Peptidase Inhibitor 15 as a potential target of miR-32 and functional consequences of PI15 overexpression to PC-3 prostate cancer cell line

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TIIVISTELMÄ

Eturauhassyöpä on miesten yleisin syöpä länsimaissa. Syövän vaarallisuuden määrää suurelta osin sen kyky tunkeutua ympäröivään kudokseen ja lähettää etäispesäkkeitä. Eturauhassyövän leviämiseen ja metastaasiin johtavat molekulaariset mekanismit tunnetaan kuitenkin yhä suurelta osin heikosti. Nämä mekanismit täytyy tuntea, jotta voidaan löytää uusia apuvälineitä eturauhassyövän diagnosointiin ja tarkemman ennusteen tekemiseen sekä suunnitella parempia terapioita syövän leviämisen pysäyttämiseksi.

Tässä työssä tutkittiin pitkälle edenneessä syövässä yli-ilmentyvän androgeenireseptorin säätelemän mikroRNA:n, miR-32:n, vaikutusta peptidaasi-inhibiittori 15:n (PI15) ilmentymiseen eturauhassyövän solulinjoissa PC-3 ja LNCaP. Tämä miRNA–kohdegeeni-pari herätti kiinnostuksen, sillä kastraatioresistentit syövät ovat tyypillisesti hyvin aggressiivisia ja peptidaaseilla ja proteaaseilla, joita PI15 inhiboi, on tähän suuri vaikutus. Hypoteesina on, että pitkälle edenneessä syövässä miR-32 vaimentaa PI15:n ilmentymisen, jolloin peptidaasien toiminta on aktiivisempaa ja syövän leviäminen kiihtynyttä.

Edellä mainittuja solulinjoja transfektoitiin *PI15* geenin sisältävällä plasmidilla saman aikaisesti pre-miR-32:lla ja vaikutuksia *PI15*:n ilmentymiseen mitattiin qRT-PCR:lla ja western blot-menetelmällä. Lisäksi tehtiin reportterivektori, jossa lusiferaasi-geeni kytkettiin *PI15*:n 3’ säätelyalueeseen, jolloin voidaan mitata miR-32:n säätelyvaikutusta lusiferaasin ilmentymiseen suoraan proteiinitasolla. Myös *PI15*:n yli-ilmentämisen vaikutuksia tutkittiin kloonaamalla *PI15* geenin koodaava alue uuteen plasmidiin, jossa on nisäkässoluissa toimiva selektiogeeni, ja transfektoimalla PC-3 solulinja pysyvästi hyödyntäen antibiootin aikaan saamaa selektiopainetta. Näin saatujen *PI15*:ttä stabiilisti yli-ilmentävien PC-3 kloonien kasvunopeutta ja solujen elinkelpoisuutta mitattiin verrattuna tyhjällä plasmidilla transfektoituihin klooneihin.

PI15:n ja miR-32:n yhtäaikaisen transfektion aiheuttama hajonta osoittautui liian suureksi pitävien johtopäätösten tekemiseksi miR-32:n vaikutuksesta *PI15*:n ilmentymiseen. Tulevaisuudessa tehtävä lusiferaasi-reportterikoe voisi vastata paremmin tähän kysymykseen. PI15:n ilmentymistä ei tämän työn puitteissa kyetty todentamaan proteiinitasolla. Geenin yli-ilmentäminen kuitenkin johti selvästi alentuneeseen kasvunopeuteen ja solujen elinkelpoisuuteen sekä fenotyypin muuttumiseen suurimmalla ilmentämisen tasolla. Jatkotutkimusta vaaditaan, jotta voidaan osoittaa PI15 proteiinin aikaan saavan nämä muutokset.

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ABSTRACT

Prostate cancer is the most common cancer in men in western countries. The most dangerous property of cancer is the ability to spread into the surrounding tissue and to metastasize. The molecular mechanisms underlying these processes are still poorly understood in prostate cancer. These mechanisms must be studied more deeply in order to find new tools for prostate cancer diagnostics and prognostics as well as to design better therapies to tackle the disease.

In this work we studied the effects of miR-32 on the expression of peptidase inhibitor 15 (PI15) in prostate cancer cell lines PC-3 and LNCaP. MiR-32 is an androgen receptor regulated microRNA that is overexpressed in castration resistant prostate cancer. Interest in this miRNA–target pair arouse because the castration resistant disease is aggressive and peptidases and proteases that PI15 inhibits have great influence on this matter. The hypothesis is that miR-32 downregulates PI15 expression leading to increased peptidase activity and enhanced invasion of cancer cells.

The aforementioned cell lines were transfected with *PI15* vector concomitantly with miR-32 and the effects on PI15 expression were observed by qRT-PCR and western blot. In addition, we constructed a luciferase reporter vector with luciferase gene integrated to *PI15* 3' UTR. In this vector, expression of luciferase is regulated by miRNAs that can bind to the 3' UTR. Finally, the effects of *PI15* overexpression were studied by cloning *PI15* open reading frame into a vector with a mammalian selection marker and then transfecting this vector stably into PC-3 cell line with the help of antibiotic selection. These stably *PI15* overexpressing cells were used to measure growth speed and cell viability in comparison to cells transfected with an empty plasmid.

The dispersion of co-transfection of *PI15* and miR-32 turned out to be too high to draw conclusions of whether miR-32 affects *PI15* expression or not. The luciferase reporter assay might give firmer answers for this matter and remains a subject of future work. Unfortunately, we could not detect PI15 in the protein level. Nevertheless, the overexpression of *PI15* significantly reduced growth and viability of the stable clones and changed the cell phenotype in the highest level of overexpression. Further research is needed to confirm that it is the PI15 protein that causes these changes.

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ABBREVIATIONS

22Rv1	22Rv1 prostate cancer cell line
ADT	androgen deprivation therapy
AGO2	protein argonaute 2
AKT1	v-akt murine thymoma viral oncogene homolog 1
AR	androgen receptor
BCL2L11	bcl-2-like protein 11
BPH	benign prostatic hyperplasia
BSA	bovine serum albumin
BTG2	BTG family member 2
CHD1	chromodomain helicase DNA binding protein 1
CDKNB1	cyclin-dependent kinase inhibitor 1B
CNA	copy number alteration
CRPC	castration resistant prostate cancer
CT	computational tomography
CYP17A1	cytochrome P450 family 17 subfamily A member 1
DGCR8	DiGeorge syndrome critical region 8
DROSHA	ribonuclease 3
DSB	double strand break
ERSPC	European randomized study of screening prostate cancer
FOXA1	forkhead box A1
GLIPR1	glioma pathogenesis-related protein 1
HGPIN	high grade prostate intraepithelial neoplasia
HOXB13	homeobox B13
miRISC	miRNA induced gene silencing complex
miRNA	microRNA
MRI	magnetic resonance imaging
MYC	v-myc avian myelocytomatosis viral oncogene homolog
NCOA2	nuclear receptor coactivator 2
NKX3-1	NK3-homeobox 1
PBS	phosphate buffered saline
PHLPP1	pH domain and leucine rich repeat protein phosphatase 1
PC	prostate cancer
PI15	peptidase inhibitor 15
PI3K	phosphoinositide-3-kinase
PIN	prostate intraepithelial neoplasia
PLCO	Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial
PSA	prostate specific antigen
PTEN	phosphatase and tensin homolog
SDS–PAGE	sodium dodecyl sulfate–polyacrylamide gel electrophoresis
SNP	single nucleotide polymorphism
SPINK1	serine peptidase inhibitor, Kazal type 1
SPOP	speckle type BTB/POZ protein
TARBP2	RISC loading complex RNA binding subunit
TCGA	the cancer genome atlas research network
TMPRSS2	transmembrane protease, serine 2
TOP2B	topoisomerase (DNA) II beta

TP53	tumor protein 53
TURP	transurethal resections of the prostate
UTR	untranslated region
WB	western blot
WT	wild type
qRT-PCR	quantitative real-time polymerase chain reaction

1. INTRODUCTION

Prostate cancer is the most common cancer in European men with approximately 417 100 new cases diagnosed in 2012 (Ferlay, Soerjomataram et al. 2015). It is also the third most common cause of cancer deaths (Ferlay, Soerjomataram et al. 2015). The inconsistency between incidence and mortality has been established during last two decades as a result of serum based test for prostate specific antigen (PSA) (Ferlay, Soerjomataram et al. 2015, Prensner, Rubin et al. 2012). Detection of elevated levels of this biomarker has allowed early detection of prostate cancers overall but identification of the high risk patients with potential to an aggressive disease remains a major clinical challenge (Shen, Abate-Shen 2010).

It is the aggressive form of the disease that would deserve better treatment solutions, as it is the main cause of death by prostate cancer. Currently, androgen deprivation therapy is still the best available treatment for locally advanced or metastatic prostate cancer, but despite the promising response during the early phase of the treatment, the disease almost invariably gains resistance and the resulting castration resistant prostate cancer (CRPC) is incurable (Shen, Abate-Shen 2010).

Overcoming these challenges requires thorough understanding of the molecular mechanisms underlying the biology of prostate cancer. One of such mechanisms is post-transcriptional microRNA mediated gene silencing (Jonas and Izaurralde 2015). Multiple studies have established dysregulation miRNA expression during prostate cancer carcinogenesis (Lu et al. 2005, Volinia et al. 2006, Porkka et al. 2007, Ambs et al. 2008, Ozen et al. 2008, Schaefer et al. 2010).

One of such potentially carcinogenesis driving miRNAs is miR-32. Its expression is regulated by the androgen receptor and it has been shown to be overexpressed in CRPC compared with benign prostatic hyperplasia (BPH) (Jalava et al. 2012). MiR-32 has also been shown to downregulate tumor protein p53 effector gene *BTG2* both *in vitro* and *in vivo* (Jalava et al. 2012) and *BCL2L11* encoding apoptotic regulator bcl-2-like protein 11 *in vitro* (Ambs et al. 2008). To further validate miR-32's connection to carcinogenesis, more target genes are being searched. The present study was motivated by the prediction of microRNA.org target prediction program (Betel et al. 2008 and 2010) that miR-32 has a potential binding site in the 3'UTR region of peptidase inhibitor 15 (*PI15*) mRNA. Early results from murine model studies have shown that expression of PI15 mRNA is indeed lower in

miR-32 overexpressing mice in comparison to wild type mice. Similar results were acquired in AR positive PCa cell line 22Rv1.

The functional role of PI15 as an extracellular peptidase inhibitor makes it an interesting possible target for miR-32: If it is downregulated by miR-32 that has been associated with more aggressive disease, it would provide one possible explanation of why CRPC is more invasive, as the absence of peptidase inhibitor would lead to higher activity of proteases and increased proteolysis in the tissue.

2. REVIEW OF THE LITERATURE

2.1 MicroRNAs

MicroRNAs are short ~18–24 nucleotide long RNA species that target mRNAs usually decreasing their expression at the post-transcriptional level. They are well conserved in evolution from nematode to mammals, which highlights their importance in the control of gene expression. There are almost 1900 putative miRNA genes in the human genome (http://www.mirbase.org; 19.2.2016, Kozomara et al. 2011), although probably not all of them are genuine. Nevertheless, based on the sequence, they can bind to most of the protein coding genes (Friedman et al. 2009) having the potential to control their expression. This is possible because many miRNAs can bind multiple different target mRNAs. Furthermore, several miRNAs can bind the same target mRNA making the network of miRNA mediated modulation even more diverse. From this context it is not surprising that miRNAs have been shown to participate in the regulation of many cellular processes that are disrupted during carcinogenesis including control of proliferation, differentiation and apoptosis (Kloosterman & Plasterk 2006).

Most miRNAs are transcribed by RNA polymerase II and 5'-capped and polyadenylated just like mRNAs (Lee et al. 2004). The pri-miRNA transcripts are then cut into shorter hairpin shaped pre-miRNA precursors in the nucleus by the microprocessor complex constituting of ribonuclease 3 (DROSHA) and DiGeorge syndrome critical region 8 (DGCR8) proteins (Gregory et al. 2004). After export to the cytoplasm by exportin 5 (Yi et al. 2003), the pre-miRNA is subject to further processing by endoribonuclease Dicer, which cleaves the pre-miRNA into mature ~22 nucleotide miRNA duplexes (Bernstein et al. 2001).

The miRNA induced gene silencing complex (miRISC), which is responsible of the actual effects of the silencing mechanism, is assembled directly after the last cleavage step. RISC-loading complex subunit TARBP2 that accompanies Dicer in the last cleavage step guides the miRNA duplex to the protein argonaute 2 (AGO2) (Chendrimada et al. 2005), which is essential for the catalytic activity of the silencing complex in mammals (Liu et al. 2004). In addition to AGO2, the miRISC includes GW182 family proteins, which guide the silencing complex into P-bodies where the miRNA mediated gene silencing takes place (Liu et al. 2005a). The miRNA biogenesis is summarized in Figure 1.

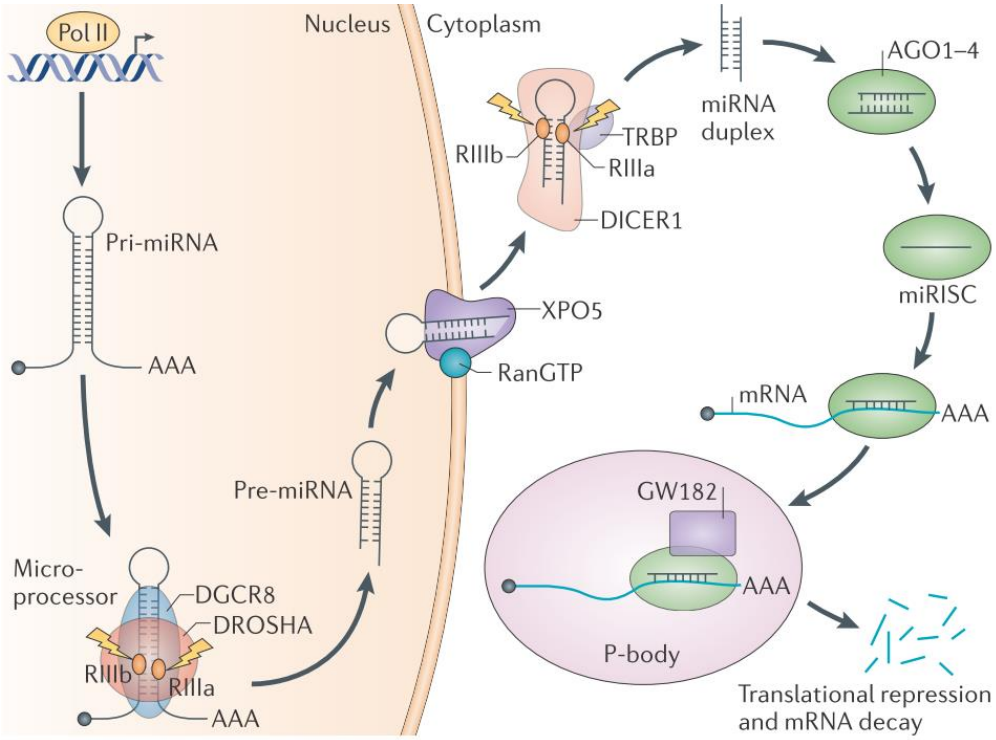

Figure 1. Canonical miRNA biogenesis pathway. Pri-miRNAs are transcripted by RNA polymerase II, and digested by microprocessor complex in the nucleus. After export to cytoplasm through exporting 5, pre-miRNAs are further cleaved by the DICER. The miRNA duplex is next separated and one of mature miRNAs is loaded into the RISC complex that mediates RNA interference. See further discussion in the text. Figure adapted from Lin and Gregory 2015.

In order to bind to its target mRNA, the miRNA guide has to be separated from the complementary passenger strand. The mechanism of this process is still unclear, although slicing of the passenger strand or a passive mismatch driven process have been suggested (Czech & Hannon 2011).

The mature RISC complex silences mRNAs complementary to the miRNA in organelles called p-bodies (Liu et al. 2005a, Liu et al. 2005b). The fate of mRNA is dependent on the degree of complementarity of miRNA to its target sequence: In mammals, miRNAs are rarely completely complementary to the mRNA, a case that leads to direct cleavage of the mRNA by AGO2 (Liu et al. 2004), but contain several mismatched nucleotides, which leads to other ways of downregulation of the target translation. The major mechanisms for downregulation of the target expression seems to be accelerated mRNA decay by deadenylation and decapping of mRNAs RISC dependently (Guo et al. 2010, Eichhorn et al. 2014). It appears to be directly connected to the common mRNA decay pathway responsible for mRNA turnover (Guo et al. 2010, Eichhorn et al. 2014).

Also other mechanisms by which miRNAs mediate regulation of gene expression exist. For example, some mRNAs are held in repressed but stable state for later use in translation, allowing rapid translational response to environmental signal (Muddasheddy et al. 2011). Additionally, in specific occasions, typically during cell cycle arrest, some miRNAs can function as translation activators (Vasudevan et al. 2007). In both cases these phenomena require specific accessory proteins that bind to AGO2 in order to change the basic behavior of the RISC complex (Muddasheddy et al. 2011, Vasudevan et al. 2007).

