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ASSESSMENT OF INTER-REGULATION OF TDP-43, FUS AND THE ANDROGEN RECEPTOR IN PROSTATE CANCER CELLS

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Degree Programme in
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ABSTRACT

Tampereen ammattikorkeakoulu
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Assessment of Inter-Regulation of TDP-43, FUS and the Androgen Receptor in Prostate Cancer Cells

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Prostate cancer is common in many western countries. Advanced prostate cancer is treated with hormonal therapy to suppress the cancer-promoting androgen and androgen receptor (AR) function. Incurable castration resistant prostate cancer (CRPC) emerges after therapy. Understanding of mechanisms leading into castration resistance is needed to discover treatment methods.

The purpose of this thesis was to determine how changing the levels of the three proteins alters the levels of each individual protein. The objective was to provide a preliminary study of their inter-regulation and to assess the required experimental methods.

Changes in the regulation of two proteins, TDP-43 and FUS, in prostate cancer cells were studied in this thesis. Their inter-regulation affected by cell line AR status was determined. The genes responsible for the proteins and AR were silenced and overexpressed with transfections in two prostate cancer cell lines. Changes in protein levels were then assessed with Western Blotting.

Some evidence was found of inter-regulation linked to sample AR status. In the cells with high AR expression, FUS overexpression increased TDP-43 levels and silencing TDP-43 decreased FUS levels. No alterations occurred in cells with low AR expression. This study forms the groundwork of further experimentation that is required to confirm and further analyse these results.

Key words: prostate cancer, TDP-43, FUS, androgen receptor, CRPC

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HÖGMAN, SINI:
TDP-43:n, FUS:n ja androgeenireseptorin yhteissäätely eturauhassyöpäsoluissa

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Eturauhassyöpä on yleisin syöpä ja toiseksi yleisin syöpäkuolemien aiheuttaja monissa länsimaissa. Pitkälle edennyttä syöpää hoidetaan hormoniterapialla, joka vaimentaa eturauhassyövälle olennaista androgeenireseptorin (AR) signalointia. Onnistuneen hormoniterapian jälkeen eturauhassyöpä kuitenkin uusiutuu aggressiivisena, hormoniterapialle resistanttina muotona (kastraatioresistantti eturauhassyöpä, CRPC), johon ei tällä hetkellä ole toimivaa hoitokeinoa. CRPC:ä estävien ja hoitavien keinojen löytämiseksi ymmärrystä kastraatioresistanssiin johtavista molekyylibiologisista mekanismeista on lisättävä.

Tässä opinnäytetyössä tutkittiin tarkemmin viimeaikaisia tutkimustuloksia proteiinitason muutoksista eturauhassyövän taudinkehityksen aikana. Työ keskittyi kahden syövän etenemiselle merkittäväksi havaituksi, proteiinitasolla vastakkaisiin suuntiin säädellyn RNA;ta sitovan proteiiniin, TDP-43:n ja FUS:n. Opinnäytetyössä selvitettiin näiden proteiinien proteiinitason mahdollista yhteissäätelyä toisiinsa nähden ja AR-tasojen sekä eturauhassyöpäsolulinjojen AR-statuksen vaikutusta siihen.

Opinnäytetyön tavoite oli tuottaa esikatsaus näiden kolmen proteiinin mahdollisesta yhteissäätelystä ja testata tutkimuksessa käytettävien protokollien sekä välineiden toimivuus. Tutkimuksen tarkoitus oli määrittää, miten kolmea proteiinia tuottavien geenien hiljentäminen sekä yliekspressio vaikuttavat muiden proteiinien tasoihin eturauhassyöpäsoluissa.

Proteiineja tuottavat geenit hiljennettiin ja yliekspressoitiin siRNA- sekä plasmiditransfektioilla eturauhassyöpäsolulinjoissa LNCaP ja PC-3, joiden AR-status eroaa toisistaan. Transfektioita seuraavat muutokset kolmen proteiinin tasoissa soluissa määritettiin Western Blottingilla.

Opinnäytetyön tulokset antoivat muun muassa viitteitä mahdollisesta TDP-43:n ja FUS:n yhteissäätelystä tavalla, joka liittyy eturauhassyöpäsolujen AR-statukseen. Korkeaa AR-statusta mallintavissa LNCaP-soluissa FUS:n yliekspressoiminen johti TDP-43-tason nousuun, kun taas TDP-43:n hiljentäminen FUS-tason laskuun. Matalan AR-ekspression PC-3-soluissa ei havaittu samaa vaikutusta. Tutkimustulosten varmistaminen ja syvempi tutkiminen kuitenkin vaativat kokeiden uusimista. Opinnäytetyön tulokset luovat pohjan jatkotutkimuksille aiheeseen.

Asiasanat: eturauhassyöpä, TDP-43, FUS, androgeenireseptori, CRPC

TABLE OF CONTENTS

ABBREVIATIONS AND TERMS

2-DE	two-dimensional gel electrophoresis
Ab	antibody
ACO2	aconitase 2
ADT	androgen deprivation therapy
Ag	antigen
ALS	amyotrophic lateral sclerosis
AR	androgen receptor
ARE	androgen response element
BPH	benign prostatic hyperplasia
BSA	bovine serum albumin
CRPC	castration resistant prostate cancer
DBD	DNA binding domain
DHEA	dehydroepiandrosterone
DHT	dihydrotestosterone
dsDNA	double-stranded DNA
ECL	enhanced chemiluminescence
ELISA	enzyme-linked immunosorbent assay
ERG	erythroblast related gene
ETS	erythroblast transformation-specific related gene
FBS	fetal bovine serum
FEV	fifth Ewing variant
FTLD	frontotemporal lobar degeneration
FUS	fused in sarcoma
HGPIN	high grade prostatic intraepithelial neoplasia
HRP	horseradish peroxide
IEF	isoelectric focusing
IP	immunoprecipitation
LB	lysogeny broth
LBD	ligand binding domain
miRNA	microRNA
mRNA	messenger RNA
MudPIT	multidimensional protein identification technology

NTD	amino terminal domain
p53	tumour suppressor phosphoprotein 53
PBS	phosphate-buffered saline
PCa	prostate cancer / prostate adenocarcinoma
pre-mRNA	precursor mRNA
PSA	prostate specific antigen
PTM	post-translational modification
RBPs	RNA-binding proteins
RISC	RNA-induced silencing complex
RNAi	RNA interference
SBMA	Kennedy's disease or spinal and bulbar muscular atrophy
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SV	splice variant
TARDBP	the gene coding for TDP-43
TBS	triton-buffered saline
TDP-43	transactive response binding protein of 43 kDa
TRIM16	tripartite motif-containing protein 16

1 INTRODUCTION

Prostate cancer is the second leading cause of cancer deaths and the most common male cancer in many western countries (Siegel, Miller & Jemal 2017). Prostate cancer progression is dependent on androgens and the signalling of the androgen receptor (AR) in prostate cells. Advanced prostate cancer is treated with hormonal androgen deprivation therapy (ADT) to suppress the AR signalling. (Watson, Arora & Sawyers 2015)

However, castration-resistant prostate cancer (CRPC) emerging after ADT treatment is lethal, aggressive and at the moment incurable. Better understanding of the molecular mechanics into castration resistance is needed to find therapeutic methods capable of preventing and treating CRPC in the future. (Shen & Abate-Shen 2010)

In this thesis, carried out at the Molecular Biology of Prostate Cancer Group at the University of Tampere Faculty of Medicine and Life Sciences, and supervised by Ph.D. Leena Latonen, changes at the protein level during prostate cancer progression are studied. Two proteins showing dysregulation at the protein level of prostate cancer and CRPC, transactive response binding protein of 43 kDa (TDP-43) and fused in sarcoma (FUS) are the focus of the thesis. Their interactions with AR and possible inter-regulation are assessed.

Silencing and overexpression of the genes responsible for TDP-43, FUS and AR are carried out with transfections. Two prostate cancer cell lines expressing AR differently are used. The following changes in the levels of the three proteins are assessed with Western Blotting.

The purpose of the thesis is to determine how changing the levels of TDP-43, FUS and AR affects the levels of the other proteins in two prostate cancer cell lines that express AR differently. The objective of the study is to assess the inter-regulation of the proteins in prostate cancer progression and to test the protocols and reagents involved in the study for further research.

