Functional effects of *SKIL* overexpression in prostate cancer cell lines

Master's Thesis

Joonas Tuominen

University of Tampere

BioMediTech

May 2015

ACKNOWLEDGEMENTS

This thesis was carried out in the Molecular Biology of Prostate Cancer group, Institute of Biomedical Technology, University of Tampere between June 2013 and August 2014. First I would like to thank our group leader Professor Tapio Visakorpi for granting me this opportunity to perform master's thesis in his group.

The person who I want to thank the most is my supervisor PhD Kati Kivinummi for her guidance. I learned a lot during my thesis but also I really enjoyed working with Kati. Also I want to thank technicians Päivi Martikainen and Marika Vähä-Jaakkola their assistance in the everyday laboratory work. Pekka Ruusuvuori helped me with cell counting and for that I want to thank him. Finally I want to thank all the other members of the Molecular Biology of Prostate Cancer group for the great working atmosphere and help they have provided.

PRO GRADU-TUTKIELMA

Paikka: Tampereen yliopisto
BioMediTech
Tekijä: TUOMINEN, JOONAS ILMARI
Otsikko: Functional effects of *SKIL* overexpression in prostate cancer cell lines
Sivut: 68
Ohjaajat: Professori Tapio Visakorpi ja FT Kati Kivinummi
Tarkastajat: Professorit Markku Kulomaa ja Tapio Visakorpi
Aika: Toukokuu 2015

TIIVISTELMÄ

Tutkimuksen tausta ja tavoitteet: Eturauhassyöpä on yksi yleisemmistä syövistä maailmanlaajuisesti ja vaikuttaa miljoonien miesten elämään. Molekulaariset mekanismit, jotka vaikuttavat eturauhassyövän taustalla, ovat edelleen melko huonosti tunnettuja. Genomin kopiolukumuutokset ja geenien fuusiot ovat yleisiä eturauhassyövässä ja esimerkiksi *TMPRSS2-ERG* geenifuusio on tunnettu ja yleinen eturauhassyövän alatyyppi. Aiemmin löysimme *TMPRSS2-SKIL* geeni fuusion eturauhassyöpäpotilaalta otetusta kliinisestä näytteestä. Samalta potilaalta ei löydetty muita tunnettuja *ETS* geenien fuusioita. Tämä viittaa siihen, että tässä tapauksessa *TMPRSS2-SKIL* fuusio voi olla syynä syövän kehittymiseen. Tämän tutkimuksen tavoitteena oli luoda eturauhassolulinjoja, jotka yli-ilmentävät *SKIL*-geeniä ja tutkia onko tällä vaikutusta näiden solujen fenotyyppiin.

Tutkimusmenetelmät: *SKIL* geenin sisältävä plasmidi ja kontrolliplasmidi ilman geeniä tilattiin Addgeneltä. DU-145 ja RWPE-1 solulinjat transfektoitiin joko *SKIL*-plasmidilla tai kontrolliplasmidilla. Molempia solulinjoja kasvatettiin mediumissa, joka sisälsi geneticin® selektiivistä antibioottia (Gibco). Selektion jälkeen DU-145 soluista tehtiin yksisolukloonеja käyttäen erittäin laimeaa (solua/ml) soluliuosta. Sekä mRNA että proteiinit kerättiin molemmista solulinjoista. Kerättyä mRNA:ta käytettiin RT-qPCR:ään ja proteiineja western blottaukseen. Proliferaatiota tutkittiin mikroskoopin ja digitaalikuva-analyysin avulla. Invaasiota tutkittiin käyttäen BD BioCoat Matrigel Invasion Chambers (BD Biosciences).

Tutkimustulokset: Tässä tutkimuksessa loimme solulinjoja, joissa on stabiilisti transfektoituna *SKIL*-plasmidi ja kontrolliplasmidi. Osoitimme, että *SKIL* on yli-ilmentynyt mRNA tasolla solulinjoissa, DU-145 yksisolukloonеissa ilmenemistasot tosin vaihtelivat suuresti. Yli-ilmenemistä ei voitu osoittaa proteiinitasolla kummassakaan solulinjassa. Osoitimme, että *SKIL*:n yli-ilmeneminen ei aiheuta proliferaatio eroja, mutta se lisää invaasiota RWPE-1 soluissa ja joissain DU-145 klooneissa.

Johtopäätökset: Tuloksemme osoittivat, että *SKIL*:n yli-ilmentyminen pystyy aiheuttamaan erilaisen fenotyypin käytetyissä solulinjoissa. Pystyimme osoittamaan selvän eron RWPE-1-soluissa ja osassa DU-145 klooneja. Tämä viittaa siihen, että *SKIL*:n yli-ilmeneminen saattaa yksistään olla tarpeeksi aiheuttamaan eturauhassyövän kasvua. Proteiinitason yli-ilmenemisen puuttuminen jättää kuitenkin kysymyksen fenotyypin aiheuttavasta mekaniikasta. Lisää tutkimusta tarvitaan, että saadaan selville *SKIL*:n tarkka rooli eturauhassyövässä ja sen kehittymisessä.

MASTER'S THESIS

Place: University of Tampere
BioMediTech
Author: TUOMINEN, JOONAS ILMARI
Title: Functional effects of *SKIL* overexpression in prostate cancer cell lines
Pages: 68
Supervisors: Professor Tapio Visakorpi and Dr. Kati Kivinummi
Reviewers: Professors Markku Kulomaa and Tapio Visakorpi
Date: May 2015

ABSTRACT

Background and aims: Prostate cancer is among the most common malignancies in the world affecting millions of men worldwide. Molecular mechanisms behind the prostate cancer are still poorly understood. Genomic copy-number alterations and gene fusions are common in prostate cancer and for example *TMPRSS2-ERG* gene fusion is one of the common known subtypes of prostate cancer. Previously we found *TMPRSS2-SKIL* gene fusion from clinical sample collected from prostate cancer patient. This patient was also negative for ETS fusions so this predicts that this cancer may be caused by *TMPRSS2-SKIL* gene fusion. The aim of this study was to generate prostate cancer cell lines with stable *SKIL* overexpression and to investigate whether *SKIL* overexpression would have any effect on the phenotype of the cells.

Methods: Plasmid with *SKIL* and control plasmid without it were ordered from Addgene. DU-145 and RWPE-1 were transfected either with *SKIL* plasmid or with control plasmid. Then cell were let to grow with geneticin® selective antibiotic (Gibco) to select only the cells with plasmid. With DU-145 cells after selection single cell clones were created by using very low cells/ml dilution. Both mRNA and proteins were collected from both cell lines and RT-qPCR were performed for mRNAs and western blotting for proteins. Proliferation assays were performed for both cell lines using microscopy and digital image analysis. Invasion assays were performed for both cell lines using BD BioCoat Matrigel Invasion Chambers (BD Biosciences).

Results: In this study we created cell lines with stable transfections of *SKIL* and control plasmid. We showed that *SKIL* is overexpressed in mRNA level when compared to control cells. Expression levels were differentiating quite a lot between DU-145 single cell clones. However we failed to show that this expression is also carried to protein level (in both DU-145 and RWPE-1 cells). We showed that *SKIL* overexpression does not induce any proliferation difference but it induces invasion in RWPE-1 cells and with some DU-145 clones.

Conclusion: Our results showed that it is possible for *SKIL* overexpression to induce different phenotype for cell lines used. In this case there was clear difference with RWPE-1 cells and with some DU-145 cells. This indicates that it is be possible that *SKIL* overexpression alone could induce prostate cancer growth. However there were no clear *SKIL* overexpression in protein level and this leaves questions about mechanisms which cause the phenotype. Further investigations needs to be carried out to find out precise functions of *SKIL* and it's overexpression in prostate cancer.

Contents

ACKNOWLEDGEMENTS 2
TIIVISTELMÄ 3
ABSTRACT 4
ABBREVIATIONS 7
1. INTRODUCTION 9
2. REVIEW OF LITERATURE 10
2.1 Prostate 10
2.2 Prostate cancer 11
2.2.1 Epidemiology 11
2.2.2 Risk factors 12
2.2.3 Pathogenesis 15
2.3 Prostate cancer genetics 16
2.3.1 Chromosomal alterations 17
2.3.2 Gene fusions 19
2.3.3 Oncogenes 20
2.3.4 Tumor suppressor genes 21
2.3.5 Epigenetics 22
2.4 TGF-β signaling 24
2.5 Role of *SKIL* in cancer progression 25
2.6 Previous results from *SKIL* studies 28
3. AIMS OF THE RESEARCH 29
4. MATERIAL AND METHODS 30
4.1 Subcloning of *SKIL* 30
4.1.1 Colony PCR 30
4.1.2 DNA sequencing 31
4.3 Cell lines 31
4.3.1 Transient transfection 32
4.3.2 Stable transfection 32
4.4 RNA extraction and RT-qPCR 33
4.5 Protein extraction and measurement of concentration 34
4.6 Western blotting 34
4.7 Growth curves 36
4.8 Matrigel invasion assay 36
5. RESULTS 38

5.1 Subcloning of *SKIL* 38
5.2 Verification of *SKIL* expression by RT-qPCR 38
5.3 The effects of *SKIL* expression on the growth of the cell lines 38
5.4 The effects of *SKIL* expression on the invasiveness of cell lines 39
5.5 Verification of *SKIL* overexpression by western blotting 39
6. DISCUSSION 45
6.1 Subcloning of *SKIL* 46
6.2 Transfection of cell lines 46
6.3 Verification of *SKIL* overexpression by RT-qPCR 47
6.4 The effects of *SKIL* expression on the growth of the cell lines 50
6.5 The effects of *SKIL* expression on the invasiveness of cell lines 51
6.6 Western blotting 53
6.7 Future perspectives 54
7. CONCLUSION 56
8. REFERENCES 57
9. APPENDIX 69

ABBREVIATIONS

ABL1	Abelson murine leukemia viral oncogene homolog
AKT	Protein kinase B
AR	Androgene receptor
BCR	Breakpoint cluster region protein
BMI	Body mass index
BPE	Bovine pituitary extract
BPH	Benign prostate hyperplasia
BSA	Bovine serum albumin
CNA	Copy number alteration
CRPC	Castrate resistant prostate cancer
DOT1L	DOT1-like
DTT	Dithiothreitol
DU-145	DU-145 human prostate cancer cell line
EGF	Epidermal growth factor
ELAC2	ElaC Ribonuclease Z 2
EP156T	EP156T human prostate cancer cell line
ERG	ETS-related gene
ERVK-24	Endogenous Retrovirus Group K, Member 24
ETS	ETS transcription factor family
ETV1/4/5	ETS translocation variant 1/4/5
FLI1	Friend leukemia integration 1 transcription factor
G418	Geneticin
HDAC	Histone deacetylases
Hep3B	Hep3B human hepatoma cell line
HES6	Transcription cofactor HES-6
HNRPA2B1	Heterogeneous nuclear ribonucleoproteins A2/B1
Hox	Paralogous homeobox
H3K18Ac	Histone H3 acetyl Lys18
H3K4	Histone H3 lysine 4
LNCaP	LNCaP human prostate cancer cell line
LuCaP	LuCaP Series of Prostate Cancer Xenografts
MIPEP	Mitochondrial intermediate peptidase
mSin3A	mSin3A chromatin modifying complex
MSR1	Macrophage scavenger receptor 1
MYC	c-Myc
N-CoR	nuclear receptor co-repressor 1
NDRG1	N-Myc Downstream Regulated 1
NKx3.1	Homeobox protein Nkx-3.1
PC-3	PC-3 human prostate cancer cell line
PIN	Prostatic intraepithelial neoplasia
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PSA	Prostate-specific antigene
PTEN	Phosphatase and tensin homolog
RB1	Retinoblastoma protein
RNASEL	Ribonuclease L

RT-qPCR	Real time quantitive reverse transcription PCR
RWPE-1	RWPE-1 epithelial cells
SKIL	SKI-like proto-oncogene
SLC45A3	Solute carrier family 45 member 3
SMRT	Nuclear Receptor Corepressor 2
TGF-β	Transforming growth factor beta
TMPRSS2	Transmembrane protease serine 2
TOP2B	DNA topoisomerase 2-beta
TP53	Tumor protein p53
VDR	Vitamin D receptor
22RV1	22RV1 human prostate cancer cell line

1. INTRODUCTION

Prostate cancer is second most common cancer among the men worldwide and most common cancer in western countries (Center *et al.* 2012). It is also sixth leading cause of cancer deaths worldwide (Center *et al.* 2012). It is known that prostate cancer incidence increases with the age and as the life expectancy is increasing, especially in the western countries, the rate of new prostate cancer cases is likely to go up in the future (Center *et al.* 2012). Although cases are increasing the mortality rate is decreasing which is mostly due the better treatment options (Center *et al.* 2012).

Prostate cancer is known to be highly heterogeneous disease, both clinically and genetically (Boyd *et al.* 2012). Fortunately most of the tumors in prostate are slowly growing, metastasize poorly and are not likely to cause death (Boyd *et al.* 2012). There are however prostate cancers which have aggressive behavior and can metastasize (Boyd *et al.* 2012). This provides the problem to identify aggressive and non-aggressive cancers from each other and indeed there are many alterations in prostate cancer which are known to cause aggressive phenotype (Boyd *et al.* 2012).

Among the known alterations are gene fusions such as *TMPRSS2*-ETS fusions (Boyd *et al.* 2012). This fusion means that ETS genes are brought under the promoter of *TMPRSS2* and this usually causes increased expression patterns of ETS genes (Boyd *et al.* 2012). Especially important fusion is *TMPRSS2-ERG* fusion which is not only the most recurrent *TMPRSS2*-ETS but there is also evidence that there is an association between the presence of this fusion and the outcome of the cancer (Boyd *et al.* 2012).

Previously novel *TMPRSS2-SKIL* fusion was found from single patient with prostate cancer (Annala *et al*. 2015). This patient also had no other common alterations known to be present in prostate cancer so this lead to hypothesis that cancer may be caused by *TMPRSS2-SKIL* fusion (Annala *et al*. 2015). Effects of *SKIL* overexpression could provide more information about role and mechanisms of *SKIL* in prostate cancer. Also there is no previous studies about *SKIL* overexpression in prostate cancer cells. In light of the found *TMPRSS2-SKIL* fusion it is important to find the functions of *SKIL* in prostate cancer cells.

2. REVIEW OF LITERATURE

2.1 Prostate

Prostate gland is part of the male reproductive system. It has roles in the production and transportation of ejaculate and male sex hormones. Main function of prostate is to secrete and store the slightly alkaline, milky prostatic fluid, which constitutes about half of the semen volume, along with the spermatozoa and seminal vesicle fluid.

The prostate is derived from the primitive endoderm, which first differentiates to cloaca. This happens during the embryogenesis. After this in humans the cloaca separates to digestive outlet and urogenital sinus, which in turns segments into the urinary bladder and the urethra (Berman *et al*. 2012). Then prostate can develop via the proliferation of epithelial buds from the urogenital sinus epithelium. After this these buds invade according to particular pattern, which directs the future development of distinct prostate zones (or lobes which happens *e.g.* in mice). Later on urogenital sinus mesenchymal cells can differentiate into stromal elements. Prostate budding occurs during the 10th week of embryogenesis (Berman *et al*. 2012).

Primary motivating force driving the prostate development, is androgen receptor (AR) signaling, which acts on the mesenchyme. This said it is important to notice that AR signaling only affects the timing of the events and has no effects on the location of events. This means that AR doesn't decide where prostate starts to develop (Podlasek *et al*. 1997). The mechanisms which direct the precise locations of prostate epithelial buds are not fully understood. However there are evidences which predict that it is related to paralogous homeobox (*Hox*) genes, as they coordinate similar processes in various tissues. Also mutations in *Hox* genes can result to changes in the prostatic branching patterns (Podlasek *et al*. 1997).

The prostate tissue in the adults is a complex tubule-alveolar gland. The regional anatomy of prostate was under the debate during the latter half of 20th century. Originally prostate was classified into lobes, but nowadays this concept is commonly replaced by zonal model. This model divides prostate into anterior, peripheral, central

and transitional zones that all have their own architectural features. Prostate is composed of epithelial and stroma. In epithelial compartment there is basal epithelial cells, intermediate cells, neuroendocrine cells and luminal secretory cells. Stromal compartment services more as a supportable structure and is consisted mostly from connective tissue, smooth muscle cells and fibroblasts (Berman *et al*. 2012).

As said androgen signaling is really important during the development of the prostate in embryogenesis but it remains important during the different stages of human life and has a role in the growth, maintenance and secretory function of prostate. In cell level androgens normal function is to drive cell differentiation (Berman *et al*. 2012).

2.2 Prostate cancer

2.2.1 Epidemiology

Prostate cancer is the most common non-skin cancer neoplasm amongst men not only in Europe (Bray *et al.* 2010) but also worldwide (Fontes *et al.* 2013). Also worldwide prostate cancer is the sixth leading cause of oncological death among men. In 2008 approximately 250000 deaths were due to prostate cancer (Fontes *et al.* 2013). In 2008 in Europe there has been almost 90000 deaths due prostate cancer (Bray *et al.* 2010). In Finland alone the mean incident cases per year was 4480 during 2001-2005. This means that Finland has one of the highest prostate cancer occurrence rates in Europe (Bray *et al.* 2010). Overall in northern Europe the disease rates are very high and unfortunately it seems that these rates are even growing. From 1990 to 1995 the increase percent concerning prostate cancer cases in Finland was 9,3% per year (Bray *et al.* 2010). This increase in prostate cancer incidence rates is mostly due to increased life expectancy, advanced diagnostic methods and PSA screening (Center *et al.* 2012). Prostate cancer is disease strongly associated with aging in United States over 70 % of all cases are diagnosed to men over 65 years old (Crawford 2003).

Also ethnicity is very high risk factor in prostate cancer. African Americans have among the highest rates of prostate cancer in the world. The incidence rate among

African Americans is 60% higher than among the white American men. Incidence rates are even lower among the Hispanics and Asians/Pacific Islanders (Clegg *et al.* 2002).

However when mortality rates are being examined things don't look that bad. During the past few decades the mortality rate in prostate cancer has steadily decreased. The 5-year relative survival proportions are approaching 100% especially with the localized disease. This may be due the overall improvements in treatment or an effect of PSA testing which helps us to detect disease early enough (Bray *et al.* 2010).

