

**An investigation into whether telomerase can be used to  
identify stem cells in the mouse**

Thesis submitted for the degree of  
Doctor of Philosophy  
at the University of Leicester

by

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September 2007

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## Abbreviations

ABC	ATP binding cassette
AJ	adherens junction
ALT	alternative lengthening of telomeres
AP	alkaline phosphatase
AP-1	activating protein-1
AP-2	activating protein-2
ATM	ataxia telangiectasia mutated
BAC	bacterial artificial chromosome
BCC	basal cell carcinoma
Bcrp	breast cancer resistance protein
bFGF	basic fibroblast growth factor
$\beta$ -gal	$\beta$ -galactosidase
BL	basal layer
BLAST	Basic local alignment search tool
bp	base pairs
BrdU	bromo-2'deoxy-uridine
cDNA	complementary DNA
CFP	cyan fluorescent protein
CHORI	childrens hospital Oakland research institute
CLP	common lymphoid progenitor
CMP	common myeloid progenitor
CNS	central nervous system
CXCL5	lipopolysaccharide-induced CXC chemokine
DKC	dyskeratosis congenital
DMEM	Dulbeccos modified eagles medium
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
Dox	doxycycline
DP	dermal papilla
dpc	days post coitum
E	embryonic day
EB	embryoid body
EC cell	embryonal carcinoma cell
EMSA	electrophoretic mobility shift assays

EPU	epidermal proliferative unit
ES cell	embryonic stem cell
ESCs	epithelial stem cells
ESTs	expressed sequence tags
FACS	fluorescence activated cell sorting
GDNF	glial cell line derived growth factor
GFAP	glial fibrillar acid protein
GFP	green fluorescent protein
GL	granular layer
GSC	germ-line stem cell
HF	hair follicle
Hox	Homeobox
HPRT	Hypoxanthine Guanine Phosphoribosyl Transferase
HSC	haemopoietic stem cell
HSV	herpes simplex virus
ICM	inner cell mass
IF	intermediate filaments
IRS	inner root sheath
ISCs	intestinal stem cells
JAKs	Janus tyrosine kinases
K15	keratin 15
K19	keratin 19
LB	Luria Bertani
LCM	laser capture microdissection
LCR	locus control region
Lhx2	Lim-homeodomain transcription factor
LI	large intestine
LIF	leukaemia inhibitory factor
LRC	label-retaining cell
LTRC	long-term repopulating cell
MCS	multiple cloning site
MEF	mouse embryo fibroblast
MSCs	mesenchymal stem cells
NCBI	national center for biotechnology information
<i>neo<sup>R</sup></i>	neomycin resistance
NFκB	nuclear factor κB
NSC	neural stem cell

ORS	outer root sheath
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFA	paraformaldehyde
PGCs	Primordial germ cells
PLCs	pulse-labelled cells
POT1	protection of telomeres 1
RNA	ribonucleic acid
SC	stratum corneum
Sca-1	stem cell antigen
SCF	stem cell factor
SDF-1	stromal cell derived factor
SDS	sodium dodecyl sulphate
Shh	sonic hedgehog
shRNA	short hairpin RNA
SI	small intestine
SL	spinous layer
SLAM	signalling lymphocytic activation molecule
Sox2	SRY-related HMG box2
SP	side population
SSC	sodium chloride-sodium citrate
SSC	spermatogonial stem cell
STO	SIM mouse embryo-derived thioguanine and ouabain resistant
STRC	short-term repopulating cell
SV40	Simian virus 40
TA	transit-amplifying
TAE	Tris-acetate-EDTA
TE	Tris-EDTA
TERC	telomerase RNA component
TERT	telomerase reverse transcriptase
TGF- $\beta$	transforming growth factor $\beta$
TK	thymidine kinase
TRAP	telomere repeat amplification protocol
TRE	tetracycline-responsive regulatory element
TRFs	telomere repeat fragments
TRF1	telomere repeat factor 1
TRF2	telomere repeat factor 2

TRFH	TRF homology domain
TUNEL	TdT-mediated dUTP nick end labelling
V	volts
v/v	volume to volume
w/v	weight to volume
YFP	yellow fluorescent protein

## Acknowledgements

Firstly, I would like to thank my supervisors Dr. Catrin Pritchard and Prof. Felix Beck for giving me the opportunity to work with them on such an interesting project. Thanks also go to all the members of the Biomedical Services involved in this project and Jenny Edwards for her histology expertise. I would also like to thank all the members of the lab both past and present. Particular thanks go to Kathryn, Susan, Emma, Catherine, Linda, Alison and Emmanuel for all their help, cake, coffee breaks and trips to the pub! I would also like to say a special thank you to Kath for keeping me running and putting a roof over our heads during the past month. In addition I would like to thank my mum and John and Linda Day for their support and encouragement. I finally want to thank Greg for looking after me and putting up with me over the past four years! I love you.

**Abstract****An investigation into whether Tert can be used to identify stem cells in the mouse**  
**Victoria Aldridge**

Telomeres are G-rich strands of DNA that protect chromosome ends from recognition as a DNA break. Telomeric DNA addition is catalysed by the reverse transcriptase telomerase which consists of a catalytic component; Tert and an RNA component Terc. *In vitro* and *in vivo* studies demonstrated that the primary mode of regulation of telomerase is through transcriptional regulation of the *Tert* gene. The *mTert* promoter driving a fluorescent reporter can recapitulate endogenous *mTert* expression in ES cells *in vitro*. Telomerase activity is a characteristic of stem cells. However, upon differentiation of stem cells, telomerase activity is switched off; resulting in the progressive shortening of telomeres until they are critically short and cellular senescence is triggered. Given that telomerase activity is determined by *Tert* promoter activity, it is hypothesised here that the *Tert* promoter could be used to mark stem cells in the adult.

A vector was generated in which the *mTert* promoter drives the expression of CFP. *In vitro* analysis of *Tert*-CFP ES clones revealed that CFP was expressed in undifferentiated cells but was down-regulated as ES cells were differentiated. Teratomas were generated from ES cells expressing the transgenic vector and CFP expression was found to correlate with regions of undifferentiation and intense proliferation.

Direct DNA microinjection generated four transgenic mouse lines containing the transgene within the germline. RT-PCR for endogenous *mTert* and CFP showed that their expression varied similarly from two transgenic lines. Histological analysis of testis and skin has shown expression in spermatogonia, basal layer and bulge cells, thus indicating activity of the reporter in these stem cells. However, reporter expression is also evident in spermatocytes of the testis and spinous and granular layers of the skin. Thus, the reporter not only identifies highly proliferative cells, it also identifies cells with a lower proliferative potential.

# 1. Introduction

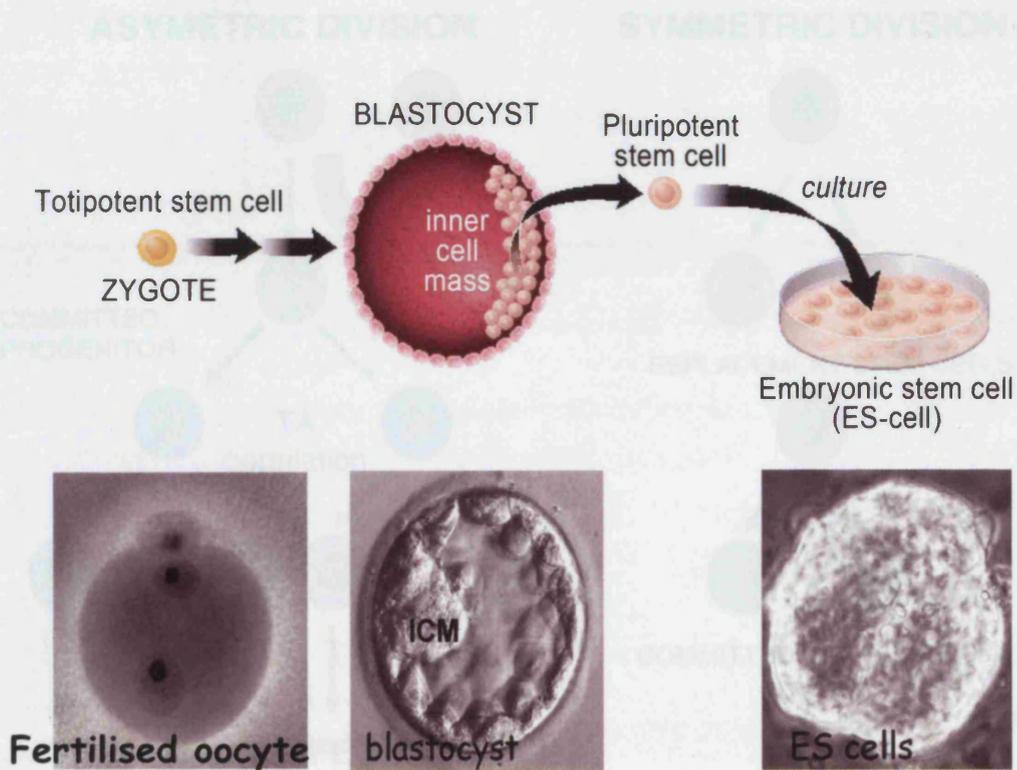
## 1.1 Stem cell characteristics

Stem cells are undifferentiated cells that renew themselves through mitotic divisions and are capable of differentiating into a diverse range of specialised cell types. There are two kinds of stem cells; embryonic stem (ES) cells and adult/somatic stem cells (Anderson *et al.*, 2001). ES cells are derived from the inner cell mass (ICM) of a blastocyst which is the source of all the tissues of the developing embryo (Figure 1.1). ES cells can therefore contribute to all three germ layers; ectoderm, endoderm and mesoderm (Alison *et al.*, 2002). Adult stem cells give rise to cell types required by a particular tissue. Both of these stem cells have the ability to give rise to at least one but often many specialised cell types whilst possessing the ability to divide without undergoing differentiation. This capacity to divide without differentiation is known as self-renewal (Alison *et al.*, 2002). This is particularly important for somatic stem cells in order to avoid exhausting stem cell reserves. The adult stem cell is able to divide asymmetrically and thus give rise to one committed progenitor cell to populate the tissue and one replacement stem cell (Alison *et al.*, 2002) (Figure 1.2). The committed progenitor cell enters the transit-amplifying (TA) compartment and becomes known as a TA cell (Figure 1.2). However, it must be noted here that not all tissues have a TA compartment. These cells then continue to divide until they become terminally differentiated and are able to perform particular functions required by the organism.

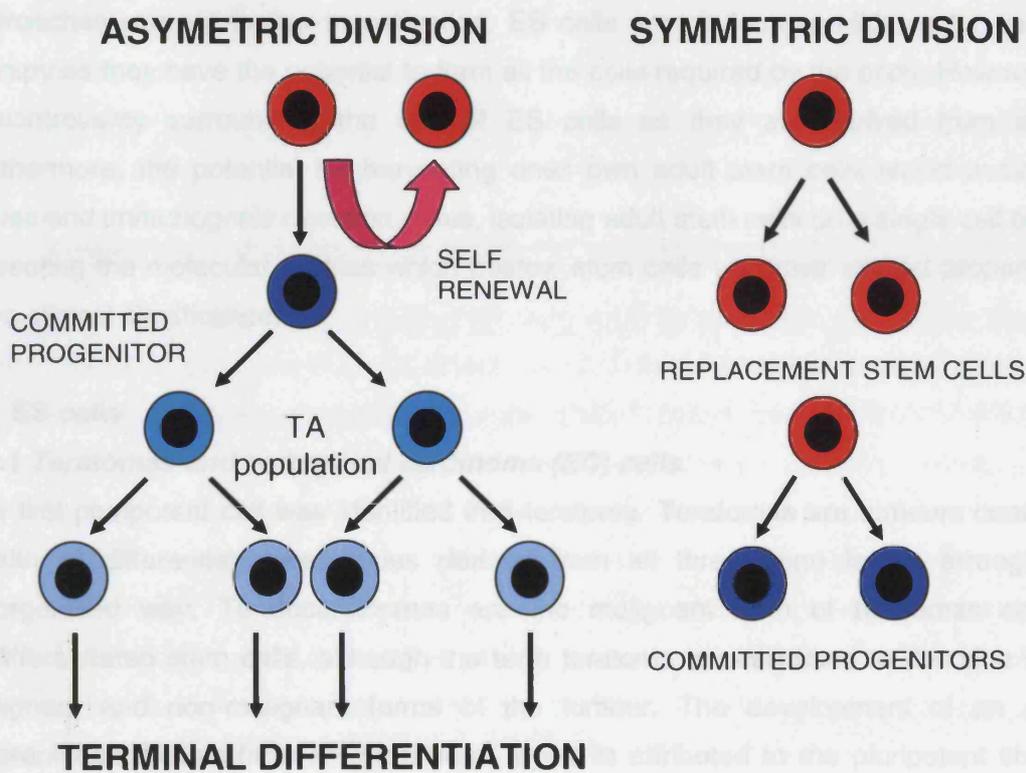
A stem cell's ability to give rise to various specialised cell types is called its potency. Totipotent stem cells form all cell types within the zygote, including the trophoblast of the placenta (Alison *et al.*, 2002) (Figure 1.1). Pluripotent cells give rise to almost all cell types except the placenta and placental tissue (Alison *et al.*, 2002). ES cells are pluripotent. Multipotent cells give rise to a limited range of cells appropriate to their location (Alison *et al.*, 2002). Adult stem cells are usually described as multipotent. However, adult stem cells can be unipotent; which means that they can only give rise one specific cell type (Alison *et al.*, 2002).

Due to their natural role in regenerating tissue and their lineage potential, stem cells are ideal candidates for cell therapy (Serakinci and Keith, 2006). For example if neural stem cells (NSCs) could be isolated and grown in culture, then they could potentially be used to treat neurological disorders such as Parkinsons and Alzheimers disease. Indeed,

**Figure 1.1** The zygote contains two pronuclei; one smaller female pronucleus and one slightly larger male pronucleus. Totipotent cells from the zygote form the blastocyst by differentiating to form the trophoblasts that become the placenta and the ICM that forms the embryo. The cells from the ICM can be collected and cultured as ES cells under particular conditions. ES cells are pluripotent cells that have the ability to form all the cells required by the organism. The cartoon (top) is taken from Anderson, *et al.* (2002). The zygote picture is taken from Krukowska, *et al* (2005). The blastocyst and ES cell pictures are taken from [www.jncasr.ac.in/vbl/](http://www.jncasr.ac.in/vbl/)



**Figure 1.2** Asymmetric and symmetric stem cell division. Asymmetric division results in the production of one committed progenitor cell which then enters the TA compartment, proliferates and terminally differentiates. Symmetric division can result in the production of two replacement stem cells or two committed progenitors, depending on the demand of the tissue.



investigators have already shown that neural stem cells harvested from green fluorescent protein (GFP) expressing transgenic mice improve neural dysfunction when transplanted into Parkinsons diseased rats (Wei *et al.*, 2007). This was postulated to be due to the fact that the transplanted NSCs express beneficial trophic factors and differentiate to replace some of the cells lost in the disease (Wei *et al.*, 2007). Putative adult stem cells from one tissue have also been used to treat disease in a different tissue. For the treatment of liver disease it has been shown that patients own putative haemopoietic stem cells (HSCs) (isolated by CD34 expression and their ability to adhere to tissue culture plastic) when injected into their portal vein or hepatic artery can increase levels of bilirubin and serum albumin which are indicators of normal liver function (Gordon *et al.*, 2006). Thus, these approaches warrant further investigation. ES cells are obvious candidates for use in cell therapy as they have the potential to form all the cells required by the body. However, there is controversy surrounding the use of ES cells as they are derived from embryos. Furthermore, the potential for harvesting ones own adult stem cells would avoid ethical issues and immunogenic rejection. Thus, isolating adult stem cells on a single cell basis and dissecting the molecular profiles which bestow stem cells with their special properties may have clinical significance.

## **1.2 ES cells**

### **1.2.1 Teratomas and embryonal carcinoma (EC) cells**

The first pluripotent cell was identified in a teratoma. Teratomas are tumours containing a wealth of differentiated cell types derived from all three germ layers arranged in a disorganised way. Teratocarcinomas are the malignant form of teratomas containing undifferentiated stem cells, although the term teratoma is often used to describe both the malignant and non-malignant forms of the tumour. The development of an array of differentiated tissues from all three germ layers is attributed to the pluripotent stem cells contained within the tumour. Teratocarcinomas were first identified to spontaneously arise in the testis of strain 129 mice (Stevens, 1958). Primordial germ cells are the cells that give rise to these tumours. Teratomas can also be made to form when 2-cell ova (Stevens, 1968) or 3-6 day embryos (Stevens, 1970) are grafted onto adult testis. The tumours were shown to be histologically identical to those that arise spontaneously and a portion of them could be transplanted into secondary recipients and maintained for prolonged periods. This was attributed to the pluripotent cells within the tumour derived from the embryo or ova.

The potent cells contained within a teratoma are known as Embryonal Carcinoma (EC) cells. Single EC cells were isolated from a teratoma and re-transplanted into mice. These EC cells gave rise to teratomas containing an assortment of differentiated cells, thus

proving the potency of these single cells (Kleinsmith and Pierce, 1964). Stem cell lines harvested from teratocarcinomas are referred to as Embryonal Carcinoma Cell (ECC) lines or EC cells and are maintained in an undifferentiated manner *in vitro* by culturing on a fibroblast feeder layer of mouse cells (Finch and Ephrussi, 1967). Undifferentiated EC cells have been shown to contribute to embryo development; although inefficiently (Brinster, 1974). The resulting mouse is a chimera, with cells originating from the host as well as the introduced EC cells (Bradley *et al.*, 1984). Many chimeras generated using EC cells developed tumours (Bradley *et al.*, 1984). Therefore although a useful tool, EC cells have some limitations.

### **1.2.2 Isolation of ES cells**

Potent cells exist in the mouse embryo as shown by the development of teratomas from the grafting of embryos onto mouse testes. The isolation of pluripotent cells directly from early embryos was achieved by two investigators in the early 1980s. Evans and Kaufmann (1981) delayed mouse blastocyst implantation by ovariectomy. The embryos hatched from the zona and remained free in the lumen of the uterus. These delayed blastocysts were then collected and cultured. After two days of culture blastocysts attached to the plastic and trophoectoderm cells grew out and differentiated. Egg cylinder-like structures then formed which were picked and cultured on gelatin-coated plates overlaid with a mitomycin-C inactivated fibroblast feeder layer. These cells resembled cultured EC cells and their potency was proved by injection into mice to form teratomas (Evans and Kaufman, 1981). Martin, (1981) isolated pluripotential cells from the ICM of blastocysts and cultured them in media conditioned by an established teratocarcinoma stem cell line (Martin, 1981). This experiment highlighted the possibility of the presence of a factor, e.g. a growth factor contained within the conditioned media important for maintaining the undifferentiated stem cell state. This factor turned out to be the cytokine Leukaemia Inhibitory Factor (LIF) (Smith *et al.*, 1988; Williams *et al.*, 1988). LIF was first identified to induce macrophage differentiation of a mouse M1 myeloid leukaemia cell line. Thus LIF performs divergent functions probably due to the differences in signalling pathways between the two cell types.

### **1.2.3 Differentiation of EC/ES cells *in vitro***

The potency of both EC and ES cells has been demonstrated by injection of these cells into mouse testis or into an extrauterine site to form teratomas (Evans and Kaufman, 1981; Kleinsmith and Pierce, 1964). Analysis of the differentiation of these cells *in vitro*, under controlled conditions is another valuable way of determining the potency of these cells as well as providing a model system in which factors can be supplied or withdrawn to identify the effects on determination/ differentiation of these cells.

The first experimental procedures that demonstrated the differentiation potential of EC cells involved the isolation of mouse teratoma aggregates and injection of these cells intraperitoneally into a healthy mouse (Pierce and Dixon, 1959). Aggregates were observed in the ascitic fluid of the host composed of a layer of visceral yolk sac overlying mesenchyme which contained EC cells embedded within it. The organisation of these aggregates mirrored that of early embryos, therefore they were called Embryoid Bodies (EBs) (Pierce and Dixon, 1959). Two types of EBs can be formed *in vivo*; simple and cystic (Martin and Evans, 1975b). Simple EBs consist of endodermal cells surrounding an EC cell core (Martin and Evans, 1975b). Cystic EBs are generally more complex and often contain EC cells, several differentiated cell types and a fluid-filled cyst (Martin and Evans, 1975b).

Differentiation of EC cells was achieved *in vitro* by culturing in the absence of feeder cells and maintaining the aggregates that formed in culture for several weeks (Martin and Evans, 1975a). Morphological analysis showed that the aggregates became rounder with a distinct outer layer of endodermal cells. EC cells were embedded in the centre of the cell aggregates which resembled the EBs formed from intraperitoneal injection as previously described (Pierce and Dixon, 1959). Histological alkaline phosphatase (AP) staining is often used to identify germ cells. AP staining was present at high levels in the EC cells but absent from the EBs, thus demonstrating their differentiated status (Bernstine *et al.*, 1973). Furthermore if the EBs were supplied fresh media, allowed to attach to plastic and cultured for several weeks; extensive differentiation was achieved.

The differentiation capacity of EC cells differentiated via EB formation *in vitro* compared to that achieved within a teratoma formed *in vivo* is similar. This was demonstrated by comparing histological sections prepared from differentiated EC cells grown on a solvent-resistant plastic Petri dish and sections prepared from a teratoma (Martin and Evans, 1975b). Almost all cell types found within the teratoma were also seen in EC cells differentiated *in vitro*.

ES cells have been demonstrated to form EBs *in vitro* when cultured in the absence of feeders for at least a week. Once formed, EBs were re-plated and allowed to attach and spread on culture plastic and many differentiated tissues were produced (Evans and Kaufman, 1981). To date, ES cells are still differentiated in the absence of feeder cells and in the absence of the cytokine LIF which is usually added to the culture media to aid self-renewal (Smith *et al.*, 1988; Williams *et al.*, 1988).

### **1.2.4 ES cell markers**

A complex transcriptional network has been found to be involved in maintaining the pluripotent state of ES cells. The three most well-known transcription factors involved in maintaining ES cell pluripotency are Oct-4, Sox2 and Nanog (Chambers *et al.*, 2003; Masui *et al.*, 2007; Rodda *et al.*, 2005; Yoshimizu *et al.*, 1999). The POU transcription factor Oct-4 is a well-known ES cell pluripotency marker (Yoshimizu *et al.*, 1999). Oct-4 is able to bind target genes containing the octamer consensus sequence ATGCAAAT. Oct-4 is expressed in the ICM cells of the blastocyst and later in development expression is restricted to the germ cells (Yoshimizu *et al.*, 1999). The germ cells continue to express Oct-4 until initiation of sexual differentiation of the gametes. Oct-4 knockout embryos are unable to form the ICM (Nichols *et al.*, 1998). Oct-4 is also expressed in ES cells *in vitro* (Niwa *et al.*, 2000). Upon differentiation of ES cells into EBs expression of Oct-4 is down-regulated (Niwa *et al.*, 2000). Thus loss of Oct-4 expression correlates with the loss of pluripotency of these cells. However, if the expression of Oct-4 is maintained in ES cells whilst LIF is removed from the media, the cells still differentiate. Therefore Oct-4 does not maintain pluripotency alone; the action of the LIF/STAT3 pathway is required (Niwa *et al.*, 2000). Nanog was discovered to maintain ES cell pluripotency independently of the LIF/STAT3 pathway by two separate investigators (Chambers *et al.*, 2003; Mitsui *et al.*, 2003). During embryonic development Nanog is expressed in pluripotent cells such as the ICM and the epiblast. During later development Nanog is only found in the germ cells (Niwa *et al.*, 1998). Nanog is expressed *in vitro* in ES cells, but this expression is down-regulated upon differentiation in a similar manner to Oct-4. The transcription factor SRY-related HMG box2 (Sox2) is a HMG-family protein involved in ES self-renewal (Masui *et al.*, 2007). Sox2 is expressed in a similar pattern to Oct-4 and acts in collaboration with Oct-4 on many of its target genes. It has been shown that there is an Oct4/Sox2 binding site in the promoter region of Nanog (Kuroda, 2005; Rodda *et al.*, 2005). Thus, Oct-4 and Sox2 are important regulators of pluripotency and potentially regulate the expression of other transcriptional components involved in maintaining pluripotency.

Oct-4 is a widely-used marker of pluripotency. Due to their expression in the primitive ICM region and in ES cells, Sox2 and Nanog also have potential as ES cell pluripotency markers.

### **1.2.5 Generation of transgenic mice**

Pronuclear injection is a technique commonly used to introduce genes into mouse embryos and involves the injection or viral infection of DNA into fertilised mouse oocytes. This technique has been successfully used to generate transgenic animals in the laboratory.

However, the generation of transgenic animals via manipulating ES cells is also frequently used and the choice of technique used depends upon the aim of the study.

In order to generate transgenic mice using pronuclear injection, fertilised oocytes for microinjection are obtained from mated, superovulated female mice. At this stage two pronuclei are visible, one female and one slightly larger male. Due to its larger size and more accessible position the male pronucleus is preferred for DNA microinjection although it has been shown that there is no difference in transgenic offspring that have been produced by injection into the female pronucleus. However, injection into the male pronucleus is slightly more efficient (Brinster *et al.*, 1985). The oocytes that are successfully injected are then transferred into a pseudo pregnant female (Figure 1.3). The embryos are allowed to develop to term and approximately 20% of the offspring can be used as transgenic founders.

The first successful microinjection experiment involved injection of simian virus 40 (SV40) DNA into the mouse blastocyst cavity and showed that this viral DNA could be identified in the resulting offspring (Jaenisch and Mintz, 1974). It was then demonstrated that small plasmid pBR322 containing pieces of Herpes Simplex Virus (HSV) and SV40 DNA could also be identified in offspring using the same technique and showed that small pieces of cloned DNA could be incorporated into the host genome. Although the DNA had incorporated into the genome and was found in most or all cells of the newborn mouse, the DNA had undergone extensive sequence rearrangement (Gordon *et al.*, 1980).

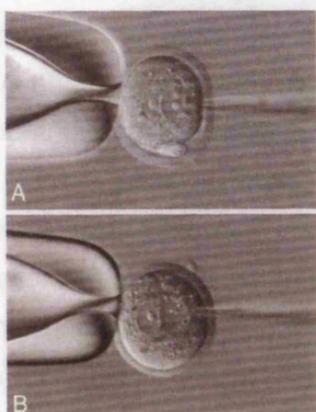
The first experiment to result in normal arrangement of injected DNA involved the introduction of two genes; the human  $\beta$ -globin gene and the thymidine kinase (*TK*) gene from HSV in a pBR322 plasmid backbone. Copies of both genes were found in embryos from different litters without any rearrangement of the DNA. It was demonstrated that functional HSV TK protein was produced in one embryo (Wagner *et al.*, 1981). Further investigations using the rabbit  $\beta$ -globin gene showed that injected DNA was present in the germ cells of the resulting mice and could therefore be transmitted to their progeny (Costantini and Lacy, 1981).

The integration of DNA into a host genome by these approaches occurs randomly. Therefore multiple copies at distinct locations within the transgenic animal can occur. However, it has been shown that multiple copies often integrate at a single site in a head-to-tail tandem array (Wagner *et al.*, 1983). Transgene expression is influenced by the position at which it integrates into the genome. However, if the transgene contains its own regulatory

**Figure 1.3** Generation of transgenic mice by direct DNA injection. Fertilised oocytes are obtained from mated superovulated female mice. (A) DNA is microinjected into the male pronucleus. (B) Microinjected oocytes are transferred into a pseudo pregnant female. (C) Offspring that possess the transgene at the DNA level are called founders and are mated to C57 or MF-1 mice to assess germline contribution. (D) Offspring that contain the transgene are F1 generation and are used to establish a transgenic line.

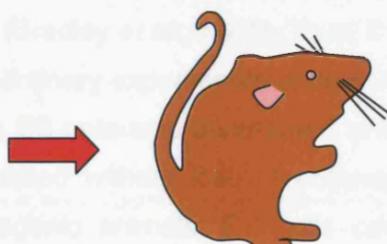
**A**

Microinjection of DNA into fertilised oocyte



**B**

Transfer of microinjected oocyte into surrogate female



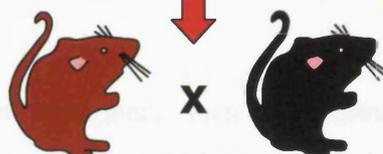
**C**



**D**



F1



F2

F3, F4 etc



elements such as a locus control region (LCR) (Grosveld *et al.*, 1987) or a CpG island (Siegfried *et al.*, 1999), positional effects are lessened. Copy number usually has no effect on transgene expression except when it contains regulatory elements as described above (Knotts *et al.*, 1995). Integration site and copy number factors mean that different expression levels between mouse lines derived from injection of the same construct can occur. Therefore all transgenic lines generated must be independently characterised.

Pluripotent EC cells have previously been shown to contribute to the cells of the developing embryo, although use of these malignant cells resulted in the development of tumours and other problems (Bradley *et al.*, 1984). The isolation of pluripotent cells from the embryo (ES cells) meant that there was a non-malignant pluripotent cell available. These cells also had the advantage of a normal karyotype (Bradley *et al.*, 1984). Thus, it was shown that ES cells can contribute to the embryo and preliminary experiments showed that over 50% of the live born animals were chimaeric and the ES cells also contributed to the germ-line (Bradley *et al.*, 1984). ES cells can be manipulated without loss of potency and therefore can be successfully used to generate transgenic animals. ES cells can be manipulated by a number of methods to allow incorporation of DNA of interest. The most common method is electroporation. This involves subjecting the ES cells and DNA to an electrical pulse, thus allowing the DNA to penetrate the ES cell (Joyner, 1999).

The first gene to be manipulated in ES cells was the human gene encoding for Hypoxanthine Guanine Phosphoribosyl Transferase (*HPRT*). This gene is X-linked; therefore disruption of a single copy in male ES cells generates an *HPRT* null genotype. This was done to provide a model system to study Lesch-Nyhan syndrome which results from *HPRT* deficiency in humans. Disruption of the gene was achieved using two approaches; one involved selecting for spontaneous mutations of *HPRT* in ES cells (Hooper *et al.*, 1987) whilst the other used retroviral vector insertion into ES cells followed by the selection for clones harbouring loss of gene activity (Kuehn *et al.*, 1987). Both approaches yielded transgenic mice deficient for *HPRT*.

The manipulation of ES cells resulting in transgenic mice harbouring gain-of-function (knock-in) or loss of function (knock-out) or more complicated targeted events is now frequently used in the laboratory. The approach used in this investigation was direct DNA injection and therefore the knock-in and knock-out approaches achieved using ES cells are not discussed here.

### 1.3 Adult stem cells

An adult stem cell or somatic stem cell resides within adult tissues and is responsible for tissue maintenance and repair upon injury (Alison *et al.*, 2002). Like their ES cell counterparts, adult stem cells are able to self-renew as well as differentiate into various cell types. Thus, adult stem cells are able to divide either asymmetrically to give rise to a replacement stem cell and one progenitor cell capable of differentiation or symmetrically to give rise to two replacement stem cells or two progenitor cells (Alison *et al.*, 2002) (Figure 1.2). The decision about whether to divide asymmetrically or symmetrically is important in maintaining stem cell numbers throughout life. The environment in which a stem cell resides is called its niche and is thought to influence this decision (Schofield, 1978).

Adult stem cells are thought to have a more limited potency than that of ES cells. It is currently accepted that adult stem cells are multipotent (or unipotent) and only give rise to cells appropriate to their location e.g. stem cells located in the brain can only give rise to neurons and other cell types present in the brain (Alison *et al.*, 2002). However, there is evidence that some adult stem cells when given the correct stimuli can alter their lineage and give rise to cell types required by another tissue. This concept is known as plasticity (Rice and Scolding, 2004).

Adult stem cells are only a small percentage of cells found within the organism e.g. the frequency of HSCs is approximately one in ten thousand of the total cells (Alison *et al.*, 2002). Therefore characterisation of tissue-specific stem cells has been difficult. HSCs were the first type of adult stem cell to be characterised and are still the best characterised to this day.

#### 1.3.1 Identifying stem cells

To date, adult stem cells can not be identified on a single cell level. Thus, in the absence of a universal marker, a combination of criteria is used to identify adult stem cells within a cell population.

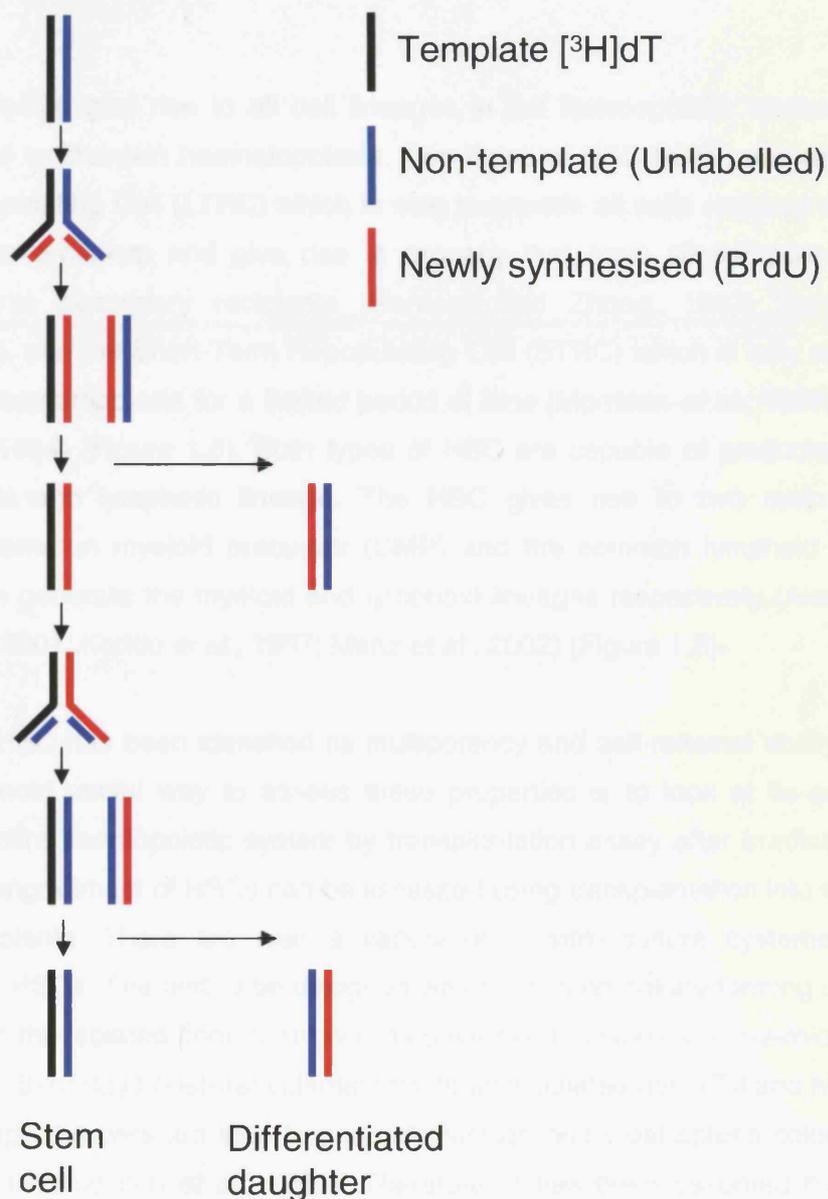
Three criteria are commonly employed to assess stem cell character. One such criterion is clonogenic potential. This uses the rationale that adult stem cells will form large self-renewing clonal colonies in culture systems whereas colonies formed from more committed cells will eventually stop proliferating, exit the cell cycle and terminally differentiate. Thus, colony-forming ability is often used as a measure of proliferation potential of putative stem cells *in vitro* (Bickenbach and Chism, 1998; Morris and Potten, 1994; Till and McCulloch,

1961). This approach was first developed to identify HSCs, although it is now also often used to identify other stem cells such as epithelial stem cells (ESCs).

The second criterion is known as the stem cell transplantation assay. This assay determines the functionality of putative stem cells and involves putting putative stem cells back into tissue whose own stem cells have been destroyed by radiation or chemical means (Brinster and Zimmermann, 1994). The ability of the putative stem cells to repopulate this tissue is then assessed. This approach is commonly used to identify cell populations containing HSCs and spermatogonial stem cells (SSCs).

The Cairns hypothesis proposed in 1975 is the third criterion (Figure 1.4) (Cairns, 1975). This hypothesis proposes that stem cells selectively retain the old DNA strands that are used as templates in DNA replication; thus providing protection for the stem cells from any replication-induced errors (Cairns, 1975). Any errors in DNA replication would thus remain in the daughter cell destined for the TA population and hence would be lost from the tissue (Figure 1.4). This hypothesis has now been employed to develop a stem cell labelling method. This method is based on the fact that although relatively quiescent to avoid the error prone division process, stem cells do divide. Therefore labelling these slowly-cycling cells would be a method for marking stem cells. Cells can be labelled by repeated injections of tritiated thymidine ( $[^3\text{H}]\text{dT}$ ) or bromo-2'deoxy-uridine (BrdU) known as the pulse to label any cells that are proliferating, followed by a prolonged interval known as the chase period in which any cells that are dividing will dilute the label and any slowly-cycling or quiescent cells will retain the label. Therefore these cells are known as label-retaining cells (LRCs) (Braun *et al.*, 2003; Cotsarelis *et al.*, 1990; Morris and Potten, 1999; Potten *et al.*, 2002). Labelling can be performed during development or following injury by radiation (post-irradiation). This method is commonly used to identify intestinal stem cells (ISCs) and ESCs, but could theoretically be applied to identify many other adult stem cell populations. LRCs can be detected in the putative stem cell position even after long experimental chase periods. If each time an LRC divides, the label is diluted by half then during long chase periods the labelled stem cells would have divided many times and greatly diluted the label to almost undetectable levels. The Cairns hypothesis provides a theory to explain the fact that LRCs can still be identified even after many rounds of stem cell division; the labelled  $[^3\text{H}]\text{dT}$  strand is selectively retained in the stem cell. Thus, stem cells are not only labelled by the LRC method because they are slowly-cycling, it also due to the selective retention of the parental DNA strand. Furthermore, it is known that small ISCs, although capable of rapid cell division, rarely acquire genetic defects such as those associated with the development of cancer. The Cairns hypothesis would provide a reasonable explanation for

**Figure 1.4** The Cairns hypothesis. Segregation of [<sup>3</sup>H]dT labelled template strands and newly synthesised BrdU labelled strands. The [<sup>3</sup>H]dT labelled template strands would be retained in the stem cells whereas the newly synthesised BrdU labelled strands would be segregated to the differentiating daughter cells and removed from the stem cells in two divisions. Adapted from Potten, *et al.* 2002.



the identification of LRCs and the fact that small intestinal cancers are rare. The caveat to this method as a stem cell labelling method is the fact that it only labels cells that were dividing during the labelling. Therefore there could be many stem cells which did not divide during this time and thus remain unlabelled.

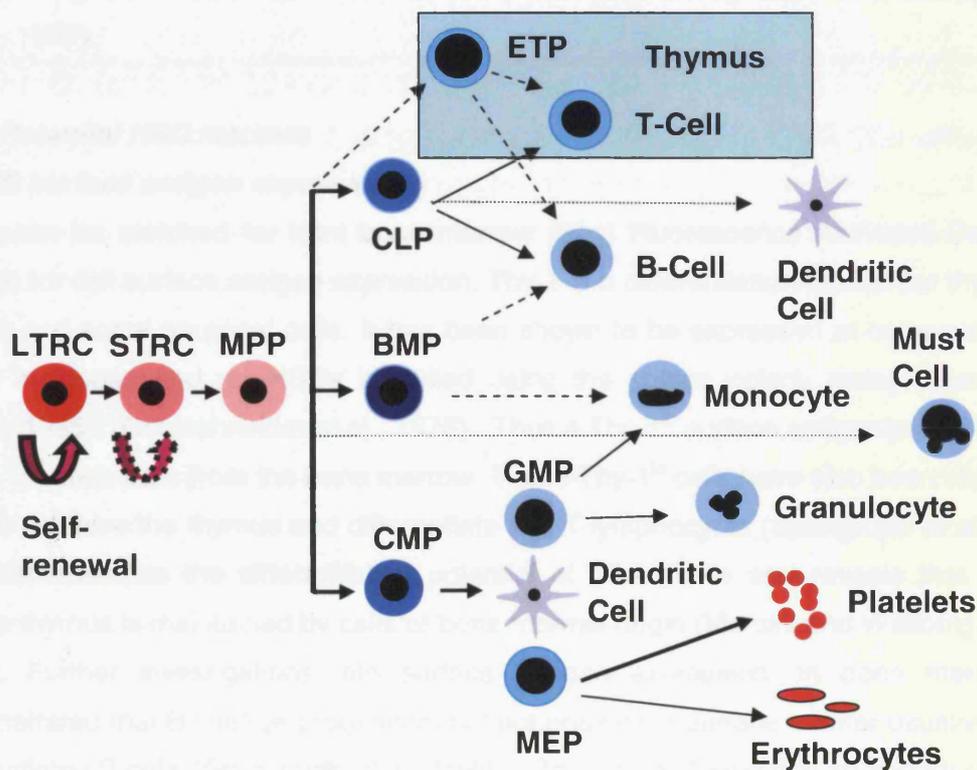
Adult stem cells are thought to reside in many adult tissues, including tissues with relatively low proliferative states such as the brain and the heart. Four types of adult stem cells will be considered here including HSCs, SSCs, ESCs and ISCs.

### 1.3.2 HSCs

HSCs are required to give rise to all cell lineages in the haemopoietic system and are therefore required to maintain haematopoiesis. Two types of HSC have been defined; the Long-Term Repopulating Cell (LTRC) which is able to provide all cells required throughout the lifetime of an organism and give rise to progeny that have similar potential upon transplantation into secondary recipients (Harrison and Zhong, 1992; Morrison and Weissman, 1994), and the Short-Term Repopulating Cell (STRC) which is only responsible for maintaining haematopoiesis for a limited period of time (Morrison *et al.*, 1997; Morrison and Weissman, 1994) (Figure 1.5). Both types of HSC are capable of producing all cells from the myeloid and lymphatic lineage. The HSC gives rise to two main types of progenitor; the common myeloid precursor (CMP) and the common lymphoid precursor (CLP) which then generate the myeloid and lymphoid lineages respectively (Akashi *et al.*, 2000; Hao *et al.*, 2001; Kondo *et al.*, 1997; Manz *et al.*, 2002) (Figure 1.5).

To prove that a HSC has been identified its multipotency and self-renewal ability must be examined. The most useful way to assess these properties is to look at its capacity to repopulate the entire haemopoietic system by transplantation assay after irradiation of the host. Long-term engraftment of HSCs can be assessed using transplantation into secondary and tertiary recipients. There are also a variety of *in vitro* culture systems used to identify/enrich for HSCs. The first to be designed was the spleen colony forming unit assay (CFU-S), in which the isolated bone marrow cells gave rise to colonies of myeloid-erythroid cells in the spleen 8-12 days post-transplantation into an irradiated host (Till and McCulloch, 1961). At this stage the cells are able to generate similar multilineal spleen colonies upon retransplantation (Siminovitch *et al.*, 1963). Therefore, it has been assumed that CFU-S ability represents pluripotential stem cell ability. However, the long-term repopulation ability of colony forming unit cells has been questioned and the cells have been shown to be unable to maintain the tissue and disappear from the spleen within 72 hours. (Magli *et al.*, 1982; Na Nakorn *et al.*, 2002). Thus, it is not clear whether these *in vitro* assays are

**Figure 1.5** Haemopoiesis. HSCs can be classified as LTRCs which are highly self-renewing and able to repopulate the haemopoietic system for the lifetime of an organism and STRCs which can repopulate, but only for a limited period of time. STRC give rise to multipotent progenitors (MPP) which give rise to lineage restricted progenitors such as CLP and CMP. CLP can only give rise to T-cells, B-cells and natural killer (NK) cells. CMP can only give rise to granulocyte/monocyte progenitor (GMP) and the megakaryocyte/erythrocyte progenitor (MEP). Alternatively, oligolineage progenitors may also arise but only at low frequencies. These include the B-cell/macrophage bipotent (BMP) progenitor and the early T-cell progenitor (ETP) in the thymus. Adapted from Bellantuono, 2004.



capable of differentiating between stem cells and progenitor cell populations. Despite this, colony-forming unit assays are still used in conjunction with other methods such as the transplantation assay to assess stem cell potential

### **1.3.3 HSC self renewal**

Self-renewal of HSCs has been demonstrated *in vivo*, using the integration of retroviral vectors as markers of stem cells, followed by analysis of these cells in the transplantation assay. It was proposed that if HSCs possessed an unlimited capacity for self-renewal then the clonal make-up of the haemopoietic system would remain stable for prolonged periods. In other words the marker (retroviral vector) would remain detectable. It was shown that the marker was indeed still detectable after periods of five (Snodgrass and Keller, 1987) and fifteen (Keller and Snodgrass, 1990) months. Furthermore secondary transplant experiments showed that the viral integration was present in blood cells from all lineages thus proving that HSCs had been marked (Keller and Snodgrass, 1990; Snodgrass and Keller, 1987).

### **1.3.4 Potential HSC markers**

#### **a) HSC surface antigen expression**

HSCs can be enriched for from bone marrow using Fluorescence Activated Cell Sorting (FACS) for cell surface antigen expression. Thy-1 is a differentiation marker for thymocytes, T-cells and some neuronal cells. It has been shown to be expressed at comparatively low levels in mouse and rat HSCs identified using the spleen colony assay, (Berman and Basch, 1985); (Goldschneider et al., 1978). Thus a Thy-1<sup>lo</sup> surface antigen profile is used to enrich for stem cells from the bone marrow. These Thy-1<sup>lo</sup> cells have also been shown to be able to colonise the thymus and differentiate into T lymphocytes (Spangrude *et al.*, 1988b). This demonstrates the differentiation potential of these cells and reveals that the adult mouse thymus is maintained by cells of bone marrow origin (Metcalf and Wakonig-Vaartaja, 1964). Further investigations into surface antigen expression on bone marrow cells demonstrated that B lineage progenitors did not possess a surface marker usually found on differentiated B cells (Spangrude *et al.*, 1988a). Thus it was found that an important criterion that identifies HSCs is that they are negative for lineage markers (Lin<sup>-</sup>). HSCs were further enriched for within the Lin<sup>-</sup> Thy-1<sup>lo</sup> cell fraction using stem cell antigen (Sca-1<sup>+</sup>) expression (Spangrude *et al.*, 1988a). Sca-1 is a member of the Ly-6 antigen family (van de Rijn *et al.*, 1989). The stem cell pool was further divided by investigations demonstrating that the tyrosine kinase family receptor c-kit was expressed in the Thy-1<sup>lo</sup> Lin<sup>-</sup> Sca-1<sup>+</sup> cell population. HSC potential was confirmed to be highest in this fraction using transplantation and CFU-S assays (Ikuta and Weissman, 1992). Therefore HSCs can be thought of as possessing a

Lin<sup>-</sup>, Thy-1.1<sup>lo</sup>, Sca-1<sup>+</sup>, c-kit<sup>+</sup> surface antigen phenotype. This narrowed the pool in which HSCs were contained, yet it is not clear whether this resulted in a pure homogenous population of HSCs.

LTRC and STRC HSCs were fractionated from this heterogeneous stem cell pool using expression of the receptor tyrosine kinase Flk-2 in order to obtain a purer population of stem cells. Isolation of the Thy-1.1<sup>lo</sup>, Flk-2<sup>-</sup> fraction provided a population capable of maintaining haematopoiesis for the life of the organism (Christensen and Weissman, 2001). An expression profile of signalling lymphocytic activation molecule (SLAM) family receptors has been shown to be able to separate HSCs, early progenitors and committed progenitors (Engel *et al.*, 2003; Kiel *et al.*, 2005). The SLAM family of receptors are arranged in a tandem array at a single locus on chromosome 1, are involved in regulating the proliferation and activation of lymphocytes and include receptors such as CD150 and CD48 (Engel *et al.*, 2003; Howie *et al.*, 2002; Wang *et al.*, 2004). HSCs were demonstrated to be CD150<sup>+</sup> CD244<sup>-</sup> CD48<sup>-</sup>, whereas early progenitors are CD150<sup>-</sup> CD244<sup>+</sup> CD48<sup>-</sup> and committed progenitors are CD150<sup>-</sup> CD244<sup>+</sup> CD48<sup>+</sup> (Kiel *et al.*, 2005). The progenitor population CMP and CLP can also be separated using surface antigen expression of IL-7R $\alpha$  within the Lin<sup>-</sup>, Thy-1.1<sup>-</sup>, Sca-1<sup>lo</sup>, c-kit<sup>lo</sup> population. Using clonogenic colony forming and transplant assays in combination with surface antigen expression, CLP progenitors are found to be IL-7R $\alpha$ <sup>+</sup>, whereas CMP progenitors are IL-7R $\alpha$ <sup>-</sup> (Akashi *et al.*, 2000; Kondo *et al.*, 1997).

The CD34 antigen is expressed on human (Civin *et al.*, 1984) and mouse (Krause *et al.*, 1994) haemopoietic stem and progenitor cells as determined using *in vitro* colony forming assays and the transplant assay and its expression has been shown to decrease as the cells become more differentiated. Thus, CD34 is often used as a marker for HSCs. This is contradicted by investigations that have shown that CD34 expression does not correlate with stem cell potential (Matsuoka *et al.*, 2001; Sato *et al.*, 1999) and that some HSCs do not express CD34 (Bhatia *et al.*, 1998). Thus it has been shown that some surface antigens are not required for stem cell function (Bellantuono, 2004).

In summary, surface antigen expression is a useful way to enrich for HSCs in combination with the transplant assay and other criteria set to identify stem cells; yet it is so far not possible to identify a HSC on a single cell level using this method. However, separation of various progenitor populations and the LTRC and STRC may prove useful as their molecular and genetic profiles may be determined which may aid in the elucidation of the differences which confer stemness or set the cell on the road to differentiation.

**b) Haemopoietic side population cells**

A small subset population of cells isolated from whole bone marrow called side population (SP) cells was discovered during cell cycle investigations using the DNA binding dye Hoechst 33342 and FACS analysis (Goodell *et al.*, 1996). Staining using Hoescht 33342 revealed a complex staining pattern showing two emission wavelengths caused by the ability of SP cells to efflux the dye. SP cells demonstrate a multi lineage differentiation capacity *in vitro* (Matsuzaki *et al.*, 2004), express HSC surface markers and are able to reconstitute irradiated animals. The ATP binding cassette (ABC) transporter superfamily member breast cancer resistance protein (*Bcrp1*) has been shown to be responsible for the SP phenotype in mice (Zhou *et al.*, 2002). Analysis of *Bcrp1*<sup>-/-</sup> mice revealed that HSCs were still present but did not display SP abilities. Furthermore it was shown that *Bcrp1*<sup>-/-</sup> HSCs were more susceptible to damage by chemical means, suggesting that the role of *Bcrp1* could be to protect stem cells from toxic damage. SP cells are also found in other tissues such as skeletal muscle (Gussoni *et al.*, 1999; Jackson *et al.*, 1999) and brain (Hulspas and Quesenberry, 2000) and may also be stem cells. Thus, the SP phenotype may have a physiological role in stem cells and it has been suggested that this role is to protect stem cells from damage induced by naturally occurring toxins. These cells are easily isolated by FACS and therefore can provide an accessible primitive cell population; however, it is still not a pure HSC population.

**c) Other potential HSC markers**

HSC self-renewal is regulated by a number of key signalling pathways and transcriptional networks. The homeobox (Hox) family of transcription factors are known to be involved in the regulation of haematopoiesis (Buske and Humphries, 2000; Magli *et al.*, 1997). A Hox family member *Hoxb4* is expressed in primitive populations of bone marrow cells. HSCs induced to overexpress *Hoxb4* by retroviral transfection demonstrated a dramatically enhanced ability to regenerate the stem cell compartment in serial transplantations (Sauvageau *et al.*, 1995). The ability of *Hoxb4* to expand HSCs was similarly demonstrated *in vitro* with *Hoxb4* transfected cells showing a 40 fold net increase in stem cells (Antonchuk *et al.*, 2002). Thus *Hoxb4* plays a role in regulating HSC self-renewal and is expressed in primitive haemopoietic populations. However, its potential as a stem cell marker needs to be more carefully examined.

The polycomb group gene *Bmi1* is involved in regulating the proliferative capacity of HSCs. *Bmi1* is expressed in primitive haemopoietic cells in both mice (Park *et al.*, 2003) and humans (Lessard *et al.*, 1999) and *Bmi1*<sup>-/-</sup> mice demonstrate a progressive haematopoietic failure, which indicates that *Bmi1* is essential for the maintenance of adult HSCs (Lessard

and Sauvageau, 2003; Park *et al.*, 2003). Thus, *Bmi1* also has potential as a stem cell marker.

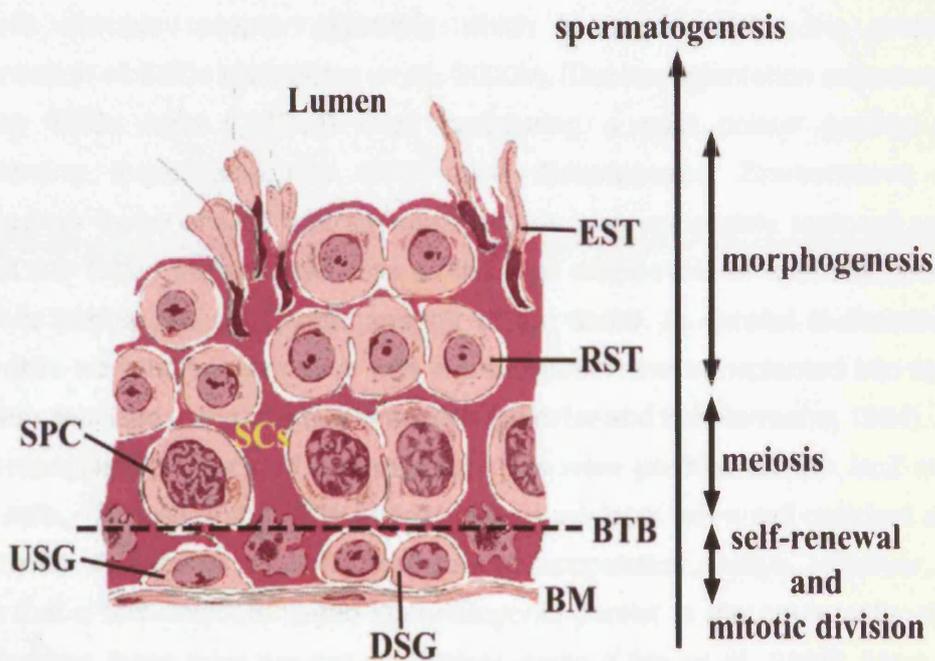
The Notch signalling pathway has been demonstrated to influence cell fate decisions in many organisms by inhibiting differentiation pathways (Artavanis-Tsakonas *et al.*, 1999; Simpson, 1998). The Notch receptor is expressed on primitive and mature haemopoietic cells, whereas its ligands are expressed on stromal cells (Varnum-Finney *et al.*, 1998; Walker *et al.*, 2001). Culture experiments with surface antigen phenotype sorted HSCs on a fibroblast cell layer expressing the Notch ligand Jagged-1 demonstrated an expansion of the HSC pool (Varnum-Finney *et al.*, 1998).

To summarise; HSCs are currently enriched to a relatively pure population using a particular surface antigen phenotype as previously discussed. Many signalling pathway components and transcription factors are expressed in primitive cell populations and therefore could aid in the enrichment of HSCs. However, none of these components have so far aided in the isolation of HSCs on a single cell level. The potential of other signalling pathway components or transcription factors as markers of HSCs needs to be examined.

### 1.3.5 SSCs

The process of sperm production in mammals is known as spermatogenesis and is essential for the maintenance of the species as genetic information is passed to the next generation via the spermatozoa. Spermatogenesis occurs in the seminiferous tubules of the testis and is maintained by proliferation and differentiation of SSCs (Figure 1.6). The SSCs are derived from primordial germ cells (PGCs) and transition to SSCs occurs between embryonic days 0 and 5 in the mouse (McLean *et al.*, 2003). The spermatogenesis cycle begins with differentiation of spermatogonia that are located on the basement membrane of the seminiferous tubule (Figure 1.6). The first spermatogonia to appear are the  $A_{\text{single}}$  ( $A_s$ ) spermatogonia (de Rooij and Grootegoed, 1998). These can give rise to differentiating progeny called  $A_{\text{paired}}$  ( $A_{\text{pr}}$ ) spermatogonia which are connected in pairs by intercellular bridges or more  $A_s$  spermatogonia to renew the stem cell population. The  $A_{\text{pr}}$  progeny continue to divide becoming chains of  $A_{\text{aligned}}$  ( $A_{\text{al}}$ ) spermatogonia which remain attached to the basement membrane of the seminiferous tubules via cytoplasmic bridges (de Rooij and Grootegoed, 1998).  $A_s$ ,  $A_{\text{pr}}$  and  $A_{\text{aligned}}$  are referred to as undifferentiated spermatogonia (Figure 1.6).  $A_{\text{aligned}}$  spermatogonia give rise to the differentiating spermatogonia A1-A4. The numbers refer to the number of mitotic divisions the spermatogonia have undergone (de Rooij and Grootegoed, 1998). A4 spermatogonia differentiate into intermediate and type B spermatogonia which then give rise to the primary and secondary spermatocytes through

**Figure 1.6** Mouse testis spermatogenesis. Undifferentiated spermatogonia (USG) are attached to the basement membrane (BM). Differentiated spermatogonia (DSG) are also shown. Sertoli cells are surrounded by spermatocytes (SPC), round spermatids (RST) and elongated spermatids (EST). The more undifferentiated cells reside on or near the BM and as cells mature they progress towards the tubule lumen. Self-renewal and mitotic division occurs in a small region between the BM and the blood-testis barrier (BTB). Taken from Tadokoro, *et al.* 2002.



the last mitotic division (Figure 1.6). Differentiation continues and produces spermatids which then transform into spermatozoa (de Rooij and Grootegoed, 1998; Olive and Cuzin, 2005) (Figure 1.6). The cell types within the seminiferous tubule are arranged in a gradient of differentiation with the most differentiated located at the lumen and the more primitive spermatogonia located at the basement membrane (Figure 1.6).

As with HSCs, the only method to prove stem cell status is repopulation of the tissue of origin. The first transplantation experiments utilised a sterile mouse model which harbours mutations on the dominant white-spotting locus on mouse chromosome 5 (*W/W*) (Handel and Eppig, 1979; Mintz and Russell, 1957). This mutation disrupts the c-kit receptor and therefore disrupts receptor signalling which is important for the proliferation and differentiation of SSCs (Shinohara *et al.*, 2000b). The transplantation experiments involved isolating SSCs from C57BL/6 mice harbouring a coat colour genetic marker and transplanting these cells into *W/W* mice (Brinster and Zimmermann, 1994). The investigators demonstrated that spermatogenesis was completely restored and this could only be due to the transplanted cells as the host was devoid of spermatogenesis and the new cells carried the coat colour marker of the donor. In parallel to these experiments, donor cells were taken from mice with a *lacZ* marker and transplanted into recipients that had been sterilised using chemical methods (Brinster and Zimmermann, 1994). Similarly the newly repopulating cells of the recipient's testis were positive for the *lacZ* marker of the donor cells. The use of the *W/W* mutant mouse model of germ cell deficient adult testis is widespread. The model is useful as a tool in repopulation assays. However, it has been shown that a few undifferentiated spermatogonia persist in the adult testis of these mice and therefore these mice are not completely sterile (Ohta *et al.*, 2003). Most experiments utilising the model use donor cells harbouring a marker and therefore can still demonstrate that it is the donor cells that are often responsible for reconstitution in this model.

### **1.3.6 Potential markers for SSCs**

#### **a) SSC surface antigen expression**

The surface antigen expression pattern of SSCs has been identified and demonstrated to be MHC-I<sup>-</sup> Thy-1<sup>+</sup> c-kit<sup>-</sup> using flow cytometric cell sorting in conjunction with the transplant assay (Kubota *et al.*, 2003). MHC-I antigens have an immunological function and the finding that SSCs are negative for MHC-I antigens substantiates findings that ES and EC cells are also negative for these antigens (Croce *et al.*, 1981; Tian *et al.*, 1997). The authors surmise that negativity for MHC-I antigens may be a common characteristic of stem cells that are able to contribute to the germ line. SSCs can be enriched for using expression of Thy-1. Thy-1 is a differentiation marker for thymocytes and other cells but is also expressed on

HSCs in the mouse and rat (Berman and Basch, 1985; Goldschneider *et al.*, 1978). The receptor tyrosine kinase c-kit is expressed on differentiating spermatogonia but not on undifferentiated spermatogonia (Schrans-Stassen *et al.*, 1999). Therefore SSCs can be enriched as c-kit negative (Shinohara *et al.*, 2000b). It has previously been shown however, that mice lacking Steel factor (c-kit ligand) have fewer stem cells than wild-type mice (Shinohara *et al.*, 2000a). It has therefore been hypothesised that there may be a small subset of stem cells that do express c-kit or that receptor expression is up regulated upon injury.

Investigators have also shown that the SSC population can be enriched for using expression of  $\beta_1$  and  $\alpha_1$  integrins (Shinohara *et al.*, 1999). Integrin expression on the surface of SSCs is thought to be involved in maintenance of the undifferentiated state by anchoring stem cells to the basement membrane of the tubule, thus anchoring stem cells within their niche. This will be discussed in more detail later.

