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University of Turku

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"*A scientist in his laboratory is not a mere technician:*
he is also a child confronting natural phenomena that impress
him as though they were fairy tales"

- Marie Skłodowska Curie

To my family

UNIVERSITY OF TURKU
Faculty of Science and Engineering, Department of Biochemistry
MARJAANA OJALILL: Prostate cancer: the role of extracellular matrix and α2β1 integrin-mediated signaling in stem cell-like cancer cells
Doctoral Dissertation, 138 pp.
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ABSTRACT

Prostate cancer, with an estimated 1.3 million new cases and 360,000 deaths every year, affects elderly men worldwide. Prostate cancer can either develop slowly without symptoms or aggressively by quickly disseminating the disease. To move, cancer cells need to adhere to the extracellular matrix (ECM) and this is crucial for the development of metastases. Prostate cancer cells metastasize mainly to nearby organs and to the bones, where the tissue microenvironment is mainly built by collagens. The stem cell-like cells in prostate cancer are known to express high levels of integrin α2β1, a collagen receptor. Here we studied the role of integrin α2β1 in the motility, survival and signaling of prostate cancer cells. In addition, we analyzed the ECM in the prostate and also the matrix produced by prostate-derived primary fibroblasts *in vitro* using mass spectrometry and proteomics.

This thesis consists of work published in two separate articles and one manuscript. The first part focuses on the role of integrin α2β1 in prostate cancer cells. In the study DU145 cells were treated with the cytotoxic drug docetaxel and analyzed by flow cytometry. Cell sorting, genome editing with CRISPR/ Cas9 and RNA sequencing were also used. In addition, 3D cell culture, migration and invasion assays were used throughout the study. The second part of this thesis focuses on the ECM of the human prostate. Surgically removed prostates were used to extract ECM proteins, which were analyzed by mass spectrometry to study the molecular composition of the matrix. Prostate tissue was also used to isolate primary fibroblasts. The fibroblasts were propagated in cell culture to study their influence on DU145 and PC3 prostate cancer cells *in vitro*. Using this approach we were able to study the ECM produced solely by fibroblasts or in co-culture with cancer cells. We also investigated further the effect of fibroblasts and the ECM on cancer cell motility and observed that fibroblasts induce DU145 cancer cell invasive capability and in co-culture enhance ECM synthesis and its active remodeling.

In conclusion, this thesis showed that α2β1 integrin is essential for cancer cell dissemination. In addition integrin α2β1 downregulates the proliferation of prostate cancer cells and due to this selection pressure favors the $\alpha2\beta1^{low/negative}$ daughter cells of the stem cell like cells. The ECM is synthesized mainly by fibroblasts and the active rearrangement of the ECM is regulated by fibroblast-cancer cell crosstalk.

KEYWORDS: prostate cancer, extracellular matrix, fibroblasts, integrin α2β1, invasion

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Molekulaaristen biotieteiden tohtoriohjelma
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TIIVISTELMÄ

Eturauhassyöpä on yleinen, pääasiassa iäkkäiden miesten tauti. Eturauhassyöpä voi olla hidas ja oireettomasti etenevä, tai nopeasti etenevä ja etäpesäkkeitä muodostava sairaus. Jotta syöpäsolut voisivat liikkua ja muodostaa etäpesäkkeitä, niiden on sitouduttava soluväliaineeseen kollageenireseptori-integriinin välityksellä. Eturauhassyöpä muodostaa etäpesäkkeitä pääasiassa eturauhasen lähellä oleviin elimiin ja luuhun, jossa solujen mikroympäristö sisältää paljon kollageenia. Eturauhassyövän kantasolukaltaiset solut ilmentävät runsaasti kollageenireseptori α2β1-integriiniä. Tässä työssä olen tutkinut α2β1-integriinin roolia eturauhassyövän solujen liikkumisessa, elonjäämisessä ja signaloinnissa. Lisäksi, eturauhasen soluväliainetta ja eturauhasesta eristettyjen fibroblastien tuottamaa soluväliainetta tutkittiin proteomiikan menetelmillä.

Väitöskirjatutkimukseni koostuu kolmesta osatyöstä, joista kaksi on julkaistu kansanvälisissä tieteellisissä julkaisuissa ja viimeinen osatyö on käsikirjoitus. Ensimmäisessä osatyössä keskityttiin α2β1-integriinin rooliin eturauhassyöpäsoluissa. Työssä käytettiin yleisesti saatavilla olevia eturauhassyövän etäpesäkkeisistä eristettyjä solulinjoja DU145 ja PC3, joita käsiteltiin doketakseli syöpälääkkeellä. Tutkimusmenetelminä käytettiin virtaussytometriaa, genomin muokkausta CRISPR/Cas9-menetelmällä sekä RNA-sekvensointia. Lisäksi koko väitöskirjatyössä käytettiin metodeina 3D-soluviljelyä ja solusferoidien migraatio- ja invaasiokokeita. Toisessa osatyössä keskityttiin eturauhasen soluväliaineeseen. Sitä tutkittiin eristämällä soluvälinaineen proteiineja kirurgisesti poistetuista eturauhasista ja analysoimalla soluväliaineen koostumusta massaspektrometrian avulla. Eturauhasista myös eristettiin fibroblasteja ja tutkittiin niiden vaikutusta DU145- ja PC3-syöpäsoluihin. Näin oli mahdollista analysoida sekä fibroblastien yksin tuottamaa soluväliainetta että niiden yhteisviljelmissä syöpäsolujen kanssa tuottamaa soluväliainetta. Lisäksi tutkimme fibroblastien ja soluvälinaineen vaikutusta syöpäsolujen liikkuvuuteen. Tuloksemme osoittavat, että yhteisviljelmissä syöpäsolujen kanssa fibroblastit lisäävät syöpäsolujen liikkuvuutta ja lisäksi soluväliaineen tuotanto ja sen muokkaus lisääntyy.

Yhteenvetona väitöskirjatyön tulokset osoittavat, että α2β1-integriinin ilmentyminen on tärkeää eturauhassyöpäsolujen liikkumiselle. Lisäksi osoitimme α2β1-integriinin hidastavan solujen jakaantumista, mikä selittää α2β1-integriinin vähäisen määrän kantasolujen kaltaisten solujen tytärsoluissa. Pääasiassa fibroblastit tuottavat eturauhasyövän soluväliaineen ja sen aktiivinen muokkaaminen tapahtuu syöpäsolujen ja fibroblastien yhteisvaikutuksesta.

AVAINSANAT: eturauhassyöpä, α2β1-integriini, soluväliaine, invaasio, fibroblastit

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ABSTRAKT

Iga aastal haigestub 1.3 miljonit ja sureb 360 000 meest eesnäärmevähi tõttu üle maailma. See on üks sagedamaid vähkkasvajaid meestel, millesse haigestumise risk kasvab vanusega. Eesnäärmevähi progresseerumine võib olla aeglane ilma sümptomiteta või vastupidiselt agressiivne ja kiiresti metastaseeruv. Eesnäärmevähk levib peamiselt lähedal asuvatesse organitesse, aga ka luudesse, mis koosnevad peamiselt kollageenist. Vähirakud kinnituvad rakuvaheainele ehk ekstratsellulaarsele maatriksile, see on vajalik nende rakkude liikumiseks kudede siseselt ning metastaaside moodustamiseks. Varasematest teadustöödest on teada, et eesnäärme tüvirakud ekspresseerivad palju kollageeni retseptorit - α2β1 integriini. Käesolevas töö peamine eesmärk oli uurida α2β1 integriini rolli eesnäärmevähi rakkude liikumises, elulemuses ja signaaliseerimises. Teiseks töö uurimisobjektiks oli rakuvaheaine: nii eesnäärmes *in vivo* toodetud rakuvaheaine, kui ka koest eraldatud fibroblastide poolt koekultuuris toodetud ekstratsellulaarne maatriks.

Käesolev doktoritöö koosneb kahest artiklist, mis on avaldatud rahvusvaheliselt tunnustatud teadusajakirjades ja ühest käsikirjast. Doktoritöö esimene osa baseerub α2β1 integriini uurimisel kommertsiaalses eesnäärmevähi rakuliinis DU145. Põhilisteks rakendatud meetodideks olid DU145 rakkude käsitlemine tsütotoksilise ravimiga Docetaxel, läbivoolu tsütomeetria analüüs, rakkude sorteerimine, genoomse DNA modifitseerimine CRISPR/Cas9 süsteemi abil ja RNA-sekvenseerimine. Lisaks eelnevale on kogu töös kasutatud 3D rakukultuuri, migratsiooni ja invasiooni eksperimente. Töö teine osa keskendub inimese eesnäärme rakuvaheaine koostise uurimisele. Selleks analüüsisin inimese eesnäärme koest isoleeritud ekstratsellulaarse maatriksi molekulaarset koostist mass spektromeetria abil. Täiendavalt uurisin *in vitro* süsteemis eesnäärme koest eraldatud fibroblastide rakuvaheaine tootmist üksi ja koos DU145 või PC3 vähi rakkudega. Lisaks uurisin põhjalikult fibroblastide ja rakuvaheaine mõju vähirakkude liikumisvõimele. Tulemuste põhjal võib väita, et fibroblastid mõjutavad positiivselt DU145 vähirakkude liikumist kollageenist geelis. Lisaks toimub ko-kultuuris rohke maatriksi valkude tootmine ning nende aktiivne remodelleerimine.

Kokkuvõtvalt, käesolev doktoritöö näitab, et integriin α2β1 on vajalik eesnäärmevähi vähirakkude liikumiseks ja samas vähendab rakkude jagunemist, seetõttu enamikes vähirakkudes ekspressioon väheneb. Eesnäärme rakuvaheaine on põhiliselt toodetud fibroblastide poolt ja selle aktiivne remodelleerimine toimub fibroblastide ja vähirakkude koostööna.

MÄRKSÕNAD: eesnäärmevähk, α2β1 integriin, rakuvaheaine, invasioon, fibroblastid

Contents

Abbreviations

Aa	Ascorbic acid
ABCB1	ATP-binding cassette sub-family B member 1
ABCG2	ATP-binding cassette sub-family G member 2
ALDH	aldehyde dehydrogenase
BC	Basal cell
bFGF	Basic fibroblast growth factor
BM	Basement membrane
CDK	Cyclin dependent kinase
CIC	Cancer initiating cell
CRPC	Castration Resistant Prostate Cancer
CSC	Cancer stem cell
ECM	Extracellular matrix
EGF	Epidermal growth factor
ERK	Extracellular signal-regulated kinase
FA	Focal adhesion
FACS	Fluorescence-activated cell sorting
FAK	Focal adhesion kinase
FC	Flow cytometry
FCS	Fetal calf serum
GAG	Glycosaminoglycan
GAPDH	Glyceraldehyde-3-phospate dehydrogenase
GFRs	Growth factor receptors
HA	Hyaluronan / Hyaluronic acid
HGF	Hepatocyte growth factor
HGFR/c-Met	Hepatocyte growth factor receptor
IGF	Insulin-like growth factor
MAPK	Mitogen-activated protein kinase
MDR1	Multidrug resistance protein 1
MMP	Metalloproteinase
MS	Mass spectrometry
MSC	Mesenchymal stem cells
NF-κB	Nuclear factor kappa B

NNGH	N-Isobutyl-N-(4-methoxyphenylsulfonyl)-glycylhydroxamic Acid
p38 MAPK	Protein 38 mitogen-activated protein kinase
PCSCs	Prostate cancer stem cells
PG	Proteoglycan
PIN	Prostate intraepithelial neoplasia
PLL	Poly-L-lysine
PRC	Prostate cancer
PSA	Prostate specific antigen
PSC	Prostate stem cell
RALP	Robotic-assisted laparoscopic prostatectomy
RTK	Receptor tyrosine kinase
SC	Stem cell
Sca1	Stem cell antigen 1
SLRP	Small leucine-rich proteoglycan
SPARC	Secreted protein, acidic and rich in cysteine
TA	Transit amplifying cell
T-ALL	T cell acute lymphoblastic leukemia
TGF-β	Transforming growth factor-β
TIMP	Tissue inhibitor of metalloproteinases
TME	Tumor microenvironment
Trop2	Tumor-associated calcium signal transducer 2
VE-cadherin	Vascular endothelial cadherin
VEGF	Vascular endothelial growth factor
VM	Vasculature mimicry
Z-FY-CHO	N-(benzyloxycarbonyl)-L-phenylalanyl-L-tyrosinal
α-SMA	α-smooth muscle actin

List of Original Publications

This thesis is based on the following original publications, which are referred to using the Roman numerals, I – III:

I Ojalill M, Parikainen M, Rappu P, Aalto E, Jokinen J, Virtanen N, Siljamäki E, Heino J. Integrin α2β1 decelerates proliferation, but promotes survival and invasion of prostate cancer cells. Oncotarget 9: 32435-32447, 2018.

II Ojalill M, Rappu P, Siljamäki E, Taimen P, Boström P, Heino J. The composition of prostate core matrisome in vivo and in vitro unveiled by mass spectrometric analysis. The Prostate. 78: 583–594, 2018.

III Ojalill M, Virtanen N, Rappu P, Siljamäki E, Taimen P, Heino J. Interaction between prostate cancer cells and prostate fibroblasts leads to the accumulation of basement membrane proteins and enhanced cell invasion. Manuscript.

1 Introduction

Adenocarcinoma of the prostate is a common cancer type in elderly men. The aggressive form of the disease, called castration-resistant prostate cancer (CRPC), has limited treatment options and remains incurable. One of the main mechanisms of invasive, treatment-resistant malignancies is suspected to be the existence of cancer stem-like cells (CSCs). Prostate tissue stem cells and also CSCs can be identified using integrin α2β1. However, the expression of integrin α2β1 has been shown to disappear in high Gleason score cases, especially in poorly differentiated adenocarcinomas with high Gleason score (Mirtti et al., 2006). As α2β1 integrin is a collagen receptor, one would assume that ECM, especially the collagenous matrix is important for CSCs.

The purpose of this study was to investigate the role of integrin α2β1 in prostate cancer. More specifically, the effect of integrin α2β1 on DU145 cell proliferation, motility, drug resistance and gene expression was analyzed by genomically alterating integrin α2. In addition matrisome of human prostate tissue was studied using mass spectrometry to determine possible ligands for integrin α2β1 in prostate ECM. For *in vitro* studies, human primary prostate fibroblast cells were isolated and primary cell lines were created. Matrisome of primary human fibroblasts, DU145 and PC3 cancer cells were studied in an individual and in a co-culture system.

