Prostate cancer stem cell fate: β-catenin and the Wnt pathway

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Abstract

A major problem in prostate cancer is the high relapse rate post therapy. This is believed to be due to the presence of a small number of prostate cancer stem cells within a tumour. Tumour initiation, maintenance and spreading have been attributed to this subpopulation of tumour cells.

Wnt activity and β-catenin signalling have been associated with different types of cancer and microarray data from our laboratory highlighted upregulation of members of the Wnt pathway in primary prostate cancer stem cells. Therefore, the aim of this study was to determine the consequences of β-catenin downregulation on prostate cancer stem cell fate and to identify whether aberrant Wnt signalling and β-catenin levels and location play a role in prostate tumour initiation, growth and dissemination.

Our strategy was to develop and use lentiviral vectors containing short-hairpin RNAs to downregulate β-catenin in prostate cancer cell lines and primary, cultured cells.

This work shows that PC-3 prostate cancer cells, infected with β-catenin-shRNA-lentiviruses (PC-3v), displayed a significant downregulation of β-catenin at the protein level. These cells also exhibited reduced growth *in vitro* as well as a significantly lower invasiveness. *In vivo*, PC-3v cells showed slower tumour onset in immunocompromised mice. To assess Wnt activity in individual cells the localisation of β-catenin in prostate cancer cell lines and cultured primary cells was determined. Cells were stained for active, dephosphorylated β-catenin and various forms of phosphorylated β-catenin using immunofluorescence. Cultured, primary prostate cells were infected with a GFP containing lentivirus to establish an optimal infection protocol for primary cells. Primary cells were also infected with shRNA-containing viruses and changes in cell morphology were observed.

This is the first study to examine the biological consequences of β-catenin downregulation by shRNAs in prostate cancer cell lines and prostate primary cells.

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Author's Declaration

I declare that this thesis represents my own unaided work, except where acknowledged otherwise in the text, and that this work has not been submitted previously in consideration for a degree at this, or any other university.

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Chapter 1

Introduction

1. Introduction

1.1 Prostate anatomy and prostatic disease

1.1.1 Prostate development and morphology

The prostate develops from the urogenital sinus (UGS), which consists of an endodermally derived epithelial layer and a mesodermally derived mesenchymal part. Initially, solid epithelial buds form as outgrowths of the UGS (Thomson and Marker, 2006). The development of the human and rodent prostate is characterised by branching morphogenesis, which is defined by epithelial invasion of the surrounding mesenchyme and by the elongation and branching of the developing ducts to form a complex secretory network.

The prostate is a structurally complex exocrine gland of the male reproductive tract in mammals, which undergoes epithelial and mesenchymal (stromal) differentiation during development (Cunha et al., 2004).

In early embryonic development, all vertebrates undergo an ambisexual period of sex differentiation where the gonads in both male and female organism are morphologically undifferentiated. The male genital tract develops from the Wolfian ducts and the UGS (Cunha et al., 1992).

As early as the 1960s it was found that organogenesis depends on epithelial-mesenchymal interactions and the prostate is no exception from this rule. One of the very early discoveries of prostatic epithelial-mesenchymal interactions was made by Cunha in 1972. He showed that UGS mesenchyme (UGM), seminal vesicle mesenchyme (SVM), UGS epithelium (UGE) and seminal vesicle epithelium (SVE) were not able to develop normally if grown in isolation from each other in the presence of adult physiological levels of androgen. However, when the UGS compartments or the seminal vesicle compartments were co-cultured, normal development of the prostate and the seminal vesicle was observed, respectively (Cunha, 1972b).

Growth and ductal branching are continuous processes, which extend from late foetal life into early adulthood (Cunha et al., 1992, Thomson and Marker, 2006) but development is most pronounced during the first half of gestation (Xue et al., 2001).

Androgenic effects on prostatic development are mediated by the androgen receptor (AR) through mesenchymal-epithelial interactions. It was shown that only urogenital mesenchyme, which expresses androgen receptor (AR), but not skin mesenchyme (no AR) can act as a mediator for the response of UGE to androgens and hence stimulate normal prostate development (Cunha, 1972a).

In rodents, AR signals via an unidentified activating or repressing mesenchymal factor to the epithelium. Although studies showed controversial results, Sonic Hedgehog (Shh) seems to play a significant role in regulating branching morphogenesis, possibly by up-regulating transcription in the epithelium (Donjacour and Cunha, 1993, Notini et al., 2005), e.g. of transcription factor NKX3.1 (Bhatia-Gaur et al., 1999, Freestone et al., 2003, Schneider et al., 2000, Tanaka et al., 2000) and the mesenchymal homeobox genes Hoxa13 and Hoxd13 to enhance prostatic duct formation (Podlasek et al., 1999, Warot et al., 1997). Mice with mutations or knockouts in these homeobox genes exhibit reduced size or missing of parts of the prostate and decreased branching morphogenesis (Podlasek et al., 1997, Podlasek et al., 1999, Warot et al., 1997). Budding and ductal branching are initiated after birth through epithelial–mesenchymal interactions. While Notch signalling can stimulate branching morphogenesis (Shou et al., 2001, Wang et al., 2004) it is inhibited by bone morphogenetic proteins BMP4 and BMP7, which are secreted by the mesenchyme (Grishina et al., 2005, Lamm et al., 2001).

TGFβ has also been shown to inhibit prostatic growth and decrease ductal tip number, leading to changes in branching pattern (Itoh et al., 1998, Tomlinson et al., 2004).

Shh expression is maintained by the interaction of fibroblast growth factors FGF7 and FGF10, which bind to the epithelial FGF receptor 2 (Donjacour et al., 2003, Guo et al., 1996, Huang et al., 2005, Sugimura et al., 1996). In addition, this process is regulated by a negative-feedback loop, as SHH is able to downregulate FGF expression (Wilhelm and Koopman, 2006). Furthermore, expression patterns of the developing prostate have implicated the importance of Activin A and Follistatin during prostate morphogenesis (Cancilla et al., 2001) as well as the polysaccharide component Hyaluronan and its receptor CD44 where it was shown that anti-CD44 antibodies were able to impair prostatic development (Gakunga et al., 1997, Marker et al., 2003). p63 is a key transcription factor which controls the differentiation of epithelial cells in the prostate and subsequently, smooth muscle cells form around the epithelium before lumen formation occurs (Kurita et al., 2004, Signoretti et al., 2000).

In rodents, Keratinocyte Growth Factor has been proposed as a mediator of androgens (Thomson et al., 1997). However, in the prostate, the androgen receptor is neither essential nor sufficient for the regulation of epithelial differentiation. Prior to and during bud formation, AR is initially only detected in the mesenchyme of the urogenital sinus, however it is undetectable in the developing buds (Cunha et al., 2004).
Androgen production, which is crucial to prostate development, initiates during the ambisexual phase and continues until after birth. Then the androgen level falls and starts to increase again during puberty, when new prostate growth is initiated. Growth ceases once adulthood is reached but can be reinitiated in old age resulting in benign prostate hyperplasia (BPH) (Cunha et al., 1992).
Most of our knowledge about prostate development is based on the rodent prostate (Figure 1), which has been extensively studied. However, there are distinct differences between the rodent and human organ. One of the main morphological differences is that, rodent UGS forms 3-4 distinct prostatic lobes, which are absent in humans (Figures 1 and 2).

Epithelial branching morphogenesis in the human prostate occurs within a solid mass of mesenchyme (stroma) and results in a compact, walnut-sized and - shaped organ (Figure 2). Three zones can be identified in the human prostate, the central, transitional and peripheral zone which consist of three distinct sets of branching ducts (Thomson and Marker, 2006).

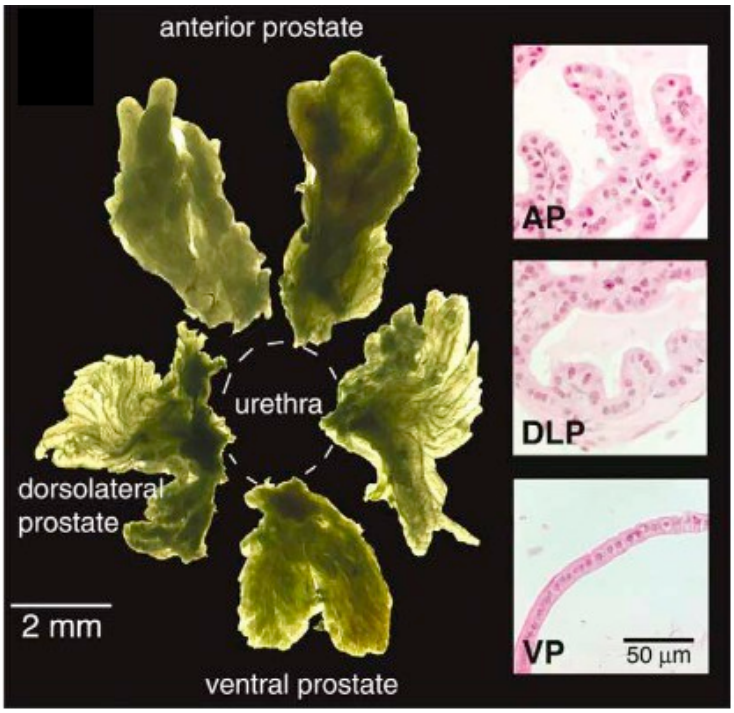


Figure 1: The rodent prostate

Lobes of the adult rodent prostate (left), each with its own distinct shape. Hematoxilin and eosin (H&E) staining (insets, right) show histological appearance of anterior (AP), dorsolateral (DLP) and ventral prostate (VP) (Marker et al., 2003).

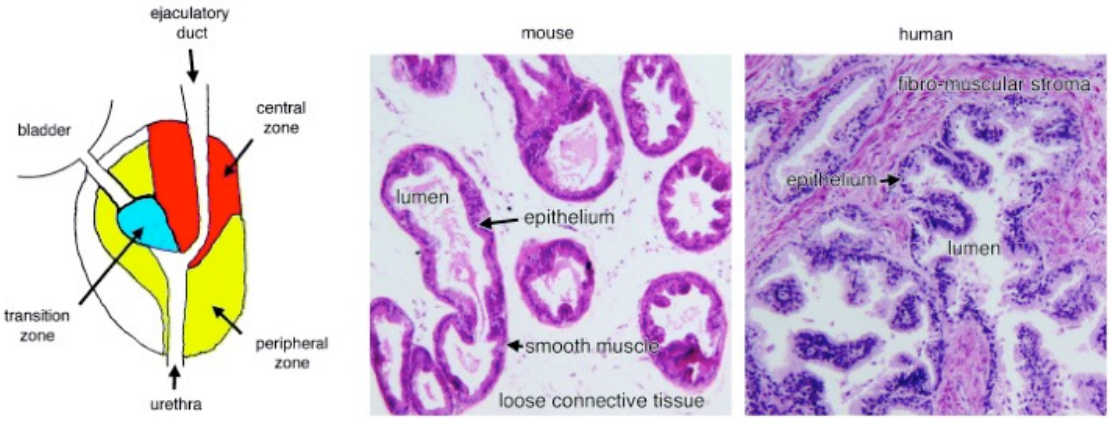


Figure 2: The human prostate

Zonal model of the human prostate and the differences between mouse and human prostate (Thomson and Marker, 2006). H&E staining of the murine prostatic duct shows that the epithelium is very closely linked to a smooth muscle compartment, whereas in the human prostate branched epithelial ducts are present which are surrounded by a solid mass of stroma.

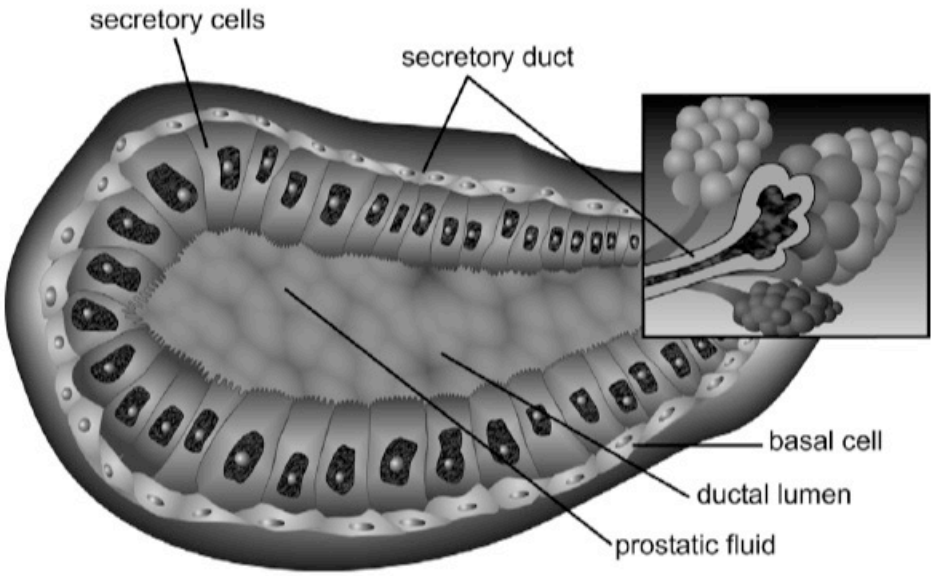


Figure 3: The organisation of the prostate epithelium.

Basal and secretory, luminal cells are illustrated in a cross-section of the prostate gland (Collins and Maitland, 2006).

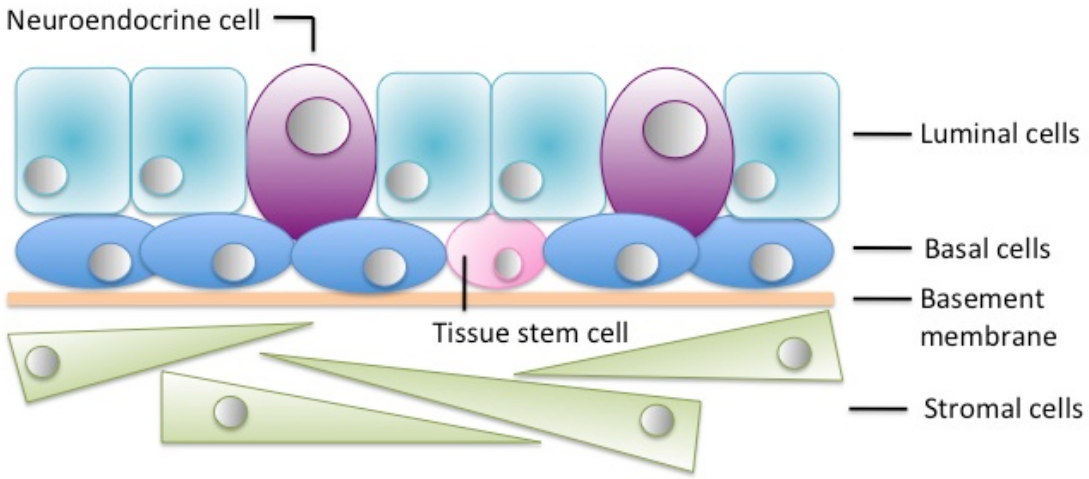


Figure 4: Schematic model of the prostate epithelium

Proposed schematic model of the prostate epithelium containing tissue stem cells among the basal layer of cells.

In the developed prostate three phenotypically distinct epithelial cell types are present: Neuroendocrine, basal and luminal cells (Figures 3 and 4) (Matusik et al., 2008). One hypothesis suggests, that neuroendocrine cells originate from the neural crest and are therefore ectodermal, in contrast to basal and luminal cells which are related by a common endodermal precursor. However, in the other hypothesis, all three cell types share a common precursor and evidence for both hypotheses are still controversial (Aumuller et al., 1999, Marker et al., 2003, Matusik et al., 2008, Xue et al., 1998).

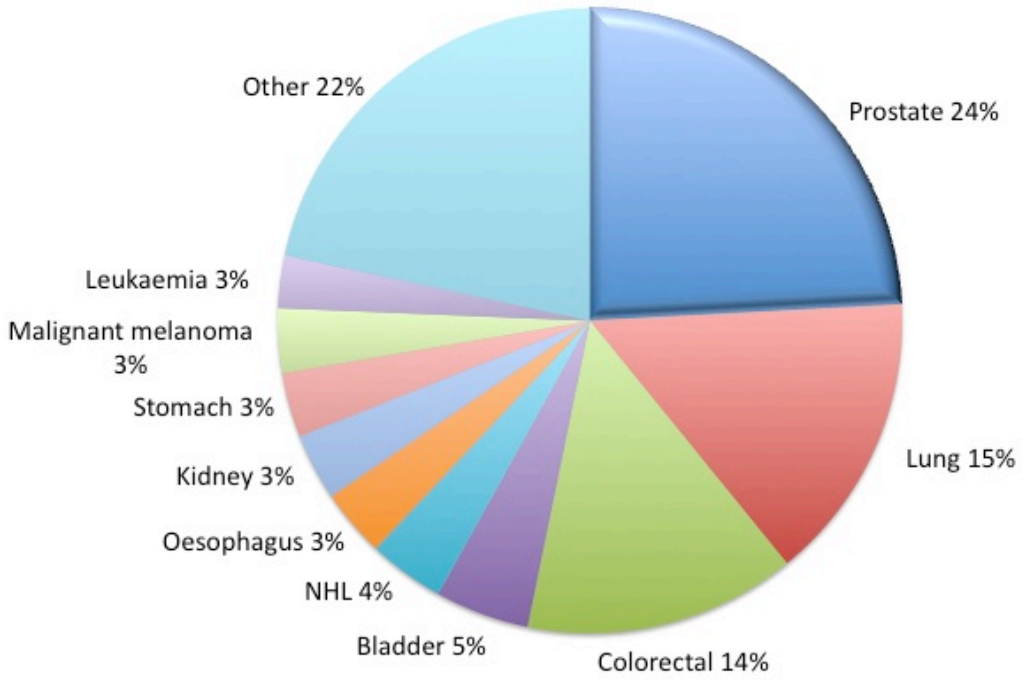
Prostate epithelial cells can be identified by the distinct expression of certain cell surface markers. Basal cells express cytokeratins CK5 and CK14 as well as Cluster Designation molecule CD44 (Islam et al., 2004, Okada et al., 1992, Terpe et al., 1994) and mediate stromal attachment, while luminal cells express CK8 and CK18 together with Prostate specific antigen (PSA) and CD57 (Islam et al., 2004, Okada et al., 1992, Terpe et al., 1994).

Neuroendocrine cells are the least common cell type and secrete Chromogranin A, seratonin and neurophysin (Bonkhoff et al., 1994, Marker et al., 2003). Prostatic epithelium was shown to consist of two functional compartments, the luminal layer with fully differentiated epithelial cells showing a high apoptotic index and the basal layer with high proliferative capacity (De Marzo et al., 2007). This proliferative compartment is androgen-independent while the luminal compartment depends on androgen, in particular dihydrotestosterone (DHT), for survival (Foster et al., 2002, Schalken and van Leenders, 2003).

1.1.2 Epidemiology of prostate cancer

Prostate cancer is the most common cancer in males in Western countries (Figure 5) and incidence rates have continuingly increased over the last years (Jemal et al., 2008, Jemal et al., 2007, Jemal et al., 2004, Weir et al., 2003).
Even though there are serum testing methods for prostate specific antigen (PSA) and improved surgical and other therapies in place, an effective cure still has to be found (Abate-Shen and Shen, 2000). Several risk factors have been associated with prostate cancer (Wigle et al., 2008). Incidence increases rapidly in men over 50 years of age and it peaks between 70 and 74 years (Figure 5). This makes age one of the main risk factors for developing prostate cancer. Prostate cancer cells have been found in most men by age 80 according to post-mortem studies (Sakr et al., 1996).

Due to geographic variation of incidence rates around the world, ethnicity is also suggested to affect the risk of developing prostate cancer. It was found that African Americans have a significantly higher risk than white Americans while Asian males have the overall lowest risk for prostate cancer (Parkin and Muir, 1992, Powell, 2007, Winter et al., 1999). Jones and Wenzel reported that it is 1.7 times more likely for African-American men to develop prostate cancer and 2-3 times more likely that these men die from the disease when compared to their white counterparts. A number of reasons like socioeconomic disparities as well as past experiences with the health-care system and cultural beliefs were suggested (Jones et al., 2005). These findings are backed up by data from UK studies where black Caribbean and black African men have a significantly higher risk of dying of prostate cancer (Jack et al., 2007). Asian men have a generally lower risk of developing the disease, however, migration studies showed that this risk can increase if these men move to or live in Western countries, suggesting a strong link with environmental risk factors (Winter et al., 1999). In a study which looked at the effects of race and ethnicity on PSA screening, it was found that black immigrants from the Caribbean were less likely to have regular, annual PSA screening performed than white Americans even though no difference was detected for initial screening incidence (Gonzalez et al., 2008)



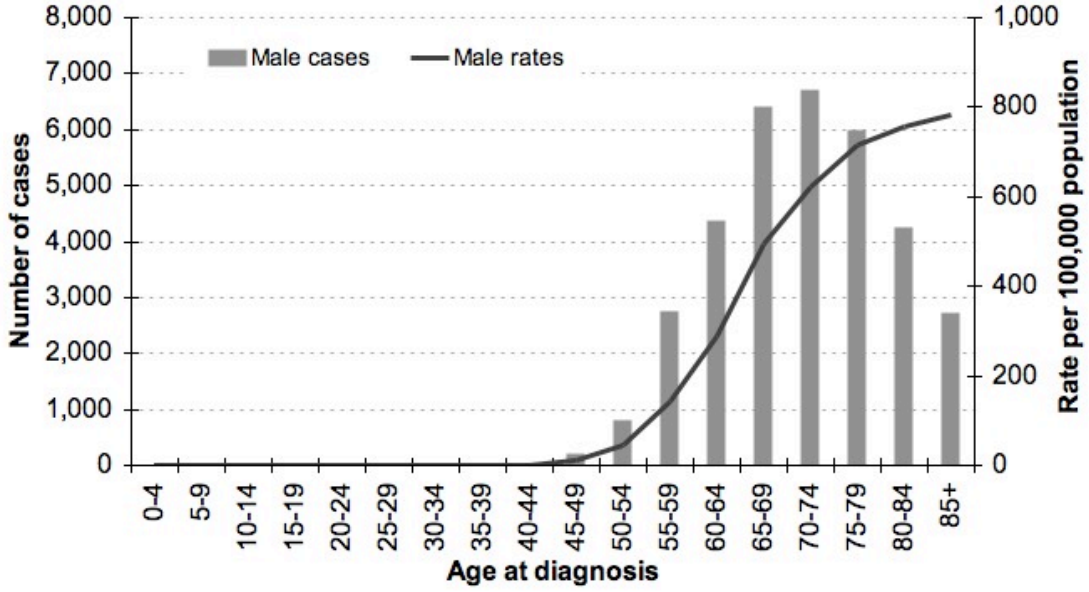


Figure 5: Prostate cancer incidence

The ten most common cancers in males in the UK, 2007 (top). Numbers of cases and age specific incidence rates for prostate cancer, UK, 2006 (bottom). Modified from Cancer Research UK[1].

[1] http://info.cancerresearchuk.org/cancerstats (accessed 09/10/2010)

Within Europe the lowest rates of prostate cancer are found in south and east Europe while Scandinavia and northern European countries show higher rates[2]. Even though socioeconomic reasons are often suggested when looking at ethnicity and cancer-risk, Mouw et al. were not able to show a relation between a risk of developing prostate cancer and lower education levels in men in the USA (Mouw et al., 2008).

In addition, several studies suggest that the diet in Western countries increases the risk of developing prostate cancer due to higher intake in alpha-linoleic acid which is contained in animal fat (Brouwer et al., 2004, Leitzmann et al., 2004). Amin et al. found that fish diets appear to reduce the risk of developing prostate cancer and at the same time, they were able to associate meat diets with a increased risk (Amin et al., 2008). In an analysis of 32 different studies van Platten et al. found that a low fat and/or vegan diet decreased PSA levels, however the usefulness of dietary supplements like lycopene remains controversial (Van Patten et al., 2008). Zlotta also pointed out the limitations when trying to pinpoint dietary factors to prostate cancer risk and evaluating the effectiveness of preventive measures (Zlotta, 2008).

Reports that alcohol can also be considered a risk factor are controversial (Bagnardi et al., 2001). However, in a recently performed metaanalysis, Middleton Fillmore et al. found that the number of drinks per day was positively associated with prostate cancer risk as heavier drinkers were more likely to develop the disease (Middleton Fillmore et al., 2009).

In addition, the effects of smoking on the development of prostate cancer are still debatable. A recent study suggests that there is no association between smoking and advanced prostate cancer (Watters et al., 2009)

In contrast to adult body mass or body growth, markers for delayed androgen action, such as delayed growth during puberty, have been assumed to decrease the risk for prostate cancer development (Giles et al., 2003a). Whitley et al. showed that anthropometric measurements in children were not strongly associated with adult cancer risk (Whitley et al., 2009). Obesity in general was found not to be a significant risk factor, however, when taking other clinical characteristics into account, such as PSA levels, obese men had a 98 % increased risk (Freedland et al., 2008).

Moreover, it was observed that, like in breast cancer, risk can increase for men with a history of prostate cancer among close relatives (Bruner et al., 2003). This is more pronounced in early-onset forms of the disease where patients are under 55 years old

[2]http://info.cancerresearchuk.org/cancerstats/types/prostate/incidence/ (accessed 09/10/2010)

(Bratt, 2002, Bratt et al., 2002, Carter et al., 1992). Therefore it was suggested that men with a family history of prostate cancer might benefit from targeted prostate cancer testing and better risk assessment (McDowell et al., 2009). Zhang et al. also reported findings where a strong family risk was associated with developing other cancers after the treatment of the initial prostate tumour, such as bladder or colorectal cancers (Zhang et al., 2009a). Genome-wide studies in the last years have highlighted genetic variants that cause a slight increase in prostate cancer risk but their contribution to a large proportion of cases is still under discussion (Amundadottir et al., 2006, Eeles et al., 2008, Haiman et al., 2007, Thomas et al., 2008, Zheng et al., 2007).

1.1.3 Prostate cancer development, treatment and therapeutic problems

The majority of prostate tumours are adenocarcinomas and share common features of other epithelial tumours such as breast and colon cancer (Abate-Shen and Shen, 2000). Aberrant cell proliferation and differentiation in the prostate can lead to benign prostate hyperplasia (BPH, Figure 6), which later can result in malignant tumours (Hudson et al., 2001).

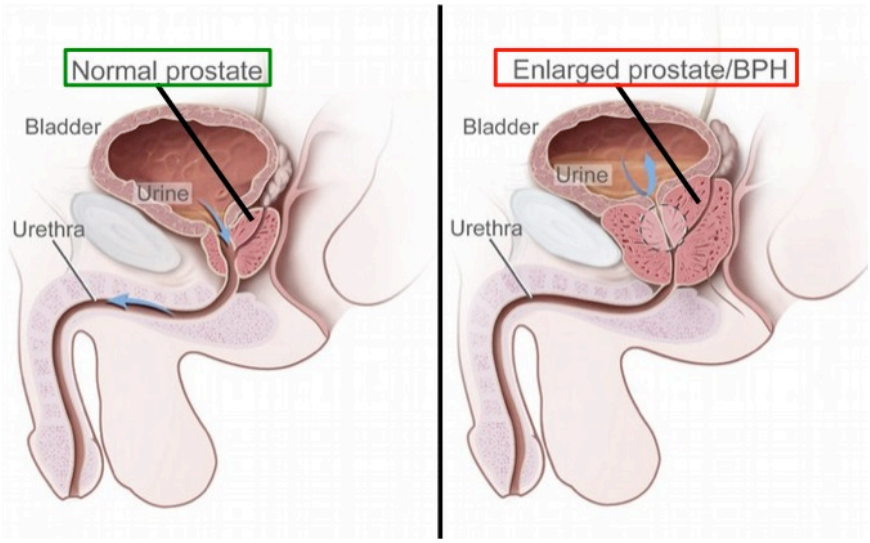


Figure 6: Schematic illustration of BPH[3].

Compared to normal prostate (left), benign (non-cancerous) cell proliferation causes the prostate to enlarge (right) from the middle part outwards. The upper part of the urethra is affected by this cell growth and patients experience reduced urinary flow (blue arrows).

Prostate cancer is graded according to the Gleason score with 1 being normal tissue and 5 the highest malignant grade (Gleason, 1966). A Gleason grade is assigned to each of the two most common areas of cancerous tissue. Both grades are added together to produce a Gleason score, e.g. 3+4, which is also used to define different stages of prostate cancer (Figures 7 and 8).

In order to improve characterisation of low and high grade prostate tumours modifications to the Gleason system have been suggested (Epstein et al., 2006, Helpap and Egevad, 2009).

[3] Modified from National Cancer Institute, http://www.cancer.gov (accessed on 09/10/2010).

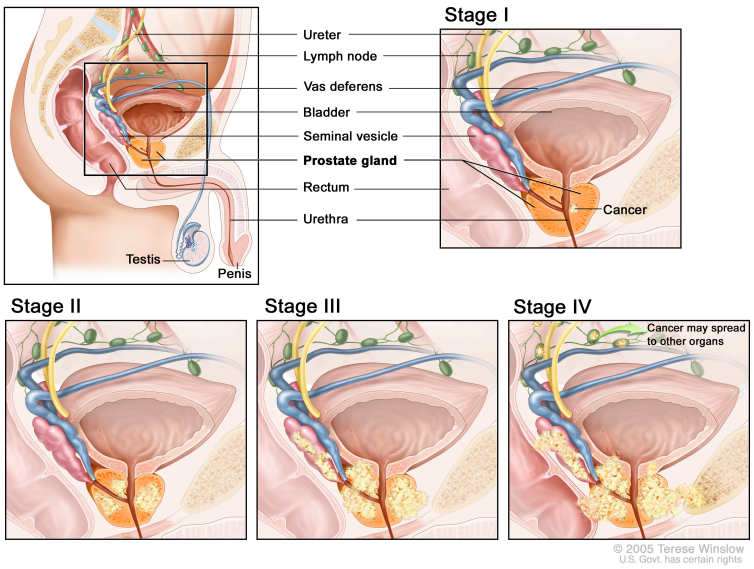


Figure 7: The four stages of prostate cancer (CaP) development[4].

Stage I - Small, localised cancer inside the prostate gland with low Gleason score. Stage II - Cancer still located inside gland but more advanced than in stage I, a hard lump might be felt during rectal examination. The Gleason score can vary from 2-10. Stage III - cancer has broken through the outer layer of the prostate to nearby tissues, e.g. seminal vesicles. Stage IV - Metastases can be found in lymph nodes and/or bladder, rectum, bones, liver, or lungs.

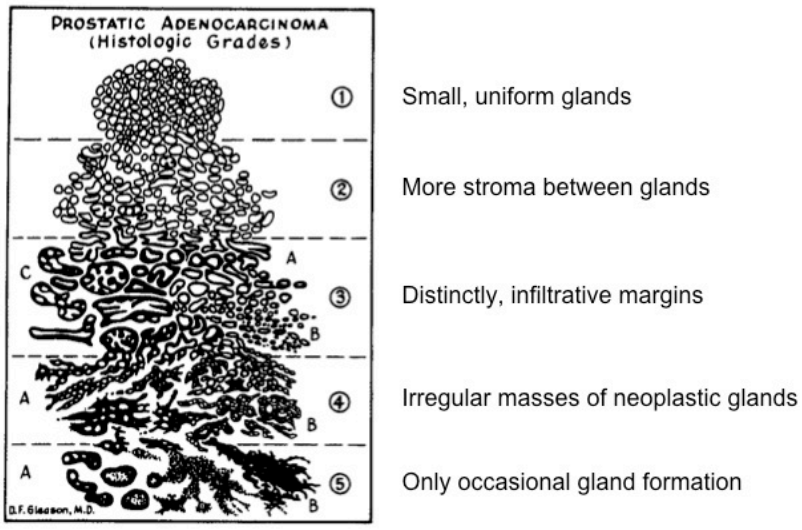


Figure 8: Gleason grading

Gleason grading system for prostate adenocarcinoma as defined by differentiation pattern within a surgical specimen (Gleason, 1966).

[4] http://www.cancerhelp.org.uk/type/prostate-cancer/treatment/the-stages-of-prostate-cancer (accessed on 17/03/2010)

De Marzo et al. suggested that prostate cancer could be caused by a combination of inflammation of the prostate gland and external elements such as dietary factors. They proposed a model where at the first stage towards the development of invasive prostate carcinoma, infiltration of inflammatory cells into the secretory cell layer occurs. This Proliferative Inflammatory Atrophy (PIA) is followed by Prostate Intraepithelial Neoplasia (PIN), which is a precancerous stage characterised by increased proliferation. The basal cell layer is still intact whereas it is lost in the invasive carcinoma stage (Figure 9) (De Marzo et al., 2007).

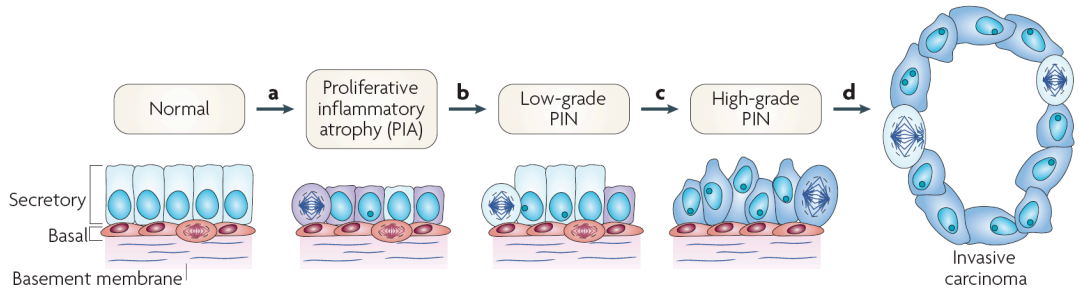


Figure 9: Development and progression of prostate adenocarcinoma

(De Marzo et al., 2007)

A number of inflammatory cytokines have been found in prostatic fluid of radical prostatectomy specimens which may be useful as prognostic or diagnostic markers in the future (Fujita et al., 2008). Furthermore, a number of anti-inflammatory drugs such as aspirin or nonaspirin nonsteroidal anti-inflammatory drugs (NSAIDs) have been suggested as potential chemopreventive treatments (Bardia et al., 2009).

Even though no single tumour suppressor gene, responsible for prostate cancer, has been found yet, a number of possible candidates have been discussed, including p53, RB and PTEN (Abate-Shen and Shen, 2000).

As the growth of prostate tumours is androgen-dependent, the cancer is initially treated by androgen ablation, e.g. by using anti-androgenic drugs or surgical treatment like castration (Culig et al., 2005). Apart from radical prostatectomy[5], external[6] and internal radiotherapy (brachytherapy[7]) can also be used for therapy (MacRae et al., 2006). A number of viral

[5] http://www.cancerresearchuk.org (accessed 09/10/2010)
[6] http://www.cancerresearchuk.org (accessed 09/10/2010)
[7] http://www.cancerresearchuk.org (accessed 09/10/2010)

and cellular gene therapy strategies for the treatment of prostate cancer are currently under discussion (MacRae et al., 2006). However, at a later stage the tumours can become androgen-independent and often metastatic to bone (Thalmann et al., 2000), and therefore other therapeutic methods have to be found (Verhagen et al., 1992).

It was proposed that the main problem with prostate cancer therapy is the lack of targeting the stem cell population that is thought to be tumour initiating and causes tumour growth (Collins et al., 2005, Collins and Maitland, 2006, Maitland et al., 2006). This could be an explanation for the reoccurrence of tumours, which were thought to be successfully treated by radiotherapy at an initial stage. Further supporting this is the fact that most stem cells highly express drug transporters such as the ABC transporter family and can therefore become resistant to chemotherapy very quickly (Dean et al., 2005).

Taking everything into account, there is a large demand for new therapeutic methods to overcome the current lack of therapeutic measures for advanced stages of prostate cancer.

1.2 Stem cells and cancer stem cells

1.2.1 Embryonic stem cells

Embryonic stem cells (ES cells) have been in the focus of extensive research since their isolation from mice in 1981 and humans in 1998 (Evans and Kaufman, 1981, Thomson et al., 1998). ES cells can be isolated from the Inner Cell Mass (ICM) of a blastocyst (Figure 10). In humans, the blastocyst stage is reached at 4 to 5 days post fertilisation. The blastocyst consists of the fluid-filled blastocoel, the trophoblast, a cell layer which surrounds the blastocoel, and the inner cell mass. ES cells are defined as undifferentiated cells which are able to give rise to all embryonic tissue (pluripotency) (Thomson et al., 1998).

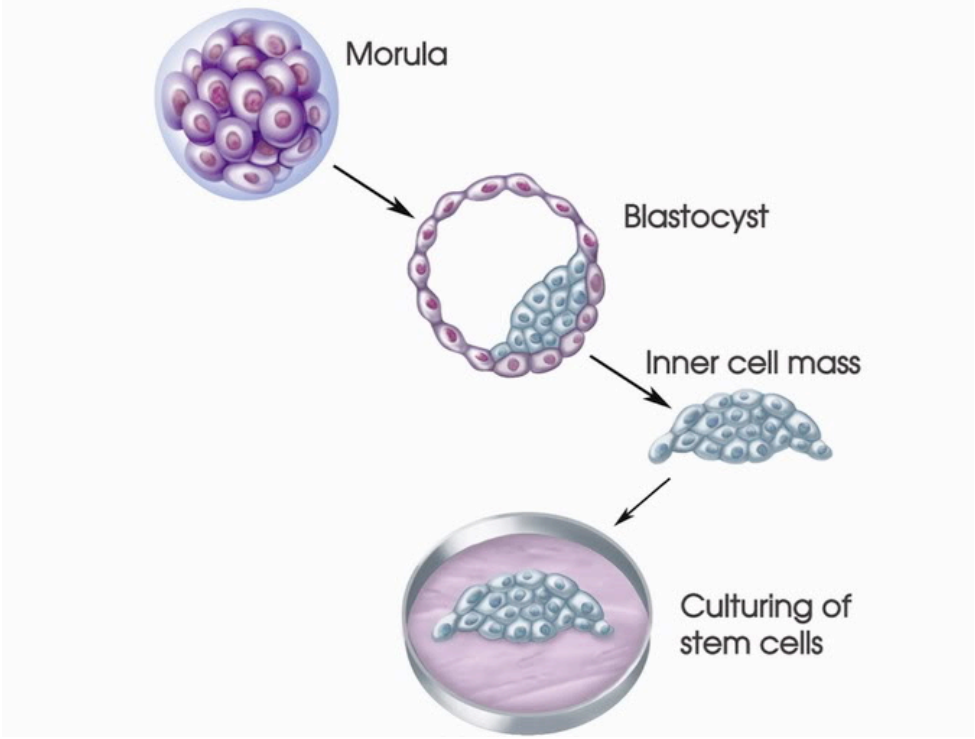


Figure 10: Isolation of ES cells from ICM of the blastocyst[8].

Stem cells, by definition, can reproduce themselves, as well as produce daughter cells which then enter a pathway of differentiation. Once the daughter cells are designated for differentiation, they become progenitor cells (also called precursor or transit amplifying

[8] Modified from http://stemcells.nih.gov (accessed 09/10/2010)

cells), which proliferate before differentiation occurs, thus multiplying the amount of specialised cells (Ying et al., 2003). Stem cells can give rise to cells of all three germ layers (Figure 11).

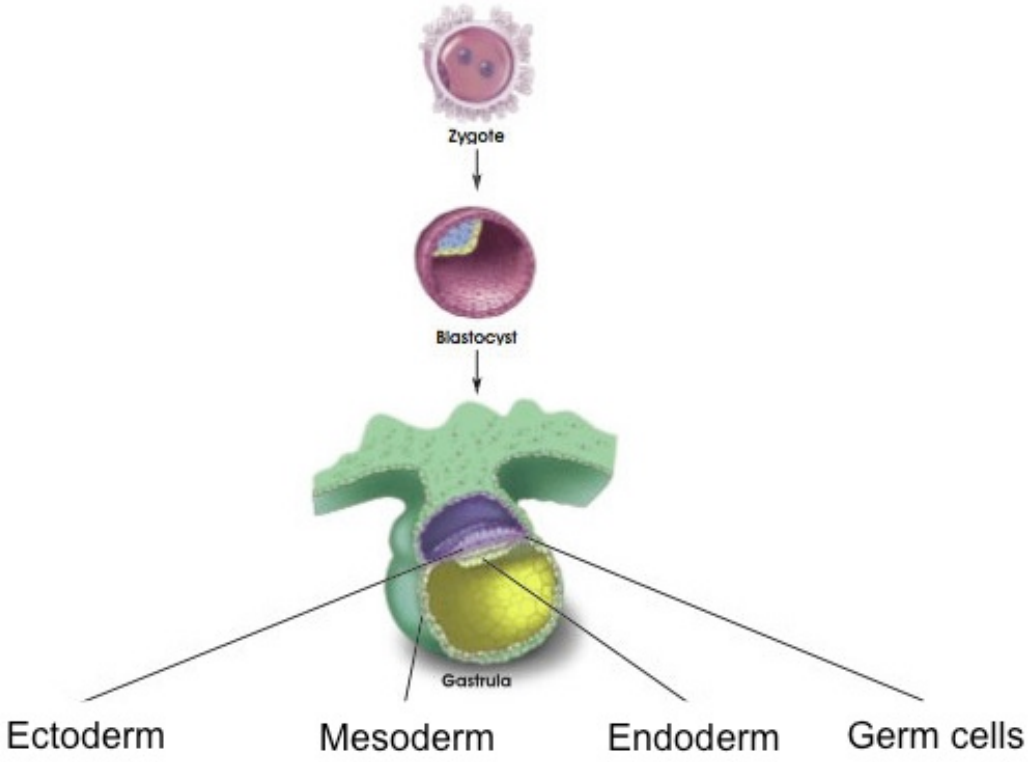


Figure 11: Pluripotency of embryonic stem cells

Pluripotent stem cells from the ICM can give rise to cells of all three germ layers, ectoderm, mesoderm and endoderm.[9]

1.2.2 Adult stem cells

It was originally thought that adult stem cells only occur in tissue with a high turnover rate, however, it has become clear that most tissues contain stem cells. Adult stem cells (tissue specific stem cells), which are derived from ES cells, are multipotent or unipotent cells producing multiple types of specialised cells within their tissue or organ or only one type of specialised cell, respectively. Adult stem cells are thought to have an unlimited capacity for self-renewal and remain in an organism for life. However, they divide slowly

[9] Modified from http://stemcells.nih.gov (accessed on 09/10/2010)

while their progenitor cells produce the high number of differentiated cells needed for tissue growth and maintenance (Raff, 2003).

Hematopoietic stem cells (HSC) were the first adult stem cells to be isolated (Spangrude et al., 1988) and a number of phenotypic markers have been used to isolate HSC from human fetal bone marrow (Baum et al., 1992). Commitment of stem cells to hematopoiesis can already be detected in the yolk sac, shortly after implantation. HSCs can differentiate into B and T lymphocytes as well as granulocytes, erythrocytes and mast cells (Huang and Auerbach, 1993). Isolated HCSs were able to reconstitute all blood cell types in lethally irradiated mice (Spangrude et al., 1988).

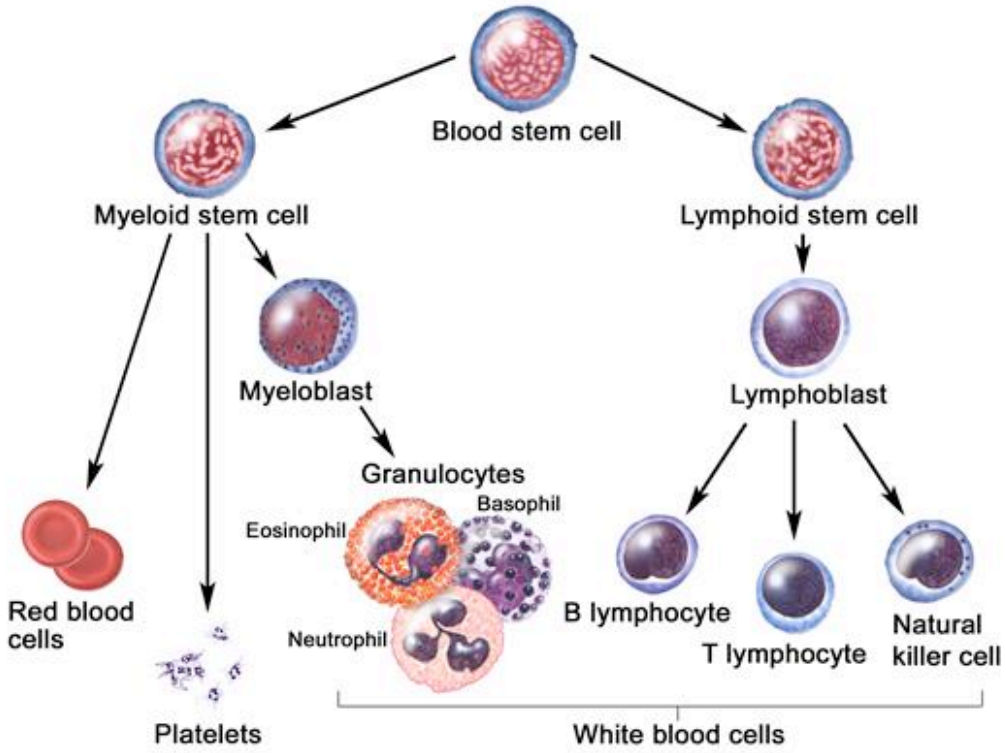


Figure 12: Hematopoietic stem cell differentiation[10].

The development of the hematopoietic model was immensely important for the understanding of mammalian tissue development and maintenance (Raff, 2003).

[10] Modified from http://stemcells.nih.gov (accessed on 09/10/2010)

1.2.3 Isolation of cancer stem cells

In recent years, stem-like cells have been isolated from a variety of cancers, such as acute myeloid leukaemia (AML), brain, breast, lung and prostate cancer (Table 1).
Leukemic stem cells were detected in a population of $CD34^{+}CD38^{-}$ cells and this subpopulation was shown to initiate human AML in NOD-SCID mice (Bonnet and Dick, 1997). Singh et al. isolated $CD133^{+}$ cells from human brain tumours, which display stem cell properties *in vitro*. The group subsequently demonstrated the ability of these cells to initiate tumour growth in a NOD-SCID mouse xenograft assay (Singh et al., 2003, Singh et al., 2004). Al-Haji and co-workers were able to identify tumorigenic cancer cells with $CD44^{+}CD24^{-/low}$ expression from breast cancer patients. It was also shown that as few as 100 cells of this phenotype formed tumours in mice (Al-Hajj et al., 2003). Bronchioaveolar stem cells, which expand *in vitro* and *in vivo* after oncogenic K-ras activation, were also recently identified (Kim et al., 2005a). Human prostate epithelial stem cells were isolated from basal cells by Collins at al. on their increased expression levels of α_2-integrin (Collins et al., 2001). Later CD133 was identified to be a marker for human prostate epithelial stem cells. $\alpha_2\beta_1^{hi}/CD133^{+}$ cells showed high proliferative potential *in vitro* and were also able to form fully differentiated acini of prostate-like structure in athymic nude mice (Richardson et al., 2004).

Breast	(Al-Hajj et al., 2003)
CNS	(Galli et al., 2004, Singh et al., 2003, Singh et al., 2004, Yuan et al., 2004)
Multiple Myeloma	(Matsui et al., 2004)
Melanoma	(Fang et al., 2005)
Prostate	(Collins et al., 2005)
HNSCC	(Prince et al., 2007)
Colon	(Dalerba et al., 2007, O'Brien et al., 2007, Ricci-Vitiani et al., 2007)
Pancreas	(Li et al., 2007)
Lung	(Kim et al., 2005a)
Ovaries	(Zhang et al., 2008a)
Cervix	(Feng et al., 2009)
Bladder	(He et al., 2009)

Table 1: Discovery of tissue and cancer stem cells

1.2.4 Cancer stem cells in prostate cancer – targets for new therapies

The problem of current cancer therapies is that they target the bulk of tumour cells. These cells are rapidly dividing and are therefore susceptible to chemotherapy. However, if the cancer stem cell hypothesis is true, the cancer stem cells will only divide sporadically and thus will not be destroyed by the anti-tumour drugs. This leads to inefficient eradication of the tumour and the possibility that it re-grows and subsequently forms metastasis. In addition, multiple drug resistance has been reported in both stem cells and cancer stem cells (Dean et al., 2005, Donnenberg and Donnenberg, 2005). Targeting of the cancer stem cell population and its subsequent manipulation to decrease or inhibit tumour growth has to be addressed in order to develop more effective drugs.

1.2.5 Signalling pathways in stem cells and cancer

A variety of pathways that have been associated with stem cell self-renewal have previously been identified to be important for oncogenesis. Questions arise whether signalling pathways which normally regulate self-renewal in stem cells can cause tumours when dysregulated, and if stem cells themselves are the targets for cancer-causing mutations as hypothesised in the cancer stem cell hypothesis (Donnenberg and Donnenberg, 2005, Pardal et al., 2003, Reya et al., 2001). There are several reasons why the cancer stem cell hypothesis is a likely model for oncogenesis. Firstly, in a lot of tumours, only a small subset of cells are able to proliferate extensively (Reya et al., 2001). In addition, normal tissues, as well a tumours, are organised as mixed, heterogeneous populations of different cell types which have a large range of differentiation potentials and varying phenotypic appearance. It is possible, that the potential of cancers for mutagenic change is based on abnormal differentiation in stem cells (Reya et al., 2001). Furthermore, stem cells are, by definition, cells that persist in the organism for a long time if not for life. They are therefore more likely to accumulate the mutations, which might give rise to cancer. Secondly, the same signalling pathways are important for self-renewal of stem cells as well as tumourigenesis. Pathways which have been associated with both stem cell self-renewal and tumourigenesis include Oct-4, Hedgehog, Wnt, Notch and Bmi-1 (Reya et al., 2001).

1.2.6 Oct-4, Hedgehog, Notch and Bmi-1 signalling

Expression of the homeobox gene Oct-4 can determine the cell fate in a level-dependent manner. While little expression results in trophectoderm differentiation, intermediate levels maintain stem cells and highest levels lead to differentiation into ectoderm. If Oct-4 expression is increased to 1.5-fold the potential of tumour formation is more than 80% as the amount of malignant cells is increased (Abate-Shen, 2003).

A number of components of the Hedgehog pathway have also been associated with cancer development. Sonic hedgehog (SHH), for example, which is needed for normal cortical development and brain function in humans, induces basal cell carcinoma of the skin if overexpressed. Furthermore, loss of function in the two genes EXT-1 and EXT-2 resulting in decreased signalling in the Hh pathway, has been associated with benign bone tumours which frequently become malignant. Loss of function mutations in PTCH1, which

activates the pathway, result in a higher-than-average rate of a number of tumours, including cerebral medulloblastomas. In mice the occurrence of these tumours is further increased in p53-/- knockouts. Another component of the Hh signalling cascade which can be linked to a variety of cancers, including glioblastoma, endometrial and prostate cancer is SU(FU) (Mullor et al., 2002). During embryogenesis, testosterone-dependent SHH signalling is particularly important for the initiation of prostate development (Podlasek et al., 1999).

Notch signalling controls inhibition of certain differentiation pathways in vertebrates and invertebrates by permitting cells only to enter specific differentiation pathways or to self-renew. If constitutively activated, immortalised cell lines can be developed from hematopoietic cells which can give rise to myeloid or lymphoid cells (Varnum-Finney et al., 2000). Furthermore, Notch signalling is responsible for the maintenance of undifferentiated, proliferative crypt progenitor cells in the intestinal epithelium and has also been associated with the proliferation of adenoma cells. The pharmacological inhibition of the Notch pathway resulted in the conversion of the adenoma cells into differentiated, post-mitotic Goblet cells (van Es and Clevers, 2005).

Another pathway suggesting a connection between stem cells and cancer is Bmi-1. It normally participates in hematopoietic development and is required for the maintenance of adult hematopoietic stem cells, but Bmi-1 can also be linked to acute myeloid leukaemia when dysregulated. Moreover, it was shown to be essential for self-renewal of leukaemia cells (Lessard and Sauvageau, 2003, Marx, 2003, Park et al., 2003).

1.2.7 The Wnt signalling pathways

The Wnt family of signalling molecules have been shown to regulate a number of different processes in animal development and have been demonstrated to control self-renewal in a variety of adult tissues. Wnt was first identified as the drosophila homolog Wingless (Int in mouse) (Nusse et al., 1991).

Since mutations in molecules involved in the Wnt-pathways can cause several hereditary diseases and mutations in adult tissues have been associated with a variety of cancers, Wnt signalling has been the focus of extensive research during the last years (Clevers, 2006, Gordon and Nusse, 2006, Klaus and Birchmeier, 2008, Nusse, 2005). Furthermore, they have been implicated to play an important role in tissue homeostasis in adult organisms (Nusse, 2005).

Wnt signalling molecules have been shown to have a number of different activities and downstream signalling pathways. One of the reasons for that is, that the Wnt family is not defined by functional properties but by amino acid sequence, which shows a characteristic cystein pattern and other conserved residues (Clevers, 2006, Gordon and Nusse, 2006, Klaus and Birchmeier, 2008, Nusse, 2005).

So far, three different pathways, which can be activated through Wnt receptor activation have been studied: The PCP (planar cell polarity) pathway (Figure 13 a), the Wnt/Ca^{2+} pathway (Figure 13 b) and the canonical Wnt/β-catenin pathway (Figure 13 c) (Clevers, 2006, Gordon and Nusse, 2006, Klaus and Birchmeier, 2008, Nusse, 2005).

PCP has been defined as tissue polarization within the epithelium and PCP signalling is involved in vertebrate development, e.g. skin patterning and movement of mesenchymal cells during gastrulation. There are similarities of PCP signalling between vertebrates and Drosophila, where PCP has been extensively studied (Jenny and Mlodzik, 2006).

The current model of Wnt-PCP-signalling, which does not involve β-catenin, uses two interlinking pathways with a presumed third one not involving Wnts as ligands (Figure 13 a). Firstly, Wnt11 can bind to Frizzled-7, which leads to the activation of Dvl. Two domains of Dvl (PDZ and DEP) then mediate convergent extension (CE), a process defined as the regulation of body axis elongation during gastrulation (Keller et al., 1985), through RhoA and Rok. It is presumed that the main outcome of CE are changes in the actin cytoskeleton. Furthermore, an interaction between Dvl and Rac activates Jun N-terminal kinase (JNK), which leads to transcriptional changes of target genes. Secondly, Wnt5 binding to the Ror2 receptor can also more directly activate JNK. One of the target genes of JNK is papc, a protocadherin which is an important mediator of CE (Tada and Kai, 2009) (Figure 13 a).

It has been noted that mutations in components of the canonical Wnt signalling pathway do not disrupt PCP. One of the reasons for this, apart from the lack of β-catenin involvement, might be the use of a different domain of Dvl in PCP signalling compared to canonical signalling (Mlodzik, 2002).

In contrast to canonical Wnt/β-catenin signalling, which depends on the effects of so-called axis inducing Wnts (like Wnt1 and Wnt3A), Wnt/Ca^{2+} signalling involves non axis inducing Wnts, predominantly Wnt5A. Similar to the PCP pathway, β-catenin is not required for Wnt/Ca^{2+} signalling. Instead, binding of Wnt5A to Fz leads to the release of intracellular Calcium through phospholipase C (PLC) and inositol-1,4,5-trisphosphate (IP3). Intracellular increase in Ca^{2+} leads to the activation of two Ca^{2+}-sensitive enzymes,

protein kinase C (PKC) and Ca^{2+}-calmodulin-dependent protein kinase II (CamKII) independently from β-catenin signalling. This results in a cellular response, modifying cell movement and adhesion (Kühl et al., 2000) (Figure 13 b).

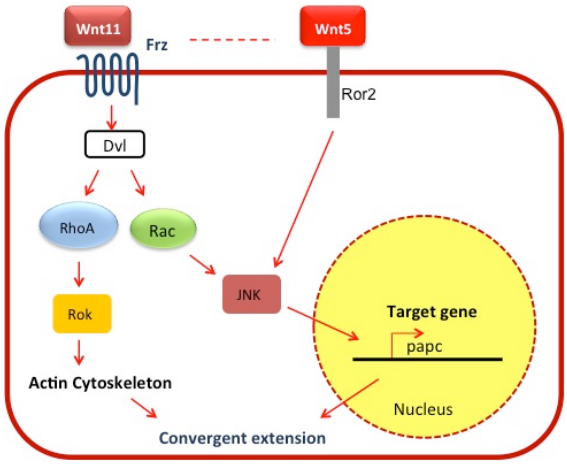
Even though Wnt ligands can play a role in different pathways as illustrated above, research has focused on canonical or β-catenin dependent Wnt signalling because of the important role this pathway plays in the regulation of cell movement, proliferation, embryonic stem cell maintenance and various types of cancers (Takahashi-Yanaga and Kahn, 2010, Takebe and Ivy, 2010). Canonical Wnt signalling is defined by the cytoplasmic accumulation of β-catenin and its activity through subsequent nuclear translocation (Gordon and Nusse, 2006) (Figure 13 c).

Upon activation of the pathway by secreted Wnt molecules, a receptor complex is formed, which consists of Frizzled (Fz) (Yang-Snyder et al., 1996) plus a low density lipoprotein receptor-related protein (LRP). Frizzled receptors belong to a family of seven transmembrane serpentine receptors with an extracellular N-terminal cystein-rich domain (CRD) (Bhanot et al., 1996). A single Wnt can bind multiple Frizzled and vice versa (Clevers, 2006). Both surface expression of Fz and LRP5/6 (Arrow in Drosophila) (Pinson et al., 2000, Tamai et al., 2000) is required to initiate Wnt signaling (Clevers, 2006).