2.2 Prostate gland

Prostate is a walnut sized organ residing just under the bladder. It is part of the male reproduction system and its main function is to produce secretions to the seminal fluid. Generally accepted anatomical model of the prostate divides the organ into four zones. Three of these zones are glandular and possess ductal anatomy whereas the remaining zone is mainly connective tissue and smooth muscle (McNeal 1981).

The glandular zones are named peripheral, central and transition zones. Central zone is different from the other two deriving from Wolffian duct, whereas the other two derive from the urogenital sinus (McNeal 1981). Histologically the central zone has denser acini and ducts (McNeal 1981, Virtanen, Hervonen 1995). The Peripheral zone is under the other two and constitutes ~70 % of the mass of prostate (McNeal 1981). The structure of the acini and ducts in the peripheral zone is less dense and simpler than in the central zone (Virtanen & Hervonen 1995, Laczkó et al. 2005). The transition zone has also smaller ducts and it is mainly distinguished from peripheral zone by being in the closer proximity to uretha between central zone and periurethal smooth muscle sphincter (McNeal 1981, Virtanen & Hervonen 1995). Benign prostatic hyperplasia (BPH) is developed almost exclusively in the transition zone, whereas most of the carcinomas arise in the peripheral zone (McNeal 1981). It has been proposed that the reason for higher rate of cancer incidence in the peripheral zone is due to higher rates of cellular proliferation in the zone (Laczkó et al. 2005). This notion is in accordance with the recent report stating that cancer risk in variety of other tissue types is attributable solely to their rate of stem cell division (Tomasetti & Vogelstein 2015).

The glandular epithelium of the prostate is pseudostratified. High columnar luminal cells are the most abundant cell type covering the lumen of the acini and ducts and are responsible for the prostate's

secretion (van Leenders & Schalken 2003). In addition to them there are basal cells, which are believed to be the stem cells for the luminal cells (van Leenders & Schalken 2003). Much rarer are the neuroendocrine cells that grow in small clusters and whose biological role in the prostate is still under debate, but they may be involved in regulation of exocrine cells in the prostate stroma (van Leenders & Schalken 2003). The prostate epithelium exchanges paracrine signals with urogenital mesenchyme during and after the prostate development (Shen & Abate-Shen 2010, van Leenders & Schalken 2003). Most of the prostate carcinomas are of the luminal phenotype (prostate acinar adenocarcinoma), but a rare and aggressive type is initiated from the neuroendocrine cells (prostate small cell carcinoma) (Shen, Abate-Shen 2010). From here on the discussion of prostate carcinoma will refer to the adenomatous type, if not otherwise mentioned.

2.3 Prostate carcinoma

2.3.1 Epidemiological factors

Prostate cancer is the second most common cancer in men globally and the most common cancer in European men (Ferlay et al. 2015). However, it is only the fifth leading cause of cancer mortality in men worldwide and the third in Europe (Ferlay et al. 2015). This gap between incidence and mortality rates is due to indolent nature of many prostate cancers. In addition, prostate cancer is frequently diagnosed at old age (Grönberg 2003), which raises the probability that the patient dies of other causes.

During the last few decades, prostate cancer incidence has been increasing around the world (Zhou et al. 2016). This is attributable to more sensitive detection of the existing cases rather than vast increases of the actual cancer incidence (Zhou et al. 2016). Especially the introduction of blood based screening for prostate cancer with prostate specific antigen (PSA) has led to early detection of the disease even in asymptomatic men (Prensner et al. 2012). This has not been completely unproblematic though, as will be discussed later.

In addition to age, the most important unmodifiable risk factors for prostate cancer are ethnic background and positive family history of the disease (Cuzick et al. 2014). For instance, the disease is more common in American men with African ancestry than in white non-Hispanic males in the USA (Merrill & Sloan 2012). Lifetime risk for white men is 15,7 % whereas for black men it is 18,5 % (Merrill & Sloan 2012). In contrast, for men with Asian origin the risk for prostate cancer specific

mortality is generally lower than in white non-Hispanic males (Chao et al. 2015). The higher incidence in African Americans has been attributed to more frequent genetic alterations in the susceptibility locus 8q24 (Gudmundsson et al. 2007).

In a large Nordic twin study (Lichtenstein et al. 2000) hereditability of prostate cancer was estimated to be as much as 42 % in Scandinavian population. However, known high risk mutations can explain only a mere fraction of prostate cancer hereditability (Attard et al. 2016). Genome wide association studies have greatly enhanced detection sensitivity over linkage mapping (Eeles et al. 2014), which has allowed to detect a total of 77 mostly low risk cancer susceptibility alleles (Attard et al. 2016). Indeed, most of the known prostate cancer susceptibility alleles are common, low penetrance single nucleotide polymorphisms (SNPs) that reside in intergenic or intronic regions and act synergistically (Eeles et al. 2014).

To date, non-synonymous germline mutations in several genes have been linked to predisposition to prostate cancer. One of these genes, *HOXB13,* has been linked to earlier onset of prostate cancer with odds ratio of 2,93; although it's mechanism of action and remains elusive (Ewing et al. 2012, Kote-Jarai et al. 2015). Clinical relevance of *HOXB13* (G84E) is limited to possible screening of members of carrier families as its effects on cancer progression, aside from earlier onset, or treatment outcomes have not been identified (Kote-Jarai et al. 2015).

In addition, mutations in several DNA damage repair genes have been linked to early onset and aggressive phenotype of prostate cancer (Attard et al. 2016). The most important of these is *BRCA2*, a double strand break repair gene that was first identified as a cause for familial breast and ovarian cancers (Breast Cancer Linkage Consortium 1999). In heterozygous carriers, deleterious mutations have been attributed to almost five-fold lifetime risk to prostate cancer (Breast Cancer Linkage Consortium 1999) and 8,6-fold increase in early (< 65 years of age) onset disease (Kote-Jarai et al. 2011a). Also the prognosis for *BRCA2* mutation carriers is poorer than to unaffected individuals as the clinical progression in the mutation carriers is faster (Kote-Jarai et al. 2011a).

There are also some rare germline variants of genes that are most frequently altered in sporadic prostate cancer. For example, Xq12 containing proto-oncogene *AR* has been identified as one possible susceptibility locus, although the changes do not affect coding region (Kote-Jarai et al. 2011b). Also

the susceptibility alleles in the aforementioned locus 8q24 have been shown to function as cell type specific enhancers for proto-oncogene *MYC in vitro* (Ahmadiyeh et al. 2010). In addition, germline variants altering the expression of PSA encoding gene *KLK3* and other kallikrein family genes have been found (Cramer et al. 2003, Kote-Jarai et al. 2011c). However, their association to risk of prostate cancer is not clear (Kote-Jarai et al. 2011c). All in all, the identified changes in these genes are quite minute, which can be explained by their vital roles in cellular activities. Major changes especially in genes that encode proteins that function in key pathways, would most probably be lethal already in early stages of embryonic development.

Although the knowledge of identified germline alterations explains about 35 % of the estimated prostate cancer hereditability, it leaves majority of the genetic susceptibility factors uncharacterized (Attard et al. 2016). It is probable that the interactions of the yet unidentified germline alterations constitute of number of alleles with complex interactions (Eeles et al. 2014).

Besides genetics, there is evidence of causality between lifestyle related risk factors such as smoking (Zu & Giovannucci 2009) and obesity (Buschemeyer & Freedland 2007) and higher incidence or poorer prognosis of prostate cancer.

2.3.2 Prostate cancer genetics

Prostate cancer is a heterogeneous malignancy that arises from mostly somatic changes in the genome of prostate epithelial cells. Traditionally changes altering function of two classes of genes have been sought-after. These two classes of driver genes include tumor suppressors that act as a barrier for uncontrollable growth, proliferation and migration, and proto-oncogenes that are commonly activators of pathways associated with these functions.

Tumor suppressors are affected by loss-of-function type of alterations, which can be caused by structure altering or expression decreasing substitutions, insertions, deletions or epigenetic changes. Tumor suppressors often require inactivation of both alleles to affect tumorigenesis. This is generally known as “two hit hypothesis” (Knudson 1971). Although in some instances loss of one allele decreases the gene dosage sufficiently to induce tumorigenicity.

Proto-oncogenes, on the other hand, are subject to activating events, often mutation or genomic rearrangement that either cause gain-of-function type of change, for example making them constitutively active or altering their substrate specificity. Often simple overexpression is enough to convert proto-oncogene into an active oncogene that is irresponsive to regulatory signals.

The knowledge of genomic alterations in prostate cancer has been vastly increasing since the establishment of high-throughput sequencing methods. The mutations vary from SNPs and other small insertions and deletions to copy number alterations (CNAs) of whole chromosomes (Taylor et al. 2010, Robinson et al. 2015). CNAs have been implicated to have more effect on PCa progression than point mutations (Taylor et al. 2010, Berger et al. 2011, Grasso et al. 2012, Baca et al. 2013, The Cancer Genome Atlas Research Network (TCGA) 2015). They even seem to occur in a chain like and coordinated manner (Baca et al 2013, Berger et al. 2011). The amount of point mutations varies from similar to lower (Vogelstein et al. 2013, TCGA 2015, Berger et al. 2011, Taylor et al. 2010) in comparison to other solid tumors, but increases with patient age and disease progression (TCGA 2015).

Relatively few recurrent mutations that affect specific proto-oncogenes and tumor suppressors are known in PCa (Taylor et al. 2010). Recently, the cancer genome atlas research network reported that many primary tumors can be classified to seven distinct subgroups according to the driver genes that are mutated (TCGA 2015). However, one fourth of the analyzed 333 tumors could not be assigned to any of these subclasses (TCGA 2015), highlighting the heterogeneity of the disease. Despite of this large group of unclassifiable cases, most PCas have mutations in certain key pathways (Taylor et al. 2010, Barbieri et al. 2013).

2.3.2.1 Key signaling pathways in PCa carcinogenesis

PI3K pathway is among the most frequently altered in cancer genomes. Defects in both tumor suppressor functions and proto-oncogenes have been reported on this pathway in PCa (Barbieri et al. 2013). PI3K signaling initiates from the cell membrane where the enzyme after which the pathway is named phosphoinositide-3-kinase (PI3K) is activated by membrane bound receptor tyrosine kinases following extracellular growth signal (Courtney et al. 2010). PI3K phosphorylates the sugar group of phosphatidylinositol glycolipid creating binding platform for v-akt murine thymoma viral oncogene homolog 1 or AKT1 (encoded by *AKT1*) (Courtney et al. 2010). After binding, AKT1 is

also phosphorylated and becomes active (Courtney et al. 2010). It has multiple cellular targets and its activation eventually leads to functions that are inherently related to the cancer phenotype, namely: growth, proliferation and antiapoptotic and pro-survival signaling (Courtney et al 2010).

Phosphatase and tensin homolog (PTEN) acts as the most important gatekeeper of the pathway by dephosphorylating phosphatidylinositol (3,4,5)-trisphosphate and thereby obstructing AKT1 activation (Courtney et al. 2010). It is also one of the most frequently deleted genes in prostate cancer (Taylor et al. 2010, Barbieri et al. 2012, Robinson et al. 2015). In addition, PHLPP1, a phosphatase that deactivates AKT, has also been found to be recurrently altered in both primary and metastatic PCa (Taylor et al 2010, Chen et al. 2011). Moreover, deletion of both *PTEN* and *PHLPP1* are more frequent in metastatic prostate cancer carrying *TP53* deletion or mutation, suggesting that the p53 acts as a barrier against mutations of both *PTEN* and *PHLPP1* or *2*, although the exact order of these events is not clear (Chen et al. 2011).

Amplification of kinases in the upstream of the pathway, especially PI3KCA, is also a common mechanism for PI3K pathway hyperactivity in PCa (Barbieri et al. 2013). By contrast AKT1 mutations are quite rare, as only about 1,4 % of PCas have been estimated to have the activating E17K mutation (Boormans et al. 2010). Finally, in the downstream of the PI3K signaling pathway another tumor suppressor, *CDKNB1*, cyclin-dependent kinase inhibitor 1B an important regulator of cell cycle progression, is among recurrently deleted loqi in PCa (Lapointe et al. 2007, Taylor et al. 2010, Barbieri et al. 2013).

From the clinical point of view, the most important signal system in prostate cancer is the AR signaling pathway, because it is frequently targeted with androgen deprivation therapy (ADT) as a treatment. AR is a nuclear receptor expressed by prostate luminal and stromal cells but not by the neuroendocrine cells and generally not by the basal cells (Zhou et al. 2015). It is activated by androgen hormones such as testosterone causing AR binding to DNA and transcriptional activation of target genes (Shen & Abate-Shen 2010, Zhou et al. 2015). In the normal prostate, AR signaling is complex interplay between stromal and epithelial cells and involves several binding partners of AR that can modulate its functions from a cell type to another (Shen & Abate-Shen 2010, Zhou et al. 2015). This complex network still remains incompletely understood, but the key functions are related to keeping

tissue homeostasis by supporting differentiation of luminal cells and promoting their survival while keeping the self-renewal of the epithelium in balance (Zhou et al. 2015).

Alterations in AR gene occur only after ADT (Visakorpi et al. 1995, Linja & Visakorpi 2004, Taylor et al. 2010). This suggests that AR itself does not initially drive carcinogenesis. However, it is required for the survival of prostate cancer cells and gain-of-function mutations become survival advantage when the selection pressure of the treatment forces to survive with minimal androgens (Barbieri et al. 2013). AR signaling may, however, play a role in early carcinogenesis via AR binding partners some of which have been found recurrently mutated in both primary and metastatic CRPCs. These genes include transcription factors forkhead box A1, *FOXA1* that modulates AR regulated transcription (Gao et al. 2003, Barbieri et al. 2012, Grasso et al. 2012) and nuclear receptor coactivator 2, *NCOA2*, which is after its name coactivator of AR (Taylor et al. 2010).

2.3.2.2 Other common somatic alterations in PCa carsinogenesis

The most usual genetic rearrangement in PCa identified by Tomlins et al. in 2005 is *TMPRSS2–ERG* gene fusion, in which the promoter of AR regulated gene *TMPRSS2* is fused with the coding region of transcriptional regulator *ERG* (Tomlins et al. 2005, Rubin et al. 2011). There are also other AR regulated 5' fusion partners and *ETS* transcription factor family members that are alternative to *TMPRSS2–ERG* fusion (Rubin et al. 2011). Jointly they are present in 50 to 70 % of PCas (Rubin et al. 2011). Because of high prevalence of *ETS* gene fusions and their molecular and phenotypic profiles it has become widely accepted that they form at least one distinct clinical entity (Rubin et al. 2011, TCGA 2015).

The commonness of seemingly such an improbable event is due to the growth benefit posed by the fusion as well as the mechanism that drives the gene fusion. *ETS* gene fusions do not occur in random. Instead, they seem to be guided by androgen receptor binding (Lin et al. 2009, Haffner et al. 2010). DNA topoisomerase TOP2B (Haffner et al. 2010) or other enzymes in the conditions of genotoxic stress (caused by exogenous agents such as radiation) can generate double strand breaks (DSBs) that can act as recombination sites (Lin et al. 2009). The probability of successful recombination during non-homologous end joining is increased by co-localization of androgen induced *TMPRSS2*, and *ERG* during androgen stimulation (Lin et al. 2009).

The oncogenic action of *ERG* has been attributed to higher activity of serine proteases (Klezovitch et al. 2008). This causes disruption of the basal membrane and formation of prostate intraepithelial neoplasia (PIN, discussed in section 2.3.3) in mice (Klezovitch et al. 2008). Although causing more invasive phenotype (Klezovitch et al. 2008), *ERG* overexpression does not lead to cancer progression without further lesions in genes such as *PTEN* of which deletion is enriched in *TMPRSS2–ERG* fusion positive cancer (King et al. 2009, Taylor et al. 2010, TCGA 2015). Together with heterogeneous loss of *Pten*, *ERG* overexpression was shown to cause rapid progression with increased proliferation and migration of PCa in mouse studies (Carver et al. 2009).

TMPRSS2–ERG fusion positive PCas also have higher occurrence of *TP53* deletions (Taylor et al. 2010). Cellular tumor antigen p53 encoded by *TP53* is a key tumor suppressor that regulates response to diverse stress situations eventually leading to DNA damage repair, cellular senescence or apoptosis. Traditionally loss of p53 function has been seen as a late event in carcinogenesis but it occurs also in primary PCa (Taylor et al. 2010), although nowhere near as frequently as in CRPC where it is prevalent in over 50 % of cases (Robinson et al. 2015).

Subset of ETS fusion negative cancers has been found to have recurrent mutations in *SPOP* (speckle type BTB/POZ protein) (Barbieri et al. 2012). Speckle-type POZ protein is the substrate binding subunit of Cullin based E3 ubiquitin ligase that regulates target proteins in multiple pathways (Zhuang et al. 2009, Barbieri et al. 2012). Like *TMPRSS2–ERG* fusion *SPOP* mutations seems to increase invasion while not affecting proliferation (Barbieri et al. 2012). Likewise, *SPOP* mutations are found in high-grade intraepithelial neoplasia (HGPIN) suggesting that the alteration is early event in PCa carcinogenesis (Barbieri et al. 2012).