#### **b) Testis SP cells**

SP cells effluxing the vital dye Hoescht 33342 have been identified in the testis as well as in haemopoietic cell populations (Kubota *et al.*, 2003; Lassalle *et al.*, 2004). *Bcrp1* has been identified to be important for this effluxing capability of HSC SP cells (Zhou *et al.*, 2002). The testis SP cells have also been shown to express *Bcrp1* mRNA along with other stem cell markers (Lassalle *et al.*, 2004). Repopulation experiments utilising the *W/W* mutant showed that these SP cells were capable of reconstituting spermatogenesis. Contradictory conclusions were drawn by other investigators whose repopulation experiments demonstrated that the SP cells that they had isolated were unable to reconstitute spermatogenesis (Kubota *et al.*, 2003). Therefore, it is currently not clear whether SSC SP cells have stem cell characteristics as the two investigations may have been performed on different starter cell populations.

#### **c) Other potential SSC markers**

SSC self-renewal is regulated by a number of signalling pathways and transcriptional networks. The transcription factors Sox3, Ngn3 and Oct-4 are expressed in undifferentiated spermatogonia and are not expressed in differentiated cell types found within the testis tubule (Pesce *et al.*, 1998; Raverot *et al.*, 2005; Yoshida *et al.*, 2004). Thus, these factors have potential as stem cell markers. Oct-4 is already a widely-used marker of pluripotency of ES cells and its expression has been shown to be down-regulated upon differentiation of ES cells as well as during oogenesis and spermatogenesis (Pesce *et al.*, 1998). Oct-4 down-regulation occurs at the time meiosis begins so it is thought that this transcription

factor may be involved in the switch from mitotic to meiotic divisions (Pesce *et al.*, 1998; Yoshimizu *et al.*, 1999). Ngn3 expression is dependent on Sox3 expression (Raverot *et al.*, 2005). The relationship between these two transcription factors is not currently clear, but both are required for controlling self-renewal versus differentiation and therefore both are potential stem cell markers.

### **1.3.7 Culture of SSCs**

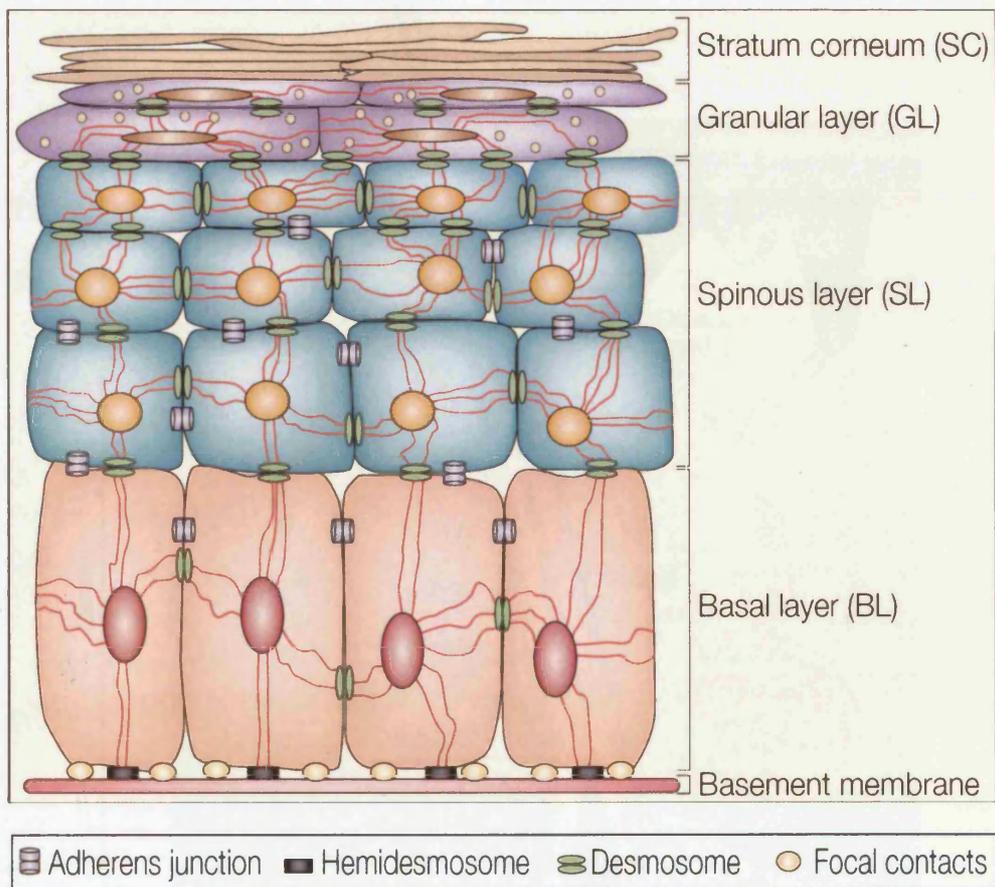
The first successful *in vitro* culture of SSCs was achieved by culturing on an STO (SIM mouse embryo-derived thioguanine and ouabain resistant) feeder layer in the presence of serum. Cells were retained in culture for four months and the presence of SSCs was determined using the transplantation assay (Nagano *et al.*, 1998). Culture conditions were improved by enriching for SSCs from the testicular cell population using expression of Thy-1 before culturing. Addition of factors involved in regulating proliferation and differentiation to the media such as glial cell line derived growth factor (GDNF) (a neurotrophic factor) its receptor GFRA1 and basic fibroblast growth factor (bFGF) also improved the culture conditions (Kubota *et al.*, 2004). This system allowed formation of stem cell colonies to be maintained in culture in the absence of serum for at least six months and potency was retained as demonstrated using the transplantation technique. (Kubota *et al.*, 2004).

### **1.3.8 Epidermal and hair follicle stem cells**

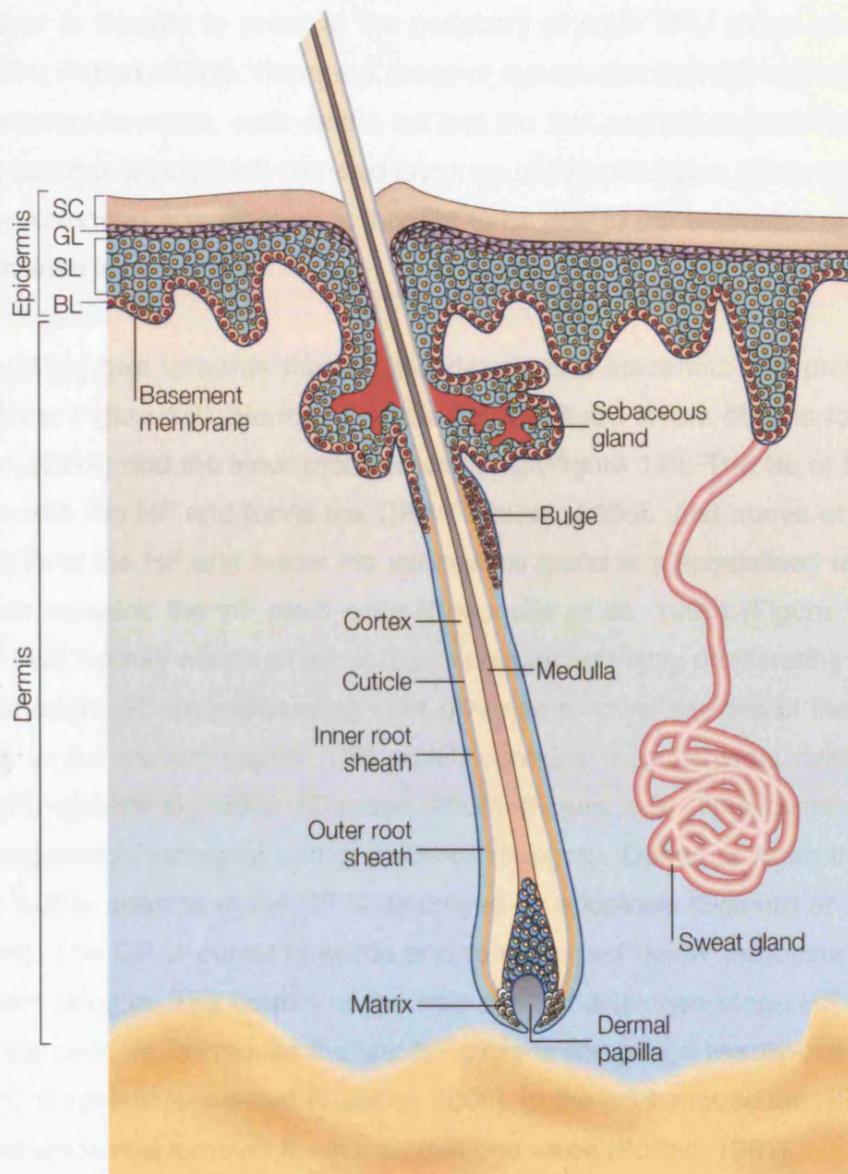
The skin is the largest organ in the body and its function is to provide a barrier against dehydration and protection from the environment. Skin is composed of a mesenchymal component called the dermis and an epidermal component called the epidermis (Tumbar, 2006). The skin can be thought of as consisting of three main components; the epidermis, the glands (sebaceous gland and sweat gland) and the hair follicle (HF) (Tumbar, 2006) (Figure 1.7 and 1.8). The HF and the glands penetrate deep into the dermis. The epidermis is the outermost layer and is a stratified keratinised epithelium that is constantly renewed. Skin epithelial cells are called keratinocytes and are mechanically strong due in large part to expression of keratins. Keratins belong to the intermediate filaments (IF) protein superfamily and can be subdivided in vertebrates into type I and type II keratins. These two subgroups are usually co-expressed in pairs with complicated expression patterns.

The epidermis is arranged in layers (Figure 1.7). The layer nearest the dermis is the basal layer (BL) and is thought to contain the ESCs; these cells have been demonstrated to express keratins 5 and 14 (Byrne *et al.*, 1994; Fuchs, 1995). The BL cells divide and migrate upwards, exit the cell cycle and give rise to the spinous layer (SL) which expresses differentiation-specific keratins 1 and 10 (Breitkreutz *et al.*, 1984; Fuchs, 1995; Fuchs and

**Figure 1.7** The mammalian epidermis. The epidermis is a stratified squamous epithelia consisting of a number of cell layers. Resting on the basement membrane are the BL cells, consisting of highly proliferative TA cells and stem cells. The differentiated SL, GL and SC are also shown. Intercellular adhesion is mediated by the desmosome and the adherens junction. Hemidesmosomes are involved in mediating the attachment to the keratin intermediate filament cytoskeleton, providing mechanical strength to the base of the epidermis and strong anchorage to the basement membrane. Focal contacts are also involved in adhesion to the basement membrane. Taken from Fuchs, *et al.* 2002.



**Figure 1.8** Mammalian skin showing the epidermis and the dermis separated by a basement membrane and the HF in detail. The epidermis consists of the BL, SL, GL and the SC. The BL cells of the epidermis rest on the basement membrane and are contiguous with the ORS of the HF. The hair bulb is at the bottom of the HF and is composed of proliferating matrix cells and houses the DP which consists of mesenchymal cells. The HF stem cells reside within their niche the bulge. Taken from Fuchs, *et al.* 2002.



Green, 1980; Moll *et al.*, 1982). Differentiation continues and the filaggrin-expressing granular layer (GL) is formed, followed by the involucrin-expressing stratum corneum (SC) (Rothnagel *et al.*, 1987; Watt and Green, 1982; Yuspa *et al.*, 1989) (Figure 1.7). The skin cells are then lost from the surface of the epidermis as squames. An array of specialised cells is also found within the epidermis; antigen presenting Langerhans cells, melanocytes responsible for skin pigmentation and Merkel cells whose function is thought to be sensory or neurosecretory (Moll *et al.*, 1996). Mouse epidermis is generally arranged in specific vertical columns (Mackenzie, 1969; Mackenzie, 1970) called epidermal proliferative units (EPUs), at the centre of which lies a single Langerhans cell. Keratinocyte migration into the spinous layer is thought to occur at the periphery of each EPU (Allen and Potten, 1974; Potten, 1974; Potten, 1981). There are areas of mouse skin that are not arranged in neatly stacked columns however, such as the tail and the foot pad (Allen and Potten, 1976). The pad of the foot has a very thick cornified layer; up to fifty cell layers (Allen and Potten, 1976) and its non-columnar arrangement is thought to be due to the increased pressure this part of the epithelium is exposed to.

The hair shaft grows upwards through the dermis and epidermis and protrudes from the skin's surface (Figure 1.8). Surrounding the hair shaft are layers of cells forming the outer root sheath (ORS) and the inner root sheath (IRS) (Figure 1.8). The BL of the epidermis is continuous with the HF and forms the ORS (Tumbar, 2006). Just above where the erector pili muscle joins the HF and below the sebaceous gland is a specialised region called the bulge which contains the HF stem cells (Cotsarelis *et al.*, 1990) (Figure 1.8). Below the bulge region is the hair matrix which contains committed highly proliferating cells thought to be of bulge origin. These proliferating cells give rise to lower regions of the HF. Within the hair matrix is the dermal papilla (DP) which contains mesenchymal cells thought to be important for growth signalling (Tumbar, 2006) (Figure 1.8). HFs cycle through growth (anagen) regression (catagen) and quiescence (telogen). During catagen the lower portion of the hair follicle apart from the DP is destroyed by apoptosis (Seiberg *et al.*, 1995; Stenn *et al.*, 1994). The DP is pulled upwards and remains just below the bulge region and the follicle enters telogen. The bottom of the hair shaft of a telogen stage HF is broad and is known as the club. In the mouse the first HF cycle is completed twenty-one days after birth after which, anagen is re-initiated (Tumbar, 2006). In the adult mouse the HFs are usually in telogen and epidermal turnover is estimated at one week (Potten, 1981).

As with other adult stem cells, ESCs are undifferentiated, few in number and are relatively slow-cycling (quiescent). The cells are capable of proliferation into a plethora of cell types required by the tissue of residence. ESC characteristics are commonly identified using three

methods; their clonogenic ability *in vitro*, their label retaining ability and their ability to form all tissues required by the tissue of origin in a transplantation assay. Transplantation assays are not as frequently used for ESCs as they are for HSCs. However, putative ESCs can be grafted onto immuno-compromised mice to assess their regeneration ability (Oshima *et al.*, 2001; Redvers *et al.*, 2006). Immuno-compromised mice are required to avoid rejection of the skin grafts. Adult stem cells, including epidermal, are also characterised as expressing particular surface antigens and residing in a particular geographical location. To assess the stem cell phenotype it is recommended to apply a combination of the criteria described above.

### **1.3.9 Evidence that clonogenic keratinocytes and LRCs are ESCs.**

Experiments have been performed that demonstrate that clonogenic keratinocytes represent multipotent ESCs (Claudinot *et al.*, 2005; Kolodka *et al.*, 1998). Human keratinocytes were maintained in culture for over thirty generations, thus demonstrating their proliferative potential. These clones were then labelled by retroviral transduction with the *lacZ* gene and grafted to immune-compromised mice. It was shown that these  $\beta$ -gal positive cells could give rise to a differentiated epidermis and that labelling was observed in distinct columns resembling EPU (Kolodka *et al.*, 1998). Similar experiments have been performed using rat whisker clonogenic keratinocytes transfected with a *lacZ* gene (Claudinot *et al.*, 2005). These marked cells were able to incorporate into part or all of the developing HF.

Label-retaining experiments have identified the bulge region as containing the HF stem cells (Braun *et al.*, 2003; Cotsarelis *et al.*, 1990; Morris and Potten, 1999). LRCs can also be detected in the interfollicular epidermis after a four week chase period. If the chase period is longer than four weeks then LRCs become undetectable in the epidermis due to dilution of the label as the epidermal LRCs proliferate more frequently than bulge LRCs (Bickenbach and Dunnwald, 2000; Bickenbach and Mackenzie, 1984; Cotsarelis *et al.*, 1999). Stem cells divide in the adult in response to growth and repair signals. LRCs have also been demonstrated to proliferate in response to phorbol esters (Braun *et al.*, 2003) and growth hormone (Ohlsson *et al.*, 1992). Thus, LRCs react upon growth induction signals as skin stem cells would be required to do.

LRCs have been shown to be highly clonogenic *in vitro* (Bickenbach and Chism, 1998; Morris and Potten, 1994). Adult epidermal LRCs were produced by repeated labelling over three days with [ $^3$ H]dT five days after birth and pulse-labelled cells (PLCs) were produced by a single injection of [ $^3$ H]dT (Morris and Potten, 1994). Pulse-labelling of cells is thought to

mark TA cells as these cells are highly proliferative and therefore would be proliferating at the time of labelling. PLCs lose their label after a few divisions, but LRCs do not. The PLCs and LRCs were trypsinised and cultured on an irradiated fibroblast cell layer. At various time points the cultures were fixed and analysed for the presence of the label. After five days in culture LRCs formed clusters exhibiting less label as determined by presence of silver grains consistent with division of these cells, whereas the PLCs were still primarily found as single cells (Morris and Potten, 1994). This demonstrates that the epidermal LRCs are clonogenic and therefore have a higher proliferative potential than PLCs. LRCs are slowly-cycling and have a high proliferative potential, thus they fulfil at least two of the criteria set to determine stem cells.

### **1.3.10 Do the epidermis and the HF have distinct stem cell populations?**

It is currently accepted that the epidermis and HF possess their own stem cells although this has been debated. Previously it was shown that HF stem cells are able to generate all hairy skin cell lineages and thus represent the multipotent stem cell capable of generating not only the HF but also the epidermis and the glands (Oshima *et al.*, 2001; Taylor *et al.*, 2000). This idea was put forward after many pulse chase experiments resulted in labelling of cells in the bulge region of the follicle but not the epidermis or the sebaceous gland suggesting that there was only one stem cell in mammalian skin residing in the follicle bulge (Cotsarelis *et al.*, 1990; Morris and Potten, 1999). However, it is possible that the chase periods used in these experiments resulted in loss of the epidermal label due to the fact that the epidermis proliferates more frequently than the bulge of the HF (Bickenbach and Dunnwald, 2000; Bickenbach and Mackenzie, 1984; Oshima *et al.*, 1999).

Taylor and colleagues marked slowly-cycling cells in the HF with BrdU and demonstrated that labelled cells moved into the epidermis in neonatal and adult mouse skin in response to a wound (Taylor *et al.*, 2000). Transplant experiments using  $\beta$ -gal-expressing upper HF (bulge) resulted in regeneration of all cell lineages typical of hairy skin in the recipient including epidermis (Oshima *et al.*, 2001). These investigations show that the HF possesses multipotent stem cells capable of reconstituting the epidermis upon injury, and transplantation. However, both experiments involved wounding of the epidermis and do not show that HF bulge cells are responsible for normal epidermal homeostasis. More recently, investigators have shown that follicle bulge cells do not contribute to normal epidermal homeostasis (Ito *et al.*, 2005; Levy *et al.*, 2005; Morris *et al.*, 2004). One investigation utilising transgenic mice with an inducible system under the control of the Keratin 1-15 promoter allowed lineage tracing of bulge cells (Morris *et al.*, 2004). It was shown that these

cells do not contribute to normal maintenance of the epidermis but are able to migrate to the epidermis and assume an epidermal phenotype upon injury (Morris *et al.*, 2004).

### **1.3.11 Potential markers for ESCs**

#### **a) ESC surface antigen expression.**

ESCs like other adult stem cells can be identified by surface antigen expression. Basal keratinocytes express adhesion molecules such as integrins which are involved in anchoring the cells to the extracellular matrix and regulating differentiation and proliferation (Jensen *et al.*, 1999; Zhu *et al.*, 1999). It has been shown that human ESCs highly express  $\beta_1$  integrin (Jones and Watt, 1993) and that the more highly expressed the integrin, the greater the proliferative potential as assessed by colony-forming efficiency. The murine HF bulge region (containing stem cells) and ESCs identified by their label-retaining capacity also express high levels of  $\beta_1$  integrin. (Huelsenken *et al.*, 2001). However, the expression was demonstrated not to be specific for the LRC population (Albert *et al.*, 2001); thus proliferative progenitors may also express the integrin. Other investigations have shown that LRCs in culture attach more readily to a multitude of substrates suggesting that adhesion is mediated through a combination of integrins (Bickenbach and Chism, 1998). Therefore although integrin expression enables enrichment of ESCs, it can not be used as a stem cell specific marker.

Keratins are expressed in specific patterns throughout the epidermis and HF, and therefore their potential as ESC markers has been investigated. Keratin 19 (K19) has been demonstrated to identify the bulge area of the HF in both mice and humans (Michel *et al.*, 1996). The investigators identified keratinocyte stem cells using the LRC method and showed that K19 expression was also evident in these cells (Michel *et al.*, 1996). Keratin 15 (K15) has been demonstrated to be specifically expressed in the bulge region of the human HF also using the LRC protocol along with co-expression of other stem cell markers K19 and  $\beta_1$  integrin (Jones and Watt, 1993; Michel *et al.*, 1996). Analysis of transgenic mice expressing *lacZ* under the control of the keratin 15 promoter showed positive  $\beta$ -gal staining and therefore K15 expression in the HF bulge of adult mouse skin (Liu *et al.*, 2003). LRC experiments using these transgenic mice demonstrated that Keratin 15 expression is specifically localised to LRCs within the bulge (Liu *et al.*, 2003). The same investigators then went on to use the keratin 15 promoter to drive expression of GFP to allow isolation of the K15-expressing bulge cells by FACs. It was shown that these cells had high proliferative capacity and could generate all required epithelial cell lineages in a reconstitution assay (Morris *et al.*, 2004).

HSCs can be enriched for using the expression of CD34 (Krause *et al.*, 1994). Using an antibody it was demonstrated that CD34 is also expressed in the bulge region of the murine HF and that this expression is co-localised with LRCs and K15 expression (Trempeus *et al.*, 2003). Bulge CD34 positive cells were demonstrated to be predominantly in the G<sub>0</sub> phase of the cell cycle, expressed higher levels of integrins and possessed a higher colony forming efficiency (Trempeus *et al.*, 2003). Thus, CD34 expression was shown to enrich for bulge cells with stem cell characteristics in this investigation. Surface antigen phenotypes are similar between adult stem cells from different tissues; murine haemopoietic and muscle SP cells both express Sca-1 (Gussoni *et al.*, 1999; Jackson *et al.*, 1999) as well as CD34 and Flk-1 (Lee *et al.*, 2000). Therefore it was further investigated whether ESCs shared the surface antigen phenotype of haemopoietic and muscle SP cells. The surface expression pattern of epidermal LRCs was compared to non-LRCs using flow cytometric analysis and it was discovered that LRCs did not express CD34, Sca-1 or Flk-1 (Albert *et al.*, 2001). The fact that LRCs did not express CD34 conflicts with the investigations conducted by Trempeus *et al.* (2003) who demonstrated that CD34 was expressed in LRCs. The caveat of the LRC protocol may explain the differences in their results; stem cells are only labelled if they divide during the time period in which the labelling is taking place, therefore many stem cells are not labelled. More investigations are needed to determine whether CD34 can enrich for ESCs.

LRCs and PLCs have been separated using surface antigen expression to try and obtain a purer putative stem cell population. It was shown that ESCs (LRCs) can be identified as highly expressing  $\alpha_6$  integrin and lowly expressing the transferrin receptor CD71;  $\alpha_6^{\text{bri}}\text{CD71}^{\text{dim}}$  (Tani *et al.*, 2000). PLCs represent the TA population as they are readily labelled with a pulse of [<sup>3</sup>H]dT or BrdU due to the fact that they are actively dividing cells. The PLC subpopulation is separated from the LRCs by expression of  $\alpha_6^{\text{bri}}\text{CD71}^{\text{bri}}$ . Differentiated epidermal cells (as identified by expression of differentiation markers keratin 10 and involucrin) were isolated as  $\alpha_6^{\text{dim}}$ . The investigators used a CD71 antibody in immunostaining to demonstrate that the bulge region of the HF cells did show low CD71 expressing cells; thus providing further evidence that ESCs can be identified using CD71 low expression (Tani *et al.*, 2000).

### **b) Skin SP cells**

SP cells have been identified in both human and mouse skin (Redvers *et al.*, 2006; Terunuma *et al.*, 2003; Triel *et al.*, 2004; Yano *et al.*, 2005). Mouse SP cells have been shown to demonstrate high levels of stem cell markers  $\alpha_6$  and  $\beta_1$  integrins, Sca1, keratins 14 and 19 and express low levels of CD71 (Jones and Watt, 1993; Michel *et al.*, 1996; Tani

*et al.*, 2000; Yano *et al.*, 2005). Furthermore, *Bcrp1* expression has been detected in the BL of the epidermis and the bulge region of the HF by *in situ* hybridisation and immunohistochemistry (Yano *et al.*, 2005). This work suggests that mouse SP cells exhibit a stem cell phenotype. However, it has been shown that mouse SP cells are a distinct and separate population from the LRC population (Triel *et al.*, 2004). The same has been shown for human SP cells (Terunuma *et al.*, 2003). The LRC technique is generally accepted as the best method to identify ESCs. However, it must be noted that this protocol will not label all of the stem cells. Therefore there could be some stem cells present in the SP population and careful interpretation of results is needed. All of the analysis of the stem cell characteristics of epidermal SP cells has thus far been based on phenotypic analysis and has not included any functional assays. Recent work by Redvers, *et al* (2006) demonstrated that SP cells exhibit phenotypic characteristics of stem cells and possess the ability to form all cell types within the epidermis on transplantation into denuded rat tracheas (Redvers *et al.*, 2006). Thus, although surface antigen expression is a useful means of enriching for HSCs and SSCs, more investigations need to be completed to achieve the same level of purification for ESCs and to determine once and for all the phenotypic and functional capacity of SP cells.

### **c) Other potential ESC markers**

Transcription factors p63, Oct-4, Lhx2 and Sox9 are involved in the maintenance of ESC fate and thus have potential as stem cell markers. The transcription factor p63 is a homologue of the tumour suppressor protein p53 and is expressed in the basal cells of the epidermis (Parsa *et al.*, 1999). This expression is lost upon differentiation (Parsa *et al.*, 1999). In the skin, Oct-4 can be detected in a small number of basal cells in the human epidermis (Tai *et al.*, 2005) and has also been found in adult murine SP cells (Redvers *et al.*, 2006). Therefore Oct-4 expression can be detected in stem cell candidates in the skin. The Lim-homeodomain transcription factor (Lhx2) has been previously demonstrated to be involved in cell fate determination of the brain (Bulchand *et al.*, 2001; Hirota and Mombaerts, 2004; Porter *et al.*, 1997). Lhx2 has since been demonstrated to be expressed in the bulge region of the HF (Rhee *et al.*, 2006). Loss of function mutants of Lhx2 result in loss of LRCs from the bulge accompanied by increased proliferation (Rhee *et al.*, 2006), suggesting that Lhx2 is involved in maintaining bulge cells in the stem cell state. Expression of the transcription factor Sox9 is restricted to the ORS and the stem cell compartment in adult mice (Athar *et al.*, 2006; Vidal *et al.*, 2005). Inactivation of Sox9 in adult mouse skin resulted in the development of multiple layers in the ORS accompanied by expression of epidermal specific markers and an absence of hair (Vidal *et al.*, 2005). Proliferative defects and complete absence of CD34-expressing bulge stem cells was also seen (Vidal *et al.*,

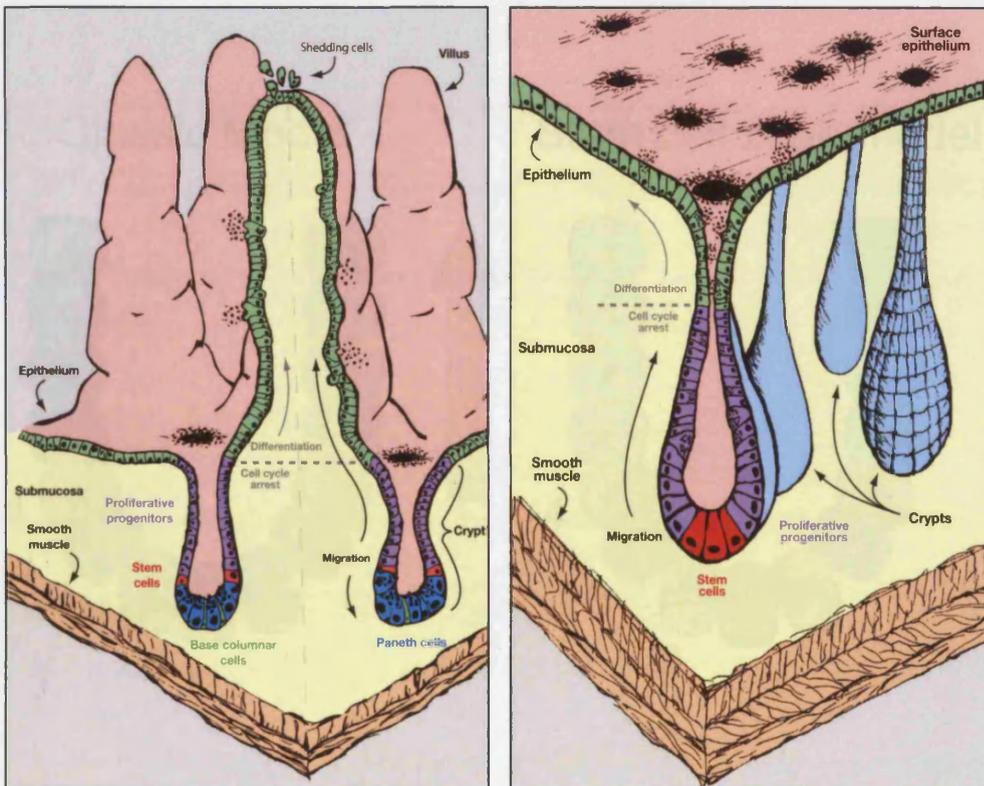
2005). The effects of Sox9 were shown to be dependent on sonic hedgehog (Shh) signalling (Vidal *et al.*, 2005). Expression of both Sox9 and Shh is increased in skin tumours such as basal cell carcinoma (BCC) (Athar *et al.*, 2006). Therefore Shh signalling and its downstream mediator Sox9 are involved in the differentiation of the HF and maintenance of the bulge stem cell compartment.

### 1.3.12 ISCs

The adult gastrointestinal tract is composed of a simple columnar epithelium arranged into invaginations called crypts. In the small intestine (SI), a number of crypts surround finger-like projections called villi. The crypt epithelium is contiguous with that of the villi, whose function is to provide a large surface area which allows for efficient absorption of nutrients from the intestinal lumen. Epithelial cells are produced in the lower regions of the crypt and migrate up through the crypt until they reach the villus tip where they are shed into the lumen (Bjerknes and Cheng, 2006; Marshman *et al.*, 2002) (Figure 1.9). There are numerous crypts supplying cells to each villus. The colon does not possess villi and so migration occurs from the bottom of the crypt to the crypt top (Figure 1.9).

There are three main functional cell types within the small intestine; the prevalent enterocytes which are columnar absorptive cells, Goblet cells which secrete mucus and trefoil factors and hormone-secreting enteroendocrine cells (Wong *et al.*, 1999). A fourth cell type lines the base of the SI crypt; the postmitotic Paneth cell which contributes to gut immunity by producing anti-microbial substances (Ayabe *et al.*, 2002). There are also M cells which are often found adjacent to Peyers patches; little is known about this cell lineage. New cells are formed by mitosis in the crypt and the cells migrate upwards to perform their functions and then are shed into the lumen generally 5-7 days after birth from division in the crypt (Marshman *et al.*, 2002). The differentiation-status of a cell can therefore be determined by its position on the crypt-villus axis. Cell replacement within the intestine is thought to be achieved by a population of stem cells in the crypt base. As with all other adult stem cells, there are no markers available to discern an ISC on a single cell basis. There are however, two models that describe the location of stem cells within the small intestinal crypt; the classic model and the zone model (Bjerknes and Cheng, 1981a; Bjerknes and Cheng, 1981b; Marshman *et al.*, 2002; Potten, 1998; Potten *et al.*, 1997a) (Figure 1.10). The zone model describes an ideal environment for stem cells in the first four or five cell positions at the crypt base. The investigators that proposed this model described proliferative putative stem cells as being dispersed among the Paneth cells of the SI at the crypt base (Bjerknes and Cheng, 1981a; Bjerknes and Cheng, 1981b). Stem cell progeny migrate out of the stem cell zone and start to differentiate above the zone in cell position 5.

**Figure 1.9** Intestine histology. The structure of the small intestine is shown on the left and that of the large intestine is shown on the right. Putative stem cells reside at the base of the large intestinal crypt whereas they reside above the Paneth cells at cell position 4-5 in the small intestinal crypt. In the small intestinal crypt, progenitor cells no longer proliferate when the crypt-villus junction is reached. Enteroendocrine, absorptive and mucosal cells migrate up the crypt whereas the Paneth cells migrate downwards. In the large intestine, proliferative progenitors comprise the bottom two thirds of the crypt and differentiation occurs in the top third of the crypt. Taken from Sancho *et al.*, 2004.

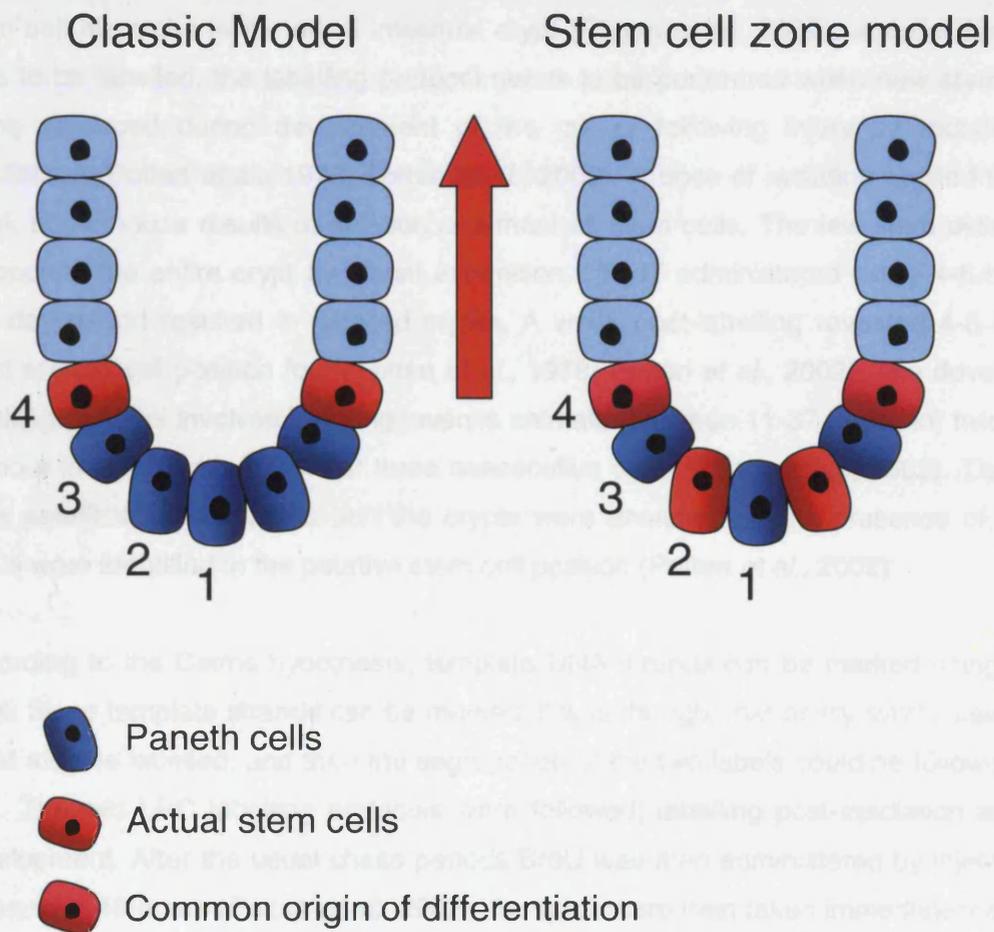


-  Paneth cells
-  Actual stem cells
-  Common origin of differentiating

**Figure 1.10** Zone and classic models of stem cell location within the small intestinal crypt. Stem cells reside at cell position 4-5 in the classic model whereas they are interspersed between the Paneth cells in the zone model. In the zone model, stem cell progeny leave the stem cell zone and begin differentiation above the zone at the common origin of differentiation at cell position 5 and above. Adapted from Bjerknes and Cheng (2006)

Increasing differentiation

Decreasing stem cell potential



Therefore this region is called the common origin of differentiation (Bjerknes and Cheng, 1981a; Bjerknes and Cheng, 1981b) (Figure 1.9). The progeny for all cell types above the common origin of differentiation migrate upwards whereas the Paneth cell progeny migrate downwards. The classic model describes the stem cells as being located above the Paneth cells, below a column of proliferating cells at approximately cell positions 4-5 (Potten *et al.*, 2002). Thus in this model stem cells are found at the common origin of differentiation in the stem cell zone model (Figure 1.10). The stem cell zone model is based on investigations by Cheng and Leblond (1974) which identified undifferentiated proliferative cells among the Paneth cells at the crypt base. The evidence for the classic model of stem cell location at cell position 4 comes from label retaining experiments in the SI (Potten *et al.*, 2002). The classic model is the most widely accepted model of small ISC position and therefore when discussing stem cell position in future it will be in reference to this model.

### **1.3.13 Label-retaining experiments identify ISCs.**

LRC experiments in the SI have contributed to the establishment of the classic model of stem cell hierarchy in the small intestinal crypt (Potten *et al.*, 2002). In order for the stem cells to be labelled, the labelling protocol needs to be performed when new stem cells are being produced during development of the gut or following injury by radiation (post-irradiation) (Potten *et al.*, 1978; Potten *et al.*, 2002). A dose of radiation applied to a 10-12 week adult mouse results in ablation of almost all stem cells. The few stem cells left then repopulate the entire crypt by clonal expansion. [<sup>3</sup>H]dT administered every 4-6 hrs over a two day period resulted in labelled crypts. A week post-labelling revealed 4-8 LRCs per crypt around cell position four (Potten *et al.*, 1978; Potten *et al.*, 2002). The developmental labelling protocol involves injecting juvenile animals (between 11-37 days old) twice daily at 12 hour intervals with [<sup>3</sup>H]dT for three consecutive days (Potten *et al.*, 2002). The animals were sacrificed at 11 weeks and the crypts were analysed for the presence of the label. LRCs were identified in the putative stem cell position (Potten *et al.*, 2002).

According to the Cairns hypothesis, template DNA strands can be marked using a [<sup>3</sup>H]dT label. Since template strands can be marked, it was thought that newly synthesised strands could also be labelled, and then the segregation of the two labels could be followed (Figure 1.4). The two LRC labelling protocols were followed; labelling post-irradiation and during development. After the usual chase periods BrdU was then administered by injection every 6 hours for 48 hours (Potten *et al.*, 2002). Samples were then taken immediately after BrdU administration and 2 to 10 days post labelling. If the newly synthesised strands are labelled with BrdU then LRCs should also be labelled with BrdU as well as [<sup>3</sup>H]dT, and it should only take two divisions after labelling for the BrdU label to be lost from the stem cell position at

which point all of the BrdU label would be retained within the TA cell and thus lost from the tissue (Figure 1.4). It was shown that approximately all of the [<sup>3</sup>H]dT LRC's were also labelled with BrdU (Potten *et al.*, 2002). BrdU labelling remained detectable for approximately three-six cell divisions after which it was lost in accordance with the theory that BrdU labelling would be lost after the second stem cell division (Potten *et al.*, 2002). Thus, the investigations undertaken by Potten and colleagues are consistent with the Cairns hypothesis that ISCs selectively retain the template [<sup>3</sup>H]dT labelled strand whereas the newly synthesised BrdU labelled strand is lost in accordance with the second stem cell division (Figure 1.4). However, the mechanisms by which this occurs are still unknown (Potten *et al.*, 2002).

#### **1.3.14 ISC numbers**

There are thought to be only 4-6 ultimate stem cells within the SI. The evidence for this comes from the LRC experiments described previously (Marshman *et al.*, 2002). The ultimate ISC is thought to divide once a day in a mouse, therefore undergoing thousands of divisions over the animal's lifetime (Martin *et al.*, 1998a; Martin *et al.*, 1998b). Stem cells are able to divide asymmetrically giving rise to one replacement stem cell and one daughter cell which enters the TA population and is capable of extensive proliferation and differentiation or symmetrically giving rise to two replacement stem cells or two daughter cells (Figure 1.2). Each time the ancestral stem cell divides, through the TA population, it is thought to result in the production of approximately 64 cells. Therefore from each crypt around 300 cells are produced per day (Marshman *et al.*, 2002).

#### **1.3.15 Regulation of ISC numbers.**

The maintenance of normal stem cell numbers is thought to be achieved via asymmetric stem cell division. Symmetric division is thought to occur in response to changes in the tissue environment. Mathematical models have proposed that symmetrical stem cell division only accounts for 5% of divisions (Marshman *et al.*, 2002). An extra stem cell produced via symmetric stem cell division would have dramatic consequences for a stem cell crypt as it is capable of producing between 64-128 cells (Marshman *et al.*, 2002). Therefore any extra stem cells need to be deleted. In normal mouse small intestinal crypts a spontaneous apoptotic rate of 5-10% can be identified. Therefore spontaneous apoptosis of extra stem cells is thought to be the mechanism by which stem cell homeostasis is regulated (Potten, 1998). This spontaneous apoptotic rate is unaffected by p53 as found by examination of p53 null mice (Merritt *et al.*, 1994; Potten *et al.*, 1997b). Altruistic apoptosis of stem cells in the SI is also thought to occur to delete any stem cells carrying errors. Studies have shown that cells in the stem cell location do not repair any DNA damage but rather initiate

apoptosis (Merritt *et al.*, 1994; Potten, 1977; Potten and Grant, 1998). The large intestine expresses the anti-apoptotic protein Bcl-2 in the crypt base at the stem cell position (Merritt *et al.*, 1995; Potten *et al.*, 1997b). Thus it is thought that more cancers arise in the large intestine due to the fact that large intestine stem cells express Bcl-2 and are therefore not deleted. This is substantiated by the observation that the incidence of spontaneous apoptosis is significantly increased in the stem cell region of the colon of Bcl-2 knockout mice, but the rate in the SI stem cell region is unchanged (Merritt *et al.*, 1995).

### **1.3.16 Potential markers for ISCs**

#### **a) Surface antigen expression/ISC markers.**

There are many lineage markers available to identify various differentiated intestinal epithelial lineages, but not for the intestinal progenitors or stem cells.  $\beta_1$  integrin is widely used for the identification of ESCs (Jones and Watt, 1993). It has also been shown to be strongly expressed in the lower third of human colonic crypts and has been used to enrich for ISCs using FACs (Fujimoto *et al.*, 2002). Enrichment for putative stem cells using surface antigen expression can be achieved for adult stem cells from various organs but to date, this is not the case for ISCs. There are however a few putative ISC markers, shown to be expressed in the stem cell regions of the intestine. Cells in the putative stem cell location, although likely candidates for stem cells have not been thus far categorically proven to be stem cells. This needs to be noted when considering putative stem cell markers.

The RNA binding protein Musashi-1 (*Msi-1*) has previously been identified to be important for the maintenance of NSCs and their differentiation (He *et al.*, 2007; Kaneko *et al.*, 2000; Sakakibara *et al.*, 2001; Sakakibara *et al.*, 2002). The RNA binding protein has now been identified as a putative ISC marker (Asai *et al.*, 2005; He *et al.*, 2007; Kayahara *et al.*, 2003; Potten *et al.*, 2003) Expression of *Msi-1* as assessed by immunohistochemistry, analysis of the mRNA patterns by *in situ* hybridisation and RT-PCR was observed in the SI crypt at regions predicted to contain the stem cells in a ring around the SI above the Paneth cells at approximately cell position four (Asai *et al.*, 2005; Kayahara *et al.*, 2003; Potten *et al.*, 2003). The transcription factor Hairy and Enhancer of split (*Hes-1*) is a downstream effector of the Notch signalling pathway. *Hes-1* has been found to be co-expressed with *Msi-1* in putative SI stem cells. Both *Hes-1* and *Msi-1* were also found to be co-expressed in between Paneth cells at the crypt base, thus suggesting that cells within the crypt base are stem cells (Kayahara *et al.*, 2003). This work agrees with the stem cell zone model for the location of stem cells in the small intestine discussed previously (Bjerknes and Cheng, 1981a; Bjerknes and Cheng, 1981b). Secreted frizzled-related protein 5 (sFRP-5) an

antagonist of Wnt signalling (Gregorieff *et al.*, 2005) and double cortin and calcium/calmodulin-dependant protein kinase-like-1 (Dcamk1) (Giannakis *et al.*, 2006) a microtubule-associated kinase have been demonstrated to be expressed in the ISC location in the SI crypt, and therefore have potential as ISC markers.

### **b) Intestinal SP cells**

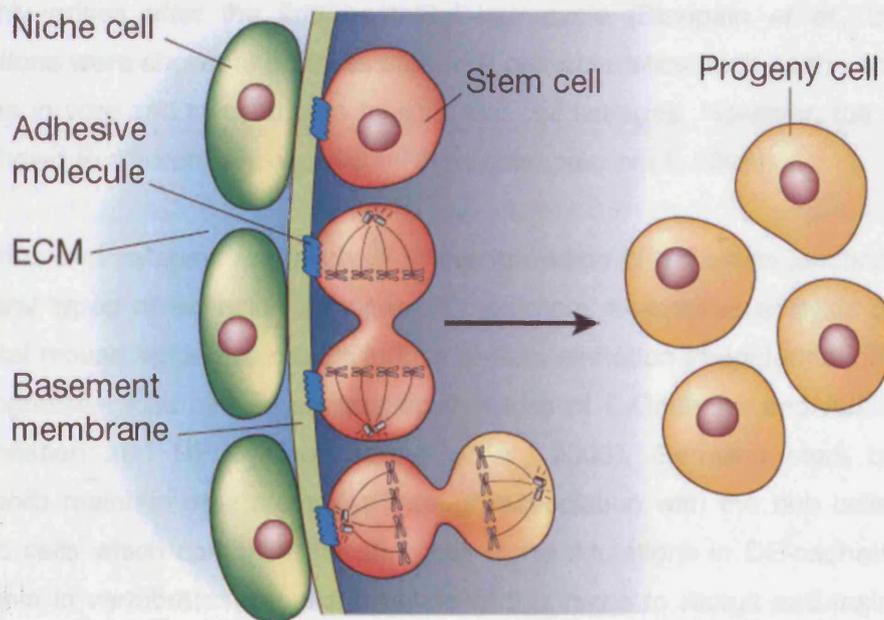
SP cells have been isolated from the intestine and shown to possess haemopoietic potential as determined by expression of the haemopoietic marker CD45 (Asakura and Rudnicki, 2002). The SP cells isolated were also demonstrated to have colony-forming efficiency, thus the cells are capable of proliferation (Asakura and Rudnicki, 2002). Other investigators have also isolated gastric SP cells and shown that the CD45 negative population of SP cells fails to attach and proliferate, suggesting that non-haemopoietic SP cells do not possess stem cell characteristics (Dekaney *et al.*, 2005). Further investigation is required to establish whether intestinal SP cells of non-haemopoietic origin can be isolated and whether these cells have stem cell potential.

## **1.4 The stem cell niche**

Stem cells reside within a particular region or environment called a niche (Schofield, 1978). A niche is composed of somatic cells and extracellular substrates that can house one or more stem cells and maintain the stem cell state by affecting self-renewal and differentiation. The importance of the stem cell niche can be appreciated when considering ES cells. When ES cells are surrounded with an unfamiliar environment of tissue *in vivo* by subcutaneous injection into a nude mouse, a teratoma is formed. Teratomas as previously discussed are tumours comprising of a wealth of cell types (Stevens, 1970). However, if ES cells are injected into a familiar environment such as a blastocyst, the ES cells resume normal behaviour and contribute to all tissues of a healthy chimeric offspring (Bradley *et al.*, 1984).

Contact of stem cells with a basement membrane or surrounding somatic cells is thought to be one mechanism by which stem cell character is maintained. A stem cell may orientate itself so that only one daughter cell retains adhesive contacts, thus enabling it to remain within the niche and become a replacement stem cell. The other daughter cell loses contact and is therefore no longer influenced by niche cells or signals and can differentiate (Figure 1.11). Thus, stem cell fate is controlled by polarising signals resulting in the segregation of stem cell and pro-differentiation determinants. Many types of adult stem cells can be enriched using integrin expression (Fujimoto *et al.*, 2002; Jones and Watt, 1993; Shinohara *et al.*, 1999). This suggests that integrins play an integral role in the maintenance of the

**Figure 1.11** The stem cell niche. Loss of contact with the basement membrane results in asymmetric division and segregation of pro-differentiation determinants in the daughter cell that has lost contact. Thus, it will begin to differentiate and form differentiated progeny cells. The daughter cell that retains contact retains pro-stem cell determinants and forms a replacement stem cell. Taken from Spradling, 2001



stem cell state. The  $\alpha_6\beta_1$  integrin is expressed on mouse SSCs and has been implicated in their attachment to laminin in the basement membrane, therefore keeping the stem cells within their niche (Shinohara *et al.*, 1999). HF stem cells within their niche (the bulge) can be identified by their ability to retain a label (LRC) as discussed previously (Braun *et al.*, 2003; Morris and Potten, 1994; Morris and Potten, 1999). Transgenic mice expressing histone (H2B) GFP controlled by a tetracycline-responsive regulatory element (TRE) were crossed with mice harbouring a K5 promoter-driven tet repressor-VP16 transgene in order to mark infrequently cycling cells in adult skin epithelium (Blanpain *et al.*, 2004). Counter-staining of these GFP containing cells with  $\alpha_6$  integrin was used to assess whether the LRC was in contact with the basal lamina, as  $\alpha_6$  is a component of the hemidesmosome which mediates this attachment. This investigation revealed that the bulge contains two distinct cell populations; one attached to the basal lamina and one which appears to be suprabasal and only arises after the first postnatal hair cycle (Blanpain *et al.*, 2004). Both cell populations were shown to possess stem cell characteristics such as the ability to generate colonies *in vitro* and to contribute to epithelial cell lineages. However, the two populations were shown to differentially express mRNAs (Blanpain *et al.*, 2004).

Cadherins and catenins are involved in the formation of adherens junctions (AJs); one of the many types of adhesion structure in mammals. Mutational analysis of E-cadherin in postnatal mouse epidermis results in loss of AJs, alteration in epidermal differentiation and the progressive loss of HFs suggesting that loss of E-Cadherin and AJs alters stem cell differentiation and HF cycling (Young *et al.*, 2003). Germline stem cells (GSCs) in *Drosophila* maintain their stemness through association with the hub cells which are the somatic cells which comprise the stem cell niche. Mutations in DE-cadherins or armadillo ( $\beta$ -catenin in vertebrates) results in failure of this niche to recruit and maintain stem cells (Fuchs *et al.*, 2004; Song *et al.*, 2002). Taken together this evidence demonstrates that adhesion mediated through integrins and cadherins is important in anchoring stem cells within their niche. The bulge stem cell investigations suggest that the niche imposes stem cell characteristics such as potency on all its residents, but allows cell polarity and changes in gene expression which may eventually overcome the stem cell character imposed by the niche, thus allowing these cells to differentiate.

Cells neighbouring stem cells are known to be important regulators of the stem cell niche in the testis. The Ets family-related molecule ERM is expressed in the Sertoli cells of murine testis (Chen *et al.*, 2005). The Ets family is a family of transcription factors that are involved in a variety of developmental processes such as regulating gene expression in haemopoietic cells (Seth *et al.*, 1992). Transgenic mice with a targeted disruption of ERM

exhibit a progressive loss of SSCs without disrupting differentiation; resulting in a Sertoli cell only syndrome (Chen *et al.*, 2005). It is currently unclear exactly how ERM regulates the SSC niche, but it is clear that factors expressed by neighbouring cells in the niche are required for the maintenance of testis stem cells.

The ISC niche comprises the lower third of the crypts of Lieberkühn (Bjerknes and Cheng, 1981a; Bjerknes and Cheng, 1981b; Marshman *et al.*, 2002; Potten, 1998; Potten *et al.*, 1997a). Mutation of TCF-4 a transcription factor involved in the Wnt signalling pathway results in disruption of proliferation and maintenance of the intestine, suggesting that this pathway is required for normal regulation of these processes in this tissue (Korinek *et al.*, 1998). The BMP pathway acts in opposition to the Wnt pathway as a negative regulator of ISC proliferation (Moore and Lemischka, 2006). Thus, both signalling pathways affect stem cells. Components of both the Wnt and BMP pathways are expressed in mesenchymal cells surrounding the intestinal crypt, thus providing further evidence for the role of neighbouring cells in regulating stem cell behaviour (Gregorieff *et al.*, 2005). The enteroendocrine cell has also been identified as a potential ISC niche regulator (Radford and Lobachevsky, 2006). The enteroendocrine cell is located at the base of the crypt (Cheng and Leblond, 1974; Evans and Potten, 1988) and produces growth-inhibitory peptides such as somatostatin (Patel, 1999). Somatostatin has been implicated in maintaining ISC quiescence as its activation can lead to increased levels of cyclin-dependant kinase inhibitors such as p21<sup>cip1</sup> (Alderton *et al.*, 2001). Furthermore, somatostatin has been shown to increase cellular adhesion to the basement membrane (Levite *et al.*, 1998; Talme *et al.*, 2001) as well as inhibiting cell migration (Pola *et al.*, 2003).

Taken together it can be seen that the stem cell state is greatly affected by the niche. Contact with basement membranes or neighbouring cells within the niche allows the segregation of pro-stem cell and pro-differentiation determinants. Signalling pathways within the niche also act to maintain stem cell character. Somatic cells often express components of signalling pathways or other molecules involved in inducing the stem cells to retain their stem cell character or to differentiate. Stem cells themselves are potentially capable of regulating their own niches. Since stem cells are able to re-colonise damaged tissue in a transplantation assay, it suggests that stem cells themselves express or secrete molecules capable of rebuilding the required niche environment.

### **1.5 Adult stem cell plasticity**

Adult stem cells were originally thought to be tissue-specific and therefore only capable of giving rise to cells required by their tissue of origin. However it is now thought that tissue-

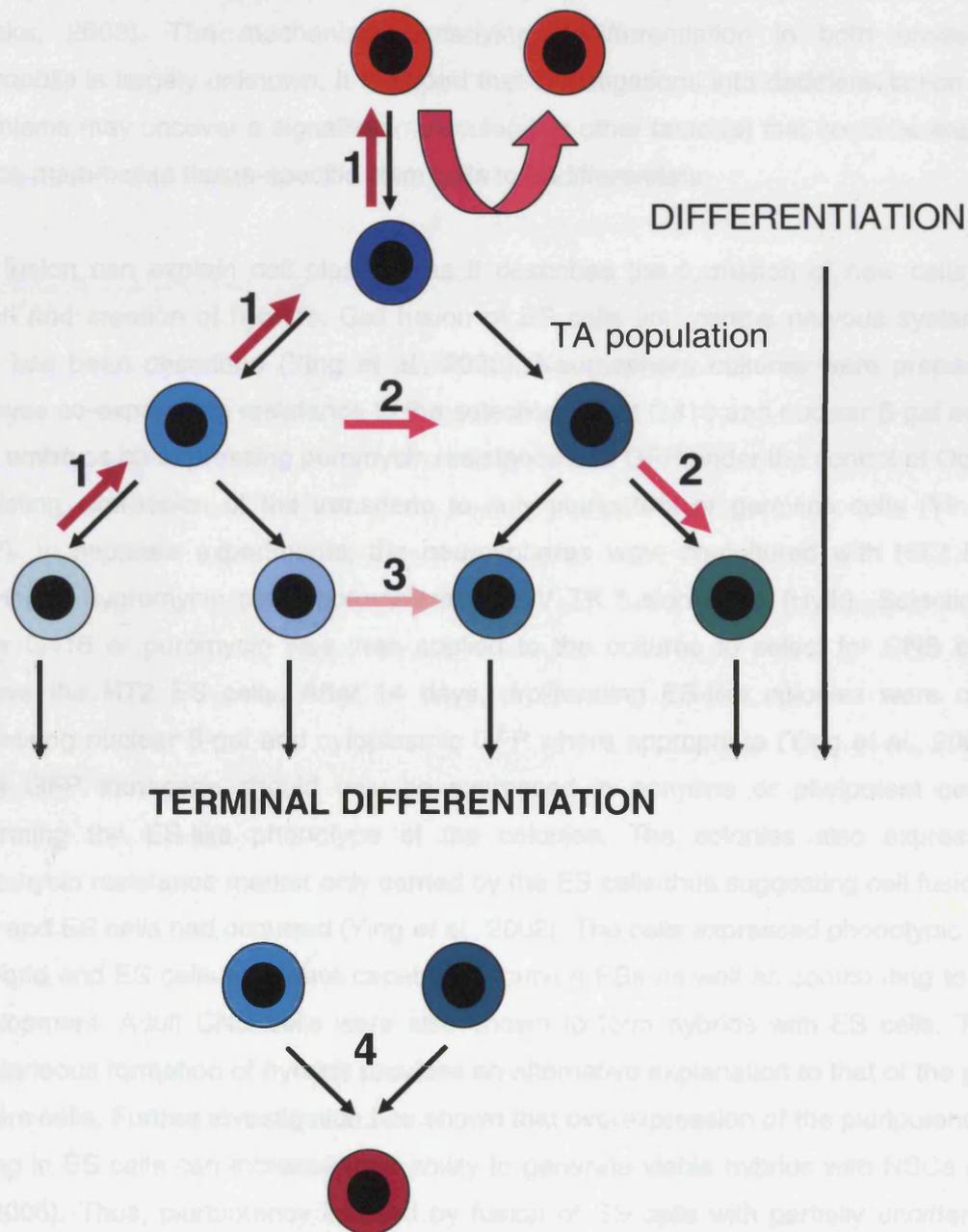
specific stem cells can also differentiate into lineages other than those of the tissue of origin.

There are four possible methods by which stem cells are proposed to alter their lineage; transdetermination, transdifferentiation, dedifferentiation and cell fusion (Figure 1.12) (Rice and Scolding, 2004). These mechanisms of stem cell plasticity are not mutually exclusive (Rice and Scolding, 2004). Transdetermination is the term employed to describe a partially committed progenitor altering its lineage to take on that of another partially committed progenitor therefore producing a separate set of differentiated cells usually found in another tissue. Transdifferentiation describes lineage switching between cells that are already committed. The terms transdifferentiation and transdetermination are often used interchangeably as it is difficult to determine whether a cell is partially committed or fully committed. Dedifferentiation describes the situation in which a partially committed progenitor reverts to the stem cell phenotype before following a new lineage path. Cell fusion can be used to explain stem cell plasticity and describes the formation of hybrids. Cell fusion suggests that no stem cell lineage switching occurs; the parent cells just fuse together. This can be determined as the resulting cell will contain the genetic information from both parental cells.

Embryonic and adult NSCs constitutively expressing  $\beta$ -gal have been shown to transdifferentiate into bone marrow using a bone marrow repopulation assay (Bjornson *et al.*, 1999). However, other investigators have failed to replicate these findings also using the transplantation assay and found that NSCs contributed only rarely to the haemopoietic lineage and that this was due to genetic changes in the cells after long periods in culture before transplantation (Morshead *et al.*, 2002). Therefore plasticity of stem cells via transdifferentiation is still widely disputed.

Transdetermination occurs naturally in *Drosophila*. In the *Drosophila* larva, imaginal disc primordia cells grow, divide and differentiate into adult structures during metamorphosis (Maves and Schubiger, 2003). The determined identity of disc cells was shown to occur in the third larval instar stage (72-96 hours of development) by fate mapping studies of mechanically cut imaginal disc fragments (Maves and Schubiger, 2003). It was further shown that if imaginal disc fragmentation was followed by a culture period, then the discs can regenerate (Maves and Schubiger, 2003; Schubiger, 1971). During this regeneration process, the discs can alter their identity and thus can undergo transdetermination and give rise to structures that normally develop from a different disc (Maves and Schubiger, 2003).

**Figure 1.12** Stem cell plasticity. 1 represents dedifferentiation of a committed cell back to the stem cell phenotype. 2 represents transdetermination of a partially committed progenitor cell switching to a partially committed progenitor cell of another lineage. 3 represents transdifferentiation of a committed cell to a committed cell of another lineage. 4 represents cell fusion of cells from different lineages



Dedifferentiation has been extensively studied in urodele amphibians as these organisms naturally regenerate their limbs and tails as well as other organs after amputation or injury (Straube and Tanaka, 2006; Tanaka, 2003). After amputation of a limb, cells dedifferentiate then proliferate and give rise to a mesenchymal growth zone known as a blastema. These cells then redifferentiate and form a replacement limb (Straube and Tanaka, 2006; Tanaka, 2003). Mature muscle fibres were shown to dedifferentiate by labelling of the endogenous muscle fibres with rhodamine-dextran and tracking during axolotl (salamander) regeneration (Echeverri *et al.*, 2001). These cell tracking experiments showed adult muscle fragmenting, dividing and populating the blastema four days post-amputation (Echeverri *et al.*, 2001; Tanaka, 2003). The mechanism underlying dedifferentiation in both urodeles and *Drosophila* is largely unknown. It is hoped that investigations into dedifferentiation in these organisms may uncover a signalling molecule(s) or other factor(s) that could be exploited to induce mammalian tissue-specific stem cells to dedifferentiate.

