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Derivation of prostate cancer from human embryonic stem cells using shRNA knock down technology

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Abstract

Prostate cancer is a disease that has increased tremendously the past years and is now the second biggest cause of cancer deaths in men. Little is known about how the cancer starts and what initiates it. The aim of this project was to develop a model system of human prostate cancer and to study what causes the initiation events of the cancer. Knocking down Retinoblastoma 1, a key prostate cancer gene, in human embryonic stem cells and recombining these cells with normal and initiated stroma forms teratomas in vivo in SCID mice. What tissues are from which cells can hold the key to how prostate cancer develops.

Keywords

Human embryonic stem cells, Mesencymal-Epithelial interaction, Prostate Cancer, Recombination, Retinoblastoma 1, shRNA, teratomas

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Derivation of prostate cancer using human embryonic stemcells and shRNA knockdown technology

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Sammanfattning

Prostata cancer är en sjukdom som drabbar ~395 000 män världen över varje år. Behovet av nya behandlingar och en bättre förståelse av sjukdomsförloppet är därför stort. Både i utvecklingen av prostatan och i det mogna organet sker epiteliala-mesenkymala signalleringar som leder till differentiering och homeostas i vävnaden. Vid prostata cancer störs eller upphör den här signallering helt.

Syftet med detta projekt var att utveckla ett modellsystem av human prostata cancer för att kunna studera signalleringen och vad som påverkar den. Därför användes humana embryonala stamceller, hESCs, med Retinoblastoma 1, en prostata cancer specifik gen, nedtystad med hjälp av transfektion av shRNA mot genen. Dessa celler rekombinerades sedan med mesenkymala celler, både normala och initierade, in vivo på SCID möss. Mesenkymet, har en förmåga att guida hESCs i deras differentiering till epiteliala celler. Används mesenkym från prostata styrs differentieringen av stamcellerna ner längs en prostata utveckling och teratomer innehållande prostata vävnad, eller prostata cancer vävnad bildas. Målet med modellsystemet är att se hur en nedreglering av Rb1 påverkar cancerutvecklingen och också förstå om en initiering i de epiteliala cellerna är tillräckligt för att cancern ska utvecklas eller om man behöver genetiska defekter i både epitelet och mesenkymet.

Examensarbete 20p i Molekylär Bioteknikprogrammet
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Abbreviations

AR	Androgen Receptor
bFGF	beta – Fibroblast Growth Factor
BPH	Benign Prostatic Hyperplasia
CAFs	Carcinoma Associated Fibroblasts
cDNA	complementary Deoxyribonucleic acid
DAB+	Diaminobenzidine
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	DiMethyl Sulfoxidase
EtOH	Ethanol
FCS	Fetal Calf Serum
GSTpi	Glutathione S-transferase pi
hESCs	human Embryonic Stem cells
HGPIN	high-grade PIN
HRP	Horse Radish Peroxidase
ICM	Inner Cell Mass
IVF	In Vitro Fertilization
LGPIN	low-grade PIN
LHRH	Luteinizing hormone-releasing hormone
mEF	mouse Embryonic Fibroblast
PAP	Prostatic Acid Phosphatase
PCR	Polymerase Chain Reaction
Pen/Strep	Penicillin & Streptomycin
PIN	Prostate Intraepithelial Neoplasia
PSA	Prostate Specific Antigen
Rb 1	Retinoblastoma 1
RNA	Ribonucleic acid
rpm	rotations per minute
SCID	Severe Combined Immunodeficiency
shRNA	small hairpin RNA
SV	Seminal Vesicle
SVM	Seminal Vesicle Mesenchyme
UGM	Urogenital Sinus Mesenchyme
UGS	Urogenital Sinus

1.0 Introduction

Every year roughly 395 000 men world wide are diagnosed with prostate cancer. Around 12000 of those are Australians and around 10000 are Swedes [1]. More then 2700 Australians and just as many Swedes die every year from the disease and the incidences of both diagnoses as well as deaths increase every year. One of the most worrying aspects with the disease is that prostate cancer usually develops without the man even recognizing any symptoms. This is because the cancer is quite slow growing and when it is finally discovered, it may have gone so far that the cancer has already spread outside the prostate. In this project, we will attempt to unravel the biological events that lead to prostate cancer. By performing recombinations between initiated human embryonic stem cells, that has lost key prostate cancer genes, with carcinoma associated fibroblasts, purified from human prostate cancer tumours, we hope to get a better understanding of the role of the stroma as well as how the cancer initiates and progresses.

1.1 The structure of the prostate gland

The prostate is a muscular, walnut-sized gland that is involved in the male reproductive system. It is located below the bladder in the pelvis and surrounds parts of the urethra. Just above the prostate sits the seminal vesicles that produce around 60% of the substances that make up the semen. The nerves that run on the outside of the prostate are the ones that control the erectile function. The main function of the prostate is to produce a quite thick fluid that forms a part of the semen. This particular fluid is produced within the duct of the prostate. The smooth muscle cells lining the ducts contract during ejaculation and press the fluid out to mix with the sperm. The fluid supplies the sperm with nutrition, such as proteins and ions, but it also protects it. The proteins supplied include acid phosphatases, citric acids, polyamines, fibrinolysin, zinc and lipids. The prostate also produces hormones and enzymes that are critical for maintenance of homeostasis.

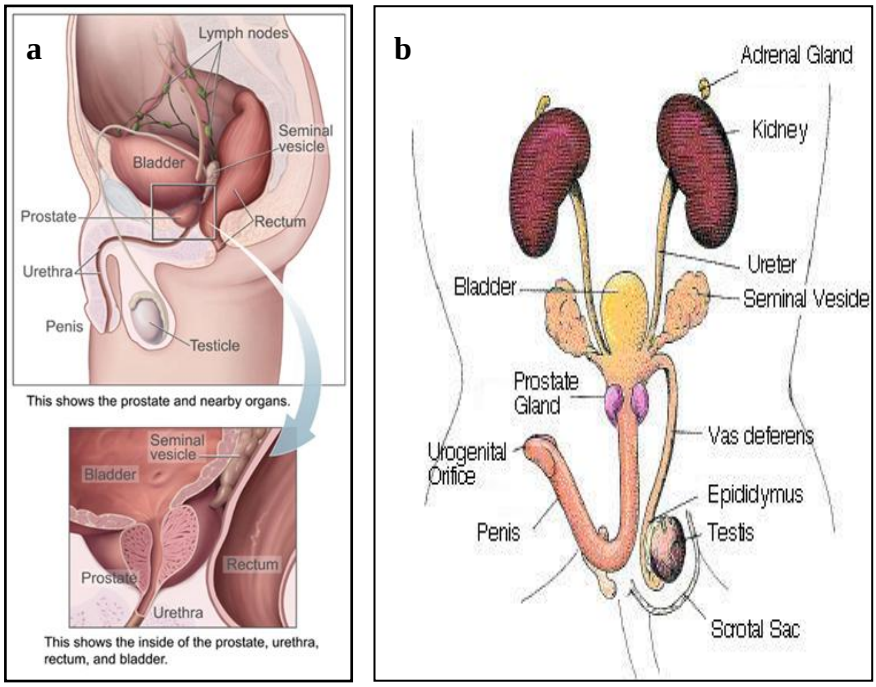

Figure 1.1 **-The anatomy of the prostate and its surrounding**
a) The prostate is located below the bladder in the pelvis and surrounds parts of the urethra.
It is divided into several lobes; the central, the peripheral and the transitional zone.
The human prostate gland is a network of glandular ducts that are embedded in fibromuscular stroma.
b) The human and the rodent prostate are built up in different ways.
The rodent prostate is multi-lobular and anatomically different. It is combined with the seminal vesicles.

a)http://www.nci.nih.gov/cancertopics/wyntk/prostate/page2
b)http://www.uic.edu/labs/prins/index.htm

The prostate gland is a network of glandular ducts that are embedded in fibromuscular stroma. The ducts are lined by epithelial cells, mainly tall columnar secretory epithelial cells. Between

the epithelial cells and the connective stroma, a layer of basal cells are found. This cell population is believed to house the adult stem cell population; approximately 1 in every 1000 basal cells is thought to be a stem cell [2]. The fibromuscular stroma is rich in nerves, blood vessels, smooth muscle, collagen, fibrous tissue and lymphatics.

1.2 Prostate disease

The prostate is dependent on the male sex hormone testosterone for its functions. Androgens produced by the testes control the growth and function of the prostate throughout life. During development of the prostate, the prostate weighs about 2g. The prostate undergoes two main periods of growth; firstly during puberty when it more than doubles its size and the weight stabilizes at around 20g, and then upon aging. Beyond the age of 40, it is common for men to experience prostate enlargement.

1.2.1 Benign Prostatic Hyperplasia (BPH)

Benign prostatic hyperplaisia (BPH; or benign prostatic hypertrophy) is enlargement of the prostate as a result of increased proliferation in both the epithelial and stromal compartments. By the age of 60, ~50% of men experience some enlargement symptoms and therefore it is the most common disease of the prostate gland. Pathologically, the growth is benign and therefore not dangerous, but inconvenient for the patient.

The outer capsule of the prostate can only expand to a certain extent. Therefore as the prostate enlarges, the inner region (central zone) will start press on the urethra and most men suffering from BPH therefore have problem with urination. In disease, both in enlargement as well as in cancer the prostate can reach a weight of about 30-50g. Urinary symptoms of BPH include being unable to urinate, having trouble with starting or stopping the urine flow, needing to urinate often especially in the night, weak flow of urine, start and stop of the urine flow and having pain or a burning feeling while urinating, but it can also cause problems with erection, give blood in the urine or semen or cause a pain in the lower back, hips or upper thighs. Also an increase in the levels of prostate specific antigen, PSA, can be seen. However these symptoms can also be caused by prostate cancer. Normally no treatment is given for BPH, but BPH and prostate cancer can be going on at the same time; therefore it is very important to be aware of the risks and to investigate each case separately.

1.2.2 Prostatitis

Prostatitis is infection or inflammation of the prostate gland. One quarter of all men suffering from urinary problems are affected by prostatitis. It is divided into four main categories; *Acute prostatitis* (bacterial), *Chronic bacterial prostatitis*, *Chronic prostatitis/chronic pelvic pain syndrome* and *Asymptomatic inflammatory prostatitis*.

Acute prostatitis is caused by bacteria such as *Escherichia Coli, Klebsiella, Proteus, Pseudomonas, Enterobacter, Enterococcus, Serratia,* and *Staphylococcus aureus*. It is characterized by symptoms such as fever, chills, pain in the lower back and genital area, frequent urination, burning or painful urination, and a demonstrable infection of the urinary tract. The condition is caused by white blood cells and bacteria in the urine but can easily be treated with antibiotics.

Chronic bacterial prostatitis is a very rare condition and is mainly caused by *E. Coli*. It is characterized by recurrent urinary tract infections caused by a chronic infection in the prostate. It expresses similar symptoms as acute prostatitis, but is usually not as severe. The condition is treated by long courses of antibiotics and sometimes also alpha-blockers.

Chronic prostatitis/chronic pelvic pain syndrome is characterized by pelvic pain of unknown cause, lasting longer than 6 months, which may also radiate to back and rectum leading to difficulties in sitting. Other symptoms are fatigue, frequent urination, increased urgency, painful ejaculation and some patients report low libido. Theories are that this condition can be caused by several factors such as autoimmunity, neurogenic inflammation & myofascial syndrome. The two last mentioned are probably caused by dysregulation of the local nervous system for some reason not totally understood. The Stanford protocol is used for treatment. It is a combination of medication, and physical & psychological therapy.

Asymptomatic inflammatory prostatitis are present in patients that have no history of urinary pain, although bacteria or leukocytosis have been reported earlier when examination for other complaints have been made. It is usually diagnosed by analyses of semen or urine to look for signs for inflammation. No treatment is needed although antibiotics are normally given. Asymptotic inflammatory prostatitis has been indicated to have a correlation with prostate cancer and therefore further and deeper examination is usually performed.

1.2.3 Prostate intraepithelial neoplasia (PIN)

Prostate Intraepithelial Neoplasia, PIN, is a pathological condition described as a precursor to prostatic carcinoma. It involves cellular proliferation within prostatic ducts, ductules, and acini. There are two grades of PIN, low-grade PIN (LGPIN) and high-grade PIN (HGPIN) but nowadays almost exclusively only the expression HGPIN is used. HGPIN is not considered to be a disease that requires therapy. However HGPIN is a potential pre-stage for the development of prostatic adenocarcinoma and some experts therefore believe that it should be treated. PIN spreads via the prostatic ducts in 3 different patterns resembling prostate cancer. The first pattern occurs when neoplastic cells replace the normal luminal secretory epithelium, but the basal layer and basement membrane is still intact. The second pattern is characterized by direct invasion via the ductal or acinar wall with disruption of the basal cell layer. In the third pattern, neoplastic cells invaginate between the basal cell layers. However, this is a rare pattern.

1.2.4 Prostate Cancer

Prostate cancer develops when cells in the prostate starts to grow and divide uncontrollably. As more and more cancer cells are formed the cells will grow into a little lump which will eventually form a tumour.

When the tumour gets in contact with blood vessels or lymph vessels it spread to other tissues and form metastases, daughter tumours. The cancer use to be divided into different stages. In the first stage the tumour grows within the prostate. In later stages it even grows outside of it and may have spread to the lymph nodes or other organs, normally the skeleton.
In over 99% of the cases the prostate cancer develops from glandular epithelial cells, the ones that make the fluid that is mixed with the semen, although there are other cells present in the prostate as well. This kind of cancer is called adenocarcinoma. Most prostate cancers grow quite slowly and it may therefore take a while before any symptoms arise. Sometimes it has gone so far that the cancer has already spread outside the prostate and sometimes it may not even affect the patient at all. It is not unusual to find prostate cancer that no one knew about before, in dead elder men, while performing an autopsy. In their 80s, 70-90% of all men have developed prostate cancer, however most men die with, not from prostate cancer.

1.2.4.1 Therapies available today for prostate cancer

The most important test used today to examine whether or not prostate cancer or disease is present or under development, is the PSA blood test. It measures the level of prostate-specific antigen, PSA, circulating in the blood. The base level for PSA is usually less then 4 mg/l but can rise to over 100 mg/l if cancer or inflammation is present. Medical doctors world wide argue whether or not an annual examination of the PSA levels should be introduced as mammography examinations are compulsory for women. This would mean that a base line of the PSA levels could be drawn and that changes within the prostate, cancer as well as disease, could be discovered at an early stage. However, this could also lead to the issue that the man stops living because of him being diagnosed with prostate cancer. It is important to keep in mind that prostate cancer does not necessarily equal a rapid death and therefore many people argue that men are better of not knowing.

The PSA levels are also useful after the discovery of the cancer to monitor the spread of the cancer as well as looking at the response to treatments. If cancer is suspected, a biopsy is usually performed. Multiple samples are taken from the prostate and examined for cancer. This is done either by insertion of a needle through the rectum or through the skin between the scrotum and rectum and into the prostate. The samples are viewed under a microscope by a pathologist to look for cancer cells. The cancer is given a Gleason score ranging between 2 and 10 with 2 being very unlikely for the tumour to spread and 10 being an aggressive cancer that easily forms metastases. Rectal examination, both using a finger and physically feel whether or not there are any changes of the prostate, or insertion of an ultrasound probe to get a monitored picture of the prostate, are used to look for changes of the prostate as well as for prostate cancer. Both these examinations used to be experienced as unpleasant for the man.

