Induced Pluripotent Stem-cell Re-programming in the elderly Prostate

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“Do not judge me by success, judge me by how many times I fell down and got back up again.” These famous words spoken by Nelson Mandela summarise my journey through my PhD study. This was indeed an arduous journey where I felt challenged, made big mistakes and fell hard on my face. At the same time I reflected on my shortcomings, improved as a scientific scholar and evolved to become a stronger person. This was a journey of blood, sweat and tears but above all it was a journey of love.

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Abstract

Prostatic differentiation is modelled through enrichment for stem-like populations through a combination of putative stem-cell markers. However, *in vitro* cultures demonstrate a phenotypic drift that abrogates normal physiology. Induced Pluripotent Stem Cell (iPSC)-reprogramming allows for any somatic cell to be transformed into an embryonic-stem-cell-like state although molecular properties as well as differentiation abilities are limited by the primary tissue type of origin. This project describes the derivation of Prostate-iPSC (Pro-iPSC) from the prostate of an individual in his sixth decade. Prostate cells were re-programmed through use of a specific Cre-Recombinase/LoxP polycistronic transduction protocol. Resultant iPS clones (14 cell lines) were checked for identical DNA fingerprinting with the parent fibroblasts and then tested for pluripotency and exogene silencing. Morphologically the Pro-iPSC are identical to human embryonic stem cells. Normal karyotyping was confirmed following which Pro-iPSC were immunostained for a panel of 6 pluripotent markers including nuclear-transcription factors Oct4 and NANOG. Messenger RNA studies confirmed a gene-expression profile that was similar to embryonic-stem cells. These Pro-iPSC are able to differentiate into all the three germ layers (embryoid body and teratoma formation) and demonstrate *in vitro* differentiation along a prostate-specific lineage when treated with specific differentiation media. Preliminary tissue recombination grafts of Pro-iPSC with the urogenital messenchyme have further demonstrated *in vivo* differentiation of these cells along a specific urological route. In conclusion a novel iPSC model has been established whereby aged prostatic fibroblasts have been progressively de-differentiated into a primitive embryonic state - this model demonstrates crucial events in prostate embryology which in turn allows the scrutiny of some complex signalling pathways as well as molecular mechanisms behind prostate carcinogenesis.

List of abbreviations

3D: three dimensional

AR: Androgen Receptor

ARE: Androgen Response Element

bFGF: basic Fibroblast growth factor

BPH: Benign prostatic hyperplasia

CAP: Comparison arm for ProtecT

DALYs: Disability-adjusted life-years

DMEM: Dulbecco's Modified Eagle's Medium

DNA: de-ocyribonucleic acid

DPX: Distrene, Plasticiser, Xylene

EB: embryoid body

EMT: Epithelial to messenchymal transition

EpCAM: Epithelial cell adhesion molecule

FACS: Fluorescence based flow cytometry

FBS: Foetal Bovine Serum

G-banding: Giemsa banding

GWAS: Genome Wide association studies

H&E: Haematoxylin and Eosin

HEA: Human Epithelial Antigen

hESC: human Embryonic Stem Cell

hTERT: human Telomerase Reverse Transcriptase

IF: Immunofluorescence

iPSC: induced Pluripotent Stem Cell

IVF: In vitro fertilisation

LHRHa: Luteinising Hormone Releasing Hormone receptor agonist

MACS: Magnetic Associated Cell Sorting

ME: mercaptoethanol

MEF: mouse embryonic fibroblast

MET: Messenchymal to epithelial transition

MOI: multiplicity of infection

mRNA: messenger RNA

NOD/SCID mouse: Non-obese diabetic/severe combined immunodeficiency mouse

NSG mouse: NOD/SCID gamma mouse

OSKM: Oct4, SOX2, Klf4, c-Myc

OSLN: Oct4, SOX2, Lin28, NANOG

p1: Passage 1

PBS: Phosphate Buffered Saline

PCAT: prostate cancer associated non-coding RNA transcripts

PCR: Polymerase Chain Reaction

PEPSC: prostatatic epithelial pluripotent-like stem cells

Pro-iPSC: Prostate Induced Pluripotent Stem cell

ProtecT: Prostate testing for cancer and treatment

PSA: Prostate specific Antigen

RNA: Ribonucleic acid

RPMI: Roswell Park Memorial Institute Medium

s/c injection: sub-cutaneous injection

shRNA: short hairpin RNA

siRNA: silencer RNA

SOX2: SRY(Sex Determining Region)-Related HMG-Box Gene 2

SSC: Spermatogonial Stem Cells

SSEA: Stage Specific Embryonic Antigen

STR: Short Tandem Repeat

SVM: Seminovesical mesenchyme

TE: Trypsin-EDTA

TGF: transforming growth factor

TGFR: transforming growth factor receptor

TU: Transduction units

TURP: transurethral resection of the prostate

UGM: Urogenital Messenchyme

UT-iPS: Urinary Tract iPS

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Chapter 1.

Introduction

1. An introduction to prostate cancer

1.1. Epidemiology of prostate cancer

Prostate cancer is a disease of increasing concern amongst developed nations and is an emerging malignancy in developing countries, it is a leading cause of death in men worldwide (Haas, Delongchamps et al. 2008) . Prostate cancer is the most frequent cancer in the USA after skin cancer (Haas, Delongchamps et al. 2008). Analyses of autopsy specimens suggest that more than 60% men will be histologically positive for prostate cancer by the age of 85 years (Haas, Delongchamps et al. 2008, Burton, Martin et al. 2013). Prostate cancer is also the commonest form of cancer in men in Europe as well as the European Union accounting for 20.3% and 24.1% of total incident cases, respectively (Ferlay, Autier et al. 2007). Along with colorectal, lung and breast, prostate cancer accounts for 10-50% of the total cancer burden in the world and is documented to be a major contributor to the total DALYs (Disability-adjusted life-years) amounting to a loss of 169.3 million years of healthy lives across the globe(Soerjomataram, Lortet-Tieulent et al. 2012). In Europe, 2.6 million new cases of prostate cancer are detected each year (Heidenreich, Aus et al. 2008) – this cancer is responsible for 9% of all cancer deaths in the EU (Black, Bray et al. 1997). In the UK, prostate cancer accounts for 24% of all new male cancers detected and 1 in every 10 men face the risk of being diagnosed with this condition in their lifetime (July, 2010). The apparent increase in the number of diagnoses has been attributed to the incidental detection of this disease following transurethral resection of the prostate (TURP) and also due to the use of prostate specific antigen (PSA) testing (NICE 2008).

The GLOBOCAN project evaluated this disease to be the second most frequently diagnosed cancer responsible for 14% of total new cancer cases and the sixth leading cause of cancer deaths in males (6% of total cancer deaths in males) (Jemal, Bray et al. 2011). In year 2010, there were more than 40,000 new cases of prostate cancer reported in the UK (Figure 1.1) (CRUK 2010). The crude rate for prostate cancer incidence in the UK was 134, meaning that for every 100,000 males there were 134 new prostate cancer cases being reported (CRUK 2010). Projections of cancer incidence are therefore important in assessing the effectiveness of current healthcare interventions and in overhauling current therapeutic regimens to improved standards (Mistry, Parkin et al. 2011). In the year 2010, the life-time risk or the risk of a new-born developing prostate cancer at some point of life was 1 in 8 (CRUK 2010). It has been documented that the increased diagnoses of new prostate cancer cases will continue over the next few decades unless appropriate lifestyle-interventions are in place (Mistry, Parkin et al. 2011, Greenberg, Wright et al. 2013). These figures emphasise on the need for management strategies that will address quality of life following disease diagnosis.

The aetiology of prostate cancer has been attributed to several factors, with perhaps the most important one being increasing age. Prostate cancer diagnosis is less than 1% in those below the age of 50 years and the incidence peaks between the ages 75-79 (CRUK 2010). This underscores the need for preclinical models in this field to be more accurate in representing this condition as a disease of ageing. The prevalence of prostate cancer refers to the number of cases of patients who have been diagnosed with prostate cancer and are still alive; although deaths due to prostate cancer has decreased by a fifth in the last 20 years prostate cancer associated deaths are on the rise due to better detection rates and increased life expectancy (CRUK 2010). The worldwide prevalence of prostate cancer in year 2008 was around 3 million (Figure 1.2) (UK 2013).

	England	Wales	Scotland	Northern Ireland	United Kingdom
Cases (thousands)	**35**	**2.5**	**2.7**	**0.9**	**41**
Crude Rate	135.5	167.4	105.9	106.5	133.7

Figure 1.1. Prostate cancer incidence statistics across the United Kingdom. The statistical data represent the number of new cases, crude and European age-standardised (AS) Incidence rates per 100,000 population age around 85 years. Table adapted from(CRUK 2010). http://www.cancerresearchuk.org/cancer-info/cancerstats/types/prostate/incidence/#source34

	1 Year Prevalence	5 Year Prevalence	10 Year Prevalence
Cases (thousands)	31	128	181

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1.4. Management of prostate cancer

As per EAU guidelines on the management of prostate cancer (NICE 2008) diagnosis of prostate cancer is based on histopathological confirmation following which the mainstays of therapy include “active surveillance” for low risk cancers (Gleason histological grade not more than 7), radical prostatectomy and radiotherapy in younger patients; whilst hormonal as well as cytotoxic therapy is reserved for late stage cancers with metastasis (Heidenreich, Aus et al. 2008). Hormonal therapy in the form of androgen deprivation offers short-term symptomatic relief in around 70% cases until the tumour progresses to a hormone insensitive phenotype (Dorkin, Robson et al. 1997).

In the UK patients with prostate cancer are risk stratified to facilitate decision making into individual management strategies (Table 1):

	PSA		**Gleason score**		**Clinical stage**
Low risk	< 10 ng/ml	and	Less than or equal to 6	and	T1-T2a
Intermediate risk	10-20 ng/ml	or	6	or	T2b-T2c
High risk	>20 ng/ml	or	8-10	or	T3-T4 (locally advanced disease)

Table.1. Risk stratification in localised prostate cancer patients (NICE 2008).

Men with low-risk, localised prostate cancer are first offered active surveillance which is particularly suitable for patients in clinical stage T1c, Gleason score 3+3, PSA density <0.15 ng/ml and in patients with less than 50% malignant foci in the total number of biopsy cores; active surveillance however is not recommended for men with high-risk localised prostate cancer (NICE 2008) A recent survey showed that in the year 2010 80% of UK oncologists expressed their approval in favour of active surveillance (Payne, Clarke et al. 2012). It has also been recommended that active surveillance should be considered for men with intermediate risk localised prostate cancer but not for men with high-risk localised prostate cancer – high-risk localised prostate cancer must be managed by radical prostatectomy or radical (conformal) radiotherapy, those with a Gleason score of 8 and higher must be considered for a minimum of 2 years of adjuvant hormonal therapy and patients with metastatic prostate cancer should be offered bilateral orchidectomy as an alternative to continuous LHRHa therapy (Guidance 2008, NICE 2008).

The benefits of screening strategies for prostate cancer and improvisation of therapeutic policies remains equivocal as of today and is a significant impediment faced by healthcare professionals across the UK (Lane, Hamdy et al. 2010). The ProtecT (Prostate testing for cancer and Treatment) trial and the CAP (Comparison Arm for ProtecT) study are two ongoing UK-based randomised controlled trials aim to measure prostate cancer mortality at 10 years as the primary outcome and overall survival, financial expenditures and quality of life as secondary outcomes (Lane, Hamdy et al. 2010). The CAP study examines the effectiveness of prostate cancer screening while the ProtecT study evaluates the efficacy of active monitoring, radiotherapy and radical prostatectomy for localised prostate cancer. In addition both these trials underscore the need for improvising better diagnostic and pharmacotherapuetic development that will facilitate management of adavanced stage

prostate cancer (Lane, Hamdy et al. 2010). Androgen refractory cases remain the biggest challenge in advanced prostate cancer management (UK 2013). Hormone-refractory stages are currently treated with cytotoxic agents such as docetaxel alone (non-metastatic cancer) or in combination with prednisone (such as in cases of symptomatic osseous metastasis)(Heidenreich, Aus et al. 2008). However, relapse to chemotherapy is not uncommon. This underscores the relevance of improved models to study the mechanism of hormone-refractory prostate cancer. It is also important to scrutinise the molecular networks and signalling pathways that regulate hormone-refractoriness. Genome-wide association studies or GWAS identify common alleles that contribute to the risk of developing prostate cancer (Lane, Hamdy et al. 2010). This reflects that an understanding of the cancer genetics and relevant molecular signalling in androgen-refractoriness can be a powerful tool to clinicians and scientists in improvising competent and effective treatment for advanced prostate cancer.

1.2. Molecular pathology of prostate cancer

The prostate relies on androgens such as testosterone and dihydrotestosterone for maintenance of its glandular structure and function. Androgen deprivation therapy offers symptomatic relief in prostate cancer until the emergence of hormone refractory disease - understanding the mechanism of hormone-refractoriness in this cancer is indispensable in elucidating the molecular pathogenesis of prostate cancer (Dorkin, Robson et al. 1997). Androgens bind to nuclear androgen receptor proteins which are then activated to further bind to androgen response elements that regulate transcription of specific target genes (Beato 1989). One such gene regulated by androgens is the prostate-specific antigen (PSA) gene which contains androgen responsive elements in its promoter region (Riegman, Vlietstra et al. 1991). Disruption in this signalling system contributes towards progression to hormone refractory disease (Dorkin, Robson et al. 1997).

A study using microarray profiling of prostate cancer xenografts concluded that a moderate increase in androgen receptor transcript was the only change that was constantly associated with the development of hormone-refractory disease and three major mechanisms have been identified behind resistance to antiandrogen therapy (Chen, Welsbie et al. 2004). Amplification of the androgen receptor gene promoting tumour growth at low serum androgen levels (Visakorpi, Hyytinen et al. 1995) and mutations of the androgen receptor gene that might provide a growth advantage following anti-androgen treatments have been reported in around 10% of prostate cancer cases (Taplin, Bubley et al. 1995, Taplin, Rajeshkumar et al. 2003). Elevated mitogen-activated protein kinase signalling such as those mediated by ERBB2 (HER-2/neu) or HRAS has also been incriminated in promoting hormone-refractory prostate cancer – forced expression of HER-2/neu results in ligand-independent growth and the androgen-receptor pathway synergises with even modest doses of androgens to hyper-activate the pathway (Craft, Shostak et al. 1999, Gioeli, Ficarro et al.

2002, Chen, Welsbie et al. 2004). A third mechanism implicated in androgen-independent prostate cancer is the androgen receptor bypass mechanism wherein alternative signalling pathways drive hormone resistance (Chen, Welsbie et al. 2004). One such pathway involves the bcl-2 oncogene that is known for its anti-apoptotic actions – bcl-2 expression is reported to be elevated following anti-androgen treatment and this has been implicated in causing the emergence of androgen-independent prostate cancer (McDonnell, Troncoso et al. 1992). It has also been documented that bcl-2 overrides apoptosis *in vitro* in LNCaP prostate cancer cells and augments hormone-refractoriness *in vivo* (Raffo, Perlman et al. 1995). Persistent activation of the hedgehog signalling pathway is also implicated in hormone refractory disease, promoting conversion of prostate progenitor cells to cancer-initiating cells (Karhadkar, Bova et al. 2004).

A study that used transplantation experiments demonstrated that conversion of a paracrine mechanism to an autocrine mechanism is instrumental behind malignant transformation of prostate epithelial cells (Gao, Arnold et al. 2001). In the normal prostate, androgen acts in a paracrine manner on different cell types – androgen receptors in prostate stromal cells produce growth factors or 'andromedins' which in turn diffuse across the basement membrane into the epithelial compartment and bind certain plasma membrane receptors thereby initiating epithelial cell growth and survival pathways (Litvinov, De Marzo et al. 2003, Isaacs and Isaacs 2004). The prostatic epithelial compartment comprises the basal and the secretory luminal epithelium along with neuroendocrine cells- basal epithelial cells do not express androgen receptor and this layer is lost in prostate cancer; however in the nuclei of secretory luminal cells, the androgen receptor initiates transcription of genes such as the prostate specific antigen and human glandular kallikrein-2 (Isaacs and Isaacs, 2004). Nevertheless, the receptor in these secretory luminal cells do not augment proliferation but act to suppress the secretory-cell growth by inhibiting andromedin-induced proliferation

(Isaacs and Isaacs 2004). In malignant cells, a switch in mechanism from stromal-cell-dependent paracrine pathways to autocrine pathways means that androgen receptors in these cancer cells directly stimulate their growth and proliferation (Gao, Arnold et al. 2001). In a hormone-escape prostatic cancer environment, molecular alterations occur that reduce the threshold of the androgen receptor ligand otherwise needed for cell growth and survival, additionally elevated levels of the androgen receptor in hormone refractory cancers also super-sensitizes these cells to androgen (Chen, Welsbie et al. 2004, Isaacs and Isaacs 2004). Indeed reducing androgen receptor expression to a level that would slow the growth of prostate cancer cells as well as trigger apoptosis has been suggested as a prostate cancer therapeutic mechanism (Isaacs and Isaacs 2004). However, this concept has not yet been studied and detailed scrutiny of the androgen receptor pathway calls for a cell biology model that would mimic the normal prostate physiology as accurately as possible.

Prensner et al evaluated the role of non-coding RNAs in prostate cancer disease progression by means of transcriptome sequencing across a prostate cancer cohort comprising 102 prostate tissues and cell lines. This group identified 121 un-annotated prostate cancer associated non-coding RNA transcripts (PCATS) of which PCAT-1 was characterised to be a prostate-specific regulator of cell proliferation as well as to be a transcriptional repressor implicated in prostate cancer (Prensner, Iyer et al. 2011). More recently, several publications have reported whole-exome sequencing to decipher the mutational configurations of prostate cancer, with one study identifying SPOP, FOXA1 and MED12 mutations to be instrumental behind carcinogenesis (Barbieri, Baca et al. 2012) and another study identifying proteins interacting with the Androgen Receptor (AR) such as FOXA1, MLL2, UTX and ASXL1 to be mutated in castration-resistant prostate cancer (Grasso, Wu et al. 2012).

The aim of this project has been to establish an ex vivo model that will eventually allow interrogation of signalling pathways that regulate the molecular pathology of advanced stage

prostate cancer. Furthermore the emerging significance of hormone-refractory prostate cancer underscores the relevance of prostate cancer stem cells which might be responsible for drug resistance (Collins and Maitland 2006).

The Androgen Receptor in Prostate Cancer

Prostate cancer progression is highly dependent on androgens, the actions of which take place through a functionally active Androgen Receptor (AR) (Linja and Visakorpi 2004). Functionally active androgen receptor signalling is critical in the development of prostate cancer since congenital syndromes such as androgen insensitivity and syndromes with reduced or absent AR signalling result in underdeveloped prostates that do not develop prostate cancer (Palmberg, Koivisto et al. 1999, Huggins and Hodges 2002). AR expression and signalling remains intact during development of androgen-insensitive disease where implications of genetic and epigenetic changes cause prostate cancers to be influenced by AR signalling (Taplin 2007).

The gene for AR is located on X chromosome Xq11-12 (Chang, Kokontis et al. 1988, Evans 1988, Taplin 2007). The AR molecule constitutes an amino-terminal activating domain and a carboxy-terminal ligand-binding domain in addition to a DNA-binding domain in the mid-region that contains two zinc-fingers (Feldman and Feldman 2001). Unligated AR molecules are cytoplasmic and remain bound to heat shock proteins 90, 70, 56 and 23, these chaperone proteins stabilize AR's tertiary conformation so as to allow for androgen binding [35; 36]. The steroid hormone androgen regulates crucial phenomenon such as those involved in prostatic development and differentiation through the formation of hormone-receptor complexes with androgens [31]. Once androgen-binding to the AR occurs, heat shock proteins dissociate from the AR, causing dimerization of AR which is followed by kinase-mediated phosphorylation – this causes AR to translocate to the nucleus [36]. AR belongs to the family of steroid-thyroid-retinoid nuclear receptor family, it is a phospho-protein where a conformational change in the ligand-binding domain occurs following androgen binding and consequent phosphorylation of the AR causes it to interact with specific androgen response elements causing activated gene expression (Brinkmann, Blok et al. 1999). The activated

DNA-bound AR homo-dimer complex recruits co-regulators to the AR complex where the activated complex involves a shift in the AR helix 12 position to create a binding site for co-activators (Feldman and Feldman 2001). These co-activators cause AR to interact with a certain complex of proteins that have the potential to either inhibit or stimulate gene transcription – this complex of proteins is known as the General Transcription Apparatus (McKenna, Lanz et al. 1999). Co-activators facilitate transcription by recruiting protein complexes to DNA which cause a change in the chromatin structure to a more activated form (Chang and McDonnell 2005). Examples of co-activators demonstrating histone acetyl transferase activity include NCOA1, NCOA2, NCOA3, PCAF, CBP, TIP60 and p300 (Dehm and Tindall 2005). Co-repressors silence transcription through chromatin condensation (Chang and McDonnell 2005), examples of such molecules include SMRT (silencing mediator of retinoid and thyroid) hormone receptors and NCOR (nuclear receptor co-repressor) (McKenna, Lanz et al. 1999). Examples of AR-regulated genes include PSA (prostate specific antigen), CDK8 (cyclin-dependent kinase 8), PIK3R1 (p85 catalytic subunit of phosphatidylinositol 3-kinase) and RAB4A (Velasco, Gillis et al. 2004). Prostate cancer growth and progression depends on the ratio of proliferating cells to the ratio of apoptotic cells – this is mediated by androgens and hence androgen ablative therapy works to control the disease progression in the initial stages by reducing cell proliferation rate and increasing rate of cell death eventually leading to elimination of prostate cancer cells (Denmeade, Lin et al. 1996, Feldman and Feldman 2001). It has also been documented that amplification of the AR gene at Xq11-q13 can account for androgen independence and consequent prostate cancer progression – Visakorpi.et al identified in vivo molecular mechanisms involving high-level amplifications of the AR that were detected in 30% recurrent tumours through FISH studies with an AR-specific probe (Visakorpi, Hyytinen et al. 1995).

The development and progression of prostate cancer results from several alterations in the AR signalling pathway (Linja and Visakorpi 2004). AR signalling in the normal prostate is very different from the malignant prostate. In the normal prostate, androgen-stimulated proliferation of the epithelium is mediated through AR positive stroma. However, in the malignant prostate androgen-mediated signalling switches to the autocrine mode whereby no interaction with the stroma is required (Gao, Arnold et al. 2001). The hormone refractory state has also been attributed to an increase in multiple androgen regulated genes (Linja and Visakorpi 2004). A cDNA microarray study that was carried out on human CWR22 prostate cancer xenografts during hormone ablation indicated the global gene expression profiles to be distinct in primary, regressing and recurrent tumours and further identified a set of androgen-responsive genes where expression levels were down-regulated initially with therapy but were later restored and up-regulated in recurrent tumours (Mousses, Wagner et al. 2001). For recurrent tumours, alteration in gene expression profiles was also noted for known targets of rapamycin as well as those that converged on the PI3K/AKT/FRAP pathway. The results from this study suggested that the combined effect of re-activation of androgen-responsive genes as well as the stimulation of rapamycin-sensitive signalling pathways could potentially lead to prostate cancer progression as well as contribute to androgen insensitive prostate cancer (Mousses, Wagner et al. 2001). Hara. et al established an androgen-insensitive MDA Pca 2b prostate cancer cell line in vitro from bone metastasis derived androgen dependent MDA PCa 2b human prostate cancer cells after 35 weeks of growth suppression through androgen depletion (Hara, Nakamura et al. 2003).These studies emphasize the relevance of androgens and the androgen receptor signalling in prostate biology.

2. Stem Cells in the Prostate

2.1 Definition of a stem-cell

Stem cells sit at the top of the lineage hierarchy and are characterised by their ability to self-renew and generate one or many specialised cell types through differentiation (Donovan and Gearhart 2001, Reya, Morrison et al. 2001). Stem cells can be embryonic or non- embryonic – whilst embryonic stem cells can differentiate into any of the three germ layers**,** the ability of non-embryonic stem cells to differentiate is much more limited (Tuch 2006). There are three types of embryonic stem cells – embryonal carcinoma cells derived from testicular tumours, embryonic stem cells derived from pre-implantation embryos and embryonic germ cells derived from primordial germ cells of post-implantation embryos (Donovan and Gearhart 2001). Alternatively, stem cells can also be defined based on their differentiation potential – totipotent stem cells sit at the top of the lineage hierarchy and can differentiate to give rise to any cells of embryonic and extra-embryonic origin ; whilst pluripotent stem cells are next in the lineage hierarchy and can differentiate into any embryonic cell types but do not differentiate to any extra-embryonic tissue (Tachibana, Sparman et al. 2012). Multi-potent stem cells are descendants of pluripotent stem cells and differentiate into several cell types along specific lineage routes while unipotent cells refers to the terminally differentiated cell types that can maintain only a single cell type or cell lineage (Hochedlinger and Plath 2009). Testicular spermatogonial stem cells (SSC) are a self-perpetuating population of germ cells that bear embryonic stem cell like characteristics and have been shown to directly trans-differentiate into reproductive as well as non-reproductive tissues of all germ layers (Simon, Ekman et al. 2009). Non embryonic stem cells also known as adults stem cells include haematopoietic stem cells and mesenchymal stem cells (Tuch 2006). A recently developed stem cell technology that enables generation of so-called induced pluripotent stem cells (or iPS) refers to stem-cells that are generated by overexpression of specific transcription factors

in somatic cells causing these cells to be reprogrammed into a pluripotent state which bears molecular and functional similarity to embryonic stem cells (Hochedlinger and Plath 2009).

It has been shown that the inner cell mass isolated from normal murine blastocysts when cultured in embryonal carcinoma cell line-conditioned medium give rise to pluripotent cell clones – these cells resembled embryonal carcinoma cell lines in morphology and expressed SSEA-1, a marker common to mouse teratocarcinoma stem cells and early embryos but absent in murine differentiated cell types; furthermore these cells demonstrated *in vitro* differentiation into cells representing all the three germ layers by forming embryoid bodies and demonstrated their pluripotential characteristics *in vivo* by forming teratocarcinomas in mice (Martin 1981). It has also been reported that *in vitro* cultures of mouse blastocycts can result in formation of established pluripotent cell lines that possess al the characteristic features of embryonic stem cells; the success of derivation of these pluripotent lines were dependent on three factors – the stage of embryonic development when the blastocysts were used for pluripotent line derivation, the number of precursor cells available from the embryos and selection of appropriate *in vitro* conditions that would facilitate proliferation of these cell lines as stable undifferentiated pluripotent cell lines (Evans and Kaufman 1981). These cells were called embryonic stem cells based on their source as opposed to the teratocarcinoma-derived embryonal carcinoma cell lines. The establishment of the first human embryonic stem cell lines defined characteristics of primate embryonal carcinoma cells to include the following: that these are derived from pre-implantataion or peri-implantation embryos, that these cells show continuous undifferentiated proliferation over long periods of time in culture and that these cells are capable of differentiating into cell types representing the endoderm, mesoderm and ectoderm irrespective of their time in culture as undifferentiated stable pluripotent cells (Thomson, Itskovitz-Eldor et al. 1998). A total of 5 distinct embryonic stem

cell lines were established from fresh or frozen cleavage stage separate embryos during *In Vitro* Fertilisation (IVF) procedures, 3 of the lines possessed a normal XY karyotype while the other two showed a normal XX karyotype (Thomson, Itskovitz-Eldor et al. 1998).

Pluripotent stem cells are identified by certain properties – morphologically they grow in colonies comprising small rounded cells with high nuclear to cytoplasmic ratio and large rounded nucleoli. They can be grown indefinitely in culture, a unique phenotype explained by high level of telomerase activity in these cells – telomerase expression is associated with immortality of cell lines in culture with high levels of telomerase expression detected in germ lines and in embryonic tissues (Wright, Piatyszek et al. 1996). On the contrary, diploid somatic cells with limited life span are marked by an limited telomerase activity and shortened telomeres that causes these cells to undergo replicative senescence following a restricted life-span *in vitro* (Allsopp, Vaziri et al. 1992, Counter, Avilion et al. 1992, Counter, Hirte et al. 1994). The pluripotent nature of these cells are further characterised by the expression of certain cell-surface antigens such as alkaline phosphatase, stage-specific embryonic antigens SSEA3, SSEA4, Tra-1-60 and Tra-1-81 (Thomson, Kalishman et al. 1995, Thomson, Itskovitz-Eldor et al. 1998, Thomson and Marshall 1998). Consistent with human embryonic carcinoma cell lines, undifferentiated pluripotent cell lines did not stain for SSEA1 – the fact that SSEA1 defines differentiated embryonic stem cell cultures in humans but undifferentiated pluripotent states in murine pluripotent stem cell lines reflects the elementary species-specific differences in development between humans and mice (Thomson, Itskovitz-Eldor et al. 1998). The functional aspects of pluripotent stem cells are defined by their extremely limited lineage restriction in that they can differentiate into a wide repertoire of terminal cell types. This is demonstrated *in vitro* through formation of 3-dimensional embryoid bodies in the absence of b-FGF. These embryoid bodies when seeded onto gelatin-

coated plates differentiate to give rise to a heterogeneous population of cell types including neuronal-like structures, myocytes (such as beating cardiomyocytes) and epithelial-like cells. Ideally, pluripotent cells such as those comprising the inner cell mass of the blastocyst and epiblast of post-implantation embryos as well as pluripotent stem cell lines should be able to contribute to chimerism *in vivo* (Trounson and Grieshammer 2012). Unfortunately, given ethical constraints chimera-formation assays cannot be carried out in the human thereby limiting an extensive application of these pluripotent cell lines in regenerative and reproductive science. Pluripotent stem cell lines generated from blastomeres, inner cell mass of blastocysts and epiblasts of post-implantation pre-gastrulation embryos all form teratomas (Chung, Klimanskaya et al. 2006, Cockburn and Rossant 2010, Nichols and Smith 2011). However, epiblast stem cells do not form chimeras; this can be attributed to the fact that either they are not pluripotent and/or to incompatibility between the host and injected cells (Trounson and Grieshammer 2012). When mouse embryonic stem cells (ESC) are injected into inner cell mass deficient tetraploid embryos these form ESC-derived embryos (Nagy, Gocza et al. 1990). This unique ability of pluripotent stem cells towards chimerism has seen this technology being extensively used to study mammalian gene function through transgenic knock-out mice (Capecchi 1989). However, very recently it has been shown that unlike murine embryonic stem cells, rhesus-derived embryonic stem cells and inner cell mass are not capable of chimera formation since they fail to incorporate into host embryos (Tachibana, Sparman et al. 2012). On the contrary, chimerism was demonstrated through monkey totipotent cells in the form of very early 4-stage embryos – raising the question whether the failure of chimera formation was due to the pluripotent cells being lineage restricted or whether they were unable to implant and utilise extra-embryonic support from the host, raising the issue of host-compatibility in such stem cell experiments (Tachibana, Sparman et al. 2012, Trounson and Grieshammer 2012).

2.2. Stem cells in the prostate - prostate epithelial stem cells and the epithelial stem cell niche

The existence of stem cells in the prostate was first reported by Isaacs . when a study in rodents demonstrated that normal prostate regenerates following several cycles of androgen ablation and replacement (Isaacs 1985, Tsujimura, Koikawa et al. 2002). A different study demonstrated glandular epithelial induction with the help of tissue recombinants prepared using adult mouse urinary bladder epithelium and mesenchyme of embryonic urogenital sinus – suggesting the possible existence of a stem cell compartment in the adult urogenital tract (Cunha, Lung et al. 1980, De Marzo, Nelson et al. 1998). An *in vivo* study further established the existence of murine prostatic epithelial stem cells in the proximal region of prostatic ducts (Tsujimura, Koikawa et al. 2002). Another research paper established prostate regeneration using adult mouse prostate epithelial cells and embryonic urogenital sinus mesenchyme (Xin, Ide et al. 2003). An *in vivo* mouse-model study demonstrated that cells that are of the Lin^{-} $Sca\text{-}1^{+}CD133^{+}CD44^{+}CD117^{+}$ phenotype are multi-potent and can generate an entire functional prostate (Leong, Wang et al. 2008). Recently it has been documented that p63 expressing cells constitute the stem cells of the developing prostate as well as the bladder (Pignon, Grisanzio et al. 2013). Another *in vivo* study analysed prostate development by investigating genetic lineage tracing and concluded that postnatal prostatic development is regulated by basal multi-potent stem cells which differentiate into basal, luminal and neuroendocrine cells as well as by uni-potent progenitor cells (both basal and luminal) (Ousset, Van Keymeulen et al. 2012). Clonal analysis revealed that while prostate development in the adult is mediated by uni-potent luminal and basal cells; there are multi-potent progenitor cells that regulate prostate development in the postnatal period (Ousset, Van Keymeulen et al. 2012). Lineage analysis of basal epithelial cells have further shown that tumour development through luminal or basal cells result in cancers that bear distinct

molecular profiles that can predict patient outcomes (Wang, Mitrofanova et al. 2013). It is the malignant transformation of basal cells which give rise to tumours with luminal phenotype; however cross-species bioinformatics analyses have shown that tumours of luminal origin are more aggressive and reflect poorer patient outcomes (Wang, Mitrofanova et al. 2013). Putative prostatic stem cells have also been isolated from human benign prostatic hyperplasia (BPH) specimens using flow cytometry-based side population sorting wherein stem cells preferentially express a multi-drug resistance protein that enables them to efflux the Hoechst dye (Bhatt, Brown et al. 2003, Tang, Patrawala et al. 2007).

Functionally, the prostate gland can be divided into several stem cell units all of which arise from a single or reserve stem cell that is located in the basal epithelial compartment (Robinson, Neal et al. 1998, Litvinov, De Marzo et al. 2003). This reserve stem cell divides to form transit amplifying cells which in turn divide and differentiate into the secretory luminal layer - a subset of basal cells constitute stem cells while the remainder form transit-amplifying cells (Hudson, O'Hare et al. 2000, Litvinov, De Marzo et al. 2003). The transit amplifying cell types have restricted ability to divide and ultimately differentiate to give rise to terminally committed and functionally active luminal secretory cells (Robinson, Neal et al. 1998). Epithelial stem cells have been implicated in the aetiology of both BPH and prostate cancer by several research groups (Isaacs and Coffey 1989, Bonkhoff and Remberger 1996, De Marzo, Nelson et al. 1998, Hudson, O'Hare et al. 2000). There is substantial ambiguity behind the etiology of both BPH and prostate cancer; however they are both diseases of aging and are dependent on androgens for growth and development (Isaacs and Coffey 1989, Coffey and Walsh 1990, De Marzo, Nelson et al. 1998). Nevertheless, BPH and prostate carcinoma are very distinct clinical conditions – firstly benign prostatic hyperplasia rarely ever transforms into malignancy, unlike prostatic intraepithelial neoplasia (PIN) and cancer genomic instabilities are rarely associated with BPH (De Marzo, Nelson et al. 1998). A

characteristic feature of BPH is the variable stromal overgrowth which in turn can cause epithelial hyperplasia through mesenchymal-epithelial interactions (Cunha 1994). One proposed model for development of prostatic intraepithelial neoplasia and eventually prostate cancer is an aberrant cell cycle effect that would cause certain cells in the secretory compartment to transiently proliferate and prevent them from differentiating into terminal cells (De Marzo, Nelson et al. 1998).

2.3. Cancer stem cell hypothesis

The concept of cancer stem cells was first put forward in haematopoietic cells by Bonnet and Dick wherein they postulated that the leukaemic clone is organised in a hierarchy (Bonnet and Dick 1997). Further to this, cancer stem cells were identified in solid tumours, including breast cancer (Al-Hajj, Wicha et al. 2003) and in brain tumours (Singh, Clarke et al. 2003). Prostate neoplasms are heterogeneous in that the cells in a single tumour display variable phenotypic characteristics and distinct proliferative and malignant potentials (Heppner 1984, Collins, Berry et al. 2005). Tumour heterogeneity suggests two possible models behind tumuorigenesis - stochastic and hierarchical (Collins and Maitland 2006). The stochastic model explains that different cancer cells within a single neoplasm would have the ability to proliferate extensively, however the probability that any such cell would enter the cell cycle is low; the hierarchical theory explains that only a subset of cells would be enriched for the ability to self-renew and would thus initiate tumourigenesis (Reya, Morrison et al. 2001). In prostate cancer, a population of cancer stem cells have been identified with a $CD44^{+}/\alpha_2\beta_1^{hi}/CD133^{+}$ phenotype – these cells show features of self-renewal, regenerate diverse populations of non-clonogenic cells that in turn express androgen receptor and prostatic acid phosphatase (Collins, Berry et al. 2005).

Establishing a prostate cancer stem cell model will serve to demonstrate the cellular and molecular mechanisms behind tumour heterogeneity (Collins and Maitland 2006). This in turn will enable specific targeting of cancer stem cells whilst considering therapy for prostate cancer and might lead to improved therapeutic efficacy.

2.4. Animal models of prostate cancer

Prostate cancer is a disease that is typically exclusive to man. The only other animal known to develop prostatic adenocarcinomas and PIN spontaneously is the dog (Waters, Sakr et al. 1998). There are several similarities between prostate cancer in humans and canines such as advanced age of disease onset, metastatic abilities of the primary tumour, apparent onset of androgen independence of advanced disease and tumour heterogeneity (Waters, Sakr et al. 1998). However, canine models are not a practical solution given that these models are economically prohibitive; the incidence of prostate cancer is incredibly low in dogs (Maini, Archer et al. 1997) and it is difficult to genetically manipulate a canine model and maintain such transgenic litters (Ghoniem and VandenBerg 1994).

Several strains of rats have been shown to develop spontaneous prostate cancer (Pylkkanen, Makela et al. 1996, Maini, Archer et al. 1997) and murine xenograft models using immunodefficient mice has been popularly used to model prostate cancer. However there are considerable differences between the mouse and the human prostate. The human prostate is alobular with a distinct central, peripheral and transitional zone. Most cancers arise in the peripheral zone of the gland, the transitional zone is rarely involved and practically none arise in the central zone (De Marzo, Platz et al. 2007). Most BPH lesions on the other hand arise in the transitional zone (De Marzo, Platz et al. 2007). The mouse prostate on the other hand has four lobes: dorsal, lateral, anterior and ventral and each lobe is divided into three regions relative to the urethra (Leong, Wang et al. 2008). Other notable differences include that the mouse prostate atrophies with advanced age while the human prostate hypertrophies with age, the mouse does not develop prostate cancer spontaneously and unlike humans the short life span of mice prevents the mouse prostate from accumulating cumulative genetic lesions (Sharma and Schreiber-Agus 1999) that may possibly be an important contributing factor in human prostate carcinoma .

2.5. Markers of "stemness" and differentiation in the prostate

Epithelial cell differentiation pathways have been demonstrated through keratin and cell adhesion molecule expression (Murant, Handley et al. 1997, van Leenders, Dijkman et al. 2000, Hudson, Guy et al. 2001) as well as through expression of androgen responsive proteins and cell proliferation (Maitland and Collins 2008). Basal cells express high levels of keratins such as K5, K14, K15, K17, K19 and p63 but express low levels of AR (androgen receptor), PSA and keratins 8 and 18. In contrast, luminal cells lack p63, K5 and K14 but express high levels of K8, K18, AR and PSA with some luminal cells also expressing K19 (Hudson, Guy et al. 2001, van Leenders, Aalders et al. 2001). Basal cells in normal, BPH and high grade intraepithelial neoplasia express p63 though this marker is rarely expressed in adenocarcinoma specimens (Parsons, Gage et al. 2001). This gene is known to play an important role in prostate development and has also been detailed as essential for normal stem-cell function in the prostate (Grisanzio and Signoretti 2008).

A number of other markers have been described as 'stemness' markers in the prostate – α2β1 integrin (Collins, Habib et al. 2001), CD133 (Richardson, Robson et al. 2004), CD44 (Patrawala, Calhoun et al. 2006), CD117 (Leong, Wang et al. 2008) and Nkx3.1 (He, Sciavolino et al. 1997, Wang, Kruithof-de Julio et al. 2009). CD133 or AC133 is also known as prominin-1 (Shmelkov, St Clair et al. 2005) is expressed by 1% of human prostate basal cells that are also positive for α2β1 integrin – these cells are characterised by high *in vitro* proliferation capacity and have the ability to generate prostatic-like acini in immuno-compromised male nude mice (Richardson, Robson et al. 2004). A study of CD44 using prostate cancer cells demonstrated that this marker is associated with increased proliferation as well as increased mRNA expression of other stem cell genes such as Oct-3/4, Bmi, β-catenin and SMO. Furthermore these cells are also more clonogenic, tumorigenic, metastatic as well as undergo asymmetric cell division in clonal analyses (Patrawala, Calhoun et al.

2006). Nkx3.1 expression marks a luminal cell population that have bipotential, possess self-renewal properties, can re-constitute prostatic ducts in renal grafts and are demonstrated by prostate regeneration assays to facilitate stem cell maintenance (Wang, Kruithof-de Julio et al. 2009) . CD117 along with Sca-1, CD-133 and CD44 has been demonstrated to mark a murine prostate stem cell population that are multi-potent and can self-renew - in fact a single such cell can reconstitute a prostate *in vivo* (Leong, Wang et al. 2008).

3. Lineage plasticity and Cell fate

3.1. Pro-iPS model to study prostate tiussue differentiation and homeostasis

The mechanism of differentiation of progenitor cells into different cellular lineages can be studied with the help of pluripotent cells. Pluripotency is defined as the ability of a cell to give rise to one of the three germ layers of the embryo. Examples of pluripotent cells include cells of the inner cell mass and its derivative, embryonic stem cells and the induced pluripotent cells. Embryoid body formation and chimera formation are characteristic features of pluripotency. Induced pluripotent cells refer to reprogrammed somatic cells by means of pluripotency factors. This was first demonstrated by Takahashi and Yamanaka when they reprogrammed mouse embryonic and adult fibroblast cultures by transducing these with Oct 3/4, Sox2, c-Myc and Klf4 under embryonic stem cell culture conditions (Takahashi and Yamanaka 2006). These initial induced pluripotent stem (iPS) cells were not germline competent and showed aberrant DNA methylation patterns until selection for Nanog expression allowed for establishment of a germline competent iPS cell line with increased embryonic stem cell-like gene expression and DNA methylation patterns comparable with chimera generating Fbx15 iPS cells (Okita, Ichisaka et al. 2007). Later, Yu. et al successfully derived iPS cell lines from human somatic cells (Yu, Vodyanik et al. 2007). Using iPS cell lines avoids some of the ethical dilemma raised with using human embryo for embryonic stem cell generation. Studying prostate induced pluripotent cell differentiation to prostate cells would enable demonstration of the genetic alterations and epigenetic modification that occur during prostate development and differentiation (Nishikawa, Goldstein et al. 2008). The availability of a stable prostate iPS cell line would also obviate current technical difficulties associated with primary culture of prostate epithelia and stroma. The presence of well characterised limited iPS cell lines would further bypass clinical variations amongst tissue specimens encountered with primary tissue culture.

Deciding on a somatic source of iPS cells that will be best committed to differentiate into prostate is important as iPS cells from different origins have different propensities to differentiate (Yamanaka 2009). Induced pluripotent stem cells generated from murine hepatocytes and murine stomach cells do not need any retroviral integration into specific sites and thus are spared of tumourigenic potential (Aoi, Yae et al. 2008). Additionally, reprogramming juvenile human primary keratinocytes is 100-fold more efficient and twice as rapid than the reprogramming of fibroblasts (Aasen, Raya et al. 2008). The aim of this project is to ascertain if non physiological skin fibroblast derived iPS cells are as efficiently committed to differentiate into prostate cells as are the iPS cells derived from the prostate.

3.2. Nuclear re-programming – and introduction

Nuclear re-programming is defined as a change in the gene expression of a cell to a different unrelated cell type (differentiated) or to that of an embryo (Gurdon and Melton 2008). Development of a fertilised egg into an adult is marked by an irreversible progressive lineage restriction during differentiation into terminal cell types that confines these cells to their committed fate; the process of re-programming is achieved by several different technologies (Figure 1.3) such as mammalian somatic cell nuclear transfer, cell fusion, induced pluripotency through forced ectopic gene expression and direct re-programming of one cell type into another (Wilmut, Schnieke et al. 1997, Rodolfa and Eggan 2006, Takahashi and Yamanaka 2006, Gurdon and Melton 2008). The process of nuclear re-programming is critical for several reasons – this technique reveals substantial information about the process of differentiation, development and about specialised gene expression and function. This process also provides an advance in cell-replacement therapy where defective cells can be replaced by 'corrected' cells of the same or a related kind but has been established from an entirely different type of cells. Fnally, nuclear re-programming allows for disease pathogenesis in a petri-dish in the sense that pluripotent cell types can be established from patients with different diseases and these cells harbour genetic information of the respective disease processes (Gurdon and Melton 2008). The different techniques used in nuclear re-programming will now be described.

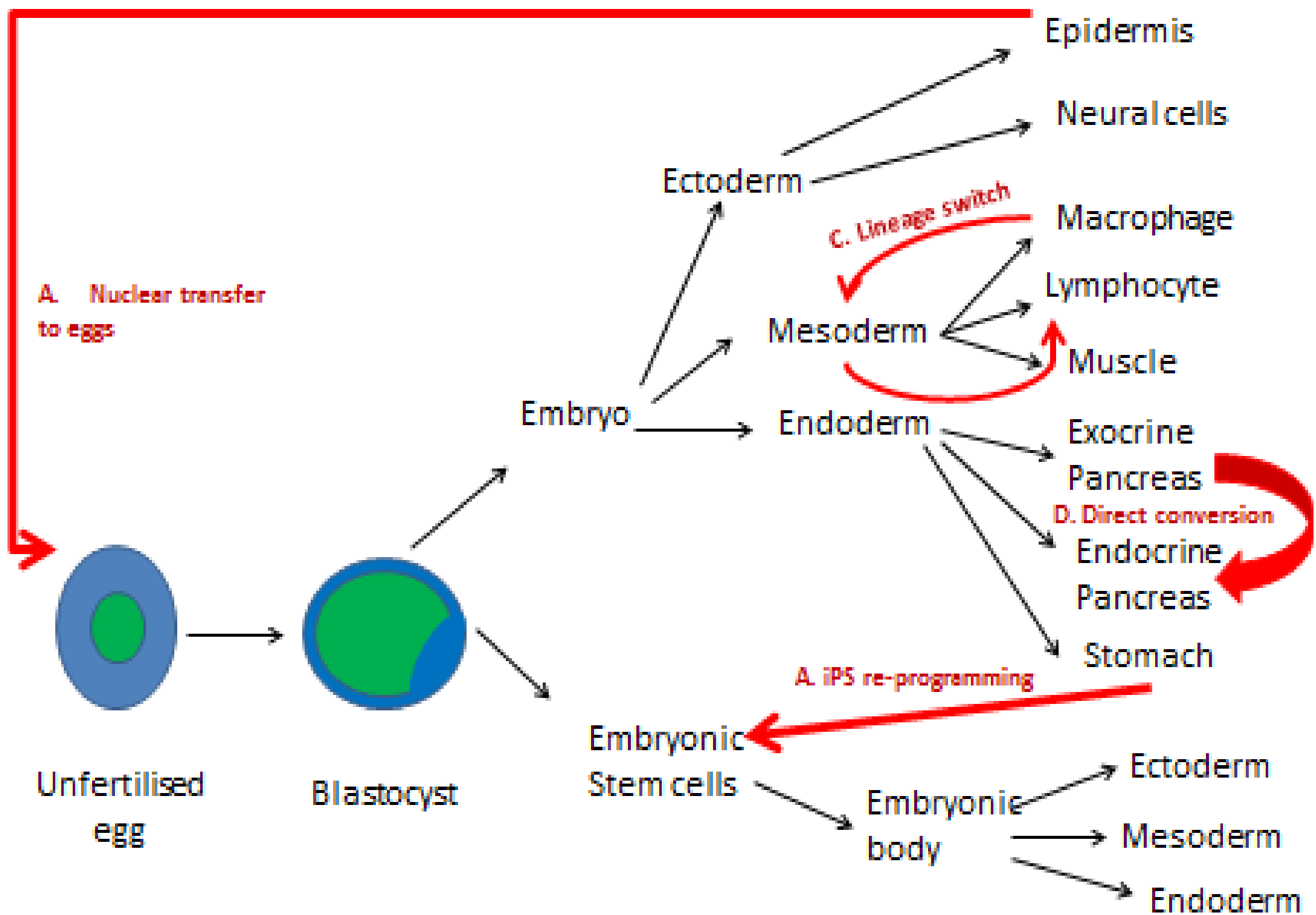


Figure 1.3. The experimental routes to nuclear re-programming: A. by means of nuclear transfer to eggs. B. By induction of pluripotency through transcription factor re-programming (iPS technology). C. By means of lineage switching back to a branch point and then again in a different direction. D. by direct conversion of one cell fate to the other. Schematic adapted from (Gurdon and Melton 2008).

3.3. Somatic cell nuclear transfer

This technology exploits the role of the nucleus in embryonic differentiation. In the 1880s, August Weissmann formulated the 'germ plasm theory' where he hypothesised that in multicellular organisms cells would differ from each other through a series of asymmetric cellular division where different cells would come to inherit varying proportions of genetic material from their progenitors (Weismann 1893). This theory was further tested by Roux and colleagues and it was established that during embryonic differentiation different blastomeres receive different kinds of nuclei which further decide upon their consequent lineage specification and cell fate.hHowever, this school of thought was later challenged with the concept that cleaved nuclei are identical and differentiation is indeed attributed to specific areas of the egg cytoplasm (Briggs and King 1952). Morgan suggested that the nuclei differentiate as a consequence of variability between different regions of the cytoplasm and such nuclear differences can further affect the cytoplasm in a reciprocal manner (Morgan 1934). The more recent studies have suggested that cytogenetic processes (Schultz 1947) and/or ciliate morphogenesis could account for nuclear differentiations (Weisz 1951). Briggs and King devised specific experiments to address these concerns by transplanting nuclei from advanced blastula cells into enucleated eggs of the amphibian *Rana pipiens* – this resulted in the production of swimming tadpoles and the study formed the earliest evidence of experimental reversal of embryonic cellular differentiation (Briggs and King 1952). These authors also concluded that the natural process of nuclear differentiation in an embryo is irreversible since nuclear transfer resulted in abnormal development when older gastrula stage cells were used in place of the blastula (Briggs and King 1957). These studies were carried forward by testing if nuclear transfer from fully differentiated cells would result in offspring.The first attempt at such a concept involved nuclear transfer from intestinal epithelium cells of feeding tadpoles where a certain proportion of transferred intestinal nuclei

gave rise to normal feeding tadpoles suggesting that at least a fraction of differentiated cells contain nuclei with pluripotent characteristics. However, 48% of intestinal nuclei failed to demonstrate cleavage following transplantation and irregular cleavage formation that resulted in abortive nuclear transfer was observed with many intestinal nuclei but with very few embryonic nuclei (Gurdon 1962). Many of the experiments also resulted in either partial cleavage and/or abnormal development (Gurdon 1962). Successful nuclear transfer from a fully differentiated 'fertile' intestinal cell into adult frogs provided conclusive evidence that during the course of differentiation processes that alter gene expression and function in directing cells to their specific fates as well as genetic mechanisms that are repressed during differentiation do not result in the irreversible alteration of genes that would be needed for differentiation of cells into other lineage/cell types.In other words, genetic information is not lost within all cells during the course of differentiation (Gurdon and Uehlinger 1966). It can therefore be deduced that during the process of embryonic differentiation there is a change in nuclear genetic expression but not in the content. Furthermore, although during the course of lineage commitment there is a change in phenotypic characteristics of a cell, its genome remains unaltered throughout lifetime (Gurdon and Melton 2008).

The next major breakthrough in nuclear transfer saw cloning of the sheep 'Dolly', when it was shown that *in vitro* cell lines isolated from sheep embryos that had been forced into quiescence through serum starvation following 6-13 passages in culture resulted in viable offspring when the nuclei of these cells were transferred into enucleated oocytes (Campbell, McWhir et al. 1996). This study suggested that induction of quiescence in donor cells brought about alterations in chromatin structure that ultimately allowed for nuclear transfer and consequent embryonic development. The same technique was repeated to facilitate nuclear cloning from three different donor populations, including terminally differentiated adult

mammary gland cells and cells from the foetus (Wilmut, Schnieke et al. 1997). A major step forward from these experiments was the nuclear cloning of monkeys to generate rhesus macaque blastocysts from adult skin fibroblasts through the process of somatic cell nuclear transfer. This generated two stable embryonic stem cells lines from the thus derived primate embryos that demonstrated all the characteristics of pluripotent cells such as the distinct morphology, expression of embryonic stem cell markers, exhibited transcriptional similarity to embryonic stem cell transcripts and differentiated into derivatives of all the three germ layers *in vitro* as well as *in vivo* (Byrne, Pedersen et al. 2007). This therefore allows us to conjecture that human cells also retain overall genomic fidelity during development and that this process can be reversed through specific genetic manipulations at the chromatin level (Gurdon and Melton 2008). Mechanisms accompanying nuclear re-programming include an increase in volume of the transferred nuclei as well as decondensation of sperm chromatin which is mediated by nucleoplasmin, an acidic immuno-depleting nuclear protein in egg cytoplasm that demonstrates histone binding functions (Philpott, Leno et al. 1991). Nuclear re-programming is also facilitated by mechanisms of histone modifications as well as through DNA demethylation on the Oct4 promoter sites (Simonsson and Gurdon 2004). Oct-4 DNA demethylation is mediated by Gadd45a interacting directly with XPG mRNA (Barreto, Schafer et al. 2007). Chromatin protein exchange of the oocyte-specific linker histone H1 by histone variants B4 or H1 also play a critical role in nuclear re-programming suggesting that maternally expressed linker histones determine nuclear dynamics in the context of embryonic toti-potency (Saeki, Ohsumi et al. 2005). One of the hindrances to nuclear re-programming is the fact that epigenetic memory of the donor cell may not get completely erased during nuclear transfer suggesting that epigenetic memory stabilizes normal development and can also lead to inefficient nuclear reprogramming (Gurdon and Melton 2008). Overexpression of the histone H3.3 has been documented to enhance parental

memory in transplanted nuclei where it interacts with the myogenic gene MyoD in non-muscle lineages. As a corollary, it has also been observed that the association of a mutated histone H3.3 (H3.3 E4) with the promoter region results in elimination of this memory (Ng and Gurdon 2008) suggesting that the mechanisms of cellular re-programming would be amenable to epigenetic manipulations.

3.4. Cell fusion and cell extracts

Re-programming by cell fusion refers to the process of fusing two different cells to form a heterokaryon and then adding a cell division inhibitor would allow for the nuclei to remain separate. The net effect sees the dominant more actively dividing cell impart its pattern of gene expression on its partner (Gurdon and Melton 2008). This phenomenon has been demonstrated for erythrocytes where its fusion with a growing cultured cell saw reactivation of the red cell nucleus along with a massive increase in its volume. The increase in volume was suggested to be due to an increase in RNA and/or DNA synthesis and was regulated by specific localizations of the chromatin on which the genetic synthesis took place (Harris 1967). Blau.et al induced the expression of muscle genes in non-muscle cells by fusing human amniocytes with differentiated murine muscle cells by means of polyethylene glycol. This generated a cell known as a heterokaryon where the parental cell nuclei remain distinct, do not divide and these cells retain a full complement of chromosomes. On the other hand, syncaryons are interspecific hybrids in which parental nuclei are combined and chromosomes are lost during cell division (Blau, Chiu et al. 1983). Primary murine muscle cells were fused with primary keratinocytes (representing ectoderm derivatives), fibroblasts (representing mesoderm derivatives) and hepatocytes (representing endoderm derivatives) with the result that all the three different types of cells were capable of activating a number of previously silent muscle gene cells, indicating that the differentiated state of a cell is amenable to genetic modifications (Blau, Chiu et al. 1985, Pomerantz and Blau 2004). This nuclear activation is believed to occur through the cytoplasm where the activators remain existent even after differentiation and are recognized by nuclei of other species (Blau, Chiu et al. 1983). In another study , human fibroblasts and foetal lung were merged with differentiated mouse muscle cells and it was observed that the enzyme MM-creatine kinase (CK) and 5.1H11 were detectable at comparable levels, irrespective of the presence or absence of the DNA synthesis

inhibitor cytosine arabinoside. Furthermore, muscle gene expression was not restricted to the G1 phase. The study thereby concluded that differentiation re-programming in heterokaryons is not dependent on the phase of cell cycle or DNA synthesis (Chiu and Blau 1984). The appeal behind the use of oocytes lies in the fact that they have the natural ability to re-programme sperm nuclei with 100% efficiency without the need for a permanent genetic change of the fertilising sperm cell or of the resultant re-programmed embryo-like cells (Gurdon and Melton 2008). However, this process is limited by severe ethical and humanitarian restrictions.