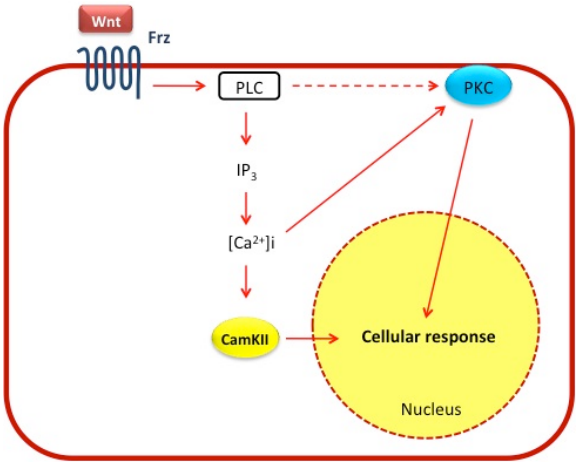
Wnt binding to the receptor complex results in inhibition of the β-catenin destruction complex. This happens when the receptor activation causes the cytoplasmic protein Dishevelled (DVL) to be activated and subsequently to dephosphorylate Axin. This decreases the capacity of Axin to form a complex with Adenomatous polyposis coli (APC) or β-catenin (CTNNB1) (Yardy and Brewster, 2006). Hypo-phosphorylated β-catenin can accumulate in the cytoplasm from where it translocates to the nucleus (Kobayashi et al., 2000). β-catenin then interacts with T-cell specific transcription factor/ Lymphoid enhancer binding factor 1 family members (TCF/LEF) and regulates target gene expression (Gordon and Nusse, 2006, van de Wetering et al., 1996, van de Wetering et al., 1991).

β-catenin therefore exists in three pools within the cell: associated at the membrane with E-cadherin and α-catenin, in the cytoplasm and in the nucleus (Chesire and Isaacs, 2003). The complex composition of the β-catenin destruction complex is not clear yet but some core proteins in the complex have been identified which include glycogen synthase kinase 3 β (GSK3B), adenomatous polyposis coli (APC) and axin (AXIN1) (Fagotto et al., 1999).

a



b



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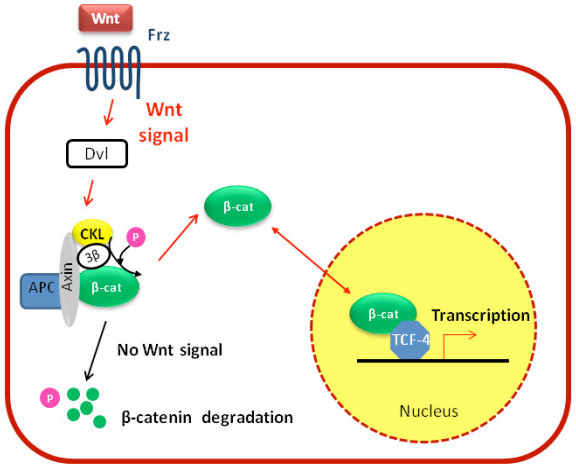


Figure 13: The different Wnt signalling pathways

a. Planar cell polarity b. Wnt/Ca^{2+} signalling c. Canonical Wnt signalling (Modified from Tada and Kai, 2009, Kühl et al., 2000 and Shitashige et al., 2008).

Under unstimulated conditions, the β-catenin destruction complex causes the phosphorylation of β-catenin by GSK3B (Dajani et al., 2003, Fearnhead et al., 2001). This results in ubiquitination of β-catenin and its subsequent degradation by the proteasome (see below for details).

Axin has been identified as a scaffolding protein but there is nothing known about conformational changes when it is bound to its partners in the complex (Kimelman and Xu, 2006).

APC is a very large protein, which is mutated in about 80% of sporadic colon cancers. It contains 3 repeat regions with the characteristic amino acid (aa) sequence serine-alanine-methionine-proline (SAMP), which mediates the interaction of APC with Axin. Furthermore there are 7 20-aa repeats and another 3 15-aa repeats, which are used for binding β-catenin (Clevers, 2006).

GSK3B binds to a central region of Axin where a single Axin helix interacts with a hydrophobic groove in the c-terminus of GSK3B. This binding does not interfere with the active site of GSK3B, which phosphorylates β-catenin (Dajani et al., 2003).

A major mechanism to inhibit GSK3B and therefore prevent β-catenin degradation is the occurrence of conformational changes within Axin (Hedgepeth et al., 1999).

Another member of the β-catenin destruction complex is Casein kinase 1 (CSNK1A1) which phosphorylates the APC 20-aa repeat and which therefore results in affinity changes for β-catenin (Ha et al., 2004, Tickenbrock et al., 2003, Xing et al., 2003).

Even though its role in the destruction complex is unclear, Protein phosphatase 2a (PPP2R4) which consists of 3 subunits, the scaffolding A subunit, a catalytic C subunit and various regulatory B subunits, was shown to bind to Axin (Hsu et al., 1999, Ratcliffe et al., 2000). However, since there is conflicting evidence, PP2A might not be essential for the destruction complex (Clevers, 2006) or even inhibit Axin/APC/GSK3B (Seeling et al., 1999).

β-catenin, the central player in the canonical Wnt pathway, is encoded by the CTNNB1 gene (Trent et al., 1995). It consists of a large central region with 12 3-helix repeats, the Armadillo repeats, and forms a superhelix (Huber et al., 1997) which provides a rigid binding scaffold for molecules such as TCF, E-cadherin, Axin and APC (Graham et al., 2002, Graham et al., 2000, Ha et al., 2004, Huber and Weis, 2001, Xing et al., 2003, Xing et al., 2004).

The N and C terminal regions of β-catenin are small and mostly flexible. When β catenin is phosphorylated on its N terminus by GSK3B, the N terminus is recognised by a

ubiquitin ligase (Kikuchi et al., 2006). The exact recognition sites are amino terminal serines which are phosphorylated (Ser 33 and Ser 37) together with an invariant aspartate (Wu et al., 2003). Another ligase subsequently transfers multiple ubiquitins to Lysine residues Lys 19 and Lys 49. This polyubiqinated β-catenin then gets degraded by the proteasome (Kimelman and Xu, 2006).

1.3 Why study β-catenin and the canonical Wnt pathway in prostate cancer?

The Wnt signalling pathway can cause tumour formation when abnormally activated. Colorectal cancer (CRC) and gastrointestinal tumours have been associated with mutations in the tumour suppressor gene APC which can mimic Wnt stimulation (Giles et al., 2003b). Up to 80% of sporadic CRC and 76% of gastric adenomas can be linked to a mutation of APC which results in loss of the binding domain for β-catenin and therefore accumulation of β-catenin in the nucleus. β-catenin itself acts on transcription factors and activates transcription (van Es and Clevers, 2005). Activation of the Wnt pathway due to β-catenin mutations has also been found in hair matrix cell tumours, hepatocellular carcinoma in Hepatitis C patients, ovarian cancer and to a low but consistent level in prostate cancer. Among childhood cancers, β-catenin mutations were detected in over 92% of hepatoblastomas (Giles et al., 2003b). In addition to that, the inhibition of Wnt using Dickkopf-1, a potent antagonist, resulted in blocking of proliferation and loss of proliferative crypts in adult mice (Kuhnert et al., 2004).

Another reason for studying β-catenin in CaP is that many β-catenin – TCF/LEF target genes have been associated with cancer, e.g. c-Myc (He et al., 1998, Yochum et al., 2010, Yochum et al., 2007) and Cyclin D1 (Tetsu and McCormick, 1999, Yochum et al., 2007). c-Myc has recently been shown to be sufficient, on its own, to induce an embryonic stem cell like programme in normal human keratinocytes, increasing the tumour initiating cells by 150-fold (Wong et al., 2008).

In colon cancer active β-catenin was associated with cancer cells at the invasive front of the tumour, at the site of potential cancer stem cells (Fodde and Brabletz, 2007).

1.3.1 A connection between β-catenin and the androgen receptor

The majority of androgen-independent prostate tumours still express androgen receptor (AR). An aberrantly activated AR pathway, in the absence of normal levels of androgen, is believed to play a role in tumour progression (Zhu et al., 2004).
One of the proteins which has been found to interact with the AR and potentiate its transcriptional activity is β-catenin, one of the major players of canonical Wnt signalling (Chesire and Isaacs, 2002, Heinlein and Chang, 2002, Pawlowski et al., 2002, Truica et al., 2000, Yang et al., 2002).
Even though activating β-catenin mutations and APC mutations are rarely found in clinical prostate tumour samples (Chesire et al., 2000, Voeller et al., 1998, Watanabe et al., 1996), they have been reported in castration resistant CaP and a link between an aberrantly activated canonical Wnt pathway and androgen independence in prostate cancer is believed to occur (Zhu et al., 2004).
High levels of both the AR protein and mRNA have been found to be associated with the transition of hormone sensitive to androgen-independent CaP (Chen et al., 2004, Koivisto et al., 1997, Linja et al., 2001, Schweizer et al., 2008). A recent study showed that while physiological levels of androgen inhibit tumour cell growth, the interaction between AR and Wnt signalling provides a growth advantage for prostate cancer cells at castration levels of androgen (Schweizer et al., 2008).

1.3.2 β-catenin and E-cadherin

Apart from its apparent role in Wnt signalling, β-catenin is also recognised as a key molecule in the E-cadherin adherens junctions and the link between Wnt signalling and adhesion through β-catenin has been investigated for some time (Moon et al., 1997). Under normal physiological condition, E-cadherin mediates cell adhesion of epithelial cells by connecting to the actin cytoskeleton via catenins in the cytoplasm (Giehl and Menke, 2008, Heasley and Petersen, 2004, Jamora and Fuchs, 2002, Morita et al., 1999, Götz, 2008) (Figure 14). Loss of this cell adhesion has been found to lead to tumour progression and formation of metastasis (Beavon, 2000).
In prostate cancer it is thought that differences in E-cadherin expression are important for the development from a non-invasive into an invasive tumour (Isaacs et al., 1994, Isaacs et al., 1995, Verras and Sun, 2006). Loss of E-cadherin or reduced expression has been

associated with more poorly differentiated tumours and therefore later stages of prostate cancer (Bussemakers et al., 1992, Luo et al., 1999, Richmond et al., 1997, Umbas et al., 1992).

The significance of this was exemplified in a study by Ewing and colleagues. By expressing α-catenin in PC-3 prostate cells, which contained a dysfunctional adhesion complex due to loss of α-catenin, they were able to re-establish a normal cell-cell adhesion pathway. This also lead to decreased tumourgenicity when the PC-3 variant was injected into nude mice, therefore establishing that normal functioning of the E-cadherin complex is required to suppress tumour growth (Ewing et al., 1995). It was also previously investigated whether loss of E-cadherin in advanced prostate cancer was resulting in increased β-catenin in the cytoplasm and nucleus (Sasaki et al., 2000).

In our study, we wanted to elucidate the importance of β-catenin in prostate cancer. Taking the relationship between β-catenin and E-cadherin into account our research was based on the following assumptions: Reduced E-cadherin due to the advanced stage of a tumour will lead to less β-catenin being required at cell junctions, which therefore will result in an increase of β-catenin availability in the cytoplasm. This in turn will increase the chances of β-catenin being transferred into the nucleus which mimics increased canonical Wnt signalling. The lentivirally delivered shRNAs against β-catenin would then target this aberrantly active signalling cascade.

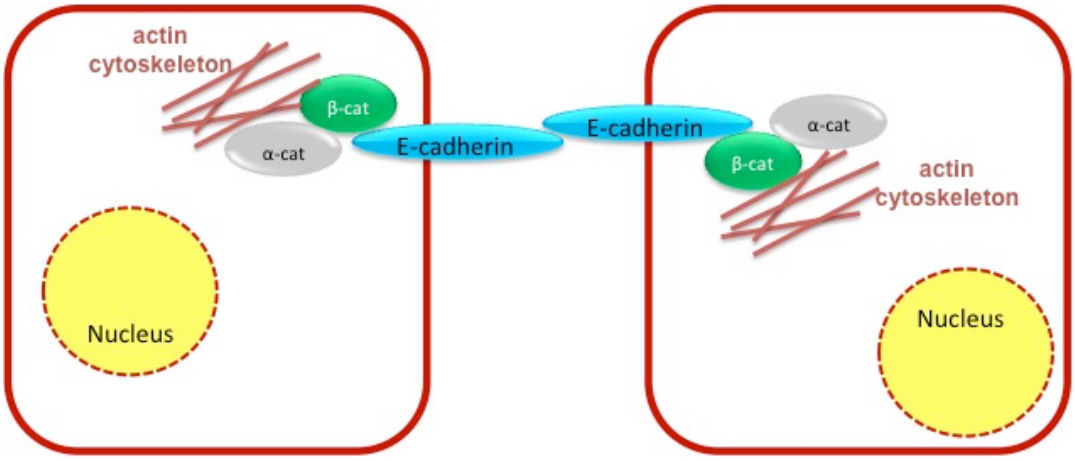


Figure 14: Basic model of an adherens junction

E-cadherin forms a complex with β-catenin and α-catenin to connect to the actin cytoskeleton within the cell (Modified from Giehl and Menke, 2008, Heasley and Petersen, 2004 and Götz, 2008).

1.4 RNA interference

In October 2006 it was announced that the Nobel Prize for Physiology and Medicine would be awarded to Andrew Z. Fire and Craig C. Mello for their discovery of RNA interference. The process of RNA interference (RNAi), also called post-transcriptional gene silencing, was first discovered in *Caenorhabditis elegans* in 1998 (Fire et al., 1998, Montgomery and Fire, 1998, Shi and Mello, 1998, Tabara et al., 1998, Timmons and Fire, 1998). It was shown that the injection of double-stranded RNA (dsRNA) into adult worms resulted in silencing of the target genes and that the silencing was more efficient that the one achieved by either corresponding single-strand. RNAi was also observed in the progeny of the injected adults (Fire et al., 1998). Double-stranded RNA was also shown to induce RNAi in plants (Voinnet et al., 1998, Waterhouse et al., 1998), *Trypanosoma brucei* (Ngô et al., 1998) and *Drosophila spec.* (Kennerdell and Carthew, 1998).

1.4.1 The RNAi mechanism

The process of RNAi is based on the enzymatic activity of the Dicer enzyme. Dicer, a dimeric enzyme, is a member of the RNase III family and contains a helicase domain, which can cleave dsRNA. The enzyme has been shown to be evolutionary conserved in *Caenorhabditis elegans*, *Drosophila melanogaster*, *Arabidopsis thaliana*, *Schizosaccharomyces pombe* and mammals including human (Bernstein et al., 2001). Members of the RNase III family are dsRNA-specific endonucleases, which need specific structurally important components from their targets in order to function efficiently. First of all, the termini of the small interfering RNAs (siRNAs) have to have an intact 5' phosphate group as well as a 3' hydroxyl group. It was shown that, if the phosphate is missing, the endonuclease activity of the enzyme will be inefficient. Additionally, it is important for Dicer, that there are two single-stranded nucleotides on either 3' end as blunt-ended siRNAs are also less efficient (Boutla et al., 2001, Caplen et al., 2000, Elbashir et al., 2001a, Elbashir et al., 2001b, Hutvagner and Zamore, 2002, Nykanen et al., 2001, Parrish and Fire, 2001).

If dsRNA enters the cell or is introduced into or produced within the cell, Dicer targets it and cleaves it into siRNAs of around 21bp, which are still double stranded. The siRNAs may differ in length according to the species used in experiments, as there may be structural differences in the RNase III domains of the Dicer homologues. This process is

ATP-dependent and therefore makes the Dicer enzyme unique among other RNase III family members, which do not need ATP. The dependence on ATP is supposed to be due to an ATP-dependent helicase domain or a RNA translocase at the amino terminus (Bernstein et al., 2001, Hutvagner and Zamore, 2002, Ketting et al., 2001, Nykanen et al., 2001, Zamore et al., 2000).

The siRNAs are then targeted by the RNA induced silencing complex (RISC). RISC, a ~ 360kDa multi-component protein/RNA complex, which is assembled using ATP, unwinds the siRNAs in an ATP-dependent manner and keeps the strand whose 5' end is less tightly attached while releasing the other strand. The bound, active guide strand is then used to target the corresponding mRNA. As soon as the complementary mRNA is bound RISC cleaves it without requiring ATP and the enzyme complex can be recycled (Dykxhoorn and Lieberman, 2006, Nykanen et al., 2001). In Drosophila cells, the protein component of the RISC complex seems to be a member of the Argonaute family, which has been shown to be essential in gene silencing in *Arabidopsis*, *Neurospora* and *C. elegans* (Hammond et al., 2001).

According to the processes described above, there is a 4 step model for the RNAi pathway (Hannon, 2002, Hutvagner and Zamore, 2002) (Figure 15).

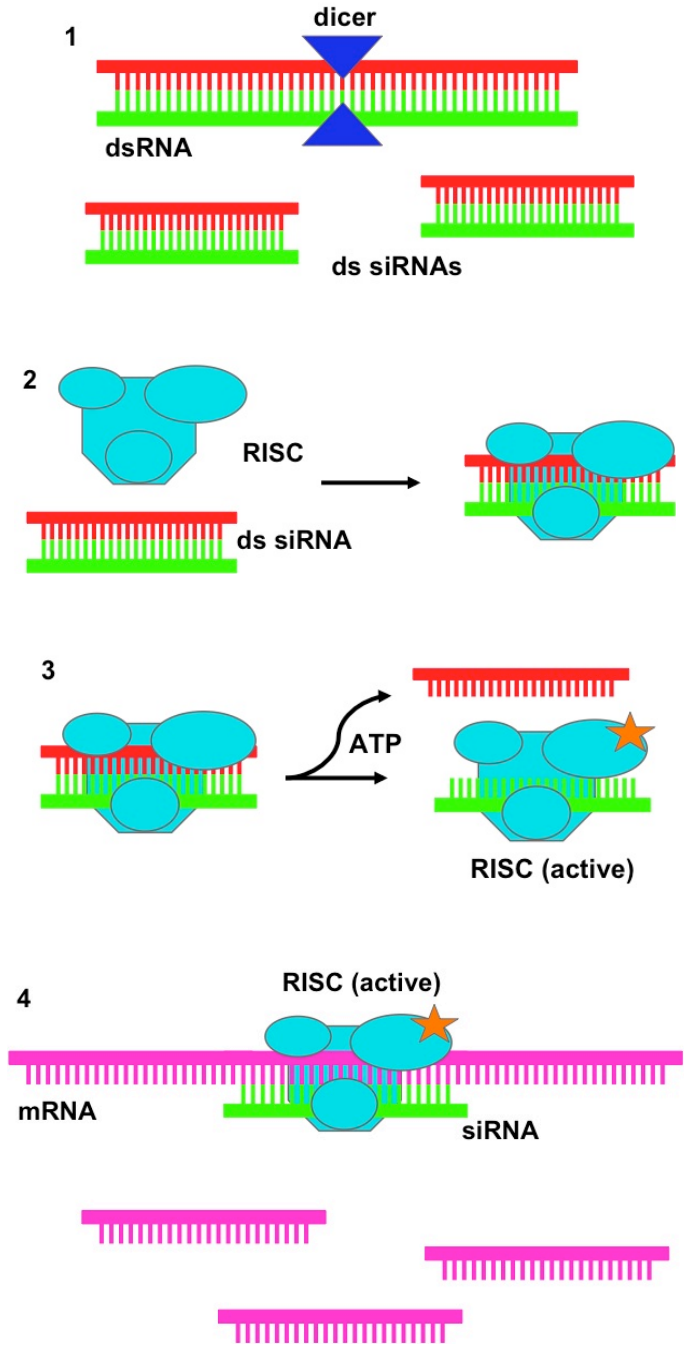


Figure 15: Four step model of RNA interference

(1) The initiation step is the cleavage of dsRNAs (green/red) into 21-25bp double stranded siRNAs by the dicer enzyme (dark blue).

(2) The next step is the incorporation of siRNAs into the inactive RISC (light blue).

(3) The third step uses ATP to unwind the RNA and activate RISC (star).

(4) Finally, in the fourth step, the target mRNA (pink) is cleaved ATP-independently.

1.4.2 RNAi in the eukaryotic cell

Even though RNAi was first thought to be a regulatory mechanism targeting mRNA in the cytoplasm, it is now clear that it also involves silencing at the genome level in the nucleus of a variety of species (Matzke et al., 2001). The effects include RNA-directed DNA methylation, DNA elimination and histone methylation. The main natural targets of RNAi are transposons and similar repeat regions, as they represent potential invasive sequences (Matzke and Birchler, 2005).

Targeting and destruction of viral dsRNA, which is a common product during virus replication, might be one of the main biological roles of the RNAi pathways. However, viruses themselves have developed a number of suppressor molecules against RNAi to guard themselves from the host cell (Voinnet, 2005).

1.4.3 Manipulating cells by RNAi

Various groups have shown that synthetic siRNAs can be recognised and taken up into the cellular RNAi pathway *in vitro* and mediate RNAi not only in Drosophila cells but also in a variety of other species including human (Elbashir et al., 2001a, Elbashir et al., 2001c, Fire et al., 1998, Hammond et al., 2000, Nykanen et al., 2001). Elbashir et al. demonstrated that synthetic 21- and 22bp dsRNAs with 3' overhangs mediate sequence-specific mRNA degradation in a Drosophila *in vitro* model (Elbashir et al., 2001c).

It has also been shown, that short hairpin RNAs (shRNAs) with a stem of 25 to 30 nucleotides, which are also recognised by the Dicer enzyme, silence target mRNA more efficiently that 21mer siRNAs (Kim et al., 2005b, Siolas et al., 2005). There are eight characteristics, which have been identified with siRNA functionality. These are mostly empirical guidelines, e.g. it was suggested that two 21 nucleotide sense and antisense oligonucleotides, which both harbour a 2-nt overhang on the 3' terminus, should be designed to target a gene of interest. In order to save the siRNA from degradation through endonucleases the overhanging bp should be dT (deoxythymidines).

Furthermore, the siRNA sequence should not target any other mRNA sequence in the cells of interest (perform BLAST search on EST libraries) and should not be directed at introns, untranslated regions (UTRs), sequences less than 75bp away from the start codon and regions with a high G+C content (over 50%). Low internal stability at the 3' end of the sense strand as well as a lack of inverted repeats are also important. In addition, sense

strand preferences at positions 3, 10, 13 and 19 should be observed, e.g. A at position 19 (Mittal, 2004, Reynolds et al., 2004). There are several online tools available for the selection of siRNA sequences (Pei and Tuschl, 2006).

RNAi is not only discussed as a powerful tool for functional genomics but also as a possibility for future therapeutic gene-silencing drugs (Shuey et al., 2002). One problem for the use of RNAi as a therapeutic method might be the activation of dsRNA-dependent protein kinase (PKR) by the introduction of short double-stranded RNA as this activation will lead to apoptosis in the target cells.

Dimerisation of PKR due to dsRNA binding leads to autophosphorylation and activation of PKR. The activated PKR can then phosphorylate substrates such as the eukaryotic translation initiation factor eIF2α. Once the small subunit of eIF2α is phosphorylated, a signalling cascade is altered which leads to apoptosis (Gil and Esteban, 2000).

For *in vivo* therapy, safe delivery of siRNAs or shRNAs is a challenging obstacle. siRNAs could be transported into cells using cholesterol-conjugation, antibody-fusion, liposomes or viral expression vectors while microinjection is only relevant for *in vitro* assays (Dykxhoorn and Lieberman, 2006). The delivery of shRNA-expressing plasmids, targeting the liver, was successfully shown in mice by hydrodynamic tail-vein injection. If transient expression in non-dividing cells is favourable, nonintegrating vectors, like adenoviruses or herpesviruses, should be chosen. However, if dividing cells are targeted and stable, long-term expression is desired, retroviruses are the appropriate choice (Snove and Rossi, 2006). Paul et al. showed that RNAi can be induced in HeLa cells by introduction of synthetic duplex RNAs of around 20bp using liposome transfection. The RNAs were expressed using a human U6 small nuclear RNA promoter (Paul et al., 2002).

Methods for promoter-based expression have already been successfully used for stable gene silencing *in vitro*. However *in vivo* application is more challenging as the silencing of specific genes might also induce unwanted side effects. Toxicity can be associated with delivery, shRNA expression or the sequence itself (Snove and Rossi, 2006). Paddison and colleagues described gene silencing in various mammalian cell lines after transfection with a plasmid containing an shRNA expression cassette under the control of a U6 promoter (Paddison et al., 2002).

The choice of promoters depends on the desired expression level. While pol II promoters are suitable for moderate expression levels, higher expression levels can be achieved by combining a pol III (U6) and a pol II (U1) promoter as the transcribed products have different export pathways. There is also the possibility of using an inducible or tissue-

specific expression system, e.g. transcriptional elements which are responsive to tetracycline or the Cre-lox system for permanent genetic changes (Snove and Rossi, 2006).

Although it was originally suggested that in human cells silencing is associated with DNA methylation (Morris et al., 2004), Ting et al. showed that effective transcriptional gene silencing in breast and colon cancer cells was independent of DNA methylation. Furthermore, it was also achieved in a cell line that was genetically modified and lacked the capacity to methylate DNA (Ting et al., 2005).

Furthermore, gene silencing by lentivirus-delivered shRNAs under a U6 promoter was successfully shown in HeLa as well as primary dendritic cells (Stewart et al., 2003). RNAi has also been used extensively in stem cell research over the last years. It was shown that the stem cell specific transcription factors Oct4 and Nanog play a significant role in stem cell self-renewal by using siRNAs or shRNAs, respectively, which were produced from lentiviral vectors to downregulate the gene expression. The promoters used were U6 and H1 respectively (Ivanova et al., 2006, Zaehres et al., 2005). Even though RNAi is a very attractive technique for functional genomics and human therapeutics, it was previously reported that oversaturation of endogenous small RNA pathways by high shRNA expression from a viral vector, lead to increased morbidity in mice (Barik, 2006, Grimm et al., 2006).

To summarise, RNA interference can be used in a variety of scientific backgrounds and future research has to be focused on applying RNAi in gene or cancer therapy.

1.5 Lentiviruses

1.5.1 Lentivirus structure and life cycle

Lentiviruses belong to the family of retroviruses and different virus species have been isolated from a variety of mammals, including humans. A summary of the lifecycle is illustrated in Figure 16. The subfamily of retroviruses is strongly associated with chronic diseases involving the immune system or the central nervous system. In contrast to the minimal genetic structure of some retroviruses, which include the gag, pol and env genes, lentiviruses contain six additional genes (Figure 17). These genes (rev, tat, nef, vif, vpr and vpu) contribute to the highly efficient infection caused by the virus (Buchschacher and Wong-Staal, 2000, Tang et al., 1999, Ailles and Naldini, 2002).

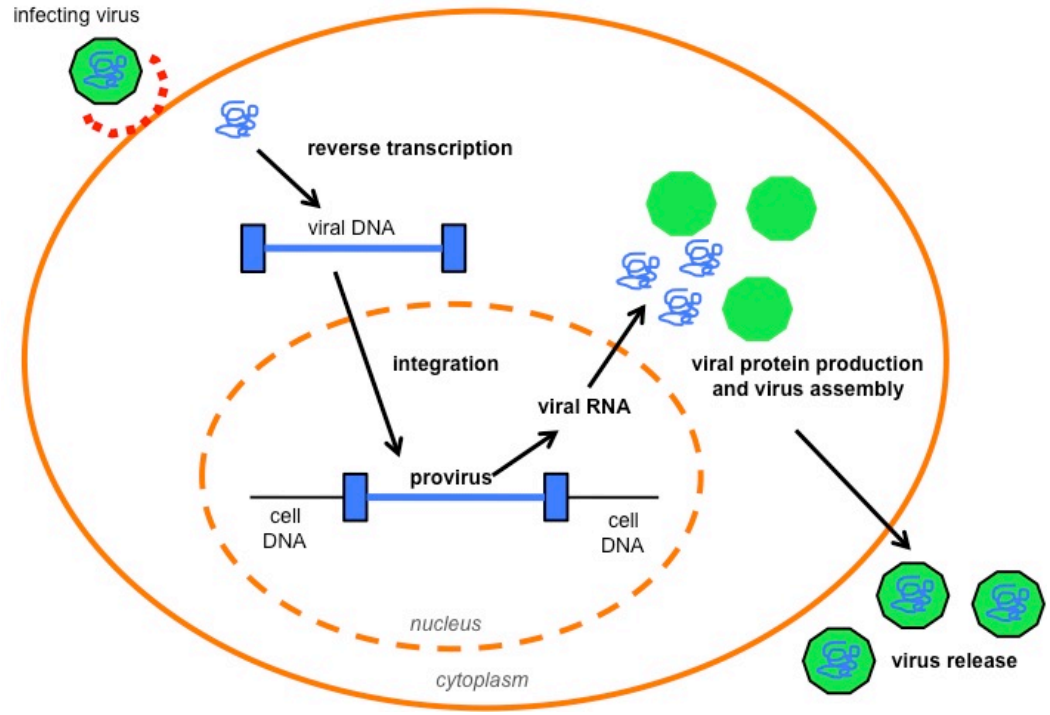


Figure 16: Life cycle of lentiviruses

After the virus has bound to the cell, the viral capsid is released into the cytoplasm. The genome is then reverse transcribed and integrated into the host cell genome. From there, viral RNA is transcribed and proteins are produced using the host cell enzymes. The viral RNA is then encapsidated and after assembly, the new viral particles are released (Buchschacher and Wong-Staal, 2000).

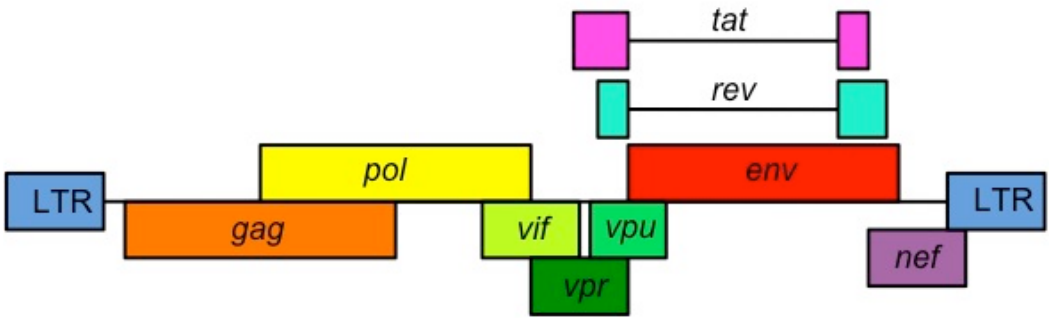


Figure 17: Genome structure of HIV-1

Genome structure of HIV-1 (Buchschacher and Wong-Staal, 2000)

One of the most studied lentiviruses is the human immunodeficiency virus type 1 (HIV-1), the causative agent of AIDS (Barre-Sinoussi et al., 1983). After the virus has entered the target cell by interacting with a cellular receptor complex, containing CD4 for HIV-1 and HIV-2, and subsequent fusion of the virion membrane with the plasma membrane of the cell, the virion is uncoated (Tang et al., 1999). The nine open reading frames in the HIV-1 genome can give rise to at least 15 different proteins. Apart from protein encoding genes, HIV-1 also possesses several cis-acting elements including the long terminal repeats (LTRs), a packaging and dimerisation signal (φ) and a polyadenylation signal (polyA) (Ailles and Naldini, 2002). The long terminal repeats consist of either U3 (unique 3' end) or U5 (unique 5' end), R (repeat region) and either the Primer Binding Site (PBS) or the Polypurine Tract (PPT) respectively (Tang et al., 1999). The env gene is responsible for the production of envelope glycoproteins, which mediate the virus-host cell interaction. After translation the Env protein is cleaved by a cellular protease into the external envelope glycoprotein and the transmembrane protein (Buchschacher and Wong-Staal, 2000). The gag gene, which encodes for the core proteins of the virion, is initially produced as a Gag-Pol fusion protein. Due to its own protease activity the polyprotein is then cleaved into Gag and Pol. Pol is then further processed to become a protease, an integrase and the reverse transcriptase (RT) (Buchschacher and Wong-Staal, 2000).

The reverse transcriptase, discovered in 1970 by Temin, Mizutani and Baltimore (Temin and Mizutani, 1970) is the defining feature of all retroviruses as this enzyme enables them not only to produce a DNA copy of their (+)-RNA genome, it also has an RNase H activity (Ailles and Naldini, 2002). The enzyme is an asymmetric heterodimer where both subunits are important, either structurally or catalytically (Katz and Skalka, 1994). One of the HIV-1 proteins that is important for efficient reverse transcription is Tat (Apolloni et al., 2003, Harrich et al., 1997). However, Tat is also a major regulator of gene expression at the transcriptional and posttranscriptional level (Dull et al., 1998).

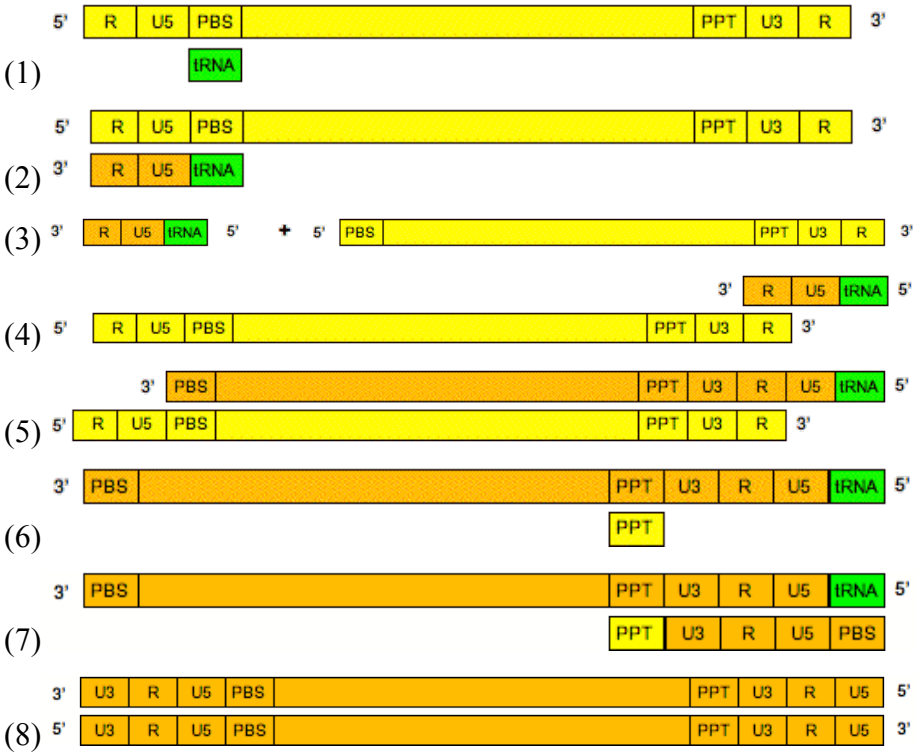


Figure 18: Reverse transcription[11]

(1) A cellular tRNA (green) acts as a primer and binds to the Primer Binding Site (PBS) of the single-stranded RNA genome (yellow).

(2) RT transcribes in 5'→3'-direction and produces a DNA copy (orange) of U5 and its flanking R element bound to the viral RNA strand

(3) RNase H activity degrades RNA, which is base-paired with DNA, resulting in a 5' end of viral RNA starting with the PBS and a free single strand consisting of a DNA copy of R, U5 and the cellular tRNA.

(4) The U5-R DNA strand can then base-pair with the R region on the 3' end of the viral RNA, a process which is called strand switch.

(5) RT can now continue synthesis producing a DNA strand with the PBS at its 3' end.

(6) Again, the RNA is degraded by RNase H activity, leaving only an RNA fragment that serves as a primer for the synthesis of the second DNA strand.

(7) This fragment stays base-paired with the polypurine tract (PPT) and elongation from this primer results in DNA copies of U3, R, U5 and PBS.

(8) After removal of the tRNA primer, the second strand switch occurs. The PBS on the 3' end can bind to the homologous sequence on the other strand, forming a circular intermediate and elongation generates a double-stranded DNA, which can then be integrated into the host cell genome The double strand contains the LTR sequence (U3-R-U5) at both termini.

[11] modified from Molecular Biology of the Gene; 5th edition; page 322-323; by Watson et al. 2004

After reverse transcription nuclear import occurs when a nucleoprotein complex called the preintegration complex (PIC) is actively imported during interphase. PIC, which includes the lentiviral genome and a number of viral proteins, offers the virus the possibility to integrate into quiescent cells that are not dividing at the time of infection. The integration of the viral ds-DNA into the host cell genome is mediated by the integrase enzyme. The integrated virus is referred to as a provirus. Although, integration occurs at random the provirus is usually found at transcriptionally active regions of the genome (Tang et al., 1999).

1.5.2 Lentiviral vectors – 1st, 2nd and 3rd generation

Lentiviruses have been at the focus of great research interest because of their infectious potential, especially since AIDS, associated with HIV-1 and HIV-2, poses a threat to human health worldwide. The development of early HIV-1 derived vectors was therefore initially intended for the study of HIV-1 biology. However, lentiviruses have proved to be a versatile tool for gene delivery *in vivo* due to the fact that they can integrate into the host cell genome and therefore they can provide stable and long-lasting gene expression. They also have a larger cloning capacity (8-9 kb) than other gene delivery systems (Ailles and Naldini, 2002). When comparing them to other possible gene therapy vectors like oncoretroviruses, which have been the primary choice for gene therapy application due to their simple genome organisation, lentiviruses have another advantage, as they are able to infect both dividing and non-dividing cells (Ailles and Naldini, 2002, Buchschacher and Wong-Staal, 2000, Lewis and Emerman, 1994).

However, the first generation vectors were never considered safe for gene therapy or similar treatments as the risk of replication-competent retroviruses (RCR) could not be diminished and there were major biosafety concerns. First generation vectors contained a constitutive promoter instead of the 5’ LTR and an SV40 polyA signal, which replaced the 3’ LTR. Apart from the env gene, which has been deleted, all other viral genes were still expressed.

For the development of second generation lentiviruses, the viral genes vif, vpr, vpu and nef were deleted as they represent important virulence factors. Their deletion did not show any significant decrease in viral particle production.

The major achievement in developing the third generation vectors was the deletion of tat, a powerful transcriptional activator, from the packaging construct. In order to compensate

for Tat, constitutive promoters like CMV or PGK, were introduced (Dull et al., 1998). It was also found that by deleting the Rev-responsive element from the vector, mobilisation rates of the vector from host cells was more than 10^4-fold lower (Lucke et al., 2005). Furthermore, rev is now supplied on a separate plasmid, thus limiting expression to the producer cell line.

However, there is still a possibility of producing a replication competent virus, e.g. when the transduced cell is subsequently infected with a wild-type HIV-1. Moreover, abnormal expression of adjacent genes after integration due to 3' LTR activity has to be addressed. For these reasons, new vectors with self-inactivating (SIN) LTRs were created, by deleting a part of the U3 sequence of the 3' LTR. After reverse transcription, this deletion is also transferred to the 5' LTR where the viral promoter is present, thus eliminating the expression of full-length viral RNA after transduction (Ailles and Naldini, 2002, Miyoshi et al., 1998, Zufferey et al., 1998). Moreover, as there is no full U3 sequence present, no recombination event would result in restoration of the wild-type U3 (Hanawa et al., 2005). SIN vectors, which were modified to harbour a cytomegalovirus (CMV) promoter in order to make them independent from Tat transcription, have also been shown to transduce target cells as efficiently as the wild type virus (Miyoshi et al., 1998). Increased biosafety was further assured when no mobilisation of SIN vectors was detected after the cells had been subsequently transduced with wild-type HIV-1 (Bukovsky et al., 1999). Additionally, recombination between transfer and packaging constructs might result in gag and pol being present in the transfer vector, however, after integration into host cells, there is no promoter present to drive expression and therefore the biosafety of this system is very high (Ailles and Naldini, 2002).

In order to enhance virus production for gene therapy vectors, Hlavaty et al. found that the WPRE (woodchuck posttranscriptional regulatory element) greatly increased transgene expression while Hsp70 5' UTR, which was also tested, did not result in a significant increase (Hlavaty et al., 2005). Another way to enhance successful gene transfer into resting or slowly dividing cells was achieved by pseudotyping lentiviruses with the envelope glycoproteins of MLV (murine leukaemia virus) and VSV-G (vesicular stomatitis virus, protein G). Pseudotyping also enables more specific targeting according to the tropism of the glycoproteins used (Stitz et al., 2000).

1.5.3 Using lentiviral vectors

Lentiviral vectors have been used as gene delivery tools on a variety of cell types during the last years. Gerolami et al. showed in 2003 that lentiviral vectors could efficiently transduce a hepatocellular carcinoma cell line with the reporter gene LacZ and also with a Herpes simplex virus thymidine kinase GFP fusion gene *in vitro* and *in vivo* (Gerolami et al., 2004). It was also shown that murine neural stem cells could be transduced *in vivo* (Consiglio et al., 2004). Prostate cancer cell lines have also been targeted using lentiviruses. Bastide at al. targeted DU145 and PC3 cells with vectors containing the marker gene EGFP. *In vitro*, the cell lines continued to express the transduced gene for 4 months while *in vivo*, the majority of the tumours kept expressing the transgene at *in vitro* levels as well (Bastide et al., 2003). When a prostate tumour specific lentivirus was administered systemically into SCID mice, the transduction levels were significantly higher at the site of the tumour than in other organs (Iyer et al., 2006). Furthermore, stable transduction of primary epithelial tissue from human prostate tumours using a recombinant retrovirus has also been shown (Maitland et al., 2001).

A major concern is of course non-specific targeting when considering gene therapy against cancers. However, Yu et al. developed an *in vitro* gene therapy system, which selectively targeted prostate cancer cell lines while non-cancer cells were not affected. The group introduced a short DNA sequence within the lentiviral vector, which is recognised by the translation initiation factor eIF4E. This initiation factor is often overexpressed in cancer cells. When transducing the cancer cell lines with a lentiviral construct harbouring the initiation factor recognition sequence in front of the suicide gene HSV thymidine kinase, cancer cells had a high sensitivity to the prodrug ganciclovir. On the other hand, non-cancer cells required 100-fold more ganciclovir in order to be killed than cancer cells. This showed an example of selective drug targeting of cancer cells (Yu et al., 2006). Another way to manipulate cancer cells is to target overexpressed genes by RNAi. There have been reports where either artificially introduced reporter genes or malignancy related genes were silenced using RNAi mediated through lentiviral vectors. Abbas-Terki et al. showed successful silencing of EGFP using a lentiviral vector, which encoded EGFP specific siRNAs under the RNA-polymerase III dependent H1 promoter (Abbas-Terki et al., 2002). In 2006 Liu et al. used lentiviral shRNA expression cassettes in a colorectal cancer cell line and a nude mice xenograft model for the knockdown of Tiam1, a metastasis related gene, which promotes tumour progression in a variety of cancers (Liu et

al., 2006). There have been a number of recent studies using lentiviruses to deliver shRNAs into cancer cells. Klier et al. succesfully downregulated cyclin D1 using a lentiviral expressed shRNA in mantle cell lymphoma (Klier et al., 2008) and Wang and colleagues used lentiviral vectors to deliver a MAT2B shRNA into hepatocellular carcinoma cells. They were able to show growth inhibition and increased apoptosis following the treatment (Wang et al., 2008b). STAT3 is also a popular target bearing in mind its role in cytokine signalling and overexpression in a number of cancers. It was shown recently that donwregulation of STAT3 by lentiviral expressed shRNAs in a glioblastoma cell line induced apoptosis (Li et al., 2009) and a similar result was shown on growth and invasive potential of pancreatic tumour cells following STAT3 knockdown (Yang et al., 2009). Studies have also used lentiviruses to deliver shRNAs into *in vivo* models of breast cancer (Krishnamachary et al., 2009) and to reverse multi-drug-resistance in a leukaemia cell culture model (Ye et al., 2009).

All these results illustrate the advantages of successful lentiviral shRNA treatments and open new ways of cancer research and, ultimately, therapy.

Chapter 2

Materials and Methods

2. Materials and Methods

2.1 DNA preparation, manipulation and analysis

2.1.1 Primer design and polymerase chain reaction (PCR)

Primers (Table 2) were obtained from Invitrogen. GC content, melting temperature, dimer and possible hairpin formation were analysed using the OligoAnalyzer 3.0 tool (Integrated DNA Technologies, http://www.idtdna.com).

Primer name	Sequence
CMV-SJ1 5’	5’ tagttattaatagtaatcaatt 3’
CMV-SJ2 5’	5’ ccgccatgcattagttattaat 3’
Fluor 3’	5’ ttacttgtacagctcgtccatg 3’
EGFP Luc-rev	5’ gatacattgatgagtttggac 3’
CMV sense	5’ gcgttgacattgattattgac 3’

Table 2: Primer sequences

Both CMV-SJ1 5’ and CMV-SJ2 5’ can be combined with either Fluor 3’ or EGFP Luc-rev. Amplification with EGFP Luc-rev results in a sequence containing a polyA signal after the fluorescent gene, whereas Fluor 3’ binds at the end of the fluorescent genes mOrange, Citrine and tdTomato, without amplifying the polyA tail.

The Expand High Fidelity PCR system (Roche Applied Science) was used for routine PCR amplifications in the Gene Amp PCR 9700 machine (Applied Biosystems). For a total reaction volume of 50 µl, 5 µl of concentrated Expand High Fidelity Buffer containing 15 mM $MgCl_2$, were combined with 1.5 µl of each primer (10 µM), 5 µl of dNTPs (2 mM) and 1 U of Expand High Fidelity Enzyme Mix. Template DNA was used at 5 ng/µl. The DNA was initially denatured for 5 min at 94° C. 30 cycles were carried out using a 1 min denaturing step at 94° C, a 1 min primer annealing step at 50° C and a 2 min elongation step at 72° C. Final extension was carried out for 7 min at 72° C, followed by storage at 4° C, if necessary.

2.1.2 Agarose gel electrophoresis and gel purification

PCR products and plasmids were analysed on 1 % (w/v) TAE agarose gels using 0.1 µl/ml SYBR Safe DNA Gel Stain (Invitrogen). Gel purification was routinely carried out at 60-80 V for a minimum of 1 h.
Bands were cut under UV light exposure and purified using the QIAquick Gel Extraction Kit (Qiagen) as instructed by the manufacturer. The DNA was eluted using 50 µl ddH_2O.

2.1.3 Insertion of fluorescent reporter gene constructs into pENTR 5' TOPO

pENTR 5' entry vectors were cloned using the pENTR 5′-TOPO TA Cloning Kit (Invitrogen) according to the manufacturer's instructions. 2 µl of PCR product (gene and promoter construct) were combined with 1 µl of linearised pENTR 5' TOPO plasmid and 1 µl salt solution. Sterile ddH_2O was added to make up the reaction mix with a total volume of 6 µl. 2 µl of this reaction mix was used for transformation into One Shot TOP10 chemically competent *E. coli* (Invitrogen) by heat shock transformation at 42° C. After 1 h incubation in SOC medium, bacteria were plated onto Luria Bertani (LB) agar plates containing 50 µg/ml kanamycin for selection. Colony growth was analysed after 24 h incubation at 37° C and DNA from transformants was analysed by restriction digest. Plasmid products were sequenced to confirm correct recombination prior to being stored as bacterial glycerol stocks.

2.1.4 Construction and selection of shRNAs

Double stranded shRNA constructs against PSCA (Table 3) were designed using the Origene RNAi collection (OriGene Technologies Inc., HuSH 29mer shRNA constucts) as a reference. The Origene sequences were modified using the Invitrogen BLOCK-iT™ RNAi Designer to combine them with the BLOCK-iT™ U6 RNAi Entry Vector Kit (pENTR/U6 plasmid, Invitrogen). All sequences were blasted against the NCBI human EST database to ensure that the constructs exclusively targeted PSCA mRNA. Each construct contained a different 29 nucleotide long stem loop. The top strands included direct cloning overhangs at the 5' end with the nucleotide sequence 'caccg'. The corresponding bottom strands contained a 3' overhang 'aaaa', to enable successful ligation

with the plasmid. The hairpin sequence in all constructs was 'cgaa' (default loop sequence).

2.1.5 Insertion of shRNA into pENTR/U6

pENTR/U6 entry vectors were cloned using the BLOCK-iT U6 RNAi Entry Vector Kit (Invitrogen) according to the manufacturer's instructions. An annealing reaction was set up and incubated at 95° C for 4 min to generate double stranded (ds) oligos from single stranded shRNAs. The resulting ds oligos were then diluted to a final concentration of 5 nM in 1x Oligo Annealing Buffer for the ligation reaction. Ds oligos were cloned into pENTR/U6, which was transformed into One Shot TOP10 chemically competent E. coli. Heat shock transformation was carried out as described under 2.1.3. DNA from transformants was analysed by restriction digest. Plasmid products were sequenced to confirm correct recombination prior to being stored as bacterial glycerol stocks.

2.1.6 Isolation of plasmid DNA

Plasmid DNA was isolated from 5 ml of *E. coli* One Shot TOP10 bacterial overnight culture using the Qiagen QIAprep Spin Miniprep according to the manufacturer's instructions. The DNA was eluted from the column using 50 µl of ddH_2O. Plasmid DNA was stored at -20°C.

2.1.7 Generation of pLenti6 expression constructs by site-specific recombination

Lentiviral expression constructs were generated using the ViraPower Promoterless Lentiviral Gateway Kit (Invitrogen). 10 fmol pENTR 5' TOPO containing a fluorescent marker gene under the constitutive CMV promoter and 10 fmol pENTR/U6 containing an shRNA construct were combined with 20 fmol destination plasmid pLenti6/R4R2/V5-DEST. After the recombination reaction was performed according to the manufacturer's instructions, pLenti6 expression vectors were obtained.

2.1.8 Restriction analysis of pENTR and pLenti6 plasmids

pENTR 5' and pENTR/U6 entry clones and pLenti6 expression clones were digested using NcoI (NEB) and NcoI/EcoRI (Promega) to confirm correct insertion of PCR products and shRNA oligos or correct recombination, respectively. 5 U (0.5 µl) of restriction enzyme was combined with 3 µl of the respective buffer, 10 µl plasmid DNA (from miniprep) and 16.5 µl water and incubated at 37° C for 2 h. Digests were examined on 1 % (w/v) TAE agarose gels using 0.1 µl/ml SYBR Safe DNA Gel Stain (Invitrogen).

2.1.9 Sequencing and sequence analysis

Sequencing was performed either by the Technology Facility at the University of York (TF) or by Cogenics (now Beckman Coulter Genomics). Plasmid DNA at the recommended concentrations (115-150 ng/µl for TF; 100 ng/µl for Cogenics) was sent for sequencing. Universal primers (M13-20; M13 rev-26; M13R), which were provided with the sequencing service, were used. pENTR/U6 plasmids, containing shRNA sequences, were sequenced using Cogenics Silver sequencing service and special conditions for hairpin DNA.

2.1.10 siRNA and shRNA constructs

Sequences of siRNA and shRNA constructs are listed in Tables 3 and 4.

shRNA name	DNA sequence
beta-actin	5’ caccgctgtccaccttccagcagatgtggatcagcgaactgatccacatctgctggaaggtggacag 3’
Luciferase	5’ caccgagttgcgcccgcgaatgatatttataatgcgaacattataaatatcattcgcgggcgcaac 3’
PSCA_S1	5’ caccgtgctgtgacaccgacttgtgcaacgccagcgaactggcgttgcacaagtcggtgtcacagca 3’
PSCA_S2	5’ caccgttcctgaggcacatcctaacgcaagtctcgaaagacttgcgttaggatgtgcctcaggaac 3’
PSCA_S3	5’ caccgtcggctctattgacacagatccgcctgcacgaatgcaggcggatctgtgtcaatagagccga 3’
PSCA_S4	5’ caccgtctatgacttgagccaggtctggtccgtgcgaacacggaccagacctggctcaagtcataga 3’
MSMB_S1	5’ caccggagattcaaccaggaaatgcatggatctcgaaagatccatgcatttcctggttgaatctcc 3’
MSMB_S2	5’ caccgccaataaactcggagtggcagactgacaacgaattgtcagtctgccactccgagtttattgg 3’
MSMB_S3	5’ caccgaagaaggaggactgcaagtatatcgtggtcgaaaccacgattaacttgcagtcctccttc 3’
MSMB_S4	5’ caccgagacctgttctgtcagtgaatggataatccgaagattatccattcactgacagaacaggtc 3’

Table 3: Sequences of shRNA top strands

Top strands to be annealed to the corresponding bottom strand and then cloned into pENTR/U6.

siRNA name	RNA sequence
PSCA_S1 (rna)	5’ (Cy3) ugcugugacaccgacuugugcaacgccagTT 3’
PSCA_S2 (rna)	5’ (Cy3) guuccugaggcacauccuaacgcaagucuTT 3’
Luciferase (rna)	5’ (Cy3) aguugcgcccgcgaaugauauuuauaaugTT 3’
siGENOME D-003697-01 PSCA (Dharmacon)	5’ gaacugcguggaugacucauu 3’
siGENOME D-003697-02 PSCA (Dharmacon)	5’ gcugugacaccgacuuguguu 3’
siGENOME D-003697-03 PSCA (Dharmacon)	5’ gcaaaggcugcagcuugaauu 3’
siGENOME D-003697-04 PSCA (Dharmacon)	5’ gcugugcuacuccugcaaauu 3’
siCONTROL D-001140-01-05 GAPDH (Dharmacon)	proprietary
siCONTROL D-001210-01 Non-targeting #1 (Dharmacon)	proprietary
AllStars Neg. siRNA Cy3 (Qiagen)	proprietary
Silencer β-actin siRNA Control (Ambion)	proprietary

Table 4: Sequences of siRNA sense strands

siRNAs were supspended in siRNA suspension buffer (Qiagen) for a final concentration of 2 µg/µl.

2.2 Transfections

2.2.1 Plasmid transfections

Cells were plated at $4x10^4$ cells/well into 24-well plates 24 h prior to transfection. Medium was removed and fresh growth medium was added before transfection. Transfection reagent (Fugene6 transfection reagent, Roche) was diluted in serum-free medium or PBS at a ratio of 1:50, according to the manufacturer's instructions. 1 µg plasmid DNA was added to the diluted reagent. The complex was incubated for 20 min at RT before it was added to the cells. Transfected cells were incubated for 48-96 h.

2.2.1 siRNA transfections using X-tremeGENE siRNA transfection reagent

Cells were plated at $2x10^4$ cells/well into 24-well plates 24 h prior to transfection according to the manufacturer's instructions (Roche). Medium was removed and fresh growth medium was added before transfection. 0.5 µl X-tremeGENE reagent per well was diluted in serum-free Opti-MEM medium. 0.5 µg siRNA (Table 4) was diluted in serum-free Opti-MEM medium before the diluted reagent was added to the siRNA solution. The complex was incubated for 20 min at room temperature (RT) before it was added to the cells. Transfected cells were incubated for 48-96 h prior to analysis.

2.3 Lentiviral infections

2.3.1 Generation of lentivirus using the Invitrogen Gateway system

After the MultiSite Gateway recombination reaction was performed, the resulting expression vector and the Packaging Mix plasmids (Invitrogen) were used to cotransfect 293FT producer cells according to the manufacturer's instruction outlined in the ViraPower Promoterless Lentiviral Gateway Kits manual (Invitrogen). For each transfection, 9 μg ViraPower Packaging Mix plasmids and 3 μg pLenti6 expression vector plasmid DNA were added to 1.5 ml serum-free OptiMEM medium and mixed. In a separate vial, 36 μl Lipofectamine 2000 was diluted in 1.5 ml serum-free OptiMEM medium. The diluted plasmid DNA was combined with the transfection reagent solution and incubated for 20 min at RT. After complexes had formed, the solution was transferred into a 10 cm cell culture dish containing 5 ml OptiMEM, before $6x10^6$ 293FT producer cells were added. Cells were incubated over night at standard culture conditions and medium was changed after 24 h. Virus containing supernatant was harvested after 48-96 h. The supernatant was centrifuged at 1350 x g (3000 rpm) for 15 min at 4° C to remove debris. Virus was then stored in aliquots at -80° C.

2.3.2 Titering of Gateway lentiviruses

One day prior to titering, HT1080 cells were plated at $2x10^5$ cells per well into a 6-well plate. A ten-fold serial dilution of lentiviral stock (from 10^{-2} to 10^{-5}) was prepared and a final volume of 1 ml (lentiviral stock in complete culture medium) was added to the cells in each well. Polybrene was also added to the cells at 6 μg/ml prior to over night incubation. Medium was replaced after a further 24 h with selection medium containing 10μg/ml Blasticidin. Selection medium was replenished every 2-3 days. After 10-12 days post selection, wells were washed with 2 ml PBS and colonies were stained with 0.5 ml/well 1 % (w/v) crystal violet (Sigma) in 10 % ethanol for 5 min. After two further washing steps, colonies were counted to determine titre. Titre was calculated by multiplying the number of colonies with the dilution factor, e.g. 12 colonies and dilution factor 10^{-2} will give titre of $12 x10^2$, or $1.2 x10^3$ TU/ml.