The primary choices of treatments for prostate cancer include radiation, surgery, hormonal therapy and cryoablation. They can be used either in combination or by themselves.
The tumours formed are stimulated by testosterone, an androgen that is produced within the testes. The most efficient method available today to slow down the development of the cancer is to castrate the man, an interference that can raise anxiety and discomfort for the patient. The castration can be performed in two different ways, either by orchiectomy or by hormonal drug therapy. Orchiectomy is surgical castration with removal of the testes. This is a form of hormonal therapy since 95% of all testosterone in the body is produced in by the testes. Orchiectomy is usually performed on patients with advanced metastatic prostate cancer and leads to deprival of testosterone for the cancer cells, resulting in shrinkage of the tumour and prevention of further growth. It is a relatively simple procedure but it is permanent and the effects can not be reversed meaning that the patients have to compensate for the low testosterone levels later on after depleted treatment. This procedure can also lead to a decreased sexual desire as well as impotence which can be very upsetting for both the patient as well as his significant other.

Hormonal drug therapy is a sort of medical castration. This is performed by injecting drugs that prevent or block the production and action of testosterone and other male hormones. There are mainly three classes of drugs used today towards prostate cancer. It is Luteinizing hormone-releasing hormone, LHRH analogs which prevent testosterone production by the testes, LHRH antagonists that also prevent testosterone production by the testes but in another way and antiandrogens that blocks the action of testosterone on the prostate. This therapy is most commonly used to treat advanced metastatic or local advanced prostate cancer.

All these drugs can be used on their own, combined with another drug or combined with for e.g. radiation therapy. Unfortunately it is common for the cancer cells to get resistant to the drugs and therefore the cancer can start progress again and other therapies are necessary.

At some occasions parts of or the whole prostate is removed to get rid of the tumour. This is a procedure that usually leads to impotence and incontinence. This is usually followed by radiation therapy where the prostate is treated with highly focused x-rays in small doses over several weeks to enable the cancer cells to grow and divide. The procedure is painless but side effects such as diarrhea and urinary problems can be experienced.

Cryoablation is yet another widely used method to try and cure prostate cancer where the tumour is exposed to extreme cold, -40˚C, to freeze the cells and destroy them. This is done using probes, delivered in liquid argon, inserted via the skin and into the diseased parts. A warming catheter is used to heat the urethra and to avoid damage of it. After the procedure is finished the tissue is thawed using helium gas. Patients usually have to spend 1-3weeks wearing a catheter and some patients experience side effects such as swelling and bruising and almost half of all the men going through cryoablation suffer from impotence afterwards. The prognosis of the cancer is affected by several factors such as the stage of the cancer when it is discovered and if it has reoccurred, the Gleason score, the PSA levels and the health and age of the patient.

Even though prostate cancer can in many cases be cured or slowed down by different therapies a lot of men suffer from serious side effects that are irreversible and affect both the patient and his significant other. More research within the area to try and understand how the cancer occurs and what is causing it could lead to a better life for many men and a lower number of deaths around the world for men suffering from the disease. Understanding the initiation of prostate cancer could also lead to development of better and more suitable drugs in the future.

1.3 Stromal-epithelial cells in the prostate gland

The prostate gland is built up by epithelial and stromal cells. Epithelial cells are usually closely packed and form the epithelium, the thin layer of cells that cover the inner and outer part of the gland. Within the prostate the epithelial cells line the ducts.
The stromal cells build up the connective tissue of the cells and surround the epithelial cells. There is a constant signalling occurring between these two cell types during development, adulthood and disease of the prostate. Prostate development is induced by androgens acting through androgen receptors, AR, found on mesencymal cells. The mesencyme in its turn act on the epithelial cells and induce prostatic epithelial development. This signalling by androgens, first and foremost by testosterone, continues also in the mature prostate to maintain functional differentiation and growth-quiescence [3].

Tissue recombination has been used to understand this communication between the two different cell types. The first recombinations were performed already in the 1980's by Cuhna and his co-workers. Their aims were to study the interactions between the different cell types in the development of the prostate[4].
Tissue recombination allows recombining stroma and epithelium, not necessarily from the same animals, and study the growth and interaction in vivo. This can answer questions about signalling between the two cell types, proliferation and functional activity as well as differentiation of the two.

This technique can be really useful when looking at what factors affect development or which genetic defects can influence cancer to develop in different organs.

1.3.1 Mesenchyme-epithelial interactions during development

Interactions between endodermal epithelium and urogenital sinus mesenchyme are what develop the prostate; mesenchymal cells produce inductive signals under the influence of androgens (primarily testosterone) to guide the epithelial cells into their fate.
Cuhna et al showed in 1992 that cell-cell interactions in the prostate development is reciprocal with the mesenchyme inducing prostate epithelial differentiation and the developed epithelium inducing smooth muscle differentiation from the mesenchymal cells [5].

Mesenchymal cells have the ability to direct differentiation of not only endodermal epithelium but also hESCs (reference Taylor et al Nature Methods).
This mechanism is what we are using in the differentiation of the tissue in this study. We recombine mesenchymal cells with hES cells to show that urogenital mesencyme can direct the differentiation of the hES cells down a prostatic lineage resulting in human prostate tissue being formed, as previously reported by Taylor and colleagues.
We also hypothesis that a carcinogenic stromal cells can convert a normal epithelial cell into a carcinogenic state or forming a carcinogenic tissue using the same signalling as in differentiation

1.3.2 Stromal-epithelial cell interactions in prostate cancer

Stromal-epithelial interactions mediate androgenic signaling in the developing and mature prostate. In prostate disease, especially cancer, this signaling is disturbed. Altered tumour stroma responds to androgenic stimulation by producing paracrine-acting mitogens that fuel a cycle of cancer cell proliferation and stromal de-differentiation. Therefore it is said that prostate cancer cells are under the control of their surrounding tumor stroma or microenvironment. These signaling between the two cell types are what maintenance the homeostasis within the tissue. Once this signaling is disrupted, adhesion, cell death and proliferation occur.

Tissue recombination is a way of studying tissue formation and its loss of homeostatic control. This can give a better insight in the transformation of a healthy tissue to a carcinogenic one and how this is controlled. If a better understanding of this can be gain perhaps a reversal process can be developed.
Cuhna et al. made recombinations between UGM and BPH-1 already in the 1980's that turned out to form human prostate tissue. To see whether carcinoma-associated fibroblasts, CAFs had the potential to transform normal epithelial cells into a malignant state or if they are just capable of progress a tumour formation in initiated cells they also recombined CAFs with normal epithelial cells as well as BPH-1 cells [6]. Cancer only formed when recombining the CAFs with the initiate BPH-1 cells indicating that an initial hit in both epithelial cells as well as in the surrounding stroma is necessary for cancer to develop. Their work is very similar to what we are trying to achieve. They were using UGM and CAFs as stromal cells just as we are doing. They are also using a non-malignant epithelial cell line for their recombinations just as us. However, the cell line that they are using to recombining their stromal cells with is BPH-1. That is a cell line purified from Benign Prostatic Hyperplasia, BPH, which is normal prostate enlargement. No studies so far show any evidence that there should be a relationship between BPH and prostate cancer, but to be able to culture this cell line in the lab, some genetic modifications had to be done. BPH-1 for e.g. has 76 chromosomes. We therefore hypothesise that the recombinations that they were doing to make their model system does not

really give a proper view of how prostate and prostate cancer develops and behaves as well as reacts upon different treatments. Using human embryonic stem cells for this purpose will give a better model that we think will behave more similar to how it is in real life, in men.

1.3.2.1 Carcinoma-associated fibroblasts (CAFs)

Fibroblasts are cells that synthesize and make up the extracellular matrix, also called the connective tissue. They make up the stroma, the framework that builds up the tissue and usually they secrete different precursors such as androgens that tell the epithelial cells what to do. This is done via cell-to-cell interactions. They have the possibility to reverse tumor cells to a normal phenotype as well as promote malignant conversion of normal cells. Therefore they are said to be able to determine the fate of epithelial cells [7].
Myofibroblasts can be found in many different tissues such as lung, brain, prostate, heart, breast etc. under normal conditions [7]. They are fibroblasts that have partially differentiated to get the phenotype of a smooth muscle cell.

CAFs, also called tumor myofibroblast, are activated fibroblasts. Myofibroblasts are only a pathological cell type when they have been activated. However when they are, they can implement malignant transformation of epithelial cells and are therefore usually located close to neoplastic epithelial cells. They seem to have key features in inflammatory conditions as well as in the cancer [7].
Tumor myofibroblast have been shown to be present in reactive stroma in many different cancers such as breast, colon and prostate cancer. The reactive stroma is characterized by increased microvessel density, inflammatory cells, modified extracellular matrix composition and CAFs. In prostate cancer, the reactive stroma is characterized by the presence of myofibroblasts and fibroblasts together with a clear reduction of smooth muscle cells. This can also be seen in Prostatic intraepithelial neoplasia, PIN [8]. As was mentioned before, the PIN is a precursor for prostate cancer. The reactive stroma therefore further implements the malignant epithelial transformation in those cells, turning the PIN into prostate cancer.
Since the CAFs are the reason for malignant tumor formation, they are a potential target for cancer therapy.

It is well defined that mesenchyme direct the differentiation of adjacent epithelium, although the exact signaling mechanisms are poorly defined [3-6, 9-16]. However, it is unclear whether or not activated prostatic tumour stroma can initiate carcinogenesis and tumour formation in a normal epithelial cell, or is it so that an initial hit need to be present in both cell types from the start. We will attempt to address this question by recombining CAFs, activated stroma, with normal hES cells as well as with $Rb^{-/-}$ cells, initiated hES cells. Recombination between CAFs and hES cells has previously been done in our lab before this study started. Teratomas were formed and when examination of the tissue was done, bone structures were found. These are still unpublished results and more recombinations need to be done before any real conclusions can be drawn. The question still remains, whether recombining CAFs with $Rb^{-/-}$ cells form prostate cancer and our aim is to be able to answer this question by performing this study.

1.4 The project – background and aim

It is very difficult to get hold of normal human prostate tissue from adult men since the prostate exhibits disease (including benign and malignant) from the age of 40 years onwards. Access to prostate tissue from young healthy men in their 20's or 30's is limited. Therefore, Professor Trounson and Dr. Taylor previously developed a model of normal human prostate from human embryonic stem cells [6]. This model is based on tissue recombination technology where urogenital mesenchyme (UGM), and/or seminal vesicle mesenchyme

(SVM), both from mice or rats, are recombined with human embryonic stem cells (hESCs). The recombinants were grown under the renal capsule of intact adult severe combined immunodeficiency, SCID, mice for up to 12 weeks. Since androgen is essential for prostate development the mice were implanted with testosterone pellets that resulted in elevated androgen levels. During this time, the mesenchymal cells were capable of guiding the hESCs to differentiate into prostate epithelial cells. Immature and mature prostatic structures were observed, and maturation of the tissue was confirmed based on the expression of prostate-specific antigen (PSA), by secretory epithelial cells surrounded by a basal cell layer. This model system is useful for studying critical systemic or local factors that influence prostate development and maturation. Understanding normal prostate biology and the transition to malignant tumour formation is critical to designing new therapeutics and potential prevention strategies of this life-threatening disease.

In the current project, we propose to use hESCs to generate prostate cancer tissue. To do this, we will attempt to silence a gene, Retinoblastoma 1, Rb1, believed to be critical in prostate cancer development using short-hairpin RNA, shRNA, and determine the phenotype following hESC differentiation. Hopefully we can graft the cells in similar ways as was done in the previous model system. If we are successful, this will provide a novel model of human prostate cancer that can be studied in the laboratory and will allow us to test different treatments for the disease.

1.4.1 Human embryonic stem cells

Human Embryonic stem cells, hES cells, are cells that are pluripotent meaning that they can develop into any cell, of the around 200 different that there is, in all the three germ layers in the body; endoderm, ectoderm and mesoderm (fig.1.2e-f). They also have self-renewal capacity.

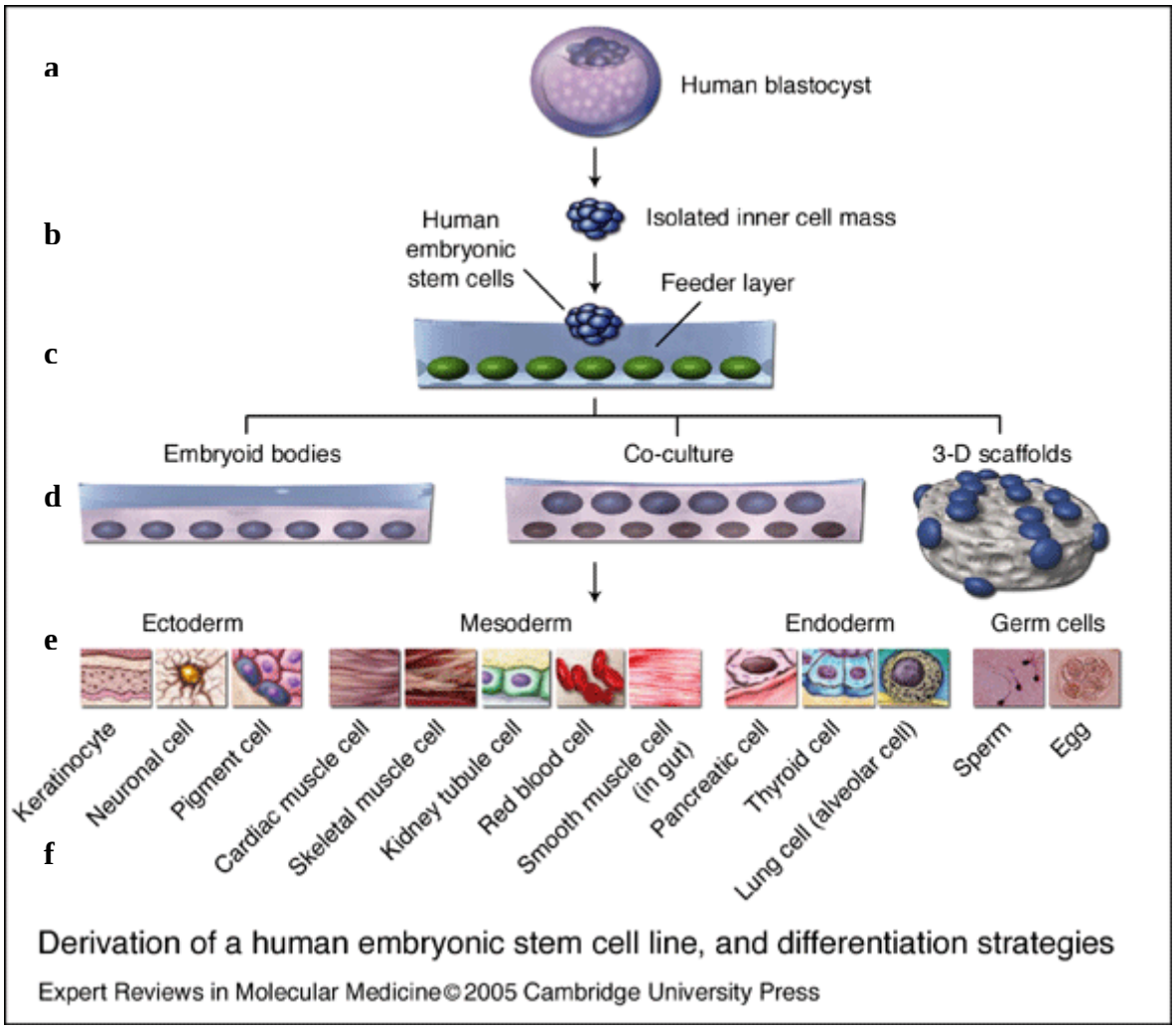

Figure 1.2 – **Overview of derivation of a human embryonic stem cell line and differentiation strategies**
Human embryonic stem cells are pluripotent and can differentiate into any cell type present in the body. In this study, prostate cells, which are differentiated further from endoderms, were derived using co-culturing and recombination with inductive cells.
a) Left over eggs from IVF studies are used to purify hES cells from. **b)** The hES cells are purified from the inner cell mass of the blastocyst that at that point is 4-5 days old. **c)** The stem cells are put on feeder cells that support them with essential nutrition. **d)** They can then be cultured in different ways, embryonic bodies, co-cultures or 3-D scaffolds. **e)** The cells are normally first differentiated down to any of the three germlayers, ectoderm, endoderm or mesoderm or into germ cells. **f)** From there any cell derived from either of the layers can be induced to form.
http://www-ermm.cbcu.cam.ac.uk/05009816h.htm

The hES cells are derived from the inner cell mass, ICM, of the blastocyst which is an early stage embryo (fig.1.2a-b). At that time the blastocyst is around 4 to 5 days old and is built up by approximately 50-150 cells. As long as no stimulation for differentiation, such as different culturing conditions, is given to the cells the hES cells will continue dividing *in vitro*, leading to each daughter cell having the same pluripotency as the mother cell. hES cells can be very useful for research in human cell and developmental biology as well as for their potential clinical application of cell replacement therapies.