2 Review of the Literature

2.1 Prostate tissue cellular organization

Prostate tissue contains epithelial acini surrounded by fibromuscular stroma. The epithelial compartment of the mature prostate consists of several cell types: differentiated luminal cells, which form the major cell type (60% of epithelial cells) and are responsible for the secretory properties producing seminal fluid; basal cells (40% of epithelial cells), and rare neuroendocrine cells that are present in both the luminal and basal cell layers. Basal cells reside on the basement membrane (BM) and can be divided into 3 sub-populations: stem cells (SC), transit amplifying (TA) cells and committed basal cells (BC). Stem cells can give rise to transit TA cells, which can further differentiate into non-secretory BC, and these are able to differentiate into luminal cells (Figure 1). The first evidence that prostate tissue contains non-differentiated progenitor cells came in the 1980s, when experiments with rodent prostates showed multiple rounds of castration-induced regression and androgen-induced regeneration of prostate tissue (English, Santen, and Isaacs 1987). A year later, this idea about progenitor cells was further supported by the discoveries that BC express low/undetectable levels of the androgen receptor (AR) and are independent of androgens, whereas differentiated luminal cells are dependent on AR signaling (Kyprianou and Isaacs 1988).

In a normal prostate, the highest expression of integrin α2β1 is detectable in basal cells, which use it to bind to BM components, including laminins and collagen IV. BC differentiation into luminal cells is accompanied by a loss of adhesion to the basal membrane and a decrease in integrin α2β1 expression. At the same time, the increased expression of AR in luminal cells supports the idea that in a normal prostate cell, adhesion to the BM and the expression of AR are separated to different cell layers. Adhesion to the BM facilitates survival and proliferation through the transduction of stromal signals, whereas androgen signaling triggers the secretion of protein and maintains the viability of luminal cells (Knudsen and Miranti 2006).

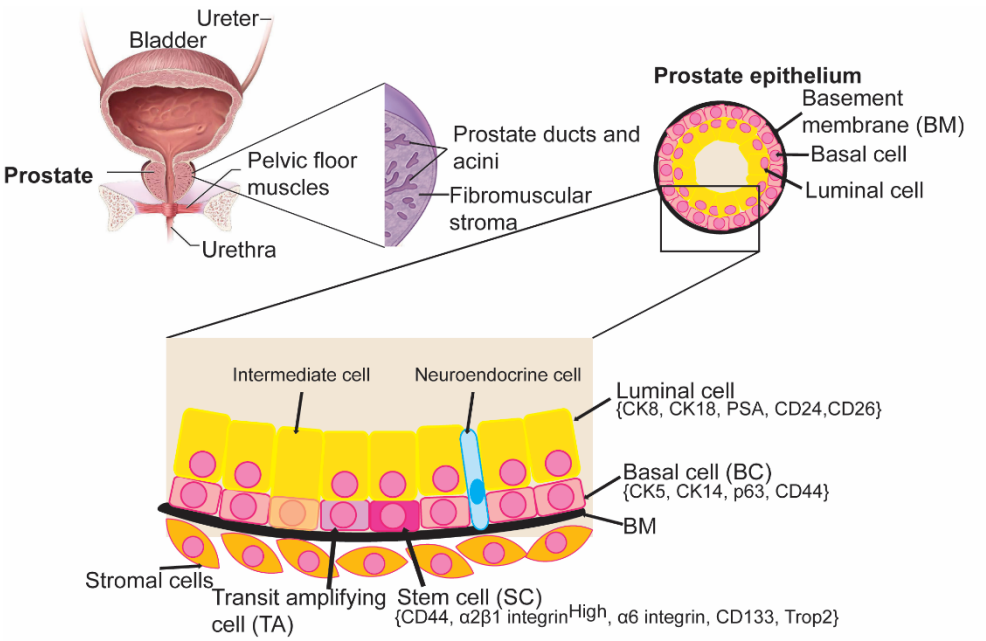

Figure 1. Schematic diagram showing the anatomical location of the prostate, tissue structure and the cellular composition of prostate epithelium. The prostate, an essential part of the male reproductive system, is located in front of the rectum and below the bladder. Prostate tissue consists of epithelial ducts and acini surrounded by fibromuscular stroma. Prostate epithelium consists of an inner layer of differentiated secretory luminal cells. Tissue stem cells (SC), transit amplifying (TA) and committed basal cells (BC) reside continuously around luminal cells in contact with the basement membrane. Neuroendocrine cells and intermediate cell (expressing both BC and luminal cell markers) are located on both layers. Modified from https://www.flickr.com/photos/ nihgov/25137782099/ and Rybak et al., 2015 .

More recently it was shown in developmental studies, that cells expressing basal progenitor marker p63 could develop the entire epithelial cell compartment, with both basal and luminal cells. This suggests that in adult tissue, p63-positive cells may also represent progenitor cells that are able to give rise to more differentiated cells when necessary due to, for example, tissue injury (Signoretti et al., 2005).

Murine and human prostates are somewhat different. In rodents, prostate SCs are located in the distal region (Kinbara et al., 1996) whereas in human, prostate SCs are located randomly throughout the acini and ducts (Collins et al., 2001). It is known that SCs are located in a specialized environment, called a niche, which supports the balance between quiescence and self-renewal through regulating asymmetric cell division. Several cell surface markers have been used to identify human prostate stem cells (PSCs), for example, a high expression of integrin α2β1 on a subset of BCs and rapid adhesion to collagen I (Collins et al., 2001). In multiple niches, β1-integrin mediated signaling has been shown to be important. For example, in the mammary gland β1-integrin is essential for maintaining the

stem cell population and regulating asymmetric division (Taddei et al., 2008). In another study, prostate sphere-forming cells were restricted to a basal phenotype and shown to express CD44, a receptor for the extracellular matrix component hyaluronan, α6 integrin, a laminin receptor, and Trop2 (Garraway et al., 2010). CD133, typically expressed by hematopoietic and neural SCs (Yin et al., 1997), has been shown to be expressed also by a subset (less than 1%) of prostate BCs. These cells generated prostate acini in vivo, consisting of epithelium with both basal and luminal cells (Richardson et al., 2004). Conclusively, murine prostate SC could be identified based on the expression of p63, Sca-1, CK5, α6 integrin, Trop2, CD133 (Lawson et al., 2006; Barclay et al., 2008; Tani et al., 2000; Kinbara et al., 1996) and human prostate SC based on the surface markers CD44, α2β1 integrinHigh, α6 integrin, CD133 and Trop2 (Collins et al., 2001; Richardson et al., 2004; Yamamoto et al., 2012; Trerotola et al., 2010; Goldstein et al., 2008; Taylor et al., 2012).

2.2 Prostate cancer statistics

The statistics for prostate cancer (PRC) show that it is a major health problem all over the world. Globally, PRC is the second most diagnosed cancer, having approximately 1.3 million new cases, and the 5th cause of cancer-related deaths in men, being responsible for 360,000 deaths per year (Bray et al., 2018). In Finland, 5,444 new cases were diagnosed in 2017 and the number of PRC-related deaths was 912 (cancerregistery.fi, Finnish Cancer Registry). In Europe approximately 417,000 citizens were diagnosed with PRC and 92,200 men died from the disease in 2012. Similarily, figures for the United States in 2018 were 164,690 new cases and 29,430 deaths (European Network of Cancer Registries). There is a strong likelihood that the high number of new incidences has been influenced by the intensive use of prostate specific antigen (PSA) testing. This early detection along with diagnostics should lead to a drop in the incidence and prevelence of new cases within a few years.

Most PRCs (about 91%) are discovered at the local stage. These cases have a good prognosis, with a 5-year survival rate of almost 100%. To ensure the best outcome for patients, it is important to distinguish slow growing cases from aggressive carcinomas to avoid overtreatment and intervene with suitable treatment as early as possible. To predict the prognosis of the patient clinicians consider the stage of the disease, histological Gleason score and the level of PSA. At later stages of the disease, when cancer cells have disseminated and formed metastasis into other tissues, the treatment is less effective and the 5-year survival rate decreases drastically to 30% (Wallace et al., 2014).

2.3 Prostate cancer progression and treatment

Prostate cancer is usually described as a stepwise process, starting from prostate intraepithelial neoplasia (PIN), followed by local prostate cancer which is found only inside of prostate capsule and locally advanced carcinoma affecting nearby organs. Invasive adenocarcinoma, in which cancer cells have disseminated into lymph nodes or metastasized into bones finally results in Castration Resistant Prostate Cancer (CRPC). The pre-malignant state of prostate cancer, called PIN, is described as hyper-proliferation of the prostate epithelial cells. Based on different studies, both luminal and basal epithelial cells can give rise to PRC (Taylor et al., 2012; Wang et al., 2014; Park et al., 2016). Prostate cancer heterogeneity can be partially explained by the fact that different cell types within the prostate epithelia can be vulnerable to mutations and through this become a cell-of-origin. However, most prostate cancers are classified as acinar adenocarcinomas with the luminal phenotype characterized by the absence of basal markers p63, cytokeratins 5 and 14. The less common PRCs include squamous cell carcinoma and small cell carcinoma (Humphrey 2012). At the initial stage of prostate adenocarcinoma, the BM is destroyed after which malignant tumor cells may directly interact with the prostatic stroma. These tumors that lie within the prostate capsule, called local prostate cancers, can be treated by radiotherapy or with radical prostatectomy (the complete removal of the prostate) and the 5-year survival prognosis for patients is almost 100%. Once cancer cells disseminate to nearby organs such as seminal vesicles and the rectum the disease is called locally advanced cancer. Metastatic prostate cancer disseminates mainly to the bones and to the lymph nodes. Hormone therapy is the first choice of treatment for disseminated prostate cancer to inhibit the proliferation of malignant cells. Although androgen deprivation therapy reduces the size of the tumor and metastatic lesions, with longer treatment periods the cancer cells often become androgen independent (Armstrong and Gao 2015). Prostate cancer is known to be a very heterogeneous disease (Robinson et al., 2015). This heterogeneity can be attributed to genomic alterations, such as copy number alterations, somatic mutations and DNA rearrangements. As part of the patient prognosis, the tumor genomic and micro-environmental heterogeneity should be considered and used (Lalonde et al., 2014). Many studies have unveiled several mechanisms that explain how cancer cells become androgen independent. These mechanisms include mutations in the AR gene or its amplification, ligand independent activation of AR by crosstalk with receptor tyrosine kinases (RTKs) and unbalanced regulation of co-receptors and -activators (Harris et al., 2009). In addition, one possible mechanism is based on cancer stem cells (CSCs) (Collins et al., 2005), which are both AR independent and also able to produce subpopulations of AR-sensitive and independent clones. This could also explain the heterogeneity of cancer cells found in autopsy samples of metastatic prostate cancer (Shah et al.,

2004). The treatment options for CRPC include microtubule targeting drugs, such as the widely used chemotherapeutics docetaxel and cabazitaxel (de Leeuw et al., 2015). However, cancer cells have mechanisms to develop drug resistance, for example by increasing the expression of nuclear factor kappa B (NF-κB) (O'Neill et al., 2011) or by increasing the efflux of drugs to the extracellular space mediated by multidrug resistance protein 1 (MDR1) or ATP-binding cassette sub-family B member 1 (ABCB1) (Armstrong and Gao 2015). Despite the many treatment options available to patients, the prognosis for metastatic CRPC is poor, which encourages further research aiming to identify new treatment options.

2.4 Cancer stem cells

The hypothesis that tumors contain a small population of cells which provide the heterogeneity, hierarchical organization and development of treatment-resistant disease has shown to be correct for many human malignancies. These cells are referred to interchangeably as cancer stem-like cells, tumorigenic cells, cancer initiating cells (CICs) or cancer stem cells (CSCs). CSCs were first identified in human acute myeloid leukemia (Bonnet and Dick 1997), after which their populations have been described in many tumors, for example in the breast (Al-Hajj et al., 2003), brain (Singh et al., 2004), lung (Ho et al., 2007), skin (Schatton et al., 2008) and prostate (Collins et al., 2005). Despite the numerous publications characterising the stemness, long survival and self-renewing capability of these cells in different cancer types, the stem-like cells in cancer are still controverial, as studies do not consistently indicate asymmetric cell-divisions and all other stem cell properties. The tumor stem cell model contradicts the stochastic model, which proposes that all cells in a tumor could initiate a new tumor, whereas the hierarchical model of cancer proposes that only CSCs are tumorigenic (Figure 2A). Malignant cells, with stem cell properties display a tumor-promoting ability, are able to self-renew during long periods and survive radio- and chemotherapy. Overall, CSCs are capable of maintaining tumor homeostasis and of generating heterogeneous sub-populations of cancer cells, to drive the progression of disease and to initiate metastases. Collectively, stem cells in cancer have enhanced plasticity, altered self-renewal and long-term tumorigenic properties.

The stem cell population in normal tissue is maintained by the balance between the renewal and quiescence of the cell. SCs are usually in quiescent state and proliferate only in cases of tissue renewal or tissue damage. It has been suggested that CSCs share this property, at least partially, which could explain the difficulties targetting them and explaining the enrichment of CSCs after chemotherapy. The quiescent cells in cancer, also called dormant or persistent cells, are often undetectable and can survive for long periods despite treatment. The dormant cells share the properties of stem cells, like low cell proliferation and resistance to drugs,

which in turn cause the enrichment of these dormant cells during drug treatment (Nan et al., 2014). Furthermore, the ability of the CSCs to be dormant or quiescent and to resist cytotoxic drugs may explain relapses after initially successful treatment (Figure 2B), even decades after the removal of a primary tumor. The dormant cells are not active in the cell cycle, instead they have been shown to remain in G0/G1 state (Touil et al., 2014).