SPOP mutant PCas have been associated also to mutations of *CHD1* encoding chromodomain helicase DNA binding protein 1 (Barbieri et al. 2012, Blattner et al. 2014, TCGA 2015). These cancers have increased levels of DNA methylation as well as homogenous gene expression profiles (TCGA 2015). Also mRNA of serine peptidase inhibitor, Kazal type 1, *SPINK1* has been found overexpressed in *SPOP/CHD1* mutation carrying tumors (TCGA 2015). However, no clinically significant features or distinctive tumor morphology have been identified with this tumor type so far (Blattner et al. 2014).

Other frequent alterations in PCa include deletion of chromosome 8p21 (Bova et al. 1993, Taylor et al. 2010, TCGA 2015). This region contains tumor suppressor gene NK3-homeobox 1, *NKX3-1* (He et al. 1997). Homeobox protein Nkx-3.1 is an androgen receptor responsive transcription factor that regulates prostate epithelial differentiation and proliferation (Bhatia-Gaur et al. 1999). Loss of *NKX3-1* expression has been correlated to PCa progression with most metastases completely lacking its expression (Bowen et al. 2000). In addition, even heterozygous deletion of the gene has been shown to cause hyperplasia and dysplasia of prostate epithelium in mouse studies (Bhatia-Gaur et al. 1999). More recently it has been shown to regulate response to DNA damage by guiding repair machinery to sites of DSBs *in vitro* (Bowen & Gelmann 2010). Thus the gene has dualistic role in PCa, favoring slight decrease, allowing proliferation and less confined differentiation state, and at the same time slight increase in the mutational rate due to less effective damage repair (Shen & Abate-Shen 2010).

In contrast, q arm of the chromosome 8 is among the most frequently amplified in PCa (Taylor et al. 2010, TCGA 2015, Robinson et al. 2015). The region contains the aforementioned AR coactivator gene *NCOA2* as well as v-myc avian myelocytomatosis viral oncogene homolog, *MYC,* encoding c-MYC, a master regulator of transcription (Taylor et al. 2010).

C-MYC is frequently overexpressed in various hematological malignancies and colorectal cancer (Dang 2012). Its various functional roles are related to proliferation and growth, for instance by promotion of cell cycle progression and replication as well as stimulation of cap dependent translation (Dang 2012). In mouse prostate, c-MYC drives carcinogenesis together with lesions such as loss of *Nkx3.1,* which is also a potential target gene of c-MYC (Ellwood-Yen et al. 2003). In PCa c-MYC overexpression has also been associated with development of treatment resistance toward ADT as one alternative to AR alterations (Bernard et al. 2003, Gundem et al. 2015). Although not located in the same chromosome, amplification of another MYC gene family member, *MYCL,* has also been linked to primary PCa in one report (Boutros et al. 2015). Despite this wealth of knowledge, *MYCs* role in PCa remains incompletely defined as it is altered only in only under 10 % of primary PCas and has not been associated with alterations of any other driver gene or disease subtype in specific (TCGA 2015).

2.3.2.3 MicroRNAs in PCa carcinogenesis

Like protein coding genes miRNAs can act as oncogenes (oncomiRs) or tumor suppressors depending on their target genes. It is not therefore surprising that expression of some miRNAs is similarly dysregulated as that of some protein coding genes in the setting of cancer. For example, miR-21 is an oncomiR overexpressed in variety of human solid tumors including PCa (Volinia et al. 2006, Ambs et al. 2008) and targets *PTEN* in hepatocellular carcinoma (Meng et al. 2007). In PCa miR-21 has been shown to be androgen inducible in AR positive cell lines and to increase cell proliferation *in vitro*, increase tumor xenograft growth in mice and even to drive androgen independent progression (Ribas et al. 2009).

Both global upregulation (Volinia et al. 2006, Ambs et al. 2008, Song et al. 2015) and downregulation (Porkka et al. 2007, Ozen et al. 2008, Martens-Uzunova et al. 2012) of miRNA expression have been reported in prostate cancer. It is tempting to think that the changes are related to changes in the miRNA biogenesis pathway. Few studies (Chiosea et al. 2006, Ambs et al. 2008, Martens-Uzunova et al. 2012) have studied expression of miRNA biogenesis pathway components in prostate cancer. Ambs et al. found moderate overexpression of microprocessor complex subunit *DGCR8* and *DICER1* (Ambs et al. 2008). Chiosea et al. also found Dicer components to be overexpressed, whereas Martens-Uzunova et al. did not find the connection (Chiosea et al. 2006, Martens-Uzunova et al. 2012). It is thus unlikely that there are major changes in miRNA biogenesis.

The miRNA expression profiling studies so far have mostly used quite small cohorts, have variability in miRNA preparation and detection platforms and use different control samples, for instance normal adjacent tissue, BPH samples or tissue from healthy individuals, which makes comparison less reliable (Fabris et al. 2016, Ozen et al. 2008, Song et al. 2015). Nevertheless, there are some miRNAs whose expression patterns in PCa remain consistent between multiple studies (listed by Fabris et al. 2016). For example, miR-145 has been found to be downregulated in total of five studies (Ambs et al. 2008, Porkka et al. 2008, Ozen et al. 2008, Martens-Uzunova et al. 2012, Larne et al. 2013) and to target oncogene *MYC* in molecular level (Sachdeva et al. 2009).

The field of miRNA profiling now most urgently needs revision of the current findings with larger cohorts. The completion of this task has been made greatly more accurate and comprehensive by the advances in the sequencing technology (Song et al. 2015). It is now possible to sequence the whole

small RNA transcriptome without any presumptions about the tissue miRNA content, which makes it possible to detect novel miRNAs and variation in the lengths of the mature miRNA (Song et al. 2015). In addition, high-throughput methods could be used to give directional evidence of miRNA–target interactions by identifying inverse correlations in expression levels in the multi-omics data.

2.3.3 Screening and diagnosis

Men with prostate cancer have typically presented to the clinic with difficulties in urination (Virtanen, Hervonen 1995). The clinical investigations that follow are serum PSA test, digital rectal examination or rectal ultrasonography and if anything suspicious is found, taking of multiple needle biopsies from the gland (Attard et al. 2016, Shen & Abate-Shen 2010).

PSA screening revolutionized prostate cancer diagnosis when it was introduced as a primary prostate cancer biomarker in the early 1990s (Prensner et al. 2012). As the serum PSA levels are very low in a healthy individual, the test is very sensitive in detecting such architectural changes in the prostate epithelium and vascularization that allow PSA leakage to the circulation (Lilja et al. 2008). However, such changes do not always occur during carcinogenesis. The prevalence of prostate cancer in biopsy among men with serum PSA levels under 4 ng/ml, a commonly used serum PSA threshold, was ~15 % in one study (Thompson et al. 2004). On the other hand the prostate architecture may change as a consequence of other pathological states including inflammation and BPH (Nadler et al. 1995).

BPH is a common disease in older men. Depending on definition and geographical location, as much as almost 3 % of men 40 – 49 years old and 23 - 30 % of men aged 80 have been estimated to have BPH (Verhamme et al. 2002, Speakman et al. 2015), which decreases the credibility of PSA as a reliable marker for early detection of prostate cancer.

Despite these problems in specificity and sensitivity, higher serum PSA levels have been significantly correlated with PCa with more advanced cancers having even more elevated levels (Stamey et al. 1987, Pinsky et al. 2007). Moreover serum PSA levels have been found to be useful predictor of prostate cancer death even ten years before when measured first under age 50 (Vickers et al. 2013). Nonetheless, only fraction of the patients can benefit from the screening, because many of the prostate cancers detected with serum PSA measurements are indolent and treatment can potentially lead

reduction of the patients' quality of life because of adverse events such as pain, anxiety and sexual dysfunction (Prensner et al. 2012).

For these reasons population based screening with PSA is not currently recommended neither in Europe or the USA. This conclusion has been reached in two ongoing longitudinal studies investigating efficacy of PSA screening (ERSPC in Europe and PLCO in USA) in preventing prostate cancer mortality even if a slight reduction in mortality in the screening group in comparison to the control group was observed in the ERSPC study (Andriole et al. 2012, Schröder et al. 2014). However screening of men who want early diagnosis should not be denied (Schröder et al. 2014).

The various imperfections associated with PSA as a prostate cancer biomarker emphasize the need of new biomarkers that would ideally be non-invasive and have better prognostic value than PSA (Prensner et al. 2012). Together with this kind of new biomarker, screening with PSA could prove to be both sensitive and selective (Lilja et al. 2008).

MiRNAs have been discussed as such new biomarkers: they are readily detectable in blood plasma, resist degradation (Michell et al. 2008) and their expression patterns change during the course of PCa (Mahn et al. 2011). The problem with miRNAs is that no single miRNA is specific enough to distinguish PCa from healthy tissue. On the other hand, it takes time and effort to validate a miRNA signature that is reproducible (Fabris et al. 2016) and if a blood based test is in question, present in blood in sufficient amounts (Mahn et al. 2011). This is also further complicated by the notions that miRNA profile of an individual changes during the course of aging (Liang et al. 2009) and the release of miRNAs is a selective process at least in some contexts (Pigati et al. 2010). Currently no such large scale studies have been conducted that the validation of this kind of panel would need, although several candidates of miRNAs for detection of the disease, distinguishing aggressive forms or biochemical recurrence after androgen deprivation therapy have been presented (Fabris et al. 2016).

Other possibilities for new prostate cancer biomarkers are also emerging including PCA3, a long noncoding RNA that is only detected in PCa and not in BPH (Prensner et al. 2012). In addition, *TMPRSS2-ERG* fusion gene mRNA detection could prove to have prognostic power (Prensner et al. 2012, Rubin et al. 2011). Despite being present only in just under half of the PCas, it is cancer specific

and the fusion is an early event in cancer progression providing enhanced ability to identify potentially more aggressive cancer cases at an early state (Rubin et al. 2011, Perner et al. 2007).

In the absence of decisive noninvasive biomarkers, prostate biopsy remains golden standard of diagnosis. There are three types of prostate biopsies: needle biopsies that are maybe the most common procedure for suspected prostate cancer cases, transurethal resections of the prostate (TURPs) that have been used to treat lower urinary track symptoms (Epstein 2010) and the prostatectomies which are not done to make a diagnosis but rather as a surgical treatment for localized prostate cancer (Heidenreich et al. 2014a). It has to be said that even needle biopsy or TURP may not be decisive because of possible sampling errors and in the prognostic point of view a higher grade cancer could be missed because of the multifocality of prostate cancer (Epstein 2010, Fabris et al. 2016).

Prostate intraepithelial neoplasia (PIN) and especially high-grade PIN (HGPIN) has been considered as the main precursor lesion of prostate carcinoma (Bostwick & Qian 2004). PIN is characterized by overgrowth of acinar or ductal luminal cells into the gland lumen as opposite to BPH where the proliferation occurs in the underlying basal cells (Bostwick & Qian 2004, Berman & Epstein 2014). In HGPIN the gland's basal cell layer can also be discontinuous, indicating change towards more invasive phenotype. The carcinoma is indeed only distinguished from HGPIN by invasion to prostate stroma (Bostwick & Qian 2004). Invasive carcinoma is also often found together with HGPIN or in subsequent biopsies of the same patient (Netto & Epstein 2006). This is especially true if the HGPIN found is *TMPRSS2-ERG* fusion positive (Park et al. 2014).

Recently Haffner et al. published evidence that at some occasions *TMPRSS2-ERG* gene fusion positive and *PTEN* negative carcinoma could form HGPIN mimicking structures in a retrograde manner (Haffner et al. 2016). This does not necessarily challenge the station of the HGPIN as a precursor of prostate cancer, but rather describes an important subset of cases misinterpreted as HGPIN. From the clinical point of view, the finding is relevant because a structure resembling a precursor lesion could actually prove to be aggressive cancer (Haffner et al. 2016).

The fundamental property of cancer and the ultimate criterion for cancer diagnosis is the ability of cancer cells to invade into surrounding tissue (Berman & Epstein 2014). This criterion is met by all malignant neoplasia, although it has to be acknowledged that not all cancers are life threatening

(Berman & Epstein 2014). As discussed previously, many prostate cancers are indolent by nature and distinguishing the aggressive cancers still remains a major clinical obstacle today (Attard et al. 2016).

2.3.4 Prognosis and treatment

One of the most widely used mean to categorize prostate tumors to make prognosis is the Gleason scoring system. It is based on pathologist's evaluation of architectural patterns of the tumor (Mellinger et al. 1967). This means the gland appearance from well differentiated separate glands to poorly differentiated glands showing spread of epithelial cells into the healthy prostate tissue (Epstein 2010). The Gleason score is obtained by adding up two of the most prominent or in the case of needle biopsy the highest and the most prominent tumor patterns (Epstein 2010). Low score cancers include all scores from two to six and generally under Gleason score six cancers are not diagnosed anymore (Epstein 2010). Low score cancers generally do not metastasize and are curable (Ross et al. 2012, Epstein 2010). On the other hand, high scoring cancers Gleason 8-10 are more aggressive and should be treated as high risk cases (Heidenreich et al. 2014a).

In addition to Gleason score, tumor stage is assessed by investigating tumor status: TNM staging reviews the size or invasiveness of the primary tumor T, status of tumor spread in the regional lymph nodes N and possible metastases M, which are detected by CT or MRI bone scans (Shen & Abate-Shen 2010, Damber & Aus 2008).

The poorly differentiated and well progressed tumors especially with distant metastases have the worst prognoses (Damber & Aus 2008). Luckily, most prostate cancers diagnosed are in the organ confined stage (Damber & Aus 2008). Treatment of these cancers varies from active surveillance without curative treatment to prostatectomy or external-beam radiotherapy or brachytherapy (internal radiotherapy) depending on patient age and the diagnostic factors such as serum PSA levels and the tumor Gleason score and TNM stage and number of positive needle biopsies (Damber & Aus 2008, Heidenreich et al. 2014a, Attard et al. 2016). In addition, newer and less frequently used treatments include high-intensity focused ultrasound and cryotherapy (Heidenreich et al. 2014a, Attard et al. 2016).

The life expectancy for patients with clinically localized prostate cancer is generally good as most prostate cancer deaths result from metastatic disease. However, locally advanced disease might be

life threatening too as it is associated with the risk of occult metastasis (Damber & Aus 2008). For these patients, adjuvant androgen deprivation therapy (ADT) is the best choice together with radical prostatectomy or radiation therapy as a primary treatment (Heidenreich et al. 2014a, Attard et al. 2016).

ADT has been the most effective available treatment for locally advanced or metastatic prostate cancer ever since it was established in the 1940s (Huggins & Hodges 1941, Damber & Aus 2008). The castration is done most often chemically nowadays, allowing reversibility and intermittency of the therapy, which reduces adverse effects (Heidenreich et al. 2014b). These drugs affect systemically by blocking the release of luteinizing hormone from the pituitary gland, which in turn prevents synthesis of androgens in the testicles (Heidenreich et al. 2014b). However, with this approach it is not possible to reach full androgen ablation as adrenal gland as well as some prostate cancer cells themselves can synthesize androgens *de novo*, independent of luteinizing hormone release (Stanbrough et al. 2006, Locke et al. 2008). Nevertheless, this does not mean that chemical castration would be inferior to other means of preventing PCa cells from using androgens, as these mechanisms of resistance probably represent only small subset of routes to castration resistance.

Antiandrogens are steroidal or nonsteroidal molecules that bind to androgen receptor without activating it. These AR-antagonists can be used in combination with or optionally to castration (Heidenreich et al. 2014b, Attard et al. 2016). However, chemical castration or combination therapy remains the preferable option as the traditional antiandrogens such as bicalutamide are not as effective as castration and there is no evidence from larger trials comparing the benefits of castration and newer antiandrogens, such as enzalutamide, as a monotherapy for hormone naïve cancer (Attard et al. 2016, Pchejetski et al. 2014). Moreover, at least AR-antagonists such as bicalutamide, cyproterone acetate and flutamide have been found to act as agonists of AR in the setting that AR is overexpressed and the balance of coactivators and corepressors has been upset (Chen et al. 2004).

In addition, there is newer drug, abiraterone acetate, that targets the synthesis of testosterone by inhibiting CYP17A1 enzyme irreversibly (Pchejetski et al. 2014, Attard et al. 2008). The greatest advantage of this drug is that it works systemically inhibiting enzymes in the testicles, adrenal cortex and the PCa cells. However, at the same time it leads to the need of steroid supplementation often in

the form of prednisone to overcome side effects of excess mineralocorticoids that are synthesized in the metabolic pathway upstream of CYP17A1 (Attard et al. 2008).

Although ADT initially leads to sound response with decreased tumor burden and relief of symptoms, the disease inevitably gains resistance to the treatment (Damber & Aus 2008). The most effective drugs in the treatment of CRPC are the previously mentioned enzalutamide and abiraterone acetate as well as cytotoxic agents such as docetaxel and cabazitaxel (Attard et al. 2016). Nevertheless, these drugs can only provide prolonged survival and temporal relieving of symptoms instead of cure for the patients, as the cancer eventually gains resistance.