2.2.2 Risk factors

There are several different risk factors related to prostate cancer. When talking about risk factors it needs to be made clear, which kind of risks we are talking about, because prostate cancer risk factors can be divided to several subgroups, such as for ''aggressive'' and ''non-aggressive'' cancers or by grade or stage. Many times however this division is not clear mainly because we still have problems in recognizing if cancer itself is "aggressive" or "nonaggressive". There are three clear factors which have clear connection to overall incident prostate cancer risk: age, positive family history and race (Giovannucci *et al.* 2007). There are also evidence which point out that higher tomato sauce intake (inversely), vitamin D and α-linolenic acid intake have also association with overall prostate cancer risk (Giovannucci *et al.* 2007). Other important subtype of risk factor is fatal prostate cancer risk. There are several different factors that can have effects on this. At least recent smoking history, taller height, higher BMI, family history, and high intakes of total energy, calcium and α-linolenic acid were associated with higher risk (Giovannucci *et al.* 2007). It seems the trend in United States (and maybe in other western countries also) is that men are diagnosed prostate cancer sooner than before. In the United States prostate cancers have significantly increased among men younger than 50 years old during the last decade. On the other hand the incidence rates among men older than 70 years have decreased at the same time. This suggests that these changes result from earlier diagnosis (Li *et al.* 2012).

Even when we are not talking about hereditary prostate cancer, a family history of prostate cancer is known to be associated with increased risk of prostate cancer diagnosis (Thomas *et al.* 2012). There are studies which suggest that there is even 2,5-

fold higher relative risk to prostate cancer when you are first-degree relatives of prostate cancer patient. This risk can increase to 3,5-fold risk when you are first-degree relatives of two prostate cancer patient (Johns & Houlston 2003). Also there is evidence that it is not only the higher risk to get prostate cancer but there is also link between disease outcome and family history. Men with first-degree relatives affected by prostate cancer are diagnosed and die at earlier age (Brandt *et al.* 2009).

As said race is important risk factor. In United States in 2007 black males had an age-adjusted annual death rate from prostate cancer 2,4 times that of whites. All though this risk factor is well known and widely accepted the reasons behind it are not clear at all. White men are screened more frequently and also they are treated more frequently after prostate cancer diagnosis. This may be at least on factor, which explains this difference but even that is not perfectly clear (Taksler *et al.* 2012). Interestingly Asian population seems to have lower prostate cancer risk than men in Europe or in United States. Some, but not all, of this risk can be explained through environmental factors like diet. This however doesn't explain the fact that when Asian men move to western countries they still have lower risk of getting the prostate cancer (Ito 2014).

In many cancers smoking is significant risk factor but its role in the prostate cancer is not as clear as in some other cancers. There is however study which suggests that current smokers have a decreased risk of nonadvanced prostate cancer. On the other hand they also have increased risk of fatal prostate cancer. Former smoking also seemed to decrease the risk of nonadvanced prostate cancer. They didn't find any association between smoking and advanced prostate cancer (Watters *et al.* 2009). In another study which was carried out as an observational study, they found out that smoking in the time of prostate cancer diagnosis increased overall mortality and cardiovascular disease and prostate cancer specific mortality and cancer recurrence. In this study also they found out that if men had stop smoking at least 10 years ago they had same risk for cancer-specific mortality as those never smoked (Kenfield *et al.* 2011).

Another common habit: consumption of alcohol has also role in risks associated to prostate cancer. There are several studies available about the subject but results are inconsistent. Cohort study in United State suggests that risk of nonadvanced prostate cancer is increased upon the increase of alcohol usage but there is no increased risk

between alcohol usage and advanced prostate cancer (Watters *et al.* 2010). In another study carried out in New Zealand results were just the opposite and they sat that consumption of alcohol lowers the risk of getting prostate cancer of any kind (Karunasinghe *et al.* 2012). In third research the outcome was that there is no difference what so ever if one consumes alcohol or not. This was true even with larger amounts of alcohol (Rota *et al.* 2012).

Vitamin D is known to have effects in preventing prostate cancer (Wang & Tenniswood 2014). There is data which demonstrates an inverse association between serum vitamin D3 levels, cancer incidence and related mortality. There is studies which suggest that vitamin D3 induces apoptosis of androgen dependent prostate cancer cell lines. Other studies on the other hand suggest that D3 only induces cell cycle arrest. Mechanism behind the vitamin D functions are not clear but recent studies have found synergistic crosstalk between the vitamin D- and androgen-mediated mRNA and miRNA expression, which adds an additional layer of post-transcriptional regulation to the known VDR- and AR-regulated gene activation (Wang & Tenniswood 2014).

There are also some other interesting risk factors which are affected by people behavior. Coffee consumption has been shown to decrease overall risk of getting prostate cancer. However this seems to be true only on high levels (Wilson *et al.* 2011). Controversially there is another study which says that there is no difference between drinkers and non-drinkers of coffee when it comes to overall risk of prostate cancer, but even this study is saying that there is difference if we talk only high Gleason grade cancers (Shafique *et al.* 2012). The omega-3 fatty acid α-linolenic acid has also been attraction of many prostate cancer studies. Despite of this it is still not clear if this fatty acid has some role or not with prostate cancer. There are many studies that suggests that there might be small increased risk to get prostate cancer upon the high usage of α-linolenic acid. These studies all however highlight the fact that more studies are needed. On the other hand there is studies which say that there is no link between α-linolenic acid and prostate cancer (De Stefani *et al.* 2000, Brouwer *et al.* 2004, Koralek *et al.* 2006, Simon *et al.* 2009).

As we can see there is many different risk factors related to prostate cancer and it is not easy to say which ones are really relevant and which ones have more minor impacts on

cancer development. Studies are often controversy and often they concentrate only to one potential risk factor. However there is clear consensus that high age and positive family history are the most important risk factors when talking about prostate cancer (Giovannucci *et al.* 2007).

2.2.3 Pathogenesis

Usually first thing appearing in prostate cancer is prostatic intraepithelial neoplasia (PIN). PINs can be divided to low grade and high grade PINs. Usually low grade PINs are not even reported if clinically found and when word "PIN" is mentioned it usually refers to high grade PIN (Bostwick & Cheng 2012). High grade PINs are more often than not accepted to be precursors of prostate cancer. It seems that the incidence of PINs increases with the age, just like with the prostate cancer. The reported high grade PINs of African Americans by decade of age between the third and eighth decades were 7%, 26%, 46%, 72%, 75% and 91% (Montironi *et al.* 2007). Also the frequency and the severity of HGPIN increases in the presence of prostate cancer and it is possible to observe the transition of high grade PIN to prostate cancer from the morphological point of view (Montironi *et al.* 2007). PINs can be described as the progressive abnormalities with genotypes and phenotypes that are an intermediate of those in benign prostate epithelium and prostate cancer. PINs are characterized by cellular proliferation within ducts and acini of prostate. Abnormalities which usually occur are nuclear and nucleolar. Also there is inversion of the normal orientation of epithelial proliferation from the basal cell compartment to the luminal surface (Bostwick & Cheng 2012).

Allthough PINs are kept as precursors of prostate cancer they are quite common they do not lead to cancer in most cases (Schoenfield *et al.* 2007, Gallo *et al.* 2008). The mean incidence of PIN cases is about 9%. This represents about 115 000 new cases of isolated PIN diagnosed each year in the United States (Bostwick & Cheng 2012).
Another common prostate disease affecting men worldwide is benign prostate hyperplasia (BPH). It is a slow progressive enlargement of the prostate gland which can lead to lower urinary tract symptoms. This happens usually in older men. It is normally characterized by hyperproliferation of epithelial and stromal cells in the transition zone

of the prostate gland. Although BPH is a clear problem it is not usually precursor of prostate cancer (Prajapati *et al.* 2013).

2.3 Prostate cancer genetics

Prostate cancer is heterogeneous disease (Boyd *et al.* 2012, Tapia-Laliena *et al.* 2014). There can be multiple cancer loci in single prostate gland and also histologically identical tumors can lead to clinically different outcomes such as latent or aggressive disease (Boyd *et al.* 2012). One thing that at least partly can explain this phenomena is genetic heterogeneity. During past several years more and more evidence points to the direction that this genetic heterogeneity is caused by genetic instability. Also it seems that genomic instability, at least in some level, can explain biological differences between the aggressive and indolent prostate cancer (Boyd *et al.* 2012, Tapia-Laliena *et al.* 2014). Although there is clear heterogenic nature in prostate cancer, there is also several recent studies, which suggests that the majority of multifocal prostate cancers may have monoclonal origins. This is contrary to that what was previously thought (Lindberg *et al.* 2013).

Heredity plays an important role in prostate cancer. However it is not perfectly clear how common heredity prostate cancer is. This is because the hereditary prostate cancer genes have not yet been cloned and the definition is based on the pedigree only. The prevalent definition includes nuclear families with 3 cases of prostate cancer, families with prostate cancer in each of 3 generations in the paternal or maternal lineage and families with 2 men diagnosed with the disease before age of 55 years (Bratt 2002, Ishak & Giri 2011). This represents about 3% to 5% of all prostate cancer cases but because it is difficult to identify female mutation carriers this method misses some of the cases. The true proportion of prostate cancer caused by mutations in dominantly inherited susceptibility genes with high penetrance is more likely 5% to 10% (Bratt 2002). However this 5% to 10% represents a vast majority of early onset diseases (Bratt 2002, Lange *et al.* 2012). Several genes such as *RNASEL, ELAC2* and *MSR1* are known to harbor mutations in hereditary prostate cancer. Also somatic mutations in these same genes usually are associated with sporadic prostate cancer (Noonan-Wheeler *et al.*

2006). But even if we are not talking about the heredity prostate cancer, positive family history is still most important risk factor (Boyd *et al.* 2012).

The ever developing new techniques such as next-generation sequencing are giving us more and more information about genetic and genomic alterations in cancer. This information is highlighting the fact that genetic basis of prostate cancer is complex. There really isn't any single alteration that is by itself enough to cause prostate cancer. It is prevalently believed that cancer is evolving through the accumulation of multiple alterations of genome in same cell. Mutations can be divided to driver and passenger mutations. Driver mutations are mutations which give cell a selective growth advantage compared to another cells. On the other hand the passenger mutations don't have effect on the growth of the cells. According to some studies, human solid tumors typically contain 40-100 coding gene alterations and 5-15 of these are usually driver mutations. (Bozic *et al.* 2010).

2.3.1 Chromosomal alterations

There are two types of massive chromosomal-damaging events which have been recently found in prostate cancer: chromothripsis and chromoplexy. There is evidence that these events can occur as a single event. In chromothripsis there is structural rearrangements which occur in a clustered fashion. This involves a single chromosome or single arm of a chromosome with 10s to 100s of rearrangements. Chromothripsis has been observed in approximately in 2% to 5% of cancers so far (Stephens *et al.* 2011, Tapia-Laliena *et al.* 2014). Chromothripsis has been associated to mutations in TP53 and an aberrant DNA damage response, but it is not clear what the mechanism is behind this phenomena (Stephens *et al.* 2011). Likely explanation is the shattering of on or few chromosome and then rejoining them randomly by DNA repair machinery. This may lead to deletions of cancer suppressor genes or amplifications of oncogenes (Tapia-Laliena *et al.* 2014).

In chromoplexy there is generation of chained patterns of chromosomal rearrangements and deletion bridges which is generated from chromosomal DNA located in multiple chromosomes. Likely explanation why chromoplexy causes tumor progression is same

as with the chromothripsis: cancer suppressor gene expression is disrupted and oncogene gene expression is increased (Baca *et al.* 2013, Tapia-Laliena *et al.* 2014). Chromoplexy is often associated with *ERG* rearrangements such as *TMPRSS2-ERG* fusion. This could even implicate that chromoplexy plays some role in cancer initiation (Baca *et al.* 2013). This two events are important also in that sense that they challenge the classical view in which cancer is developed through the accumulation of mutations and alterations over a prolonged period (Tapia-Laliena *et al.* 2014).

Another common chromosomal alteration in cancer is aneuploidy which means numerical whole chromosome aberrations. Aneuploidy can also be found from other malignancies and common feature of aneuploidy is the increasing of it with stage. This leads to the fact that virtually all CRPCs are aneuploid (Tapia-Laliena *et al.* 2014). Aneuploidy is often driven by centrosomal abnormalities which are very common founding in prostate cancer. These centrosomal abnormalities can be found from 100% of metastatic lesions and also more than 90% of localized prostate cancer (Pihan *et al.* 2001). However it is important to remember that not always centrosomal abnormalities lead to aneuploidy. Interestingly as with the chromoplexy the aneuploidy has also been linked to *ERG* rearrangements (Saramaki & Visakorpi 2007, Magistroni *et al.* 2011)(Saramaki, Visakorpi 2007, Magistroni, Mologni et al. 2011)(Saramaki & Visakorpi 2007, Magistroni *et al.* 2011)(merged).

There are also several copy number alterations (CNAs) in prostate cancer both gains and losses. Common losses found in prostate cancer are losses of 2q21-22, 5q13-21, 6q14-21, 8p21-23, 10q23-25, 13q14-22, 16q13-24, 18q12-23 and 21q22. Common gains are the gains of 3q23-33, 7q21-33, 8q12-23, 17q24-25 and Xq11-23 (Saramaki & Visakorpi 2007, Cheng *et al.* 2012). Alterations in chromosome 8 are quite common (both gains and losses) and they have been known for almost two decades already (Matsuyama *et al.* 1994, Van Den Berg *et al.* 1995). With chromosome 8 alterations it is interesting that the prevalence of these alterations comes much more common along the progression of prostate cancer. When the stage of the prostate cancer increases the chromosome alterations of chromosome 8 follow and in the hormone refractory prostate cancer there is chromosome 8p deletion and 8q gain simultaneously in 60% of cases (El Gammal *et al.* 2010). There are some known genes associated with cancer in areas

of deletion and gain in chromosome 8. Notable are at least NKx3.1 which is lost from 8p21.2 and MYC which is gained in 8q24.21 (Boyd *et al.* 2012).

2.3.2 Gene fusions

Important factors in prostate factor which are also important factors of this master thesis are gene fusions. A fusion gene is a hybrid gene formed from two previously separate genes (Mitelman *et al.* 2007). Good known example of these fusions in other cancers is BCR-ABL1 gene fusion in leukemia (Rana *et al.* 2013). This fusion is good example of long known fusion which has high clinical relevance in treatment of decease (Rana *et al.* 2013). In prostate cancer there is recurrent chromosomal translocation between the ETS transcription factor family of genes and the *TMPRSS2* gene. As consequence of this gene fusion, the expression of ETS genes is now controlled by *TMPRSS2* promoter (Boyd *et al.* 2012). Especially important *TMPRSS2*:ETS fusion is the fusion between *ERG* and *TMPRSS2* genes. This is not only the most recurrent *TMPRSS2*:ETS but there is also evidence that there is an association between the presence of this fusion and the outcome of the cancer. There is evidence that *TMPRSS2-ERG* fusion is early or even initial fusion in some prostate cancers. This fusion is detected less frequently in PINs than in tumor lesions but it is detected frequently in PINs which lead to fusion-positive tumors (Boyd *et al.* 2012). The mechanism behind this gene fusion is not fully understood, however the proximity between *TMPRSS2* and *ERG* can be induced in both malignant and nonmalignant prostate cells following androgen treatment, suggesting an early role in prostate carcinogenesis (Boyd *et al.* 2012). After the suggestion that fusion induction results from gene colocalization and double-strand break (DSB) generation it was found that androgen signaling promotes corecruitment of AR and DNA topoisomerase 2-β (TOP2B) to sites of *TMPRSS2-ERG* genomic breakpoints, this in turns can trigger a recombigenic TOP2B mediated DSBs (Haffner *et al.* 2010, Boyd *et al.* 2012).

There are also other possible fusion partners detected for the *TMPRSS2* gene. *ETS* genes—such as *ETV1*, *ETV4*, *ETV5*, and *FLI1*—have been identified as *TMPRSS2* 3' fusion gene partners. In addition, a number of 5' partners—including *SLC45A3*, *ERVK-24* (also known as *HERVK_22q11.3*), *HNRPA2B1*, *C150RF21*, and *NDRG1*—have

been identified in ETS gene fusions so not only the *TMPRSS2* provides promoter regions for genes related prostate cancer (Boyd *et al.* 2012).

Because *TMPRSS2* is important factor in my master's thesis I will talk about it little bit more. *TMPRSS2* is androgen regulated gene and it is preferentially expressed in prostate. *TMPRSS2* is normally expressed exclusively in the normal basal cell population (Lin *et al.* 1999, Gasi Tandefelt *et al.* 2014). In androgen sensitivity cell line LNCaP addition of androgen results in increased *TMPRSS2* expression which demonstrates the fact that *TMPRSS2* is androgen regulated. Also when the expression of *TMPRSS2* is examined from xenografts that recapitulate the androgen-dependent and subsequent androgen-independent characteristics of human prostate cancer growth, it is seen that *TMPRSS2* is expressed also in cells which have reached the androgen-independent state. This indicates possible dysregulation of *TMPRSS2* control (Lin *et al.* 1999). *TMPRSS2* is located on chromosomal band 21q22 common *fusion* companion *ERG* is also in the same band and distance between them is only 3 Mb. Proximity of these genes makes sense because they are so common fusion partners (Gasi Tandefelt *et al.* 2014). However *SKIL* is located to different chromosome 3q26 (Deheuninck & Luo 2009). This might sound problematic in terms of fusion to happen but there are many other cases such as *ERG* fusion to SLC45A3 which happen despite the fact that they are not in genomic proximity of each other (Gasi Tandefelt *et al.* 2014).

There are also gene fusions which don't include either *ERG* or *TMPRSS2*. For example *DOT1L-HES6* fusion is showed to drive prostate cancer growth in androgen independent manner (Annala *et al.* 2014). In this study it was found that there was an interchromosomal rearrangement that fused intron 9 of *DOT1L* with a position 4 kb upstream of *HES6*, resulting in *HES6* overexpression, which however wasn't showed on protein level (Annala *et al.* 2014).

2.3.3 Oncogenes

Genes with ability to contribute cancer with gain-of-function mutations are called proto-oncogenes. As name suggests proto-oncogenes can become oncogenes. This happens typically through point-mutations, amplifications or rearrangements. Typically

oncogenes have great impact on signaling pathways. There are oncogenes that are important factors in various different cancers and some that are specific only in some types of cancers.

When talking about cancer specific proto-oncogenes there is pretty good example in prostate cancer: *AR*. *AR* codes for androgen receptor which is a nuclear receptor that has many functions in human development and physiology. Unfortunately *AR* is also involved strongly in prostate cancer where it plays role usually after the hormone therapy (Li & Al-Azzawi 2009). The transition from proto-oncogene to oncogene usually happens during the prostate cancer progression (Visakorpi *et al.* 1995, Han *et al.* 2005). *AR* is activated in most cases through the amplifications in the DNA. Gain-of-function mutations are quite rare in this case (Visakorpi *et al.* 1995). Because growth of the prostate cancer is heavily dependent on androgens it seems that high levels of androgen receptors can help cells to sustain growth even when there is little androgen present (Visakorpi *et al.* 1995). Allthough *AR* is usually related to prostate cancer it also seems that it may have oncogenic role in the breast cancer (surprisingly) but this needs more evidence before we can be sure about it (Hickey *et al.* 2012).

