TRANSGENE VERIFICATION AND TECHNICAL IMPROVEMENT IN THE ESTABLISHMENT OF TRANSGENIC MIR-32 MOUSE LINE

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Master's Thesis
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Preface

I want to thank the University of Tampere, BioMediTech and professor Tapio Visakorpi for providing me an opportunity to do my master thesis work in the Molecular Biology of Prostate Cancer group. I am especially thankful for my supervisor Leena Latonen, who was more than understanding during the summer I was working. Thanks to Leena for the preceding handling of the mice prostates and histopathology images, and to Marika Vähä-Jaakkola for sample preparation. I am also grateful for all the other people, who provided me help and guidance in the laboratory whenever it was needed. I express my thankfulness to Pirkanmaa hospital district for the financial support in my work. Finally, I want to thank my now already late father, who let me stay and sleep at his apartment the whole summer I was working and supported me in my work despite of his own condition. He always encouraged me to study and to have an education, and the day before he passed away I promised to finish this work and graduate.

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Abstract of Master´s Thesis

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Abstract: The main challenge to be faced with prostate cancer (PCa) is the segregation and treatment of patients with life-threatening disease. As current prognostic, diagnostic and therapeutic tools are insufficient, solution is tried to be found from new biomarkers. One of these could be microRNA miR-32, which is possible up-regulated target of androgen receptor in aggressive PCa. Btg2 and Klf2 have further been suggested to be potential down-regulated miR-32 targets. To study hypothesized miR-32 oncogenicity *in vivo*, transgenic mice over-expressing miR-32 specifically in the prostate are established. Establishment of transgenic mouse line is a prolonged process, in which transgene functionality needs to be verified. Additionally, more than one so called founder line needs to be created from the same gene construct to confirm the phenotype. At the time of this study one line with verified miR-32 expression was established and more founder lines were screened for. In the first part of this study, transgene functionality i.e. expression and its effect on possible target genes was examined with quantitative PCR in the prostate samples of one new founder line. In the second part of this study, the suitability of PaxGene method for future studies was piloted. Molecular fixative PaxGene was used for the first time for prostate fixation in a desire to preserve both tissue morphology and integrity of nucleic acids from the same sample enabling one prostate to be used for both histopathological examination and molecular analyses. Traditionally these have been done from different prostates, as morphology preserving fixation methods have degraded RNA. Molecular analyses have further been done separately from different prostate lobes. In the one prostate method not only financial benefits but also ethical improvements would be achieved, as mouse individuals can be spared. Importantly, the transgene-phenotype conclusions would be more reliable too. To study PaxGenes feasibility, its RNA preservation ability was assessed by surveying the RNA integrity with BioAnalyzer. In addition, as the whole prostate cross-section with different lobes and adjacent tissues were now included in the RNA extraction as well, different tissue and prostate markers were used to study the possible effects of these issues on transgene detection. Clear transgene expression was detected only in two samples, whereas in rest of the samples the expression was of low level. Further, down-regulation of neither Btg2 nor Klf2 was observed in the transgenic samples. However, the RNA integrity PaxGene provided was on the same sufficient level that the traditional Trizol method constantly provides. Prostate marker expression profiles reveal that varying prostate lobe ratios may affect to transgene detection and be the reason for the low transgene expression values. However, prostate adjacent tissues most likely do not disturb the detection. It is also highly possible that the PaxGene procedure itself does not have an influence, but instead transgene is silenced. As a conclusion, PaxGene is a possible choice of method for future studies, if constant transgene detection can be verified even with varying prostate lobe ratios. PaxGene provides sufficient intact RNA and thus it could be possible used in *in situ*-hybridization based expression detection as well. In any case, not all the transgenic samples here have the desired transgene over-expression and thus it can not be verified that the transgene functions properly. For this reason, additional samples need to be examined in subsequent founder lines. Only with verified transgene over-expression reliable conclusions of the connections between the transgene and PCa can be made. In addition, possible down-regulation of Btg2 and Klf2 should be examined again in subsequent samples, before any final conclusions of their role as miR-32 targets are made.

Keywords: Prostate cancer, microRNA, transgenic mouse, PaxGene

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Tiivistelmä: Suurin haaste eturauhassyöpään liittyen on erottaa ja hoitaa ne potilaat, joiden syöpä on henkeä uhkaava. Tämän hetkiset prognostiset, diagnostiset ja terapeuttiset menetelmät ovat riittämättömiä, minkä vuoksi ratkaisua yritetään etsiä uusista biomarkkereista. Yksi tällainen saattaa olla mikroRNA miR-32, joka mahdollisesti on androgeenireseptorin yli-ilmennetty kohdegeeni aggressiivisessa eturauhassyövässä. Tämän lisäksi Btg2 ja Klf2 ovat potentiaalisia ali-ilmennettyjä miR-32:n kohdegeenejä. miR-32:n mahdollista onkogeenisyyttä tutkitaan nyt *in vivo* transgeenisillä hiirillä, jotka yli-ilmentävät miR-32:ta spesifisesti eturauhasessa. Transgeenisen hiirilinjan luominen on pitkällinen prosessi, jossa transgeenin ilmeneminen tulee verifioida. Tämän lisäksi yhdestä transgeenikonstruktista tulee luoda useampi niin kutsuttu perustajalinja, jotta voidaan varmistua transgeenin fenotyypissä aiheuttamista muutoksista. Tämän tutkimuksen aikoihin transgeenin toimivuus oli varmistettu yhdestä perustajalinjasta ja seuraavia linjoja seulottiin. Tämän työn ensimmäisessä osassa tutkittiin transgeenin ilmentymistä ja sen vaikutusta mahdollisiin kohdegeeneihin kvantitatiivisen PCR:n avulla yhden uuden perustajalinjan hiirien eturauhasissa. Työn toisessa osassa testattiin PaxGene menetelmän soveltuvuutta jatkokäyttöä varten. Hiirten eturauhaset oli fiksattu PaxGene:n molekyylifiksatiivilla, joka lupaa säilöä sekä näytteen morfologian että nukleiinihapot tarkoittaen, että samaa eturauhasta voidaan käyttää sekä histopatologisiin että molekulaarisiin tutkimuksiin. Perinteisesti nämä tutkimukset on tehty eri eturauhasista, sillä morfologian säilöneet fiksaatiomenetelmät ovat hajottaneet RNA:n. Lisäksi molekulaariset tutkimukset on tehty jokaisesta eturauhaslohkosta erikseen. Yhden eturauhasen käyttäminen toisi taloudellisen edun lisäksi eettisiä etuja, kun käytettyjen hiiriyksilöiden määrä vähenisi. Tärkeä seikka on myös, että johtopäätökset transgeenin ja fenotyypin suhteesta olisivat luotettavampia kuin aiemmin. PaxGene:n käyttökelpoisuuden selvittämiseksi sen RNA:n säilömiskykyä arvioitiin tutkimalla RNA:n eheys BioAnalyzer:lla. Lisäksi eri kudos- ja eturauhasmarkkereiden avulla pyrittiin selvittämään vaikuttavatko eturauhasläpileikkeiden muut kudokset tai vaihtelevat eturauhaslohkokoostumukset transgeenin havainnointiin, sillä myös nämä olivat mukana RNA:n eristyksessä. Transgeenin selkeä ilmeneminen havaittiin vain kahdessa näytteessä, kun lopuissa näytteistä se oli hyvin matalaa. Transgeenisissä näytteissä ei myöskään havaittu Btg2:n tai Klf2:n alisäätelyä. Eheydeltään PaxGene näytteiden RNA oli kuitenkin samaa riittävää luokkaa kuin mitä Trizol jatkuvasti tarjoaa. Eturauhasmarkkereiden ekspressioprofiilit paljastavat, että vaihtelevat lohkokoostumukset saattavat vaikuttaa transgeenin havainnointiin ja olla syy matalille transgeenin ekspressioarvoille, mutta leikkeiden muut kudokset eivät kuitenkaan näyttäisi häiritsevän transgeenin detektiota. On kuitenkin myös hyvin mahdollista, ettei menettelytavalla ole vaikutusta havainnointiin, vaan transgeeni itsessään on hiljennetty. Johtopäätöksenä voidaan sanoa, että PaxGene on potentiaalinen menetelmä jatkoa ajatellen, mikäli transgeenin näkyminen pystytään varmentamaan, vaikka eri eturauhaslohkojen suhteelliset määrät vaihtelevat. PaxGene:llä saadaan joka tapauksessa riittävän eheää RNA:ta, minkä vuoksi kyseessä olevia näytteitä voisi mahdollisesti käyttää myös *in situ*-hybridisaatioon perustuvassa transgeenin havainnoinnissa. Loppujen lopuksi transgeenin ei voida kuitenkaan verifioida toimivan asianmukaisesti, sillä haluttu transgeenin yli-ilmeneminen ei näy kaikissa transgeenisissä näytteissä. Tämän vuoksi näytteitä on tutkittava lisää, sillä luotettavia johtopäätöksiä transgeenin ja eturauhassyövän yhteydestä voidaan tehdä ainoastaan transgeenin ollessa verifioitu. Lisäksi Btg2:n ja Klf2:n mahdollinen alisäätely tulisi tutkia uudelleen, ennen kuin lopullisia johtopäätöksiä näiden roolista miR-32:n kohdegeeneinä voidaan tehdä.

Avainsanat: Eturauhassyöpä, mikroRNA, transgeeninen hiiri, PaxGene

Table of Contents

Abbreviations

Adipoq Adiponectin

AR Androgen Receptor

PBSN Probasin

Btg2 B-cell translocation gene 2

Ckm Creatine kinase, muscle

Cnn1 Calponin 1

Klf2 Krüppel-like Factor 2

Krt18 Keratin 18

miRNA Micro-RNA

PCa Prostate cancer

PSA Prostate specific antigen

PSP94 Prostatic secretory protein-94

PTEN Phosphatase and Tensin homolog

qPCR Quantitative polymerase chain reaction

TBP TATA-binding protein

1. Introduction

Prostate cancer (PCa) remains one of the most common cancers among men (Globocan 2012: Estimated Cancer Incidence, Mortality and Prevalence Worldwide in 2012). Despite of its frequency, it does not cause deaths in the same prevalence. The main reason for this disparity is that most of the patients are older men, whose disease stays moderate during their lifetime and does not evolve into life-threatening form (Damber & Aus 2008; Shen & Abate-Shen 2010). However, part of the patients develop an aggressive and life-threatening cancer, and thus the main challenge remains to be in distinguishing and treating particularly these patients.

Currently, widely used prostate specific antigen (PSA) testing reveals cancer cases earlier and more than before, but its use as an aggressiveness predictor together with Gleason score grading system is insufficient (Damber & Aus 2008; Shen & Abate-Shen 2010). PSA testing has indeed increased the incidence rate, but studies have been controversial whether it has decreased the mortality significantly. Instead, over-diagnosis and over-treatment of low-risk patients are the main concerns of PSA testing, as the life quality of these patients suffers more from diagnostic and treatment side-effects than the disease itself (Loeb et al 2014). Thus, present general opinion seems to incline towards not to use PSA testing for a large scale PCa screening, but despite of its deficiencies it still is an essential part of diagnosis and prognosis, as no substitutive method is available (LeFevre & Moyer 2013; Loeb et al 2014). Consequently, better and more accurate diagnostic and especially prognostic tools revealing the aggressive cancer cases early on are urgently needed (Damber & Aus 2008; Shen & Abate-Shen 2010).