Mammalian development is marked by a unidirectional progressive loss of developmental potential that causes a unicellular zygote to differentiate to give rise to a wide repertoire of 220 specialized cell types in the mammalian body (Hochedlinger and Plath 2009). The process of induced pluripotency refers to re-programming a terminally differentiated cell type against its differentiation gradient to an immature pluripotent phenotype. The newly re-programmed cells now acquires the characteristics of an embryonic stem cell in terms of morphology and differentiation ability (Takahashi and Yamanaka 2006, Takahashi, Tanabe et al. 2007, Yu, Vodyanik et al. 2007, Hochedlinger and Plath 2009). Induced pluripotent cells are also known as ‘iPS’ cells and were first established from mouse tail fibroblasts by Prof. Shinya Yamanaka’s group in Tokyo. Genetic manipulation of these somatic fibroblasts with a cocktail of 4 out of 24 factors tested defined pluripotency transcripts – Oct4, SOX2, Klf4 and C-Myc (OSKM). This 4 factor combination saw fibroblast cells gradually lose their somatic phenotype in that the cells no longer demonstrated the distinct elongated phenotype and the characteristic gene-expression pattern of a fibroblast was lost (Takahashi and Yamanaka 2006). These results were later reproduced using human adult fibroblasts to generate human iPS cells that would have extensive applications in research as well as regenerative medicine

(Takahashi, Tanabe et al. 2007). Since these latter iPS cells were formed through selection using the factor Fbx15 these iPS cells were termed Fbx15 iPS cells.I Ironically, in spite of their ability to form embryoid bodies and teratomas these cells demonstrated certain differences to embryonic stem cells in terms of embryonic stem cell transcript expression and failure to produce chimeras. To address this issue, Nanog selection (a factor previously declared as redundant in pluripotency re-programming) was used to generate Nanog iPS cells that resulted in viable chimeras as well as being more identical to human embryonic stem cells with respect to gene-expression profiling and DNA methylation patterns (Okita, Ichisaka et al. 2007). Nanog is one of the three core transcriptional factors in the pluripotency circuitry of embryonic stem cells (Boyer, Lee et al. 2005). Nanog deficiency results in abortive epiblasts and Nanog-deficient embryonic stem cells lose their characteristic pluripotent phenotype and differentiate into extra-embryonic endodermal lineage (Mitsui, Tokuzawa et al. 2003). In addition, all the transgenes of Oct4, SOX2, Klf4 and C-Myc were strongly silenced in Nanog selected clones. However, 20% of the Nanog iPS-generated offspring developed tumours that were attributed to the reactivation of C-Myc gene (Okita, Ichisaka et al. 2007, Okita, Nakagawa et al. 2008). Although C-Myc plays an important role in maintaining murine embryonic stem cell self-renewal and pluripotency through the LIF/STAT3 pathway (Cartwright, McLean et al. 2005) it has been documented in human embryonic stem cells that it adversely affects pluripotency by causing apoptosis and differentiation in a transcriptional activity dependent manner (Sumi, Tsuneyoshi et al. 2007). Therefore, attempts were made at the induction of pluripotency in human cells without C-Myc (Nakagawa, Koyanagi et al. 2008) by using only Oct4, SOX2 and Klf4 (Wernig, Meissner et al. 2008) and by using a slightly different combination of transcription factors, namely – Oct4, SOX2, Lin28 and Nanog (Yu, Vodyanik et al. 2007, Nakagawa, Koyanagi et

al. 2008). Hence, iPS generation without C-Myc in humans has been described to be more specific although the process is less efficient (Nakagawa, Koyanagi et al. 2008).

Transgene integration has been another major cause for concern in using the iPS cell technology. The first iPS cell lines were established by mean of retroviral transduction in mouse (Takahashi and Yamanaka 2006) as well as human fibroblasts (Takahashi, Tanabe et al. 2007, Huangfu, Osafune et al. 2008, Lowry, Richter et al. 2008). *In vitro* transduction of cells with retrovirus has been shown to result in subsequent mutations that lead to the formation of neoplastic clones.I It has also been observed that SCID patients cured by means of retroviral gene therapy have consequently developed leukaemia thereby inducing genotoxicity associated with retroviral integration (Nienhuis, Dunbar et al. 2006). Lentiviral transduction protocols for iPS generation (Yu, Vodyanik et al. 2007, Stadtfeld, Brennand et al. 2008, Sommer, Stadtfeld et al. 2009, Anokye-Danso, Trivedi et al. 2011) target both dividing and non-dividing cells and have not been associated with the slow kinetics that is often seen with retroviral transduction methods that can only transduce actively dividing cells (Robinton and Daley 2012). Lentiviral transductions however are also associated with genomic integration as well as inefficient proviral silencing (Yu, Vodyanik et al. 2007, Stadtfeld, Brennand et al. 2008, Sommer, Stadtfeld et al. 2009, Anokye-Danso, Trivedi et al. 2011, Robinton and Daley 2012). A study by Judson and colleagues showed that the introduction of microRNAs, miR-291-3p, miR-294 and miR-295 increase the efficiency of re-programming in mouse through all four of the OSKM factors, most specifically through C-Myc which binds the promoter of the miRNAs (Judson, Babiarz et al. 2009). The transcriptional activation of Oct 4 and SOX2 regulated miR-302 and translational inhibition of its target, Cyclin D1 which is an important regulator of the G1 phase of cell cycle suggests a putative role for Oct4 and SOX2 in pluripotent cell cycle regulation (Card, Hebbar et al. 2008). As

expected, miRNA (302/367)-mediated iPS induction is highly efficient and rapid. The efficiency of this miRNA iPS induction can be further improved in conjunction with addition of the small molecule valproate and suppression of HDAC2 (Anokye-Danso, Trivedi et al. 2011). Efficiency of lentiviral transduction can also be improved by adapting an inducible transduction protocol, this further allows for controlled expression of factors (Maherali, Ahfeldt et al. 2008, Stadtfeld, Maherali et al. 2008). An inducible lentiviral transduction protocol enabled the scrutiny of iPS induction, demonstrating for the first time that following 10 days of ectopic transcript expression the successfully re-programmed cells start producing their own endogenous pluripotent transcript and enter a stable pluripotent state when the exogenous transcripts become redundant (Stadtfeld, Maherali et al. 2008). The doxycycline-inducible lentiviral system also proved that doxycycline addition to human-iPS-derived differentiated cells resulted in secondary h-iPS cell lines with a 100 fold increased frequency, with a more rapid induction in keratinocytes when compared to fibroblasts (Maherali, Ahfeldt et al. 2008). To address the issue of lentiviral integration, Okita and colleagues used an adenovirus mediated transduction protocol and were initially unable to generate iPS cells using the OSKM factors in 4 separate adenoviral vectors. However, iPS cell lines were successfully generated in mouse embryonic fibroblasts through repeated plasmid transfections of two different vectors, one containing the transcription factors Oct4, SOX2 and Klf4 in a single construct and the other containing C-Myc alone (Okita, Ichisaka et al. 2007). iPS generation through adenoviral transduction was also shown to be successful using a varied source of starting cell types such as tail tip fibroblasts, foetal liver cells and hepatocytes (Stadtfeld, Nagaya et al. 2008). Adenoviral iPS induction protocols and plasmid transfections are transient procedures and do limit exogene integration. However, they are associated with very low transduction efficiencies, a technical hindrance addressed by PiggyBac transposition re-programming (Robinton and Daley 2012). The piggyBac, host-

independent transposon technology catalyses the insertion of doxycycline-inducible pluripotent exogenes through the transposase enzyme. This phenomenon is transient and is benefitted by the fact that the piggyBac transposons possess natural ability to be excised once the pluripotent lines have been generated (Woltjen, Michael et al. 2009). Another excisable system saw use of a floxed lentiviral transgene construct that could be excised through Cre-recombinase once the stable pluripotent cell lines expressing endogenous embryonic stem cell genes were established (Somers, Jean et al. 2010). Using this strategy several normal and disease-specific (cystic fibrosis and α 1-AT deficiency as well as from individuals suffering from scleroderma and sickle cell disease) iPS lines were established through a single lentiviral stem-transcript cassette that encoded all the four pluripotent transcripts (Oct4, SOX2, Klf4 and C-Myc). Both the piggyBac transposon and the lentiviral floxed stem cell cassette resulted in iPS induction with much improved efficiencies (from 0.001% in adenoviral systems to 0.1-1% in both the excisable systems) as well as resulting in lines that are devoid of any genomic integration (Somers, Jean et al. 2010, Robinton and Daley 2012). However, a common problem with both the excisable systems is that the technique involves the industrious process of screening of the excised lines to confirm transgene removal (Woltjen, Michael et al. 2009, Somers, Jean et al. 2010, Robinton and Daley 2012). Yet another approach to iPS generation involves using viral-free vectors such as small molecules (Kim, Kim et al. 2009), protein particles (Zhou, Wu et al. 2009), synthetic mRNA particles (Warren, Manos et al. 2010) and mature microRNA particles (Miyoshi, Ishii et al. 2011). Proteins such as cell-penetrating peptides provide direct delivery of re-programming factors to target fibroblasts to generate stable iPS lines and this technique obviates the use of genome-integrating and/or mutagenic materials (Kim, Kim et al. 2009). Another study generated iPS lines from mouse embryonic fibroblasts by using recombinant cell-penetrating proteins; however the protein-based delivery techniques have been associated with low

efficiency and requirements for generation of large quantities of pure proteins (Zhou, Wu et al. 2009, Robinton and Daley 2012). Synthetic mRNA molecules can also be used to re-programme differentiated cells to RNA-iPS cells. The very same methodology can again be used to differentiate the RNA-iPS cells into desired lineage cell types (Warren, Manos et al. 2010). To summarise, the traditional methods for iPS generation are DNA-based (retroviral (Takahashi and Yamanaka 2006, Takahashi, Tanabe et al. 2007, Huangfu, Osafune et al. 2008, Lowry, Richter et al. 2008), lentiviral (Yu, Vodyanik et al. 2007, Sommer, Stadtfeld et al. 2009, Somers, Jean et al. 2010, Anokye-Danso, Trivedi et al. 2011) , piggyBac (Woltjen, Michael et al. 2009) as well as plasmid based induction methods (Okita, Nakagawa et al. 2008)) ; DNA-free re-programming methods are mostly dependent on RNA-based delivery systems and include induction through cell-penetrating proteins (Kim, Kim et al. 2009, Zhou, Wu et al. 2009), synthetic mRNAs (Warren, Manos et al. 2010) , mature microRNA (Miyoshi, Ishii et al. 2011) and through the RNA-free Sendai virus (Fusaki, Ban et al. 2009).

3.5. Mechanism of lineage re-programming

Early studies proved the process of re-programming to be a stochastic process whereby all the cells were amenable to being re-programmed to pluripotency on constant growth and transcription factor expression (Hanna, Saha et al. 2009). The other model demonstrating the re-programming mechanism comprised the elite model where only a small number of cells can be re-programmed either partially or to the fully re-programmed state, this can again either refer to the induced elite model or the pre-determined elite state (Yamanaka 2009). Evidence for the pre-determined elite state comes from studies that used un-differentiated stem-cells and showed that cells that were not yet lineage restricted were more amenable to the re-programming process. Specific examples include the study on nuclear re-programming where it was observed that higher re-programming efficiencies were obtained from the nuclei of immature cells (such as neural-stem cells and embryonic stem cells) than from terminally differentiated nuclei (such as from lymphocytes differentiated from neuronal progenitor cells) (Blelloch, Wang et al. 2006). Multi-lineage differentiating stress-enduring or 'Muse' cells were identified as a population of SSEA3+/CD105+ stress-resistant cells that can self-renew, grow in colonies that show a pluripotent stem cell gene expression profile, represent the three germ layers *in vitro* and *in vivo* these homed into damaged skin, muscle and liver cells to ultimately differentiate into cytokeratin 14+ (ectoderm), dystrophin+ (mesoderm) and albumin+ (hepatic-endoderm) cells respectively. In addition, these Muse cells were primarily isolated from cultured skin fibroblasts, bone marrow stromal cells or bone marrow aspirates (Kuroda, Kitada et al. 2010). Muse cells are more amenable to re-programming suggesting that human fibroblast cells contain a proportion of adult stem cells that show greater propensity towards being induced to a fully re-programmed state.I It has also been seen that iPS-like cells generated from the non-Muse cells were not fully re-programmed (Wakao,

Kitada et al. 2011). The pre-determined elite model of re-programming also raises the question of whether during re-programming there is an actual re-setting of a terminally differentiated cell's phenotypic and genotypic constitution to an embryonic state or whether the process simply involves mere de-differentiation of an already immature cell towards greater lineage-plasticity (Yamanaka 2009).

However, it has been recently shown that re-programming in mouse comprises an early stochastic and a late hierarchical stage. The early stochastic stage comprises a marked variation between individual cells with respect to their gene expression profiles with Esrrb, Utf1, Lin28 and Dppa2 acting as markers that reliably predict cells most prone to the re-programming process. This is followed by activation of Sox2 that initiates a later deterministic hierarchical phase marked by the up-regulation of factors not including Oct2, Sox2, Klf4, Nanog but involving a cohort of other genes , including Esrrb, Sall4 and Lin28. These downstream factors are sufficient to activate the pluripotent circuitry and result in stable iPS lines (Buganim, Faddah et al. 2012).

Transcription factor-induced re-programming has been shown to involve epigenetic modifications whereby the epigenome of a differentiated somatic cell is altered to that of an embryonic stem cell-like state, X-chromosome reactivation was seen in female iPS lines which again showed random X-inactivation upon differentiation (Maherali, Sridharan et al. 2007). H3K4 trimethylation is associated with active transcription of genes (Bernstein, Kamal et al. 2005, Kim, Barrera et al. 2005) while H3K27 trimethylation is associated with the silencing of genes (Boyer, Plath et al. 2006, Lee, Jenner et al. 2006). Genome-wide analysis of these two key histone elements confirmed that successful iPS induction involves the epigenetics of the target cells to be reset to an immature state (Maherali, Sridharan et al.

2007). However, partially re-programmed cells show reactivation of only certain embryonic transcripts, incomplete suppression of lineage-specifying transcripts (which can be addressed by the use of RNA inhibitors) and DNA hypermethylation at pluripotency-loci, suggesting that DNA methyltransferase inhibitors can be used to facilitate the re-programming process (Mikkelsen, Hanna et al. 2008). Furthermore, in partially re-programmed cells genes co-occupied by C-Myc and any of SOX2, Oct4 and Klf4 factors show pluripotent cell binding pattern and the relevant expression profile. However, genes that are co-bound by Oct4, SOX2 and Klf4 in embryonic stem cells and those that encode pluripotency regulators lack binding and are not transcriptionally activated in incomplete re-programming. It has been suggested that exogenous c-Myc acts before activation of the pluripotency determinants (Sridharan, Tchieu et al. 2009). Upon retroviral transduction, increased expression of SSEA1 and down-regulation of Thy1 allowed identification of cells during the different phases of re-programming.Whilst Thy1 down-regulation occurs in most of the cells, activation of SSEA1 and other pluripotency regulators occurs at very low frequency suggesting the role of epigenetic barriers in the iPS induction protocol (Sridharan, Tchieu et al. 2009).

The re-programming process in mice is marked by three phases – initiation, maturation and stabilisation with the initiation phase being chiefly dominated by a mesenchymal-epithelial transition (MET) phase (Samavarchi-Tehrani, Golipour et al. 2010).The initiation phase is a reversible phase marked by an up-regulation of epithelial genes such as Cdh1, Epcam, Crb3 and Ocln and a down-regulation in mesenchymal genes such as Snail, Slug, ZEB1 and ZEB2 (Li, Liang et al. 2010, Samavarchi-Tehrani, Golipour et al. 2010). The MET phase in mice appears before SSEA-1 can be detected and is marked by a change in morphology of the cells from elongated spindle-shaped fibroblasts to rounded cobble-stone shaped clusters of cells that form well-defined intercellular junctions and express increased cytokeratin from day 5

post-transduction. The induction of MET in mouse fibroblasts was more rapid with OSKM than with Oct4, SOX2 and Klf4 combination (Li, Liang et al. 2010). Oct4, SOX2, Klf4 and c-Myc also down-regulate miRNAs, miR-155 and miR-10b, which are associated with EMT (Ma, Teruya-Feldstein et al. 2007, Kong, Yang et al. 2008). The same transcription factors are associated with the down-regulation of miR-205 and -429 which bring about a MET by targeting transcription factors, Zeb1, Zeb2 and Sip1 (Gregory, Bert et al. 2008, Park, Gaur et al. 2008, Li, Liang et al. 2010). This transcript change was further reflected by a change in the behaviour of the cells that acquired a marked reduction in invasiveness following MET (Li, Liang et al. 2010). A well-known EMT inducer TGF-β, that partly acts through Snail (Peinado, Quintanilla et al. 2003) has been documented to act as an obstacle to nuclear reprogramming and iPS induction (Maherali and Hochedlinger 2009). As expected, it has also been proved that TGF-β 1, TGF-β 2 and TGF-β 3 all hinder the initiation phase of iPS induction by preventing MET. Activin-A however, which also belongs to the TGF-β family was noted to have no effects on iPS induction (Li, Liang et al. 2010). TGF-β cytokines bind to TGFβR-2 leading to recruitment of TGFβR-1 to the complex , this ligand binding event causes autophosphorylation of the complex (Shi and Massagué 2003) and triggers a signalling cascade that activates Snail.I In this whole process TGFβR-3 acts as a co-receptor (Li, Liang et al. 2010). Yamanaka factors, OSKM brings about a reduction in levels of TGFβR-2 and TGFβR-3, although levels of TGFβR-1 remain unchanged. SOX2 and Oct4 supress TGFβR-3. Oct4 and Klf4 supress TGFβ-3 and c-Myc supresses TGFβ-1 and TGFβR-2. However, it has been seen that c-Myc is indispensable in the complete repression of the TGFβ pathway (Takahashi and Yamanaka 2006, Li, Liang et al. 2010). The initial 8 days of fibroblast re-programming is also marked by a reduction in fibroblast specific genes such as Thy1, following which there is a gradual increase in levels of SSEA1. However, the process of re-programming is not yet irreversible at this stage and very few of the $Thy^{low}SSEA1^{hi}$

cells move on through maturation to the stabilisation phase (Stadtfeld, Maherali et al. 2008, Li, Liang et al. 2010). Reduction in levels of Thy1 is the earliest detectable change during re-programming followed by an up-regulation in levels of SSEA-1 which increases once the cells become independent of the exogene. The transition phase in mouse is marked by re-activation of embryonic transcripts Fbx15, Nanog, Oct4, Sox2, telomerase and also by X-inactivation (Brambrink, Foreman et al. 2008, Stadtfeld, Maherali et al. 2008). Also, fully re-programmed cells are dependent on the ectopic pluripotent transcripts during the first 12 days of iPS induction following which they start producing their own endogenous pluripotent genes and do not rely anymore on the exogenes. The cells now enter the stabilisation phase (Brambrink, Foreman et al. 2008). The 'pre-determined' elite model however has been challenged by the fact that beyond fibroblasts, lineage-committed cells can also be re-programmed to a pluripotent state (Yamanaka 2009). Skin keratinocytes have proved to re-programme with higher efficiencies (Aasen, Raya et al. 2008) and it has also been shown that iPS cells can be derived from several types of adult somatic cells such as hepatocytes (Aoi, Yae et al. 2008, Lee, Seo et al. 2012), gastric mucosa cells (Aoi, Yae et al. 2008), pancreatic β cells (Bar-Nur, Russ et al. 2011) and terminally differentiated B lymphocytes (Hanna, Markoulaki et al. 2008). These models argue more in favour of the stochastic re-programming phenomenon. The induced elite model hypothesises that several genes besides the 4 iPS induction factors need to be induced or silenced through viral integration into the host genome (Yamanaka 2009). However, this very clause defines viral integration to be a critical process for iPS induction which is controversial given that iPS re-programming can be carried out through several non-genome-integrating transduction mechanisms such as through adenovirus (Stadtfeld, Nagaya et al. 2008, Zhou and Freed 2009), plasmids (Okita, Nakagawa et al. 2008), small molecules (Warren, Manos et al. 2010, Chen, Gulbranson et al. 2011) as well as DNA-free RNA-based transcription-factor delivery systems (Fusaki, Ban et

al. 2009, Kim, Kim et al. 2009, Zhou, Wu et al. 2009, Warren, Manos et al. 2010, Miyoshi, Ishii et al. 2011) . Also, it has been shown that epithelial cell and other types of cells from liver, gastric mucosa and skin are less prone to viral integration (Aasen, Raya et al. 2008, Aoi, Yae et al. 2008). These cells did not show any common viral genome-integration sites diminishing kudos for the induced elite models. Furthermore, re-programming techniques that use episomal DNA vectors have seen the episomal DNA to disappear spontaneously over time (Yu, Hu et al. 2009) . Nevertheless, the high efficiency seen with retroviral transduction protocols (Robinton and Daley 2012) would suggest that viral genome integration does contribute towards accelerating the iPS induction process. Insertional mutagenesis does promote iPS induction possibly by affecting several other endogenous genes that ultimately lead to increased proliferation, decreased apoptosis as well as increased iPS induction rates (Hargrove, Kepes et al. 2008, Hawley 2008, Robinton and Daley 2012).

The stochastic iPS induction model states that most cells in the body are susceptible to the re-programming process given that the pluripotency re-programming transcripts collectively re-set the epigenetics of the differentiated cells to an embryonic state (Hanna, Saha et al. 2009). During the process of development, normal cells roll down the slope of differentiation to their destined lineage. However, embryonic stem cells possess a special epigenetic status that prevents them from differentiating through specific epigenetic road-blocks that allow the cells to move from a totipotent to a pluripotent state but obstruct their journey further down to the multipotent and terminally differentiated unipotent states (Hochedlinger and Plath 2009). There are two critical factors for re-programming to be successful –firstly the expression of the four pluripotency determining transcripts must be at a level that is sufficient to move the cells towards the right direction and secondly that the epigenetic road-block is indispensable in confining the cells to the particular pluripotent state even after the expression of the

exogenes are silenced (Hochedlinger and Plath 2009). Both these events are however controlled stochastically as it is impossible to control the expression levels of the pluripotency transcripts as well as to achieve the necessary epigenetic road-block events through the re-programming factors (Hochedlinger and Plath 2009). However, this underscores the role that epigenetic mechanisms such as DNA methylation and histone modifications would play in the whole re-programming process (Hochedlinger and Plath 2009).

The next important rate-limiting step in the re-programming process is the stoichiometry between the different pluripotency transcripts during iPS induction. If the balance between the re-programming factors is not correct then this results in failed re-programming and/or formation of partially re-programmed cells (Carey, Markoulaki et al. 2011). Cells from different organs show varying plasticity with respect to the re-programming process. One of the reasons for this is that different cells carry different levels of endogenous pluripotency transcripts. For instance, excess levels of Oct4 and SOX2 are detrimental to the maintainence of pluripotency (Radzisheuskaya, Le Bin Chia et al. 2013). Furthermore, it has been observed in neural stem cells that already contain higher levels of endogenous SOX2, that iPS induction is a lot more efficient without the addition of SOX2 exogene transcript to the re-programming cocktail (Eminli, Utikal et al. 2008, Kim, Zaehres et al. 2008). Improper balance between the different pluripotent factors leads to incomplete re-programming and senescence/apoptosis (Carey, Markoulaki et al. 2011). Another factor that influences the re-programming process is the integration pattern following re-programming. This, along with the amount, stoichiometry, continuity and silencing of the exogenes can be controlled by the gene delivery system (Yamanaka 2009). Also a favourable integration pattern can be attained by using cells that would already contain a re-programming competent integration pattern

(Maherali, Ahfeldt et al. 2008, Wernig, Lengner et al. 2008). This was demonstrated by the observation that fibroblast cells obtained through differentiation of iPS cells were a lot more amenable to enable ‘secondary iPS’ cell line generation (Wernig, Lengner et al. 2008). Furthermore, the piggyBac system of iPS generation has shown the efficiency of re-programming to be approximately 20%, further underscoring the relevance of attaining appropriate transgene integration for successful pluripotency re-programming (Woltjen, Michael et al. 2009).

The various mechanistic steps in iPS-reprogramming is summarised in Figure 1.4

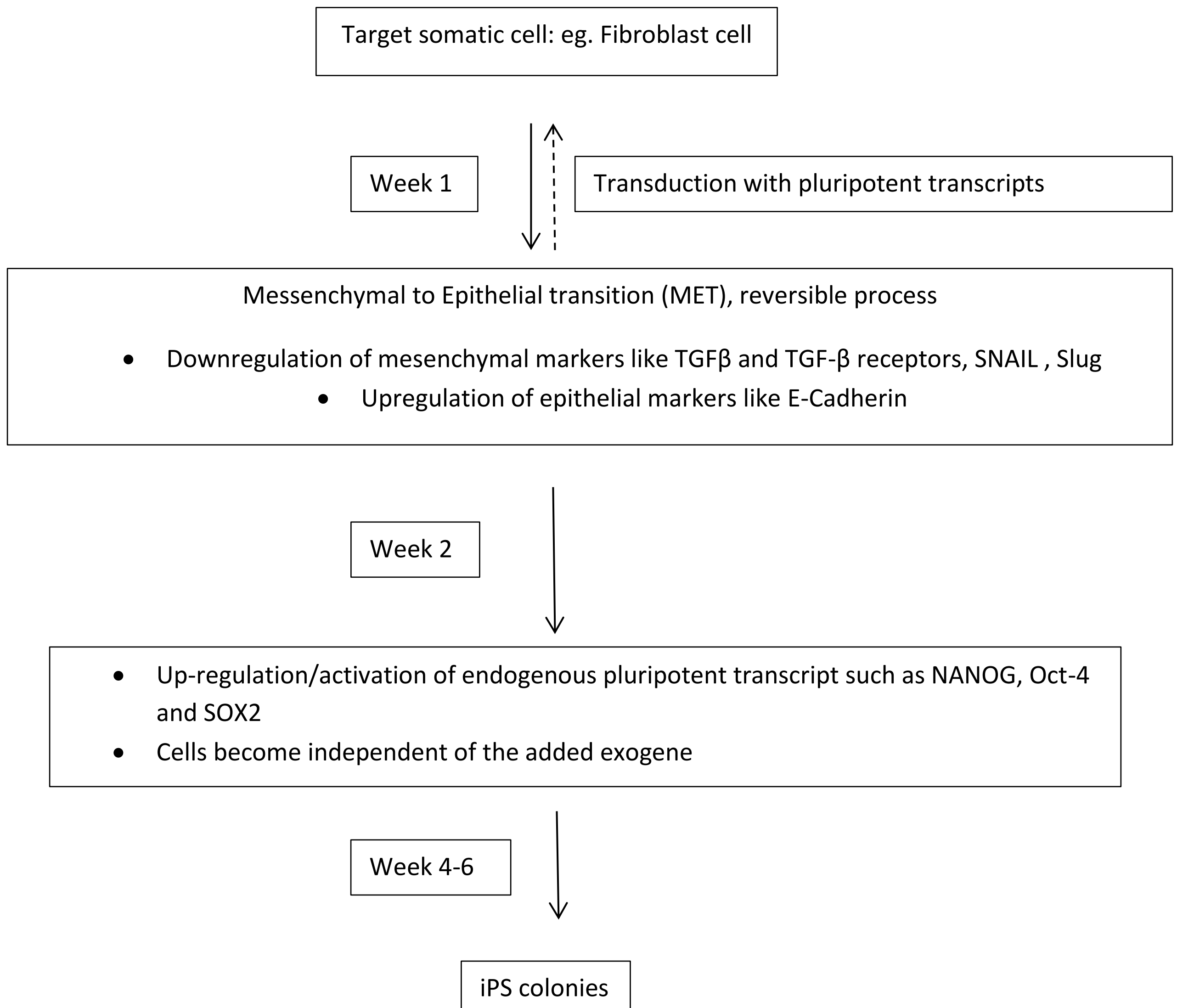


Figure.1.4. Flowchart summarising the mechanism of iPS reprogramming

3.6.Epigenetics of iPS re-programming

Based on their epigenetic potential, stem cells have been assigned different names that reflect their differentiation abilities – each specific stem cell population (eg: totipotent cells, pluripotent cells, multipotent cells and unipotent cells) will have a distinct epigenetic pattern that will determine its lineage plasticity (Figure 1.4) (Yamanaka 2009). It is therefore absolutely essential that when moving from a differentiated epigenetic state to the embryonic state, the initial de-differentiated status is inactivated (Stadtfeld, Maherali et al. 2008, Hochedlinger and Plath 2009). Lineage specific genes include (although are not limited to) Gata6, SOX9 and Pax7 and failure to completely inactivate these genes results in partial re-programming (Mikkelsen, Hanna et al. 2008). Knockdown of these lineage-specific genes results in efficient transition from the partial to the fully re-programmed state. Treating partially re-programmed cells with extra-cellular signal-related kinase (ERK) and glycogen synthase kinase-3 (GSK3)/ Wnt signalling cascades also promotes partially re-programmed cells to attain the fully re-programmed state (Silva, Barrandon et al. 2008, Ying, Wray et al. 2008). Another critical factor is the repression of differentiation-associated HOX cluster of genes by the SET domain protein, Blimp1 for the maintenance of primordial germ cells *in vivo*. This further suggests the importance of silencing differentiation specific genes in attaining the fully re-programmed state (Ohinata, Payer et al. 2005). The fully re-programmed iPS state can be only achieved once the endogenous loci of the relevant pluripotency factors are indefinitely activated through proper DNA methylation. Failing this, the cells would roll down the epigenetic slope back to their initial differentiated state (Yamanaka 2009). The promoter regions of most pluripotency genes are methylated in somatic cells but are hypomethylated in embryonic stem cells. DNA demethylation of these regions is very important for successful iPS re-programming (Imamura, Miura et al. 2006, Mikkelsen, Hanna et al. 2008, Yamanaka 2009). The OSKM/OSLN factors do not have

intrinsic demethylation properties and therefore iPS generation can be enhanced by using demethylation-promoting agents such as 5-azacytidine (Mikkelsen, Hanna et al. 2008). Histone modifications are also important in iPS reprogramming – in all pluripotent cells hsitones H3 and H4 are hyperacetylated in the promoter regions of pluripotency associated genes (Yamanaka 2009). Un-differentiated target cells must undergo critical histone modification from their initial hypoacetylated state to a hyperacetylated state (Imamura, Miura et al. 2006, Yamanaka 2009). This may be brought about by c-Myc which has the ability to recruit histone acetyltransferases to target genes (Knoepfler, Zhang et al. 2006). Valproic acid is also a histone deacetylase inhibitor which has been proven to enhance the efficiency of iPS generation (Huangfu, Osafune et al. 2008).

Another important observation is the fact that silencing of the differentiation-related genes occurs prior to up-regulation of pluripotency genes. This suggests that the differentiation state of the cell does influence its susceptibility to iPS re-programming (Hanna, Markoulaki et al. 2008, Hochedlinger and Plath 2009). One of the reasons why stem cells such as adult stem cells may re-programme with higher efficiency can be explained by the fact that they share transcriptional regulators such as Zinc finger protein X-linked (Zfx) and SOX2 with embryonic stem cells (Galan-Caridad, Harel et al. 2007, Hochedlinger and Plath 2009). Also, neural stem cells and keratinocyte stem cells give rise to cloned mice more readily than do mature fibroblasts, neurons and transit amplifying cells (Aasen, Raya et al. 2008, Eminli, Utikal et al. 2008, Kim, Zaehres et al. 2008). However, it has also been shown that in the haematopoietic system, nuclei of differentiated granulocytes are more competent donor cells for nuclear transfer than haematopoietic stem cells (Inoue, Ogonuki et al. 2006, Sung, Gao et al. 2006). The identity of the starting cell that gives rise to the iPS cell as yet remains ambiguous. To address this issue, two sets of experiments were cperformed in two different systems - in the pancreas and in the haematopoietic systems (Hochedlinger and Plath 2009).

Hanna et al. attempted to re-programme B-lymphocytes into iPS cells, B cells carry differentiation-associated DNA rearrangements that identify their differentiation state. It was seen that expression of the OSKM exogenes alone was ineffective in re-programming the cells to an iPS state (Hanna, Markoulaki et al. 2008). This was even true for 'secondary iPS' lines that would have potentially contained at least competent integration sites for complete re-programming (Wernig, Lengner et al. 2008) . The only way iPS lines were generated was through either additional overexpression of Cebpα or by knock-down of Pax5 as an addition to OSKM transduction (Hanna, Markoulaki et al. 2008). Ectopic expression of Cebpα leads to downregulation of Pax5 and reduced levels of Pax5 have been associated with multipotency in B cells (Nutt, Heavey et al. 1999). Pro-B cells were re-programmed with higher efficiency and this study showed that even in the haematopoietic system the differentiation state influences iPS re-programming (Hanna, Markoulaki et al. 2008). In a different study that used terminally differentiated pancreatic β cells as target cells for re-programming, successful re-programming of the differentiated cells using 4 pluripotency transcripts was achieved. The authors concluded that de-differentiated cells are not selectively targeted during the re-programming process (Stadtfeld, Brennand et al. 2008). This latter result is consistent with Sung's work where they asserted that their study unambiguously confirmed that contrary to conventional belief the differentiation state of a cell does not play any role in cellular re-programming (Sung, Gao et al. 2006). However, pancreatic β cells belong to the endodermal lineage and it has been suggested that cells of the endodermal lineage (including cells such as liver and gastric mucosa cells) are more susceptible to pluripotency re-programming than cells like fibroblasts and haematopoietic cells which are of mesodermal origin (Hochedlinger and Plath 2009). Also, systems with definitive cellular hierarchy are more difficult to re-programme as the cellshave to be pushed

through several tiers of the lineage before they can reach pluripotency, as opposed to cells that multiply through self-duplication (Hochedlinger and Plath 2009).

During transcriptional activation transcription factors bind to the distal enhancer and promoter elements which lead to recruitment of co-activators that facilitate binding of the general transcriptional machinery and assembly of RNA polymerase-II-containing pre-initiation complex (PIC) at the core promoter (Hochedlinger and Plath 2009, Yamanaka 2009, Robinton and Daley 2012). Packaging of DNA into nucleosomes also influences transcriptional events such as transcription factor binding, PIC formation as well as transcriptional elongation. Transcription factor binding is further affected by chromatin structure such as DNA methylation, histone modifications and variants as well as ATP-dependent chromatin remodelling (Hochedlinger and Plath 2009). In differentiated cells, the loci for pluripotency remains in an unfavourable conformation for transcription factor binding (Hochedlinger and Plath 2009). However, it is believed that the reprogramming factors (most specifically OSKM) bind closed chromatin and induce favourable conformation changes before transcriptional changes are in place (Sridharan, Tchieu et al. 2009). Reprogramming is primarily a multistep process requiring cell division whereby target cells go through a number of transitional states to finally reach the pluripotent state (Samavarchi-Tehrani, Golipour et al. 2010, Buganim, Faddah et al. 2013). Each transition is marked by a distinct gene-expression profile (Stadtfeld, Maherali et al. 2008, Robinton and Daley 2012). When dealing with heterogeneous cell populations it has been shown that the efficiency of re-programing can be improved by selecting for cells that would be more poised to reach the 'ground state' (Robinton and Daley 2012). This can be attained by selecting for cells expressing certain cell-surface marker-profiles and by selectively advancing these cells through to the final maturation and stabilisation phases (Robinton and Daley 2012). Markers

that become down-regulated include tissue-specific or differentiation-specific markers such as Thy1 (for fibroblasts). In the study herein, it has been shown that the prostatic marker, androgen receptor becomes down-regulated during the course of prostate-specific iPSC generation (Moad, Pal et al. , Stadtfeld, Maherali et al. 2008).

The initial stages of iPS re-programming tend to be reversible and a majority of cells that pass through the initial transition tend to regress back through differentiation. A very small proportion of target cells attain the stable ‘pluripotent state’(Li, Liang et al. 2010). The fate of a cell through to the pluripotent stages is marked by distinct epigenetic changes and a characteristic gene expression profile. In murine cells, SSEA1 positive cells tend to be incompletely re-programmed in that they often tend to regress back to a Thy1 positive phenotype (Stadtfeld, Maherali et al. 2008). In human cells it has been reported that alkaline phosphatase, Gdf3, hTERT and NANOG do not identify with the fully re-programmed state (Chan, Ratanasirintrawoot et al. 2009). The initial stage of iPS induction in fibroblast cells is marked by a mesenchymal-epithelial transition that is identified by a down-regulation in mesenchymal transcripts such as SNAI1, SNAI2, Zeb1 and Zeb2 and an up-regulation in epithelial markers such as E-Cadherin, EpCam and Ocln (Li, Liang et al. 2010, Samavarchi-Tehrani, Golipour et al. 2010). These changes are accompanied by morphological alterations such as reduction in cell size, increase in nuclear-cytoplasmic ratio, increased proliferation and formation of cell clusters (Moad, Pal et al.). TGF-β is also known to play an important role in the initiation phase whereby TGF-β inhibition promotes the initiation phase in iPS induction by replacing SOX2 and cMyc. E-616452 (RepSOX or replacer of SOX2), a small molecule inhibitor of TGFbR1/alk5 kinase inhibitor has been shown to promote iPS induction by replacing SOX2 through NANOG induction (Ichida, Blanchard et al. 2009, Maherali and Hochedlinger 2009). This MET (mesenchymal-epithelial transition) phase is then followed

by a gradual up-regulation in embryonic stem cell genes. In mice, NANOG and Sall4 are up-regulated at a later stage while Utf1 and endogenous SOX2 are induced right at the end of the iPS re-programming process (Samavarchi-Tehrani, Golipour et al. 2010, Buganim, Faddah et al. 2013). In humans, the first step of iPS re-programming involves down-regulation of the differentiation marker CD13 on day 2 of the induction protocol. This is followed by an up-regulation in SSEA-4 on day 2 to day 6 and up-regulation of Tra-1-60 and NANOG on day 6-13 (Chan, Ratanasirintrawoot et al. 2009) . In human cells it has been noted that an up-regulation of nuclear NANOG as well as SSEA4 does not denote a completely re-programmed state, in fact the majority of SSEA4^{+}Tra-1-60^{+} cells tend to either undergo senescence or apoptosis or remain in an incompletely re-programmed state. It has also been shown that pro-viral silencing as well a Hoerscht^{dim} cell phenotype identifies more strongly with complete re-programming. This is in addition to the cells up-regulating three other pluripotency markers – Tra-1-60, DNMT3b and REX1 (Chan, Ratanasirintrawoot et al. 2009).

Pro-viral silencing is an important step in the re-programming process. Once cells are completely re-programmed they start producing their own endogenous pluripotency transcripts; this usually occurs at around day 14 of re-programming when exogene dependence is no longer important, and exogene silencing is critical at this stage (Chan, Ratanasirintrawoot et al. 2009). Another crucial step in iPS re-programming to the fully embryonic ground state is reactivation of the silent X chromosome. This occurs late in the iPS induction process and is identifiable with fully re-programmed cells (Maherali, Sridharan et al. 2007, Stadtfeld, Maherali et al. 2008).

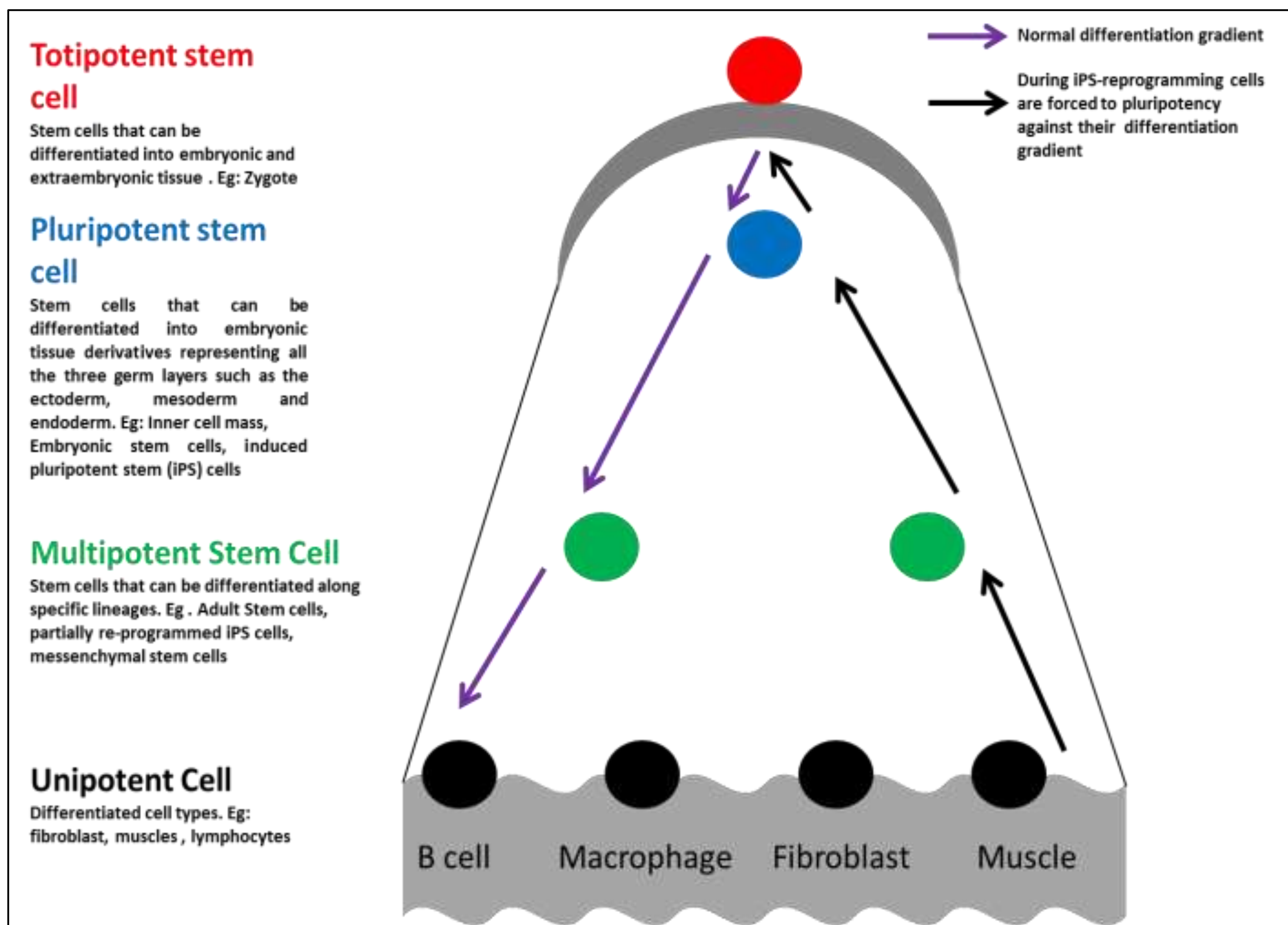


Figure 1.4. The development potential of stem cells at different stages of development. The natural tendency for cells is to flow downwards along their differentiation gradient. Hence, it is very difficult to arrest cells when pushed up against their natural differentiation gradient. This can be achieved only once some sort of epigenetic road-block has been applied that would retain the re-programmed cells in their de-differentiation state. Figure adapted from (Hochedlinger and Plath 2009) .

3.6.Applications of iPS technology

The iPS technology has found wide-spread applications in both research and clinics. In research, it has contributed significantly to the understanding of developmental biology most specifically with regards to transcriptional and epigenetic events that regulate development in human and murine-based models. It has greatly expanded our knowledge in analysing certain canonical signalling pathways, for example the Wnt pathway (Menendez, Yatskievych et al. 2011), TGF-β signalling and epithelial-mesenchymal-transition signalling pathways, p53 signalling, role of Oct4, SOX2 and NANOG as the core pluripotency network, activin/nodal signalling (Vallier, Touboul et al. 2009), role of SMAD signalling in directing differentiation towards a neural route (Chambers, Fasano et al. 2009, Menendez, Yatskievych et al. 2011) as well as elaborating the role of several transcriptional factors (such as Oct4, NANOG, Lin28, c-Myc, Klf4, Fbx15, EpCam, E-Cadherin and hTERT to name a few. The iPS technology also serves as a model to evaluate epigenetic events regulating normal development. For example, it suggests that all somatic cells in principle retain considerable developmental potential. The inherent ability of a cell to be returned to a primitive lineage-unrestricted state is further dependent upon several criteria such as its inherent plasticity, its molecular make-up with respect to endogenous transcripts as well as its *in vitro* robustness against apoptosis and senescence. Re-programming somatic cells to an embryonic stem cell state reveals critical information with regards to DNA methylation patterns, chromatin remodelling in cellular development and lineage plasticity. Also, it has now been shown that most somatic cells retain developmental potential not only to a generic embryonic phenotype but intercellular transitions are also possible through direct trans-differentiation-based reprogramming. This would hint that perhaps there is an alternative pathway in the

developmental hierarchy that can lead a differentiated cell type to a very different cell type without having to first go through embryonic lineage plasticity (Ieda, Fu et al. 2010). However, recently fibroblast cells have been directly re-programmed to an angioblast-like stage by first pursuing a state of intermediate plasticity when the cells have been transduced by the traditional OSKM factors but have absent pluripotent marker expression and are identifiable with a mesodermal progenitor-like state. These $CD34^+$ cells are then able to directly give rise to functional endothelial and smooth muscle cells (Kurian, Sancho-Martinez et al. 2013). There are several papers demonstrating direct conversion of fibroblasts cells into cortical excitatory neurons through Ascl1, Brn2 and Myt1l (Vierbuchen, Ostermeier et al. 2010) , direct conversion of pancreatic exocrine cells into insulin-producing endocrine cells (Zhou, Brown et al. 2008) and conversion of fibroblast cells to macrophage cells by means of PU.1 and C/EBPα/β (Feng, Desbordes et al. 2008). Another study demonstrated that by means of three combinations of two transcription factors, namely Hnf4α and either of FoxA1, FoxA2 or FoxA3 mouse embryonic and adult fibroblasts can be directly converted into hepatocyte-like cells (Sekiya and Suzuki 2011). Also, cardiac as well as dermal fibroblast cells can be directly trans-differentiated into functional cardiomyocytes by a combination of merely three transcriptional factors – Gata4, Mef2c and Tbx5 (Ieda, Fu et al. 2010). A very recent development to the lineage-re-programming technology has been *in vivo* re-programming of cells, the re-programming of non-cardiomyocytes into functional cardiomyocytes *in vivo* (Hansson and Chien 2012, Song, Nam et al. 2012) with one study demonstrating this through only three transcription factors GMT or Gata4, Mef2c and tbx5 (Qian, Huang et al. 2012).

A critical application of the iPS technology has been in the field of clinical and translational research where these cells have been used to model several disease states. Disease-specific

iPS models potentially provide information on the natural history and progression of diseases (Robinton and Daley 2012). They reveal a substantial amount of information on the genetic and signalling aberrations instrumental in pathogenesis of disease states and thereby provide *in vitro* models for drug modelling as well as drug screening (Park, Arora et al. 2008, Somers, Jean et al. 2010, Robinton and Daley 2012). They further serve as tools on which therapeutic efficacy as well as potential toxicities can be evaluated in the first instance. These cells also serve as suitable models to test potential gene therapies since genetically faulted cells can be re-programmed, the gene defect can be corrected and the modified cells can then be differentiated to study molecular effects of gene therapy. Figure 1.5 describes the various disease-specific iPS models. These cells also have immense value in regenerative medicine where they can be used for tissue regeneration in disease-states such as organ failure as well as tissue reconstruction following injury and following surgical resection in malignancies (Soldner, Hockemeyer et al. 2009) .

System	Disease	Molecular defect of donor cell	Cell type differentiated from iPS cell
Neurological Disease	Amyotrophic lateral saclerosis	Heterozygous Leu144Phe mutation in SOD1	• Motor neurons • Glial cells
	Spinal Muscular Atrophy	Mutations in SMN1	• Neurons • Astrocytes • Mature motor neurons
	Parkinson's Disease	Mutations in • LRRK2 • SNCA	Dopaminergic neurons
	Huntingtons disease	72 CAG repeats in the Huntington gene	None
	Down's syndrome	Trisomy 21	Teratoma with tissue from each of the three germ layers
	Schizophrenia	Complex trait	Neurons
Haematological Disease	Fanconi's Anaemia	FAA and FAD2 corrected	Haematopoietic cells
	Sickle Cell Anaemia	Homozygous HbS mutation	None
	B-thalassaemia	Homozygous deletion in the β-globin gene	
	Polycythemia Vera	Heterozygous Val617Phe	Haematopoietic

		mutation in JAK2	progenitors ($CD34^+CD35^+$)
Metabolic Disease	Type I diabetes	Multifactorial, unknown	β-Cell-like cells (express somatostatin, glucagon and insulin; glucose-responsive)
	Gaucher's disease, type III	Mutation in GBA	None
	A1-antitrypsin deficiency	Homozygous mutation in the α1-antitrypsin gene	Hepatocyte-like cells (foetal)
	Type1 Long QT syndrome	Dominant mutation in KCNQ1	Cardiomyocytes
Cardiovascular Disease	Type II Long QT syndroma	Dominant mutation in KCNH2	Cardiomyocytes
	SCID or leaky SCID	Mutation in RAG1	None
Primary Immunodefficiency	Herpes Simplex Encephalitis	Mutation in STAT1 or TLR3	Mature cell types of CNS
	Duchenne musculodystrophy	Deletion in dystrophin gene	None

Other category	Becker Muscular Dystrophy	Unidentified mutation in dystrophin	None
	Cystic fibrosis	Homozygous deletion in CFTR	None
	Friedreich's Ataxia	Trinucleotide GAA repeat expansion in FXN	• Sensory neurons • Peripheral neurons cardiomyocytes
	Retinitis Pigmentosa	Mutations in • RP9 • RP1 • PRPH2 OR RHO	• Retinal progenitors • Photoreceptor precursors • Retinal-pigment epithelial cells • Rod photoreceptor cells
	Scleroderma	• Unknown	• None
	Prostate	Prostate cancer associated fibroblasts	None

Figure 1.5. Suumary of the different disease-specific iPS models. iPS cells have the ability to arguably retain some disease-specific characteristics during the course of reprogramming. This feature of the technology finds use in creating *in vitro* as well as *in vivo* models for

various disease pathogenesis. These models also have clinical relevance as they highlight the possibility of disease genotype correction through gene therapy. Table and date adapted from (Robinton and Daley 2012, Vêncio, Nelson et al. 2012).

3.7.Tissue-specificity in iPS re-programming

Several studies have documented that immature cell types with increase lineage plasticity are easier to re-programme than their more differentiated counterparts (Eminli, Utikal et al. 2008). It has also been widely suggested that during the course of re-programming iPS cells retain the memory of their original tissue type of origin or in other words the relevant somatic epigenetic memory remains intact in very low-passage iPS cells (Kim, Doi et al. 2010, Polo, Liu et al. 2010). There is evidence to suggest that during the course of re-programming adult mouse cells from various somatic sources retain the DNA methylation changes characteristic of the parental tissue of origin and this in fact makes the derived iPS cells more prone to differentiate back to the original cell of origin (Moad, Pal et al. , Kim, Doi et al. 2010). In a study that used distinct cell types from genetically matched individuals it was concluded that iPS cells from the different cell types are transcriptionally distinct, demonstrate distinguishable epigenetic patterns and show varied predelictions towards differentiation. These molecular properties, transcriptional and epigenetic patterns and functional characteristics however get erased with higher passages (Polo, Liu et al. 2010). However, the differentiation and methylation patterns of pluripotent cells derived by means of nuclear transfer have been noted to be closer to embryonic stem cells than iPS cells. This epigenetic memory can nevertheless be erased and modified through treatment of iPS cells with chromatin-modifying drugs (Onder, Kara et al. 2012). It has also been seen that embryonic tissues are more easily re-programmable to an embryonic stem cell state than cells that are more lineage committed. Cells from aged donors have higher levels of Ink4/Arf (Li, Collado et al. 2009). The Ink4/Arf is a tumour suppressor locus that encodes $p16^{INK4a}$, $p15^{INK4b}$ and

Arf and these three molecules are expressed by differentiated cells and are potent inhibitors of cellular proliferation and are critically regulated by *in vitro* culture conditions as well as being up-regulated in response to abnormal mitogenic signals (Li, Collado et al. 2009). The INK4/Arf locus is up-regulated in ageing organisms (Krishnamurthy, Torrice et al. 2004) and acts as an impediment to iPS induction in aged cells. The re-programming efficiency in these circumstances can be improved by inhibiting the locus with the relevant shRNA (Li, Collado et al. 2009). Incidentally, it has also been noted that senescent cells are impossible to re-programme with the traditional 4 factor re-programming cocktail.I Instead, a cocktail of six factors (Oct4, SOX2, Klf4, c-Myc, NANOG, Lin28) is essential in re-programming these aberrant cell types (Lapasset, Milhavet et al. 2011).

Heterogeneity in iPS induction is also well-noted based on the tissue of origin. For example, keratinocytes re-programme with faster kinetics than fibroblasts (Maherali, Ahfeldt et al. 2008). It has also been noted that stomach and liver cells when re-programmed to iPS cells show lower pro-viral integration thereby demonstrating that these cells need lower levels of pluripotency transcripts for re-programming purposes (Aoi, Yae et al. 2008). Another study that used mouse cells noted a variation in teratoma forming abilities of secondary neurospheres that were derived from varying tissue sources (Miura, Okada et al. 2009, Kim, Doi et al. 2010). Neurospheres from tail tip fibroblasts showed highest propensity towards teratoma formation, whereas iPS derived from MEF cells and gastric epithelial cells were least inclined towards teratoma generation while hepatocyte iPS cells showed an intermediate tendency towards teratoma generation (Miura, Okada et al. 2009). It was also observed that embryonic fibroblast derived neurospheres behaved similarly to embryonic stem cells in forming teratomas (Miura, Okada et al. 2009).

One of the explanations behind the persistence of such an epigenetic memory has been the fact that demethylation is inefficient and slow in factor-based re-programming. This persistent, tissue-based methylation signature is believed to impart iPS cells with the epigenetic imprint characteristic of the cell of origin (Kim, Doi et al. 2010). A study carried out exclusively on hepatocytes, saw iPS induction in three different lineage states – hepatoblast derived iPS cells, adult hepatocyte derived iPS cells and embryonic fibroblasts (Lee, Seo et al. 2012). It was observed that at low passages that hepatocyte-derived iPS cells showed a transcriptional pattern that was very similar to embryonic stem cells but nevertheless was also characteristic of the respective parental lineage state (Lee, Seo et al. 2012). This hierarchical-specific hepatocyte memory may have been potentially responsible for the tendency of these low-passage lineage-specific iPS cells to differentiate into the parental cell type with greater propensity than the MEF-iPS cells (Lee, Seo et al. 2012). The hepatocyte-specific-transcriptional imprint does however gets erased in iPS cells at higher passages when they apparently seem to lose their lineage fidelity towards differentiation (Polo, Liu et al. 2010, Lee, Seo et al. 2012). It has been further suggested that variation between the different types of iPS cells towards hepatic differentiation can be modulated by donor differences where the genetic make-up of the individual patient can be more influential than the specific cell type and route of re-programming (Kajiwara, Aoi et al. 2012).

Based on previous literature, it was decided that prostate development could potentially be modelled through a prostate-derived iPS model. Genetic changes during prostatic development reflect the aberrant signalling mechanisms that result in prostate cancer. There are no existing models that describe the genetic landmarks of prostatic differentiation and the cellular hierarchies in the human prostate remain as yet open to conjecture. Currently, stem cell models that represent prostatic development are confined to the murine prostate and there

are significant deviations of the mouse prostate from the human prostate, especially given mice never develop prostate cancer. Human stem cell models are derived through stem cell enrichments by means of certain putative stem cell markers. These cells are sparse, not sustainable in culture, laborious to grow and the properties of the resultant cells heavily vary between patients, quality of primary tissue as well as *in vitro* culture techniques. Furthermore, these cells are subject to *in vitro* genetic drifts and prove difficult to disentangle, based on their ambiguity with respect to stem markers. Although there are several stem cell markers that identify stem cell populations, this process is not deterministic.

This thesis presents the establishment of a prostatic iPS cell (iPSC) model that de-differentiates prostate stroma into an embryonic phenotype through a mesenchymal-epithelial transition state. These cells have been derived through a lentiviral transduction protocol that involved the use of 4 different pluripotent transcripts in a single polycistronic construct. The cDNA was further 'Floxed' by LoxP sites to ensure deletion of the viral genome once the cells attained their 'ground-state'. Following transduction, the first 7 days of the re-programming process was marked by a mesenchymal-epithelial transition phase. The cells were then subjected to an optimized protocol involving the use of pluripotent-cell as well as feeder cell-conditioned media. A total of 14 different cell lines have been generated that represent de-differentiation of the human prostate to an embryonic state. The resultant cells have been substantiated with a diploid 46 XY karyotype and their credibility were further corroborated by means of 'paternity' tests showing an identical DNA identity match between parent and the resultant 'Pros-iPSC' cells for a panel of microsatellite markers, including sex marker Amelogenin.

All the Pros-iPS cells expressed embryonic stem cell characteristics. The cells were checked for endogenous stem transcript expression and expression of these transcripts was confirmed to be at a similar level or higher than that of human embryonic stem cells. Next, these cells were differentiated through conventional differentiation protocols *in vitro* to embryoid bodies which were further differentiated to give cells representing all the 3 germ layers. When injected in mice, Pros-iPS cells formed teratomas confirming their ability to differentiate into ectoderm, mesoderm and endoderm *in vivo*. It was also shown that these prostate-iPSC possess a greater propensity towards prostatic differentiation suggesting that a tissue-specific molecular and epigenetic imprint may be inherent during iPSC re-programming. This would imply that certain canonical pathways in prostate-specific metabolism may well persist during adult life. There may be certain transcriptional and epigenetic patterns that get silenced in adult life, which nevertheless retain the ability to become activated under distinct stimuli and/or niches and aberration of these canonical signals may be detrimental potentially leading to carcinogenesis.

Hypothesis, Aims and objectives:

Hypothesis

Prostate-derived induced pluripotent stem cells (Pro-iPSC) will show a greater predilection and commitment towards lineage-specific differentiation than the conventional skin-derived iPSC (skin-iPSC).

Aims and Objectives

The objectives of this project are:

1. To establish novel iPS cell clones from the prostate (termed Pro-iPSC) and to compare these with skin-iPSC.
2. To validate differentiation and tumourigenic potential of Pro-iPSC and skin-iPSC.
3. To establish a prostate-specific stem cell model that will accurately mirror mechanisms instrumental in prostatic differentiation and carcinogenesis.

Chapter 2.