2.3.4 Tumor suppressor genes

There are genes which in case of loss-of-function mutation can drive cells towards the development of cancer. These cells are called tumor suppressor genes. There are several ways which can cause the loss-of-function mutations. Typical ways are by chromosomal deletions, point mutations or by epigenetic mechanisms. Good thing however is the fact that usually there needs to be alterations in both alleles of the gene before any development towards cancer happens (Alberts 2008). There are some typical features which are common with different suppressor genes. Typical functions that are regulated by tumor suppressor genes are the detection and repair of DNA damage, protein ubiquitination and degradation and cell cycle checkpoint responses (Sherr 2004).

Common loss-of-function tumor suppressor gene in prostate cancer and in many other cancers is *TP53* which codes p53 protein. Normally *TP53* is activated in response to

cellular stress such as DNA damage, and when activated p53 gets phosphorylated and it acts as a transcription factor. The activation of the p53 pathway can result in cell cycle arrest, cell senescence and apoptosis (Levine 2011). Docetaxel is a drug which is commonly used in the treatment of the castration resistant prostate cancer. In recent study it was found out that this drug induces the phosphorylation of p53. In accordance with this study the docetaxel sensitivity of prostate cancer is gratly affected by the mutational status of p53 (Liu *et al.* 2013). Very well-known example of effects of mutation in *TP53* is Li-Fraumeni syndrome (Kamihara *et al.* 2014).

PTEN is another well-known tumor suppressor gene. *PTEN* is known to be inactivated in many different cancers such as glioma, melanoma and carcinoma of the endometrium, kidney, breast, lung, upper respiratory track and prostate cancers (Li *et al.* 1997, Pourmand *et al.* 2007). *PTEN*, which is located in 10q23, codes for dual-specificity phosphatase and is known to act as a part of the PI3K-PTEN-AKT signaling pathway. This is a pathway which, as in many other cases with pathways associated with cancer, is known to regulate many important cellular processes such as apoptosis, cell metabolism, cell proliferation and cell growth (Pourmand *et al.* 2007). *PTEN* is normally a negative regulatory factor of PI3K–AKT pathway, so the loss of *PTEN* function promotes ell growth, survival and proliferation (de Muga *et al.* 2010). Inactivation of *PTEN* is frequent event also in prostate cancer progression and the lack of *PTEN* expression is known to correlate with advanced pathological state and with a high Gleason score (de Muga *et al.* 2010). There is frequently *PTEN* mutation in metastatic prostate cancer but how frequent it is not clear and varies between different studies in range from 20 to 60% (Pourmand *et al.* 2007, de Muga *et al.* 2010).

2.3.5 Epigenetics

Word “epigenetics” means changes in DNA which don’t alter the sequence of DNA. They induce conformational changes in the DNA double helix and with that they modify the access of transcription factors to the DNA. Epigenome comprises all the changes in DNA methylation, histone modification, nucleosome remodeling and RNA-associated silencing. In cancer there is often if not always epigenetic changes in addition to genetic changes. In the carcinogenesis of prostate cancer somatic epigenetic

alterations appear earlier and more frequently than genetic sequence changes. Already there is multiple genes which are known to be silenced in prostate cancer. These genes provide new possible biomarkers and can help us to understand prostate cancer mechanisms better (Albany *et al.* 2011).

DNA methylation changes are common in prostate cancer. DNA methylation means covalent chemical modification of DNA by adding the methyl ($-CH_3$) group at the carbon-5 position of the cytosine ring. Usually promoters are unmethylated however in prostate cancer there is often hypermethylation of promoters (Albany *et al.* 2011). This modification can silence many classical tumor suppressor genes involved things like hormone signaling, DNA repair, cell adhesion, cell-cycle control, and apoptosis (Maruyama *et al.* 2002, Yegnasubramanian *et al.* 2004, Jeronimo *et al.* 2004). Interestingly tumor suppressor genes such as *PTEN, RB1* and *TP53* that are frequently altered in many other common cancers are usually not hypermethylated in prostate cancer (Albany *et al.* 2011). Another common methylation change in prostate cancer is CpG hypermethylation of GSPT1. This methylation is even used as biomarker for prostate cancer (Wright & Lange 2007).

AR which is the most studied transcriptional activator in prostate cancer seems also to be controlled at least in some degree by the epigenetic mechanisms. When comparing castrate resistance prostate cancer (CRPC) to untreated tissue it is found out that hypermethylation of AR is more frequent in CRPC (28%) than in untreated tissue (10%). This suggests that hypermethylation may have important roles in regulation of AR (Nakayama *et al.* 2000). Also histone acetylation is known to play important role in the regulation of AR-pathway (Nakayama *et al.* 2000). There has also been studies where the main interested has been in possible reversibility of castrate resistance in PC cell lines. This was done by using the hypomethylating agent azacitidine in combination with the antiandrogen bicalutamide. Results showed that this method worked with PC-3 cells but failed to work in 22RV1 cells. Also in another study hypomethylation was studied as a therapeutic option to counteract resistance to androgen deprivation in both *AR* positive and negative cell lines. However other models needs to be used to get more reliable results in this matter (Gravina *et al.* 2008, Gravina *et al.* 2010, Chen *et al.* 2010, Alva *et al.* 2011).

In addition to hypermethylation there is of course chance of hypomethylation of DNA. Indeed there are several malignancies including prostate cancer where hypomethylation can be found (Albany *et al.* 2011). It seems that there is usually first hypermethylation and only after that hypomethylation can be detected in DNA. This means that usually hypomethylation can be detected only from higher stage cancers and it seems to occur heterogeneously during prostate cancer progression and metastatic dissemination (Nelson *et al.* 2007). There are several mechanisms which are hypothesized to contribute the oncogenesis of hypomethylation. Suggested mechanisms are at least activation on oncogenes, activation of latent retrotransposons and contribution to genomic instability (Kulis & Esteller 2010).

Also it seems that histone modification plays important role in prostate cancer tumorigenesis. The global level changes in individual's histone modifications are showed to be predictive in clinical outcome of prostate cancer independently of other features such as tumor stage, preoperative prostate-specific antigen levels, and capsule invasion (Seligson *et al.* 2005). There are two global methylations of *H3K4* and histone H3 lysine 18 acetylation (*H3K18Ac*) which are independent predictor of recurrence in low-grade prostate cancer (Seligson *et al.* 2005, Zhou *et al.* 2010).

2.4 TGF-β signaling

Transforming growth factor β is a ubiquitous cytokine that has effects in various different biological processes also the functions of TGF-β signaling are important in my master thesis. In many epithelial cells TGF-β is negative growth factor and loss of this growth inhibition is a hallmark of many different cancer types (Liu *et al.* 2001). TGF-β causes cells to accumulate in mid-to-late G1 phase of the cell cycle by blocking the transition from G1 to S. TGF-β binds to type II receptor on the cell surface and this leads to the recruiting of type I receptors. Both of these receptors are ser./thr. kinases. This means that type II receptors phosphorylate the type I receptors and then type I receptors in turn phosphorylate its intracellular substrates, which are Smad2 and Smad3. After this the two proteins can bind with Smad4 and form a complex. This complex can then move to nucleus and functionally collaborate with different

transcription factors (Liu *et al.* 2001). Cell cycle progression through the G1/S transition requires activation of the cyclin E/cdk2 complex (Liu *et al.* 2001).

There are two major pathways which Smad complex can use to inhibit the cyclin E/cdk2 complex which activity is needed cells to get past the G1 restriction point. First it can activate transcription of two inhibitors of cyclin-dependent protein kinases, $p21^{Cip1}$ and $p15^{INK4B}$ (Liu *et al.* 2001). In this quite complicated mechanism increase in levels of $p15^{INK4B}$ cause it to displace $p27^{Kip1}$ from the complex of cyclin D/cdk4/6, then in turn $p27^{Kip1}$ is free to bind cyclin E/cdk2 complex. Binding of $p27^{Kip1}$ inactivates the kinase activity of cyclin E/cdk2 complex and this leads to cell cycle arrest. Smad complex can also repress the transcription of c-myc. Myc is able to induce the transcription of cdc25a which is phosphatase that causes the dephosphorylation cdk2. This is necessary step for cdk2 kinase activation. Many different mutations have been found which can disturb the normal activity of TGF-β signal cascade inside of the cell. For example mutation in Smad4 which causes its inactivation can be found from up to 50% of all pancreatic tumors. Mutations in Smad2 are found in colorectal carcinomas and alterations of type II receptor occur in 90% of colon cancer cells with microsatellite instability (Liu *et al.* 2001). Also crosstalk between different signaling pathways can lead to TGF-β signaling inhibition. One way this to happen overexpression of the proto-oncogenes *ski*, *sno* or *e*v*i*-1 (Liu *et al.* 2001).

2.5 Role of *SKIL* in cancer progression

Gene *ski* was originally discovered in the avian Sloan–Kettering viruses that formed during the passaging of a transformation-defective avian leukosis virus. This is called *v-ski* which is found to arise from cellular gene *c-ski.* Cellular homologs of *c-ski* has been found from many species: at least human, mouse, chicken and zebra fish homologs are known to exist (Liu *et al.* 2001). Normal function of *ski* seems to be related to differentiation of cells. This was found *ski* knock-out models were made with mice and there were multiple defects in the central nervous system and in skeletal muscle development. Another gene *SKIL* (also known as *sno,* ski-related novel gene) was later found when probing human cDNA libraries using *v-ski*. There are several isoforms of

this gene in human: *snoA*, *snoN (SKIL)*, *snoN*2 and *snoI* (Pearson-White 1993, Pearson-White & Crittenden 1997).

Several things support the oncogenic nature of *SKIL*. First it seems that level of SnoN, protein encoded from *SKIL* are elevated in many different cancers such as esophageal squamous cell carcinoma (Imoto *et al.* 2001a, Fukuchi *et al.* 2004), melanoma (Chen *et al.* 2003, Reed *et al.* 2005), estrogen receptor-positive breast carcinoma (Zhang *et al.* 2003), colorectal carcinoma (Buess *et al.* 2004) and leukemia (Pearson-White *et al.* 1995, Ritter *et al.* 2006). In addition to that as earlier said *SKIL* is located in 3q26. This locus is frequently amplified in many tumors, including cancers of the lung (Racz *et al.* 1999, Sugita *et al.* 2000), esophagus (Imoto *et al.* 2001b), head and neck (Singh *et al.* 2001, Lin *et al.* 2005), cervix (Sugita *et al.* 2000, Hopman *et al.* 2006), ovary (Sugita *et al.* 2000, Nanjundan *et al.* 2007) and prostate (Jung *et al.* 2006). It is important to note however that this locus has also other genes which are known to be oncogenes so the amplification of this locus alone is not sufficient proof for *SKIL* to be oncogene. Stronger evidences come from the studies where siRNAs are used to decrease the expression of SnoN in human lung or breast cancers and this inhibits the tumor growth both *in vitro* and *in vivo* (Zhu *et al.* 2007). Also the down regulation of *SKIL* in pancreatic cancer cells reduces the tumor growth (Heider *et al.* 2007). Interestingly siRNA treatment targeting *SKIL* in lung and breast cancer has no effect on the transforming activity of these cells. This may mean that tumor-promoting activity of SnoN is restricted to certain type of cancers only (Zhu *et al.* 2007, Deheuninck & Luo 2009).

When *ski* oncogene was discovered it took many years to find out the molecular mechanism behind this molecule. It seemed that *ski* had many effects on transcription but it also seemed that it couldn't bind the DNA. Then simultaneously several groups found out that *ski* can directly bind to Smad3/4 complex. This means that cells can't respond normally to the TGF-β stimulation. More precise studies showed that it is Smad3 which can bind the SnoN protein in human. It was also shown that this binding of SnoN to Smad3 happens only after TGF-β stimulation in several different cultured cells (Liu *et al.* 2001). Previously there had been attempts to identify DNA binding site for Ski and by using the in vitro selection protocol (Nicol & Stavnezer 1998). They found that DNA binding site for Ski is GTCTAGAC. Later another group found that

the binding site for Smad3 and Smad4 is exactly the same GTCTAGAC. This lead to hypothesize that Ski can only bind to DNA indirectly when bound to Smad3 and indeed this was showed to be true later. Interestingly this Smad3-Ski/SnoN complex doesn't activate the Smad-responsive genes but represses them (Nicol & Stavnezer 1998, Liu *et al.* 2001). This explains in which way the overexpression of Ski/SnoN antagonizes the normal effects of TGF-β stimulation. Also the overexpression of Smad3 and Smad4 can reverse the effects of Ski/SnoN overexpression. This suggests that there is antagonistic relationship between the Smad3/4 and Ski/SnoN (Liu *et al.* 2001).

Cells can normally control the levels of Ski and SnoN. This also happens through the TGF-β stimulation. Cells treated with TGF-β show decreased levels of Ski and even more decreased levels of SnoN. However it is possible to inhibit this loss of Ski and SnoN. This can be done by pretreating cells with MG132, which is proteasome inhibitor that can block the protein degradation via a proteasome-mediated degradation pathway. This indicates that TGF-β signaling pathway can somehow cause proteasome-mediated degradation of Ski and Sno (Sun *et al.* 1999). Interestingly there is also study which shows that with Hep3B cells short exposure of TGF-β indeed decreases the levels of SnoN but longer exposure lead to clearly increased levels of SnoN. It was showed that this is due to increased mRNA levels of SnoN upon 2h treatment with TGF-β. Because of these results it is suggested that SnoN is a nuclear corepressor for Smad4 to maintain TGF-b responsive gene expression at a basal level (Stroschein *et al.* 1999). So when the Smad proteins are phosphorylated it triggers the nuclear translocation which then can trigger the SMad3-dependent degradation of SnoN thus allowing activation of TGF-β pathway downstream genes. After this the transcriptional activation of SnoN steadily starts to increase the steady state levels of SnoN and this eventually leads to inhibition of TGF-β pathway. Same study also found out that more stable mutant of SnoN can more effectively turn of the transcriptional activation of TGF-β responsive genes. This result is also consistent with earlier findings (Stroschein *et al.* 1999, Liu *et al.* 2001).

All though the TGF-β inhibition seems to be the most important pathway connected to oncogene feature of SnoN and Ski, there is also other cellular targets for these proteins. Ski can directly bind to N-CoR/SMRT and mSin3A to form a complex with HDAC. This means that when Ski is bind to Smad2/3/4 complex which is activated by TGF-β

signaling it can same time bind to N-CoR/SMRT which in turn can recruit HDAC. HDAC can promote histone deacetylation, resulting in shutting down the transcription (Nomura *et al.* 1999, Liu *et al.* 2001). Deletion of binding site of N-CoR clearly decreases the inhibition activity of Ski which is consistent with other results (Nomura *et al.* 1999).

2.6 Previous results from *SKIL* studies

Starting point for this study was the identification of novel *TMPRSS2-SKIL* gene fusion from fresh frozen tissues acquired from the Tampere University Hospital (Tampere, Finland). There were tissue from 12 benign prostatic hyperplasias (BPH), 28 untreated prostate cancers (PC), and 13 castration resistant prostate cancers (CRPC) in the cohort. We then screened 76 additional tumors and 22 LuCaP xenografts with qRT-PCR, and identified *SKIL* overexpression in one xenograft and one clinical sample. These samples had different fusion partners *MIPEP* for the clinical and *SLC45A3* for the LuCaP-77. Also none of the coding sequences disrupted the protein coding sequence of *SKIL*, suggesting that *SKIL* is still functional. We also found that inhibition of *SKIL* with siRNA in PC-3 and LNCaP cell lines decreased the growth rates of the cells and increased the invasion of cells with PC-3 cells.

3. AIMS OF THE RESEARCH

The goal of this Master's thesis was to clarify the functional role of the *SKIL* overexpression in prostate cancer. There are already several studies which suggests that *SKIL* is potential oncogene in several cancers. Previous studies performed with *SKIL* showed that *SKIL* might be important factor in some subtypes of prostate cancer. Aims of this study were:

1) Construction of prostate cancer cell line steadily overexpressing *SKIL*.
2) Analyzing the effects of overexpression of *SKIL* in prostate cancer cells.

4. MATERIAL AND METHODS

4.1 Subcloning of *SKIL*

Starting materials for the subcloning of *SKIL* were pCMV6-XL4/5/6 vector (Origene) with full *SKIL* gene and empty pcDNA3.1(+) vector (Invitrogen). Enzymes used with subcloning were EcoR1 and NotI (Thermo scientific). Restriction reactions with both plasmids were performed as double digestion in Buffer O (Thermo Scientific) at 37 °C for 4,5 hours after which inactivation was performed at 80 °C for 20 minutes. After restriction 1,5% agarose gel was used to separate the *SKIL* insert and linearized pCMV6-XL4/5/6 plasmid. The *SKIL* insert was cut out from the gel and restricted using the QIAquick Gel Extraction Kit (Qiagen). The linearized pcDNA3.1(+) vector was purified using QIAquick PCR Purification Kit (Qiagen). After purifications ligation reaction was performed using T4-DNA ligase T4-buffer (Fermentas). Reaction was performed at room temperature for 10 minutes and inactivated at 70 °C for 5 minutes.

Constructs were then transformed into One Shot® chemically competent TOP10 *e. coli* cells. Then transformed *e. coli* cells were cultured overnight at 37 °C on LB plates containing 50 µl/ml of ampicillin. Multiple colonies were taken and cultured in 200 µl of LB medium for 4 hours. Colony PCR was performed as described below on all recultured colonies with *SKIL* specific primers. Possible *SKIL* positive colonies were then cultured in 4 ml of LB medium containing 50 µl/ml of ampicillin. Next day plasmid DNA was extracted by using the GenElute™ Plasmid Miniprep Kit (Sigma-Aldrich). This DNA was then sequenced as described below.

4.1.1 Colony PCR

Following components per each reaction was pipetted: 20 µl PCR-water, 4 µl 5x Phusion GC buffer (Thermo Scientific), 0,4 µl 10 mM dNTPs (Thermo Scientific), 0,5 µl both 50 µM forward and reverse primer, 0,5 µl of Phusion DNA polymerase (Thermo Scientific) and 2 µl of LB medium with bacteria. Samples were then moved to PCR-machine and following program was ran: initial denaturation 98 °C for 5 minutes, denaturation 98 °C for 10 seconds, annealing 65 °C for 30 seconds, extension 72 °C for

30 seconds then 34 times to denaturation step then final extension at 72 °C for 10 minutes.