The first stable human embryonic stem cell lines, H1, H13, H14 (XY karyotype) and H7, H9 (XX karyotype), were derived by James Thomson and his team at University of Wisconsin-Madison in 1998 [17]. Since then more lines have been generated from fertilized eggs left over from in vitro fertilizations, IVF. Rebineuff and Pera amongst others generated the lines that are used today at MISCL [18-20]. The company, ES International, that nowadays has the ownership for the cell lines, was once a spin-off from Monash University. The cell lines were

derived in Singapore since back then it was illegal to generate embryonic stem cells cultures in Australia. The lines are called hES1, hES2, hES3 (Chinese, XX karyotype), hES4, hES5 (Caucasian, XY karyotype) and hES6 (Caucasian, XX karyotype).

At the moment, a new stem cell line is derived at MISCL called MISCES-01. The difference between that cell line and the others are that it has only been grown on human feeders, instead of on mice feeders, meaning that they might eventually be useful for clinical applications. The cells have to be grown on a feeder layer of some kind (fig.1.2c-d). This enables the cells to attach to the surface of the organ culture dish. The feeders also provide the hES cells with nutrients essential for their survival.

1.4.2 Retinoblastoma 1 gene

Retinoblastoma 1, Rb1, is a tumor suppressor gene involved in cell cycle control, maintainance of chromosomal integrity, survival of epithelial cells and cellular differentiation. Loss of its activity has been shown in all sorts of tumors through mutations or in some cases a total loss of the gene [20]. It is perhaps not as important for prostate cancer development as it is for cells in general to become carcinogenic. Knockout of the gene leads to enhanced susceptibility of the tissue to undergo further genetic change, eventually leading to cancer as previously reported for lung, breast, eye and prostate cancer etc [21].

In an hypophosphorylated state its gene product acts as a transcriptional co-repressor that inhibits the function the E2F family genes. E2F is a family of transcription factors that play a major roll in the G1/S transition in the mammalian cell cycle. Rb1, in its normal state, bind the E2F-1 transcription factor and prevents it from interacting with the cells transcription machinery. If the Rb1, however, is inactivated, the E2F-1 mediates trans-activation of its target genes leading to DNA replication and cell division which eventually might lead to a tumor formation.

Findings, made by Reed et al. suggest that deregulation of the specific Rb1 targets can contribute to altered chemo sensitivity [21]. The same group also showed that inhibition of Rb1 in a human lung cancer cell line, by RNA interference via transfection of a vector, led to increased proliferation in vitro. When injecting these cells into Balb/c athymic mice, an increase tumor growth was seen compared to when injecting control cells. The control cells were the same human lung cell line transfected with an empty vector instead.

Homozygous deletion of the *RB* gene is lethal in embryonic mice at ~E13. Several years ago, Wang and co-workers rescued the prostates of $Rb^{-/-}$ animals by sub-renal grafting of the pelvic visceral rudiments and observed normal prostate differentiation [15]. Deletion of *Rb* had no discernible effect on prostatic histodifferentiation in rescued $Rb^{-/-}$ tissues or UGM + $Rb^{-/-}$ tissue recombinants [15]. Importantly, they demonstrated that deletion of the *Rb* gene predisposed prostate epithelium to hyperplasia and increased proliferative activity and promoted the progression to malignancy.

In a separate study, conditional somatic deletion of a single *Rb* allele in the epithelial cells of the mouse prostate caused focal hyperplasia as a result of the loss of *RB*-mediated cell cycle control, but not prostate cancer, even out to 52 weeks of age [22]. These studies have thoroughly investigated the effects of *Rb* loss in mouse prostate epithelial cells. It appears that loss of *Rb* does not alter differentiation of prostate epithelia, but increases proliferation resulting in a pre-cancerous phenotype. The result of this is increased susceptibility to malignant transformation following a carcinogenic insult such as hormonal carcinogenesis.

We hypothesis that going for this gene will make the hES cells used in this study more susceptible to undergo further genetic change, eventually leading to cancer.

Our aim with this project is to develop a model system of human prostate cancer using initiated human embryonic stem cells and recombine them with carcinoma associated fibroblast. We hope that this will give us a better understanding of the initiation and progression of the cancer as well as help us pinpoint what is actually causing it.
This will be the first time ever that human embryonic stem cells are used in these kind of experiments.

2.0 Materials and Methods

2.1 Culture of human embryonic stem cells

Human embryonic stem cells (hESCs) were cultured on mouse feeder fibroblasts as previously described [18] using two different methods, 1) organ culture of individual colonies and 2) bulk culture.

2.1.1 Preparation of mouse embryonic fibroblasts

Mouse embryonic fibroblasts (MEFs) were derived for mouse embryos at days E12.5-13.5 of gestation. In this study, the MTKneo2 strain was used. Embryonic fibroblasts were prepared and cultured in T175 flasks until 80-90% confluency when they were split into new flasks (~3 million cells per each flask). After 4 passages, the cells were inactivated using γ-radiation to prevent further proliferation. Excess irradiated mEFs were frozen down, in small quantities, in liquid nitrogen to be used for hESC culture.

2.1.2 Organ culture of individual hESC colonies

hES2 cells were routinely cultured on normal density ($1.65*10^5$ cells/cm^2) mouse embryonic fibroblasts (MEFs; MTKneo2 strain) as individual colonies on organ culture dishes using the following hES media formulation: KnockOut Delbecos Modified Essential Media (DMEM), 20% KnockOut Serum replacement (KOSR), 0.5x Penicillin/streptomycin, 1x Non-essential Amino Acids, basic-Fibroblast Growth Factor (bFGF) (4 ng/ml), 1x Glutamax and B-mercaptoethanol (1.8%). A cutting pipette was used to split the colonies into equal sized pieces and good quality undifferentiated pieces from the colonies were taken for transfer. The pieces were rinsed in an organ culture dish containing hES media before 8-10 pieces were transferred, using a 20 µl pipette, to each new mEF plate (pre-equilibrated with hES media). The pieces were spread out equally over the plate. The media was changed every day and the colonies were split every 7^{th} day.

2.1.3 Bulk culture of hESCs

hES2 cells were routinely cultured on one third normal density ($2*10^4$ cells/cm^2) mouse embryonic fibroblasts (MEFs; MNTKneo strain) as bulk cultures using the following media formulation: KnockOut Delbecos Modified Essential Media (DMEM), 20% KnockOut Serum replacement (KOSR), 0.5x Penicillin/streptomycin, 1x Non-essential Amino Acids, basic-Fibroblast Growth Factor (bFGF) (4 ng/ml), 1x Glutamax and B-mercaptoethanol (1.8%). Passages were performed with a brief Phosphate Buffered Saline (PBS) wash followed by Cell dissociation solution (Sigma-Aldrich, C5914). Cell Dissociation Solution was added for 4 minutes at 37ºC. Colonies were detached using a tapping technique (that selectively leaves MEFs behind). Cells were collected in hES media and the cell suspension was spun down at 2000 rpm for 2 minutes. The supernatant was discarded and the hESCs were resuspended in an appropriate volume of media. Alternatively colonies were passaged using collagenase. Following a brief PBS wash, cells were incubated in collagenase (4mg/ml) for 20-30 minutes at 37ºC until the boarders of the colonies started to curl up. The collagenase was aspirated off and the colonies were gently washed off the flask. The cell suspension was dispersed into pieces of approximately 50-100 cells. The suspension was spun down at 1800 rpm for 3 minutes. The supernatant was discarded and the cells were resuspended in an appropriate volume of media. An appropriate split was (~$1*10^6$ cells per T25 flask, ~$3*10^6$ cells per T75 flask, ~$6*10^6$ cells per T175 flask) made and the cells were seeded in flasks containing media. This was comparable to a 1:4 to split. Experiments used hESC passage numbers 50 – 99 (no higher than 30 in bulk culture).

2.2 Characterisation of human embryonic stem cells

2.2.1 Immunostaining of hESCs

In order to confirm that our hESC colonies were undifferentiated, we performed indirect immunofluorescence for Oct ¾ and CD30 proteins. The colonies were fixed in cold 100% ethanol for 10min and then air dried. Following washes in PBS, FCS was added to the colonies for 15min to block non-specific binding. Colonies were then incubated in primary antibodies at room temperature for 30min. Antibodies included monoclonal mouse anti-human CD30 antibody (DAKO Cytomation, M0751; 1:30 dilution) and mouse monoclonal IgG2b Oct-3/4 (C-10) antibody (Santa Cruz Biotechnology, SC5279; 1:50 dilution). Following washes in PBS, colonies were then incubated in donkey anti-mouse-FITC secondary antibodies (1:3000 dilution) at room temperature for 30 min. Detection was visualised under fluorescent light for FITC.

2.2.2 Real time PCR analysis

RNA purification was performed using the PicoPure™RNA isolation kit (ARCTURUS, USA) and the protocol addressed by the manufacturer was used. cDNA synthesis was performed using the High-Capacity cDNA Archive Kit (Applied Biosystems, Australia) and the protocol addressed by the manufacturer was used.

In order to determine gene expression of key stem cell genes and our genes of interest in relation to prostate cancer, we performed real-time PCR on undifferentiated and differentiated hESCs. Gene expression analysis was performed using the Pre-Developed TaqMan® Assay Reagents (Applied Biosystems, Australia) and the protocol addressed by the manufacturer was used. The TaqMan primers for the Retinoblastoma 1, Glutatione-S-transferase P1, Oct-42, Nanog and IPF-1 genes were pre-designed and made by Assays-on-Demand Gene expression TaqMan® primers (Applied Biosystems, Australia).

2.3 short hairpin RNA in human embryonic stem cells

2.3.1 Preparation of hESCs for transfection

hES2 cells were harvested from n=4 T25 flasks using collagenase. hESC colonies were resuspended in 50% hES media and 50% condition MEF media (taken from n=2 T75 flasks with mEF grown in hES media, see appendix). The cells were seeded in 6 well plates that were previously coated in Matrigel at a concentration of 0.0347mg/cm^2 and incubated at 37ºC (see appendix).

2.3.2 Viral transfection

The transfection of viral particles and selection of transfected cells was implemented during a week. The hES2 cells were transfected with the virus that acted on the cells for 24h before they were washed away. The antibiotic selection started 2 days post-removal of virus. This was to allow the cells to recover and stabilize from the start of the knockdown of the gene. The selection went on for 3 days before the cells were harvested and moved to T25 flasks with 1/3 mEFs.

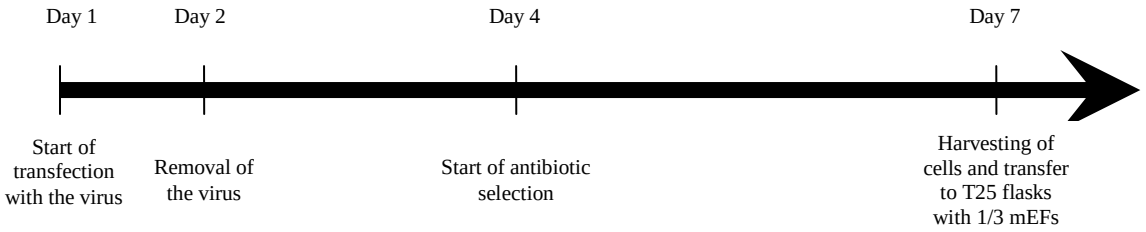

Figure 2.1 – **Time line of the transfection**
The transfection was started and was left to progress for 24h at 37˚C and 5% CO_2 before the virus was removed. The cells were left to stabilize and recover for 2 days before antibiotics, 2µg/ml puromycin was added to the target plates and 2µg/ml blastocydin was added to the control plates. The selection went on for 3 days for the target plates and for 1week for the control plates before harvesting and transfer of the cells to T25 flasks with 1/3 mEFs was performed.

One day after the hESCs were plated on matrigel, cells were incubated in PolyGreen (Sigma Aldrish, 0.1% in conditioned media). PolyGreen neutralized the negative charges in the membranes of the cells so the virus could bind easier and start the transfection. The shRNA's to target retinoblastoma 1 were prepared by Sigma according to the TRC system. TRC is an RNAi consortium with collaboration between the Broad Institute of MIT and Harvard. 11 institutes are involved, working to create lentiviral shRNA libraries targeting around 15000 human and just as many mouse genes within the next three years.
Each set contains 4-5 constructs per gene to get an ultimate silencing of the gene of interest in both primary and non-dividing cells. The shRNA is cloned into lentiviral vectors and come supplied as viral particles. The virus was thawed and added to the 6 well plates at 1:5, 1:50, 1:500 and 1:5000. Controls included no viral particles and GFP shRNA non-specific targets. Five targets were added for the Rb1 gene spanning different regions of the gene (Fig.2.2). The 6 well plates were left in an isolated incubator for virus transfections and were incubated at 37ºC over night.