General Methods

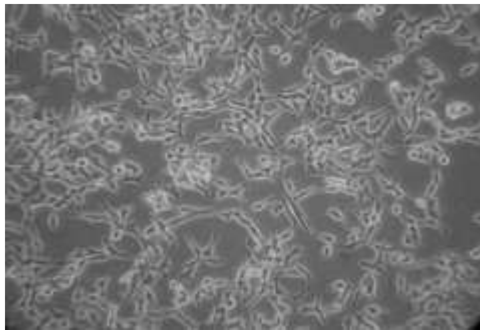
1. Cell biology techniques

1.1. Cell line culture – prostate cancer cell lines

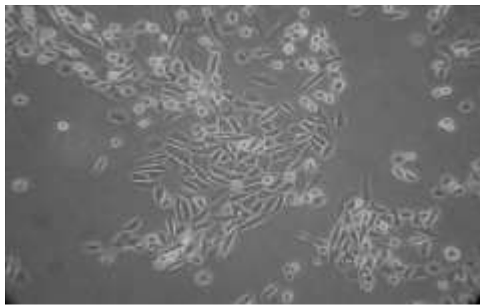
Four different prostate cancer cell lines were used (Figure 2.1) – LNCaP (from lymph node metastasis of prostate cancer); an androgen-independent LNCaP subline (LNCaP-AI); PC3 (derived from prostate cancer metastatic deposits in the vertebrate) and DU145 (derived from brain metastatic lesions in prostate cancer) (van Bokhoven, Varella-Garcia et al. 2003). LNCaP, PC3 and DU145 cell lines were obtained from ATCC (Rockville, MD, USA) and were cultured in 75cm^2 and 150 cm^2 polystyrene tissue culture flasks with canted neck and 0.2 µm vented-caps (430641, 431079, Corning Incorporated, New York, USA). The cells were maintained in RPMI-1640 medium with Hepes Modification (R5886, SIGMA®, Steinheim, Germany) supplemented with 10% foetal bovine serum (FBS) (F7524, Sigma Chemical Co., USA) and 2mM L-Glutamine (G7513, SIGMA®). Androgen-independent LNCaP subline, LNCaP-AI was generated by culturing LNCaP cultures for approximately 20 passages in regular RPMI-1640 medium where regular FBS was substituted with 10% dextran-charcoal-treated steroid-depleted FBS (SH30068.03, HYCLONE, Utah, USA). This cell line represents an *in vitro* model of androgen-independent prostate cancer (Gustavsson, Welen et al. 2005, Wiltshire, Singh et al. 2010). Tissue culture conditions were optimised at 37°C with 100% humidity and 5% CO2. Cells were frozen at relatively early subcultures in culture media supplemented with 5% FBS (F7524, Sigma Chemical Co.) and 5% DMSO (D2650, SIGMA®) to ensure cell line stocks between passages 2-50. Cells were sub-cultured in a split ratio of 1:4 at 75% confluence by means of trypsinisation with 1XTrypsin-EDTA

(T4174, SIGMA®). Cultures were regularly monitored for adherence and bacterial contamination and maintained as mycoplasma negative.

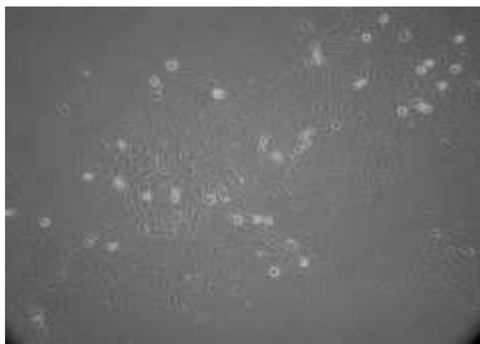
The main purpose for culturing cell lines was to study stem cell and prostate-specific markers in these cell lines, with the ultimate aim to optimise primer sets as well as conditions for real time RT-PCR. LNCaP, DU145 and PC3 are regarded as the three ‘classical’ prostate cancer cell lines. LNCaP cells are known to express androgen receptor as well as multiple androgen regulated genes (eg. PSA) at both the mRNA and protein level. Both PC3 and DU145 serve as negative controls for AR and PSA mRNA expressions (van Bokhoven, Varella-Garcia et al. 2003).



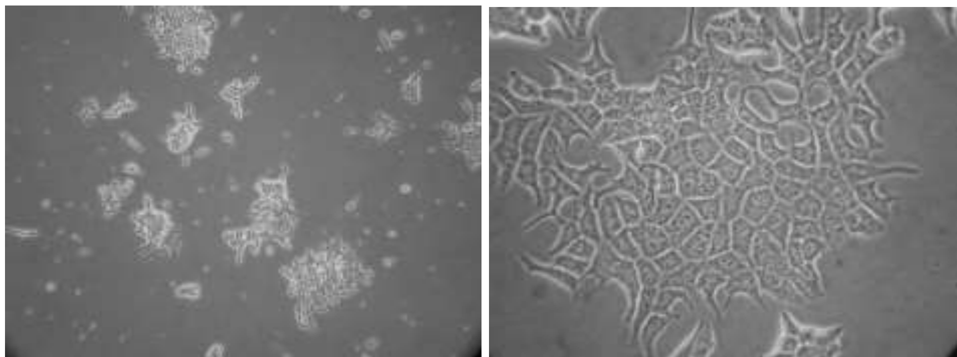
LNCaP cells



PC3 cells



DU 145 cells



LNCaP-AI cells: right picture at higher magnification

Figure 2.1. Light microscopy images for LNCaP, PC3, DU 145 and LNCaP-AI cells. At X10 magnification

1.2.Cell line culture : Feeder cell lines

1.2.1. Mouse STO cells

Murine STO embryonic fibroblast cells were used as feeder cells in order to provide mechanical as well as paracrine support to prostate epithelial cultures. Murine STO (embryonic fibroblast) cells were obtained from Dr. Stuart Williamson and were maintained in culture in DMEM medium with Hepes Modification (R5886, SIGMA®, Steinheim, Germany) supplemented with 10% FBS (F7524, Sigma Chemical Co., USA) and 2mM L-Glutamine (G7513, SIGMA®). Cells were routinely monitored for adhesion and contamination and were passaged every 48-72 hours at 75% confluence in a 1:10 ratio. These cells were maintained as mycoplasma negative.

Cells were irradiated prior to use at 310 Amps 10kV for 20 minutes (4000 rads) or were inactivated by means of Mitomycin C. Mitomycin C (SIGMA®) was reconstituted in STO-culture media at a concentration of 10µg/ml. Cells were incubated with the Mitomycin C containing media for 2 hours at 37˚C , 5% CO2 and 100% relative humidity following which they were washed three times in 1X PBS and trypsinised. The pellet was again washed a further 3 times in 1X PBS to ensure near-complete removal of any traces of Mitomycin-C. These cells were then seeded onto prostate primary epithelial culture at an initial confluence of ~65% or 100,000 cells per 75cc flask. After 5 days, once the prostate primary epithelial cells were seen to proliferate in a stable manner, the confluence of feeder cells was increased to ~75% or 500,000 cells per 75 cc flask. The reason for maintaining a moderate initial STO density was to prevent any potential competition from these freshly inactivated feeder cells during the early days of establishing epithelial cultures.

1.2.2. Mouse Embryonic Fibroblast cells

1.2.2.1. Preparing gelatin coated plates

These cells were seeded onto 0.1% Gelatin coated plates to improve adhesion and attachment of these cells. To prepare the gelatin solution, 10 grams of gelatin was added to 1L distilled sterile water. This was autoclaved for 30 minutes, following which this 1% stock was stored at -20oC in 50 ml aliquots. To make 0.1% Gelatin, 50 ml of 1% Gelatin was dissolved in 450 ml sterile distilled water. 2ml of this 0.1% gelatin was added to each well of a 6 well plate and the plates were incubated overnight at 37oC. Immediately prior to use, the gelatin was completely aspirated from the plates and MEF cells were seeded.

1.2.2.2. Culturing MEF cells

Mouse Embryonic Fibroblast cells or MEFs were used as feeder cells for pluripotent stem cell cultures. CF-2 (mouse strain) irradiated mouse embryonic fibroblast cells were purchased from VhBio and were directly seeded onto 0.1% Gelatin coated plates in DMEM medium with Hepes Modification (SIGMA®) supplemented with 10% FBS (Sigma Chemical Co.) and 2mM L-Glutamine (G7513, SIGMA®). Non-inactivated cell lines were also maintained in culture so as to generate feeder-conditioned media. These cells were routinely monitored for adhesion and for any potential mould and/or bacterial contamination and were maintained as mycoplasma negative.

Irradiated cells were seeded at a density of 50,000 cells/cc and used immediately (within a maximum of 24 hours). In cases where there were unforeseeable delays in using the MEF cells within 24 hours of seeding, the DMEM media described above was removed from the adherent cultures and the cells were washed twice with 1XPBS. Following this, Embryonic Stem Cell Media was added onto the MEF cultures. This conditioned the media with the

MEF cells secreted factors, however in all circumstances the seeded MEF plates were used within a maximum of 48 hours. Irradiated cells were used as a feeder layer for a maximum of 7-10 days. Unlike with the STO cells, iPS cells were seeded onto these cells. it was observed that adding irradiated MEF cells onto established iPS cultures failed to offer appropriate support and resulted in death and/or differentiation of the pluripotent cells. In all circumstances the iPS cultures were transferred onto freshly seeded MEF-plates every 7 to 10 (maximum) days.

1.3. Culturing cell lines: Normal Human Dermal Fibroblast (NHDF) cells

NHDF cells were kindly donated by Prof. Majlinda Lako and were cultured in DMEM medium with Hepes Modification (SIGMA®) supplemented with 10% FBS (Sigma Chemical Co.) and 2mM L-Glutamine (G7513, SIGMA®). They were split in a 1:10 ratio every 5-7 days and were given a media change every 48 hours. These cells were used as the control cell line for iPS induction and cells were used at very low passages. Hence they were frozen down at very low passages and were not propagated in culture beyond passage 20. The cells were routinely monitored for adhesion and for any potential mould and/or bacterial contamination. The cells were maintained as mycoplasma negative cells.

1.4. Freezing and thawing cell lines.

Freezing media was prepared fresh and to do so the respective growth media was reconstituted with an additional 10% FBS (Sigma Chemical Co.) and 10% Dimethyl Sulphoxide (D2650, DMSO Hybri-MAX®, Sigma®). Cells were counted trypsinised and resuspended in the freezing media at 2 million cells / ml and stored at -80˚C for short-term storage.

To retrieve frozen cells, thawed cell vials were immediately reconstituted in the respective culture media and centrifuged at 1500 rpm X5 minutes. The cells were washed once more in their respective media and were then plated out in T75 culture flasks. After 24 hours, cells were monitored for adherence and potential mould and/or bacterial contamination. The cells were washed with 1X PBS and fed with fresh media.

1.4. Primary tissue culture in the prostate

In total, human prostatic tissue was collected from more than 60 anonymised patients (aged 50-85 years) who underwent trans-urethral resection of the prostate (TURP) for BPH or cystoprostatectomy for bladder cancer. All samples were obtained with patient consent and after conforming to ethical guidelines and ethical permission.

I. Collagenase I digestion of prostatic tissue

Collagenase I digestion was performed to release epithelial structures and stromal organoids (Collins, Robinson et al. 1996, Robinson, Neal et al. 1998, Heer, Robson et al. 2007). All tissue samples were washed in PBS to reduce operation theatre contaminants. Each sample was dissected into small cubes measuring 3mmX3mmX3mm so as to ensure increased surface area for digestion. Samples were incubated in 200 IU/ml strength Type I Collagenase (LS004196, Worthington, Lorne Laboratories, Reading, UK) solution for a period of 20 hours. Collagenase I solution was prepared by dissolving appropriate amount of Collagenase IV powder in RPMI 1640 media supplemented with 5% FBS (Sigma Chemical Co.,USA), 2mM L-Glutamine(SIGMA®) and 1% Penicillin-Streptomycin (SIGMA®). Tissue digestion was performed overnight in an oven at 37°C.

II. Separating prostate epithelia from prostate stroma

Following collagenase digestion, the digest was passed through a 21 gauge needle to homogenise and increase yield. Cells were then washed at least five times in PBS. Epithelial cells were separated from their stromal counterparts through repeated differential gravity centrifugation at 800rpm for 1 minute – this alone has been reported to yield epithelial fractions to 98% purity (Robinson, Neal et al. 1998, Heer, Robson et al. 2007). Epithelial acini were further treated with 1X trypsin (SIGMA®) at 37°C for 30 minutes to disaggregate

organoids and release single cells (Collins, Habib et al. 2001, Heer, Robson et al. 2007). Cells from the epithelial fraction were further sorted using magnetic separation with the help of MACS microbeads linked to an antibody against the HEA (Human Epithelial Antigen/CD326/EpCAM/) antigen (Miltenyl Biotech Ltd., Surrey, UK).

III. MACS sort

Epithelial cell adhesion molecule (EpCAM) or CD326 is expressed by epithelial cells as well as by epithelial-tumour cells (Moldenhauer, Momburg et al. 1987). Cells were magnetically labelled with CD326 microbeads at 4°C for an hour, and then made to pass through a MACS column placed in a magnetic field. Epithelial antigen expressing cells bind to these ferritin labelled microbeads and are retained in the column when made to pass through. The labelled cells were subsequently collected in MACS buffer (2mM EDTA in PBS and 0.25% FBS) and cultured as epithelial cells.

IV. Culturing prostate epithelia

Prostate epithelial cells were routinely cultured in 25 cm^2 Collagen I coated flasks (BD Biosciences, Oxford, UK) in serum-free, low-calcium containing keratinocyte basal medium (17005, GIBCO®, Invitrogen Ltd., Paisley, UK) supplemented with 25mg of bovine pituitary extract (13028, GIBCO®) and 2.5μg of recombinant epidermal growth factor (10450, GIBCO®). The medium was further supplemented with 100ng/ml of cholera toxin (C8052-1MG, SIGMA-ALDRICH®, Steinheim, Germany), 0.2 ng/ml of LIF (L5283, SIGMA-ALDRICH®), 0.1 ng/ml of GM-CSF (G5035, SIGMA-ALDRICH®), 0.2 ng/ml of SCF (S7901, SIGMA-ALDRICH®). Presence of bovine pituitary extract, low calcium concentration, cholera toxin and other growth factors have been demonstrated to facilitate proliferation as well allow for higher serial passages of these epithelial cultures (Chaproniere and McKeehan 1986).

A ten minutes rapid adhesion and a twenty-minutes adhesion was used to enrich for stem cell populations and viable cell populations, respectively (Collins, Habib et al. 2001, Heer, Robson et al. 2007). Mitomycin C inactivated murine STO cells (mouse embryonic fibroblast cell line) were used as feeder cells in epithelial culture. These cells provided mechanical support and provided stromal-derived factors which are both essential for epithelial culture (Collins, Habib et al. 2001, Lang, Stark et al. 2001). It was noted in this study that since the initial yield of epithelial cells was most often very low, culturing in 25 cm^2 flasks in place of previously used 75 cm^2 flasks ensured that the epithelial cells were in close proximity which in turn increased culture success rate.

Epithelial cells were washed with 1XPBS (GIBCO) and fed with epithelial culture medium every 48 hours. Mitomycin C inactivated feeder cells had to be replenished at least once a week. These cells were split once colony formation was observed, cell clusters containing > 32 cells were designated as colonies (Collins, Habib et al. 2001). Cells were split using 1Xtrypsin-EDTA (SIGMA®) and the trypsin was neutralised using 10% FBS (Sigma Chemical Co.) in place of regular medium. Using 10% FBS in place of conventional culture medium prevented occurrence of stromal contamination in epithelial cultures as well as facilitating epithelial growth (Chaproniere and McKeehan 1986).

V. Culturing primary prostate stroma

Prostate stromal cells were separated from their epithelial counterparts by first washing the cells from the prostate collagenase I digest (please refer Step I) in 1X PBS (GIBCO) and then re-suspending the cells in stromal culture medium comprising RPMI 1640 media supplemented (SIGMA®) with 5% FBS (Sigma Chemical Co.), 2mM L-Glutamine (SIGMA®) and 1% Penicillin-Streptomycin (SIGMA®). This suspension was then centrifuged at 800rpm for 1 minute – the resultant supernatant was enriched with stromal

cells (Lang, Stark et al. 2001) and was cultured in 75cm^2 regular tissue-culture flasks (Corning Incorporated). Cells were regularly monitored for adherence and fed thrice a week with stromal culture medium. Cells were split by means of trypsinisation at 75% confluence in a split ratio of 1:5.

1.3. 3-D culture of prostate primary cells

It has been observed that development of a functional and anatomical appropriate prostate relies on the presence of extracellular matrix (Lang, Stark et al. 2001). To culture primary cells in 3-dimensional structures, a total of 2000 cells were re-suspended in 100 μl of BD Matrigel ™ basement membrane (35428, BD Biosciences, Bedford, UK). BD Matrigel is stored frozen at -20°C and has to be thawed overnight at 4°C before use. Re-suspended cells were incubated in 24 well tissue culture plates (Corning incorporated) at 37°C in a tissue culture incubator for 30 minutes. Once the Matrigel had set, 50 μL of appropriate culture medium was added.

For stromal cells, stromal culture media was used whilst for epithelial cells epithelial culture media was further enriched with stromal-derived factors. The latter was obtained by culturing inactivated mouse STO cells in epithelial growth medium for 2 hours following which the stromal factors-enriched media was aspirated and filter sterilised to remove dead STO cells. For 3-D culture, cells were fed with respective media every 48 hours.

1.4. Pluripotent Stem Cell Culture

1.4.1. Culturing Pro-iPS cells

Human pluripotent stem cells were grown on MEF cells at 50,000 MEF cells/cc and maintained in 6-well plates. These cells grew in colonies and the colonies were monitored daily for evidence of death, differentiation as well as fungal and/or bacterial contamination. The health of the surrounding MEF cells were also regularly monitored as considerable variation was observed between different batches of MEF cells. Depending on the health and density of the surrounding MEF cells, Pro-iPS colonies were passaged every 5-7 days.

A healthy Pro-iPS colony would be denoted by a tight compact morphology lacking any evidence of central umbilical differentiation and/or peripheral fibroblast-like differentiation. Cells were maintained in pluripotent stem cell culture media that comprised the following:

1. 80% Knockout™ D-MEM (10829-018, GIBCO, Life Technologies, Paisley, UK)
2. 20% Knockout™ Serum Replacement (10828-028, GIBCO, Life Technologies, Paisley, UK)
3. FGF-basic Recombinant Human (13256-029, GIBCO, Life Technologies, Paisley, UK) at 8ng/ml
4. 100mM MEM non-essential amino acids (11140-035, Life Technologies, Paisley, UK)
5. 200 mM GlutaMAX™-I Supplement (35050-038, Life Technologies, Paisley, UK)
6. 1% Penicillin-streptomycin (Sigma, Germany)
7. 0.1 mM β-mercaptoethanol (Sigma, Germany)

Differentiation at the centre of the colonies were identified and removed with the help of P20/200 sterile plastic Gilson tip. If peripheral differentiation causing irregular ill-defined colony borders were observed then the colonies were passaged and healthy areas of each colony was broken into small cells clumps of 50-60 cells and transferred onto fresh MEF cells. In this case, the colonies were subjected to mechanical and enzymatic dissociation.

1.4.2. Passaging Pro-iPS cells

To detach the Pro-iPS colonies from the surrounding MEF cells, the colonies were treated with Collagenase IV solution (Gibco, Life Technologies, Paisley, UK) at 1mg/ml for a maximum of 5 minutes. This resulted in curling of the colony-borders. At this point the Collagenase IV solution was removed from the cells and fresh pluripotent stem cell culture media was added. The cells were observed under a dissection microscope and were dissected on a stage heated to 37˚C.

Firstly, any areas of differentiation (this appeared as brown discoloration while the healthy pluripotent cells looked like translucent areas) were removed manually with the help of a P20 Gilson pipette. Next the tip of the P20 was run over the remainder colony to break it up into small bits of 50-60 cells and these were gently sucked up in the pipette and transferred onto fresh MEF feeder plates which were placed in the incubator for 24-48hrs before changing the media. The colonies were split in a ratio of 1:8 every 5 days.

It was essential to ensure that the colonies were broken into appropriate sized cell clusters – pluripotent stem cells do not survive as single cells and therefore if colonies are too small then they will fail to grow. However, failure to dissect a colony into cell clusters smaller than 60 cells would result in excessive and early differentiation of the transferred colonies. Also, excessive precaution must be taken during Collagenase IV treatment since this does not get

neutralised by serum. Complete removal of the Collagenase IV solution is essential as otherwise this will cause death of pluripotent cells.

Improper maintenance and passaging of pluripotent cells result in karyotypic abnormalities. Enzymatic digestions through Trypsin-EDTA as well as through Collagenase IV treatment can result in cytogenetic aberrations. Such anomalies can be minimised through mechanical dissociation whereby no enzymatic processes are involved and the colonies are exclusively removed manually with the help of a firepolished sterilised glass pipette tip.

1.4.3. Freezing Pro-iPS cells

Pro-iPS cultures were frozen at 85% confluence. Cells were collected as clumps through Collagenase IV digestion, cell pellet was collected after centrifuging the cells at 200g X 5 minutes. Freezing media was made up in media constituting equal volumes of pluripotent-stem cell culture media and cryopreservation media. Cryopreservation media was made up with 60% pluripotent stem cell culture media, 20% FCS and 20% DMSO. 1 6-well plate containing ~300 healthy pluripotent colonies were collected in 200 µL of the freezing media, to this 10µM Y-molecule (Stemolecule ™ Y27632, Stemgent, MA, USA) was added and the cells were frozen at -80˚C for short term storage and were later transferred to liquid nitrogen for long term storage.

1.4.4. Thawing Pro-iPS cells and recovering them back to culture

Pro-iPS cells were thawed onto MEF feeders on BD Falcon™ 4 well In Vitro Fertilization (IVF) Plate (353654, BD Biosciences, Oxford, UK). Contents of 1 6-well plate were thawed onto 4 wells of a 4 well IVF plate. The cells were allowed to stick down onto MEF cells for 48 hours following which their growth was monitored every 24 hours. Recovery was around

80% and colonies were transferred onto 6-well plates (from 1 4 well IVF plate to 15 wells of a 6 well plate) after one week.

1.4.5. Embryoid Body (EB) formation and differentiation

Pro-iPS cell clumps were collected in EBformation media (Knockout-DMEM 80%, Knockout Serum Replacement 20%, GlutaMAX™-I Supplement 200 mM, 1% MEM non-essential amino acids, 1% Penicillin-streptomycin) and cultured on low-adhesion plates for 7 days. Once embryoid bodies were formed these were subsequently differentiated to test the pluripotency of the Pro-iPS.

To differentiate the embryoid bodies, these were transferred onto 0.01% Gelatin-coated plates (each plate contained around 10-20 embryoid bodies) and were allowed to differentiate in EB-formation media for a further 10-15 days.

2.1. Lentiviral transduction using OSKM and OSLN factors

Several transduction protocols and Lentiviral kits were tried and the most efficient human-iPS specific generation methodology was optimised through repeated attempts at the process. Two different types of lentiviral constructs were used - individual Oct4, SOX2, Lin28 and NANOG lentiviral vectors (all from ST000005, STEMGENT, San Diego, USA) in one system and the use of a 4-in-1 Floxed lentiviral system (ABP-SC-LVI4in1, Allele Biotechnology, San Diego, USA) in another protocol. Two different iPS reprogramming cocktails were tried – the traditional Yamanaka OSKM factors and the Thomson OSLN factors. As per literature, the Yamanaka OSKM factors have been associated with faster kinetics and higher efficiency (Robinton and Daley 2012). Although they have been used to re-programme human cells, the sustained activation of c-Myc is known to cause death and differentiation in human pluripotent cells (Sumi, Tsuneyoshi et al. 2007, Yu, Vodyanik et al. 2007). Therefore, it was decided that the transduction protocol would be optimised initially using Thomson's OSLN factors. Incidentally, two different protocols were followed with the two different re-programming cocktails. A range of MOI (Multiplicity of Infection) was tested from 2 to 10 so as to optimise the maximal efficiency at minimal toxicity. For both the transduction protocols, Polybrene (TR-1003-G, Millipore, Massachusetts, USA) was used to aid entry of the lentivirus into the cells.

For all transductions the transduction media was made up fresh and used immediately. Cells were transduced for 48 hours and from week 1 onwards they were cultured in embryonic stem cell-like culture conditions.

2.2. Analysis of optimal transduction efficiency

The optimal transduction efficiency was regarded as the MOI that would be sufficient to transduce the majority of the target cells. Given that iPS induction is a multi-step process, the number of cells passing from one transition to the other progressively reduces with a very small proportion of the initially transduced cells reaching the ultimate 'ground state'. Due to certain epigenetic events that are as yet equivocal, very few cells progress beyond the initial MET (or mesenchymal-epithelial transition) phase. Many transduced cells that have acquired an epithelial phenotype are still prone to either perish or revert back to the mesenchymal phenotype (Buganim, Faddah et al. 2013). Also, it is essential that the amount of virus used is kept to a minimum so as to prevent multiple integral sites and to ensure silencing of the transgene.

Target cells were seeded at 75% confluence in a 96-well plate. A range of MOI was tested: 0.5, 1.0, 2.5, 5.0, and 10.0. Cells were transduced with mWasabi GFP empty lentiviral vector (Allele Biotech., USA) in their respective growth media for 48 hours after which GFP expression was analysed and compared against a polybrene-only control (or MOI = 0). All FACS analysis was done on the FACS Calibur using Fl1-H channel for GFP detection and Cyflogic for analysis and interpretation of results.

3. Cytogenetics: Karyotyping

Karyotypic analysis was carried out so as to ensure that Pro-iPS cells did not suffer from any aneuploidies. Pro-iPS cell lines were karyotyped at passage 18-20. Karyotyping was done through the following three steps:

3.1. Harvesting cells and Metaphase arrest – protocol courtesy, Mr. Arman Esfandiari

3.1.1. Growth and Harvest of Pro-iPS cells

Pro-iPS cells were grown on irradiated MEF feeder cells and were harvested for analysis only at 85% confluence. 6 wells of a 6 well plate full of Pro-iPS colonies were treated with the mitotic spindle poison Colcemid (kindly donated by Mr. Arman Esfandiari and Prof. John Lunec) for a period of 120 minutes. The duration of Colcemid treatment is dependent on the cell cycle of the relevant cell lines. Cells that multiply quickly and have short cell cycles (example: embryonic stem cells and other pluripotent stem cells) need shorter Colcemid treatment while cells with longer cell cycles need longer Colcemid treatment to facilitate appropriate Metaphase arrest. It is important to treat the cells for the correct duration of Colcemid ; lengthy incubations with Colcemid will result in the generation of tightly compacted chromosomes on G-banding which in turn would complicate inspection for interchromosomal and subchromosomal aberrations. The incubation period for Pro-iPS cells was optimised to 2 hours of treatment which resulted in a well-spread out metaphase with precise G-banding of the chromosomes.

Following Colcemid treatment, Pro-iPS colonies were collected by means of collagenase treatment. Collagenase treatment exclusively allows for lifting of pluripotent stem cell colonies while the feeder MEF cells remain mostly unaffected. Once the colonies were collected, the suspension was allowed to stand for another 10 minutes which further ensured

the separation of any accompanying MEF cells from the Pro-iPS colonies. The iPS colonies were then washed in 1XPBS and subjected to a brief 1% trypsin digestion for 5 minutes at 37˚C after which the trypsin was neutralised with 10% FCS-PBS. This was then centrifuged at 1500 rpm X 5 minutes following which most of the FCS-PBS was aspirated from the cell pellet leaving behind 200 µl. The pellet was gently flicked to resuspend the mitotic cells.

3.1.2. Addition of hypotonic solution

To lyse the cells, 1ml of hypotonic solution (1:1 0.4% KCl + 0.4% Sodium Citrate) was added against the side of the tube to the mitotic cells, at this stage the cells were resuspended and then the total final volume was made up to 2ml with the hypotonic solution. This was incubated at 37˚C for 7 minutes following which the cells were centrifuged at 1500 rpm X 6 minutes.

3.1.3 Fixation

5 drops of fixative (3:1 Methanol and Acetic Acid) was added along the sides of the tube to the cell pellet to make up a final volume to 2 mL. The cells were resuspended in the fixative and were fixed at room temperature for 30 minutes. This was centrifuged as before and the pellet obtained was again resuspended in fresh fixative made up to 2 mL and the solution was incubated at room temperature for a further 20 minutes. The cells were then dropped onto slides or frozen at -20˚C.

3.1.4. Dropping cells and preparing slides

Slides were rinsed with ice cold water and were then rinsed with the fixative. Appropriate air humidity in the room for this step is 50%-60% (in a dry day this can be achieved by placing the slide over a beaker of water). In case frozen cells were used, these were washed once in the fixative. Using a plastic transpipet, 2-3 drops of the cells were dropped at 45˚ angle onto

the slides following which a tongue-shaped smear was attained. The optimal cell density was ascertained by observing each smear under the phase contrast microscope – the best cell density would be the one showing appropriate lysis of the cell and a well-spread out chromosomal pattern immediately next to the cell body. The slides were then aged at room temperature for at least 24 hours.

3.2. G-banding of chromosomes and generation of Karyogram, protocol courtesy Dr. Claire Schwab

3.2.1. G-banding

Pots were arranged in the following order (reagents kindly provided by Dr. Claire Schwab):

a. 1ml Trypsin in 25 ml saline/25 ml Leishman's buffer , pH 6.8
b. 50 ml Saline
c. 50 ml Saline
d. Staining solution: Giemsa and Leishman's staining solutions, 0.4 ml Giemsa Staining solution added to 8ml Leishman's stain and 40 ml Leishman's buffer
e. 100+ ml pot of cold distilled water

One slide was processed at a time. The slide was placed in trypsin solution (a) for 15 seconds and then transferred immediately to the saline solution (b). This was then rinsed in the third saline pot (c). The slide was next placed in the Staining solution (d) for 5 minutes and then thoroughly rinsed in distilled water (e). The stained slides were then mounted with coverslip using DPX and analysed using the Kario software or Cytovision®.

3.2.2. Analysis and generation of Karyogram

A brightfield image was opened on the software and an appropriate human cell, based on the lysis, metaphase spread and G-banding pattern was chosen. Human chromosomes were specifically and exclusively chosen for analysis, this was confirmed through morphology with the help of a cytogeneticist (Dr. Claire Schwab). Extrachromosomal objects such as cell debris were disregarded. Next, the chromosomes were separated from each other when overlapped; this was most specifically common nearer the centromeres. This procedure was

continued until the software read each chromosome as a single entity. The total number of chromosomes was recorded. Next, the chromosomes were aligned with the help of a standardised normal human karyogram and these were labelled. Any interchromosomal and/or subchromosomal re-arrangements and/or aberrations were noted.

4. DNA fingerprinting

DNA fingerprinting was carried out to corroborate authenticity of the Pro-iPS cells and to rule out any possibility that these could have been generated as a result of cross-contamination with other pluripotent cell lines. Cell pellets were collected from both the parental fibroblasts and from the generated Pro-iPS cells. These samples were sent off to Northern Molecular Genetics Service, UK and were fingerprinted for a set of 16 different microsatellites including the sex-marker Amelogenin through the Promega PowerPlex® 16 system. The kit was specific for human cells alone and would not amplify murine DNA.

The PowerPlex® 16 is a STR (Short Tandem Repeat) analysis technology whereby a set of different loci are co-amplified and detected through a three colour system. One primer for each of the loci Penta E, D18S51, D21S11, TH01 and D3S1358 is labelled with fluorescin (FL); one primer for each of the loci FGA, TPOX, D8S1179, yWA and Amelogenin is labelled with carboxy-tetramethylrhodamine (TMR) and one primer for each of the loci Penta D, CSF1PO, D16S539, D7S820, D13S317 and D5S818 is labelled with 6-carboxy-4'5'-dichloro-2', 7'-dimethoxy-fluorescein (JOE). All the loci are then then amplified simultaneously in a single tube and analysed in a single gel lane. The results were analysed using the ABI377 sequence detector using Genotype software (Applied Biosystems), courtesy Dr. Helen Powell.

5. Live cell staining and Imaging

hESC colonies were stained with 1:100 dilutions of Anti-TRA-1-60, Clone TRA-1-60-FITC conjugate (Millipore) and Anti-SSEA-4, Clone MC-813-70-PE conjugate (Millipore). The hESC colonies were incubated with these antibodies at 37oC for 2 hours following which the anitibody-containing hESC media was removed and hoercsht hESC media solution was added at a final concentration of hoercsht at 0.5µg/ml. After 10 minutes incubation, the respective wells were washed twice with hESC media to ensure that all traces of the toxic hoercsht dye were removed completely. The colonies were then imaged in hESC media under an Nikon eclipse TE2000U inverted microscope using the software 'NIS elements – BR 3.0' (Figure 2.2 demonstartes the exposure time for the different filters).

Filter (the light that you actually see)	Staining colour(your colony will show this colour)	Exposure time	Marker
green	red	1s	SSEA4
Blue(cobalt - like)	green	600-800ms	Tra-1-60
Purple (Prussian blue like)	Blue	60-80ms	Hoercsht

Figure 2.2 Exposure times as optimised for the three laser filters. Following acquisition of the images, the media was replaced with fresh hESC media containing 10µM Y-molecule (Stemolecule ™ Y27632, Stemgent, MA, USA).

6. Alkaline phosphatase staining

Pros-iPS cells were cultured for 5 days and were analysed for alkaline phosphatase activity at 50-70% confluence. The cells were fixed in 10% formalin for 2 minutes and were then rinsed with 1XTBST buffer (20mM Tris-HCL, pH 7.4, 0.15 NaCl, 0.05% Tween-20). The cells were then treated with Fast Red Violet, AS-BI phosphate solution and distilled water in a 2:1:1 ratio for 15 minutes in the dark at room temperature after which they were washed in 1XTBST Buffer. The numbers of colonies expressing alkaline phosphatase were counted in 1XPBS.

7. Immunofluorescence staining fixed cells

4% PFA fixed cells were blocked in appropriate blocking agent and incubated with respective primary antibody overnight at 4˚C. Next, the cells were washed in 1XPBS and incubated with the respective Alexa-Fluor conjugated secondary antibody. The cells were mounted in VECTASHIELD® Hard-set mounting media with DAPI and analysed through confocal microscopic imaging (for antibody details see supplementary information).

8. Teratoma assays

Teratoma assays were carried out using NOD/SCID mice. This procedure wasconducted using the following steps.

8.1. Preparation of cells for injection

The day before injection, BD Matrigel ™ basement membrane (35428, BD Biosciences, Bedford, UK) was defrosted on ice. Matrigel was defrosted overnight below 3˚C. Syringes, needles as well as tips were also kept on ice to ensure that while handling Matrigel the temperature was maintained below 3˚C since higher temperatures would cause Matrigel to set.

Pro-iPS colonies were collected through Collagenase digestion and were separated from the surrounding MEF cells by letting the disrupted colonies sediment through gravity instead of centrifuging the cells. The supernatant containing MEF cells was carefully aspirated and the Pro-iPS colony pellet was washed once in 1XPBS and then trypsinized to single cells. The cells were counted and made up to a concentration of 5 million cells per ml in the pluripotent stem cell media. This was placed on ice and brought down to a temperature of ???. For the injection, aliquots were prepared with 100ul of the cell suspension (100ul of cell suspension) and 100ul of thawed Matrigel.

8.2. Injecting cells and measuring tumours

Cells were injected subcutaneously in the right thigh of each mouse. One cell line was used per mice and in total each cell line was replicated through three mice. All injections were performed by Dr. Lyle Armstrong. Each mouse was identified through ear notches. Prior to injecting the mice it is recommended that the respective areas are shaved as this results in a greater precision for the injections. Failure to maintain precision can result in intra-abdominal injections in place of subcutaneous injections and this can cause intra-abdominal growths which fail to mature into teratomas and can result in improperly differentiated teratomas.

Tumours were looked out for and they were measured every week commencing from Week 3 following injection. Measurements were taken in both vertical and horizontal dimensions and the mice were euthanized based on the following guidelines:

1. 1-1.5 cm^2

2. If any dimension exceeds 15mm

3. Tumours reach 5% body weight

4. Weight loss reaches 20% of initial body weight

8.3. Harvesting the tumours

Mice were euthanized by Mrs. Shirley Dodd by cervical dislocation. The tumours were then provided to be harvested. Any overgrown hair was clipped and the skin over the tumour was lifted with the help of forceps. A small nick was made which was then extended to expose the tumour. The tumour was dissected away after dissecting it away from surrounding tissues including vascular supply. The tumours were measured, weighed and collected in formalin.

8.4. Fixation and dehydration of teratomas

Tumours were fixed in both Bouins fluid and 4% paraformaldehyde (PFA). Bouin's fluid fixation was used for trichrome stains while 4% PFA was used for immunohistochemistry. Accordingly, the tissues were either fixed in 20X volume of Bouins fixative for 24 hours, or in 20X volume of PFA for 12 hours at 4˚C. Next, the fixative was removed from the samples and the tissue was washed three times in water. 30-40ml of 70% ethanol was added to the tissue and this was left on for two hours. The procedure was repeated with 80%, 90% and then 95% ethanol. Tissues were stored in 95% ethanol until ready for processing.

3. General Molecular biology techniques

3.1.Optimising real time PCR primers for 12 stem-cell and prostate-specific differentiation genes

12 genes were selected based on their ability to demonstrate 'stemness' and prostate-specific differentiation. These included four pluripotency genes – Oct-4, SOX-2, NANOG, LIN28; four prostate stem cell genes – CD133, CD44, CD117, PSCA; and four androgen responsive genes – AR, PSA, Nkx3.1 and p63. Expression of these genes was used to characterise *in vitro* inherent plasticity in prostate primary samples. The aim here was to optimise the PCR technique and primers in terms of appropriate standard curves and primer specificity. LNCaP cell line was used a positive control for androgen responsive genes and both PC3 and DU 145 were used as negative controls for AR and PSA (van Bokhoven, Varella-Garcia et al. 2003). All experiments were performed in triplicates.

3.2. RNA isolation

RNA was isolated from four different prostate cancer cell lines using RNeasy® Micro-kit (Qiagen, West Sussex, UK) (Figure 2.3 demonstrates an example of RNA extraction from three replicates of each cell line tested. Samples were ensured for purity through their 260/280 ratio) . The protocol followed was as described in the manufacturer's handbook (RNeasy® Micro Handbook, Qiagen). Cells were trypsinised and 100,000 cells were collected for RNA isolation. The first step involved disrupting cells in guanidine-thiocyanate containing RLT Lysis buffer which was further supplemented with 10µl of β-mercaptoethanol per ml of lysis buffer used. This step was performed in a fume hood and appropriate face protection was used. Following this, an equal volume of RNase-free 70% ethanol was added to the lysate and the sample was directly transferred to an RNeasy MinElute spin column placed in a 2 ml collection tube. Ethanol facilitates selective binding

of RNA to the silica membrane found in the spin columns. Following a sample-wash with RW1 wash buffer the cells were treated with DNase I for 15 minutes at room temperature to degrade DNA contaminants and ensure purity of resultant RNA. The sample was washed again in RW1 buffer and following this step another wash with buffer RPE was performed. The spin column was subsequently washed with RNase-free 80% ethanol after which the column was carefully removed from the collecting tube and placed in a new collecting tube. Care was taken for the column not to touch the flow-through from the earlier step since carryover of ethanol affects down-stream reactions steps. The spin columns were centrifuged at full speed for 5 minutes to ensure complete removal of any traces of ethanol. RNA from the columns was eluted using 14 µl of RNase-free water.

All the above steps were performed using RNase-free products and frequent change of gloves during the procedure minimised occurrence of contamination as well as improved yield by eliminating contaminant-RNases. RNA concentration was measured using a nano-drop spectrophotometer and RNA preparations that demonstrated 260/280 ratios close to 2were regarded as suitable for downstream analysis. Resultant RNA was either used immediately or stored at -80°C for future use. Storing at -80°C is essential to minimise degradation and maintain RNA integrity. Each RNA extraction experiment was repeated thrice with three different samples harvested on different days to account for variations in cell cycle events. All cells were harvested at 75% confluency.

Cell Line sample	RNA concentration (ng/µl)	260/280 ratio
LNCaP 1	1963.94	2.08
LNCaP 2	2132.28	2.08
LNCaP 3	1958.45	2.05
LNCaP-AI 1	2810.20	2.04
LNCaP-AI 2	1784.53	2.05
LNCaP-AI 3	1504.48	2.07
PC 3	2950.93	2.03
PC 3	1925.23	2.05
PC 3	1743.45	2.07
DU 145	1011.71	2.01
DU 145	2138.29	2.04
DU 145	964.86	2.08

Figure 2.3. RNA concentrations and 260/280 ratio of samples harvested from prostate cancer cell lines. A 260/280 ratio of 2 indicates good quality RNA with high purity.

2.1.2. Reverse transcriptase PCR

mRNA was converted to cDNA using reverse transcription PCR. 1µg of RNA was made up to a volume of 12.7µl using RNase-free water. This RNA solution was then incubated at 65°C for 5 minutes to remove any secondary structure following which it was incubated at 37°C for two minutes. 7.3 µl of reverse transcriptase cocktail (see below for ingredients) was added next and the mixture was incubated at 37°C for one hour. At the end of an hour the reaction was stopped by inactivating enzyme activity by incubating at 95°C for five minutes. Resultant cDNA was either used immediately or stored at -20°C for future use.

Reverse transcription cocktail:

1. 0.3 µL of MMLV Reverse Transcriptase(Promega, Madison, USA)

2. 4µl of 5X MMLV RT buffer(Promega),

3. 2 µl of 4mM dNTPs(Promega)

4. 1µL of 50µM Oligo dT15(Promega)

2.1.3. Real time PCR

SYBR green (Platinum®Sybr®Green qPCR supermix-UDG with ROX, Invitrogen) reporter was used wherein Sybr green dye binds to the minor groove of double-stranded DNA and the fluorescence emitted is directly proportional to the amount of amplicons produced. 9µl of Sybr green mixture and 1µl of respective cDNA was loaded into wells of a 384 well plate. The Sybr green mix consisted of 5 µl Sybr green, 0.4 µl each of forward and reverse primers (see table for primer sequences). The mixture was made up to a volume of 9µl using RNase-free water. The plates were sealed and centrifuged at 1000 rcf for 1 minute and loaded in a 7900 HT real time PCR system (Applied Biosystems, California, USA) with predefined PCR programme. Gene expression profiles were analysed using ABI 7900 HT SDS 2.2 software (Applied Biosystems, Copyright 2003). All transcript levels were normalised to the housekeeping gene GAPDH (Figure 2.4 depicts an amplification curved for the house-keeping gene GAPDH). No variation in GAPDH levels were noted between different samples. In samples with equal RNA concentration, the cycle time of GAPDH was unchanged (between 16-17 cycles). Measurements for each gene was evaluated with the help of a standard curve (Figure 2.5)

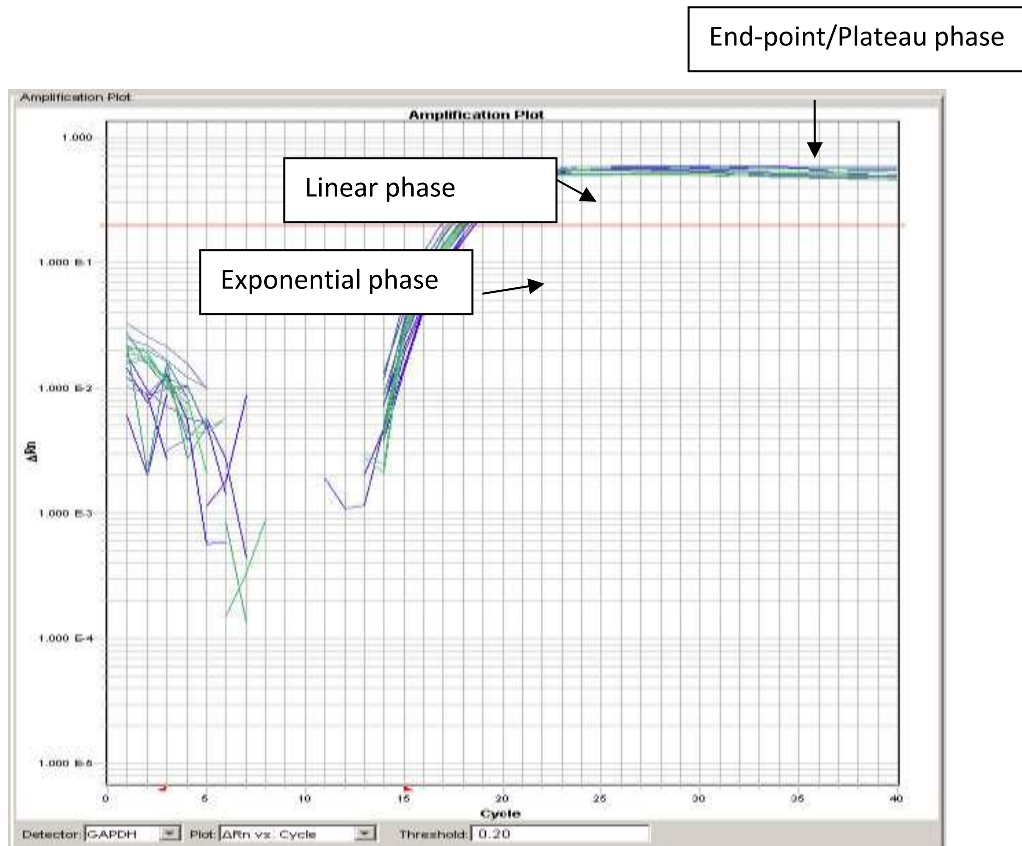


Figure 2.4. Amplification curve for housekeeping gene GAPDH for the different cell lines. mRNA expression for all other genes were normalised to GAPDH. Plot depicts amplicon accumulation against the number of amplification cycles. Quantitation of amplicon in real-time PCR occurs in the exponential phase of the PCR cycle as opposed to traditional PCR methods (such as quantitation by agarose gels) that use PCR end-point product for analysis. Exponential phase in PCR amplification is the optimal point for analysing data, hence real-time PCR is more precise than Agarose-gel PCR methods.

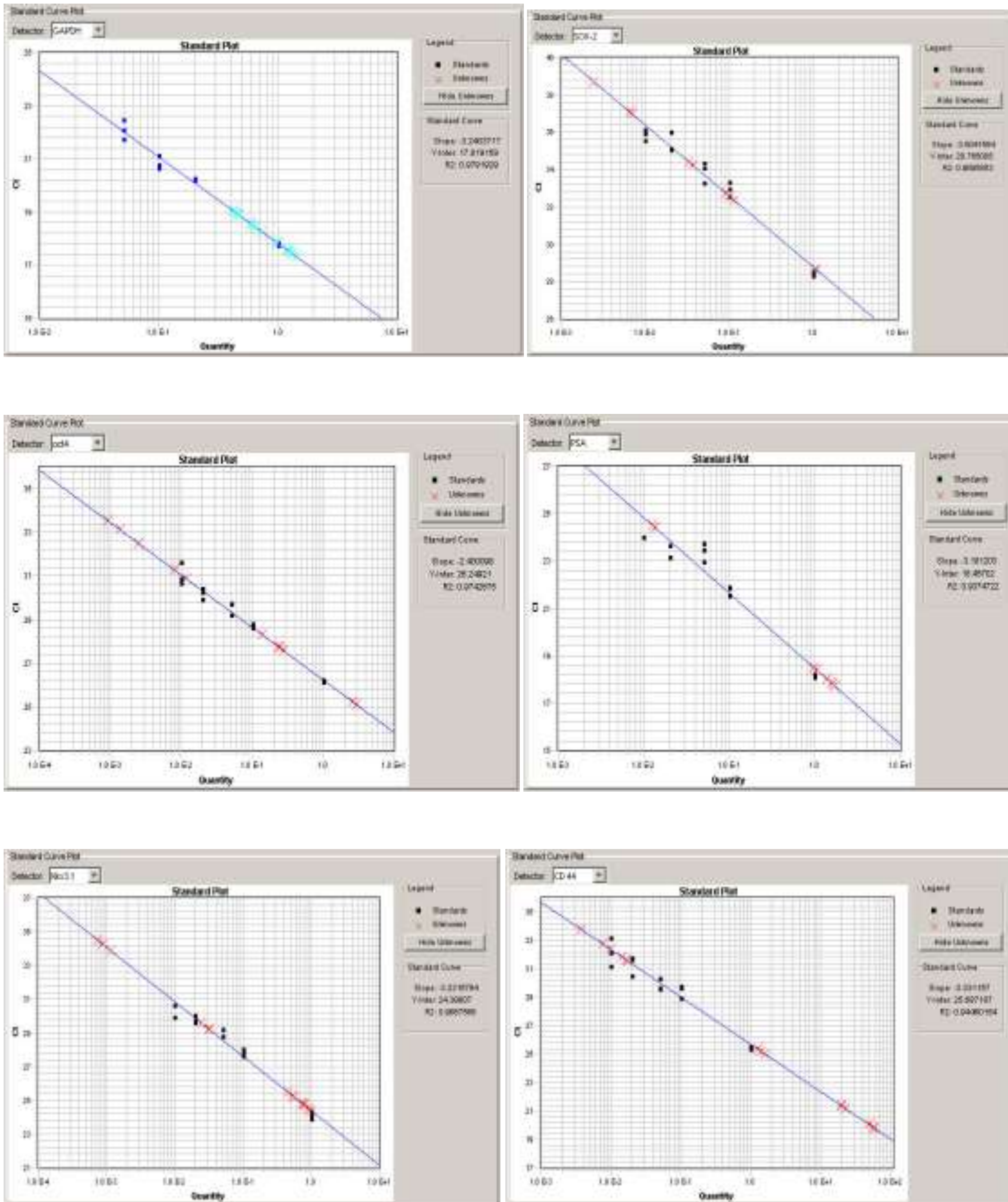


Figure 2.5. An example of the standard curves used to determine gene expression levels. An R^2 value of around 1 and a slope of around -3.2 were aimed for in each experiment. Standard concentrations were prepared such that unknown samples would fall closely within the ranges of values for known samples. This reduced possible errors that might arise due to extrapolation of the curve.

2.1.4. Designing primers

Primers were designed through the primer designing software Primer Express. They were then checked for specificity on NCBI Nucleotide BLAST and through NCBI Primer BLAST. Sequences are enlisted in Figure 2.6

SOX-2	F = 5'-CCCGGCGGAAAACCAA-3' R=5'-CGGGCAGCGTGTACTTATCC-3'
Oct-3/4	F = 5'-GCAGCGCAACGCCCT-3' R = 5'-GAATGTTGGCTCCCATGCC-5'
NANOG	F = 5'-CATGAGTGTGGATCCAGTTTG-3' R = 5'-CCTGAATAAGCAGATCCATGG
LIN 28	F = 5'-CTGCACCTTGGGTCCCAC R = 5'-CACACAGCTAGTGCAGTTGGC
CD 133	F = 5'-AACAGCGATCAAGGAGACCAAA-3' R = 5'-AAGGTGCTGTTCATGTTCTCCA-3'
CD117	F = 5'-CCAGCCTTCAAAGCTGTGC-3' R = 5'-AAGATAGCTTGCTTTGGACACA-3'
CD 44	F = 5'-AGAAGGTGTGGGCAGAAGAA-3' R = 5'-AAATGCACCATTTCCTGAGA-3'

PSCA	F = 5’-CAGGTGAGCAACGAGGAC-3’ R = 5’-GTTCTTCTTGCCCACGTAGT-3’
Nkx3.1	F = 5'- AGCCAGAAAGGCACTTGGG-3' R = 5'-GGCGCCTGAAGTGTTTTCA-3
P63	F = 5’-GCAGCGCAACGCCCT-3’ R = 5’-GAATGTTGGCTCCCATGCC-3’
AR	F = 5’-CTGGACACGACAACAACCAG-3’ R = 5’-CAGATCAGGGGCGAAGTAGA-3’
PSA	F = 5’-CAATGACGTGTGTGCGCAA-3’ R = 5’-CGTGATACCTTGAAGCACACCA-3’
GAPDH	F = 5’-CGACCACTTTGTCAAGCTCA-3’ R = 5’-GGGTCTTACTCCTTGGAGGC-3’

Table 2.6. Primer sequences for real-time RT-PCR. Primer sequences were designed using Primer Express 2.0 (Applied Biosystems) software. All sequences other than that for LIN 28 and Nkx3.1 were obtained from Dr. S. Williamson; Nkx3.1 was provided from Dr. K. Coffey; Primer sequences for LIN28 were designed in this project. All other primer sequences were verified using the primer design software.

2.1.5. Statistical analyses

All experiments were performed at least in triplicates and the average was considered. All graphs plotted show the respective standard error. Relevant statistical significance was evaluated through T-Test analysis.

Chapter 3.

Establishing and characterising primary cell culture of prostate stroma and epithelia

3.1 Introduction

Prostate primary cultures were established for both stroma and epithelia. Healthy and robust cells are absolutely essential for successful lentiviral transduction and for pluripotency reprogramming. In addition, the re-programming protocol is a long procedure spanning between 2-4 weeks before cells become fully re-programmed. Hence, the culture protocols were optimised so that healthy primary cell cultures could be established that could further be propogated in culture over longer periods of time. It was also absolutely essential to establish pure populations of stroma and epithelia. With stromal cells it was seen that the initial cultures were heavily contaminated with blood cells and haematopoietic cells. However, purity of cultures was established with higher passages. Epithelial cells were purified by means of the EpCAM (CD324) MACS sort and purity was subsequentlysubsequently confirmed through transcript studies.

3.2 Aims

a. To establish robust primary cultures of prostate stroma and epithelia

b. To ensure that cultures were devoid of endothelial and haematopoietic contaminants prior to transduction

c. To characterise endogenous levels of pluripotency transcripts in prostate primary cells

3.3 Results

3.3.1 Primary culture in the prostate – culturing prostate stroma and prostate epithelia

Primary tissue culture was attempted on at least 50 independent samples. Benign prostate samples were obtained from either TURP or cysto-prostatectomy specimens. All samples were obtained following patient consent and as per ethical approval. Single cell suspensions were harvested from these samples which were further separated for stromal and epithelial cell fractions.

3.3.2 Culture of the prostate epithelia

A total of 55 samples were processed for derivation of prostate epithelial cultures. Out of all the samples tried, a total of 8 samples failed to grow and were regarded as "abortive" thereby giving a success rate of 85% for growing and culturing primary prostate epithelia. The success rate for epithelial primary culture in the author's hands has varied between 75%-85% (Figure 3.1). All cultures were grown for at least 4 weeks after which they were regarded as either successful or 'abortive'. Cultures showing epithelial cell colonies with at least 32 cells were considered successful and were further sub-cultured. However, the fraction of samples that reached higher subcultures was limited indicating that the survival and proliferation of primary prostate epithelial attenuated with increased time and passage *in vitro* (Figure 3.1). This reflects the tedious nature of primary epithelial culture of the human prostate and emphasises on the need for a simplified *in vitro* model system that will facilitate the study of biology of the prostatic epithelium. Figure 3.1. demonstrates the success rates of culturing primary prostate epithelium at the different passages. 83% of samples grew such that they could be harvested at least at passage 1. Higher subcultures were attained with difficulty with only 2% of cultures reaching beyond passage 4. Only 1 out of all the 55 samples that were cultured reached passage 6.

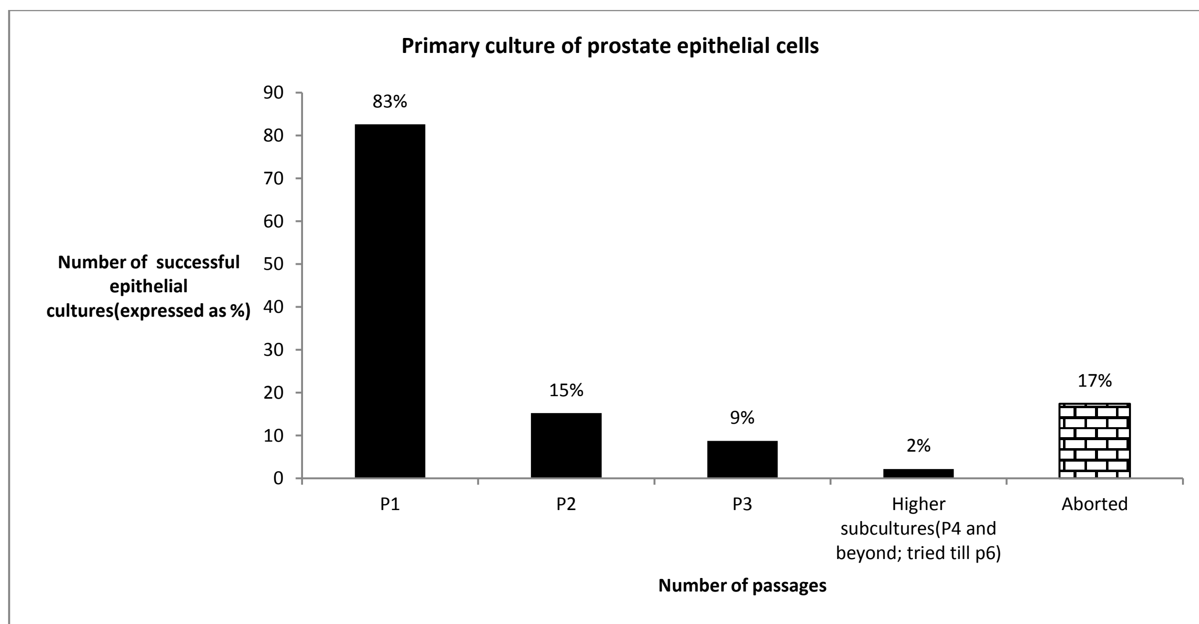


Figure 3.1. This figure demonstrates the success of primary prostate epithelia in the author's hands. The success rate of epithelial cultures is indicated against the respective passage number. Higher subcultures refer to passage numbers beyond P4 and up to P6 (highest passage number that was obtained by the author). 17% of total samples failed to grow *in vitro*; these cultures contained less than 32 cells and were regarded as 'aborted cultures'.

Out of the attempted culturing of the 55 samples, epithelial cell fractions from 35 samples were further purified through MACS separation using CD326 microbeads (see Chapter 2). It was observed that following CD326 enrichment the cultures survived higher passages better (Figure 3.2). In addition more than twice the number of purified samples reached higher passages as compared to the unsorted samples (Figure 3.2 and Figure 3.3). Furthermore, unsorted samples showed a failure rate three-fold higher than that of the enriched samples (Figure 3.4).