Cell fusion can explain cell plasticity as it describes the formation of new cells through fusion and creation of hybrids. Cell fusion of ES cells and central nervous system (CNS) cells has been described (Ying *et al.*, 2002). Neurosphere cultures were prepared from embryos co-expressing resistance to the selection agent G418 and nuclear  $\beta$ -gal as well as from embryos co-expressing puromycin resistance and GFP under the control of Oct-4, thus restricting expression of the transgene to only pluripotent or germline cells (Ying *et al.*, 2002). In separate experiments, the neurospheres were co-cultured with HT2 ES cells carrying a hygromycin phosphotransferase HSV TK fusion gene (Hytk). Selection using either G418 or puromycin was then applied to the cultures to select for CNS cells and remove the HT2 ES cells. After 14 days, proliferating ES-like colonies were observed expressing nuclear  $\beta$ -gal and cytoplasmic GFP where appropriate (Ying *et al.*, 2002). The Oct-4 GFP transgene should only be expressed in germline or pluripotent cells, thus confirming the ES-like phenotype of the colonies. The colonies also expressed the hygromycin resistance marker only carried by the ES cells thus suggesting cell fusion of the CNS and ES cells had occurred (Ying *et al.*, 2002). The cells expressed phenotypic markers of hybrid and ES cells and were capable of forming EBs as well as contributing to embryo development. Adult CNS cells were also shown to form hybrids with ES cells. Thus the spontaneous formation of hybrids provides an alternative explanation to that of the plasticity of stem cells. Further investigation has shown that overexpression of the pluripotency factor Nanog in ES cells can increase their ability to generate viable hybrids with NSCs (Silva *et al.*, 2006). Thus, pluripotency induced by fusion of ES cells with partially undifferentiated somatic cells or tissue-specific stem cells seems to be mediated by the pluripotency factor Nanog. Further investigations are needed to elucidate whether this or other similar factors

could be used to reinstate pluripotency to cells, thus furnishing them with therapeutic potential.

If adult stem cells exhibit plasticity and are therefore able to change fate when provided with the right stimuli the therapeutic potential would be enormous. It would bypass the need for embryos to obtain stem cells and would mean that a well characterised adult stem cell such as the HSC could be harvested and cultured to provide replacement cells for potentially any organ of the body. Controversy surrounds the plasticity field as many researchers have attempted to replicate published data without success. It has also been shown that cell fusion occurs and could be one explanation for the supposed plasticity of adult stem cells. Indeed ES cell induced pluripotency by fusion is becoming an established concept.

### 1.6 Stemness markers

Adult stem cells can be enriched from many organs as SP cells, or by the expression of particular surface antigens. Indeed, all adult tissues covered here seem to harbour stem cells with an SP phenotype. Furthermore, many adult stem cells seem to express similar surface antigens such as CD34 or c-Kit. However, to date there is no common marker capable of identifying all known adult stem cells. If it were possible to identify adult stem cells on a single cell basis then their therapeutic potential would be infinite. Since determination of surface antigen profiles and other approaches have not uncovered the common stem cell marker, attention has turned to transcriptional analysis of tissue-specific stem cells. As adult stem cells are affected by similar signalling pathways and possess similar characteristics such as the ability to self-renew, it is possible that all adult stem cells will express the same or similar genes which regulate these processes.

Transcriptional analysis performed by two independent groups in 2002, compared the transcriptional profiles of HSCs, NSCs and ES cells using oligonucleotide microarrays (Ivanova *et al.*, 2002; Ramalho-Santos *et al.*, 2002). Both investigations revealed upregulation of between 200-300 genes in all three stem cell types. These included members of signalling pathways known to be important for regulating the stem cell state such as transforming growth factor  $\beta$  (TGF- $\beta$ ) and Notch as well as upregulation of integrins and components of the cell cycle. Ramalho-santos *et al* (2002), found only 2 out of the 216 upregulated genes discovered were specific to stem cells and were completely absent from differentiated cell populations. These were uridine phosphorylase and suppressor of Lec15. Two Expressed Sequence Tags (ESTs) were also found (Ramalho-Santos *et al.*, 2002). Therefore it seems that most genes seemingly enriched in stem cells are also found in differentiated cell populations. A further study compared the transcriptional profiles of

embryonic, neural and retinal stem cells and identified 385 genes expressed in all three stem cell types (Fortunel *et al.*, 2003). This group then analysed the data from (Ramalho-Santos *et al.*, 2002) and (Ivanova *et al.*, 2002) along with their own data and only uncovered one upregulated gene in all types of stem cell in all three investigations; the integrin  $\alpha_6$  gene (Fortunel *et al.*, 2003). This finding is interesting as integrins are already used as a marker to enrich for many adult stem cells (Fujimoto *et al.*, 2002; Jones and Watt, 1993; Shinohara *et al.*, 1999). Integrins are also thought to maintain stem cell character by anchoring stem cells within their niche. The fact that only one upregulated gene was common to all stem cells in all three studies puts in doubt whether transcriptional analysis is a useful method for determining common stem cell characteristics.

There are caveats to the approaches which should be considered when analysing these data. Firstly, there are differences in the technical applications; one group analysed three mouse genome chips whereas the other analysed only one. The starter cell populations were isolated using different methods in each study. There may also be differences in the computational analysis employed to determine differences in gene expression. It is also possible that true stemness genes were not on the chips used in either investigation, or that the genes are only transiently expressed and therefore would not be detected as upregulated in the analysis. There is also the fact that there are no markers available as yet which can identify adult stem cells on a single cell basis, and therefore the analyses were probably conducted on heterogeneous cell populations. Most of these caveats apply to all transcriptional investigations of adult stem cells.

Transcriptional analysis of a stem cell deficient microenvironment may be informative as to what is missing and therefore should normally be present to maintain the niche. To this end, microarray analysis was performed on the ERM deficient (thus SSC deficient) and wild-type testis (Chen *et al.*, 2005). The microarray analysis revealed that the expression of the chemokines stromal cell-derived factor (SDF-1) and lipopolysaccharide-induced CXC chemokine (CXCL5) were greatly reduced in ERM deficient testis (Chen *et al.*, 2005). These chemokines have previously been implicated in regulating the haemopoietic niche (Choong *et al.*, 2004; Christensen *et al.*, 2004). Specifically, SDF-1 was demonstrated to be involved in HSC migration and self-renewal and CXCL5 was implicated in maintenance of HSCs (Choong *et al.*, 2004; Christensen *et al.*, 2004). Therefore these chemokines may be generally involved in maintaining adult stem cell niches and are potential adult stem cell markers.

Transcriptional analysis of a stem cell rich environment has also been performed. Transgenic mice whose small intestinal Paneth cells have been ablated harbour an increase in the fractional representation of undifferentiated cells within the crypt (Garabedian *et al.*, 1997). Using these transgenic mice intestinal progenitors have been harvested using laser capture microdissection (LCM) and their molecular profiles determined (Stappenbeck *et al.*, 2003). The profile of intestinal progenitor cells included transcripts encoding many proteins involved in regulating c-myc transcription, stability and transactivation. The canonical Wnt signalling pathway target c-Myc has previously been implicated in maintenance of the stem cell state (He *et al.*, 1998; Satoh *et al.*, 2004; Waikel *et al.*, 2001). Components of the cell cycle such as cyclin-dependent kinase regulatory subunits 1 and 2 along with RNA processing, trafficking and translocation proteins were also found to be upregulated in the intestinal progenitor cell populations (Stappenbeck *et al.*, 2003). The transcriptional profile of intestinal progenitors and that of gastric progenitors was then compared to databases obtained from mouse HSC, NSC and ES cell gene anatomy projects (Giannakis *et al.*, 2006; Mills *et al.*, 2002). It was found that these stem cells also expressed many identical or functionally equivalent proteins involved in mRNA processing, localisation and translation (Giannakis *et al.*, 2006). Interestingly, such control of mRNA processes is thought to be important in regulating the communication of progenitor cells with other cellular components of their niches and may also influence asymmetric division possibly by instigating polarized migration of progenitor offspring (Stappenbeck *et al.*, 2003). Thus, downstream effectors of the canonical Wnt pathway and RNA processing proteins are stem cell marker candidates, although further investigations are required to confirm this.

Isolation of putative stem cells from the bulge region of the HF using GFP expression driven by the K15 promoter has allowed transcriptional analysis of these cells (Morris *et al.*, 2004; Tumber *et al.*, 2004). Microarray analysis of GFP positive bulge cells revealed an upregulation of many stem cell-associated mRNAs including stem cell factor (SCF), ephrin tyrosine kinase receptors (Ephs) and CD34 (Holmberg *et al.*, 2006; Trempus *et al.*, 2003; Tumber *et al.*, 2004; Ueda *et al.*, 2000). Reverse Transcription Polymerase Chain Reaction (RT-PCR) and immunofluorescence was used to confirm that expression of these up-regulated genes was restricted to the bulge region of the HF, thus confirming their stem cell niche preferred location (Tumber *et al.*, 2004). Ephrins and their receptors the Ephs are involved in maintaining the spatial organisation of the ISC niche (Holmberg *et al.*, 2006). Since Eph mRNA is upregulated in the bulge stem cell niche it is possible that Ephs play a similar role in the HF. CD34 and SCF have also been implicated to be involved in the maintenance of HSCs. Thus, Ephs, CD34 and SCF are potential common adult stem cell

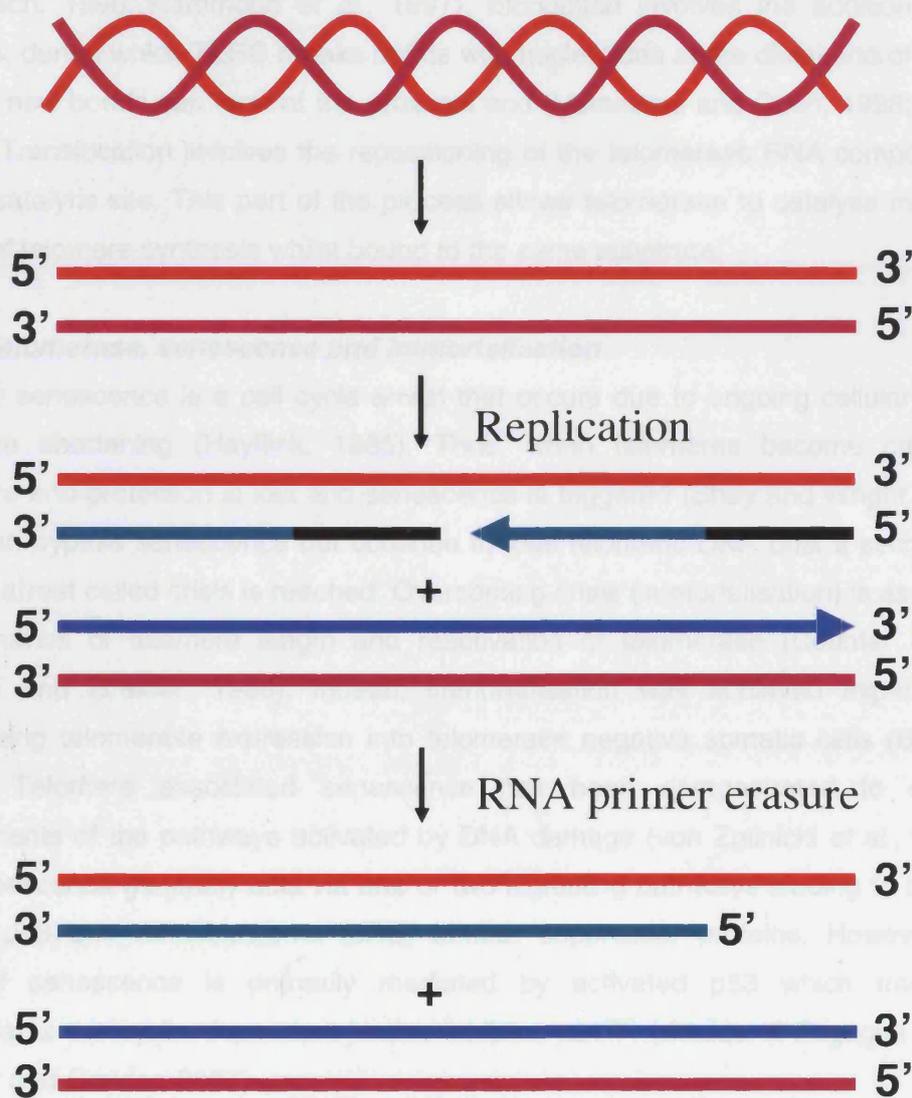
markers. Furthermore, the Wnt pathway has been implicated in controlling gradients of Ephs, which provides more evidence that the Wnt pathway is a key stem cell regulatory pathway (Holmberg *et al.*, 2006). A similar transcriptional analysis of bulge stem cells revealed upregulation of equivalent genes as well as genes associated with inhibition of proliferation and differentiation such as “inhibitor of DNA binding” genes (*Idb2* and *Idb4*), indicating that these GFP expressing cells are slowly cycling quiescent stem cells (Morris *et al.*, 2004).

In summary, transcriptional analysis of stem cells has revealed that several signalling pathways and components involved in processes such as RNA processing and the cell cycle are involved in the maintenance of the stem cell state for a number of tissue-specific stem cells; thus identifying potential adult stem cell markers. However, there are caveats to this kind of approach and further investigations into the stem cell marker potential of each candidate should be conducted. The transcriptional investigations have also shown vast differences in the results obtained. This could be due to some of the issues discussed previously, but it could also be possible that stem cells can only be enriched for by a combination of genes expressed relative to differentiated populations rather than expression of a particular gene only expressed in stem cells. To date, isolation of stem cells on a single cell basis using expression of a particular gene has proven unsuccessful.

### 1.7 Telomeres

Telomeres are specialised DNA strands consisting of a tandem array of TTAGGG repeats that cap the ends of eukaryotic chromosomes (Blackburn, 1991). Telomeres consist of duplex DNA with a 3' single-stranded overhang which can become tucked back into the telomere by base pairing into the duplex DNA forming a T-loop (Griffith *et al.*, 1999; Munoz-Jordan *et al.*, 2001). The function of telomeres is to provide genetic stability for cells by preventing the ends of chromosomes from fusion and from being recognised as DNA breaks. Telomeres allow complete replication of chromosomes without erosion of important coding information by solving the end replication problem (Figure 1.13). This problem is due to the inability of DNA polymerase to fully replicate the 3' end of the lagging strands of linear DNA molecules. Thus, with each cell division telomeres progressively shorten until a critically short telomere length is reached (known as the Hayflick limit) and replicative senescence is triggered (Hayflick and Moorhead, 1961). It has been suggested that the progressive shortening of telomeres can act as a molecular clock monitoring the replicative history of cells (Shay and Wright, 2000).

**Figure 1.13** The end replication problem. DNA polymerase is unable to synthesise in the 3'-5' direction. Thus, removal of the RNA primer (in black) on the lagging strand (green) results in the production of a daughter cell with an eroded DNA strand. The parental DNA is shown in pink and red and the continuously synthesised leading strand is shown in blue. Adapted from Stryer, L. 4<sup>th</sup> Ed. 1995.



### 12.3 Telomeres length maintenance: the telomerase cap

The length of telomeric repeat sequences is heterocorrelant even within a single cell, yet it is usually maintained within a particular limit, to prevent the initiation of cell (cancer) immortality. Telomere length is thought to be monitored by either alk wing or delaying which stress occurs

### **1.7.1 Telomerase**

Telomerase is a reverse transcriptase responsible for the elongation of telomeres (Greider and Blackburn, 1985). Telomerase consists of a catalytic component called telomerase reverse transcriptase (TERT) and an RNA component called TERC, TER or TR. Telomere lengthening occurs by the addition of one nucleotide at a time using the integral RNA component of telomerase as a template (Greider and Blackburn, 1985; Greider and Blackburn, 1987). Telomerase action can be divided into three steps; recognition, elongation and translocation (Figure 1.14). Recognition involves hybridization of TERC with the substrate and anchorage via a site thought to be within the catalytic subunit (Hammond and Cech, 1998; Hammond *et al.*, 1997). Elongation involves the addition of telomere repeats, during which TERC breaks bonds with nucleotides at the distal end of the template so that new bonds can form at the proximal end (Hammond and Cech, 1998; Wang *et al.*, 1998). Translocation involves the repositioning of the telomerase RNA component relative to the catalytic site. This part of the process allows telomerase to catalyse more than one round of telomere synthesis whilst bound to the same substrate.

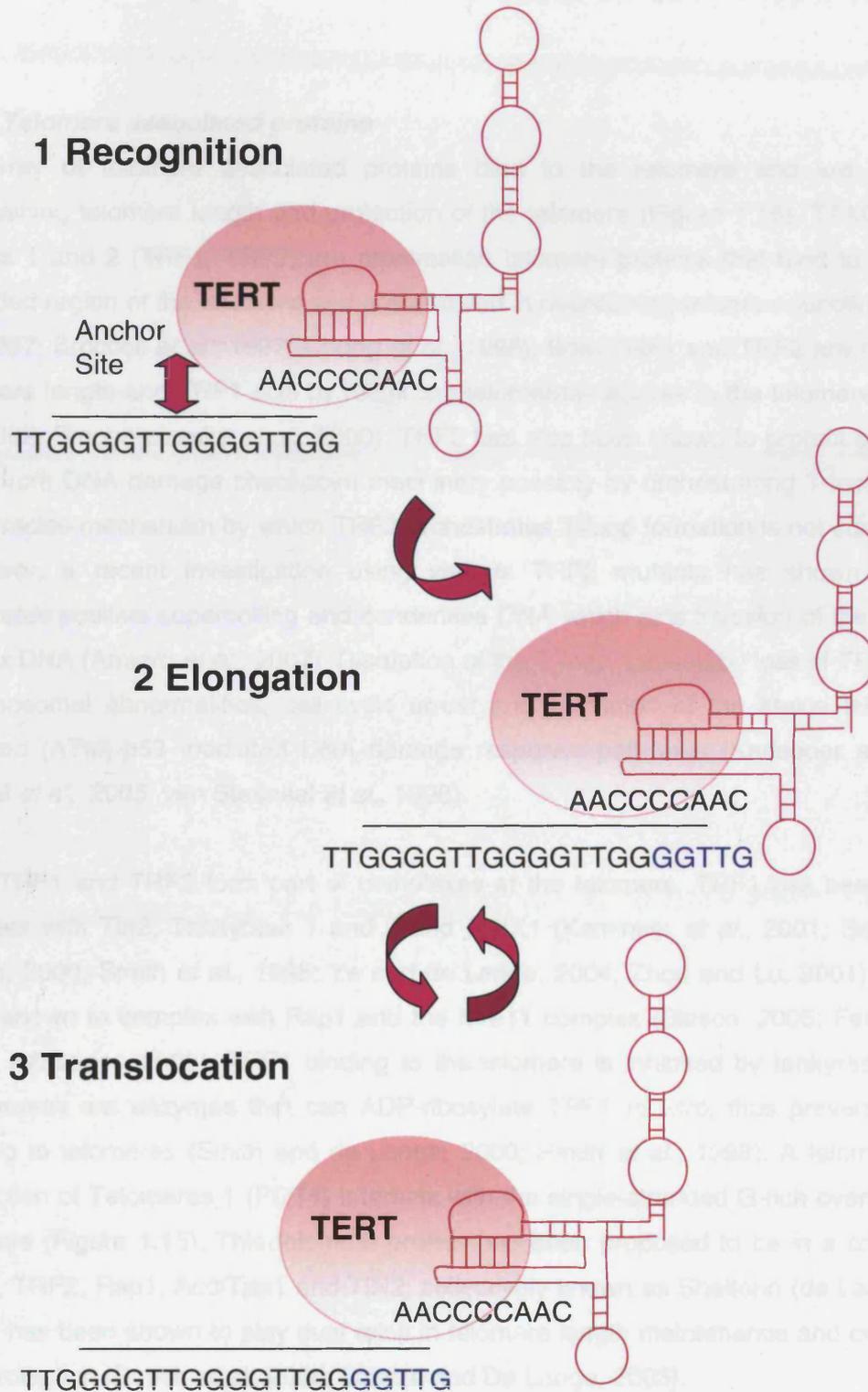
### **1.7.2 Telomerase, senescence and immortalisation**

Cellular senescence is a cell cycle arrest that occurs due to ongoing cellular division and telomere shortening (Hayflick, 1965). Thus, when telomeres become critically short, telomere end-protection is lost and senescence is triggered (Shay and Wright, 2005). Rare cells can bypass senescence but continue to lose telomeric DNA until a second phase of growth arrest called crisis is reached. Overcoming crisis (immortalisation) is associated with maintenance of telomere length and reactivation of telomerase (Counter *et al.*, 1992; Prowse and Greider, 1995). Indeed, immortalisation was achieved experimentally by introducing telomerase expression into telomerase negative somatic cells (Bodnar *et al.*, 1998). Telomere associated senescence has been demonstrated to share many components of the pathways activated by DNA damage (von Zglinicki *et al.*, 2005). Thus, cell senescence generally acts via one or two signalling pathways leading to the activation of the p53 and retinoblastoma (pRB) tumour suppressor proteins. However, telomere induced senescence is primarily mediated by activated p53 which transcriptionally upregulates the cyclin dependant kinase inhibitor p21<sup>cip1</sup> (d'Adda di Fagagna *et al.*, 2004; Feldser and Greider, 2007).

### **1.7.3 Telomere length maintenance; the telomere cap**

The length of telomere repeat sequences is heterogeneous even within a single cell, yet it is usually maintained within a particular limit, to prevent the initiation of cellular senescence. Telomere length is thought to be monitored by either allowing or denying telomerase access

**Figure 1.14** Telomerase mode of action. The first step is recognition of the substrate by TERC, and anchorage at an anchor site thought to be within the telomerase catalytic subunit. Elongation of the telomere then occurs, followed by translocation and repositioning of TERC on the substrate. Adapted from Harrington, 2003.



to the telomere. This is thought to be achieved by the telomere cap which is composed of an array of telomeric proteins (Chan and Blackburn, 2004; Ferreira *et al.*, 2004). The temporarily capped telomere denies telomerase access to the telomere and therefore successive erosion of telomeric DNA ensues. As the telomere becomes shorter it uncaps and telomerase can gain access to the telomere and catalyse the addition of telomeric DNA.

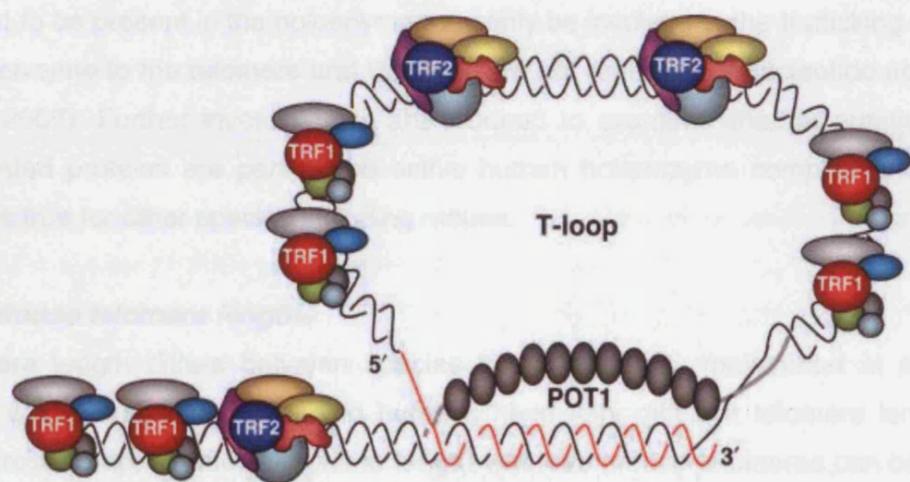
#### **1.7.4 Telomere associated proteins**

An array of telomere associated proteins bind to the telomere and are involved in maintaining telomere length and protection of the telomere (Figure 1.15). TTAGGG repeat factors 1 and 2 (TRF1, TRF2) are mammalian telomere proteins that bind to the double-stranded region of the telomere and are involved in maintaining telomere function (Bilaud *et al.*, 1997; Broccoli *et al.*, 1997; Chong *et al.*, 1995). Both TRF1 and TRF2 are regulators of telomere length and TRF1 acts by restricting telomerase access to the telomere (Ancelin *et al.*, 2002; Smogorzewska *et al.*, 2000). TRF2 has also been shown to protect chromosome ends from DNA damage checkpoint machinery possibly by orchestrating T-loop formation. The precise mechanism by which TRF2 orchestrates T-loop formation is not currently clear. However, a recent investigation using various TRF2 mutants has shown that TRF2 generates positive supercoiling and condenses DNA which aids invasion of the T-loop into duplex DNA (Amiard *et al.*, 2007). Disruption of the T-loop, caused by loss of TRF2 leads to chromosomal abnormalities, cell cycle arrest and activation of the ataxia telangiectasia mutated (ATM)-p53 mediated DNA damage response pathways (Karlseder *et al.*, 1999; Lechel *et al.*, 2005; van Steensel *et al.*, 1998).

Both TRF1 and TRF2 form part of complexes at the telomere. TRF1 has been shown to complex with Tin2, Tankyrase 1 and 2 and PINX1 (Kaminker *et al.*, 2001; Smith and de Lange, 2000; Smith *et al.*, 1998; Ye and de Lange, 2004; Zhou and Lu, 2001). TRF2 has been shown to complex with Rap1 and the Mre11 complex (Blasco, 2005; Ferreira *et al.*, 2004; Karlseder, 2003). TRF1 binding to the telomere is inhibited by tankyrase 1 and 2. Tankyrases are enzymes that can ADP-ribosylate TRF1 *in vitro*, thus preventing it from binding to telomeres (Smith and de Lange, 2000; Smith *et al.*, 1998). A telomere protein Protection of Telomeres 1 (POT1) interacts with the single-stranded G-rich overhang of the telomere (Figure 1.15). This telomere protein has been proposed to be in a complex with TRF1, TRF2, Rap1, Acd/Tpp1 and TIN2; collectively known as Shelterin (de Lange, 2005). POT1 has been shown to play dual roles in telomere length maintenance and chromosome end protection (Bunch *et al.*, 2005; Loayza and De Lange, 2003).

1.7.4 Telomere associated proteins

**Figure 1.15** Telomere associated proteins. The telomere is shown in a T-loop conformation. TRF1 and 2 play roles in telomere length maintenance and TRF2 is involved in telomere end protection. Protein complexes build upon both TRF1 and TRF2. Taken from Blasco 2005



### **1.7.5 Telomerase associated proteins**

Telomerase catalytic activity is thought to be reliant upon formation of a holoenzyme complex containing many associated proteins (Aisner *et al.*, 2002; Cheong *et al.*, 2003). Approximately 30 proteins have been implicated in complexing with human telomerase. Proteins thought to bind to the RNA component include; TEP1, dyskerin, L22 and Stau (Harrington *et al.*, 1997; Le *et al.*, 2000; Mitchell *et al.*, 1999). Proteins thought to bind to the catalytic component include chaperones such as hsp90 and p23 as well as 14-3-3 which is thought to be involved in nuclear localisation (Holt *et al.*, 1999; Seimiya *et al.*, 2000). A recent investigation measured the size of the active human telomerase holoenzyme complex in immortalised cell lines from a variety of cancer cells (Cohen *et al.*, 2007). The active complex was then purified and analysed and was found to consist of only hTERC, hTERT and dyskerin (Cohen *et al.*, 2007). The authors hypothesise that many proteins thought to be present in the holoenzyme may only be involved in the trafficking or recruitment of the enzyme to the telomere and that they are not required for nucleotide addition (Cohen *et al.*, 2007). Further investigations are required to examine whether putative telomerase associated proteins are part of the active human holoenzyme complex and whether the same is true for other species including mouse.

### **1.7.6 Mouse telomere lengths**

Telomere length differs between species but is generally maintained at an equilibrium length within a species. Mice and humans have very different telomere lengths. Human telomeres are approximately 10kb in length whereas mouse telomeres can be up to 150kb long (Kipling and Cooke, 1990). There are also differences in telomere length between established inbred mouse strains (derived more than 60 years ago) and wild-derived mouse species such as *Mus spretus*. The established inbred mouse strains have hypervariable telomere lengths of between 30 and 150kb whereas the *Mus spretus* strain has telomere lengths similar to humans at around 10kb (Coviello-McLaughlin and Prowse, 1997; Hemann and Greider, 2000; Prowse and Greider, 1995; Starling *et al.*, 1990). Wild-derived inbred mouse strains (inbred within the last 20-30 years) have shorter telomere lengths than the established inbred strains suggesting that there is no requirement for hypervariable long telomeres in mice. Furthermore, short telomeres have been shown not to affect the lifespan of the mouse (Hemann and Greider, 2000). The reason that established inbred mice have long telomeres remains uncertain but could be due to the confined breeding environment and therefore long telomeres are just a consequence of no competition. A cross between a mouse with long telomeres and one with short telomeres results in offspring with long telomeres suggesting a generalised propensity for longer telomeres (Hemann and Greider, 2000). The mouse strain needs to be taken into account when comparing

telomere/telomerase mouse models as different strains may have different telomere lengths which may affect the phenotypes produced.

### **1.7.7 *mTerc* mouse models**

*mTerc*<sup>-/-</sup> mice are viable, can be bred for six generations and lack telomerase activity as assayed by the telomere repeat amplification protocol (TRAP); a PCR based assay which amplifies products containing the TTAGGG repeat sequence (Blasco *et al.*, 1995; Blasco *et al.*, 1997; Kim *et al.*, 1994; Lee *et al.*, 1998). Telomere length progressively shortens in these animals and cells derived from animals from the fourth generation were devoid of telomere repeats and exhibited chromosomal abnormalities such as aneuploidy and fusion (Blasco *et al.*, 1995). Thus *mTerc* and therefore telomerase is required for telomere length maintenance.

Phenotypes were thought not to appear in these animals until later generations due to the long telomeres possessed by the *Mus musculus* strain laboratory mice used in this investigation (Blasco *et al.*, 1995; Kipling and Cooke, 1990). Fourth generation intercrosses of *mTerc*<sup>+/-</sup> resulted in a significant reduction in litter size which was partly attributed to the fact that a subset of embryos failed to close the neural tube at the forebrain and midbrain (Herrera *et al.*, 1999; Lee *et al.*, 1998). Intercrosses continued to be productive and generate viable offspring until the sixth generation when no offspring were produced (Lee *et al.*, 1998). The size and weight of the testis of sixth generation animals was up to 80% reduced and histological analysis of testis from these mice revealed a marked loss of proliferative germ cells with only the Sertoli and Leydig cells remaining (Lee *et al.*, 1998). Biochemical analysis of testis from sixth generation animals showed that there was a marked reduction in proliferation as assessed by BrdU incorporation and an increase in apoptosis as measured by the TdT-mediated dUTP nick end labelling (TUNEL) assay (Lee *et al.*, 1998). *mTerc*<sup>-/-</sup> mice also exhibited proliferative defects in the haemopoietic cells of the bone marrow and spleen (Lee *et al.*, 1998). The mice exhibited signs of premature ageing in highly proliferative organs e.g. defects in liver regeneration and a decreased stress response when compared to ageing controls (Rudolph *et al.*, 1999). Together the data generated from analysis of *mTerc*<sup>-/-</sup> mice suggest that telomerase activity is important for genomic integrity and the proliferation and long-term viability of germ cells and highly proliferative cells such as those in stem cell compartments (Lee *et al.*, 1998).

### **1.7.8 *mTert* mouse models**

Mice deficient for the catalytic component of telomerase have also been reported (Liu *et al.*, 2000; Yuan *et al.*, 1999). Both investigators mutated *mTert* by replacement of exons coding

for motifs known to be highly conserved among reverse transcriptases, as well as a motif specific for TERT proteins of various species; thus compromising the catalytic core of the enzyme (Liu *et al.*, 2000; Yuan *et al.*, 1999). The precise exons targeted by the two investigators differed slightly, yet both approaches resulted in transgenic animals exhibiting no telomerase activity. Yuan *et al.* (1999) assayed telomerase activity in the testis, liver and MEF cell lines using a quantitative stretch assay and found an intermediate level of telomerase activity in the *mTert*<sup>+/-</sup>. Conversely, the *mTert*<sup>+/-</sup> generated by Liu *et al.* (2000) revealed comparable telomerase activity to that found in the wild-type animals. Both investigators could not detect telomerase activity in their *mTert*<sup>-/-</sup> animals. The *mTert*<sup>-/-</sup> mice produced by Yuan *et al.* (1999) demonstrated no macroscopic or microscopic phenotype in the first two generations which were the only generations studied. This finding is comparable to the situation in the *mTerc*<sup>-/-</sup> mice which only demonstrate phenotypic changes in later generations (Blasco *et al.*, 1995; Blasco *et al.*, 1997; Lee *et al.*, 1998). Liu *et al.* (2000) found that their second generation *mTert*<sup>-/-</sup> mice exhibited a significant reduction in telomere length when compared to wild-type littermates and first generation *mTert*<sup>-/-</sup> animals. Thus, mTert and therefore telomerase activity is required for telomere length maintenance.

### 1.7.9 *mTerc*, *mTert* and telomere elongation

The telomerase mouse models have been used to determine whether mTerc or mTert are limiting for telomere elongation. Flow-cytometry and fluorescence *in situ* hybridisation (Flow-FISH) was used to analyse telomere length in late passage *mTert*<sup>-/-</sup> and *mTert*<sup>+/-</sup> ES clones (Liu *et al.*, 2000). It was demonstrated that *mTert*<sup>-/-</sup> and *mTert*<sup>+/-</sup> ES clones both exhibited loss of telomeric DNA, suggesting that loss of one *mTert* allele is sufficient to affect telomere length *in vitro*. Aneuploidy was observed in some of the late passage *mTert*<sup>-/-</sup> and *mTert*<sup>+/-</sup> ES clones, but end-to-end fusions were only observed in *mTert*<sup>-/-</sup> ES clones after approximately 300 population doublings (Liu *et al.*, 2000). Furthermore, it has been shown that *mTert*<sup>+/-</sup> ES cell lines maintain a minimal telomere length at chromosome ends, thus protecting them from genetic instability and fusion (Liu *et al.*, 2002). In contrast to *mTert*<sup>+/-</sup> ES clones, *mTerc*<sup>+/-</sup> ES clones exhibit no telomere length alterations (Niida *et al.*, 1998). This suggests that the catalytic component Tert not Terc is limiting for telomere length maintenance. However, the mouse and ES strains in which the experiments have been undertaken need to be considered when analysing the resultant phenotypes. Interspecies crosses with the *mTerc*<sup>-/-</sup> mice and *Mus musculus castaneus* (CAST/Ei) or *Mus Spretus* (SPRET/Ei) mouse strains with naturally shorter telomeres has shown that haploinsufficiency of *mTerc* can be achieved *in vivo* (Chiang *et al.*, 2004; Hathcock *et al.*, 2002).

Thus, there is evidence for both *Tert* and *Terc* to be limiting in terms of telomere elongation. The various investigations have been undertaken in a variety of mouse strains and therefore it is difficult to directly compare these results. Knockout of either *mTerc* or *mTert* is sufficient to abolish telomerase activity and therefore both may be required for proper telomerase function. Furthermore, both *Tert* and *Terc* may be required to ensure normal telomere elongation and maintenance.

#### **1.7.10 *Tert* is the main factor determining telomerase activity**

##### **a) *Tert* and *Terc* expression**

Human somatic tissues and primary cell lines possess low or undetectable levels of telomerase activity (Meyerson *et al.*, 1997; Nakamura *et al.*, 1997). In contrast, somatic mouse tissues express a moderate and detectable level of telomerase activity. This is thought to be the reason that there is a higher incidence of spontaneous cancer in the mouse and that mouse cells more readily become immortalized (Greenberg *et al.*, 1998; Prowse and Greider, 1995).

Expression of hTERC has been shown not to correlate with telomerase activity. This was demonstrated by identification of *hTERC* expression using Northern blot analysis in cell strains that do not have telomerase activity (Avilion *et al.*, 1996). Furthermore, *TERC* expression has been detected in normal human tissues which possess low or undetectable telomerase activity (Feng *et al.*, 1995). Moreover, analysis of normal and tumour tissues has shown an increase in *hTERC* expression in tumour samples when compared to normal tissues, although again, levels did not parallel telomerase activity (Avilion *et al.*, 1996; Takakura *et al.*, 1998).

Expression levels of *mTerc* have been assessed in two different transgenic mouse models of multi-stage tumorigenesis (Blasco *et al.*, 1996). Telomerase activity was only detected in late-developing tumours, yet *mTerc* expression was detectable at early stages and continued to increase during tumour progression (Blasco *et al.*, 1996). Taken together it can be seen that telomerase activity is not reflected by either *mTerc* or *hTERC* expression.

hTERT expression has mostly been investigated at the RNA level (Meyerson *et al.*, 1997; Nakamura *et al.*, 1997; Takakura *et al.*, 1998). Analysis of hTERT and hTERC mRNA expression in cervical cancer and normal samples revealed that *TERC* was expressed in all samples whereas *TERT* was only expressed in the cervical cancer samples and cell lines (Takakura *et al.*, 1998). Furthermore, there was a strong correlation between telomerase activity in these samples and *hTERT* expression. A further investigation showed that the

*hTERT* message is up-regulated in association with the activation of telomerase during the immortalisation of cells in culture and down-regulated during *in vitro* differentiation of cells (Meyerson *et al.*, 1997). Thus, these investigations indicate that *hTERT* expression is closely linked to telomerase activity.

Several splice variants of *hTERT* have been described (Colgin *et al.*, 2000; Kilian *et al.*, 1997; Saeboe-Larssen *et al.*, 2006; Ulaner *et al.*, 1998). Controlled expression of these variants contributes to the control of telomerase activity. Thus, when examining *hTERT* expression and telomerase activity knowledge of the various *hTERT* isoforms and their functions is vital. The *hTERT* gene has 16 exons and spans approximately 37kb of genomic DNA of which approximately 33kb is intronic sequence and 4kb corresponds to the *hTERT* mRNA transcript. Two of the several reported *hTERT* isoforms arise from in-frame deletions of exonic sequence (Ulaner *et al.*, 1998). The  $\alpha$ -splice site is within exon 6 and causes a 36bp deletion of one of the reverse transcriptase motifs which is required for the formation of an active transcriptase. The  $\beta$ -splice site generates an 182bp deletion which results in a truncated protein missing some other important reverse transcriptase motifs (Ulaner *et al.*, 1998). Overexpression of the *hTERT* $\alpha$  variant in telomerase positive immortal cell lines has shown that this isoform is a dominant-negative inhibitor of telomerase activity (Colgin *et al.*, 2000). There are other *hTERT* splice variants formed by exon deletions or insertion of intron sequences. One common splice variant exhibits deletions of exons 7 and 8. A further six novel variants of *hTERT* have recently been identified (Saeboe-Larssen *et al.*, 2006). The finding that numerous splice variants of *hTERT* exist, including a dominant-negative form suggests that alternative splicing represents an important method for controlling *hTERT* expression. This is also evident as different transcripts are expressed in different cells at various stages of development. Thus, it is possible that as with the  $\alpha$ -variant, expression of the truncated transcripts results in translation of proteins that are involved in regulating telomerase activity (Ulaner *et al.*, 1998). However, telomerase negative cells often exhibit full-length mRNA suggesting that other downstream regulatory mechanisms are also involved (Saeboe-Larssen *et al.*, 2006).

*mTert* has been shown to be expressed in embryonic and adult tissues using RNA protection assays. However, *mTert* transcripts are generally in low abundance when compared to an internal control such as  $\beta$ -actin (Greenberg *et al.*, 1998). *mTert* RNA transcripts were found to be at their highest in embryos between E9.5 dpc and E15.5 dpc and in adult tissues with detectable telomerase activity such as the thymus and in highly proliferative tissues such as the intestine and testes (Greenberg *et al.*, 1998). *mTert* expression was also detected in tissues with a high regenerative ability such as the liver

and tissues that develop postnatally such as the lung. No detectable *mTert* transcripts were found in the brain, heart muscle or kidney (Greenberg *et al.*, 1998). Another investigator also analysed *mTert* expression in embryos and adult tissues using *in situ hybridisation* and RT-PCR. In this investigation, embryos between E9.0 dpc and E12.0 dpc demonstrated a widespread expression of *mTert* with high expression in highly proliferative areas such as the limbs, the tip of the tail and the nares (Martin-Rivera *et al.*, 1998). Analysis of mouse adult tissues revealed that *mTert* mRNA was found in all tissues regardless of telomerase activity status. However, mRNA levels were higher in tissues previously shown to have telomerase activity (Martin-Rivera *et al.*, 1998). mTert protein expression was detected in highly proliferative tissues such as thymus, testis, spleen and liver, but was not detected in muscle, brain, intestine or heart. The identification of *mTert* mRNA, but not mTert protein in all tissues analysed suggests that like hTERT, the expression of mTert may be regulated at many levels. Alternative splicing may be one mechanism which is involved in regulating mTert expression, however there is currently no evidence to suggest this. *mTert* expression was also analysed in *mTerc* knockout embryos and adult mice. It was found that mTert expression was not dependent on mTerc expression suggesting that mTert is expressed independently of the formation of active telomerase complexes (Martin-Rivera *et al.*, 1998).

In summary, telomerase activity is not reflected by either *hTERC* or *mTerc* expression. Indeed expression of hTERT and mTert has been more closely linked to telomerase activity. Furthermore, these investigations have revealed that TERT expression is highest in tumour samples, immortalised cells and somatic tissues with high proliferative capacity.

### **b) Reconstitution of telomerase activity by introduction of TERT**

As previously discussed, the expression of *hTERT* at the RNA level is closely linked to telomerase activity, suggesting that TERT expression is the rate limiting determinant of the enzymatic activity of human telomerase (Meyerson *et al.*, 1997; Nakamura *et al.*, 1997; Takakura *et al.*, 1998). These investigations have been substantiated by reconstitution of telomerase activity by the introduction of TERT into telomerase negative cells (Bodnar *et al.*, 1998; Nakayama *et al.*, 1998; Weinrich *et al.*, 1997). One such investigation involved the *in vitro* transcription and translation of *hTERT* co-synthesised or mixed with *hTERC* (Weinrich *et al.*, 1997). It was shown that this was sufficient to reconstitute telomerase activity (Weinrich *et al.*, 1997). The investigators then generated *hTERT* mutants with alterations in the conserved reverse transcriptase motifs and showed that expression of these mutants was sufficient to reduce or abolish telomerase activity (Weinrich *et al.*, 1997). This approach provides direct evidence that hTERT is the catalytic component of human telomerase.

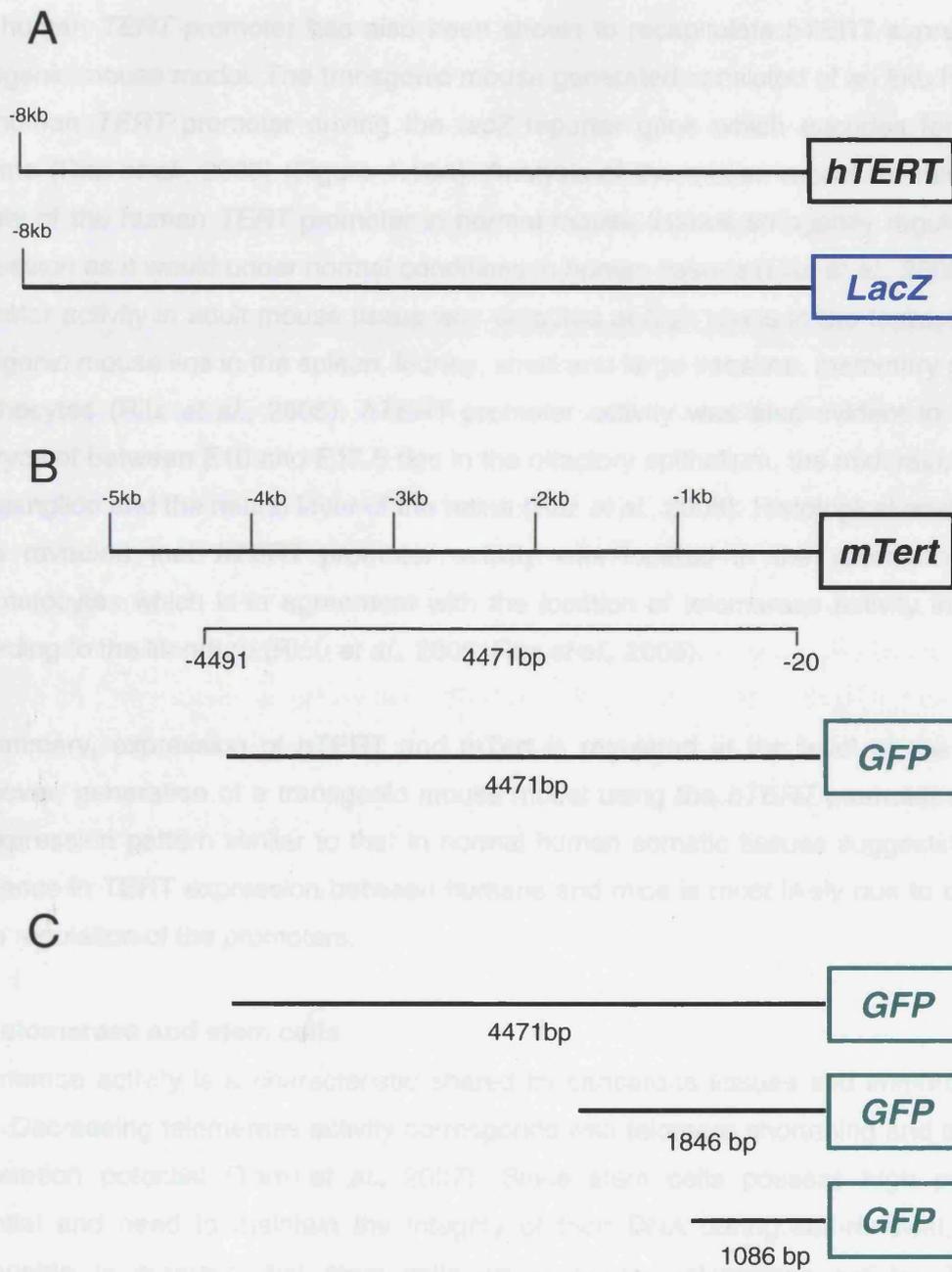
### 1.7.11 *Tert* expression is primarily regulated at the level of the promoter

There are differences in *TERT* gene expression between human and mouse. Somatic human tissues tightly regulate expression of hTERT compared to mTert which is more widely expressed. However, *TERT* mRNA and telomerase activity are upregulated in both human and mouse tumours, demonstrating that telomerase is involved in tumorigenesis. Thus it has been suggested that there may be regulatory mechanisms conserved between human and mouse, and that the differences in expression in several tissues may be due to differences in promoter elements (Ritz *et al.*, 2005). Indeed, the generation of *Tert* promoter mouse models and an *in vitro* promoter model have provided evidence that *Tert* expression is primarily regulated at the level of the promoter (Figure 1.16) (Armstrong *et al.*, 2000; Pericuesta *et al.*, 2006; Ritz *et al.*, 2005)

The first reporter promoter model to be generated consisted of a 4.4kb (referred to as 5kb) region of the *mTert* promoter driving the expression of GFP (Armstrong *et al.*, 2000) (Figure 1.16B). This reporter cassette was stably transfected into ES cells and was shown to mimic mTert expression *in vitro* (Armstrong *et al.*, 2000). Reporter expression was also shown to correlate with telomerase activity (Armstrong *et al.*, 2000). This investigation demonstrates that the 5kb promoter region is sufficient for mTert expression and mTert expression is sufficient for telomerase activity.

The mouse *Tert* promoter has been shown to regulate mTert expression *in vivo* using transgenic mouse models. Three transgenic mouse models using 1kb, 2kb (1.8kb) and 5kb (also 4.4kb) regions of the *mTert* promoter to drive expression of GFP have been generated by the same investigators (Pericuesta *et al.*, 2006) (Figure 1.16C). GFP expression was detectable at the blastocyst stage and in the germinal ring of E13.5 dpc transgenic embryos (Pericuesta *et al.*, 2006). GFP expression was also evident in stem cell lines derived *in vitro* from embryonic, foetal and adult transgenic tissues (Pericuesta *et al.*, 2006). The GFP-expressing stem cell lines generated included new ES cell lines, GSCs generated from neonatal and adult testis and cultures generated from E14 dpc foetus brain. FACs analysis of the cells derived from transgenic brains revealed that a more intensive fluorescence was evident in the 5kb transgenic cells (Pericuesta *et al.*, 2006). RT-PCR analysis for GFP in adult mouse tissues revealed the presence of mRNA in liver, spleen, kidney, heart, testis, ovary and brain; although it was lower than endogenous *mTert* expression (Pericuesta *et al.*, 2006). Thus, this investigation also provides evidence for *Tert* expression being regulated at the level of the promoter. However, no GFP expression could be detected using fluorescent microscopy or Western blot analysis in any tissues of the transgenic neonates or adult mice generated (Pericuesta *et al.*, 2006). This could be because the

**Figure 1.16** Five Tert promoter models generated. (A) *hTERT* promoter model comprising an 8kb region of the promoter driving expression of the LacZ gene, compared to endogenous *hTERT* promoter. (B) Region of *mTert* promoter used in the *in vitro* investigation by Armstrong *et al*, (2000), compared to endogenous *mTert* promoter. (C) Regions of *mTert* promoter used to generate *in vivo* mouse models by Pericuesta *et al* (2006).



transgene is only expressed in stem cells and these are scarce in the total population of cells within adult tissues; making detection difficult. Alternatively, the fact that the transgene could only be detected in adult tissues by deriving stem cells lines could be due to the *in vitro* culture conditions that possibly stimulate proliferation and dedifferentiation. Thus, the authors suggest that *mTert* activity is recovered *in vitro* due to loss of repression mechanisms which are in place under normal physiological conditions (Pericuesta *et al.*, 2006).

The human *TERT* promoter has also been shown to recapitulate hTERT expression in a transgenic mouse model. The transgenic mouse generated consisted of an 8kb fragment of the human *TERT* promoter driving the *lacZ* reporter gene which encodes for the  $\beta$ -gal enzyme (Ritz *et al.*, 2005) (Figure 1.16A). Analysis of the mouse model revealed that the activity of the human *TERT* promoter in normal mouse tissues stringently regulates TERT expression as it would under normal conditions in human tissues (Ritz *et al.*, 2005). *hTERT* promoter activity in adult mouse tissue was detected at high levels in the testis, and in one transgenic mouse line in the spleen, kidney, small and large intestine, mammary glands and lymphocytes (Ritz *et al.*, 2005). *hTERT* promoter activity was also evident in transgenic embryos of between E10 and E17.5 dpc in the olfactory epithelium, the midbrain, the dorsal root ganglion and the neural layer of the retina (Ritz *et al.*, 2005). Histological analysis of the testis revealed that *hTERT* promoter activity was located in the spermatogonia and spermatocytes which is in agreement with the location of telomerase activity in the testis according to the literature (Riou *et al.*, 2005; Ritz *et al.*, 2005).

In summary, expression of hTERT and mTert is regulated at the level of the promoter. Moreover, generation of a transgenic mouse model using the *hTERT* promoter resulted in an expression pattern similar to that in normal human somatic tissues suggesting that the difference in TERT expression between humans and mice is most likely due to differences in the regulation of the promoters.

### 1.8 Telomerase and stem cells

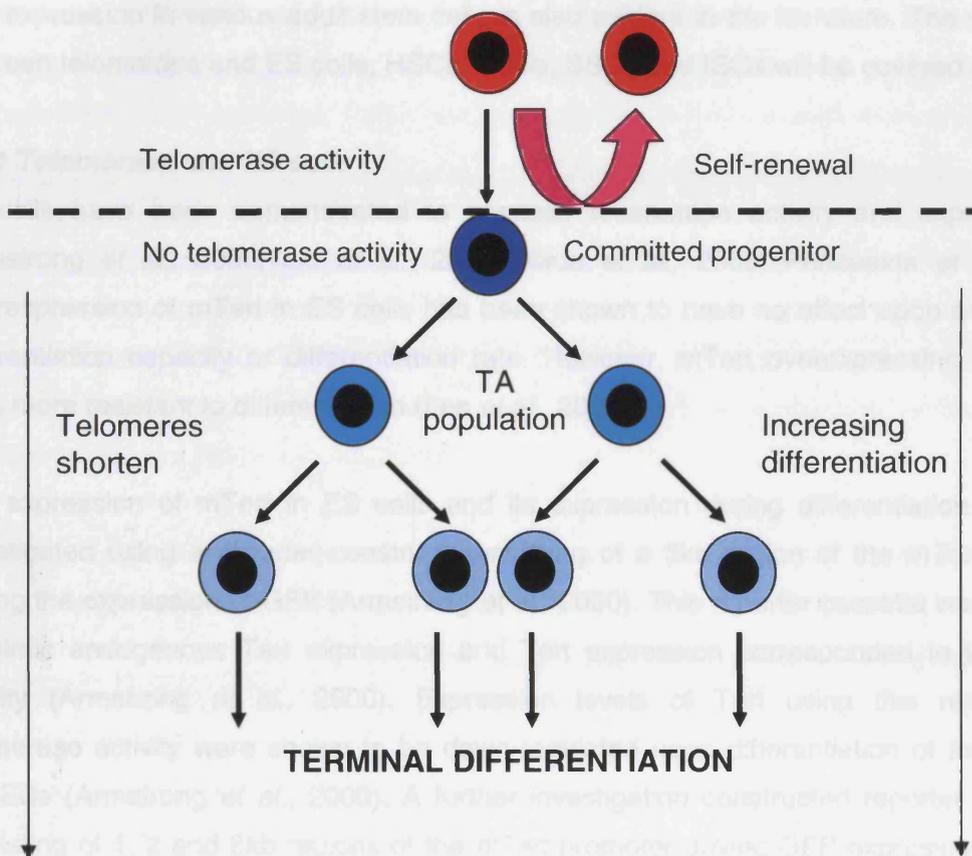
Telomerase activity is a characteristic shared by cancerous tissues and immortalised cell lines. Decreasing telomerase activity corresponds with telomere shortening and decreasing proliferation potential (Tam *et al.*, 2007). Since stem cells possess high proliferative potential and need to maintain the integrity of their DNA during self-renewal, it seems reasonable to suppose that stem cells would require telomerase activity. Thus it is hypothesised here that stem cells possess telomerase activity which is turned off when stem cells give rise to TA cells, resulting in telomere shortening and a decrease in

proliferative potential. As the TA cells continue to differentiate, the telomeres continue to shorten until terminal differentiation is achieved (Figure 1.17). Since Tert expression closely correlates with telomerase activity the expression of Tert may be a useful general stem cell marker.

The literature contains several lines of evidence to support the hypothesis that telomerase is expressed in highly proliferative putative stem cells. The first line of evidence comes from the study of diseases such as cancer, ageing, dyskeratosis congenital (DKC) and aplastic anaemia. Telomerase is expressed in a variety of human and mouse cancers and is thought to be involved in maintaining the proliferation of cells within the tumour. Telomerase has also been described as playing a role in cellular ageing also due to its role in regulating proliferation (Blasco, 2005). Both cellular ageing and cancer have been described as stem cell diseases; cancer can originate from the transformation of progenitor or normal tissue stem cells (often referred to as cancer stem cells) and ageing is thought to occur due to a decline in the functionality of tissue stem cells (Blasco, 2005; Hiyama and Hiyama, 2007). DKC arises due to a defect in TERC which then results in premature telomere shortening and loss of telomerase activity (Mitchell *et al.*, 1999). The patients exhibit problems in organs that require constant replenishment and thus often suffer from bone marrow failure and skin abnormalities. A link with defects in TERC has also been found for aplastic anaemia (Vulliamy *et al.*, 2002). This disease also results from bone marrow failure and is thought to be due to damage or loss of HSCs (Hiyama and Hiyama, 2007). Thus, both diseases are mediated by failure to maintain the HSC compartment. The link with aplastic anaemia and telomerase is further strengthened with the observation that telomere length in peripheral blood granulocytes and monocytes is decreased in patients with the disease compared to age-matched controls (Hiyama and Hiyama, 2007; Lee *et al.*, 2001).

A second line of evidence comes from transfection of telomerase into various putative adult stem cells. These experiments have shown that the addition of telomerase to stem cells can extend their proliferative life-span whilst not altering their capacity to differentiate into the required cell types (Roy *et al.*, 2004; Serakinci and Keith, 2006; Simonsen *et al.*, 2002). For example, mesenchymal stem cells (MSCs) transfected with hTERT were shown to have increased proliferative potential when compared to controls which underwent senescence-associated proliferation arrest (Simonsen *et al.*, 2002). Transplantation experiments were used to demonstrate the functionality of the cells (Simonsen *et al.*, 2002). Furthermore, immortalisation of stem cells, whilst not disrupting differentiation capacity, may be of benefit in manufacturing large scale cultures of adult stem cells for use in cell therapy or other medical applications (Serakinci and Keith, 2006).

**Figure 1.17** The project hypothesis. The stem cells shown in red possess telomerase activity and are able to maintain their telomere lengths. When the stem cell gives rise to a progenitor cell, this telomerase activity is switched off. As the progenitor cells differentiate the telomeres become shorter and shorter. Thus it is hypothesised that telomerase is a potential marker of stem cells.



More evidence for the association of telomerase and proliferation potential comes from the study of long-lived animals (Klapper *et al.*, 1998a; Klapper *et al.*, 1998b). In contrast to mammals, some fish species and lobster continuously grow throughout their lives and exhibit little or no senescence (Klapper *et al.*, 1998a; Klapper *et al.*, 1998b). Therefore, their somatic cells must require a high proliferative capacity and must have relatively high telomerase activity. Indeed it has been demonstrated using the TRAP assay that rainbow trout and lobster possess high telomerase activity in all organs analysed (Klapper *et al.*, 1998a; Klapper *et al.*, 1998b).

Thus, it can be seen that telomerase is associated with high proliferation potential and increased cellular lifespan. Further evidence for the importance of telomerase activity and Tert expression in various adult stem cells is also evident in the literature. The relationship between telomerase and ES cells, HSCs, ESCs, SSCs and ISCs will be covered here.

### **1.8.1 Telomerase and ES cells**

ES cells have been demonstrated to possess telomerase activity and express mTert (Armstrong *et al.*, 2000; Liu *et al.*, 2000; Niida *et al.*, 2000; Pericuesta *et al.*, 2006). Overexpression of mTert in ES cells has been shown to have no effect upon proliferation, differentiation capacity or differentiation rate. However, mTert overexpressing ES clones were more resistant to differentiation (Lee *et al.*, 2005).

The expression of mTert in ES cells and its expression during differentiation has been investigated using a reporter construct consisting of a 5kb region of the *mTert* promoter driving the expression of GFP (Armstrong *et al.*, 2000). This reporter cassette was sufficient to mimic endogenous Tert expression and Tert expression corresponded to telomerase activity (Armstrong *et al.*, 2000). Expression levels of Tert using this reporter and telomerase activity were shown to be down-regulated upon differentiation of the ES cells into EBs (Armstrong *et al.*, 2000). A further investigation constructed reporter constructs consisting of 1, 2 and 5kb regions of the *mTert* promoter driving GFP expression and also showed that this expression mimicked mTert expression, which was subsequently lost upon differentiation (Pericuesta *et al.*, 2006).

A novel transcription factor Zap3 has been reported that binds to the *mTert* promoter and negatively regulates mTert expression (Armstrong *et al.*, 2004). Overexpression of this transcription factor in ES cells results in down-regulation of *mTert*, telomerase activity and telomere length during differentiation (Armstrong *et al.*, 2004). Thus, Zap3 is an important regulator of mTert expression during differentiation of ES cells.

In summary, *mTert* expression is regulated by its promoter and its expression corresponds with telomerase activity. ES cells express *mTert* and possess telomerase activity. However, *mTert* expression and telomerase activity are down-regulated upon ES cell differentiation and this is potentially regulated by the Zap3 transcription factor. Thus, mTert can be associated with the ES cell phenotype.

### **1.8.2 Telomerase and HSCs**

Telomerase activity has been identified at low levels in primitive haemopoietic cells (Broccoli *et al.*, 1995; Chiu *et al.*, 1996; Counter *et al.*, 1995; Engelhardt *et al.*, 1997; Hiyama *et al.*, 1995). However, Southern analysis of telomere repeat fragments (TRFs) has shown that HSCs still exhibit progressive telomere shortening and loss of proliferative potential. Thus the telomerase activity detected in HSCs is at insufficient levels to prevent telomere shortening or plays a different role in these cells. *In vivo* bone marrow transplantation and transplant experiments using HSCs have shown that telomeres also shorten with successive transplantation (Allsopp *et al.*, 2001; Notaro *et al.*, 1997). Significant shortening of approximately 7kb was shown after two rounds of serial transplantation by southern analysis of TRFs in donor HSCs (Allsopp, *et al.*, 2001). Additional serial transplantation experiments using telomerase deficient HSCs have shown that telomerase is required to partially counter the telomere reduction during serial transplantation, thus partially preventing loss of telomere function and endowing some replicative capacity (Allsopp *et al.*, 2003).

The association between telomerase expression in HSCs at four stages of differentiation and proliferative potential has been investigated using a modified single cell TRAP assay. It was found that the cells that possessed the greatest self-renewal potential exhibited the highest telomerase activity.

In summary, HSCs and progenitors have been shown to possess telomerase activity. This telomerase activity slows telomere shortening with successive divisions, but does not prevent it. Furthermore, high telomerase activity has been associated with high proliferative potential and thus with the HSC phenotype.

### **1.8.3 Telomerase and ESCs**

Telomerase activity can be detected in the growth (anagen) phase and not in the resting (telogen) phase of the HF in both mouse and human (Ramirez *et al.*, 1997; Sarin *et al.*, 2005). Furthermore, telomerase activity has been found to be associated with areas

containing cells with high mitotic activity such as progenitor cells (Ramirez *et al.*, 1997; Sarin *et al.*, 2005).

The presence of putative stem cells (LRCs) in the bulge was assessed in *mTerc*<sup>-/-</sup> to determine whether telomerase activity is important for the maintenance of the bulge stem cell compartment (Flores *et al.*, 2005). Third generation *mTerc*<sup>-/-</sup> mice with short telomeres demonstrated a substantial increase in the number of LRCs within the bulge; suggesting that a decrease in telomere length as a consequence of no telomerase activity results in accumulation of stem cells within their niche (Flores *et al.*, 2005). Clonogenic assays showed that *mTerc*<sup>-/-</sup> keratinocytes formed fewer and smaller colonies when compared to wild-type controls suggesting a decrease in proliferation potential with mTerc deficiency (Flores *et al.*, 2005). However, potency was unaffected as the keratinocytes were able to fully differentiate.

