Influence of cell type of origin to the differentiation potential of induced pluripotent stem cells derived from human urinary tract cells

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Northern Institute for Cancer Research

2015

Contents

Acknowledgements

In the first place I would like to express the deepest appreciation to my supervisors Dr. Rakesh Heer and Prof. Craig Robson for their guidance, patience, immense knowledge, and persistent help from the very early stage of this research. I could not have imagined having better advisors for my Ph.D study. I am indebted to them more than they know.

Besides my advisors, many thanks go in particular to Dr Anastasia Hepburn for her valuable advice and supervision; she always kindly grants me her time even for answering some of my unintelligent questions. Furthermore, I would like to thank, Deepali Pal, Stuart Williamson, Luke Gaughan, Kelly Coffey, Laura Wilson and all members of the Solid Tumour Target Discovery group for their help in the laboratory and for giving me such a pleasant time when working together with them.

I would like to extend my gratitude to my PhD assessment panel Prof. Deborah Tweddle and Dr Annette Meeson for their encouragement, insightful comments, and hard questions. It is a pleasure to express my gratitude wholeheartedly to Prof Josef Vormoor and Prof. Andrew Hall for housing me at the Northern Institute for Cancer Research. I am very grateful to the Syrian ministry of higher education for sponsoring me and supporting me during my PhD study.

Where would I be without my family? My parents deserve special mention for their inseparable support and prayers. My Father Mahmoud Moad and my mother Huda who sincerely raised me with their caring and gently love. Mervat, Maher, Manal, Marwa, Marwan and Mira, thanks for being supportive and caring siblings.

Words fail me to express my appreciation to my wife, Hiba whose dedication, love and persistent confidence in me, has taken the load off my shoulder. She was always there cheering me up and stood by me through the good times and bad. She is my rock, and I dedicate this thesis to her. Therefore, I would also thank my in laws; Abd Al-Malik and Hana for letting me take her hand in marriage, and accepting me as a member of the family, warmly. I also thank my wonderful children: Zaid and Rayia for always making me smile and for understanding on those weekends when I was working in the lab instead of playing with them.

Abstract

Background: Direct reprogramming of human somatic cells to pluripotent embryonic stem (ES) cell -like cells, termed induced pluripotent stem (iPS) cells, can be achieved by expression of defined transcription factors. The potential use of iPS cells derived from the urinary tract provides a substantial opportunity in developing new disease models, drug screening and tissue engineering. We aimed to generate, for the first time, human induced pluripotent stem cells derived from the urinary tract (UT-iPS) cells and to assess capacity for directed differentiation into bladder lineages.

Methods: Human primary culture cells derived from benign bladder and ureters were transduced with *OCT4, SOX2, KLF4* and *C-MYC* genes to generate human UT-iPS cells. Generated cells were characterised using RT-PCR and immunofluorescence. Differentiation capacity was evaluated by embryoid body formation in vitro and teratoma assay in vivo. Established co-culture based directed differentiation into bladder cells was assessed in comparison with classical skin-derived iPS cells.

Results: We demonstrated successful re-programming of adult urinary tract cells from both bladder and ureter into human UT-iPS cells. Most of the clones showed efficient transgene silencing and maintained a normal diploid karyotype. Specifically, we showed expression of ES cell markers and functional pluripotency by the generation of endodermal, ectodermal and mesodermal lineages. Differentiation into bladder lineages was demonstrated by expression of urothelial-specific markers, uroplakins (*UPIb, UPII, UPIIIa, and UPIIIb*), claudins (*CLD1* and *CLD5*) and cytokeratin (*CK7*); and stromal smooth muscle markers *a-SMA*, *calponin*, and *desmin*. Human UT-iPS cells were shown to be more efficient than skin-derived iPS cells in undergoing bladder differentiation, underlining the importance of the origin of the parent cell for re-programming.

Conclusions: We demonstrated that the induction of human urinary tract cells into iPS cells is possible, offering a new exciting opportunity for tissue engineering and for the study of bladder disease.

List of abbreviations:

3D: Three dimensional
ADSC: Adipose-derived stem cells
ALP: Alkaline phosphatase
ALS: Amyotrophic lateral sclerosis
ATP: Adenosine triphosphate
AUM: Asymmetric unit membrane
BAM: bladder acellular matrix
BAMG: Bladder acellular matrix grafts
bFGF: Basic fibroblast growth factor
BiPS: iPS cells derived from human beta cells
BMP: Bone morphogenetic protein
BMSCs: Bone marrow mesenchymal stem cells
BrdU: 5-bromo-2'-deoxyuridine
cDNA: Complementary DNA
Cdx2: Caudal type homeobox 2
CKs: Cytokeratins
CLD: Claudin
CM: Conditioned medium
Crb3: Crumbs homolog 3
DAPI: 4’, 6-diamidino-2-phenylindole
DEPC: Diethyl pyrocarbonate
DMEM: Dulbecco's Modified Eagle's Medium
DMSO: Dimethyl sulfoxide
DNA: De-ocyribonucleic acid
DPPA2: Developmental pluripotency-associated 2
EB: Embryoid body
EBLM: Embryonic bladder mesenchyme
EC: Embryonal carcinoma
ECM: Extracellular matrix
EDTA: Ethylenediaminetetraacetic acid
EdU: 5-ethynyl-2- deoxyuridine
EG: Embryonic germ
EGFP: Enhanced green fluorescent protein

EMT: Epithelial to mesenchymal transition

EpCAM: Epithelial cell adhesion molecule

EpiS cells: Epiblast derived stem cells

ERK: Extracellular-signal-regulated kinase

ES: Embryonic stem

ESCC: Embryonic stem cell cycle

ESG1: Embryonic cell specific gene 1

Esrrb: Oestrogen-related receptor beta

FA: Fanconi Anemia

FACS: Fluorescence based flow cytometry

FBS: Foetal Bovine Serum

FBS: Foetal bovine serum

FGF4: Fibroblast growth factor 4

FOXA1: Forkhead box A1

GAPDH: Glyceraldehyde-3-phosphate dehydrogenise

G-banding: Giemsa banding

GDF3: Growth and differentiation factor 3

GFP: Green fluorescent protein

H&E: Haematoxylin and Eosin

H3K27: Histone H3 lysine 27 methylation

H3K4: Histone H3 lysine 4 methylation

HDF: Human dermal fibroblasts

HEA: Human Epithelial Antigen

hESC: Human Embryonic Stem Cell

hHFCs: Human hair follicle cells

HPs: Hematopoietic progenitors

hTERT: Human Telomerase Reverse Transcriptase

ICM: Inner cell mass

IF: Immunofluorescence

iPS cell: Induced Pluripotent Stem Cell

IVF: In vitro fertilisation

JAM: Junctional adhesion molecule

KLF4: Kruppel-like factor 4

KSFMc: Complete Keratinocyte Serum-Free Medium

LIF: leukemia inhibitory factor

LRC: label retaining cells

LUT: lower urinary tract

MACS: Magnetic activated cell sorting

ME: Mercaptoethanol

MEF: Mouse embryonic fibroblast

MET: Mesenchymal to epithelial transition

miRNA: microRNA

M-MLV RT: Moloney Murine Leukaemia Virus Reverse Transcriptase

MOI: Multiplicity of infection

mRNA: messenger RNA

MSCs: Mesenchymal stem cells

Muse: Multilineage differentiating stress-enduring

NFκB: Nuclear Factor Kappa-lightchain- enhancer of activated B cells

NO: Nitric oxide

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

NSCs: Neural stem cells

NSG mouse: NOD/SCID gamma mouse

OCT4: Octamer-binding transcription factor 4

OSKM: Oct4, SOX2, Klf4, c-Myc

OSLN: Oct4, SOX2, Lin28, NANOG

p1: Passage 1

PBS: Phosphate Buffered Saline

PCR: Polymerase Chain Reaction

PDGF-BB: Platelet-derived growth factor BB

PI: Propidium iodide

piPS: Protein-induced pluripotent stem

Pro-iPSC: Prostate Induced Pluripotent Stem cell

REX1: Reduced expression 1

RNA: Ribonucleic acid

ROCK: Rho-associated Kinase

SCC: Squamous cell carcinoma

SCNT: Somatic cell nuclear transfer

shRNA: Short hairpin RNA

siRNA: Silencer RNA

SIS: Small intestinal submucosa

SMA: Smooth muscle α-actin

SMCs: Smooth muscle cells

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

SSC: Spermatogonial Stem Cells

SSEA: Stage-specific embryonic antigen

STAP: Stimulus-triggered acquisition of pluripotency

STR: Short tandem repeat

TCA: Trichostatin A

TCC: Transitional cell carcinoma

TDGF-1: Teratocarcinoma-derived growth factor-1

TE: Trypsin-EDTA

TGF: Transforming growth factor

TGFR: Transforming growth factor receptor

TGFβ: Transforming growth factor β

TRA: Tumour-related antigen

UGM: Urogenital Mesenchyme

UPCs: Urine-derived progenitor cells

UPs: Uroplakins

Utf1: Undifferentiated embryonic cell transcription factor 1

UT-iPS: Urinary Tract iPS

vWF: Von Willebrand factor

ZO: Zonular occludens

List of Figures and tables:

1 Chapter 1. Introduction

1.1 Clinical need for bladder reconstruction

The normal function of the bladder and associated structures of the lower urinary tract may be diseased for many reasons and people from all races, ages and ethnic groups suffer from poor quality of life even with access to the best medical care. Some conditions are primary congenital abnormalities and become lifelong health concerns of the genitourinary system. The most common birth abnormalities include hypospadias, where the urethral opening develops inferior to its normal location, and bladder exstrophy, where the bladder develops on the outer surface of the abdomen. These conditions have a considerable impact on social and psychological development. Secondary conditions are much more common and create acute and chronic medical problems such as bladder malignancies, trauma and neuropathy (Atala, 2008; Ferlay *et al.*, 2008) and are a leading cause of urinary symptoms (including incontinence), kidney failure, pelvic pain, urinary tract infection and death.

In particular, carcinoma of the bladder is a significant clinical problem that is a common and a serious healthcare problem throughout the world. Recent statistics estimate that over 380,000 persons are diagnosed with the disease worldwide each year (Ferlay J, 2010). In the UK, bladder cancer ranks as the fourth and eleventh most frequently diagnosed cancer in men and women, respectively. It is estimated that about 10,300 cases are diagnosed each year, and 4,500 patients die annually, that is around 96 people every week (CRUK-CancerStats, 2010). Bladder cancer is typically seen in people over 65, with an incidence nearly four times higher in men than in women. Histologically, the majority of bladder cancer patients present with transitional cell carcinoma (TCC, 90%), whilst 5% present with squamous cell carcinoma (SCC) and less than 2% with adenocarcinoma (*Bladder cancer statistics* July 2008). To determine the stage of bladder cancer, many factors must be considered; how deeply the tumour is and whether it has invaded the bladder wall (T-staging) (Figure 1-1), the presence of tumour in the lymph nodes (N-staging), and whether there are any metastases (M-staging) (Barentsz *et al.*, 1993; MacVicar, 2000). TCC presents as non-muscle invasive disease (superficial), i.e. (stage Tis, Ta, T1) in nearly 80% of patients, and as muscle invasive (stage T2) or

extravesical bladder invasive (stage T3-4) in the remaining 20% (Kirkali *et al.*, 2005; Said and Theodorescu, 2009). Approximately 80% of patients with superficial transitional cell malignancy survive the disease for at least five years after diagnosis, however, with invasive tumours, the overall survival rate is approximately 50% even when aggressive multimodal treatments are combined (Crawford *et al.*, 1991; de Wit, 2003). The verities of health problems associated with the bladder highlights the urgent need to expand bladder health-related research and to develop better management, and treatment of bladder diseases.

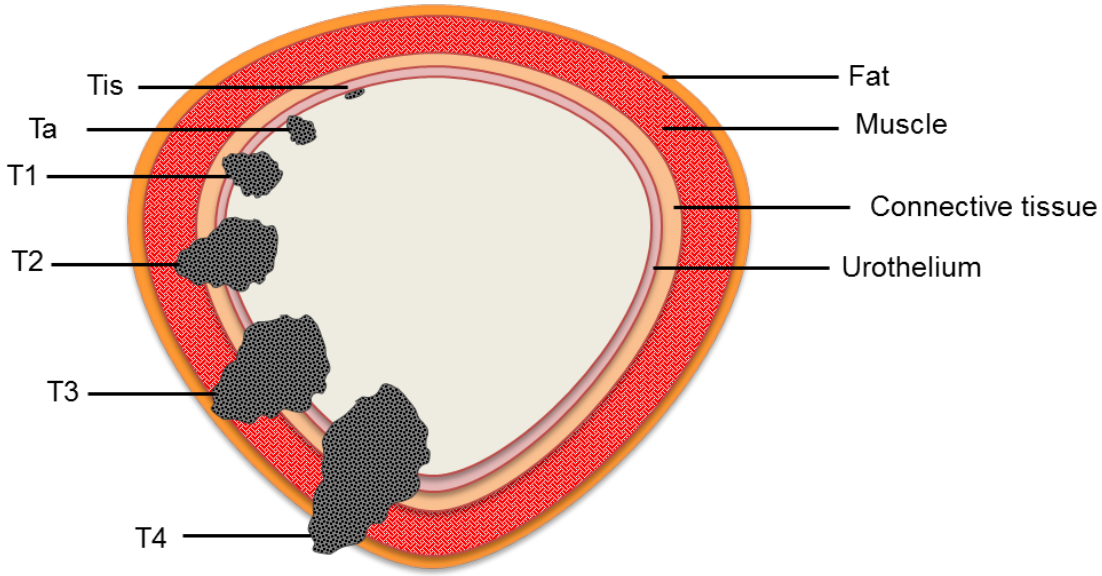


Figure 1-1: Stages of Urinary Bladder Cancer. Tis: Carcinoma *in situ*, Ta: cancer is just in the innermost layer of the bladder lining, T1: cancer limited to lamina propria, T2: cancer invades the muscle, T3: cancer has grown through the muscle into the fat layer, T4: cancer has spread outside the bladder. Modified from (Bladder cancer stage and grade, 2013)

Currently, using gastrointestinal segments of the patient's own intestine, also termed Enterocystoplasty is the most commonly performed procedure for bladder replacement or repair (Bolland and Southgate, 2008). However, because the bladder and intestine have different functions, this procedure has been associated with multiple complications such as infection, stone formation, metabolic disturbances, and malignancy (Atala *et al.*, 1993; Ali-El-Dein *et al.*, 2002). Therefore, new regenerative methods and also new models (human specific tools that accurately reflect normal physiology) to study disease initiation and progression are required.

1.2 Embryology of the lower urinary tract

To better understand normal and pathologic function of the lower urinary tract it is important to understand its embryology. In human development, the blastocyst, a structure containing an inner cell mass (ICM) and formed five days after fertilisation, gives rise to all three germ layers of the embryo (Figure 1-2). By the second week of gestation, the ectoderm and the endoderm are developed from the ICM and by week three, a third layer called mesoderm develops in between the previous two layers (Sadler, 1985). It is from the mesoderm and endoderm germ layers that the bladder develops.

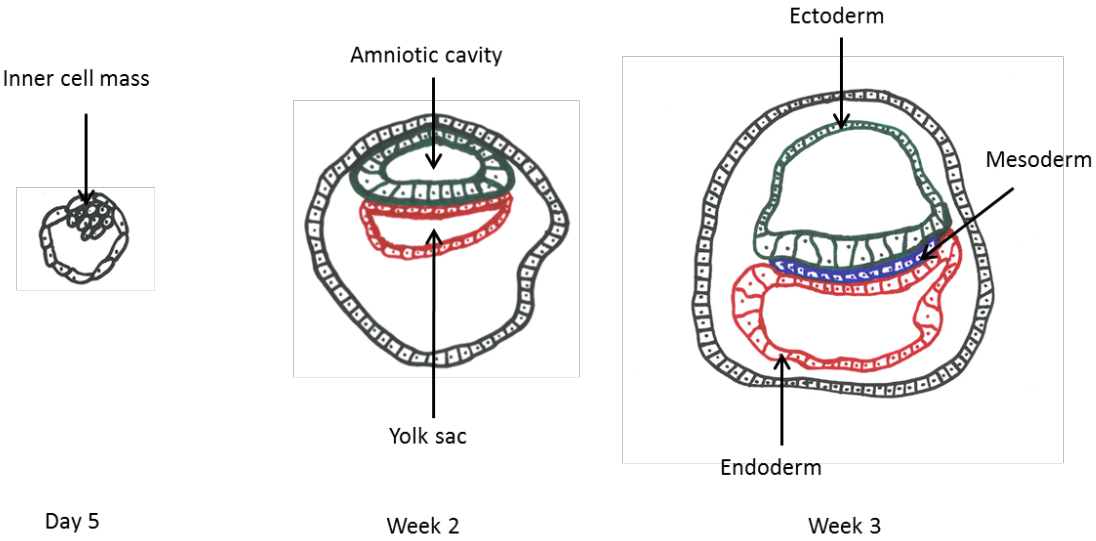


Figure 1-2: Schematic diagram showing the development of the germ layers. By the second week of gestation the inner cell mass differentiates to form two cell layers: ectoderm (green) and endoderm (red). During the 3rd week of gestation, a third layer, mesoderm (blue) develops in between the ectoderm and endoderm.

3.1.1 Development of the bladder

The cloacal membrane is formed at the caudal end of the embryo, differential growth of the mesenchyme near the cloacal membrane leads to the generation of the cloaca, which is a chamber formed by folding the caudal end of the embryo onto itself. Between the 4th and the 7th week of gestation, the urorectal septum grows caudally dividing the cloacal membrane into the urogenital

membrane and the anal membrane, and the cloaca into the urogenital sinus and the rectum. From this point the urogenital sinus can be divided on morphological basis into three sections. The first and largest section will form the urinary bladder. The second and pelvic section will form the prostatic and membranous urethra in males and the third section will form the urethra and the external genitalia (Figure 1-3) (Stephens, 1963; Schick, 2008). The mesonephric duct develops from the mesoderm adjacent to the coelom, called the primitive peritoneum. Once the mesonephric duct extends caudally to reach the urogenital sinus, a diverticulum grows cranially from the mesonephric duct forming the ureteric bud. The excretory duct is formed from stretching the mesonephric duct and merge together to form the primitive trigone. However, it has been suggested that the trigone may be formed through vitamin A-mediated apoptosis of the common nephric duct rather than fusion of the mesonephric ducts but (Batourina *et al.*, 2005; Viana *et al.*, 2007) other reports also suggested the endodermal origin of the trigone rather than the mesodermal origin (Oswald *et al.*, 2006; Tanaka *et al.*, 2010).

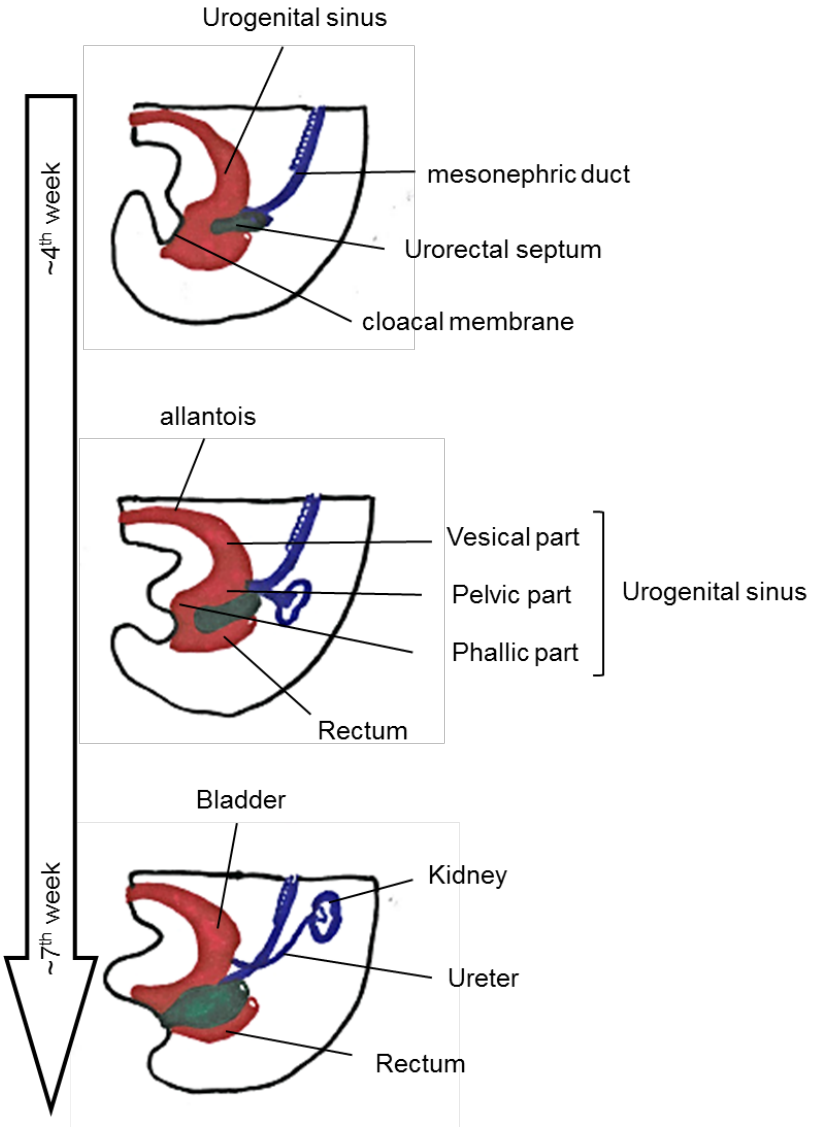


Figure 1-3: Schematic diagram showing the development of the bladder. During gestational week 4, the urorectal septum divides the cloaca into the urogenital sinus and the rectum. During gestational week 7, the urogenital sinus can be further subdivided into three sections. The first and largest section will form the urinary bladder. The second and pelvic section will form the prostatic and membranous urethra in males and the third section will form the urethra and the external genitalia.

Around the 6th week of gestation, the urogenital sinus expands cranially to form the primitive bladder and caudally to form the future prostate, urethra, and external genitalia. At this stage, the bladder wall is composed primarily of connective tissue. However, by the 17th week of gestation, the bladder has three muscle layers: inner and outer longitudinal layers, and a middle circular layer. The urothelium also undergoes extensive development during this time.

The urothelium of the bladder wall, excluding the trigone and the urethra is thought to be derived from the endodermal layer, while the urothelium of the renal pelvis, ureter, and trigone region of the bladder (the area of the bladder that forms a triangle between the insertion of the two ureters and the point where the urethra begins) is derived from the mesodermal layer. However, histologically, the urothelium derived from mesoderm is indistinguishable from that derived from endoderm. In addition, no significant cellular, structural and functional differences are found among the urothelial cells of different regions of the urinary tract (Staack *et al.*, 2005). The urothelium differentiates gradually starting from a simple cuboidal epithelium with smooth luminal surface and continues to end with a stratified transitional epithelium with asymmetric unit membrane plaques and mature fusiform vesicles (Staack *et al.*, 2005; Ersoy *et al.*, 2006).

3.1.2 Anatomy of the post-natal human bladder

The lower urinary tract (LUT) consists of two ureters, the bladder, and the urethra and is responsible for the storage and evacuation of the urine. Urine comes from the kidneys through two ureters which measure from 25 to 30 cm in length and have a tubular structure that begin at the renal pelvis, pierce the posterior wall of the bladder, and run indirectly through it for about 2 cm at the level of the vesico–ureteral junction. Crossing the bladder wall obliquely is believed to work as a valve mechanism, and prevents the ureteric reflux during increases in bladder pressure. The wall of the ureter has two distinct layers of smooth muscle: an inner, longitudinal and an outer, circular layer. The bladder is a balloon-like organ that stores and expels urine. The normal capacity of the bladder is about 400ml of urine. When the bladder is filled, it sends a signal through the nerves to the brain that the bladder is getting full. When the signal comes back from the brain to void, the detrusor muscle in the bladder contracts and the sphincter relaxes to push the urine through the urethra and out of the body. From an inside view, three distinct orifices in the bladder wall that delimit the trigonal region of the bladder can be observed: two ureteral orifices and the internal urethral orifice (Lang *et al.*, 2006; Drake, 2007; Clare J. Fowler, 2008).

I. The detrusor muscle

Functionally, the bladder has a complex imbrication of smooth muscle fibres for efficient bladder emptying and elasticity that permits low-pressure urine storage. Anatomically, the wall of the bladder consists of three smooth muscular layers called the detrusor muscle. The lumen of the bladder is covered by a layer of transitional epithelium, called the urothelium. Deep to this, a thick layer of connective tissue traversed by numerous capillaries, lymph vessels, and nerves called lamina propria lies between the urothelium and the detrusor muscle. This layer provides the bladder with a highly variable shape and allows it to expand and collapse during filling and emptying (Haab F, 2001; Schick, 2008).

II. The urothelium

The majority of the urinary tract, including the renal pelvis, ureters, bladder, and proximal urethra is lined by transitional epithelium that occurs nowhere else in the body, also known as “Urothelium”. The urothelium consists of a basal, intermediate, and a superficial cell layer (Lewis, 2000). Although similar to epithelial cells in other type of tissues, the urothelium has unique properties. In addition to its role as a highly effective barrier between the urine and the underlying connective tissue, the urothelium modulates the movement of ions, solutes, and water across the mucosal surface of the bladder, and protects the underlying tissue from pathogens (Hicks, 1975; Marceau, 1990; Limas, 1993; Baskin *et al.*, 1997; Apodaca, 2004). Furthermore, it has been reported that the smooth muscle layers under the urothelium need an epithelial signal to differentiate from the mesenchyme (Baskin *et al.*, 1996; Cao *et al.*, 2008).

3.1.3 Urothelial histology

Typically, the urothelium is composed of three cell layers: basal, intermediate, and umbrella superficial cell layer (Figure 1-4) (Lewis, 2000; Apodaca, 2004). The basal layer is found immediately above the basement membrane and consists of a single layer of small (with diameters of ~10μm) and polygonal cells. The intermediate cells are pyriform (10–25 μm in diameter) and can form numerous cell layers. The luminal surface of the bladder is lined by a single layer of cuboidal, large (25–250 μm in diameter), occasionally multi-nucleated, and terminally differentiated cells (known as umbrella or superficial cells) with

distinct apical and basolateral membrane domains demarcated by tight junctions. The morphology and size of these cells are changed according to the filling condition of the bladder. In empty bladders, umbrella cells are roughly cuboidal, whilst in filled bladders, these cells become stretched (Truschel *et al.*, 2002; Acharya *et al.*, 2004; Varley *et al.*, 2006; Khandelwal *et al.*, 2009).

The scalloped appearance of the apical surface of umbrella cells which comprises of raised hinges (also called micro-plicae) and intervening regions called plaques are considered one of the most identifiable features of umbrella cells (Hicks, 1965; Kachar *et al.*, 1999). Urothelial plaques provide the urothelium with a very high transepithelial electrical resistance making the urothelium a very constant permeability barrier with inherent flexibility to accommodate significant changes in surface area (Lewis and de Moura, 1982; Wu *et al.*, 2009; Ho *et al.*, 2012). In the plaque regions, the luminal leaflet of the plasma membrane is twice as thick as the cytoplasmic leaflet, forming an asymmetric unit membrane (AUM) (Porter *et al.*, 1967; Romih *et al.*, 2005). These AUM plaques are formed mainly by the interactions between a family of transmembrane proteins called uroplakins (UPs) (Wu *et al.*, 1990; Wu and Sun, 1993; Southgate *et al.*, 1994; Wu *et al.*, 1994; Southgate *et al.*, 1999a; Truschel *et al.*, 1999).

The intermediate cell layer exists directly under the umbrella cell layer and comprises of variable cell layers of pear-shaped cells. These cells are connected to each other, to the upper layer, and to the basal cell layer by desmosomes (Hicks, 1975; Jost *et al.*, 1989). Notably, the intermediate cells just below the superficial cells are partially differentiated, can also express UPs and have the ability to rapidly differentiate in case of the loss of the superficial cells (Martin, 1972; Hicks, 1975). The basal cell layer includes a single layer of mononucleate cells connected to a continuous basement membrane by hemidesmosomes (Southgate *et al.*, 1994; Jones, 2001; Southgate *et al.*, 2007; Khandelwal *et al.*, 2009; Wu *et al.*, 2009)

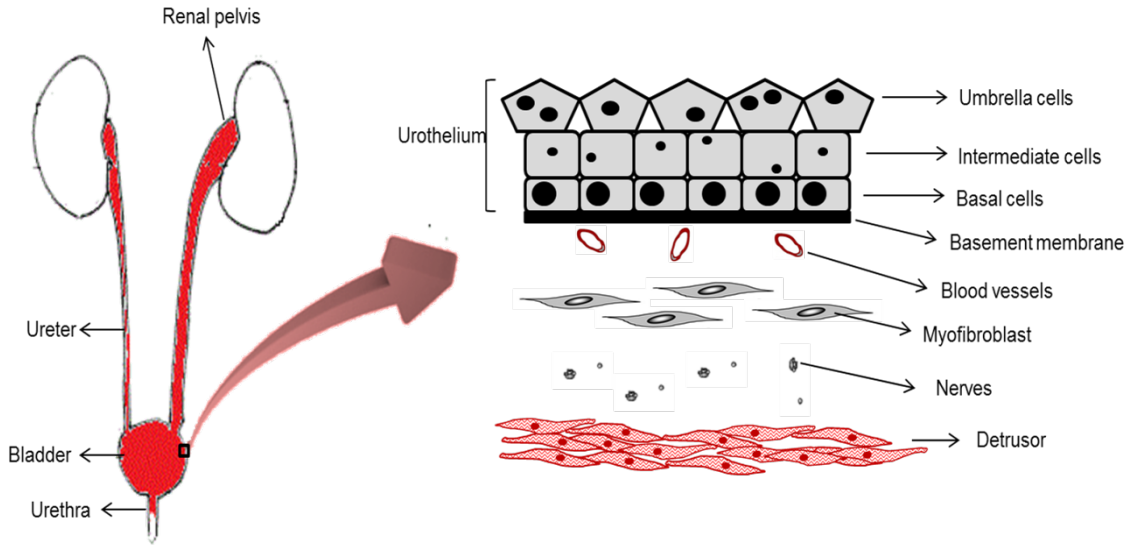


Figure 1-4: Diagram showing that the urothelium expands to cover the renal pelvis, ureters, and bladder (red). Bladder urothelium: A three-layered epithelium is apparent, consisting of large, binucleated superficial cells overlying an intermediate and a basal cell layer. This is separated by a basement membrane from a suburothelial layer that contains blood vessels, nerves and myofibroblasts.

3.1.4 The urothelium differentiation

The urothelium expresses various types of cytokeratins (CKs) (Moll *et al.*, 1988; Schaafsma *et al.*, 1989; Southgate *et al.*, 1999b; Romih *et al.*, 2005). Immunohistochemical analysis of normal human urothelium has shown that cytokeratin 13 is expressed in both intermediate and basal layers, whereas cytokeratin 5, 10, and 17 are only expressed by the basal cells (Figure 1-5). All urothelial layers express cytokeratin 7, 8, 18, and 19, whereas cytokeratin 20 is restricted to the fully differentiated umbrella cells (Moll *et al.*, 1988; Southgate *et al.*, 1999a; Southgate *et al.*, 1999b; Romih *et al.*, 2005; Varley *et al.*, 2006; Southgate *et al.*, 2007).

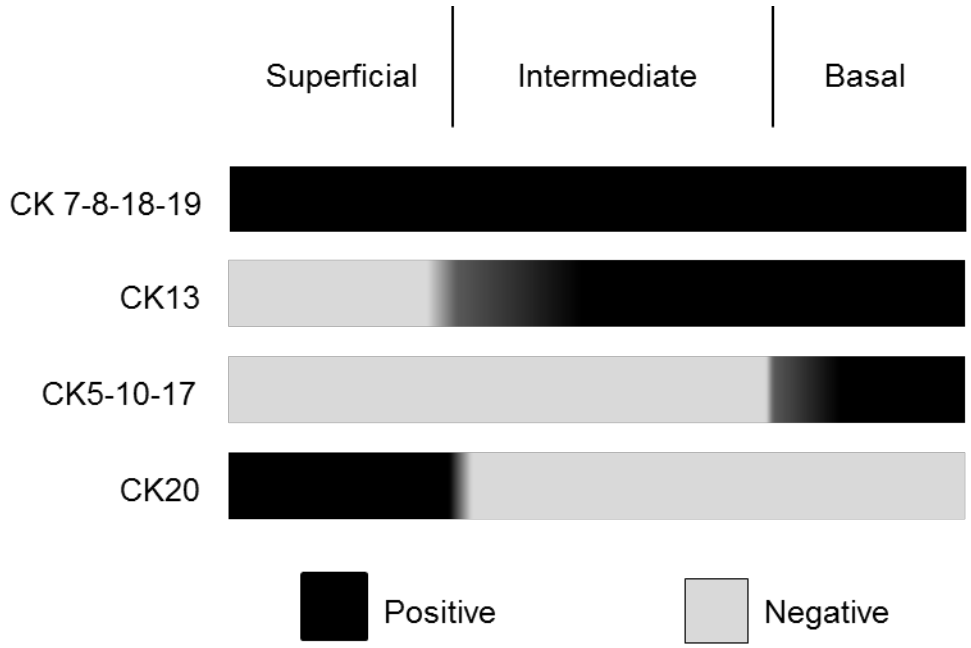


Figure 1-5: Distribution of various types of CKs in the urothelium layers

Another panel of urothelial differentiation markers are the UPs, a group of integral transmembrane proteins that design AUM plaques. As mentioned before, five types of UPs (UPIa, UPIb, UPII, UPIIIa, and UPIIIb) have been described and their expression provides excellent markers for studying the urothelial differentiation as they are only expressed in urothelial cells during advanced stages of differentiation (Wu *et al.*, 1994; Yu *et al.*, 1994; Olsburgh *et al.*, 2003; Romih *et al.*, 2005; Wu *et al.*, 2009).

The UP proteins have many functions, contributing towards the barrier and permeability to enable solute and water to flow across the apical membrane (Hu *et al.*, 2002). UPs are also targets of bacterial and fungal toxins (Zhou *et al.*, 2001). In normal human urothelium UPIa, UPII, UPIIIa, and UPIIIb are only expressed in umbrella cells (Figure 1-6); while UPIb is found in intermediate cells suggesting that UPIb expression might be correlated with less differentiation (Lobban *et al.*, 1998; Olsburgh *et al.*, 2003).

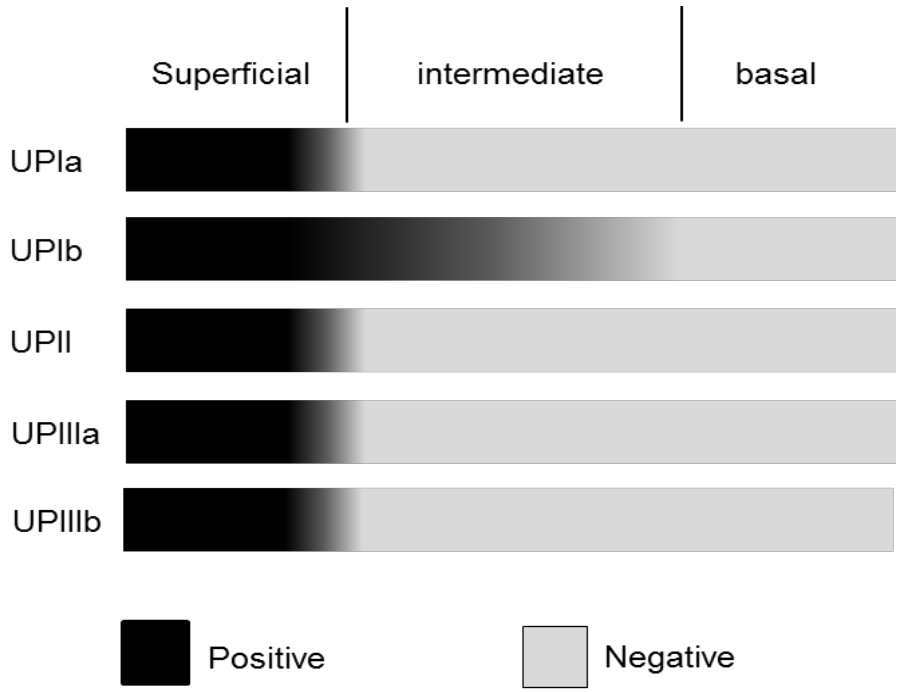


Figure 1-6: Distribution of selected uroplakins in the normal urothelium.

The paracellular barrier function of the urothelium is controlled by intercellular tight junctions located between juxtaposed umbrella cells. These tight junctions consist of cytoplasmic plaque proteins, the zonular occludens (ZO), that connect the tight junction to the cytoskeleton and integral transmembrane proteins, such as occludins, junctional adhesion molecule (JAM) and claudins (Varley and Southgate, 2008).

Claudins are a group of 24 proteins, which regulate the paracellular transport and are involved in the structure of the tight junctions of all epithelial cells (Kiuchi-Saishin *et al.*, 2002). In 2006, Varley *et al* (Varley *et al.*, 2006) examined the expression of claudins in human urothelium using probes for *claudin-1* to -*10* and found that human ureteric urothelium expressed *claudin-3, 4, 5*, and *7*, whilst the basolateral surface of the umbrella cells layer expressed *claudin-5*. Also, *claudin-4* was distributed at the intercellular borders of all urothelial cell layers (Figure 1-7).

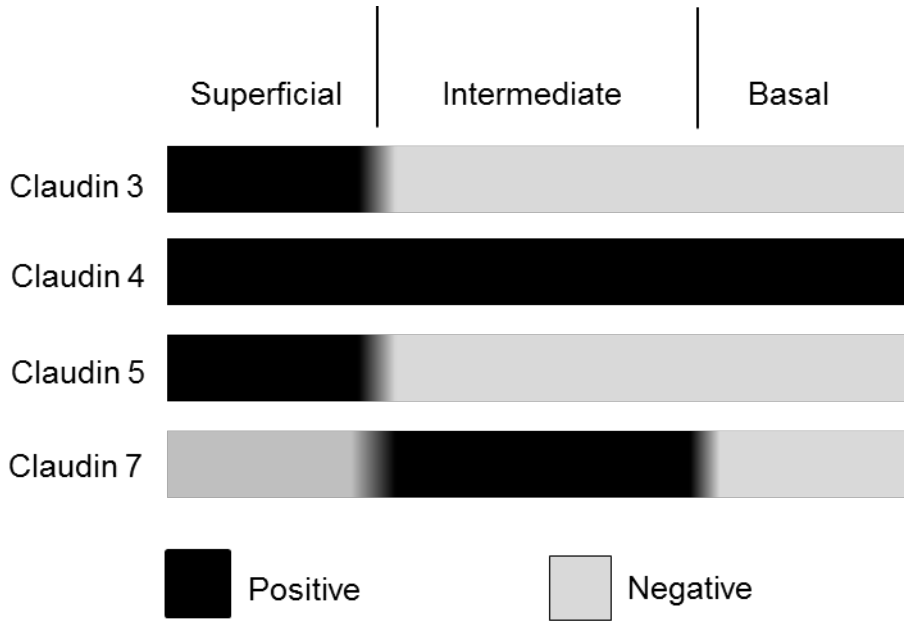


Figure 1-7: Distribution of various types of claudins in the urothelium layers.

1.3 Urinary bladder replacement and tissue engineering strategies

Diseased urinary tract may require either replacement or augmentation, usually with bowel segments as the current mainstay of treatment. Although this procedure can improve bladder capacity and continence, incorporating bowel into the urinary tract can be associated with several relatively common and potentially serious complications such as low grade bacteriuria, stone formation, and malignant transformation (Bolland and Southgate, 2008) as the intestinal lining is not adapted to prolonged contact with urine (Turner *et al.*, 2011).

Experimental animal models have shown that even augmentation with de-epithelialised bowel segments is associated with fibrosis and shrinkage (Bolland and Southgate, 2008). These observations suggest that the ideal material would be the use of urothelium and the compliance afforded by its associated stroma containing smooth muscle. The use of native cells has been explored in a number of experimental approaches involving *ex vivo* expansion of autologous cells that would avoid rejection. The main strategies include engraftment of urothelium onto de-epithelialised bowel (composite enterocystoplasty) or engraftment of both urothelial and smooth muscle stromal cells into acellular biomaterials such as for reconstruction (Turner *et al.*, 2011).

3.1.5 Composite enterocystoplasty:

Composite enterocystoplasty is a cell engineering approach in which the bowel epithelium is replaced with an *in vitro* -generated autologous urothelium therefore enabling utilisation of the available vascularised and compliant smooth muscle of the bowel. However, preclinical models of composite enterocystoplasty show that this technique can be compromised by graft contraction and poor urothelial coverage (Fraser et al., 2004).

3.1.6 Biomaterials and Cell-seeded constructs

Biomaterials can be either natural or synthetic (Grise, 2002). The outcomes of the incorporation of synthetic materials such as polyglycolic acid (PGA), polyethylene and polyvinyl into the bladder have been unfavorable due to biomechanical failure or biological incompatibility resulting in recurrent infection, scar generation and urinary stone formation (Elbahnasy *et al.*, 1998). Natural tissue matrices derived from various types of tissue, including small intestinal submucosa (SIS), porcine dermis (Kimuli *et al.*, 2004), and the urinary bladder itself have been developed and investigated in both *in vitro* and *in vivo* settings. In animal models, using SIS bioscaffold for bladder regeneration resulted in rapid cellular infiltration with the resultant tissue similar to that of the native organ. However, the level of the bladder damage appears to affect the success of bladder reconstruction using SIS (Zhang *et al.*, 2006). Bladder acellular matrix grafts (BAMG) are naturally derived from dissected split thickness bladders and also from full-thickness bladders. Previous studies using BAMG in animal bladder studies have demonstrated their bladder regeneration potential. However, the use of this matrix has been associated with many problems including poor vascularisation, graft shrinkage and incomplete or disorganised smooth muscle development (Bolland and Southgate, 2008). In 2006, Atala and colleagues reported the first human clinical trial with engineered bladders, using autologous cells onto biomaterials (Atala *et al.*, 2006). However, these strategies rely on *ex vivo* cell culture to generate sufficient quantities and quality of autologous cells and patients with tissue loss and end-organ cellular damage are not ideal candidates. Although even small biopsies of normal urothelium can be readily expanded before undergoing senescence, this is significantly restricted in diseased tissue (Subramaniam *et al.*, 2011). Moreover, the use of

this material may be inherently compromised by the disease itself and in the case of malignancy the use of macroscopically normal urothelium would be of concern due to genetically primed field-characterisation affecting the entire bladder (Jones *et al.*, 2005). Given the limitations described, increasing attention has focussed on the use of stem cells that may provide a more readily expandable source of cells with their ability for sustained self-renewal (Becker and Jakse, 2007).

1.4 Stem cells and urinary bladder tissue regeneration

3.1.7 Stem cells

The ability to continually self-renew through mitosis and to specialize to a certain tissue makes stem cells unique. Based on their ability to grow and their potential to generate differentiated cell types, stem cells can be classified as: (a) totipotent stem cells, which exist at the earliest stage of organism development (fertilized egg, zygote and the first 2, 4, 8, 16 blastomeres from the early embryo), and are capable to form all three major germ layers required for embryo development (ectoderm, endoderm, and mesoderm) as well as the extra-embryonic tissues, such as the placenta, (b) pluripotent stem cells derived from the ICM of the blastocyst and have the potential to generate any of the three germ layers, but they are unable to produce extra-embryonic tissues,(c) multipotent stem cells are observed later in development and have the ability to form a small number of tissues, multipotent stem cells are necessary for tissue renewal and believed to exist in all adult tissue and (d) unipotent stem cells which reside in adult organisms and give rise to just one cell type under normal conditions (Wagers and Weissman, 2004). Based on their source of origin, stem cells are also categorized into embryonic or adult stem cells.

1.4.1.1 Stem cells derived from early embryos

Embryonic stem (ES) cell research began with the study of spontaneous tumours that occur in the testes or ovaries of certain strains of mice and arise from the germ cells, known as teratocarcinomas. Histological analyses revealed a mixture of tissues derived from the three embryo germ layers such as bone, skin, and muscles. In the 1970s, Stevens found that embryos from the blastocyst grafted into ectopic sites in host mice could also form spontaneous

teratocarcinomas (Stevens, 1970). These spontaneous tumours contain many kinds of embryonic, immature, and adult tissues. They realized that the small population of undifferentiated cells in the tumour mass termed embryonal carcinoma (EC) cells were mainly responsible for growth of teratocarcinomas. These cells are pluripotent stem cells that can proliferate indefinitely and differentiate in culture into derivatives of the three germ layers even after serial transplantation (Stevens, 1970; Martin, 1981). Although EC cells could contribute to the development of completely normal adult mice when introduced into blastocyst-stage embryos (Brinster, 1974; Papaioannou *et al.*, 1975), these cells maintained a tumour generating phenotype and showed phenotypic and chromosomal aberrations (Solter *et al.*, 1970). A need for non-cancer derived, pluripotent cells that could be used for developmental studies and clinical applications was necessary. In 1981, pluripotent cells were successfully isolated directly from the ICM of pre-implantation mouse embryos by two independent laboratories. These cells were termed mouse embryonic stem (ES) cells to distinguish their origin from EC cells derived from teratocarcinomas (Evans and Kaufman, 1981; Martin, 1981).

Like mouse ES cells, human ES cell lines were first isolated from the ICM of pre-implantation embryos in 1998 (Thomson and Marshall, 1998). Significantly, in the unmanipulated embryo, cells from the ICM function as precursor cells, but not as stem cells as they have limited life-spans before they become committed to form the primary germinal layers. However, these cells can maintain their features and self-renew as undifferentiated cells when maintained in optimal conditions (Martin, 1981; Thomson and Marshall, 1998).

Human ES cell lines have also been derived from parthenogenetic embryos (Lin *et al.*, 2007), single- cell blastomeres (Chung *et al.*, 2006) earlier stage embryos (morula) (Strelchenko *et al.*, 2004) and later blastocyst embryos (Stojkovic *et al.*, 2004). The ability of transplanted gonadal ridges to form teratocarcinomas suggested that ES–like cells could be isolated from primordial germ cells before they differentiated to specialized gametes. Subsequently, embryonic germ (EG) cell lines were derived from murine (Matsui *et al.*, 1992; Resnick *et al.*, 1992) and human (Shamblott *et al.*, 1998) primordial germ cells prior to their migration in gastrulating embryos or following their arrival in the genital ridges. Like ES

cells, these pluripotent cell lines have the ability to differentiate *in vitro* and *in vitro* (Rohwedel *et al.*, 1996), as well as the ability to contribute to the germ line of chimeric mice (Stewart *et al.*, 1994). These observations indicate that the germline lineage maintains the capacity to establish pluripotent cells. Indeed, pluripotent stem cells were isolated from spermatogonial cells of newborn and adult mice. Analysis of mouse spermatogonial stem cells demonstrated that they have the morphology of ES-like cells, express the pluripotency marker genes, induce mature teratomas after transplantation into nude mice and form germline chimeras *in vivo* (Kanatsu-Shinohara *et al.*, 2004; Guan *et al.*, 2006).

1.4.1.2 Adult stem cells

Adult stem cells (or somatic stem cells) are undifferentiated cells that are found in a differentiated tissue. Adult stem cells are mostly multipotent cells, and tend to be tissue specific. Their primary function is to maintain and repair the organ system in which they exist because they are able to renew themselves during the lifetime of the organism and to generate differentiated daughter cells. Of these, the most studied are haematopoietic stem cells isolated from bone marrow (de Haan, 2002), and mesenchymal stem cells isolated from bone marrow stroma (Kim and Cho, 2013). Within the last decade many other types of tissue-specific stem cells have been identified and studied in detail such as retina (Tropepe *et al.*, 2000), brain (Okano, 2002), skin (Watt, 2001), pancreas (Ramiya *et al.*, 2000), and prostate (Bhatt *et al.*, 2003; Richardson *et al.*, 2004).

Adult stem cells can be found in a specific microenvironment in each organ known as the “niche” which has been proposed to regulate their behaviour. Unfortunately, only low numbers of adult stem cells can be found in each tissue and once removed from their normal cellular environment they lose their capacity to divide continually (Yoshida *et al.*, 2007); the reason why propagation of adult stem cells in culture is still challenging. Typically, adult stem cells generate intermediate cell types (precursor or progenitor cells) which in turn divide and give rise to fully differentiated cells (Marcus and Woodbury, 2008). In most tissues, adult stem cells may remain in a metabolically quiescent state for long periods of time before they are triggered by tissue injury. However, even in normal situations they constantly generate new cells to maintain tissues. Isolation and characterization of tissue-specific stem cells has been possible

through the use of a variety of methods to track cell fate *in vivo* or *in vitro*. Of these, the most fundamental tools are fluorescence-activated cell sorting (FACS) and monoclonal antibody production (Spangrude *et al.*, 1988; Baum *et al.*, 1992), while methods of *in vivo* lineage tracing include the incorporation of DNA nucleoside analogs and cellular marking through genetic reporter strategies.

The classification into embryonic or adult stem cells is very important due to the ethical issues associated with the destruction of an embryo to harvest cells and to use in research. Although adult stem cells are identified to be restricted in their potency by the tissue from which they arose, there is a hot debate in the literature about this idea. During the last decade, it has been suggested that these cells are inherently plastic, purporting that under certain conditions, adult stem cells may be much more flexible than previously envisioned and they could cross boundaries to differentiate into cells of unrelated tissue. This phenomenon is referred to as trans-differentiation and has been reported for a variety of cell types. For example, neural stem cells isolated from cloned mice show the ability to differentiate into all cell types of haematopoietic lineage (Bjornson *et al.*, 1999), and haematopoietic stem cells can differentiate into liver cells (Petersen *et al.*, 1999). Haematopoietic stem cells and mesenchymal stem cells have also been reported to possess the ability to give rise to non-haematopoietic cells such as muscle, liver, and lung (Quesenberry *et al.*, 2010). More recently, other studies have suggested that this process is a result of fusion between implanted cells and host somatic/precursor cells instead of actual trans-differentiation. Hence, the exact molecular mechanisms responsible for this phenomenon are poorly understood (Nygren *et al.*, 2004; Gruh and Martin, 2009; Jopling *et al.*, 2011; Peran *et al.*, 2011).

Although adult stem cells do not require the destruction of an embryo and can be used in autologous therapies, there are challenges involved in employing them in a wider field. Principally, these cells are usually present in a very limited numbers within the adult organs and isolating and purifying them has also proven to be quite challenging (Mimeault and Batra, 2008). In addition, great difficulty has been encountered in maintaining and expanding long term cultures.

1.4.1.3 Bladder urothelial stem cell

Urology is lagging behind other fields in terms of stem cell research. While the characteristics and location of stem cells in other organs and tissues have been well known for years, and those cells have been cultured, expanded, and even differentiated, the isolation, morphological and biochemical characterization of urinary tract stem cells has not yet been possible. In the case of the urothelium, this may be due to the fact that it is a poorly understood epithelium: very few biochemical markers of differentiation have been found, and the growth regulation mechanisms are not known. It is only recently that research has explored the possible applications of stem cells in the urology field and a number of studies now support the existence of urothelial stem cells (Pastor-Navarro *et al.*, 2010).

The mature urothelium renews itself very slowly with turnover interval being estimated as up to 12-24 weeks (Jost, 1989; Khandelwal *et al.*, 2009). However, the urothelium shows remarkable ability to proliferate and regenerate itself following injury or pathological damage resulting in its very rapid and full restoration (Kreft *et al.*, 2005; Mysorekar *et al.*, 2009). Furthermore, several studies report the plasticity of urothelial cells to undergo different patterns of differentiation (Kvist *et al.*, 1992; Staack *et al.*, 2005). Overall, the presence of resident committed urothelial progenitor or stem cell populations has been suggested.

Epithelial stem cells have been found to reside in a specialized and well-protected geographical niche that may reduce their exposure to trauma. For example, corneal stem cells are found to reside in the basal layer of the limbus, sequestered in the peripheral cornea (Cotsarelis *et al.*, 1989; Pellegrini *et al.*, 2001; Majo *et al.*, 2008). In the epidermis, stem cells are found at the bottom of the hair follicles in area called 'the bulge' (Oshima *et al.*, 2001; Morris *et al.*, 2004). However, in the case of the urothelium, the exact location of urothelial stem cells has not been identified.

A particularly powerful method to identify the location of stem cells takes advantage of their slow-cycling characteristics (Potten and Morris, 1988; Terskikh *et al.*, 2012). Once stem cells are exposed to labelled nucleosides,

such as 3H-thymidine or 5-bromo-2'-deoxyuridine (BrdU), they retain that label for a long period of time, while the more rapidly-cycling cells incorporate the label faster, mature, and die. Thus, these slowly cycling cells known as label retaining cells (LRC) are thought to represent the stem cell subpopulation. Using this technique, stem cells have been identified in the bulge region of the hair follicle (Watt *et al.*, 2006), the limbus of the cornea, the endometrium of the uterus, the crypts of intestine, and the proximal region of prostatic ducts (Bickenbach, 1981; Chan and Gargett, 2006). Recently, Kurzrock *et al* have adapted this technique and pulse-chased labelled rats with BrdU (Kurzrock *et al.*, 2008). One year after the administration of BrdU, 9% of bladder urothelium basal cells retained the label. Further analyses of this bladder LRC population indicated that these cells are characterized by small size (5–10 μM), low granularity, high β4 integrin expression and superior clonogenic and proliferative ability compared with unlabelled epithelial cells. Furthermore, these cells specifically expressed cytokeratin 5 and 6, which are basal cell-specific markers in the lung, prostate, and other epithelia cells. However, the ability of these LRC to differentiate into urothelial cells remains to be proven.

Consistent with this result, Gaisa *et al.* used naturally occurring mitochrondrial DNA mutations as markers of clonal expansion in an attempt to identify the location of the urothelial stem cell niche in humans. In this study, the authors identified patches of intermediate and umbrella cells that had been extended from monoclonal proliferative units originated in basal cells. However, superficial cells could not be identified in all monoclonal proliferative units (Gaisa *et al.*, 2011). Moreover, *in vivo* lineage tracing studies in mice suggested that basal cells of the urothelium may contain stem cells that can give rise to all other layers (Shin *et al.*, 2011). These data from rodents and humans support the urothelial stem cell to localise within the basal layer. However, controversial data have recently been obtained using LRC method with a different synthetic nucleoside-5-ethynyl-2- deoxyuridine (EdU) to identify potential stem cells in neonatal rat bladders. In this study, the distribution of the LRC in the urothelium was mostly random with no clear preferential labelling of basal cells (Zhang *et al.*, 2012). One possible explanation could be the differences in labelling efficiency and chase period. Also the identification of BrdU – label retaining cells is technically challenging and thus prone to error. In addition, finding that

umbrella cells are capable to divide suggested that they may represent another pool of stem cells in the urothelium (English *et al.*, 1987; Evans and Chandler, 1987).

One such study by Signoretti *et al.* used the p63-/- mouse as a tool to study urothelial development. *p63*, also known as transformation-related protein 63 is a well-documented marker of basal and intermediate cell layers of bladder urothelium (Yang *et al.*, 1998). In this study, bladders of *p63* deficient chimera only contained umbrella cells suggesting that functional *p63* basal cells are not required for the development of umbrella cells (Signoretti *et al.*, 2005). Further support to the hypothesis that basal cells are not the only origin of urothelial stem cells that can give rise to mature urothelial cells came from another study which reported the development of abnormal urothelium containing only umbrella cells in adult bladders of *p63*-null mice. Notably, these cells were positive for uroplakin II expression (Karni-Schmidt *et al.*, 2011; Ho *et al.*, 2012).

An original study by Nguyen *et al.* (Nguyen *et al.*, 2007) demonstrated that cells harvested from rat caudal bladder segments have significantly higher colony-forming efficiency than those from cephalic bladder segments, suggesting that more clonogenic cells exist in the lower bladder. On the other hand, Ki-67 staining showed no geographical difference in cell proliferation under normal homeostatic *in vivo* conditions. Although these data demonstrated that progenitor cells distribution pertains to a region, the authors argued that this does not necessarily reflect a stem cell niche and the proliferative capacity presented *in vitro* might be caused by transit amplifying population, which is still undefined in the urothelium.

Further evidence for the existence of urothelial stem cells comes from *in vivo* studies that have characterized a population of spheroids obtained from human bladder specimens with self-replicative potential, termed bladder spheres. Spheroids were mechanically dissociated into single cells and re-plated to produce secondary cultures. This procedure was repeated every week for 2 months and most of the cultures maintained a good proliferation rate implying that they might represent a stem cell population in the bladder (Fierabracci *et al.*, 2007). Zhang *et al* have recently described the isolation of a progenitor cell population (About 0.2%) from urine specimens, termed urine-derived progenitor

cells (UPCs) (Zhang *et al.*, 2008). These UPCs can be induced to undergo multilineage differentiation into urothelial, smooth muscle and even endothelial and interstitial cells. In addition, these UPCs express stem/progenitor cell markers, such as c-Kit (interstitial stem cell marker), and *SSEA4* an ES cell marker. *In vitro*, UPCs gave rise to multiple lineages that express cell markers of urothelium, endothelial, smooth muscle, and interstitial. These UPCs expressed *CD44* (a marker for cells developed from the basal layer) and *CK13* (an intracellular protein marker for basal cell) suggesting that UPCs are most likely derived from basal cells of the urothelium. In addition, karyotype analysis was performed to test the chromosomal stability of urine derived cells after serial subcultures and all passages exhibited a normal diploid complement of autosomes. However, the exact source of these cells is still undetermined.

3.1.8 Tissue engineering of urinary bladder using stem cells

Over the past decade, an increased number of studies have investigated the utility of stem cells such as MSCs, ES cells, EG cells, and amniotic fluid-derived stem cells in the field of regenerative urology (Yu and Estrada, 2010). In addition, several differentiation protocols have been utilized to direct stem cell differentiation to bladder tissue. However, the most powerful described method utilises tissue recombinant xenografts of embryonic bladder mesenchyme (EBLM) (Baskin *et al.*, 1996; Oottamasathien *et al.*, 2006; Oottamasathien *et al.*, 2007). Interesting results have been achieved using ES cells. Oottamasathien *et al*, showed that mouse ES cells can differentiate to bladder cells when associated with embryonic rat bladder mesenchyme and implanted under the kidney capsule for up to 42 days. The endodermal markers of Foxa1 and Foxa2, but not uroplakin were first detected at day 7 after grafting. By 42 days, optimized number of cells resulted in pure urothelial cells with mature bladder tissues derived from the ES cells that was evident by hematoxylin and eosin staining. Maturation was evident based on expression of uroplakin, a selective marker for urothelial cell differentiation and the basal cell marker p63, whereas smooth muscle α-actin (SMA); was used as a marker to identify smooth muscle cells (SMCs) (Oottamasathien *et al.*, 2006; Oottamasathien *et al.*, 2007).

However, there are ethical and immunological debates about using this procedure in humans. In addition, the differences observed between murine ES cells and human ES cells regarding molecular and developmental properties may represent an obstacle for direct translation to humans. Therefore, increasing attention has been paid to the use of adult stem cells which are less controversial, but equally promising cells in particular MSCs due to their versatility and their ability to differentiate into wide range of adult tissue cell types (Caplan, 2007; da Silva Meirelles *et al.*, 2008), including muscle (Luttun *et al.*, 2006; Crisan *et al.*, 2008), liver (Mimeault and Batra, 2008), lungs (Nolen-Walston *et al.*, 2008), neuronal (Duan *et al.*, 2007) and gut tissue (Jiang *et al.*, 2002). Utilizing the same model, Anumanthan and his colleagues used a recombinant xenograft of MSCs with EBLM to differentiate mouse MSCs toward mature bladder cells. Histological examination showed a bladder tissue structure with expression of uroplakin, SMA and desmin (Oottamasathien *et al.*, 2007). MSCs and human EG cell-derived cells seeded on porcine small intestinal submucosa grafts were also found to enhance bladder reconstitution in animal models. Three months after augmentation, only the stem cell seeded biohybrid displayed normal bladder structure with both urothelial and SMCs exhibiting gene expression levels similar to those of sham-operated animals (Chung *et al.*, 2005; Frimberger *et al.*, 2005). However, cytotoxic effects of the commercially available small intestinal submucosa (SIS) on urothelial cells have been reported (Feil *et al.*, 2006).

Tian *et al.* reported that bone marrow mesenchymal stem cells (BMSCs) can be differentiated into urothelial cells and SMCs *in vitro* and *in vivo* when co-cultured with bladder cells or conditioned media derived from bladder cell culture (Tian *et al.*, 2010b). Later, the same group published that BMSCs could be induced to differentiate into bladder SMCs and urothelial cells when seeded on a highly porous PLLA scaffold and treated with several key growth factors including platelet-derived growth factor BB (PDGF-BB) and transforming growth factor β1 (TGF-β1) (Tian *et al.*, 2010a; Petrovic *et al.*, 2011). However the clinical utility of BMSC is currently limited mainly because of their extremely low frequency, the intricacy and pain of the process, and difficulty in maintaining them in culture (Arai *et al.*, 2002).

Another source of autologous adult stem cells has been obtained from stromal elements of adipose tissue (referred to as adipose-derived stem cells ,ADSC) and have been successfully used to tissue engineer the smooth muscle of the urinary bladder in rat (Zuk *et al.*, 2001; Jack *et al.*, 2009).

In an attempt to overcome problems concerning appropriate cells sources for tissue regeneration of the bladder, Drewa *at al* showed that stem cells from rat hair follicle seeded on a bladder acellular matrix (BAM) scaffold and grafted into a surgically created defect within the anterior bladder wall were able to reconstruct both the urothelial and the muscle layers into surgically created defects. However, they did not demonstrate any uroplakin expression and the urothelial cells showed incomplete differentiation with weak expression of CK7. Most importantly, they couldn't control the differentiation of the hair follicle stem cells after transplantation. Muscle layers were thick in bladders reconstructed with cell-seeded grafts and very thin in acellular grafts. Again, obtaining sufficient cell numbers posed a major challenge regarding the use of these cells (Drewa, 2008; Petrovic *et al.*, 2011).

Recently, Zhang *et al.* isolated a subpopulation of cells with progenitor cell characteristics from urine samples. These cells showed the ability to differentiate *in vitro* into multiple lineages that expressed cell markers of urothelial, endothelial, smooth muscle, and interstitial cells and maintained normal karyotype even after several passages (Zhang *et al.*, 2008).

Although many trials documented great therapeutic potential of adult stem cells, experimental studies reported that adult stem cells are able to form other cell types by fusion with them rather than trans-differentiation which might produce cells with karyotypic abnormalities (Terada *et al.*, 2002; Ying *et al.*, 2002; Sievert *et al.*, 2007). Alternatively, pluripotent stem cells by their ability to proliferate indefinitely and to differentiate into any of the cell types in the body represent a serious alternative and major avenue in the regenerative medicine

An exciting and recent advancement in stem cell research involves that of viral overexpression of specific transcriptional factors *OCT4*, *SOX2*, *KLF4*, and *C-MYC* which can reprogram the differentiated cells back to pluripotency termed induced pluripotent stem (iPS) cells (Takahashi and Yamanaka, 2006;

Takahashi *et al.*, 2007). These iPS cells exhibit embryonic stem cell characteristics and can differentiate into all 3 embryonic germ layers. Disease-specific and patient-specific iPS cells have also been generated, thus providing a new method to evaluate potential therapeutics and to gain mechanistic insight into a variety of diseases (Park *et al.*, 2008). Because production of iPS cells doesn't involve the use of embryos or oocytes, they overcome the ethical restrictions that clearly obstruct the isolation, study, and use of ES cells, therefore they hold the general promise of ES cells, that is, pluripotency, and thus the ability to form any desired tissue (Yu and Estrada, 2010; Robinton and Daley, 2012). In this project, we aim to generate iPS cells from adult human urinary tract cells by lentiviral transduction. Such cells have great application potential for tissue engineering, understanding the mechanisms of bladder disease and drug screening.

1.5 Pluripotent stem cell characteristics

Irrespective of the cell origin, all pluripotent stem cells have the same main biological characteristics which considerably differ from those of normal somatic cells: the ability to proliferate indefinitely and the ability to differentiate into multiple somatic and germ cells *in vitro* and *in vivo* (Murry and Keller, 2008).

1.1.1 Pluripotent stem cells morphology and cell cycle

In culture, undifferentiated pluripotent stem cells grow in relatively flat, compact colonies with defined edges containing cells with a high ratio of nucleus to cytoplasm and prominent nucleoli (Thomson *et al.*, 1995; Thomson and Marshall, 1998). Currently, the majority of human ES cell lines are maintained on a feeder layer of mouse embryonic fibroblasts (MEFs), in medium that lacks serum but includes other exogenous peptide growth factors (Thomson and Marshall, 1998). However, the use of feeder layers and animal serum products can produce variable and heterogeneous results and precludes clinical applications (Sakamoto *et al.*, 2007). Great progress in the culture systems of human ES cells has been recently achieved by Amit *et al*. who reported long-term culture of human ES cells under serum-free conditions (Amit *et al.*, 2000), Richard *el al.* who replaced MEFs with human feeder layers (Richards *et al.*, 2002), and Xu *et al.* who reported the first attempt to produced feeder-free

cultures of human ES cells using Matrigel, or laminin substrates in medium conditioned by MEFs (Xu *et al.*, 2001). In addition, iPS and ES cell lines have been successfully established using a number of serum and feeder replacement media formulations including KnockOut Serum replacement, defined serum-free media (Cheng *et al.*, 2004), and mTeSR as specific media which provides serum-free and feeder-free conditions to culture human iPS and ES cells (Ludwig *et al.*, 2006). A very common method for monitoring ES cells culture is visual observation. Observation of ES cell morphology may give an indication of the state of differentiation in routine cultures (Thomson *et al.*, 1995; Thomson and Marshall, 1998). Despite limitations in terms of quantification and sensitivity, this approach is one of the most effective and inexpensive means to reveal any changes in the undifferentiated cells in culture (Schatten *et al.*, 2005). Pluripotent stem cells have rapid growth supported by brief G1 cell cycle phase whilst cells remain in S-phase for most of the cell cycle (Becker *et al.*, 2006).