The best therapeutic tool for PCa is prostatectomy, which means surgical removal of the prostate (Damber & Aus 2008). However, this removal can only be accomplished, if the cancer is found early enough. Unfortunately, more aggressive cases, meaning typically castration resistant prostate cancer, which is an advanced stage of the disease, are often not

curable (Shen & Abate-Shen 2010). Thus, in addition to new prognostic and diagnostic tools, also new curable therapeutic tools for life-threatening cancer cases are needed. To fulfill these needs, one has to better understand the molecular pathways behind the PCa development and progression. Only that way novel biomarkers i.e. tools for prognosis, diagnosis and therapy can be found (Bickers & Aukim-Hastie 2009; Liu et al 2012).

Prostate together with seminal vesicles and bulbourethral glands form the accessory sexual glands which belong to a male reproductive system (figure 1) (Campbell et al 2008, pp 373-374). The function of these accessory glands is to produce the nutritional fluid for semen, which helps to keep the spermatozoa vital and thus contributes to fertilization. The human prostate is a size of a walnut and it lies just under the bladder and in front of the rectum (figure 1) (Oh et al 2003). The composition of it is fibromuscular stroma and branching glands with two different kinds of epithelial cells, basal and secretory, the latter releasing the excretions into the ducts of the prostate. Additionally, periprostatic adipose tissue surrounds the prostate (Ribeiro et al 2012).

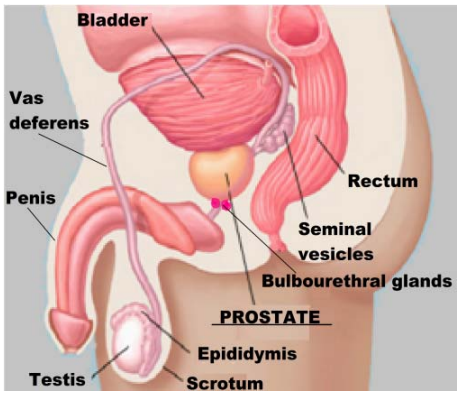

Figure 1. Prostate location in male reproductive system. (modified from WebMD)

Cancer develops when cells lose their normal growth control and become malignant (Campbell et al 2008, pp 373-374). Typical consequence is an apparent tumor which has the potential to spread in the body. At molecular level, several genetic alterations in so called proto-oncogenes and/or tumor suppressor genes are the fundamental reason for

cancer. PCa results from malignant epithelial cell growth and behind the malignancy is an extensive mutational heterogeneity (Barbieri et al 2013). In addition to some common and well-known cancer mutations which disturb e.g. cell cycle regulation, prostate-specific defects have been identified behind the PCa. Those affecting the androgen signaling pathway have especially a major role.

Androgens are male sex hormones which are produced mainly in the testicles (Feldman & Feldman 2001). One of their normal functions is to regulate the differentiation and survival of prostate cells by binding to androgen receptor (AR), which acts as a transcription factor (figure 2). In other words, the ligand-receptor complex binds to androgen responsive elements in DNA and regulates transcription of target genes, which are responsible for growth and survival of prostate cells. Defects in this regulation system are of great importance not only in the PCa development, but in the progression as well (Barbieri et al 2013). Lesions like gene amplification, point mutations and abnormal splice variants of the AR are the reason for active signaling in metastatic castration resistant prostate cancer, an advanced stage of the disease, in which a patient has become resistant to androgen ablation therapy i.e. castration, and metastases have occurred (Barbieri et al 2013; Feldman & Feldman 2001). Castration is a typically used method for advanced PCa and it usually efficiently regress progression of the disease for a while, as the growth of the cancer is dependable on androgens (Damber & Aus 2008; Feldman & Feldman 2001). The norm however seems to be that sooner or later the resistance appears, after which hormone therapy is useless and PCa tends to continue to progress predicting short life expectancy. AR lesions have been found to be almost totally absent in localized PCas and thus it is highly plausible that they only emerge in response to hormonal therapy and do not play a role in cancer initiation (Barbieri et al 2013).

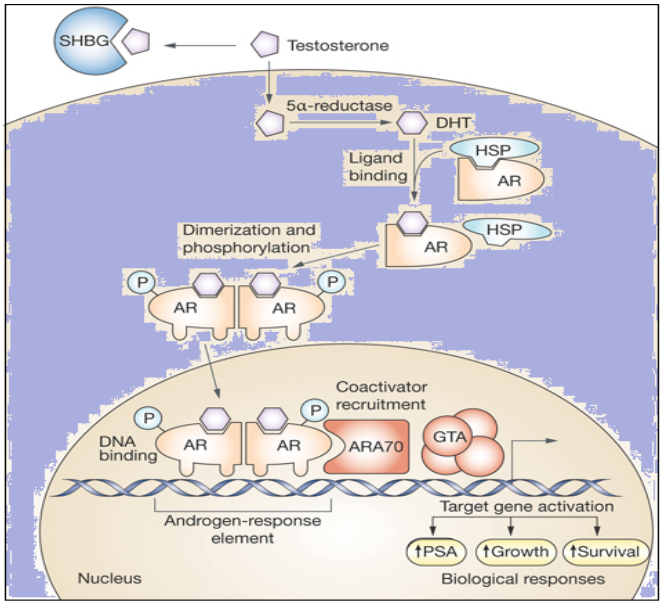

Figure 2. Androgen signaling pathway in prostate cells. Androgen hormone testosterone is transformed in a form of dihydrotestosterone before it can bind to androgen receptor. (modified from Harris et al 2009)

Despite of now knowing the significant role of AR signaling pathway in the development and progression of PCa, the key target genes of AR, that drive the formation of certain cancer types, are poorly understood (Jalava et al 2012; Jariwala et al 2007). Some AR targets are well known, like ACPP, KLK3, TMPRSS2:ERG and NKX3-1, but their expressions do not significantly differ from each other in local and advanced cancer cases (Jalava et al 2012; Waltering 2010). Identified key targets could serve as potential future biomarkers and therapeutic targets for aggressive disease, which raises the probability that PCa one day is a disease under control. Research is ongoing in this area and certain micro-RNAs (miRNAs) have been demonstrated to be androgen regulated and possible targets of AR (Waltering et al 2011). Micro-RNAs are small non-coding RNAs, which function as gene expression regulators through mRNA degradation and translation inhibition and they influence in cell growth and survival process (Bushati & Cohen 2007; Kim 2005). Since the discovery of miRNAs, there has been a growing evidence of their role in cancers as oncogenes and tumor suppressors. Clinical and *in vitro* studies have suggested that androgen-regulated microRNA miR-32 is up-regulated in castration resistant prostate cancer and involved in the cancer progression (Jalava et al 2012). In addition, Btg2 (B-cell translocation gene 2) has been shown to be a possible target of miR-32 and down-regulated

in PCa and castration resistant prostate cancer. Thus, preliminary studies suggest that miR-32 is an oncogene in PCa. Consequently, the research has been taken into a next level to study the possible oncogenicity *in vivo* and for this purpose transgenic mice, expressing miR-32 specifically in the prostate epithelium of post-puberty mice, are established.

Despite of some deficiencies, mice are one of the best animals to model human cancers and thus also widely used in prostate cancer research (Valkenburg & Williams 2011; Abate-Shen & Shen 2002). Mice offer the opportunity to study the genetic alterations, initially found in *in vitro* studies, in living organisms and they are, no doubt, irreplaceable in finding new therapies for currently lethal human diseases. Naturally, mice are never entirely equivalent to human and for instance the anatomy of mouse and human prostate is different. Mouse prostate consists of four different lobes; ventral, dorsal, lateral (=core prostate) and anterior, whereas human prostate lacks the lobular structure, but is divided in three different zones; central, transitional and peripheral (figure 3). Mouse dorsolateral lobe has been said to be most similar with human peripheral zone, in which majority of human PCas are found. However, no consensus exists of this. According to one classification PCa mouse models can be divided in five categories: xenograft models, transgenic T antigen models, other transgenic models, traditional knockout models and conditional knockout models (Valkenburg & Williams 2011). The last four are considered as genetically engineered mouse models. Transgenic mice represent a model, in which foreign DNA, for instance a potential oncogene, is transferred into the genome of a mouse to examine the influence of its expression.

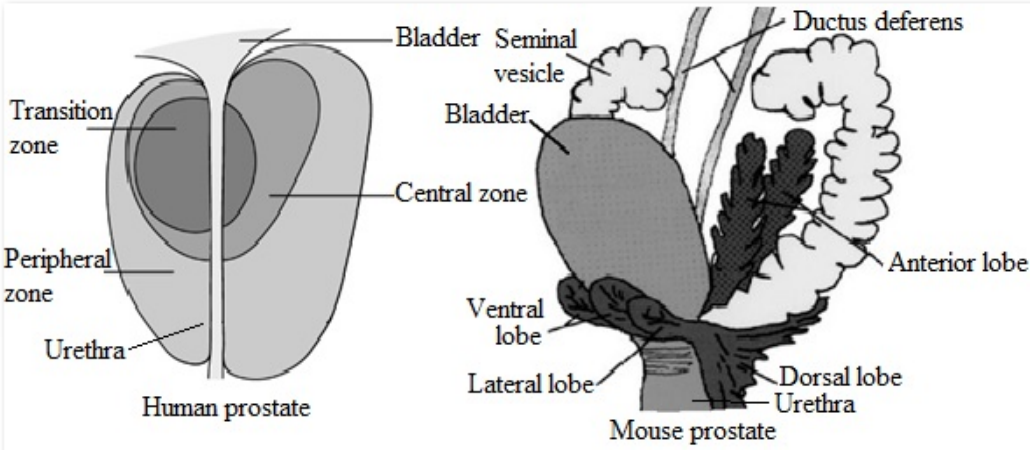

Figure 3. Anatomical structures of human and mouse prostate. (modified from Canadian Cancer Society website; Valkenburg & Williams 2011)

All in all, establishment of transgenic mice is a prolonged process, in which transgene functionality needs to be verified (Cho et al 2009; Haruyama et al 2009, Gordon 1997). First of all, if tissue specific expression is the aim, transgene is located in a plasmid under a tissue specific promoter. Next, in pronuclear microinjection method the purified and linearized gene construct is microinjected into the male pronucleus and the zygotes are implanted into a foster mother mouse. After that the born mice are genotyped to identify the founder mice (F0), which carry the transgene. The founder mice are crossbred with wild-type mice to create different founder lines with unique insertion sites. The wild-type strain used for crossbreeding is recommended to be the same from which the founders are generated to avoid mixed backgrounds, as different backgrounds may result in different phenotypes. Next, transmission of the transgene into the next generations (F1, F2 and so on) as well as proper spatial and temporal expression is verified. Occasionally, founder mice are mosaic, in which case transgene transmission via germline might fail. Mosaic founder mice are only partially transgenic i.e. only part of their cells has the transgene due to unoptimal microinjection timing and thus, if the germline lacks the transgene, it will not proceed into next generations. However, most of the founders are presumed to transfer the transgene forward. Typically, a minimum of two or three founder lines generated from the same gene construct are established and analyzed to confirm the phenotype. If more than one line shows similar phenotype, it is more unlikely that the phenotype is a result of unknown mutation possible arising from the random integration of the transgene. In

addition to mosaicism and random integration, several other things, like gene silencing, may complicate the desired function of the transgene and thus establishment of transgenic mouse lines.