gctcagttgccgggcgggggagggcgcgtccggtttttctcaggggacgttgaaattatttttgtaacgggagtcgggagaggacggggcgtgccccgacgtgcgcgcgcgtcgtcctccccggcgctcctccacagctcgctggctccc
gccgcggaaaggcgtcatgccgcccaaaaccccccgaaaaacggccgccaccgccgccgctgccgccgcggaaccccggcaccgccgccgccgcccccctcctgaggaggacccagagcaggaca gcggcccggaggacctg
cctctcgtcaggcttgagtttgaagaaacagaagaacctgattttactgcattatgtcagaaattaaagataccagatcatgtcagagagagagcttggttaacttgggagaaagtttcatctgtggatggagtattgggaggttatattcaaaaga
aaaaggaactgtggggaatctgtatctttattgcagcagttgacctagatgagatgtcgttcactttactgagctacagaaaaacatagaaatcagtgtccataaattctttaacttactaaaa gaaattgataccagtaccaaagttgataatgctat
gtcaagactgttgaagaagtatgatgttattgtttgcactcttcagcaaattggaaaggacatgtgaacttatatatttgacacaacccagcagttcgatatctactgaaataaattctgcattggtgctaaaagtttcttggatcacatttttatt agctaa
aggggaagtattacaaatggaagatgatctggtgatttcatttcagttaatgctatgtgtccttgactattttattaaactctca cctcccatgttgctcaaagaa ccatataaaacagctgttatacccattaatggttcacctcgaacacccaggcga
ggtcagaacaggagtgcacggatagcaaaacaactagaaaatgatacaagaattattgaagttctctgtaaagaacatgaatgtaatatagatgaggtgaaaaatgtttatttcaaaaattttat accttttatgaattctcttggacttgtaacatcta
atggacttccagaggttgaaaatctttctaaacgatacgaagaaatttatcttaaaaataaagatctagatgcaagattatttttggatcatgataaaactcttcagactgattctatagacagttttgaaacacagagaacaccacgaaaaagtaacc
ttgatgaagaggtgaatgtaattcctccacacactccagttaggactgttatgaacactatccaacaattaatgatgattttaaattcagcaagtgatcaaccttcagaaaatctgatttcctat tttaacaactgcacagtgaatccaaaagaaagtat
actgaaaagagtgaaggatatagatacatctttaaagagaaatttgctaaagctgtgggacagggttgtgtcgaaattggatcacagcgata caaacttggagttcgcttgtattaccgagtaatggaatccatgcttaaatcagaagaagaac
gattatccattcaaaatttagcaaacttctgaatgacaacatttttcatatgtctttattggcgtgcgctcttgaggttgtaatggccacatatagcagaagtacatctcagaatcttgattctggaacagatttgtctttcccatggattctgaatgtgctt
aatttaaaagccttgatttttacaaagtgatcgaaagttttatcaaagcagaaggcaacttgacaagagaaatgataaaacatttagaacgatgtgaacatcgaatcatggaatcccttgcatg gctctcagattcaccttttatttgatcttattaaaca
atcaaaggaccgagaaggaccaactgatcaccttgaatctgcttgtcctcttaatcttcctctccagaataatcacactgcagcagatatgtatctttctcctgtaagatctccaaagaaaaaaggttcaacta cgcgtgtaaattctactgcaaatg
cagagacacaagcaacctcagccttccagacccagaagccattgaaatctacctctctttcactgttttataaaaaagtgtatcggctagcctatctc cggctaaatacactttgtgaa cgccttctgtctgagcacccagaattagaacatatcatc
tggaccctttccagcacacccctgcagaatgagtatgaactcatgagagacaggcatttggaccaaattatgatgttccatgtatggcatatgcaaagtgaagaatatagaccttaaattcaa aatcattgtaacagcatacaaggatcttcctc
atgctgttcaggagacattcaaacgtgttttgatcaaagaagaggagtatgattctattatagtattctataactcggtcttcatgcagagactgaaaacaaatattttgcagtatgcttccaccaggccccctaccttgtcaccaatacctcacattcc
tcgaagcccttacaagtttcctagttcacccttacggattcctggagggaacatctatatttcaccccgaagagtccatataaaatttcagaaggtctgccaacaccaacaaaaatgactccaa gatcaagaatcttagtatcaattggtgaatcat
tcgggacttctgagaagttccagaaaataaatcagatggtatgtaacagcgaccgtgtgctcaaaagaagtgctgaaggaagcaaccctcctaaaccactgaaaaaactacgctttgatattgaaggatcag atgaagcagatggaagtaaac
atctcccaggagagtccaaatttcagcagaaaactggcagaaatgacttctactcgaacacgaatgcaaaagcagaaaatgaatgatagcatggatacctcaaacaaggaa gagaaatgaggatctcaggaccttggtggacactgtgtacac
ctctggattcattgtctctcacagatgtgactgtataacttccccaggttctgtttatggccacatttaatatcttcagctctttttgtggatataaaatgtgcagatgcaattgtttgggtgattcctaagccacttgaaatgttagtcattgttatttatacaa
gattgaaaatcttgtgtaaatcctgccatttaaaaagttgtagcagattgtttcctcttccaaagtaaaattgctgtgctttatggatagtaagaatggcccctagagtgggagtcctgataacccaggcctgtctgactacttttgccttctttttgtagcata
taggtgatgtttgctcttgtttttattaatttatatgtatatttttttaatttaacatgaacacccttagaaaatgtgtcctatctatcttccaaatgcaatttgattgactgcccattcaccaa aattatcctgaactcttctgcaaaaatggatattattagaaatt
agaaaaaaattactaatttttacacattagattttatttactattggaatctgatatactgtgtgcttgttttataaaatttgcttttaattaaatataaagctggaagcaaagtataaccatat gatactatcatactactgaaacagatttcatacctcagaat
gtaaaagaacttactgattattttcttcatccaacttatgttttaaatgaggattattgatagtactcttggttttataccattcagatcactgaatttataaagtacccatctagtacttgaaaaagtaaagtgttctgccagatcttaggtatagaggacc
ctaacacagtatatcccaagtgcacttttctaatgtttctgggtcctgaagaattaagatacaaattaattttactccataaacagactgttaattataggagccttaatttttttttcatagagatttgtctaattgcatctcaaaattattctgccctccttaatt
tgggaaggtttgtgttttctctggaatggtacatgtcttccatgtatctttgaactggcaattgtctatttatctttttattttttaagtcagtatggtctaacactggcatgttcaaag ccacattatttctagtccaaaa attacaagtaatcaaggggtcattat
gggttaggcattaatgtttctatctgattttgtgcaaaagcttcaaattaaaacagctgcattagaaaaagaggcgcttctcccctccccctacacctaaaggtgtatttaaactatcttgtgtgattaacttatttagagatgctgtaacttaaaatagggg
gatatttaaggtagcttcagctagcttttaggaaaatcactttgtctaactcagaattatttttaaaaagaaatctggtcttgttagaaaacaaaatttttattttgtgctcatttaagtttcaaa cttactatttgacagttatttttgataacaatgacactagaa
aacttgactccatttcatcattgtttctgcatgaatatcatacaaatcagttagttttaggtcaagggcttactatttctgggtcttttgctactaagttcacattagaattagtgccagaattttag gaacttcagagatcgtgtattgagattcttaaataa
tgcttcagatattattgctttattgctttttgtattggttaaaactgtacatttaaaattgctatgttactatttttctacaattaatagtttgtctatttttaaaataaattagttgttaagagtcttaa

TRCN0000040163 - 01
CCGGCCACATTATTTCTAGTCCAAACTCGAGTTTGGACTAGAAATAATGTGG**TTTTTG**

TRCN0000040164 - 02
CCGGCGCGTGTAAATTCTACTGCAACTCGAGTTGCAGTAGAATTTACACGCG**TTTTTG**

TRCN0000010417 – 03
CCGGTATTGCACGAGTTGACCTAGACTCGAGTCTAGGTCAACTCGTGCAATA**TTTTTG**

TRCN0000040165 - 04
CCGGCGGCTAAATACACTTTGTGAACTCGAGTTCACAAAGTGTATTTAGCCG**TTTTTG**

TRCN0000010418 – 05
CCGGACTTCTACTCGAACACGAATCTCGAGATTCGTGTTCGAGTAGAAGTC**TTTTTG**

TRCN0000040166 – 06
CCGGCCTCCCATGTTGCTCAAAGAACTCGAGTTCTTTGAGCAACATGGGAGG**TTTTTG**

TRCN0000010419 – 07
CCGGCAGAGATCGTGTATTGAGATTCTCGAGAATCTCAATACACGATCTCTG**TTTTTG**

TRCN0000040167 – 08
CCGGCGAAATTGGATCACAGCGATACTCGAGTATCGCTGTGATCCAATTTCG**TTTTTG**

Figure 2.2 – **Alignment of target sequences to the Retinoblastoma 1 gene**
8 shRNA targets were available to knockdown the Retinoblastoma 1, Rb1 gene. A kit of 5 targets could be bought from Sigma-Aldrish and TRCN0000040163 - 01, TRCN0000040164 - 02, TRCN0000040165 - 04, TRCN0000040166 - 06 and TRCN0000040167 - 08 was used when performing the knock down of the gene.

The following day, the media containing the viral particles was removed and cells were washed with hES media. Cells were then incubated in conditioned mEF media and incubated at 37ºC over night. Conditioned mEF media was replaced the following day and again the cells were incubated 37ºC over night.

On day 4 post-transfection, the hESCs that had integrated the shRNA were selected under antibiotic resistance. The Rb1 shRNAs were selected under puromycin resistance (2µg/ml in conditioned mEF media) whilst the GFP non-specific shRNA was selected under blastocydin resistance (5µg/ml Blastocydin in conditioned mEF media). Cells transfected with the different vectors could produce the antibiotic resistance and therefore survived living in media containing the different antibiotics, resulting in positive selection of transfected cells.
The plates were incubated at 37ºC for 3-7 days.

2.3.3 Stabilizing the transfected cells

When the selection was complete, the cells were stabilized and harvested using Cell Dissociation Solution. Each well was transferred to its own T25 containing mEF cells in hES media and incubated at 37ºC over night. Following the return to feeder layers in bulk culture, the shRNA cells were maintained as normal hESCs. Some organ culture colonies were produced from the first passage and maintained as individual colonies.

2.3.4 Confirmation of the gene silencing

In order to confirm the silencing of Rb1 in hES 2 cells by the five individual targets, western blotting analysis for protein and real time PCR for mRNA analysis was preformed. Unfortunately due to time constraints, this work was conducted after the end of this project.

2.4 Tissue recombination

2.4.1 Animals

Timed pregnant Balb/C mice were obtained from Monash University central Animal Services, killed at 16 days gestation (plug day = day 0). Urogenital sinuses were obtained from male embryos as previously described [23]. All animal handling and procedures were carried out in accordance with National Health and Medical Research Council (NHMRC) guidelines for the

Care and Use of Laboratory Animal Act and according to the Animal Experimentation and Ethics Committee at Monash Medical Centre, Clayton, Australia.

2.4.2 Tissue Separation

Using a dissecting microscope (SZX12, Olympus Corporation, Tokyo, Japan) and dissecting tools, urogenital tracts were removed from male pups. The bladder and the genital tract were removed including the wolffian duct, the Urogenital Sinus, UGS, the bladder and the Urethrea were dissected out. The tracts were trimmed to reveal the UGS. Microdissections were performed in a modified watch glass (maximov depression slide; San Francisco, CA), in the presence of dissecting media (basal medium of Dulbecco's Modified Eagels Media (DMEM) and Hams F-12 (1:1 vol/vol) supplemented with penicillin and streptomycin (5mls/ltr) and fungizome (20µg/ml) at pH 7.3). Urogenital mesenchyme (UGM) were obtained by mechanical separation following digestion in 1% trypsin (Difco, Detroit, MI) in Hank's calcium and magnesium free Balanced Salt Solution (HBSS; Gibco, Invitrogen, Vic, Australia) for 60 minutes. The UGE was separated from the UGM using a graefe separation tool.

2.4.3 Recombination of UGM and hESCs

hESCs were prepared from bulk culture and therefore as colonies in cell suspension. $Rb^{-/-}$ cells were harvested using Cell Dissociation Solution.

For recombinations between UGM and $Rb^{-/-}$, 40000 $Rb^{-/-}$ cells were used. For the $Rb^{-/-}$ alone controls, only 120000 cells were used.
The $Rb^{-/-}$ cells were put in appropriate volumes of setting solution. They were spun for 5min at 1800 rpm and the supernatant was discarded. The pellet was responded in 1 µl collagen+setting solution per number of recombinations made. The mix was put as 1 µl droplets in a dish and was incubated for 15min at 37ºC to solidify. Incubation media (RPMI + 5% FCS + 10^{-8}M Testosterone) was added on top of the samples.

Tissue recombinants were generated by combining a collagen hESC piece with 4 pieces of UGM. Tissue recombinants were incubated for 24 hours at 37 ºC on a solidified agar medium consisting of 1% agar (Oxoid Ltd, Hampshire, England) in 2x Dulbecco's Modified Eagle's Medium (DMEM; Gibco. NY, USA) with 10% (v/v) heat-inactivated foetal calf serum (FCS) (PA Biologicals Co. Pty Ltd, NSW, Australia) and antibiotics (100UI/ml penicillin and 10g/ml streptomycin; CSL Ltd, Parkville, Vic, Australia).

2.4.4 Recombination of CAF and hESCs

hESCs, CAFs and NPFs were prepared from bulk culture. $Rb^{-/-}$ cells were harvested using Cell Dissociation Solution whereas the others were harvested using trypsin.

In the recombinations, 10000 CAFs, 50000 NPFs, 10000 $Rb^{-/-}$, 10000 BPH-1 and 2 pieces from ENVY colonies were used.
When grafting the cells alone, 50000 CAFs, 30000 $Rb^{-/-}$, 50000 NPFs and 35000 BPH-1 cells were used.
The cells to be recombined were mixed in setting solution. They were spun for 5min at 1800 rpm and the supernatant was discarded. The pellet was responded in 50 µl collagen+setting solution. The mix was put as a droplet in a dish and was incubated for 15min at 37ºC or until it had solidified. Incubation media (RPMI + 5% FCS + 10^{-8}M Testosterone) was added on top of the samples.

Tissue recombinants were incubated for 24 hours at 37 ºC on a solidified agar medium consisting of 1% agar (Oxoid Ltd, Hampshire, England) in 2x Dulbecco's Modified Eagle's Medium (DMEM; Gibco. NY, USA) with 10% (v/v) heat-inactivated foetal calf serum (FCS) (PA Biologicals Co. Pty Ltd, NSW, Australia) and antibiotics (100UI/ml penicillin and 10g/ml streptomycin; CSL Ltd, Parkville, Vic, Australia).

Several controls were set up. CAFs were recombined with BPH-1 cells to confirm that the CAFs worked as previously described [5]. They were also recombined with ENVY cells, normal hES cells, to see the difference between normal and initiated stem cells.
Normal prostste fibroblasts, NPFs were recombined with BPH-1 cells as well as with $Rb^{-/-}$ to see the difference between initiated and normal stroma.
All cells were also prepared alone to be able to check for contamination.

2.4.5 Grafting

The procedures sub-renal grafting were performed as previously described [16].
Briefly, heterospecific tissue recombinants were grafted under the kidney capsule of adult male immune-deficient SCID mice bearing subcutaneous 10mg testosterone implants to augment androgen levels [5, 6]. The mice were sent to sleep using appropriate injections of Avertin. Two recombinations were grafted on each kidney. Recombinants were grown in host mice for 3-5 weeks. UGM was grafted alone as a method of detecting contamination by UGE. CAFs, NPFs and BPH-1 cells were also grafted alone to detect contamination. hESC were grafted alone to confirm pluripotency.

2.4.6 Harvesting mice

Harvesting tissue recombinants was done 3.7 weeks post-grafting for UGM and $Rb^{-/-}$ and 5 weeks post-grafting for CAFs and $Rb^{-/-}$.
The host mice were sent to sleep using appropriate levels of Avertin before they were decapitated. The kidneys were taken out and the grafts were removed using dissecting tools. The ventral and the anterior prostate as well as the seminal vesicles were also removed to look for normal growth and development of them.
The grafts were measured and weighed and put in Bouins solution, for different time spans depending on size, to be fixed. They were moved to 70% ethanol to be stored for further use.

2.4.7 Examination of teratomas

Fixed teratomas were sent to a histology lab to be processed to paraffin wax.
Paraffin embedding was performed by putting the processed tissues in forms and adding melted wax on top. The forms were left to solidify on ice.
The tissues were cut into 5µm sections in a microtome. Every 20^{th} or 50^{th} section of the teratoma was mounted onto glass slides. Several different sets were made. All slides were baked at 47ºC for 2h or at 37 ºC over night to properly melt sections onto the slides.