1.1.2 Pluripotent stem cells gene expression and epigenetics

All pluripotent stem cell lines share similar gene expression patterns, which significantly distinguish them from other cells with lower developmental potency. These cells express a panel of protein markers that has been commonly used to characterize them in cultures such as stage-specific embryonic antigens 3 and 4 (SSEA-3 and SSEA-4), high molecular weight glycoproteins TRA-1-60 and TRA-1-81, and alkaline phosphatase (Draper *et al.*, 2002; Henderson *et al.*, 2002). They also express a number of genes that have now been found to be closely associated with the pluripotent state including Pou5F1/OCT3/4 (POU domain, class 5, transcription factor 1/Octamer binding transcription factor 3/4), SOX2, NANOG, teratocarcinoma-derived growth factor-1 (*TDGF-1*), *DNMT3B*, and growth and differentiation factor 3 (*GDF3*), together with the lack of differentiation markers. Furthermore, pluripotent stem cells have long telomeres due to high telomerase activity, an enzyme associated with immortal cell lines (Thomson *et al.*, 1995; Thomson and Marshall, 1998; Adewumi *et al.*, 2007). Telomerase is a ribonucleoprotein responsible for the maintenance of chromosome length by adding telomere repeats to chromosome ends. The high telomerase activity expressed by the ES cells provides them with long

replicative life span and long-term maintenance in culture and as the cell differentiates the telomerase activity is reduced (Marion and Blasco, 2010).

The International Stem Cell Initiative has analyzed 59 human ES cell lines obtained and maintained in 17 laboratories worldwide for expression of different potential markers of the undifferentiated stem cells. They found that all of these ES cell lines expressed a specific set of marker antigens and genes that can generally be used to screen pluripotent stem cells (Adewumi *et al.*, 2007).

Epigenetically, ES cells also have distinct chromatin signatures consisting of bivalent domains. These regions harbour both a “repressive” and an “activating” chromatin modification and function to silence developmental genes in ES cells while keeping them poised for induction upon initiation of specific developmental pathways (Bernstein *et al.*, 2006).

1.1.3 Functional assays of pluripotency

I. *In vitro* differentiation potential of pluripotent stem cells

Several tests have been developed to characterise pluripotent cell lines. The pluripotency of ES cells *in vitro* can be evaluated by the ability of these cells to generate embryoid bodies (EBs) comprising cells that represent the three germ layers. When cultivated in suspension, both mouse and human ES cells form multicellular aggregates which have been termed “embryoid bodies” (EBs) , and upon further differentiation on adherent culture the EBs produce a wide range of cell types derived from all three embryonic germ layers However, EBs do not exactly simulate the structural design perceived in the embryo. Several studies have routinely used this assay to test pluripotency *in vitro* (Martin, 1981; Itskovitz-Eldor *et al.*, 2000). Direct differentiation of ES cells to form the lineage of interest can also be used to test their ability to generate cells that represent each embryonic layer (Trounson, 2006). In these protocols, cells are transfected with ubiquitously expressing transcription factors, co-cultured with cell types capable of lineage induction or exposed to specific culture conditions and selected growth factors to enhance the differentiation towards a specific lineage.

II. *In vivo* differentiation potential of pluripotent stem cells

The most convincing proof for pluripotency can be achieved by demonstrating the ability of stem cells to give rise to all three embryonic germ layers, including germ cells, in chimeras formed by mixing ES cells with mouse blastomeres or blastocysts (Nagy *et al.*, 1993). However, because chimera formation is unethical using human ES cells, this has only been successfully established with mouse ES cells. For testing human ES cells, the most irrevocable test available is to demonstrate their ability to produce benign teratomas containing differentiated cells representing all three embryonic germ layers in immunodeficient mice (Thomson and Marshall, 1998; Gertow *et al.*, 2004). Teratomas are generated by the introduction of the cells into immunodeficient mice beneath the testis or kidney capsules (Thomson and Marshall, 1998), intramuscularly (Tzukerman *et al.*, 2003), subcutaneously (Levenberg *et al.*, 2002), or in the liver (Cooke *et al.*, 2006).

1.6 Signalling pathways to maintain pluripotency

The first isolated mouse ES cells were cultured onto a feeder layer of mitotically inactivated mouse primary embryonic fibroblasts using media conditioned by teratocarcinoma cells supplemented with foetal bovine serum and exogenous proteins. Until recently, this was the most effective system for derivation, propagation, and expansion of ES cell lines of different species (Martin, 1981). The feeder layer supports ES cell growth and prevents spontaneous differentiation of ES cells during culture. In the absence of feeder layers, conditioned medium (CM) collected from embryonic fibroblasts was found to support undifferentiated mouse ES cell culture (Smith and Hooper, 1983). Smith and Williams analysed the CM collected from embryonic fibroblasts and found that the key factor to inhibit the differentiation was leukemia inhibitory factor (LIF) (Smith *et al.*, 1988; Williams *et al.*, 1988). LIF enhanced the expression of specific target genes important for sustaining the undifferentiated state of the mouse ES cell by activating the Jak/Stat3 signalling pathways (Burdon *et al.*, 2002). Subsequently, other extrinsic factors involved in self-renewal and pluripotency of ES cells have been detected. Animal sera such as foetal bovine serum (FBS) provide all of these extrinsic factors, except LIF.

The bone morphogenetic protein (BMP) signalling pathway was found to cooperate with LIF to maintain self-renewal of mouse ES cells and activate differentiation inhibitor genes in serum-free culture conditions (Ying *et al.*, 2003). More recently, a combination of LIF and small-molecule inhibitors of ERK has been reported to repress the differentiation proteins and enhance the proliferation of the ES cell propagation (Ying *et al.*, 2008). Other factors such as vitamin A (Chen and Khillan, 2010), threonine (Wang *et al.*, 2009), and a decreased oxidation state (Yanes *et al.*, 2010) have also been reported to maintain undifferentiated mouse ES cells.

Although human and mouse ES cells are similar with respect to their self-renewal and differentiation capacity, growth factor requirements for maintaining mouse ES cell culture were unable to support long-term self-renewal of human ES cell lines (Bongso *et al.*, 1994; Xu *et al.*, 2002). For instance, human ES cells do not require LIF signalling for their derivation or propagation. Indeed, the LIF-JAK-STAT3 signalling is not active in human ES cells (Brandenberger *et al.*, 2004) but is essential for self-renewal of mouse ES cells. Further confirmation of the fundamental differences between mouse and human ES cells comes from studies by Xu *et al*. In this work, the authors found that BMP-4 signalling pathways which have been shown to enhance the self-renewal of mouse ES cells, could stimulate human ES cells to differentiate into trophoblast cells or mesodermal precursors (Xu *et al.*, 2002; Pera *et al.*, 2004).

In contrast, human ES cells require elevated basic fibroblast growth factor (bFGF) in the culture medium to maintain their pluripotency and undifferentiated state in the absence of fibroblasts or fibroblast-conditioned medium (Vallier *et al.*, 2005; Xu *et al.*, 2005a; Xu *et al.*, 2005b). A study by Wang *et al.* demonstrated for the first time that a combination of BMP antagonist and high concentrations of bFGF can help to maintain the pluripotency of human ES cells in the absence of feeder layers (Wang *et al.*, 2005). However, in mouse ES cells, FGF signalling cascades have been shown to promote differentiation by stimulation of ERK1/2 signalling (Mayshar *et al.*, 2008).

Other essential factors in combination with bFGF are likely to be involved in growth enhancement and preserve the undifferentiated state of human ES cells such as transforming growth factor β (TGFβ), activin and Nodal (Beattie *et al.*,

2005; James *et al.*, 2005). It has been suggested that the TGFβ/Activin/Nodal and BMP/GDF/MIS subfamilies control the cell fate of human ES cell through the Smad pathway. In human ES cells, the activity of Smad1/5/8 pathway is repressed and Smad2/3 pathway is stimulated whereas upon differentiation Smad 2/3 pathway is repressed and Smad 1/5/8 pathway is stimulated (Miyazawa *et al.*, 2002; Shi and Massague, 2003). The ERK, PI3K/Akt and Nuclear Factor Kappa-lightchain- enhancer of activated B cells (NFκB) pathways have also been suggested to have a role in the maintenance of human ES cells (Armstrong *et al.*, 2006). Inhibition of the ERK and PI3K/Akt pathways has been shown to enhance the differentiation and induce the apoptosis in human ES cells (Li *et al.*, 2007b). Similarly, the inhibition of NFκB pathway induces differentiation and increases cell death in human ES cells (Armstrong *et al.*, 2006).

Activation of Wnt signalling is sufficient to maintain and support the growth of mouse and human ES cells (Sato *et al.*, 2004). Wnt, a biological inhibitor of Gsk3β, is endogenously activated in both mouse (Nordin *et al.*, 2008) and human (Okoye *et al.*, 2008) ES cells. A previous study by Sato *et al*, reported that the activation of Wnt pathway maintained the expression of pluripotency markers OCT4, REX-1 and NANOG and promoted self-renewal in both types of ES cells whilst the usual differentiation program could be induced by removing the compound (Sato *et al.*, 2004). However, contradictory results have been reported by Dravid *et al.* in 2005, using either Wnt3a or Wnt antagonists. They found that Wnt activation could enhance the proliferation of human ES cells but was not sufficient to maintain and expand their undifferentiated state (Dravid *et al.*, 2005). On the other hand, Wnt signalling is also required to promote the differentiation of mouse (Lindsley *et al.*, 2006), and human ES cells (Wang and Nakayama, 2009). Therefore, further research is required to understand the precise roles of Wnts on ES cell proliferation and maintenance.

1.7 Transcriptional network for maintenance of pluripotency

Cellular pluripotency is governed by specific molecular signalling pathways as well as maintained by specific gene expression patterns that are characterized by activation of genes that support and sustain an undifferentiated cellular state and repression of those that promote differentiation. Several transcription

factors have been recognized as key regulators of cell pluripotency. Amongst them, OCT4, SOX2, and NANOG are proposed to form a core regulatory circuitry regulating pluripotency and self-renewal in human (Boyer *et al.*, 2005), and mouse (Chen *et al.*, 2008) ES cells. However, the reprogramming of mouse and human somatic cells to a pluripotent state using just four transcription factors OCT4, SOX2, C-MYC and KLF4 (Takahashi and Yamanaka, 2006; Takahashi *et al.*, 2007) or OCT4, SOX2, NANOG and LIN28 (Yu *et al.*, 2007) endorsed the central roles of these genes in regulating cell pluripotency.

OCT4:

OCT4 (octamer-binding transcription factor 4), also known as POU5F1 is the master regulator of the pluripotent state of germ cells, EG, and ES cells. OCT4 is an octamer-binding homeobox transcriptional factor that belongs to the class V family of transcription factors containing the POU DNA binding domain. In the mouse embryo, OCT4 protein is only expressed in the ICM cells after cavitation whilst it's downregulated in the differentiated trophectoderm (TE). Therefore, OCT4 is essential marker for stem cell pluripotency. *In vitro*, OCT4 is expressed in undifferentiated EC, EG and ES cell lines (Niwa *et al.*, 2000; Takahashi and Yamanaka, 2006). OCT4-deficient embryos fail to form the ICM and die shortly at pre implantation stage. *In vitro* culture of OCT4-negative embryos produced non-pluripotent ICM cells (Nichols *et al.*, 1998), suggesting that OCT4 function is an absolute requirement for the establishment of the ICM pluripotency and it's necessary to maintain self-renewal of pluripotent ES cells. Furthermore, OCT4 can directly repress the main regulators of trophectoderm differentiation Cdx2, Eomes and hCG. Hence, a component of OCT4's role is as a gatekeeper that blocks the differentiation into the trophectoderm lineage and maintains the pluripotency in the ICM (Liu *et al.*, 1997; Niwa *et al.*, 2000). Importantly, a precise level of OCT4 seems to be critical for the fate decisions of ES cells. In support of this, previous studies have demonstrated that overexpression of OCT4 resulted in differentiation into primitive endoderm and mesoderm, In contrast, reduction in OCT4 levels result in dedifferentiation to trophectoderm (Niwa *et al.*, 2000; Zeineddine *et al.*, 2006).

SOX2:

In mouse embryo, SOX2 expression is found in epiblast, and germ cells. Unlike OCT4, SOX2 expression is also persistent in multipotent cells of extra-embryonic ectoderm. Knockout of SOX2 in mice resulted in embryonic lethality after implantation due to defects in epiblast formation (Avilion *et al.*, 2003). *In vitro*, SOX2 expression is detected in pluripotent ES cells and neural progenitor cells (Li *et al.*, 1998). SiRNA-mediated knockdown of SOX2 results in the differentiation of ES cells into trophoectoderm. However, forced expression of OCT4 can successfully rescue SOX2-null pluripotent cells indicating that the essential function of SOX2 is related to the transcriptional activation of OCT4. SOX2 represents an important binding partner for OCT4 to enhance the expression of most pluripotency-associated genes, including FGF4, UTF1, Fbxo15, Lefty1, and NANOG. Furthermore, the OCT–SOX enhancers can stimulate the expression of OCT4 and SOX2 through a positive-feedback loop (Kuroda *et al.*, 2005; Masui *et al.*, 2007).

NANOG:

The homeodomain transcription factor NANOG is another important pluripotency-related factor in mouse and human pluripotent and teratocarcinoma cells. Similar to OCT4, the *in vivo* expression of NANOG is specific to the ICM, epiblast and germ cells. *In vitro*, NANOG is expressed in both mouse and human pluripotent cell lines (Chambers *et al.*, 2003). Mouse embryos lacking NANOG fail to form ICM and die shortly after implantation. Similarly, removing NANOG from mouse (Mitsui *et al.*, 2003) and human (Hyslop *et al.*, 2005) ES cells has been shown to incite differentiation to multi-lineage cells, while elevated levels of NANOG can enhance the self-renewal and prevent the differentiation of human ES cells (Darr *et al.*, 2006), and mouse ES cells in the absence of LIF, or even in the presence of LIF antagonists (Chambers *et al.*, 2003). Notably, NANOG expression failed to maintain the pluripotency of mouse ES cells in the absence of OCT4 (Mitsui *et al.*, 2003; Hart *et al.*, 2004). NANOG is also found to interact in concert with OCT4 and SOX2 in the maintenance of pluripotency of mouse and human ES cells (Chen *et al.*, 2008). In this aspect OCT4, SOX2, and NANOG together can also form interconnected autoregulatory and feedforward loops by binding to the

promoters of their own genes (Boyer *et al.*, 2005; Wang *et al.*, 2006). In addition, these transcription factors interact with several other genes to repress the differentiation of ICM into extra-embryonic lineages–trophoblast and extra-embryonic endoderm (Niwa *et al.*, 2009). A recent study by Chambers *et al*. (2007) suggested a different view where the authors reported that NANOG-null ES cells maintain the expression of pluripotency markers and the ability to differentiate to multi-lineage cells *in vitro* and *in vivo* (Chambers *et al.*, 2007). Collectively, NANOG appears to play a significant role in regulating pluripotency but unlike OCT4 and SOX2, it is not critical in the transcriptional housekeeping machinery of pluripotency.

KLF4:

KLF4 belongs to the Krüppel-like factor family of zinc finger transcription factors. It has been found that overexpression of OCT4, SOX2 and C-MYC, KLF4 can reprogram somatic cells back to an embryonic-like state (Takahashi and Yamanaka, 2006; Takahashi *et al.*, 2007). In mouse, KLF4 overexpression was found to block the differentiation of ES cells into erythroid progenitors (Li *et al.*, 2005). KLF4 knockout mice preserved their pluripotent stem cell populations. Similarly, siRNA-mediated knockdown of KLF4 doesn't affect the general phenotype of mouse ES cells which might be due to the functional corporation among other KLF family members in ES cells. Indeed, triple knockdown of KLF2, KLF4 and KLF45 induced differentiation in mouse ES cells and caused more changes in expression compared to single or double knockdown (Jiang *et al.*, 2008). Moreover, somatic cells were successfully reprogramed after replacing KLF4 with other KLF family members such as KLF1, KLF2 and KLF5 (Nakagawa *et al.*, 2008). However, successful generation of iPS cells without using KLF4 suggested that KLF4 acts as a supporter rather than an essential factor in the reprogramming process (Yu *et al.*, 2007). The requirement for LIF to maintain pluripotency can be sufficiently replaced by artificial expression of KLF4 or Tbx3. Remarkably, in the absence of LIF, overexpression of NANOG supports self-renewal of mouse ES cells while sustaining the OCT4 activity even without KLF4 and Tbx3 activity (Niwa *et al.*, 2009). In human ES cells, KLF4 expression was found to prevent differentiation by activating the NANOG promoter (Chan *et al.*, 2009b).

LIN28

LIN28 encodes a cytoplasmic RNA binding protein that was identified as a key regulator of developmental timing in *Caenorhabditis elegans* (Polesskaya *et al.*, 2007). Overexpression of LIN28 was associated with very rapid cell proliferation in mouse ES cells (Xu *et al.*, 2009a) while it seemed to decrease cell proliferation in human ES cells (Darr and Benvenisty, 2009). Indeed at present, the exact role of LIN28 in human and mouse ES cell is still intriguing. Although LIN28 was used together with OCT4, SOX2 and NANOG to reprogram human somatic cells back to a pluripotent state (Yu *et al.*, 2007), its role was not critical for reprogramming and it can even be replaced by other factors, suggesting that it may not be an essential factor in maintaining pluripotency but rather, serves as a secondary factor to enhance pluripotency, much like KLF4 (Takahashi *et al.*, 2007).

C-MYC:

MYC is a proto-oncogene whose overexpression is frequently observed in human tumours and is involved in transformation and tumorigenesis (Kendall *et al.*, 2006). Overexpression of C-MYC is reported during the blastocyst stage and several studies have demonstrated the central role of C-MYC in early mouse and human embryo (Paria *et al.*, 1992; Naz *et al.*, 1994). Overexpression of C-MYC in mouse ES cells has been found to prevent differentiation while downregulation of C-MYC antagonized self-renewal and caused differentiation (Cartwright *et al.*, 2005). On the other hand, overexpression of C-MYC in human ES cells resulted in apoptosis and differentiation into extraembryonic endoderm and trophectoderm. To explain the conflicting roles of C-MYC in both proliferation and apoptosis, the “dual signal” model has been proposed in which C-MYC is involved in both proliferation and growth arrest pathways (Pucci *et al.*, 2000). Although C-MYC was one of the four factors that Yamanaka used to reprogram both mouse and human somatic cells (Takahashi and Yamanaka, 2006; Takahashi *et al.*, 2007), subsequent studies have reported the generation of iPS cells without using C-MYC. However, the efficiency was significantly reduced (Nakagawa *et al.*, 2008; Wernig *et al.*, 2008b) Indeed, several studies have suggested a different role for C-MYC in comparison to other pluripotency factors. During the reprogramming

process, C-MYC was proposed to mainly down-regulate somatic gene expressions rather than activate that of pluripotency regulators (Sridharan *et al.*, 2009). C-MYC is also proposed to support the self-renewal of stem cells by inducing a cell cycle program and enhancing cell proliferation (Vermeulen *et al.*, 2003).

In conclusion, pluripotency cannot simply be characterized by just the expression of a particular set of genes or signalling pathways. Instead, multiple signalling pathways and transcriptional networks interact together to maintain ES cell pluripotency (Figure 1-8).

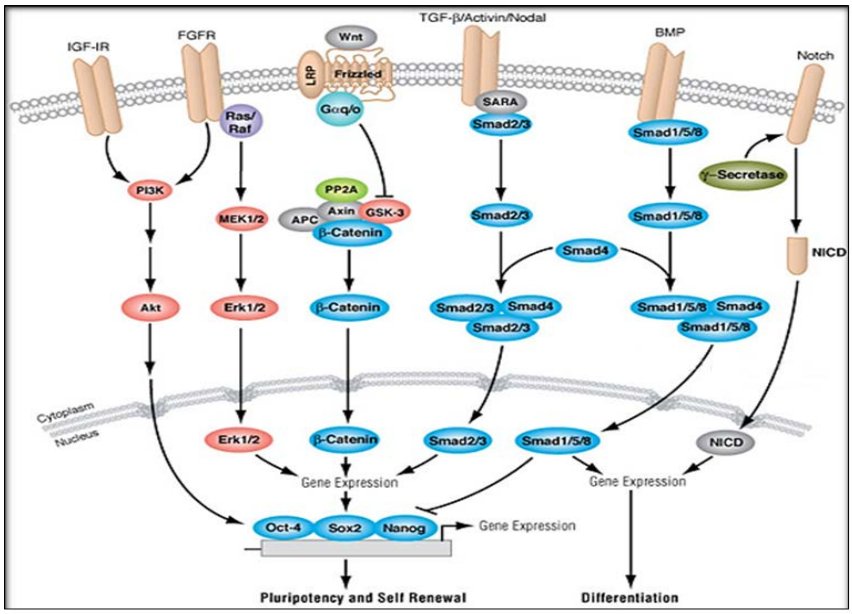


Figure 1-8: Signalling pathways involved in pluripotency and self-renewal. Modified from: (http://www.pharmatutor.org/articles/molecules-involved-in-regulation-of-stem-cell-differentiation).

1.8 Reprogramming and induced pluripotency

Despite their immense potential for differentiation and expansion, the application of human ES cells in research, clinical therapies, and pharma-screening is still controversial due to the destruction of human embryos, immunologic rejection of allogeneic ES cell-derived grafts and the unavailability of patient-specific cells (Johnson, 2008). Adult stem cells have also gained serious interest as they circumvent some of the ethical and immunologic issues of human ES cells, but, they have a rather limited differentiation and expansion potential (Wagers *et al.*, 2002; Murry *et al.*, 2004). Consequently, great effort

has gone into developing functional equivalents of human ES cells without using embryonic material. In particular, research has focused on generating pluripotent stem cells directly from somatic cells which finally led in 2006 to the ground breaking work by Shinya Yamanaka who demonstrated the induction of pluripotency in differentiated cells. These cells are called induced pluripotent stem (iPS) cells and are generated from a patient's own cells, thus bypassing the immune rejection and the ethical issues associated with the use of human embryos (Takahashi and Yamanaka, 2006). Reversal of the differentiated state to a state of developmental pluripotency is defined as "reprogramming." A number of techniques have been established over the years to reprogram somatic cells to a pluripotent state including cloning or somatic cell nuclear transfer (SCNT), cellular fusion, cell extracts or defined media, and direct reprogramming using exogenous factors (Figure 1-9).

1.1.4 Somatic cell nuclear transfer (SCNT)

SCNT is a pioneering technique first used by John Gurdon to generate Dolly the sheep, the first mammal to be cloned, whereby he transferred the somatic cell nucleus into an enucleated oocyte arrested at metaphase II stage (Gurdon and Melton, 2008). Upon transfer, the epigenome of the somatic cell is reprogrammed by the cellular factors in the egg. The resultant entity is assumed to have the same developmental potential as a fertilized zygote and can give rise to a cloned organism (Wilmut *et al.*, 1997). Hereby this work constituted the first evidence that the differentiation process is not a permanent change and that the somatic cell can be reprogrammed to an embryonic-like stage and more importantly acquires all genetic materials required to create a mature organism (Hanley *et al.*, 2010). Following Dolly, other animals including mouse (Tsunoda *et al.*, 1987), cow (Prather *et al.*, 1987), goat (Willadsen, 1986), pig (Prather *et al.*, 1989), cat (Imsoonthornruksa *et al.*, 2012) and rabbit (Matsuda *et al.*, 2002) were successfully generated by SCNT.

SCNT bestowed a powerful tool to study the molecular mechanisms controlling early development and raised the possibility of using them for autologous transplantation without the risk of immunologic rejection. There are two types of cloning, reproductive and therapeutic cloning. Reproductive cloning is used to create genetically identical embryo to the donor nucleus. Then this embryo is

grown into the uterus to generate an infant that is a clone of the donor. However, the application of this type of cloning for humans is denied in most countries. The other type of cloning is therapeutic cloning which has also been called somatic cell nuclear transfer. This type is also used to form blastocysts from an embryo that has identical genetic material to its cell origin. However, blastocysts are cultured *in vitro* with the goal of generating an autologous ES cell line (Kawase *et al.*, 2000; Hochedlinger and Jaenisch, 2003; Byrne *et al.*, 2007). Importantly, these cell lines are similar to normal ES cell lines with respect to morphology and gene expression profile (Brambrink *et al.*, 2006).

The pluripotency of ES cells derived from SCNT was confirmed by their ability to form teratomas after injection into immunocompromised mice (Munsie *et al.*, 2000), and their ability to contribute to mouse diploid chimeras, suggesting that they can be used as an alternative source of transplantable cells that are identical to the patient's own cells (Hochedlinger and Jaenisch, 2002). The therapeutic effects of nuclear transfer derived ES cells have been reported in mouse models of severe combined immunodeficiency (Rideout *et al.*, 2002), and Parkinson's disease (Barberi *et al.*, 2003). Most recently, SCNT human blastocysts were produced for the first time using somatic adult donor nuclei reprogrammed by human oocytes (French *et al.*, 2008). Nevertheless the efficiency of generating ES cell lines from nuclear-transferred embryos is significantly low where very few clones survive after implantation, and even those have severe malformations (Ogonuki *et al.*, 2002; Byrne *et al.*, 2007; Atala, 2011).

Interestingly, cloning with a less differentiated somatic cell as a nuclear donor is significantly more efficient than with terminally differentiated somatic cells (Blelloch *et al.*, 2006). Consistently, skin stem cells and neural stem cells create cloned animals more easily than do keratinocytes and neurons, respectively (Inoue *et al.*, 2007; Li *et al.*, 2007a). Donor cell cycle stage has also been reported to impact the efficiency of SCNT (Cibelli *et al.*, 1998). Following SCNT, histone modifications and DNA demethylation seem to be essential for developmental potential of cloned embryos (Dean *et al.*, 2001). DNA methylation and histone acetylation are also directly involved in X chromosome inactivation in female SCNT embryos (Xiong *et al.*, 2005). Bortvin *at al* reported

that the failure of deriving cloned embryos is often correlated to an incomplete reactivation of genes functioning in the pluripotent cells of the preimplantation embryo, in particular, OCT4 which is only expressed in pluripotent cells of the early embryo and the germline (Bortvin *et al.*, 2003). Although this approach has recently been expanded to other mammals (Gurdon and Melton, 2008), the use of SCNT in research and therapy however is hampered by many technical limits associated with the cloning process including low efficiency, incomplete remodelling, high percentage of abnormalities and low birth rate, and ethical limits surrounding the use of human eggs (Gurdon and Melton, 2008). Nevertheless, SCNT trials provide the proof-of-concept that paved the way to identify the main key factors required for nuclear reprogramming.

1.1.5 Cellular fusion

Cellular fusion has been used previously to study the plasticity of differentiated cells (Blau and Blakely, 1999). In a pioneering study, mouse EC cells were fused with thymocytes and the resultant pluripotent hybrid cells showed the ability to induce teratomas upon injection into immunocomprimised mice (Miller and Ruddle, 1976). More recent research has revealed that ES and EG cells also possess the ability to reprogram somatic cells (Han and Sidhu, 2008). Matveeva *et al.* reported for the first time the ability of mouse ES cells to reprogram somatic cells. In this work, hybrid cells with pluripotent properties were obtained by fusion of mouse ES cells with the spleen cells of an adult female mouse (Matveeva *et al.*, 1998). Reactivation of OCT4–enhanced green fluorescent protein (EGFP) transgene and silencing of X chromosome were detected in the ES hybrid cells (Tada *et al.*, 2001). Microarray analysis showed that a mouse embryonic fibroblast /ES cell hybrid contains unique expression profiles that are similar to normal ES cells (Ambrosi *et al.*, 2007). Similarly, Eggan's group artificially fused human ES cells with human fibroblasts through the merging of their membranes to form a stable cell hybrid. The resultant cell contained both the somatic and human ES cell chromosomes in a single nucleus, had a phenotype similar to the parental ES cell, and grew in appearance similar to a human ES cell. Reprogramming of the somatic genome in hybrid cells to an embryonic state is demonstrated by the silencing of the somatic-specific genes, demethylation and reactivation of several pluripotency genes including OCT4, NANOG, and REX1, reactivation of the inactive X

chromosome in fused female somatic cells and the ability to differentiate into all three germ layers *in vitro* and *in vivo* (Cowan *et al.*, 2005). Comparable results have been obtained with human ES cells and haematopoietic cells. The resultant hybrid cells showed reactivation of OCT4–EGFP and expression of ES cell-specific genes at a level comparable to that in diploid human ES cells. Furthermore, the hybrid cells had the ability to form the three germ layers suggesting functional nuclear reprogramming (Yu *et al.*, 2006). However, this approach is still limited by technical and ethical barriers that have restricted the use of ES cells in research including low efficiency and chromosomal and genetic instability of the hybrid which can lead to malignant transformation (Vasilkova *et al.*, 2007; Nowak-Imialek *et al.*, 2010). Furthermore, although the somatic cell genome is reprogrammed, the tetraploid DNA content presents an additional technical hurdle before this process could be used in customized cell therapy as transplanting these tetraploid hybrids into the somatic donor will probably result in immune rejection caused by the existence of the foreign genome.

1.1.6 Cell extracts and defined media

Based on observations from SCNT and fusion experiment, it has been suggested that reprogramming may be induced by incubation of somatic cells with ES nuclear and cytoplasmic extracts (Taranger *et al.*, 2005). Xenopus egg extract has been used to reprogram human lymphocytes. Egg extract-treated cells showed increased expression of endogenous pluripotent gene OCT4 whilst somatic genes were inhibited (Hansis *et al.*, 2004). Similarly, exposing 293Tand NIH3T3 cells to extracts of mouse EC or ES cells resulted in demethylation of OCT4 and NANOG promoters concomitant with down regulation of differentiation genes. Moreover, generated cells exhibited the ability to differentiate towards several cell lineages including neurogenic, adipogenic, osteogenic, and endothelial lineage (Taranger *et al.*, 2005; Freberg *et al.*, 2007). However, employing this technique is hindered by the instability of the reprogramming outcomes which may lead to cancer development and the very low yield of extracts even with large numbers of oocytes.

Another technique to produce reprogrammed cells is based on exposing cells *in vitro* to a specific mixture of growth factors. Under standard ES cell culture

conditions, adult testis cells can gain ES cell characteristics (Guan *et al.*, 2006). Cellular dedifferentiation of murine C2C12 myoblasts can also be induced using A2,6-disubstituted purine, reversine (Chen *et al.*, 2004). To date the mechanisms by which these defined factors function are still elusive.

1.1.7 Direct reprogramming using exogenous factors

In 2006, Shinya Yamanaka and his team presented a new concept of reprogramming mouse somatic cells using retroviral-mediated overexpression of key transcription factors (Takahashi and Yamanaka, 2006). The generated cells called induced pluripotent stem (iPS) cells are almost identical to ES cells in terms of morphology, molecular characteristics, and teratoma formation ability. These results were later reproduced on human adult fibroblasts to generate human iPS cells that would serve as another source of patient-specific pluripotent stem cells that circumvent some of the problems associated with ES cells, hence bear promise to advance biomedical research and regenerative medicine (Takahashi *et al.*, 2007; Yu *et al.*, 2007).

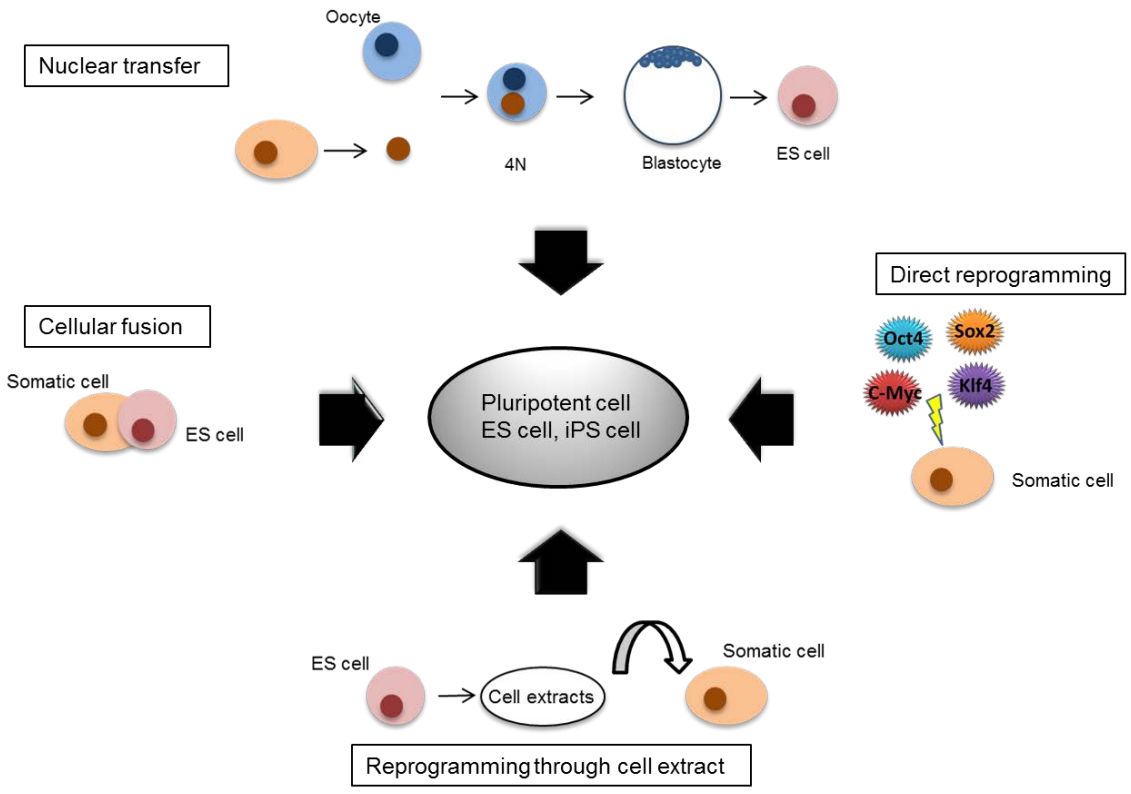


Figure 1-9: Mechanisms to induce nuclear reprogramming.

1.8.1.1 Induction of pluripotent stem cells from murine fibroblasts

The induction of pluripotent stem cells from murine fibroblasts by Shinya Yamanaka and Kazutoshi Takahashi represents a hallmark in stem cell research (Takahashi and Yamanaka, 2006). They first selected a combination of 24 candidate genes due to their suggested role in production and maintenance of the ICM in the blastocyst and also in maintaining the pluripotent state of ES cells (Figure 1-10). These factors were delivered as a pool by retroviral transduction to MEFs engineered to express neomycin resistance from the Fbx15 locus, a downstream target of the pluripotency associated factor OCT4. The transfected cells were cultured in mouse ES cell environment and colony resistance to G418 as a selective antibiotic was used to control the induction of the Fbx15 locus which is only expressed in embryos and mouse ES cells. Cells transduced with a single gene were unable to confer G418 resistance while cells transduced with all 24 genes formed colonies with mouse ES cell characteristics after about 25 days. Then exogenous factors were eliminated one by one while monitoring the efficiency and timeline of ES-like colony formation until they demonstrated that only four transcription factors, OCT4, SOX2, KLF4, and C-MYC, were required to form a G418-resistant colony and to bestow ES cell-like properties on fibroblasts. Established iPS cells were positive for alkaline phosphatase and SSEA-1 (stage-specific embryonic antigen 1). Moreover, iPS cells reactivated the silenced X chromosome in female cells and restored telomerase activity. *In vitro*, iPS cells exhibited the ability to form embryoid bodies, which in turn exhibited the ability to differentiate into cell types of the three germ layers. Teratoma formation resulting from subcutaneous injection of iPS cells into nude mice demonstrated their pluripotency *in vivo*.

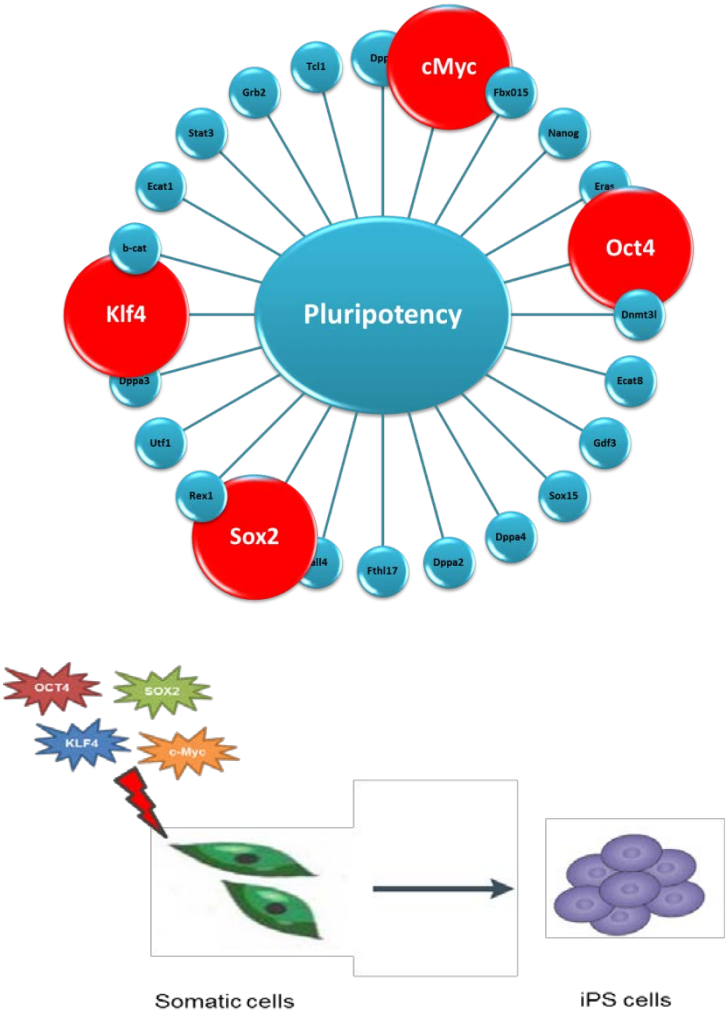


Figure 1-10: Generation of iPS cells. Yamanaka and colleagues demonstrated that retroviral-mediated over expression of a combination of just four transcription factors was sufficient to convert somatic cells into pluripotent stem cells, called induced pluripotent stem (iPS) cells.

Nevertheless, these early attempts generated iPS cells that didn't show global gene-expression patterns similar to those of ES cells, and failed to produce viable chimeras upon injection into a developing blastocyst suggesting that the reprogramming was incomplete. However, replacing the FBX15-neo selection with GFP-IRES-puro cassette introduced into the endogenous OCT4 (OCT4-neo) or NANOG locus (NANOG-neo) in MEFs allows the generation of iPS cells that were similar to ES cells with respect to DNA methylation, gene expression profiles and the chromatin state of the reprogrammed cells (Maherali *et al.*, 2007; Okita *et al.*, 2007). Both OCT4 and NANOG promoters showed demethylation in NANOG -selected iPS cells that were almost identical to global

histone methylation patterns (H3K4 and H3K27) in ES cells. FBX15 selected iPS cells had significantly higher expression levels of the four transgenes and lower levels of endogenous OCT4, SOX2, and NANOG gene expression compared with NANOG-iPS cells. Importantly, OCT4 -selected iPS cells could be maintained through endogenous expression of OCT4, whilst FBX15 selected iPS cells required continuous expression of the exogenously provided factors to maintain their self-renewal and pluripotency (Takahashi and Yamanaka, 2006). These cells were able to form teratomas containing differentiated cell types representing all three embryonic germ layers and importantly could contribute to the germ line of chimeric offspring following blastocyst injection (Maherali *et al.*, 2007; Okita *et al.*, 2007) suggesting that the reprogramming was complete. Subsequently, mouse iPS cells passed the most stringent *in vivo* assay of pluripotency by showing the ability to give rise to full-term embryos by tetraploid complementation, thus becoming functionally highly similar to mouse ES cells (Boland *et al.*, 2009; Zhao *et al.*, 2009). On the other hand, Meissner *at al* suggested that the incomplete reprogramming in the original Yamanaka work was caused by the early selection for Fbx15 activation and postulated that selection for Fbx15 activation at later times would generate fully reprogrammed iPS cells based on the finding that the reprogramming process is slow and gradual (Meissner *et al.*, 2007). IPS cells have also been derived from unmodified somatic cells based upon morphological criteria rather than selection for drug resistance. The overall efficiency of reprogramming using morphological selection was approximately 5–10 times higher when compared to drug-selection strategies. This result endorsed the supposition that reprogramming is a gradual process where selection for drug may exclude cells before reaching the ultimate pluripotency state.

1.8.1.2 Induction of pluripotent stem cells from human fibroblasts

One year later, two groups succeeded in generating iPS cells from adult human dermal fibroblasts (HDF) by the introduction of either the same four transcription factors (Takahashi *et al.*, 2007) or a slightly different combination (Yu *et al.*, 2007). Similar to human ES cells, human iPS cells formed tightly-packed flat colonies. Disaggregation of these colonies resulted in cells similar to human ES cells in morphology characterized by large nuclei and scant cytoplasm. Human iPS cells expressed human ES cell-specific surface antigens, including SSEA-3,

SSEA-4, and tumour-related antigen (TRA)-1-60, TRA-1-81, and alkaline phosphatase. RT-PCR also showed that human iPS cells expressed many undifferentiated ES cell-marker genes, such as OCT4, SOX2, NANOG, GDF3, fibroblast growth factor 4 (FGF4), embryonic cell specific gene 1 (ESG1), developmental pluripotency-associated 2 (DPPA2), DPPA4, and telomerase reverse transcriptase (hTERT) at levels similar to those in human ES and EC cell lines as well as an epigenetic status similar to that observed in human ES cells. The OCT4 and NANOG promoters were demethylated and in an active state, and exhibited H3K4 methylation and H3K27 demethylation patterns. Furthermore, these cells possessed the ability to differentiate into cell types of the three germ layers both *in vitro* and *in vivo*. However, Yamanaka *et al.* found that each iPS clone contained some retroviral integration, which may increase the risk of tumorigenesis. Around 20% of mice derived from iPS cells exhibited tumours, which was partially ascribed to reactivation of the C-MYC retrovirus (Takahashi and Yamanaka, 2006). Thomson *et al.* reported that human somatic cells can be converted to pluripotent cells using a similar, but distinct combination of genes (OCT4, SOX2, NANOG, and LIN28) without compromising iPS efficiency. KLF4 and C-MYC therefore may not be required for the reprogramming process in human cells. These data demonstrated that iPS cells can be generated not only from mouse, but also from human fibroblast cultures. After the successful generation of iPS cells from animal and human fibroblasts, other cell types have been successfully reprogrammed either in animal such as stomach cells (Aoi *et al.*, 2008) and B cells (Hanna *et al.*, 2008), or in human such as cord blood (Nishishita *et al.*, 2011), keratinocytes (Aasen *et al.*, 2008), adipose tissues (Sun *et al.*, 2009), neural progenitor cells (Kim *et al.*, 2009b), platelets (Gekas and Graf, 2010), prostate and bladder cells (Moad *et al.*, 2013), and the number of cell types that can be used to generate iPS cells is growing steadily. Similar to human ES cells, human iPS cells should be very useful for studying the development and function of human tissues, for discovering and testing new drugs and for transplantation medicine.

1.8.1.3 Mechanistic insights to reprogramming

Several studies have reported that the reprogramming process involves a series of transcriptional modifications. Recently, it has been indicated that acquisition of epithelial characteristics through mesenchymal to epithelial transition (MET)

is an important early step required for the successful reprogramming of a mouse fibroblast cell into a pluripotent stem cell. MET takes place before the acquisition of ES cell-like properties and is characterised by epithelial-like morphological alterations including size reduction, compacting cells with well-defined intercellular junctions and high cytokeratin expression from day 5 post-transduction, activation of epithelial-associated genes such as E-cadherin (Cdh1), Claudins 3, 4, 7, 11, Occludin, epithelial cell adhesion molecule (EpCAM), and crumbs homolog 3 (Crb3), as well as repression of mesenchymal associated genes, such as SNAIL1/2, SLUG, ZEB1 and ZEB2 (Li *et al.*, 2010; Samavarchi-Tehrani *et al.*, 2010). Consistent with these, reprogramming can be improved using factors facilitating MET such as TGF-b inhibitors, Cdh1, BMPs, microRNA miR200s and miR302/367 (Liao *et al.*, 2011; Liang and Zhang, 2013). OCT4, SOX2, KLF4 and C-MYC reprogramming factors have a suppressive effect on TGF-b signalling and miRNAs miR-155, miR-10b, which are associated with EMT (Peinado *et al.*, 2003; Kong *et al.*, 2008). Subsequently, OSKM were more effective in generation of iPS cells than OSK which might be due to the inefficient block of the TGF-b pathway mediated by C-MYC, indicating that MET is essential for the establishment of pluripotency.

However, acquisition of the epithelial status doesn't guarantee reaching the iPS cell fate, since cells undergoing reprogramming will still require continuous expression of reprogramming factors to reach bona fide pluripotency found in ES cells (Smith *et al.*, 2010). Concurrent with acquiring epithelial cell characteristics, reprogrammed cells increase their proliferation rate and escape cell cycle arrest. Consistently, knocking down p53-p21 or inhibiting Ink4a/Arf has been found to promote the iPS generation by enhancing cell proliferation (Smith *et al.*, 2010; Liang and Zhang, 2013).

Metabolic change is also an important step for inducing pluripotency. Studying the metabolome profiles of iPS cells relative to ES cells and to their somatic cells of origin showed that iPS cells and ES cells have similar but not an identical metabolomic signature (Panopoulos *et al.*, 2012). In addition, the metabolome profile of differentiated cells which prefer oxidative phosphorylation is converted to pluripotent glycolytic metabolism which fuels induction of

pluripotency (Folmes *et al.*, 2011). Previous studies reported that C-MYC is involved in metabolic gene regulation and cell cycle acceleration, indicating that C-MYC plays a critical role in the early phase of the reprogramming process rather than activation of pluripotency regulators (Sridharan *et al.*, 2009). However, successful generation of iPS cells without C-MYC suggests that metabolic changes can be achieved by other reprogramming factors (Liang and Zhang, 2013).

The next step in reprogramming involves the activation of the pluripotency circuitry. Pioneering studies by two groups reported the first insights into the mechanisms of reprogramming fibroblasts into iPS cells using doxycycline-controlled lentiviral vectors encoding the four reprogramming factors OCT4, SOX2, KLF4 and C-MYC to infect MEFs with a knock in of GFP into the endogenous OCT4 allele (OCT4-GFP) (Brambrink *et al.*, 2008; Stadtfeld *et al.*, 2008b). Stably reprogrammed cells were produced after treating with doxycycline for at least 8 days. Addition of doxycycline for 10, 11, 12, and 13 days increased the number of iPS colonies, sequentially. Cell sorting with Thy1 (expressed in fibroblasts and other differentiated cell types) and SSEA1 markers (expressed in mouse ES cells) showed that the Thy+/SSEA1− phenotype in fibroblasts starts to shift gradually after 3 days of doxycycline withdrawal, where Thy1 down-regulation preceded the SSEA-1 upregulation. However, the phenotype reversal to Thy1−/SSEA1+ was not significantly detected until the doxycycline was removed from the cultures at day 12, suggesting that surface markers Thy1 and SSEA1 distinguish early intermediates of the reprogramming process and only few Thy1−/SSEA1+ cells can pass to the stabilisation phase (Stadtfeld *et al.*, 2008b; Li *et al.*, 2010; Liang and Zhang, 2013).

Previous studies also revealed that direct reprogramming is a slow and gradual process encompassing the sequential activation of various pluripotency associated markers where alkaline phosphatase appears at day 3 of transgene expression followed by expression of the surface marker SSEA1 on day 9. On the other hand, endogenous expression of OCT4 or NANOG was first detectable on day 16, suggesting that SSEA1+ cells may mark an intermediate step of reprogramming whereas the endogenous expression of OCT4 or

NANOG was postulated as a marker for fully reprogrammed cells. Retroviral silencing also appeared to be a gradual process that started early but finished coinciding with the acquisition of late pluripotency gene expression in iPS cells.

In a more advanced system Wernig *et al* generated doxycycline-inducible secondary iPS cells. Firstly, reprogramming factors were expressed using doxycycline-inducible lentiviruses to generate primary iPS cells which were then used to generate chimeric mice. Addition of doxycycline to the MEF population selected from these chimeric mice resulted in the generation of secondary iPS cells with higher efficiency compared to primary iPS cells but was not 100% as one might expect (Wernig *et al.*, 2008a). Yamanaka gave explanation for this discrepancy in his recent comment about the two models for iPS cell generation (Yamanaka, 2009a). First, the elite model, presupposes that only a certain population of cells, such as stem/progenitor cells, can be successfully reprogrammed into a pluripotent state. Evidence for this model comes from a study by Kuroda *et al* where they reported the existence of “multilineage differentiating stress-enduring” (Muse) cells, a type of cell in adult human mesenchymal cells such as dermal fibroblasts and bone marrow stromal cells. Muse cells were found initially to be stress-tolerant and SSEA3+/CD105+ and showed pluripotency characteristics such as self-renewal ability, expression of pluripotency markers (OCT4, SOX2, and NANOG) and ability to differentiate into the three germ layers *in vitro* and *in vivo,* while at the same time they also exhibited characteristics of mesenchymal cells (Kuroda *et al.*, 2010). These Muse-cells have been found to be more amenable to generating iPS cells when transduced with the four factors OCT4, SOX2, KLF4, and C-MYC, while no iPS cells could be established from non-Muse cells indicating that human fibroblast cells contain a population of adult stem cells that primarily contributes to iPS cell generation (Wakao *et al.*, 2011). However, such a model cannot explain the existence of the partially reprogrammed cells that acquire epithelial properties but without activation of the pluripotency genes.

The other model, the stochastic model proposed that most differentiated cells can be reprogrammed after infected with the reprogramming factors. However, genetic and epigenetic obstacles must be overcome to reach successful reprogramming. This model may integrally depict the reprogramming process

and most data generated appear to support it (Hanna *et al.*, 2009; Yamanaka, 2009a).

A study by Tehrani *et al* revealed three phases of reprogramming termed initiation, maturation, and stabilization where the Initiation phase is mainly marked by MET. Importantly, the authors found that reactivation of some pluripotency markers such as NANOG and SALL4 mark the transition to the maturation phase (Samavarchi-Tehrani *et al.*, 2010). NANOG is also found to play an important role in initiating the pluripotency state, specifically by driving the partially reprogrammed iPS cells to reach pluripotency (Silva *et al.*, 2009) and therefore may help in setting up the whole pluripotency circuitry.

Recently, single-cell gene expression analysis and clonal retrospective tracing of cells derived from early time points, intermediate cells, and fully reprogrammed iPS cells showed that reprogramming in mouse comprises an early stochastic and a late hierarchical stage. Gene expression of Esrrb, Utf1, Lin28, and Dppa2 were found to stringently predict successful generation of stable iPS cells lines before the pluripotency core circuitry is activated (Buganim *et al.*, 2012). Moreover, up-regulation of pluripotency genes such as Fbxo15, Fgf4, and unexpectedly OCT4 doesn't strictly associate with successful reprogramming. In the same study, the authors found that the expression of SOX2 significantly activated the most upstream pluripotency genes and derived a hierarchical activation of key pluripotency genes making it a potential late marker. Such a hierarchy proposes that fully reprogramming can be induced from multiple entry points and even without any of the original Yamanaka factors. Interestingly, generation of iPS cells has been reported by a combination of Lin28, Sall4, Esrrb and Dppa2 suggesting specific gene expression and hierarchical activation of key pluripotency genes mediate the activation of the pluripotency circuitry during the late phase in reprogramming (Liang and Zhang, 2013). Upon activation of the pluripotency circuitry, reprogramming cells gain the ability for self-renewal independently of the exogene expression (Guenther *et al.*, 2010; Newman and Cooper, 2010; Bock *et al.*, 2011).

Dramatic changes in the gene-expression patterns without alterations in DNA sequences are referred to as "epigenetic" changes, mainly including DNA

methylation, genomic imprinting and histone modification. Epigenetic changes allow pluripotent cells to differentiate into tissue specific cells during the normal development. Epigenetic changes, such as DNA methylation and histone modifications, are also believed to play a critical role in the process of reprogramming somatic cells to an undifferentiated state and maintaining stem cell pluripotency. Successful reprogramming requires silencing of differentiation specific genes and activation of gene expression patterns unique to pluripotent cells (Han and Sidhu, 2008). Lysine methylation and acetylation are two of the most frequently studied histone post-translational modifications. Changes in histone modifications including a deposition of the histone H3 dimethylated at lysine 4 (H3K4me2) mark and a gradual depletion of H3K27me3 is observed immediately after induction whereas DNA demethylation and X-chromosome reactivation can be seen late in the reprogramming process (Buganim *et al.*, 2013). Collectively, these studies provide deep insights into the molecular events that occur during the reprogramming process using integrating viral vectors.

1.8.1.4 Potential application of iPS cells

Overcoming both immunological rejection and the ethical issues relating to ES cells, together with the unique ability to continuously self-renew and differentiate into all cell types in the human body gives iPS cells the potential to revolutionize the earliest steps of disease modelling and treatment. Therefore, the potential use of iPS cells as treatments for various disorders has been investigated *in vitro* and *in vivo*.

A proof of principle study for potential clinical applications of iPS cells with encouraging results was described by Hanna *et al* in 2007. Tail-tip fibroblasts isolated from a humanized knock-in mouse model of sickle cell anemia were transduced with retroviruses encoding for OCT4, SOX2, KLF4, and C-MYC transcription factors to generate iPS cells. Then the β-sickle mutation in generated iPS cells was corrected by homologous recombination with a human *β*A wild-type globin gene. Haematopoietic progenitors (HPs) derived *in vitro* from gene-corrected iPS transplanted into irradiated mice were able to reconstitute the haematopoietic system of sickle mice and correct their disease phenotype (Hanna *et al.*, 2007).

More recently, neural precursors derived from mouse iPS cells have been shown to migrate into various brain regions and differentiate into glia and neurons when grafted into the embryonic cerebral ventricles of parkinsonian rats. Successfully implanted animals showed functional recovery and significant improvement in the disease phenotype (Wernig *et al.*, 2008c). Another therapeutic application of iPS has been evaluated using FVIII expressing endothelial/endothelial progenitor cells derived from wild-type mouse iPS cells to treat haemophilia A mutant animals. Transplantation of these iPS-derived cells into the liver of a preclinical mouse model of haemophilia A resulted in phenotypic correction of the bleeding disorder. Monitoring plasma levels of FVIII revealed long-term functional engraftment and structural integrity of iPS-derived donor cells (Xu *et al.*, 2009b).

Additional technical challenges may be expected when applying these therapeutic approaches into human diseases therapies. However, several trials to generate disease-specific iPS cells are presently in progress. Initial experiments by Dimos *et al* showed that disease-specific iPS cells can be produced utilizing skin fibroblasts cells obtained from an 82-year-old patient diagnosed with a familial form of amyotrophic lateral sclerosis (ALS). Generated iPS cells could further be successfully directed to differentiate into motor neurons, the cell type involved in ALS pathology, providing a potential therapeutic model and suggesting that iPS generation is not restricted by the age and the source of the cells (Dimos *et al.*, 2008).

IPS cells were subsequently generated to study disease and drug development. IPS cells were successfully generated from patients with 10 different single-gene disorders including Gaucher disease type III, Shwachman-Bodian-Diamond syndrome, Huntington disease, Lesch–Nyhan syndrome and Parkinson's disease. The mutation to the wild-type was corrected before transplantation by gene targeting. The resulting cells were analysed to confirm that gene repair was perfect and specific, thereby decreasing the safety concerns of random, viral-mediated gene therapy. Furthermore, all generated iPS cell lines expressed markers including OCT4, SOX2, NANOG, REX1, GDF3 and hTERT and possessed the ability to differentiate into the three germ layers *in vitro* (Park *et al.*, 2008). Similarly, primary dermal keratinocytes and

fibroblasts were obtained from Fanconi Anemia (FA) patients (Raya *et al.*, 2009). Somatic cells were used either directly or after genetic correction to generate iPS- cells. IPS cells generated from genetically corrected FA somatic cells showed disease-free phenotype with fully functional FA pathway. Furthermore, haematopoietic progenitors of the erythroid and myeloid lineages were successfully obtained from FA-iPS cells and maintained the disease-free phenotype of FA-iPS cells. However, iPS cells could not be generated from patient's cells before repairing the genetic alteration. Recently Zou *et al* reported the first successful gene targeting by homologous recombination in human iPS cells using an engineered gene-targeting vector expressing a zinc finger nuclease. This technique increased the efficiency of HR-mediated gene targeting by almost 200-fold in human ES and iPS cells without detrimental effects on either cell karyotypes or pluripotency. Therefore, this study provided a solid foundation to enhance the development of future therapeutic gene targeting to efficiently create or correct specific mutations in patient-specific iPS cells (Zou *et al.*, 2009).

Human iPS cells can also be used to model a specific pathology seen in a genetically inherited disease. Fibroblast cells isolated from a child with spinal muscular atrophy were used to generate iPS cells. IPS derived neurons retained the disease genotype and showed selective deficits compared to those derived from the patient's healthy mother (Ebert *et al.*, 2009). Disease-specific iPS cells can also help as a small molecule screening platform for drug development. IPS cells established from familial dysautonomia patients (FD-iPS) were differentiated towards neural crest lineages to model functional characteristics of pathogenesis *in vitro* (Lee *et al.*, 2009). This model enabled the authors to identify a new drug candidate termed ''kinetin,'' for the treatment of FD, and further supported the potential use of iPS cell technology in disease modelling and cell therapy. Professor Schwartz and his colleagues reported new, positive data in a paper in Lancet from their clinical trials using retinal pigmented epithelial cells (RPEs) made from human ES cells for treatment of different forms of macular degeneration (MD). Importantly so far bleeding and some adverse side effects were reported and appeared related to the delivery procedure itself and to immunosuppression (Schwartz et al., 2014). Recently, a Japanese woman in her seventies who had retinal damage owing to a condition

known as age-related macular degeneration has been received for the first time ever tissue derived from iPS cells. Cells from the patient's skin were reprogrammed to produce iPS cells. Then those cells were differentiated into retinal pigment epithelium cells and grown into a sheet for implantation. No serious side effects have been reported. However, researchers around the world are watching to see whether the cells stop the retina from deteriorating further and whether any side effects develop (Cyranoski, 2014).

Overall, iPS cells present a potentially unlimited source of cells that may be directed to differentiate into all cell types within the body and used in tissue engineering, cell replacement therapies and for regenerative medicine applications. A potential major advantage here is that iPS cells have the ability to generate other cell types such as neural, endothelial and a smooth muscle cell which makes them a valuable cell source for urological tissue engineering compared with other cell types. These are integral cell types that make up the complete organ.

1.8.1.5 The limitation of using iPS cells in regenerative medicine

The unique nature of iPS cells lies in their capability, when cultured, for unlimited self-renewal and reproduction of all cell types of the body in the course of their differentiation; therefore they represent invaluable tools for research into the mechanism of tissue formation. However, there are also significant problems associated with the use of these cells in tissue engineering (Ibarretxe et al., 2012). Pluripotent stem cells present a safety concern because of their potential to form tumours. When these cells are transplanted in the undifferentiated state, they form teratomas, tumours derived from all three germ layers. Currently, the only way to ensure that teratomas do not form is to differentiate the pluripotent stem cells, enrich for the desired cell type, and screen for the presence of undifferentiated cells (Zhu et al., 2011). Another serious problem is the use of potentially harmful genome integrating viruses to deliver reprogramming factor transgenes. Most iPS cells are prepared by viral vectors that integrate the reprogramming factors into host genomes, increasing the risk of tumor formation (Takahashi and Yamanaka, 2006; Brambrink et al., 2008). The residual presence of integrated transgenes following the derivation of IPS cells is highly undesirable. The four genes used for the induction of

pluripotency are recognised oncogenes, thus pose a theoretical risk of neoplastic development from cells derived from iPS cells. There are substantial grounds to state that the process of nuclear reprogramming by virus-assisted factor insertion in the cell genome increases the risk of carcinogenesis (Miura et al., 2009). This high risk of carcinogenesis is largely, but not exclusively, related to the integration of c-MYC transgenes (Nakagawa et al., 2008). Several possible strategies exist for resolving the above mentioned problems including the development of delivery protocols for non-integrated genetic constructs (adenoviruses, plasmid transfection, doxycycline-inducible excisable piggyBac (PB) transposon system), and minimizing the number of genes required for reprogramming. Another way to reprogram somatic cells consists of delivery of recombinant proteins rather than genes into the cells to be reprogrammed (Zhou et al., 2009) or the induction of reprogramming by chemical stimulation and screening/selection of effective small molecules, thus reducing the amount of factors delivered to cells (Lyssiotis et al., 2009). The opportunity to obtain patient-specific iPS cells has brought a big hope on the prospect of future tissue engineering regenerative therapies by cell transplant since these new pluripotent cells circumvent two of the main problems traditionally associated with human ES cells (ethical issues and the possibility of rejection of the transplanted cells by the host immune system). One can confidently state that both iPS cells and their derivatives are potent instruments applicable in cell replacement therapy. Their use for tissue regeneration, however, poses considerable health risks, with research into their clinical value still in the earliest stages (Medvedev et al., 2010).

1.8.1.6 "Retentive" memory of reprogrammed cells

Although iPS cells were shown to be similar to ES cells with respect to gene expression of pluripotency markers and the ability to differentiate into cell types from the three embryonic germ layers both *in vitro* and in teratoma assays, differences between iPS cells and ES cells in their gene expression profiles (Chin *et al.*, 2009), differentiation abilities (Feng *et al.*, 2010), and persistence of donor-cell gene expression (Ghosh *et al.*, 2010; Bar-Nur *et al.*, 2011) have been recently documented. It has been shown that iPS cells at low passages display an epigenetic memory inherited from the original somatic cell that will likely favour iPS cell differentiation towards lineages related to that cell (Chin *et al.*,

2009; Marchetto *et al.*, 2009; Kim *et al.*, 2010). Genome wide expression analysis of human iPS cells and their embryo derived counterparts showed that early- and late-passage iPS cells have different gene expression signatures, where late-passage appeared to be much more similar to their embryo-derived counterparts than early-passage iPS cells. Analysing the expression differences between early-passage iPS cells lines and their related human ES cells showed that most of the genes highly expressed in iPS cells versus ES cells were associated with differentiation. Although extended passaging significantly reduced these transcriptional differences, late passage iPS cells were still distinguishable from ES cells. These transcriptional differences didn't appear to be due to differences in histone modification patterns (Guenther *et al.*, 2010) suggesting insufficient suppression of somatic genes or insufficient induction of pluripotent genes (Chin *et al.*, 2009; Marchetto *et al.*, 2009). Other studies also revealed unique DNA methylation and gene expression patterns that are inherited from a parental cell following reprogramming in both human and mouse iPS (Kim *et al.*, 2010; Lister *et al.*, 2011; Ohi *et al.*, 2011).

Mouse iPS cells in very low-passage were found to maintain a DNA methylation memory of their somatic tissue of origin that may influence their differentiation propensity toward tissue specific fates related to that origin, and limiting other cell destinies (Kim *et al.*, 2010; Polo *et al.*, 2010). These data are consistent with previous reports, which indicate that cell origin influences reprogramming efficiency (Aoi *et al.*, 2008; Maherali *et al.*, 2008; Miura *et al.*, 2009). Importantly, this epigenetic memory may only appear upon differentiation when the particular loci that retained epigenetic marks are expressed and not in the pluripotent state. Methylation, faulty restoration of bivalent domains and loss of key factors that mediate repression of genes expressed only in differentiated cells constitute suggested mechanisms of epigenetic memory in iPS cells (Kim *et al.*, 2010; Polo *et al.*, 2010). In accordance with these findings, gene expression and DNA methylation analysis of human iPS cells established from different cell origins showed that iPS cells retain a residual transcriptional memory of the original somatic cell which can be partially identified by inefficient promoter DNA methylation and silencing of somatic genes. Inhibition of C9orf64, one of the main incompletely silenced genes in iPS cells significantly decreased the efficiency of iPS generation suggesting that such genes may be

required during reprogramming therefore continue to be expressed in nascent iPS cells. Notably most of these findings were associated with early passage iPS cells (Ohi *et al.*, 2011). However, much additional research will be necessary to understand how this memory varies among different cell types and tissues. Interestingly, the presence of a tissue-specific epigenetic memory might be of benefit rather than a hindrance through generation of iPS cells that retain the ability to differentiate into a specific cell type.

Recently Bar-Nur *et al* utilised a genetic lineage-tracing approach for monitoring the origin of reprogrammed cells and evaluating the differentiation potential of iPS cells derived from human beta cells (BiPS). Generated BiPS cells were found to retain an epigenetic memory during their expansion *in vitro* that may preferentially drive their differentiation more readily into insulin producing cells. BiPS cell lines showed a typical ES-like morphology, expressed most pluripotency markers at both RNA and protein levels, silenced the retroviral transgenes, maintained a normal diploid karyotype and generated cells from all three embryonic germ layers. Importantly, the linear correlation coefficient between BiPS cells and ES cells was very similar to the correlation coefficient between two different ES cell lines (R = 0.94). These observations collectively indicated that BiPS cells were truly reprogrammed pluripotent cells. Chromatin immunoprecipitation showed epigenetic imprint preserved in the INSULIN and PDX1 gene promoters in BiPS cell lines at similar levels to those of beta cell-derived (BCD) progeny, while not detected in non-beta pancreatic iPS (PiPS) cell lines, iPS cells derived from fibroblasts, or ES cells of similar passage numbers. Similar epigenetic memory was observed at the DNA methylation level with unique DNA methylation signature in BiPS cells that segregated them from BCD, PiPS cells lines, iPS cells, and ES cells. The authors next investigated if that observed epigenetic memory may skew the differentiation of BiPS cells into insulin producing cells and found that differentiated cells derived from BiPS cells expressed higher levels of INSULIN, PDX1, and FOXA2 compared to differentiated cells derived from PiPS cell lines, iPS cells, and ES cells of similar passage numbers, suggesting preferential lineage-specific differentiation in BiPS (Bar-Nur *et al.*, 2011). Therefore, it is essential to generate human iPS cells from different tissues and compare their safety and differentiation capacities.