During the course of this study, one transgenic mouse line with verified miR-32 expression in the prostate was established and at that time more founder lines were screened for. The prostate specific expression of miR-32 was achieved with a rat probasin (PBSN) promoter androgen-responsively. Probasin is a gene that functions in prostate only and thus its regulatory elements can be exploited to achieve prostate specific transgene expression (Johnson et al 2000). At the same time it serves as a good marker of prostate differentiation and for elucidation of androgen action. Here, I studied the expression levels of miR-32 from the prostate cross-sections of founder line 036 F2 and F3 generation mice to observe whether the transgene was expressed in the first place. Potential down-regulation of Btg2 and Klf2 (Krüppel-like Factor 2), which are likely target genes of miR-32 and functionally related to the cancer, was determined as well. F2 mice were derived from cross-breeding transgenic heterozygous miR-32 F1 mouse with wild type mouse, whereas F3 mice were derived from cross-breeding heterozygous miR-32 F2 mouse with heterozygous PTEN (Phosphatase and Tensin homolog) knockout mouse. PTEN is a tumor suppressor gene and thus breeding with PTEN knockout mice provokes premalignant prostatic intraepithelial neoplasia lesions in mice, in which they usually appear with low frequency (Wang et al 2003; Di Cristofano et al 1998). PTEN expression in F3 was studied to assess the knockout success. The samples in this study were treated for the first time with a different, so called PaxGene, method, and here I also piloted the suitability of this method for future studies. Until the time of this study, histopathology studies were done from different samples, meaning different prostates in the case of mice due to small size of the prostate, than gene expression studies, as good morphology requires the use of fixation methods, which as for do not preserve RNA. Naturally, this is not ideal as the observed expression is not totally equivalent to the observed phenotype. Now, PaxGene method was tested for the first time in a desire to find a solution to this problem. So called molecular fixative by PaxGene (PreAnalytiX) should not degrade RNA like typically used formaldehyde does. Thus, it should preserve both tissue morphology and the integrity of nucleic acids allowing both

histopathological and molecular analyses from the same tissue specimens i.e. from the same prostate in this case. Prostates of the mice were removed at post-puberty age of three months, cut and then placed in the molecular fixative, after which they were stabilized, paraffin embedded and sectioned. Part of the sections was hematoxylin + eosin stained and imaged to examine possible neoplastic or other prostatic lesions and other part was kept for molecular analysis. Here, I used the latter for the expression analyses and RNA preservation examination. Previously, molecular analysis was done separately from different prostate lobes, but now with the PaxGene method the whole cross-section of core prostate with different lobes and other adjacent tissues was part of the molecular analysis as well because of the histopathological requirements (figure 4). In addition to prostate, composed of epithelium, stroma and adipose tissue, cross-sections were predicted to contain also smooth and skeletal muscle, the former arising from bladder and urethra and the latter from urethra (Andersson & Arner 2004). It was of concern whether transgene expression was intense enough to be clearly detected despite of the other tissues in the sections. In other words, it was of concern whether transgenic miR-32 expression could arise clearly above the possible intrinsic expression of the other tissues, so that a clear difference in the expression between the sample and control group could be observed. It was also of interest to know whether tissue and prostate lobe ratios vary between the samples, depending on the site of the prostate the sample was taken, and whether this variance affect to transgene detection. For this purpose different prostate and tissue markers were analyzed. At the time of this study it was thought that, if RNA and tissue morphology are preserved, and transgene expression is intense enough, PaxGene could become a substitutive method for future studies. The interest was to have a simpler procedure, save time and spare mouse individuals, as PaxGene enables the use of one prostate instead of two. The observed transgene expression would be also more equivalent to the observed phenotype, which would make the transgene-phenotype relationship more reliable.

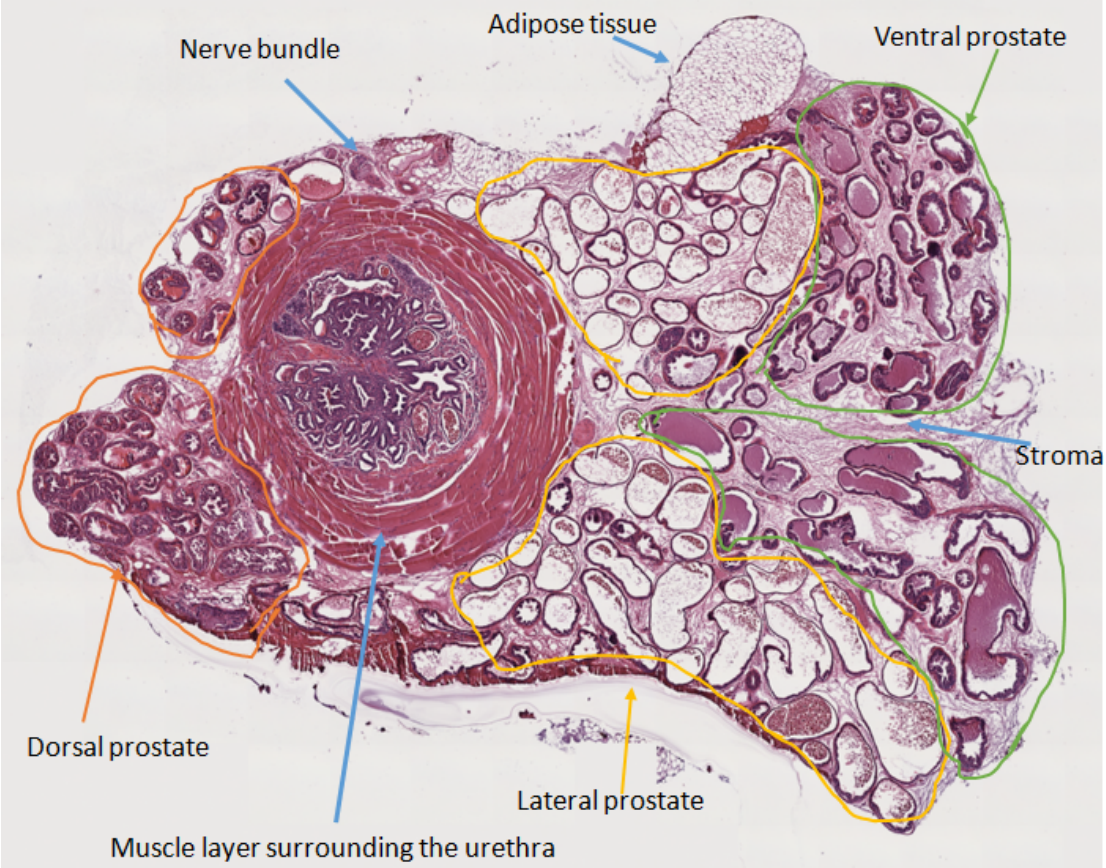

Figure 4. Cross-section of the whole PaxGene-fixed and paraffin-embedded mouse prostate with different lobes and adjacent tissues. Image courtesy of LL.

2. Aims of the Study

This study had two main aims. The first one was to examine transgene expression and its influence on potential target genes Btg2 and Klf2. In other words it was studied whether the transgene was functional and whether the possible targets of miR-32 were down-regulated in the case of transgene over-expression. The second aim was to pilot the suitability of new method, PaxGene, for future studies in a desire of receiving better, more efficient and mouse sparing technique. Thus, it was surveyed whether PaxGene preserved RNA and whether transgene expression was intense enough to be clearly detected even though RNA now was extracted from the whole prostate instead of prostate lobes, in which case also other tissues were included in the cross-sections. Different prostate and tissue

marker expressions were examined to assess whether this procedure affect on and vary the transgene gene expression results. The suitability of PaxGene for tissue morphology preservation was studied elsewhere.

3. Materials and Methods

3.1. Mice

3.1.1. Ethical issues

The use of mice material in this study was approved by animal experiment permission number ESAVI/6271.

3.1.2. Sample material

The prostate material i.e. mainly prostate core, including ventral, dorsal and lateral lobes, was derived from the founderline UTU 186/036 F2 and F3 generation mice individuals with FVB/N strain background. All together seven F2 mice samples were investigated; four of them were transgene miR-32 heterozygous and three of them were controls for the former with transgene negativity. Heterozygous F2 miR-32 mice and heterozygous PTEN knockout mice were cross-bred to receive F3 generation with four different types of genetics. Total of 15 F3 mice samples were investigated; three of them were transgene miR-32 heterozygous and PTEN homozygous, and four of them were controls for the former with transgene negativity and PTEN homozygosity. Five F3 mice samples were transgene miR-32 and PTEN heterozygotes, and three were controls for the former with transgene negativity and PTEN heterozygosity. Mice breeding and crossing was

accomplished by personnel in the University of Turku. Additionally, resectioning, fixing and stabilizing the prostates as well as tissue embedding and sectioning were accomplished by others. Every tenth paraffin section was transferred into an eppendorf tube for RNA extraction and each tube contained 10 sections and one prostate was divided in more than one tube. The tubes were maintained in the freezer until the time of RNA extraction. Rest of the sections was transferred onto glasses for histopathology examination, which was accomplished by others. All together three adjacent sections were put on one glass, and at that stage only one glass was HE-stained and imaged, and the other two glasses were kept for other stainings. HE-stained sections were adjacent to the RNA extraction section. See schematic flow of the procedure in figure 5.

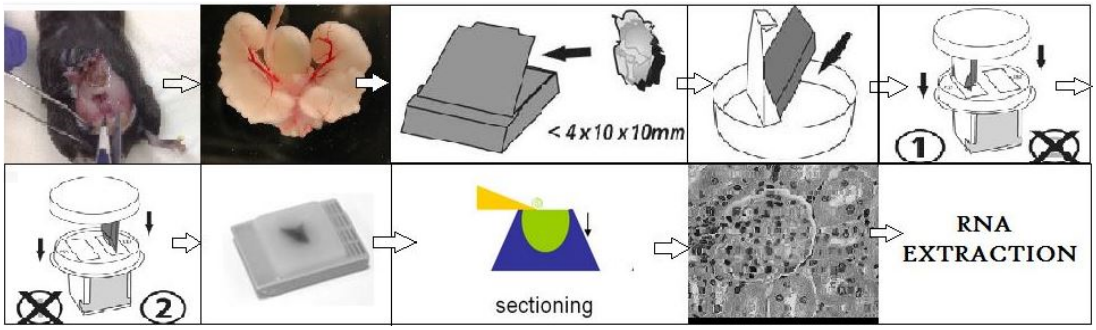

Figure 5. Procedure from prostate resectioning until the time of RNA extraction. Resectioned prostate was cut in suitable pieces and placed into a tissue cassette. The cassette was attached to a rack and placed into PaxGene Tissue Containers first chamber, which contained the molecular fixative. After fixation, the rack was transferred into a second chamber, which contained the stabilizer. Finally, the tissue specimen was paraffin embedded and sectioned. One part of the sections was HE-stained and imaged, one part was kept for other stainings and rest was freezed until the time of RNA extraction. (modified from youtube video; Visible Mouse Project website; PAXgene Tissue Container Product Circular handbook 2013 and Max Planck Institute of Neurobiology website)

3.1.3. Control tissues

Bladder (containing smooth muscle), ventral prostate (epithelium and stroma), adipose tissue and skeletal muscle served as control tissues in the study. Tissues were kept in a RNA preserving liquid (RNALater) in a fridge until the time of RNA extraction. Control tissues were used in this study for qPCR (quantitative polymerase chain reaction) standard

in expression analyses of tissue markers. Additionally, miR-32 expression was studied from these tissues, and one of them, ventral prostate, was used in Bioanalyzer RNA integrity analysis.