K5-*mTert* transgenic mice have been generated using the keratin 5 promoter to target expression of mTert to basal keratinocytes (Gonzalez-Suarez *et al.*, 2001; Tumber *et al.*, 2004). These mice have increased telomerase activity and Tert expression in the skin (including the bulge), as well as an increased susceptibility to skin tumorigenesis (Gonzalez-Suarez *et al.*, 2001). Overexpression of mTert in the K5-*mTert* mice results in lower LRC numbers compared to wild-type controls (Flores *et al.*, 2005). After chemical treatment the K5-*mTert* mice exhibited increased mobilisation of LRCs from the niche when compared to controls. K5-*mTert* keratinocytes also exhibited a high colony-forming efficiency suggesting an increased proliferative potential of these cells (Flores *et al.*, 2005).

Thus, mTert overexpression promotes LRC mobilisation and increased proliferation potential whereas lack of mTerc and therefore telomerase results in accumulation of LRCs in the bulge and reduced mobilisation of the stem cells upon proliferation induction (Flores *et al.*, 2005; Gonzalez-Suarez *et al.*, 2001).

These findings have been substantiated using conditional expression of Tert in mouse skin epithelium by administration of the tetracycline analogue doxycycline (Dox) in the drinking water of transgenic mice containing *Tert* cDNA under the control of a tetracycline responsive promoter (Sarin *et al.*, 2005). Induction of the transgene results in increased telomerase activity in the skin and manifests phenotypically as an abnormal increased hair growth. Skin biopsies of the induced (i-Tert) mice revealed that this was due to a promotion of anagen from telogen; thus resulting in new growth (Sarin *et al.*, 2005). Bulge stem cells were labelled using an LRC protocol with BrdU in both i-Tert and control animals. Double

immunostaining for BrdU and CD34 was used to identify the label and the putative stem cell population. Upon dox treatment the i-Tert mice stained positively for CD34 in the bulge, but the BrdU label was greatly diminished, suggesting that Tert overexpression induces bulge stem cells to proliferate which is noted by depletion of the BrdU label in the stem cell niche. However, due to the CD34 population remaining within the niche, Tert may promote self-renewal of the stem cell population as well as increasing mobilisation of cells out of the niche (Sarin *et al.*, 2005).

Intercrosses of the i-Tert mice with *mTerc*<sup>+/-</sup> showed that the effects of Tert in the bulge are not dependent on the formation of active telomerase complexes and are therefore independent of its role in maintaining telomere length (Sarin *et al.*, 2005). The phenotypes found in this investigation depend upon overexpression of Tert, and therefore the endogenous role of Tert needs to be carefully elucidated. The precise mechanism by which Tert may affect the stem cell compartment in skin is currently unknown.

#### **1.8.4 Telomerase and SSCs**

Telomerase activity can be detected in cell extracts from both human and mouse testis (Kim *et al.*, 1994). Moreover, telomerase activity is important for the progression of normal spermatogenesis as defects in spermatogenesis are apparent after successive breeding of *mTerc*<sup>-/-</sup> animals (Lee *et al.*, 1998). Telomerase activity has been shown to be specific for spermatogonia and meiotic spermatocytes (Eisenhauer *et al.*, 1997; Wright *et al.*, 1996). This telomerase activity decreases as spermiogenesis progresses until the telomerase negative epididymal spermatozoa are formed (Eisenhauer *et al.*, 1997; Ravindranath *et al.*, 1997; Wright *et al.*, 1996). The testis α<sub>6</sub> positive SP has high telomerase activity and has been demonstrated to contain primarily spermatogonia and GSCs (Bastos *et al.*, 2005; Lassalle *et al.*, 2004; Riou *et al.*, 2005). Thus, telomerase activity within the testis is highest in the most undifferentiated cell types and is progressively lost throughout differentiation.

Expression of mTert has been demonstrated in the testis using mRNA analysis (Greenberg *et al.*, 1998; Martin-Rivera *et al.*, 1998; Pericuesta *et al.*, 2006; Ritz *et al.*, 2005). This has been substantiated by analysis of the *hTERT-lacZ* transgenic mouse model described previously (Ritz *et al.*, 2005). In this investigation, a sensitive radioactive RT-PCR method revealed that in all three transgenic mouse lines generated the promoter activity was highest in the testis (Ritz *et al.*, 2005). Furthermore, immunohistochemistry using a β-gal antibody revealed that *hTERT* promoter activity in the adult testis is restricted to the spermatogonia and primary spermatocytes (Ritz *et al.*, 2005). The *mTert*-GFP mouse model described previously also demonstrated that the *mTert* promoter is active in GSCs by

## 2 Materials and Methods

### 2.1 Molecular Biology

All restriction endonucleases were obtained from New England Biolabs unless otherwise stated. All chemicals were obtained from Sigma unless otherwise stated.

#### 2.1.1. *Plasmids*

The plasmids used for this project were as follows;

pECFP-C1 (BD Biosciences; Clontech; Figure 2.1 A)

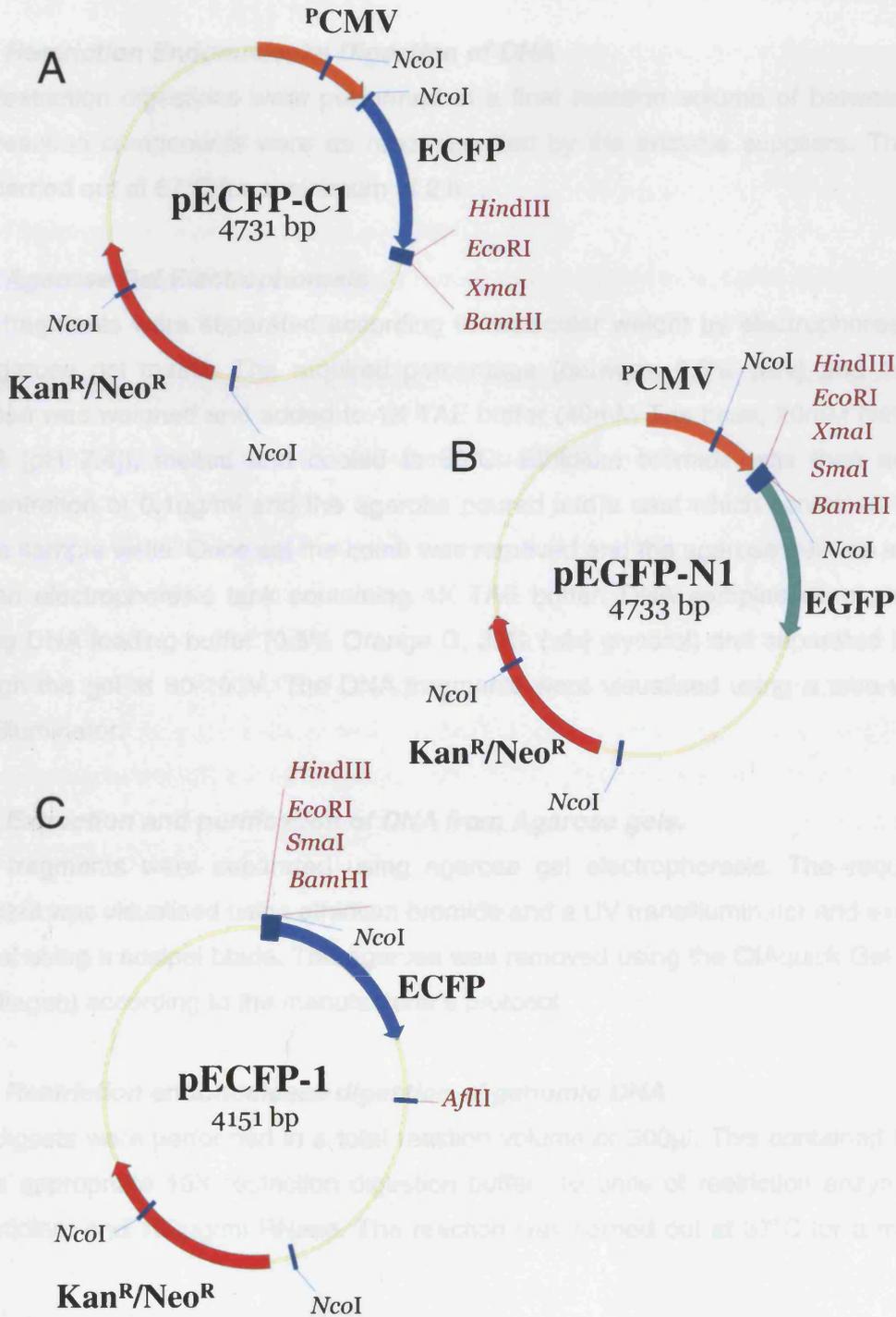
pEGFP-N1 (BD Biosciences; Clontech; Figure 2.1 B)

pECFP-1 (BD Biosciences; Clontech; Figure 2.1 C)

2.1.2 DNA purification

DNA of abundance followed. The DNA was collected as a pellet by centrifugation at 12,000 rpm (Heraeus Biofuge 15) for 40 s at 4°C. The supernatant was removed by aspiration and the pellet washed with 100 µl of ethanol. The DNA was air-dried and resuspended in 10 µl of TE (10 mM Tris-HCl [pH 8.0], 0.1 mM diethylamine hydrochloride and 1 mM EDTA) at sterile 37°C.

**Figure 2.1.** Plasmid maps. (A) pECFP-C1 (B) pEGFP-N1 (C) pECFP-1



### **2.1.2 DNA precipitation**

DNA was precipitated by the addition of 0.1 volumes of 3M NaOAc, pH 5.5 and 3 volumes of absolute ethanol. The DNA was collected as a pellet by centrifugation at 13,000rpm (Heraeus Biofuge) for 15 min at 4°C. The supernatant was removed by aspiration and the pellet washed with 70% (v/v) ethanol. The DNA was air-dried and resuspended in either TE (10mM Tris-HCl [pH 8.0], 0.1mM diaminoethane-tetra acetic acid [EDTA]) or sterile d.H<sub>2</sub>O.

### **2.1.3 Restriction Endonuclease Digestion of DNA**

The restriction digestions were performed in a final reaction volume of between 20-50µl. The reaction components were as recommended by the enzyme suppliers. The reaction was carried out at 37°C for a minimum of 2 h.

### **2.1.4 Agarose Gel Electrophoresis**

DNA fragments were separated according to molecular weight by electrophoresis through an agarose gel matrix. The required percentage (between 0.8% (w/v) and 1.5% (w/v)) agarose was weighed and added to 1X TAE buffer (40mM Tris base, 20mM NaOAc, 1mM EDTA [pH 7.4]), melted and cooled to 60°C. Ethidium bromide was then added at a concentration of 0.1µg/ml and the agarose poured into a cast which contained a comb to create sample wells. Once set the comb was removed and the agarose gel was submerged into an electrophoresis tank containing 1X TAE buffer. DNA samples were prepared by adding DNA loading buffer (0.5% Orange G, 30% (v/v) glycerol) and separated by running through the gel at 80-100V. The DNA fragments were visualised using a ultra-violet (UV) transilluminator.

### **2.1.5 Extraction and purification of DNA from Agarose gels.**

DNA fragments were separated using agarose gel electrophoresis. The required DNA fragment was visualised using ethidium bromide and a UV transilluminator and excised from the gel using a scalpel blade. The agarose was removed using the QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's protocol.

### **2.1.6 Restriction endonuclease digestion of genomic DNA**

The digests were performed in a total reaction volume of 300µl. This contained DNA, 30µl of the appropriate 10X restriction digestion buffer, 10 units of restriction enzyme, 2.5mM spermidine, and 100µg/ml RNase. The reaction was carried out at 37°C for a minimum of 16 h.

### **2.1.7 Alkaline phosphatase treatment of DNA**

Alkaline phosphatase treatment was used to remove the free phosphate groups from the ends of linearised plasmids. DNA was resuspended in 1X enzyme buffer and 0.5 units of Calf Intestinal Phosphatase (CIP) per  $\mu\text{g}$  of DNA was added to the reaction and incubated for 30 mins at 37°C. The fragment was then gel purified.

### **2.1.8 DNA ligation**

DNA was ligated in a total reaction volume of 20 $\mu\text{l}$ . Linearised vector DNA was used at a 3:1 ratio to insert DNA. The reaction also contained 0.1 volumes of 10X T4 ligase buffer and 1 unit of T4 DNA ligase. The ligation reaction was incubated at 16°C overnight.

### **2.1.9 Preparation of competent *E.coli* (DH5 $\alpha$ ) cells**

A single colony of DH5 $\alpha$  *E.coli* was used to inoculate 5ml Luria Bertani (LB) (10g bactotryptone, 5g bacto yeast extract, 10g NaCl up to 1 litre d.H<sub>2</sub>O and autoclaved) media and grown at 37°C overnight. 1ml of the overnight culture was then used to inoculate 100ml pre-warmed LB which was then grown at 37°C until the OD<sub>600</sub> was equal to 0.2. MgCl<sub>2</sub> was then added to a final concentration of 20mM (2ml of 1M MgCl<sub>2</sub>). The cells were then left to grow to an OD<sub>600</sub> = 0.45-0.55 and were decanted into 50ml falcon tubes and incubated on ice for 2hrs. The cells were pelleted by centrifugation at 4000rpm for 5min, the supernatant discarded, the pellet gently re-suspended in 50ml prechilled Ca<sup>2+</sup>Mn<sup>2+</sup> solution (40mM NaOAc.3H<sub>2</sub>O, 100mM CaCl<sub>2</sub>.6H<sub>2</sub>O, 70mM MnCl<sub>2</sub>.4H<sub>2</sub>O, pH5.5) and the cells incubated on ice for 45min. The cells were pelleted at 4000rpm for 5min, the supernatant discarded and the pellet gently resuspended in 5ml Ca<sup>2+</sup>Mn<sup>2+</sup> solution containing 15% (v/v) glycerol. The cells were aliquoted (200 $\mu\text{l}$ ) quickly into prechilled eppendorf tubes and flash frozen in liquid nitrogen. Competent cells were stored at -80°C.

### **2.1.10 Bacterial transformation of plasmid DNA**

Competent DH5 $\alpha$  (50 $\mu\text{l}$ ) were thawed on ice and mixed with 5 $\mu\text{l}$  of ligation reaction in a polypropylene tube and incubated on ice for 30mins. The bacteria were then heat shocked for 45sec at 42°C and transferred back to ice for 1min. LB medium (900 $\mu\text{l}$ ) was added and the reaction incubated in a shaking incubator at 37°C for 1 hour. Next, 200 $\mu\text{l}$  was spread onto an LB agar (LB with 15g agar up to 1 litre d.H<sub>2</sub>O and autoclaved) plate containing 50 $\mu\text{g}/\text{ml}$  ampicillin (or appropriate antibiotic). The plate was then inverted and incubated overnight at 37°C to allow the formation of bacterial colonies.

### **2.1.11 Minipreparation of plasmid DNA**

A bacterial colony was picked for screening using a toothpick/sterile yellow tip into a 5ml LB

culture containing appropriate antibiotic and grown at 37°C in a shaking incubator at 37°C overnight. 1.5ml of the culture was then transferred to an eppendorf tube and centrifuged (Heraeus Biofuge) at 13000rpm, 4°C for 30 seconds. The supernatant was aspirated and discarded and the bacterial pellet re-suspended by vortexing in 100µl ice cold Buffer P1 (50mM Tris-HCl [pH 8.0], 10mM EDTA, 100µg/ml ribonuclease [RNase]). 200µl of Solution 2 (200mM NaOH, 1 % (w/v) sodium dodecyl sulphate [SDS]) was added and the tube inverted to mix followed by incubation at room temperature to allow lysis to occur. 150µl of Solution 3 (3M KOAc [pH5.5]) was added and the tubes inverted five times to neutralise. The unwanted cell debris was removed by centrifugation at 13000rpm at 4°C for 10min. The supernatant was transferred to a fresh tube and the DNA precipitated by centrifugation at 13000rpm for 10mins after the addition of 400µl ice-cold isopropanol. The supernatant was discarded and the DNA pellet washed with 70% (v/v) ethanol and air-dried. The pellet was then re-suspended in 50µl TE and stored at 4°C until diagnostic restriction digests could be performed to identify plasmids with the correct insert.

#### **2.1.12 Caesium chloride preparation of plasmid DNA**

The remainder of the overnight culture from the small scale prep of plasmid DNA was used to inoculate 400ml of LB plus ampicillin (or appropriate antibiotic) and incubated at 37°C in a shaking incubator overnight. The overnight culture was then centrifuged at 6000rpm (Sorvall evolution RC) for 10min at 4°C and the pellet resuspended in 10ml buffer P1. Freshly prepared buffer P2 was added and the mixture incubated on ice for 5min to lyse the cells. Neutralisation was then performed by the addition of 15ml buffer P3 and a further 5min incubation on ice. Following mixing, the precipitate was removed by centrifugation at 9000rpm (Sorvall evolution RC) for 15min at 4°C and the supernatant transferred into a clean tube. The DNA was precipitated by the addition of 50ml ice cold isopropanol and centrifugation at 9000rpm (Sorvall evolution RC) for 15min. The DNA pellet was air-dried and resuspended in 6ml TE and transferred to a 12ml centrifuge tube. To the 12ml tube, 6.0g of CsCl<sub>2</sub> and 550µl of 5mg/ml ethidium bromide was added and dissolved before centrifuging at 4000rpm (Eppendorf 5810R) for 5min. The solution was then transferred to two Beckman Quickseal centrifugation tubes and heat-sealed. The tubes were centrifuged at 100,000rpm for 12-16h overnight at 20°C (Beckman Optima ultracentrifuge). The following day the lower plasmid band was removed using a 21G needle and the ethidium bromide extracted 3-4 times using equal volumes of water-saturated isobutanol. The DNA was then precipitated using 2 volumes of water and 2 volumes of absolute ethanol and centrifuged at 11000rpm (Sorvall evolution RC) for 15min. The DNA pellet was washed using 70% (v/v) ethanol and air-dried before being re-suspended in 500µl TE. Diagnostic restriction digests were then performed to check the DNA and the concentration measured

by optical density at 260nm, assuming that 1 absorbance unit = 50µg/µl DNA.

### **2.1.13 Maxipreparation of Endotoxin free DNA**

100ml of LB media was inoculated with bacteria and cultured overnight at 37°C with shaking at 300rpm. The plasmid endotoxin-free maxi kit (Qiagen) was used to purify the plasmid following the manufacturer's protocol. Any glassware used in the procedure was baked overnight at 180°C to destroy endotoxins. Pyrogen-free plasticware was also used.

### **2.1.14 Generation of Transgenic mice**

Plasmid DNA was prepared using an endotoxin-free maxi kit (Qiagen) (see **2.1.13**). Restriction endonuclease digestion and sequence analysis was performed to ensure the correct plasmid had been purified. Removal of the plasmid backbone using restriction endonuclease digestion was then performed and the DNA injected into mouse oocytes by the Transgenic Unit, University of Leicester, following Home Office regulations under Project License number: 80/1899.

### **2.1.15 DNA sequencing and primer design**

All sequencing was performed by the University of Leicester Protein and Nucleic Acid Laboratory (PNAACL). Sequencing was analysed using the program Chromas v1.45. All primers were designed using the Primer 3 program ([http://frodo.wi.mit.edu/cgi-bin/primer3/primer3\\_www.cgi](http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi)) and synthesised by Invitrogen.

### **2.1.16 Genomic DNA isolation from mouse ears/tails for genotyping analysis**

70µl 0.05M NaOH was added to 2mm ear/tail snips and incubated for 15min at 95°C. Next 7µl 1M Tris buffer was added and the samples were ready for analysis.

### **2.1.17 Genomic DNA isolation from ES cells**

ES cells were grown until confluent on 12 well plates. The media was removed and the cells washed in phosphate buffered saline (PBS; 137mM NaCl, 8.1mM Na<sub>2</sub>HPO<sub>4</sub>, 2.7mM KCl, 1.5mM KH<sub>2</sub>PO<sub>4</sub> and autoclaved). The cells were lysed in 500µl of DNA lysis buffer (50mM Tris-HCl [pH 7.6], 1mM EDTA, 100mM NaCl, 0.2% (w/v) SDS, 100µg/ml proteinase K), per well at 37°C overnight. The DNA was spooled into a tube containing 2 volumes of absolute ethanol and 0.3 volumes of sodium acetate and precipitated by centrifuging at 13000rpm for 1min. The DNA pellet was then washed using 70% (v/v) ethanol and resuspended in an appropriate volume of TE.

### **2.1.18 Southern analysis**

The DNA fragments were separated on a 0.8% (w/v) agarose gel by electrophoresis at 20V overnight. The gel was post-stained in ethidium bromide at a concentration of 0.1µg/ml. The DNA was visualised on a UV transilluminator and the gel nicked near the size markers for orientation. The gel was then soaked in 0.25M HCl for 5 min to depurinate the DNA, rinsed in sterile d.H<sub>2</sub>O, and soaked in 0.4M NaOH for 30mins. The DNA was transferred onto Zeta-Probe GT membrane (Bio-Rad) by capillary action. This was achieved using the following transfer apparatus set-up; sponges pre-rinsed in d.H<sub>2</sub>O were placed into a tray and soaked in 0.4M NaOH transfer buffer. A thick piece of gel blot paper bigger than the area of the gel pre-soaked in 0.4M NaOH was placed on top of the sponges, the inverted gel was then placed on top of the paper and any air-bubbles removed. Zeta probe membrane cut to the same size as the gel was placed on top of the gel followed by three more sheets of gel blot paper. A stack of paper towels and a weight to aid capillary action was placed onto the stack. More transfer buffer was added and any further air-bubbles removed. Saran wrap was placed over the apparatus to avoid evaporation of the transfer buffer. Transfer was left to occur for a minimum of 12 hours. Once transfer had occurred the markers that were nicked in the gel were copied onto the corresponding place on the membrane using a pencil. The membrane was then rinsed in 2XSSC (diluted from a 20X stock; 3M NaCl, 0.3M trisodium citrate [pH 7.0]) and baked for 2 hours at 80°C. The membrane was then ready for hybridisation.

### **2.1.19 Preparation of P<sup>32</sup> labelled DNA probes, hybridisation and post-hybridisation washing process**

Radioactive probes were used to identify transgene copy number in DNA extracted from mice as well as ES cell clones. The DNA to be used as probe (25-50ng in a reaction volume of no more than 45µl) was denatured for 5mins at 95°C then immediately placed on ice for 2 minutes, centrifuged briefly then added to a DNA labelling bead (Ready-To-Go DNA Labelling Beads (-dCTP) Amersham Biosciences). After the labelling bead dissolved, 5µl [ $\alpha$ -P<sup>32</sup>] dCTP (Amersham Biosciences) was added and the reaction mix incubated at 37°C for 30 minutes. The probe was then purified through a G-50 microcolumn (ProbeQuant, G-50 microcolumns, Amersham Bioscience) to remove unincorporated nucleotides. The labelled DNA probe was denatured at 95°C for 10 minutes before addition to the Zeta-Probe membrane that had been pre-hybridised with 20ml Church buffer (0.5M sodium phosphate [pH7.2], 7% (w/v) SDS, 0.5M EDTA [pH 8.0]) for a minimum of 3hrs at 65°C, then 13mls of fresh Church buffer added prior to addition of probe. Hybridisation occurred overnight at 65°C in a rotating oven (Shake n Stack, Hybaid). Post hybridisation, the blot was washed 5 times with 50ml 0.2XSSC/0.1% SDS at 65°C. The membrane was exposed to film (Kodak)

for various times required to produce the correct band intensity at  $-80^{\circ}\text{C}$  in the presence of an intensifying screen.

#### **2.1.20 Stripping Southern blots for re-probing**

Stripping buffer (0.1 X SSC, 0.5 X SDS) was boiled, poured over the blot and left to cool at room temperature for approximately 30mins. This was repeated then the filter washed with 2X SSC, before being pre hybridised and hybridised as normal.

#### **2.1.21 Isolation of RNA from cells**

Cells were homogenised using a QIAshredder column (QIAGEN RNeasy plant mini kit). Total RNA was isolated using the RNeasy total RNA purification kit and spin protocol (QIAGEN).

#### **2.1.22 Isolation of RNA from mouse tissue**

Tissue was excised from the animal and if not used immediately stabilised in RNAlater (Qiagen) and stored at  $-80^{\circ}\text{C}$  for long term storage. A maximum amount of 30mg of tissue was homogenised using a rotor-stator homogenizer and RNA extracted using the RNeasy mini kit and protocol (QIAGEN). The amount of RNA extracted from each tissue was determined using a spectrophotometer (Biophotometer, Eppendorf).

#### **2.1.23 Reverse Transcriptase Polymerase Chain Reaction.**

The amount of RNA used in the reverse transcription reaction was normalised so that equal amounts of RNA from each tissue was used in the reaction. The appropriate amount of RNA, 1 $\mu\text{l}$  oligo dT primer and  $\text{H}_2\text{O}$  up to a total volume of 13 $\mu\text{l}$  was incubated at  $70^{\circ}\text{C}$  for 5min, then placed on ice. The following components were then added; 1 $\mu\text{l}$  ribonuclease inhibitor, 5 $\mu\text{l}$  5X transcription reaction buffer, 1 $\mu\text{l}$  dNTPs, 2.5 $\mu\text{l}$   $\text{MgCl}_2$  and 3 $\mu\text{l}$  reverse transcriptase (not in control). The transcription reaction was carried out at  $42^{\circ}\text{C}$  in the presence or absence (control) of an appropriate reverse transcriptase. 1 $\mu\text{l}$  of cDNA was then used in a Polymerase Chain Reaction (PCR) reaction.

#### **2.1.24 Standard Polymerase Chain Reaction (PCR)**

Standard PCR was performed in a final reaction volume of 10 $\mu\text{l}$  containing 8 $\mu\text{l}$  ReddyMix (ABgene) and 1 $\mu\text{l}$  of 20pm/ $\mu\text{l}$  of each forward and reverse primer. The samples were overlaid with a drop of mineral oil to prevent evaporation of the sample. The reaction was performed in a G-Storm PCR machine (GeneTechnologies Ltd). Standard PCR conditions were as follows;

DNA was denatured at 95°C for 5 minutes followed by 35 cycles of the following three steps;

**Step 1** 94°C for 1min

**Step 2** X°C for 1min (see Table 2.1)

**Step 3** 72°C for 1min per kb of product

Final extension at 72°C for 10min.

Primer Name	Sequence 5'-3'	Annealing temperature
mTertpromfor	GCCAGTGGAGCCAAGCTTCCAGCAAACCAGTTAG	64°C
mTertpromrev	GCGCATCCGCACACTAGGCATTGGGCAACCA	64°C
mTertpromforscreen	ATCCCGGCCTTGAGCACAAT	60°C
mTertpromrevscreen	GGACACGCTGAACTTGTGGC	60°C
mTertpromforprobe	GAAAGCTGAAGGCACCAAAG	60°C
mTertpromrevprobe	TTGTGGTGC GACCATACT	60°C
93	GGCGTTCTCTTTGGAAAGGTGTTT	62°C
94	CTCGAACCACATCCTTCTT	62°C
CFP-1	TGAACCGCATCGAGCTGAAGGG	64°C
CFP-2	TCCAGCAGGACCATGTGATCGC	64°C
CFP-3	CCT ACGGCAAGCTGACCCTGA	57°C
CFP-4	ACGCTGCCGTCCTCGATGT	57°C
mTertRTPCRfor	ATGGCGTTCCTGAGTATGGGTGC	55°C
mTertRTPCRrev	ACTTCAACCGCAAGACCGACAGG	55°C
OCP174	AGGTCGGTGTGAACGGATTTG	55°C
OCP175	TGTAGACCATGTAGTTGAGGTCA	55°C
NeoF1	TTTGTCAAGACCGACCTGTC	50°C
NeoR1	TCAAGAAGGCGATAGAAGGC	50°C

**Table 2.1** A list of all primers used and their corresponding annealing temperatures.

### 2.1.25 Real-time PCR (qPCR)

cDNA was prepared as outlined in 2.1.23. qPCR was performed in a final reaction volume of 25µl per well. All samples were analysed in triplicate. The reaction was composed of 12.5µl of SYBR Green (Bio-rad), 300nm of each primer, appropriate concentration of cDNA (usually 1µl) and distilled water up to final volume. The reaction was performed using the MiniOpticon System (Bio-rad). The reaction conditions varied according to samples but the following template was followed:

**Step 1** Incubate 95°C for 5min

**Step 2** Incubate 95°C for 20sec

**Step 3** Incubate 60°C for 1min

**Step 4** PLATE READ

**Step 5** Go to step 2, 39 more times.

**Step 6** Melting curve from 55°C - 95°C, read every 1.0°C, hold for 1sec.

END

## **2.2 Cell Culture**

All cell culture reagents were supplied by GIBCO and plasticware supplied by Helena Biosciences unless otherwise stated.

### **2.2.1 General cell maintenance**

#### **ES media**

For 500ml; Dulbecco's modified Eagle's medium (DMEM) plus L-Glutamine, 4500mg/Lglucose

15 % (v/v) Fetal calf serum (FCS)

20 mM L-Glutamine

10 mM Sodium Pyruvate

1 mM Non-essential amino acids

100 U/ml penicillin

100 µg/ml streptomycin

115 µM β-mercaptoethanol.

Leukaemia Inhibitory Factor (LIF) at a final concentration of 1000u/ml, provided by S.Munsen, Geneta, University of Leicester.

#### **Media used in the maintenance of NIH 3T3 cells and MEF feeders**

For 500ml; DMEM (4500mg/l glucose)

15 % (v/v) FCS

200 mM L-Glutamine

100 U/ml penicillin

100 µg/ml streptomycin

### **2.2.2 Thawing of cells**

Cells were thawed quickly in cryo-vials at 37°C then transferred to 10ml growth media and centrifuged at 1500rpm for 2min. The supernatant was then removed and discarded and the pellet resuspended in fresh growth media and transferred to a 6cm plate containing an MEF feeder layer if required. Cells were then left to adhere at 37°C and 5% CO<sub>2</sub>.

### **2.2.3 Freezing of cells**

Confluent cells were trypsinised and then transferred to a 12ml centrifuge tube and pelleted at 1250rpm for 4min (Eppendorf 5810R). The pellet was then re-suspended in freezing mix

(10% dimethylsulphoxide [DMSO] (v/v)) in complete medium and 0.5ml transferred to pre-labelled cryo-vials. The cells were frozen slowly in freezing chambers at  $-80^{\circ}\text{C}$  and then transferred to liquid nitrogen for long-term storage.

#### **2.2.4 Maintenance of NIH 3T3 cells and MEFs**

Cells were maintained at  $37^{\circ}\text{C}$  and 10%  $\text{CO}_2$ . Cells were passaged when confluent by aspiration of the media, washing with PBS, followed by a 5min incubation at  $37^{\circ}\text{C}$  in an appropriate volume of 0.5mg/ml trypsin to achieve a single cell suspension. Addition of complete media neutralised the trypsin.

#### **2.2.5 Nucleofection of NIH3T3 cells**

NIH3T3 cells were harvested by trypsinisation, washed with PBS and counted to determine cell density. The required number of cells ( $2 \times 10^5$ - $2 \times 10^6$  per nucleofection sample) were pelleted by centrifugation at 200xg for 10min. The pellet was then resuspended in room temperature Nucleofector Solution as recommended by the manufacturer (Amaxa), to a final concentration of  $2 \times 10^5$ - $2 \times 10^6$  cells per 100 $\mu\text{l}$ . The cell suspension was then mixed with 5-10 $\mu\text{g}$  caesium chloride prepared DNA and transferred into a cuvette. The cuvette was inserted into the Nucleofector and the appropriate program selected. After nucleofection the cuvette was immediately removed and 500 $\mu\text{l}$  of pre-warmed media added. The cells were removed using a plastic pipette provided by the manufacturer into a 6-well plate containing more pre-warmed complete media and incubated at  $37^{\circ}\text{C}$  and 10% $\text{CO}_2$ .

#### **2.2.6 Maintenance of ES cells**

E14.1a ES cell lines were maintained at  $37^{\circ}\text{C}$  and 10%  $\text{CO}_2$  in a humidifying chamber. Cells were grown on tissue culture plates pre-coated with a 0.1% (v/v) gelatin layer and a mitomycin-C treated MEF feeder layer. When 70% confluent, the cells were passaged by aspiration of the media, washed with PBS, followed by a 5 min incubation at  $37^{\circ}\text{C}$  in an appropriate volume of 0.5mg/ml trypsin to achieve a single cell suspension. The trypsin was then neutralised by addition of an equal volume of complete media.

#### **2.2.7 Electroporation of ES cells**

ES cells ( $5 \times 10^7$  –  $1 \times 10^8$ ) were harvested by trypsinisation and centrifuged at 1300rpm (Eppendorf 5810R) for 4min, washed twice in PBS and finally resuspended in a total volume of 1.6ml of PBS. 20 $\mu\text{g}$  of caesium chloride/endotoxin-free prepared DNA was added to the cells and then the mixture was divided between two electroporation cuvettes. The cells were electroporated with a Bio-Rad Gene Pulser at a capacitance of 500 $\mu\text{F}$  and 0.25V. After electroporation the cells were left to stand in the cuvette for a few minutes at room

temperature before being transferred to 10ml of ES media and plated onto an appropriate number of plates containing mitomycin C treated MEFs. The cells were left overnight in a 37°C incubator to recover. The following day the media was removed and the cells were washed in PBS. Selective ES media (15ml) was added containing 250µg/ml of G418 (or other appropriate selection). Fresh selective media was added to the cells every day until ES clones started to appear (7-10 days). The ES clones were washed using PBS and picked in 12ml of PBS using a pipette. Each clone was picked into 30µl of 0.5mg/ml trypsin in a 96 well tissue culture plate and incubated at 37°C for 30min before being transferred to a 48 well tissue culture plate previously coated with 0.1% (w/v) gelatin and mitomycin C treated MEFs and containing ES cell complete media. The colonies were propagated until 60% confluent, at which point individual clones were frozen down and also grown for DNA isolation.

### **2.2.8 Freezing ES cell clones**

The ES clones in 48 well tissue culture plates once confluent were washed using PBS and trypsinised using 80µl 1X trypsin at 37°C for 5min. The cells were mixed by gently pipetting until a single cell suspension was achieved, followed by inactivation of the trypsin by addition of 80µl Fetal Calf Serum (FCS). 100µl of this mixture was then transferred to a well on a 96 well plate for freezing, whilst the other 60µl was transferred to a 12 well plate to grow for DNA isolation. To the 96 well plate, 100µl of 2X freezing mix (1ml DMSO + 4ml ES media) was added and mixed and the 96 well plate sealed well and frozen in polystyrene boxes at -80°C.

### **2.2.9 Generation of primary MEFs from E14.5 mouse embryos**

Embryos were harvested and embryonic liver removed. The embryo dissections were performed by S.Giblett, Department of Biochemistry, University of Leicester. The remaining tissue was sliced with a scalpel and forceps, collected and placed in 5ml PBS in a 15ml centrifuge tube. The tissue was allowed to settle to the bottom of the tube and the excess PBS removed. 2ml of 0.25% (v/v) trypsin was added and the tissue incubated at 4°C for 6-18hrs. The tissue was transferred for a further incubation at 37°C for 20-30mins before being centrifuged at 1250rpm for 5mins. The trypsin was aspirated and the pellet resuspended in 5ml complete media. The cells were transferred to a 10cm Petri dish and a further 5ml of complete media added. The cells were incubated at 37°C until confluent.

### **2.2.10 Treating primary MEFs with mitomycin C**

Primary MEFs were generated as in 2.2.9. When the required number of plates were confluent, the media was removed and replaced with media containing mitomycin C at a

final concentration of 2µg/ml. After 2-3hrs incubation, the cells were washed with PBS, harvested by trypsinisation and counted using a haemocytometer. The MEFs were then either aliquoted and frozen down or plated out on the appropriate tissue culture dish coated with 0.1% (w/v) gelatin.

Plate size	Numer of MEFs
10 cm	$1 \times 10^6$
6 cm	$6 \times 10^5$
6-well	$4 \times 10^5$
12-well	$2.5 \times 10^5$
24-well	$1 \times 10^5$
48-well	$4 \times 10^4$

**Table 2.2** Number of Mitomycin C treated MEFs to be plated out on different sized tissue culture plates in order to give the correct density monolayer.

### **2.2.11 ES cell differentiation into EBs**

A 10X concentrated stock of Poly (2-hydroxyethylmethacrylate) or Poly (HEMA) was made by dissolving 6g in 50ml 95% ethanol overnight at room temperature. Any undissolved material was removed by centrifugation and the stock diluted 1 in 10 with 95% ethanol. Each well of a 24 well tissue culture plate was coated using 200µl of the diluted polyHEMA and left to dry for 48 hours at 37°C. Once coated the plates could be stored at room temperature indefinitely. Before use the wells were washed with PBS. ES cells were trypsinised and seeded onto the plate at a density of  $5 \times 10^4$  cells/well in 1ml of media plus LIF. The following day 1ml of media minus LIF is added to each well. On the third day, the plate was tilted to allow the forming embryoid bodies to sink to the front of the well, and then most of the media was aspirated leaving approximately 100µl of media containing the embryoid bodies. On subsequent days feeding the embryoid bodies with 1ml of media minus LIF was alternated with aspiration and media change. Embryoid bodies formed after approximately 8 days of culture.

### **2.2.12 Differentiated ES cell culture**

Firstly, the embryoid bodies were transferred to a 0.1% (w/v) gelatin-coated 10cm tissue culture plate and allowed to attach and spread. For long-term maintenance the embryoid bodies were disaggregated using 1X trypsin and cultured on gelatin coated tissue culture dishes using ES cell media minus LIF.

### **2.2.13 Teratoma formation**

ES cells were cultured, washed with PBS and trypsinised. A total volume of 100µl at a density of  $5 \times 10^6$  cells was injected subcutaneously into the flank of nude mice following

Home Office regulations under Project License number: 80/1899. The formation of the teratoma was monitored and harvested from the animal if it reached more than 10% of the animals total body weight. Upon harvesting the teratomas were fixed in 4% (w/v) PFA, embedded in paraffin and 5µm sections were prepared for analysis.

#### **2.2.14 Indirect Immunofluorescence of ES cells and differentiated ES cells.**

Cells were grown on sterilised coverslips coated with 0.1% (w/v) gelatin in 8-well plates at an approximate density of  $5 \times 10^4$  cells. The cells were washed and fixed using 2% (w/v) PFA for 10mins at room temperature. Cells were permeabilised using 0.2% (v/v) Triton-X-100 for 2-5min and blocked in 1%BSA/PBS overnight. The cells were then incubated with the primary antibody for one hour at room temperature. After washing the cells were incubated with a fluorescent-conjugated secondary antibody, and counterstained with 4'-6 diamidino-2 phenylindole (Dapi) (Molecular Probes). The coverslips were mounted on slides using an antifade reagent (Biomedica) and viewed using a fluorescence microscope Eclipse TE300 (Nikon).

### **2.3 Histology**

#### **2.3.1 Preparation of tissue for histology**

The required tissue was dissected from an animal, rinsed in PBS and fixed in 4% (w/v) PFA overnight at room temperature with shaking. Tissue pieces 2-3mm in thickness were fixed in approximately 20 X volume of fixative. When fixed the tissue was transferred to 70% (v/v) ethanol and stored at 4°C until processed.

#### **2.3.2 Tissue processing**

Tissues were impregnated with wax using a Shandon Citadel 2000 following the manufacturer's protocol. Once processed the tissues were embedded in a wax block.

#### **2.3.3 Preparation of slides to allow wax sections to adhere**

Uncoated microscope slides (Raymond Lamb) were placed into metal or plastic racks and soaked overnight in 5% (v/v) Decon. The slides were then washed in hot running H<sub>2</sub>O for 30mins followed by rinsing in Milli-Q H<sub>2</sub>O five times for five minutes each and dried in the oven for at least 30mins. The slides were submerged for one minute in subbing solution (2% 3-aminopropyltriethoxysilane in acetone) followed by immersion in two lots of acetone and washed in two tanks of Milli- Q H<sub>2</sub>O. The slides were then dried in the oven and replaced in boxes ready for use.

### 2.3.4 Immunohistochemical analysis of paraffin sections

Primary antibody	Dilution	Company
Oct-4 (goat polyclonal)	1:200-1:400	Santa-Cruz
GFP (rabbit polyclonal)	1:200-1:600	Molecular Probes
Cytokeratin-1 (CK-1)	1:200	Abcam
Glial Fibrillar Acid Protein (GFAP)	1:200	Abcam
Ki67	1:200	Neo markers.

**Table 2.3** Primary antibodies and their appropriate dilutions used in immunohistochemical analysis.

5µm paraffin tissue sections were cut using a Leica RM2135 microtome. The majority of sections were cut by S.Giblett, Department of Biochemistry, University of Leicester. Once dry, sections were de-waxed by immersion in xylene for a minimum of 20 min, and then dehydrated through a descending ethanol series. Endogenous peroxidase activity was blocked by incubating the slides for 10 min in 2.4ml of 30% (w/v) hydrogen peroxide in 400ml methanol. Antigen retrieval was achieved by microwaving in 0.01M citrate buffer pH 6 (2.94g trisodium citrate in 1 litre H<sub>2</sub>O, pH adjusted with acetic acid) for 20 min. Slides were washed in water and rinsed in PBS before blocking by incubating with serum (from the animal in which the secondary antibody was raised in) diluted 1:25 in PBS for one hour. The slides were then incubated with the primary antibody (see Table 2.3) diluted as required in serum/PBS at 4°C overnight in a humidifying chamber. The next day the slides were washed in PBS and incubated for one hour in the appropriate biotinylated secondary antibody. The slides were then re-washed in PBS and incubated in Streptavidin-Peroxidase (DakoCytomation) for a minimum of 30 minutes. After further washing, slides were incubated in 3, 3'-diaminobenzidine (DAB) reagent (Vector laboratories) until the colour had developed sufficiently (2-10minutes). The slides were then dehydrated through an ethanol series, cleared by incubation in xylene and mounted in DPX-mountant (Raymond Lamb). The sections were examined using a Leica DMLB light microscope and images were captured using an Imaging for Windows program supplied by the microscope manufacturer.

### 2.3.5 Immunofluorescent analysis of paraffin sections

5µm paraffin sections were cut using a Leica RM2135 microtome. The majority of sections were cut by S.Giblett, Department of Biochemistry, University of Leicester. The sections were dewaxed by immersion in xylene and dehydrated through an ethanol series as in 2.3.4, then rehydrated through the series into water. Slides were microwaved in 0.01M

Citrate buffer for 20 minutes, washed in water then rinsed in PBS followed by incubation for one hour in serum (from the animal the secondary antibody was raised in ) diluted 1:25 in PBS. The slides were then incubated in primary antibody diluted as required in serum/PBS overnight at 4°C in a humidifying chamber. The following day the slides were washed with PBS for one hour with wash changes every ten minutes. The slides were then incubated with a fluorescent-conjugated secondary antibody for two hours at room temperature in the dark. The slides were then washed three times for ten minutes with PBS followed by counterstaining with Dapi (Molecular Probes) at a 1:5000 dilution in PBS. After a further three washes for ten minutes each the slides were mounted using Vectorshield Hardset mountant (Vector Laboratories). The samples were viewed using a Leica confocal microscope.

### **2.3.6 Histological staining of paraffin sections**

#### **i) Haematoxylin and Eosin**

All routine H&E staining was done using a Shandon Varistain staining machine following the manufacturer's instructions.

### 3. Generation of an *mTert* promoter reporter mouse

#### 3.1 Introduction

##### 3.1.1 *Tert* expression is regulated by its promoter

As discussed in chapter 1, *Tert* expression is primarily regulated at the level of the promoter in both mouse and human. The evidence for this comes from the generation of *in vitro* and *in vivo* models using the *Tert* promoter driving expression of a reporter cassette (Armstrong *et al.*, 2000; Pericuesta *et al.*, 2006; Ritz *et al.*, 2005). Initial investigations reported that 5kb upstream of *mTert* was required to recapitulate *mTert* expression and that *mTert* expression correlated with telomerase activity (Armstrong *et al.*, 2000).

##### 3.1.2 *Tert* promoter regulation

There are differences between *hTERT* and *mTert* expression in somatic tissues and this is thought to be due to differences in regulation of the promoters. Promoters are regulated by transcription factors, although the presence of a transcription factor binding site in a potential promoter region does not necessarily indicate functionality. In order to elucidate the mechanisms of regulation of both the human and mouse *Tert* promoters, there have been a number of investigations analysing the presence and function of various transcription factor binding sites and other potential regulatory elements. Many of these investigations employed luciferase reporter assays to evaluate promoter activity. This is a commonly used assay to elucidate promoter function and simply involves the fusion of the promoter region of interest to the luciferase coding region. Thus, luciferase activity acts as a read-out of promoter function.

The transcription factor activator protein 1 (AP1) is a complex of the Jun and Fos family of proteins and its activation is involved in cell proliferation, apoptosis, differentiation and carcinogenesis (Shaulian and Karin, 2002). There are binding sites for AP1 in both the mouse and human *Tert* promoters (Takakura *et al.*, 2005). It has been demonstrated that overexpression of AP1 in cancerous cells results in transcriptional repression of *hTERT* (Takakura *et al.*, 2005). Transcriptional repression of *hTERT* was measured by a decrease in *hTERT* mRNA assessed by RT-PCR and a decrease in telomerase activity assessed by the TRAP assay (Takakura *et al.*, 2005). Mutations of the AP1 sites in the *hTERT* promoter rescued this repression. Conversely, overexpression of AP1 in mouse fibroblasts had no effect upon *mTert* promoter activity, assessed using qRT-PCR and telomerase activity (Takakura *et al.*, 2005). Thus, regulation of the *Tert* promoter by AP1 may provide one explanation for the differences between *mTert* and *hTERT* expression.

Other investigations have uncovered additional promoter elements potentially responsible for the differences between human and mouse *Tert* expression (Armstrong *et al.*, 2004; Horikawa *et al.*, 2005). One such investigation employed site-directed mutations within the conserved and non-conserved potential regulatory elements in both the *mTert* and *hTERT* promoters and used the luciferase assay to assess the effect on promoter function (Horikawa *et al.*, 2005). Using this approach it was found that a non-conserved GC-box within the *hTERT* promoter was responsible for the human-specific repression of *hTERT* (Horikawa *et al.*, 2005). GC boxes are GC-rich regions which act as binding sites for the transcription factor Sp1 (Gidoni *et al.*, 1984; Kadonaga *et al.*, 1987; Kadonaga *et al.*, 1988). Sp1 is a ubiquitous transcription factor whose expression level is regulated during development suggesting that it plays an important role in regulating development and differentiation (Saffer *et al.*, 1991). Transcriptional regulation of the *mTert* promoter by Sp1 has been studied during muscle differentiation (Nozawa *et al.*, 2001). It was found that a core region of the promoter was responsible for the reduction of *mTert* transcription during myogenesis. This region was shown to contain GC boxes which are binding sites for Sp1 and other family members such as Sp3 (Nozawa *et al.*, 2001). The DNA binding activities of Sp1 and Sp3 were shown to be down-regulated during muscle differentiation suggesting that this family is important for activation of *mTert* (Nozawa *et al.*, 2001). A further investigation using electrophoretic mobility shift assays (EMSA) also demonstrated that Sp1 and Sp3 are activators of *mTert* in ES cells. However, during the differentiation of ES cells into EBs the DNA binding activities of these transcription factors were unaltered. Thus, regulation of *mTert* by Sp1 during differentiation seems to be dependent upon cell type. Thus, whether Sp1 and Sp3 are involved in down regulation of *mTert* in ES cells is not clear.

Binding sites for the transcription factor c-Myc have been found in both the human and mouse *Tert* promoters (Armstrong *et al.*, 2004; Nozawa *et al.*, 2001; Wu *et al.*, 1999). This transcription factor has previously been shown to regulate cell proliferation and differentiation (Henriksson and Luscher, 1996). Construction of a luciferase reporter utilising the region of the *hTERT* promoter containing the c-MYC/MAX binding sites showed that c-MYC activates *hTERT* transcription (Wu *et al.*, 1999). Furthermore, co-transfection of 293T cells with the luciferase reporter and a c-MYC expression vector resulted in a four-fold increase in reporter gene activity. Mutation of the c-MYC/MAX binding sites in the reporter ablated the effect on promoter activity (Wu *et al.*, 1999). Thus c-MYC activates *hTERT* expression by binding to the promoter. The investigations into *mTert* transcriptional regulation during differentiation of muscle and ES cells have demonstrated that c-Myc is a transactivator of *mTert* transcription (Armstrong *et al.*, 2004; Nozawa *et al.*, 2001). As with

the Sp transcription factors, c-Myc was shown to be down-regulated during differentiation of muscle, but was unaffected during differentiation of ES cells.

Binding sites for NF- $\kappa$ B have been identified in the human and mouse *Tert* promoter regions (Yin *et al.*, 2000). The *mTert* promoter NF- $\kappa$ B binding site was detected using EMSA. Activation of the *mTert* promoter by this transcription factor was demonstrated using a luciferase reporter assay (Yin *et al.*, 2000). This reporter activation was further stimulated by expression of an NF- $\kappa$ B activator. However, in this investigation no activity for the NF- $\kappa$ B binding site in the *hTERT* promoter could be detected (Yin *et al.*, 2000).

To summarise, both the *mTert* and *hTERT* promoters are regulated by transcription factors. Some of these transcription factor binding sites are conserved between the two species. However, binding of transcription factors to these conserved sites does not always result in similar regulation; they may have species-specific effects.

### **3.1.3 Considerations for the generation of transgenic mice by pronuclear injection**

The generation of transgenic mice by direct DNA injection into 1dpc oocytes was discussed in Chapter 1. This approach results in random integration of the transgene within the mouse genome. Therefore, the site or number of integrations cannot be predicted or controlled. This can be beneficial as multiple copies of a reporter may result in amplification of expression. However, this does not always follow as integration may have occurred within a region of the genome that prevents transgene expression. Thus, integration site and copy number factors can both affect transgene expression. Furthermore, integration can occur before the first cleavage, or after the first cleavage in approximately 20-30% of progeny resulting in mosaics (Wilkie *et al.*, 1986). The result of this is that not all of the cells will carry the transgene and the resulting animal is mosaic. However, many animals are produced from direct DNA injection in a relatively short period of time; therefore the chance of obtaining a transmitting transgenic is relatively high. Transgenic mice can also be generated using ES cells as discussed in Chapter 1. One of the major advantages of this approach is that the ES clones can be well-characterised before being used to generate transgenic animals. Thus, the transgene copy number (if relevant) can be determined along with the expression pattern of the transgene. However, this analysis may take time. Thus, the direct DNA injection approach was chosen as the preferred method in this investigation.

### 3.2 Aims

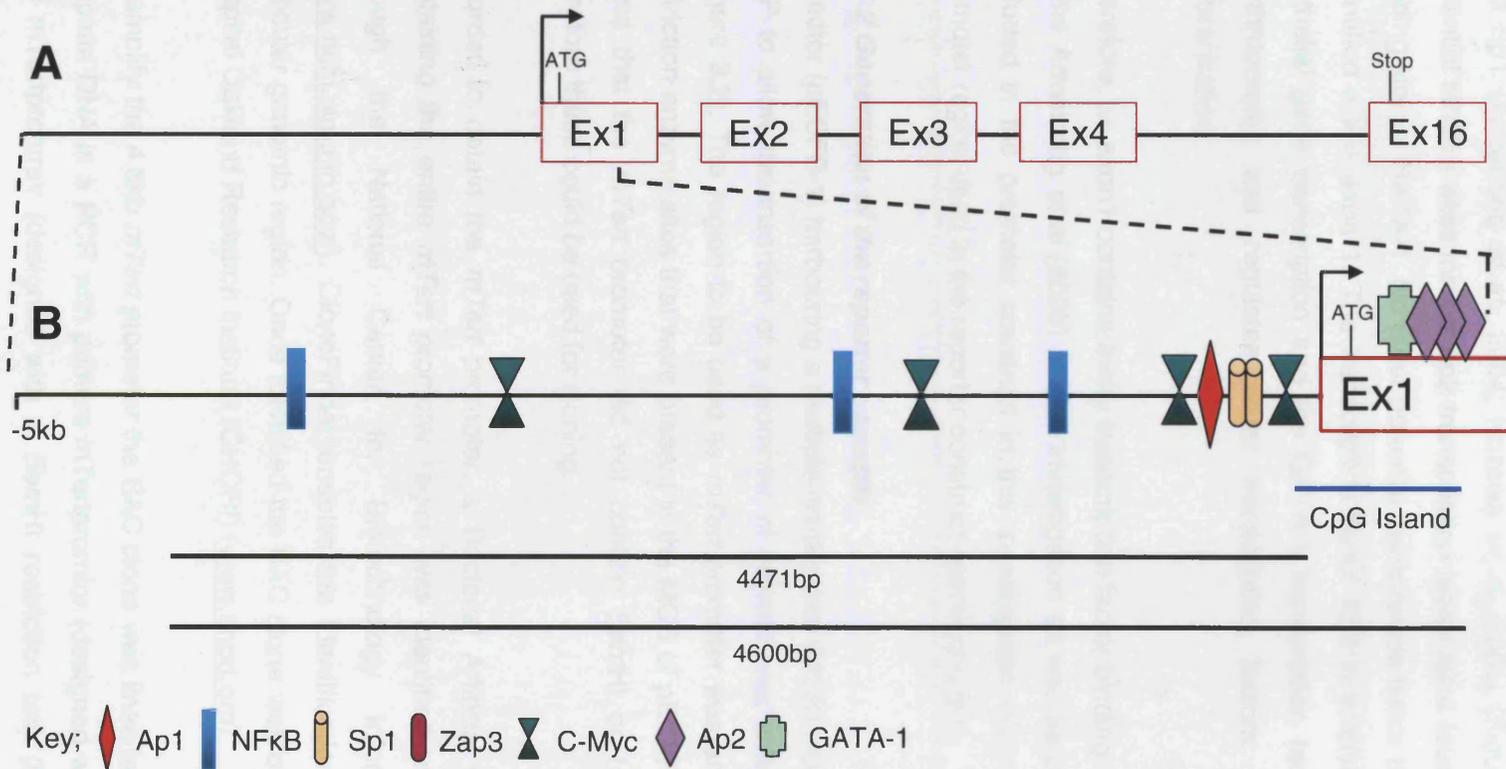
There is a wealth of evidence to suggest that telomerase, through its catalytic component, is important for the proliferation of certain cells including stem cells. *mTert* expression is closely linked to telomerase activity; thus it would be of interest to generate a transgenic reporter mouse using the catalytic component of telomerase. *mTert* is regulated at the level of the promoter and a previous report has generated a reporter cassette consisting of the *mTert* promoter driving GFP expression and shown that this was sufficient to mimic *mTert* expression and telomerase activity *in vitro* (Armstrong *et al.*, 2000). However, at the time of starting the project there had been no reports in the literature of the production of such a reporter mouse. Thus, it was decided that the *mTert* promoter region described by Armstrong *et al.* (2000) would be included in the reporter construct chosen here, but the nucleotide sequence surrounding the putative *mTert* promoter region would be further characterised for any additional potential regulatory elements using computational analysis. The main aim was then to generate a reporter mouse using the *mTert* promoter region driving expression of a cyan fluorescent protein (CFP). A fluorescent reporter was chosen over a gene-based reporter such as the bacterial gene *LacZ*, due to the fact that visualisation of a fluorescent reporter is non-invasive and does not require chromogenic substrates. Furthermore, the fluorescence can be monitored in real-time *in vivo* and thus can allow quantification of expression. The CFP variant was chosen over the GFP variant as it offers the possibility of investigating the overlapping expression of two reporters with overlapping spectra e.g. yellow fluorescent protein (YFP).

### 3.3 Results

#### 3.3.1 *mTert* promoter analysis

The nucleotide sequence of the 5kb *mTert* promoter region and exon 1 of *mTert* were analysed for transcription factor binding sites and CpG islands using publicly available web-based programs. Exon 1 was included in the analysis as core promoter elements often reside within the first exon and therefore it often forms part of the promoter (Werner, 2000). GrailEXP analysis of the promoter nucleotide sequence was performed to identify any potential CpG islands (<http://compbio.ornl.gov/grailexp/>). GrailEXP allows analysis of DNA sequence for specific features such as CpG islands or exon predictions. One potential CpG island was identified using GrailEXP. The island was found to span part of exon 1 of *mTert* including the initiating ATG (Figure 3.1). No other potential CpG islands were identified.

The transcription factor binding sites were analysed using MatInspector, a program available through the Genomatix (Understanding gene regulation) website ([www.genomatix.de](http://www.genomatix.de)) (Cartharius *et al.*, 2005). MatInspector is a web-based program that



**Figure 3.1** *mTert* promoter analysis. **(A)** The *mTert* gene which has 16 exons. Transcription start site and initiating ATG are shown. Region analysed in promoter analysis is indicated by dotted lines. **(B)** The transcription factor binding site analysis undertaken for this investigation using MatInspector. The most relevant sites found in the analysis are shown. The CpG island was identified using GrailEXP. The black lines represent the 4471bp promoter region used by Armstrong et al (2000) and the promoter region used in this investigation.

identifies transcription factor binding sites from inputted nucleotide sequence. The potential transcription factor binding sites identified in the MatInspector analysis included sites previously identified to be important for *mTert* promoter regulation such as c-Myc, NF- $\kappa$ B and Sp1 (Armstrong *et al.*, 2004; Nozawa *et al.*, 2001) (Figure 3.1). Furthermore, three potential binding sites for the Ap2 transcription factor were found in exon 1 along with one binding site for GATA-1. No other potential transcription factor binding sites of interest were identified within exon 1. The transcription factor Ap2 is involved in regulating neural and epithelial gene transcription and the GATA-1 transcription factor is essential for normal erythropoiesis and regulates other transcription factors involved in haemopoietic differentiation.

Therefore, as exon1 contains many transcription factor binding sites, the entire region used in the Armstrong *et al* (2000) *in vitro* investigation as well as part of exon 1 of *mTert* was included in the promoter construct in this investigation (Figure 3.1). In total, the *mTert* promoter region used in the reporter construct spanned 4.6kb.

### **3.3.2 Generation of the reporter cassette.**

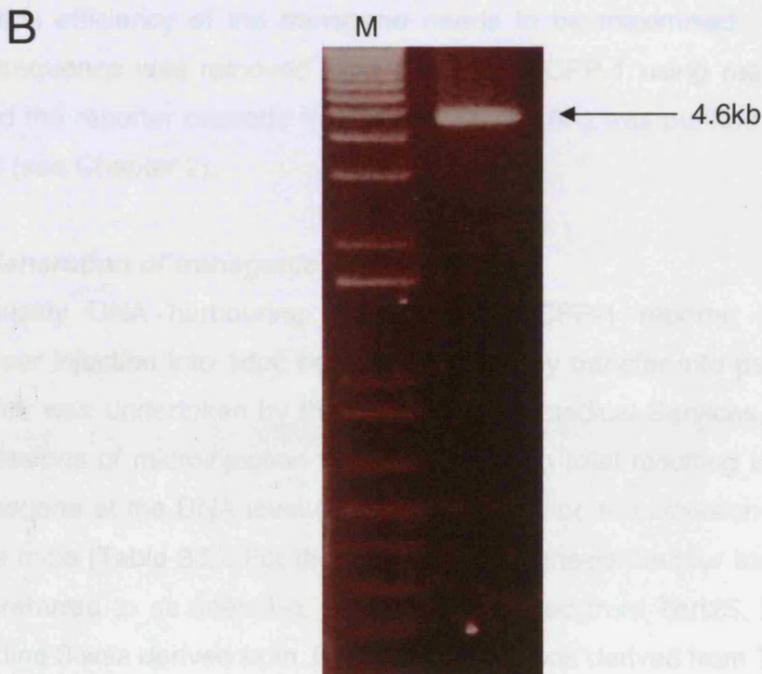
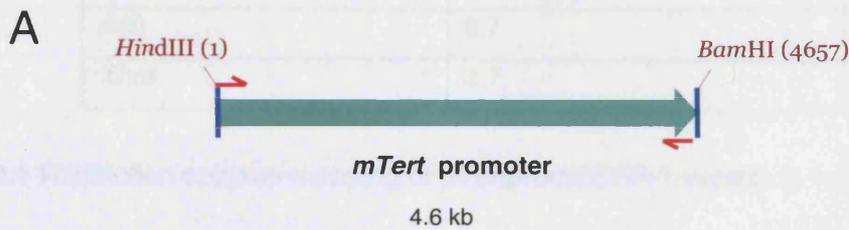
A vector (pECFP-1) harbouring a multiple cloning site (MCS) next to the coding sequence of CFP to allow the insertion of a promoter of interest was obtained from BD Biosciences (Figure 3.2). The region to be used as *mTert* promoter was analysed for the presence of restriction enzyme sites that were present in the MCS of pECFP-1 using Vector NTI. It was found that the *mTert* promoter did not contain *Bam*HI or *Hind*III restriction sites and therefore these could be used for cloning.

In order to obtain the *mTert* promoter, a Bacterial Artificial Chromosome (BAC clone) containing the entire *mTert* promoter region was identified using CloneFinder available through the National Center for Biotechnology Information (NCBI) website ([www.ncbi.nlm.nih.gov/](http://www.ncbi.nlm.nih.gov/)). CloneFinder enables the identification of clones that contain a particular genomic region. Once identified the BAC clone was obtained from the Children's Hospital Oakland Research Institute (CHORI) ([www.chori.org](http://www.chori.org)).

To amplify the 4.6kb *mTert* promoter the BAC clone was linearised using *Xho*I and used as template DNA in a PCR with primers mTertpromfor (designed with a *Hind*III restriction tail) and mTertpromrev (designed with a *Bam*HI restriction tail) (Figure 3.2). The promoter fragment was then isolated by gel purification and ligated into *Bam*HI and *Hind*III digested pECFP-1 by "sticky-end" cloning. Restriction enzyme mapping using several restriction enzymes and sequencing was performed to ensure that the vector obtained was correct

(Figure 3.2). The amplified fragments (1000 bp) are outlined in Table 3.1. The resulting vectors were named mTert-pECFP-1 (Figure 3.2) and mTert-pEGFP-2 (Figure 3.2).