Consistent with these results iPS cells derived from murine ventricular myocytes showed a dramatically higher tendency to re-differentiate back to cardiovascular progenitors and contribute to functionally beating cardiomyocytes as compared to genetically matched ES cells or iPS cells derived from tail-tip fibroblasts (Xu *et al.*, 2012). Global gene expression and DNA methylation analysis of these iPS cells showed a distinct transcriptional and epigenetic signature that may potentially be involved in directing iPS cells to ventricular myocytes fate. Later Lee *et al* extended these studies to demonstrate that the differentiation potential of iPS cells may depend on the lineage stage-specific differentiation state of donor cells. iPS cell lines established from hepatic lineage cells at an early stage (hepatoblast) can differentiate more effectively toward hepatocytic lineage as compared to iPS cells established from adult hepatocyte (late stage), mouse embryonic fibroblasts, or mouse ES cells (Lee *et al.*, 2012). All the generated iPS cell lines showed ES cell-like morphology, expressed pluripotency markers and underwent multilineage differentiation *in vitro* and *in vivo* demonstrating their complete reprogramming. Moreover, a global gene expression analysis of hepatoblast derived iPS cells also exhibited a unique gene expression signature, which clearly differentiated them from iPS cells established from adult hepatocyte as well as mouse ES cells. These differences in gene expression were suggested to be responsible for the variability observed in differentiation potency. Comparison of the gene expression profiles of the hepatoblast derived iPS cells and parental cells allowed identification of 24 genes (7 upregulated and 17 downregulated genes) as the hepatoblast derived-iPS cells specific donor memory genes.

Based on this literature, we hypothesised that iPS cells generated from urinary tract specific tissue is better able to regenerate differentiated bladder tissue than conventional skin fibroblast derived iPS cells. To this end, stromal cell isolated from the lower urinary tract were transduced with lentiviral vectors encoding the four pluripotency-inducing factors, OCT4, SOX2, KLF4, and C-MYC 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’. Transduced cells were subjected to an optimized protocol involving the use of pluripotent-cell as well as feeder cell-conditioned media. A total of 31 clones from 5 different patients have been generated. The resultant

cells silenced the lentiviral transgenes, and maintained a normal diploid karyotype and their credibility were further corroborated by means of ‘paternity’ tests showing an identical DNA identity match between parent and the resultant UT-iPS cells for a panel of microsatellite markers. All the UT-iPS cells expressed embryonic stem-cell characteristics. The cells were expressed endogenous pluripotency genes in comparable levels to human ES cells and gave rise to differentiated cells representing all the three germ layers *in vitro*, as judged by EB formation assay, and *in vivo*, as evidenced by teratoma formation assay. Taken together, these results demonstrate that UT-iPS cells are truly reprogrammed pluripotent cells. We have also found that these UT-iPS cells show preferred differentiation towards bladder tissues suggesting that a tissue-specific molecular and epigenetic imprint may remain contained during iPS cells reprogramming.

Hypotheses:

IPS cells generated from urinary tract specific tissue (UT-iPS) are better able to regenerate differentiated bladder tissue than conventional skin fibroblast derived iPS cells (skin-iPS).

Aims:

- Generation of induced pluripotent stem cells from stromal/ urothelial cells isolated form the urinary tract tissue (UT-iPS) cells.

- Evaluate the potential of UT-iPS cells to differentiate into bladder specific cells compared with the conventional skin-iPS cells.

2 Chapter 2. Materials and methods

2.1 Cell culturing and maintaining

3.1.9 Cell line culture: Normal Human Dermal Fibroblast (NHDF) cells

NHDF cells were kindly donated by Prof. Majlinda Lako (Institute of Genetic Medicine, Newcastle University). Cells were cultured in RPMI1640 medium with HEPES modification (Sigma) supplemented with 10% heat inactivated foetal bovine serum (FBS) (GIBCO, Life Technologies) and 1% L-glutamine (2mM) (Sigma). Cells were passaged approximately every week and the medium was changed every 48 hours. The main purpose for growing these cells is to be used as control cell line for iPS induction.

3.1.10 Primary tissue culture

The urothelium is defined as the epithelial cells lining the surface of the renal pelvis, ureter, bladder, and proximal urethra; therefore, we used the urothelium isolated from human bladder and ureter as a model for studying the urothelial cells. All surgical specimens were collected after conforming to ethical guidelines and had full patient consent. Samples of bladder and ureter were obtained from patients undergoing various urological procedures with no history of urothelial dysplasia or malignancy (Department of Urology, Freeman Hospital, Newcastle Upon Tyne, UK). Samples were taken to the laboratory as promptly as possible. All procedures were performed under sterile conditions.

2.1.1.1 Isolation and culturing of human urothelial cells

Two methods were used to isolate and grow human urothelial cells *in vitro*, primary explant culture, and enzymatic digestion method described by Southgate *et al* (Jennifer Southgate, 2002; Southgate *et al.*, 2007).

I. Primary explant culture

Ureter samples were opened longitudinally and the urothelium attached to underlying tissues was separated mechanically using fine curved scissor and forceps (sterilized tools were used to prevent any cross-contamination), and cut into small pieces (≈2x2 mm). Tissue pieces were placed into 90 mm Petri dish (Figure 2-1) (approx 5 pieces/dish) containing 2-3 ml of keratinocyte serum-free

medium (KSFM) supplemented with epidermal growth factor (EGF, 5 ng/ml) and bovine pituitary extract (BPE, 50 pg/ml) as provided by the manufacturer (GIBCO, Life Technologies) as well as 1% Penicillin-Streptomycin (Sigma), the medium was further supplemented with 30 ng/ml of cholera toxin (Sigma) to improve cell attachment, this medium was called Complete Keratinocyte Serum-Free Medium (KSFMc). Using low calcium concentration, bovine pituitary extract, and other supplementary growth factors have been found to promote the growth of epithelial cells and enable for higher serial passages in culture (Chaproniere and McKeehan, 1986). Cultures were kept at 37°C and in a humidified atmosphere of 5% CO_2. The medium was replaced every 2-3 days. Explants outgrowth was maintained until a significant number of cells were obtained. Cells were passaged as follows: after removing the explants and medium from the dish, 1.5 ml of 0.05% trypsin (Sigma) was added and incubated at 37°C for about 5 minutes. Once all the cells were lifted off, the trypsin was diluted by adding 5 ml of KSFMc. Cells were centrifuged at 1200 x rpm for 4 minutes, re-suspended in fresh medium, and seeded into flasks or Petri dishes depending on the density of the cells.

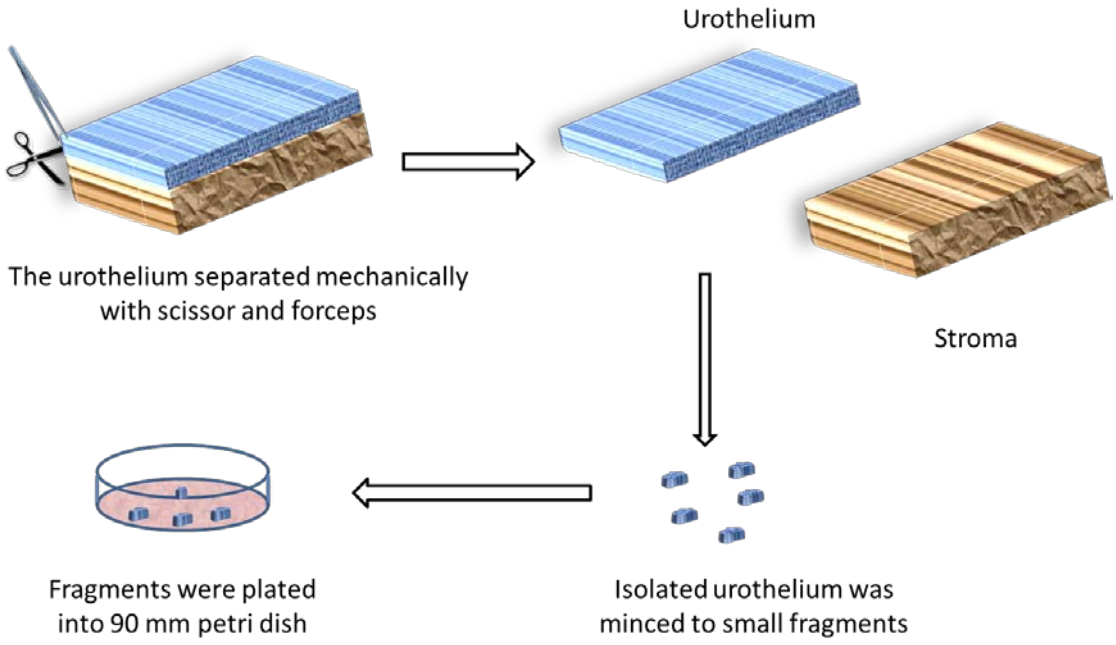


Figure 2-1: A schematic drawing for the initial steps of the primary explant culture.

II. Enzymatic digestion

Samples were dissected into approximately 1-2 cm^2 pieces. Each piece was placed into 10 ml of stripper medium (Table 2-1) for 4 hours at 37°C or overnight at 4°C to detach the urothelial cell sheets from the underlying stroma. The urothelium sheets were then scraped gently from their stromal counterparts using forceps and centrifuged at 1200 x rpm for 4 minutes. The resulting pellet was further disaggregated by treating with 2ml of collagenase type IV 100 U/ml (Sigma) for 20 minutes at 37°C. Cells were centrifuged and were further purified through MACS 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). CD326 positive urothelial cells were seeded into 60mm tissue culture dish and maintained in KSFMc at 37°C and in a humidified atmosphere of 5% CO_2 in air. Because the initial yield of urothelial cells was in most cases very low, we found that culturing cells initially into 60mm tissue culture dish rather than 90mm culture dishes increased culture success rate since it ensured that the cells are more close to each other. Cultures were passaged as follow: the medium was removed and the cells were treated with phosphate buffered saline (PBS) containing 0.1% (w/v) EDTA for 5 minutes at 37°C, followed by incubation in 1 ml of trypsin-versene (containing 0.25% (w/v) trypsin and 0.02% (w/v) EDTA) to detach the cells. Cells were re-suspended in KSFMc containing trypsin inhibitor (Sigma) and collected by centrifugation at 1200 x rpm for 4 minutes. The supernatant was aspirated and the cells were resuspended in KSFMc medium and plated into two 60mm tissue culture dish.

Stripping solution

Components	***Quantity***	***Supplier***
Hanks' Balanced Salt Solution (HBSS) without Ca^{2+} and Mg^{2+}	500ml	Invitrogen
1% (w/v) EDTA	50ml	Sigma
1 M HEPES buffer pH 7.6	5ml	Sigma
Aprotinin(500,000-KIU)	1ml (20 KIU)	Sigma

Table 2-1: Stripping solution composition.

III. CD326 magnetic activated cell sorting (MACS)

We used the MACS Kit provided by Miltenyi Biotec and CD326 microbeads to enrich for epithelial cells. Briefly, cells were incubated in 300 µl MACS buffer (1xPBS, 0.25%FCS, and 2mM EDTA), 100 µl FcR Blocking Reagent human (to increase the specificity of labelling with MACS Micro Beads), and 100 µl of CD236 MicroBeads conjugated to monoclonal antibody (Miltenyi Biotech, Bergisch Gladbach, Germany) for 30 minutes at 4 °C. Following incubation, cells were washed with cold MACS buffer, and centrifuged at 300 x g for 10 minutes at 4°C. Finally, cells were re-suspended in 500 µl of MACS buffer. For cell sorting, the magnetic separation MS column was placed in the magnetic field and prepared by rinsing with 500 µl of MACS buffer. The cell suspension was applied onto the column. CD326 positive cells remained on the column in the magnetic field whereas unlabeled cells (CD326 negative cells) flowed through and were collected in a universal tube. Immediately after removing the column from the separator, the labelled cells were flushed out to another universal tube with 1 ml MACS buffer and plunger.

2.1.1.2 Isolation and culturing of human urinary tract stromal cells

All stromal cells were propagated and maintained in RPMI1640 media with HEPES modification (Sigma) supplemented with 10% FBS, 1% L-glutamine (2mM), and 1% Penicillin-Streptomycin (Sigma). This medium was called full RPMI1640 medium. Firstly, the stroma layer from which the urothelium had been detached was minced with sterile scissors to ensure increased surface area for digestion (Figure 2-2). Then, resulting tissue pieces were digested by incubating with 250 U/mg of collagenase Type I (LS004196, Worthington, Lorne Laboratories, Reading, UK) overnight at 37°C with stirring. Collagenase I solution was prepared by dissolving 20mg of Collagenase IV powder in 20 ml of full medium RPMI1640. The cell suspension was mechanically disrupted by aspiration back and forth through a 21 gauge (21G) needle to homogenise and increase yield. Cells were then washed with PBS and centrifuged at 1500 x rpm for 5 minutes. Finally, cells were seeded in 25cm^2 flask containing 5ml of full medium RPMI1640. Stromal cells were passaged when approximately 85% confluent using either 75cm^2 or 175cm^2 flasks (Corning, UK) and maintained at 37°C in a humidified atmosphere of 5% CO_2 in air. Subculture was achieved as

follows: after removing the medium, cells were washed 1 x with PBS and detached from culture surface enzymatically by treatment with 2-3 ml of 0.05% trypsin solution and incubating at 37°C for 5 minutes. Then trypsin was neutralised by adding 8 ml of full medium RPMI1640. Cells were centrifuged at 1500 x rpm for 5 minutes and re-suspended in appropriate amount of full medium RPMI1640 depending upon flask size.

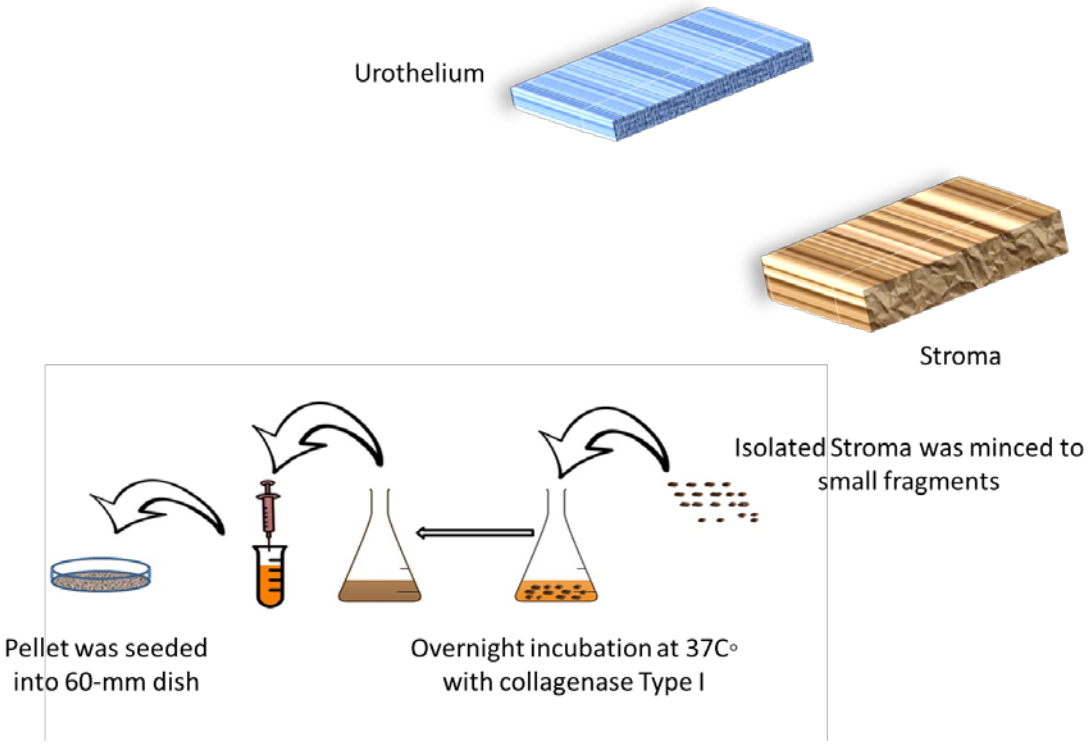


Figure 2-2: A schematic drawing of the initial steps in isolation and culturing the urinary tract stroma.

2.1.1.3 Freezing and thawing human urinary tract cells

We couldn't freeze the urothelial cells because the yield of these cells was in most cases very low. 80-90% confluent T175 flask of stromal cells was washed with PBS and incubated with 5 ml of 0.05% trypsin for 5 minutes at 37°C. The cells centrifuged at 1500 x rpm for 5 minutes, and the supernatant was discarded and replaced with 1 ml of fresh prepared freezing medium (Table 2-2) per original T175 flask of stromal cells. Cell suspension was transferred to cryopreservation cryovials (Nunc) and stored in a -80°C freezer. To retrieve frozen cells, a cryovial was rapidly transferred into 37°C water until most of the contents were thawed. Thawed cells were immediately washed with 9 ml pre-warmed RPMI1640 full medium and centrifuged at 1500 x rpm for 5 minutes at

room temperature. The cells pellet was re-suspended in 10 ml of RPMI1640 full medium and plated into T75 culture flasks. After 24 hours, the medium was changed and the cells were monitored for adherence and potential contamination.

Freezing medium

Components	*Quantity*	*Supplier*
RPMI1640	8 ml	Sigma
Dimethyl sulfoxide (DMSO)	1 ml	Sigma
Heat treated foetal bovine serum (FBS)	1 ml	Invitrogen

Table 2-2: Freezing Mixture Composition.

3.1.11 Pluripotent stem cell culture

2.1.1.4 Culturing UT-iPS cells on MEF feeder cells.

Human UT-iPS cells were routinely cultured in a 0.1% gelatin coated 6-well plate (Corning, UK) and were maintained on a layer of mouse embryonic fibroblast (MEF) feeder cells in human ES cell medium (Table 2-3). UT-iPS cells were checked microscopically to assure cell growth and sterility and maintained as mycoplasma negative.

Human Embryonic stem cell medium

Components	*Quantity*	*Supplier*
Knockout-Dulbecco's Modified Eagle's Medium (KO-DMEM)	120ml – 80%	Invitrogen
Knockout-Serum Replacement (KO-SR)	30ml – 20%	Invitrogen
MEM Non-Essential Amino Acids (100X), liquid	1.5ml – 1%	Invitrogen
Penicillin-Streptomycin Solution	1.5ml – 1%	Sigma
Basic Fibroblast Growth Factor (B-FGF)	120µl- 8ng/ml	Invitrogen
GlutaMAX™-I Supplement, 200 mM	1.5ml– 2mM	Invitrogen
β-mercaptoethanol	2µl- 100µM	Sigma

Table 2-3: Human Embryonic Stem Cell Media Composition.

2.1.1.4.1 Preparing gelatin coated culture plates

To improve their adhesion and attachment, MEF cells were plated onto 0.1% gelatin-coated plates. To prepare the gelatin solution, 1 gram of gelatin powder was dissolved to 100ml of distilled sterile water. This was autoclaved, dispensed into 50ml of 1% gelatin aliquots, and stored at -20ºC. To make 0.1% gelatin, 50 ml of 1% gelatin was dissolved in 450 ml sterile distilled water. A gelatin-coated 6-well plate was prepared by placing 1 ml of this 0.1% gelatin into each well. These gelatinised plates were incubated in a sterile environment for 30 minutes at room temperature or for 2 hours at 37°C, and the gelatin solution was only removed immediately prior to plating of irradiated MEFs.

2.1.1.4.2 Thawing and preparing MEF feeder plates

UT-iPS cells were maintained on irradiated MEF cells, passage 3, purchased from VhBio. MEF cells were derived from CF-1 mouse embryos at day 13 and mitotically inactivated by treatment with irradiation. The cells can be directly plated onto gelatin coated plates. Frozen stocks of MEFs were stored at -80º. At a time point 24 hours prior to use, cells were thawed as quickly as possible in a 37°C water bath, with gentle shaking by hand and transferred to a 20 ml universal centrifuge tube (Sterilin, UK) and washed by adding 10 ml of complete MEF medium (DMEM medium with HEPES modification supplemented with 10% FBS and 2mM L-Glutamine) dropwise, slowly with swirling and centrifuged at 1500 x rpm for 5 minutes. The cells pellet was re-suspended with an appropriate volume of complete MEF medium and directly plated at a concentration of 5 x 10^4 cells / cm^2 on pre-gelatinised 6-well plates. The MEF coated plates were ready to use 24 hours after plating and always used within 5 days.

2.1.1.4.3 Preparation of MEF- conditioned medium (MEF-CM)

MEF cells were plated at a density of 5 x 10^4 cells / cm^2 on pre-gelatinised 6-well plates in complete MEF medium. The following day, the MEF medium with 2ml/well of human ES cell medium was replaced, and incubated at 37°C, and 5% CO_2. The MEF-CM was collected from the wells every 24 hours and sterilized by passing it through 0.22 µM disposable filter. MEF-CM was collected for up to seven days using this procedure. MEF-CM was used fresh (within 3

days), otherwise frozen at -80º. Non-inactivated cell lines were also maintained in culture so as to generate feeder-conditioned media. These cells were routinely checked to assure cell growth and sterility and were maintained as mycoplasma negative.

2.1.1.4.4 Passaging UT-iPS cells onto MEF feeder cells

A plate which contained UT-iPS colonies that cover 80-90% of each well was ready to be passaged in a ratio of 1 to 3. One day before splitting UT-iPS cells, a new MEF plate was prepared. The complete MEF medium was replaced on the day of UT-iPS cells culture with 2 ml of human ES cell medium. UT-iPS cultures were split using mechanical and enzymatic dissociation passaging using a dissecting microscope within a laminar flow cabinet. UT-iPS colonies were washed with 1XPBS and treated with 1ml per well of prewarmed Collagenase IV solution (1mg/ml, GIBCO, Life Technologies) for 5-8 minutes at 37ºC until the edge of the colonies could be clearly defined and appeared slightly raised from the surrounding MEF cells. At this point the Collagenase was replaced with 2 ml of fresh human ES cell medium. To maintain the general undifferentiated state of the culture, the differentiated regions were identified morphologically and excised manually using a dissecting microscope within a laminar flow cabinet with P20/200 sterile plastic Gilson tip. The remaining undifferentiated colonies were scored with a 29 gauge (29G) needle or a P20 Gilson pipette tip into small clumps and transferred with a P200 pipette to new 6-well MEF plates and mixed gently to spread the clumps evenly over the well. The plate was incubated at 37°C in 5% CO_2 for 24 hours to allow cells to adhere to the bottom. Medium was changed the following day and every second day thereafter and colonies were monitored daily with any differentiated area of cells removed. Care was taken to avoid disaggregation of UT-iPS colonies to single cells during passaging, as this will significantly decrease their survival efficiency. On average, UT-iPS colonies were plated at a density of ~250-300 colonies per well/6-well plate. 5-7 days post-passage, the colonies reached 80-90% confluent within the wells and were then transferred into fresh MEF plates.

2.1.1.5 Culturing UT-iPS cells under feeder-free conditions

Human UT-iPS cells were cultured in 6-well culture plates coated with BD Matrigel™ human ES cell-qualified Matrix (354277, BD Biosciences, Bedford, UK) and maintained in a defined serum-free media mTeSR™1(05850, StemCell Technologies.) that was changed every 24 hours. To prepare the mTeSR1 medium, the 100ml mTeSR1 5X supplement was thawed overnight at 2 - 8°C and added to 400 ml basal medium for a total volume of 500 ml. The complete mTeSR™ medium was dispensed into working aliquots and stored at -20°C for up to 6 months. Thawed aliquots were stored at 4°C and used within two weeks. Under these conditions, UT-iPS cells were grown in colonies and passaged about every 5-7 days at split ratio of 1:6.

2.1.1.5.1 Preparing Matrigel-coated culture plates

The BD Matrigel™ matrix was thawed overnight at 4°C on ice. Once thawed, the vial was swirled to ensure the Matrigel was evenly dispersed. The content was aliquoted into smaller working volumes (160 µl) and stored at -80°C for up to 6 months. BD Matrigel was kept on ice at all times while handling, aliquoted as quickly as possible using pre-cooled tips and refrozen immediately to prevent it from gelling. Multi freeze- thaw was avoided. According to the dilution factor provided on the certificate of analysis, each aliquot can be used to coat two 6-well plates. To coat the plate, the BD Matrigel aliquot was thawed on ice and mixed with 13 ml of cold DMEM/F-12 (Sigma). Immediately, the wells were fully coated with the diluted BD Matrigel solution using 1ml/ well of a 6 well plate. The plates were incubated at room temperature for at least 1 hour before use. Otherwise the plates were sealed with Parafilm to prevent evaporation of the BD Matrigel solution and stored at 4°C for up to 7 days after coating. Stored coated plates were kept at room temperature for 30 minutes before use. Just before plating the UT-iPS cells, the BD Matrigel solution was carefully removed using a pipette without scratching the coated surface followed by adding 2ml of complete mTeSR1 medium.

2.1.1.5.2 Passaging UT-iPS cells under feeder-free conditions

UT-iPS colonies were passaged when about 80% of the culture surface was covered with colonies. Before passaging, differentiation areas were visually detected and removed by scraping with a pipette tip using an inverted microscope in a laminar flow cabinet. To passage, undifferentiated UT-iPS colonies were washed with DMEM/F-12 medium (2 mL/well) and loosened from the Matrigel by incubation with 1 ml/well of dispase (07923, StemCell Technologies) at a concentration of 1 mg/ml at 37°C for 5-7 minutes. Once the edges of the colonies start curling up, the dispase solution was removed and the cells were gently washed with 2 ml/well of DMEM/F-12 medium to dilute away any remaining dispase. Then 2 ml/well of mTeSR1 medium was added and the colonies were gently scraped from the substrate with a glass pipette and broken into pieces. These pieces were transferred onto new Matrigel-coated culture wells and grown in mTeSR1 medium. The new plates were then placed in an incubator and agitated a few times to disperse the colonies evenly over the whole surface. Starting 24 hours after passaging, the cells were monitored daily and the medium was changed every other day.

2.1.1.6 Freezing and thawing of UT-iPS cells

IPS cells have a low viability when split to single cells and are therefore routinely passaged as aggregates or clumps of cells. Unfortunately, when these cells are frozen, their viability upon thawing is particularly low making freezing and reconstituting of iPS cells a real challenge. Cryopreservation was done at the time when they were ready for passaging where wells are 80 % confluent. Before freezing, the UT-iPS cell culture was checked to ensure that it was at a suitable confluency, healthy situation and free of contamination. UT-iPS clumps were stored using both slow (Cryovial Cryopreservation) and fast (Vitrification) freezing methods. Vitrification is a rapid process and usually provides better post-thaw recovery rate of iPS cells compared to that in slow freezing procedure. However, the latter one is generally more conveniently performed and hence desired.

2.1.1.6.1 Slow freezing of the UT-iPS cells

I. Cryopreserving using DMSO:

Cell aggregates were collected as per routine UT-iPS cells passaging and kept as big as possible. Cells were pelleted at 200g for 4 minutes at room temperature. During the spin, the freezing medium was prepared, which contains: 50% human ES cell medium and 50% cryopreservation medium (Table 2-4). The supernatant was aspirated and the cells pellet was reconstituted in 200µl of freezing medium and mixed quickly and gently. It has been previously shown that the addition of Rho-associated Kinase (ROCK) inhibitor Y-27632 to the freezing medium minimises cell apoptosis and significantly enhances survival by as much as 27 fold (Watanabe *et al.*, 2007). Cells harvested from one 6-well plates were frozen in 1 cryovial, to this 10µM of ROCK inhibitor (Stemolecule ™ Y27632, Stemgent) was added and the cryovials were placed in a -80˚C freezer for short term storage and were later transferred to liquid nitrogen (-196°C) for long-term storage.

Cryopreservation medium(x10)

Components	***Quantity***	***Supplier***
Heat Treated Foetal Bovine Serum (FBS)	600µl- 60%	Invitrogen
DMSO	200µl- 20%	Sigma
HESC Media	200µl- 20%	-
Y-27632 Rho-associated Kinase (ROCK) inhibitor	10µM	Stemgent

Table 2-4: Cryopreservation medium composition.

II. Cryopreserving using STEM-CELLBANKER:

Cells were harvested as per routine UT-iPS cells passaging. Cell clumps were centrifuged at 200g for 4 minutes at room temperature, the supernatant was removed and cells were gently suspended with 1 ml STEM-CELLBANKER cryopreservation medium (11890, AMSBIO LLC) with no other supplements and transferred to one cryovial. Cryovials were directly placed in a -80˚C for storage. For every well of a 6-well plate being frozen 1 mL of STEM-CELLBANKER was used.

2.1.1.6.2 Cryovial cryopreservation thawing

To thaw the UT-iPS cells, we removed the frozen UT-iPS Cryovial from the liquid nitrogen storage tank and quickly thawed the cells by swirling gently in a 37°C water bath. The vial was wiped with 70% Ethanol and the cells were washed with 10 ml of human ES cell medium. Cells were centrifuged at 200 x g for 4 minutes at room temperature and the cells pellet was resuspended in 2 ml of fresh human ES cells medium containing 10 µm ROCK inhibitor and plated in one well of a freshly prepared 6-well culture plate. The medium was changed the following day, and subsequently every 2 days. Small colonies initially appeared after one week. In our hands, unfortunately, only 1-5 colonies can often be recovered when used 10% DMSO to freeze the UT-iPS cells. However, we found that about 50% of the UT-iPS cells cryopreserved with STEM-CELLBANKER were recovered.

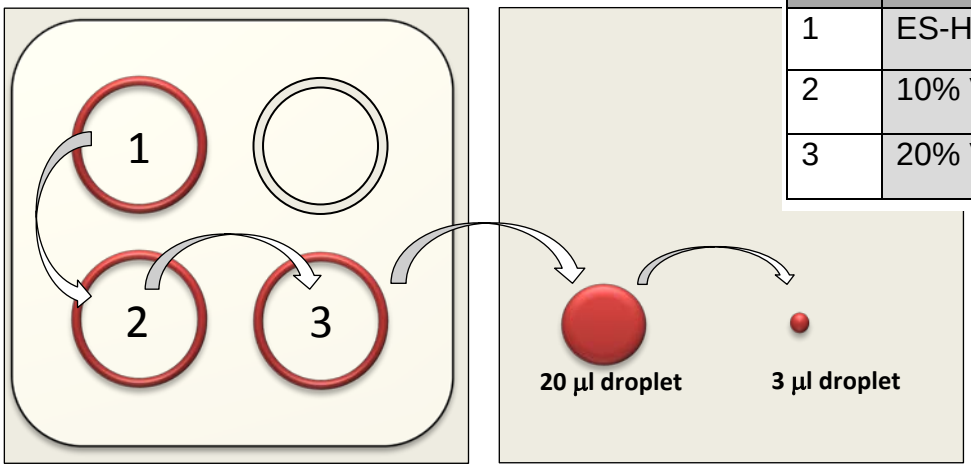
2.1.1.6.3 Fast freezing of UT-iPS cells

Protocol courtesy of Dr. Sun Yung and Professor Majlinda Lako (Institute of Genetic Medicine, Newcastle University, International Centre for Life.).

I. Open straw vitrification freezing:

This procedure allows high cooling and warming speeds which helps to avoid chilling injury and minimise toxic and osmotic harm to the cells. Collagenase/dispase treated UT-iPS colonies were collected as pieces that are larger than those used for passaging and placed into well number 1 (Figure 2-3) of a 4-well vitrification plate (TKT-190-130V, Thermo Scientific Nunc) containing 500 µl of ES-HEPES solution (Table 2-5) for 1 minute at 37°C. 8 to 10 colony pieces were transferred to well number 2 which contains 500 µl of 10% vitrification solution (Table 2-6) and incubated for 1 minute at 37°C. During this minute, a 20 µl drop of 20% vitrification solution (Table 2-8) was placed on the inside of the lid of the plate, one per straw to be frozen. The colony pieces were transferred to well number 3 containing 500 µl of 20% vitrification solution (Figure 2-3) and incubated for 25 seconds at 37°C. The colony pieces were transferred to the 20 µl drop of 20% vitrification solution. The colony fragments were aspirated in a 3 µl volume from the 20 µl drop and deposited as a

separate small, peaked droplet on the lid (Vajta *et al.*, 1998). The narrow end of the vitrification straw was immediately inserted into the side of the 3 µl drop at a 30° angle to the plane of the dish, and the pieces were sucked up into the straw by capillary action. The straw was plunged into liquid nitrogen at a 45° angle and stored in a labelled tube within a nitrogen storage canister.



Well	*Solution/Media*
1	ES-HEPES
2	10% Vitrification
3	20% Vitrification

Figure 2-3: Open straw vitrification plate – freezing.

ES-HEPES solution

Components	*Quantity*	*Supplier*
Knock Out-Dulbecco's Modified Eagle's Medium (KO-DMEM)	15.6ml	Invitrogen
Heat Treated Foetal Bovine Serum (FBS)	4ml	Invitrogen
HEPES (1M)	0.4ml	Sigma

Table 2-5: ES-HEPES solution composition. Stored at 4°C for up to one week.

10% Vitrification solution

Components	*Quantity*	*Supplier*
ES-HEPES Solution	2ml	-
Ethylene Glycol	0.25ml	Sigma
DMSO	0.25ml	Sigma

Table 2-6:10% Vitrification solution composition. Stored at 4°C. Unused solution discarded after each day.

1M sucrose stock

Components	*Quantity*	*Supplier*
Sucrose	3.42g	Sigma
ES-HEPES Solution	14ml	-
Heat Treated Foetal Bovine Serum (FBS)	2ml	Invitrogen

Table 2-7: 1M sucrose stock composition. Stored at 4°C. Unused solution discarded after each day.

20% Vitrification Solution

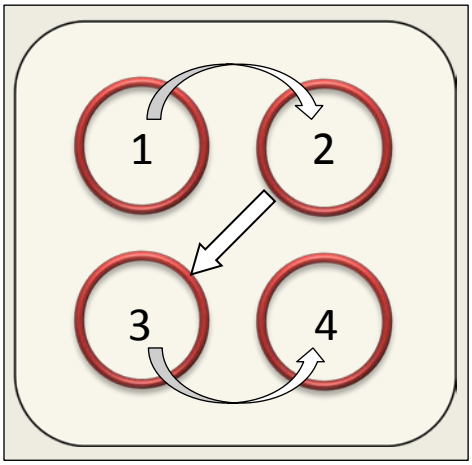
Components	*Quantity*	*Supplier*
ES-HEPES Solution	0.75ml	-
1M Sucrose Stock	0.75ml	-
Ethylene Glycol	0.5ml	Sigma
DMSO	0.5ml	Sigma

Table 2-8: 20% Vitrification solution composition. Stored at 4°C. Unused solution discarded after each day.

II. Open straw vitrification thawing

A 4 well vitrification thawing plate was prepared as follow:
500µl of 0.2M sucrose solution (Table 2-10) was pipetted into well number 1 (Figure 2-4), 500µl of 0.1M sucrose solution (Table 2-9) was pipetted into well number 2, and 500µl of ES-HEPES solution (Table 2-5) was pipetted into wells number 3 and 4. The tube that containing the vitrification straw in vitrification solution was collected from nitrogen storage in a receptacle containing liquid nitrogen. The straw was removed using forceps and immediately the narrow end was submerged into the first well containing 0.2M sucrose solution

(Figure 2-4) at 30° angle to the plane of the plate. As soon as the liquid column melts, a 10 µl pipette was placed at the top end of the straw and the liquid was gently pipetted out. After 1 minute at 37°C, the colony pieces were transferred to the next well containing 0.1 M sucrose solution and incubated for 5 minutes at 37°C. The colony pieces were transferred to the next well containing ES-HEPES medium and incubated for 5 minutes at 37°C, and then the pieces were moved to ES-HEPES solution in well number 4 and incubated for a final 5 minutes at 37°C. The fragments were harvested and placed into a single well of a 4-well plate which contained MEF cells in 500 µl of human ES cell medium.



Well	*Solution/Media*
1	0.2M Sucrose
2	0.1M Sucrose
3	ES-HEPES
4	ES-HEPES

Figure 2-4: Open straw vitrification plate - thawing.

0.1M sucrose solution

Components	*Quantity*	*Supplier*
ES-HEPES Solution	4.5ml	-
1M Sucrose Stock	0.5ml	-

Table 2-9: 0.1M sucrose solution composition. Stored at 4°C. Unused solution discarded after each day.

0.2M sucrose solution

Components	*Quantity*	*Supplier*
ES-HEPES Solution	4ml	-
1M Sucrose Stock	1ml	-

Table 2-10: 0.2M Sucrose solution composition. Stored at 4°C. Unused solution discarded after each day.

2.2 Optimizing the transduction efficiency

The optimal transduction efficiency was regarded as the MOI that would be sufficient to transduce the majority of the target cells with the least toxicity. Urinary tract stromal cells were seeded at 50,000 cells/well of a 6-well plate and transduced with MOIs of 2, 5, 10, 20, and 30 using mWasabi GFP empty lentiviral vector (Allele Biotech, USA) in the presence of polybrene 10µg/ml (TR-1003-G, Millipore, Massachusetts, USA) for 48 hours after which GFP expression was analysed and compared against a polybrene-only control (or MOI = 0). All FACS analysis was performed on FACSCalibur system using FL1-H channel for GFP detection and Cyflogic for analysis and interpretation of results.

2.3 Cell viability analysis by flow cytometry

Cells were exposed to polybrene at concentrations ranging from 0 to 20µg/ml for 48 hours. Cells were collected and resuspended in 500µl of MACS buffer. Immediately before injection into the flow cytometry, 20µl of 1mg/ml propidium iodide (PI, Sigma) was added to each sample. Samples with low PI staining were considered viable, while PI-high cells were considered unviable.

2.4 Alkaline phosphatase staining

Alkaline phosphatase (ALP) activity was analysed in the UT-iPS cells after five days in culture and at low to medium density. The cells were fixed with 4% Paraformaldehyde in PBS for 1-2 minutes maximum as fixing for longer period will result in the inactivation of alkaline phosphatase. Fixed colonies were washed with 1 X wash Buffer (20mM Tris-HCL, pH 7.4, 0.15 NaCl, 0.05% Tween-20). The colonies were then treated with Fast Red Violet, AS-BI phosphate solution and distilled water in a 2:1:1 ratio and incubated in the dark at room temperature for 15 minutes after which the staining solution was removed and the colonies were washed again with 1 X wash buffer. The cells were covered with 1 X PBS to prevent drying and observed under observed under light microscope for AP expression.

2.5 Live immunofluorescence staining

Live cell imaging of emerging colonies was performed by staining 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 (SCR001, Millipore). Cells were incubated with 1ml/well of human ES cell medium containing these antibodies. After 2 hours of incubation at 37ºC, Hoechst dye was added to wells at a final concentration of 0.5µg/ml. After 10 minutes of incubation at 37ºC, cells were washed twice with 1ml/well of human ES cell medium and 500µl of medium were added before viewing under fluorescent Nikon TE2000-4 inverted microscope using NIS elements – BR 3.0 software.

2.6 Immunofluorescence

Cells were fixed in 4% Paraformaldehyde/PBS for 15 minutes at room temperature. After washing with PBS, cells were permeabilized with 0.1% Triton X-100/PBS for 10 minutes at room temperature and blocked in PBS with 4% goat serum for 30 minutes followed by incubation with primary antibody overnight at 4ºC, and with secondary antibodies for 1 hour at room temperature. Primary antibodies included anti-OCT4 (1:100, Millipore); anti-NANOG (1:100, Cell Signaling Technology); anti-SSEA-4, anti-TRA-1-60 and anti-TRA-1-81 (1:100; Millipore); anti-CD31 (1:100, BD Pharmingen), anti-βIII-Tubulin (1:100; Covance), anti-αFP (1:100, Sigma), and anti-UPIb (1:100, Santa Cruz). Secondary antibodies used were Alexa546-conjugated goat anti-mouse, Alexa488-conjugated goat anti-mouse, and Alexa568-conjugated rabbit anti-goat (all at 1:400, Invitrogen). The nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) and cells were mounted (Vectashield; Vector Laboratories Inc, Burlingame, CA, USA). Images were obtained using a confocal laser scanning microscopy system (Nikon Corp, Tokyo, Japan).

2.7 Karyotyping of human UT-iPS cells

UT-iPS cells were karyotyped at passage 25. Karyotyping was performed based on the following protocol:

1) collect cells and arrest at metaphase:

Once UT-iPS colonies reached 85% confluence, they were treated with the mitotic spindle poison colcemid (kindly donated by Mr. Arman Esfandiari and Prof. John Lunec) for 2 hours at 37˚C. Following colcemid treatment, the medium was collected and stored in 37ºC. UT-iPS colonies were harvested as per routine UT-iPS cells passaging, re-suspended in the previously collected medium and allowed to stand for another 10 minutes which further ensured the separation of any remaining MEF cells from the UT-iPS colonies. The iPS colonies were then washed in 1XPBS and broken down to single cell suspensions by treating with 1% trypsin for 5 minutes at 37˚C after which the trypsin was inactivated with 10% FBS-PBS. These were then centrifuged at 1500 rpm for 5 minutes and the supernatant was carefully removed by aspiration leaving approx. 200µl of supernatant to re-suspend the mitotic cells in by gently flicking the side of the tube.

2) Addition of hypotonic solution

To swell the cells and separate the chromosomes, 1ml of ice-cold hypotonic solution (1:1 0.4% KCl + 0.4% sodium citrate) was added to the side of the tube to make up the final volume to 2ml. Cells were incubated at 37˚C for 7 minutes following which the cells were centrifuged at 1500 rpm for 6 minutes and re-suspended in the remaining 200µl supernatant.

3) Fix the cells

Drops of fixative (3:1 methanol and acetic acid) were added slowly to the suspension to make up a final volume to 2 mL while gently tapping the tube. After 30 minutes of incubation at room temperature, cells were again centrifuged, the supernatant was removed and the pellet was resuspended in the remaining fluid. The previous step was repeated but with 20 minutes incubation at room temperature. At this point, the fixed cells were either dropped onto slides or stored at -20˚C.

4) Prepare the slides

The glass slides were rinsed with ice cold water and were then rinsed with the fixative. The cells fixed earlier were washed once in the fixative. Using a plastic transpipet, 2-3 drops of the cell suspension were dropped at 45˚ angle onto the slides. The slides were left to dry at room temperature for at least 24 hours.

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

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 deionized water

The slides were placed in freshly prepared trypsin solution (a) for 15 seconds. At end of this time period, slides were immediately immersed in the saline solution (b). These were then rinsed twice in the third saline pot (c) and immersed in the Staining solution (d) for 4-5 minutes. The stained slides were then rinsed in deionized water (e), mounted with coverslip using DPX and analysed using the Kario software or Cytovision®. Karyogram analysis was performed by Dr.Sarah Fordham).

2.8 DNA fingerprinting:

DNA fingerprinting was carried out to confirm the genetic source of the generated UT-iPS cell to their parent stromal cells and to rule out any possibility of contamination with skin iPS cell line that was cultured concurrently in our lab. Cells were harvested as per routine passaging. The cells pellet of UT-iPS cells, parental urinary tract stromal cells and skin-iPS cells were sent off to Northern Molecular Genetics Service, (Newcastle Upon Tyne, UK) where the total genomic DNA was extracted and amplified through the Promega PowerPlex® 16 system for the detection of 16 different human microsatellite markers. This system is designed specifically for co-amplification and three-color detection of sixteen human loci (fifteen STR loci and Amelogenin): Penta E, D18S51, D21S11, TH01, D3S1358, FGA, TPOX, D8S1179, vWA, Amelogenin, Penta D, CSF1PO, D16S539, D7S820, D13S317 and D5S818. One primer for each of the Penta E, D18S51, D21S11, TH01 and D3S1358 loci is labeled with fluorescein (FL); one primer for each of the FGA, TPOX, D8S1179, vWA and Amelogenin loci is labeled with carboxy-tetramethylrhodamine (TMR); and one primer for each of the Penta D, CSF1PO, D16S539, D7S820, D13S317 and

D5S818 loci is labeled with 6-carboxy-4´,5´-dichloro-2´,7´-dimethoxy-fluorescein (JOE). The results were analysed on an ABI 377 sequence detector using Genotype software (Applied Biosystems, Foster City, CA).

2.9 Embryoid body (EB) formation from UT-iPS cells

Using a sterile pipette tip or needle, UT-iPS cells were collected using the same protocol for passaging. Gently, the colony pieces were placed into low-adhesion culture plate (Corning, UK) containing standard EB differentiation media (Table 2-11) and incubated at 37°C with 5% CO_2 with medium changed every 3 days. To change the medium, the EBs were transferred to a 15ml conical tube and allowed to settle to the bottom of the tube for 10 minutes. The supernatant was removed leaving about 20% and replaced with fresh EB media into a new low-adhesion culture plate.

EB differentiation medium

Components	***Quantity***	***Supplier***
Knockout-Dulbecco's Modified Eagle's Medium	120ml – 80%	Invitrogen
Heat Treated Foetal Bovine Serum (FBS)	30ml – 20%	Invitrogen
MEM Non-Essential Amino Acids (100X), liquid	1.5ml – 1%	Invitrogen
Penicillin-Streptomycin Solution	1.5ml – 1%	Sigma
GlutaMAX™-I Supplement, 200 mM	1.5ml– 2mM	Invitrogen

Table 2-11: EB basic differentiation medium composition

2.10 Teratoma formation assay

Teratoma formation assay was performed using immunodeficient NSG mice as recipient. 24 hours before injection.

1. Preparing UT-iPS cell for injection

Cells were harvested according to the normal protocol for UT-iPS cells passaging and placed in a 15-ml conical tube which was placed upright for 4-5 minutes to separate the disrupted colonies from MEF cells through gravity rather than centrifuging the cells. Then the supernatant was carefully removed and the UT-iPS cells were broken down to single cells by trypsinization. Cells were then collected, counted, and resuspended in sufficient human ES cell

medium at a concentration of 5 million cells per ml. Each injection was prepared by mixing 100µl of cells for each cell line with 100µl of Matrigel.

2. Injecting cells

Injection procedure was performed by Dr. Lyle Armstrong. Cells were injected subcutaneously into the right flanks of each mouse. One cell line was used per mice and in total each cell line was replicated through three mice. Ear notching was used to identify each mouse.

3. Harvesting and fixation of teratomas

Mice were euthanized by Mrs. Shirley Dodd by cervical dislocation after 6–12 weeks. Xenografts were extracted and fixed for 24 hours in 20X volume of Bouin's fluid. Bouin's fixation provides good penetration and morphological/structural preservation of tissues. The following day, tissues were washed three times in water and placed in 30-40ml of 70% ethanol for 2 hours. The procedure was repeated with 80%, 90% and 95% ethanol. Tissues were stored in 95% ethanol until ready for processing.

2.11 Induce differentiation of bladder specific cells from human UT-iPS cells *in vitro*.

To induce differentiation, both UT-iPS cells and skin-iPS cells were treated with conditioned media (CM) for 14 days. Two main types of CM were used in this study: urothelial cells derived CM (U-CM) collected from cultured human urothelial cells and stroma cells derived CM (S-CM) collected form human urinary tract stromal cells. CM was collected After 24 hours, centrifugation was performed at 1500 x rpm for 5 minutes to remove cells, filtrated with a 0.2-µm syringe membrane filter and diluted with one-third the volume of DMEM with serum at a final concentration of 2% (U-CM), or with an equal volume of RPMI 10% FCS (S-CM).

2.12 Lentiviral transduction

Stromal cells were seeded in a 6-well plate at 5 x 10^4 cells per well one day before transduction. Cells were transduced using a single polycistronic lentiviral vector (ABP-SC-LVI4in1, Allele Biotechnology, San Diego, USA) at MOI=10 in the presence of 10µg/ml polybrene. On day 2, the transduction medium was replaced with standard stromal medium. On day 6, cells were harvested and

plated onto previously prepared MEF 6-well plate in human ES cell medium and maintained till day 10. From this point, the transduced cells were cultured in MEF-CM supplemented with human iPS culture medium collected from cultivated skin-iPS cells. ES cell-like colonies were manually picked based on morphology and transferred to a new plate on MEF cells.

2.13 Lentivirus production

PLenti- EF1α-citrine lentivirus was constructed by Dr.Fiona Frame (YCR Cancer Research Unit, Department of Biology, University of York). The lentiviral particles stock was generated by cotransfecting the pLenti- EF1a-citrine construct and the optimized packaging plasmid mix (GIBCO, Life Technologies) into the 293T cell line. One day prior to transfection (Day 1), 5 x 10^6 293T cells were plated onto a 10cm tissue culture plate and incubated overnight in 10ml of full RPMI1460 without antibiotic. The next day (Day 2), the culture medium was replaced with 5 ml of OPTI-MEM I medium. For each transfection sample, DNA-Lipofectamine™ 2000 complexes was prepared as follows: 9 µg of the ViraPower lentivirus packaging mix and 3 µg of pLenti expression plasmid DNA were added to 1.5 ml of OPTI-MEM I medium without serum and mixed gently in 5 ml tube. In a separate sterile tube, 36 µl of Lipofectamine 2000 with 1.5ml of OPTI-MEM I medium without serum was added, mixed gently by finger tapping and incubated for 5 minutes at room temperature. After the incubation, the diluted DNA was mixed gently with the Lipofectamine containing solution, and incubated for 20 minutes at room temperature to allow the DNA Lipofectamine 2000 complexes to form. The DNA-Lipofectamine 2000 complexes were added dropwise to each plate of cells and swirled gently. After 24 hours of incubation at 37°C in a humidified 5% CO_2 incubator (day3), the transfection mixture was replaced with 10 ml of full RPMI1460 without antibiotic and incubated for another 24 hours at 37°C in a humidified 5% CO_2 incubator. Virus-containing medium was collected 48 hours post-transfection (Day 4) and filtered using a 0.45 µm pore-size polyethersulfone syringe filter to remove remaining cells. To yield a higher titration, the viral supernatant was concentrated 10-fold by centrifugation at 120,000g for 2 hours at 4°C in an Optima XL-100K ultracentrifuge (Beckman Coulter). The supernatant was

discarded and the viral pellet was resuspended in DMEM-F12 medium and stored as aliquots at -80°C.

2.14 Transduction and establishment of transgenic UT-iPS cell lines

3.1.12 Lentiviral transduction of UT-iPS cells

Undifferentiated UT-iPS cells were detached from the Matrigel by incubation with dispase for 5 -7 minutes at 37°C. The detached aggregates were then plated into a 6-well matrigel-coated culture plate with an overall confluency of <40%. 24 hours after plating, the medium was replaced with the virus supernatant diluted in mTeSR1 medium in the presence of 6 µg/ml polybrene. The following day, the virus suspension was replaced with fresh mTeSR1 medium. 5 days post transduction, blasticidin was added at final concentration of 1 µg/ml. Selection with blasticidin lasted 12 days with medium and blasticidin changes every 2 days.

3.1.13 FACS analysis and cell sorting of transduced UT-iPS cells

FACS analysis and sorting of β-actin-mOrange and EF1a- mWasabi/Citrine expressing cells was performed on a BD FACSAria III system, according to their fluorescent emission. Undifferentiated UT-iPS cells were used to set the background level of fluorescence. UT-iPS cultures were pre-treated with 10µM Y-27632 ROCK inhibitor for one hour prior to analysis and sorting by FACS and subsequently sorted as follow: UT-iPS clumps were dissociated to single-cell suspensions by 7-10 minutes of incubation with Accumax solution (SCR006, Chemicon) at 37°C. Cells were subsequently resuspended in mTeSR1 medium containing 5mM EDTA at a concentration of $5x10^6$ cells/ml. Cells were sorted at approximately 20 PSI using a 100-micron nozzle at an average acquisition rate of 3,000 cells per second into mTeSR1 medium. Following centrifugation at 200xg for 4 minutes, the sorted cells were resuspended in mTeSR1 medium containing 10µM of Y27632 ROCK and plated onto 6-well Matrigel coated plate at a concentration of ~ 1-2 x 10^6 cells/well. 1% Penicillin/Streptomycin was added to the medium for one week after sorting to avoid bacterial contamination.

2.15 RNA extraction and analysis

3.1.14 RNA isolation

All products used for RNA extraction were from certified RNAse free sources. During the procedure, all samples, reagents, and plasticware were handled with gloved hands to avoid any contamination from RNases found on human skin. The choice of RNA extraction procedure and the kit to use were determined depending on the cell number. RNA was extracted from cells ≥ $5x10^5$ using EZ-RNA (Biological Industries, Beit haemek, Israel). RNA from cells ≤ $5x10^5$ was extracted using RNeasy® Micro-kit (Qiagen, West Sussex, UK). Steps were performed in a fume hood and appropriate face protection was used when required.

2.15.1.1 RNA isolation with EZ-RNA kit

Cells were lysed in 0.5ml of denaturing solution, vortexed until complete lysis and incubated for 5 minutes at room temperature. 0.5ml of extraction solution was added to the homogenate, shaken vigorously for 15 seconds, and incubated for 10 minutes at room temperature. Sample was centrifuged at 12,000 x g for 15 minutes at 4°C. The upper aqueous phase was carefully transferred to a fresh RNase-free microcentrifuge tube without disturbing the white precipitate layer, which contains DNA and protein and 0.5ml of analytical grade isopropanol (Sigma) was added to precipitate the RNA. To increase yield, the sample was stored at this point at -20°C overnight. After incubation, samples were centrifuged at 12,000 x g for 8 minutes at 4°C and the isopropanol was carefully removed leaving a small amount behind in order to avoid disturbing the pellet. The pellet was washed with 1ml of 75% ethanol, and centrifuged at 7,500 x g for 5 minutes. The ethanol was removed completely and the pellet air dried for 5-7 minutes at room temperature to remove any residual ethanol. RNA was dissolved in DEPC-treated water by incubating at 55ºC for 15 minutes. RNA was either used immediately or stored at −80°C to minimise degradation and maintain RNA integrity.

2.15.1.2 RNA isolation with Qiagen Micro RNeasy extraction kit

The Kit included all the required buffers apart from the ethanol. The protocol followed was as described in the manufacturer's handbook (RNeasy® Micro Handbook, Qiagen). Cell pellet was lysed in 350µl of lysis buffer (guanidine-thiocyanate containing RLT buffer supplemented with 1% of β-mercaptoethanol), briefly vortexed until complete lysis. Following this, 350µl of RNase-free 70% ethanol was added to the lysed cell, mixed well, and directly pipetted to an RNeasy MinElute spin column placed in a 2 ml collection tube and centrifuged at 10,000 x rpm for 15 seconds. Ethanol promotes selective binding of RNA to the spin columns silica membrane. The flow through was discarded and the samples were washed with 350µl of RW1 wash buffer. The spin columns were centrifuged and the flow through was discarded. Samples were then treated with DNase I solution (70µl of RDD buffer and 10µl of DNase I stock) for 15 minutes at room temperature to degrade DNA contaminants and ensure purity of resultant RNA. The sample was washed again by adding 350µl of buffer RW1 onto the RNeasy MinElute Spin Column and centrifuged at 10,000 x rpm for 15 seconds. The column was transferred to a new 2 ml collection tube and another wash with 500µl buffer RPE was performed. Then, 500µl of RNase-free 80% ethanol was pipetted to the RNeasy column before spinning the tube for 2 minutes at 10,000 x rpm. Following the centrifugation, the RNeasy MinElute Spin Column was carefully removed from the collection tube and placed into a new 2 ml collection tube. The membrane of the column was dried by centrifuging the column with the lid opened at full speed for 5 min. RNA was eluted by pipetting 14µl of RNase-free water directly onto the centre of the silica-gel membrane and spinning for 1 minute at full speed. RNA was either used immediately or stored at −80°C to minimise degradation and maintain RNA integrity.

3.1.15 Quantification of RNA

RNA concentrations of samples were quantified using NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, Delaware, USA). The data was logged in and recorded through the supplied NanoDrop software which controls the machine and runs from a desktop computer. To clean the lower and upper surface of the sample retention platform of the Spectrophotometer, 1µl of DEPC treated water was pipetted onto both surfaces, the lever arm closed and then the surfaces wiped with a clean fresh laboratory wipe. The machine was set up and calibrated according to the manufacturer's instructions. DEPC treated water was used as a blank reference. The optical surfaces were firstly cleaned with a fresh clean laboratory wipe. 1.2µl of RNA was pipetted onto the end of the optical surface and the lever arm was closed to bring the second surface into contact with the liquid sample making the sample link the optical ends together before taking the measurement with the NanoDrop software. Before applying the next sample the optical surfaces were cleaned again. The ratio of sample absorbance at 230nm, 260nm and 280nm was used to measure the purity of RNA. The resultant RNA should be free of DNA, proteins, or other contaminants for which a ratio of ~2.0 for the 260/280 ratio and 1.8-2.2 for the 260/230 ratio should be expected. Resultant RNA was either used immediately or stored at −80°C to minimise degradation and maintain RNA integrity

2.16 Reverse Transcriptase and cDNA synthesis

To prepare the isolated RNA for the production of cDNA, 1 - 2 µg of total RNA from each sample was diluted in DEPC treated water to a final volume of 12.7µl and incubated for five minutes at 65°C to remove the secondary structure. Reaction mixture containing: 4µl 5X Moloney Murine Leukaemia Virus Reverse Transcriptase (M-MLV RT) buffer (Promega), 2µl 4mM dNTPs (Promega), 1µl of 50µM Oligo dT16 primers (Applied Biosystems) and 0.3µl M-MLV RT enzyme (Promega) was added to RNA and incubated for 1 hour at 37°C. Reverse transcription was terminated by incubating at 95°C for 5 min. Resultant cDNA was either used immediately or stored at −20°C.

2.17 Real-time PCR

Real-time polymerase chain reaction (PCR) was performed using an ABI PRISM® 7900 HT sequence detection system according to the manufacturer's instructions (Applied Biosystems, UK). 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. Reactions were performed in an optical 384-well plate containing 9µl of reaction mixture (Table 2-12) + 1µl of cDNA in each well. The reaction plate was sealed with a transparent adhesive cover and centrifuged briefly at 1000 x rcf for 1 minute to spin down the contents and eliminate any air bubbles from the solutions. The plate was placed in the 7900HT real time PCR system where it went through the following PCR temperature cycles, 50°C for 2 minutes, 95ºC for 10 minutes, and 45 cycles of 95°C for 15 seconds and 60°C for 1 minute. The reaction was finished with dissociation step which consisted of 15 seconds at 95ºC, 15 seconds at 60ºC and 15 seconds at 95ºC. A single peak in the resultant dissociation plot represented a single specific product. Each sample was performed in triplicate, and negative control reactions without cDNA were included in each experiment. Glyceraldehyde-3-phosphate dehydrogenise (GAPDH) was used as a housekeeping gene to normalise expression. Serial dilutions of cDNA that were known to express transcripts of interest were used to quantify the relative amounts of cDNA produced in the PCR reactions. Data were analysed using ABI 7900 HT SDS 2.3 software (Applied Biosystems). Absolute quantities were measured for each gene, then subsequently normalised to GAPDH to account for differences in loading the samples.

Real-time PCR reaction mixture

Components	***Quantity***	***Supplier***
SYBR green	5µl	Invitrogen
Forward primers	0.4µl	Sigma
Reverse primers	0.4µl	Sigma
DEPC treated water	3.2µl	-

Table 2-12: Real-time PCR reaction mixture.

The following primers (Table 2-13) were used for evaluating genes expression:

Genes	**Forward primer (5′−3′)**	**Reverse primer (5′−3′)**
GAPDH	CGACCACTTTGTCAAGCTCA	GGGTCTTACTCCTTGGAGGC
CD24	TGAAGAACATGTGAGAGGTTTG	GAAAACTGAATCTCCATTCCAC
vWF	ACTGAAGCGTGATGAGACGC	TTCATCAAAGGGTGGGCAGC
CD45	GAAATTGTTCCTCGTCTGAT	CTTTGCCCTGTCACAAATAC
α-SMA	CCGACCGAATGCAGAAGGA	ACAGAGTATTTGCGTCCGAA
CD90	CACACARACCGCTCCCGAACC	GCTGATGCCCTCACACTT
ENDO-OCT4	GCAAGCCCTCATTTCACCAGGCC	AGGATCAACCCAGCCCGGCT
ENDO-SOX2	TCACATGTCCCAGCACTACC	CCCATTTCCCTCGTTTTTCT
NANOG	CCAAATTCTCCTGCCAGTGAC	CACGTGGTTTCCAAACAAGAAA
GDF3	CTTATGCTACGTAAAGGAGCTGGG	GTGCCAACCCAGGTCCCGGAAGTT
REX1	CGTACGCAAATTAAAGTCCAGA	CAGCATCCTAAACAGCTCGCAGAAT
DNMT3B	TGCTGCTCACAGGGCCC	TCCTTTCGAGCTCAGTGCACC

	GATACTTC	ACAAAAC
AR	CTGGACACGACAACAACCAG	CAGATCAGGGGCGAAGTAGA
PSA	CAATGACGTGTGTGCGCAA	CGTGATACCTTGAAGCACACCA
UPIb	GGGACAGACAAGGTGCCTGTTAT	TATTGGCTGGCTTGCTTCTCTCCA
UPII	CAGTGCCTCACCTTCCAACA	TGGTAAAATGGGAGGAAAGTCAA
UPIIIa	TCACTGGCACCCACGAGGTCT	CGTTGAGCCCAGTGGGGTGTT
UPIIIb	CCCTGGCCCTGGACCCTATCG	CCACAGGCTGGAGAAGCGCA
Calponin	TTTGAGGCCAACGACCTGTT	CCTTTCGTCTTCGCCATGCT
Desmin	CCATCGCGGCTAAGAACATT	TCGGAAGTTGAGGGCAGAGTA
Claudin1	ATGGAAAGGGTGTTGGCATTGGTG	AATGCCTTGCTCAAACACAGACGG
Claudin5	CTGTTTCCATAGGCAGAGCG	AAGCAGATTCTTAGCCTTCC
CK7	TGTGGTGCTGAAGAAGGATGTGGA	TGTCAACTCCGTCTCATTGAGGGT
Transgene	TGCTGCCAAGAGGGTCAAG	AGCCATACGGGAAGCAATAG

Table 2-13: List of primer sets and sequences.

2.18 Statistical analyses

All experiments were independently repeated at least 3 times and the average values were considered. All graphs plotted show the respective standard error of the mean. A paired t-test analysis was used to evaluate the statistical significance between two independent variables.

* $P \leq 0.05$, **$P \leq 0.01$, *** $P \leq 0.001$.

Results

3 Chapter 3. Establishing and characterising cell cultures of primary human urothelial and stromal cells

3.1 Introduction

Histological examination confirmed the absence of urothelial dysplasia or malignancy. Both urothelial and stromal single cell suspensions were prepared from the primary tissue samples. Primary culture protocols were optimised to obtain healthy and homogeneous primary cultures of both urothelial and stromal cells, which is critical for effective transduction and successful reprogramming. Isolated urothelial cells were purified via CD326 (EpCAM) Magnetic-activated cell sorting (MACS) sort while homogeneous stromal cells were obtained through multiple passages. Cells were characterized according to morphology, growth characteristics, and mRNA expression of a panel of cell lineage markers.

3.2 Aims

1. To establish viable primary cultures of human urothelial and stromal cells.
2. To produce highly pure cultures of primary urothelial and stromal cells.
3. To evaluate the mRNA expression levels of pluripotency markers in pure primary cultures.

3.3 Results

3.3.1 Isolation and culture of human urothelial cells

Primary cultures were established for both urothelial and stromal cells using either the outgrowths from explanted tissues, ‘explant culture method’, (Fischer et al., 1980; Reznikoff et al., 1983; Jing et al., 2011) or by dissociating the tissue with EDTA, trypsin and collagenase following protocol described by Southgate, ‘the enzymatic digestion method’ (Southgate J, 2002; Southgate et al., 2007).

3.3.1.1 Isolation and culture of human urothelial cells using explant culture method

The primary explant method was tested on 15 patient samples. Cultures were initiated on a 90mm diameter culture dish in KSFMc medium. 11 out of 15 samples exhibited outgrowth, giving an overall success rate of about 73% for growing and culturing primary urothelial cells using this method. Passage 1 was reached by most of the samples. However, urothelial cells showed low propensity to survive when subcultured, with only 26% of cultures reaching passage 2, after which all cultures eventually died (Figure 3-1). This might be due to the toxic effects of trypsin which was used to detach the cells from the plate. About 65-75% of the explants attached to the growth surface of dishes within 24 to 36 hours after plating and eventually gave rise to urothelial cells. Frequent observation of the culture was avoided particularly on the first day as this may cause explant detachment from the surface of the culture dish.

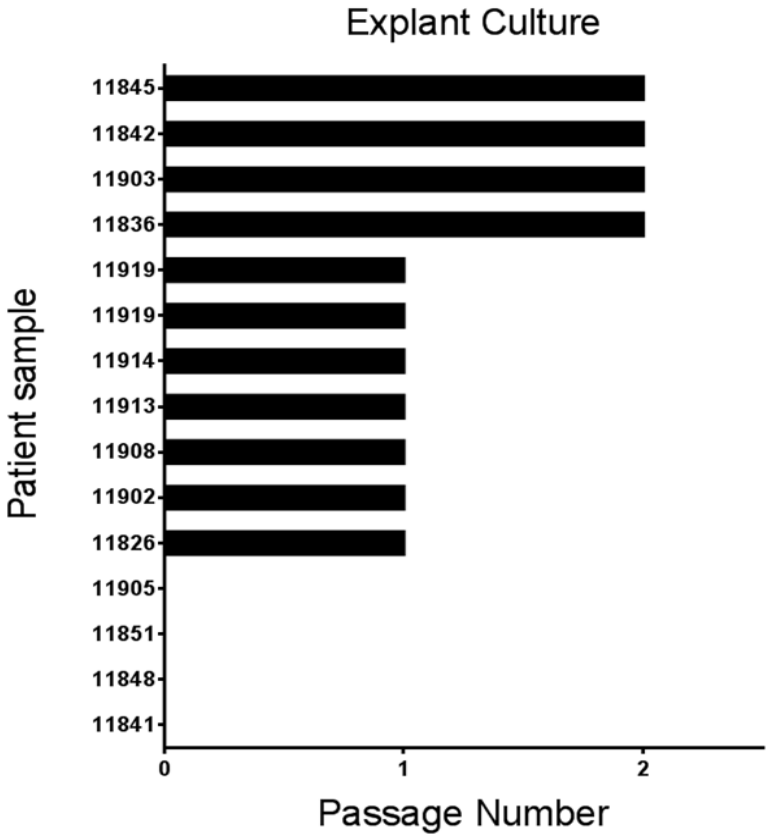


Figure 3-1: A summary of primary urothelial explant cultures. About 73% of samples grew successfully in culture. Only 4 of 15 samples could be maintained to passage 2. Y-axis represents the patient samples ID. X-axis represents the passage number.

