cells including prostate cancer cell lines. However, an *in vitro* colony formation assay with VCaP and DuCaP cells was not possible as these cells grow above 50 % confluency and not as single cells. Multiple methods to facilitate the formation of colonies were tested (data not shown) with no success. The methods included gelatine, collagen, laminin, fibronectin and poly-lysine-coated plates, 3D growth in Matrigel™ matrix (BD) or collagen, growth on a monolayer of fibroblasts or VCaP/DuCaP cells. Also the addition of conditioned medium (10 - 50%) in combination with increasing concentrations of DHT (0 to 100 nM) failed to potentiate colony formation of VCaP and DuCaP cells. Thus alternative assays were used in this work to test the effect of IR.

In this chapter further evidence is presented, which supports the hypothesis that *TMPRSS2-ERG* transcript confers proliferation advantage and new data that sheds light on the role of *TMPRSS2-ERG* in response to radiotherapy. The analysis of cell morphology showed differences in the growth patterns of ERG-knockdown and ERG-expressing cells. In order to exclude subjective assessment of morphological changes, viability and cell number were also evaluated. ERG-positive VCaP and DuCaP cells demonstrated proliferative advantage over ERG-knockdown cells. Exposure to dose-escalating radiation however, caused a decrease in cell number and cell viability regardless of *TMPRSS2-ERG* status. This effect was seen in both VCaP and DuCaP cells and suggested that radiotherapy *per se* might be sufficient to restrain cell growth independently of *TMPRSS2-ERG* status. This raises the question whether patients need to be treated differently and what the optimal disease management would be. Recently it was suggested that ERG-positive tumours mediate better response to ADT (Karnes et al. 2010). In addition, *TMPRSS2-ERG* presence has recently been proposed as a predictive biomarker for abiraterone-based ADT (Attard et al. 2009).

We undertook research to evaluate the effect of androgens and castration on response to radiation in VCaP cell line. Interestingly the growth of VCaP cells in CSS medium did not demonstrate clear proliferative advantage for cells expressing the ERG protein Figure 4.2.4-1 and Figure 4.2.4-2. Furthermore, growth in CSS medium did not cause a decrease in viability, which means that even though cells do not proliferate normally, they might be capable of keeping constant physiological activity. The treatment of CSS-grown cells with IR lead to further change in their viability, which was equally affected irrespective of ERG status. On the other hand, VCaP cells grown in DHT-supplemented medium showed a growth pattern that was similar to those of cells grown in standard culture medium. It appeared that androgens in the culture medium

might promote radio-protective effect to ERG-positive cells, especially after two weeks of exposure to androgens (Figure 4.2.4-1 and Figure 4.2.4-2). However the overall data did not support this statement. A limitation of the experiment was the need for regular replacement of medium with fresh DHT as seen from the fluctuations in cell proliferation caused by depletion and addition of DHT, which has shorter half-life then some synthetic substances used in similar studies. It is noteworthy that the overall levels of viability did not exceed 1.5 fold increase, which was lower than the viability recorded for cells grown in standard culture medium (Figure 4.2.3-1 and Figure 4.2.3-2) and this may suggest that apart from androgens there might be additional substances influencing the proliferation of VCaP cells.

Taken together this data indicates that ERG-positive tumours may indeed grow more aggressively than ERG-negative tumours. However, the *in vitro* experiments presented in this chapter suggest that androgen-deprivation alone may be sufficient to neutralise the growth advantage of ERG-positive tumours, and that androgens may play an important role in the radioresponse of ERG-positive tumours. Furthermore, VCaP and DuCaP cells grown in standard growth medium demonstrated similar proliferation levels independent of ERG status, although ERG presence showed to influence in part response to radiation. Therefore, it appears that *TMPRSS2-ERG* has no effect on radioresistance. Future work needs to investigate the effect of ERG expression on cell motility using video microscopy and cell motility assay including scratch assays or transmembrane assays.

Chapter 5. Molecular effect of TMPRSS2-ERG on the radioresponse of prostate cancer cells

5.1 Introduction

Defects in the DSB repair system can cause cell reproductive death, chromosomal rearrangements or deletions, and mutations, which in turn may result in cancer (Jeggo & Löbrich 2007; McKinnon & Caldecott 2007). IR exposure causes DSBs and induce DNA lesions that can be repaired by NHEJ, where two DNA ends are ligated without the use of a homologous template, or by homologous recombination. Due to their importance for cell survival these processes are closely monitored by DSB repair pathways including the key component γ-H2AX, which accumulates around the break site within 30 minutes of exposure to IR and facilitates efficient DNA repair. Previously H2AX deficiency was linked to radiosensitivity and genome instability (Bassing et al. 2002). Presumably γ-H2AX mediates a more efficient response to DNA damage by acting as a docking site concentrating repair proteins to nuclear foci (Bouquet et al. 2006; Petersen et al. 2001; Celeste et al. 2003). Each γ-H2AX focus comprises several hundred γ-H2AX molecules and the foci number corresponds to DSBs, which suggests that the two are parallel during the early stages of DNA repair (Rothkamm & Löbrich 2003; Sedelnikova et al. 2004; Rogakou 1999; Sedelnikova et al. 2002). With the advance of DNA repair, however, γ-H2AX foci become dephosphorylated and disappear (Olive & Banáth 2004; Ivashkevich et al. 2011). Generally the half-life of γ-H2AX is around 2-7 hours depending on the

cell line (Bouquet et al. 2006; Macphail et al. 2003). In addition the initial number of small foci decreases but the size increases as the damage repair pathway progresses (Mah et al. 2010).

The quantification of γ-H2AX using immunofluorescence is one of the most sensitive method to detect a single focus in the nucleus (Qvarnström et al. 2004). The method is based on an *in situ* antibody recognition. Using immunofluorescence microscopy it was demonstrated that despite variations in the steady state levels of γ-H2AX across different tissues, there was a significant increase in the corresponding tumour sample Figure 4.2.4-1 (Bartkova et al. 2005; Gorgoulis et al. 2005). Often a limitation of this method is

a cross-reactivity reaction with other types of tumours or in some cases with normal tissues.