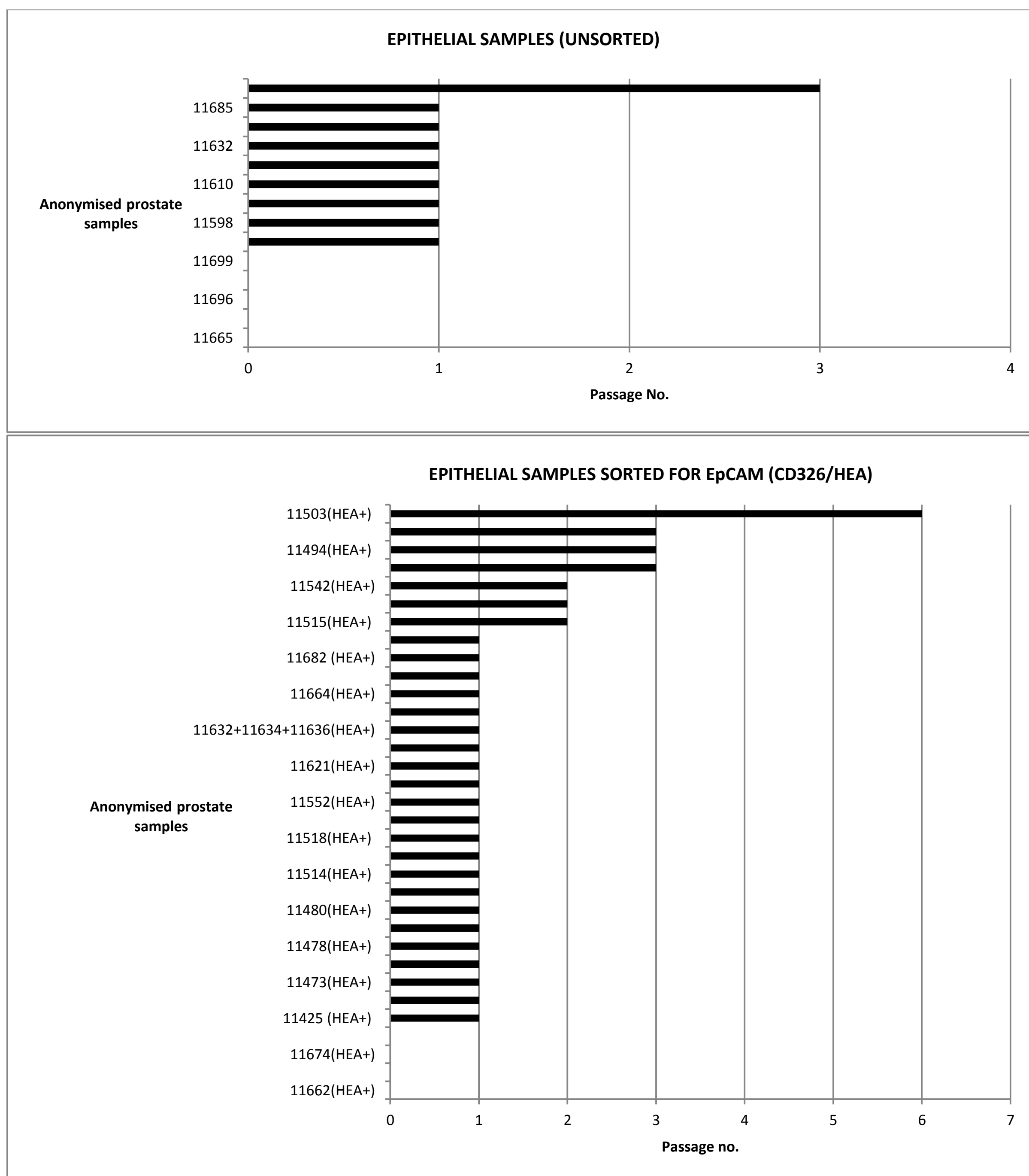


Figure 3.2. Table demonstrating the success rate of primary prostate epithelial culture with and without CD326 EpCAM sort. 20% of sorted samples could be cultured in vitro beyond the first passage. 11% of sorted cultures did not grow in culture. Only 1 of 20 unsorted cultured could be maintained beyond passage 1 and 25% of unsorted cultures abortedA summary of sorted and unsorted primary epithelial cultures. Y-axis shows the anonymised patient-ID; HEA+ = Samples sorted for Human Epithelial Antigen

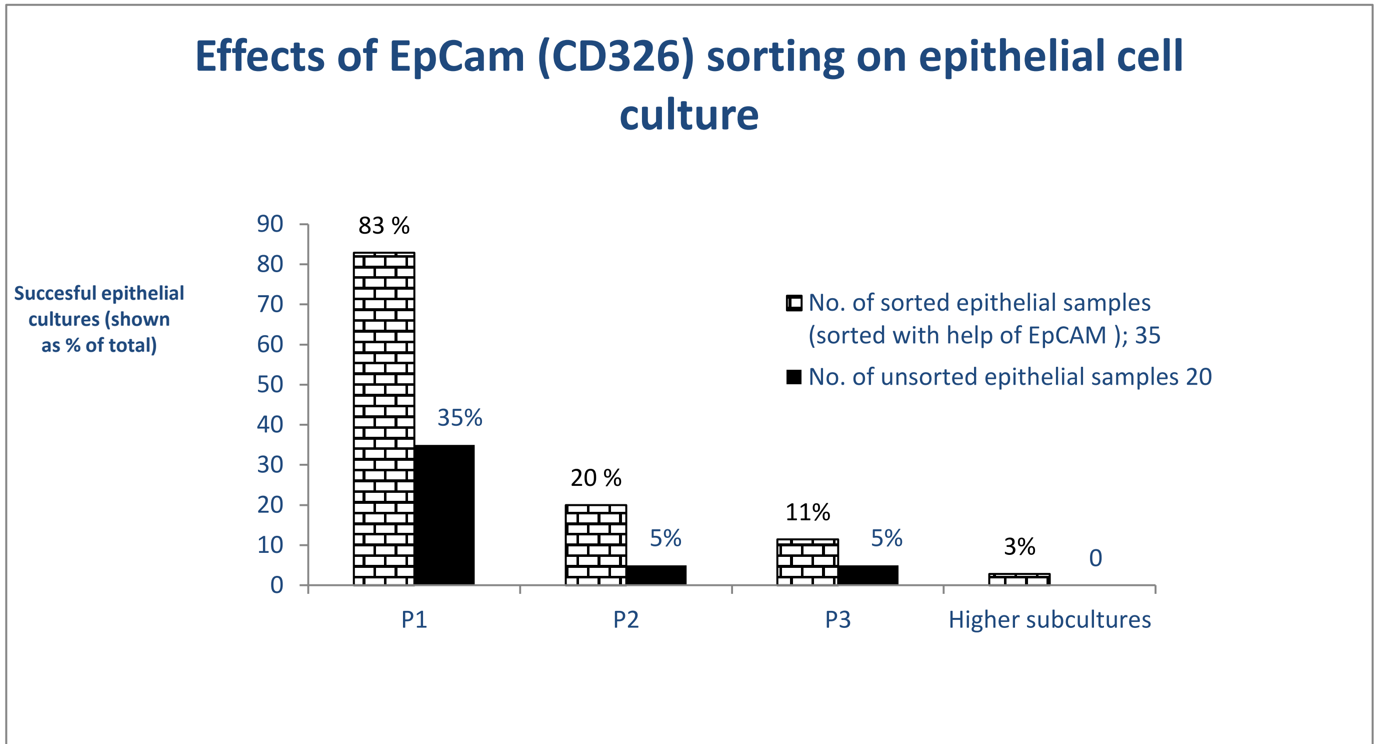


Figure 3.3. Effects of EpCam (CD326) sort on epithelial cell culture. Sorting cells with the CD326 antibody through MACS allows for enrichment of prostate epithelial cells. It is possible that pure populations of epithelia survive better than when they have not been enriched due to contaminating cell types not overtaking the culture. It is also possible that CD326 sort enriches for a stem-cell population that tends to survive better in culture (Sundberg, Jansson et al. 2009).

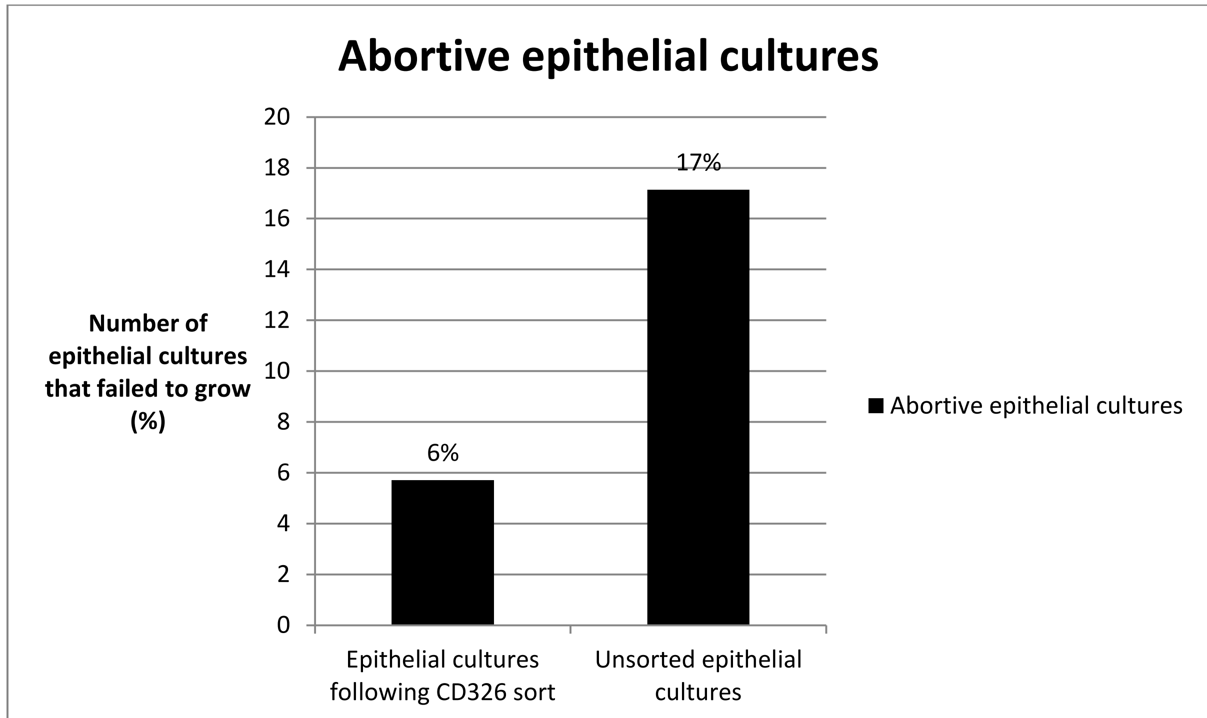
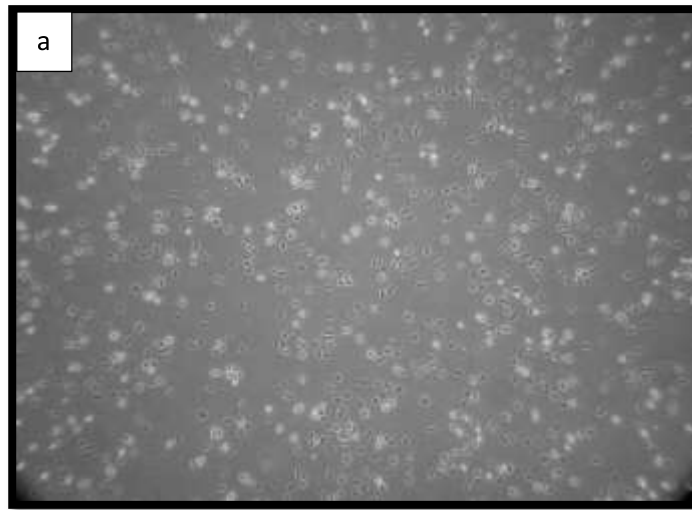


Figure 3.4. Figure showing abortive colonies without CD326 sort and after CD326 sort. CD326 sorting allows for better cell survival and the number of abortive colonies is reduced by half (from 17% to 6%).

Overall, it was noticed that epithelial cultures struggled to reach higher subcultures.In addition it was noted that Human Epithelial Antigen played a crucial role in facilitating robustness of epithelial cultures. One explanation for this might be that although differential gravity centrifugation yields an epithelial purity of >95% (Robinson, Neal et al. 1998, Heer, Robson et al. 2007); the cultures at that point are contaminated with blood cells as well as endothelial cells. It is possible that these contaminants compete with epithelial cells for nutrition and possibly outgrow them and cause these epithelial cultures to die out.

3.3.2.1 Morphology of epithelial cells

Prostate epithelial cells grew as colonies and demonstrated a characteristic cobble-stone morphology (Figure 3.1-3.9) . Cells started to cluster and showed colony formation from week 1 of culture (Figure 3.5 and 3.6). Well established colonies were observed by week 4 (Figure 3.7 and 3.8). The colonies demonstrated variable morphology with some colonies depicting a distinct border and densely clustered cells and some others depicting irregular borders and loosely-clustered cells (Figure 3.7 and 3.8). Cultures were most often passaged at this point. Colony size did not increase beyond week 6. At this point un-passaged colonies started to demonstrate an altered morphology. The compact borders started to become irregular and indistinct and the cells started to disperse and die out within the colony (Figure 3.10). Cells showed altered morphology with an increase in cell size and altered cytoplasmic-nuclear ratio. Irregularity in cell shapes and vacuolation was also noted. Cultures that were not subcultured at this point detached off the floor and eventually perished. Cells rarely grew as monolayers. Subculture before attainment of maximal-density has been documented as an essential factor favouring successful serial passages (Chaproniere and McKeehan 1986).



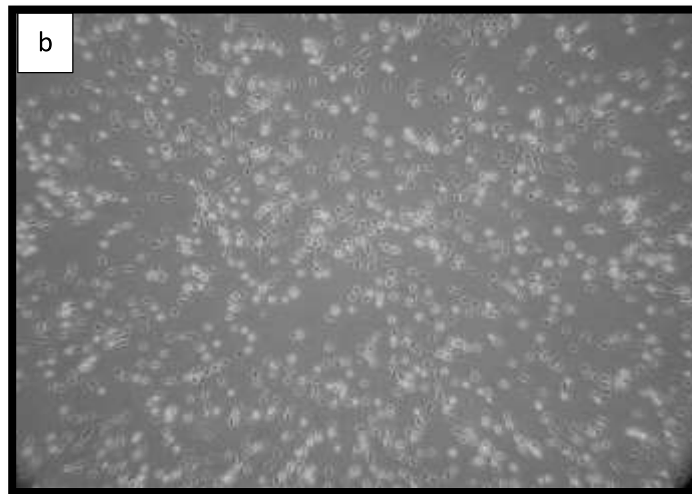
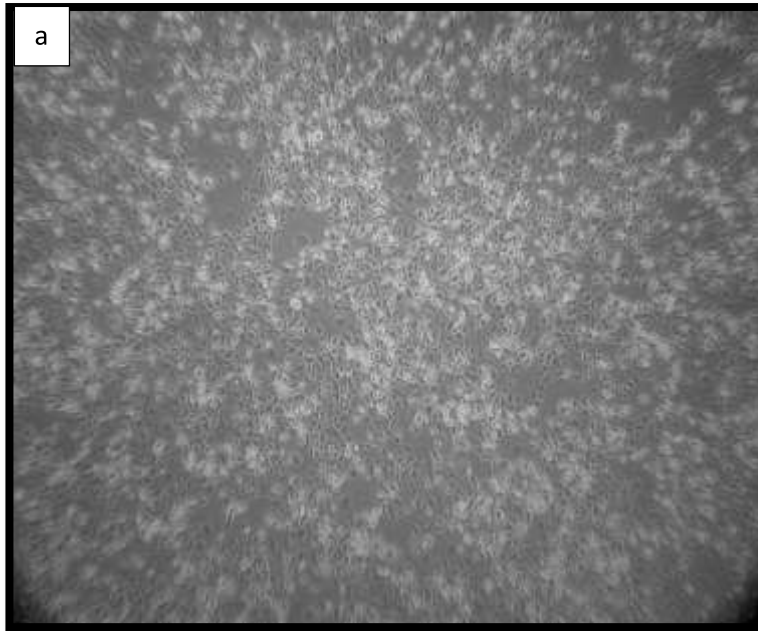


Figure 3.5. Epithelial cells immediately following extraction and prior to addition of feeder cells. a. The cells are initially not very confluent, are relatively sparse and do not yet form colonies. b. However at week 1 epithelial cells start to aggregate in clusters



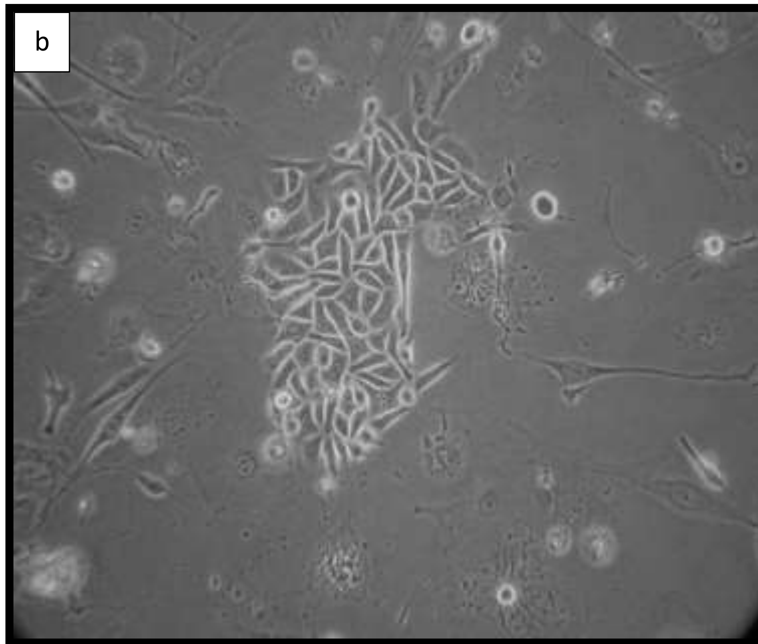


Figure 3.6. Epithelial cells at week1 – a. cells start to cluster and b. gradually form colonies. Cells have been seeded on a layer of inactivated STO feeder cells

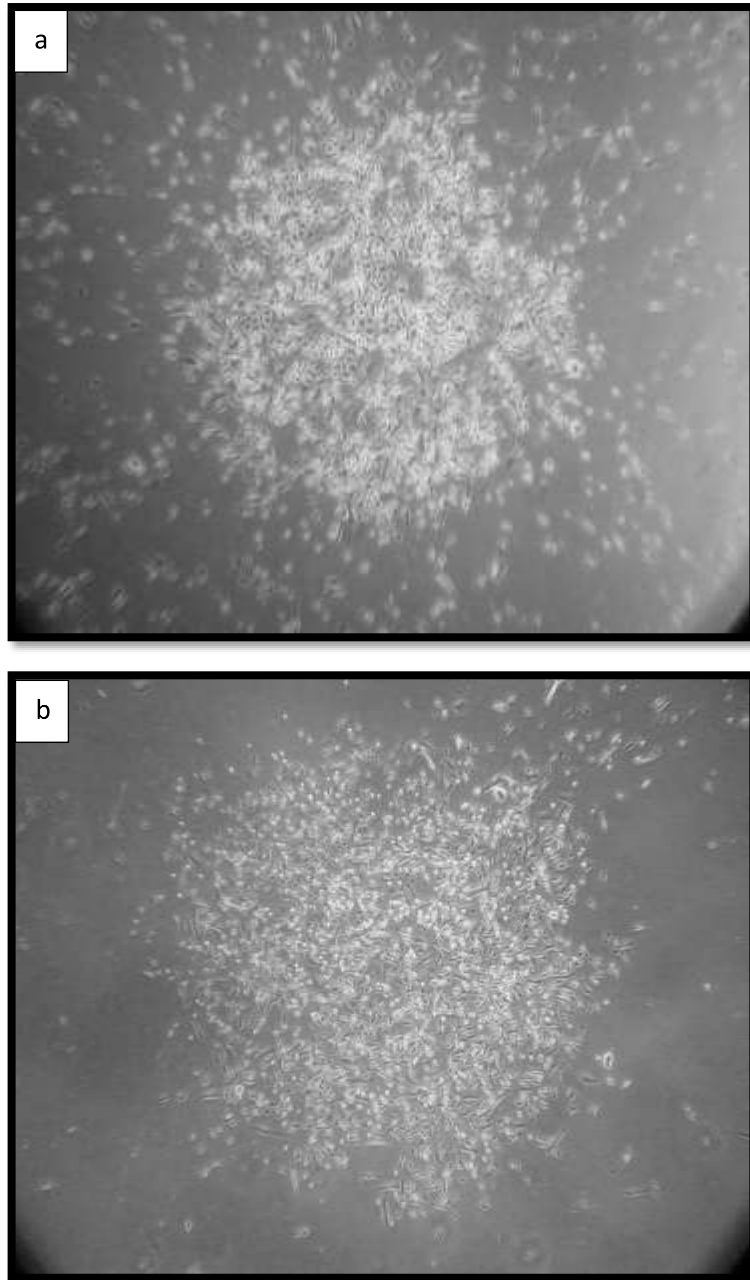


Figure 3.7. Figure demonstrating epithelial cell colonies at week 3. a. Epithelial colony with irregular borders and dispersed morphology. b. Epithelial colony that is comparatively more densely packed and demonstrates distinct border.

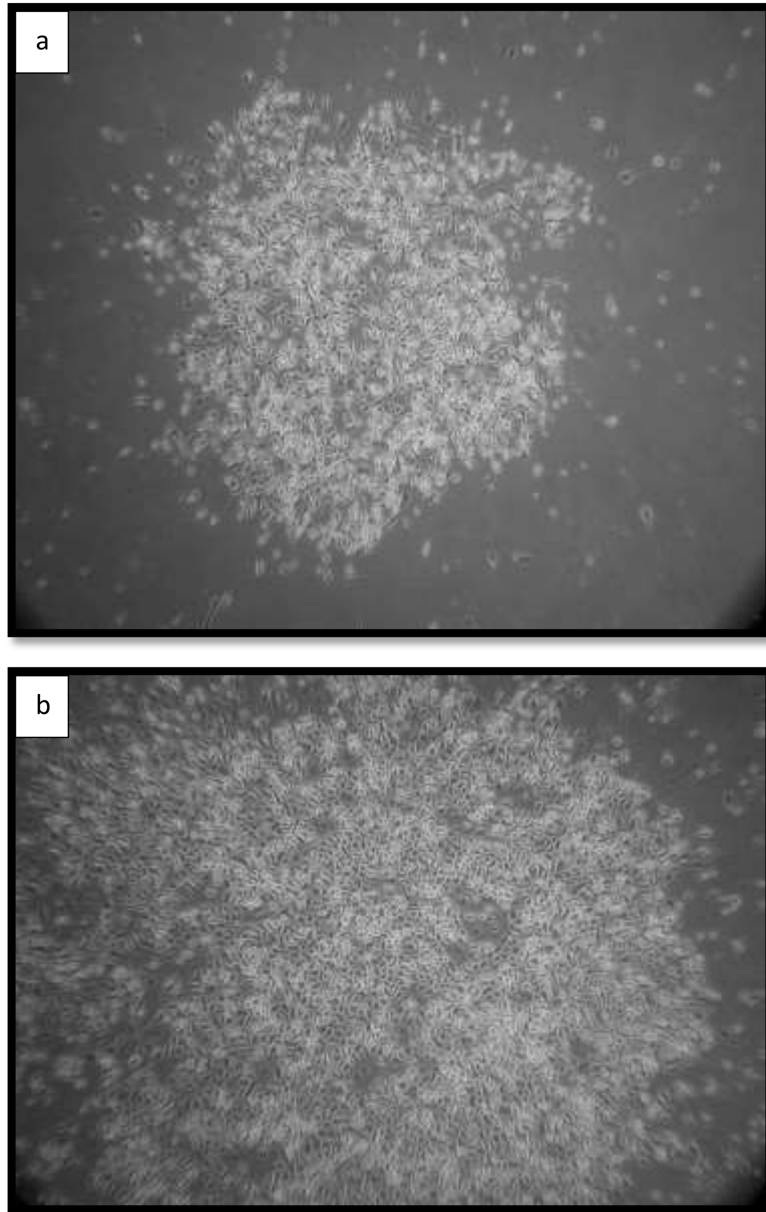


Figure 3.8. Epithelial cell colony at week 4. a. The colonies start to acquire a more compact morphology although some of them depict irregular borders. X4 magnification. b. Epithelial cell cultures grow in a cobble-stone morphology at higher magnification . X10 magnification

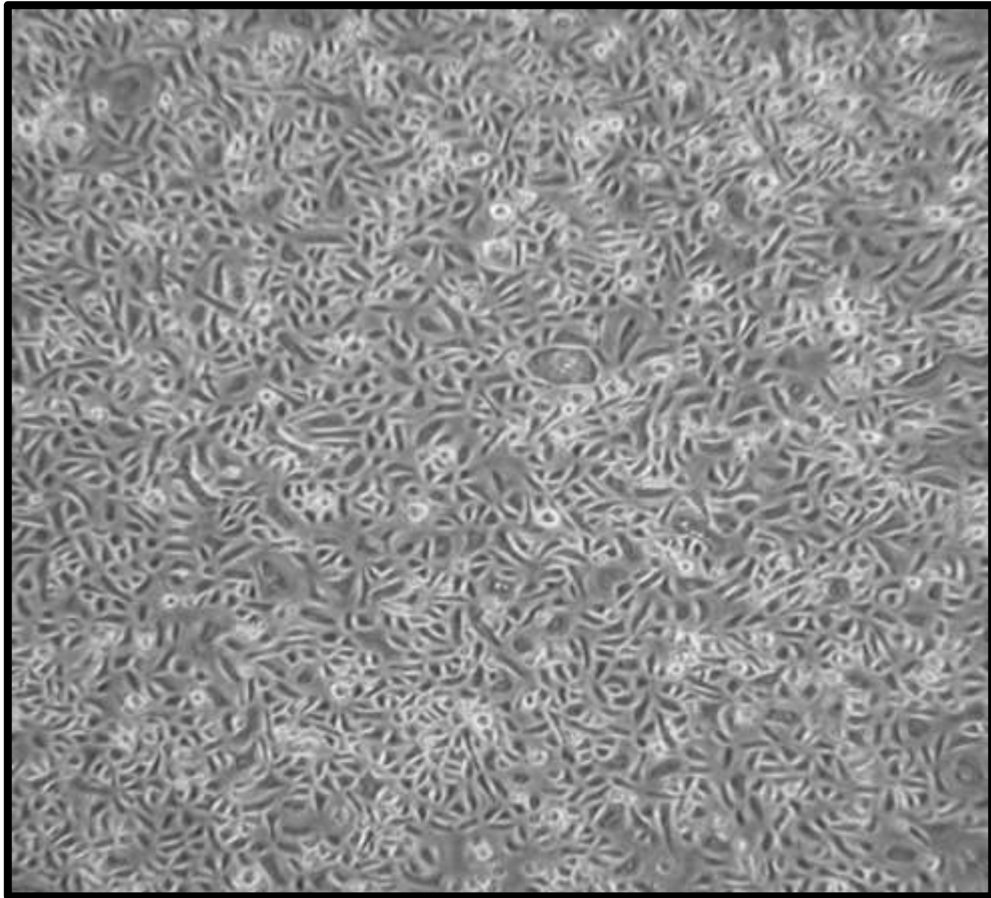


Figure 3.9. Primary prostate epithelial cells showing cobble-stone morphology. Magnification X40

It was also noted that epithelial cultures were not sustainable over higher subcultures. Cultures did not show any overt phenotypic variation till passage 1. However cultures beyond P1 showed an increased tendency towards differentiation and cultures were increasingly difficult to establish (Figures 3.10-3.12). This suggests that primary culture of epithelia from solid tissues is difficult to establish and maintain over longer time intervals thereby underscoring the need for a more robust and convenient *in vitro* model that will simulate prostatic environment and physiology as accurately as possible.

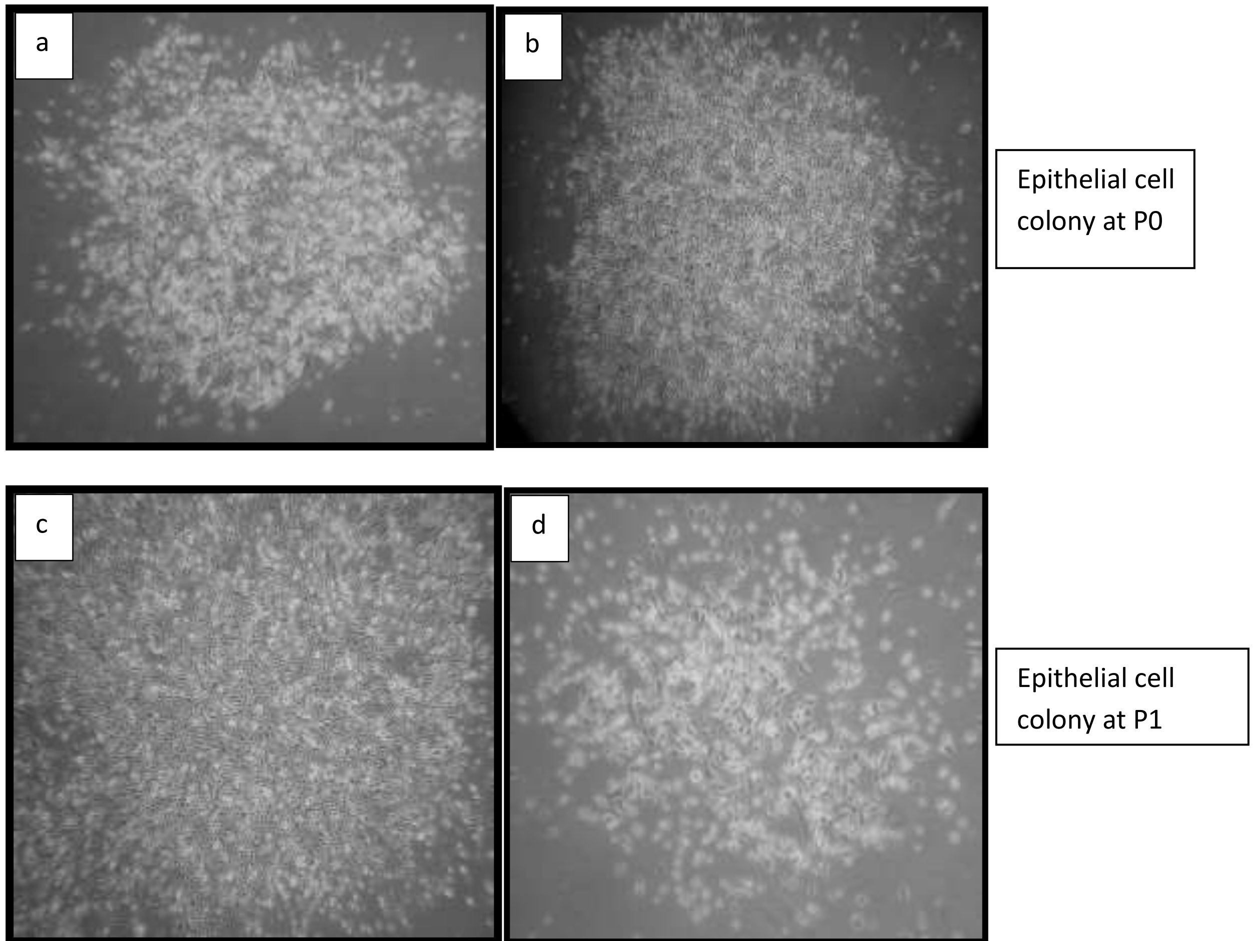


Figure 3.10. Epithelial cells at P0 (a. and b.) and P1 (c. and d.), colonies tend to get more and more differentiated with increase in passage number.

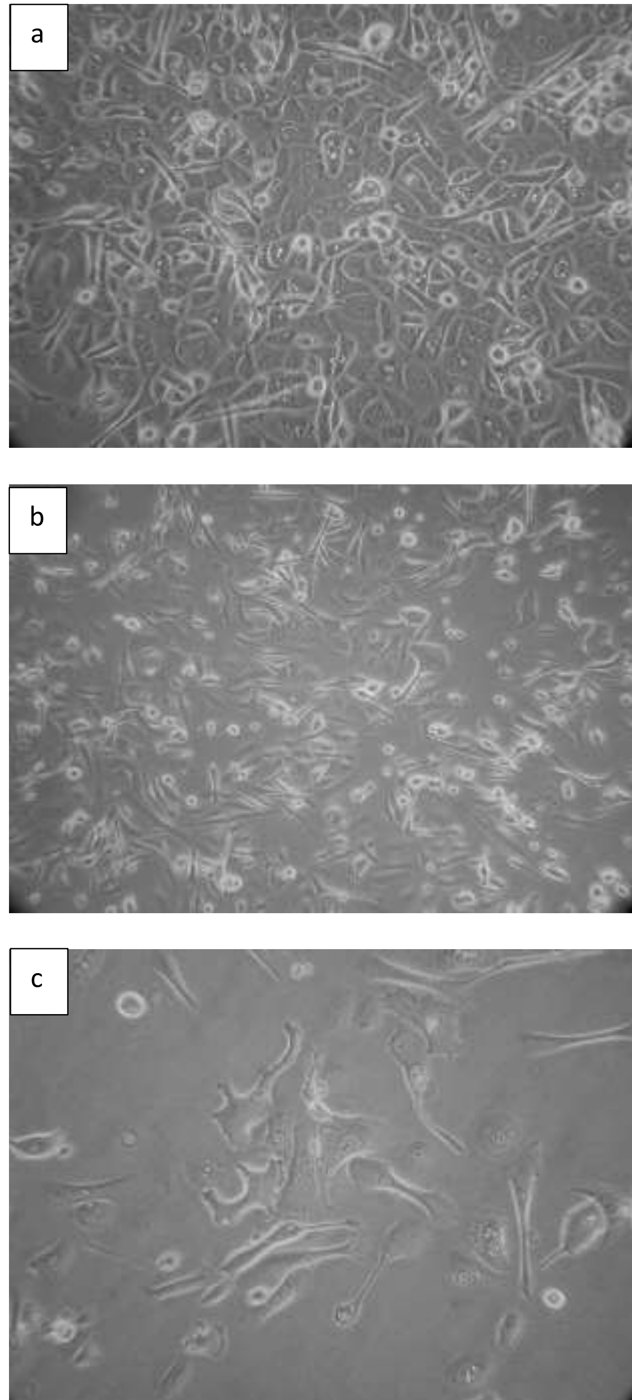


Figure 3.11. Morphology of prostate primary epithelia beyond passage 1. a. Altered morphology of epithelial cells at P2. b. and c. At higher magnification the cells look senescent and vacuolated and start to lose their cobble-stone morphology.

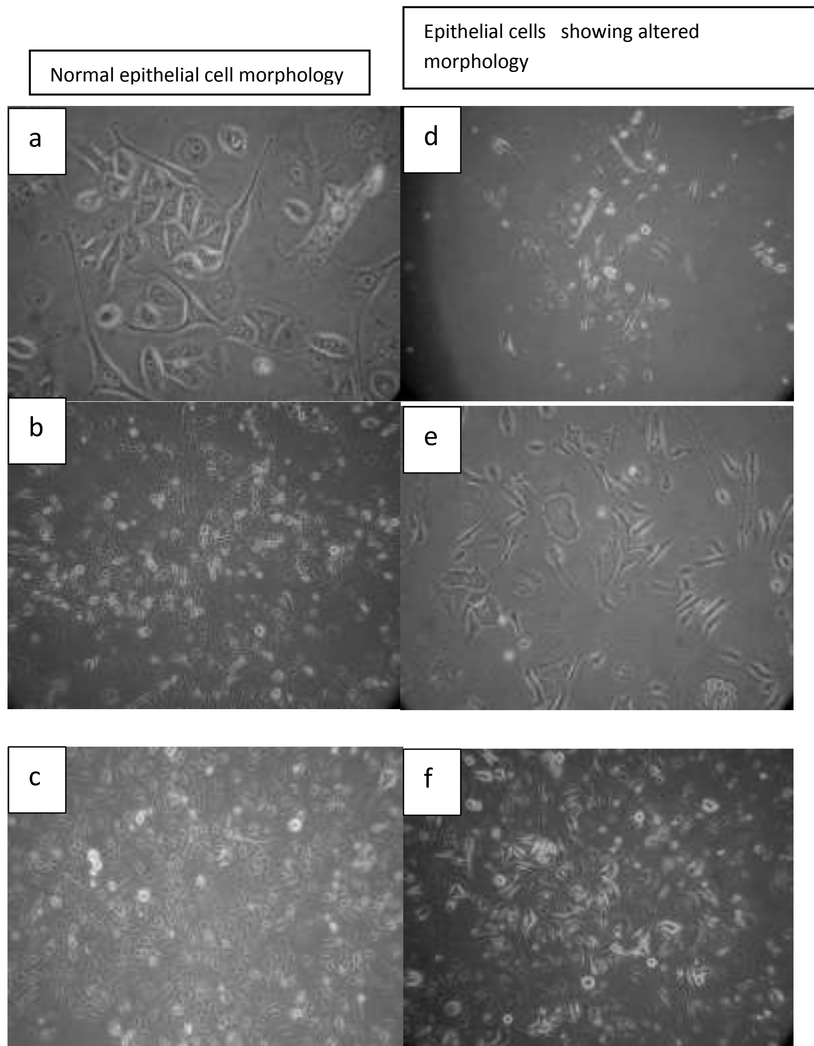
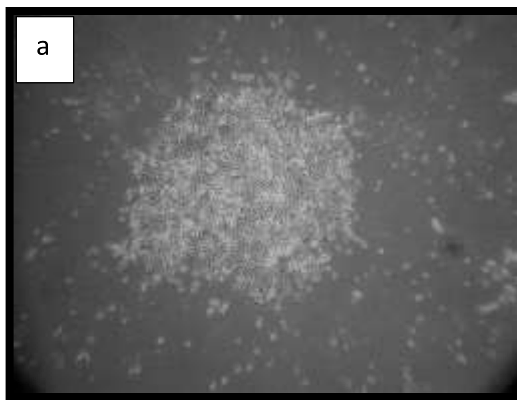


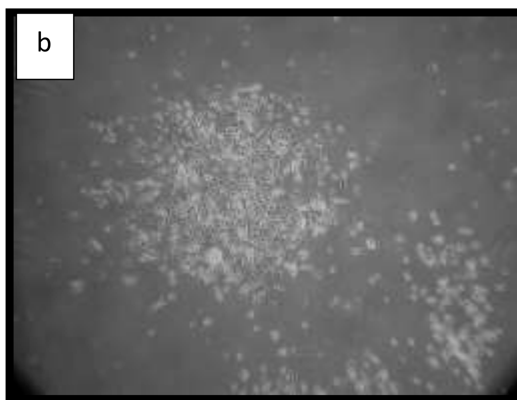
Figure 3.12. Epithelial cell morphology over long time periods in culture. Healthy epithelial cell culture (a. b. and c.). Figures d. e. and f. shows cultures that were not sub-cultured beyond week 6, cells here show altered morphology with increased cell size and vacuolation.

3.3.2.2 Epithelial cell colonies

Three types of colonies were identified with primary epithelial cell culture namely the holoclones, paraclones and the intermediate meroclone-type colonies (Figure 3.13). Holoclones are described as large colonies with smooth rounded perimeters; paraclones are smaller colonies with an irregular perimeter. Meroclones are an intermediate variety with serrated borders (Barrandon and Green 1987). Distinct colony formation based on individual proliferation abilities was first described in keratinocytes (Barrandon and Green 1987); a similar description of prostate epithelial colonies has also been described prostate epithelial culture (Collins, Habib et al. 2001). Holoclones are regarded to have the highest growth potential and contain small rapidly multiplying stem-cell like cells whilst paraclones show limited proliferation and rapid terminal differentiation (Barrandon and Green 1987). Meroclones or type II colonies demonstrate heterogeneity and contain both small and terminally differentiating cells (Barrandon and Green 1987, Collins, Habib et al. 2001).

In our cultures, all the three colony types were noted. Culture by means of rapid type I collagen adhesion highly selected for holoclones comprising stem-cell like cells as compared to a 20 minutes type I collagen adhesion which selected primarily for viable cells. Also, cultures following rapid-adhesion resulted in higher success rates survived higher subcultures better than the ‘viable’ cells.





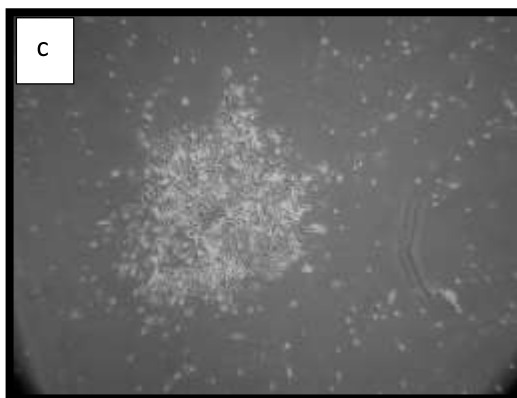
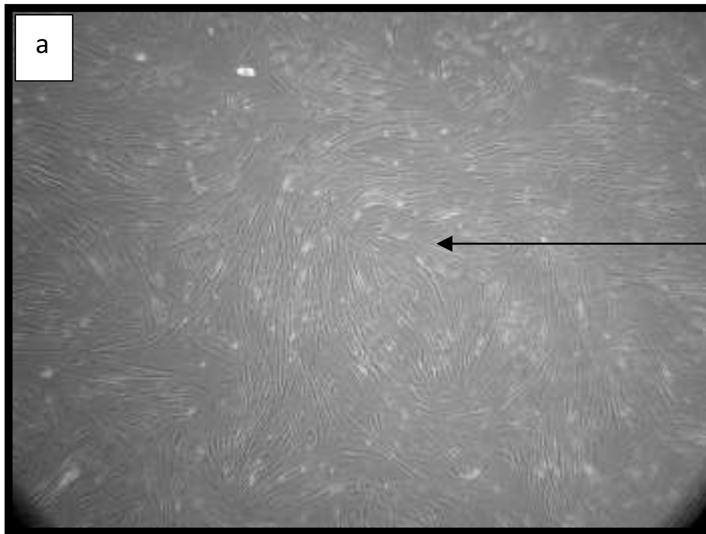


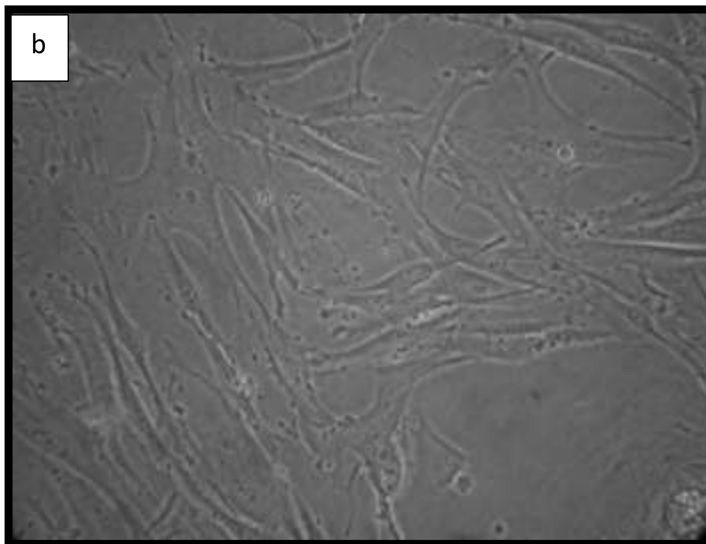
Figure 3.13. Colony architecture in prostate stem epithelial cell types. Holoclones, meroclones and paraclones in epithelail cell culture. a. Holoclones are circular colonies with a relatively tight border and compact colony architecture. Holoclones mostly comprise undifferentiated cell types. b. Meroclones are an intermediate colony type between the holoclones and paraclones. c. Paraclones are colonies that are dispersed and lack definitive cell border, these colonies contain the largest number of differentiated cell types.

3.3. Culture of prostate stroma

A total of 40 stromal cultures were grown and success was nearly 100%. These cells are distinct from their epithelial counterparts in terms of cell size, shape and culture-type. Primary prostate stromal cultures grew as monolayers rather than colonies and demonstrated *in vitro* formation of whorl-like structures (Figure 3.14.a.). Individual stromal cells showed elongated and spindle-shaped cell morphology (Figure 3.14 b). Lamellae formation between cells was also noted. Stromal cultures survived higher serial passages better than their epithelial counterparts and were a lot more durable. These cultures have been split upto at least 13 times and there has been no visible change in morphology with higher subcultures (Figure 3.15).



Primary prostate stroma showing whorl-like patterns



Less confluent culture of primary prostate stroma showing lamellae-like structures (at higher magnification X40)

Figure 3.14. Primary prostate stromal culture. a. These cells grow in monolayers. X4 magnification b. Individually prostate stroma are elongated spindle shaped cells that grow in whorl-shaped patterns. X40 magnification.

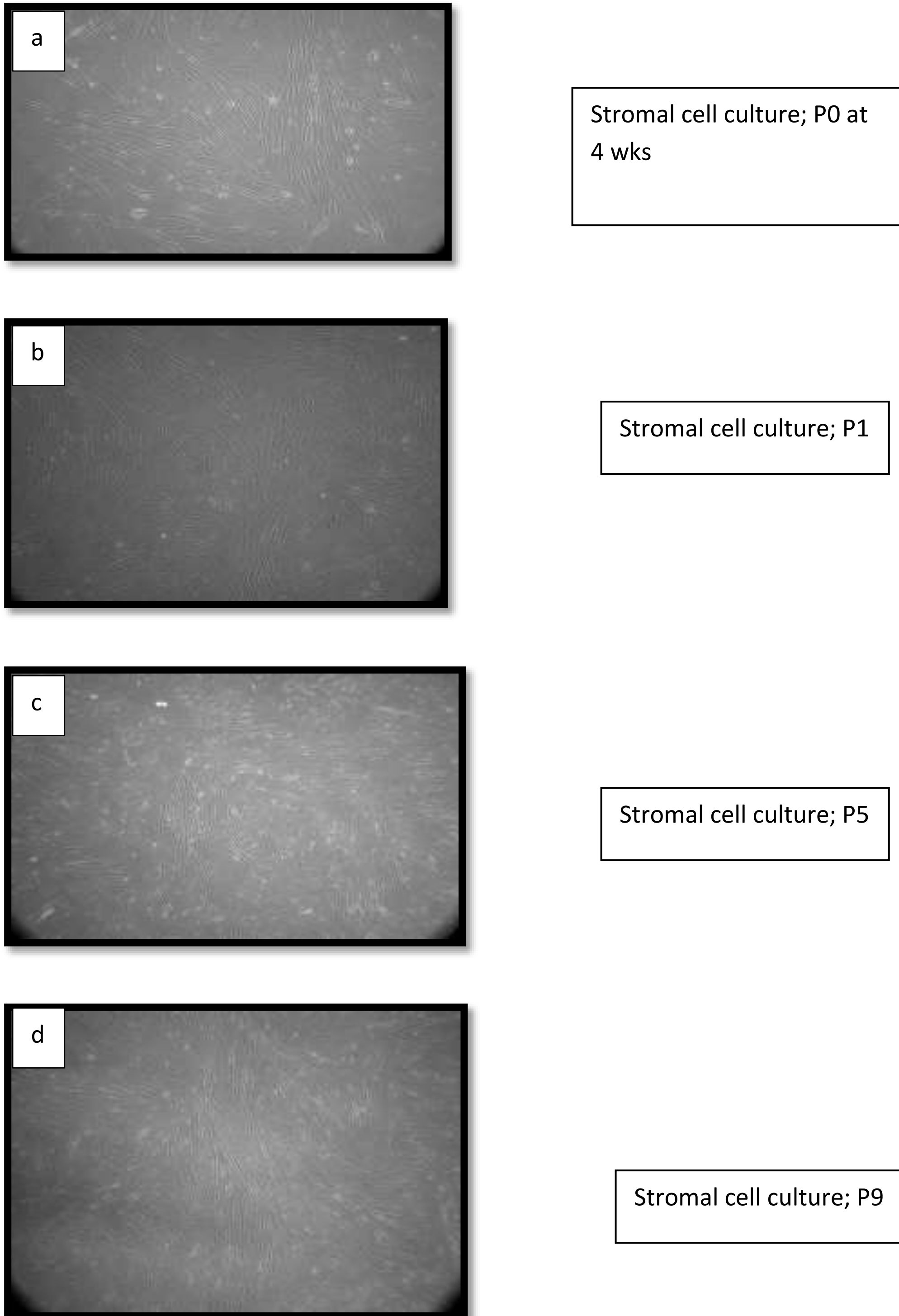


Figure 3.15. Primary prostate stromal cultures at different subcultures. The cells tend to grow well in culture and do not show any aberrant morphology over long time culture.

3.3.4. 3-D culture of prostate primaries

In vitro cell culture studies are two-dimensional whilst in real life tissues and organs are three-dimensional. 3-D studies bridge the gap between conventional in vitro studies and whole-animal physiology. Some of the advantages of 3-D cultures include preservation of epithelial cell polarity and cell shape, gene expression profiles that more closely simulate in vivo systems and 3-D matrix induced modulation of cell growth, gland branching and cell differentiation (Yamada and Cukierman 2007). 3-D culture systems have been established in the prostate in the hope of establishing a model that would better emulate prostate microenvironment and physiology (Lang, Stark et al. 2001, Lang, Smith et al. 2006).

In the 3-D cultures established by us, we noticed distinct 3-D epithelial and stromal morphologies. Firstly, it was observed that in general prostate primary cells grew and proliferated more rapidly in Matrigel than in 2-D. Prostate primary cells when seeded into Matrigel three dimensional formed spheroids (Figure 3.16). In contrast, primary stromal cells grew as lamellar matrices that apparently formed mesh-like structures and seemingly resembled a 2D-layered morphology (Figure 3.17). Epithelial cells showed increased commitment towards 3-D cultures compared to stromal cells which failed to show a typical 3-D morphology.

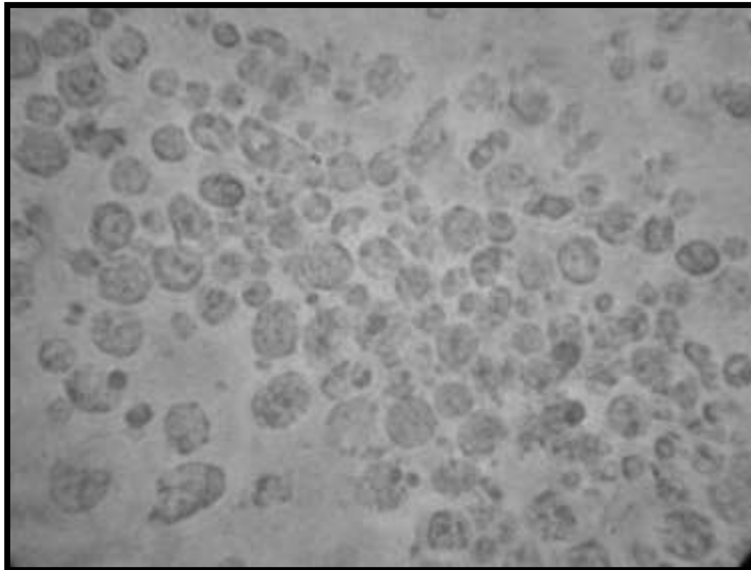
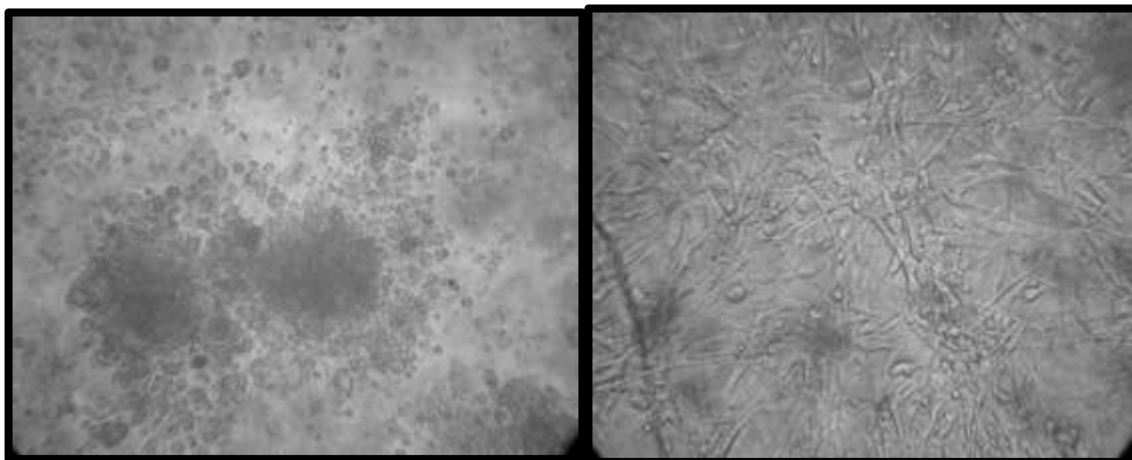


Figure. 3.16 Primary prostate epithelial cells in 3-D Matrigel culture. Cells grow in spheroid shaped structures.



Epithelial spheroid bodies – clustering to form spheres

Prostate stromal cells grow in Matrigel as lamellar matrices and do not show spheroid

Figure 3.17 Prostate epithelia and prostate stroma in Matrigel cultures. Prostate epithelia form spheroids while prostate stroma grow in lamellar matices that seem to disperse through he entire matrigel plug

Prostate cancer cell lines are not comprehensive with respect to prostate cancer phenotypes – they do not represent primary prostate adenocarcinomas (Peehl 2005). Also cell lines do not represent the full breath of prostatic differentiation and thus do not serve as ideal models to study prostate growth and development. Furthermore, long-term culture of cell lines results in alteration of their biological properties (Peehl 2005) which adversely affects quality of research. Most of the limitations seen with cell line culture are resolved by means of primary culture which can be regarded as the gold-standard technique for studying development and differentiation in organ-systems. Although prostate primary culture techniques have been significantly optimised by several studies (Chaproniere and McKeehan 1986, Robinson, Neal et al. 1998, Collins, Habib et al. 2001); this technique is met with several hurdles such as tedious handling and low yield. Furthermore, this study observed variations between cultures as well over long durations of the same culture. Clinical variations between patients account for differences between cultures thereby hindering standardisation of experimental techniques. 3-D cultures are a development on primary 2-D cultures – however experimentation that can be performed with these cultures is limited.

In this study it has been observed that factors that facilitate primary prostate epithelial culture growth and proliferation are:

1. Sorting with the help of Human Epithelial antigen

2. Presence of cell-cell contact and

3. Use of extracellular matrix supplemented with stromal-derived factors. While comparing epithelial cell culture to stromal cell culture it was observed that although stromal cells proliferated rapidly in 2-D cultures, they did not show significant 3-D anatomy in matrigel cultures.

3.3.5 Optimisation of primers for stem-cell and prostate-specific-differentiation gene expression profile in prostate cancer cell lines

Real time RT-PCR was used to evaluate mRNA expression of 12 different genes in four different prostate cancer cell lines – LNCaP; LNCaP-AI; PC3 and DU 145 (Figure 3.18). The primer sets for all the genes were thus optimised and confirmed to have been working. Non-specific binding and possibility of primer-dimers were ruled out through analysis of the dissociation curves. A single melt peak in the dissociation curves ruled out possibilities of primer-dimers and ensured that a single product was being amplified. Gene expression profiles defining three levels of plasticity were studied – those defining pluripotency, those defining adult stem cells with restricted differentiation and those that are terminally differentiated.

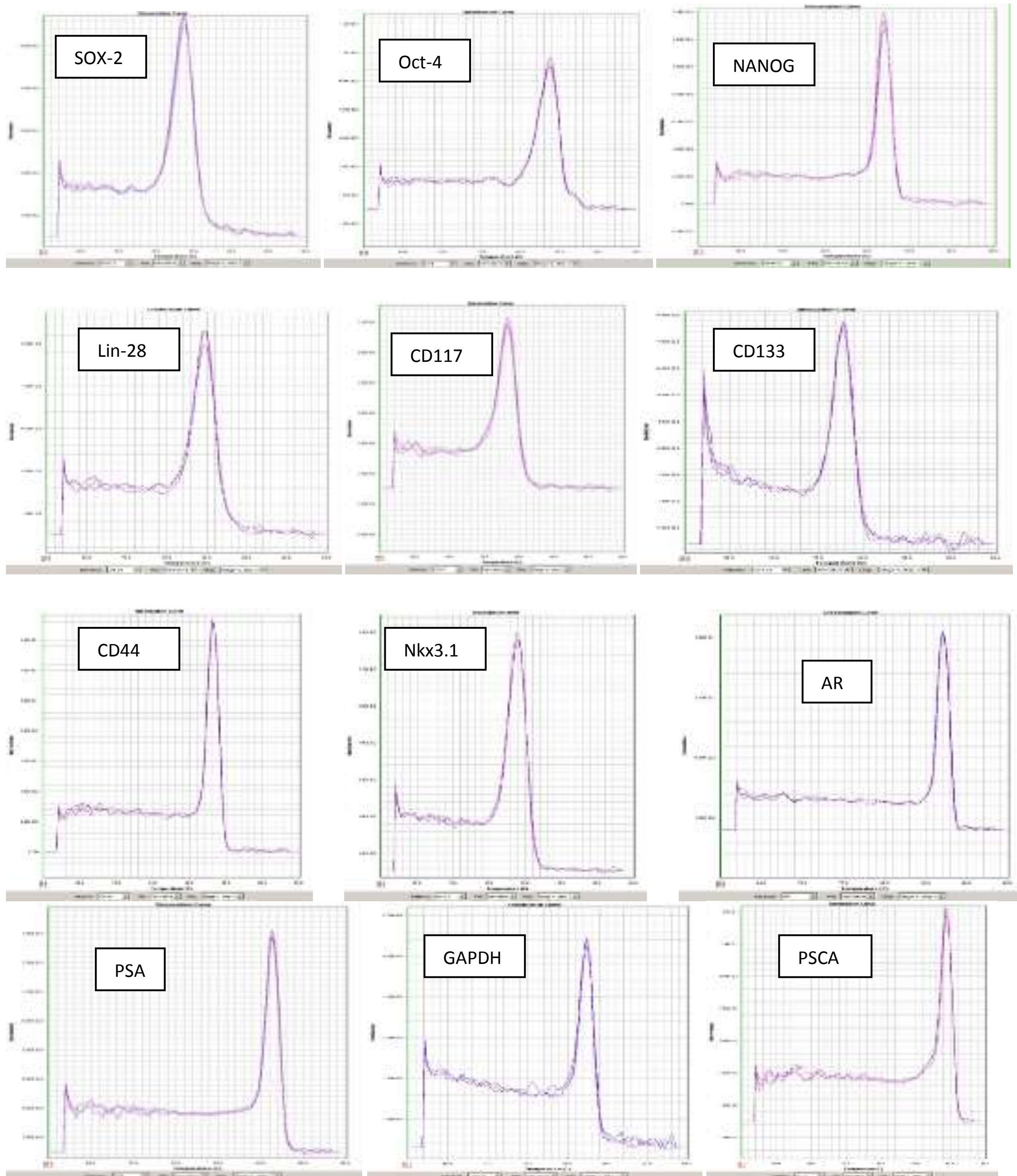


Figure 3.18. The dissociation curves for all the different primers showing a single melting point curve for all samples. Plot shows amplification against temperature.