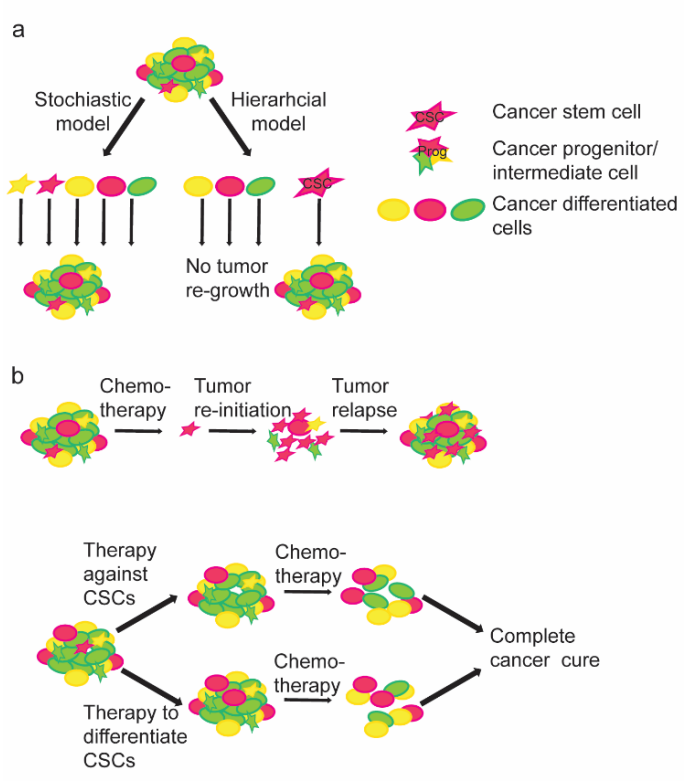

Figure 2. Tumor heterogeneity and CSCs.

A, two models, stochastic and hierarchical, have been developed to explain the cellular heterogeneity of tumors. In the stochastic model all cancer cells are equal in their tumorigenic potential. In the hierarchical model CSCs are on top of hierarchy, being able to grow new tumors and cause heterogeneity by division generating progenitor cells, which further divide into differentiated cancer cells.

B, CSCs may be dormant, resistant to chemotherapy and tumorigenic, which makes them the major reason for relapse. Targeting existing therapies for CSCs or directing CSCs to differentiate would create a novel type of therapy.

To exit the dormant state, cells need external stimuli and the activation of cyclin dependent kinases (CDKs). The signals needed for dormant CSCs to start to proliferate are poorly understood. It is likely that the balance between stimulatory and inhibitory signals has to be disrupted to trigger proliferation. Like normal tissue SCs, it has been proposed that CSCs are located in specialized areas of the tumor microenvironment, called niches (Plaks et al., 2015) (Figure 3). Studies have shown that certain growth factors, for example transforming growth factor β (TGF-β) (Salm et al., 2005) and hepatocyte growth factor (HGF) signaling (Nishida et al., 2013) are important for prostate CSCs. Still, the niche of CSC in the prostate is poorly understood. CSCs are similar to tissue stem cells in that they are considered to be capable of symmetric and asymmetric divisions. The importance of Notch

signaling has been indicated in colon CSCs (Bu et al., 2013) and distribution of CD133 between daughter cells determines the symmetric or asymmetric division (Lathia et al., 2011). Cell plasticity (epithelial to mesenchymal transition) and the microenvironment may also induce the de-differentiation of a malignant cell and this process provides another potential source for CSCs (Chaffer et al., 2011; Mani et al., 2008). CSCs should be considered as non-static populations of cells with a certain potential to contribute to cancer development (Shackleton et al., 2009). Eliminating CSCs or preventing the differentiation of CSCs could be a successful approach in many cancers. In addition, specifically targeting prostate CSCs by using cell surface marker proteins has been proposed as a novel therapeutic strategy.

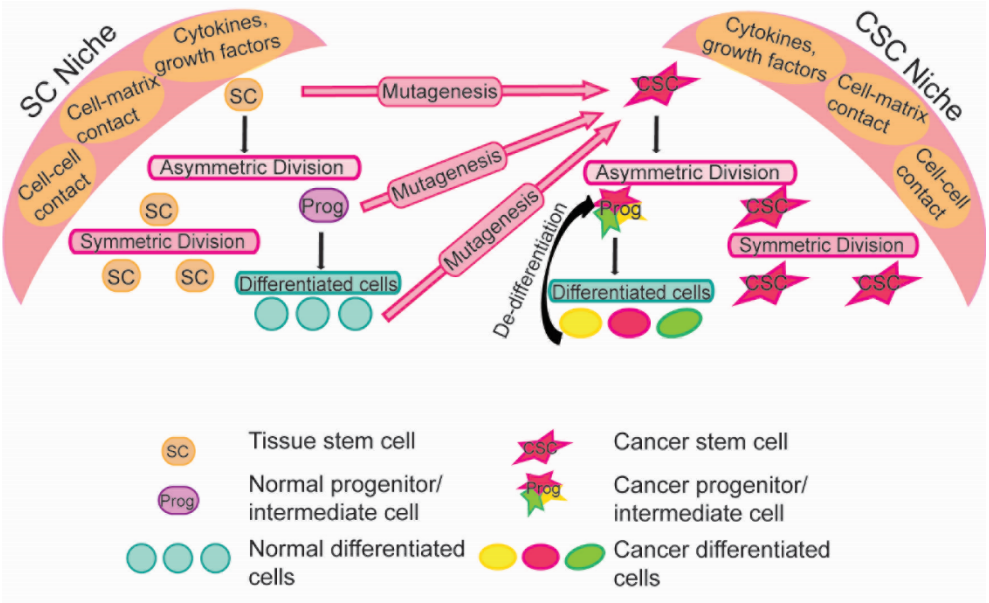

Figure 3. Stem cells (SC) and cancer stem cells (CSC) are supported by the niche. The phenotype and both symmetric and asymmetric cell division could be regulated by the niche. All cells, both normal and cancerous, may be progenitors of CSCs through mutagenesis.

2.4.1 Prostate cancer stem cells

Prostate cancer stem cells (PCSCs) share many marker proteins with prostate tissue SCs. These include the cell surface proteins: CD44, α2β1 integrinHigh, CD133, and Trop2, which have been used alone and in combination. Other markers proposed for PCSCs are CD166, aldehyde dehydrogenase (ALDH) and the ATP-binding cassette sub-family G member 2 (ABCG2), PSA, NANOG or “side populations” identified based on Hoechst dye staining (Skvortsov et al., 2018).

Methodologies, such as fluorescence-activated cell sorting (FACS) and magnetic cell sorting (MACS), have mainly contributed to the research into marker protein-based CSC research. However, many shortcomings presist in CSC and their niche research, which can predominantly be attributed to technological challenges. The problem with using cell surface marker proteins to identify PCSCs is that these proteins are not exclusively expressed only by PCSCs, but also by normal tissue stem cells and on cancer cells of another type. Furthermore, the extensively used transplantation assays used to show long-term survival and the re-population of tissue have several problems (Plaks et al., 2015).

2.5 Integrins

Adhesion to the surrounding ECM is essential for many physiological processes, like embryonic development and tissue homeostasis, and often altered under pathological conditions, such as inflammation or tumor development. Integrins are a group of transmembrane adhesion receptors that consist of 18 α-subunits and 8 β-subunits. Each subunit is a glycoprotein consisting of a large globular extracellular domain, transmembrane domain and usually a small intracellular tail. Humans have 24 different functional integrin receptors, which are composed of one α and β subunit linked by non-covalent interactions. The heterodimers can be divided into subfamilies based on their ligands or on their phylogenic origin. The subfamilies comprise 1) collagen-binding integrins, 2) laminin-binding integrins, 3) RGD-motif binding integrins and 4) leukocyte specific integrins 5) a subgroup formed by α4 and α9 integrins (Johnson et al., 2009). Based on the structure of the α-subunit head domain, integrins can be divided into 2 groups: integrins containing an αI-domain and itegrins without it. Integrin αI-domain is also reffered to as the von Willebrand factor A-like domain or shortly as the αA domain based on the similarity. Integrins with an αI-domain bind to their ligands through the αI-domain whereas integrins which lack an αI-domain bind to their ligands using the interface of the βI-domain and the β-propeller domain in the α-subunit (Campbell and Humphries 2011). Although some integrins have only one known ligand (for example α6β4 binds to laminin), many integrins can bind to several ECM proteins (for example α2β1 binds to Collagen I, II, III, IV, V, VII, VIII, IX, X, XVI, XXIII, laminins, osteopontin and tenascin) (Heino 2007). The expression pattern of integrins on a cell surface and the selection of the ligands bound can determine the cell behavior in response to the surrounding microenvironment. As soon as the extracellular part of an integrin is bound to an ECM protein and the integrin tail is connected to the cytoskeleton the cells can obtain mechanical and chemical signals that regulate cellular responses, such as, growth, migration, differentiation and survival.

Ligand binding induces conformational changes in the receptor structure and consequently the intracellular parts of α and β units, also calles “legs” move apart from each other. Small cytoplasmic tails of integrin subunits are well conserved and crucial for regulating the activation of integrins (Calderwood 2004). The space created by the β-subunit conformational change between the tails of the α and β subunits enables various intracellular signaling proteins and cytoskeletal adaptor proteins to bind. The activation of integrins can happen either by binding the extracellular ligand and signaling outside-in or the integrin can be activated from the inside to bind the ligand on the outside of the cell (Campbell and Humphries 2011). Once integrins are activated by ligand binding they can cluster to form focal adhesions.

Integrin-based macromolecular complexes “bridge” ECM to the actin and through actin microfilaments connect it to a nuclear envelop. This is one of the mechanisms through which integrins mediate mechanical signals into cells. Integrins are also known to use chemical signals in cell regulation, for example, they activate focal adhesion kinase (FAK), tyrosinekinase Src and phosphoinositide 3-kinase (PI3K) with their downstream signaling pathways. The mechanosensing and chemical signals mediated by integrins and focal adhesions also regulate the responses to the extracellular force and stiffness of the matrix. Cells continuously modify the extracellular mileu based on these signals.

2.5.1 The collagen binding integrins

The subfamily of collagen-binding integrins consists of four α-subunits: α1, α2, α10 and α11. All four have an αI-domain in their α-subunit and can only form heterodimers with β1-subunits. As the name collagen-binding indicates, the main ligands for all four receptors are members of the large collagen family. These receptors use their αI-domain to recognize distinct collagens with varying affinities, but all of them can specifically recognize GXX´GER-like motifs, such as GFOGER (single letter amino acid nomenclature, O = hydroxyproline) motif in collagen triple helices (Knight et al., 2000; Zhang et al., 2003). Integrins α1β1 and α2β1 are the main collagen receptors, whereas α10β1 and α11β1 were discovered later and knowledge about these integrins is updated continuously. While α2β1 is expressed on platelets, endothelial, epithelial, some mesenchymal and certain inflammatory cells, integrin α1β1is expressed on fibroblasts, smooth muscle cells, lymphocytes and chondrocytes. Integrin α10β1 is also expressed by chondrocytes (Camper, Hellman, and Lundgren-Åkerlund 1998). Integrin α11β1 is known to be expressed on mesenchymal stem cells (MSCs) (Tiger et al., 2001) and to be an important integrin for fibroblastic cells (Zeltz et al., 2014). Integrin α11β1 expression on cancer associated fibroblasts regulates matrix stiffening in response to TGF-β (Carracedo et al., 2010). Recent studies support the role of integrin

α11β1 in several malignancies, including lung, breast and prostate cancer progression (Smeland et al., 2019; Reigstad et al., 2016; Navab et al., 2016).

When comparing the similarity of collagen-binding integrin α-subunits, then α1 and α2 are most closely related; α11 share the highest similarity to α10 (42%) and form a separate branch in the protein phylogenetic tree based on the alignment of the protein amino acid sequences (Velling et al., 1999). However, in ligand preferences α1β1 and α10β1 resemble each other and bind to BM collagen IV with higher affinity than they bind to fibril-forming collagens. In contrast, α2β1 and α11β1 tend to prefer binding to fibril-forming collagens (Tuckwell, et al., 1995; Tiger et al., 2001; Jokinen et al., 2004; Tulla et al., 2008; Lahti et al., 2011).

Studies on knockout mice for all four collagen-binding integrin α-subunits have been published. Integrin α1 was the first for which a knockout was made. Mice without integrin α1 develop normally and are fertile. The isolated embryonic fibroblasts (EFs) fail to attach to type IV collagen and have decreased binding to laminin, but no defects in spreading on collagen I. The results from this study indicated that to a great extent, the attachment to type IV collagen is mediated by α1β1, but for binding to collagen I EFs might have another integrin in addition to α1β1 and α2β1 (Gardner et al., 1996). Next, knockout mice for integrin α2 were generated; these were also viable, fertile and seemed to develop normal. However, some abnormalities have been discovered, for example markedly reduced mammary gland development (J. Chen et al., 2002) and increased breast cancer metastasis which have been connected with the diminished expression of integrin α2β1 (Ramirez et al., 2011). Integrin α2β1 is expressed on platelets, so it has been assumed that the knockout of α2 integrin has a major role in hemostasis. However, platelets from α2 knockout show delayed, but not reduced attachment to collagen type I. This suggests that integrin α2β1 has a supportive, not essential role in normal homeostasis (Holtkötter et al., 2002). The mild cartilage defects found in α10β1 knockout mice were assumed, since chondrocytes primarily express this integrin in vivo (Bengtsson et al., 2005). More recently, the nonsense mutation in the ITGA10 gene was discovered to cause the diminished expression of integrin α10β1 resulting in chondrodysplasia in dogs (Kyöstilä et al., 2013). Integrin α11β1 knockout mice are viable but display dwarfism with increased mortality (Popova et al., 2007). Integrin α11β1 is expressed mainly on MSCs and on a subset of fibroblasts. TGF-β binding to its receptor induces Acta2 (which encodes αSMA), connected with myofibroblast differentiation and also up-regulates α11β1 integrin, which is needed to stabilize α-SMA protein expression (Carracedo et al., 2010). In addition, integrin α11β1 participates in collagen reorganization, which explains the wound healing defects indicated in α11β1 knockout mice (Schulz et al., 2015). Thus, collagen-binding integrins have no functions that are critical for normal homeostasis; however in conditions such as inflammation, wound healing and

tumorigenesis collagen-binding integrins may play a more crucial role (Zeltz and Gullberg 2016).

2.5.2 Integrin α2β1 mediated signaling

Although integrin α2β1 does not have kinase activity, it is able to activate several kinases, for example, FAK, Akt/protein kinase B, PI3K (Holtkötter et al., 2002) and protein 38 mitogen-activated protein kinase (p38 MAPK) (Ivaska, et al., 1999). In addition, integrin α2β1 also activates protein serine/threonine phosphatase 2A (PPA2), p27KIP1 and the Rho family GTPases (Ivaska et al., 2002; Henriet et al., 2000). The following shortly explains how integrins activate kinases and their importance on cell regulation.

