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„Persistant prostate inflammation and its ability to transform prostate but without invasive character“

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Eva Ladenhauf (B.Sc.)

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Ao. Univ.-Prof. Mag. Dr. Pavel Kovarik

Externe Betreuung

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Mag. Dr. Andreas Birbach

Ort

Department of Vascular Biology and Thrombosis
Research

Center for Physiology and Pharmacology

Medical University of Vienna

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1 Introduction

1.1 Anatomy of the prostate gland

Mouse prostate consists out of four different lobes: the dorsal and lateral lobe originates dorsally at the base of seminal vesicles bilaterally and partly wrap bilaterally around the ventral urethra. Ventral prostate lobe, originates ventrally and wraps incompletely around the urethra dorsally. Fourth lobe is the anterior lobe located to the slighter curvature of the seminal vesicles.

Human prostate consists out of three zones: First, the central zone that encircles ejaculatory ducts and terminates in prostatic urethra. The peripheral zone is surrounding the transitional zone and the central zone. Third lobe is the transitional zone surrounding the proximal urethra¹ (Fig.1).

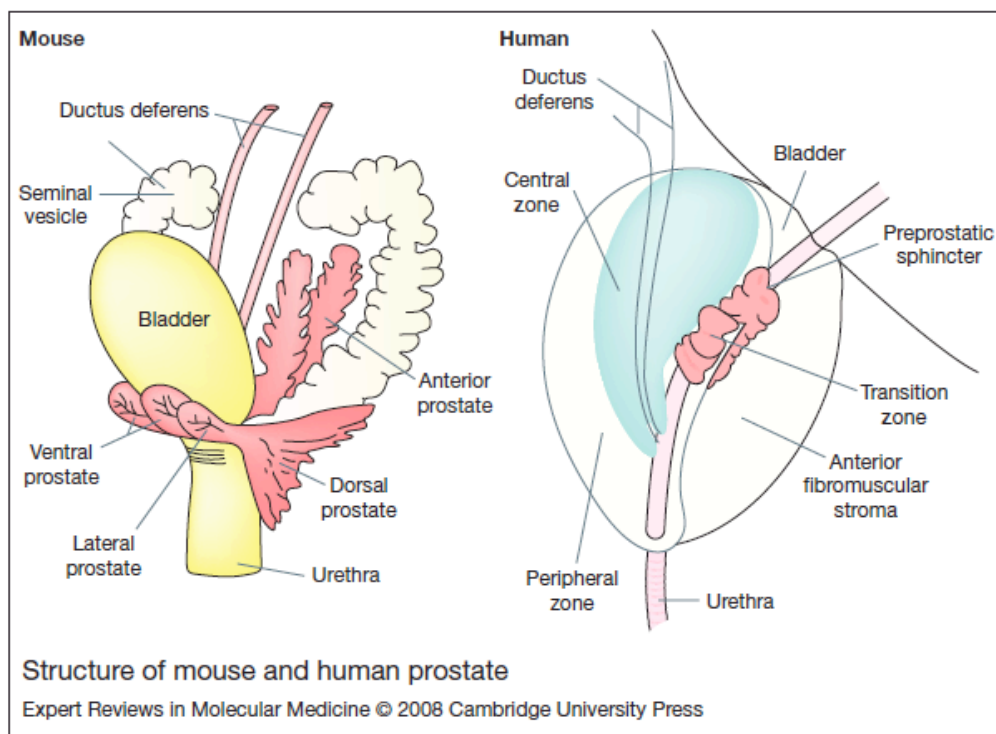


Figure 1: Comparison of a mouse and human prostate. Taken from: Ahmad, I., Sansom, O.J. & Leung, H.Y. Advances in mouse models of prostate cancer. *Expert Rev Mol Med* 10, e16.

1.2 Prostate cancer

Besides lung and colon cancer, prostate cancer is the most common death cause in man all over the world. Although there is a variety of research done in this field, the prevention and treatment of this cancer type is a medical challenge. Progression of this cancer type, as well as other cancer types, depends on genetic and epigenetic changes resulting from mutated or inactivated tumor suppressor genes and the activation of oncogenes^{2,3}. It is assumed that there is a huge number of additional factors involved in prostate cancer progression, like hormonal changes and environmental factors like physical trauma and probably dietary habits⁴. Interestingly the risk of prostate cancer seems to underlie a geographic variation: For example men in South East Asian countries have the lowest incidence of prostate cancer, while men from Western countries have a much higher tendency to get this disease. It has been shown that men from South East Asian countries after migration to western countries develop higher prostate cancer risks within one generation⁵, arguing as well for a strong environmental component. Moreover epidemiological, histopathological and molecular pathological studies hypothesize that inflammation plays an important role in developing this disease having a tumor-promoting effect⁶. Although there is a lot of research in this field, the relationship between inflammation and prostate cancer is not completely understood and requires further examination to define the link between prostate inflammation and cancer.

1.3 Inflammation and Prostate cancer

It was estimated that infections and inflammatory responses are linked to 15-20% of all deaths from cancer worldwide⁷.

Further it is known that a high number of people that suffer from chronic prostatitis have an increased risk to develop prostate cancer at a later age. Cancer progression is assumed to start with proliferative inflammatory atrophy [PIA] characterized by immune cell infiltration, downregulation of genes important for cell cycle progression and upregulation of stress responsive genes. PIA is known to be quite frequently merged with high-grade prostatic intraepithelial neoplasia (PIN) characterized by genetic instability and accumulation of genetic changes⁸ and known as the ultimate precursor of invasive carcinoma.

Therefore it is assumed that a permanent inflammation of the prostatic duct is linked to a higher risk of prostate cancer. This is underlined by studies showing that bacterial infections could be able to promote cancer progression via permanent inflammatory stimulation of the prostate. It was shown that mice with a chronic *Escherichia coli* infection, investigated after 5 days, 12 and 26 weeks develop inflammatory changes in stromal and epithelial cells already after 12 weeks. 26 weeks after bacterial infiltration, mouse prostates show a phenotype related to prostatic intraepithelial neoplasia and high-grade dysplasia, showing increased signs of oxidative DNA damage too. This finding supports the hypothesis of an existing link between inflammation and prostate cancer⁹. Next to infectious agents such as bacteria and viruses, urine reflux is assumed to be causative for a permanent inflammatory stimulation⁴. Thereby immunological cascades such as the NF κ B pathway including its key mediator I κ B kinase 2 (IKK2) are triggered. Interestingly it is

documented that long time use of aspirin, known as an inhibitor of IKK2¹⁰, seem to decrease prostate cancer risk. Although it is not exactly known what molecular processes are involved in the linkage of inflammation and cancer, the NFκB pathway and therein the IKK2 kinase seem to be an excellent target for further research.

1.4 From Proliferative inflammatory atrophy (PIA) to Invasive carcinoma

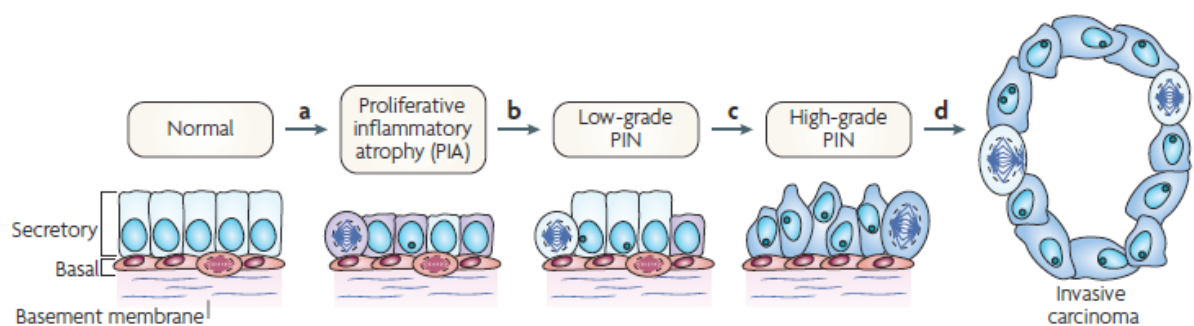


Figure 2: Cellular and molecular model of early prostate neoplasia progression. Taken from: De Marzo, A.M., *et al.* Inflammation in prostate carcinogenesis. *Nat Rev Cancer* 7, 256-269

Although the exact reasons for development of prostate cancer and how it is linked to inflammation are not yet clear, several hypotheses have been put forward speculating how inflamed prostate tissue could develop invasive carcinoma. It seems that the progression of this disease starts with an infiltration of immune cells such as lymphocytes, macrophages and neutrophils stimulated by several factors like repeated infections, dietary factors or onset of autoimmunity. These immune cells produce cytokines and chemokines promoting further immunological processes. Phagocytes release reactive oxygen and nitrogen species causing cell injury followed by DNA damage resulting in mutation of genes involved in cell metabolism,

proliferation and apoptosis. A complicated interplay of these changes in DNA structure is assumed to lead finally to invasive carcinoma formation (Fig.2).

1.5 Factors linked to inflammation

As mentioned above environmental factors but also autoimmune reactions seem to stimulate and promote inflammation and prostate cancer. Inflammation is a very complex process in which many cell types and proteins are involved to interact with each other resulting in an increased production of cytokines such as $\text{TNF}\alpha$ that further activate immune cells, chemokines such as CXCL5, CXCL2, CXCL10 and CXCL15 or intercellular adhesion molecules such as ICAM I that attract these cells.

1.5.1 NF κ B pathway

One major molecular process stimulated within inflammation is the NF κ B pathway with its key mediator IKK2 kinase (IKK β). IKK2 is part of a heterotrimeric complex additionally containing alpha (IKK α) and gamma (IKK γ /NEMO) subunits. IKK α and IKK2 (IKK β) have kinase activity whereas IKK γ works as a regulatory subunit. Together these proteins play an essential role in the NF κ B pathway responsible for activation of many factors involved in inflammation and many other physiological processes such as cell growth, differentiation, proliferation, cell survival and apoptosis.

1.5.1.1 Molecularbiological background of NF κ B pathway

Initiated by a variety of factors, ligands such as IL1 and $\text{TNF}\alpha$ bind to respective receptors leading to a cascade of protein interactions that result in the phosphorylation and activation of the IKK2 kinase¹¹. Hence IKK2 phosphorylates Inhibitor of kappaB (I κ B) that is consequently linked to polyubiquitin chains followed

by proteasomal degradation and release of NF κ B transcription factor (Fig.3).

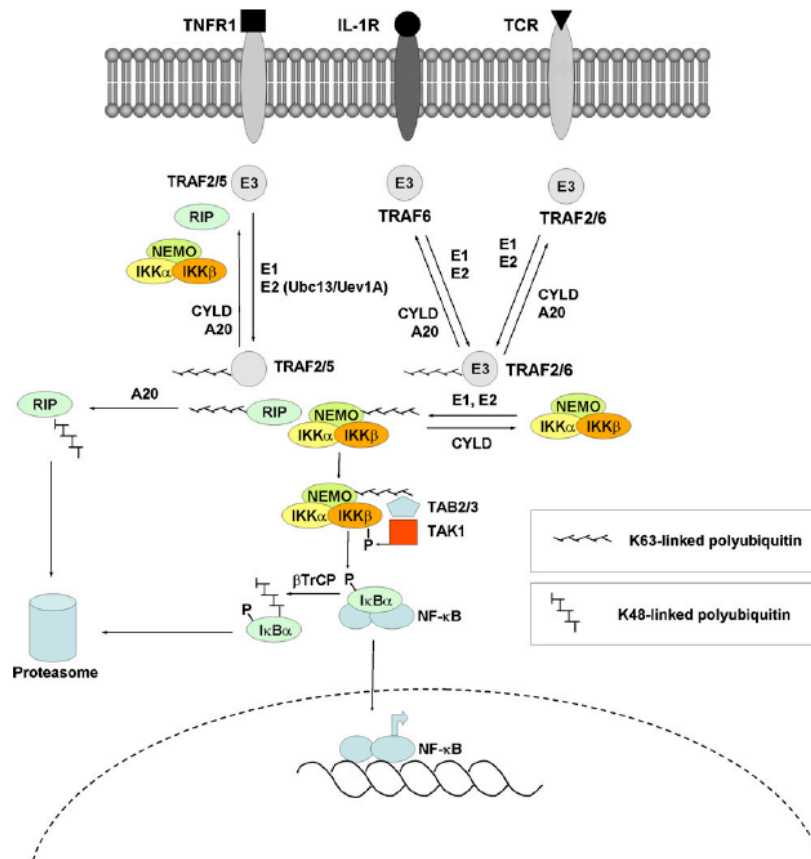


Figure 3: An illustration of the inflammatory NF κ B pathway. Taken from: Schmid, J.A. & Birbach, A. I κ B kinase beta (IKK β /IKK2/IKK β)--a key molecule in signaling to the transcription factor NF- κ B. *Cytokine Growth Factor Rev* 19, 157-165

1.6 Tumor suppressor genes

1.6.1 Phosphatase and tensin homolog (PTEN)

Besides inflammation and a permanent activation of the I κ B/IKK2 axis, another component assumed to play a role in prostate cancer development is phosphatase and tensin homologue PTEN. PTEN mutations or deletions are found in 30% of primary prostate cancers¹² and in 63% of metastatic prostate tissue samples¹³ demonstrating that the tumor suppressor gene PTEN is a quite frequent factor involved in prostate cancer development and progression. It is documented that in mouse models PTEN deficiency results in transformation of prostate tissue

depending on PTEN activity¹⁴. Monoallelic deletions of PTEN lead to low grade PIN late in mouse life whereas full deletions lead to high grade PIN with ability to form invasive and possibly metastasizing carcinomas at a late age¹⁵.

1.6.1.1 Molecular biological background of PTEN

The main function of PTEN is as a phosphatase causing the dephosphorylation of phosphatidylinositol 3,4,5-triphosphate (PIP3) resulting in the biphosphate product phosphatidylinositol 4,5-biphosphate (PIP2) and thereby negatively regulating the PI3K/AKT pathway¹⁶. Loss of PTEN results in accumulation of PIP3 and activation of its downstream effectors such as the serine/threonine kinase AKT that is responsible for cell cycle progression and cell survival¹⁷.

1.7 Factors that promote invasive tumor formation

1.7.1 Basal cell layer

It is documented that malignant glands in prostate adenocarcinoma lack the basal cell layer when acquiring an invasive character. Therefore antibodies targeting the basal cell marker p63 are quite frequently used for detection of loss of basal cell layer¹⁸.

1.7.2 Smooth muscle cells

Smooth Muscle Actin (SMA) and Smooth Muscle Myosin Heavy Chain (MYH11), the main components in smooth muscle cells, are used as differentiation markers in prostate cancer. It is known that there is a reduction of smooth muscle correlating with the histological grade of prostate tumors and that this tissue is frequently lost in malignant tumors^{15,19}.

1.7.3 Nkx3.1 and Androgen Receptor

Nkx3.1 is known as a homeodomain transcription factor that functions as a prostate specific tumor suppressor. In mouse models with bacterial prostatitis it was shown that the Nkx3.1 level was profoundly reduced in infected prostate lobes²⁰. Loss of Nkx3.1 leads to epithelial hyperplasia and dysplasia that worsens with age²⁰. These data correlate with findings from human prostate cancer²¹. Moreover it is hypothesized that diminished Nkx3.1 could lead to an abnormal expression of antioxidant and peroxidant enzymes leading to an increase in DNA damage that consequently promotes neoplastic transformation²². Interestingly it was shown that the downregulation of Nkx3.1 correlates with the reduction of the androgen receptor (AR) that is assumed to regulate Nkx3.1²⁰. This correlation gives an impression of how the Nkx3.1 level could be controlled.

1.7.4 Spink3

Spink3, homolog of human Spink1 gene, has serin protease inhibitor function and is documented to be a biomarker upregulated in a subset of aggressive ETS (E-twenty six) negative prostate cancers and assumed to be involved in invasiveness of prostate tumors²³.

1.8 Other proteins known to be involved to prostate carcinogenesis

1.8.1 SFRPI/ IV

Secreted frizzled related proteins are known to be an antagonists of Wnt signalling, a pathway that is involved in regulation of cell proliferation, differentiation, apoptosis and negatively regulates bone formation^{24,25}. In further research it was shown that

SFRPI treatment of prostate cells or tissue leads to increased proliferation, decreased apoptosis, and decreased signalling through the Wnt/beta-catenin pathway in vitro and increased proliferation in vivo²⁶. Further it was revealed that in vitro overexpression of SFRP4 is linked to a decrease in cell proliferation and invasiveness²⁴.

1.8.2 Hepsin

Another gene that is documented to be overexpressed in prostate cancer is the trypsin-like serine protease Hepsin²⁷. It was reported that Hepsin overexpression in a mouse model of nonmetastasizing prostate cancer causes disorganization of the basement membrane and promotes primary prostate cancer progression and metastasis²⁸. But what cellular and molecular mechanisms are involved therein is still unclear and warrants further investigation.

1.9 Aim of the study

As mentioned above prostate cancer is the most common cancer type in men all over the world. Although this disease is responsible for 10% of death in male population possible causes and progression are rather unclear. It has been reported that inflammation is a causative event in 20% of human cancers and there is a growing amount of evidence that suggests that inflammation and inflammatory signalling are involved in the tumorigenic process of prostate cancer.

In this study we wanted to demonstrate this link using a genetic model. This model harbours a heterozygote deletion of the PTEN gene and expresses a constitutively active IKK2 kinase. Probasin–Cre mice expressing CRE recombinase gene under the control of a prostate specific promoter were first crossed with mice harbouring an inducible IKK2 gene with an upstream loxP-flanked (“floxed”) stop cassette to

investigate a permanent inflammatory stimulation in the prostate tissue. Second Probasin-Cre mice were crossed with mice carrying a floxed version of the tumor suppressor PTEN that is known to be one of the most mutated genes in prostate carcinogenesis and documented to cause the late precursor lesion PIN (prostate intraepithelial neoplasia). Third we wanted to investigate what consequences the combination of both factors might have in the progression of prostate carcinogenesis. For this reason we established double transgenic mice harbouring Probasin-Cre background, IKK2 gene with an upstream floxed stop cassette and the floxed PTEN gene.

Starting with chip analysis to get an overview of up and down regulated genes we used real-time RT PCR, immunoblot analysis and insitu hybridisation technique to investigate how inflammation could lead to prostate cancer and which molecular processes are involved.

2 Methods

2.1 Mice

Mouse breeding was performed by the project manager Andreas Birbach creating the different mouse models for the project described below.

2.1.1 Probasin–Cre mice

Probasin–Cre (PB-Cre4) (Fig.4) mice were obtained from the NCI Fredrick mouse repository and express the CRE recombinase specifically in the prostate epithelium. The Cre gen is under the control of the composite promoter ARR2PB which is a derivative of the rat prostate-specific probasin (PB) promoter²⁹.



Figure 4: CRE recombinase gene under the control of the ARR2PB promotor expressed in Probasin-Cre mice.

2.1.2 Mice with a floxed PTEN allele

Mice with a floxed PTEN allele¹⁵ (Fig.5) were obtained from Prof. Tak Mak (Ontario Cancer Institute, Toronto, Canada) via Dr. Gernot Schabbauer (Department of Vascular Biology, Medical University of Vienna). Mice with a floxed PTEN allele will express less PTEN after crossing with Probasin-Cre mice using the well-described CRE-Lox system.