4.1.2 DNA sequencing

Sequencing of plasmid DNA was performed by using the Sanger's method using BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) and ABI-3130xl genetic analyzer (Applied Biosystems). Prior to sequencing DNA was amplified by using the Bio-Rad C1000TM Thermal Cycler (Bio-Rad). In each sequencing reaction 1 µl of BigDye Terminator reaction mix (Applied Biosystems), 1,5 µl of 5x sequencing buffer, 1 µl of 5 µM primer and 100-300 ng of DNA was added. The total volume of all reactions were adjusted to 10 µl by using sterile, deionized water. Primers which were used to sequencing are shown in the table 1. Reactions were denatured at 95 °C for 3 minutes, followed by 45 s at 95 °C, 10 s at 50 °C and 4 min at 60 °C followed by 25 cycles of 15 s at 98 °C, 10 s at 50 °C and 4 min at 60 °C. After amplification DNA was precipitated by adding 25 µl absolute ethanol and 1 µl of 3 M sodium acetate (pH 5.2). Reactions were incubated 15 minutes at room temperature. Then reactions were centrifuged at 2000 g for 45 minutes, followed by immediately centrifugation at 700 g for 1 min upside-down. This was done to discard the supernatant perfectly. DNA was washed with 125 µl of 70% ethanol and the pelleted again with centrifugation at 2000 g for 15 minutes. Ethanol was removed again with 700 g centrifugation for 1 min upside-down. DNA pellets were then resuspended to 12,5 µl of Hi-Di^{TM} formamide (Applied Biosystems). DNA was denatured by incubating at 95 °C for 3 minutes. Samples were then briefly centrifuged and sequenced with ABI-3130xl genetic analyzer (Applied Biosystems).

4.3 Cell lines

Cell lines used in this study are PC-3, DU-145, EP156T and RWPE-1. These were obtained from the American Type Culture Collection (ATCC). All the cells were cultured at 37 °C and 5% CO_2 all the time. All the cell cultures were subcultured every three to four days if not mentioned differently. The basal media for PC-3 cells was Ham's F12 with 10% fetal bovine serum and 2 mM L-glutamine. For DU-145 cells

basal media was Eagle's Minimum Essential Medium with 10% fetal bovine serum and 2 mM L-glutamine. For EP156T cells basal media was Keratinocyte Serum Free Medium with 0.05 mg/ml BPE, 5 ng/ml EGF and 1nM DHT. For RWPE-1 cells basal media was Keratinocyte Serum Free Medium with 0.05 mg/ml bovine pituitary extract (BPE) and 5 ng/ml epidermal growth factor (EGF). Basal media and fetal bovine serum were purchased from Lonza.

4.3.1 Transient transfection

Transfection of prostate cancer cells with *SKIL* construct were performed using the jetPRIME® transfection reagent (Polyplus-transfection). 100 000 cells were seeded on the 6-well plate and then incubated 24 hours at 37 °C and 5% CO2. Transfections were performed according to manufacturer's instructions. All the control cells were transfected with the expression vectors lacking the *SKIL* insert. RNA and proteins were extracted from the cells within 24 hours.

4.3.2 Stable transfection

Transfection of prostate cancer cells leading to stable transfection were performed using the jetPRIME® transfection reagent (Polyplus-transfection). 250 000 cells were seeded to T75 cell culture flasks (Sigma Aldrich) and then incubated 24 hours at 37 °C and 5% CO2. Again transfections were performed according to manufacturer's instructions. All the control cells were transfected with the expression vectors lacking the *SKIL* insert. 24 hours from the transfection medium (described under the "cell lines") of the cells was changed to that containing 150 µg/ml geneticin® selective antibiotic (also known as G418 or G-418). After this cells were let to grow in the medium for about 4 weeks. Medium was changed every three days and cells were frequently monitored. During this time most of the cells first died because of geneticin but after about three weeks cells started slowly grow again. After cells started growing normally amount of geneticin in medium was reduced to 100 µg/ml just to restrain the vectors in the cells.

After this DU145 cells with stable *SKIL* insert were used to create single-cell clones. First cells were detached from flask as described under the "cell lines". Mixed

population of cells was diluted 30 cells/ml to extablish monoclonal lines. Dilution was seeded to 96-well plate (total volume of 500 µl per well) and put to incubator. Again medium was carefully changed every three days and cells were frequently monitored. After cells started to grow normally again they were subcultured to bigger T75 flasks. With this technique we got some wells were all the cells were originally from one cell only thus they are all clones.

4.4 RNA extraction and RT-qPCR

RNA was extracted from cells by using the TRI Reagent® (Sigma). This RNA extraction was performed according the manufacturer's instructions. Before the extraction cells were seeded on 6-well plate containing 50 000 cells/plate. For real time quantitative PCR (RT-qPCR) RNA was first reverse transcribed by using the Maxima Reverse Transcriptase (Thermo Scientific) and random hexamer primers (Thermo Scientific). First 1 µg of RNA and 200 ng of random hexamer primers are adjusted to 12,5 µl using sterile, deionized water. Reactions are then incubated at 65 °C for 5 minutes. Next master mix with 4 µl of Maxima 5x buffer (Thermo Scientific), 2 µl of 10mM dNTP mix (Thermo Scientific), 0,5 µl of RNase inhibitor (Thermo Scientific) and 1 µl of Maxima Reverse Transcriptase enzyme (Reverse Transcriptase). After that 25 °C for 10 min, 50 °C for 30 min and 85 °C for 5 min. Standard curves were prepared from the RNA extracted from the non-transfected PC-3 cells by using the 5-fold dilution series. All expression values were normalized by using the housekeeping gene *TBP* (TATA binding protein). Sterile water was used as a negative control. The Real time quantitive reverse transcription PCR (RT-qPCR) itself was carried out by using the Bio-Rad CFX96™ Real-Time PCR Detection System (Bio-Rad) with MaximaTM SYBR Green/ROX qPCR Master Mix (Fermentas). Reactions were prepared by using the manufacturer's instructions and reaction conditions were optimized for primers used. Primers which were used are shown in table 1. RT-qPCR results were analyzed by using the CFX Manager Software.

4.5 Protein extraction and measurement of concentration

Cells were seeded either on 6-well plate or on the T75 flasks. Proteins were harvested when cells were75-85 % confluent. Cells were first washed three times with the ice cold PBS (10 ml for the T75 flask and 2 ml for 6-well plate) while on ice. Then small amount of PBS (750 µl for the T75 flask and 500 µl for the 6-well plate). Cells were then detached by using the cell scraper. PBS with the cells was then harvested. This was repeated to ensure that all the cells are collected. Cell were centrifuged at 200 g for 5 minutes at 4 °C. Supernatant was discarded. Cells were then resuspended to 50 µl of hypotonic buffer. Hypotonic Buffer has 10 mM Hepes (pH 7,9), 1,5 mM $MgCl_2$, 10 mM KCl, 0,2 mM PMSF, 0,5 mM DTT and 40 µl of complete protease inhibitor (Roche)/1 ml. Cells were incubated on ice for 10 minutes and centrifuged at 2000 g for 15 minutes at 4 °C. Supernatant containing the proteins was collected.

Protein concentration was measured by using the reagents from the Bio-Rad. First standard curve was prepared by using the 1 mg/ml BSA, water and Bradford reagent (Bio-Rad). 2-fold dilutions were used. Protein samples were prepared to 1:100 and 1:50 dilutions using the water and Bradford reagent (Bio-Rad). All the dilutions were done as the triplicates on the 96-well plate with flat bottoms. After pipetting plate was shaken and incubated at RT for 15 minutes. Protein concentration was measured using the EnVision® 2104 multilabel reader.

4.6 Western blotting

For western blotting proteins extracted were first prepared in the following way. About 20 µg of protein was added to 9 µl of 3xSS and 1 µl of 1,25 M DTT. Total volume was adjusted to 30 µl. Samples were denatured at 99 °C for 5 minute and chilled on ice. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate the proteins according the size. SDS-PAGE is made in a way that first there is lower gel which is pipetted first followed by less concentrated upper gel. Lower gel was prepared in following way: 2,95 ml of water, 2,5 ml of 30% acrylamide mix (BIO-RAD), 1,9 ml of 1,5 M Tris (pH8,8), 0,075 ml of 10% SDS, 0,075 ml of 10% APS and 0,003 ml of TEMED (Sigma Aldrich). Upper gel was prepared in following way: 1,7

ml of water, 0,415 ml of 30% acrylamide mix (BIO-RAD), 0,315 ml of 1,5 M Tris (pH8,8), 0,025 ml of 10% SDS, 0,025 ml of 10% APS and 0,0025 ml of TEMED (Sigma Aldrich). Gel was then loaded with samples and molecular markers. Markers used were PageRuler™ Prestained Protein LadderPlus (Fermentas) and ColorPlus™ Prestained Protein Ladder, Broad Range (New England Biolabs). Gel was run first 100 V for 15 minutes and then 150 V for 1 hour.

Western blotting was performed using Trans-Blot® SD Semi-Dry Transfer Cell system (BIO-RAD). First nitrocellulose membrane or polyvinylidene difluoride (PVDF) membrane was prepared. Nitrocellulose membrane was dipped to methanol and straight to the water. Membrane was then moved to transferring buffer (25 mM Tris, 192 mM glycine, 10% methanol). Also six Whatman filters (Sigma Aldrich) were incubated in the transferring buffer. PVDF membrane was prepared in the same way expect it was not dipped to the methanol first. Blotting machine was assembled in the way that first it was slightly moistened with transferring buffer. Three Whatman filters were laid and on the top of them membrane, gel and three more filters, respectively. Blotting was performed in 1 hour using 12 V and 80 mA. After blotting membrane was checked to see if protein size markers were transferred to membrane. After blotting membranes were blocked in 3% BSA/0,1% Tween/PBS for 1 hour at RT on shaker. Membranes where then transferred to primary antibody which was 5% BSA/0,1% Tween/0,1% NaN_3 and 1:1000 SnoN antibody (Cell Signaling Technology) in BSA. Membranes were then incubated at 4 °C over night in shaker. Membranes were washed three times first with 0,5% Tween in BSA then 0,1% Tween in BSA and finally 0,05% Tween in BSA. Membranes were then transferred to secondary antibody, which was 0,1% Tween and 1:5000 anti-rabbit (P0217, Dako) in BSA. Membranes were incubated for 1 hour at RT in shaker. Membranes were washed again as before. After final wash the membranes were rinsed with BSA and moved to transparent film. Then mixture (1:4) of Immunocruz Western Blotting Luminol Reagent SC-2018 (Santa Cruz) and SuperSignal® West Femto Maximum Sensitivity Substrate (Thermo Scientific) was added to the top of the membrane. Membranes were incubated for 30 seconds. After this the membrane was moved to Kodak cassette (Kodak) which can be sealed in the way that no light can enter. Next steps were performed at the dark room. There X-ray film was exposed to the membrane. Exposure time was varied between 5 seconds and

5 minutes to find the optimal result. After exposure X-ray films were developed by using the CP1000 table top film processor (Agfa).

4.7 Growth curves

Growth curves were made out of DU145, EP156T and RWPE-1 cell lines. Both stabile and transient transfections were used in growth curves. Growth curves were performed using the 24-well plates. First cells were removed from the bottom of the flask as with the cell splitting. Then cells were counted using the Moxi^{Z} (Orflo). Then 20 000 cells per well were seeded to each well. Cells were seeded as triplicates. Then cell growth was quantified by using the light microscopy every day after seeding. Pictures were taken using the Olympus IX71 microscope and using Surveyor microscope software user interference. Pictures were taken as long as the cells didn't occupied the whole growth area. This usually takes between 4 to 6 days.

4.8 Matrigel invasion assay

Matrigel invasion assays were performed with cells using BD BioCoat Matrigel Invasion Chambers (BD Biosciences). 24-well plate (BD Falcon TC Companion Tissue Culture Plates). All the samples and controls were prepared as triplicates. Chambers were first removed from the -20 °C and allowed to come to room temperature. Then 0,5 ml of Eagle's Minimum Essential Medium (EMEM, Lonza) was added to interior of inserts and to bottom of the wells. This was allowed to rehydrate for 2 hours at 37 °C and 5% CO_2. All the medium was carefully removed. For RWPE-1 cells media with no BPE and EGF was prepared. Then concentration of cells in that media was adjusted to 50 000 cells/ml. 0,5 ml of this medium was added to the interior of inserts. 0,75 ml of normal RWPE-1 media was added to the bottom of the wells. Inserts were carefully transferred to the wells to avoid any air bubbles between bottom of the insert and media in the well. Chambers were incubated for 24 hours at 37 °C and 5% CO_2. Medium was removed from the insert and after that non-invaded cells were removed by scrubbing. This was performed in the way that cotton tipped swab was inserted into insert and it was moved there with gentle but firm pressure. This was repeated with a second swab moistened with medium.

After this invasive cells were stained. Inserts were first incubated in methanol for 2 minutes and then in 1% toluidine blue in 1% borax for 2 minutes. After this chambers were rinsed twice with plenty of distilled water. Inserts were let to air dry. Membrane was removed from the insert with sharp scalpel and membranes were placed to the microscope slide with drop of immersion oil. Another drop was added and cover slip was placed to the top with gentle pressure to remove the air bubbles.

5. RESULTS

5.1 Subcloning of *SKIL*

We failed to subclone *SKIL* into empty pcDNA3.1(+) vector (Invitrogen). We got very few colonies and when those were sequenced they were all negative. Because of this we decided to buy commercial plasmids pCI-Neo HA-hSnoN which is a gift from Robert Weinberg, Addgene plasmid #10908 (Sun *et al.* 1999) and universal empty, pCI-Neo backbone pUNIV-plasmid which is a gift from Cynthia Czajkowski, Addgene plasmid #24705 (Venkatachalan *et al.* 2007).

5.2 Verification of *SKIL* expression by RT-qPCR

We used commercial plasmids pCI-Neo HA-hSnoN and pUNIV backbone from Addgene to overexpress *SKIL* in prostate cancer cells. Overexpression of *SKIL* mRNA in cell lines was verified using RT-qPCR. The expression levels of *SKIL* in different cell lines are presented in the figure 1. The results show that in RWPE-1 cells mRNA levels of *SKIL* are about three fold higher in cells transfected with *SKIL* vector compared to those transfected with empty vectors. With DU145 cell line after the transfection cells were used to create single cell clones either with *SKIL* vector or empty vector. Clear expression differences between different clones can be seen in the figure 1 B. However clones with empty vector (pUNIV A1 and pUNIV B1) are among clones with low *SKIL* expression. There was also clones with clearly higher *SKIL* expression (A2, A6, B3 and B4). These clones showed about 4-10 fold higher *SKIL* expression than the ones with the empty vector. We also transfected PC-3 cells with *SKIL* siRNA to inhibit the *SKIL* mRNA in this cell line. We detected about three fold reduction in *SKIL* mRNA expression. These results can be seen in appendix picture 1.

5.3 The effects of *SKIL* expression on the growth of the cell lines

The effects of the *SKIL* expression for the growth of the cell lines was tested by using the RWPE-1 and DU-145 cells. Growth curves were created by growing cells on 24-well plates and pictures were taken using the Olympus IX71 microscope and using

Surveyor microscope software user interference. Figure 2 A shows results for cell growth experiments performed by using the stable transfected RWPE-1 cells. It seems that there are no difference in growth rates between cell transfected with *SKIL* vector and cells transfected with empty vector. Figure 2 B shows results for cell growth experiments performed by using the stable transfected single cell clones of DU145 cells. In this case there are little differences which can be seen between cells transfected with *SKIL* and cells transfected with empty vector, but the difference is not statistically significant ($p>0.05$). Also it is not the clones with *SKIL* overexpression but the control clones, which grow faster.

5.4 The effects of *SKIL* expression on the invasiveness of cell lines

Invasion efficiency of cells was tested using the matrigel invasion assay (BD biosciences). Cell lines used were again RWPE-1 and DU-145. In figure 3 B there is shown cell invasion results when using the RWPE-1 cells. From there it can be seen that cells overexpressing *SKIL* seem to have higher invasiveness than those with empty control vector. Difference is also statistically significant ($p>0.05$).

Figure 3 B shows results for invasion experiment made with DU-145. Cells which showed the highest *SKIL* expression in mRNA level were chosen for this experiment. Results show that there are clear differences between different clones of DU-145 transfected with *SKIL* vector. There are two clones with empty control vector and one of those (A1) shows very little invasiveness capability, but other (B1) seems to show as much if not more invasiveness capability than clones transfected with *SKIL*. However there is one clone (A6) which shows clearly increased invasiveness capability. This clone was also the only one which showed statistically significant difference ($p<0.05$).

5.5 Verification of *SKIL* overexpression by western blotting

We wanted also know the expression levels of *SKIL* in protein level. This was done using semi-dry western blotting. Proteins derived from RWPE-1, DU-145 and PC-3 were used in western blotting. Figure 4 shows results from each different western blot.

Western blot created with the RWPE-1 cells fails to show any difference between cells transfected with *SKIL* and cells transfected with empty vector. As can be seen from the picture antibody used is not that specific and there is lot of bands that should not be there. Proteins extracted from DU-145 cells also gave similar results than those with RWPE-1. There is lot of unspecific bands and they fail to show any real difference between cells transfected with *SKIL* and cells transfected with empty vector. PC-3 cells again differ from the RWPE-1 and DU-145 cells in a sense that they are not stabile but transient transfected. Despite from that it again seems that western blotting fails to show any difference between cells transfected with *SKIL* and cells transfected with empty vector and there is still many unspecific bands present. All the western blots were repeated by using another primary antibody (Rabbit polyclonal anti-*SKIL* antibody, TA312882, Origene) but the results were similar with the ones showed here.

Table 1: List of primers used in sequencing and in RT-qPCR

Primers used in sequencing have "SEQ" in their name.