FAK is an intracellular non-receptor tyrosine kinase, which associates with receptors at the plasma membrane. The classical mechanism of FAK activation is initiated by integrin clustering and the formation of focal adhesion complex after cells have bound to the ECM (Mitra and Schlaepfer 2006). The activation of FAK requires its dimerization and auto-phosphorylation at Y397 (Brami-Cherrier et al., 2014). FAK phosphorylation at Y397 allows Src-kinase and PI3K to bind through the SH2-domain. Further phosphorylation of the FAK residues 576 and 577 is induced by Src to create more binding sites and trigger downstream signaling. Other proteins which can bind to FAK are paxillin, talin vinculin, Grb2, p130Cas, and Shc, which become activated through interaction with FAK and participate in the regulation of cell migration and proliferation. Besides the important role of FAK as a kinase, it also has crucial scaffolding properties. FAK-mediated cell cycle regulation and responses to stress take place through its interactions with p53 and MDM2 (Golubovskaya et al., 2008, 2013). FAK can activate the Akt pathway, but β1 integrins also directly induce the phosphorylation of Akt at Ser473 and Thr308 in a PI3K-dependent manner (Velling et al., 2004).

The MAPK pathway connects integrin α2β1 to the regulation of the cell cycle. As such, the balance between the stress-regulated p38 pathway and mitogen activated ERK signaling can regulate cell cycle progression and switch from dormancy to active proliferation (Aguirre-Ghiso et al., 2001). Recently it was shown that one specific subtype of p38 MAPK, namely gamma (p38γ), could act as a CDK-like kinase and cooperate with CDKs to regulate entry into the cell cycle (Tomás-Loba et al., 2019).

Integrins crosstalk with many growth factor receptors (GFRs). Well-characterized examples are the epidermal growth factor (EGF), the vasculature endothelial growth factor (VEGF), the insulin-like growth factor (IGF), and the hepatocyte growth factor receptor (HGFR / c-Met) (Ivaska and Heino 2011). Several types of crosstalk exist. Integrins have shown to directly bind to GFRs, and as well as having the potential to activate GFRs even when the GF itself is not

present, integrins can also amplify GFR-induced signals through binding to the ECM proteins and activating the same signaling pathway in addition to GFRs. An example of such crosstalk is the interplay between TGF-βR and integrin α2β1, in which integrin-collagen interaction changes growth factor signaling (Garamszegi et al., 2010). In addition, many GFs can also induce the expression of the integrins and in this way regulate cell-ECM signaling. HGF signaling through its receptor c-Met is known to induce integrin α2 expression (Chiu et al., 2002) and the interaction between integrin α2β1 with c-Met regulates innate immunity (Mccall-Culbreath et al., 2008). Anchorage independent growth can also be induced by interaction between α2β1 integrin with c-Met to facilitate tumorigenesis (Barrow-McGee et al., 2016). Intergins also crosstalk with non-classical GFRs, for example the Eph receptor. Ephrin signaling induces the adhesion of PC3 cells on collagen type I through β1 integrin (Yu et al., 2015).

The signals induced by GFR and integrins can be GF and matrix protein specific. Although the binding of integrin α5β1 to fibronectin results in cell cycle progression, when integrin α2β1 binds to the laminin in the same cells, it induces growth arrest (Mettouchi et al., 2001).

In normal cells, integrins mediate strong survival signals and the GF-induced signal can remain hidden. Once integrin signals are removed cells undergo growth arrest and anoikis because of the impaired signals from GFs and cytokines (Danen and Yamada 2001). To avoid anoikis, the necessary signals include the interaction of the integrins with ECM proteins, the activation of FAK and the downstream signaling of the PI3K-Akt pathway (Frisch and Ruoslahti 1997). Recent studies have shown that integrins can also activate FAK in endosomes to help cells avoid anoikis (Alanko et al., 2015). Studies into tumor cells have opened up the signaling to avoid anoikis. In contrast to normal cells, a population of invasive cancer cells is resistant to anoikis. For these cancer cells detachment from the ECM and neighboring cells does not induce cell death, while the cell leaves the primary site and travels through the lymphatic or vasculature vessels to a distant site. Metastatic malignant cells have developed numerous ways to escape from anoikis, a hallmark of metastatic malignancy. These mechanisms are somewhat similar to those seen in drug resistance and include the upregulation of kinases (Douma et al., 2004) and scaffolding molecules (Parsons et al., 2009), the suppression of apoptotic pathways (Simpson, Anyiwe, and Schimmer 2008), and the downregulation of critical anoikis regulators seen in normal epithelial cells (Rohwer et al., 2008). Furthermore, cells that are able to survive anoikis, have been shown to be harder to target due to the decreased levels of p53 in the cells detached from the ECM (Truong et al., 2003).

2.5.3 Regulation of α2β1 integrin in prostate cancer

The fact that bone matrix is composed of various collagens makes it one of the main metastatic locations in prostate cancer. Integrin α2β1, the main cellular receptor for collagens, is very attractive as a target for disseminating prostate cancer. However, published data about the expression of integrin α2 during prostate cancer development is controversial.

Firstly, Bonkhoff and colleagues indicated that α2 integrin expression is downregulated in Grade I and II PC; in Grade III integrin expression was shown to be highly heterogeneous while corresponding lymph node metastasis had an increased expression of integrin α2 (Bonkhoff et al., 1993). Our group has also shown that integrin α2β1 expression at an mRNA level decreases during cancer progression, being lowest in high-grade carcinomas. At protein level, the same effect was shown, the strongest α2 staining was seen in luminal epithelial cells and staining decreased in PIN and further in parallel with poor differentiation of the prostate carcinomas (Mirtti et al., 2006). The possible differential expression of α2 integrin could be obtained by epigenetic regulation (Chin et al., 2015). Lymph node metastasis of prostate cancer also has controversial findings. A noticeable expression of integrin α2β1 in disseminated cancer cells was reported by (Bonkhoff et al., 1993). Conversely, prostate cancer lymph node metastasis have been indicated to have low integrin α2β1 expression levels (Pontes-Junior et al., 2009) in another study. Functionally the role of integrin α2β1 could be tissue specific as studies have confirmed that prostate cancer cells that are able to initiate micro-metastasis have to have integrin α2β1 expression (Kostenuik et al., 1996; Sottnik et al., 2013). However, in breast cancer the integrin α2β1 has been proposed to have a metastasis-suppressor role (Ramirez et al., 2011).

2.5.4 α2β1 integrin in prostate cancer stem cells

Several studies have indicated that integrin α2β1 is essential in prostate cancer dissemination. The information that integrin α2 expression is downregulated in prostate tumors does not exclude the possibility that a high expression in a subset of tumor cells, especially CSCs, is important for metastatic behavior, especially in collagen I rich bone microenvironment.

2.6 Tumor microenvironment

Cancer cells are not isolated from the surrounding tissue, called tumor stroma or the tumor microenvironment (TME). The TME is continuously evolving together with oncogenic signals from malignant cells and with the normal cells present in the area of malignant growth. One cannot underestimate the role of TME in

metastasis formation from the very first transformed cell division. Cancer cells secrete cytokines and chemokines to support their own growth in an autocrine manner. They activate nearby stromal cells, recruit immune cells and trigger angiogenesis. These events alter the normal homeostasis of the tissue and modify the TME. The TME is a niche for cancer cells to proliferate and to create local or distant metastasis. Figure 4 depicts the TME with all the included cell types. The contribution of these cells and processes regulating cancer growth and spreading have been covered in numerous reviews (Hanahan and Weinberg, 2011; Balkwill et al., 2012).

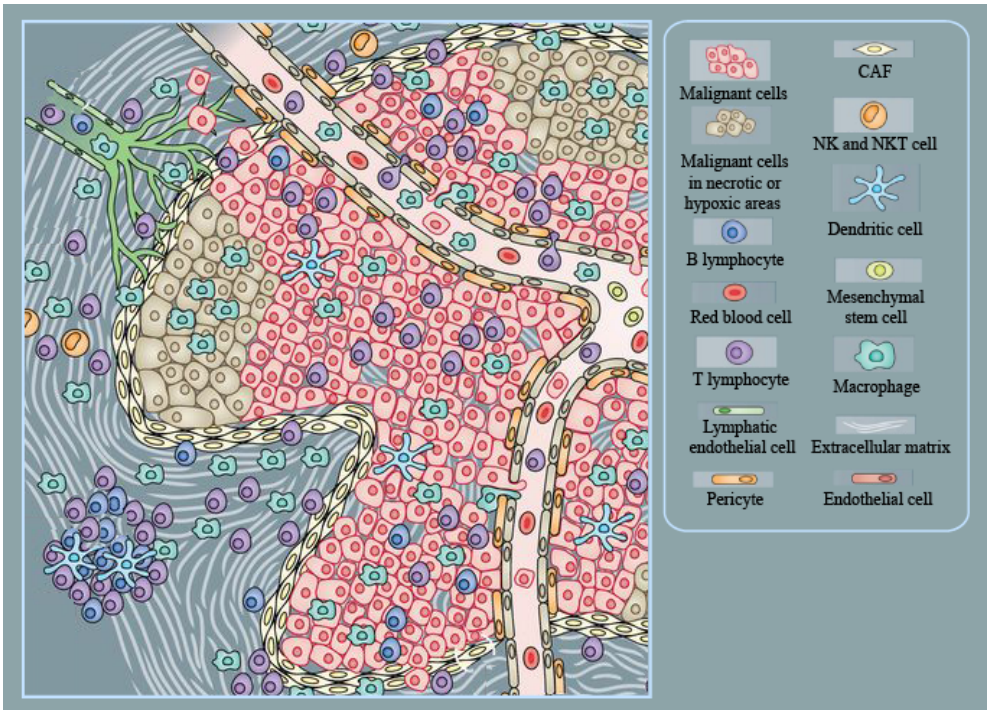

Figure 4. Tumor microenvironment. Several sub-populations of malignant cells can be found in tumors. Cancer cells (pink), cancer cells from hypoxic or necrotic areas (brown) and cancer stem-like cells (not shown). Stromal cells as cancer associated fibroblasts (CAFs) (light yellow) and mesenchymal stem cells (dark yellow). The lymphatic and neovasculature system consisting of endothelial cells (dark pink), lymphatic endothelial cells (light green), pericytes (orange) and blood cells (red). Immune system cells: natural killer (NK) cells (darker orange), macrophages (darker green), dendritic cells (light blue) and T- (purple) and B-lymphocytes (dark blue). Non-cellular part as extracelluar matrix (ECM) (gray waves). Figure modified with permission from Balkwill et al., 2012.

2.6.1 Fibroblasts

Fibroblasts are a population of elongated, spindle-shaped cells typically located in the stroma. They are considered to be of mesenchymal origin and they share similarities with smooth muscle cells. Since there are no specific markers for a fibroblast, this cell type is recognized based on shape, the co-expression of mesenchymal and smooth muscle markers and the absence of a marker for other cell types. The most common markers used to identify fibroblasts are α-smooth muscle actin (α-SMA), desmin, fibroblast specific marker 1 (FSP1) and α1β1 integrin (Kalluri and Zeisberg 2006). Fibroblasts are remarkably activated during embryonic development, when all the matrices in tissues are produced. Later, they are quiescent in normal adult tissues and activated in cases of inflammation, wound healing or tumorigenic growth (Kalluri 2016). The deposition of the ECM is one mechanism for fibroblasts to help them to maintain the homeostasis of tissues. The ECM produced by stromal fibroblasts regulates the phenotype and behavior of epithelial cells and modifies inflammation. Fibroblasts can be isolated from various tissues and cultured. In cell culture studies, fibroblasts are frequently used but they become immediately activated in cell culture conditions (Attieh and Vignjevic 2016). Quiescent fibroblasts have the ability to respond to growth factors and become activated. This leads to proliferation, migration, the production of growth factors and the active synthesis/remodeling of the ECM. Fibroblasts from distant tissues exhibit distinct and characteristic gene expression patterns and should be considered individual cell lines, although activation is occurring, some original properties remain even *in vitro* (Chang et al., 2002).

2.6.2 Activated fibroblasts

Fibroblasts are activated as a consequence of altered gene expression in cancer cells. Cytokines and growth factors mediate the effects of malignant cells (Kalluri and Zeisberg 2006). Activated fibroblasts, also called myofibroblasts, or in the context of cancer peritumoral fibroblasts, reactive stromal fibroblasts, cancer associated fibroblasts (CAFs) or tumor associated fibroblasts, are highly heterogeneous and may originate from different cell types (Micallef et al., 2012; Kalluri 2016). Activated fibroblasts were first detected during wound healing based on their expression of α-SMA (Micallef et al., 2012). α-SMA is so far the most prominent marker used to detect activated fibroblasts. Once fibroblastic cells are activated, their phenotype changes, for example, the nucleus is usually enlarged, the ER and Golgi become more apparent and protein synthesis is activated (Kalluri and Zeisberg 2006). In contrast to non-activated fibroblasts, which are mostly quiescent or in slow-cycle self-renewal, myofibroblasts actively proliferate.

For a long time, it was thought that the activated phenotype of fibroblasts is only a consequence of tumor growth. However, it is now known that CAFs actively participate in the progression of cancer. CAFs, immune cells and cancer cells collectively contribute to the formation of a cancer specific ECM and in tumor growth. In many cancer types, the altered, stiffer and directional organization of the ECM has been shown to facilitate cancer cell invasion (Attieh et al., 2017). In the prostate, fibroblasts become activated even in the precancerous PIN state (Tuxhorn et al., 2002).

2.6.3 ECM

The ECM is the non-cellular component of the tissue microenvironment. Consisting of a variety of matrix proteins, the ECM provides a scaffold for cells and integrity for tissues. The exact composition and structure of the ECM varies in different tissues and depends on certain processes, such as development, wound healing, tumor growth or ageing. The ECM provides more than just a scaffold, it has a far more complex role, since it is crucial for stem cell niches and in development. Alterations of the ECM in cancer development has received considerable attention (Richard O Hynes 2009).

The ECM can be divided into loose connective tissue and the more specialized matrix “basal lamina”. Basal lamina or BM is a thin (40-120 nm) flexible sheet of well interconnected proteins that separates epithelial structures from connective tissue. In addition to its separation function, BM also determines cell polarity and regulates cell survival, proliferation and differentiation. Although the basal laminas in different tissues may have a slightly distinct composition, the typical components of the BM include collagen IV, laminins, nidogen 1and 2, perlecan, agrin and collagen XV and XVIII (Theocharis et al., 2016). The BM is synthesized co-operatively by cells on both sides of the membrane, the laminin network being on the epithelial side and collagen IV network localizing on the stromal side. Epithelial cells bind to the BM and connect it to the intracellular intermediate filaments using integrin-mediated attachment structures - hemidesmosomes. Hemidesmosomes are responsible for anchoring the epithelial cell layers to the BM, regulating cell polarity and have tumor suppressive function (De Arcangelis et al., 2017).