Figure 5: Transgenic gene cassette for CRE-mediated deletion of PTEN

2.1.3 R26StopIIKK2 mice

R26StopIIKK2 (Fig.6) mice were obtained from Dr. Marc Schmidt-Suppran (Max Planck institute for Biochemistry, Martinsried, Germany). This strain constitutes a mouse model that expresses a constitutively active IKK2 kinase upon excision of floxed Stop cassette ³⁰.



Figure 6: Transgenic gene cassette for CRE-mediated expression of IKK2 gene

2.2 Genotype of mouse models for this project

Mice were bred on a C57BL/6 background, yielding different genotypes we used for our prostate cancer project (Fig.7). In this study the shortcut PE stands for prostate epithelium, meaning that the transgene is only expressed in epithelial cells of the prostate.

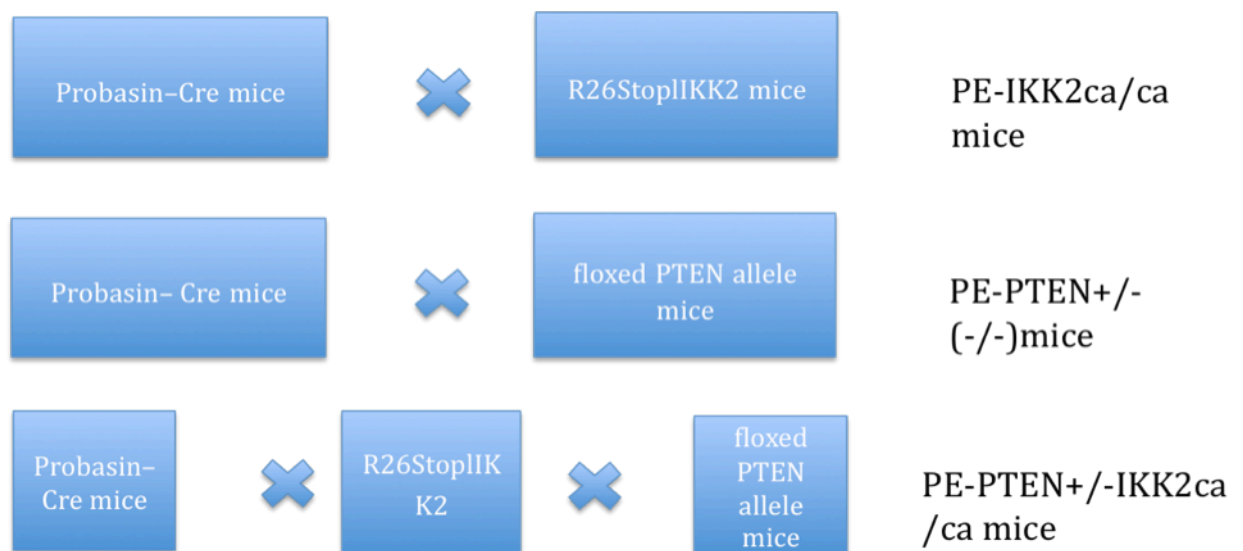


Figure 7: Genetic background of IKK2ca/ca, PE-PTEN+/-(-/-) and PE-PTEN+/--IKK2ca/ca mice

2.3 Genomic DNA isolation

Solutions and Buffer

Lysis buffer

For verifying the genotype of transgenic mice genomic DNA was isolated from tail biopsies. In samples with tail biopsies 500 μ l lysis buffer, 3 μ l Proteinase K (20 mg/ml) was added and they were incubated at 58°C over night. After adding 200 μ l 5M NaCl and mixing, samples were centrifuged at 16000g at room temperature for 5 min. 650 μ l of the supernatants were transferred to a new sample and 650 μ l Isopropanol (concentrated) was added to each sample before centrifugation at 16000g for 30min. The supernatants were discarded and 1ml of ice cold 70% ethanol was added and samples were again centrifuged at 16000g at 4°C. The pellets were dried for 10 min at room temperature and resuspended in 100 μ l of 10 mM Tris pH8.

2.4 Genotyping

2.4.1 Polymerase chain reaction (PCR)

Solutions and Buffer

Taq Polymerase (5 units/ microliter) and 10x Buffer (Peq Lab)

For genotyping we added 2.5 μ l buffer, 0,5 μ l dNTPs (10 mM), 0,5 μ l forward and reverse primer (10 μ M), 0,5 μ l taqPolymerase and 20 μ l ddH₂O to 1 μ l template solution.

Gene (symbol) or amplicon	Primer sequences (sense and antisense, 5'-> 3')
CRE	5'-CGGTCGATGCAACGAGTGATGAGG-3' 5'-CCAGAGACGGAAATCCAT CGCTCG-3'
PTEN	5'-CTCCTCTACTCCATTCTTCCC-3' 5'-ACTCCCACCAATGAACAAAC-3'
rNeo/IKK2	5'-GAGCTGCAGTGGAGTAGGCG-3' 5'-GCCTTCTTGACGAGTTCTTC-3'
ROSA	5'-CCAGATGACTACCTATCCTC-3' 5'-GAGCTGCAGTGGAGTAGGCG-3'

Gene (symbol) or amplicon	Primer sequences (sense and antisense, 5'-> 3')
PTENex	5'-ACTCCCACCAATGAACAAAC-3' 5'-GGTAGACTAGTCGGTACTCAG-3'

Table 1: Primer sequences used for Polymerase chain reaction

Step	Temperature/Time	Cycle
	95°C 10min	1
Denaturation	95°C 30 sec	40
Annealing	55°C 30 sec	
Elongation	72°C 1 min	
Final Elongation	72°C 5 min	1

Table 2: Polymerase chain reaction (PCR) cycle periods and temperatures

2.5 RNA extraction

2.5.1 RNA isolation kit

Solutions and Buffer

RNA isolation kit (Qiagen, Hilden)

Total RNA kit (Peqlab, Erlangen)

For RNA extraction prostate tissue/cells was homogenized in RNA later using a Polytron homogenizer and purified on RNA binding columns using RNA isolation kit RNeasy or Total RNA kit. Isolation instructions were used from respective manufacturers.

2.5.2 RNA isolation from cells with TRIZOL

Solutions and Buffer

Trizol: Carl Roth GmbH

Chloroform: Rotipuran® ≥ 99% p.a. (Carl Roth GmbH)

The media was removed and cells were washed with 1xPBS before adding 1ml TRIZOL per 0,1 -1g cells, transferred to samples and incubated for 5 minutes at 15-30°C. After adding 0,2ml of chloroform for each 1ml TRIZOL used in the beginning, the samples were vortexed for 15sec and incubated for 2-3 min at 15-30°C. Then the samples were centrifuged at no more than 12 000g for 15 min at 2-8°C to get an aquatic, an organic and a central protein phase. Aquatic phases, including RNA,

were transferred to new tubes, 0,5ml Isopropyl alcohol was added for each 1ml TRIZOL that was used in the beginning and incubated at 15-30°C for 10min. After incubation samples were centrifuged at no more than 12 000g for 10 min at 2-8°C. The supernatants were removed and the pellets were washed with 1ml 75% ethanol for each 1ml TRIZOL used in the beginning. Again the samples were centrifuged this time at no more than 7 500g for 5 min at 2-8°C. Afterwards the supernatants were removed. The pellets were dried for around 5-10min at room temperature and 10 μ l RNase free ddH₂O was added to each sample.

2.6 cDNA synthesis

Solutions and Buffer

RevertAid H Minus First standard cDNA synthesis kit (Fermentas, St. Leon-Rot)

2.7 Real-time RT-PCR

Solutions and Buffer

TaqPolymerase (5 units/ microliter) and 10x Buffer (Peqlab)

DMSO (Sigma)

SYBR green (Invitrogen)

For real-time RT-PCR we added 2,5 μ l buffer, 2,375 μ l DMSO (1:100 dilution), 0,125 μ l SYBR green, 0.5 μ l dNTPs (10mM), 0,5 μ l forward and reverse primer (10 μ M), 0,1 μ l taqPolymerase, 17,5 μ l ddH₂O to 1 μ l cDNA.

For amplification we used a StepOne real-time PCR cycler (Applied Biosystems, Vienna, Austria). HPRT was used as housekeeping gene.

Gene (symbol) or amplicon	Primer sequences (sense and antisense, 5'→3')
CXCL2	GCGCCCAGACAGAAGTCATAG AGCCTTGCCTTTGTTTCAGTATC
CXCL5	GAAAGCTAAGCGGAATGCAC GGGACAATGGTTTCCCTTTT
CXCL10	GCTCCTGCATCAGCACCAGC CTTGAACGACGACGACTTTGG
CXCL15	CCA TGG GTG AAG GCT ACT GT TCT CAG GTC TCC CAA ATG AAA
Cytokeratin18	CAGCCAGCGTCTATGCAGG CTTTCTCGGTCTGGATTCCAC
ICAM1	TGC GTT TTG GAG CTA GCG GAC CA CGA GGA CCA TAC AGC ACG TGC AG
TNF	TAGCCAGGAGGGAGAACAGA TTTTCTGGAGGGAGATGTGG
SFRP1	CAACGTGGGCTACAAGAAGAT GGCCAGTAGAAGCCGAAGAAC
SFRP4	AGAAGGTCCATACAGTGGGAAG GTTACTGCGACTGGTGCGA
Nkx3.1	ATGCTTAGGGTAGCGGAGC TGCGGATTGCCTGAGTGTC

Gene (symbol) or amplicon	Primer sequences (sense and antisense, 5'→3')
Spink3	ATG AAG GTG GCT GTC ATC TTT C TCA GCA AGG CCC ACC TTT TCG
Smooth muscle actin	GTCCCAGACATCAGGGAGTAA TCGGATACTTCAGCGTCAGGA
MYH11	AAGCTGCGGCTAGAGGTCA CCCTCCCTTTGATGGCTGAG
HPRT	CAA ATC AAA AGT CTG GGG ACG C GCT TGC TGG TGA AAA GGA CCT C

Table 3: List of oligonucleotids used for real-time RT-PCR

Step	Temperature/Time	Cycle
	95°C 10min	1
Denaturation	95°C 15 sec	40
Annealing	55°C 15 sec	
Elongation	72°C 1 min	
Final Elongation	72°C 5 min	1

Table 4: Real-time RT PCR cycle periods and temperatures

2.8 Tissue sectioning and immunostaining

2.8.1 Paraffin sections

Solutions and Buffer

Xylol substitute: Roticlear® (Carl Roth)

Antigen Retrieval Solution: Target Retrieval Solution (Dako)

10xPBS:

80g NaCl

2g KCl

14,4g Na₂NPO₄

2,4g KH₂PO₄

pH 7,4

→ 1000ml with ddH₂O

For paraffin sections, harvested tissue was fixed over night in 4% paraformaldehyde.

After being dehydrated tissues were paraffin-embedded in a small tissue blocks, cut in 2 μ m sections using a Leica microtome and were collected on SuperFrost Plus slides (Thermo Scientific, Braunschweig, Germany).

Paraffin sections were first deparaffinised two times for 20 minutes in xylol substitute than rehydrated in descending ethanol solutions (100%, 96%, 80%, 70%, 50%) for 30 seconds each. Afterwards the slides were incubated for 5 minutes in ddH₂O and then the antigen retrieval was started using a steamer for simmering the slides in antigen retrieval solution for 40min. Slides were cooled down and washed with 1xPBS.

2.8.2 Cryosections

For frozen sections, harvested tissue was directly frozen in isopentane/liquid nitrogen and frozen until cutting. Cryosections were cut on a cryostat in 8 μ m sections, dried at room temperature for 20min or longer and processed or stored at -80°C (-20°C if not possible otherwise) until needed. Cryosections were incubated in -20°C cold acetone at room temperature for another 3min and washed with 1xPBS for 5min.

2.9 Immunostaining

Solutions and Buffer

Goat serum:

Avidin/ Biotin blocking kit (Vector laboratories, Burlingame, CA)

DAB tablets (Sigma)

Hemalaun (Mayers Hemalaun)

In Situ Cell Death Detection Kit (Roche, Vienna, Austria)

Tissues were encircled by a DakoPen and incubated in 5% goat blocking serum in 1xPBS for 1hr. Then slides were incubated in a wet chamber with first antibody over night at 4°C in respective dilutions made in 5% goat serum. Next morning they were washed 3 times for 5min in 1xPBS, incubated with secondary antibody in respective dilutions made in 5% goat serum for 30min to 1hr and washed another 3 times with 1xPBS. In case of using biotinylated antibodies sections were additionally incubated with Avidin/ Biotin blocking kit before incubation with primary antibody. Slides were treated with fluorescent secondary antibodies, protected from sunlight and mounted in PBS/glycerol until observation. Slides treated with antibodies linked to HRP were observed under a light microscope using DAB Tablets. Enzymatic reaction was stopped by ddH₂O after 7min and slides were additionally stained with Hemalaun for 3- 5min. Antibodies were used as described below. Apoptotic cells were assessed using the TUNEL method and the In Situ Cell Death Detection Kit.

Antibody / Antigen	Source (catalog # or equivalent)	Use, dilution
F4/80	eBioscience 13-4801	IF-F 1:200
CD11b	eBioscience 13-0112	IF-F 1:200
Ki67	Neomarkers RM-9106-S1	IF-P 1:200
Nkx3-1	Santa Cruz sc-15022	IB 1:400
Smooth muscle actin	Sigma C6198	IF-P 1:400 IF-F 1:400
Beta-Tubulin	Santa Cruz sc-9104	IB 1:400
A555 goat anti rabbit	Invitrogen A-21428	IF-P, IF-F 1:2000
Biotin anti rabbit	Vector Laboratories BA-1000	IHC-P 1:400
Biotin anti goat	Vector Laboratories BA-9500	IHC-P 1:400
Biotin anti mouse	Vector Laboratories BA-9200	IHC-P 1:400
Streptavidin, Alexa Fluor 555-conjugate	Invitrogen S32355	IF-F 1:500
Abbreviations: IF immunofluorescence, IHC, immunohistochemistry F: frozen sections, P: paraffin sections, IB: Immunoblot,		

Table 5: List of antibodies used in this study

2.10 Immunoblot analysis

2.10.1 Protein Isolation from prostate tissue

Solutions and Buffer

5x Loading dye:

0,5ml glycerol

0,5ml 20% SDS

0,11ml Tris HCl pH 6.8

1mg bromophenol blue

2x dye was produced by dilution from 5x dye, and 2% mercaptoethanol was added before use.

For protein isolation from prostate tissue, 800µl of ice-cold acetone was added to 200µl of flow-through from RNA isolation. This solution was incubated for 10 min at -20°C and centrifuged for another 10 min for 16000g at 4°C. To the protein pellets 100µl of 75% ethanol was added and samples were centrifuged for 5 min at full speed (4°C). Finally the pellet was dried for 10min at room temperature and resuspended in 2x protein loading dye and heated at 95°C to load the suspension on the SDS page.

2.10.2 SDS PAGE

Solutions and Buffer

Running Buffer (5x)

15.1g Tris

94g Glycin

→900ml with ddH₂O

50ml 10% SDS

→1000ml with ddH₂O

Resolving gel/I (10ml/15%)

4ml ddH₂O

3,3ml 30% acrylamide mix

2,5 ml 1,5 M Tris pH 8,8

0,1ml 10% SDS

0,1ml 10% ammonium persulfate

0,004ml TEMED

Resolving gel/II (10ml/12%)

4,3ml ddH₂O
3ml 30% acrylamide mix
2,5ml 1,5 M Tris pH 8,8
0,1ml 10% SDS
0,1ml 10% ammonium persulfate
0,004ml TEMED

Stacking gel (4ml)

2,7ml ddH₂O
0,67 30% acrylamide mix
0,5 ml 1 M Tris pH 6,8
0,04ml 10% SDS
0,04ml 10% ammonium persulfate
0,004ml TEMED

10x TBS

24,4g Tris
80gNaCl

TBST

1xTBS
0,1% Tween

Isolated proteins were loaded on 12% or 15% SDS gels and were dissolved at 180V for 1 1/2hr.

2.10.3 Western blotting**Solutions and Buffer****Transfer Buffer**

3g Tris
14,4g Glycin

→800ml with ddH₂O
200ml pure methanol

For blotting we used PVDF membrane (6,2x 7,6cm) and it was performed at room temperature at 0,16- 0,2 Ampere for 90min.

2.10.4 Ponceau S Staining

Solutions and Buffer

Ponceau S solution

0,5g Ponceau S

1ml acetic acid

99ml ddH₂O

Membrane was incubated for 3min in Ponceau S Staining solution and afterwards washed 3-4 times with ddH₂O.

2.10.5 Antibody treatment

Solutions and Buffer

Pierce SuperSignal West Femto (Pierce)

Blots were blocked using either in 5% milk powder diluted 1xTBST or 1% BSA diluted in 1xTBST and incubated with primary antibody diluted in blocking solution over night. Next morning blots were washed 3 times 5min in 1xTBST, then incubated with secondary antibody diluted in equal solution for 30min for 1hr and washed again 3 times 5min with 1xTBST. For developing the blots we used Pierce SuperSignal West Femto.

3 Results

3.1 mRNA expression analysis

To reveal the molecular biological link between inflammation and cancer Andreas Birbach started the project with Microarray analysis to get an overview of up and down regulated genes located to the prostate epithelium in PE-PTEN^{+/-}-IKK2ca/ca vs. PTEN^{+/-} mice and continued with real-time RT-PCR analysis to confirm differences in gene expression to explain phenotypic differences of PE-PTEN^{+/-}-IKK2ca/ca, PE-PTEN^{+/-}, wildtype and additionally IKK2ca/ca mouse prostates (Fig.8).

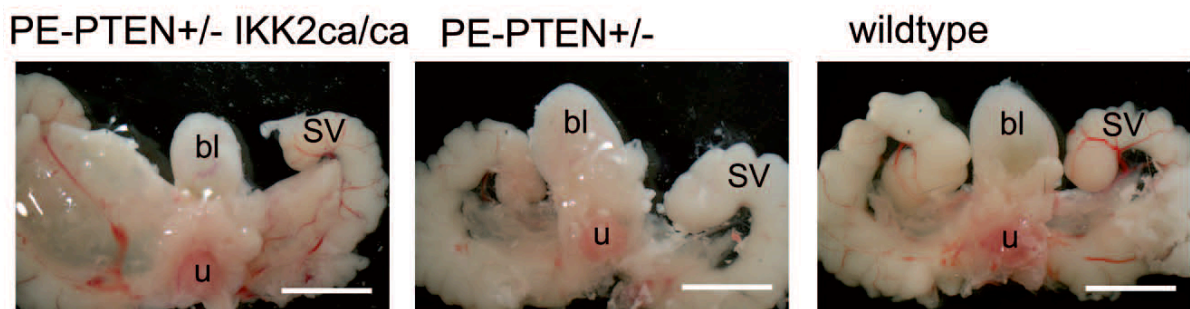


Figure 8: Stereomicroscopical images of PE-PTEN^{+/-}-IKK2ca/ca, PE-PTEN^{+/-} and wildtype littermates showing the structures surrounding the urethra (u), bladder (bl) and seminal vesicles (SV) from 12months- old mice. Prostate lobes are seen between the noted structures³¹.