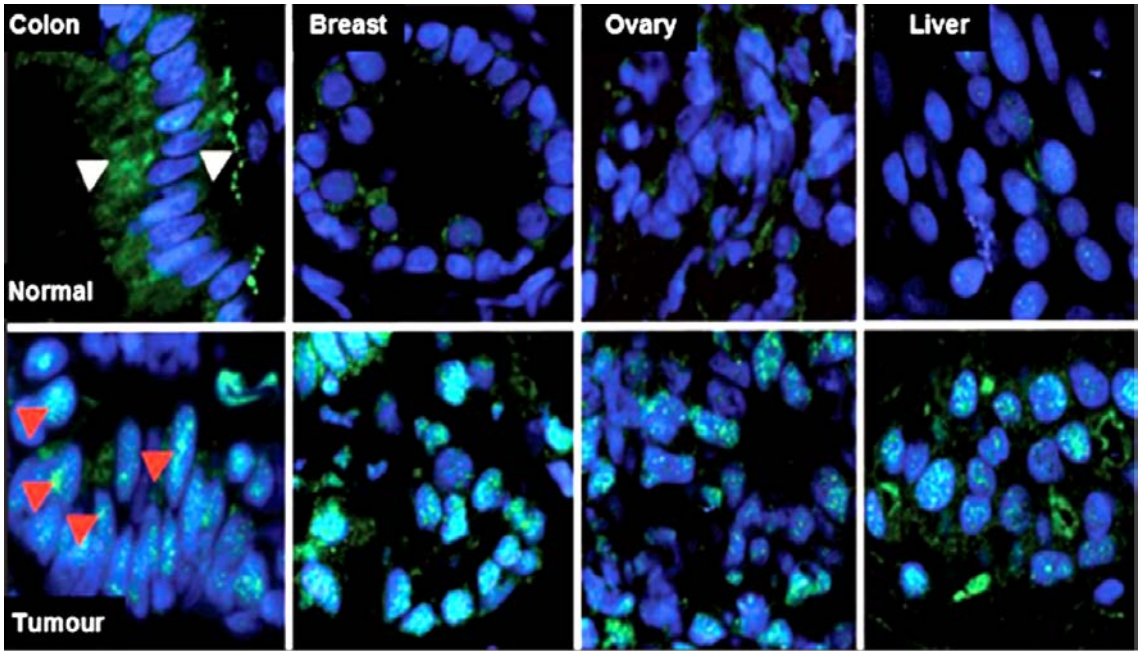


Figure 4.2.4-1 Steady-state levels of γ-H2AX in various tissues. The top row shows sections from normal human tissue samples from colon, breast, ovary and liver tissue. The bottom row shows adjacent tumour tissue corresponding to the normal sections. White arrows point to unspecific cross-reaction and red arrows point to γ-H2AX foci. Figure adapted from Sedelnikova & Bonner 2006.

In clinical practice, it would be beneficial to measure DSBs in each patient and thus determine the appropriate doses of radiation that can be administered. To date there are no clinically validated methods that can directly measure DSBs in response to different levels of IR. γ-H2AX is used in basic research to measure the levels of DSBs following radio- and chemotherapy. For PCa the number of γ-H2AX increases more than 8 times following exposure to IR. In addition the quantification of γ-H2AX in normal tissue might be used as an indication for toxicity of the treatment (Taneja et al. 2004; Downs 2007).

Additional proteins are also recruited to γ-H2AX foci including 53BP1, Mdc1, Mre11, Rad50, Nbs1, Rad17 and Brca1 (Iwabuchi et al. 2003; Jeyapalan et al. 2007; Sedelnikova et al. 2008; Stucki & Jackson 2006; Spycher et al. 2008). However the detection of DSB using 53BP1 and other surrogate markers is not canonically equivalent to γ-H2AX (Sedelnikova et al. 2008; Porcedda et al. 2006; Mailand et al. 2007). In addition the phosphorylation of H2AX is a *de novo* event while 53BP1 already exists in the nucleus before concentrating at the lesion site (Mailand et al. 2007; Xie et al. 2007; Joubert et al. 2008).

As mentioned in the chapter four, the effect of ERG on radioresponse in PCa has not been clarified. The study by Swanson and colleagues from 2011 demonstrated a contrasting role for ERG leading to radiosensitivity in PC3 cells and radioresistance in DU145 cells. In 2013 Dal Pra and colleagues on the other hand focused on samples from intermediate-risk patients and reported no correlation between *TMPRSS2-ERG* and radioresistance. Other studies suggested an indirect role for ERG in HR and NHEJ through interaction with poly(ADP-ribose) polymerase 1 (PARP1) (Brenner et al. 2011). This interaction

suggested that ERG status may be associated with radioresistance in ERG-transduced PC3 and DU145 cell models and that this effect is weaker following PARP inhibition (Han et al. 2013). Furthermore the number of γ-H2AX foci in Du145 and PC3 cells resolved quicker in the presence of ERG, which may suggest extended long-term DNA repair.

The data presented in chapter four suggested that ERG and radioresistance are not linked in *TMPRSS2-ERG* expressing cell models. However the effect of IR was measured by means of physiological assays. In this chapter, the molecular changes occurring after exposure to IR were studied in order to confirm the previous observations at the molecular level.

5.2 Results

5.2.1 Radiotherapy is not linked to different levels of proteins associated with DNA damage repair in ERG-positive and ERG-knockdown cells

