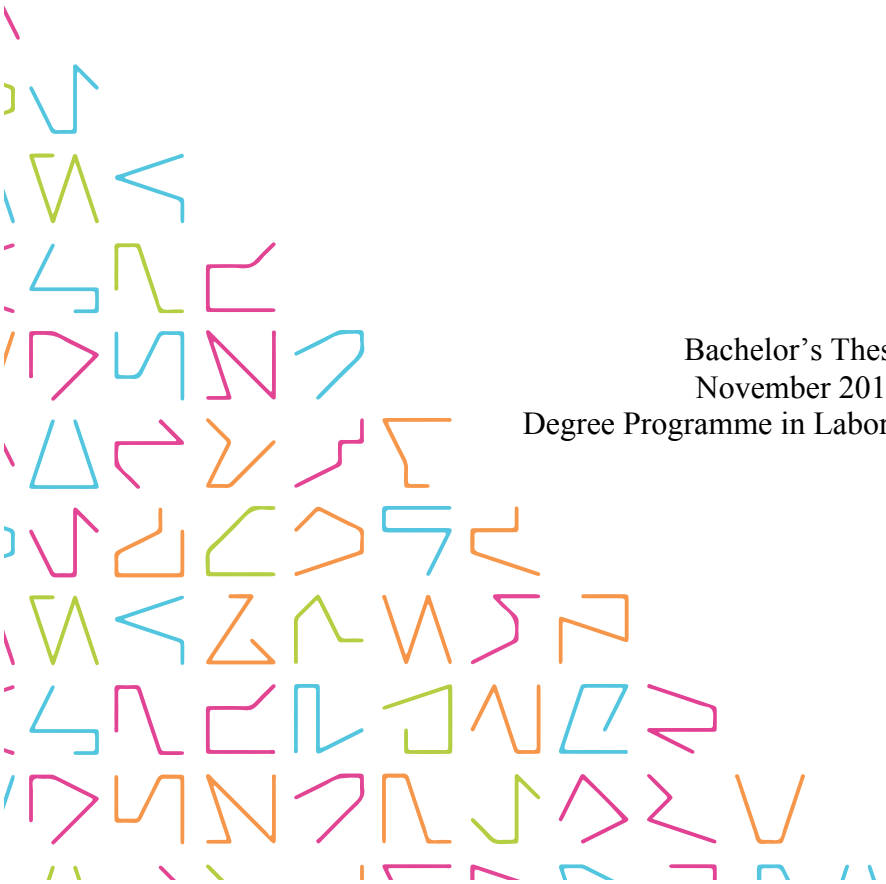
TAMPEREEN
AMMATTIKORKEAKOULU

ASSESSING GOBLET CELL METAPLASIA AND EXPRESSION OF PEPTIDASE INHIBITOR 15 IN MOUSE PROSTATE TISSUE

Sonja Koivukoski



Bachelor's Thesis
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ABSTRACT

Tampereen ammattikorkeakoulu
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KOIVUKOSKI, SONJA:
Assessing Goblet Cell Metaplasia and Expression of Peptidase Inhibitor 15 in Mouse Prostate Tissue

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Prostate cancer is the second most common cancer and leading cause of cancer death in men. It is caused by malignant growth of the prostate gland. The largest risk factor is age, but it has one of the lowest fatality rates among cancers and most patients die of other causes. Prostate cancer is usually diagnosed together with high prostate specific antigen level. It can be treated with radio- or hormonal therapy depending how far the cancer has progressed. However, sometimes the cancer progresses to a stage where it is resistant to the treatment and will eventually lead to death.

Histochemistry is the study of chemical components in tissues. In this thesis, the objective was to use three types of stainings to study goblet cell metaplasia and the expression of peptidase inhibitor 15 (PI15) in mouse prostate tissue. The purpose was to assess goblet cell metaplasia in aged and miR-32 transgenic mouse prostate tissue and to establish a protocol for staining of PI15. Haematoxylin-eosin staining was used to find areas of interest, which were then further stained with periodic acid-Schiff (PAS) staining to detect goblet cells by the mucin they secrete. For staining of peptidase inhibitor 15, an immunohistochemical protocol was established. PI15 is a gene in a family of secretory proteins expressed in some glandular structures, such as the prostate.

In the tissues studied here, goblet cell metaplasia was found by the mucin inside the goblet cells and some by the mucin they secrete to the lumen inside the gland. The metaplasia could also be seen in the epithelial cells, which were taller than average. The metaplasia was located in the ventral and dorsal lobes of the tissue, close to the urethra, and often in several glands rather than just one, which indicated that the metaplasia had spread. No difference was found in the metaplasia between transgenic and wild type mouse prostate tissues. To further study the metaplasia, more tissue samples should be examined to see where exactly it is located, as well as to study the metaplasia in human prostate tissue.

PI15 was expressed in the tissues and no large difference was found between the two fixatives tested. PAXgene fixation did show slightly better results in the whole tissue samples, which could be caused by the cross-linking formalin fixation causes. In addition, two different buffers were tested to optimise antigen retrieval. The buffers showed a larger difference and PI15 was better visible in the pH 9 buffer. In the future, also transgenic mouse prostate and human prostate should be tested with staining of PI15 to see whether PI15 has a role in prostate cancer development.

Key words: prostate cancer, immunohistochemistry, peptidase inhibitor 15, PI15, goblet cell

TIIVISTELMÄ

Tampereen ammattikorkeakoulu
Laboratorioalan koulutus

KOIVUKOSKI, SONJA:
Pikarisolujen metaplasian ja peptidaasi-inhibiittori 15:n ekspression arvioiminen hiiren eturauhaskudoksessa

Opinnäytetyö 45 sivua, joista liitteitä 2 sivua
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Eturauhassyöpä on miesten toiseksi yleisin syöpä ja aiheuttaa eniten syöpäkuolemia miehillä. Syöpä johtuu eturauhasen pahanlaatuisesta kasvusta ja vanhemmilla miehillä on iän takia suurempi riski sairastua eturauhassyöpään. Eturauhassyövällä on kuitenkin yksi alhaisimmista kuolleisuuksista ja suurin osa sairastuneista kuolee muiden syiden takia. Eturauhassyöpä diagnosoidaan usein prostataspesifisen antigeenin korkealla arvolla. Sitä voidaan hoitaa säde- tai hormonihoidolla riippuen siitä, kuinka pitkälle syöpä on edennyt. Osa syövistä etenee kuitenkin vaiheeseen, jossa syöpä jatkaa leviämistä hoidoista huolimatta johtaen lopulla kuolemaan.

Histokemia on kudosten kemiallisten komponenttien oppi. Opinnäytetyön tavoitteena oli kolmea värjäystekniikkaa apuna käyttäen tutkia pikarisolujen metaplasiaa ja peptidaasi-inhibiittori 15 (PI15) ekspressiota hiiren eturauhaskudoksessa. Tarkoituksena oli arvioida pikarisolujen metaplasiaa vanhoissa ja miR-32-transgeenisissä hiiren eturauhaskudoksissa ja kehittää värjäysprotokolla PI15:lle. Hematoksyliini-eosiini-värjäystä käytettiin kiinnostavien kudosten löytämiseen, joita sitten värjättiin perjodihappo-Schiff-värjäyksellä pikarisolujen löytämiseksi niiden erittämän musiinin avulla. Peptidaasi-inhibiittori 15:n värjäykseen kehitettiin immunohistokemiallinen värjäysmenetelmä. PI15 on geeni sekretoristen proteiinien perheessä, joka ekspressoituu rauhasrakenteissa, esimerkiksi eturauhasessa.

Tutkituissa kudoksissa pikarisolumetaplasia löydettiin pikarisolujen erittämän musiinin avulla, joko itse solujen sisältä tai rauhasonteloista. Metaplasia voitiin myös havaita epiteelisoluissa, jotka olivat korkeampia kuin normaalisti. Metaplasia sijaitsi kudoksen ventraali- ja dorsaalilohkoissa, virtsaputken lähellä, ja usein useammassa kuin yhdessä rauhasessa. Tämä osoitti metaplasian levinneen. Transgeenisten ja villityyppien välillä metaplasiassa ei huomattu eroavaisuutta. Jatkossa metaplasiaa voitaisiin tutkia useammissa kudosnäytteissä, jotta voitaisiin selvittää, missä se tarkalleen sijaitsee, sekä tutkia metaplasiaa ihmisen eturauhaskudoksessa.

PI15 oli ekspressoitu kudoksissa ja suurta eroavaisuutta ei löydetty kahden testatun fiksatiivin välillä. PAXgene-fiksaatiolla saatiin hieman parempia tuloksia kokokudosnäytteissä, mikä saattaa johtua formaliinin aiheuttamasta silloittamisesta. Lisäksi testattiin kahta eri puskuria antigeenin paljastuksen optimointiin. Puskureissa näkyi suurempi eroavaisuus: PI15 näkyi paremmin pH 9-puskuria käytettäessä. Tulevaisuudessa myös transgeenisten hiirten sekä ihmisten eturauhaskudoksia tulisi testata PI15-värjäyksellä, jotta voitaisiin selvittää, onko PI15:llä roolia eturauhassyövän kehityksessä.

Asiasanat: eturauhassyöpä, immunohistokemia, PI15, peptidaasi inhibiittori 15, pikarisolu

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ABBREVIATIONS

ADT	Androgen deprivation therapy
AR	Androgen receptor
BPH	Benign prostatic hyperplasia
CRISP	Cysteine-rich secretory proteins
CRPC	Castration resistant prostate cancer
DAB	3,3α-diaminobenzidine tetrahydrochloride
FFPE	Formalin fixation and paraffin embedment
HRP	Horseradish peroxidase
H&E	Haematoxylin eosin
IgG	Immunoglobulin G
IHC	Immunohistochemistry
NBF	Neutral buffered formalin
PAS	Periodic acid-Schiff
PCLC	Paneth cell-like change
PFPE	PAXgene fixation and paraffin embedment
PIN	Prostatic intraepithelial neoplasia
PI15	Peptidase inhibitor 15
PSA	Prostate-specific antigen
TNM	Tumour, lymph node and metastasis category based prostate cancer classification system

1 INTRODUCTION

Prostate cancer is malignant growth of the prostate gland. It is the most common cancer in men after skin cancer and the second leading cause of death from cancer in men. The largest risk factor is age as the occurrence rises radically after the age of 55. One in ten men will get prostate cancer at some point in their lives. The PSA test has however made it possible to detect prostate cancer early. (Kellokumpu-Lehtinen, Joensuu & Tammela 2013, 562–563.)

The objective of this thesis is to study goblet cell metaplasia in mouse prostate tissue, as well as to establish a protocol for one possible biomarker. The purpose is to examine goblet cell metaplasia with the help of periodic acid-Schiff staining in aged and transgenic mouse prostate tissue. Periodic acid-Schiff staining detects mucin, which is normally not secreted by the prostate. The second purpose is to establish a protocol for detecting peptidase inhibitor 15 by immunohistochemistry in mouse prostate tissue.

This study is carried out for the Molecular Biology of Prostate Cancer group in Prostate Cancer Research Center in the Institute of Biosciences and Medical Technology (University of Tampere) for postdoctoral fellow Leena Latonen. Professor Tapio Visakorpi leads the group. There are seven different research groups in Prostate Cancer Research Center all with a common goal, which is to find answers regarding the etiology of the disease, the identification of clinically relevant diseases, personalised cancer therapy, and new treatments for the disease (University of Tampere 2015).

2 THEORETICAL BACKGROUND

2.1 Prostate cancer

2.1.1 Prostate

The prostate is a walnut-sized accessory sex gland of the male reproductive system. Its main function is to produce an alkaline fluid that is a part of the seminal fluid. It is located below the bladder, surrounding the urethra. The prostate can be divided into four zones: anterior, central, transitional, and peripheral, as can be seen in figure 1. (O'Rourke & Loughrey 2004, 311; Pawlina & Ross 2011, 808–809; Kujala 2012, 800.)