3.2. RNA extraction

3.2.1. PaxGene protocol

The total RNA, including miRNA, of the paraffin embedded sample prostate tissue sections was isolated and purified with the PaxGene™ Tissue miRNA Kit (PreAnalytiX QIAGEN/BD Company, Lot No. 142331209) according to the PaxGene® Tissue miRNA Kit Handbook (PreAnalytiX QIAGEN/BD Company 10/2010). The kit is intended to be used in association with PaxGene Tissue Container system, and binding and washing conditions of isolation and purification are optimized to receive all RNA molecules longer than 18 nucleotides, including miRNAs, with high purity. After the isolation, concentration and purity of total RNA was determined with NanoDrop ND-1000 spectrophometer. The long-term storage of the isolated RNA took place in – 80 ^{o}C freezer.

3.2.2. Trizol protocol

The total RNAs of the control tissues were isolated with TRIzol® Reagent (Life Technologies) according to manufacturer's instructions. Concentration and purity measurements as well as long-term storage of the RNAs were performed as above.

3.3. RNA quality

3.3.1. Purity

Purity of all the extracted total RNAs was determined with NanoDrop ND-1000 spectrophotometer. Absorbance maximum of nucleic acids is at 260 nm, and ratio A260/A280 is generally used to estimate the purity of both DNA and RNA isolations (Thermo Scientific NanoDrop Technical Bulletins 2012 & 2011).

3.3.2. Integrity

The Agilent 2100 bioanalyzer together with Agilent RNA 6000 NanoKit and Expert 2100 software was used according to the Agilent RNA 6000 NanoKit Quick Start Guide (by Agilent Technologies) to determine the integrity of selected PaxGene extracted RNAs. Also Trizol extracted RNA from ventral prostate (G) was included in the integrity analysis as for point of comparison. The highest amount of RNA was taken into account when the samples were selected for BioAnalyzer quality analysis.

The principle of BioAnalyzer is basicly same as the one of slab gel electrophoresis (Agilent 2100 Bioanalyzer Expert User's Guide 2005). With BioAnalyzer samples are loaded in a special chip with gel, ladder and fluorescence dye, after which the chip is placed into a power supply of the equipment. Voltage gradient drives charged biomolecules, like RNA, electrophoretically towards opposite charge resulting in biomolecule separation by size, as smaller fragments migrate faster and thus further than the larger ones. The biomolecules are connected with dyes, which fluoresce when laser-beam hits them, and this fluorescence data is transformed into informative electropherograms and gel-like images. RNA integrity can be evaluated from the peaks of

electropherograms, but so called RIN (RNA Integrity Number) application has been specifically developed to facilitate and to standardize integrity assessment (Mueller et al 2004). This application automatically grades intactness of the RNA in a scale 1-10, 1 meaning totally degraded RNA and 10 totally intact RNA. Thus subjective person dependent variation in evaluation can be minimized. However, the software creates RIN values from the electrophoretic trace and for this reason it is important to understand the electropherograms as well. Attention should be paid in certain regions and peaks, which are related to RNA integrity (figure 6). The more intact the RNA, the more the electropherogram reminds the uppermost in figure 7. Two intensive and clearly detectable peaks, which indicate ribosomal subunits 18S and 28S, are seen, whereas other regions intensities are of low level. Vice versa, more degraded the RNA, more intensive other regions than 18S and 28S become (figure 7, middle electropherogram). In addition, peak intensity increases towards left when degradation proceeds as smaller fragments become more and more abundant (figure 7, bottom electropherogram).

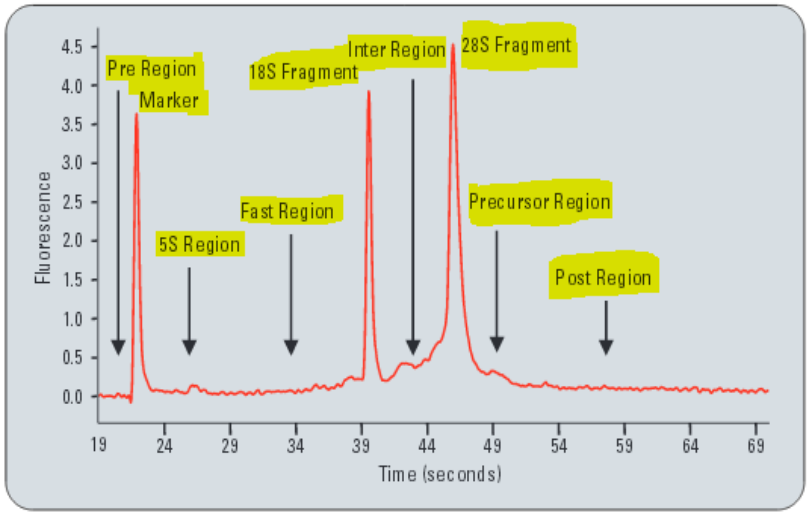

Figure 6. RNA integrity can be interpreted from certain regions and peaks of electropherogram, which shows fluorescence in a function of time. More intensive the fluorescence, more abundant the certain size RNA fragment is. The smaller the fragment, the faster it appears in the electropherogram, as smaller fragments migrate fastest and furthest in the gel. Especially the intensities of 18S and 28S ribosomal fragments are indicative of RNA integrity. (Modified from Mueller et al 2004)

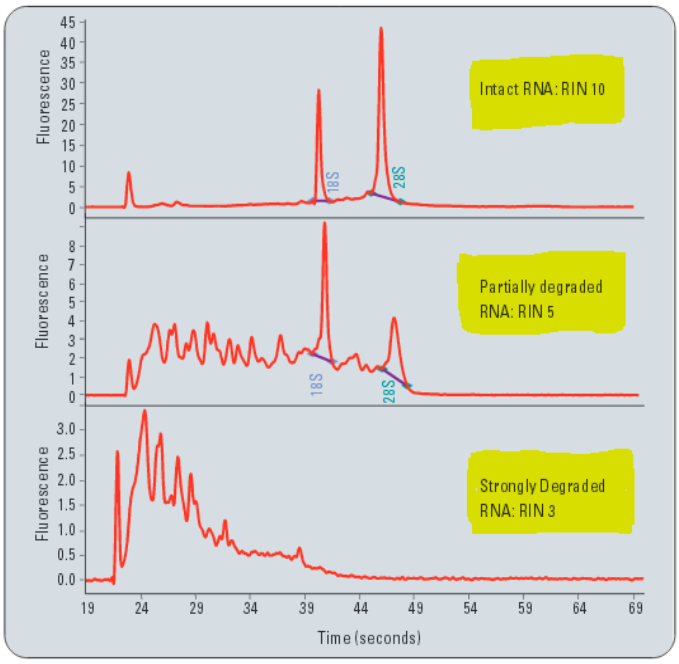

Figure 7. Electropherograms indicating different levels of RNA integrity. More degraded the RNA, more intensive the peaks are at the beginning of the electropherogram, as bigger subunits 18S and 28S degrade into smaller fragments. (Modified from Mueller et al 2004)

3.4. cDNA synthesis, qPCR and agarose gel electrophoresis

3.4.1. MicroRNA

MicroRNAs miR-32 and RNU6B of every extracted RNA, both sample and control tissues, were reverse transcribed to cDNA with the TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems by Life Technologies, Lot No. 1203074) and then used in qPCR. RNU6B served as a housekeeping gene in the expression analysis of miR-32. Both phases were performed according to the TaqMan® MicroRNA Assays Protocol (Applied Biosystems by Life Technologies 01/2011). 22Rv1 RNA (extracted previously) was reverse transcribed as well to receive standard for qPCR.

3.4.2. Other RNA

Sample RNA for other expression analyses in this study was reverse transcribed to cDNA with Maxima RT (Thermo Scientific) according to manufacturer's instructions, whereas qPCR was performed with SYBRGreen (Fermentas) according to manufacturer's instructions. Expression analyses were accomplished from prostate markers PBSN and PSP94 (prostatic secretory protein-94), prospective miR-32 targets Btg2 and Klf2, PTEN and finally from tissue markers Krt18 (keratin 18), Cnn1 (calponin 1), Ckm (creatine kinase, muscle) and Adipoq (adiponectin). β-actin and TBP (TATA-binding protein) served as housekeeping genes. Primers (Oligomer Oy) of the targets mentioned above are shown in table 1. To verify purity of the products after qPCR, they were run on 1 % agarose gel and imaged with a Syngene imaging device.

Table 1. Primer sequences for genes examined in this study.

Target	Primer
PBSN	mPbsn_F (5'-TGACGGGCCTTGGCAAACAA-3') mPbsn_R (5'-TGCCAAAACTCCAGCACTCGT-3')
PSP94	mPSP94_F (5'-TTGGGCCCACACGAAGCACAT-3') mPSP94_R (5'-GGAAGCTTGGCTGGGCAGTCT-3')
Btg2	mBtg2_F (5'-GTGGGTTTCCTCTCCAGTCT-3') mBtg2_R (5'-CGGATACAGCGATAGCCAGA-3')
Klf2	mKlf2_F (5'-CACCAAGAGCTCGCACCTAA-3') mKlf2_R (5'-GGCACTGAAAGGGTCTGTGA-3')
PTEN	mPten_ex5_F (5'-GGCACAAGAGGCCCTAGATT-3') mPten_ex7b_R (5'-TCACCTTTAGCTGGCAGACC-3')
Krt18	mKrt18_F (5'-CCGGGAACATCTGGAGAAGAA-3') mKrt18_R (5'-ACTGGCGCATGGCTAGTTC-3')
Cnn1	mCnn1_F (5'-GTTGCGCTTGTCTGTGTCAT-3') mCnn1_R (5'-CTCCCGCTGATGGTCGTATT-3')
Adipoq	mAdipoq_F (5'-TGTCAGTGGATCTGACGACAC-3') mAdipoq_R (5'-TGCCATCCAACCTGCACAAG-3')
β-actin	mBACTIN_ex4_F (5'-CGAGCGGTTCCGATGCCCTG-3') mBACTIN_ex6_R (5'-ACGCAGCTCAGTAACAGTCCGC-3')
TBP	mTBP_ex5b_F (5'-AGCTCTGGAATTGTACCGCA-3') mTBP_ex7_R (5'-AATCAACGCAGTTGTCCGTG-3')

4. Results and Conclusions

4.1. RNA quality

4.1.1. Purity

260/280 purity values obtained with NanoDrop spectrophometer range from 1.63 to 2.19 (mean 2.05) in PaxGene extracted samples, whereas purity value for Trizol extracted RNA is 1.94 (table 1). Only one RNA extraction (mouse 81) seems not to be acceptable pure, as the 260/280 ratio shows value of 1.63. Thus, it is possible that not all the chemicals used in the extraction procedure were removed sufficiently, or alternatively some protein had remained in the sample. Outside of this one exception, the purity was in acceptable level. In addition, there was no difference in the purity of PaxGene extracted RNAs and Trizol extracted RNAs.

4.1.2. Integrity

PaxGene extracted RNA RIN-values range from 3.80 to 5.00 (mean value 4.37), whereas Trizol extracted RNA show RIN value of 5.10 (table 2 and figure 8). Trizol provides traditionally RIN-values of 4-6 and therefore the Trizol RIN-value obtained here is alike. RIN-value of 3 represents strongly degraded RNA and 5 partially degraded RNA, as shown previously in figure 6, and therefore the RIN values obtained in this study fall approximately in between these definitions. Electropherograms seem to be consistent with the RIN values, as fluorescence intensities of smaller fragments have increased, whereas intensities of 18S and 28S ribosomal subunits have decreased (figure 8). So, all the extracted RNAs were at least partially degraded. However, it is known that Trizol provides consistently RNA of uniform and sufficient quality for used downstream applications, in

which case it can be concluded that the RNA preservation ability of PaxGene is as good as the one of Trizols, as the RIN values are greatly alike.