DuCaP cells were exposed to IR and cell lysates collected at specific time points were used for Western Blot analysis. The expression levels of proteins related to androgen signalling and DNA damage repair were calculated using the band intensity of the blots. The levels of ERG proteins were determined in DuCaP cells treated with ERG-specific siRNA or control siRNA. On Figure 5.2.1-1 time point zero indicates the introduction of IR which was done 48 hours after transfection of the DuCaP cells with siRNA against ERG. Throughout the course of the

experiment, persistent downregulation of the ERG protein levels was recorded ranging from 80 to 70%. When the effects of IR on ERG-positive and ERG-knockdown cells were compared, surprisingly a significant downregulation of both AR and PSA was found after one hour of radiotherapy. This effect was not persistent throughout the experiment but it demonstrated the coordinated downregulation of ERG, AR and PSA in DuCaP cells 1 hour after exposure to IR. Afterwards, the levels of AR and PSA returned to normal and there were no substantial changes that were observed later. To evaluate the effect of IR, the levels of p53 and γ-H2AX were compared between ERG-positive and ERG-knockdown cells. As shown on Figure 5.2.1-1 the levels of p53 seemed to increase after 0.5, 24 and 48 hours, but the variation of the data did not allow links to be made between the changes in p53 expression and specific pattern of radioresponse. The overall levels of γ-H2AX appeared to be similar in ERG-knockdown and ERG-expressing DuCaP cells thus suggesting similar response. In addition it appeared that a difference between the ERG-positive and ERG-knockdown DuCaP cells in the expression levels of p53 and particularly γ-H2AX was already present at time point zero. This suggested that ERG knockdown alone might be associated with specific signalling pathways involved in DNA damage recognition and response. Overall, analysis of the protein levels after radiation, showed downregulation of AR and PSA after 1 hour. There was no significant difference in the levels of p53 and γ-H2AX between ERG-knockdown and ERG-expressing cells.

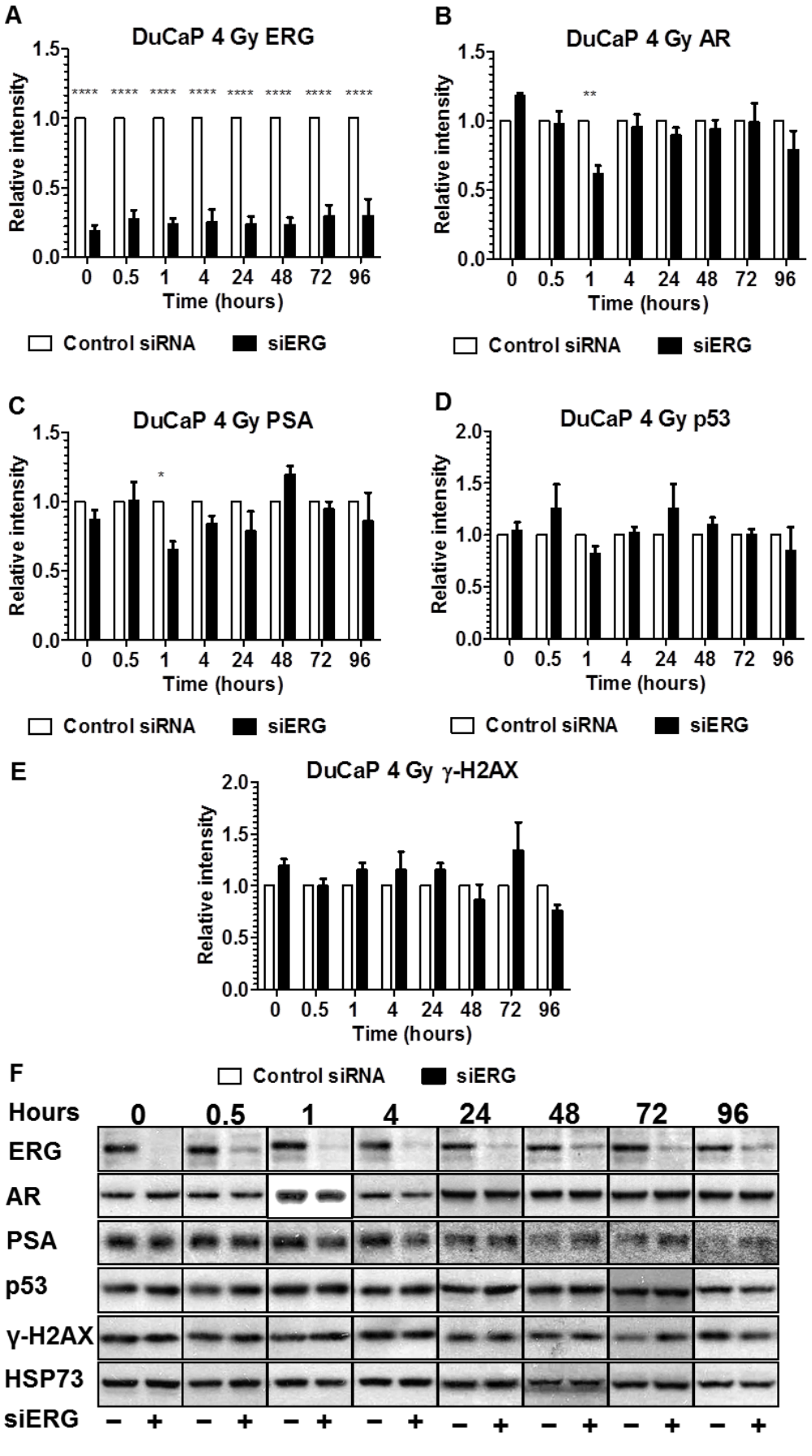

A
DuCaP 4 Gy ERG
Relative intensity
Time (hours)
Control siRNA
siERG
B
DuCaP 4 Gy AR
C
DuCaP 4 Gy PSA
D
DuCaP 4 Gy p53
E
DuCaP 4 Gy γ-H2AX
F
Hours
ERG
AR
PSA
p53
γ-H2AX
HSP73
siERG

Figure 5.2.1-1 ERG knockdown in DuCaP cells followed by radiotherapy. ERG expression was silenced in DuCaP and cells were subjected to 4 Gy IR. Cell lysates were collected at time points as indicated. For each time point, the siERG cells were normalised to the control ERG-expressing cells. Protein levels were measured using Western Blot and subsequently quantified using ImageJ software. Data represents three independent experiments.