Viruses were diluted according to the following scheme:

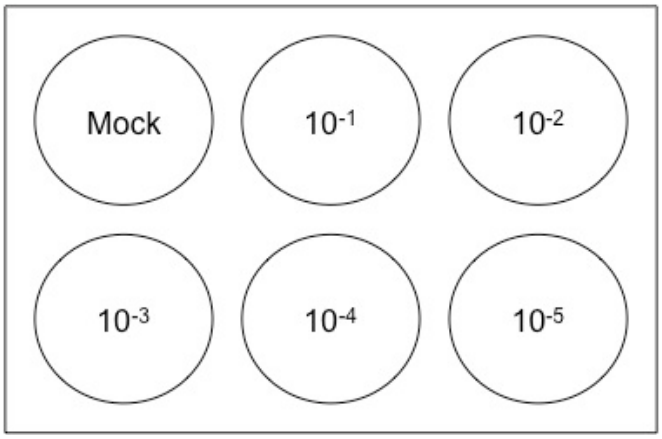


Figure 19: Titration dilution layout

2.3.3 Lentiviral infection using Sigma shRNA lentiviruses

Cell lines were plated at $2x10^3$ cells per 24-well. After 24 h, medium was replaced with new culture medium containing 8 µg/ml polybrene, prior to infection. Lentiviruses were added to the cells at a multiplicity of infection (MOI) of 10. Cells were incubated at 37° C, and medium was changed after 24 h. If long term, stable transduction was desired, the selection medium, containing 2 µg/ml puromycin, was added 48-72 h post infection. Medium was changed every 2-3 days for a minimum of 14 days.

Primary cells were cultured to approximately 70-80% confluency prior to infection in order to reduce the relative amount of mouse feeder cells present in the culture. Lentiviruses were added to $2x10^4$ cells in suspension (1 ml stem cell medium, SCM, without polybrene) at an MOI of 10. The cell suspension was then incubated at RT or 37° C on a rotating spinner for 45 min. Cells were plated onto collagen-coated 35 mm cell culture dishes with irradiated mouse feeder cells and incubated at 37° C. SCM was changed after 24 h and more feeder cells were added, if necessary. If stably transfected cells were desired, selection medium, containing 0.5 µg/ml puromycin, was added 72 h post infection. Medium was changed every 2-3 days for 10 days and feeder cells were added as required. Afterwards, cells were placed into normal culture medium (SCM).

2.3.4 Puromycin Kill Curve

A puromycin kill curve was performed to determine the minimum concentration for 100 % cell death. Cells were plated at $1.6x10^4$ cells per well of a 96-well plate. Medium was exchanged with 120 µl puromycin-containing medium per well one day post plating. Puromycin was added at the following concentrations, in triplicate: 0.5 µg/ml, 2 µg/ml, 4 µg/ml, 6 µg/ml, 8 µg/ml and 10 µg/ml. Cell lines were selected for a minimum of 14 days. For primary cells, which were plated at $5x10^4$ cells per well, the concentrations were 1 µg/ml, 0.5 µg/ml, 0.3 µg/ml and 0.2 µg/ml, respectively. Primary cells were selected for a maximum of 10 days. Medium was changed every 3 days and surviving cells were visually examined every 2 days.

2.4 Cell culture

2.4.1 Bacterial cell culture

E. coli One Shot TOP10 cells were cultured on LB agar plates or in LB medium supplemented with antibiotics at 37° C if applicable. Liquid cultures were incubated under agitation at 180 rpm in an orbital shaker incubator. For cryopreservation *E. coli* were frozen at -80° C in LB medium containing 35 % glycerol.

2.4.2 Primary cell culture

For culturing primary prostate epithelial cells, collagen coated plasticware (BioCoat, BD Bioscience) was used. Cells were cultured in stem cell medium (SCM) with the addition of irradiated or mitomycin C treated mouse feeder cells (STOs). Feeders were replaced if necessary, and the medium was changed every two days. Cells were subcultured at a ratio of 1:2-1:4 once they reached 80-100 % confluence.
Prostate fibroblasts (stroma) were cultured in R10 containing 1 x Antibiotic-Antimycotic (Gibco by Invitrogen). Medium was exchanged initially after 5-7 days and then every 3-4 days until confluency.
SCM consists of KSFM medium supplemented with 2 ng/ml Leukaemia Inhibitory Factor (LIF), 2 ng/ml Stem Cell Factor (SCF), 100 ng/ml cholera toxin (CT) and 1 ng/ml Granulocyte Macrophage Colony-Stimulating Factor (GM-CSF).

2.4.3 Mammalian cell lines and culture conditions

Cell line	Medium	Subculture ratio	Medium components
293FT	D10 plus	1:6 every 2-3 days	D10 plus 0.1 mM MEM non-essential amino acids; 1 mM MEM sodium pyruvate; supplemented with 500 ng/µl G418 in culture
BPH-1	R5	1:6 every 3-4 days	RPMI (GIBCO, Invitrogen); 5 % FBS; 6 mM L-Glutamine
Caco-2	DR10	1:10 every 2-3 days	1:1 mix of R10 and D10
HeLa	D10	1:8 every 2-3 days	DMEM (GIBCO, Invitrogen); 10 % FBS; 6 mM L-Glutamine
HT1080	ATCC MEM (E10)	1:10 every 3-4 days	Minimum essential medium, Eagle's (ATCC); 10 % FBS; 6 mM L-Glutamine
LNCap	R10	1:4 every 2-3 days	RPMI (GIBCO, Invitrogen); 10 % FBS; 6 mM L-Glutamine
MCF-7	D10	1:4 every 2-3 days	DMEM (GIBCO, Invitrogen); 10 % FBS; 6 mM L-Glutamine

Cell line	Medium	Subculture ratio	Medium components
P4E6	K2	1:3 every 2-3 days	Defined Keratinocyte-SFM (GIBCO, Invitrogen); 2 % FBS; 6 mM L-Glutamine; K2 supplements EGF and bovine pituitary extract (supplied with medium)
PC-3	H7	1:4 every 2-3 days	Ham's F12 (GIBCO, Invitrogen), 7 % FBS; 6 mM L-Glutamine
PNT2-C2	R10	1:5 every 2-3 days	RPMI (GIBCO, Invitrogen); 10 % FBS; 6 mM L-Glutamine
SaOS-2	αDMEM	1:4 every 3-4 days	Invitrogen #A10490-01
STO	D10	1:10 to 1:20 every 3-4 days	DMEM (GIBCO, Invitrogen); 10 % FBS; 6 mM L-Glutamine
SW480	DR5	1:2 to 1:8 every 3-4 days	1:1 mix of R5 and D5 (DMEM; 5 % FBS; 6 mM L-Glutamine)

Table 5: Medium and subculture requirements for cell lines

All cells were cultured in humidified atmosphere with 5 % CO_2 at 37° C.

2.5 Invasion assay

Cell culture inserts (BD Biosciences) were coated with matrigel (dilution in KSFM, approximately 750 µg/ml) and left to polymerise for 1 h at 37° C. Cells were seeded into matrigel-coated cell culture inserts and non-matrigel control inserts at a density of 2 x 10^4 cells per insert. The inserts were transferred onto a plate containing conditioned medium from stromal cultures or R10 as the attractant and incubated for 48 h at 37° C. Cells were washed twice in cold PBS and fixed in ice-cold methanol at -20° C for 20 min. The bottom membrane of the inserts was cut out, mounted bottom-side up, using Vectashield mounting medium with Dapi (Vector Laboratories) and examined under a fluorescent microsope. 4 random images per membrane were taken at 20 x magnification, avoiding the edges of the membrane. Nuclei were counted either by eye or using the cell count analysis function of ImageJ or Nikon NIS Elements software. The number of invasive cells in each cell line was compared. Invasion-motility-ratios were calculated to determine the number of invasive cells relative to migratory cells.

2.6 Immunomagnetic isolation of CD133 cell population

Cultured primary cells were subjected to immunomagnetic separation once they reached approximately 80 % confluency. To isolate CD133-expressing cells from the culture, the direct CD133 Cell Isolation Kit (Miltenyi Biotec) was used.

Cells were washed in PBS, trypsinised and pelleted. The cell pellet was washed again twice before the cell suspension was passed through a 40 µm cell strainer (BD Bioscience) in order to remove cell clumps. Cells were pelleted again and suspended in 300 µl MACS buffer. Magnetic cell labelling and separation over MACS MS columns was performed according to the manufacturer's instructions. To increase purity of the CD133 population, this procedure was repeated over a second column. Separated cell populations were the used for IF.

2.7 Injection of PC-3 cells into mice

Stably transfected PC-3 cells (PC-3v45, PC-3_2V) and untransfected PC-3 cells were injected subcutaneously into the left flank of GC-1-RAG2 γc -/- mice to measure tumour induction and growth. Cells were counted and 5 x 10^5 cells per mouse were suspended in matrigel (BD Bioscience). Cells were kept on ice prior to injection. For each animal, an aliquot of 50 µl cells-in-matrigel suspension was used, with 3 replicates in each group. The procedure was carried out by Dr Anne Collins, Paul Berry and Katy Hyde. Tumour growth was measured every 2-4 days from the time point of first, visible tumour detection. Mice were sacrificed once tumours reached 15 mm in length or earlier, if tumour size was inhibiting movement or when tumours started to break up. Tumours were then subjected to depletion to remove hematopoietic cells and infiltrated mouse cells, followed by Western Blot for β-catenin.

2.8 Depletion of hematopoietic cells and mouse cells from PC-3 tumours

Due to possible infiltration of murine cells into subcutaneous PC-3 tumours, depletion by magnetic separation was performed. During this procedure, mature hematopoietic cells (T cells, B cells, NK cells, dendritic cells, monocytes, granulocytes, erythroid cells) are removed using a lineage depletion kit (Miltenyi Biotech). Afterwards, the remaining cells are depleted from any infiltrated murine cells, using a mouse-specific CD31 antibody.

Firstly, fresh collagenase was weighed out for a final concentration of 200 IU/ml, dissolved in 2.5 ml KSFM per tumour (up to 1 g of tissue). 5 ml serum containing medium (R10 with ABM) was added. Tissue was placed into a 10 cm petri dish and washed in PBS. A small piece (2 mm x 2 mm x 1 mm, approximately) was cut off for fixation in 10% formalin and stored at 4 °C. The collagenase solution was added to the remaining tumour, which was then diced into small pieces using tweezers and a scalpel. The tissue-collagenase mixture was then transferred into a 125 ml sterile Erlenmeyer flask and incubated at 37 °C overnight in a small orbital shaker for digestion (80 rpm).

Following the digestion, repeated pipetting was used to break up any remaining pieces of tumour, before the mixture was centrifuged at 670 x g (2000 rpm) for 10 min to sediment the cells. The pellet was resuspended in 10 ml of PBS to wash out any remaining

collagenase, followed by further centrifugation at 670 x g for 10mins. Washing and centrifugation were then repeated once more.

For further digestion, the pellet was resuspended in 10 ml of 1 x trypsin and incubated at 37 °C for 30 min in a small orbital shaker (80 rpm). The digestion was stopped by adding 10 ml of R10. After short centrifugation (300 x g for 4 min) and another washing step in PBS, the pellet was resuspended in 500 µl pre-cooled (4 °C) MACS buffer and kept on ice for the whole procedure. Cells were passed through a 40 µm cell sieve before lineage depletion was carried using the MACS Lineage Cell Depletion Kit human (Miltenyi Biotech) as outlined in the manufacturer's instructions.

80 µl of MACS buffer were used to resuspend the pellet before 20 µl of Biotin-Antibody Cocktail were added, mixed and the mixture then incubated for 10 min at 4 °C on a rotating spinner. 60 µl of MACS buffer, followed by 40 µl of Anti-Biotin MicroBeads were added, for incubation for 15 min at 4 °C on a rotating spinner. Cells were washed by adding 4 ml of buffer and pelleted by centrifugation (300 x g for 4 min).

For magnetic separation, cells were resuspended in 500 µl of MACS buffer. An LS column was placed in the magnetic field of a MACS Separator and washed using 3 ml of MACS buffer. Cells were applied to the column to collect the unlabelled, enriched, lineage negative fraction (Lin- cells). Further collection of Lin- cells was carried out after washing the column 3 times with 3 ml of buffer. The retained cells (Lin+) were collected outside the magnetic field. Cells were then subjected to magnetic separation of stromal and endothelial cells using a mouse-specific biotin-CD31 antibody (AbD Serotec).

Pelleted Lin- cells were resuspended in 90 µl of buffer. 10 µl of CD31 antibody were added and the mixture incubated for 10 min at 4 °C on a rotating spinner. Following incubation, 60 µl of buffer and 40µl of Anti-Biotin MicroBeads were added, for incubation for 15 min at 4 °C on a rotating spinner. Cells were washed by adding 4 ml of buffer and pelleted by centrifugation (300 x g for 4 min). The pellet was resuspended in 500 µl of buffer and cells were subjected to magentic separation as outlined above. The unlabelled, collected fraction represented (human) CD31- cells, while the retained cells were mouse CD31+. CD31- cells were pelleted and either stored frozen at -20 °C or used immediately lysed for Western Blot using CytoBuster (see 2.14.1).

2.9 Wnt pathway activation

Cells were activated using 20 mM final concentration of LiCl 24 h post plating, for 24 h. NaCl served as the negative control, according to the protocol used by Spencer and colleagues (Spencer et al., 2006). Alternatively, Wnt1 protein (Abcam) was added to cells at 100 ng/ml to induce Wnt signalling.

2.10 Fixation techniques

Adherent cells were cultured in chamber slides (BD Falcon™ 8-well CultureSlides; Nunc Lab-Tek™ 8-well Chamber Slide; BD BioCoat™ Collagen I 8-well CultureSlides). Cells were washed twice with PBS prior to fixation. 200 µl 4 % paraformaldehyde (PFA) or 4 % formaldehyde solution (Formalin, FA) was added per well and cells were incubated for 20 min at RT. Fixed cells were washed 3 x with PBS before chamber slides were dried for approximately 30 min at RT. Unless fixing was directly followed by immunofluorescence staining, slides were wrapped in parafilm and stored at -20 °C.

2.11 Immunofluorescence for β-catenin

Fixed cells were washed 3 x with PBS before they were permeabilised using 0.5 % Triton X-100 in PBS for 10-20 min at RT. Blocking was performed in 10 % normal goat serum (Sigma) in PBS for 30 min at 37° C. The primary antibody was diluted in blocking solution at appropriate concentrations (Table 6) and incubated for 1 h at RT or 37° C. Cells were washed 3 x with PBS before the secondary antibody (Alexa Fluor 488 goat anti-rabbit IgG, A11034; Alexa Fluor 568 goat anti-rabbit IgG, A11036; Alexa Fluor 568 goat anti-mouse IgG, A11031; all Invitrogen) was added at a dilution of 1:1000 in blocking solution. Cells were washed 3 x with PBS, then mounted using Vectashield mounting medium with Dapi (Vector Laboratories). Slides were examined under a Nikon TE300fluorescent microsope or a multiphoton Zeiss LSM 510 NLO meta confocal microscope. Slides were kept at 4° C if necessary.

Antibody name	Company & Cat. No.	Dilution
Anti-β-catenin antibody	Sigma; C2206	1:1000
β-Catenin (6B3) Rabbit monoclonal antibody	Cell Signaling; 9582	1:1000
Phospho-β-Catenin (Ser552) Antibody	Cell Signaling; 9566	1:1000
Phospho-β-Catenin (Ser675) Antibody	Cell Signaling; 9567	1:1000
Phospho-β-Catenin (Ser33/37/Thr41) Antibody	Cell Signaling; 9561	1:1000
Phospho-β-Catenin (Thr41/Ser45) Antibody	Cell Signaling; 9565	1:1000
Anti-Active-β-Catenin (anti-ABC), clone 8E7	Millipore; 05-665 lot no. DAM1487766 & TR1510175	1:50, 1:100

Table 6: Antibodies used for immunofluorescence

2.12 Colocalisation

IF images of β-catenin (red) and DAPI (blue) staining were opened in ImageJ software. If necessary, a stack of images was separated into 2 images (2 channels). From the 'Plugins' menu, 'Colocalization' was selected and 'Channel 1 (red)' was assigned the β-catenin image, 'Channel 2 (green)' the DAPI image. The resulting red/green colocalisation image type was changed into a 32-bit image (black and white). Colocalisation of β-catenin and DAPI was seen as white dots within the nucleus.

2.13 TOPFlash reporter assay

Prostate cell lines PC-3, P4E6 and PNT2-C2 (as well as SW480 and Hela control lines) were plated at 200 and 500 cells per 96-well. 24 h post plating, cells were transfected with 0.1 µg reporter plasmid MO50 (Super8XTOPFlash), MO51 (Super8XFOPFlash) or MO72

(Super16XTOPFlash), respectively, using 0.28 µl/well TransIT-LT1 (Mirus) transfection reagent, according to the manufacturers instructions. If desired, cells were activated with 20 mM LiCl 24 h post transfection. The luciferase assay was performed 48 h post transfection.

2.14 Luciferase Assay

Luciferase assays were performed using the Bright-Glo Luciferase Assay System (Promega). The Glo Lysis Buffer was equilibrated to RT before use. Cells were washed with PBS prior to the addition of 50 µl of Glo Lysis Buffer to each well of a 96-well plate. To ensure complete and equal coverage of the cells, the plate was slowly rocked several times. After 5 min incubation at RT to allow for cell lysis, the lysate was transferred to black-out luminometer plates and 50 µl Bright-Glo Assay Reagent was added. Luminescence was measured using a microplate reader (BMG Labtech POLARstar OPTIMA).

2.15 Cell cycle analysis

Cells from one 6-well or the equivalent amount from a larger culture container, were trypsinised and suspended in 5 ml R10. Cells were centrifuged at 280 x g (1300 rpm) for 5 min. The cell pellet was then resuspended in 1 ml PBS. 2.5 ml absolute ethanol was added to fix the cells and the suspension was immediately mixed to prevent clustering of cells. Cells were incubated on ice for 15 min or at -20 °C for up to one month. On the day of the FACS analysis, cells were centrifuged at 280 x g for 5 min. The pellet was resuspended in 500 µl propidium iodide (PI) solution and incubated at 37 °C for 40 min. 3 ml PBS were added and the suspension was again centrifuged at 280 x g for 5 min. Most of the supernatant was removed, leaving approximately 500 µl for resuspension prior to FACS analysis.

2.16 Protein analysis

2.16.1 Cell lysis

Cells were washed with PBS and the appropriate amount of CytoBuster Protein Extraction Reagent (Millipore), depending on cell culture surface area or number of cells, was added, according to the manufacturer's instructions. When producing lysate from adherent cells in culture, cells were scraped off to maximise recovery, after the initial incubation of 5 min at RT. The solution was transferred to a 1.5 ml tube and centrifuged for 4 min at 670 x g (2000 rpm). Supernatant was transferred to a fresh tube. 1x complete Protease Inhibitor Cocktail, PIC, (Roche Applied Science) was added to the supernatant which was then stored at -80 °C.

2.16.2 Bradford assay

Bradford assays were performed using the 'Protein Bradford' application on the Nanodrop 1000 software (Thermo Scientific). A serial dilution of protein standards of 0, 2000, 1000, 500, 250, 125, 62.5, 31.25, 15.625, 7.8125 and 3.9063 µg/ml BSA was prepared and mixed in a 1:50 dilution with Coomassie Protein Assay Reagent (Thermo Scientific Pierce). After production of a standard curve, 1:50 sample dilutions in Coomassie reagent were prepared and measured.

2.16.3 SDS-PAGE -Gel-electrophoresis

10 % acrylamide gels were prepared and left for polymerisation for a minimum of 2.5 h or overnight before loading. SDS running buffer was used with these gels. Alternatively, 10 % pre-cast Precise Protein Gels (Thermo Scientific) were pre-run at 60 V in Tris-HEPES-SDS buffer prior to sample loading.
Samples were mixed with SDS loading dye (final concentration of approximately 1x) and incubated at 100 °C for 15 min. Per lane of marker, 5 µl Kaleidoscope Prestained Standards (Bio-Rad) and 2 µl Biotinylated Protein Ladder (Cell Signaling) were mixed with 8 µl SDS loading dye and incubated at 100 °C for 5 min. 20 µl sample or 15 µl marker mix were added per lane. Gels were run at 60-100 V for up to 1 h or up to 2.5 h

(non-pre-cast gels). Gels were then used for wet or semi-dry transfer of proteins onto PVDF membranes.

2.16.4 Wet transfer

Wet transfer was performed using the Mini Trans-Blot Electrophoretic Transfer Cell system (BioRad). Membranes were wet in Methanol and washed with ddH_2O before being soaked in pre-cooled transfer buffer for approximately 5 min. The gel holder cassette was loaded while submerged in transfer buffer to minimise bubbles starting from the black side of the cassette. One ScotchBrite foam pad and two pieces of pre-soaked 3 mm Western Blotting paper were laid onto the black side of the cassette before the gel, followed by the membrane were carefully laid onto the card. On top of the membrane another two pieces of blotting paper and one ScotchBrite foam pad were added. The cassette was firmly closed and placed in the transfer cell tank. Transfer was performed at 4 °C at 100 V for 1 h. If necessary, dried membranes were stored in between Western Blotting paper at 4 °C.

2.16.5 Semi-dry transfer

Using a Trans-Blot SD cell (Bio-Rad), proteins were transferred from gels onto PVDF membrane by semi-dry blotting. Membranes were pre-soaked in Methanol, washed in ddH_2O and then incubated in transfer buffer B. Gels were equilibrated for 10-15 min in transfer buffer B. Onto the bottom plate of the Trans-Blot cells 2 sheets of Extra Thick Blot Paper (Bio-Rad), pre-soaked in transfer buffer A and 2 sheets, pre-soaked in buffer B, were added. Air bubbles were removed between each step by rolling a pipette across the stack. The membrane, followed by the gel and 4 sheets of pre-soaked blot paper (buffer B) were added on top, before semi-dry transfer was performed at 200 mA using a Power Pac 200 (Bio-Rad), for 45 min. After disassembly, membranes were stored dry in between Western Blotting paper, or re-wet in methanol prior to protein detection.

2.16.6 Western Blot

All washes and incubations were performed while rocking. Membranes were wet in Methanol, rinsed in dH_2O and washed in TBST for 10 min prior to blocking. Membranes

were blocked in 3 % Marvel or 3 % BSA for a minimum of 1 h at RT or over night at 4 °C. Primary antibodies were added and incubated according to Table 7.

After washing 3 times in TBST for 10 min each, the respective secondary antibody (Table 8) was added. The anti-biotin antibody to detect biotinylated marker bands on the membrane was added together with the secondary antibody. Incubation of secondary antibody was performed at room temperature. After another washing step (3x 10 min) in TBST, a chemiluminescent substrate mix (Roche) was added. Blots were developed using the Kodak® BioMax™ system (GBX developer, GBX fixer) and Amersham Hyperfilm ECL (GE Healthcare).

If necessary, western blot membranes were stripped by incubating them in stripping buffer at 56 °C for 45-60 min. Membranes were washed in TBST 3 times. In general, stripping of blots was only performed to re-probe for β-actin as a control.

Antibody name	**Company & Cat. No.**	**Dilution**	**Incubation Time**
PSCA polyclonal antibody (A01)	Abnova; H00008000-A01	1:750; 1:1250	2 h
PSCA antibody	Abcam; ab53159	1:500, 1:1000	2 h
Anti-β-catenin antibody	Sigma; C2206	1:2000	1 h or o. n.
β-Catenin (6B3) Rabbit monoclonal antibody	Cell Signaling; 9582	1:1000	2 h or o. n.
Phospho-β-Catenin (Ser33/37/Thr41) Antibody	Cell Signaling; 9561	1:1000	2 h
Monoclonal Anti-Actin clone AC-40 produced in mouse	Sigma; A3853	1:10000	1 h
Monoclonal Anti-β-Actin antibody produced in mouse clone AC-74	Sigma; A5316	1:5000	1 h

Table 7: Primary antibodies for Western Blots

Antibody name	Company & Cat. No.	Dilution	Incubation Time
Anti-Rabbit IgG (whole molecule) - Peroxidase antibody	Sigma; A0545	1:5000	1 h
Polyclonal Rabbit Anti-Mouse Immunoglobulins/HRP	Dako; P0260	1:5000	1 h
Anti-Rabbit IgG (whole molecule), F(ab′)2 fragment−Peroxidase	Sigma; A6667	1:5000	1 h
Anti-rabbit IgG, HRP-linked Antibody	Cell Signaling; 7074	1:1000	1 h
Anti-biotin, HRP-linked Antibody	Cell Signaling; 7075	1:5000	1 h

Table 8: Secondary antibodies for Western Blots

2.16.7 Western Blot using the Snap i.d. system

Western Blots for β-actin (with antibody A5316, Sigma), were routinely performed using the Snap i.d. system (Millipore) according to the manufacturer's instructions. 0.3 % Marvel was used for blocking and the antibody was added in a 1:1000 dilution in blocking solution, instead of 1:5000 for standard Western Blots. The total volume of antibody mix was 3 ml per blot in single blot holders. The secondary antibody (P0260, Dako) was also used at a 1:1000 dilution in blocking solution.

The Snap i.d. system was initially also used with β-catenin antibodies C2206 (Sigma) at 1:500, and 6B3 (9582, Cell Signaling) at 1:250, together with secondary antibodies A6667 or A0545 for C2206, at 1:1000, or 7074 (Cell Signaling) at 1:250, respectively.

Chapter 3

Aims and Hypothesis

3. Aims and hypothesis

Currently, there is no effective treatment for the late stages of prostate cancer due to tumours becoming resistant to standard therapeutic approaches. The aim of this project was to evaluate the use of lentiviral delivery of shRNAs into prostate cancer cells, to target overexpressed genes. In principle, there are two potential cell populations within a prostate tumour which could be targeted: the committed basal (CB) population, which forms the mass of the tumour, or the far rarer proposed cancer stem cell (CSC) population, which expresses the surface marker CD133 (Collins et al., 2005, Richardson et al., 2004).

The aim of this study was to establish a method for lentiviral transfer of shRNAs into primary prostate epithelial cells, and if possible, the cancer stem cell population to elucidate the role of the overexpressed shRNA target within prostate tumours. To test the feasibility of the method, we initially developed a cell line model (based on PC-3 cells) and targeted PSCA, an established marker for prostate basal differentiation (Tran et al., 2002). PSCA has also been shown to be overexpressed in a large number of prostate tumours (Reiter et al., 1998, Zhigang and Wenlv, 2004) and its expression is associated with higher Gleason score and more advanced tumours (Gu et al., 2000). Furthermore, xenografted animals treated with anti-PSCA antibody displayed decreased tumour growth and increased survival (Saffran et al., 2001). In addition, PSCA has been shown to be higher expressed in CB cells during a microarray study on primary tissue, with a mean fold expression change of 20.11 in committed versus stem cells (Birnie et al., 2008). PSCA has also been under discussion as a potential target for T-cell-based immunotherapy (Dannull et al., 2000). It therefore provided an interesting shRNA target for downregulation within a selected tumour population.

The hypothesis was that successful stable downregulation of such a highly expressed gene by shRNAs will alter the biological tumour properties, such as growth, invasion and metastasis formation.

The cell line model was approached from two different angles. Firstly, ‘home-made’ lentiviruses targeting PSCA were constructed but use of these was abandoned due to insufficient titres. Secondly, commercial viruses were used to infect PC-3 cells and generate stable shRNA-cell lines.

As the cell line model was only a proof of principle and the ultimate aim was to use shRNA-lentiviruses on primary prostate epithelial cells from patient samples, and more specifically, the prostate cancer stem cell population within them, PSCA was abandoned as a target in favour of β-catenin. This decision was based on microarray expression data obtained in our laboratory, where patient tissue was analysed (Birnie et al., 2008). Of a total of 54618 probes in the array, about 17000 showed high enough expression levels for statistical analysis. The first β-catenin probe was at place 2409 and there were two further probes above the expression threshold (at position 2087 and 11762). Furthermore, TCF-4, a direct interaction partner of β-catenin was also found to be differentially expressed (positions 4828, 6485, 8939 and 10575). In the Affimetrix array, TCF-4 showed an average 1.37 or 1.30 fold change between stem versus committed and malignant versus benign, respectively. This data was confirmed by RT-PCR (Birnie et al., 2008).

Our interest in the role of β-catenin in prostate cancer, and prostate CSCs, was based on its widely known importance in stem cell self renewal pathways (O'Brien et al., 2010), as well as its role in other types of cancers, with colon cancer being the most prominent example (Kanwar et al., 2010, Kolligs et al., 2002, Morin et al., 1997). In the majority of colon and gastrointestinal cancers, canonical Wnt signalling is abnormally activated due to mutations in the tumour suppressor gene APC (Giles et al., 2003b, van Es and Clevers, 2005). Although mutations of β-catenin in prostate cancer are relatively rare, they have been found to contribute to aberrant activation of Wnt signalling in hepatocellular, ovarian and hair matrix cell tumours (Giles et al., 2003b).

For prostate cancer, it is still under discussion whether β-catenin is an important contributor to cancer formation and if so to what degree and in which type of prostate tumours. There are varying reports about β-catenin mutation, expression and its cellular location in prostate (cancer) tissue (Chesire et al., 2000, Shah et al., 2009, Voeller et al., 1998), and interaction between β-catenin and the androgen receptor has been documented (Chesire and Isaacs, 2002, Schweizer et al., 2008, Wang et al., 2008a).

Being the essential mediator between extracellular Wnt signals and the TCF family of transcription factors, β-catenin has been shown to alter gene expression of a large number of genes. Some of these, like c-myc, Cyclin D1 and Cyclin D2 have been implicated in cancer development or progression (Cole et al., 2010, Jung et al., 2001, Tetsu and McCormick, 1999, Yochum et al., 2010, He et al., 1998).

The aim of infecting prostate cancer primary cells with sh-β-catenin lentiviruses was based on the hypothesis that high expression of β-catenin might provide the cancer cells

with certain advantages regarding growth, invasion and metastasis formation. Downregulation of such an important gene would therefore interfere with these important tumour properties and provide a new anchor point for future therapeutical approaches.
Considering the encouraging results in the cell line model, where sh-β-catenin lentiviruses did interfere with growth and invasion, as well as tumour onset in vivo, β-catenin remains an interesting target in the study of prostate cancer.

Chapter 4

Results

4. Results

4.1 Generation of reporter-shRNA-lentiviruses

4.1.1 Amplification of fluorescent genes and CMV promoter for cloning into pENTR 5' TOPO

The Invitrogen Gateway system has been designed to quickly produce vectors with different combinations of promoters and genes without traditional, restriction enzyme based cloning steps. We wanted to adapt this system for the production of lentiviral vectors carrying an shRNA and a fluorescent marker plus their respective promoters (Figure 20). The fluorescent marker would allow for easy monitoring of the infection and its effects on prostate cancer cells.

Plasmids, which contained one of three fluorescent reporter genes, mOrange, mCitrine or tdTomato (Table 9) (Shaner et al., 2005) were used to amplify each gene and its promoter (CMV).

Reporter genes were cloned into the Gateway pENTR 5' TOPO plasmid under the control of a CMV promoter. Constructs were amplified by PCR from pCMVmOrange, pCMVcitrine or pCMVtdTomato, respectively (Hager et al., 2008), with or without the polyA sequence following the fluorescent gene (Figure 19, Table 10). Gelelecrophoresis was performed to confirm the PCR products (Figure 21, see Table 10 for product sizes). Since tdTomato is a dimer, two bands were visible on the gel. CMVmOrangeA and CMVcitrineA were used for cloning into pENTR 5' TOPO.

Protein	Origin	Structure	Relative brightness compared to EGFP [%]
mOrange	DsRed	Monomer	146
mCitrine	YFP	Monomer	174
tdTomato	mRFP1	Dimer	142

Table 9: Fluorescent proteins

Listing of fluorescent proteins used in this study, which proteins they were derived from, their structure (monomer or dimer), their relative brightness compared to EGFP (Campbell et al., 2002, Griesbeck et al., 2001, Shaner et al., 2004, Shaner et al., 2005, Zacharias et al., 2002).

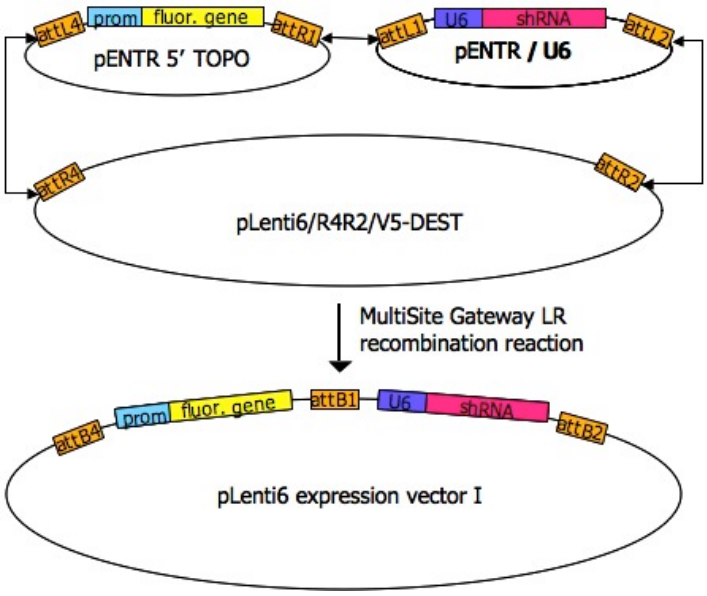


Figure 20: Schematic drawing of the MutiSite Gateway recombination reaction

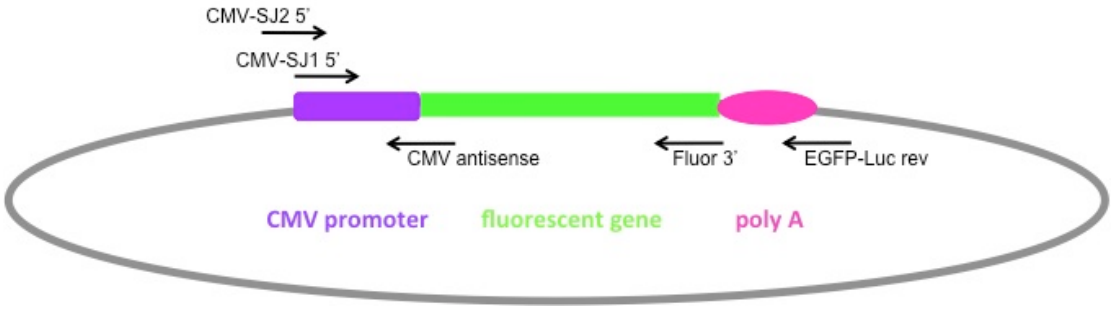


Figure 21: Position of PCR primers

Primer 1	**Amplified product using CMV-SJ1 5' or CMV-SJ2 5' as Primer 2**	**Size in bp (approximate)**
CMV antisense	CMV promoter	589
Fluor 3'	CMV promoter + mOrange	1325
Fluor 3'	CMV promoter + citrine	1332
Fluor 3'	CMV promoter + tdTomato	2043
EGFP-Luc rev	CMV promoter + mOrange + polyA	1525
EGFP-Luc rev	CMV promoter + citrine + polyA	1532
EGFP-Luc rev	CMV promoter + tdTomato + polyA	2243

Table 10: Products amplified by PCR using different primer combinations

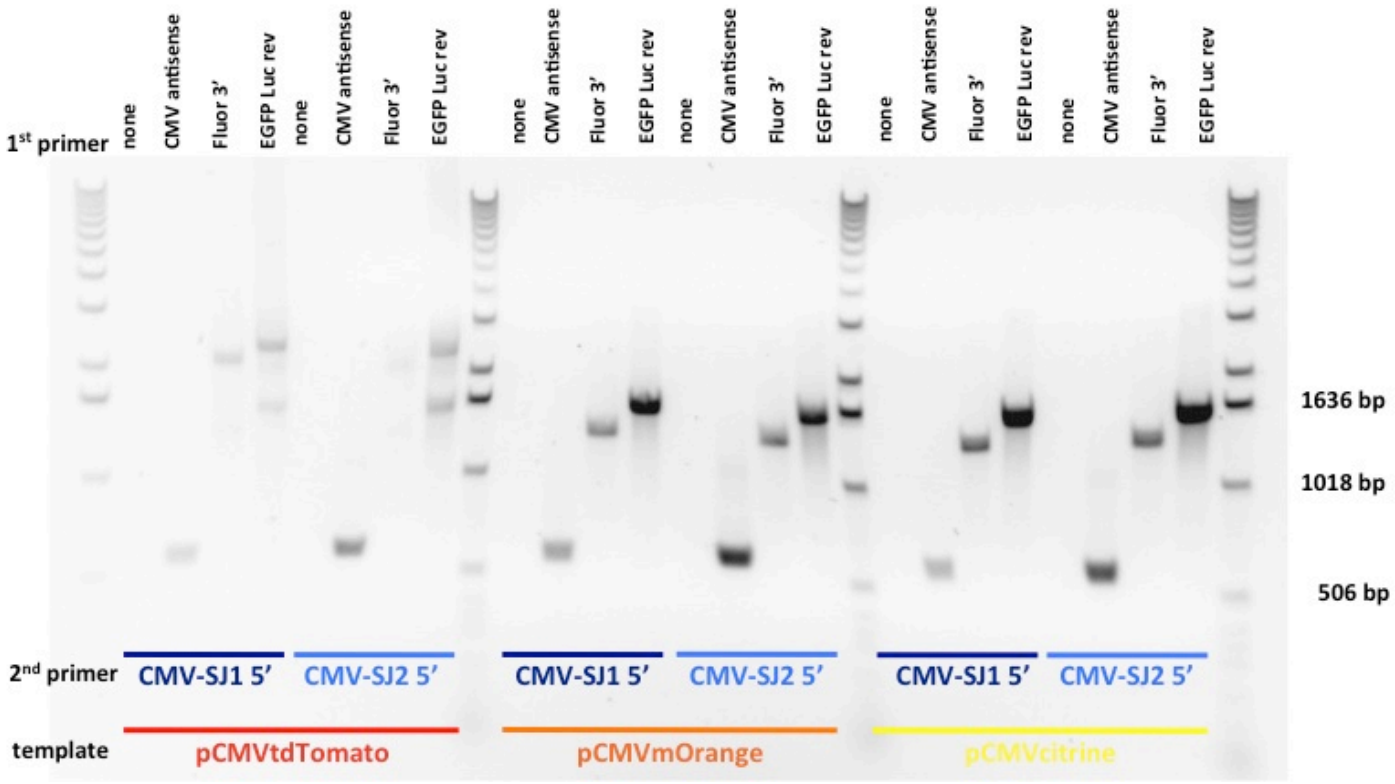


Figure 22: Gelelectrophoresis of PCR products after amplification

Gelelectrophoresis of PCR products after amplification of CMV, CMVtdTomato, CMVtdTomatoA, CMVmOrange, CMVmOrangeA, CMVcitrine and CMVcitrineA using primers CMV-SJ1 5' and CMV-SJ2 5' with either CMV antisense, Fluor 3' or EGFP-Luc rev, respectively.

4.1.2 Cloning of reporter expression cassettes into pENTR 5' TOPO

CMVmOrangeA and CMVcitrineA were cloned into pENTR 5' TOPO. NcoI restriction digests were performed to confirm the successful generation of the entry clones pENTR-CMVmOrangeA and pENTR-CMVcitrineA (Figure 23). Successful cloning followed by NcoI digest of pENTR-CMVmOrangeA should have resulted in 3 bands: 2833 bp, 663 bp and 484 bp, or 2916 bp, 663 bp and 401 bp, depending on orientation of the fragment post integration. There should be 2 bands for pENTR-CMVcitrineA (3496 bp and 484 bp, or 2779 bp and 1201 bp, respectively).

For pENTR-CMVmOrangeA, DNA gel electrophoresis resulted in 6 correct clones (1, 2, 4, 5, 9, 10) (Figure 23), which showed bands around the 3000 bp marker, as well as around 650 bp and 450 bp. As the digest made it difficult to distinguish between a possible band at 484 bp or 401 bp, further confirmation was necessary. Sequencing as well as a functionality test of the construct via mOrange expression post transfection was performed. The digest also resulted in an additional band around 250 bp, which also appeared in one of the incorrect clones (clone 3), and might therefore have been a contamination. However, it was not visible in the three clones without an insert (clones 6, 7 and 8).

The NcoI digest of pENTR-CMVcitrineA resulted in 7 correct clones (2, 3, 4, 7, 8, 9,10) (Figure 23). They all showed 2 bands after DNA gel electrophoresis, one around the 3000 bp marker, the other at 1200 bp. This confirmed that the construct had integrated in reverse orientation, compared to the reading frames of pUC origin and the kanamycin resistance gene.

As the tdTomato construct (Figure 22) resulted in double bands after PCR amplification, due to tdTomato being a tandem-dimer, only pENTR-CMVmOrangeA and pENTR-CMVcitrineA were chosen for further cloning.

To confirm functionality of the fluorescent marker, 293 cells were transfected with pENTR-CMVmOrangeA and pENTR-CMVcitrineA plasmids. All transfections resulted in strong fluorescence after 48 h and visually estimated transfection efficiencies were between 40 and 50% (Figures 24 and 25).

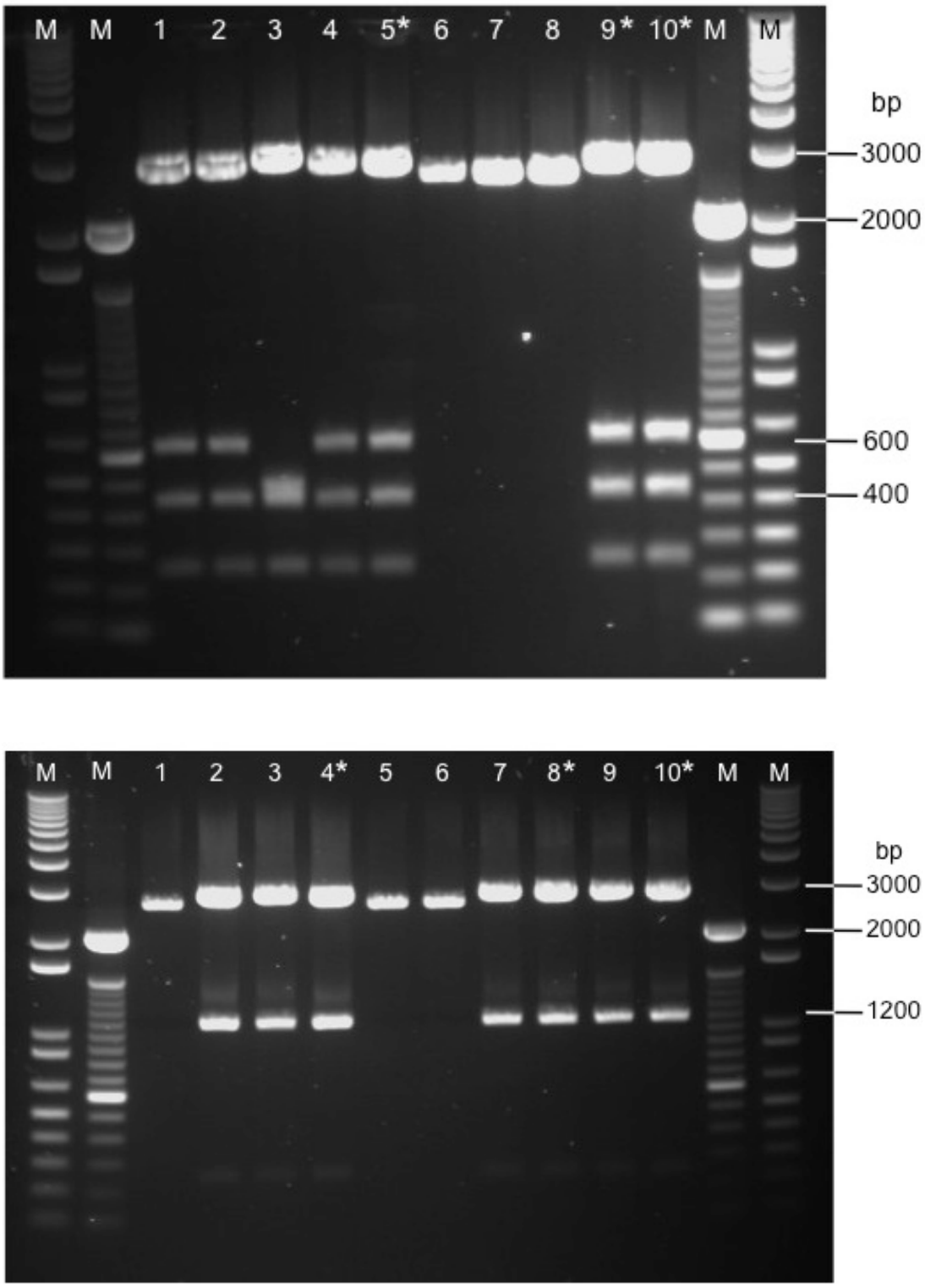


Figure 23: NcoI digest of pENTR-CMVmOrangeA and pENTR-CMVcitrineA

NcoI digest of pENTR-CMVmOrangeA (top) and pENTR-CMVcitrineA (bottom). Clones indicated by asterisk (*) were sequenced.

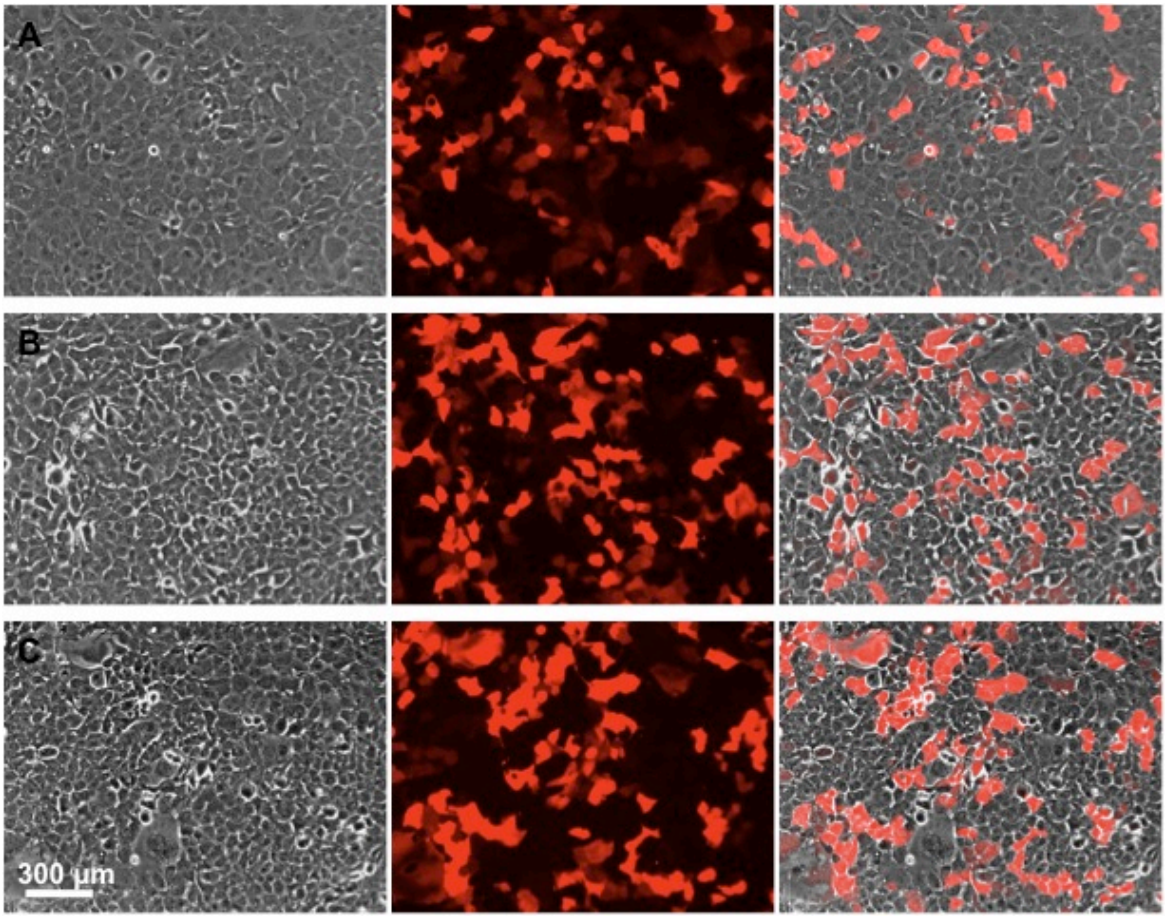


Figure 24: Transfection of 293 cells with pENTR-CMVmOrangeA

Transfection of 293 cells with pENTR-CMVmOrangeA clone 5 (A), clone 9 (B) and clone 10 (C) at 48 h post transfection.

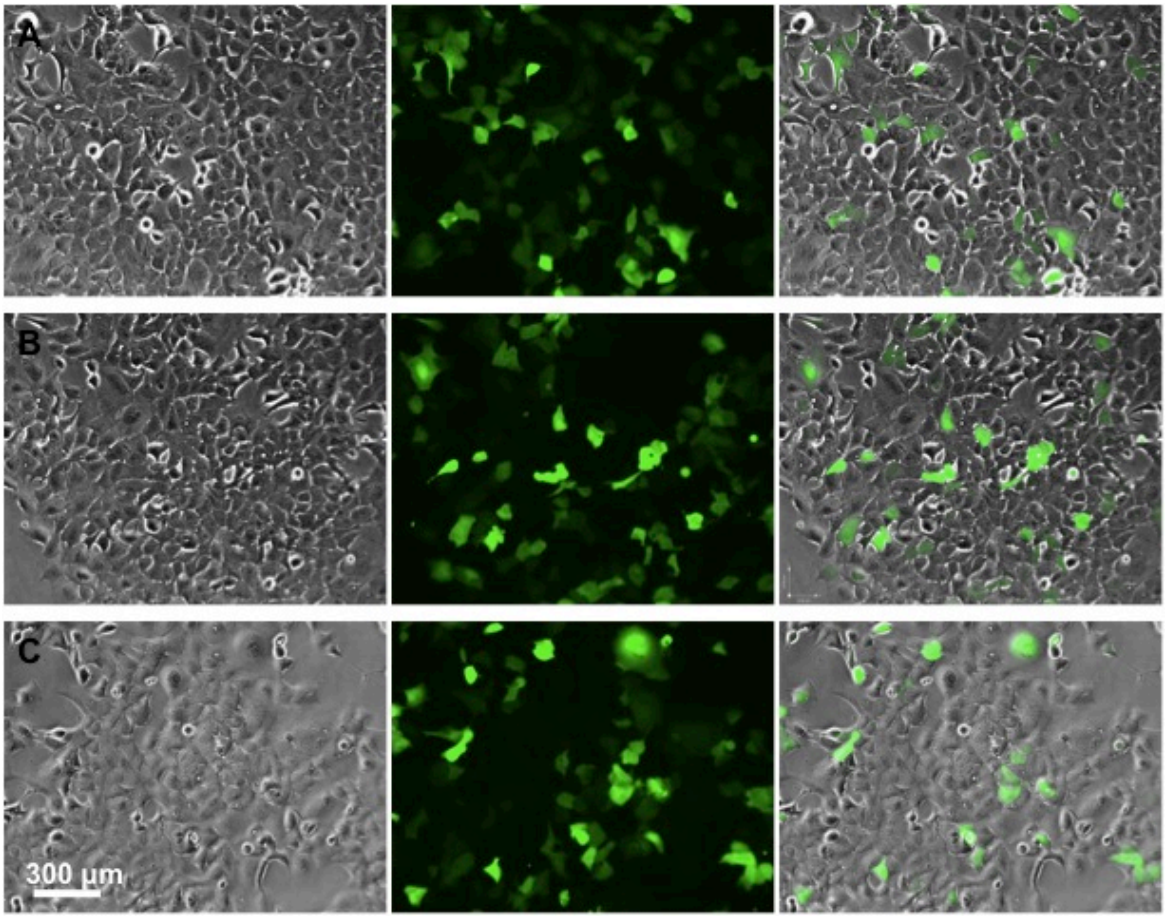


Figure 25: Transfection of 293 cells with pENTR-CMVcitrineA

Transfection of 293 cells with pENTR-CMVcitrineA clone 4 (A), clone 8 (B) and clone 10 (C) at 48 h post transfection.

4.1.3 Sequencing analysis of promoter and fluorescent reporter construct

Sequences were analysed and aligned using Vector NTI software (Invitrogen). As differentiation between the fragments after NcoI digest was not possible, sequencing confirmed that pENTR-CMVmOrangeA clones 5, 9 and 10 contained the insert in reverse orientation (Figures 26 and 27). The sequence of the CMV promoter was also confirmed as correct (Figure 26). Sequencing also confirmed the result of the NcoI digest of pENTR-CMVcitrineA clones 4, 8 and 10. These clones contained the insert in reverse orientation (Figure 28).

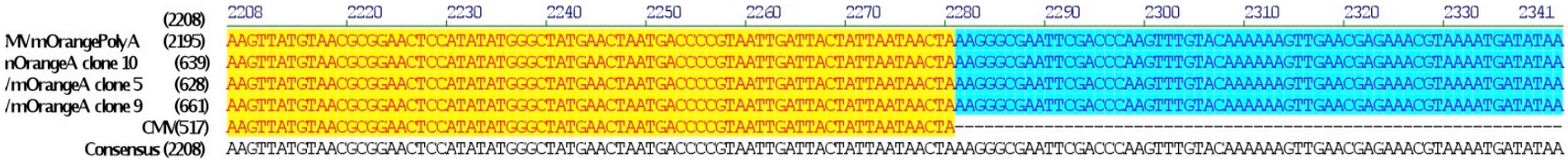


Figure 26: Sequence alignment of pENTR-CMVmOrangeA clones

Sequence alignment of pENTR-CMVmOrangeA clones 5, 9 and 10 using primer M13rev-26. Partial CMV promoter sequence shown in yellow and plasmid backbone in blue.

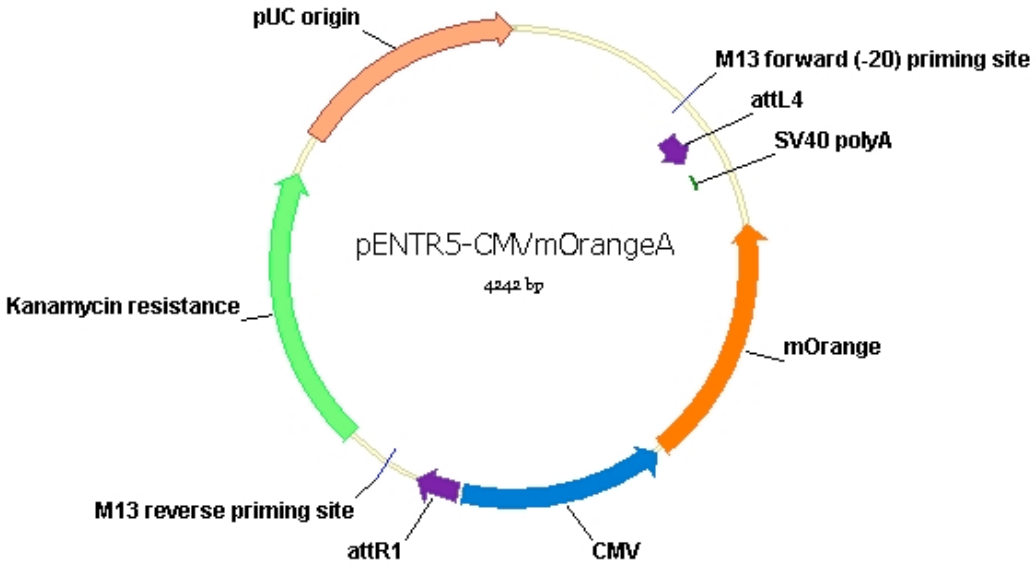


Figure 27: Confirmed layout of pENTR5-CMVmOrangeA after sequencing analysis

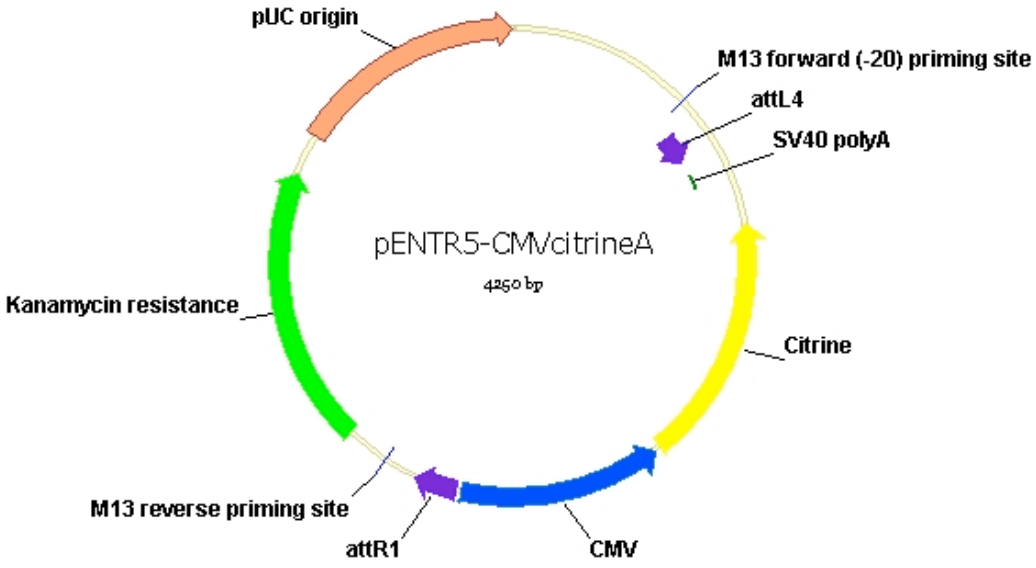


Figure 28: Confirmed layout of pENTR5-CMVcitrineA after sequencing analysis

4.1.4 Cloning of shRNAs for PSCA, β-actin and luciferase into pENTR U6

The U6 promoter is an RNA polymerase III type 3 promoter, which is responsible for the transcription of small nuclear RNAs. It is one of the promoters commonly used to express shRNAs (Bannister et al., 2007) and it is present in a number of commercially available vectors.
The aim of this part of the project was to develop a model system for lentiviral shRNA transfer into prostate cancer cells before later modifying the system to use in cultured prostate primary cells and cancer stem cells. The chosen gene for downregulation was PSCA (Reiter et al., 1998), which has been found to be upregulated in a large proportion of prostate cancers (Zhigang and Wenlv, 2004). According to a recent microarray study, PSCA is over-expressed in the malignant transit-amplifying population of prostate samples (Birnie et al., 2008), and therefore provided a suitable model target for downregulation in a prostate cancer cell line.