2 THEORETICAL BACKGROUND

2.1 Prostate Cancer

Prostate carcinoma is cancer, malignant growth resulting in tumour formation, occurring in the prostate. Prostate cancer has a strong correlation with high age, mean age of diagnosis being 66 years (American Cancer Society 2018). It causes problems in urinating, erection and ejaculation, as well as discomfort or pain, bowel problems and fatigue that lower life quality. Prostate cancer can also be symptomless and continue unnoticed for long. Ultimately in advanced cancer, painful bone metastases and death are a cause of concern for patients. (Cancer Treatment Centers of America 2016)

The normal prostate gland is a walnut sized male gland located at the base of the bladder, surrounding the urethra (Shen & Abate-Shen 2010). Its function is to produce prostatic fluid into seminal fluid and to aid in ejaculation by contracting. Prostatic fluid contains high amounts of citrate and zinc which maintain an electrochemical balance, and enzymes such as prostate specific antigen (PSA). (Costello & Franklin 2009)

The prostate has a zonal architecture, shown in figure 1 together with the prostate location. As shown in the figure, the prostate is comprised of the central zone, the fibromuscular zone or stroma, the transition zone, the peripheral zone and the periurethral zone. The outermost peripheral zone is where most prostate cancers and prostatitis originate, whereas the transition zone is the most common site of benign prostate hyperplasia (BPH). (Aaron, Franco & Hayward 2016)

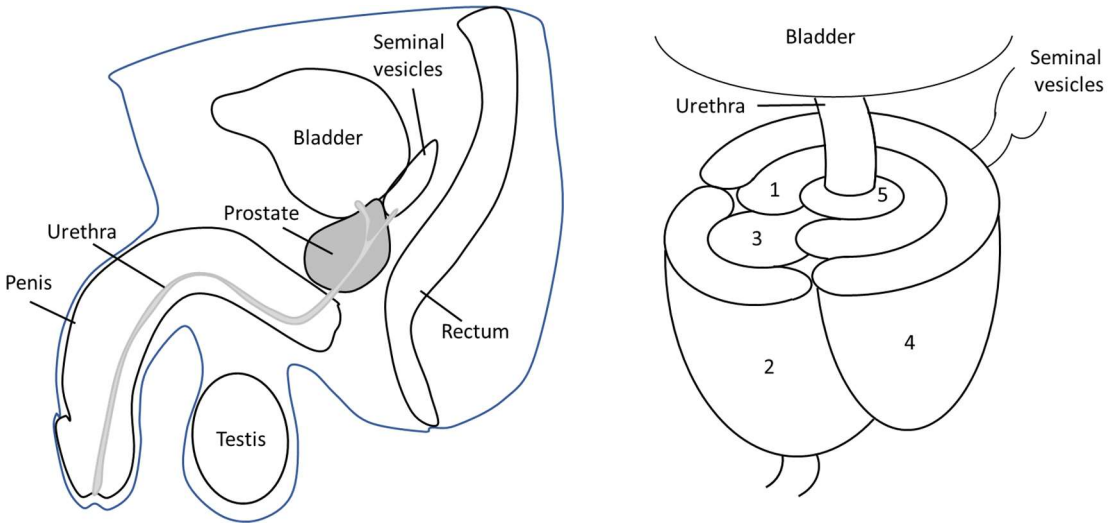


FIGURE 1. Anatomical location (left) and zones (right) of the prostate, cross-section. 1: central zone, 2: fibromuscular zone, 3: transition zone, 4: peripheral zone and 5: periurethral zone (Edited from Hoffmann M. 2008 (left) and De Marzo et al. 2007 (right))

Prostatitis and BPH are benign forms of prostate problems that cause prostate enlargement. Prostatitis, prostate inflammation, is usually caused by bacterial infection and is curable. Causes of BPH are more complex, and its symptoms can be eased but not completely cured. (Collins, Stafford, O'Leary & Barry 1999)

Prostate tissue is comprised of luminal and basal epithelial cells surrounded by stromal tissue. There is also a rare cell type, neuroendocrine cells, present in the prostate tissue in few numbers. Epithelial cells are the sheet-forming cells that form exterior surfaces and surround glands and cavities of the body. The epithelial cells of the prostate are organ-surrounding glandular cells that produce secretions. (Schrecengost & Knudsen 2013)

Prostate cancer is preceded by high-grade prostatic intraepithelial neoplasia (HGPIN) which is diagnosed by biopsy. HGPIN causes multifocal lesions with abnormal growth and differentiation of luminal cells as well as reduction of basal cells. Benign prostate hyperplasia and prostatitis in turn are not precursors of cancer. (Schrecengost & Knudsen 2013) Figure 2 illustrates how the prostatic epithelial tissue is altered along the development of cancer from HGPIN to metastatic cancer.

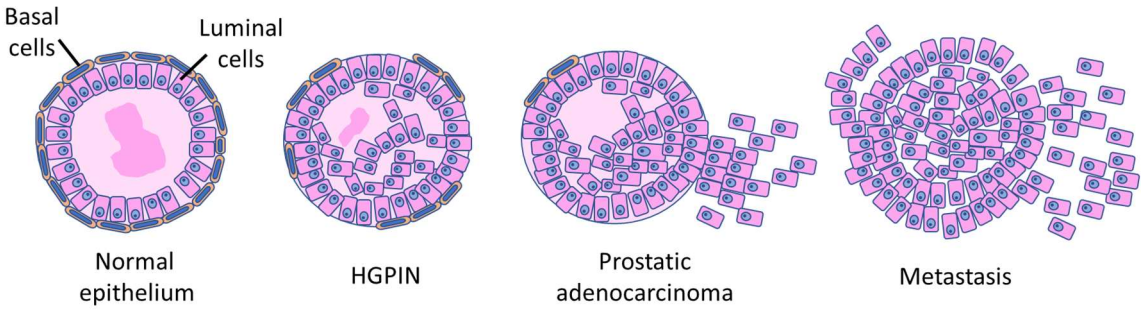


Figure 2. Changes in the prostate epithelium during the development of prostate cancer (Edited from Shen & Abate-Shen 2010)

Prostatic abnormalities lead to an increase in PSA levels in serum, which is used for diagnosis. PSA is a serine protease enzyme which liquefies seminal fluid. As a result of any prostate disruption, it is released into blood. Unfortunately, PSA testing cannot differentiate between benign and malign prostate conditions. This has lead to the problem of overtreatment and inability to direct treatment to patients most in need of it. (Pérez-Ibave, Burciaga-Flores & Elizondo-Riojas 2018)

Patients with increased PSA levels undergo biopsy, where tissue samples are gathered and histopathologically examined under the microscope. The tissue is graded according to the Gleason score method to evaluate the severity of the abnormalities. An evaluation of five cellular pattern categories is performed and the grades combined to get a score of 2 to 10, where a score of 7 to 10 indicates aggressive cancer. (Humphrey 2004)

Prostate cancers are mostly adenocarcinomas which originate in glandular epithelial cells (Hartmann & Friess 2017). Characteristics of prostate cancer include a luminal phenotype and bone metastasis. Prostate cancer is dependent upon circulating androgens such as testosterone and their action occurring through the hormonal androgen receptor (AR) in prostate cells. (Shen & Abate-Shen 2010)

Prostate cancer shows a luminal phenotype that is promoted by AR. Luminal epithelial cells, which have high AR expression, are increased and basal epithelial cells reduced in prostate cancer. This change is already seen during HGPIN formation. The abundance of luminal cells could be due to basal cells differentiating into luminal cells in cancer. (Xin 2013)

Primary prostate cancer, localised only to its primary site without metastasis, is treated with prostatectomy, chemotherapy and radiotherapy. Prostatectomy is the surgical removal of the tumour or the whole prostate. Radiotherapy aims to destroy cancer cells by high-energy radiation. (Zhen & Zhao 2013)

Advanced prostate cancer refers to cancer that is incurable by surgery or radiation, and can be metastatic or non-metastatic. Metastasis, a feature of advanced cancer, means the spread of cancer to a secondary site in the body. Prostate cancer forms metastases into bone which are bone-forming, osteoblastic, in contrast to osteolytic, bone-consuming metastases formed by many other cancers. (Logothetis & Lin 2005)