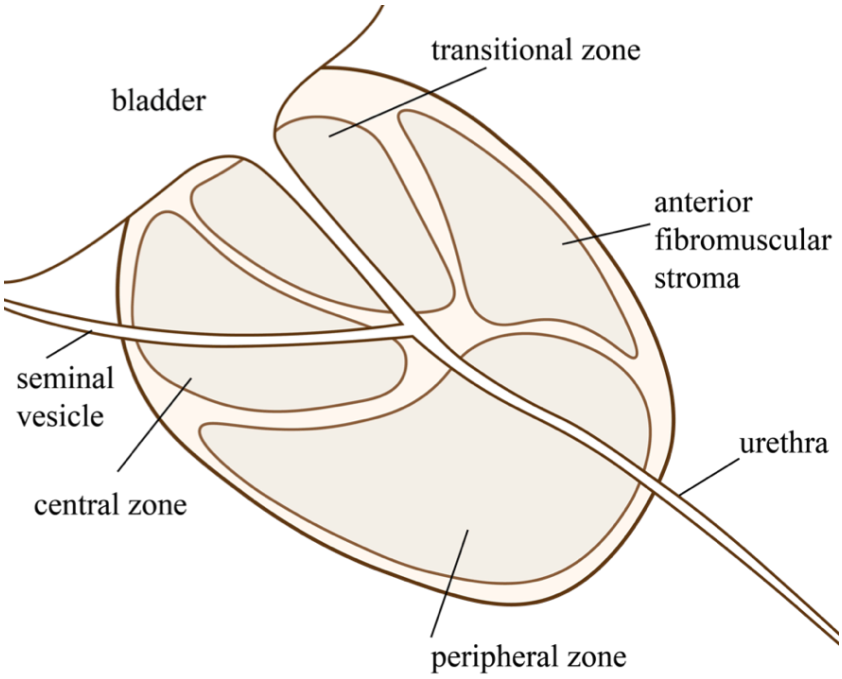

FIGURE 1. Prostatic zones (Koivukoski 2015, adapted from O'Rourke & Loughrey 2004, 312)

The central zone surrounds the ejaculatory ducts and forms approximately 25 % of the prostate gland. Its histological features differ from the rest of the prostate: it is more similar to the ejaculatory ducts and seminal vesicles, whereas the rest of the prostate gland derives from the urogenital sinus. The central zone is resistant to pathological abnormality, and tumours originating from it have very different behaviours and outcomes than tumours originating from other zones. (O'Rourke & Loughrey 2004, 311; Pawlina & Ross 2011, 809; Vargas et al. 2012.)

The peripheral zone, which is formed around the central zone, contains around 70 % of the glandular tissue. 80-90 % of adenocarcinomas originate from the peripheral zone, which is also prone to inflammation. The glands in this zone are small and simple. The anterior fibromuscular stroma is located in the front surface of the prostate gland, and it is mainly composed of smooth muscle fibers and very few glands. (O'Rourke & Loughrey 2004, 311; Pawlina & Ross 2011, 809–810; Kellokumpu-Lehtinen et al. 2013, 563.)

The transitional zone, which surrounds the urethra, forms approximately 5 % of the prostatic glandular tissue, and contains mucosal glands. This region often undergoes hyperplasia as the man ages. It can cause problems in urination due to its close proximity to the urethra. This condition, called benign prostatic hyperplasia (BPH), is very common in elderly men. (O'Rourke & Loughrey 2004, 311; Pawlina & Ross 2011, 809; Kujala 2012, 802.)

2.1.2 Metaplasia and goblet cells

Metaplasia is the change from one adult cell type to another. It is often benign at first, but can sometimes develop into malignancy and cancer if affected cells continue to grow and spread. Metaplasia is found in 0.6 % of prostates. There are many types of metaplasia in the prostate, the most common being urothelial and squamous metaplasia. Other more rare types of metaplasia found in the prostate include mucinous and Paneth cell-like metaplasia. (Harik & O'Toole 2012; Bostwick, Qian & Hossain 2014, 403; Kumar et al. 2014, 38; Trpkov 2014, 58.)

Urothelial metaplasia is also known as transitional metaplasia, as it often occurs in the transitional zone of the prostate, in cells that line the urethra. It can also be found in the peripheral zone. Urothelial metaplasia is the replacement of benign epithelium or peripheral ducts and acini with benign urothelium (cells lining the urethra). Its location varies widely, making it sometimes difficult to identify it from small biopsy samples. (Humphrey 2008, 379; Harik & O'Toole 2012; Bostwick et al. 2014, 407; Trpkov 2014, 58.)

Squamous metaplasia is when the normal prostatic epithelium is replaced with benign squamous epithelium. It often lacks malignant atypia. Squamous metaplasia usually occurs in normal prostatic glands, but is also found in prostates treated with hormonal or radiation therapy, or adjacent to prostatic infarct. (Humphrey 2008, 379; Harik & O'Toole 2012; Bostwick et al. 2014, 404; Trpkov 2014, 58–59.)

Mucinous metaplasia is the replacement of epithelial cells with mucin-secretory cells, such as goblet cells. The cells have basal nuclei and the cytoplasm is filled with mucin. Mucinous metaplasia is usually focal and it can involve a varying number of acini from individual cells to even clusters. (Humphrey 2008, 379; Bostwick et al. 2014, 405–406; Trpkov 2014, 58.)

Paneth cell-like metaplasia or change (PCLC) is a microscopic cellular alteration to neuroendocrine cells (cells that receive neuronal input and release message molecules to the blood) with large, eosinophilic (stain with eosin) granules. They usually appear as isolated cells or in clusters. This type of change is often linked with a good prognosis as most cancers with PCLC are not aggressive. PCLC is very rare in prostatic adenocarcinomas, and is instread more common in its subtype, ductal adenocarcinoma of the prostate. (Humphrey 2008, 379; Lee, Miller & Epstein 2010; Mortal et al. 2010; Bostwick et al. 2014, 404, 406–407; Trpkov 2014, 59–61.)

Goblet cells are unicellular, mucin-secreting glands that appear in mucinous metaplasia. The mucins are high molecular weight, large glycoproteins. The structure of a goblet cell can be seen in figure 2. The cell is shaped like a glass goblet with mucinogen granules at the apex of the cell and a stem-like basal portion. The basal portion contains the nucleus, mitochondria, Golgi apparatus, and endoplasmic reticulum. The microvilli are on the surface of the cell. (Pawlina & Ross 2011, 589, 592–593; Kim & Khan 2013.)

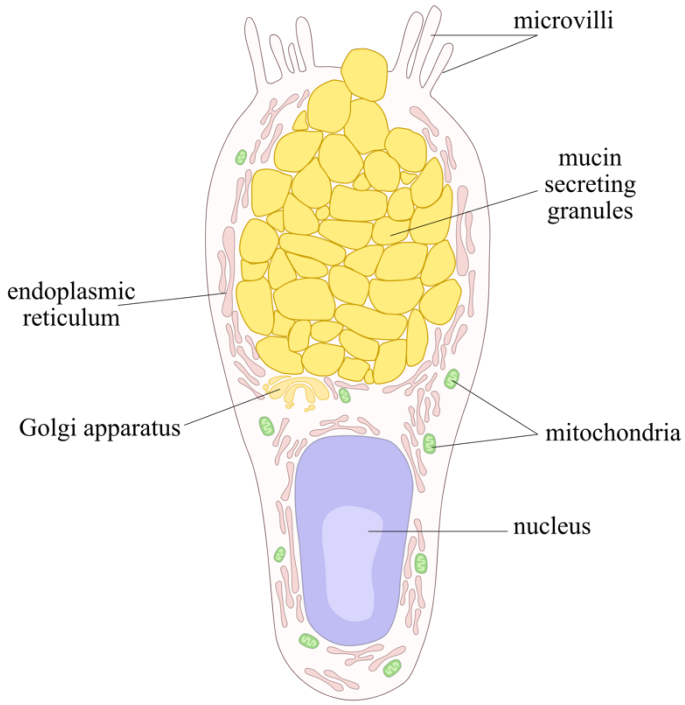

FIGURE 2. Structure of a goblet cell (Koivukoski 2015, adapted from Pawlina & Ross 2011, 593)

Goblet cells are components of the mucosal epithelia in the gastrointestinal tract, the airways, and the conjunctival epithelium in the eye. There they produce mucin to maintain a stable environment in the tissue due to external fluctuations. Mucins are used as lubrication in all three tissues, but have further tissue-specific functions: mucus provides a bacteria-free environment in the small intestine, protects the respiratory epithelium in the airways, and is a component of tears in the eye. (Kim & Khan 2013; McCauley & Guasch 2015.)

2.1.3 Cancer background and causes

Prostate cancer is the most common cancer in men in Finland and the second most common cause of death among cancers. Over 98 % of prostate cancers are adenocarcinomas, which are defined as neoplasia formulating in epithelial cells with a glandular origin. In addition to prostate adenocarcinoma, there are also other types of cancer that can occur in the prostate, such as ductal adenocarcinoma, urothelial cancer, squamous cell cancer and mucinous cancer. These are all however extremely rare, some making up only less than 1 % of all prostate carcinomas. Lymphomas or leukaemia can some-

times initiate from the prostate, as well as secondary tumours from other organs. (O'Rourke & Loughrey 2004, 315; Kujala 2012, 800, 803–804; Marcus et al. 2012; Kellokumpu-Lehtinen et al. 2013, 563.)

There are many known risk factors in prostate cancer. The most common factor is age: two thirds of prostate cancers are found in men over 65 years old, while it is rare in men under 50. There are also large differences between different ethnic groups in the incidence of prostate cancer. A genetic variant found in higher frequencies in men of African American descent has been found to be associated with prostate cancer. There are also studies showing that lifestyle, nutrition, and physical exercise are linked with the risk of prostate cancer. (Damber & Aus 2008; Kujala 2012, 804.)

Prostate adenocarcinoma has one of the lowest fatality rates among cancers, and it has a 5-year survival rate of almost 99 % in the United States. Approximately 15 % of men will be diagnosed with prostate cancer at some point in their lives. The median age at diagnosis is 66 and 80 at death. Most men, however, die of causes other than prostate cancer. (VanderWeele 2014; SEER 2015.)

2.1.4 Diagnosis

Prostate cancer can be tested with a prostate-specific antigen (PSA). PSA is a protein produced in the prostate gland, and it is released into the blood in abnormal conditions in the prostate structure. The normal PSA level is under 4.0 ng/ml but it rises with age. The PSA test has become very common in recent years, and its development has made it possible to detect prostate cancer much earlier. This has, however, also resulted in the increasing treatment of clinically insignificant prostate cancers and overdiagnosis. On average, PSA levels start increasing approximately seven years before the cancer has progressed to a clinical state. PSA is specific to the prostate but not prostate cancer. Some benign diseases, such as BPH, also release PSA into the blood, hence an increased PSA level does not always mean prostate cancer. (O'Rourke & Loughrey 2004, 313; Damber & Aus 2008; Lilja, Ulmert & Vickers 2008; Shen & Abate-Shen 2010; Kujala 2012, 804; National Cancer Institute 2012; Kellokumpu-Lehtinen et al. 2013, 565; Sfoungaristos, Katafigiotis & Perimenis 2013.)

Those found with high PSA levels are advised to get a biopsy from which a diagnosis can be made. Multiple biopsies are taken and they are graded with a Gleason score. The two most common histological patterns in the biopsy samples are graded from 1 to 5, with 5 being the most aggressive form. The worst grade should always be included. The result is a Gleason score from 2 to 10, the most common scores being 6 and 7. (Damber & Aus 2008; Shen & Abate-Shen 2010; Kellokumpu-Lehtinen et al. 2013, 563.)

TNM staging is also used in the classification of prostate cancer. It is based on three key points: primary tumour (T), lymph nodes (N), and distant metastasis (M). The tumour is categorised from 0 to 4, depending on the size and growth. Lymph nodes and metastasis are categorised as either 0 or 1, with 0 indicating that there is no spreading to lymph nodes or distant metastasis, and 1 indicating that there is. The TNM stage, PSA level, and Gleason score are used to determine the stage (from I to IV) of the cancer. The stage grouping categories and requirements can be seen below in table 1. (Kellokumpu-Lehtinen et al. 2013, 564–565; American Cancer Society 2015.)