The use of these most promising drugs, enzalutamide and abiraterone acetate, in the setting of hormone naïve patients would be the next rational approach to improve treatment of prostate cancer despite their expenses (Pchejetski et al. 2014, Attard et al 2016). In addition, docetaxel may benefit these patients (Vosboynik et al. 2014).

The fact that the advanced disease previously perceived as "androgen independent" commonly remains androgen dependent has given hope of finding new androgen signaling targeting drugs that would not allow development of resistance to the treatment. Nonetheless, it seems that these bulk treatments may not be viable options for all the patients, for example those possessing *AR* gene mutations (Robinson et al. 2015) and for this reason further research focusing into the molecular mechanisms of prostate cancer has to be continued.

However, there probably is not another "one drug for all prostate cancers" type of solution as it is the case with androgen signaling pathway targeting drugs. The heterogeneity of PCa forces to find targets that not necessarily all cases share, which raises the cost and effort of tackling the disease. While on the other hand this may be compensated by the possibility of finding drugs that are effective in limited subtypes of cancer of multiple different organs (Courtney et al. 2010). Nevertheless, with the ever improving knowledge of the biology of prostate cancer it is possible to take more effective and personalized approach into PCa management. And there certainly is not lack of alternative targets. For example, alterations in PI3K and Wnt signaling could be targeted with existing drugs as well (Courtney et al. 2010, TCGA 2015, Robinson et al. 2015). The unquestionable benefits of this

approach have already been proven in a trial in which patients with mutations in DNA damage repair genes were treated with DNA damage repair impairing drug olaparib (Mateo et al. 2015).

3. OBJECTIVES

The purposes of this study were to assess whether miR-32 regulates expression of PI15 and how PI15 overexpression affects to the growth and viability of PCa cells. Exogenous source of both PI15 (in the form of plasmid vector) and miR-32 (in the form of pre-miR) were used to have higher levels of expression and thus bigger changes that would be easier to monitor both in the RNA and protein level.

In addition, two new vectors were needed for the generation of stable PI15 overexpressing cell lines and for luciferase reporter assay. The first would need to have marker for antibiotic selection in mammalian cells and the second the coding region of luciferase marker gene with the 3'UTR of *PI15* to allow possible binding of miR-32.

Thus, the objectives were:

1. To assess whether PI15 is miR-32 target gene
2. To construct suitable vectors for generating PCa cell lines stably expressing PI15 and for the luciferase assay
3. To observe effects of PI15 overexpression on PCa cell lines' growth and cell viability

4. MATERIALS AND METHODS

4.1 Sanger sequencing

Sequencing was used to confirm that the commercial pCMV6-XL4 PI15 plasmid (Origene technologies, Rockville, MD USA) was correct and contained the whole gene with the long 3' UTR region (full length 6732 bp, refseq id: NM_015886.3). Sequencing primers were generated using the Primer BLAST tool by NCBI (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) and the best primers were selected on the preference that they did not have excessive secondary structures within the primer sequence as predicted by mFold web tool (Zuker et al. 2003). In addition, both *PI15* ORF and *luc2P* cloning was validated by sequencing.

Samples were prepared for the sequencing by amplification with master mix containing fluorescent terminator nucleotides. Simple master mix was prepared as follows: 1 µl BigDye terminator mix (Thermo Fisher Scientific, Waltham, MA USA); 1,5 µl of 5x BigDye buffer and 5,5 µl of nuclease free water. Each reaction was supplied with 300 ng of sample DNA in the volume of 1 µl and 1 µl of 5 µM sequencing primers. The PCR reactions were thermally cycled according to reagent manufacturer's instructions. Sequencing primers are denoted with "seq" in the primer name in table 1. List of primers. In addition, primers VP1.5 and XL39 provided by Origene Technologies were used for sequencing.

Table 1. List of primers

#	Primer name	Primer sequence
1.	VP1.5	GGACTTTCCAAAATGTCG
2.	PI15_Seq_for2	GGCCAAAATCTATCTGTACG
3.	PI15_Seq_for3	TGTGCTAATCTTGTTTTCCTC
4.	PI15_Seq_for4	CTTATTTGAGTCCACCAAAGG
5.	PI15_Seq_for5	GATGGAGAGCTTCAGAATGG
6.	PI15_Seq_for6	TCAGGTTGTGTAATGCTCCCG
7.	PI15_Seq_for7	AGATGGCTACACTAAGTTCC
8.	PI15_Seq_for8	ACACACTTAGGCAATAGTCC
9.	PI15_Seq_for9	GACACAGTCTTAATGTTTCTGG
10.	PI15_Seq_for10	ACTTTGGTGTAGGTTCTTCC
11.	PI15_Seq_for11	TGTTGATGGCTTTAGTAATGCTCC
12.	PI15_Seq_for12	CCATGATTTTCCAGTTCTGAGGC
13.	PI15_Seq_for13	CACATGCAAAACTCCAACCTGT
14.	PI15_Seq_for14	GTTAGCAGTCTTTCAGTTTGG
15.	PI15_Seq_rev1	ATGAACATGGTACCCCTACT

16.	PI15_Seq_rev2	AATGTGTGTTAAGAAGTCCCA
17.	PI15_Seq_rev3	TGGCTTATTACTTGCTCCAC
18.	PI15_Seq_rev4	GGAGCATTACACAACCTGAA
19.	PI15_Seq_rev5	ATTCCATGAATAAGCTTCCCT
20.	PI15_Seq_rev6	ACATTTTAGGTGCTGTCAGA
21.	PI15_Seq_rev7	ACAAAAGAGATTACACCCAGT
22.	PI15_Seq_rev8	ATATGTATCCAAGCACCAGC
23.	XL39	ATTAGGACAAGGCTGGTGGG
24.	PI15_NheI	AATTATGCTAGCCCACCCCTCAAAATGATAGC
25.	PI15_BamHI	ATAAATGGATCCGTAAACTTATTTAAACCAGTACAGG
26.	Luc2P_EcoRI_for2	AACTATGAATTCTGATAATATGGCCACATGGAAG
27.	Luc2P_HpaI_rev	ATATGAGTTAACAATTATTACACGGCGATCTTGC
28.	Luc2P_PstI_rev	ATATGACTGCAGAATTATTACACGGCGATCTTGC
29.	T7 promoter	TAATACGACTCACTATAGGG
30.	BGH_rev	TAGAAGGCACAGTCGAGG
31.	Luc_for	CGCACATATCGAGGTGGACA
32.	Luc_rev	AACTTGCCGGTCAGTCCTTT
33.	PI15_F	GGAAGCGCTACATTTCGCAG
34.	PI15_R	CCAAGCCTCTGCCGATTTTG
35.	TBP3 rev	GAGCCATTACGTCGTCTTCC
36.	TBP3 for	GGGGAGCTGTGATGTGAAGT

After the PCR the samples were precipitated by centrifugation with absolute ethanol supplied with sodium acetate. The precipitated samples were washed with 70 % ethanol and dissolved into Hi-Di formamide (Thermo Fisher Scientific). The sequencing was done by the University of Tampere core facility in ABI3130xl Genetic Analyzer (Applied Biosystems/ Thermo Fisher Scientific).

4.2 Subcloning of PI15 and luc2P

To observe the effects of overexpression of *PI15* gene, the genes' open reading frame (ORF) from the pCMV6-XL4 PI15 plasmid was subcloned into pcDNA3.1 (+) plasmid that contains *neo* antibiotic resistance gene against Geneticin. In addition, another plasmid was constructed for luciferase reporter assay. The pCMV6-XL4 plasmid was used as the backbone of this reporter plasmid in which the *PI15* coding region was replaced by *luc2P* gene from pHIV luciferase plasmid (https://www.addgene.org/21375/; 11.4.2016) kindly provided by prof. Anne Kalllioniemi's group (BioMediTech, University of Tampere, Tampere, Finland). The subcloning of the *luciferase* gene was designed by analyzing the *PI15* 3' UTR with the University of Waterloo web tool WatCut (http://watcut.uwaterloo.ca/template.php?act=restriction_new; 11.4.2016) for suitable cut sites. Two different versions of the plasmid were designed to preserve different lengths of the UTR. This was

done by using two different *luc2P* reverse primers with different cut sites for the 3' end of the *PI15* 3' UTR region where the miR-32's supposed binding site (Figure 2) is located.

```
     3' acgUUGAAUCAUUACACGUUAu 5' hsa-miR-32
           || || ||  ||||||||
4407:5' uaaAAAUUUGU--UGUGCAAUa 3' PI15
```

Figure 2. Hsa-miR-32/PI15 Alignment. Alignment from microrna.org (Betel et al. 2008 and 2010).

Mutation primers were designed for both cloning works manually by searching for sufficiently long stretches of DNA with preferentially 2 or 3 purines at the end of the 3' end. In addition to the sequence complementary with *PI15*, cut sites were added for restriction enzymes NheI and BamHI for the 5' end and 3' end primer respectively. For *luc2P* primers, cut sites for EcoRI and PstI or HpaI were added to the 5' end and 3' end primers respectively. In addition, 6 random nucleotides were added to the primers before cut site to ensure proper function of the restriction endonucleases.

To ensure that the PCR is as specific as possible the primers were assessed with sequence based bioinformatics tools for approximately equal melting temperature: Oligo calc (Kibbe et al. 2007), melting temperatures of possible secondary structures within primer sequence: mFold (Zuker et al. 2003) and formation of primer dimers: Multiple Primer Analyser by Thermo Fisher Scientific (https://www.thermofisher.com/fi/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/multiple-primer-analyzer.html#; 11.4.2016, Breslauer et al. 1986). Primer sequences are listed in table 1 with order numbers 24 and 25 for *PI15* ORF cloning primers and 26, 27 and 28 for *luc2P* cloning primers.

The PI15 ORF was subloned by traditional restriction–ligation method. PCR conditions were optimized for Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific) to minimize errors during amplification of the inserts. The PCR products and the empty pcDNA3.1 (+) plasmids where double digested with the BamHI and NheI restriction enzymes according to enzyme supplier protocol (New England Biolabs, Ipswich, MA USA) and extracted from 1 % agarose gel after electrophoresis with QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) to check right size of the amplicons and to get rid of any unspecific product.

PI15 ORF was ligated to the pcDNA3-1(+) vector with T4 DNA ligase (Thermo Fisher Scientific) according to manufacturer's protocol. Next 4 µl of the ligation mix was applied on One Shot® TOP10 Chemically Competent *E. coli* cells (Thermo Fisher Scientific) and after 15 min incubation the cells were transformed by 45 s heat shock in 42 °C water bath. The transformed bacteria were incubated 2 min on ice and supplied with 150 µl of SOC medium (Thermo Fisher Scientific) and streaked on LB-agar plates with 50 µg/ml ampicillin and cultured overnight in 37 °C.

On the next day, four colonies were picked and grown in LB-broth with 100 µg/ml ampicillin. Plasmids were extracted from the bacteria with GenElute™ Plasmid Miniprep Kit (Sigma-Aldrich, St. Louis, MO, USA) and the plasmids were checked for the insert by PCR with primer pair 29 and 15 (Table 1.), 2 and 30 and 29 and 30, the amplified regions spanning the 5' cloning site, 3' cloning site and the whole inserted region respectively. The presence of right sized products was checked first by agarose gel electrophoresis and the insert region was then sequenced, as previously described, with primers 2, 16, 29 and 30 to confirm that the gene had been successfully subcloned.

TOPO-TA cloning was used for the *luc2P* subcloning. The insert was first amplified with the primers 26 and 27 or 28 as described previously for *PI15* ORF. However, for TOPO-TA cloning another amplification was done for these products with Maxima HS Taq polymerase (Thermo Fisher Scientific) both to increase yield and to generate the TA overhangs required for the cloning technique. Subcloning to the pCR-XL-TOPO vector was carried out by TOPO-TA Cloning Kit (Invitrogen / Thermo Fisher Scientific) after manufacturer's instructions and TOP 10 chemically competent cells were transformed as described previously with the following exception: As the selection marker of pCR-XL-TOPO plasmid is bactericidal antibiotic kanamycin, the bacteria were incubated 1 h in 37 °C in the SOC medium before streaking onto the LB-agar kanamycin plates to allow expression of the resistance gene before introducing them to the antibiotic.

Subcloning was confirmed with colony PCR and restriction analysis. The primer pair 31 and 32 was designed to amplify 1458 bp region within the *luc2P* gene and the restriction enzymes EcoRI, PstI and HpaI (Thermo Fisher Scientific) were used both alone for linearization and in pairs (EcoRI and PstI, EcoRI and HpaI) to separate the inserts. After the digestion, the inserts and similarly digested pCMV6-XL4 vectors containing the *PI15* 3' UTR were extracted from gel and ligated with T4 DNA ligase with an elongated ligation time of 4 h. After transformation and liquid culturing of the positive

clones, the new plasmids were again tested with colony PCR with primer pair 31 and 32 (*luc2P*) and 29 and 20 (region spanning the whole insert region including cloning sites). The plasmid was also sequenced with the primers 20, 21, 29, 31 and 32.

4.3 Cell lines

PC-3 and LNCaP from American Type Cell Collection (Manassas, VA USA), were the cell lines used in the experiments of this work. PC-3 cells were cultured in Ham's F-12 (Lonza, Basel, Switzerland) and LNCaPs in RPMI-1640 (Lonza) medium supplemented with 10 % fetal bovine serum and 1 % of L-glutamine in 37 °C and presence of 5 % CO_2. The cells were passaged twice a week and were not allowed to reach full confluency.

4.4 Transfections and generation of stable cell lines

The expression vectors were checked by transfecting PC-3 and LNCaP cell lines with either pCMV6-XL4 PI15 or pcDNA3.1(+) PI15 ORF and by comparing *PI15* expression of these cells into empty vector transfected controls. The effects of miR-32 on *PI15* expression was studied in co-transfection experiments, where the commercial pCMV6-XL4 PI15 vector or empty control vector and pre-miR-32 or pre-miR control (Ambion / Thermo Fisher Scientific) were used. Transfections were also made to generate cell lines with *PI15* gene stably integrated into their genomes.

Mono-transfections were done with the following protocol. 400 000 cells in 2 mls of medium were first seeded into six well plates. The cells were then transfected with mix containing 2 µgs of plasmid vectors and 4 µls of jetPEI DNA transfection reagent (Polyplus-transfection, Illkirch, France) in 200 µl of 150 mM NaCl.

In the case of stable transfection, the plasmid vectors (pcDNA3.1 (+) PI15) were linearized with BglII restriction enzyme (Thermo Fisher Scientific) before transfection to improve efficiency of genome integration. Also the transfection was scaled up by using two million cells, 10 µgs of the plasmid and 20 µls of jetPEI to overcome the antibiotic selection burden.

The antibiotic selection was started on day three after the transfection with 500 µM Geneticin (Gibco / Thermo Fisher Scientific) for PC-3 and 300 µM Geneticin for LNCaP cells. The selection medium

was carefully replaced at least every third day until visible colonies were detected. The colonies were ring cloned and passaged onto bigger culture formats until sufficient amount for growth curve and metabolic rate analysis was reached.

For co-transfections 250 000 cell were first seeded into six well plates in three replicates. In the next day the cells were transfected with mix containing 0,5 µgs of plasmid vector; 1,0 µls of 20 µM pre-miR (for final concentration of 10 nM in 2 mls of medium) and 4 µls of jetPRIME (Polyplus-transfection) in 200 µls of jetPRIME buffer (Polyplus-transfection). The co-transfections where repeated twice for RNA extraction.

4.5 Growth curves and cell viability assays

For the growth rate analysis 10 000 cells of each stable PI15 clone and control clone were seeded into 24 well plates in six replicates. The cells were imaged on days 1, 3 and 4 after seeding with 10 x magnification with Olympus IX71 microscope with integrated OASIS automation control system and Surveyor imaging software version 5.5.5.26 (Objective Imaging Ltd., Cambridge, UK). The area of the cells in each well was then analyzed with ImageJ software (NIH, Bethesda, MD USA). The experiment was repeated three times for the PI15 overexpressing clones, however the two initial experiments did not contain proper empty vector transfected controls.

AlamarBlue® Cell Viability Reagent (Thermo Fisher Scientific) was used as another measure to monitor growth and viability of the clones. The cells were seeded as with the imaging experiment, but there were two plates with six replicates for each clone because of the setup of the experiment. On day one after seeding the first six replicates were supplied with 50 µl AlamarBlue to the 500 µl of growth medium, put into the incubator and 100 µl samples were collected after one and two hours after the addition of the reagent. Same was repeated for the remaining six replicates on day four after seeding. The samples were stored in -20 °C, protected from light until fluorescence emission at 590 nm following excitation at 560 nm was measured with Envision multilabel plate reader spectrophotometer (PerkinElmer, Waltham, MA USA). The fluorescence values of the day one samples were used to normalize the values acquired for the day four samples.