Table 2. The purity and integrity of the extracted RNAs. 260/280 values of ~ 2 indicate pure RNA, whereas RIN values indicate RNA intactness in a scale 1-10, 1 for completely degraded RNA and 10 for intact RNA.

		260/280	RIN
PaxGene	**mouse 361**	2.10	
	mouse 362	2.10	4.00
	mouse 363	2.00	
	mouse 364	2.04	
	mouse 365	2.09	
	mouse 366	2.18	
	mouse 367	2.19	4.10
	mouse 62	2.13	
	mouse 63	2.15	
	mouse 64	2.15	4.80
	mouse 65	1.83	
	mouse 75	1.99	5.00
	mouse 76	2.03	
	mouse 78	2.06	4.50
	mouse 79	1.96	
	mouse 80	2.23	
	mouse 81	1.63	
	mouse 86	2.07	
	mouse 87	1.90	3.80
	mouse 88	2.07	
	mouse 89	2.07	
	mouse 90	2.10	
	Mean	2.05	4.37
Trizol	**Ventral prostate**	1.94	5.10
	Fat	1.93	
	Bladder	1.93	
	Skeletal Muscle	1.95	

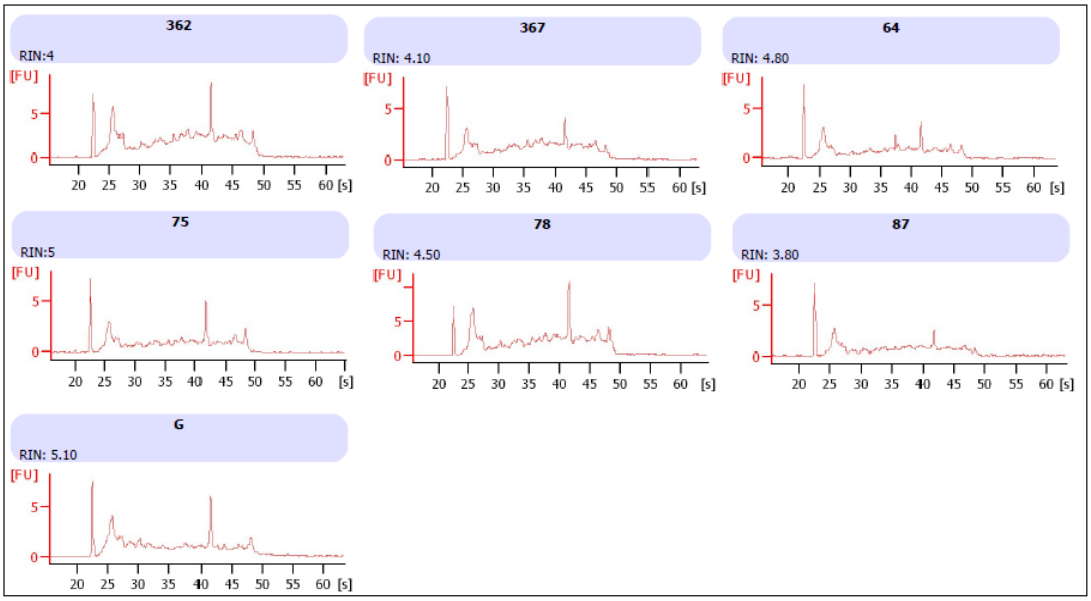

Figure 8. Electropherograms and corresponding RIN values of the extracted RNAs, which were included in the BioAnalyzer quality assessment.

4.2. Tissue Markers

Different tissue marker expressions were studied to see how much the samples differentiated from each other by the relative amounts of prostate epithelium, smooth muscle, skeletal muscle and adipose tissue and to see whether these tissues could affect to the transgene expression detection. Tissue marker Krt 18 is a one type of keratin gene family and it is expressed in simple epithelia, for instance in glandular cells of male tissues and thus it can be used as a marker for presence of this kind of cells (The Human Protein Atlas). Cnn 1 instead participates specifically in smooth muscle contraction regulation and modulation and thus can be used as a marker for the presence of smooth muscle (UniProt[1]). Tissue marker Ckm is a protein kinase, which transfers phosphate between ATP and creatine and plays an important role i.e. in skeletal muscle and thus may indicate the presence of this tissue (UniProt[2]). Finally, Adipoq is involved in fat metabolism and is exclusively synthesized by adipocytes and thus presence of adipoq indicates the presence of adipose tissue (UniProt3). The expressions of all of the tissue markers vary between the samples meaning that there is variation in the tissue composition of different samples

(figures 9-12). Most varying components seem to be skeletal muscle and adipose tissue, whereas epithelium and smooth muscle appear in more constant fashion (table 3). Highest expressions of Krt18 indicate relatively large epithelium component in the extracted sections and highest expressions of Cnn1, Ckm and Adipoq relatively large smooth muscle, skeletal muscle and adipose tissue component, respectively. Different tissues of prostate cross-sections are illustrated in few HE-stained images (figures 13 and 14).

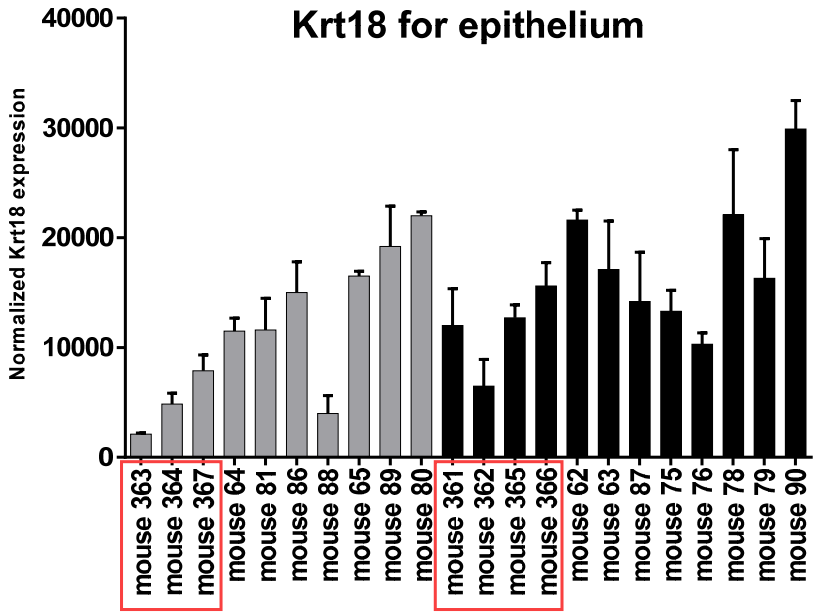

Figure 9. Normalized Krt18 expression values indicate the relative presence of prostate epithelium in different prostate samples. Normalization was done against TBP + β-actin. Grey=control group, black=transgenic group. Red box=F2 generation, no box=F3 generation.

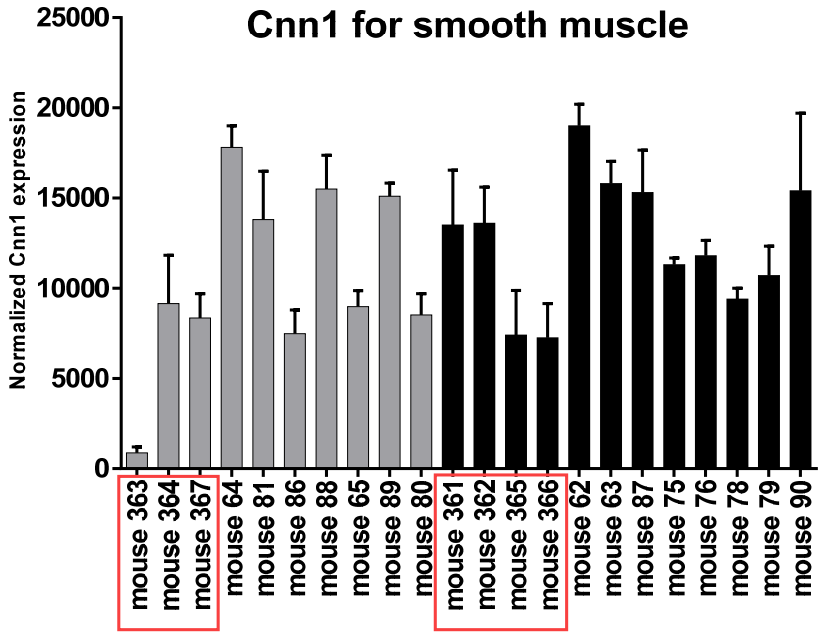

Figure 10. Normalized Cnn1 expression values indicate the relative presence of smooth muscle in different prostate samples. Normalization was done against TBP + β-actin. Grey=control group, black=transgenic group. Red box=F2 generation, no box=F3 generation.

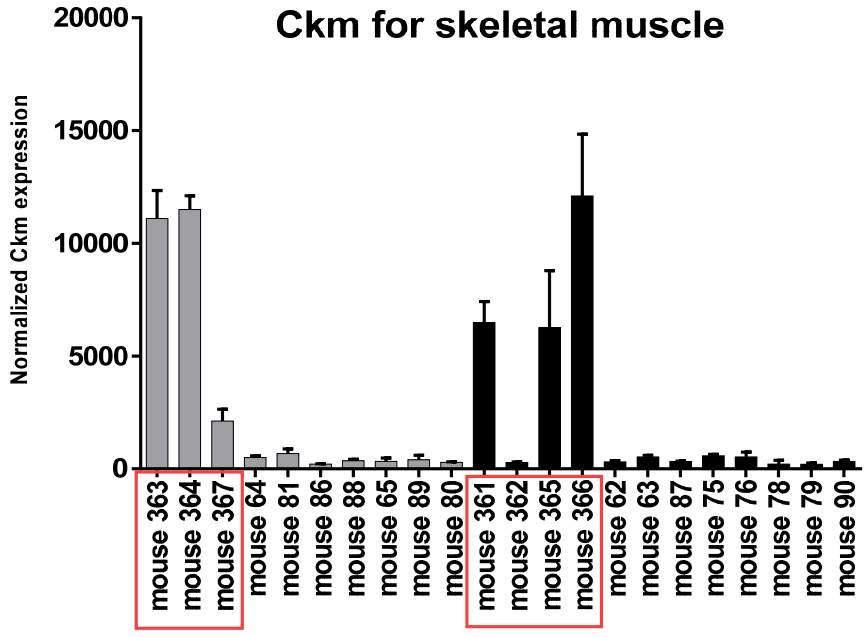

Figure 11. Normalized Ckm expression values indicate the relative presence of skeletal muscle in different prostate samples. Normalization was done against TBP + β-actin. Grey=control group, black=transgenic group. Red box=F2 generation, no box=F3 generation.