Advanced prostate cancer is treated with hormonal ADT which aims to suppress the presence of androgens in the body. ADT is achieved through either surgical or chemical castration. Chemical castration is induced with antiandrogens such as bicalutamide or gonadotropin-releasing hormone (gnRH) agonists or antagonists. In combined androgen blockade these medications are used together for more efficient AR suppression. (Watson, Arora & Sawyers 2015)

Androgen deprivation therapy however causes side effects that lower the life quality of patients (Mohile et al. 2009). Moreover, a recurring, lethal form of prostate cancer emerges in close to all cases after ADT with rising PSA levels and symptoms. In CRPC, cancer cells have acquired resistance to hormonal therapy, bypassing the suppression of androgens and AR signalling. (Hotte & Saad 2010) Although the survival estimate of patients with CRPC has significantly improved, no completely curative treatment is available. To find therapeutic methods capable of treating CRPC, the molecular mechanisms underlying progression into castration resistance must be elucidated. (Wong, Ferraldeschi, Attard & de Bono 2014)

2.1.1 Androgen Receptor

Androgen receptor (AR) is the nuclear steroid hormone receptor responsible for mediating the effect of androgens on the prostate. Formation and development of the prostate, as well as prostate cancer progression, are dependent upon it. (Lonergan & Tindall 2011) Figure 3 illustrates the structure and location of the *AR* gene located on position 11 to 12 on the longer arm of the chromosome X (q11-12). It also shows the structural parts of the AR protein: NTD, the amino terminal domain, DBD, the DNA binding domain and LBD, the ligand binding domain.

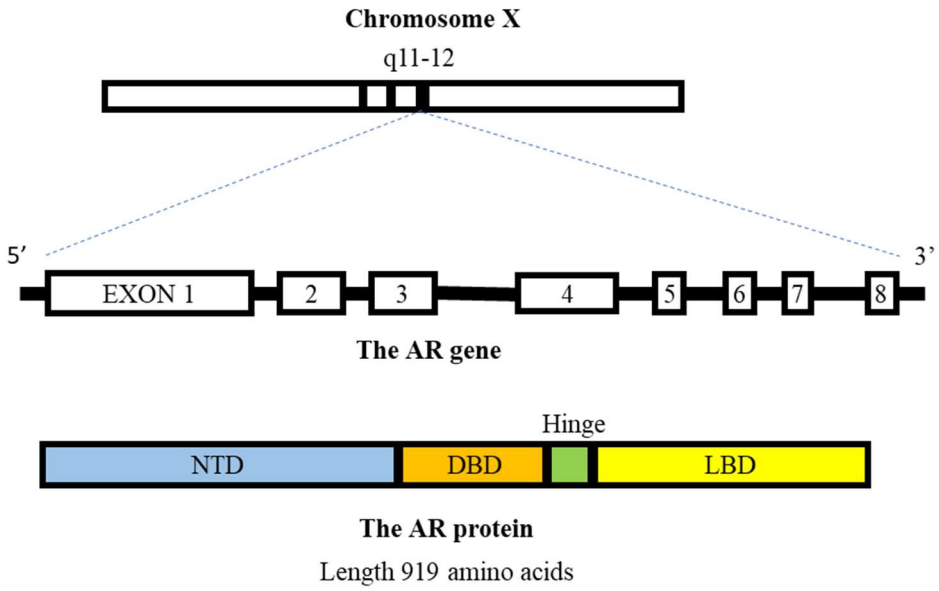


FIGURE 3. Chromosomal location (Xq11-12) and structure of the AR gene (above). Structure of the AR protein (below), where NTD is the amino terminal domain, DBD the DNA binding domain and LBD the ligand binding domain (Edited from Lonergan & Tindall 2011)

Androgens are steroid hormones produced by testes and the adrenal gland. They regulate the development of male characteristics. Testosterone is the most abundant one in the body and is metabolized into more potent dihydrotestosterone (DHT). In the prostate, DHT has a higher affinity for AR and therefore is effective at lower concentrations. (Watson et al. 2015)

The effects of androgens are mediated through the transcriptional activity of AR. Androgens bind to the AR ligand binding domain (LBD) and cause AR to bind to androgen

response elements (AREs) in DNA. AR then transcriptionally activates androgen-targeted genes such as the PSA gene. (Xu et al. 2009)

The dependence of the prostate on androgens can be seen as prostate cell apoptosis following ADT. However, androgen-dependent prostate cell lines do not undergo apoptosis after hormone depletion but only cease division and growth. This indicates that apoptosis signal requires induction that the prostatic stroma normally provides. (Shen & Abate-Shen 2010)

One of AR's functions in normal prostate tissue is to inhibit proliferation. In cancer AR instead inhibits basal cell proliferation and promotes growth of luminal cells which have high AR expression. This causes the strongly luminal phenotype of prostate cancer. AR also is the metastasis-promoting factor in prostate cancer. (Schrecengost & Knudsen 2013)

2.1.2 Reactivation of AR Signalling in CRPC

Many theories attempt to explain how prostate cancer cells are able to maintain and reactivate AR signalling in castration resistance. AR reactivation can occur through *AR* amplification and mutations, AR protein overexpression, splice variants (SVs), posttranslational modifications (PTMs) and prostatic intratumoural androgen synthesis. (Watson et al. 2015)

Intratumoural androgen synthesis refers to AR signalling sustained by trace amounts of residual androgens and other steroid hormones remaining after castration. For example, dehydroepiandrosterone (DHEA) produced by the adrenal gland is a metabolic intermediate of testosterone. DHEA is a weak androgen, meaning that it only mildly stimulates AR in high concentrations. Normally adrenal hormones are only a minor source of testosterone. About 90 per cent of all testosterone is produced by the testes. (Cai & Balk 2011)

After ADT, the weak adrenal androgens become an increasing source of testosterone that continue to maintain prostate cancer. In CRPC, DHT levels remain high or are even increased compared to normal tissue. The enzymes required for synthesis of testosterone

and DHT from weak androgens show increased expression in CRPC as well. (Montgomery et al. 2008)

The prostate stroma normally secretes paracrine growth factors, maintaining the hormonal regulation of androgen production. With intratumoural androgen synthesis, a shift to an autocrine, self-controlled, production by the cancerous epithelial cells is observed. This is coupled with overexpression of paracrine growth factors. (Marques et al. 2010)

AR amplifications or gains increase *AR* copy number resulting in AR protein overexpression. Overexpression sensitizes prostate cells to androgens. Amplification is found in around 30 per cent of CRPC cases. (Edwards et al. 2003) *AR* mutations, present in around 10 to 30 per cent of recurring cancer cases, are mostly gain-of-function point mutations produced by selection after ADT. Treatment with androgens causes selective pressure, where mutations that allow survival despite AR suppression are favoured. (Waltering, Urbanucci & Visakorpi 2012)

Mutations are observed mostly in the *AR* LBD occurring in the codons 736 to 771. LBD mutations can increase sensitivity to AR or reduce specificity of ligand binding, allowing activation by other ligands than androgens. (Eisermann et al. 2013) They are mostly point mutations which alter one nucleotide base in DNA (Hartwell et al. 2016, 208). One point mutation reducing ligand specificity present in CRPC was discovered in *AR* codon 877 in 1995 (Taplin et al.). It is also present in the AR expressing prostate cancer cell line LNCaP. This mutation, referred to as T877A, generates AR that can be activated by oestrogen, progesterone and the anti-androgen flutamide. Cells exhibiting this mutation are not inhibited but in turn activated by anti-androgens. T877A promotes survival and growth of cancer cells and resistance to apoptosis. (Sun et al. 2006)

Proteomic and transcriptional causes of castration resistance include splice variants and post-translational modifications. Post-translational modifications (PTMs) are modifications that alter proteins after their translation. PTMs of AR include methylation, ubiquitylation and phosphorylation of the protein. Splice variants (SVs) are different forms of proteins created by alternative splicing. Introns, parts of genes unused for RNA synthesis, can be removed in different ways to produce multiple RNA products from one gene. (Hartwell et al. 2016, 272, 280) Splice variants of *AR* may provide alternative transcriptional pathways for prostate cancer. They promote AR activation through a variety of

mechanisms, such as increasing transcriptional activity or binding to DNA. (Schrecengost & Knudsen 2013)

