The regulation and impact of telomerase in human embryonic stem cells and their neural derivatives

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Declaration of Originality

All experiments included in this thesis were performed by me unless otherwise stated in the text.

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Abstract

Telomeres cap the ends of eukaryotic chromosomes, masking the end from DNA repair processes and preventing end-to-end fusions. Telomeres shorten progressively with each cell division, eventually becoming dysfunctional and producing a signal that results in cellular senescence. Telomere shortening can be counteracted by telomerase, a ribonucleoprotein complex with reverse transcriptase activity that catalyses the addition of nucleotide repeats to the end of the telomere. During normal human development, telomerase expression is restricted to the germ cells, some adult stem cells and the early embryo. Correspondingly, telomerase is highly expressed in human embryonic stem cells (hESCs), which are derived from the inner cell mass of the blastocyst and are able to self-renew indefinitely and differentiate into cells of all three germ layers. hESCs provide a useful model for studying telomerase biology during human development therefore I aimed to study the regulation of telomerase in hESCs and their neural progeny.

The research in this thesis revealed that whilst telomerase was downregulated during hESC neural differentiation and telomeres of the derived neural progenitor/stem cells (NPSCs) shortened extensively, telomerase became reactivated in the NPSCs following extended culture, leading to telomere elongation. Furthermore, this reactivation occurred in the absence of cellular transformation. Telomerase reverse transcriptase (hTERT) expression in hESCs and their derived NPSCs was found to be regulated by dynamic DNA methylation of the hTERT promoter and this methylation regulated hTERT transcription by blocking the binding of the transcriptional activator Sp1.

The impact of telomerase expression on hESC self-renewal and differentiation was studied using hESCs that constitutively expressed hTERT. This showed that while hTERT expression only resulted in a minor delay in hESC differentiation, NPSCs derived from these hESCs had a compromised differentiation potential, suggesting that overexpression of hTERT has a more profound effect in somatic stem cells than it does in embryonic stem cells.

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Abbreviations

ALT	Alternative lengthening of telomeres
ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia and Rad3 related
bFGF	Basic fibroblast growth factor
BMP	Bone morphogenetic protein
bp	Base-pair
BrdU	5'-bromo-2'-deoxyuridine
CDK	Cyclin dependent kinase
cDNA	Complementary DNA
ChIP	Chromatin immunoprecipitation
DAPI	4,6-diamidino-2-phenylindole
DIG	Digoxigenin
DMEM	Dulbecco's modified Eagles' medium
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
EDTA	Ethylene-dinitrilo tetraacetic acid
EGF	Epidermal growth factor receptor
ER	Oestrogen receptor
ESC	Embryonic stem cell
FBS	Foetal bovine serum
GF	Growth factor
GFAP	Glial fibrillary acidic protein
hESC	Human ESC

HRP	Horseradish peroxidase
HSC	Haematopoietic stem cell
hTERT	Human TERT
IC	Internal control
ICM	Inner cell mass
iPSC	Induced pluripotent stem cell
IR	Ionising radiation
kb	Kilobase
KSR	Knockout serum replacement
MEF	Mouse embryonic fibroblast
mESCs	Mouse ESC
mRNA	Messenger RNA
MSC	Mesenchymal stem cell
MSP	Methylation specific PCR
mTERT	Mouse TERT
mTR	Mouse TR
NPSC	Neural progenitor/stem cell
NSC	Neural stem cell
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PGK	Phosphoglycerate kinase
PI	Propidium iodide
PLL	Poly-L-Lysine
qRT-PCR	Quantitative reverse transcription PCR

RNA	Ribonucleic acid
RT	Reverse transcription
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
TERT	Telomerase reverse transcriptase
TR	Telomerase RNA
TRAP	Telomere repeat amplification protocol
TRF	Telomere restriction fragment
Ts	transcriptional start site
UTR	Untranslated region
UV	Ultraviolet
wk	Week

Chapter 1. General Introduction

1.1 Telomeres

The interest in telomeres can be traced back to the experiments carried out in the 1930's by two remarkable geneticists: Barbara McClintock and Hermann Muller. Working independently with different organisms, they discovered that chromosomes bore a special component at their ends that discriminates them from intrachromosomal breaks and provides them with stability (McClintock, 1941; Muller, 1938). This special component at the ends of eukaryotic chromosomes is termed 'telomere', from the Greek nouns for 'end' (telos) and 'part' (meros). However, the exact components of the telomere remained a mystery until the 1970's when the work of Elizabeth Blackburn and colleagues began to unveil the nature of this special structure (Blackburn, 1984; Blackburn and Challoner, 1984; Blackburn and Gall, 1978). We now know that telomeres are comprised of DNA repeats bound by a well conserved set of proteins. They distinguish chromosomal ends from intrachromosome breaks and protect them from the DNA damage response machinery, thereby contributing to the integrity of the genome (Blackburn et al., 2006).

1.1.1 Telomeric DNA

Telomeric DNA is composed of tandem non-coding DNA repeats. The repetitive sequence of the telomere is conserved in all vertebrates as TTAGGG. The majority of telomeric DNA is double-stranded, with the GT-rich sequence paired with a CA-rich complement, but the G-rich strand protrudes at the 3' end with a single-stranded region, which is referred to as the G-rich 3' overhang (Palm and de Lange, 2008). Mammalian telomere lengths are genetically inherited, depending on the species and individual (Clark et al., 2003). In humans, telomeres

consist of 10-15 kilobases (kb) of double-stranded DNA at birth, ending in 50-500 nucleotides of single-stranded DNA. In sheep, telomere lengths are typically around 20 kb at birth whereas in laboratory mice, telomeres often reach 50 kb. It remains to be determined what factors make telomere lengths different between species.

1.1.2 Shelterin

The multiple proteins that bind telomeric DNA, directly and indirectly, form a complex which has been named 'shelterin' (de Lange, 2005). The shelterin complex consists of six protein subunits in mammalian cells (Figure 1.1A): the telomere repeat-binding factors TRF1 and TRF2, Protection of telomeres 1 (POT1), TRF1-interacting nuclear protein 2 (TIN2), TPP1 and Repressor and Activator Protein 1 (Rap1). TRF1 and TRF2 bind to double-stranded telomeric DNA (Smogorzewska et al., 2000), while POT1 binds to the single-stranded G-rich 3' overhang (Baumann and Cech, 2001). The other three proteins bind to the telomere indirectly via interactions with one of the above three shelterin proteins. Rap1 binds to TRF2 while TPP1 is a POT1 binding partner and TIN2 bridges TRF1 and TRF2. Together they play an essential role in the formation of the structure of the telomere and in the regulation of telomere lengths.

Both TRF1 and TRF2 contain TRF homology domains, which form the central part of their protein sequence and mediate homodimerisation (Fairall et al., 2001), and Myb-like DNA binding domains at their C-termini, which are highly specific for double-stranded telomeric DNA sequences (Broccoli et al., 1997; Chapman et al., 2004). However, TRF1 and TRF2 contain different N-terminal domains as well as different peptide-docking hinge regions, indicating that they may have different biological functions (Palm and de Lange, 2008). POT1 contains two OB-folds that form the DNA binding domain, which has high specificity

and affinity for single-stranded telomeric DNA sequences (Lei et al., 2004). Unlike TRF1 and TRF2, which bind to telomeres as homodimers, POT1 binds as a monomer. TPP1 binds to POT1 and this is essential for recruitment of POT1 to the telomere (Hockemeyer et al., 2007; Wang et al., 2007). TIN2 binds to both TRF1 and TRF2, linking them. Other than this TIN2-mediated interaction, TRF1 and TRF2 do not interact with each other. TIN2 also binds to TPP1 thereby forming the centre of the shelterin complex, linking TRF1/TRF2 to TPP1/POT1 (Fairall et al., 2001; Kim et al., 1999; Ye et al., 2004a; Ye et al., 2004b). It should be noted that there are conflicting data around whether all shelterin components are part of one single complex or if sub-complexes exist (Liu et al., 2004; Takai et al., 2010).

1.1.3 The structure of the telomere

Telomeres have a unique structure that is thought to be integral to their function. The 3' overhang of the telomere loops back and invades the double-stranded duplex, forming a higher order lariat structure which has been visualised by electron microscopy and is referred to as the T-loop (Griffith et al., 1999) (Figure 1.1B). This was first observed using an *in vitro* artificial telomeric DNA model, which was comprised of a unique 3 kb DNA sequence followed by several kilobases of double-stranded TTAGGG repeats, ending with a 100-200 nucleotide 3' overhang generated by 5' exonuclease-mediated resection. When this artificial telomeric DNA was incubated with purified TRF2 and visualised by electron microscopy, lasso-like structures were observed at the end of the telomere, with TRF2 protein localised at the beginning of the loop. Loop formation was dependent upon both incubation with TRF2 and the presence of the single-stranded overhang. Furthermore, TRF1 was unable to induce loop-formation as TRF2 did. Microscopic analysis of genomic DNA extracted from HeLa cells demonstrated an abundance of T-loops and these were also seen in primary cells from

humans and mice. T-loop size was correlated with mean telomere length. After incubation with purified single-stranded binding (SSB) protein, SSB protein complexes were observed at the loop-tail junction, suggesting that the single-stranded telomere overhang was located at the junction and possibly invaded the telomeric duplex, stabilising the T-loop (Griffith et al., 1999).

This study was followed-up by another which used a similar telomere model. Incubation with TRF2 and visualisation by electron microscopy showed the telomeric DNA folded back at one end into a 200-500 base-pair (bp) loop, with TRF2 present at the junction. As before, the presence of the 3' overhang was necessary for T-loop formation however, the length of the overhang did not determine the frequency of T-loop observation. Modifying the DNA sequence of the overhang demonstrated that the TTAGGG sequence is essential for TRF2 localisation to the T-loop junction. Furthermore, moving double-stranded TTAGGG to internal sites within the DNA, replacing double-stranded TTAGGG at the end with non-telomeric sequence, prevented binding of TRF2 to the DNA terminus and inhibited T-loop formation (Stansel et al., 2001). T-loops have also been observed in isolated telomeric chromatin (Nikitina and Woodcock, 2004).

TRF2, but not TRF1, enhances the binding of telomeric oligonucleotide probes to double-stranded telomeric DNA and increases the sensitivity of that DNA to nuclease digestion. It has also been shown to induce positive supercoiling, resulting in a conformational change of the double helix. Taken together, these data suggest that TRF2 promotes unwinding of the telomeric duplex and invasion by the single-stranded overhang (Amiard et al., 2007). TRF2 neutralises the negative charge of nucleosomes through its basic N-terminal region, leading to nucleosomal compaction and promotion of single-strand invasion of the telomeric duplex (Baker et al., 2011). This property is also present in TRF1 however, it is inhibited by the protein's acidic domain (Poulet et al., 2012). Telomeric DNA is packaged similarly to

constitutive heterochromatin, with repressive epigenetic histone modifications (Blasco, 2007), and was traditionally thought to be transcriptionally silent. However, the recent discovery of telomeric transcripts intimately associated with the telomere has shown that this is not the case (Azzalin et al., 2007; Schoeftner and Blasco, 2008).

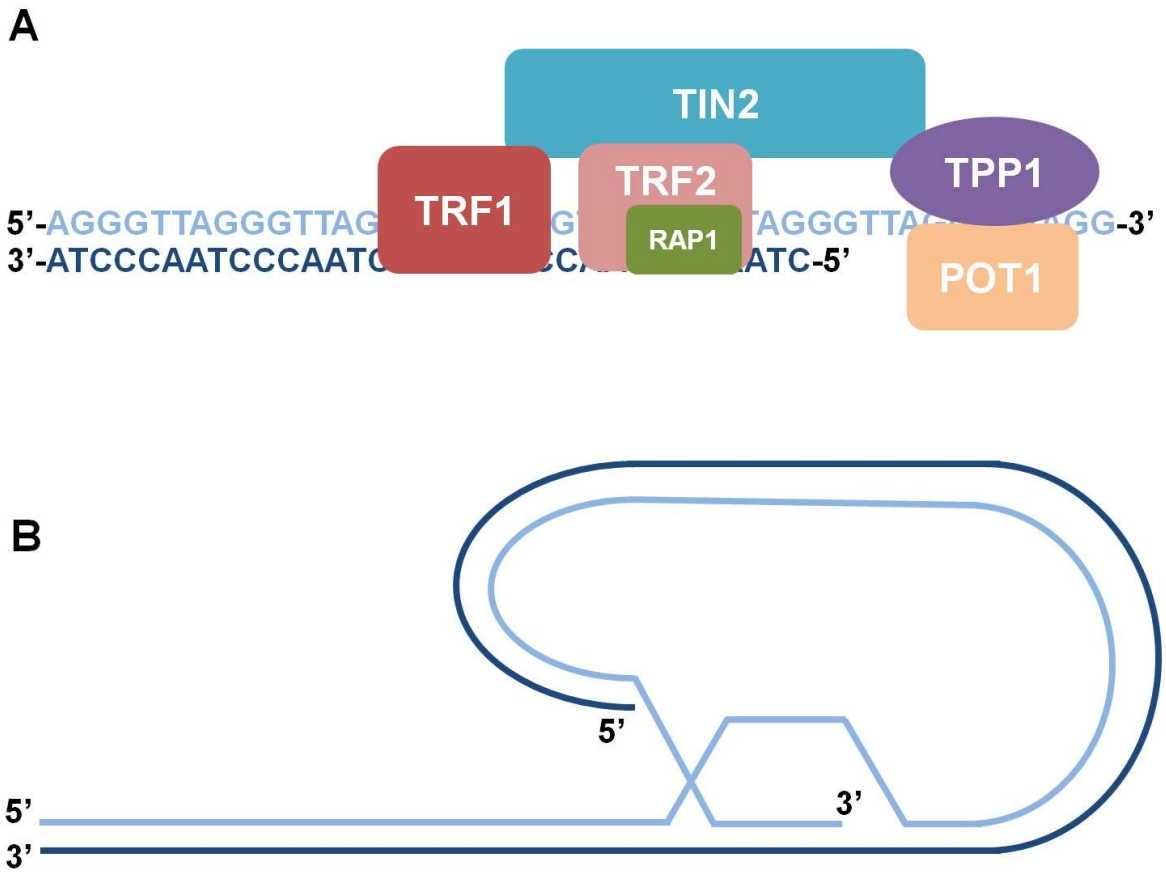


Figure **1.1: Depiction of the shelterin complex and telomere T-loop structure** **(A)** The shelterin complex is composed of six protein subunits. TRF1 and TRF2 bind to double stranded telomere repeats while POT1 binds to the single stranded repeats of the telomere overhang. Rap1 binds to the telomere via its interaction with TRF2. TIN2 links TRF1 and TRF2 and binds TPP1, which also binds to POT1. **(B)** At the telomere terminus, the G-rich strand has a 3' overhang which loops back to invade the double stranded duplex.

1.2 Function of telomeres

The main function of telomeres is to cap the chromosomal end, protecting it from nuclease-mediated degradation and masking the end of the DNA from DNA repair complexes, which would otherwise recognise the end as a double strand break (de Lange, 2009). Thereby, the telomere prevents chromosomal end-to-end fusions that would result from inappropriate DNA repair activities. Functional telomeres are essential for the maintenance of chromosomal integrity and genome stability.

1.2.1 The end replication problem

Formation of functional telomeres requires the telomere to be sufficiently long and the minimum length required appears to be cell type- and species-dependent. The question of how to maintain a functional telomere length had been an interesting research area for many years, particularly the question of how telomeric DNA repeats are replicated during cell division, and this had puzzled researchers for a long time because it was realised that the mechanism of DNA replication prevents the ends of linear DNA from being fully copied. This was referred to as the 'end replication problem'. During DNA replication, the leading strand, once initiated, is synthesised by DNA polymerase in a highly processive and continuous manner which can potentially replicate through to the terminus of a linear DNA template. However, the lagging strand is synthesised as discrete Okazaki fragments due to the requirement of multiple RNA primers to initiate synthesis in the 5'-3' direction. The fragments are then ligated to form a continuous strand. All the RNA primers require DNA as a template and without DNA to serve as the template for a RNA primer at the end of linear DNA, the replication machinery cannot synthesise the lagging strand completely (Levy et al.,

1992); thereby, telomeric DNA gets shorter with each cell division (Harley et al., 1990). In this way, telomeric DNA acts as a buffer, preventing the erosion of coding DNA.

1.2.2 Telomeres and replicative senescence

Telomeres shorten with each round of replication, eventually reaching a critically short length; at this point the cell is forced into senescence (Espejel and Blasco, 2002), a permanent state of cell cycle arrest but continued viability. This process is referred to as replicative senescence and explains why primary cells in culture can only divide a limited number of times, referred to as the Hayflick limit (Hayflick and Moorhead, 1961). It is thought that telomeres therefore act as a mitotic clock and that replicative senescence underlies organismal ageing, an idea supported by the observation that peripheral blood cell telomere lengths are inversely correlated with subject age (Hastie et al., 1990).

The precise mechanisms that trigger replicative senescence are still largely unclear. Critically short telomeres, termed dysfunctional telomeres, accumulate DNA damage response factors commonly found at DNA double-stand breaks - including phosphorylated histone 2A.X (γ-H2AX), p53-binding protein (53BP1) and ataxia telangiectasia mutated (ATM) – together referred to as telomere-induced foci (d'Adda di Fagagna et al., 2003; Takai et al., 2003). It is thought that dysfunctional telomeres are too short to form proper T-loops, resulting in exposure of the chromosome end, somehow causing the phosphorylation and activation of ATM and ATR, which leads via Chk2/Chk1 to phosphorylation and activation of p53 (Gire et al., 2004; Guo et al., 2007). p53 then activates transcription of the Cip/WAF CDK inhibitor p21 which prevents entry into S-phase and induces senescence. This event can be recapitulated using a dominant negative DNA binding mutant of TRF2 which strips the telomeres of endogenous TRF2, exposing them to DNA damage response proteins

(Smogorzewska and de Lange, 2002). Experimental models of telomere biology often utilise molecular biology techniques to induce telomere dysfunction. However, differences exist between this and the telomere deprotection that results from physiological telomere shortening. For example, inhibition of TRF2 leads to ligase IV-dependent chromosomal fusions, mediated by classical non-homologous end joining. In contrast, physiological telomere shortening leads to chromosomal fusions which occur independently of ligase IV and DNA PKcs (through alternative non-homologous end joining) (Rai et al., 2010).

It has been demonstrated that individual shelterin components play key and specific roles in preventing the DNA damage response at telomeres, with TRF2 specifically inhibiting ATM signalling and POT1 specifically inhibiting ATR signalling. Therefore, dysfunctional telomere-induced senescence likely involves a change in shelterin function (Denchi and de Lange, 2007). Conditional deletion of TRF1 and TRF2 demonstrates that they are able to prevent six types of DNA damage response pathway: ATM signalling, ATR signalling, DNA resection, homology-directed repair, classical non-homologous end joining and alternative non-homologous end joining (Sfeir and de Lange, 2012). In S. Pombe, deletion of Taz1 (the TRF homologue) leads to the recruitment of repair proteins and checkpoint sensors to telomeres. However, Crb2 (53BP1) is not recruited. 53BP1 is required for Chk1-mediated cell cycle arrest. Association of 53BP1 to telomeres requires H4K20me2 and γH2AX and S. Pombe telomeres were shown to be deficient for H4K20me2, in contrast to other chromosomal regions. Furthermore, the POT1 interaction with Ccq1, an additional component of the shelterin complex in fission yeast, blocks Crb2 recruitment to telomeres in a H4K20me2-dependent manner (Carneiro et al., 2010).

1.3 Telomerase

Telomere shortening can be counteracted by the action of a ribonucleoprotein complex called telomerase. The key components of telomerase consist of a reverse transcriptase catalytic subunit (TERT) (Harrington et al., 1997; Meyerson et al., 1997; Nakamura et al., 1997) and the telomerase RNA moiety (TR), containing a CUAACCCUAAC sequence which is complementary to the telomere repeat unit (Feng et al., 1995). The RNA subunit TR acts as a template for the addition of nucleotides to the 3' telomere overhang by the catalytic subunit TERT. DNA polymerase then catalyses the addition of nucleotides to the complementary strand (Figure 1.2). Biochemical studies have shown that *in vivo* human telomerase consists of two hTERT subunits and binds two telomeric DNA substrates. Furthermore, the two hTERT subunits function catalytically as a dimer (Sauerwald et al., 2013). During one single round of the cell cycle, 60 nucleotides are added per telomere by telomerase (Zhao et al., 2011). POT1-TPP1 stimulates telomerase processivity, the ability of telomerase to consecutively add nucleotides to the growing telomere 3' strand (Figure 1.2). The mechanism by which this is achieved involves the fact that POT1-TPP1 decreases the rate of primer dissociation and also enhances template translocation (Latrick and Cech, 2010).

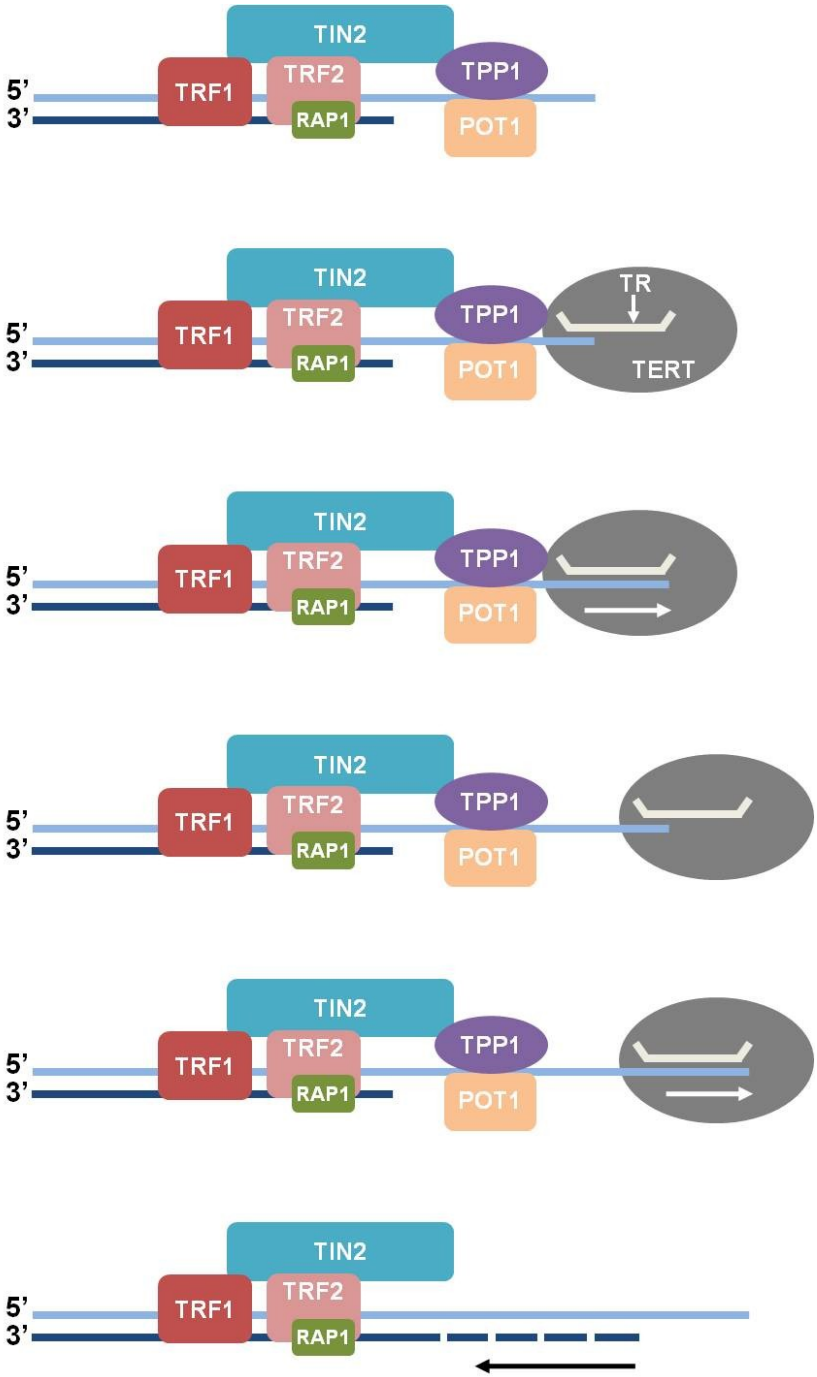


Figure 1.2: Mechanism of action of telomerase

Telomerase is recruited to the telomere via the interaction of TERT with TPP1. The telomerase RNA subunit (TR) contains a region with sequence complementarity to the telomere G-rich repeats, acting as a template for the addition of telomere repeats to the overhang by TERT. As TERT processively adds new telomere repeats to the overhang in a POT1-TPP1-dependent manner, the 3'-end of the telomere is extended. Canonical DNA polymerase then extends the 5'-strand.