3.3.7.Optimisation of prostate stem cell gene primers

CD133 mRNA transcript was not detected in DU 145 cells. Both PC3 and DU 145 cells showed higher levels of CD 44 expression. Similar to pluripotency genes, relative mRNA expression was lower in androgen-independent LNCaP cells (Figure 3.19).

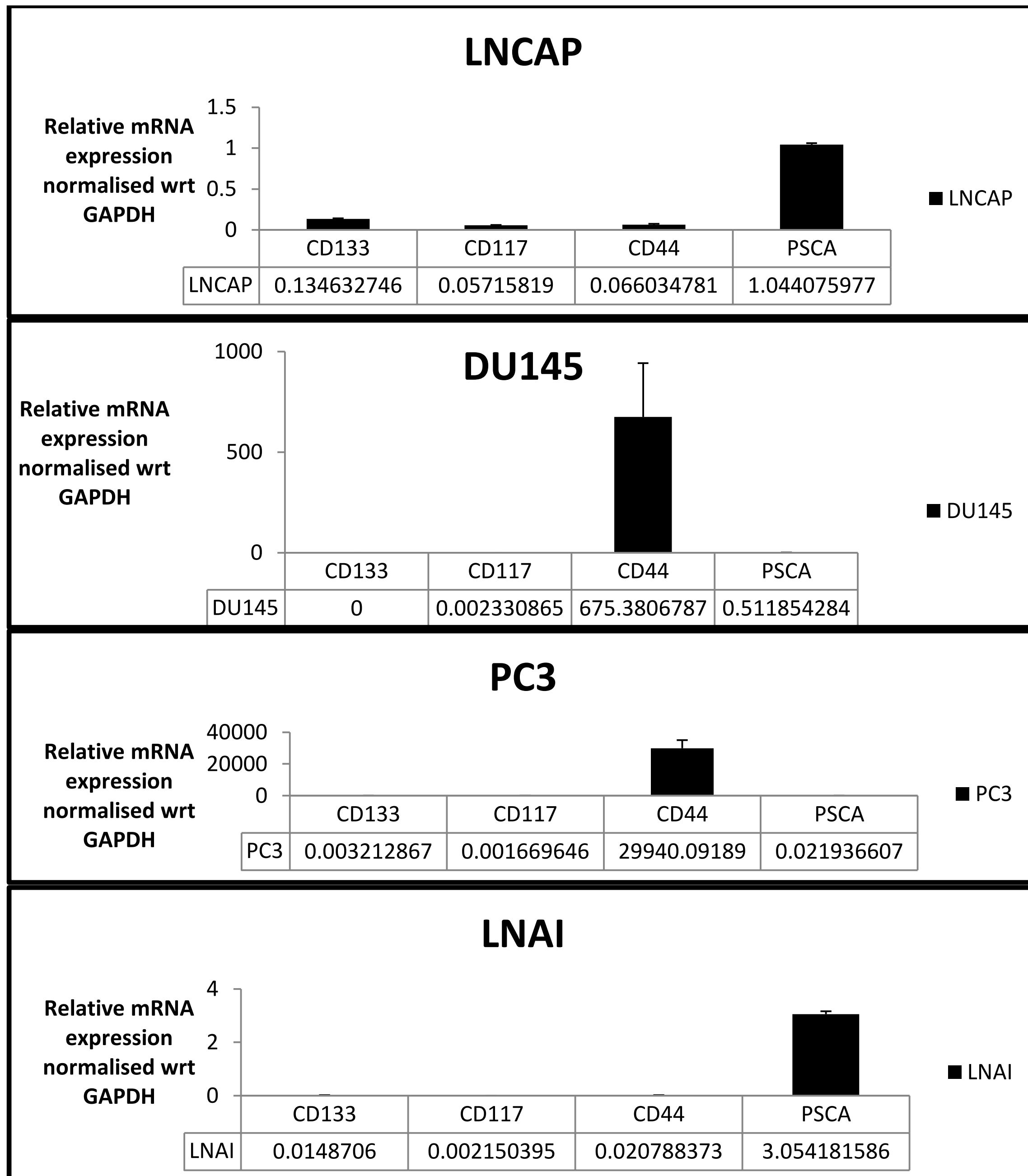


Figure 3.19 Prostate stem cell gene expression in prostate cancer cell lines. Expression of the various stem-cell markers are variable across the different cancer cell lines

1.3.8. Expression of Androgen responsive elements in prostate cancer cell lines

DU 145 and PC3 cell lines serve as negative controls for AR and PSA; these genes were not expressed in these two cell lines. Also it was noticed that though AR mRNA transcripts were relatively upregulated (as compared to LNCaP cells) in LNCaP-AI cells, these cells did not show any expression of PSA (Figure 3.20).

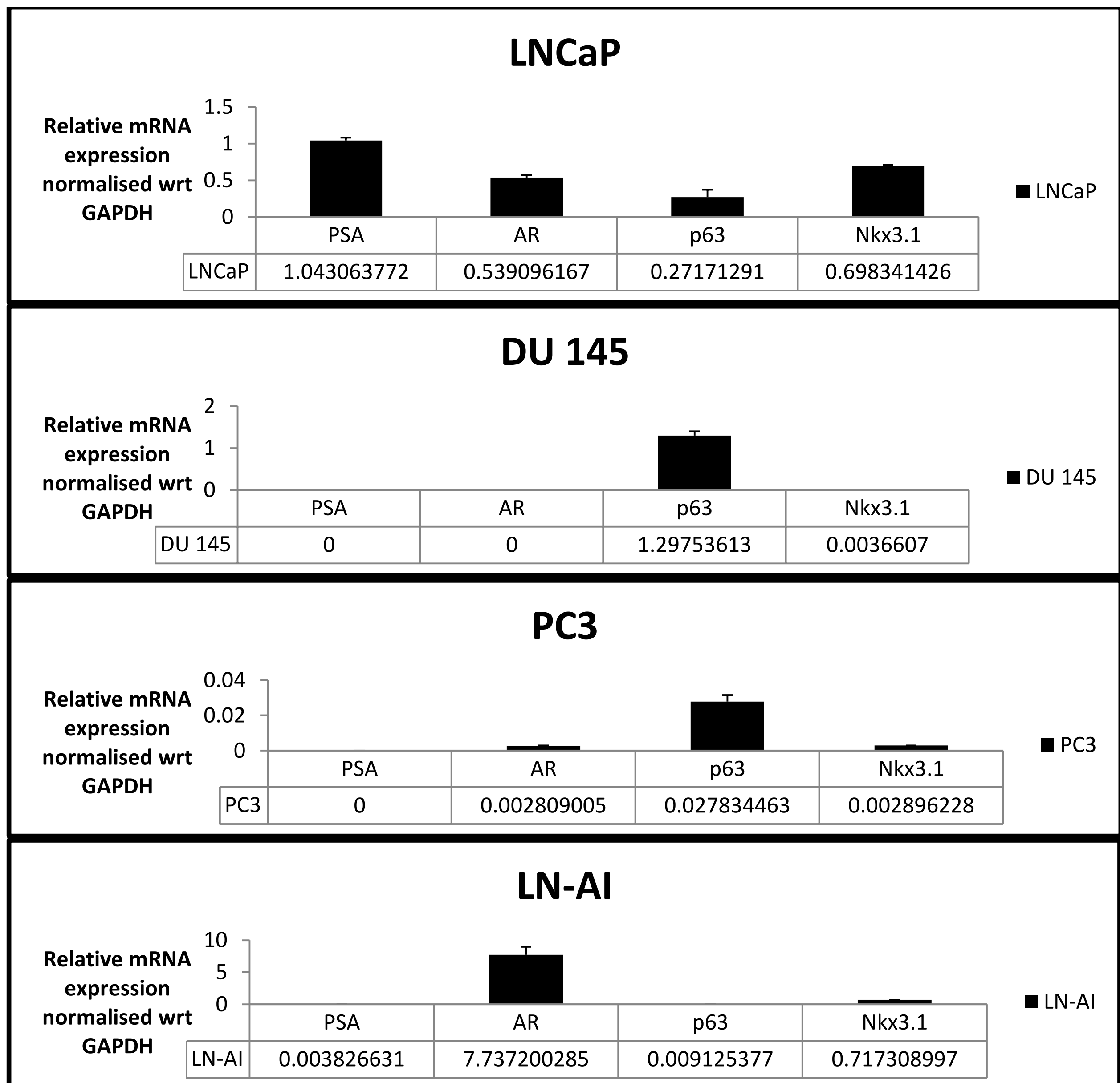


Figure 3.20 Expression of androgen regulated genes in prostate cancer cell lines. Note, no expression of AR and PSA in cell lines PC3 and DU145.

3.3.9. Characterisation of prostate primaries

3.3.9.1. Characterisation of prostate primary stroma

Prostate stroma was cultured in monolayers and was analysed at initial and late passages. Initial passages showed evidence of endothelial contamination (through presence of CD 146 +ve cells), epithelial contamination (through presence of CD24 +ve cells) and haematopoietic cells (through presence of CD45 +ve cells) (Figure 3.21-3.23). However as these cells were passaged, it was noted that the contamination level was significantly reduced at passage 1

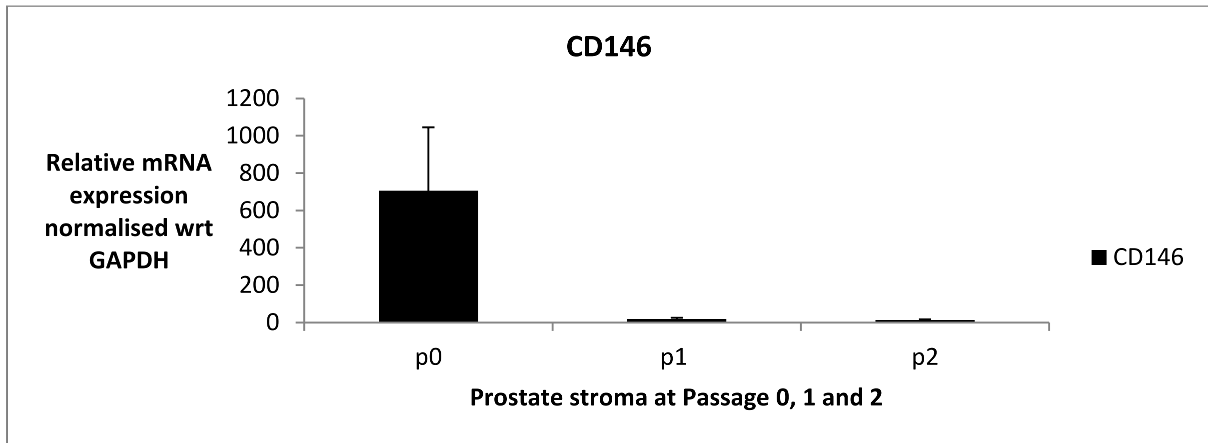


Figure 3.21. Prostate primary stroma at p0, p1 and p2 showing absence of endothelial contamination at p1 passage

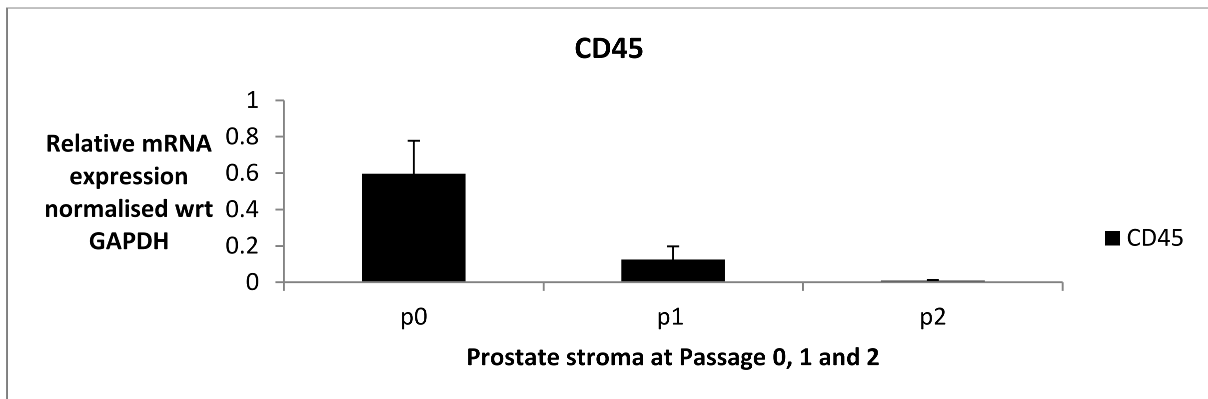


Figure 3.22. Prostate primary stroma at p0, p1 and p2 showing significant reduction of haematopoietic contamination at p1 passage

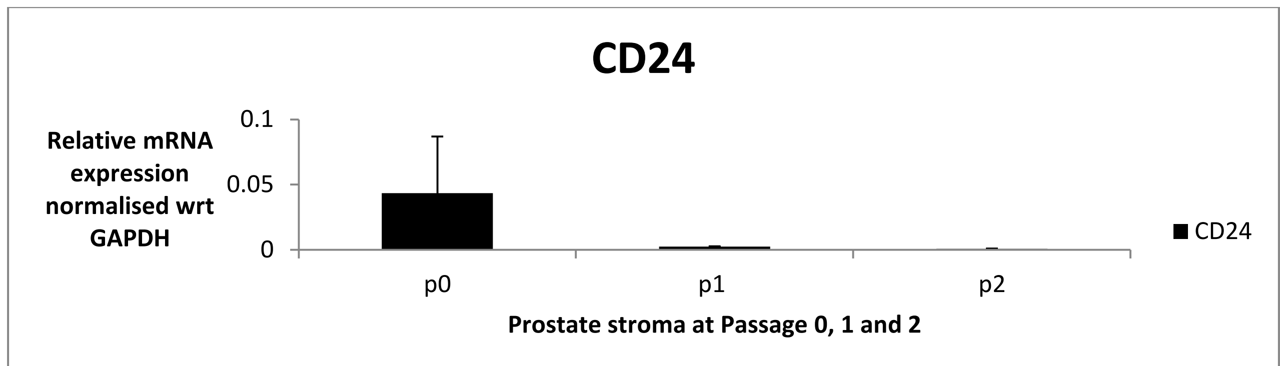


Figure 3.23. Prostate primary stroma at p0, p1 and p2 showing significant reduction of epithelial contamination at p1 passage

Next, the cells were analysed for levels of endogenous pluripotent transcript (Figure 3.24). It is well reported in literature that the expression of certain pluripotent transcripts is not confined to pluripotent and stem cells; varying levels of pluripotent transcript expression is seen in certain cancer cells (Gu, Yuan et al. 2007, Rodriguez-Pinilla, Sarrio et al. 2007, Chiou, Wang et al. 2010, Rajasekhar, Studer et al. 2011) and somatic cells . It is important to know the levels of endogenous pluripotent expression since re-programming factor stoichiometry is critical in the achievement of a pluripotent status (Carey, Markoulaki et al. 2011).

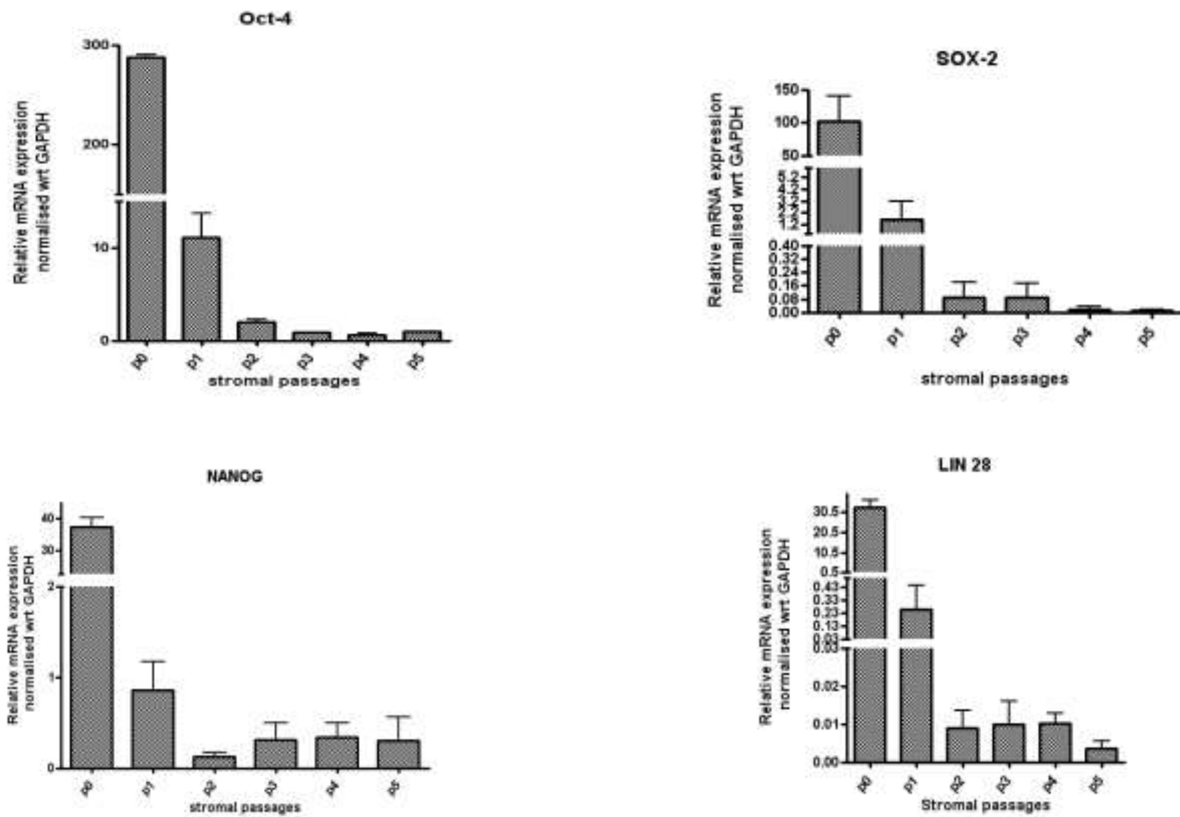


Figure 3.24 Endogenous expression of Oct4, SOX2, NANOG and Lin 28 in prostate stroma at different passage numbers.

Transcript analyses revealed that basal expression of pluripotency transcripts is observed in prostate primary cells; however the expression levels went down at higher passages. There was very high expression in the cells at harvest, but given the high levels of contamination seen with cells at p0 it is possible that the high levels of expression can be explained by the contaminating cells as opposed to the prostate cells themselves. P1 cultures were chosen for transduction since at this passage the cells were relatively pure and demonstrated moderate to high relative levels of pluripotency transcripts. These experiments were later re-considered after comparing them against pluripotent transcript expression levels in H9 human embryonic stem cells .

Next, these prostate cells were studied for expression of AR and PSA (Figure 3.25). P0 stroma expressed both AR and PSA suggesting that these cells contain functionally active AR. However, this data is against current literature which suggests that AR in prostate stroma is not functional and that these cells do not express any PSA. This discrepancy in PSA expression seen with p0 stroma can again be explained by the fact that these cells contained fractions of epithelial cells that are known to express functionally active AR and thus PSA. Reduction in AR levels with higher passage is unexpected and this cannot be explained at this stage of this study.

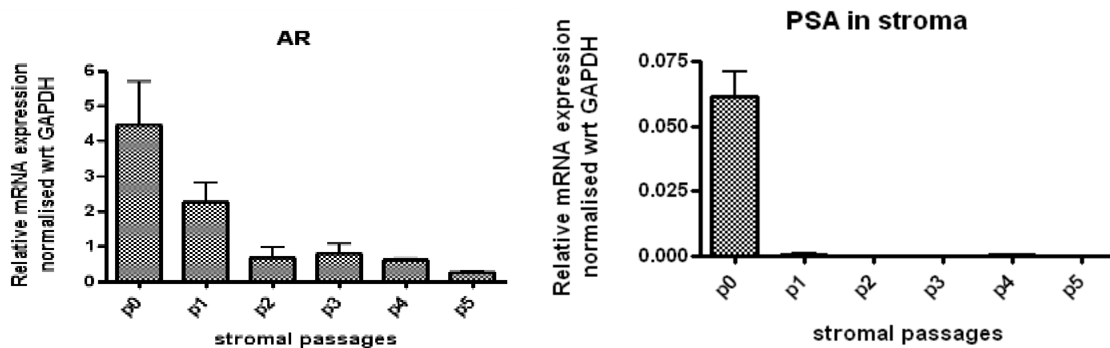


Figure 3.25 AR and PSA expression in prostate primary stroma at initial harvest and at higher cultures.

1.3.9.2. Characterisation of prostate primary epithelia

Endogenous embryonic stem-cell transcripts were also analysed in prostate primary epithelia (Figure 3.26). Epithelial cells were purified with the help of EpCAM (CD324) MACS sort prior to culture and analysis. MACS separation through EpCAM antigen has been established as a robust method towards establishment of pure epithelial cultures (Williamson, Hepburn et al. 2012). Hence p0 epithelial cultures have been confirmed to be pure epithelial fractions and any transcript expression seen in these cells is attributed to prostate epithelia alone. Like prostatic stroma, these cells did show endogenous expression of embryonic transcripts and the levels here decreased with increasing passage number. Once again the exact reason behind this remains equivocal.

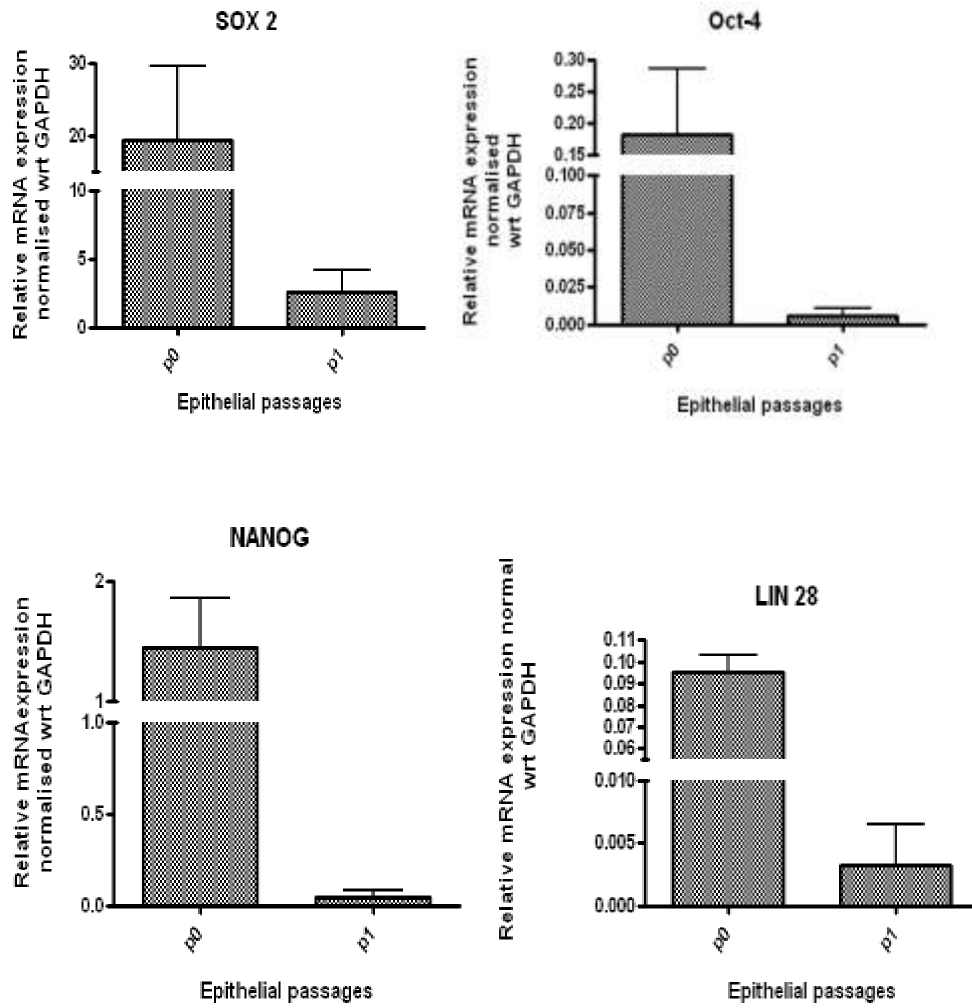


Figure 3.26. Endogenous expression of Oct4, SOX2, NANOG and Lin 28 in prostate stroma at different passages

AR and PSA expression was also measured in epithelia cells (Figure 3.27 and 3.28). Since the epithelial cells had been EpCAM sorted, only p1 cultures were analysed and compared against the prostate stroma.

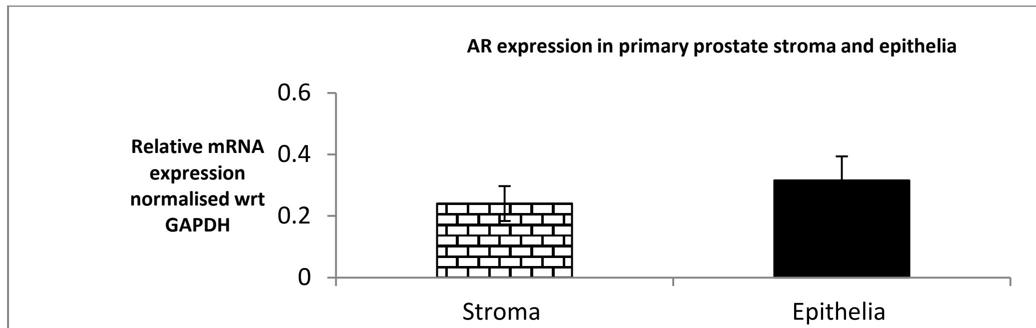


Figure 3.27. AR expression in prostate primary stroma and epithelia

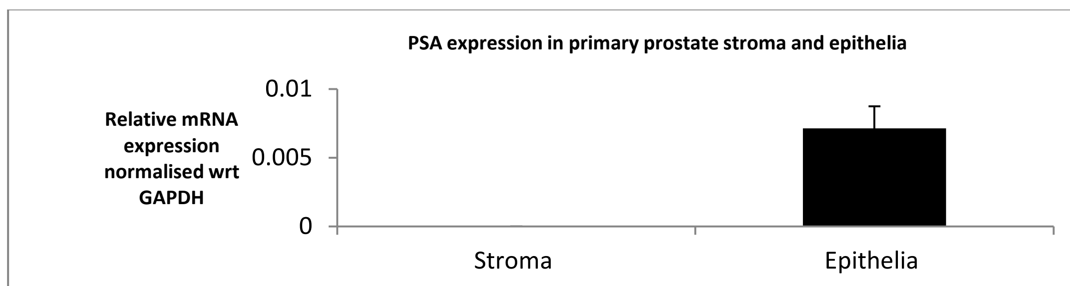


Figure 3.28. PSA expression in prostate epithelia and stroma. PSA was expressed only by prostate epithelia thereby corroborating the presence of functional AR in our epithelia.

As expected, epithelial cells showed AR and PSA expression but only AR expression was observed for their respective stromal counterparts. This is consistent with the current literature and corroborates that the epithelia used in this project expressed functionally active AR as is the case with prostate-specific epithelia. PSA expression was absent at very high passage. This may be explained by the fact that the epithelial cultures were established through an EpCAM sort and were further subjected to the rapid adhesion assay. Both the techniques specifically enrich for stem cell populations. It has also been shown that AR and PSA expression is reduced in prostate stem cells (Williamson, Hepburn et al. 2012).

3.4 Conclusion

Robust and healthy cells are critical for successful iPS re-programming. Hence, a great deal of emphasis was given to this part of the project in optimising the protocol so as to ensure healthy and sustainable cells in culture. Furthermore, it was absolutely essential to confirm quality control in terms of cell characterisation and purity. The main purpose of establishing the Pro-iPS model was to study prostate-specific growth and development; given the containment of a tissue-specific epigenetic memory in iPS cells it was important to ascertain the prostatic quality of the initial target cells.

Chapter 4

4. Lentiviral transduction of prostate primary stroma and epithelia

4.1. Introduction

This chapter will describe the protocol that was adapted to re-programme primary prostate stroma and epithelia by means of lentiviral transduction. Two different protocols were tested. Both protocols involved lentiviral transduction. One system was based on the Thomson re-programming cocktails, namely OSLN (Oct4, SOX2, Lin28 and NANOG) and used the four different pluripotency factors in four independent lentiviral constructs. The other system was more sophisticated and used a single polycistronic construct that contained all the 4 OSKM transcripts in one element. Each of the different transcripts is released as individual polypeptides through the '2A' peptide cleavage sites. Furthermore, this system also incorporated a Cre-recombinase technology that allowed for manual removal of the exogene following iPS transduction. Both the OSKM and OSLN systems have their own advantages and disadvantages as discussed below. However, the most important factor that best describes the suitability of the two different re-programming manoeuvres was their ability to successfully introduce the pluripotent transcripts at the appropriate level into the target cells. This conclusion is based on the fact that neonatal foreskin was used as positive control. These cells are relatively easy to re-programme and were used alongside prostate cells to decide upon the most suitable iPS-induction protocol.

4.2. Aims

1. To induce iPS-phenotype in prostate primary stroma

2. To induce iPS-phenotype in prostate primary epithelia

3. To re-programme human neonatal foreskin fibroblast cells into skin-iPS cells

4.3. Specific methods and Results

4.3.1. iPS induction – OSLN factors

4.3.1.1. Transduction Media

This protocol used 4 separate iPS-induction viral vectors - Stemgent® Reprogramming Lentiviruses: Human SOX2 (ST070012, Stemgent®, USA), Human Oct4 (ST070013, Stemgent®, USA), Human Nanog (ST070017, Stemgent®, USA) and Human Lin 28 (ST070016, Stemgent®, USA). The concentrations of each of the different viruses are:

1. Human SOX2 = 2.4 x 10^6 TU/mL
2. Human Oct4 = 7.2 x 10^6 TU/mL
3. Human Nanog = 4.4 x 10^6 TU/mL
4. Human Lin 28 = 4.1 x 10^6 TU/mL

Transduction media contained the respective parental media in 10% FCS and 1% Pen-Strep and the relevant amount of virus so as to target an MOI of 10. Multiplicity of infection refers to the ratio of infectious agents (in this case virus) to the number of cells. MOI depends on the transducibility of different cell lines/cell types and must be optimised accordingly. While very low MOI would result in inefficient transduction and thus a failed iPS induction, a very high MOI can result in multiple integration sites and persistence of the exogene. The volume of virus to be added to the transduction media was calculated using the following formulae:

Transduction units (TU) = MOI X Number of target cells. Eg. To re-programme 100,000 cells using an MOI of 10 (10 viral particles will target 1 primary cell) 1x10^6 transduction units (TU) of the respective virus will be needed.

Volume of each virus = TU ÷ concentration of each virus

100,000 cells were transduced with an MOI of 10. For each of the 4 virus constructs the number of transduction units worked out to be $1x10^6$. The volume of each virus used was thus:

1. hSOX2 (Lot 1722):

$1x10^6/2.4 \times 10^6 = 417$ µl

2. hOct4 (Lot 1608):

$1x10^6/7.2 \times 10^6 = 139$ µl

3. hLIN28 (Lot 1611):

$1x10^6/4.1 \times 10^6 = 244$ µl

4. hNanog (Lot 1612):

$1x10^6/4.4 \times 10^6 = 228$ µl

Appropriate viral volumes were added to 2mL of transduction media, for this example the calculations were:

2000 + 417 + 139 + 244 + 228 = 3028 µl of total transduction media

Lastly polybrene was added to a final concentration of 0.6ug/ml. Again for the given example the volume of polybrene required was:

(0.66x3028)/(1000) = 1.8 µg of polybrene

The transduction media was made up fresh and was used immediately

4.3.1.2. Transduction of primary prostate stroma

100,000 prostate stroma cells were seeded onto each well of a 6-well plate. Patint (aged ~ 60 years) sample was collected following consent and ethical approval. The patient's histoptaholigical analysis report showed no evidence of malignant foci in the sample. 24 hours later, the cells were washed once in 1XPBS and the freshly prepared transduction media was added onto the cells. The cells were kept in transduction media for 48 hours. One well containing polybrene in media alone was used as a control well. After 48 hours, the viruses were removed and the cells were fed with 10% FCS-containing RPMI 1640. The plates were incubated for 7 days at 37˚C. No change in morphology was noticed. The cells grew to confluence without showing evidence of any obvious apoptosis when compared to the polybrene control.

24.3.1.3. Culturing prostate stroma cells post-transduction

The first 7 days following transduction is detailed as the initial phase of re-programming when the cells go through the first transition phase and acquire a more epithelial phenotype (Li, Liang et al. 2010). A fraction of these cells would then be expected to move on to producing their own endogenous pluripotency factors and therefore become independent of the exogene(s). However, when using the OSLN system no change in morphology was noticed, the cells retained their mesenchyme phenotype and grew to confluence. On the 7th post-transduction day, 6-well plates were coated with Matrigel as described below.

Materials:

BD Matrigel™ hESC-qualified Matrix, 5ml Vial

Method:

Dilution factor: dilution is calculated for each lot based on the protein concentration. Aliquots are prepared according to the dilution factor provided on the Certificate of Analysis. Aliquots were stored at -70°C for up to 6 months, freeze-thaw cycles were avoided. One day before use, Matrigel aliquots were thawed overnight on ice. The solution was made up to the appropriate dilution and 1ml was used per well of a 6-well plate. Plates were incubated overnight at 4°C. Matrigel was aspirated immediately prior to use, the plates were incubated for 30 minutes with 2ml of the relevant cell culture medium.

On the 8th day post-transduction, the stroma fibroblasts were trypsinised and seeded at a density of 10,000/well onto gelatin coated plates in Nutristem™ XF/FF Culture Medium

(Stemgent Cat. No. 01-0005). Nutristem media is a pluripotent stem cell specific media recommended by the manufacturer (Stemgent) and is detailed to contain all the factors required for stem cell culture and proliferation. The cells were allowed to adhere for 24 hours following which the media was changed and 2ml of fresh Nutristem media was added. Media was changed every 48 hours for the next 3-4 weeks.

Around week 3-week 4 post transduction, colony-formation was noticed. Small irregular shaped colonies started to appear and these were transferred onto MEF-feeder plates and cultured for another 4-6 weeks. Although it was expected that these colonies would grow and multiply to generate iPS cell-lines, no such proliferation was noted. The colonies did however grow in size, but demonstrated only a 20% increase in their size over 4 weeks. When these were divided into smaller clumps they they did not show any significant proliferation, the individual clumps failed to grow significantly. Given the irregular shape of the colonies, it was decided that these would be categorized as either partially re-programmed or abortive colonies. Consequently, this protocol using the OSLN re-programming factors was discontinued.

4.3.2. iPS induction using OSKM factors

Although c-Myc is a well characterised oncogene and is known to cause death and differentiation in human pluripotent stem cells (Sumi, Tsuneyoshi et al. 2007) the OSKM factors are more efficient in re-programming human fibroblasts and also offer faster kinetics of iPS induction (Robinton and Daley 2012). Hence, it was decided that the OSLN cocktail would be replaced with the OSKM cocktail for Pro-iPS generation. The new kit used contained all the 4 OSKM factors in a single construct separated by a self-cleaving 2A peptide (Figure 4.1) – this helps to ensure that all successfully transduced cells would receive equal amounts of each of the four transcription factors, thereby improving the stoichiometry between the 4 re-programming factors. This in turn would improve the efficiency of iPS-induction (Carey, Markoulaki et al. 2011). Furthermore, the construct was floxed at both ends by LoxP sites that would further enable removal of the exogene through a Cre-recombinase technology. Hence, this system offered the capacity for cMyc removal in case excessive death and apoptosis was noticed in the Pro-iPS.

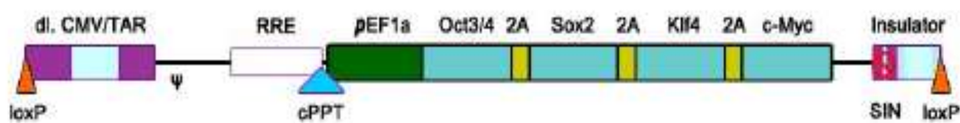


Figure 4.1. OSKM 4 in1 construct used to generate Pro-iPS. All the four pluripotent transcripts are present in a single construct. The construct is flanked by two LoxP sites at either ends to facilitate removal of the exogenous transcript through Cre-recombinase

technology. Courtesy Allele biotechnology: http://www.allelebiotech.com/4-in-1-lentiviral-particles-for-ipscs-generation-human-oskm/

The initial step was to optimise the transduction efficiency by means of a GFP-tagged empty vector control (Figure 4.2). The mWasabi GFP virus stock was transduced into prostate fibroblast cells at different MOIs.

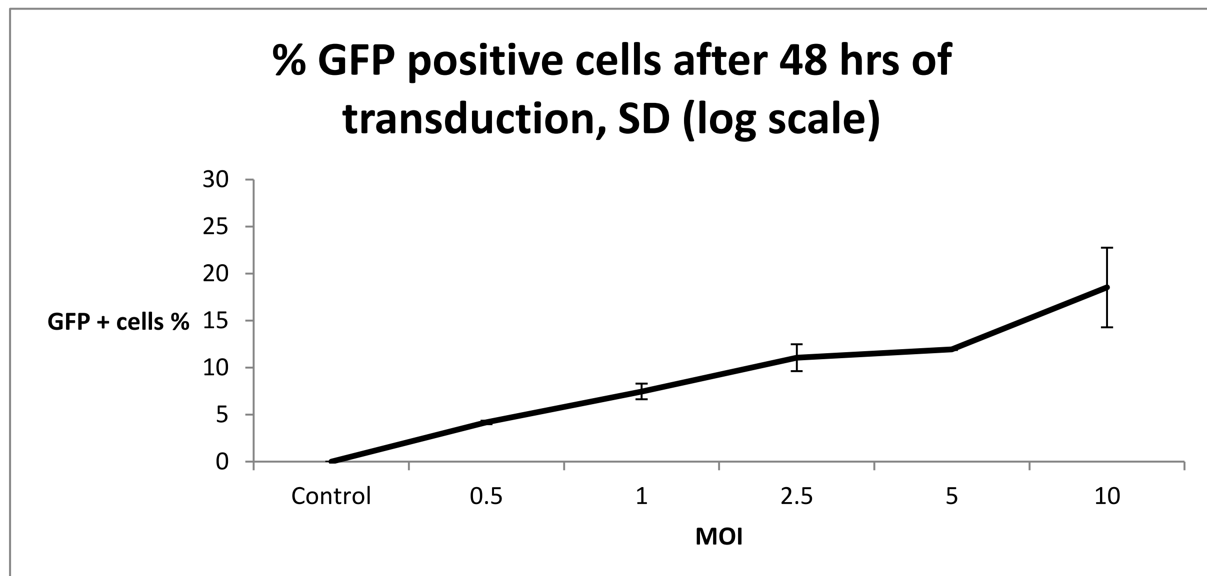


Figure 4.2. Optimising MOI for the iPS induction in prostate. Primary prostate stroma was used at passage 2. Cells were transduced with the mWasabi GFP-tagged empty viral vector and the percentage of GFP positive cells was determined through FACS analysis.

It was noted that at an MOI of 10, the net transduction efficiency was near to 20% (Figure 4.2). MOI is defined as the number of infective particles (in this case virus containing OSKM) per target cell. Hence, in theory an MOI of 10 would mean that each of the target prostate fibroblasts is being aimed with 10 viral particles. It would also indicate that in theory all transduced cells would contain more than 1 integration site since the number of integrations

sites is also dependant on the MOI.For example an MOI of 0.3 would predict that 3% of the total target cell population may contain multiple integration sites. Hence, transduction efficiency should potentially be 100% for MOI 10. Nevertheless, the poor transduction efficiencies observed even at higher MOI suggested that these fibroblast cells were not easily amenable to the transduction protocol and with this system very few cells were getting successfully transduced. This may have been due to several reasons, including general resistance of the cells to lentiviral transduction, poor health of the target cell cultures or it is also possible that the viral titres were not as potent as described on the stock vials. However, the prostate fibroblast cells were never sorted for enrichment nor were they analysed to assess presence of varying phenotypes within the general population. It is possible that the stroma cell cultures contained cell fractions within them that were more amenable to the transduction procedure and these cells were transduced more successfully than the others. It has been well documented that stem cells and/or cells with a primitive de-differentiated phenotype are more amenable to iPS induction than more mature differentiated cells (Wakao, Kitada et al. 2011). If this is indeed the case then multiple exogene integration sites would be a limitation, but this can be tackled by monitoring transgene expression and by manually removing the exogene once stable Pro-iPS lines have been established.

The iPS cell induction protocol with the OSKM 4 in 1 construct was then subjected to certain alterations. In the OSLN protocol, cells were seeded onto Matrigel-coated plates on Day 7 of transduction. It is more difficult to grow human pluripotent stem cells in the absence of MEF feeder cells and thus it was decided that on Day 7 of transduction the prostate fibroblasts would be directly seeded onto MEF feeder plates. Further, the pluripotent stem cell culture media was changed from Nutristem to a KO-DMEM-based pluripotent stem cell culture media. Hypoxia is known to regulate stem cell renewal and it also activates signalling

pathways such as Notch and increases the expression of Oct4 (Keith and Simon 2007). Keeping this in mind, it was decided that in the absence of a hypoxic incubator, a reducing agent such as β-mercaptoethanol would be added to the media that would act to mop up any oxygen free radicals and thus potentially reduce the likelihood of oxidative stress.

In the first 7 days following transduction the cells were closely monitored for evidence of altered morphology as well as cell death. No significant cell death was seen and the cells grew to confluence. However, a change in morphology was noted at this time. In certain regions, cell clusters started to aggregate and these cells showed a reduction in cell size, they started acquiring a rounded shape and also demonstrated a cobble-stone like morphology. This suggested that a mesenchymal-epithelial transition (MET) was potentially occurring in a fraction of the transduced cells. Details of this MET-like phase during iPS re-programming in the prostate has been described in details in Chapter 5. The cells were maintained on the feeder plates for a maximum of 14 days and were fed with fresh pluripotent stem cells media (Chapter 2) every 48-72 hours. After 14 days of culture, the cells were trypsinised and transferred onto fresh MEF feeder plates and weret maintained in such a manner for 6-8 weeks. No iPS colony formation was seen, but small irregular colonies started to appear which were picked and cultured for another 4-8 weeks. These are described in Chapter 5.

It was decided that the protocol would be modified to include the use of conditioned media since this has been shown to improve the efficiency of iPS generation (Tilgner. K. 2010). For this purpose, two types of conditioned media were generated, a MEF feeder conditioned media and a human pluripotent stem cell conditioned media. MEF feeder media was generated by treating MEF cells at a density of 50,000/ml with pluripotent stem cell culture media and by collecting this media every 48 hours and by filter-sterilising it before use. Pluripotent stem cell culture media was generated in a similar fashion, by treating skin iPS cells with pluripotent stem cell culture media and by collecting the media every 48 hours. The

skin iPS conditioned media was then centrifuged at 1500 rpm X 5 minutes to precipitate any floating skin iPS cells and the supernatant media was collected and passed twice through a 0.2 µm filter.

The conditioned media were collected after treating the relevant cells for 24-48 hours at 37˚C. Given that the pluripotent stem cell culture media is stable only at 4˚C, some of the components of the media would likely have been depleted or degraded at 37˚C. Hence, it was decided that the conditioned media would be used in conjunction with freshly prepared pluripotent stem cell culture media. This way it was possible to utilise the paracrine factors secreted by pluripotent stem cells that may putatively contain growth factors that are critical in promoting pluripotent stem cell maintenance and enhanced transition of transduced cell fractions into iPS cells by means of a positive loop mechanism (Tilgner. K. 2010). It has been documented that during iPS transduction in human neonatal foreskin fibroblast cells, cultures treated with hES-conditioned media and MEF-conditioned media in a 1:1 ratio results in an increase in iPS colony generation that is 6-fold higher than in cultures that were treated with MEF-conditioned media alone (Tilgner. K. 2010). For Pro-iPS generation, freshly prepared pluripotent stem cell culture media was used in conjunction with MEF-conditioned media and pluripotent stem cell culture media in a 1:1:1 ratio. Cells were treated with this reprogramming media from Day 10 post-transduction until stable Pro-iPS cell lines were generated. Stable Pro-iPS cell lines were established between weeks 4 and 6 post transduction (Moad, Pal et al.). In this manner a total of 14 cell lines were generated and were expanded for characterisation.

4.3.3. Morphological description of Pro-iPS

Pro-iPS cells were similar to hES cells in morphology. At early passages, the colonies were not as compact and had irregular borders (Figure 4.3.b and 4.3.c.). They showed an inclination to rapidly differentiate at the centre and around the borders and were difficult to culture. This may be explained by the fact that iPS induction forces cells against their differentiation gradient and during the initial stages there would be a tendency for the newly re-programmed cells to roll down the epigenetic gradient to their original differentiated state (Hochedlinger and Plath 2009). However, it was noticed that with a few more passages and with increased culture time the cells gradually adapted to culture conditions and started showing a morphology characteristic of human embryonic stem cells (Figure 4.4, 4.5 and 4.6) (Moad, Pal et al.).

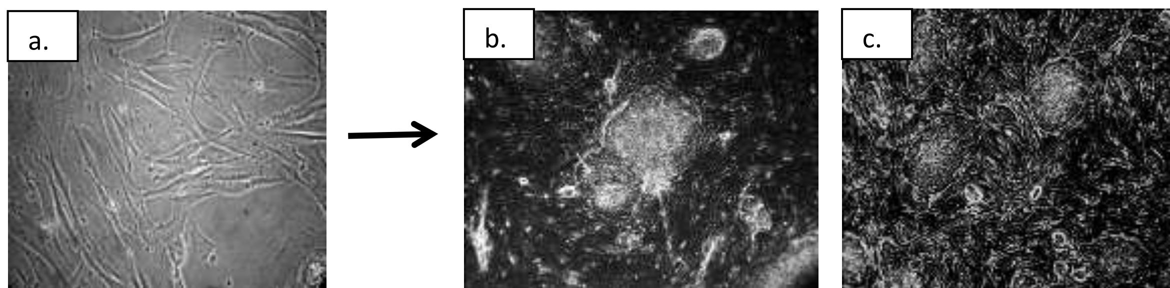


Figure 4.3. iPS induction in human primary fibroblast cells. a. Prostate primary fibroblast cells showing the characteristic spindle-shaped mesenchymal morphology. b. Pro-iPS colonies at P0, irregular-shaped colonies with poor morphology and tendency to undergo spontaneous differentiation at the centre and around the borders. c. Pro-iPS colony at P6, morphology is more similar to embryonic-stem cells, borders look more well-defined and cells are more compact than at early passages.

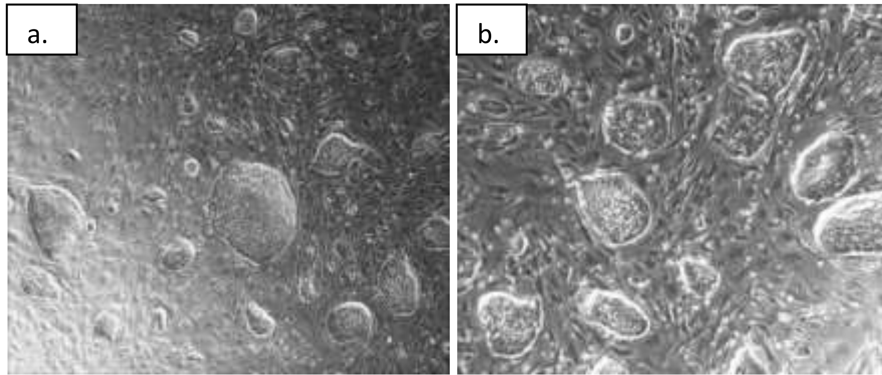


Figure 4.4. Pro-iPS cultures at higher passages. Between P8-P15 these colonies adapt better in culture and demonstrate greater resemblance to the human embryonic stem cell morphology (Moad, Pal et al.). a. At p10 and b. at p15.

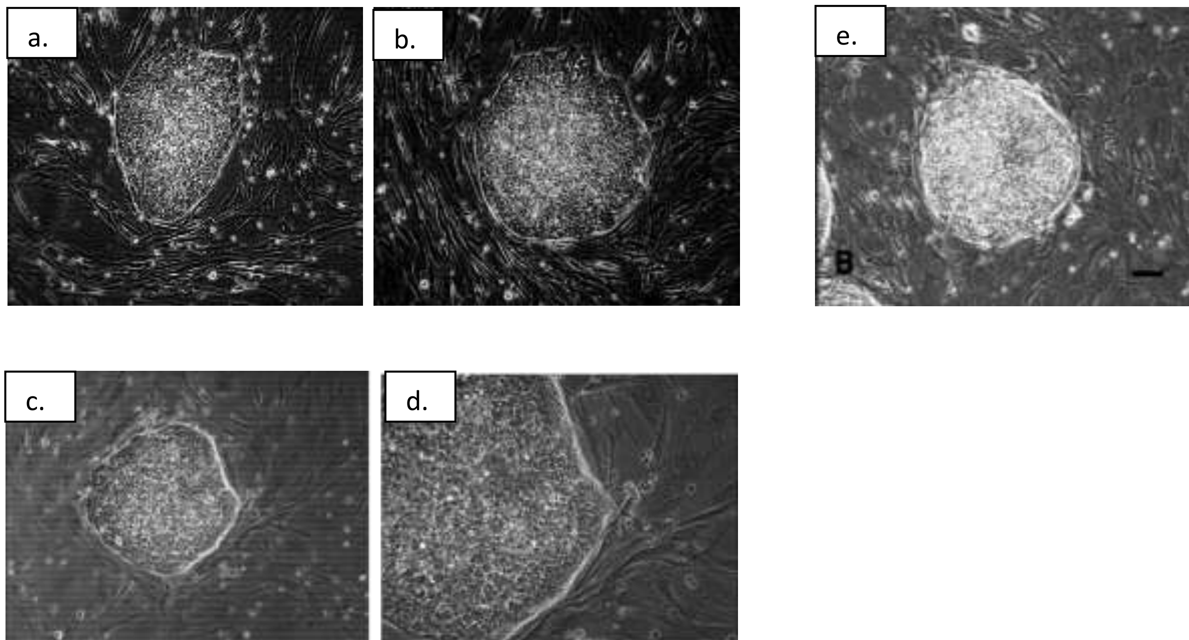


Figure 4.5. Pro-iPS colony morphology compared to H9 ES cells a. (X20), b.(X20), c.(X20) and d. (X40) morphology of Pro-iPS colonies against e. H9 human embryonic stem cell colony morphology. Human embryonic stem cell-colony image have been adapted from (Thomson, Itskovitz-Eldor et al. 1998).

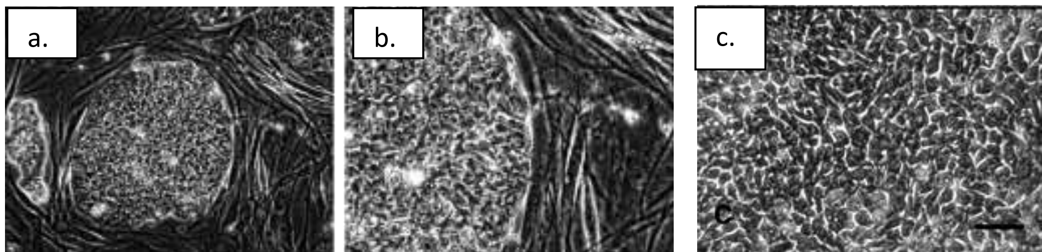


Figure 4.6. Pro-iPS colony and cellular morphology a. and b. at X40 and X60 magnification, respectively (Moad, Pal et al.) display similar morphology with c. H9 human embryonic stem cell-morphology (Thomson, Itskovitz-Eldor et al. 1998). Both the colonies comprise small round cells with high nuclear-cytoplasmic ratio.

4.3.4. DNA fingerprinting of Pro-iPS

DNA fingerprinting was essential to confirm authenticity of the cell lines and to confirm the absence of cross-contamination between the Pro-iPS with any other pluripotent cell line. In order to do this, 11 different satellite markers were tested and an identical match was confirmed between the parental fibroblast cells and resultant Pro-iPS cells thereby corroborating the Pro-iPS cells were derivative of primary prostate fibroblasts and were not due to cross-contaminaton (Table 4.1).

	MARKERS TESTED	**Pro-iPSC**	**Prostate parent fibroblast**
1	**Amelogenin**	**XY**	**XY**
2	**THO1**	**9.3 - 9.3**	**9.3 - 9.3**
3	**D21S11**	**30 - 31.2**	**30 - 31.2**
4	**D18S51**	**15 - 17**	**15 - 17**
5	**D13S317**	**8 - 12**	**8 - 12**
6	**D7S820**	**9 - 11**	**9 - 11**
7	**D16S539**	**11 - 13**	**11 - 13**
8	**PentaD**	**9 - 11**	**9 - 11**
9	**D8S1179**	**13 - 14**	**13 - 14**
10	**TPOX**	**8 - 11**	**8 - 11**
11	**FGA**	**21 - 25**	**21 - 25**

Table 4.1. DNA fingerprinting results confirm an identical match between parental prostate fibroblast cells and Pro-iPS clones for the 11 microsatellites tested. Amelogenin is a sex marker and the presence of two bands denotes the sample being male XY.

4.3.5. Karyotyping

Pro-iPS colonies were karyotyped between P10-P15 passage numbers and were tested for the presence of any aneuploidies. 26 karyograms were analysed and the average result was considered. No major aneuplodies were noted and the karyotype was confirmed to be 46 XY (Figure 4.7).

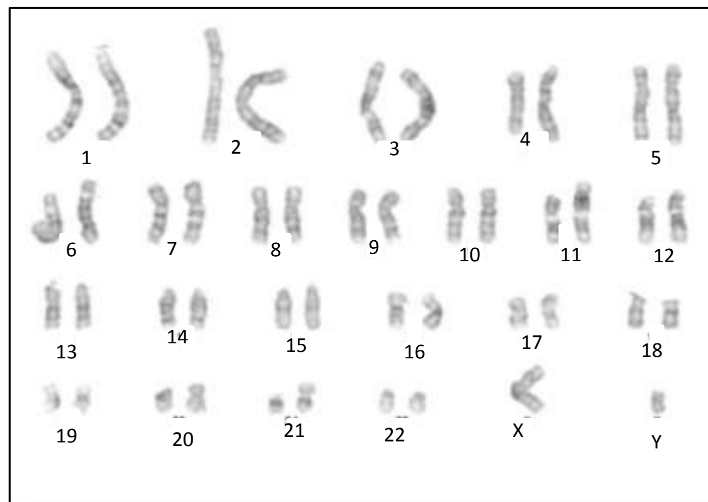


Figure 4.7. Karyogram of human Pro-iPS showing a diploid 46 XY karyotypic status (Moad, Pal et al.). No major chromosomal aneuploidies were noted.

4.4. Discussion and Conclusion

In this chapter, the optimisation of the iPS-induction protocol has been described along with the initial characterisation of the generated Pro-iPS cell lines. These cell lines were genotyped to corroborate authenticity and eliminate redundancy and were karyotyped to rule out any aneuploidies. The initial characterisation suggested that the Pro-iPS model thus generated would potentially present an appropriate model for non-diseased prostate growth and development. The next step was to corroborate these cell lines in terms of their pluripotency. To this end, two series of characterisation experiments were undertaken – phenotypic characterisation by means of expression-marker and transcript analyses and functional characterisation. These will be described in Chapters 6 and 7.

An important question that remains as yet equivocal is the mechanism of iPS induction. It has been documented that iPS induction chiefly occurs in three stages – initiation, maturation and stabilisation with fewer cells transiting from one stage to the other so as to finally reach the 'ground state' or the fully re-programmed state (Samavarchi-Tehrani, Golipour et al. 2010). Chapter 4 looks at this aspect of re-programming in the prostate and particularly at the role of epithelial to mesenchyme transition in prostatic de-differentiation.

Chapter 5.

Mechanisms of prostate de-differentiation and formation of partial -iPS colonies in the human prostate

5.1. Introduction

In this chapter, the molecular changes at each of the different steps during iPS re-programming in the prostate will be described. The changes observed in prostate fibroblast re-programming is characterised by an initial induction phase marked by a mesenchymal-epithelial-transition phase. Prostate epithelia did not survive the transduction process, and failed to form iPS colonies and therefore induction of pluripotency could not be evaluated. However, when primary prostate epithelial were seeded onto MEF-feeder plates in pluripotent stem cell media they expressed an arguable de-differentiated status.

5.2. Aims

1. To analyse the different mechanisms of iPS-induction in prostate

2. To re-programme prostate epithelia

5.3. Specific methods and results

5.3.1. Transduction of prostate epithelia

Prostate epithelia were cultured as described in Chapter 3. Once confluent, they were trypsinised and seeded onto MEF feeder plates and cultured in pluripotent stem cells media. Colonies started forming on the MEF feeders at 7 days after seeding them out. Once substantial sized colonies were noticed these were transduced with the OSKM 4 in 1 construct (Allele Biotech., USA) and 0.6 µg/ml of polybrene. After 48 hours of transduction, the media was replaced with fresh pluripotent stem cell media and the cells were cultured in this media for another 4 weeks. No iPS colonies were noted.

An alternative strategy of transducing prostate epithelia in KSFM media (see Chapter 3) for 48 hours followed by culturing these prostate cells in KSFM media for the first 7 days was also tried. However, when using this method the prostate epithelial cells either seemed to die out and/or seemed to undergo a senescent and/or mesenchymal-like morphology.

5.3.2. MET in prostate re-programming

5.3.2.1. MET changes in prostate primary fibroblasts

Prostate fibroblast cells were transduced with the 4 in 1 OSKM virus and 48 hours after transduction they were maintained in their original RPMI-1640 media (see chapter 3) for a total of 7 days. On the 7th day post-transduction the cells were collected and analysed for any change in gene expression profile. The cells demonstrated an altered morphology following Day 7 of transduction. Prostate stroma fibroblast cells are spindle shaped in morphology and grow as monolayers (Chapter 3 and Figure 5.1.a and 5.1.b.) . Following transduction, it was noted that the cells started to acquire a rounded morphology with each cells depicting a high nuclear-cytoplasmic ratio and a cobble-stoned patter reminiscent of epithelial cells (Figure 5.1.c. and d.)