2.2 The Proteome of Prostate Cancer

Proteomics is the study of the proteome and individual proteins. The proteome refers to the combination of all proteins in a biological entity such as a cell. It consists of the final products of all active genes at a given time. Proteomic research is concerned not only in the protein content but also structure, structural modifications, activity, interactions with other proteins and molecules as well as localisation in cells. (Zhang et al. 2013)

Figure 4 illustrates the stages of how the information of DNA is activated through proteins, and the terms involved. DNA is transcribed into precursor mRNA (pre-mRNA), which is processed into messenger RNA (mRNA) by removing introns in splicing and adding protective and regulatory features. mRNA is translated into proteins which undergo among all post-translational modifications to form the final gene products. (Hartwell et al. 2016, 264–280)

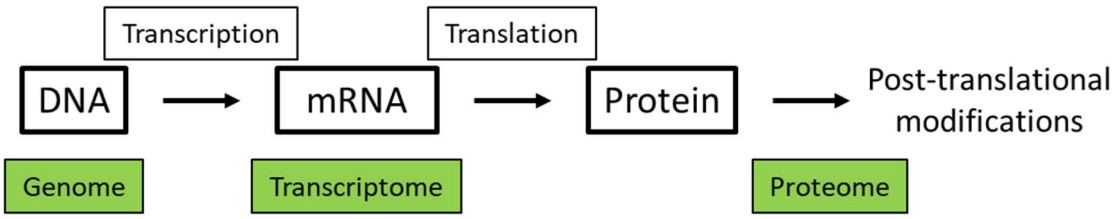


FIGURE 4. The flow of genetic information from the genome to the proteome, and the related terms. (Edited from Graves & Haystead 2002)

The messenger RNA content of cells, referred to as the transcriptome, and the genome do not always directly translate to the proteome. Therefore, assessing molecular mechanics at the genomic and transcriptomic level is not enough to elucidate the whole picture of the molecular biology of cancer and other disease. Proteins are not only regulated at the transcriptome level. Their expression does not always reflect the levels of their corresponding mRNA. This is why studies of the proteome of cancer are needed to understand the disease. (Iglesias-Gato et al. 2016)

Different products from the same gene can occur at the level of transcription, translation or modifications of synthesized mRNA and proteins. For example, alternative splicing of RNA and post-translational modifications applied after protein translation create different proteins from the same genes. Protein modifications are important features of diseases and the mechanisms underlying them. (Graves & Haystead 2002) Proteins consist of 20 amino acids which provide greater diversity than the four nucleotide bases that determine DNA sequence (Herrmann, Liotta & Petricoin III 2001).

The proteome is a complex set of biomolecules under constant change stemming from its environment. Studying the proteome involves examining the protein assembly and its components in their immediate and momentarily surroundings. Changes in the cell environment are reflected on the proteome. Proteins can be modified, re-localised or degraded in response to various stimuli. (Harper & Bennett 2016)

Development of disease such as cancer affects the protein content of tissues. This can be used to find new diagnostic methods. (Gallego & Gavin 2007) Proteins are also the primary targets of validated drugs, and contain binding sites related to disease mechanics where the drug molecules can interact (Bull & Doig 2015). Many biomarkers, indicators of biological conditions used to diagnose disease, are proteins (Herrmann et al. 2001).

Dysregulation in the expression of proteins related to growth, survival and function of normal cells promotes cancer. Dysregulation involves changes in protein expression level or misguided production of proteins. (The Human Protein Atlas n.d.) Structural alteration of proteins such as misfolding can cause protein aggregation in cells leading to various diseases. If misfolding occurs in proteins that have functions in growth and differentiation, cancer can be the result. (Chaudhuri & Paul 2006) For example, inactivation of the gene producing the tumour suppressor phosphoprotein 53 (p53) is present in more than half of human cancers (Nikolova et al. 2000).

The proteome of cells is significantly altered during prostate cancer. Genomic events such as DNA methylation, mutations and gene copy number alterations influence mRNA but not always the proteome in prostate cancer. Most proteins match levels of their corresponding mRNA in prostate cancer, but the correlation is lower in CRPC. The proteomic profiles of BPH, primary prostate cancer and CRPC are very distinctive. Comparing the proteomic profiles with each other can reveal molecular level changes occurring with

cancer initiation, progression and development into castration resistance. (Latonen et al. 2018)

Some proteins are dysregulated at the protein level of prostate cancer. Among the ones dysregulated at the protein level are TDP-43 and FUS, RNA-binding proteins (RBPs) similar in structure and function. Primary prostate cancer with low AR expression and CRPC with high AR expression show distinct TDP-43 and FUS protein expression profiles with a negative correlation with each other. The expression data suggests a significance of TDP-43 and FUS in CRPC that is linked to the AR level of the cancer cells. (Latonen 2017)

Moreover, TDP-43 and FUS have previously been linked to each other in neurodegenerative disease. In amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration (FTLD), mutated and misfolded forms of these proteins accumulate in neurons into inclusions that deteriorate nervous system function. TDP-43 and FUS proteinopathies is the nomenclature for these conditions. (Da Cruz & Cleveland 2011) The connection to neurodegeneration exists with AR in Kennedy's disease or spinal and bulbar muscular atrophy (SBMA). Death of motor neurons in the central nervous system caused by SBMA has been linked to inclusions of mutated AR. (Monks et al. 2008)

There is some evidence that some cancers are related to TDP-43 and FUS. The study of Zeng et al. (2017) shows that TDP-43 is overexpressed and regulates cancer growth as well as metastasis in melanoma, skin cancer. TDP-43 promotes the survival of glioblastoma, a type of brain cancer (Chang & Lin 2014). In neuroblastoma, a type of nerve tissue cancer, and in breast cancer, high TDP-43 expression together with a tumour suppressor, tripartite motif-containing protein 16 (TRIM16), inhibits cancer growth. (Kim et al. 2016).

According to Shing et al. (2003), *FUS* gene fusions with *erythroblast transformation-specific (ETS) related gene* (*ERG*) are related to rare cases of myeloid leukaemia, bone marrow cancer. Similarly, fusions of *FUS* and the *fifth Ewing variant* (*FEV)* gene are present in sarcomas, cancers of connective tissue (Ng et al. 2007). FUS has also been linked to regulation of breast cancer, with FUS down-regulation and interaction with other cancer suppressing factors leading to cancer cell death (Ke et al. 2016).

Based on a study of Brooke et al. (2011), FUS is down-regulated by androgens and a mediator of androgen signalling and prostate cancer progression. FUS has been found to be a co-activator of AR in prostate cancer cells, where it enhances AR transcriptional activity (Haile et al. 2011).

2.3 Methods of Protein Research

Proteomic methods are concerned with obtaining proteins from various complex biological samples and enabling their analysis. Proteomics faces the challenge of assessing the significance, function, interaction and structural variation of these diverse biomolecules. Reflecting the complicated nature of proteomic mechanics, numerous research methods exist and they are under constant development. In this chapter, a brief look into some examples of frequently applied methods will be provided.

Total protein concentration is determined with spectrophotometric techniques such as Lowry and Bradford protein assays. High-performance liquid chromatography (HPLC) and liquid chromatography-mass spectrometry (LC-MS) can detect the presence and amount of a specific protein automatedly. Antibody-based immunoassays such as enzyme-linked immunosorbent assay (ELISA), immunoprecipitation (IP) and Western Blotting are used to detect the presence, concentration as well as interactions of proteins.