Several studies in the last decade indicate that telomerase, particularly the catalytic subunit TERT, has extra-telomeric functions, such as promoting cell proliferation. Telomerase deficient mouse models suffer from defective hair growth due to a failure to mobilise the hair follicle stem cells (Flores et al., 2005; Sarin et al., 2005). In contrast, conditional upregulation of TERT in mouse keratinocytes leads to activation and proliferation of the stem cell compartment and consequently hair follicle growth. This effect was also shown to be independent of TR and therefore unrelated to telomerase activity and telomeres (Choi et al., 2008). Moreover, activation of stem cell compartments could be achieved by overexpression of a catalytically inactive mutant of TERT, which was explained by the observation that TERT can be found co-localised with chromatin remodelling complexes at Wnt-dependent gene promoters, where it enhanced transcription (Park et al., 2009). However, separate data on extra-telomeric functions of telomerase are conflicting. Gene expression profiling of liver tissue from mTERT and mTR knockout mice showed no significant differences compared with wild type (Vidal-Cardenas and Greider, 2010). Furthermore, Strong and colleagues did not observe any differences in Wnt-dependent reporter gene activation in mTERT-deficient mice compared with wild type (Strong et al., 2011). Further investigations are required to clarify this issue.

1.3.1 Recruitment of telomerase to telomere

Shelterin proteins TRF1 and TRF2 have been identified as negative regulators of telomeres and inhibition of them can lead to elongation of telomeres (Kim et al., 1999; Loayza and De Lange, 2003). In contrast, TPP1 mutants lacking the OB DNA binding domain (TPP1ΔOB) do not lead to telomere elongation. Instead, they fail to recruit telomerase. TPP1-TERT association has been shown by co-immunoprecipitation and this association is lost with

TPP1ΔOB mutants (Xin et al., 2007). Furthermore, TPP1ΔOB mutants and TPP1 knockouts inhibit the recruitment of hTERT to telomeres in HeLa cells (Abreu et al., 2010). Conditional knockouts of TPP1 in mouse embryonic fibroblasts (MEFs) led to the ablation of TERT localisation at telomeres. This was associated with increased chromosomal instability and accumulation of DNA damage at telomeres (Tejera et al., 2010).

TERT recruitment to telomeres occurs specifically in S-phase but the precise mechanism by which TERT recruitment is cell cycle-regulated is unknown. ATM and ATR are recruited to telomeres during S-phase (Denchi and de Lange, 2007) and deletion of the homologues of either in S. Pombe (Tel1 and Rad3, respectively) leads to a failure to recruit telomerase (Moser et al., 2009). Telomerase recruitment in S. Pombe is mediated by Ccq1 and this process requires the phosphorylation of threonine 93 of Ccq1 (Moser et al., 2011; Yamazaki et al., 2012). Interestingly, Tel1 and Rad3 phosphorylate threonine 93 of Ccq1. This phosphorylation is inhibited by the Taz1-Rap1-Poz1 complex (the S. Pombe TRF1/2-Rap1-TIN2-like complex); since shorter telomeres are bound to less Taz1-Rap-Poz1 complexes and have more phosphorylated Ccq1 (Moser et al., 2011), this provides a possible negative feedback mechanism whereby longer telomeres inhibit the recruitment of telomerase.

1.3.2 Telomerase, telomere maintenance and their impact in ageing and cancer

Telomere maintenance is a prerequisite for cancer cell growth; hTERT is upregulated and high telomerase activity is found in ninety percent of cancers (Blasco and Hahn, 2003; Kim et al., 1994). The telomerase RNA component TR is ubiquitously expressed in most cells but is also upregulated in some cancers (Soder et al., 1997). Inhibition of telomerase prevents cancer cell growth (Hahn et al., 1999) thus telomerase has been proposed as a potential target for anti-cancer therapy (Parkinson and Minty, 2007). In the small proportion of telomerase-

negative cancers, a telomerase-independent mechanism for telomere maintenance exists. This mechanism is referred to as alternative lengthening of telomeres or the ALT pathway and involves homologous recombination (Cesare and Reddel, 2010).

Mouse knockout models (of either TERT or TR) are viable however, after a limited number of generations defects become apparent, such as infertility, reduced lymphocyte proliferation and reduced wound healing (Blasco, 2005). Many of these phenotypes involve a decline in stem cell function, including compromised tissue regeneration, and mimic human premature ageing syndromes. Inbred laboratory mice have unnaturally long telomeres, therefore, telomerase knockout mouse models require several generations for extensive telomere shortening to occur and for phenotypes to become apparent. Zebrafish, on the other hand, have shorter telomeres. TERT knockout zebrafish die prematurely by the first generation, develop degenerative phenotypes, including premature infertility and gastrointestinal atrophy, have impaired cell proliferation and accumulation of DNA damage markers, followed by accumulation of senescent cells (Henriques et al., 2013).

Several human diseases map to a telomerase component mutation: Dyskeratosis congenita involves a mutated Dyskerin protein (one element of the telomerase complex) and the autosomal dominant form is caused by a mutation in the human TR (hTR) gene (Vulliamy et al., 2001). The disease causes skin and nail abnormalities and progressive bone marrow failure. Aplastic anaemia (resulting in low blood cell counts) patients have hTERT or hTR mutations and idiopathic pulmonary fibrosis involves TERT mutations resulting in telomere shortening in lung stem cells leading to a loss of lung alveolae.

Ectopic expression of the human TERT (hTERT) gene in primary cells is able to avert telomere shortening, preventing replicative senescence and extending cellular lifespan indefinitely (Bodnar et al., 1998). This tool has been extensively used, particularly for *ex vivo*

expansion of progenitor cells with potential therapeutic applications. While overexpressing TERT in mice results in an increased incidence of cancer (Blasco, 2003; Gonzalez-Suarez et al., 2001), it extends lifespan when these mice are crossed to a cancer resistant background to achieve higher expression levels of key tumour suppressor genes (Tomas-Loba et al., 2008). Moreover, reactivating TERT in telomerase knockout mice for just four weeks results in telomere elongation, attenuation of the DNA damage response, restoration of stem cell functionality and a reversal of the ageing phenotype, ultimately leading to increased lifespan (Jaskelioff et al., 2010). All of these studies provide compelling evidence for the importance of telomerase in organismal ageing.

1.3.3 Telomerase expression during development and after birth

Human oocytes do not express telomerase and undergo telomere shortening from foetal oogenesis and over the course of a women's life; this has been linked with female reproductive ageing (Kalmbach et al., 2013). In contrast, spermatogonia have high telomerase activity and telomere length of sperm cells has been shown to be positively correlated with age (Eisenberg et al., 2012). Despite this, telomerase is not expressed by the zygote (Kalmbach et al., 2013) although extensive telomere elongation does occur in the early embryo in a telomerase-independent manner, presumably through the ALT pathway (Liu et al., 2007). Telomerase activity and TERT expression is detected around the morula stage and peaks at the blastocyst stage. Telomerase activity is downregulated after twenty weeks of gestation (Wright et al., 1996). In the adult organism, telomerase activity and TERT expression is only found in male germ cells and at low levels in activated lymphocytes and some adult stem cell populations (Collins and Mitchell, 2002; Wright et al., 1996).

B-lymphocytes have longer telomere lengths compared with T-lymphocytes. Furthermore, memory B-lymphocytes were observed to have longer telomeres than naive B-lymphocytes. Consistently, activated B-lymphocytes had low levels of telomerase activity whereas telomerase was undetectable in non-activated B-lymphocytes (Martens et al., 2002). Similarly, low to undetectable levels of telomerase in peripheral T-lymphocytes were found to be upregulated upon T-cell activation (Weng et al., 1996). Telomerase activity has also been detected in CD34+CD71+ haematopoietic progenitor cells, whereas it was absent in mature CD34- haematopoietic progenitor cells. However, this low and transient activation of telomerase activity was insufficient to maintain telomere lengths as telomere shortening was observed in cultured haematopoietic progenitor cells (Chiu et al., 1996). Telomerase activity has been detected in keratinocytes from the basal epidermal layer of the skin (Harle-Bachor and Boukamp, 1996) and also in oesophageal crypts, the region where putative stem cells reside (Hiyama et al., 2001). These observations suggest that telomerase may play a role in somatic progenitor/stem cells that continuously regenerate adult tissues.

1.3.4. Regulation of telomerase expression

The molecular basis for the regulation of telomerase expression and activity is very complex and many signalling pathways and transcription factors have been reported to regulate telomerase expression (Cairney and Keith, 2008; Cifuentes-Rojas and Shippen, 2012). Since expression of the catalytic subunit TERT gene is more dynamic during normal development and cancer and the level of TERT expression appears to be a limiting factor for telomerase activity in cells and tissues, many studies have been carried out to explore its regulation.

Mounting evidence has demonstrated that TERT expression is regulated predominantly at the transcriptional level (Bodnar et al., 1998; Cui et al., 2002) as the level of telomerase activity

correlates well with TERT mRNA levels. It is now generally accepted that hTERT transcription is regulated by both transcriptional and epigenetic factors which bind to the hTERT promoter region and subsequently regulate hTERT expression (Cifuentes-Rojas and Shippen, 2012; Kyo et al., 2008).

1.3.4.1 Transcription factors

The hTERT gene is located on the short arm of chromosome 5, is more than 40 kb long and consists of 15 introns and 16 exons (Bryce et al., 2000; Cong et al., 1999; Takakura et al., 1999). The hTERT promoter was first cloned in 1999 and lacks TATA and CAAT boxes but is CG-rich. The core hTERT promoter was defined by deletion analysis using luciferase reporter assays in cancer cell lines and is ~300 bp, spanning ~200 bp upstream and ~100 bp downstream of the transcriptional start site (Cong et al., 1999; Takakura et al., 1999). This core promoter has since been the focus of many studies and has been shown to contain multiple putative binding sites for a variety of transcription factors (Figure 1.3) (Horikawa et al., 1999; Takakura et al., 1999), several of which have been reported to regulate hTERT expression by either activating or repressingits transcription.

ETS2 has been shown to activate hTERT transcription in breast cancer cells, in complex with c-myc, (Xu et al., 2008).and in colon cancer cells (Flavin et al., 2011). In addition, HER2 signalling in breast cancer cells results in binding of the ETS family member ETV1 to two sites within the hTERT promoter: 288 and 390 bp downstream of the transcriptional start site. This leads to the subsequent activation of hTERT transcription (Goueli and Janknecht, 2004). Furthermore, ETV1 activation of hTERT trasnscription has been confirmed in HER2-positive breast carcinoma tissue (Vageli et al., 2009). The transcription factor AP-2 has been shown to bind to the hTERT promoter in lung tumours, activating hTERT transcription (Deng et al.,

2007). Hypoxia inducible factor (HIF) is upregulated in response to decreased oxygen levels, often in poorly vascularised tumours, and the hTERT promoter contains HIF binding sites. Both hypoxia and HIF-1 overexpression have been shown to upregulate hTERT expression (Nishi et al., 2004; Yatabe et al., 2004) and HIF-2a increases hTERT expression in renal cell carcinoma cells (Lou et al., 2007). However, HIF-2a represses hTERT expression in glioma cells (Lou et al., 2007) and HIF-1 has been shown to repress hTERT in colon cancer cells (Koshiji et al., 2004).

Other transcription factors known to inhibit hTERT expression include WT1, MEN1, SIP and MAD1. WT1 is a well reported transcriptional repressor of hTERT that has been shown to bind the hTERT promoter and repress transcription (Oh et al., 1999; Sitaram et al., 2010), for example during differentiation of promyelocytic leukaemia cells and in renal tissue (compared to renal cell carcinoma) (Azouz et al., 2010; Sitaram et al., 2010). MEN1, SIP and MAD1 were all identified as transcriptional repressors of hTERT through an enhanced retroviral mutagen screen in HeLa cells (Lin and Elledge, 2003), although the function of MEN1 as an hTERT repressor has been challenged by another study (Hashimoto et al., 2008); MAD1 competes with c-myc for binding to their shared binding partner Max and subsequently the hTERT promoter (Xu et al., 2001). MAD1 also recruits histone deacetylases to the hTERT promoter, leading to epigenetic repression (Ge et al., 2010). Finally, E2F1 has been shown to repress hTERT in squamous carcinoma cells (Crowe et al., 2001) and overexpression of Jun represses hTERT in HeLa cells via binding to AP-1 sites in the promoter (Takakura et al., 2005) .

In addition to regulation of hTERT expression by transcription factors, it has also been shown that overexpression of the polycomb complex protein Bmi1 in mammary epithelial cells led to activation of hTERT transcription and induction of telomerase activity (Dimri et al., 2002).

Of all the transcriptional regulators of hTERT, c-myc and Sp1 have probably drawn the most attention.

1.3.4.1.1 c-myc

The most well characterised transcriptional activator of hTERT is c-myc. c-myc is a basic helix-loop-helix transctiption factor and known proto-oncogene. The core hTERT promoter contains two E-box motifs (CANNTG or CACGTG) to which Myc family transcription factors can bind (Figure 1.3) (Takakura et al., 1999). c-myc expression has been shown to positively correlate with telomerase activity and hTERT expression levels in cancer cells, and c-myc protein can bind to the E-boxes in the hTERT promoter and to induce hTERT transcription and activate telomerase activity in normal epithelial cells (Kyo et al., 2000; Wang et al., 1998; Wu et al., 1999). In addition, the binding of c-myc to the hTERT promoter plays a role in activating hTERT transcription and telomerase activity during SIRT1-mediated immortalisation of normal human umbillical cord fibroblasts (Yamashita et al., 2012).

1.3.4.1.2 Sp1

The core hTERT promoter contains five GC-boxes, a consensus motif with the sequence GGGCGG. The zinc finger transcription factor Sp1 (Suske, 1999) has been shown by electrophoretic mobility shift assay (EMSA) to bind to each of these (Figure 1.3) (Xu et al., 2001). Furthermore, sequential mutation of each of the five GC-boxes in the hTERT promoter leads to progressive hTERT downregulation in cancer cells, demonstrating that Sp1 binding to each of the GC-boxes synergistically enhances hTERT transcription (Kyo et al., 2000). In addition, overexpression of Sp1 results in increased hTERT transcription in EBV-immortalised B-cells (Wu et al., 1999). Sp1 has been shown to act in concert with MCAF1 to activate hTERT transcription in cancer cells (Liu et al., 2009) and the anti-cancer compound butylidenephthalide suppresses telomerase expression via Sp1 downregulation (Lin et al.,

2011). During HPV E6 protein-mediated keratinocyte immortalisation, hTERT upregulation occurs via increased Sp1 binding to the hTERT promoter (Xu et al., 2013). In activated T cells, NFAT cooperates with Sp1 to activate hTERT transcription (Chebel et al., 2009). Furthermore, NFAT leads to the induction of c-myc expression, further contrbuting to hTERT expression in these cells (Buchholz et al., 2006). It is of note that Sp1 expression is often present in normal cells. Therefore, additional factors and mechanisms must exist to activate and repress hTERT transcription in cancer cells and in normal cells, respectively (Horikawa et al., 2002).

1.3.4.2 Signalling pathways

A number of extracellular signalling proteins have been reported to regulate hTERT transcription, including hormones, cytokines and growth factors (Figure 1.3). Ligand-bound oestrogen receptors translocate from the cell membrane to the nucleus and bind to either of two oestrogen-responsive elements within the hTERT promoter, increasing hTERT transcription (Cukusić et al., 2009; Kyo et al., 1999; Misiti et al., 2000). In progesterone receptor-positive breast cancer cells, progesterone activates hTERT transcription via MAP kinase signalling (Wang et al., 2000). In T-cells, IFN-α leads to hTERT downregulation via activation of the hTERT transcriptional repressor E2F (Lanna et al., 2013). TGF-ß represses hTERT transcription (Yang et al., 2001) either via indirect downregulation of c-Myc expression (Hu et al., 2005) or via direct Smad3-mediated interference with c-Myc activity (Li et al., 2006). The MAP kinase pathway appears to be a key mediator of hTERT activation by extracellular signalling. hTERT activation by HER2 signalling and ETV1 binding to the promoter (as described above) occurs via the MAP kinase pathway and upstream Ras and Raf-1 are essential for this (Goueli and Janknecht, 2004). Finally, EGF signalling is known to

activate hTERT transcription by activating ETS2, c-myc and Sp1, via MAP kinase signalling (Bermudez et al., 2008; Maida et al., 2002).

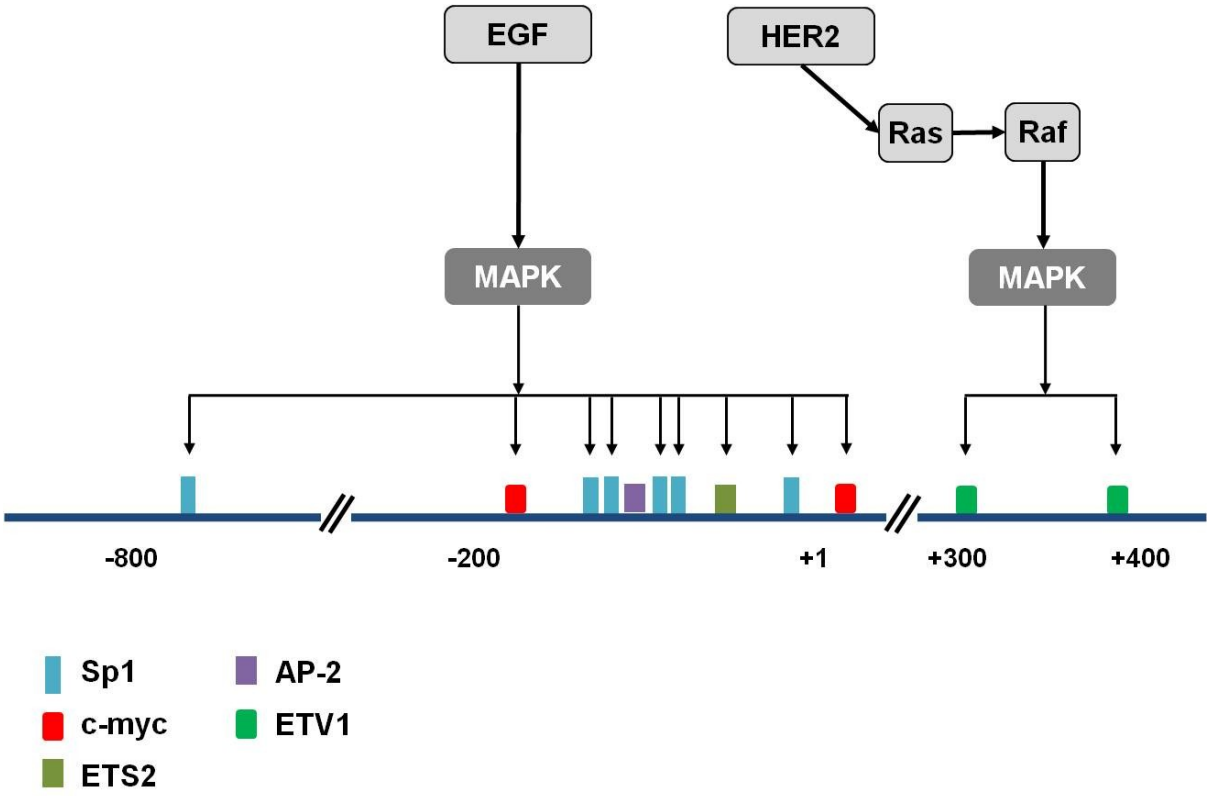


Figure 1.3: Transcriptional activation of the hTERT promoter

The hTERT promoter contains a number of putative binding sites for known transcription factors. Several of these have been shown to bind to their respective binding site, activting transcription of hTERT, including Sp1, c-myc, ETS2, AP-2 and ETV1. It has also been demonstrated that HER2 and EGF signalling activate hTERT transcription via ETV1 and Sp1/c-myc/ETS2, respectively.

1.3.4.3 Epigenetic regulation of hTERT transcription

1.3.4.3.1 CpG methylation

Methylation of cytosine residues in CpG dinucleotides within gene promoters has been associated with transcriptional silencing. CpG methylation is thought to affect gene transcription by inhibiting the binding of transcription factors and by recruiting corepressor proteins containing methyl-CpG-binding domains (Bird and Wolffe, 1999; Boyes and Bird, 1991). Genome-wide changes in methylation occur during development and CpG methylation is thought to play a role in regulating many cellular processes (Carlson et al., 1992; Santos et al., 2002).

Several techniques have been developed to detect methylated regions of the genome, which include: 1) Methylation-sensitive endonuclease assays such as the HELP (**H**paII tiny fragment **E**nrichment by **L**igation-mediated **PCR**) assay (Figueroa et al., 2009). These methods take advantage of specific restriction endonucleases that are unable to cleave their target DNA sequence if it contains a methylated CpG. 2) Methylated DNA immunoprecipitation (MeDIP), an analogue of ChIP (chromatin immunoprecipitation), in which methylated DNA is immunoprecipated with a specific antibody and then sequenced or subjected to microarray analysis (Mohn et al., 2009). 3) Bisulphite sequencing, which involves chemical conversion of unmethylated cytosines to uracil, while methylated cytosines remain unconverted (Patterson et al., 2011). Specific regions of the genome are then sequenced and the generated sequence is compared with the known sequence of the region of interest, in order to assess the methylation status of specific cytosine residues (within the context of CpG dinucleotides). 4) Methylation-Specific PCR (MSP), in which bisulphite conversion is combined with PCR, using primers complementary for a particular CpG-containing sequence, as it would exist following bisulphite conversion if methylated (Herman et al., 1996).

The hTERT promoter is very CG-rich and contains a cluster of CpG sites, which form a large CpG island. Therefore, research into hTERT regulation by CpG methylation at the promoter has been carried out by a number of groups, with conflicting results. The majority of studies of hTERT promoter methylation have been carried out in cancer cell lines and have focussed on the core promoter region. CpG methylation at gene promoters is typically associated with gene silencing and indeed, some studies have shown methylation of the hTERT promoter to be associated with hTERT silencing, for example in B-cell lymphocytic leukaemia and in normal human oral keratinocytes (Bechter et al., 2002; Shin et al., 2003). In contrast, there are reports of hTERT-positive cancer cells having dense methylation at the promoter (de Wilde et al., 2010). In those cells, the hTERT promoter is heavily methylated, except a small region close to the transcriptional start site where CpG dinucleotides are unmethylated (Devereux et al., 1999; Zinn et al., 2007). It has been shown that the hTERT promoter is hypermethylated in many cancer cells and that this is associated with elevated hTERT transcription, while demethylation resulted in repression of hTERT transcription (Renaud et al., 2007). However, complete methylation of the hTERT promoter led to hTERT silencing and hypomethylation of a region within the core promoter, around the transcriptional start site, was essential for hTERT transcription (Renaud et al., 2007). The emerging view is that in numerous telomerase-positive cancer cell types, hTERT promoter methylation functions to block the binding of the transcriptional repressors which silence hTERT in non-transformed somatic cells, whilst unmethylated promoter regions proximal to the transcriptional start site allow for a low level of hTERT transcriptional activation (Chatagnon et al., 2009). In addition, hTERT promoter methylation has been positively correlated with hTERT mRNA levels in leukocytes taken from patients with clinical atherosclerosis (Zhang et al., 2013).

1.3.4.3.2 Histone modification

Modification of histone proteins by acetylation and methylation is known to regulate the structure of chromatin and thereby affect gene transcription (Stein et al., 2000). In line with this, histone modification has been shown to play a role in the regulation of hTERT expression, and hTERT transcription has been associated with hyperacetylation of histone H3 and H4 and methylation of histone H3 lysine 4. Conversely, hTERT repression was associated with histone H3 and H4 hypoacetylation and methylation of histone H3 lysine 9 (Atkinson et al., 2005). Interestingly, Sp1 has been shown to interact with Hmga2, which inhibits recruitment of an histone deacetylase enzyme to the hTERT promoter, thereby promoting hTERT transcription (Li et al., 2011). In contrast, acetylation of histone H3 localised to the first hTERT exon induced by sulforaphane has been reported as leading to the binding of MAD1, CTCF and other transcriptional repressors to the promoter, resulting in hTERT repression (Meeran et al., 2010). Therefore, the effect of histone modification on hTERT expression is complex and depends upon its localisation at the hTERT promoter/enhancer and upon cell type.

1.4 Stem Cells

Stem cells are defined as unspecified cells which possess two important properties: 1) Self-renewal, being able to generate at least one daughter cell the same as themselves; 2) Differentiation, capable of producing more specified cell types (Smith, 2006). Stem cells can be categorised into two main groups: somatic stem cells and embryonic stem cells.

1.4.1 Somatic stem cells

Somatic stem cells, also called adult stem cells, can be found throughout our body but often resident in specific locations of their tissues of origin (Joseph and Morrison, 2005). For example, intestine stem cells are located around the bottom of the intestinal crypt (Korinek et al., 1998); hair follicle stem cells are located in the bulge region of the follicle (Nishimura et al., 2002; Oshima et al., 2001), haematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs) are found in the bone marrow (Pittenger et al., 1999). These stem cells are able to self-renewal throughout our lifespan and their self-renewal is supported by the stem cell niche, the microenvironment provided by their surroundings (Moore and Lemischka, 2006). Somatic stem cells are also able to differentiate into multiple cell types of their lineage but are unable to produce cell types of other lineages. For example, mesenchymal stem cells are capable of differentiating into multiple cells of the mesenchymal lineage, including bone, cartilage and fat cells, but cannot generate neurons or liver cells (Nardi and da Silva Meirelles, 2006). Somatic stem cells are responsible for repairing any damaged tissues and replacing non-functional cells and are therefore essential for our body to carry out normal physiological tasks.