NAME OF THE PRIMER	SEQUENCE
*SKIL*_SEQ1_FOR	5'-TCCTTGAAGGTAACTGGGCA-3'
*SKIL*_SEQ1_REV	5'-TCCAGGGTCAATGCAATGGT-3'
*SKIL*_SEQ2_FOR	5'-AATGGGATGGGAGATGATGGC-3'
*SKIL*_SEQ2_REV	5'-GCGACATGCTTTCTTGGGAA-3'
*SKIL*_SEQ3_FOR	5'-TTGCCACTGGGGCTTTGAAT-3'
*SKIL*_SEQ3_REV	5'-CTGTGAGCCTTCTCTGACTGT-3'
*SKIL*_SEQ4_FOR	5'-GACAGGAACGGGAAGCAAGA-3'
*SKIL*_SEQ4_REV	5'-TGCCTAGTTATCGTCATGCAG-3'
T7_SEQ_FOR	5-'TAATACGACTCACTATAGGG-3'
TBP_FOR	5'-GAATATAATCCCAAGCGGTTTG-3'
TBP_REV	5'-ACTTCACATCACAGCTCCCC-3'
*SKIL*_FOR	5'-AGAGGCTGAATATGCAGGACA-3'
*SKIL*_REV	5'-CCAAAGCAAGCAACAAACAA-3'

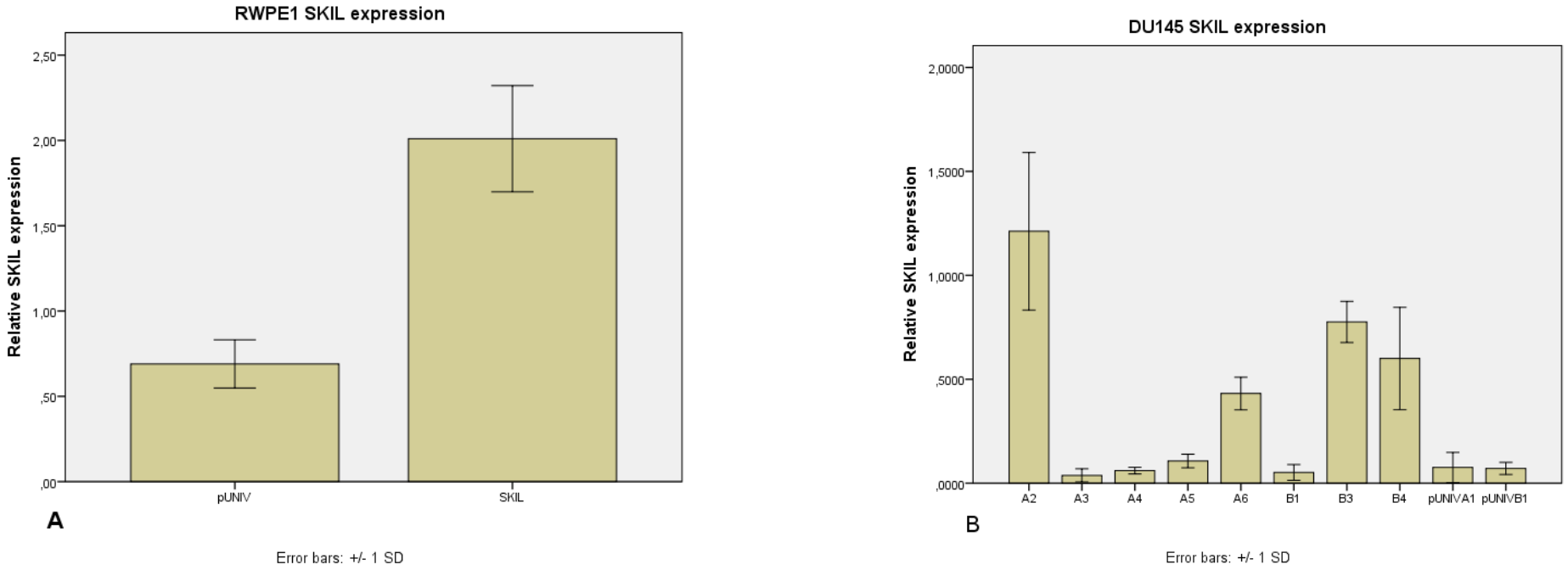


Figure 1: Relative mRNA expression levels of *SKIL*

A) The expression of *SKIL* mRNA in RWPE-1 cells stable transfected with *SKIL* or with empty expression vector. Error bars represent standard deviations of RT-qPCR replicates. P<0.05 (unpaired T-test). B) The expression of *SKIL* mRNA in single cell cloned DU145 cell lines with *SKIL* or with empty expression vector. Error bars represent standard deviations of RT-qPCR replicates. P<0.05 when A2, A6 or B3 is compared to pUNIVA1 or to pUNIVB1 (unpaired T-test).

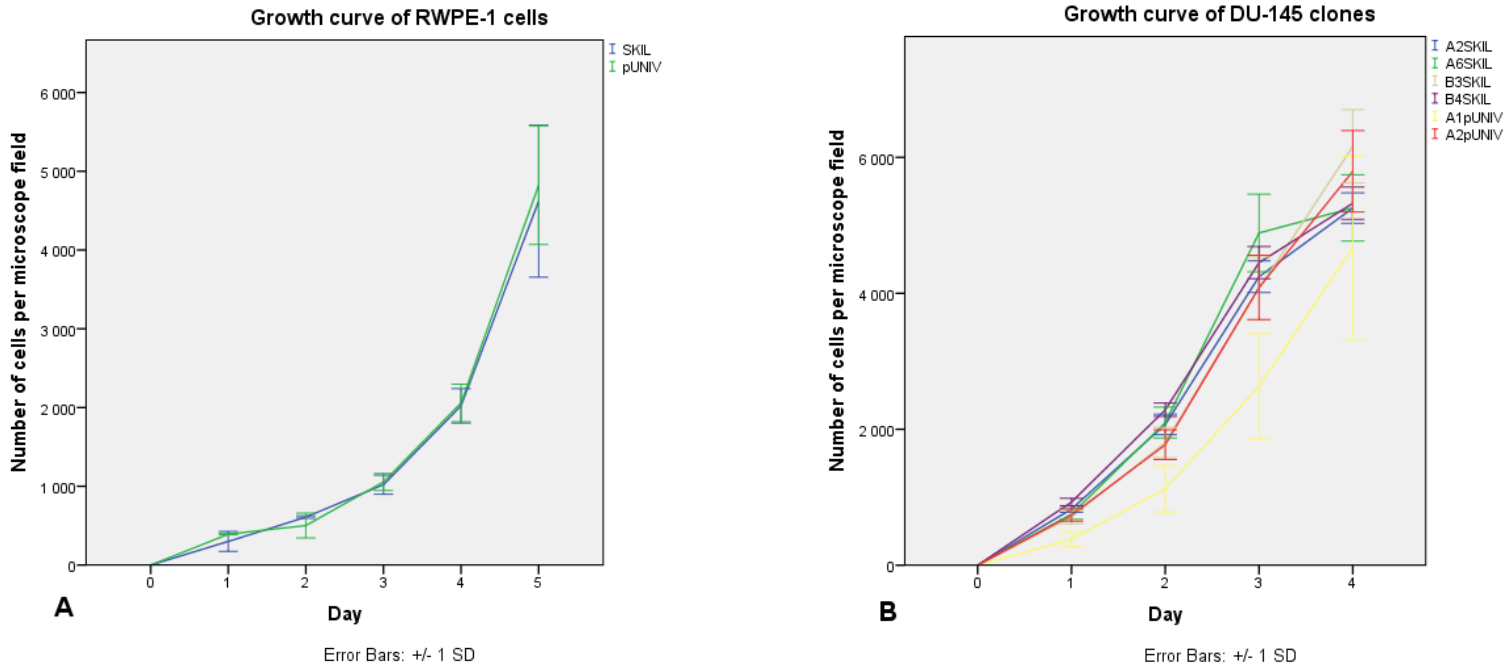


Figure 2: Growth curves of RWPE-1 and DU-145 cells
A) Growth curves of RWPE-1 cells stable transfected with *SKIL* and empty control vector. Error bars represent standard deviation of replicate wells. B) Growth curves of DU-145 cells stable transfected with *SKIL* and empty control vector. Error bars represent standard deviation of replicate wells.

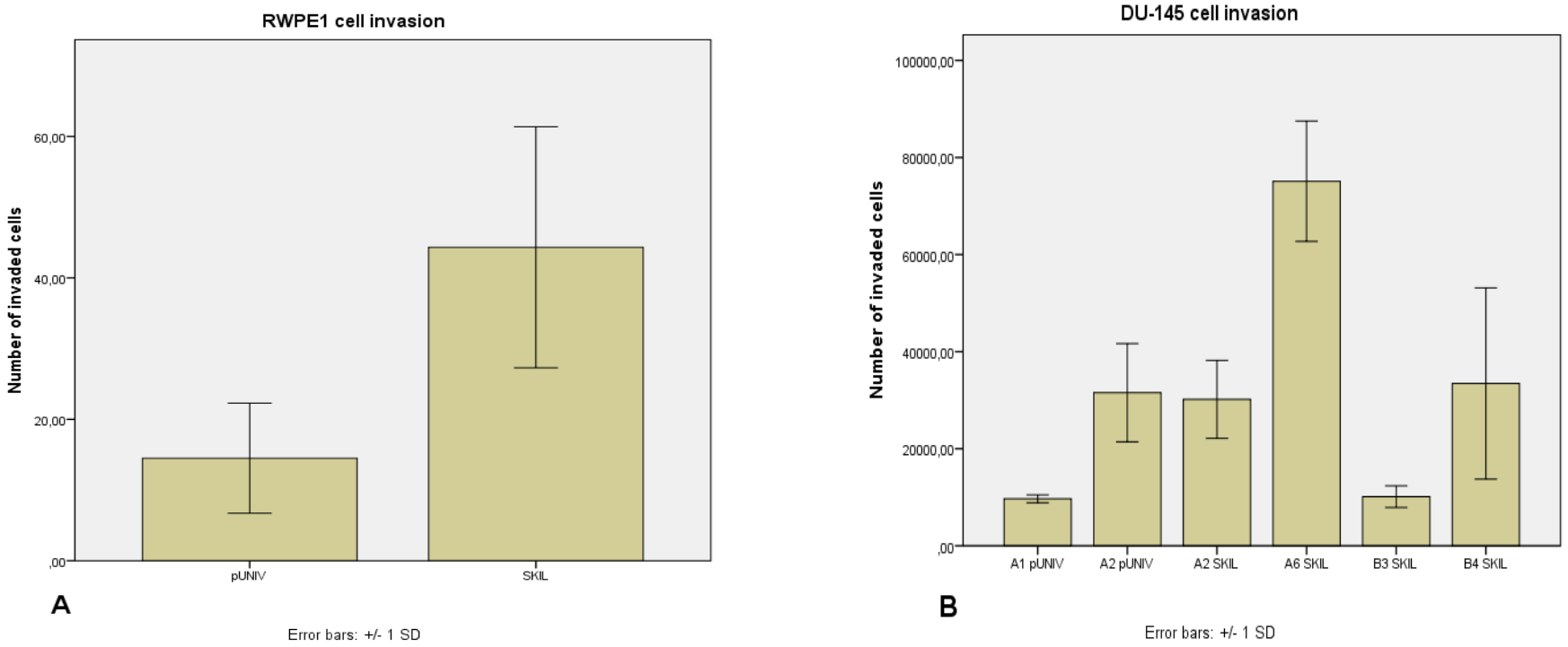


Figure 3: Number of cells invaded in Matrigel invasion assay
A) Number of RWPE-1 cells invaded during the Matrigel invasion assay. Error bars represent standard deviations of invaded cells between different Matrigel invasion chambers. B) Number of DU-145 cells invaded during the Matrigel invasion assay. Error bars represent standard deviations of invaded cells between different Matrigel invasion chambers.

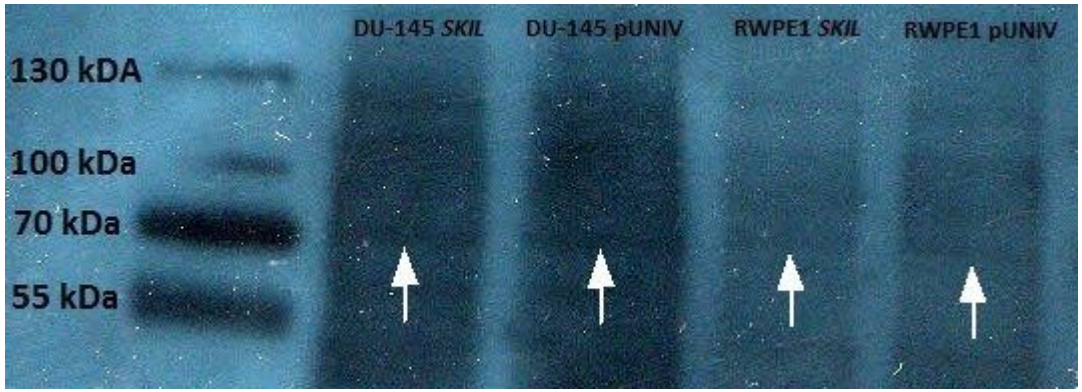


Figure 4: X-ray film developed from western blotting membrane.
On the left there is molecular marker which shows the size of bands in kilodaltons (kDa). Arrows point the SnoN bands or places where SnoN band should be. There is western results from stable cell lines of DU-145 and RWPE-1 transfected with *SKIL* and control

6. DISCUSSION

In this study we created cell lines with overexpression of *SKIL* and studied the functional differences between cells with or without *SKIL* overexpression. Previous studies carried out by our group have showed that there are some cases of prostate cancer where there is clear overexpression of *SKIL* but no overexpression of any ETS genes (Annala *et al.* 2015). Further studies showed that there is gene fusion between *SKIL* and *TMPRSS2* genes. Earlier studies strongly suggest that *SKIL* has oncogenic features (Pearson-White *et al.* 1995, Sun *et al.* 1999, Liu *et al.* 2001, Imoto *et al.* 2001a, Chen *et al.* 2003, Buess *et al.* 2004, Reed *et al.* 2005, Deheuninck & Luo 2009, Hagerstrand *et al.* 2013). Also because there is no overexpression or fusion in ETS genes, which is common fusion gene in prostate cancer it seems likely that they don't have any impact in progression of those cancers with *SKIL* fusion (Gasi Tandefelt *et al.* 2014). Our study focused on the possible cancer driving role of *SKIL* in prostate cancer and in normal prostate cells. Although *SKIL* is known to be likely oncogene there are no evidence that it has any role in prostate cancer.

According the previous studies *SKIL* has important role as inhibitor in TGF-β pathway. This pathway is known to be potent inhibitor of epithelial cell proliferation (Stroschein *et al.* 1999, Liu *et al.* 2001, Zhang *et al.* 2003, Deheuninck & Luo 2009). Inhibition of this pathway may be driving cause for developing of different cancers (Pearson-White *et al.* 1995, Stroschein *et al.* 1999, Liu *et al.* 2001). TGF-β is likely mechanistic explanation if overexpression of *SKIL* can induce cancer in prostate cells. Another recent study has proposed that *SKIL* can interact with and promote the activity of estrogen receptor α in the nuclei of breast carcinoma cells. This interaction can occur via two highly conserved nuclear receptor binding LxxLL-like motifs in *SKIL* (Band & Laiho 2012). This finding is intriguing as it suggests a potential interaction between *SKIL* and androgen receptor, as some LxxLL motifs have ability to bind with the ligand binding domain of AR (Dubbink *et al.* 2006).

6.1 Subcloning of *SKIL*

As mentioned we failed to subclone the *SKIL* into empty pcDNA3.1(+) vector (Invitrogen). We tried many different solutions to this problem including changing the ratio of empty plasmid and *SKIL* gene, different gel isolation procedures and different bacterial cells (QIAquick Gel Extraction Kit (Qiagen) and GeneJET Gel Extraction Kit (Thermo scientific) were tried as gel extraction and One Shot® TOP10 chemically competent *E. coli* and NEB 5-alpha competent *E. coli* (New England Biolabs) were tried as competent cells). Also we made new agar plates for growing bacteria and also got new enzymes. Nothing of this worked so we decided to buy commercial plasmid with *SKIL* and control vector for that (pCI-Neo HA-hSnoN and pUNIV backbone from Addgene). There are some possible explanations which caused the failing of subcloning. One is the GC-rich zone in the *SKIL*, this provides problems in cloning and in PCR generally (Frey *et al.* 2008). Also we noticed afterwards that the patch of antibiotic which was used, might have been too old.

6.2 Transfection of cell lines

RWPE-1 and DU-145 cell lines were both transfected in order to create cell lines with stable overexpression of *SKIL* and cell lines with control stable control vectors. We wanted to create single cell clones with both of those cell lines. With DU-145 we managed to create several clones with stable transfection with *SKIL* or empty vector. When we tried to create single cell clone by using RWPE-1 cell line in same manner, we found out that these cells won't start growing from single cell. This is however expected because RWPE-1 cells are created to mimic normal prostate cells (Webber *et al.* 1997). These prostate epithelial cells are usually not dividing so this also explains the problems in creating single cell clones (Frank & Miranti 2013). This lead us to conclusion that only option is to use cell pool with *SKIL* overexpression in case of the RWPE-1 cells.

6.3 Verification of *SKIL* overexpression by RT-qPCR

Our goal was to make sure that there is significant overexpression of *SKIL* in mRNA level in all of the cell lines used. To do this we extracted total mRNA from all the cells and reverse transcribed it to cDNA using random hexamers. Then we used *SKIL* specific primers to perform qPCR to found out the relative *SKIL* mRNA levels of RWPE-1, DU-145 and PC-3 cells. Levels were compared in a way that cells with *SKIL* vector were compared with cells containing empty vector (*e.g.*no information about. expression levels between RWPE-1 and DU-145 cells).

With DU-145 cells the overexpression between the clones was variable: we got some clones with quite high overexpression (about 10 times higher) and some clones have little or even nonexistent overexpression. This was expected because it is known that between single cell clones variance can be pretty high. We chose the clones with highest overexpression of *SKIL* in mRNA level. Although choosing the clones with highest expression is pretty standards procedure, one could argue that we are choosing only results which are good to us. To defend against that first thing to notice is the difference between cell pool and single cell clones. With cell pool we cannot be sure that every cell is identical in terms of expression levels of different genes and in our case specifically in terms of *SKIL* expression. With single cell clones we can be surer about the expression of genes to be more identical between cells. But when we then see expression differences between the different clones we cannot be sure what is causing the difference, this is why many clones are needed when using this kind of single cell clones. There are many potential reasons why expression levels of clones vary so much. Because all the cell are surviving in the environment with selection antibiotics, it is very likely that all the clones have the copy of the plasmid in them. So if we know that we have a vector in the cells and still there is no expression on explanation is that there is some problem with the plasmid. There can be problems for example with the promoter region of the gene of interest in our case with promoter of *SKIL*. Promoter region is likely region to be corrupted in some way because small changes in promoter region can cause big differences in expression levels of gene. CMV-promoter should be very strong promoter for protein expression in mammalian cells so changes there could indeed have effects for gene expression (Andersen *et al.* 2011). Number of plasmids

inside the cell is another factor which is not easy to control. If there is too many copies of the plasmid in the cell this can cause too much expression for the gene of interest.

Of course it is possible that there are some mutations also in the gene of interest. This is however not that likely because even if there is some mutations in gene mRNA will still be transcribed and if mRNA is transcribed RT-qPCR should be able to detect this mRNA. So if there are mutations in gene itself they should be in the regions where primers used in the RT-qPCR should hybridize or the whole sequence between the primers used could be deleted. These are possible but unlikely scenarios which might explain some of the clones with no over expression but seems unlikely that they could explain all the cases. Of course it is possible that the whole gene has deleted from the vector and therefore there is no sign of overexpression. Reason why I think it is most likely that either there is no *SKIL* gene at all or there is some mutations which enable its promoter is the fact that expression levels are almost exactly the same as with the control clones. This indicates that the expression detected is coming from the cell's own copy of the *SKIL* gene.

It is known that too high expression of oncogene can cause apoptosis in cells (Lowe *et al.* 2004). So it is also possible that in some clones there were so high expression that it lead cells to apoptosis and so only the ones with low *SKIL* expression survived. Also there is mechanisms in cells which can lead to rapid mRNA degradation (Shim & Karin 2002). This might also be the case in some of the clones. Epigenetic alterations in the plasmid could also cause the expression levels of *SKIL* to be similar with the control clones (Albany *et al.* 2011).