The field of proteomics can be divided roughly into the six areas of expression profiling, modification research, protein-protein interactions, structure studies, functional proteomics and proteome mining. Functional proteomics refers to the study of target proteins instead of complete proteomes. Proteome mining searches for possible drug targets (Graves & Haystead 2002)

To assess which proteins are present in a sample, the proteins have to be rendered into an analysable state. Studying proteomics involves extracting the proteins of interest from samples, purification with column chromatography and separation with electrophoresis. Further analysis and choice of methods is highly varied depending on the viewpoint. (Graves & Haystead 2002)

Proteins have to be released from biological sample material, usually by homogenisation of the sample by for example sonication and centrifugation, and using buffers that lyse the cell membranes. Purification removes contaminants with column chromatography, which can be for example size-exclusion, affinity, ion-exchange or HPLC. Reverse Phase HPLC is the method of choice for uncharged molecules and often used in proteomics. (Campbell & Farrell 2012, 117)

The traditional method of protein separation is gel electrophoresis. It is based on the principle of charged molecules moving towards the oppositely charged electrode of an electric field. A charge is applied to proteins that is proportional to their molecular size. The movement of a protein in a gel placed in an electric field is relative to its size as a result. Electrophoresis is used to separate the proteins present in a sample according to their molecular sizes for further analysis. (Rabilloud, Chevallet, Luche & Lelong 2010)

In sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), proteins of interest are denatured and given a charge relative to size by SDS. In native electrophoresis, proteins are separated in their native form. In proteomic research this is used when a protein needs to be studied in its natural conformation. (Campbell & Farrell 2012, 126)

Isoelectric focusing (IEF) is electrophoresis which takes advantage of the isoelectric points (pI) of proteins. At various pH values, proteins have different charges according to their pI value. In IEF, a pH gradient is applied in the separating gel placed in the electric field. This way, proteins traveling along the gel will generate a charge, become neutral when the pH equals their pI and become immobilised in a characteristic way. (Pergande & Cologna 2017)

Two-dimensional gel electrophoresis (2-DE) combines SDS-PAGE and IEF. In 2-DE proteins are separated along first by their pI into one direction and secondly according to their size into another direction. (Adams and Gallagher 2004) The advantage of 2-DE is that post-translational modifications (PTMs) can be studied with it. Modifications to protein structure cause changes in the charge and mass of the protein. These changes affect the 2-DE migration pattern of proteins. Another advantage is use in expression profiling. The presence or absence of protein spots on 2-DE gels enables quantitative and qualitative comparison between protein samples. (Graves & Haystead 2002)

Some methods aim to determining the identity of a protein by assessing its primary structure, the amino acid sequence. Usually the first step is to hydrolyse the separated protein and to identify and quantify all the amino acids present in it. Automated amino acid analysers with HPLC are frequently used. Finally, the protein is fragmented into smaller parts to enable sequence determination with Edman degradation. The protein is degraded stepwise starting at the amino-terminal. (Weder & Belitz 2003, 4807)

Mass spectrometry (MS) techniques are used to determine amino acid sequences of proteins. MS methods provide good sensitivity, low limits of detection, high throughput and the ability to analyse complex mixtures of proteins. Protein identity of individual, proteolysed 2-DE bands is provided by mass finger printing analysis combined with peptide sequencing. (Andersen & Mann 2000)

Tandem mass spectrometry (MS/MS) techniques provide the ability to identify proteins from complex samples through fragmentation data. Proteins separated from complex sample matrices can be analysed with for example a quadrupole ion trap MS/MS. MS/MS counts the peptide mass, fragments the peptides and determines the resulting ion fragments. Identification is based on characteristic fragmentation fingerprint data, with the use of algorithms that search databases for identity. (Graves & Haystead 2002)

Modern methods applied to study the proteome usually involve shotgun (bottom-up) proteome analysis, where all amino acids in a protein mixture are determined. In contrast, in a top-down proteomics method, whole intact proteins can be studied. Middle-down analysis combines bottom-up and top-down techniques to provide a wider capability of recognizing proteins. In shotgun proteomics analysis, peptides in a protein mixture are released by proteolysis, fractionated and analysed by liquid chromatography tandem mass spectrometry (LC-MS/MS). (Zhang et. al 2013)

2.3.1 Immunological Methods

Immunological protein analytics function on the principle of antibody-antigen interaction. Antibodies or immunoglobulins are proteins produced by the immune system. Antigens are proteins that activate immune reactions. They are present for example on the surface of pathogens that cause illness. Antibodies recognise and bind to antigens by a specific site, the epitope, present on the surface of the antigen. This enables the targeting and neutralisation of pathogens. (Campbell & Farrell 2012, 411)

The antibody-antigen interaction can be utilised to recognise proteins. Specifically designed antibodies bind to the epitope of the protein of interest. The detection of proteins with antibodies is referred to as immunodetection. Western Blotting, or immunoblotting, is a method that is used to study relative levels of proteins in biological samples. It involves separation of proteins with SDS-PAGE from sample lysates. Following separation, the proteins are transferred onto a solid support membrane that allows immunodetection. (Whitford 2005, 337–339)

Immunodetection is performed with primary antibodies that bind to the protein and secondary antibodies that bind to the primary antibody to enhance signal. The secondary antibody usually is conjugated with a molecule that allows detection. The most common detection methods are fluorescent and enzymatic. Enzymatic methods, where a substrate such as horseradish peroxide (HRP) is conjugated to the secondary antibody, include colorimetric, colour-producing, and chemiluminescent, light-producing, reactions. (Whitford 2005, 337–339)

2.3.2 Protein Expression Experiments

Expression of genes at the right level at the correct developmental stage of an organism is vital for the function of the organism. Several molecular mechanisms work together to regulate genes so that they are only active when appropriate. Too much or too little expression of a gene can be detrimental to the organism. Proteins are coded for by genes through mRNA to mediate gene function, and expressed as required in response to various stimuli. Protein expression experiments are used to study how altering the levels of certain proteins affects cellular mechanisms. (Gregory 2012)

The genes responsible for the proteins are manipulated at gene level to induce expression changes. Exploiting overexpression, causing a specific gene to overproduce its gene product, and assessing the following changes, allows studying various pathways and molecular mechanisms. Overexpression can be achieved with specifically designed plasmid vectors containing an insert that expresses the gene coding for the protein. (Douglas et al. 2012) Gene silencing in turn can be achieved with RNA interference (RNAi) (Heino & Vuento 2017, 78).

Ribonucleic acid interference (RNAi) is a method to induce sequence specific gene silencing. It is a cellular defence mechanism and developmental gene regulation mechanism in eukaryotes. It is activated for example when exogenous genetic material (DNA or viral RNA) enters a cell. In gene technology, siRNA constructs are designed to bind to and degrade specific mRNA targets, prevent their function and induce gene silencing. (Wilson & Doudna 2013)

RNAi occurs through small noncoding RNAs (small ncRNAs), including microRNAs (miRNAs) and small interfering RNAs (siRNAs), 20 to 30 nucleotides long RNAs that regulate gene expression. The function of small ncRNAs is to direct the action of Argonaute family effector proteins to specific sequences in the targeted genetic material. Argonaute proteins are enzymes, mostly endonucleases, that catalyse the cleavage of molecules. (Carthew & Sontheimer 2009)

siRNAs are formed from foreign genetic material such as viral DNA, in contrast to miRNAs which originate in the host genome. In the siRNA pathway, a complex containing the RNase III enzyme Drosha processes the foreign double-stranded DNA (dsDNA) material in the nucleus into double-stranded siRNAs. The products are released into the cytoplasm, where the double-stranded siRNAs are cleaved by Dicer, an endoribonuclease enzyme. (Shabalina & Koonin 2008)

The following elimination process occurs by degradation through the RNA-induced silencing complex (RISC). The complex is formed by an Argonaute protein and one strand of the siRNA working as the recognition template in the cytoplasm. The sequence of the siRNA will convey specificity for the foreign mRNA and direct the Argonaute protein to degrade it. (Liu et al. 2004)

Transfections are used to introduce the silencing and overexpression inducing constructs into mammalian cells. Biological, chemical and physical methods can be applied to deliver the genetic material into cells. Biological methods include using viruses, chemical methods cationic polymers and physical methods electroporation. Transfections can be either transient, temporary, or stable, long-lasting, depending on the duration of the effect on gene function. (Kim & Eberwine 2010)

3 AIMS OF THE STUDY

The purpose of this thesis is to determine how changing the levels of TDP-43, FUS and AR proteins alters the levels of each other individual protein. This will improve understanding of protein level mechanics of these proteins in prostate cancer and test hypotheses formed from previous research. Determining whether the inter-regulation shows any difference with sample AR status helps reveal if these proteins are related to the formation of castration resistance.