1.4.1.1 Telomerase expression in somatic stem cells

As mentioned in section 1.3.3, telomerase has been found to be expressed in some stem cell compartments *in vivo* after birth and its expression is thought to play a role in the self-renewal of these cells. However, the results are conflicting regarding telomerase expression when separating these cells from their *in vivo* niche. For example, MSCs derived from the bone marrow of adult donors have been found to have low levels of telomerase activity which decreased during subsequent *in vitro* expansion (Parsch et al., 2004). This is in contrast to

another report of bone marrow-derived MSCs that found no detectable telomerase activity, either before or after expansion (Zimmermann et al., 2003). Moreover, three further reports of bone marrow-derived MSCs demonstrated that these cells did not have telomerase activity, that their telomere lengths decreased during expansion (Banfi et al., 2002; Samsonraj et al.) and finally that they underwent replicative senescence (Bernardo et al., 2007). Interestingly, one study has demonstrated that while adult bone marrow-derived MSCs had undetectable telomerase activity and short telomeres, comparable to a somatic control cell type, MSCs derived from foetal blood, liver and bone marrow had higher telomerase activity and longer telomeres. The foetal cells also proliferated faster than the adult cells (Guillot et al., 2007). Nevertheless, these cells eventually also ceased proliferation and became senescent due to telomere shortening. These results suggest that telomerase cannot maintain telomere length in MSCs cultured *in vitro*.

1.4.1.2 Telomere shortening in somatic stem cells

Since the majority of the studies on telomere regulation by telomerase in humans have been done in cancer cells, limited information is available regarding telomere length in somatic stem cells. However, it was shown that telomere lengths in various tissues were shorter in older donors (Verfaillie et al., 2002). Senescent cells have been observed to accumulate in various tissues during ageing of different species, including humans (Dimri et al., 1995; Krishnamurthy et al., 2004; Rossi et al., 2007; Rube et al., 2011). In addition, the accumulation of DNA damage markers and increased rates of apoptosis have also been observed (Pollack et al., 2002). Telomere shortening and DNA damage accumulation occurs in HSCs during ageing (Sperka et al., 2012). DNA damage can result in the differentiation of somatic stem cells, such as HSCs (Wang et al., 2012), melanocytic stem cells of the hair

follicle (Inomata et al., 2009) and intestinal epithelial stem cells (Paulus et al., 1992). Differentiation as a response to DNA damage could be a checkpoint to limit the self-renewal of damaged stem cells potentially carrying oncogenic mutations. It could be hypothesised that telomere dysfunction in stem cells could have the same effect as DNA damage and telomere dysfunction in HSCs has been observed to lead to lymphoid differentiation (Wang et al., 2012). Interestingly, during human ageing the number of lymphoid-competent HSCs is reduced and there is skewing towards myeloid differentiation, resulting in the decreased immune function observed during ageing (Beerman et al., 2010; Dykstra et al., 2011; Wang et al., 2011). Of note, depletion of p53, one of the major effectors of the DNA damage response, increases the self-renewal capacity of HSCs, as well as that of liver progenitors and neural stem cells (Katz et al., 2012; Meletis et al., 2006; TeKippe et al., 2003). Furthermore, depletion of p53 in differentiated cells increases the efficiency of their reprogramming to the undifferentiated, pluripotent state (Marion et al., 2009a).

1.4.2 Embryonic stem cells

Embryonic stem cells (ESCs) were first derived in 1981 from the inner cell mass (ICM) of blastocyst-stage mouse embryos (Evans and Kaufman, 1981). Building on this research and the later derivation of non-human primate ESCs, the first human ESC (hESC) lines were finally derived in 1998 (Thomson et al., 1998), also from the ICM of pre-implantation blastocyst-stage embryos. hESCs, like mouse ESCs (mESCs), have a large nuclear to cytoplasmic ratio and grow as colonies. However, their colonies are different compared to those of mESCs, having a distinct morphology.

All ESCs are defined by two essential properties. Firstly, ESCs can have unlimited self-renewal, dividing symmetrically at each cell division to maintain their undifferentiated,

pluripotent state. ESC self-renewal is governed by key intrinsic factors - such as the transcription factors Oct4 and Nanog - and extrinsic factors - such as extracellular growth factors like bFGF. Secondly, ESCs are pluripotent, with the capacity to differentiate into the three embryonic germ layers (endoderm, ectoderm and mesoderm), from which all cell types in the body are generated. This pluripotency can be demonstrated *in vitro* by the differentiation via embryoid body formation, a process that recapitulates embryo development to an extent, or *in vivo*, by the formation of teratomas when ESCs are injected into severe combined immunodeficient (SCID) mice, and chimera production (only for rodent ESCs) when ESCs are injected into pre-implantation embryos.

The derivation of pluripotent hESCs has led to two major potential applications; hESCs have great potential for use in cell-based therapies and also provide a useful model for studying early developmental processes. In addition to embryo-derived ESCs, pluripotent stem cells have also been derived from somatic cells in two ways. Somatic cell nuclear transfer involves replacing the nucleus of oocyte with that of a donor adult cell. The fused cell can then be stimulated to divide, forming an embryo from which ESCs can be harvested (Tachibana et al., 2013). More importantly, it has been shown that a somatic cell can be reprogrammed and induced to become an undifferentiated, pluripotent cell type (induced pluripotent stem cell [iPSC]) by the introduction of four transcription factors: c-myc, Klf4, Oct4 and Sox2 (Takahashi et al., 2007). Both approaches allow the production of person/patient-specific pluripotent stem cells (French et al., 2006).

1.4.2.1 Telomerase and telomeres in embryonic stem cells

As in its *in vivo* counterpart, the ICM of the blastocyst (Wright et al., 1996), telomerase is also highly expressed in undifferentiated hESCs (Thomson et al., 1998), resulting in high telomerase activity, which allows hESCs to maintain long telomeres throughout successive cell divisions and therefore to self-renew indefinitely. Interestingly, telomerase is upregulated during the reprogramming of fibroblasts to iPSCs and telomeres are elongated in a telomerase-dependent manner (Marion et al., 2009b). Similar to during embryonic development, telomerase expression is robustly downregulated upon differentiation of hESCs (Gerrard et al., 2005; Hay et al., 2008; Thomson et al., 1998) and this was found to be accompanied by the loss of histone acetylation, a mark of transcriptional activation, at the hTERT promoter. In parallel, telomerase activity is downregulated and telomere lengths decrease with cell division (Saretzki et al., 2008). These results demonstrated that pluripotent stem cells are among the few normal cell types to express endogenous telomerase and that telomerase expression is dynamically regulated during the differentiation of these cells, resembling that which occurs during normal development. Therefore, these cells provide a valuable cell model with which to investigate the molecular mechanisms that regulate telomerase expression and to study its role during early human development.

1.4.2.2 Telomerase expression during neural differentiation of hESCs

In an experiment of neural differentiation of hESCs, it was reported that high hTERT expression of hESCs was downregulated to a low level in hESC-derived neural stem cells (NSCs) which was then further downregulated to undetectable levels in beta-III-tubulin-positive neurons derived from the NSCs. Furthermore, the level of hTERT was found to be

positively and negatively correlated with permissive and repressive histone modifications, respectively (Golebiewska et al., 2009).

Work in our laboratory has led to the development of a protocol for the simple, monolayer differentiation of hESCs down the neural lineage. Using this protocol, neural progenitor/stem cells (NPSCs) have been derived, which have a bipolar morphology and express NPSC markers (Gerrard et al., 2005). During this neural differentiation, hTERT expression in hESCs has been observed to be dramatically downregulated (Gerrard et al., 2005), consistent with other studies of hESC differentiation (Golebiewska et al., 2009; Hay et al., 2008; Saretzki et al., 2008), to neural and other lineages.

1.4.2.3 Role of telomerase in hESCs

It is unknown whether hTERT downregulation is a requirement for or merely a consequence of hESC differentiation. Studies in mouse embryonic stem cells (mESCs) have been contradictory. While one study has found no effect of ectopic mTERT expression on differentiation but a conferred growth advantage and resistance to apoptosis (Lee et al., 2005), another reported a greater tendency towards haematopoietic differentiation even though the same growth and survival advantages were found (Armstrong et al., 2005). By contrast, a third study has shown that ectopic TERT expression delays neural differentiation of mESCs (Schwob et al., 2008).

In hESCs, overexpression of hTERT caused cells to progress through the cell cycle more rapidly, leading to faster cell proliferation in culture (Yang et al., 2008), which is consistent with that observed in mESCs. While *in vivo* differentiation of the hTERT-overexpressing hESCs produced larger teratomas, evidence of quicker differentiation, the *in vitro*

differentiation of these cells appeared much slower than the controls. Embryoid bodies derived from hTERT-overexpressing hESCs were found to contain more neural and haematopoietic progenitors and less mature cells, suggesting a delay in differentiation.

In addition to the unclear role of telomerase in ESC differentiation, data about its role in the regulation of self-renewal and pluripotency are confusing. Telomerase expression appeared to be unnecessary for the maintenance of mESC self-renewal and pluripotency, since both telomerase knockout ESCs and mice are able to survive and differentiate/develop. However, in hESCs, transient hTERT knockdown led to a loss of pluripotency and induction of differentiation (Yang et al., 2008). Since hESCs and mESCs have distinct telomere lengths, it is unclear whether telomerase has a direct function in regulating ESC pluripotency or if the phenotypes observed were a result of telomere erosion.

1.5 Aims of the project

The overall aim of my project is to use hESCs and their neural derivatives to study the function and regulation of telomerase during normal cellular development. I attempt to achieve this overall aim by targeting three objectives.

I) hESCs provide a valuable tool for studying telomerase and telomere biology in the context of normal development and in a non-transformed cell type. Our neural differentiation protocol offers a monolayer method that can be easily visualised and monitored and produces a homogenous population of NPSCs. In the first part of the project, I aim to study how telomerase and telomeres change in hESCs, during their neural differentiation and in the culture of NPSCs, using our established protocol. I will examine hTERT expression, telomerase activity, telomere length dynamics and genomic stability during neural differentiation of hESCs, as well as during the culture of NPSCs and during the differentiation of these to postmitotic neurons and glial cells. The information obtained could help us to understand more about telomere and telomerase biology in development, health and diseases.

II) Telomerase is dynamically expressed during neural differentiation of hESCs, which provides us with a model to explore the molecular mechanisms that regulate telomerase expression. In the second part of my project, I aim to examine the genetic and epigenetic factors that regulate hTERT expression in hESCs versus differentiated neural progenitors and postmitotic neural cells.

III) Due to the robust and rapid downregulation of hTERT during hESC differentiation and the ubiquity of silencing of hTERT among differentiated cell types, I hypothesised that forced expression of hTERT in hESCs might inhibit their ability to differentiate, particularly to postmitotic functional cell types. Therefore, in the third part of the study I aim to test the

effect of ectopic hTERT expression in hESCs upon their self-renewal and differentiation using our previous established stably transfected hESC clones.

Chapter 2. Materials and Methods

2.1 Cell culture materials

Item	Supplier	Catalogue No.
β-mercaptoethanol	Invitrogen	31350-010
Apo-transferin	Sigma	T1147
B27	Invitrogen	17504-044
BSA	Sigma	A9576
Collagenase	Invitrogen	17104-019
DMEM (liquid)	Invitrogen	11960-044
DMEM (powder)	Invitrogen	12100061
DMEM/F12 (liquid)	Invitrogen	21331-020
DMEM/F12 (powder)	Invitrogen	12500062
0.02% EDTA	Sigma	E8008
EGF	Peprotech	100-15
FBS	Sigma	F7524
FGF2	Peprotech	100-18C
Gelatin	Sigma	G1393
Insulin	Sigma	I1882
KO-DMEM	Invitrogen	10829-018
KSR	Invitrogen	10828-028
100X L-Glutamine	Invitrogen	25030-024
Mouse laminin	Sigma	L2020
Matrigel	Invitrogen	12760021
Neurobasal medium	Invitrogen	21103-049
Noggin	R&D systems	719-NG-050
NEAA	Invitrogen	11140-035
100X P/S	Invitrogen	15140-122
Poly-L-lysine	Sigma	P4707
Progesterone	Sigma	P6149
Putrescine	Sigma	P5780
Sodium Selenite	Sigma	S5261
Trypsin-EDTA	Sigma	T3924
TrypLE Express	Invitrogen	12604-013

KSR Formulation	Final Concentration
KO-DMEM (400ml)	--
KO-SR	20%
FGF2	4 ng/ml
100X NEAA	1X
2-mercaptoethanol	0.1 mM
100X L-Glutamine	1X
100X P/S	1X

100X N2	Final Concentration
10 mg/ml insulin in 0.01M HCL	500 μg/ml
100 mg/ml transferin in dH2O	10 mg/ml
75 mg/ml BSA in PBS	5 μg/ml
0.6 mg/ml progesterone in ethanol	2 μg/ml
160 mg/ml putrescine in dH2O	1.6 mg/ml
1 mg/ml selenite in dH2O	0.5 μg/ml
DMEM/F12	--

2.2 Cell culture methods

2.2.1 Preparation of MEF-CM

MEFs were expanded in DMEM + 10% FBS + 1X P/S + 1X L-Glutamine and when they had reached the required number and density, they were trypsinised and collected in centrifuge tubes. The cells were exposed to 40 gray of ionising radiation, centrifuged and then plated onto 0.5% gelatine-coated T225 flasks, at a density of 1.88-2x10^7 cells per flask. These were left in the incubator at 37°c overnight in the same medium as before. The next day, the medium was replaced with KSR medium, supplemented with 4 ng/ml bFGF. One day later, the medium, now MEF-conditioned medium (MEF-CM) was collected and stored at -80°c for future use. The cells were given fresh KSR medium and the collection procedure repeated over the next seven days.

2.2.2 Culture of hESCs

The hESCs used in this thesis were the H1 and H7 lines, two of the first hESC lines established and originally derived in 1998 (Thomson et al., 1998). hESCs were maintained in matrigel-coated 6-well plates in MEF-CM supplemented with 8 ng/ml bFGF. The medium was changed daily. The hESCs were routinely passaged by incubating for 5-10 minutes with 200 U/ml collagenase and scraping with a 5 ml pipette to lift up the hESC colonies, which were then split to new plates at a 1:3 ratio.

2.2.3 Differentiation of hESCs

hESCs were differentiated down the neural lineage using the lab's standard protocol (Gerrard et al., 2005). Briefly, confluent hESCs were incubated with EDTA to promote cell detachment. EDTA was removed and the cells were flushed with N2B27 medium and triturated into small clumps, which were then split at a 1:5 ratio and plated into PLL/laminin-coated 6-well plates (passage 1). The differentiating cells were cultured in N2B27 medium (1:1 mix of DMEM/F12 and neurobasal medium plus 1xN2 and 1xB27, P/S and L-glutamine) supplemented with 100 ng/ml noggin. Upon confluency, the differentiating cells were split 1:3 using collagenase and scraping (as with hESC culture). This was repeated for passage 2 cells and then from passage 3 onwards, the cells were split using TrypLE Express to dissociate them into single cells. After passage 3, noggin was withdrawn from the medium and by passage 5, the cells, now N2 stage neural progenitors, were supplemented with bFGF and EGF, both at 20 ng/ml.

2.2.4 Culture and differentiation of NPSCs

The hESC-derived neural progenitor/stem cells (NPSCs) were cultured in PLL/laminin-coated 6-well plates in N2B27 medium +bFGF and EGF. When confluent, they were treated with TrypLE Express at 37°c for 5 minutes and transferred to centrifuge tubes, counted, centrifuged and resuspended in fresh medium. Typically, $5x10^5$ cells were plated to each well of a 6-well plate. To induce differentiation, cells were plated into PLL/laminin-coated 6- or 24-well plates, at $4\text{-}5x10^5$ or $8\text{-}10x10^4$ cells per well respectively. bFGF/EGF were withdrawn and the cells cultured in N2B27 alone.

2.2.5 Culture of primary and cancer cells

Primary Hs27 human fibroblasts, HepG2 and HeLa cancer cell lines were all maintained in DMEM + 10% FBS + 1x P/S + 1x L-glutamine. When confluent, the cells were passaged by incubating with 1x Trypsin-EDTA for approximately 5-10 minutes to detach them from the plate and then splitting then at a 1:10 ratio to fresh plate.

2.3 Molecular biology materials

Item	Supplier	Catalogue. No.
100 bp DNA ladder	Invitrogen	15628019
BCA protein assay kit	Pierce	23225
CL-XPosure file	Fisher	34090
Enhanced chemiluminescence substrate	Pierce	32209
DH5α Competent Cells	Active motif	11096
DNase I	Sigma	AMPD1-1KT
Immobilon-P membrane, PVDF	Millipore	IPVH-000-10
Oligo dT 12 18 Primer	Invitrogen	18418012
Protease Inhibitor	Sigma	P8340
Protein ladder	Fermentas	SM1811
RNase OUT	Invitrogen	10777019
SuperScript II RT	Invitrogen	18064014
SYBR Green Jumpstart Readymix	Sigma	S9430
Propidium Iodide	Sigma	P4864
BrdU	Sigma	B5002
DAPI	Sigma	D9542
Ethidium Bromide	Sigma	E1510
Trizol	Invitrogen	15596-026
Glycoblue	Ambion	AM9515
Colcemid	Invitrogen	15212012
Protein-A-agarose	Santa Cruz	Sc-2001
Proteinase K	Sigma	P4850
RNase A	Sigma	R6513
Taq Polymerase	Invitrogen	10342053
Jumpstart Taq Polymerase	Sigma	D4184
Methylcode Bisulphite Conversion Kit	Invitrogen	MECOV50
pGEM-T-Easy cloning Kit	Promega	A1360
TRAPeze Assay	Millipore	S7700
SYBR Green I DNA Stain	Sigma	S9430
	Roche	12209136001

2.4 Molecular biology methods

2.4.1 Irradiation of cells

Subconfluent cells were exposed to 6 gray ionising radiation and then returned to the incubator. Cells were harvested 3 hours later for protein lysates, 48 hours later for PI staining or at various time points for cell number counts. For this, cells were grown in triplicate wells of a 12-well plate.

2.4.2 Immunocytochemistry

Cells grown on coverslips were washed with PBS, fixed for 10 minutes at room temperature with 4% paraformaldehyde and washed again. Cells were blocked and permeabilised by incubating with 10% goat serum + 0.1% Triton-X-100 in PBS for 1 hour at room temperature and then stained overnight at 4ºC with primary antibody diluted in 1% goat serum in PBS. The primary antibodies used are listed in the appendix. Cells were then washed and incubated with secondary antibody (Alexa fluor-conjugated goat anti-rabbit IgG or anti-mouse IgG or IgM, diluted in PBS) for 30 minutes at room temperature in the dark, washed, stained with DAPI (diluted 1:5000 in PBS) for 1 minute, washed for a final time and the coverslips were then mounted onto microscope slides with Mowiol mounting medium. Fluorescently labelled cells were visualised using a Nikon TE2000-U.

2.4.3 BrdU labelling

BrdU was added to the culture medium to a final concentration of 10 μM and the cells incubated at 37ºC for 2 hours. The cells were fixed and their DNA denatured by incubating at 37ºC for 1 hour with 2M hydrochloric acid. After extensive (6x5minutes) washing with PBS, immunocytochemistry was performed as described using an anti-BrdU antibody. For cell counting, representative areas from triplicate wells were counted.

2.4.4 Propidium iodide (PI) staining

Cells were harvested with Trypsin-EDTA and fixed with ice cold 70% ethanol. This was washed out with PBS before staining with PI buffer for 20 minutes on ice. Cells were declumped by pushing through a needle before flow cytometry was carried out using a FACSCalibur machine (BD biosciences) with analysis using CellQuest software.

2.4.5 Soft agar assay

2x DMEM or 2x DMEM/F12 was prepared from powdered medium, dissolving in cell culture tested water. This was filter sterilised and used to make the appropriate growth medium for NPSCs or HeLa cells, only with twice the concentration of growth factors, supplements or serum. This medium was tested on the relevant cells by diluting 1:1 with water and added to the cell culture in place of the normal medium. This confirmed that the medium prepared from powder was not toxic. Cells were harvested with Trypsin-EDTA/TrypLE Express and then resuspended in a 1:1 mix of the double strength medium with 0.5% agar, producing a 0.25% agar solution. The cells in this mixture were then plated into 35 mm dishes with a base layer of 0.35% agar, $5x10^5$ cells per dish. They were covered

with liquid medium, which was changed every few days, and left in the incubator for two weeks before assessing the number of colonies under the microscope.

2.4.6 Preparation of metaphase spreads

Cells were split to new plates and 24 hours later (so that they were in the log growth phase) were incubated with 80 ng/ml colcemid for 4 hours. The cells were harvested with Trypsin-EDTA and resuspended in a warm, hypotonic solution of 0.56% potassium chloride. They were left to swell for 15 minutes before being fixed with a 3:1 mixture of methanol and glacial acetic acid.

2.4.7 Genomic DNA extraction

Cells were harvested with Trypsin-EDTA and centrifuged. The cell pellets were washed with PBS and then resuspended in 500 ul TNE buffer + 20 ug/ml RNase A + 200 ug/ml Proteinase K before overnight incubation at 45°c to digest RNA and protein. The sample was transferred to a phase-lock-gel (PLG) tube, an equal volume of phenol:chloroform:isoamyl alcohol was added and the sample mixed by thorough inversion. The tubes were then centrifuged at 14000 rpm for 10 minutes at 4°c. The upper aqueous phase was transferred to a 1.5 ml tube and 1 ml 100% ethanol was added + 15 ug/ml glycoblue. The tubes were gently inverted and left overnight at -20°c. They were then centrifuged for 25 minutes at 14000 rpm at 4°c, the supernatant discarded and the pellet washed with 1 ml ice-cold 70% ethanol by centrifugation for 5 minutes. After discarding the ethanol, the pellet was air-dried and then resuspended in 5—150 ul TE buffer. The DNA was left overnight at 4°c to ensure it had completely dissolved. The DNA concentration was determined using a Nanodrop spectrophotometer. All

pipetting was done using Gilson P1000 tips with the end to cut off, to ensure the genomic DNA did not become sheared.

2.4.8 RNA extraction

Cells were scraped in Trizol and RNA was isolated as per the manufacturer's instructions. To 1 ml lysates, 0.2 ml chloroform was added and mixed by thorough shaking. The tubes were centrifuged at 12,000xg for 15 minutes at 4ºC. The aqueous phase was transferred to a fresh tube and 0.5 ml isopropanol added. The tubes were mixed by inversion and left for 10 minutes at room temperature to precipitate RNA. The tubes were centrifuged at 12,000×g for 10 minutes, the supernatant discarded and the RNA pellet washed with 75% ethanol by centrifugation for 5 minutes at 7500xg. After air-drying the pellet, it was resuspended in TE buffer and treated with DNase I to remove any traces of genomic DNA: 10x DNase + 10x reaction buffer were added to the RNA sample at 1:10 dilutions. The samples were left for 15 minutes at room temperature, 10x stop buffer was added at a 1:10 dilution and the samples heated at 70°c for 10 minutes to inactivate the DNase. RNA was stored at -80°c until use.

2.4.9 Reverse transcription

2 ug of total RNA was mixed with oligo dT primers and 10 mM dNTPs. Alternatively, gene specific primers were used for hTR. After denaturing the RNA at 65°c for 5 minutes, DTT and RNase OUT was added along with reaction buffer and Superscript II reverse transcriptase (RT) in a total volume of 20 ul. First-strand cDNA synthesis was carried out for 1 hour at 42°c. The cDNA was diluted 1:10 in water and typically 5 ul of this used for standard PCR and 2 ul for real-time PCR.

2.4.10 Polymerase chain reaction (PCR)

cDNA was PCR amplified using gene specific primers designed with NCBI's PrimerBlast program. cDNA was mixed with 2.5 mM dNTPs, magnesium, forward and reverse primers, reaction buffer and Taq polymerase in a total volume of 50 ul. The PCR reaction was carried out using a thermal cycler with the following steps: 94°C for 2 minutes then 25-45 cycles of 94°C for 30 seconds, annealing temperature for 30 seconds, 72°C for 45 seconds, ending with 72°C for 5 minutes. Once the reaction was complete, the reaction mixture was mixed with loading dye and one tenth run on a 1.5% agarose gel (along with a 100 bp molecular weight ladder) containing 0.5 ug/ml ethidium bromide, which was then visualised on a UV transilluminator. PCR for β-actin gene expression was run for all cDNA samples as a loading control whilst non-RT samples were also subjected to PCR, typically using primers for the Oct4 gene, to check for genomic DNA contamination.

Real-time quantitative RT-PCR (qRT-PCR) was also performed in some experiments. 2 ul of cDNA were mixed with 1.8 ul of a 10 uM mix of forward and reverse primers. 15 ul of SYBR Green JumpStart Taq ReadyMix were added and the reaction run in optical 96-well plates using the Opticon Monitor real-time PCR machine (Biorad). For every cDNA sample, β-actin amplification was also performed in parallel. Ct values for particular genes were normalised to the β-actin Ct values and the Δ ΔCt method was used to derive fold change values for each sample, relative to a reference sample.