3.1.1 Chip Analysis

Microarray experiment revealed that constitutive active IKK2 gene on a monoallelic PTEN background (PE-PTEN^{+/-}-IKK2ca/ca) leads to a much higher upregulation of factors known to be linked to inflammation and cancer development as it was seen in PTEN^{+/-} mice (PE-PTEN^{+/-}). We summarized the most upregulated and downregulated genes and related them to functional groups to get an idea in which direction we should go in further experiments.

The gene expression profile of double transgenic mice showed a quite intense upregulation of factors associated with immune cell attraction, activation and stimulation. Interestingly some factors, known to be linked to invasiveness, such as smooth muscle cell markers, serine protease inhibitors and some prostate specific genes are downregulated. All together this experiment gave us a starting point of the transgenic mouse models we wanted to use in our prostate cancer project.

3.1.2 Real-time RT-PCR

Using real-time RT PCR we wanted to know if the Flag- tagged KK2 gene expression, following excision of the Stop cassette in Rosa26-FIStop-IKK2ca/ca mice, is expressed in prostate tissue. Furthermore we wanted to know if there is a difference in the expression level of the transgene in lateral (LP), anterior (AP), dorsal (DP) and ventral prostate lobes (VP).

Further we performed expression analysis on PE-PTEN^{+/-} IKK2ca/ca, PE-PTEN^{+/-}, PE-IKK2ca/ca and wildtype lateral prostates to compare again the influence of the different genotypes on the expression level of transgenic mice, to get an idea of molecular link between inflammation and cancer.

3.1.2.1 Expression level of the constitutive active IKK2 gene

On mRNA level it was shown by Andreas Birbach that the transgene is expressed in the different lobes and that the transgene expression was highest in the lateral prostate (LP), followed by the anterior (AP) and dorsal prostate (DP) with lowest expression level in the ventral prostate (VP)

3.1.2.2 Upregulated genes in transgenic prostate tissue

3.1.2.2.1 Chemokines

Consistent with Chip Analysis the mRNA level of the chemokines CXCL5, CXCL2, CXCL15 and CXCL10 was increased in PE-PTEN^{+/-} IKK2^{ca/ca} lateral prostates in contrast to PTEN^{+/-}, IKK2^{ca/ca} and wildtype (Fig.9-12). These genes are known to be upregulated in inflammatory processes, attracting immune cells to the affected tissue. Particularly neutrophil granulocytes and monocytes/macrophages interact with these molecules to reach the inflamed tissue.

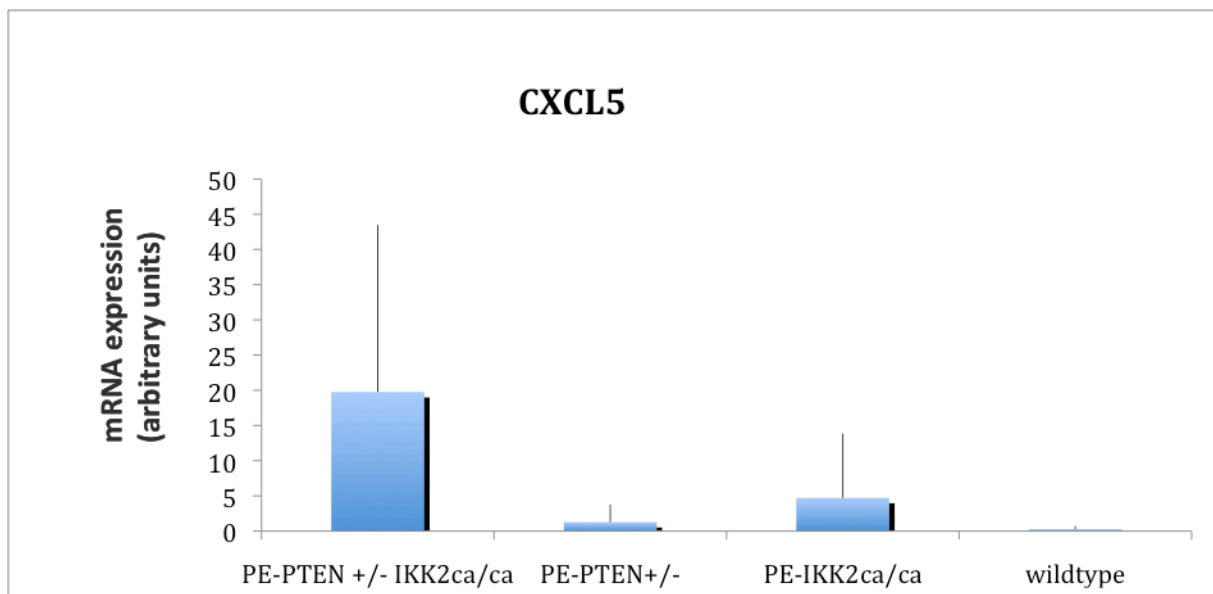


Figure 9: mRNA expression level of inflammatory cytokine CXCL5 in lateral prostate tissue of transgenic mice and wildtype mice.

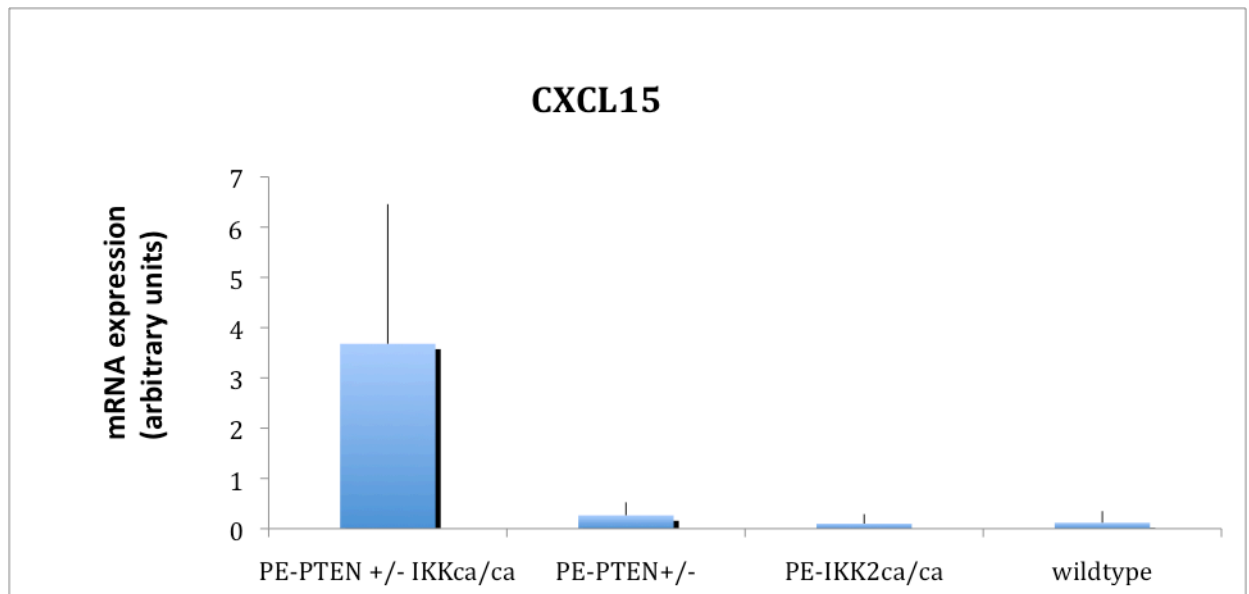


Figure 10: mRNA expression level of inflammatory cytokine CXCL15 in lateral prostate tissue of transgenic mice and wildtype mice.

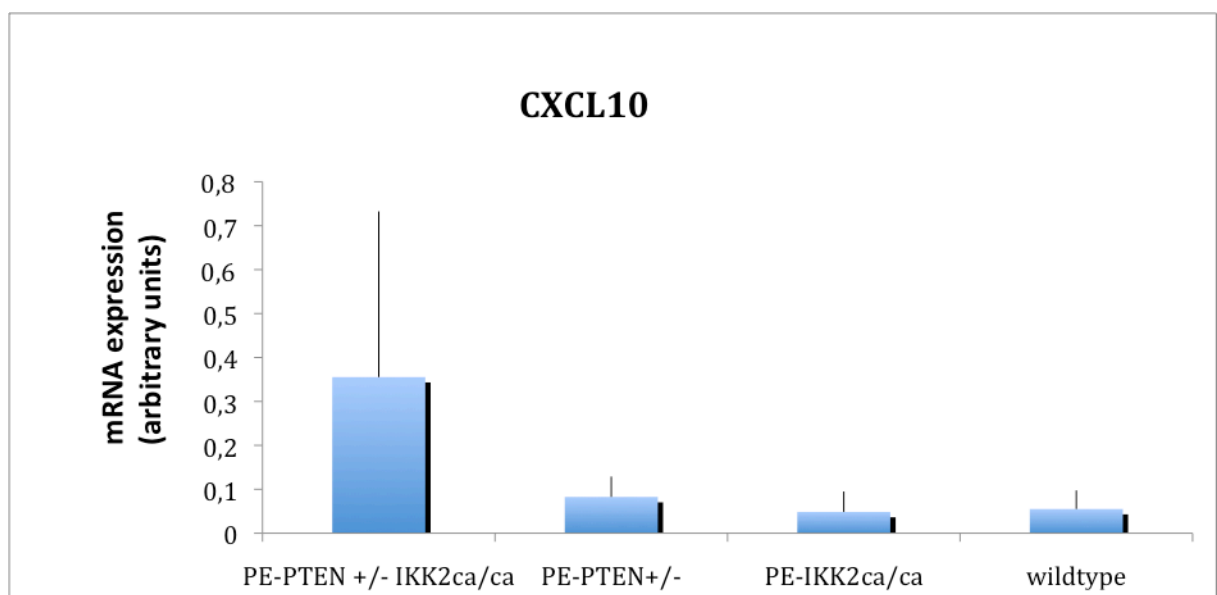


Figure 11: mRNA expression level of inflammatory cytokine CXCL10 in lateral prostate tissue of transgenic mice and wildtype mice.

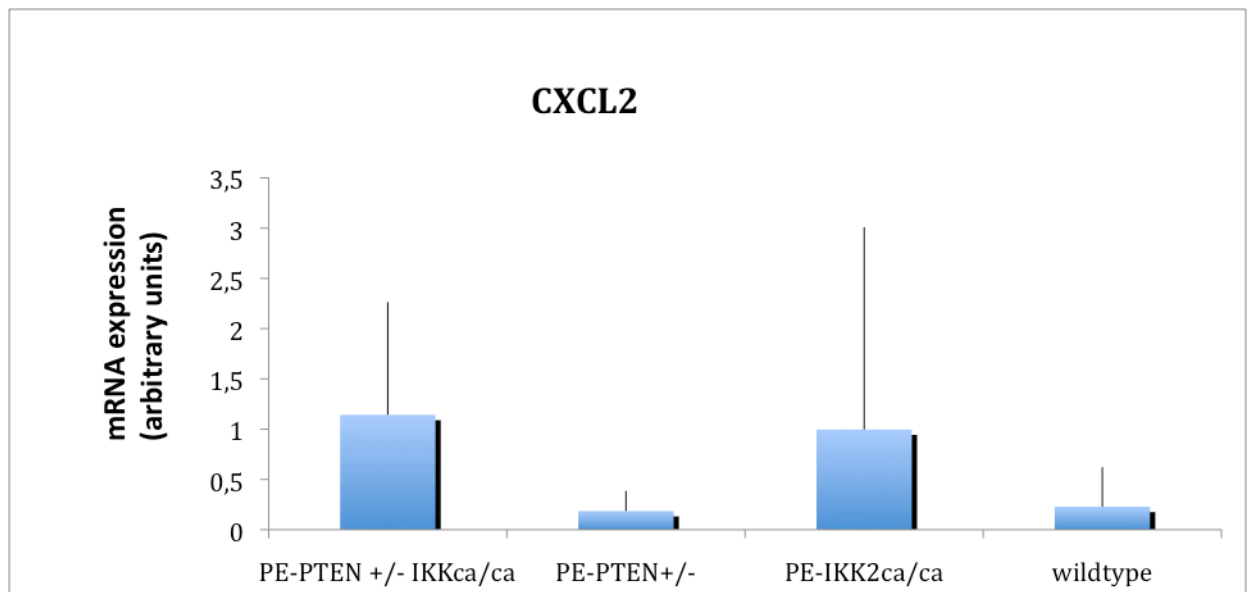


Figure 12: mRNA expression level of inflammatory cytokine CXCL2 in lateral prostate tissue of transgenic mice and wildtype mice.

3.1.2.2.2 Cytokines and NF κ B target genes

Another group of overexpressed genes in PE-PTEN+/-IKK2ca/ca mice are cytokines, particularly TNF α (Fig.13) an NF κ B target molecule indicating an inflammatory process going on in the prostate tissue. Another overexpressed molecule among NF κ B target genes is the intercellular adhesion molecule ICAM1 (Fig.14).

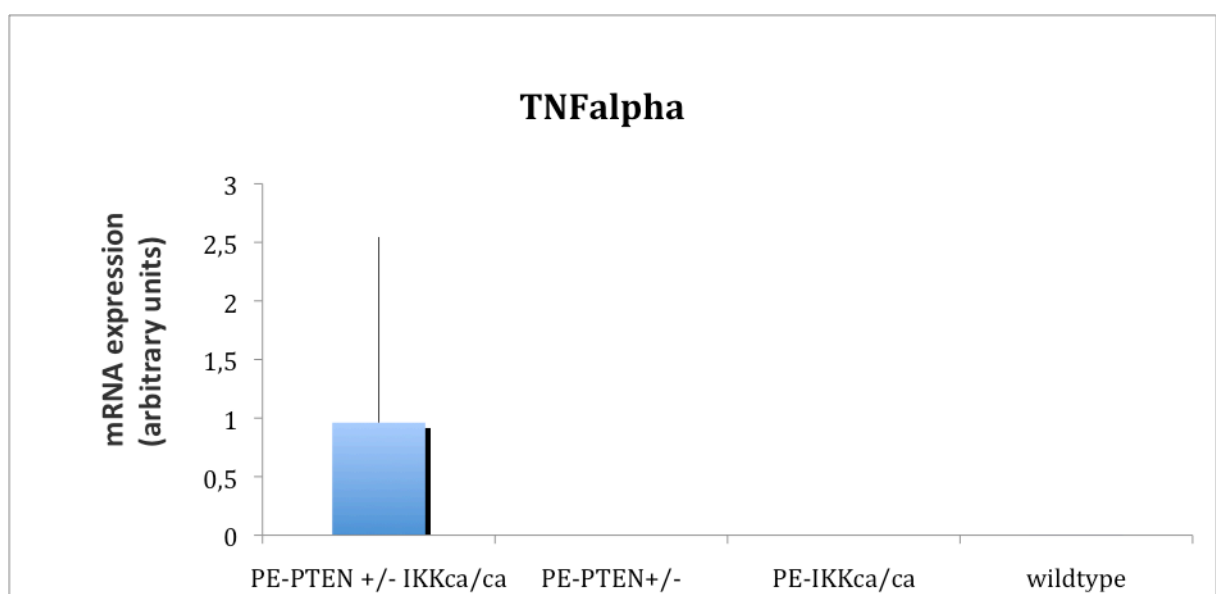


Figure 13: mRNA expression level of inflammatory chemokine TNF α in lateral prostate tissue of transgenic mice and wildtype mice.

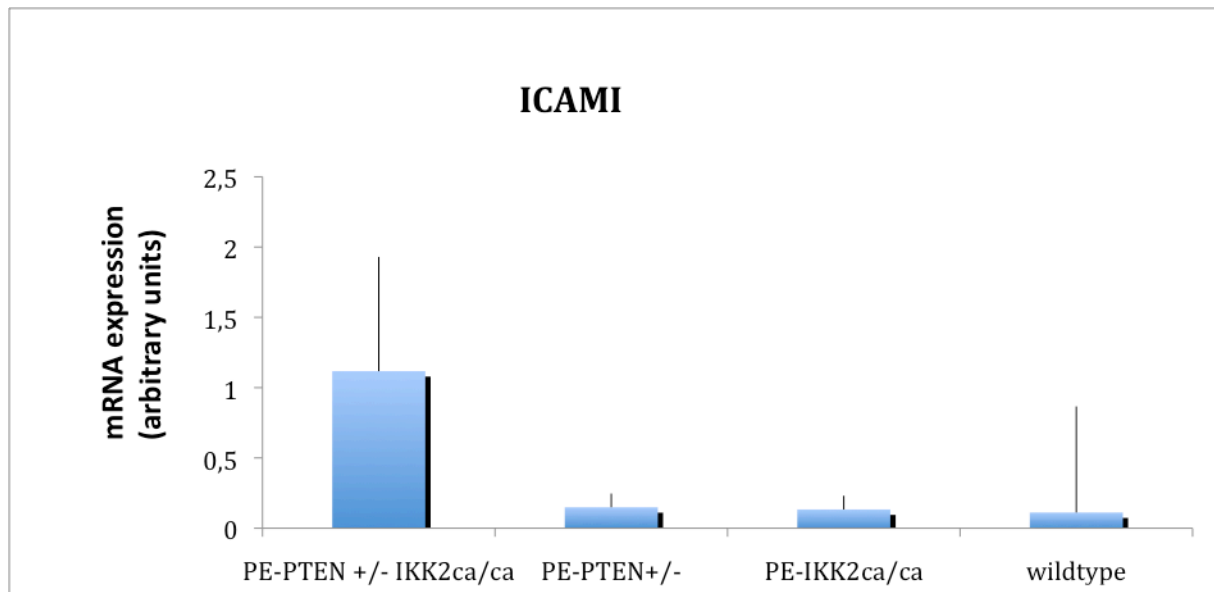


Figure 14 mRNA expression level of intercellular adhesion molecule ICAM I in lateral prostate tissue of transgenic and wildtype mice.

3.1.2.2.3 Secreted frizzled-related proteins

Additionally we could confirm the upregulation of the secreted frizzle related proteins 1 and 4 (SFRP1 and SFRP4) (Fig.15, 16) in PE-PTEN+/-IKK2ca/ca mice. SFRP1 has been described as a candidate gene expressed by the stroma to modulate the epithelium in prostate cancer progression²⁶, showing that the activated stroma in our intraepithelial tumors bears resemblance to human prostate cancer stroma.