TABLE 1. Stage grouping in prostate cancer (American Cancer Society 2015)

Stage	tumour	lymph nodes	metastasis	Gleason Score	PSA (ng/l)
I	1	0	0	≤ 6	< 10
	2a	0	0	≤ 6	< 10
IIa	1	0	0	7	< 20
	1	0	0	≤ 6	10 - 20
	2a or 2b	0	0	≤ 7	< 20
IIb	2c	0	0	any	any
	1 or 2	0	0	any	≥ 20
	1 or 2	0	0	≥ 8	any
III	3	0	0	any	any
IV	4	0	0	any	any
	any	1	0	any	any
	any	any	1	any	any

2.1.5 Cancer progression and treatment

Prostate cancer is considered to be a multifocal cancer where there are multiple tumours in one organ. The cancer can originate from several different cells in the prostate. The progression of prostate adenocarcinoma can be seen below in figure 3. The normal pros-

tatic epithelium has two basal layers: basal cells and basal lamina. Throughout the cancer progression, both of these layers are lost partially or completely, depending on how far the cancer progresses. (de Werra et al. 2008, 129; Shen & Abate-Shen 2010.)

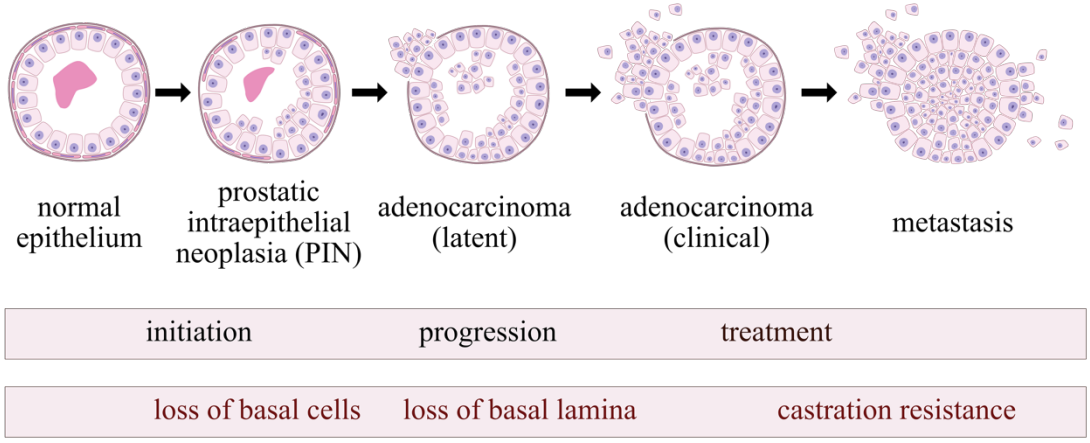

FIGURE 3. Cancer progression (Koivukoski 2015, adapted from Abate-Shen & Shen 2000; Shen & Abate-Shen 2010)

Prostatic intraepithelial neoplasia (PIN) is often said to be a precursor of prostate adenocarcinoma. It is characterised as prostate epithelial hyperplasia. PIN has two different stages: low grade and high grade. The only way to diagnose PIN is to take a biopsy as it does not raise PSA levels or have any macroscopical changes. (O'Rourke & Loughrey 2004, 313; Shen & Abate-Shen 2010; Kujala 2012, 808.)

Latent prostate adenocarcinoma is defined as a cancer that is not active and does not show clinical symptoms. Clinical adenocarcinoma is a cancer, which has progressed to the stage where the tumour can be clinically found. Latent adenocarcinoma has been found in men in their 20s, 30s and 40s even though prostate cancer is extremely rare in younger men. These results suggest that cancer initiation can start at a relatively early age, but it does not develop into a cancer until at a later age. Most studies say that latent prostate cancer is an early stage of prostate cancer. The tumours found in latent prostate cancers are often Gleason score 1-4, very rarely 5, and the growth rate is slower. In clinical cancers, the tumours are often Gleason scores 2 to 5. (Takahashi et al. 2006; Shen & Abate-Shen 2010.)

There are many different treatment options for prostate cancer. For localised or latent prostate cancer, it is sometimes best to not treat it at all and instead to actively monitor

the cancer progression. The latter is often the most suitable option, especially when the patient has a life expectancy of less than 10 years and the test results do not change. (Damber & Aus 2008; Shen & Abate-Shen 2010; National Cancer Institute 2015.)

Other treatment options for localised prostate cancer (stages I-IIb) are radical prostatectomy and radiotherapy. In radical prostatectomy, the entire prostate, including the tumour, is surgically removed. Radical prostatectomy has been found especially useful in patients under 65, and it is often performed only when the disease is curable and the patient has a life expectancy of more than 10 years. On the contrary, radiotherapy is used when surgery is not suitable, and it can be used either by itself or alongside with other treatments when the cancer has progressed to a higher stage. (O'Rourke & Loughrey 2004, 316; Kellokumpu-Lehtinen et al. 2013, 566–569.)

Androgen deprivation therapy (ADT) is also a commonly used treatment method in prostate cancer. Androgen receptor (AR) is a nuclear hormone receptor, which is activated by androgenic hormone binding. It is essential to normal prostate and prostate cancer development. In the beginning of prostate cancer, the cells are very dependent on testosterone, so when the androgen is deprived, the cells start apoptosis, which is why the treatment is very efficient. It is also the most common and effective treatment method for metastatic prostate cancer. There are different ways of performing the treatment and all of them have various side effects. (Montgomery et al. 2008; Shen & Abate-Shen 2010; Jalava et al. 2012; Kellokumpu-Lehtinen et al. 2013, 570–571; Latonen 2014.)

Prostate cancer often metastasises to the bone. Other common sites include the lung, the liver, and the pleura. In the bone, it forms characteristic osteoblastic (bone forming) lesions. Metastatic prostate cancer is often treated with many treatment methods, ADT for multiple months and a short period of radiotherapy in the end, for example. (Logothetis & Lin, 2005; Shen & Abate-Shen 2010; Kellokumpu-Lehtinen et al. 2013, 575.)

Prostate cancer is defined as castration resistant when the cancer keeps progressing even though castration (ADT) has been performed. Castration resistant prostate cancer (CRPC) occurs in 80 % of the patients with metastasised prostate cancer. It is more aggressive and androgen-independent, meaning the AR signalling pathway still works and the cancer progresses. CRPC is incurable and will eventually lead to death. At this stage, patients often only receive treatment to relieve pain caused by the bone metasta-

sises. (Jalava et al. 2012; Kellokumpu-Lehtinen et al. 2013, 576–577; Yuan et al. 2013; Latonen 2014; Saad & Miller, 2015.)

2.1.6 Peptidase inhibitor 15

Peptidase inhibitor 15 (PI15) is a member of the CRISP (cysteine-rich secretory proteins) family. CRISPs function as venoms or toxins in some plants, insects, and vertebrates. They are expressed in human secretory glands, such as reproductive, salivary, and thyroid glands. The PI15 gene is expressed in mouse and human prostate tissue, skeletal and smooth muscle, ovaries and aortic wall. (Smith et al. 2001; Gibbs, Roelants & O'Bryan 2008; Falak et al. 2014; NCBI 2015.)

PI15 might have a role in the drug resistance regulation in ovarian cancer, therefore it could serve as a potential target in targeted therapies. One study found out that PI15 interacts with 11 genes, 5 of which are oncogenes, which could further indicate a role for it in cancer. (Zou et al. 2015.) In addition, PI15 expression was detected in the chicken during organogenesis, which might mean it also has a role in the development of internal organs (Falak et al. 2014). PI15 is also a part of the trypsin inhibitor family. It has been reported that this family's proteases have a role in gynaecological cancers, but no further mechanistic studies or therapeutic strategies have been developed. (Giri et al. 2014.)

PI15 is secreted and localized in the extracellular matrix in multiple species, therefore it might have a role in the regulation of the modification of the extracellular matrix. There are studies showing an increased production of PI15 in cancer cell lines and weak trypsin inhibitory activity, which could mean the PI15 has a role in cancer pathogenesis. However, neither of these theories has yet to be confirmed. (Gibbs et al. 2008.)

2.2 Histology of mouse prostate

In contrast to human prostate, which is a single structure, the mouse prostate is divided into four lobes: ventral, dorsal, lateral, and anterior. Three of these lobes can be seen in figure 4 below. The dorsal lobe surrounds the urethra. The ventral lobe is located above the urethra, in between the lateral prostate lobes. The anterior lobes are located cranial to the other lobes, curving upwards along the seminal vesicles. The mouse prostate has less stroma than the human prostate. The glands are irregularly shaped and surrounded by the connective tissue. (Knoblaugh & True 2011, 296–300.)

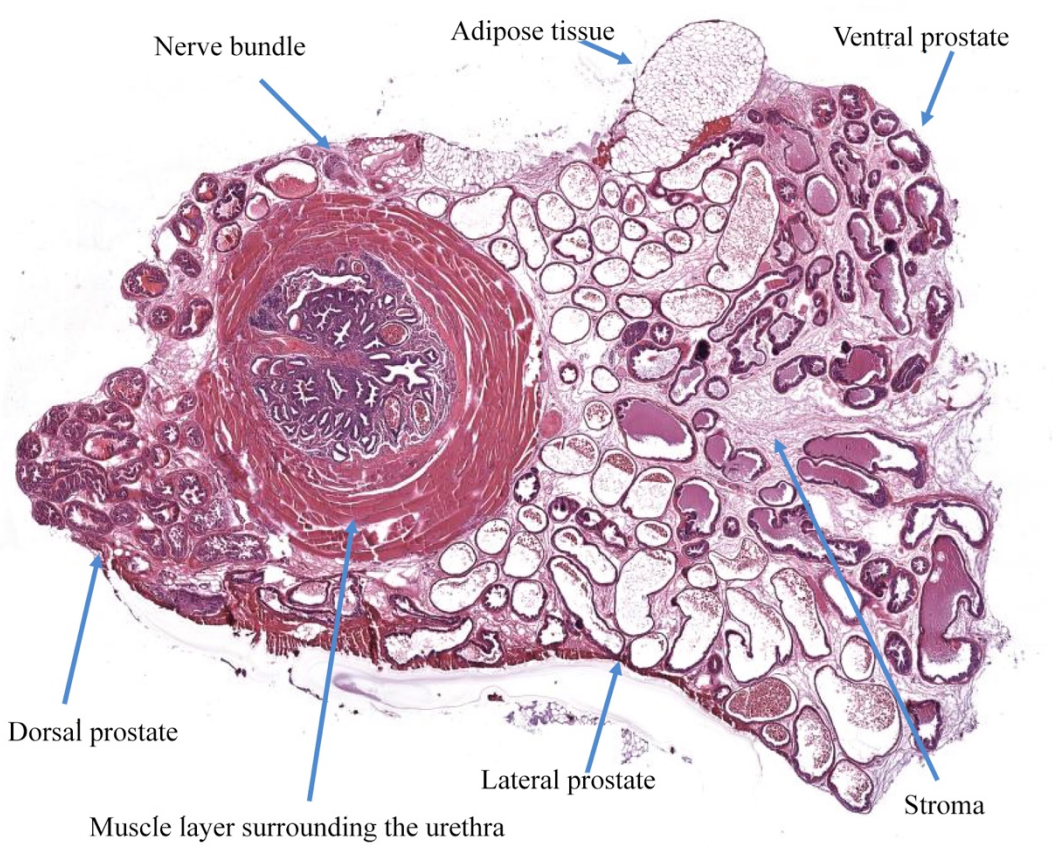

FIGURE 4. Histology of mouse prostate (Latonen 2014, modified)

Dorsal lobe has small or moderate glands, dense stroma, and eosinophilic secretion. The epithelium is columnar, and the nuclei are basally or centrally located. Lateral prostate has sparse infoldings in the epithelium, the secretion is pale and amorphous, and the stroma is thin. The epithelium is more cuboidal and the nuclei are more basal than in the dorsal prostate. Ventral prostate reminds of lateral prostate, but it has larger glands. In addition, the secretions are homogenous and paler than in the lateral prostate. (Knoblaugh & True 2011, 296–300; Latonen 2014.)

2.3 Histochemistry

Histochemistry is the study of chemical components in tissues. Histochemical methods maintain the whole structure of the tissue, meaning both the localization and the cells can be studied. It uses an array of different methods, such as staining and light microscopy. There are many different staining methods, which highlight different components of the tissue and cell: nucleus, various enzymes, and extracellular components for instance. (Pawlina & Ross 2011, 1–5; Thermo Fisher Scientific 2015.)