2.4.11 Chromatin immunoprecipitation

Cells were grown to subconfluence in 10 cm dishes and cross-linked by addition of formaldehyde into the medium at a final concentration of 1% for 10 minutes at 37°c. Cross-linking was quenched by adding glycine to a final concentration of 0.125M for 5 minutes at room temperature and the cells were then washed with ice cold PBS. Lysis buffer was added to the cells and left for 10 minutes before scraping and transferring to a dounce homogeniser and homogenising the lysate 50x to isolate the nuclei. The lysate was then centrifuged at 4500 rpm for 5 minutes at 4°c. The supernatant was discarded and the pellet resuspended in 2 ml of nuclear lysis buffer and divided between two 1.5 ml tubes. These were then sonicated for two hours at 60 seconds on, 30 seconds off, producing chromatin fragments between 200 and 1000 bp. The crude chromatin was centrifuged and the supernatant transferred to fresh tubes, to remove insoluble material.

For immunoprecipitation, 100 ul (equivalent to $1x10^6$ cells) of chromatin was transferred to a new tube and this was topped up with 900 ul of sonication buffer. 4 ug of antibody was added and the tubes were left on a rotating wheel at 4°c overnight. 30 ul of protein-A-agarose beads were added and the samples left rotating at 4° for 3 hours. The tubes were then centrifuged and the supernatant discarded. The beads (with bound immune complexes) were washed for 4-5 minutes with different buffers: 1 wash with sonication buffer, 2 washes with buffer A, 2 washes with buffer B and 2 washes with TE buffer. After the final wash and centrifugation, the beads were resuspended in 250 ul elution buffer + 1%SDS, heated at 65°c for 5 minutes, left at room temperature on a spinning wheel for 15 minutes and centrifuged. The eluate was transferred to a 1.5 ml tube and the beads resuspended in a further 250 ul of elution buffer, repeating the elution step and pooling the eluates at the end. NaCl was added to a concentration of 160 mM and RNase A to a concentration of 20 ug/ml and the eluates left at

65°c overnight to reverse the cross-links. EDTA and proteinase K were added to concentrations of 4 mM and 200 ug/ml and the sample left at 45°c for 2 hours. DNA was phenol:chloroform extracted as describe above. The DNA pellet was resuspended in 30 ul TE buffer and 2 ul was used for PCR analysis. The fold enrichment method was used to determine the level of enrichment of the protein-of-interest at a particular region of DNA, taking into account the total chromatin (input) and any non-specific binding (normal IgG).

2.4.12 Bisulphite sequencing

Genomic DNA was bisulphite treated using the Methylcode Bisulphite Conversion Kit (Invitrogen). 500 ng DNA in a volume of 20 ul was mixed with the bisulphite conversion reagent. The sample was heated at 98°c for 10 minutes followed by 64°c for 2.5 hours. The sample was then cleaned and the converted DNA eluted in a volume of 10 ul using the spin columns provided with the kit. 2 ul of this was used to PCR amplify the regions of interest, using primers specific for the bisulphite converted DNA sequences and amplifying for 45 cycles. The PCR products were run on a 1% agarose gel and then extracted from the gel. The extracted PCR products were ligated into the pGEM-T-Easy vector and this was then used to transform competent DH5α E.Coli. Transformed cells were plated onto agar plates containing antibiotics and X-Gal for selection and incubated overnight at 37°C. Individual white colonies were picked and expanded in LB broth + antibiotic. The plasmid DNA was then isolated by miniprep and sent for sequencing (MRC CSC Genomics Facility) with a Sp6 primer. The sequencing results were aligned with the original sequence, comparing cytosine residues that were part of CpG dinucleotides. CpG cytosines that remained unchanged in the bisulphite converted sequences indicated methylation of that CpG.

2.4.13 Methylation specific PCR

2 ul of bisulphite converted DNA was PCR amplified using primers specific for the methylated bisulphite converted hTERT promoter BS1 region (27 cycles) or primers specific for the unmethylated bisulphite converted GAPDH promoter (30 cycles). PCR products were run on a 1.5% agarose gel containing ethidium bromide and visualised on a UV transilluminator.

2.4.14 Western blotting

Cells were washed with PBS and lysed in pre-heated 1% SDS (typically ~200ul per well of a 6-well plate). The total protein concentration of the cell lysates was determined using the BCA assay. 40 ug of total protein was mixed with the appropriate volume of sample buffer (with β-mercaptoethanol) and boiled at 95-100°c for 5 minutes before loading (along with a pre-stained molecular weight ladder) onto a small vertical 10-13% (depending on the molecular weight of the protein-of-interest) polyacrylamide gel that had been pre-run at 200V for 20 minutes. The gel was then run at 200V in fresh 1xSDS running buffer for approximately 1 hour. The gel was then sandwiched with PVDF membrane and soaked in transfer buffer and the protein blotted to the membrane using a semi-dry transfer apparatus, at 20V for 1 hour. After transfer, membranes were blocked in 5% milk (in TBS-T) for 1 hour at room temperature before incubating with primary antibody diluted in 5% milk overnight at 4°c. Membranes were washed in TBS-T, incubated with HRP-conjugated secondary antibody, diluted 1:2000 in 5% milk, for 1 hour at room temperature, washed again and then ECL substrate solution was applied. Membranes were exposed to x-ray film.

2.4.15 Telomere Restriction Fragment (TRF) length analysis

Average telomere lengths were determined by TRF analysis using the TeloTAGGG telomere length assay kit (Roche), according to the protocol. 2 µg of genomic DNA was digested with 0.75 µl each of *Rsa*I and *Hinf*I restriction enzymes in a total volume of 25 µl at 37ºC overnight, cleaving all DNA except for telomeric sequences into small fragments. These were separated on a 0.8% agarose gel, run at 100V for 20 minutes then 30V for ~16 hours. The gel was depurinated in 0.25M HCl, denatured in 1.5M NaCl/0.5M NaOH, neutralised in 0.5M Tris/1.5M NaCl and blotted onto a positively charged nylon membrane overnight by wet transfer. The membrane was cross-linked to the DNA with UV, pre-hybridized at 42ºC for 1 hour and hybridised to a DIG-labelled telomere-specific probe overnight. After stringent washes, non-specific antibody binding was blocked by incubation with blocking reagent for 30 minutes and then the membrane was incubated with a sheep anti-DIG antibody (diluted 1:10,000) conjugated to alkaline phosphatase. After washing, the membrane was treated with a chemiluminescent alkaline phosphatase substrate (CDP-Star) and exposed to X-ray film.

2.4.16 Telomere Repeat Amplification Protocol – TRAP assay

Telomerase activity was measured using the TRAPeze TRAP assay kit (Millipore), according to the manufacturer's protocol. Cell pellets were snap frozen with liquid nitrogen and stored at -80ºC. When needed, pellets were resuspended in 200 µl CHAPS lysis buffer, incubated on ice and then centrifuged. An aliquot of the lysate was used to determine the total protein concentration (using the BCA protein assay, Pierce, and a standard curve derived from serial dilutions of BSA) and then 1µl was diluted to 10 ng/µl with CHAPS buffer. Two dilutions were made for each sample and one was boiled at 90ºC for 30 minutes to denature any telomerase present (for use as the heat inactivation control). For the TRAP reactions, 1 µl of

diluted lysate was added to an RNase-free tube along with 1 µl TS primer (a telomerase substrate oligonucleotide) and 1 µl primer mix (the TS reverse primer plus an unrelated oligo with a primer specific for it which produces a 36 bp band, serving as a loading and a PCR efficiency control). dNTPs, reaction buffer, hot-start Taq polymerase (JumpStart Taq, Sigma) and water up to 50µl. The mixture was heated to 30ºC for 30 minutes (the telomerase extension step, producing a 6 bp ladder of TS extension products from 50 bp upwards) and then PCR amplified using 30 cycles of 94ºC for 30 seconds, 60ºC for 30 seconds and 72 ºC for 1 minute. The TRAP reaction was resolved on a 10% vertical polyacrylamide gel which was run at 400V for 1.5 hours in 0.5xTBE buffer and then stained with SYBR Green I. The gel was scanned with a phosphoimager (set up for fluorescence) and the image semi-quantified by densitometry, using Quantity One software (Biorad).

Chapter 3. Telomerase and telomeres in human embryonic stem cells and their derived neural progenitor/stem cells

3.1 Introduction

Pluripotent human stem cell lines, including hESCs, are among the few, if not the only normal cell types in culture that express telomerase and can proliferate indefinitely. In addition, telomerase expression in these cells is dynamically regulated, depending on their differentiation state: Telomerase is highly expressed in undifferentiated hESCs but dramatically downregulated upon cell differentiation as described in Chapter 1.4.2.1. Therefore, hESCs may provide an invaluable cell model to investigate the regulatory mechanisms of telomerase expression. In our laboratory, we have found that treatment of hESCs with noggin in a neural-supportive medium in a monolayer culture system results in an efficient neural differentiation (Gerrard et al., 2005; Wu et al., 2010). Neural initiation (N1 stage), occurring during the first one-two weeks, leads to the appearance of neural rosettes and structures resembling the neural tube, seen during neuro-ectodermal development of the embryo *in vivo*. By three to four weeks of differentiation, noggin is withdrawn from the culture and the cells develop a typically bipolar neural progenitor appearance, which is referred to as the N2 stage (Figure 3.1). In the absence of the growth factors bFGF and EGF, these neural progenitors are predominantly differentiated into postmitotic β-III-tubulin-positive neurons with very few GFAP-positive glia. However, in the presence of bFGF and EGF, the cells are able to continuously grow in culture and gradually change their morphology from a bipolar shape to a triangular shape. After approximately fifteen weeks of culture (and ten passages) the cell population develops a homogenous triangular morphology and their differentiation potential gradually changes from exclusively neurogenic to more gliogenic. Nevertheless, the cells are multipotent and clonally isolated neural progenitor cells

of this stage are able to differentiate into both neurons and glia. Thereby, the cells are referred to as N3 stage neural/progenitor stem cells (NPSCs) (Wu et al., 2010).

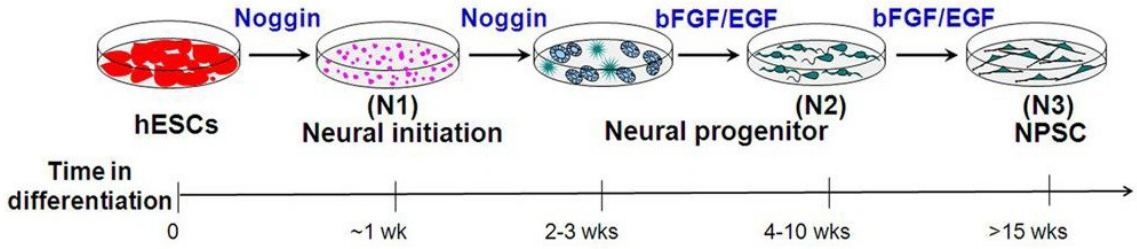


Figure 3.1: Schematic representation of the different stages of neural differentiation of hESCs. Taken from Wu et al, 2010. During the first week of hESC neural differentiation, cells are supplemented with the BMP antagonist noggin and appear as small clumps as they progress through the neural initiation (N1) stage. At two to three weeks of differentiation, neural tube-like structures and neural rosettes become visible, followed by the appearance of neural progenitor cells and the replacement of noggin with bFGF, with or without EGF (N2 stage). At this stage, neural progenitors mainly produce neurons upon further differentiation. During culture, neural progenitor cells gradually change morphology from bipolar to more triangular and after 15 weeks, the cells become homogenously triangular and produce both neurons and glial cells, and thus are termed neural progenitor/stem cells (NPSC) (N3 stage).

This differentiation protocol has been employed on wild-type hESCs many times, with very reproducible results. As hTERT expression and telomerase activity are known to be strongly downregulated during hESC differentiation, including neural differentiation with this protocol (Gerrard et al., 2005; Golebiewska et al., 2009; Hay et al., 2008), we had expected the resulting NPSCs to enter senescence at some point in their extended culture. Therefore, we continuously cultured two of the N3-NPSC populations (one derived from H1 and one from H7 hESCs) for an extended, long period of time. However, after more than one year of continuous culture (and more than fifty passages), the NPSCs derived from both H1 and H7 hESC lines were still proliferating vigorously. This sustained proliferation and lack of senescence was surprising therefore I examined more closely the telomerase status and telomere length of the long-term cultured NPSCs at different stages of culture.

3.2 Results

3.2.1 Reactivation of telomerase in long-term cultured NPSCs

Firstly, I examined mRNA expression of telomerase genes in H7 hESCs and their derived NPSCs of different passages by quantitative (q)RT-PCR. The results showed that hTERT mRNA was indeed dramatically downregulated after hESC neural differentiation, similar to that previously reported (Gerrard et al., 2005; Wu et al., 2010), and this low/undetectable hTERT mRNA expression was maintained throughout early passages (before passage 30) of the long-term cultured NPSCs. Similarly, hTR was also downregulated in early passage cells after neural differentiation, as was Dyskerin gene expression (Figure 3.2A-C). Unexpectedly, after continuous propagation, hTERT mRNA was upregulated in the NPSCs at a later stage of culture. Meanwhile, the expression of hTR and Dyskerin mRNA was also increased (Figure 3.2A-C).

In order to assess whether the dynamic changes in telomerase expression during hESC neural differentiation and the culture of their NPSCs have any effect on telomerase function, I next carried out the TRAP assay of telomerase activity. In agreement with telomerase gene expression, telomerase activity was downregulated in early-passage NPSCs derived from H7 hESCs but increased considerably around passage 30 (Figure 3.2D). In addition, hTERT mRNA expression and telomerase activity exhibited a similar dynamic pattern in H1 hESCs and their NPSCs (Figures 3.2E & F), indicating that these changes are cell type- rather than cell line-specific, and not a technical artefact.

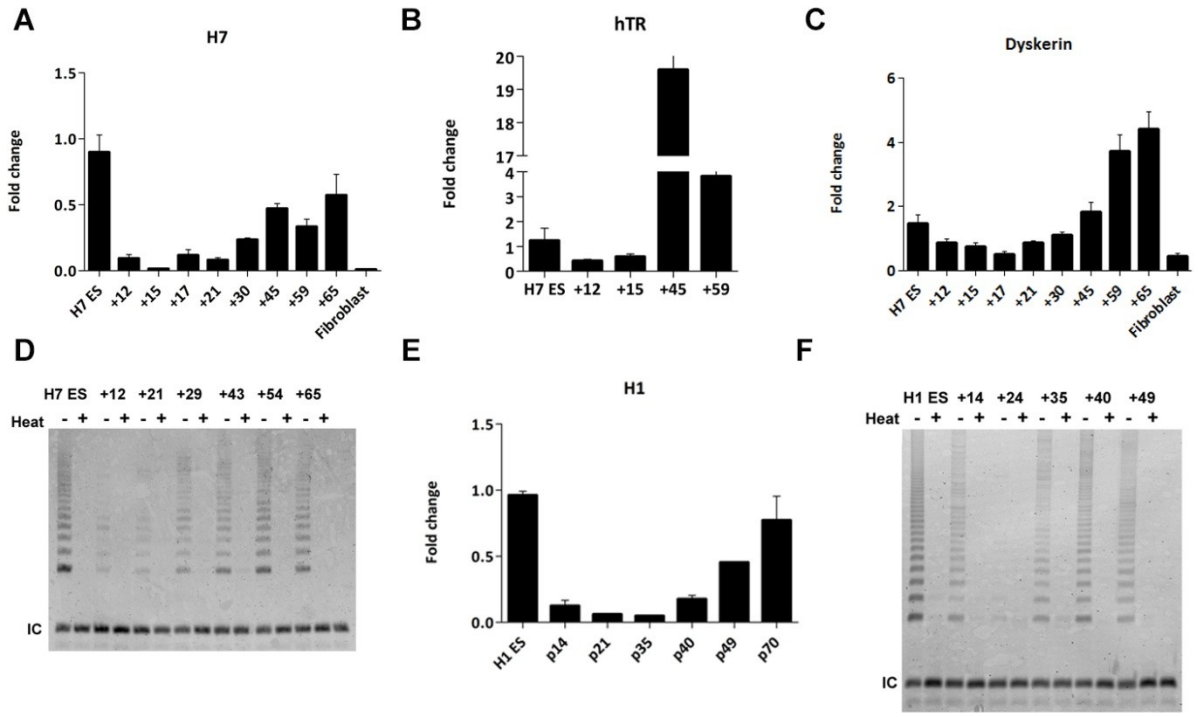


Figure 3.2: Telomerase in NPSCs during long-term culture

(A-D) Telomerase expression and activity during neural differentiation of H7 hESCs. mRNA of telomerase subunit genes hTERT (A), hTR (B) and Dyskerin (C) was downregulated in early passage NPSCs. At later passages, expression of these genes became upregulated again. High telomerase activity in H7 hESCs was greatly reduced in early passage (p12, p21) NPSCs but increased again in later passage NPSCs (D). **(E-F)** The dynamic pattern of hTERT expression and telomerase activity seen in H7 hESCs and NPSCs was also observed in H1 hESCs and NPSCs. mRNA expression and telomerase activity were assessed by qRT-PCR and TRAP assay, respectively, in hESCs and their derived NPSCs, at consecutive passages in culture. mRNA expression was calculated as described in Chapter 2.4.10, normalised to one undifferentiated hESC value and presented as mean ± S.E.M. of three PCR experiments. IC in D & F represents internal control of TRAP PCR.

Furthermore, measurement of telomere length by telomere restriction fragment (TRF) Southern blot showed that telomeres were long in both H1 and H7 undifferentiated hESCs but shortened in the early passages after neural differentiation, with cell proliferation. However, following telomerase reactivation, telomeres were elongated to a relatively long length (albeit shorter than hESC telomeres, Figure 3.3). These data suggest that the upregulation of telomerase in the long-term cultured NPSCs has a functional consequence and that this reactivation of telomerase and elongation of telomeres accounts for the sustained proliferation of hESC-derived NPSCs.

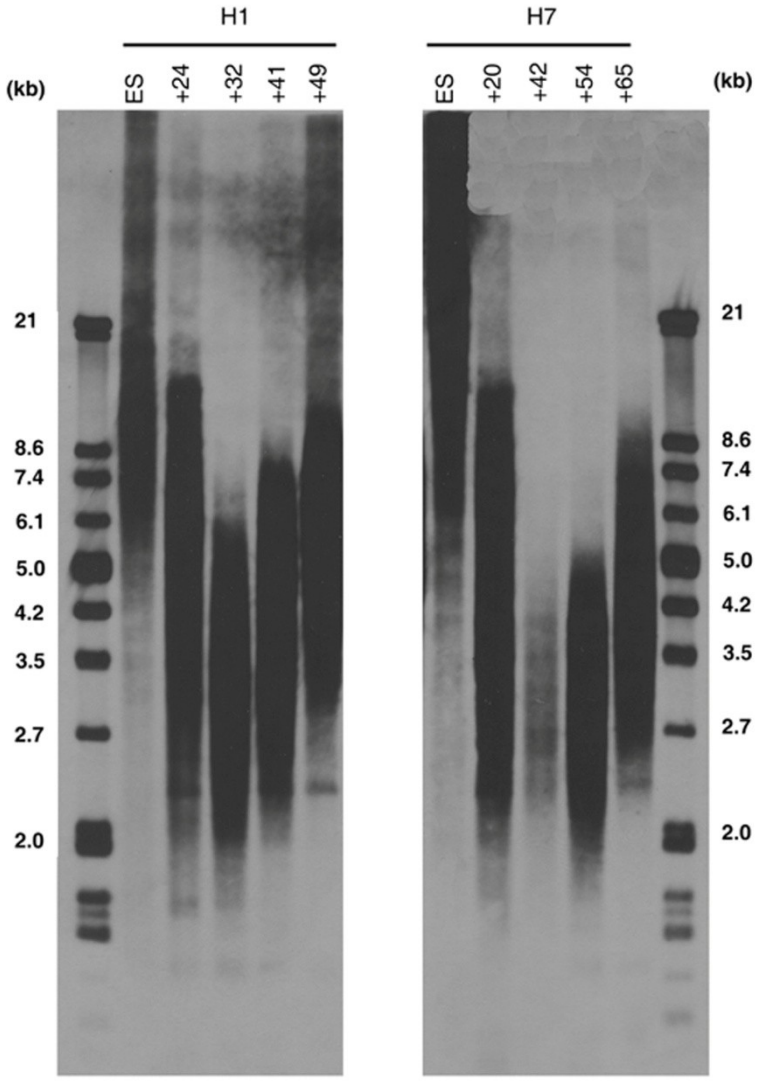


Figure 3.3: Telomere lengths in NPSCs during long-term culture

H1 (left) and H7 (right) hESCs had long telomeres which, following neural differentiation, shortened progressively in early passage NPSCs. However, in later passage NPSCs, telomeres became elongated, as shown by TRF Southern blot of genomic DNA extracted from hESCs and their derived NPSCs, at consecutive passages in culture. Molecular weight ladder is present at the left and right of the blot.

It could be possible that the re-expression of telomerase observed is not in fact due to an intrinsic reactivation within the NPSCs but rather due to a culture selection event. There exists the possibility that after hESC differentiation, a rare subpopulation of cells could have retained telomerase expression and during long-term NPSC culture, this telomerase positive subpopulation could have been expanded preferentially due to a growth advantage, overtaking the whole population and leading to the increase in hTERT expression and telomerase activity that was seen. However, during the transition from telomerase low to high, the cells' properties did not change; they displayed the same morphology, proliferation rate, marker expression and differentiation potential (see section 3.2.2) and are a very homogeneous population. In addition, selection of a telomerase-high subpopulation would likely involve loss of the main telomerase-low population due to telomere dysfunction, resulting in either senescence or apoptosis. However, during the long term NPSC culture, cells with a senescent morphology were never observed and neither was significant cell death.

It can be assumed that a subpopulation of cells retaining high telomerase expression would maintain longer telomeres than the rest of the population and this subpopulation of long telomeres would most likely be visible on a TRF Southern blot, probably greater than 7 kb, as the TRF blot is quite sensitive. Indeed, when NPSCs with long telomeres (p73) are mixed with NPSCs with short telomeres (p40), the subpopulation of long telomeres was visible on the blot even when comprising just 1% of the whole population (Figure 3.4). However, the NPSC TRF blot (Figure 3.3) does not display this; instead, it showed gradual telomere shortening in the early passage cells followed by telomere lengthening later. The cells with the average shortest telomeres (passage 42) have telomeres ranging from 2-5 kb with an average of 3.5 kb. There is no telomeric DNA visible above 5 kb. Certainly, the reappearance of telomerase activity in the TRAP assay precedes the appearance of longer telomeres on the

TRF blot, indicating that the long telomeres in the late NPSC population arose through telomere elongation dependent upon reactivated telomerase, rather than expansion of a constitutive telomerase positive subpopulation.

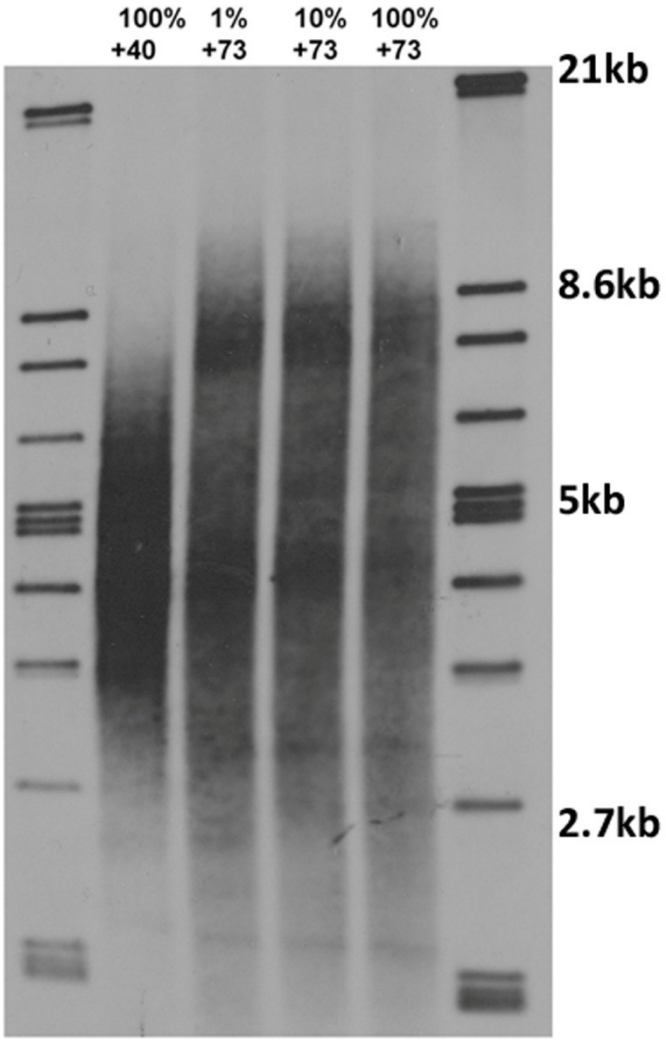


Figure 3.4: TRF assay in mixed long- and -short-telomere NPSCs

Mixing long- and short-telomere NPSC populations showed that long telomeres were visible on a TRF blot even at small proportions: TRF Southern blot of genomic DNA extracted from H7 NPSCs of different passages, mixed at various ratios. Lane 1: 100% p40 NPSCs, lane 2: 99% p40 1%p73, lane 3: 90% p40 10% p73, lane 4: 100% p73.