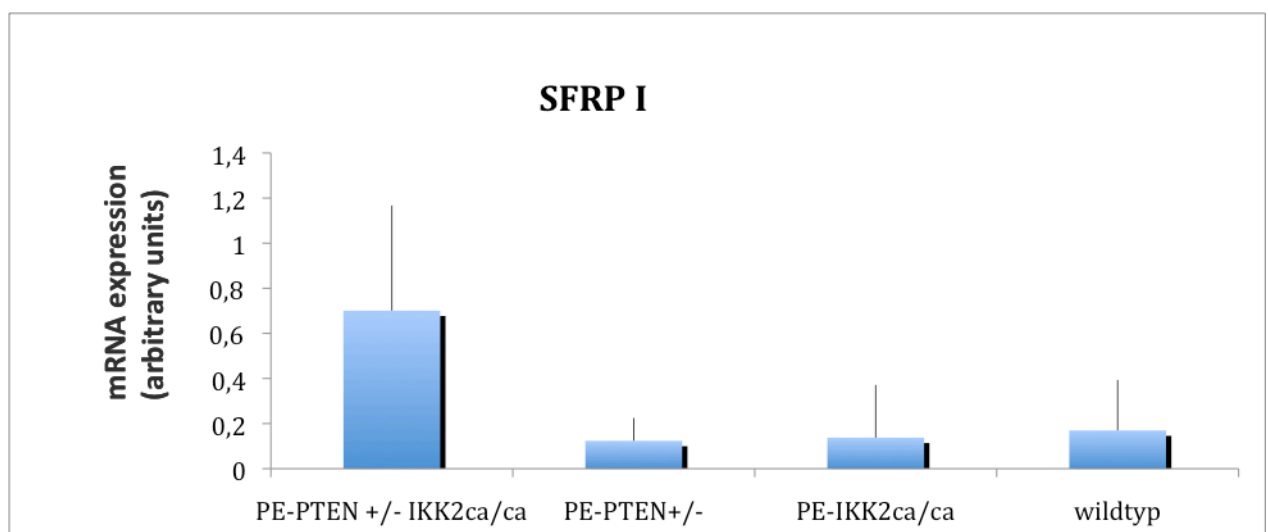


Figure 15 mRNA expression level of SFRP I in lateral prostate tissue of transgenic and wildtype mice

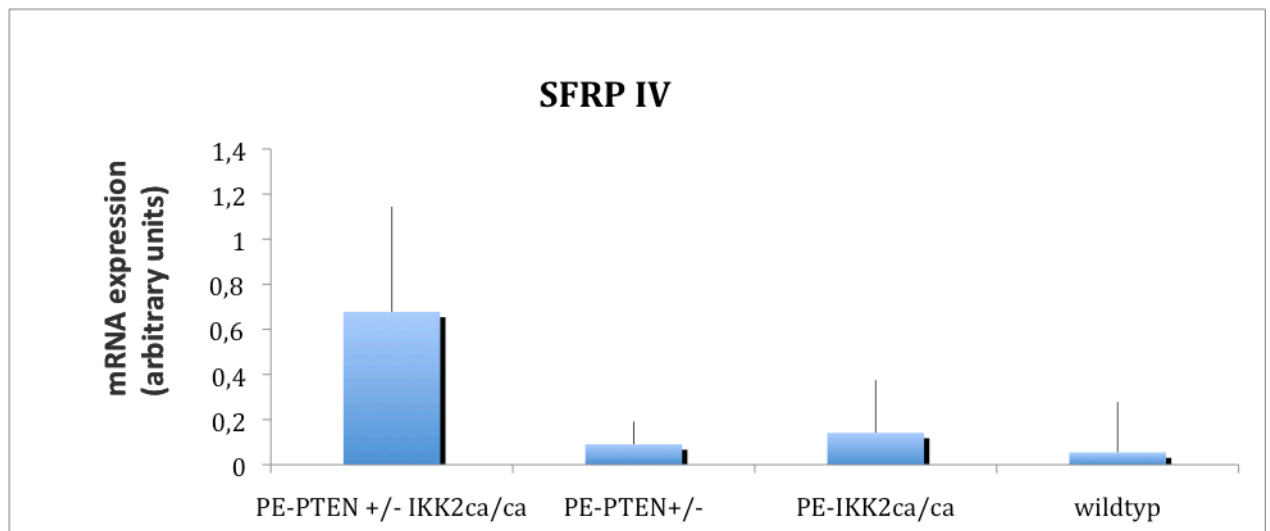


Figure 16: mRNA expression level of SFRP IV in lateral prostate tissue of transgenic and wildtype mice

3.1.2.2.4 Others

Another upregulated gene is the trypsin-like serine protease Hepsin known to be overexpressed in many human prostate cancers ³³(Fig.17)

In addition the neutrophil marker Lipocalin2 could be verified as an upregulated gene in the transgenic prostate tissue (Fig.18).

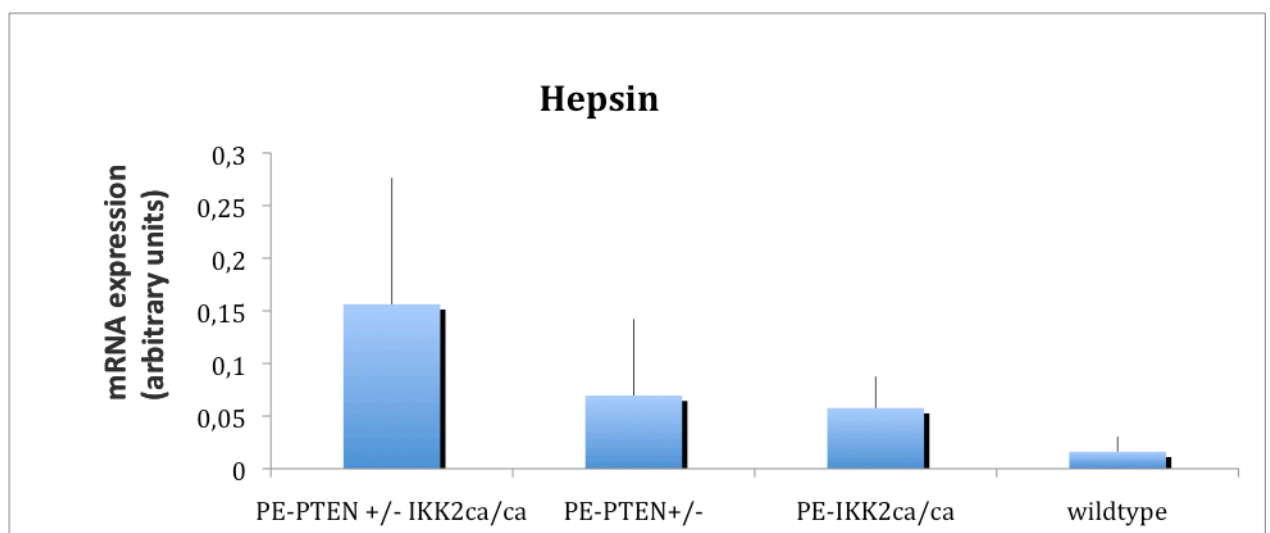


Figure 17: mRNA expression level of trypsin-like serine protease Hepsin in lateral prostate tissue of transgenic and wildtype mice

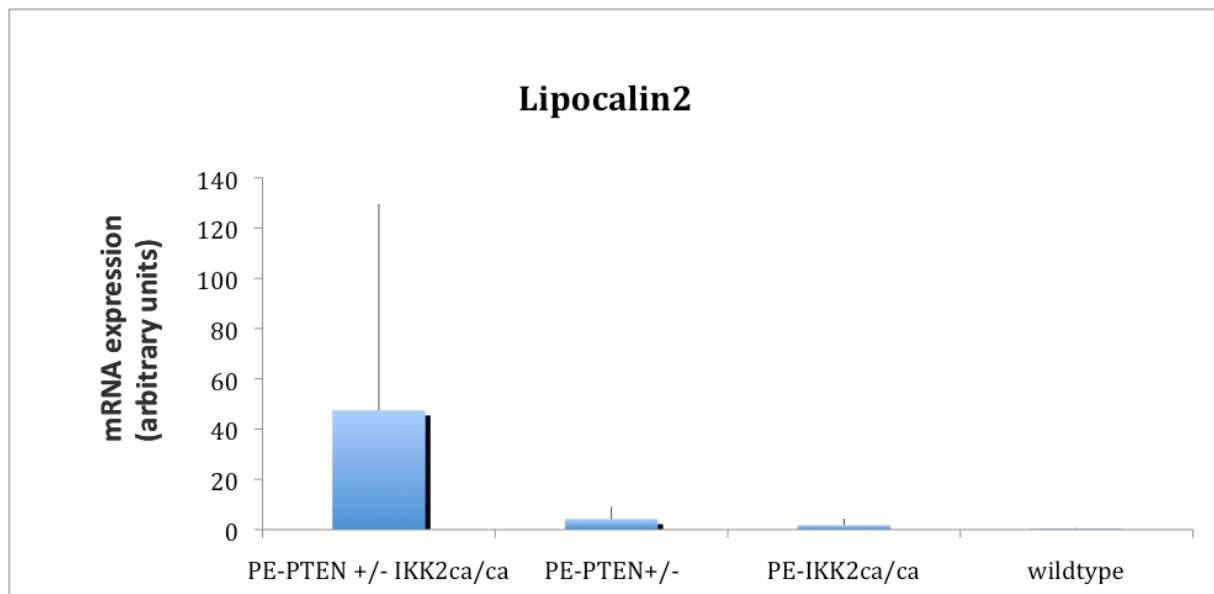


Figure 18: mRNA expression level of trypsin-like serine protease Lipocalin2 in lateral prostate tissue of transgenic and wildtype mice

3.1.2.3 Downregulated genes in transgenic prostate tissue

3.1.2.3.1 Genes linked to Invasiveness

3.1.2.3.1.1 Smooth muscle cell markers

In line with the results from Chip Analysis the most downregulated genes are smooth cell markers Myosin Heavy Chain 11 (MYH11) (Fig.19) and Smooth Muscle Actin (SMA) (Fig.20), loss of these proteins have been used as indicators for invasive carcinoma formation^{15,34}. Furthermore microarray data revealed that Calponin 1 is downregulated, a protein that is known to regulate the actin/myosin interaction in smooth-muscle cells³⁵.

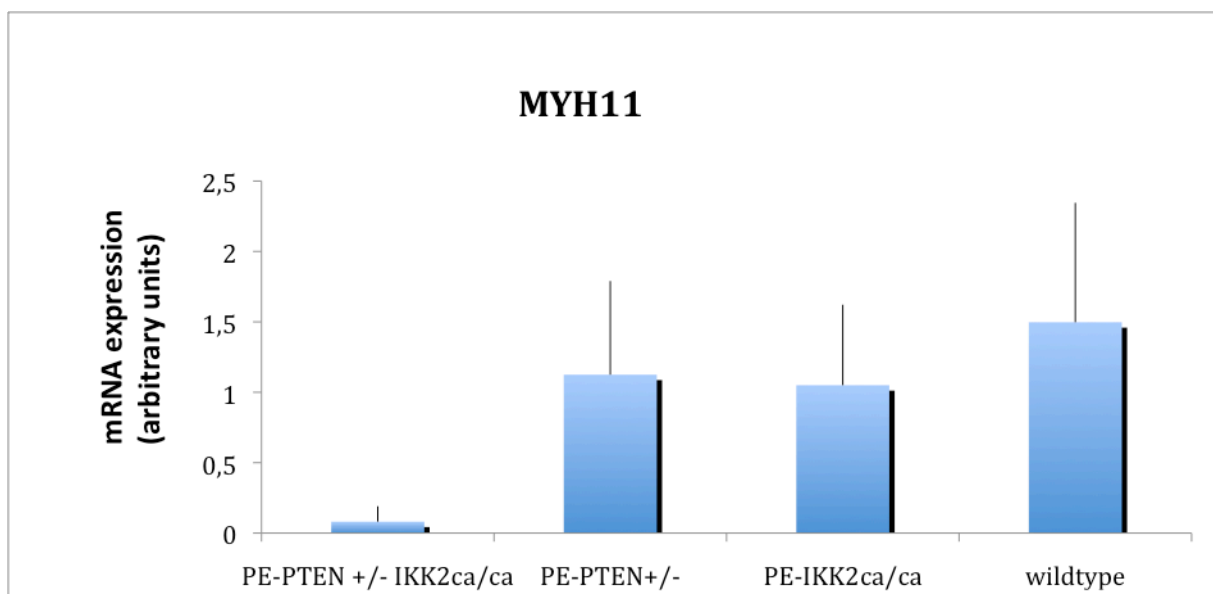


Figure 19: mRNA expression level of smooth muscle cell marker MYH11 in lateral prostate tissue of transgenic and wildtype mice

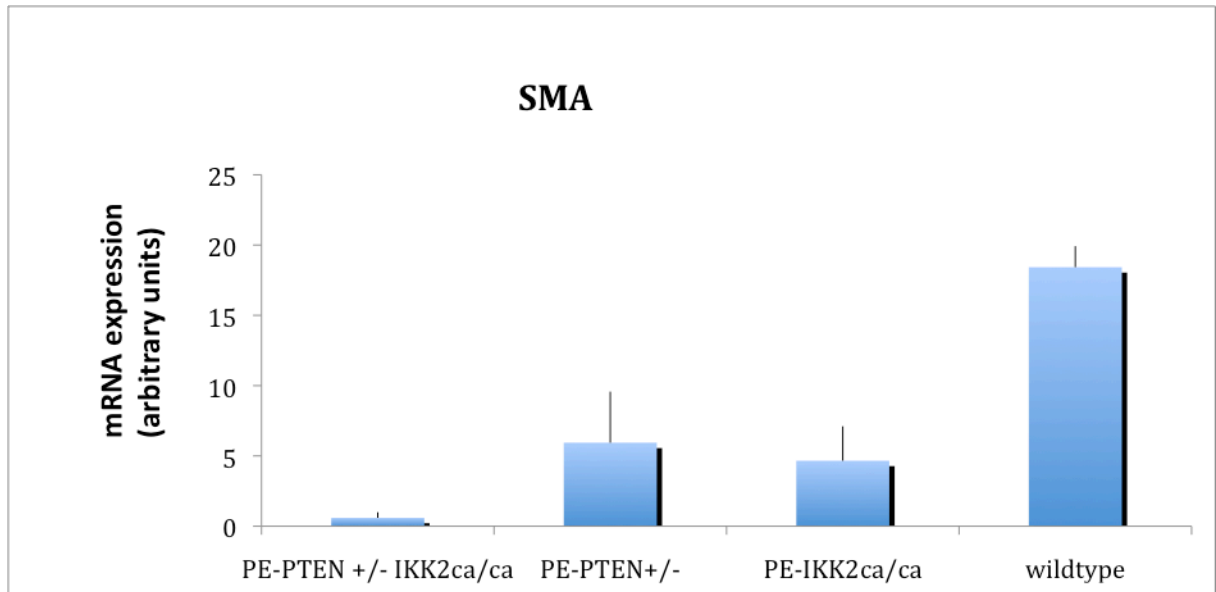


Figure 20: mRNA expression level of smooth muscle cell marker SMA in lateral prostate tissue of transgenic and wildtype mice

3.1.2.3.1.2 Serin protease inhibitors

Chip analysis showed that a group of serin protease inhibitors is downregulated in PE-PTEN+/-IKK2ca/ca mice, most of all Serine Protease Inhibitor 3 (Spink3). Via real-time RT-PCR we could confirm this result (Fig.21). Spink3 is documented to be a biomarker upregulated in a subset of aggressive ETS (E-twenty six) negative prostate cancers²³ and could be an indication for a lack of invasiveness in our mouse model.

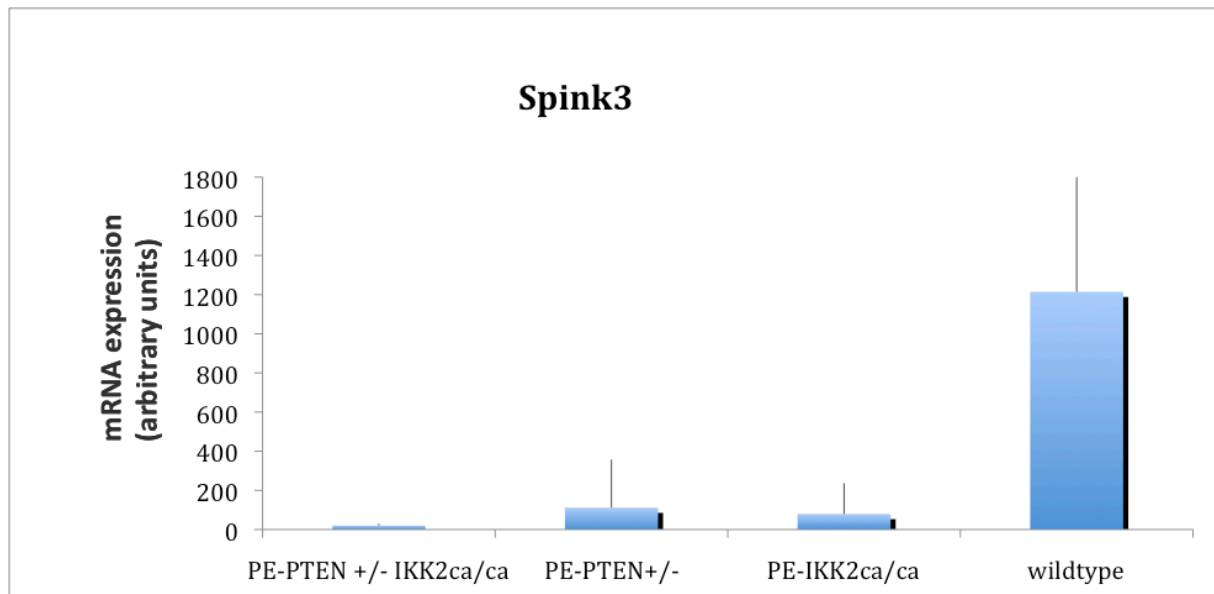


Figure 21: mRNA expression level of Serin Protease Inhibitor Spink3 in lateral prostate tissue of transgenic and wildtype mice

3.1.2.3.1.3 Prostate specific genes

Verifying Chip Analysis we could show that the transcription factor NK3 (Nkx3.1) is downregulated in PE-PTEN+/-IKK2ca/ca mice (Fig.22). Nkx3.1 is a protein frequently lost in human prostate cancer and its loss in mouse models leads to hyperplasia and dysplasia that worsens with age^{20,36,37}

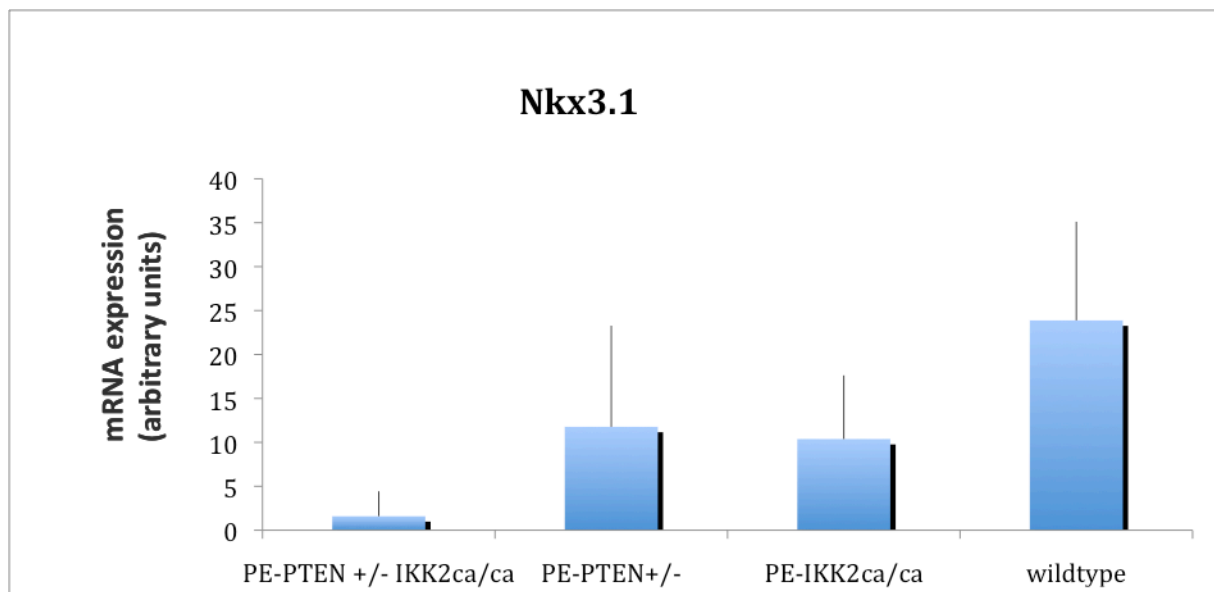


Figure 22: mRNA expression level of the transcription factor Nkx3.1 in lateral prostate tissue of transgenic and wildtype mice

3.1.2.4 Variation in Genexpression of transgenic Epithelial and Stromal cells

For this experiments stromal and epithelial cells of PE-PTEN^{+/-}-IKK2^{ca/ca} transgenic mice and wildtype littermates were established by Andreas Birbach by explant cultures (Fig.23). These cell lines were used to check whether the Flag-IKK2 transgene is expressed in the transgenic epithelium and if the inflammatory cytokines and chemokines are expressed in the whole prostate tissue, or produced by the transgenic epithelium only. Moreover we wanted to compare gene expression profiles of the different cell lines and additionally we wanted to know if and in which way the transgenic epithelium influences the stromal cell gene expression.