Single stranded shRNA oligos for PSCA, β-actin (as a positive control) and luciferase (as a negative control), were annealed and the double stranded shRNAs were then cloned into pENTR/U6 (Figure 29).
Even though plasmids were initially digested using MluI, the short sequence of the shRNA insert (66 bp or 67 bp), made it impossible to distinguish between clones with successfully integrated shRNA (fragment size 1988/1989 bp and 932 bp) and empty backbones (fragment size 1922 bp and 932 bp) (Figure 30). Using BanII for restriction analysis (fragment size of backbone 2100 bp, with insert 830 bp, without 760 bp) did also not improve resolution to a high enough level to sort negative from positive clones (Figure 31). BanII digests were also examined on VisiGel (Stratagene) in an attempt to enhance visibility (Figure 31). There was no suitable restriction site common to all shRNA sequences, which could have been used. This made it necessary to have a reliable method for sequencing shRNA clones to confirm correctness of inserts.

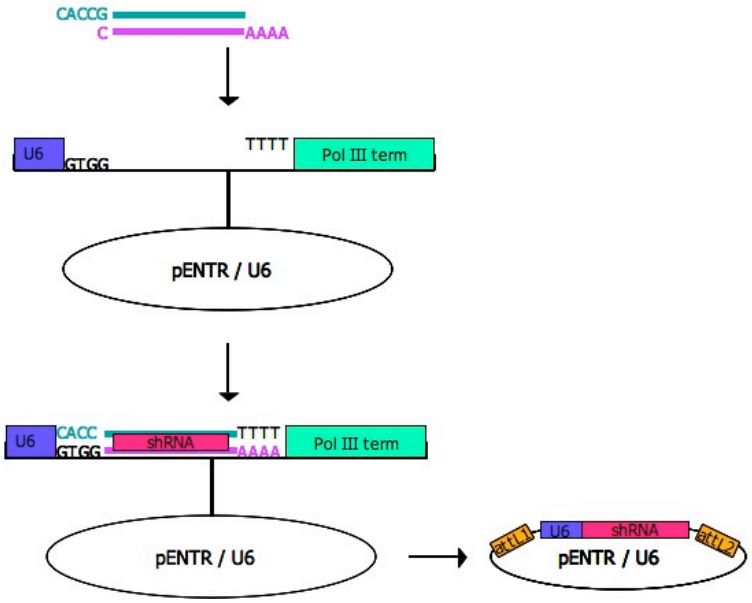


Figure 29: Construction of pENTR/U6

Construction of pENTR/U6 plasmids using the Invitrogen BLOCK-iT™ U6 RNAi Entry Vector Kit

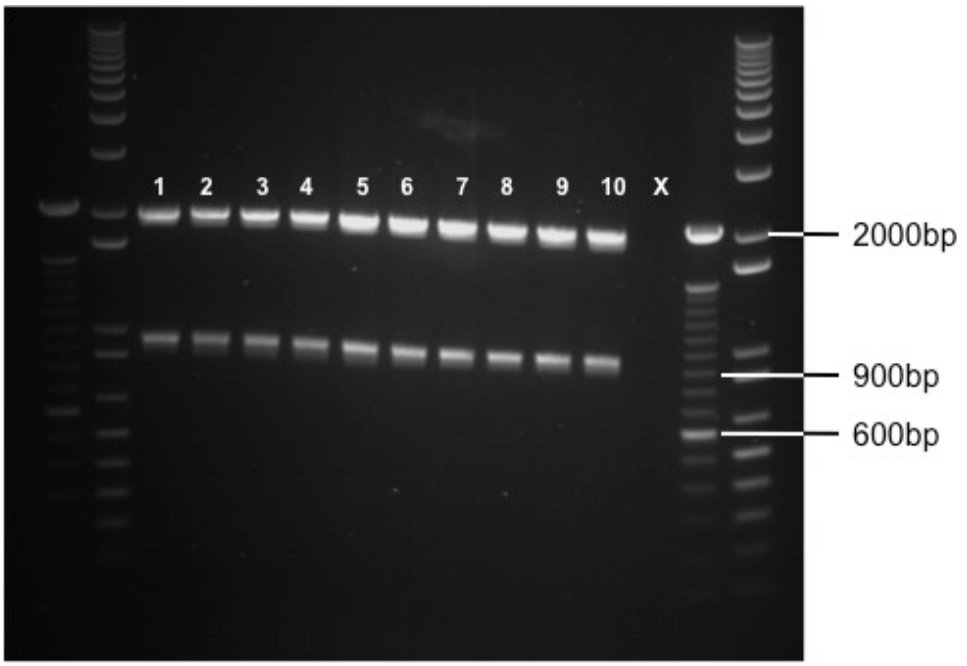


Figure 30: MluI restriction digest of pENTR/U6

MluI restriction digest of pENTR/U6 shRNA clones (1-10; negative control X) with MluI.

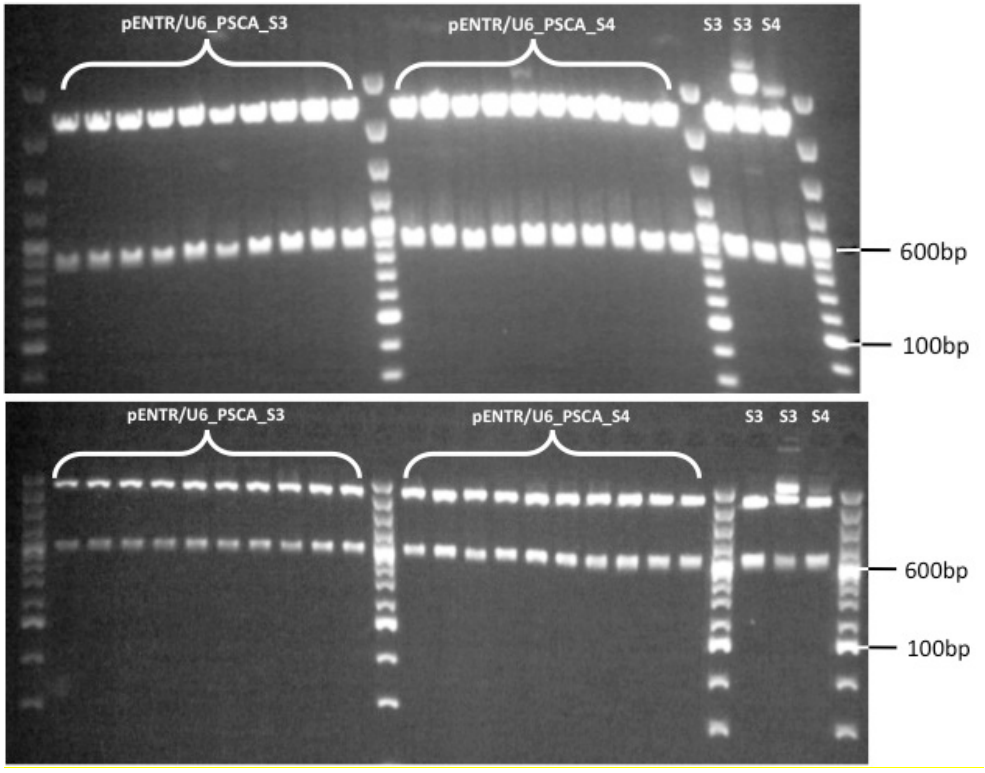


Figure 31: BanII restriction digest of pENTR/U6

Restriction digest of pENTR/U6 shRNA clones with BanII on agarose gel (top) and Visigel (bottom).

Sequencing was optimised to confirm correctly inserted shRNA cassettes, as the hairpins within the constructs caused problems during standard sequencing procedures. This is a common phenomenon when sequencing hairpin structures and has been reported previously (Devroe and Silver, 2002, Guo et al., 2005, McIntyre and Fanning, 2006, Miyagishi et al., 2004, Yu et al., 2003). Different strategies have been discussed to overcome this shortfall (Taxman et al., 2006).

Initial sequencing of the pENTR/U6 clones with primer M13R resulted in short read outs around 150 bp with a break off after a few bases into the shRNA sequence. The only exception was clone 24_9 (with shRNA PSCA_S2) with a read out of around 700 bp. However, in this clone there was a 3 bp deletion within the shRNA. The primer M13F also gave a low read out of about 400 bp. The promoter was confirmed in all clones without detectable mutations but the read out stopped a few base pairs into the shRNA sequence, similar to the results from M13R (Figure 32)

After the initial problems, successful sequencing across the hairpin using Silver sequencing service with special conditions (SAM plus, Cogenics), confirmed correct sequences for pENTR/U6_PSCA_S2, pENTR/U6_PSCA_S3, pENTR/U6_PSCA_S4, as well as the positive control pENTR/U6_PSCA_sh-β-actin and the negative control pENTR/U6_PSCA_sh-luciferase. Sequences were analysed and aligned using Vector NTI software (Invitrogen). Even though the read out still dropped slightly at the beginning of the hairpin structure in all constructs, sequencing was of good enough quality for analysis (Figure 33).

Analysis of the sequences confirmed positive clones for all pENTR/U6 constructs (Figure 34 A, B, D-F) except the clones containing PSCA_S1 shRNA. None of the 10 sequenced pENTR/U6_PSCA_S1 clones was positive and they all showed the same pattern of sequence disruption (Figure 34 C).

It was decided that, for lentivirus production, pENTR/U6_PSCA_S3 clone 2 and pENTR/U6_PSCA_S4 clone 10 would be used initially.

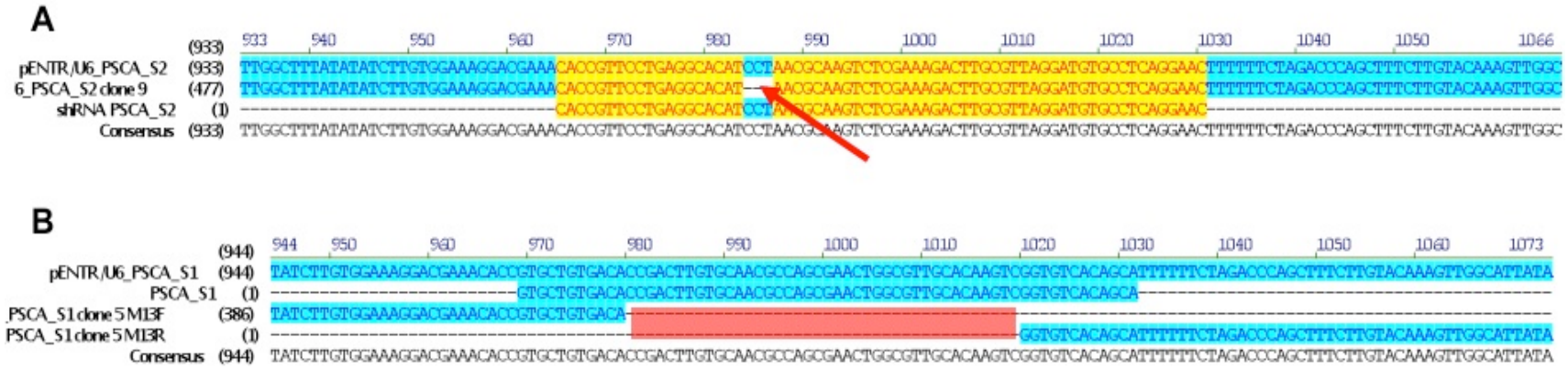


Figure 32: Sequence alignment of pENTR/U6 clones

A. Alignment result of pENTR/U6_PSCA_S2 clone 9 (bottom line) with its hypothetical correct sequence (complete plasmid: top line, actual clone sequence with 3 bp deletion (red arrow): 2nd line from top, shRNA insert only: 3rd line from top).

B. pENTR/U6_PSCA_S1 clone 5 with break off of the read out a few bases into the shRNA sequence, illustrated by red rectangle (complete plasmid: top line, shRNA insert only: 2nd line from top, M13F forward read out: 3rd line from bottom; M13R reverse complement read out: 2nd line from bottom).

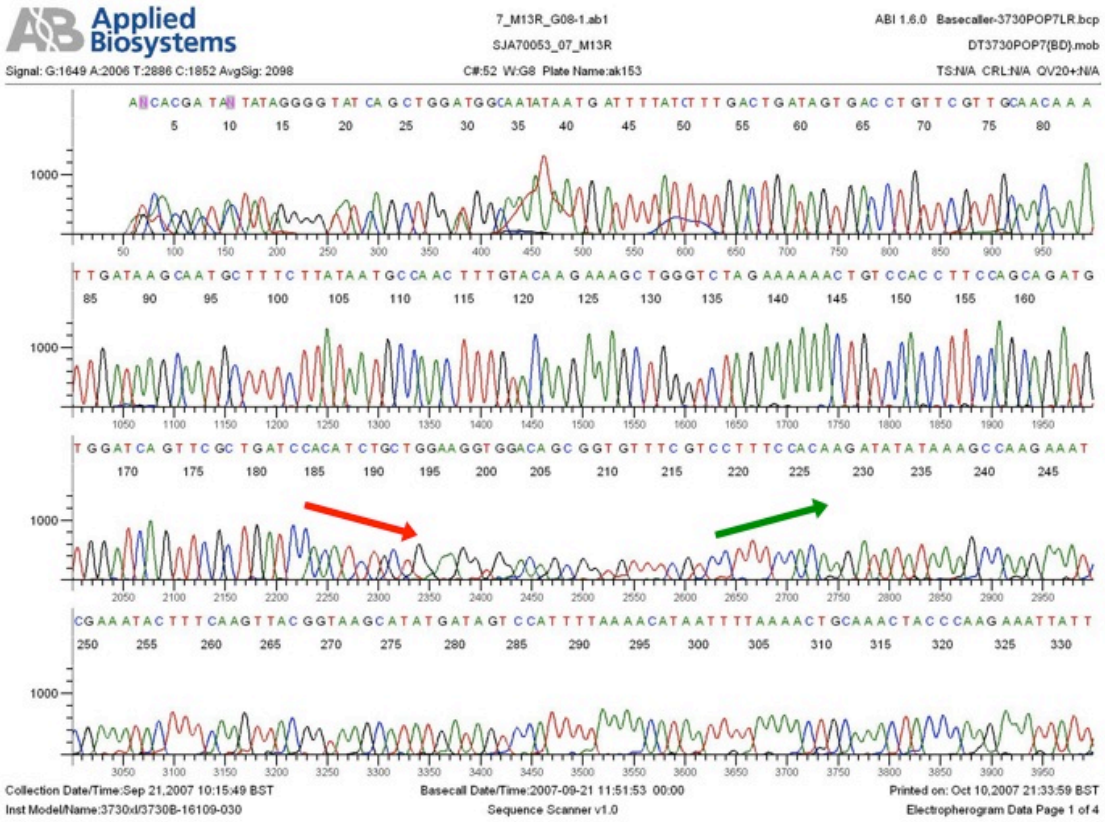
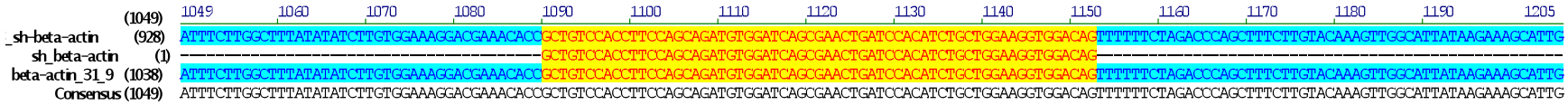


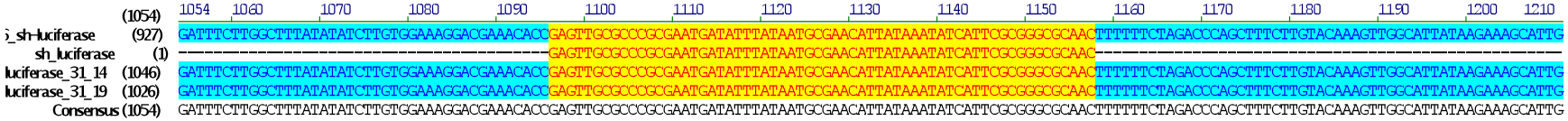
Figure 33: Example of sequencing spectrum

Example of read-out with drop after the start of the shRNA (red arrow). The read-out improves later (green arrow). The sequence shown is from pENTR/U6_PSCA_S3 clone 7.

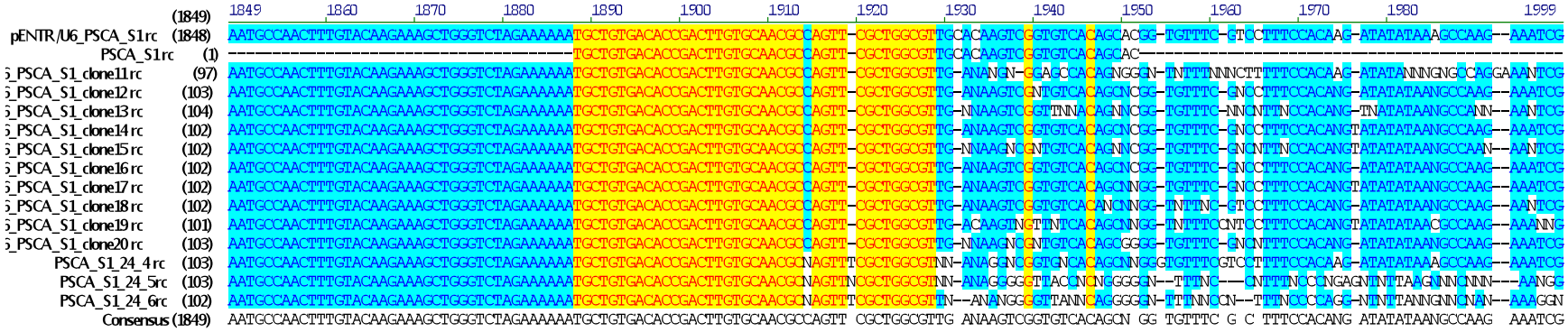
(A) pENTR/U6_sh-β-actin



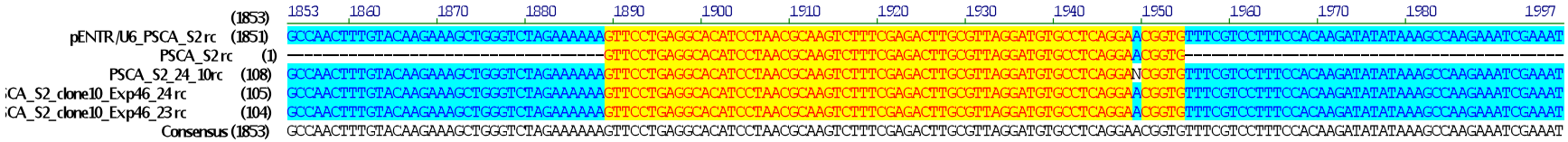
(B) pENTR/U6_sh-luciferase



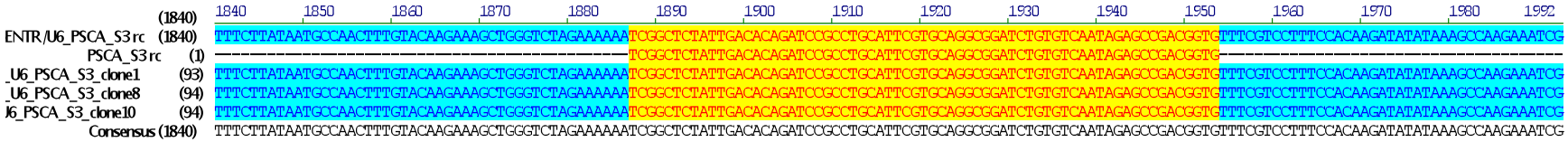
(C) pENTR/U6_PSCA_S1



(D) pENTR/U6_PSCA_S2



(E) s pENTR/U6_PSCA_S3



(F) pENTR/U6_PSCA_S4

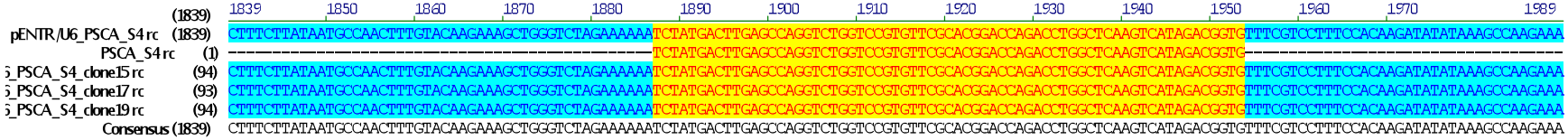


Figure 34: Sequence alignments of pENTR/U6 clones containing shRNAs

The location of the shRNA sequences is indicated in yellow, the hypothetical correct sequence is listed first, with the shRNA sequence alone underneath, followed by the different clones and the consensus sequence on the bottom line.

4.1.5 Generation of pLenti6 expression plasmids

The MultiSite Gateway LR recombination reaction using the LR Clonase II Plus Enzyme Mix occurs between the att-sites of the different entry vectors and the destination vector. It results in an expression vector carrying both fluorescent gene and shRNA with their respective promoters (Figure 35). Expression plasmids were constructed using site specific recombination (Hartley et al., 2000) between pENTR-CMVmOrangeA, pENTR/U6 entry clones containing an shRNA and the destination vector pLenti6/R4R2/V5-DEST (referred to as pLenti6 below) (Figure 35).

The resulting pLenti6 expression constructs were confirmed by restriction digest with EcoRI and NcoI (Figures 36, 38 and 39).

To confirm the functionality of the fluorescent reporter gene in the context of the lentiviral construct, pLenti6 expression clones were transfected into PC-3 and P4E6 cells. High levels of mOrange expression were detected after 72h for pLenti6mOrangePSCA_S3 and pLenti6mOrangePSCA_S4 as well as the positive and negative controls, pLenti6mOrangeβ-actin and pLenti6mOrangeLuc, respectively (Figure 37).

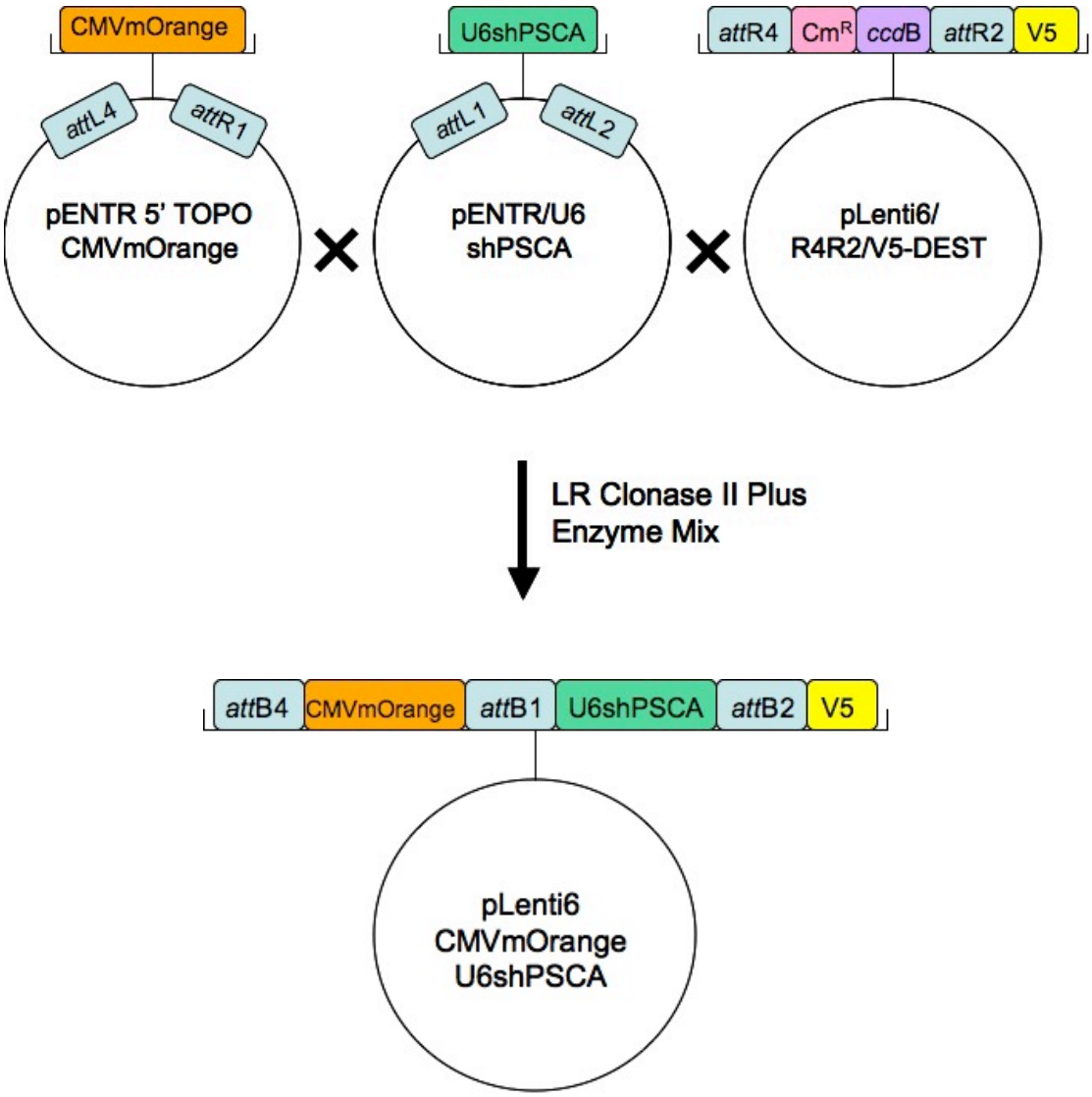


Figure 35: Recombination reaction to generate pLenti6 plasmids

pENTR 5' TOPO containing the fluorescent gene mOrange under the control of the constitutive promoter CMV and pENTR/U6 containing an shRNA under the control of the U6 promoter were combined with the pLenti6 destination vector.

Plasmid clones	Virus names
pLenti6mOrangePSCA_S3 clone 2	Lenti6mOrangePSCA_S3
pLenti6mOrangePSCA_S4 clone 10	Lenti6mOrangePSCA_S4
pLenti6mOrangeLuc clone 1	Lenti6mOrangeLuc
pLenti6mOrangeβ-actin clone 1	Lenti6mOrangeβ-actin

Table 11: Plasmid clones chosen for virus production with corresponding virus names

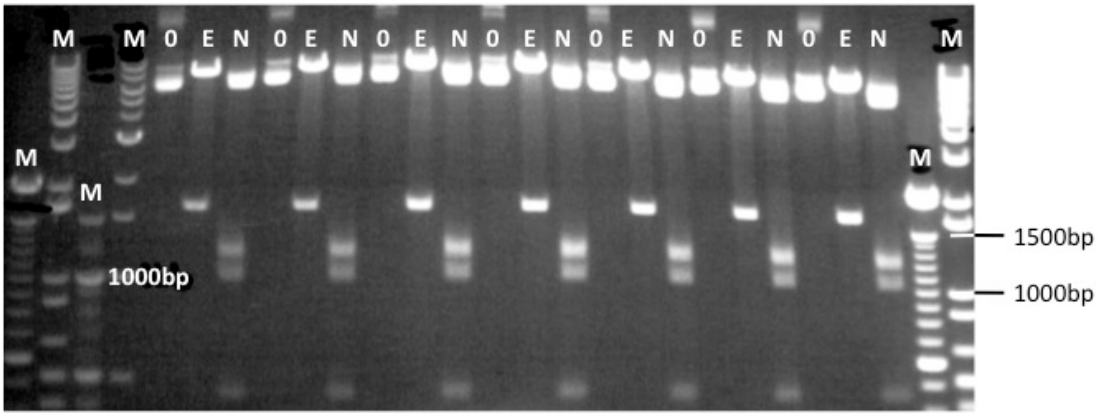


Figure 36: EcoRI and NcoI digests of pLenti6mOrangePSCA

Digest of pLenti6mOrangePSCA_S3 clones with EcoRI (E) and NcoI (N). Undigested (0). CMVmOrangePSCA_S3 was cut out using EcoRI (approx. 1580bp).

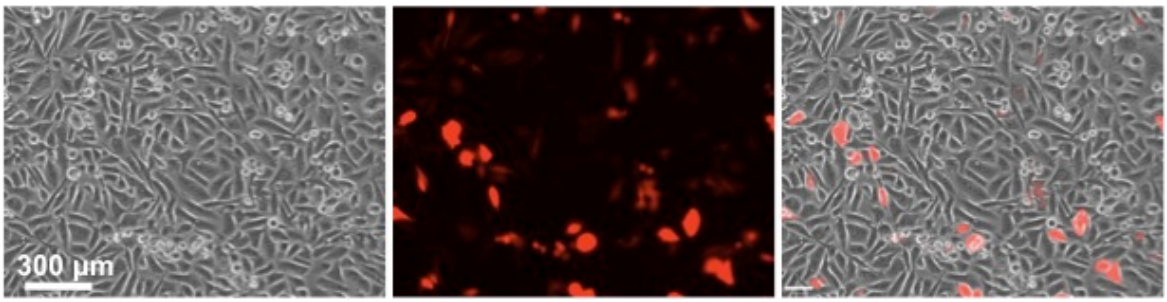


Figure 37: Transfection of PC-3 with pLenti6mOrangePSCA

Representative example of PC-3 cells transfected with pLenti6mOrangePSCA_S4 at 72 h post transfection.

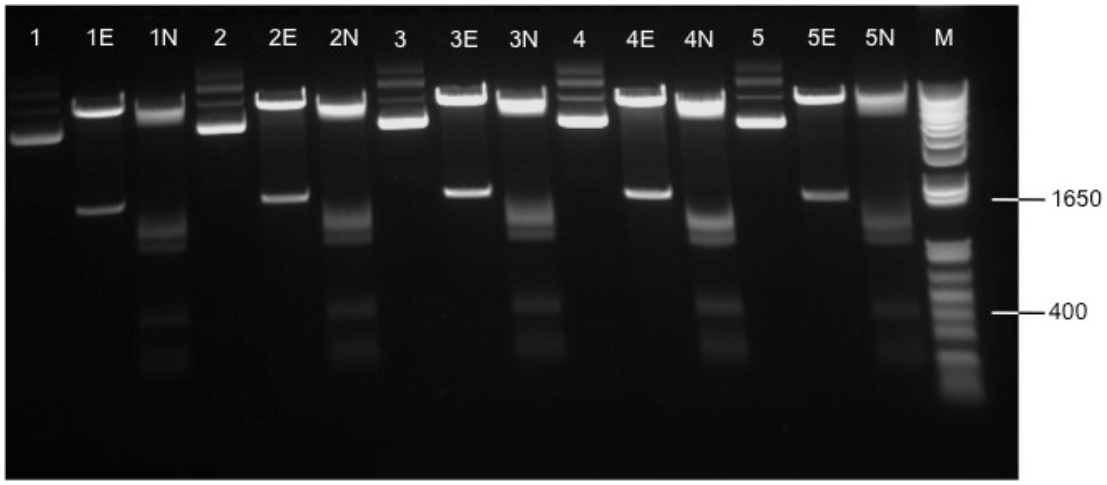


Figure 38: EcoRI and NcoI digests of pLenti6mOrangeβ-actin

Digest of pLenti6mOrangeβ-actin clones 1-5 with EcoRI (E) and NcoI (N). CMVmOrangeβ-actinA was cut out using EcoRI (approx. 1580bp).

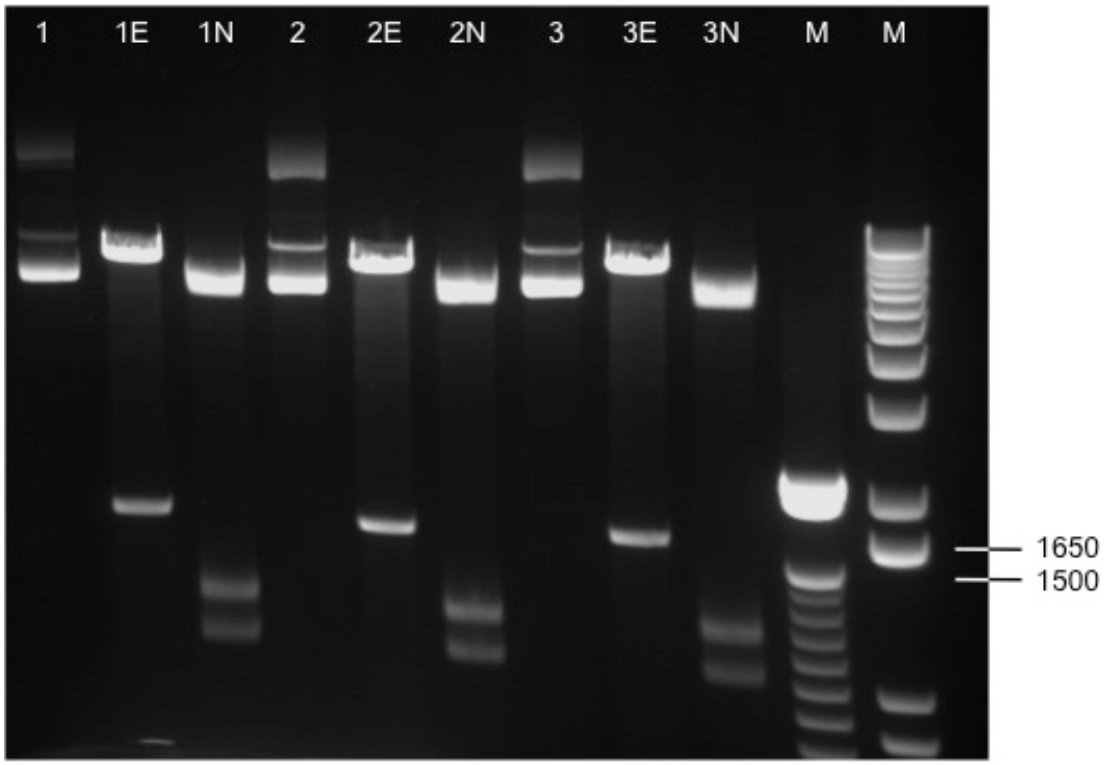


Figure 39: EcoRI and NcoI digests of pLenti6mOrangeLuc

Digest of pLenti6mOrangeLuc clones 1-3 with EcoRI (E) and NcoI (N). CMVmOrangeLucA was cut out using EcoRI (approx. 1580bp).

4.1.6 Production and titering of recombinant lentiviruses

A cotransfection of the pLenti6 expression constructs and the ViraPower Packaging Mix into 293FT producer cells was performed to generate functional, VSV-G pseudotyped lentiviruses, which has been described previously (Hager et al., 2008).

In general, viral particle numbers, obtained from titration in HT1080 cells, were very low, ranging from 0.9×10^2 to 5.4×10^2. For the initial titration of Lenti6mOrangePSCA_S3, Lenti6mOrangePSCA_S4, Lenti6mOrangeLuc and Lenti6mOrangeβ-actin, virus-containing supernatant was harvested at 72 h, according to manufacturer's recommendations. In order to improve viral yield, virus production was repeated with Lenti6mOrangePSCA_S3 harvested at 48 h, 72 h and 96 h, respectively (Figure 40).

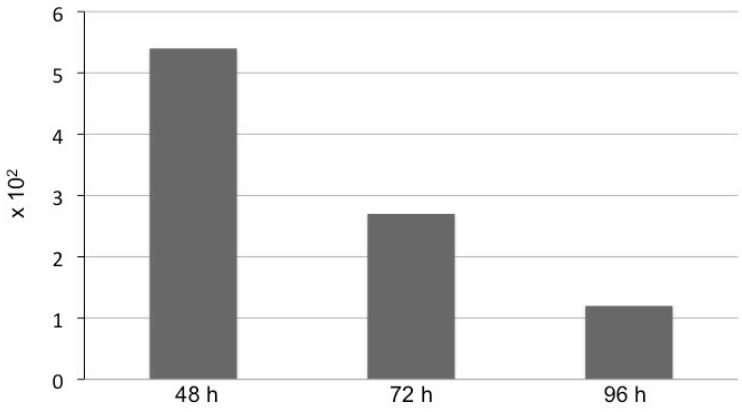
Titres obtained at 48 h, were higher than at 72 h and 96 h. However, the maximum titre achieved after optimisation was still only 5.4×10^2. According to the manufacturer, titers between 5×10^5 to 2×10^7 should be obtained[12]. Repeated communication with Invitrogen did not result in additional information to improve results.

Since work by Stefanie Hager and Fiona Frame had also shown the limitations of the Invitrogen lentivirus production system, especially for primary prostate cancer cells (Hager et al., 2008), this work was not continued.

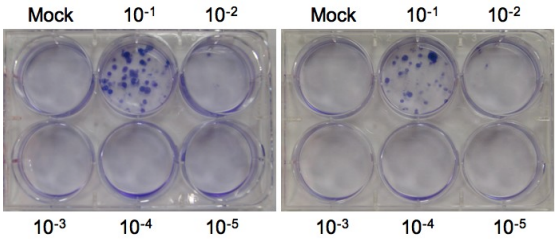
Instead, it was decided to use custom made shRNA-lentiviral particles, which had become commercially available (Sigma) and focus on over-expressed genes from the prostate cancer stem cell population according to microarray data provided by Birnie and colleagues (Birnie et al., 2008).

[12] Invitrogen ViraPower™ Promoterless Lentiviral Gateway® Kits manual, Version C, 23 August 2007, for catalog nos. K591-10 and K5910-00

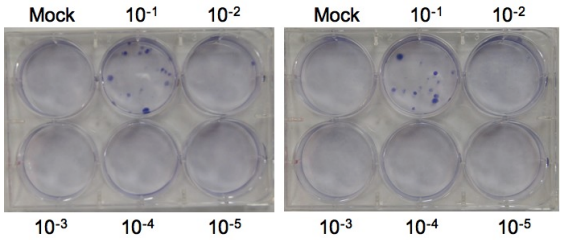
(A)



(B)



(C)



(D)

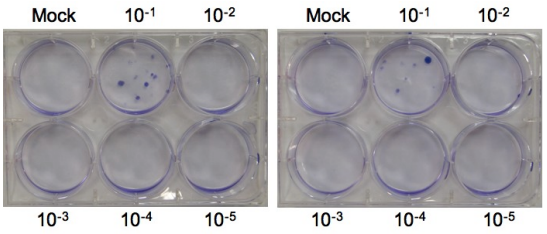


Figure 40: Titer of Lenti6mOrangePSCA viruses

Titer of Lenti6mOrangePSCA_S3 virus, harvested at different time points. Virus was harvested from 293FT producer cells at 48 h (B), 72 h (C) and 96 h (D). (A) Titres obtained: 5.4 x 10^2 (48 h), 2.7 x 10^2 (72 h) and 1.2 x 10^2 (96 h).

4.2 β-catenin studies in prostate cancer cell lines

One of the signalling pathways, that has long been associated with cancer formation due to its important role in various stages of embryonic development, is the Wnt signalling pathway. It was named after its upstream ligands (Wnts). The key molecule of this pathway, β-catenin, has been associated with a variety of different cancers, most prominently, colon cancer (Giles et al., 2003b). Results from a recent microarray analysis, which compared gene expression in the committed basal and stem cell populations of prostate patient samples, found an overrepresentation of genes involved in Wnt signalling (Birnie et al., 2008). Therefore, we wished to investigate the role of β-catenin in prostate tumour initiation, growth and dissemination.

We used lentiviral delivered shRNAs to knock down β-catenin expression in a prostate cancer cell line (PC-3). Following the generation of stable cell lines, the effects of reduced β-catenin expression on the biological properties of tumour cells were studied.

4.2.1 Generation of PC-3 cell lines with stable knockdown of β-catenin (PC-3v)

To determine the long-term effects of a constitutively expressed shRNA targeting β-catenin, PC-3 cells were infected with shRNA-containing lentiviruses. As lentiviruses integrate into their host cell genome, the transported shRNA is stably expressed in the cell line. After having technical difficulties with producing suitable high titre shRNA-lentiviruses ourselves, we purchased commercially available lentiviral particles (Sigma), as these offered a time- and cost-effective tool for our study.

4.2.1.1 Puromycin selection of prostate cell lines

Before stable cell lines were generated, a kill curve experiment was performed to determine the minimum concentration of puromycin that caused complete cell death after 3-5 days. This concentration was then used to select lentivirus-infected cells. Prostate cancer cell lines PC-3 and LNCaP as well as the normal prostate cell line PNT2-C2 were used for puromycin titration.

PC-3 cells were sensitive to puromycin at 2 μg/ml and showed extensive cell death at day 5. By day 8 there were no viable cells left (Figure 41). Both LNCaP and PNT2-C2 cell lines were more sensitive to puromycin than PC-3 cells, and extensive cell death was

observed with 0.5 μg/ml at day 5 (Figure 42). Cells were cultured in selection medium for 14 days. Even though some PC-3 cells remained at the optimal concentration of 2 μg/ml, these cells did not start growing when selection medium treatment was terminated and cells were placed in normal growth medium.

Since PC-3 cells presented as the most robust cell line, whilst still being susceptible to puromycin, they were chosen for lentivirus infection.

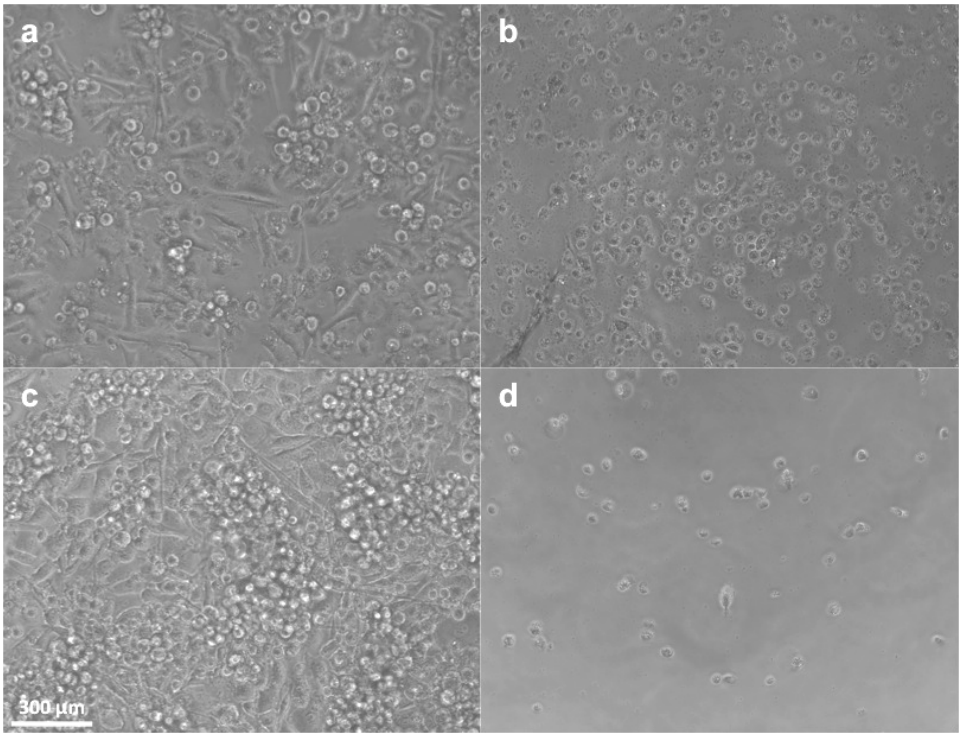


Figure 41: Puromycin susceptibility in PC-3 cells

PC-3 cells at day 5 (a, b) and day 8 (c, d) of puromycin selection. The medium contained 0.5 µg/ml (a, c) or 2 µg/ml (b, d) puromycin, respectively.

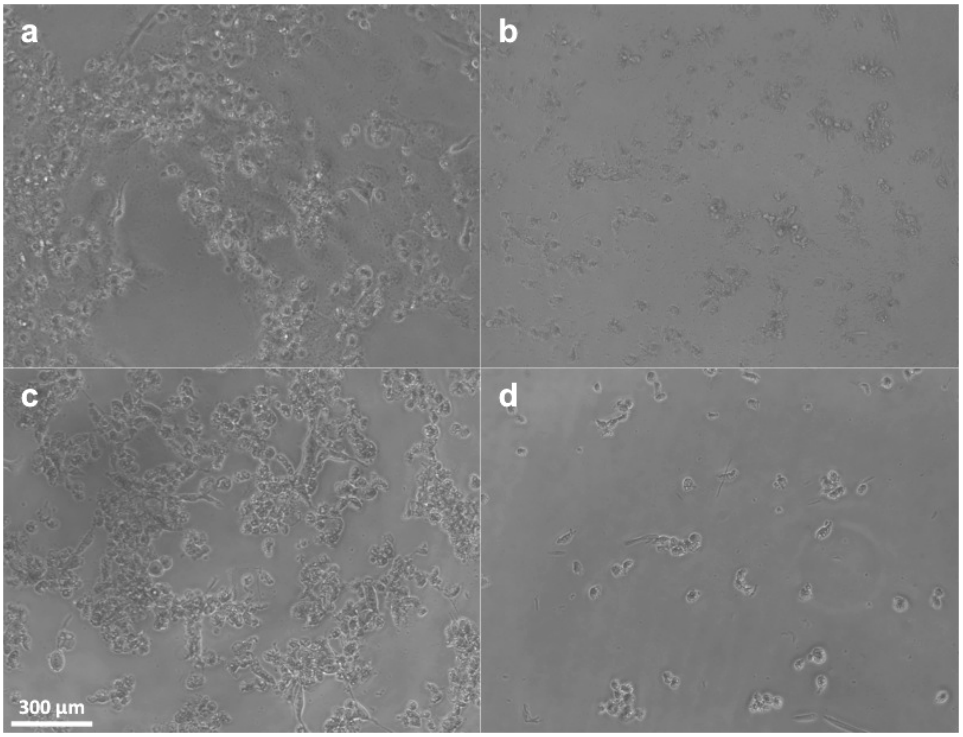


Figure 42: Puromycin susceptibility in PNT2-C2 and LNCaP cells

PNT2-C2 (a, b) and LNCaP (c, d) at day 5 with 0.5 µg/ml (a, c) or 2 µg/ml (b, d) puromycin.

4.2.2 Infection of PC-3 prostate cancer cells and selection of stable clones

PC-3 cells were infected with four lentiviruses (Sigma, Clone IDs TRCN0000003843, TRCN0000003844, TRCN0000003845 and TRCN0000003846; subsequently referred to as virus 43, 44, 45 and 46, respectively). All four lentiviruses contained a different shRNA targeting β-catenin. PC-3 cells were infected at MOI = 10 in the presence of media alone or media supplemented with polybrene at 8 μg/ml. Following an initial medium change at approximately 15 h post infection, the selection was started 24 h post infection. 2 μg/ml puromycin was used for selection, as this was the lowest concentration, which effectively killed untransfected PC-3 cells (Figure 41). The selection medium was changed every 2-3 days and the first colonies were usually observed 6 days post infection. At 15 days post infection, cells were split from one 24-well into two 6-wells to culture with and without puromycin, in order to determine if puromycin itself had any adverse effect on the growth of positively selected cells. Puromycin did not have any obvious negative effects on cell morphology or cell growth (Figure 43) and selection was concluded to not interfere with the later experiments on β-catenin knockdown.

Stable PC-3 cell lines resulting from virus infection were named PC-3v or PC-3v43, PC-3v44, PC-3v45 and PC-3v46 respectively. Cells cultured in selection at the time of experiment, are indicated with a "+", e.g. PC-3v43+.

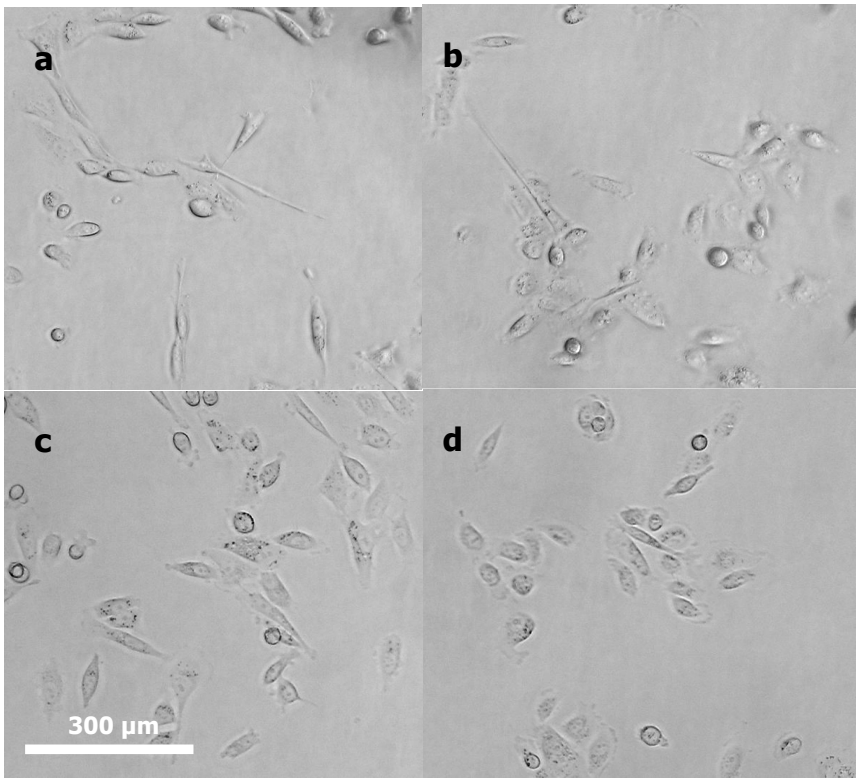


Figure 43: Puromycin has no effect on cell morphology

Positively selected PC-3v43 (a, b) and PC-3v45 (c, d) cells were cultured with (b, d) and without (a, c) puromycin.

4.2.3 Determination of β-catenin protein expression

Prostate cell lines PC-3, LNCaP, P4E6, PNT2-C2 and BPH-1 as well as the colon cancer cell line Caco-2 were used to determine endogenous β-catenin protein levels before knockdown experiments were initiated (Figure 44). In addition to the predominant β-catenin band at 94 kDa, some nonspecific binding at lower molecular weight was observed, probably due to overloading of the gel.

This was done to ensure that protein levels in the negative control (untreated cells) were at a detectable level for Western Blot. We postulated that β-catenin signalling may be responsible for giving prostate cancer cells stem cell like abilities, like enhanced proliferation and long-term self-renewal, which would enable them to be more successful during tumour establishment, invasion and metastasis formation.

There has already been some evidence that this is an important feature in other cancers, including colon (Le et al., 2008). Therefore, PC-3 cells, which were originally derived from a prostate cancer bone metastasis, represented a suitable model for our experiments.

β-catenin protein levels were compared in cell lines infected with 4 lentiviruses, all carrying a different shRNA targeting β-catenin (virus 43, 44, 45 and 46).

48 h after lentiviral infection of PC-3 cells, lysates were made to determine any changes in β-catenin protein. In PC-3 cells viruses 43, 44 and 45 showed a reduction of β-catenin protein level. Apart from the main band for full-size β-catenin at 94 kDa, there was also a fainter, smaller second band observed in all samples (Figure 45).

A puromycin selection was performed to generate a stable cell line, in order to analyse the long-term effects of β-catenin shRNAs in PC-3 cells. Infected cells were selected with puromycin for 8 - 9 passages post infection before cell lysates were produced. PC-3v45 cells consistently showed a reduction in β-catenin protein level (at 94 kDa) while the levels in all other infected cells were variable. Statistical analysis was performed to determine the level of β-catenin knockdown in PC-3v cells (Figures 46 and 47). Band intensity was measured using ImageJ software and averages as well as standard deviation were calculated. The reduction of β-catenin protein in PC-3v45+ was highly significant compared to the uninfected PC-3 control with $P < 0.01$ and a downregulation of > 50 %. PC-3v43 and PC-3v44 cells exhibited reduced levels of the protein, however, this was not statistically significant.

These experiments suggest that lentivirus 45 was most effective at reducing β-catenin protein levels in PC-3 prostate cancer cells. This virus was therefore the choice when infecting cultured primary cells from patient samples at a later stage.

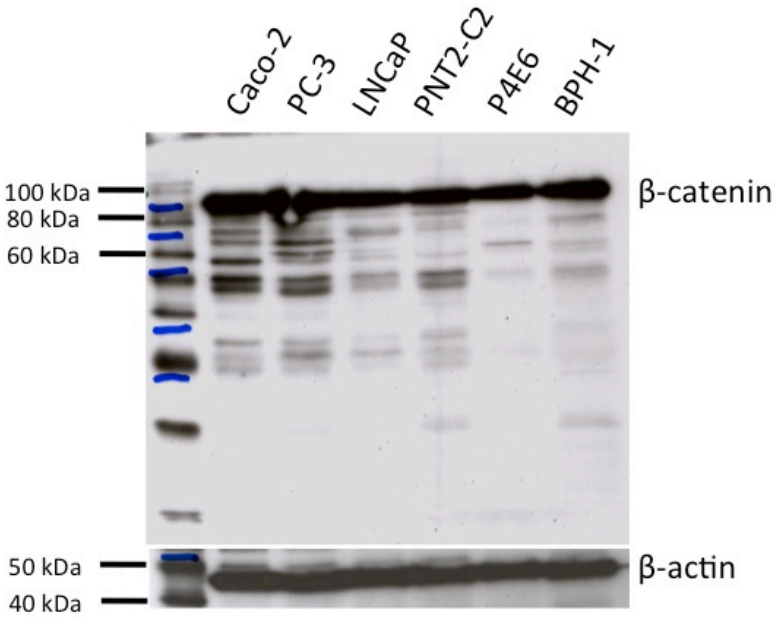


Figure 44: β-catenin expression in different cell lines

Identification of β-catenin protein (94 kDa) in whole protein lysates with antibody C2206 (Sigma). β-actin loading control at 42 kDa.

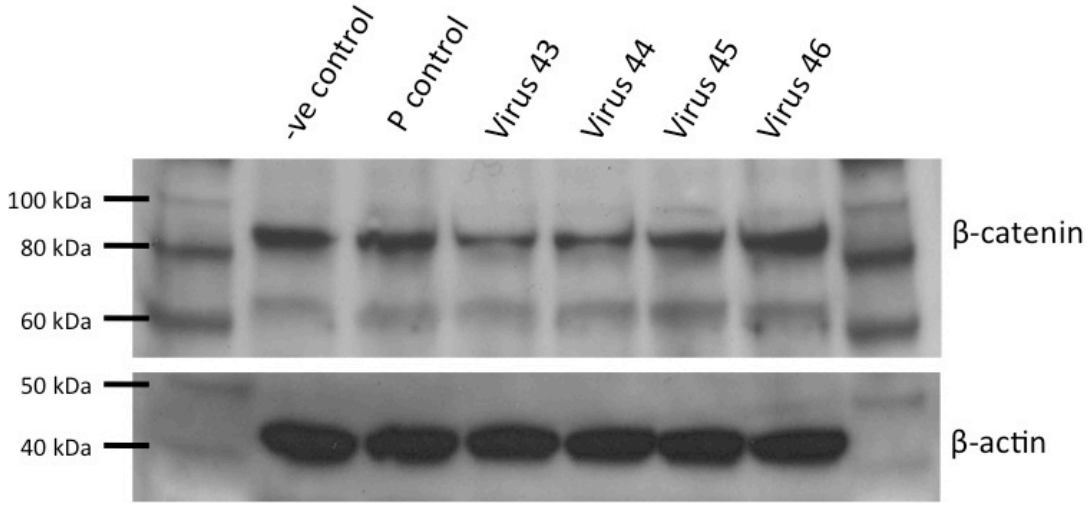


Figure 45: β-catenin knockdown in PC-3 cells 48 h post infection

Western Blot in PC-3 using antibody C2206 (Sigma) for the detection of total β-catenin (94 kDa). β-actin loading control at 42 kDa.

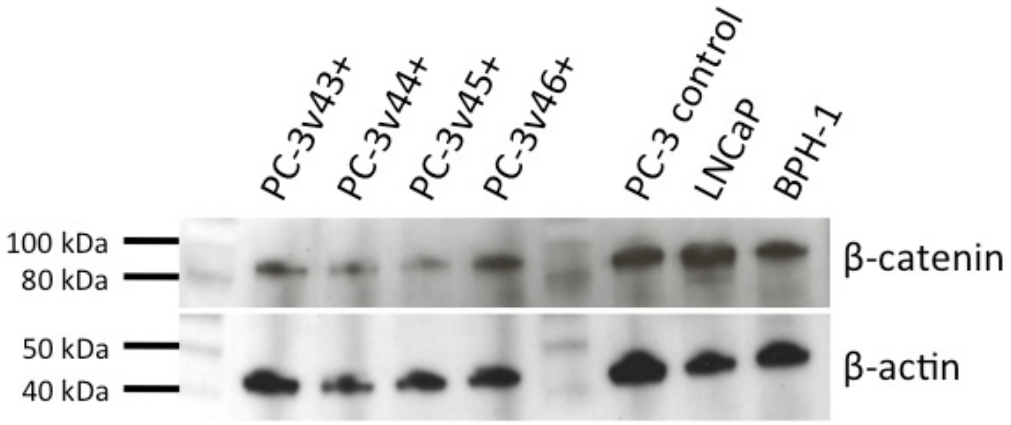


Figure 46: β-catenin expression in stable transfected PC-3v cells

Representative immunoblot with β-catenin (94 kDa) targeting antibody C2206 (Sigma) and β-actin loading control (42 kDa). Whole protein lysates of PC-3 cells infected with lentiviruses 43, 44, 45 and 46 and under selection at time of experiment (+). Cell line controls: (uninfected) PC-3, LNCaP and BPH-1, plus primary epithelial (PE) culture 07/021. For statistical analysis see figure 47.