2.4.8 Haematoxylin & Eosin Staining

Haematoxylin & Eosin staining was performed to look at the morphology of the teratomas. The slides were washed twice in Solvent 3B-2026 (HiChem Industries Pty Ltd, Australia) for 4 min each. They were moved twice to 100% EtOH for 4 min and then to 70% EtOH for 4 min. The slides were rinsed in tap water for 4 min. The sections were stained in Mayers-Haematoxylin Solution (Amber Scientific, Australia) for 2 min and were rinsed in tap water for 8 min. They were also washed in Scotts tap water substitute (Amber Scientific, Australia) for 1 min before they were moved to tap water again for another 4 min. The slides were stained in Eosin 1% (Amber Scientific, Australia) for 1 min and were dipped twice in water.

The slides were washed in 70% EtOH for 2 minand then twice in 100% EtOH for 2 min each, before they were left in Histosolve Solution (HD Scientific Supplies Pty Ltd, Australia). Cover glasses were mounted over the sections using DPX neutral mounting medium (LabChem, Australia) and the slides were left in a hood to dry.

2.4.9 Immunohistochemistry

In order to determine the cell types and which receptors were present within the tissue recombinants, immunohistochemistry with different antibodies was performed using the DAKO Autostainer Universal Staining System (DAKO A/S, Denmark). Paraffin sections were dewaxed in graded ethanols. Following washing in PBS, antigen retrieval was performed in order to reveal antigen sites removed by crosslinking from fixation. Antigen retrieval involved boiling the sections in 0.01 M Citric Buffer ph 6.0 in a microwave for 5 min at high temperature, 15 min at medium low temperature followed by 20 min in the warm water.

The UGM/$Rb^{-/-}$, UGM alone and $Rb^{-/-}$ alone sections were stained for Androgen Receptor, AR (N-20) (Santa Cruz Biotechnology, INC, SC-816, 1:300 dilution), Mouse Anti-human Mitochondria Monoclonal Antibody (Chemicon International, MAB 1273, 1:100 dilution) and Polyclonal Rabbit Anti-human Prostate-Specific Antigen (DAKO Cytomation, A0562, 1:1200 dilution).
Rabbit Immunoglobulin Fraction (Solid phase absorbed) (DAKO Cytomation, X936, 1: 1:5000 dilution) and Mouse IgG1 Negative Control (DAKO Cytomation, X0931, 1:50 dilution) were used as negative controls.
Androgen Receptor, AR, as well as Prostate Specific Antigen, PSA, were used to show on prostate tissue. Mitochondria was used to show on human origin of the tissue.

The CAFs/$Rb^{-/-}$, CAFs/BPH-1, CAFs/envy, NPF/ $Rb^{-/-}$, NPF/BPH-1, $Rb^{-/-}$ alone and NPF alone were all stained for Cytokeratin (8/18) Mouse Monoclonal antibody (Novocastra Laboratories Ltd, NCL-5D3, 1:200 dilution), Monoclonal Mouse-Anti-Human Cytokeratin High Molecular Weight Clone 34βE12 (DAKO Cytomation, M0630, 1:300 dilution) and Monoclonal Anti-α Smooth Muscle Actin Clone 1A4 (SIGMA , A2547, 1:2000 dilution).
BPH-1 alone was only stained for Cytokeratin (8/18) Mouse Monoclonal antibody and Monoclonal Mouse-Anti-Human Cytokeratin High Molecular Weight Clone 34βE12 whereas CAFs anlone was only stained for Monoclonal Anti-α Smooth Muscle Actin Clone 1A4 and Monoclonal Anti-Vimentin Clone LN-6.
The CAFs/envy was also stained for Rabbit Anti-GFP Antibodies IgG Fraction (Invitrogen, A11122, 1:300 dilution).
Negative Control Mouse IgG_2a (DAKO Cytomation, X0943, 1: 50 dilution), Negative Control Rabbit Immunoglobulin Fraction (Solid Phase Absorbed) (DAKO Cytomation, X0936, 1:200 dilution) and Mouse IgG1 Negative Control (DAKO Cytomation, X0931, 1:50 dilution) was used as negative controls.

Yet another antigen retrieval step as well as two blocking steps, the first one with H_2O_2 and the other one with CAS Block was programmed to minimize background staining. The rest was performed according to the guidelines for the DAKO Envision + ™ System kit (DAKO A/S, Denmark).

After finalized staining program in the machine, the slides were counterstained with haematoxylin. A dehydration step was performed in graded ethanols and the slides were

washed 3 times in histolene-1 for 5min. The slides were mounted with cover glasses using DPX mounting medium.

3.0 Results

3.1 Human Embryonic Stem Cell culturing

Culturing of human embryonic stem cells is a difficult technique that labs around the world are still trying to learn how to master. The overall goal is to handle the cells in such as way to prevent spontaneous differentiation.

hES cells were routinely cultured on feeder layer cells, usually mouse embryonic fibroblasts, that provided the hES cells with attachment to the surface where they were grown as well as with important nutrition needed for the survival of the hES cells, thereof the name feeders.

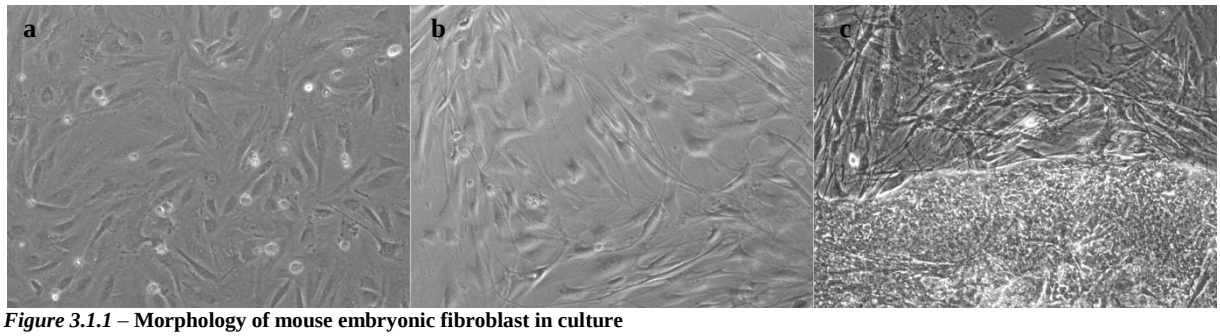

Figure 3.1.1 – **Morphology of mouse embryonic fibroblast in culture**
Human embryonic stem cells were routinely cultured on mouse feeder fibroblasts, mEFs. The mEFs used in this study were derived for mouse embryos at days E12.5-13.5 of gestation from the MTKneo2 strain.
The mEFs were pre-equilibrated with basic fibroblast growth factor, bFGF which activates them. A difference in morphology can be seen when this occurs. The mEFs elongates as a response to addition of bFGF. This is a sign for usage of good quality feeders.
a) mEFs growing in media with the absence of bFGF. **b)** mEFS growing in media supplemented with 4ng/ml bFGF. **c)** mEFs and human embryonic stem cells co-cultured in media supplemented with 4ng/ml bFGF.

A special media was used for the culturing of the cells. This media contained all necessities needed to make the conditions optimal for the hES cells to not go through differentiation, but to survive and proliferate. The media used was supplemented with basic fibroblast growth factor, bFGF. bFGF support un-differentiating growth. It also activates the feeders which elongates as a response to it [24]. This is a sign for usage of good quality feeders which is a key feature for successful hES cell culturing (Fig. 3.1.1).
hES cells were cultured in serum replacement media. The base media knockout-DMEM (Gibco) is a basal cell culture media developed to optimize the conditions for hES cells and to improve their morphology and performance. Addition of knockout serum replacement further improves the growth and maintenance of an un-differentiated state. The media used was also supplemented with amino acids, glutamax, penicillin/streptomycin, insulin-transferrin-selenium and β-mercaptoethanol.

The parental stem cells lines available at Monash Immunology and Stem Cell Laboratory, MISCL, hES1-6 (NIH Code: ES01-06) were routinely checked for chromosomal stability, characteristic human embryonic stem cell markers such as Nanog, Oct-4 and SOX2, as well as for spontaneous differentiation in vivo at regular intervals. This was to make sure that we were always working with high quality hES cells.

There were mainly two different ways that stem cells were cultured, in bulk cultures or on organ culture dishes. The two ways differed significantly both when it came to procedures in the lab when handling the hES cells as well as for the stress caused on them during these procedures.

hES cells were normally started up growing on organ culture dishes. It was easier to have control over and eliminate the differentiation of the cells when using this culturing technique. Only the best bits and peaces from the colonies were cut and used when passaging them. Even the number of cells per lump transferred could be better controlled when cutting from a plate. When splitting cells from a bulk using a chemical solution one was dependent on the solution to not break down the colonies in too small pieces. The optimal number of cells per lump transferred was 50-100 and absolutely not less then 20. This could be difficult to control and if it happened, the chances of the hES cells to differentiate, die or go through genetic modifications was high.
Growing hES cells in bulk cultures allowed a larger number of colonies to grow on the same time (Fig. 3.1.2 d). Expansion of the population could be done easily unlike with organ culture dish growth where one could decide which bits to continue with after passaging the cells, it was more difficult to control what continued to the next bulk since one had to trust in different solutions to only bring up the undifferentiated parts.

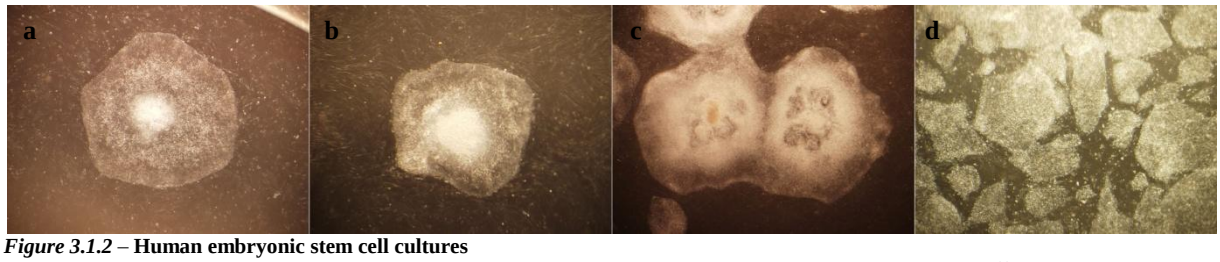

Figure 3.1.2 – **Human embryonic stem cell cultures**
Human embryonic stem cells were either cultured on organ culture dishes or in bulk cultures. The two techniques differed in the way the cells grew. On organ culture dishes the cells grew in nice colonies separated from one another whereas in bulk cultres the colonies grew more closely to one another.
By looking at the colonies scores regarding if the colonies were good or bad was determined. **a)** Good quality hES cells had defined boarders and a round and uniform shape. **b)** Some colonies could be scored as partially good. Those colonies could contain regions that were better than others. For example could good quality stem cells be seen in some parts whereas differentiation or cystic formations could be seen in others. The good parts from these colonies could still be used for passaging. **c)** Bad quality colonies that had differentiated usually looked blown up and contained craters within the colonies. Cystic formations could also occur. **d)** Stem cells growing in bulk culture.

The score of what was considered a good or a bad quality hES cell colony was made by looking at the colonies by eye. Good quality hES cells had defined boarders and a round and uniform shape (Fig. 3.1.2 a).
Some colonies could contain parts that were differentiated. Within those colonies one could cut from the good bits, but one had be to be aware of the fact that even though bits close to the differentiated area looked good, they might have started to go abnormal or bad, therefore a safety margin was used to minimize the risk of transferring bad pieces (Fig. 3.1.2 b).
Colonies that had differentiated usually looked blown up and contained craters within the colonies. Cystic formations could also occur (Fig. 3.1.2 c).

The stem cells used during this project was established on organ culture dishes before expansion in bulk cultures were done. In both these systems the hES cells were grown on mouse embryonic fibroblasts, mEFs, immortalized using γ-radiation.

3.2 Characterisation of gene expression in human embryonic stem cells

In order to determine the level of mRNA expression of particular genes in undifferentiated and differentiated hESCs, we performed real time RT-PCR analysis. We were particularly interested in the level of retinoblastoma expression post-differentiation. We also determined the gene expression level of another key prostate cancer gene, Glutathione-S-transferase pi, for future reference.

Other genes we examined were Oct42 which is a marker of undifferentiated stem cells and IPF1 which is a marker for differentiated cells. IPF1 is upregulated in the pancreas lineage and was therefore used to see that the differentiated samples were actually differentiated. A control gene included β-Actin as a house-keeping gene to make sure that all samples expressed it and were amplified equally. We also used dH2O as a negative control for all different genes used.

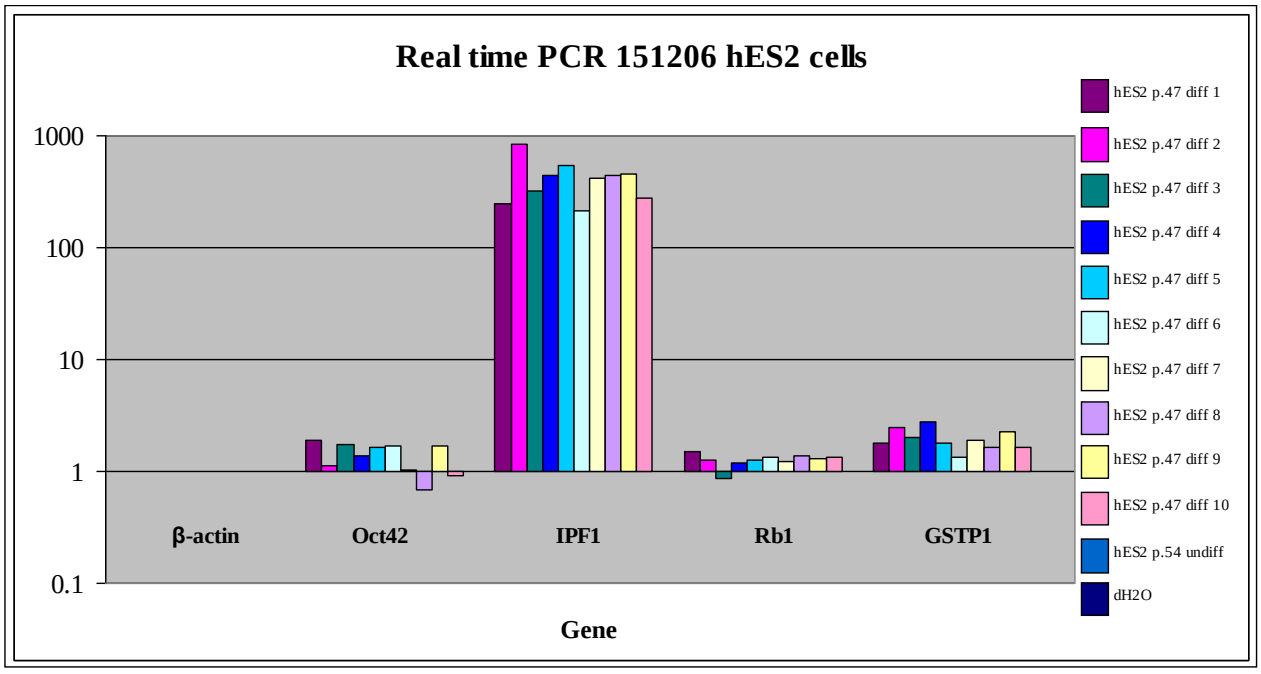

Figure 3.2 – **Analysis of up or down regulation of the Oct 42, IPF1, Rb1 and GSTpi genes in differentiated cells compared to in human embryonic stem cells**
Real time PCR studies were performed on cDNA converted from RNA purified from hES cell colonies and cells left to spontaneously differentiate for 4 weeks.
hES cells were used as calibrator and the genes Oct42, IPF1, Rb1 and GSTpi was checked for up or down regulation in differentiated cells. That IPF1 was up regulated in differentiated cells did not come as a surprise considering IPF1 is a gene only expressed in pancreatic cells, a highly differentiated cell type (Fig.1.2 f). The up regulation of Oct42 was unexpected. Oct42 is a stem cell marker known to down regulate as hES cells differentiate. Considering the differentiated cells are really differentiated, conclusions drawn from the strong up regulation of IPF1, we hypothesis that some cells had still stayed in an undifferentiated state even after 4weeks.
It appears as Rb1 and GSTpi was both up regulated in differentiated cells.