**Figure 3.2** Amplification of *mTert* promoter. **(A)** Diagram of *mTert* promoter region amplified by PCR using primers mTertpromfor (with *Hind*III restriction tail) and mTertpromrev (with *Bam*HI restriction tail) shown in red. Restriction enzyme tails allow cloning into pECFP-1. **(B)** PCR amplification of 4.6kb *mTert* promoter using primers specified above. M = 1kb plus DNA marker



(Figure 3.3). The expected fragment sizes are outlined in Table 3.1. The resulting vector was named pTertpromECFP-1 (Figure 3.3).

Restriction enzyme	Fragments expected (kb)
<i>Nco</i> I	3.5, 2.6, 1.8, 0.7
<i>Sac</i> I	2, 6.7
<i>Hind</i> III	8.7
<i>Not</i> I	8.7
<i>Bam</i> HI/ <i>Hind</i> III	4.6, 4.1
<i>Afl</i> II	8.7
<i>Xho</i> I	8.7

**Table 3.1** Restriction enzyme mapping of pTertpromECFP-1 vector.

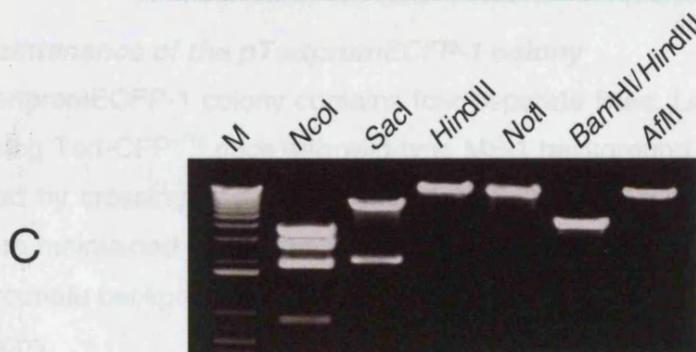
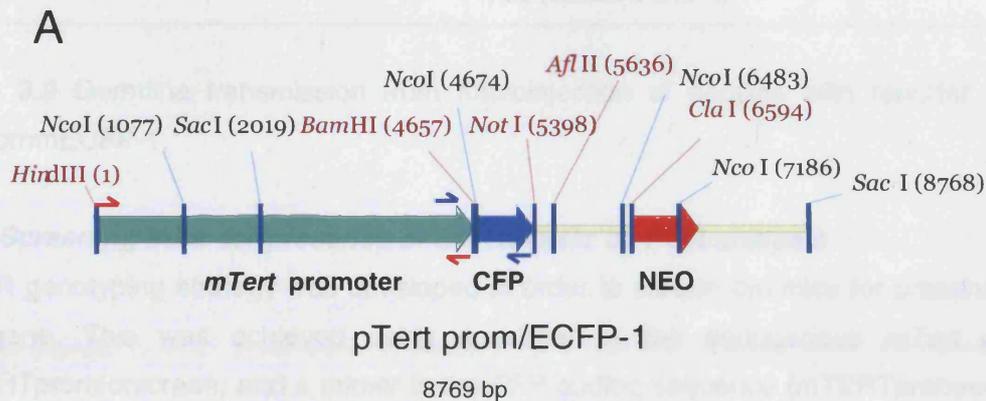
### 3.3.3 Preparation of the DNA for microinjection

In order for the generation of transgenic mice by direct DNA injection into 1dpc oocytes, the integration efficiency of the transgene needs to be maximised. Therefore the backbone vector sequence was removed from pTertpromECFP-1 using restriction sites *Hind*III and *Afl*II and the reporter cassette fragment (Figure 3.6A) was purified using an Endotoxin-free method (see Chapter 2).

### 3.3.4 Generation of transgenic mice

High quality DNA harbouring the pTertpromECFP-1 reporter cassette was used for pronuclear injection into 1dpc oocytes, followed by transfer into pseudo pregnant females. This work was undertaken by the Division of Biomedical Services, University of Leicester. Nine sessions of microinjection were performed in total resulting in seven mice containing the transgene at the DNA level (Table 3.2). Germline transmission was achieved from four of these mice (Table 3.2). For the purpose of this thesis the four transgenic lines generated will be referred to as lines 1-4. Line 1 was derived from Tert25, line 2 was derived from Tert26, line 3 was derived from Tert42 and line 4 was derived from Tert46 (Table 3.2)

**Figure 3.3** (A) Final pTertpromECFP-1 vector. Primers shown in red were those used to amplify the *mTert* promoter. (B) Restriction analysis of bacterial colonies. DNA was linearised using *Xho*I. Colonies identified as containing the transgene can be seen in lanes 8, 10, 11 and 12. (C) Further restriction digestion of no8 confirms that cloning was successful. (D) An example of sequencing which was performed with primers shown in A. M = 1kb plus DNA marker.



This chapter describes the successful generation of pTertpromECFP-1 transgenic mice. As the control transgene, the *lacZ* gene was used. The *lacZ* gene was chosen as an mTert promoter reporter because it has been extensively characterised. However, it had been reported using an *in vitro* reporter system that the region 440T-200bp upstream of the initiating ATG region was sufficient to

Mice with transgene detected by PCR	Germline transmission achieved?
Tert 3	No
Tert 20	No
Tert 25	Yes (created line 1)
Tert 26	Yes (created line 2)
Tert 32	No
Tert 42	Yes (created line 3)
Tert 46	Yes (created line 4)

**Table 3.2** Germline transmission from microinjection of oocytes with reporter cassette pTertpromECFP-1.

### 3.3.5 Screening mice for presence of the reporter by PCR analysis

A PCR genotyping strategy was developed in order to screen the mice for presence of the transgene. This was achieved using a primer in the endogenous *mTert* promoter (mTERTpromforscreen) and a primer in the CFP coding sequence (mTERTpromrevscreen) (Figure 3.4). Thus, if the transgene was present a 300bp PCR product would be amplified (Figure 3.4B). Diluted pTertpromECFP-1 vector was used as a positive control. Since the reverse primer sequence corresponded to the CFP coding sequence, no amplification would occur from endogenous genes. The transgene was detected in all four of the transgenic lines generated. The transgenic mice generated were named Tert-CFP<sup>+Tg</sup>.

### 3.3.6 Maintenance of the pTertpromECFP-1 colony

The pTertpromECFP-1 colony contains four separate lines. Lines 1 and 2 were generated by crossing Tert-CFP<sup>+Tg</sup> mice with wild-type MF-1 background mice and lines 3 and 4 were generated by crossing Tert-CFP<sup>+Tg</sup> mice with wild-type C57BL/6 background mice. The 4 lines were maintained by continual crossing of the Tert-CFP<sup>+Tg</sup> with wild-type animals from the appropriate background (Figure 3.5). Currently the mice have been bred for 4 backcross generations.

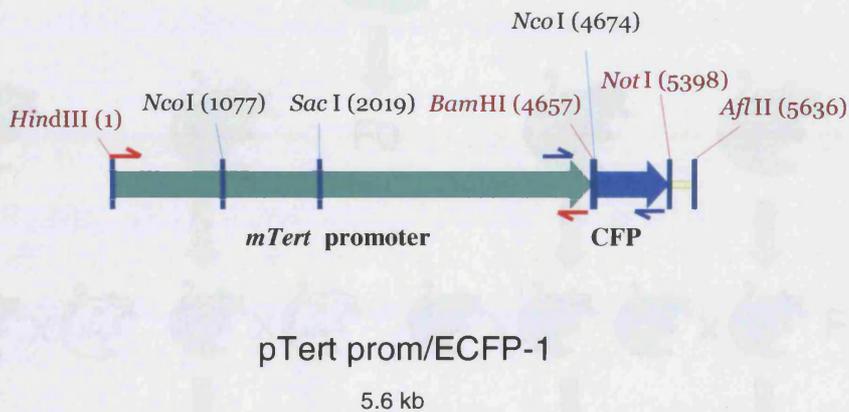
## 3.4 Discussion

This chapter describes the successful generation of pTertpromECFP-1 transgenic mice. At the construct designing stage of the project there had been no reports of an *mTert* promoter reporter mouse model. Indeed, the *mTert* promoter, unlike the *hTERT* promoter had not been extensively characterised. However, it had been reported using an *in vitro* reporter system that the region 4491-20bp upstream of the initiating ATG codon was sufficient to

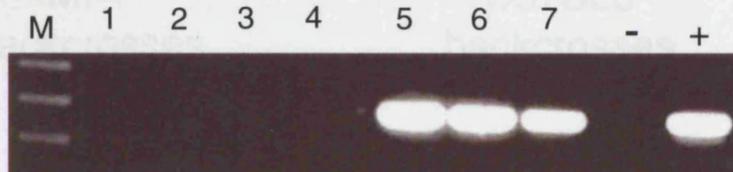
Figure 3.4 Generation of four Tert-CFP transgenic lines by direct DNA

**Figure 3.4 (A)** Restriction map of the region of pTertpromECFP-1 between restriction endonuclease sites *HindIII* and *AflII* used to inject into oocytes to generate transgenic mice. **(B)** Colony genotyping performed using mTertpromforscreen (primer in the endogenous mTert promoter) and mTertpromrevscreen (primer in CFP coding sequence) primers shown in blue, generating a band of 300bp if the transgene is present at the DNA level. 3 mice from line4 (samples 5, 6 and 7) possess the transgene at the DNA level. M= 1kb plus DNA marker, + = positive control, - = negative control.

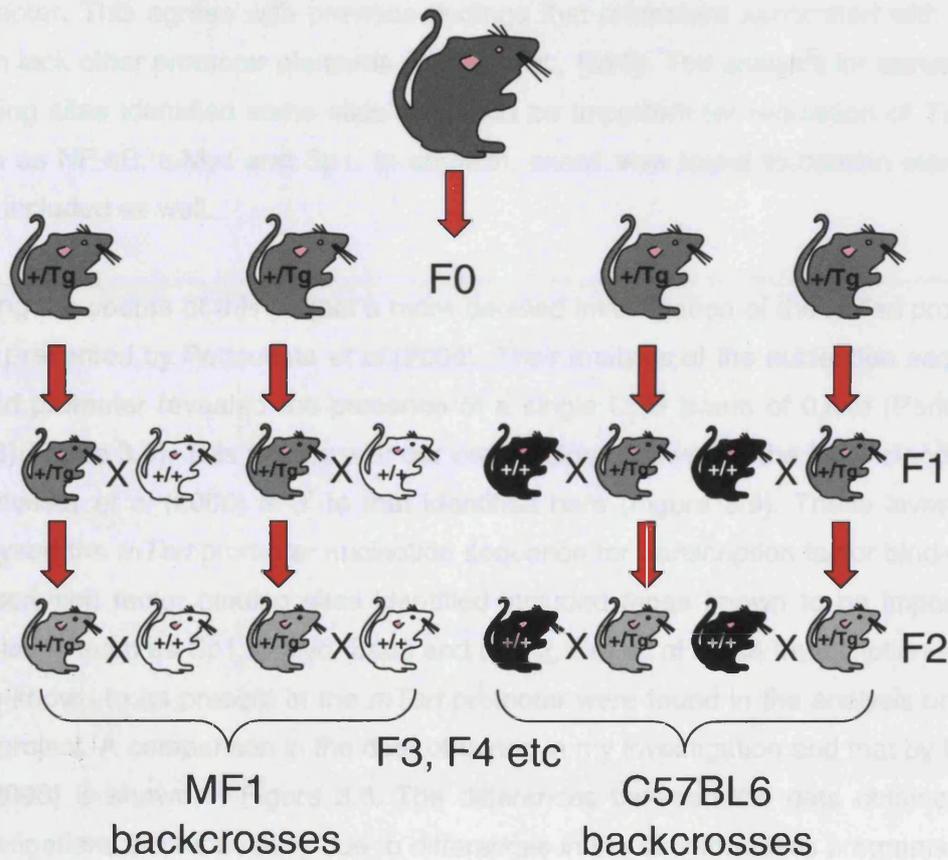
A



B



**Figure 3.5.** Generation of four Tert-CFP transgenic lines by direct DNA microinjection. From the surrogate mother, four transgenic founders (F0) were produced that were capable of transmitting the transgene through the germline. Two of these founders were mated to MF-1 mice (white) and two were mated to C57 mice (black). The heterozygote offspring were continually mated to generate F2, F3 etc generations.

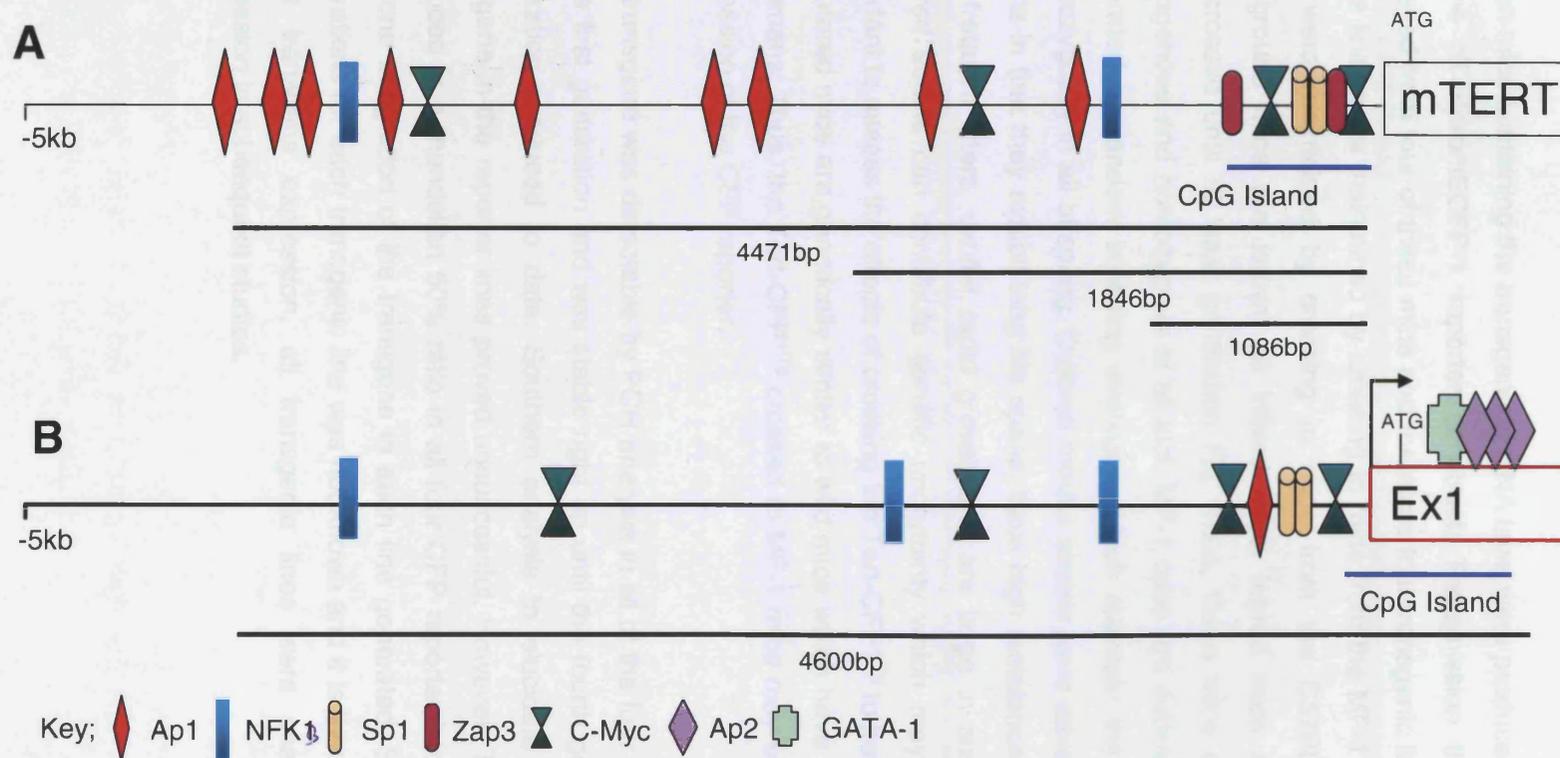


drive *mTert* expression. Armstrong *et al* (2000) used this region to drive expression of GFP and showed that this reporter correlated with *mTert* expression and telomerase activity in ES cells. Moreover, expression of the reporter was lost during differentiation of ES cells. As a result of this finding this region of the promoter was included in the reporter construct used in this investigation.

In this investigation, analysis of exon1 of *mTert* and its promoter had revealed a potential CpG island encompassing the transcription start site and the initiating ATG of *mTert* (Figure 3.1). TATA box analysis revealed that there were no putative TATA boxes in the *mTert* promoter. This agrees with previous findings that promoters associated with CpG islands often lack other promoter elements (Blake *et al.*, 1990). The analysis for transcription factor binding sites identified some sites known to be important for regulation of *Tert* promoters such as NF- $\kappa$ B, c-Myc and Sp1. In addition, exon1 was found to contain elements so this was included as well.

During the course of this project a more detailed investigation of the *mTert* promoter region was presented by Pericuesta *et al* (2006). Their analysis of the nucleotide sequence of the *mTert* promoter revealed the presence of a single CpG island of 0.4kb (Pericuesta *et al.*, 2006)(Figure 3.9). This agrees with our own findings. However, the CpG island identified by Pericuesta *et al* (2006) is 3' to that identified here (Figure 3.9). These investigators also analysed the *mTert* promoter nucleotide sequence for transcription factor binding sites. The transcription factor binding sites identified included those known to be important for *Tert* regulation such as Sp1, c-Myc, Zap3 and MZF2. Not all of these transcription factor binding sites known to be present in the *mTert* promoter were found in the analysis undertaken for this project. A comparison in the data obtained in my investigation and that by Pericuesta *et al* (2006) is shown in Figure 3.6. The differences between the data obtained in the two investigations are most likely due to differences in the bioinformatics programs used for the analysis. Bioinformatics programs assign transcription factor binding sites using various parameters which are unique to each program. Since Pericuesta *et al* (2006) used a different bioinformatics program to determine transcription factor binding sites then that program would have assigned sites on the basis of certain parameters which would be different to those used by the MatInspector program used here. Furthermore, identification of a binding site does not mean that it is a functional site.

Pericuesta *et al* (2006) generated three *in vivo* mouse models using different regions of the *mTert* promoter. The three models consisted of 1kb, 2kb (1.8kb) and 5kb (4.4kb) regions of the *mTert* promoter driving GFP expression (see Chapter 1; Figure 1.16). Analysis of these



**Figure 3.6** Comparison of *mTert* promoters. **(A)** A representation of the transcription factor binding sites identified in the *mTert* promoter region by Pericuesta *et al* (2006). The three regions used to generate the three reporter models are shown. The CpG island identified in their analysis is also shown. **(B)** The transcription factor binding site analysis undertaken for this investigation using MatInspector. The most relevant sites found in the analysis are shown. The CpG island was identified using GrailEXP. The region included in the promoter construct is indicated.

models has revealed that as little as 1kb upstream of the *mTert* gene is sufficient for *mTert* expression and telomerase activity.

Seven mice containing the transgene at DNA level were produced from direct DNA injection of the pTertpromEGFP-1 reporter construct. Transmission through the germline was achieved from four of these mice and therefore four transgenic lines were established. Two of the lines were maintained by crossing to mice from the MF-1 background and the other two were maintained by crossing to mice from the C57BL/6 background. C57BL/6 background mice are known as inbred mice. Inbred mice are mice that have been intercrossed until at least generation F<sub>20</sub>. Thus, these mice are said to be genetically homogenous and homozygous at all loci. MF-1 mice are outbred mice. Outbred mice are generated by random breeding strategies which maintain the maximal level of genetic heterozygosity in all offspring. Outbred mouse strains have advantages over inbred mouse strains in that they exhibit long life spans, have high resistance to disease, produce large and frequent litters, exhibit rapid growth and are large in size. However, random-bred outbred strains can contribute genetic uncertainty which may affect phenotype. It was important to assess the effects of crossing the Tert-CFP<sup>+Tg</sup> to both inbred and outbred mice as outbred mice are genetically similar to wild mice which have telomeres similar in length to humans. Thus, the Tert-CFP<sup>+Tg</sup> crossed to MF-1 mice may exhibit a different pattern of expression of the CFP reporter.

The transgene was detectable by PCR analysis in all of the four transgenic lines generated in the first generation and was stable right up until the fourth generation which is the last generation produced to date. Southern analysis to elucidate the copy number of the transgene in the reporter lines proved unsuccessful. However, Tert-CFP<sup>+Tg</sup> offspring were produced in a mendelian 50% ratio in all four CFP reporter lines implying that there was only one integration of the transgene in each line generated. Since the exact number of integrations for each transgenic line was not known and it is thought that copy number can affect transgene expression, all transgenic lines were characterised for transgene expression in subsequent studies.

## 4. Transgene expression analysis and correlation with the stem cell phenotype *in vitro*.

### 4.1 Introduction

#### 4.1.1 *Tert* expression in ES cells

A number of investigators have shown that ES cells express *mTert* and possess telomerase activity (Armstrong *et al.*, 2000; Liu *et al.*, 2000; Niida *et al.*, 2000; Pericuesta *et al.*, 2006). Analysis of *mTert*<sup>-/-</sup> ES clones has shown that loss of *mTert* results in a loss of telomeric DNA (Liu *et al.*, 2000). Furthermore, late passage *mTert*<sup>-/-</sup> ES clones exhibit aneuploidy and chromosomal end-to-end fusions (Liu *et al.*, 2000). Overexpression of mTert in ES cells bestows a survival advantage upon them and protects from stress-induced apoptosis (Lee *et al.*, 2005). However, overexpression does not affect proliferation, differentiation capacity or differentiation rate (Lee *et al.*, 2005).

Armstrong *et al* (2000) reported the generation of a reporter cassette consisting of a 4.4kb region of the *mTert* promoter driving expression of GFP. This reporter was stably transfected into murine ES cells and was shown to recapitulate mTert expression and correlate with telomerase activity. Pericuesta *et al* (2006) also described the generation of reporters comprising the *mTert* promoter driving GFP. These reporters also recapitulated *mTert* expression and were expressed in ES cells. Expression of these reporters was also visualised at the blastocyst stage of mouse development (Pericuesta *et al.*, 2006).

#### 4.1.2 Down-regulation of *mTert* during differentiation of ES cells

Both Armstrong *et al* (2000) and Pericuesta *et al* (2006) analysed expression of their reporters during differentiation of ES cells. Both investigators differentiated their ES cells into EBs and treated the aggregates with media without LIF (see Chapter 1). During the initial stages of formation of the EB, the expression of the EGFP reporter increased (Armstrong *et al.*, 2000; Pericuesta *et al.*, 2006). This was proposed to be due to the increased proliferation of the cells during the formation of the EB (Armstrong *et al.*, 2000). Upon further culture, expression of the reporter decreased in accordance with substantial differentiation of the cells (Armstrong *et al.*, 2000; Pericuesta *et al.*, 2006). The same results were obtained in different ES cell lines, demonstrating that this was not a phenomenon specific to one ES cell line (Pericuesta *et al.*, 2006).

## 4.2 Aims

The initial aim of this part of the project was to analyse the expression of the Tert-CFP gene generated in Chapter 3 in ES cells. If transgene expression could be detected in ES cells, the second aim was then to determine whether this expression was down-regulated upon differentiation of ES cells into EBs. The final aim for this part of the project was to analyse the differentiation potential of transgenic ES clones via teratoma formation and to determine the expression pattern within the teratoma.

## 4.3 Results

### 4.3.1. Electroporation of the pTertpromECFP-1 reporter into ES cells.

The construction of the pTertpromECFP-1 reporter was described in Chapter 3. 60µg of pTertpromECFP-1 was linearised using *Afl* II and then electroporated into  $1 \times 10^8$  E14.1a ES cells as previously described (see Chapter 2). After one week of G418 selection, 50 G418 resistant clones were picked and grown up. A portion of each cell culture was frozen and the remainder was prepared for DNA analysis.

### 4.3.2 Detection of the transgene in ES clones by PCR screening

The primers mTertpromforscreen and mTertpromrevscreen were used for PCR screening of ES clones to identify the transgene (Figure 4.1A). mTertpromforscreen is located within the *mTert* promoter and mTertpromrevscreen is located within the CFP coding sequence (Figure 4.1A). PCR analysis of ES clones using these primers generated a product of 300bp if the transgene was present at the DNA level (Figure 4.1B). Diluted pTertpromECFP-1 plasmid DNA was used as a positive control for the PCR. Eleven positive clones were identified from the 50 analysed by PCR screening (clone numbers 2, 5, 8, 17, 20, 22, 23, 25, 36, 38 and 40). Since the clones were selected for using G418 selection, all clones would be expected to contain the transgene. The reasons that this was not the case include unstable integration of the transgene, or perhaps the efficiency of the selection.

### 4.3.3 Identification of copy number of pTertpromECFP-1 in ES clones by Southern analysis

The first five ES clones that tested positive by PCR (clones 2, 5, 8, 17 and 20) were further assessed by Southern blot analysis. ES genomic DNA was digested with *Bam*HI, electrophoresed through an agarose gel and transferred by blotting onto a nitrocellulose membrane. The membrane was probed with a short sequence (approximately 500bp) corresponding to the *neo<sup>R</sup>* gene. If the ES clones contained the transgene then a band would be detected. The number of bands detected generally corresponds to the number of integrations of the transgene, although if there are tandem integrations, a more intense

## 4.3.4 Identification of GFP reporter expression by direct visualization

band of approximately 300 bp would be expected with a 1 kb fragment with the genomic DNA. The positive control would be at least 4 kb (Figure 4.2B). ES clones 17, 2, 5, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192, 193, 194, 195, 196, 197, 198, 199, 200, 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219, 220, 221, 222, 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1185, 1186, 1187, 1188, 1189, 1190, 1191, 1192, 1193, 1194, 1195, 1196, 1197, 1198, 1199, 1200, 1201, 1202, 1203, 1204, 1205, 1206, 1207, 1208, 1209, 1210, 1211, 1212, 1213, 1214, 1215, 1216, 1217, 1218, 1219, 1220, 1221, 1222, 1223, 1224, 1225, 1226, 1227, 1228, 1229, 1230, 1231, 1232, 1233, 1234, 1235, 1236, 1237, 1238, 1239, 1240, 1241, 1242, 1243, 1244, 1245, 1246, 1247, 1248, 1249, 1250, 1251, 1252, 1253, 1254, 1255, 1256, 1257, 1258, 1259, 1260, 1261, 1262, 1263, 1264, 1265, 1266, 1267, 1268, 1269, 1270, 1271, 1272, 1273, 1274, 1275, 1276, 1277, 1278, 1279, 1280, 1281, 1282, 1283, 1284, 1285, 1286, 1287, 1288, 1289, 1290, 1291, 1292, 1293, 1294, 1295, 1296, 1297, 1298, 1299, 1300, 1301, 1302, 1303, 1304, 1305, 1306, 1307, 1308, 1309, 1310, 1311, 1312, 1313, 1314, 1315, 1316, 1317, 1318, 1319, 1320, 1321, 1322, 1323, 1324, 1325, 1326, 1327, 1328, 1329, 1330, 1331, 1332, 1333, 1334, 1335, 1336, 1337, 1338, 1339, 1340, 1341, 1342, 1343, 1344, 1345, 1346, 1347, 1348, 1349, 1350, 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1849, 1850, 1851, 1852, 1853, 1854, 1855, 1856, 1857, 1858, 1859, 1860, 1861, 1862, 1863, 1864, 1865, 1866, 1867, 1868, 1869, 1870, 1871, 1872, 1873, 1874, 1875, 1876, 1877, 1878, 1879, 1880, 1881, 1882, 1883, 1884, 1885, 1886, 1887, 1888, 1889, 1890, 1891, 1892, 1893, 1894, 1895, 1896, 1897, 1898, 1899, 1900, 1901, 1902, 1903, 1904, 1905, 1906, 1907, 1908, 1909, 1910, 1911, 1912, 1913, 1914, 1915, 1916, 1917, 1918, 1919, 1920, 1921, 1922, 1923, 1924, 1925, 1926, 1927, 1928, 1929, 1930, 1931, 1932, 1933, 1934, 1935, 1936, 1937, 1938, 1939, 1940, 1941, 1942, 1943, 1944, 1945, 1946, 1947, 1948, 1949, 1950, 1951, 1952, 1953, 1954, 1955, 1956, 1957, 1958, 1959, 1960, 1961, 1962, 1963, 1964, 1965, 1966, 1967, 1968, 1969, 1970, 1971, 1972, 1973, 1974, 1975, 1976, 1977, 1978, 1979, 1980, 1981, 1982, 1983, 1984, 1985, 1986, 1987, 1988, 1989, 1990, 1991, 1992, 1993, 1994, 1995, 1996, 1997, 1998, 1999, 2000, 2001, 2002, 2003, 2004, 2005, 2006, 2007, 2008, 2009, 2010, 2011, 2012, 2013, 2014, 2015, 2016, 2017, 2018, 2019, 2020, 2021, 2022, 2023, 2024, 2025, 2026, 2027, 2028, 2029, 2030, 2031, 2032, 2033, 2034, 2035, 2036, 2037, 2038, 2039, 2040, 2041, 2042, 2043, 2044, 2045, 2046, 2047, 2048, 2049, 2050, 2051, 2052, 2053, 2054, 2055, 2056, 2057, 2058, 2059, 2060, 2061, 2062, 2063, 2064, 2065, 2066, 2067, 2068, 2069, 2070, 2071, 2072, 2073, 2074, 2075, 2076, 2077, 2078, 2079, 2080, 2081, 2082, 2083, 2084, 2085, 2086, 2087, 2088, 2089, 2090, 2091, 2092, 2093, 2094, 2095, 2096, 2097, 2098, 2099, 2100, 2101, 2102, 2103, 2104, 2105, 2106, 2107, 2108, 2109, 2110, 2111, 2112, 2113, 2114, 2115, 2116, 2117, 2118, 2119, 2120, 2121, 2122, 2123, 2124, 2125, 2126, 2127, 2128, 2129, 2130, 2131, 2132, 2133, 2134, 2135, 2136, 2137, 2138, 2139, 2140, 2141, 2142, 2143, 2144, 2145, 2146, 2147, 2148, 2149, 2150, 2151, 2152, 2153, 2154, 2155, 2156, 2157, 2158, 2159, 2160, 2161, 2162, 2163, 2164, 2165, 2166, 2167, 2168, 2169, 2170, 2171, 2172, 2173, 2174, 2175, 2176, 2177, 2178, 2179, 2180, 2181, 2182, 2183, 2184, 2185, 2186, 2187, 2188, 2189, 2190, 2191, 2192, 2193, 2194, 2195, 2196, 2197, 2198, 2199, 2200, 2201, 2202, 2203, 2204, 2205, 2206, 2207, 2208, 2209, 2210, 2211, 2212, 2213, 2214, 2215, 2216, 2217, 2218, 2219, 2220, 2221, 2222, 2223, 2224, 2225, 2226, 2227, 2228, 2229, 223

band of approximately 8.7kb would be expected plus a junction fragment with the genomic DNA. The junction fragment would be at least 4.1kb (Figure 4.2B). ES clone 17 seems to have tandem integrations of the transgene as there is a thick band at approximately 8.7kb. One of the other sets of bands larger than 4.1kb could correspond to the junction fragment. There are two other sets of bands suggesting that there are two other integrations. The number of integrations detected in the ES clones is summarised in Table 4.1

ES clone number	Number of integrations
2	1
5	3
8	1
17	3 (1 tandem plus 2 others)
20	1

**Table 4.1.** The number of integrations in ES clones as detected by Southern analysis using a *neo<sup>R</sup>* probe.

#### **4.3.4 Identification of CFP reporter expression by direct visualisation**

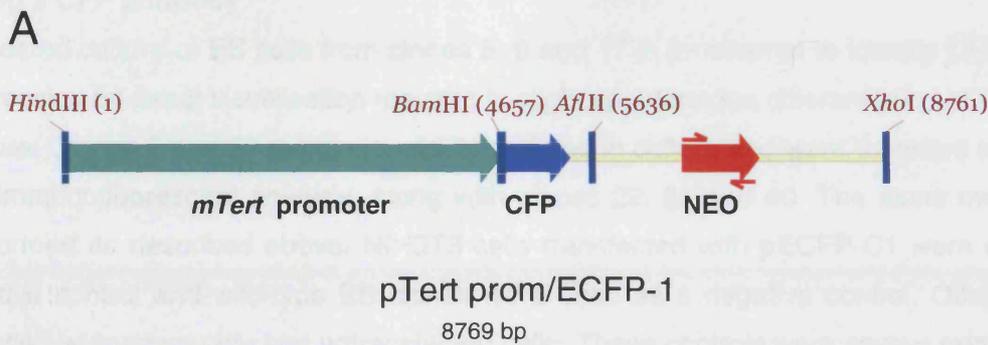
ES clones 2, 5, 8, 17 and 20 were analysed for expression of the reporter under the correct wavelength of light (approx 433nm) to excite CFP. Emission from the reporter should be discernable at approximately 475nm. However, no expression was seen using a standard fluorescence microscope or a confocal microscope in any of the clones analysed (data not shown). NIH3T3 fibroblast cells transfected with a CFP plasmid (pECFP-C1) were used as positive controls to determine CFP expression. In this plasmid, expression of the CFP coding region is driven by the human CMV promoter. CFP expression was detected but was weak. The reason for this is due to the fact that CFP is generally more difficult to detect than GFP. The emission spectrum needs to be very precise in order to obtain a weak CFP fluorescent signal.

#### **4.3.5 Identification of CFP expression by immunofluorescence using a GFP antibody.**

As CFP fluorescence could not be visualised directly, I determined whether a rabbit polyclonal GFP antibody could be used to identify CFP reporter expression. An immunofluorescence experiment was performed using NIH3T3 fibroblast cells expressing either a GFP plasmid (pEGFP-N1) or a CFP plasmid (pECFP-C1). Both of these plasmids express their fluorescent coding sequences under the direction of the human CMV promoter (see Chapter 2). The cells were transfected using the nucleofector method (see Chapter 2).

Only transfected cells were then transferred onto coverslips and used for immunofluorescence using an antibody against GFP. Strong signal was observed in the cells transfected with pTertprom/ECFP-1, suggesting that the reporter was expressed in these cells.

**Figure 4.2** Southern analysis of ES clones using *neo<sup>R</sup>* probe to assess copy number. **(A)** pTertpromECFP-1 reporter showing primers NeoF1 and NeoF2 (shown in red) used to amplify *neo<sup>R</sup>* probe from *neo<sup>R</sup>* cassette. **(B)** Southern analysis of ES clones 2, 5, 8, 17 and 20. The number of bands represents the number of integrations of the reporter, although in the case of clone 17, multiple integrations are observed.



Once transfected, the cells were then transferred onto coverslips and used for immunofluorescence using an antibody against GFP. A strong signal was obtained with both the cells transfected with pEGFP-N1 and those transfected with pECFP-C1 suggesting that the GFP antibody could be used to detect expression of the CFP coding sequence as well as the GFP coding sequence (Figure 4.3). The specificity of the signal was further shown by the lack of fluorescence obtained with the secondary only control and untransfected cells. Thus the GFP antibody will be referred to as a CFP antibody.

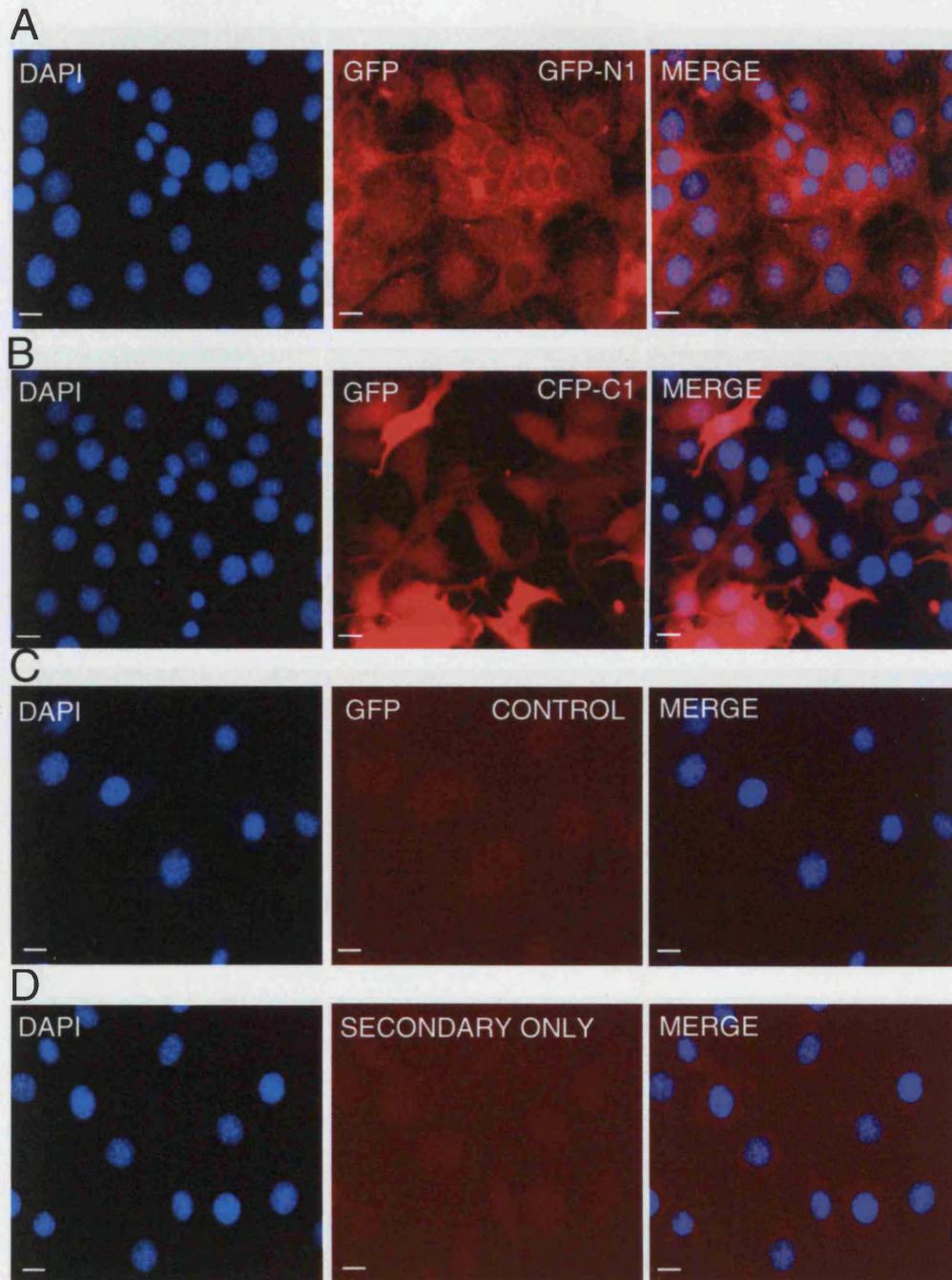
#### ***4.3.6 Analysis of CFP reporter expression in ES clones by immunofluorescence using a CFP antibody***

Repeated culture of ES cells from clones 5, 8 and 17 in an attempt to identify CFP reporter expression by direct visualisation resulted in slight spontaneous differentiation of the cells in culture. Clones 2 and 20 remained undifferentiated in culture and were therefore included in the immunofluorescent analysis, along with clones 22, 38 and 40. The same method was performed as described above. NIH3T3 cells transfected with pECFP-C1 were used as a positive control and wild-type ES clones were used as a negative control. Other controls included secondary only and untransfected cells. These controls were always negative. The experiments were repeated a minimum of three times. Furthermore all microscope/computer settings were kept the same where possible when analysing ES cells. The immunofluorescence data shows that CFP reporter expression can be detected in ES clones transfected with pTertpromECFP-1 (Figure 4.4). The signal distribution suggests that the reporter is expressed both in the nucleus and the cytoplasm of the ES clones. There seemed to be no obvious difference in strength of signal between any of the ES clones analysed.

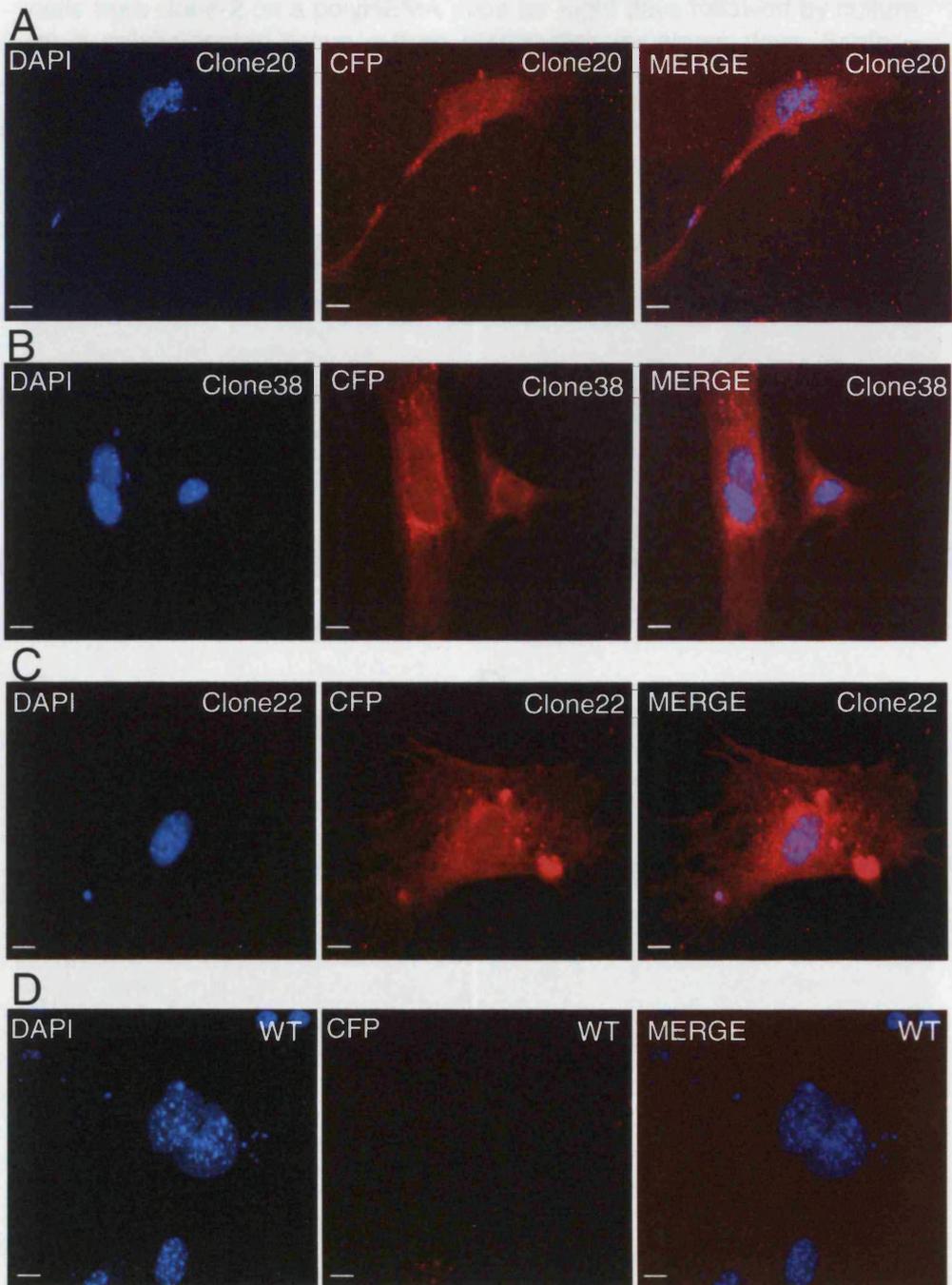
#### ***4.3.7 Differentiation of ES cells and analysis of CFP reporter expression by immunofluorescence.***

ES cells from clones 2, 22 and 40 were differentiated into EBs by culturing in media minus LIF on a PolyHEMA-coated dish for eight days. The EBs were then cultured for a further seven days on gelatin-coated dishes (Figure 4.5). Differentiated fibroblast-looking cells can be seen moving out from the EBs. Furthermore, some of the EBs were seen to beat during culture suggesting that some cardiac cells were formed within the EBs. The differentiated cells were then transferred to coverslips for use in immunofluorescence experiments. The corresponding undifferentiated ES clones were analysed in conjunction with the differentiated cells. DAPI staining was used to identify the nucleus of the cells, the CFP antibody was used to analyse CFP reporter expression and an Oct-4 antibody was used as a marker for pluripotency. Controls for the secondary antibodies are also shown (Figure

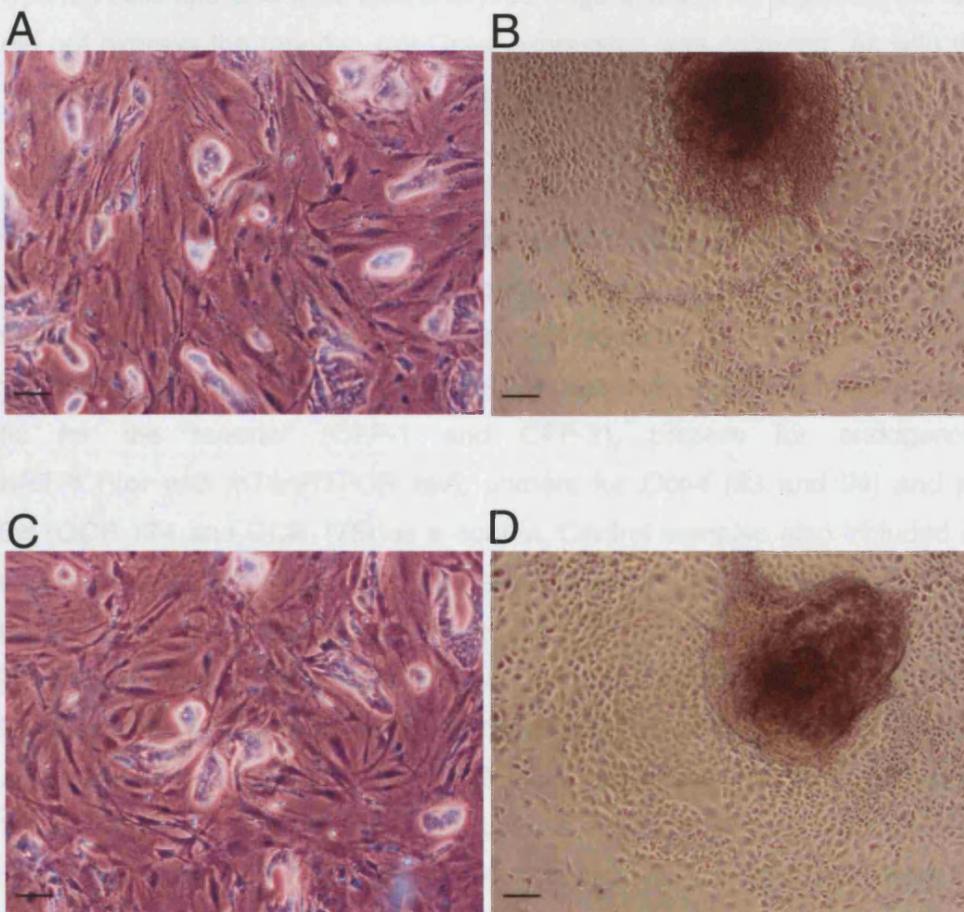
**Figure 4.3.** Immunofluorescence of NIH3T3 cells. **(A)** Immunofluorescence of NIH3T3 cells expressing GFP (pEGFP-N1) using GFP antibody. **(B)** Immunofluorescence of NIH3T3 cells expressing CFP (pECFP-C1) using GFP antibody. **(C)** Immunofluorescence of untransfected NIH3T3 cells using GFP antibody **(D)** Immunofluorescence of NIH3T3 cells expressing GFP using only secondary antibody. Scale = 15 $\mu$ m



**Figure 4.4.** Immunofluorescence of ES clones. **(A)** Immunofluorescence of ES cells from CFP reporter clone 20 using the CFP antibody. **(B)** Immunofluorescence of ES reporter clone 38 using the CFP antibody. **(C)** Immunofluorescence of ES reporter clone 22 using the CFP antibody. **(D)** Immunofluorescence of wild-type ES cells using CFP antibody. Scale = 15 $\mu$ m



**Figure 4.5.** (A) Wild-type ES cells grown on an MEF feeder layer. (B) wild-type EBs photographed after culturing ES cells on a polyHEMA plate for eight days followed by culture on a gelatin-coated tissue culture plastic dish for seven days. (C) Transgenic ES cells from clone 2 grown on an MEF feeder layer. (D) Transgenic EBs photographed after culturing ES cells from clone 2 on a polyHEMA plate for eight days followed by culture on a gelatin-coated tissue culture plastic dish for seven days. Scale = 100 $\mu$ m



4.6A). The experiments were repeated a minimum of three times and the microscope/computer setting were kept the same where possible.

The immunofluorescence data shows that expression of the reporter as detected with the CFP antibody is consistently down-regulated upon differentiation of ES cells via EB formation (Figure 4.6B and C). Oct-4 expression in ES clones as detected using the Oct-4 antibody is both nuclear and cytoplasmic. However, the staining observed in these experiments shows that the expression of Oct-4 is stronger in the nucleus as might be expected for a transcription factor. Expression of Oct-4 is down-regulated in the differentiated cells; showing that the differentiation protocol was successful (Figure 4.6). Wild-type ES cells and EBs were also analysed (Figure 4.6C). As expected the wild-type ES cells did not express the reporter, but Oct-4 expression was detected. As with the reporter cells, Oct-4 expression was down-regulated upon differentiation.

#### **4.3.8 CFP reporter expression analysis using RT-PCR**

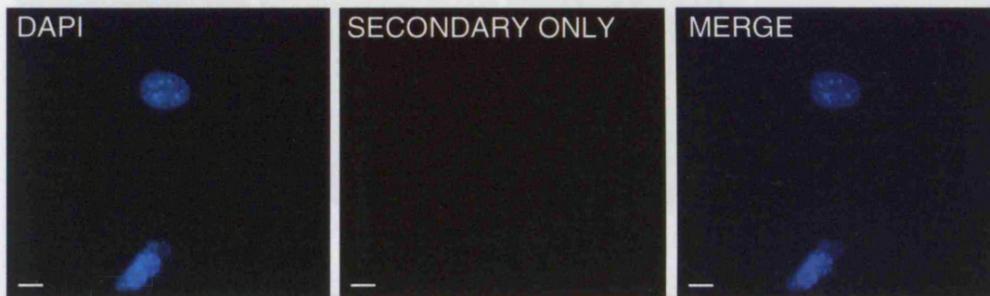
Analysis of CFP reporter expression using immunofluorescence suggests that expression is down-regulated upon differentiation of ES cells. In order to confirm these data, the level of reporter expression at the RNA level was determined using RT-PCR from ES clone 2 and a wild-type ES cell and corresponding EBs. cDNA was used in a PCR reaction using primers specific for the reporter (CFP-1 and CFP-2), primers for endogenous *mTert* (*mTert*RTPCRfor and *mTert*RTPCR rev), primers for *Oct-4* (93 and 94) and primers for *GAPDH* (OCP 174 and OCP 175) as a control. Control samples also included no reverse transcriptase samples in which no cDNA should be synthesised. These controls were all negative as expected. As expected, reporter expression was only observed in transgenic ES clone 2 (Figure 4.7). This expression was lost upon differentiation. Endogenous *mTert* and *Oct-4* expression was seen in the transgenic ES clone and wild-type ES cell (Figure 4.7). *mTert* and *Oct-4* expression were both down-regulated with differentiation of both the reporter ES clone and the wild-type ES cell as expected (Figure 4.7). However, some residual expression of both *mTert* and *Oct-4* remained with the EB formed from the reporter ES clone, suggesting that in this particular experiment the differentiation achieved for the reporter ES clone was not as efficient as that achieved for the wild-type ES cell or the efficiency of the PCR reaction was different in these samples.

#### **4.3.9 CFP reporter expression analysis during the differentiation of ES clones during teratoma formation**

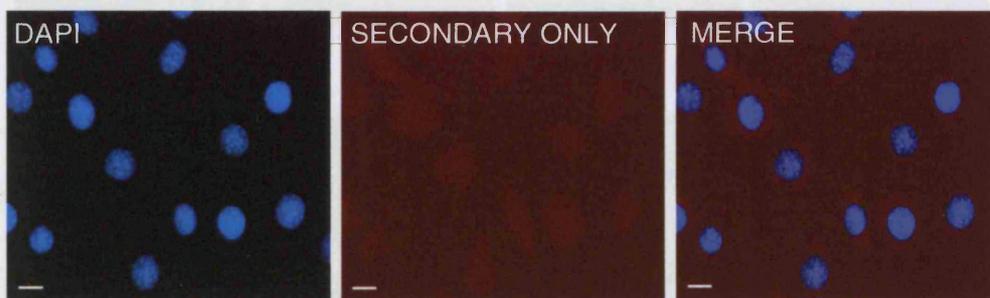
The differentiation potential of ES cells can be extensively studied during the formation of teratomas. Teratomas are formed by the subcutaneous injection of ES cells into syngeneic

**Figure 4.6A.** Immunofluorescence controls. **(A)** Immunofluorescence of wild-type ES cells using only Alexa Fluor® anti goat secondary antibody used to detect Oct-4 primary. **(B)** Immunofluorescence of ES cells expressing pTertpromECFP-1 using only Alexa Fluor® anti rabbit secondary antibody used to detect GFP primary. Scale = 15µm

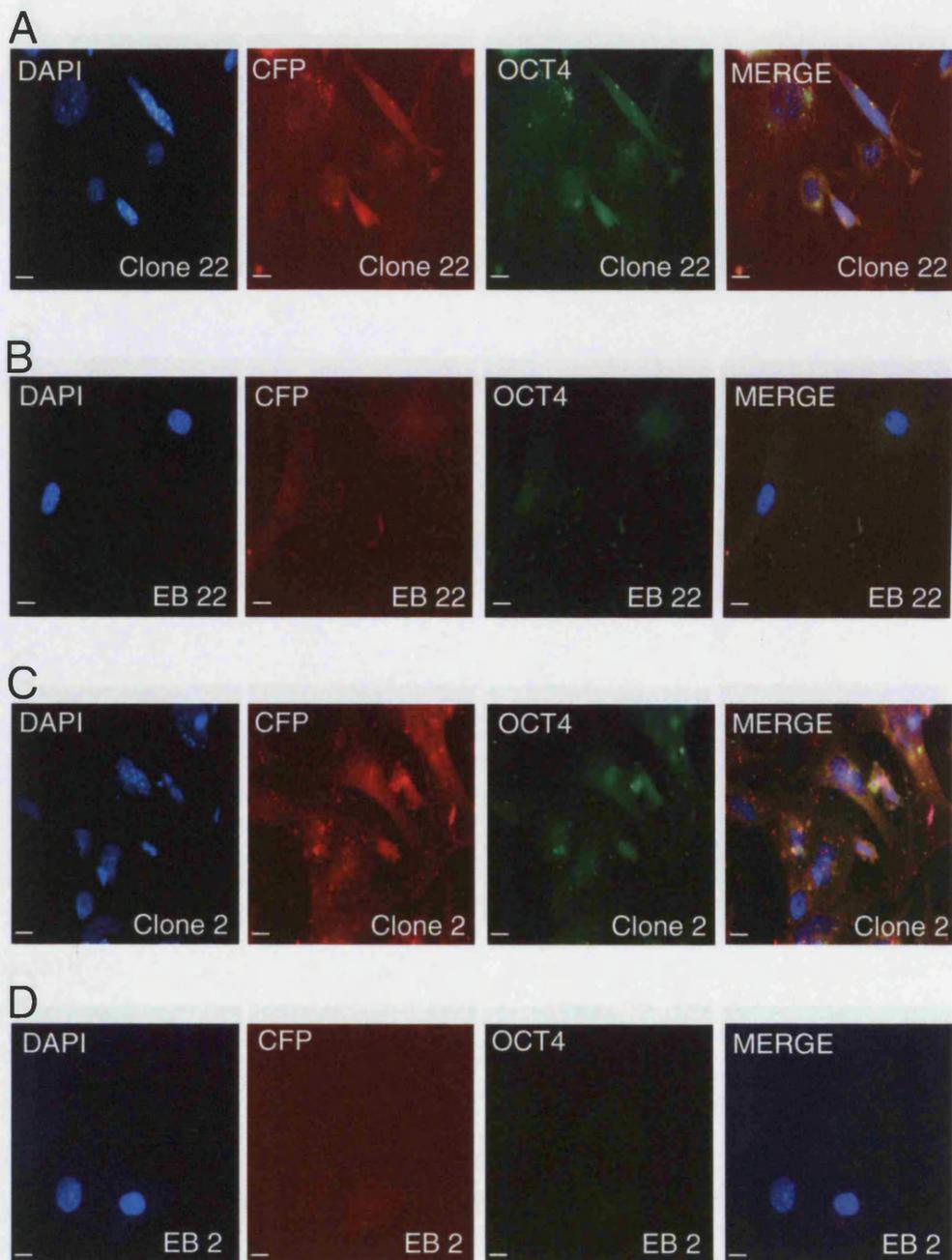
**A**



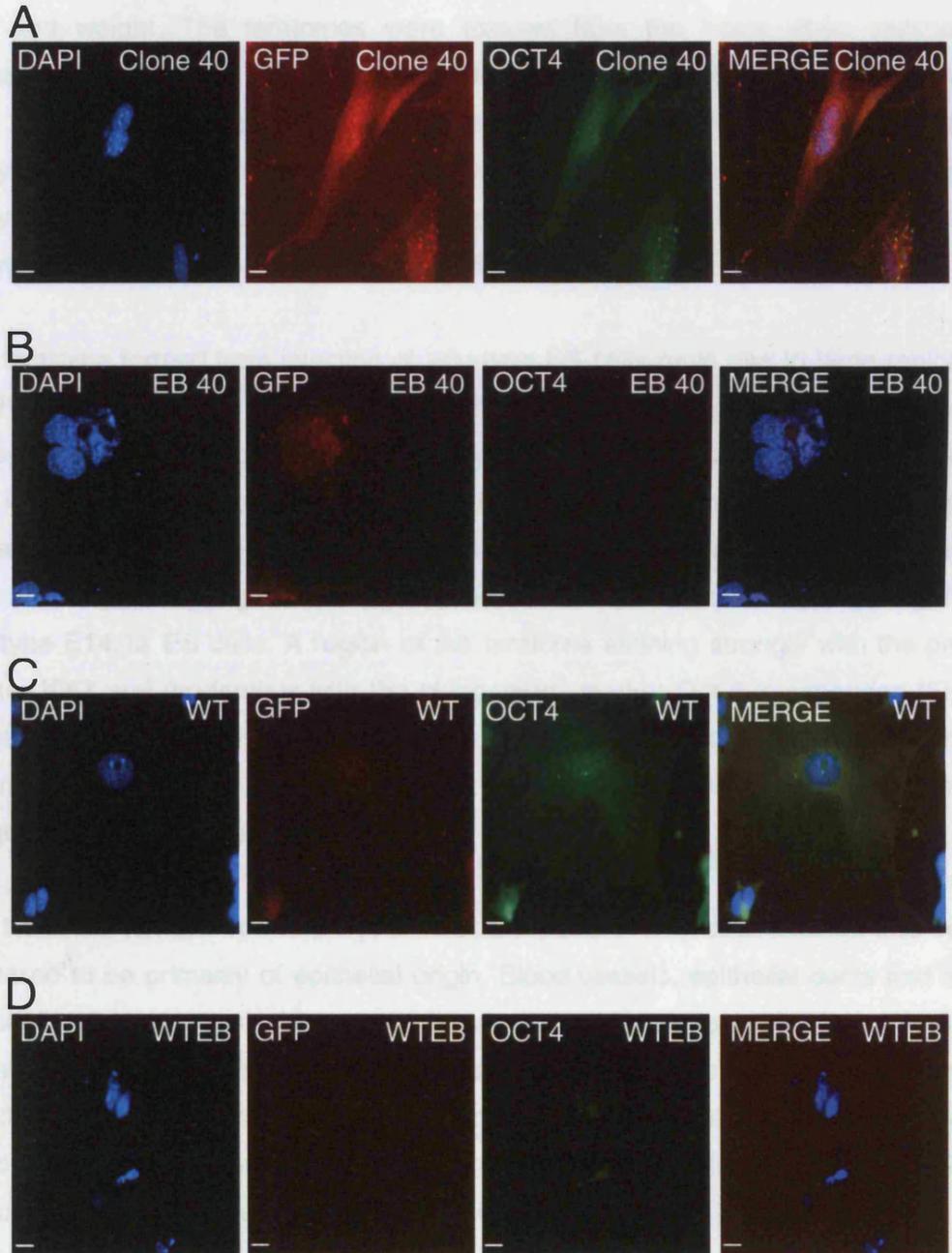
**B**



**Figure 4.6B.** Immunofluorescence of ES clones and EBs. **(A)** Immunofluorescence of ES clone 22 using CFP and Oct-4 antibodies. **(B)** Immunofluorescence of EB derived from ES clone 22 using CFP and Oct-4 antibodies. **(C)** Immunofluorescence of ES clone 2 using CFP and Oct-4 antibodies. **(D)** Immunofluorescence of EB derived from ES clone 2 using GFP and Oct-4 antibodies. Scale = 15 $\mu$ m



**Figure 4.6C.** Immunofluorescence of ES clones and EBs. **(A)** Immunofluorescence of ES clone 40 using CFP and Oct-4 antibodies. **(B)** Immunofluorescence of EB derived from ES clone 40 using CFP and Oct-4 antibodies. **(C)** Immunofluorescence of wild-type ES cell using CFP and Oct-4 antibodies. **(D)** Immunofluorescence of EB derived from wild-type ES cells using CFP and Oct-4 antibodies. Scale = 15µm

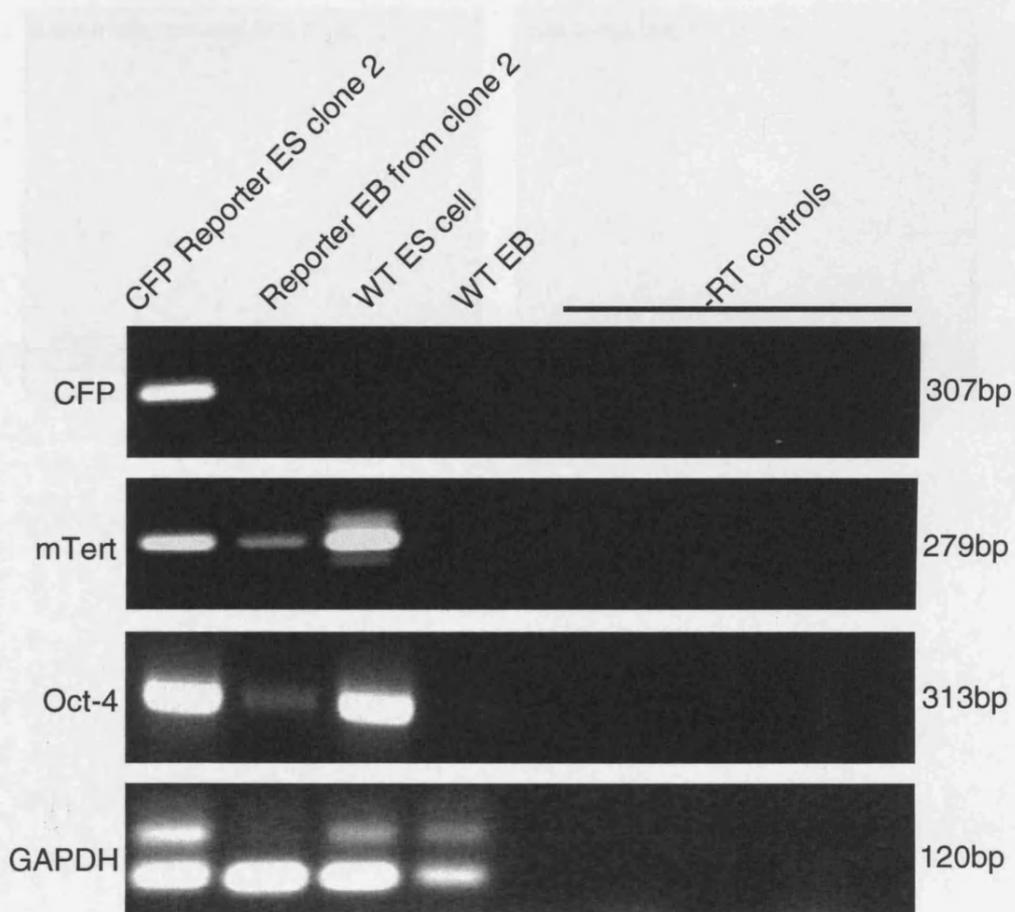


hosts. A benign solid teratoma containing derivatives of all three germ layers is then formed. Wild-type E14.1a ES cells and ES cells from clones 2 and 38 were injected subcutaneously into syngeneic hosts where they formed teratomas. Clone 2 was chosen for teratoma formation as it was one of the most well characterised ES clones and still remained markedly undifferentiated in culture. Clone 38 was chosen to be analysed during teratoma formation as it had been demonstrated to express the CFP reporter (Figure 4.4) and remained significantly undifferentiated in culture. All teratomas formed were of expected size and weight. The teratomas were excised from the hosts when appropriate and prepared for histological analysis by embedding in paraffin-wax to allow analysis of paraffin-wax embedded sections. 5µm serial sections were prepared using a microtome and analysed using the labelled streptavidin biotin (LSAB) method. The immunohistochemical analysis was repeated at least three times. As with immunofluorescence, secondary only controls were performed but showed no staining (Figure 4.8).