Histological staining is based on a chemical procedure in the tissue, such as specific binding or enzymatic activity. (Pawlina & Ross 2011, 3.) Before the tissues can be stained and studied further, they must be processed. The processing steps can be seen in figure 5 below.

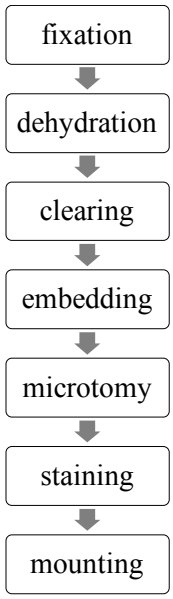

FIGURE 5. Sample processing steps

2.3.1 Tissue processing

The most common tissue processing protocol is formalin-fixed and paraffin embedded (FFPE) tissue blocks. The purpose of fixation is to stop enzyme activity and metabolism, while still preserving the morphology of tissue samples. The most commonly used chemical fixative is 10 % neutral buffered formalin (NBF) with a pH of 7.0. 10 % NBF fixes the tissues by penetrating the tissue and forming cross linkages between the reac-

tive glycol and proteins. (Cook 2006, 11, 13–14; Colley & Stead 2013, 21, 23; Human Protein Atlas 2015.)

Another tissue processing protocol option is PAXgene fixation and paraffin embedment (PFPE). The fixation is a commercial fixation kit by PreAnalytiX (Switzerland). It has two containers: the fixative, which contains methanol, acetic acid, and a soluble organic compound, and the stabilising solution, which contains different alcohols, such as ethanol. It is currently researched whether or not PFPE is a better option than FFPE. PFPE is meant to improve the preservation of morphology, nucleic acids and proteins better than FFPE but it is much more expensive. (Kap et al. 2011; Belloni et al. 2012; PreAnalytiX 2015.)

There are other chemical fixative solutions, such as different alcohols and acetic acid. All these, however, have different qualities that make them not as suitable for histochemistry, such as price or toxicity and tissue swelling, shrinking or hardening. In addition to chemical fixation, heat, microwaves or ultrasound technology can be used, with or without chemical solutions. Chemical fixation is, however, the most common fixation protocol used in histology. The used fixation method is chosen according to what is needed from the tissue sample and, for example, which cellular components are being studied. (Cook 2006, 12–17; Colley & Stead 2013, 24–25; Rhodes 2013, 70–71.)

During tissue processing, wax is used to replace fixation reagents containing water. This is done in two stages: dehydration and clearing. Dehydration removes the water by slowly diffusing the water out and replacing it with the solvent used in dehydration. It is done in multiple steps by increasing the solvent concentration all the way up to absolute. Clearing clears the alcohol out of the tissue sample and replaces it with the solvent, which has to be miscible with the wax. (Cook 2006, 22–24, 26; Colley & Stead 2013, 25.)

After the clearing, the solvent is replaced with molten wax (melting point 50-60 °C). The solid wax has a similar hardness to tissues, which supports the tissue both internally and externally. Once the processing is done, the tissues are embedded in paraffin blocks. The tissue remains stable in the blocks, which can be easily handled and stored for many years without deterioration. (Cook 2006, 21–23, 29–30; Colley & Stead 2013, 25.)

The blocks are cut into 3-10 µm sections using a microtome. The sections cut are floated in room temperature water and picked up to glass slides. After this, the sections are floated in warm water (around 5-9 °C cooler than the wax melting point), which allows the sections to fully expand and straighten to the original dimensions. When the sections have dried off of excess water, they are heated in an incubator from 2 to 16 hours in over 37 °C to achieve complete adhesion. (Cook 2006, 36–37, 40–41; Rolls 2010, 19–20; Colley & Stead 2013, 25; Human Protein Atlas 2015.)

After the adhesion, the wax in the tissues must be completely removed so all the reagents, antibodies, and probes can adhere to the tissue. This is done by immersing the slides in a dewaxing solution, such as xylene or hexane. After this, the dewaxing agent is removed and replaced with water by gradually decreasing the alcohol content. (Colley & Stead 2013, 25–26.)

2.3.2 Staining methods

Haematoxylin-eosin staining (H&E) is the most widely used staining technique for histological samples because of its ability to widely demonstrate different types of structures. There are many different ways of preparing the haematoxylin solution, but all of them have the same main purpose: to stain the nuclei blue. Eosin is used as a counterstain and it stains the cytoplasm, as well as most connective tissues, pink or reddish. (Cook 2006, 226–227; Bancroft & Layton 2013, 173.)

Haematein is the oxidation product of haematoxylin and it is a natural dye that is responsible for the staining. Haematein is anionic, but with the presence of a mordant, it has a positive charge and so it can be bound to the anionic sites of the nuclei. There are dozens of different haematoxylin solutions, but the most common ones used in H&E staining are alum based. These stain the nuclei red, but when subjected to a weak alkali solution, they gradually convert to blue. (Bancroft & Layton 2013, 174.)

Eosin is used to show the general architecture of a tissue. If differentiated properly, it can be used to distinguish different types of cells and connective tissues. Eosin, which is a xanthene dye, can be found in multiple different types, the most common one being

eosin Y. It is used as a 0.5-1 % solution in distilled water. Tap water is used for further differentiation with eosin. (Bancroft & Layton 2013, 173.)

Periodic acid-Schiff staining (PAS) is used to detect molecules with a high carbohydrate content including mucin, fungi, and glycogen. It is the most important technique on detecting carbohydrates due to its ability to detect most of the neutral saccharide containing materials. The PAS technique is a two-step staining protocol with two chemical reactions: an oxidation reaction with periodic acid, and a reaction with Schiff's reagent. It is used to detect whether a cell is PAS-negative or -positive. (Cook 2006, 112–113; Bisen 2014, 357.)

The periodic acid acts as an oxidising agent: it creates dialdehydes from carbohydrates. Periodic acid (HIO_4) is used as a 0.5 or 1 % aqueous solution. It is the most commonly used oxidising agent in PAS staining since it does not continue oxidising after the creation of aldehydes. The dialdehydes are then exposed to Schiff's reagent, which creates an insoluble magenta compound. Schiff's reagent is very sensitive and specific to aldehydes; even ketones do not react with it in PAS method. Haematoxylin is often used as a counterstain and it stains the nuclei blue. (Cook 2006, 113–114; Bisen 2014, 357.)

2.3.3 Immunohistochemistry

Immunohistochemistry (IHC) is a protocol where an immunological reaction is used to identify antigens in tissue samples. In immunoperoxidase methods, antibody binds to an enzyme, which then creates an insoluble coloured substance. This, with the help of a countestain, can then be used for highly specific immunohistochemistry when specific antibodies are used. The staining protocol steps for IHC are shown in figure 6 below. (Cook 2006, 188; Jackson & Blythe 2013, 382.)

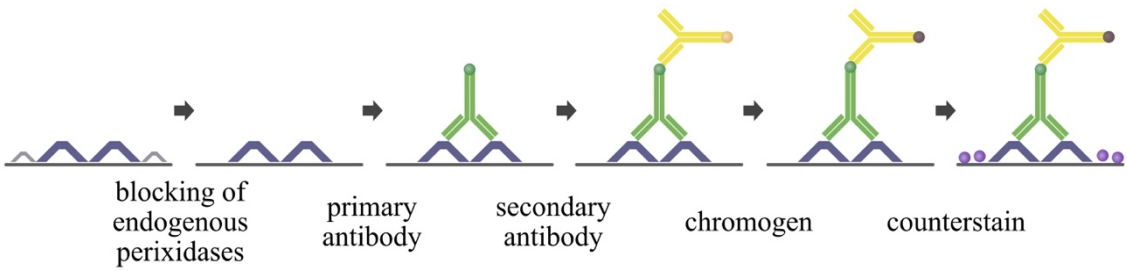

FIGURE 6. IHC staining process (Koivukoski 2015)

Fixation sometimes alters the structure of the antigens, but most of these changes are reversible. In FFPE tissue samples, antigen retrieval is needed to undo the crosslinking between some molecules to recover the antigens for the antibodies to bind to. This is done with heat, enzymes or a combination of these two. All of these treatments break down the crosslinks in antigens. Endogenous peroxidases are blocked with a solution, 3 % hydrogen peroxidase or a commercial blocking solution for example, to avoid background staining. (Chen, Cho & Yang 2010; Jackson & Blythe 2013, 390–391; Taylor 2013, 12; Human Protein Atlas 2015.)

Antibodies, also known as immunoglobulins, are found in blood and tissue fluids. They are part of the immune system, and they are produced in B-lymphocytes and plasma cells when a foreign structure is detected. The produced antibodies invade foreign structures by binding to them strongly. There are many types of antibodies, but the most common ones used in immunohistochemistry are immunoglobulin G (IgG). (Cook 2006, 180; Jackson & Blythe 2013, 390–391.)

Antibodies can be polyclonal or monoclonal. Polyclonal antibodies are produced in animals, whereas monoclonal can be produced in cell culture. Polyclonal antibodies are a heterogeneous mix of antibodies and they will bind to multiple different epitopes. They are more likely to create at least some kind of background noise, but they also have a higher chance of binding to the target molecule. Monoclonal antibodies bind to a specific epitope and they are more identical as they only contain one antibody. The staining results of polyclonal antibodies can vary due to their heterogeneity, but monoclonal antibodies produce a more uniform staining. (Cook 2006, 182–183; Jackson & Blythe 2013, 384; Human Protein Atlas 2015.)

The primary antibody binds to the specific antigen of interest, while the secondary antibody binds to the primary antibody. The secondary antibody is often labelled with an enzyme, which produces the coloured product when incubated with a suitable chromogen. The most commonly used enzyme is horseradish peroxidase (HRP) because it works well with a common chromogen, DAB (3,3α-diaminobenzidine tetrahydrochloride). DAB forms an insoluble brown substrate in the presence of a peroxidase. Haematoxylin is often used as a counterstain. (Jackson & Blythe 2013, 382, 384–385; Taylor 2013, 12; Vector Laboratories 2014.)

3 AIM OF THE STUDY

The objective of the study is to assess goblet cell metaplasia and to establish a protocol for a possible biomarker in mouse prostate tissue. The results will benefit prostate cancer research from a histochemical angle: whether the biomarker has a role in prostate cancer and how the goblet cells act histologically.

The purpose of this study is to examine goblet cell metaplasia in aged and miR-32 transgenic mouse prostate tissue by performing periodic acid-Schiff staining to detect mucin not normally secreted by the prostate. The second purpose is to test and validate an immunohistochemistry protocol to detect PI15 in mouse prostate tissue.

4 MATERIALS AND METHODS

4.1 Sample preparation

There were two types of samples used in this study: wild type and miR-32 transgenic mouse prostate. The samples were received fixed and in their embedding cassettes. Most of the samples were fixed in 10 % NBF (FF-Chemicals) for 2 to 14 days, with the average being 3 to 4 days. The remaining selected samples were fixed with PAXgene™ Tissue Containers (Qiagen). PAXgene™ samples were fixed in a mixture of methanol and acetic acid for 5 to 7 hours and then stabilised in ethanol for 5 days. After fixation, all the samples were processed in Shandon Citadel 2000 tissue processor. FFPE and PFPE samples were processed separately. The protocol used in the processing of both fixatives is shown in table 2. More information about the solutions used in tissue processing can be found in appendix 1.

TABLE 2. Sample processing protocol

solution	t (h)
70 % EtOH	2
96 % EtOH	1.5
96 % EtOH	1
Absolute EtOH	1.5
Absolute EtOH	1.5
Absolute EtOH	1.5
Xylene	1.5
Xylene	1.5
Xylene	1.5
Paraffin (59 °C)	1.5
Paraffin (59 °C)	1 -

After the tissues were processed, the cassettes were moved from the molten paraffin to the Sakura Tissue-Tek® TEC™ Tissue embedding console for embedding. The samples were embedded so that the urethra was facing upwards in the mold. The sample was then covered in molten paraffin, the cassette placed on top of it, and then placed on a cold plate (– 4 °C) in order for the wax to set. When the wax had set, the molds were removed and the samples stored in + 8 °C until sectioning.