3.2.2 Late-passage telomerase-high cells maintain NPSC characteristics

The telomerase-low earlier passage (p10-25) and telomerase-high later passage (>p30) NPSCs derived from H7 hESCs are referred to as early and late NPSCs, respectively, from here onwards, and are all N3 stage cells as described in section 3.1. In order to verify that the late passage telomerase-high cells are indeed NPSCs, maintaining their respective characteristics, I studied their neural progenitor/stem cell properties in comparison to the early NPSCs. The late telomerase-high (hTERT-H) NPSCs exhibited a similar neural stem cell morphology compared with early telomerase-low (hTERT-L) NPSCs (Figure 3.5A). They also showed an equally strong expression of the neural stem cell markers Nestin and Sox2 as the early NPSCs (Figure 3.5B). qRT-PCR (Figure 3.5C) showed that both early and late NPSCs lacked expression of the pluripotency-associated transcription factors Oct4 and Nanog, which were expressed highly in hESCs, as expected. Concurrently, they expressed high levels of the neural transcription factors Sox1 and Pax6, although the expression levels were even higher in early NPSCs than in the late NPSCs, which could explain the lower tendency for neuronal differentiation of the late NPSCs.

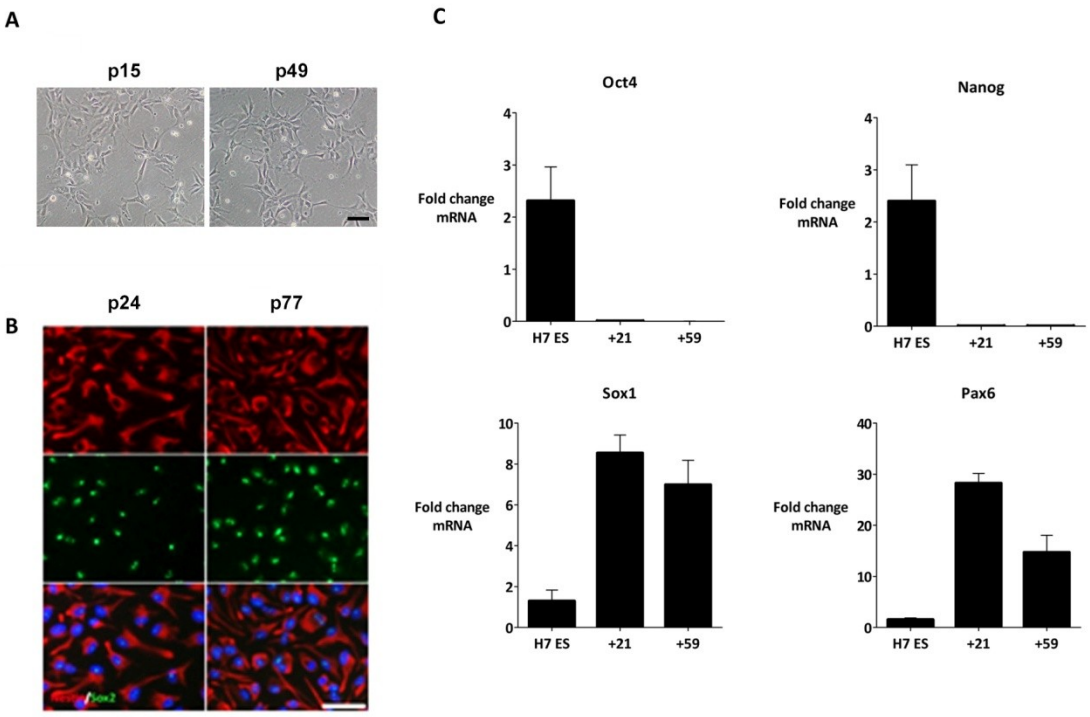


Figure 3.5: Morphology and marker expression in cultured NPSCs

(A) Early passage (p15) and late passage (p49) NPSCs exhibited a similar morphology, as viewed under a light microscope; scale bar represents 50 µm for both images. **(B)** Early passage (p24) and late passage (p77) NPSCs both strongly expressed the neural stem cell markers Nestin and Sox2, as shown by immunocytochemical staining; Nestin (red), Sox2 (green), DAPI counterstain (blue); scale bar represents 50 µm for all images. **(C)** The hESC-specific transcription factors Oct4 and Nanog were highly expressed in H7 hESCs (H7 ES) but absent in early (p21) and late (p59) passage NPSCs, as shown by qRT-PCR for mRNA expression. The neural-specific transcription factors Sox1 and Pax6 were expressed at a very low level in H7 hESCs and highly upregulated in both early and late passage NPSCs. Data presented as mean ± S.E.M. of three PCR experiments.

The NPSCs were maintained in culture in N2B27 medium supplemented with bFGF and EGF. It has been previously demonstrated that when cultured in N2B27 medium supplemented with bFGF and EGF, NPSCs are able to self-renew and proliferate whereas upon withdrawal of the growth factors, the cells further differentiate into postmitotic neuronal and glial cells (Wu et al., 2010). In order to verify that the late NPSCs maintained the ability to generate postmitotic neurons and glia, I cultured these cells in N2B27 medium in the presence or absence of bFGF and EGF and examined their proliferation and differentiation properties.

I used the BrdU incorporation assay to study the proliferation of the cells. This showed that in the presence of growth factors, 41% of early (p24) NPSCs and 43% of late (p71) NPSCs became labelled after a two hour incubation with BrdU whereas in the absence of growth factors, there was a total lack of BrdU labelling, indicating that the cells had exited the cell-cycle and become postmitotic (Figure 3.6A). Furthermore, after withdrawal of the growth factors, these late NPSCs efficiently differentiated down the neuronal and glial lineages, losing their triangular morphology and developing neurite-like processes and upregulating the neuronal marker β-III-tubulin and the glial marker GFAP (shown by immunocytochemistry, Figure 3.6B). Taken together, these data demonstrate that the late NPSCs maintain neural progenitor/stem cell properties.

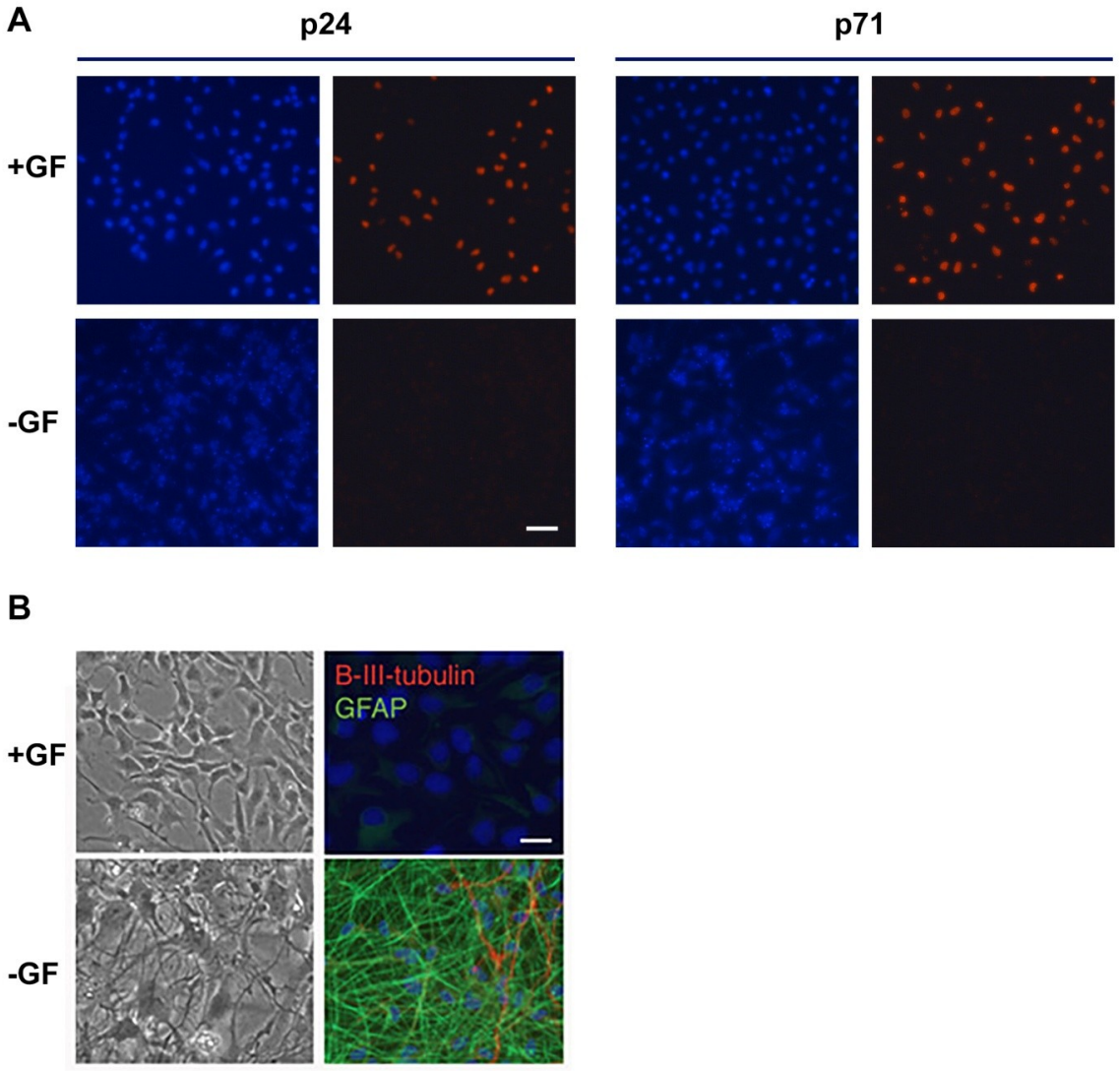


Figure 3.6: Proliferation and differentiation of NPSCs

(A) Early (p24) and late (p71) passage NPSCs were highly proliferative in the presence of growth factors (+GF) but completely ceased proliferation after growth factor withdrawal (-GF), as shown by BrdU labelling (details in Chapter 2.4.3); BrdU, red; DAPI counterstain, blue; scale bar represents 50 µm for all images. **(B)** Late passage (p57) NPSCs displayed the typical triangular morphology in the presence of growth factors (+GF) but changed to a neuronal morphology after growth factor withdrawal (-GF), as shown by light microscopy (left panels); the cells were negative for neuron marker β-III-tubulin and astrocyte marker GFAP in the presence of growth factors (+GF) and these markers were strongly upregulated after growth factor withdrawal (-GF), as shown by immunocytochemistry, right panels; β-III-tubulin, red; GFAP, green; DAPI, blue; scale bar represents 100 µm for all images.

3.2.3 Are the late NPSCs transformed?

Telomerase is generally repressed in somatic cells, where telomere shortening acts as a potent tumour suppressor mechanism. In contrast, telomere maintenance is prerequisite for tumour development and the majority of cancer cells express telomerase. Therefore, the reactivation of telomerase in the NPSCs raised the question of whether the cells were still normal or if they had become transformed during their long-term culture. Transformation of cells in culture is usually preceded by a crisis event; cells proliferate until telomere shortening activates a DNA damage response resulting in senescence or apoptosis and mass cell death. Rare surviving cells then emerge out of this crisis having been transformed and activating a telomere maintenance pathway (Degerman et al., 2010; Montalto et al., 1999). However, the NPSCs have proliferated stably during their extended time in culture and nothing resembling a crisis event was ever observed. Furthermore, they respond normally to the withdrawal of growth factors, ceasing proliferation and differentiating to glia and neurons (Figure 3.6), indicating they are not transformed. Nevertheless, I tested the cells for certain properties relating to transformation in order to rule this possibility out.

One of the hallmarks of cancer cells is the disruption of negative-feedback mechanisms that attenuate proliferative signalling (Hahn and Weinberg, 2002). Contact inhibition plays an important role in regulating the growth of normal cells and is often lost in transformed cells (Chen et al., 1997; Dietrich et al., 1997; Kato et al., 1997). To test this, early and late NPSCs were grown to confluency and the cells were then left for three days before labelling with BrdU to assess their proliferation rate. Immunocytochemistry with an anti-BrdU antibody followed by cell counting under a fluorescence microscope showed that after reaching confluency, the percentage of cells undergoing DNA replication significantly decreased in both the early and late NPSCs. Also, the percentage of subconfluent cells that incorporated BrdU was similar for both cell stages. In contrast, HepG2 cancer cells continued to proliferate

after becoming confluent (Figure 3.7A). Cell cycle analysis of the late NPSCs by PI staining and flow cytometry showed that the proportion of cells in the S- and G2-phases was clearly reduced upon confluency whereas the proportion in G1-phase was increased, suggesting that the cells became blocked in G1 phase of the cell-cycle when confluent (Figure 3.7B).

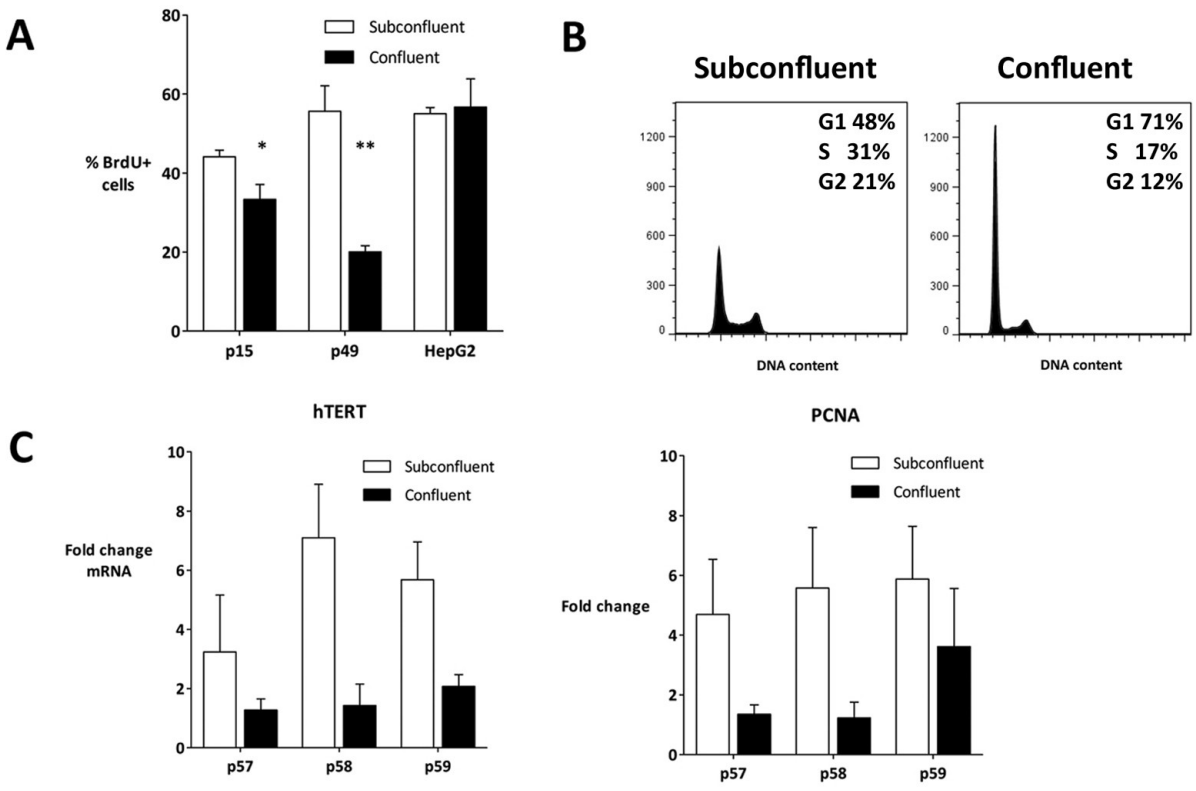


Figure 3.7: Both early and late NPSCs exhibit contact inhibition

(A) Reduction of BrdU labelling after NPSC confluency. Upon reaching confluency, the proportion of early (p15) and late (p49) passage NPSCs showing BrdU incorporation was significantly reduced, in contrast to HepG2 cells. Open and closed bars represent subconfluent and over-confluent cells, respectively. Bars represent mean ± S.E.M. of 3 independent experiments; * $p<0.05$, ** $p<0.005$. **(B)** Cell cycle analysis of late passage (p60) NPSCs showed that upon confluency, the proportion in G1 and S-phase increased and decreased, respectively. Subconfluent (left) or confluent (right) cells were stained with propidium iodide and analysed by flow cytometry. **(C)** hTERT and PCNA mRNA was downregulated upon confluency in late passage NPSCs, as shown by qRT-PCR in subconfluent (open bars) or over-confluent (closed bars) cells of three consecutive passages; bars represent mean ± S.E.M. of 3 independent experiments.

These data show that NPSCs are subject to contact inhibition, one of the main criteria used to distinguish normal from transformed cells. In addition to proliferation, hTERT mRNA was downregulated upon confluency and upregulated again upon passaging. Late stage NPSCs were allowed to become over-confluent and then split to a new plate. This was repeated twice and RNA was collected for NPSCs that were subconfluent or confluent over three consecutive passages. qRT-PCR revealed that hTERT was downregulated at confluency and then re-expressed upon splitting the cells. PCNA expression was used as a marker of proliferation and this followed the same pattern as hTERT (Figure 3.7C). The confluency-dependent hTERT downregulation exhibited by the NPSCs is consistent with published data on Swiss 3T3 cell contact inhibition (Holt et al., 1996) and suggests that in the NPSCs, hTERT expression is tightly regulated.

Another important criterion for normal versus transformed cells is growth anchorage-dependency; the requirement of a solid support, typically the bottom of a cell culture dish, to grow (Lee et al., 2011; Mehta et al., 1986; Polyak et al., 1994). Early (p22) and late (p46) passage NPSCs, along with HeLa cells used as a positive control, were plated into soft agar. Two weeks later, the plates were evaluated for the presence of colonies. Whilst HeLa cells were able to proliferate, forming colonies, the NPSCs were not, producing almost no colonies (Figure 3.8), indicating that the growth of NPSCs is anchorage-dependent and that they are not transformed.

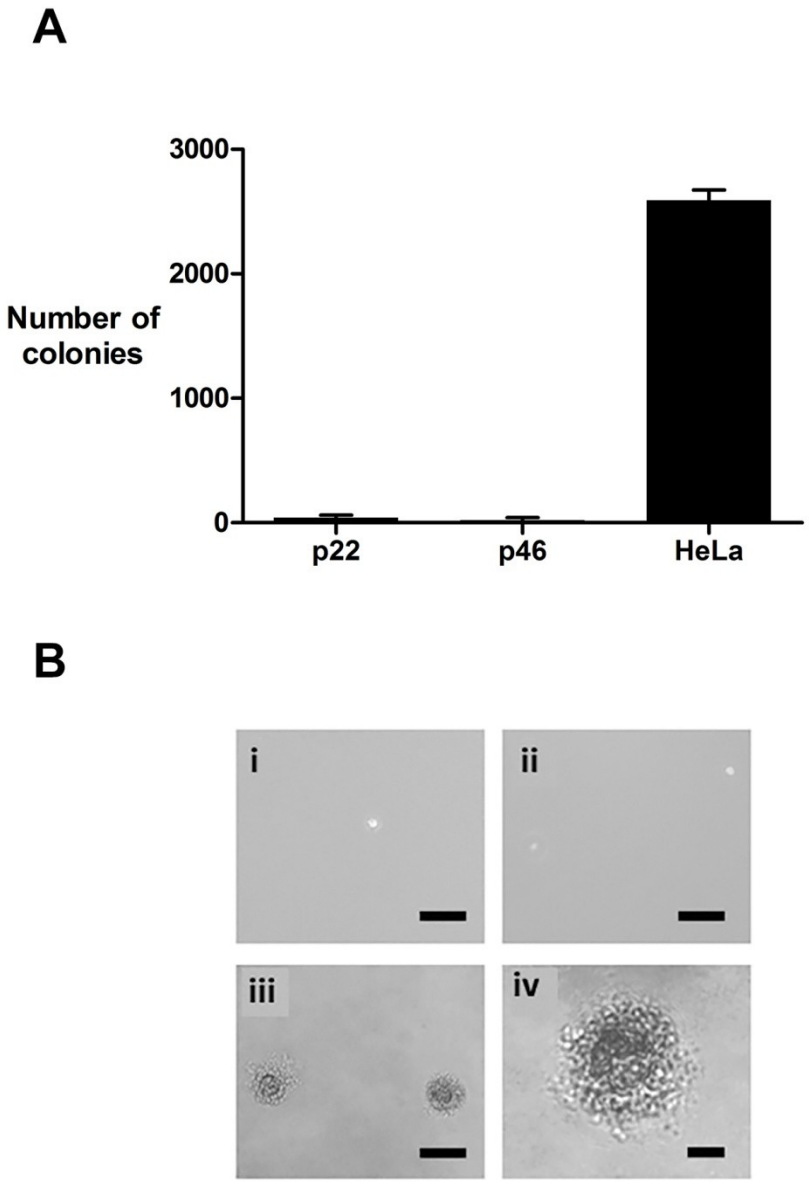


Figure 3.8: Anchorage-dependent growth of NPSCs

(A) Early (p22) and late (p46) passage NPSCs formed no colonies when plated in soft agar, in contrast to HeLa cells. Cells were plated in soft agar and after two weeks, the number of colonies was counted. Values are the average of triplicate plates ± S.E.M. **(B)** Representative phase-contrast images of early (i) and late (ii) passage NPSCs and HeLa cells (iii, iv) two weeks after plating in soft agar. Scale bar represents 50 μm in i-iii, 10 μm in iv.

Transformation is usually accompanied by genomic instability therefore late stage NPSCs (p40) were metaphase arrested, harvested, fixed and sent for karyotyping. Karyotype analysis was carried out by TDL Genetics, London. The analysis showed that 18/20 metaphase spreads had a normal diploid karyotype whereas the other two each showed a unique karyotypic abnormality (Figure 3.9 and Appendix Figure A1). However, these abnormalities were not present when the same cells were examined again ten passages later, indicating that these chromosomal aberrations were non-transmissible and that the small number of cells with chromosomal changes were unable to be expanded and subsequently died. It can be concluded from this that overall the NPSCs are karyotypically normal.

A

Karyotype	Number of metaphases
46,XX	18
46,XX,add(15)(q25)	1
46,XX,t(13;17)(p13;q21.3)	1

B

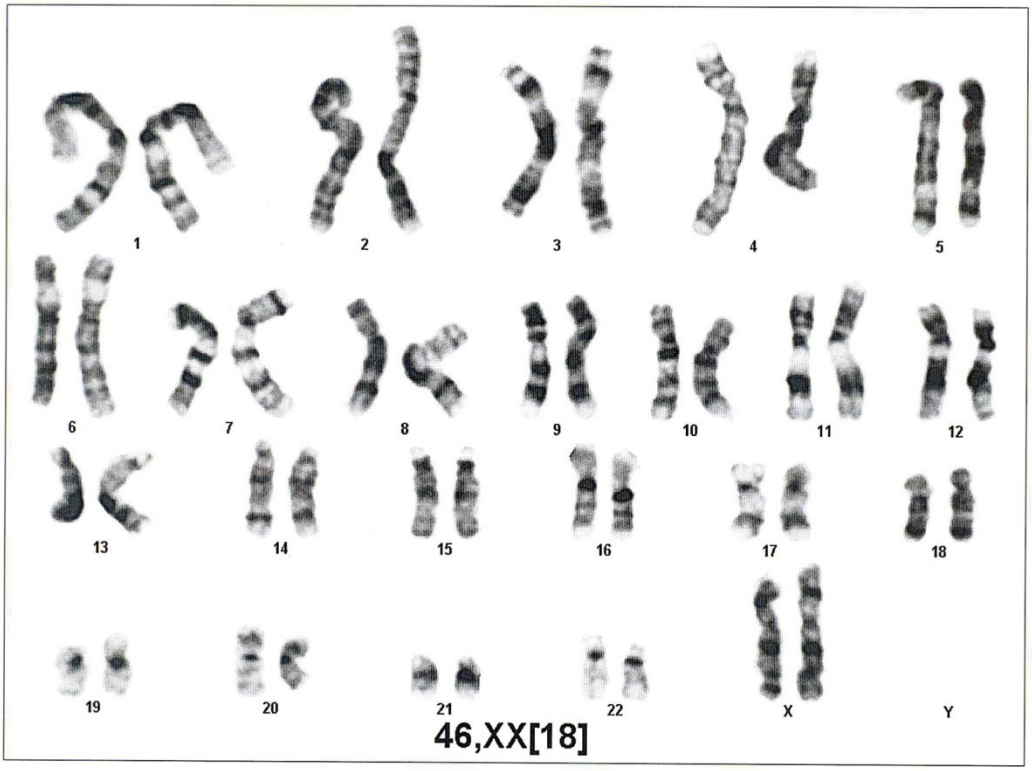


Figure 3.9: Karyotype of late passage NPSCs

(A) 18/20 p40 NPSC metaphase spreads showed a normal diploid XX karyotype; representative spread shown in **(B)**. Two metaphase spreads showed an abnormal karyotype (see Figure A1 in Appendix).