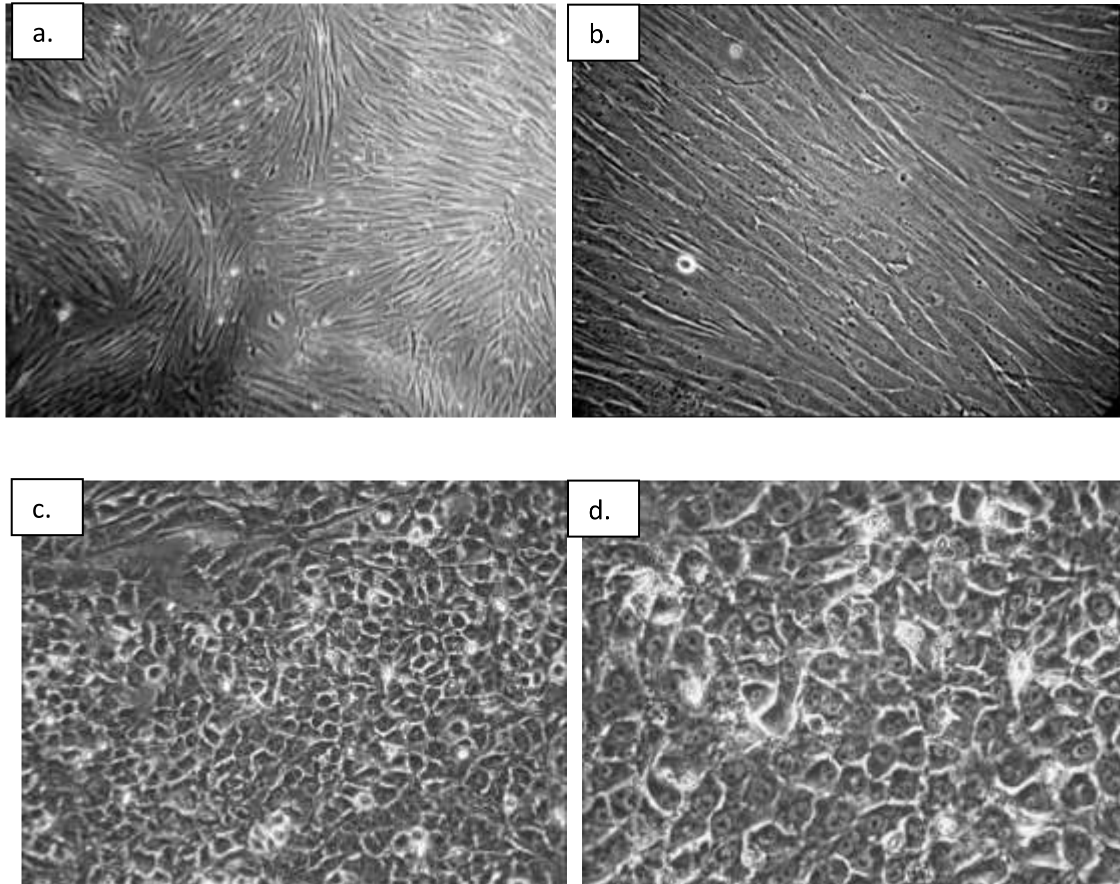


Figure 5.1. MET transition in prostate fibroblast cells is noted in the first 7 days following transduction. a. Prostate fibroblast cells X10. b. Prostate fibroblast cells X 40. c. Prostate fibroblast cells post transduction X10. d. Prostate fibroblast cells post transduction X40

A number of mesenchymal and epithelial mRNA transcript levels were examined in these cells. The first step was to evaluate the TGF-receptor status in the two cell types. Inhibition of TGF-β signalling enhances iPS re-programming in mice by facilitating a higher efficiency of iPS induction and by also promoting a faster kinetics. The activation of the TGF-β receptor does the opposite (Maherali and Hochedlinger 2009). Also, the inhibition of the TGF-β receptor allows for the replacement of exogenous c-Myc and SOX2 in the re-programming cocktail (Maherali and Hochedlinger 2009).

TheTGFf-receptor (TGF-R) status was evaluated in prostate primary cells and in LNCaP prostate cancer cells (Figure 5.2). As expected, the highest level of expression was observed in primary prostate stroma. TGF-R1 and TGF-R2 can only be differentiated by peptide binding and both these receptors have high affinity for TGF-β 1 (Cheifetz and Massagué 1989) while TGF-R3 binds TGFβ 1 and TGFβ2 with high affinity (Cheifetz, Andres et al. 1988). It has been documented that an increase in the levels of TGFβ1 and TGFβ2 reduced efficiency of iPS colony formation from 0.017% to 0.0013% (Maherali and Hochedlinger 2009). Levels of TGF-R3 were similar in prostate epithelia and LNCaP cells. However, TGF-R1 and TGF-R2 expression levels were a lot higher in prostate epithelial cells than LNCaP cells. This would suggest that compared to LNCaP cells the cultured prostate epithelia potentially possess more mesenchymal properties. This may be due to the fact that these cells are prone to an epithelial to mesenchymal transition in the current culture conditions. This aspect of research needs further overhaul but is beyond the scope of this project. These results suggested that prostate stroma may be more resistant to iPS induction than prostate primary epithelia.

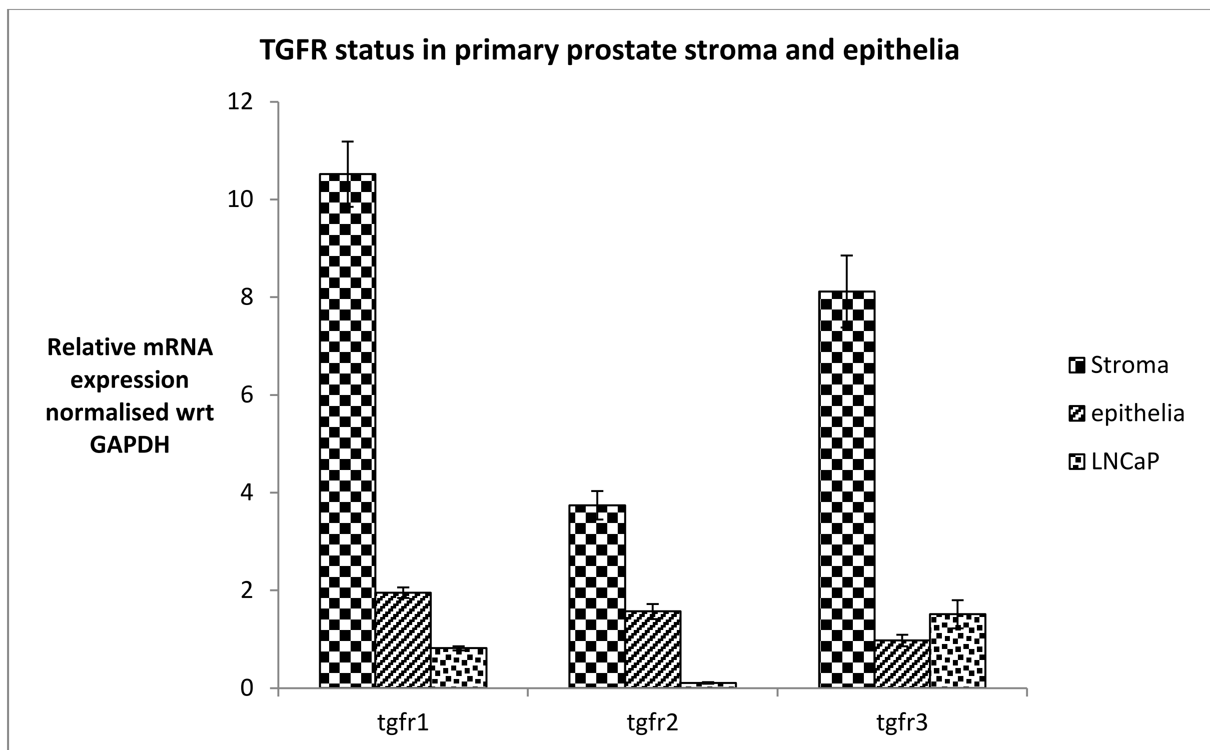


Figure 5.2. Expression of TGF-receptor (TGF-R1, TGF-R2 and TGF-R3) in prostate stroma, epithelia and in LNCaP cells.

The total levels of the pluripotency transcripts (endogenous and exogenous transcripts) were checked in the prostate primary fibroblasts so as to ensure that the cells were being transduced. An increase in all three transcription factors, Oct4, SOX2 and NANOG further suggested that these cells were indeed being successfully transduced (Figure 5.3-5.5). Since the re-programming cocktail did not contain any NANOG, an increase in NANOG levels re-assured that the cells were beginning to induce their own endogeneous transcription factor mRNA expression. Confirmation of an increase in endogenous transcripts for the other pluripotency factors were later monitored through primers encoding the 3'UTR end of the respective genes (see Chapter 6) . Three more players in EMT were subsequently analysed, namely mesenchymal markers SNAIL, SLUG and E-Cad these have been known to play a role in the MET phase of iPS induction (Li, Liang et al. 2010, Samavarchi-Tehrani, Golipour et al. 2010). Prostate primary stroma cells were analysed 7 days after transduction and it was seen that there was a significant decrease ($p<0.05$) in levels of SNAIL and SLUG following transduction with the iPS-re-programming cocktail (Figure 5.6) (Moad, Pal et al.). In addition, it was observed that levels of E-Cadherin were up-regulated in the prostate stroma following transduction (Figure 5.6).This indicated that the OSKM re-programming factors were inducing a mesenchymal-epithelial-transition (MET) in prostate stroma within the first 7 days post-transduction.

In this project, no tracking methods were employed hence it cannot be ascertained whether the cells that underwent the MET phase indeed were the ones that were ultimately re-programmed. However, based on documented evidence it is highly likely that the first transition in iPS induction in prostate primary fibroblast cells is the MET.

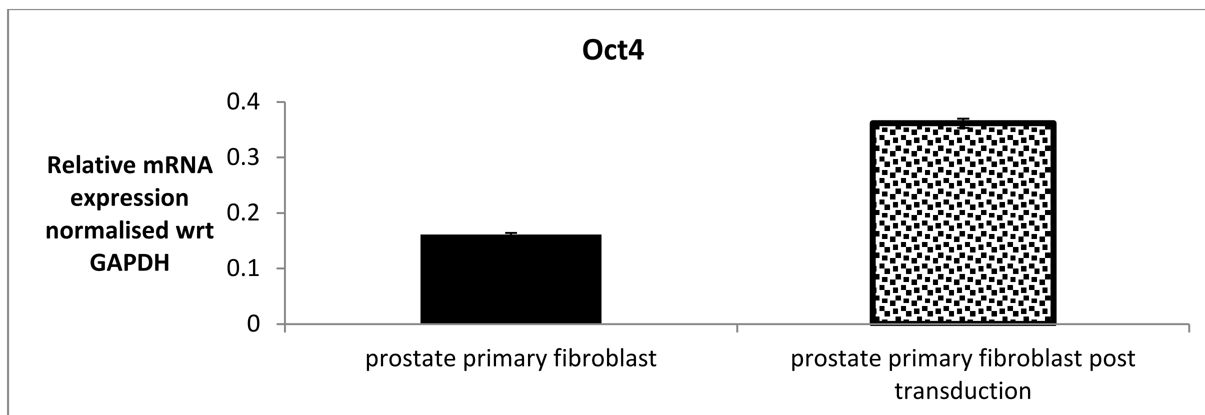


Figure 5.3. Increase in Oct4 levels in prostate primary fibroblasts at 7 days post transduction.

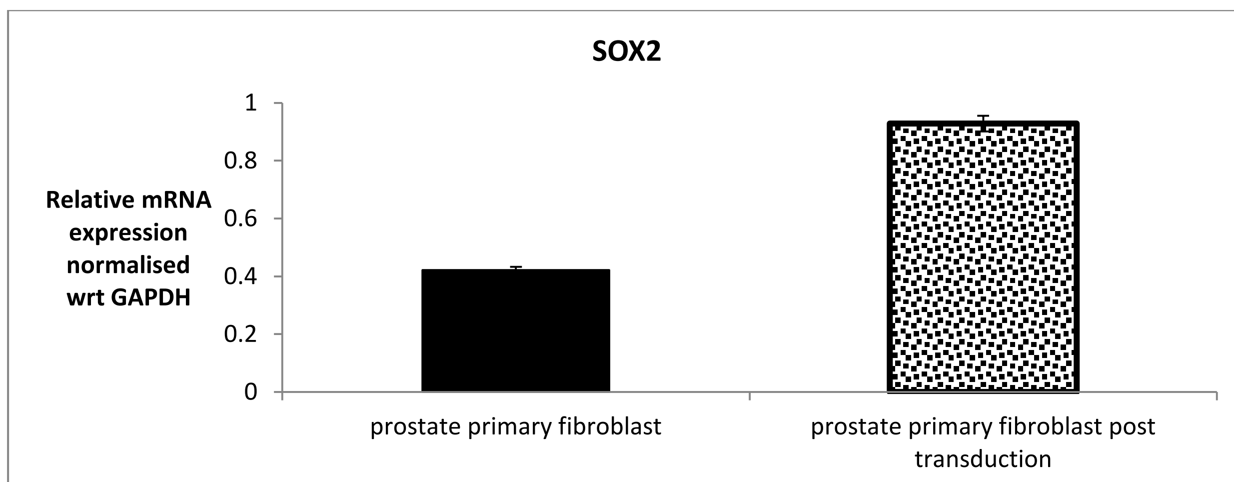


Figure 5.4. Increase in SOX2 levels in prostate primary fibroblasts at 7 days post transduction

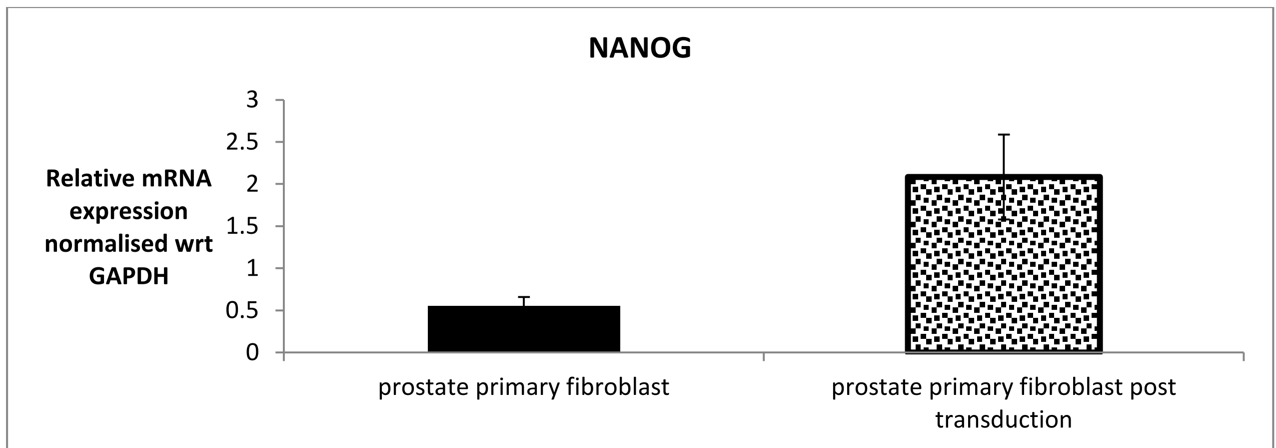


Figure 5.5. Increase in NANOG levels in prostate primary fibroblasts at 7 days post transduction

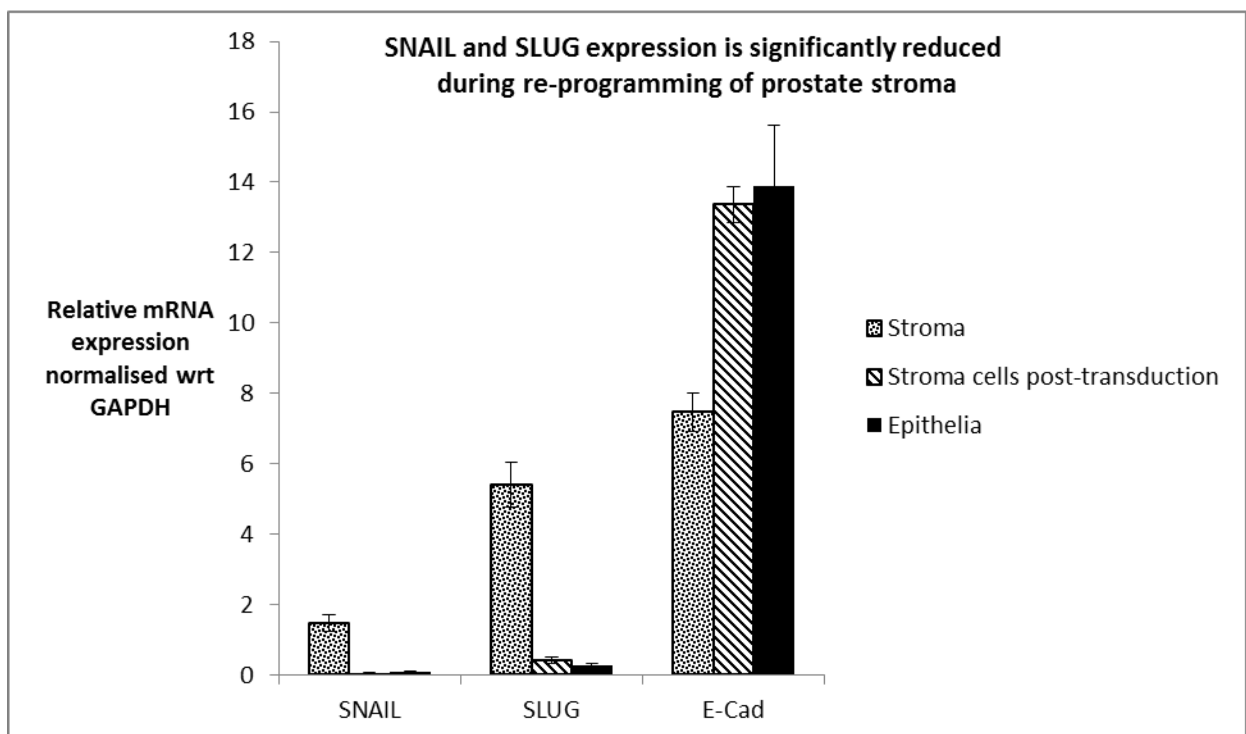


Figure 5.6. MET changes in prostate primary stroma post transduction. Following transduction prostate stroma down-regulate SNAIL and SLUG levels and up-regulate levels of E-Cadherin.

5.3.2.2. EMT transcript changes in prostate primary epithelia

Transduction of prostate epithelial cells proved to be challenging in that the cells did not survive the transduction process and owing to technical limitations analysis of these cells proved to be extremely difficult. The preliminary analysis demonstrated that there was an initial rise in the levels of E-Cadherin (after Day 7 post transduction)(Figure 5.7). However, unlike the case for prostate stroma, the mesenchymal transcripts, Slug and Snail were not down-regulated (Figure 5.8 and 5.9). This may potentially suggest a block in the MET process causing failure of iPS induction in these cells. A more detailed scrutiny of this phenomenon including analysis of an extended set of pluripotency mRNA transcripts and other EMT markers at different time points is needed, but this is beyond the scope of this study.

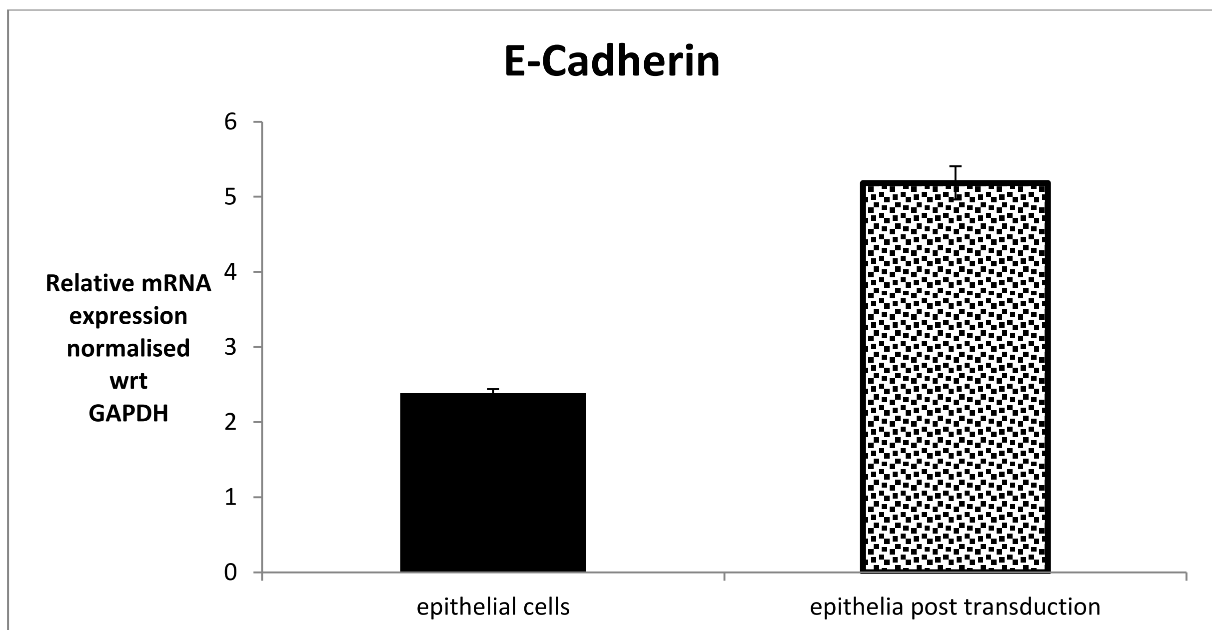


Figure 5.7. E-Cadherin expression is up-regulated in prostate epithelial cells post transduction

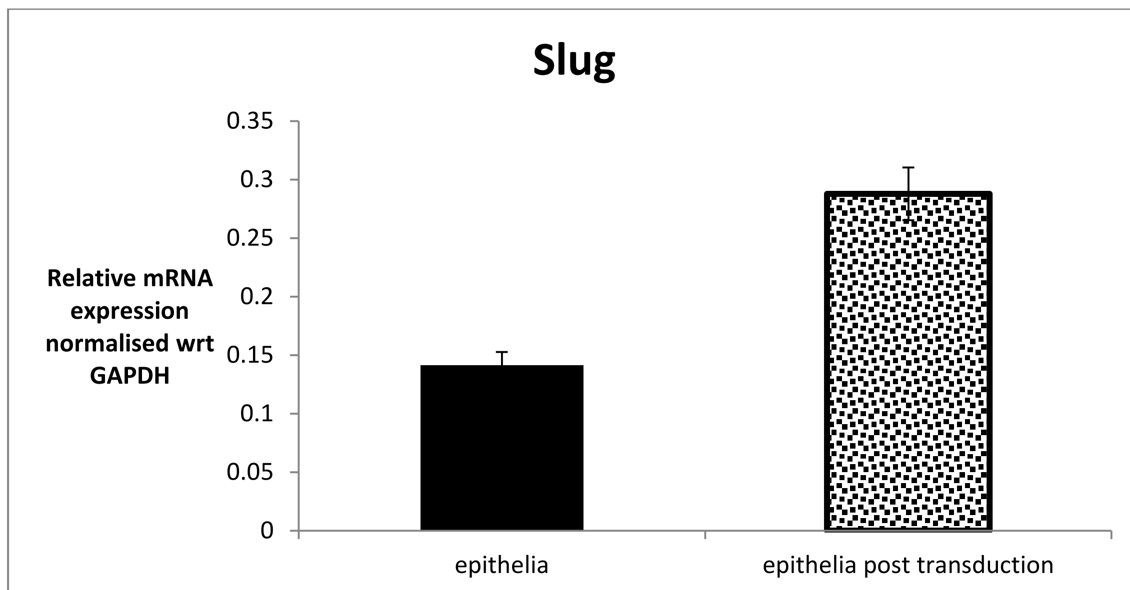


Figure 5.8. Mesenchymal marker Slug is not down-regulated following transduction (Day 7) with OSKM factors.

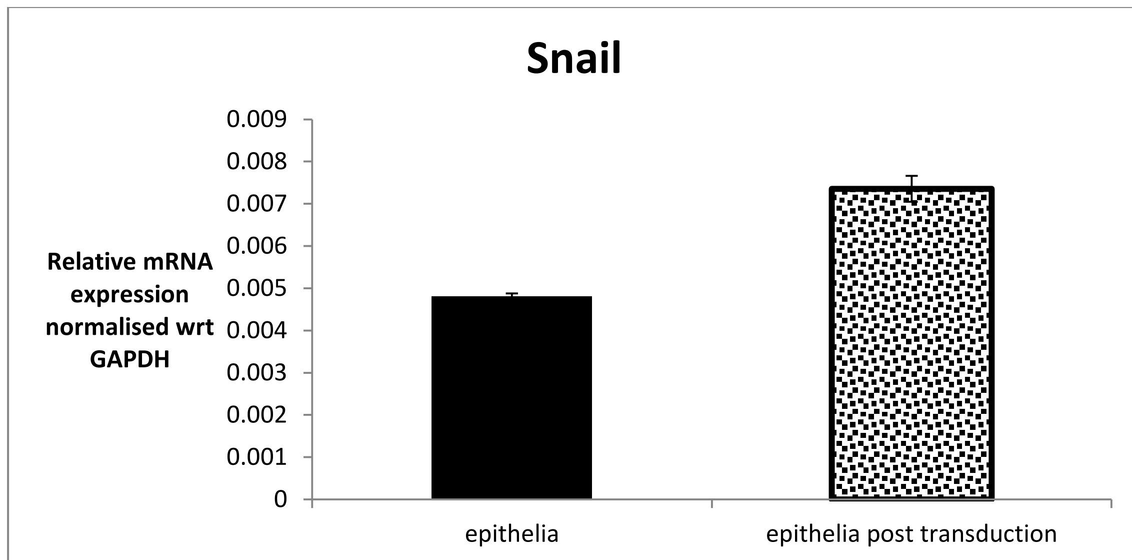


Figure 5.9. Mesenchymal marker Snail fails to be down-regulated following transduction (Day 7) with OSKM factors.

Given the transcript changes were not favourable, the prostate epithelia did not survive the transduction process and additionally there were associated technical limitations, this may help to explain why the prostate epithelia were not transduced with the re-programming cocktail.; The characterisation was however continued using the prostate-derived fibroblast cells.

5.3.3. De-differentiation in prostate primary epithelia

Prostate epithelia were exposed to a pluripotent stem cell niche with the intention of de-differentiating them to a primitive phenotype. Prostate primary epithelia were cultured (as described in Chapter 3) and a MACS sort was conducted to enrich for CD324+ve (EpCAM positive) cells. These cells were directly seeded onto MEF feeder plates and were cultured in pluripotent stem cells media until small rounded colonies started to appear. These were cultured for 3-4 weeks and the colonies thereby formed were denoted, prostatic epithelial pluripotent-like stem cells (PEPSC). After 7 days of initial culture, these cells were stained with SSEA4, Tra-1-60 and Hoerscht (see chapter 4). A live cell imaging for the surface markers without fixing the cells allowed these colonies to be cultured following the staining process and to observe their progress over time. 7 days following culture the colonies did not show evidence of any SSEA4+ve or Tra-1-60 +ve staining. Hoercsht is a cytotoxic dye and is excluded from the cells by the transmembrane ABCG2 transporter protein. Stem cells have a higher ability to exclude this dye since they have higher levels of ABCG2 while non-stem cells fail to exclude this toxic dye and stain blue (Scharenberg, Harkey et al. 2002). It was noted that the pluripotency markers were positive at 2 weeks following culture in the pluripotent stem cell niche (Figure 5.10). In addition the PEPSC cells up-regulated stem-cell transcripts namely CD133, CD44, NANOG and Oct4 and down-regulated AR, PSA and Nkx3.1 (Figure 5.11). These colonies however failed to expand and were thus deemed as partially de-differentiated.

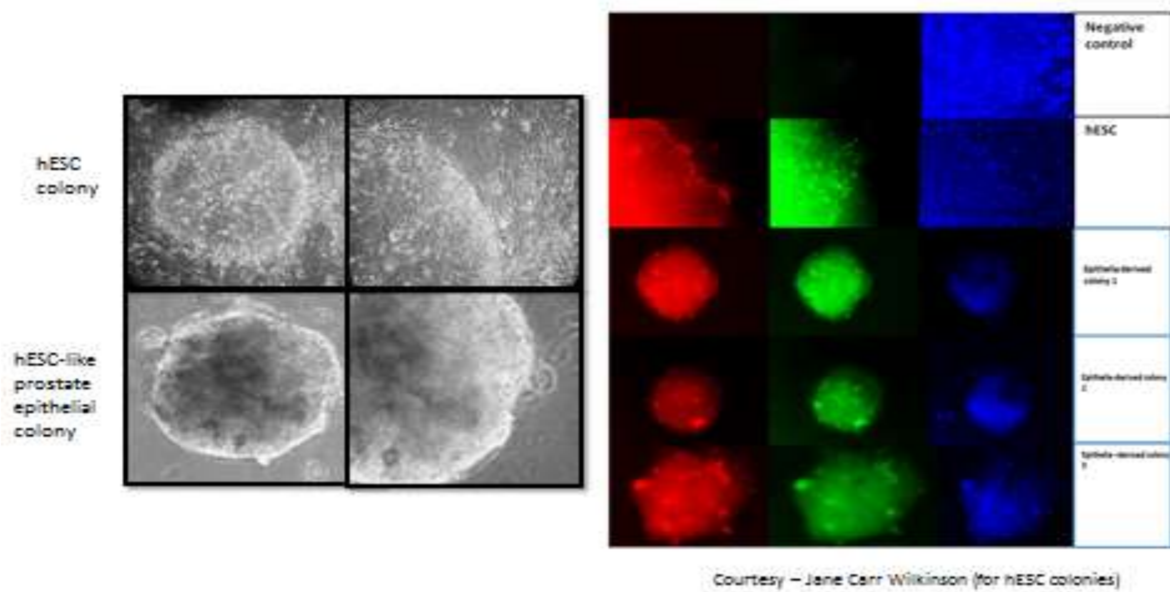


Figure 5.10. Prostate epithelial cells grown in specific culture conditions form hESC-like colonies (left panel). Staining patterns for hESC markers in negative and positive control alongside stroma-derived stem cell colonies

	Epithelia	Prostatic epithelial pluripotent-like stem cell (PEPSC)
AR		
PSA		
Nkx 3.1		
CD133		
CD44		
CD117		
NANOG		
Oct 4		

Legend	
Up-regulated	
Down-regulated	

Figure 5.11. A schematic heat-map representing prostate stem markers and androgen regulated genes in prostate epithelia and prostate epithelial pluripotent-like stem cells. CD133, CD44, CD117, NANOG and Oct4 are upregulated in the prostatatic epithelial pluripotent-like stem cells (PEPSC)

These colonies were fixed and stained for nuclear transcript, Oct4. However, it was noted that although Oct4 was expressed in these cells it was not localised to the nucleus, but instead was present in the cytoplasm (Figure 5.12). This expression pattern is consistent with a multipotent stem cells phenotype (Zuk 2009).

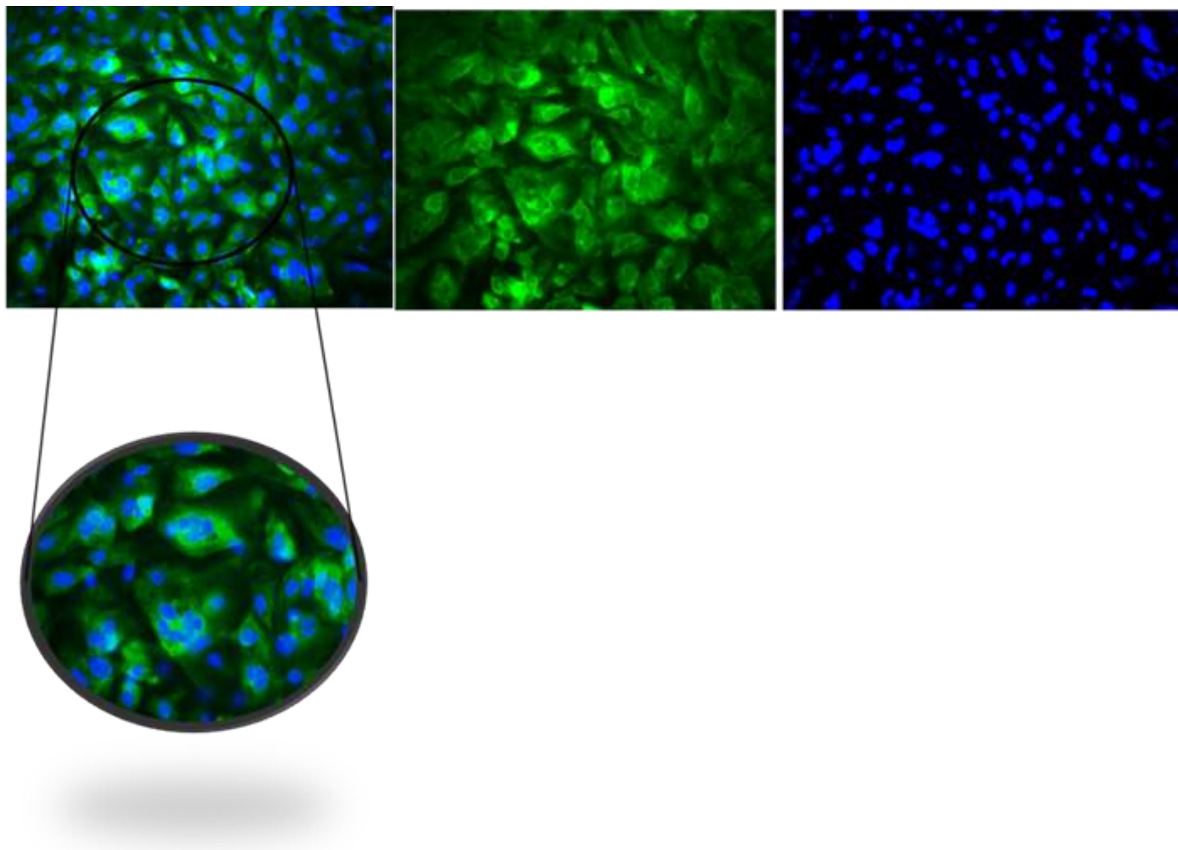


Figure 5.12. PEPSC colonies stain for Oct4 but this marker is localised to the cytoplasm instead of the nucleus.

65.3.4. Partial Pro-iPS colonies

Prostate stroma at week 3-4, following transduction with the OSKM/OSLN factors formed small irregular colonies that stained very brightly for SSEA4 and Tra-1-60 and were arguably Hoercsht positive (Figure 5.13) . However, these colonies failed to expand in size and did not show the characteristic morphology of human pluripotent stem cells and were thus regarded as partially re-programmed iPS colonies.

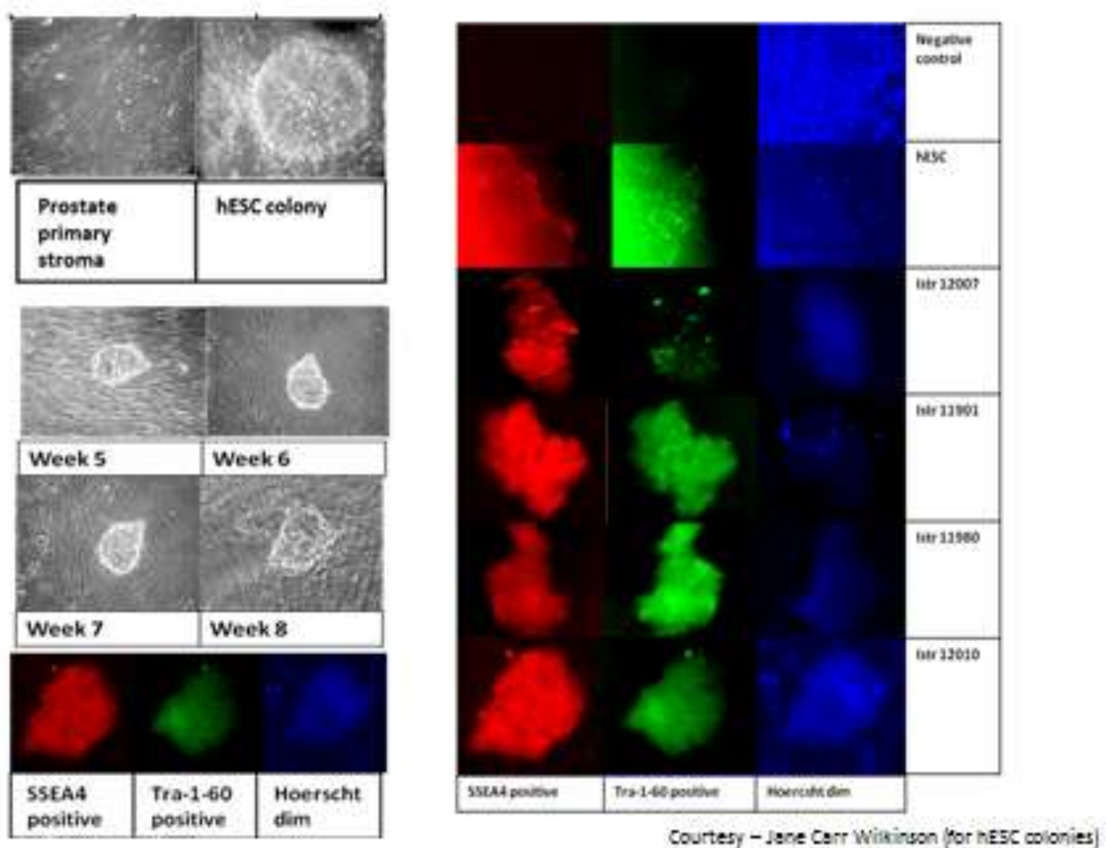


Figure 5.13. Staining patterns in H9 hESC and in prostate-derived iPS-like colonies. Primary stoma (negative control), hESC colony (positive control) (top left panel). Stroma-derived stem cell colony along with staining pattern (bottom left panel). Staining patterns for hESC markers in negative and positive controls alongside stroma-derived stem cell colonies.

35.4 Discussion and Conclusion

In summary, this chapter provides an insight for some of the possible mechanisms that drive pluripotency and de-differentiation in the prostate cells (Figure 5.14). A mesenchymal to epithelial transition-like phase seems to drive pluripotency in primary prostate fibroblast cells while the prostate epithelia seemed resistant to this MET phase but on the other hand seemed to become more invasive. There seems to be an aberrant signalling pathway in prostate epithelia that acts to block the re-programming process . This mechanism needs a further scrutiny, of possible aberrant epigenetic mechanisms occurring in the re-programming process. Several signalling pathways regulate iPS re-programming and the Initiation stage in the re-programming is controlled by several factors (Figure 5.15).Cellular tracking and single-cell analysis of prostate stroma and epithelia during the re-programming will be essential in elucidating the possible road-blocks instrumental in pluripotency induction in the human prostate.

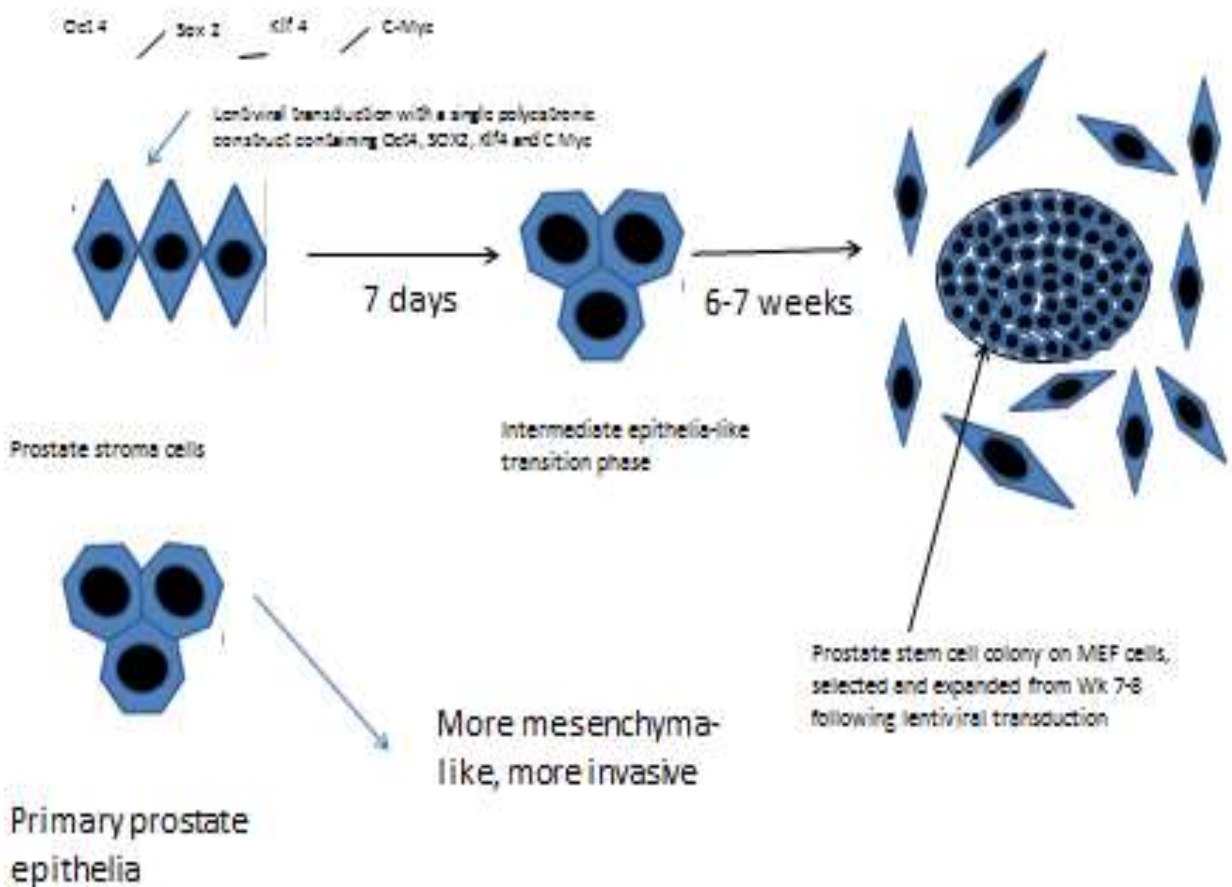


Figure 5.14. A schematic diagram representing the MET process during iPS induction in the prostate. Transduced prostate stroma are observed to undergo a mesenchymal-epithelial transition at 7 days following transduction. After another 6-7 weeks, these cells form Pro-iPS colonies. In contrast, primary prostate epithelia do not form completely re-programmed iPS colonies (Zhao, Sun et al. 2013). We noted that these cells acquired higher levels of mesenchymal transcripts.

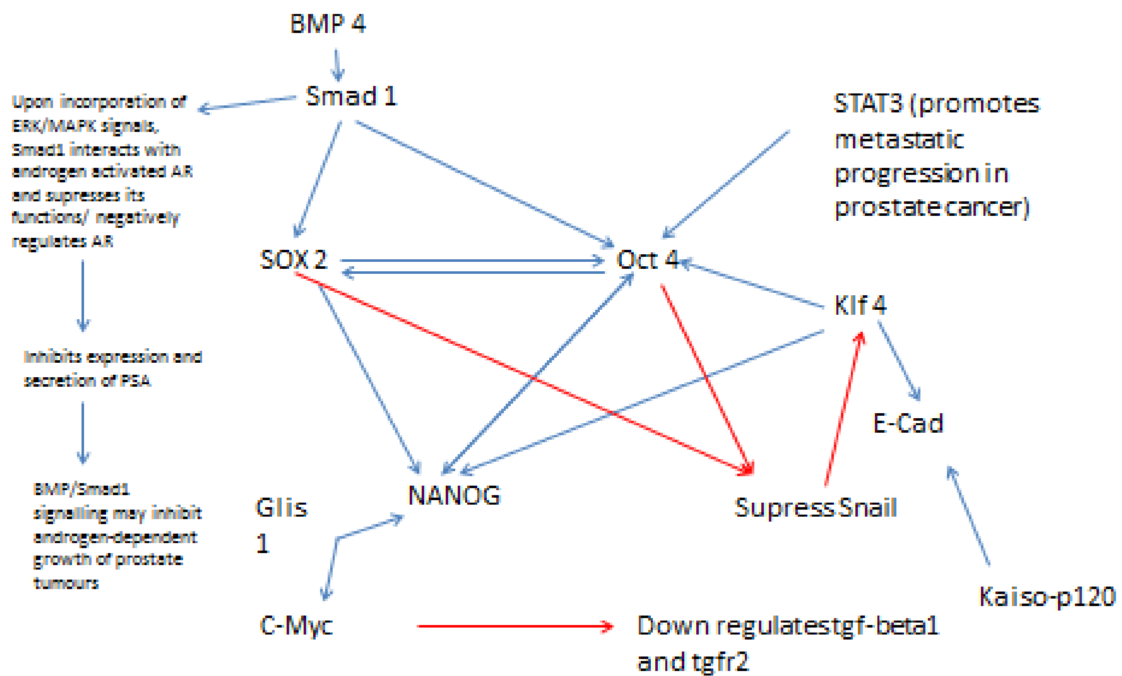


Figure 5.15 Schematic diagram demonstrating the transcriptional regulatory network in embryonic stem cells. Synergism of BMP with the OSKM factors during the Intial MET phase may be potentially mediated through interactions of Smad1, Oct4 and SOX2 to regulate the iPS induction process

Chapter 6

6. Phenotypic characterisation of Pro-iPS cells

6. 1. Introduction

This chapter will describe the phenotypic characterisation of Pro-iPS cells. The prostate-derived pluripotent stem cell lines were characterised through expression marker analyses. Gene expression analysis was carried out for a panel of 6 embryonic transcripts. Exogene silencing was confirmed through real-time PCR using a primer sequence was specifically directed against the lentiviral backbone. iPS colonies express endogenous transcripts around Day13-14 post-transduction (Stadtfeld, Maherali et al. 2008). Endogenous pluripotent transcript expression was checked in the Pro-iPS lines by designing primers specifically coding the 3' untranslated region of the gene. The panel of genes tested included Oct4, SOX2, NANOG, gdf3, Dnmt3b and Rex1. All of these genes were expressed at levels similar to and/or higher than the embryonic stem cell line H9 (Moad, Pal et al.).

Immunofluorescence staining was performed using a panel of 5 different markers including, surface markers SSEA4, Tra-1-81 and Tra-1-60 and the nuclear markers NANOG and Oct4. Immunocytochemistry was also applied to detect levels of alkaline phosphatase because undifferentiated pluripotent stem cells are demonstrated to express high levels of alkaline phosphatase (Thomson, Itskovitz-Eldor et al. 1998, Takahashi, Tanabe et al. 2007, Yu, Vodyanik et al. 2007). Pro-iPS cells expressed all the pluripotent surface markers and demonstrated nuclear localisation for all the nuclear markers (Moad, Pal et al.) further corroborating their pluripotent status over simply multipotency (Zuk 2009).

6.2. Aims

1. To characterise Pro-iPS cell lines for presence of pluripotent transcripts and to compare these transcripts expression against H9 human embryonic stem cells

2. To confirm the expression of pluripotent markers in the Pro-iPS cells

6.3. Specific Methods and Results

6.3.1. Transcript analyses

Pro-iPS cells expressed endogenous transcripts SOX2 and Oct4 at levels that were similar to or higher than human H9 cells (Figure 6.1 and 6.2) (Moad, Pal et al.). Figures 6.1 and 6.2 summarise SOX2 and Oct4 expression levels in prostate stroma, prostate stroma 6 days following transduction, H9 human embryonic stem cells, skin-iPS cells and 14 clones of Pro-iPS cells. For both SOX2 and Oct4 transcripts the endogenous gene levels were measured using primers that specifically encoded detected the 3' untranslated region of the gene. Parental prostate stroma showed negligible expression of endogenous Oct4 and SOX2 when compared against Pro-iPS clones. Prostate fibroblasts collected 7 days following transduction showed very high expression of the endogenous transcripts. This demonstrates that, in prostate cells the endogenous transcripts are switched on as early as Day 7 post transduction and the levels of these genes remain the same and/or increase following the complete re-programming process.

All 14 Pro-iPS clones expressed transgene-independent SOX2 (Figure 6.1). For clones 1 and 4 the levels of these were very similar to levels in the H9 cells. However, SOX2 levels were on an average 5 folds higher in Pro-iPS clones 2,3,5,6,7,8,10,11,12,13 and 14. Clone 9 expressed SOX2 at levels that were 9 fold higher than H9 cells. Non-transduced fibroblasts were used as negative control. Endogenous SOX2 levels were also measured in prostate stroma at an early post-transduction stage, around day 10. An increase in endogenous gene expression at this stage confirmed that the cells had not only been successfully transduced by the virus but also that the iPS-induction phase had already started at this time. Transcript levels were also measured in skin-iPS cells (kindly provided by Prof. L Lako, Newcastle

University) since conventionally iPS cells are derived from skin and therefore these cells were used for comparison against Pro-iPS to test the role of epigenetic imprinting towards lineage fidelity.

A larger number of the Pro-iPS clones, namely clones 2, 3, 5, 6, 7, 9, 11, 12, 13 and 14 expressed endogenous Oct4 that were very similar to levels seen in the H9 cells (Figure 6.2). However, Oct4 levels were very high in Pro-iPS clones 1, 4, 8 and 9. The endogenous expression for early transduced prostate stroma for Oct4 is very similar to H9 cells and this may be associated with a greater number of Pro-iPS clones acquiring an expression profile that was more consistent with the H9 cells than the scenario for SOX2. Since all the clones were derived from the same patient, it is possible that there is an association between prostate and Oct4 that causes the expression profile for this particular gene to be more embryonic stem cell-like than the expression profile for SOX2.

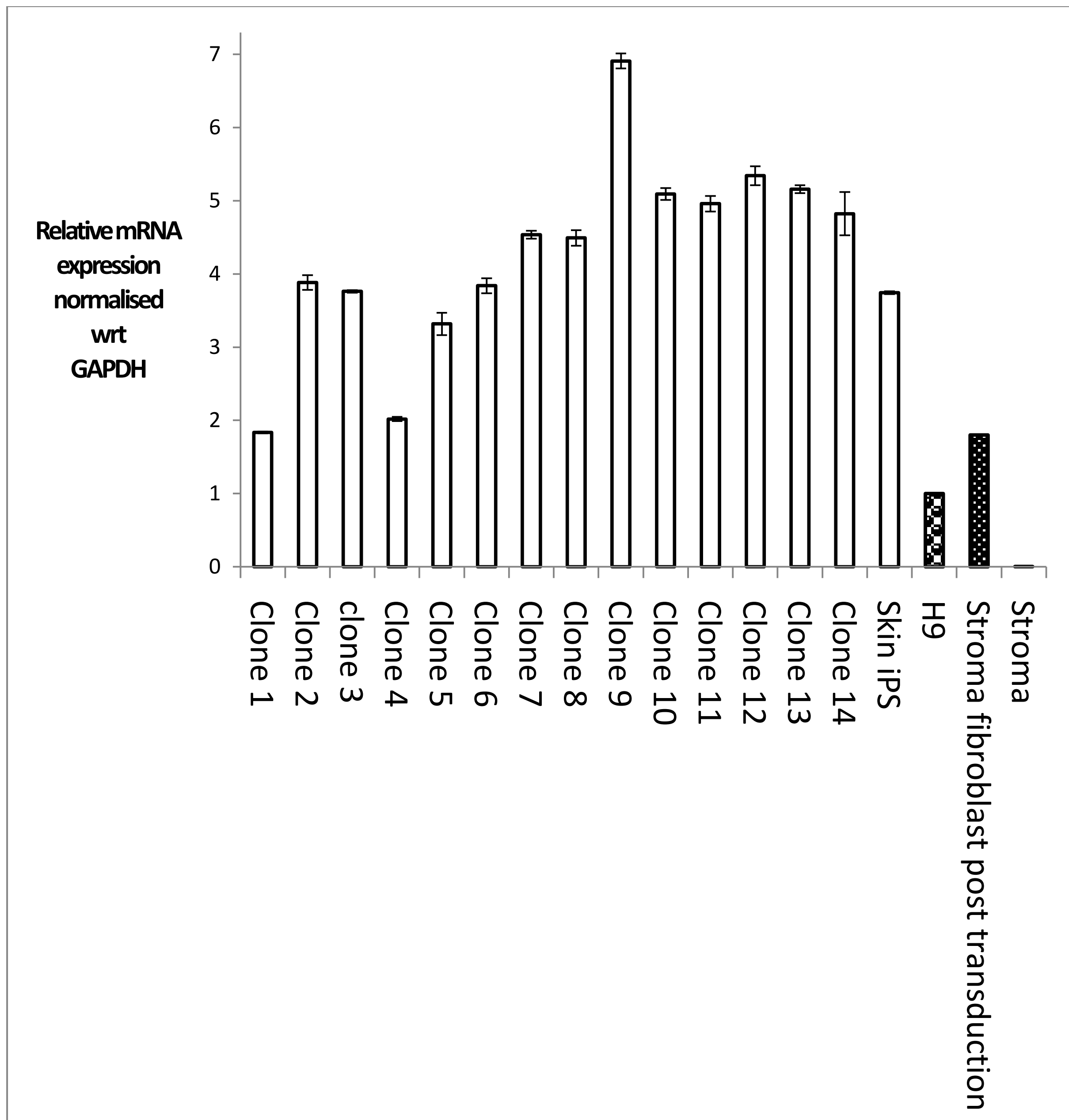


Figure 6.1. Endogenous SOX2 levels in the 14 Pro-iPS clones, H9, stroma cells post transduction and parental stroma cells. Endogenous SOX2 expression was minimal in non-transduced prostate stroma. Within 7 days of transduction, levels of endogenous SOX2 were up-regulated. Both the skin-iPS and Pro-iPS cells expressed SOX2 that were at a similar level to or higher than SOX2

levels in the H9 cell line.

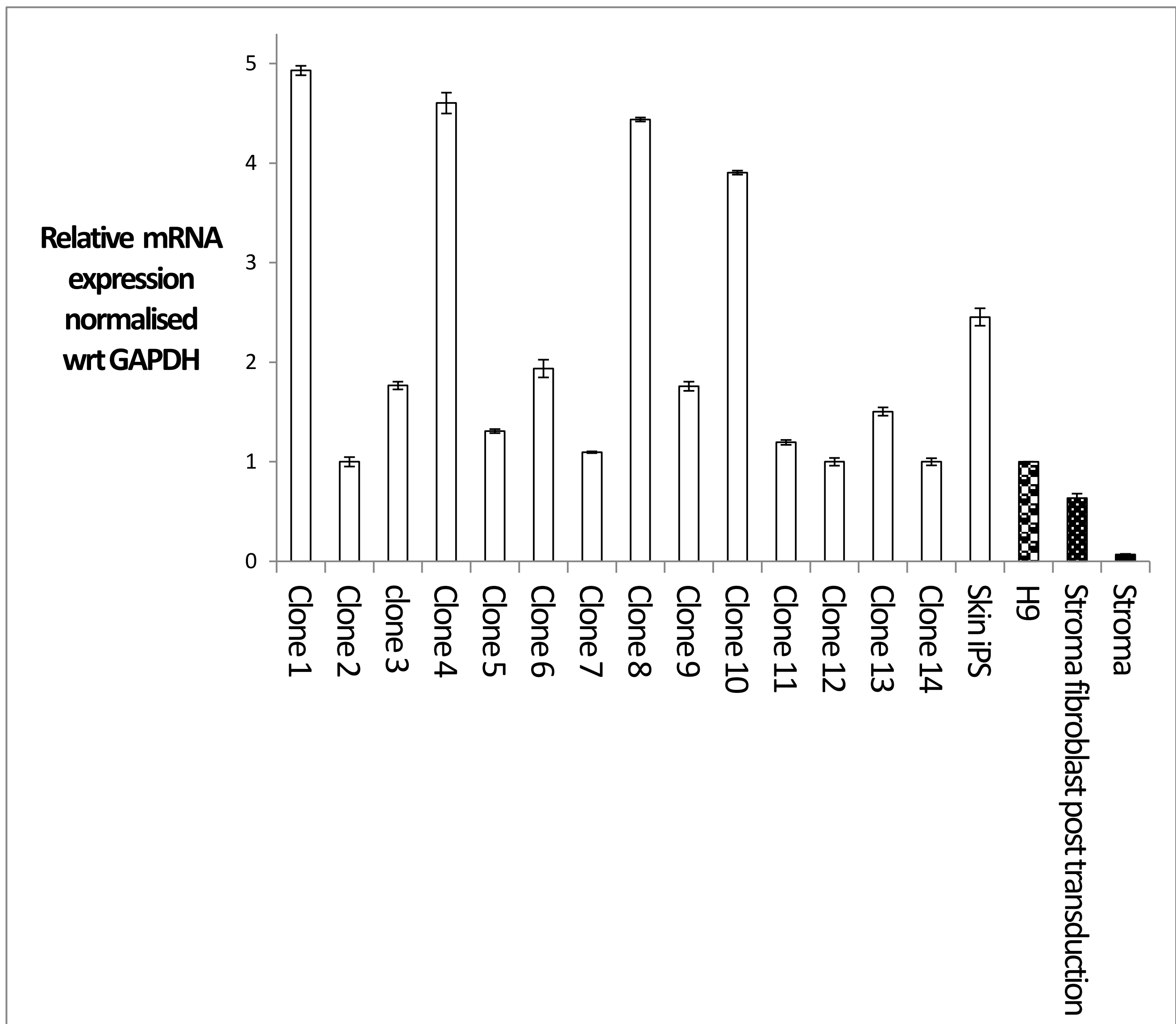


Figure 6.2. Endogenous Oct4 levels in the 14 Pro-iPS clones, H9, stroma cells post transduction and parental stroma cells. Endogenous Oct4 levels were upregulated by prostate stroma at 7 days following transduction and the expression levels remained elevated in the fully formed Pro-iPS cell lines. Furthermore, Oct-4 expression levels were variable amongst the different cell lines. This may be a consequence of multiple integrations of the transgene and/or due to a variation in the integration site. Both these events can have downstream implications and affect the genetic machinery of the transduced prostate cells. H9 cDNA was kindly provided by Prof. L Lako, Newcastle University.