The connective tissue ECM is synthesized by the cells resident in the matrix. In many tissues these cells are fibroblasts, in specialized tissues other mesenchymal cells produce the matrix, for example, in bone these cells are osteoblasts. The typical loose connective tissue ECM is composed of collagens, which together with fibronectin provide the mechanical strength of the tissue and from proteoglycans, which provide the GF and cytokine binding sites and interconnections of ECM proteins (Erler and Weaver 2009).

Each organ of the human body under normal conditions has optimal, well-regulated tissue stiffness. During cancer development, however, the ECM changes, for example its stiffness, because of the excessive synthesis of ECM components and the remodeling of the ECM structure by proteolytic enzymes. A good example of tissue stiffness due to increased ECM and CAFs is breast cancer, which can be discovered because of the presence of a lump in the breast. This lump might reflect the tumorigenic growth of cancer cells, an increased number of CAFs and the accumulated connective tissue around it, called desmoplasia. Desmoplasia is an extra fibrous matrix synthesized and remodeled by CAFs. The increased number of CAFs and ECM in invasive ductal carcinoma is called fibrotic foci, which is connected with a more aggressive disease and metastatic potential (Hasebe et al., 2001).

2.6.3.1 Proteoglycans

Proteoglycans (PG) are proteins which have linked glycosaminoglycan (GAG) chains. Large GAG chains fill the extracellular space and help to form a porous hydrated gel-like structure, which is resistant to compressive forces. Hyaluronan (HA) is a linear GAG, it is synthesized on the cell surface. HA can be either protein -free or as non-covalently attached to other PGs. HA polymers can be various sizes and act as signaling molecules. HA is produced by hyaluronan synthases (HAS) and the degradation of HA is performed by hyaluronidases (HYALs), which can degrade HA into low molecular weight fragments (Theocharis et al., 2016). Proteoglycans can be classified into four families: extracellular, intracellular, BM and cell-surface ones. Extracellular PGs can be divided again into 2 sub-families. First, HA- and lectin-binding PGs (hyalectans), from which the most common proteins are aggrecan and versican, and secondly, small leucine-rich PGs (SLRPs). Decorin, biglycan and lumican are some examples of SLRPs, which can participate in several biological processes. SLRPs can interact with collagens and participate in collagen fibrillogenesis and by binding to RTKs they can modulate cell signaling pathways (Iozzo and Schaefer 2015). The BM PG family consists of perlecan, agrin and collagen XV and XVIII, all of which participate in the BM organization. Perlecan and agrin share a structurally organized C-terminus, endorepellin, which can be cleaved by metalloproteinases (MMPs). Collagen XV and XVIII also share some structural properties and be cleaved by MMPs and other proteinases. Cell surface PGs, as the name indicates, are attached to cell membranes. Syndecans and glypicans are the two main sub-families. Syndecans can interact with fibronectin, laminins and collagens. Syndecan-1 is a co-receptor for collagen with integrin α2β1 and known to induce the transcription of MMP1 (Vuoriluoto et al., 2008). Glypicans are a family which consists of six glycosylphosphatidylinositol (GPI)-anchored members, glypican 1-6. Glypicans can bind and modulate several GFs,

for example Wnt signaling (Theocharis et al., 2016). In addition to their structural role, most proteoglycans are able to modulate the signaling between the cells via regulating the availability of growth factors.

2.6.3.2 Collagens

Collagens are abundant and constitute up to 30% of the total human proteins. The collagen superfamily consists of 28 different collagen types, which are expressed through the body. Collagen I is a major structural element for tissues such as bone, dermis and tendon (Heino 2007). Collagens differ from each other in their structure, properties and the tissues where they are expressed. Collagens can be classified into seven categories as fibrillar collagens, network-forming collagens, FACITs (fibril-assiciated collagens with interupted triple helices), MACITs (membrane-associated collagens with interupted triple helices), anchoring fibrils, beaded-filament-forming collagens, and MULTIPLEXIN (multiple triple-helix domains and interruptions) (Theocharis et al., 2016).

2.6.3.3 Glycoproteins

The fiber-forming protein fibronectin (FN) is expressed by several cell types. FN is encoded by a single gene, but due to alternative splicing, the 20 distinct proteins exist in humans. FN can either be soluble plasma FN or cellular FN, which is assembled into fibers. *In vitro* cells are able to produce their own FN, secrete it and then assemble it. FN consists of two covalently linked subunits (250 kDa), which contain three types of repeating modules. FN contains 12 type I repeats, two type II repeats and 15-17 type III repeats. The FN protein has fibrin-, collagen/gelatin and fibrillin binding sites. It can interact with proangiogenic factors VEGF and HGF (R.O. Hynes et al., 2002). The main integrin-binding site RGD and second integrin-binding LDV are used by cells to bind to FN (Richard O Hynes 2009).

Another group of glycoproteins are laminins. These proteins consist of one α, one β and one γ subunit, each of which are transcribed from an individual gene. It is known that there are 5 α genes (*LAMA1-5*), 3 β genes (*LAMB1-3*) and 3 γ genes (*LAMC1-3*) and *LAMA3* has 2 isoforms, long and short, which gives six possible α genes although there would be possibly 51 potential trimers, only sixteen have been found in human (Durbeej 2010). According to the newer nomenclature the names of laminins are based on the heterotrimers formed, for example α1, β1 and γ1 form laminin-111 (Aumailley et al., 2005). Laminins bind each other as well as other ECM proteins. Binding sites for nidogens, fibulins, perlecan, heparin, sulfatides and agrin are known. The main cellular receptors for laminins are integrins and syndecans. Laminins are crucial for embryonic development and organogenesis (Durbeej 2010).

2.6.3.4 Matricellular proteins

The ECM also contains proteins, which do not have a structural role, but contribute to the integrity of the ECM by modulating the cell-cell and cell-ECM interactions. These proteins have been categorized as matricellular. They interact with the structural ECM proteins, bind to a large variety of receptors on the cell surface and regulate the cell-matrix interactions (Bornstein and Sage 2002). The matricellular proteins group includes CCN proteins, galectins, thrombospondin 1 and 2, SPARC (secreted protein, acidic and rich in cysteine; also known as osteonectin), the SIBLING family (small integrin-binding ligand N-linked glycoproteins), periostin (POSTN), TINAGL, tenascin C and X (Bornstein and Sage 2002; Li et al., 2007; Thakur and Mishra 2016). Matricellular proteins in adult tissues show diminished to moderate expression levels. However, in developing tissues, and pathological conditions such as tumor growth, their expression increases notably (Wong and Rustgi 2013).

2.6.4 Proteolytic regulation of ECM composition.

The proteolytic turnover of the ECM is required for normal physiological processes like development and wound healing, as well as in tumor growth and metastasis. Based on their catalytic activity and cofactor requirements, enzymes degrading ECM can be divided into four groups: aspartic, cysteine and serine proteinases, metalloproteinases (MMPs). MMPs are a family of zinc-dependent endopeptidases which can regulate ECM composition, since they can degrade collagens, matrix glycoproteins, and proteoglycans, and ECM properties by regulating growth factors and their receptors, as well as cytokines and chemokines in ECM (Sternlicht and Werb 2001). MMPs have been associated with tumor progression and cancer cell invasion (Gialeli, Theocharis, and Karamanos 2011). MMPs can be divided into several subgroups, which have been thoroughly reviewed (Reunanen and Kähäri 2002). In the context of this work, the most interesting subgroups are collagenases and transmembrane MMPs. The group of collagenases contains three members MMP1, MMP8 and MMP13. These major proteinases can cleave fibrillar collagens. MMP1 can degrade several components of the ECM, including collagen types I, II, III, VII, VIII and X, aggrecan, fibronectin, perlecan and tenascin (Sternlicht and Werb 2001). MMP1 has been shown to be crucial for prostate cancer DU145 cells to invade (Yoneda et al., 2010) and its synergy with integrin $\alpha 2\beta 1$ has also been reported (Dumin et al., 2001; Riikonen et al., 1995).

Membrane type MMP1 (MT-MMP1/ MMP14) belongs to the membrane-bound MMP subfamily. In addition to many other cancer types, MMP14 is found in prostate carcinoma, where it is expressed either by cancer cells or CAFs. Macrophages and endothelial cells have been shown to express MMP14 as well

(Rowe and Weiss 2009). MMP14 has many substrates, including fibronectin, collagen type I, tenascin and laminin-332 (Sternlicht and Werb 2001).

Four endogenous protease inhibitors form the family of Tissue inhibitors of metalloproteinases (TIMPs). Classically, TIMPs inhibit the MMP-mediated degradation of ECM and additionally have other roles in regulating apoptosis and angiogenesis. TIMP1 has been the most intensively investigated TIMP. In colorectal cancer, TIMP1 is exclusively expressed by CAFs at the invasive front of the tumor (Holten-Andersen et al., 2005). Whereas, in prostate cancer TIMP-1 is expressed by both CAFs and prostate cancer cells (Gong et al., 2013). These results suggest that TIMP-1 expression and role might be tumor type specific.

3 Aims of the Study

The aims for this thesis were set in agreement with the Marie Curie Initial Training Network CAFFEIN. The goal was to increase knowledge about the function of cancer associated fibroblasts in tumor expansion and invasion. Based on this, the specific aims were:

1. To study the composition of the human prostate extracellular matrix (ECM) by mass spectrometry and proteomics.
2. To isolate human prostate-derived fibroblast cell lines in order to study their ECM in *in vitro* 2D and 3D systems and to compare it to the *in vivo* produced ECM.
3. To study the role of integrin α2β1 in DU145 prostate cancer cells by creating an α2 knockout with Crispr/Cas9 and to perform functional studies on invasion, proliferation and gene expression.

4 Materials and Methods

The extensively used materials and methods applied in this thesis work are listed here and have been described in detail in the original publications. The specific methods used for this study have been explained in detail below.

Table 1. Methods

Method	Used in
Flow cytometry	I
Flow assisted cell sorting	I
Confocal microscopy	I-III
Live microscopy	II, III
Mass spectrometry and proteomics	II,III
Western Blot	I,III
Real time cell adhesion measurement with xCelligence system	III
Out from spheroid migration and invasion assay	I,III
RT-qPCR	I,II
Matrigel transwell invasion assay	I
Cell proliferation analysis	I, II
Cell cycle analysis	I
Colony survival assay	I
Extraction of ECM proteins from human prostate tissue	II
Immunohistochemistry	III
3D spheroid culture system	I-III
RNA sequencing	I
Immunocytochemistry	I-III
Image analysis	I-III

4.1 Human prostate tissue samples

In order to study the in vivo composition of the human prostate ECM the matrix proteins were extracted from 12 human prostates tissue samples, from 6 cancerous and 6 non-cancerous locations. In addition, prostate tissue samples were used to isolate human primary cells to initiate fibroblast cell lines (27 cell lines were

created during the study). The tissue samples were obtained from surgically removed prostates using robotic-assisted laparoscopic prostatectomy (RALP) at the Turku University Hospital during 2013-2014. All patients signed the informed consent to allow the use of their tissue material for scientific research conducted by the Turku Prostate Cancer Consortium Study (TPCCS). This study has been approved by the ethics committee of the Hospital District of Southwest Finland (ETMK: 3/180/2013).

4.2 Cell cultures

Human androgen independent prostate cancer cell lines DU145 and PC3 were purchased from the American Type Culture Collection (ATCC). DU145 cells The origin of DU145 cells are prostate cancer metastasizing to brain and PC3 cells from a metastatic bone lesion. Human prostate-derived fibroblastic cultures were developed in-house according to the published protocol (Le et al., 2006). All cells were cultivated in RPMI 1640 medium, supplemented with 2mM ultraglutamine, 10% fetal calf serum (FCS), 100U/ml penicillin and streptomycin. For serum-free conditions the FCS was omitted and serum-free keratinocyte medium (KSF) with 5 ng/ml human recombinant EGF, 50 µg/ml bovine pituitary extract, supplemented with 2 ng/ml recombinant human leukemia inhibitory factor and 2 ng/ml stem cell factor (Sigma Aldrich) was used in indicated experiments. Cells were routinely screened with the MycoAlert™ PLUS mycoplasma detection kit.

4.3 Genome editing for knock-out and rescue of α2 integrin

To create integrin α2 negative DU145 cells Crispr/ Cas9 genome editing was applied. A commercial all-in-one vector (Sigma, HS0000253951 gRNA sequence: GTTACTGGTTGGTTCACCCTGG) was transfected into the DU145 cells. A transient transfection was confirmed and a positive population of transfected cells was sorted based on their expression of GFP. The GFP expressing cells represented a mixed population of ITGA2 knockout and α2 integrin positive cells. Therefore, the cells were stained against α2 integrin, the negative population was identified and sorted separatly. These cells were labelled as DU145KO. To create cells with rescued integrin α2 expression (called DU145KO+α2 cells), DU145KO cells were stably transfected with the pAWneo2 vector carrying α2 integrin cDNA (Ivaska, et al., 1999) or with the empty pAWneo2 vector to create a proper α2 negative control cell line (DU145KO+vector). To ensure the stable expression of the vector constructs the cells were cultured in the presence of 250 µg/ml of G418.