5.2.2 The presence of TMPRSS2-ERG might be linked to changes in radioresponse of DuCaP cells

In order to investigate the radioresponse of ERG-knockdown and control DuCaP cells, a foci formation assay was performed, and a comparison of the γ-H2AX foci following radiotherapy was conducted. As shown on Figure 5.2.2-1 the number of γ-H2AX appeared to be higher in ERG-knockdown cells before radiotherapy and interestingly the steady-state levels of foci in both DuCaP populations were also higher than anticipated. One hour after 4 Gy IR the average number of γ-H2AX foci per cell doubled in number and demonstrated significantly higher presence in ERG-knockdown DuCaP cells. At this point the numbers of γ-H2AX are usually associated with the introduced DNA damage, whereas at later points lower number of foci is associated with the efficiency of the cellular DNA damage repair machinery to resolve them. Furthermore, even after 48 hours the number of γ-H2AX foci remained substantially different between the two populations of DuCaP cells, albeit the overall number decreased. This effect seemed to be persistent up to 96 hours of IR exposure and initially suggested that the overall phosphorylation of γ-H2AX in ERG-knockdown cells might be higher compared to the control siRNA cells, which may imply decrease in the efficacy of DNA damage repair. However, the difference was present at time point 0, which may indicate difference in the endogenous levels of γ-H2AX expression, but not in response to radiotherapy.

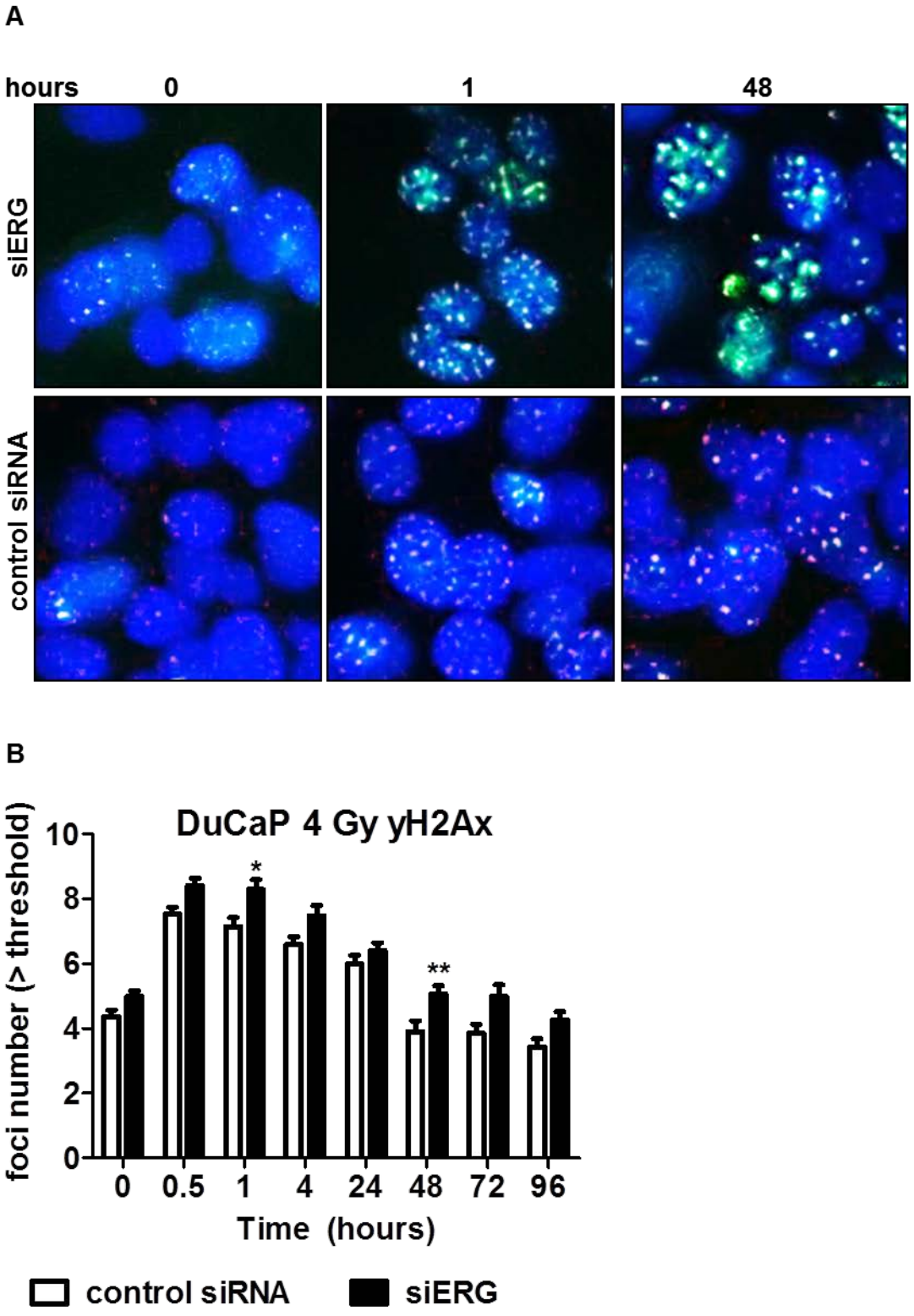


Figure 5.2.2-1 γ-H2AX foci formation in DuCaP cells following IR. DuCaP cells were transfected with ERG-targeting siRNA or control siRNA and subsequently irradiated. The formation of γ-H2AX foci was observed until 96 hours and the data was evaluated using InCell analyser software. **A.** Fluorescence microscopy images of DuCaP cells. **B.** Representation of average foci number per cell after applying filter based on cell foci brightness and cell shape.

53BP1 was subsequently used as a surrogate marker of DSBs, in order to test the effects of radiation in ERG-knockdown and in control siRNA DuCaP cells. Using the same time points as in the γ-H2AX assay experiments were undertaken to determine whether the recruitment of 53BP1 to γ-H2AX foci will reveal similar patterns of response to radiotherapy. As shown on Figure 5.2.2-2 the overall increase in 53BP1 foci doubled in number by four hours of radiotherapy. Although the number of 53BP1 foci in ERG-knockdown DuCaP cells appeared to be higher compared to the control siRNA population, there was no significant difference between the two. More specifically the number of 53BP1 foci appeared to be higher in the ERG-knockdown cells after half an hour and after 48 hours but the *P value* was not statistically significant. Furthermore, the overall appearance of 53BP1 foci was rather random across all the cells, whereby some cells showed almost undetectable 53BP1 recruitment and others showed very clear and bright 53BP1 foci. Overall, the formation of 53BP1 appeared to mimic the localisation of γ-H2AX in some cells but not in others and the overall higher levels of 53BP1 foci in ERG-knockdown cells were less prominent and were not found to be statistically significant.