The sectioning was done with three different microtomes, a rotation microtome Leica RM2235 which was used to cut 7 µm sections, and two sliding microtomes, Leica SM2010 R and Leica SM2000 R, which were used to cut 5 µm sections. From each sample, the entire prostate was sectioned. Three sections were placed on one Superfrost® Plus slide by floating them in room temperature distilled water. The sections were then straightened in + 52 °C distilled H_2O and placed in an upright position to dry out the excess water overnight. After drying, the slides were incubated in + 62 °C from 2 hours to overnight.

4.2 Histochemical stainings

From the slides prepared, every third one was H&E stained with Kedee KD-RS3 Slide Stainer using the protocol showed in table 3. The rest of the slides were stored in + 8 °C. After the slides were stained, coverslips were added to the slides using a Dako Coverslipper by putting the slides soaked in xylene to the coverslipper. The bubbles underneath the coverslip were gently pushed out to avoid interference during microscopy. DPX mountant medium (VWR) was used as a mounting medium.

TABLE 3. H&E staining protocol

solution	t (min)
n-Hexane	3
n-Hexane	3
Absolute EtOH	2
Absolute EtOH	1
96 % EtOH	2
70 % EtOH	1
distilled H_2O	30 sec
Mayer's Haematoxylin	6
H_2O	7
distilled H_2O	40 sec
Eosin	1
H_2O	1
distilled H_2O	30 sec
96 % EtOH	2
Absolute EtOH	2
Absolute EtOH	2
Absolute EtOH	2
Xylene	2

From the H&E staining results, selected slides were chosen for PAS staining. The staining was done by hand using the protocol shown below in table 4. The periodic acid solution was prepared by dissolving 1.00 g of periodic acid into 200 ml of water. The remaining of the solutions used in this process were commercial (Appendix 1). The slides were then covered using the same protocol as in H&E staining.

TABLE 4. PAS staining protocol

solution	t (min)
n-Hexane	4
n-Hexane	4
Absolute EtOH	2
96 % EtOH	2
70 % EtOH	2
distilled H_2O	1
0.5 % Periodic Acid	5
distilled H_2O	30 sec
Schiff's reagent	7
H_2O	5
Mayer’s Haematoxylin	1
H_2O	5
distilled H_2O	1
70 % EtOH	2
96 % EtOH	2
Absolute EtOH	2
Xylene	2
Xylene	2

4.3 Immunohistochemical stainings

Suitable slides were chosen for staining with PI15 antibody. First, the slides were deparaffinized using the protocol shown in table 5. After deparaffinization, the slides were air dried and put in a slide chamber with buffer solution for antigen retreival. Two different buffers were tested: pH 9 Tris-EDTA buffer and pH 6 Citrate buffer (Appendix 2). Both chambers were placed in 2100 Retriever, which heats them up to 121 °C for 2 minutes in high pressure, and then gradually cooled down to room temperature. The buffer solutions are replaced with 1x TBS Tween to keep them from drying when placed in the Autostainer. 1x TBS Tween was diluted 1:10 from 10x TBS Tween (Appendix 2).

TABLE 5. Deparaffinization

solution	t (min)
Hexane	4
Hexane	4
Absolute EtOH	1

After the antigen retrieval, the slides were immunostained using Lab Vision™ Autostainer 480. The protocol used in the staining was created using old antibody staining protocols for various different antibodies, which have been found suitable. The protocol can be seen in table 6. Each slide was treated with 300 µl of each reagent except for washes where the slides were washed thoroughly. Anti-PI15 (OriGene) was diluted with Normal Antibody Diluent (ImmunoLogic) by first making a 1:100 dilution and from that a 1:10 dilution. DAB was diluted by adding 15 µl of ImmPACT™ DAB Chromogen (Vector Laboratories) concentrate to every 1 ml of ImmPACT™ DAB Diluent (Vector Laboratories). More information about the solutions used with the autostainer can be found in appendix 1.

TABLE 6. PI15 staining protocol

solution	t (min)	wash
TBS Tween	-	1x
3 % H_2O_2	5	-
TBS Tween	-	1x
anti-PI15 1:1000	30	-
TBS Tween	-	3x
UIP Max	30	-
TBS Tween	-	3x
ImmPACT™ DAB	5	-
distilled H_2O	-	2x
TBS Tween	-	1x
Mayer's Haematoxylin	2	-
TBS Tween	-	1x
distilled H_2O	-	1x

After the immunostaining, the slides were rinsed quickly in distilled water, and dehydrated using the protocol shown in table 7. The slides were then covered with coverslips using the same protocol as for H&E staining.

TABLE 7. Dehydration

solution	t (min)
70 % EtOH	2
96 % EtOH	2
Absolute EtOH	2
Xylene	4
Xylene	4

All the stained slides (H&E, PAS and IHC) were scanned with an Olympus BX51 microscope. First, the entire slide was scanned with an Olympus Plan N 4x objective to detect all the parts with tissue. Then, the scanner would determine and focus 3 to 6 focus points in each tissue sample with a 20x magnification using Olympus UPlan Apo 20x objective. After all the focus points had been focused, the scanner would scan the tissue samples with the 20x objective.

5 RESULTS

5.1 Goblet cell metaplasia

All the stained slides were studied and examined for goblet cells. Goblet cells can be found by the mucin they secrete. In each of the figures 7 and 8 we can see two different samples, one sample is H&E and the other PAS stained. Both figure 7 sections are from a wild type mouse prostate, while the figure 8 ones are from a transgenic mouse prostate. The left columns in both figures are H&E stained and right columns PAS stained, with the bottom ones being larger magnifications of the rectangles shown in the top row. In figure 9, we can see PAS stainings of two different sections of the same tissue with approximately 250 μm of tissue separating them. All of the three samples shown are processed with FFPE protocol.

In the H&E stainings in figures 7C and 8C, the white arrows are pointing to a slightly lighter shade of pink than the cell cytoplasm which is the mucin inside the goblet cells. In the larger magnification in figure 8C, the goblet cells can be seen very clearly. In the PAS staining, the mucin the goblet cells secrete makes the lumen inside the epithelial cells much darker than average, which makes finding the goblet cells in PAS stained samples much easier. This can be seen in figures 7B and 8B (black arrows versus white arrows). Goblet cells can be seen in figures 7D and 8D as well as in both 9A and 9B (black arrows). The mucin inside the goblet cells is stained deep purple, therefore it differs from the normal epithelial cells, which are just different shades of blue.

In figures 7C and 9B, the metaplasia can be seen in the change of the cell size. The grey arrow in figure 7C is showing much shorter cells than the black arrows. The whole epithelium, when compared to the two top glands, is also much thinner in the bottom gland. The nuclei are much taller and oval shaped in the upper glands, whereas in the bottom gland they are mostly round. The same can be seen in figure 9B: the epithelium is much taller in the upper part of the gland (black arrows) when comparing to the lower part of the gland and the gland on the left (white arrows). This metaplasia becomes very clear in figure 7D, in which the top two glands have much darker lumen and much more goblet cells in the epithelium than in the bottom gland, in which only a few light purple spots can be seen in the epithelium.

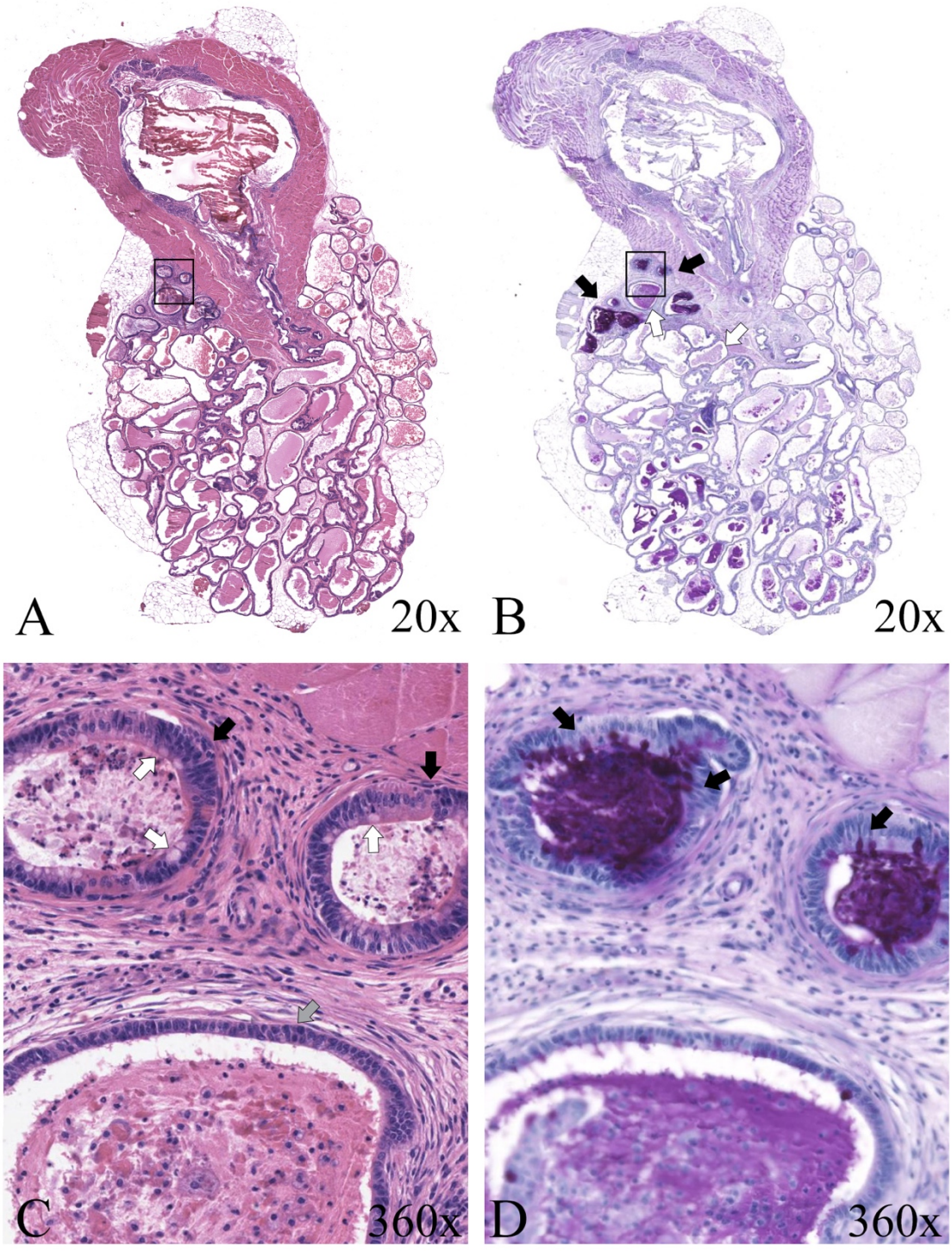

FIGURE 7. Histological stainings of a wild type mouse prostate (FFPE, 18 months) A. H&E staining, and B. PAS staining of a whole mouse prostate with 20x magnification. C. H&E staining, and D. PAS staining of a mouse prostate with 360x magnification