4.4 Gene expression analysis

Table 2. Primers and probes used for qPCR

Gene	Forward primer	Reverse primer	Probe
CHD5	tgggctacatggatgagaaa	ctcactctccactctatccaagg	11
CDH5	aagcctctgattggcacagt	gactcggaagaactggccc	58
KIF26B	cgtgttcttcacactgcacat	ctgcgacctccagacattc	58
LGI1	tcactaaccaaactgacattcctaa	acacgtccccttttcactgag	88
PKP1	aaacaggcacgtctggca	aagccatcatacatggaacctc	68
RBP1	acgctgagcacttttaggaact	atgcctgtcagatcctcctc	12
SCARA5	tccaagctgaacctgtgtga	agaatcaggaagaccagcag	56
SDC2	aaacggacagaagtcctagcag	ccttcatccttctttctcatgc	18
SVEP1	tctctgttggtttgcccata	atggagcccacaaaagactc	26
SYK	aaagacaaatggaaagttcctga	ctttgtcgatgcgatagtgc	40
VWA2	gggtttgcagaggttgactg	ctgcggtaccaccaggac	76
MMP1	aagatgaaacgtggaccaacaatt	ccaagagaatggaagagttc	*
ACTA2	cctatccccgggactaagac	aggcagtgctgtcctcttct	78
FAP	tggcgatgaacaatatcctaga	atccgaacaacgggattctt	19
COL1A1	gggattccctggacctaaag	ggaacacctcgctctcca	67
COL3A1	ctggaccccagggtcttc	gaccatctgatccagggtttc	20
KRT8	tcaacaacaagtttgcctcct	atgttgtccatgttgcttcg	1
KRT 18	ggaagatggcgaggacttta	atggtttgcatggagttgct	9
POSTN	aatgccaacagttactatgac	cttgataccagttcttacagg	9
GAPDH	acccactcctccacctttga	ttgctgtagccaaattcgttgt	**

* 5'-FAM-cagagagtacaacttacatcgtgttgcggctc-TAMRA-3'
** 5'-FAM-acgaccactttgtcaagctcatttcctggt-TAMRA-3'

4.5 Immunoassays

Table 3. List of antibodies used in this study

Target protein	Product code	Company	Method	Used in
Integrin α2	12F1, BD5555668	BD Pharmingen	FC, FACS	I
Integrin α2	Mab12332	R&D Systems	IF	I
CD44-FITC	Ab19622	Abcam	FC, FACS	I
β-actin	A1978	Merck	WB	I, III
Hsp90	4877S	Cell Signaling Technology	WB	I
p38 MAPK, pT180/Y182	9215L	Cell Signaling Technology	WB	I
FAK pY397	3283S	Cell Signaling Technology	WB	I
ERK 1/2, pT204/pT187	5726S	Cell Signaling Technology	WB	I
CREB, pS133	MAB6906	BD Biosciences	WB	I
FAK	610088	BD Biosciences	WB	I
ERK	9102S	Cell Signaling Technology	WB	I
Trop2	MAB650	BD Biosciences	FC	I
αSMA	ab32575	Abcam	IF	II
Pan-cytokeratin (1,4,5,6,8,10,18,19)	C2562	Merck	IF	II
Collagen I	ab6308	Abcam	IF	II
Fibronectin	ab6328	Abcam	IF	II
Laminin5	ab14509	Abcam	WB	III
Endostatin	PA1-601	Thermo Fisher Scientific	WB	III
MMP14	AB6004	Merck	WB	III
Collagen XVIII	N2, Ref	homemade	WB	III

WB: Western blot, IF: Immunofluorescence, FC: Flow cytometry, FACS: Fluorescence-activated cell sorting

4.6 Reagents

Table 4. List of ECM proteins used in this study

Protein	Company, product code	Used in
Collagen I (Bovine)	Advanced BioMatrix, 5010-50	I-III
Fibronectin	Merck, F1141	I-III
Recombinant Human Endostatin	Peprotech, 150-01	III
Laminin-332	Biolamina	III
Poly-l-lysine	Merck, P9155	I

Table 5. List of reagents used in the original publications.

Regent	Type	Company, product code	Used in
Fugene 6	Transfection regent	Promega	I
CellTracker™ Orange CMRA Dye	Fluorescent stain	Thermo Fisher Scientific, C34551	II-III
CellTracker™ Green CMFDA Dye	Fluorescent stain	Thermo Fisher Scientific, C7025	II-III
N-Isobutyl-N-(4-methoxyphenylsulfonyl)-glycylhydroxamic Acid, NNGH	MMP inhibitor	Merck	I, III
N-(benzyloxycarbonyl)-L-phenylalanyl-L-tyrosinal, Z-FY-CHO	Cathepsin L inhibitor	Santa Cruz Biotechnology	III
3,4-Dichloro-N-(1-methylbutyl)-benzamide, 3,4-Dichloro-N-(pentan-2-yl)benzamide, NSC 405020	MT1-MMP inhibitor	Merck	III
NucleoSpin® RNA	RNA extraction kit	Macherey-Nagel	I
4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1H-imidazole, SB 203580	p38 MAPK inhibitor	Merck	I
SensiFAST™ cDNA Synthesis Kit	cDNA synthesis kit	Bioline	I, II
ABI TaqMan Universal Master Mix II	PCR mastermix	Applied Biosystems	I, II

4.7 Data and statistical analysis

Statistical analyses were performed using IBM SPSS statistics 22 software. The normal distribution of data was checked with Shapiro-Wilk test. A paired Student's t-test was used to determine the statistical significance between the two treatments. Several treatment samples were compared with a one way ANOVA test and pairwise comparisons were made with the Tukey HSD and Dunnett T3 post hoc test. The statistical significance was considered for p-values less than 0.05.

5 Results

5.1 Integrin α2, CD44 and Trop2 expression on DU145 and PC3 cells (I)

The DU145 and PC3 cell lines were used as a model system for the cancer stem cells in this study. The expression of the stem cell markers α2 integrin, CD44 and Trop2 was studied using flow cytometry.The analysis of the markers expressed on the cells surfaces for both cell lines are shown in Figure 5.

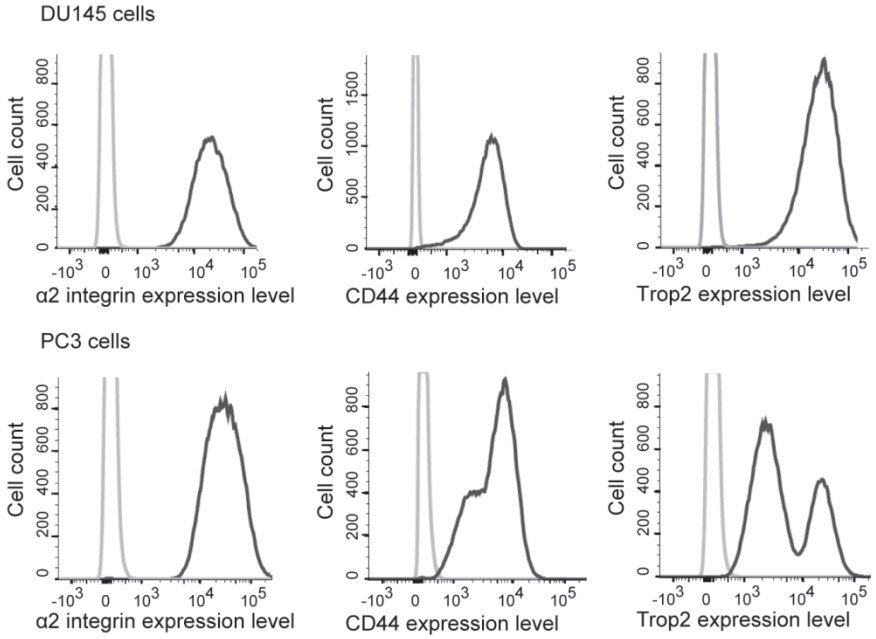

Figure 5. PCSC markers: α2 integrin, CD44 and Trop2 expression levels on the DU145 and PC3 cell surface were determined by flow cytometry using human CD49b, CD44 and Trop2 antibodies. Both, DU145 and PC3 cells express all three marker proteins on cells surface. The 5 * 10^4 cells were analyzed using BD LSRFortessa™ cell analyzer, data were analyzed with FlowJo v10 software.

5.2 Docetaxel resistant cells show high prostate cancer stem cell marker expression (I)

We first studied whether ECM or certain ECM proteins have protective properties for DU145 and PC3 cancer cells. The cancer cells were plated on the human prostate primary fibroblast-derived ECM or surface coated with the matrix proteins fibronectin or collagen I, and treated with docetaxel. Based on these data from our group, the ECM in 2D cell culture system does not have a protective role for cancer cells (Elina Aalto, Master's thesis 2015). Next, we studied the cells which survived the docetaxel treatment. We discovered that the stem cell markers α2 integrin and the CD44 cell surface levels were higher on the DU145 cells treated with docetaxel than on the corresponding untreated cells (I, Figure 1A-B). The same effect of increased integrin α2 was seen with PC3 cells (I, Supplementary Figure 3A-B). As anticipated, the surviving cells were mostly arrested at the G2/M of the cell cycle (I, Supplementary Figure1).

To study the α2 integrin high and low sub-populations, the DU145 and PC3 cells were sorted using fluorescence-activated cells sorting (FACS) (I, Figure 1C). The sorted cells kept the expression profile for 5-7 passages. The levels of α2 integrin, CD44 and Trop2 were studied at the 5th passage after sorting. The DU145WTα2High cells also expressed more CD44 than the DU145WTα2Low cells, suggesting that these stem cell markers are connected. There was no difference in the Trop2 expression on the DU145WTα2High and the DU145WTα2Low cells (I, Figure 1D). Surprisingly, the PC3 cells with a high expression of α2 integrin had lower levels of CD44 when compared to the PC3α2Low cells. Another interesting observation was that the PC3α2High population had a positive and negative population of Trop2 expressing cells whereas PC3α2Low cells were negative for Trop2 expression (I, Supplementary Figure 3C). The proliferation assay indicated that the DU145WTα2High cells had a reduced ability to multiply on collagen I, fibronectin and poly-L-lysine (PLL) when compared to the DU145WTα2Low cells (I, Figure 1E). This explains the enrichment of the cells possessing more α2 integrin and CD44 expression after the docetaxel treatment. Docetaxel targets cells at mitosis. Cells with a higher α2 integrin expression divide less frequently and are therefore more resistant to drug treatment. These promising results justified continuingt the study to describe in depth the role of integrin α2β1 in cancer cell proliferation and survival by creating α2 integrin knockout lines for DU145 cells.

5.3 The role of α2 integrin in DU145 cells (I)

We successfully applied the CrispR/Cas9 system to target the α2 integrin at gene level in the DU145 cells and to create the knockout cell line DU145KO. The

knockout was confirmed by sequencing PCR amplified genomic DNA around the guide RNA binding area (I, Supplementary Figure 2). The DU145KO cells were used to generate two more cells lines: DU145KO+α2 cells, which have a rescued expression of α2 integrin, and DU145KO+vector line, integrin α2 negative cells, which carry an empty vector. The expression of α2 integrin was confirmed by Western blot (I, Figure 2B) and the presence of α2β1 on the surface of the DU145KO+α2 cells was determined by flow cytometry (I, Figure 2A). Additionally, the CD44 expression levels were controlled by flow cytometry in all cell lines as well. The results showed that the overexpression of α2 integrin did not affect the CD44 expression, indicating that these two stem cells markers are not regulated by each other.

5.3.1 Integrin α2 regulates proliferation and resistance to docetaxel (I)

Since α2β1 integrin signaling is thought to be more relevant in a 3D environment (Heino 2014) and DU145 cells are able to grow as spheroids in non-adherent conditions, the majority of the following work was carried out in a 3D spheroid system. The proliferation of the α2 positive DU145KO+α2 and the α2 negative DU145KO+vector cells was analyzed by measuring the increase in the DNA content in the spheroids. The α2 negative cells formed more compact spheroids and appeared smaller. However, the amount of DNA in the spheroid increased significantly faster, presenting a higher proliferation rate of the cells (I, Figure 2E). In the colony survival assay on collagen the DU145KO+α2 cells were significantly more resistant to docetaxel when compared to their α2 negative counterparts (I, Figure 2F), indicating the connection between reduced proliferation and increased survival.

5.3.2 Integrin α2 regulates motility of cancer cells (I)

The 3D spheroids consisting of α2 positive DU145KO+α2 cells had a loosened appearance compared to the α2 negative cell spheroids, and one of the explanation for this might be the increased motility of cells. To study the cell motility, the invasion of cancer cells through a matrigel coated transwell chamber was monitored. The results revealed that the DU145KO+α2 cells had a significantly higher invasion capacity compared to the α2 negative DU145KO+vector and the DU145WT cells (I, Figure 3A). The same assay was repeated with PC3α2High and PC3α2Low cells (I, Supplementary Figure 3D), where again α2β1 integrin high expressing cells had an advantage in their ability to invade through the matrigel transwell chambers. All the results supported the belief that the ability of cancer cells to invade is connected to their α2β1 integrin levels. To confirm that invasive prostate cancer cells have to express α2β1 integrin in order to move on or through

the collagen rich matrix, a spheroid-based migration and invasion assay were developed.

Spheroids for the migration and invasion assay, composed of α2 positive DU145KO+α2 or α2 negative cells, were grown for 72 hours. For the migration assay, the spheroids were placed onto collagen I coated plates and for the invasion assay the spheroids were embedded into the collagen. The size of the surface area covered by cells was measured in both assays daily during 96 hours. The DU145KO+α2 cells had a significantly higher migration (I, Figure 3B) and invasion rate (I, Figure 3C) when compared to the α2 negative cells.

5.3.3 Integrin α2β1 suppresses cell growth and promotes motility by activation of p38 MAPK. (I)

Although integrin α2β1 does not have kinase activity itself, it still activates several signaling pathways. To understand α2 integrin specific signaling we used our α2β1 integrin positive and α2 negative DU145KO+vector cells and measured the activation of FAK, ERK and p38MAPK (I, Figure 4A). The phosphorylation of all three proteins was significantly lower in the DU145KO+vector cells when compared to the DU145KO+α2 cells. These cells were plated onto a collagen I coated surface. The most significant effect was seen in the case of the p38 phosphorylation. Therefore, we decided to clarify whether the inhibition of p38 MAPK would induce an increase in DU145KO+α2 cells proliferation. The p38 specific inhibitor SB203580 at a concentration of 10 μg/ml significantly increased the proliferation of the DU145KO+α2 cells. It also slightly increased the proliferation of the DU145KO+vector cells, but the difference was not statistically significant (I, Figure 4B). In addition, the p38 inhibitor had an enormous effect on the DU145KO+α2 cell migration (I, Figure 4C) and invasion (I, Figure 4D), indicating that the p38 MAPK pathway is important in DU145 cancer cell proliferation and motility regulation.

5.3.4 Integrin α2β1 regulates genes previously associated with cancer progression (I)

RNA sequencing was used to identify differentially expressed genes in DU145WT, DU145KO, DU145KO+α2 and DU145KO+vector cancer cells. Table 6 shows a list of genes expressed differentially in cells overexpressing α2 when compared to the knockout (DU145KO) and to the α2 integrin negative (DU145KO+vector) cells. Four biological replicates were used in order to increase the credibility of the analyzed gene list. The biological processes which were potentially affected by the differential gene expression between the α2 positive and the negative cells (DU145KO and DU145KO+vector) were identified (I, Figure5B).