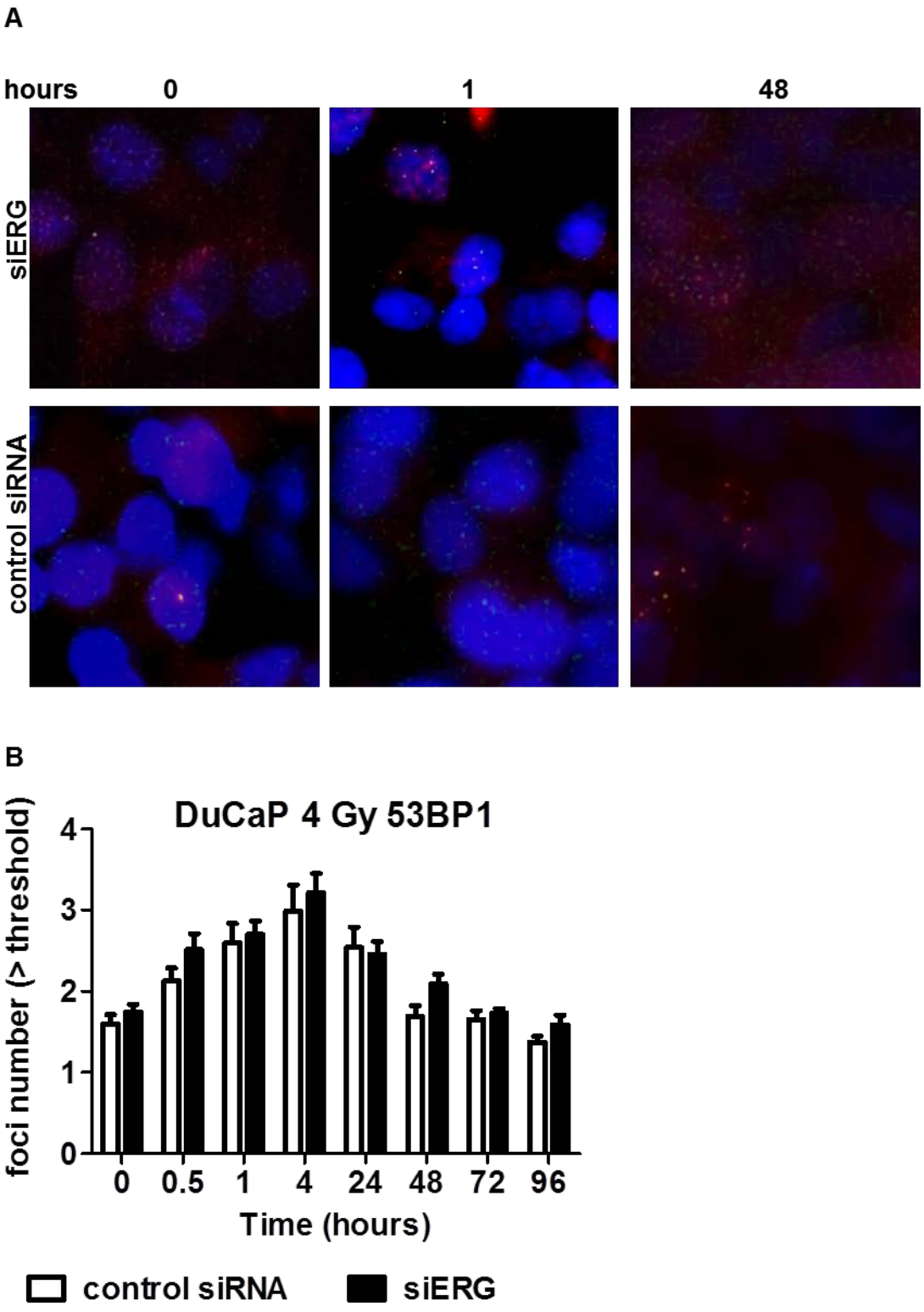


Figure 5.2.2-2 53BP1 foci formation in DuCaP cells after radiotherapy. The figure shows response of DuCaP cells to 4 Gy IR in the presence or absence of ERG. **A.** Fluorescence microscopy images. **B.** Bar chart indicating time points of cell fixation after radiotherapy and the average number of 53BP1 foci per cell nucleus.

5.3 Discussion

The aim of this chapter was to reveal the molecular processes involved in the response of ERG positive cell models to IR by comparing ERG-expressing and ERG-knockdown cells. The overall amount of p53 and γ-H2AX did not differ between ERG expressing and ERG-knockdown cells after IR.

However, the expression of PSA and AR was reduced significantly after one hour from IR in ERG-knockdown cells compared to control ERG expressing cells. The reason for this effect might be related to a potential role of AR in the regulation of key DNA damage repair proteins other than p53 and γ-H2AX. Two recent papers analysed the role of AR in mediating response to radiation independently, and found that AR facilitated the activity of DNA damage repair proteins including DNAPK, XRCC2, and XRCC3. In addition it was shown that DNAPK can sequentially facilitate the expression of AR (Goodwin et al. 2013). Another interesting point is the role of Poly (ADP-ribose) polymerase 1 (PARP1) for the activity of *TMPRSS2-ERG* in PCa. Previous studies demonstrated that PARP1 is essential for *TMPRSS2-ERG* activity and for the mediation of AR activity by binding to the sites of AR targets and recruiting more AR (Schiewer et al. 2012; Brenner et al. 2011). Thus it seems possible that radiation increased AR levels along with androgen-dependent genes in ERG expressing cells, but not in ERG-knockdown cells.

Next, the formation of γ-H2AX and 53BP1 foci was examined in DuCaP cells. Thorough evaluation of the rate at which the foci were resolved (Figure 5.2.2-1 and Figure 5.2.2-2) in ERG-knockdown and control cells, it emerged that the presence of ERG may not be related to the efficacy of response to radiotherapy.