As said earlier, it is fair to say that clones have the vector in them, because they can survive in medium with geneticin. However there is possibility that some clones have created an ability to survive in medium with geneticin even without the geneticin resistance gene existing in the vector. This explanation however also seems unlikely, because human cells don't have any genes which could be easily mutated to give geneticin resistance. It is possible that cells survive without resistance, but when taken account the concentration of geneticin (150 µg/ml during the selection and 100 µg/ml after that) there really shouldn't be any cells like that.

As mentioned with RWPE-1 cells we weren't able to create single cell clones because cells don't survive the selection procedure. We got pretty nice overexpression in this cell pool (about 3 times higher in *SKIL* transfected), but the overexpression wasn't as high as with the some of the single cell clones created using DU-145. Again there are several reasons which could explain this difference in expression differences between these two cell lines. First it is likely that because we have pool of cells the expression levels of individual cells differ more from each other than with the single cell clones. So with single cell clones we might get couple of clones with pretty high expression but with cell pool even if we have some cells with high expression levels we might lose this information because cells with somewhat lower expression levels bring the overall expression levels of pool lower.

In addition to lower overall expression level we noticed that longer we kept growing the transfected RWPE-1 cells the lower was also the *SKIL* expression compared to cells transfected with empty vector. This change in expression during the time could not be seen in single cell clones made from DU-145 cells, although it is possible that it occur also there. With this kind of chance of expression during the time it is clear that this cell line is not that stabile after all. However right after stabilizing the cell line we created several cell ampules to storage in the liquid nitrogen. Right after we realized that there is change in expression levels of the cells we started using cells from the nitrogen in our experiments. These cells still had the same expression levels which were comparable with the ones detected right after cell pool was stabilized.

Changes in the expression levels of course raise a question what is causing this. As mentioned there is probably cells with different expression levels within this cell pool. If high expression of *SKIL* causes some kind of growth disadvantage it means that there might be evolutional effects which cause the change in expression levels. Basically this means that cells with lower *SKIL* expression grow slightly faster. This causes the problem that every time cells are split there is little less cells with higher than average *SKIL* expression. If cells are being grown too long this eventually starts to affect the expression levels detected from the whole cell pool.

Other possible explanation is that in the beginning there is not that much difference between the expression levels of different cells inside the RWPE-1 cell pool. Again if

over expression of *SKIL* causes some kind of growth disadvantage to the cells during the time then it is logical that cells will eventually somehow reduce the *SKIL* expression again because evolutional reasons (cells with original *SKIL* expression grow slower and are eliminated). I think however that the first option where cells have different expression to start with is more viable. I think so because with DU145 cells we can see that single cell clones have very different expression levels when compared with each other's. If RWPE-1 cells act anything like that it seems logical to say that there is cells with expression levels clearly differing from each other's.

6.4 The effects of *SKIL* expression on the growth of the cell lines

Increased growth rates can sometimes be detected when cells are transforming more towards the cancerous phenotype. We wanted to check if this happens with *SKIL* overexpression and with the cells we are using. However we already known from previous studies of the Hagerstrand *et al.* (2013) that they didn't get increases growth rate when using immortalized human mammary epithelial cells with *SKIL* overexpression (Hagerstrand *et al.* 2013). However previously we knocked *SKIL*-expression down in PC-3 cells using siRNAs (Annala *et al.* 2015). There we saw that *SKIL* inhibition decreased cell growth. This hints that *SKIL* might have role in cell growth also (Annala *et al.* 2015).

Results from the DU-145 single cell clones showed no increased growth rate in clones with *SKIL* overexpression. Actually when looking the results it seems that control clones are the ones which grow little faster, but the difference is not statistically significant. It is not expected that overexpression actually slows the growth rates of the cells. There is not any obvious mechanism which could explain this slowed growth rate, but if the overexpression is really high this might already trigger some mechanisms which slow the growth of cells. That however shouldn't be the case since overexpression is not that high in RT-qPCR. Because the difference is not statistically significant it may be that there is no real difference and no explanation is needed. Results are consistent with Hagerstrand *et al.* so in that sense these results are expected.

Results from RWPE-1 cell pool showed no increased growth rate in pool with *SKIL* overexpression. When comparing these two growth curves we can see that they are almost identical. There is little more deviation with *SKIL* overexpressing cell pool but nothing significant. We are missing results from day 3 because of failure in saving of the pictures taken on day 3. These results as the ones with the DU-145 are consistent with Hagerstrand *et al.* and they are expected.

6.5 The effects of *SKIL* expression on the invasiveness of cell lines

Although overexpression of *SKIL* didn't have any effects on the growth rates of cells we wanted to see if it had some effects on the invasiveness of cells. In fact it would be more interesting if we had effects on invasiveness because it is usually considered to be stronger indicator of cancer causing abilities of the gene. Furthermore there is already another study which studied overexpression of *SKIL* in immortalized human mammary epithelial cells. Their study came to the conclusion that when expression levels of *SKIL* grow higher it doesn't have any effect on the growth rate of the cells but instead increases the invasiveness capability of them (Hagerstrand *et al.* 2013). If the *SKIL* has similar effects on the prostate cells as in the mammary cells it seems likely that there should be changes in invasiveness although growth rates stayed the same.

Results from the matrigel invasion assay done with the DU-145 single cell clones showed high variation. We saw that ¾ clones showed no significant difference when compared to control in terms of invasion efficiency. There is one clone (clone A6) which showed significantly higher invasion efficiency than other clones. However there were also clone which (clone B3) had even lower invasion efficiency than control clones with empty vector. It is expected that there is variation between the clones. This could be already seen in the mRNA levels of *SKIL*. It seems reasonable to predict that highest invasiveness rates would be in the clones which show highest *SKIL* expression levels. However when looking at the results we see that this is not in fact the case.

Explanation to this is not that easy to found. There could be some mechanisms which cause too high *SKIL* expression actually to decrease the invasiveness of cells, so the cells with little bit higher *SKIL* expression than the control cells would get invasiveness

advantage and the ones with even higher *SKIL* expression would again lose it. However there is not any, easy to find, well known mechanisms which supports this idea. It is also possible that these variations are due to some random factors that have nothing or little to do with the levels of *SKIL* expression.

Results from the matrigel invasion assay done with the RWPE-1 cell pool showed more consistent results than ones with the DU-145 single cell clones. This is expected because DU-145 cells are aggressive and usually they harbor more random mutations which can cause the heterogeneity (Stone *et al.* 1978). We can see that cells which are overexpressing *SKIL* have significantly higher invasiveness rates than ones transfected with control vector. Results were expected and are consistent with (Hagerstrand *et al.* 2013). However it is important to notice that number of cells which invaded through the matrigel are significantly lower with RWPE-1 cells than with the DU-145 cells (tens of cells in RWPE-1 versus thousands of cells with DU145). This can be explained by the fact that DU-145 cells are cancerous cells and RWPE-1 cells are not. Basically RWPE-1 cells should not invade at all (Bello *et al.* 1997). Cancer cells are usually more invasiveness than cells immortalized by other means and these results are consistent with that (Stone *et al.* 1978, Bello *et al.* 1997).

Mechanism which causes this increase in invasiveness is not perfectly clear but it is likely that this happens due to increased inhibition in TGF-β pathway. There are couple of reasons which support this theory. First TGF-β pathway is best known target for the *SKIL* overexpression to have effects on. Also it is known that inhibition of TGF-β pathway has effects on the cell invasiveness (Liu *et al.* 2001, Deheuninck & Luo 2009). Controversially there is also some cell line as LNCaP which are induced comprehensive morphology changes by TGF-β signaling (Yang *et al.* 2014). Second evidence which supports the TGF-β pathway inhibition to be the mechanisms behind the increased invasiveness is results from the Hagerstrand *et al.* As mentioned before they showed that in immortalized human mammary epithelial cells *SKIL* overexpression induces invasion. Also they showed that SMAD4, which is the protein inhibited by the *SKIL*, when inhibited induces the same effect as *SKIL* overexpression (Hagerstrand *et al.* 2013).

6.6 Western blotting

Western blotting was carried out in purpose of verifying the *SKIL* overexpression also in protein level. This is important for us because our hypothesis predicts that *SKIL* overexpression may drive cancer development in cells via the TGF-β pathway. For this to be possible there should be more *SKIL* presence in protein level because only then it can bind to SMAD4 in increased rates and inhibit the TGF-β pathway. There are not any known mechanisms with *SKIL* mRNA to carry out so also in that sense there should be expression changes in protein level also. In their study Hagerstrand *et al.* were able to show *SKIL* overexpression in the immortalized human mammary epithelial cells also in protein level (Hagerstrand *et al.* 2013).

Our results from both cell lines DU-145 and RWPE-1 showed no clear overexpression of *SKIL* in protein level. With both cell lines we can see that there are many bands which tells us that there has happened unspecific binding during the antibody incubations. However with both cell lines we can see that there is also band which represents the size *SKIL* should be. This predicts that antibody is able to bind right protein but it is unspecific and this leads to several bands seen in western blotting. We tried with two different antibodies and several different dilutions (1:4, 1:3, 1:2, 1:1 only Immunocruz and only SuperSignal®) with the Immunocruz Western Blotting Luminol Reagent SC-2018 (Santa Cruz) and SuperSignal® West Femto Maximum Sensitivity Substrate (Thermo Scientific). All results were similar in the sense that there were always unspecific bands present in the western blotting. Only time when amount of unspecific bands was low was when we used only Immunocruz Western Blotting Luminol Reagent SC-2018 (Santa Cruz) with short exposure time. This time however there were no clear bands representing *SKIL* (results not shown). We had had similar results with *SKIL* antibodies in our group before so it didn't come as a surprise that this western blotting is problematic.

The fact that we couldn't show overexpression in protein level leaves us with some unanswered questions: there is change in phenotype of cells but is it due to *SKIL*? There is overexpression in mRNA level but is it enough to cause overexpression also in protein level? The reason why we can't see the overexpression, or can't even say with

confidence that there is none, is most likely the quality of antibodies. There is chance that problems are rising from some other source than antibody but in our lab there has been successful western blotting with same reagents so it doesn't seem likely.

6.7 Future perspectives

Our goal was to create stable cell lines with *SKIL* overexpression and see the effects of *SKIL* overexpression in phenotype. We were able to get mRNA levels of *SKIL* up and got difference in the invasion levels of cells overexpressing *SKIL* and in control cells. We failed however to show this overexpression in protein level. First thing to do would be find a way to show the overexpression in protein level. One way to do that would be to create cell lines with more *SKIL* overexpression seen already in mRNA level. In our cells we got only about twice as high *SKIL* mRNA overexpression when compared to control cells. If these experiments would be repeated in the future we could use different prostate cancer cell lines or treat these cell lines with some cofactors to increase the gene expression. However there are not that many prostate cell lines so alternatives are limited. We could use non-prostate cell lines but that wouldn't be that relevant. Other way of increasing the gene expression could be using lentiviral transduction in which lentiviruses are used as vectors when transforming cells. This should provide higher transfection efficiency and thus could increase the *SKIL* expression seen in cells compared to control cells. Other possible solutions are usage of different plasmid with different promoter or even the same plasmid but different promoter. At the moment plasmid has CMV promoter which is considered to be very strong promoter in mammalian cells (Andersen *et al.* 2011). However it is possible that this promoter is not that strong in cell lines used. This might be true with RWPE-1 cells as there is no articles clearly proving that CMV is a strong promoter in this cell line. With DU-145 and PC-3 cells there is strong evidence that CMV promoter should create strong expression levels in the gene it is attached to (Zhang *et al.* 2002). When these facts are taken into consideration it seems unlikely that changing only the promoter would be enough to create higher expression in cell lines. Thus it seems more reasonable to believe that lentiviral transduction should be more efficient solution in this case since lentiviral expression system integrades gene in the genome itself. Also it would be

possible to use tet-off or tet-on system. This would allow us to turn gene expression on and measure the expression right away.

In longer term it would be nice to be able to create *in vivo* model for the *SKIL* overexpression. This could be done using mice as it is standard model animal when studying prostate cancer. First thing to do with *in vivo* studies could be injection of LNCaP prostate cancer cells subcutaneously into nude mice. These LNCaP cells should first be transfected with plasmid overexpressing *SKIL* and other cells with control plasmid. Growth of the cancer could then be monitored during the period of time desired. This should provide us more information how *SKIL* effects development and growing of cancer *in vivo.* If this experiments provides positive results for the role of *SKIL* in cancer development mice experiments could be taken even further. It is possible to create mice lines which are genetically modified in the way that *SKIL* is overexpressed and this overexpression takes place only in the prostate. This should provide most reliable model for development of prostate cancer and through that it might be possible to understand the development in more detailed matter. For example it would be really interesting to find out gene expression levels in prostate before there is clear cancer development in prostate. This could provide us important information how this precise cancer develops. Also this can provide us information of gene expression levels of fully developed cancer. This is important, because if this *SKIL* overexpression represents new previously unknown subtype of prostate cancer, it helps us to recognize the expression patterns which can be found in real patience. This kind of studies leads us towards the personalized medicines and thus better treatment for prostate cancer.

7. CONCLUSION

Our aim in this study was to create cell lines with stable overexpression of *SKIL* and analyze the effects of overexpression to cell's phenotype. *SKIL* is potential prostate cancer inducing gene which was found earlier by our group. We transfected DU-145 and RWPE-1 cells with plasmid containing *SKIL*-gene and control cells with empty vector. We cultured these cells with geneticin® (Gibco) to create stable transfections. Single cell clones were then created from DU-145 cells. After this we performed proliferation and invasion assays and also measured the mRNA and protein levels of *SKIL*. We wanted to see if overexpression of *SKIL* has effects on proliferation and invasion as these are typical qualities in cancer cells.

Our results showed that it is possible for *SKIL* overexpression to induce different phenotype for cell lines used. We found out that proliferation is not changed in any of the cell lines but invasion effectivity of cells is changed. With RWPE-1 cells *SKIL* overexpression caused higher invasion rate. With DU-145 cells results were variable between the different clones. This indicates that it is be possible that *SKIL* overexpression alone could induce prostate cancer growth. However we could not get a clear *SKIL* overexpression in protein level and this leaves questions about mechanisms which cause the phenotype. Further investigations needs to be carried out to find out precise functions of *SKIL* and it's overexpression in prostate cancer. We need to find precise gene expression patterns caused by *SKIL* overexpression. However these studies already suggest that there is link between *SKIL* and cancer also this provides good foundation and starting point for further studies concerning *SKIL*.

8. REFERENCES

Albany, C., Alva, A.S., Aparicio, A.M., Singal, R., Yellapragada, S., Sonpavde, G. & Hahn, N.M. 2011, "Epigenetics in prostate cancer", *Prostate cancer,* vol. 2011, pp. 580318.

Alberts, B., 2008, *Molecular biology of the cell,* Garland Science, New York.

Alva, A.S., Hahn, N.M., Aparicio, A.M., Singal, R., Yellapragada, S. & Sonpavde, G. 2011, "Hypomethylating agents for urologic cancers", *Future oncology (London, England),* vol. 7, no. 3, pp. 447-463.

Andersen, C.R., Nielsen, L.S., Baer, A., Tolstrup, A.B. & Weilguny, D. 2011, "Efficient expression from one CMV enhancer controlling two core promoters", *Molecular biotechnology,* vol. 48, no. 2, pp. 128-137.

Annala, M., Kivinummi, K., Leinonen, K., Tuominen, J., Zhang, W., Visakorpi, T. & Nykter, M. 2014, "DOT1L-HES6 fusion drives androgen independent growth in prostate cancer", *EMBO molecular medicine,* vol. 6, no. 9, pp. 1121-1123.

Annala, M., Kivinummi, K., Tuominen, J., Karakurt, S., Granberg, K., Latonen, L., Ylipaa, A., Sjoblom, L., Ruusuvuori, P., Saramaki, O., Kaukoniemi, K.M., Yli-Harja, O., Vessella, R.L., Tammela, T.L., Zhang, W., Visakorpi, T. & Nykter, M. 2015, "Recurrent *SKIL*-activating rearrangements in ETS-negative prostate cancer", *Oncotarget,* .

Baca, S.C., Prandi, D., Lawrence, M.S., Mosquera, J.M., Romanel, A., Drier, Y., Park, K., Kitabayashi, N., MacDonald, T.Y., Ghandi, M., Van Allen, E., Kryukov, G.V., Sboner, A., Theurillat, J.P., Soong, T.D., Nickerson, E., Auclair, D., Tewari, A., Beltran, H., Onofrio, R.C., Boysen, G., Guiducci, C., Barbieri, C.E., Cibulskis, K., Sivachenko, A., Carter, S.L., Saksena, G., Voet, D., Ramos, A.H., Winckler, W., Cipicchio, M., Ardlie, K., Kantoff, P.W., Berger, M.F., Gabriel, S.B., Golub, T.R., Meyerson, M., Lander, E.S., Elemento, O., Getz, G., Demichelis, F., Rubin, M.A. & Garraway, L.A. 2013, "Punctuated evolution of prostate cancer genomes", *Cell,* vol. 153, no. 3, pp. 666-677.

Band, A.M. & Laiho, M. 2012, "SnoN oncoprotein enhances estrogen receptor-alpha transcriptional activity", *Cellular signalling,* vol. 24, no. 4, pp. 922-930.

Bello, D., Webber, M.M., Kleinman, H.K., Wartinger, D.D. & Rhim, J.S. 1997, "Androgen responsive adult human prostatic epithelial cell lines immortalized by human papillomavirus 18", *Carcinogenesis,* vol. 18, no. 6, pp. 1215-1223.

Berman, D.M., Rodriguez, R. & Veltri, R.W. 2012 " Development, Molecular Biology, and Physiology of the Prostate", in Wein, A.J., Kavoussi, L.R., Novick, A.C., Partin, A.W. and Peters, C.A. (ed.) *Campbell-Walsh Urology 10th edition,*Philadelphia: Elsevier Inc.

Bostwick, D.G. & Cheng, L. 2012, "Precursors of prostate cancer", *Histopathology,* vol. 60, no. 1, pp. 4-27.

Boyd, L.K., Mao, X. & Lu, Y.J. 2012, "The complexity of prostate cancer: genomic alterations and heterogeneity", *Nature reviews.Urology*, vol. 9, no. 11, pp. 652-664.

Bozic, I., Antal, T., Ohtsuki, H., Carter, H., Kim, D., Chen, S., Karchin, R., Kinzler, K.W., Vogelstein, B. & Nowak, M.A. 2010, "Accumulation of driver and passenger mutations during tumor progression", *Proceedings of the National Academy of Sciences of the United States of America*, vol. 107, no. 43, pp. 18545-18550.