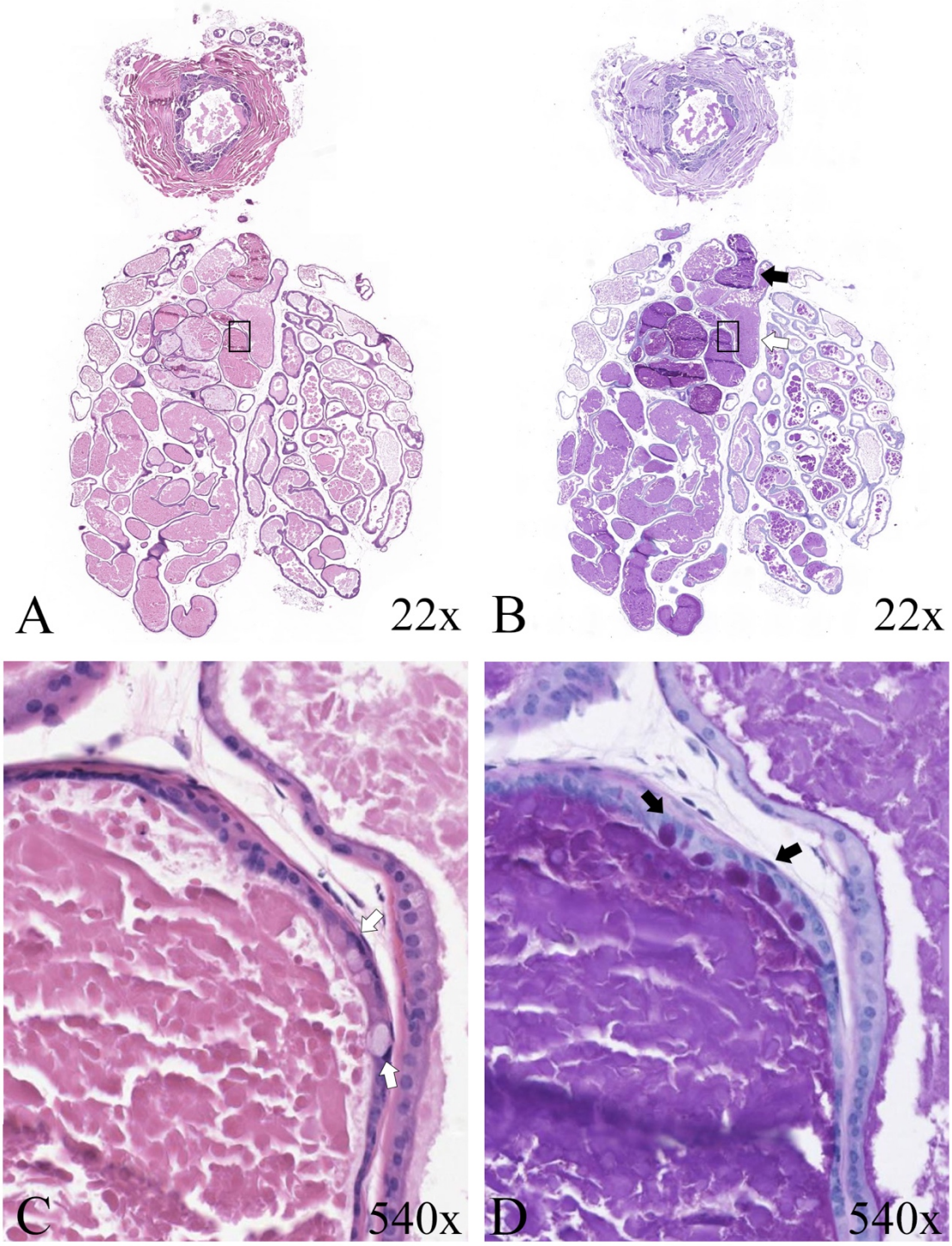

FIGURE 8. Histological stainings of a transgenic mouse prostate (FFPE, 23 months) A. H&E staining, and B. PAS staining of a whole mouse prostate with 22x magnification. C. H&E staining, and D. PAS staining of a mouse prostate with 540x magnification

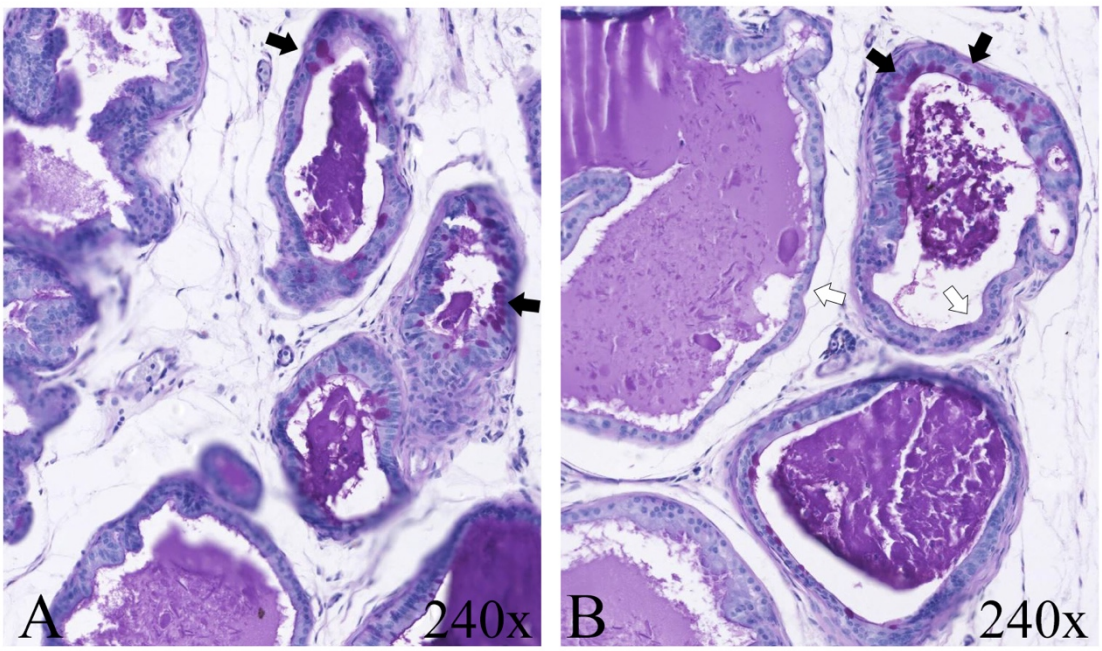

FIGURE 9. PAS staining of a transgenic mouse prostate (FFPE, 23 months) with 240x magnification. The difference between the sections in A and B is approximately 250 µm

5.2 Peptidase inhibitor 15 expression

For staining of PI15, only a necessary number of samples were stained, one for each buffer from both fixating-techniques. The whole sections can be seen in figure 10. The top row was fixed using the FFPE method and the bottom row was fixed using the PFPE method. In both cases, the left column has pH 9 Tris-EDTA buffer used in antigen retrieval, whereas the right column has pH 6 Citrate buffer. A larger magnification of the rectangles shown in figure 10 can be seen in figure 11.

In figure 10, it is very difficult to see any difference at all between 10A and 10B. There is a slight difference in the lumen shown with the black arrows, as in 10A it is slightly darker than in 10B in some glands. The white arrows are however showing that the lumen in 10B would be a bit darker than in 10A. This could either be caused by the buffers used for antigen retrieval or, because the difference is so small, simply due to the fact that the sections are from a slightly different part of the prostate and the lumen has a different density in that part.

In 10C and 10D, the difference can be seen very clearly. 10C is much darker overall and clearly showing the PI15 expression all over the tissue, whereas in 10D it is only visible around the urethra (white arrows) as well as in some of the glands close to the urethra. The black arrows in 10C and 10D show us the larger difference between the two buffers, with 10C showing us almost completely brown glands, with a slight tint to blue, whereas the glands in 10D are almost completely stained with haematoxylin.

In figure 11, the difference in PI15 expression can be seen more clearer in both 11A and 11B as well as 11C and 11D, when compared to figure 10. The expression of PI15 can be seen lining the muscle layer in 11A (black arrow), whereas it cannot be seen in 11B. The epithelial cells in both 11A and 11B (white arrows) are both stained with DAB, with the 11A ones being a slightly darker shade.

In 11C and 11D the difference is again larger. The connective tissue and the fibromuscular stroma are stained in both 11C and 11D (black arrows). The white arrows, which show us the epithelial cell lining in 11C, show us that it’s stained almost throughout the gland, whereas this cannot be seen in 11D at all.

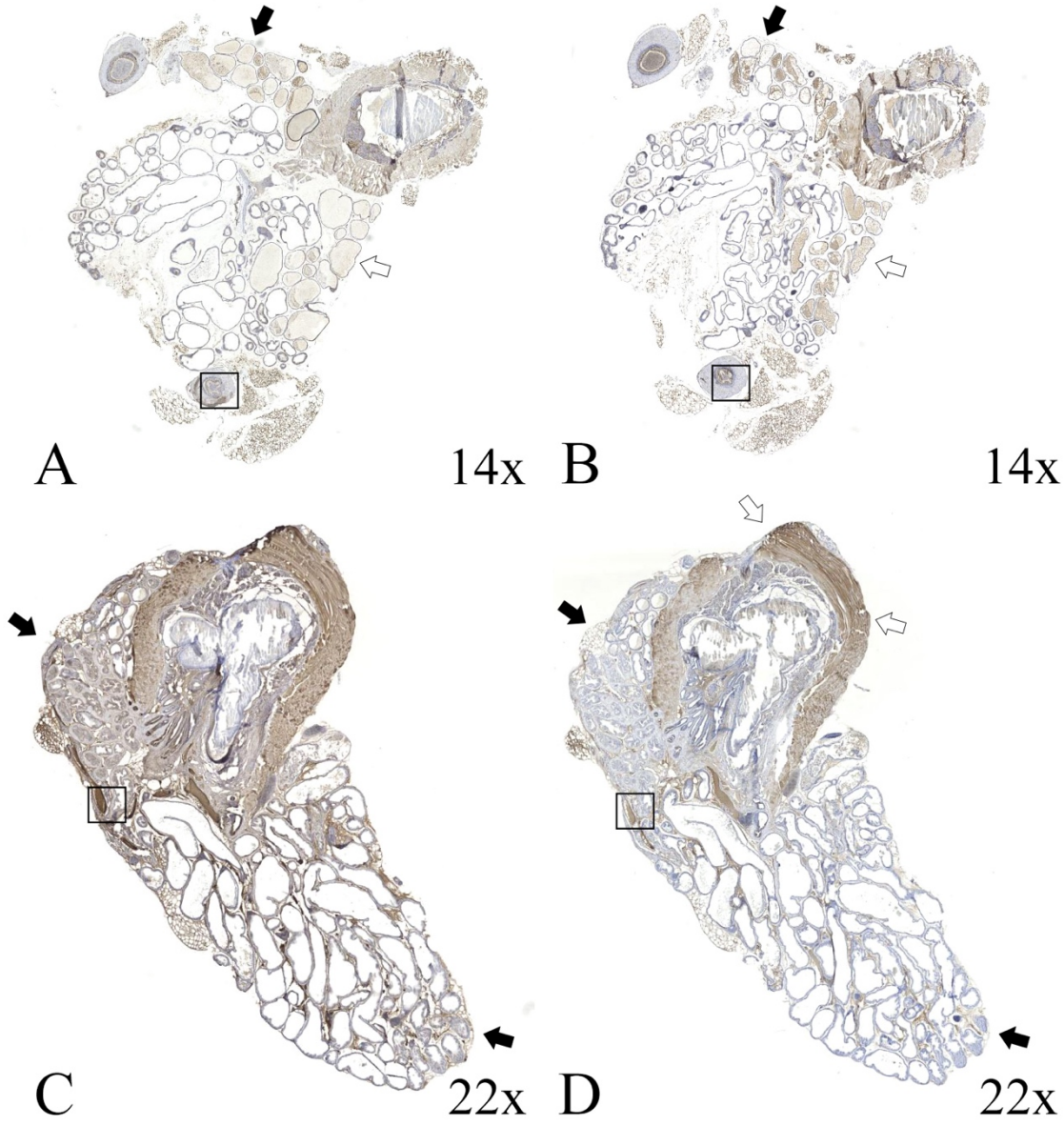

FIGURE 10. Immunohistological stanining of PI15 of a whole mouse prostate. A-B. FFPE, wild type (11 months) C-D. PFPE, wild type (15 months) A, C. pH 9 Tris-EDTA Buffer. B, D. pH 6 Citrate buffer.