To discriminate between mechanisms of ERG-mediated DNA damage DuCaP cells were fixed and stained for γ-H2AX and 53BP1 foci after 0, 0.5, 1, 4, 24, 48 and 96 hours exposure to IR, thus reflecting the changes occurring during the initial DNA damage recognition and later stages of DNA damage repair. Initially it appeared that γ-H2AX foci formation was higher in DuCaP cells with downregulated ERG expression. However the number of γ-H2AX foci in ERG-knockdown cells appeared to be already higher than those in ERG expressing cells before introduction of radiotherapy and remained higher up to 48 hours, which might suggest increased genomic instability in ERG-knockdown cells. In addition persistently higher levels of γ-H2AX may indicate inability of the cell to cope with the DNA lesions and possibly resulting in cellular senescence. The formation of 53BP1 foci did not show significant difference between ERG-knockdown and ERG expressing cells.

Overall knockdown of ERG resulted in decreased AR and PSA expression after 1 hour of IR suggesting a role for ERG in response to radiation. In addition higher number of γ-H2AX foci was observed in ERG knockdown cells after 1 hour and 48 hours of radiation. After taking into consideration the difference between the two cell populations at 0 hours, one could suggest no difference at all. Therefore, it appears that ERG expression has no effect on radioresistance.

Chapter 6. General discussion and future work

The *TMPRSS2-ERG* fusion gene was found to be present in about 50% of localised and CRPC cases (Scheble et al. 2010; Tu et al. 2007; Iljin et al. 2006; Rajput et al. 2007; Tomlins et al. 2005; Tomlins et al. 2009; Mosquera et al. 2009). This is the most frequent chromosomal aberration in solid tumours, which appears to be an early molecular event detected already in PIN (Klezovitch et al. 2008; Perner et al. 2007), but insufficient to initiate carcinoma (Zong et al. 2009; Carver et al. 2009). Furthermore, it is not clear yet how the presence of this fusion gene affects the response to standard PCa treatments and in particular radiotherapy. Previously *in vitro* studies have reported different roles of *TMPRSS2-ERG* in chemo- and radioresponse depending on the isoform. ERG-transfected PC3 cells showed increased radiosensitivity and decreased paclitaxel sensitivity, whilst ERG-transfected Du145 cells showed mixed effects of ERG on radioresistance and no effect on response to chemotherapy (Swanson et al. 2011). It was also reported that ERG interacts with DNA damage repair protein PARP1 and that inhibition of PARP1 reverses the effect of radioresistance mediated by ERG in transfected PC3 and Du145 cells (Han et al. 2013). Similarly earlier study involving ERG and PARP1 interaction, reported association between *TMPRSS2-ERG* overexpression and DNA damage, which was facilitated by PARP1 inhibition. The same study also reported increased steady-state levels of γ-H2AX and 53BP1 foci in VCaP cells and linked the overexpression of ERG to genomic instability (Brenner et al. 2011). Investigation at the DNA level of pre-treatment biopsies of individuals with intermediate-risk

PCa treated with radiation demonstrated no prognostic value for *TMPRSS2-ERG* (Dal Pra et al. 2013). This thesis aims to clarify the role of *TMPRSS2-ERG* in radio- and chemoresponse using cell lines derived from the different metastatic sites of the same patient and harbouring the *TMPRSS2-ERG* transcript. Given the importance of ADT in clinical practice, the effect of androgen-deprived versus androgen-supplemented medium was also tested.

6.1 Androgen-deprivation causes a decrease in ERG levels and an increase in both AR and DNA damage response proteins in TMPRSS2-ERG cell models

In this study evidence has been presented suggesting that the status of *TMPRSS2-ERG* is linked to variations in expressions of genes related not only to androgen signalling but also to DNA damage response. Across a vast number of *in vitro* models only two cell lines, VCaP and DuCaP, demonstrated distinct overexpression of ERG protein as a result of the *TMPRSS2-ERG* fusion gene. This work also confirms simultaneous expression of the *TMPRSS2-ERG* transcript and the wild-type *ERG* gene. Overexpression of the ERG protein was associated with increased levels of AR and PSA in both VCaP and DuCaP cells. DuCaP cells demonstrated lower ERG expression on protein and gene levels compared to VCaP cells, but substantially higher overexpression of AR. To better understand this phenomenon the effect of castrate androgen levels in culture medium was researched and these experiments brought about interesting results. At a gross protein expression level the use of androgen-deprived medium lacking androgens caused a substantial decrease in the levels of ERG in VCaP

and DuCaP cells. Surprisingly the levels of AR were in fact elevated and this was in contrast with the levels of PSA, a known critical downstream target gene of AR. In line with this observation previous studies have related the reactivation of AR in castrate serum androgen levels to relapse of ADT and cancer progression (Cai et al. 2009). Furthermore when the two cancer cell lines were stimulated by the addition of various concentrations of DHT, it was established that gross levels of ERG increased rapidly even at low DHT concentrations. Similarly, the levels of PSA also increased, while those of AR decreased noticeably. This inverse co-regulation of AR and PSA was in contrast with the traditional understanding that androgen deprivation leads to regression of AR-dependent tumours and that this process is accompanied by a decrease in PSA levels (Huggins 1967; Huggins & Hodges 1972). Nevertheless it was observed that androgen deprivation caused an increase in AR and decrease in PSA expression, which could be linked to the *TMPRSS2-ERG* transcript and the resultant overexpression of transcription factor ERG. Previous research suggested that PSA might transactivate AR and this could represent a new model of AR regulation under the conditions of accumulating PSA (Saxena et al. 2012; Niu et al. 2008). This could explain the decrease in AR after DHT stimulation and PSA increase but in the same study the silencing of PSA caused almost complete ablation of AR in PCa C4-2B and VCaP cells (Niu et al. 2008). The data presented in this thesis suggests that downregulation of PSA does not correlate with downregulation of AR. It appears that ERG expression or alternative proteins may be involved in the repression of AR production in the presence of androgens. In fact in 2011 Cai and colleagues proposed that AR bound with ligand affects AR activity and other genes via a negative feedback loop. In accordance with this model, the negative feedback

loop decreases AR signalling in the presence of high levels of androgens. At the same time, the negative feedback loop allows elevated AR levels and androgen production in CRPC. In addition, lower AR levels in CRPC may relieve AR suppression of different genes involved in DNA synthesis and proliferation. This in turn may lead to increased tumour growth. Different actions of AR on enhancer or suppressor sequences may cause selective alteration of the AR transcriptional repressor function and thus postpone development of CRPC (Cai et al. 2011).