Table 6. List of genes most differentially expressed in α2 overexpressed DU145KO+α2 cells when compared to DU145KO and DU145KO+vector cells indicated in fold change (FC).

Gene symbol	Description	DU145KO+ α2 vs DU145KO FC	DU145KO+ α2 vs DU145KO +vector FC
CDH5	cadherin 5, VE-cadherin	7,646	67,204
SCARA5	scavenger receptor class A, member 5	5,487	15,744
ZNF853	zinc finger protein 853	5,386	12,975
LGI1	leucine-rich, glioma inactivated 1	10,466	10,179
JAM3	junctional adhesion molecule 3	2,059	6,726
MMP-1	matrix metalloproteinase 1	8,76	5,268
LINC00421	long intergenic non-protein coding RNA 421	4,952	4,816
AMPH	amphiphysin	2,47	4,501
SVEP1	sushi, von Willebrand factor type A, EGF and pentraxin domain containing 1	3,195	4,224
FOXL1	forkhead box L1	2,543	4,057
SLC18A2	solute carrier family 18 (vesicular monoamine transporter), member 2	2,929	4,053
DQX1	DEAQ box RNA-dependent ATPase 1	3,002	3,857
ALX1	ALX homeobox 1	3,046	3,768
CACNA1D	calcium channel, voltage-dependent, L type, alpha 1D subunit	2,029	3,417
POM121 L9P	POM121 transmembrane nucleoporin-like 9, pseudogene	2,273	3,293
BEAN1	brain expressed, associated with NEDD4, 1	2,052	3,187
KIF26B	kinesin family member 26B	2,771	3,033
DDX60	DEAD (Asp-Glu-Ala-Asp) box polypeptide 60	-2,35	-3,037
VWA2	von Willebrand factor A domain containing 2	-3,49	-3,639
CYP4F3	cytochrome P450, family 4, subfamily F, polypeptide 3	-3,60	-3,046
UBA7	ubiquitin-like modifier activating enzyme 7	-3,71	-3,252
RASSF10	Ras association (RalGDS/AF-6) domain family member 10	-4,20	-3,055
SYK	spleen tyrosine kinase	-4,92	-4,519
RBP1	retinol binding protein 1, cellular	-5,18	-3,328
C1orf116	chromosome 1 open reading frame 116	-6,62	-7,178
PKP1	plakophilin 1	-6,66	-4,748
CHD5	chromodomain helicase DNA binding protein 5	-7,14	-9,988
SLC15A3	solute carrier family 15 (oligopeptide transp.) member 3	-9,36	-7,288
BST1	bone marrow stromal cell antigen 1	-9,88	-14,72
SDC2	syndecan 2	-28,55	-11,773

Taking into account the previous connection to cancer progression described in the literature, 12 genes were selected (marked with light blue in the Table 6). To confirm the RNA sequencing results, the expression of these 12 genes in DU145KO+α2 and DU145KO+vector cells was analyzed using qPCR (I, Figure 5D).

The results indicate integrin α2 as a regulator of several genes associated with cancer progression, many of which have previously been related to prostate cancer or other malignancies. Substantial downregulation was seen in syndecan 2 expression, which has been shown to be present in the majority of prostate cancers and is upregulated in high Gleason score cases (Popović et al., 2010). As α2β1 integrin expression decreases in Gleason 3-5 prostate cancers (Mirtti et al., 2006) this opposite direction of syndecan 2 expression might have a role in vivo as well. Also, the expression of PKP1 and CDH5 genes was downregulated by integrin α2β1, both genes are transcribed to proteins with known tumor-suppressive properties. The diminished expression of PKP1 has been associated with a metastatic phenotype of prostate cancer (Yang et al., 2013). In contrast, the expression of two tumor suppressors, namely SCARA5 and LGI1, was induced, which is in accordance to the anti-proliferative action of integrin α2β1 in prostate cancer cells. The highest increase in gene expression was seen in cadherin 5, also known as vascular endothelial cadherin (CDH5 / VE-cadherin). Our group has previously shown the connection between α2β1 integrin and MMP-1 expression in other cells (Riikonen et al., 1995). Here, one of the remarkably upregulated genes in α2 positive cells was also MMP1 and since these cells had increased motility on the surface and in collagen I gel, the MMP1 degradation of collagen could be one reason for the ability of α2 positive cells to move through the collagen rich matrix as well as on its surface. We tested the MMP inhibitor, N-Isobutyl-N-(4-methoxyphenylsulfonyl) glycyl hydroxamic acid (NNGH), in spheroid migration and invasion assays. The MMP inhibition, implementing 1 μM of NNGH, significantly reduced the migration of the DU145KO+α2 and DU145WT cells on collagen; however, invasion was not influenced by this concentration (I, Supplementary Figure 5). These results showed that MMPs are at least partially involved in the motility of the prostate cancer cells expressing α2 integrin.

5.4 ECM in human prostate tissue (II)

In order to study the ECM composition of the human prostate tissue, a 3 step matrix extraction protocol, developed initially for the isolation of the ECM from a human aorta (Didangelos et al., 2010) was applied. Based on the proteomics data obtained with mass spectrometry (MS), altogether 120 reliable recognitions were observed and categorized as ECM proteins. MS data enabled the composition of ECM to be established in the human prostate (II, Figure 4A, Supplementary Table

S3). The prostate loose connective tissue was composed of fibril forming collagens I, III and V, and fibril-associated collagens XII and XIV, in addition fibril-binding proteoglycans decorin and biglycan. BM components, such as collagen IV chains α1 and α2, laminin chains α2, α4, α5, β1, β2, and γ1, nidogen 1and 2, perlecan, fibulin 1, and collagens XV and XVIII were also recognized. Fibronectin, tenascin C and XB, collagen VI, fibulin-1 and -2, and proteoglycans lumican and osteoglysin were identified in several samples. In order to study in depth the cells contributing to the ECM production and the possibility to use ECM in vitro we continued with isolation of fibroblastic cells from human prostate tissue.

5.5 Prostate-derived fibroblastic cells (II, III)

Prostate-derived fibroblasts were isolated from cancerous and non-cancerous tissue samples from radical prostatectomy specimens obtained from Turku Prostate Cancer Consortium in collaboration with Auria Biobank and the Pathology unit of Turku University Hospital.

5.5.1 Fibroblasts become activated by culture conditions (II)

During the study 27 cell-lines were created of which cells had the characteristic appearance of fibroblastic cells in culture. To confirm their mesenchymal origin, cells were immunostained for widely used marker proteins. Vimentin, α-SMA and FAP, together with negative staining for cytokeratin, were used as markers for the fibroblasts and activated fibroblasts. The created fibroblast cell-lines were positive for vimentin, α-SMA and FAP, independently from the cancerous or non-cancerous origin (Figure 6). The expression levels of the two activation markers, α-SMA and FAP, correlated with each other in relative mRNA levels measured by the qPCR analysis (II, Figure 1D-E). A minor tumor or non-transformed epithelial cell contamination was observed (II, Figure 1B-C); however, it was considered not to interfere with further studies.

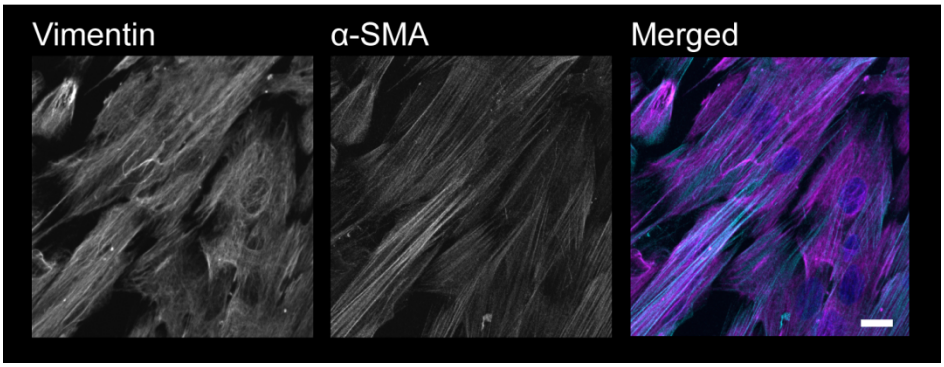

Figure 6. Cell cultures initiated from human prostate tissue showed the positive staining for mesenchymal marker vimentin and activated fibroblast marker α-SMA. Scale bar is 20 µm.

5.5.2 Fibroblasts produce functional ECM (II)

Fibroblasts were allowed to produce ECM proteins on the plastic cell culture dish during the 7 days cultivation in the presence of ascorbic acid (aa, 50 µg/ml) in order to provide the performance of prolyl hydroxylases and the synthesis of stable collagens. The presence of the ECM proteins was identified by immunofluorescence staining of the abundant matrix proteins fibronectin and collagen I (II, Figure 2A). Apparent staining was confirmed in both conditions with or without cells after the hypotonic lysis of the fibroblasts. The fibrillary structures in the ECM were visualized by scanning electron microscopy (SEM) (II, Figure 2B)

The cell-free ECM was subjected to further studies with the DU145 and PC3 prostate cancer cells. Cancer cells attached and spread on the ECM as on the collagen I or fibronectin coated plates. We discovered that the DU145 cells show a significantly decreased proliferation rate on the ECM when compared to fibronectin and collagen I coated surfaces (II, Figure 2C).

ECM proteins produced in 2D monolayer cultures by 6 cancerous and accordingly 6 non-cancerous fibroblastic cell-lines were collected. After collection the ECMs were subjected to mass spectrometry analysis to identify the synthesized ECM proteins. We identified 164 matrisome proteins which have been listed as “core matrisome” or “matrisome associated” in the Matrisome database (Naba et al., 2012). The detected 132 proteins out of 164 were identified in the produced matrix at least in one of the cell-lines in both groups (II, Supplementary Table2). This finding is at least partially related to the fact that both cancer associated fibroblasts (CAFs) and non-cancer-derived fibroblasts were activated in the cell culture conditions. Thirteen proteins were identified in the CAF produced matrix and not present in the non-cancerous matrix. Nineteen proteins were solely identified in some of non-cancerous fibroblasts matrix, without identification in the

CAF matrix. However, these differences were not statistically significant once compared between the groups defined as tumor and non-cancerous fibroblasts. Our results are in accordance with the outcome of studies analyzing and describing the considerably high person-to-person protein expression variation. Furthermore, even the fibroblasts isolated from the same patient can be remarkably heterogeneous in their gene expression pattern despite having been demonstrated to keep their gene expression for several passages in cell culture (Chang et al., 2002).

We also analysed the expression of three matrix proteins at mRNA levels, namely collagen α1 (I), collagen α1 (III), and periostin (II, Figure 3A-C). The mRNA levels do not always reflect the actual protein level in matrix, but based on this it is possible to investigate their expression. We noted that collagen α1 (III) mRNA levels were lower in 5 out of 6 cases in the CAFs compared to the non-affected fibroblasts in the same prostate ($P = 0.026$). We also compared the expression of these matrix proteins with the activation level of the fibroblasts and discovered that collagen α1 (I) or α1 (III) mRNA levels did not correlate with α-SMA mRNA levels (II, Figures 3D-E), but periostin and α-SMA had a statistically significant correlation ($P = 0.005$) (II, Figure 3F). Based on our results all fibroblasts become activated in the cell-culture conditions, but they may preserve some properties related to the origin of the tissue, i.e. the CAFs versus normal tissue fibroblasts. To understand better how the 2D cell culture conditions changed the matrix produced by fibroblasts, we decided to use a fibroblastic cell-line (II, Table 1, Patient 2 cancer location isolated fibroblasts) in a 3D spheroid culture system with and without the DU145 prostate cancer cells (II, Figure 5). The results revealed that only 62 proteins were shared within the matrix produced by same fibroblastic cell-line in the monolayer and in the spheroid (II, Figure 6A). Surprisingly, we identified 86 ECM proteins specific to either the 2D or 3D culture condition. Most differences were in the group of “ECM regulators”, followed by “secreted factors” and then “glycoproteins”. The formation of basal lamina was alternatively regulated since collagen VII, laminin α3 and γ2 chains were identified only in spheroids. Lysyl oxidase (LOX) was only found in the spheroids, suggesting that the assembly of collagen fibrils is induced in the 3D culture system.

5.5.3 ECM components produced by cancer cells or by fibroblasts and in co-culture (III)

Based on mass spectrometry and proteomics analysis, 64 matrisome proteins were identified in the spheroid cultures for the DU145 or PC3 prostate cancer cells, indicating that without fibroblasts cancer cells synthesize little ECM. The DU145 cells did not express core matrisome structural proteins when cultivated alone, whereas PC3 cells did produce the laminin chains alpha3, beta3 and gamma 2 (known as LN332/ laminin-332), as well as perlecan and collagen type VI (III,

Supplementary Table 1.) Thirty-two matrisomal proteins were shared between the DU145 and PC3 cells, 4 proteins were identified only in the DU145 matrisome and the PC3 cells had 28 unique matrix proteins (III, Figure1A, and Supplementary Table 1.). The proteins common between the DU145 and PC3 cells could be categorized into several regulatory protein families, like cathepsins and ADAM proteinases, annexins, S100 and galectin family members and procollagen lysine hydroxylases (PLOD) (III, Supplementary Table 1.). Interestingly, the presence of PLODs in the ECM is in accordance with the recently reported extracellular function in lung cancer (Y. Chen et al., 2016). These findings propose that collagen may be modified in the extracellular space, not only in endoplasmic reticulum. PLOD2 is required for collagen fiber formation and alignment, and through this function it contributes to ECM stiffening. The overexpression of PLOD2 leads to fibrosis (Gilkes et al., 2013).

The experimental data from the second study described fibroblasts to be the main cell type predominantly synthesizing the core ECM proteins. Another fibroblast cell-line generated (II, Table 1, Patient 3 cancerous tissue sample) was cultured in the spheroids alone or alongside the fibroblasts/DU145 and fibroblasts/PC3 cells (III, Figure 1B, Table 2). In the fibroblast spheroids, most of the collagenous ECM components were identified: both α1 and α2 chains of fibril forming collagen I, fibril associated collagen XII and beaded filament forming collagen VI, as well as fibronectin, tenascin C and decorin. The fibroblasts alone express laminin β1 and γ1 chain (III, Supplementary Table 1.); however, in the co-culture with cancer cells a prominent accumulation of the laminins (α3, α5, β2 and β3) was observed (III, Table 2.). The synthesis of BM components, namely perlecan and collagen XVIII had increased expression in both the fibroblast/DU145 and fibroblast/PC3 spheroids (III, Table 2.). The downregulation of certain ECM proteins was identified in the co-cultures, particularly fibrillin-2, fibulin-1 and collagen XIV. HGF was identified only in the fibroblast spheroids,but it remained undetectable in the co-culture samples (III, Table 2.).