From the real time RT-PCR analysis, we observed that all the genes, including Rb1 and GSTpi were upregulated in differentiated cells compared to the undifferentiated. There was strong evidence of differentiation based on an up regulation of the IPF1 gene. However the up regulation of the Oct42 gene rose a bit of a question mark considering we would expect that gene to get down regulated as the cells differentiate. The reason for this result is probably that when purifying RNA from the different samples, the entire dish of cells was used from the differentiated cells and only small bits and pieces from the undifferentiated ones. This might have lead to that among the differentiated cells there were still a lot of undifferentiated ones, more than what was used in the undifferentiated samples. We therefore guess that this is the reason for the appearance of the up regulation.

Up regulation of both Rb1 and GSTpi meant that a high expression of the gene could be seen in differentiated cells. This was one requirement for a successful knock down.
A low expression of the gene in differentiated cells would not have been efficient to knockdown. That would have meant that the impact would not have been great and that the whole point of silencing the gene would have been unnecessary.

Rb1 is as mentioned a tumour suppressor gene. A low expression of the gene in differentiated cells would have raised questions about the normality of the cells. Since a high expression was achieved we can be certain that we are working with good quality cells. The same goes for GSTpi which is transcriptionally silenced in prostate cancer due to hypermethylation of the gene.

3.3 shRNA knockdown of retinoblastoma gene

Knockdown of the Retinoblastoma 1 gene was performed using intergration of shRNA into the genome via viral transfection. Transfection was performed in 6-well plates coated with MatriGel in the absence of mEFs. Transfected cells were selected using different concentrations of antibiotics for 3-7 days. The cells were transferred to T25 flasks with mEFs to stabilize. A good cell survival was achieved although some differentiation was seen. By the end of this project the cells had still not stabilized and grown confluent enough to do western blot analysis to see the percentage of knockdown of the retinoblastoma 1 gene.

Retinoblastoma 1, Rb1, a tumour suppressor gene known to be involved in prostate cancer as well as other cancers, was knocked down in human embryonic stem cells. What we wanted to know was if there need to be an initial hit in both the epithelial cells, the Rb cells, as well as in the mesenchymal cells for prostate cancer to form in vivo.

Due to time limitations, we were unable to use the cells that we ourselves generated from shRNA of RB1 as the characterization of the knockdown was ongoing after the end of this study. Previously, our collaborator at the Australian Stem cell centre Dr. Ernst Wolvetang (Australian Stem Cell Centre, Dept of Anatomy and Cell Biology, Monash University) had attempted to knock down this particular gene in hESCs. During that study, Rb1 was only reduced to 80% protein expression (personal communication, Dr. Ernst Wolvetang). Therefore, in order to complete the tissue recombination studies, we used the knockdown cells provided by Dr. Ernst Wolvetang.

Whether the low down regulation of the gene was due to a bad outcome of the knockdown or if that was the limit for what cells could handle before it was lethal is still unknown. Unfortunately the outcome of our own knockdown is still not known and therefore no conclusions can be drawn in comparison to the two knockdowns.

3.4 Differentiation of $Rb^{-/-}$ cells using tissue recombination

Initiation and progression of prostate cancer is not clearly understood. Using tissue recombination with initiated human embryonic stem cells recombined with either urogenital mesenchyme or carcinoma associated fibroblasts can be a way of getting a better understanding of the question.

3.4.1 Normal differentiation

In order to examine if a genetic defect in the epithelial cells were enough to form prostate cancer in vivo, urogenital mesencyme, UGM, was recombined with $Rb^{-/-}$ cells.
UGM purified from day E17.5 male rat pups was classified as normal mesencyme.
The different cell types were recombined in collagen and were grafted under the kidney capsule of Severe combined immunodeficient, SCID mice for 3.7 weeks before they were harvested and examined.

Harvesting tissue recombinants 3.7 weeks post-grafting revealed that all recombinants survived and grew in the host mouse. All teratomas formed were supplied with blood vessels from the mouse. The teratomas formed by $Rb^{-/-}$ cells alone were larger than the other ones and as could be seen by the eye contained small liquid filled cysts.
The UGM alone grew as well which might be an indication that there was contamination of UGE left among the mesencymal cells meaning that rat prostate was formed.
The recombinants between $Rb^{-/-}$ cells and UGM were more tissue like and not as fluid filled as the $Rb^{-/-}$ teratomas.

3.4.1.1 Histology

Analyses of all the grafts are necessary to see what kind of tissue they contain.

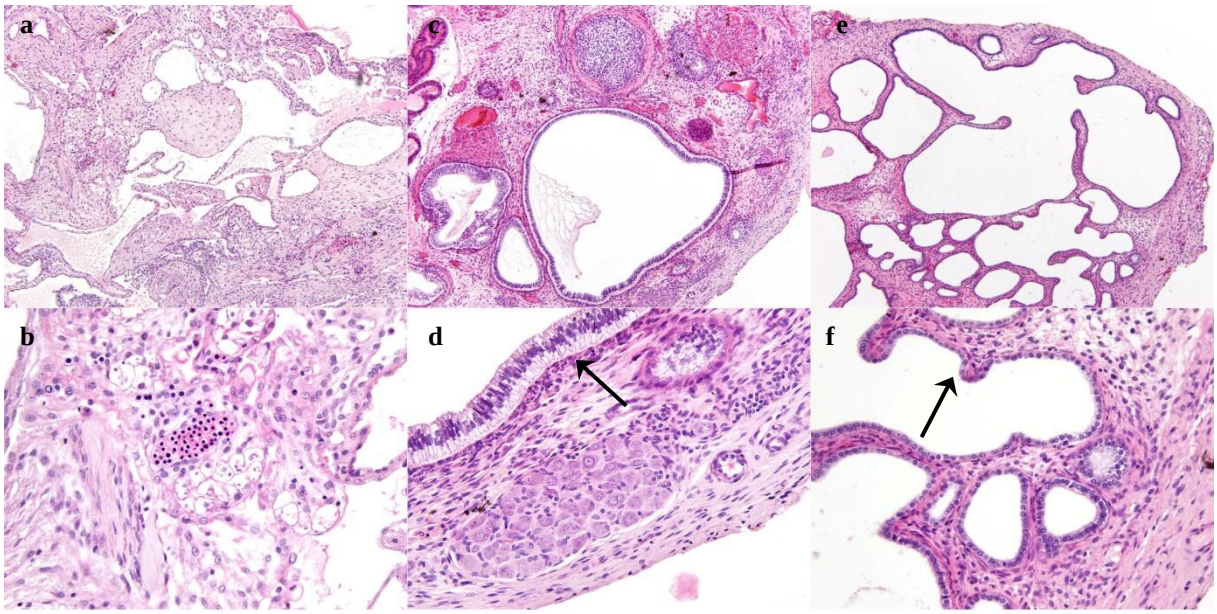

Figure 3.4.1.1 **– H&E staining of UGM recombined with $Rb^{-/-}$ cells and the controls used**
The recombinations were grafted for 3.7 weeks in SCID mice. To look at the morphology of the teratomas formed H&E staining was performed of sections from the tumours.
a) An overview of the morphology of the $Rb^{-/-}$ alone grafts. Lots of different cell types and structures could be seen within the tissue as expected. **b)** A close up of the $Rb^{-/-}$ tissue. **c)** An overview of the UGM/$Rb^{-/-}$ tissue. Ducts as well as other structures were seen. **d)** A close up look of the ducts formed in the UGM/$Rb^{-/-}$ tissue. The epithelial cells (indicated by the arrow) lining the ducts resemble cells found in normal human prostate ducts. However the placements of the nuclei in human prostate ducts are closer to the basal cells seen here lining the ducts in between the epithelial cells and the stroma. **e)** An overview of the morphology of the UGM alone grafts. A lot of duct structures could be seen with different morphology compared to the ducts found in the UGM/$Rb^{-/-}$ grafts. **f)** A close up of the ducts found within the UGM alone tissue. The cells found lining the ducts (indicate by the arrow) within this tissue looked very different compared to the ones found within the UGM/$Rb^{-/-}$ tissue, and is probably due to UGE contamination.
The overview pictures were taken with a 10x magnification whereas the close ups have a 40x magnification.

There was a clear difference in morphology between the $Rb^{-/-}$/UGM teratomas and the $Rb^{-/-}$ alone as well as UGM alone. As could be seen the teratomas developed by $Rb^{-/-}$ cells alone formed a bunch of different kinds of structures which was expected (Fig. 3.4.2 a-b). Since $Rb^{-/-}$ cells are still hES cells they are pluripotent and have the ability to form any type of cell found in the body. Within the tissues a few ductal structures could be found.
The teratomas formed by UGM alone mainly contain contamination of UGE (Fig. 3.4.2 e-f). The ducts and structures formed within the tissue were due to epithelial rat cells that easily took over and formed rat prostate. A better purification and separation of the UGM from the UGE could have avoided this. This was probably also why we could see such nice duct structures in the $Rb^{-/-}$/UGM grafts. However other structures could also be seen in the teratoma. There were several prostate like structures formed within the tissues (Fig. 3.4.2 c-d).

The morphology of those duct were not perfect though. As could be seen the nuclei in the epithelial cells lining the duct was situated somewhere in the middle of the cells. They were also quite disorganized with the different cells having their nuclei situated in different parts of the cells. In normal human prostate ducts those nuclei are found in the bottom of the cells as close to the basal cells as possible. This pattern was probably due to immaturities of the tissue when it probably takes longer for the organization of the duct structure to occur.
The prostate like ducts appeared to be lined by basal cells and surrounded by smooth muscle tissue just as expected.

To be able to see what had developed from hES cells and what was prostate like we performed immunohistochemistry. Immunohistochemistry is a technique that is used to identify different proteins, expressed by cells, under the microscope. When looking at the staining from the immunohistochemistry one get a good view of where the different cells expressing these proteins came from.
This was how we identified what type of tissue had developed when performing our recombinations. To see whether or not cancer had developed only a professional eye was used. Cancer is usually characterized by a disorganized tissue structure, lack of duct formation or an overgrowth of epithelial cells within the ducts formed. Nuclei enlargement, and sometimes also necrosis and invasion of the kidney is found.

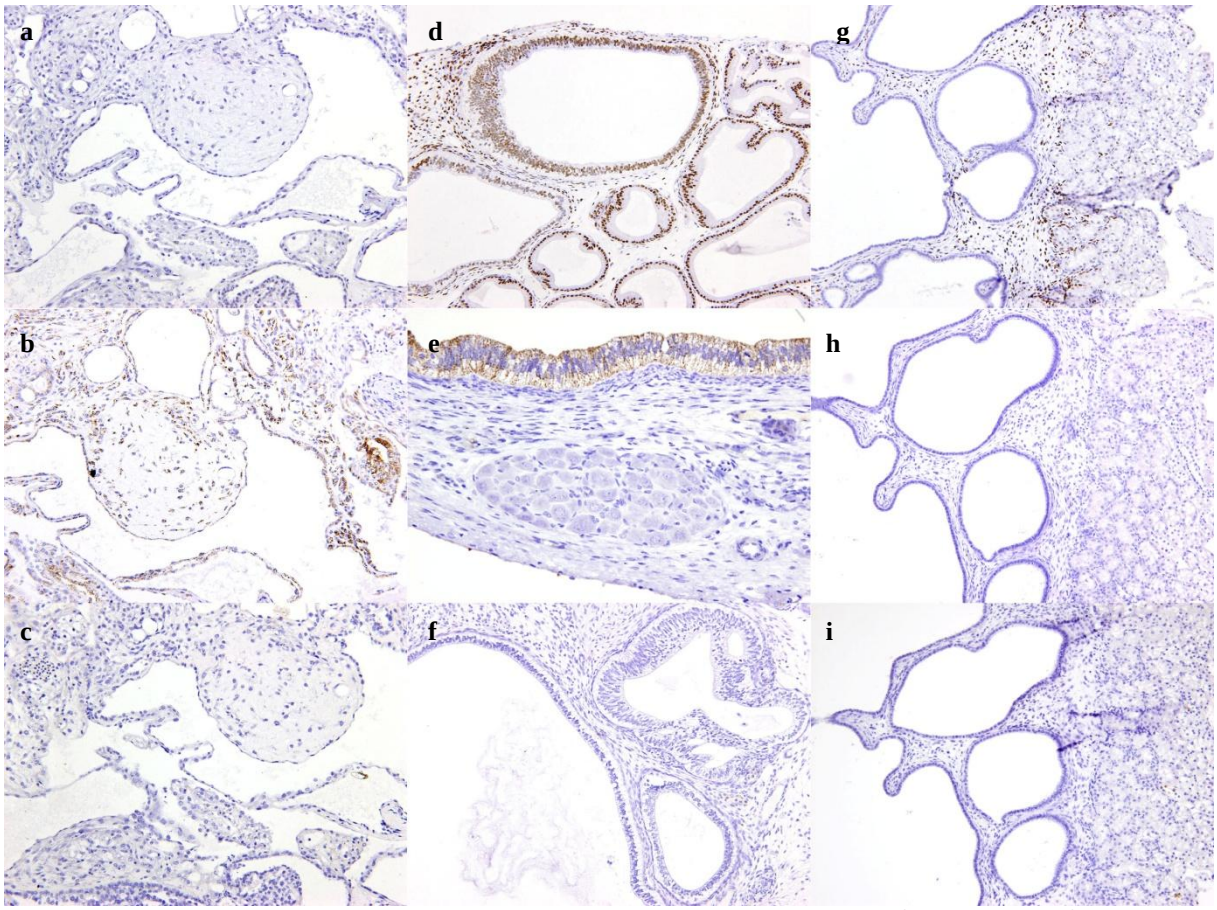

Figure 3.4.1.2 – **Immunostaining by androgen receptor, AR, mitochondria, mito and prostate-specific antigen, PSA, of UGM recombined with $Rb^{-/-}$ cells and the controls used**
Staining by mitochondria was done to show that the tissue structures developed was from human material, the hES cells. AR staining gave an indication that the cells positive for it are prostate like. If the cells also express PSA they were classified mature prostate cells.
The $Rb^{-/-}$ tissue stained positive negative for AR (**a**) and PSA (**c**) but positive for mito (**b**) as expected, meaning that spontaneous differentiation of the hES cells occurred.
The UGM/$Rb^{-/-}$ tissue stained positive for AR (**d**) and mito (**e**) but not for PSA (**f**) indicating that a immature prostate like tissue was formed by directed differentiation induced by the mesenchymal cells.

The UGM tissue stained positive for AR (**g**) but not for mito (**h**) or PSA (**i**) showing that the ducts formed are not of human origin although they are prostate like, indicating that our theory of UGE contamination is probably true.
All photos have a 20x magnification except **e** that has a 4ox due to lake of 20x.