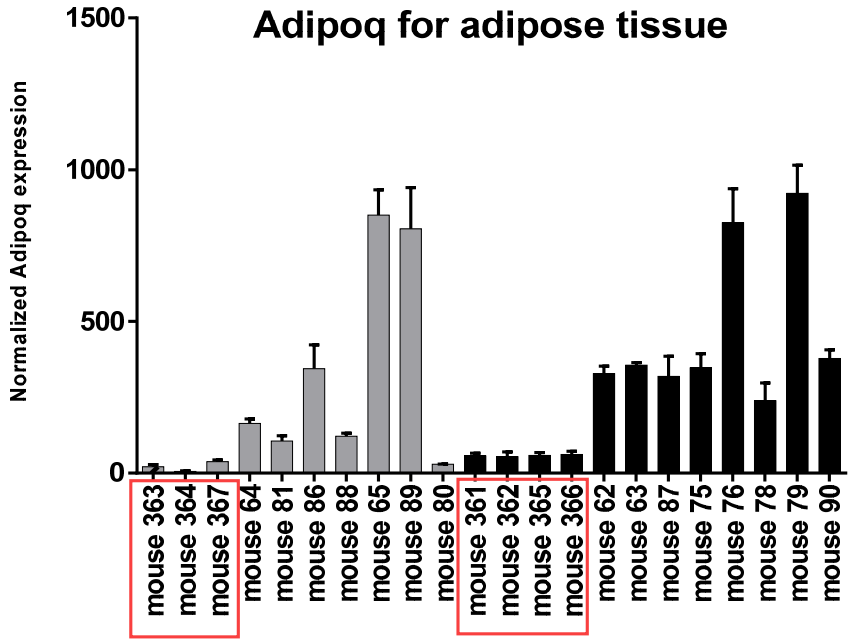

Figure 12. Normalized Adipoq expression values indicate the relative presence of adipose tissue in different prostate samples. Normalization was done against TBP + β-actin. Grey=control group, black=transgenic group. Red box=F2 generation, no box=F3 generation.

Table 3. Standard deviation (SD) values of different tissue markers indicate that there is variation in the amounts of predicted tissue types between the samples. Percentual SD values indicate that the amounts of skeletal muscle (Ckm) and adipose tissue (Adipoq) vary most in the samples, whereas the proportions of epithelium (Krt18) and smooth muscle (Cnn1) are more constant across the samples.

Tissue marker	Average expression	Standard deviation (SD)	% SD
Krt18	13900	6705	48 %
Cnn1	11600	4292	37 %
Ckm	2530	4080	161 %
Adipoq	292	298	102 %

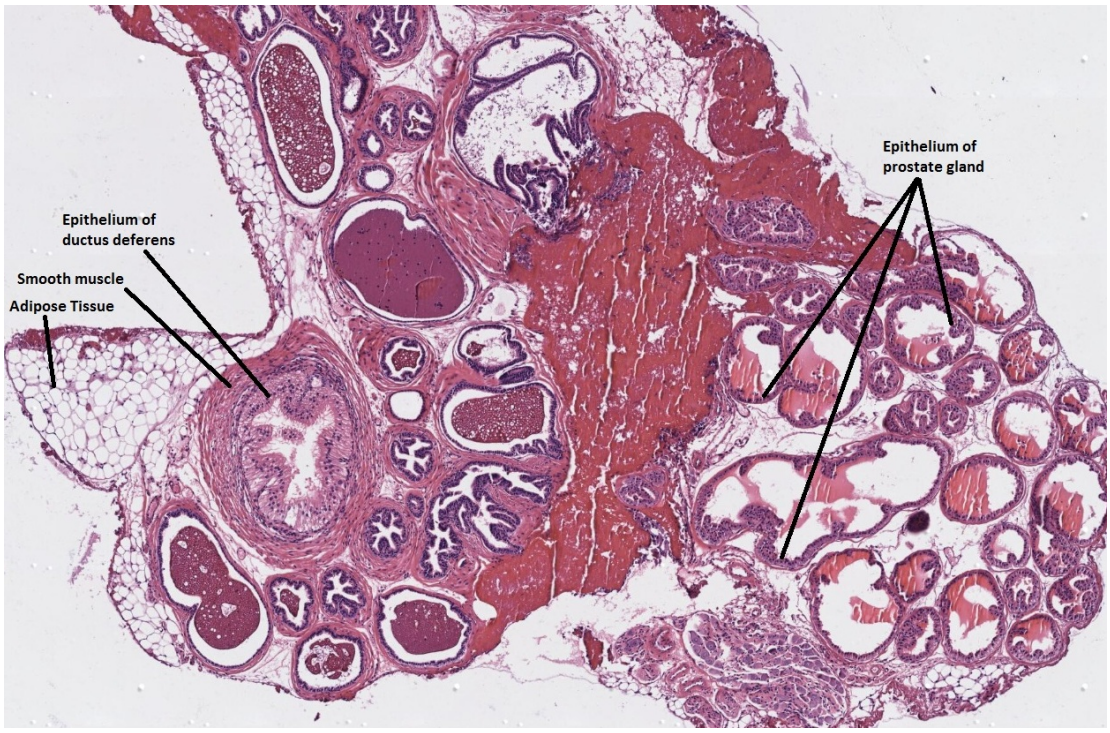

Figure 13. An example of a cross-section of mouse sample 363. In addition to prostate epithelium also other tissues and components are present. In this section for instance epithelium and smooth muscle of ductus deferens, which carries spermatozoa, are detectable. Adipose tissue is seen as a netlike pattern. Image from UTA JVS microscope website and used with permission of Leena Latonen.

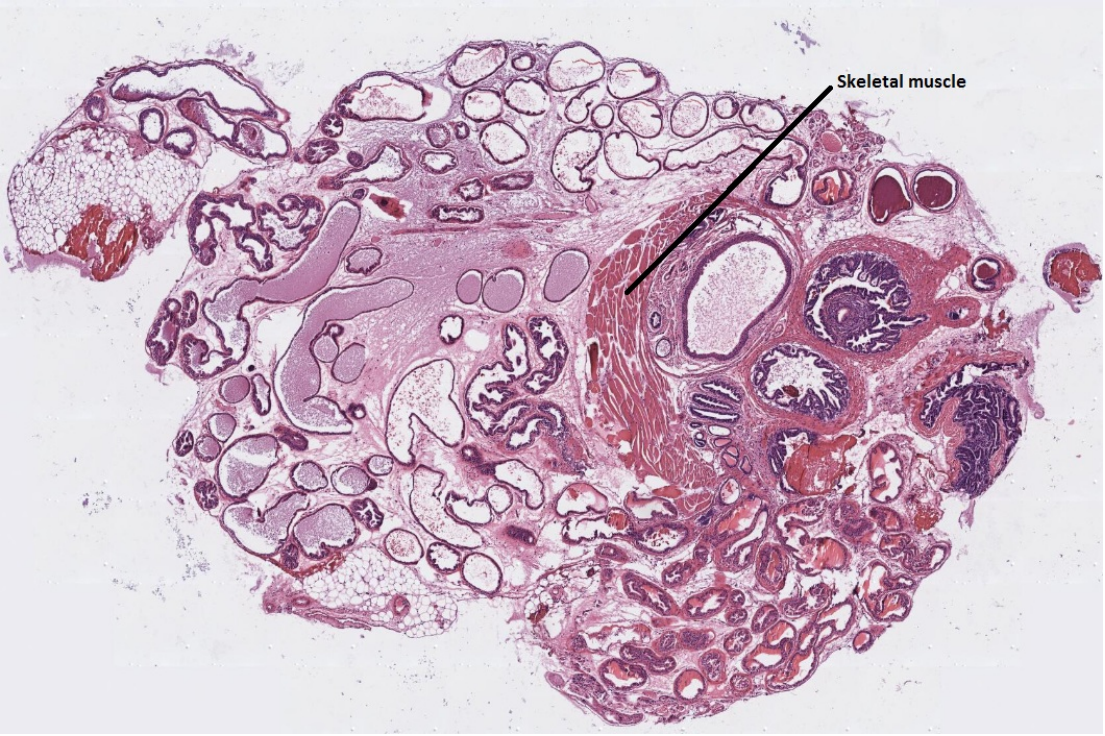

Figure 14. An example of a cross-section of mouse sample 364, which illustrates the skeletal muscle surrounding the urethra. Image from UTA JVS microscope website and used with permission of Leena Latonen.

4.3. Prostate Markers

All the samples express prostate markers PBSN and PSP94 (figures 15 and 16), which verifies that prostate epithelium existed in the samples, as PBSN and PSP94 expressions are known to be prostate-specific (Thota et al 2003; Johnson et al 2000). Highest PBSN expression is known to occur in dorsal prostate, the second highest in lateral prostate and the lowest expression in ventral prostate. Instead, highest PSP94 expression occurs in lateral prostate, the second highest in ventral prostate and only a minimal expression in dorsal prostate. Thus, behind the varying levels of prostate marker expressions in the samples likely are different prostate lobe ratios depending on which site of the prostate the sample was taken. For instance, the highest expressions of PBSN likely indicate relatively large dorsal prostate component, whereas the highest expressions of PSP94 instead indicate relatively large lateral prostate component.

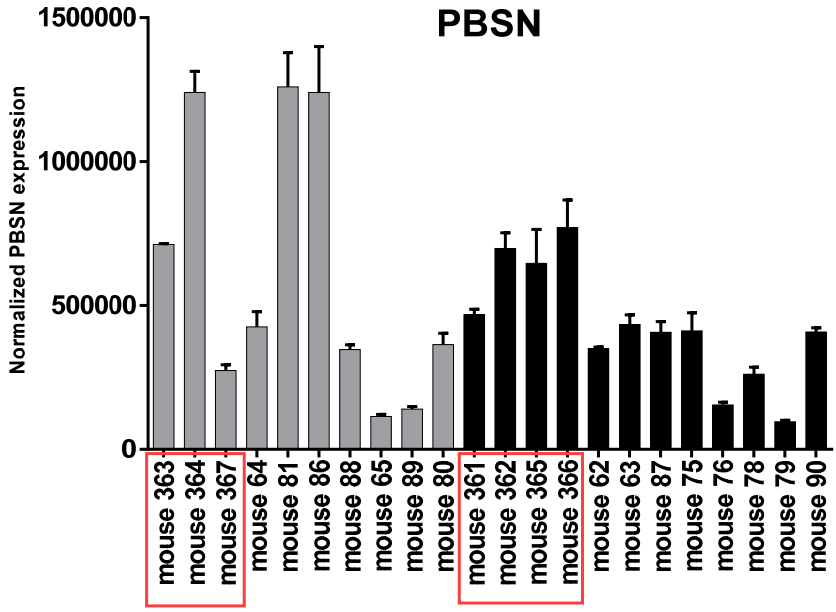

Figure 15. PBSN expression is detectable in all of the samples, which verifies the presence of prostate epithelium. Different levels of expression indicate different ratios of prostate lobes in the samples. Normalization was done against TBP + β-actin. Grey=control group, black=transgenic group. Red box=F2 generation, no box=F3 generation.

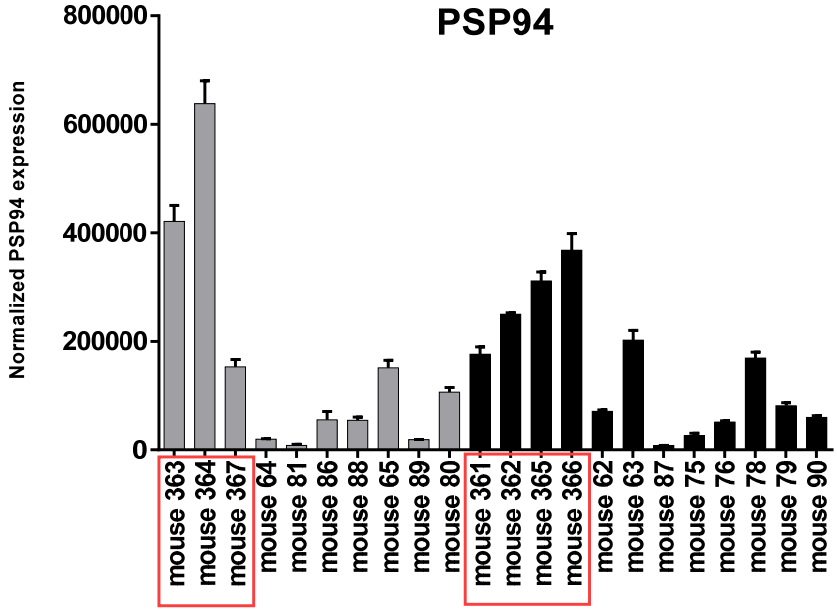

Figure 16. PSP94 expression is detectable in all of the samples, which further verifies the presence of prostate epithelium. Different levels of expression indicate different ratios of prostate lobes in the samples. Normalization was done against TBP + β-actin. Grey=control group, black=transgenic group. Red box=F2 generation, no box=F3 generation.