High levels of endogene expression is a favourable characteristic of a pluripotent stem cell phenotype since this suggests that the cells have been successfully reprogrammed and that they do not need to rely on the transgene for pluripotency and survival (Stadtfeld, Maherali et al. 2008). However, one major concern with such high levels of expression is that it may potentially indicate viral genome integration as well as aberrant and incomplete transgene silencing. Hence, the next step was to check for the expression of the transgene in the Pro-iPS clones.

6.3.2 Testing exogene expression in Pro-iPS cells

Exogene levels were tested in all the 14 Pro-iPS cell lines (Figure 6.3). Expression of the endogene along with the absent exogene expression is consistent with the fully re-programmed or Class II iPS cells (Hotta and Ellis 2008). There is evidence to suggest that in retroviral-transduction, the exogene becomes silenced once the iPS cells are independent of the transgene stimulation by virtue of their own endogenous pluripotent transcripts (Maherali, Sridharan et al. 2007, Okita, Ichisaka et al. 2007, Wernig, Meissner et al. 2007).

In order to examine the persistence of the transgene, PCR was performed using a primer pair encoding sequences that specifically target the lentiviral backbone. H9 embryonic stem cells were used as the negative control while prostate fibroblast at Day10 post-transduction was used as the negative control. Clones 1, 2, 3, 9, 10 and 13 showed negligible transgene expression while Clones 4, 5, 6, 7, 8, 11, 12 and 14 showed almost absent transgene expression. These results appeared to suggest that re-programming in the Pro-iPS cells was potentially complete with possible silencing of the transgene. Confirmation of the transgene however can only be attained through analysis of the exogene-integration sites in the re-programmed cells.

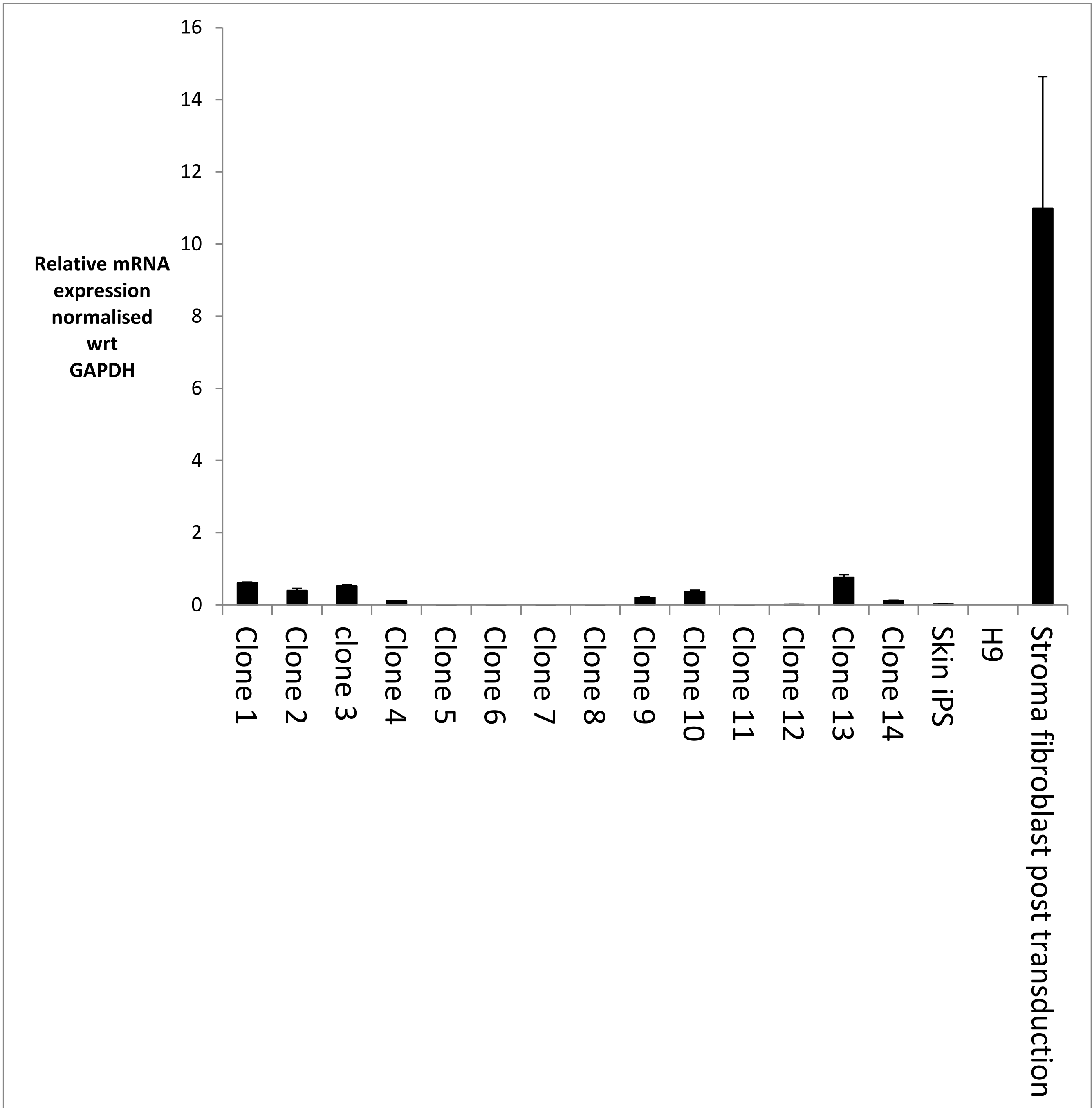


Figure 6.3. Exogene expression in Pro-iPS cell lines. Transgene levels were measured by using a primer pair that specifically amplified a sequence in the lentiviral backbone of the exogene construct. H9 embryonic stem cells were used as a negative control since they dol not express any exogenous pluripotent transcripts. Stroma cells at 7 days post transduction expressed very high levels of the transgene and this was used as a positive control.

6.3.3. Expression of other pluripotent transcripts in Pro-iPS cells

Levels of four other pluripotent transcripts, namely NANOG, dnmt3b, gdf3 and Zfp42 were tested and compared with the H9 embryonic cells (Figure 6.4). For these experiments, Clone 4 was chosen as the representative clone for the Pro-iPS cells. Pro-iPS cells expressed these transcripts at levels very similar to the H9 cells. Along with pro-viral silencing, expression of dnmt3b and REX1 is associated with the fully re-programmed state (Chan, Ratanasirintrawoot et al. 2009).

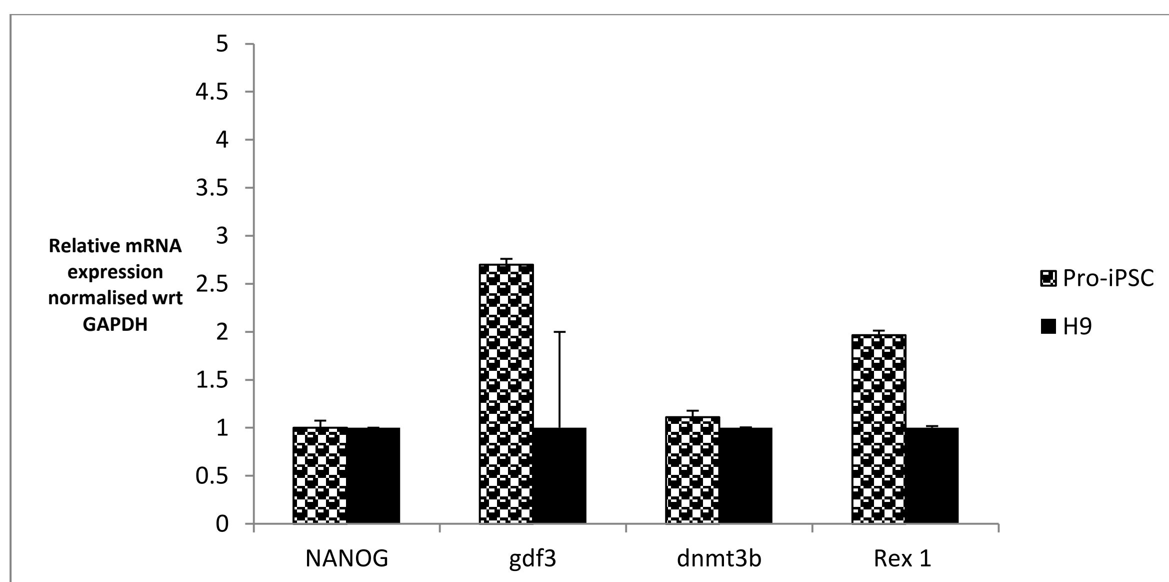


Figure 6.4. Expression of pluripotent transcripts NANOG, gdf3, dnmt3b and Rex1 (Zfp42) in Pro-iPS clone 4 and H9 cells. NANOG and dnmt3b levels were remarkably similar between Pro-iPS and H9 cells. Gene expression levels here refer to the total gene levels which represents the endogenous transcript levels.

46.3.4. Expression marker analysis in Pro-iPS cells

The Pro-iPS cells stained positive for alkaline phosphatase (Figure 6.5), a surface marker that is expressed by pluripotent cells (International Stem Cell, Adewumi et al. 2007, O'Connor, Kardel et al. 2008, Ramirez, Gerbal-Chaloin et al. 2011) . Alkaline phosphates is a marker for pluripotency but is not associated with the fully re-programmed state and is insufficient on its own to denote complete re-programming (Chan, Ratanasirintrawoot et al. 2009).

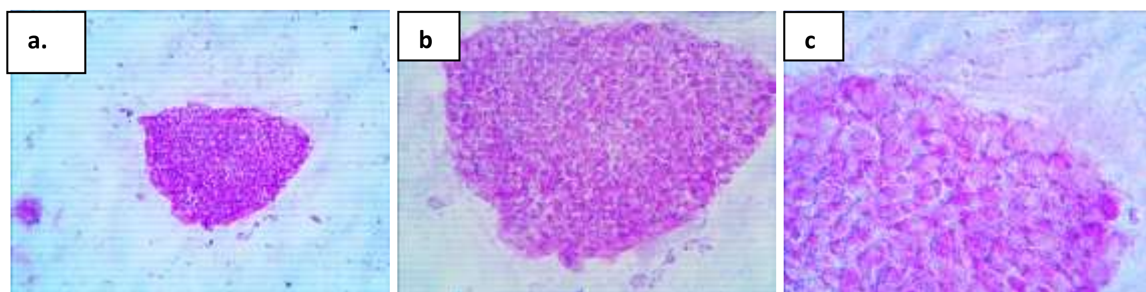


Figure 6.5. Pro-iPS cells express the pluripotent surface marker alkaline phosphatase a. X10 magnification b. X20 magnification and c. X 40 magnification

Pro-iPS cells also expressed surface markers SSEA4, Tra-1-81 and Tra-1-60 as well as the nuclear markers Oct4 and NANOG (Moad, Pal et al.). This has been demonstrated in figures 6.6 and 6.7. Figure 6.6 demonstrates the expression of SSEA4, TRA-1-81 and Oct4 in Pro-iPS cells. The Pro-iPS colonies express the surface markers SSEA4 and Tra-1-81 in a honey-comb pattern. The staining also further corroborates the colony morphology to be tightly packed, without any evidence of central and/or peripheral differentiation which would be evident as areas of poor/absent staining. The colonies can be seen to be surrounded by MEF feeder cells that stain for nuclear marker DAPI but not, as expected for the pluripotency markers. Figure 6.7 depicts the pluripotent markers expression at a higher magnification. While the expression of SSEA4, Tra-1-81 and Tra-1-60 is on the cell surface, Oct4 and NANOG expression localises to the nucleus. Nuclear expression of Oct4, NANOG and expression of Tra-1-60 is associated with the fully re-programmed state (Chan, Ratanasirintrawoot et al. 2009). Together, these staining patterns validate the credibility of our thus formed Pro-iPS cells lines as being fully re-programmed to an embryonic stem cell level (Moad, Pal et al.).

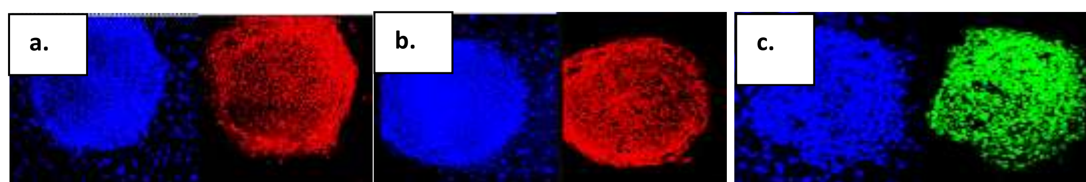


Figure 6.6. Expression of pluripotent markers in Pro-iPS colonies with absent staining in non-pluripotent feeder MEF cells. MEF cells outside the colonies were used as negative control and these cells did not express any of the pluripotent markers. a. SSEA4. b. TRA-1-81 and c. Oct4. Colonies shown here are all at X10 magnification.

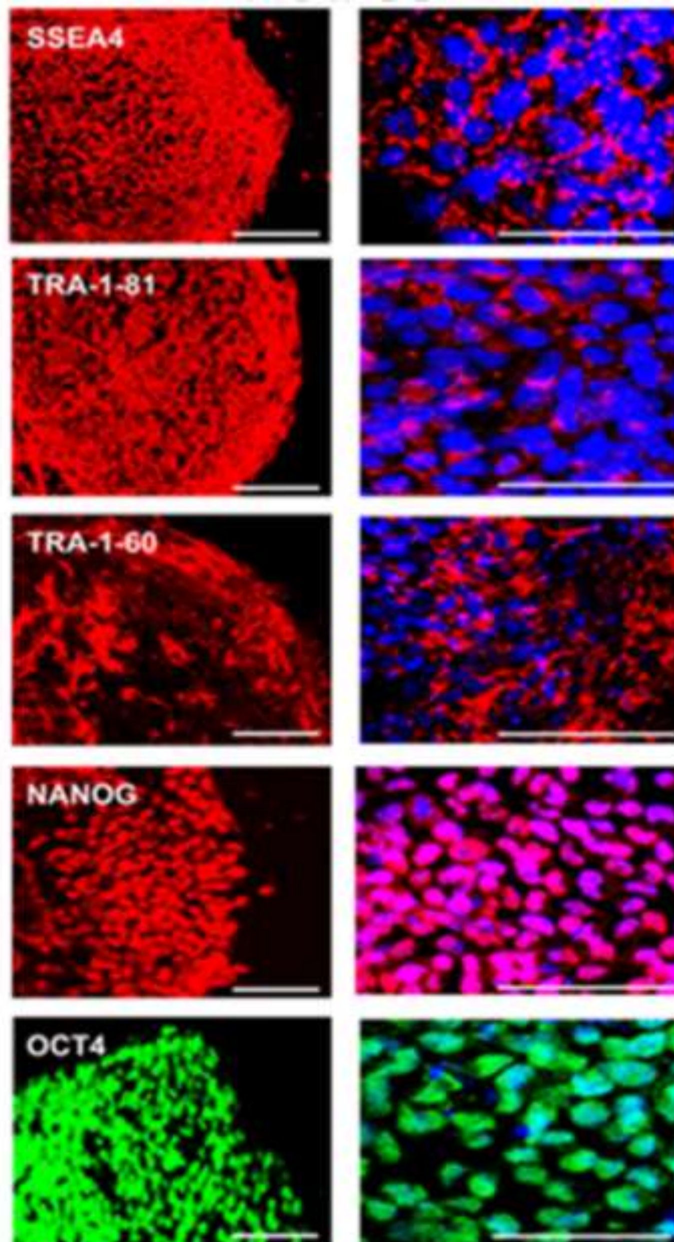


Figure 6.7. Pro-iPS colonies express a panel of 3 surface markers, SSEA4, TRA-1-81 and TRA-1-60 and a panel of 2 nuclear markers, NANOG and Oct4. All images in the left column are at X 20 magnification and in the right panel are at X40 magnification. In addition, the left panel depicts the antibody staining without DAPI while the right-hand panel depicts the respective antibody staining merged with DAPI.

6.4. Discussion and conclusion

In this chapter the phenotypic characterisation of the Pro-iPS has been described. Transcript analyses confirmed that the gene expression profile of the Pro-iPS cells with respect to pluripotency was comparable to the H9 embryonic stem cell line. Transgene silencing, expression of Tra-1-60, Dnmt3b and Zfp42 are identified as markers of the fully re-programmed state. In contrast, GDF3, SSEA4, NANOG as well as alkaline phosphatase expression are not sufficient ,arkers of the fullt re-programmed iPS state (Moad, Pal et al. , Chan, Ratanasirintrawoot et al. 2009). The Pro-iPS cell lines also expressed pluripotent markers in a an embryonic stem cell-specific manner, where core pluripotency molecules Oct4 and NANOG were localised to the nucleus alone (Moad, Pal et al.).

After confirming the phenotypic characterisation of these cells, the next obvious step would be to test their functional properties in terms of their lineage plasticity and differentiation capacities. This is detailed in Chapter 7.

Chapter 7.

Functional characterisation of Pro-iPS cells

7.1. Introduction

The functional characterisation of Pro-iPS cells was carried out with two major objectives: firstly to test whether the Pro-iPS cells would differentiate into all the three germ-layer derivatives namely ectoderm, mesoderm and endoderm. The most irrevocable test of pluripotency of a cell is its germline competence, a feature that is demonstrated in murine pluripotent cells by means of chimera formation (Okita, Ichisaka et al. 2007). It is not possible to demonstrate chimeras in human iPS cells and therefore pluripotent functionality has been demonstrated in human iPS cells by means of *in vivo* teratoma formation (Takahashi, Tanabe et al. 2007, Yu, Vodyanik et al. 2007).

The next objective is to test the core hypothesis of this thesis, that is, the lineage fidelity of the Pro-iPS cells in terms of their tendency to differentiate into prostate. The justification behind sourcing iPS cells from the prostate rather than the more accessible skin tissue was based on the speculation that the retained epigenetic memory in prostate derived iPS cells would make these cells more inclined towards a prostate-specific differentiation route.

7.2. Aims

1. To test whether Pro-iPS cells would differentiate into all the three germ layers – ectoderm, mesoderm and endoderm

2. To compare lineage fidelity of Pro-iPS cells and skin-iPS cells towards prostatic phenotype.

7.3. Specific methods and results

7.3.1. AR levels in Pro-iPS cells

One of the characteristics of iPS cell induction is the fact that fully re-programmed skin-iPS cells fail to express fibroblast specific marker – CD90/Thy 1. In other words, the fully re-programmed state is characterised by loss of expression of lineage or differentiation markers (Stadtfeld, Maherali et al. 2008). Hence, AR levels were checked during re-programming of prostate and it was observed that during the course of de-differentiation the prostate fibroblasts down-regulated AR expression (Figure 7.1). Since no PSA expression is apparent in prostate stroma it was decided not to evaluate the status of this gene. PSA is an androgen responsive gene and is secreted by following activation of functional AR by ligand. Hence, in the scenario of decreasing AR levels it is unlikely to correlate with an increase in PSA expression by the re-programming fibroblasts. AR expression was also noted in skin-iPS cells (Figure 7.1) and this can be accounted for by the fact that AR expression is previously demonstrated in skin cells (Keenan, Meyer Iii et al. 1975). Both prostate epithelia and prostate stroma were found to express similar levels of AR (Figure 7.1). AR levels were observed to reduce by approximately half their initial value during the first 6 days post transduction. This was followed by a further 5-fold reduction in AR in the Pro-iPS cells (Figure 7.1) .

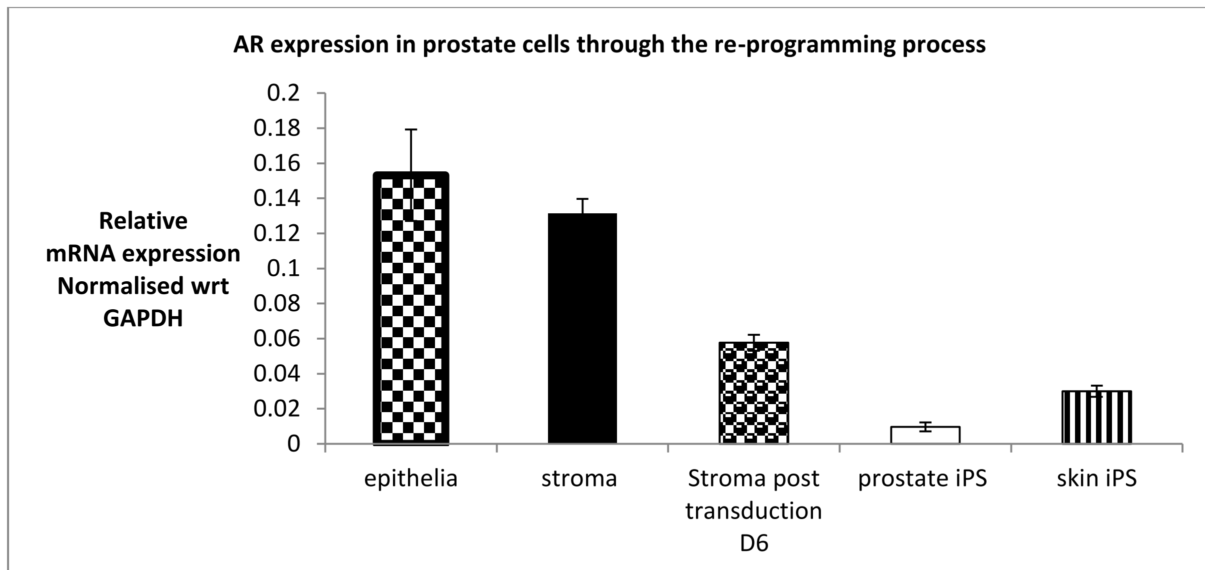


Figure 7.1. Changes in AR expression levels in prostate cells following viral transduction. Relative AR levels in primary prostate stroma, epithelia, stroma cells at 6 days following transduction and prostate iPS cells were compared with each other and against skin iPS cells. AR expression was observed to be reduced in the prostate stroma during the course of iPS induction.

7.3.2. *In vitro* differentiation – Embryoid body formation

The Pro-iPS cells were examined to demonstrate their ability to differentiate into the three germ layers *in vitro* by means of embryoid body formation in human pluripotent stem cell media in the absence of bFGF (Figure 7.2). Pro-iPS cell clumps when cultured in the absence of b-FGF on low-adhesion plates for 7-10 days were noted to form three dimensional structures. It was also observed that these three-dimensional embryoid bodies contained areas that were apparently darker than adjacent areas (Figure 7.2). It is possible that these areas

represent foci of necrosis due to insufficient gas and nutrient transfer. It is also possible that these areas represented regions where the constituent embryoid body forming cells were undergoing various differentiation processes. These bodies were transferred onto 0.1% gelatin coated plates for another 7-10 days in order to facilitate adherence and to demonstrate differentiation into cells representing the ectoderm, mesoderm and endoderm (Moad, Pal et al.). The differentiation of Pro-iPS cell-derived embryoid bodies into the three embryonic lineages is demonstrated in Figure 7.3. Example of cells representing the ectoderm includes those of the peripheral nervous system, that is, neuronal like cells. Cells representing the mesoderm include mesenchyme myocyte-like cells while cells representing the endodermal lineage include the cobble-stoned epitheloid cells (Figure 7.3). The differentiated germ layers were further characterised for their respective markers (Figures 7.4- 7.9).

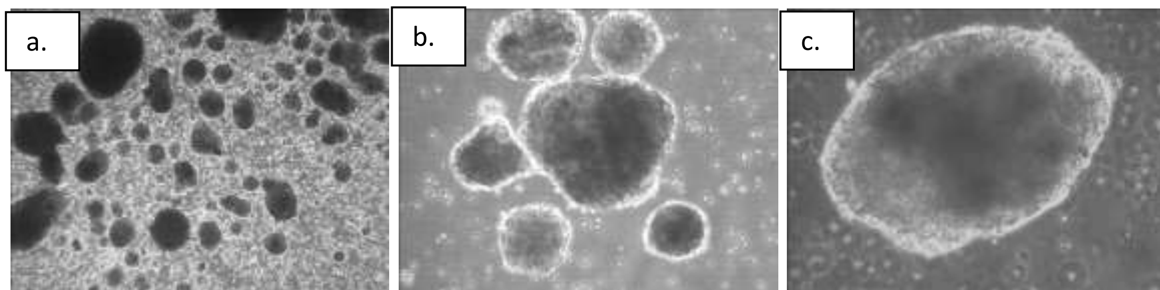


Figure 7.2. Pro-iPS cells when cultured in the absence of b-FGF on low-adhesion plates form spherical structures that resemble embryoid bodies. a. X 10 magnification . b. X20 magnification. C. X 40 magnification.

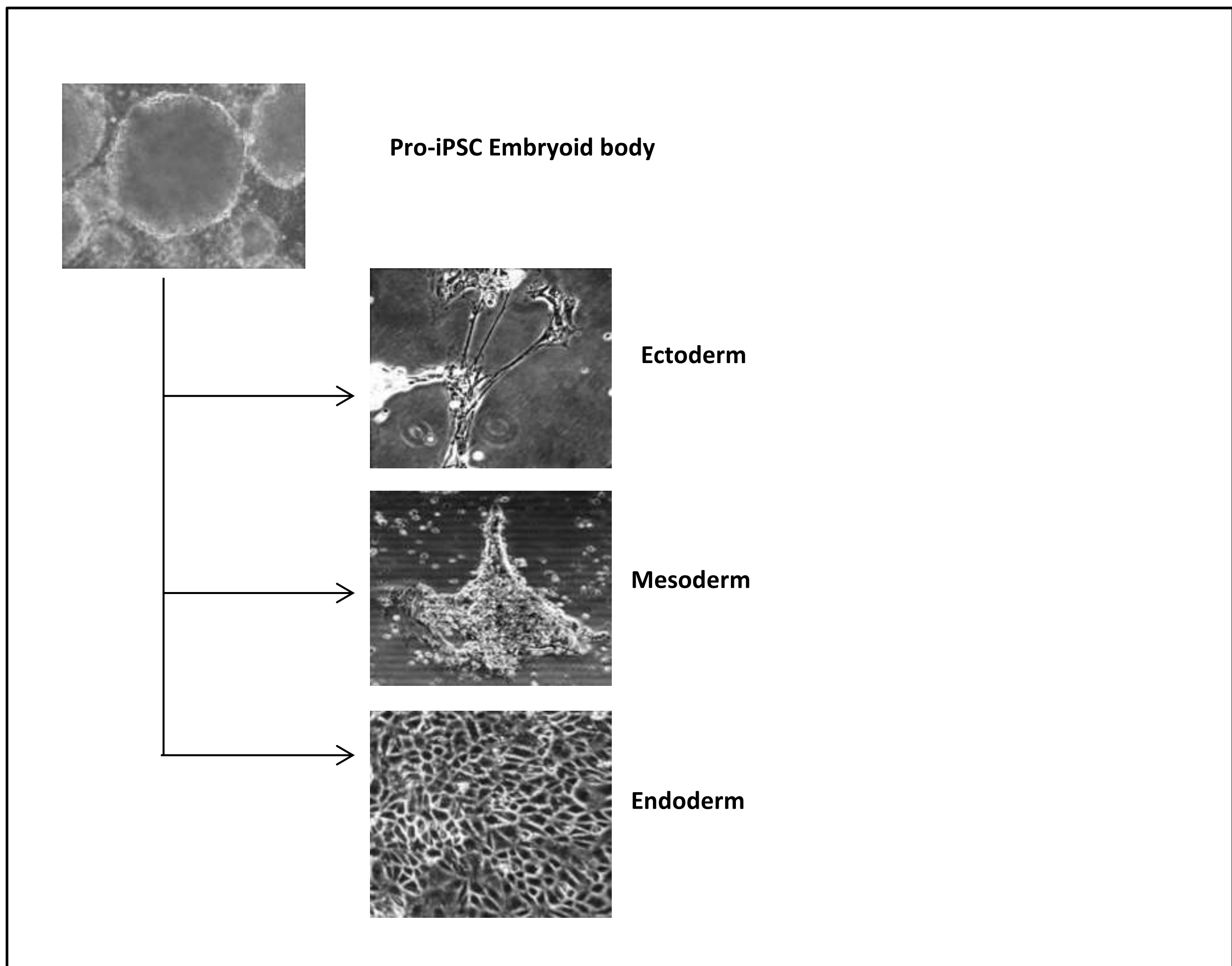


Figure 7.3. Pro-iPS derived embryoid bodies differentiate into all three germ layers – the Ectoderm, Mesoderm and Endoderm. Examples of ectoderm-derived cells includes neurons and differentiation into this germ layer was depicted by the presence of neuronal-like offshoots arising from the Pro-iPS embryoid bodies.

The next step was to analyse the Pro-iPS differentiated cells in order to confirm their lineage and to this end 1-2 transcript specific for each of the three lineages were employed, namely PAX6 (Figure 7.4) and β-III tubulin (Figure 7.9) for ectoderm, αSMA (Figure 7.5) and CD 31 (Figure 7.9) for mesoderm and α-feto protein for endoderm (Figure 7.6 and Figure 7.9). As expected, Pro-iPS embryoid body-differentiated cells expressed their specific lineage transcripts (Moad, Pal et al.). It would be expected that during the course of differentiation into the three germ layer derivatives that the embryoid-body derived cells should down-regulate pluripotent transcripts. Failure to do so may potentially indicate the persistence and possible integration of the provirus (Stadtfeld, Maherali et al. 2008). Pro-iPS embryoid bodies were found to down-regulate pluripotent transcripts Oct4 and SOX2 during differentiation (Figure 7.7 and 7.8), further corroborating the RT-PCR results in Chapter 6 that suggested the potential silencing of the provirus (Moad, Pal et al.).

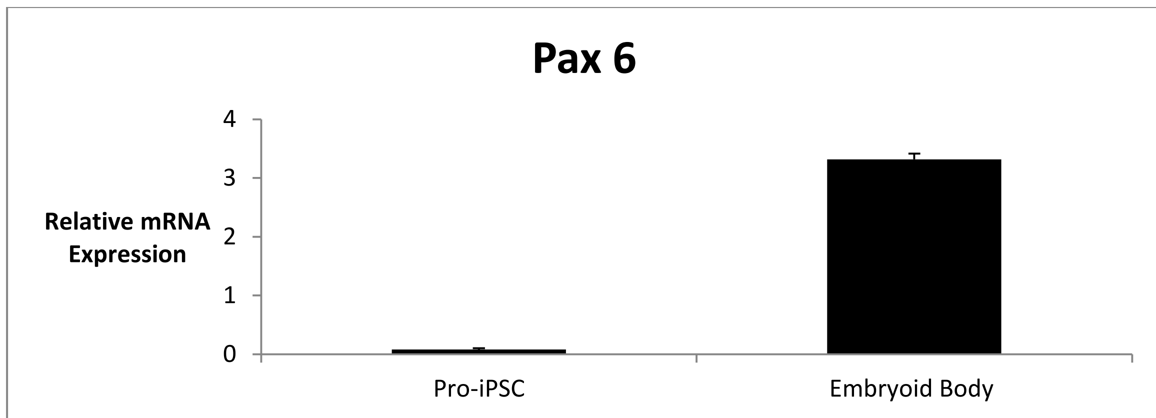


Figure 7.4. Pro-iPS derived embryoid bodies differentiate into cells of ectodermal lineage. PAX6 expression (ectoderm-specific marker) is up-regulated by Pro-iPS cells during differentiation of embryoid bodies.

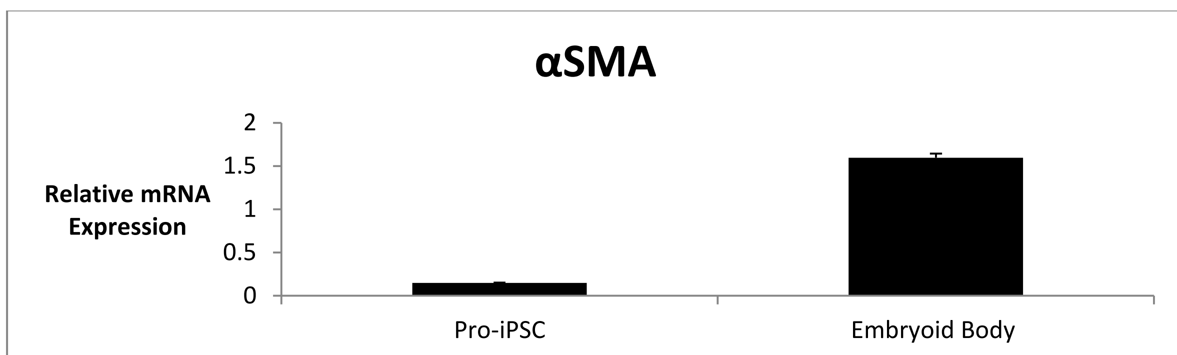


Figure 7.5. Pro-iPS derived embryoid bodies differentiate into cells of mesodermal lineage. αSMA expression (mesoderm-specific marker) is up-regulated by Pro-iPS cells during differentiation of embryoid bodies.

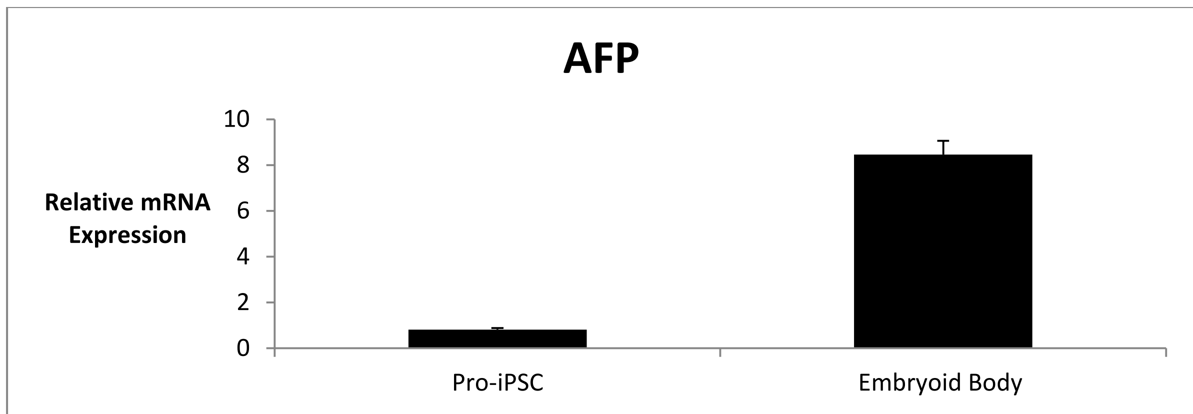


Figure 7.6. Pro-iPS derived embryoid bodies differentiate into cells of endodermal lineage. AFP expression (endoderm-specific marker) is up-regulated by Pro-iPS cells during differentiation of embryoid bodies.

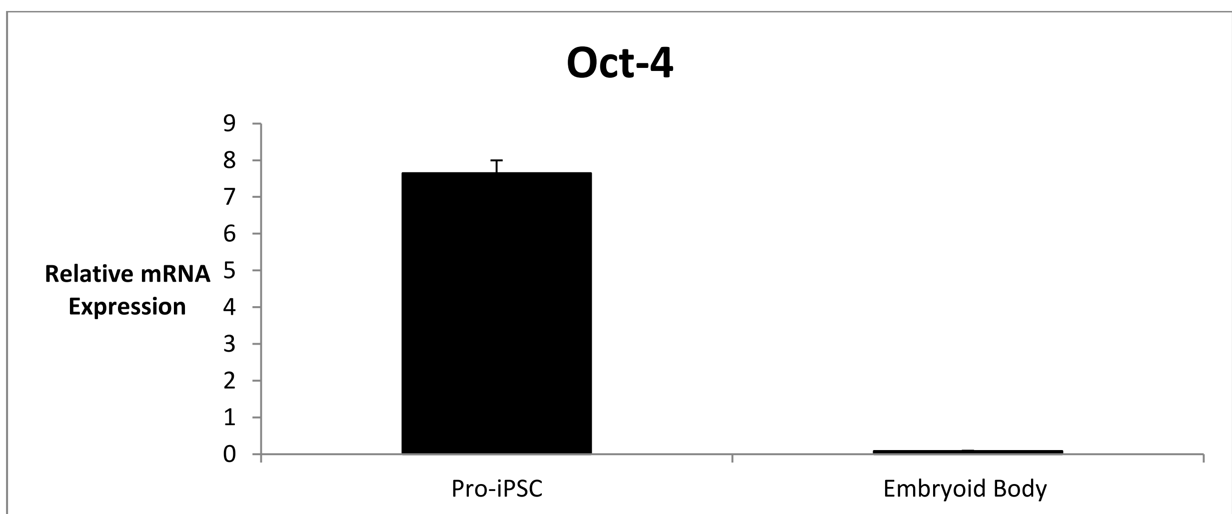


Figure 7.7. Following differentiation of Pro-iPS to embryoid bodies, these differentiated cells down-regulate the core pluripotency factor Oct-4. This further corroborates data from Chapter 6 suggesting silencing of the exogenous transcript (Figure 6.3)

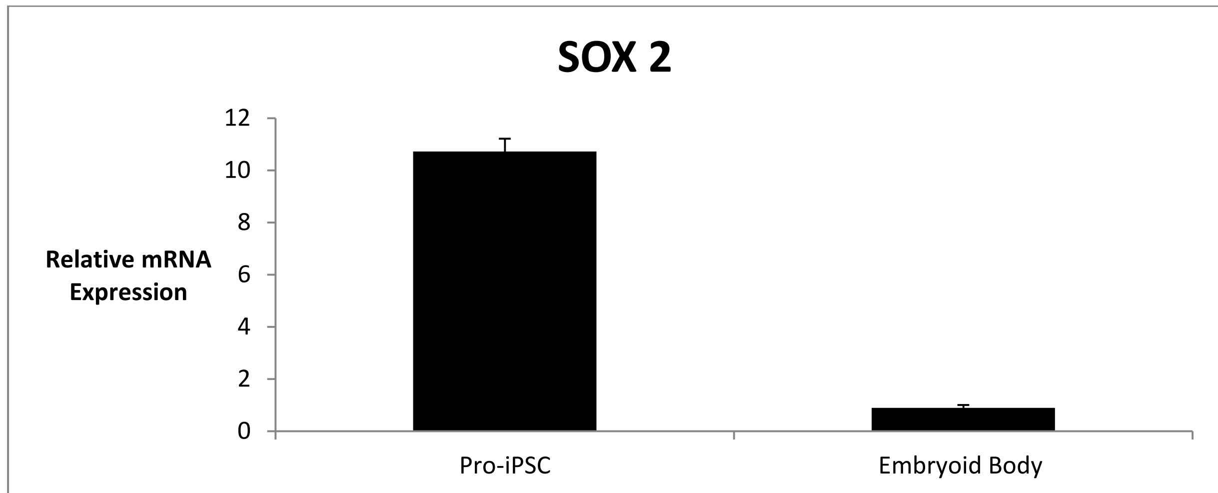


Figure 7.8. Following differentiation of Pro-iPS to embryoid bodies, these differentiated cells down-regulate the core pluripotency factor SOX2. This experiment further validates silencing of the exogenous transcripts that was discussed in Chapter 6 (Figure 6.3)

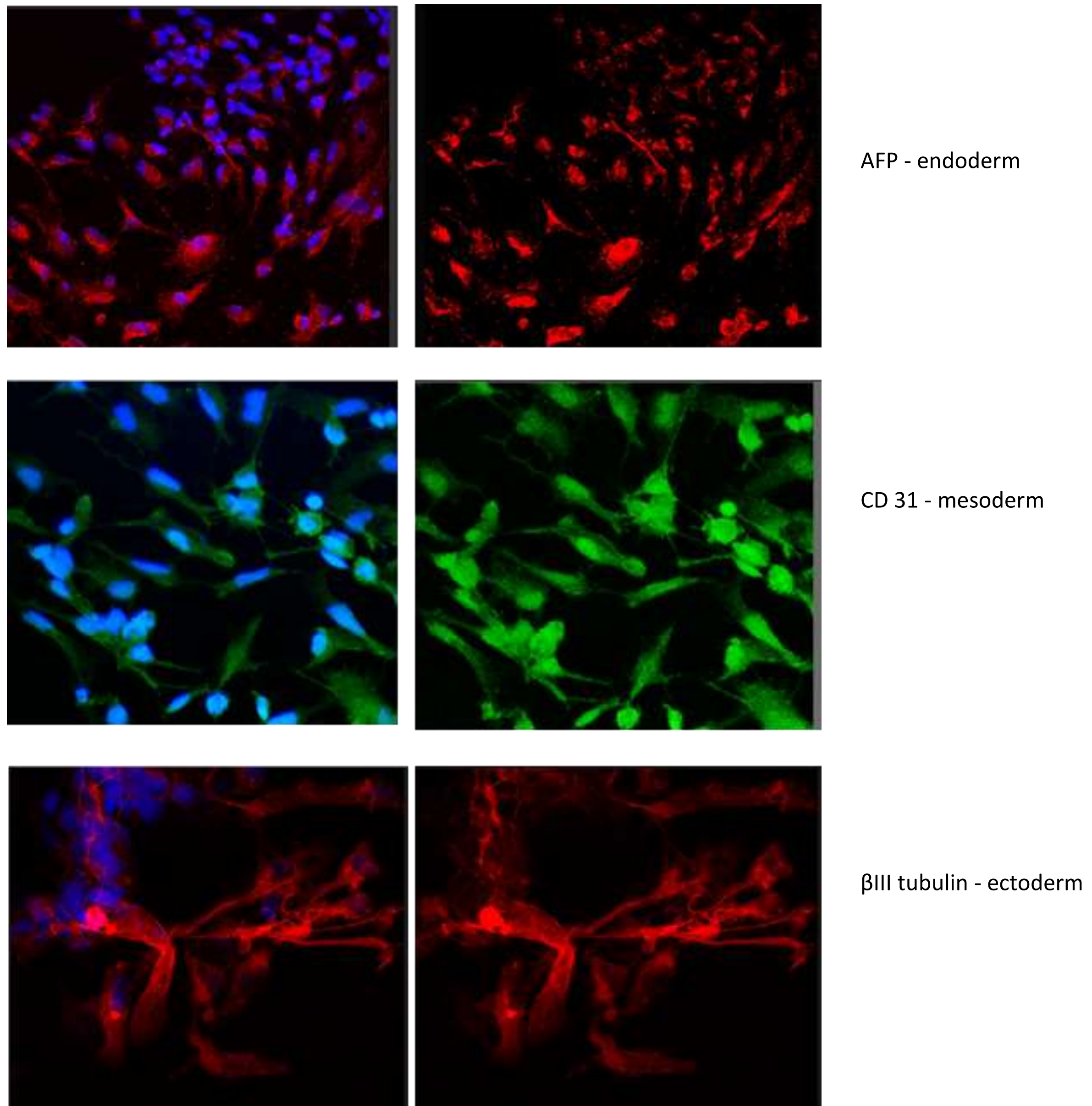


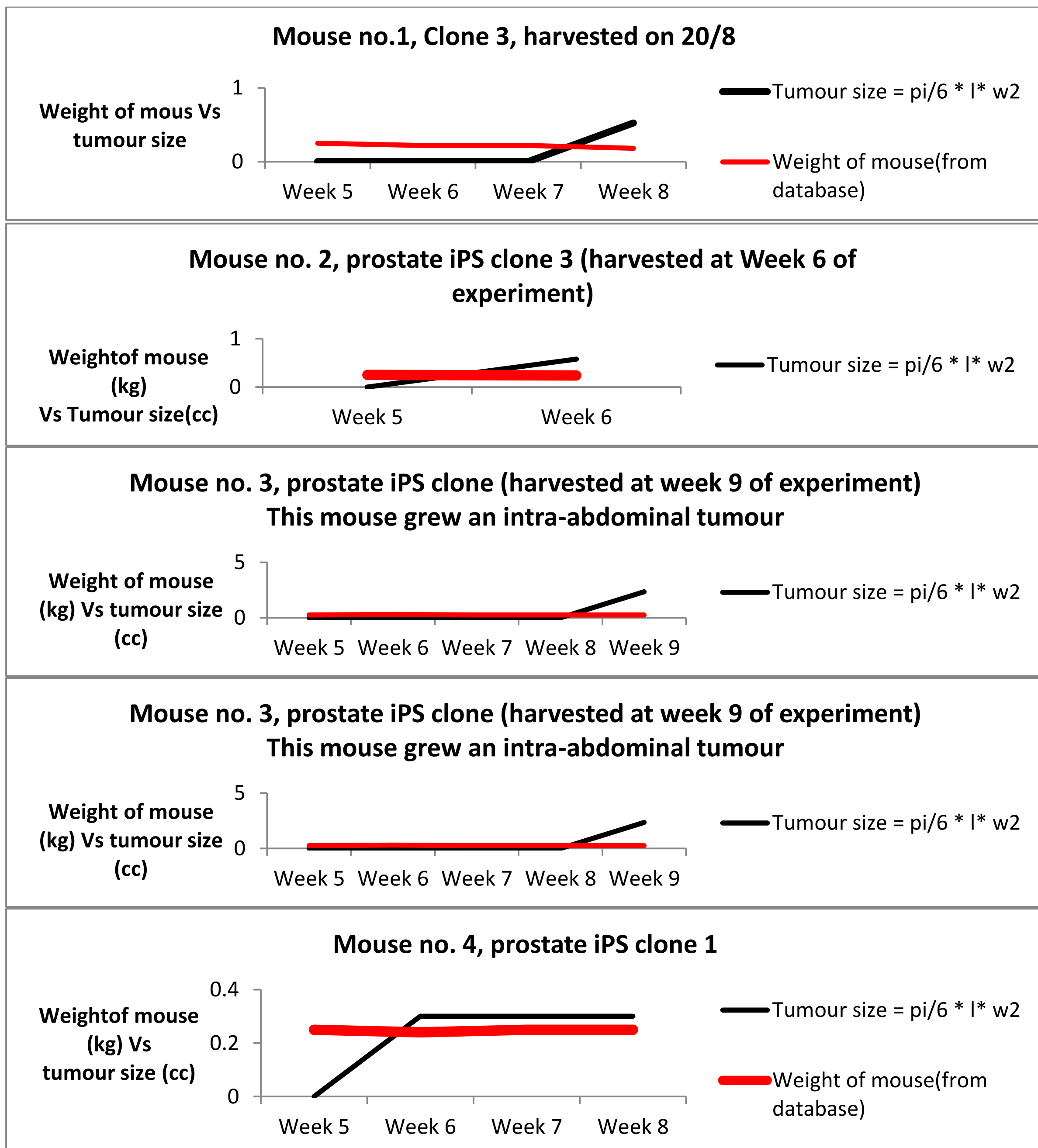
Figure 7.9. Characterisation of Pro-iPS embryoid body differentiation through immunofluorescence staining. Following differentiation, Pro-iPS-derived embryoid bodies express βIII-tubulin (marker for ectoderm), CD 31 (marker for mesoderm) and AFP (α-feto protein marker for endoderm). The panels on the left show the individual antibody staining merged with DAPI (for localisation of the nucleus) while the panels on the right shows the individual antibody staining without DAPI. All the three markers shown demonstrate a cytosolic expression pattern that is consistent with the literature

7.3.3. *In vivo* differentiation – teratoma formation

The ability of Pro-iPS cells to differentiate into the three germ layers *in vivo* was depicted by the formation of teratomas at 3 weeks after s/c injection into NOD/SCID Y mice (Figure 7.10 and Figure 7.11). Pro-iPS cells formed small solid tumours at the site of injection and these demonstrated rapid growth over the next week. The mice were euthanised as per the relevant license and tumours were dissected out, weighed, measured and processed either for immunohistochemistry (fixed in 4% PFA) or for trichrome stains (fixed in Buoin's fluid). A total of 9 mice were injected, 7 of them showed formation of solid tumours as shown in Figure 7.10 below. The dimensions of the tumours were plotted alongside the body weight of each mice against time following injection. Generally, it was noted that over the course of the experiments, the weight of the mice showed a tendency to remain constant while there was in most cases a rapid growth in tumour size. Formation of solid tumours that grow rapidly over time at the site of injection suggest that these would be more likely to be consistent with a teratoma on the grounds of histopathology (Gertow, Przyborski et al. 2007).

For the above detailed experiments, three Pro-iPS clones were tested (clones-1, -3 and -4 based on their gene expression profile) and each clone was injected into three mice (Figure 7.10). All three mice injected with Pro-iPS clones-1 and -3 formed tumours, while only one mouse injected with Pro-iPS clone-4 exhibited tumour formation. One of the three mice injected with Pro-iPS clone-3, grew an intra-abdominal tumour – the tumour could not be seen externally. The mouse was euthanised as it was doing very poorly and became inactive. On dissection an intra-abdominal tumour was noted that measured 2.4 cm3 in volume. The tumour had an osseous/cartilagenous feel to it and was fixed in 4% PFA/Bouins fluid. Teratoma formation by these Pro-iPS cells has also been described by Dr Simon Hayward's group at Vanderbilt University Medical Centre, TN, USA (Figure 7.11) (Moad, Pal et al.). Figure 7.11 in addition shows teratoma formation by UT-iPS cells, whereby all the three

germ layers have been depicted. The possibility of carcinomatous change in urological-iPS-dervied teratomas has been appropriately critiqued as a limitation of the iPS technology (Wezel and Southgate 2013). Our research group acknowledge this as a valid risk and recognise future emphasis should be on improving methods of re-programming such as transgene-free re-programming approaches as well as xeno-free culture methods (Pal, Moad et al. 2013).



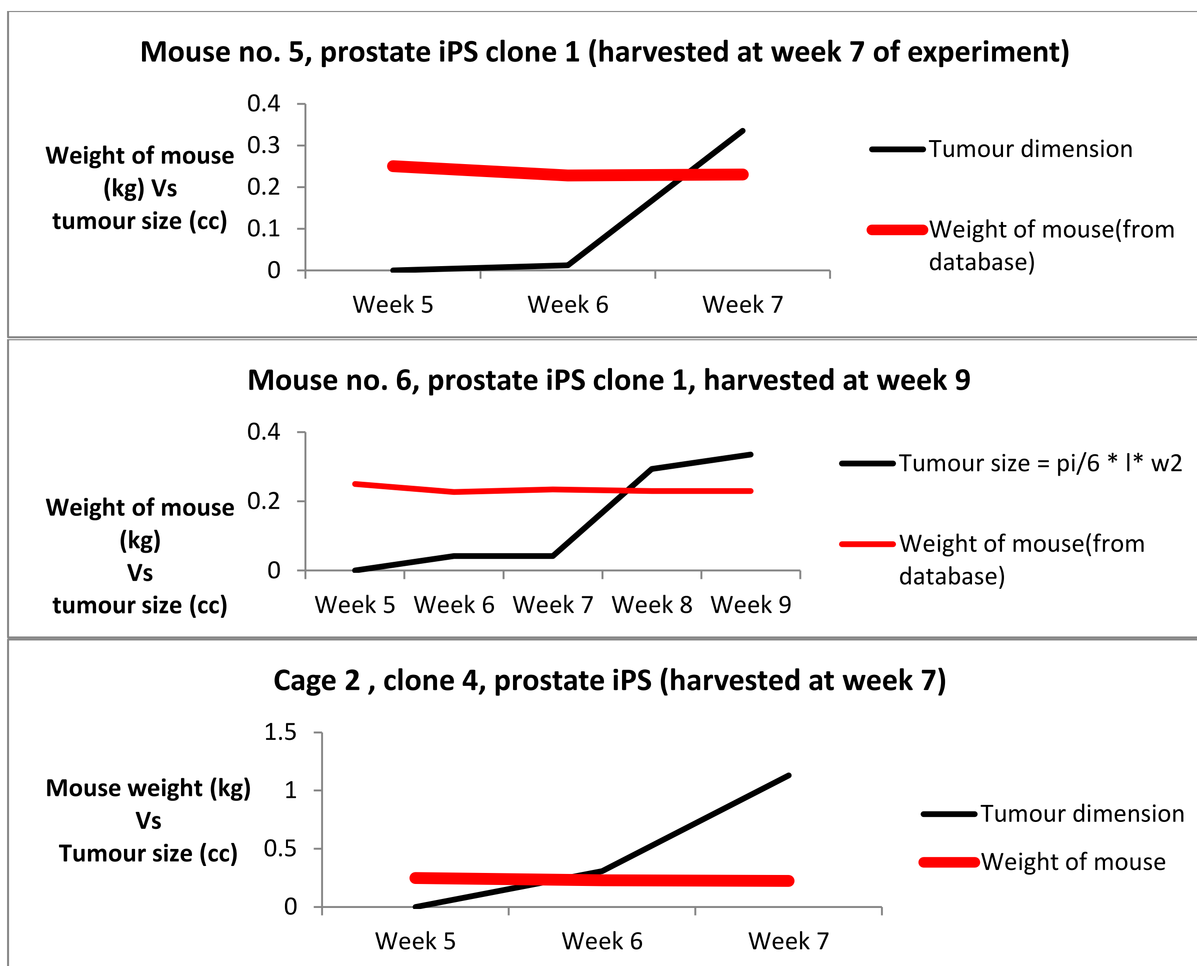


Figure 7.10. Formation of teratomas in Pro-iPS cells. Pro-iPS clone 3 when injected s/c into NOD/SCID Y mice formed detectable tumours at week 7, week 5 and week 8 for mouse 1, 2 and 3, respectively. Once externally visible they grew rapidly over the next week. Mouse 3 formed an intra-abdominal tumour possibly as a result of the injection going beyond the subcutaneous region to the abdomen. Pro-iPS clone 1 when injected s/c into NOD/SCID Y mice formed tumours at week 5, week 6 and week 5 for mouse 4, mouse 5 and mouse 6, respectively. Once externally visible they grew rapidly over the next week. Pro-iPS clone 4 formed only one tumour from one of the three mice at around week 5 and was harvested at week 7.

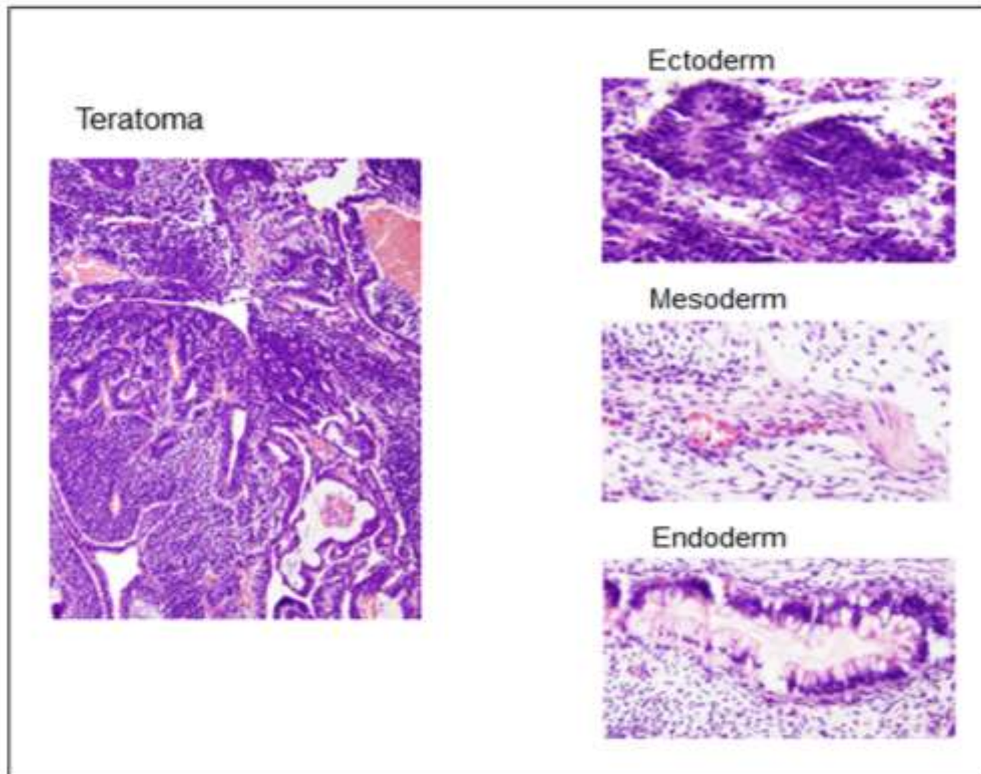


Figure 7.11. Histology from a teratoma formed by Pro-iPS cells. The histopathological analyses was kindly performed by Prof. Simon Hayward, Vanderbilt University, USA. Pro-iPS derived teratoma demonstrated neuronal epithelial differentiation, UT-iPS (Urinary Tract-iPS) demonstrate ddifferentiation into all the three germ layers (Moad, Pal et al.).

7.3.4. Lineage-specific differentiation of Pro-iPS and skin-iPS towards a prostate-specific route

It is well established that the prostate mesenchyme regulates prostate epithelial differentiation and function and this concept was utilised to analyse prostatic differentiation through stroma-conditioned media (Moad, Pal et al. , Lawrence, Taylor et al. 2013, Pal, Moad et al. 2013). Skin-iPS cells were used as control. The aim here was to evaluate whether prostate-derived iPS cells would show greater lineage fidelity by virtue of their primary epigenetic memory and whether this would make them more inclined towards prostatic differentiation. The first step was to establish culture conditions that would promote a prostatic differentiation. To this end, prostate stroma-conditioned media was generated by feeding prostate stroma cells with RPMI-1640 medium supplemented with 10% FCS and 10nM dihydrotestosterone. Dihydrotestosterone was used to stimulate the prostate stroma cells with the expectation that this would facilitate the differentiation process. The prostate primary fibroblast cells were fed with the stroma-conditioned media every 48 hours. Conditioned media was filtered through a 0.2 micron filter and further supplemented with 10nM dihydrotestosterone prior to use.