The teratomas formed in the first recombination between UGM and $Rb^{-/-}$ was stained for mitochondria, prostate-specific antigen, PSA and androgen receptor, AR.
A positive staining by mitochondria showed that the tissue structure had developed from human material, the hES cells. AR staining indicate that the cells positive for it were prostate like cells and if the cells express PSA they were classified as mature prostate cells.
The ducts within the $Rb^{-/-}$ alone teratomas stained positive for mitochondria meaning that they had developed from hES cells as expected (Fig. 3.4.1.2 b).
As could also be seen the ducts shown within the $Rb^{-/-}$/UGM recombinations also stained positive for this antibody meaning that they as well had arose from the hES cells (Fig. 3.4.2.1 e).
The AR staining showed that the basal cells fond between the epithelial ducts and the surrounding stroma in the $Rb^{-/-}$/UGM recombinations expressed androgen receptor, which is a characteristic marker for prostatic stroma (Fig. 3.4.2.1 d).
None of the three different recombs, $Rb^{-/-}$/UGM, $Rb^{-/-}$ alone or UGM alone, were stained positive for PSA indicating that only a prostate like tissue had been formed, not perfect normal human prostate (Fig. 3.4.2.1 c,f & i).

3.4.2 Carcinogenesis

In order to examine if an initial hit was necessary in both the epithelial as well as the mesenchymal cells for prostate cancer to form in vivo, $Rb^{-/-}$ cells were recombined with carcinoma associated fibroblasts, CAFs.
CAFs are activated fibroblasts purified from human prostate cancer tumours. We classified them as initiated stroma.
In 1999, Olumni et al made recombinations between CAFs and BPH-1 cells [5]. The outcome was prostate cancer. The CAF cells used in that project was from the same cell line used by us.
BPH-1 cells are human cells purified from benign prostate hyperplasia, BPH-1. In order to culture these cells in the lab, genetic modification had to be made, leaving the BPH-1 cell line with for e.g. 76 chromosomes.
We hypothesised that recombining CAFs with $Rb^{-/-}$ cells instead, would make a better model system with features more equivalent to human prostate cancer.

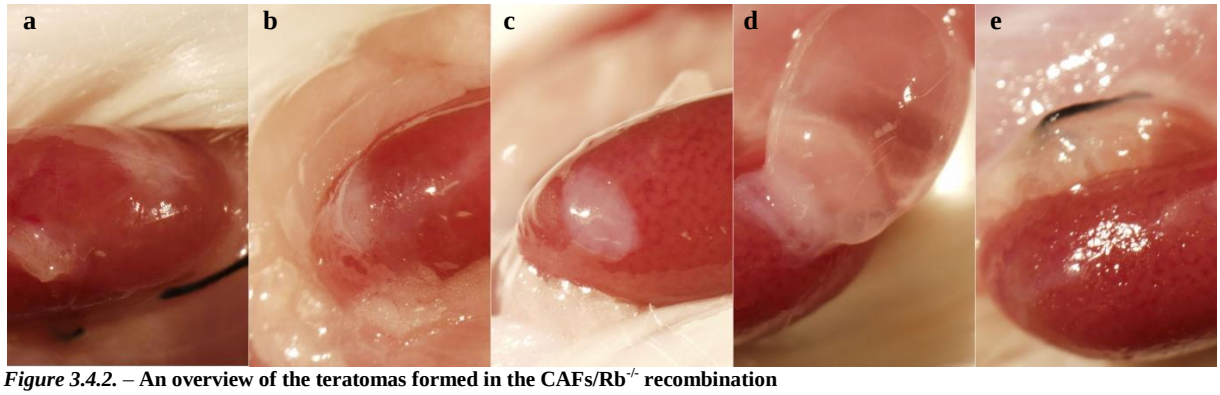

Figure 3.4.2. **– An overview of the teratomas formed in the CAFs/$Rb^{-/-}$ recombination**
The recombinations were grafted for 5 weeks in SCID mice before the teratomas were harvested.
a) The CAFs/BPH-1 teratoma. No particular growth could be seen. **b)** The BPH-1 alone teratoma. No particular growth could be seen. **c)** The CAFs/Rb-/- teratoma. No particular growth could be seen. **d)** The $Rb^{-/-}$ alone teratoma was large and fluid filled. **e)** The CAFs alone teratoma. No particular growth could be seen.
The pictures were taken with a 1.6x magnification.

The recombination between the CAFs and the $Rb^{-/-}$ cells was grafted for 5 weeks but was not successful. Teratomas was only formed for one out of four $Rb^{-/-}$ alone grafts the growth of all the other implants was minimal.

The teratomas formed in the recombination between CAFs and $Rb^{-/-}$ cells were very small (Fig. 3.4.2 c). Only one out of four $Rb^{-/-}$ alone controls grew (Fig. 3.4.2 d) and none of the CAFs and BPH-1 controls grew (Fig. 3.4.2 a) and.
This indicates bad quality and low survival rate of the cells used.
CAFs recombined with BPH-1 cells have previously been reported, by Olumni et al, to form prostate cancer [5]. The CAFs and BPH-1 cells used in the recombinations reported here were from the same cell lines used by Olumni et al in their experiment. Therefore this recombination was used as a control to show that the CAFs did work. Obviously this was not the case.

3.4.3 Carcinogenic histology

As can be seen in the pictures from the sectioned tissues of the different recombinations no epithelial structures were formed in the CAFs/BPH-1 or CAFs/ $Rb^{-/-}$ recombinations (Fig. 3.4.3.1 c-d). Nor were there any difference in morphology between the BPH-1 cells grafted alone (Fig. 3.4.2 a-b) compared to the BPH-1 recombined with CAFs indicating that the CAFs had no action.
The only graft of $Rb^{-/-}$ cells alone that grew behaved like hES cells as expected and went through spontaneous differentiation into different cell types (Fig. 3.4.2 g-h).
No growth or prostate tissue structures could be seen in any of the other recombinations performed.

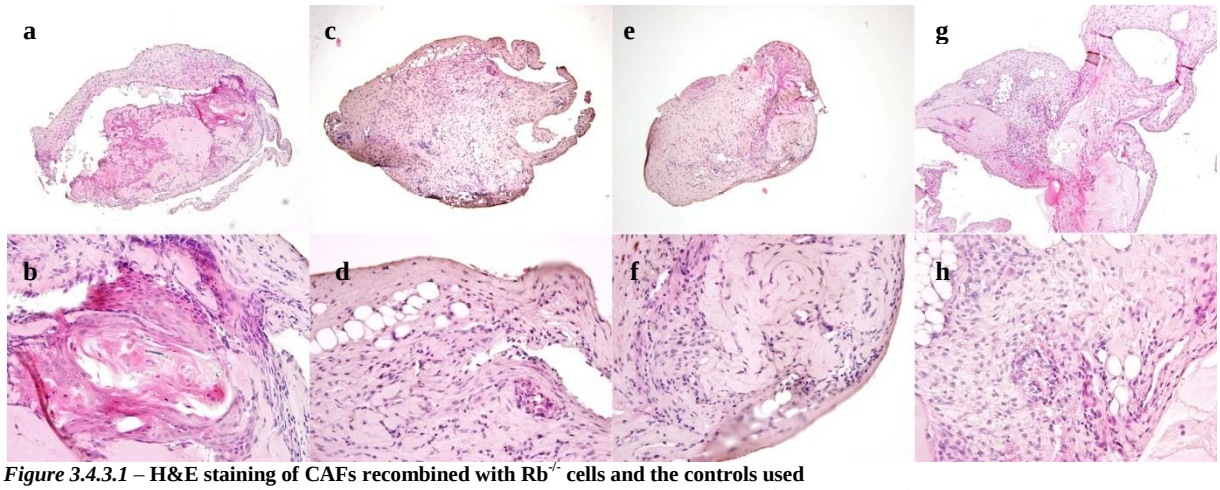

Figure 3.4.3.1 – **H&E staining of CAFs recombined with $Rb^{-/-}$ cells and the controls used**
The recombinations were grafted for 5 weeks in SCID mice. To look at the morphology of the teratomas formed H&E staining was performed of sections from the tumours.
a) An overview of the morphology of the BPH-1 alone grafts. **b)** A close up of the BPH-1 tissue. A lot of squamous tissue could be seen. **c)** An overview of the CAFs/$Rb^{-/-}$ tissue. No duct formations could be seen. However some fat structures was observed. **d)** A close up look of the tissue formed in the CAFs/Rb-/- tissue. The tissue was very uniform with unorganized stroma. It appeared as if only the CAFs survived. **e)** An overview of the morphology of the CAFs alone grafts. **f)** A close up of the CAFs alone tissue. The morphology of this tissue compared to the CAFs/Rb-/- tissue was very similar, further indicating that the Rb-/- cells did not survive when grafting them. **g)** An overview of the morphology of the $Rb^{-/-}$ alone grafts. A lot of different structures were seen **h)** A close up of the $Rb^{-/-}$ alone tissue. Spontaneous differentiation of the hES cells had occurred.
The CAFs/BPH-1 tissue showed similar structures as the BPH-1 tissue (data not shown here) indicating that the CAFs had no action as differentiation inducers. Similar patterns could also be seen with the NPF when recombining them with either BPH-1 or $Rb^{-/-}$ cells (data not shown here).
The overview pictures were taken with a 10x magnification whereas the close ups have a 40x magnification.

Immunostaining of the tissues were anyhow performed in order to see if differentiation, although not expansion, had occurred within the teratomas.
The tissues were stained for cytokeratines, vimentin, α-actin and GFP.
Cytokeratines are intermediate filament keratins. They are expressed in epithelial tissue and antibodies against them will therefore stain positive in cells that arouse from the $Rb^{-/-}$ or the BPH-1 cells.
Vimentin and Actin are both different kinds of filaments. They are expressed in stroma cells and will therefore stain positive in cells that aroused from the CAFs and NPFs.
Since the ENVY cells are a fluorescent cell line, they will stain positive for the GFP antibody.

The CAFs/$Rb^{-/-}$, CAFs/BPH-1, CAFs/envy, NPF/ $Rb^{-/-}$, NPF/BPH-1, and $Rb^{-/-}$ alone were all stained for Cytokeratin (8/18), Cytokeratin High Molecular Weight, α Smooth Muscle Actin and Vimentin.
BPH-1 alone was only stained for Cytokeratin (8/18) and Cytokeratin High Molecular Weight whereas CAFs alone was only stained for α Smooth Muscle Actin Clone 1A4 and Vimentin.
The CAFs/envy was also stained for GFP

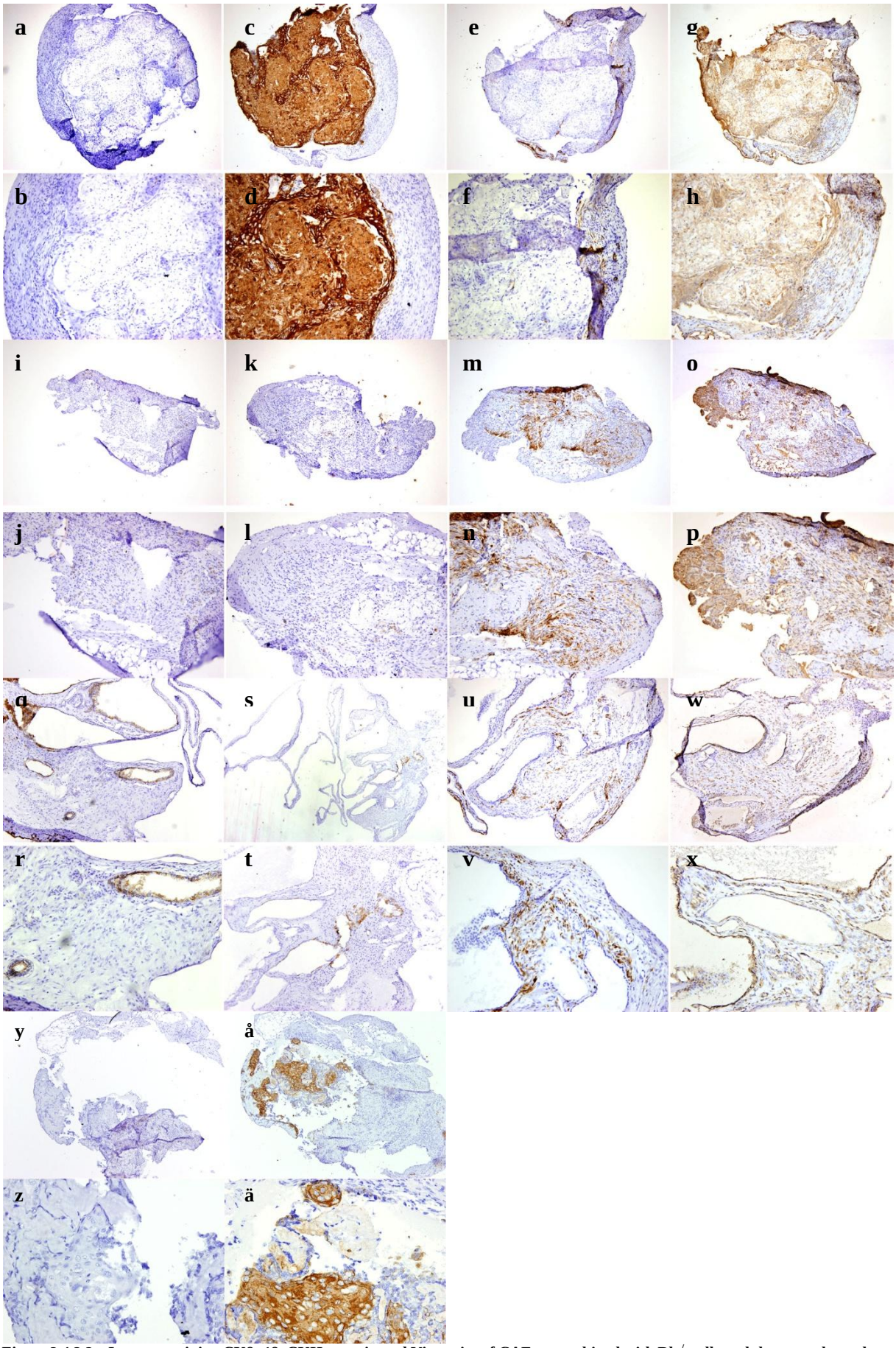

Figure 3.4.3.2 **– Immunostaining CK8+18, CKH, α-actin and Vimentin, of CAFs recombined with $Rb^{-/-}$ cells and the controls used**

Staining by cytokeratines both 8+18 and the heavy ones was done to show that the tissue had an epithelial origin, so to say from the $Rb^{-/-}$ or the BPH-1 cells. Staining by α-actin and vimentin was done to show that the tissue formed had a stromal origin, so to say from the CAFs or the NPFs.
The squamous found in the CAFs/BPH-1 tissue stained positive for CKH (**c** and **d**) and vimentin (**g** and **h**). The tissue was stained negative for the other markers (**a**,**b**,**e** and **f**). This indicates that both cell types survived although the morphology of the tissue was not what we expected.
The CAFs/$Rb^{-/-}$ tissue stained positive for α-actin (**m** and **n**) and vimentin (**o** and **p**) and negative for the other markers (**i**, **j**, **k** and **l**). This confirmed what we suspected that the $Rb^{-/-}$ cells did not survive the grafting.
A lot of different structures were seen within the Rb-/- alone tissue. The sections stained positive for all the markers looked at (**q**-**x**) as expected meaning that the teratomas contained both epithelial cells as well as stromal ones.
The BPH-1 tissues alone was only stained for CK8+18 and CKH. The tissue only expressed CKH (**å** and **ä**) and not within the whole teratoma. No staining of the CK8+18 could be seen (**y** and **z**). A lot of fat tissue could be seen around the graft and probably has some of that grown together with the implant as well.
The CAFs alone stained positive for vimentin and α-actin and negative for the cytokeratines as expected (data not shown).
Similar patterns could also be seen with the NPF when recombining them with either BPH-1 or $Rb^{-/-}$ cells (data not shown here).
All photos have a 20x magnification except **e** that has a 4ox due to lake of 20x.