3.3.1.1.1 Morphology of epithelial cells

Following 48h, polygonal cells began to migrate from the explants and increased gradually in number over the following days (Figure 3-2). Cells at the leading edge of growth often exhibited rounded surface with ruffles, while in confluent regions, cells exhibited more cuboidal appearance. Fresh samples (collected and processed within 24-48 hours) grew more readily than old ones (collected and processed after 48 hours). Although the conditioned culture medium was used to inhibit the growth of stromal cells and to stimulate the growth of urothelial cells, few stromal cells were seen in some cases. All cultures identified with fibroblastoid growing cells were discarded. Generally, growing cells migrated from the tissue and formed a crown of confluent cells around it. At higher magnification, polygonal cells grew with a cobble-stone pattern. These cells were uniform in size and shape and exhibited epithelial morphology. The morphology and the growth characteristics of the derived urothelial cells using ureter or bladder as tissue source were comparable (Figure 3-3).

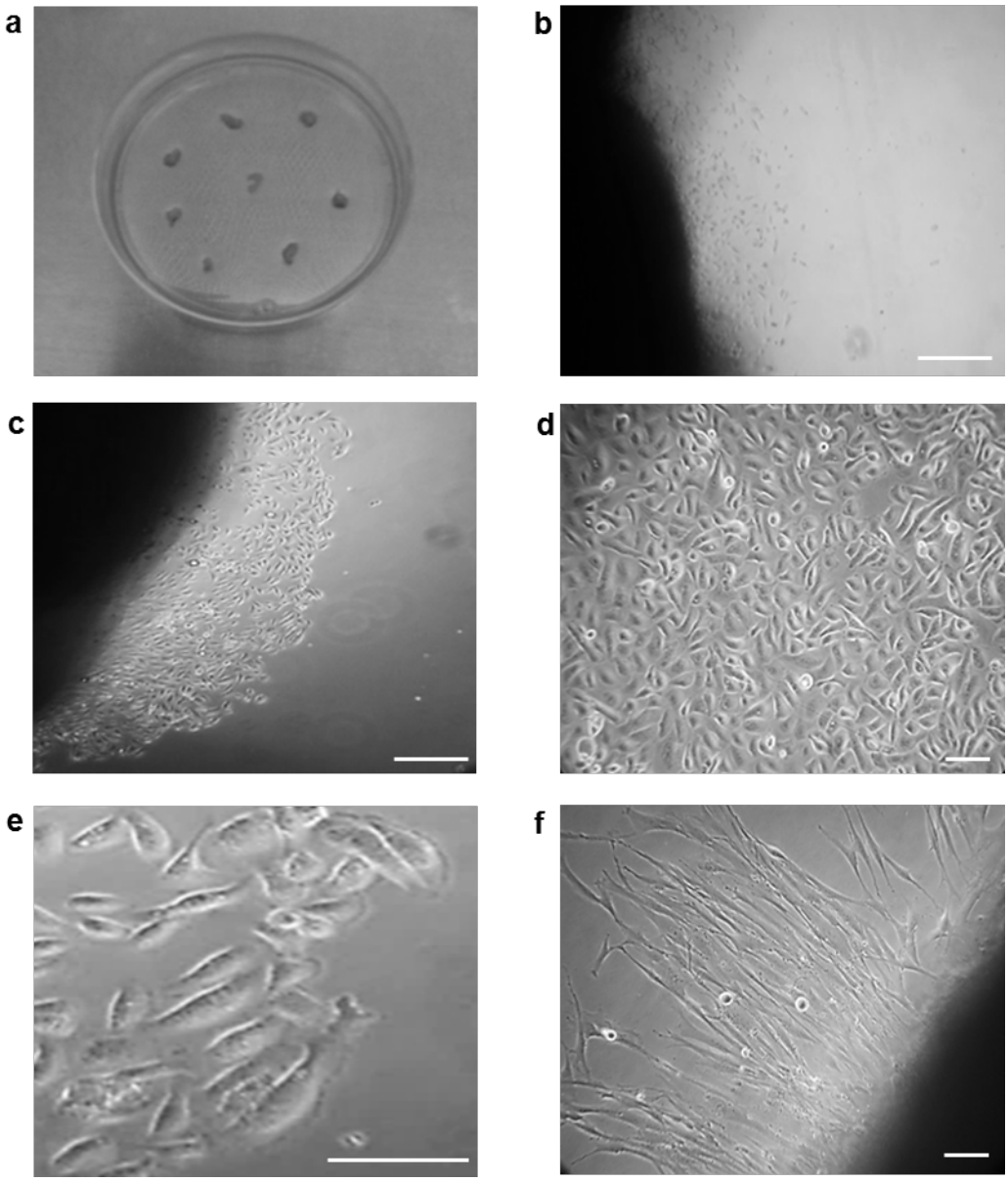


Figure 3-2: Procurement of human urothelial cells. (a) The explants were plated onto 90mm culture dish and maintained in KSFMc medium. (b, c) Phase contrast photomicrograph showing normal urothelial cells migrated away from the ureteral explant at days 2 and 5, respectively. (d) Cells adhere closely together and yield a cobblestone epithelial morphology in a more confluent layer. (e) Membranes of cells at the outer regions of the growth are often ruffled. (f) Example of non-urothelial cells contamination. Scale bars: 50 µm (b, c); 10 µm (d, e, f).

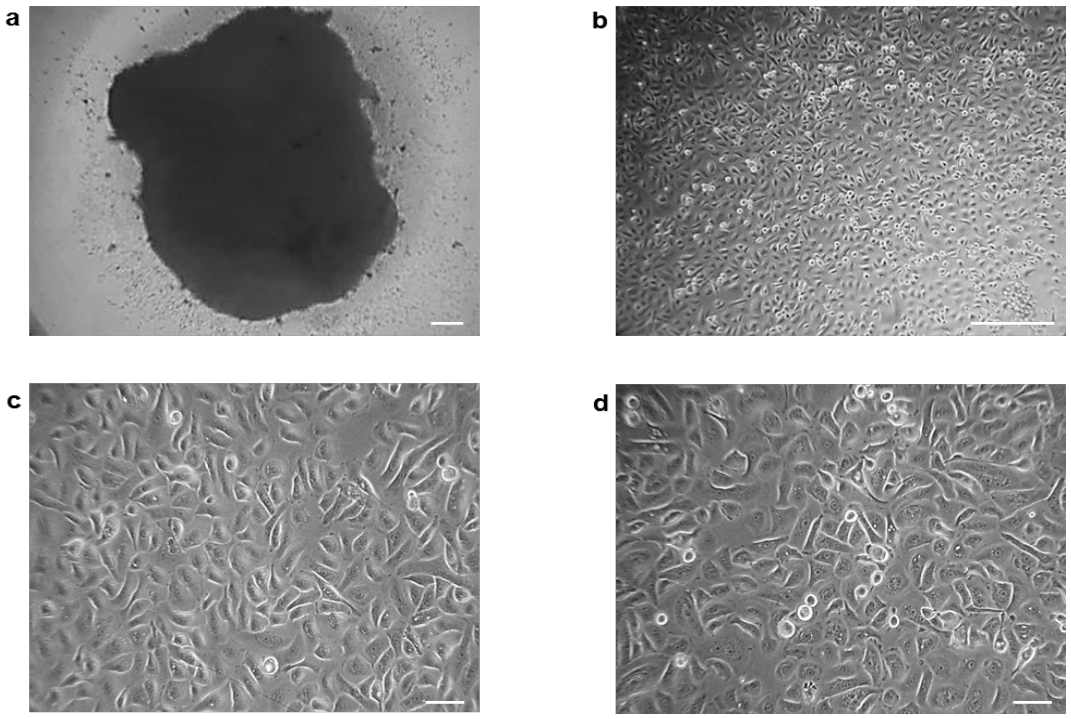


Figure 3-3: Explant culture of human urothelial cells. (a) Explant of normal ureter growing on 90mm culture dish at day 10. Outgrowths continuously expanded from the tissue resembling a ring around of confluent cells around the explants. (b) Higher magnification shows the cobble-stone morphology. (c) Urothelial cells derived from ureter tissue. (d) Urothelial cells derived from bladder tissue. Scale bars: 100 µm (a, b); 10 µm (c, d).

As an alternative to repeated subculture, the potential of re-plating the explant tissue was investigated (Figure 3-4). In two experiments, three explants were re-plated three times and each time a new confluent culture of cells was reinitiated that continued to express epithelial morphology. The survival of these cells may be related to the gradual release in culture of growth factors known to be present in the extracellular matrix, or produced by mature cells of the urothelium. However, this technique of initiating new cultures by re-plating explants was not quantitative, whilst the purity of these cells and the effect of serial re-plating were not investigated. The number of viable cells isolated per sample was higher when more than one tissue piece was plated in the same well (6- 8 tissue pieces per well). Culturing one tissue piece per well had a suppressive effect on the growth efficiency and most of the explants failed to produce outgrowing cells (Figure 3-5) suggesting that the interactions between these tissues may play a role in stimulating the growth of urothelial cells, or the outgrowth from one tissue may enhance the growth from other tissues.

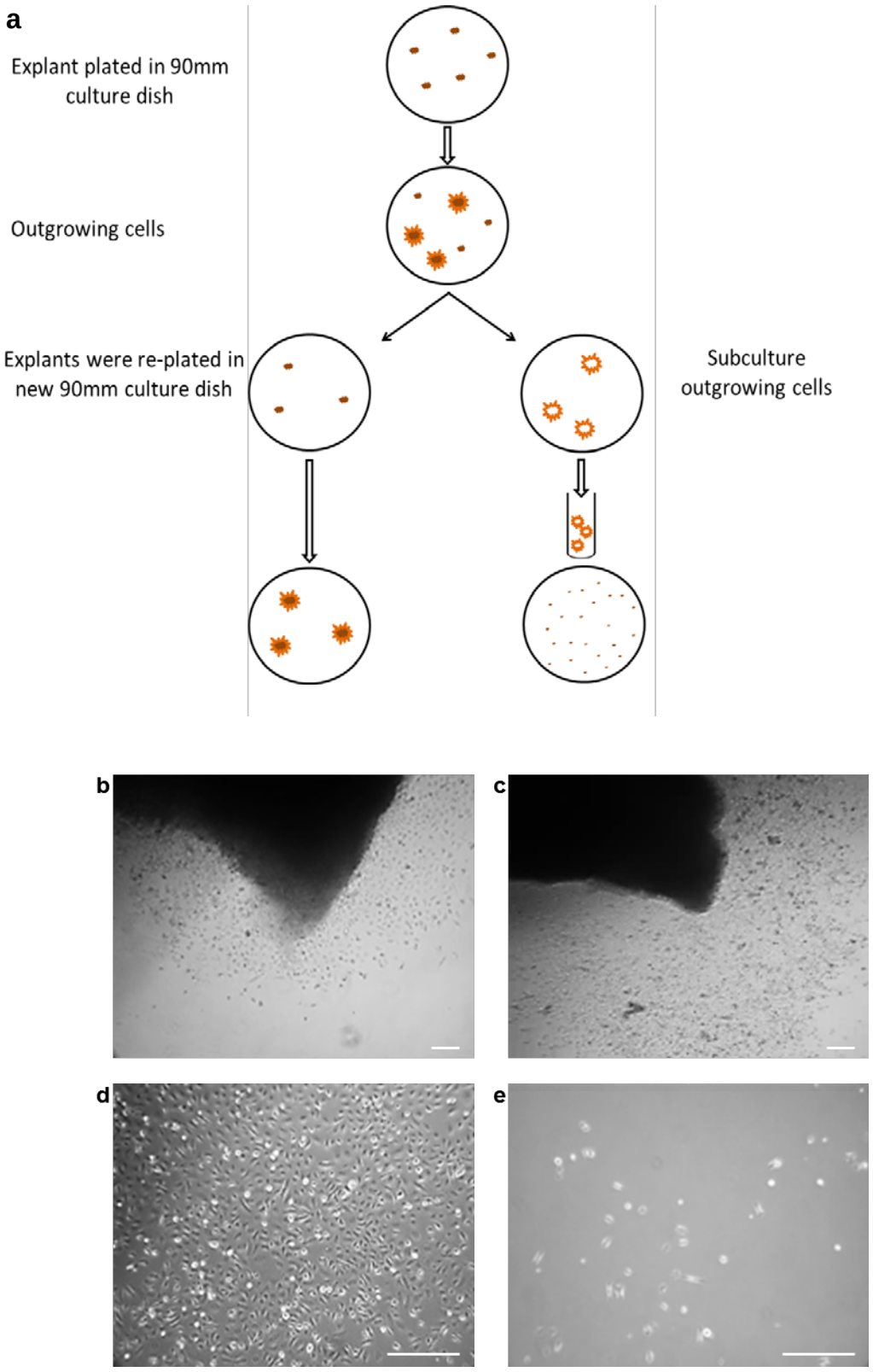


Figure 3-4: (a) Schematic diagram outlining the technique used to re-plate tissue explants. Repeated urothelium outgrowth from tissue explants, (b) Day 5. (c) Day 10. Continuous cell outgrowth was observed after replating the explants onto 90mm diameter culture dish under the same conditions. (d) Cell outgrowth from a recycled explant has a similar growth pattern to the initial culture. (e) Outgrowing cells subcultured from explants (1st passage) day7. Scale bars: 100 µm.

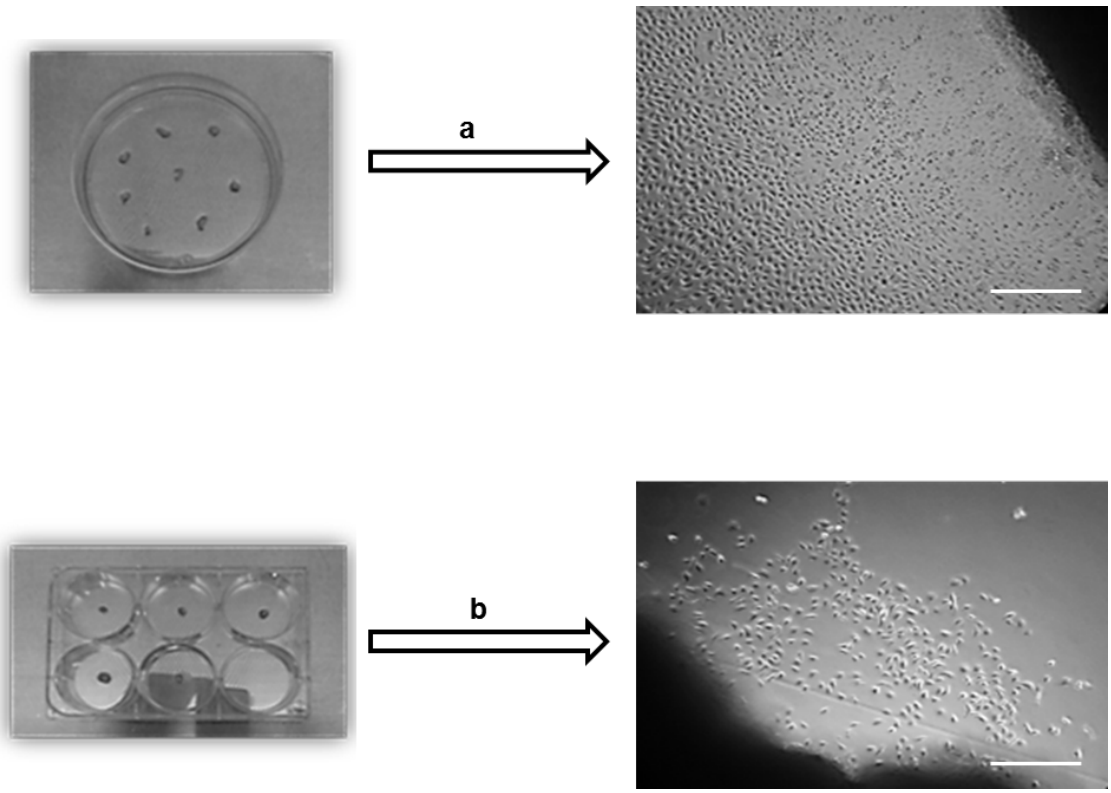


Figure 3-5: Outgrowing cells from explants tissues, Day 8. (a) On 90mm culture dish. (b) On 6-wells plate. Scale bars: 100 µm.

3.3.1.1.2 Purity of urothelial cells from explant primary culture

Although validating the epithelial cells by appearance is a well-accepted method, some epithelia might exhibit greater variations in shape. Hence, to confirm their origin and to check their purity, mRNA expression of cell lineage markers in primary outgrowing cells from tissue explants (*CD24* as marker for epithelial cells (Gracz *et al.*, 2013), *CD45* as marker for haematopoietic cells (Altin and Sloan, 1997), *CD146* as marker for endothelial cells (Elshal *et al.*, 2005), and α-SMA as marker for stromal cells (Lazard *et al.*, 1993)) was tested using real-time PCR assay (Figure 3-6). Although morphologically outgrowth cells were epithelioid, only one sample expressed *CD24* (epithelial cell marker), but also co-expressed *α-SMA* and *CD146*, suggesting that growing cells contained both epithelial and nonepithelial cells. No expression of *CD45* was detected in any of the samples so excluding any haematopoietic contamination.

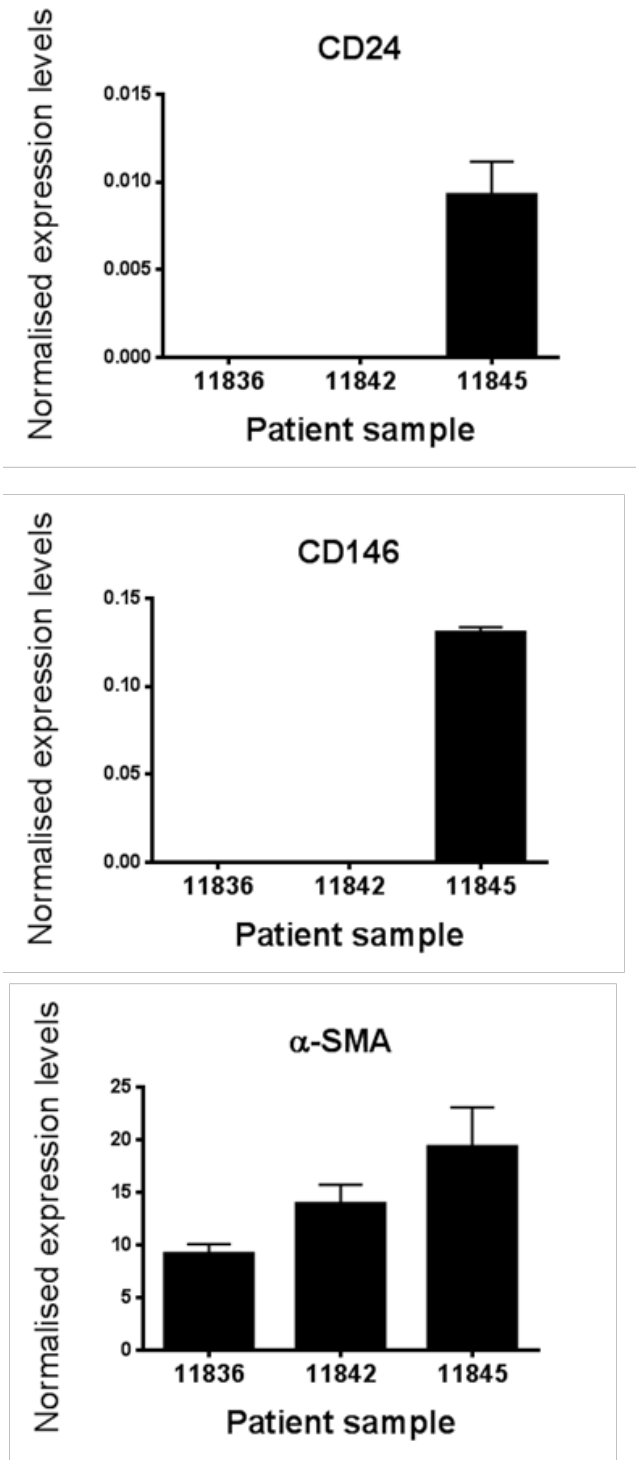


Figure 3-6: Explant outgrowth showing haematopoietic and stromal contamination. Real time-PCR for mRNA expression of *CD24* (epithelial cell marker), *CD146* (endothelial cell marker), and *α-SMA* (stromal cell marker) in explant outgrowth. Y-axis depicts expression of the cell marker normalized to *GAPDH* expression. X-axis depicts the patient samples ID. Error bars represent standard error of the mean (SEM).

3.3.1.2 Isolation and culture of urothelial cells using enzymatic digestion method

Because real time-PCR showed that the outgrowing cells from explant tissue were not pure epithelial cells, an alternative technique to isolate human urothelial cells was investigated. A total of 36 samples were prepared according to the method of Southgate *et al*. Isolated cells were further purified through MACS separation using CD326 microbeads. The CD326 antigen (also known as human epithelial antigen (HEA), epithelial-specific antigen (ESA), and EpCAM) is a 40 kDa transmembrane glycoprotein that is extensively expressed by normal and tumour epithelial cells. 7 out of 36 samples failed to grow giving a success rate of about 80% for growing and culturing primary urothelial cells using this technique. Higher subcultures were attained with difficulty with only three samples reaching passage 5 (Figure 3-7). After selection, both CD326 positive and negative cells were cultured. Following 24 hours in culture, satisfactory growth by CD326 positive cells was observed whilst only a small number of CD326 negative cells grew (Figure 3-8).

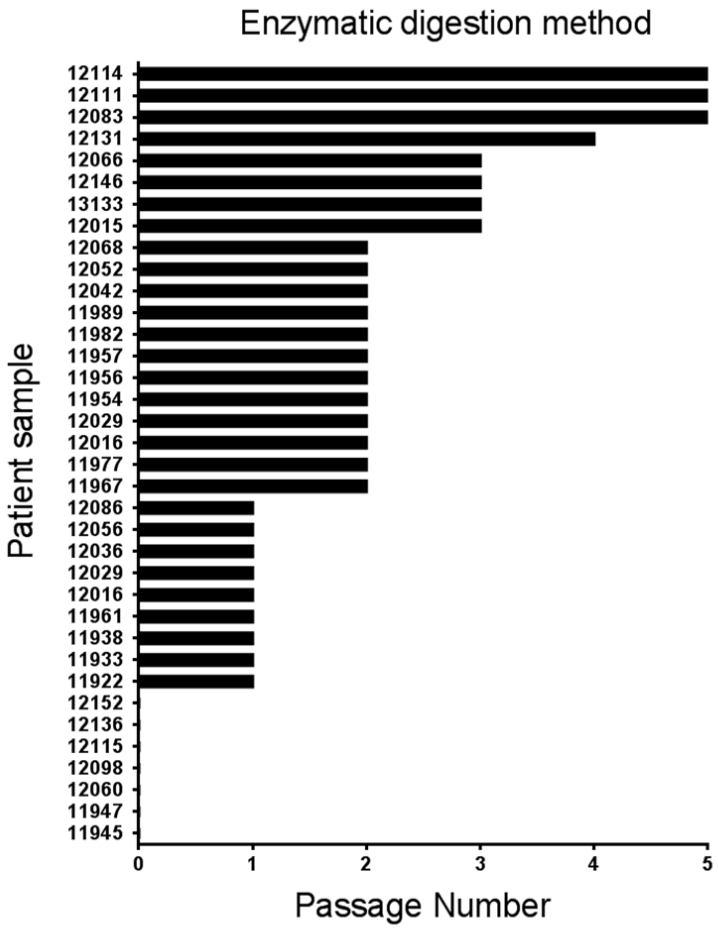


Figure 3-7: A summary of primary urothelial cultures using enzymatic digestion method. About 20% of samples did not grow in culture. Y-axis represents the patient samples ID. X-axis represents the passage number.

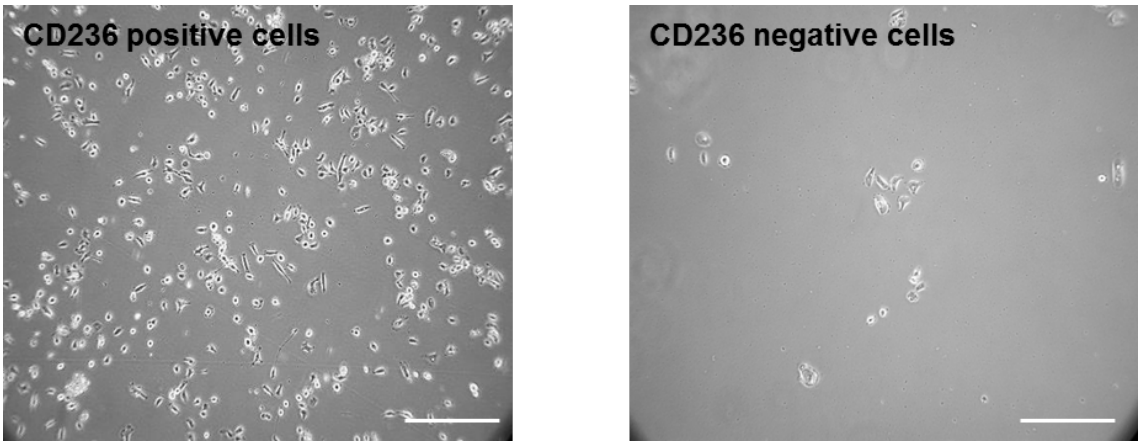


Figure 3-8: Phase contrast photomicrographs of CD326 positive and negative cells after 24 hours in culture. Scale bars: 100 µm.

3.3.1.2.1 Morphology of epithelial cells

Cells started to cluster and form colonies after 1 week in culture and took about 2-3 weeks to reach 70-80% confluency. At this point, cultures were subcultured as cell differentiation might occur progressively after that point. The urothelial cells derived from both normal bladder and ureter tissues showed similar epithelial appearance. Cells grew as colonies of compact cells with tight borders. Typically, cultures contained near isodiametric and cuboidal cells that arranged themselves in a cobblestone pattern (Figure 3-9). No fibroblast contamination was visually observed in these cultures.

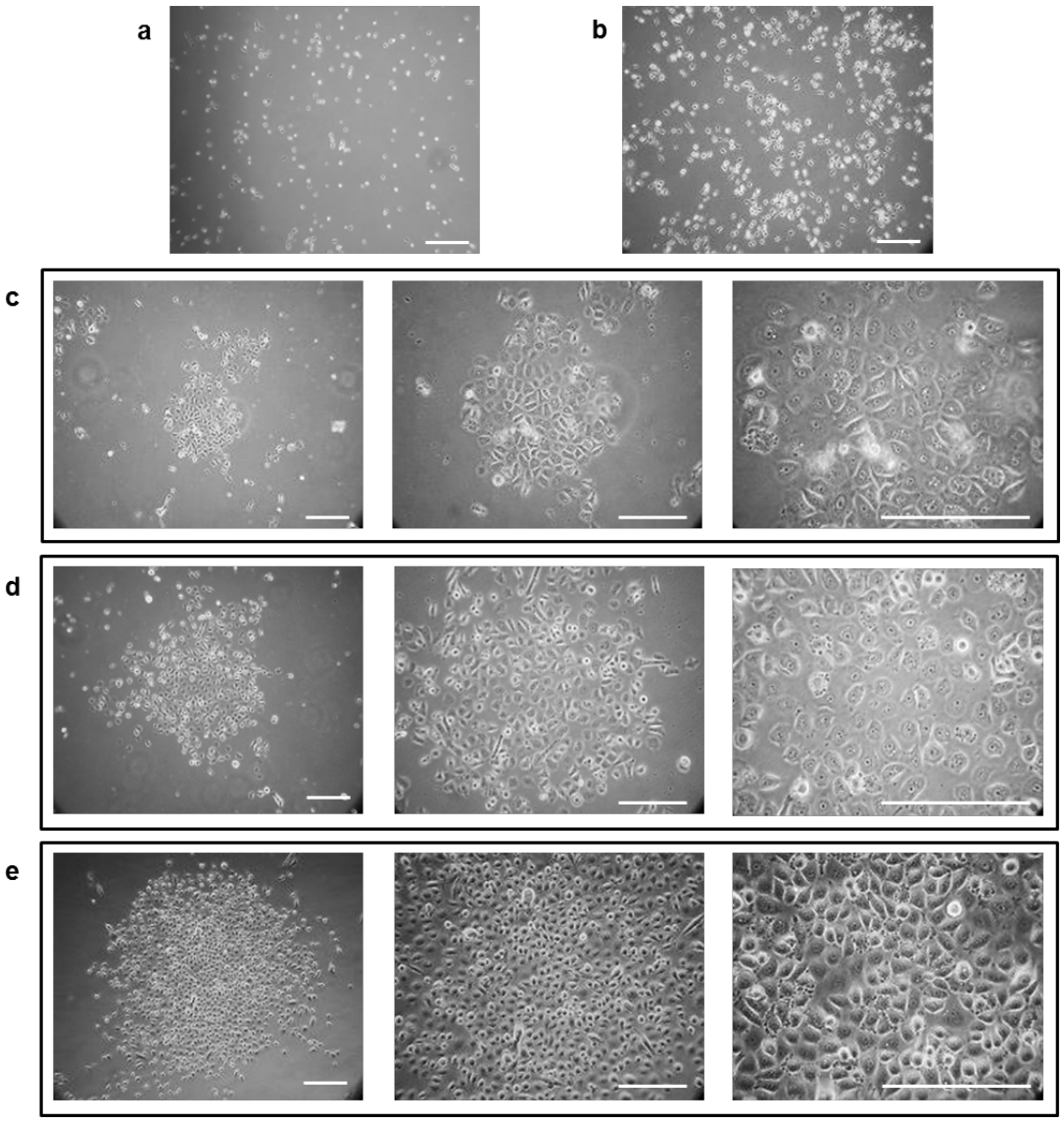


Figure 3-9: Phase contrast photomicrographs of normal human urothelial cells (CD326+) passage 1 growing on 60mm dish in KSFMc medium. (a) day 1, (b) day 3, (c) day 6, (d) day 12, and (e) day 19. Higher magnification showed compacted cells growing in colonies with a cobblestone pattern. Scale bars: 50 µm.

Beyond week 3, no notable increase in colony size could be observed; instead cells showed an increased tendency towards differentiation and senescence represented by an increase in cell size, shape irregularities and cytoplasmic vacuolation. It was also observed that in cultures beyond Passage 3, urothelial cells started to diffuse over the whole dish rather than form well-circumscribed patches, lost their cobblestone appearance and were made up of cells with high level of morphological heterogeneity (Figure 3-10). Southgate et al (Southgate *et al*., 2007) demonstrated separation of the urothelium lining from the outer layer following incubation in the stripper medium either for 4 hours at 37ºC or overnight at 4ºC. In my experiments I observed that the viability was higher in the shorter incubation time (N=10).

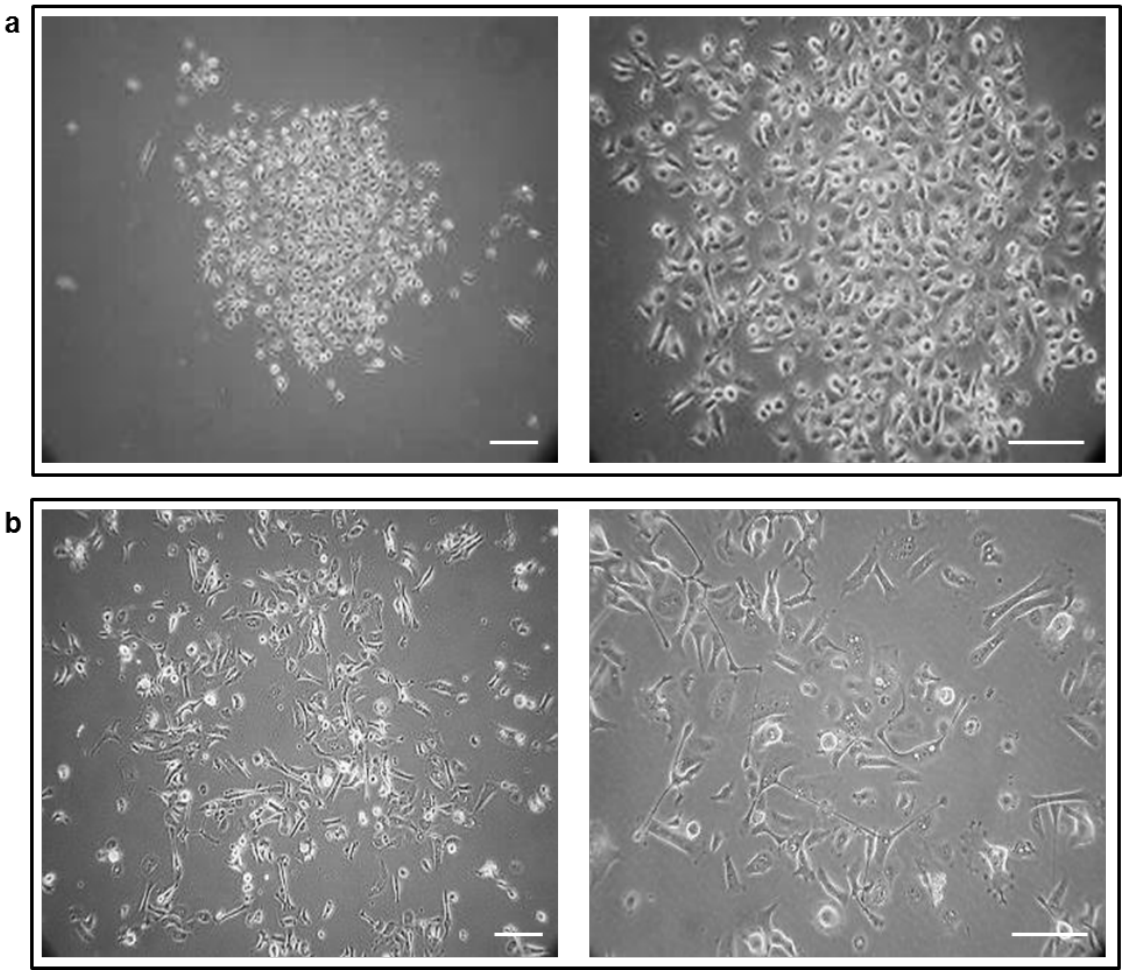


Figure 3-10: Phase contrast photomicrographs of urothelial cells at day 7. (a) Passage 2. (b) Passage 4, cell population shows high degree of morphologic heterogeneity and shape irregularities. Scale bars: 50 µm.

3.3.1.2.2 Purity of urothelial cells cultured following the enzymatic digestion protocol

To confirm the enrichment of cultured urothelial cells following the enzymatic digestion protocol, mRNA expression of cell lineage markers (*α-SMA, CD90, CD24, CD45; CD146*; and Von Willebrand factor (*vWF*)) was assessed by real time-PCR in both CD326 positive and negative fractions separated by MACS technique. Expression of all genes was investigated in cells from passages P0, P1, and P2. As shown in (Figure 3-11), CD326 positive cells overexpressed epithelial cell marker (*CD24*) suggesting their epithelial origin. Importantly, none of the samples exhibited expression of stromal cell markers (*α-SMA*, *CD90*), endothelial cell markers (*CD146*, *vWF*) or haematopoietic cell marker, (*CD45*) therefore excluding any obvious contamination. In CD326 negative cells, real time-PCR showed expression of *CD24* which might be due to the existence of some epithelial cells as MACS enriches for epithelial cells. However, these cells also co-expressed *CD45*, and *vWF*. This data indicated that isolating urothelial cells via enzymatic digestion followed by MACS selection for CD326+ cells resulted in highly pure urothelial cells.

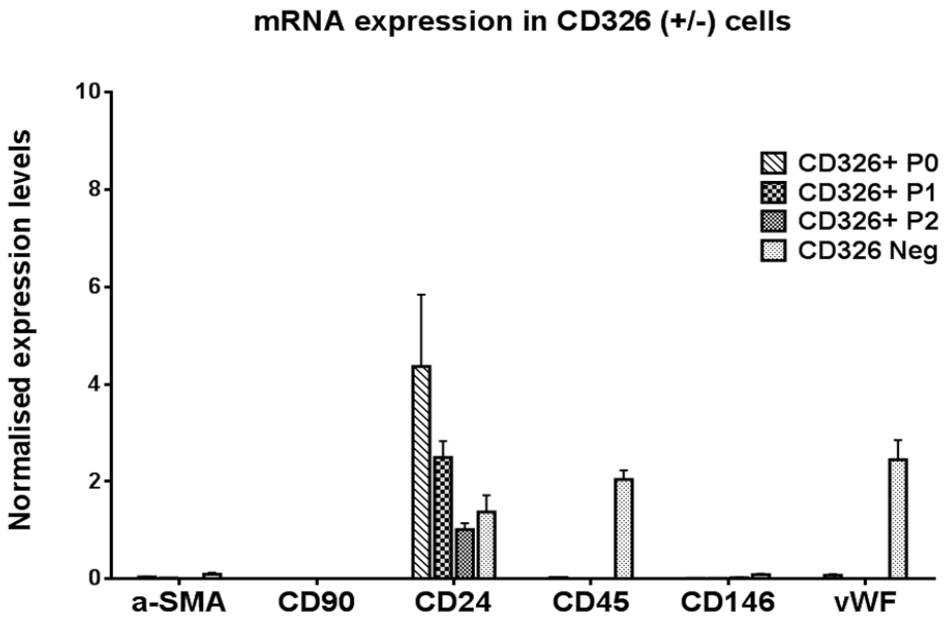


Figure 3-11: Primary urothelial cells showing absence of stromal haematopoietic and endothelial contamination. Real time-PCR for mRNA expression of *α-SMA* and *CD90* (stromal cell markers), *CD24* (epithelial cell marker), *CD45* (haematopoietic marker), and *vWF* and *CD146* (endothelial cell markers) in CD326 positive and negative cells after MACS selection. Error bars show standard error of the mean (SEM) for N=4.

3.3.1.2.3 Cell identification

The optimised protocol we used to isolate the urothelial cells from the underlying tissue ensures that only epithelial cells become established in culture. However, to establish the identity of cultured cells more objectively, the expression of differentiation-specific markers including *α6 integrin*, *β4 integrin*, *CK13*, *CK14*, claudin7 (*CLD7*), and *UPIb* was investigated. *CK13* is present in all but the superficial cell layer of the urothelium. Uroplakin is a selective marker for urothelial cell differentiation. In particular, *UPIb* is expressed by the superficial and the intermediate cells. In addition, normal human urothelial cells express the *α6 β4* integrin *in vitro*. Real time-PCR showed that urothelial cells expressed these markers at variable levels (Figure 3-12). Expression of *CK14* was higher than that of *CK13* which may be an indicator that cells in culture tend towards a more squamous phenotype (Southgate *et al.*, 1994; R. Ian Freshney, 2002). Expression of UPIb marker was further validated at the protein level; immunofluorescence showed that isolated cultured cells exhibited typical UPIb expression. Altogether, these results verified that upon isolation and culture, a homogeneous population with typical epithelial morphology of primary urothelial cells could be obtained and further subcultured.

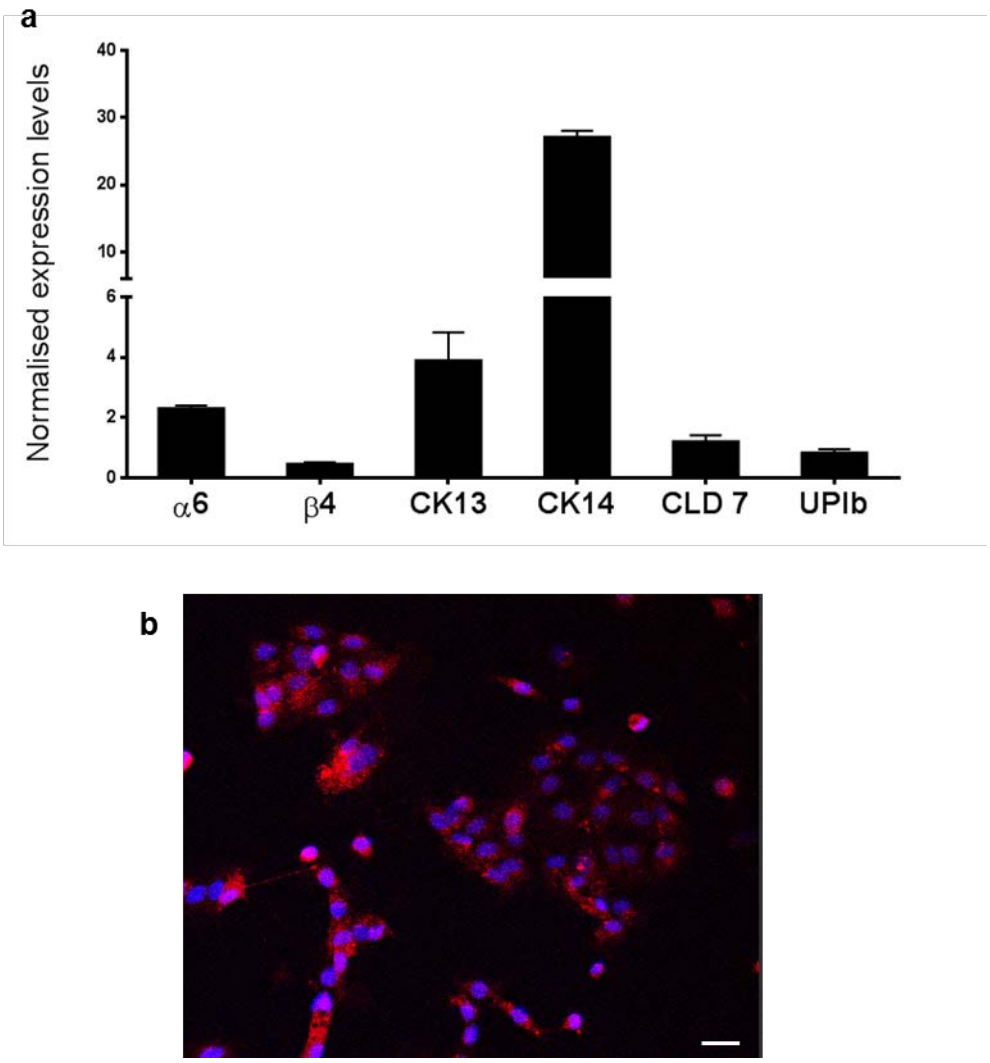


Figure 3-12: Expression of selected differentiation markers by human urothelium. (a) Real time-PCR for mRNA expression of differentiation-specific markers in normal human urothelial cells. Y-axis depicts expression of differentiation-specific markers normalized to *GAPDH* expression. X-axis depicts expression of individual differentiation-specific markers. Error bars show SEM for N=3. (b) Normal urothelial cells immunostained for UPIb (red), cell nuclei were counterstained with DAPI (blue). Scale bar: 10 µm.

3.3.2 Isolation and culture of human urinary tract (UT) stromal cells

After culturing the urothelial cells using either explant or enzymatic digestion method, their associated stromal cells were cultured using the protocol previously described in the methods (Chapter 2). A total of 45 stroma samples were cultured with almost 95% success rate. Stromal cultures appeared more able to survive higher serial subcultures than their epithelial counterparts. The first passage was performed on stromal cells growing in 25 cm^2 flask from which they were transferred into 75 cm^2 and 175 cm^2 flasks for further propagation. Stromal cells could be serially subcultured for at least ten passages after which they were frozen and stored at -80°C.

3.3.2.1 Morphology of UT-stromal cells

Initial culture of stromal cells from tissues following isolation of the urothelium using the explant method didn't exhibit typical morphology of stroma (Figure 3-13). Clumps of cells with a sunburst-like outgrowth and some cells with epithelioid appearance were observed. This may be expected as separating the urothelium mechanically from the stroma was very difficult and subsequent contamination could be possible. In time, the clumped cells tended to scatter and epithelioid growth gradually diminished. In advanced subcultures, only cells with stromal morphology were seen. On the other hand, stroma cultures derived from tissues after isolating the urothelium using enzymatic digestion exhibited typical stromal morphology even from the initial cultures. Cells showed distinct morphology from their epithelial counterparts in terms of cell size, shape and culture-type. Unlike urothelial cells, primary UT-stromal cells grew as monolayers rather than colonies (Figure 3-14). In preconfluent culture, cells exhibited a spindle-shaped, elongated, and fibroblast-like morphology with whorl-like patterns associated with greater confluence. No visible changes in morphology between the initial cultures and subcultures were observed (Figure 3-15).

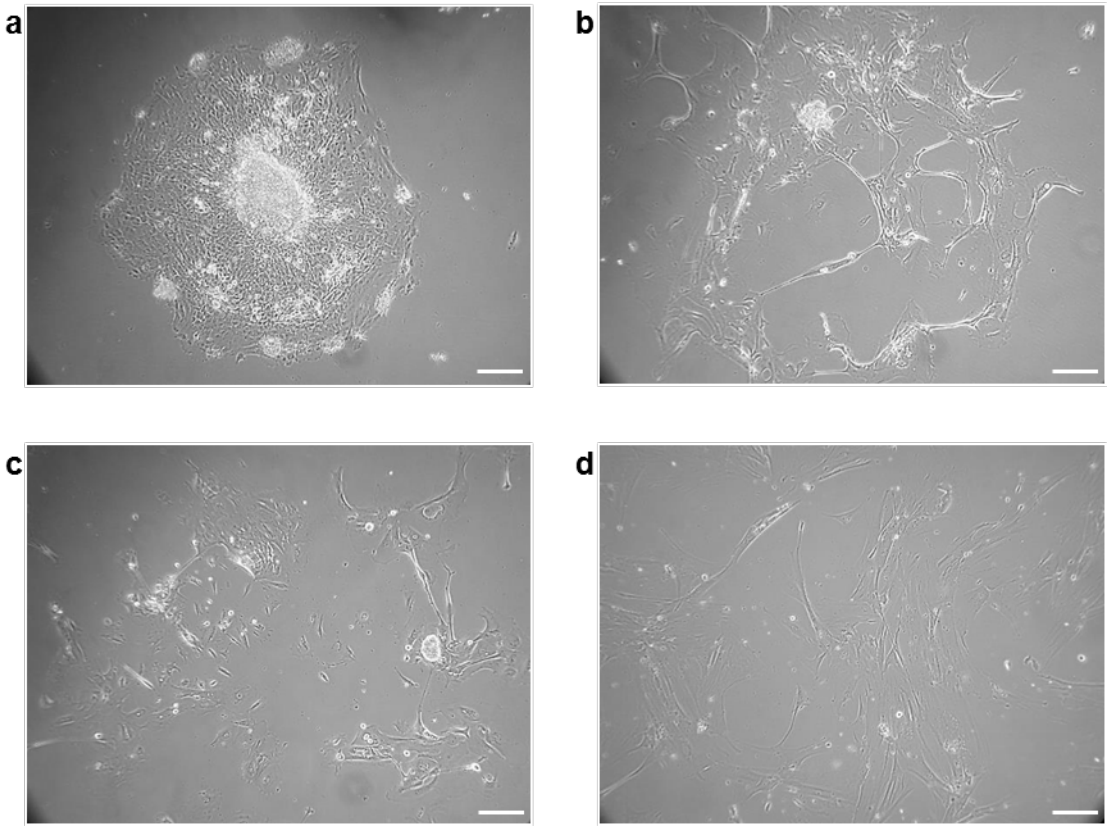


Figure 3-13: Phase contrast photomicrographs of human UT-stroma culture after isolating the urothelium using explant culture. (a, b, c, and d) represent stroma culture at day 2, 7, 14, and 21 days, respectively. Scale bars: 50 µm.

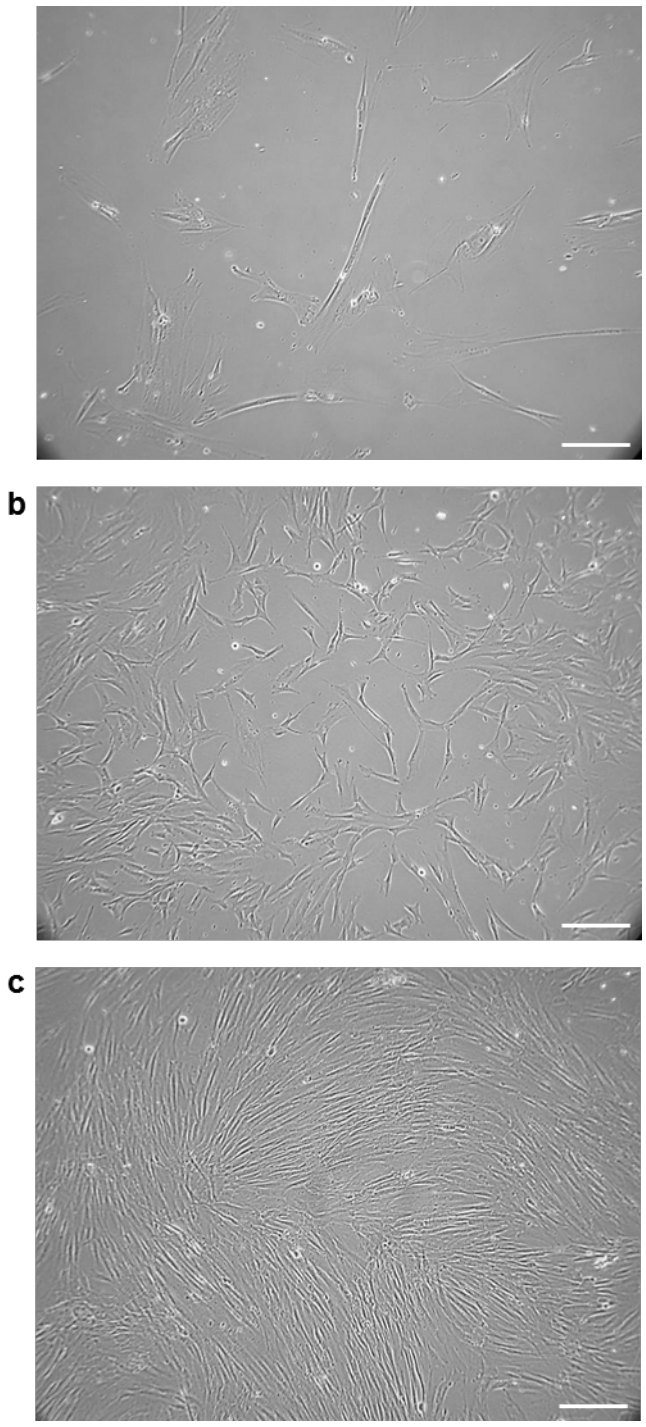


Figure 3-14: Phase contrast photomicrographs of UT-stroma culture from tissues after isolating the urothelium using enzymatic digestion showing typical morphology of stromal cells. (a) Day 2. (b) Day 7. (c) Day 10. Stromal cells were fibroblastic in appearance, in the more confluent culture the stromal cells exhibited “streaming,” forming loops and whorls. Scale bars: 100 µm.

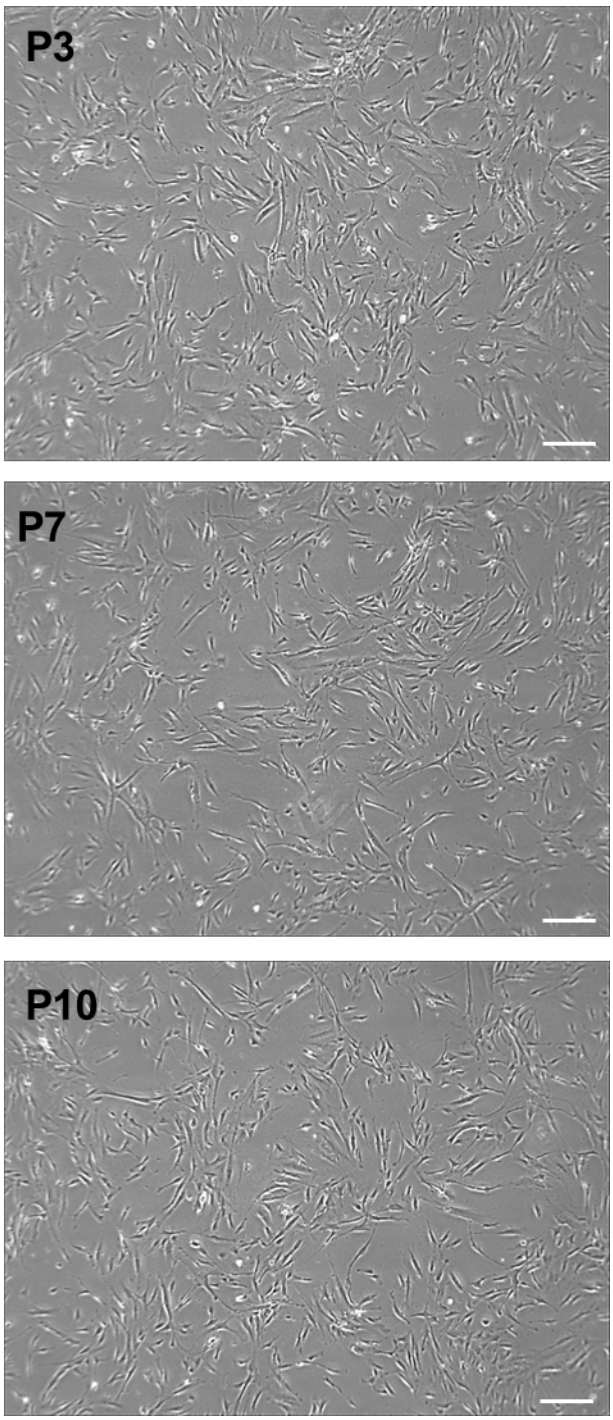


Figure 3-15: Phase contrast photomicrographs showing typical morphology of the stromal cells at different subcultures. Scale bars: 100 µm.

Another observed difference in UT-stroma cultures following urothelium isolation using explant method or enzymatic digestion method was that stromal cultures following explant method required ~33-37 days to reach 80-90% confluency, whilst stromal cultures following enzymatic method only required 8-12 days (Figure 3-16). However, this difference was not observed in subcultures, suggesting that stromal cell proliferation might be repressed in the presence of the more slowly dividing epithelial cells.

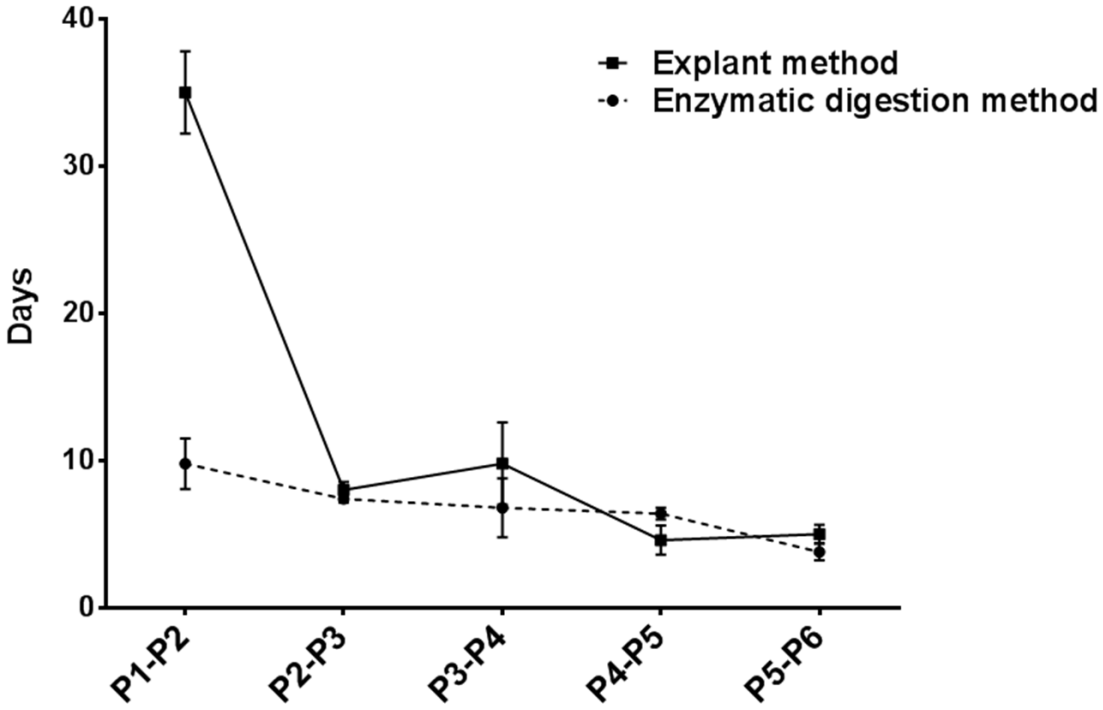


Figure 3-16: Days required for reaching 80-90% confluence culture of UT-stromal cells after isolating the urothelium using two different methods (explants and enzymatic digestion method). Y-axis depicts days between two passages. X-axis depicts passage number. Error bars show standard error of the mean (SEM) for N=5.

3.3.2.2 Purity of stromal cells

Morphologically, stromal cells isolated via enzymatic digestion method exhibited typical fibroblast structure. The purity of cultured UT-stromal cells was checked by investigating the mRNA expression of a panel of cell lineage markers (*α-SMA; CD90; CD24; CD45*; and *vWF*). Primary stromal cultures at initial passages (P0, P1) showed evidence of contamination with epithelial cells (through positive expression of *CD24*), haematopoietic cells (through positive expression of *CD45*), and endothelial cells (through positive expression of *vWF*). As these cells were progressively passaged (P2, P3), cells expressed stromal cell markers *α-SMA* and *CD90*, with no significant expression of epithelial cell marker *CD24*, haematopoietic cell marker *CD45*, and endothelial cell marker *vWF* (Figure 3-17). Collectively, these data indicated that passaging was enriching for stromal cells and cells at P2 were apparently pure stromal cells.

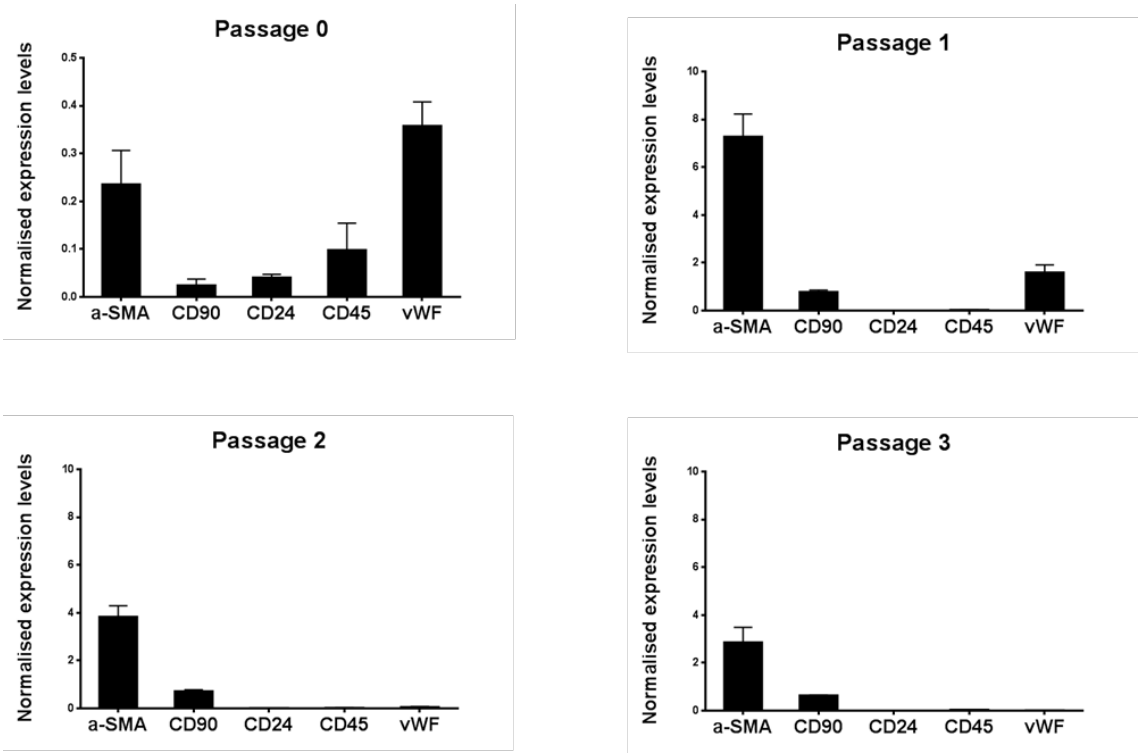


Figure 3-17: Primary UT-stromal cells at passage 0, 1, 2, and 3 showing significant reduction of epithelial, haematopoietic, and endothelial contamination. Real time-PCR for mRNA expression of *α-SMA* and *CD90* (stromal cell markers), *CD24* (epithelial cell marker), *CD45* (haematopoietic marker), and *vWF* (endothelial cell markers) in UT-stromal cells from serial subcultures (P0; P1; P2; and P3). Error bars show standard error of the mean (SEM) for N=3.

3.3.3 Evaluation of pluripotency markers at different passages

The endogenous expression of certain reprogramming factors in different cell types has permitted their exclusion from the factor cocktail (Maherali and Hochedlinger, 2008). Generally, generating iPS cells from somatic cells that already express endogenous reprogramming factors tends to be easier and may require less exogenes. Therefore, the expression of pluripotency markers in pure urothelial and stromal cultures was evaluated.

3.3.3.1 Evaluation of pluripotency markers in normal human urinary tract cells

Real time-PCR showed that mRNA expression of *OCT4*, *SOX2*, and *NANOG* was detectable in cells at P0. This expression was significantly reduced with increasing passage number (Figure 3-18).

As shown previously, stroma cells at P0 and P1 were not pure, therefore the pluripotent transcript expression at this stage were not assessed. Transcript expression of *OCT4*; *SOX2*; and *NANOG* was detectable at P2, and P3; however the expression levels decreased with increasing passage number. Previous study showed that the pluripotency-associated *OCT4A* is not expressed by normal or malignant human urothelium, but indicated the presence of alternative isoforms (*OCT4B*, and *OCT4B1*) or potentially translated pseudogenes (Wezel et al., 2013). Therefore we studied the expression of the alternatively spliced variant *OCT4B1*.

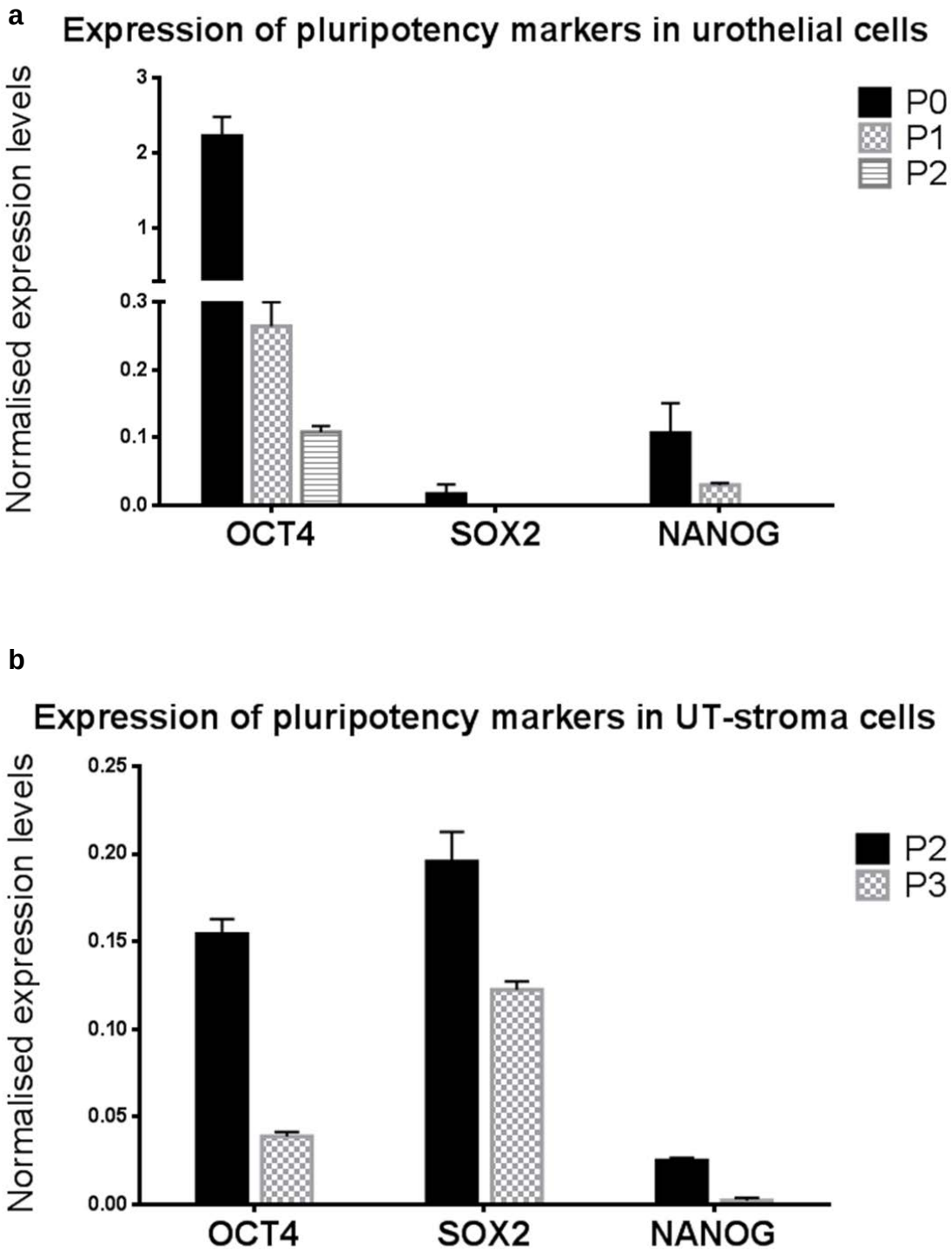


Figure 3-18: Expression of pluripotency markers in normal human urinary tract cells at different passages. (a) mRNA expression of (*OCT4*, *SOX2*, and *NANOG*) in pure urothelial cells at (P0; P1; P2) of the same sample. (b) mRNA expression of (*OCT4*, *SOX2*, and *NANOG*) in pure UT-stroma cells at (P2; P3) of the same sample. Y-axis depicts expression of pluripotency markers normalized to *GAPDH* expression. X-axis depicts the expression of individual pluripotency markers. Error bars show standard error of the mean (SEM) for N=3.