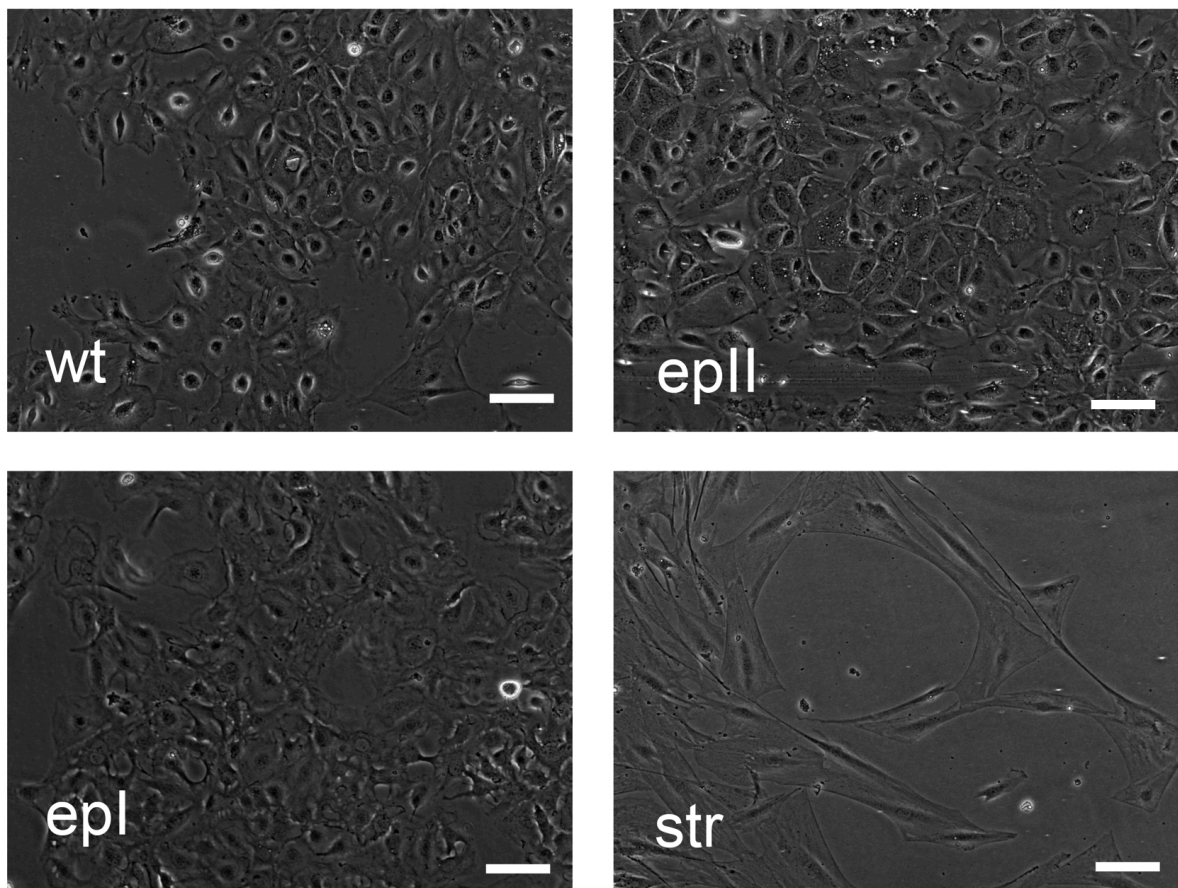


Figure 23: Explant cultures of epithelial and stromal cells from PE-PTEN^{+/-}-IKK2^{ca/ca} and wild type prostate tissues³¹.

3.1.2.4.1 Expression of the constitutively active IKK2 gene

In epithelial lines from PE-PTEN^{+/-}-IKK2^{ca/ca} mice the Flag-IKK2 transgene is only expressed in this cell line (Fig.24).

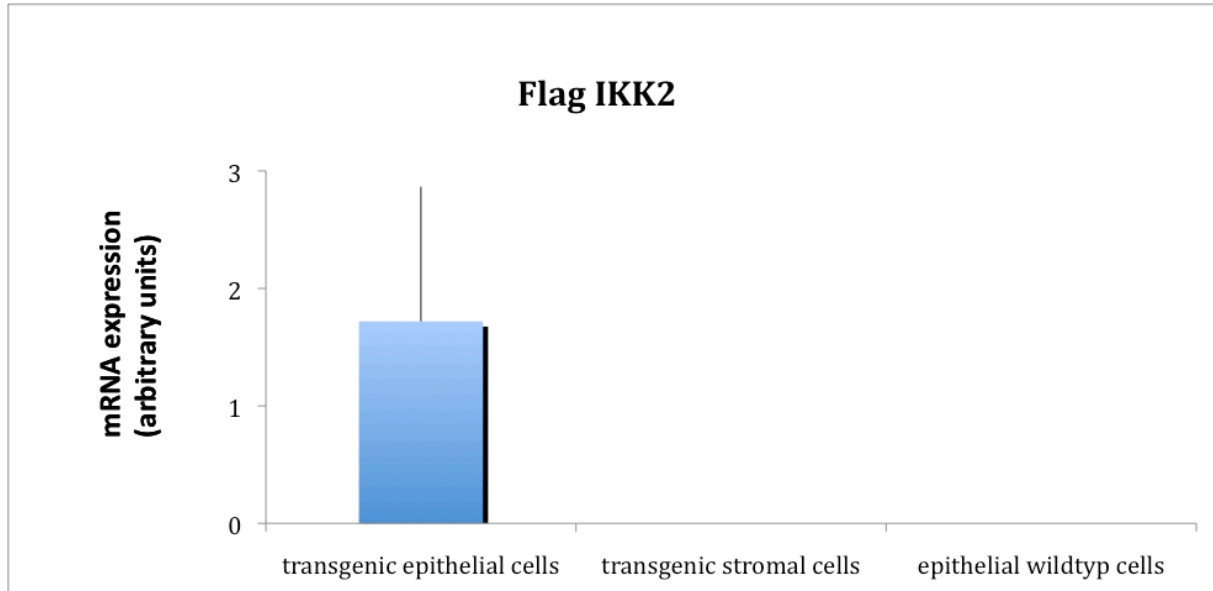


Figure 24: mRNA expression level of the Flag-tag of IKK2 in lateral prostate cells of PE-PTEN^{+/-}-IKK2^{ca/ca} mice.

3.1.2.4.2 Upregulated genes in transgenic epithelial cells in contrast to wild type epithelial cells compared to gene expression of transgenic stromal cells

In this part of the experiment we wanted to confirm that transgenic epithelial cells from PE-PTEN^{+/-}-IKK2^{ca/ca} mice prostates show an upregulation of factors involved in inflammation and prostate cancer progression in contrast to non-transgenic epithelial cells (wildtype epithelial cells). Furthermore we wanted to compare the gene expression profile of transgenic epithelial cells to transgenic stromal cells both isolated from PE-PTEN^{+/-}-IKK2^{ca/ca} mice prostates.

3.1.2.4.2.1 Chemokines

We could show that the chemokines CXCL5 and CXCL15 were significant and CXCL10 and CXCL2 moderate upregulated in transgenic epithelial cells in contrast to non-transgenic epithelial cells. Furthermore we could show that chemokine overexpression is only located to the transgenic epithelium and not to the stroma (Fig.25-28).

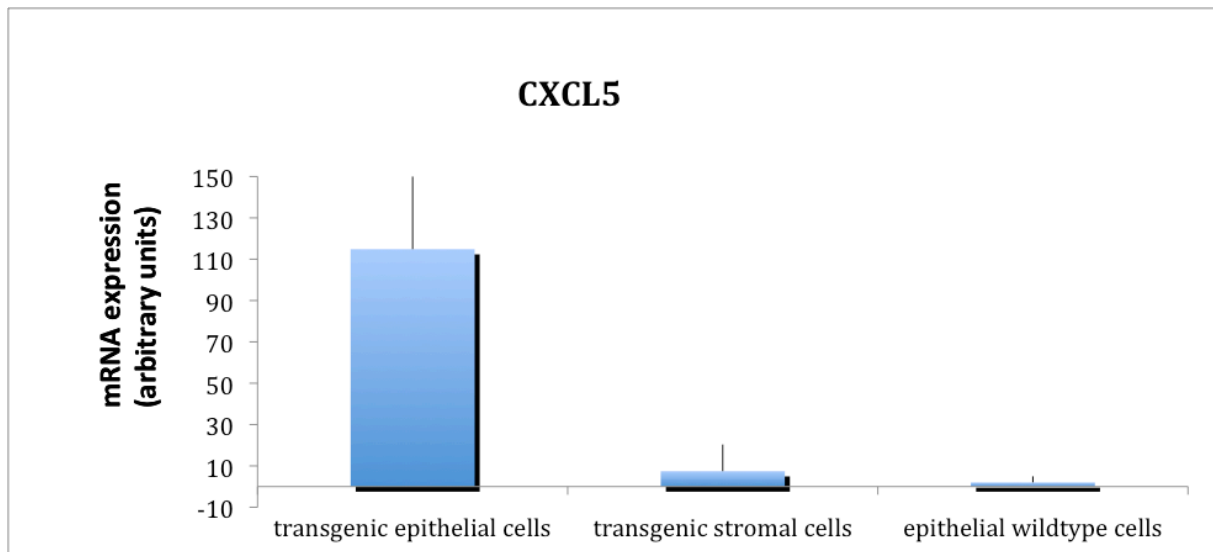


Figure 25: Comparison of mRNA expression level of inflammatory chemokine CXCL5 in transgenic epithelial cells isolated from PE-PTEN+/-IKK2ca/ca lateral prostates to non-transgenic (wildtype) epithelial cells and contrast of mRNA expression level of CXCL5 in transgenic-epithelial and -stromal cells both isolated from PE-PTEN+/-IKK2ca/ca lateral prostates.

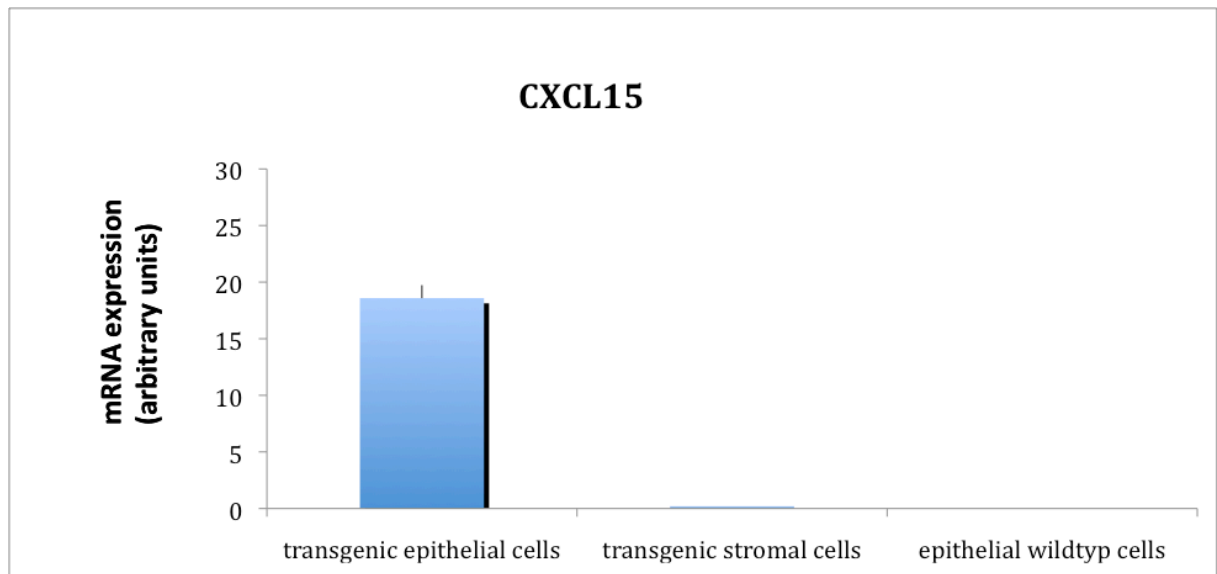


Figure 26: Comparison of mRNA expression level of inflammatory chemokine CXCL15 in transgenic epithelial cells isolated from PE-PTEN+/-IKK2ca/ca lateral prostates to non-transgenic (wildtype) epithelial cells and contrast of mRNA expression level of CXCL15 in transgenic-epithelial and -stromal cells both isolated from PE-PTEN+/-IKK2ca/ca lateral prostates.

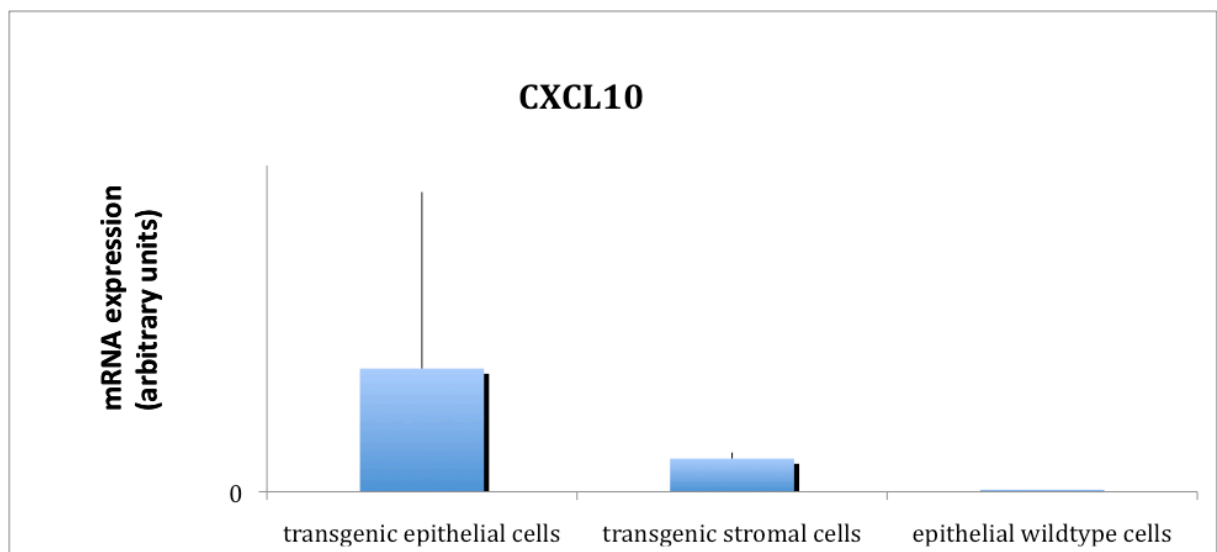


Figure 27: Comparison of mRNA expression level of inflammatory chemokine CXCL10 in transgenic epithelial cells isolated from PE-PTEN+/-IKK2ca/ca lateral prostates to non-transgenic (wildtype) epithelial cells and contrast of mRNA expression level of CXCL10 in transgenic-epithelial and -stromal cells both isolated from PE-PTEN+/-IKK2ca/ca lateral prostates.

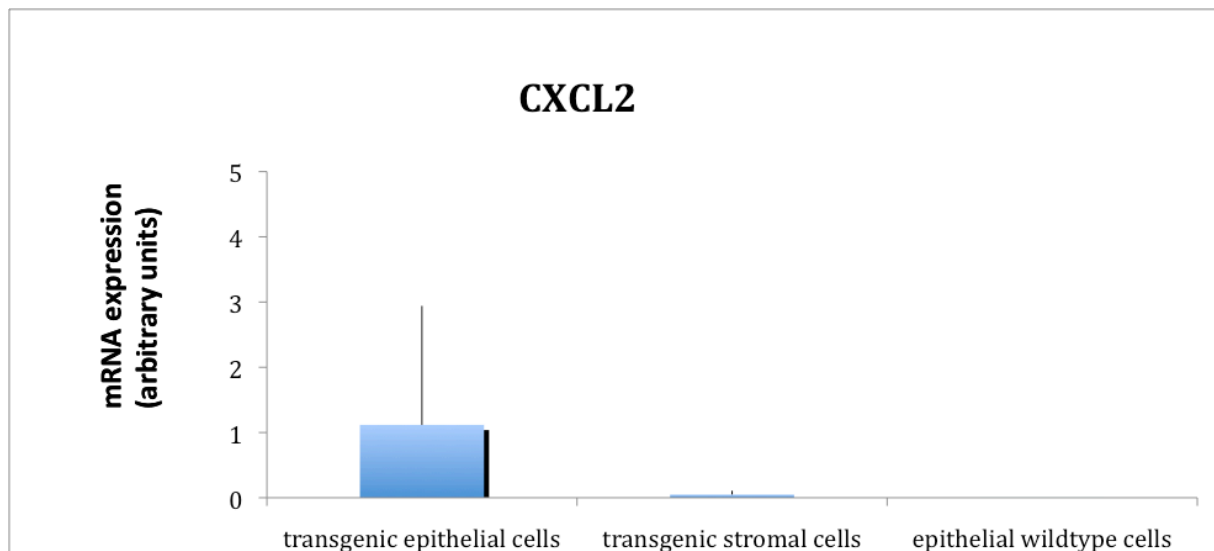


Figure 28: Comparison of mRNA expression level of inflammatory chemokine CXCL2 in transgenic epithelial cells isolated from PE-PTEN+/-IKK2ca/ca lateral prostates to non-transgenic (wildtype) epithelial cells and contrast of mRNA expression level of CXCL2 in transgenic-epithelial and -stromal cells both isolated from PE-PTEN+/-IKK2ca/ca lateral prostates.