The CAFs/BPH-1 teratoma had a large area of squamous tissue in the middle. This area showed a positive staining for CKH (Fig. 3.4.1.2 c-d) and vimentin (Fig. 3.4.1.2 g-h) but negative for the other markers (Fig. 3.4.1.2 a,b,e and f) indicating that both cell types survived. However the morphology of the tissue did not resemble the tissue formed by Olumni et al [5].
The sections from the CAFs/$Rb^{-/-}$ teratoma stained positive for α-actin (Fig. 3.4.1.2 m-n) and vimentin (Fig. 3.4.1.2 o-p) and negative for the other markers (Fig. 3.4.1.2 i-l). This confirmed what we suspected that the $Rb^{-/-}$ cells did not survive the grafting.
Within the $Rb^{-/-}$ alone teratomas a lot of different structures could be seen as expected. The tissue stained positive for all the markers looked at (Fig. 3.4.1.2 q-x), meaning that the teratomas contained both epithelial cells as well as stromal ones.
The BPH-1 alone tissue only expressed CKH (Fig. 3.4.1.2 å-ä). No staining of the CK8+18 could be seen (Fig. 3.4.1.2 y-z).
The CAFs alone stained positive for vimentin and α-actin as expected (data not shown).
Similar patterns could also be seen with the NPF when recombining them with BPH-1 or $Rb^{-/-}$ cells (data not shown here).

4.0 Discussion

Prostate cancer is a disease that approximately 395 000 men world wide are diagnosed with every year. Drugs slowing down the progression of the cancer are available on the market and treatments such as surgery can be performed. Usually both these treatments cause severe side effects with for e.g. castration, impotence, and urination problems as a result.
Unravel the biological events that lead to prostate cancer could eventually lead to better treatments of the disease.
We hypothesis that recombining initiated human embryonic stem cells, that has lost key prostate cancer genes, with carcinoma associated fibroblasts, purified from human prostate cancer tumours, will form human prostate cancer in vivo. We also hope to get a better understanding of the role of the stroma as well as how the cancer initiates and progresses.

hES2 cell colonies were established on mouse embryonic fibroblasts, mEFs, from the MTKneo2 strain. The cells were initiated from the original hES2 line (NIH code: ES-02) available at Monash Immunology and Stem Cell Laboratory. The cells were checked for Oct ¾ and CD30 proteins in order to confirm that our hESC colonies had an undifferentiated state, and that good quality cells were used.
Successful expansion of the cells in bulk cultures was achieved.

Retinoblastoma 1, Rb1, is a tumor suppressor gene known to be involved in maintainance of chromosomal integrity, cell cycle control, cellular differentiation and survival of epithelial cells. Knockout of the gene leads to enhanced susceptibility of the tissue to undergo further genetic change and has been shown to be involved in all sorts of cancers including prostate [21].
We hypothesis that silencing this gene using shRNA targeting will form human prostate cancer when recombining $Rb^{-/-}$ cells with either urogenital mesencyme or carcinoma associated fibroblasts in vivo.
In order to silence the Retinoblastoma 1 gene, the cells from the bulk cultures were transfected with shRNA vectors delivered by lentiviral particles. The cells were resuspended in 50% hES media and 50% condition MEF media and were moved to 6-well plates coated with MatriGel. The transfection went on for 24h. After two day of stabilization selection by antibiotics was started and went on for 3-7 days.
A high survival rate of the cells was achieved although some differentiation could be seen. After finalized selection, the cells were moved back to T25 flasks with 1/3 mEFs. To eliminate transfer of the differentiated cells over to the bulks, cell dissociation solution was used when splitting. This solution was chooses since it did only bring up undifferentiated cells and did not split the colonies into too small lumps. At the end of this project the cells looked really good. Expansion of the cells could not be done though due to time limitations and therefore western blotting to look at protein downregulation is still to be determined.

Due to time limitations we were also unable to use our own $Rb^{-/-}$ cells in this study. Our collaborator at the Australian Stem cell centre, Dr. Ernst Wolvetang (Australian Stem Cell Centre, Dept of Anatomy and Cell Biology, Monash University) has previously attempted to knock down the Rb1 gene in hESCs. During that study, the protein expression was down regulated 20% (personal communication, Dr. Ernst Wolvetang). Therefore, in order to complete the tissue recombination studies, we used the knockdown cells provided by Dr. Ernst Wolvetang.

By recombining two different cell types the signalling between them can be studied. One can also study the impact and guidance one cell type has on the other, not only in differentiation but also in conversion of a normal cell state to a carcinogenic.
We set out to recombine normal stromal cells as well as carcinogenic stroma with initiated human embryonic stem cells to see whether or not an initiation in the epithelial cells is enough to form adenocarcinoma or if genetic changes need to be present in both cell types. Human embryonic stem cells with the retinoblastoma 1 gene knocked down was used for the recombination. When recombining the $Rb^{-/-}$ cells with stromal cells they will differentiate into epithelial cells.
As normal stroma, urogenital mesencyme, purified from day E13.5 rat pups. Carcinoma associated fibroblasts purified from human prostate cancer tumours were used as initiated stroma.
Recombination between $Rb^{-/-}$ cells and UGM formed nice big teratomas when grafting them in vivo for 3.7 weeks. When staining the tissues with Haematoxylin and Eosin, to looking at the morphology of them, a prostate like tissue with duct structures, very much resembling the ducts found in the human prostate was formed. However, the epithelial cells lining the ducts did not look like they do in a normal human prostate. The nuclei of the cells are in normal human prostate tissue situated at the inner part of the cells, very close to the basal cell layer. The nuclei of the epithelial cells in the tissues formed during the UGM/$Rb^{-/-}$ recombinations were situated much closer to the middle of the cells and were quite disorganized. Immunohistochemistry showed that they didn't express PSA which is probably a consequence of immaturation of the tissue. It takes 15 years for a human man to start develop PSA. Taylor et al showed in 2006 that in human prostate tissue formed via recombination of hES3 cells and UGM produced PSA after grafting in vivo for 12 weeks [9]. Probably 3.7 weeks of grafting, which was the case for the $Rb^{-/-}$/UGM recombinations, was to short for the PSA expression to start.
In the $Rb^{-/-}$ teratomas, many different structures and duct formations could be seen. This was expected from the human embryonic stem cells since they were left to spontaneously differentiate.
The UGM/$Rb^{-/-}$ teratomas formed a more uniform tissue compared to the morphology seen in the $Rb^{-/-}$ alone teratomas. A few duct formation that strongly resembled the ducts fond in a human prostate was seen, all surrounded by basal cells, stroma and muscle tissue. This suggests a directed differentiation of the $Rb^{-/-}$ cells by the UGM. Why a perfect prostate tissue was not formed is not fully understood. Perhaps genetically modified hESCs do not behave like parental cells do.
The kidney ducts found contaminating the tissue is probably a result of the inductive force that the UGM has on other cells and tissues. UGM gives a very strong guidance to the hES cells leading to a directed differentiation. A hypothesis is that the UGM can also act on the kidney if there is not enough surviving hES cells to act on, leading to duct formation. It is easy though to exclude these glands since they are negative for mitocondria. Therfore we could without a problem only focus on the human derived glands.There is also UGE contamination found within the teratomas, both in the UGM/$Rb^{-/-}$ recombinations but also in the UGM alone recombinations. Those ducts stained positive for AR but negative for mitochondria confirming that they had not arouse from the $Rb^{-/-}$ but indeed came from UGE. Since UGE is very easily guided by the UGM and only a few cells are needed to form nice rat prostate it easily takes over and form rat prostate ducts when guided by the UGM. This could have been avoided by more careful disections. However, rat prostate can easily be excluded as being human prostate because they stain negatively for the mitochondrial marker.

The BPH-1 cells as well as the CAFs were the same cells used in the recombinations made by Olumni et. al in 1999 [5]. They were sent to us from Simon Hayward's lab in USA (Vanderbilt University).
If the transportation of the cells from USA to Australia had an effect on the cells is not clear. It might have lead to a decrease in cell potency, but it can also be that it didn't affect them at all. Experiments to test their functionslity are ongoing in the lab.
The CAF/BPH-1 recombinations that were set up were used as a control to see that the CAFs still worked. We knew what we expected as an outcome of that recombination since those data already had been published [6]. However, as it seems, the tissue formed was not even near what Olumni et. al saw in 1999.
We can only speculate in the matter of this being due to bad CAF cells or a low viability of the cells used. Due to a short time frame and in the end time limitations repeating experiments could not be performed.
Immunohistochemistry was performed to look at what cell types were present within the tissue.
The CAFs/BPH-1 teratomas grew and stained positive for both CKH and vimentin. Since one marker is for epithelial cells and the other is for stromal we can conclude that both cell types did indead survive. However, the tissue formed did not resemble the tissue formed by Olumni et al [6]. Their tissue was more unorganized, contained enlarged nuclei and had invasive migration into the kidney. The tissue formed in or recomibnations was more organized and contained a large area with squamous tissue.
The CAFs/$Rb^{-/-}$ teratoma only stained positive for α-actin and vimentin. This indicate what we suspected, that only the CAFs survived and that the Rb-/- cells did die during grafting. An outcome in the study of wheteher or not an initiation in both the epitelial cells as well as in their surrounding stroma can therefore not be drawn.
The $Rb^{-/-}$ alone teratomas went through spontaneous differentiation and stained positive for all the markers tested. A lot of different structures containing bothe epithelial as well as mesenchymal cells was formed as expected.
Why the $Rb^{-/-}$ cells survived when grafted alone but not recombined with CAFs or NPFs is probably due to the difference in cell number used. For the $Rb^{-/-}$ alone grafts 120,000 cells were used whereas for the recombinations only 40,000 cells were used.
We stained the the BPH-1 cells for epithelial markers and the NPFs and the CAFs for stromal markers, with succesful results, to confirm their cell state.

Considering the CAF/BPH-1 recombinations had quite unreliable results, not much conclusions could be drawn from the CAF/$Rb^{-/-}$ recombinations.
The $Rb^{-/-}$ cells used in the second experiment were from the same cell line used in the first recombination. They were passaged four more times in between the two recombinations that could have lead to differences within them. Stem cells have the ability to easily go through genetic modifications or differentiation if they are not treated properly and perhaps some sort of changes had started to occur here. However, teratomas formation with different cell types formed is a result of in vivo grafting of human embryonic stem cells and therefore one can imply that good quality cells were used.

4.1 Future aspects

A new knockdown of the Rb1 gene has already been made. Due to time limitations in this project, confirmation of gene silencing by performing western blotting had to be conducted after the end of this project. However if the percentage of silencing appears to be higher then within the $Rb^{-/-}$ cell line used here, with the Rb1 gene knocked down by Dr. Ernst Wolvetang at ASCC, it would be interesting to see if the outcome of the recombinations performed

within this project, differ with the new cells. One can also conclude whether or not the 20% knock down, of the Rb1 gene in the cell line used here, is due to lethalness or bad outcome of silencing of the gene.
Other genes that might be interested to silence to see whether or not that will give a different outcome of the recombinations might be Glutatione-S-transferase pi and p10.

Glutathione S-transferase pi, GSTpi, is CpG island hypermethylated in nearly all prostate carcinomas and its promoter is deoxycytidine methylated in more than 90% of all prostate cancers. This leads to transcriptional inactivation of the gene.
The primary function of GSTpi is to conjugate toxins with glutathione. When the gene is silenced it is likely that the individual renders an increased susceptibility to cancer causing xenobiotics.

p10 is a member of family of tumor suppressor genes. It is a cyclin-dependent kinase inhibitor that constitutes the active part of the caspaces. The caspaces are natural proteases that mediate apoptosis.
p10 is mutated in many types of cancers such as melanoma, colon cancer, lymphoma, kidney cancer, leukemia, breast cancer, pancreatic cancer, prostate cancer, bladder cancer etc.
A knockdown of this gene inhibits the cells ability to go through natural cell death and will therefore enable an uncontrolled growth.

Perhaps a combination of gene knockdowns is necessary in the same cell lines to be able to form human prostate cancer.

When the model system of human prostate cancer is successful different treatment can be tested on theses animals. Since the teratomas formed in these recombinations were provided with blood vessels from the kidney, drugs taken up by the blood could be tested to see how the cancer reacts.
We also hope to gain a better understanding of the signaling in the development of the cancer. We hope to find answers to questions like; is this due to a basal stem cell turning carcinogenic? Or is this due to an initial hit in the stroma that acts on the epithelial cells and converts them into a malignant phenotype? Or even; is an initial hit in both stromal cells as well as in the epithelial cells necessary for the cancer to initiate and progress?
One can also speculate about the cancer having started out as an inflammation of some kind, like in prostatitis, which leads to tissue neoplasia, as in PIN, eventually leading to prostate cancer.
Many questions are still to be answered and hopefully this model system, when it is fully developed, can help to eliminate some of them.
This could lead to identification of a more suitable antigen or protein to target when developing new drugs.

5.0 Conclusions

In this project we managed to establish good quality hESC colonies from the original hES2 line (ES-02).
We succesfully performed shRNA transfection to knock down the Retinoblastoma 1, Rb 1, in those cells, although the outcome of the down regulation of the Rb protein could not be determined due to time limitations.
Therefore a cell line with the Rb 1 gene knocked down to only 20 % by our collaborator Dr. Ernst Wolvetang at the Australian Stem Cell Center, ASCC, was used for all the recombinations performed during this project.
After performing tissue recombinations between $Rb^{-/-}$ cells with urogenital mesencyme, UGM, we could conclude that a 20% knockdown of the Rb1, gene was not sufficient enough to form cancer in vivo. However, a prostate like tissue could be seen.
No conclusions could be drawn from the recombination between $Rb^{-/-}$ cells and CAFs due to low cell viability.
Repeated recombinations as well as recombinations using other stem cells with other genes knocked down or with a better silencing of the Rb1 gene are therefore suggested.

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8.0 Appendix

Medias used throughout the project

mEF media – F-MEM

MEM-α
10% FCS
0.5% Penicillin/Streptomycin

hES media

KnockOut DMEM
20% KnockOut Serum
1% Non-Essential Amino Acids
1% GlutaMax
1% Penicillin/Streptomycin
1% ITS
55 mM β-Mercaptoethanol
4 ng/ml bFGF
.

ES-HEPES

D-MEM
FBS
1 M HEPES

10% Vitrification Solution

ES-HEPES
Ethylene Glycol
DMSO

20% Vitrification Solution

ES-HEPES
1 M Sucrose Solution
Ethylene Glycol
DMSO

Condition media

Condition media is hES media added to T75 flasks of full mEFs for 24h before it was used. All condition media was filtered through a 0.20µm filter before use.
Antibiotics, Puromycin and Blastocydin was also added when selection of transduced cells were performed

MatriGel coating of 6-well plates

The MatriGel was thawed on ice over night at a shaker. Six 6-well plates were coated at a concentration of 0.0347mg/cm^2. Since each well had a surface area of 9.6cm^2 and each plate therefore had a total area of 57.6cm^2, six 6-well plates end up having 345.6cm^2 to coat. Therefore (0.0347mg/cm^2)*(345.6cm^2) = 12mg MatriGel was used. Since the stock concentration was 10mg/ml a total volume of 1200µl was used. This was diluted in 72ml DMEM-F12 (Invitrogen, Australia). 2ml was then added to each well. All the plates were incubated at 37ºC over night.

Virus transfection

When working with the virus it is very important to be careful and work sterile. Extra cautions were made and gloves, coat and mouth protection was worn at all occasions. Everything that came in contact with the virus was put in bleach. A special incubator had to be used when the transfection was made to make sure that no contamination to other cell lines could occur.