Brandt, A., Bermejo, J.L., Sundquist, J. & Hemminki, K. 2009, "Age at diagnosis and age at death in familial prostate cancer", *The oncologist*, vol. 14, no. 12, pp. 1209-1217.
Bratt, O. 2002, "Hereditary prostate cancer: clinical aspects", *The Journal of urology*, vol. 168, no. 3, pp. 906-913.

Bray, F., Lortet-Tieulent, J., Ferlay, J., Forman, D. & Auvinen, A. 2010, "Prostate cancer incidence and mortality trends in 37 European countries: an overview", *European journal of cancer (Oxford, England : 1990)*, vol. 46, no. 17, pp. 3040-3052.

Brouwer, I.A., Katan, M.B. & Zock, P.L. 2004, "Dietary alpha-linolenic acid is associated with reduced risk of fatal coronary heart disease, but increased prostate cancer risk: a meta-analysis", *The Journal of nutrition*, vol. 134, no. 4, pp. 919-922.

Buess, M., Terracciano, L., Reuter, J., Ballabeni, P., Boulay, J.L., Laffer, U., Metzger, U., Herrmann, R. & Rochlitz, C. 2004, "Amplification of SKI is a prognostic marker in early colorectal cancer", *Neoplasia (New York, N.Y.)*, vol. 6, no. 3, pp. 207-212.

Center, M.M., Jemal, A., Lortet-Tieulent, J., Ward, E., Ferlay, J., Brawley, O. & Bray, F. 2012, "International Variation in Prostate Cancer Incidence and Mortality Rates", *European urology*, vol. 61, no. 6, pp. 1079-1092.

Chen, D., Xu, W., Bales, E., Colmenares, C., Conacci-Sorrell, M., Ishii, S., Stavnezer, E., Campisi, J., Fisher, D.E., Ben-Ze'ev, A. & Medrano, E.E. 2003, "SKI activates Wnt/beta-catenin signaling in human melanoma", *Cancer research*, vol. 63, no. 20, pp. 6626-6634.

Chen, M.F., Chen, W.C., Chang, Y.J., Wu, C.F. & Wu, C.T. 2010, "Role of DNA methyltransferase 1 in hormone-resistant prostate cancer", *Journal of Molecular Medicine (Berlin, Germany)*, vol. 88, no. 9, pp. 953-962.

Cheng, I., Levin, A.M., Tai, Y.C., Plummer, S., Chen, G.K., Neslund-Dudas, C., Casey, G., Rybicki, B.A. & Witte, J.S. 2012, "Copy number alterations in prostate tumors and disease aggressiveness", *Genes, chromosomes & cancer*, vol. 51, no. 1, pp. 66-76.

Clegg, L.X., Li, F.P., Hankey, B.F., Chu, K. & Edwards, B.K. 2002, "Cancer survival among US whites and minorities: a SEER (Surveillance, Epidemiology, and End Results) Program population-based study", *Archives of Internal Medicine*, vol. 162, no. 17, pp. 1985-1993.

Crawford, E.D. 2003, "Epidemiology of prostate cancer", *Urology*, vol. 62, no. 6 Suppl 1, pp. 3-12.

de Muga, S., Hernandez, S., Agell, L., Salido, M., Juanpere, N., Lorenzo, M., Lorente, J.A., Serrano, S. & Lloreta, J. 2010, "Molecular alterations of EGFR and PTEN in prostate cancer: association with high-grade and advanced-stage carcinomas", *Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc*, vol. 23, no. 5, pp. 703-712.

De Stefani, E., Deneo-Pellegrini, H., Boffetta, P., Ronco, A. & Mendilaharsu, M. 2000, "Alpha-linolenic acid and risk of prostate cancer: a case-control study in Uruguay", *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology*, vol. 9, no. 3, pp. 335-338.

Deheuninck, J. & Luo, K. 2009, "Ski and SnoN, potent negative regulators of TGF-beta signaling", *Cell research*, vol. 19, no. 1, pp. 47-57.

Dubbink, H.J., Hersmus, R., Pike, A.C., Molier, M., Brinkmann, A.O., Jenster, G. & Trapman, J. 2006, "Androgen receptor ligand-binding domain interaction and nuclear receptor specificity of FXXLF and LXXLL motifs as determined by L/F swapping", *Molecular endocrinology (Baltimore, Md.)*, vol. 20, no. 8, pp. 1742-1755.

El Gammal, A.T., Bruchmann, M., Zustin, J., Isbarn, H., Hellwinkel, O.J., Kollermann, J., Sauter, G., Simon, R., Wilczak, W., Schwarz, J., Bokemeyer, C., Brummendorf, T.H., Izbicki, J.R., Yekebas, E., Fisch, M., Huland, H., Graefen, M. & Schlomm, T. 2010, "Chromosome 8p deletions and 8q gains are associated with tumor progression and poor prognosis in prostate cancer", *Clinical cancer research : an official journal of the American Association for Cancer Research*, vol. 16, no. 1, pp. 56-64.

Fontes, F., Severo, M., Castro, C., Lourenco, S., Gomes, S., Botelho, F., La Vecchia, C. & Lunet, N. 2013, "Model-based patterns in prostate cancer mortality worldwide", *British journal of cancer*, vol. 108, no. 11, pp. 2354-2366.

Frank, S.B. & Miranti, C.K. 2013, "Disruption of prostate epithelial differentiation pathways and prostate cancer development", *Frontiers in oncology*, vol. 3, pp. 273.

Frey, U.H., Bachmann, H.S., Peters, J. & Siffert, W. 2008, "PCR-amplification of GC-rich regions: 'slowdown PCR'", *Nature protocols*, vol. 3, no. 8, pp. 1312-1317.

Fukuchi, M., Nakajima, M., Fukai, Y., Miyazaki, T., Masuda, N., Sohda, M., Manda, R., Tsukada, K., Kato, H. & Kuwano, H. 2004, "Increased expression of c-Ski as a co-repressor in transforming growth factor-beta signaling correlates with progression of esophageal squamous cell carcinoma", *International journal of cancer.Journal international du cancer*, vol. 108, no. 6, pp. 818-824.

Gallo, F., Chiono, L., Gastaldi, E., Venturino, E. & Giberti, C. 2008, "Prognostic significance of high-grade prostatic intraepithelial neoplasia (HGPIN): risk of prostatic cancer on repeat biopsies", *Urology*, vol. 72, no. 3, pp. 628-632.

Gasi Tandefelt, D., Boormans, J., Hermans, K. & Trapman, J. 2014, "ETS fusion genes in prostate cancer", *Endocrine-related cancer*, vol. 21, no. 3, pp. R143-52.

Giovannucci, E., Liu, Y., Platz, E.A., Stampfer, M.J. & Willett, W.C. 2007, "Risk factors for prostate cancer incidence and progression in the health professionals follow-up study", *International journal of cancer.Journal international du cancer*, vol. 121, no. 7, pp. 1571-1578.

Goeman, L., Joniau, S., Ponette, D., Van der Aa, F., Roskams, T., Oyen, R. & Van Poppel, H. 2003, "Is low-grade prostatic intraepithelial neoplasia a risk factor for cancer?", *Prostate cancer and prostatic diseases*, vol. 6, no. 4, pp. 305-310.

Gravina, G.L., Festuccia, C., Millimaggi, D., Dolo, V., Tombolini, V., de Vito, M., Vicentini, C. & Bologna, M. 2008, "Chronic azacitidine treatment results in differentiating effects, sensitizes against bicalutamide in androgen-independent prostate cancer cells", *The Prostate*, vol. 68, no. 7, pp. 793-801.

Gravina, G.L., Marampon, F., Di Staso, M., Bonfili, P., Vitturini, A., Jannini, E.A., Pestell, R.G., Tombolini, V. & Festuccia, C. 2010, "5-Azacitidine restores and amplifies the bicalutamide response on preclinical models of androgen receptor expressing or deficient prostate tumors", *The Prostate*, vol. 70, no. 11, pp. 1166-1178.

Haffner, M.C., Aryee, M.J., Toubaji, A., Esopi, D.M., Albadine, R., Gurel, B., Isaacs, W.B., Bova, G.S., Liu, W., Xu, J., Meeker, A.K., Netto, G., De Marzo, A.M., Nelson, W.G. & Yegnasubramanian, S. 2010, "Androgen-induced TOP2B-mediated double-strand breaks and prostate cancer gene rearrangements", *Nature genetics*, vol. 42, no. 8, pp. 668-675.

Hagerstrand, D., Tong, A., Schumacher, S.E., Ilic, N., Shen, R.R., Cheung, H.W., Vazquez, F., Shrestha, Y., Kim, S.Y., Giacomelli, A.O., Rosenbluh, J., Schinzel, A.C., Spardy, N.A., Barbie, D.A., Mermel, C.H., Weir, B.A., Garraway, L.A., Tamayo, P., Mesirov, J.P., Beroukhim, R. & Hahn, W.C. 2013, "Systematic interrogation of 3q26 identifies TLOC1 and *SKIL* as cancer drivers", *Cancer discovery*, vol. 3, no. 9, pp. 1044-1057.

Han, G., Buchanan, G., Ittmann, M., Harris, J.M., Yu, X., Demayo, F.J., Tilley, W. & Greenberg, N.M. 2005, "Mutation of the androgen receptor causes oncogenic transformation of the prostate", *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 4, pp. 1151-1156.

Heider, T.R., Lyman, S., Schoonhoven, R. & Behrns, K.E. 2007, "Ski promotes tumor growth through abrogation of transforming growth factor-beta signaling in pancreatic cancer", *Annals of Surgery*, vol. 246, no. 1, pp. 61-68.

Hickey, T.E., Robinson, J.L., Carroll, J.S. & Tilley, W.D. 2012, "Minireview: The androgen receptor in breast tissues: growth inhibitor, tumor suppressor, oncogene?", *Molecular endocrinology (Baltimore, Md.)*, vol. 26, no. 8, pp. 1252-1267.

Hopman, A.H., Theelen, W., Hommelberg, P.P., Kamps, M.A., Herrington, C.S., Morrison, L.E., Speel, E.J., Smedts, F. & Ramaekers, F.C. 2006, "Genomic integration of oncogenic HPV and gain of the human telomerase gene TERC at 3q26 are strongly

associated events in the progression of uterine cervical dysplasia to invasive cancer", *The Journal of pathology,* vol. 210, no. 4, pp. 412-419.

Imoto, I., Pimkhaokham, A., Fukuda, Y., Yang, Z.Q., Shimada, Y., Nomura, N., Hirai, H., Imamura, M. & Inazawa, J. 2001, "SNO is a probable target for gene amplification at 3q26 in squamous-cell carcinomas of the esophagus", *Biochemical and biophysical research communications,* vol. 286, no. 3, pp. 559-565.

Ishak, M.B. & Giri, V.N. 2011, "A systematic review of replication studies of prostate cancer susceptibility genetic variants in high-risk men originally identified from genome-wide association studies", *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology,* vol. 20, no. 8, pp. 1599-1610.

Ito, K. 2014, "Prostate cancer in Asian men", *Nature reviews.Urology,* vol. 11, no. 4, pp. 197-212.

Jahchan, N.S., Ouyang, G. & Luo, K. 2013, "Expression profiles of SnoN in normal and cancerous human tissues support its tumor suppressor role in human cancer", *PloS one,* vol. 8, no. 2, pp. e55794.

Jeronimo, C., Henrique, R., Hoque, M.O., Mambo, E., Ribeiro, F.R., Varzim, G., Oliveira, J., Teixeira, M.R., Lopes, C. & Sidransky, D. 2004, "A quantitative promoter methylation profile of prostate cancer", *Clinical cancer research : an official journal of the American Association for Cancer Research,* vol. 10, no. 24, pp. 8472-8478.

Johns, L.E. & Houlston, R.S. 2003, "A systematic review and meta-analysis of familial prostate cancer risk", *BJU international,* vol. 91, no. 9, pp. 789-794.

Jung, V., Kindich, R., Kamradt, J., Jung, M., Muller, M., Schulz, W.A., Engers, R., Unteregger, G., Stockle, M., Zimmermann, R. & Wullich, B. 2006, "Genomic and expression analysis of the 3q25-q26 amplification unit reveals TLOC1/SEC62 as a probable target gene in prostate cancer", *Molecular cancer research : MCR,* vol. 4, no. 3, pp. 169-176.

Kamihara, J., Rana, H.Q. & Garber, J.E. 2014, "Germline TP53 mutations and the changing landscape of Li-Fraumeni syndrome", *Human mutation,* vol. 35, no. 6, pp. 654-662.

Karunasinghe, N., Han, D.Y., Goudie, M., Zhu, S., Bishop, K., Wang, A., Duan, H., Lange, K., Ko, S., Medhora, R., Kan, S.T., Masters, J. & Ferguson, L.R. 2012, "Prostate disease risk factors among a New Zealand cohort", *Journal of nutrigenetics and nutrigenomics,* vol. 5, no. 6, pp. 339-351.

Kenfield, S.A., Stampfer, M.J., Chan, J.M. & Giovannucci, E. 2011, "Smoking and prostate cancer survival and recurrence", *Jama,* vol. 305, no. 24, pp. 2548-2555.

Koralek, D.O., Peters, U., Andriole, G., Reding, D., Kirsh, V., Subar, A., Schatzkin, A., Hayes, R. & Leitzmann, M.F. 2006, "A prospective study of dietary alpha-linolenic

acid and the risk of prostate cancer (United States)", *Cancer causes & control : CCC*, vol. 17, no. 6, pp. 783-791.

Kulis, M. & Esteller, M. 2010, "DNA methylation and cancer", *Advances in Genetics*, vol. 70, pp. 27-56.

Lange, E.M., Salinas, C.A., Zuhlke, K.A., Ray, A.M., Wang, Y., Lu, Y., Ho, L.A., Luo, J. & Cooney, K.A. 2012, "Early onset prostate cancer has a significant genetic component", *The Prostate*, vol. 72, no. 2, pp. 147-156.

Levine, A.J. 2011, "Introduction: The Changing Directions of p53 Research", *Genes & cancer*, vol. 2, no. 4, pp. 382-384.

Li, J. & Al-Azzawi, F. 2009, "Mechanism of androgen receptor action", *Maturitas*, vol. 63, no. 2, pp. 142-148.

Li, J., Djenaba, J.A., Soman, A., Rim, S.H. & Master, V.A. 2012, "Recent trends in prostate cancer incidence by age, cancer stage, and grade, the United States, 2001-2007", *Prostate cancer*, vol. 2012, pp. 691380.

Li, J., Yen, C., Liaw, D., Podsypanina, K., Bose, S., Wang, S.I., Puc, J., Miliaresis, C., Rodgers, L., McCombie, R., Bigner, S.H., Giovanella, B.C., Ittmann, M., Tycko, B., Hibshoosh, H., Wigler, M.H. & Parsons, R. 1997, "PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer", *Science (New York, N.Y.)*, vol. 275, no. 5308, pp. 1943-1947.

Lin, B., Ferguson, C., White, J.T., Wang, S., Vessella, R., True, L.D., Hood, L. & Nelson, P.S. 1999, "Prostate-localized and androgen-regulated expression of the membrane-bound serine protease TMPRSS2", *Cancer research*, vol. 59, no. 17, pp. 4180-4184.

Lin, S.C., Liu, C.J., Ko, S.Y., Chang, H.C., Liu, T.Y. & Chang, K.W. 2005, "Copy number amplification of 3q26-27 oncogenes in microdissected oral squamous cell carcinoma and oral brushed samples from areca chewers", *The Journal of pathology*, vol. 206, no. 4, pp. 417-422.

Lindberg, J., Klevebring, D., Liu, W., Neiman, M., Xu, J., Wiklund, P., Wiklund, F., Mills, I.G., Egevad, L. & Gronberg, H. 2013, "Exome sequencing of prostate cancer supports the hypothesis of independent tumour origins", *European urology*, vol. 63, no. 2, pp. 347-353.

Liu, C., Zhu, Y., Lou, W., Nadiminty, N., Chen, X., Zhou, Q., Shi, X.B., deVere White, R.W. & Gao, A.C. 2013, "Functional p53 determines docetaxel sensitivity in prostate cancer cells", *The Prostate*, vol. 73, no. 4, pp. 418-427.

Liu, X., Sun, Y., Weinberg, R.A. & Lodish, H.F. 2001, "Ski/Sno and TGF-beta signaling", *Cytokine & growth factor reviews*, vol. 12, no. 1, pp. 1-8.

Lowe, S.W., Cepero, E. & Evan, G. 2004, "Intrinsic tumour suppression", *Nature*, vol. 432, no. 7015, pp. 307-315.

Magistroni, V., Mologni, L., Sanselicio, S., Reid, J.F., Redaelli, S., Piazza, R., Viltadi, M., Bovo, G., Strada, G., Grasso, M., Gariboldi, M. & Gambacorti-Passerini, C. 2011, "ERG deregulation induces PIM1 over-expression and aneuploidy in prostate epithelial cells", *PloS one,* vol. 6, no. 11, pp. e28162.

Maruyama, R., Toyooka, S., Toyooka, K.O., Virmani, A.K., Zochbauer-Muller, S., Farinas, A.J., Minna, J.D., McConnell, J., Frenkel, E.P. & Gazdar, A.F. 2002, "Aberrant promoter methylation profile of prostate cancers and its relationship to clinicopathological features", *Clinical cancer research : an official journal of the American Association for Cancer Research,* vol. 8, no. 2, pp. 514-519.

Matsuyama, H., Pan, Y., Skoog, L., Tribukait, B., Naito, K., Ekman, P., Lichter, P. & Bergerheim, U.S. 1994, "Deletion mapping of chromosome 8p in prostate cancer by fluorescence in situ hybridization", *Oncogene,* vol. 9, no. 10, pp. 3071-3076.

Mitelman, F., Johansson, B. & Mertens, F. 2007, "The impact of translocations and gene fusions on cancer causation", *Nature reviews.Cancer,* vol. 7, no. 4, pp. 233-245.

Montironi, R., Mazzucchelli, R., Lopez-Beltran, A., Cheng, L. & Scarpelli, M. 2007, "Mechanisms of disease: high-grade prostatic intraepithelial neoplasia and other proposed preneoplastic lesions in the prostate", *Nature clinical practice.Urology,* vol. 4, no. 6, pp. 321-332.

Nakayama, T., Watanabe, M., Suzuki, H., Toyota, M., Sekita, N., Hirokawa, Y., Mizokami, A., Ito, H., Yatani, R. & Shiraishi, T. 2000, "Epigenetic regulation of androgen receptor gene expression in human prostate cancers", *Laboratory investigation; a journal of technical methods and pathology,* vol. 80, no. 12, pp. 1789-1796.

Nanjundan, M., Nakayama, Y., Cheng, K.W., Lahad, J., Liu, J., Lu, K., Kuo, W.L., Smith-McCune, K., Fishman, D., Gray, J.W. & Mills, G.B. 2007, "Amplification of MDS1/EVI1 and EVI1, located in the 3q26.2 amplicon, is associated with favorable patient prognosis in ovarian cancer", *Cancer research,* vol. 67, no. 7, pp. 3074-3084.