4.4. miR-32 expression

4.4.1. Control tissues

miR-32 expression was surveyed from Trizol extracted control tissues i.e. ventral prostate, bladder, skeletal muscle and adipose tissue. The control tissues were tissues, which were presumed to be present in the sample cross-sections according to the known mouse anatomy. The control tissues without transgene exposure show varying levels of naturally occurring miR-32 expression (figure 17), which verifies that miR-32 is expressed intrinsically in these tissues. Adipose tissue seems to express miR-32 relatively most, after which come bladder, skeletal muscle and ventral prostate.

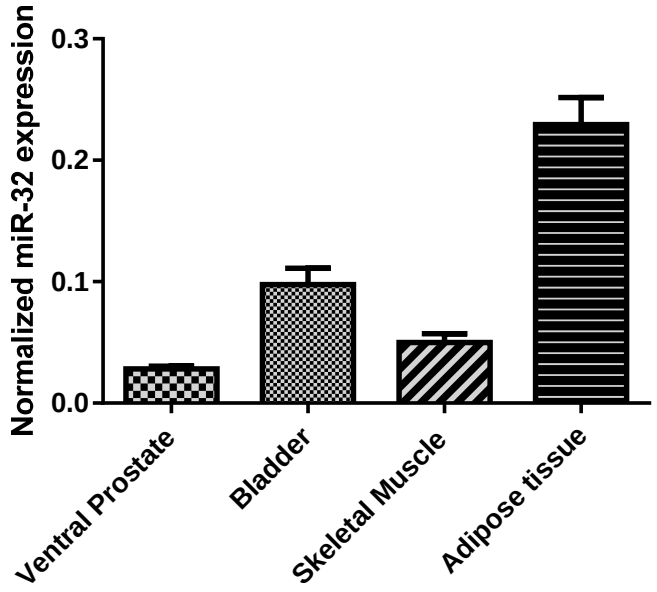

Figure 17. The normalized expression of miR-32 in control tissues, which have no transgene exposure. In normal conditions, miR-32 is expressed most in adipose tissue and least in ventral prostate. Normalization was done against RNU6B.

4.4.2. Samples

To observe transgene function, miR-32 expression was examined from PaxGene fixed and extracted transgenic prostate samples and compared to corresponding controls. Desired transgene over-expression is detectable in F2 generation on average level, but in reality only two transgenic samples, 362 and 365, clearly over-express miR-32 (figure 18). In F3 generation the average expression of transgenic samples is little higher than the one of controls, but the difference is notably smaller than in F2 generation. In F3 generation too, only two transgenic samples have slightly higher expressions, which increase the average value. In rest of the transgenic F2 and F3 samples miR-32 expression does not significantly differ from the one of controls, in which the expression is very low or absent. Low expression in some of the controls likely results from the intrinsic miR-32 expression, which was proved to occur previously.

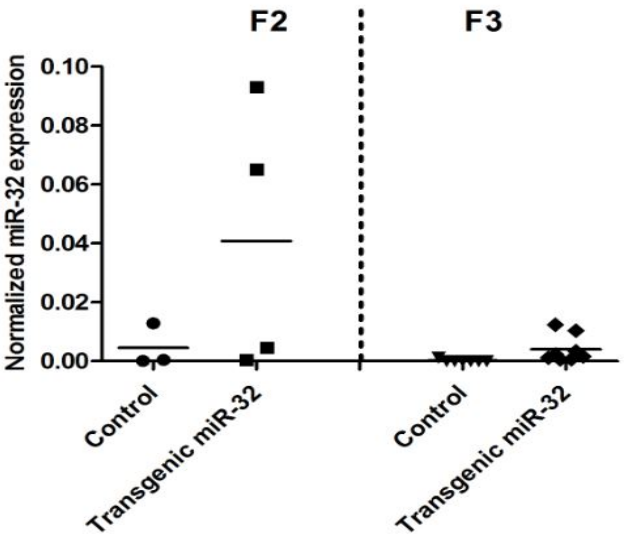

Figure 18. The normalized expression of miR-32 transgene in F2 and F3 generations. Normalization was done against RNU6B. Desired transgene over-expression is only seen in two transgenic F2 samples, 362 and 365.

4.5. PTEN

Transgenic miR-32 F2 mice were bred with PTEN knockout mice to provoke lesion formation in F3 generation mice. Genetically half of the F3 generation was PTEN homozygous and other half PTEN heterozygous. The expression of PTEN in F3 generation was surveyed to see whether the knockout was successful i.e. whether the PTEN expression of heterozygous mice was only half of the expression of homozygous. The expression of knockout mice was 54 % of the expression of wild-type mice (figure 19). Thus, PTEN expression in knockout mice was approximately half of the expression in wild-type mice, which is statistically significant result (P value < 0.01).

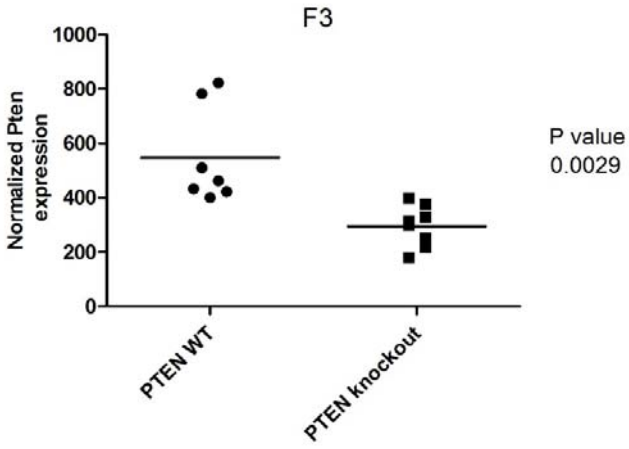

Figure 19. Wild-type versus knockout PTEN expression in F3 generation. PTEN expression was normalized against TBP + β-actin. Statistical analysis was performed with GraphPad Prism5. T-test with 95 % confident interval gives P value of 0.0029, which is statistically significant (< 0.01).

4.6. Possible Targets of miR-32

qPCR was performed for Btg2 and Klf2, which were hypothesized to be possible targets of miR-32 and down-regulated, when miR-32 is over-expressed. However, in F2 generation both of these are expressed more in the transgenic than in the control group (figure 20-22).

In F3 generation both are expressed approximately at the same level in control and transgenic group (figures 20-22).

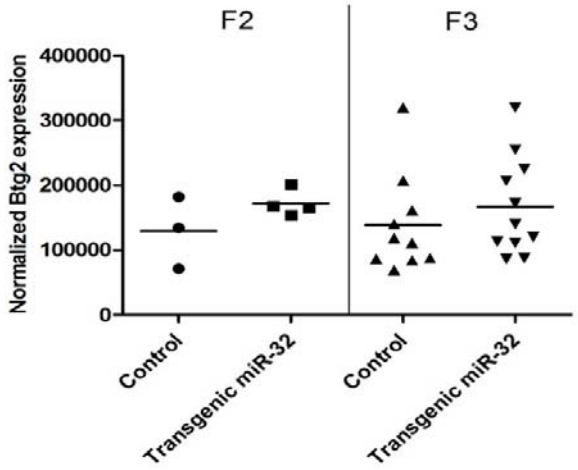

Figure 20. Normalized Btg2 expression in F2 and F3 generations. Normalization was done against TBP + β-actin. No hypothesized down-regulation is observed in transgenic samples.

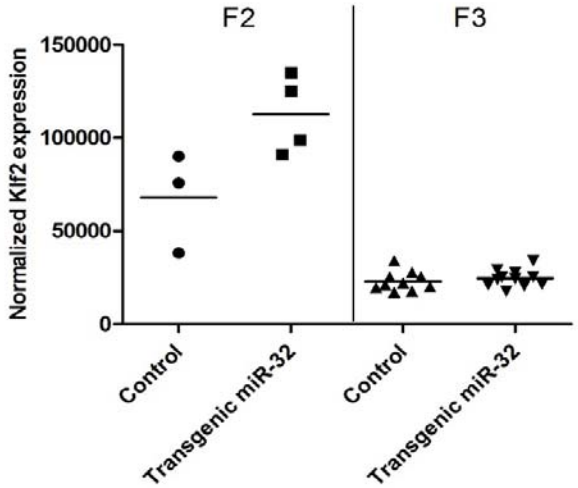

Figure 21. Normalized Klf2 expression in F2 and F3 generations. Normalization was done against TBP + β-actin. No hypothesized down-regulation is observed in transgenic samples.

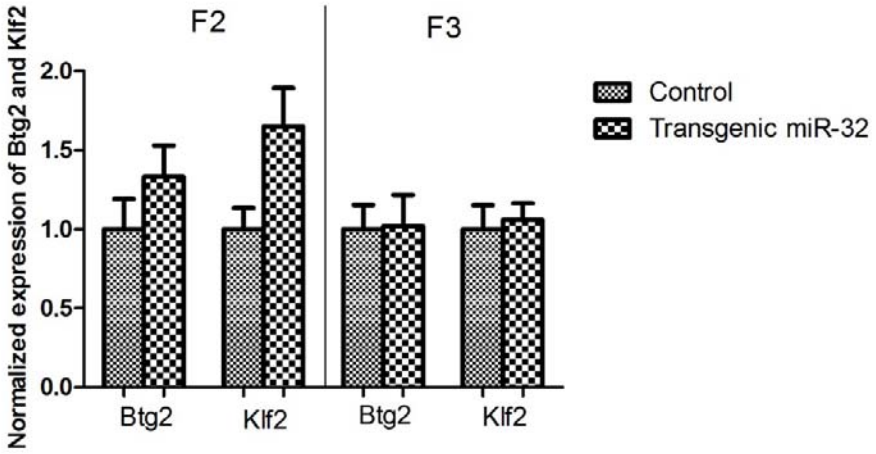

Figure 22. Btg2 and Klf2 expressions are brought to the same level so that value one has been given to the control.

5. Discussion

Downstream applications, like gene expression studies, normally require the use of good quality RNA in order to receive meaningful data (Peirson & Butler 2007; Fleige & Pfaffl 2006; Mueller et al 2004; Thermo Fisher Scientific General Article). Thus, common habit is to evaluate the extracted RNA in means of quantity, purity and integrity, before one proceeds further. A ratio of ~2.0 is commonly accepted pure for RNA and values lower than that normally indicate residual chemical or protein contamination (Thermo Scientific NanoDrop Technical Bulletins 2012 & 2011). Chemical contamination arises from reagents used in the extraction protocol or in some cases low ratio may be caused by low concentration of nucleic acid (<10 ng/ul). Instead, high ratio values do not automatically indicate problems, but very high ratio may be a consequence of poor quality blank. There is no consensus of the acceptable upper or lower limit for pure RNA, but it is known that pH and ionic strength of the used buffer may influence the ratio +/- 0.2-0.3. However, one has to keep in mind that the ratio only is indicative, but needs to be taken into account if problems appear with downstream applications. In this study, the purity of the extracted RNAs was generally in acceptable level and thus it can be concluded that extraction and purification were performed accurately enough, as no contaminations were observed. It

also can be concluded that reagents used in the extraction and purification procedure provided sufficient purity, and thus PaxGene Tissue miRNA Kit holds its promise in providing pure total RNA. The purity assessment is important, because contaminations such as residual chemical contamination may interrupt downstream applications like RNA reverse transcription to cDNA in RT-PCR (Fleige & Pfaffl 2006). As purity level was good in this study, it can be speculated that extraction and purification procedure did not bring any cDNA synthesis interrupting contaminations and therefore cDNA products were used directly in qPCR reactions. Gel analysis for reverse transcription success could be performed, but its information value is limited.