4.6 RNA extractions and reverse transcription

Cells were collected approximately 48 hours after transfections by applying appropriate amount (8 ml for T75 flask, 1 ml for well of a six well plate) of TRI Reagent (Thermo Fisher Scientific) onto them while keeping them on ice. All RNA extractions were carried out with TRI Reagent according to manufacturer's instructions. Glycogen (RNA grade) (Thermo Fisher Scientific) was added to the sample with isopropanol when extracting RNA from samples cultured in 6 well plate format for enhanced precipitation of the RNA.

Reverse transcription was used to generate cDNA suitable for qRT-PCR. Random hexamer oligonucleotides were used as primers to reverse transcribe the whole pool of extracted RNAs. 1000 ng of RNA, as measured with the NanoDrop 1000 (Thermo Fisher Scientific) instrument, was used as starting amount of total RNA. The reactions were done with Maxima Reverse Transcriptase (Thermo Fisher Scientific) according to the manufacturer's instructions.

4.7 SYBR-green and TaqMan qRT-PCR assays

SYBR-green qRT-PCR method was used to measure the amount of *PI15* transcript in transfected PC-3 and LNCaP cells (primer pair 33–34). Data was normalized against the house keeping gene *TBP* (primer pair 35–36). For the test run, a pool of PC-3, LNCaP and 22Rv1 total RNA in 1:5 dilution series was used as a standard, but as the samples transfected with the *PI15* plasmid were consistently over the scale of these wild type samples' signal, total RNA pool from these transfected cells were used as a standard for the subsequent qPCRs.

Single SYBR-green master mix was prepared by adding 10 µls of 2x Maxima SYBR Green (Thermo Fisher Scientific) and 0,1 µls of 10 µM forward and reverse primers into 7,8 µls of water. 2 µls of each sample, standard and water each in duplicates were added and the plate was cycled with program with a suitable annealing temperature for each primer pair and the fluorescent signal was read after each thermal cycle in CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA USA).

In addition to SYBR-green qRT-PCRs, TaqMan microRNA assay for quantitation of the amount of miR-32 in each sample was performed. RNU6B was used to normalize the amount of miR-32. PCRs for TaqMan were done in two parts according to Applied Biosystems' (Thermo Fisher Scientific)

TaqMan MicroRNA assays protocol. First, 25 ng/µl dilutions of the samples' and 250 ng/µl dilutions of the standards' total RNA were used for reverse transcription. Single reverse transcription master mix contains 9,15 µl of nuclease free water, 1,5 µl of 10 x Reverse transcription buffer 0,15 µl of 10 mM dNTPs, 0,2 µls of RNase inhibitor, 1 µl of RT primer and 1 µl of Multiscribe Reverse Transcriptase. 2 µl of sample were added to the master mix and the following PCR program was used for reverse transcription: 30 min at 16 °C (priming), 30 min at 42 °C (transcription) and 5 min at 85 °C (denaturation of transcriptase).

In the next step TaqMan qRT-PCR was performed to quantify miR-32 in the samples. Master mix for single sample contains 7 µls of nuclease free water, 10 µl of 2x universal PCR master mix and 1 µl of TaqMan MicroRNA Assay 20x. Reverse transcribed samples were diluted in 1:15 and 2 µls of the dilutions were used for each reaction. Following program was run with the CFX96 Real-Time PCR Detection System: initial denaturation 10 min at 95°C, 40 cycles of 15 s at 95 °C, 60 s at °60. No duplicates of the samples were run for the TaqMan assay because of the large amount of samples.

4.8 Protein extraction and determination of protein concentration

Cells for protein extraction and western blot were collected 72 to 96 h after transfections. Cells were first rinsed with fresh phosphate buffered saline (PBS). 500 µl of ice cold PBS with 2x concentration of cOmplete protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland) were applied onto the cells and they were scraped off and collected. The cells were then centrifuged in 800g for 3 min and the supernatant discarded.

The cells were lysed by resuspending them with 80 µl of Triton X-100 lysis buffer (0,5 % Triton-X 100, 300 mM NaCl in 50 mM Tris-HCL pH 7,4) supplied with 40 µl/ml 25 x cOmplete protease inhibitor cocktail, 10 µl/ml of 100mM DTT (Sigma-Aldrich), 10 µl/ml of 100 mM PMSF (Sigma-Aldrich), and incubating them 15–30 min on ice. Next the suspensions were further degraded by ultra-sonication with Bioruptor Sonicator (Diagenode, Liège, Belgium) the instrument set on high frequency, four times 30 seconds on and off. The suspensions where then centrifuged (10 min, 16 000 g) to remove cell debris and supernatant containing the proteins was passed to clean tube.

Because PI15 is a secreted protein, the culture medium was also collected as a sample for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and western blot (WB). The first

attempt of WB with medium containing serum lead to big smears in the membrane with no apparent marks of proteins the size of PI15 (~25 kDa). For this reason, in the second experiment the growth medium was replaced with 800 µl of serumless growth medium 24 h before collection. Before experiment the medium samples were centrifuged (10 min, 16 000 g). Undiluted medium was then used as sample for SDS-PAGE. Protein concentration was not measured from these growth medium samples.

The protein concentration was measured with DC™ Protein Assay (Bio-Rad), according to kit manufacturer's instructions, although in some instances the BSA standard series was much more concentrated (0,5 mg/ml to 10 mg/ml) than instructed by Bio-Rad (0,2 mg/ml to 1,5 mg/ml). The absorbances of the samples were measured in 96 well plate in the Envision multilabel plate reader spectrophotometer at 750 nm.

4.9 SDS-PAGE and western blot

After measurement of the protein concentrations, the samples were diluted into the same concentration and prepared for PAGE with Red Loading Buffer Pack (New England Biolabs), (1/10 volume of 1,25 M DTT as a reducing agent) according to the reagent supplier's instructions. Details of the samples in each experiment are listed in Table 2. Western blot samples.

Table 2. Western blot samples. Each column represents individual experiment. *Same samples as in the previous experiment. † The clone is stably transfected with PI15 plasmid but does not overexpress PI15 as assessed by qRT-PCR

Cell lines	LNCaP, PC-3	LNCaP, PC-3	LNCaP, PC-3	LNCaP*, PC-3*	PC-3 PI15
Medium sample	-	medium	serumless medium	serumless medium*	-
Controls	empty vector	empty vector	empty vector + untransfected	empty vector + untransfected*	non overexpressing PC-3 PI15 clone†
Trans-fection	pCMV6-XL4 PI15	pCMV6-XL4 PI15	pcDNA3.1 (+) PI15	pcDNA3.1 (+) PI15	Stable pcDNA3.1 (+) PI15
Amount of protein	7,5 µg / well	21 µg / well	13 µg / well	13 µg / well	20 µg / well
Antibody	polyclonal rabbit-α-PI15	polyclonal rabbit-α-PI15	polyclonal rabbit-α-PI15	monoclonal mouse-α-PI15	both

The samples were loaded into premade 12,5 % polyacrylamide gels (1 x running gel prepared by mixing 2,8 ml H_2O; 2,25 ml of 1,5 M Tris buffer with pH 8,8; 180 µl 10 % W/V SDS solution; 3,75 ml 30 % Acrylamide/Bis Solution (Bio-Rad), 10µl TEMED and 40 µl 10 % ammonium persulfate). In some instances, visible puffs of sample from well to another occurred, but their effect was minimized by leaving empty well between PI15 transfected and control (untransfected or empty plasmid) samples. The samples were run in Mini-PROTEAN® Tetra Cell Systems chamber (Bio-Rad) ~30 min with 50 V to drive the samples into the border of stacking and running gels and then ~1,5 h in 100 V until the edge of the sample buffer color marker emerged to the running buffer.

The proteins were transferred into immobilon®-P (Merck & Co., Inc., Kenilworth, NJ USA) PVDF membrane for detection with PI15 antibodies. Membrane was activated with methanol, and semidry electro blotting was done by loading the Trans-Blot Semi-Dry System (Bio-Rad) apparatus with three whattmans that were dipped into semi dry transfer buffer, the membrane was then applied onto the whattmans, followed by the polyacrylamide gel and three more wet whattmans. Air bubbles where then removed by rolling with a measuring pipette gently over the top of the stack. At last the proteins were transferred by applying 80 mA current for 1 h.

The membrane was rinsed with PBS and rocked for ~2 min in Ponceau S stain to check transfer of proteins and the stain was rinsed off with ddH_2O. Next the membrane was blocked with 3 % bovine serum albumin (BSA) in PBS for 1h in room temperature or overnight in +4°C. Rabbit polyclonal (TA308594, Origene technologies) or mouse monoclonal (H00051050-M02, Abnova Corporation, Taipei city, Taiwan) antibody (0,5 µg/ml dilution with 1 % BSA; 0,1 % Tween-20 and 0,1 % NaN_3 in BPS) against PI15 was then used to bind the protein by rolling in tube for 1h in RT. Membrane was washed by rinsing with PBS and rocking 3 x 5 min in PBS and 0,1 % Tween-20. The membrane was then rolled in secondary antibody, which was either swine-α-rabbit-HRP or rabbit-α-mouse-HRP, (both Dako / Agilent Tehcnologies, Santa Clara, CA USA) (1:5000 dilutions in BPS with 1 % BSA; 0,1 % Tween-20) and washed similarly as before secondary antibody.

Detection was done with Western Blotting Luminol Reagent (Santa Cruz Biotechnology inc., Dallas, TX USA) or SuperSignal™ West Pico Chemiluminescent Substrate (Thermo Fisher Scientific) and x-ray film.

4.10 Statistical testing

All statistical testing was performed with IBM SPSS software version 20. Testing was applied to growth rate and cell viability assays to study statistical differences in these properties between PI15 transfected clones and vector transfected controls.

5. RESULTS

5.1 Subcloning of PI15 ORF

To study the effects of stable *PI15* overexpression to PCa cell lines, a plasmid containing the gene and a mammalian selection marker was needed. *PI15* ORF from the commercial pCMV6-XL4 PI15 vector was successfully subcloned to pcDNA3.1 (+) plasmid into NheI and BamHI restriction sites. The plasmid sequence spanning both restriction sites and the insert region were confirmed by Sanger sequencing.

5.2 Subcloning of luciferase

To investigate with the luciferase reporter assay whether the predicted interaction of miR-32 with the *PI15* 3'UTR has an effect on the protein expression, the luciferase gene *luc2P* was also cloned to the pCMV6-XL4 PI15 plasmid in the place of *PI15* ORF. Two constructs with different lengths of *PI15* 3'UTR region preserved (~1600 bps with HpaI restriction site and ~2150 bps with PstI restriction site in the 3' end of the *PI15* gene) were attempted. The cloning was successful with the 3' primer containing PstI restriction site and this was confirmed by sequencing. Partial sequence (~81 %) of the coding region of luciferase gene was covered by the sequencing. In addition, the sequencing covered most of the 3'UTR of the *PI15* gene from the *luc2P* gene towards the putative miR-32 binding site, but unfortunately not the putative binding site itself.

The *luc2P* gene of the constructed vector was found to contain one non-synonymous mutation: 424A<G or 142K>E. As to our knowledge this mutation is not present in any of the sequences deposited into the NCBI GenBank sequencing database. Because the sequence is critical for the protein structure, and in the case of luciferase reporter assay, a mutation may even compromise the usage of the plasmid, we next analyzed the sequence context of the mutated amino acid to assess its effects on the functionality of the mature protein.

5.3 Effects of the luc2P mutation to the protein conformation

The *luc2P* gene is a codon optimized luciferase developed by Promega corporation (Fitchburg, WI USA) based on the *Photinus pyralis* luciferase. Using the roentgen crystallography based structure of WT *P. pyralis* luciferase (PDB structure: 4g36, Sundlov et al. 2012) the mutated amino acid was loca-

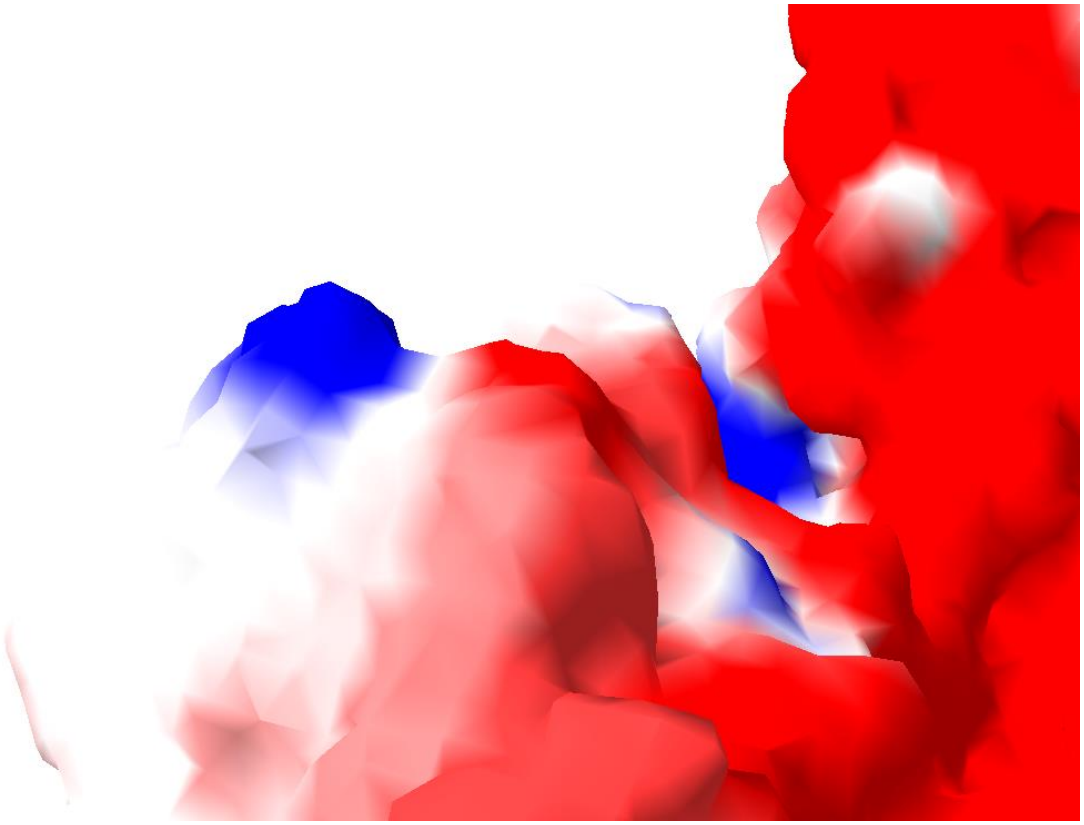

Figure 3. Mutation site (WT) in the luciferase enzyme. The mutation site 142K is the blue (positive charge) bulge in the middle of the picture. The amino acid is fully exposed to the solution in this roentgen crystallographic model. Structure PDB coordinates 4g36 (Sundlov et al. 2012). Image from DeepView - Swiss-PdbViewer v4.1 (Guex et al. 1997).

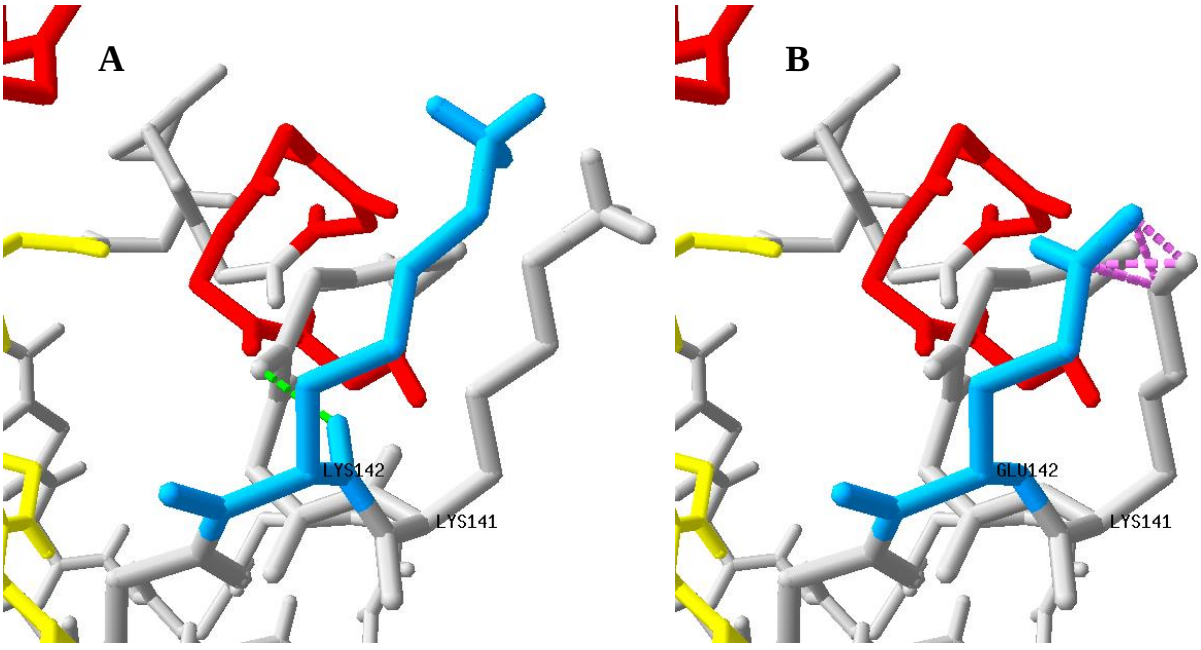

Figure 4. Stick model of the mutation site in the luciferase enzyme. A The WT structure of the luciferase. **B** The same structure with the mutation 142K>E. In the mutant enzyme the Glu 142 might form ionic bond with Lys 141. Structure A PDB coordinates 4g36 (Sundlov et al. 2012), structure B modified from 4g36. Image from DeepView - Swiss-PdbViewer v4.1 (Guex et al. 1997).

ted to the protein surface (Figure 3). It is not part of any secondary structure in this PDB structure and seems to interact only with the preceding amino acid Lys141, which is also positively charged (see Figure 4A). When the lysine 142 is replaced by glutamate (Figure 4B) the two amino acids could form an ion pair, potentially even stabilizing the unstructured region in some conditions.