3.4 Discussion

Primary culture is the early *in vitro* culture of cells isolated from donor tissue. Such cultures are widely used for basic research as they are assumed to display key characteristics similar to those seen *in vivo*. Usually, maintaining primary cell culture requires specific media with several supplementary components due to their limited life time and their tendency to change their differentiated characteristics with time in culture. Specifically, establishing and maintaining primary culture of normal human epithelial cells *in vitro* has been deemed to be difficult and challenging (Reznikoff *et al.*, 1983; Michael Aschner, 2011). Herein, primary cultures of urothelial and stromal cells from human ureter and bladder biopsies were established using two different techniques. Although the time-honoured method to validate epithelial cells in culture is by morphology, some cell types can show greater plasticity in shape, for example endothelial cells derived from mesenchyme can display epithelioid shape in a confluent culture (R. Ian Freshney, 2002). Urothelial and UT-stroma cells were subjected to real time-PCR analysis using a panel of cell specific or differentiation-specific markers. Markers were selected as most representative of the class of cell type concerned. Morphology and growth characteristics were also utilized to monitor and follow culture progress of both cell types *in vitro* after separation.

Initiating cells using explant culture is an old technique and it has been used to culture epithelial cells from various tissues *in vitro* (Stonington and Hemmingsen, 1971; Fischer *et al.*, 1980). Although the explant culture method showed that it might be a simple technique, outgrowth cells show low viability and limited application, since mixed cultures of urothelial and stromal cells are produced. Minor contamination of stromal cells can eventually overtake the urothelial cells, even under improved conditions for urothelial growth, since stromal cells have higher proliferation rate compared with that of urothelial cells. In recent years, culture systems have been developed to achieve high purity of homogeneous primary urothelial cultures. In particular, Southgate and colleges dissociated the urothelium from the basement membrane using EDTA. This method permits high cell yields that show high plating efficiencies (Southgate *et al.*, 1994; Southgate J, 2002). In our hands, isolating urothelial cells using this

technique followed by MACS selection for CD326 was found to produce almost pure urothelial cells. Although urothelial cells showed extended culture lifetime many fold as compared to explant culture, their overall growth potential is still limited (Southgate *et al.*, 1994; Southgate J, 2002; Southgate *et al.*, 2007). Notably, due to limitation in the number of cells that can be extracted and expanded from a small biopsy of clinical material from the urinary tract, we didn't examine the doubling time which can give a more intuitive sense of the long-term impact of growth. On the other hand, UT-stroma cells displayed typical fibroblastic characteristics including robustness and proliferative capacity and could be successfully thawed and subcultured after freezing. We accept the limitation of not showing the protein expression to confirm the origin of the stomal cells. However, showing that cells have mRNA expression of stomal markers and no expression of epithelial, haematopoietic, and endothelial markers strongly suggested the stromal origin and the purity of cultured cells. Moreover, the protocols were directly learnt from a placement in Prof Jenny Southgate's lab, who has extensively characterised the nature of these cells from human tissue (Southgate J, 2002; Southgate et al., 2007), including protein expressions.

After producing highly pure cultures of primary urothelial and stromal cells, as quantified by real time-PCR, the expression of the main pluripotency markers in these cultures at different passages were assessed. Previous reports showed that reprogramming somatic cells which already express high endogenous levels of reprogramming factors is easier and may require fewer ectopic factors (Kim *et al.*, 2008; Kim *et al.*, 2009b). Thus in general, the higher the expression of reprogramming factors in the cells the more easily they are to reprogram. In addition, cultured cells might change qualitatively over time (Utikal *et al.*, 2009). By real time-PCR, higher expression levels of *OCT4*, *SOX2*, and *NANOG* at P0 and P2 of urothelial and stromal cells, respectively was observed. Therefore, urothelial cells at P0 and stromal cells at P2 were selected for transduction.

4 Chapter 4. Generation of iPS cells from normal human urinary tract cells

4.1 Introduction

This chapter will describe in detail the experimental protocol designed to produce iPS cells from primary UT-stroma and urothelial cells by means of lentiviral overexpression of *OCT4*, *SOX2*, *KLF4*, and *C-MYC* (OSKM). Lentiviruses provide particularly attractive vectors for iPS generation as they can stably integrate into the genome without incurring cellular toxicity and maintain sustained expression of the transgene during prolonged proliferation and subsequent differentiation. Additional advantages of lentiviruses are their ability to transduce both proliferating and non-proliferating cells at high efficiency. Reprogramming into iPS cells was mediated by a single polycistronic construct encoding the transcription factors that are involved in maintaining the pluripotent state. The reprogramming factors in this vector are driven by the elongation factor 1 alpha (EF1a) promoter and fused in-frame into a single open reading frame (ORF) via self-cleaving 2A sequences flanked by loxP sites (Figure 4-1). These advances in vector design have significantly improved vector safety and reprogramming efficiency, ensuring that all transduced cells receive equal amounts of each of the four transcription factors, and enables excision of the exogene through a Cre-recombinase technology (Ma *et al.*, 2003; Shao *et al.*, 2009).

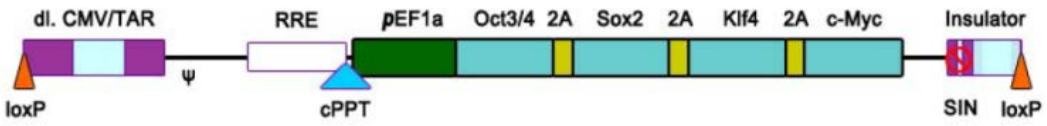


Figure 4-1: Schematic representation of the lentiviral construct.

4.2 Aims

- To induce iPS-phenotype in normal human primary UT-stromal cells.
- To induce iPS-phenotype in normal human primary urothelial cells.

4.3 Results

4.3.1 Transduction of human UT-stromal cells

Having confirmed the homogeneity of the stromal cells by real time-PCR using a panel of cell lineage markers, the lentiviral transduction was subsequently optimised.

4.3.1.1 Determination of optimal Polybrene concentration for transduction

One method to improve the very low transduction efficiency is to use an additive substance in the transduction cocktail such as polybrene (hexadimethrine bromide). Polybrene is a cationic polymer that is usually used with viruses to improve the transduction efficiency (Toyoshima and Vogt, 1969; Lin *et al.*, 2011a). Polybrene acts by neutralizing the negative electrostatic repulsion between the viral particles and the surface of their target cells leading to enhanced absorption of the virus by the cells (Davis *et al.*, 2002; Lin *et al.*, 2011a). Polybrene is tolerated well at low concentrations. However, at concentrations greater than 10 µg/mL, significant inhibition of cell proliferation has been reported in some cell types, such as keratinocytes (Seitz *et al.*, 1998) and human mesenchymal stem cells (Lin *et al.*, 2011a). To investigate the effect that different concentrations of polybrene have on stroma cell viability, cells were exposed to polybrene at concentrations ranging from 0 to 20 µg/ml for 48 hours and apoptosis was analysed by flow cytometry and propidium iodide (PI) staining. PI staining was analysed and compared against control (vehicle control). Under standard culture conditions, the percentage of PI-positive cells (approximately 6%) were variously increased after a polybrene treatment (Figure 4-2). Increasing the concentration of polybrene dramatically increased its unfavourable impact on cell viability especially at the concentration of 20µg/ml and that was in accordance with the morphological observations (Figure 4-3). Low cytotoxic effects with polybrene at 10ug/ml is to be expected and accepted, therefore this concentration was selected for further experiments.

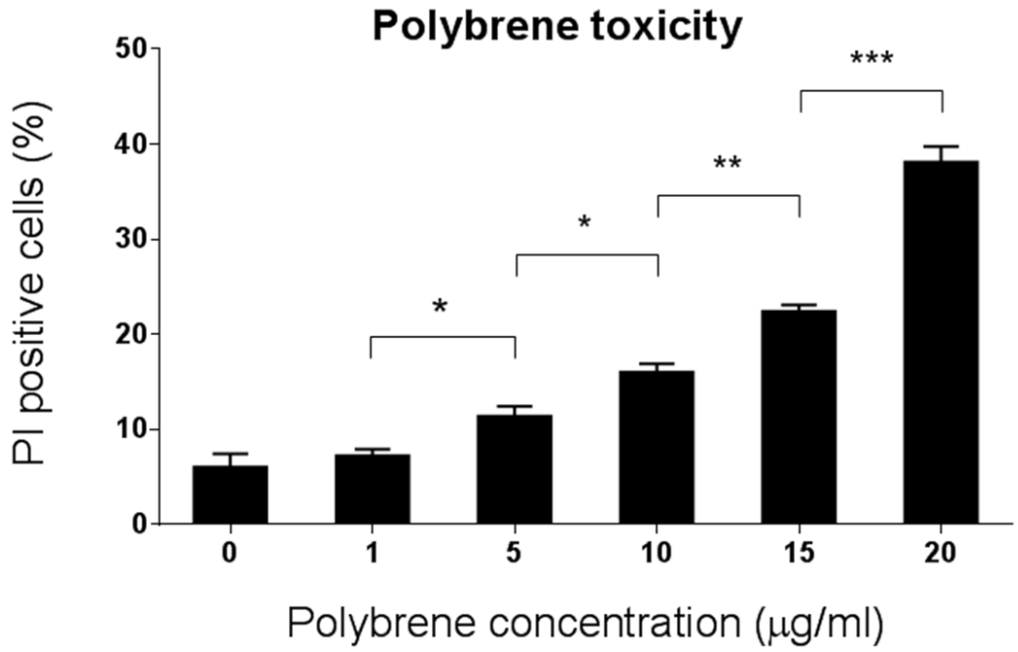


Figure 4-2: Polybrene induced apoptosis of UT-stroma cells. The graph represents % apoptosis in UT-stroma cells after polybrene exposure (0, 1µg/ml, 5µg/ml, 10µg/ml, 15µg/ml, and 20µg/ml) for 48 hours. Y-axis depicts percent PI positive cells. X-axis depicts different concentrations of polybrene. Error bars show SEM for N=3. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

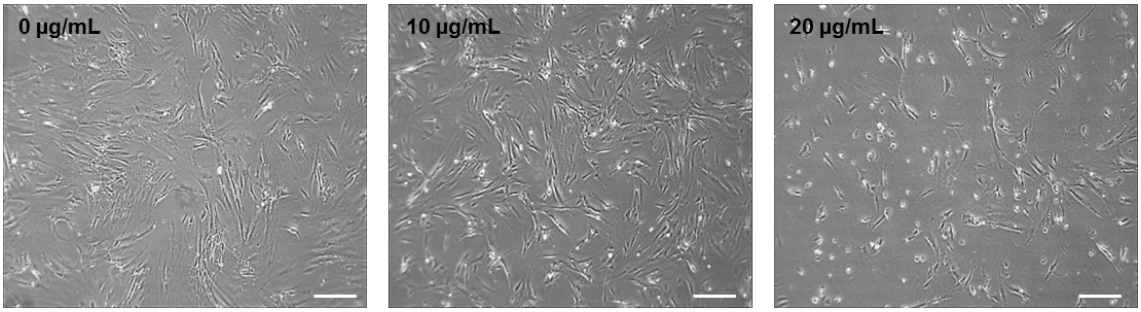


Figure 4-3: Phase- contrast micrographs of cultured stroma cells either not exposed or exposed to 10 and 20ug/ml of polybrene for 48 hours. Control cells (0ug/ml) showing healthy spindle-shape cells with long, delicate processes. Incubation with 10ug/ml of polybrene results in low cytoxicity. Incubation with 20ug/ml of polybrene results in high cytotoxicity. Scale bars: 100 µm.

4.3.1.2 Determination of optimal MOI for transduction

To determine the most efficient multiplicity of infection (MOI) with the least toxicity for transduction of stromal cells a range of MOIs were investigated using mWasab GFP control transduction particles. 5×10^4 stromal cells seeded on 6 well plate in 2 ml of complete RPMI1640 medium were transduced with MOIs of 2, 5, 10, 20, and 30 in the presence of polybrene 10µg/ml for 48 hours. GFP expression was analysed and compared against control (MOI = 0). The percentage of GFP positive cells increased in a linear fashion from ~5% to ~45% as MOI was increased from 2 to 30, respectively (Figure 4-4). At lower MOIs, an only minimal difference in cell viability was observed, as determined by PI dye exclusion (Figure 4-4). With MOIs of more than 10, a more dramatic effect was apparent with an approximate 50% decrease in surviving cells. 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. Therefore, MOI=10 and polybrene = 10µg/ml were determined as optimal conditions in subsequent experiments.

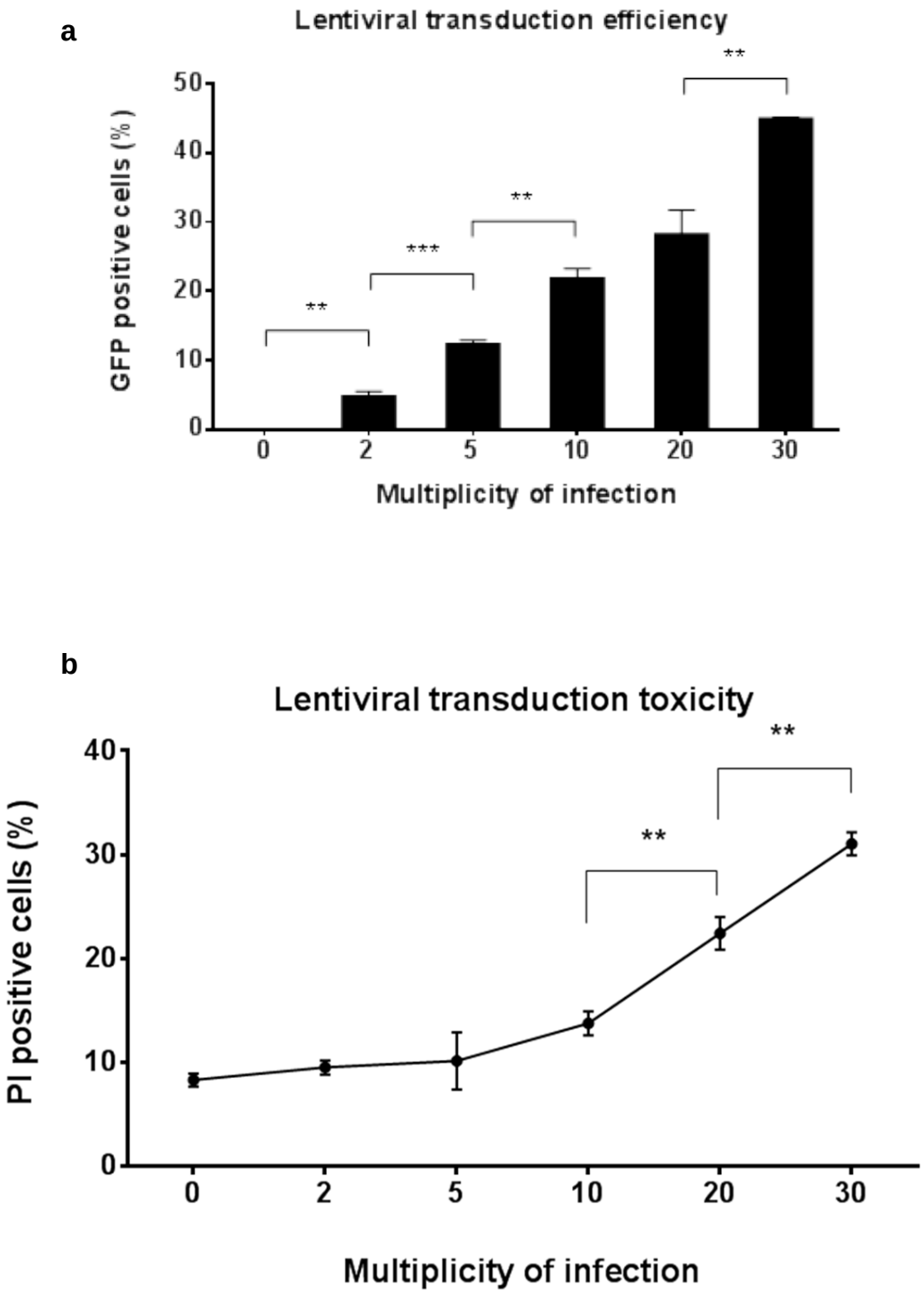


Figure 4-4: Determination of optimal MOI for transduction. (a) Transduction efficiency of UT-stroma cells by control lentivirus particles mWasabi (EF1a). Flow cytometry analysis of transduced cells, bar graph representation of the efficiency of transduction at increasing MOIs, as indicated on the x axis.
(b) Cells viability after 48h of transduction with different MOI. At higher MOI cell death was observed more frequently. Error bars show SEM for N=3

4.3.1.3 Transduced cells underwent a mesenchymal to epithelial transition

It has been shown that reprogramming of fibroblast cells to iPS cells inevitably involves an early event termed mesenchymal to epithelial transition (MET); during which cells undergo changes in morphology and gene expression. The characterisation of MET had become a recognised and central phenomena in the generation of iPSCs from mesenchymal cells (Li et al., 2010; Samavarchi-Tehrani et al., 2010). We undertook a preliminary assessment of this process using transcript expression of markers that are strongly indicative of this process. we accept that further characterisations with protein levels of these markers would be stronger evidence, however, given that definitive proof of iPS cells in generating teratomas and embryoid bodies is presented, the further descriptions of MET was not considered to be critical. Overall, the picture of MET was apparent given the transcript changes and final functional proof of iPS cells. Phase-contrast photographs taken at day 10 post transduction showed few cells that became aggregated and had acquired a rounded shape (Figure 4-5). Real-time PCR analysis of transduced cells at day 10 post transduction validated the upregulation of epithelial gene expression (*E-cadherin*, and *Ep-CAM*), and showed a concomitant downregulation of mesenchymal gene gene (*slug, snail, twist1,* and *vimentin*), indicating that the exogenous factors initiated the MET program in stromal cells.

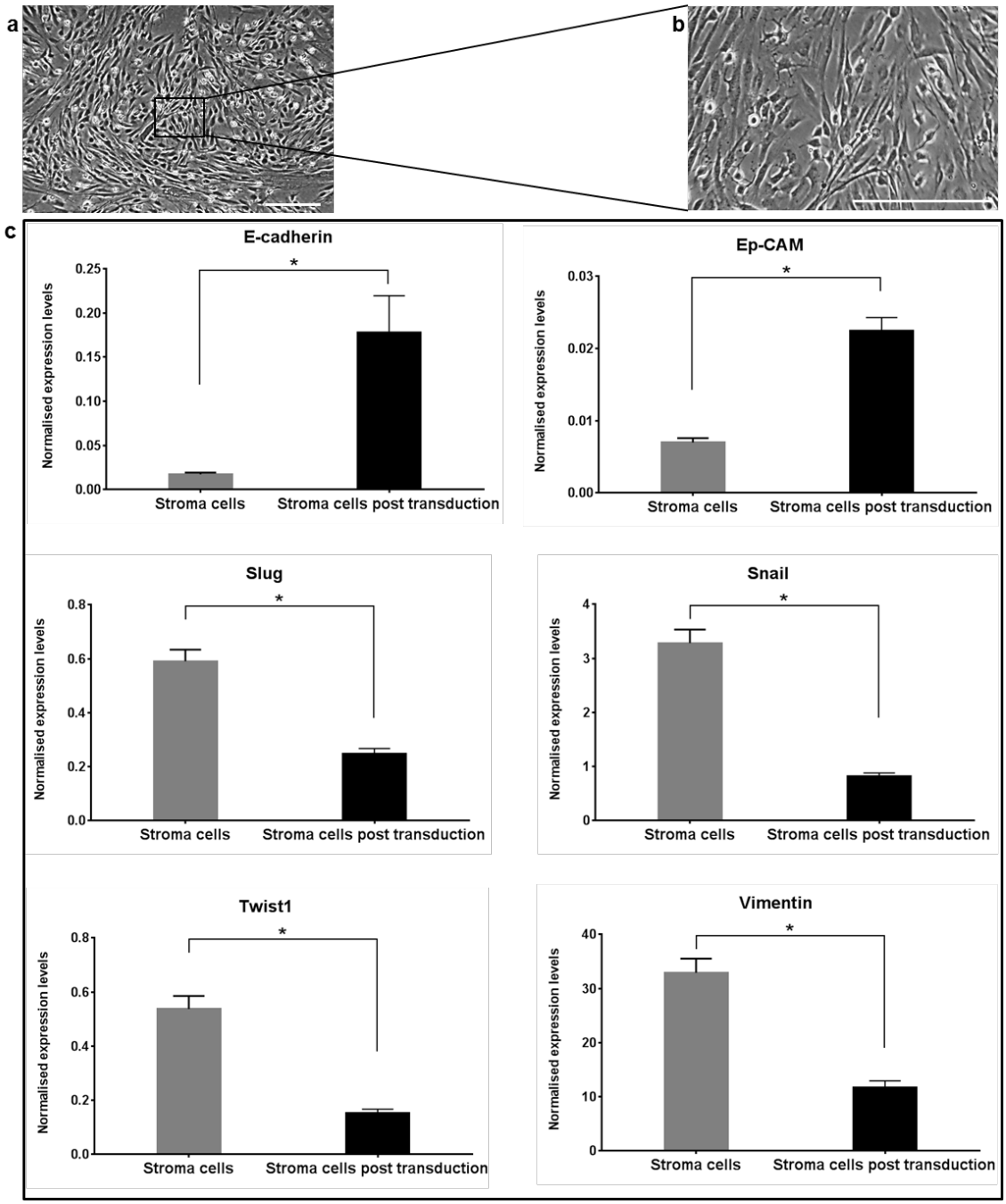


Figure 4-5: MET changes in primary UT-stroma post transduction. (a) Phase-contrast micrographs of transduced stromal cells 10 days post transduction; cells displayed an altered epithelial morphology. (b) Higher magnification, x100 (c): Real time PCR analysis showed upregulation in epithelial markers (*E-cadherin* and *Ep-CAM* accompanied by downregulation of mesenchyme markers (*Slug, Snail, Twist1, and Vimentin*) in stromal cells 10 day post transduction suggesting the occurrence of an MET. Error bars show SEM for N=3. Scale bars: 100 µm. * $P \leq 0.05$, **$P \leq 0.01$, *** $P \leq 0.001$.

4.3.1.4 Generation of iPS cells from human UT- stroma cells

Our protocol for human UT-stroma cells transduction is summarized in (Figure 4-6). One day prior to transduction, 5×10^4 cells were seeded onto a 6-well plate and incubated overnight in complete RPMI 1640. The following day, cells were washed once in 1XPBS and freshly prepared lentiviral transduction mixture containing OSKM virus MOI=10 and 10µg/ml of polybrene was added to the cells. 'media only' and 'media and polybrene' controls were used. After 48 hours, the transduction media was replaced with fresh complete RPMI 1640 medium and the cells were cultured for another 4 days.

The cells grew normally and appeared healthy against the control. On day 6, cells were transferred to 6-well plate seeded with inactivated mouse embryonic fibroblasts (MEFs) in human ES cell medium. On day 10, the transduced cells were cultured in human ES cell medium conditioned from inactivated MEFs (MEF-CM) and supplemented with human iPS culture medium and maintained in such a manner for 4-6 weeks. MEF-conditioned ES cell medium was prepared by treating MEF cells at a density of 50,000 cells/well with human ES cell medium. Media was collected every 24h for 5 to 7 days after plating and was filtered through 0.2 µm filter before use. Similarly, human iPS culture medium was collected from skin-iPS culture 3-5 days after plating, centrifuged for 5 min at 1500 x rpm to remove cells, and the supernatant media was collected and filtered through 0.2 µm filter.

Because the conditioned medium was collected after treating the relevant cells for 24h at 37˚C, some of the media chemicals components might be broken down, therefore, freshly prepared human ES cell medium was added to the MEF-conditioned ES cell medium and human Skin-iPS culture medium in a 1:1:1 ratio. MEF-and ES cell conditioned medium have been shown to increase the reprogramming efficiency at least 10 times by enhancing the transition of pre-iPS cell colonies to a fully reprogrammed state. Combination of different factors provided by MEFs and pluripotent stem cells has been found to help the transduced cells undergoing reprogramming to survive and gain pluripotent state (Katarzyna Tilgner, 2010). The reprogramming progress was monitored on a daily basis by light microscopy to identify any presumptive colonies.

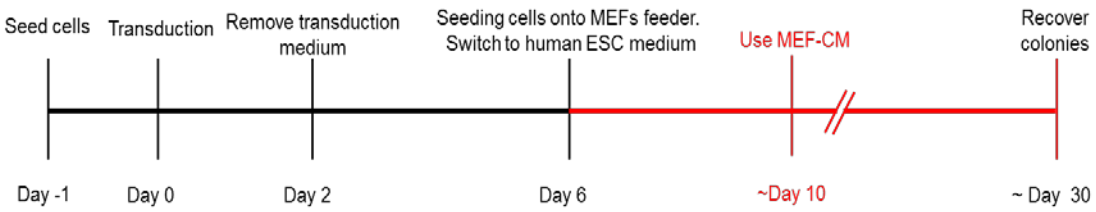


Figure 4-6: Time line for UT-iPS cell generation.

Four weeks after transduction with the lentivirus, several small, tight cell colonies with dark appearance were detected that grew slowly and were distinct from both ES cells and parental stromal cells in morphology. By week 6 after transduction, rapidly growing colonies displaying morphology similar to that of human ES cells were observed (tight and flat colonies with clear-cut edges comprising of small cells with a high nucleus-to-cytoplasm ratio) (Figure 4-7).

In this manner a total of thirty one ES cell-like colonies (17 bladder and 14 ureter derived) were successfully expanded and stably maintained throughout the cell passages (>50 passages, >10 months). Patient details from which successful iPS cell lines were established are summarised in (Table 4-1). Since each colony represents one separate human iPS cell line, these colonies were individually picked out and each was placed into a separate wall for expansion and identity analyses.

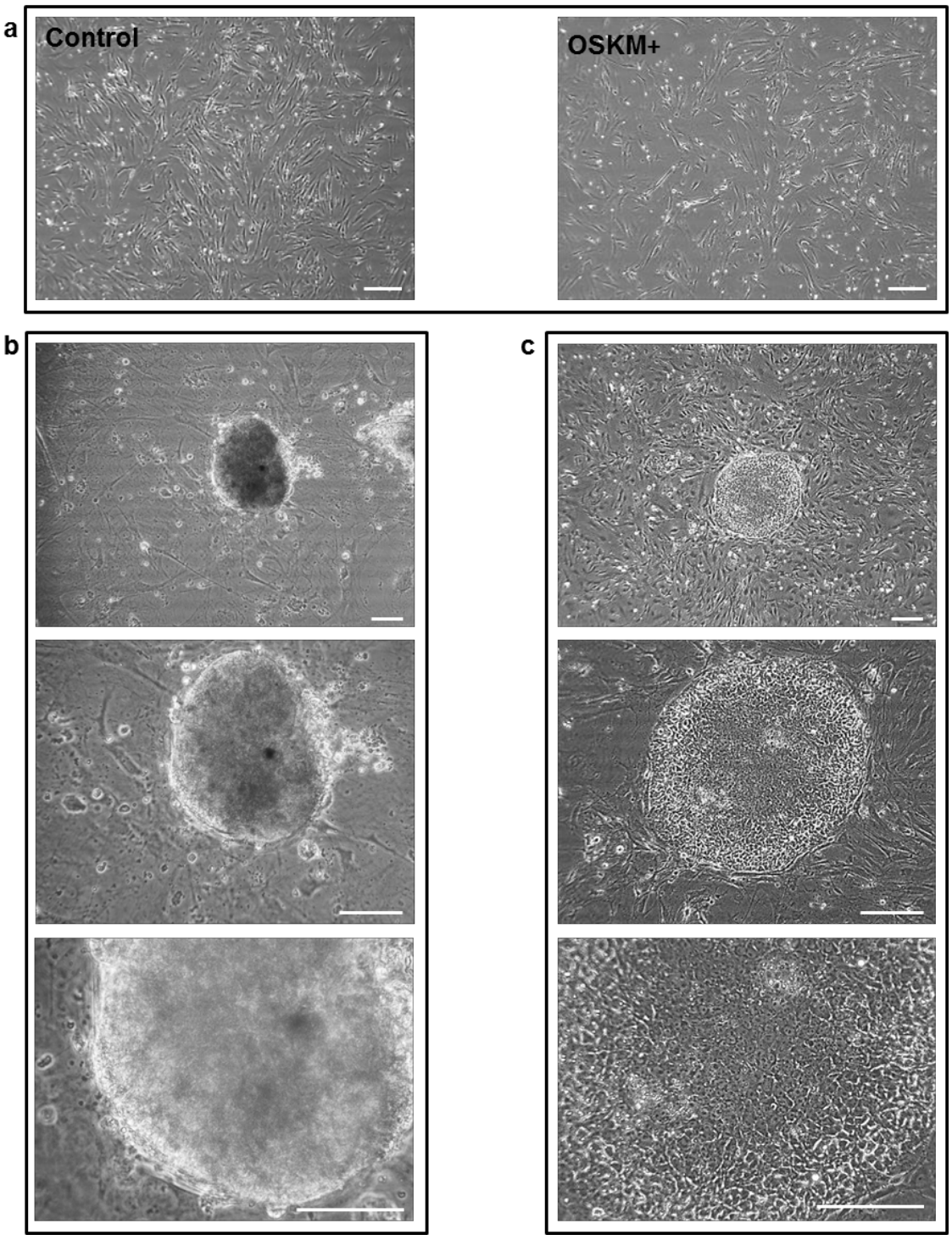


Figure 4-7: (a) Phase- contrast micrographs of UT-stroma cells 48h post transduction. The control represent untransduced cells, OSKM+ represent cells transduced with lentivirus at MOI = 10 and Polybrene = 10ug/ml. (b) Example of small and tight cell colonies observed 4 weeks after transduction. (c) Example of established UT-iPS colonies on MEFs feeder layer showing standard hallmarks of human ES cell colonies, including sharp borders and tightly packed cells within the colonies. Scale bars: 100 µm

Patent identifier	Age	Gender	History of patient	iPS clones
12380	65	Female	Ureter from radical nephrectomy for renal cell carcinoma	4
12459	66	Male	Ureter from Cystoprostatectomy for benign functional disorder	7
12491	48	Male	Bladder from Cystoprostatectomy for benign functional neurological disorder	10
12502	54	Male	Ureter from radical nephrectomy	3
12506	56	Male	Bladder from Cystoprostatectomy for benign functional neurological disorder	7

Table 4-1: Patient details from which UT-iPS cell lines were established.

4.3.1.5 Characterization of generated UT-iPS cells

4.3.1.5.1 Morphological analysis of UT-iPS cells

Colony and cellular morphological characteristics were assessed by phase-contrast microscopy. Some of the early forming colonies appeared with slightly fuzzy margins with a high frequency to undergo differentiation (Figure 4-8). This might be expected since the nascent reprogrammed cells are highly prone to differentiation, especially within the first few passages (Hochedlinger and Plath, 2009). However, as they expanded, colonies became uniformly round or oval shaped with well-defined margins and flatter appearance (Figure 4-8).

The cells within these colonies showed large round nuclei with prominent nucleoli and displayed a large nuclear to cytoplasmic ratio and phase-bright borders. Within the same colony a variety of cell shapes and sizes were observed but most of the cells were typically trapezoidal. As a colony increased in size, the cells packed more tightly causing the cells to appear smaller. Therefore, cells in smaller colonies looked larger than those in big colonies. In addition, cells in the centre of the colony appeared smaller than those at the

edge. While maintaining the human iPS cells in culture, a fraction of the cells underwent spontaneous differentiation.

Typically, 10% spontaneous differentiation was seen in human ES cell cultures. Differentiation is represented by loss of border integrity and the appearance of obvious different cell types. The two frequent types of differentiation observed included central differentiation at the top of the colonies and peripheral differentiation where undifferentiated UT-iPS cells were surrounded by a ring of differentiated cells as shown in (Figure 4-9).

During each passage, differentiated cells were carefully scraped and removed from the culture to avoid deterioration of cell quality. To maintain sterility of the iPS cell cultures, this process was performed using an inverted light microscope in a tissue culture hood. Notably, long intervals between passages or incubating with culture media for more than 48h resulted in increased differentiation of the cells. The size and confluence of iPS colonies were visually observed and used as a guide for assessing when to passage. In general, UT-iPS cell cultures were regularly passaged approximately every 5 to 7 days.

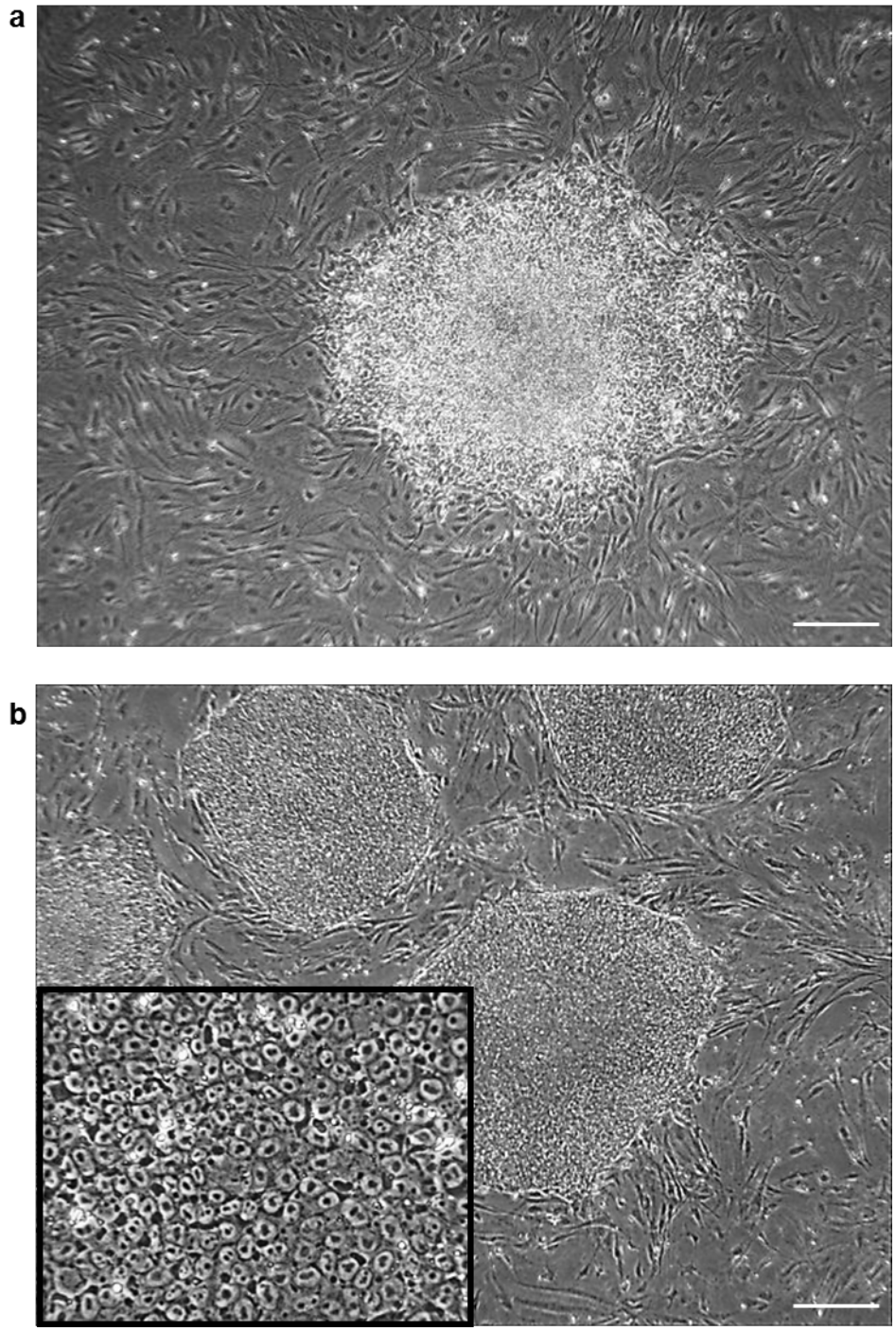


Figure 4-8: Phase- contrast micrographs of established UT-iPS colonies on a feeder layers. (a) Passage 1. (b) Passage 7, Inserts show higher magnification. Scale bars: 100 µm.

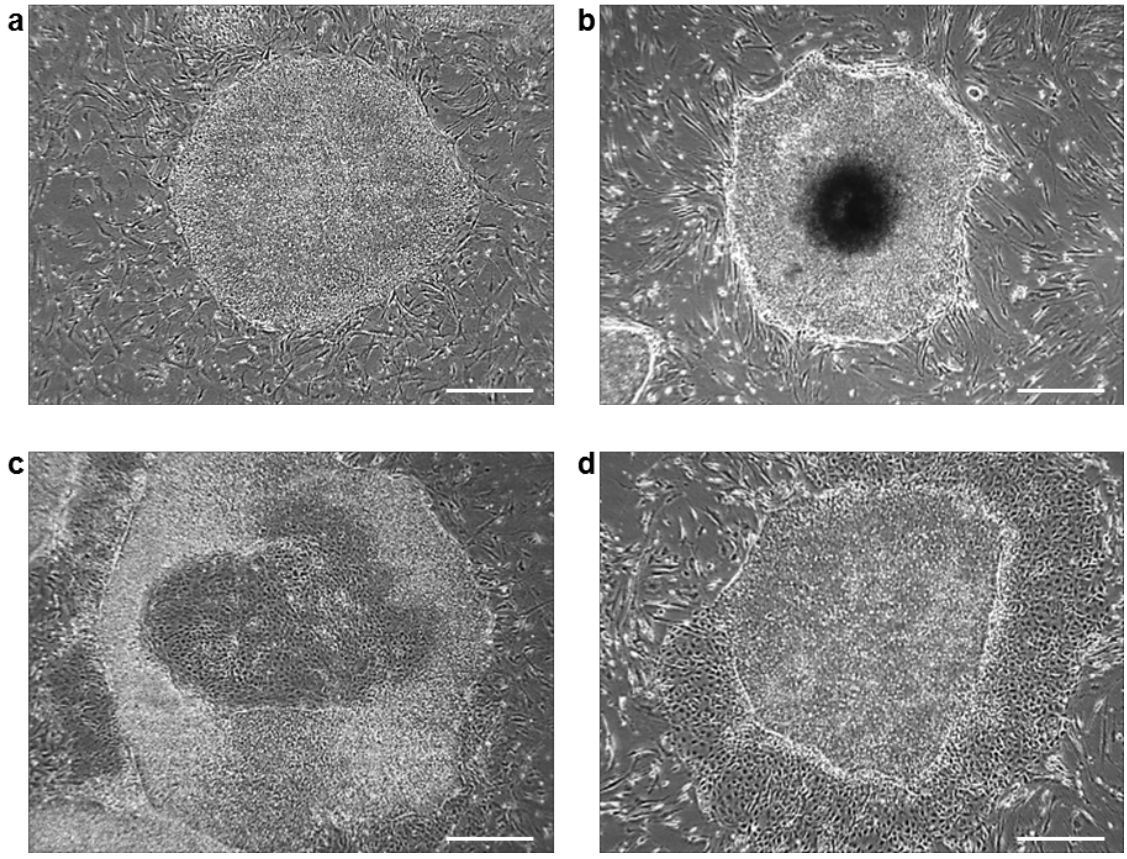


Figure 4-9: Visual inspection of human UT-iPS cell culture. (a) Example of undifferentiated UT-iPS colony. Types of differentiation observed during propagation of UT-iPS cell colonies vary, but they include: (b, c) Central differentiation and (d) Peripheral differentiation where an undifferentiated UT-iPS colony is surrounded by a ring of differentiated cells. Scale bars: 100 µm.

4.3.1.5.2 Genetic analysis of UT-iPS cells (Surface marker and gene expression)

Clones were expanded by standard human ES cell culture procedures on MEF feeder cells and gave rise to cell lines with human ES cell–like morphology. Three clones were selected for further analyses and the remaining clones were frozen and stored in liquid nitrogen.

I. Surface marker analysis in UT-iPS cells

Consistent with their human ES cell-like morphology, immunostaining using antibodies for the surface antigen stage–specific embryonic antigen SSEA-4, tumour rejection antigen TRA-1-81 and TRA-1-60 and also transcription factors OCT4, and NANOG showed positive staining for these iPS markers (Figure 4-10). Furthermore, alkaline phosphatase activity typical of an ES cell-phenotype was demonstrated in the induced cells (Figure 4-10).

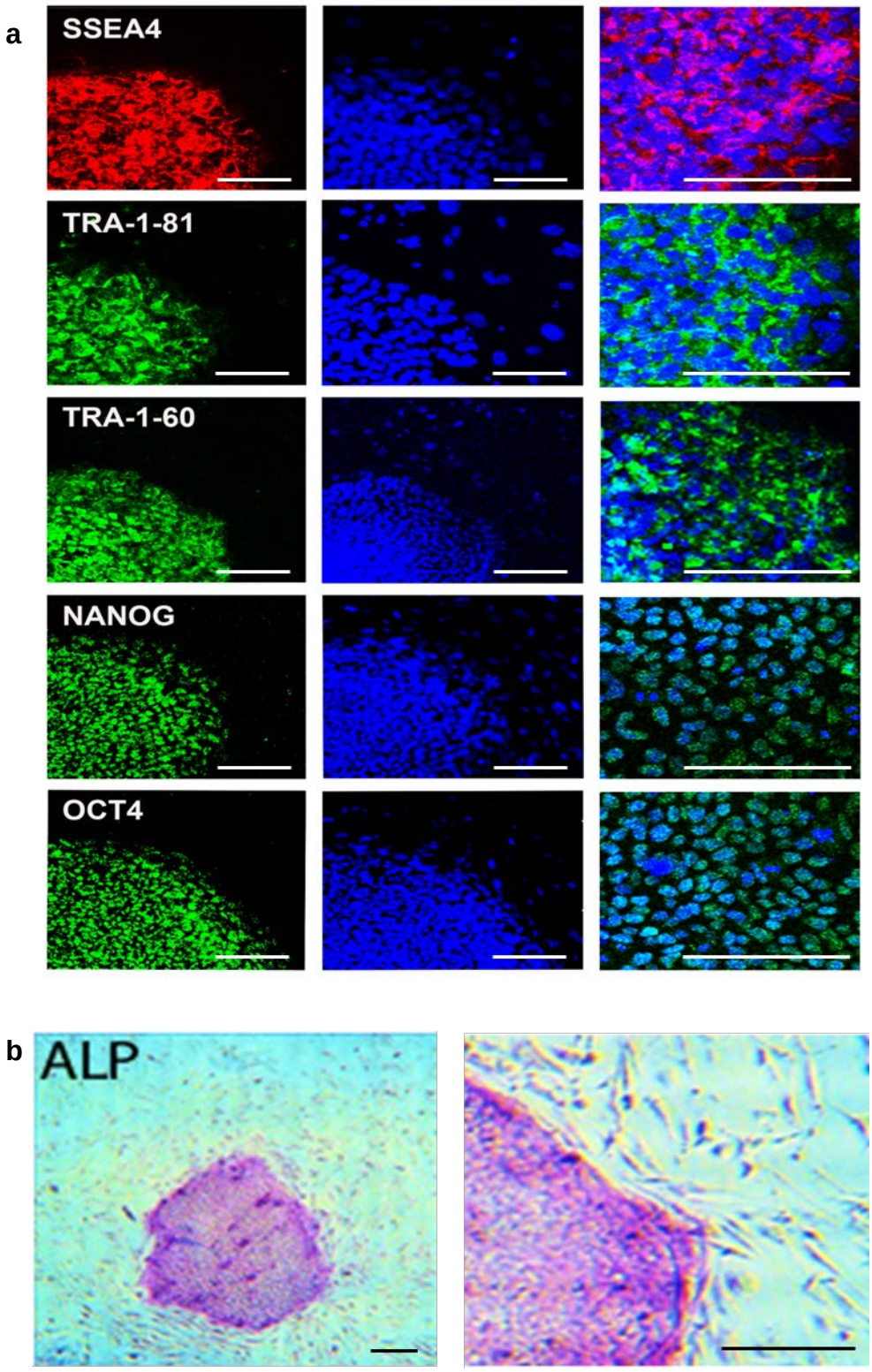


Figure 4-10: (a) Immunofluorescence of generated UT-iPS cells for the expression of specific human ES cell surface markers, SSEA-4, TRA-1-81, TRA-1-60, and nuclear transcription factors NANOG and OCT4. Nuclei were stained with DAPI (blue). (b) Alkaline phosphatase staining of UT-iPS cell colonies. MEF feeder cells served as the negative control. Scale bars: 100 µm.

II. Testing transgene expression in UT-iPS cells

Transgene silencing is associated with the generation of iPS cells, where there is a critical switch to endogenous expression of key ES cell regulatory factors such as *OCT4*, *SOX2* and *NANOG*. Expression level of the exogenous factors was analysed in the induced cells by real time-PCR using primers specific for lentiviral transcripts and demonstrated that transgenic expression of these defined genes had ceased in UT-iPS clones (Figure 4-11).

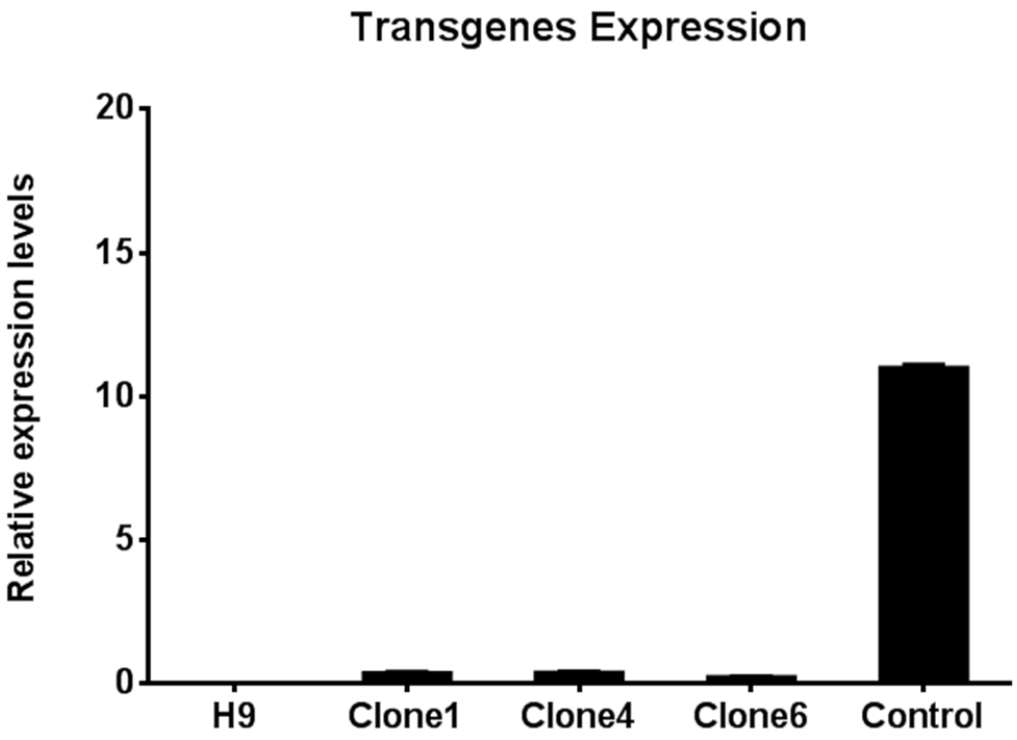


Figure 4-11: Real time-PCR using primers specific for the transgenes, and not detecting endogenous gene expression levels confirm lentiviral transgene silencing in UT-iPS cells (Passage 5). Control represents parental stromal cells 6 days after transduction. H9 human embryonic stem cell line was used as a negative control. Clone1, clone4, and clone6 are three different clones of UT-iPS cells. Error bars show SEM for N=3.

III. Transcript analyses

Furthermore, endogenous expression of the pluripotency markers *OCT4*, *SOX2*, and *NANOG*, was consistent with that of H9 human ES cells (cDNA was kindly donated by Prof. Majlinda Lako (Institute of Genetic Medicine, Newcastle University)) and markedly increased compared with that in the parental stromal cell population (Figure 4-12), whilst fibroblast lineage specific genes *α-SMA*, *calponin*, and *desmin* were down-regulated (Figure 4-13).

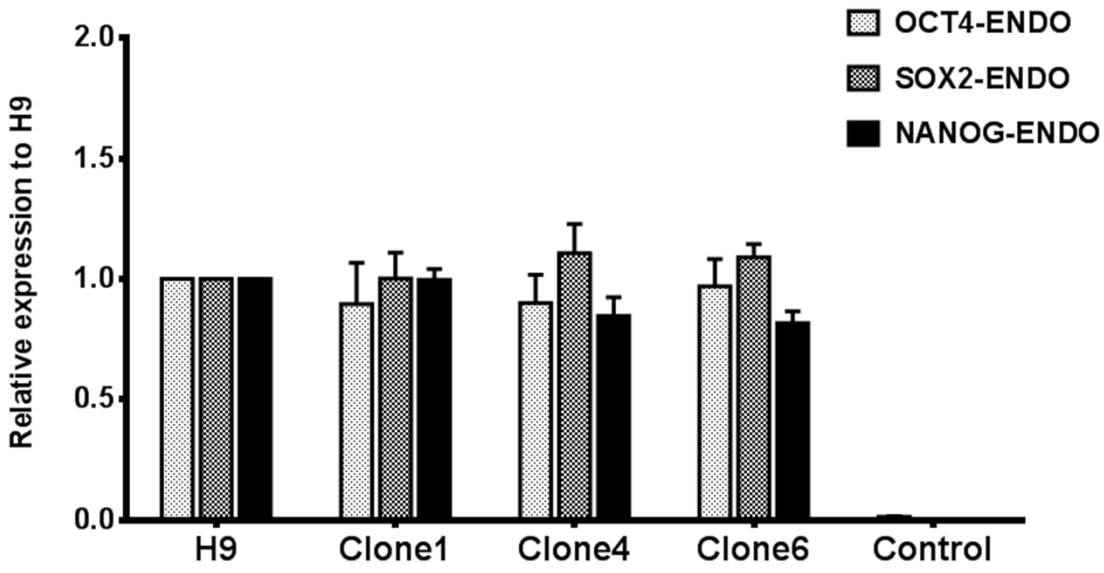


Figure 4-12: Real time-PCR analysis for expression of endogenous *OCT4* and *SOX2* and *NANOG* in three different clones of UT-iPS cells (Passage 5). Control represents parental stromal cells before transduction. All values were calculated with respect to the value for H9 human ES cell which was set to 1.

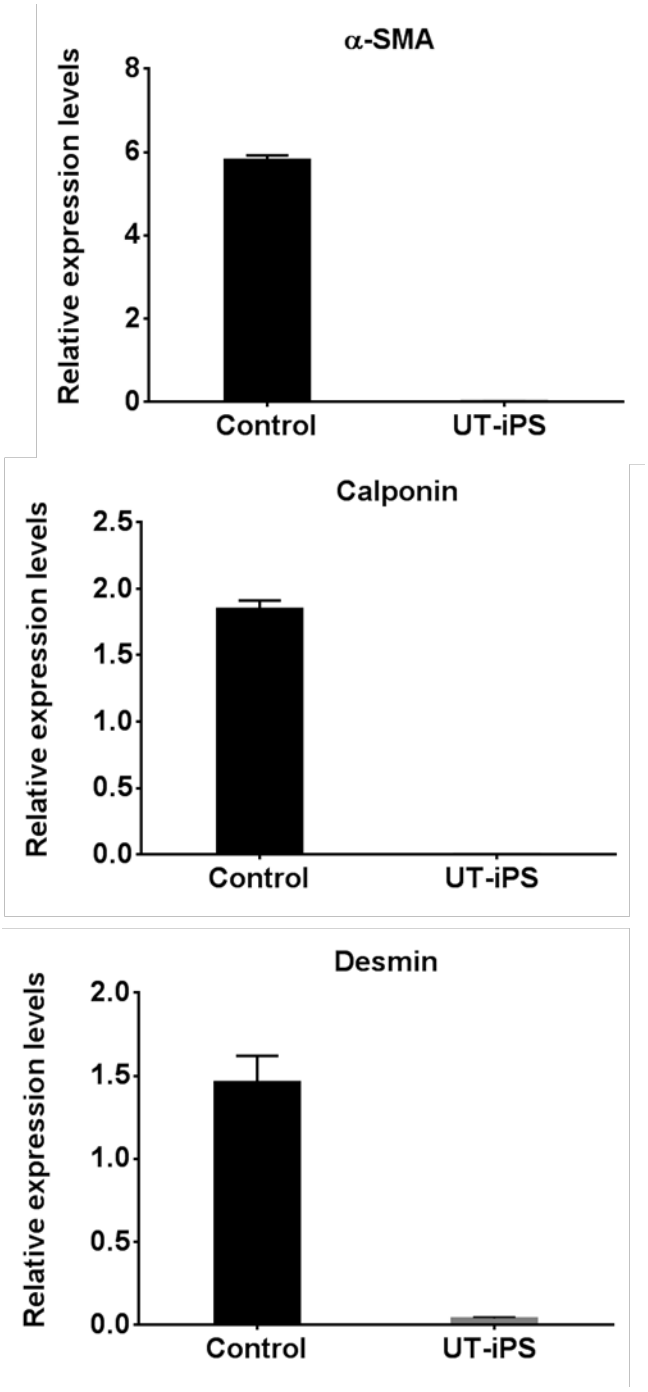


Figure 4-13: Real time-PCR analysis for fibroblast lineage specific genes *α-SMA*, *calponin*, and *desmin* in UT-iPS cells (Passage 5). Control represents parental stromal cells before transduction. Error bars show SEM for N=3.

IV. Expression of other pluripotent transcripts in UT-iPS cells

A recent publication reported that the expression of *DNMT3B*, *GDF3*, and *REX1* markers can distinguish the *bona fide* iPS cells from partially reprogrammed cells (Chan *et al.*, 2009a). The generated UT-iPS clonal cells expressed these markers at comparable levels to human ES cells, confirming their full reprogramming capacity (Figure 4-14). The UT-iPS cell lines derived from bladder and ureter were shown to be identical in terms of ES cell-like morphology, proliferation and gene-expression signatures.

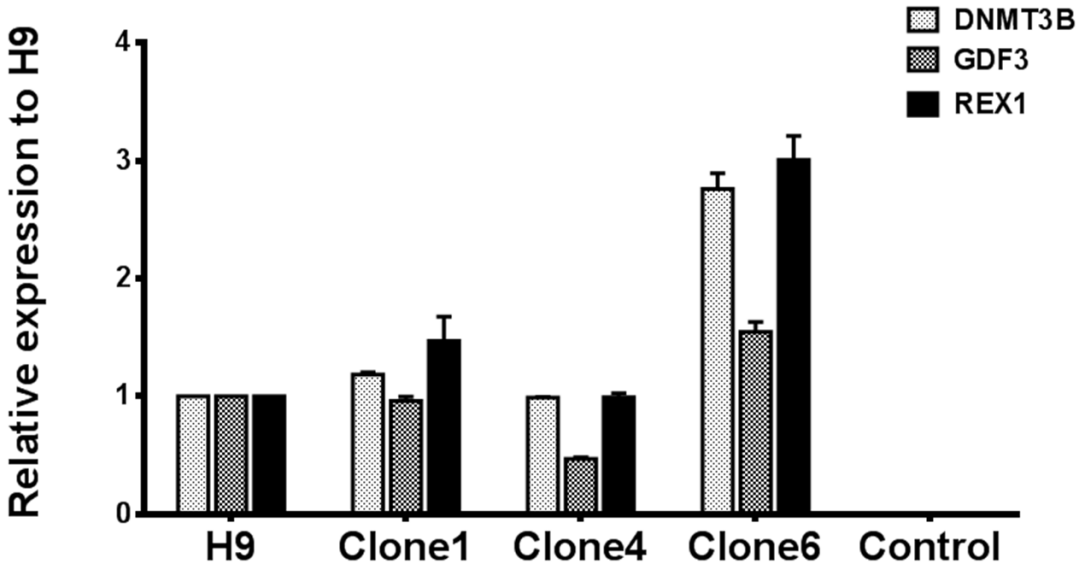


Figure 4-14: Real time-PCR analysis of stem cell marker genes in three different clones of UT-iPS cells for expression of *DNMT3B, GDF3,* and *REX1*. Control represents stromal cells before transduction. All values were calculated with respect to the value for H9 human ES cell which was set to 1.

4.3.1.5.3 Identity testing of UT-iPS cells

Authentication of UT-iPS cells derivation from parental stromal cells was confirmed using DNA fingerprinting. The patterns of 16 short tandem repeat (STR) markers were completely identical between UT-iPS clones and the parental stromal cells. In addition, these patterns differed from skin-iPS line that was cultured concurrently in the laboratory ruling out any contamination of our iPS cell lines by pre-existing skin-iPS cells (Table 4-2).

Name	Parental cells	UT-iPS	Skin-iPS
Amelogenin	XY	XY	XY
D3S1358	12 17	12 17	17 18
THO1	6 9.3	6 9.3	6 9
D21S11	29 30	29 30	29 30
D18S51	13 15	13 15	12 13
PentaE	5 15	5 15	7 12
D5S818	10 12	10 12	11 12
D13S317	11 13	11 13	9 9
D7S829	8 9	8 9	10 11
D16S539	10 13	10 13	11 11
CSF1PO	12 12	12 12	10 12
PentaD	9 10	9 10	10 10
vWA	14 14	14 14	16 17
D8S1179	12 12	12 12	13 15
TPOX	8 10	8 10	8 9
FGA	21 24	21 24	21 27

Table 4-2: DNA fingerprinting showing that UT-iPS cells have DNA genetic profiles matched to their parental stromal cells and differ from that of pre-existing skin-iPS cells.

4.3.1.5.4 Karyotyping of UT-iPS cells

Karyotyping was performed to demonstrate that the generated UT-iPS cells maintain a stable karyotype after serial passages. UT-iPS cells showed a normal diploid 46, XY chromosome arrangement with the absence of any aneuploidy (loss or duplication of chromosome) and heteroploidy (having abnormal numbers of chromosomes) (Figure 4-15).

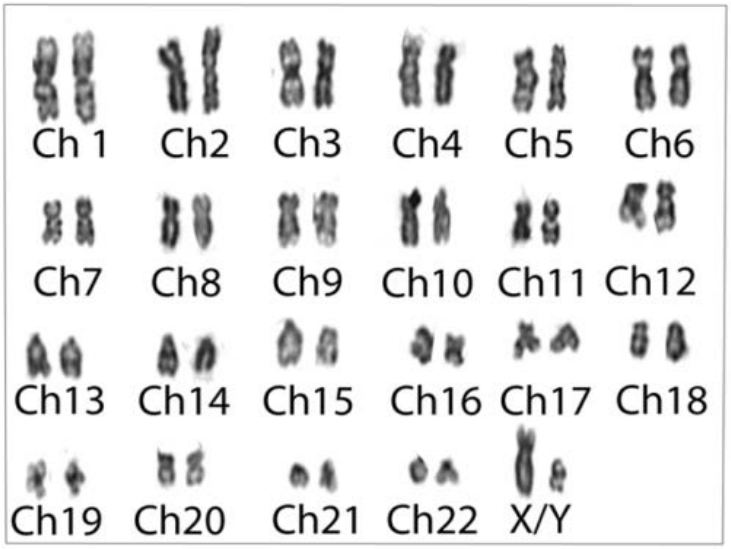


Figure 4-15: Karyotype analysis shows normal karyotype of established UT-iPS cells at passage 25.

4.3.1.6 In vitro differentiation capacity of UT-iPS cells

As mentioned before, the pluripotent stem cells are characterized by their ability to differentiate into representative derivatives of all three embryonic germs. Although the iPS cells were very similar to human ES cells with respect to morphology, proliferation, antigen markers and gene expression markers, it remained unclear whether these generated cells are true pluripotent cells with full differentiation capacity. To investigate this, the differentiation potential of these putative iPS clones was evaluated *in vitro* using floating cultivation to form embryoid bodies (EBs). Typically, within a suspension culture, pluripotent stem cells aggregate and form three-dimensional structures or spheroids called EBs because they mimic many features of normal embryonic development. UT-iPS cells formed ball-shaped EB-like structures after 8 days in suspension culture with differentiation medium (Figure 4-16). These EBs were transferred to gelatin-coated cell culture plates and cultured for a further 8-10 days.

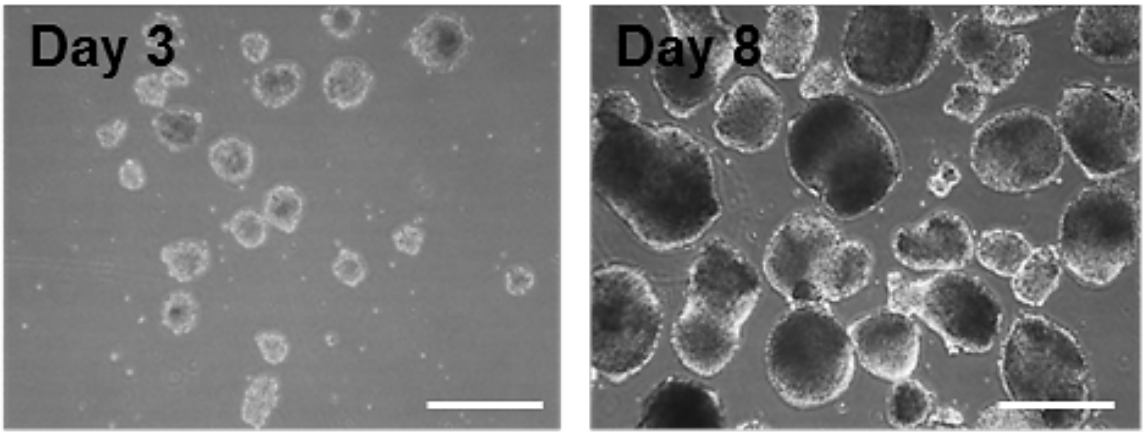


Figure 4-16: UT-iPS cells formed EBs in suspension culture. Phase- contrast micrographs of EBs created by human UT-iPS cells at day 3, and 8. Scale bars: 500 µm.

The ability to differentiate to cells derived from three germ-cell layers was examined by immunofluorescence. Outgrowth cells were detected to be positive for CD31 (mesoderm marker), alpha-fetoprotein (endoderm marker), and βIII tubulin (ectoderm marker) (Figure 4-17).

Differentiation to three germ-cell layers was further confirmed by real time-PCR. Differentiated cells showed marker gene expression for all three embryonic germ layers: *alpha-fetoprotein* (endoderm marker), *PAX6* (ectoderm marker), and *α-SMA* and *vimentin* (mesoderm marker) (Figure 4-18).

Moreover, following iPS cells differentiation into the three germ layers, the pluripotent transcripts should be down regulated, otherwise potential integrated proviruses might be present (Stadtfeld *et al.*, 2008b). UT-iPS embryoid-body derived cells showed down regulation of the endogenous expression of *OCT4*, *SOX2*, and *NANOG* (Figure 4-19).

Although integrated transcription factors become transcriptionally silenced over time, spontaneous reactivation of these exogenous factors during cell culture and upon differentiation into various lineages have been reported (Nakagawa et al., 2008; Shao and Wu, 2010). Therefore the expression of the exogenous factors in UT-iPS embryoid-body derived cells was analysed by real time PCR and this demonstrated that transgenic expression of these defined genes is still silenced (Figure 4-20). Together, these results indicate that these ES cell-like colonies not only expressed pluripotency markers, but also could differentiate into ectoderm, mesoderm, and endoderm-derived germ layers *in vitro*.

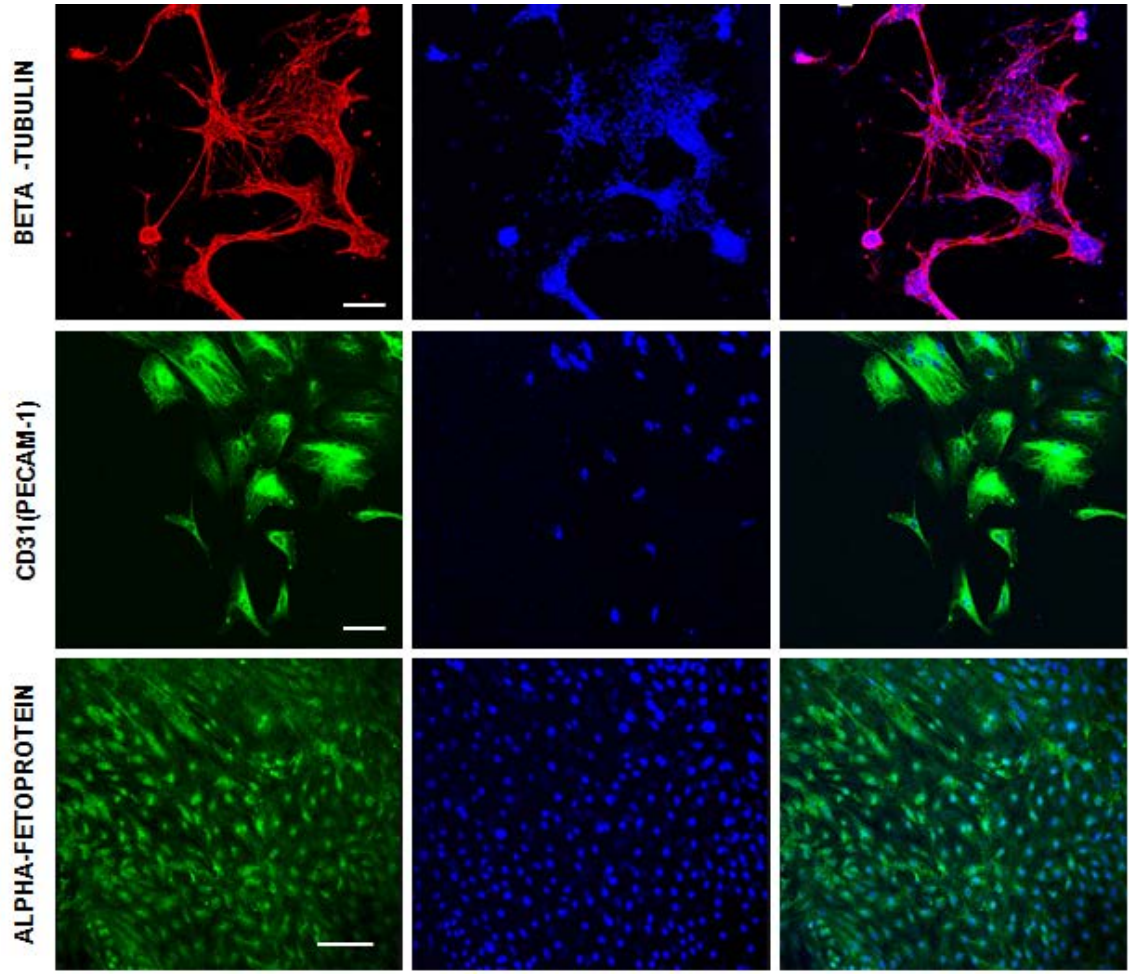


Figure 4-17: Characterisation of Pro-iPS embryoid body differentiation through immunofluorescence staining. Immunofluorescence analysis of EBs derived from UT-iPS cells shows expression of the lineage markers beta-tubulin (ectodermal marker; Red), CD31 (mesodermal marker; Green) and alpha-fetoprotein (AFP) (endodermal marker; Green). Nuclei were stained with DAPI (blue) (scale bar = 100µm).