3.1.2.4.2.2 Cytokines and NF κ B target genes

Moreover we could show that the inflammatory cytokine and NF κ B target gene TNF α is upregulated in transgenic epithelial cells in contrast to wildtype cells (Fig.29) and that its expression is only increased in transgenic epithelial cells not in transgenic stromal cells. Additionally it was confirmed by David Eisenbarth that the cell adhesion molecule ICAM1 is upregulated in transgenic epithelial cells in contrast to epithelial wildtype cells and that it's overexpression is located to the transgenic epithelium too. ICAM1 is a protein described as a neutrophil granulocyte receptor³² and therefore involved in immunological responses.

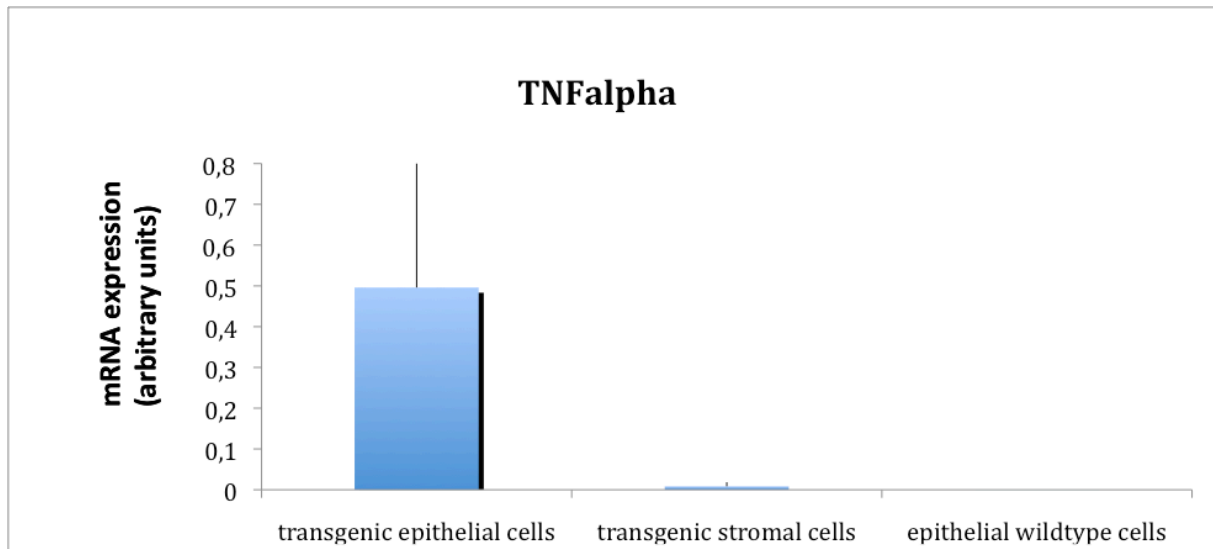


Figure 29: Comparison of mRNA expression level of $TNF\alpha$ in transgenic epithelial cells isolated from PE-PTEN+/-IKK2ca/ca lateral prostates to non- transgenic (wildtype) epithelial cells and contrast of mRNA expression level of $TNF\alpha$ in transgenic-epithelial and –stromal cells both isolated from PE-PTEN+/-IKK2ca/ca lateral prostates.

3.1.2.4.2.3 Others

Futhermore the type-1 transmembrane cell-cell adhesion glycoproteinprotein E-cadherin, shown by David Eisenbarth and the luminal cell marker Cytokeratin18 (Fig.30) were upregulated. In one wildtype and one transgenic epithelial line (epII) we could verify an overexpression of the cytoskeletal Cytoceratin 14 gene while in another transgenic line (epI) not. Suggesting that epII has a more intermediate character while epI shows a more luminal phenotype. As well we could detect an upregulation of the neutrophil marker Lipocalin2 (Fig.31) documented as upregulated in several epithelial cancers such as prostate cancer³⁸.

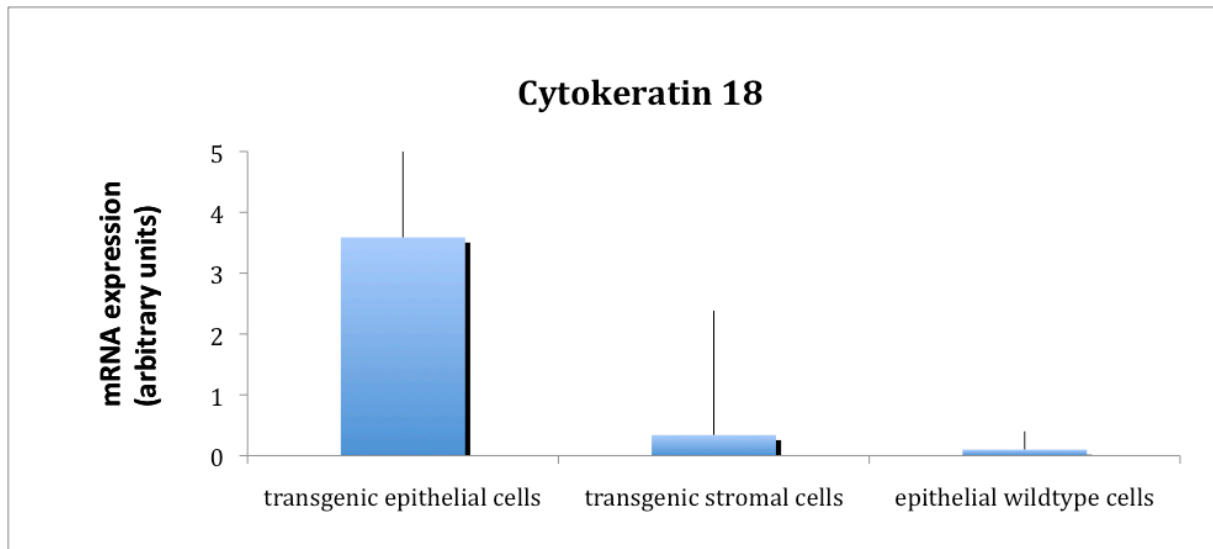


Figure 30: Comparison of mRNA expression level of Cytokeratin18 in transgenic epithelial cells isolated from PE-PTEN+/-IKK2ca/ca lateral prostates to non-transgenic (wildtype) epithelial cells and contrast of mRNA expression level of Cytokeratin18 in transgenic-epithelial and -stromal cells both isolated from PE-PTEN+/-IKK2ca/ca lateral prostates.

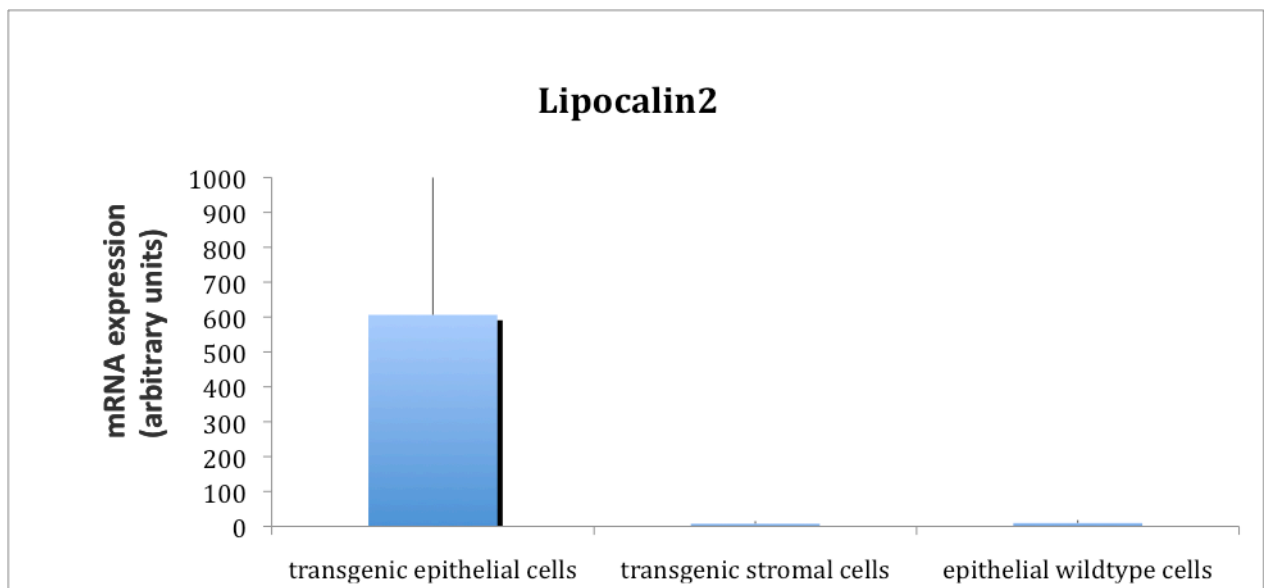


Figure 31: Comparison of mRNA expression level of Lipocalin2 in transgenic epithelial cells isolated from PE-PTEN+/-IKK2ca/ca lateral prostates to non-transgenic (wildtype) epithelial cells and contrast of mRNA expression level of Lipocalin2 in transgenic-epithelial and -stromal cells both isolated from PE-PTEN+/-IKK2ca/ca lateral prostates.

3.1.2.4.3 Upregulated genes in transgenic stromal cells in contrast to wild type epithelial cells compared to gene expression of transgenic epithelial cells

3.1.2.4.3.1 Frizzles related proteins

Still we could show that the frizzles related proteins SFRP1 and SFRP4 are upregulated (Fig.32, 33). SFRP1 has been documented to be involved to the modulation of the epithelium in prostate cancer progression.²⁶

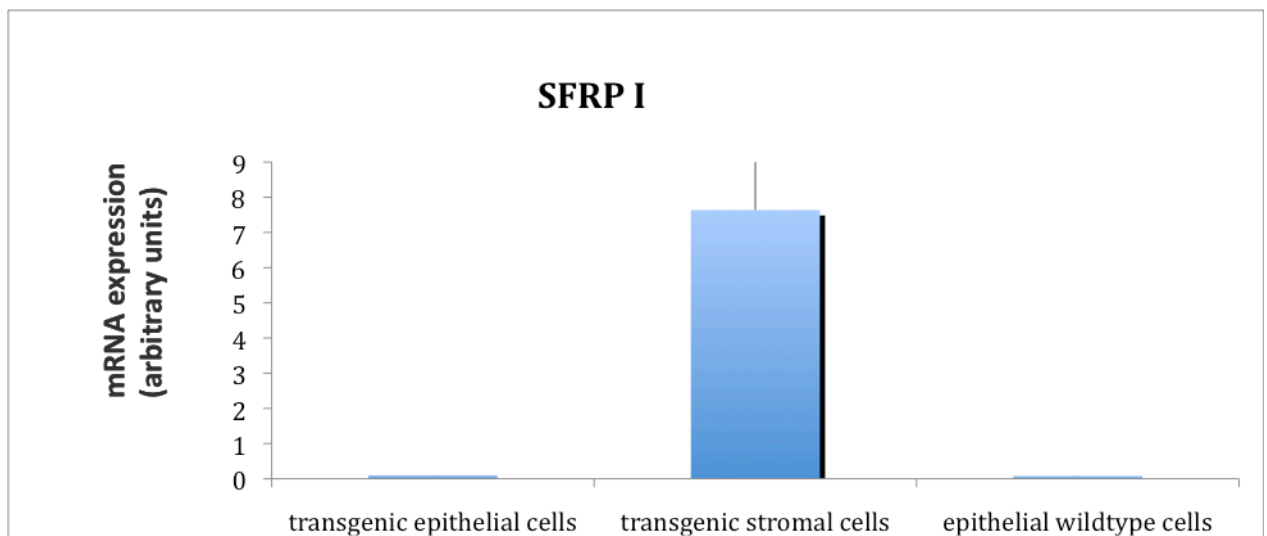


Figure 32: Comparison of mRNA expression level of SFRP1 in transgenic epithelial cells isolated from PE-PTEN+/-IKK2ca/ca lateral prostates to non- transgenic (wildtype) epithelial cells and contrast of mRNA expression level of SFRP1 in transgenic- epithelial and –stromal cells both isolated from PE-PTEN+/-IKK2ca/ca lateral prostates.

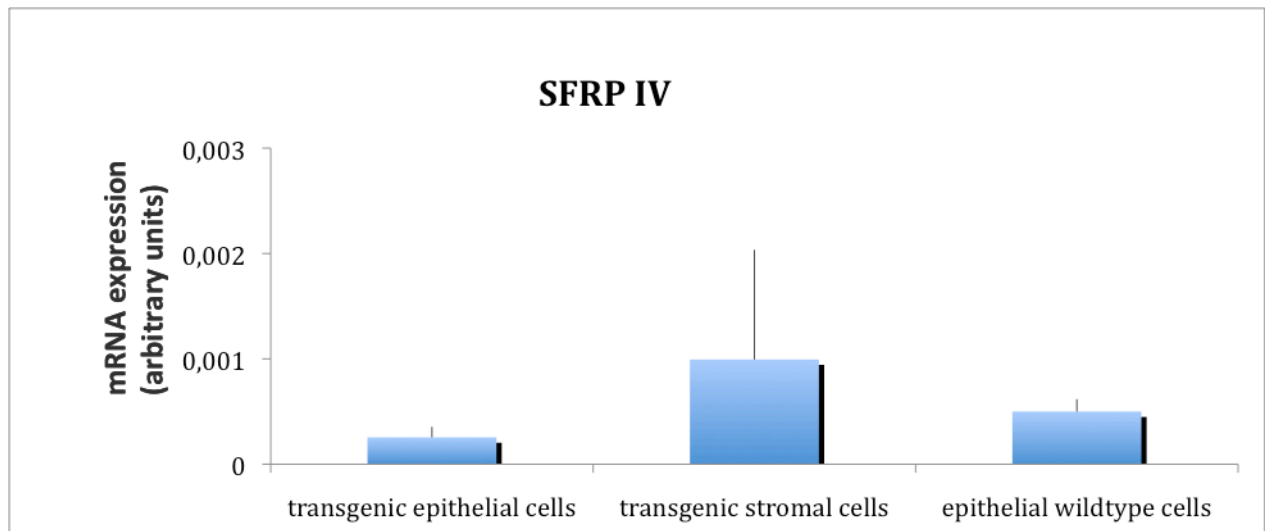


Figure 33: Comparison of mRNA expression level of SFRP IV in transgenic epithelial cells isolated from PE-PTEN+/-IKK2ca/ca lateral prostates to non- transgenic (wildtype) epithelial cells and contrast of mRNA expression level of SFRP IV in transgenic- epithelial and –stromal cells both isolated from PE-PTEN+/-IKK2ca/ca lateral prostates.

3.1.2.4.4 Stromal transgenic cultures stimulated with $TNF\alpha$

It was shown by David Eisenbarth that the stroma of transgenic mice prostates could be activated from cytokines produced by the transgenic epithelium and hence is able to express immune cell attracting chemokines. Stromal cell line of PE-PTEN+/-IKK2ca/ca mouse prostate was stimulated with $TNF\alpha$ and the chemokine level was checked after 24 and 48h. Certainly CXCL2 and to a minor extent CXCL10, but surprisingly not CXCL5 or CXCL15, were overexpressed after $TNF\alpha$ treatment.

3.1.2.4.5 Epithelial transgenic cell line stimulated with IKK2/ NF κ B inhibitor

BMS-345541

In this experiment we wanted to prove that the transgenic activation of the IKK2/ NF κ B axis is responsible for overexpression of cytokines and chemokines in the prostate tissue. In the transgenic (PTEN $\pm$ IKK2ca/ca) epithelial cell line epl the TNF α gene is significantly downregulated after BMS treatment (Fig.34) and we could show a downregulation of CXCL5 (Fig.35), one of the most upregulated chemokines in double transgenic prostate tissue.

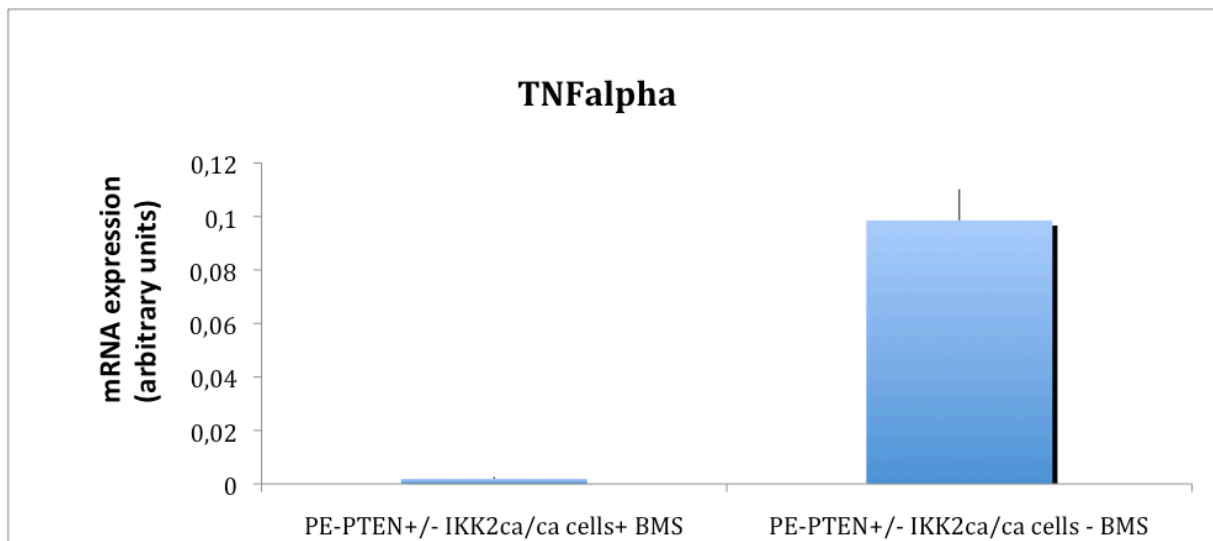


Figure 34: mRNA expression level of inflammatory cytokine TNF α in epithelial cell line treated with mock control or BMS-345541 for 72 hours.

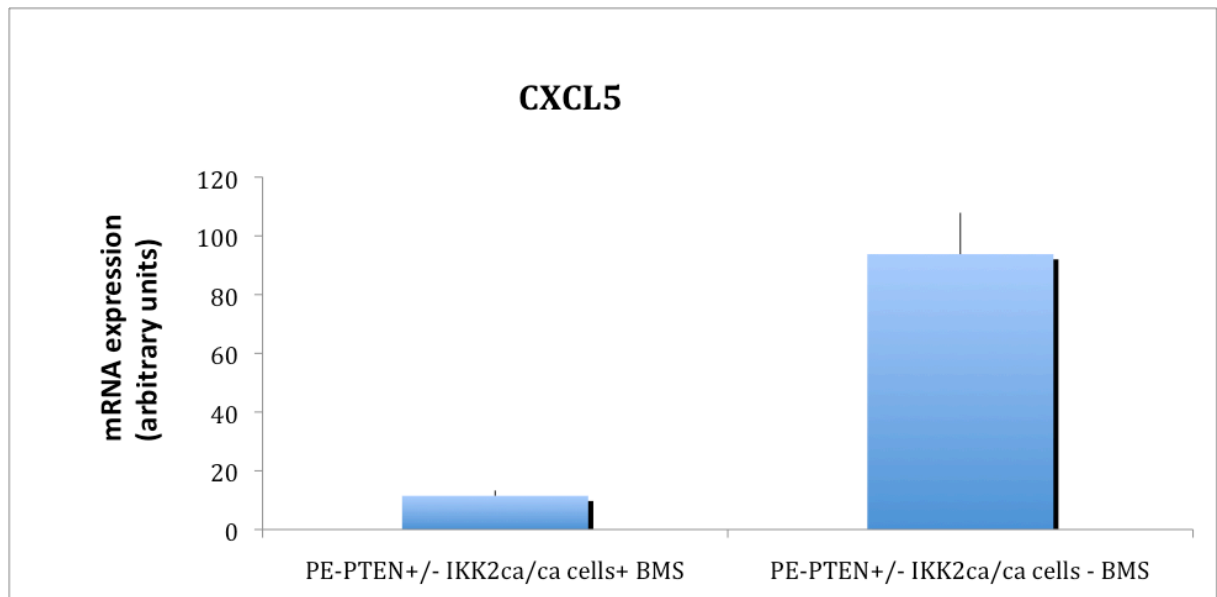


Figure 35: mRNA expression level of inflammatory chemokine CXCL5 in epithelial cell line treated with mock control or BMS-345541 for 72 hours.