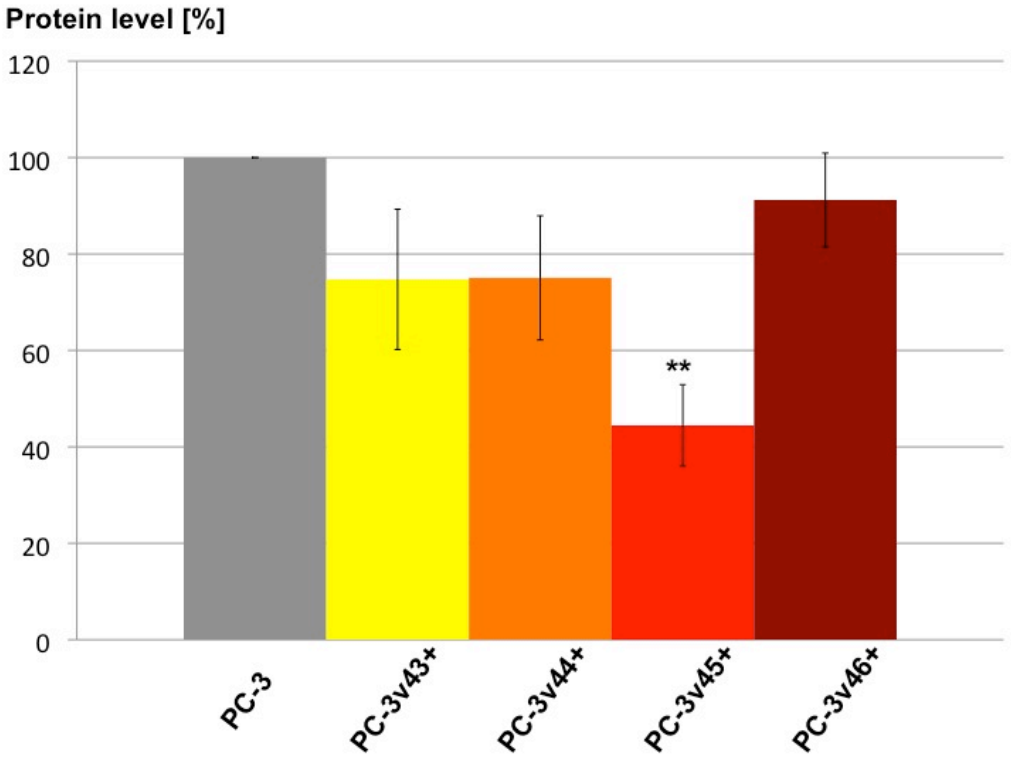


Figure 47: PC-3v45+ showed a significant reduction of β-catenin

Statistical analysis of a triplicate of Western Blot experiments (see Figure 46) to determine β-catenin protein levels in stable transfected cell lines. p-value of PC-3v45+ was p = 0.0079.

4.2.4 Lentiviral infections of P4E6 and LNCaP prostate cancer cells

In order to have a wider variety of stably transfected prostate cancer cell lines, two more cell lines were subjected to infection experiments. P4E6 cells, derived from primary prostate cancer cells after transfection of the HPV E6 gene (Maitland et al., 2001) and the androgen-sensitive LNCaP, originally derived from a lymph node metastasis, were both chosen for these experiments. These two cell lines represent different stages of tumour differentiation and would therefore give valuable insight into the importance of β-catenin at different times in tumour development. Furthermore, P4E6 cells contain a CD133+ subpopulation which has previously been established as a model for prostate cancer stem cell studies using lentiviruses (Hager et al., 2008).
In parallel, PC-3, P4E6 and LNCaP were infected at MOI=10 with shRNA-containing and control viruses. These control viruses included the TurboGFP® expression lentivirus (Sigma, MISSION Control Transduction Particle SHC003V; referred to as 3V below), a scrambled shRNA control (2V) and an empty vector without shRNA (1V).

First, whole cell lysates were taken 3 days post infection. Cells were also selected using 2 µg/ml puromycin. No stable cell lines were obtained from LNCaP cells.
Lysates of selected PC-3 and P4E6 were made at P. 3 or 4, between 19 and 23 days post infection, depending on growth. Protein knockdown was observed in PC-3 and P4E6 cells (Figure 48).
Cells infected with the shRNA - β-catenin virus 45 exhibited a downregulation of β-catenin, confirming the results of earlier experiments. Control viruses 1V (empty vector), 2V (scrambled shRNA) and 3V (GFP) showed minor reductions at P. 1 post infection, which was no longer detectable in the stable PC-3v cells at P. 16/17 post infection (Figure 48). Similarly, P4E6 cells infected with control viruses showed minor reductions. We observed a strong decrease of β-catenin protein in cells infected with shRNA - β-catenin virus 45 (Figure 48).
Using GFP expression from the 3V virus as a measure of successful infection, it could be shown that 100% of puromycin-selected P4E6 cells had taken up the viral genome (Figure 49) and had a strong expression of GFP.
To conclude, the approach used for infecting prostate cancer cell lines PC-3 and P4E6 was successful. We were able to obtain stable cell lines, incorporating lentivirally delivered

shRNAs, which were selected using puromycin. We could also show that downregulation of β-catenin at the protein level was stable over a long period of time and several passaging events.

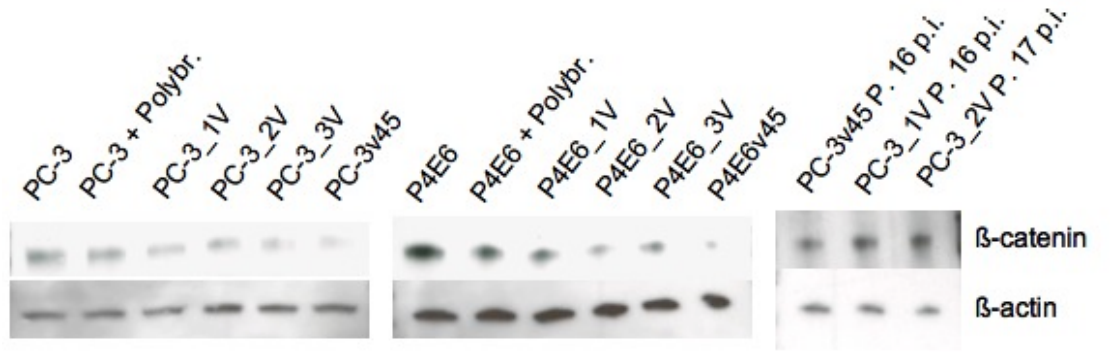


Figure 48: β-catenin protein detected by C2206 antibody

Western blot from PC-3v and P4E6v P.1 p.i. (left and middle, respectively) and stable PC-3v post selection (right).

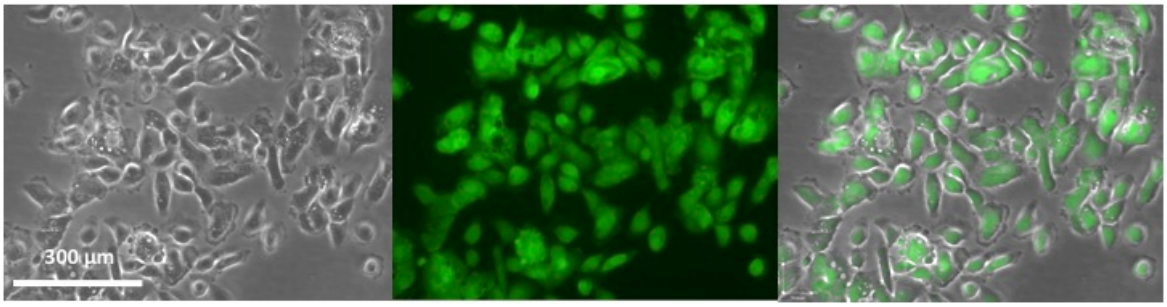


Figure 49: GFP expressing P4E6_3V cells

Cells at passage 3 post infection were under selection medium containing 2 µg/ml puromycin.

4.2.5 Analysis of β-catenin knockdown in stable transfected cells

4.2.5.1 Cell growth in PC-3v cell lines

In normal development and growth, the canonical Wnt signalling pathway plays an important role (Peifer and Polakis, 2000). It has been shown previously, that siRNA-mediated knockdown of β-catenin reduced the viability of hepatocellular carcinoma cells and compromised proliferation (Zeng et al., 2007). It has also been reported that hematopoietic stem cells from conditional β-catenin knockout mice showed reduced long-term growth (Zhao et al., 2007).

To determine if the reduction in β-catenin protein level had an effect on cell growth, PC-3, PC-3v43 and PC-3v45 were plated at 1000 cells per well into a 6-well plate. Cells of one well were counted every 24 h over a period of 6 days to establish a growth curve. Cells were not confluent up to and including day 6.

The experiment was repeated, using two plates per cell line and analysing the average of these replicates (Experiment 1 and 2, Figure 50).

Both transfected cell lines showed reduced growth. PC-3v45 showed the largest reduction in growth compared to PC-3v43 and non-infected PC-3. At day 6 both PC-3v45 and PC-3v43 reached a similar growth level which was about 30% lower than that of the PC-3 control, however, the reduction in PC-3v43 was not statistically significant (Experiment 1, Figure 50). PC-3 cells grew about 50% more than the lentivirus infected cells. The difference between PC-3 and PC-3v45 was significant with a p-value of $p \leq 0.05$ (Experiment 2, Figure 50).

Therefore, we can conclude, that reduced levels of β-catenin reduces growth of PC-3 cells.

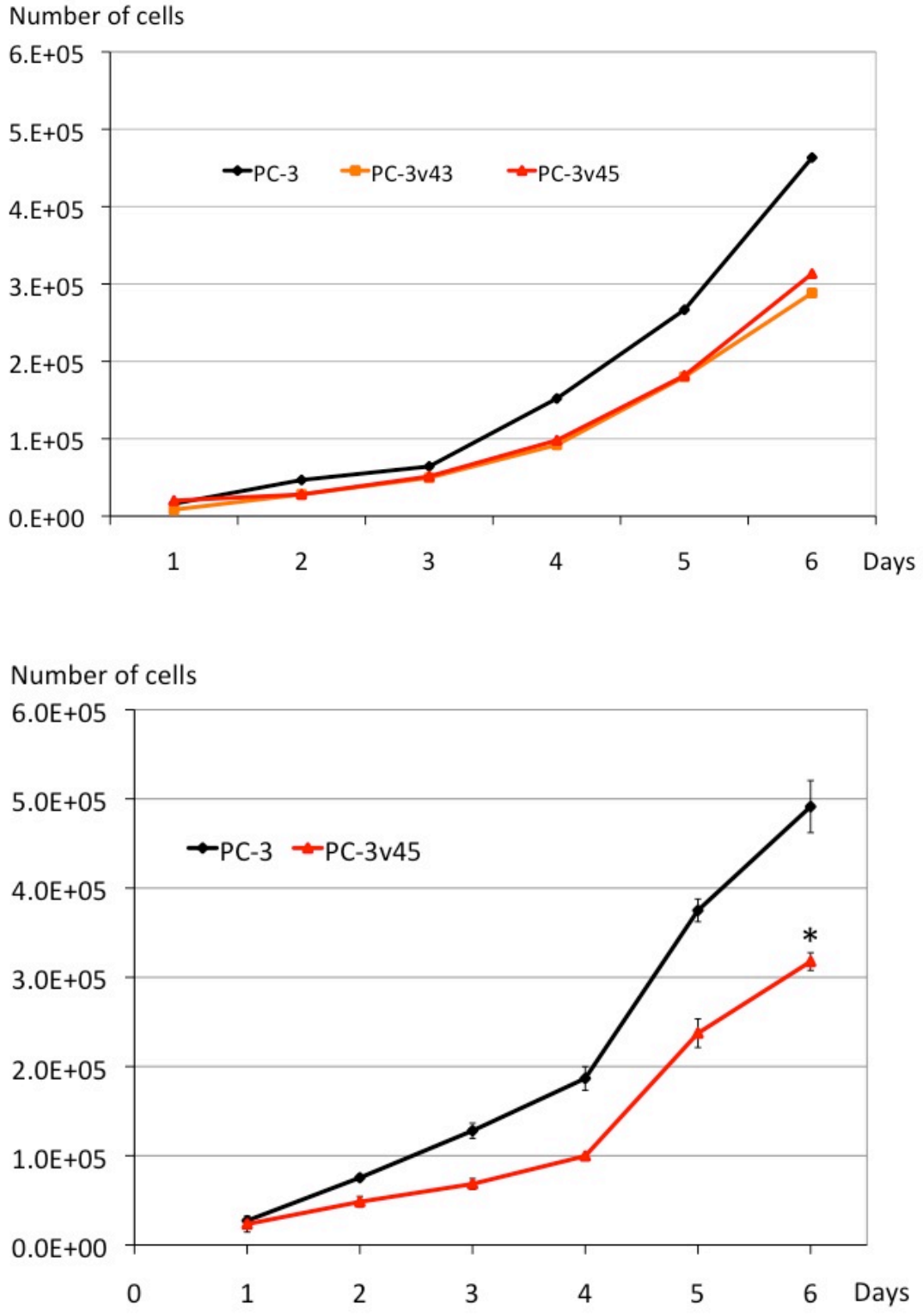


Figure 50: Growth analysis of PC-3v43 and PC-3v45

Experiment 1 (top) showed that lentiviral transfected PC-3v43 and PC-3v45 cell lines exhibited reduced growth. Experiment 2 (bottom) showed that growth reduction in PC-3v45 is reproducible and statistically significant compared to uninfected PC-3 cells.

4.2.5.2 Effect of β-catenin knockdown on the cell cycle of PC-3v cells

As a transcriptional regulator, β-catenin has long been shown to be responsible for the expression of cyclin D1. A mutated or abnormally regulated β-catenin could therefore lead to accumulation of cyclin D1 and a disruption of the normal cell cycle (Tetsu and McCormick, 1999).

Initial FACS analysis of PC-3v cells at passage 16 p. i., using propidium iodide staining, did not show any changes in the cell cycle. The experiment was repeated with cells at P. 1, 5 and 11 p. i. to confirm results. Cells were selected with puromycin for a minimum of 14 days but placed under non-selective medium conditions for a minimum of 1 day prior to analysis. Cells in each experiment were at similar levels of confluency of around 60%.
The analysis revealed no significant changes in the cell cycle (Figure 51). At passage 1 p. i. there was no difference detectable (Figure 51). At passages 5 and 11 p. i. slight changes were observed with more PC-3v45 cells in the S-phase and less in G0/G1, however these changes were consistently very small (around 5%) and not significant enough to explain the previously observed reduction in growth (Figure 51).

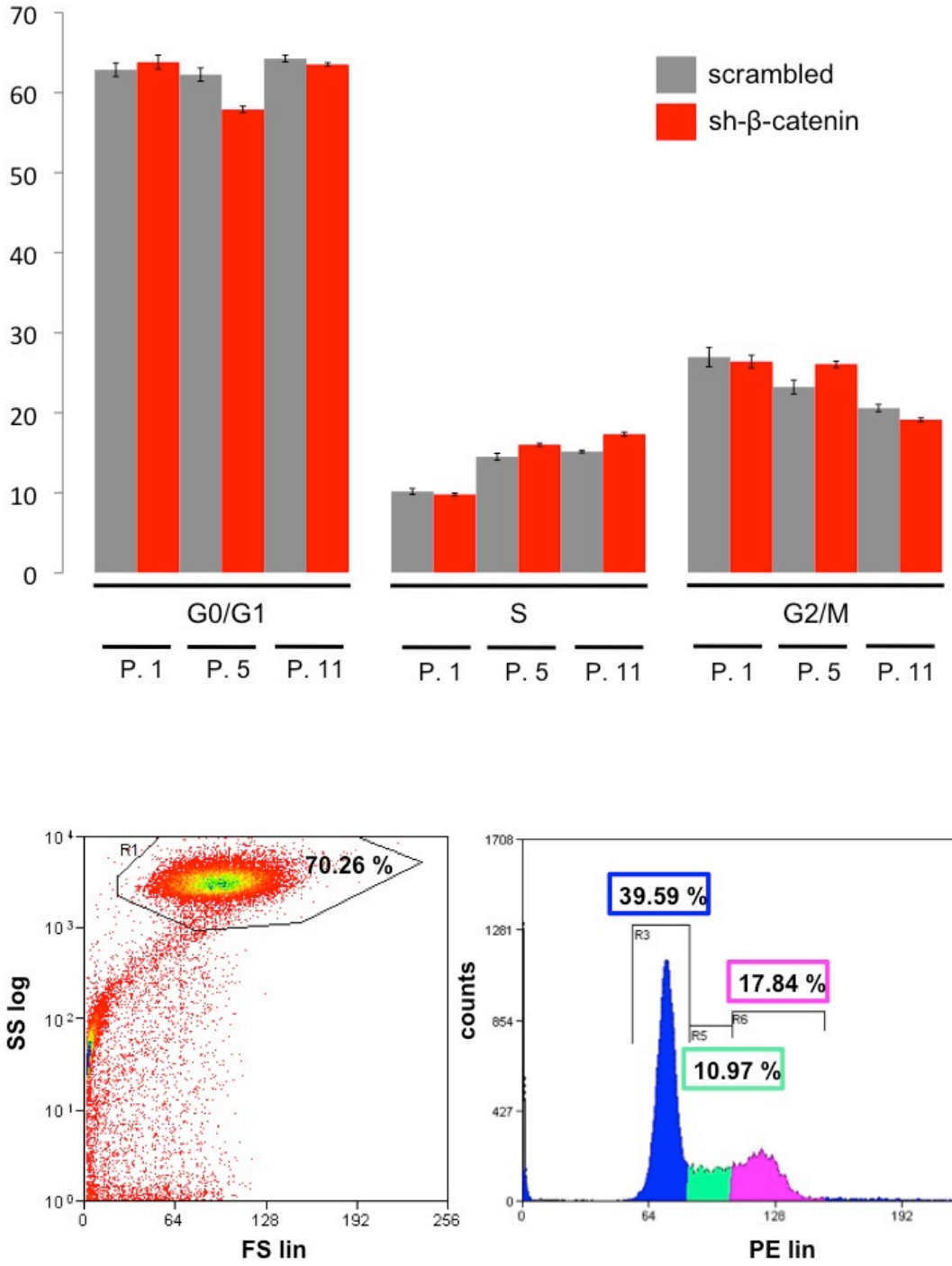


Figure 51: Cell cycle analysis of PC-3v cells

Percentages of PC-3v cells at passage 1, 5 and 11 post infection, in different phases of the cell cycle (top). Representative example of cell cycle analysis FACS trace from PC-3v cells at P. 5 p. i. Gate (R1) was used to remove dead cells from analysis (bottom left). Cells in G0/G1 phase (blue), S phase (green) and G2/M phase (pink) (bottom right).

4.2.6 Wnt activation and immunofluorescence β-catenin staining

Wnt ligands cause the inhibition of the β-catenin destruction complex, which results in β-catenin being able to accumulate in the cytoplasm from where it translocates to the nucleus (Kobayashi et al., 2000). The predominant interaction partners of β-catenin in the nucleus are transcription factors of the TCF/LEF family (Gordon and Nusse, 2006). Staining for β-catenin therefore gives an indication on the status of the Wnt signalling pathway in the examined cells.

In all experiments, β-catenin staining was detected in the plasma membrane. This was expected as β-catenin localises with E-cadherin (McCrea and Gumbiner, 1991, McCrea et al., 1991, Nagafuchi and Takeichi, 1989). The β-catenin-E-cadherin-complex is an essential part of cell adhesion and plays an important role in the correct formation of adherens junctions. It is also linked to the cytoskeleton via α-catenin (Aberle et al., 1994, Gooding et al., 2004, Hülsken et al., 1994, Jou et al., 1995).

To optimise immunofluorescent staining of β-catenin localisation within the cell, a reliable positive control was required. To increase reliability and homogeneity during immunofluorescence, artificial activation of the canonical Wnt signalling pathway using 20 mM LiCl or recombinant Wnt1 protein was used, as described previously (Spencer et al., 2006). LiCl inhibits the kinase GSK3β, which forms part of the β-catenin destruction complex. This inhibition allows β-catenin to accumulate and translocate to the nucleus (Klein and Melton, 1996).
The osteosarcoma cell line SaOS-2, which was recommended as a positive control for Wnt activation (personal communication Paul Genever), was activated for 24 h with 20 mM LiCl. SaOS-2 cells showed nuclear β-catenin staining following activation (Figure 52). Using the imaging software ImageJ, the amount of colocalisation of β-catenin with the nuclear DAPI stain was visualised (Figure 52). Activated SaOS-2 cells, treated for 24 h with LiCl, showed a considerable increase in colocalisation, compared to the negative control, which was treated with NaCl. It was noted that some SaOS-2 cells exhibited a weaker response to activating conditions (Figure 52). These results confirmed that immunofluorescence can be used to detect active Wnt signalling.

Wnt activation experiments were also performed in prostate cell lines PC-3, LNCaP and PNT2-C2 cells. No nuclear β-catenin staining was observed in PC-3 cells under activating conditions (Figure 53). LNCaP cells did not attach firmly to slides and most cells were either lost or destroyed during the staining procedure (Figure 54). In PNT2-C2 cells, some nuclear staining was observed but results remained inconclusive (Figure 54).
Caco-2 cells were also stained for β-catenin in order to determine whether they can be used as a positive control for nuclear staining. However, the cells grew in very tightly packed colonies, and did not show any nuclear staining. Membrane staining was observed (Figure 55).

The results obtained from prostate cell lines might give an indication of the importance of β-catenin with regard to Wnt signalling, as no nuclear staining (active canonical Wnt pathway) was observed in most samples. They also suggest that another function of the protein might be a prominent factor in prostate cancer, such as the E-cadherin-β-catenin complex, which is not directly influenced by LiCl activation.

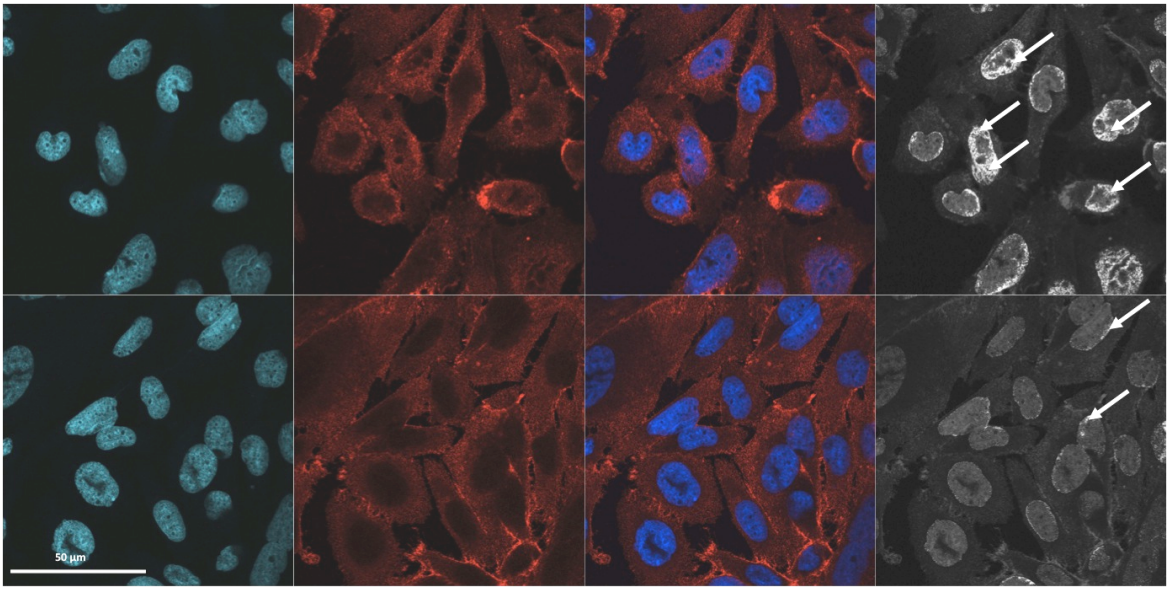


Figure 52: β-catenin staining in activated SaOS-2 cells

SaOS-2 were activated for 24 h with 20 mM LiCl (top), 20 mM NaCl served as a negative control (bottom). Colocalisation of β-catenin (red, Alexa568) staining in the area of the nucleus (blue, DAPI) indicated as white dots on corresponding colocalisation images (far right) by arrows.

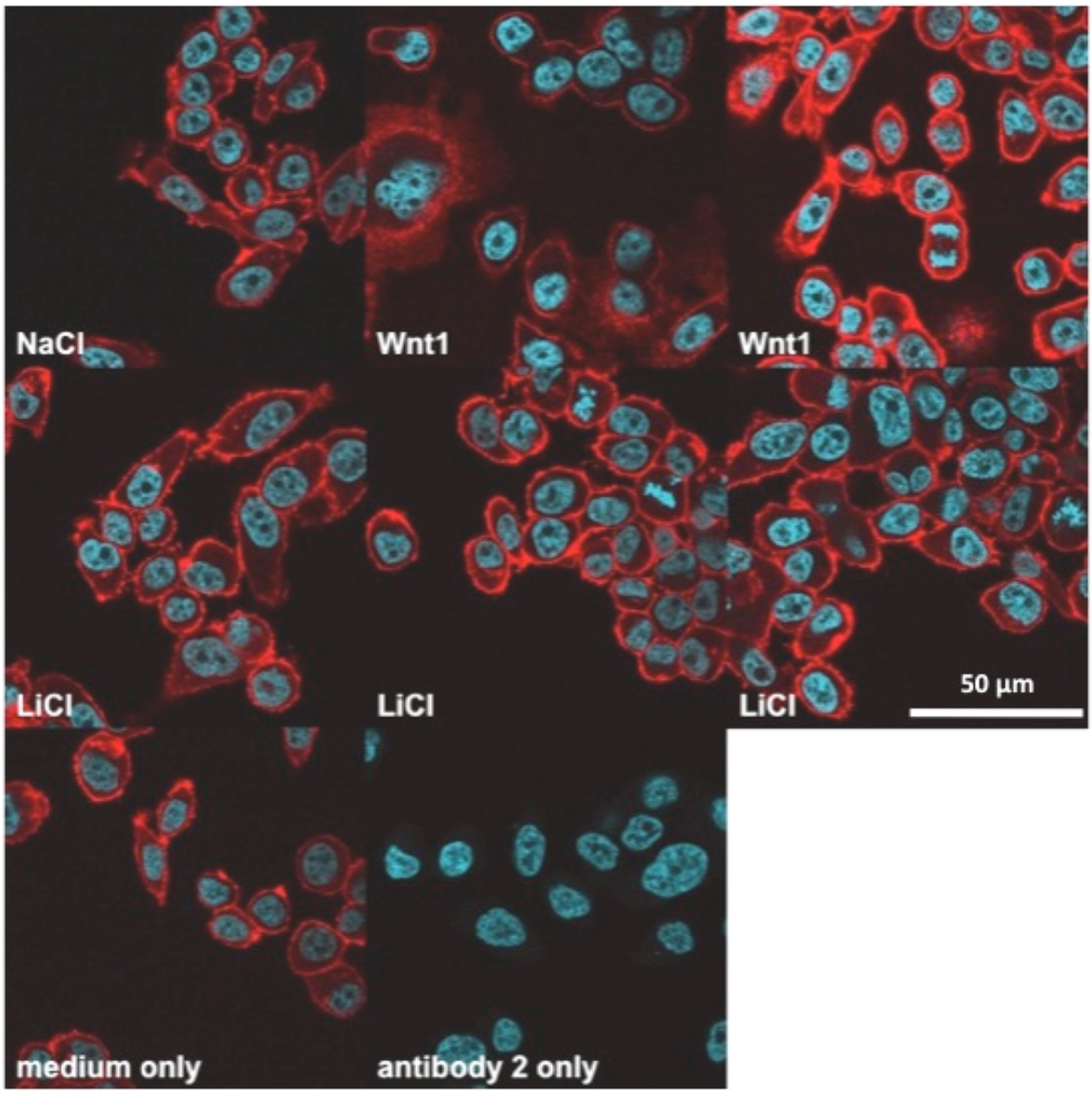


Figure 53: β-catenin staining in activated PC-3 cells

PC-3 were activated for 24 h with 100 ng/ml Wnt1 protein or 20 mM LiCl for 24 h, according to the method used by Spencer and colleagues (Spencer et al., 2006). Negative controls included 24 h incubation with medium containing 20 mM NaCl, medium only and staining with secondary antibody only.

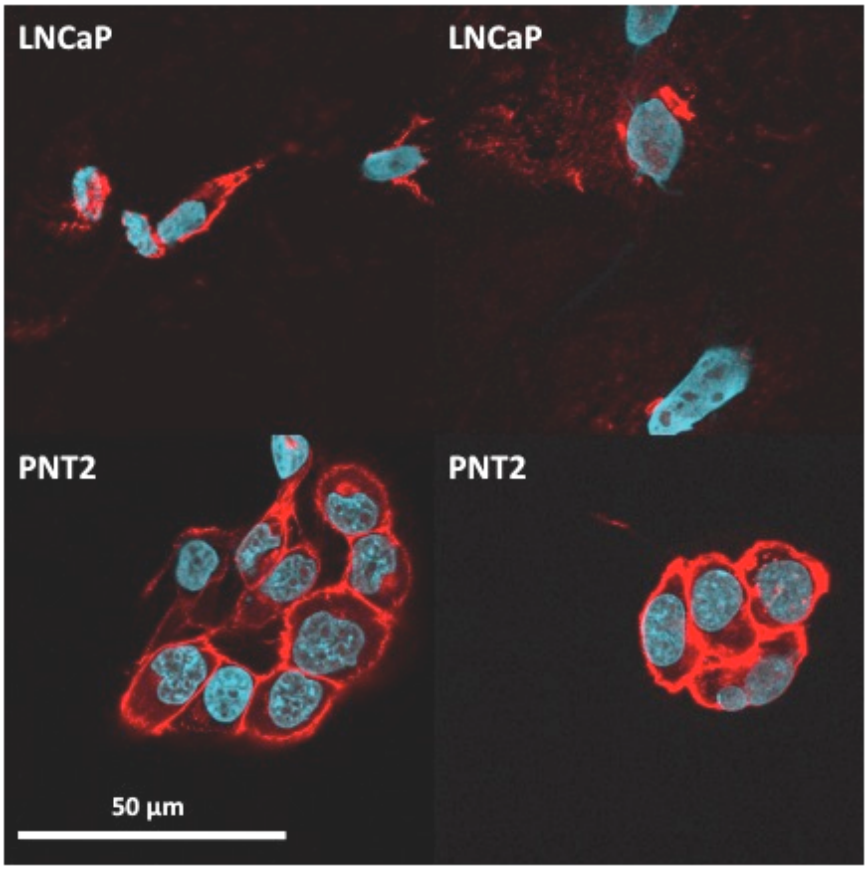


Figure 54: β-catenin staining in activated LNCaP and PNT2-C2

Prostate cancer cell line LNCaP and normal prostate cell line PNT2-C2 were activated with 100 ng/ml recombinant Wnt1 protein for 24 h.

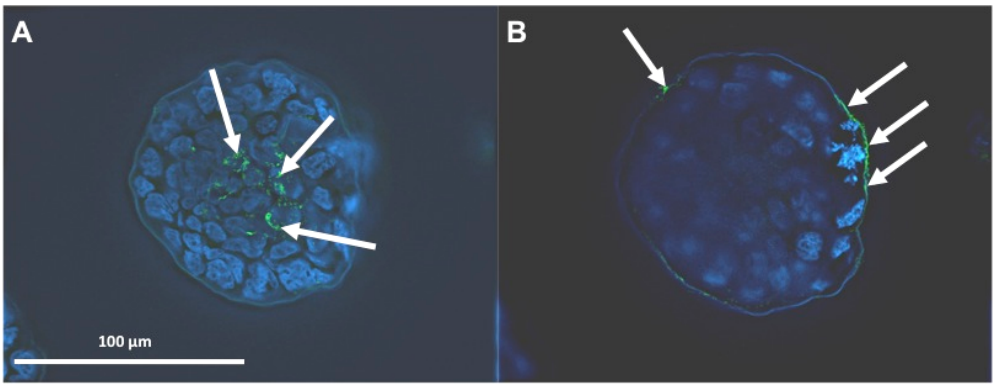


Figure 55: β-catenin staining of Caco-2 colony

Z-series of a Caco-2 colony stained for β-catenin (green) and nuclei visualized with DAPI (blue). The cells grow very close together and β-catenin is observed in the middle (A, arrows) and on the edges of the colony (B, arrows) as a membranous staining.

4.2.7 TOPFlash reporter assay to monitor Wnt activity

TOP-flash reporter assays (Molenaar et al., 1996) and the later developed SuperTOPFlash reporter plasmids have been widely used. These plasmids are luciferase reporters of β-catenin-mediated transcriptional activation, and therefore able to detect active Wnt signalling. We used three reporter plasmids, each based on the pTA-Luc vector (Clontech), harbouring a TA viral promoter to drive the expression of the firefly luciferase gene. Each plasmid also contains multiple TCF/LEF recognition sites (sequence: AGATCAAAGG, followed by a spacer gggta), where β-catenin can bind to. The plasmid MO50 (Super8XTOPFlash) contains 8 recognition sites, while its partner MO51 (Super8XFOPFlash), containes 8 mutated sites (Veeman et al., 2003), in order to be used as a negative control. MO72 (Super16XTOPFlash) contains 16 recognition sites (DasGupta et al., 2005).

Prostate cell lines PC-3, P4E6 and PNT2-C2 (as well as SW480 and Hela control lines) were transfected with reporter plasmids MO50, MO51 and MO72. The cells were activated with 20 mM LiCl 24 h post transfection, or 20 mM NaCl (negative control) and a luciferase assay was performed 24 h post activation (Figure 56, 57 and 58).

Activated PC-3 cells exhibited a 12-22 fold change in luciferase with MO50 and MO72 respectively, compared to the luciferase expression in cells transfected with the negative control plasmid MO51, which was normalised to 1. Without activation, however, PC-3 cells did not show β-catenin activity above the background level (Figure 56 and 58). This is probably due to the expression of the endogenous Wnt inhibitor Dkk-1 in PC-3 cells (Emami and Corey, 2007, Hall et al., 2005). The fold-change in Hela cells was in a similar range (Figure 56).
LiCl-treated SW480 showed a much higher luciferase activity than PC-3 cells with a fold change of over 900 (Figure 57). In contrast to PC-3 cells, SW480 showed a high background activity of β-catenin even in non-activated conditions (data not shown).
It was concluded therefore that there were no or very low levels of residual active β-catenin in PC-3 cells.

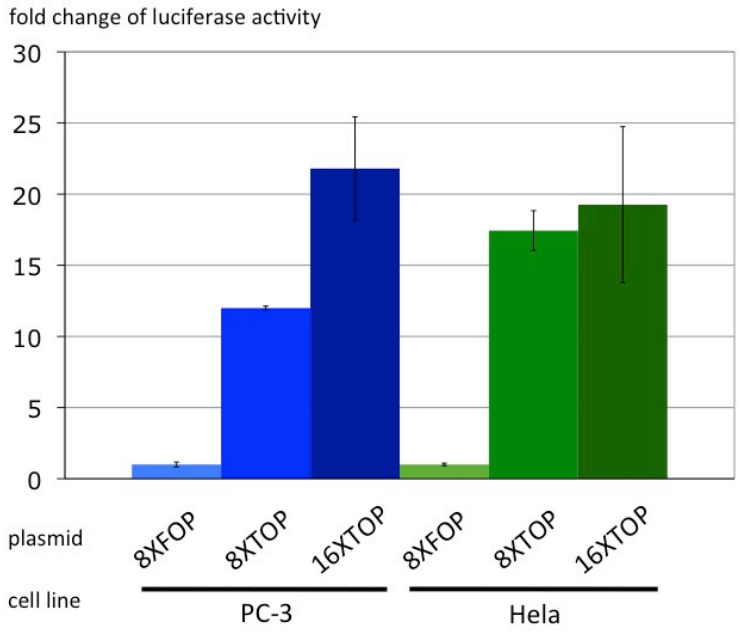


Figure 56: TOPFlash assay in PC-3 and Hela cells

Cells were activated with 20 mM LiCl. Fold change in luciferase activity normalised to negative control plasmid Super8XFOPFlash (MO51), set to 1.

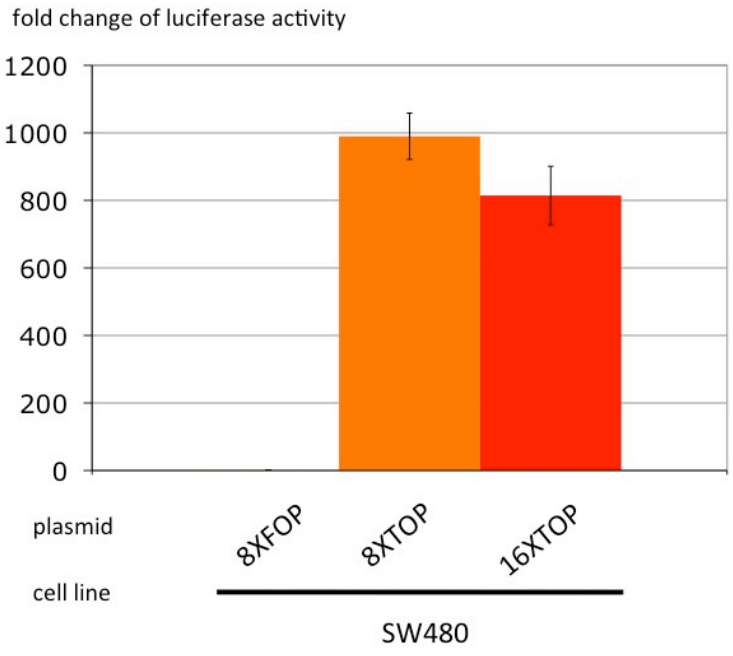


Figure 57: TOPFlash assay in SW480 cells

Cells were activated with 20 mM LiCl. Fold change in luciferase activity normalised to negative control plasmid Super8XFOPFlash (MO51), set to 1.

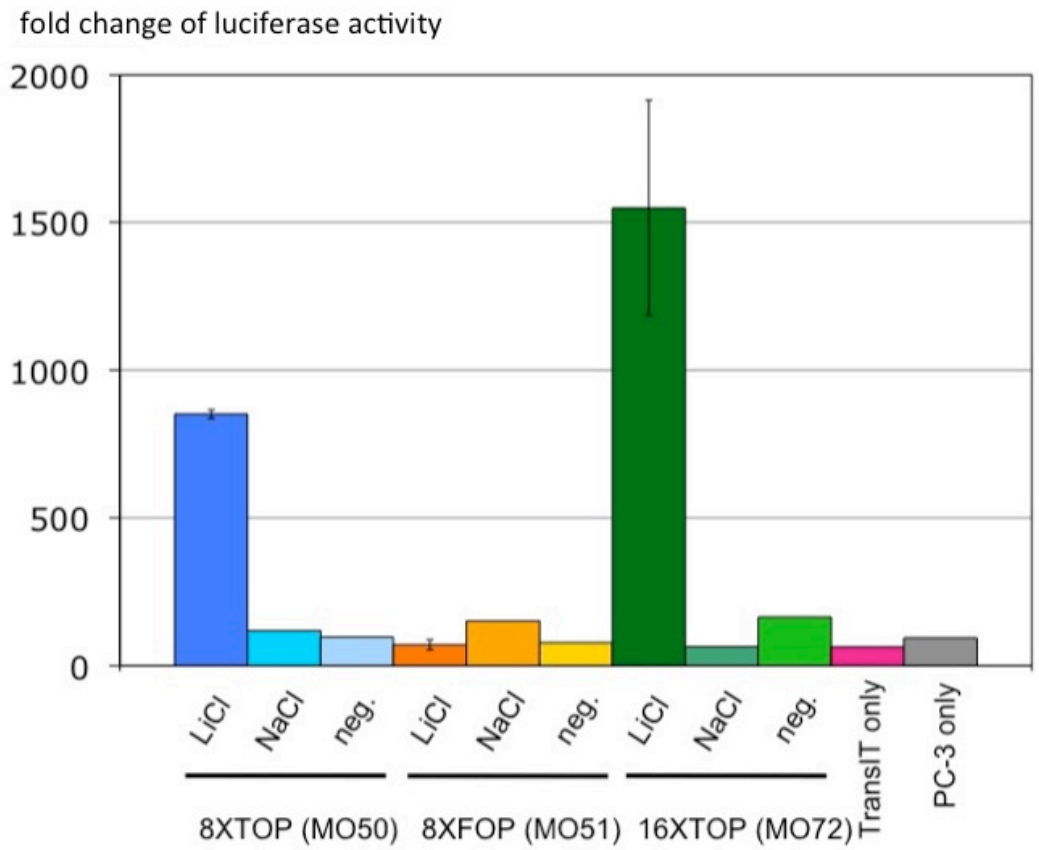


Figure 58: Luciferase activity measured in TOPFlash assay in PC-3 cells

PC-3 cells transfected with TOPFlash plasmids 8XTOP (MO50), 8XFOP (MO51) or 16XTOP (MO72) were activated with 20 mM LiCl or 20 mM NaCl, and compared to transfected but not activated cells (neg.), PC-3 cells only treated with TransIT trasnfection reagent and PC-3 cells only. Values shown are units of luciferase activity.

To summarise, we have seen that active β-catenin levels in PC-3 cells are very low, but we were able to successfully activate these cells using LiCl. Upon activation, the TOPFlash reporter assay was used to detect the level of Wnt signalling activity. These results confirm observations made using IF, where we were not able to detect nuclear β-catenin staining in PC-3 cells.

4.2.8 Reduction of invasive potential in PC-3v cells

Currently, there is no cure for metastatic prostate cancer, which shortens patient survival and negatively impacts on quality of life. As such, metastatic prostate cancer is a priority for novel therapeutic strategies (Moro et al., 2008).

Therefore, studying the invasive potential of cancer cells is important to determine how successful they are at escaping the confines of the original tumour and metastasising to other parts of the body. During the process of epithelial-mesenchymal transition (EMT), among other changes, gene mutations and variation in expression in cadherins and catenins, occur in epithelial tumour cells. This facilitates their ability to invade surrounding tissue (Friedl and Wolf, 2003).

In order to determine the invasive potential of sh-β-catenin (PC-3v45) cells, which exhibited reduced β-catenin protein levels, invasion assays were performed. In brief, cells were plated into matrigel coated inserts and placed into wells containing conditioned medium for an incubation period of 48 h. Inserts without the addition of matrigel served as a control for measuring motility (see Figure 59 for a schematic of the experimental layout).

Cells that had migrated through the matrigel to the bottom side of the insert's PET membrane (Figure 60) were counted by eye using a cell count analysis tool (ImageJ) (Figure 61). Images were taken of 4 random fields per membrane and cell counts were averaged. After optimisation of positive and negative control cell lines, it was decided to use normal, non-invasive prostate epithelial cells (PNT1A) as a negative control and the highly invasive, metastatic breast cancer cell line MDA-MB-231 as a positive control.

One of the important technical aspects of the invasion assay experiments was the reliability of cell counting, to determine the number of cells, which had invaded through the matrigel and/or the membrane. Automated counting using the NIS Elements software (Nikon) was abandoned in favour of manual counting. After comparing both methods, it was found that the latter gave more consistent results. In our experience, the NIS software is a good way of counting a whole membrane with small to medium amounts of invasive cells. However, if the cell density is too high, the software did not distinguish between clumps of cells and debris. Modifications to the set-up are possible, for example, to exclude large conglomerates of nuclei or oddly shaped (not round) nuclei, however, this then reduced the number of actually counted cells. This reduced the number of counted

cells to a falsely lower number. Depending on the individual membrane, results were therefore often biased in favour of highly invasive cell lines.

After analysing the number of invasive cells, PC-3v45 cells showed a significant reduction compared to the scrambled control (Figures 62, 63, 64, 65 and 67). Numbers of cells that invaded varied between experiments though they always showed the same trend.

It is advisable to take motility of cells into account when looking at their invasiveness. Highly motile cells can still be non-invasive if they do not express the necessary proteases to digest matrigel. In addition, invasive cells which are less motile than another invasive cell line, will take longer to invade and consequently, might show lower numbers in the invasion assay. Therefore it is reasonable to analyse invasiveness with respect to motility of each cell line and look at these ratios in addition to invasion-only numbers (Figures 66 and 68). However, in contrast to the reduction of invasive capacity by β-catenin shRNA, there was no significant difference observed in motility.

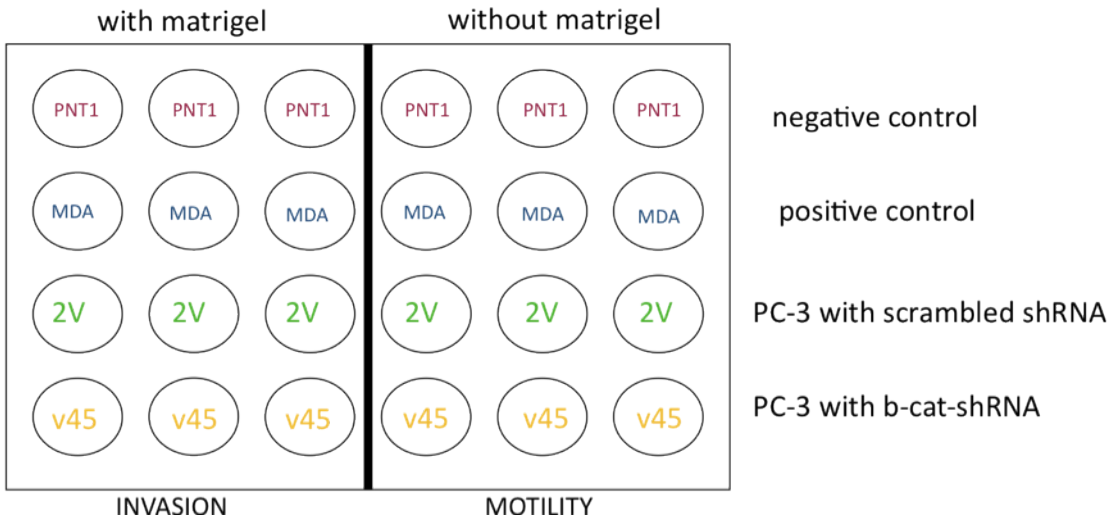


Figure 59: Invasion assay experimental layout

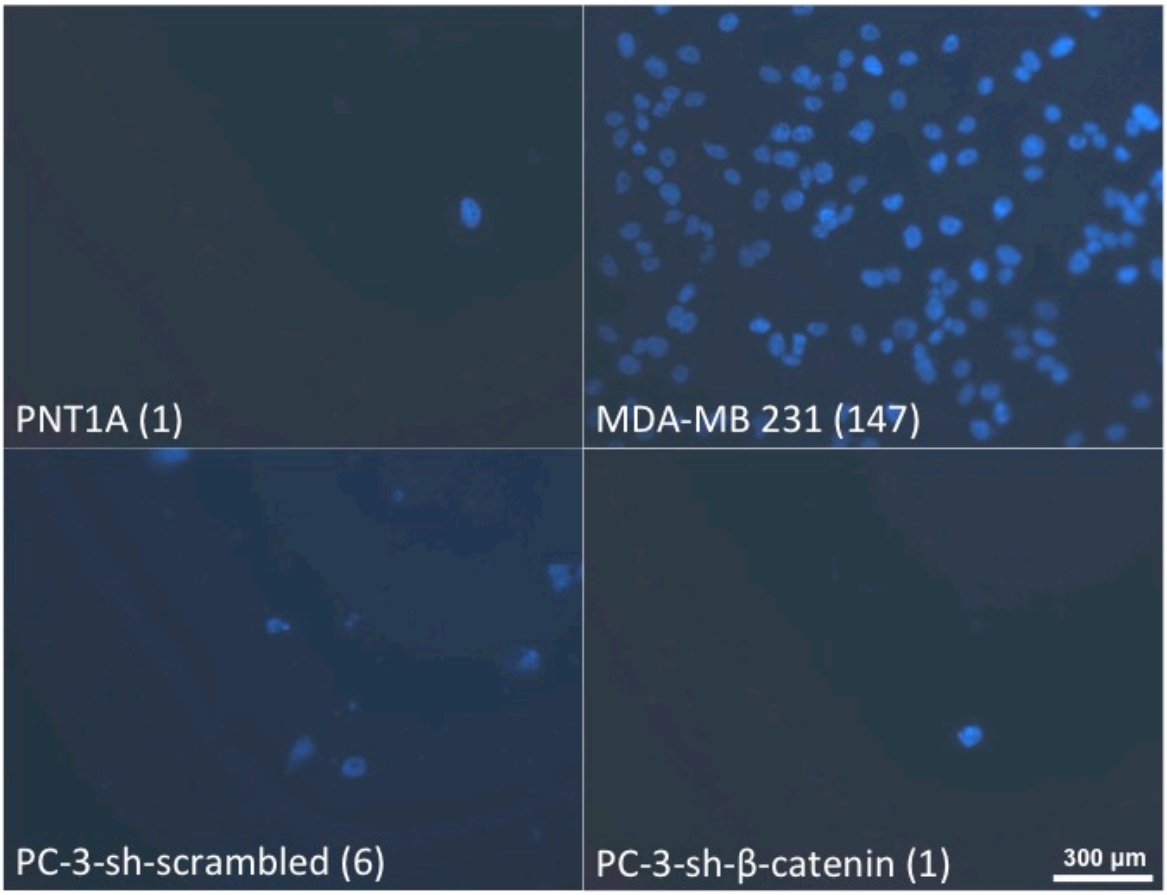


Figure 60: Invasion assay cell counts

Representative examples of images from invasion assay membranes with Matrigel of PNT1A, MDB-MB 231 PC-3-sh-scrambled (PC-3_2V) and PC-3-sh-β-catenin (PC-3v45) with counted cell numbers in brackets, e.g. (6). Cell nuclei shown in blue (DAPI).

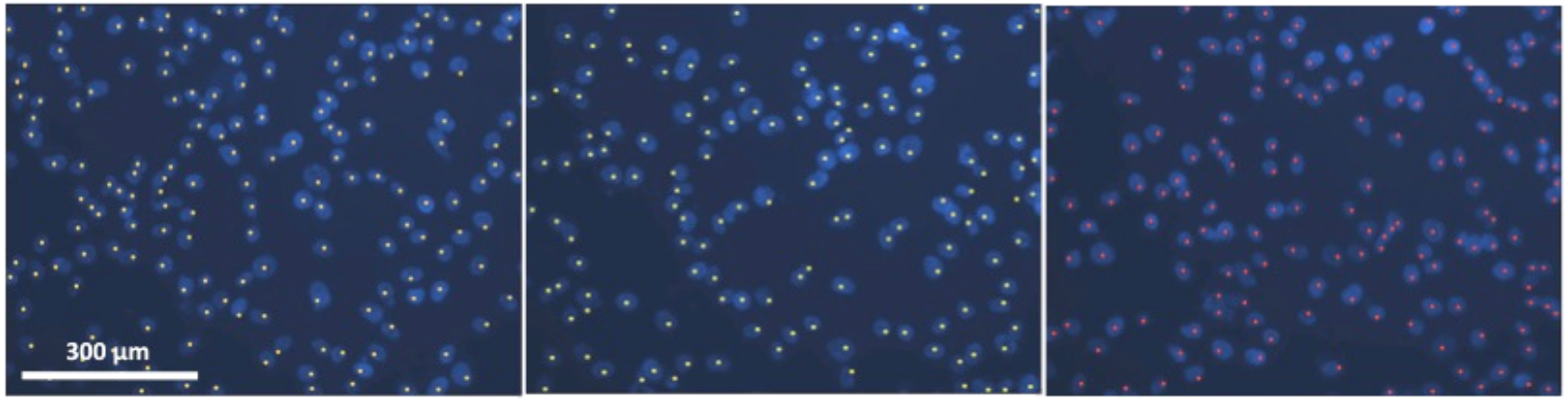


Figure 61: Invasion assay cell counting with ImageJ

Representative examples of counting highly invasive cells using ImageJ cell counter tool. Each cell was marked manually (coloured dots) and was then automatically added to the cell count by the software.

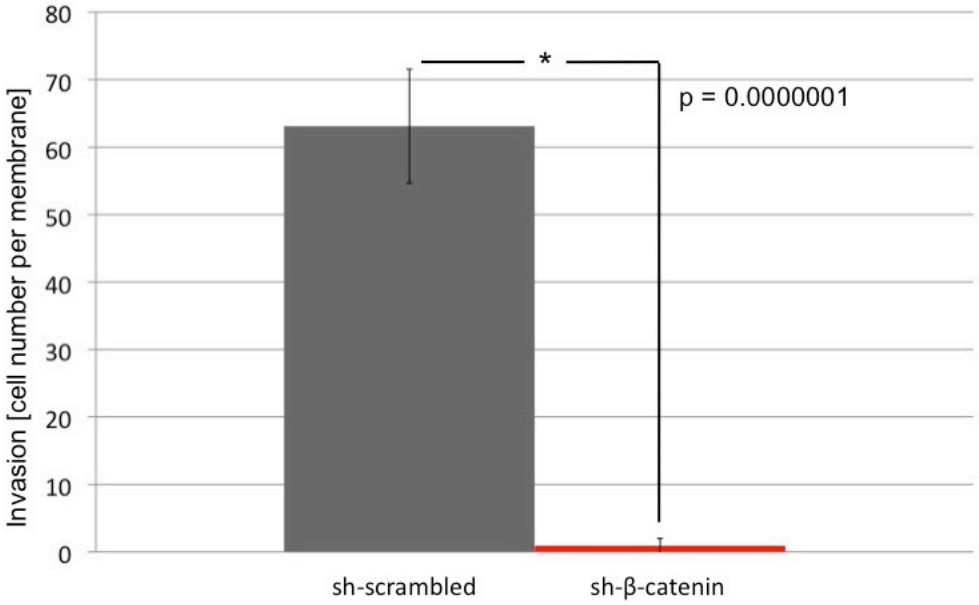


Figure 62: Invasion sh-β-catenin vs. sh-scrambled control cells (I)

Invasion capacity of sh-β-catenin (PC-3v45) cells compared to sh-scrambled control cells (PC-3_2V), measured as cell number per membrane, not normalised to motility.

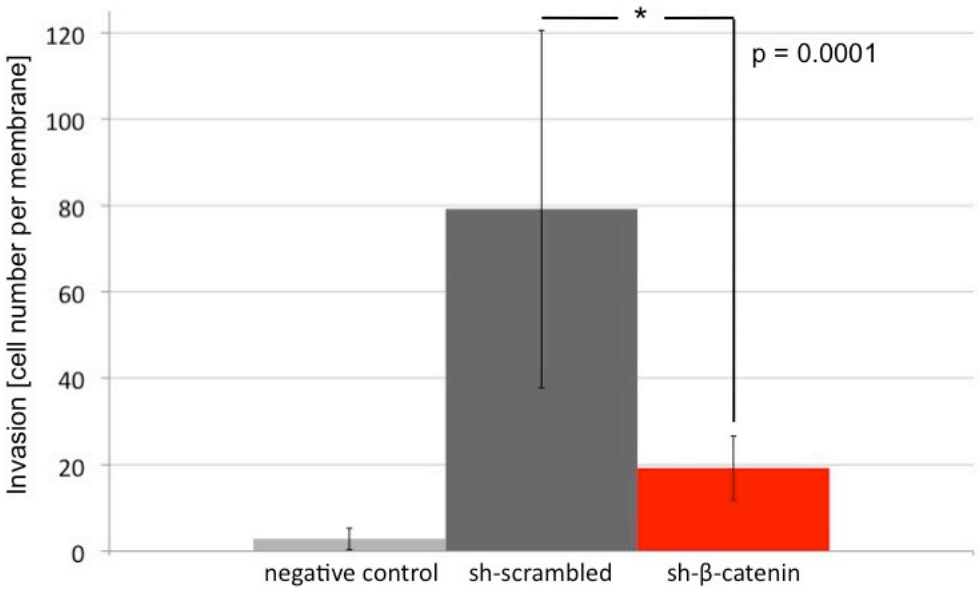


Figure 63: Invasion sh-β-catenin vs. sh-scrambled control cells (II)

Invasion capacity of sh-β-catenin (PC-3v45) cells compared to sh-scrambled control cells (PC-3_2V) and the negative, non-invasive PNT2-C2 cells, measured as cell number per membrane, not normalised to motility.