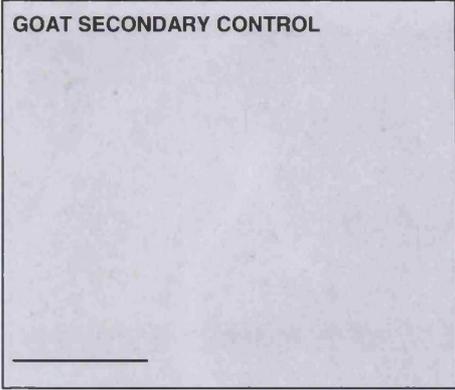
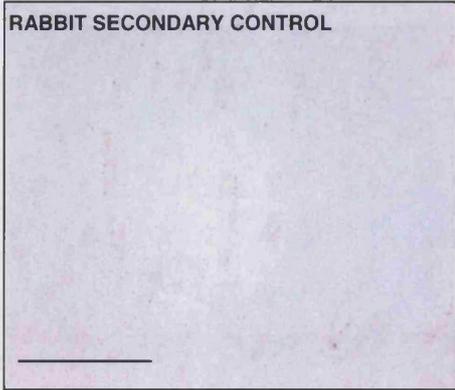
The teratoma formed from injection of wild-type ES cells gave rise to large regions of cells which appeared to be of epithelial origin. There are also patches of cells which may represent ducts or glands (Figure 4.9). However, a range of markers would be required to fully identify the range of cell types formed. Immunohistochemical staining was performed on serial sections of the wild-type teratoma (Figure 4.9). No staining was observed with the CFP antibody in any region of the paraffin-wax embedded teratoma formed by injection of wild-type E14.1a ES cells. A region of the teratoma staining strongly with the proliferation marker Ki67 and moderately with the pluripotency marker Oct-4 is expanded (Figure 4.9). Overall, no regions stained very strongly for Oct-4 suggesting that the majority of the teratoma was partly or fully differentiated. However, the presence of Ki67 positive cells suggests that some cells with proliferative potential remained.

The teratoma formed from the injection of ES clone 2 only differentiated into cells which appeared to be primarily of epithelial origin. Blood vessels, epithelial ducts and areas with morphology appearing similar to smooth muscle could also be seen (Figure 4.10). As with the wild-type teratoma, immunohistochemistry using a range of markers would be required to fully identify the tissue types formed. Immunohistochemical staining was performed on serial sections of the teratoma formed by injection of ES cells derived from ES clone 2. Immunostaining using a GFAP antibody which is a marker of astrocytes and the CK-1 antibody which identifies spinous and granular layer cells found within the epithelium of the skin was negative suggesting that no neural tissue or skin epidermis was formed within this teratoma (data not shown). No other regions of specific interest were identified within the teratoma. Immunostaining using the Ki67 antibody indicated that proliferation within this

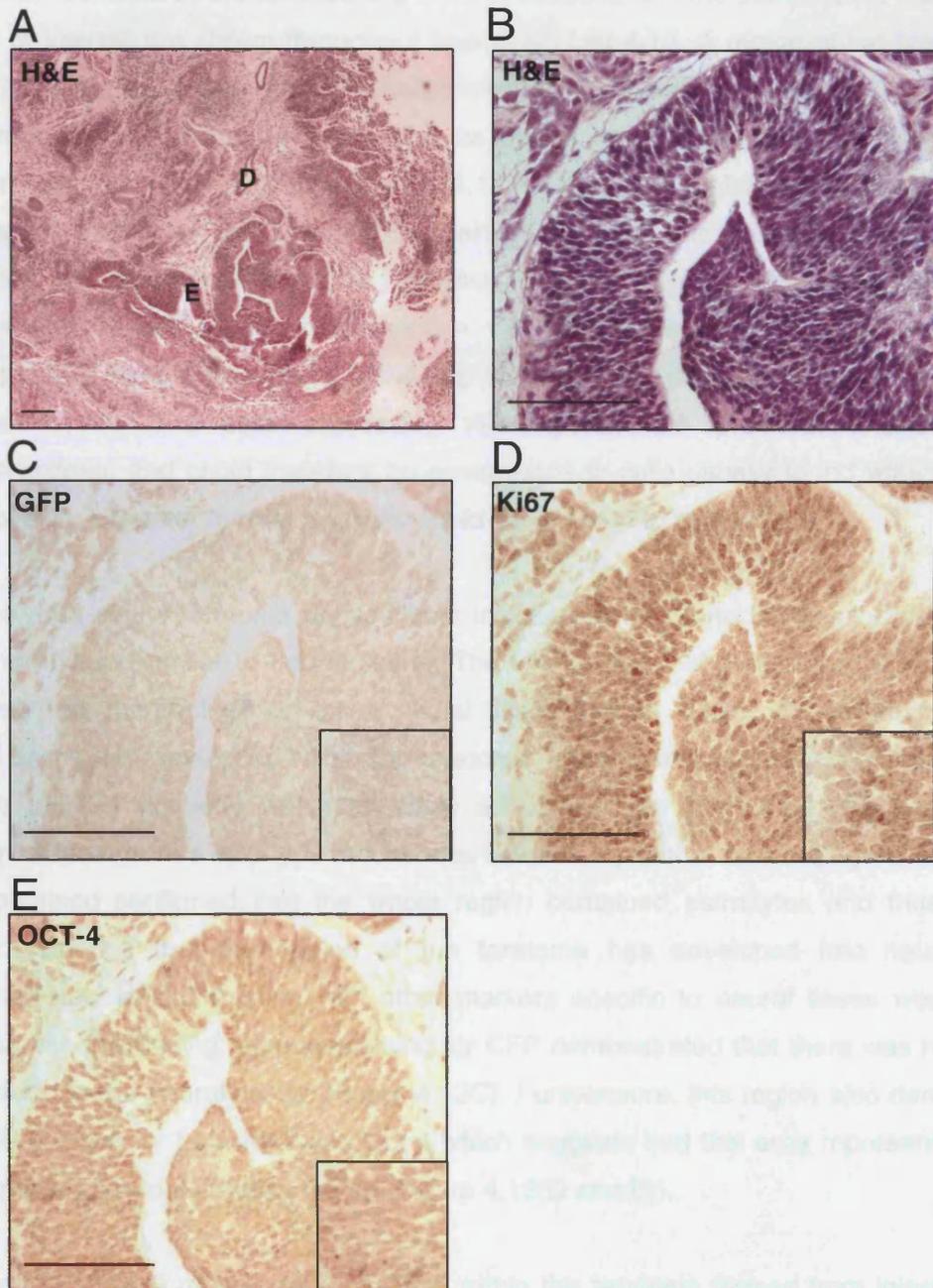
**Figure 4.7** Semi-quantitative RT-PCR analysis of ES clones and EBs. cDNA was prepared from a wild-type ES cell and corresponding wild-type EB and reporter ES clone2 and corresponding EB and RT-PCR performed. RT-PCR for CFP with primers CFP-1 and CFP-2 which produce a product of 307bp identified reporter expression. RT-PCR for endogenous mTert was performed using mTertRTPCRfor and mTertRTPCRrev which generates a band of 279bp. RT-PCR using OCP174 and OCP 175 identifies GAPDH expression at 120bp which was used as a control.



**Figure 4.8** Teratoma immunohistochemical controls. (A) Anti-rabbit secondary antibody control. (B) Anti-goat secondary antibody control. Scale = 100 $\mu$ m



**Figure 4.9** Immunohistochemical analysis of teratoma derived from subcutaneous injection of nude mouse with wild-type ES cells. **(A)** H&E staining overview. D = duct E= epithelia. **(B)** H&E of epithelial region of teratoma. Teratoma 5 $\mu$ m serial sections were immunostained with GFP **(C)**, Ki67 **(D)**, and Oct-4 **(E)**. Scale = 100 $\mu$ m



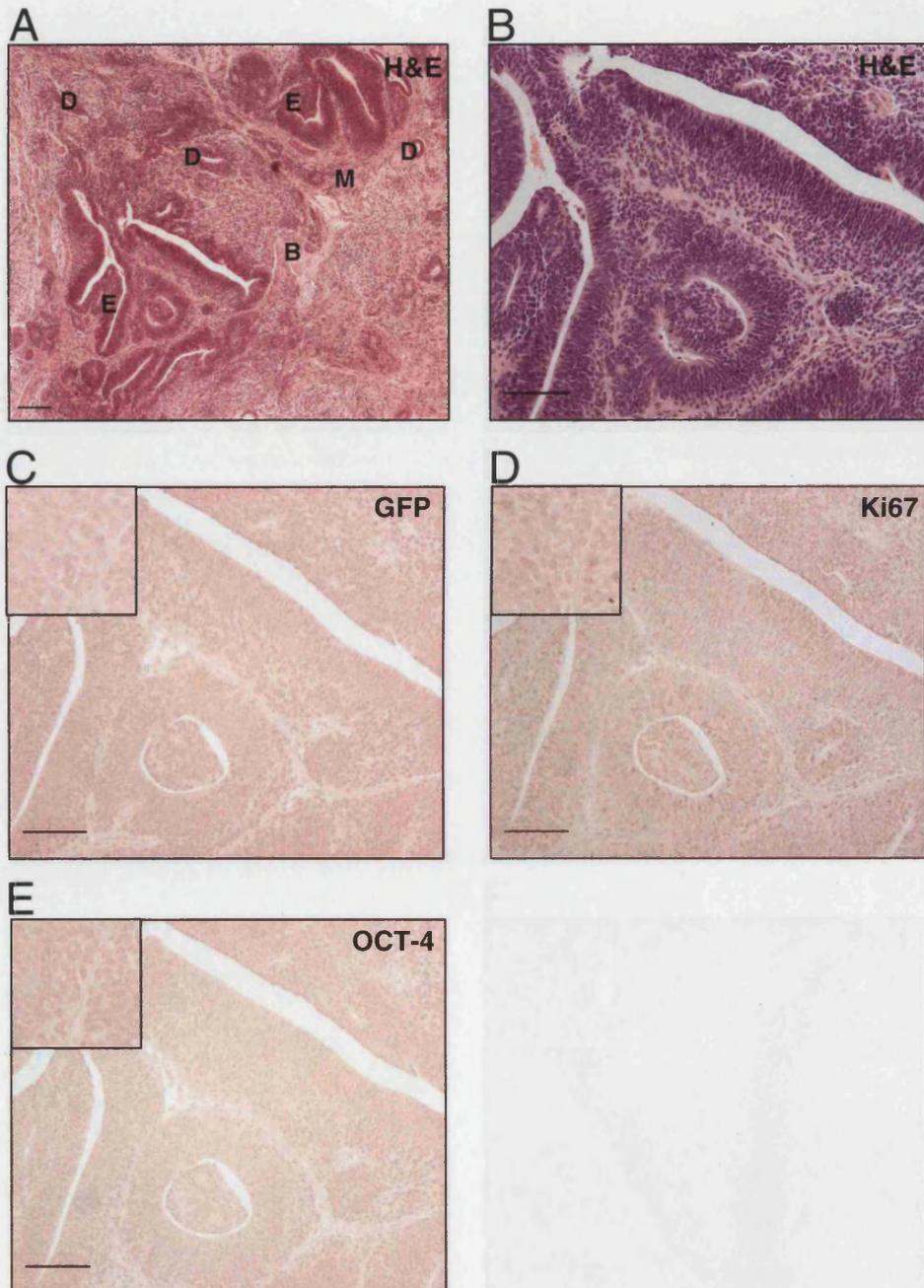
teratoma was minimal (Figure 4.10D). Furthermore, immunostaining using the Oct-4 antibody did not reveal any significant patches of undifferentiated cells (Figure 4.10E). Reporter expression analysis using the CFP antibody revealed that minimal to no expression of the reporter was obvious within this teratoma (Figure 4.10C).

The teratoma formed from the injection of ES clone 38 gave rise to a variety of tissues including neural tissue, skin epidermis and skeletal muscle. Patches of undifferentiated cells were also identified by immunostaining of serial sections with the pluripotency marker Oct-4. Areas of interest are shown throughout figures 4.11 to 4.15. A region of the teratoma was identified with morphology similar to keratinised stratified-squamous epithelium. This region demonstrated some reporter expression as determined by immunostaining of a parallel section with the CFP antibody (Figure 4.11). This immunostaining correlated with the expression pattern of the proliferation marker Ki67 and the pluripotency marker Oct-4 identified by immunohistochemical analysis of serial sections (Figure 4.11(D and E)). Immunohistochemistry of a serial section with the spinous/granular cell marker CK-1 indicated that there were cells of spinous/granular origin within this region of the teratoma (Figure 4.11F). The cells expressing the reporter are proliferative and relatively undifferentiated and could therefore be comparable to cells usually found within the basal layer of skin. However, further analysis would be required to confirm this.

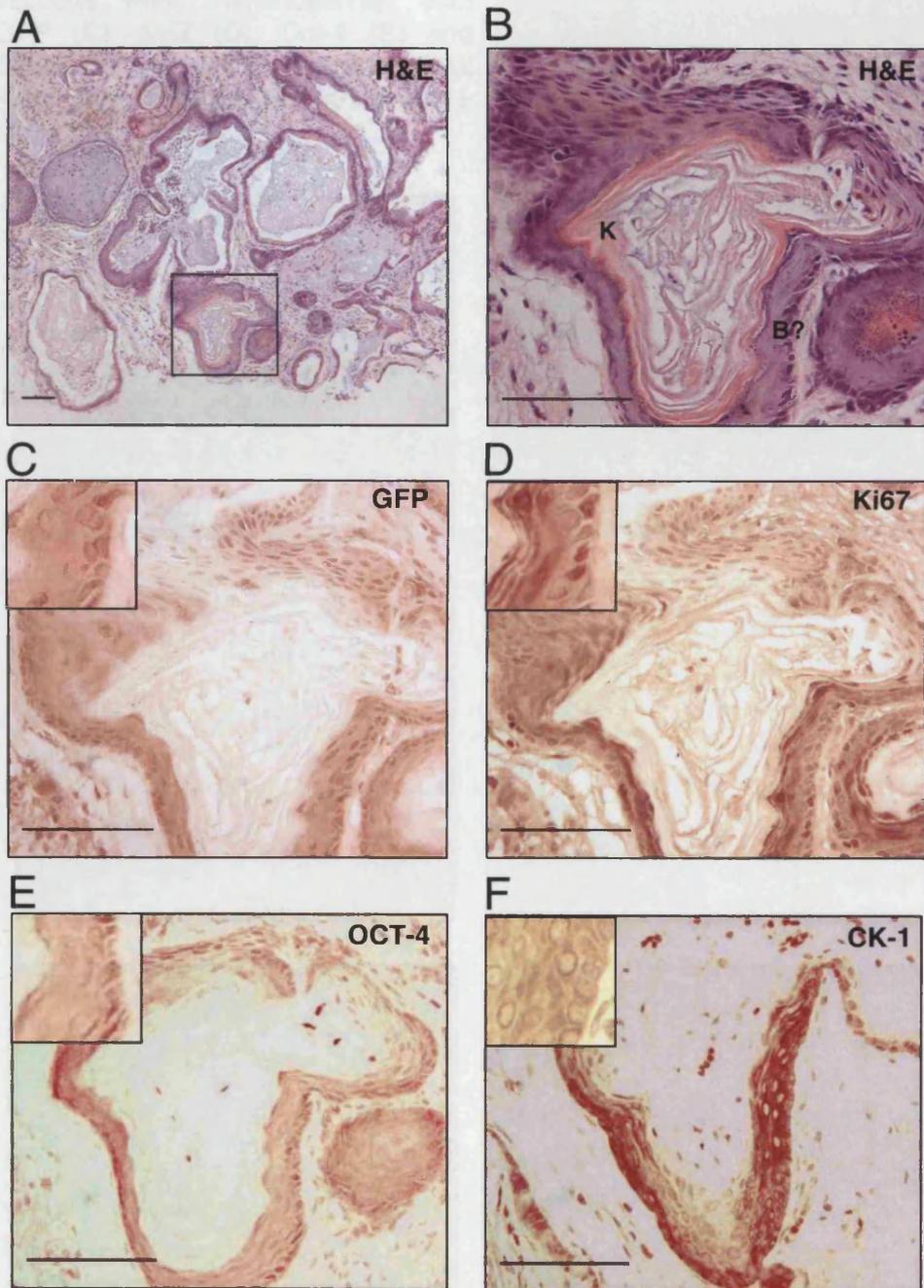
A large area of the teratoma derived from injection of ES clone 38 developed into tissue with morphology similar to neural tissue. The whole region shown in the H&E low power overview has morphology similar to neural tissue (Figure 4.12A). To determine whether neural tissue had developed within the teratoma, immunostaining using a glial fibrillar acid protein (GFAP) antibody which identifies astrocytes was performed (Figure 4.12F). A section of brain from a wild-type mouse was used as a positive control (Figure 4.12G). The data obtained confirmed that the whole region contained astrocytes and thus provides further evidence that this region of the teratoma has developed into neural tissue. Immunohistochemical staining with other markers specific to neural tissue would further substantiate this finding. Immunostaining for CFP demonstrated that there was no reporter expression in the neural tissue (Figure 4.12C). Furthermore, this region also demonstrated minimal staining for both Ki67 and Oct-4 which suggests that this area represents a highly differentiated, low proliferative region (Figure 4.12(D and E)).

A region of skeletal muscle was identified within the teratoma formed from injection of ES clone 38 using H&E histological staining (Figure 4.13(A and B)). The muscle fibre exhibited little CFP reporter expression (Figure 4.13C). A moderate level of proliferation was identified

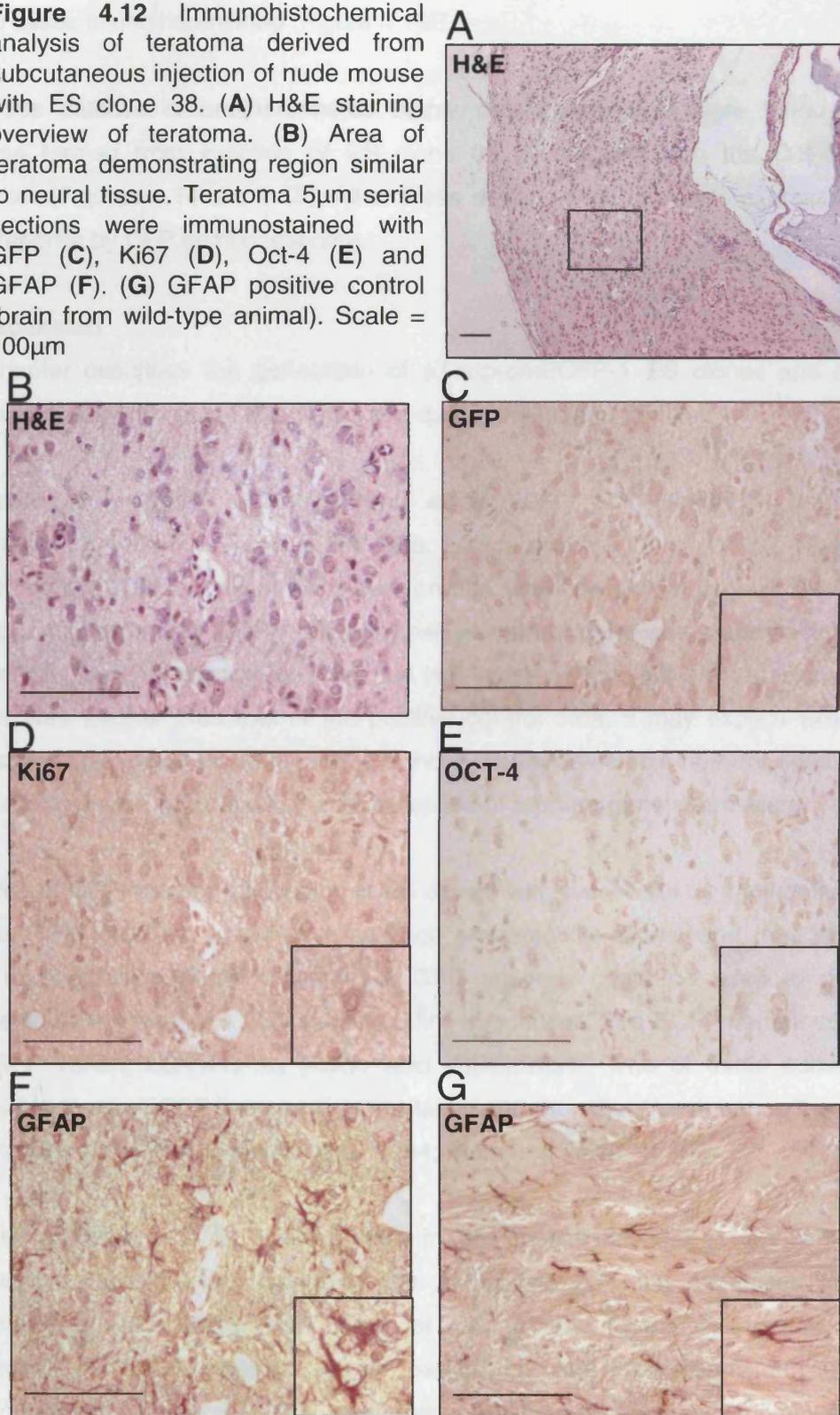
**Figure 4.10** Immunohistochemical analysis of teratoma derived from subcutaneous injection of nude mouse with ES clone 2. **(A)** H&E staining overview of teratoma. D = duct, M = muscle, E = epithelia and B = blood vessel. Teratoma 5 $\mu$ m serial sections were immunostained with GFP **(C)**, Ki67 **(D)** and Oct-4 **(E)**. Scale = 100 $\mu$ m



**Figure 4.11** Immunohistochemical analysis of teratoma derived from subcutaneous injection of nude mouse with ES clone 38. **(A)** H&E staining overview of teratoma. **(B)** H&E of area of teratoma demonstrating region of keratinised stratified-squamous epithelia. K = keratinised layer, B = basal layer. Teratoma 5 $\mu$ m serial sections were immunostained with GFP **(C)**, Ki67 **(D)**, Oct-4 **(E)** and CK-1 **(F)**. Scale = 100 $\mu$ m



**Figure 4.12** Immunohistochemical analysis of teratoma derived from subcutaneous injection of nude mouse with ES clone 38. (A) H&E staining overview of teratoma. (B) Area of teratoma demonstrating region similar to neural tissue. Teratoma 5 $\mu$ m serial sections were immunostained with GFP (C), Ki67 (D), Oct-4 (E) and GFAP (F). (G) GFAP positive control (brain from wild-type animal). Scale = 100 $\mu$ m



within the muscle fibre using the Ki67 proliferation marker (Figure 4.13D). Moreover a high level of Oct-4 immunostaining was identified along the muscle fibre; suggesting that the muscle tissue is undifferentiated (Figure 4.13E).

Numerous patches of undifferentiated highly proliferative cells were found within the teratoma formed from injection of ES clone 38 by staining with the Oct-4 and Ki67 antibodies (Figures 4.14 and 4.15). All of these regions were shown to express high levels of the reporter by CFP immunostaining.

#### 4.4 Discussion

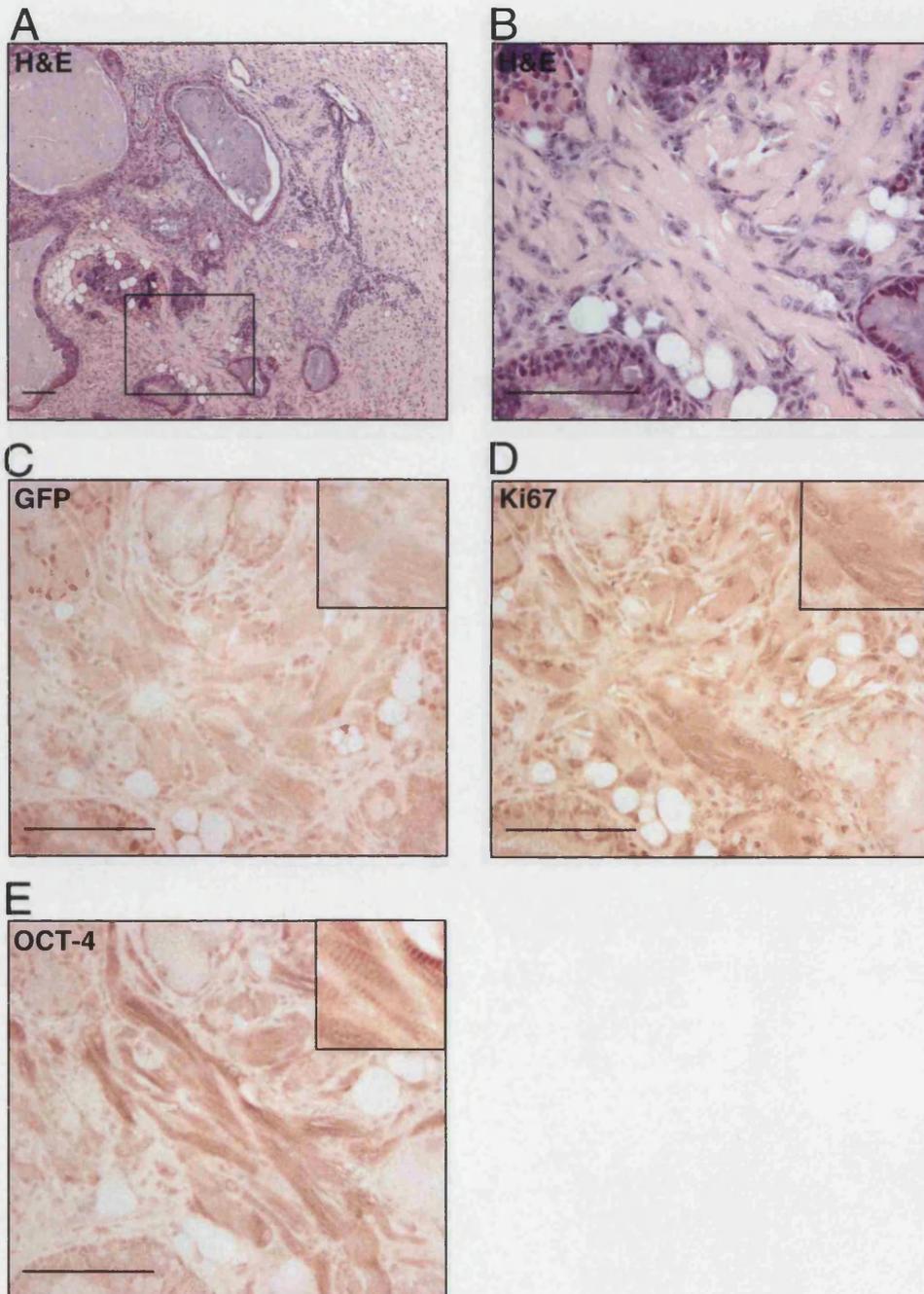
This chapter describes the generation of pTertpromECFP-1 ES clones and analysis of reporter expression in these ES clones and during their differentiation.

Pericuesta *et al* (2006) and Armstrong *et al* (2000) both described the successful visualisation of a GFP reporter in ES cells. Direct visualisation of the pTertpromECFP-1 reporter constructed in this investigation proved unsuccessful in any of the ES clones analysed. Expression of CFP from the positive control cells was discernable but weak. Furthermore, the CFP expression bleached very quickly. Thus, if the expression of the CFP reporter was weaker than that of the positive control cells, it may explain why no direct visualisation was identified. In hindsight, a more readily detectable reporter such as GFP or an inducible system would aid in the identification of reporter gene expression.

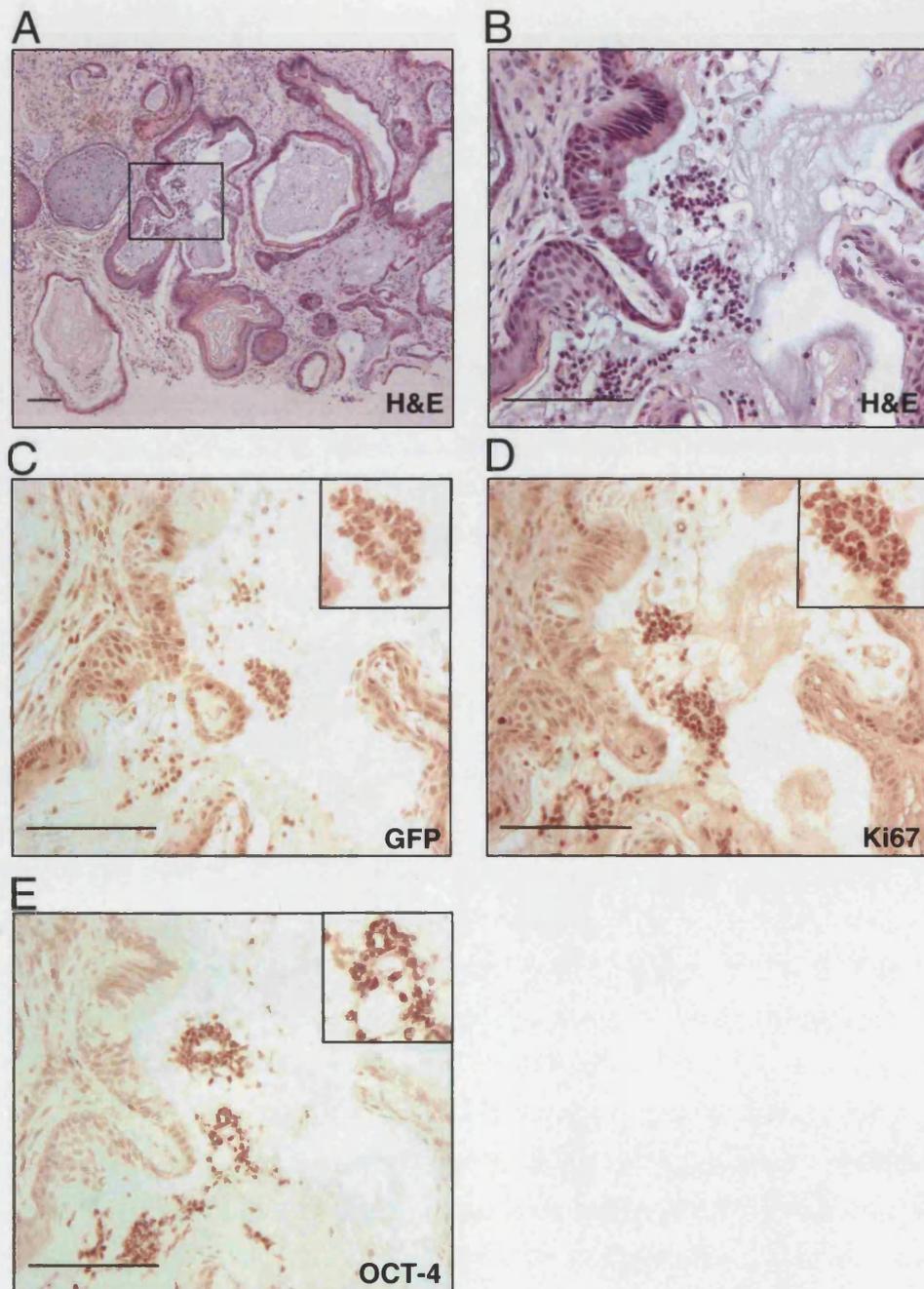
Detection of CFP reporter expression in ES clones was successful by immunofluorescence using a GFP antibody. Immunofluorescence amplifies the signal and thus enables low levels of expression to be detected. A GFP antibody could be used to detect CFP expression as the two have very similar coding sequences. The ECFP variant compared to the EGFP variant contains six amino acid substitutions. One of these substitutions is responsible for the ECFP fluorescence excitation and the other five enhance the brightness and solubility of the protein (Heim *et al.*, 1994; Heim and Tsien, 1996).

Reporter expression in ES clones seen in this investigation is in line with previous observations that reporters driven by the *mTert* promoter are expressed in ES cells (Armstrong *et al.*, 2000; Pericuesta *et al.*, 2006). Upon differentiation of the pTertpromECFP-1 ES clones, reporter expression was down-regulated as seen by immunofluorescence and RT-PCR. Again this correlates with previous findings using similar *mTert* promoter reporters (Armstrong *et al.*, 2000; Pericuesta *et al.*, 2006).

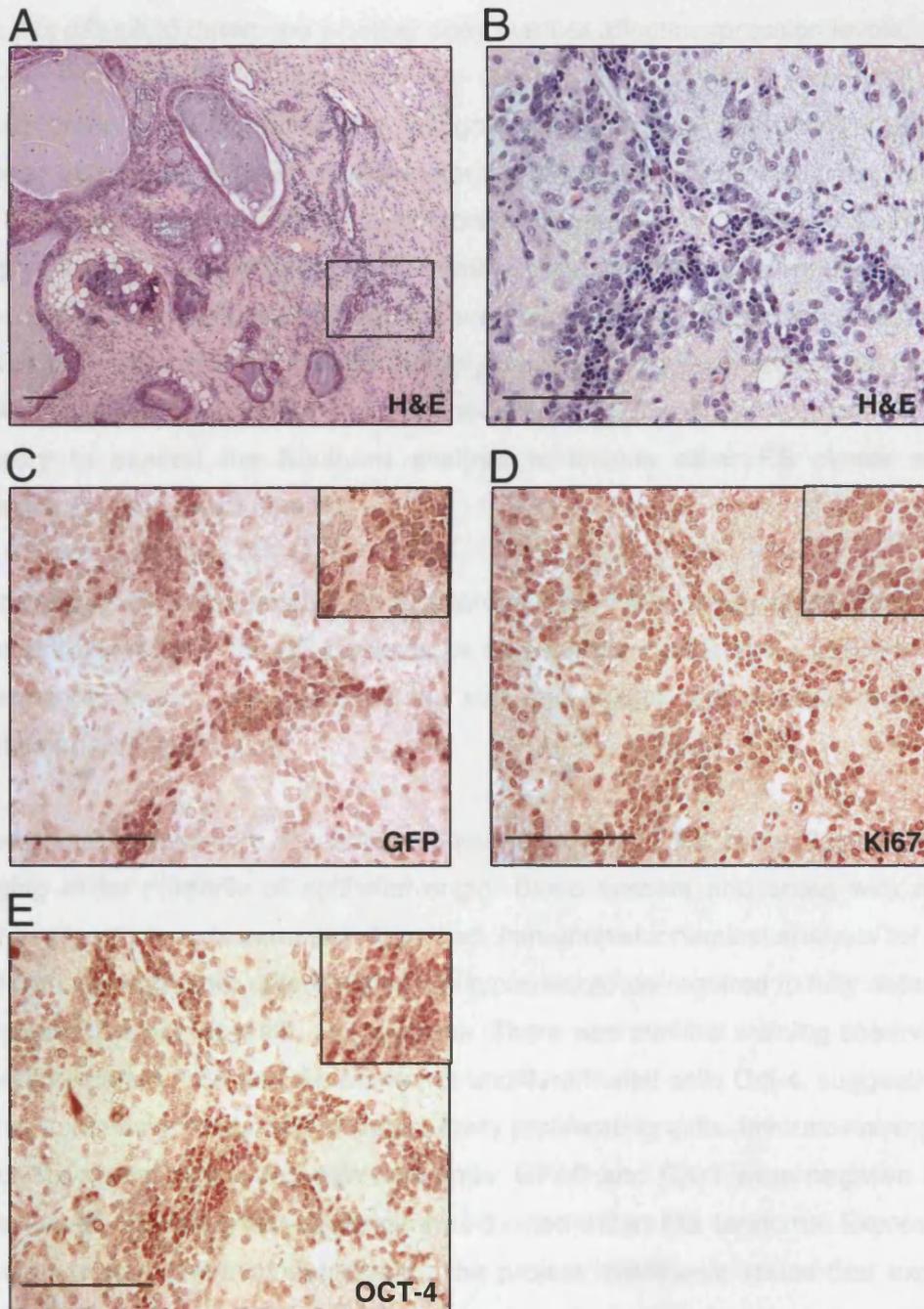
**Figure 4.13** Immunohistochemical analysis of teratoma derived from subcutaneous injection of nude mouse with ES clone 38. **(A)** H&E staining overview of teratoma **(B)** Area of teratoma demonstrating muscle morphology. Teratoma 5 $\mu$ m serial sections were immunostained with GFP **(C)**, Ki67 **(D)** and Oct-4 **(E)**. Scale = 100 $\mu$ m



**Figure 4.14** Immunohistochemical analysis of teratoma derived from subcutaneous injection of nude mouse with ES clone 38. **(A)** H&E staining overview of teratoma, **(B)** H&E staining of region of interest within teratoma. Teratoma 5 $\mu$ m serial sections were immunostained with GFP **(C)**, Ki67 **(D)** and Oct-4 **(E)**. Scale = 100 $\mu$ m



**Figure 4.15** Immunohistochemical analysis of teratoma derived from subcutaneous injection of nude mouse with ES clone 38. (A) H&E staining overview of teratoma (B) H&E staining of region of interest within teratoma. Teratoma 5 $\mu$ m serial sections were immunostained with GFP (C), Ki67 (D) and Oct-4 (E). Scale = 100 $\mu$ m



Copy number analysis of the ES clones using the *neo<sup>R</sup>* probe revealed a difference in the number of integrations of the pTertpromECFP-1 reporter. Of the clones analysed, clone number 17 had the most integrations. The clones analysed by Southern were studied for visualisation of the CFP reporter by direct visualisation. Thus, these clones were continuously cultured which led to slight spontaneous differentiation of some of these clones. Therefore different clones were analysed for expression of the CFP reporter by immunofluorescence. Since the copy number of the reporter was not known for these clones it is difficult to determine whether copy number affects expression levels.

Previous work has suggested that integration site rather than copy number affects transgene expression (Palmiter and Brinster., 1986). A number of ES clones were analysed for CFP reporter expression by immunofluorescence and no difference in expression levels was apparent. This suggests that copy number may not affect CFP reporter expression as determined by immunofluorescence. However, since immunofluorescence results in signal amplification it is perhaps difficult to quantify reporter expression levels using this method. In order to determine whether copy number affects CFP reporter expression it would be necessary to expand the Southern analysis to include other ES clones analysed in immunofluorescence experiments.

The analysis of the differentiation of pTertpromECFP-1 ES clones during the formation of teratomas revealed that the ES clones were able to differentiate into a variety of cell types. The teratomas formed were of expected size and weight and all produced a variety of differentiated cell types.

The teratoma formed from the subcutaneous injection of ES clone 2 gave rise to cells appearing to be primarily of epithelial origin. Blood vessels and areas with morphology similar to smooth muscle were also identified. Immunohistochemical analysis for markers of smooth muscle and other differentiated cell types would be required to fully determine what tissue types had formed within this teratoma. There was minimal staining observed with the proliferation marker Ki67 and the marker of undifferentiated cells Oct-4, suggesting that this teratoma contained mostly differentiated, lowly proliferating cells. Immunostaining using the markers for neural tissue and skin epidermis; GFAP and CK-1 were negative suggesting that neither neural tissue nor epidermis was formed within this teratoma. Expression of the CFP reporter was low to undetectable. The project hypothesis states that expression of *mTert* should correlate with the undifferentiated/highly proliferative phenotype. The fact that the teratoma derived from ES clone 2 expresses little to no reporter correlates with the finding that this teratoma is relatively unproliferative and highly differentiated.

The teratoma formed from ES clone 38 formed a variety of differentiated tissue types including keratinised stratified-squamous epithelia (similar to that found in skin), neural tissue and muscle. These were identified using H&E histochemical staining. Immunostaining with GFAP further identified the region thought to be neural tissue. Neural tissue comprised approximately 40% of the total teratoma. Immunostaining using the CK-1 antibody (a marker of spinous/granular cells found within the epidermis of skin) identified regions of skin (that contained spinous/granular cells) within the teratoma. Many regions of keratinisation could also be easily discerned within the teratoma by H&E staining although many of these did not possess other cell types usually found within the epidermis and therefore these areas are not shown. Expression of the reporter was identified in basal layer-like cells found within the region of keratinised stratified-squamous epithelia. This expression correlated with expression of the proliferation marker Ki67 and the marker of undifferentiated cells Oct-4. Immunostaining with the CK-1 antibody has given high background. There are some spinous/granular layer cells in the region of interest. However, the cells expressing the reporter, Ki67 and Oct-4 do not seem to be expressing CK-1. This suggests that they are undifferentiated cells and are possibly basal layer cells. Future work could be to determine whether these cells are basal layer cells by immunostaining with a cytokeratin 5 or cytokeratin 14 (CK-5, CK-14) antibody as CK-5 and CK-14 are expressed within the basal layer cells of the epidermis. Previous studies have shown that *mTert* overexpression increases stem cell mobilisation from the bulge of the hair follicle and promotes stem cells self-renewal independently of its role in maintaining telomere length (Flores *et al.*, 2005; Sarin *et al.*, 2005). Thus, the finding that the CFP reporter is expressed within basal-layer like cells is in line with the role suggested for *mTert* in the skin.

Many regions of undifferentiated/highly proliferative cells were identified within the teratoma using the markers Ki67 and Oct-4. These regions all expressed high levels of the reporter. No reporter expression was identified in the area of neural tissue identified within the teratoma. This region was highly differentiated and unproliferative as identified by Ki67 and Oct-4 immunostaining.

The skeletal muscle identified within teratoma 38 expressed the reporter very weakly. The Ki67 staining was moderate suggesting that the skeletal muscle is moderately proliferating. The Oct-4 immunohistochemical staining was strong and was specific for the muscle fibre. The fact that the muscle fibre expresses the reporter weakly is in line with the fact that it is a differentiated tissue. However, the moderate and strong levels of Ki67 and Oct-4 expression are confusing. Immunohistochemical analysis with a marker for fully differentiated muscle such as myosin or a marker for Desmin which is expressed in the muscle precursor

(myoblast) would perhaps elucidate whether the muscle tissue formed within this teratoma is fully differentiated (Chen and Goldhamer, 2003). It is also possible that the Oct-4 staining is an artefact. Future work could include determining the precise nature of the skeletal muscle present perhaps by performing immunohistochemical analysis with a range of markers such as myosin or desmin in order to fully explain the results obtained. Immunohistochemical analysis for the homeobox gene *Pax7* could also be performed in order to identify whether any satellite cells (the adult stem cell of skeletal muscle) can be identified and whether these cells express the reporter (Chen and Goldhamer, 2003).

The immunohistochemical analysis of teratoma parallel sections indicates that the CFP reporter, Ki67 and Oct-4 are expressed in many of the same cells. Since the immunohistochemical analysis for each of these antibodies is performed on different sections it is impossible to state definitively that particular single cells are expressing all three. A more definitive method for determining whether these three markers are co-expressed would be co-immunofluorescence on the teratoma tissue sections. Fluorescent immunohistochemistry on tissue sections was not routinely performed in our laboratory. During the later stages of the project, analysis of the intestine by fluorescent immunohistochemistry using the Ki67 antibody proved successful (see Chapter 6). However, co-immunofluorescence on tissue sections has still not been achieved. Thus, future experiments could involve setting-up co-immunofluorescence experiments in order to ultimately determine whether the CFP reporter, Ki67 and Oct-4 are expressed in the same cells.

Taken together the data from this chapter is line with the project hypothesis and suggests that the *mTert* promoter reporter correlates with the stem cell phenotype. Furthermore, it suggests that this expression is down-regulated upon the differentiation of ES cells via EB formation and upon the differentiation of ES cells via teratoma formation into tissues such as neural tissue. However, the reporter can still be detected in regions of intense proliferation, and in relatively undifferentiated cell populations within specific tissues types such as the basal layer-like cells within the skin formed within the teratoma.

## 5. Transgene expression analysis at the RNA level

### 5.1 Introduction

#### 5.1.1 *Tert* expression at the RNA level

hTERT has been shown to be expressed in immortalised cell lines and cancer cells but not in somatic cells (Meyerson *et al.*, 1997; Nakamura *et al.*, 1997). In contrast; mTert is not as tightly regulated as hTERT and is expressed more widely in inbred mice (Greenberg *et al.*, 1998; Prowse and Greider, 1995). *mTert* mRNA has been shown to be at its highest level in embryos around E9.0 until E15.5 dpc (Greenberg *et al.*, 1998; Martin-Rivera *et al.*, 1998). Furthermore, analysis of embryos using *in situ* hybridisation has demonstrated that *mTert* mRNA is expressed throughout the embryo, but is strongly detectable in highly proliferative regions such as the limbs, the tip of the tail and the nares (Martin-Rivera *et al.*, 1998). RT-PCR analysis of adult tissues has shown that *mTert* mRNA is highest in tissues shown to have telomerase activity such as the thymus and in highly proliferative tissues such as the intestine, testis and the liver (Greenberg *et al.*, 1998; Martin-Rivera *et al.*, 1998).

### 5.2 Aims

The *mTert* expression pattern has been extensively characterised at the RNA level. Thus, the aim of this chapter was to characterise the expression pattern of both endogenous *mTert* and the reporter at the RNA level and determine whether expression of the two correlates. This was achieved using semi-quantitative RT-PCR. The pattern of expression of endogenous *mTert* was also characterised using qPCR. However, due to time limitations, conditions for qPCR for CFP reporter expression levels could not be developed.

### 5.3 Results

#### 5.3.1 RT-PCR analysis of wild-type and transgenic adult tissue

As discussed previously, the expression of *mTert* has been mostly characterised at the RNA level. For this reason, pTertpromECFP-1 reporter expression in adult tissue was assessed by RT-PCR. RNA was prepared from a range of adult tissues from mice from lines 3 and 4 that tested positive for the reporter at the DNA level by PCR analysis. RNA was also prepared from a wild-type mouse as a control. The range of tissues analysed included those known to express *mTert* such as the testis and those thought to express low levels of *mTert* such as the brain. The RNA was treated with DNase in order to remove contaminating DNA followed by treatment with a DNase inactivation reagent in order to deactivate the DNase. Next, a reverse transcription reaction was performed to generate cDNA which was then used as template DNA in PCR analysis. PCR was performed using

primers specific for the reporter (CFP-1, CFP-2 and another primer pair CFP-3 and CFP-4), primers for endogenous *mTert* (mTertRTPCRfor and mTertRTPCRrev) and primers for *GAPDH* (OCP174 and OCP175) as a control. The RT-PCR analysis was semi-quantitative as the number of cycles performed ensured that the PCR was in the linear range of amplification. Two different sets of primers were used to assess the pTertpromECFP-1 expression to ensure that the reporter was being detected and also in case one set of primers proved more sensitive than the other. Experimental controls included cDNA from ES clone 2 transfected with pTertpromECFP-1 which had been shown to express the reporter by immunofluorescence; thus this was used as a positive control (Chapter 4). Negative controls included cDNA from a wild-type animal which should not express CFP and a control for each tissue made using no reverse transcriptase enzyme; thus no cDNA should be synthesised. All controls were run simultaneously with samples of interest. Minimal contamination was observed in the samples made with no reverse transcriptase with all primer sets (Figures 5.1-5.3).

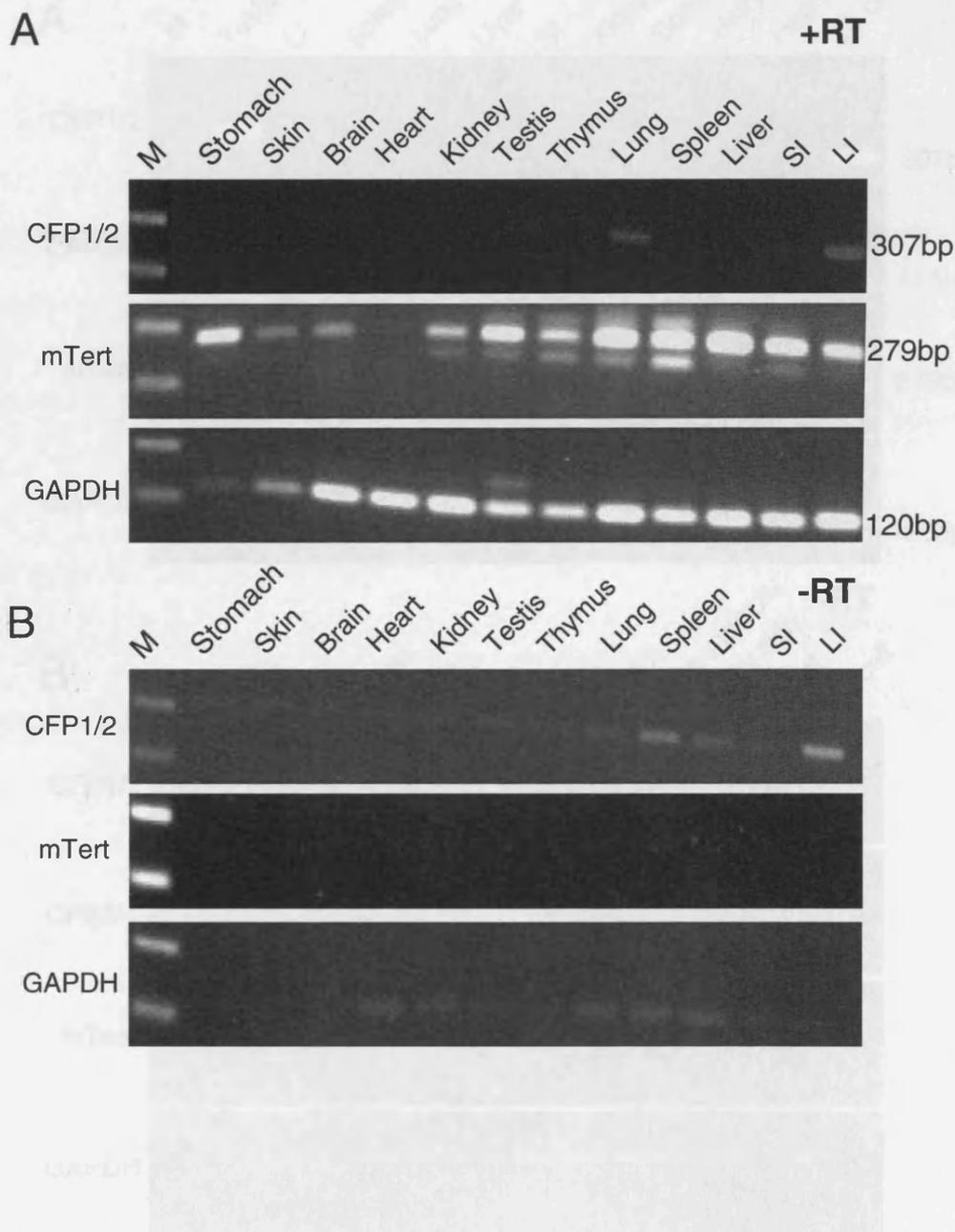
There was no CFP reporter expression in the wild-type samples as expected (Figure 5.1). Endogenous *mTert* expression was widely expressed in the wild-type tissue (Figure 5.1). The general pattern of expression is similar to that reported in the literature.

The expression pattern of the CFP reporter as assessed by RT-PCR in lines 3 and 4 is outlined in Table 5.1 and Figures 5.2 and 5.3. Expression of the reporter was highest in the testis of both transgenic lines analysed (Figures 5.2 and 5.3). In line 3, as well as in the testis, strong CFP reporter expression was detected in the kidney and SI (Figure 5.2/Table 5.1). The pattern of reporter expression pattern determined using the two primer sets generally correlated. However, moderate reporter expression was identified using one set of reporter primers (CFP3/4) in the LI but not detected at all in this tissue using the other set (Figure 5.2). Low expression of the reporter was found in the spleen, skin, heart and liver (Figure 5.2/Table 5.1). No expression was detected in the lung.

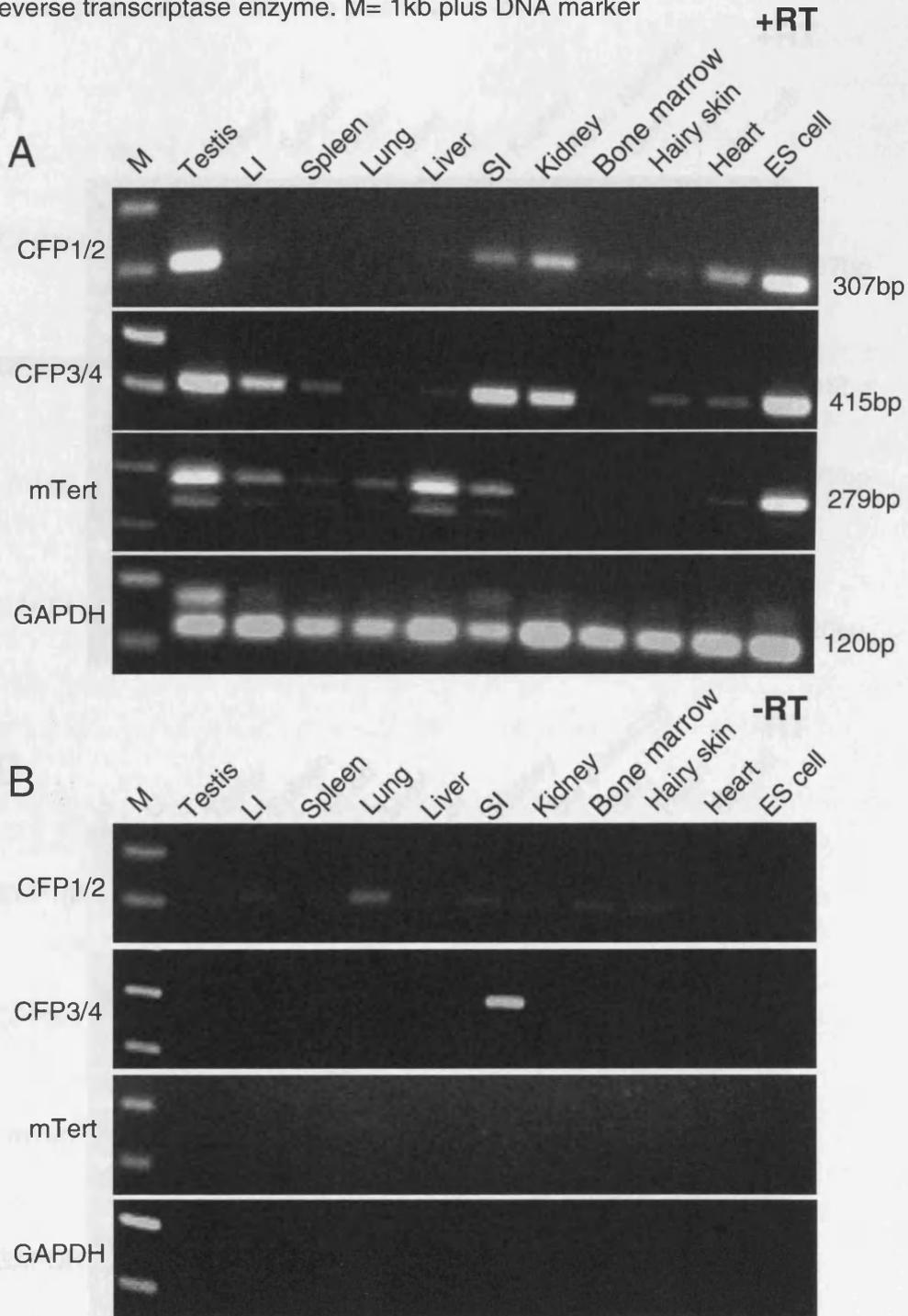
Generally transgene expression correlated with *mTert* expression. However, a high to moderate level of reporter expression was detected in the kidney of this line, but no endogenous *mTert* expression could be detected. Furthermore only a low level of reporter expression was detectable in the liver, yet this tissue expresses a high level of endogenous *mTert* (Figure 5.2).