Given the importance of androgen signalling axis in the context of ERG expression, the next experiments sought to explore the effect of ERG knockdown on radioresponse in presence and absence of androgens. Prior to IR, the androgen-deprived medium caused striking upregulation in the levels of p53 and γ-H2AX, two proteins linked respectively to DNA damage repair or and to DNA damage recognition. Interestingly both VCaP and DuCaP harbour different mutations in the p53 gene (van Bokhoven et al. 2003), which may indicate functional deficiency. The increase of p53 and γ-H2AX indicated that the effect of androgen deprivation *per se* was sufficient to cause a genotoxic effect in the cells and trigger DNA damage response. In addition, a gradual increase in the amount of androgens in the culture medium caused the opposite effect, specifically a rapid decrease in protein levels of p53 and γ-H2AX. The data in this work demonstrated a potential link between androgen deprivation and DNA damage response in ERG positive models. Previous studies demonstrated that ADT used in PCa treatment decreased the 7-year prevalence of PCa while also leading to an increase of aggressive tumours with Gleason Score 7-10 (Thompson et al. 2003). The work presented in this thesis indicates that ADT alone in androgen sensitive models triggers DNA damage responses and increased levels of AR in

TMPRSS2-ERG positive cells. This observation may suggest a better radioresponse in ERG positive models exposed to androgen deprivation. To analyse this effect it will be necessary to evaluate cell viability, the activity of p53 and cell cycle arrest, and the proportion of senescent cells. This may reveal a potential effect where cells accumulate mutations and give rise to aggressive populations.

6.2 Lower endogenous ERG levels might suggest a better response to chemotherapy with taxanes

One of the potential targets in the treatment of metastatic PCa is the inhibition of the dynamics of mitotic microtubules by means of taxane based chemotherapy. At the cellular level, this causes blockage on intracellular transport, which is key to tumour survival. Although taxanes are the only chemotherapy drugs found to improve overall survival, tumour cells can develop resistance.

To explore potential mechanisms of resistance to taxanes within certain tumour subtypes, the viability of cell models harbouring the *TMPRSS2-ERG* transcript was analysed. The selected cell lines, VCaP and DuCaP, both originate from the same patient but from vertebral and brain metastases respectively. The fact that VCaP cells express significantly higher levels of ERG compared to DuCaP cells makes them relevant models for studies on response to taxanes in ERG positive tumours. This could be related to the clinical practice in the cases of patients that are resistant to one taxane class but respond well to another. To help the better understanding of individual respond to a specific taxane type, two classes of taxanes, paclitaxel and docetaxel, were selected. The results that were obtained

indicated a dramatic difference in the response of VCaP and DuCaP cells to treatment with paclitaxel or docetaxel. The treatment with paclitaxel induced a stronger decrease in the cell viability of DuCaP cells compared to VCaP cells. Although both cell lines appeared to be responsive to treatment with this taxane class, it was noticeable that after 72 hours the viability of VCaP cells decreased down to 80%, while the viability of DuCaP cells decreased to 25%. Paclitaxel is known to cause apoptosis in various tumour types and low levels of the drug were previously linked to p53-induced cell cycle arrest in G1 before entering the S phase. Cells with G1 DNA content were viable for at least 10 days without further proliferation (Demidenko et al. 2008). Future work needs to determine the mechanism by which DuCaP cells were affected more severely than VCaP cells. To test the response to another taxane, the cell models were treated with docetaxel. The effect that was observed recapitulated to a certain extent the effect seen with paclitaxel. DuCaP cells appeared to be much more sensitive to chemotherapy with docetaxel than VCaP cells. In a similar fashion DuCaP cells demonstrated a lower tolerance to concentrations as little as 10 nM, whereas VCaP cells remained almost unaffected even by the highest concentration used. In fact while the viability of DuCaP cells dropped down to 50% after 48 hours and to 30% after 72 hours, the viability of VCaP cells barely decreased to around 85% after 24 hours and did not change significantly after 72 hours. In general VCaP cells tend to be more resistant to treatment with docetaxel than with paclitaxel. Likewise, DuCaP cells responded better and quicker to treatment with paclitaxel. The data in this work indicates that response to taxanes in *TMPRSS2-ERG* positive metastatic tumour subtypes may be linked to the levels of endogenous ERG. However, a limitation of this study was the short time for observation of the

cell viability, which did not account for the difference in the doubling time of VCaP and DuCaP cells. Also both cell lines are unable to form colonies thus excluding the possibility of using colony formation assay in this thesis. In the future, work needs to be carried out to address the question as to how chemotherapy with taxanes will affect cytotoxicity and cell death. Also since VCaP and DuCaP cells showed different response to taxanes it will be important to investigate whether the reason is linked to the different levels of endogenous ERG expression. In order to do this it is planned to use VCaP and DuCaP cells for apoptotic assays based on the detection of caspase-3 and caspase-9 activity. Also, it will be important to determine the cytotoxic effect of taxanes by evaluating the levels of cytotoxic biomarkers such as lactate dehydrogenase, which is released in the culture medium, or by labelling the DNA of cells with permeable membranes with a specific dye. This would allow for a more detailed understanding of the mechanisms underlying taxane-based treatment.

6.3 Combined androgen deprivation therapy and radiotherapy may be the most adequate treatment for TMPRSS2-ERG positive tumours

Previous studies have found increased disease free and overall survival for treatment of high- and intermediate-risk PCa when radiotherapy is administrated along with ADT instead of radiotherapy alone (Bolla et al. 1997; Jones et al. 2011). Consequently, the combination of ADT and radiotherapy has been the standard treatment for the past 20 years, although the mechanisms by which this effect is achieved remains unknown. The literature suggests that addition of ADT

to radiotherapy drives cell death (Wo & Zietman 2008) which might be due to reduced DNA repair (Al-Ubaidi et al. 2013) or reduced hypoxia in cancer cells (Jain et al. 1998). Recently the relationship between AR and DNA damage repair was investigated in more detail and a role for AR in PCa translocations after IR was proposed (Lin et al. 2009). In addition, the DNA damage repair protein PARP1 was found to be a key cofactor in AR function as a transcription factor (Schiewer et al. 2012). Given the advance in the development of second-generation antiandrogens for treatment of CRPC (de Bono et al. 2011), it becomes more and more important for clinicians to understand the processes by which ADT interacts with radiotherapy.