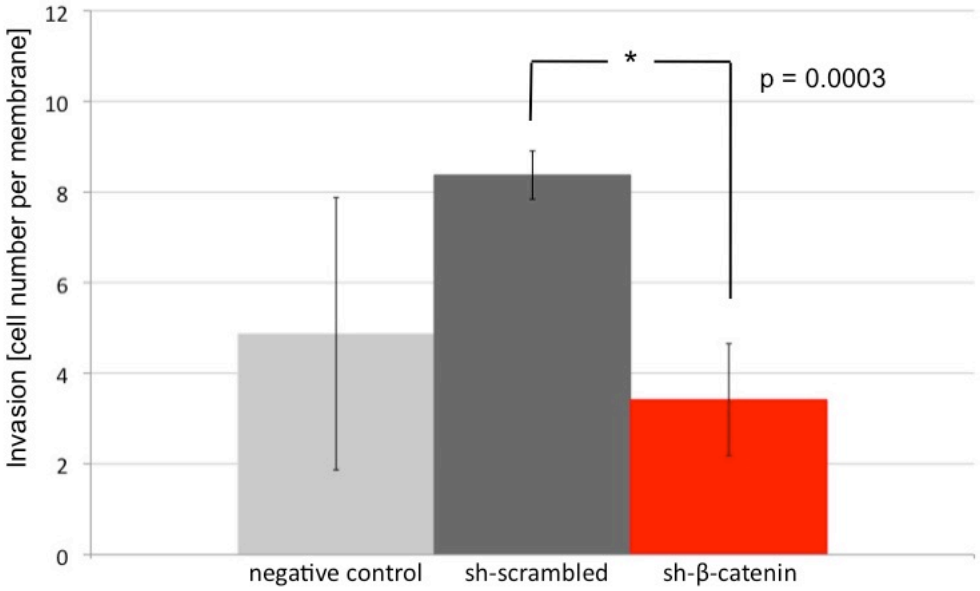


Figure 64: Invasion sh-β-catenin vs. sh-scrambled control cells (III)

Invasion capacity of sh-β-catenin (PC-3v45) cells compared to sh-scrambled control cells (PC-3_2V) and the negative, non-invasive PNT2-C2 cells, measured as cell number per membrane, not normalised to motility.

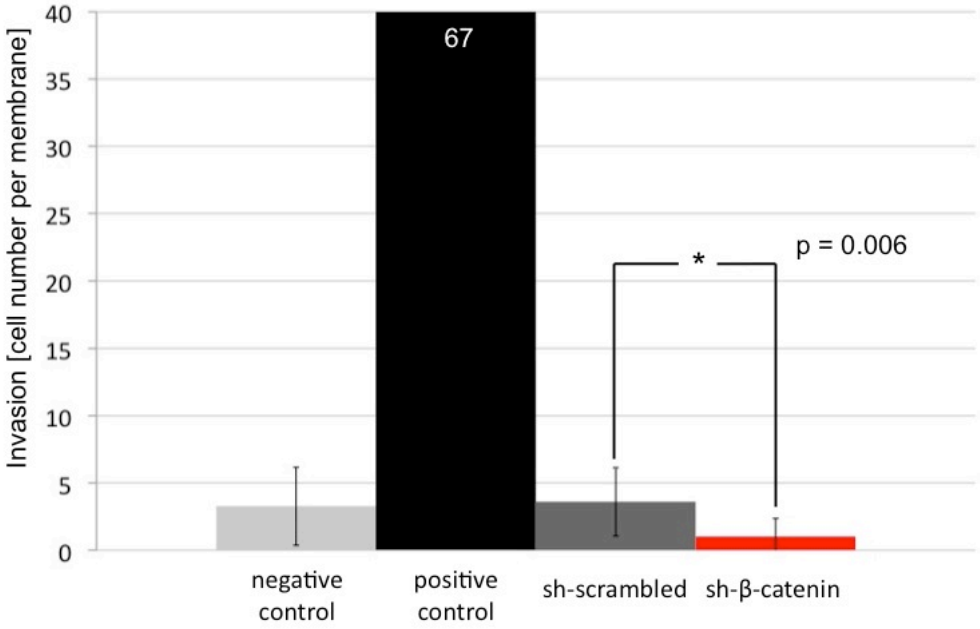


Figure 65: Invasion sh-β-catenin vs. sh-scrambled control cells (IV)

Invasion capacity of sh-β-catenin (PC-3v45) cells compared to sh-scrambled control cells (PC-3_2V), negative, non-invasive PNT1A cells and positive, highly invasive MDA-MB-231 cells, measured as cell number per membrane, not normalised to motility.

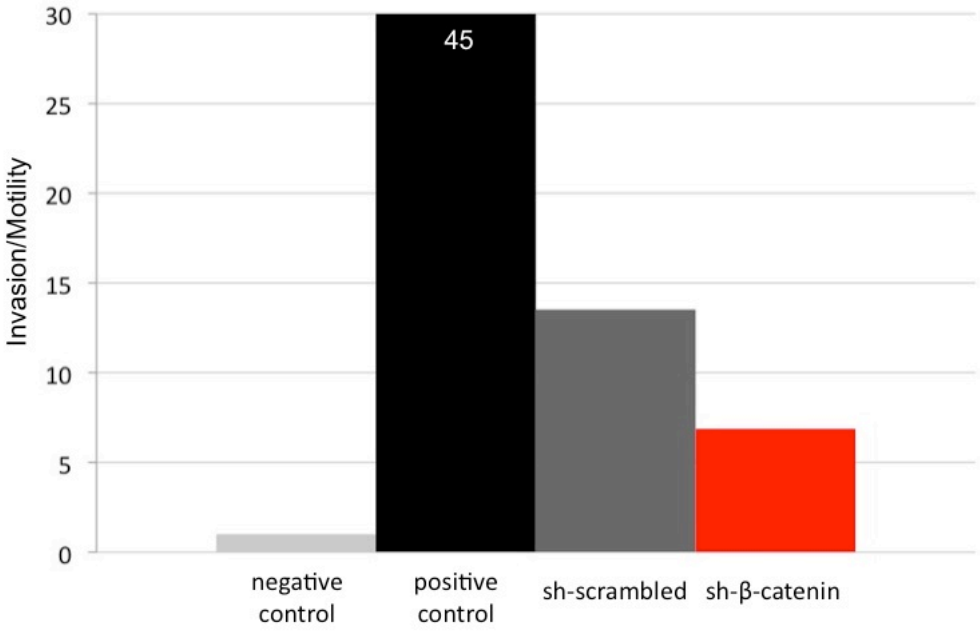


Figure 66: Invasion vs. motility in sh-β-catenin cells (I)

Invasion capacity normalised to motility (in %) of sh-β-catenin (PC-3v45) cells compared to sh-scrambled control cells (PC-3_2V), negative, non-invasive PNT1A cells and positive, highly invasive MDA-MB-231 cells.

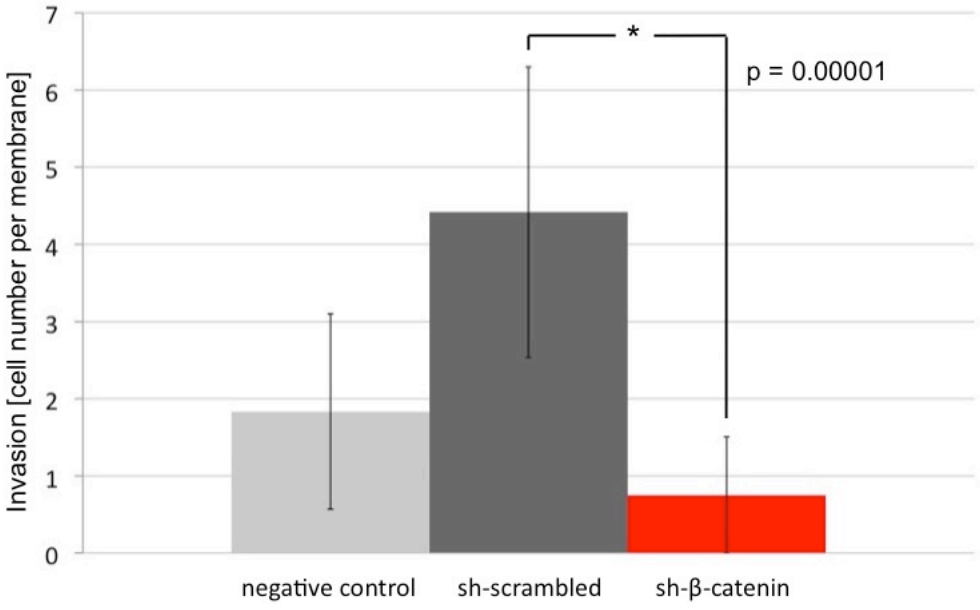


Figure 67: Invasion sh-β-catenin vs. sh-scrambled control cells (V)

Invasion capacity of sh-β-catenin (PC-3v45) cells compared to sh-scrambled control cells (PC-3_2V) and negative, non-invasive PNT1A cells, measured as cell number per membrane, not normalised to motility.

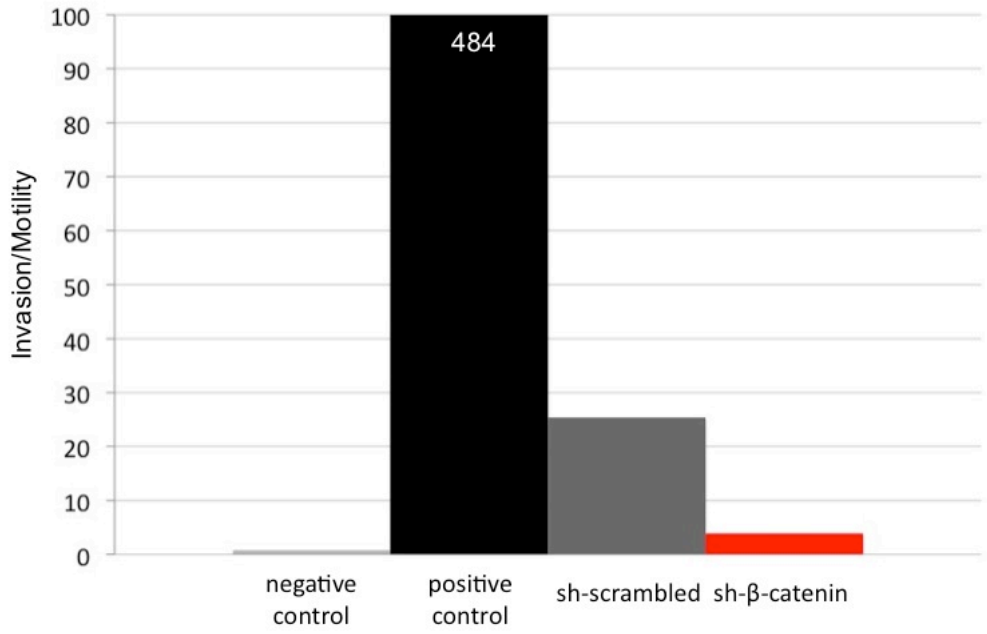


Figure 68: Invasion vs. motility in sh-β-catenin cells (II)

Invasion capacity normalised to motility (in %) of sh-β-catenin (PC-3v45) cells compared to sh-scrambled control cells (PC-3_2V), negative, non-invasive PNT1A cells and positive, highly invasive MDA-MB-231 cells.

To summarise, we have shown here that β-catenin knockdown also reduced invasion in PC-3 cells. It did not however have an effect on the motility of cells. It is therefore possible to hypothesise that β-catenin plays an important part in (metastatic) prostate cancer, where a high invasion capacity is a major growth advantage for tumour cells. Even if β-catenin expression is at a relatively low level, e.g. PC-3 cells compared to MDA-MB 231 cells, knockdown of β-catenin has a dramatic and significant effect on invasion.

4.2.9 Effects of β-catenin knockdown in xenografts

4.2.9.1 PC-3v tumour growth in mice

In order to examine tumour induction and growth *in vivo*, the stably transfected PC-3 cell lines PC-3v45 (sh-β-catenin) and PC-3_2V (sh-scrambled) as well as untransfected PC-3 cells were injected subcutaneously into GC-1-RAG2 γc -/- mice. Each group consisted of 3 animals and tumour growth was measured at regular intervals from the time point of first, visible tumour detection. The procedure was carried out by Dr Anne Collins, Paul Berry and Katy Hyde.

In the PC-3 control, the first tumour was detected on day 16 post injection with a lengths of 5.8 mm. By day 21 all three mice had measurable tumours of 5.0-5.8 mm. The average size of PC-3 tumours at day 65 post injection was 14.95 mm (Figure 69). The scrambled shRNA control cells produced a tumour in one mouse by day 16 (5.8 mm) and the second tumour developed by day 23 (6.0 mm). The final mouse showed a detectable tumour on day 28 (5.0 mm). The third tumour was considerably smaller than those in the other two mice and its growth rate varied more than in any of the other xenografts. There was also some regression of growth observed. Tumours from the scrambled control grew to an average size of 12.67 mm at day 65 post injection, with two tumours at 15 mm and the third tumour at 8 mm (Figure 70).

In contrast to both controls, the sh-β-catenin cell line PC-3v45 did not produce measurable tumours until day 18, and the first tumour was 4 mm in length, and therefore smaller than the initial tumours in both controls. The second and third mice exhibited measurable tumour growth on day 23, when all tumours were 5 mm in length (Figure 71). However, the growth of sh-β-catenin xenografts had caught up with the controls by day 25 (Figure 72). The average size of PC-3v45 tumours was 16.48 mm on day 65 post injection. This indicated a delay in tumour induction with reduction of β-catenin (Figure 72, insert).

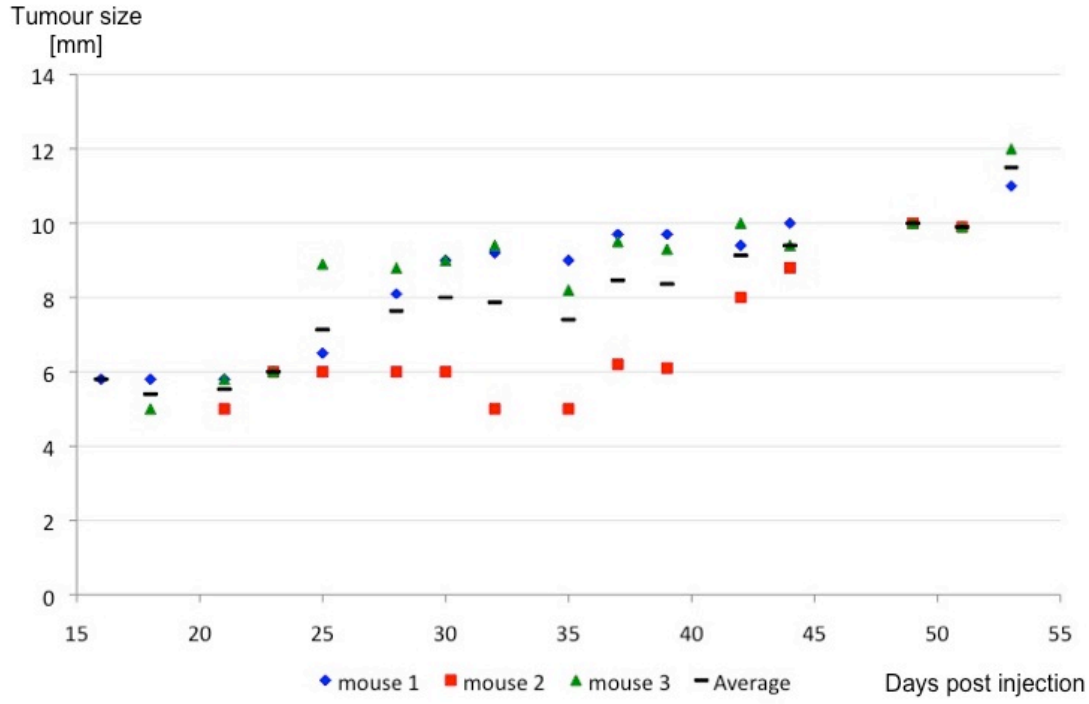


Figure 69: Tumour growth in mice injected with PC-3 control cells

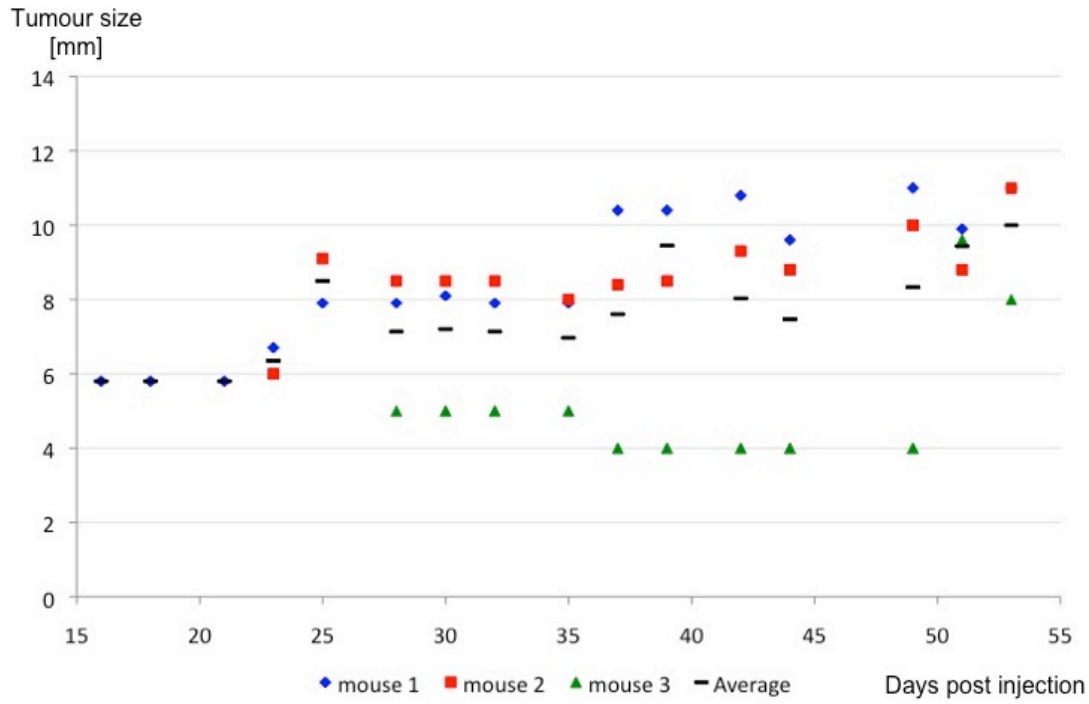


Figure 70: Tumour growth in mice injected with sh-scrambled (PC-3_2V) control cells.

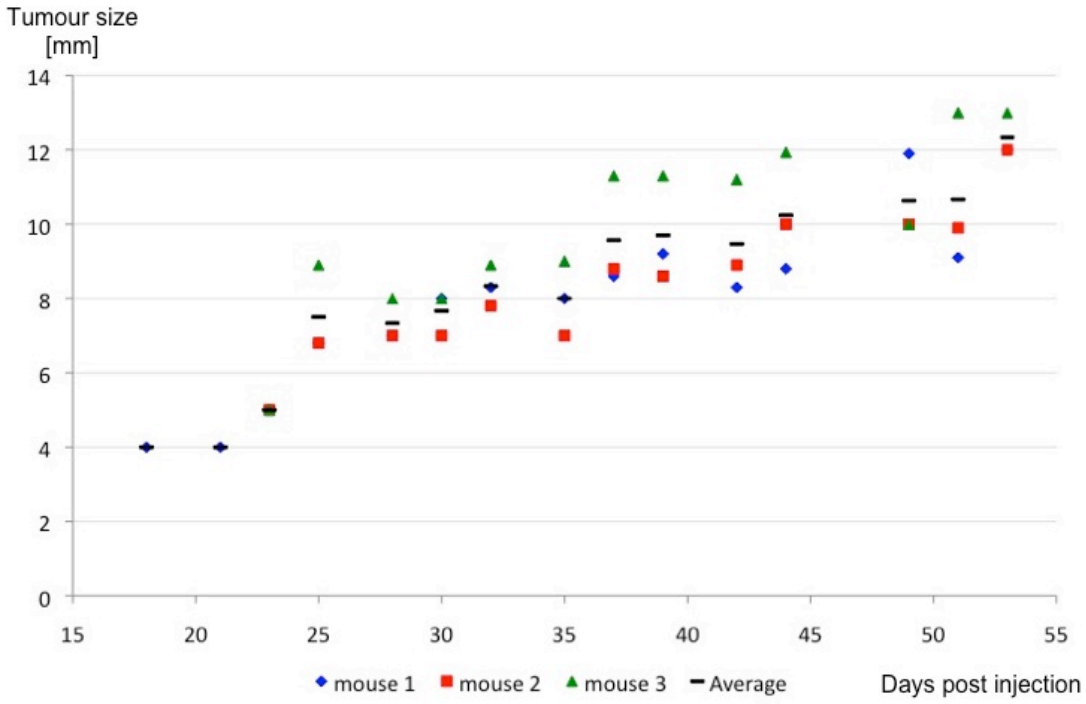


Figure 71: Tumour growth in mice injected with sh-β-catenin (PC-3v45) cells.

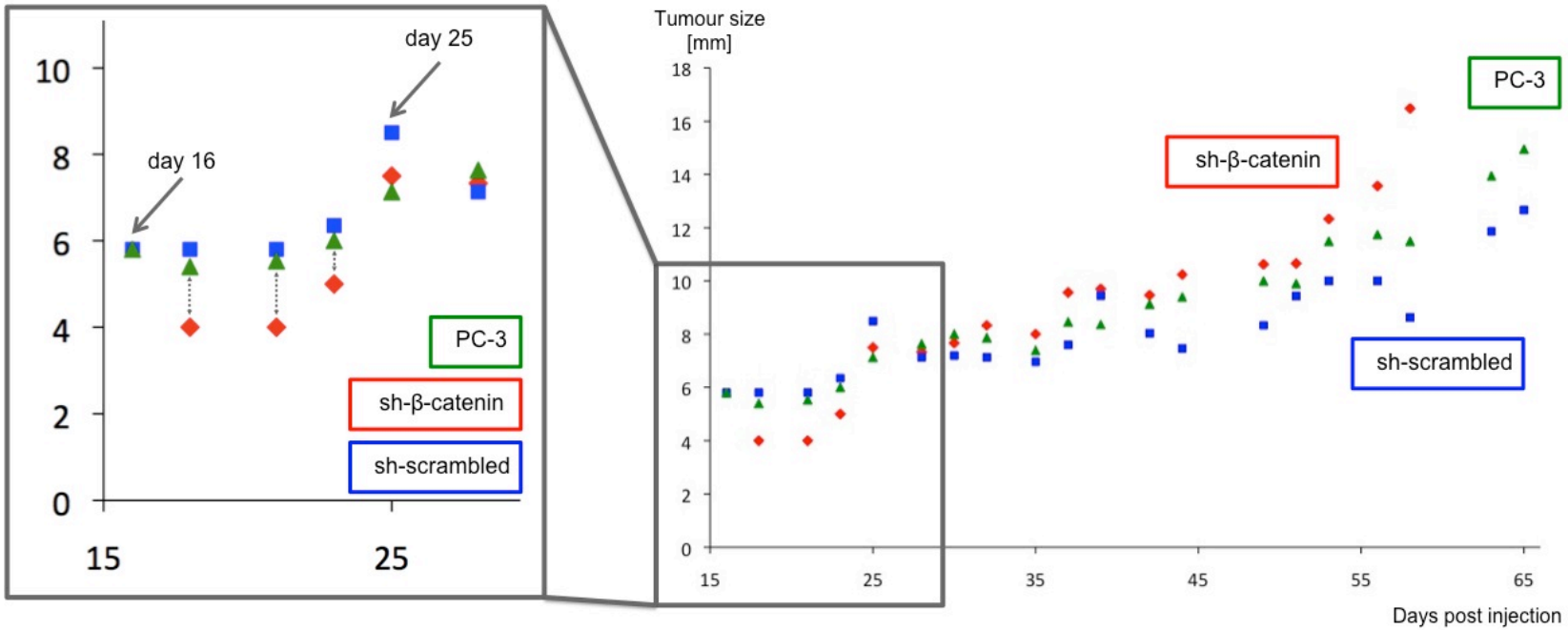


Figure 72: Tumour growth in mice compared

Tumour size was averaged per time point. Tumour growth after injection of sh-β-catenin (PC-3v45) cells was delayed and did not catch up with controls until day 25 (see arrows in insert).

To conclude, although sh-β-catenin (PC-3v45) xenografts produced tumours of the same size as scrambled and negative control cells, these tumours occured at a later time point. This is a promising result, as it indicated that the knockdown of β-catenin in sh-β-catenin (PC-3v45) tumours caused changes in the tumours cells, which were significant enough to result in delayed tumour induction. To study this phenomenon in more detail, xenografting a larger cohort of mice would be advantageous.

4.2.9.2 Relation between β-catenin knockdown and β-actin protein expression

Xenografted mice were sacrificed once tumours reached 15 mm in length or were restricting movement of the animals. Tumours were then processed to deplete them from any infiltrated mouse cells. Non-epithelial cells, e.g. blood cells, were also extracted to gain a pure human epithelial cell population. Lysates were made of the depleted xenograft cells, which were then subjected to Western Blot, to detect β-catenin. As in previous Western Blot experiments, β-actin was used as a loading control. We were not able to detect β-actin in sh-β-catenin lysates from mouse A and F (Figure 73). Therefore β-catenin knockdown could not be measured in relation to the loading control β-actin.

At this point, no further conclusions could be drawn as the results above were preliminary and only covered a small number of xenografted tumours. For future experiments, alternative loading controls which are not related to the β-catenin/E-cadherin/β-actin cytoskeletal complex, such as GAPDH, should be used.

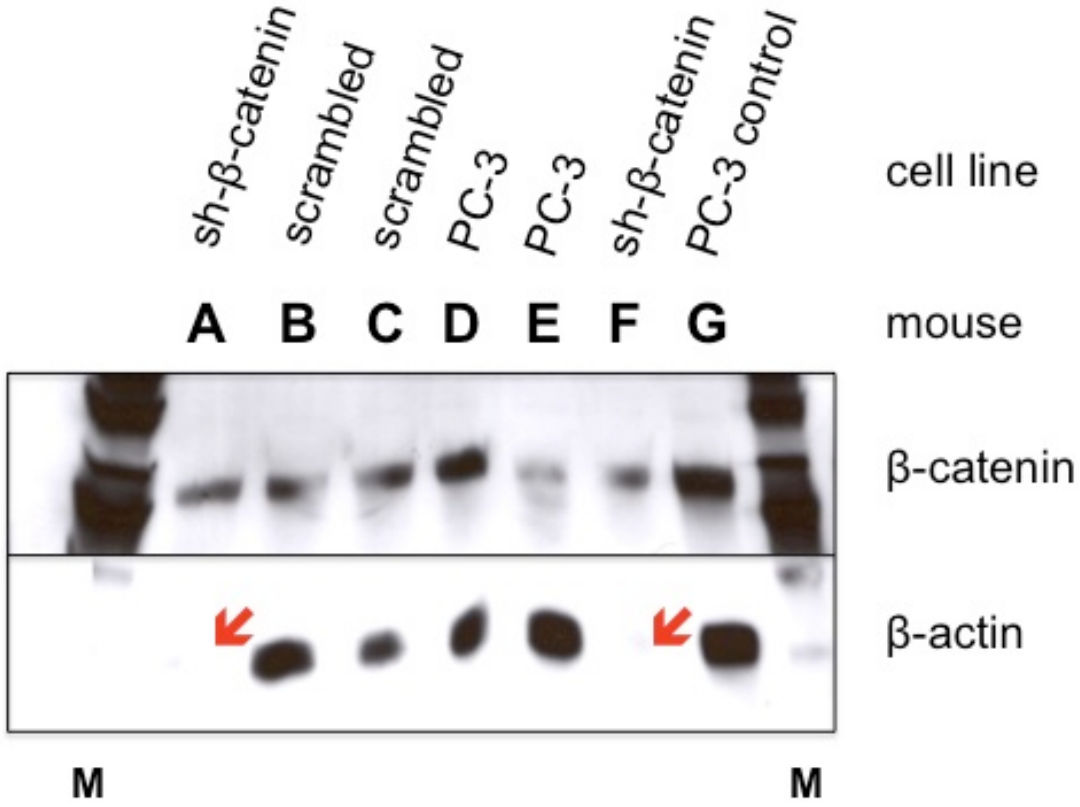


Figure 73: Western Blots from xenograft tumours

Western Blot of cell lysates made from xenograft tumours. No β-actin bands were detected in sh-β-catenin lysates (mouse A and F, red arrows).

4.3 β-catenin studies in primary prostate cells

4.3.1 Reanalysis of microarray data

For most cancers, it is widely accepted now, that there is a small population of tumour initiating stem cells, the cancer stem cells, which are responsible for tumour maintenance, formation of metastasis and recurrence post therapy. Genetic profiles of tissue stem cells and various tumours, including prostate, have been obtained over the last years, to elucidate the genetic events, which lead to tumour initiation (Birnie et al., 2008, Forsberg et al., 2006, Hwang-Verslues et al., 2008, Lukacs et al., 2008, Marquardt and Thorgeirsson, 2010, Menicanin et al., 2009, Nelson, 2004).

For this project, microarray data obtained in our laboratory and published by Birnie et al. was reanalysed to examine the expression of genes involved in Wnt signalling. We were particularly interested in any expression differences between the stem cell population and the committed basal population of malignant prostate tumours. We focussed on samples with a Gleason score of 7 and above, because gene expression differences were very clear. When Gleason 6 was included the differences were not clear.

Even though there was considerable variation in the data obtained from primary tumour samples, there was a clear trend for higher β-catenin expression in the proposed prostate cancer stem cell population (CSC; CD133+ phenotype), than in the malignant committed basal population (CB; α2β1low phenotype) (Figure 74, Table 12). The average expression level in CSC was more than 60% higher than in the CB population.

The same trend was also observed in the expression of two different microarray probes for TCF4, the direct downstream target of activated β-catenin. TCF4 was expressed to a lesser extent in the CB cells than in the CSC population. Expression differed between 30 % and 50 % (Figure 74, Table 12).

Since mutations in the tumour suppressor gene APC are rarely found in prostate cancer (Brewster et al., 1994, Watanabe et al., 1996), it was not surprising to note that APC expression results from the microarray did not differ between the two populations (Table 12). Similarly to the analysis of β-catenin expression, the variation of expression levels between the same populations of different tumour samples was high. Expression was also analysed for GSK3B, the kinase, which phosphorylates β-catenin prior to degradation. GSK3B expression varied among the different probes and no clear trend was seen. It also showed 10 % to 15 % variation between the different populations (Table 12). Expression

levels of Axin, one of the major proteins of the β-catenin destruction complex, remained the same between the two populations with only a minor difference of about 6 % (Table 12).

Target genes of β-catenin mediated Wnt signalling include MYC (c-myc) and CCND1 (Cyclin D1) (Chien et al., 2009). Cyclin D1 showed a very high expression level. It was expressed to a slightly higher extent in the CB population although the variation between samples was again very high (Table 12). MYC expression remained the same in CSC and CB cells with a minor difference of about 0.7 % (Table 12).

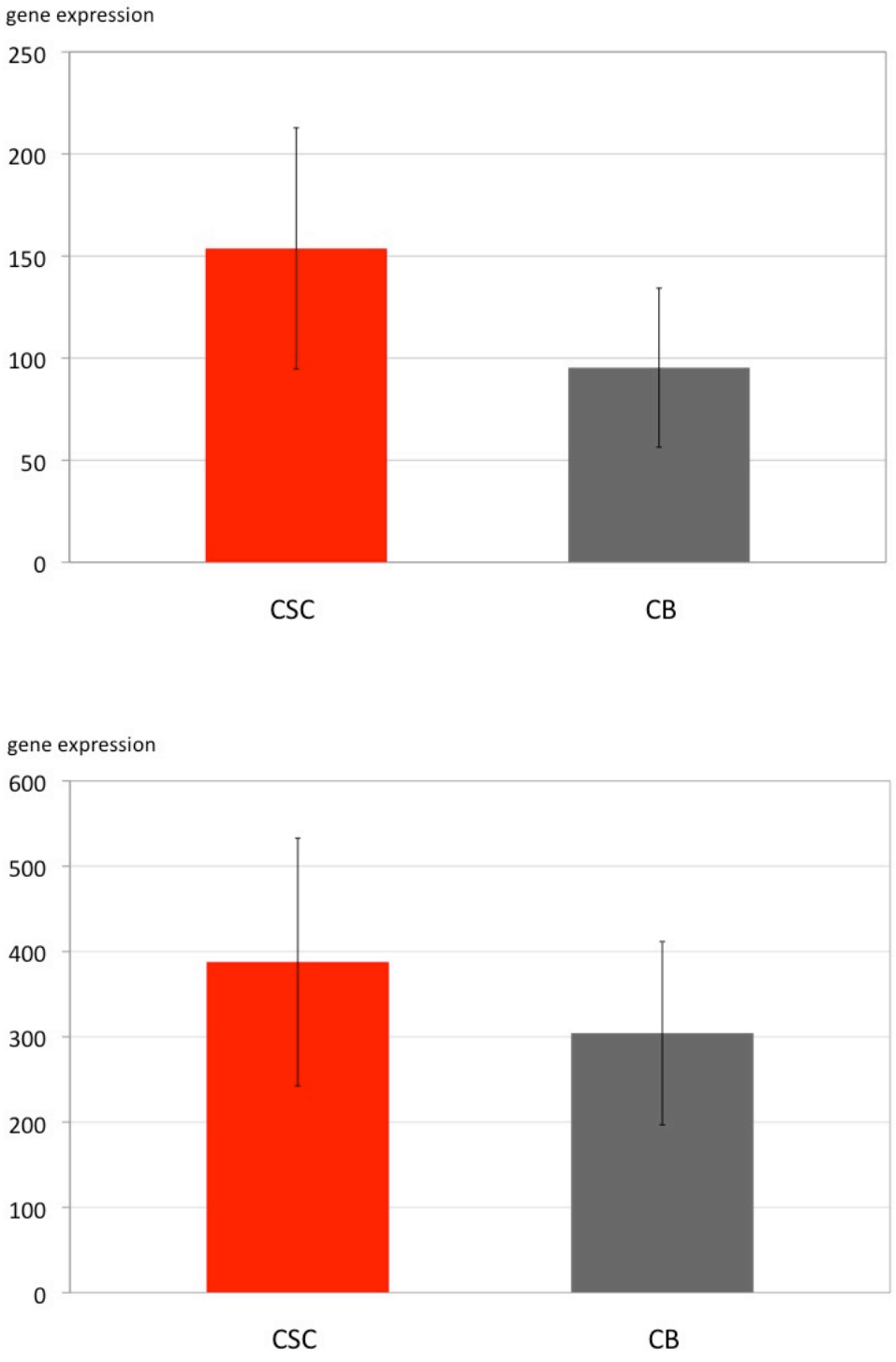


Figure 74: Gene expression of β-catenin (CTNNB1) and TCF4 in CSC and CB cells

Differences in gene expression of β-catenin (top) and TCF4 (bottom) between CSC and CB cells from from 9 prostate tumour samples (Gleason 7 and above). Reanalysed Affimetrix gene expression microarray data (Birnie et al., 2008).

Gene	Name	CSC	CB	Change in expression
APC	Adenomatous polyposis coli	246.0	238.0	3.4%
AXIN1	Axin 1	85.1	80.3	6.0%
GSK3B	Glycogen synthase kinase 3 β	940.3	1063.3	-11.6%
		592.6	514.0	15.3%
CTNNB1	β-catenin	153.7	95.3	61.3%
TCF4	T cell factor 4	387.7	304.2	27.4%
		238.3	157.5	51.3%
		465.0	515.9	-9.9%
		365.3	435.7	-16.2%
CCND1	Cyclin D1	2087.6	2363.1	-11.7%
		683.0	780.3	-12.5%
MYC	c-Myc	894.8	888.8	0.7%

Table 12: Gene expression of genes involved in canonical Wnt signalling

Average gene expression levels according to microarray data from 9 prostate tumour samples (Gleason 7 and above). Levels are given for the CD133$^+$ CSC population as well as the $\alpha_2\beta_1^{low}$ CB population. The percentage (change of expression) indicates to what extent the gene is higher or lower (-) expressed in CSCs compared to CB cells, where expression was set to 100%. Rows indicate expression levels of separate microarray probes of the same gene (Birnie et al., 2008).

4.3.2 Immunofluorescence for β-catenin in primary prostate cultures

As microarray results indicated that β-catenin was differentially expressed in the committed basal (CB) and stem cell (CSC) populations of prostate tumours ($\alpha_2\beta_1^{low}$ / $CD133^-$ vs. $\alpha_2\beta_1^{hi}$ / $CD133^+$), one of the aims of this project was to determine the effect of downregulation of β-catenin in the prostate CSC population, using shRNA-lentiviruses. The scientific question we wanted to answer was if downregulation of β-catenin changed CSCs with regard to growth, invasive properties and metastases formation.

Downregulation at the protein level had to be visualised in order to evaluate experimental results. However, since the CSC population consists of a very small number of cells (about 0.1% of the whole tumour), standard Western Blot, was not feasible, as this method requires large amounts of cells.

Cultured primary tumour cells were therefore examined with immunofluorescence for β-catenin. Cells were stained with antibodies for total β-catenin (C2206; 6B3), dephosphorylated, active β-catenin (anti-ABC clone 8e7) and phosphorylated β-catenin (Phospho-β-Catenin (Ser33/37/Thr41); Phospho-β-Catenin (Ser552)).

4.3.2.1 Immunofluorescence for total β-catenin

Primary prostate tumour samples and benign prostate hyperplasia (BPH) samples were stained to identify β-catenin location within the cells. Depending on its function within the cell, either the regulation of cell adhesion, or signal transduction through the Wnt pathway, there are two different pools for β-catenin in the cytoplasm.

When bound to PSEN1 and cadherin, β-catenin is associated with the actin cytoskeleton and therefore serves as part of the cell adhesion complex. If, however, β-catenin is associated with AXIN, APC and GSK3B, it is phosphorylated and subsequently degraded in the proteasome, as part of an inactive Wnt signalling pathway. This signalling cascade becomes active through Wnt ligands, which results in a stabilised, dephosphorylated β-catenin, which translocates to the nucleus, where it binds to members of the TCF/LEF-1 family of transcriptional regulators (Shitashige et al., 2008) (Figure 75). Therefore, determining the location of β-catenin, already gives an indication of its activity and function within a cell.

Cultured, primary cells were plated onto collagen-coated chamberslides without the addition of STO feeder cells. Cells were fixed after 2 h-4 h to ensure adherence and minimise any effects of the non-feeder culture conditions. Two secondary antibodies (Alexa 488, green and Alexa 568, red) were tested in combination with primary antibody C2206 for immunofluorescence. β-catenin was detected in the cell membrane with both secondary antibodies, however, Alexa 568 gave a slightly stronger signal and was therefore used in subsequent experiments (Figure 76). β-catenin was clearly detectable on the cell membrane in all samples, indicating that it was serving its function in the cell adhesion complex (Figures 77 and 78).

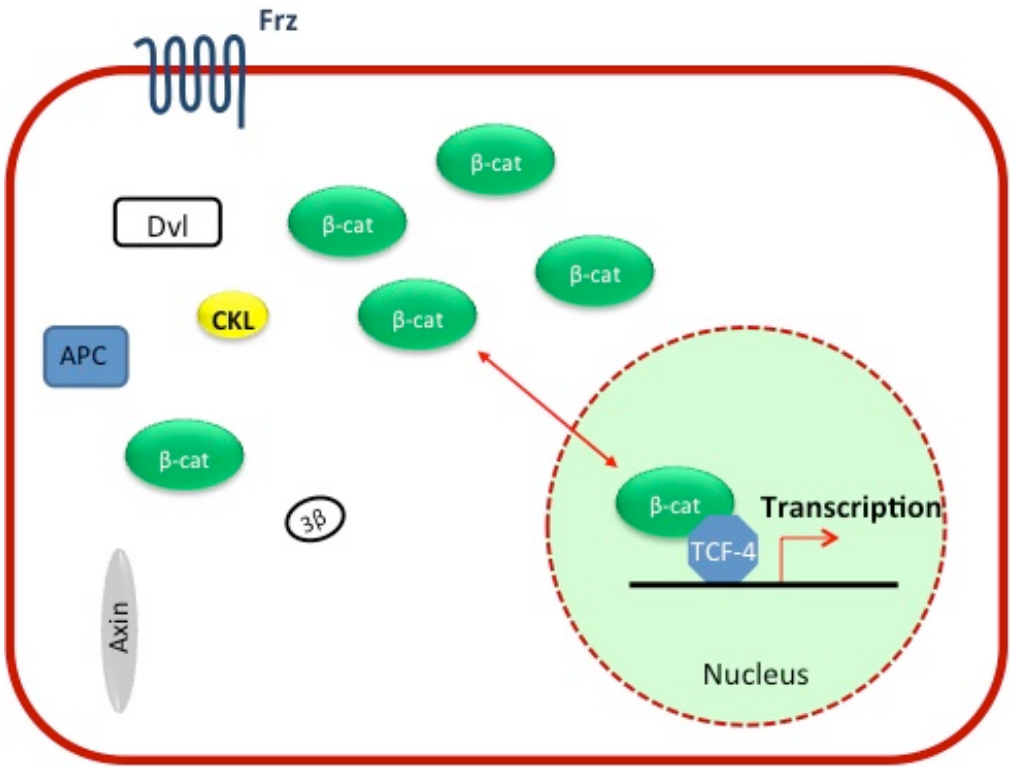


Figure 75: β-catenin localisation within the cell

Aberrantly active Wnt signalling can cause β-catenin to accumulate in the cytoplasm and nucleus, where it can be detected by immunofluorescent staining (Modified from Shitashige et al. 2008).

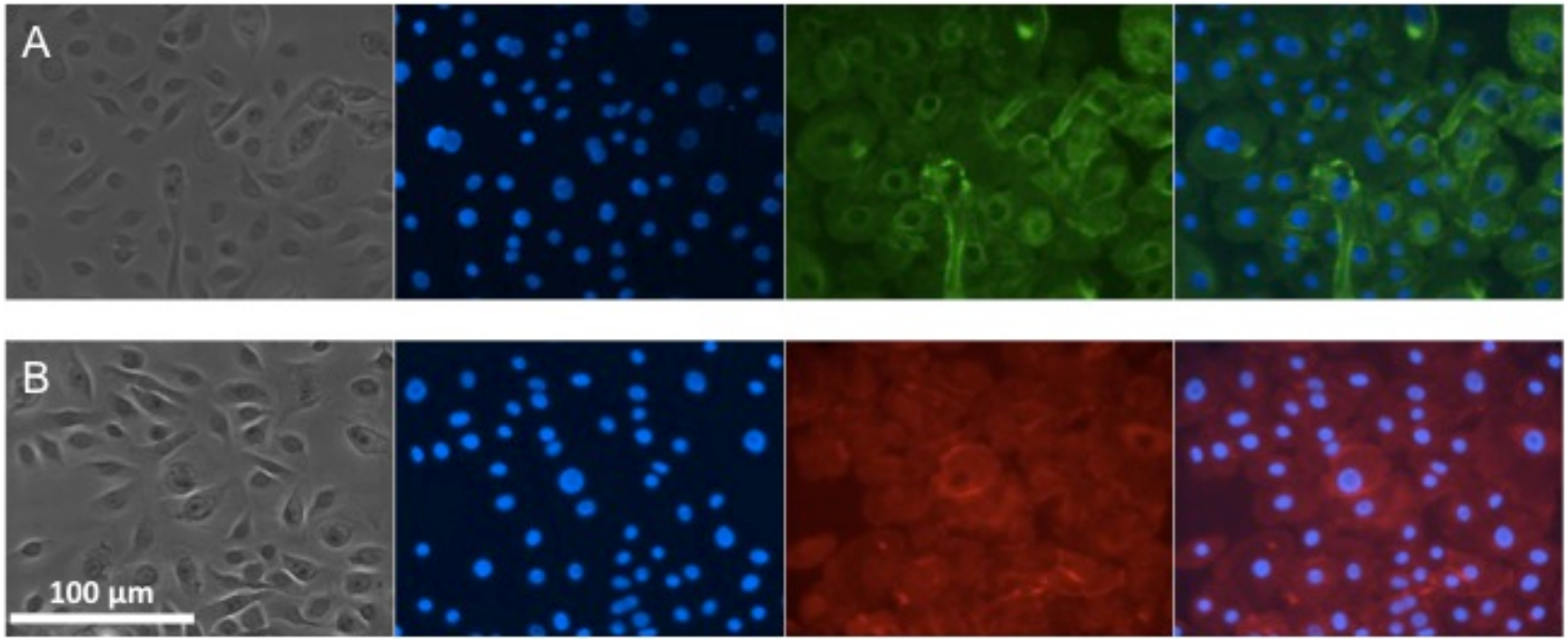


Figure 76: β-catenin staining in sample 007/08 (I)

Images taken of BPH sample 007/08 fixed in 4% FA solution and stained for β-catenin using antibody C2206 and secondary antibody Alexa 488 (A, green) or Alexa 568 (B, red). Cell nuclei were stained with DAPI (blue).

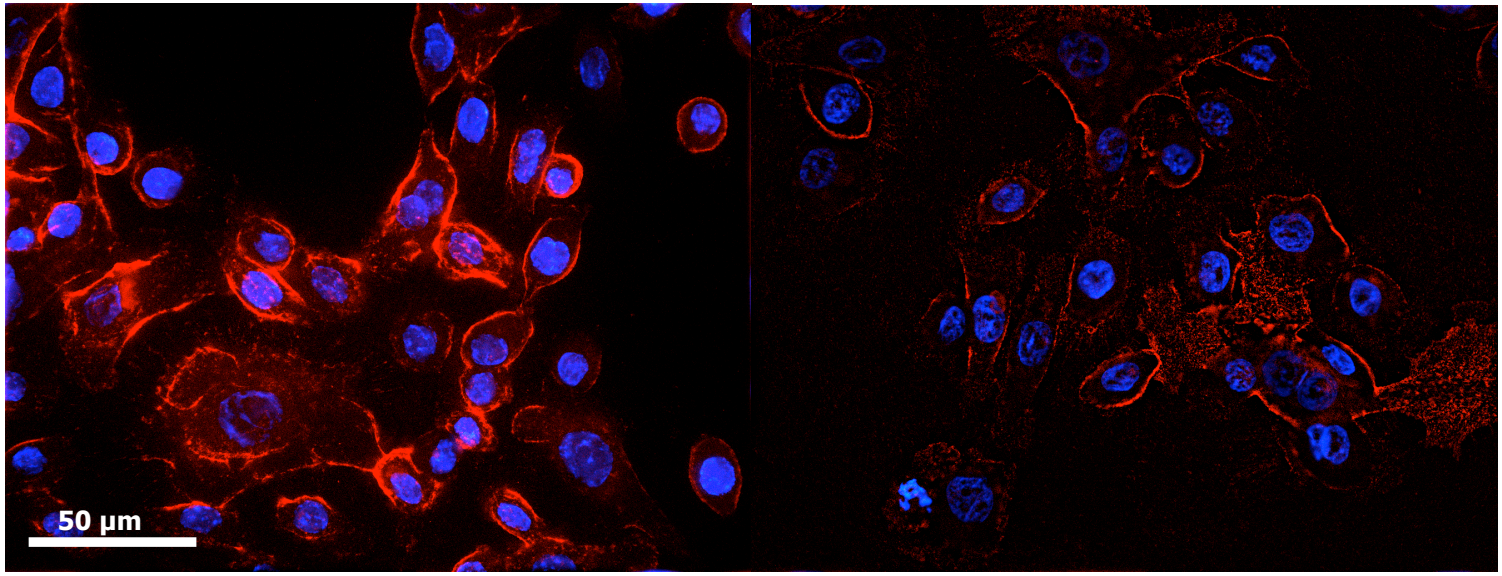


Figure 77: β-catenin staining in sample 010/08

Confocal micrograph of sample 010/08 stained for β-catenin (C2206, red). Extended focus image (left) and image of middle section (right) of z-series, nuclei stained with DAPI (blue).

Figure 78: β-catenin staining in sample 007/08 (II)

Confocal micrograph of BPH sample 007/08 stained for β-catenin (C2206, red), localised at the membrane, nuclei stained with DAPI (blue).

As we wanted to examine the location of β-catenin in the proposed prostate cancer stem cell subpopulation (CD133+), immunofluorescence experiments were extended to cultured primary cells, which had been fractionated by immunomagnetic selection. Cells were selected, then plated onto collagen-coated chamberslides without the addition of STO feeder cells and fixed within 2-4h, prior to staining.

We observed mainly cytoplasmic staining for β-catenin, but also membranous staining in both the CSC and CB population (Figures 79 and 81). Using ImageJ software for colocalisation, β-catenin was found to localise with the nuclear DAPI stain in most cells of the CSC subpopulation (Figure 80). There was also a considerable size difference between most of the CSC and the CB cells. Many CSC appeared 'flattened' out and bigger than the other populations, which indicated differentiation (Figure 80). This is a common phenomenon when cells are plated without feeder cells. Colocalisation images revealed that there was nuclear staining in both populations (Figures 80 and 81), but that there was a higher percentage of cells with nuclear β-catenin in the CSC population than in the CB population, when counted by eye. CB cells generally were more variable in the extent of their nuclear β-catenin staining (Figure 81).

Results imply that β-catenin is still fulfilling its different functions within the cells, being found both in the cytoplasm and on the cell membrane. The variable but still slightly higher appearance of nuclear β-catenin in the CSC population compared to the CB population suggests that β-catenin's function as a transcriptional activator might be more important there, than its role as an adhesion molecule.
However, variable results were observed for both populations and β-catenin might therefore also be a feature of different stages of differentiation of cells within the same subpopulation.

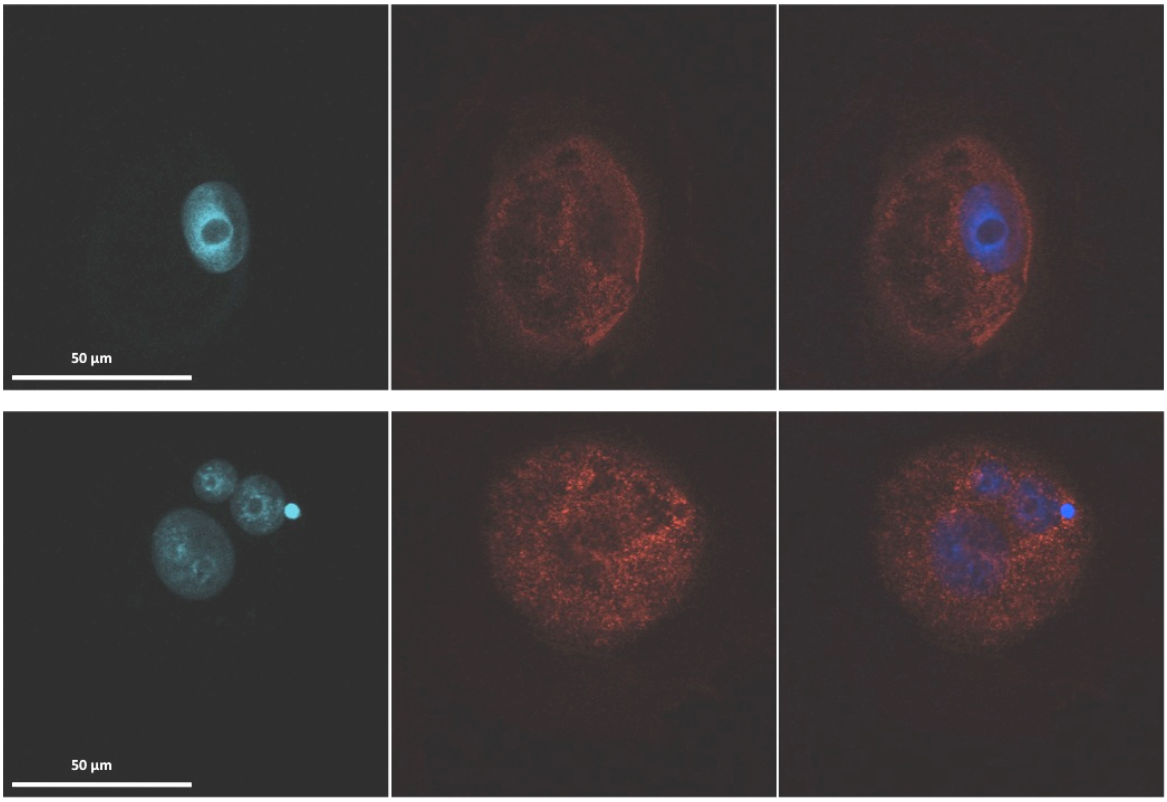


Figure 79: β-catenin staining in CSC subpopulation

Representative confocal micrographs of CSC from primary sample 525 stained for total β-catenin (red), using antibody C2206. Nuclei stained with DAPI (blue).

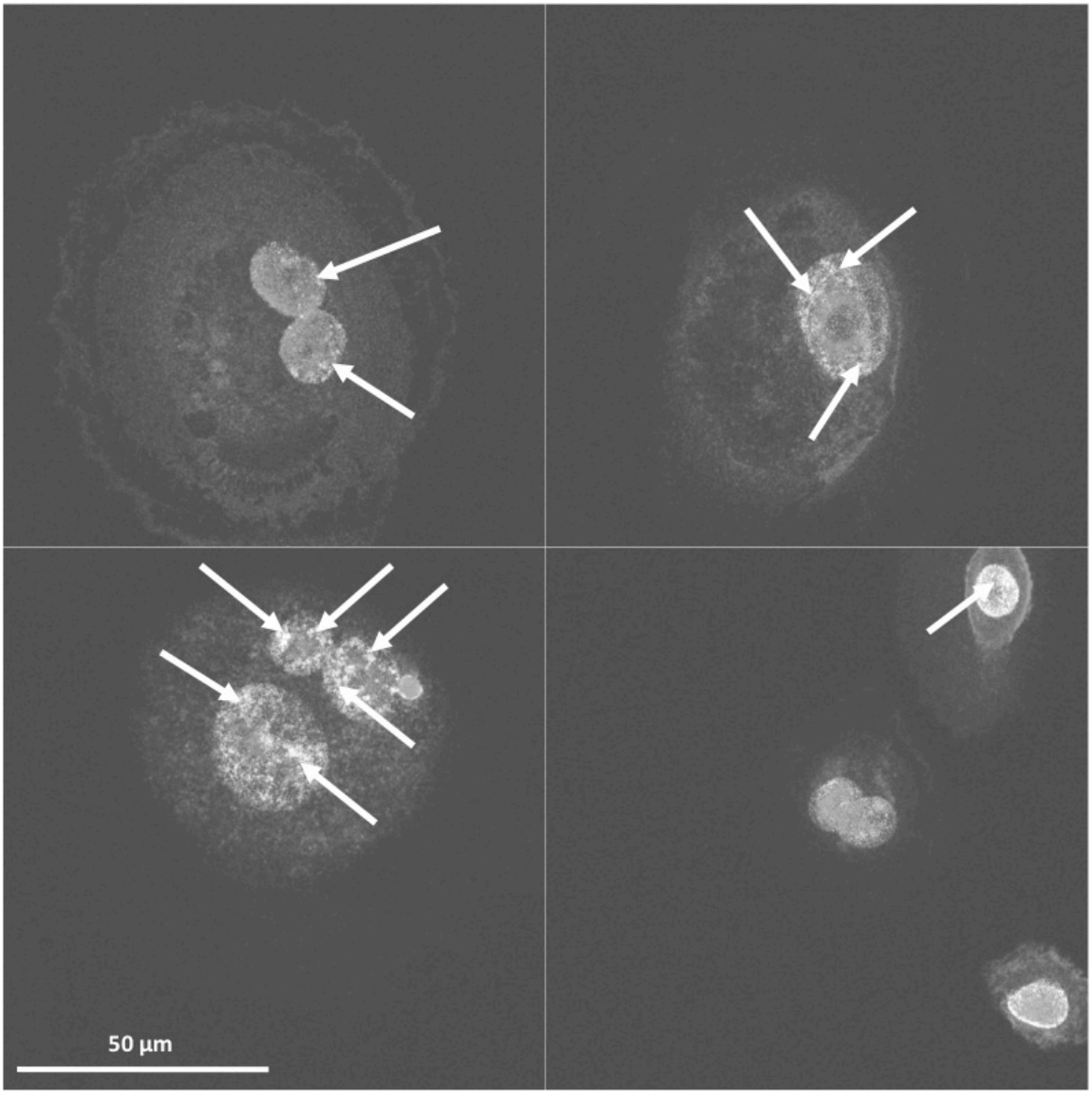


Figure 80: Colocalisation of β-catenin within the nucleus in CSC subpopulation

Colocalised image of cells from primary sample 525 stained for total β-catenin, using antibody C2206 (see previous figure). Colocalised image points are indicated as white spots within nucleus (arrows).