Pro-iPS cell clumps were suspended in the prostate stroma-conditioned media and grown on low adhesion dishes for 7-10 days. This resulted in the formation of 3-D structures that were likened to potential prostatospheres. Some of these structures were fixed in formalin and paraffin embedded and stained with H&E in order to determine the presence of any possible luminal architecture. Some other structures were transferred onto 0.1% gelatin coated plates and maintained in the stroma-conditioned medium as described above. On Day 21 the cells started to emerge from the spheroids. There were mesenchymal and epitheloid cell types noticed. In particular, clusters of epitheloid cells surrounded by mesenchymal cells were noted (Figure 7.12). Clusters of epitheloid differentiation was observed as 60-70% differentiation in Pro-iPS cells. Cells treated with RPMI-1640 media were used as a control

arm and no epitheloid structures were noted in the absence of conditioned media. Subsequently, the cells were trypsinised and were put through a CD324 (HEA/EpCaM) sort and the positive cells were cultured for another 5 days to enable them to seed out on the plates. The cells were then characterised for the epithelial marker CD 24 (Figure 7.13) and prostate markers (AR and PSA) (7.14-7.16). The same protocol was duplicated for skin-iPS cells. It was observed that when skin-iPS clumps were cultured in prostate stroma conditioned media, they did not possess the same predilection towards prostatic differentiation as was observed for the Pro-iPS cells. After treating skin-iPS and Pro-iPS with stroma-conditioned media, a 2-fold increase was noted in AR transcripts.I In contrast, the Pro-iPS cells demonstrated a 270 fold increase in AR levels following treatment with stroma-conditioned media (Figure 7.14) (Moad, Pal et al.). In addition, PSA expression was only induced in the Pro-iPS cells following treatment with stroma-conditioned media (Figure 7.15) (Moad, Pal et al.). Immunofluorescence staining of the Pro-iPS-derived cells further confirmed localisation of AR in the nucleus supporting its functional status, while PSA being a secretory protein was localised to the cytoplasm (Figure 7.16) (Moad, Pal et al.).

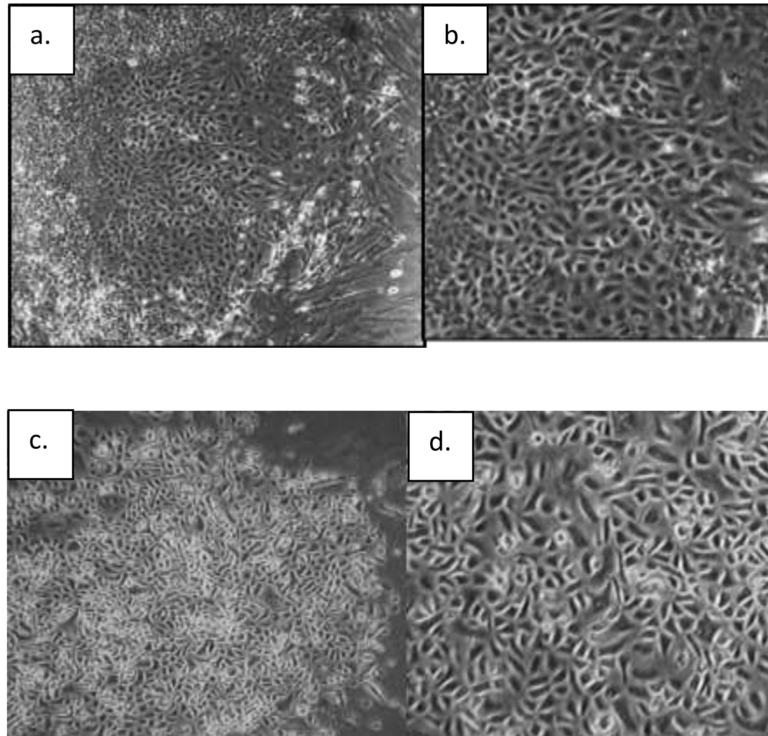


Figure 7.12.Effect of prostate-stroma conditioned media on Pro-iPS-derived spheroids. Pro-iPS cell clumps formed spheroids when cultured in stroma-conditioned media. These spheroids, when seeded onto gelatin coated flasks differentiated to form clusters of epitheloid cells surrounded by mesenchymal cells. a. Pro-iPS in stroma conditioned media after 3 weeks in culture X10. b. Pro-iPS differentiated epitheloid cells at week 3 of culture X20 magnification. c. Prostate epithelia primary culture X10. d. Prostate epithelia primary culture X20.

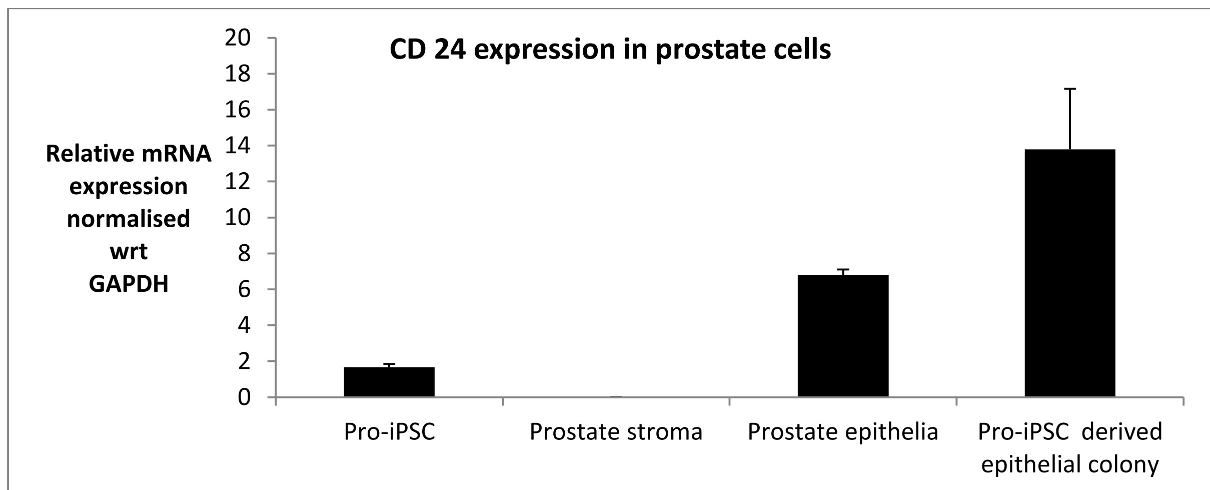


Figure 7.13. Expression of CD 24 epithelial marker in prostate cells. Prostate stroma was used as negative control. CD 24 expression was noted in Pro-iPS cells. This is not unexpected since iPS cells are epitheloid and are known to express epithelial markers (Li, Liang et al. 2010, Samavarchi-Tehrani, Golipour et al. 2010). Prostate epithelial cells were used as positive control. Pro-iPS-derived cells up-regulated CD 24 expression as compared to Pro-iPS suggesting that they were possibly epitheloid.

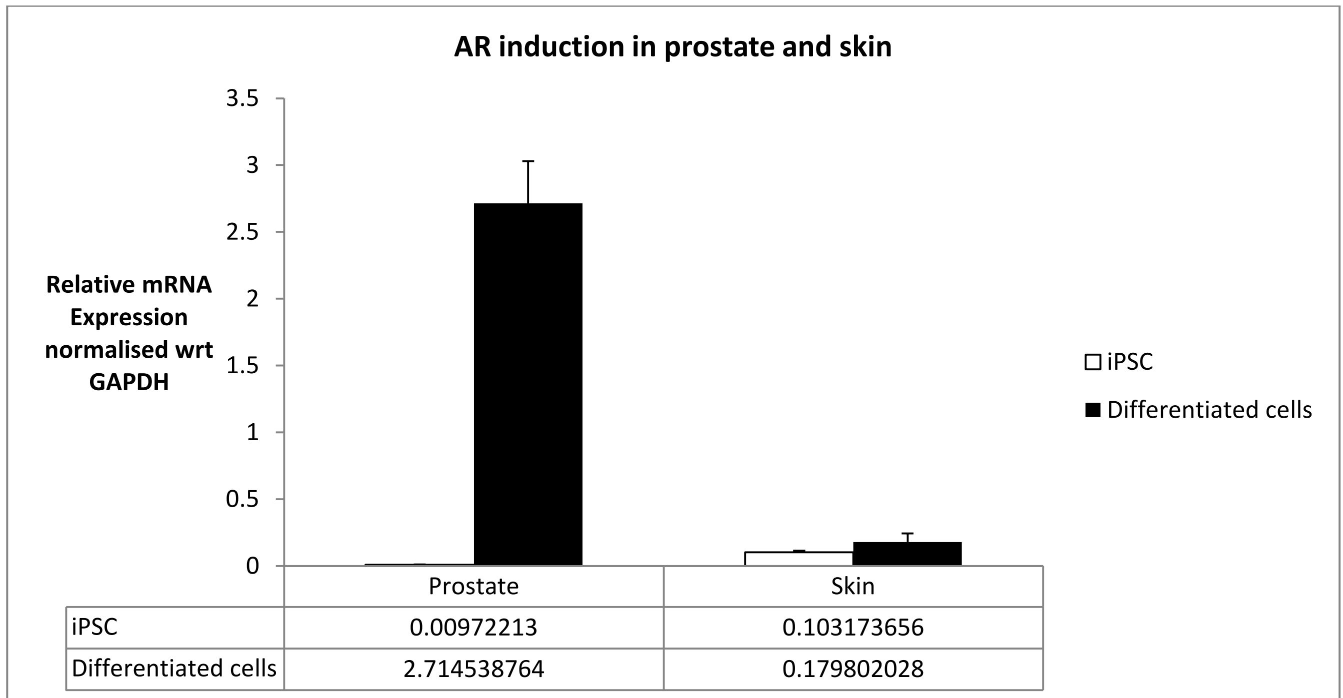


Figure 7.14. AR induction in Pro-iPS and skin-iPS following treatment with prostate-stroma conditioned media. AR induction was elevated around 270 fold in prostate while it is only elevated 2-fold in skin iPS cells.

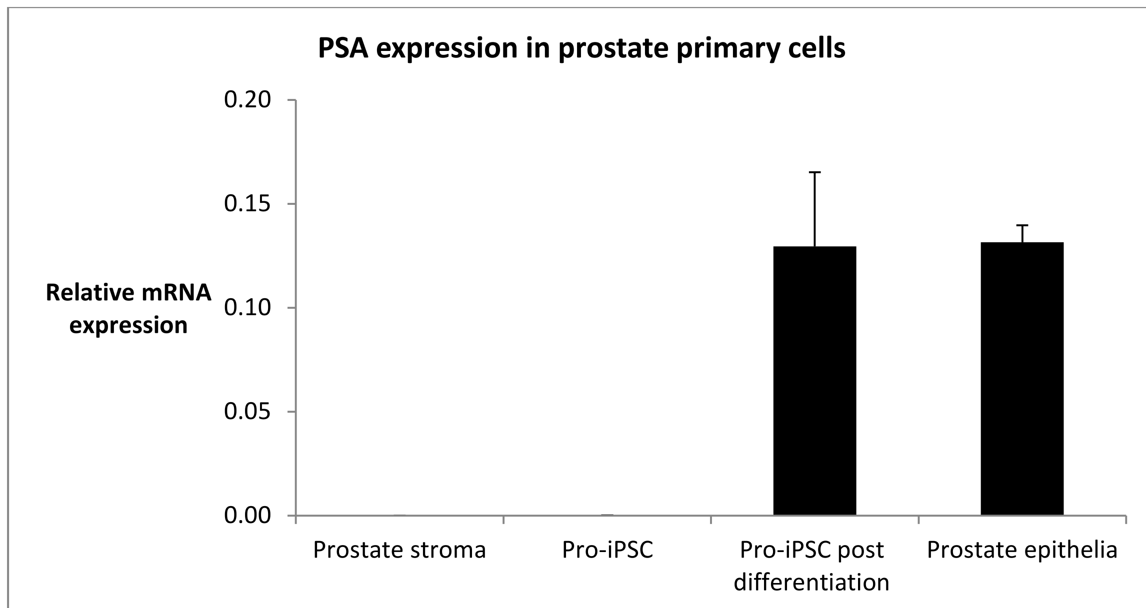
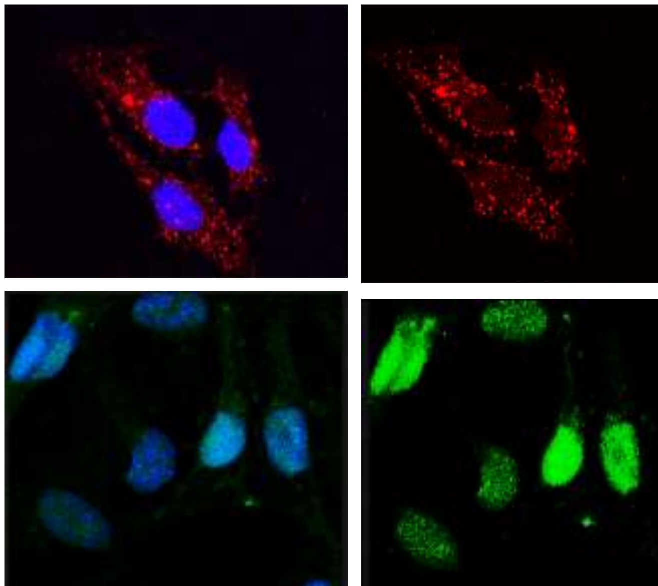


Figure 7.15. Pro-iPS epithelioid cells express PSA. Prostate stroma was used as negative control. Pro-iPS cells did not express any PSA denoting that the AR in Pro-iPS cells is functionally inactive. Post differentiation PSA levels in Pro-iPS-derived cells and prostate epithelia were comparable.



PSA staining in pros-iPSC following differentiation

AR staining in pros-iPSC following differentiation

Figure 7.16. Immunofluorescent staning of Pro-iPS-derived epitheloid cultures demonstrate expression of AR and PSA. PSA being a secreted protein was evident in the cytoplasm of the differentiated cells. Nuclear localisation of AR in these cells further endorsed the AR protein was functionally active. Panels on left show DAPI staining merged with AR/PSA. Right hand panel are stained for either PSA or AR alone.

The author was keen to evaluate 3-dimensional differentiation in the putative prostatic spheroids was next examined. To this end, Pro-iPS clumps were cultured in prostate stroma-conditioned media for 10 days and the resulting spheroids were paraffin embedded, sectioned and stained with Haematoxylin and Eosin. Results demonstrated possible luminal architecture (Figure 7.17). However, the possibility of necrotic pockets forming within the spheroids due to inefficient and uneven mass transfer cannot be ruled out as accounting for the apparent lumen. Staining the spheroids for AR and PSA should be conducted to validate the 3-dimensional differentiation in these spheroids. Alternatively, 3-dimensional differentiation may be achieved by culturing Pro-iPS cells in 3-dimensional synthetic scaffolds. The architecture of these scaffolds is such that the 200 micron thickness (example: Alvetex, Reinnervate) simulates an *in vivo* environment and allows for uniform gas and nutrient amongst individual cells. The scaffolds can then be paraffin embedded, sectioned and evaluated for architecture as well as expression of prostate-specific markers. It is well established that 3-dimensional cell culture improves cell function and demonstrates differentiation in a more efficient manner (Maltman and Przyborski 2010, Xia, Nivet et al. 2013).

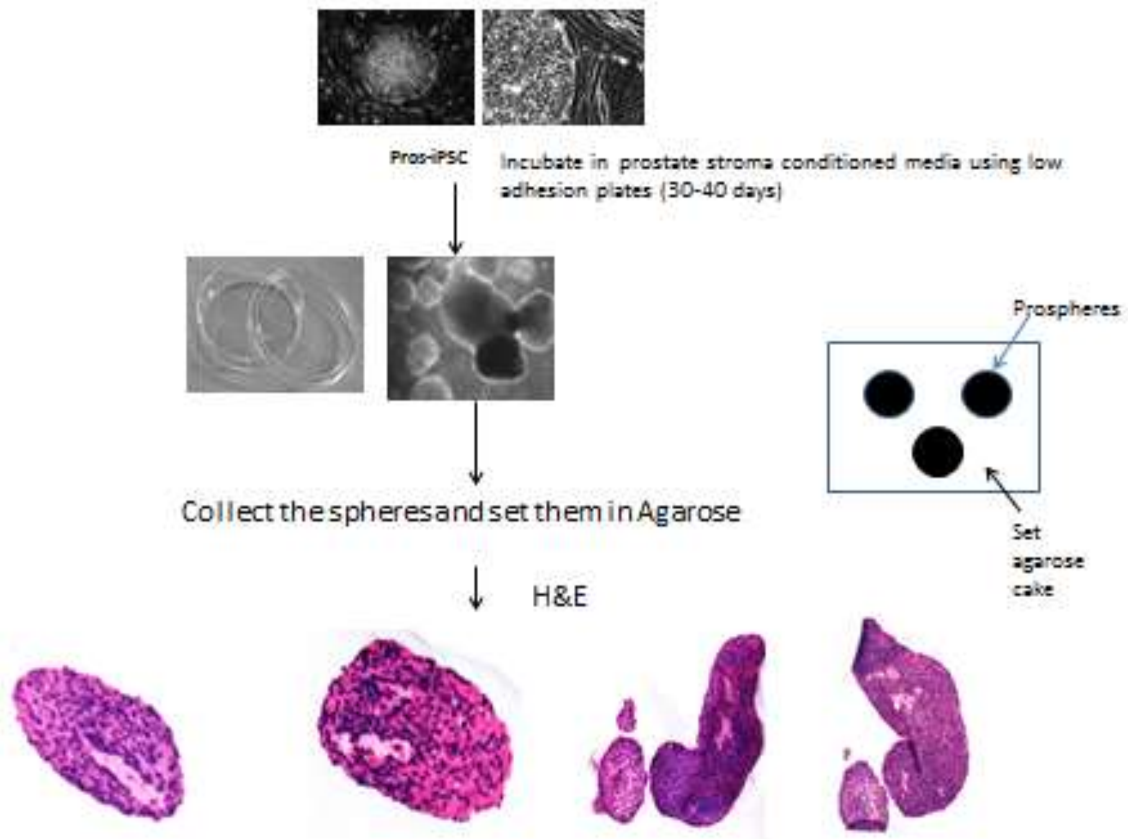


Figure 7.17. Analysis of Pro-iPS-spheroids following exposure to stroma-conditioned media. Pro-iPS-derived spheroids show possible luminal-type architecture on H&E. However, the methodology behind the spheroid culture did not take into account use of matrix or scaffold and therefore it is possible that the conjectured 'luminal' areas are in fact necrotic pockets.

5.4 Discussion and Conclusion

In this chapter, the functional properties of Pro-iPS with respect to pluripotency has been described through *in vitro* formation of embryoid bodies and *in vivo* formation of teratomas (Moad, Pal et al.). The Pro-iPS cells differentiated into cells representing all the three germ layers, namely ectoderm, mesoderm and endoderm. It has also been shown that Pro-iPS cells retained lineage fidelity with respect to prostate-specific differentiation (Moad, Pal et al.). This further substantiates the concept of retained epigenetic memory during stem-cell re-programming (Kim, Doi et al. 2010, Polo, Liu et al. 2010, Bar-Nur, Russ et al. 2011).

Chapter 8

Discussion and Conclusion

The Pro-iPS cell model provides a pertinent model to decipher some of the canonical signalling pathways in prostate lineage plasticity that may regulate prostate development and differentiation. It also provides a new vehicle to test epigenetic signatures in the androgen-receptor signalling during the process of prostatic differentiation. A major critique of the Pro-iPS and UT-iPS cell model (Moad, Pal et al.) is that it has been assumed that imprinted gene signature of the tissue of origin infers incompletely reprogramming of cells (Wezel and Southgate). It is well characterised that *de facto* completely reprogrammed germ-line-competent murine iPS cells and pluripotent human iPS cells retain epigenetic memory during somatic cell reprogramming (Aasen, Raya et al. 2008, Aoi, Yae et al. 2008, Miura, Okada et al. 2009, Kim, Doi et al. 2010, Polo, Liu et al. 2010, Bar-Nur, Russ et al. 2011, Lister, Pelizzola et al. 2011, Lee, Seo et al. 2012). However, this epigenetic memory is transient and gets erased with increased passage number (Polo, Liu et al. 2010). UT-iPS and Pro-iPS cells were used at passage numbers below 50 and it is therefore justifiable that the origin-tissue specific epigenetic memory will be retained in these cells at this stage of early passage (Kim, Doi et al. 2010). Whether this tissue-specific imprinting would get erased at higher passages remains unknown and needs further investigation. This further calls for scrutiny of epigenetic signatures in the iPS cells and underscores the need for individual cellular tracking at the molecular level. It will be interesting to track epigenetic signatures at single cell levels through the process of de-differentiation and differentiation of these urological cells – this would further clarify currently unanswered questions on the epigenetic status of somatic cell re-programming and their consequent differentiation into target lineages.

The most definitive test for pluripotency is formation of chimeras that prove the generated iPS cells to be germ-line competent (Okita, Ichisaka et al. 2007). However, this test is not

feasible in humans and therefore the gold-standard for demonstrating that a cell has been fully re-programmed in human-iPS cell lines is by means of teratoma formation (Takahashi, Tanabe et al. 2007, Yu, Vodyanik et al. 2007). The teratomas shown (Figure 8.1) reflect uncoordinated differentiation but the main objective here is to show pluripotency, which is what the terotomas demonstrated. *In vivo* engraftments with either bladder or urogenital sinus mesenchyme were undertaken but these experiments were not optimised to investigate directed differentiation, and the mesenchyme served to enhance engraftment rates and growth (Kanai, Ishii et al. 2008, Lawrence, Taylor et al. 2013). Although the directed differentiation to generate prostate and bladder-like structures *in vivo* would further support our finding, we do demonstrate the ability for terminal epithelial differentiation with induced expression of AR and PSA in the prostate derived iPS cells and induction of uroplakins and claudins in the urinary tract derived iPS cells. The use of conditioned medium to induce differentiation was criticised (Wezel and Southgate) but these are established and well characterised techniques (Baskin, Hayward et al. 1996, Bayne, Ross et al. 1998, Hashiba, Noguchi et al. 2000, Tian, Bharadwaj et al. 2010, Taylor, Toivanen et al. 2012, Lawrence, Taylor et al. 2013). The need to assess the effect of conditioned media from various sources on the UT-iPS and Pro-iPS cells would further clarify their inherent *in vitro* differentiation abilities as well as lineage commitments. Although this is beyond the scope of the study herein, the study of the iPS cell lines generated by us pave a foundation for such assessments. Our group with other colleagues have recently noted that UT-iPS and Pro-iPS cells when differentiated *in vivo* with bladder or prostate instructive mesenchyme differentiate into urological lineage. These studies confirm that in the absence of any tissue-specific-instructions, the UT-iPS and Pro-iPS cells differentiate to represent all the three germ layers whereas they retain an inherent ability towards urological differentiation when subjected to lineage-specific differentiation

strategies. This suggests an inherent fidelity of these cells towards tissue-specific differentiation that may be directed by an epigenetic status – this needs further investigation.

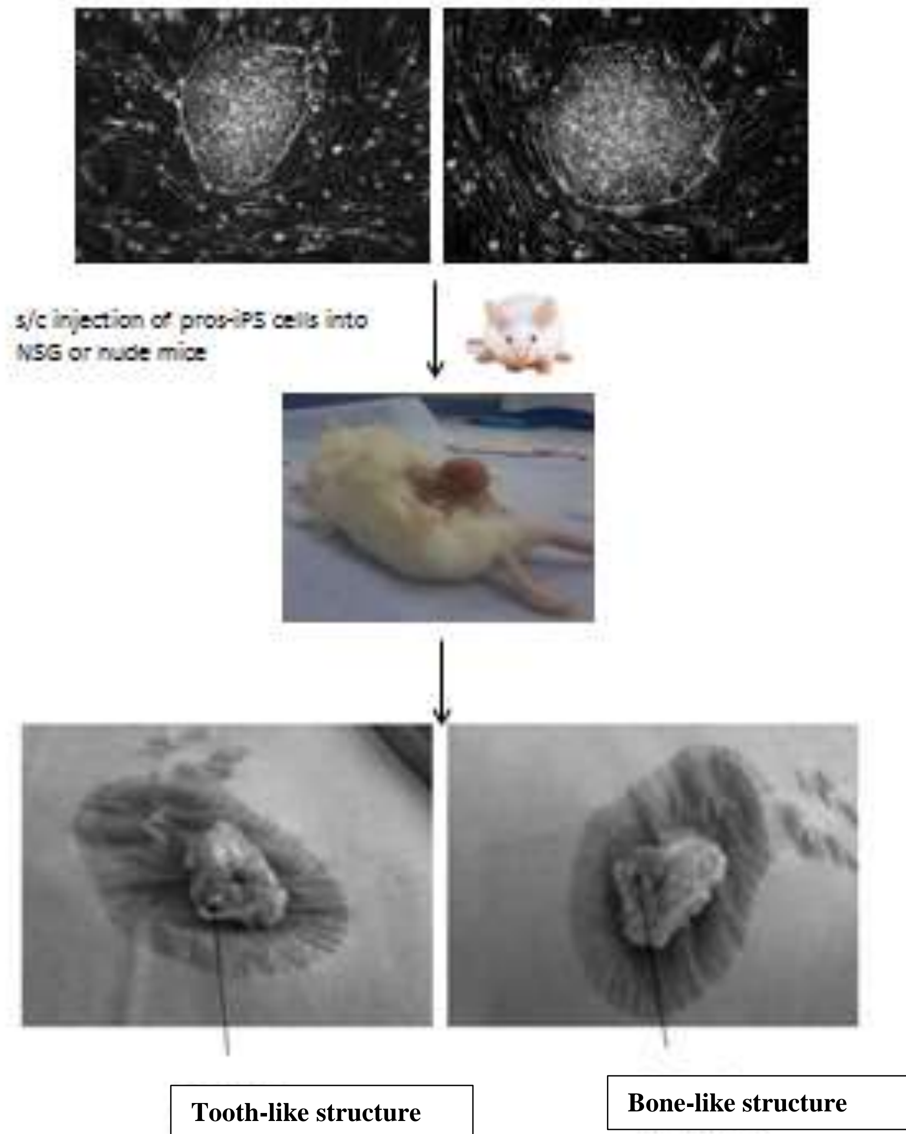


Figure 8.1. Teratoma formation in Pro-iPS cells. Pro-iPS cells were injected in NOD/SCID mice. Cells were injected subcutaneously and the mice were closely observed for the formation of any tumours. Tumours were measured and harvested when they were of desired

size (Chapter 2). On examining the gross morphology, some tumours depicted tooth-like structures and cartilage/bone-like structures within them.

Media conditioned by the respective organ-specific stroma directed a lineage specific differentiation only in the respective organ-specific iPS cells; the same conditioned media failed to induce the same extent of lineage-specific differentiation in skin-iPS cells (Moad, Pal et al.). Our data emphasise the potential importance of the source from which iPS cells are generated as a consideration for organ-specific development studies. The role of urological mesenchyme in maintaining the respective tissue homeostasis has been well documented (Taylor and Risbridger 2008) . Although histologically, all mesenchyme are highly similar to each other it has been reported that the genetic make-up of each type of mesenchyme is distinct with the net result that both bladder and prostate mesenchyme have their own characteristic gene expression profile and are phenotypically distinct (Goo, Goodlett et al. 2005). This corroborates the role of mesenchyme in regulating lineage fidelity and this principle was used by us in the *in vitro* differentiation of UT-iPS and Pro-iPS cells (Moad, Pal et al.). It was our observation that media conditioned by the respective organ-specific stroma directed a lineage specific differentiation only in the respective organ-specific iPS cells (Figure 8.2); the same conditioned media failed to induce the same extent of lineage-specific differentiation in skin-iPS cells (Moad, Pal et al.). A detailed analysis of tissue-specific mesenchyme conditioned media is warranted although this is at present beyond the scope of our study. The need to assess the effect of conditioned media from various sources on the UT-iPS and Pro-iPS cells will further clarify their inherent *in vitro* differentiation abilities as well as lineage commitments. Although this is beyond the scope of our study the iPS cell lines generated by us pave a foundation for such assessments. Analysis of *in vitro* UT-iPS and Pro-iPS differentiation models will also facilitate the scrutiny of

various canonical pathways as well as epigenetic mechanisms that potentially regulate prostate development and carcinogenesis.

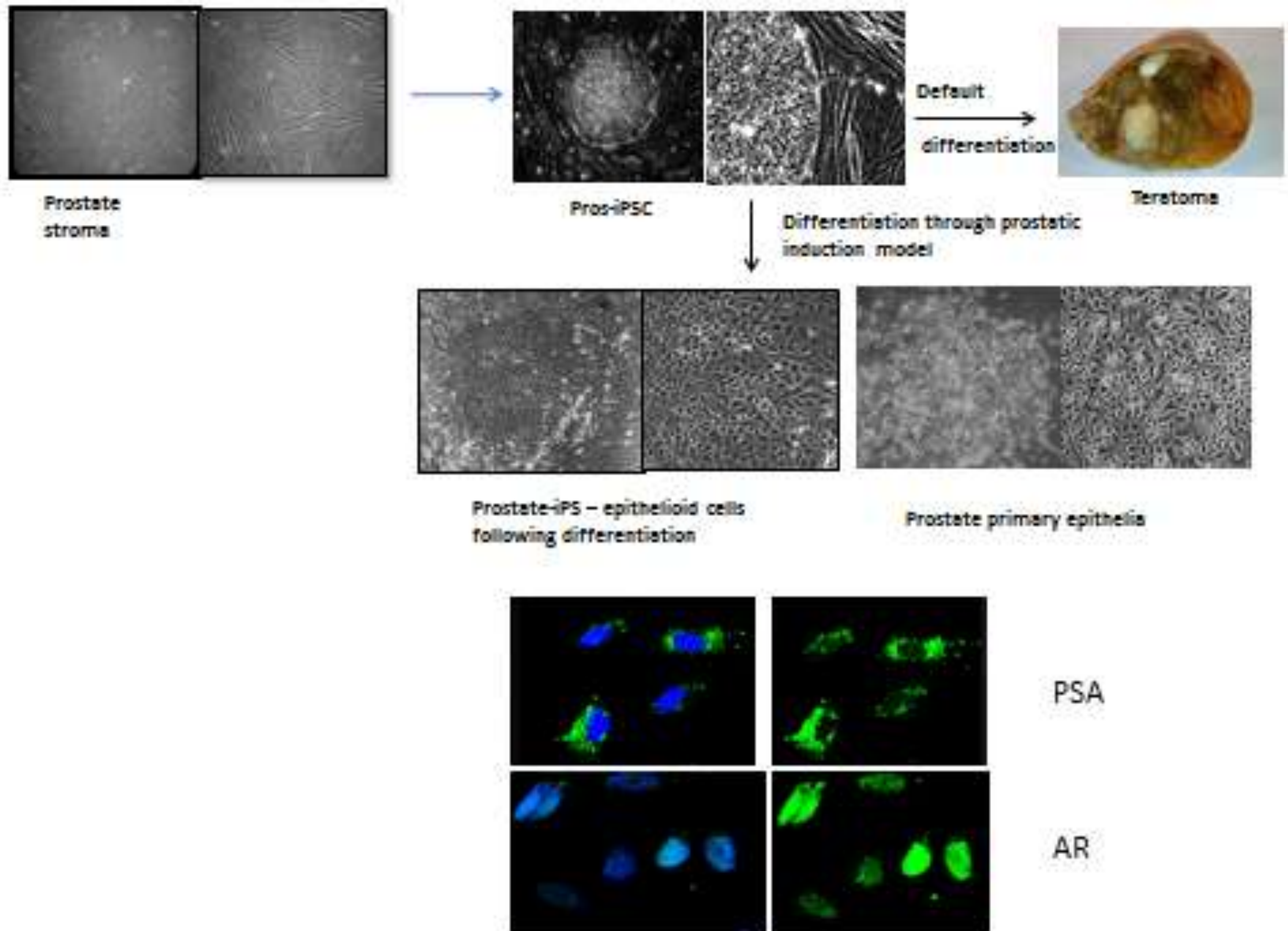


Figure 8.2. Differentiation of Pro-iPS in the presence and absence instructive mesenchyme-derived factors. Differentiation of Pro-iPS cells into teratomas in the absence of instructive mesenchyme; however when exposed to prostate mesenchyme conditioned media these differentiate into prostatic lineage

The fact that UT-iPS and Pro-iPS cells are able to differentiate into epithelial cells further corroborates their extent of pluripotency since it demonstrates that cells derived from an ectodermal lineage are able to give rise to endodermal cells. The fully re-programmed iPS cell state is marked by a phenotype that silences the parental fibroblast gene expression profile (Maherali, Sridharan et al. 2007, Maherali, Ahfeldt et al. 2008). The Pro-iPS cells demonstrated silencing of the parental tissue genes thereby suggesting that these cells were indeed devoid of the respective parental phenotype and instead had acquired an embryonic identity distinct from their initial cell type of origin. The fact that they nevertheless retain a parental epigenetic imprint may be explained by the fact that during the process of transcription factor-mediated re-programming the cells have been pushed against a differentiation gradient to an embryonic pluripotent state and thereby have the natural predilection to roll down the differentiation gradient unless restricted to the state of pluripotency by an epigenetic road-block (Hochedlinger and Plath 2009). Many concepts here as yet remain ambiguous and must be scrutinised in a step-wise fashion through single-cell tracking during the process of differentiation. Also as suggested, these cells should be subjected to different unrelated tissue-specific conditioned media to confirm their lineage commitments. A step-wise cellular-level analysis of molecular and epigenetic events will help to clarify and elaborate the precise mechanisms of de-differentiation and consequent re-differentiation in these cells.

It will be important to induce iPS cell generation in prostate and urinary tract epithelia and to track the molecular mechanisms behind somatic cell reprogramming in these cells (Wezel and Southgate). Epithelial cells are technically challenging cell cultures and often have limited life span *in vitro*. Poor cell quality and limited life span means that these cells may not be amenable to re-programming even when enriched for stem cell populations through surface markers (Richardson, Robson et al. 2004) which as yet are not necessarily

deterministic in purifying stem cell populations (Collins and Maitland 2009). For this purpose our iPS cell models are in place to serve as a starting population of pluripotent cells that can be made to differentiate to a multipotent adult stem cell state. This will unarguably need a lot more urological stem cell phenotypic characterisation. One strategy will be to use Pro-iPS and UT-iPS derived epithelial cell types as the starting population for iPS induction since it is well documented that these cells would be more susceptible to iPS cell induction due to favourable integration sites (Hochedlinger and Plath 2009, Robinton and Daley 2012). However, it has also been documented that there is no integration site dependent clonal selection during lentiviral re-programming and somatic cell re-programming by mean of lentiviral transduction is not dependant on insertional activation or deletion of genes or gene clusters (Winkler, Cantilena et al. 2010).

The methodology of iPS re-programming has evolved over the years. The early attempts at re-programming involved of self-silencing Moloney-based retroviral vectors (Maherali and Hochedlinger 2008). This method is associated with several limitations such as the viral transduction infectivity is restrained to dividing cells thereby resulting in lower transduction efficiency (Miller, Adam et al. 1990). The re-programming efficiency is further limited by the fact that the transgene gets gradually silenced during the iPS-induction process itself (Stadtfeld, Maherali et al. 2008). Finally retrovirus transduction protocols are associated with transgene integration and this maintenance of the viral-gene expression further limits the utility the derived iPS cell lines (Dimos, Rodolfa et al. 2008, Park, Zhao et al. 2008). Lentiviral transduction protocols are more favourable than retroviral methods in that lentiviruses can infect non-dividing cell types (Naldini, Blömer et al. 1996). However lentiviral transduction are also associated with poor transgene silencing (Lois, Hong et al. 2002) which limits the use of derived cell lines most specifically in terms of their clinical application. Drug-inducible lentiviral transduction protocols provide temporal control over

transcript factor expression and are therefore useful to analyse mechanism of iPS-re-programming (Brambrink, Foreman et al. 2008, Stadtfeld, Maherali et al. 2008). More recent advances in iPS re-programming include the use of non-integrating viral vectors such as the adenovirus (Stadtfeld, Nagaya et al. 2008), virus-free iPS generation through expression plasmids containing complementary DNA strands (Okita, Nakagawa et al. 2008), iPS generation facilitated by the use of small molecules such as Valproic acid (Huangfu, Maehr et al. 2008) and by direct delivery of transcription factors through protein transduction (Gump and Dowdy 2007, Bosnali and Edenhofer 2008).

The generation of Pro-iPS cell lines was a tedious and challenging technique. Prostate cancer is a hetrogenous tumour and clinical variation between patient samples meant that the course of iPS-reprogramming in the prostate was not consistent. Improved technologies in iPS-reprogramming are called for so that several lines of Pro-iPS can be established from different patient samples. Several road-blocks have been identified that slow-down the iPS re-programming process. It is well documented that inhibiting the tgf-β signalling pathway improves the efficiency of iPS-reprogramming, in particular it facilitates the initiation phase of iPS induction which is characterised by the messsenchymal to epithelial transition phase (Chapter 5) (Ichida, Blanchard et al. 2009, Maherali and Hochedlinger 2009, Massague 2012) . Hence it will be interesting to evaluate whether blocking the tgf-β signalling in human prostate epithelial and stroma re-programming would facilitate the initiation phase. In addition, it has also been documented that iPS-induction is also facilitated by the transcription factor Glis1(Glis family Zinc Finger 1) which positively influences multiple re-programming networks involving c-Myc, Lin 28, NANOG, Wnt, ESrrb and the messencymal to epithelial transition phase (Maekawa, Yamaguchi et al. 2011). Furthermore, it has been recently revealed that Mbd3/NuRD (nucleosome re-modelling and deacetylation) repressor complex acts as a brier in iPS re-programming (Rais, Zviran et al. 2013). The levels of Mbd3

roadblock expression is significantly higher in skin than prostate (Rais, Zviran et al. 2013) and thus depleting MbD3 may improve iPS-generation efficiency in the human prostate. It has been demonstrated that depleting Mbd3 along with the OSKM transduction protocol result in near 100% efficiency within seven days from human and mouse cells (Rais, Zviran et al. 2013). Given, the iPS-induction in human prostate was between 4-6 weeks long, it will be useful to explore these developments to generate Pro-iPS cell lines.

Another strategic approach and development in iPS technology is the advent of in vivo re-programming. It has been demonstrated that iPS re-programming can be attained in vivo through the OSKM factors whereby the in vivo iPS cells are extremely similar to in vivo ES cells but distinct from in vitro iPS cells (Abad, Mosteiro et al. 2013). It will be interesting to assess in vivo cellular plasticity of the human prostate as that may reveal important information regarding prostate development as well as the role of epigenetic alterations during prostatic development and differentiation. In vivo generated iPS cells are more primitive and plastic and possess the differentiation status comparable to a totipotent stem cells in that they can be differentiated into embryonic as well as extraembryonic tissue derivatives (Abad, Mosteiro et al. 2013).

An important step forwards from this work would be to assess the behaviour of the iPS cells from skin and prostate when co-cultured with instructive mesenchyme *in vivo* (Figure 8.3). This could reveal how the iPS cells would behave in a three-dimensional context and results will potentially provide information on the potential of these cells with respect to clinical use as well as in the context of other bio-clinical applications such as disease modelling, drug modelling as well as biomarker screening (Wezel and Southgate). It will also be important to establish a mean of tracking prostate stem cell differentiation *in vitro*. This can be achieved by lentiviral tagging of the Pro-iPS cells using a fluorescent protein. The differentiation of the Pro-iPS can then be examined uder different condition or in the presence of different

environments. Clonal evolution of prostate cancer can be further scrutinised through ectopic xenograft models using genetically barcoded Pro-iPS cells. The purpose of our model was to establish a means to facilitate the study of development of urological tissues and to capture an urologically-derived cell in a transient embryonic state such that it can be manoeuvred to re-differentiate into its original tissue-specific lineage. This in turn has created a urological model albeit at its very incipient stage for further scrutiny of human growth and tissue-specific development.

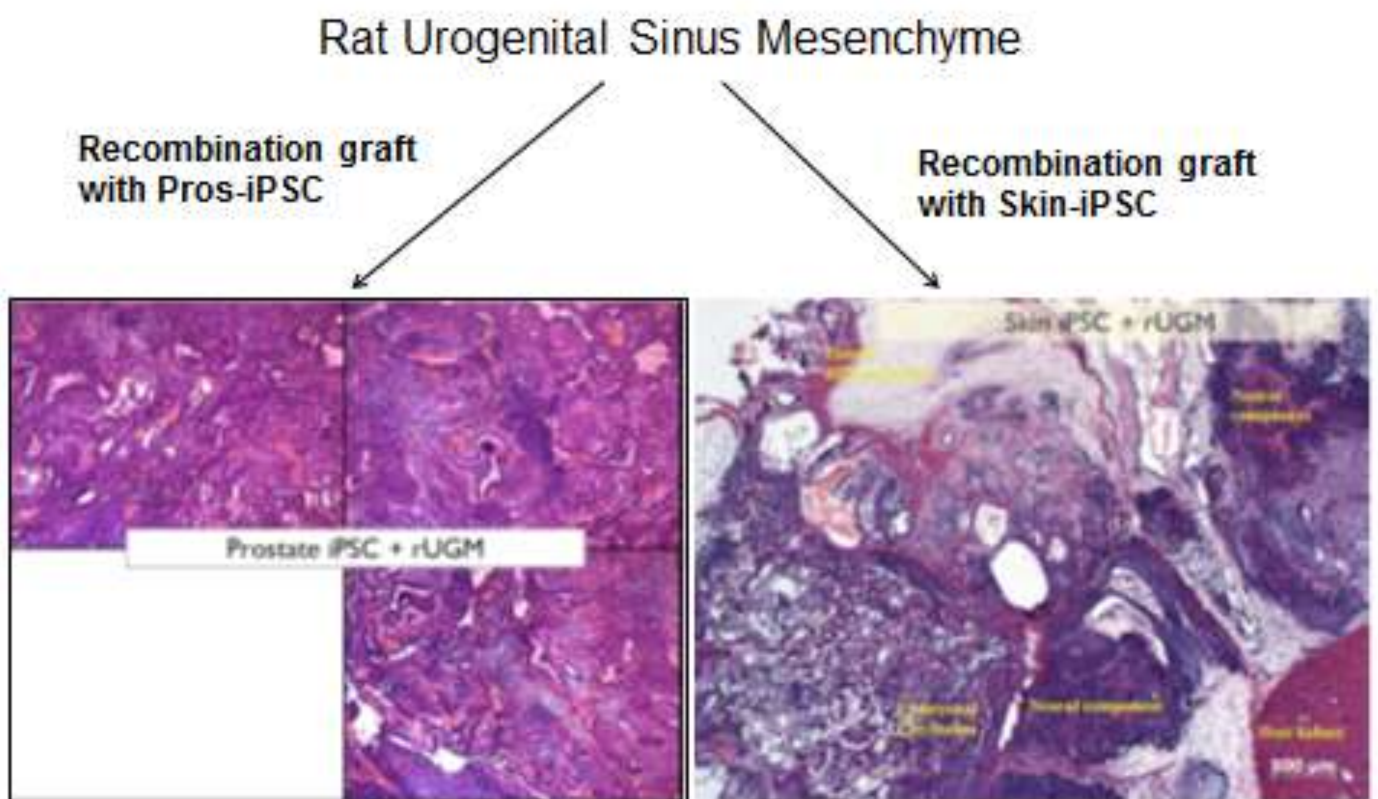


Figure 8.3. Skin-iPS and Pro-iPS cells co-cultured with rat urogenital mesenchyme (prostate-instructive mesenchyme) *in vivo*. Courtesy Prof. Simon Hayward and Dr. Omar Franco.

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Zhao, H., N. Sun, S. R. Young, R. Nolley, J. Santos, J. C. Wu and D. M. Peehl (2013). "Induced Pluripotency of Human Prostatic Epithelial Cells." PLoS ONE **8**(5): e64503.

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Zhou, Q., J. Brown, A. Kanarek, J. Rajagopal and D. A. Melton (2008). "In vivo reprogramming of adult pancreatic exocrine cells to [bgr]-cells." Nature **455**(7213): 627-632.

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Appendix

Publications: first author

1. A novel model of urinary tract differentiation, tissue regeneration and disease: Reprogramming of human prostate and bladder cells into induced pluripotent stem cells.

Mohammad Moada#, Deepali Pala#, Anastasia C Hepburna, Stuart C Williamsona, Laura Wilsona, Majlinda Lakob, Lyle Armstrongb, Simon W Haywardc, Omar E Francoc, Justin M Catesc, Sarah E Fordhama, Stefan Przyborskid, Jane Carr-Wilkinsona, Craig N Robsona*¥, Rakesh Heera¥.

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#Both authors contributed equally to this work.

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Keywords: Prostate, Bladder, differentiation, pluripotent, stem cells, tissue engineering, ureter, urothelium, androgen receptor, OCT4, SOX2, KLF4, cMYC and NANOG.

Abstract

Background: Primary culture, animal and cell-line models of prostate and bladder development have limitations in describing human biology and novel strategies that describe the full spectrum of differentiation from foetal through to ageing tissue are required. Recent advances in biology demonstrate that direct reprogramming of somatic cells into pluripotent embryonic stem cell (ESC)-like cells is possible. These cells, termed induced pluripotent stem cells (iPSCs), could theoretically generate adult prostate and bladder, providing an alternative strategy to study differentiation.

Objective: To generate human iPSCs derived from normal ageing human prostates (Pro-iPSCs) and urinary tract (UT-iPSCs) and to assess their capacity for lineage directed differentiation.

Design, setting, and participants: Prostate and urinary tract stroma were transduced with OCT4, SOX2, KLF4 and cMYC genes to generate iPSCs.

Outcome Measurements and Statistical Analysis: The potential for differentiation into prostate and bladder lineages was compared with classical skin-derived iPSCs. Student's t test was used.

Results and limitations: Successful reprogramming of prostate into Pro-iPSCs and bladder and ureter into UT-iPSCs was demonstrated by characteristic ESC morphology, marker expression and functional pluripotency in generating all three germ layer lineages. In contrast to conventional skin-derived iPSCs, Pro-iPSCs showed a vastly increased ability to generate prostate epithelial-specific differentiation as characterised by androgen receptor and PSA induction. Similarly, UT-iPSCs were shown to be more efficient than skin-derived iPSCs in undergoing bladder differentiation as demonstrated by expression of urothelial-specific markers, uroplakins, claudins and cytokeratin; and stromal smooth muscle markers α-SMA, calponin, and desmin. These disparities are likely to represent epigenetic differences between individual iPSC lines and highlight the importance of organ-specific iPSCs for tissue-specific studies.

Conclusions: iPSCs provide an exciting new model to characterise mechanisms regulating prostate and bladder differentiation and to develop novel approaches to disease modelling. Regeneration of bladder cells also provides an exceptional opportunity for translational tissue engineering.

Publication: First author letter to Editor

Reply from Authors re: "Reprogramming Stromal Cell from the Urinary Tract and Prostate: A Trip to Pluripotency and Back?" (2013)

Author(s): Pal D, Moad M, Hepburn AC, Williamson SC, Robson CN, Heer R

Journal: European Urology

Publication type: Editorial

Bibliographic status: In Press

Reply from Authors re: "Reprogramming Stromal Cell from the Urinary Tract and Prostate: A Trip to Pluripotency and Back?"

Publication: Co-author

Human α2β1HI CD133+VE Epithelial Prostate Stem Cells Express Low Levels of Active Androgen Receptor (2012)

Author(s): Coffey K; Robson CN; Heer R; Hepburn AC; Pal D; Wilson L; Williamson SC; Ryan-Munden CA; Leung HY

Date: 07-11-2012

Journal: PLoS One

Volume: 7

Issue: 11

Pages: e48944

Publisher: Public Library of Science

Publication type: Article

Bibliographic status: Published

Publication: Co-author

Prostate specific antigen enhances the innate defence of prostatic epithelium against Escherichia coli infection (2013)

Author(s): Townes CL, Ali A, Gross N, Pal D, Williamson SC, Heer R, Robson CN, Pickard R, Hall J

Journal: The Prostate

Publication type: Article

Bibliographic status: In Press

Publication: Co-author, clinical audit

Secondary haemorrhage following transurethral resection of bladder tumour — is it always related to infection? (2012)

Author(s): Johnson MI; Heer R; Rix D; Pal D; Glendinning RJ; Nesbitt CN; Menezes P

Date: 18-05-2011

Journal: British Journal of Medical and Surgical Urology

Volume: 5

Issue: 2

Pages: 61-66

Publisher: Elsevier Ltd

Publication type: Article

Bibliographic status: Published

Publication: Conference proceedings, oral presentation

Characterising potential for pluripotency induction in human prostate tissue (2011)

Author(s): Pal D, Williamson SC, Robson CN, Rigas AC, Heer R

Date: 5-6 January 2011

Conference Name: British Journal of Surgery: Annual Meeting of the Society of Academic and Research Surgery

Volume: 98 (s2)

Pages: 47

Publisher: John Wiley & Sons Ltd.

Publication type: Conference Proceedings (inc. abstract)

Bibliographic status: Published

Publication: Conference proceedings, oral presentation

Characterising potential for pluripotency induction in human prostate tissue (2011)

Author(s): Pal D, Williamson SC, Robson CN, Rigas AC, Heer R

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Volume: 98 (s2)

Pages: 47

Publisher: John Wiley & Sons Ltd.

Publication type: Conference Proceedings (inc. abstract)

Bibliographic status: Published

Publication: Conference proceedings, oral presentation

Stem-cell regulatory protein Piwil2 enriches for known stem-cell and cancer stem-cell markers in prostate cancer cells (2011)

Author(s): Pal D, Robson CN, Williamson SC, Nayernia K, Soleimanpour-Lichaei HR, Heer R

Date: 5-6 January 2011

Conference Name: British Journal of Surgery: Annual Meeting of the Society of Academic and Research Surgery

Volume: 98 (s2)

Pages: 47

Publisher: John Wiley & Sons Ltd.

Publication type: Conference Proceedings (inc. abstract)

Bibliographic status: Published

Publication: Conference proceedings, oral presentation

Induced pluripotent stem cell (iPSC) re-programming in the human prostate (2012)

Author(s): Pal D, Rigas AC, Williamson SC, Moad M, Carr-Wilkinson J, Lako L, Robson CN, Heer R

Date: 4-5 January 2012

Conference Name: Annual Meeting of the Society of Academic and Research Surgery

Volume: 99 (s4)

Pages: 56-56

Publisher: John Wiley & Sons Ltd.

Publication type: Conference Proceedings (inc. abstract)

Bibliographic status: Published

Publication: Conference proceedings, oral presentation

The role of epithelial to mesenchymal transition (EMT) in iPS induction of human prostate (2012)

Author(s): Pal D, Rigas AC, Williamson SC, Moad M, Robson CN, Heer R

Date: 4-5 January 2012

Conference Name: Annual Meeting of the Society of Academic and Research Surgery

Volume: 99 (s4)

Pages: 57-57

Publisher: John Wiley & Sons Ltd.

Publication type: Conference Proceedings (inc. abstract)

Bibliographic status: Published

CV Deepali Pal

Academic Qualifications

a) **MSc Medical Genetics 2008-2009:** Institute of Human Genetics, Newcastle University, Newcastle upon Tyne, UK. Result: ***Merit***

b) **Bachelor of Medicine and Bachelor of Surgery (MBBS) 2002-2008 (Including Foundation programme/Internship):** Manipal University, India. Result: First Class in all the years. Overall result = First Class

Awards and achievements:

1. **ARTP award for the best paper** (oral paper) in Prostate Cancer Research at the 20th Meeting of the European Association of Urology Section of Urological Research, October 2013.
2. **National Cancer Research Institute Prize award 2012** , Liverpool, UK
3. Travel award at the 20th Meeting of the EAU Section of Urological Research, 2012, Strasbourg
4. Travel award at the 9th World congress on Urological Research, 2011. The role of EMT in iPS induction of human prostate
5. Travel Bursary at the Academic Urology section of the Annual SARS (Society of Academic and Research Surgery) meeting, 2012.

Publications:

1. Moad M, **Pal D (joint first author),** Hepburn AC, Williamson SC, Wilson L, Lako M, Armstrong L, Hayward SW, Franco O, Cates J, Fordham SE, Przyborskid S, Carr-Wilkinson J, Robson CN, Heer R. A novel model of urinary tract differentiation, tissue regeneration and disease: Reprogramming of human prostate and bladder cells into induced pluripotent stem cells. European Urology 2013. In Press. **Article selected as Platinum Priority, accompanied and highlighted by a Platinum Priority editorial article**. **Impact factor = 10.5**
2. **Pal D**, Moad M, Hepburn AC, Williamson SC, Robson CN, Heer R. Reply from Authors re: "Reprogramming Stromal Cell from the Urinary Tract and Prostate: A Trip to Pluripotency and Back?". European Urology 2013. In Press. **Impact factor = 10.5**
3. Townes.C.L., Ali.A., Gross.N. , **Pal.D**., Williamson.S.C., Heer.R., Robson.C.N., Pickard.R., Hall.J. PSA enhances antimicrobial response of prostate epithelium against E.coli'. The Prostate, 2013. In Press. **Impact factor = 3.8**
4. Singhapol.C, **Pal.D**., Porika.M., Nelson.G. , Saretzki.S.C. Mitochondrial telomerase protects cancer cells from nuclear DNA damage. **PLOS One 2012. In press. Impact factor = 3.7**
5. Williamson.S.C., Hepburn.A.C., Coffey.K., Wilson.L, **Pal.D**., Leung.H.Y, Robson.C.N., Heer.R. Human α2β1HI CD133+VE epithelial prostate stem cells

express low levels of active androgen receptor. **PLOS One, 2012. Impact factor = 3.7**

6. Heer R, Glendinning RJ, Nesbitt CN, **Pal D**, Rix D, Menezes P, Johnson MI. Secondary haemorrhage following transurethral resection of bladder tumour — is it always related to infection?. British Journal of Medical and Surgical Urology 2011

7. Carr-Wilkinson.J., Prathalingam.N., **Pal.D**., Moad.M., Lee.N., Sundaresh.A., Lako.M., Murdoch.A. P., Herbert. H and Tweddle.D.A. Differentiation of human embryonic stem cells to sympathetic neurons: A model for understanding neuroblastoma pathogenesis. 2013. Submitted for Publication in Stem Cells Translational Medicine, sister journal of Stem Cells which has an impact factor of 7.7.

Conferences attended and level of participation:

1. **8th NCRI Cancer Conference, Liverpool, November, 2012**:

a. **Invited speaker in proffered paper session** The Cancer cells and Model Systems II: Induced pluripotent stem cell (iPSC) reprogramming of the elderly human prostate into a primitive embryonic stem cell state - a model for prostatic development and carcinogenesis

b. Poster presentation: A mesenchymal-epithelial transition in the prostate stroma marks the initiation of prostatic reprogramming to an embryonic-stem-cell state

2. **20th Meeting of the EAU Section of Urological Research, 25-27 October 2012, Strasbourg: One of the 4 Marie Curie fellows (out of total 20) selected to give a talk** at the PRO-NEST symposium of this conference: Induced pluripotent stem cell reprogramming of the elderly human prostate.

3. Annual Meeting of the Society of Academic and Research Surgery. 2012, Nottingham, UK:

a. **Oral presentation**: The role of EMT in iPSC induction of prostate

b. **Oral presentation**: iPSC re-programming in the Human prostate

4. NEUS (North-East association of Urological Surgeons). 2012. Newcastle, UK

Oral presentation: Can't find prostate stem cells? – Let's make one!.

5. North-East Post graduate conference. 2012. Newcastle, UK

Oral presentation: Induced pluripotent stem-cell reprogramming in the human prostate.

6. 9th World Congress on Urological Research, Innsbruck, Austria, 2011

Poster presentation: The role of EMT in iPS induction of human prostate

7. Annual Meeting of the Society of Academic and Research Surgery. 2011, Dublin, Ireland

a. **Oral presentation**: Characterising potential for pluripotency induction in human prostate tissue.

b. **Oral presentation**: Stem-cell regulatory protein Piwil2 enriches for known stem-cell and cancer stem-cell markers in prostate cancer cells.

8. 8th NCRI Cancer Conference, Liverpool, November, 2010

a. Poster presentation: Piwil2 expression selects for known stem-cell and cancer stem-cell markers in LNCaP prostate cancer cell line

b. Poster presentation: A study of pluripotency potential within the human prostate tissue and establishment of a model to understand prostate growth, development and carcinogenesis

Leadership, Management and Communication skills:

1. NICR Teaching and Training Committee Postgraduate representative, Newcastle University: 2010 – 2012. Key responsibilities: Represent all students at the institute, student welfare, first point of communication between students and faculty
2. Graduate School Committee Representative, Medical School, Newcastle University: 2011. Key responsibilities: Represent students across all institutes at faculty level, participate in academic board meetings so as to plan, review and enforce administrative policies for student welfare and training.
3. Clinical and research demonstrator: demonstrating clinical skill to junior medical students and laboratory skills to BSc and PhD students.

Research Skills;

•<u>Establishing primary culture of patient-derived cells</u> – epithelia, stroma. Subculturing primary cells over time

•<u>Characterisation and genetic manipulation</u> of human primary cells – normal and tumorigenic

•<u>3D models</u> and analysis, developing prostatospheres

•<u>Karyotyping</u> and karyotypic analysis using relevant software

•<u>Lentiviral transduction of patient –derived primary cell types</u> eg: stroma, epithelia and foreskin fibroblast cells

•<u>Human Pluripotent stem cell culture</u> (hES and iPS culture and propagation)

•Immunofluorescence and immunocytochemistry – confocal and live cell imaging

•Molecular biology techniques: handling RNA, mRNA synthesis, RT-PCR, qPCR

•FACS

•Animal studies – teratoma assays (witnessed and assisted in procedures involving injection of genetically manipulated primary cells, daily observation of tumour growth, harvesting and dissecting tumours under ethical guidelines and animal-welfare regulations, orthotopic xenograft assays). Awareness of animal welfare regulations whilst experimentation.

•DNA fingerprinting

1. **Research assistant/associate** in the **Leukemic Stem Cell Group** under the direct supervision of **Prof. Josef Vormoor** and **Prof. Olaf Heidenreich**, Newcastle University: 2013 –2014.

Research Skills:

1. Project : Self-renewal genes and pathways in Lymphoid malignancies
    - Culture of Leukaemic cells lines , suspension cultures
    - Transduction of Leukaemic cell lines
    - Culture of murine stromal cell lines – for co-culture purposes
    - FACS
    - Basic molecular biology technique: DNA extraction and handling, PCR amplification, cloning, validation, Analytical Gel analysis
    - Awareness in basic bioinformatics analyses: GSEA, pathway mapping
2. Project (Pilot): **Ex vivo culture models to grow patient-derived B-ALL cells**
    - Culture of patient-derived primary messenchymal stem cells (hMSC)
    - Osteoblastic andEndothelial differentiation of hMSC
    - Culture and differentiation of hMSC in 3D scaffolds
    - Co-culture of Leukaemic cell lines with hMSC/hMSC-derived cells in 3D scaffolds
    - PFA fixation, paraffin embedding and sectioning of 3D scaffolds for the purposes of immunostaining