In this work, the role of *TMPRSS2-ERG* as a response modifier in radiotherapy was studied. The morphological changes accompanying the culture of a ERG-positive *in vitro* model in androgen-free medium and radiotherapy were first evaluated. The data demonstrated a well-defined difference in the growth patterns of VCaP cells grown in androgen-free versus DHT-supplemented or standard growth medium. Androgen deprivation distinctly impaired the growth of VCaP cells, while both DHT-supplemented and standard growth medium allowed regular cell growth resulting in increased cell confluency (Figure 4.2.1-1). In addition, the number of cells with irregular shape in androgen-free medium was visibly higher. The treatment with radiation did not show changes between irradiated versus non-irradiated cells and it appeared that androgen deprivation caused the strongest inhibition of cell growth in the short-term (Figure 4.2.1-1).

Subsequently experiments were focused on evaluating the effect of radiotherapy alone and sought to find a relation to *TMPRSS2-ERG* status by using ERG-positive and ERG-knockdown *in vitro* models grown in standard culture medium

with regular androgen levels. In line with previous reports, the non-irradiated ERG-positive cells appeared to become confluent faster than the ERG-knockdown cells. This difference was noticed in the early days of the experiments but became obvious by day 14. The irradiated cell on the other hand were proliferating more slowly than the non-irradiated, but no visible differences between ERG-positive and ERG-knockdown cells were observed in the early days of the experiment. Nevertheless by day 14 the number of rounded cells, likely dead cells, in the ERG-knockdown cells was noticeably higher along with lower cell confluency. This suggested that the presence of androgens may benefit ERG-positive cells following radiotherapy.

To determine the feasibility of this hypothesis the total number of VCaP and DuCaP cells exposed to IR was studied. In agreement with previous studies and the morphological observations, the total cell number of non-irradiated ERG-positive VCaP and DuCaP cells increased significantly faster than the number of ERG-knockdown VCaP and DuCaP cells. This was also reflected in the increased number of dead cells. Nevertheless, the irradiation of the cells caused decrease in the numbers of both VCaP and DuCaP irrespective of their ERG status.

To further elaborate on these results, the cell viability of ERG-positive and ERG-knockdown cells was determined. It is worth noting that in this experimental setup, the dose of irradiation was escalated from 0, 2, 4, 6 to 8 Gy. Once again the results demonstrated a proliferative advantage for non-irradiated ERG-positive VCaP and DuCaP cells compared to ERG-knockdown cell populations. Nevertheless irradiation as low as 2 Gy caused apparent decrease in cell viability and neutralised the proliferative advantage ERG-positive VCaP and DuCaP cells. Further escalation of the irradiation dose seemed to reduce the time of the dose-

dependent lag phase and intensify the reduction of cell viability throughout the whole experiment, which suggested that the IR may have caused irreparable DNA damage. Irradiated ERG-positive VCaP and DuCaP cells demonstrated a proliferative advantage compared with the knockdown cells on day 5 and 7 respectively after 4 Gy. We confirmed this data with the following experiment where instead of standard culture medium, an androgen-free medium with 10% charcoal stripped serum versus DHT-supplemented medium was used. Also the experimental duration was extended to 15 days in order to detect potential effects of radiotherapy that might occur at a later stage (Figure 4.2.4-1 and Figure 4.2.4-2). However, the data did not demonstrate strong evidence that there is a difference between ERG-positive and ERG-negative VCaP cells. Some minor differences could be identified at the end of the experiment and became more apparent with the increase of the irradiation dose. This might suggest that there could be a difference in the long-term but this statement needs further confirmation.

The study has some limitations that should be addressed in future work. The use of DHT proved to have practical difficulties as its half-life is only a couple of days, thus making it less adequate for use with VCaP cells than a synthetic androgen such as R1881. Since both VCaP and DuCaP cells are slow growing cells compared to other cancer cell lines, and even PCa cell lines, it may be necessary to further extend the duration of the experiments. Therefore the use of a synthetic androgen, such as R1881, will be more adequate in future experiments. In addition, it would be useful to conduct post-irradiation experiments that will determine cell senescence, cell cycle arrest and mitotic cell death, thus revealing

a better understanding of the underlying mechanisms. Future experiments should also include *in vivo* testing.

Chapter 7. Final remarks

This work provides evidence that the expression of AR and DNA damage proteins are related in ERG-positive cell models. However this could not be linked to specific molecular events underlying the response to IR. Despite the established difference in the number of γ-H2AX foci of ERG-positive versus ERG-knockdown cells, this could not be associated with more efficient DNA damage recognition and repair. The study of ADT and dose-escalating radiation in *in vitro* models endogenously overexpressing ERG, revealed an important partnership between the two types of treatments. The use of androgen-containing standard culture medium showed a proliferative advantage for ERG-positive over ERG-knockdown cells. The use of ADT and IR however caused decrease in cell number and viability irrespective of their ERG status. Interestingly, a weak proliferation advantage was observed in ERG-positive cells after 15 days exposure to IR and in the presence of DHT-supplemented medium. Such effect was not observed in ERG-positive cells grown in androgen-free medium following IR. Nevertheless, this observation requires further investigation in order to exclude random effect. Therefore future work needs to explore the long-term effect of irradiation on ERG-positive cells.

This work also demonstrates a potential alternative chemotherapeutic strategy to target ERG-positive tumours. The use of taxanes in *TMPRSS2-ERG* positive cells with low endogenous levels or ERG, showed very low tolerance to both taxane classes used in this study. On the other hand *TMPRSS2-ERG* harbouring cells with higher levels of ERG showed minor response to docetaxel and slightly better response to paclitaxel.

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