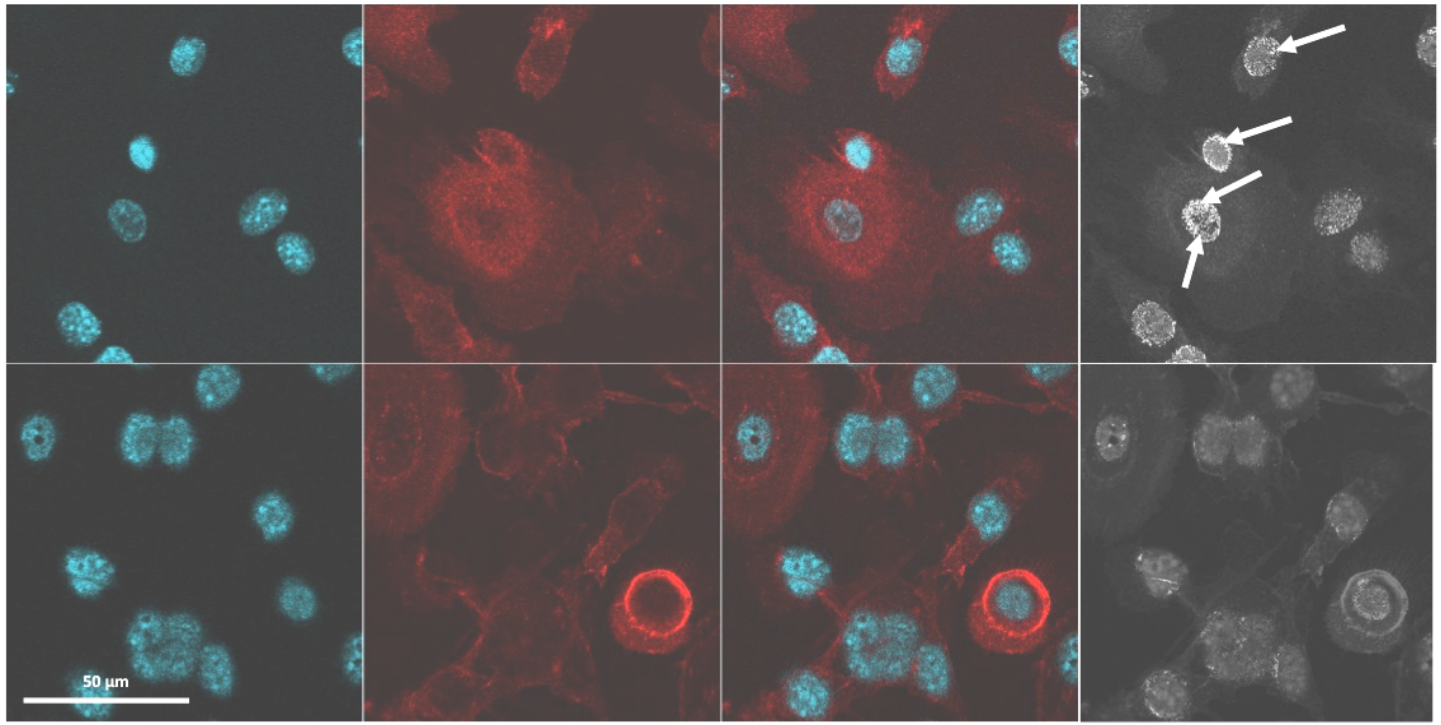


Figure 81: β-catenin staining in CB subpopulation

CB population of primary sample 525 stained with C2206 β-catenin antibody (red). Colocalisation of β-catenin with nuclear DAPI stain (blue) (image on far right, arrows).

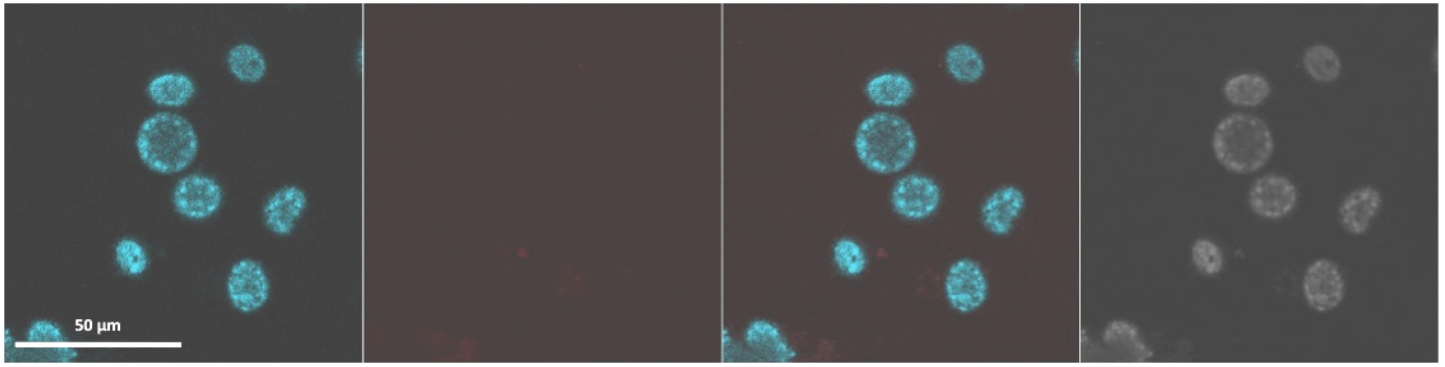


Figure 82: β-catenin staining in CB subpopulation (secondary antibody control)

CB population of primary sample 525, secondary antibody control, including colocalisation (image on far right).

4.3.2.2 Immunofluorescence for dephosphorylated and phosphorylated β-catenin

The phosphorylation status of β-catenin is controlled via the canonical Wnt signalling cascade (van Noort et al., 2002). Therefore, Staal and colleagues developed an antibody to specifically detect β-catenin, when it is dephosphorylated at Serine 37 and Threonine 41 (Staal et al., 2002). The antibody anti-active β-catenin (anti-ABC) has since been made commercially available through Millipore (hybridoma clone 8E7).

As previous dilutions of anti-ABC antibody (1:50 and 1:100) were not successful, a dilution of 1:25 was used on selected CB cells of samples 042/08 and 045/08 (Figures 83 and 84). We observed nuclear β-catenin in cells treated with anti-ABC antibody but as with previous results, the cells exhibited some heterogeneity even within the same subpopulation and sample (Figures 83 and 84).

Apart from anti-ABC, three other antibodies were used for staining, 6B3 (total β-catenin), Phospho-β-Catenin (Ser33/37/Thr41) and Phospho-β-Catenin (Ser552) (see Table 13 for overview).

Phospho-β-Catenin (Ser33/37/Thr41) specifically detects endogenous levels of β-catenin when it is phosphorylated at serines 33, 37 or threonine 41. Phosphorylation is catalysed by GSK-3β and happens after the phosphorylation of β-catenin by CK1 on Ser45 (Amit et al., 2002, Liu et al., 2002, Morin et al., 1997, Yanagawa et al., 2002, Yost et al., 1996).

Phospho-β-Catenin (Ser552) antibody specifically detects β-catenin, which is phosphorylated at Ser552. This phosphorylation occurs through either Akt or PKA and leads to β-catenin accumulation in the nucleus, which increases its transcriptional activity (Fang et al., 2007, He et al., 2007, Taurin et al., 2006).

In the primary patient samples examined, antibody 6B3 resulted in cytoplasmic staining in the majority of cells, however, extensive nuclear staining was also observed. There was some distinct peri-nuclear staining but no membranous staining observed (Figure 85).

Phospho-β-Catenin (Ser552) antibody staining was observed in the area of the nucleus, which indicated its potential for increasing β-catenin transcpriptional activation. Furthermore, there was also clear cytoplasmic staining detected in almost all cells (Figure 86).

The staining observed with antibody Phospho-β-Catenin (Ser33/37/Thr41) was predominately cytoplasmic and nuclear (Figure 87). These phosphorylation sites (Serine 33, Serine 37 and Threonin 41) are used by GSK-3β to destabilise β-catenin prior to its degradation in the cytoplasm (Yost et al., 1996). Even though the nuclear distribution was

contradictory to the current literature, which suggests that phosphorylated β-catenin is rapidly degraded, it has been reported previously, in invasive breast cancer cell lines (Nakopoulou et al., 2006). In one of our samples, staining was particularly weak and few conclusions could be drawn from these results (Figure 87).

Name	Detection	Function	Results	References
anti-active β-catenin (anti-ABC) clone 8E7	β-catenin dephosphorylated at Ser37 or Thr41	Active form (Wnt signalling pathway)	Weak nuclear, some cytoplasmic	Staal et al. 2002
6B3	Total β-catenin	Various	Cytoplasmic, nuclear, peri-nuclear, membranous	http://www.cellsignal.com/products/9582.html
Phospho-β-catenin (Ser33/37/Thr41)	β-catenin phosphorylated at serines 33, 37 or threonine 41	GSK-3β destabilises β-catenin for degradation	Some nuclear, inconclusive	Amit et al. 2002; Liu et al. 2002; Yanagawa et al. 2002; Yost et al. 1996; Morin et al. 1997
Phospho-β-catenin (Ser552)	β-catenin phosphorylated at Ser552.	Induction of β-catenin accumulation in the nucleus and increase in transcriptional activity	Nuclear, cytoplasmic	Taurin et al. 2006; Fang et al. 2007; He et al. 2007

Table 13: Phospho-β-catenin antibodies, their function and IF results

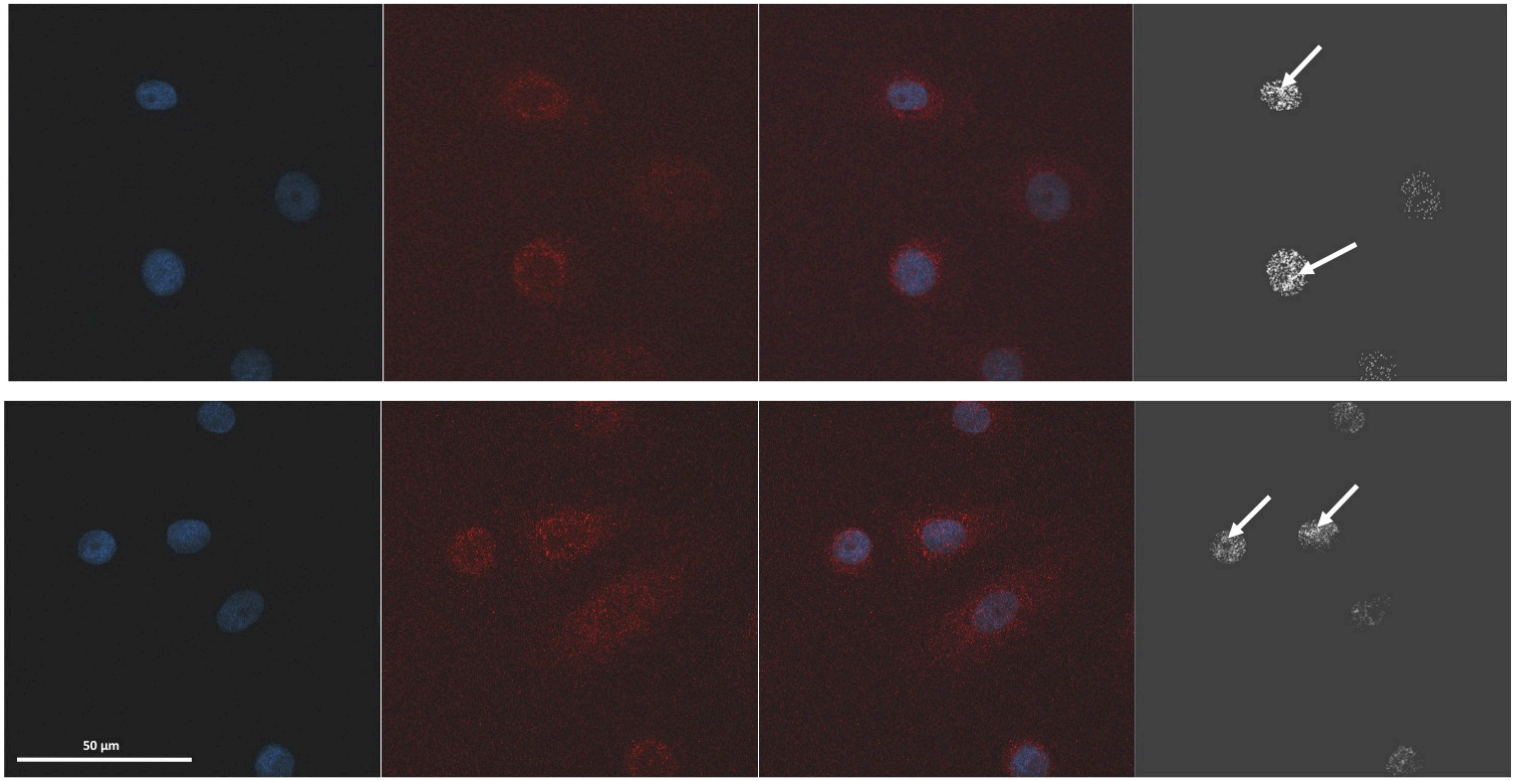


Figure 83: CB cells stained with anti-ABC antibody

Confocal images of sample 045/08, stained for active β-catenin (red), including colocalisation of β-catenin with nuclear DAPI stain (blue) (image on far right, white arrows).

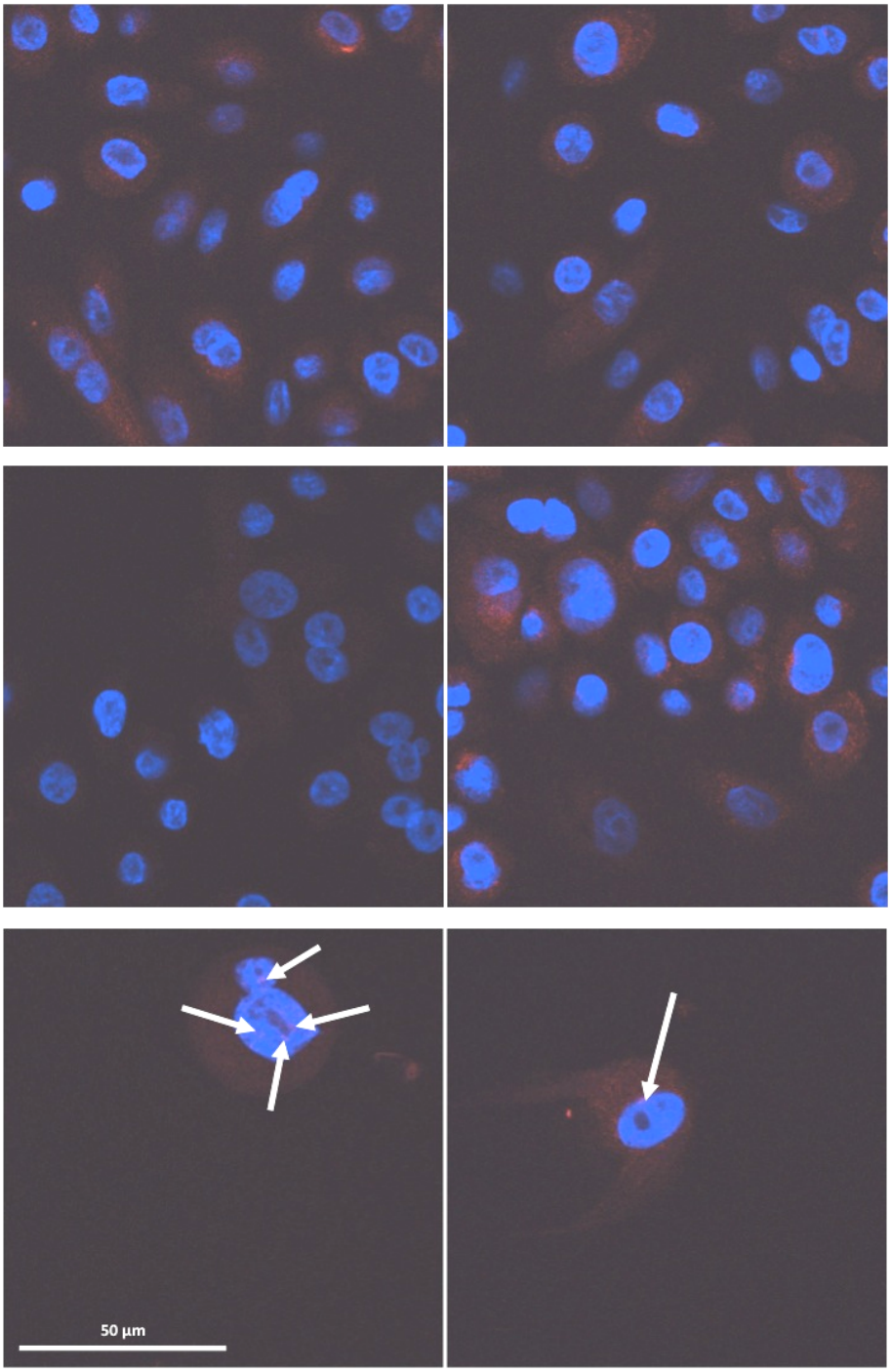


Figure 84: CSC and CB cells stained with anti-ABC antibody

Nuclei stained with DAPI (blue). Nuclear staining of β-catenin (red) indicated by white arrows. CSC (bottom row) and CB (top and middle row), secondary antibody control (middle left).

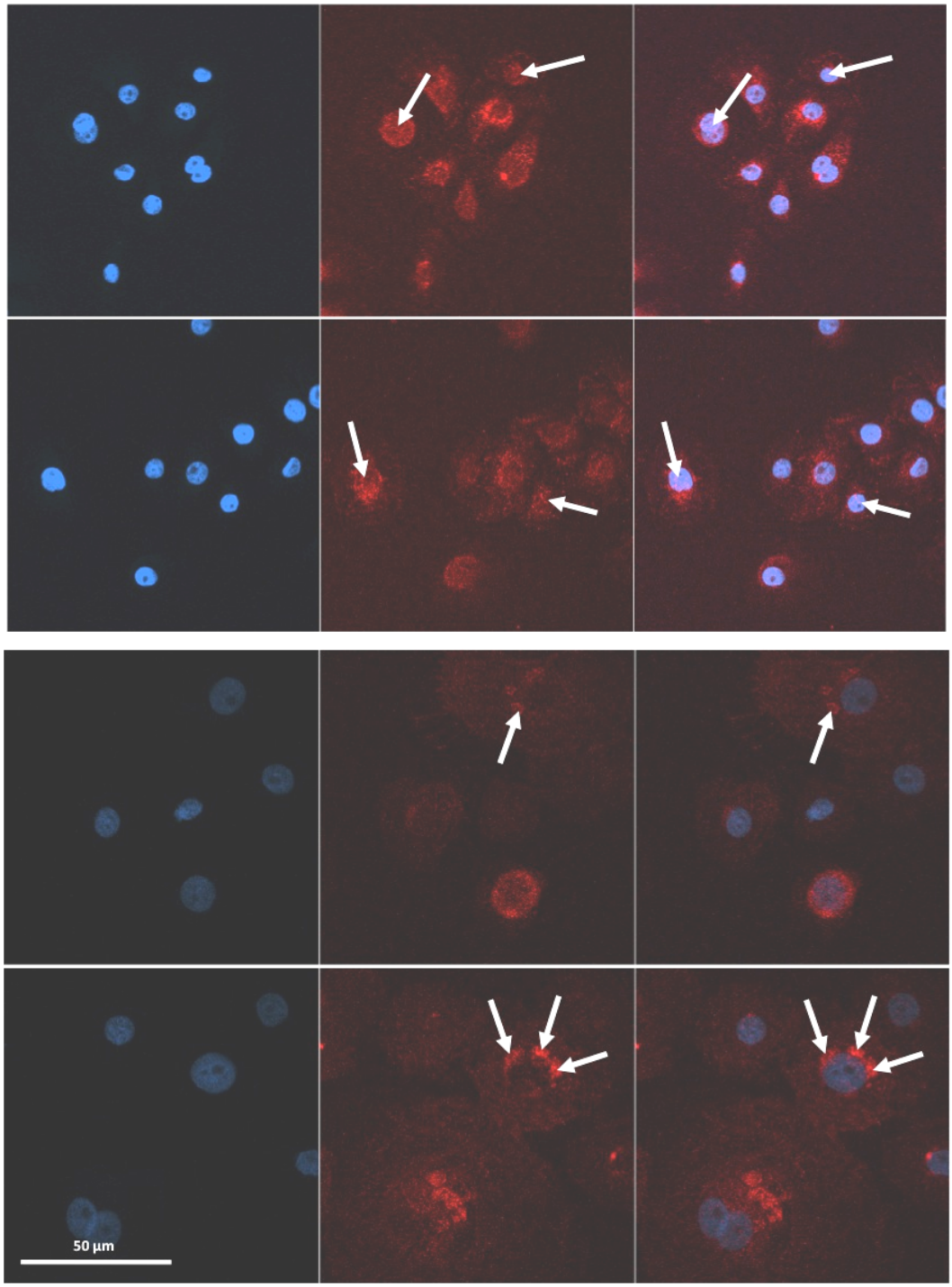


Figure 85: β-catenin (6B3) staining in CB subpopulation of primary sample 042/08 and 045/08

Confocal micrograph of cells stained for total β-catenin (red), using monoclonal antibody 6B3. Nuclei stained with DAPI (blue). Sample 042/08 (top, 2 rows) and 045/08 (bottom, 2 rows). Arrows indicating nuclear and peri-nuclear staining.

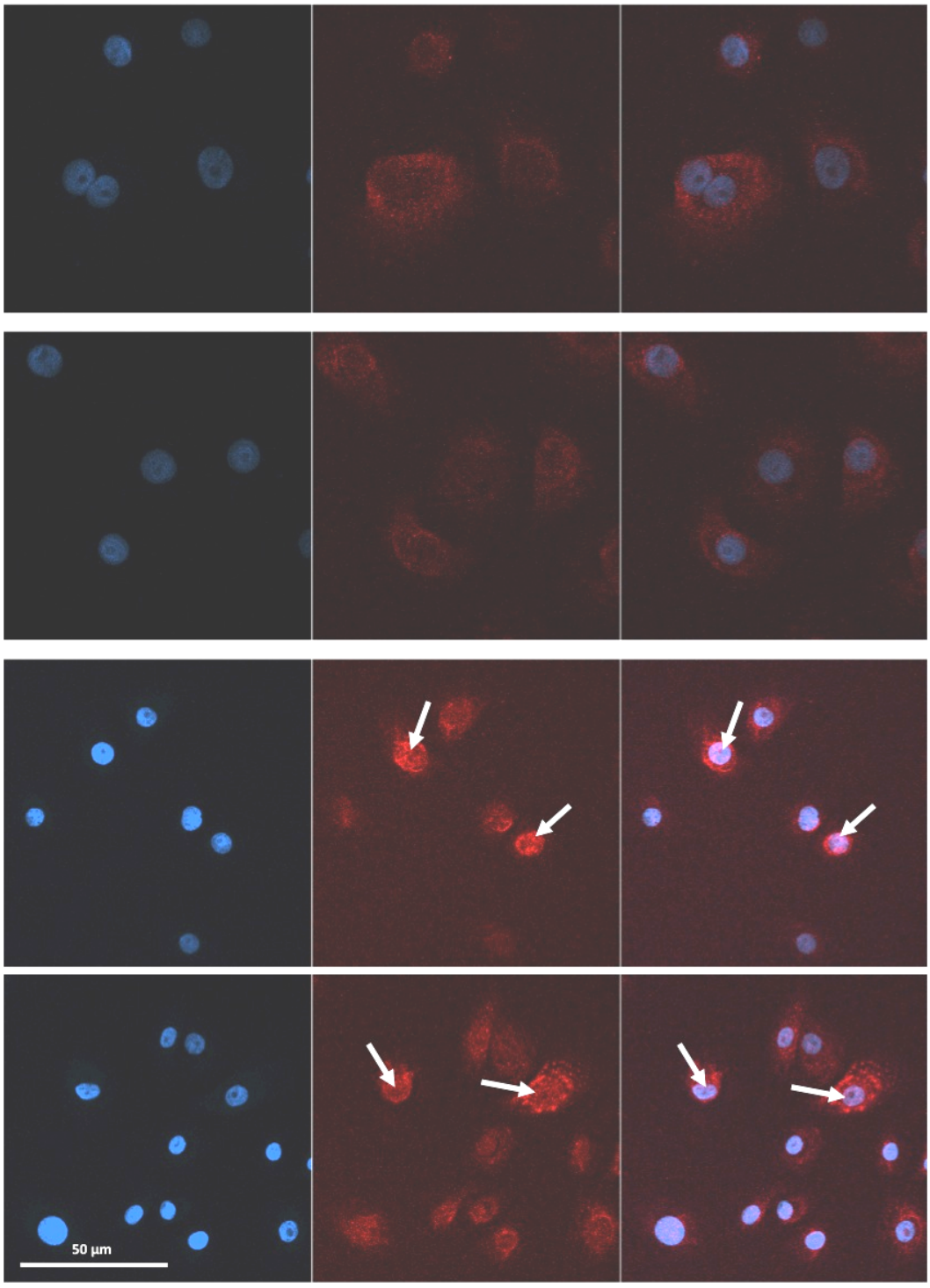


Figure 86: Phospho-β-catenin (Ser552) staining in CB subpopulation of primary sample 042/08 and 045/08

Confocal micrograph of cells stained for Ser552-phosphorylated β-catenin (red). Nuclei stained with DAPI (blue). Sample 042/08 (bottom, 2 rows) and 045/08 (top, 2 rows). Arrows indicating nuclear staining.

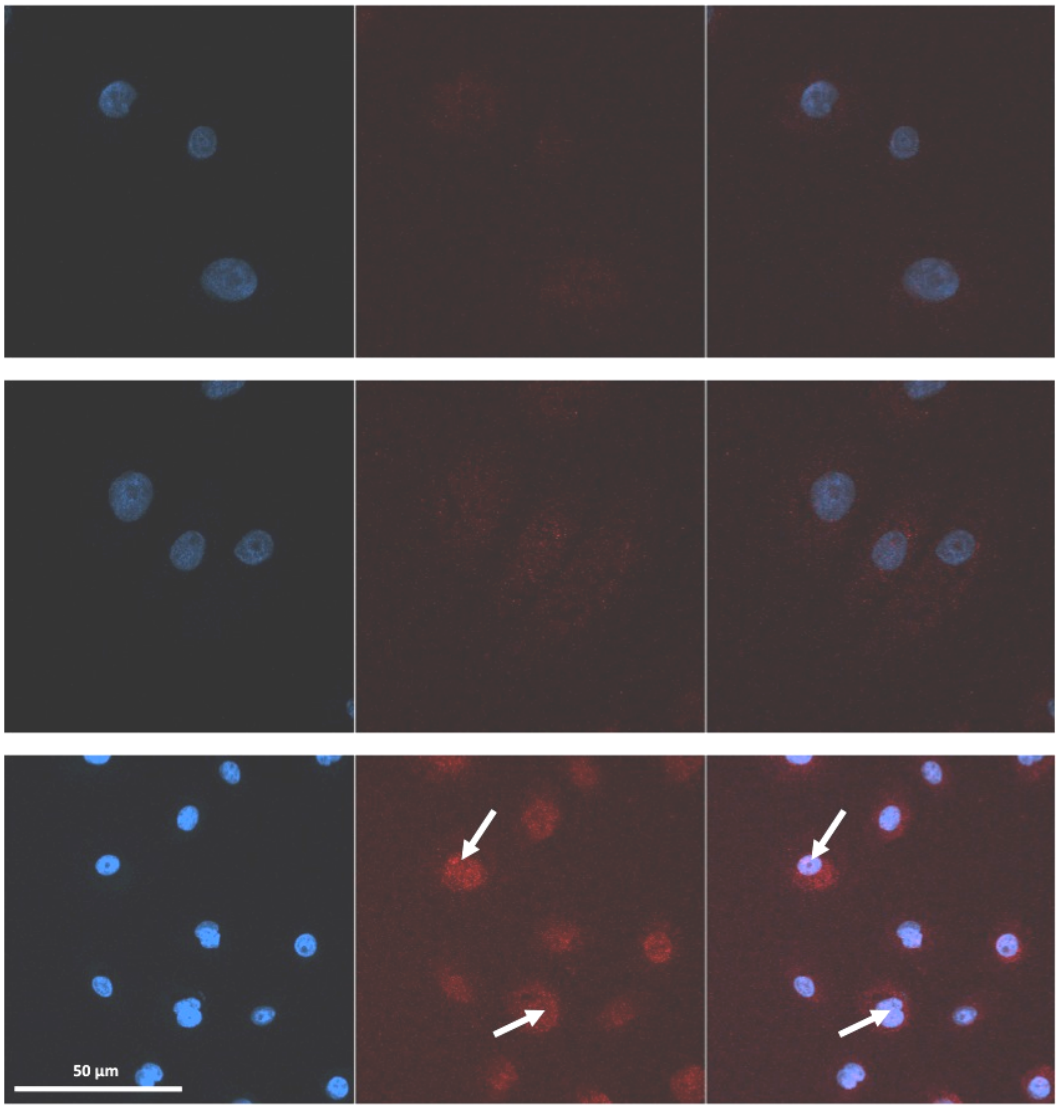


Figure 87: Phospho-β-catenin (Ser33, 37, Thr41) staining in CB subpopulation of primary sample 042/08 and 045/08

Confocal micrograph of cells stained for phosphorylated β-catenin, at Ser33, Ser37 or Thr41 (red). Nuclei stained with DAPI (blue). Sample 042/08 (bottom, 1 row) and 045/08 (top, 2 rows). Arrows indicating nuclear staining.

4.3.3 Infection of primary prostate epithelial cells with shRNA-lentiviruses

4.3.3.1 Targeting lentiviruses to primary cells

Our experiments on PC-3 cells had shown successful application of commercially available lentiviruses for knocking down β-catenin, resulting in slower growth in culture and reduced tumour take in mice. Just like gene-expression profiles which should ideally be produced from primary tumour cells, and not cell lines (Pardal et al. 2003), we wanted to repeat the infection experiments on cultured primary cells to obtain data which would be more closely related to actual conditions in the patient.

Using a gfp expressing lentivirus (3V), the transfection efficiency of commercially available shRNA-lentiviruses in primary prostate epithelium was examined. Cells were selected in puromycin-containing medium from day 3 post infection (p. i.) and they were monitored during selection (Figure 88, day 2 and figure 89, day 7). Most were flattening out to some extent from early in the selection (Figure 88) and the selection was continued for a total of 22 days.

FACS was performed at day 18 p. i. (Figures 90 and 91). The rate of successful infection as measured by gfp expression was low, between 1.6 and 1.7 % (Figure 91). Selection did not influence the number of cells expressing gfp and no extensive cell death was observed in gfp-expressing cells, as measured by DAPI staining (Figure 90).

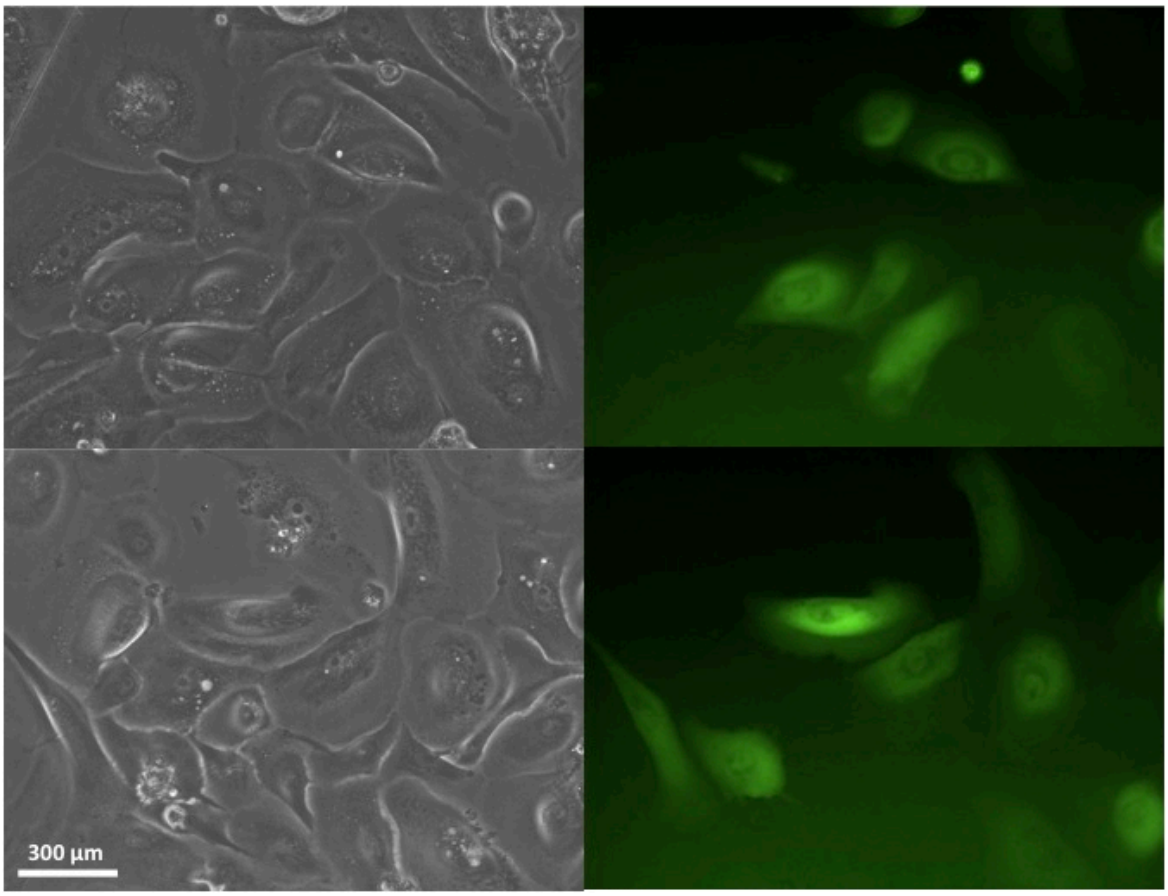


Figure 88: Primary sample 030/09 infected with gfp-expressing control lentivirus (3V), day 2 of puromycin selection.

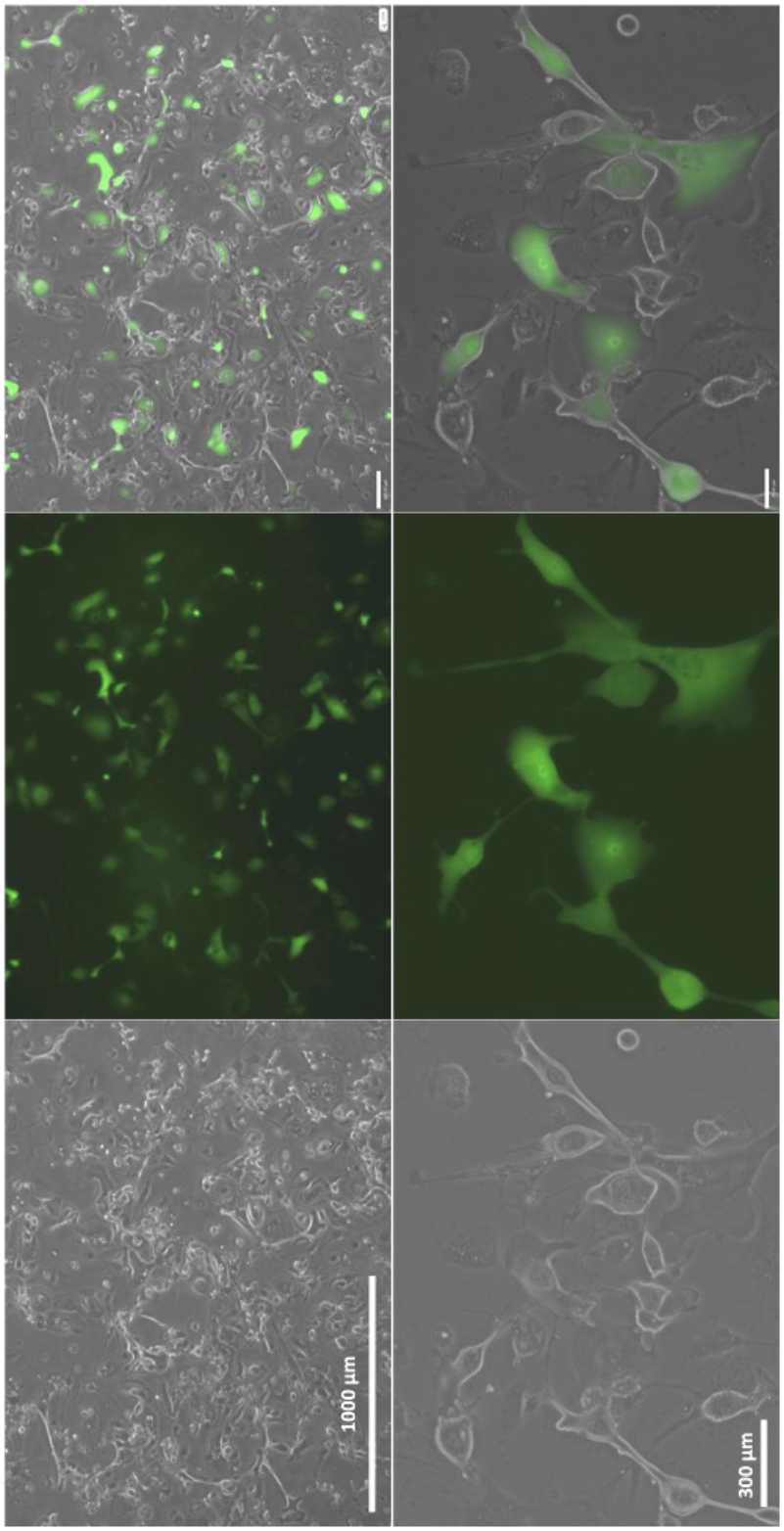


Figure 89: Primary sample 030/09 infected with gfp-expressing control lentivirus (3V), day 7 of puromycin selection

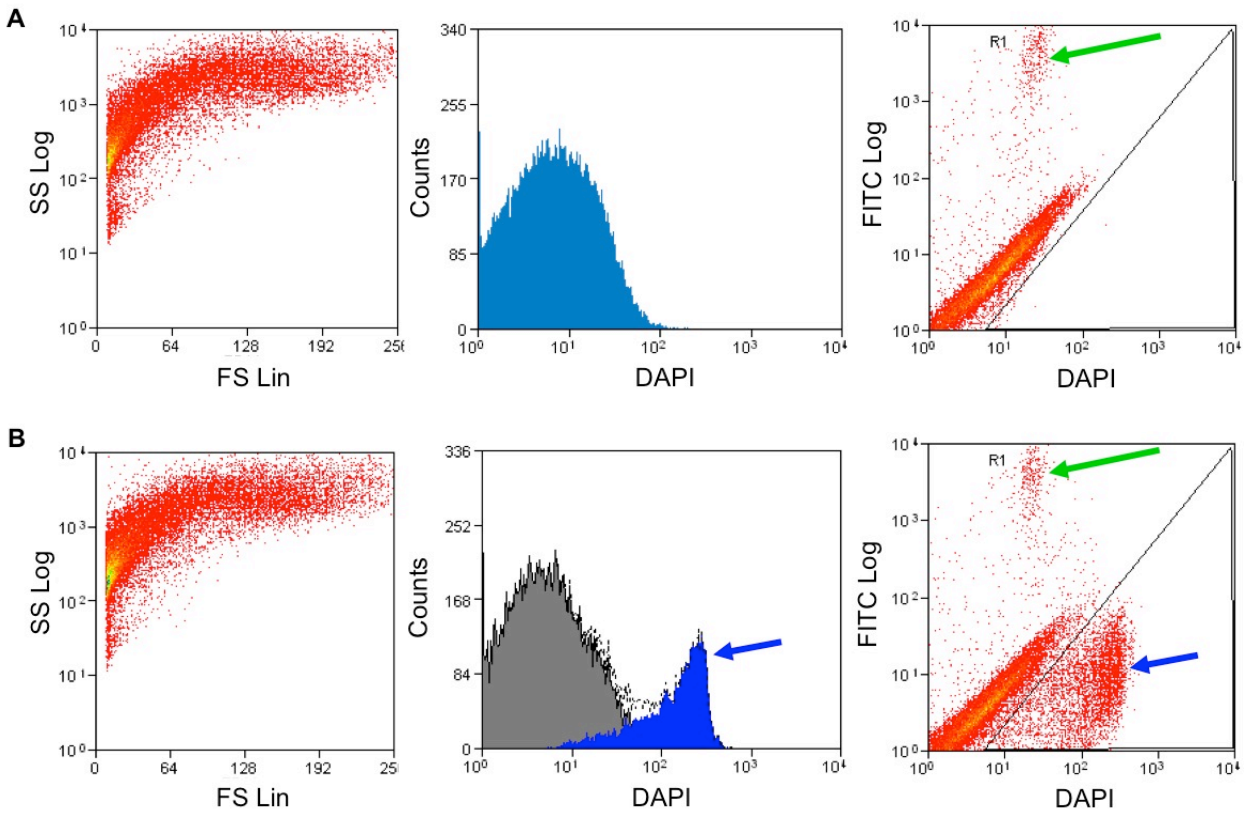


Figure 90: FACS analysis of primary sample 030/09 (I)

Sample 030/09 infected with gfp-expressing control lentivirus (3V, green arrows) at day 18 of puromycin selection, with DAPI (B, blue arrows) and without DAPI (A).

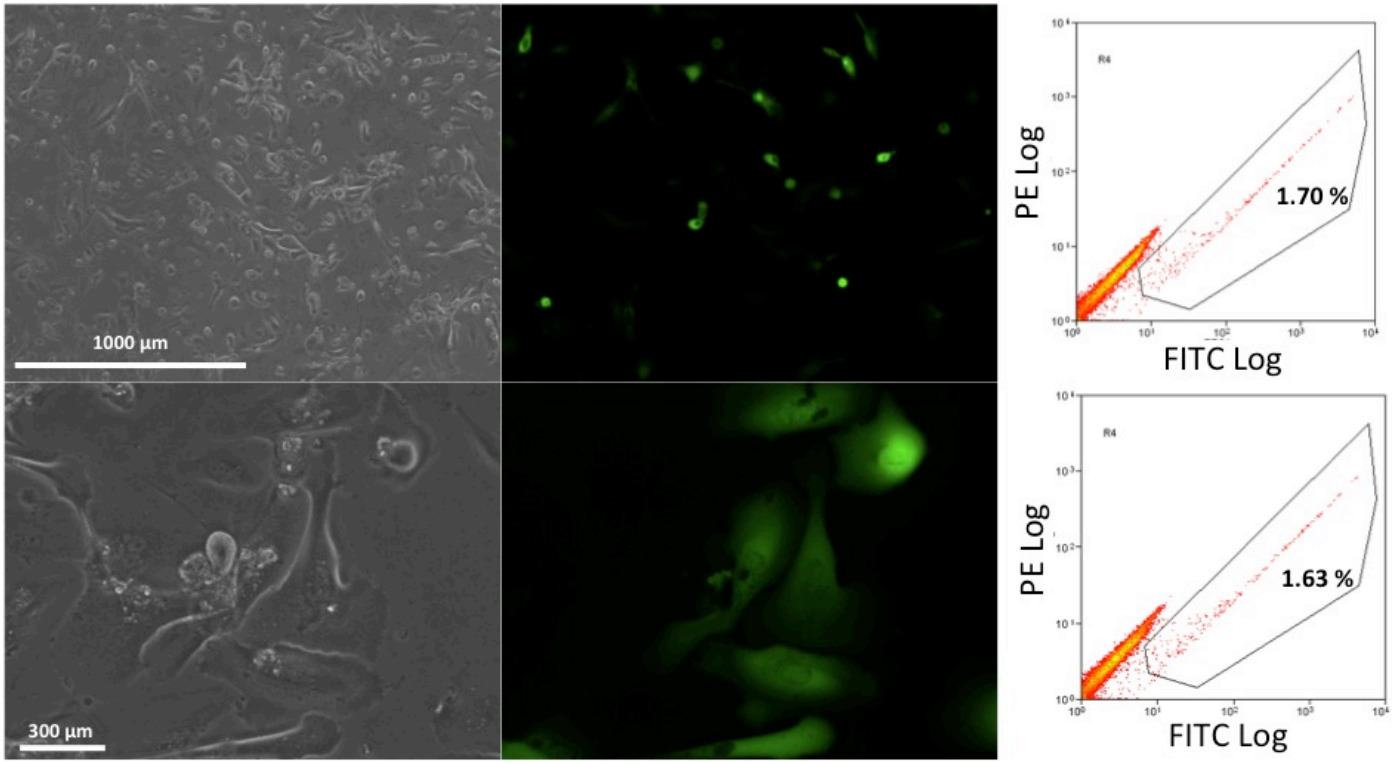


Figure 91: FACS analysis of primary sample 030/09 (II)

Micrographs of sample 030/09 (pre FACS analysis) and FACS analysis (right) to determine infection efficiency of gfp-expressing control lentivirus (3V) at day 18 of puromycin selection (right).

4.3.3.2 Phenotypic changes in shRNA-lentiviruses infected primary cells

During consistent visual monitoring of primary cells infected with shRNA-lentiviruses, we observed, that undifferentiated colonies of epithelial cells did not express gfp. These colonies consisted of small, tightly-packed cells with a distinct light edge and could be clearly distinguished form the more differentiated, flattened epithelial cells (indicated by white arrows in figures), which did express gfp (Figures 92 and 93).

Non-infected cells, which were always monitored as an additional control during infections, showed mostly tightly-packed colonies (Figure 94), while cells infected with sh-β-catenin-lentivirus and the scrambled control virus (Figures 95 and 96) usually exhibited more flattened cells as well as some distinct colonies (indicated by red circles in figures).

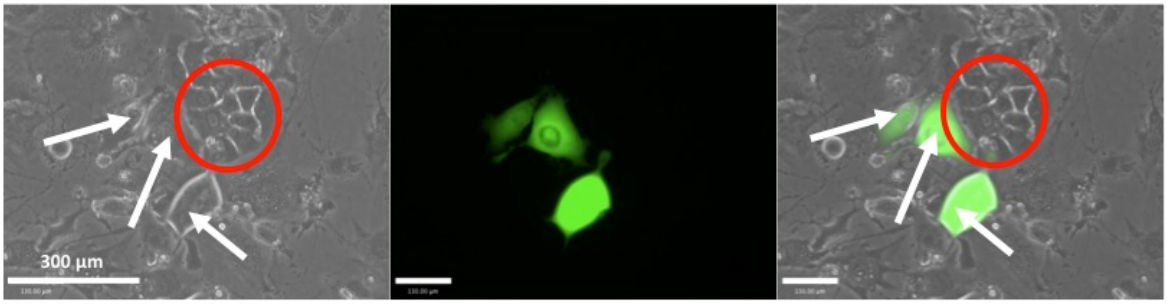


Figure 92: Phenotypic changes in lentiviruses infected primary prostate epithelium under puromycin selection (I)

Sample PE Y 061/09 infected with 3V lentivirus (GFP) at day 3 of puromycin selection, showing undifferentiated colonies (red circles) and more differentiated, gfp expressing cells (arrows).

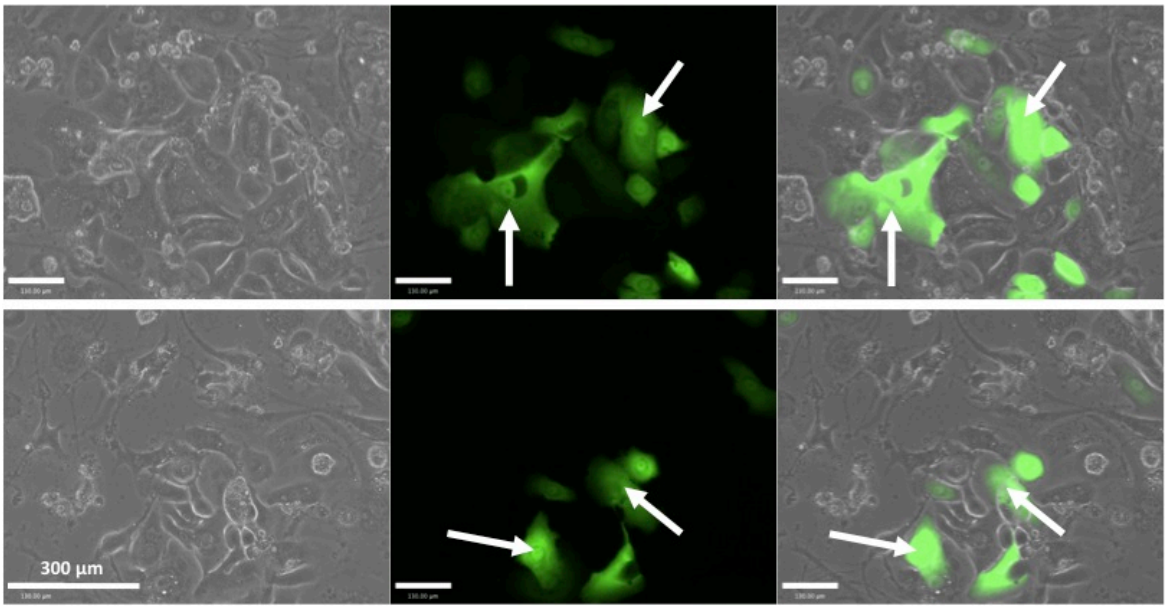


Figure 93: Phenotypic changes in lentiviruses infected primary prostate epithelium in SCM (I)

Sample PE Y 061/09 infected with 3V lentivirus (GFP) post puromycin selection showing flattening of cells (arrows).

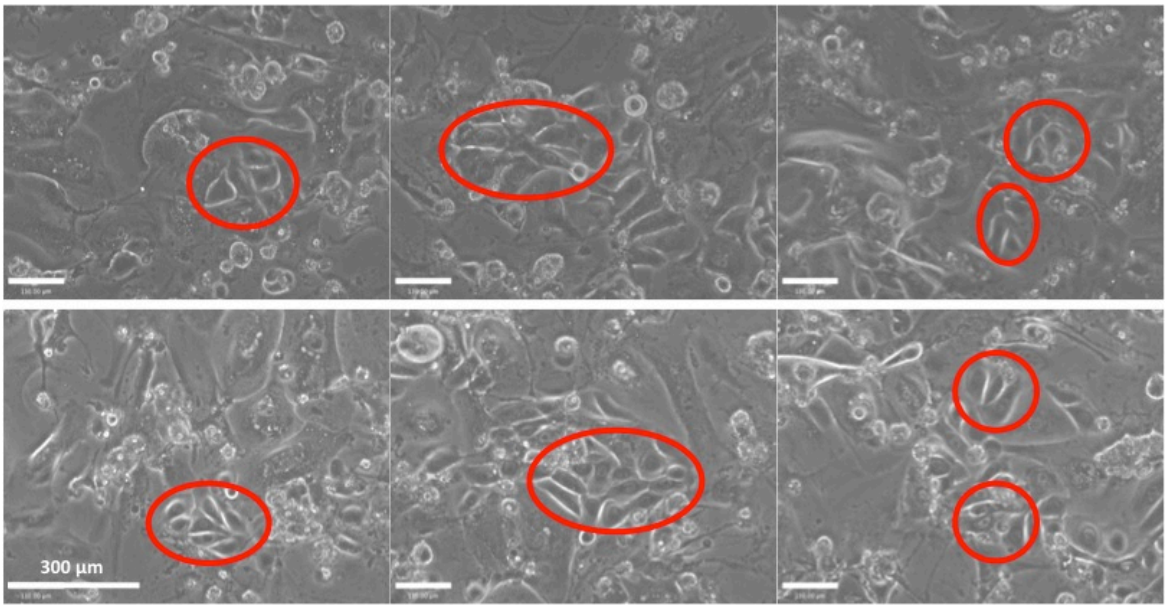


Figure 94: Phenotypic changes in lentiviruses infected primary prostate epithelium in SCM (II)

Sample PE Y 061/09 non-infected cells, showing undifferentiated colonies (red circles).

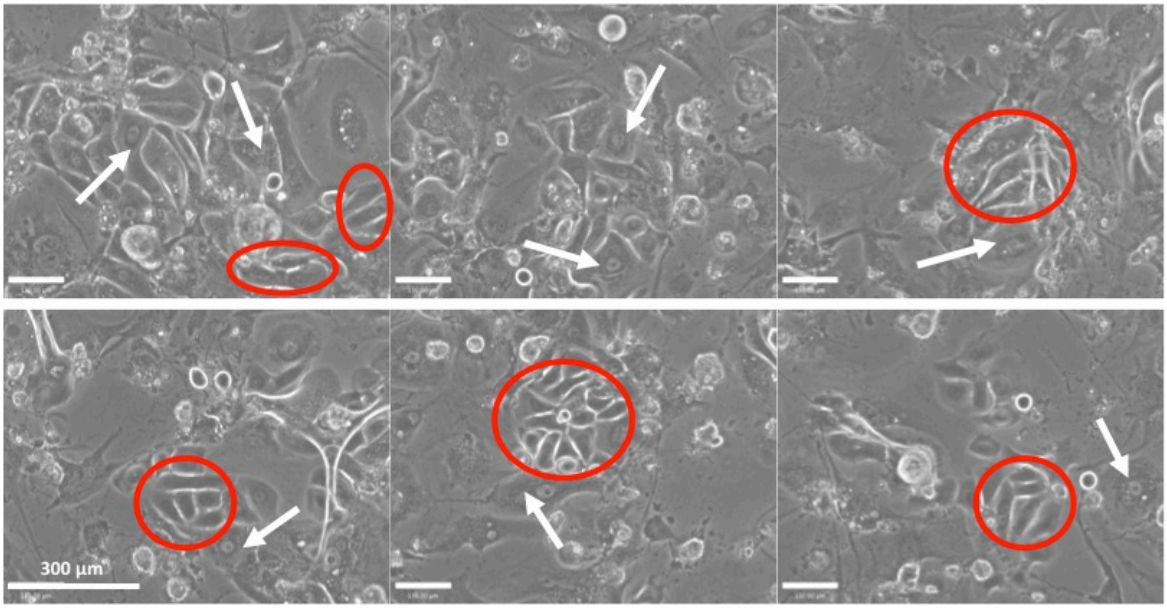


Figure 95: Phenotypic changes in lentiviruses infected primary prostate epithelium in SCM (III)

Sample PE Y 061/09 cells infected with scrambled control sh-lentivirus (top) and sh-β-catenin-lentivirus (bottom), showing undifferentiated colonies (red circles) and some flattened out, more differentiated cells (white arrows).

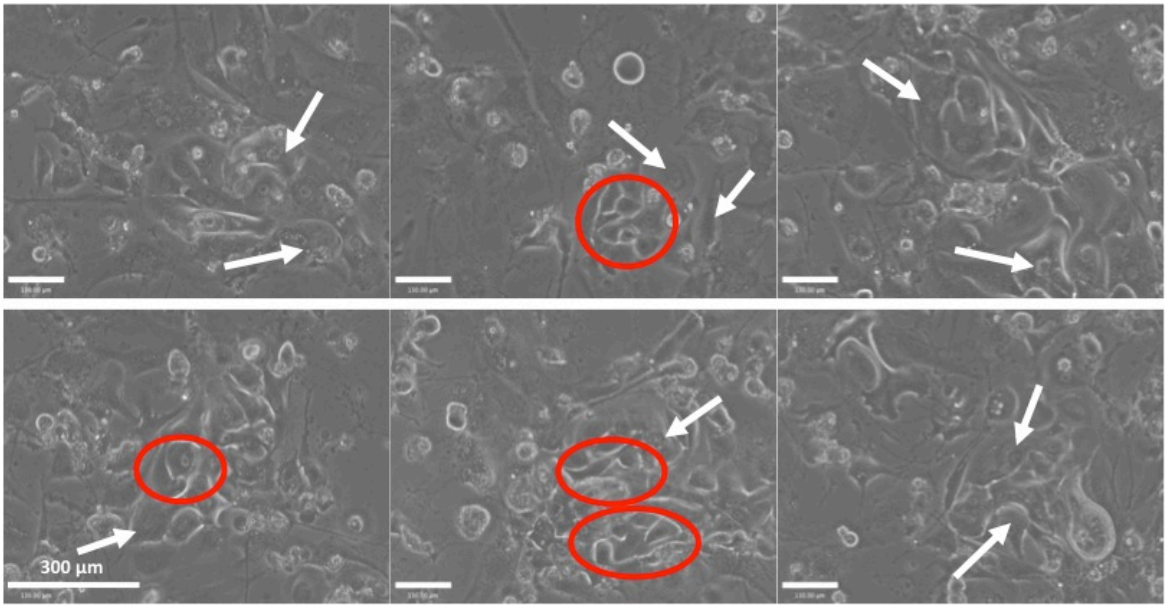


Figure 96: Phenotypic changes in lentiviruses infected primary prostate epithelium under puromycin selection (II)

Sample PE Y 061/09 cells infected with scrambled control sh-lentivirus (top) and sh-β-catenin-lentivirus (bottom), showing mostly flattened out, more differentiated cells (white arrows) and some undifferentiated colonies (red circles).

Cultured primary prostate cells were repeatedly, successfully infected with sh-lentiviruses. They were also successfully drug-selected, however, their growth eventually ceased and no further experiments (examining invasion or cell death, for example) could be performed due to lack of cell numbers. Most infected primary cells also grew larger and flattened out during the selection process, which indicated terminal differentiation. Nevertheless, the potential for valuable data obtained from primary cancer cells is very important and future attempts should focus on improved infection conditions or modified viruses to overcome these obstacles.

Chapter 5

Discussion

5. Discussion

The overall aim of this study was to develop a tool to target prostate cancer cells using shRNA-containing lentiviruses, with a view to study the outcome of targeted gene knockdown in the prostate cancer stem cell (CSC) subpopulation. CSCs represent promising targets for new therapies, as they are the population within a tumour that shows a high potential for tumour initiation (Maitland and Collins, 2008a). Similar properties to those of normal stem cells, such as self-renewal and longevity, have also been attributed to CSCs. Recently, Bae and colleagues found that factors which are normally expressed in pluripotent stem cells, including OCT3/4, Nanog and SOX2, could be found in potential prostate tumour initiating cells (Bae et al., 2010). Semiquantitative RT-PCR was used on DU145 and PC-3 prostate cancer cell lines and patient tissue to evaluate the expression of several reprogramming factors. The cells, which were enriched by FACS for E-cadherin were confirmed to have cancer stem cell-like characteristics in soft agar, spheroid and tumorigenicity assays. The potential prostate tumour initiating cells from DU145 and PC-3 also showed a high tumourigenicity in mice. *In vivo* growth was inhibited by the application of shRNA knockdown of OCT3/4 or SOX2 (Bae et al., 2010).

As a proof of principle, we developed fluorescent reporter gene-containing shRNA-lentiviruses to target PSCA, a highly expressed gene in the abundant committed basal (CB) subpopulation of prostate cancers.
However, thanks to the commercial availability of custom-made shRNA-lentiviral particles, we were able to study the biological effects of β-catenin knockdown in the prostate cancer cell line PC-3. To achieve this, we successfully infected PC-3 cells and generated stable knockdown cell lines, which showed reduced growth, decreased invasiveness and slower tumour growth kinetics in mice. Furthermore, we achieved infection of cultured primary prostate cells with commercial shRNA-lentiviruses.