5.5.4 Co-culturing of fibroblasts with DU145 or PC3 cells leads to enhanced ECM proteins degradation (III)

Although the PC3 cells could independently produce some BM components in the spheroid cultures, the DU145 cells were not capable of doing so. However, the co-cultivation of cancer cells along with fibroblasts lead to an accumulation of the proteins required to form the BM (III, Table 2). Concurrently, the degradation of the ECM proteins seemed to be enhanced in the co-culture spheroids. The accumulation of laminin-332 and collagen XVIII noticed in the MS and proteomics analysis was controlled by Western blot. Using an anti-human laminin-332 antibody confirmed the accumulation of this BM component and also revealed

several smaller bands detected only in the fibroblast/DU145 and fibroblast/PC3 spheroid samples (III, Figure 3). The collagen XVIII was significantly processed into endostatin, as the peptides identified in the MS belonged to the endostatin part of the protein and enhanced endostatin protein bands were detected also by Western blot (III, Figure 4). The active degradation of laminin-332 into smaller peptides was also noticed in Western blot analysis. The enhanced turnover of the ECM, detected in co-cultures, is probably mediated through the co-operation of several degrading enzymes. Presumable candidates include regulators such as MMP14, expressed by fibroblasts, or cathepsins and the ADAM family MMPs produced by cancer cells. Utilizing broad MMP inhibitor and MMP-14 specific inhibitor revealed that MMPs are needed for invasion of DU145 cancer cells in "out of spheroid" assay, however MMP inhibition did not affect the formation of laminin-332 and endostatin containing fragments. Endostatin formation was decreased when cells in spheroids were treated with cathepsin L inhibitor.

6 Discussion and General Perspectives

6.1 Integrin α2β1 regulates proliferation, invasion and gene expression of prostate cancer cells

The complexity of integrin α2β1 in prostate cancer is reflected by the controversial findings that integrin α2β1 is downregulated during prostate cancer progression (Bonkhoff, Stein, and Remberger 1993b; Mirtti et al., 2006) and at the same time is a cancer stem cell marker (Collins et al., 2005; Trerotola et al., 2010). Previous works have also suggested the importance of integrin α2β1 in prostate cancer cell metastasization into bone (Sottnik et al., 2013; Ricci et al., 2013). These assertions do not fit perfectly to the traditional tumor progression cascade, unless the cancer stem-cell theory is included. This theory suggests that most invasive cells have stem cell properties and are resistant to all environmental challenges. Questions regarding the role of integrin α2β1 in prostate cancer cells and its ability to regulate gene expression and motility have previously remained unanswered. The findings obtained within this current doctoral thesis significantly extend the previously known properties of integrin α2β1 in cancer cell motility and as additional features, also include the regulation of proliferation and the control of gene expression.

6.1.1 Docetaxel treatment enriched the population of DU145 cells with higher expression of α2β1integrin and CD44

The outcome of our first attempts to study the role of the ECM or its certain components as a protective factor against the chemotherapeutic drug docetaxel did not agree with some previously published data (Thomas et al., 2010), since the ECM had no direct effect on cell survival. However, other studies have clearly indicated integrin-mediated protection against chemotherapeutics. In one study, integrins α2β1 and α5β1 expression on MDA-MB-231 breast cancer cells was shown to decrease apoptosis induced by paclitaxel. However, these receptors did not have the same effect on MDA-MB-435 cells. In the last case α6β1 integrin and the attachment to laminin was indicated to be responsible for a reduction in paclitaxel induced cell death. In both cell lines the integrins were shown to activate

PI3K/Akt signaling, which induced the expression of Bcl-2 and inhibited the release of cytochrome c to mediate survival (Aoudjit and Vuori 2001). In another study, integrin α2β1 expression on T cell acute lymphoblastic leukemia (T-ALL) cells and attachment to collagen I promoted survival against doxorubicin through the activation of MAPK/ERK (Naci et al., 2012). These results indicate that the integrin-mediated attachment and survival enhancing effects may be receptor and cell type specific. Although, in our study the protective effect of ECM proteins on docetaxel induced cell death was not noted, we decided to study the surviving cells in more detail. We discovered the enrichment of the integrin α2β1 and CD44 high cells among surviving cells. One of the mechanisms of cancer stem like cells to survive cytotoxic drug treatments could be the rapid efflux of drugs. CD44 has been shown to participate in the induction of MDR1 expression and the regulation of drug resistance (Misra et al., 2005). Another explanation could be the decreased proliferation of cells with a high expression of integrin α2β1 and CD44 which provide a better survival against drugs that target highly proliferative cells. To test this hypothesis, DU145 cells were sorted into high and low α2β1 integrin expressing cells and their proliferation and survival were tested. The results created a link between the higher expression of integrin α2β1 and better survival. Nonetheless, it was impossible to claim whether the higher expression is the reason or the consequence. To test the integrin α2β1 induced stem cell characteristics of DU145 cells Crispr/cas9 technology was applied.

6.1.2 Proliferation and motility of prostate cancer DU145 cells is regulated by α2β1 integrin through p38 MAPK pathway

The study revealed that integrin α2β1 decelerates proliferation since DU145KO+vector cells had increased proliferation compared to α2 positive DU145KO+ α2 cells. A decrease in the proliferation of α2β1 integrin expressing cells could be the reason for the decrease in their population during prostate cancer progression and would explain the previous report by Mirtti et al, 2006.

Still, the α2 positive DU145KO+ α2 cells had remarkably increased motility compared to the α2 negative cells, which demonstrates the importance of α2β1 integrin expression in a metastatic cancer cell population. It has been shown that PC3 cells with suppressed or reduced α2β1 integrin on cells have significantly less tumorigenic potential and metastases capability (Ryu, Park, and Lee 2016; Lee et al., 2009). Furthermore, we discovered that the p38 MAPK pathway is one main regulator of invasion and proliferation downstream from α2β1 integrin in DU145 prostate cancer cells. The regulation of DU145 cell motility by p38 has also been studied earlier (Shen et al., 2010). Moreover, we showed that the inhibition of p38 MAPK with specific inhibitor SB203580 significantly increased the proliferation

of α2 integrin positive cells, whereas the treatment on α2 negtaive cells was ineffective. Also, the treatment with an inhibitor resulted with the diminished invasion capability of α2 positive cells to the level of α2 negative cells. These findings warrant studying α2β1 integrin-targeted therapy as a potent approach to treat chemotherapy resistant and metastatic prostate cancer cells.

6.1.3 Integrin α2 regulates gene expression related to cancer progression

The α2β1 positive cells had a significantly higher MMP-1 expression. This is in accordance with earlier study where Riikonen et al. showed that α2β1 integrin induces the expression of MMP-1. Considering the motile phenotype of α2 integrin positive cells, high MMP-1 expression enables the local degradation of the ECM to further facilitate dissemination.

The induction of cadherin 5 (CDH5) expression was also observed in α2 integrin expressing cells compared to knockout cells. The cell-cell adhesion proteins, cadherins, have been shown to be dysregulated in many malignancies. Cadherin 5, also known as vascular endothelial cadherin (VE-cadherin) is not expressed in normal epithelium, but is expressed in several tumors. Cancer cells expressing cadherin 5 might easily take part in vasculature mimicry (VM). VM is described as cancer cells interacting with endothelium or even replacing endothelial cells which form mosaic vessels. VM vessels are clinically significant and increase the risk of metastatic disease. In gastric cancer CDH5 is a marker for poor survival (Higuchi et al., 2017) and the phosphorylation of cadherin 5 in a co-culture of breast cancer cells with HUVECs was induced by α2β1 integrin (Haidari et al., 2012). In addition it has been proposed that the matricellular proteins produced by CAFs, the proteolytic processing of laminin-332 and the dense 3D collagen-network existing in the TME could trigger the formation of VM vessels (Eble and Niland 2019).

Concomitantly, integrin α2β1 downregulates several tumor suppressors, including plakophilin1 (PKP1), a protein participating in desmosome formation. Syndecan 2 was up-regulated in α2 integrin knockout cells. In high Gleason score prostate cancers, where overall expression of integrin α2β1 is decreased, syndecan 2 is known to be increased (Popović et al., 2010). The bi-dierctional expression of syndecan 2 and integrin α2β1could be studied in more detail.

The majority of genes (7 outr of 12) that were up or down regulated by the expression of integrin α2β1 on the cell surface, were also regulated by integrin α2β1 binding to its ligand, collagen I. However, when α2 positive and negative cells were plated on collagen and fibronectin some of the effects were evident on both surfaces leaving the option that α2β1may also have ligand independent functions. It has been proposed earlier that integrins may have ligand independent

functions (Ferraris et al., 2014) and that α2β1integrin with a loss-of-function mutation at α2E336A is able to induce FAK phosporylation and downstream signaling (Salmela et al., 2017). However, the ligand independent functions of integrins have not been studied in detail.

Collectively, based on our results, α2β1 integrin expression on cancer cells contributes to the gene expression pattern influencing tumor evolvement.

6.2 Prostate fibroblasts mainly produce the components of ECM, crosstalk with cancer cells enhances remodelling

Alterations in the ECM composition and organization have been proposed to induce tumour burden and dissemination (Erler and Weaver, 2009; Rowe and Weiss, 2009; Lu et al., 2012). CAFs as common cells in cancerous stroma are known to synthesize and organize the ECM in the TME. Interestingly, it was shown that another integrin, namely α3β1 with its interaction to laminin-332 maintains the CAF phenotype and facilitates the invasion of pancreatic adenocarcinoma cells (Cavaco et al., 2018). Based on our results, prostate-derived fibroblasts in *in vitro* cell culture are able to synthesize many ECM proteins. These include several structural proteins such as collagens, FN, tenascin C, and also GFs embedded into the matrix, for example FGF2 and HGF. In addition the fibroblasts in the spheroid culture were able to synthesize some components of the BM, for example laminin β1 and γ1 chain, collagen IV and XV. Prostate cancer cell lines DU145 and PC3 differ in their ability to produce ECM proteins. When DU145 cells are unable to synthesize any core matrisome proteins, then the PC3 cells can produce several components of the BM and many matricellular proteins. However, in co-cultures of cancer cells with fibroblasts, ECM protein synthesis is increased and it seems that the cells collectively produce all the components needed for the BM formation. The induced expression of several laminins, perlecan and collagen XVIII was observed in both the DU145 and PC3 co-cultures with fibroblasts. Laminin-332 protein expression has been reported to be diminished in prostate cancer. The same report, however, indicates that the corresponding mRNA levels remain normal (Hao et al., 2001). In the *in vitro* system we not only saw the induction of laminin-332 protein expression, but simutanously the degredation of the BM components laminin-332 and collagen XVIII were also observed. An intensive degradation of proteins rather than reduced gene expression may explain the findings by Hao *et al.,* 2001. Depending on the cancer type, laminin-332 has been shown to be either down or up regulated with the opposite effect on cancer cell dissemination. In colorectal carcinoma, breast cancer, prostate carcinoma, and oral squamous cell carcinoma (SCC) the protein levels of laminin-332 are diminished and these tumors are thought to have a tumor suppresive role. Whereas,

in pancreatic carcinoma, lung adenocarcinoma, malignant glioma, gastric cancer, SCC elevated laminin-332 protein levels have been reported (Tsuruta et al., 2008). In some of these cancer types elevated laminin-332 is connected with the increased dissemination of cancer cells. Degradation of basement membrane proteins can produce biologically active peptides which can regulate behavior of malignant cells and other cells present in local microenvironment. Our results indicated that MMPs are needed for dissemination of DU145 cells in "out of spheroid" assay, but are not responsible for formation of endostatin and laminin-332 fragments. Whereas cathepsin L, expressed by DU145 and PC3 cells is responsible at least partially for formation of endostatin containing fragments from collagen XVIII.

7 Conclusion and Future Prospects

The tumor microenvironment, including malignant cells as well as activated stromal and immune cells and the scaffolding ECM, has been proposed to contribute to cancer progression. This thesis provides new knowledge about the ECM synthesized by cancer associated fibroblasts and the adhesion molecule integrin α2β1 in human prostate cancer. This thesis work demonstrates the following findings:

I. Integrin α2β1 is essential for cancer cell dissemination in the collagen rich ECM
II. Integrin α2β1 regulates the proliferation and motility of DU145 prostate cancer cells through the p38 MAPK pathway
III. The ECM is mainly produced by fibroblast cells in *in vitro* cell culture conditions and *in vivo* in human prostate
IV. The co-culture of cancer cells with activated fibroblasts enhances the synthesis and proteolytic processing of ECM proteins.

Integrin α2β1 is a marker protein of both prostate stem cells and prostate cancer stem cells. The adhesion protein α2β1 mediates the cell attachment to collagens and to several other ECM proteins. The attachment to ECM proteins through the integrin regulates the behavior of the cell including its processes such as proliferation, motility and survival.

The results obtained in this thesis project explain the previous controversy related to the role of α2β1 integrin in prostate cancer. Firstly, the finding that integrin α2β1 inhibits prostate cancer cell proliferation explains why the bulk of non-tumorigenic cells in tumors have reduced levels of integrin α2β1 expression. Secondly, integrin α2β1 seems to protect the phenotype and survival of cancer stem like cells. And thirdly, the most important cells for cancer progression and dissemination are tumorigenic cancer stem cell like cells, which remain integrin α2β1 positive.

ECM is a complex scaffold and reservoir of growth factors and active peptides. In tumorigenesis, the ECM is continuously remodeled. Cancer cells use matrix proteins to move and release growth factors to induce their own proliferation. The

results support the findings that fibroblasts are the main source of ECM proteins in the *in vitro* models used in this thesis. Due to the restrictions of the cell culture models, the composition of the ECM *in vitro* is slightly different from the ECM found *in vivo* in the human prostate. However, based on the similarity of the core matrisome components it could be said that fibroblasts are the main source of ECM proteins also in human prostate tissue. The effects of the insoluble matrix on cancer cells have been investigated less extensively. This thesis aimed to bring new knowledge into this topic also.

In the future, the 3D co-culture model applied extensively in this thesis could be used to investigate the ECM in other cancer types. It would be interesting to study whether cancer stem cell markers could be used to specifically target this population of cancer cells or could certain matrix proteins be used to target a microenvironment. This could improve the cancer treatment options already available.

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