3.2 Protein level verification in transgenic prostate tissue and cell lines

3.2.1 Immunoblot analysis

We did Immunoblot analysis to confirm the upregulation or downregulation of genes identified by Chip Analysis or real-time RT-PCR on protein level.

3.2.1.1 Verification of protein level in transgenic prostate tissues

3.2.1.1.1 Nkx3.1

This blot shows the lower protein amount of Nkx3.1 in PE-PTEN^{+/-} IKK2^{ca/ca} mouse prostates in contrast to wild type mice. By analysing data of the housekeeping gene β -Tubulin it was shown that although there is most amount of protein loaded in PE-PTEN^{+/-}-IKK2^{ca/ca} mice slots, the concentration of Nkx3.1 is lower in contrast to wildtype littermates. Interestingly the amount of housekeeping gene Actin is highest in wildtype prostates, exactly the other way around as it was revealed with β -Tubulin on the same blot (Fig.36). This result confirms the downregulation of Smooth Muscle Actin (SMA) detected in Chip Analysis and real-time RT PCR analysis and Tissue staining.

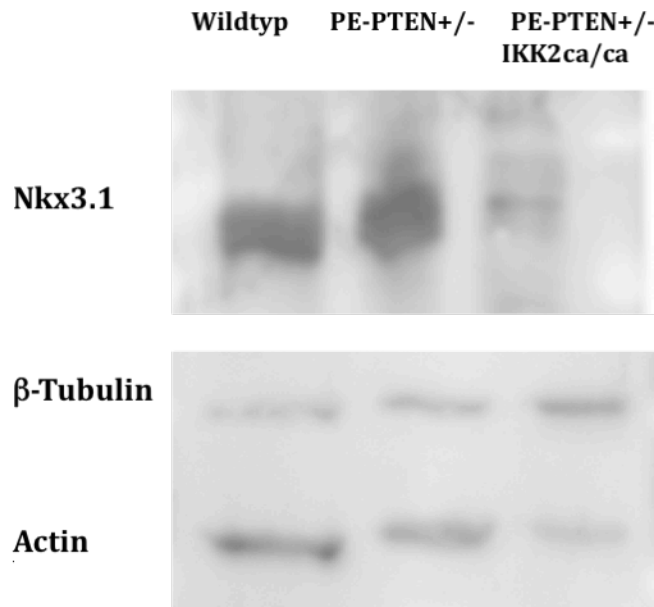


Figure 36: Immunoblot analysis from protein isolates of transgenic prostate tissue and wildtype targeting Nkx3.1.

3.2.1.2 Verification of protein level in explant cell cultures of transgenic prostate tissues

3.2.1.2.1 *IκBα*

Via Immunoblot we could show that *IκB* level is highest in wildtype and in PE-PTEN^{-/-} mice and decreased in PE-PTEN^{+/-}-IKK2^{ca/ca} mice. This should be due to phosphorylation by IKK2 kinase and subsequent ubiquitinylation and degradation of *IκB* in case of an inflammatory stimulation in double transgenic mice. This finding is consistent with other results in this project and stands for an intense activation of the NFκB pathway based on constitutive active IKK2 kinase. Unfortunately it was not possible to detect the phosphorylated version of *IκB* (pIκB), we speculate that the turn over rate of pIκB is quite fast so that we were not able to detect this protein (Fig. 37).

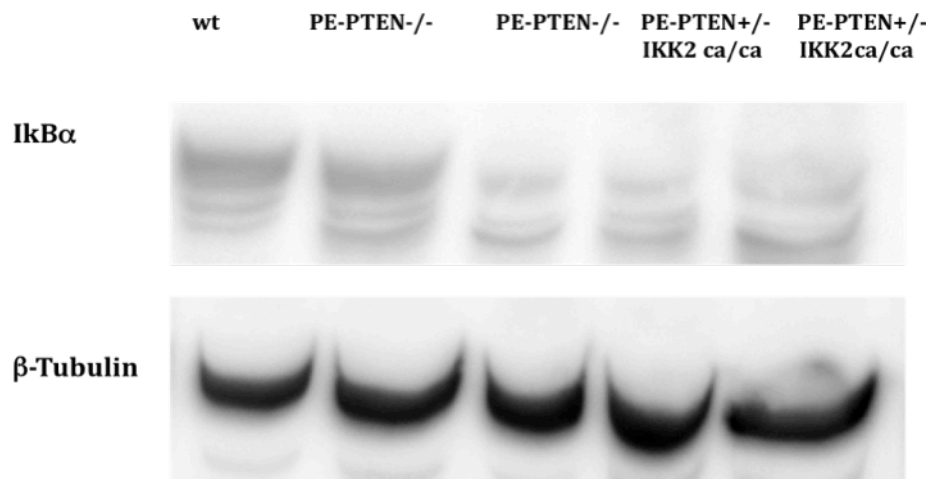


Figure 37: Immunoblot Analysis from transgenic explant cell cultures of transgenic prostate tissue showing protein level of IκBα. β-Tubulin was used as housekeeping gene.

In the second blot targeting the same protein we wanted to verify differences in NFκB pathway activation through degradation of IκBα in the different prostate lobes (Fig.38). Unfortunately the second immuno blot concerning IκBα was not that meaningful as we expected. In Fact the IκBα concentration is slightly decreased in PE-PTEN^{+/-}-IKK2^{ca/ca} mice in comparison to wildtype but the protein level should be much more decreased in transgenic mice through the constitutive IKK2 kinase.

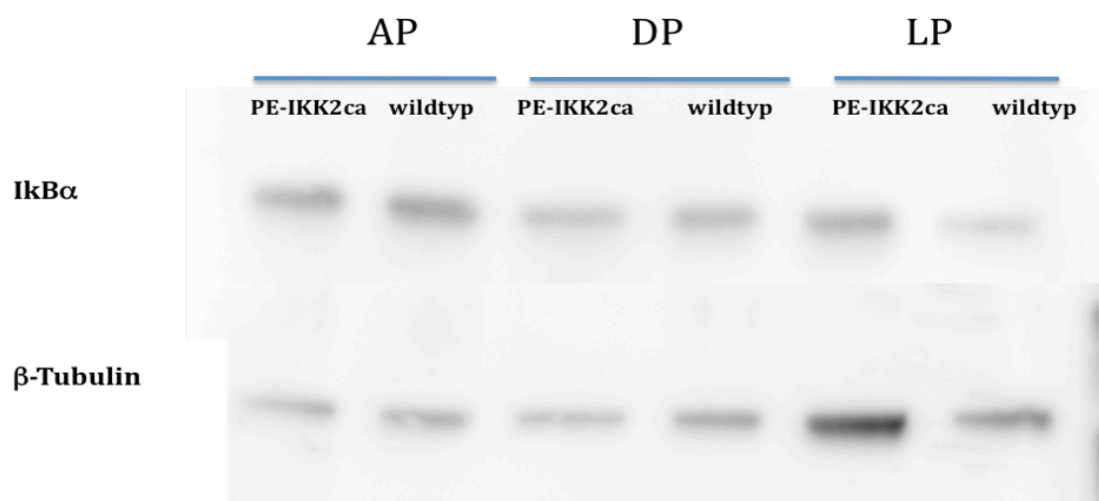


Figure 38: Immunoblot analysis from transgenic explant cell cultures of transgenic prostate tissue showing protein level of IκBα in AP; DP and LP of PE-PTEN IKK2^{ca/ca} in comparison to wildtype prostate cells. β-Tubulin was used as housekeeping gene.

3.2.1.2.2 pI κ B α

In PE-PTEN^{+/}-IKK2^{ca/ca} mice I κ B α should be present in its phosphorylated form to be ubiquitinated and degraded permitting NF κ B transcription factor to be translocated to the nucleus. Unfortunately it was not possible to demonstrate that the phosphorylated version of I κ B α (pI κ B α) is present in PE-PTEN^{+/}-IKK2^{ca/ca} mice, we speculate that the turnover rate of pI κ B α makes it quite difficult to detect it.

3.3 In situ analysis of transgenic prostate tissue

Previously main parts of experiments insitu were performed by Andreas Birbach assisted by Diplomastudent David Eisenbarth and parts of them were repeated by myself. Pictures that are not shown in my thesis can be found in the paper “Persistent inflammation leads to proliferative neoplasia and loss of smooth muscle cells in a prostate tumor model³¹.

3.3.1 Tissue staining

With tissue staining we wanted to show in situ that the Flag-tagged IKK2 transgene is definitely expressed in the epithelium. Furthermore we wanted to show that there is an increase in tumor size and epithelial and stromal proliferation in PE-PTEN+/- IKK2ca/ca in comparison to PTEN+/-, PE-IKK2ca/ca and wildtype mice.

Additionally we wanted to confirm that there is an inflammatory process going on as well as an immune cell infiltration to the affected tissue. Moreover we tried to field the question whether there are invasive cells and factors that could probably be involved in this process.

3.3.1.1 In situ detection of constitutive active IKK2 gene

It was shown by Andreas Birbach that the Flag-tag of the IKK2 protein is expressed and restricted to the transgenic epithelium using an antibody specific for Flag (Fig.39). Question was now if the constitutive active IKK2 gene in epithelial cells of the prostate is able to transform prostate tissue.

Therefore histological assessment of prostate lobes from the diverse transgenic prostate tissues at different time points was performed but there were no histological relevant changes in mice expressing the constitutive active IKK2 gene after 4(n=3), 8(n=4) or 12(n=10) months of age, neither in animals expressing IKK2 (IKK2ca) from

a single allele nor in animals expressing the transgene from both alleles (IKK2ca/ca). Hence it was concluded that a constitutive active IKK2 and therein permanent active NF κ B pathway is not able to transform prostate tissue.

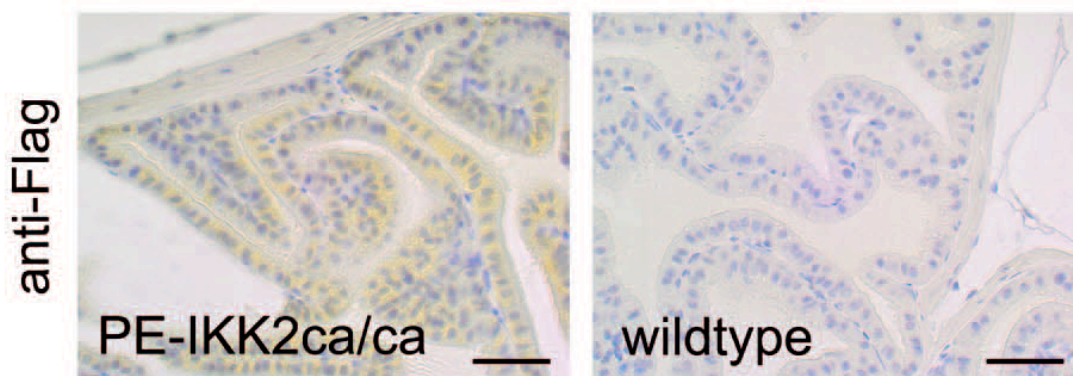


Figure 39: Anti Flag stain in PE-IKK2ca/ca and wildtype mice³¹.

3.3.1.2 Prostate-specific monoallelic loss of PTEN combined with IKK2ca/ca expression

It was shown that permanent active NF κ B/IKK2 axis on a PTEN $\pm$ background leads to a visible increase in prostate size after 12 months and significant alternations after 8 months. After 4 months no alternations have been noticed in prostate tissue. Mice expressing the IKK2 transgene on both alleles (PE-PTEN $\pm$ IKK2ca/ca) showed enlarged tissue in all lobes after 8 months. Older mice expressing the transgene on one allele (PE-PTEN $\pm$ IKK2ca) only showed enlarged lateral prostates where the transgene expression was shown to be highest.

3.3.1.3 Prostate size and changes in the transgenic tissue

Performing immunohistochemistry it was illustrated that PE-PTEN $\pm$ IKK2ca/ca mouse prostates show severe hyperplastic changes which often led to cribriform structures. They had equal incidence of nuclear atypia as PE-PTEN $\pm$ mice epithelium. PE-PTEN $\pm$ mouse prostates showed hyperplasia and focal nuclear

abnormalities also referred as prostatic intraepithelial neoplasia (PIN). Wildtype mouse prostates showed normal, smooth epithelium lacking any hyperplastic or noticeable abnormalities. In addition PE-PTEN^{+/-} IKK2^{ca/ca} mouse prostates showed a clear indication for stromal changes with numerous collagen fibers in comparison to the loose stroma with a few fibers in PE-PTEN^{+/-}, PE-IKK2^{ca/ca} and wildtype.

3.3.1.4 Cell proliferation and apoptosis in transgenic tissue

Coincident with mentioned changes in prostate tissue we could show an increased level of proliferation marker³² Ki67 positive epithelial and stromal cells (Fig.41). TUNEL-staining, which is a method to show apoptotic cell processes, was low and not significantly changed in one of the genotypes, revealing indeed an increase of cell proliferation in PE-PTEN^{+/-}-IKK2^{ca/ca} mice but not in apoptosis (Fig. not shown).

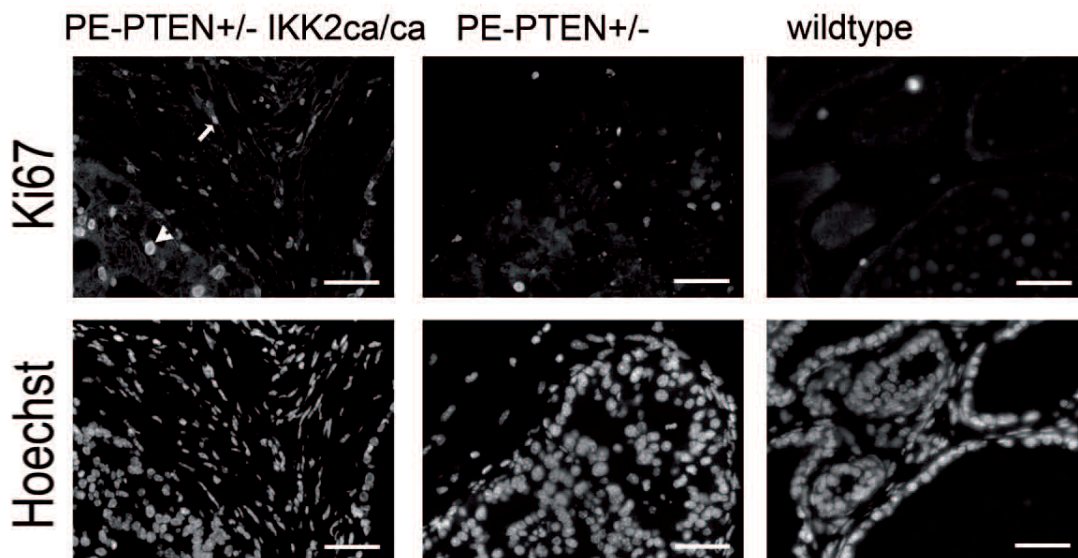


Figure 40: paraffin sections of lateral prostates (12months) stained for proliferation marker Ki67. Positive epithelial cells (arrowhead) and stromal cells (arrow) were seen³¹.

3.3.1.5 Inflammatory processes in transgenic tissue

It was shown that PE-PTEN^{+/-} IKK2^{ca/ca} mice had an increased tendency of severe prostatitis in disparity to young PE-PTEN^{+/-} (4months:n=3, 8months: n=5) and

IKK2ca/ca mice where no signs of prostatitis occurred. Anyway a number of PE-PTEN+/- mice developed signs of severe prostatitis with around 12 months (Fig. not shown).

3.3.1.6 Infiltration of immune cells to the transgenic tissue

As we expected the transgenic tissue, especially PE-PTEN+/-IKK2ca/ca mice showed an increased rate of immune cell infiltration. This was shown by staining for the neutrophil marker Lipocalin2 and it was seen that neutrophil granulocytes infiltrate the stroma and the epithelium where they could be well spotted in lumina of cribriform structures.

In Immunofluorescence analysis we could moreover demonstrate an infiltration of macrophages/monocytes, neutrophils and to a lesser extent leukocytes in PE-PTEN+/-IKK2ca/ca mice in contrast to control tissues. For this purpose we used the macrophages/monocytes antigen CD11b, the macrophage marker F4/80 and the neutrophil marker Gr-1 (Fig.43).