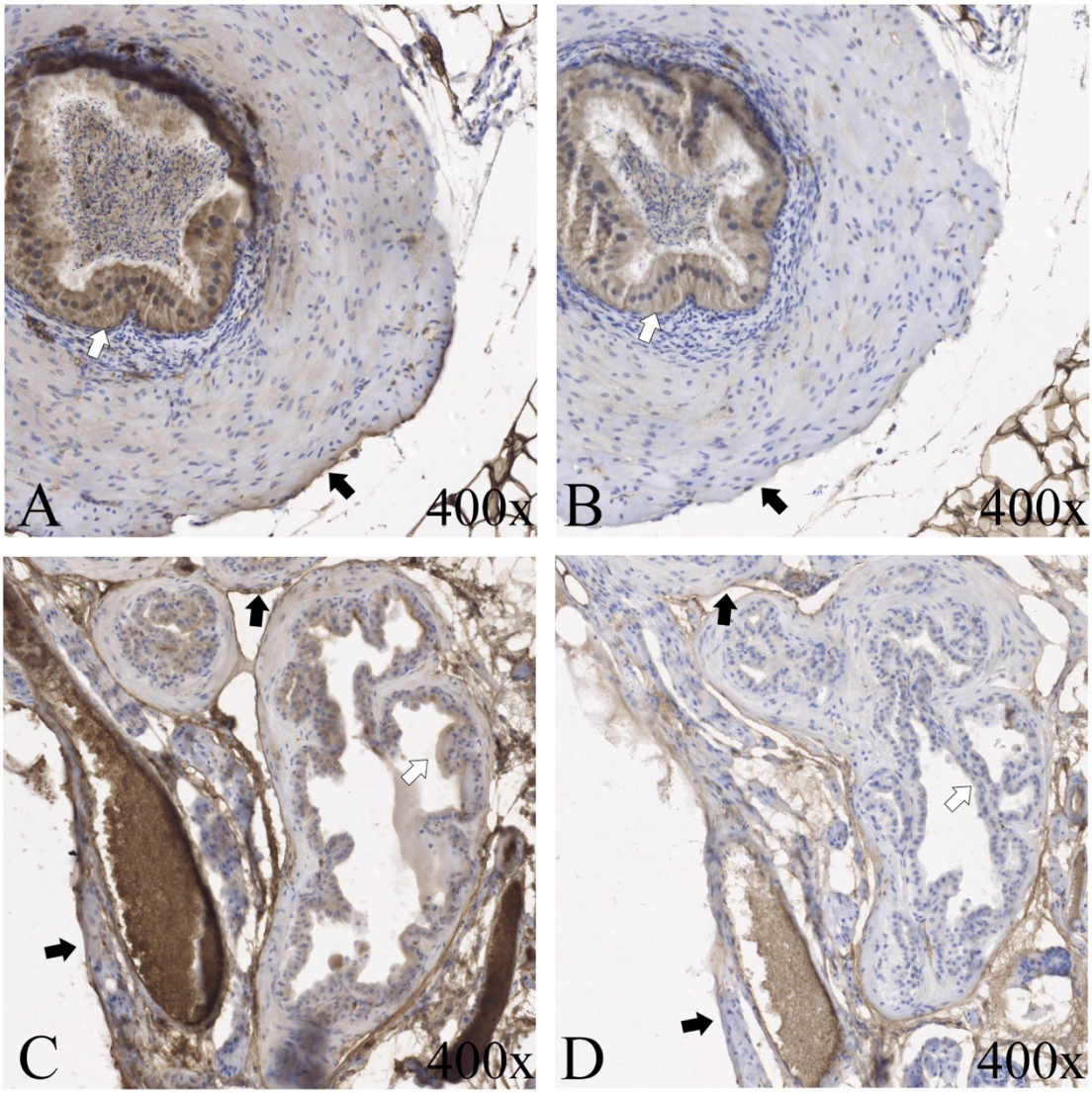

FIGURE 11. Immunohistological stanining of PI15 of a mouse prostate with 400x magnification. A-B. FFPE, wild type (11 months) C-D. PFPE, wild type (15 months) A, C. pH 9 Tris-EDTA Buffer. B, D. pH 6 Citrate buffer.

6 DISCUSSION

The objective of this study was to assess goblet cell metaplasia and to establish a protocol for one biomarker in mouse prostate tissue. The purpose of this study was to examine goblet cell metaplasia in aged and transgenic mouse prostate tissue with the help of PAS staining to detect mucin not normally secreted by the prostate. The second purpose was to test and validate an immunohistochemistry protocol to detect PI15 in mouse prostate tissue.

The first sign of metaplasia in the epithelial cells is the increase in size. They appear much taller than in other glands where the goblet cells cannot be seen. In addition, the nuclei are oval shaped. This is very prominent in the tallest and most altered epithelial cells. Additionally, goblet cell metaplasia is occurring in several glands in the same area in all of the studied samples, which means that the metaplasia has spread from one gland to another. This is shown in figure 9A (page 31), where four glands with goblet cell metaplasia can be seen, all very close to one another.

The goblet cells often appear close to other goblet cells in the epithelium. This indicates that the metaplasia starts from one or more focal points and starts spreading from there. This can be seen clearly in figure 9B (page 31), in which the black arrows show goblet cells, but in the same gland there are also normal epithelial cells (white arrows). The goblet cells and the abnormal epithelial cells are only in the upper part of the gland, whereas the bottom is still mostly normal.

The more goblet cells there are in the epithelium, the taller the epithelial cells seem to be. In figure 8D (page 30), there is only a small amount of goblet cells and the epithelial cells are not much taller than normal, whereas in figures 7D, 9A and 9B (pages 29 and 31) there is a larger amount of goblet cells in most of the visible glands and the epithelium is also much taller. This, again, indicates that the more the cells are altered in the tissue, the more goblet cells appear and the more prominent the metaplasia is.

Even a small amount of goblet cells is enough to stain the lumen in the gland much darker than in the glands nearby. In figure 9B (page 31), there are two glands with darker lumen than in the other two visible in the figure, but in the lower gland there are only

a few small goblet cells visible and yet the lumen in still much darker than in the two normal glands. This indicates that either the small amount of goblet cells have secreted a large amount of mucin to the lumen and it is enough to stain it as dark as the mucin still inside the goblet cells in the epithelium or most of the goblet cells are in other parts of the gland and not visible in the section shown.

Mucinous metaplasia has been previously found in the dorsolateral lobe of the mouse prostate. This study, however, did not separate goblet cells in the metaplasia, but it only referred to tall columnar cells that secrete mucin. (Suwa et al. 2002.) The goblet cells appear close to the urethra, in either the ventral or dorsal lobe in the studied tissues so the results show some similarity to the study by Suwa et al. (2002).

No clear difference between the goblet cells in transgenic and wild type mouse prostate tissues is found, at least in the studied tissues. Both secrete the mucin to the lumen and so stain it darker in the PAS staining. They are similar in size and shape, and the epithelial cell metaplasia seems to be of similar structure as well. They were all located close to the urethra, mostly in the same lobes. The only varieties in the lobes were between two transgenic mice.

To study goblet cell metaplasia further, more samples should be examined to see exactly which lobe or lobes the metaplasia occurs in. Possibly study a bit younger mice as well to see how the metaplasia starts and which processes initiate the metaplasia in the first place. Further down the line, it should be examined in human prostate tissue in mucinous metaplasia as well as in mucinous prostate cancer.

As Gibbs et al. (2008) said, PI15 is expressed in the glandular structure of the prostate. No big difference is found in the expression of PI15 between formalin and the PAXgene fixation, especially between the larger magnifications. The small difference in the visibility could have been caused by the different thickness of the sections or the actual fixative. Formalin is known to cross-link some molecules in the tissue (Jackson & Blythe 2013, 390), which is why the antigen retrieval is needed. The PFPE processing protocol does not always require antigen retrieval for some antigens (Kap et al. 2011), which could also explain the small difference in results between these two fixatives. PI15 might still require antigen retrieval, but the antigens in the tissue could be easier

accessible and the antigen retrieval could bring out the antigens slightly better in the PAXgene fixed tissues when compared to formalin fixed tissues.

The larger difference will more likely be found in the DNA and RNA extraction results. Thus, the selection on which fixation method to use with PI15 expression should mainly be based on the extraction results and whether it is worth the much higher price rather than the IHC staining results, at least in the case of PI15.

As for choosing the best buffer to use with PI15, it was clearly better visible in the pH 9 Tris-EDTA buffer. PI15 can be seen in the lumen of the glands in both buffers (figures 10 and 11 on pages 33 and 34) but it is much more prominent in the lining of the epithelial cells in the tissues where pH 9 Tris-EDTA buffer was used to retrieve the antigens.

To perfectly optimise the PI15 staining method, it should be tested with equally thick sections from both fixation methods to see if there is a critical difference. In addition, the DNA and/or RNA should be extracted and the results evaluated. When the staining method has been optimised, it should be examined in transgenic mice prostate as well. Then, sometime in the future when it has been researched further, it should be tested in human prostate tissues as well and whether it can be utilised in prostate cancer diagnosis or whether PI15 has a role in prostate cancer development.

When dealing with animal tissue, the ethics of using them must always be considered. The studied tissues were received fixed in containers and the dissection process was done elsewhere. The animals were treated and euthanized according to the legislations and regulations on using animals in research.

To conclude, the purpose of the study was mostly accomplished. Goblet cell metaplasia was largely assessed in both transgenic and wild type prostate tissue and the staining of PI15 were tested out with both buffers and fixation methods. The original purpose was to also examine if the staining of PI15 is altered in transgenic mouse prostate, as well as to assess the staining in human prostate tissue, both of which were left out of this study due to time constraints.

As miR-32 is a possible contributor to prostate cancer (Latonen 2014), these results could benefit the research in that area since miR-32 transgenic mice were studied. These

results can also be used in studying goblet cell and mucinous metaplasia, and, sometime further down the line, maybe help finding what initiates the metaplasia. As for PI15, the results could help the research on whether or not it has a part in prostate cancer development or initiation.

REFERENCES

Abate-Shen, C. & Shen, M.M. 2000. Molecular genetics of prostate cancer. Genes & Development, 14(19): 2410–2434.

American Cancer Society. 2015. How is prostate cancer staged? Read 28.8.2015 http://www.cancer.org/cancer/prostatecancer/detailedguide/prostate-cancer-staging

Bancroft, J.D. & Layton, C. 2013. The hematoxylins and eosin. 7th ed. In Suvarna, K.S., Layton, C. & Bancroft, J. D. (ed.) Bancroft's theory and practice of histological techniques. Oxford: Elsevier Science Health Science div, 173–186.

Belloni, B., Lambertini, C., Nuciforo, P., Phillips, J., Bruening, E., Wong, S. & Dummer, R. 2012. Will PAXgene substitute formalin? A morphological and molecular comparative study using a new fixative system. Journal of Clinical Pathology, 66(2): 124–135.

Bisen, P.S. 2014. Laboratory protocols in applied life sciences. Boca Raton: CRC Press.

Bostwick, D.G., Qian, J. & Hossain, D. 2014. Non-neoplastic Diseases of the Prostate. In Bostwick D. G. & Cheng, L. (ed.) Urologic Surgical Pathology. United States: Saunders: 381–442.

Chen, X., Cho, D.-B. & Yang, P.-C. 2010. Double staining immunohistochemistry. North American Journal of Medical Sciences, 2(5): 241–245.

Colley, E.C. & Stead, R.H. 2013. Fixation and Other Pre-Analytical Factors. 6th Edition. In Taylor, C. R. & Rudbeck, L. (ed.) Immunohistochemical Staining Methods. Denmark: Dako Denmark A/S, 21–29.

Cook, D.J. 2006. Cellular Pathology: Introduction to Techniques and Applications. 2nd ed. Oxfordshire: Scion Publishing.

Damber, J.-E. & Aus, G. 2008. Prostate cancer. The Lancet, 371(9625): 1710–1721.

De Werra, C., Donzelli, I., Perone, M., Di Micco, R. & Orabona, G. 2008. Chapter 9. In Renda, A. (ed.) Multiple primary malignancies. Milan: Springer Verlag: 129–142.

Falak, S., Schafer, S., Baud, A., Hummel, O., Schulz, H., Gauguier, D., Hubner, N. & Osborne-Pellegrin, M. 2014. Protease inhibitor 15, a candidate gene for abdominal aortic internal elastic lamina ruptures in the rat. Physiological Genomics, 46(12): 418–428.

Gibbs, G.M., Roelants, K. & O'Bryan, M.K. 2008. The CAP Superfamily: Cysteine-Rich Secretory Proteins, Antigen 5, and Pathogenesis-Related 1 Proteins - Roles in Reproduction, Cancer, and Immune Defense. Endocrine Reviews, 29(7): 865–897.

Giri, K., Shameer, K., Zimmermann, M.T., Saha, S., Chakraborty, P.K., Sharma, A., Arvizo, R.R., Madden, B.J., Mccormick, D. J., Kocher, J.-P.A., Bhattacharya, R. & Mukherjee, P. 2014. Understanding Protein–Nanoparticle Interaction: A New Gateway to Disease Therapeutics. Bioconjugate Chemistry, 2014(25): 1078–1090.

Harik, L.R. & O'Toole, K.M. 2012. Nonneoplastic Lesions of the Prostate and Bladder. Archives of Pathology & Laboratory Medicine, 136(7): 721–734.