In line 4 CFP reporter expression was moderate in the spleen and liver and weak in the SI, bone marrow, skin and heart (Figure 5.3/Table 5.1). Tissues in which reporter was absent

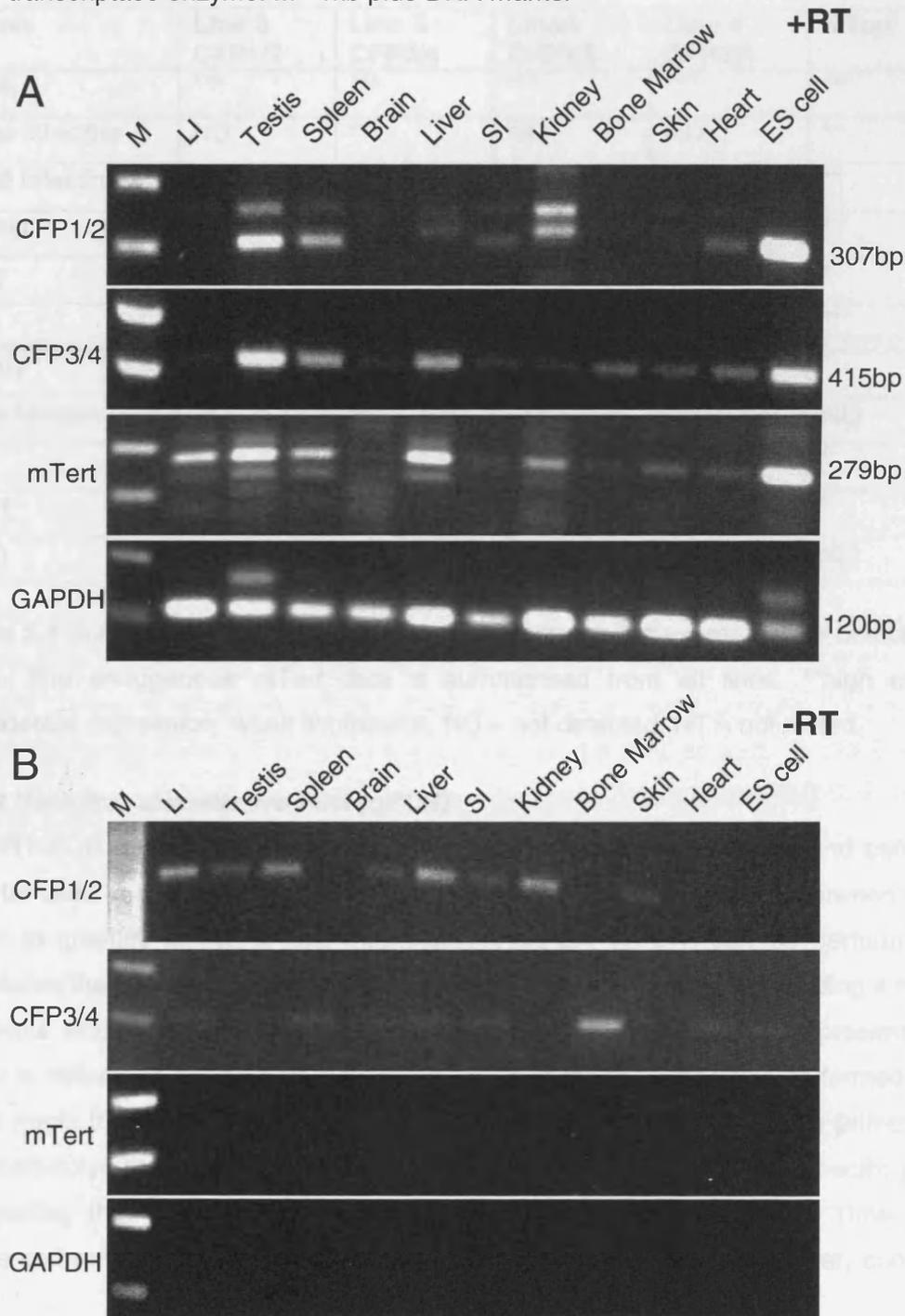
**Figure 5.1** Semi-quantitative RT-PCR of C57BL/6 wild-type mouse adult tissues. **(A)** RT-PCR for CFP which produces a product of 307bp using primers CFP-1 and CFP-2 (top panel), RT-PCR for endogenous *mTert* using primers mTertRTPCRfor and mTertRTPCRrev generating a product of 279bp (middle panel) and RT-PCR for GAPDH as a loading control producing a product of 120bp using primers OCP174 and OCP175 (bottom panel). **(B)** Control samples for each tissue made using no reverse transcriptase enzyme. M = 1kb plus DNA marker



**Figure 5.2** Semi-quantitative RT-PCR of adult transgenic mouse tissues from line 3. **(A)** RT-PCR for CFP with primers CFP-1 and CFP-2 which generate a product of 307bp (top panel), RT-PCR for CFP using primers CFP-3 and CFP-4 (second top panel) generating a 415bp product, RT-PCR for endogenous *mTert* using primers mTertRTPCRfor and mTertRTPCRrev generating a 279bp product (second to bottom panel) and GAPDH as a loading control generating a product of 120bp using primers OCP 174 and OCP 175 (bottom panel). **(B)** Control samples for each tissue made using no reverse transcriptase enzyme. M= 1kb plus DNA marker



**Figure 5.3** Semi-quantitative RT-PCR of adult transgenic mouse tissues from line 4. **(A)** RT-PCR for CFP with primers CFP-1 and CFP-2 which generate a product of 307bp (top panel), RT-PCR for CFP using primers CFP-3 and CFP-4 (second top panel) generating a 415bp product, RT-PCR for endogenous *mTert* using primers mTertRTPCRfor and mTertRTPCRrev generating a 279bp product (second bottom panel) and GAPDH as a loading control generating a product of 120bp using primers OCP 174 and OCP 175 (bottom panel) **(B)** Control samples for each tissue made using no reverse transcriptase enzyme. M= 1kb plus DNA marker



include the LI and brain. The CFP reporter expression pattern determined by CFP1/2 was very similar to that obtained using CFP3/4. Furthermore, in line 4, expression of the transgene generally correlates well with expression of endogenous *mTert* (Figure 5.3). However, a moderate level of endogenous *mTert* was detected in the LI, but no transgene expression was detected.

Tissue	Line 3 CFP1/2	Line 3 CFP3/4	Line4 CFP1/2	Line 4 CFP3/4	mTert
Testis	***	***	***	***	***
Large Intestine	ND	**	ND	ND	**
Small Intestine	**	**	*	*	**
Spleen	ND	*	**	**	**
Lung	ND	ND	NT	NT	*
Liver	ND	*	ND	**	***
Kidney	**	**	ND	*	*
Bone Marrow	ND	ND	ND	*	ND
Skin	*	*	ND	*	*
Heart	**	*	*	*	*
Brain	NT	NT	ND	ND	ND

**Table 5.1** Summary of CFP reporter and endogenous *mTert* expression as detected by RT-PCR. The endogenous *mTert* data is summarised from all lines. \*\*high expression, \*\*moderate expression, \*weak expression. ND = not detected. NT = not tested.

### 5.3.2 Real-time quantitative PCR (qPCR)

The RT-PCR described in section 5.3.1 is not completely quantitative and can therefore only be used to give an indication of the difference in expression levels between tissues. In order to quantify mRNA levels, quantitative RT-PCR (qPCR) can be performed. qPCR measures the accumulation of amplified product. This is achieved by including a fluorescent molecule whose signal increases proportionally to the amount of DNA present. Thus, in order to determine the precise expression levels of *mTert*, qPCR was performed. Attempts were made to quantify reporter expression levels by qPCR using various primer sets, but the melt-curve produced in the initial experiments did not give rise to a specific peak; thus suggesting that none of the primers were specifically detecting GFP. Time limitations prevented further attempts to develop conditions for CFP qPCR. However, conditions for

mTert were established. The primers mTertRTPCRfor and mTertRTPCRrev and the cDNA from line 3 used for semi-quantitative RT-PCR were also used for *mTert* qPCR.

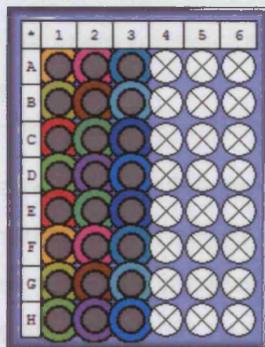
In order to explain the qPCR data, the principles of qPCR must be discussed. The qPCR reaction can be split into two phases. The first phase is the exponential phase during which the amount of PCR product approximately doubles each cycle. As the qPCR reaction continues, reaction components are used up resulting in a slowing of the reaction into the plateau phase. During the initial stages of the exponential stage of the reaction, the fluorescence remains at background levels until enough product accumulates to yield a detectable fluorescent signal. The cycle number at which this occurs is known as the threshold cycle ( $C_T$ ).

In order to perform a qPCR experiment, the parameters of the assay need to be optimised. Optimisation begins with an annealing temperature gradient to determine at which annealing temperature the assay should be performed. To verify the result obtained all qPCR reactions are performed in triplicate. ES cell cDNA from a wild-type animal was used in a temperature gradient from 72°C to 60°C in order to determine the optimal annealing temperature for the *mTert* qPCR (Figure 5.4). The specificity of the reaction products can also be checked at this stage by assessing the melt-curve data graph. The melt-curve data graph should exhibit a single peak which corresponds to a single product produced in the reaction. As can be seen from Figure 5.4, the melt-curve shows one peak corresponding to the specific amplification of *mTert* by mTertRTPCRfor and mTertRTPCRrev. The most efficient annealing temperature is the one that gives the lowest  $C_T$  value. The gradient did not show much difference between the  $C_T$  values obtained at each temperature (Figure 5.4). However, there was a trend that the  $C_T$  values were decreasing with decreasing temperature. Thus, 60°C was chosen as the optimal temperature for the qPCR assay.

The next step in optimisation of a qPCR assay involves running serial dilutions of the template at the optimal annealing temperature and constructing a standard curve. The standard curve is constructed by plotting the log of the starting quantity of template or the dilution factor against the  $C_T$  value obtained during amplification of each dilution. The equation of the linear regression line along with the coefficient of determination ( $R^2$ ) can be used to determine whether the assay is optimised. The  $R^2$  value reflects how linear the data are, which gives an indication of the variability across triplicate samples. Thus, the  $R^2$  value should be >0.98. The serial dilution series is also used to calculate the amplification efficiency of the reaction. The amplification efficiency should be between 90-105% as this

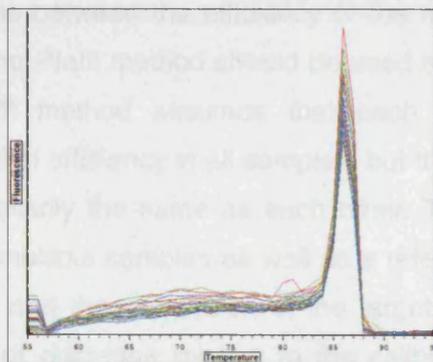
**Figure 5.4.** qPCR *mTert* gradient. 72°C-60°C (A) Microtitre plate and tabled data. A1-A3 triplicate samples at the same annealing temperature. B1-B3 triplicate samples at different annealing temperature etc. Average  $C_T$  values obtained at each temperature are shown (B) Melt-curve showing one peak corresponding to the specific amplification of *mTert*. Amplification curves obtained during the gradient are shown. The dotted line represents the Threshold line at which point the curves begin to linearise. The 60°C temperature gave the lowest  $C_T$  value and therefore was selected as the optimal annealing temperature for the qPCR assay.

A

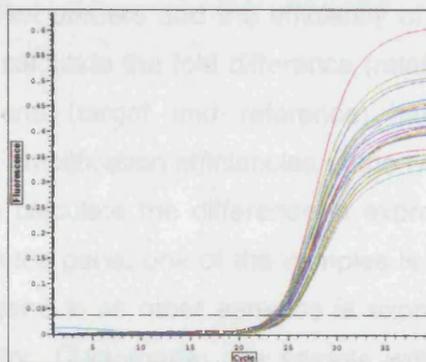


Sample lanes	Temp	Average $C_T$
A1-A3	72°C	24.64
B1-B3	71.2°C	25.05
C1-C3	69.7°C	24.49
D1-D3	67.5°C	24.14
E1-E3	64.8°C	23.64
F1-F3	62.6°C	24.19
G1-G3	60.9°C	23.94
H1-H3	60°C	23.53

B



Melt-curve



Amplification curves

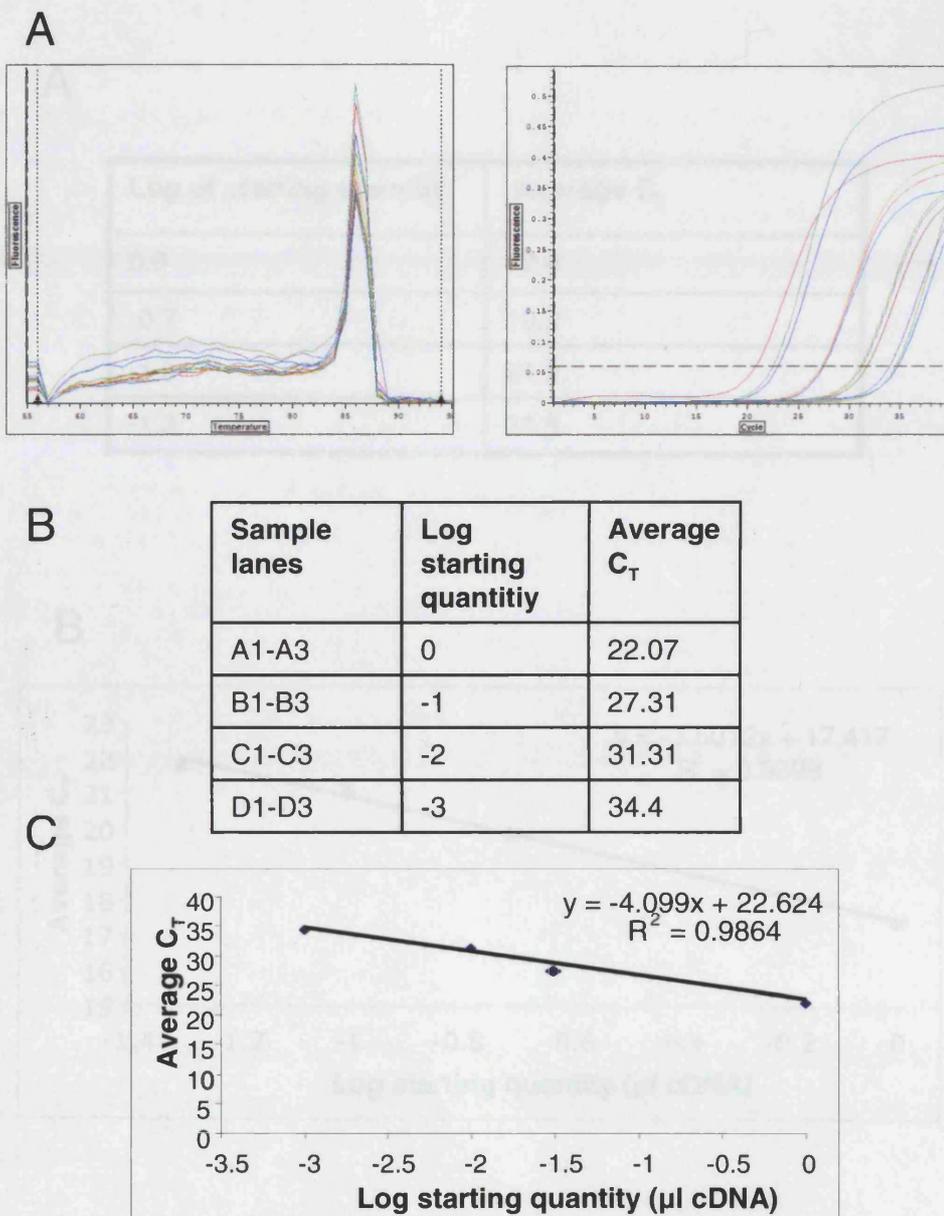
indicates that the amount of PCR product perfectly doubles during each cycle of exponential amplification.

The wild-type ES cell cDNA was used to run a serial dilution series at 60°C for optimisation of the qPCR assay. A 10-fold dilution series was performed in triplicate. The four dilutions used to generate the standard curve and the corresponding melt-curve and amplification curves are shown in Figure 5.5. The standard curve is shown in Figure 5.5. The  $R^2$  value was found to be  $>0.986$ . The efficiency of the *mTert* qPCR reaction was calculated using the following formula;  $E = 10^{-1/\text{slope}}$  where the slope = -4.099. The efficiency of the *mTert* primers was calculated to be 75%, and therefore the amplification of *mTert* is not perfectly exponential. When this occurs the primers can be redesigned to achieve a higher amplification efficiency. However, in this case due to time limitations, the Pfaffl method can be used to calculate expression levels as this takes account of the amplification efficiency of the primers.

Since the amount of cDNA present in the reaction will affect the quantitation of *mTert*, the data obtained by *mTert* qPCR needs to be compared to that obtained for a reference gene (in this case *GAPDH*) for each tissue. The *GAPDH* primers used in this investigation for semi-quantitative RT-PCR had been optimised for qPCR by Dr E. Debrand, Department of Biochemistry, University of Leicester. The efficiency of the *GAPDH* primers was found to be 93% and the  $R^2$  value was 0.99 (Figure 5.6).

The *mTert* qPCR experiment was performed using cDNA from tissues from reporter line 3 and ES clone 2. The tissues analysed included liver, SI, LI, testis and skin. Since there are differences between the efficiency of the *mTert* primers and the efficiency of the *GAPDH* primers the Pfaffl method should be used to calculate the fold difference (relative amount). The Pfaffl method assumes that each gene (target and reference) has the same amplification efficiency in all samples, but the amplification efficiencies of the two genes are not necessarily the same as each other. To calculate the difference in expression levels between multiple samples as well as a reference gene, one of the samples is chosen as a calibrator and the expression of the target gene in all other samples is expressed as an increase or decrease relative to the calibrator. Customarily, the sample with the lowest expected expression of the target gene is chosen as the calibrator. The sample presumed to have the lowest expression level of *mTert* in this investigation was skin. This assumption was based on RT-PCR data obtained in this investigation. The -RT controls were also run with the samples and no reproducible amplification was seen (Figure 5.7). The melt-curve

**Figure 5.5.** *mTert* dilution series. **(A)** Melt-curves and amplification curves produced by serial dilution series. One peak corresponding to specific amplification of *mTert* is shown. **(B)** Average  $C_T$  values and log of starting quantity used to plot standard curve. **(C)** *mTert* standard curve with the average  $C_T$  plotted against the log of the starting quantity of template for each dilution. The equation for the regression line and the  $R^2$  value are shown. The calculated amplification efficiency was 75%.

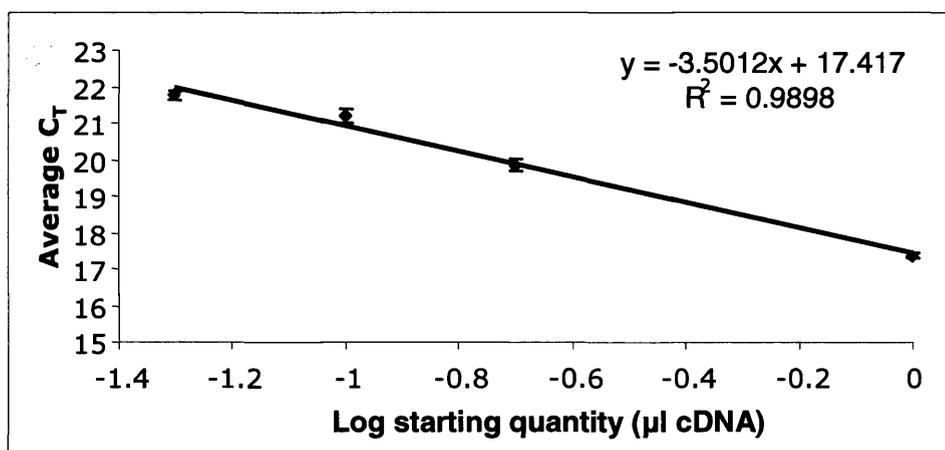


**Figure 5.6.** GAPDH standard curve. **(A)** Dilutions used to calculate the GAPDH standard curve. The average  $C_T$  values for the various dilutions of template plotted to generate the standard curve are shown. **(B)** GAPDH standard curve with the average  $C_T$  plotted against the log of the starting quantity of template for each dilution. The equation for the regression line and the  $R^2$  value are shown. The calculated amplification efficiency was 93%.

**A**

Log of starting quantity	Average $C_T$
0.0	17.4
-0.7	19.9
-1.0	21.2
-1.3	21.8

**B**



and amplification curves obtained during the *mTert* sample analysis are shown in Figure 5.8. These data show that specific amplification of *mTert* was achieved.

The equation used to calculate the fold difference in expression levels between *mTert* and *GAPDH* is as follows;

$$\text{Ratio} = \frac{(E_{\text{target}})^{\Delta\text{CT, target (calibrator-test)}}}{(E_{\text{ref}})^{\Delta\text{CT, ref (calibrator-test)}}$$

Where the  $E_{\text{target}}$  is equal to the efficiency of the primers used to amplify the gene of interest (in this case *mTert*), and  $E_{\text{ref}}$  is equal to the efficiency of the primers used to amplify the reference gene (in this case *GAPDH*). The calibrator in this case is the skin sample. The  $C_T$  values used to calculate the fold difference are outlined in Table 5.2. The fold difference in expression of *mTert* is outlined in table 5.2 and shown in Figure 5.9.

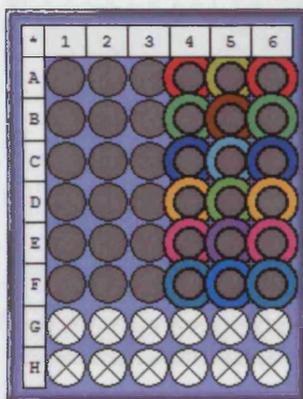
Sample	<i>mTert</i> $C_T$	<i>GAPDH</i> $C_T$	Fold difference	Standard Deviation
ES cell	26.61	17.43	20	0.61
LI	33.07	22.02	6.6	0.58
Liver	29.7	19.98	14.5	0.55
SI	33.4	23.8	15.5	0.51
Testis	28.54	27.88	2303	0.58
Skin	36.43	24.14	0 (calibrator)	0.52

**Table 5.2**  $C_T$  values and fold difference of *mTert* expression in the ES cell and various tissues.

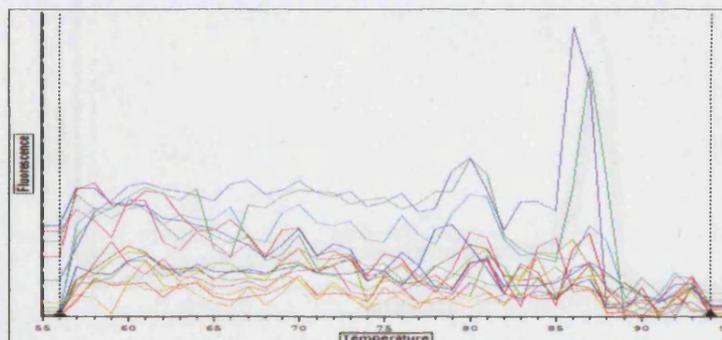
The expression pattern of *mTert* as assessed using qPCR closely correlates with that identified using semi-quantitative RT-PCR. The *mTert* expression in the testis is 2000X that found in the skin. The standard deviations calculated for each tissue are shown in Table 5.2 and Figure 5.9.

**Figure 5.7.** qPCR analysis of *mTert* expression in various tissues. **(A)** Microtitre plate showing -RT control samples. These samples were run in triplicate. **(B)** Melt-curve analysis of -RT control samples showing two peaks probably corresponding to experimental contamination. **(C)** Data graph of *mTert* -RT control samples.

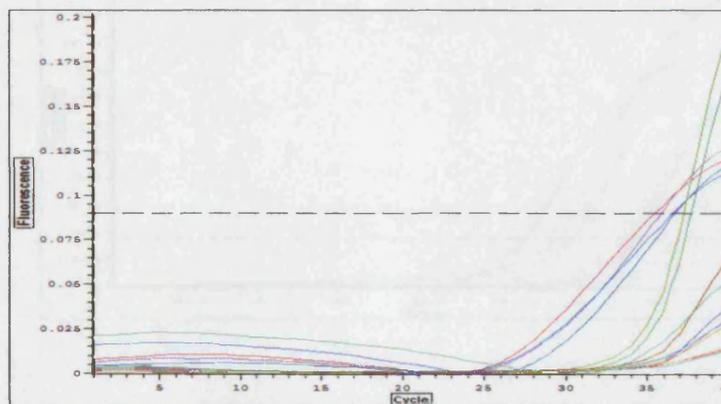
**A**



**B**

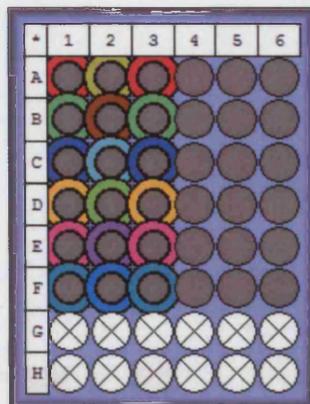


**C**

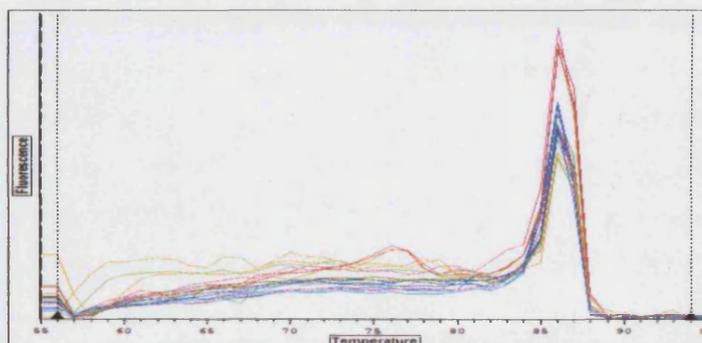


**Figure 5.8.** qPCR analysis of *mTert* expression in various tissues. **(A)** Microtitre plate showing tissue samples and ES sample wells. The samples were run in triplicate. **(B)** Melt-curve showing one peak corresponding to specific amplification of *mTert*. **(C)** Data graph of *mTert* amplification curves obtained during qPCR analysis of tissue and ES cell cDNA.

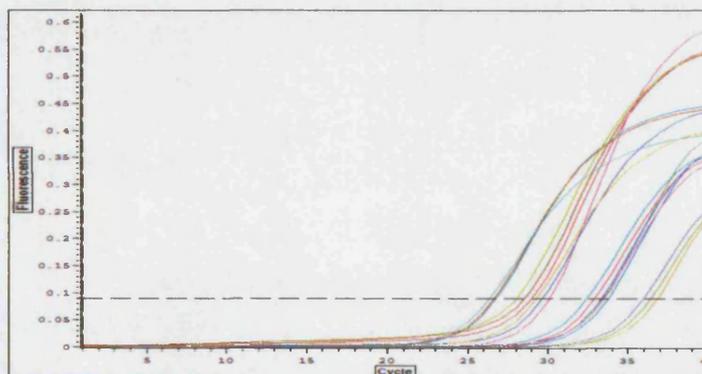
**A**



**B**



**C**





#### 5.4 Discussion

This chapter describes the analysis of adult mouse tissue for the expression of the pTertpromECFP-1 reporter and endogenous *mTert* by semi-quantitative RT-PCR and qPCR.

Previous analysis of *mTert* expression using RT-PCR has been performed (Greenberg *et al.*, 1998; Martin-Rivera *et al.*, 1998; Pericuesta *et al.*, 2006; Ritz *et al.*, 2005). Expression of *mTert* as assessed by mRNA levels in a panel of adult tissues was found to be consistently high in the testis and liver in all previous investigations. Adult tissues consistently exhibiting moderate to weak levels of *mTert* mRNA included spleen, intestine and ovary. Tissues with a moderate to weak expression of *mTert* mRNA included lung, kidney, heart, brain and muscle. The analysis of endogenous *mTert* expression by RT-PCR performed in this investigation for the most part agrees with previous published observations. High *mTert* mRNA levels were consistently detected in the testis and liver and moderate levels were detected in the intestine and spleen. The precise level of *mTert* expression in adult tissues was determined by qPCR. The general expression pattern of *mTert* is as would be expected; the testis has the highest level of expression followed by the ES cell, the SI, the liver and then the LI. However, the level of *mTert* expression in the testis as detected by qPCR was extremely high. The calculated fold difference of *mTert* expression in the testis was 2303. Thus, the testis has 2000X more *mTert* in comparison to the calibrator – the skin. This implies that the expression level of *mTert* in the skin is extremely low. However, reporter expression was detectable in the skin (Chapter 6) suggesting that although expression is low, it is still detectable by immunohistochemistry. The *mTert* levels in the testis according to these data are extremely high. *mTert* and *hTERT* are known to be expressed in the testis (Greenberg *et al.*, 1998; Martin-Rivera *et al.*, 1998). Furthermore, it has previously been reported that sperm have long telomeres and that these are required in order to maintain telomere length during division-rich development (Allsopp *et al.*, 1992; Coviello-McLaughlin and Prowse, 1997; Prowse and Greider, 1995). Thus, high levels of *mTert* within the testis found in this investigation are in line with previous findings.

The RT-PCR analysis was only performed on lines 3 and 4 which are maintained by backcrossing to C57BL/6 inbred mice. It would be of interest to analyse the expression pattern at the RNA level in lines 1 and 2 which are maintained on the outbred MF-1 background. The mice maintained on the outbred background are more like wild-mice and possess telomeres similar in length to humans. Thus, the expression pattern of the CFP reporter may be more restricted in these lines and more like that found in human tissues.

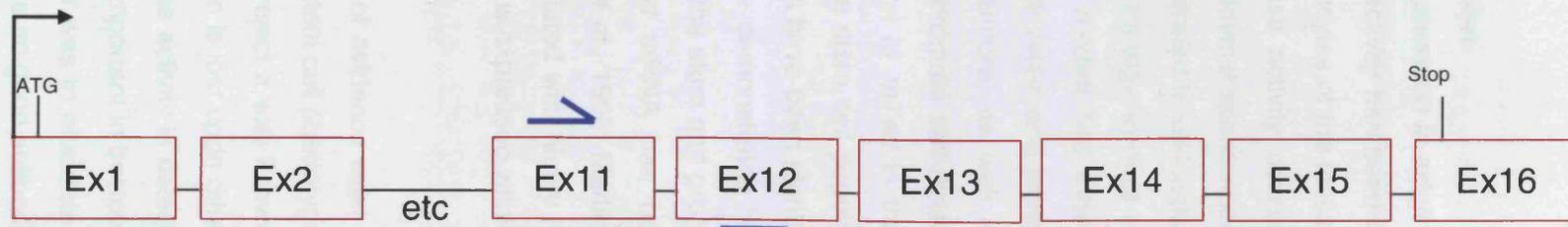
Whilst this work was being undertaken, two groups reported the generation of similar *Tert* promoter mouse models. Ritz *et al* (2005) reported the generation of an 8kb fragment of the *hTERT* promoter driving the expression of the *LacZ* gene. The investigators performed RT-PCR with primers specific for the reporter and thus measured *LacZ* gene expression in the three transgenic lines generated (Ritz *et al.*, 2005). In two of the three transgenic lines generated, reporter expression as assessed by RT-PCR was restricted to the testis. In the other transgenic line low level expression was also detected in the spleen, kidney, intestine, mammary glands and brain (Ritz *et al.*, 2005). Thus, it was found that generally expression of the reporter correlated with the expected expression pattern of *hTERT* found in human tissues. Pericuesta *et al* (2006) reported the generation of three different mouse models comprising three regions of the *mTert* promoter driving the GFP coding sequence (see Chapter 1). RT-PCR analysis for the GFP reporter was carried out on six transgenic lines (two for each reporter construct) (Pericuesta *et al.*, 2006). It was demonstrated that overall the GFP mRNA levels were lower than those identified for endogenous *mTert*, but the pattern obtained generally recapitulated the endogenous *mTert* transcription pattern (Pericuesta *et al.*, 2006).

In this investigation, the expression of the CFP reporter was determined using RT-PCR primers for CFP. The expression of the reporter as detected using the two different CFP primer pairs generally correlated. However, the primer set generating a band of 415bp proved to be more sensitive, thus allowing very low levels of expression to be detected. Reporter expression was found to be consistently high in the testis, liver and intestine. In general, reporter expression correlated with the endogenous *mTert* expression pattern. However, in reporter line 4 a high to moderate level of endogenous *mTert* was detected in the LI, but no transgene expression was identified. Furthermore, a low level of reporter expression was detected in the liver of transgenic line 3, yet this line expresses a high level of endogenous *mTert*. Moreover, in line 3 a high level of transgene expression was detected in the kidney, yet no endogenous *mTert* was identified. These data are similar to the data presented by Pericuesta *et al* (2006). These investigators reported that the transgene expression recapitulated that of endogenous *mTert*. However, in their data for some tissues endogenous *mTert* was detected, but there was no expression of the reporter and vice versa. Furthermore, as with the data presented here, there are slight differences in reporter gene expression between the different transgenic lines generated.

The regulation of telomerase activity is complex and involves many mechanisms including transcriptional control, post-translational modification and alternative splicing. Thus, when evaluating TERT gene expression and telomerase activity it is important to consider the fact

that hTERT is known to exist as various isoforms, and it is not currently clear whether this is the case for mTert (Forsyth *et al.*, 2002). It is also important to note that a dominant-negative variant of hTERT exists which acts as an inhibitor of telomerase activity and that many telomerase-negative cells possess full-length *hTERT* mRNA (Colgin *et al.*, 2000; Saeboe-Larssen *et al.*, 2006). Therefore the identification of mRNA does not necessarily indicate telomerase activity. Thus, when evaluating cells and tissues for telomerase activity, the TRAP assay should be employed alongside any other experimental methods used.

The primers used to detect endogenous *mTert* mRNA in this investigation, were in exons 11 and 12 (Figure 5.10). It is not known whether alternative splice variants for mTert exist and therefore it is difficult to predict whether the primers used would have identified any splice variants. The known *hTERT* variants mostly have insertions or deletions within the early part of the gene, e.g. there are three variants which involve splicing of intron 2 (Saeboe-Larssen *et al.*, 2006). Since there are similarities between the gene structure of *hTERT* and *mTert* (e.g. they both have sixteen exons), they may have similar splice sites. If this is the case then the location of the primers used in this investigation probably couldn't be used to discriminate between splice variants. More detailed investigation is required to determine whether splice variants of *mTert* exist and their functions in order to aid the understanding of telomerase function and regulation.



**Figure 5.10** Position of the primers used to identify *mTert* mRNA in this investigation. The *mTert* gene has 16 exons, the primers are positioned within exons 11 and 12.

## 6. Transgene expression analysis and correlation with the stem cell phenotype *in vivo*

### 6.1 Introduction

#### 6.1.1 *Tert* expression in adult tissues

Telomerase activity has been demonstrated to be at its highest within the spermatogonia and spermatocytes of the testis (Eisenhauer *et al.*, 1997; Wright *et al.*, 1996). Furthermore, this telomerase activity decreases as spermiogenesis progresses until the telomerase negative epididymal spermatozoa are formed (Ravindranath *et al.*, 1997). *mTert* mRNA has been demonstrated to co-localise with potential stem cells (LRCs) in the bulge region of the hair follicle in transgenic mice overexpressing *mTert* (Sarin *et al.*, 2005). Further analysis of *mTert* mouse models has shown that *mTert* overexpression promotes mobilisation of stem cells from their niche and increases proliferative potential (Flores *et al.*, 2005; Sarin *et al.*, 2005). Furthermore, as well as increase mobilisation, *mTert* overexpression has been proposed to promote self-renewal of the stem cell population (Sarin *et al.*, 2005). Thus, overexpression of *mTert* in the skin is associated with increased proliferation and self-renewal of the stem cell population. In the intestine, low levels of telomerase activity and *hTERT* mRNA have been identified (Hiyama *et al.*, 2001). Immunohistochemical analysis of the colon has demonstrated hTERT protein expression in the lower third of the colonic crypts where the stem and progenitor cells of the colon reside (Hiyama *et al.*, 2001). In the mouse, *mTert* mRNA has been identified, but no protein expression was detected (Greenberg *et al.*, 1998; Martin-Rivera *et al.*, 1998). Thus, in humans hTERT expression can be associated with highly proliferative cells of the colon, but it is still unclear whether mTert protein is expressed within the mouse intestine.

### 6.2 Aims

Several lines of evidence exist to suggest that telomerase activity via *mTert* expression is linked to the stem cell phenotype. Most of these were discussed in Chapter 1. At the time of starting the project it was known for example that *mTert* is expressed in ES cells and that this expression is lost upon differentiation. Furthermore there was evidence in the literature that telomerase activity is detectable in adult stem cells such as HSCs and this expression seems to be important in bestowing the stem cell with a high proliferative potential. The aim of this chapter was to elucidate whether *mTert* can be used as an adult stem cell marker. Since there are no good antibodies commercially available for mTert, a CFP reporter mouse using the *mTert* promoter was generated with the hope of identifying adult stem cells. Thus, the aim of this chapter was to characterise expression of the CFP reporter in adult tissues

and correlate this with the geographical location of stem cells. This was achieved using immunohistochemical analysis to determine the specific cellular localisation of reporter gene expression.

### 6.3 Results

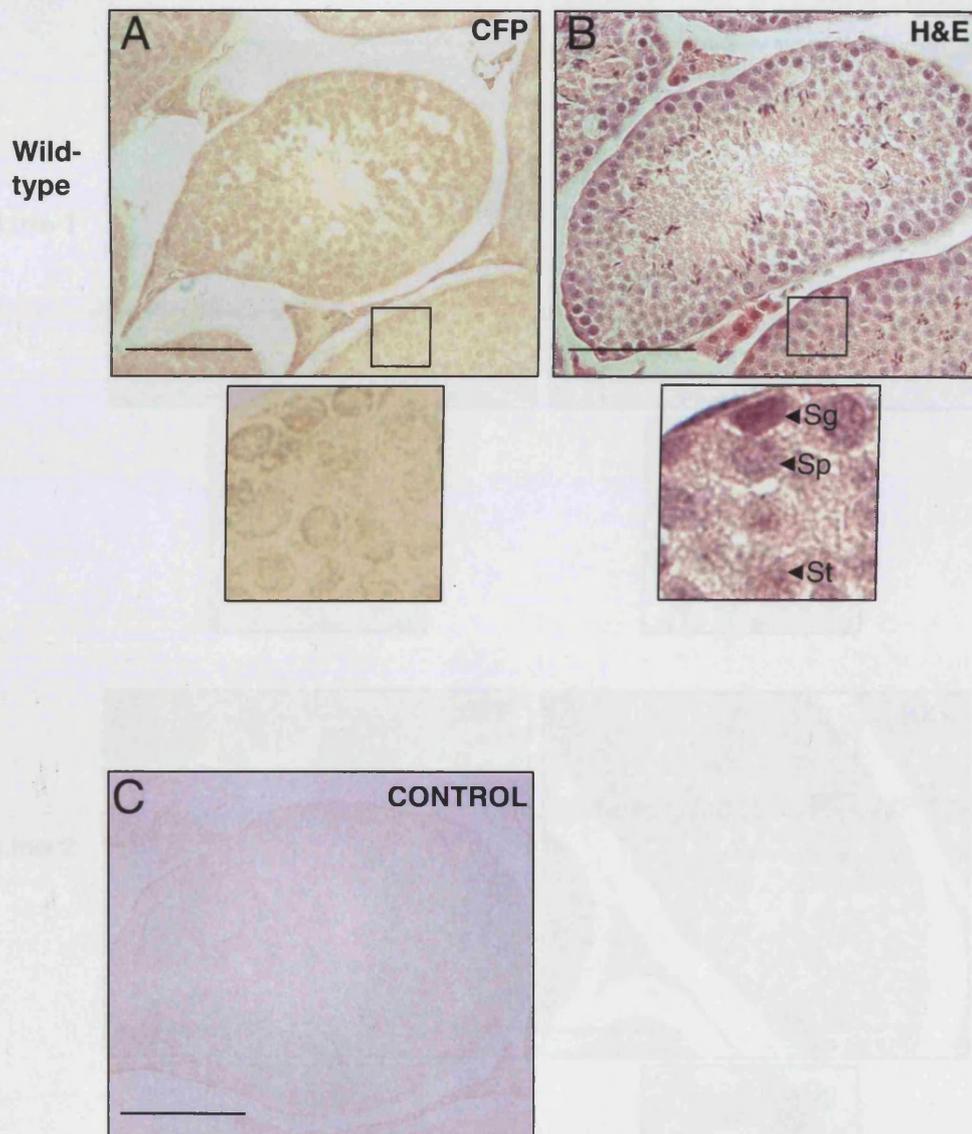
#### 6.3.1 Immunohistochemical analysis of the testis

RT-PCR analysis described in Chapter 5 revealed that the CFP reporter was expressed at high levels in the testis in both line 3 and line 4, which were both maintained on the inbred C57BL/6 background. In order to determine the cellular localisation of CFP reporter expression, immunohistochemical analysis using a CFP antibody was performed, on tissue sections from these mice. Lines 1 and 2 which were maintained on the outbred MF-1 background were also analysed for CFP reporter expression in the testis.

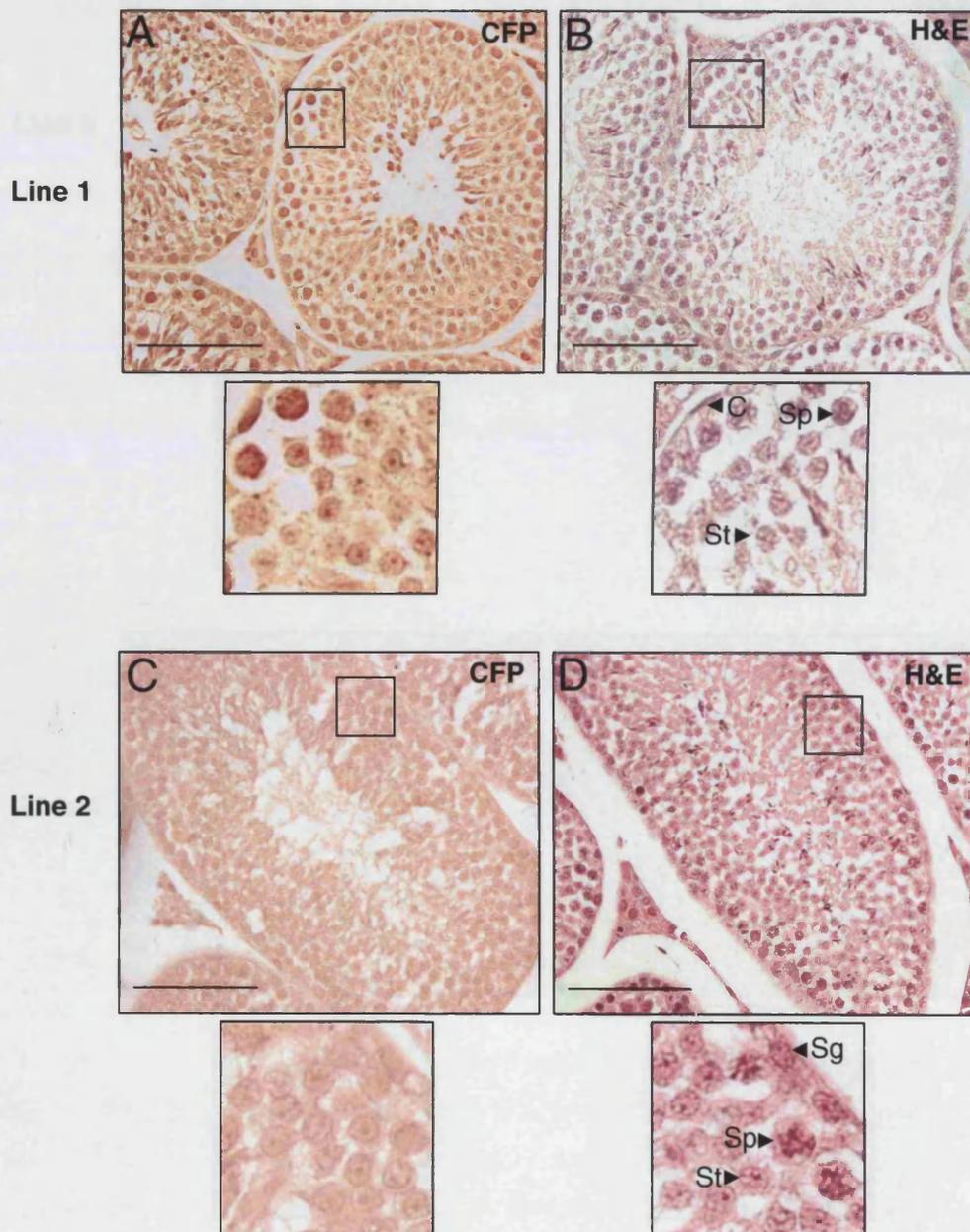
Testes were dissected from male mice from each reporter line and a wild-type mouse and embedded in a paraffin-wax block. Each mouse processed from each reporter line was re-genotyped by PCR analysis using primers mTertpromforscreen and mTertpromrevscreen in order to ensure that the reporter was present at the DNA level. 5µm sections were cut from the wax block using a microtome and then dewaxed and used for immunohistochemistry. The labelled streptavidin biotin (LSAB) method was employed to analyse the testis paraffin-wax sections. All sections were analysed together and the experiment was repeated at least three times. Experimental negative controls included a secondary only control, ruling out signal production from the secondary antibody. Sections from the wild-type mouse were also used in each experiment to prove the specificity of the antibody. Furthermore, where possible, all microscope/computer settings were kept the same when analysing sections from each reporter line and the wild-type animal. Haematoxylin and Eosin (H&E) staining was performed on serial sections to identify cell types within the testis.

No CFP reporter expression was detected in any control testis including the wild-type mouse and the secondary only controls (Figure 6.1). Strong reporter expression was detected in the spermatogonia located at the periphery of the testis tubule and in the primary spermatocytes in reporter lines 1, 3 and 4 but not 2 (Figures 6.2 and 6.3). Reporter expression was also evident at a weaker level in the round spermatids (Figures 6.2 and 6.3). Thus, reporter expression seems to be more widespread than the expected expression pattern of *mTert*. No reporter expression was detected in the Sertoli cells. Expression of the CFP reporter is nuclear which is in line with the expression pattern found for the ES cells in which the expression was both nuclear and cytoplasmic (Chapter 4).

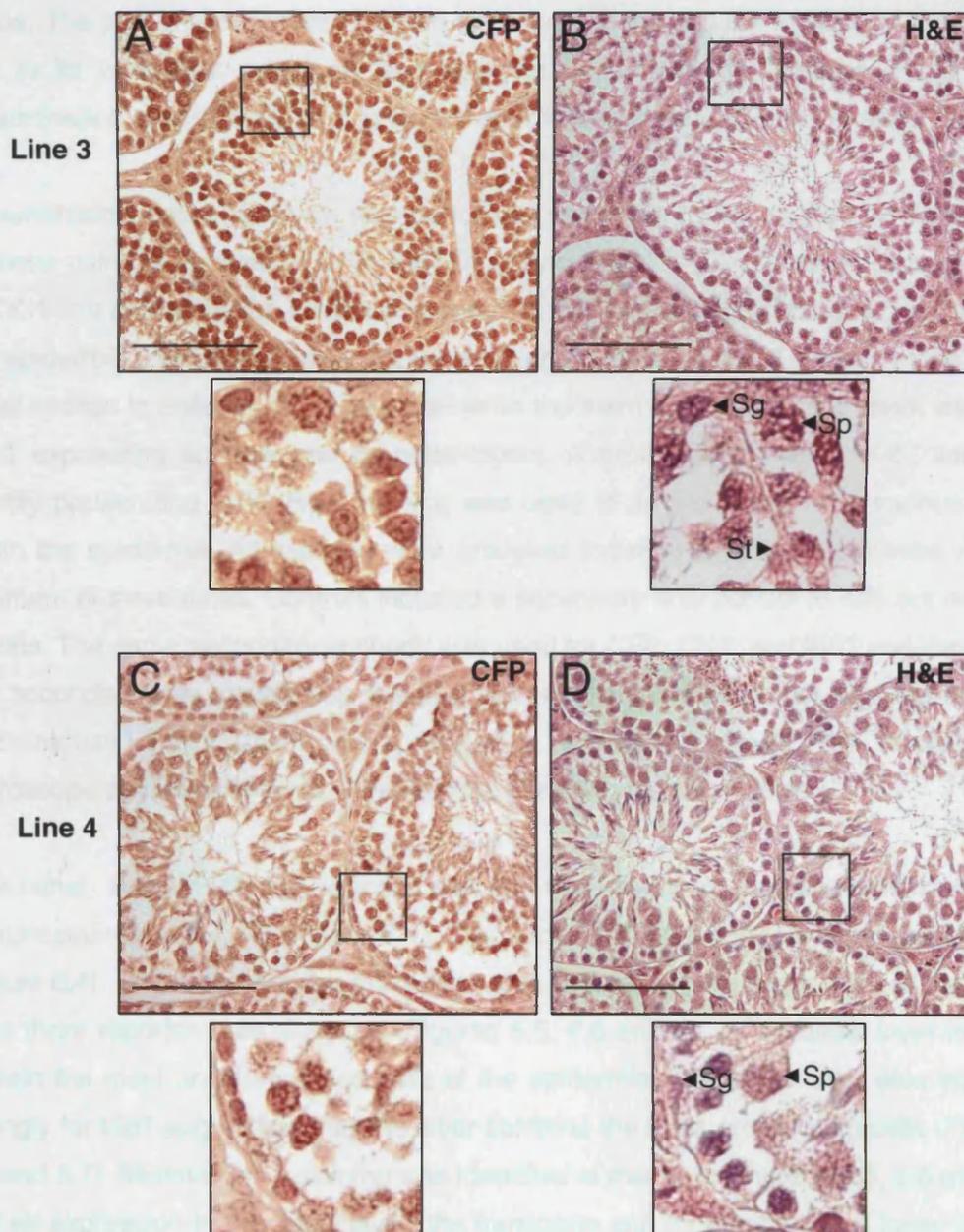
**Figure 6.1** Testis immunohistochemistry. Lower panels represent enlarged images of boxed regions. **(A)** Staining of testis section from wild-type mouse using CFP antibody. No staining was observed **(B)** H&E histochemical staining of testis serial section matching region shown in **(A)**. Sg = spermatogonia, Sp = spermatocytes, St = round spermatids. **(C)** Secondary only control. Scale = 100 $\mu$ m



**Figure 6.2** Testis immunohistochemistry. Lower panels represent enlarged images of boxed regions. **(A)** Staining of testis section from transgenic mouse line 1 using CFP antibody. **(B)** H&E serial section matching region shown in **(A)**. Sg = spermatogonia, Sp = spermatocytes, St = round spermatids, C = capsule. **(C)** Immunostaining of testis section from transgenic mouse line 2 using CFP antibody. No staining was observed. **(D)** H&E serial section from transgenic mouse matching region shown in **(C)**. Scale = 100 $\mu$ m



**Figure 6.3** Testis immunohistochemistry. Lower panels represent enlarged images of boxed regions. (A) Staining of testis section from transgenic mouse line 3 using CFP antibody. (B) H&E serial section matching region shown in (A) Sg = spermatogonia, Sp = spermatocytes, St = round spermatids. (C) Staining of testis section from transgenic mouse line 4 using CFP antibody. (D) H&E serial section matching region shown in (C). Scale = 100 $\mu$ m



Line 2 did not express the reporter as assessed by immunohistochemistry (Figure 6.2). However, in reporter lines 1, 3 and 4 the CFP reporter is expressed and the pattern of expression is the same in all three lines. Given that the CFP reporter is not expressed in line 2, the remainder of immunohistochemical analysis was only carried out on the three CFP reporter lines shown to express the reporter; lines 1, 3 and 4.

### **6.3.2 Immunohistochemical analysis of non-hairy skin from the pad of the foot**

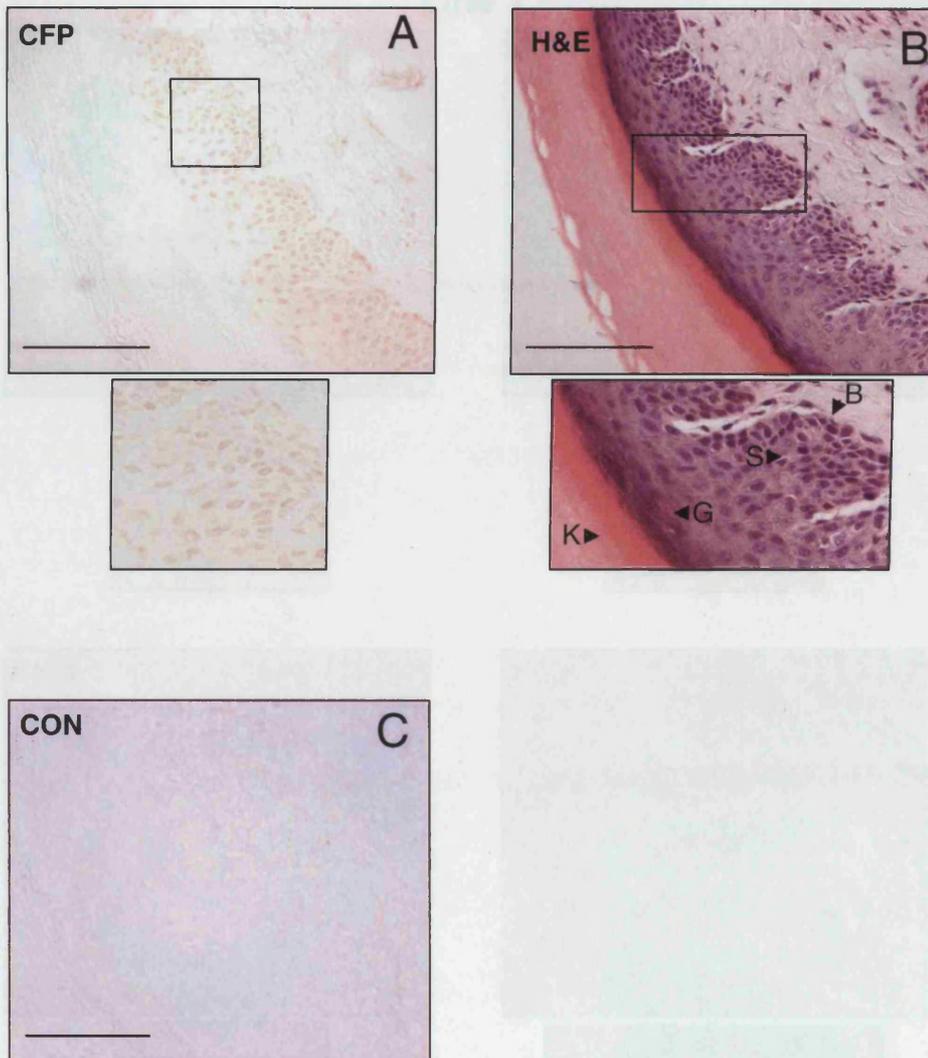
RT-PCR analysis of skin had indicated that the reporter was only expressed weakly in this tissue. The pad of the foot was chosen to determine the cellular location of this expression due to its very thick epidermis and absence of hair. These features would aid in the determination of CFP reporter expression specifically in the epidermis of skin.

Immunohistochemical analysis was performed using the LSAB method on 5µm serial skin sections using antibodies to CFP, Ki67, and cytokeratin-1 (CK-1). As discussed in Chapter 1, CK-1 is a differentiation marker expressed in the differentiating spinous/granular layers of the epidermis (Fuchs and Green, 1980; Moll *et al.*, 1982). A CK-1 antibody was used on a serial section in order to differentiate between the stem cell - containing basal layer and the CK-1 expressing spinous and granular layers. A proliferation marker Ki67 was used to identify proliferating cells. H&E staining was used to further identify the various cell types within the epidermis. All sections were analysed together and the experiment repeated a minimum of three times. Controls included a secondary only control to rule out non-specific signals. The same secondary antibody was used for CFP, CK-1 and Ki67 and therefore only one secondary only control was required per experiment. Pad of foot sections from a wild-type mouse were used as a negative control. As with the testis sections, microscope/computer settings were kept the same where appropriate.

A minimal amount of non-specific staining was seen in the wild-type control when immunostained with CFP (Figure 6.4). The secondary only controls were always negative (Figure 6.4). Maximum reporter expression was detected in the basal layer of the epidermis in all three reporter lines analysed (Figures 6.5, 6.6 and 6.7). The basal layer is known to contain the most undifferentiated cells of the epidermis. The basal layer also stained very strongly for Ki67 suggesting that this layer contains the most proliferating cells (Figures 6.5, 6.6 and 6.7). Minimal CK-1 staining was identified in this layer (Figures 6.5, 6.6 and 6.7). As well as expression in the basal layer, the transgene can be detected at a lower level in the more differentiated layers such as the spinous and granular layers (Figures 6.5, 6.6 and 6.7). Thus, the reporter is expressed at the highest level in the most undifferentiated cell

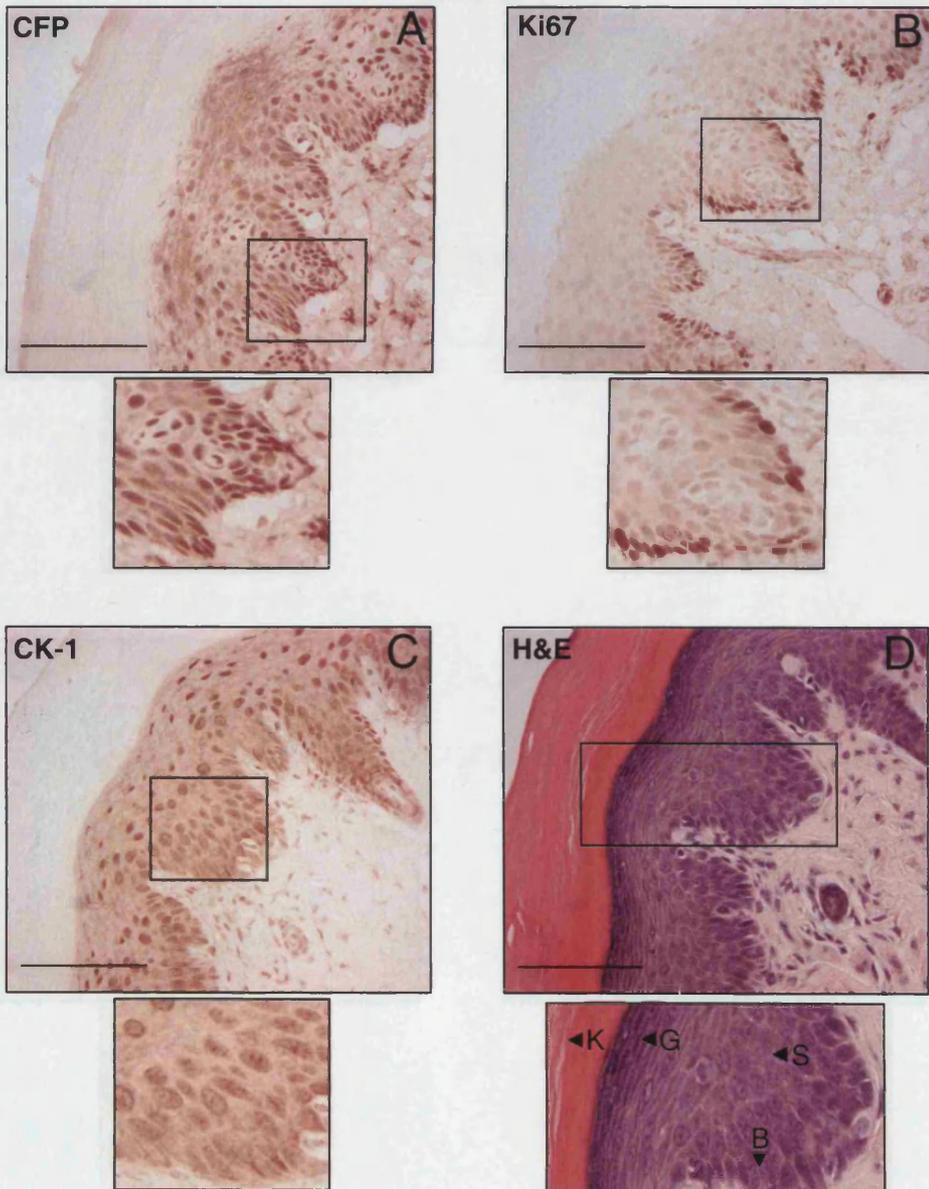
**Figure 6.4** Immunohistochemistry of wild-type (C57/BL6) pad of the foot. Lower panels represent enlarged images of boxed regions. **(A)** Staining of pad of the foot section using CFP antibody. **(B)** H&E staining of a serial section. B = basal layer, S = spinous layer, G = granular layer, K = keratinised layer. **(C)** Secondary only control. Scale = 100 $\mu$ m

### Wild-type



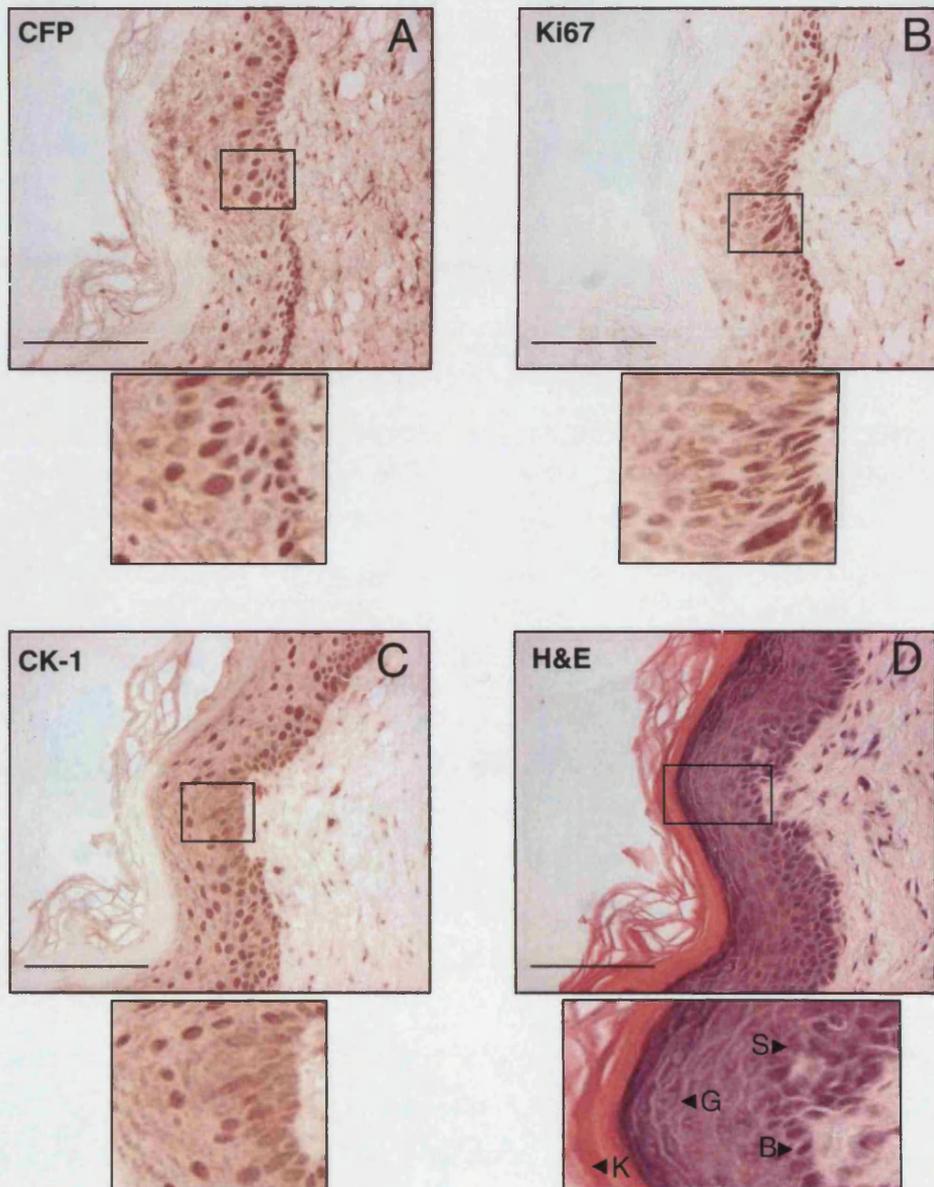
**Figure 6.5** Immunohistochemistry of the pad of the foot on CFP reporter line 1 animal. Lower panels represent enlarged images of boxed regions (A) Staining of the pad of the foot section using CFP antibody. (B) Ki67 staining of a serial section. (C) CK-1 staining of serial section. (D) H&E staining of a serial section. B = basal layer, S = spinous layer, G = granular layer, K = keratinised layer. Scale = 100 $\mu$ m

**Line 1**



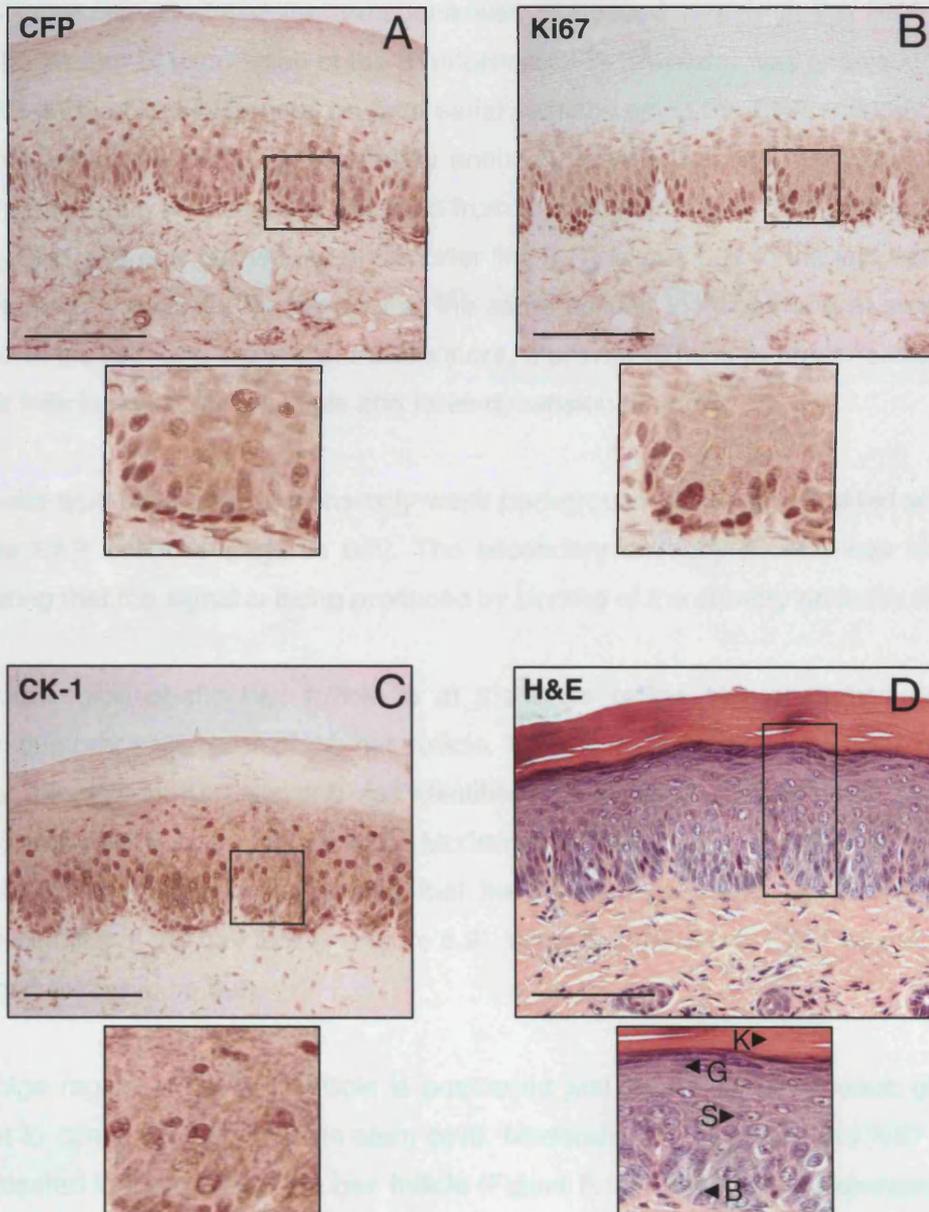
**Figure 6.6** Immunohistochemistry of the pad of the foot on CFP reporter line 3 animal. Lower panels represent enlarged images of boxed regions. **(A)** Staining of the pad of the foot section using CFP antibody. **(B)** Ki67 staining of a serial section. **(C)** CK-1 staining of serial section. **(D)** H&E staining of a serial section. B = basal layer, S = spinous layer, G = granular layer, K = keratinised layer. Scale = 100µm

**Line 3**



**Figure 6.7** Immunohistochemistry of the pad of the on CFP reporter line 4 animal. Lower panels represent enlarged images of boxed regions. (A) Staining of the pad of the foot section using CFP antibody. (B) Ki67 staining of a serial section. (C) CK-1 staining of serial section. (D) H&E staining of a serial section. B = basal layer, S = spinous layer, G = granular layer, K = keratinised layer. Scale = 100 $\mu$ m

### Line 4



types within the skin and this expression gradually decreases as the cells become more differentiated.