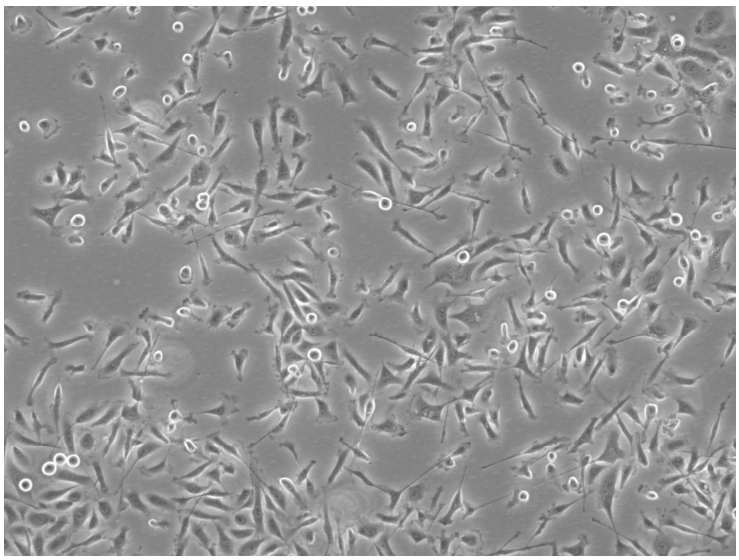
The objective is to provide a preliminary study of the inter-regulation of TDP-43 and FUS and how it is related to AR. The objective is also to test the required experimental methods, protocols and reagents, such as the siRNA constructs and overexpression plasmids. Further studies into the subject by the research group will benefit from this study and use the results to plan further experiments.

4 MATERIALS AND METHODS

4.1 Cell Culture

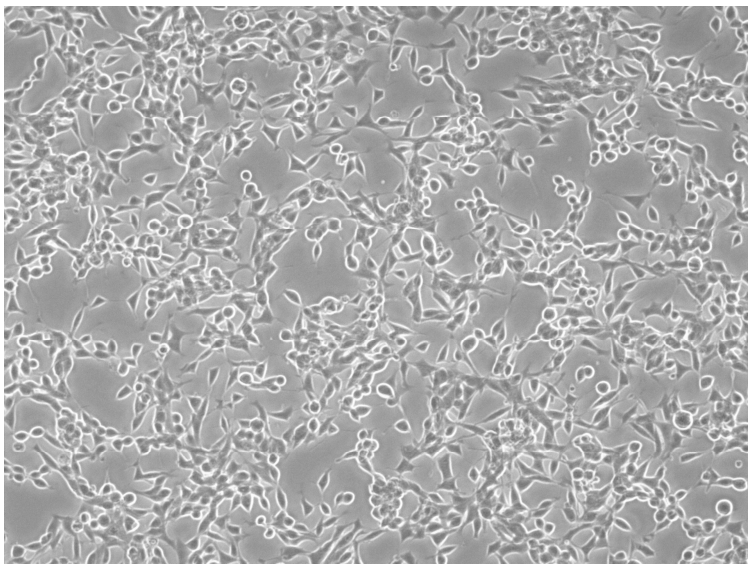
Cell lines used in this study were PC-3 and LNCaP, two lines commonly used in prostate cancer research. PC-3 cells are adherent tumorigenic epithelial cells acquired from a prostate cancer bone metastasis. PC-3 cells lack AR expression and are metastatic. (ATCC 2016)

In this study, PC-3 cells were used to model prostate cancer cells with low AR expression. PC-3 cells were cultured in Ham's F-12K medium (Lonza) supplemented with L-Glutamine, fetal bovine serum (FBS) and penicillin-streptomycin in humidified incubators at 37 ℃, 5 % CO_2. Picture 1 shows PC-3 cells cultured during the experiments.



PICTURE 1. PC-3 cells in culture

Lymph node carcinoma of the prostate (LNCaP) cells are derived from a prostate cancer metastasis of a lymph node. These cells have stable AR expression, are very androgen sensitive and have very low metastatic ability. (ATCC 2016) LNCaP cells express an AR amplification present in prostate cancer cells after ADT, the T877A mutation (Brooke et al. 2015).A cell culture of LNCaP used in the study is shown in Picture 2.



PICTURE 2. LNCaP cells in culture

LNCaP cells were used in this study to model CRPC with high AR expression. LNCaP cells were cultured in RPMI 1640 medium (Gibco) supplemented with L-Glutamine, FBS and penicillin-streptomycin in humidified incubators at 37 ℃, 5 % CO_2.

4.2 Transfections

The proteins TDP-43, FUS and AR were removed from the sample cells with siRNA induced RNAi of the genes producing the proteins, and overexpressed with overexpression plasmids. The siRNA constructs used for transfections were Thermo Fisher Scientific Silencer Select constructs that are described in Table 1. The negative siRNA control was Thermo Fisher Scientific Silencer Select Negative Control No. 1 siRNA. AR silencing was performed only to the AR expressing LNCaP cells.

TABLE 1. Thermo Fisher Scientific Silencer Select constructs used in siRNA transfections

Protein	siRNA 1	siRNA 2
AR	s1539	s1538
FUS	s5401	s5403
TDP-43	s23829	s23830

For the overexpression transfections, TDP-43 and FUS plasmids were ordered from the Addgene plasmid repository. *TAR DBP* overexpression was induced with TDP43 NO-TAG1, plasmid number 28206 gifted by Zuoshang Xu and published by Yang et al.

(2010). *FUS* overexpressions were carried out with Addgene plasmid number 21828, TLS 2: hTLS.pCDNA1 gifted by David Ron, introduced by Crozat, Aman, Mandahl & Ron (1993). AR overexpression was performed to the PC-3 cells with low AR expression. Overexpression was induced with the pcDNA 3.1 (+) plasmid (Invitrogen) with an AR insert, published by Waltering et al. (2009). The empty control was the pcDNA 3.1 (+) plasmid without an insert.

The plasmids for TDP-43 and FUS were received as agar stabs. They were transformed into *Escherichia coli* strains under antibiotic selection with ampicillin and incubated overnight with constant shaking. The plasmids were extracted with Nucleospin Plasmid Separation and a NucleoBond Xtra Midi/Maxi Extraction kits (Macherey-Nagel) according to the manufacturer's protocols. Their concentration, purity and identity were confirmed with a Nanodrop 2000 spectrophotometer unit (Thermo Fisher Scientific) and restriction enzyme digestion.

Transfections were carried out using the transfection reagents INTERFERin for silencing and JetPEI PolyPlus for overexpression (PolyPlus-Transfection SA) according to the manufacturer's protocol. Cells were seeded onto nine cell culture dishes per experiment, one for each protein to be affected, two non-treated controls and the negative controls. The number of cells seeded for transfection per dish was $1{,}5 \cdot 10^6$ for LNCaP and $8 \cdot 10^5$ for PC-3. Cell densities were determined using Luna cell counting haemocytometer slides and a Luna II automated cell counter unit (Logos Biosystems).

Transfections were performed on the same day according to the reverse transfection protocol. The siRNA constructs or plasmids were diluted into Opti-MEM (Gibco). INTERFERin or jetPEI reagent was added and the mixture incubated for 10 minutes for INTERFERin and 15 minutes for jetPEI to allow complex formation. The transfection complexes were added onto the cells. The cultures were incubated at 37 ℃ until the cells had achieved sufficient growth.

4.3 Preparation of Protein Lysates

After incubation, the cells were collected by scraping and pelleted by centrifugation. The required amount of triton lysis buffer according to pellet size (200 µl for PC-3 and 400 µl

for LNCaP) was supplemented with 10 µl per ml 25x protease inhibitor cocktail (Roche), 10 µl per ml $0{,}1\,\frac{\text{mol}}{\text{l}}$ DTT and 5 µl per ml $0{,}2\,\frac{\text{mol}}{\text{l}}$ PMSF. The lysis buffer solution was added onto the pellets and allowed 30 minutes incubation on ice. The lysates were sonicated with a Bioruptor Ultrasonicator (Diagenode) four times for 30 seconds on high power, centrifuged at 16000 g for 10 minutes and the supernatant was gathered.