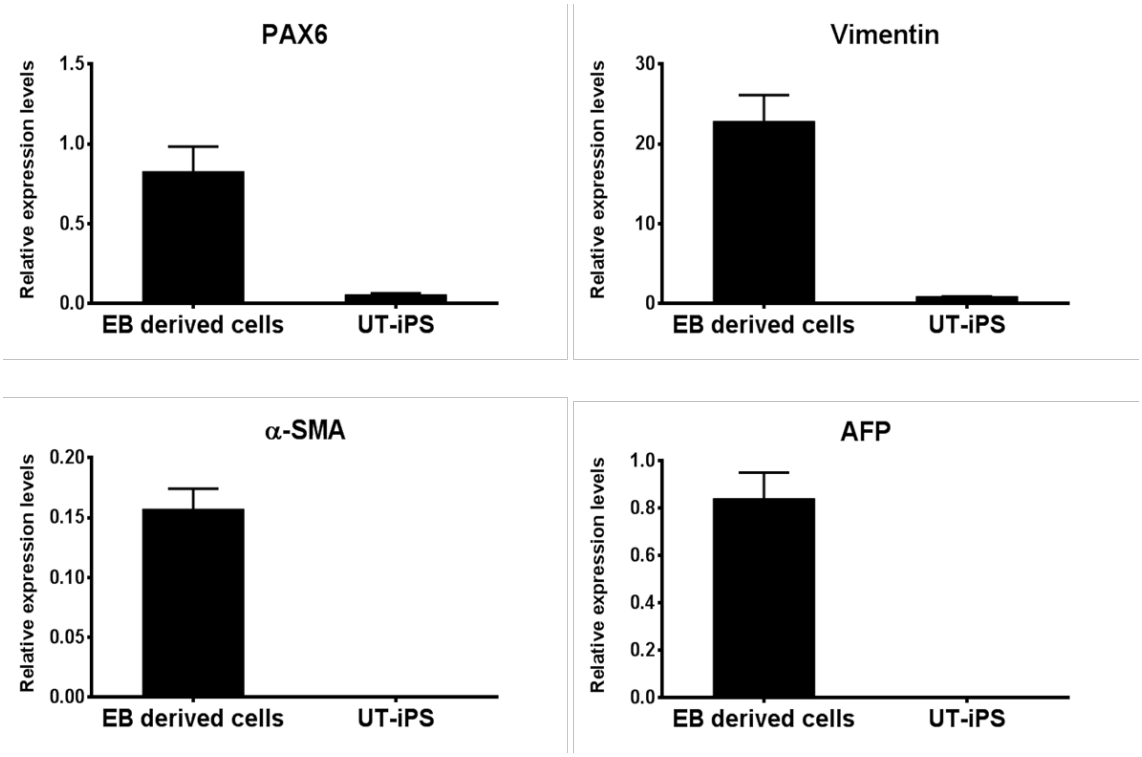


Figure 4-18: UT-iPS derived embryoid bodies differentiate into cells of ectodermal, mesodermal, and endodermal lineage. Real time-PCR analysis for mRNA expression of *PAX6*, and vimentin (ectodermal marker), *α-SMA* (mesodermal marker), and *AFP* (endodermal) in EBs derived from UT-iPS cells compared to undifferentiated UT-iPS cells. Error bars show SEM for N=3.

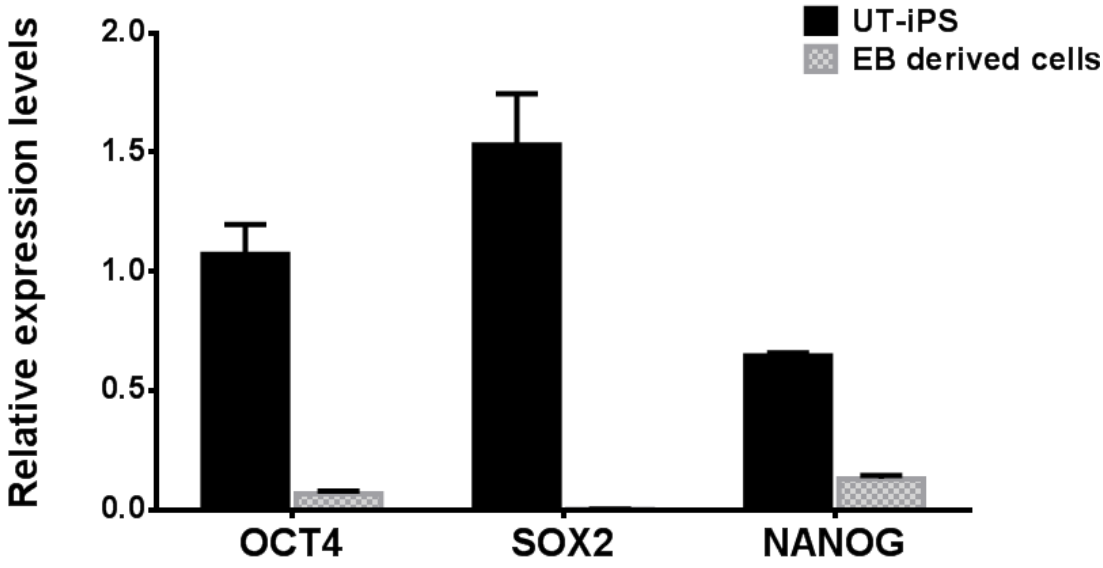


Figure 4-19: Real time-PCR analysis for endogenous expression of *OCT4*, *SOX2*, and *NANOG* in EBs derived from UT-iPS cells shows down-regulation of these markers. Error bars show SEM for N=3.

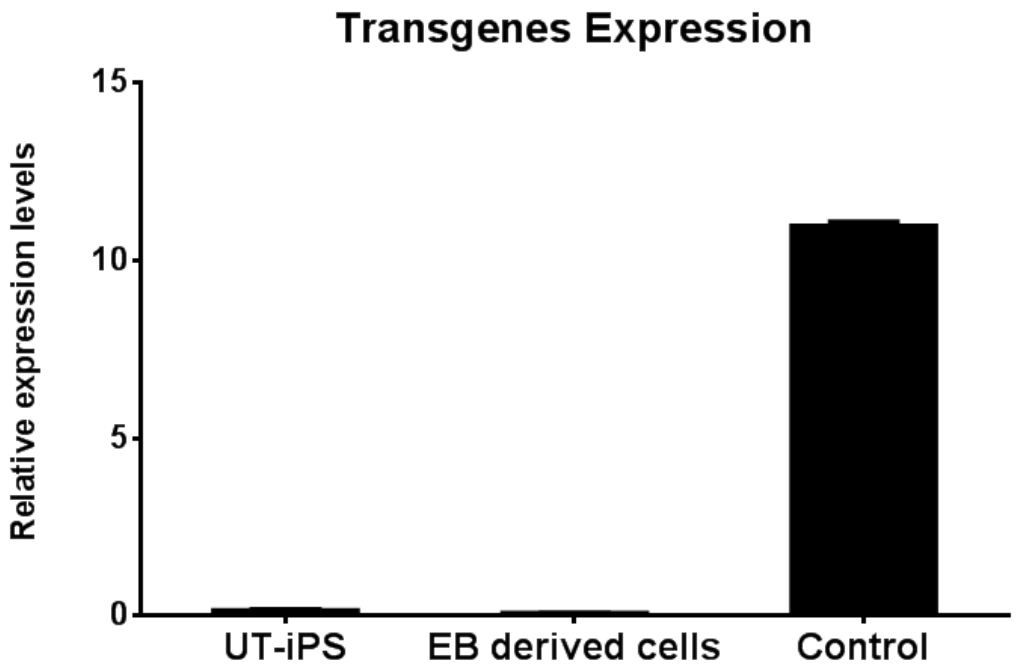


Figure 4-20: Real time-PCR using primers specific for the transgenes confirm that lentiviral transgene is still silenced in EBs derived from UT-iPS cells (week 4). Control represents parental stromal cells 6 days after transduction. Error bars show SEM for N=3.

4.3.1.7 In vivo differentiation capacity of UT-iPS cells

The most instructive *in vivo* test of pluripotency is the ability to contribute to all cell types, including germ cells, in chimeric offspring generated by mixing ES cells with mouse blastocysts (Evans and Kaufman, 1981; Martin, 1981). However, due to obvious ethical concerns, human ES cells cannot be subjected to the same definitive test. Therefore, the gold standard to assess the developmental potency of human ES cells is the formation of teratomas in immunodeficient murine hosts.

Approximately 5 x 10^5 UT-iPS cells for three clones were injected subcutaneously to immunodeficient NSG mice. Similarly, 5 x 10^5 human ES cells (H9) were used as a positive control. Teratomas that developed from grafted UT-iPS cells were surgically removed, fixed in Bouin's, and paraffin embedded. The tissues in the teratoma were analyzed histologically and confirmed that UT-iPS cells formed tissues derived from all three embryonic germ layers confirming their pluripotency. Areas of germ layer specific differentiation were evident with sections containing structures consistent with endoderm, mesoderm and ectoderm lineages (Figure 4-21).

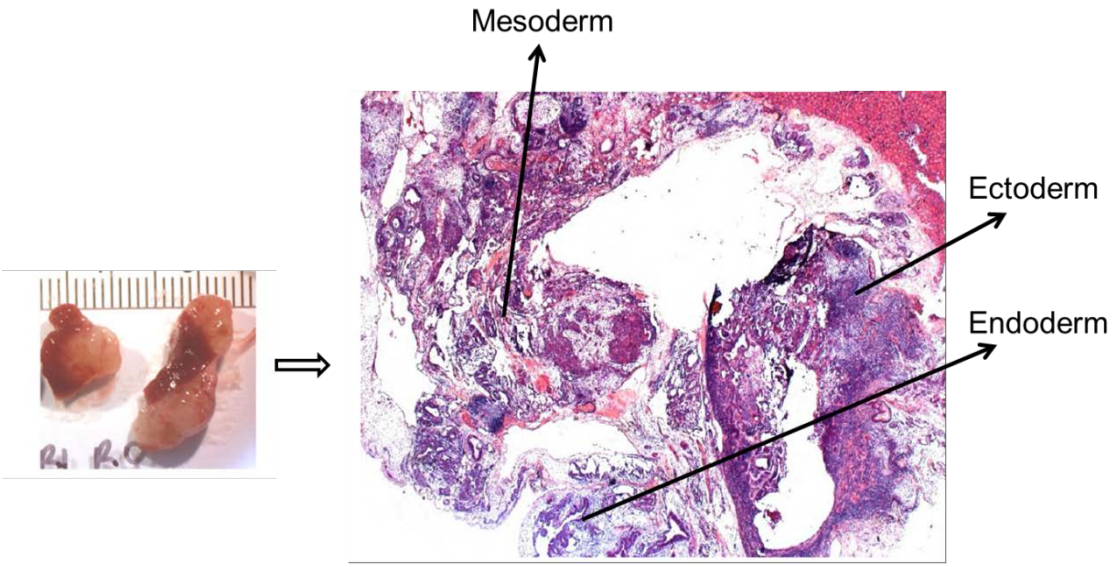


Figure 4-21: Histological sections of identified cells within teratoma formed by UT-iPS cells representing all three embryonic germ layers: ectoderm (neuronal rosette-like structures), endoderm (intestinal epithelial-like cells) and mesoderm (muscle-like tissue). The histopathological analyses was kindly performed by Prof. Simon Hayward, Vanderbilt University, USA.

4.3.2 Transduction of human urothelial cells

Urothelial cells were cultured as described in the previous chapter. Once confluent, the media was replaced with freshly prepared transduction media containing the OSKM 4 in 1 construct in the presence of polybrene 10 µg/ml final concentration. Prior transduction, mRNA expression of cell lineage markers (*α-SMA; CD90; CD24; CD45;* and *vWF*) was assessed in urothelial cells by real time-PCR to confirm their purity (Figure 4-22).

All samples expressed the epithelial cell marker, *CD24* while no expression of stromal, haematopoietic and endothelial cell markers was detected. Urothelial cells were transduced using the same protocol as for stroma cells and at day 14 post-transduction, small and dense colonies were observed (Figure 4-22).

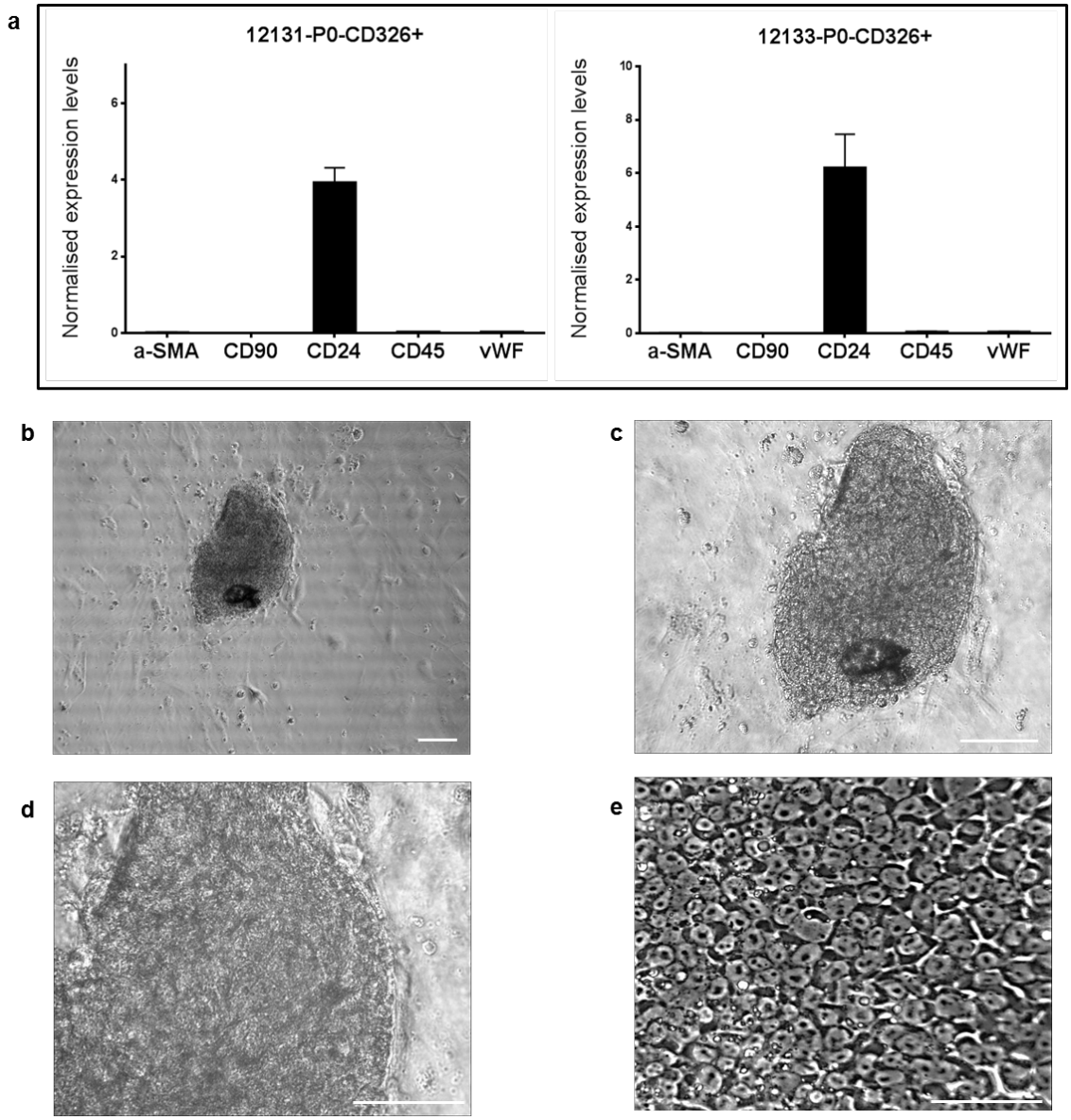


Figure 4-22: Transduction of human urothelial cells. Urothelial cells were transduced with OSKM lentivirus vectors, and plated onto MEFs feeder cells in human ES cell medium. (a) Real time-PCR for mRNA expression of *α-SMA* and *CD90* (stromal cell markers), *CD24* (epithelial cell marker), *CD45* (haematopoietic cell marker), and *vWF* (endothelial cell marker) for two samples of urothelial CD326+ cells at P0. (b, c, d). Phase-contrast photomicrographs of urothelial colonies in ES cell environment at different magnifications. These cells didn't exhibit ES/iPS cells morphological features including small round cells with large nuclei, notable nucleoli, and spaces between cells. (e) Control phase-contrast photomicrograph of UT-iPS cells. Scale bars: 100 µm (b, c), 50 µm (d, e).

4.3.3 Live- cell staining of transduced urothelial cells

A live-cell staining of urothelial colonies using anti SSEA-4 and TRA-1-60 antibody was performed in conjunction with Hoechst 33342 nuclear staining, as previously described. SSEA-4 expression is particularly interesting as it seems to be expressed more rapidly than other commonly used markers (Enver et al., 2005; Stewart et al., 2006) while the TRA-1-60 antigen exhibits an intermediate behaviour (Draper et al., 2002). Moreover, these markers are located on the exterior surfaces of the cell membrane; therefore they can be detected by live cell imaging and allow us to culture these colonies following the staining process and further observe their progress over time without damaging the cells. Interestingly, analysis of the photomicrographs revealed that some of these colonies exhibit SSEA-4 and TRA-1-60 positive phenotype (Figure 4-23).

Although it was expected that these colonies would grow, no such proliferation was observed. Even after dividing into smaller clumps and transferring to fresh MEFs, it was not possible to detect any noteworthy proliferation in the resulting clumps. These nascent reprogrammed cells might fail to reach the fully reprogrammed state and possibly reverted back or simply died. Since these colonies were easily distinguishable by behaviour from human ES cell–like iPS colonies, it was decided that these would be categorized as either partially re-programmed or abortive colonies.

On the other hand, control cells (un-transduced cells) started to form colonies on the MEFs feeder layer at day 5-7 after plating (Figure 4-24). However, staining for SSEA-4 and TRA-1-60 was undetectable in any of these colonies (Figure 4-25). Once substantial-sized colonies were noticed, these were split into smaller fragments and transferred to fresh MEFs. However, they didn’t grow as fast as the parental colonies and remained negative for SSEA-4 and TRA-1-60. Most of these colonies eventually died or stopped proliferating.

Alternatively, epithelial cells were directly seeded after MACS sorting onto MEFs in human ES cell medium and once substantial-sized colonies were noticed these were transduced with the OSKM 4 in 1 construct and 10 µg/ml of polybrene. Growing prostate epithelial cancer cells in such an environment has been shown to upregulate the transcription factors (OCT4, SOX2, NANOG) in

these cells which in turn might increase the reprogramming efficiency (Anastasia Hepburn, NICR, unpublished data). In addition, this may avoid the cells the stress resulting from replating process. After 48 hours of transduction, the media was replaced with fresh human ES cell medium and the cells were cultured in this media for another 4 weeks. No iPS colonies were noted.

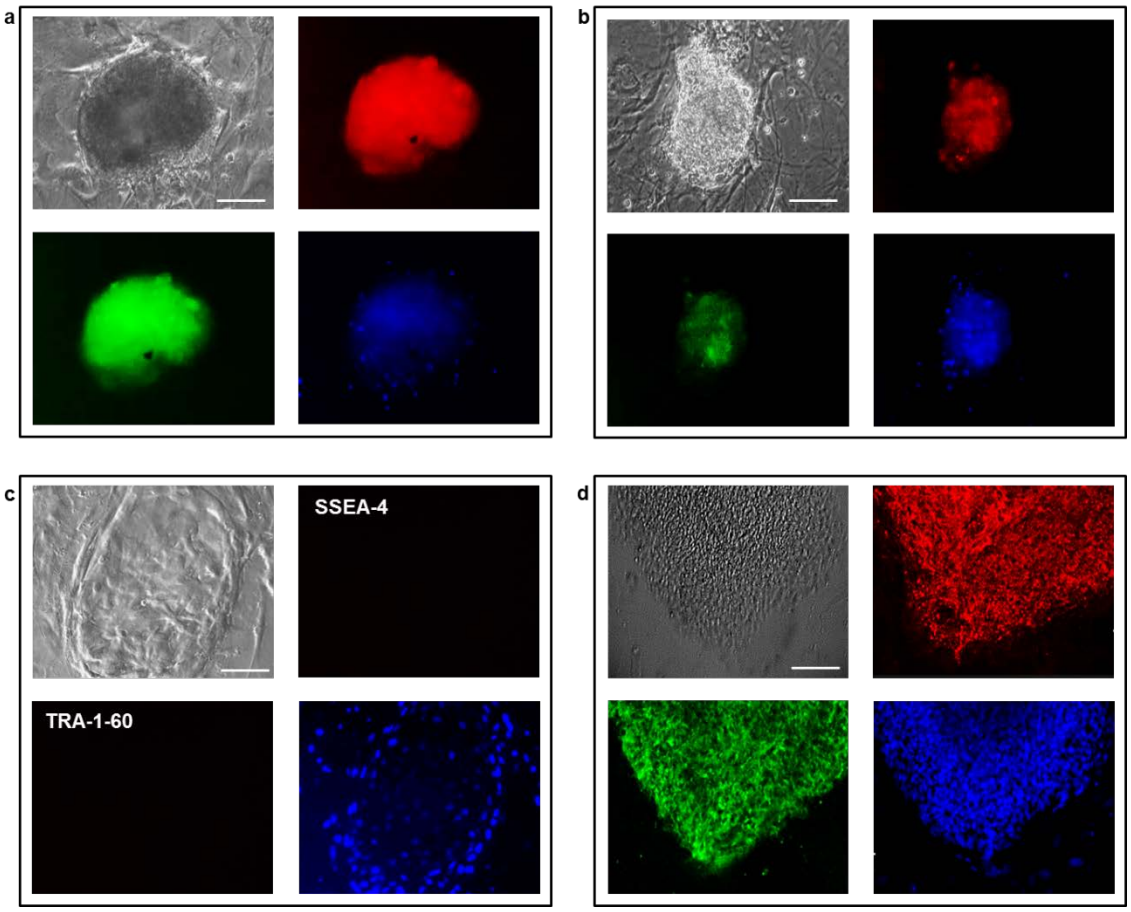


Figure 4-23: Live cell imaging analysis of transduced urothelial cells (12165). (a) Day 14. (b) Day 30 post transduction. (c) Primary urothelial cells (negative control). (d) Human skin iPS cell colony (positive control). Colony was analysed by phase contrast (top left) and fluorescence microscopy for expression of pluripotency markers SSEA-4 (red), and TRA-1-60 (green); nuclei were stained with Hoechst 33342 (blue). Scale bars: 100 µm (a, b), 50 µm (c, d).

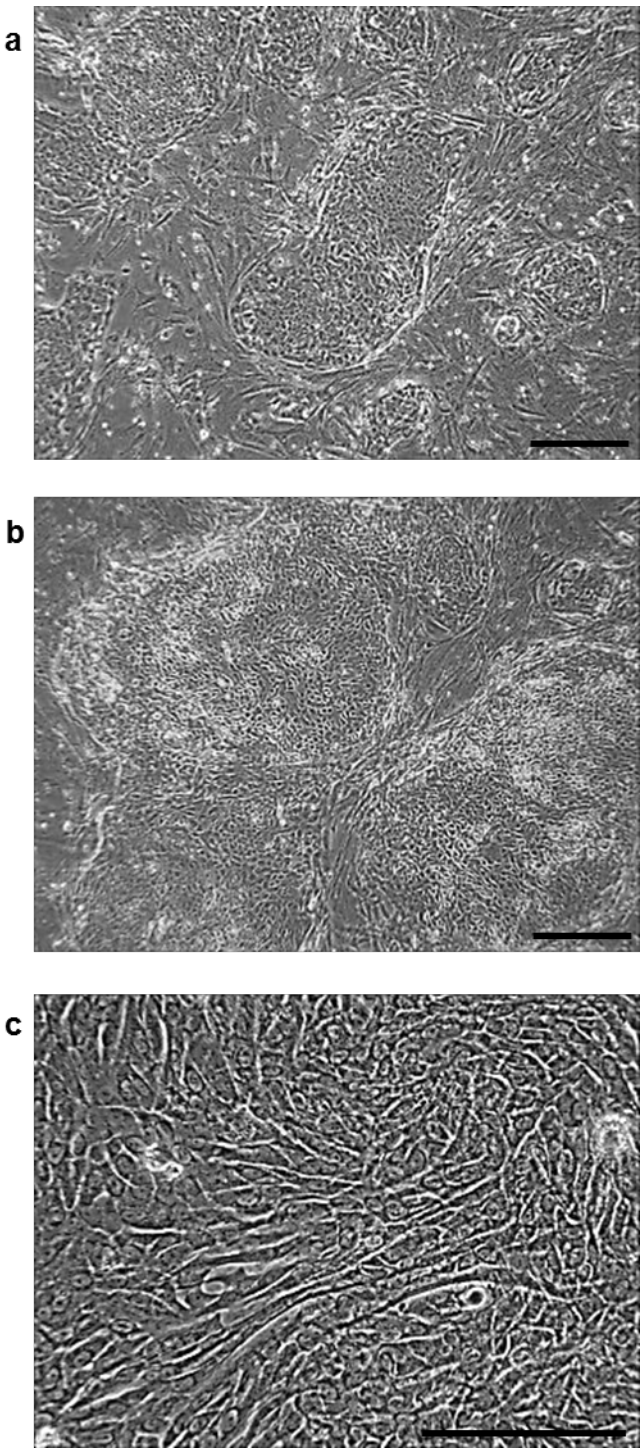


Figure 4-24: Phase-contrast photomicrographs of colonies formed by untransduced urothelial cells cultured in ES cell environment. (a) Day 8. (b) Day 15. Colonies on low power magnification showed tight borders and smooth surface. Scale bars: 100 µm.

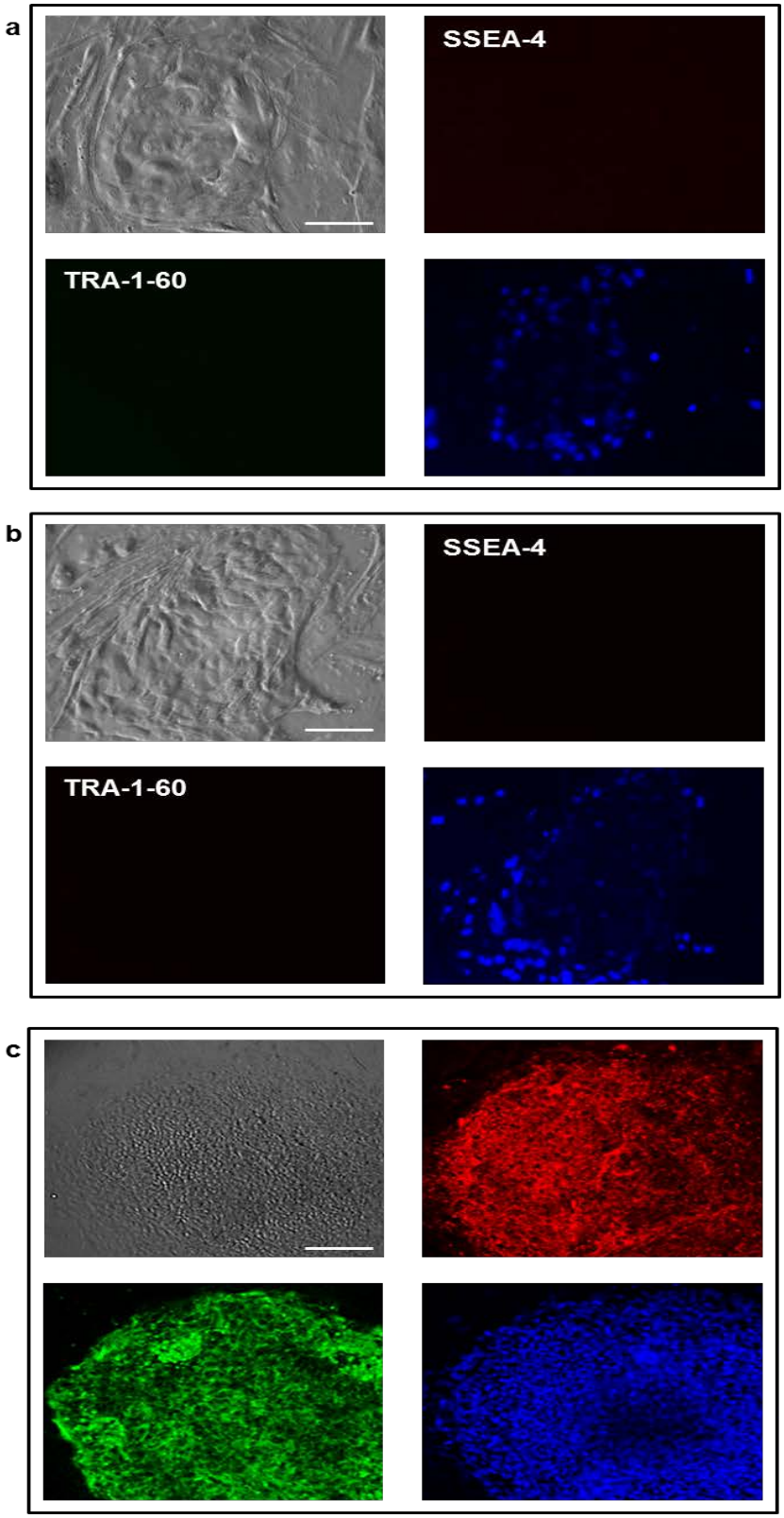


Figure 4-25: Live cell imaging analysis of untransduced urothelial cells. (a) Day 10. (b) Day 20 post transduction. (c) Human skin iPS cell colony (positive control). Colony was analyzed by phase contrast and fluorescence microscopy for expression of pluripotency markers SSEA-4 (red), and TRA-1-60 (green); phase contrast (top left), nuclei were stained with Hoechst 33342 (blue). Scale bars: 50 µm.

4.4 Discussion

Since iPS cells may retain an epigenetic memory of their original cell types (Chin *et al.*, 2009; Marchetto *et al.*, 2009; Kim *et al.*, 2010), it is important to establish human iPS cells from different tissue origins and compare their differentiation potentials. In this chapter successful reprogramming of adult human stromal cells isolated from both bladder and ureter into iPS cells (UT-iPS) is successfully described following the method developed in the Yamanaka laboratory, with modifications described by Tilgner and colleges (Katarzyna Tilgner, 2010). In addition to exhibiting ES cell morphology, the newly derived UT-iPS cells highly expressed the pluripotency markers OCT4, SOX2, NANOG, SSEA-4, TRA-1-60 and TRA-1-81 and alkaline phosphatase, showed efficient transgene silencing and maintained a normal diploid karyotype. It should be noted that karyotyping relies on G-band quality and resolution and poor quality chromosomes increase the risk of missing small subtle abnormality may not detect mosaicism. Therefore more sensitive technique such as CGH Microarray testing (array CGH), MLPA (multiple ligation-dependent probe amplification) or FISH (fluorescence in-situ hybridisation) that can detect copy number change (deletion or duplication) in the genome at a higher resolution than G-band analysis should be performed in the future.

Furthermore, human UT-iPS cells showed functional pluripotency by the generation of endodermal, ectodermal and mesodermal lineages *in vitro* and *in vivo*. UT-iPS colonies were morphologically selected by microscopic observation, which is consistent with previous reports that drug selection with pluripotent markers is not essential for iPS cells derivation (Meissner *et al.*, 2007; Nakagawa *et al.*, 2008; Ohnuki *et al.*, 2009). This result is also consistent with the findings that reprogramming to pluripotency is a slow and gradual process where cells that have not yet reached the pluripotency state may be eliminated by drug selection. In addition, using a selection system will need genetic modification which may constitute a potential drawback for therapeutic application of similar approaches (Lyssiotis, 2009).

Yamanaka in his original work used multiple individual retroviral vectors to deliver each transcription factor to generate iPS cells (Takahashi and Yamanaka, 2006; Takahashi *et al.*, 2007) which can lead to high number of

genomic integrations. In addition, each of the reprogramming factors was individually integrated into different sites within the genome leading to an increased risk of gene mutagenesis and genomic instability. In this project, to avoid these problems, cells were transduced using a single, self-inactivating (SIN), polycistronic lentiviral vector encoding for the four transcription factors separated by 2A sequences with a loxP site in truncated 3` LTR and controlled by the EF1α promoter. This system improves the reprogramming efficiency and silencing of transduced exogenous and ensures that all transduced cells will receive equal amounts of each of the four transcription factors (Shao *et al.*, 2009). Most importantly the integrated provirus can be deleted from the iPS cell genome through transient expression of Cre-recombinase in transduced cell and therefore allows the derivation of transgene-free human iPS cells (Oh *et al.*, 2012; Awe *et al.*, 2013). Although C-MYC is a cancer-causing gene which is known to cause death and differentiation in human ES cells (Sumi *et al.*, 2007); it markedly increased the reprogramming efficiency and OSKM factors have been shown to be more efficient to reprogram human fibroblasts compared to OSK, and OSLN (Robinton and Daley, 2012). Hence, OSKM set was used for the establishment of UT-iPS cells.

5 Chapter 5. Induced differentiation of bladder specific cells from UT-iPS cells in vitro

5.1 Introduction

Pluripotent stem cells are functionally characterized by their ability to self-renew and differentiate into all cell types derived from all 3 germ layers. In this project, the generated UT-iPS cells are proven to be pluripotent and must be able to differentiate to any cell type including urothelial and stromal cells. However, recent evidence revealed that during the reprogramming process, iPS cells retain an epigenetic memory of the starting cell type that increase their propensity to redifferentiate back into the parental cell types (Kim *et al.*, 2010; Bar-Nur *et al.*, 2011; Ohi *et al.*, 2011; Lee *et al.*, 2012; Xu *et al.*, 2012), the exact mechanisms behind this donor memory are not fully understood. This raises the question whether UT-iPS cells will exhibit higher propensity to differentiate *in vitro* into bladder lineages as compared to classical skin-derived iPS cells. To address this question, both UT-iPS and skin-iPS cells were differentiated using the protocol described by Tian *et al* with minor modifications (Tian *et al.*, 2010b).

5.2 Aim:

To investigate the basic ability of the UT-iPS cells to differentiate into urothelial and stromal like cells by using CM derived from bladder cells and comparison with conventional skin derived iPS cells for potential use in urological tissue engineering and regeneration.

5.3 Results:

Primary urothelial and stromal cells from urinary bladders or ureters were isolated and cultured in accordance with the methods described in the previous chapter. Two different protocols were used to induce the differentiation of UT-iPS and skin-iPS cells into urothelial and stromal cells (Figure 5-1).
UT-iPS cell were collected as clumps and cultured on low adhesion dishes in a urothelial cell-derived CM (U-CM) collected from cultured human urothelial cells and alternatively on a stromal cell-derived CM (S-CM) collected from human urinary tract stromal cells where they were observed to round up into embryoid

body-like masses and were referred to as bcm-EB (bladder conditioned medium embryoid body). After 10 to 14 days of suspension culture, bcm-EBs were transferred onto gelatin coated plates and further cultured in the same CM for another 3 weeks. The other protocol used was previously described by Tian et al (Tian *et al.*, 2010b), but with minor modifications. Briefly, iPS cells were initially plated in a 6-well plate for 5-7 days in human ES cell medium. Then 70-80% confluent iPS cell cultures were washed with 1XPBS and incubated in various differentiated media for a further 2 weeks. For urothelial cell differentiation, U-CM was collected from cultured human bladder urothelium and diluted to one-third volume with fresh DMEM. For stormal cells differentiation, S-CM was collected from cultured human bladder stromal cells and diluted with an equal volume of RPMI1640 10% FCS. The medium was changed at 24 h prior to collection and the filtered CM was also stored at -80^{o}C to retain the biological activity of the secreted factors. Differentiated cells were analysed for a panel of urothelium and stromal smooth muscle specific genes using real time-PCR. The expression of specific markers in the differentiated cells derived from UT-iPS cells and skin-iPS cells were compared after the same planned period of culture and using the same CM. UT-iPS that were not induced were processed in parallel as a negative control.

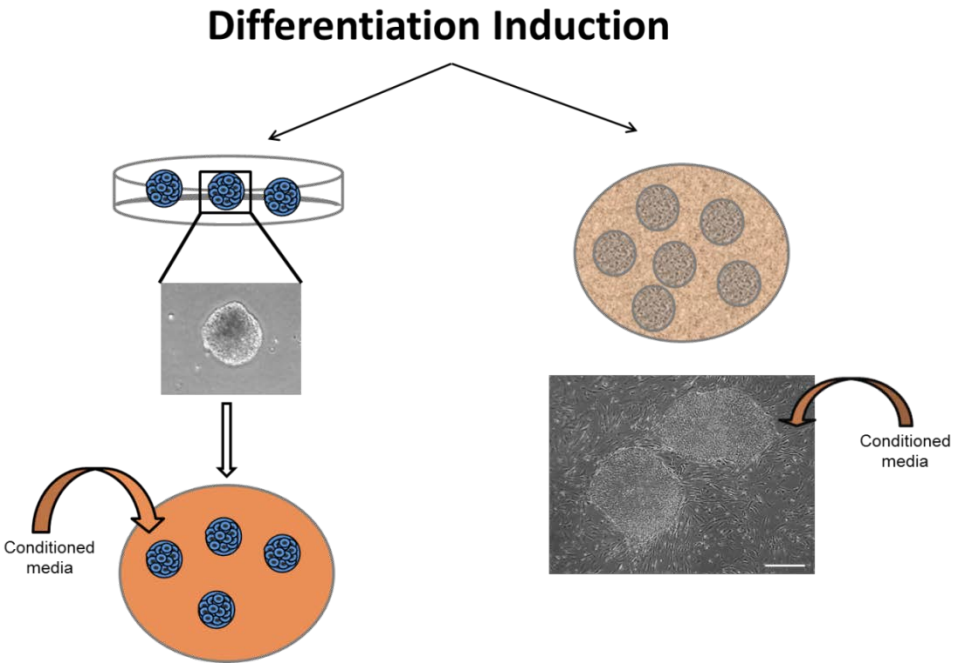


Figure 5-1: General outline of the two protocols used for iPS differentiation. Scale bars: 100 µm

5.3.1 Characteristics of newly differentiated urothelia/stroma-like cells

Morphological examination of the differentiation cells derived from UT-iPS cells showed characteristically small, epithelioid cells. To investigate the ability of generated UT-iPS to differentiate into bladder tissues, the mRNA expression of urothelium differentiation specific genes (UPIb, UPII, UPIIIa, UPIIIb, CLD1, CLD5, and CK7) and stromal smooth muscle cells specific markers (a-SMA, calponin, and desmin) in differentiated cells derived from UT-iPS cell line was analysed and compared to that derived from the skin-iPS cell line.

5.3.2 Differentiated UT-iPS cells expressed urothelial and stromal-specific genes

Real time-PCR was performed on UT-iPS and skin-iPS cells before and after induction. Amplification of urothelium differentiation specific genes (*UPIb, UPII*, *UPIIIa*, and *UPIIIb*, *CLD1*, *CLD5*, *CK7*) and stromal smooth muscle cells specific markers (a-SMA, calponin, and desmin) showed specific products in induced cells from both UT-iPS cells and skin-iPS cells using both U-CM and S-CM although some variation was observed between expression of uroplakins. However, mRNA expression of uroplakins was significantly higher in induced UT-iPS cells using both S-CM and U-CM, when compared to that in non-induced UT-iPS cells and induced skin-iPS cells (Figure 5-2). Similarly, induced UT-iPS cells particularly after treating with U-CM showed higher levels of other markers for epithelial cells, *CLD1*, *CLD5*, and *CK7* (approximately 15 to 20 fold), as compared to non-induced UT-iPS cells and induced skin-iPS cells. In addition, all urothelium differentiation specific genes were more strongly expressed when UT-iPS cells were treated with U-CM compared to those treated with S-CM.

Stromal smooth muscle cell-specific transcripts (*a-SMA, calponin,* and *desmin*) also showed a significant increase in induced UT-iPS cells compared to non-induced UT-iPS cells and induced skin-iPS cells. Amongst the stromal smooth muscle markers, there was a massive increase in *a-SMA* transcription (150-200 fold) but about 5-10 fold induction in calponin and desmin transcription compared to the non-induced UT-iPS cells and induced skin-iPS cells.

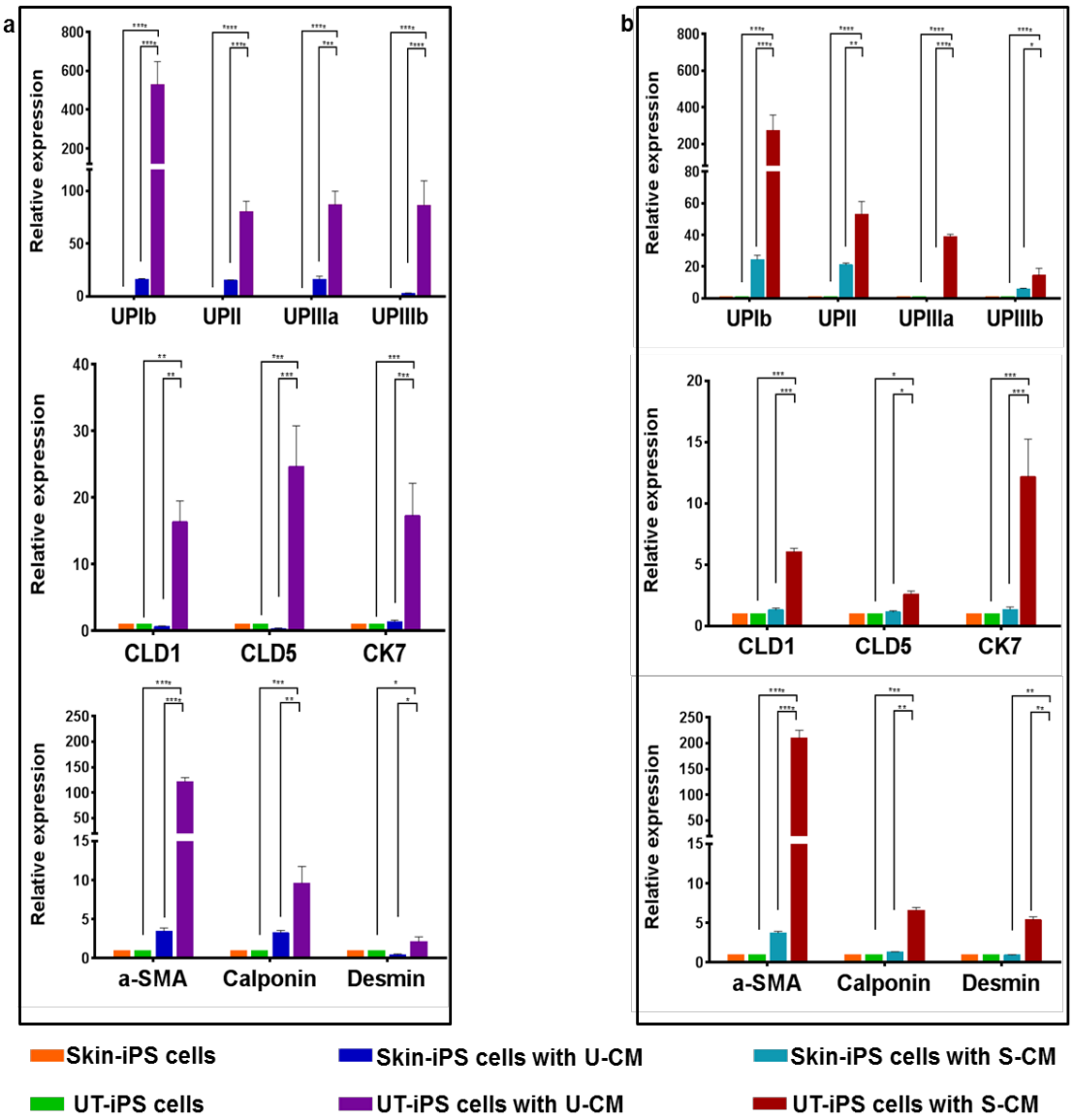


Figure 5-2: Expression of urothelial and smooth muscle lineage specific transcripts in differentiated cells derived from UT-iPS cells and Skin-iPS cells at day 14. The mRNA levels are shown as a fold change relative to control (undifferentiated cells) (N=3). (a) Differentiation induction with conditioned medium from urinary tract urothelium. (b) Differentiation induction with conditioned medium from urinary tract stroma.
* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

5.3.3 Urothelial marker expression in differentiated UT-iPS cells

The above findings using real time-PCR have demonstrated that differentiated UT-iPS cells express a panel of urothelial specific genes. To further examine whether these cells had differentiated into urothelial cells, the expression of UPIb was measured at the protein level by immunofluorescence. Cells immuno-positive for UPIb were seen in differentiated cells derived from UT-iPS cells after 2 weeks in U-CM as shown in (Figure 5-3)

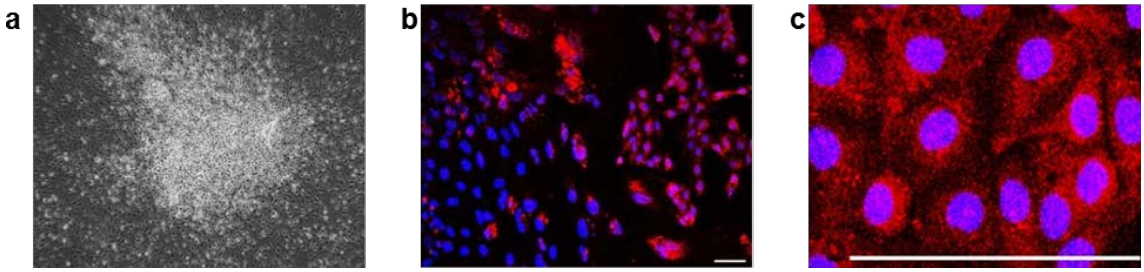


Figure 5-3:Immunofluorescence of differentiated cells derived from UT-iPS cells treated with conditioned medium at day 14, showing (a) Bright field; (b) Positive staining for UPIb (red) juxtaposed with an area of UPIb negative staining, with DAPI nuclear counterstain (blue); and (c) High magnification of UPIb immunostaining. (scale bar = 100µm).

5.4 Discussion

Stem cell fate is mainly controlled by the extracellular environment represented by the cells and secreted proteins (Moore and Lemischka, 2006; Liu *et al.*, 2009a). Therefore, the local environment is likely to be a critical factor in defining stem cell features (Van Vranken *et al.*, 2005). Our results demonstrated that CM from bladder tissue may provide a niche that is favourable for urothelial differentiation of iPS cells *in vitro.* The mammalian bladder is composed of a three layer arrangement of serosa, smooth muscle and urothelium. The urothelium consists of three cell layers, basal, intermediate, and umbrella superficial cell zones and contains a group of integral membrane proteins called uroplakins, UPIa/b, UPII and UPIIIa/b, which represent highly sensitive and specific markers for mature urothelium. Moreover, cytokeratins (CKs) and claudins are expressed by different types of epithelial cells. Specifically, Urothelial cells in culture express various types of CKs and claudins which also provide useful markers to identify the urothelial cells (Varley and Southgate, 2008; Liu *et al.*, 2009a). The results showed that urothelium and stromal smooth muscle gene expression was detected in differentiated UT-iPS cells after treating with either U-CM or S-CM. Differentiation into urothelial like cells was further confirmed by showing that UT-iPS cells can give rise to cells expressing UPIB (which is one of the most commonly used urothelial cell markers) on the protein level. Its expression is very specific and many experts in the field would consider the expression of this marker at a protein level to be definitive of urothelium. Ideally additional markers could be assessed for urothelial and smooth muscle cell phenotypic characterisation. In addition further studies to determine whether UT-iPS cells can differentiate into functional urothelial cells and smooth muscle cells for potential use in tissue engineering would be desired (Southgate et al., 1994; Wezel et al., 2014). This experiment is also limited by the fact that UT-iPS and skin iPS cell lines are not derived from the same patient. Hence it will be interesting to investigate the differentiation potential between two different iPS cell lines derived from the same patient.

Previous study reported that CM derived from bladder cells was able to induce the differentiation of human bone marrow mesenchymal stem cells into smooth muscle cells and urothelium-like cells (Tian *et al.*, 2010b). UT-iPS cells were

also shown to be more proficient than skin-derived iPS cells in generating urothelial and stromal like cells which was demonstrated by expression of urothelial-specific markers including uroplakins, claudins, and cytokeratin and stromal smooth muscle markers including *α-SMA*, *calponin*, and *desmin*. These disparities highlight the epigenetic differences between individual iPS lines and represent the importance of organ-specific iPS cells for tissue-specific studies and justify sourcing iPS cells from the urinary tract tissue rather than the more accessible skin tissue for potential applications in clinical regenerative medicine and modelling urinary tract disease. Furthermore, these results suggest that the growth factors might be sufficient to induce differentiation of iPS cell along the urothelial and stromal lineage and that physical contact between iPS cells and the inductor cells is not always a requisite.

6 Chapter 6. Induce differentiation of bladder specific cells from UT-iPS cells *in vivo*

6.1 Introduction

In the previous chapter it was demonstrated that the generated UT-iPS cells have skewed ability to differentiate into bladder cells *in vitro*. The next step was to investigate the ability of these cells to differentiate into bladder specific tissues *in vivo*. Previous experiments reported that embryonic bladder mesenchyma (EBLM) is an appropriate inductor that regulates differentiation of mouse ES cells and bone marrow derived mesenchymal stem cells toward mature bladder tissue (Oottamasathien *et al.*, 2006; Oottamasathien *et al.*, 2007). Therefore, the behaviour of the UT-iPS cells was assessed when co-cultured with appropriate inductive mesenchyme *in vivo*. Inducing the differentiating of UT-iPS cells into mature bladder tissue *in vivo* might be a major step towards the clinical use of iPS cells in regenerative medicine and tissue engineering of urological organs and also may lead to a better understanding and studying of human bladder embryogenesis and diseases. To this end, it was decided to establish a feeder free culture using a matrigel matrix with optimised human ES cell media. Thereafter, to enable tracking their differentiation *in vitro* and *in vivo*, UT-iPS cells were labelled with a fluorescent marker. Such a model might enable us to capture early events involved in bladder development and also facilitate the ability to identify bladder progenitor cells.

6.2 Aims

- Adapting and maintaining UT-iPS cells to feeder-free culture.
- Generate stable transfectant iPS clones using lentiviral vectors encoding for fluorescent marker genes.
- Investigate the potential of UT-iPS cells to undergo complex differentiation to form mature bladder tissue under the inductive signaling environment provided by EBLM.

6.3 Results:

6.3.1 Feeder-free adaptation, culture and passaging of human UT-iPS cells

To avoid loss of lentivirus into the feeder layer and to exclude the effect of Blasticidin on MEFs, attempts were made to establish UT-iPS cells feeder-free culture. UT-iPS cells were seeded and maintained in culture dishes coated with BD Matrigel human ES cell qualified matrix in optimised human ES cell media-mTeSR1. BD Matrigel human ES cell qualified matrix is a soluble basement membrane extract optimized for stem cell research mainly consisting of laminin, collagen IV, entactin, and heparan sulfate proteoglycan (Kleinman *et al.*, 1982). MTeSR1 is complete, serum-free standardized media for feeder-free maintenance of human ES and iPS cells in culture (Yu *et al.*, 2007; Sun *et al.*, 2009). No adaptation step is required when switching iPS cells from feeder to mTeSR1. Simply, at the time of passaging, undifferentiated UT-iPS aggregates were scraped from the surface of the plate under a low power microscope, washed with DMEM/F-12 and seeded in appropriate volume of mTeSR1 on BD Matrigel-coated plates. High magnification images demonstrated that UT-iPS cells grew as compact and multicellular colonies with a distinct border and displayed prominent nucleoli with a high nuclear-to-cytoplasm ratio (Figure 6-1). Cells at the margins of the colony appeared larger than the cells in the colony centre. Healthy colonies were multilayered in the centre, resulting in clusters of phase-bright cells.

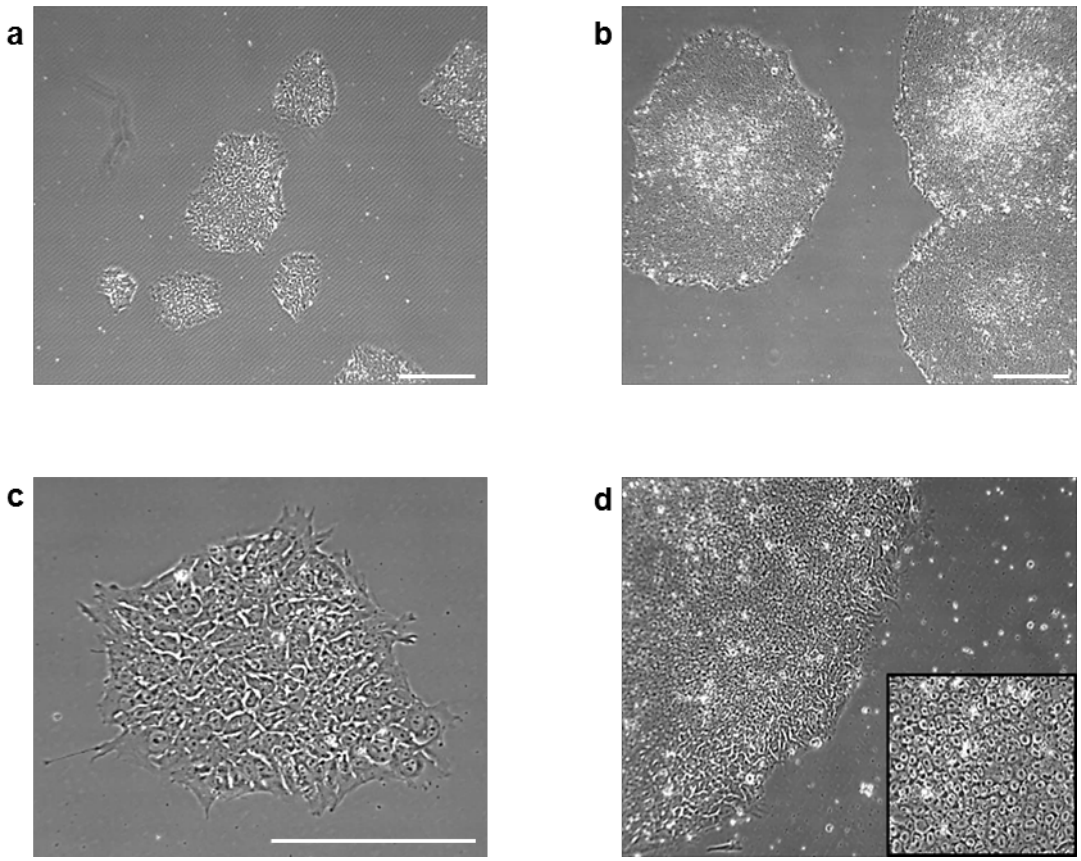


Figure 6-1: Morphology of human UT-iPS cells cultured on a Matrigel coated plate in mTeSR1 medium. (a) An undifferentiated human UT-iPS colony at day 1. (b) and day 5. (c) Higher magnification, feeder-free colonies appear to have a flatter morphology, whereby individual cells are more easily observed within the colonies. (d) Cells at the margins of the colony appear larger than the cells in the colony centre. Insert shows UT-iPS cells with high nuclear to cytoplasmic ratio and prominent nucleoli. Scale bars = 100µm.

As human iPS cells are maintained in culture, a subset of the cells spontaneously differentiate. Differentiation is characterized by loss of border integrity, and the appearance of obvious different cell types as shown in (Figure 6-2). These differentiated cells were scraped off the dish surface and removed before each passage. In our hands, the percentage of the differentiated cells didn't exceed 2%.

To ensure continued quality with optimum attachment and continued undifferentiated proliferation, iPS cells should be passaged at the proper time. Extending the time between passaging in feeder free system results in overgrowing and increased differentiation of the iPS cells and cannot be rescued. Passaging too early, however, resulted in reduced attachment and poor survival. During the first few passages after transferring to feeder free culture, UT-iPS cells grew slowly and they therefore passaged to matrigel plates at a 1:1 ratio. Later, cells were split using 1:6 ratio.

Cells were passaged when the colonies became large with a dense and phase-bright centre compared to their edges when viewed using phase contrast microscopy and when the colonies start to touch and fuse with one another (Figure 6-3). This generally happened around 5–7 days after seeding. UT-iPS cells were passaged using dispase at a concentration of 1 mg/ml at 37°C for 7 minutes until the edges of the colony appear slightly folded back (Figure 6-3). All cultures were observed in the days immediately following passage to ensure that they already passaged at the appropriate time. In the first two days after seeding colonies may not be very densely packed with cells. However, the density of the colonies increased quickly after this time point. UT-iPS cells were phenotypically homogeneous and could be maintained and expanded in undifferentiated state for more than 30 passages.

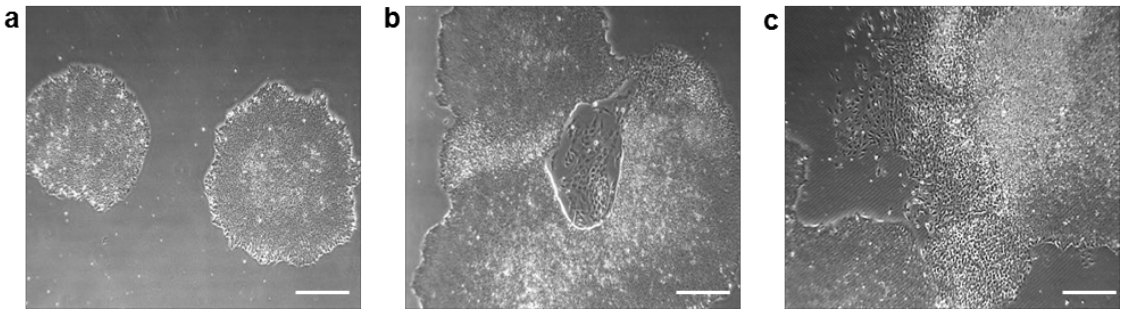


Figure 6-2: (a) An undifferentiated human UT-iPS colony day 5. (b) UT-iPS colony showing an area of differentiation in the centre. (c) Area of differentiation between 2 undifferentiated human UT-iPS colonies. Scale bars = 100µm.

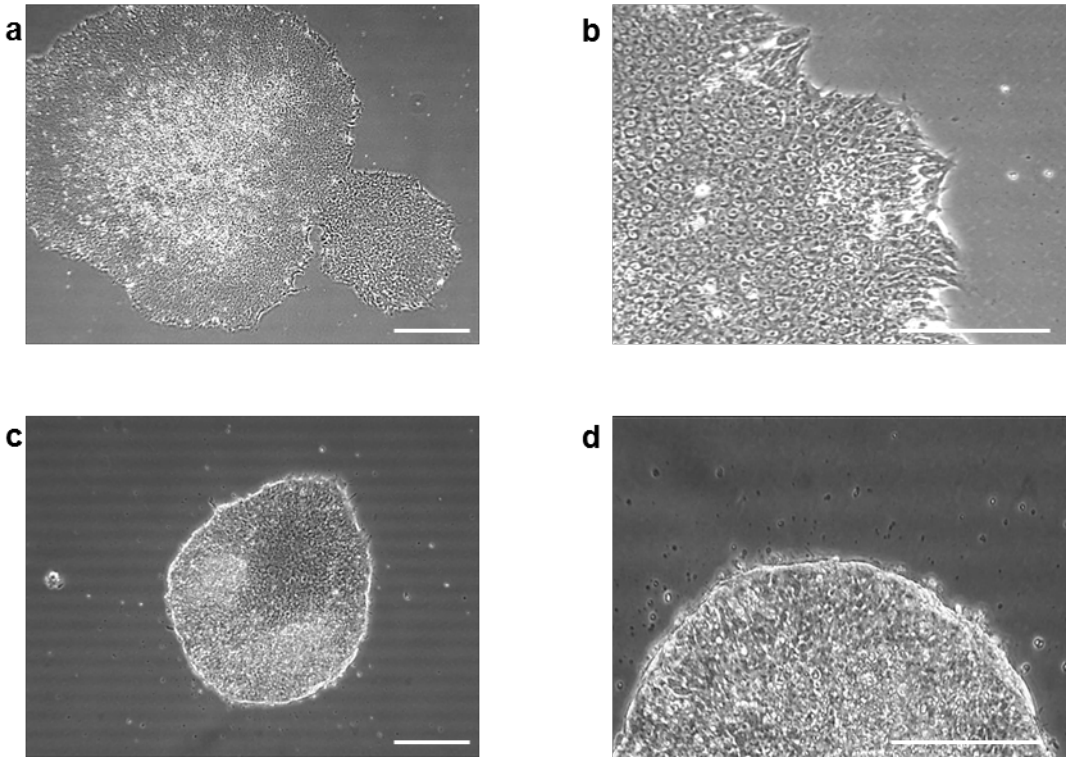


Figure 6-3: Passaging of UT-iPS cells on feeder - free culture. (a) UT-iPS cells ready to be passaged. (b) UT-iPS colonies growing on Matrigel have a very flat appearance with the edges of the colonies tightly adhering to the Matrigel coated dish surface. (C) Exposure to dispase for ~7 minutes results in the curling of the colony edges but the colonies should remain attached to the plate. (d) Higher magnification. Scale bars = 100µm.

6.3.2 Generation of stable transfectant UT-iPS cell lines

The second aim of this part of project was to generate stable transfectant UT-iPS clones to enable tracking them through and after their differentiation. To achieve this, UT-iPS cells were transduced with β-actin-mOrange self-inactivating lentiviral vectors and blasticidin selected (Figure 6-4) (gifted by Norman J. Maitland, YCR Cancer Research Unit, York, UK). The m-Orange marker and the selectable marker are driven by β-actin and SV40 promoters, respectively. Drug selection is a simple and widely used approach to separate the stable transfectant cells from other cells that have not integrated the vector DNA into their chromosomes. Drug selection performs two important roles, both to remove untransduced cells, but also to force integration of the vector. The lowest dose of blasticidin that would completely kill 100% of un-transduced UT-iPS cells by 12 days after drug addition was taken as a starting point for selection trials.

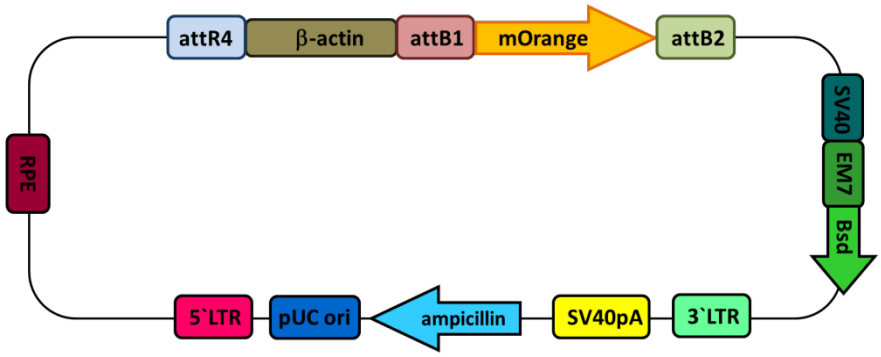


Figure 6-4: Diagram of mOrange (β-actin)-Bsd lentiviral particles lentivirus construct. Modified from (Frame et al., 2010).

In brief, undifferentiated iPS cells were plated at a confluency of <40% per well into a 6-well culture plate, taking care to maintain cells as aggregates. After 24h, media was replaced with the desired amount of virus particles diluted in mTeSR1 medium supplemented with 6µg/ml of polybrene. Medium was changed after 24h. 5 days post transduction, blasticidin was added at a final concentration of 1 µg/ml, as determined previously through kill curves. Selection lasted 12 days. Medium and selective antibiotic were replenished every 2 days. After blasticidin selection the mOrange expression was analysed by fluorescence microscopy and by flow cytometry. A control experiment was first carried out to validate the virus stock, in which an easy to handle cell line (HEK293) was transduced with 10, and 100 µl of virus using the same protocol. Three days after transduction, few fluorescent positive cells were observed confirming that the virus was working well (Figure 6-5).

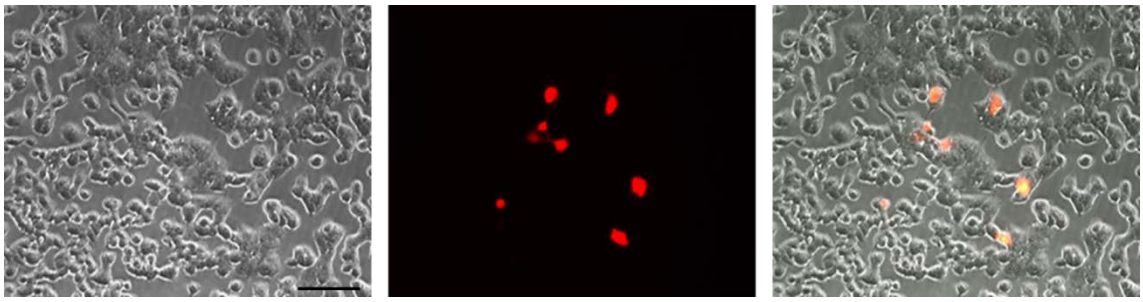


Figure 6-5: HEK293 cells transduced with β-actin-mOrange lentivirus, day 3. Phase contrast (left), Fluorescence (middle), and merged (right) micrographs are shown. Scale bar = 100µm.

6.3.2.1 Promoter activity in undifferentiated UT-iPS cells

As the concentration of the virus was not known, the MOI couldn't be determined, therefore, the initial transduction of UT-iPS was carried out using different amounts of virus (100, 500, and 1000µl). Undifferentiated UT-iPS cells were prepared for transduction as described previously; transduced cells were subsequently subjected to blasticidin selection (1 µg/ml for 12 days). Five days after transduction, mOrange was constitutively expressed in very few cells (Figure 6-6). An attempt was made to clone the positive cells. However, colonies of lentivirus-infected UT-iPS cells showed silencing of the β-actin promoter in blasticidin-selected cells (Figure 6-7).