There was no obvious difference in reporter expression levels between lines in the pad of the foot analysis. Thus, only line 3 was analysed for the expression pattern of the reporter in hairy skin.

### **6.3.3 Immunohistochemical analysis of hairy skin**

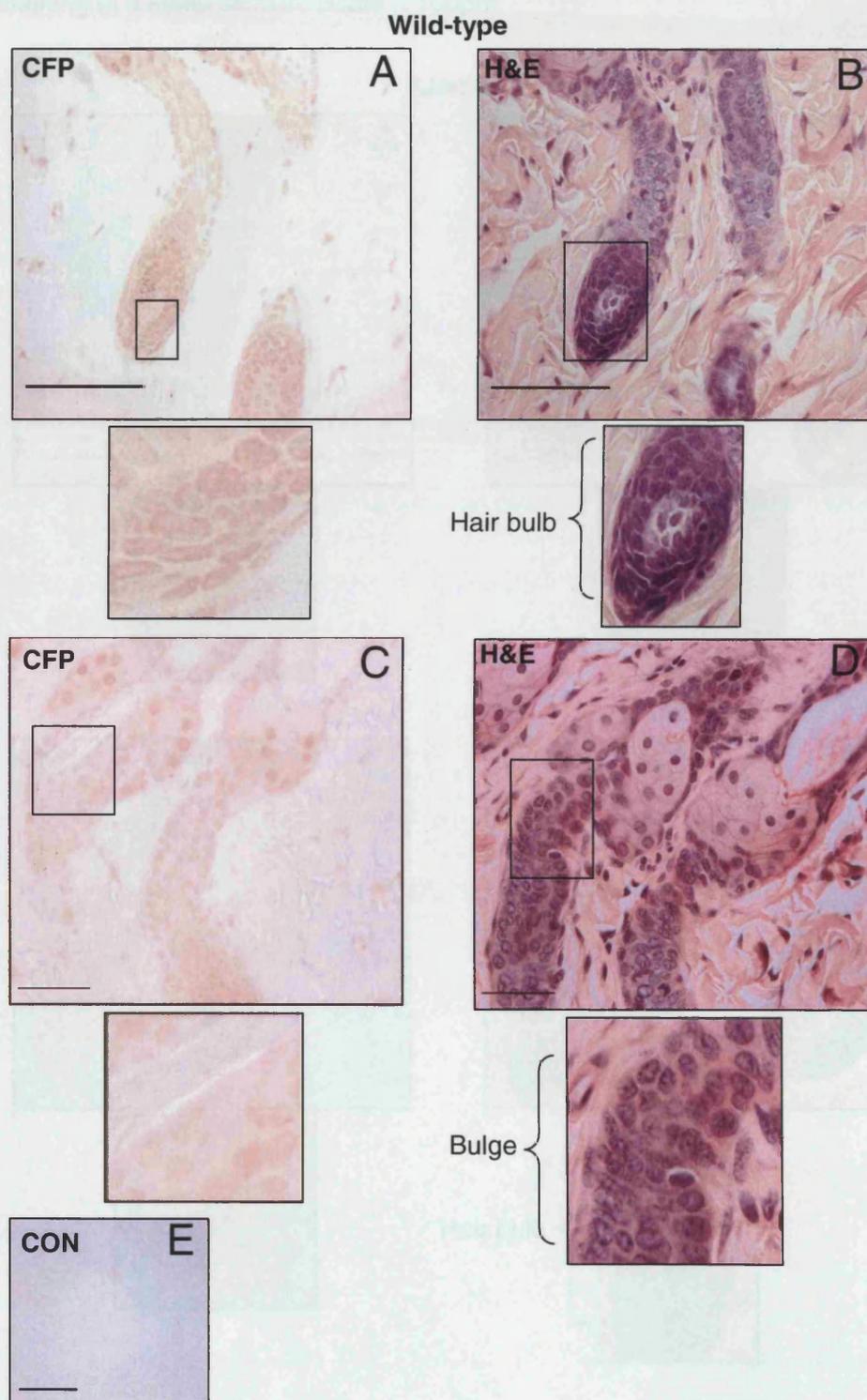
RT-PCR demonstrated that the transgene was expressed weakly at the RNA level in the skin. The pattern of expression of the pTertpromECFP-1 reporter was analysed in hairy skin by LSAB immunohistochemistry on 5µm serial sections using the CFP antibody. Similarly to the other IHC experiments, a secondary antibody only control was always included in the experiments along with sections obtained from a wild-type mouse. The histological analysis of hairy skin was only carried out on reporter line 3. This was due to the fact that all reporter lines seemed to express the reporter at the same cellular locations and at similar levels in the pad of the foot and the testis. Furthermore, it proved difficult to obtain serial sections of the hair follicle due to its thickness and three dimensional nature.

In the wild-type hairy skin sections only weak background level was obtained when stained with the CFP antibody (Figures 6.8). The secondary antibody control was always clean suggesting that the signal is being produced by binding of the primary antibody (Figure 6.8).

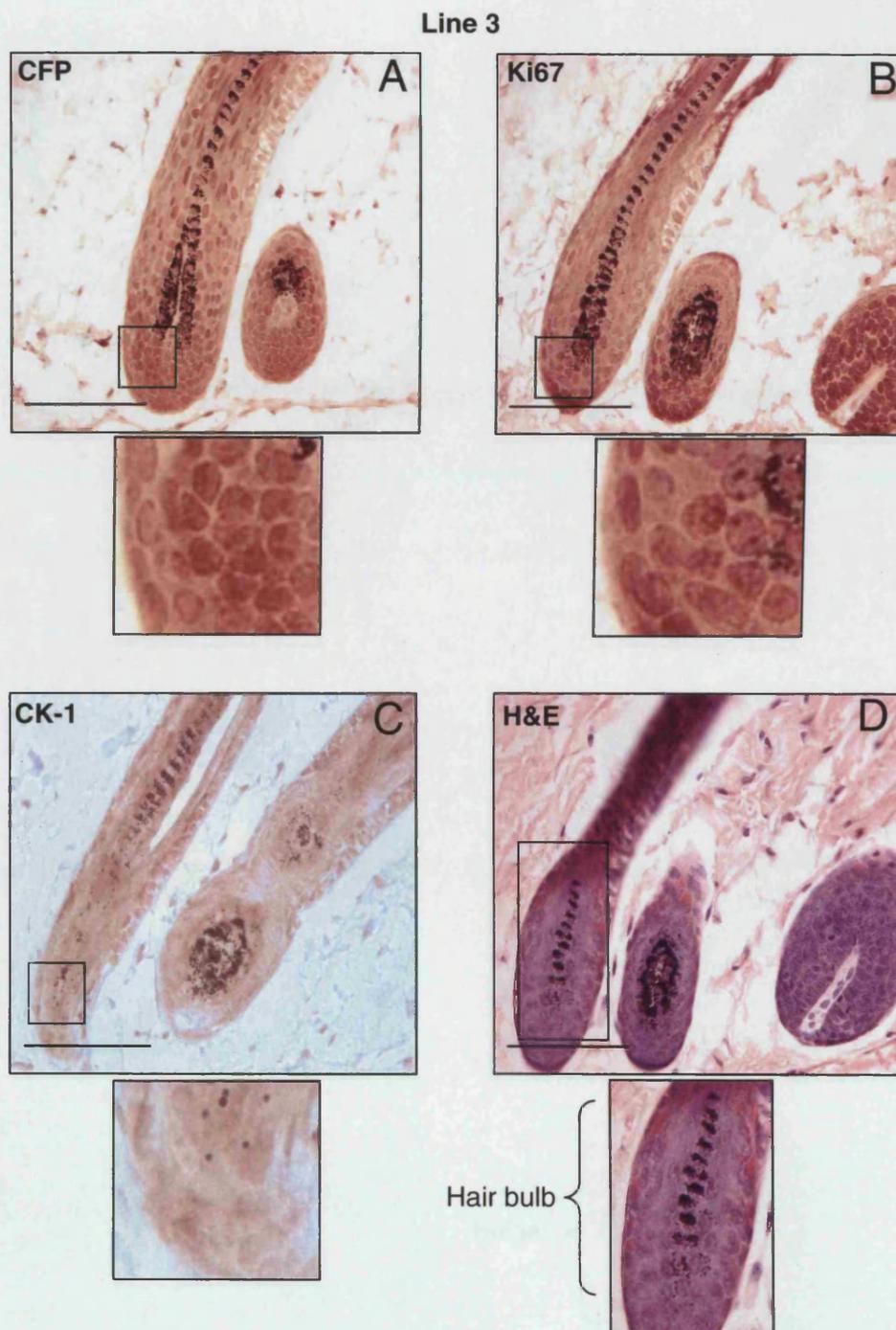
The bulb region of the hair follicle is at the base of the hair shaft and contains the proliferating progenitor cells of the hair follicle. Thus, this region of the hair was analysed for reporter expression. Ki67 staining was identified at a moderate to weak intensity in the hair bulb of the hair follicle (Figure 6.9). Moderate to weak CFP reporter expression was detected in the hair bulb suggesting that the reporter is expressed in the proliferating progenitor cells of the hair follicle (Figure 6.9). Weak expression of CK-1 was also identified in the hair bulb (Figure 6.9).

The bulge region of the hair follicle is positioned just below the sebaceous gland and is thought to contain the hair follicle stem cells. Moderate CFP reporter and Ki67 expression was detected in the bulge of the hair follicle (Figure 6.10). Weak CK-1 expression was also identified in the hair bulge. This suggests that the CFP reporter is expressed at the same geographical location as the bulge stem cells.

**Figure 6.8** Immunohistochemistry of hairy skin from a wild-type mouse. Lower panels represent enlarged images of boxed regions. (A) Staining of the hair bulb region using CFP antibody. (B) H&E staining of a serial section. (C) Staining of bulge region using CFP antibody. (D) H&E staining of a serial section. (E) Secondary only control. Scale = 100 $\mu$ m

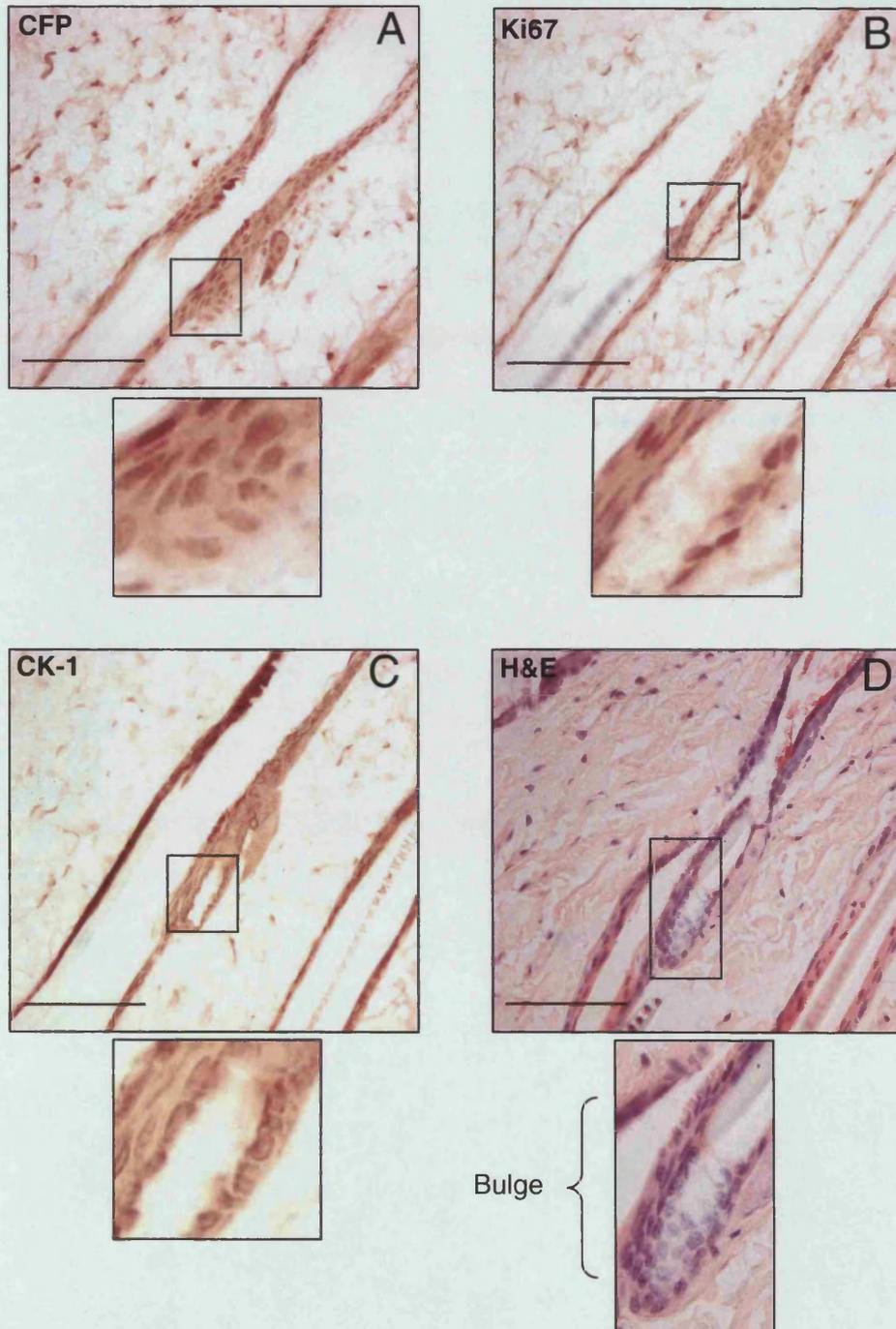


**Figure 6.9** Immunohistochemistry of hairy skin from a mouse from CFP reporter line 3. Lower panels represent enlarged images of boxed regions. (A) Staining of the hair bulb region of the HF using CFP antibody. (B) Ki67 staining of a serial section. (C) CK-1 staining of serial section. (D) H&E staining of a serial section. Scale = 100 $\mu$ m



**Figure 6.10** Immunohistochemical analysis of hairy skin from a CFP reporter mouse from line 3. Lower panels represent enlarged images of boxed regions. (A) Staining of the bulge region of the HF using CFP antibody. (B) Ki67 staining of a serial section. (C) CK-1 staining of serial section. (D) H&E staining of a serial section. Scale = 100 $\mu$ m

Line 3



#### **6.3.4 Immunohistochemical analysis of intestine**

RT-PCR analysis indicated that there was no reporter expression in the LI in line 4, but there was moderate expression in line 3. Analysis of SI indicated that there was weak expression in line 4, yet moderate to strong expression in line 3. Thus, line 3 was analysed for expression of the reporter by LSAB immunohistochemistry. Sections of the SI and LI were dissected from adult mice and embedded in paraffin wax. 5µm transverse sections were cut using a microtome and the sections were dewaxed and used in immunohistochemistry. All sections were analysed together and the experiment was repeated a minimum of three times. Experimental controls again included a secondary only control and sections from a wild-type mouse. Microscope/computer setting were kept the same where appropriate.

In the wild-type SI and LI sections, the background staining levels were low with the CFP antibody (Figure 6.11).

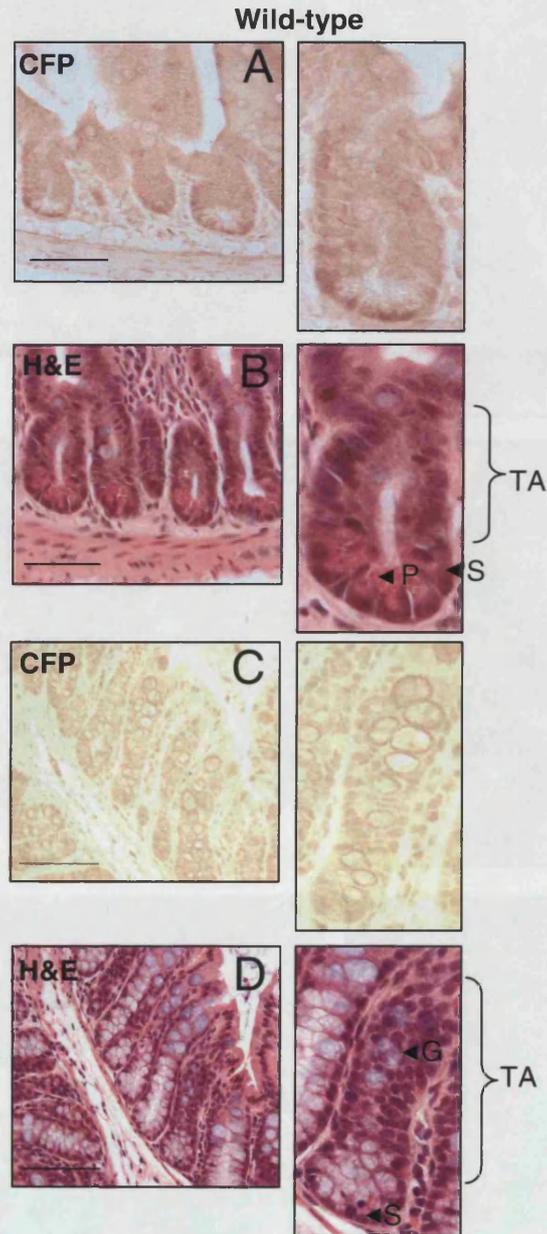
The proliferation marker Ki67 stained the proliferative regions of the SI and LI crypts (Figures 6.12 and 6.13). Thus, it stains both the stem cells and the proliferative progenitor cells. CFP reporter expression as detected by the CFP antibody was difficult to determine in both the LI and the SI. However, in the SI, there is discernible staining which is stronger near the bottom of the crypts at around the stem cell position (Figure 6.12). Staining in the proliferative TA region of the SI crypt is also detectable (Figure 6.12). However, the staining pattern gradually decreases further up the crypt. This suggests that the CFP reporter is expressed most strongly in the stem cell position and at a lower level in the progenitor cells of the SI.

In the LI, CFP reporter expression again seems to be stronger in the bottom of the crypt in the stem cell position and this expression gradually decrease as you go up the crypt into the progenitor cell position (Figure 6.13). Similarly to the SI, this suggests that the CFP reporter is expressed most strongly in the stem cell position and is expressed at a lower level in the progenitor cell population. Results obtained for one CFP reporter line are shown as similar results were obtained with all CFP reporter lines analysed.

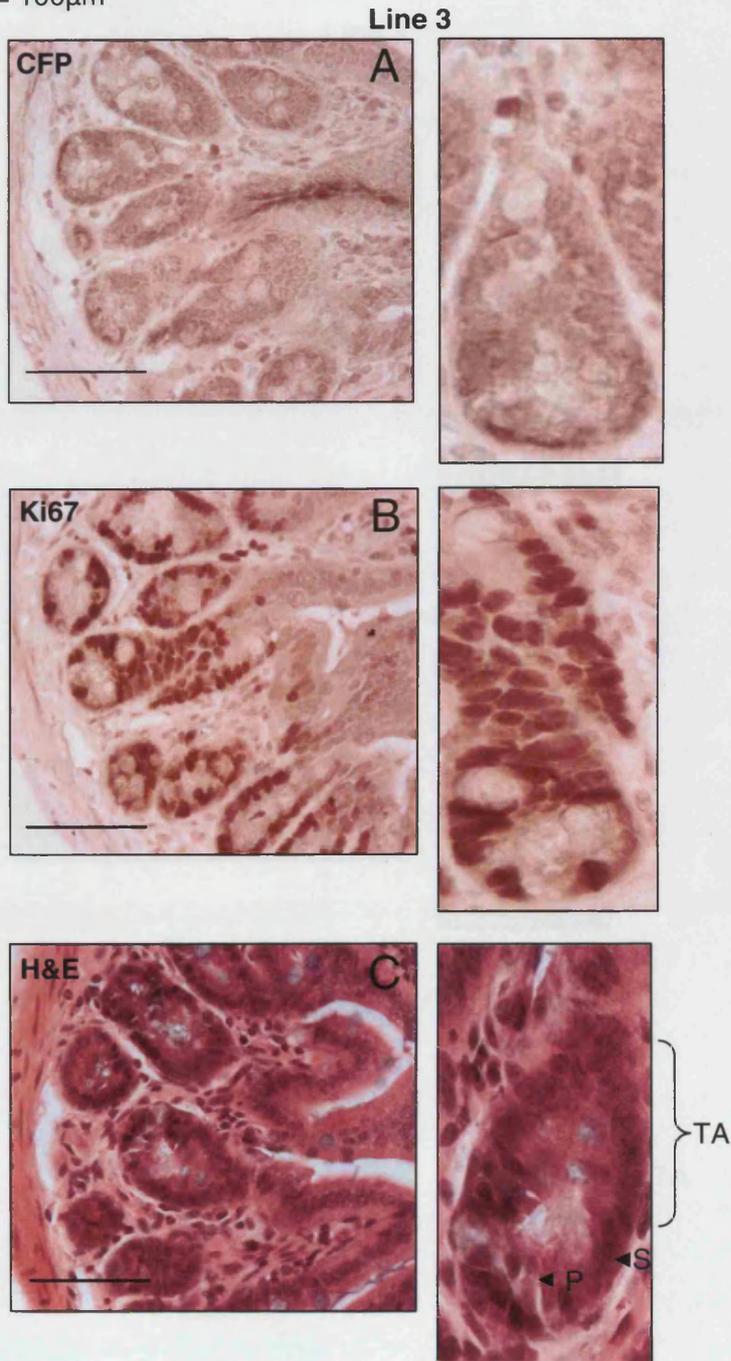
#### **6.3.5 Immunofluorescence of the intestine**

Immunofluorescence on ES clones expressing the pTertpromECFP-1 reporter had proved successful (see Chapter 4). The secondary antibody used in this case was an Alexa Flour® anti rabbit dye-conjugated secondary antibody which emits a red-orange colour at 603nm when excited at 568nm. Thus, immunofluorescence using this antibody in combination with

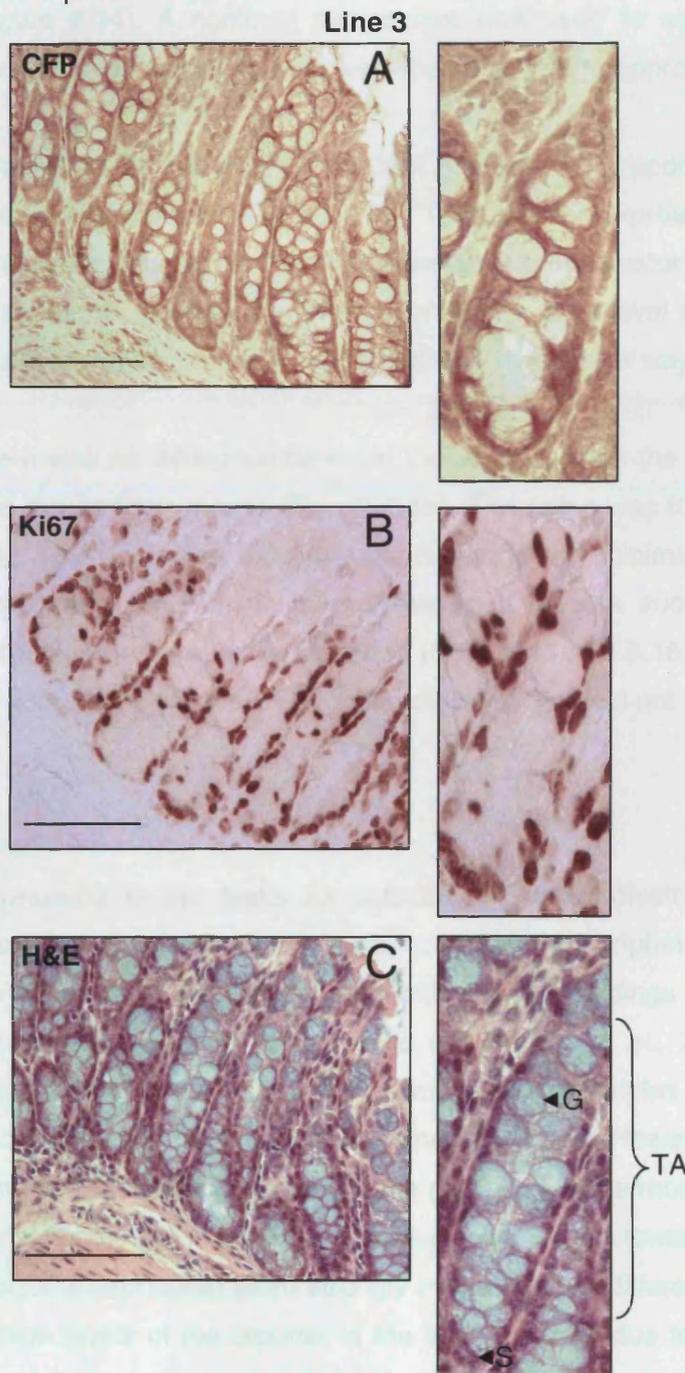
**Figure 6.11** Immunohistochemical analysis of the small and large intestine from a wild-type animal. Enlarged images of whole crypts are shown on the right. (A) Staining of the SI using a CFP antibody. (B) H&E staining of a serial section. (C) Staining of the LI using a CFP antibody. (D) H&E staining of a serial section. P = Paneth cells, S = stem cell position, TA = transit-amplifying population. G = goblet cells. Scale = 100 $\mu$ m



**Figure 6.12** Immunohistochemical analysis of the small intestine. Enlarged images of whole crypts are shown on the right. (A) Immunostaining of the SI from an animal from CFP reporter line 3 using a CFP antibody (B) Ki67 immunostaining of a serial section. (C) H&E staining of a serial section. P = Paneth cells, S = stem cell position, TA = transit-amplifying population. Scale = 100 $\mu$ m



**Figure 6.13** Immunohistochemical analysis of the large intestine. Enlarged images of crypts are shown on the right. (A) Immunostaining of the LI from an animal from reporter line 3 using a CFP antibody. (B) Ki67 immunostaining of a serial section (C) H&E staining of a serial section. S = stem cell position, TA = transit-amplifying population, G = Goblet cell. Scale = 100 $\mu$ m



the CFP primary antibody was attempted on paraffin-wax intestine sections in order to determine a more obvious reporter expression pattern. Sections from the pad of the foot from line 3 were also analysed. Ki67 immunofluorescence was performed in parallel in order to identify proliferating cells within the gut and the pad of the foot and in order to ensure that the protocol was successful (Figures 6.14 – 6.16). Sections of LI, SI and pad of foot from a wild-type animal were used as a negative control. A secondary only control was also included (Figure 6.14). A confocal microscope was used to analyse the sections. The microscope and computer setting were kept the same where appropriate.

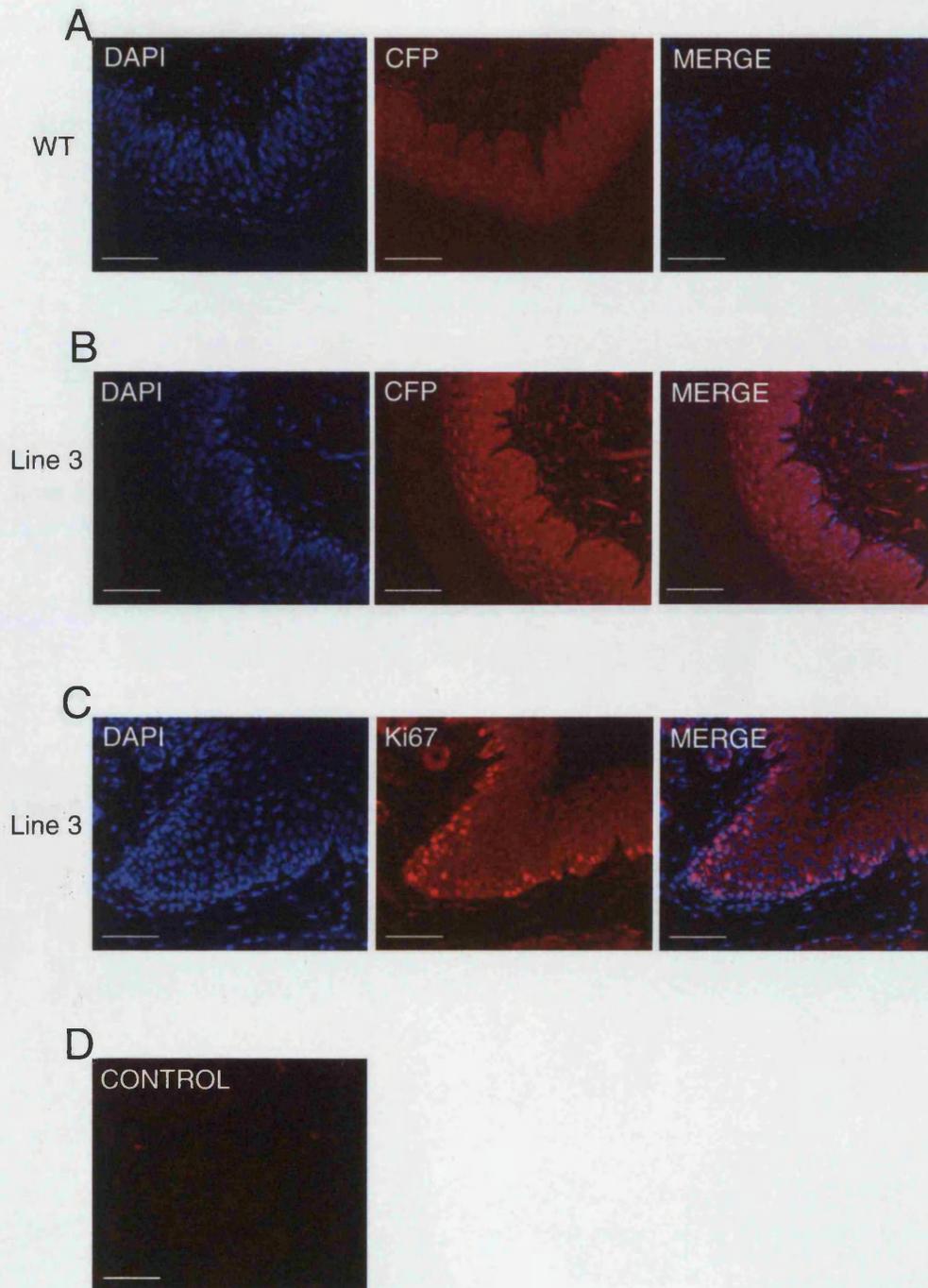
Immunofluorescence of the pad of the foot showed that reporter expression could be discerned using this method (Figure 6.14). The reporter expression pattern identified in immunofluorescence was similar to that obtained by immunohistochemistry. The basal layer cells of the epidermis express the CFP reporter at a high level and this expression level decreases as the cells become more differentiated, in a similar way to Ki67.

In the SI, there was no difference between the sections from the wild-type mouse and the sections from the reporter mouse (Figure 6.15). The same was found to be true for the LI (Figure 6.16). The secondary antibody control exhibited minimal staining (Figure 6.14). Immunostaining with the marker of proliferation Ki67 was successful and single cells expressing the marker were easily identified (6.14, 6.15 and 6.16). Thus, the fluorescence method was successful, yet the CFP immunofluorescence did not give a clear result for the intestine.

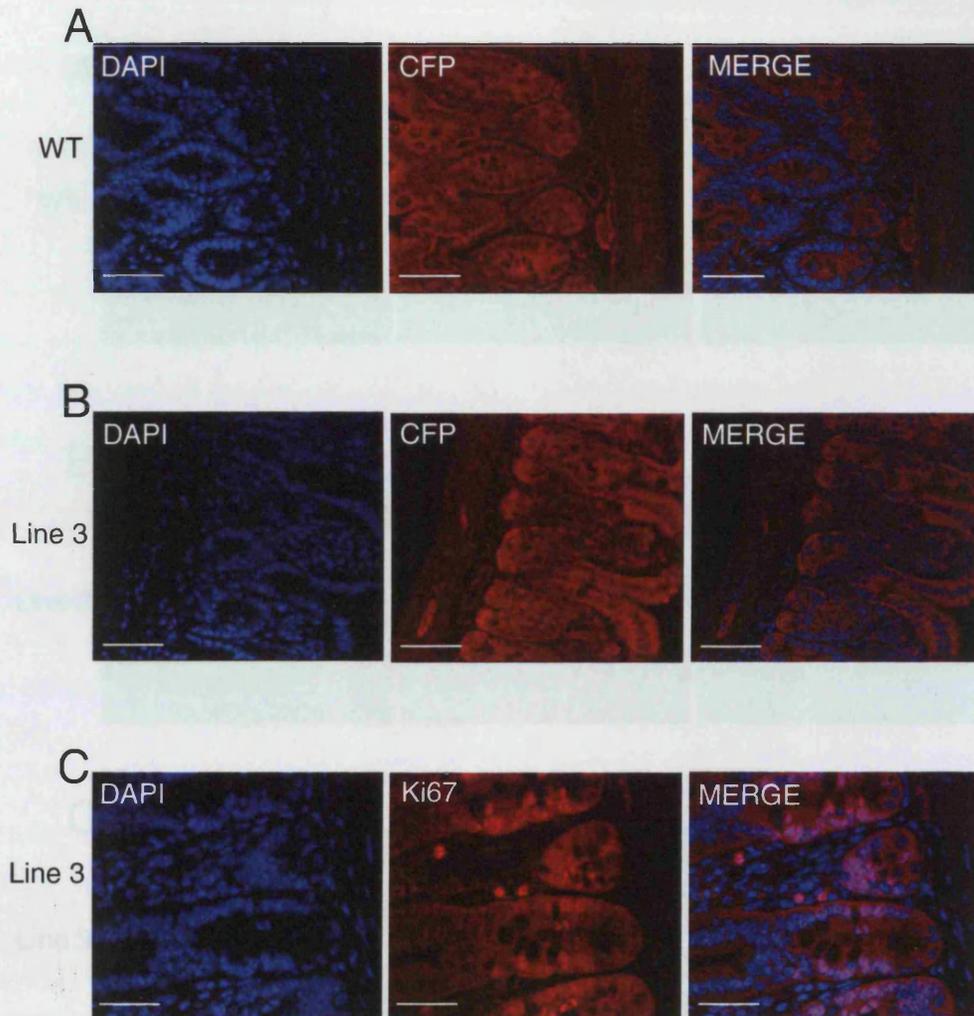
#### **6.4 Discussion**

Reporter expression in the testis as detected by immunohistochemistry using a CFP antibody was evident in the spermatogonia located at the periphery of the testis tubule and in the primary spermatocytes. This agrees with previous findings that *Tert* is expressed in the most undifferentiated cells in the testis (Pericuesta *et al.*, 2006; Ritz *et al.*, 2005). Reporter expression was also evident in the round spermatocytes at a lower level and thus the expression pattern is more widespread than expected. However, the Sertoli cells and the developing spermatids do not express the reporter. Furthermore the staining is stronger nearer to the periphery of the tubule and gradually decreases towards the lumen suggesting that the reporter is expressed more strongly in the most undifferentiated cells of the testis tubule. The high levels of the reporter in the testis may be due to the requirement for the maintenance of chromosome ends in preparation for division-rich development.

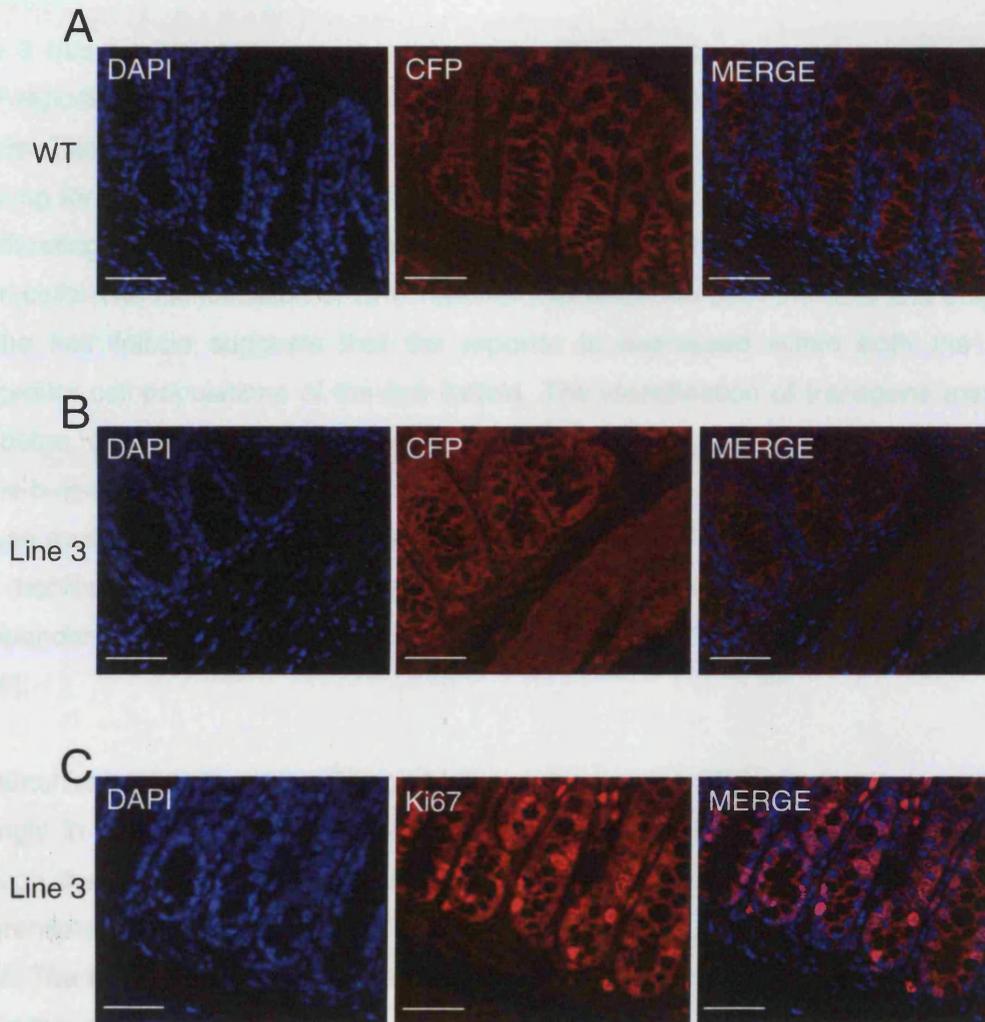
**Figure 6.14** Pad of foot immunofluorescence (A) Wild-type pad of foot immunostained with CFP and counter-stained with DAPI. (B) Line 3 pad of foot immunostained with CFP and counter-stained with DAPI. (C) Line 3 pad of foot immunostained with Ki67 and counter-stained with DAPI. (D) Secondary only control for both Ki67 and GFP. Scale = 50 $\mu$ m



**Figure 6.15** SI immunofluorescence. (A) Wild-type SI immunostained with CFP and counter-stained with DAPI (B) SI from CFP reporter line 3 immunostained with CFP and counter-stained with DAPI. (C) SI from reporter line 3 immunostained with Ki67 and counter-stained with DAPI. Scale = 50 $\mu$ m



**Figure 6.16** LI immunofluorescence. (A) Wild-type LI immunostained with CFP and counter-stained with DAPI. (B) LI from reporter line 3 immunostained with CFP and counter-stained with DAPI. (C) LI from reporter line 3 immunostained with Ki67 and counter-stained with DAPI. Scale = 50 $\mu$ m



The immunofluorescence analysis of LI in mice showed that there was no CFP expression in reporter line 2, which was maintained by long-term breeding using the mutant  $\beta$ -casein transgene (transgene 2), which was also maintained by backcrossing to  $\beta$ -casein null mice. It was shown to express the reporter in the mammary gland, but the expression pattern was distinct from mammary glands of humans. Thus, the reporter expression pattern in this mouse may be expected to more closely resemble that reported for endogenous  $\beta$ -casein. Endogenous  $\beta$ -casein has been demonstrated to be expressed in the mammary gland by Ishii et al. (2005) using a reporter mouse. Thus, the  $\beta$ -casein promoter driving the CFP reporter expression pattern in line 2 is identified in the mammary gland by

In the epidermis, reporter expression as detected by immunohistochemistry was most evident in the cells of the basal layer in all three lines analysed. This correlates with expression of the proliferation marker Ki67 which was very strong in the basal layer cells. As expected CK-1 expression was minimal in this layer, suggesting that the layer is the basal layer and does not contain any CK-1 expressing spinous or granular layer cells.

Line 3 was the only reporter line analysed for CFP reporter expression in the hair follicle. CFP reporter expression was identified in the hair bulb and the bulge region of the adult hair follicle. The staining obtained in the hair follicle was weak for both Ki67 and CFP, yet the staining for both antibodies was found in comparable locations. The hair bulb contains the proliferating progenitor cells and the hair bulge region is thought to contain the hair follicle stem cells. The identification of CFP reporter expression in both the bulb and bulge regions of the hair follicle suggests that the reporter is expressed within both the stem and progenitor cell populations of the hair follicle. The identification of transgene expression in the bulge region of the hair follicle correlates with previous findings that *mTert* is expressed in the bulge of the hair follicle (Sarin *et al.*, 2005). Furthermore, analysis of the hair follicle in mouse models overexpressing *mTert* have shown that *mTert* expression is related to stem cell mobilisation from the bulge and stem cell self-renewal and that this relationship is independent of its role in maintaining telomere length (Flores *et al.*, 2005; Sarin *et al.*, 2005).

Immunohistochemistry of the SI and LI suggests that the CFP reporter is expressed more strongly in the stem cell positions of the intestine and this expression is gradually lost through the progenitor cell population. Thus, the cells higher up the crypt that are more differentiated express less reporter than the more undifferentiated cells at the bottom of the crypt. The immunofluorescence was unsuccessful in determining CFP reporter expression within the intestine and would need to be repeated in order to obtain definitive results.

The immunohistochemical analysis of the testis revealed that there was no CFP expression in reporter line 2, which was maintained by continual backcrossing to the outbred MF-1 strain of mice. However, line 1 which was also maintained by backcrossing to MF-1 mice was shown to express the reporter in the testis. Outbred mice are similar to wild-mice and exhibit similar telomere lengths to humans. Thus, the reporter expression pattern in MF-1 mice may be expected to more closely resemble that expected for endogenous *hTERT*. Endogenous *hTERT* has been demonstrated to be expressed in the spermatogonia of the testis by Ritz *et al* (2005) using a reporter model. Thus, this is in line with our findings. The CFP reporter expression pattern in line 1 as identified in the non-hairy skin by

immunohistochemistry seems to be similar to that obtained for lines 3 and 4, which were maintained by backcrossing to the inbred C57BL/6 strain. Thus, there seems to be no difference in the CFP reporter identified in any of the three reporter lines (1, 3 and 4). However, in order to determine that there is no difference in expression pattern between the lines maintained on different backgrounds, all tissues in all three reporter lines should be analysed.

As previously mentioned, during the course of this project, three *mTert* promoter GFP reporter mouse models have been generated (Pericuesta *et al.*, 2006). Direct fluorescent GFP reporter expression could not be identified in any adult tissues of the three models, which is consistent with the findings in this investigation. CFP reporter expression was identified in this investigation using immunohistochemistry. The CFP reporter expression pattern identified in adult tissues suggests that the reporter marks not only the stem cell population, but also the proliferating progenitor cell population. This suggests that telomerase activity is not switched off when stem cells give rise to TA cells, but instead decreases in a gradient as the cells become more differentiated.

## 7 Summary and Discussion

### **7.1 Generation of an *mTert* promoter reporter mouse**

When this project began, Armstrong *et al* (2000) had reported that a 4.4kb region of the *mTert* promoter could recapitulate *mTert* expression. The investigators had further described the expression of this reporter in ES cells and shown that upon differentiation of the ES cells, this expression was lost. However, at the start of the project no *in vivo* promoter mouse model had been developed.

In order to generate an *mTert* promoter reporter mouse, the *mTert* promoter needed to be investigated. The 4.4kb region used in the Armstrong investigation was included in the promoter construct as this region had been shown previously to recapitulate *mTert* expression. In addition, a CpG island encompassing part of exon 1 of the *mTert* gene was identified in the promoter analysis. This, coupled with the discovery of transcription factor binding sites, led to the inclusion of part of exon 1 in the *mTert* promoter construct. Once the promoter region had been decided upon, it was amplified by PCR and cloned into a promoterless CFP plasmid. Thus, the expression of CFP was reliant upon the functionality of the *mTert* promoter. Once constructed, the transgenic vector was injected into the pronucleus of 1 dpc oocytes, which were then transferred into pseudo pregnant females. Seven mice resulted from the microinjections, of which four were able to transmit the transgene through the germline.

### **7.2 Characterisation of CFP reporter expression in ES cells and differentiated ES cells**

The transgenic vector was electroporated into ES cells in order to characterise expression in this stem cell type. Several clones expressing the CFP reporter at the DNA level were identified by PCR analysis. Direct visualisation of the CFP reporter proved unsuccessful in these clones. Visualisation of a positive control CFP plasmid also proved difficult and therefore in hindsight a more readily detectable reporter such as GFP or *LacZ* could have been chosen as the reporter. Immunofluorescence using a GFP antibody was successfully employed to detect CFP reporter expression in ES cells. The identification of reporter expression found in this investigation is in line with previous findings that reporters driven by the *mTert* promoter are expressed in ES cells (Armstrong *et al.*, 2000; Pericuesta *et al.*, 2006). There seemed to be no difference in reporter expression levels between any of the ES clones analysed, suggesting that copy

number in this case has not affected expression level. However, expansion of the Southern analysis to include all clones that were used in immunofluorescence analysis would substantiate this finding.

Previous investigations have shown that reporters driven by the *mTert* promoter are down-regulated during the differentiation of ES cells (Armstrong *et al.*, 2000). In order to replicate these findings reporter ES clones were differentiated via the formation of EBs. The efficiency of differentiation was first observed during the culture of the cells as fibroblast-like cells were observed along with clumps of 'beating' cells indicating the presence of cardiac cells within the culture. Oct-4, a marker of pluripotency, was also used to monitor the efficiency of differentiation. In both immunofluorescence studies and RT-PCR analysis the expression of Oct-4 was down-regulated upon differentiation of the cells indicating that the differentiation protocol was successful. CFP reporter expression was also shown to be consistently down-regulated during differentiation by immunofluorescence and RT-PCR analysis. Thus, these data are in line with previous observations and confirm that *Tert* is linked with the ES cell phenotype *in vitro*.

### **7.3 Characterisation of CFP reporter expression during teratoma formation**

Teratoma formation from ES clone 38 gave rise to a variety of tissues including skin epidermis, neural tissue and skeletal muscle. Numerous patches of undifferentiated cells were also identified by Oct-4 immunostaining. CFP reporter expression was found in all regions positively stained with the Oct-4 antibody suggesting that reporter expression correlates with the undifferentiated/pluripotent cell phenotype. Reporter expression also correlated with regions of intense proliferation as assessed by immunohistochemical staining with the proliferation marker Ki67. In the epidermis formed within the teratoma, basal layer-like cells were identified by correlation of Ki67/Oct-4 immunostaining and the lack of CK-1 staining. These cells also expressed the reporter suggesting that the reporter correlates with the stem cell phenotype in the skin. The teratoma formed a large area of neural tissue as identified by immunohistochemical staining with GFAP which is an astrocyte marker. Immunostaining with Oct-4 and Ki67 demonstrated that this portion of the teratoma was relatively unproliferative and differentiated. The reporter was not expressed within this region. The fact that the CFP reporter is not expressed within the neural tissue supports the idea that the reporter should only be expressed in undifferentiated stem cell populations. Furthermore, this finding is in line with RT-PCR data presented both in this project and in a previous investigation (Greenberg *et al.*, 1998).

Thus, overall, the immunohistochemistry suggests that the CFP reporter is able to identify undifferentiated cells. However, co-immunofluorescence on teratoma sections with the CFP/Oct-4 and Ki67 markers would be an improved approach to this project, allowing the expression of all three markers to be co-localised on a single-cell basis.

#### **7.4 Characterisation of CFP reporter expression in the testis**

Semi-quantitative RT-PCR analysis consistently showed high CFP reporter expression in the testis, which correlated with the high levels of expression of endogenous *mTert* also found by RT-PCR. These data were substantiated by qPCR for endogenous *mTert* which demonstrated an extremely high level of *mTert* in the testis as determined by calculations using the expression of *mTert* in the skin as a calibrator. Immunohistochemical analysis identified CFP reporter expression in the spermatogonia and primary spermatocytes, which are the most undifferentiated cells of the testis. However in this investigation, expression was also evident in the secondary spermatocytes. The high levels of *mTert* and the reporter in the testis may be due to the requirement for the maintenance of chromosome ends in preparation for division-rich development.

#### **7.5 Characterisation of CFP reporter expression in the skin**

RT-PCR analysis of the skin revealed that the CFP reporter was expressed in this tissue. Indeed, reporter expression was identified in the basal layer cells of the epidermis of the pad of the foot by immunohistochemistry. This layer contains the cells with the most proliferation potential. Expression was also detected at lower levels in the cells of the spinous and granular layers of the epidermis. CFP reporter expression was also detected in the bulb and the bulge regions of the hair follicle. This expression also correlated with the expression of the proliferation marker Ki67. These data suggest that the CFP reporter marks not only the undifferentiated cells of the skin, but also the progenitor cell population but at a lower level.

The correlation between *mTert* and proliferation within the stem cell compartments of the skin suggested here is in line with that made in previous investigations. It has previously been demonstrated that *mTerc*<sup>-/-</sup> mice exhibit an accumulation of LRCs in the bulge of the hair follicle. *mTert* overexpression has the converse effect of increasing LRC mobilisation from the niche resulting in less LRCs remaining within the niche (Flores *et al.*, 2005). Intercrosses of *mTert* overexpressing mice with *mTerc*<sup>+/-</sup> has shown that the effects of *mTert* on the stem cell niche are not dependent on its ability to form active telomerase complexes. It has also been suggested that *mTert* may promote

stem cell self-renewal as well as increasing mobilisation of cells out of the niche (Sarin *et al.*, 2005). Thus, these data show that *mTert* plays a role in regulating stem cell proliferation in the skin.

### **7.6 Characterisation of CFP reporter expression in the intestine**

RT-PCR analysis indicated reporter expression within the mouse intestine. Immunohistochemical analysis of both the SI and the LI demonstrated some stronger staining in the stem cell position at the bottom of the crypts, which gradually decreases up the crypt. Thus these data suggest that the reporter is expressed in the TA population as well as the stem cell population. Immunofluorescence of the intestine was performed in order to identify a more obvious reporter expression pattern. However, the results obtained showed that the CFP reporter could not be identified in the intestine using this method. The analysis of younger animals may aid in the elucidation of a more obvious CFP reporter expression pattern as the higher turnover of the intestine in younger animals may lead to an increase in the number of stem/progenitor cells.

### **7.7 Other *mTert* promoter models**

During the course of this project a number of *Tert* promoter mouse models have been presented. Pericuesta *et al* (2006), generated three different *mTert* promoter reporter models utilising three regions of the *mTert* promoter as outlined in Chapter 1, and have shown that as little as 1kb of the promoter is sufficient to recapitulate *mTert* expression and telomerase activity (Pericuesta *et al.*, 2006). Analysis of the mouse models generated by Pericuesta *et al* (2006) revealed that expression of the *mTert* promoter GFP reporter could not be detected in adult tissues from any of the three models by direct visualisation. However, expression of the GFP reporter was observed in GSCs generated from neonatal and adult testis and in neurospheres generated from E14dpc fetus brain (Pericuesta *et al.*, 2006). This suggests that in this model, the *mTert* promoter reporter can be correlated with the stem cell phenotype.

Ritz *et al* (2005) generated a mouse model using the *hTERT* promoter driving expression of the bacterial *LacZ* gene. Analysis of this model has shown that expression of the reporter was restricted to the testis in two out of three lines generated and that this expression was specific to the spermatogonia and spermatocytes (Ritz *et al.*, 2005). This is in line with the data presented here.

### 7.8 Further work

The data presented here indicate that CFP reporter expression can be correlated with highly proliferative relatively undifferentiated cells. Expression can also be identified in the proliferative progenitor cell population. It would be desirable to continue analysis of the mouse model for expression of the CFP reporter. Tissues still to test include those found to express the reporter by RT-PCR such as the liver and spleen. Younger animals could be analysed for CFP reporter expression in the intestine. Furthermore, the expression pattern in embryos could also be investigated. It would also be beneficial to set-up dual fluorescence in order to co-localise expression of the CFP reporter and other putative stem cell markers in adult tissue and teratoma sections. Expansion of the qPCR data to include quantification of the CFP reporter in adult tissues and embryos would also be of interest.

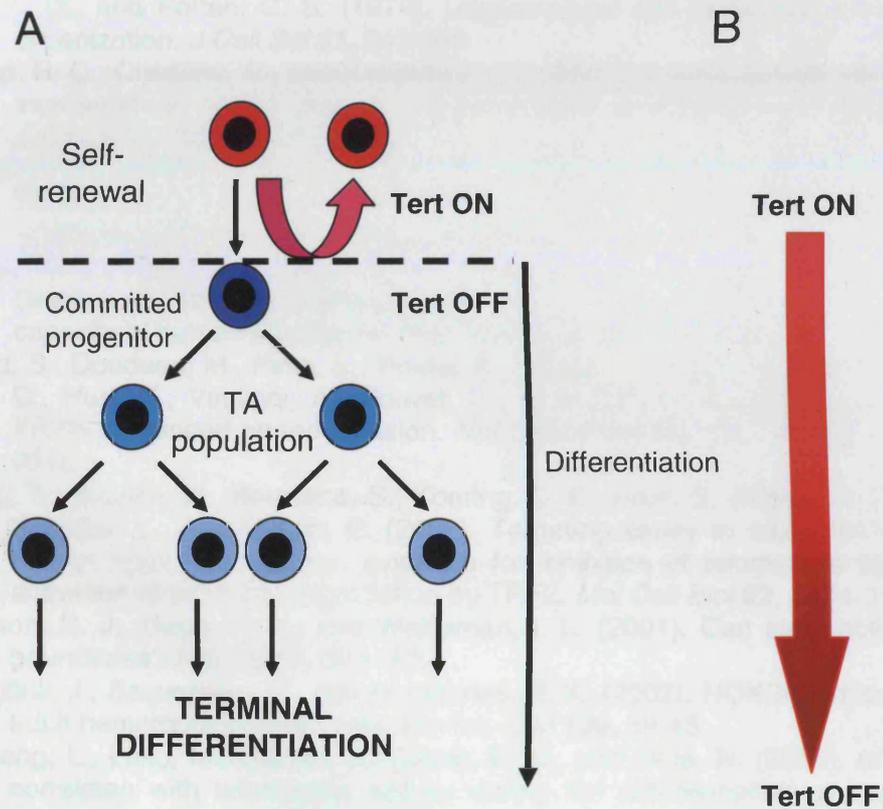
Once the CFP reporter expression pattern has been fully characterised, LRC experiments could be performed and the location of expression of the label correlated with the expression of the CFP reporter. This would provide further evidence for the correlation of reporter expression and the stem cell phenotype. However, the only way of determining whether the CFP reporter could be used to identify stem cells/progenitor cells would be to isolate CFP expressing cells (perhaps by FACS) and use them in repopulation and clonogenic assays.

### 7.9 Conclusion

The generation and analysis of an *mTert* promoter mouse model has provided evidence that *mTert* expression as regulated by its promoter can be associated with the highly proliferative stem cell phenotype. Expression of the CFP reporter in putative stem cell locations such as the basal layer cells of the epidermis, the bulge region of the hair follicle and the spermatogonia of the testis tubule has provided evidence that *mTert* expression can be correlated with the geographical location of stem cells. Analysis of reporter expression in ES cells and teratomas and correlation with markers of proliferation such as Ki67 and pluripotency such as Oct-4 also indicate that *mTert* through its promoter can be correlated with proliferative pluripotent cells. However, the analysis of adult tissues has shown that the CFP reporter is also expressed in the progenitor cell populations. Thus, the project hypothesis that *mTert* expression would be switched off as the stem cells give rise to TA cells is not quite accurate. Instead the data presented here would suggest that *mTert* expression as regulated by its promoter is down-regulated upon differentiation cells into TA cells and thus gradually decreases in a gradient as the cells become more differentiated (Figure 7.1B). Thus, the usefulness of

*mTert* as a stem cell marker is questionable as it demonstrates similar patterns of expression as other markers that identify proliferating cells e.g. Ki67.

**Figure 7.1** The project hypothesis. **(A)** The original project hypothesis which states that the stem cells shown in red possess telomerase activity, express mTert and are able to maintain their telomere lengths. When the stem cell gives rise to a progenitor cell, this telomerase activity is switched off. As the progenitor cells differentiate the telomeres become shorter and shorter, until terminal differentiation is achieved. **(B)** The revised project hypothesis which suggests that stem cells possess telomerase activity and express mTert, but this expression is gradually reduced upon differentiation of cells and is eventually switched off in terminally differentiated cells.



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