Protein concentrations of the lysates were determined with a Detergent Compatible (DC) colorimetric Protein Assay kit (Bio-Rad) on a 96-well plate using five sample replicates. A bovine serum albumin (BSA) standard series with concentrations from 1 to 2 $\frac{\text{mg}}{\text{ml}}$ was used. The colorimetric reaction was measured spectrophotometrically at 690 nm using an EnVision 2105 Multimode Plate Reader unit (Perkin Elmer). Protein concentrations were determined by linear regression from a standard curve using Microsoft Excel software.

4.4 Western Blotting

Relative levels of AR, FUS, and TDP-43 following transfections were determined with Western Blotting. Aconitase 2 (ACO2) and pan-actin, house-keeping genes expressed at the same level in all cells, were used as the load controls. Molecular sizes of the proteins and the antibodies (Ab) used for their detection are shown in Table 2.

TABLE 2. Molecular sizes of the proteins (kDa) and antibodies

Protein	Size (kDa)	Primary Ab	Secondary Ab
AR	99	AR monoclonal Ab 441 (mouse) 0,3 µg/µl (SCBT)	rabbit-anti-mouse-HRP
TDP-43	44,7	TARDBP Ab ARP38942_T100 (rabbit) 1:2000 (Aviva Biosystems)	swine-anti-rabbit-HRP
FUS	53	FUS/TLS Ab 4H11 (mouse) 1:200 (SCBT)	rabbit-anti-mouse-HRP
ACO2	85	Anti-ACO2 Ab (rabbit) 1:1000 (Sigma Aldrich)	swine-anti-rabbit-HRP
pan-actin	42	pan-actin Ab (mouse) 1:1000 (Neomarkers)	rabbit-anti-mouse-HRP

Proteins were separated with sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). 20 µg of protein was loaded per lane. Color Prestained Protein Standard Broad Range ladder (New England Biolabs) was used for size standardization. The proteins were transferred to an Immobilon P PVDF membrane (Merck Millipore). After transfer, immunodetection was performed according to the incubation protocol described in Table 3. All secondary antibodies (Dako) were diluted to 1: 5000.

TABLE 3. Immunodetection incubation protocol

Step	Reagent	Incubation time
Rinse	1x TBS	5 min
Blocking	3 % BSA in 1x TBS	1 hour / overnight at 4 ℃
Primary probe	Primary Ab in 1x TBS + 1% BSA + 0.01% NaN_3	1 hour / overnight at 4 ℃
Wash 1	1x TBS	5 min
Wash 2	0.1 % Tween-20 in 1x TBS	3x 10 min
Wash 3	1x TBS	5 min
Secondary probe	Secondary Ab in 1 % BSA	30 min to 1 hour
Wash 1	1x TBS	5 min
Wash 2	0.1 % Tween-20 in 1x TBS	3x 10 min
Wash 3	1x TBS	5 min

A Clarity Western Enhanced Chemiluminescence (ECL) kit (Bio-Rad), that utilises the horseradish peroxide (HRP)-luminol system, was used for visualization with autoradiography. The membrane was imaged using Kodak BioMax Cassettes, FujiFilm Medical X-ray film 100NIF Super RX and an AGFA CP-1000 Automatic X-Ray Film Processor unit.

5 RESULTS

5.1 siRNA Experiments

The genes responsible for TDP-43, FUS and AR were silenced with siRNA transfections in two experiments. All siRNA constructs were functional, and FUS level was decreased by AR silencing in LNCaP. The results are shown in figure 5.

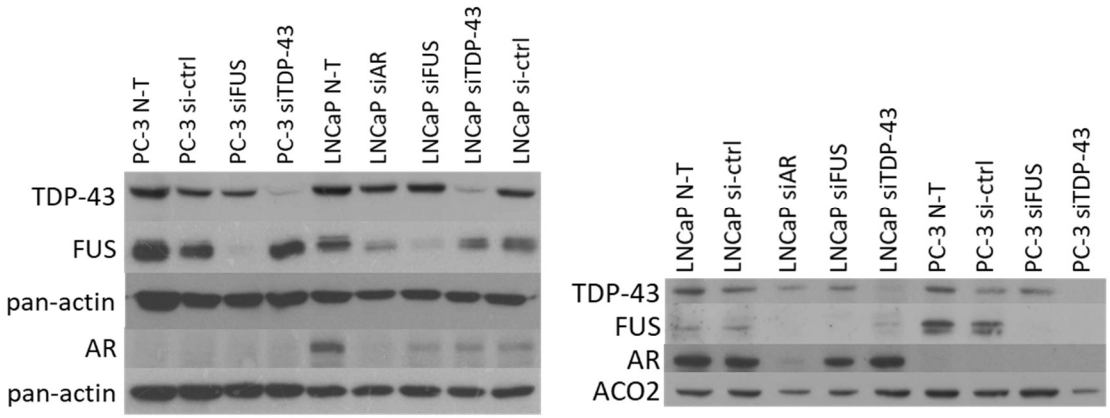


FIGURE 5. siRNA transfection results. Abbreviations: N-T: non-treated control, si-ctrl: the negative control siRNA, siAR: *AR* knockdown, siFUS: *FUS* knockdown and siTDP-43: *TARDBP* knockdown.

The observed bands were compared to the siRNA control bands to estimate changes in band strength. The targeted genes were silenced and their corresponding protein products absent from the cells as shown by missing bands. FUS and TDP-43 bands were missing or significantly reduced at their corresponding siRNA lanes. PC-3 normally exhibits no AR expression, so all PC-3 lanes showed no AR bands. The AR band was missing in the LNCaP siAR lane.

In the second experiment, shown on the right in figure 5, some technical difficulties were encountered. The LNCaP siAR lane was loaded with less protein because the concentration was very low. The PC-3 siTDP-43 well leaked and most of the loaded protein was lost. The results from these lanes are therefore unreliable. The load control results show that some protein was present. The exact amount is however unknown, so the results are not eligible. PC-3 load control bands are thicker, this can be due to uneven loading but also the high actin protein content of PC-3 cells.

5.2 Overexpression Experiments

The proteins TDP-43, FUS and AR were overexpressed with plasmid transfections in two experiments. AR and FUS overexpression plasmids were confirmed to work as intended. The function of the TDP-43 plasmid could not be confirmed. The results are shown in figure 6.

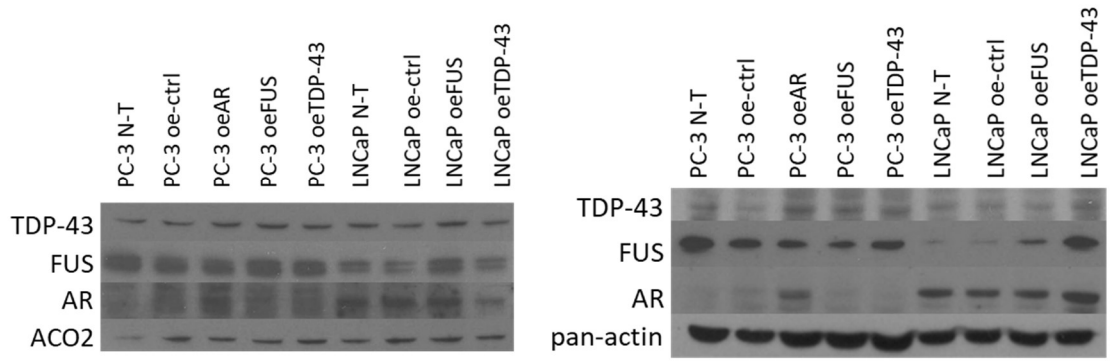


FIGURE 6. Overexpression transfection results. Abbreviations: N-T: non-treated control, oe-ctrl: the negative control plasmid, oeAR: AR overexpression, oeFUS: FUS overexpression and oeTDP-43: TDP-43 overexpression.

The AR band was missing from all PC-3 lanes as expected. A possible increase can be seen in the PC-3 oeAR lane of the first experiment. In the second experiment PC-3 oeAR clearly shows an AR band. In the first experiment oeFUS lanes showed thicker bands, but not in the second experiment. TDP-43 was not reliably increased in either experiment. The LNCaP oeTDP-43 lane shows some increase compared to other bands.