An additional important negative-feedback mechanism in non-transformed cells is their ability to respond appropriately to DNA damage. In a normal cell, when DNA damage is sensed, DNA damage signalling pathways are activated, involving upregulation and activation of the tumour suppressor protein p53 (Lee et al., 2011; Orford et al., 1999), which then effects cell cycle arrest, allowing time for DNA repair to occur (or alternatively p53 will mediate senescence or apoptosis). In many transformed cell types, this DNA damage response is lost, often due to mutation or deletion of p53 (Baker et al., 1990; Mackay et al., 1988; Nigro et al., 1989; Tsai et al., 1990). Therefore, I aimed to test the ability of the NPSCs to respond to DNA damage. Exposing the NPSCs, early and late passage, to ionising radiation (IR) activated a potent DNA damage response. p53 protein was strongly upregulated, as was its downstream effector, the cell cycle inhibitor p21 (Figure 3.10). This also occurred in primary human fibroblasts, which were used as a positive control.

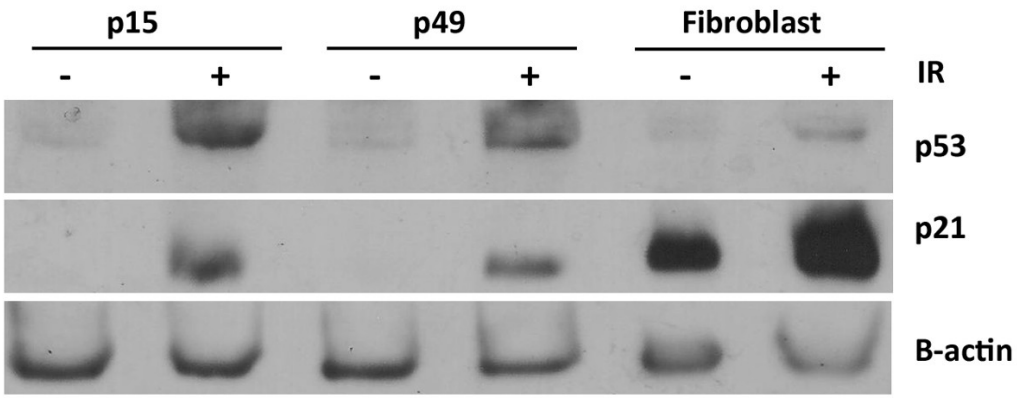


Figure 3.10: NPSCs retain the ability to respond to DNA damage

After exposure to ionising radiation (IR), p53 and p21 were upregulated in early (p15) and late (p49) NPSCs, as well as in primary human fibroblasts (used as a positive control), as shown by Western blotting of cell lysates before and 3 hours after 6 gray IR. β-actin is used as a loading control for total protein.

Consistent with this, cell cycle analysis by PI staining and flow cytometry demonstrated that the NPSCs became cell cycle arrested at G2/M phase, showing a decrease in the proportion of cells in G1-phase and a strong increase in the proportion of cells in G2-phase of the cell-cycle (Figure 3.11A). Furthermore, continuous culture of the cells after exposure to IR and counting the cells at various time points showed that, while control non-irradiated cells proliferated well with increases in cell number, the irradiated cells displayed decreases in cell number with their time in culture (Figure 3.11B). Together, these data indicated that NPSCs respond to IR through G2-phase cell cycle arrest and then cell death.

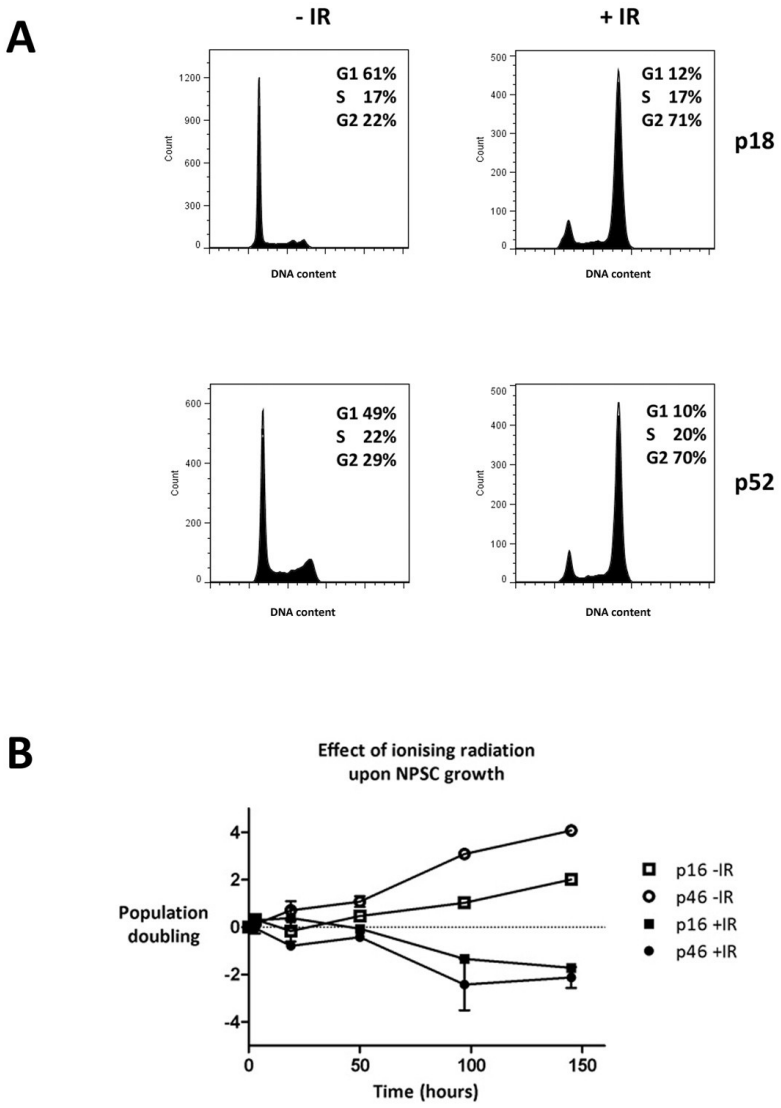


Figure 3.11: Reduced NPSC proliferation following ionising radiation

(A) 48 hours after exposure to 6 gray of ionising radiation (IR), early (p18) and late (p52) NPSCs became arrested in G2-phase of the cell cycle, as shown by cell cycle analysis. Cells were stained for DNA content with propidium iodide and analysed by flow cytometry. **(B)** After IR exposure, early (p16) and late (p46) NPSCs stopped proliferating and died, as shown by a growth curve of NPSCs before or after IR. Population doubling is plotted against time (hours after exposure to IR). Values are averages of cell counts from triplicate wells with error bars representing S.E.M.

3.2.4 Changes to telomeres in hESCs and NPSCs

During the long-term culture of the NPSCs, I observed striking changes in telomere length; the cells underwent significant telomere shortening followed by elongation mediated by telomerase. These differential telomere dynamics make this cell system a very interesting model for studying telomere biology within the context of a normal somatic stem cell. One of the most important aspects of telomere biology is the shelterin complex of telomere binding proteins. Shelterin gene expression has been shown to change during tumourigenesis and with cellular ageing (Bellon et al., 2006; Chebel, 2009; Oh et al., 2005) TRF1 and TRF2 are two key proteins of the shelterin complex and have been reported to be negative regulators of telomere length (Smogorzewska et al., 2000). Therefore, I examined the expression of TRF1 and TRF2 during hESC neural differentiation and in the long-term culture of the derived NPSCs. In both H1 and H7 hESC lines, TRF1 mRNA was highly expressed in hESCs but then dramatically downregulated upon differentiation; this lower level was then maintained throughout the long-term NPSC culture (Figure 3.12A, left panel). TRF1 protein levels were also reduced after neural differentiation but to a lesser extent (Figure 3.12B).

In contrast, although TRF2 mRNA expression fluctuated during hESC neural differentiation and throughout the long-term culture of NPSCs (Figure 3.12A, right panel), TRF2 protein was observed to be expressed at a surprisingly low level in hESCs but greatly upregulated in early NPSCs and then remaining at much higher levels than in hESCs, regardless of telomere length (Figure 3.12, Figure 3.3). These data indicate that TRF2 may have a role in neural differentiation of hESCs independent of its function at telomeres. Since my project was mainly focussed on the role of telomerase and telomeres, this line of the research is being pursued by other researchers.

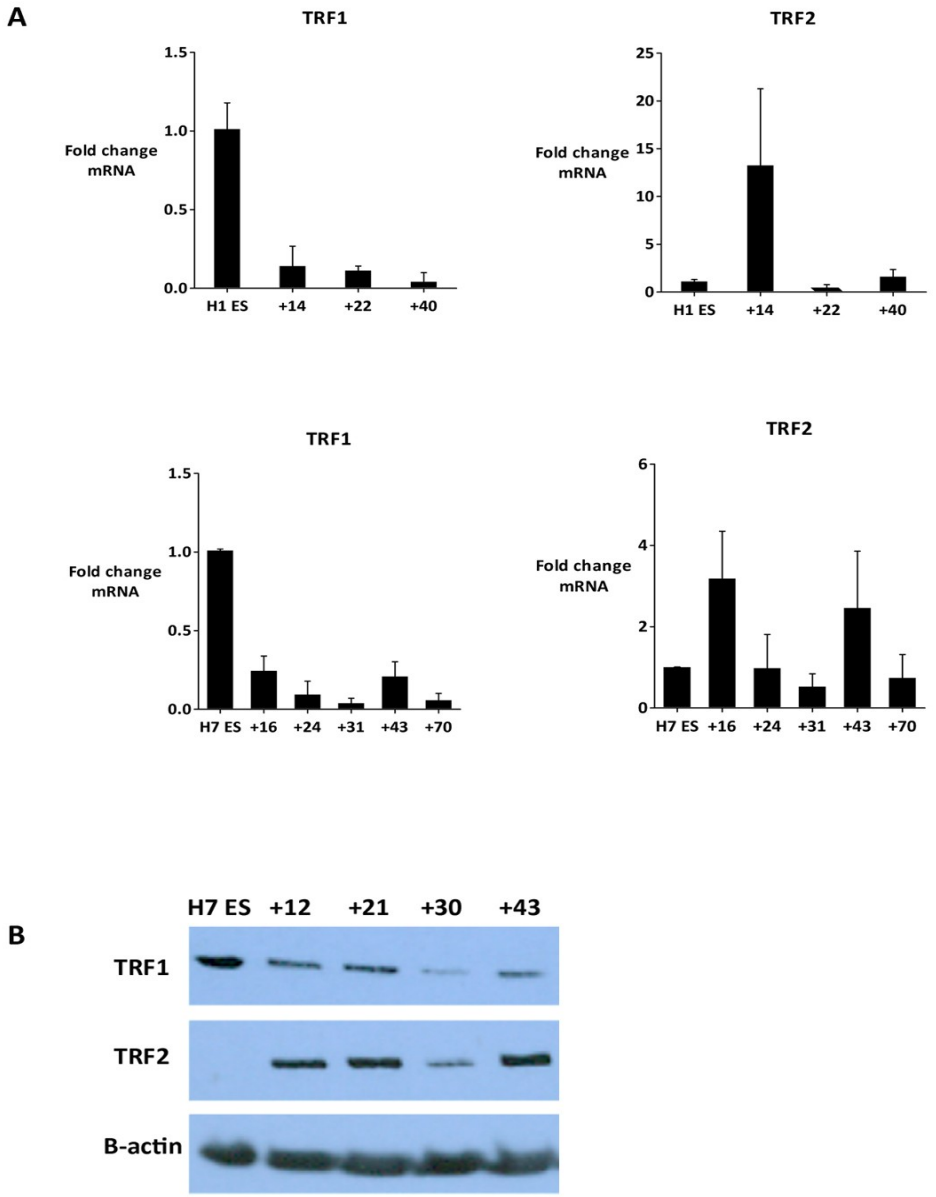


Figure 3.12: Expression of TRF1 and TRF2 in hESCs and NPSCs. **(A)** qRT-PCR analyses of H1 (left) and H7 (right) cells showed that TRF1 mRNA was highly expressed in hESCs and considerably downregulated after neural differentiation whereas TRF2 mRNA levels fluctuated during hESC neural differentiation and NPSC culture. Passage numbers of NPSCs are indicated. Bars represent the mean ± S.E.M. (n=3) **(B)** Western blotting of TRF1 and TRF2 protein in H7 hESCs and NPSCs showed that TRF1 protein levels were reduced after differentiation. TRF2 protein expression was low in H7 hESCs but strongly upregulated in early NPSCs and maintained at higher levels during NPSC culture. β-actin was used as a loading control for total protein. Experiments performed in collaboration with Sarah Testori.

Another aspect of telomere biology that has received increasing attention in recent years is the chromatin status of telomeres and the epigenetic regulation of telomere length and structure. It has been demonstrated that the chromatin status of subtelomeric and telomeric regions can be regulated by telomere length. In normal cells with long telomeres, these regions are in a heterochromatic state, enriched for trimethylation of histone H3 lysine residues 9 and 27 and lacking histone H3 acetylation; when telomeres become critically short, the heterochromatin histone marks become reduced at these regions (Benetti et al., 2007). On the other hand, the epigenetic factors that affect subtelomeric chromatin could have an effect on the regulation of telomere length in tumour cells (Vera et al., 2008).

In an attempt to assess whether the chromatin environment of telomeres in the NPSCs was affected by changes in telomere length, I measured acetylation of histone H3 (a mark of open euchromatin) at the proximal subtelomere of the X chromosome in the H7 hESC line using XpYp specific primers. Immunoprecipitation was performed on chromatin extracted from H7 hESCs and NPSCs - p15 (long telomeres) and p45 (short telomeres) - using an antibody specific for acetylated histone H3 protein. The precipitated DNA was analysed by PCR for the presence of XpYp subtelomeric DNA (Figure 3.13A). A low level of enrichment was seen for all cells, with very little difference between them, demonstrating that subtelomeric histone H3 in hESCs and NPSCs is hypoacetylated, consistent with published data demonstrating that normal telomeres harbour very low levels of histone acetylation (Benetti et al., 2007; Martin et al., 2004).

Since epigenetic modifications in the subtelomere are known to be associated with telomere length, I also studied DNA methylation at a CpG island in the same subtelomeric region. Genomic DNA was extracted from hESCs, NPSCs of p16 (long telomeres), p37 (short telomeres) and p68 (long telomeres) and primary fibroblasts. After bisulphite conversion, a small CpG rich region close to the subtelomere/telomere boundary of the X chromosome was

sequenced (Figure 3.13B). This revealed that in hESCs, the vast majority of CpGs (97.5%) were methylated. In NPSCs the methylation pattern was more mosaic. 50% of CpGs were methylated in both p16 and p68 telomeres whereas in p37 NPSCs, 72% of CpGs were methylated. In fibroblasts, 79% of CpGs were methylated. These data are in agreement with published reports indicating that subtelomeric CpGs are heavily methylated (Brock et al., 1999) however, no association between telomere length and subtelomeric CpG methylation can be observed. The data do suggest though that undifferentiated hESCs have a higher proportion of subtelomeric CpG methylation than differentiated cells.

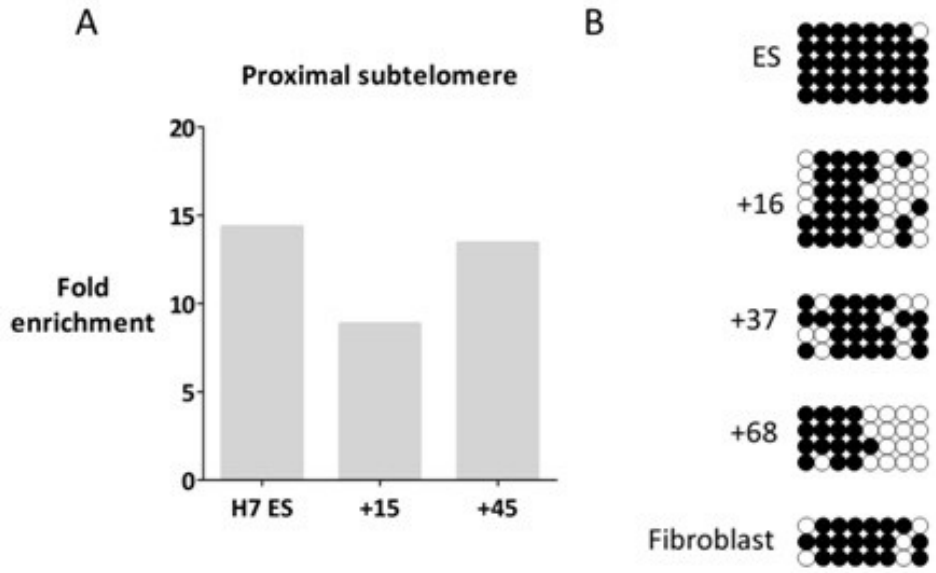


Figure 3.13: Epigenetic status of telomeres in hESCs and NPSCs

(A) Histone H3 acetylation at the XpYp subtelomere region. ChIP analysis showed a low level of histone H3 acetylation at the XpYp subtelomere of H7 hESCs, p15 NPSCs (long telomeres) and p45 NPSCs (short telomeres), with little difference between them. Bars represent the fold enrichment of acetyl-H3 over normal IgG at the subtelomeric DNA. **(B)** DNA methylation at the XpYp subtelomere/telomere boundary. Bisulphite sequencing of a CpG-rich region close to the XpYp subtelomere/telomere boundary showed high levels of CpG methylation in H7 hESCs, p16 NPSCs (long telomeres), p37 NPSCs (short telomeres), p68 NPSCs (long telomeres) and primary human fibroblasts. hESCs had the highest proportion of methylated CpGs and there was no clear difference between early and late NPSCs. Closed circles represent methylated CpGs, horizontal rows of CpGs represent individual clones.

3.3 Discussion

In this chapter, I report the first instance of a non-transformed cell type displaying reactivation of functional telomerase and subsequent telomere homeostasis. This cell system displays a dynamic pattern of hTERT expression, telomerase activity and telomere length. Focussing on the hTERT expression pattern, this can be divided into two phases: 1) high expression in hESCs which is then strongly downregulated after neural differentiation and maintained at a very low level throughout the early passages of NPSCs. 2) hTERT reactivation in late passage NPSCs, after extended culture.

Telomerase expression was robustly downregulated after differentiation of hESCs, consistent with previous data from our laboratory and others (Gerrard et al., 2005; Hay et al., 2008; Thomson et al., 1998). hTERT expression during hESC differentiation has been found to be associated with changes in histone modifications at the hTERT promoter (Golebiewska et al., 2009; Saretzki et al., 2008). Downregulation of hTERT expression during hESC differentiation parallels the hTERT silencing observed during embryonic development. Thus, the hTERT downregulation observed in our cell system makes it a good model for studying the mechanisms that regulate hTERT silencing in normal somatic cells.

In early passage NPSCs, with the expression of telomerase repressed, the cells' telomeres shortened over successive passages, as expected and in agreement with other reports of hESC neural differentiation, in addition to studies of primary human neural precursor cells (Ostenfeld et al., 2000). However, following many population doublings and once the average telomere length had reached near to that previously reported in senescent fibroblasts (Espejel and Blasco, 2002), telomerase became reactivated in the NPSCs. This telomerase reactivation occurred in the absence of any genetic modification and was accompanied by

elongation of the telomeres, which were then maintained at a relatively long length. Thereby, these cells bypassed replicative senescence.

hESC-derived lineage-specific cell types have great potential for use in regenerative medicine. However, the ability to expand these cells enough *in vitro* for clinical use has been hampered by replicative senescence, hence much effort has been made to artificially immortalise cells which hold promise for therapeutic application, particularly neural stem cells. The NPSCs derived through our protocol are able to naturally expand without the limit of replicative senescence, whilst retaining tight regulation of their proliferation, therefore giving them a clinical advantage.

Telomerase reactivation has been widely reported in cancer and expression of hTERT has been shown to increase the likelihood of transformation (Hahn, 2003), resulting in its classification by some as an oncogenic risk factor. Elevated levels of telomerase in cancer cells have been demonstrated to increase their metastatic properties, conferring the ability to form colonies in soft-agar and eliminating contact-inhibition. However, I confirmed that our cells were non-transformed as these telomerase-reactivated NPSCs exhibited the normal negative feedback machinery, such as contact inhibition and DNA damage responsiveness, and they also maintained anchorage-dependent growth and karyotypic stability (Figure 3.7-11). The data in this chapter therefore indicate that increased telomerase in NPSCs does not affect these properties, lending support to the idea that hTERT is not an oncogene. Furthermore, reactivation of telomerase in these NPSCs did not appear to affect their neural progenitor properties. The NPSCs require stimulation by bFGF/EGF growth factor signalling to proliferate and are capable of generating both neurons and glia upon withdrawal of the growth factors (Figure 3.5-6).

Telomerase expression in hESC-derived NPSCs has been previously reported, but in that study, telomerase seems to exhibit a continuous expression from hESCs to NPSCs (Koch et al., 2009). Also, telomerase has been reported to be expressed in *ex vivo* NPSCs. However, this was associated with tumourigenicity (Casalbore et al., 2009). Here, we showed a dynamic expression pattern of telomerase in hESCs, during their neural differentiation and extended culture of NPSCs.

It is noticeable that telomerase expression was reactivated during the extended culture of NPSCs. It has been proposed that telomerase reactivation is a very late event in multi-stage tumourigenesis (Hahn and Weinberg, 2002). However, this hypothesis has not been corroborated in somatic cells in culture. It only has been possible to generate a tumour cell from a normal somatic cell by pre-introducing telomerase expression into the cells by genetic manipulation (Hahn et al., 1999). Our data presented here represent the first report of spontaneous reactivation of telomerase in cell culture normal. The telomerase-reactivated NPSCs had a normal, untransformed phenotype and exhibited telomere homeostasis, suggesting that telomerase reactivation may not necessarily require other transformation events in neural progenitor/stem cells, such as activation of oncogenes or inhibition of tumour suppressor genes. Our cell system provides a useful model for studying the mechanisms underlying reactivation of hTERT. Furthermore, hESC-derived NPSCs will provide a model for studying the transcriptional and epigenetic regulation of hTERT activation in the context of a normal cell type.

Chapter 4. The regulation of hTERT expression in neural progenitor/stem cells

4.1 Introduction

Although telomerase plays an important role in maintaining normal cell function, organism ageing and diseases, the mechanisms that regulate its expression still remain unclear (Dwyer et al., 2007). The hESC-derived NPSCs described in Chapter 3 displayed a dynamic pattern of hTERT expression. hTERT was highly expressed in hESCs, downregulated after neural differentiation and then maintained at a very low level throughout the early passages of NPSCs. Following extended culture and telomere shortening, hTERT was reactivated in late passage NPSCs. Most previous studies of the regulation of telomerase expression have mainly compared normal and cancer cells of different genetic backgrounds and different cell types/lineages; these factors have the potential to confound results. The differential hTERT expression observed during neural differentiation of hESCs and extended culture of the derived NPSCs occurred within the same group of cells, from the same source; thus, any possible differences or interference due to genetic background has been eliminated. Furthermore, the observed hTERT reactivation occurred in NPSCs, which share characteristics similar to hTERT-low NPSCs, thereby reducing interference from cell type differences. Taken together, this cell system provides us with a useful model for studying the regulation of hTERT transcription in normal somatic cells and the mechanisms underlying hTERT reactivation. Therefore, in this chapter I endeavour to explore the mechanisms that regulate hTERT expression, especially its reactivation, using the NPSC culture system.

The results in Chapter 3 confirmed the previous findings that the level of telomerase activity in cells and tissues is closely correlated with the level of hTERT mRNA, indicating that hTERT expression is predominantly regulated at the transcriptional level (Cifuentes-Rojas

and Shippen, 2012; Cui et al., 2003; Oh et al., 2001). The 2 kb region upstream of the transcriptional start site (Ts) in the hTERT gene is considered to be the promoter region and luciferase reporter expression under the control of this region has been shown to reflect endogenous hTERT expression in cancer and normal cells (Horikawa et al., 1999). As reviewed in Chapter 1.3.4, the promoter contains numerous consensus motifs which are predicted to act as binding sites for a variety of transcription factors. The most well studied activator of hTERT transcription is the classical proto-oncogene c-myc. (Wang et al., 1998; Wu et al., 1999) and the zinc finger transcription factor Sp1 (Kyo et al., 2000; Xu et al., 2001). In addition to transcription factors, it has also been shown that hTERT expression is affected by various signalling pathways that control the translocation of DNA binding factors to regulate hTERT gene expression (Bermudez et al., 2008; Goueli and Janknecht, 2004; Lanna et al., 2013). Furthermore, the hTERT promoter is extremely CG-rich, located within a large CpG island, which has been shown to be subject to CpG DNA methylation. Despite several studies conducted to determine if and how hTERT promoter CpG methylation correlates with hTERT expression, the results have been conflicting (Bechter et al., 2002; de Wilde et al., 2010; Shin et al., 2003; Zhang et al., 2013; Zinn et al., 2007).