Nelson, W.G., Yegnasubramanian, S., Agoston, A.T., Bastian, P.J., Lee, B.H., Nakayama, M. & De Marzo, A.M. 2007, "Abnormal DNA methylation, epigenetics, and prostate cancer", *Frontiers in bioscience : a journal and virtual library,* vol. 12, pp. 4254-4266.

Nicol, R. & Stavnezer, E. 1998, "Transcriptional repression by v-Ski and c-Ski mediated by a specific DNA binding site", *The Journal of biological chemistry,* vol. 273, no. 6, pp. 3588-3597.

Nomura, T., Khan, M.M., Kaul, S.C., Dong, H.D., Wadhwa, R., Colmenares, C., Kohno, I. & Ishii, S. 1999, "Ski is a component of the histone deacetylase complex required for transcriptional repression by Mad and thyroid hormone receptor", *Genes & development,* vol. 13, no. 4, pp. 412-423.

Noonan-Wheeler, F.C., Wu, W., Roehl, K.A., Klim, A., Haugen, J., Suarez, B.K. & Kibel, A.S. 2006, "Association of hereditary prostate cancer gene polymorphic variants with sporadic aggressive prostate carcinoma", *The Prostate,* vol. 66, no. 1, pp. 49-56.

Paone, A., Galli, R. & Fabbri, M. 2011, "MicroRNAs as New Characters in the Plot between Epigenetics and Prostate Cancer", *Frontiers in genetics,* vol. 2, pp. 62.

Pearson-White, S. 1993, "SnoI, a novel alternatively spliced isoform of the ski protooncogene homolog, sno", *Nucleic acids research,* vol. 21, no. 19, pp. 4632-4638.

Pearson-White, S. & Crittenden, R. 1997, "Proto-oncogene Sno expression, alternative isoforms and immediate early serum response", *Nucleic acids research,* vol. 25, no. 14, pp. 2930-2937.

Pearson-White, S., Deacon, D., Crittenden, R., Brady, G., Iscove, N. & Quesenberry, P.J. 1995, "The ski/sno protooncogene family in hematopoietic development", *Blood,* vol. 86, no. 6, pp. 2146-2155.

Pihan, G.A., Purohit, A., Wallace, J., Malhotra, R., Liotta, L. & Doxsey, S.J. 2001, "Centrosome defects can account for cellular and genetic changes that characterize prostate cancer progression", *Cancer research,* vol. 61, no. 5, pp. 2212-2219.

Podlasek, C.A., Duboule, D. & Bushman, W. 1997, "Male accessory sex organ morphogenesis is altered by loss of function of Hoxd-13", *Developmental dynamics : an official publication of the American Association of Anatomists,* vol. 208, no. 4, pp. 454-465.

Pourmand, G., Ziaee, A.A., Abedi, A.R., Mehrsai, A., Alavi, H.A., Ahmadi, A. & Saadati, H.R. 2007, "Role of PTEN gene in progression of prostate cancer", *Urology journal,* vol. 4, no. 2, pp. 95-100.

Prajapati, A., Gupta, S., Mistry, B. & Gupta, S. 2013, "Prostate stem cells in the development of benign prostate hyperplasia and prostate cancer: emerging role and concepts", *BioMed research international,* vol. 2013, pp. 107954.

Racz, A., Brass, N., Heckel, D., Pahl, S., Remberger, K. & Meese, E. 1999, "Expression analysis of genes at 3q26-q27 involved in frequent amplification in squamous cell lung carcinoma", *European journal of cancer (Oxford, England : 1990),* vol. 35, no. 4, pp. 641-646.

Rana, A., Ali, G.M., Ali, S., Khan, A., Mansoor, S., Malik, S. & Farooqi, A.A. 2013, "BCR-ABL1 in leukemia: disguise master outplays riding shotgun", *Journal of cancer research and therapeutics,* vol. 9, no. 1, pp. 6-10.

Reed, J.A., Lin, Q., Chen, D., Mian, I.S. & Medrano, E.E. 2005, "SKI pathways inducing progression of human melanoma", *Cancer metastasis reviews,* vol. 24, no. 2, pp. 265-272.

Ritter, M., Kattmann, D., Teichler, S., Hartmann, O., Samuelsson, M.K., Burchert, A., Bach, J.P., Kim, T.D., Berwanger, B., Thiede, C., Jager, R., Ehninger, G., Schafer, H.,

Ueki, N., Hayman, M.J., Eilers, M. & Neubauer, A. 2006, "Inhibition of retinoic acid receptor signaling by Ski in acute myeloid leukemia", *Leukemia,* vol. 20, no. 3, pp. 437-443.

Rota, M., Scotti, L., Turati, F., Tramacere, I., Islami, F., Bellocco, R., Negri, E., Corrao, G., Boffetta, P., La Vecchia, C. & Bagnardi, V. 2012, "Alcohol consumption and prostate cancer risk: a meta-analysis of the dose-risk relation", *European journal of cancer prevention : the official journal of the European Cancer Prevention Organisation (ECP),* vol. 21, no. 4, pp. 350-359.

Saramaki, O. & Visakorpi, T. 2007, "Chromosomal aberrations in prostate cancer", *Frontiers in bioscience : a journal and virtual library,* vol. 12, pp. 3287-3301.

Schoenfield, L., Jones, J.S., Zippe, C.D., Reuther, A.M., Klein, E., Zhou, M. & Magi-Galluzzi, C. 2007, "The incidence of high-grade prostatic intraepithelial neoplasia and atypical glands suspicious for carcinoma on first-time saturation needle biopsy, and the subsequent risk of cancer", *BJU international,* vol. 99, no. 4, pp. 770-774.

Seligson, D.B., Horvath, S., Shi, T., Yu, H., Tze, S., Grunstein, M. & Kurdistani, S.K. 2005, "Global histone modification patterns predict risk of prostate cancer recurrence", *Nature,* vol. 435, no. 7046, pp. 1262-1266.

Shafique, K., McLoone, P., Qureshi, K., Leung, H., Hart, C. & Morrison, D.S. 2012, "Coffee consumption and prostate cancer risk: further evidence for inverse relationship", *Nutrition journal,* vol. 11, pp. 42-2891-11-42.

Sherr, C.J. 2004, "Principles of tumor suppression", *Cell,* vol. 116, no. 2, pp. 235-246.
Shim, J. & Karin, M. 2002, "The control of mRNA stability in response to extracellular stimuli", *Molecules and cells,* vol. 14, no. 3, pp. 323-331.

Simon, J.A., Chen, Y.H. & Bent, S. 2009, "The relation of alpha-linolenic acid to the risk of prostate cancer: a systematic review and meta-analysis", *The American Journal of Clinical Nutrition,* vol. 89, no. 5, pp. 1558S-1564S.

Singh, B., Gogineni, S.K., Sacks, P.G., Shaha, A.R., Shah, J.P., Stoffel, A. & Rao, P.H. 2001, "Molecular cytogenetic characterization of head and neck squamous cell carcinoma and refinement of 3q amplification", *Cancer research,* vol. 61, no. 11, pp. 4506-4513.

Singh, M., Jha, R., Melamed, J., Shapiro, E., Hayward, S.W. & Lee, P. 2014, "Stromal Androgen Receptor in Prostate Development and Cancer", *The American journal of pathology,* .

Stephens, P.J., Greenman, C.D., Fu, B., Yang, F., Bignell, G.R., Mudie, L.J., Pleasance, E.D., Lau, K.W., Beare, D., Stebbings, L.A., McLaren, S., Lin, M.L., McBride, D.J., Varela, I., Nik-Zainal, S., Leroy, C., Jia, M., Menzies, A., Butler, A.P., Teague, J.W., Quail, M.A., Burton, J., Swerdlow, H., Carter, N.P., Morsberger, L.A., Iacobuzio-Donahue, C., Follows, G.A., Green, A.R., Flanagan, A.M., Stratton, M.R., Futreal, P.A. & Campbell, P.J. 2011, "Massive genomic rearrangement acquired in a single catastrophic event during cancer development", *Cell,* vol. 144, no. 1, pp. 27-40.

Stone, K.R., Mickey, D.D., Wunderli, H., Mickey, G.H. & Paulson, D.F. 1978, "Isolation of a human prostate carcinoma cell line (DU 145)", *International journal of cancer.Journal international du cancer*, vol. 21, no. 3, pp. 274-281.

Stroschein, S.L., Wang, W., Zhou, S., Zhou, Q. & Luo, K. 1999, "Negative feedback regulation of TGF-beta signaling by the SnoN oncoprotein", *Science (New York, N.Y.)*, vol. 286, no. 5440, pp. 771-774.

Sugita, M., Tanaka, N., Davidson, S., Sekiya, S., Varella-Garcia, M., West, J., Drabkin, H.A. & Gemmill, R.M. 2000, "Molecular definition of a small amplification domain within 3q26 in tumors of cervix, ovary, and lung", *Cancer genetics and cytogenetics*, vol. 117, no. 1, pp. 9-18.

Sun, Y., Liu, X., Ng-Eaton, E., Lodish, H.F. & Weinberg, R.A. 1999, "SnoN and Ski protooncoproteins are rapidly degraded in response to transforming growth factor beta signaling", *Proceedings of the National Academy of Sciences of the United States of America*, vol. 96, no. 22, pp. 12442-12447.

Sun, Y., Liu, X., Ng-Eaton, E., Lodish, H.F. & Weinberg, R.A. 1999, "SnoN and Ski protooncoproteins are rapidly degraded in response to transforming growth factor beta signaling", *Proceedings of the National Academy of Sciences of the United States of America*, vol. 96, no. 22, pp. 12442-12447.

Taksler, G.B., Keating, N.L. & Cutler, D.M. 2012, "Explaining racial differences in prostate cancer mortality", *Cancer*, vol. 118, no. 17, pp. 4280-4289.

Tapia-Laliena, M.A., Korzeniewski, N., Hohenfellner, M. & Duensing, S. 2014, "High-risk prostate cancer: A disease of genomic instability", *Urologic oncology*, .

Thomas, J.A.,2nd, Gerber, L., Moreira, D.M., Hamilton, R.J., Banez, L.L., Castro-Santamaria, R., Andriole, G.L., Isaacs, W.B., Xu, J. & Freedland, S.J. 2012, "Prostate cancer risk in men with prostate and breast cancer family history: results from the REDUCE study (R1)", *Journal of internal medicine*, vol. 272, no. 1, pp. 85-92.

Van Den Berg, C., Guan, X.Y., Von Hoff, D., Jenkins, R., Bittner, Griffin, C., Kallioniemi, O., Visakorpi, McGill & Herath, J. 1995, "DNA sequence amplification in human prostate cancer identified by chromosome microdissection: potential prognostic implications", *Clinical cancer research : an official journal of the American Association for Cancer Research*, vol. 1, no. 1, pp. 11-18.

Venkatachalan, S.P., Bushman, J.D., Mercado, J.L., Sancar, F., Christopherson, K.R. & Boileau, A.J. 2007, "Optimized expression vector for ion channel studies in Xenopus oocytes and mammalian cells using alfalfa mosaic virus", *Pflugers Archiv : European journal of physiology*, vol. 454, no. 1, pp. 155-163.

Visakorpi, T., Hyytinen, E., Koivisto, P., Tanner, M., Keinanen, R., Palmberg, C., Palotie, A., Tammela, T., Isola, J. & Kallioniemi, O.P. 1995, "In vivo amplification of the androgen receptor gene and progression of human prostate cancer", *Nature genetics*, vol. 9, no. 4, pp. 401-406.

Wang, W.L. & Tenniswood, M. 2014, "Vitamin D, intermediary metabolism and prostate cancer tumor progression", *Frontiers in physiology,* vol. 5, pp. 183.

Watters, J.L., Park, Y., Hollenbeck, A., Schatzkin, A. & Albanes, D. 2010, "Alcoholic beverages and prostate cancer in a prospective US cohort study", *American Journal of Epidemiology,* vol. 172, no. 7, pp. 773-780.

Watters, J.L., Park, Y., Hollenbeck, A., Schatzkin, A. & Albanes, D. 2009, "Cigarette smoking and prostate cancer in a prospective US cohort study", *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology,* vol. 18, no. 9, pp. 2427-2435.

Webber, M.M., Bello, D., Kleinman, H.K. & Hoffman, M.P. 1997, "Acinar differentiation by non-malignant immortalized human prostatic epithelial cells and its loss by malignant cells", *Carcinogenesis,* vol. 18, no. 6, pp. 1225-1231.

Wilson, K.M., Kasperzyk, J.L., Rider, J.R., Kenfield, S., van Dam, R.M., Stampfer, M.J., Giovannucci, E. & Mucci, L.A. 2011, "Coffee consumption and prostate cancer risk and progression in the Health Professionals Follow-up Study", *Journal of the National Cancer Institute,* vol. 103, no. 11, pp. 876-884.

Wright, J.L. & Lange, P.H. 2007, "Newer potential biomarkers in prostate cancer", *Reviews in urology,* vol. 9, no. 4, pp. 207-213.

Yang, F., Chen, Y., Shen, T., Guo, D., Dakhova, O., Ittmann, M.M., Creighton, C.J., Zhang, Y., Dang, T.D. & Rowley, D.R. 2014, "Stromal TGF-beta signaling induces AR activation in prostate cancer", *Oncotarget,* vol. 5, no. 21, pp. 10854-10869.

Yegnasubramanian, S., Kowalski, J., Gonzalgo, M.L., Zahurak, M., Piantadosi, S., Walsh, P.C., Bova, G.S., De Marzo, A.M., Isaacs, W.B. & Nelson, W.G. 2004, "Hypermethylation of CpG islands in primary and metastatic human prostate cancer", *Cancer research,* vol. 64, no. 6, pp. 1975-1986.

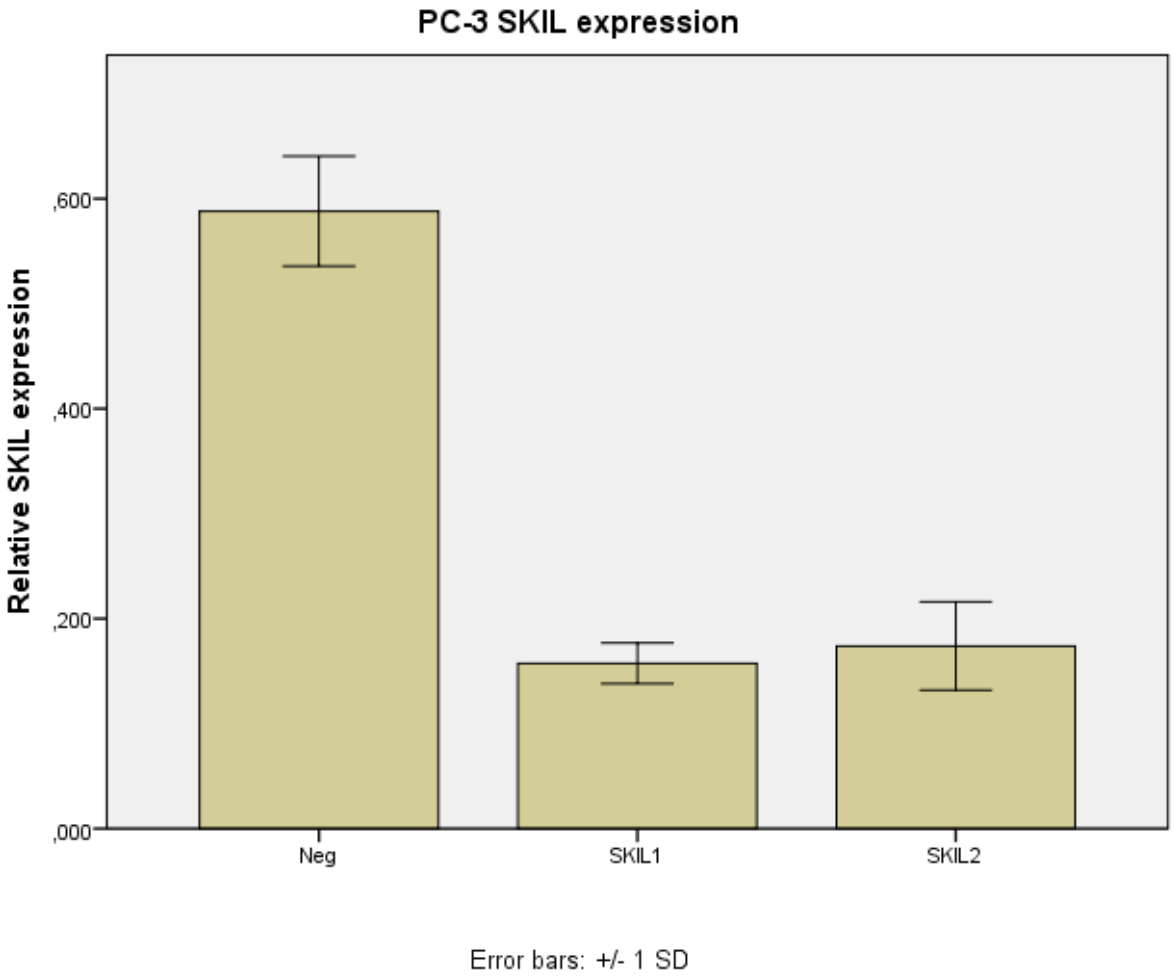
Zhang, F., Lundin, M., Ristimaki, A., Heikkila, P., Lundin, J., Isola, J., Joensuu, H. & Laiho, M. 2003, "Ski-related novel protein N (SnoN), a negative controller of transforming growth factor-beta signaling, is a prognostic marker in estrogen receptor-positive breast carcinomas", *Cancer research,* vol. 63, no. 16, pp. 5005-5010.

Zhang, S., Gu, J., Yang, N.S., Kao, C., Gardner, T.A., Eble, J.N. & Cheng, L. 2002, "Relative promoter strengths in four human prostate cancer cell lines evaluated by particle bombardment-mediated gene transfer", *The Prostate,* vol. 51, no. 4, pp. 286-292.

Zhou, L.X., Li, T., Huang, Y.R., Sha, J.J., Sun, P. & Li, D. 2010, "Application of histone modification in the risk prediction of the biochemical recurrence after radical prostatectomy", *Asian Journal of Andrology,* vol. 12, no. 2, pp. 171-179.

Zhu, Q., Krakowski, A.R., Dunham, E.E., Wang, L., Bandyopadhyay, A., Berdeaux, R., Martin, G.S., Sun, L. & Luo, K. 2007, "Dual role of SnoN in mammalian tumorigenesis", *Molecular and cellular biology*, vol. 27, no. 1, pp. 324-339.

9. APPENDIX



Appendix picture 1: Relative mRNA expression levels of *SKIL*.
The expression of *SKIL* mRNA in PC-3 cells transfected with either of two different *SKIL* siRNAs or with control siRNA. Error bars represent standard deviations of RT-qPCR replicates.