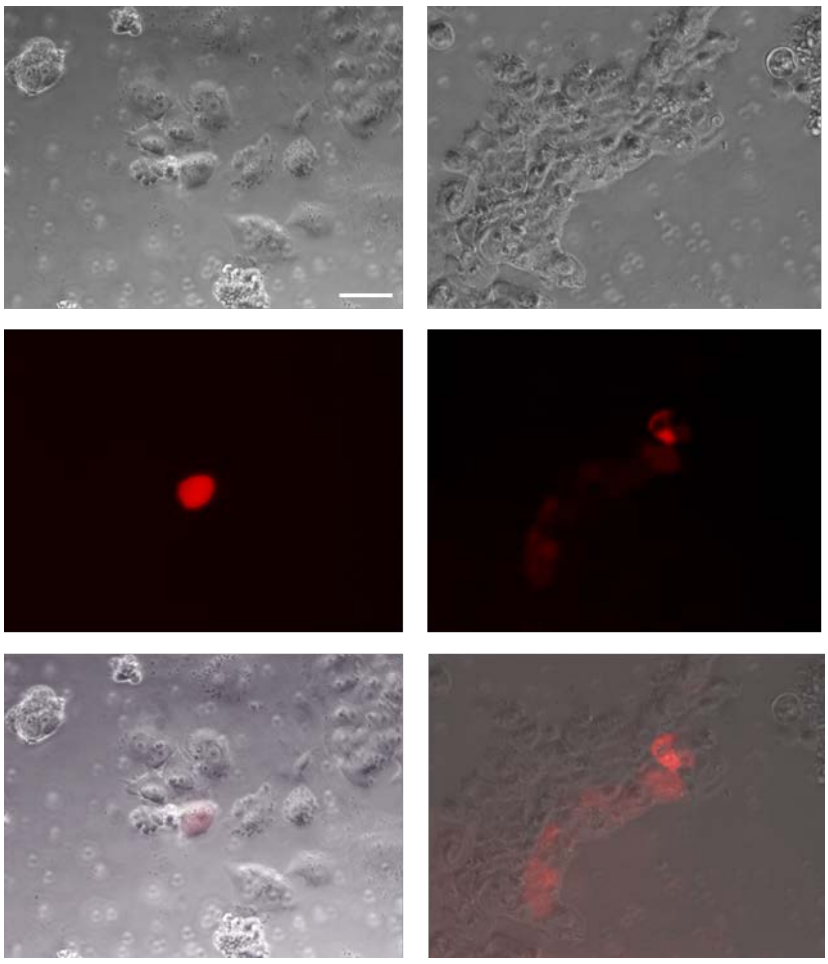


Figure 6-6: UT-iPS cells transduced with β-actin-mOrange lentivirus. Five days after transduction, very few mOrange-positive cells were detected. Phase contrast (top row) fluorescence (middle row), and merged (bottom row) micrographs are shown. Scale bar = 10µm.

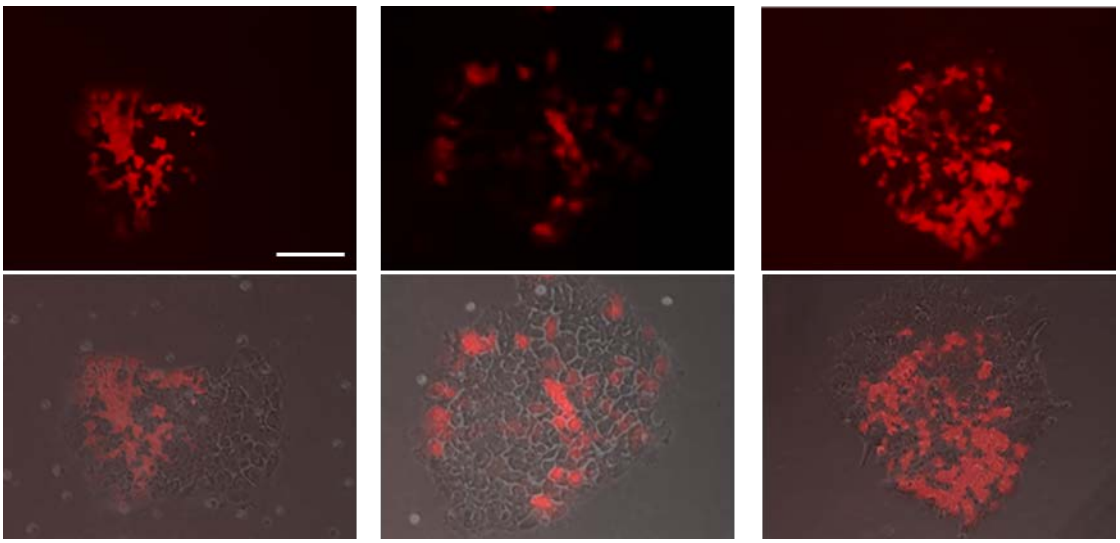


Figure 6-7: Colonies of mOrange(β-actin) lentivirus- transduced UT-iPS cells showing silencing of the β-actin promoter in blasticidin-selected cells. Fluorescence (top row) and merged (bottom row) micrographs are shown. Scale bar = 50µm.

Lentivirus (β-actin)-transduced UT-iPS cells were analysed after blasticidin selection, to quantify the proportion of cells expressing the mOrange fluorescent proteins and to isolate the positive cells. Flow cytometry analysis demonstrated that mOrange was negative in about 99% of the cells (Figure 6-8). mOrange positive cells were isolated and cultured for 10 days. Colonies formed from these sorted cells showed heterogeneity in mOrange expression and remained blasticidin-resistant. After three weeks in culture, the flow cytometry was repeated and it was observed that less than 1% of the sorted cells expressed the mOrange marker (Figure 6-9). In addition, differentiated cells derived from transduced UT-iPS cells did not constitutively express the mOrange marker (Figure 6-10).

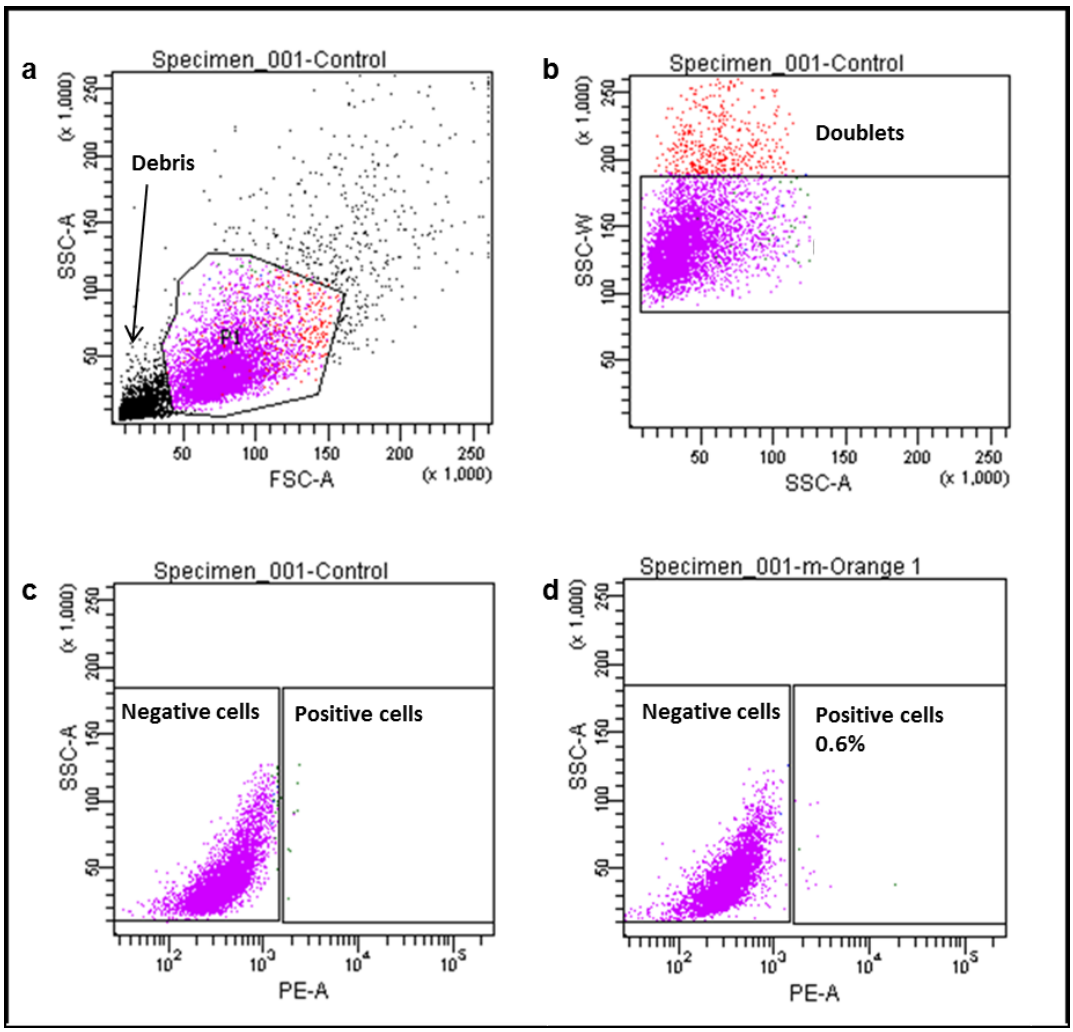


Figure 6-8: Flow cytometry of mOrange (β-actin) lentivirus- transduced UT-iPS cells. (a) P1 gated the cells after ruling out the cellular debris. (b) Doublets discrimination. (c) Control untransduced cells. (d) Lentivirus- transduced UT-iPS cells, ~ 99% of the cells were mOrange negative. UT-iPS cells control cells, mOrange negative (purple events); mOrange positive cells (yellow events); Doublets (red events).

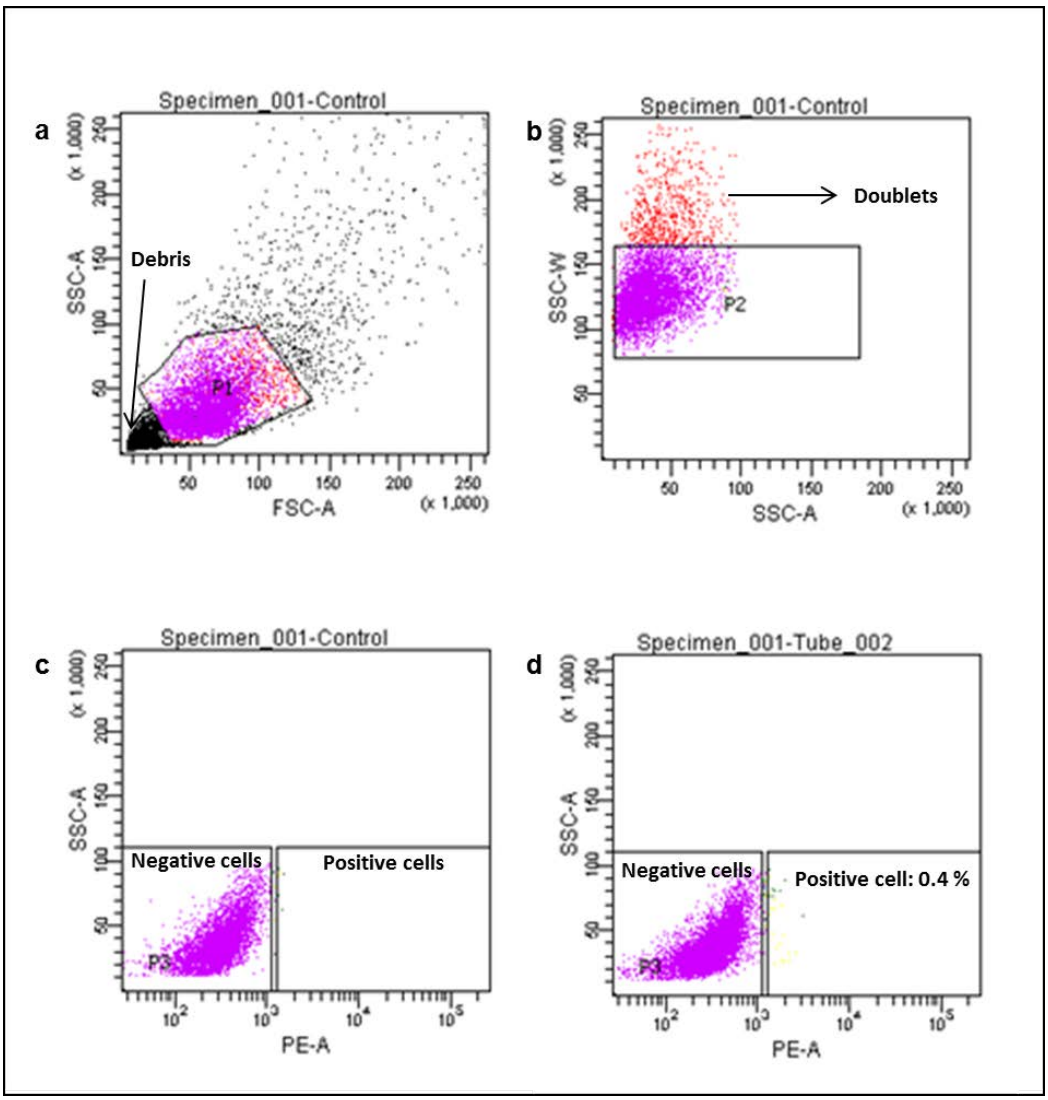


Figure 6-9: Flow cytometry of mOrange (β-actin) lentivirus-transduced UT-iPS cells 3 weeks after selection. (a) P1 gated the cells after ruling out the cellular debris. (b) Doublets discrimination. (c) Control untransduced cells. (d) Lentivirus-transduced UT-iPS cells, less that 1% of the cells are mOrange positive (green events). UT-iPS cells control cells, mOrange negative (purple events). Doublets (red events).

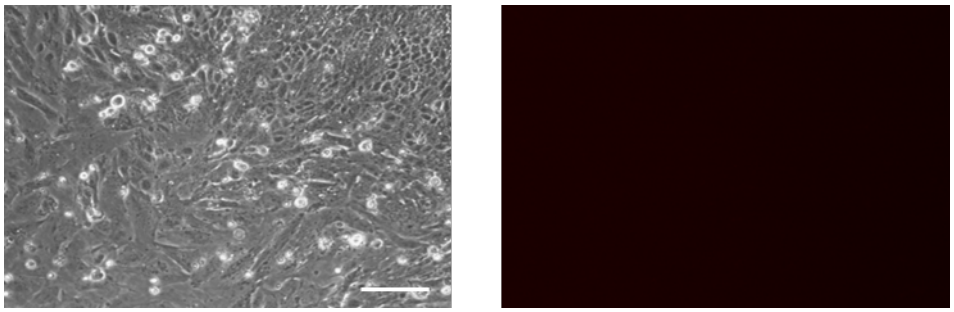


Figure 6-10: Phase contrast (left) and fluorescence (right) micrographs of mOrange(β-actin)lentivirus-transduced UT-iPS cells showed silencing of the β-actin promoter upon differentiation. Scale bar = 100µm.

As a result of this, fluorescent markers driven by human elongation factor 1-alpha (EFIα) constitutive promoter were alternatively used in further studies. UT-iPS cells were transduced with EF1α-mWasabi or EF1α-Citrine self-inactivating lentiviral vectors following the same protocol described above. 3 days post transduction; iPS cells were identified demonstrating mWasabi or citrine expression by fluorescence microscopy (Figure 6-11).

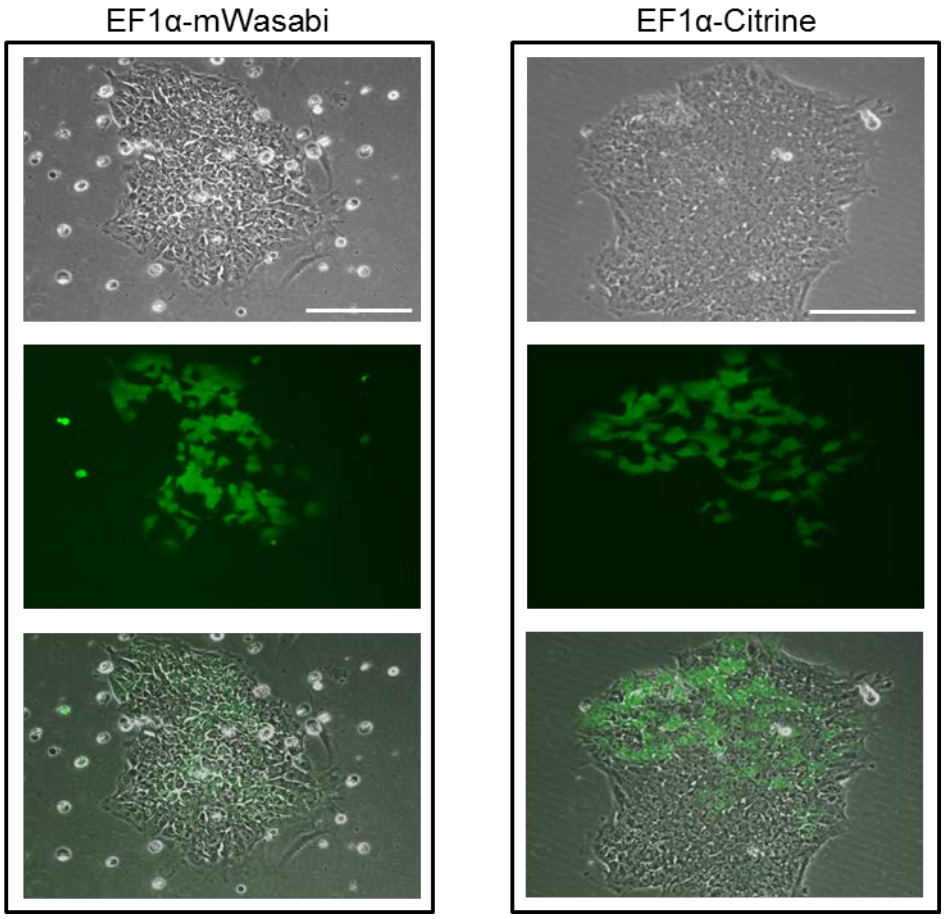


Figure 6-11: UT-iPS cells transduced with EF1α-mWasabi (left column) or EF1α-Citrine lentivirus (right column). Phase contrast (top row) fluorescence (middle row), and merged (bottom row) micrographs at day 3 are shown. Scale bar = 100μm.

To generate relatively homogeneous cell populations, positive cells were isolated by FACS and cultured on matrigel coated plates in mTeSR1 medium. Human iPS cells prefer close cell-to cell contacts, and will rarely survive as single cells. It was found that the density of initial plating after sorting was critical for sorted cells recovery; therefore, to maximize their recovery, wasabi+ or citrine+ sorted cells were seeded at a very high density (1–2x10^6 cells/well of a 6-well plate). In addition, as widely performed, a pre-incubation with the Rho kinase (ROCK) inhibitor before harvesting the cells for FACS was found to enhance the cell survival following sorting. mWasabi and Citrine positive sorted cells exhibited characteristic human ES cells morphology and maintained the fluorescent expression upon extended iPS cell culture (Figure 6-12). FACS analysis of transduced cell lines indicated that the purity of sorted mWasabi- and Citrine-expressing cells were 98% (Figure 6-13) and 90.4%, respectively (Figure 6-14) even after 10 passages when cultured in normal proliferation conditions.

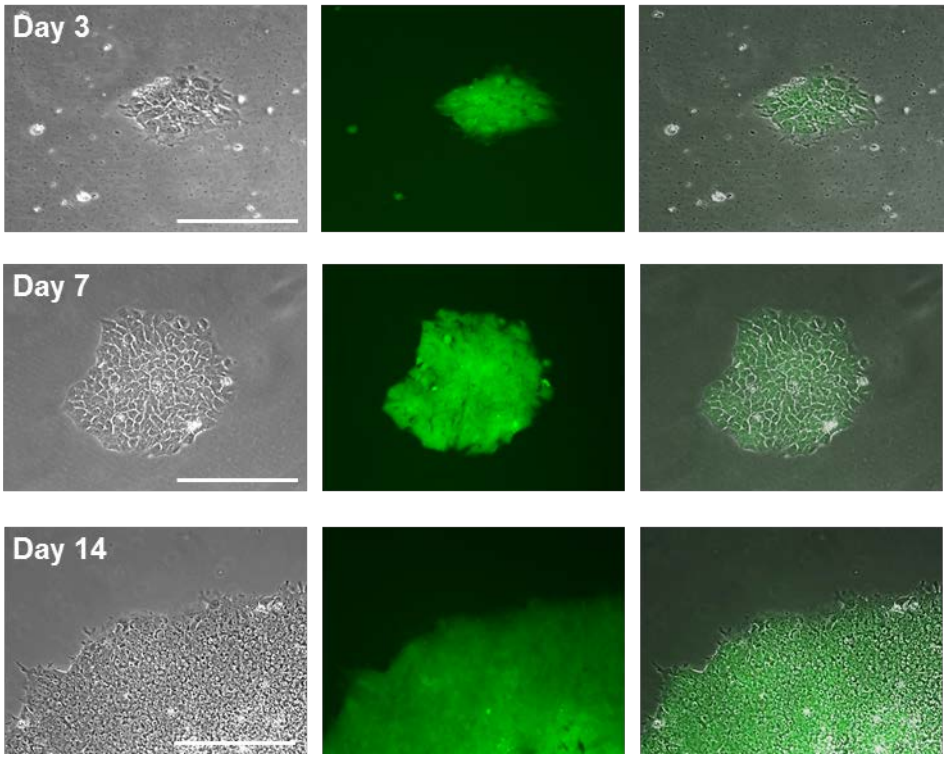


Figure 6-12: Clonal populations of UT-iPS cells transduced with EF1α-mWasabi lentivirus at day 3 (top row), day 7 (middle row), and day 14 (bottom row). Phase contrast (left column), fluorescence (middle column), and merged (right column) micrographs are shown. Scale bar = 100μm.

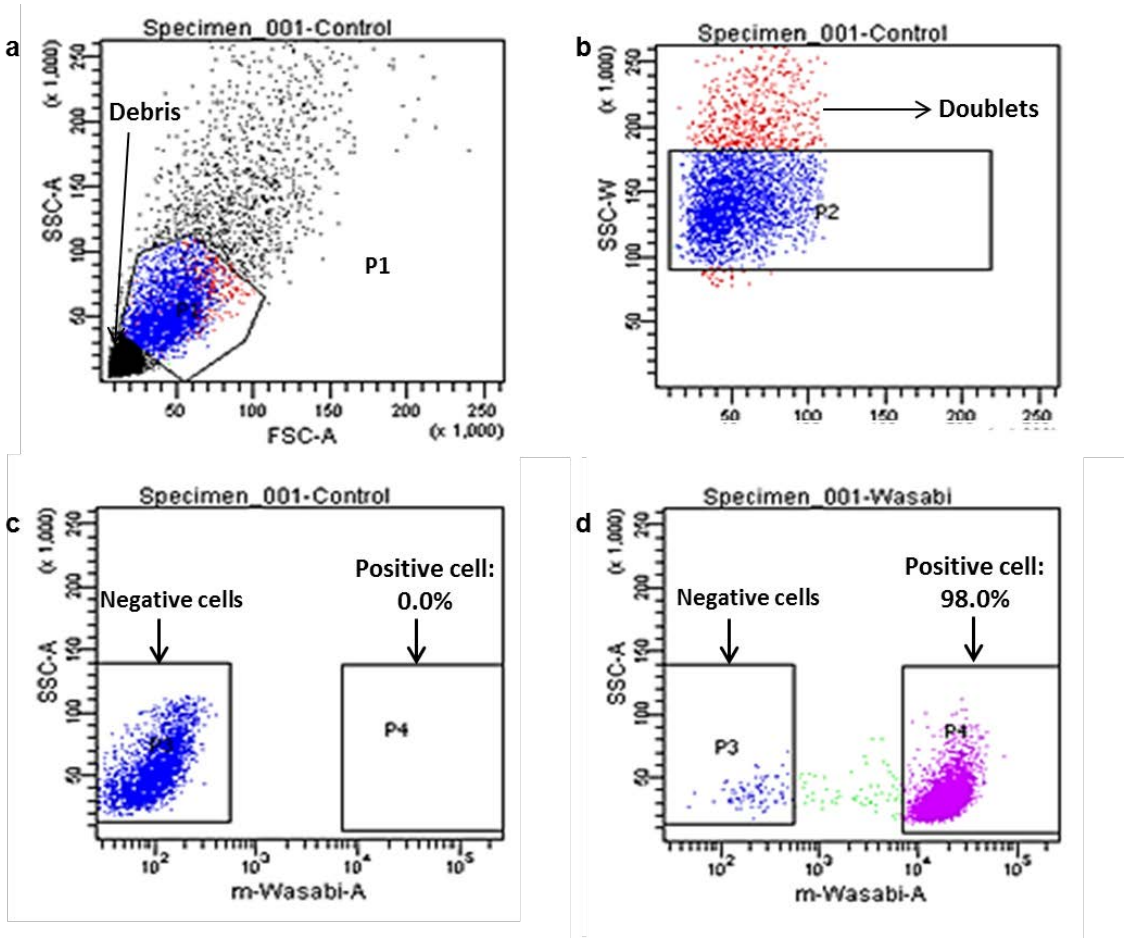


Figure 6-13: Flow cytometry of UT-iPS transduced with EF1α-mWasabi lentivirus. (a) P1 gated the cells after ruling out the cellular debris. (b) Doublets discrimination. (c) Control untransduced cells. (d) Lentivirus-transduced UT-iPS cells, 98% of the cells are positive. mWasabi positive (purple events). Doublets (red events).

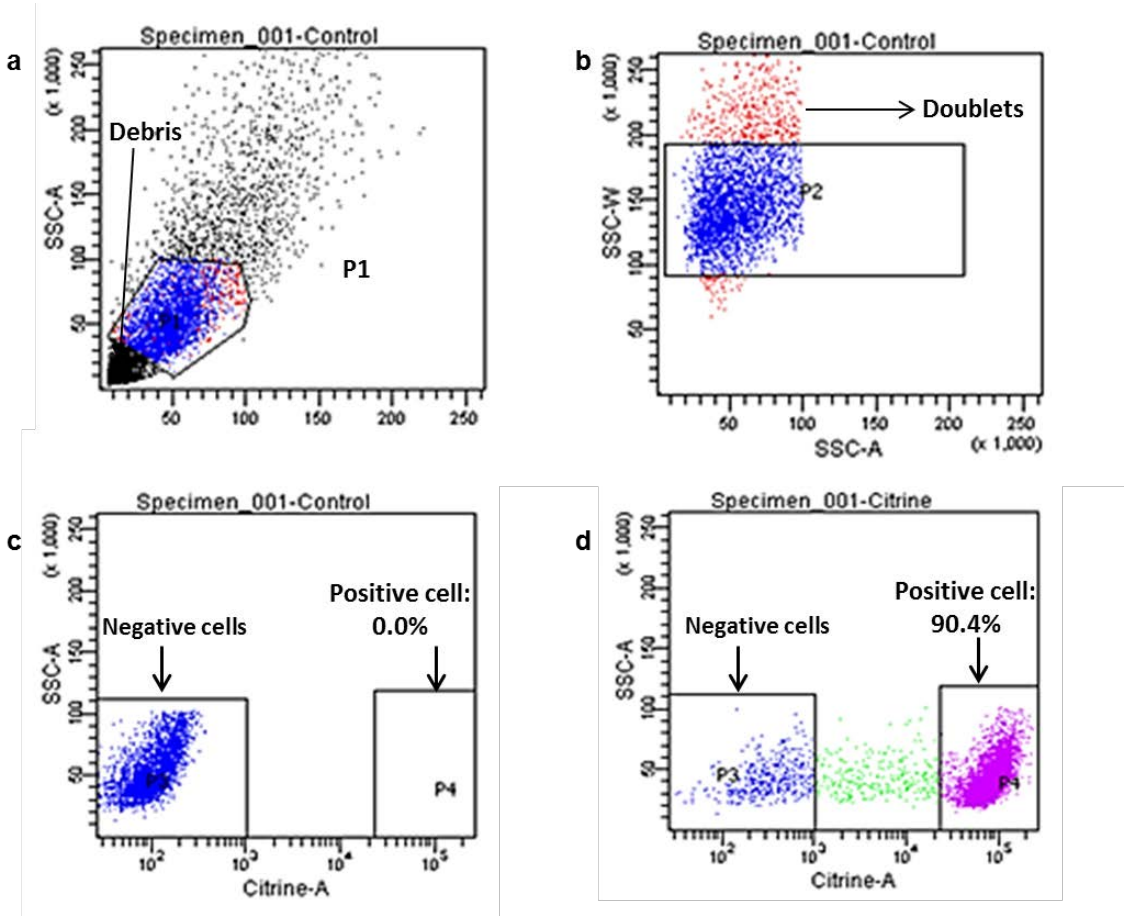


Figure 6-14: Flow cytometry of UT-iPS transduced with EF1α-Citrine lentivirus. (a) P1 gated the cells after ruling out the cellular debris. (b) Doublets discrimination. (c) Control untransduced cells. (d) Lentivirus-transduced UT-iPS cells, 90.4% of the cells are positive. Citrine positive (purple events). Doublets (red events).

6.3.2.2 Promoter activity in differentiated UT-iPS cells

Although promoter silencing was not detectable in undifferentiated cells, it might occur during iPS cell differentiation. Thus, it was considered whether mWasabi fluorescence would be maintained during differentiation of the transduced UT-iPS cell line. Differentiation was induced by culturing EF1α/mWasabi-transduced UT-iPS cells as embryoid body for 2 weeks followed by replating and culture on gelatin coated plate for 2 weeks. As shown in (Figure 6-15), the cells maintained their mWasabi- expression during differentiation suggesting that EF1a is a stable promoter during differentiation.

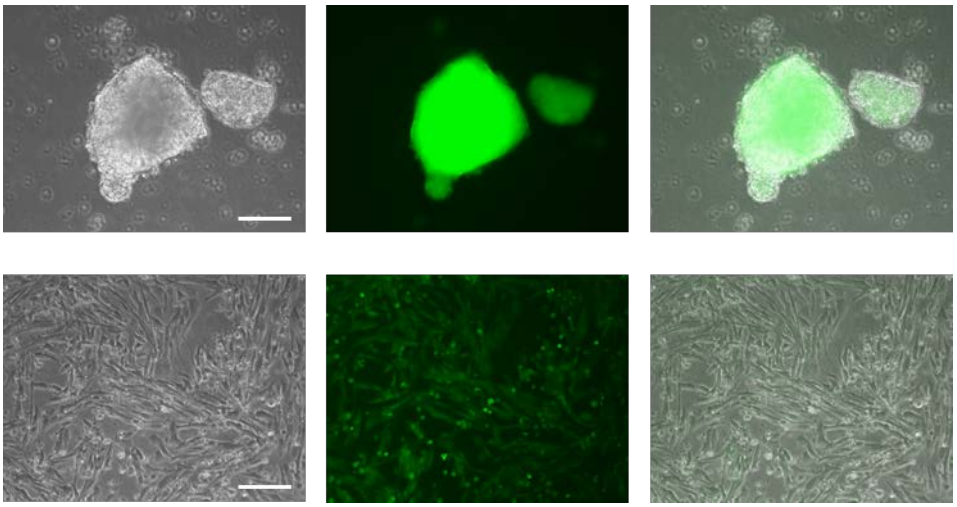


Figure 6-15: lentivirus-transduced UT-iPS cells maintained their mWasabi-expression upon differentiation. mWasabi expression in embryoid bodies of UT-iPS cells at day 14 (top row). mWasabi expression in embryoid bodies outgrowth (bottom row). Phase contrast (left column) fluorescence (middle column), and merged (right column) micrographs are shown. Scale bar = 100µm.

6.3.3 Tissue Recombination Grafts of UT-iPS cells with mouse EBLM

To investigate the ability of UT-iPS cells to undergo complex differentiation and form mature urothelium with bladder tissue formation under the inductive signalling environment provided by EBLM, initial experiments with other colleagues from the department of urologic surgery, Vanderbilt university were carried out using UT-iPS cells recombined with EBLM and injected under the kidney capsule of male athymic nude mice (CD-1 nu/nu Charles River) aged 7–8 weeks. Grafts were harvested at 42 days post *in vivo* incubation and tissues were processed, paraffin embedded, and sectioned for staining. All the recombinants grew under the kidney capsule and no gross invasion outside of the renal capsule or into the renal parenchyma was observed. However, these experiments were not optimised and multiple differentiated structures representing a teratoma were observed with no evidence of bladder tissue formation (Figure 6-16). Oottamasathien *et al* reported similar problem while trying to differentiate mouse ES cells into bladder tissues. The authors found that using 1000 ES cells + one EBLM shell per graft was not sufficient to avoid teratoma formation and yield pure bladder tissue while simply using four shells of EBLM in each graft, along with increasing the number of ES cells to 1500 cells resulted in only pure bladder structures in each graft with no evidence of teratoma formation. Therefore, it is evident that further optimisation of both the number of EBLM shells in each graft, and the number of UT-iPS cells is required.

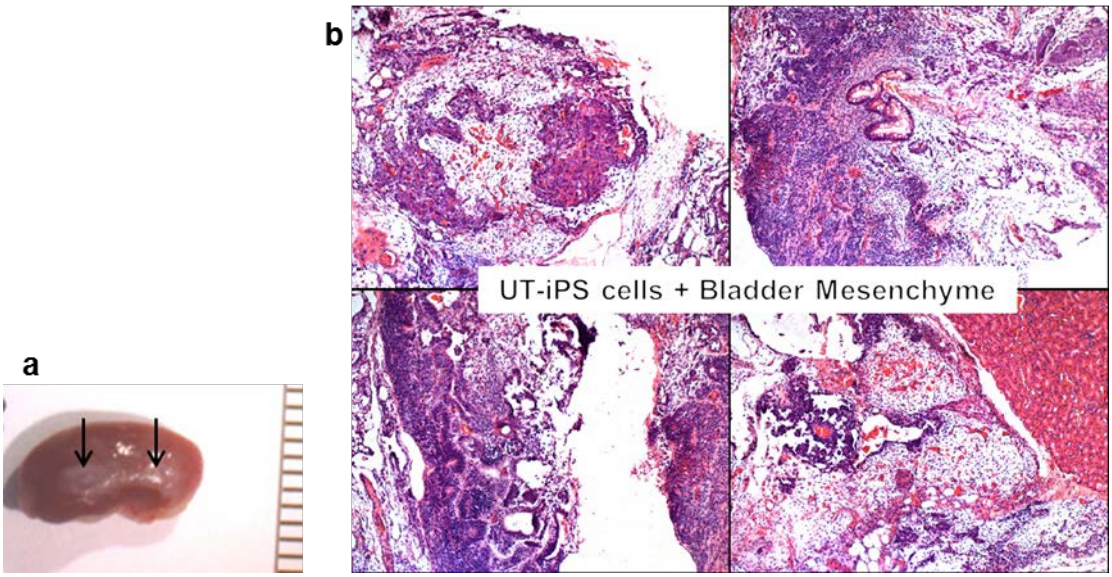


Figure 6-16: (a) Gross appearance of xenografts on host mouse kidney; Two grafts were placed beneath the renal capsule of each kidney. (b) H&E staining showing complex structures representing a teratoma.

6.4 Discussion

Human iPS cells are unique in their dual ability to continuously self-renew and differentiate into any cell in the adult body (Kreft *et al.*) without immune rejection or the ethical issues involved in destroying human embryos. These characteristics give iPS cells the potential to be suitable in many different aspects of basic and clinical research, including use as an *ex vivo* source for cellular transplantation; and producing cells for use in studying new drug candidates and assessing their toxicity; and as an *in vitro* system for modelling human development and disease. However, some of the most promising applications of iPS cells in research are restricted by the difficulty in generating genetically modified iPS cell lines. This chapter describes feeder-free adaptation, culture and passaging of human UT-iPS cells and the generation of stable transgenic UT-iPS cell lines, specifically for use in fluorescent lineage tracking.

Although studies have previously reported successful integration of exogenous DNA into ES cells, isolation of stable transfectant cell lines is still inefficient because of the poor integration of the construct into the genome and high tendency of the exogenous gene to be silenced (Eiges *et al.*, 2001; Liew *et al.*, 2007). UT-iPS cells were cultured onto BD Matrigel hESC qualified Matrix in

mTeSR1 medium. Previous reports showed that ES cells and iPS cells cultured and maintained in this environment have homogeneous phenotype, maintain a normal karyotype, high levels of appropriate ES cell markers expression, including OCT4, TRA-1-60, TRA-1-81, SSEA-4 and the ability to form all three germ layers both *in vitro* and *in vivo (*Yu *et al*., 2007; Sun *et al*., 2009*).* iPS cells were initially transduced with lentivirus vector that contains a gene encoding mOrange, under the control of the β-actin and the blasticidin resistance gene under control of SV40. Since iPS cells grow as colonies and prefer close cell-to cell contacts, starting the antibiotic selection too early, may isolate the stably transfected cells and reduce their survival rates. Therefore, the addition of selective antibiotics was postponed until day 5 post transduction. In this way, resistant cells are afforded time to form small colony of daughter resistant cells before selection initiates. However, it was found that UT-iPS cells transduced with mOrange driven by β-actin promoter showed silencing of this promoter in both undifferentiated and differentiated cells while cells transduced with either mWasabi or citrine under the control of the constitutive promoter EFIα showed a purity of more than 90% even after long term culture.

Fluorescent tracking of cells using lentivirus vectors has recently been used successfully in other tissues (Naldini *et al.*, 1996; Frame *et al.*, 2010). Actually, many constitutive promoters have successfully been reported to conserve stable transgene expression in stem cells and are therefore good candidates in generation of fluorescent reporter cell lines. Previous studies reported promoter silencing with lentivirus constructs (Xia *et al.*, 2007) and suggested that EF1a promoter acts as a strong and stable promoter for transgene fluorescent expression in human ES cells (Kim *et al.*, 2007). Our results demonstrate that lentiviral transduction can successfully be used to produce stable transfectants in iPS cells with good viability. Moreover, consistent with previous studies, it was found that EF1a is a stable promoter during differentiation of iPS cells and therefore is suitable for long term transgene expression. Notably, lentivirus vectors have the potential to activate oncogenes or inactivate tumour suppressor genes in the modified cells since they randomly integrate into the genome which raises concerns regarding clinical applications.

7 Chapter 7. General discussion and conclusion

The urinary tract is subject to damage from a variety of different diseases and conditions such as congenital and neuropathic disorders, and malignancies. Mature, differentiated native cells collected from the patient remain one of the few tools available for replacement and repair of the urinary tract. Nevertheless, the shortage of native tissue in congenital disorders and malignancies, and the limited regenerative potential of fully differentiated mature cells, even if available, is still a crucial drawback (Lakshmanan et al., 2005; Atala, 2008). Therefore, cellular systems simulating urinary tract characteristics are in urgent need for development of disease specific models for investigations of new therapeutic targets as well as for surgical treatment through regenerative medicine and transplantation therapies.

7.1 Stem cells for bladder tissue regeneration

Numerous trials have attempted to differentiate both embryonic and adult stem cells into bladder specific tissue *in vitro* and *in vivo* (Liu et al., 2009a; Tian et al., 2010a; Tian et al., 2010b; Ning et al., 2011). Early studies using both mouse ES cells and human BMSCs have raised the possibility to generate bladder-like lineages by xenografting ES cells with EBLM *in vivo* (Oottamasathien et al., 2006; Oottamasathien et al., 2007; Anumanthan et al., 2008). However, ethical issues associated with the destruction of an embryo and the difficulty in isolating and growing adult stem cells limited their use in human therapy. It is also important to distinguish between mouse and human studies. Despite being a useful and powerful model organism, mouse models cannot always completely mimic human disorders and promising results with preclinical trials in animal models are not always replicated in human clinical trials. About 1% of mouse genes have no detectable homologues in the human genome. In addition, obvious differences between species have been found in morphology, gestation period, and the spatial and temporal regulation of gene expression during embryonic development (Zhu and Huangfu, 2013).

7.2 Potential advantages of iPS cells

Human iPS cells present a unique and potential source of cells for tissue repair or regeneration since they have the ability to propagate themselves through self-renewal, differentiate into multiple lineages and importantly overcome the ethical barriers that have limited human ES cell research, since oocytes and embryos are not required. In addition, autologous iPS cells can be derived directly from patients, such patient-specific iPS cell lines would be compatible with the immune system thus provide a potential cell source for cellular therapy and give the opportunity to develop diseases models for the study and treatment of human diseases (Yamanaka, 2009b; Trounson et al., 2012). This pluripotent nature of iPS cells makes them very attractive as a potentially inexhaustible source of various cell types that could be used in regenerative medicine, drug discovery, disease modeling, and pharmaceutical applications (Hochedlinger and Plath, 2009; Onder and Daley, 2012).

In this project for the first time, successful reprogramming of cells isolated from adult human urinary tract tissue to an ES cell-like pluripotent state is reported. These cells were validated as *de facto* iPS cells by confirming their ability for sustained self-renewal, silencing of transgenes, expression of ES cell-specific genes such as NANOG, reactivating of endogenous OCT4 and SOX2 to levels comparable to those found in human ES cells, and pluripotent differentiation into cell types from the three embryonic germ layers both *in vitro* and *in vivo*. Furthermore, within the appropriate inductive environment, UT-iPS cell differentiation could be directed into bladder-specific lineages allowing for enormous scope in the future for studies of tissue engineering, disease mechanisms and drug treatments. UT-iPS cells have remarkable potential for regenerative medicine applications and studying the biochemical and physiological features of the human urinary tract system.

7.3 Generating iPS cells from UT-stromal cells

Since the reprogramming efficiency might be affected by the heterogeneity of the targeted cellular population and differentiation state (Stadtfeld and Hochedlinger, 2010) we started with producing highly pure cultures of primary cells, as quantified by real time-PCR.

We next aimed to establish iPS cells from UT-stroma cells using the four classical Yamanaka factors (OCT4, SOX2, KLF4, and C-MYC) and following the protocol described before. ES cell-like colonies were first observed around week 5-6 post transduction. Subsequently, these generated cells passed through a series of checkpoints to ascertain that genuine fully reprogrammed UT-iPS cells have been obtained. Morphologically, UT-iPS cells looked indistinguishable from human ES cells and demonstrate unlimited self-renewal. On a molecular level, UT-iPS cells showed high expression levels of key pluripotency markers at both transcript and protein levels, and ES cell-specific surface antigens (SSEA-4, TRA-1-81, TRA-1-60) with a concomitant downregulation of lineage-specific genes associated with the cell of origin. Silencing of exogenous genes was demonstrated in UT-iPS clones indicating that they become factor-independent. On a functional level, UT-iPS cells showed the ability to differentiate into lineages from all three embryonic germ layers *in vitro* and *in vivo*.

7.3.1 Identification of UT-iPS cell colonies

Non-reprogrammed cells can be easily distinguished from reprogrammed ones. However, differentiating between partially reprogrammed cells and fully reprogrammed cells is more challenging. Previous studies reported that partially reprogrammed cells have clearly distinct gene expression from both parental and iPS cells. Although they can be morphologically similar to ES cells and can reactivate many pluripotency genes, they fail to express many genes that are directly related to pluripotency, including OCT4 and NANOG, and showed incomplete silencing of somatic genes, and DNA hypermethylation at the promoters of pluripotency-related genes (Mikkelsen *et al*., 2008; Plath and Lowry, 2011). Chan *et al.* reported that SSEA-4, GDF3, hTERT and NANOG cannot sufficiently distinguish the partially reprogrammed from *bona fide* iPS cell lines whereas silencing the transgenes and expression of TRA-1-60, DNMT3B and REX1 are validated as sufficient markers (Chan *et al.*, 2009a). Generated UT-iPS cells were positive for many pluripotency factors including SSEA4, TRA-1-81, TRA-1-60, OCT4, NANOG, GDF3, DNMT3B, and REX1 at comparable levels to human ES cells. In addition, most of the clones showed

efficient transgene silencing demonstrating that these cell lines are completely reprogrammed into *bona fida* induced pluripotent stem cells.

7.3.2 Mechanisms underlying iPS cell generation

7.3.2.1 Acquiring epithelial properties

Gene expression profile analyses proposed that the reprogramming process could be divided into 3 phases, termed initiation, maturation and stabilization; the initial phase is delineated by a mesenchymal-to-epithelial transition (MET), while the maturation and stabilisation phases are marked by the activation of a subset of pluripotency related genes (Li et al., 2010; Samavarchi-Tehrani et al., 2010). Initiating and maintaining the reprogramming of fibroblasts inevitably requires a process called MET, in which cells undergo morphological changes toward epithelial-like cells and epithelial-associated genes are activated while mesenchymal genes (such as Snail1, Snail2, Zeb1, and Zeb2) are robustly suppressed (Mikkelsen et al., 2008; Li et al., 2010; Samavarchi-Tehrani et al., 2010; Smith et al., 2010). Around day 7, we observed that some transduced UT-stromal cells undergo epithelial-like morphological changes. Real time PCR analysis showed overexpression of MET genes in accordance with suppression of the important inducers of EMT, such as transcription factors Snail, Slug and Twist1, consistent with the occurrence of a MET.

Cells should go through MET before moving on to the next step, termed maturation which is characterized by activation of the pluripotency marker genes including NANOG, Sall4, and OCT4. However, the regulatory network controlling pluripotency is not completely activated until the late stabilization phase (Samavarchi-Tehrani et al., 2010). By tracking clonally derived cells, researchers were able to analyse the late stages of reprogramming including late maturation and stabilization phases for understanding the global changes that occur in cells during reprogramming and gaining mechanistic insights of reprogramming. Golipour *et al.* found that transgenes silencing is essential for the transition from the maturation to the stabilization phase and becoming pluripotent (Golipour et al., 2012).

Another study used genome-wide analyses to examine intermediate cell populations poised to become iPS cells (Polo et al., 2012) reported major gene

activity in two distinct waves during iPS cell formation: the first wave occurred in all cells between days 0 and 3, mostly mediated by MYC and was identified by the upregulation of genes involved in proliferation, metabolism, and cytoskeleton organization and downregulation of genes related to development, while the second wave was observed after day 9 and was more restricted to reprogrammable cells. The second wave was characterized by the activation of genes responsible for embryonic development and stem cell maintenance, specifically OCT4 and SOX2. KLF4 was involved in both phases by downregulating the differentiation related genes in the first phase and by promoting the expression of pluripotency genes during the second one.

Gene expression analysis of 48 genes included those related to pluripotency, proliferation, epigenetic modification, and ES cell-maintaining pathways in single cells derived from early time points, intermediate cells, and fully reprogrammed iPS cells, revealed that the four factors induce the somatic cells to acquire early stochastic gene expression changes. These stochastic epigenetic expression changes are followed by a late ‘deterministic’ or more ‘hierarchical’ phase that leads to activation of the pluripotency circuitry with SOX2 being the triggering factor in a gene expression hierarchy (Figure 7-1).

During the early stage of reprogramming, cells will undergo MET, increase proliferation, undergo alterations in DNA methylation and histone modifications in specific genes, and activate DNA repair and RNA processing. The reprogrammable cells will then pass to an intermediate phase with an unknown rate-limiting step that delays their progress toward a fully reprogrammed state and therefore supposed to be responsible for the low efficiency of the reprogramming process. In some rare cases, the stochastic gene expression can trigger the activation of ‘‘predictive markers’’ like undifferentiated embryonic cell transcription factor 1 (Utf1), oestrogen-related receptor beta (Esrrb), developmental pluripotency associated 2 (Dppa2) and Lin28, which then identify the cells that have a higher probability to move on to the second phase, starting with the activation of SOX2 which in turn will trigger a series of deterministic events that eventually leads to stabilise the cells into the pluripotent state. This late hierarchic phase includes silencing of transgene expression, activation of the core pluripotency circuit, remodelling of the cytoskeleton to an ES cell-like

state, and resetting the epigenome of a somatic cell to a pluripotent state. (Buganim et al., 2013).

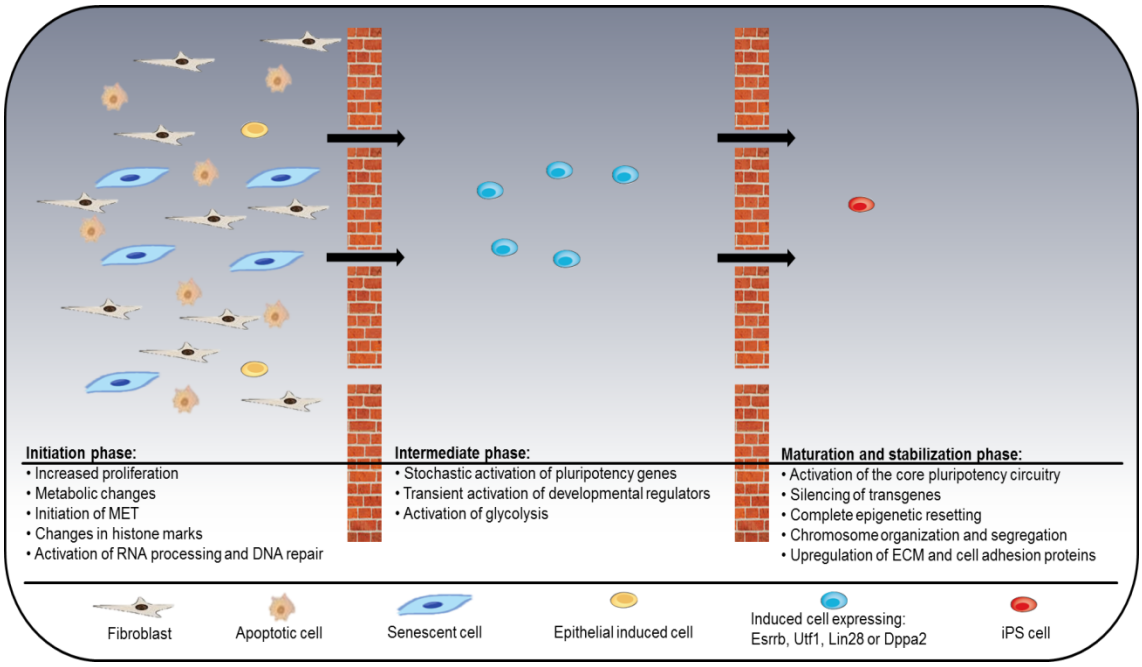


Figure 7-1: Phases of the reprogramming process. Modified from (Buganim et al., 2013).

7.3.2.2 Stochastic versus elite model

The overall efficiency in generating UT-iPS cells was low (~0.03%), but is comparable to the reported efficiency of iPS cells generated from human dermal fibroblasts (Takahashi et al., 2007). The so-called ‘‘elite’’ or ‘‘deterministic’’ model can explain the low efficiency of iPS cell derivation by proposing that only the few somatic stem or progenitor cells that present naturally in a somatic cell culture are amenable to reprogramming (Yamanaka, 2009a). However, the elite model cannot explain the successful reprogramming of somatic cells derived from different starting tissues at similar efficiency rates, including fully differentiated B and T lymphocytes (Hanna et al. 2008; Eminli et al. 2009) as well as pancreatic b cells (Stadtfeld et al. 2008a). In addition, long-term analysis of reprogramming monoclonal populations of early B cells and monocytes showed that nearly all donor cells are amenable to attain the fully reprogrammed pluripotent state albeit, at different times due to different latencies (Hanna et al., 2009). In contrast, the ‘‘stochastic’’ model proposes that all somatic cells regardless of their tissue origin, are equally susceptible to

reprogramming upon continuous overexpression of stemness-related genes, but have to transit through sequential stochastic epigenetic events toward the pluripotent state. Only a few cells may pass all of these roadblocks, resulting in the overall low efficiency (Yamanaka, 2009a; Stadtfeld and Hochedlinger, 2010). Indeed, current evidence revealed that induction of the pluripotent state is a multistep procedure, in which somatic cells have to go through sequential gene expression steps to acquire pluripotency. Failure to move between any of these steps would result in blocked or incomplete reprogramming (Hanna et al., 2009; Papp and Plath, 2013). Utilizing specific surface marker combinations, it has been found that successfully reprogramming cells first downregulate the fibroblast-associated marker Thy1 followed by activation of the embryonic marker SSEA1 and, eventually, reaching the fully pluripotency state (Brambrink et al., 2008; Stadtfeld et al., 2008b; Polo et al., 2012). The silencing of Thy1 was detected in the majority of the starting cell population. However, only a small portion of Thy1-negative cells showed subsequent gain of SSEA1 and only a small subset of the SSEA1-positive cells completed the reprogramming process and became iPS cells (Wernig et al., 2008a; Stadtfeld and Hochedlinger, 2010). These finding might explain somehow why efficiency of iPS cell generation is typically so low. It was also found that cells which remain positive for Thy1 upon OSKM expression become refractory to reprogramming. Notably, during the first two weeks, SSEA1 positive cells are phenotypically still elastic and may revert to earlier reprogramming state, later on in reprogramming, they exhibit much more commitment to a stable pluripotent cell fate (Polo et al., 2012; Papp and Plath, 2013). Accordingly, following clonal populations of early B cells and monocytes revealed that almost all starting cells ultimately have the potential to form iPS cells even though the reprogramming process may take more than 6 months (Hanna et al., 2009). However, the differentiation phase itself has been suggested to influence the efficiency of reprogramming. Indeed, establishing iPS cells from adult progenitor and stem cells have been found to be more efficient and take less time as compared to mature, differentiated cells (Eminli et al., 2009; Galende et al., 2010). Hence, recent studies suggested a modified stochastic model that integrates an elite component to help explain the low efficiency of reprogramming (Smith et al., 2010; Stadtfeld and Hochedlinger, 2010).

7.3.3 Transduction efficiency

Transduction efficiency of lentivirus for reprogramming factors is critical for iPS generation. The ratio of active virus to target cell is referred to as the multiplicity of infection (MOI). As there are substantial differences in cell types that affect their susceptibility to transduction, MOI should be optimized for each cell line before transduction. Transduced cells with a very high MOI can lead to multiple vector copy integration and persistence of the exogenes, on the other hand transduction with very low MOI would result in ineffective transduction and thus failed iPS induction; therefore we modified the MOI during the initial transduction step to achieve efficient transduction with fewer copies per cell. We found that an MOI of 30 resulted in transduction efficiency of approximately 45%. However, with this MOI the lentivirus was very toxic to stromal cells with about 30 % of the cells dying, while the percentage death dropped down to 13% with an MOI of 10. Taking in mind to use the minimal amount of virus particles to minimise the number of integrating sites and to ensure silencing of the transgene, an MOI of 10 was selected despite the lower infection efficiency. Growth properties of the target cells are also important for iPS cell generation. Transduction efficiency is significantly decreased when senescent cells are used for transduction. Therefore, fresh and pure stromal cells were transduced as early as possible for iPS cell production.

7.3.4 Culturing and maintaining UT-iPS cells

The generated UT-iPS were initially cultured and maintained in feeder-dependent culture. Quality of the MEFs has been found to play an important role to help maintain successful culture of human pluripotent cells (Amit et al., 2003). The quality of UT-iPS culture varied with different batches of MEFs since each batch varies in its capacity to support these cells. UT-iPS cells cultured onto low-quality MEFs showed excessive differentiation and poor colony morphology. The optimum plating density of MEFs is also critical to reduce the differentiation and maintain appropriate proliferation. We found that iPS cells plated on 50,000 cells/cm^2 MEFs density produced healthy cultures with good colony morphology. UT-iPS were also cultured and maintained successfully in feeder free culture. Human ES cells were cultured in feeder free conditions for the first time by Xu et al (Xu et al., 2001). This system allowed for long-term

maintenance of stem cells with low level of spontaneous differentiation even at high passages. Recently, several human ES and iPS cell lines have also been cultured in the absence of feeder cells (Amit et al., 2000; Sjogren-Jansson et al., 2005; Warren et al., 2012; Fukusumi et al., 2013). The move to feeder free culture has several advantages; first and foremost, it reduces the exposure of human ES and iPS cells to animal pathogens. In addition, it eliminates the need to prepare and preserve an additional cell type (MEFs), attains better visualization of iPS colonies, and excludes a source of potentially confounding cells especially during iPS cell differentiation. Regardless of the culture system, feeder dependent or independent, to preserve the passaging efficiency and pluripotency of the iPS culture, it is significant that the iPS cells are transferred as small fragments (200 to 300 cells/clump). Triturating the colonies down to very small fragments or single cells will significantly increase the differentiation and reduce the plating efficiencies. Colony fragments that are too large however result in poor attachment. Moreover, the period of time from colony scrapping to re-plating is also critical and should be kept to a minimum. Appropriate passage timing and plating densities are also essential to maintain a constant undifferentiated state and optimum attachment. Passaging cells too late will stimulate the differentiation and reduce the quality of the culture. In contrast, replating the cells too early may results in low plating efficiencies. In our hands, iPS cultures were passaged every 5 to 7 days, while passaging we tried to obtain clumps of the same, near-optimal size and spread them throughout the well. UT-iPS cells lines were maintained using a combination of both the mechanical and enzymatic transfer procedures which allows for mass production of undifferentiated iPS cells by manually excluding differentiated colonies prior to enzyme treatment (Oh et al., 2005; Schatten et al., 2005).

Since the first descriptions of iPS cells in 2006, most reports have focused on the generation of iPS cells from a range of normal and diseased tissues. Only more recently researchers have started to further explore the differences in the ability for terminal tissue-specific differentiation between the iPS cell lines derived from different organs and found that not all iPS cells are the same in this respect (Kim et al., 2010; Polo et al., 2010; Ohi et al., 2011). Interestingly, several studies have demonstrated that *de facto* iPS cells may carry an “epigenetic memory” of their cell type of origin. Consequently, iPS cells with

source cell memory have higher tendency to differentiate back to their parental cell type than to other cell fates (Lee et al., 2012; Xu et al., 2012). Moreover, genetically identical iPS cell lines derived from different somatic cell types showed histone methylation patterns and transcription profiles unique to their tissue of origin (Polo et al., 2010). Whether retained DNA methylation marks, histone modifications, or a combination of both do contribute to iPS cell-somatic donor memory is still unclear (Kim et al., 2010; Ohi et al., 2011; Xu et al., 2012). Therefore, the functional differences and differentiation potential of UT-iPS cells to more efficiently generate bladder tissue cells compared with iPS cells derived from skin fibroblasts were further analysed.

7.3.5 UT-iPS cells exhibit higher capacity for bladder tissues differentiation than skin-iPS cells

We compared the differentiation potential of UT-iPS cells with skin-iPS cells in generating urothelial and smooth muscle stromal cells and our results demonstrated vast differences in their capabilities for bladder specific differentiation. UT-iPS cells have a greater propensity for bladder differentiation compared to skin-iPS cells under the same conditions. This observation serves to emphasise the importance of tissue specific iPS cells for the study of urinary tract differentiation, and is likely to be related to established mechanisms of epigenetic imprinting restricting differentiation potential.

Although the epigenetic memory was observed in early passage iPS cells and can be gradually erased upon extended iPS cell culture (Kim et al., 2010; Polo et al., 2010), UT-iPS cells were used at passage numbers below 50 and it is therefore justifiable that these cells still retain some epigenetic signature of their somatic-cell provenance at this stage. Whether this source cell memory can be erased by continued passaging remains unknown and requires further investigation. This further underscores the need for understanding how this memory varies among different cell types and tissues.

In urothelial and smooth muscle stromal cells differentiation of UT-iPS cells, U-CM treatment appears more efficient in inducing UT-iPS cells differentiation into urothelium when comparing treatment with S-CM. In previous studies, U-CM was shown to be more efficient in inducing urothelial differentiation whilst S-CM

was more efficient at inducing smooth muscle stromal cell differentiation (Mudge and Klumpp, 2005); however, further studies of UT-iPS cells differentiation would be required to disentangle relative contributions to epithelial or stromal differentiation in our model. Nevertheless, our findings show that either U-CM and S-CM were able to guide UT-iPS cells to differentiate into both urothelial and SM cells consistent with evidence that stromal-epithelial interactions are necessary for their development and maintenance (Baskin et al., 1996).

Moreover, a reciprocal cell-cell signalling relationship between the stromal and epithelial compartments during differentiation would be consistent with the simultaneous induction of both cell types as observed in our model. Contemporary evidence has shown that mesoderm (Wolffian duct) does not contribute to trigone development (Viana et al., 2007; Tanaka et al., 2010) and that endodermal derivatives of the urogenital tract could differentiate to form prostate and only mesodermal derivatives of the urogenital tract could differentiate to form seminal vesicle (Tanaka et al., 2010). However, this usual pattern of restricted differentiation depending on the germ layer origin of the epithelium can be overcome by iPS cell generation as both ureteric (mesodermal derivative) and bladder (endodermal derivative) were able to demonstrate bladder specific differentiation. This is consistent with de-differentiation into a pluripotent ES cell-like state preceding gastrulation; where endoderm and mesoderm arise from the transient mesendoderm common precursor cell population.

In addition, we know that organ-specific mesenchyme, from which we took conditioned media, can enforce lineage commitment and alter terminal differentiation of adult epithelia across endodermal and ectodermal boundaries (Taylor et al., 2009). This may also explain why there is plasticity in mesenchymal stem cells (MSCs), such as those derived from bone, that are able to undergo endodermal differentiation into bladder tissue (Anumanthan et al., 2008). Pre-clinical studies of MSCs in the urinary tract have shown considerable promise (Anumanthan et al., 2008; Tian et al., 2010b). However, in comparison to human ES cell derived progenitors, MSCs display substantially decreased tissue formation (Peppo et al., 2012) and furthermore the

regenerative capacities of MSCs can be enhanced by inducing the expression of iPS cell-related genes NANOG and OCT4 (Liu et al., 2009c). The exact contribution of MSCs to fully differentiated bladder regeneration requires further assessment and a direct comparison with the UT-iPS cells would be of interest.

Interestingly, the expression of a-SMA was substantially higher than that of calponin and desmin. a-SMA is considered as an early marker of smooth muscle differentiation, while calponin and desmin are highly specific markers confined to fully differentiated contractile smooth muscle cells (Jack et al., 2009). The expression of a-SMA has also been found to precede the expression of other smooth muscle markers, including calponin and desmin during the myofibrillar development in the early development of rat and chicken hearts (Ruzicka and Schwartz, 1988; Ya et al., 1997). Therefore, the presence of differences in the expression of these markers in differentiated UT-iPS cells might reveal differences in the developmental timing of myocyte maturation.

Early investigations proved that various sources of stem cells (embryonic stem cells (ESCs), BM-derived SCs) have the ability to differentiate into bladder cells. Oottamasathien et al, showed that mouse ES cells can differentiate to bladder cells when associated with embryonic rat bladder mesenchyme and implanted under the kidney capsule for up to 42 days. The endodermal markers of Foxa1 and Foxa2, but not uroplakin were first detected at day 7 after grafting. By 42 days, optimized number of cells resulted in pure urothelial cells with mature bladder tissues derived from the ES cells that was evident by hematoxylin and eosin staining. Maturation was evident based on expression of uroplakin, a selective marker for urothelial cell differentiation and the basal cell marker p63, whereas smooth muscle α-actin (SMA); was used as a marker to identify smooth muscle cells (SMCs) (Oottamasathien et al., 2006; Oottamasathien et al., 2007). However, there are ethical and immunological debates about using this procedure in humans. In addition, the differences observed between murine ES cells and human ES cells regarding molecular and developmental properties may represent an obstacle for direct translation to humans. Utilizing the same model, Anumanthan and his colleagues used a recombinant xenograft of MSCs with EBLM to differentiate mouse MSCs toward mature bladder cells. Histological examination showed a bladder tissue structure with expression of

uroplakin, SMA and desmin (Oottamasathien et al., 2007). The primary limitation of using adult stem cell derived urothelium hinges on a poorly understood differentiation process that typically occurs through either transdifferentiation or cell fusion. Furthermore, adult cells have limited proliferation potential in vitro (Ning et al., 2011). While the murine model provided evidence of feasibility, only recently has the differentiation of human urothelium from pluripotent stem cells been reported. Osborn *et al* described the induction of human urothelium from ES cells and iPS cells using a developmentally directed culture system, where urothelium is induced through a definitive endoderm step. The system efficiently differentiated urothelium through a process that appeared to mimic development of the bladder epithelium during embryogenesis (Osborn et al., 2014). Osborn data also supported our finding human iPS cells be efficiently differentiated in vitro into urothelial cells in the absence of cell contact (Moad et al., 2013).

Several studies have also addressed the question of true differentiation versus fusion of stem cells (Terada et al., 2002; Ying et al., 2002). In this study, using cell-free conditioned medium to treat the UT-iPS cells does confirm that they have undergone true differentiation.

7.3.6 Establishment of UT-iPS transgenic cell line

To identify the UT-iPS cells and their cell lineage *in vitro* and *in vivo*, UT-iPS cells were transduced with lentiviral vectors encoding for fluorescent marker gene. Lentiviral mediated gene transfer was selected for gene delivery into UT-iPS cells since it provides stable transgene integration, efficient transduction (Gropp et al., 2003; Ma et al., 2003; Norrman et al., 2010), and less tendency for silencing during propagation and differentiation (Cherry et al., 2000), while other non-integrating viral systems, such as adenovirus can only provide transient expression of the transgene (Suzuki et al., 2008). Fluorescent proteins are genetically encoded which allows to monitor and track the cells easily, and make it possible to image certain type of cells in real time within living cells and in animal grafting experiments (Miyawaki et al., 2003; Verkhusha and Lukyanov, 2004; Giepmans et al., 2006). Although the low viral transgene expression is a common phenomenon in human ES cells (Xia et al., 2007), several viral promoters have successfully been reported for efficient stable expression of

transgenes in human ES cells. We started with a lentivirus vector expressing mOrange under the control of the β-actin promoter. This lentiviral vector also carries blasticidin resistance, which was used to select for the transduced cells. However, mOrange (β-actin)- transduced UT-iPS cells lost most of their mOrange expression in subsequent culturing and during their *in vitro* differentiation as confirmed by fluorescent microscopy and flow cytometry and remained blasticidin-resistant. Similar and complementary results were reported previously using the same construct (Frame et al., 2010). It is well known that lentiviral silencing is usually associated with certain promoters (Xia et al., 2007; Mao et al., 2008). Although human ES cell lines expressing robust levels of EGFP both in undifferentiated and differentiated cells has been achieved using a human β-actin promoter-driven EGFP gene (Costa et al., 2005), the transgene expression in the transduced cells might be affected by potential alterations in activity of the promoter sequence of the fluorescent gene. Indeed promoter silencing associated with both the promoter-fluorescent gene combination and cell type have been reported previously (Tao et al., 2007; Frame et al., 2010). The observed difference in β-actin promoter activity may also be explained by the differences in lentiviral vector design. As a result of this, the constitutively active promoter EF1a was subsequently investigated. Previous studies reported long-term stable expression of most transgenes under the control of EF1a by lentiviral vectors in ES cells *in vitro* and *in vivo* (Chung et al., 2002; Hong et al., 2007; Kim et al., 2007; Liu et al., 2009b; Qin et al., 2010). UT-iPS cells were transduced with a lentiviral vector coding for mWasabi or citrine under the control of EF1a. mWasabi is a monomeric green fluorescent protein derived from mTFP1 and it is approximately 2-fold brighter than EGFP. We found that mWasabi and citrine expression under the control of EF1a promoter were stably maintained for more than 10 passages in undifferentiated UT-iPS cells with no obvious cytotoxic side effects. This is in line with previous data demonstrating constant, robust EF1a promoter activity in human ES cells for up to 60 days in culture (Ma et al., 2003). In addition, transgene expression is sustained during *in vitro* differentiation. Such genetically modified iPS cell lines will provide new tools for tracking cells transplanted into animal models and hence a better understanding of human development. However, the long term activity of EF1a promoter in differentiated UT-iPS cell lines has not been extensively studied. In addition, the behaviour of

this promoter during *in vivo* UT-iPS cell differentiation remains unknown. It is worth noting that differentiating iPS cells will result in heterogeneous cell populations at the fully differentiated stage, including different types of cells, therefore it is still unclear whether the EF1a promoter may exhibit various transcriptional activities in different cell lineages.