5.4 PI15 and miR-32 co-transfections

The effect of miR-32 on *PI15* expression was studied by transiently transfecting the original pCMV6-XL4 PI15 plasmid and pre-miR-32 or negative control miR into LNCaP and PC-3 cells and observing the effects on *PI15* mRNA expression. At first, we checked by mono-transfections and qRT-PCR that the plasmid worked (data not shown). The RNA level expression was analyzed with qRT-PCR and the data was analyzed by the comparative C_T method (Livak & Schmittgen 2001, Schmittgen et al. 2008). The co-transfection protocol was also working, as control samples (both WT and empty vector) present no to little *PI15* expression, whereas the samples transfected with the gene containing plasmid have higher levels of expression (See figures 5 and 6A). Transfections were repeated, because some samples had low RNA concentrations after the extraction on the first attempt (see figure 5). However, the SYBR-green qRT-PCR did not provide clear evidence of whether miR-32 controls the expression of *PI15* or not.

To check whether the transfection protocol was working also on behalf of miR-32, we conducted a TaqMan assay to determine miR-32 expression in each sample. Unfortunately, signal was detected only from the LNCaP cells of the second experiment (Figure 6B). However, the success of the transfection was again confirmed with the TaqMan data, as the samples transfected with pre-miR-32 presented much higher expression of miR-32. Although the pattern of the last two samples in Figure 6B looks promising, there is not enough samples to state whether miR-32 regulates the expression of *PI15*.

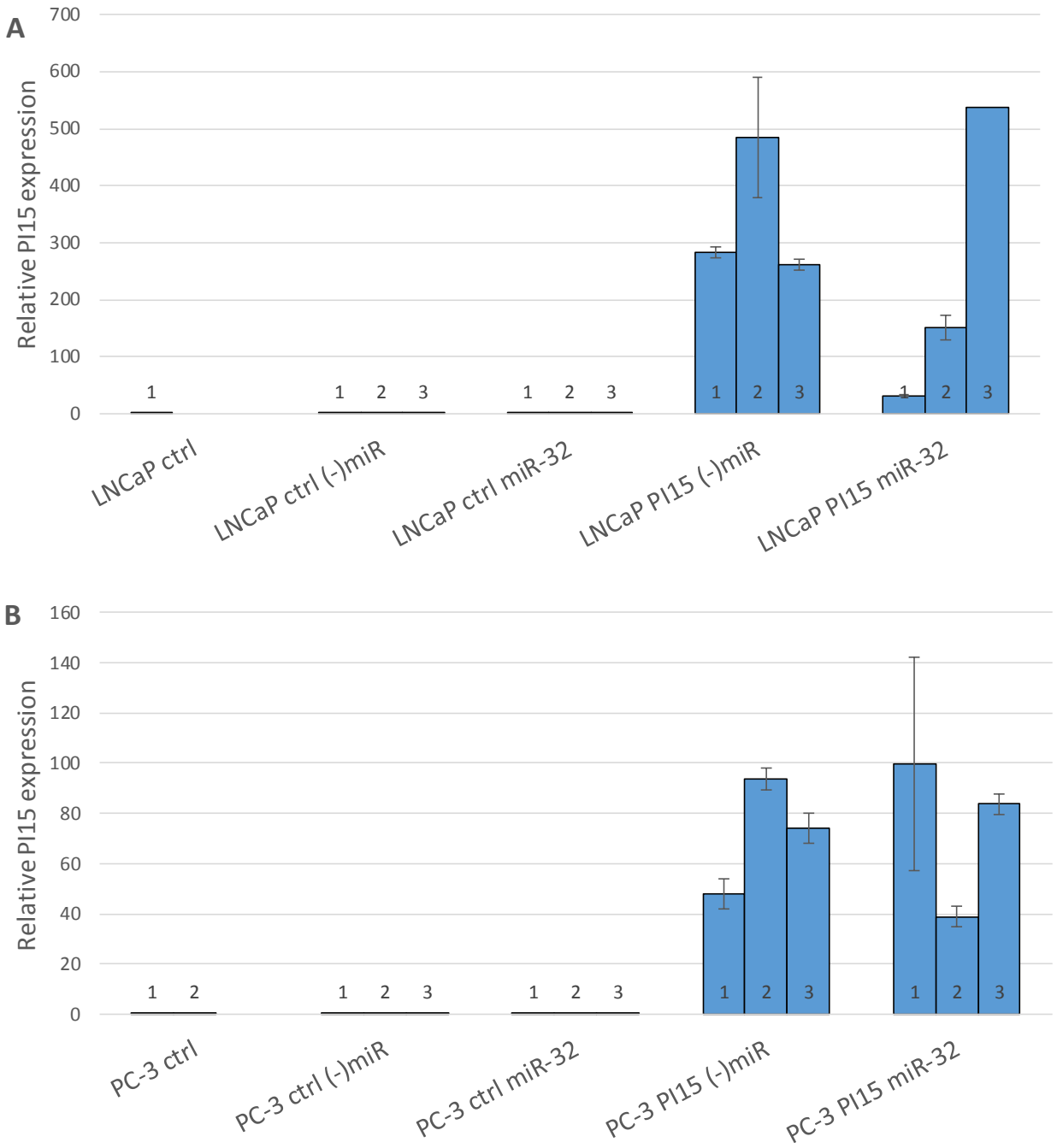

Figure 5. *PI15* expression in the mRNA level in co-transfected LNCaP (A) and PC-3 (B) cells, first experiment. The first samples shown on the left side of the graphs are WT controls. Next are the cells transfected with control vector or *PI15* and negative control miR or pre-miR-32. There were three replicates in each transfection (separate columns denoted with numbers) and each of these had two replicates in qRT-PCR. Relative expressions were calculated with comparative C_T method with *TBP* as an internal control gene. Error bars (± standard deviations, s.ds.) are calculated for qRT-PCR replicates. **A** The missing replicates from transfection (LNCaP ctrl 2 and 3) are due to low concentration of RNA from the extraction. The qRT-PCR replicate of the last sample (LNCaP PI15 miR-32 3) is missing because of a pipetting error. **B** The missing replicate from transfection (PC-3 ctrl 3) is due to low concentration of RNA from the extraction.

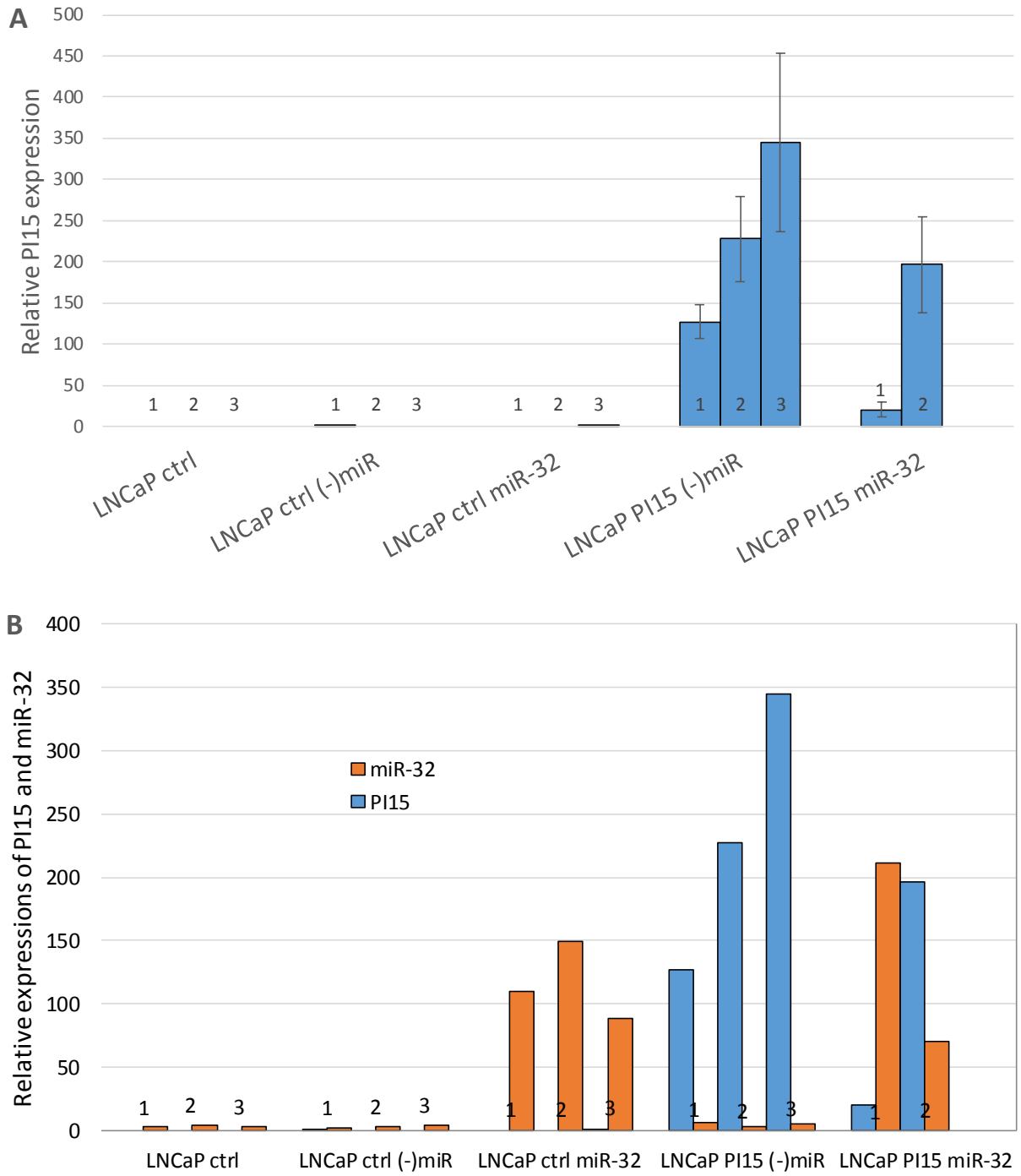

Figure 6. *PI15* expression in the mRNA level in co-transfected LNCaP cells, second experiment. There were three replicates in transfection (denoted with numbers) **A** Error bars ± standard deviation from the qRT-PCR replicates. Sample LNCaP PI15 miR-32 3 was omitted because of pipetting errors in qRT-PCR. **B** Same chart with relative miR-32 expression as determined with TaqMan assay. There were no qRT-PCR replicates for the TaqMan assay so the error bars could not be calculated.

We also tried to detect PI15 expression in the protein level by using both cell lysates and growth medium samples extracted from cells transfected both transiently (with pCMV6-XL4 PI15) and stably (with pcDNA3.1(+) PI15 ORF) and with two different primary antibodies (rabbit-polyclonal

and mouse monoclonal antibody). Unfortunately, PI15 was not detected in any experiment. There were bright bands of proteins both bigger (45 and 75 kDa) and smaller (15 kDa) than PI15. There were also some dim bands near the 25 kDa, which is the size of PI15, but they were consistently as dim or even dimmer in the samples transfected with *PI15* than in the controls. The two antibodies detected different unspecific proteins with the brightest bands above 70 kDa for the monoclonal antibody and below 15 kDa for the polyclonal.

5.5 Morphology, growth rate and cell viability of stable PI15 clones

To study the effects of PI15 overexpression on PCa cell lines, stably *PI15* expressing cell lines were grown by transfecting PC-3 and LNCaP cells with the pcDNA3.1 (+) PI15 ORF vector. Both cell lines were grown under antibiotic (Geneticin) selection. However, only PC-3 cells survived the selection and thus, growth rate and cell viability was measured from that cell line only. Four different clones of PC-3 cells and two control clones transfected with empty vector were used for the assays. The overexpression of the PC-3 clones was assessed at the mRNA level with qRT-PCR (Figure 7). For this qRT-PCR measurement there was no empty vector transfected controls available so WT controls were used as a comparison.

Because there were only two replicates in the qRT-PCR and no error was noticed during the pipetting, some of the samples, namely 6000- and 300-fold PI15 overexpression clones, have very high standard deviations. These clones throughout the results and in the Appendix Figures will therefore be marked with an asterisk (*) after their names. In both cases the erroneous pipetting or other error occurred in the *PI15* qRT-PCR experiment. Although the standard deviation is large, in each case both of the replicates indicated high overexpression. For the 6000-fold overexpression clone the overexpression is 2000–17 000-fold and for the 300-fold overexpression clone 60–1350-fold, if calculated with the raw data of individual replicates of the *PI15* experiment normalized with the averages of the housekeeping genes' data.

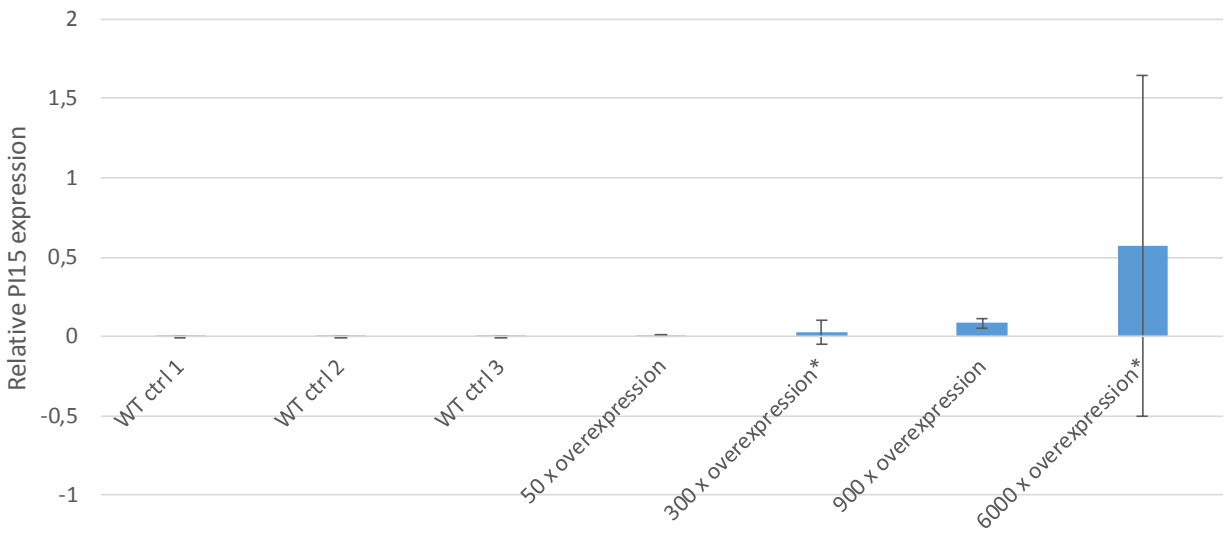

Figure 7. *PI15* expression of the stable PC-3 clones. Error bars ± s.ds. The labels indicate rounded up fold changes compared to the PC-3 wild type controls. *The C_T values of the qRT-PCR replicates of these samples in the PI15 experiment were very far from each other so the s.ds. (error bars) became high. The relative expressions of PI15 were calculated by the comparative C_T method with *TBP* as an internal control gene.

The clones acquired differ in their morphology as assessed by light microscopy (Figure 8). Because the first control clone does not look like common round PC-3 in morphology, but instead adheres tightly onto the surface and there are lots of dead cells in the culture medium, it was excluded from the growth and cell viability analysis. The clone with the highest (6000-fold) overexpression of *PI15* also looks quite different from the other clones in that it too adheres flat on the surface and has filopodia like extensions protruding around the cells. The cells also contain small vesicles that are mostly absent in the other clones.