In the following chapter, the outcomes of this study will be discussed, highlighting potential future uses with respect to studying β-catenin knockdown in primary prostate cells and the potential to develop future therapeutic tools.

5.1 Development of lentiviral vectors with reporter-shRNA-constructs

The aim of this study was to develop functional lentiviral particles that contained a fluorescent reporter gene as well as an shRNA against a chosen target. At the beginning of our study commercial vectors combining shRNAs and fluorescent reporters were not available, which made it necessary for us to develop our own constructs.

Due to the small number of cancer stem cells within the bulk population of a tumour (0.3 – 1.6 %), (Collins et al., 2005), the need to develop methods to monitor this population during development of new therapeutic tools becomes necessary.

Fluorescent reporters have been used extensively to track not only cancer cells both *in vitro* and *in vivo*, but they have also been applied for cell tracking of embryonic and adult stem cells (Consiglio et al., 2004, Stuelten et al., 2007, Sun et al., 2009, Wu et al., 2006). Previous studies from our laboratory have shown that prostate CSCs could be tracked using lentiviral constructs containing the fluorescent reporter genes mOrange and citrine (Frame et al., 2010, Hager et al., 2008).

Delivery of genetic material *in vivo* is often problematic, however, dual-targeted lentiviruses have been shown to successfully infect prostate cancer bone metastases in SCID mice. Transgene expression in the liver was 190 times lower, emphasising the site-specific transgene expression possible with these vectors (Pariente et al., 2007). When C4-2 prostate tumour xenografts were treated intratumorally or intravenously with trastuzumab-bound lentivirus harbouring a prostate specific promoter, targeting was highly specific as shown via viral gene expression in xenografted tumours (Zhang et al., 2009b).

Furthermore, the application of RNA interference tools gives hope for the development of cancer treatments, which are highly specific and could one day potentially become patient-specific as well (Mullenders and Bernards, 2009, Rolle et al., 2010, Stege et al., 2010).

For this study, the more abundant CB population was used initially to establish a working methodology, before moving on to CSCs. Microarray data was available, which highlighted strong expression of the glycosylphosphatidylinositol-anchored cell membrane glycoprotein PSCA in the large population of CB cells in patient tumours (Birnie et al., 2008). Due to PSCA expression being upregulated in a large proportion of both androgen-dependent and –independent prostate tumours (Reiter et al., 1998), PSCA was chosen as our initial target for shRNA-mediated downregulation.

We therefore developed shRNA-containing lentiviruses harbouring three different sequences to target PSCA. The Gateway technology (Hartley et al., 2000) was employed to generate VSV-G pseudotyped self-inactivating lentiviruses containing a fluorescent reporter construct (mOrange or citrine) and a PSCA- or control-shRNA under the control of the U6 promoter.

The fluorescent reporter constructs (CMV promoter, fluorescent gene, polyA) were cloned into the pENTR 5' TOPO vector. The downstream polyadenylation signal (polyA) from simian virus 40 (SV40), used as part of the reporter expression cassettes, has been shown to aid stability of transgene expression (Maxwell et al., 1991, Narita et al., 2000, Shimotohno and Temin, 1981). In our laboratory, it increased fluorescent reporter expression in producer cells as well as target cells infected with CMV-mOrange lentiviruses (Hager et al., 2008). Dual gene expression from lentiviral vectors, using transcriptional units which included a polyA signal, have been succesfully used in epidermal keratinocytes, bone marrow mesenchymal stem cells and hair follicle stem cells (Tian and Andreadis, 2009).

However, it has also been shown that the polyA signal, which can cause truncation of the full-length viral genome (Shimotohno and Temin, 1981), reduces lentiviral titer in CMV and inducible promoter containing constructs (Hager et al., 2008, Liu et al., 2010b). Ultimately, increase in fluorescent protein expression was deemed to be the most important feature for tracking purposes.

After confirmation of the correct inserts for pENTR-CMVmOrangeA and pENTR-CMVcitrineA, sequencing confirmed that the expression cassette was located in opposite orientation to the remaining reading frames on the plasmid backbone (3.1.1). This meant that following recombination with the shRNA-construct-containing plasmid, the resulting expression vector contained both expression cassettes in opposite orientations.

It has long been understood that transcriptional interference can cause a decrease in expression levels in tandem promoter constructs (Greger et al., 1998, Proudfoot, 1986). Having the two promoters in opposite orientation could therefore be beneficial as it was previously shown that, to yield satisfactory expression levels, expression constructs could be arranged in opposite reading frame orientation (Maetzig et al., 2010).

However, Eszterhas and colleagues found that there was a difference between convergent and divergent arrangement of promoters which were facing in opposite directions. In

addition, the integration site as well as the gene's orientation within the integration site played a role in suppressing expression in double constructs (Eszterhas et al., 2002).
To optimise constructs for future studies, vectors could be modified to harbour so-called insulator sequences (Bell et al., 2001). Under natural circumstances, promoter interference is avoided by separating genes using these DNA sequences. Insulators have been shown to reduce the effect of transcriptional interference between two promoters within the same construct, including in lentiviral vectors (Hasegawa and Nakatsuji, 2002, Tian and Andreadis, 2009). Examples of insulators include MAR (beta-interferon matrix attachment region) (Girod et al., 2007), H19ICR (H19 imprinting control region) (Yoon et al., 2007) and cHS4 (chicken hypersensitive site 4) (Arumugam et al., 2007, Arumugam et al., 2009, Urbinati et al., 2009).

Our aim was to use shRNAs with a stem loop of 29 nucleotides, which had the advantage of increased specificity over 21mer constructs (Kim et al., 2005b, Siolas et al., 2005). This could potentially reduce the number of side effects due to off-target effects, if constructs were to be used in patients in the future. Established design protocols (Invitrogen BLOCK-iT™ RNAi Designer) were applied to generate shRNA constructs using published sequences (Origene HuSH 29mer RNAi collection).
Confirmation of hairpin constructs by sequencing can prove challenging and these challenges have been reported widely in the literature (Devroe and Silver, 2002, Guo et al., 2005, McIntyre and Fanning, 2006, Miyagishi et al., 2004, Yu et al., 2003). Taxman and colleagues developed strategies for optimal sequencing of such constructs (Taxman et al., 2006). Some problems during sequencing were also observed in this study in the first instance.
Initial sequencing attempts resulted in break off after a few base pairs into the hairpin structure. After consulting the sequencing company about alternative methods, sequencing conditions were successfully optimised, and sequencing confirmed the correctness of our constructs. We achieved the generation of multiple clones for most plasmids.
Plasmids were then tested for functionality of the fluorescent reporter, which was confirmed by transfection into prostate cells, pre and post recombination. Following this we produced infectious lentiviral particles. The titres obtained were considerably lower than suggested by the manufacturer. We successfully improved lentiviral titres by time course titration, using the Invitrogen Lentiviral Gateway Kit. However, titres obtained were still too low to conduct successful infection experiments. With the present titres, a

larger volume of crude virus supernatant than was available at the time would have to be used in order to gain a suitable MOI. Poluri and Sutton also described the low titre phenomenon in shRNA-lentiviruses. They found that HIV-based vectors encoding for shRNAs produced a 30-fold lower titre than the comparable empty vector controls (Poluri and Sutton, 2008). Even after much trouble-shooting and discussion with Invitrogen by colleagues and myself regarding optimal virus production, no reassurance was given that titers could be improved.

Suggestions for improving lentiviral titres are readily available in the literature and span a variety of methods. First of all, inhibitors of the dicer-dependent RNAi pathway could be employed. One example for increasing titres was to include the adenovirus VA.1 RNA (Andersson et al., 2005) or the nodamura virus B2 protein (Sullivan and Ganem, 2005). Including these components increased titres to levels similar to the empty vector control (Poluri and Sutton, 2008). Co-expression of RNA-silencing suppressors of viral and plant origin (VP35, E3L, NS1 or P19, respectively) have been used to increase HIV-1 virus yield in HEK293 cells (de Vries et al., 2008, Haasnoot et al., 2007).

A similar line of thought uses (over-) saturation of the RNAi pathway in the cell. In accordance with the above findings, Liu and colleagues reported recently, that saturation of the RNAi pathway could be used to rescue vector production (Liu et al., 2010b). Furthermore, the amount of shRNAs within one plasmid could be increased to 5, or plasmids harbouring an shRNA target decoy, with the corresponding shRNA sequence, could be co-transfected into virus producing cells (Liu et al., 2010b). To inhibit the RNAi pathway in producer cells directly, siRNAs targeting Dicer (Paddison et al., 2002), or shRNAs against Drosha (Liu et al., 2010b) have been investigated. It is still controversial whether self-targeting in shRNA vectors, is a major cause for titer reduction (Poluri and Sutton, 2008, Zhou et al., 2009), due to the fact that different lentiviral vector systems have been studied, thus preventing direct comparison of the results.

Lastly, promoters used for expression of genes contained on lentiviral vectors have been under discussion in the literature for some time now, including their role in titre reduction of RNA vectors. In one study it was found that the lentiviral production system used for miRNA vectors seemed to be incompatible with the CMV promoter. Replacement of this promoter with a tetracycline inducible promoter increased titres up to 400-fold (Liu et al., 2010b). Improvements could therefore be achievable by exchanging the CMV promoter for another universal, e.g. eF1α or β-actin, or prostate tissue specific promoter (PSA/Pb), as has been shown in a recent study (Hager et al., 2008).

Even though improvement of viral titres was achieved through protocol optimisation, no high titre stocks could be produced. However, commercial shRNA-lentiviruses (Sigma Mission® shRNA lentiviral particles) became available at that time and we chose to use these particles, in order to move on to studies of biologically relevant functions in prostate cells treated with shRNA lentiviruses. This enabled us to focus on the main aim of this study – the investigation of pathways relevant in prostate CSCs. β-catenin was chosen for developing this study further, because of its relevance in signalling and cell-adhesion in cancers and its role in cell differentiation in embryonic stem cells (Birnie et al., 2008, Cleton-Jansen et al., 2009, Jiang et al., 2007, Kanwar et al., 2010, Lu et al., 2009, Mishra et al., 2009, Takahashi-Yanaga and Kahn, 2010, Wang et al., 2010).

5.2 Generating prostate cancer cell lines with stable β-catenin knockdown

We were interested in how β-catenin and Wnt signalling affect prostate tumours and especially their relevance to the prostate CSC population. Our primary aim was to produce a suitable cell line model for the knockdown of β-catenin to study its biological effects and to investigate possibilities to carry these experiments forward into cultured primary cells, for the first time.

It has been shown in colon cancer that invasiveness might be the principal trait most commonly associated with potential CSCs. Fodde and Brabletz showed that the cells at the invasive front of a tumour contained more nuclear β-catenin than the rest of the tumour cells, thus providing evidence that β-catenin/Wnt-signalling might have an important role in the invasive property of CSCs (Fodde and Brabletz, 2007).

The PC-3 prostate cancer cell line was chosen, as these cells were derived from a bone metastasis (Kaighn et al., 1979) and therefore represented an aggressive form of prostate cancer, providing us with a good model in which to study biological features such as invasiveness. Additionally, tumour growth from PC-3 cells can also be studied in immunocompromised mice, where they can give rise to metastases (Waters et al., 1995).

In addition, the prostate cancer cell lines LNCaP and P4E6, representing different stages of prostate cancer differentiation (Horoszewicz et al., 1980, Horoszewicz et al., 1983, Maitland et al., 2001) were also used in this study. As PC-3 cells were the most robust and gave highly reproducibly results, these cells were taken further into biological studies. Another reason for choosing PC-3 cells was that they represent an androgen-independent prostate cancer model (Kaighn et al., 1979). This was important as we wanted to continue our studies in primary prostate cancer cells, particularly the CSC population, which do not express androgen receptor (Maitland and Collins, 2008b). PC-3 cells were therefore closer to our long-term study model than other cell lines, such as the androgen responsive LNCaP cells (Horoszewicz et al., 1980, Horoszewicz et al., 1983).

We successfully generated stable cell lines harbouring an shRNA against β-catenin or a scrambled control, respectively, the former showing stable knockdown at the protein level over the course of antibiotic selection and long-term culture.

The stability of knockdown achieved (3.2.4), due to the integration of lentivirally transferred expression cassettes into the DNA of infected cells, permitted experiments on long-term cultures.

5.2.1 Lentiviruses as the vector of choice

Lentiviruses integrate into their host cell genome upon infection, therefore providing a tool for long-term studies. They are also able to infect both dividing and non-dividing cells, which gives them an advantage over other viral vectors (Buchschacher and Wong-Staal, 2000). As (cancer) stem cells are either dormant or only very slowly dividing, and we decided to target the CSC population in prostate cancer as the ultimate aim of this study, lentiviruses seemed an obvious choice. Furthermore, lentiviruses containing shRNAs have recently been used to study the Wnt signalling pathway in different cell lines, including prostate cancer cells (Fiorentino et al., 2008, Yochum et al., 2010, Zeng et al., 2008).

The commercial viruses we used for this study (Sigma Mission® shRNA lentiviruses) have now been used extensively in cancer research. They have been shown to infect CSCs in human glioma (Bao et al., 2008), leading to growth arrest during a kinase screening study (Yang and Stockwell, 2008) and were able to suppress growth and metastasis formation in pancreatic cancer (Wei et al., 2008). Sigma lentiviruses have been used in prostate cancer cell lines, including PC-3 and LNCaP, to study growth arrest (Gray et al., 2007), apoptosis (Elis et al., 2008) and hormone refractory prostate cancer. The latter showed that shRNAs targeting the ErbB3-binding protein and AR corepressor EBP1 in stable transfected LNCaP cells promoted a hormone refractory phenotype. Knockdown also cancelled out growth arrest caused by ErbB3 ligand heregulin (HRG), in cells which would normally undergo apoptosis upon HRG treatment (Zhang et al., 2008b).

When PC-3 cells were infected with shRNA-lentiviruses for β-catenin a consistant knockdown of β-catenin was observed at the protein level in puromycin selected stable cells. Stable knockdown of β-catenin resulted in decreased cell growth in prostate cells but no changes to the cell cycle.

The double bands, which we observed at ~ 75 kDa and ~ 92 kDa during Western Blot experiments using total β-catenin antibody C2206 (Sigma), were reported previously by Rios-Doria and colleagues as a characteristic of metastatic prostate tumours, as well as

several prostate cancer cell lines, including PC-3 (see 4.2.3, figure 45). The smaller (75 kDa) fragment represents a proteolytic β-catenin fragment, which was closely associated with expression of calpain, a calcium-dependent protease. Calpain caused proteolytic cleavage of the N-terminal regulatory domain of β-catenin. This takes place in metatstatic prostate cancer and might be a mechanism by which β-catenin is activated during tumour expansion. This would also be one explanation why in most prostate, and also breast cancers, β-catenin mutations are rare but β-catenin activity has been widely reported (Rios-Doria et al., 2004). Initial observations during the transient infection phase before the establishment of stable clones, indicated an shRNA induced slower growth in treated cells, so we examined this more closely in several experiments. Growth of cancer cells can give some indication of their aggressiveness and any growth reduction in treated cells would therefore be a positive result of the treatment.

After the generation of stable shRNA expressing cell lines, growth curve experiments to compare PC-3-sh-β-catenin cells (PC-3v45) and the scrambled control were carried out. For these experiments, cells were no longer under puromycin-containing selection medium, but they had been selected prior to the experiment, to ensure that only cells which express the lentivirally transfected constructs, would be examined.

β-catenin knockdown did indeed affect proliferation of stable PC-3-sh-β-catenin cells, and a statistically significant growth reduction compared to the scrambled control was observed ($p \leq 0.05$). This is in line with recently published reports (Jiang et al., 2009, Liu et al., 2010a). Jiang and colleagues used shRNA expression plasmids, with the shRNAs under a U6 promoter, which were transfected into cells with Lipofectamine. They examined the knockdown of β-catenin in human gastric carcinoma cell lines, e.g. AGS, and observed suppression of cell proliferation (Jiang et al., 2009). They were also able to use the treatment to induce apoptosis, which is in line with further reports by Liu et al. It was shown that β-catenin knockdown not only inhibited proliferation in malignant glioma cells, but it also caused the induction of apoptosis and a cell cycle arrest in the G0/G1 phase (Liu et al., 2010a).

Based on the observed reduced growth in the stable knockdown cell line PC-3v45, changes in the cell cycle in these cells were measured next. However, no major effect, such as a cell cycle arrest, which could have explained the growth reduction seen in the stable cell line, was found.

To summarise, the experiments showed that lentivirus targeted β-catenin expression knockdown, caused a reduction in cell growth in PC-3 cells. As lentiviral infection and expression of small dsRNA can by itself perturb cell metabolism, e.g. by induction of an interferon response (Bridge et al., 2003, Stark et al., 1998), experiments always included a non-targeting, scrambled shRNA lentivirus control.

In the future, an apoptosis assay could determine whether the reduced cell counts, which were observed here, were due to increased apoptosis. This is unlikely however, as no obvious increase in cell death was observed in PC-3v cells compared to the scrambled control when cells were visually examined every 1-2 days during the experiment. This suggests that induction of apoptosis may not have been the predominant factor of growth reduction as reported elsewhere (Jiang et al., 2009, Liu et al., 2010a).

5.2.2 Monitoring Wnt pathway activity in PC-3 cells

PC-3 cells were stained for total β-catenin in native and activated state. Compared to the positive control (SaOS-2 cells), PC-3 cells did not show nuclear β-catenin staining.
This absent Wnt activity in PC-3 cells is in line with reports on how canonical Wnt signalling contributes to bone metastases in prostate cancer. Active Wnt signalling causes new bone formation and osteoblastic metastases. However, PC-3 cells generally form osteolytic bone lesions. The cells have been shown to express not only a number of different Wnts but also high amounts of Wnt antagonist Dkk1 (Hall et al., 2005, Li et al., 2008b), resulting not in osteoblastic, but osteolytic phenotypes. In addition, experiments to modify Wnt signalling by various methods (natural and synthetic inhibitors or Wnt antagonists, receptor modifications, adenoviral gene therapy approach) showed a suppression of growth and invasiveness in these cells (Davies et al., 2000, Fishman et al., 2003, Giladi et al., 2007, Grandy et al., 2009, Lu et al., 2010, Zi et al., 2005). This highlights the importance of the Wnt signalling pathway and its antagonists in androgen-independent prostate cancer cells like PC-3.
In order to confirm the immunofluorescence results, the Topflash assay, which is commonly used for examining β-catenin *activity* (Korinek et al., 1997), was successfully employed to measure Wnt activity in PC-3 cells. We found that, through external activation using LiCl, PC-3 cells transfected with either 8XTOPFlash or 16XTOPFlash plasmids, responded with a luciferase read-out of 12-22 fold higher than the negative control (NaCl) (3.2.7). However, if PC-3 cells were not artificially activated, β-catenin activity was too low to be detected by this assay. As reviewed above, due to the abundance of Wnt inhibitor Dkk1 (Hall et al., 2005, Li et al., 2008b), this was an expected result and confirmed previous immunofluorescence experiments.

5.2.3 Decreased invasion in stable sh-β-catenin cells

To determine the cause of growth reduction we saw in sh-β-catenin (PC-3v45) cells, the role of β-catenin, which did not directly relate to the Wnt pathway, was investigated next. Apart from being the major molecule of the canonical Wnt signalling pathway, β-catenin also plays an important role in cell adhesion and control of E-cadherin (Hülsken et al., 1994).

As cell motility requires the involvement of extra-cellular matrix molecules like E-cadherin, it seemed logical to examine motility and also invasiveness in our model.
Although there were no differences in motility found between PC-3v45 and the scrambled control, the differences in invasiveness were significant. PC-3v45 cells showed much less invasiveness than the control cells in a matrigel invasion assay.
The observations are in line with other reports where inhibition or modification of the Wnt pathway lead to a reduction of invasiveness (Davies et al., 2000). Zi and colleagues showed, for example, that treating PC-3 cells with Wnt antagonist sFRP3 (soluble Frizzled-related protein 3), resulted in growth reduction and decreased invasiveness. They also showed that cell-cell contacts were upregulated upon treatment and the cells expressed more epithelial markers. From these observations, it was concluded that treatment with a Wnt antagonist reversed epithelial to mesenchymal transition (EMT). In accordance with this, lower expression of mesenchymal markers was found (Zi et al., 2005). Similar observations of a reversal of EMT in prostate cancer have also been reported by several other groups (Acevedo et al., 2007, Jiang et al., 2007, Shah et al., 2009, Yee et al., 2010).
Invasion assays also showed that there was no significant difference in motility between PC-3v45 and control cells. However, measuring motility as well as invasiveness gave us the advantage of ruling out any 'masking' effects due to incomparable motility of different cell lines (3.2.8). Highly motile cells statistically have a higher chance than their less motile counterparts to find pores in the invasion assay membrane. Even if the expression of matrigel degrading enzymes in those cells was lower than in a more invasive cell line, motility would give them some advantage.

5.2.4 Delayed tumour onset in mice and protein expression in xenografts

Having seen significant changes in growth and invasiveness, an initial, small-scale *in vivo* experiment was carried out. As PC-3 cells readily form subcutaneous tumours in immunocompromised mice (Havens et al., 2008), this method was chosen as our next experiment, to gather valuable *in vivo* data for tumour growth.
After injecting PC-3v45 and control cells (scrambled, uninfected) subcutaneously into immunocompromised mice, tumour growth was monitored and tumour size measured. Here, a delayed onset of tumour growth in the PC-3v45 mice was seen when compared to controls. This was a noteworthy result, as reduced tumour growth in animal studies is

often the first step towards future clinical studies for cancer therapies (Fishman et al., 2003). As each group of mice in our pilot study only consisted of 3 animals, no statistical analysis was possible.

To analyse the amount of β-catenin protein in tumours taken from mice, lysates were made of the depleted xenograft cells and subjected to Western Blot. As with the previous Western Blot experiments, β-actin was used as a loading control. In two mice tested, no β-actin was detected along β-catenin, while in the remaining mice both proteins were clearly visible on the blots. This observation raised the question whether downregulation of β-catenin was interfering with the actin cytoskeleton in tumour cells.

Apart from its essential function in canonical Wnt signalling, β-catenin is also part of the E-cadherin transmembrane complex in epithelial cells. β-catenin binds to the highly phosphorylated intracellular domain of E-cadherin, thus making this region vital to the correct functioning of the protein. As part of this complex, β-catenin can also bind α-catenin, therefore linking E-cadherin cell-junctions with actin-containing cytoskeletal filaments, which are often adjacent to adherens junctions in epithelial cells (Aberle et al., 1994, Gooding et al., 2004, Hülsken et al., 1994). Loss of function or reduced expression of E-cadherin has been shown to contribute to cancer progression and metastasis (Beavon, 2000, Hazan et al., 2004, Wijnhoven et al., 2000). This has also often been linked to genetic alterations of β-catenin, which reduced or abolished interaction with the E-cadherin/α-catenin complex (Kawanishi et al., 1995, Oyama et al., 1994). Highly metastatic tumour cell lines are known to usually express low levels of E-cadherin (Bukholm et al., 1998, Pignatelli et al., 1994) and they also often show a more fibroblast like morphology, mostly due to poor differentiation (Wijnhoven et al., 2000).

Future experiments could therefore include knockdown experiments of β-catenin followed by dual or triple IF staining for β-actin/E-cadherin or β-actin/β-catenin/E-cadherin, respectively.

5.3 Targeting primary prostate cells with shRNA-lentiviruses

Following the successful β-catenin knockdown experiments in the PC-3 cell line, the last aim was to apply the now established protocol to primary prostate cells, which were cultured directly from patient tumours. This was done to see whether any effects seen in cell lines were applicable or relevant in patients. Accordingly, primary cells were infected with commercial lentiviruses.

The integration of lentiviral cDNA into the host cell genome is not only the first step in the natural life cycle of retroviruses, it is also one of the most challenging aspects when employing lentiviral gene transfer as a therapeutic method. Side effects due to integration site position have been reported regularly, since the first human gene therapy trials (Baum et al., 2003, Seggewiss et al., 2006). Retroviruses do not select their integration site at random. A clear preference has been shown for transcriptionally active sites or regulatory regions, which are relevant to cell growth, differentiation and development, thus increasing the possibility for unwanted side effects (Felice et al., 2009, Montini et al., 2009).

In general, lentiviruses are suitable for infecting and replicating in non-dividing or slowly dividing cells, like adult stem cells. However, one of the hurdles of infecting primary cells, especially stem cells, with lentiviruses is the tendency of the cells to differentiate when placed in culture and also their fragility (Paré and Sherley, 2006). This fragility could be enhanced even further through immunomagnetic selection of the CSC population. Ideally, the prostate CSCs would be selected from the total tumour population and infected to compare the effects of silencing to CB cells. Due to the fragility of the cells however, whole populations of primary prostate tumour cells, including CSC and CB cells were infected. This mimics the situation in a tumour, when mixed cell populations would have to be transduced.

To overcome any integration site specific effects, various methods have been suggested, which were shown to work in haematopoietic stem cells and progenitor cells. These include using a ubiquitously acting chromatin opening element, to enhance stable transgene expression (Zhang et al., 2007). Compared to CMV promoter containing controls, silencing of the transgene was also reduced (Zhang et al., 2007).

It has also been reported that external factors, such as media composition with or without serum, multiplicity of infection and duration of transduction were important to achieve transgene delivery without compromising self-renewal or multipotency of stem cells (Millington et al., 2009).
To receive more consistent results, it is possible to use clonal populations of primary cells obtained through serial replating. The behaviour of clonal populations is usually more consistent than that of a mixed population (personal communication S. Hager).

5.3.1 Wnt pathway microarray analysis in prostate cancer patients

The basis for our study of β-catenin in primary cells was the reanalysis of microarray data obtained by Birnie and colleagues (Birnie et al., 2008). This led to the hypothesis, that Wnt signalling and possible deregulation of β-catenin might play an important part in prostate CSC maintenance and growth. In addition, as discussed above, higher amounts of β-catenin would also influence cell-cell-contacts and adherence through E-cadherin related activity (Gooding et al., 2004), and would therefore influence migration and invasion.
Furthermore, the large number of β-catenin target genes makes it challenging to study individual outcomes and usually prominent examples like Cyclin D1 or cMyc are chosen to be examined first.
A trend for higher expression of β-catenin was observed in the proposed prostate cancer stem cell population (CSC; CD133+ phenotype), compared to the malignant committed basal population (CB; α2β1low phenotype), although its statistical significance was limited by inter-patient variability. To cover prominent components of Wnt signalling, expression of APC, AXIN, GSK3B and TCF4 as well as of β-catenin target genes c-Myc and Cyclin D1 was also analysed.
Although consistent differences in expression in both β-catenin and TCF4, which were both higher expressed in CSC than CB cells of primary prostate cancer samples, were found, no major expression differences in APC, AXIN or the two chosen β-catenin target genes were seen. This is in line with the literature, as mutations in APC or AXIN, which are very common in colorectal cancer, for example, are rarely detected in prostate cancer and these genes might therefore not be differentially expressed. This is especially true since β-catenin has been shown to be influenced during EMT and it is still under discussion whether this is due to a direct correlation between active Wnt signalling and

EMT (Jiang et al., 2007), or due to its interaction with E-cadherin in the adhesion complex.

It has been shown recently, that one of the nuclear transducers of Wnt signalling, Lef-1, exists in two different transcripts in pancreatic cancer. On the one hand, transient expression of the shorter form resulted in inhibition of E-cadherin expression, which happened in a β-catenin independent manner. On the other hand, the full-length protein caused the induction of cell-cycle regulators c-Myc and Cyclin D1 (Jesse et al., 2010).

Expression of Cyclin D1 is not directly affected, even if APC mutations occur, but its expression changes at a later stage. However, APC mutations seem to have a more imminent effect on Cyclin D2. It has been shown that Cyclin D2 plays an essential role in tumourigenesis post APC loss in colon cancer (Cole et al., 2010). It would therefore be interesting to follow up gene expression of Cyclin D2 in prostate samples and establish if it is affected in CSCs compared to CB cells and determine APC status of the samples at the same time.

For our analysis, we plotted the average expression of 9 different patient samples and calculated standard deviation. One technical disadvantage when analysing and comparing gene expression from patient samples is, that any statistics applied, like standard deviation, will result in high variability, due to the high variation between individual patients (Pereira et al., 2010, Sontrop et al., 2009). This has to be taken into account when comparing results from a cohort of patients or tissue samples. However, we were still able to determine expression trends, especially in genes like β-catenin, where differences were high between the different cell populations. To reduce variation and therefore decrease the range of standard deviation, a larger cohort of patient samples could be analysed. However, depending on the availability of patient samples and the time needed for growth and subculturing of primary prostate cancer cells, which often show variable growth, further studies would have to be designed on a more long-term scale. Factors which have to be taken into account in any future studies, include defining tumour status by Gleason Grade, invasive potential of samples (invasion assay) and any pre-treatment patients received prior to donating tissue. Another factor which could influence data gathering is the low dynamic range of Affymetrix microarray chips, which can lead to small yet significant under-representations of fold changes in gene expression (Chang et al., 2000).

To follow on from the microarray analysis, which only provided mRNA expression data, protein arrays or ChIP assays could be performed to enhance and compare data. ChIP assays have become a popular tool for Wnt signalling pathway studies (Lee et al., 2010).

ChIP would also provide a tool to monitor protein-DNA interactions and could clarify if there is more interaction of β-catenin with genes related to E-cadherin rather than cell cycle regulators, for example.

5.3.2 Detection of activated β-catenin

Staal and colleagues have shown dephosphorylation and nuclear localisation of β-catenin, which provided a tool for studying Wnt signalling pathway activity (Staal et al., 2002). In our study, the detection of nuclear β-catenin was not reliable when using an antibody, which has been marketed as detecting 'active' β-catenin (anti-ABC, Millipore). The present observations are in line with those reported by the manufacturer, that state that the antibody has been used for IF to detect β-catenin in the cytosol and membrane. However, this is a contradiction of the nuclear localisation of 'active' β-catenin. After optimisation and application of higher than recommended antibody concentrations with very limited success, we decided to omit using this particular antibody. Results were confirmed with colleagues from a neighbouring lab, who had discovered the same drawbacks using the anti-ABC antibody (personal communication Lisa Kirkwood, Southgate lab, University of York). These observations were in contrast to the original publication and the observations made by the group who developed the antibody (Staal et al., 2002).

To overcome the limitations seen with the anti-ABC antibody, colocalisation with nuclear DAPI staining was used to visualise how much nuclear β-catenin was present in cells, using an antibody for total β-catenin (C2206, Sigma). We showed that although colocalisation varies both in the CSC and CB populations, higher levels of colocalisation were detected in CSCs. In order to provide statistical analysis for these experiments, larger numbers of CSCs would be necessary. The varability observed might reflect that even after selection for CSCs, and especially in the CB population, there might still be a mixed population present. One reason could be that cells at different stages of differentiation might express different levels of total β-catenin, which then in turn increases or decreases the percentage found in the nuclear compartment. Following on from this, co-staining for Cyclin D1 or D2 as well as staining for an established proliferation marker like Ki67 (Fiorentino et al., 2010) or a promising new proliferation marker like SOX9 (Thomsen et al., 2010), could be employed. This has been shown to point to regions of low proliferation in colorectal adenocarcinomas (Jung et al., 2001).

5.3.3 Appearance of infected primary colonies

Observations included a striking difference in appearance between infected cells, which expressed the GFP-expressing control lentivirus and those in the same culture, that did not. The latter were small and tightly packed, and colonies generally looked healthy and undifferentiated. Round, densely packed colonies are suggestive of being derived directly from stem cells (Barrandon and Green, 1987, Hudson et al., 2001). In contrast to that, GFP expressing cells looked flatter, more spread out and differentiated than their healthy looking counterparts.

This raised the question, if the lentiviral infection itself, the selection using puromycin, or the expression of GFP is the reason for this phenomenon. Some cells might switch off the expression of GFP or the whole integrated lentiviral genome.

That the lentivirus itself being solely responsibly for the effect observed, is not likely, as the healthy looking, tight colonies survived the selection. This should not have happened if they did not carry the resistance transfected as a transgene within the lentiviral genome.

One medium component, which might influence the appearance of primary epithelial cells following lentivirus infection is the serum contained in the viral supernatant. Primary cells, which are normally cultured without any serum present, would undergo differentiation following exposure. Due to the high titres of commercial lentiviruses and therefore the very low amounts of supernatant used per infection, this should not be a problem. However, it has to be taken into account when 'home-made' lentiviruses are produced (see chapter 3.1.6).

Of course, the expression of GFP alone could be a problem to the cells' metabolism. However, without the aid of fluorescent proteins, there is no possibility to visualise or track infected cells. Other fluorescent proteins have been tried on primary prostate cells with similar effects of differentiation or growth arrest (Hager et al., 2008).

Multiplicity of infection could contribute to the problem as well. It might be that one or a few integrated lentiviral vectors are enough for transferring resistance to puromycin, but that more integrated vectors are needed to microscopically detect GFP expression to a sufficient level. In addition to that, multiple integration events might cause cells to switch on differentiation or cell death pathways.

The changing shape of infected cells could also be due to insertion of the lentivirus into particular genes or regions, or a potential interaction between β-catenin and E-Cadherin,

or the downregulation thereof, which has been reported in the literature (Nelson and Nusse, 2004).

Primary cells infected with sh-β-catenin-lentivirus also displayed heterogeneity in cultured colonies. However, as these viruses do not carry a fluorescent reported gene, expression of lentiviral genome in different colonies could not be confirmed in the same way.

Clonal heterogeneity might also be displayed in the culture, resulting in differences in GFP expression while maintaining puromycin resistance. This phenomenon has been reported in primary cultures of keratinocytes (Barrandon and Green, 1987), but also in primary prostate epithelium (Collins et al., 2001, Hudson et al., 2001, Richardson et al., 2004) and in holoclones formed from potential tumour-initiating cells selected from the PC-3 cell line (Li et al., 2008a).

5.4 Conclusions and Outlook

Nuclear β-catenin has been suggested as a prognostic factor in colorectal cancer and as a means of identifying tumour initating (stem) cells (Elzagheid et al., 2008, Fodde and Brabletz, 2007). This thesis produced some promising results, which indicated that β-catenin might also play an important role in prostate cancer. However, improvements are needed to develop a more reliable method for a virally transmitted knockdown, especially in primary cells.

Multicistronic cotranscription has been used for some time in lentiviral systems and it has been shown that shRNAs expressed within a microRNA context produced efficient knockdown as well as providing cotranscription of a reporter gene (Stegmeier et al., 2005). This could be employed to provide a tool for multiple targets within one transcript. Such a method has been developed using adenoviral delivery (Junn et al., 2010).

One idea for multiple targets is based on a recent report by Shah and colleagues. They studied the calcitonin-calcitonin receptor axis in prostate cancer and found correlations between nuclear β-catenin accumulation, higher amounts of calcitonin mRNA in higher Gleason grade tumours and destabilization of cell-cell junctions, resulting in EMT (Shah et al., 2009). Targeting calcitonin and β-catenin together, using a multicistronic vector, could therefore provide insight into this interaction and might improve any future therapeutic applications.

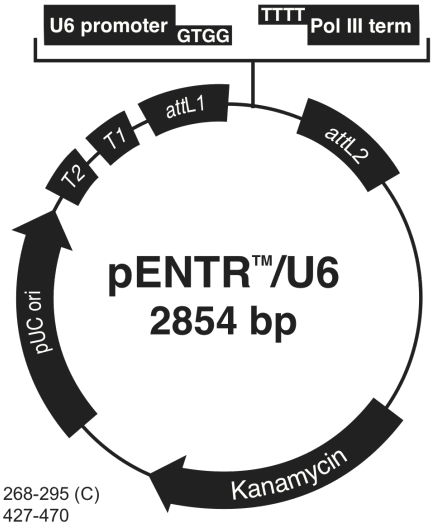
As a therapeutic delivery system, lentiviral vectors could be further improved by increased viral titres and infection efficiency. Recently, reports suggested that a new envelope glycoprotein could help attachment of virions to prostate cells (Sakuma et al., 2010). The XMRV (xenotropic murine leukemia virus-related virus) envelope glycoprotein is a promising candidate for this approach. While many tissues express XPR1, the virus' cell surface receptor (Battini et al., 1999, Tailor et al., 1999), it has been found to be strongly associated with prostate cancer patients with a familial background of the disease (Urisman et al., 2006). There is also evidence that XMRV primarily infects malignant prostate cells, and is associated with tumours of a high Gleason grade (Schlaberg et al., 2009). XPR1 is believed to be the most important port of entry for this virus (Bhosle et al., 2010), making a lentiviral vector pseudoyped with the XMRV envelope an ideal candidate for a promising new viral approach. In addition, the association between infections with gammaretrovirus XMRV and prostate cancer has major implications on how researchers

approach prostate carcinogenesis and could potentially link tumour development to a sexually transmitted disease (Trottier and Fleshner, 2010).
Alternatively, the lentiviral approach could be altered in favour of other viral vectors. Oncolytic viruses, such as herpes simplex, have been used to target cells with strong β-catenin/TCF-signalling (Kuroda et al., 2006) and a similar approach was used, combining oncolysis with shRNA knockdown of IL-8 in breast cancer, which resulted in growth reduction and antiangiogenesis (Yoo et al., 2008).
In addition, the structure of shRNAs used could be improved as recent reports suggest that directly delivered shorter shRNAs with minimal length offer a suitable alternative, potentially decreasing any side effects during therapeutic use (Ge et al., 2010).
Furthermore, another Wnt protein, not part of the canonical signalling cascade, Wnt5a, has recently had more attention in relation to its importance in prostate cancer. However, reports are conflicting, as Wnt5a has been shown to be more highly expressed in the potential prostate CSC population, alongside β-catenin (Birnie et al., 2008), while others reported exclusive expression of either β-catenin or Wnt5a (Yamamoto et al., 2010). Reports are consistent in suggesting an important role for Wnt5a in prostate cancer.
This is in line with our results, which suggest that although β-catenin probably plays an important part in prostate cancer growth and invasiveness, its role in canonical Wnt signalling might not be of primary importance.

To summarise, it was shown that β-catenin plays a role in regulating growth and invasiveness in the prostate cancer cell line PC-3. A stable knockdown cell line also delayed tumour induction in immunocompromised mice. Additionally, experiments showed encouraging results as well as some limitations when using shRNA-lentiviruses in primary prostate epithelial cell cultures.
Our results are a promising start to elucidate the role of β-catenin in prostate cancer but there is still a lot of work to be done.

Appendices

Appendix 1: Plasmid maps



Comments for pENTR™/U6
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*rrn*B T2 transcription terminator: bases 268-295 (C)
*rrn*B T1 transcription terminator: bases 427-470
M13 forward (-20) priming site: bases 537-552
*att*L1: bases 569-668 (C)
U6 promoter: bases 705-968
U6 forward priming site: bases 890-909
5′ overhang: bases 965-968 (C)
5′ overhang: bases 969-972
Pol III transcription terminator: bases 969-974
*att*L2: bases 979-1078
M13 reverse priming site: bases 1119-1135
Kanamycin resistance gene: bases 1248-2057
pUC origin: bases 2178-2851

(C) = complementary strand

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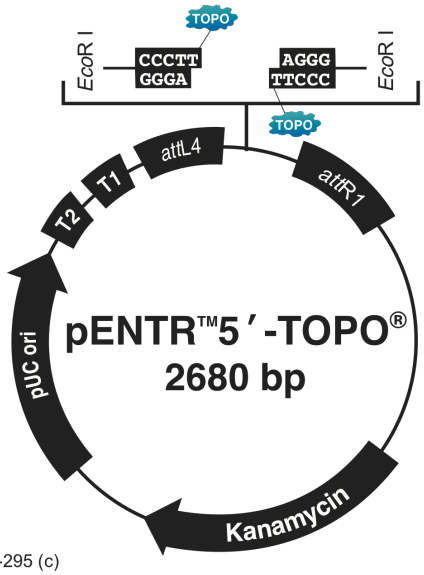

Comments for pENTR™5′-TOPO®
2680 nucleotides

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*rrn*B T1 transcription terminator: bases 427-470 (c)
M13 forward (-20) priming site: bases 537-552
attL4: bases 592-688
GW1 priming site: bases 630-654
TOPO® recognition site 1: bases 701-705
TOPO® recognition site 2: bases 706-710
attR1: bases 721-845
GW3 priming site: bases 752-781
M13 reverse priming site: bases 945-961
Kanamycin resistance gene: 1074-1883
pUC origin: bases 2004-2677

(c) = complementary strand

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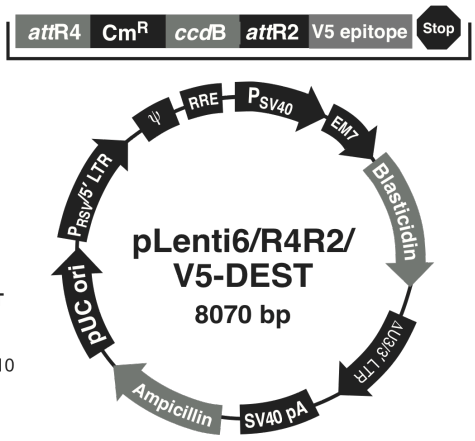

Comments for pLenti6/R4R2/V5-DEST
8070 nucleotides

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RSV promoter: bases 1-229
HIV-1 5′ LTR: bases 230-410
5′ splice donor: base 520
HIV-1 psi (ψ) packaging signal: bases 521-565
HIV-1 Rev response element (RRE): bases 1075-1308
3′ splice acceptor: base 1656
3′ splice acceptor: base 1684
*att*R4 site: bases 1823-1947
Chloramphenicol resistance gene (Cm^R): bases 2055-2714
*ccd*B gene: bases 3056-3361
*att*R2 site: bases 3402-3526
V5 epitope: bases 3579-3620
SV40 early promoter and origin: bases 3675-3984
EM7 promoter: bases 4039-4105
Blasticidin resistance gene: bases 4106-4504
ΔU3/3′ LTR: bases 4590-4824
ΔU3: bases 4590-4643
3′ LTR: bases 4644-4824
SV40 polyadenylation signal: bases 4896-5030
bla promoter: bases 5886-5984
Ampicillin (*bla*) resistance gene: bases 5985-6845
pUC origin: bases 6990-7663

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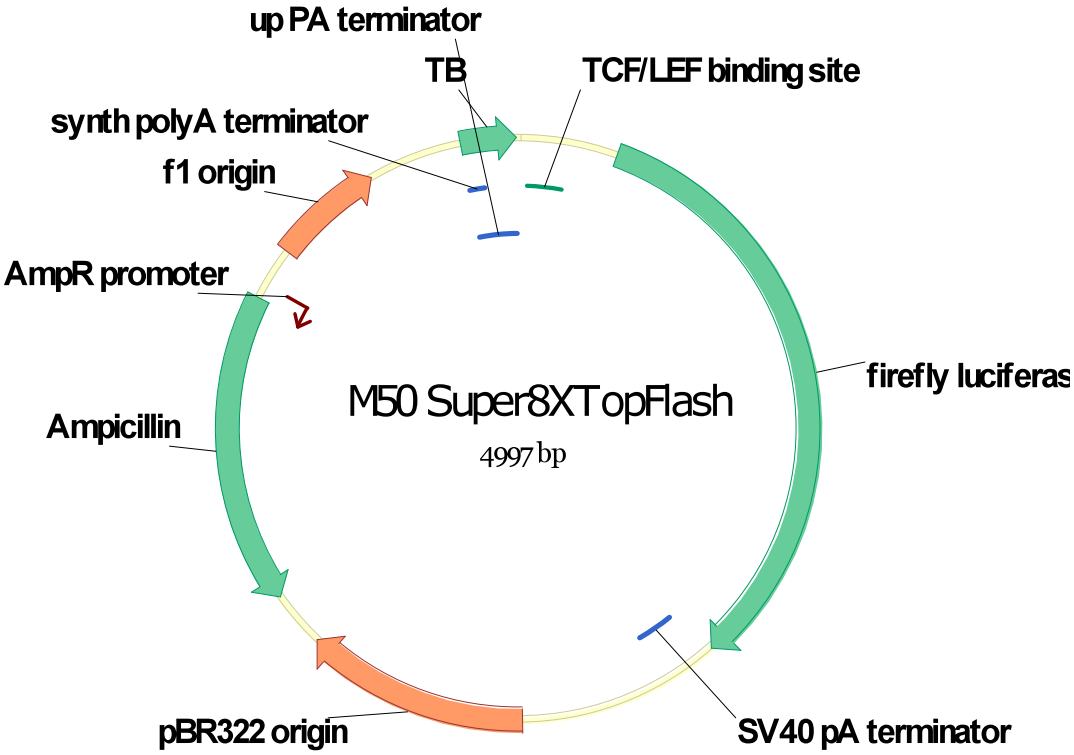
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TB
TCF/LEF binding site
synth polyA terminator
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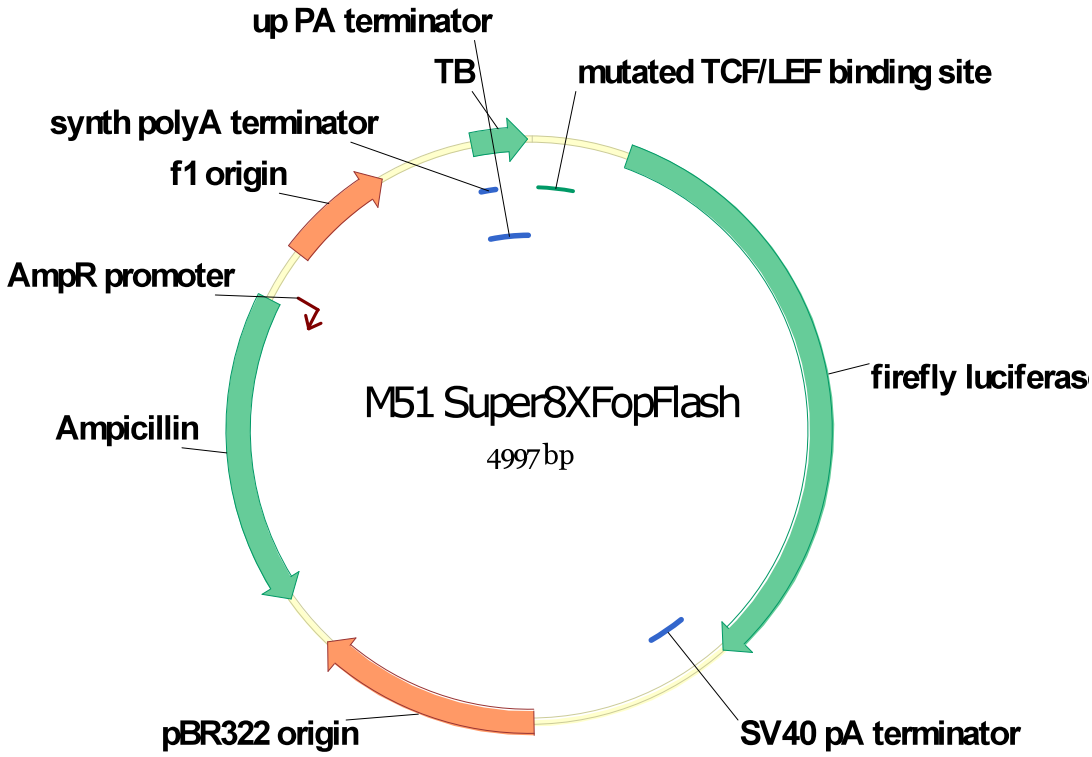
firefly luciferas
Ampicillin
pBR322 origin
SV40 pA terminator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TB
mutated TCF/LEF binding site
synth polyA terminator
f1 origin
AmpR promoter
M51 Super8XFopFlash
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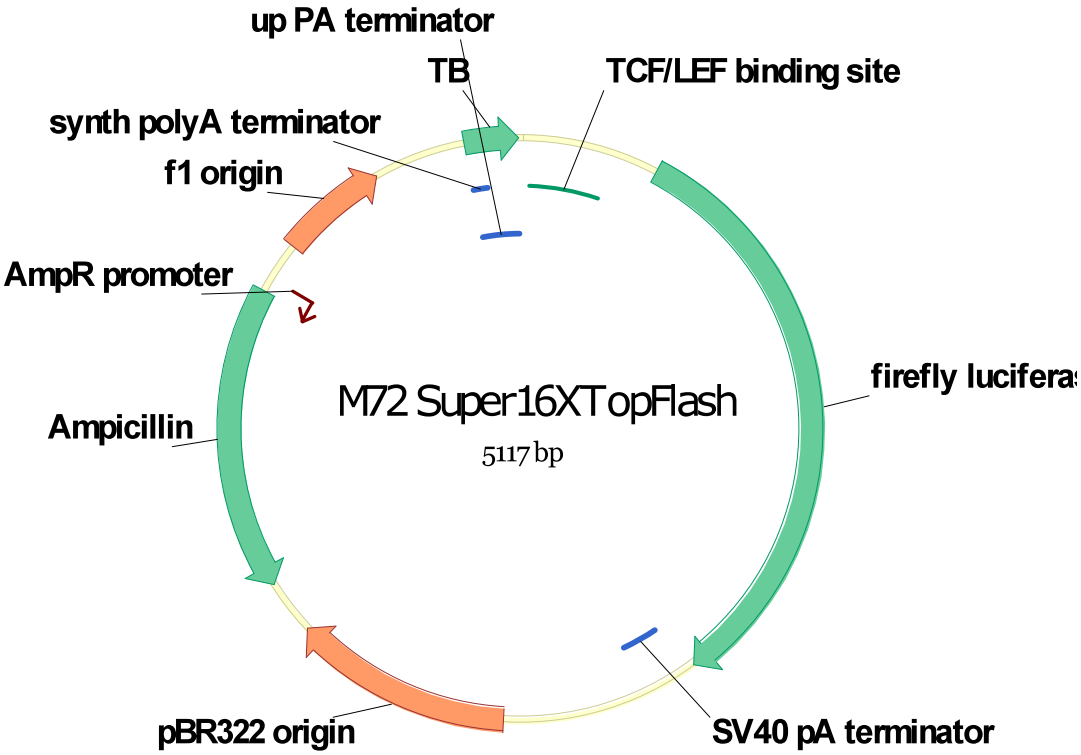
firefly luciferas
Ampicillin
pBR322 origin
SV40 pA terminator


up PA terminator
TB
TCF/LEF binding site
synth polyA terminator
f1 origin
AmpR promoter
M72 Super16XTopFlash
5117 bp
firefly lucifera
Ampicillin
pBR322 origin
SV40 pA terminator

Appendix 2: Composition of buffers and solutions

General molecular biology

1 x TE buffer
10 mM Tris-HCl, 1 mM EDTA, pH 8.0

1 x TAE buffer
40 mM Tris-acetate, 1 mM EDTA, pH 8.0

6 x agarose gel-loading buffer
0.25 % (w/v) bromophenol blue, 0.25 % (w/v) xylene cyanol FF, 30 % (v/v) glycerol in H_2O

Magnetic cell separation

MACS buffer
PBS, pH 7.2, supplemented with 0.5% foetal calf serum and 2 mM EDTA

Flow cytometry

FACS buffer
2 mM EDTA in phosphate buffered saline, pH 7.2

SDS-Page and Semi-dry transfer

10x SDS running buffer
250 mM Tris, 1.92 M glycine, 1 % SDS

Transfer buffer A
300mM Tris, 20 % MeOH

Transfer buffer B
25mM Tris, 20 % MeOH

Immunocytochemistry

TBS
50 mM Tris-HCl, 150 mM NaCl

TBST
TBS supplemented with 0.4 % (v/v) Triton X-100

Reagents obtained with commercial kits

● Gateway cloning

Salt solution
1.2 M NaCl, 0.06 M MgCl2

Proteinase K solution
2 µg/µl Proteinase K in 10 mM Tris-HCl, pH 7.5, 20 mM CaCl2, 50% (v/v) glycerol

● Restriction buffers

NEBuffer 3 (1x)
50 mM Tris-HCl, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, pH 7.9

Promega Buffer D (1x)
6 mM Tris-HCl, 150 mM NaCl, 6 mM $MgCl_2$, 1 mM DTT, pH 7.9

Promega Buffer H (1x)
90 mM Tris-HCl, 50 mM NaCl, 10 mM $MgCl_2$, pH 7.5

● DNA elution and storage

Buffer EB
10 mM Tris-HCl, pH 8.5

● Ligation
T4 DNA Ligase Buffer (10x)
300 mM Tris-HCl (pH 7.8), 100 mM MgCl2, 100 mM DTT, 10 mM ATP

● shRNA annealing
10 x Oligo Annealing Buffer
100 mM Tris-HCl (pH 8.0), 10 mM EDTA (pH 8.0), 1 M NaCl

Media and solutions for bacterial work

S.O.C. Medium
2% Tryptone, 0.5% Yeast Extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose

LB medium
10 g/l bacto-tryptone, 5 g/l bacto-yeast extract, 10 g/l NaCl in ddH2O

LB agar
10 g/l bacto-tryptone, 5 g/l bacto-yeast extract, 10 g/l NaCl, 15 g/l agar in ddH2O

Abbreviations

AIDS	Acquired immunodeficiency syndrome
anti-ABC	Anti active β-catenin
APC	Adenomatous polyposis coli
AR	Androgen receptor
AXIN1	Axin 1
bp	Base pairs
BPH	Benign prostatic hyperplasia
CamKII	Ca^{2+}-calmodulin-dependent protein kinase II
CaP	Carcinoma of the prostate
CCND1	Cyclin D1
CE	Convergent extension
CD	Cluster of differentiation
ChIP	Chromatin immunoprecipitation
CMV	Cytomegalovirus
CRU	Cancer research unit
CSC	Cancer stem cell
CSK1A1	Casein kinase 1 alpha
CTBP1	C terminal binding protein 1
CTNNB1	β-catenin
Da	Dalton
DAPI	4',6-diamidino-2-phenylindole
DHT	Dihydrotestosterone
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxynucleoside triphosphate
ds	Double stranded
dT	Deoxythymidine
dTTP	Deoxythymidine triphosphate
dUTP	Deoxyuridine triphosphate
EDTA	Ethylenediaminetetraacetic acid

EF1α	Elongation factor 1α
EGFP	Enhanced green fluorescent protein
ELISA	Enzyme-linked immunosorbent assay
ES cell	Embryonic stem cell
FACS	Fluorescence-activated cell sorting
FITC	Fluorescein isothiocyanate
fmol	Femtomole
FS	Forward scatter
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green fluorescent protein
GSK3B	Glycogen synthase kinase 3 β
Hh	Hedgehog
HIV	Human immunodeficiency virus
HRP	Horseradish peroxidase
HSC	Hematopoietic stem cell
ICM	Inner cell mass
IF	Immunofluorescence
IP3	Inositol-1, 4, 5 –triphosphate
JNK	Jun N-terminal kinase
kb	Kilobases
KSFM	Keratinocyte serum-free medium
LB	Lysogeny broth or Luria-Bertani broth
LTR	Long terminal repeat
m	Monomer
M	Molar
MHC	Major histocompatibility complex
MLV	Murine leukaemia virus
min	Minute
MOI	Multiplicity of infection
MOPS	3-(N-morpholino)propanesulfonic acid
mRFP	Monomeric red fluorescent protein
mRNA	Messenger ribonucleic acid
MYC	c-Myc
NF-κB	Nuclear factor-kappa B

NOD/SCID	Nonobese diabetic/severe combined immunodeficiency
NSAIDs	Nonaspirin nonsteroidal anti-inflammatory drugs
PAP	Prostatic acid phosphatase
PBS	Phosphate-buffered saline
PBS	Primer binding site
PCP	Planar cell polarity
PCR	Polymerase chain reaction
PE	Prostate epithelium
PET	Polyethylene terephthalate
PF	Prostate fibroblasts
PFA	Paraformaldehyde
PGK	Phosphoglycerate kinase
p.i.	Post infection
PIA	Proliferative inflammatory atrophy
PIN	Prostate intraepithelial neoplasia
PIC	Preintegration complex
PIC	Protease inhibitor cocktail
PIN	Prostate intraepithelial neoplasia
PKC	Protein kinase C
PLC	Phopholipase C
poly(A) signal	Polyadenylation signal
PPT	Polypurine tract
PSA	Prostate-specific antigen
PSCA	Prostate stem cell antigen
PSEN1	Presenilin 1
PTEN	Phosphatase and tensin homolog deleted on chromosome 10
PVDF	Polyvinylidene fluoride
RNA	Ribonucleic acid
RNAi	RNA interference
RNase	Ribonuclease
RRE	Rev-responsive element
rRNA	Ribosomal ribonucleic acid

RT-PCR	Reverse-transcription polymerase chain reaction
Sca	Stem cell antigen
SCM	Stem cell medium
SDS	Sodium dodecyl sulfate
Shh	Sonic hedgehog
shRNA	Short hairpin ribonucleic acid
SIN	Self-inactivating
siRNA	Short interfering ribonucleic acid
SP	Side population
SS	Side scatter
STO	SIM (sandos inbred mice) derived embryonic fibroblasts resistant to thioguanine and ouabain
SV40	Simian virus 40
SVE	Seminal vesicle epithelium
TBS	Tris-buffered saline
TBST	Tris-buffered saline Triton-X 100
TCF4	T cell factor 4
td	Tandem-dimer
TF	Technology Facility
TGF	Transforming growth factor
UGE	Urogenital sinus epithelium
UGM	Urogenital sinus mesenchyme
UGS	Urogenital sinus
UV	Ultraviolet
U	Units
V	Volt
v/v	Volume per volume
VSV-G	Vesicular stomatitis virus G-glycoprotein
WPRE	Woodchuck posttranscriptional regulatory element
w/v	Weight per volume
XMRV	Xenotropic murine leukaemia virus-related virus
XPR1	Xenotropic and polytropic murine leukaemia virus receptor

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