A typical feature of RNA is its vulnerability to degrade mainly because of RNases, which are present not only in tissues and cells themselves, but in the surrounding environment too (Peirson & Butler 2007; Fleige & Pfaffl 2006; Mueller et al 2004; Thermo Fisher Scientific General Article). Even though RNases are challenging in RNA isolation procedure, they have reasonable function in nature in protecting organisms against pathogens. However, all the possible degradation in RNA extraction procedure as well as in handling before and after the isolation is tried to be minimized. Thus, certain issues need to be considered in order to receive the most intact RNA possible. First of all, all the chemicals which are in contact with RNA must naturally preserve it. In addition, outside RNase contamination is avoided by using RNase-free materials and appropriate protective clothing, like lab coat and gloves, and also by taking care of sufficient purity of working environment. Perhaps one of the most important and critical things prior to RNA isolation is to homogenize the sample fast and well enough in appropriate lysis buffer, so that the cell contents become in touch with RNA protecting agents as quickly as possible.

In this study, new PaxGene method with molecular fixative and PaxGene Tissue miRNA Kit for extraction and purification of RNA was used for the first time in a desire to preserve both tissue morphology and RNA from the same mouse prostate. So, it was of interest to know RNA quality in terms of RNA integrity to find out PaxGenes RNA preservation ability. Results indicate that all the RNAs obtained with PaxGene were at

least partially degraded. Degradation of RNA is tried to be avoided, but it is almost impossible to receive totally intact RNA just because of the nature of the procedure. It takes certain time before the prostates are cut and put in a fixative and thus RNA may already be partially degraded at this point. Additionally, degradation may occur for instance due to insufficient fixation or during the extraction, if RNases are present. However, as the integrity of PaxGene and Trizol RNAs were on the same level and as it is known that Trizol produces adequate intact RNA, it can be concluded that RNA preservation ability of PaxGene is as good as the one of Trizols. This means that PaxGene RNA was of sufficient quality for the downstream applications.

Even though the extracted RNA is not completely intact, it can be used in certain downstream applications (Fleige & Pfaffl 2006; Mueller et al 2004). It strongly depends on type of a downstream study, which level of intactness is enough. For instance, RNA may be too degraded for genome-wide microarray analysis, but sufficiently intact for qPCR as smaller regions of RNA are analyzed in qPCR. Moderately degraded RNA may result in same gene expression profile as high quality intact RNA, and thus the best indicator of sufficient RNA integrity is its usefulness in a downstream application i.e. ability to obtain meaningful results.

miR-32 over-expression was seen clearly only in F2 generation, and more precisely only in two transgenic samples, whereas in F3 generation the expression was almost absent. As sample 362 with RIN value of 4 was one of the two samples with clear miR-32 expression, it seems that small miRNA expression can be detected even though RNA is partially degraded and not of best quality. Additionally, RIN values of samples 75 and 78 were 5.0 and 4.5, respectively, i.e. somewhat better than of sample 362 and despite of this no clear transgene expression was detected in these samples. Thus, partial degradation of RNA was not likely the reason for lack of transgene expression, which further verifies that PaxGene provided RNA was of sufficient integrity. Instead, it is possible that transgene expression was silenced from F2 generation to F3 generation, as this kind of age-dependent transgene silencing is known to occur possible through epigenetic mechanisms (Robertson et al

1996). However, silencing usually appears in later generations and not in early as in this study. It is known too that short multi-copy transgenes are especially vulnerable for silencing phenomenon and thus microRNAs may well be in group at risk for down-regulation (Calero-Nieto et al 2010). One alternative possibility is that the yield of small microRNAs in the purification step was not successful with all of the samples.

Additionally, composition of the prostate cross-sections may affect transgene detection. As transgene expression is known to occur most in ventral prostate, second most in lateral prostate and least in dorsal prostate, relatively large dorsal prostate component in the sections may result in low transgene expression. However, transgenic samples, in which transgene expression is of low level, seem not to have relatively large dorsal prostate component according to the expression values of PBSN, which are not of the highest level in the low expression level transgenic samples. Instead, expressions of PSP94 indicate quite the opposite. Based on these values, it seems that F3 generation transgenic mice samples are in general more abundant with dorsal prostate than F2 generation transgenic mice samples, as PSP94 expression is relatively lowest in F3 generation. Thus, if PSP94 expression information is correct, relatively larger ventral and/or lateral prostate components in samples 362 and 365 could explain why transgene expression is seen clearly only in these samples. In that case, there would not possible be fault in the transgene itself. However, the detection of the transgene would be dependable on the prostate lobe ratios in the cross-sections meaning that a clear transgene expression could not be observed if dorsal prostate was too dominant. In addition to prostate itself, meaning prostate epithelium, stroma and adipose tissue, the cross-sections were predicted to contain also other adjacent tissues based on the known mouse prostate anatomy. Practically, these adjacent tissues meant skeletal muscle and smooth muscle, and tissue marker expression analysis verified the prediction to be true. It was of concern whether these tissues would disturb the detection of the transgene or influence on the transgene expression values. First of all, the amount of prostate epithelium seems to be quite constant across the samples, even though the cross-sections were taken from different sites of the prostates. Thus, at least samples 362 and 365 do not have relatively larger epithelium component, which could have explained the higher transgene expression values of these samples. In fact,

samples 362 and 365 have little less epithelium than transgenic F3 samples in general, so even with relatively smaller epithelium component transgene detection is possible. Despite the transgenic F3 group have relatively larger epithelium only low transgene expression is detected. The smooth muscle component seems to vary relatively least in the samples meaning that the amount of smooth muscle is also quite constant across the samples regardless of the sites the cross-sections were taken. Even though smooth muscle appears, it seems not to be disruptive factor in transgene detection, as in samples 362 and 365 miR-32 expression is clearly higher than in the corresponding control group. Thus, even though smooth muscle has the second highest intrinsic miR-32 expression compared to other control tissues, it does not reach the level of the transgene. Skeletal muscle component varies most in the samples and the F2 samples are in general much more abundant with it than the F3 samples. This likely is due to different sites of the prostates the cross-sections were taken. Thus, even relatively large skeletal muscle component in sample 365 does not disturb the transgene detection i.e. the intrinsic miR-32 expression, which is relatively of low level, does not reach the level of the transgene. Finally, adipose tissue varies second most in the samples. Variation likely results from the sites the cross-sections were taken too, but partial influence may have how well the adipose tissue has succeeded to be removed around the prostates, when they were sectioned. F3 samples have relatively much more adipose tissue than F2 samples in general level, which possible could be transgene detection disturbing factor, as the adipose has relatively highest intrinsic miR-32 expression. However, at least the relatively small amount of adipose tissue in samples 362 and 365 does not prevent detecting the transgene. Large adipose tissue component may equalize the expression differences seen between the transgenic and control group, but it does not explain here the low expression values of the transgene. In conclusion, adjacent tissues most likely do not disturb the transgene detection even in larger proportions. However, as information PBSN and PSP94 provide is inconsistent, one not can say with certainty whether the virtually non-existent expression is due to transgene itself or inability to detect the transgene because of the nature of the new procedure, in which whole prostate cross-section with different lobes was included in RNA extraction.

In the presence of miR-32 over-expression in F2 generation no down-regulation of either Btg2 or Klf2 was observed. Instead, Btg2 and Klf2 were expressed more in transgenic F2 group than in the control F2 group. In F3 generation expression was averagely on the same level with the control group. Similar expression patterns of Btg2 and Klf2 raised a doubt that there might be variation in RNA integrity, but RIN values of samples included in the integrity analysis however indicate that RNA quality was on the same level. Different public algorithms predict as much as thousands of different targets for miR-32, which makes it challenging to find the real targets. Naturally, not all the suggested can be the targets of miR-32 and thus the possible ones need to be verified. Hence, it is possible that Btg2 and Klf2 are not miR-32 targets at all, at least in the mouse prostate, even though preliminary studies suggested that. In future, other possible targets could be searched for from the samples used in this study.

Finally, PTEN expression analysis of F3 generation indicated that mice with PTEN knockout genotype expressed PTEN approximately half less than mice with PTEN wild type genotype and the result was statistically significant. Thus it can be concluded that PTEN knockout was successful. The original purpose was to observe whether transgene miR-32 over-expression further increases neoplasia in PTEN knockout mice, but as no transgene over-expression was detected in F3 generation, the neoplasia possible seen in F3 generation likely results from PTEN knockout only.

In conclusion, accomplished molecular analyses here suggest that PaxGene is a possible choice of method for future studies. The RNA preservation ability of PaxGene was of sufficient level, which means that gene expression analyses were performed with adequate intact RNA. It is however possible that transgene detection suffers from the nature of the procedure in that all the different lobes are included in the cross-sections. Instead, adjacent tissues seem not to disturb the detection. The additional chance is that the transgene silencing has occurred, in which case the problem is in the functionality of the transgene itself. No down-regulation of possible miR-32 targets, Btg2 and Klf2, was observed, which may indicate that these are not targets for miR-32 at all. Subsequent transgenic samples

could be examined with PaxGene to see whether differentiating prostate lobe ratio really affect to the transgene detection. If clear and constant transgene detection can not be achieved with PaxGene procedure, its use is limited meaning that it is not appropriate for qPCR based gene expression observation. However, more transgenic samples need to be examined anyway to verify the transgene functionality. Only with verified transgene function reliable conclusions of its relation to PCa can be made. As the desired function of transgene is not always straightforward i.e. repeatability is poor and as the possible cancer changes have to be seen in more than one founder line, the whole process can be time-consuming.

In future, other interesting possibilities than qPCR for gene expression observation exist too and one of these is *in situ* hybridization (ISH). The aim of ISH is to localize RNAs like miRNAs in tissue sections by using labeled complementary RNA i.e. probe, which is detected with light or electron microscope (Tsai & Harding 2013; Thermo Fisher Scientific Tech Note). The benefit of this method is the precise localization of gene expression within a tissue and thus in transgene expression observation this would be a great advantage over qPCR. However, optimization of this method requires many steps. Not only in qPCR based expression analysis, but in ISH too, the RNA has to be preserved and for this purpose PaxGene molecular fixative could be one possible choice of method. Alternatively, frozen tissue sections i.e. cryosections for miRNA ISH is an option (Silahtaroglu et al 2007). Extra degradation and other disadvantages occurring in the RNA isolation procedure required for qPCR is however avoided in ISH, as no extraction is performed. An additional challenge in microRNA *in situ* detection has been the low affinity of probes to miRNAs due to small size of the miRNAs (Song et al 2010). Nevertheless, if challenges and problems are to overcome, *in situ* hybridization could provide an efficient method for miRNA transgene expression observation in future.

6. References

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FIGURES

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