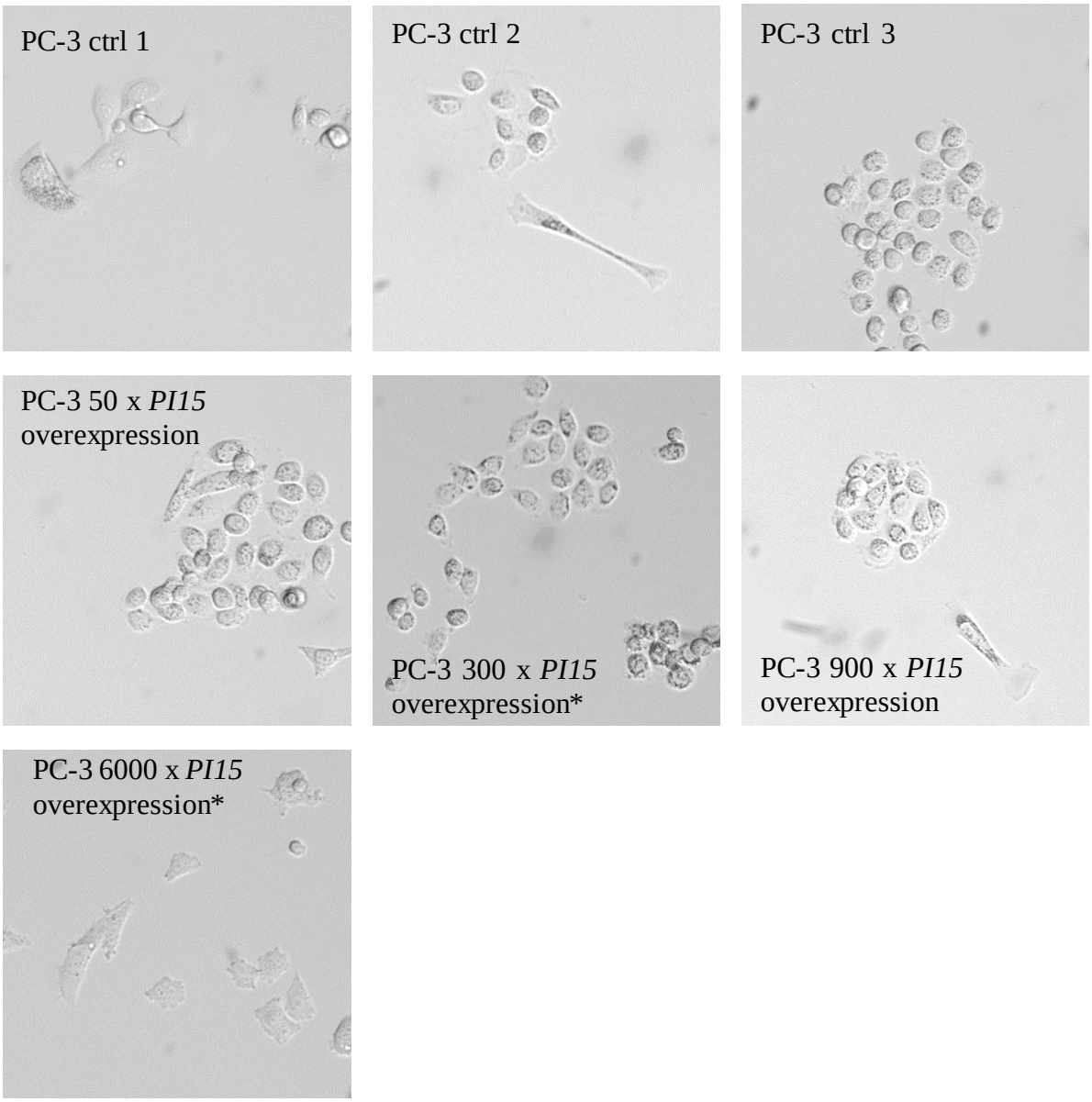

Figure 8. Image panel of the stable PC-3 PI15 clones. Morphology of each stable clone is shown. Controls (top row) are stably transfected with empty vector. Images taken in 10 x magnification with Olympus IX71 microscope.

Growth of PC-3 PI15 clones was next analyzed. Transfected controls were found to grow in four days statistically significantly more slowly than the vector transfected controls (Mann-Whitney U test $p < 0,001$) (Figure 9). If individual *PI15* overexpressing clones are considered, only 900- and 6000-fold overexpression clones grow statistically significantly more slowly than the control clones together (Mann-Whitney U test, $p < 0,01$ and $p < 0,001$).

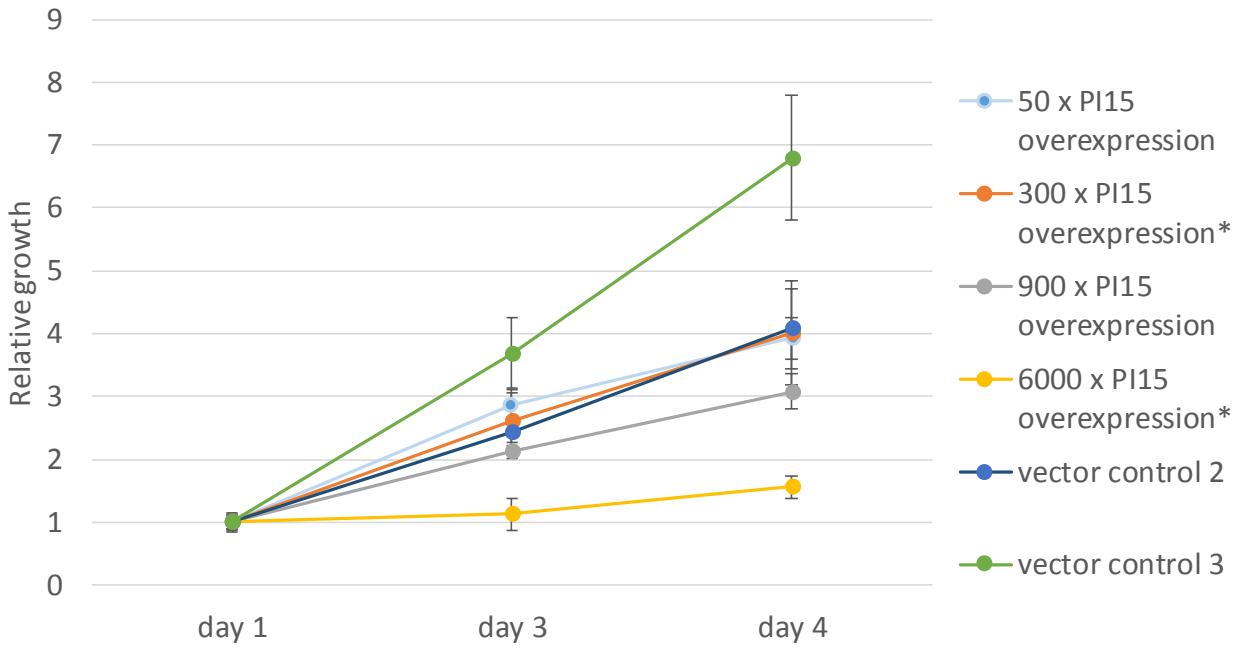

Figure 9. Growth curves of stable PC-3 PI15 clones. Each clone was seeded in six replicate wells and images were taken on days 1, 3 and 4 after seeding. Relative growth is the surface area of day three or day four in comparison to area measured on the day one (baseline). Error bars ± s.ds. Cells transfected with *PI15* grew statistically significantly slower than vector transfected clones as assessed by Mann-Whitney U test for the day 4 data ($p < 0{,}001$). When analyzed separately, 900 and 6000 x PI15 overexpression clones were the only ones to reach the statistical significance with Mann Whitney U test as compared to both vector controls ($p < 0{,}01$ and $p < 0{,}001$) respectively.

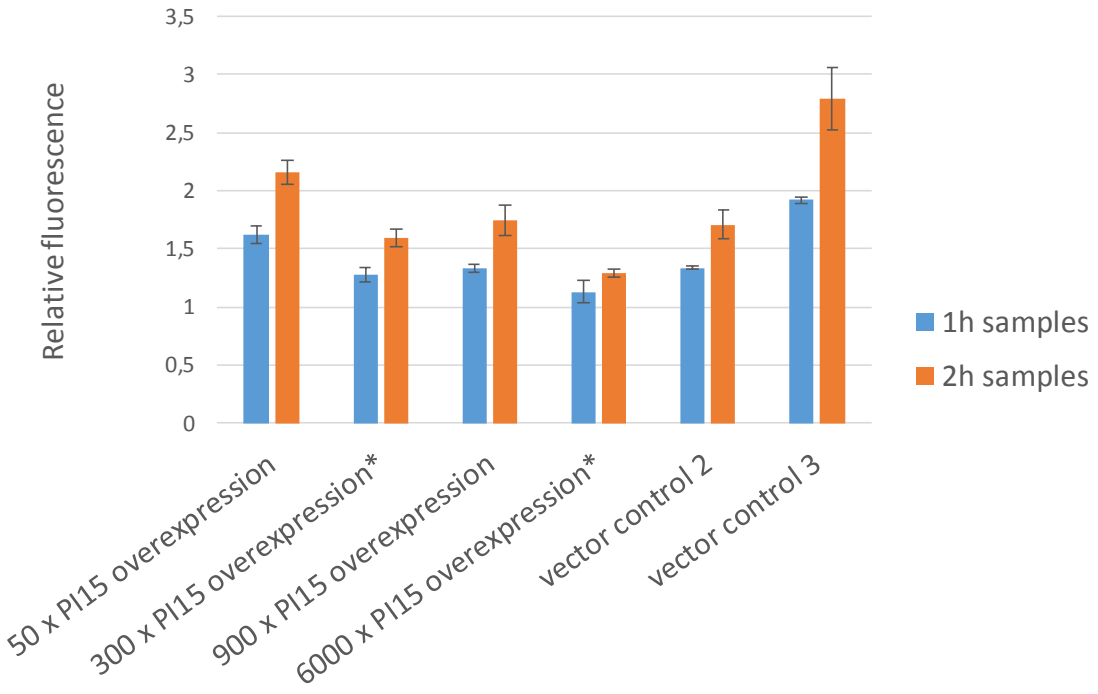

Figure 10. Cell viability of stable PC-3 PI15 clones. Each clone was seeded into six replicate wells in two sets. The samples were collected 1 h and 2 h after addition of the AlamarBlue reagent on days one (set one)

and four (set two) after seeding. The values presented in the graph are the fourth day values normalized against the first day values (baseline). The viability in both measurements was statistically significantly lower in *PI15* transfected cells in comparison to vector transfected cells (Mann-Whitney U test, $p < 0,05$). Error bars ± s.ds.

In the data shown in Figure 9, the clones are approximately in the inverse order in growth rate in comparison to overexpression level. However, in two preliminary experiments done with the PI15 clones (without vector transfected controls), the order is not as consistent (see Appendix Figure 1). The biggest difference in the preliminary experiments (Appendix Figure 1) was that the 50 x PI15 overexpression clone grows much more slowly than in the final experiment (Figure 9). Although not so clearly, the same trend can be seen also in the Figure 9: The 50-fold overexpression clone is growing faster than the 300-fold overexpression clone on day three, but already on day four it starts to fall behind.

Throughout the experiments, the 6000-fold overexpression clone grows the slowest. Moreover, in the data shown in Figure 9, the clone grows statistically significantly more slowly than any of the other PI15 overexpression clone (Mann-Whitney U test, $p < 0,01$). The growth rate of 900-fold PI15 overexpression clone was also statistically significantly slower than that of 300- and 50-fold overexpression clones (Mann-Whitney U test, $p < 0,05$ and $p < 0,01$; respectively). On the other hand, the growth speed of vector control 3 over 2 also reached statistical significance (Mann-Whitney U test, $p < 0,01$).

Finally, the cell number was assessed indirectly with the AlamarBlue cell viability reagent (Thermo Fisher Scientific) (Figure 10). The assay is based on the rate of the metabolism in the sample (cells on the culture vessel), which is directly proportional to the amount of live cells. The cells were seeded in six replicates and the samples were collected on the days one and four after the seeding. The fluorescence that was measured for the day four samples was normalized against the fluorescence of the samples from the day one, so the clones with a high growth rate should appear as highly viable in the assay.

Indeed, like in the growth rate analysis, the 6000-fold PI15 overexpression clone has the lowest viability and the vector control 3 is the most viable. The other clones fall in between these extremes. Measurements with both incubation times agreed that the controls have statistically significantly better cell viability than the PI15 overexpression clones (Mann-Whitney U test, $p < 0,05$ for both

measurements). The 6000-fold overexpression clone was less viable than the control clones for both incubation times (Mann-Whitney U test, $p < 0,001$). Other overexpression clones were statistically significantly less viable only compared to the vector control 3 (Mann-Whitney U test, $p < 0,05$ for 50-fold overexpression clone in 1h measurement and $p < 0,01$ for all other comparisons). Again, also the vector control 3's viability was statistically significantly higher than vector control 2's (Mann-Whitney U test, $p < 0,01$).

As with the growth rate analysis, there are some differences between the results of different cell viability assays as well. In the final experiment, the cell viability of the 50-fold overexpression clone is larger than that of the other *PI15* overexpressing clones, but in the preliminary experiments, the same clone had either same, or lower cell viability than the 300- and 900-fold *PI15* overexpression clones (see Appendix Figure 2).

6. DISCUSSION

6.1 Context of the results

Invasiveness to the surrounding tissue and eventually to the circulation and distant sites is the most lethal characteristic of cancer. Proteolytic cascades in the extracellular space of tumor tissue are key in these processes (Joyce & Pollard 2009, Mason & Joyce 2011). Moreover, the balance between renewal and degradation of extracellular matrix is set by the balance of proteases and their inhibitors (Kessenbrock et al. 2010). For example, activity of MMPs 2, 7 and 9 is increased during PCa progression in SV40 T antigen (a viral oncogene) induced small cell carcinoma of the prostate in mice (Littlepage et al. 2010).

This work was motivated by the notion that miR-32, an androgen regulated miRNA that has been associated with CRPC (Jalava et al. 2012), might regulate expression of *PI15*, which encodes peptidase inhibitor 15. The hypothesis is that if miR-32 downregulates PI15, the activity of proteases increases, leading to enhanced invasion of the tumor cells.

MiR-32 is by far more studied than *PI15* and there are several studies stating its overexpression in PCa (Volinia et al. 2005, Ambs et al. 2008, Waltering et al. 2011, Jalava et al. 2012, Martens-Uzunova et al. 2012, Leite et al. 2013) although there are also studies that have found the opposite (Mahn et al. 2011). This may be because miR-32 has a role especially in advanced PCa (Jalava et al. 2012, Leite et al. 2013) and Mahn et al. used only localized PCas in the part of their analysis which concerned miRNA expression in tissues. Currently known miR-32 target genes in PCa include *BTG2* and *BCL2L11*, (Jalava et al. 2012, Ambs et al. 2008). The functions of these genes are related most closely to apoptotic signaling and no validated target gene of miR-32 is related to the regulation of cellular migration or invasion (http://amigo.geneontology.org/amigo/search/annotation; 22.6.2016, Carbon et al. 2009).

There are few publications about *PI15*. Two studies have shown PI15 expression in brain cancer cells. It is described as a novel protein with weak trypsin inhibiting activity and it is secreted by human glioblastoma cell line T98G (Koshikawa et al. 1996). The study did not state a role for PI15 in the carcinogenesis. In the second study *PI15* mRNA was also found to be expressed in multiple neuroblastoma cell lines (Yamakawa et al. 1998), but again no connection to the carcinogenesis was

studied. It was stated though that PI15 has homology with GLIPR1, glioma pathogenesis-related protein 1 (Yamakawa et al. 1998) that has proapoptotic effects in prostate cancer cells (Ren et al. 2004). In addition, more recent mass spectrometric study found that PI15 is secreted from prostate and detectable from urine after digital rectal examination among other prostatic exosomal secreted proteins (Principe et al. 2013). Authors report over two-fold signal of PI15 in the urine of low grade PCa patients compared to healthy individuals using label free quantification (Principe et al. 2013). All this data nevertheless does not give much idea about the role of PI15 in the context of the vast network of proteases that are expressed from the human genome and in the prostate tissue. Our work with the stably transfected cell lines gives some more information about the physiological effects of this gene to the prostate cells.

6.2 Co-transfections with PI15 and pre-miR-32

We aimed to assess if miR-32 regulates expression of *PI15* post transcriptionally. Due to the low level of expression of the endogenous *PI15* in PCa cell lines (http://www.betastasis.com/prostate_cancer/prensner_et_al_2011/gene_expression_barplot/; Gene: PI15; 21.4.2016; Prensner et al. 2011), this was approached by co-transfecting AR positive LNCaP cells and AR negative PC-3 cells with pre-miR-32 and *PI15* containing plasmid. These cell lines were selected for the study due to their different metastatic backgrounds. PC-3 is originally extracted from bone and LNCaP from a lymph node metastasis. LNCaP cells are androgen sensitive, whereas PC-3 cells are not.

The co-transfection experiments of this work did not produce conclusive results to say if miR-32 affects *PI15* expression or not and the reasons are diverse. First of all, the transient transfection we used to assess these effects led to high variation in the transfection outcome. This can be seen from Figures 5 and 6 as replicates from transfection show high variation in the expression levels of PI15 (Figure 5 and 6A) and miR-32 (Figure 6B). The variation in the expression levels could result from variation in transfection efficiency (Hollon et al. 1989). Gene dosages in some cells may be very high and in others very low. In the optimal situation it would have been useful to assess the transfection efficiency, but it was not possible due to the schedule and lack of suitable marker plasmid. It is also possible that the transfected agents have not been evenly distributed into all cells. In this case the effect of miRNA could be masked by the high *PI15* expression in the cells, which have acquired more plasmid in comparison to miRNA from the transfection.