In this chapter, I aimed to explore the molecular mechanisms underlying the dynamic expression of telomerase in our hESC-NPSC cell system and focused my study on the regulation of the hTERT gene. I examined the transcriptional and epigenetic factors that may function to regulate hTERT expression, particularly at the promoter, and I investigated how these factors play a role in the repression of hTERT in early NPSCs and how they regulate the later hTERT reactivation.

4.2 Results

4.2.1 The role of growth factors in hTERT reactivation

Neural differentiation of hESCs was carried out in serum-free medium. The growth factors bFGF and EGF were added into the medium soon after neural progenitor cells emerged from the differentiation (usually before passage 5) and the resulting NPSCs have been maintained in this medium throughout their extended culture. These growth factors are essential for the NPSCs to proliferate and self-renew and in the absence of bFGF/EGF, NPSCs further differentiate into postmitotic neurons and glia. However, EGF signalling has been previously reported to activate hTERT transcription through MAP kinase signalling-mediated activation of hTERT activators ETS2 (Maida et al., 2002), c-myc and Sp1 (Bermudez et al., 2008). Therefore, it would seem plausible that supplementing EGF into the culture could have resulted in the hTERT reactivation observed. Although hTERT reactivation occurred much later than EGF addition, it is possible that prolonged exposure resulted in sensitisation of the NPSCs to EGF, for example via upregulation of the EGF receptor.

To test this possibility, EGF was withdrawn from the medium and the cells were cultured continuously for over two weeks with bFGF alone. Telomerase activity in these cells was examined at two time points during the culture and compared with that in parallel cells cultured with both bFGF and EGF. Although telomerase activity was slightly reduced in the absence of EGF at individual time points compared to the parallel sample with EGF, which is consistent with the published data, it was clearly positive. Furthermore, after 17 days of EGF withdrawal, no overall decrease in telomerase activity was observed compared to 5 days (Figure 4.1A). Consistent with the maintained telomerase activity, no telomere shortening

was detected in the NPSCs after 26 days without EGF and their telomeres were a similar length compared with the cells cultured with both bFGF and EGF (Figure 4.1B).

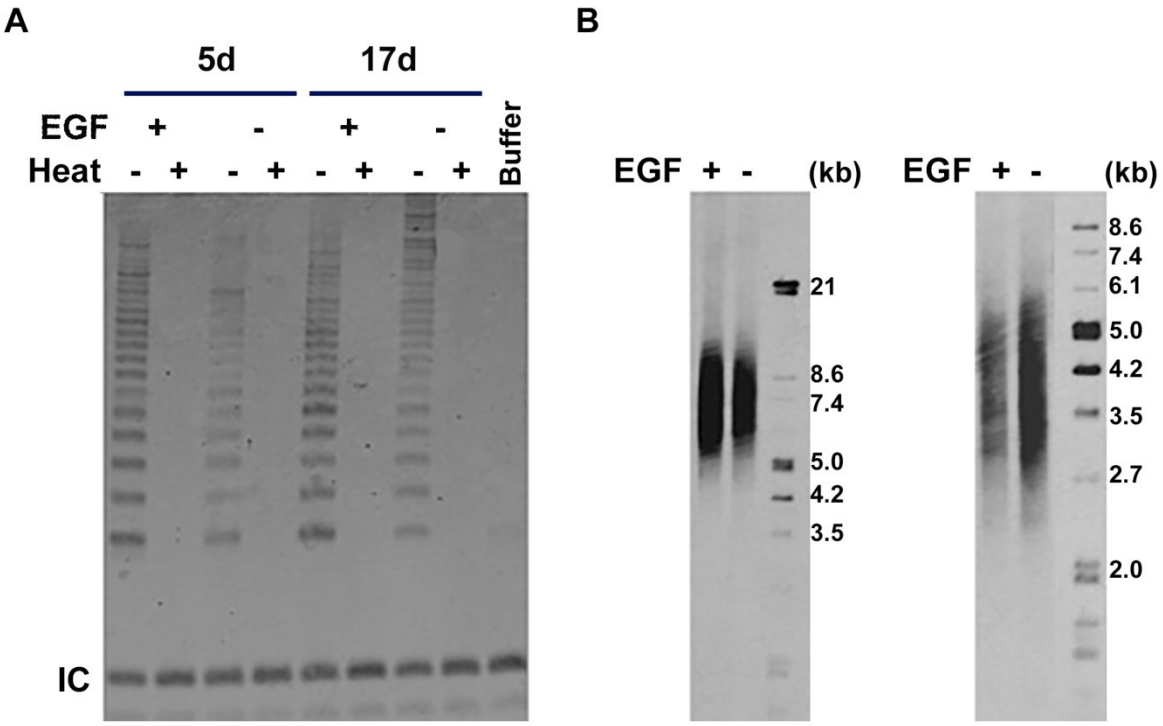


Figure 4.1: The effect of EGF withdrawal on telomerase expression and telomere length in late NPSCs

(A) Withdrawal of EGF from the culture medium of late passage H7 NPSCs had no overall long-term effect on telomerase activity, although a slight decrease was observed. H7 p70 NPSCs were cultured in medium supplemented with bFGF in the presence or absence of EGF for the indicated time and telomerase activity was measured by TRAP assay. IC, internal control. **(B)** Withdrawal of EGF from late passage (p70) H1 (left) and H7 (right) NPSCs for 26 days had no effect on telomere length, as shown by TRF Southern blotting.

To further confirm that EGF was not responsible for the reactivation of hTERT expression, NPSCs that had never been given exogenous EGF were serially propagated (in bFGF only). These cells also exhibited telomerase reactivation and passage 30, similar to the NPSCs differentiated in the presence of both bFGF and EGF, demonstrating that EGF is not required for the reactivation of telomerase (Figure 4.2).

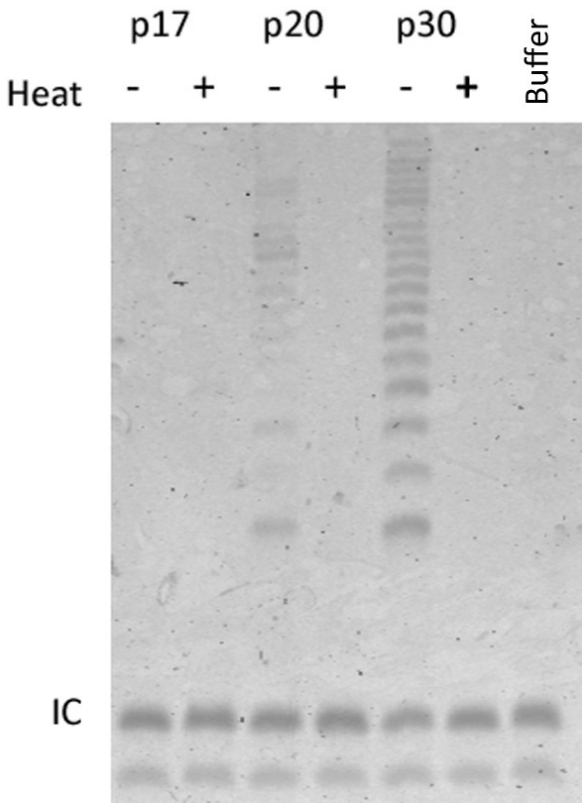


Figure 4.2: Telomerase reactivation in NPSCs cultured without EGF

Early NPSCs which had never been cultured with EGF displayed telomerase reactivation, as shown by TRAP assay of EGF-naive NPSCs at different passages. IC, internal control.

Telomerase has also been reported to be regulated by bFGF. Addition of bFGF to mouse neural precursor cells has been shown to increase telomerase activity (without affecting levels of mTERT) (Haik et al., 2000). Additionally, synoviocytes from rheumatoid arthritis patients display raised telomerase compared to those from osteoarthritis patients and these telomerase levels can be increased further by bFGF treatment (Tsumuki et al., 2000). To test the effect of bFGF upon telomerase in the NPSCs, growth factors were withdrawn. As shown in Chapter 3, when growth factors are withdrawn, NPSCs further differentiate towards neuronal and glial cells. After one week without growth factors, cell cycle exit and glial differentiation were reflected at the mRNA expression level by downregulation of PCNA and upregulation of GFAP, respectively, as shown by qRT-PCR (Figure 4.3A). Furthermore, hTERT mRNA was robustly downregulated and maintained at a low level. Consistent with this, telomerase activity became undetectable (Figure 4.3B).

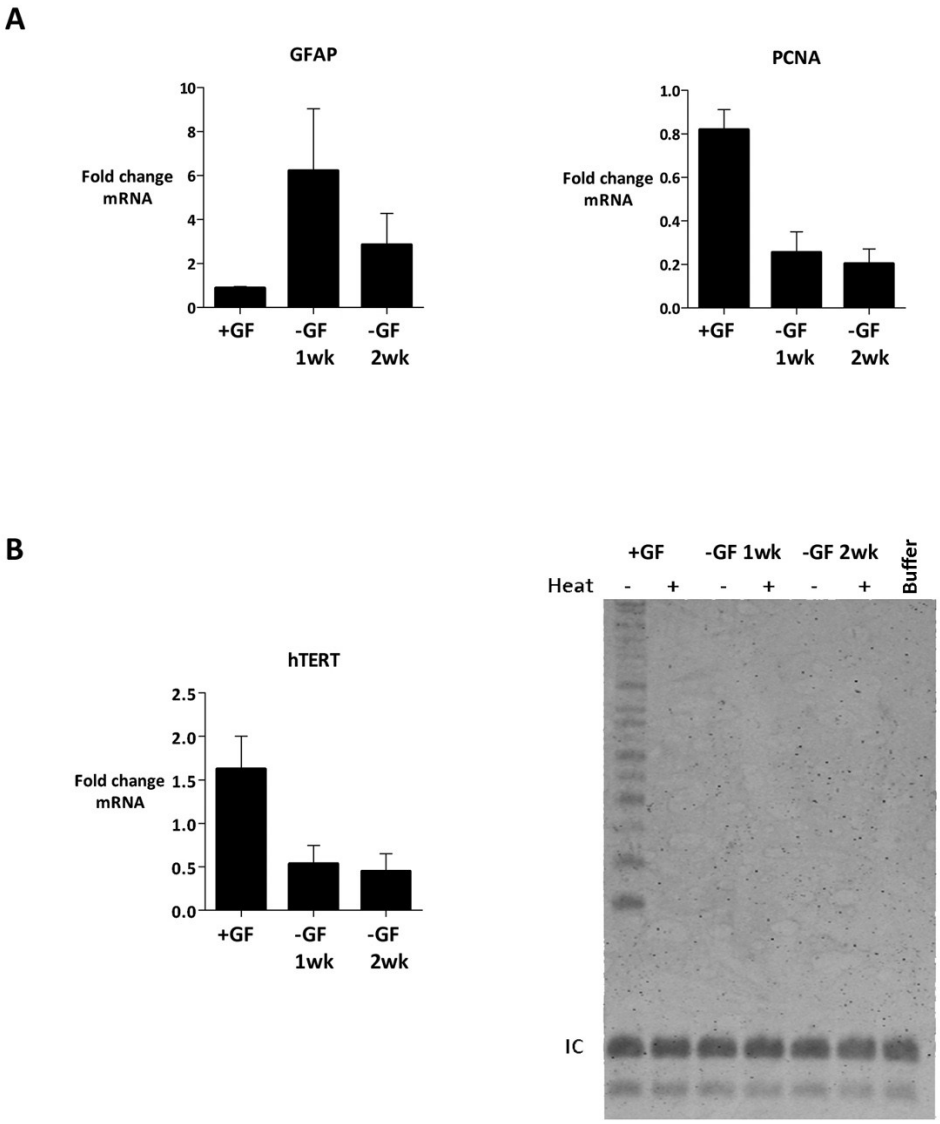


Figure 4.3: Telomerase expression after withdrawal of growth factors

(A) Following growth factor (GF) withdrawal for 1 and 2 weeks from late passage (p57) NPSCs, GFAP (left) and PCNA (right) mRNA was upregulated and downregulated, respectively. Data are presented as mean ± S.E.M. (n=3). **(B)** hTERT mRNA was downregulated, as shown by qRT-PCR (left) which was accompanied by a reduction in telomerase activity, as shown by TRAP assay (right). An internal control sequence (IC) shows the relative PCR efficiency of each reaction.

Since downregulation of hTERT occurred when the combination of bFGF and EGF were withdrawn from the NPSCs and since EGF withdrawal alone did not have the same effect, it is logical that bFGF may directly signal to potentiate hTERT transcription in these cells. However, bFGF is required for the maintenance of NPSC properties and essential to block further differentiation of the NPSCs. Although withdrawal of bFGF from the NPSC culture led to downregulation of telomerase, it also resulted in terminal differentiation, which involved changes in many genes, making it difficult to dissect the direct role of bFGF in the regulation of hTERT expression. However, bFGF was included in the NPSC culture from a very early stage during their differentiation, when hTERT expression was hardly detectable. Similarly, supplementing bFGF into fibroblast cultures did not result in increased hTERT expression. Therefore, it is clear that bFGF signalling alone cannot activate hTERT expression and other factors may be involved. Taken together, it seems that both bFGF and EGF signalling pathways are unlikely to account for the reactivation of telomerase expression in extended cultured NPSCs.

4.2.2 Transcriptional regulation of hTERT in NPSCs

Transcription factors play a critical role in regulation of gene expression. In order to gain insight into the genetic regulation of hTERT in the hESC-NPSC system, I examined the mRNA levels of a panel of candidate activators and repressors of hTERT transcription in hESCs and NPSCs at different stages of culture, and therefore with different hTERT expression levels. The transcription factors studied came from the published literature and also from the preliminary results of a siRNA screen for hTERT regulators performed by our collaborator, Nick Popov of Dr. Jesus Gil's laboratory at the MRC Clinical Science Centre, Imperial College London (unpublished data). This screen identified a number of genes as

putative activators (among them NAB2) and repressors (among them AP2-B) of hTERT in HeLa cells.

I aimed to see if the expression of the candidate hTERT regulators correlated with hTERT expression in the cells, using qRT-PCR. The results (Figure 4.4) showed that of the potential activators, Bmi1 was steadily expressed in hESCs and NPSCs and appeared to gradually increase throughout NPSC culture. c-myc and ETS2 showed high expression in hESCs but were then dramatically downregulated in NPSCs and retained at low levels throughout the NPSC culture. AP2-A and NAB2 expression fluctuated in hESCs and NPSCs. Notably, the expression pattern of ETV1 appeared to follow that of hTERT very closely. However, it remains to be further studied whether ETV1 functions to regulate the expression of hTERT in hESCs and their neural progeny. It is possible that hTERT expression is controlled by different mechanisms in hESCs and NPSCs. For example, c-myc could be an important activator in hESCs whereas different factors may regulate hTERT expression in NPSCs.

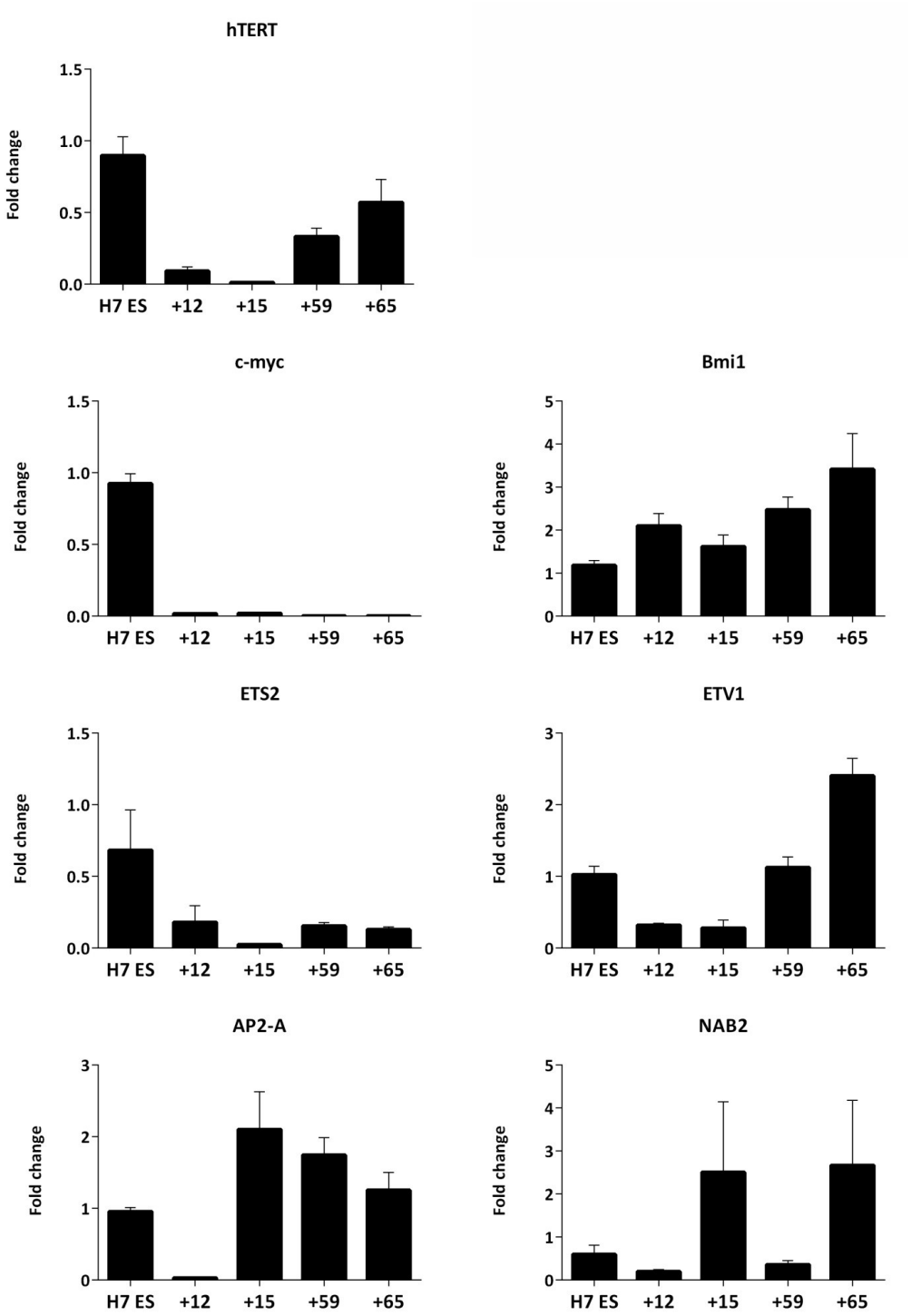


Figure 4.4: Expression pattern of hTERT transcriptional activators in hESCs and NPSCs of different passages

qRT-PCR showing mRNA expression of a panel of candidate hTERT transcriptional activators in hESCs, telomerase-low NPSCs (p12 & p15) and telomerase-high NPSCs (p59 & p65). Data are presented as mean ± S.E.M. (n=3).

Similarly, using qRT-PCR to examine potential repressor expression (Figure 4.5), I have found that WT1 was expressed in hESCs and then dramatically downregulated in all NPSC stages. The expression of MAD1 and E2F1 was consistent in hESCs and NPSCs of all stages. The expression of SIP1 and MEN1 was highest in hESCs and lower in NPSCs. Jun gradually increased from hESCs to NPSCs and with NPSC culture. However, its expression was very low in p12 NPSCs, when hTERT expression is very low. AP2-B mRNA was relatively high in hESCs and p12 NPSCs and thereafter was undetectable. None of the repressors studied exhibited negative correlation with hTERT expression in NPSCs therefore may not play a critical role in the regulation of hTERT expression in these cells.

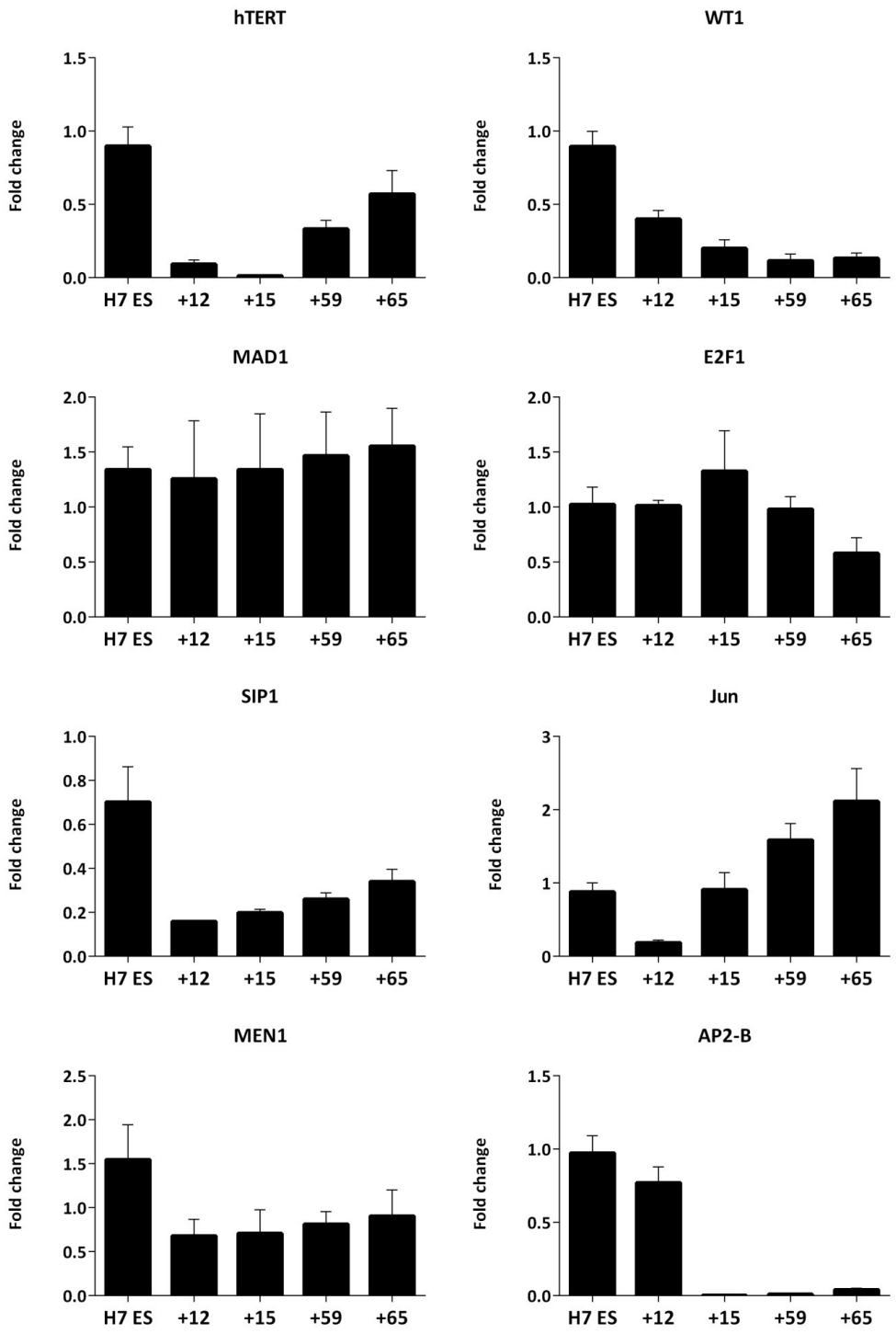


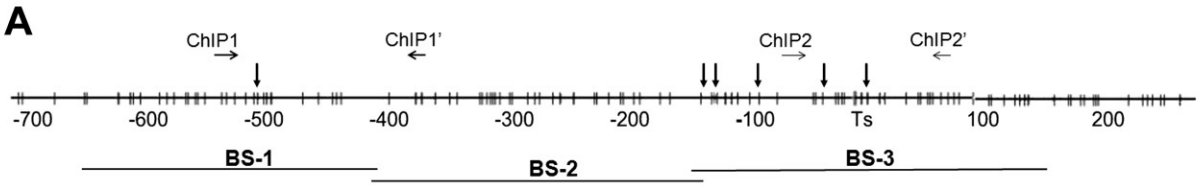
Figure 4.5: Expression pattern of hTERT transcriptional repressors in hESCs and NPSCs of different passages

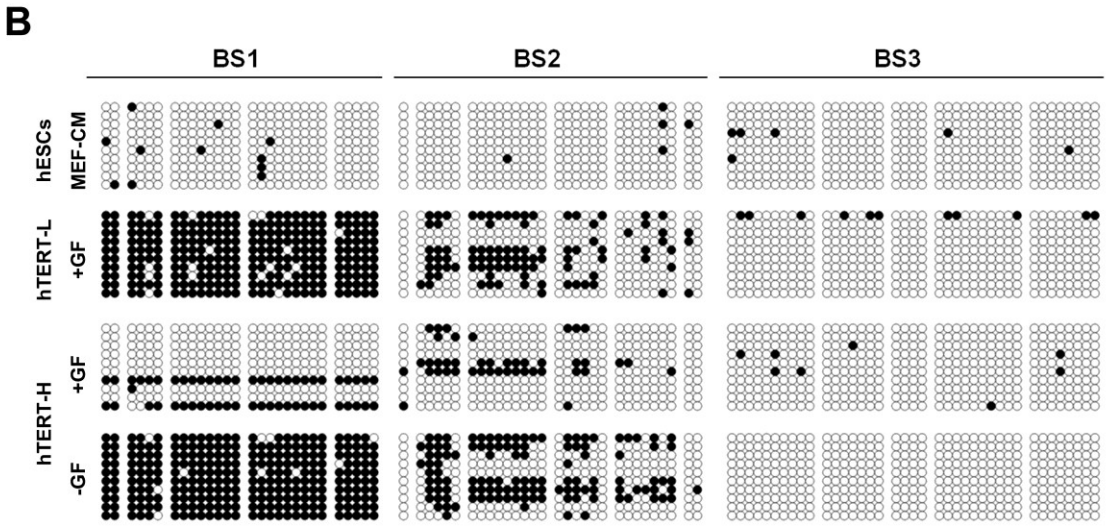
qRT-PCR showing mRNA expression of a panel of candidate hTERT transcriptional repressors in hESCs, telomerase-low NPSCs (p12 & p15) and telomerase-high NPSCs (p59 & p65). Data are presented as mean ± S.E.M. (n=3).