7.4 Transduction of human urothelial cells

The induction protocol used for UT-stromal cells was also applied to the primary culture of human urothelial cells. However, we undertook unsuccessful preliminary attempts at inducing urothelium. It is conceivable that epithelial cells *per se* are more amenable to reprogramming, perhaps because, unlike stromal cells, they may not need to undergo an initial MET to yield iPS cells. However, primary urothelial cells might not be healthy enough for reprogramming after the cell sorting and replating processes, taking into account that primary urothelial cells even without the stress of sorting are technically challenging cell cultures and often have limited life span *in vitro*. Although a few colonies that were positive for the TRA-1-60 and SSEA4 antigen were detected, these were non-proliferative and the morphology was not similar to that of ES cells.

Previous study reported early TRA-1-60 and SSEA-4 positive cells detected in human fibroblasts transduced with the four reprogramming factors (OCT4, SOX2, KLF4 and C-MYC) that were not on a trajectory to reach a fully reprogrammed state, but instead senesced, died or remained partially reprogrammed (Chan *et al.*, 2009a). Consistently, a very recent paper from the Yamanaka lab (Tanabe et al., 2013) in which the fate of transduced cells was monitored during the reprogramming process demonstrated that although 20% of the transduced cells exhibited TRA-1-60 positive staining, about 99% of these nascent reprogrammed cells failed to reach the pluripotency state and turned back to be negative again during the subsequent culture. Small-molecule treatment has been suggested to induce these reversed cells to convert to the next reprogramming stage and subsequently to iPS cells more efficiently (Ichida et al., 2009; Esteban et al., 2010; Plath and Lowry, 2011).

Further reduction of toxicity involving the optimisation of MOI, reduction of polybrene and timing of transfer to ES cell medium with MEFs may also be

required. Further experimentation was not pursued given that the stroma based UT-iPS cells demonstrated robust ability to differentiate into urothelium. Furthermore, a proof of principle in animal studies was already established where mouse ES cells were shown to undergo endodermal lineage transformation into mature urothelium (Oottamasathien et al., 2007). This phenomenon also provides the additional attraction of using an alternative, genetically normal, tissue source in urothelial malignancies and thus avoids the hazards of using diseased cells.

7.5 Challenges of iPS cells

Future challenges include developing viral- and transgene-free reprogramming approaches and xeno-free culture methods in building towards clinical translation.

7.5.1 Gene delivery methods

Since the first generation of iPS cells using retroviral transduction there has been remarkable progress toward reprogramming technologies, optimizing the delivery of the reprogramming factors into somatic cells, and improving reprogramming efficiency (Figure 7-2).

7.5.2 Viral delivery system

The basic method developed by Yamanaka and colleagues used constitutively active retroviral vectors in mouse (Takahashi and Yamanaka, 2006) as well as human fibroblasts (Takahashi et al., 2007; Lowry et al., 2008). Because they tend to be completely silenced and provide extended expression of the transgene, retroviruses are considered as effective gene transfer vehicles (Hotta and Ellis, 2008). However, subsequent mutations that contributed to neoplastic clone formation have been identified in retrovirus transduced cells (Nienhuis et al., 2006; Takahashi and Yamanaka, 2006). Later on, iPS cells from various cell types have been generated successfully using lentiviruses, a subclass of retroviruses. Unlike retroviruses, lentiviral vectors are able to target both dividing and nondividing cells which increases the rate of cell transduction (Yu et al., 2007; Sommer et al., 2009; Sun et al., 2009) and can be used with constitutive or inducible expression system (Brambrink et al., 2008;

Hockemeyer et al., 2008; Stadtfeld et al., 2008a), but they seem to be associated with inefficient proviral silencing (Robinton and Daley, 2012). These two systems have been shown to be robust, reproducible and very efficient at delivering genes. However, the risks of permanent transgene integration into the genome and the possible reactivation of the transgene expression leading to tumour formation pose serious clinical concerns (Ton-That et al., 1997; Stadtfeld et al., 2008a; Sommer et al., 2009; Anokye-Danso et al., 2011). Additionally, the protocol efficiency which uses retro/lentiviruses is low, with reported reprogramming rates of 0.001% to 1% (Maherali et al., 2007; Wernig et al., 2007). Since the most exciting prospect for the use of iPS cell technology is for human therapeutic applications, alternative methods to derive iPS cells free of transgenic sequences have been now demonstrated. Generally integration-free iPS cell lines have been established using integrating vectors that can be subsequently excised from the genome, non-integrating viruses, and non-viral delivery system (Stadtfeld et al., 2008c; Zou et al., 2009).

To reduce the tumour formation potential of iPS cells, the exogenous factors can be excised from genomic integration sites in iPS cells by Cre-loxP recombination and piggyBac (PB) transposition. Reprogramming factors flanked by loxP sites can be excised by transient expression of CRE recombinase. The Cre protein is a site-specific DNA recombinase that catalyzes the recombination of DNA between loxP sequences which contain binding sites for Cre (Shi et al., 2008; Soldner et al., 2009). However, transgene excision leaves behind the entire long terminal repeat, which contains a loxP site and that could potentially lead to genomic instability and genome rearrangements.

To overcome the multiple genomic integrations, a single polycistronic vector, containing the four reprogramming factors connected with 2A peptide linkers was established (Chang et al., 2009; Shao et al., 2009). However, a loxP site and vector DNA external to the loxP sites still remains in the genome after Cre-mediated excision, retaining the possibility of interrupting promoters, coding sequences and regulatory elements.

In contract, the PB transposon removes itself without leaving any remnants of exogenous DNA in the cell genome (Woltjen et al., 2009). Woltjen, Kaji, and colleagues reported successful and efficient generation of transgene-free

murine and human iPS cells using doxycycline-inducible transcription factors delivered by PB transposition which can be excised once pluripotent cell lines become established. The transduction efficiency using this approach was similar to the retroviral methods (Yusa et al., 2009).

Adenovirus is a non-integrating vector that remains in an epichromosomal form in cells and provides high-level expression of exogenous genes. The first iPS cells free of exogenous gene integration were generated from mouse fibroblasts and liver cells using adenoviral vectors containing OCT4, SOX2, KLF4 and C-MYC genes without permanent genetic integration and at an efficiency of about 0.0005% (Stadtfeld et al., 2008c). Human fibroblasts have also been reprogrammed into iPS cells with adenoviral vectors (Zhou and Freed, 2009). However, the limited availability of primary cellular receptor required for binding and internalization decrease the efficiency of gene transfer (Freimuth et al., 2008). In addition, repeated transfection might be required for efficient reprogramming of certain cells (Stadtfeld et al., 2008c).

Fusaki et al, reported efficient generation of viral-free iPS cells from human fibroblast cells with Sendai virus (SeV) vectors (Fusaki et al., 2009). Sendai virus is an RNA virus which does not integrate into chromosomes of the target cells.

7.5.3 Non-viral delivery system

The transgene silencing after retrovirus transduction and successful reprogramming with a nonintegrating system indicated that transgene integration into the genome is not essential for direct reprogramming. Therefore researchers tried to generate iPS cells using non-viral delivery approaches.

Plasmid vectors are a non-viral delivery system in which the encapsulated exogenes are carried on plasmids and transfected into the cells to be reprogrammed. Plasmids are episomally retained and usually exhibit short duration of gene expression. This approach was used for the first time by Okita et al (Okita et al., 2008) to transfect mouse embryonic fibroblast. No integration was identified within the iPS cells generated using the transient transfection nucleofection protocol. However, plasmid incorporation into the host genome

was detected at rates as low as approximately 5.5% using the serial transfection method. Compared with regular plasmid vectors which provide a short window of gene expression, episomal plasmid vectors show longer and more stable expression in target cells and can be controlled in transfected cells through drug selection to remove the untransduced cells (Nanbo et al., 2007). Nevertheless, the reprogramming efficiency of this strategy was extremely low (3–6 colonies per 10^6 somatic cells) comparing to that of viral systems (Kaji et al., 2009; Yu et al., 2009).

However, all the previous strategies still target the cells with foreign genetic materials, which may still produce unpredicted alterations within the genome. Thus, reprogramming strategies entirely free of DNA-based vectors are a major future goal. In 2009, Zhou et al. and Kim et al. reported the possibility of obtaining mouse and human transgene-free iPS cells using cell-permeable recombinant proteins of the four factors (OCT4, SOX2, KLF4 and C-MYC) rather than forcing their transcription through transgenes (Kim et al., 2009a; Zhou et al., 2009). 5×10^4 mouse embryonic fibroblast cells were subjected to four rounds of protein supplementation, and treated with the histone deacytelase inhibitor, valproic acid (VPA). After 30–35 days in culture, three iPS cell clones that were morphologically similar to mouse ES cells were obtained. In addition, the generated protein-induced pluripotent stem (piPS) cells were shown to have global gene expression similar to that of mouse ES cells and demonstrated an ability to differentiate into cells of the three primary germ layers *in vitro* and *in vivo*. However, it is not clear if adult cells can be transduced successfully using recombinant cell-penetrating reprogramming proteins which have proven to be more refractory to reprogramming than embryonic cells. Moreover, due to the short half-life of the recombinant proteins, repeated protein transductions might be required for successful reprogramming (Gump and Dowdy, 2007; Lai et al., 2011).

More recently, Warren and colleagues showed that they were able to efficiently generate iPS cell lines from multiple human differentiated cells using modified mRNA encoding reprogramming factors (Warren et al., 2010). Using mRNAs for reprogramming has many advantages over other methods including high efficiency as they are much smaller than DNA; high activity as they will be

translated into proteins by the host cell, and better ability to control the amount of each factor (Plews et al., 2010). However, mRNA provides only a short duration of gene expression (2–3 days), thus multiple transfections are required for complete reprogramming and innate immune response must be suppressed alongside mRNA transfection (Wang and Na, 2011).

One major drawback to these approaches, however, is the very low reprogramming efficiency (0.01–0.1%), making them currently far from being technically mature. Finding that microRNA (miRNA)-deficient ES cells are defective in differentiation and proliferation highlighted the importance of miRNAs in the control of pluripotent stem cells.

Recently, Judson *et al*. reported the first successful generation of iPS cells from mouse embryonic fibroblasts using miRNAs specific to ES cells (Judson et al., 2009). Cells were exposed to a subset of the miR-290 cluster, known as the embryonic stem cell cycle (ESCC) regulating miRNA in the presence of OCT4, SOX2 and KLF4 factors. MicroR-294 was found to be the most effective miRNA and increased the reprogramming efficiency to 0.1–0.3%. MicroR-302 which is the human homologous miR-291/294/295 family is also abundantly present in mouse and human pluripotent cells but not in somatic cells, thus miR-302 can be potentially used to enhance the reprogramming efficiency of differentiated cells. The miR302/367 cluster has been found to be a direct target of OCT4 and SOX2 (Card et al., 2008). It was also found that the introduction of the miR-302/miR-372 cluster improved the reprogramming efficiency through enhancing the kinetics of MET during reprogramming (Nie et al., 2012) confirming its critical role in self-renewal ability and the maintenance of pluripotency. As expected, the miR302/367 cluster possessed the ability to directly reprogram mouse and human somatic cells to an iPS cell phenotype without the expression of the known reprogramming factors (Anokye-Danso et al., 2011; Miyoshi et al., 2011). Similarly, Lin *et al.* reported successful generation of iPS cells from human hair follicle cells (hHFCs) by inducing miR-302 expression beyond 1.3-fold of the concentration in human ES cells (Lin *et al.*, 2011b). Reprogramming by miRNAs produced iPS cells indistinguishable from those generated by transcription factors with respect to colony morphology, expression of stemness markers, differentiation potential, and germline

transmission (in mice). In addition, this reprogramming technique has been found to be highly efficient and rapid compared with that by the transcriptional factors and most importantly it requires neither using transcription factors nor manipulating the cell genomes. However, how exactly miRNAs can induce the whole network governing pluripotency and the practical use and robustness of this approach is yet to be revealed (Wang and Na, 2011).

Stimulus-triggered acquisition of pluripotency (also known as STAP) is a new cellular reprogramming approach which describes the ability to reprogram the somatic cells by exposing these cells to harsh external stimuli such as a transient low-pH stressor without the need for nuclear transfer or the introduction of transcription factors. However, since this report was published, scientists in other prominent stem-cell laboratories have been unable to produce “STAP” cells using the protocols reported in Obokata’s publications (Obokata et al., 2014).

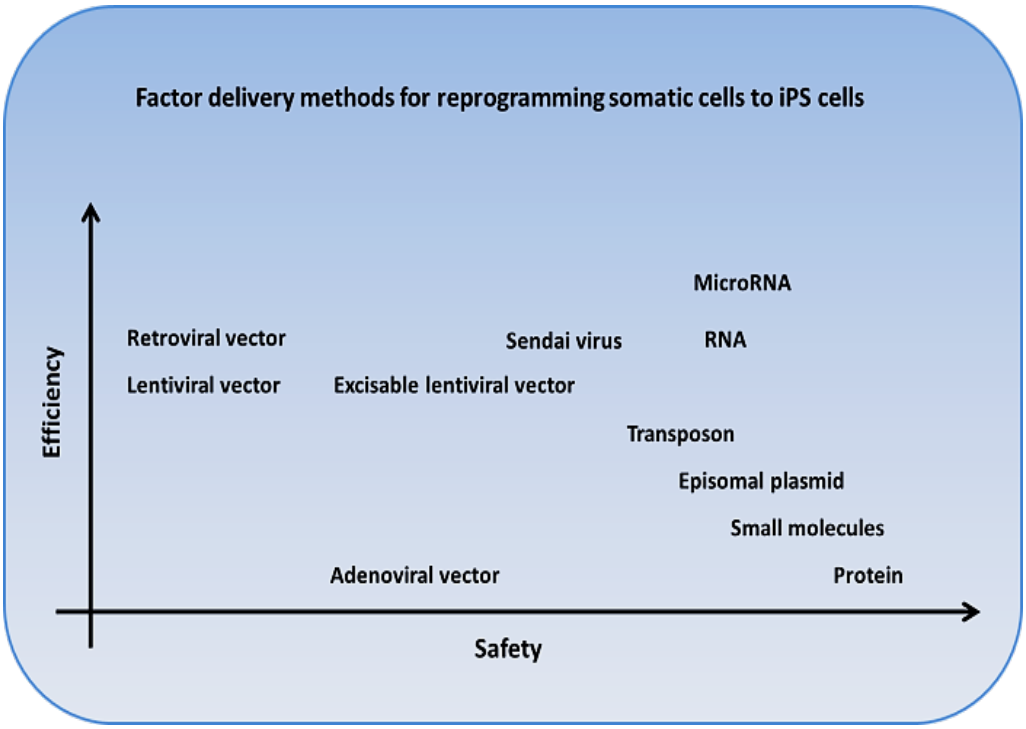


Figure 7-2: Factor delivery methods for reprogramming somatic cells to iPS cells. Based on (Gonzalez *et al.*, 2011).

7.6 Factors and strategies to enhance reprogramming

To enhance the efficiency of DNA reprogramming several modifications have been tried such as adding small molecules and chemical compounds including vitamin C which has been found to significantly enhance the generation of both mouse and human iPS cells by suppressing p53 and minimizing the cellular senescence resulting from the stress of DNA damage (Esteban et al., 2010), and valproic acid, a histone deacetylase inhibitor which increased the reprogramming efficiency by more than a hundred fold (Huangfu et al., 2008a; Mikkelsen et al., 2008; Feng et al., 2009b). The efficiency of lentiviral transduction can also be enhanced using a drug inducible transgenic system, this system further permits temporal control over factor expression (Maherali et al., 2008; Stadtfeld et al., 2008b).

A recent study by Liao *et al.* improved the efficiency of producing iPS cells from human differentiated cells using a combination of 6 transcription factors, OCT4, NANOG, SOX2, LIN28, C-MYC and KLF4 (Liao et al., 2008). Human newborn foreskin fibroblasts were transduced with the lentivirus carrying GFP (as negative control), a cocktail of lentivirus carrying 4 factors (OCT4, NANOG, SOX2, and LIN28), or 6 factors (OCT4, NANOG, SOX2, LIN28, C-MYC and KLF4). Colonies with human ES cell-like morphology were observed after 12 days from transduction with 4 factors, and after only 7 days from transduction with 6 factors.

More recently, deterministic reprogramming of mouse and human differentiated cells back to an undifferentiated state similar to ES cells at efficiencies nearing 100% within seven days has been achieved by OSKM factors together with inhibiting Mbd3, a core member of the Mbd3/NuRD (nucleosome re-modelling and deacetylation) (Rais et al., 2013). In this study Mbd3/NuRD was found to supress iPS cells induction through silencing the OSKM target genes that are required to boost the reprogramming process. However, contrary to previous reports (Luo et al., 2013), depleting Mbd3 expression couldn’t induce the formation of iPS cell in somatic cells without exogenous overexpression of OSKM. It is worth exploring if depleting Mbd3 may improve iPS-generation efficiency in the human urinary tract cells.

Another major drawback with Yamanaka's original transduction protocol however, was the use of C-MYC oncogene as a reprogramming factor. Although retrovirally delivered, C-MYC is strongly silenced in *bona fide* iPS cells, reactivation of the C-MYC transgene induced tumour formation in about 20% of iPS - derived progeny (Okita et al., 2007).

At the same time, Thomson and co-workers were successfully generating human iPS cells utilizing lentiviral vectors with OCT4, and SOX2 in combination with NANOG and LIN28 genes instead of C-MYC and KLF4 (Yu et al., 2007). The reprogrammed human somatic cells met all defining criteria for ES cells suggesting that different transcription factors can stimulate each other's synthesis which in turn leads to a common pluripotent ground state, or, alternatively, different routes can lead to the pluripotency state (Stadtfeld and Hochedlinger, 2010).

Nakagawa *et al.* examined whether iPS cells can be generated without the MYC transgene (Nakagawa et al., 2008). Mouse and human fibroblast were transduced with three factors devoid of MYC and cultured without selection for at least 2 or 3 weeks. NANOG-GFP expressing colonies appeared ~30 days after transduction and NANOG-selected iPS cells displayed human ES-cell markers, and differentiated into cell types from the three germ layers. Importantly, generated chimeric mice didn't develop tumours indicating that C-MYC is not essential for direct reprogramming of somatic cells into iPS cells albeit at the cost of reduced reprogramming efficiency and speed. Omitting C-MYC also selected for high standard iPS cells, reduced the high number of background cells and significantly reduced the risk of tumourigenicity in chimeras (Nakagawa et al., 2008; Wernig et al., 2008b).

In subsequent studies the number of genes required for reprogramming was further reduced. Both mouse (Kim *et al.*, 2008) and human (Hester et al., 2009) neural stem cells (NSCs) which endogenously express high levels of SOX2 and C-MYC compared to ES cells have been reprogrammed into a pluripotent state using only OCT4/ KLF4 or OCT4/C-MYC, though at lower efficiency than with four factors. A single factor reprogramming of human and mouse neural stem cells with only OCT4 has been also reported (Kim et al., 2009b; Kim et al., 2009c). Similarly, dermal papilla (DP) cells from mouse hair follicles which

express high endogenous levels SOX2, C-MYC, and KLF4 can be reprogrammed into iPS cells with the single transcription factor OCT4 (Tsai et al., 2011).

Successful reprogramming has also been achieved using subtypes of transcription factors, albeit with a decrease in reprogramming efficiency, for example SOX1 and SOX3 can be used instead of SOX2, L-MYC and N-MYC instead of c-MYC, and KLF2 instead of KLF4 (Blelloch et al., 2007; Okita et al., 2007; Lai et al., 2011). Direct reprogramming of somatic cells has been also achieved by replacing some of the reprogramming transcription factors with small molecules. For example, the histone deacetylase inhibitor, valproic acid enables efficient reprogramming of human fibroblasts without using KLF4 and C-MYC (Huangfu et al., 2008b). Feng *at al.* reported successful reprogramming of MEFs using orphan nuclear receptor, Esrrb in the presence of OCT4, SOX2 and C-MYC (Feng et al., 2009a). Even OCT4, which was considered to be essential for reprograming, has been replaced with an orphan nuclear receptor gene, Nr5a2 in iPS cells generation in the presence of ectopic expression of SKM. However, Nr5a2 upregulates OCT4 and NANOG through direct binding to their promoters, indicating that its ability to promote reprogramming was still through the OCT4 route (Heng et al., 2010). Moreover, using chemical components as an alternative to transcription factors significantly decreased the number of generated iPS cell clones, suggesting that none of these compounds can completely substitute the function of a transcription factor (Stadtfeld and Hochedlinger, 2010).

Another challenge before human iPS cells can be used for therapy is developing a serum-free, xeno-free, and chemically defined medium, suitable for derivation and maintenance of these cells. At first, scientists commonly used human ES cell medium containing foetal bovine serum (FBS) supplemented with conditioned secretory factors from MEFs (Thomson et al., 1998), but later, more standardized and better-defined medium to overcome xenogeneic -related problems was described. Knock-out serum replacement with basic fibroblast growth factor (bFGF) has been successfully used to support feeder-based human iPS cell culture. This medium provides more standardized and better defined culture conditions compared with serum-

containing medium. However, it is not an animal free medium as it contains lipid-rich bovine serum albumin (Koivisto et al., 2004; Chaudhry et al., 2008). Later on, Tenneille Ludwig and colleagues developed serum-free, xeno-free culture medium (termed TeSR1) with high levels of FGF-2, lithium chloride (LiCl), gamma-aminobutyric acid (GABA), TGF-beta, and pipeolic acid for use in feeder-free conditions (Ludwig et al., 2006).

Currently, the majority of human ES and iPS cell lines are maintained on a feeder layer of MEFs because they provide robust growth of their colonies. However, since MEFs have complex, undefined, and xenogeneic properties, various human cell types have been substituted to support human ES and iPS cell growth (Chen et al., 2014). Great progress has been achieved by Richard *el al.* who replaced MEFs with human foetal and adult fibroblast feeders (Richards et al., 2002), and Mallon *et al*. who used human cells from adult tissues such as foreskin, uterine endometrium, and marrow-derived stromal cells to grow human ES cells (Mallon et al., 2006). However, the use of feeders is not suitable for clinical application. Hence, considerable effort has been made to develop complete xeno-free environment for derivation and maintenance of iPS cells.

Xu *et al.* reported the first attempt to produced feeder-free cultures of human ES cells using Matrigel, an animal based extracellular matrix (ECM) preparation, or laminin substrates in medium conditioned by MEFs (Xu et al., 2001). Currently well-defined and xenogeneic- free extracellular components for the maintenance of human ES cells and iPS cells under feeder-free conditions are commercially available including CELLstart, StemAdhere, and Synthemax- R Surface (Chen *et al.*, 2014).

7.7 Conclusion and future directions

In summary, iPS cells seem to meet virtually all the defining criteria of true pluripotent stem cells but without the ethical and immunological concerns that have limited progress with human ES cells. Furthermore, alternative approaches are now becoming established to produce virus/integration free iPS cell lines, thereby improving the prospects of clinical translation. Nevertheless, the field of iPS cells remains in its infancy and a better understanding of the reprogramming process and its effects on the cells is needed in order to

develop safer and more quick and efficient approaches for pluripotency induction.

Development of a robust protocol for the differentiation and derivation of a pure pool of desired cells to be used in the development of cellular-based therapies is also required. Our study, for the first time, provides proof of principle for the direct reprogramming of adult human stromal cells derived from urinary tract to iPS cells (UT-iPS cells). These UT-iPS cells exhibited typical pluripotent stem cell characteristics and showed more propensity toward urothelial and smooth muscle stromal cell differentiation as compared to iPS cells obtained from human skin fibroblast. As such, UT-iPS cells provide a cellular platform toward the development of differentiation protocols and show great promise in clinical regenerative medicine and modelling urinary tract disease.

To direct the differentiation of these cells *in vivo* to form bladder structures using the inductive signalling properties of appropriate mesenchyme would be a great step forward from this work as this will potentially reveal information about the process of bladder development and bladder stem cells, and also offers the ability to characterize these cells with respect to their potential use for regenerative medicine and other bio clinical applications such as disease modelling, and drug screening.

8 References

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9 Appendix

Publications: first author

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

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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 and 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 tissue, providing an alternative strategy to study differentiation.

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

Design, setting, and participants: Prostate and urinary tract stroma were transduced with POU class 5 homeobox 1 (POU5F1; formerly OCT4), SRY (sex determining region Y)-box 2 (SOX2), Kruppel-like factor 4 (gut) (KLF4), and v-myc myelocytomatosis viral oncogene homolog (avian) (MYC, formerly C-MYC) 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. The student t test was used.

Results and limitations: Successful reprogramming of prostate tissue 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 prostate-specific antigen induction. Simi- larly, 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: a-smooth-muscle actin, 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: Co-author

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: Published

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

Publication: Conference, Poster presentation

Generation of induced pluripotent stem cells from human urinary tract cells (2013)

Author(s): M. Moad, A. Hepburn, M. Lako, D. Pal, S. Williamson, C. Robson, R. Heer

Article first published online: 25 APR 2013

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

Volume: 100 (s4)

Pages: 67

Publisher: John Wiley & Sons Ltd.

Publication type: Conference Proceedings (inc. abstract)

Bibliographic status: Published

PRESENTATIONS

- Oral presentation in NESCI research day, Durham, 18 May, 2012. Abstract: Induction of pluripotent stem cells from adult human urinary tract cells.

- A poster presentation at the ISSCR 11th Annual Meeting, June 12-15, 2013 at the Boston Convention and Exhibition Center (BCEC), Boston, USA. Abstract: Human induced pluripotent stem cells from the human urinary tract cells are able to regenerate bladder cells

- A poster presentation at the FIRM Symposium, 30 Sept - 4 Oct 2013, Girona, Spain. Abstract: Preferential lineage-specific differentiation in induced pluripotent stem cells derived from human urinary tract cells.

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A Novel Model of Urinary Tract Differentiation, Tissue Regeneration, and Disease: Reprogramming Human Prostate and Bladder Cells into Induced Pluripotent Stem Cells

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Article info

Article history:
Accepted March 25, 2013
Published online ahead of print on April 6, 2013

Keywords:
Prostate
Bladder
Differentiation
Pluripotent
Stem cells
Tissue engineering
Ureter
Urothelium
Androgen receptor
POU5F1 (formerly OCT4)
SOX2
KLF4
MYC (formerly cMYC)
NANOG

Abstract

Background: Primary culture and 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 tissue, providing an alternative strategy to study differentiation.
Objective: To generate human iPSCs derived from normal, ageing, human prostate (Pro-iPSC), and urinary tract (UT-iPSC) tissue and to assess their capacity for lineage-directed differentiation.
Design, setting, and participants: Prostate and urinary tract stroma were transduced with POU class 5 homeobox 1 (POU5F1; formerly OCT4), SRY (sex determining region Y)-box 2 (SOX2), Kruppel-like factor 4 (gut) (KLF4), and v-myc myelocytomatosis viral oncogene homolog (avian) (MYC, formerly C-MYC) 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. The student *t* test was used.
Results and limitations: Successful reprogramming of prostate tissue 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 prostate-specific antigen 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-smooth-muscle actin, 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.

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0302-2838/$ – see back matter
http://dx.doi.org/10.1016/j.eururo.2013.03.054

Please cite this article in press as: Moad M, et al. A Novel Model of Urinary Tract Differentiation, Tissue Regeneration, and Disease: Reprogramming Human Prostate and Bladder Cells into Induced Pluripotent Stem Cells. Eur Urol (2013), http://dx.doi.org/10.1016/j.eururo.2013.03.054

1. Introduction

Mechanisms involved in prostate and bladder development and differentiation have been implicated in disease [1–3]. Established animal models of prostate and bladder development are well characterised; however, despite providing key insights for differentiation, there are limitations in translating these findings to the human setting. Similarly, there are constraints on studying cells lines that forcibly maintain expression of immortalising genes. Approaches to human primary cell culture can overcome some of these issues, although this can be challenging due to the limited life-span of cultures, biologic variability between patients, limited material despite cell expansion, and changes in the phenotype associated with in vitro culture adaptation [4–6]. An alternative strategy is the use of embryonic stem cells (ESCs) that can generate foetal and adult prostate and bladder tissue [7,8], but this approach is limited by ethics associated with the source of material. However, a recent landmark discovery by Yamanaka and colleagues demonstrated that somatic cells can be reset to an embryonic-like state, termed *induced pluripotent stem cells* (iPSCs), by the expression of defined factors [9,10]. Such cells offer an unparalleled opportunity for regenerative therapies, disease modelling, and drug screening [11]. However, iPSCs appear to retain epigenetic imprinting associated with their tissue type of origin. This phenomenon results in restricted terminal differentiation into other cell types [12–14].

In this study, we generated, for the first time, iPSCs derived from human prostate (Pro-iPSCs) and urinary tract cells (bladder and ureter) (UT-iPSCs). Furthermore, our data showed that Pro-iPSCs and UT-iPSCs are more efficient in differentiating into respective prostate and bladder lineages relative to established skin fibroblast-derived iPSCs, confirming the importance of the organ of origin on the differentiation potential of the reprogrammed cell.

2. Materials and methods

2.1. Cell culture

All surgical specimens were collected according to local ethical and regulatory guidelines and included written, informed patient consent (Freeman Hospital, Newcastle Upon Tyne, UK). Patient details from which successful iPSC lines were established are summarised in Table 1. Histologic examination confirmed the absence of dysplasia or malignancy. Prostate primary culture was undertaken according to previously optimised protocols to separate purified epithelial and stromal cells [15,16]. Cell cultures of urothelium and associated urinary tract stroma were established using a protocol described by Southgate et al. [17]. Detailed protocols for cell culture are provided in Supplement 1. The homogeneity of the stromal cells that were subsequently transduced were confirmed by real-time reverse transcription-polymerase chain reaction (RT-PCR) using a panel of cell lineage markers (CD24 epithelial, CD45 haematopoietic, von Willebrand factor endothelial, CD146 endothelial, α-smooth-muscle actin [SMA] stromal smooth muscle, and Thy-1 cell surface antigen [CD90] stromal cells).

2.2. Lentivirus transduction

Pure cultures of 5×10^4 prostate, bladder, and ureter stromal cells were transduced using a polycistronic lentiviral vector (POU class 5 homeobox 1 [POU5F1, formerly OCT4], SRY [sex determining region Y]-box 2 [SOX2], Kruppel-like factor 4 (gut) [KLF4], and v-myc myelocytomatosis viral oncogene homolog [avian] [MYC, formerly C-MYC]; Allele Biotech, San Diego, CA, USA) at a multiplicity of infection of 10 in the presence of polybrene (10 μg/ml) and transduction medium (RPMI1640 medium with HEPES modification; Sigma-Aldrich Co, St. Louis, MO, USA) containing 10% foetal calf serum (Sigma-Aldrich Co, St. Louis, MO, USA), L-glutamine (2 mM), and 1% penicillin and streptomycin (Invitrogen Corp, Carlsbad, CA, USA). On day 2, the transduction medium (including lentiviral vectors) was replaced with standard stroma culture medium. On day 6, cells were seeded onto gelatine-coated plates with a feeder layer of irradiated CF-1 mouse embryonic fibroblasts (MEFs) (MTI-GlobalStem, Rockville, MD, USA) in human ESC medium (Knockout Dulbecco's modified Eagle's medium, 1 mM L-glutamine, 100 mM nonessential amino acids, 20% serum replacement, and 8 ng/ml fibroblast growth factor [FGF] 2 [Invitrogen Corp, Carlsbad, CA, USA]). Additional details on optimisation of these protocols are available in Supplement 1. After an additional week, cells were cultured in MEF-conditioned ESC medium. ESC-like colonies were manually selected based on morphology between 4 and 6 wk. The medium was changed every 48 h. A similar protocol was applied to epithelial cells but was unsuccessful in iPSCs generation; it is described in Supplement 1.

2.3. Characterisations by polymerase chain reaction, DNA fingerprinting, karyotyping, immunofluorescence, and alkaline phosphatase staining

RNA isolation and real time RT-PCR was normalised to glyceraldehyde 3-phosphate dehydrogenase according to protocols described in Supplement 1. DNA fingerprinting was based on microsatellite markers for short tandem repeats and karyotyping was determined by Giemsa banding. Details about this and about alkaline phosphatase activity detection and immunofluorescence are described in Supplement 1.

2.4. Assays of pluripotency

In vitro and in vivo differentiation assays, and embryoid body and teratoma formation were undertaken using established protocols and are detailed in Supplement 1.

Table 1 – Details of patients from whom induced pluripotent stem cells lines were established

Patent identifier	Age, yr	Sex	Nature of tissue biopsy
12380	65	Female	Ureter biopsy from radical nephrectomy for renal cell carcinoma
12459	66	Male	Bladder biopsy from cystoprostatectomy undertaken as part of urinary diversion for benign functional disorder (secondary to urethral injury and bowel surgery)
12491	48	Male	Bladder biopsy from cystoprostatectomy for benign, functional, neurologic disorder
12502	54	Male	Ureter biopsy from radical nephrectomy for renal cell carcinoma
12506	56	Male	Bladder biopsy from cystoprostatectomy for benign functional neurologic disorder
11901	66	Male	Prostate biopsy from cystoprostatectomy for bladder cancer

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2.5. Lineage-specific differentiation of human induced pluripotent stem cells in vitro

MEFs were removed for differentiation studies. For the induction of prostate differentiation, Pro-iPSCs were cultured with primary prostate stroma-conditioned medium. For bladder differentiation, UT-iPSCs were cultured with conditioned medium for 14 d using a previously established protocol [18]. Details of the conditioned medium from either cultured human urothelial cells or stroma cells are described in Supplement 1. Comparison of differentiation potential was drawn against conventional human-skin iPSCs, which is a pre-established cell line [19].

2.6. Statistical analysis

All results are expressed as mean plus or minus the standard error, and statistical differences assessed using the student t test with p values $\leq$0.05 considered significant.

3. Results

3.1. Generation of induced pluripotent stem cells from normal ageing prostate and human urinary tract cells

Once primary cultures of prostate, bladder, and ureter stromal cells were established, we examined the purity of fibroblast lineage. Following passage, there was an inherent culture-based selection for fibroblasts based on cell morphology and lineage-marker expressions. Morphologically, cells were consistent with primary, prostate stromal fibroblasts and were confirmed to be devoid of endothelial, epithelial, and haematopoietic contamination following the first passage (Supplemental Fig. S1A and S1B). Pure bladder and ureter stromal cells expressing smooth muscle and

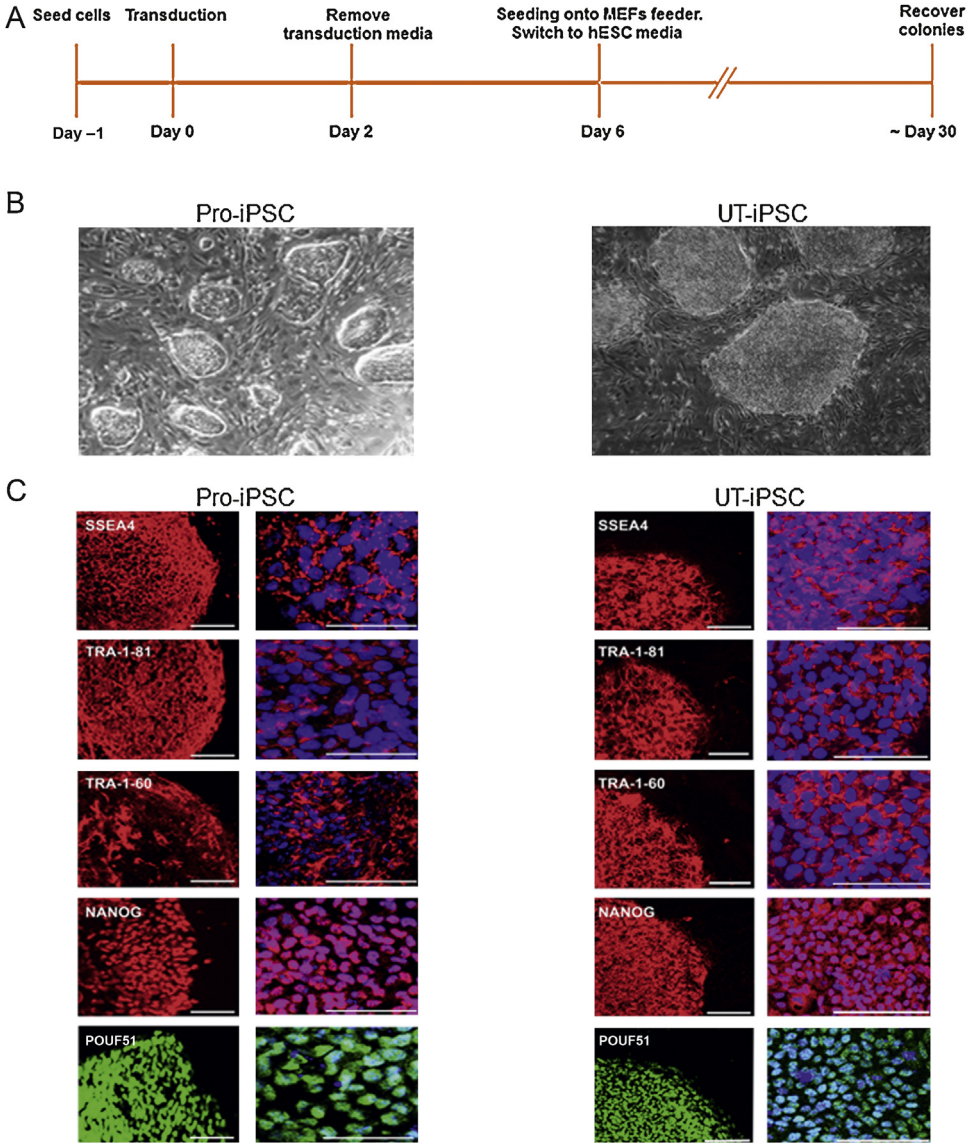

Fig. 1 – (A) Timeline for induced pluripotent stem cell (iPSC) generation. (B) Example of established iPSC colonies growing on a feeder layer with human embryonic stem cell (ESC)–like morphology. (C) Immunofluorescence of generated iPSCs for the expression of specific human ESC surface markers: stage-specific embryonic antigen-4 (SSEA4), tumour rejection antigen (TRA)-1-81, TRA-1-60, and nuclear transcription factors Nanog homeobox (NANOG) and POU class 5 homeobox 1(POU5F1, formerly OCT4). Note mouse embryonic fibroblast (MEF) cells at the periphery of the colonies are negative for stem cell marker expression. Nuclei were stained with 4′,6-diamidino-2-phenylindole (blue) (scale bar = 100 μm), and specific cellular localisation of the stem cell markers are shown in their expected distribution.
hESC = human embryonic stem cell; Pro-iPSC = prostate induced pluripotent stem cell; UT-iPSC = urinary tract induced pluripotent stem cell.

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myofibroblastic markers α-SMA and CD90 were confirmed at second passage, associated with typical stromal-cell appearance (Supplemental Fig. S2A and S2B). Polycistronic lentiviral vectors containing the four transcription factors POU5F1, SOX2, KLF4, and MYC were transduced into pure stromal cells. A schedule for human iPSC reprogramming is summarised in Figure 1A. Seven days following lentiviral transduction, fibroblasts demonstrated features of mesenchymal to epithelial transition, which is typical of early reprogramming [20]. Mesenchymal to epithelial transition was characterised by a change in the prostate fibroblast morphology from spindle-shaped cells to classic, epitheloid, cobblestone colonies, and was confirmed by marker expression showing upregulation of epithelial marker E-cadherin and downregulation of mesenchymal markers snail homolog 1 and 2 (Drosophila) (dubbed Snail and Slug, respectively) ($p < 0.05$) (Supplemental Fig. S1C). Four week to 5 wk after transduction with the lentivirus, several small and tight cell colonies were detected; however, they grew slowly and so were not consistent with ESC-like cells (Supplemental Fig. S2C). By week 6 after transduction, rapidly growing colonies displaying morphology similar to that of human ESCs were observed (tight and flat colonies with clear-cut edges composed of small cells with a high nucleus-to-cytoplasm ratio) (Fig. 1B, Supplemental Fig. S1D and S2D). Eleven prostate and 31 urinary tract ESC-like colonies (17 bladder and 14 ureter) were successfully expanded and stably maintained throughout culture passages (>50 passages, >10 mo). The overall efficiency in generating Pro-iPSCs and UT-iPSCs was low (0.02–0.04% of all stromal cells transfected), but was comparable to the reported efficiency of iPSCs generated from human dermal fibroblasts [21]. Karyotyping confirmed a diploid 46,XY chromosome arrangement (Supplemental Fig. S1E and S2E). Authentication of Pro-iPSCs and UT-iPSCs derivation from parental stromal cells was confirmed using DNA fingerprinting (Supplemental Fig. S1F and S2F).

3.2. *Characterisation of generated induced pluripotent stem cells*

Immunofluorescence for human, ESC-specific, surface markers—stage-specific embryonic antigen-4, tumour rejection antigen (TRA)-1-81, and TRA-1-60, and also

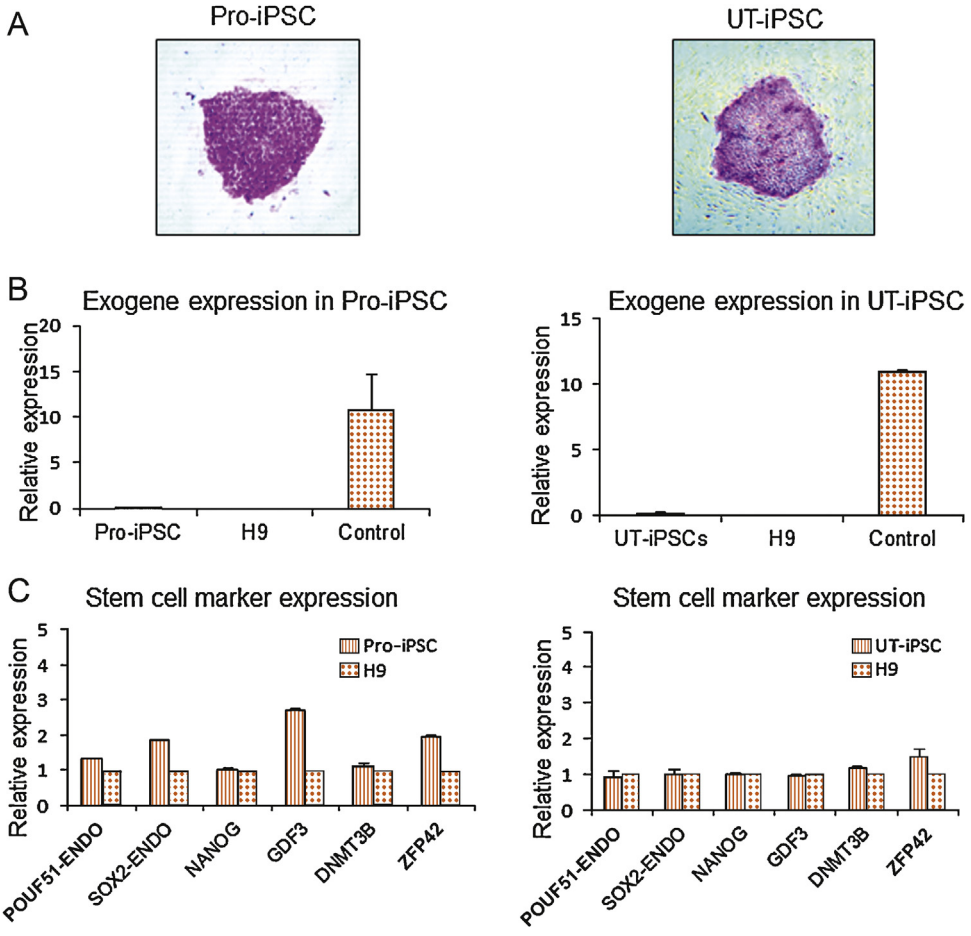

Fig. 2 – (A) Alkaline phosphatase staining of induced pluripotent stem cell (iPSC) colonies. Note underlying mouse embryonic fibroblast cells act as negative control cells with no staining. (B) Reverse transcription–polymerase chain reaction (RT-PCR) analysis demonstrating silencing of exogenous transgene expression in iPSCs. Control represents stromal cells 6 d after transduction. (C) Real-time RT-PCR analysis for expression of endogenous POU class 5 homeobox 1 (*POU5F1*, formerly OCT4), SRY (sex determining region Y)-box 2 (*SOX2*), and other stem cell marker genes: Nanog homeobox (*NANOG*), growth differentiation factor 3 (*GDF3*), DNA (cytosine-5-)-methyltransferase 3 beta (*DNMT3B*) and ZFP42 zinc finger protein (*ZFP42*, formerly REX1) in iPSCs. All values were calculated with respect to the value for H9 human embryonic stem cell, which was set to 1.
Pro-iPSC = prostate induced pluripotent stem cell; UT-iPSC = urinary tract induced pluripotent stem cell; ENDO = endothelial.

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transcription factors Nanog homeobox (*NANOG*) and *POU5F1*—was confirmed (Fig. 1C). Additionally, alkaline phosphatase activity, typical of an ESC phenotype, was demonstrated in the induced cells (Fig. 2A). Exogenous transgene silencing is associated with the generation of iPSCs, where there is a critical switch to endogenous expression of key ESC regulatory factors such as *POU5F1*, *SOX2*, and *NANOG*. Real-time RT-PCR using primers specific for lentiviral transcripts demonstrated that exogenous transgene expression had ceased in both Pro-iPSC and UT-iPSC clones (Fig. 2B). Furthermore, endogenous expression of the pluripotency markers *POU5F1*, *SOX2*, and *NANOG*, in addition to ESC markers growth differentiation factor 3 (*GDF3*), DNA (cytosine-5-)-methyltransferase 3 beta (*DNMT3B*), and ZFP42 zinc finger protein (*ZFP42*, formerly REX1) in both Pro-iPSCs and UT-iPSCs was consistent with expression levels in the control human-ESC line H9 (Fig. 2C). The iPSC clones were identical in terms of ESC-like morphology, proliferation, and gene-expression signatures (data not shown).

3.3. Pluripotency of generated induced pluripotent stem cells

When cultured in suspension on low-adhesion plates in the absence of basic FGF, both Pro-iPSCs and UT-iPSCs formed embryoid bodies containing all three germ-layer derivatives, as demonstrated by immunofluorescence of lineage-specific markers βIII tubulin (ectoderm), CD31 (mesoderm), and α-fetoprotein (endoderm) (Fig. 3A). Xenografts from the in vivo teratoma-forming assay also confirmed ectoderm, mesoderm, and endoderm lineage histology (Fig. 3B).

3.4. Prostate induced pluripotent stem cells differentiate into androgen receptor and prostate-specific antigen–expressing cells

We compared the differentiation potential of Pro-iPSCs with conventional skin-derived iPSCs (skin-iPSCs). Following induced differentiation by prostate stromal-cell conditioned medium for 3 wk, both Pro-iPSCs and skin-iPSCs displayed epithelioid cell morphology. Transcripts of epithelial marker CD24 were detected at comparable levels to primary prostate epithelium (data not shown). When characterised with the prostate differentiation markers androgen receptor (AR) and prostate-specific antigen (PSA), the prostate-specific phenotype was restricted to Pro-iPSCs (Fig. 4). AR expression was present in the differentiated Pro-iPSCs and the degree of immunofluorescence was at levels comparable with mature prostate epithelium in early primary culture (day 10), whereas only minimal levels of AR were detected in skin-iPSCs (Fig. 4A). Functional AR was confirmed by PSA transcript and protein expression at levels in keeping with primary, cultured, prostate epithelia, whereas no PSA expression was detected in the differentiation medium-treated skin-iPSCs (Fig. 4B).

3.5. Urinary tract-induced pluripotent stem cells differentiate into uroplakin-expressing cells

Lineage-specific differentiation of the UT-iPSCs into bladder lineages was assessed using established coculture methods

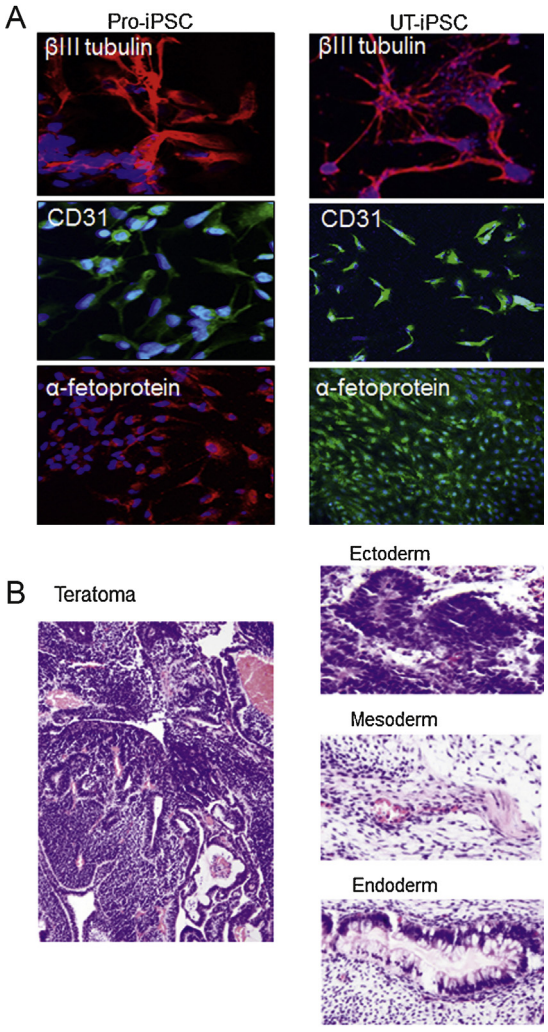

Fig. 3 – Pluripotency in prostate induced pluripotent stem cells (Pro-iPSCs) and urinary tract induced pluripotent stem cells (UT-iPSCs). (A) Immunofluorescence analysis of embryoid bodies derived from iPSCs shows expression of the lineage markers βIII-tubulin (ectodermal marker; red), CD31 (mesodermal marker; green), and α-fetoprotein (endodermal marker). Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (blue) (scale bar = 100 μm). (B) Left: Histologic section of teratoma formed from Pro-iPSCs showing neuronal epithelial differentiation. Right: UT-iPSCs representing all three embryonic germ layers: ectoderm (neuronal rosette-like structures), mesoderm (muscle-like tissue), and endoderm (intestinal epithelial-like cells).

[18]. In comparison to cells derived from skin-iPSCs, UT-iPSCs were significantly more efficient at inducing bladder-specific differentiation, as demonstrated by urothelial differentiation-specific genes (UPIb, UPII, UPIIIa, and UPIIIb; claudin 1 [CLD1], claudin 5 [CLD5] and keratin 7 [CK7]) and markers specific for smooth-muscle cells (α-SMA, calponin, and desmin) (Fig. 5A). Furthermore, the effect of conditioned medium from urothelia was compared with that of conditioned medium from stromal cells (Fig. 5B). Similar to the embryogenesis of the urinary tract, where there is a reciprocal differentiation of epithelial and mesenchymal fractions [22], both urothelial and stromal differentiation was concurrently induced using either urothelial- or stromal-conditioned medium (Fig. 5A and 5B). UPIb protein

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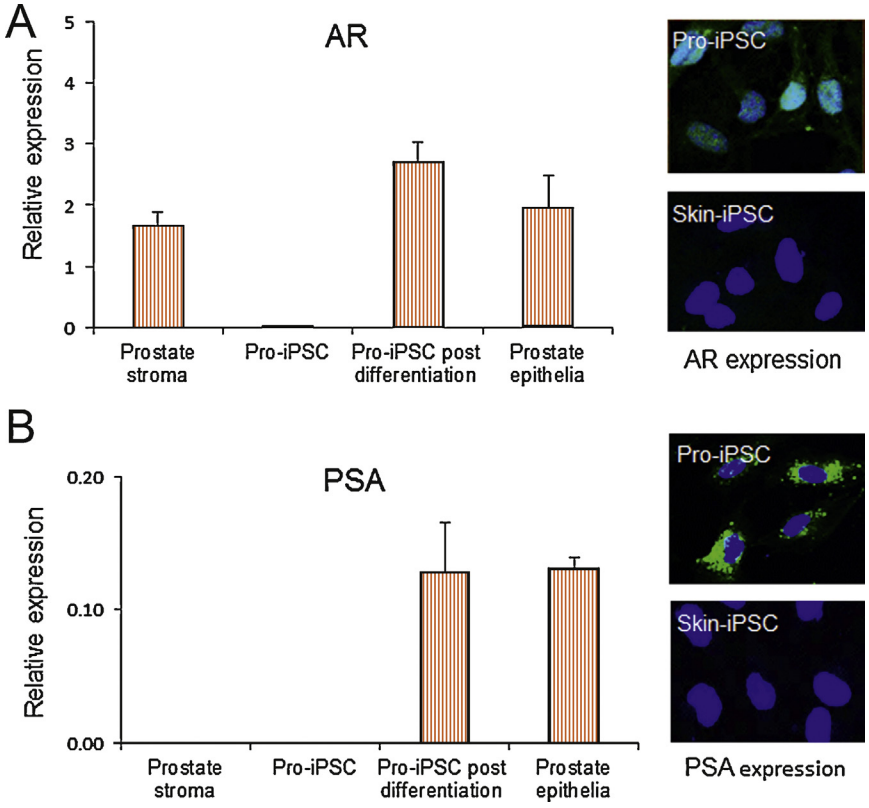

Fig. 4 – Prostate-specific differentiation of prostate induced pluripotent stem cells (Pro-iPSCs). (A) Relative messenger RNA (mRNA) expression of androgen receptor (AR) in Pro-iPSCs before and after differentiation (control levels in primary prostate stroma and epithelia shown) (n = 6). Right: Immunofluorescence staining for AR (green) shown in Pro-iPSCs and skin-iPSCs. AR is induced in Pro-iPSCs only, with functional activity suggested by nuclear AR localisations (blue 4′,6-diamidino-2-phenylindole [DAPI] counterstain). (B) Relative mRNA expression of prostate-specific antigen (PSA) in Pro-iPSCs before and after differentiation (control levels in primary prostate stroma and epithelia shown) (n = 3). Immunofluorescence staining for PSA (green) shown in Pro-iPSCs and skin-iPSCs. PSA is induced in Pro-iPSCs only (blue DAPI counterstain).

expression indicated by immunofluorescence was also confirmed in differentiated cells derived from UT-iPSCs after 2 wk in urothelial-conditioned medium (Fig. 5C).

4. Discussion

We report for the first time successful reprogramming of normal, human, ageing prostate, bladder, and ureter stromal fibroblasts to an ESC-like pluripotent state. These cells were validated as de facto iPSCs by confirming their ability for sustained self renewal, silencing of exogenous transgenes, expression of ESC-specific genes, and pluripotent differentiation into all three germ lineages. Furthermore, within the appropriate inductive environment, prostate epithelial-specific differentiation with induced functional AR, as characterised by PSA expression, was demonstrated and UT-iPSC differentiation could be directed into urothelial-specific lineages. These models allow for enormous scope in future studies of differentiation, tissue engineering, disease mechanisms, and drug development.

Since the first descriptions of iPSCs in 2006, most reports have focused on the generation of iPSCs from a range of normal and diseased tissues. In our work, we generated multiple clones of UT-iPSCs from multiple donors and sites (bladder and ureter), and multiple clones of Pro-iPSCs from a single donor. All clones were comparable to both skin-iPSCs and ESCs in terms of ESC-marker expression and pluripotency potential, supporting the generalisability of these methods to urologic tissues. Researchers exploring further differences in ability for terminal organ-specific differentiation among the iPSC lines derived from different organs have found that not all iPSCs are the same in this respect [13]. We compared the differentiation potential of Pro-iPSCs and UT-iPSCs with skin-iPSCs and our results demonstrated vast differences in their capabilities for prostate- and bladder-specific differentiation. These findings are consistent with emerging evidence that epigenetic imprinting, specific to the tissue type of iPSC origin, remains intact throughout the mechanism of stem cell reprogramming and may limit the potential for terminal differentiation into all cell types [12–14]. Our data emphasise the importance of the source from which iPSCs are generated as a consideration for organ-specific development studies.

Although we confirmed prostate-specific differentiation, additional work is required to determine whether Pro-iPSCs can generate the full breadth of epithelial differentiation. In addition, it would be of interest to investigate if these cells differentiate through somatic stem cell phenotypes or directly into basal and luminal cells, in keeping with an alternative model that proposes that epithelial stem cells

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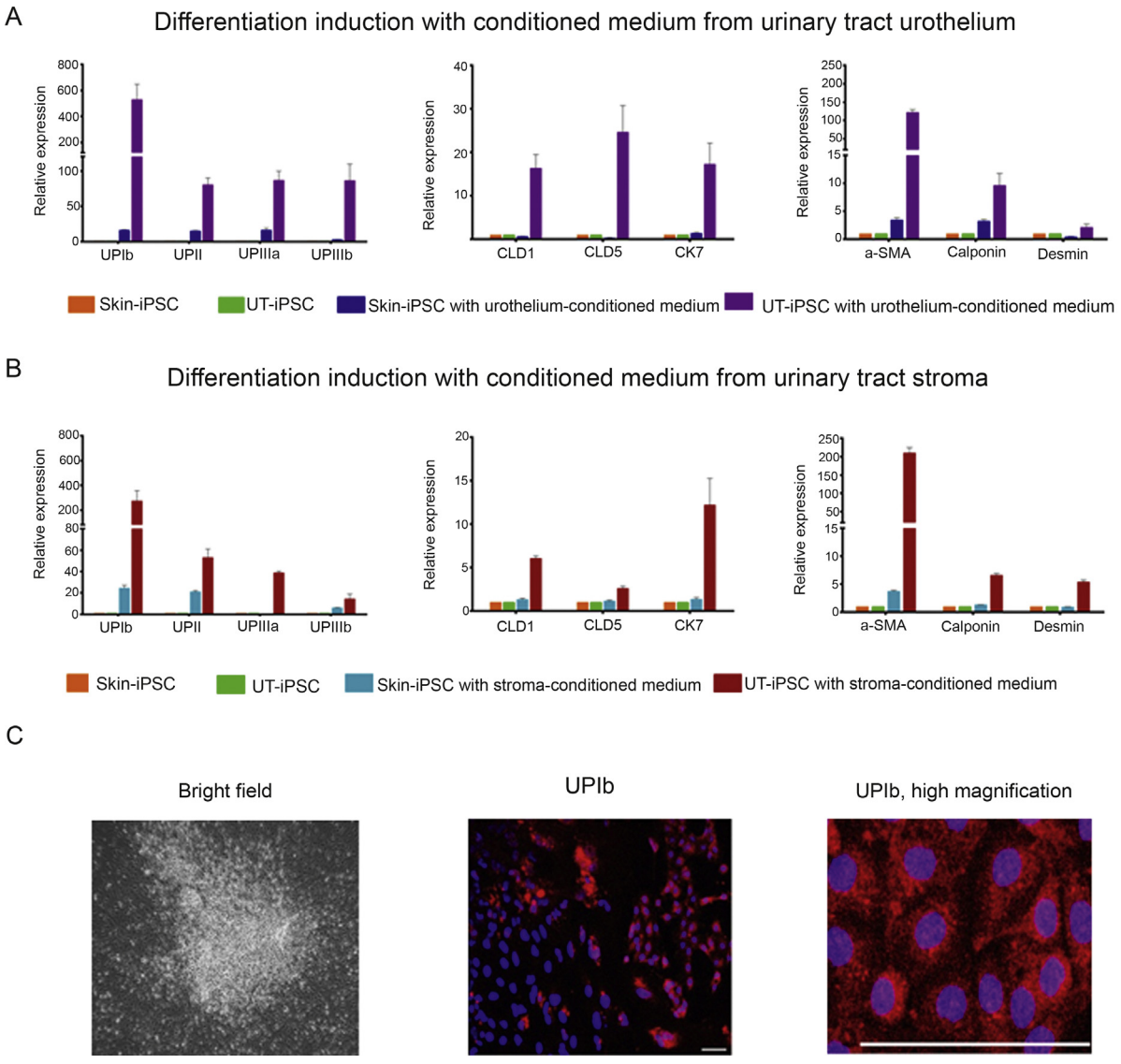

Fig. 5 – Expression of urothelial and smooth-muscle lineage-specific transcripts in differentiated cells derived from urinary tract induced pluripotent stem cells (UT-iPSCs) and skin-iPSCs by reverse transcription-polymerase chain reaction on day 14. Messenger RNA levels are shown as a fold change relative to control (undifferentiated cells) (*n* = 3). (A) Expression of urothelial-specific markers (UPIb, UPII, UPIIIa, UPIIIb, claudin 1 [*CLD1*], claudin 5 [*CLD5*], keratin 7 [*CK7*]) and smooth-muscle-specific markers (α-smooth muscle actin [a-SMA], calponin, desmin) induced with conditioned medium from urothelium. (B) Expression of urothelial- and smooth-muscle–specific markers induced with conditioned medium from stroma. (C) Immunofluorescence of differentiated cells derived from UT-iPSCs treated with conditioned medium, at day 14, showing (left) bright field; (centre) positive staining for UPIb (red) juxtaposed with an area of UPIb-negative staining, with 4′,6-diamidino-2-phenylindole nuclear counterstain (blue); and high magnification of UPIb immunostaining (scale bar = 100 μm).

maintain the phenotype of the original embryonic progenitor of the prostate (urogenital sinus epithelium) [23]. Also of interest would be further in vivo study of prostate epithelial development and organisation from iPSCs, especially given the considerable debate about the nature of the somatic stem cell in prostate epithelium. Recent evidence has shown that in early, postnatal, mouse prostate development, epithelial homeostasis is maintained by basal multipotent stem cells that differentiate into basal, luminal, and neuroendocrine cells, as well as by unipotent basal and luminal progenitors [24]. In contrast, in situ assays in the human adult setting have revealed that epithelial hierarchical organisation is based on a common stem cell [25]. The Pro-iPSC model is well placed to explore these differences with lineage tracking studies. Furthermore, the generation of ESC-like Pro-iPSCs are of particular interest to the study of prostate disease, where benign prostatic hyperplasia has been associated with models of embryonic reawakening [26] and prostate cancer can be associated with ESC-marker expressions [27].

Atala and colleagues reported the first human clinical trial with engraftment of both urothelial and smooth-muscle stromal cells into acellular biomaterials for bladder engineering and reconstruction [28]. However, these strategies rely on ex vivo cell culture to generate sufficient quantities and quality of autologous cells. Although even small biopsies of normal urothelium can be readily expanded before undergoing senescence, this is significantly restricted in diseased tissue [29]. We were unsuccessful in preliminary

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attempts at inducing urothelium. Further experimentation was not pursued given that the stroma-based UT-iPSCs demonstrated ability to differentiate into urothelium. In clinical applications for regenerative medicine, this phenomenon also provides the additional attraction of using an alternative, genetically normal tissue source in urothelial malignancies for reconstruction. However, concerns about induction of malignancies from iPSCs persist and are being cautiously tackled with refinements in induction methods that include virus-free and transgene-free reprogramming and xeno-free approaches [30]. Alternative approaches are now becoming established to realise this ambition, including vector-free human-iPSC generation using episomal-factor delivery, and feeder-free and albumin-free culture [31]. As such, UT-iPSCs show great promise in clinical regenerative medicine and modelling urinary tract disease.

5. Conclusions

Human prostate and urinary tract tissue can be used to generate iPSCs that can be differentiated back into their parent organ lineages. The generation of Pro-iPSCs and UT-iPSCs provides a convenient ready-to-access model that offers considerable potential for studies of normal and diseased prostate and bladder biology.

Author contributions: Craig Robson had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study concept and design: Robson, Heer.
Acquisition of data: Moad, Pal, Hepburn, Williamson, Franco, Fordham.
Analysis and interpretation of data: Robson, Heer, Moad, Pal, Hepburn, Williamson, Hayward, Franco, Lako, Armstrong, Fordham, Przyborski.
Drafting of the manuscript: Robson, Heer, Pal, Moad.
Critical revision of the manuscript for important intellectual content: Moad, Pal, Hepburn, Williamson, Wilson, Lako, Armstrong, Hayward, Franco, Cates, Fordham, Przyborski, Carr-Wilkinson, Robson, Heer.
Statistical analysis: Moad.
Obtaining funding: Robson, Heer.
Administrative, technical, or material support: Robson, Heer.
Supervision: Robson, Heer.
Other (specify): None.

Financial disclosures: Craig Robson certifies that all conflicts of interest, including specific financial interests and relationships and affiliations relevant to the subject matter or materials discussed in the manuscript (eg, employment/affiliation, grants or funding, consultancies, honoraria, stock ownership or options, expert testimony, royalties, or patents filed, received, or pending), are the following: None.

Funding/Support and role of the sponsor: This work was supported by grants from the JGWP Foundation, Cancer Research UK, EU FP7 Marie Curie Integrated Training Network (PRO-NEST), and the NIH (R01 DK067049). Cancer Research UK, PRO-NEST, Newcastle University, was involved in the design and conduct of the study.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.eururo.2013.03.054.

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