The problem would have been partially solved also by stable transfection. However, because the plasmid we used did not contain selection marker compatible with mammalian cells, stable transfection would have needed co-transfection with additional plasmid with the selection marker or cloning of the whole *PI15* gene to a different plasmid. The former technique is not optimal as it produces low level of positive clones. We did not try to clone the whole gene to the pcDNA3.1(+) either, because the ORF was better suited for the PI15 overexpression experiments (this will be discussed in more detail below). Moreover, the transient co-transfections of pre-miR and target gene containing vector have successfully provided consistent results at least with luciferase reporter assays in our group (Jalava et al. 2012, Kaukoniemi et al. 2015) and in other groups (Ambs et al. 2008, Noonan et al. 2009, Saini et al. 2011). Also due to the schedule of the project, transient transfection was a more feasible method. Otherwise it might have been reasonable to subclone the whole gene and generate stable cell lines for these experiments as well, although a plasmid with a long gene might be more challenging to get transfected.

There might be biological explanations for the modest success as well. If miR-32 binding to PI15 3'UTR is weak and thus the overall effect on PI15 expression is mild, the change in PI15 expression is hard to detect given the high variation in the expression of PI15 resulting from transfection. Moreover, some miRNAs downregulate expression of their target genes mostly by translational repression, and possibly stabilize the targets (Ambs et al. 2008, Muddasheddy et al. 2011). If miR-32 binds and represses *PI15* without causing degradation of the mRNA, the stable transfection without protein level information would have been insufficient for conclusion.

6.3 qRT-PCR experiments

qRT-PCR experiments of this work demonstrated that the used *PI15* plasmids pCMV6-XL4 PI15 and pcDNA3.1(+) PI15 ORF were working fine as the cDNA prepared from RNA of untransfected control cells or cells transfected with empty plasmid did not present nearly as high signals in the SYBR green based experiments.

Mostly the experiments with qRT-PCR were technically successful and the expression levels could be determined. However, there was whole PC-3 series (from the second co-transfection experiment) of which qRT-PCR results could not be used as all three replicates from transfection (PC-3 PI15 miR-

32) had too high variation between the two qRT-PCR replicates presumably due to inaccurate pipetting or adequate mixing of the samples. In this case the experiment would have benefited from more replicates of each sample. Same was evident with the stable *PI15* overexpressing clones (Figure 7) some of which had lot of variation between the samples. In addition, in those co-transfection experiments that could be analyzed there were single samples from transfection that were excluded from the analysis because of high standard deviation between qRT-PCR-replicates.

In addition, the TaqMan experiment was not fully successful as there was not enough signal from the samples of the first co-transfection experiment. The reason for this could be the overall lower yields of RNA from the first experiment in comparison to the second round. In addition, it would have been preferable to have replicates for this experiment as well. Now there is a chance of unnoticeable pipetting errors in the data despite careful pipetting.

6.4 Western blot

Despite continued efforts, there was no success in the WB experiments. The most probable reason for this is that neither of the antibodies we tried in PI15 detection did not bind the protein properly. PI15 is not much studied, as discussed previously, and thus there are no literature where to look for the best available antibodies for PI15. Both of the antibody suppliers, Origene technologies for the rabbit polyclonal antibody and Abnova Corporation for the mouse monoclonal antibody, have used synthetic peptides with partial sequences of PI15 as immunogens. It may be that the mature PI15 does not have the epitopes in the same conformation as they are in those peptide. In this case it would not be surprising that the protein is not identified correctly.

Other less important reasons for the lack of success in the WB experiments are the possible inaccuracies of protein concentration measurement due to use of too concentrated standards in some instances. This has affected to the amount of protein pipetted into the SDS-PAGE gel. However, it does not explain why the PI15 was not detected at all, whereas there were bands marking other proteins from every experiment. If new experiments with a working antibody were made, new protein concentration measurements should still be made. Additionally, measurements with a house keeping gene as an internal control for quantifying the actual expression levels in each sample could be added. Yet another means to try to improve the experiment would be to concentrate the collected culture medium to have more protein to detect.

We cannot exclude the possibility that despite that the transcription was working (as assessed by qRT-PCR) translation was somehow obstructed. However, the constructs had intact Kozak sequences (Kozak 1987) and both expression vectors are well tested with other genes so there should not be anything wrong with the sequences. Thus, this possibility seems unlikely.

6.5 Stable cell lines

Transient transfection is not optimal way to study the effects of gene of interest. In transient transfection the vectors are unevenly distributed into the cell population and hence the observed effects are not consistent throughout the cell culture. For this reason, stable transfection is used when more consistent results are needed.

The establishment of cell lines with stable *PI15* expression required subcloning of the *PI15* gene to a selection marker containing plasmid. We decided to clone *PI15* ORF because it is shorter sequence and thus, easier to transfect stably and contains no regulatory sequences like miRNA binding sites that might interfere with the desired overexpression. Subcloning of the gene was successful and the right clones were achieved already from the first transformation as confirmed by Sanger sequencing.

Selection of stably transfected cells was a half success. Although the viable PC-3 clones were plentiful, none of the LNCaP clones survived the antibiotic pressure, so those cells would probably have benefited for optimization of the old selection protocol with previously determined antibiotic concentrations. The consequent lack of point of comparison for the PC-3 cells is unfortunate for the results we gained with that cell line.

The results on simple microscopy, growth rate analysis and the cell viability assay give some reason to believe that *PI15* has an effect to the biology of the PC-3 cell line. The 6000-fold *PI15* overexpression clone presented significantly different phenotype from common PC-3, which usually have round shape with some extensions required for migration. The cells (Figure 8, PC-3, 6000 x *PI15* overexpression) were adhering to the surface of the culture vessel and presented sharp edged protrusions as well as small granular structures, possibly resulting from high burden to the exocytose machinery of these cells. The cells also had reduced cell viability (Figure 10) and consistently lower growth rate (Figure 9) in comparison to all other clones.

It needs to be stated though, that to have an effect on biology of the cells the overexpression needs to be quite substantial. This is indicated by two observations. Firstly, the clones with more moderate overexpression of *PI15* have not strictly consistent order in growth rate and cell viability assays as can be seen from the preliminary experiments shown in the Appendix Figures 1 and 2. Secondly, the effect on cell morphology is restricted to the clone with the most prominent *PI15* overexpression.

The fact that the control 2 is in the same level with the 50- and 300-fold overexpression clones in growth rate and in the level with 900-fold overexpression clone in the cell viability also show that the effects can be caused by changes that are not restricted to the PI15 expression. We did not detect PI15 in the protein level, which leaves open the possibility that the effects seen are not related to the PI15. Thus, further work with the protein detection is needed before it can be stated that PI15 protein is causing the observed effects.

6.6 Subcloning of Luciferase

The luciferase reporter assay is the conventional way to study if a miRNA regulates its target by binding to the 3' UTR. Thus, the cloning of the luciferase gene would have been an important part of the project even if the co-transfections would have been a total success. The luciferase proved to be quite a difficult target to enhance by PCR. There were multiple bands close to each other in size in the gel after agarose gel electrophoresis and for this reason the right product was difficult to purify and the yield of the product was low and probably still contained some unspecific product in it. Probably due to these reasons cloning was not successful by the traditional restriction–ligation method. Hence, we used TOPO-TA cloning.

The gene was amplified with Maxima HS Tac polymerase enzyme, but luckily this polymerase proved to be more specific than the originally used HS-Phusion polymerase and there were much less unspecific products in the agarose gel. The reason for this might be a contamination in the Phusion polymerase tube. The reaction of the TOPO-TA based technique is more efficient than T4 DNA ligase because of the differences in the reaction mechanisms of the enzymes. Positive colonies of the luciferase construct with the PstI restriction site were acquired on the first attempt of TOPO-TA cloning. The construct with the HpaI site however was much too short in agarose gel and the lane contained additional ~250 bp product indicating that there may have been some unspecific star

activity during the 1h restriction, although a double digestion protocol suggested by the reagent manufacturer (Thermo Fisher Scientific) was used.

The *Luc2P* gene was still subcloned from the pCR-XL-TOPO vector to the pCMV6-XL4 PI15 vector. This was done by the traditional cloning, but this time the luciferase gene with the required restriction site could be amplified in bacteria and enough specific product was easier to purify from the gel after restriction. This made the yield much better than before and the ligation with the T4 DNA ligase was successful. It is possible that restriction and ligation of the Maxima HS Tac amplified product itself would have provided the same result without the additional TOPO-TA cloning step.

Possibly due to the use of non-proofreading Taq polymerase, a mutation occurred in the during cloning. We cannot exclude the possibility that the mutation was in the original pHIV luciferase vector as it was not sequenced prior use. Regardless of the source, the mutation affects the amino acid sequence of the protein and is not present in the sequences deposited to NCBI GenBank database. The mutation site is in the surface of the protein in PDB structure 4g36 (Sundlov et al. 2012) and the affected lysine 142 residue is next to another lysine 141. Because the change into glutamic acid balances the charge in this part of the protein surface it possibly stabilizes the enzyme. Whether this is the case or not may be clarified in the future experiments with the reporter vector construct.

6.7 Future perspectives

Indeed, to assess the miR-32 *PI15* interaction further, the best options to continue would be to try the reporter assay with a non-regulated control gene (*R. reniformis* lusiferase) as a normalizer. Luciferase assay measures the changes directly in the protein level as luciferase activity and thus fluorescence intensity in the presence of a substrate is directly proportional to the expression of the gene. This would bypass the possible problem of mRNA stabilization upon miRNA binding. In addition, the internal control gene would allow to assess transfection efficacy, which could not be done by the present experiments.

Another strategy to assess the regulatory role of miR-32 would be to simply use some other non-prostate cell line with higher levels of endogenous *PI15* expression. The problem is that the gene is not well characterized so the data especially of the protein level expression is scarce. However, there are some possibilities. For example, melanoma cell line SK-MEL-30 expresses the gene in mRNA

level according to Protein Atlas database (http://www.proteinatlas.org/ENSG00000137558-PI15/cell/SK-MEL-30; 24.5.2016). If downregulation of luciferase activity or PI15 expression is detected with either experiment, the exact binding site should be also identified. This would be done by mutating the putative miR-32 binding site with site-directed mutagenesis, for example by QuickChange, and measuring the luciferase activity or *PI15* expression after new transfection experiment.

With the stable *PI15* overexpressing clones we should also continue by testing their migration and invasion capabilities in wound healing and basement membrane invasion assays respectively to test the latter part of our hypothesis, which states that *PI15* decreases these abilities. Nevertheless, detection of protein PI15 and assessing its overexpression in protein level would be the top priority for the future work concentrating to the biological effects of PI15. Also further characterization of the PI15 would come into question. As PI15 is not alone responsible for any physiological effects that we know of, but instead inhibits function of peptidases, it would be really important to know which these enzymes exactly were and what are their roles in the maintenance of tissue homeostasis.

7. CONCLUSION

In this work, we aimed to assess whether *PI15* is target of miR-32. This was studied by co-transfecting PC-3 and LNCaP prostate cancer cells transiently with these genes and analyzing changes in *PI15* expression by qRT-PCR. Despite the efforts, we did not achieve conclusive results for the matter and further research is needed on the subject.

In addition, luciferase reporter vector containing *PI15* 3' UTR was constructed to further investigate the relationship of *PI15* and miR-32. The cloning proved to be challenging due to which the project time run out before luciferase reporter assays could be conducted. The subcloning of the reporter vector was, however, completed and is available for future studies. The luciferase reporter experiments might be a more robust way to assess effects of miR-32 to PI15 expression. This is because the luciferase reporter assay measures changes directly in the protein level, whereas the data so far was gained on the mRNA level.

We also studied the effects of *PI15* overexpression to the PC-3 cells. For this purpose, *PI15* ORF was successfully subcloned to a vector with a selection marker gene for mammalian cells. With this vector, stable *PI15* expressing clones were selected for growth rate and cell viability analysis. High overexpression of PI15 was found to slow down growth of the PC-3 cell line, and to reduce viability of these cells. Interestingly, the high overexpression also resulted into significant phenotypic changes in these cells: the cells became more adherent and contained small granules probably due to exocytotic processes. In the future, it would be really interesting to see how these changes affect to migration and invasion capabilities of these cells.

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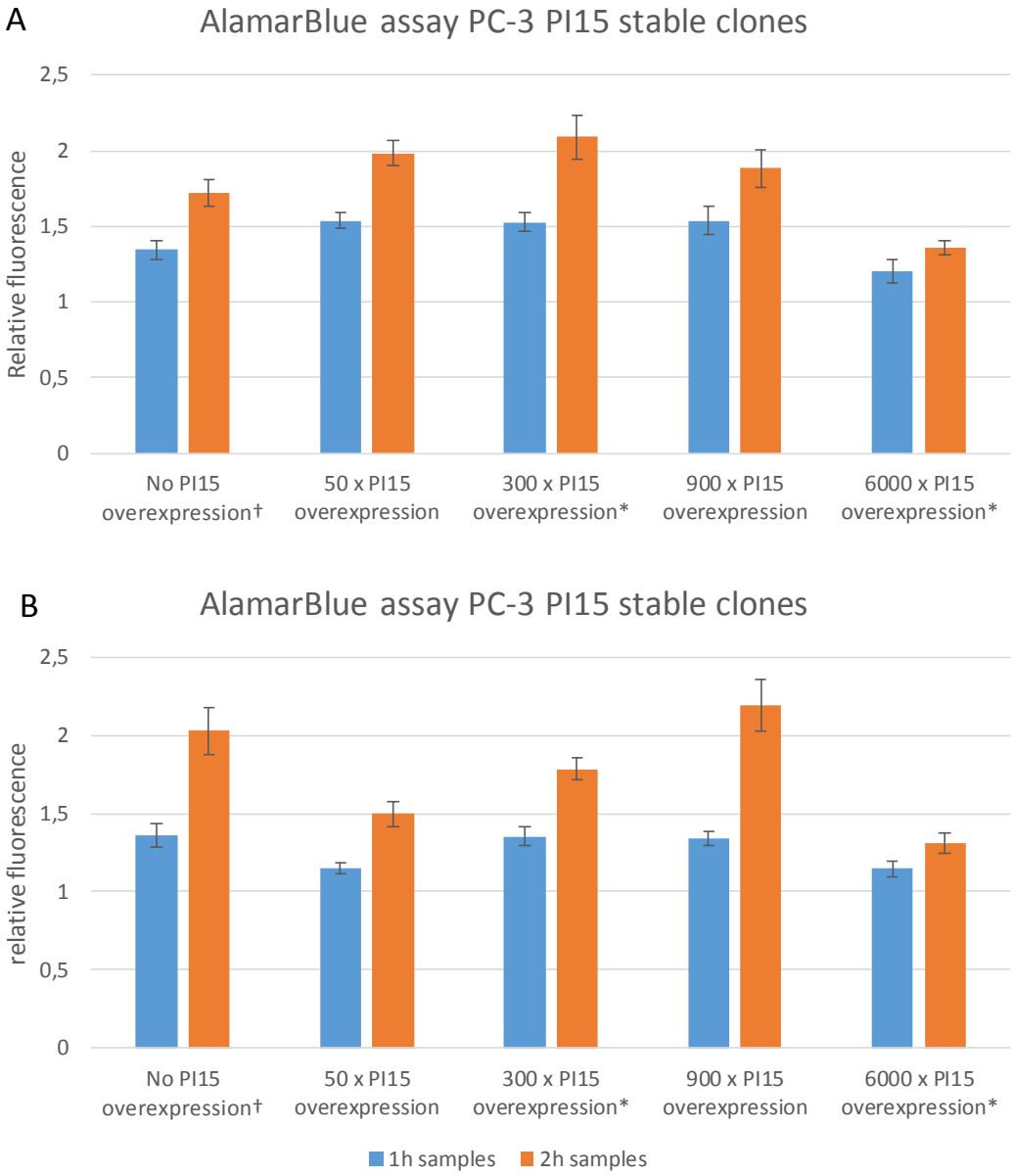
9. APPENDIX

Appendix 1. Growth curve of stable PC-3 PI15 clones. Each clone was seeded in six replicate wells. Relative growth is the surface area of day three or day four in comparison to area measured on the day one (baseline). Error bars ± s.ds. **A** and **B** results are from different experiments. † No PI15 overexpression clone is transfected with *PI15* plasmid but does not overexpress *PI15* as assessed by qRT-PCR.



Appendix 2. Cell viability of stable PC-3 PI15 clones. Each clone was seeded in six replicate wells. The samples were collected on days one and four after seeding. Samples collecctd 1 h after addition of the reagent are shown with blue bars and the ones collected after 2 h are shown in orange. The values presented in the graph are the fourth day values normalized against the first day values (baseline). Error bars ± s.ds. **A** and **B** are results from different experiments. † The first clone is a stable clone transfected with *PI15* plasmid, but does not express *PI15* compared to WT controls as assessed by qRT-PCR.