Load volumes were even except for the first PC-3 N-T control lane, which had received slightly less protein as shown by a thinner band. The PC-3 sample lanes showed thicker bands in the second experiment. The FUS control lanes of the second experiment unexpectedly showed thinner bands, which sometimes occurs in transfections. There was a lot of interference for AR in the first experiment (left in figure 6) and for TDP-43 in the second one (right in figure 6). Because of this, it is challenging to interpret the results from these lanes.

6 DISCUSSION

In this thesis, one objective was to test the protocols and reagents used in the study. The siRNA constructs and antibodies were all confirmed to work, and the protocols required for the expression experiments were functional. FUS and AR overexpression plasmids were working as intended, but the function of the TDP-43 plasmid could not be confirmed.

The main purpose of the study was to assess how changing the levels of TDP-43, FUS or AR would affect the other proteins in cell lines with high AR expression, LNCaP, and low AR expression, PC-3. The experiments showed promising preliminary biological results. In the first siRNA experiment, the band for FUS was also fainter in the LNCaP siAR lane, which suggests that knocking down *AR* might lower FUS levels.

The results showed down-regulation of FUS when *TAR DBP* is silenced in LNCaP, but not in PC-3 cells. A slight decrease in the strength of the FUS band in the LNCaP siTDP-43 lane was seen in both siRNA experiments. As for PC-3, the siTDP-43 lane FUS band showed no change in strength in the first experiment. These results suggest that silencing *TAR DBP* in the presence of AR could lead to FUS down-regulation. LNCaP cells which represented CRPC in this study have high AR expression, whereas PC-3 cells representing prostate cancer before hormonal treatment have low AR expression. A change in only LNCaP FUS levels suggests that the observed downregulation could be tied to the AR status of the samples.

In a previous study (Latonen et al. 2018), primary prostate cancer samples with low AR expression and CPRC samples with high AR expression were grouped by their protein level expression data of TDP-43 and FUS. TDP-43 and FUS expression levels had a negative correlation separating the groups. The results showing FUS downregulation by TDP-43 silencing only in AR expressing cells point to some AR related inter-regulation being present. No confirmed change was observed in TDP-43 or AR levels following the other knockdowns in PC-3. A very slight decrease in AR level and a slight increase in TDP-43 level could be seen when FUS was knocked down in LNCaP.

Down-regulation of FUS by androgens and FUS mediating androgen signalling has been reported (Brooke et al. 2011). FUS has also been shown to be a co-activator of AR in prostate cancer cells, enhancing AR transcriptional activity (Haile et al. 2011). These previous studies suggest an interaction between AR and FUS. The results of this thesis suggest some connection does exist. The connection of TDP-43 and AR has not been studied earlier. TDP-43 and FUS have been connected in neurodegeneration (Hanson et al. 2012), pointing that they might have a connection.

The overexpression experiments proved more challenging. The TDP-43 overexpressions were not successful. Overexpressing AR in PC-3 lead to a slight increase in TDP-43 level in the second experiment. FUS overexpression lead to a very slight TDP-43 increase in LNCaP in one experiment. An increase of the same weight in TDP-43 can possibly be seen in the second PC-3 oeFUS.

With alterations this small in band strength and with no confirmation by multiple repeats due to technical challenges and time limits, the results require more testing. It should be noted that many more repeats than two are required to validate these results. The results present important starting points for further experimentation.

In the last siRNA experiment, the siTDP-43 lane was near empty due to well leak, so the experiment has to be repeated for confirmation. The LNCaP siAR lane received less protein than the other lanes in the same experiment. Especially FUS downregulation resulting from AR silencing should be investigated in further experiments. There was a lot of interference in the AR and TDP-43 lanes in some of the overexpression blots and it is difficult to judge the exact weight of the bands.

Considerable time was invested in learning and practicing the methods, as Western Blotting is a time-consuming multi-step technique that can prove challenging. Cell cultures had to be established and allowed to achieve adept growth for transfections. The cells used in the second experiments were slower to grow, which caused delay. Further repeats of the experiments are required to investigate whether the results achieved in this study are reliable.

The technical difficulties were related to cell growth, protein yield and SDS-PAGE loading. Transfections cause damage to cells and can cause their growth rate to slow down.

Transfected cells should be regularly examined under the microscope to determine if they can be gathered. Enough time must be allowed for them to grow before gathering. Collected too early on, they will not yield enough cellular material and protein for Western Blotting. However, transient transfections were used, so their effects will cease with too much time, so collection timing is vital.

The observed unreliability of the TDP-43 plasmid could be due to multiple reasons. Transfection efficiency cannot be easily predicted and inexperience can easily cause failure. Success of the other plasmids suggests however that the plasmid itself could be non-functional. This can be explained by the plasmid not targeting the *TARDBP* sequence as intended, or an unexpected mechanism occurring during transcription or translation of TDP-43. The plasmid should be tested with for example DNA sequencing to further ensure its functionality.

Gene sequences can vary to some extent in cells (Latonen 2018), which could explain why the plasmid does not function as expected. The function of another TDP-43 overexpression plasmid could be tested with the same experiment. Explanations at RNA or protein level could be the formation of a protein product that was not recognised by the TDP-43 antibody. This could be due to alternative splicing and translational modifications of TDP-43. Cells used in the second experiments were from another origin. As cell cultures can withstand only a certain number of passages, new cells had to be provided after initial testing and the first experiments. The change of cells should also be considered when judging the results, as cells of different origin can have slightly different qualities.

Overexpression transfection experiments can be hard to accomplish, so the reason could simply be a failed transfection. Poor cell growth was seen in some cultures after transfection, and allowing for a day or two more growth time for the cells might have improved the results. Allowing cell growth permits the effects of transfections to take place, and the accumulation of cellular material and protein needed for Western Blotting. Transfection efficiency also varies unexpectedly with experiment and cell culture.

Western Blotting and autoradiography can also cause challenge in interpreting the results. Antibodies and luminol reagents used in autoradiography lose their effectiveness over time, causing poor signal. Using fresh reagents or strengthening existing ones is important for success. Bacterial contamination of antibodies, membranes and blocking reagents can

cause interference that makes blots difficult to read. All reagents need to be stored in low temperature and new ones prepared as required. Adjusting exposure times in autoradiography can be challenging if some bands show strong and some poor signal. Appropriate washing is key in Western Blotting to prevent background interference from unnecessary bands.

Interpreting the results requires experience with Western Blotting. It can be challenging to reliably determine if a slight change in band strength is present, especially from consequent scans of the x-rays. The bands need to be compared to the control lanes and the load control bands. Based on all this it needs to be judged if band strength variation is caused by uneven loading, exposure time or actual protein level changes. Very small variations are challenging to judge in an objective way.

The possible biological results of the experiments create guidelines for what to look for in the next experiments, where all tests should be repeated. Especially the partially failed LNCaP siAR and PC-3 siTDP-43 experiments need further assessment. Particular interest should be invested in further testing if knocking down *AR* lowers FUS levels, and if the down-regulation of FUS occurs when *TAR DBP* is silenced in LNCaP. RNA levels should also be determined to investigate the results as well as the failures. For example, assessing if silencing or overexpression has occurred on the RNA level when it has failed on the protein level could help understand where the problem lies.

Silencing AR in LNCaP caused FUS to decrease. In turn, AR overexpression in PC-3 caused TDP-43 to increase. These results oppose each other regarding AR and, if they can be confirmed, provide a hypothesis to continue from. They suggest that inter-regulation into opposite directions could exist and that it could be related to AR status and changing it. This is in line with the findings that TDP-43 and FUS have a negative correlation at the protein level (Latonen 2017). Also, TDP-43 decreasing with siFUS in only LNCaP points at AR having a significance in the inter-regulation of these proteins.

AR status of prostate cancer is altered when castration resistance develops. CRPC cells have high AR expression and can possibly synthesise AR independently. (Lonergan & Tindell 2012) By determining changes in protein level of certain proteins, such as TDP-43 and FUS, how they interact and how they are affected by AR, mechanisms of CRPC

could be elucidated. If AR was found to be reduced by affecting the proteins, new drug action targets to suppress AR might be developed.

This thesis is a preliminary look into the inter-regulation of TDP-43, FUS and AR in prostate cancer cells, and helps continue research into the subject. With further assessment, the inter-regulation of TDP-43 and FUS in relation to AR in prostate cancer, as well as the significance of this interaction to cancer development, can be better understood.

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