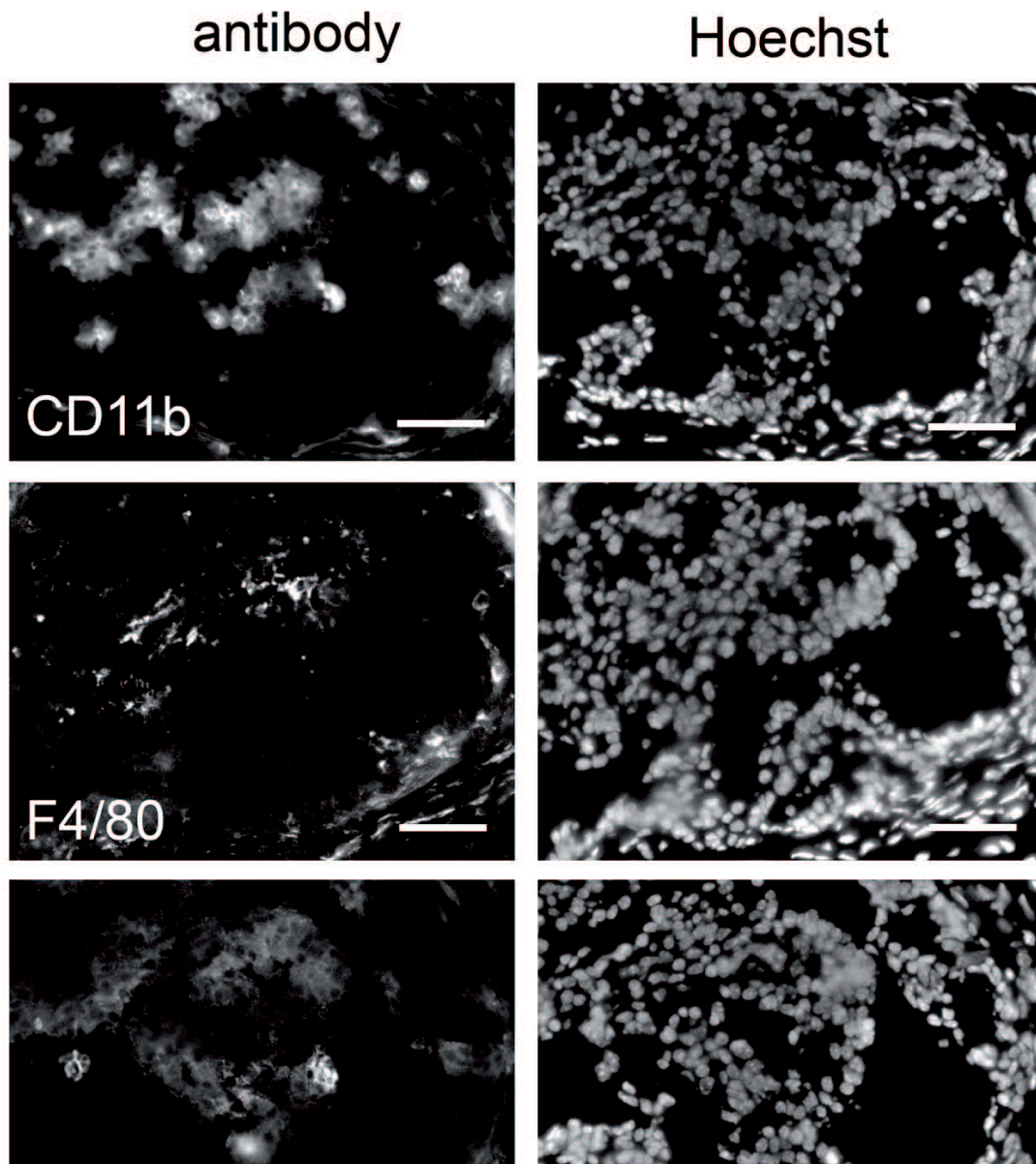


Figure 41: Antibodystaining of cryosections from dorsal prostates of PE-PTEN+/-IKK2ca/ca mice using CD11b, F4/80 and Gr-1 antibodies³¹.

3.3.1.7 Androgen receptor (AR)

Tissue staining with an antibody against the prostate specific transcription factor AR revealed a lower nuclear activated level of androgen receptor in PE-PTEN+/- IKK2ca/ca mice in contrast to wildtype prostate epithelial cells (Fig.44). Decrease of AR activity was already indicated in Chip Analysis by a downregulation of other androgen-responsive genes such as Probasin. Furthermore AR is known being linked to the expression of prostate specific transcription factor Nkx3.1 that is well established to be decisive for cell proliferation in prostate²⁰.

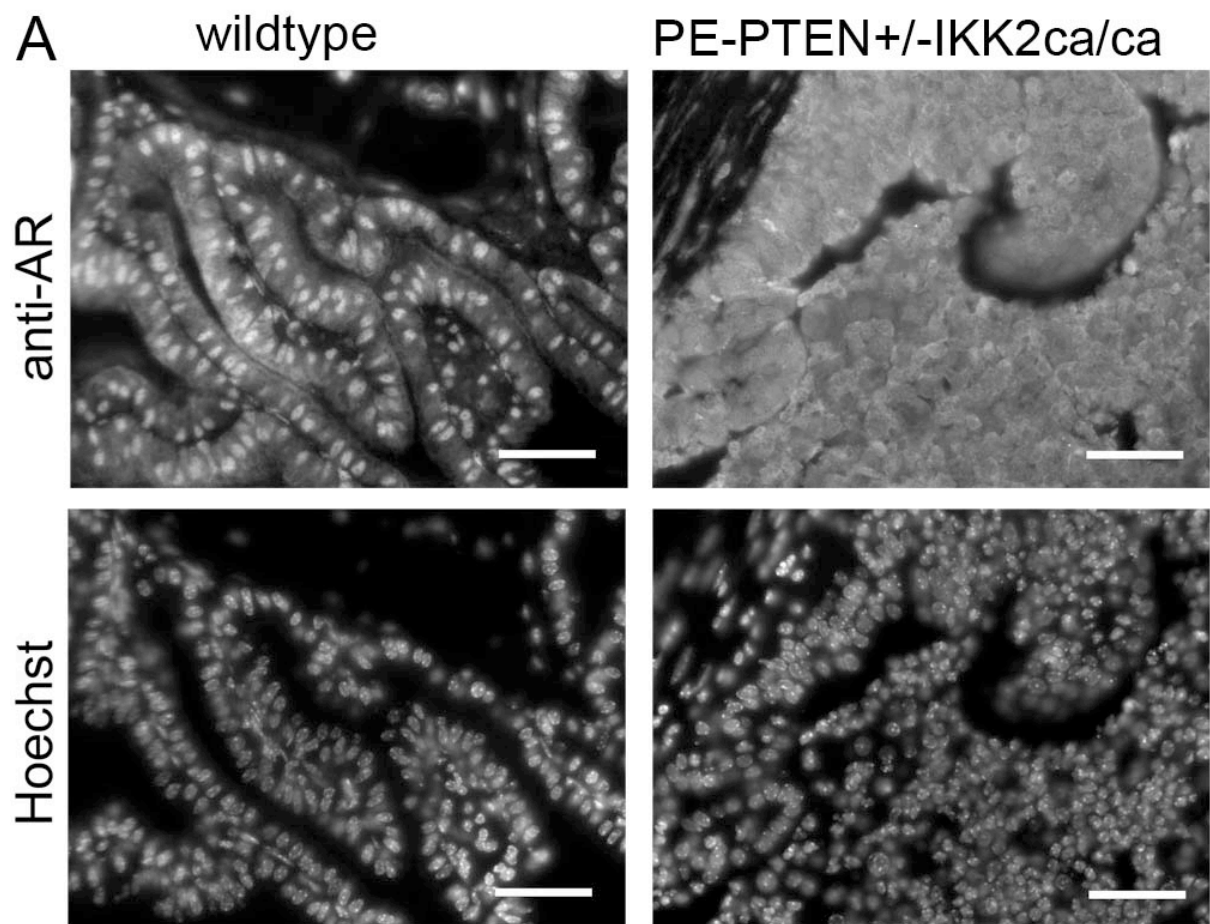


Figure 42: Paraffin sections of lateral prostates (12months) stained for Androgen Receptor (AR). Same tissues were stained for Hoechst to indicate tissue structure³¹.

3.3.1.8 Factors involved in Invasiveness

So far dramatic changes in stroma of the transgenic prostate tissue were investigated by histological staining and revealed by real-time RT-PCR presuming a phenotype that might show signs of invasiveness.

3.3.1.8.1 Smooth muscle cell markers

Results concerning the downregulation of smooth cell markers, smooth muscle myosin heavy chain (MYH11) and Smooth Muscle Actin (SMA) in PE-PTEN^{+/-}-IKK2^{ca/ca} prostates compared to PE-PTEN^{+/-}, PE-IKK2^{ca/ca} and wildtype were confirmed by staining lateral prostates with SMA antibody. Staining revealed that in PE-PTEN^{+/-}-IKK2^{ca/ca} prostates, Smooth Muscle Cells around many prostatic ducts were lost.

3.3.1.8.2 Basal cell marker

The loss of basal cells in the epithelium is an indication for invasive tumor formation. By Co-staining of smooth muscle marker and the basal cell marker p63 it was shown that although there was even in the glands with a complete smooth muscle cell loss a preserved basal cell expression. The larger tumors in PE-PTEN^{+/-}-IKK2^{ca/ca} prostates showed no signs of “dripping off” of smaller epithelial structures or even invasion to neighbouring tissue.

4 Discussion

In this study we wanted to define a gene expression profile that differs in prostate cancer tissue in contrast to normal tissue with special focus on a permanent inflammatory stimulation and the link to cancer progression. For this reason we used a mouse model that is able to express a constitutive active form of the key mediator of the inflammatory NF κ B pathway IKK2, using the well-described Cre/LoxP system. Further we wanted to reveal what phenotype results out of an additional PTEN deficiency. PTEN is a gene that is documented to be mutated in prostate carcinogenesis in a frequent manner¹³⁻¹⁵. Using these tools we wanted to get an idea of how prostate cancer development and inflammation could be linked and which factors could be involved therein.

Interestingly no histological relevant changes in mice expressing the constitutive active IKK2 gene were seen. Beside we could not reveal significant changes in gene expression profile. For this reason we conclude that a constitutive active IKK2 and therein permanent active NF κ B pathway is not able to transform prostate tissue in this mouse model. This is curious because IKK2 has been documented as an oncogenic kinase and results in double transgenic mice in an obvious phenotype with increased inflammation and activated stroma, hyperplasia as well as appropriate changes in gene expression, being constitutive active on a PE- PTEN $\pm$ background. Beside the fact that IKK2 is known as an onkogenic kinase, it was recognized that long time treatment of Aspirin, an inhibitor of IKK2, decreases prostate cancer risk arguing for an important role therein.

Furthermore we speculate that the reason for a lacking inflammatory phenotype in PE-IKK2ca/ca mice could be that NF κ B transcription factor is activating promoters in

proliferating epithelium that cannot be activated in quiescent epithelium such as the prostate epithelium. Reason for that speculation is that Andreas Birbach bred mice that express the same constitutive version of IKK2 on same genetic background but in keratinocytes of the skin a proliferating epithelium resulting a clear phenotype (Birbach et al. unpublished). Furthermore Probasin Cre mice excise flanked gene sides only in adult mice harbouring a already quiescent prostate epithelium. This is consistent with the finding that PE-PTEN^{+/-}-IKK2 develop a phenotype described above.

Double transgenic mouse prostates are characterized by an infiltration of neutrophil granulocytes and macrophages/monocytes indicated by staining with antibodies against Gr-1, CD11b and F4/80. This immune cell infiltration is founded in NFkB-dependent overexpression of cytokines, primarily IL1b and TNF α , responsible for stimulation of immune cells and production of chemokines such as CXCL5, CXCL15, CXCL3, CXCL10 and CXCL2 responsible for immune cell attraction assumingly via binding of chemokine receptors and selectines. Stimulation of transgenic stromal cells with TNF α indicated that immune cells could be attracted directly via transgenic epithelial cells and their produced chemokines or indirect by cytokines produced by the epithelium activating chemokine production in the stroma cells.

Immune cell infiltration is much lower in PE-PTEN^{+/-} mice and in wildtype in contrast to PE-PTEN^{+/-}-IKK2^{ca/ca}, a finding that is coherent with expression profile and histological data of these mouse models. These data show that inflammation processes going on in transgenic prostate tissue is the basic source of the phenotype of transgenic mice we worked with.

Moreover the adhesions molecule ICAM1, that is known to be produced by stimulation of cytokines and responsible for immune cell attraction too.

Double transgenic mice did not show any signs of invasive tumor formation although many factors that are known to be involved in prostate cancer progression are deregulated, such as secreted frizzled related proteins SFRPI and SFRPIV. These genes, expressed by stromal cells, could contribute to abnormal proliferation in prostate epithelium and could support prostate cancer progression²⁶. Another frequent mutated gene in Prostate cancer is hepsin, a serine protease that is documented to promote primary prostate cancer progression and metastasis²⁸

Furthermore smooth muscle actin (SMA), myosin heavy chain 11 (MYH11) and Calponin 1 were significantly downregulated indicating a loss of smooth muscle cells frequently lost in prostate cancer. This result was confirmed by histologic staining showing loss of smooth muscle around stratified epithelium.

We speculate that probably the remaining basal cell layer indicated by p63 staining could be a reason why tumors are not invasive although smooth muscle cells were lost.

Furthermore the androgen receptor target gene Nkx3.1, only downregulated in double transgenic mice, but known to be frequently lost in prostate cancer could be a potential reason for lacking of invasiveness in PE-PTEN+/-IKK2 ca/ca mice. Nkx3.1 downregulation moreover correlates with reduced activity of AR that could be influenced by modified stromal-to-epithelial signalling that is perhaps influenced among others via SFRP1.

Additionally Spink3 is downregulated in double transgenic mice and is assumed to be involved to invasiveness of prostate tumors if it is overexpressed. Downregulation of this serine protease inhibitor could also account for a lack of invasive character in double transgenic mice.

In total our results give an impression of how inflammation could be linked to prostate cancer formation although the final transforming step in mice models we used in this project was not detected. We think that tumors could become invasive in case of a longterm study under same condition postulating additional genomic mutations caused by events subsequent to inflammatory stimulation.

5 Appendage

5.1 Literature

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5.3 Summary

Prostate cancer is one of the most common cancer types in man all over the world. In former investigations it was shown that genetic as well as epigenetic factors could be involved in prostate cancer progression. It was also shown that bacterial and viral infections could trigger inflammation, which later could lead to an increase in tumor formation. Noticeable is that there seems to be a correlation between inflammation and tumor formation at a later time point.

In our studies we wanted to investigate this correlation using a mouse model expressing a constitutively active I κ B kinase (IKK2) in epithelial cells of the mouse prostate. IKK2 is a component of the NF κ B pathway and is involved in triggering immune responses activated by several stimuli. However, overexpression of this protein is not sufficient to induce prostate cancer formation or even changes in prostate tissue. But constitutive active IKK2 kinase in combination with heterozygote loss of phosphatase and tensin homologue (PTEN) in the same cell type, leads to an increase of tumor size in epithelial and stromal proliferation in prostate tissue.

Starting with microarray analysis we revealed several up and down regulated genes that were later investigated using real time RT PCR, immunoblot and immunohistochemical analysis. We could show a clear infiltration of immune cells into the prostate tissue, mainly macrophages/monocytes and neutrophils attracted by several chemokines. This inflammatory phenotype is accompanied by hyperplastic and dysplastic lesions but without invasive character.

Furthermore we could reveal the downregulation of smooth muscle cell markers, known to be lost in invasive tumors, correlating with inflammation around many prostatic ducts. In our mouse model the invasive character is lacking, reason could

be the genetic background because C57BL/6 is known to have a low tumor tendency. Anyway this mouse model shows how inflammation in combination with genetic mutations could lead to tumor formation in time.

5.4 Zusammenfassung

Prostatakrebs ist in der männlichen Bevölkerung die häufigste Krebsart und es herrscht reges Interesse die Hintergründe und Ursachen dieser Krankheit aufzudecken. Unlängst haben Forschungen ergeben, dass die vermutlich ausschlaggebende Komponente zur Entstehung von Prostatakrebs in der Genetik und Epigenetik zu suchen ist.

Weiters wurde gezeigt, dass auch bakterielle und virale Infektionen Entzündungen auslösen können, die in weiterer Folge in Verdacht stehen in chronischer Form krebsauslösend zu sein. Es scheint also einen Zusammenhang zwischen lange andauernden Entzündungen und Prostatakrebs zu geben.

In diesem Projekt haben wir versucht dieser These nachzugehen indem wir auf der einen Seite mit einem Mausmodell (PE-IKK2ca/ca) gearbeitet haben, das eine konstitutiv aktive Form der Kinase IKK2 in Prostata Epithelzellen exprimiert. IKK2 ist eine Kinase im so genannten NFkB pathway die maßgeblich an immunologischen Prozessen, aktiviert durch eine Vielzahl von Stimuli, beteiligt ist. Wir konnten zeigen, dass die permanente entzündliche Stimulierung durch die konstitutive Aktivierung von IKK2 im oben angesprochenen Mausmodell nicht zu wesentlichen Veränderungen des Prostatagewebes führt. Des weiteren arbeiteten wir mit einem doppelt transgenen Mausmodell (PE-PTEN IKK2ca/ca), das ebenfalls die konstitutiv aktive Form der Kinase IKK2 in Prostata Epithelzellen exprimiert, aber zusätzlich Phosphatase und Tensin homolog (PTEN) im selben Zelltyp heterozygot ausgeknockt hat. Hier konnten wir eine Vergrößerung des Tumorgewebes, epitheliale und stromale Proliferation und ein verändertes Genexpressionsprofil feststellen.

Beginnend mit einem Microarray Experiment konnten einige interessante hinauf und hinunter regulierte Gene detektiert werden auf die wir in real-time RT-PCR Experimenten, Immunoblot-Analysen und histologischen Stainings näher eingegangen sind. Es konnte eine stark erhöhte Immunzellinfiltrierung, hauptsächlich Makrophagen/Monozyten und Neutrophile, des Prostatagewebes die vermutlich durch eine erhöhte Anzahl von Chemokinen angelockt wurden, nachgewiesen werden. Doppelt transgene Mäuse zeigten des weiteren einen Prostata Phenotyp der durch Hyperplasie und dysplastisches Gewebe, das jedoch keinen invasiven Charakter zeigt, gezeichnet ist.

Interessanterweise sind Faktoren wie zum Beispiel Gene spezifisch für Zellen der glatten Muskulatur, die bei der Formation von invasiven Tumoren verloren gehen, stark herunterreguliert. Dennoch fehlt der invasive Charakter, was eventuell durch den C57BL/6 Background zu begründen ist, der dafür bekannt ist, eine niedrige Tumortendenz zu haben.

Nichts desto trotz stellt dieses Mausmodell ein interessantes Objekt dar, um Veränderungen im Prostatagewebe, herbeigeführt durch chronische Entzündung und die heterozygote Mutation von PTEN, zu erforschen. Gegebenenfalls könnte es bei einer Langzeitstudie, herbeigeführt durch weitere Genmutationen, in diesem Mausmodell zur Karzinombildung kommen.

5.5 Curriculum Vitae



PERSON

Name	Eva Ladenhauf
Address	Rabensburgerstraße 17, Top 65, 1020 Vienna, Austria
Mobile Phone	+436649493353
E-Mail	e.ladenhauf@gmail.com
Nationality	Austria
Date of Birth	01.04.1985

EDUCATION

year	1995- 2009
2009- October 2011	Master Microbiology and Immunobiology (Universität Wien)
2007-2009	Molecular Life Science (Universität Wien)
2005-2007	Molecular Life Science (Karl-Franzens-Universität Graz)
2004-2005	Biodiversity and Ecology (Karl-Franzens-Universität Graz)
1995-2004	Elementary School (WIKU)

WORK EXPERIENCE

year	2004- 2010
2010-2011	Vascular Biology/ Oncology, 12 months Diploma thesis; (Med. Universität Wien)
2008	Internship (Immoconsult Leasing)
2005	Technical Chemistry, 4 months Internship (Austriamikrosystems AG)
2004-2008	Promotion (Kronen Zeitung)
2004	Human Resources, Internship/ Holiday replacement (Austriamicrosystems AG)
2003- 2004	Promotion (Kleine Zeitung)
2002	Officemanagement, Internship (Austriamicrosystems)

PUBLICATIONS

Birbach et al. Persistent Inflammation Leads to Proliferative Neoplasia and Loss of Smooth Muscle Cells in a Prostate Tumor Model. Neoplasia, 11-524 (2011)

PERSONAL SKILLS AND TALENTS

LANGUAGES	German (first language) English (business fluent) Italian (basics)
RESIDENCE	
2000	3 weeks Eastbourne/England
2002	2 weeks Dublin/Ireland
SOCIAL SKILLS	2002-2004 ambulance (Rotes Kreuz)

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