Human Protein Atlas. 2015. Immunohistochemistry. Read 21.8.2015 http://www.proteinatlas.org/learn/method/immunohistochemistry

Humphrey, P.A. 2008. Prostate. In Humphrey, P. A., Dehner, L. P. & Pfeifer, J. D. (ed.) The Washington Manual of Surgical Pathology. Philadelphia: Lippincott Williams and Wilkins: 375–389.

Humphrey, P.A. 2011. Histological variants of prostatic carcinoma and their significance. Histopathology, 60(1): 59–74.

Jackson, P. & Blythe, D. 2013. Immunohistochemical techniques. In Suvarna, K. S., Layton, C. & Bancroft, J. D. (ed.) Bancroft's theory and practice of histological techniques. Oxford: Elsevier Science Health Science div, 381–426.

Jalava, S.E., Urbanucci, A., Latonen, L., Waltering, K.K., Sahu, B., Jänne, O.A., Seppälä, J., Lähdesmäki, H., Tammela, T.L.J. & Visakorpi, T. 2012. Androgen-regulated miR-32 targets BTG2 and is overexpressed in castration-resistant prostate cancer. Oncogene, 31(41): 4460–4471.

Kap, M., Smedts, F., Oosterhuis, W., Winther, R., Christensen, N., Reischauer, B., Viertler, C., Groelz, D., Becker, K.-F., Zatloukal, K., Langer, R., Slotta-Huspenina, J., Bodo, K., de Jong, B., Oelmuller, U. & Riegman, P. 2011. Histological Assessment of PAXgene Tissue Fixation and Stabilization Reagents. Moura, I. C. (ed.) PLoS ONE, 6(11): e27704.

Kellokumpu-Lehtinen, P.-L., Joensuu, T. & Tammela, T. 2013. Eturauhassyöpä. In Joensuu, H., Roberts, P. J., Kellokumpu-Lehtinen, P.-L., Jyrkkiö, S., Kouri, M., & Teppo, L. (ed.) Syöpätaudit. 5th edition. Helsinki: Duodecim, 562–580.

Kim, J.J. & Khan, W.I. 2013. Goblet Cells and Mucins: Role in Innate Defense in Enteric Infections. Pathogens, 2(1): 55–70.

Knoblaugh, S. & True, L. 2011. Male Reproductive System. In Treuting, P., Dintzis, S. & Liggitt, D. (ed.) Comparative Anatomy and Histology: A Mouse and Human Atlas. Amsterdam: Elsevier/Academic Press: 285–308.

Kujala, P. 2012. Eturauhanen. In Mäkinen, M., Carpén, O., Kosma, V.-M., Lehto, V.-P., Paavonen, T. & Stenbäck, F. (ed.) Patologia. Helsinki: Duodecim, 800–809.

Kumar, V., Perkins, J.A., Abbas, A.K. & Aster, J.C. 2014. Robbins & Cotran Pathologic Basis of Disease. 9th ed. United States: Elsevier - Health Sciences Division.

Latonen, L. 2014. Modelling human prostate cancer in mice - transgenic miR-32 expression in mouse prostate. University of Helsinki. Palmenia. Pathology Training Program in Disease Modelling. Project Report.

Lee, T.K., Miller, J.S. & Epstein, J.I. 2010. Rare histological patterns of prostatitc ductal adenocarcinoma. Pathology, 42(4): 319–324.

Lilja, H., Ulmert, D. & Vickers, A.J. 2008. Prostate-specific antigen and prostate cancer: prediction, detection and monitoring. Nature Reviews Cancer, 8(5): 268–278.

Logothetis, C.J. & Lin, S.-H. 2005. Osteoblasts in prostate cancer metastasis to bone. Nature Reviews Cancer, 5(1): 21–28.

Marcus, D.M., Goodman, M., Jani, A.B., Osunkoya, A.O. & Rossi, P.J. 2012. A comprehensive review of incidence and survival in patients with rare histological variants of prostate cancer in the United States from 1973 to 2008. Prostate Cancer and Prostatic Diseases, 15(3): 283–288.

McCauley, H.A. & Guasch, G. 2015. Three cheers for the goblet cell: maintaining homeostasis in mucosal epithelia. Trends in Molecular Medicine, 21(8): 492–503.

Montgomery, R.B., Mostaghel, E.A., Vessella, R., Hess, D.L., Kalhorn, T.F., Higano, C.S., True, L.D. & Nelson, P.S. 2008. Maintenance of Intratumoral Androgens in Metastatic Prostate Cancer: A Mechanism for Castration-Resistant Tumor Growth. Cancer Research, 68(11): 4447–4454.

Morgan, T.M., Welty, C.J., Vakar-Lopez, F., Lin, D.W. & Wright, J.L. 2010. Ductal Adenocarcinoma of the Prostate: Increased Mortality Risk and Decreased Serum Prostate Specific Antigen. The Journal of Urology, 184(6): 2303–2307.

National Cancer Institute. 2012. Prostate-Specific Antigen (PSA) Test. Read 19.8.2015. http://www.cancer.gov/types/prostate/psa-fact-sheet

National Cancer Institute. 2015. Prostate Cancer Treatment. Read 25.8.2015 http://www.cancer.gov/types/prostate/patient/prostate-treatment-pdq

NCBI National Center of Biotechnology Information. 2015. PI15 Homo sapiens peptidase inhibitor 15 [(human)]. Read 28.9.2015. http://www.ncbi.nlm.nih.gov/gene?cmd=Retrieve&dopt=Graphics&list_uids=51050

O'Rourke, D.M. & Loughrey, M.B. 2004. Prostate. In Allen, D. C. & Cameron, I. R. (ed.) Histopathology specimens: clinical, pathological and laboratory aspects. New York: Springer London, 311–322.

Pawlina, W. & Ross, M.H. 2011. Histology: A Text and Atlas: With Correlated Cell and Molecular Biology. 6th ed. Philadelphia, PA: Lippincott Williams and Wilkins.

PreAnalytiX. 2015. PAXgene Tissue Container. Read 23.9.2015. http://www.preanalytix.com/products/tissue/fixation-stabilization/paxgene-tissue-container

Rhodes, A. 2013. Fixation of Tissues. 7th ed. In Suvarna, K.S., Layton, C. & Bancroft, J. D. (ed.) Bancroft's theory and practice of histological techniques. Oxford: Elsevier Science Health Science div: 69–93.

Rolls, G. 2010. Microtomy and Paraffin Section Preparation. Leica Microsystems.

Saad, F. & Miller, K. 2015. Current and Emerging Immunotherapies for Castration-resistant Prostate Cancer. Urology, 85(5): 976–986.

SEER Surveillance, Epidemiology, and End Results Program. 2015. SEER Stat Fact Sheets: Prostate Cancer. Read 29.9.2015. http://seer.cancer.gov/statfacts/html/prost.html

Sfoungaristos, S., Katafigiotis, I. & Perimenis, P. 2013. The role of PSA density to predict a pathological tumour upgrade between needle biopsy and radical prostatectomy for low risk clinical prostate cancer in the modified Gleason system era. Canadian Urological Association Journal, 7(11-12): E722–E727.

Shen, M.M. & Abate-Shen, C. 2010. Molecular genetics of prostate cancer: new prospects for old challenges. Genes & Development, 24(18): 1967–2000.

Smith, D.M., Collins-Racie, L.A., Marigo, V.A., Roberts, D.J., Davis, N.M., Hartmann, C., Schweitzer, R., LaVallie, E.R., Gamer, L., McCoy, J. & Tabin, C.J. 2001. Cloning and expression of a novel cysteine-rich secreted protein family member expressed in thyroid and pancreatic mesoderm within the chicken embryo. Mechanisms of development, (102): 223–226.

Suwa, T., Nyska, A., Haseman, J.K., Mahler, J.F. & Maronpot, R.R. 2002. Spontaneous Lesions in Control B6C3F 1 Mice and Recommended Sectioning of Male Accessory Sex Organs. Toxicologic Pathology, 30(2): 228–234.

Takahashi, S., Suzuki, S., Inaguma, S., Asamoto, M. & Shirai, T. 2006. Differences between latent and clinical prostate carcinomas: Lower cell proliferation activity in latent cases. The Prostate, 66(2): 211–217.

Taylor, C.R. 2013. Introduction to Immunohistochemistry. In Taylor, C. R. & Rudbeck, L. (ed.) Immunohistochemical Staining Methods. Denmark: Dako Denmark A/S: 10–19.

Thermo Fisher Scientific. 2015. Immunohistochemistry vs Immunocytochemistry. Read 8.9.2015. https://www.thermofisher.com/fi/en/home/life-science/protein-biology/protein-biology-learning-center/protein-biology-resource-library/pierce-protein-methods/immunohistochemistry-immunocytochemistry.html

Trpkov, K. 2014. Nonneoplastic diseases of the prostate. In Zhou, M. & Magi-Galluzzi, C. (ed.) Genitourinary Pathology: A Volume in the Series: Foundations in Diagnostic Pathology. United States: Elsevier Health Sciences: 1–68.

University of Tampere. 2015. PCRC research Projects - Prostate Cancer Research Center. Read 14.10.2015. http://www.uta.fi/pcrc/group_descriptions.html

VanderWeele, D.J., Brown, C.D., Taxy, J.B., Gillard, M., Hatcher, D.M., Tom, W.R., Stadler, W.M. & White, K.P. 2014. Low-grade prostate cancer diverges early from high grade and metastatic disease. Cancer Science, 105(8): 1079–1085.

Vargas, H.A., Akin, O., Franiel, T., Goldman, D.A., Udo, K., Touijer, K.A., Reuter, V.E. & Hricak, H. 2012. Normal Central Zone of the Prostate and Central Zone Involvement by Prostate Cancer: Clinical and MR Imaging Implications. Radiology, 262(3): 894–902.

Vector Laboratories. 2014. ImmPACT DAB Peroxidase Substrate. [PDF document]. Read 4.9.2015. https://vectorlabs.com/data/protocols/SK–4105.pdf

Yuan, X., Cai, C., Chen, S., Yu, Z. & Balk, S.P. 2013. Androgen receptor functions in castration-resistant prostate cancer and mechanisms of resistance to new agents targeting the androgen axis. Oncogene, 33(22): 2815–2825.

Zou, J., Yin, F., Wang, Q., Zhang, W. & Li, L. 2015. Analysis of microarray-identified genes and microRNAs associated with drug resistance in ovarian cancer. International journal of clinical and experimental pathology, 6(8).

APPENDICES

Appendix 1. Solutions

solution	manufacturer
70 % EtOH	diluted from absolute ethanol: 3500 ml of ethanol to 1500 ml of distilled H_2O
96 % EtOH	Ethanol 96 % vol (VWR Chemicals)
Absolute EtOH	Ethanol absolute (VWR Chemicals)
Xylene	Technical Xylene (VWR Chemicals)
Paraffin	Histoplast (Thermo Scientific)
n-Hexane	n-Hexane for HPLC (VWR Chemicals)
Mayer Haematoxylin	Histolab Mayers HTX
Eosin	Histolab Eosin 0.2 %
Schiff's reagent	Schiff's reagent for microscopy (Merck)
PI15	Rabbit Polyclonal antibody to Protease Inhibitor (peptidase inhibitor 15) TA308594 (OriGene Technologies)
Antibody diluent	ImmunoLogic Normal Antibody Diluent
UIP Max	Histofine Simple Stain MAX PO (MULTI) Universal Immunoperoxidase Polymer Anti-Mouse and -Rabbit
ImmPACT™ DAB	ImmPACT™ DAB Peroxidase Substrate (Diluent and Chromogen concentrate) SK-4105 (Vector Laboratories)

Appendix 2. Buffers

pH 9 Tris-EDTA buffer
0.05 M Tris (Sigma)
0.001 M EDTA Titriplex III (Merck)
pH of 9.0 with 2 M hydrochlorid acid
0.05 % Tween 20 (Sigma)

pH 6 Citrate buffer
0.01 M Sodium Citrate
pH of 6.0 with 2 M HCl
0.05 % Tween 20 (Sigma)

10x TBS 0.5 % Tween
0.2 M Tris (Trizma base) (Sigma)
1.5 M Sodium Chloride (J. T. Baker)
pH of 7.6 with 37 % hydrochloric acid
0.5 % Tween 20 (Sigma)