4.2.3 Epigenetic regulation of hTERT in NPSCs

4.2.3.1 CpG methylation at the hTERT promoter

DNA methylation at gene promoter and enhancer regions is one of the key mechanisms regulating gene expression (Bird and Wolffe, 1999; Boyes and Bird, 1991). As mentioned above, the hTERT promoter region is very CG-rich and embedded in a large CpG island. Therefore, CpG methylation could affect hTERT transcription. To further study epigenetic regulation of hTERT in the NPSCs, I examined CpG methylation at the core hTERT promoter using bisulphite DNA sequencing (Figure 4.6). In hESCs, which have the highest hTERT expression, the promoter was almost completely unmethylated. In contrast, early telomerase-low (p16) NPSCs showed abundant CpG methylation (37.8%) and this was specifically localised to the BS1 region -400 to -665 bp upstream of the transcriptional start site, with 94.0% of CpG dinucleotides methylated (Figure 4.6B,C). However, in late NPSCs (p31) where hTERT was reactivated, this methylation had dramatically diminished to 10.9% overall and 19.6% in the BS1 fragment. Furthermore, when the growth factors were withdrawn from late passage NPSCs, leading them to further differentiate and downregulate hTERT, abundant CpG methylation was again seen localised to the same BS1 region of the promoter (96.4%). These data indicate that CpG methylation levels at the hTERT core promoter, particularly the -400 to -665 bp region, negatively correlate with hTERT mRNA levels.





C

Position		BS1 (-665 → -400)	BS2 (-399 → -135)	BS3 (-134 → +150)	Total (-665 → +150)
No. of CpG		28	30	39	97
% of methylation	hESCs	3.9%	1.7%	1.5%	2.3%
	hTERT-L +GF	94.0%	31.0%	2.8%	37.8%
	hTERT-H +GF	19.6%%	14.3%	2.1%	10.9%
	hTERT-H -GF	96.4%	40%	0	40.2%

Figure 4.6: DNA methylation at the hTERT promoter in hESCs and NPSCs

(A) Schematic map of the hTERT promoter. The nucleotides in the promoter are numbered relative to the transcriptional start site (Ts) and each short bar represents a CpG dinucleotide. The promoter is divided into three regions - BS1, BS2 and BS3 - and their locations are marked. Arrows point to Sp1 binding motifs in the hTERT promoter and the primers used for the ChIP assay are illustrated. **(B)** CpG methylation was examined by bisulphite sequencing in hESCs, telomerase-low NPSCs (hTERT-L) and telomerase-high NPSCs (hTERT-H), with or without growth factors (+GF/-GF). Open and closed circles represent unmethylated and methylated CpGs, respectively. **(C)** Quantification of the results in (B). Experiments performed in collaboration with Kelly Makarona

4.2.3.2 *Histone acetylation at the hTERT promoter*

Compelling evidence has demonstrated that histone modifications are important for the regulation of gene expression, including histone methylation and acetylation. The level of acetylation of histone proteins within and around specific gene loci has been associated with the level of transcription of those genes. Hyperacetylation has been associated with active genes whilst hypoacetylation has been associated with silenced genes (Bernstein et al., 2007; Shahbazian and Grunstein, 2007). In order to try and identify any epigenetic mechanisms regulating hTERT expression in our cell system, I examined the degree of histone acetylation at the core hTERT promoter.

ChIP was performed in hESCs and early (p15) and late passage (p45) NPSCs using an antibody specific for acetylated histone H3 protein (pan-acetylation, non-amino acid-specific). The precipitated DNA was analysed by qPCR for the presence of the core hTERT promoter using primers which amplified the BS1 fragment 400-665 bp upstream of the Ts. Telomerase-high p45 NPSCs showed a strikingly higher level of acetylated histone H3 than telomerase-low p15 NPSCs (Figure 4.7), indicating that the hTERT promoter in telomerase-high NPSCs is more acetylated and in an open and transcriptionally accessible state, which could account for the higher expression of hTERT. However, undifferentiated hESCs that have the highest expression levels of hTERT showed similar acetylation of histone H3 compared with p15 NPSCs, which express the lowest levels of hTERT, suggesting that there is no clear correlation between histone H3 acetylation at these sequences and hTERT expression. Even so, the regulation of hTERT expression could occur through different mechanisms in different cell types. hESCs and NPSCs have very different gene expression profiles, including critical transcription factors such as c-myc and ETS2 (section 4.2.2), which can bind to different sequences in the hTERT promoter, resulting in different chromatin configurations. Further analysis needs to be done to have a better picture of the

chromatin status throughout the whole 600 bp promoter, not only on acetylation but also on methylation of histone H3.

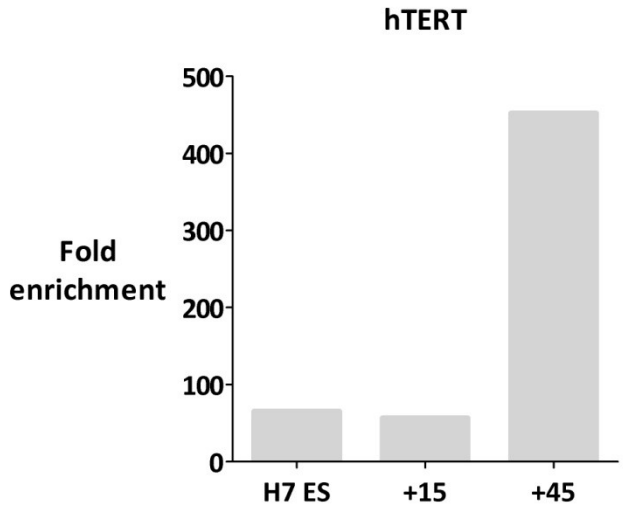


Figure 4.7: Histone H3 acetylation at the hTERT promoter in hESCs and NPSCs

ChIP analysis showed similar levels of histone H3 acetylation at the core hTERT promoter in hESCs and early (p15) NPSCs but substantially greater levels in late (p45) NPSCs. Bars represent the fold enrichment of acetyl-H3 over normal IgG at the hTERT promoter, 400-665 bp upstream of the transcriptional start site.

4.2.4 Sp1 regulation of hTERT transcription in hESCs and NPSCs

Although the exact role of DNA methylation in gene expression is unknown, its appearance in gene promoters is often correlated with transcriptional silencing, perhaps by blocking sites within the promoters at which activating transcription factors bind. Since CpG methylation levels at the BS1 hTERT promoter region are inversely correlated with hTERT mRNA expression so closely, I hypothesised that transcriptional activation of hTERT could be mediated by hTERT activators whose binding sites are particularly localised to the BS1 region of the hTERT promoter. Hence, CpG methylation in the early stage NPSCs could block the binding of these hTERT activators, accounting for the hTERT downregulation.

A search in the Transcription Element Search System (TESS) database for transcription factor binding sites within the hTERT core promoter revealed that several known regulators of hTERT transcription have binding sites within the BS1 region, in particular c-myc and Sp1, two well studied hTERT activators (Kyo et al., 2000), each of which has one binding site within the BS1 region. However, as the predicted c-myc binding site does not contain a CpG dinucleotide, its binding should not be affected by methylation. Furthermore, despite its high expression in hESCs, c-myc was hardly detectable in hESC-derived NPSCs at both the mRNA and protein levels, regardless of telomerase expression (Figure 4.4, 4.8), indicating that c-myc is unlikely to account for the reactivation of the hTERT gene in NPSCs. Sp1 has been shown to bind to the hTERT promoter in multiple cell types (Lin et al., 2011; Liu et al., 2011; Marconett et al., 2011) and to activate reporters driven by core hTERT promoter fragments (Kyo et al., 2000). Therefore, I studied this factor in more detail within our cell system.

4.2.4.1 Sp1 expression

I first examined Sp1 mRNA expression. It appeared that Sp1 mRNA was consistently expressed in NPSCs at all stages of culture, albeit with slight fluctuations in expression level, and it was also expressed in hESCs (Figure 4.8A). Even after growth factor withdrawal leading to hTERT downregulation, Sp1 mRNA expression remained fairly consistent (Figure 4.8B). Furthermore, Western blotting confirmed Sp1 protein expression in hESCs and showed that its protein levels were even higher in NPSCs, both early (p15) and late (p45) passages (Figure 4.8C). These data suggest that the level of Sp1 expression does not control hTERT expression in the NPSCs.

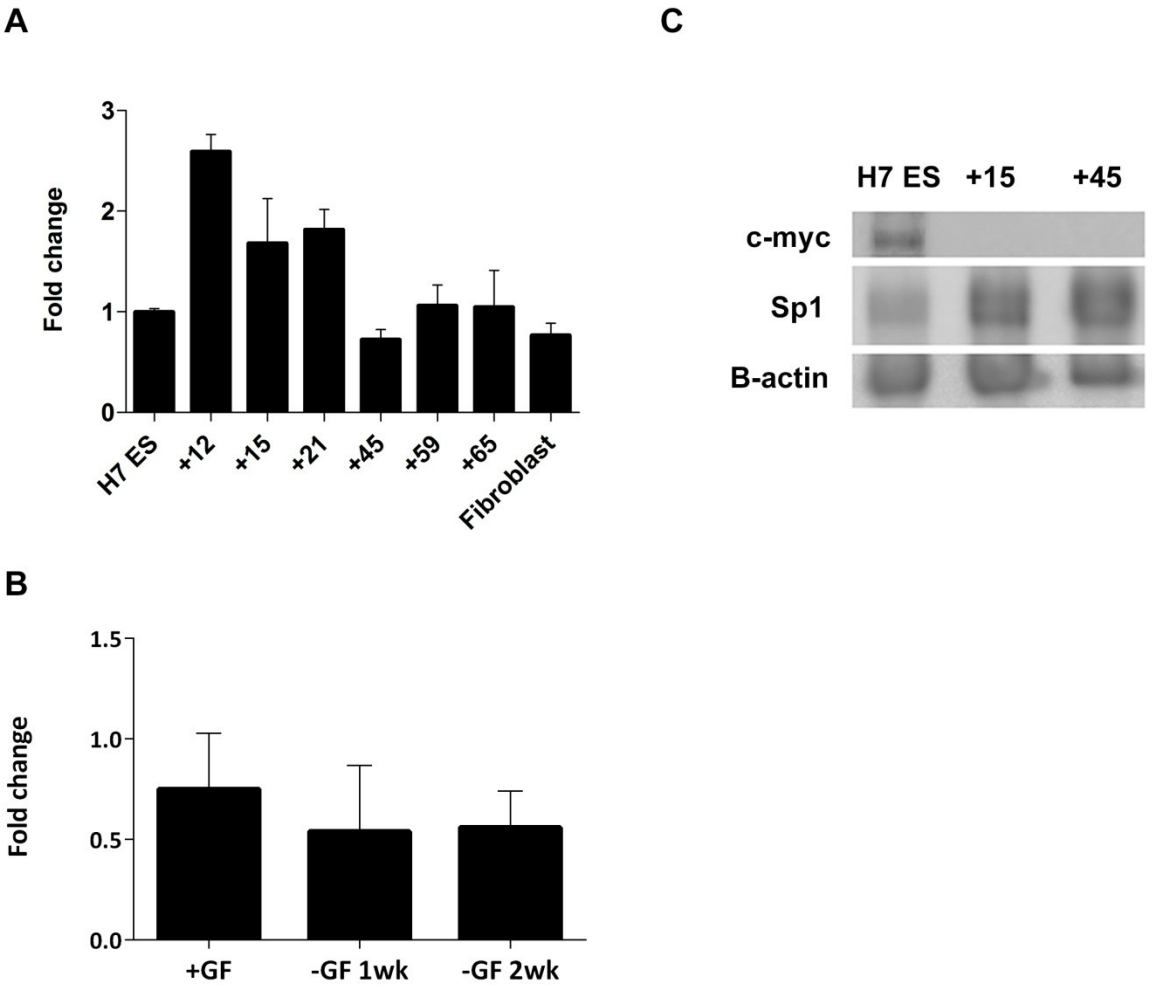


Figure 4.8: Sp1 expression in NPSCs

(A) Sp1 mRNA was expressed in H7 hESCs and their derived NPSCs at various passages, as shown by qRT-PCR. **(B)** Sp1 mRNA expression did not change significantly after growth factor (GF) withdrawal in late (p57) passage NPSCs. **(C)** c-myc protein was highly expressed in H7 hESCs but was absent in early (p15) and late (p45) NPSCs, as shown by Western blotting. Sp1 protein was expressed by both hESCs and early and late NPSCs. β-actin is used as a loading control for total protein.

4.2.4.2 Sp1 binding to the hTERT promoter

To decipher whether the binding of Sp1 could have any role in regulation of hTERT expression in NPSCs, I carried out Sp1 ChIP experiments to determine if Sp1 was bound to the hTERT promoter BS1 region in hESCs and/or NPSCs and if the binding was affected by CpG DNA methylation. DNA precipitated by an antibody against Sp1 from hESCs, early NPSCs and late NPSCs were quantified by qPCR using primers which amplified the BS1 promoter region. Since Sp1 also has several binding sites at the BS3 region of the hTERT promoter, which was constitutively hypomethylated in early and late NPSCs, I also performed qPCR with the same DNA using primers specific for the BS3 region (Figure 4.9A). These experiments produced a high fold enrichment of Sp1 over normal IgG at the hTERT promoter BS1 region in hESCs. Sp1 protein bound to the BS1 region was detected 2-fold greater in the telomerase-high late NPSCs than in the telomerase-low early NPSCs, ($P < 0.005$, Figure 4.9A), echoing the expression of hTERT but inversely correlated with CpG methylation; in contrast, Sp1 binding in hESCs, telomerase-low NPSCs and telomerase-high NPSCs showed no difference at the BS3 region, which exhibited consistently low DNA methylation (Figure 4.9A). To ensure that the DNA methylation patterns in these cells faithfully reflected that detected by bisulphite sequencing, I also used methylation-specific BS1 primers to examine the genomic DNA isolated from those cells. The results showed that methylation was only detected in the early NPSCs (p15) but not hESCs or late NPSCs (p45) (Figure 4.9B), which is in agreement with the results of bisulphite sequencing. These results indicate that Sp1 is bound to the hTERT promoter in hESCs and NPSCs but to a much lesser extent in early passage NPSCs, where its binding is likely inhibited by CpG methylation.

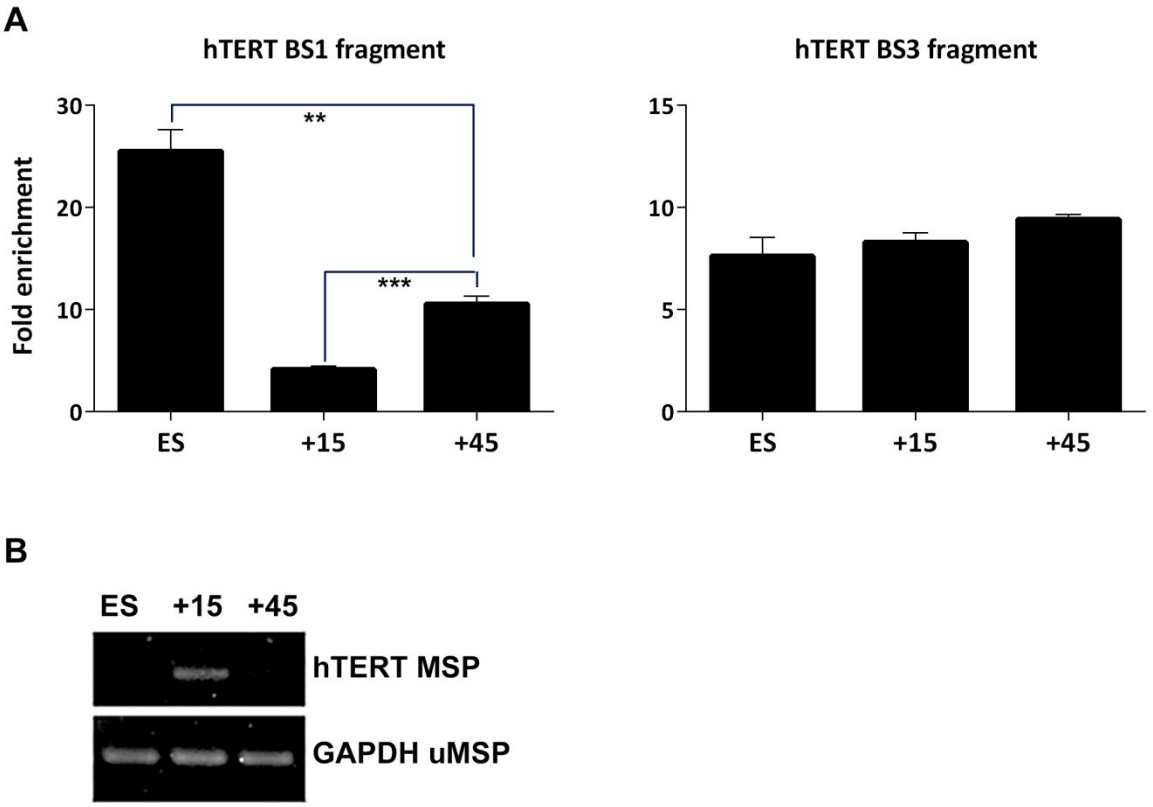


Figure 4.9: Binding of Sp1 to the hTERT promoter in hESCs and NPSCs

(A) ChIP assays showed that Sp1 binding to the hTERT BS1 promoter region (left) was significantly higher in late (p45) NPSCs than in early (p15) NPSCs and Sp1 binding was even more abundant in H7 hESCs. In contrast, Sp1 binding to the hTERT BS3 promoter region (right) was similar in all three cell types. Sp1-bound BS1 and BS3 DNA fragments were amplified with ChIP1 and ChIP2 primers, respectively, as indicated in Figure 4.6A. The data are normalised to the input value after subtracting the IgG value and presented as mean ± S.E.M from three experiments. **, $p<0.01$; ***, $p<0.005$ (Student's *t* test). **(B)** Methylation specific PCR confirmed that the BS1 fragment of the core hTERT promoter was methylated only in p15 NPSCs, not in H7 hESCs or p45 NPSCs (hTERT MSP). Primers specific for a GAPDH promoter sequence with unmethylated CpGs (GAPDH uMSP) were used as a loading control for total bisulphite converted genomic DNA.

4.3 Discussion

As mentioned in section 4.1, it remains unclear how telomerase expression is regulated during cell differentiation and proliferation, as well as in ageing and cancer. We have shown that telomerase activity and expression is dynamically regulated in hESCs, during their neural differentiation and the culture of NPSCs. Here, we take the advantage of this cell system to explore the possible mechanisms that regulate hTERT transcription. We were particularly interested in the regulatory mechanisms underlying the reactivation of hTERT expression during extended NPSC culture.

To this end, I examined the expression of a panel of transcriptional regulators of hTERT in hESCs and NPSCs at different stages of culture. Data from these experiments indicated that AP2-A, NAB2, MAD1 and E2F1 are unlikely to play a role in the regulation of hTERT in our cell system, as their expression patterns do not reflect that of hTERT. The reported hTERT repressors WT1, SIP1 and MEN1 had the highest expression in hESCs, where hTERT expression is also highest. Therefore, these transcription factors are unlikely to play a role in hTERT regulation in these cells. Jun has been reported to repress hTERT (Takakura et al., 2005) however its expression mirrored hTERT expression in our cell system, so it is unlikely to regulate hTERT in these cells. c-myc and ETS2 were highly expressed and therefore may help to activate hTERT in hESCs. However, they do not seem to play a role in hTERT reactivation due to their low expression in NPSCs. Finally, the expression of ETV1, which has been reported to activate hTERT transcription in breast cancer cells (Goueli and Janknecht, 2004), was closely correlated with hTERT expression in hESCs and NPSCs of different stages in culture. Therefore, ETV1 is a possible transcriptional activator of hTERT in our cell system and investigation of its protein expression pattern by Western blot is

warranted. If this mirrors the mRNA expression pattern, ChIP assay and siRNA experiments should be carried out to investigate the role of ETV1 in hTERT regulation in these cells.

The most interesting finding in this study is the negative correlation between CpG-methylation of the hTERT promoter BS1 region and hTERT mRNA expression levels in this cell system. Bisulphite DNA sequencing analysis revealed that in hESCs and telomerase-high NPSCs, the core hTERT promoter was almost completely unmethylated. In contrast, early passage telomerase-low NPSCs and further differentiated late passage NPSCs with downregulated hTERT displayed abundant methylation at a small 200 bp fragment within the core promoter, distal to the transcriptional start site. These results are in agreement with the idea that promoter CpG methylation often results in transcriptional silencing of genes, the generally accepted role of DNA methylation in epigenetic regulation. The data also suggest that in these cells, hTERT transcription is regulated by dynamic and specific methylation of the promoter.

Methylation of the hTERT promoter has been studied by several laboratories. However, most of these studies focused on the BS2-3 region of the hTERT promoter (-300 to +100 bp) and the results are paradoxical and complicated. Some showed that methylation of the hTERT promoter is associated with reduced hTERT expression (Bechter et al., 2002; Shin et al., 2003) and that complete methylation led to hTERT silencing (Renaud et al., 2007). In contrast, the others reported hypermethylation in a number of cancer cell types with higher telomerase activity and hypomethylation in somatic non-transformed cells with lower telomerase (de Wilde et al., 2010; Devereux et al., 1999; Zinn et al., 2007). Our data demonstrated that the BS2-3 region, particularly the BS3 region of the hTERT promoter, was consistently hypomethylated during neural differentiation of hESCs and the culture of the NPSCs and that hypomethylation of this region is even maintained in postmitotic neurons and glia. The methylation pattern of this proximal hTERT promoter region does not show any

correlation with telomerase activity in these cells. However, we found that hTERT expression and consequent telomerase activity are inversely correlated with CpG methylation levels in the BS1 hTERT promoter region (-665 to -400 bp) further upstream of the core promoter region. These results suggest that regulation of telomerase expression in progenitor stem cells may be distinct from that of cancer cells.

The c-myc and Sp1 transcription factors are two well studied hTERT activators (Kyo et al., 2000) that have multiple binding sites in the core hTERT promoter region. My results showed that c-myc is highly expressed in undifferentiated hESCs, at both the mRNA and protein level, but its expression became almost undetectable after neural differentiation and did not show upregulation after extended culture (Figures 4.4 & 4.8). Therefore, c-myc is unlikely to account for the reactivation of the hTERT gene in NPSCs. The expression of Sp1 showed no difference between telomerase-low and -high NPSCs, but its binding to the -665 to -400 bp BS1 region of the hTERT promoter increased in the long-term cultured telomerase-high NPSCs, most likely as a result of DNA demethylation. Importantly, the level of Sp1 binding to the BS1 region of the hTERT promoter mirrors hTERT expression in hESCs and their neural progenitor progeny. The binding of Sp1 to this region has been reported to activate hTERT expression in human ovarian cancer cells upon EGF stimulation (Bermudez et al., 2008). Given that in the NPSC cultures, Sp1 can also bind to the hTERT promoter near the transcription start site, in areas that are not affected by DNA methylation, the significance of Sp1 binding to the -665 to -400 bp region in the regulation of hTERT transcription needs further investigation.

Taken together, the data presented here suggest a model for Sp1-mediated regulation of hTERT in NPSCs. In hESCs, hTERT expression is driven by the transcription factor Sp1, which binds to the core promoter, particularly in the distal region, promoting hTERT transcription. During hESC differentiation, the distal promoter region becomes heavily

methylated at CpG dinucleotides, preventing Sp1 binding at this region thereby vastly reducing hTERT transcription. Later, the CpG methylation disappears due to unknown mechanisms, resulting in increased Sp1 binding and reactivation of hTERT transcription.

It remains largely unknown what regulates CpG methylation of the hTERT promoter. However, we have noticed that hTERT reactivation in the late passage NPSCs derived from both hESC lines happened after telomeres shortened to around 4 kb in average length, which is about the length that triggers human fibroblasts to enter senescence (Allsopp and Harley, 1995). The hTERT gene is located at subtelomeric band 5p15.33 of chromosome 5 (Bryce et al., 2000) and it is known that genes inserted adjacent to telomeres become silenced by a phenomenon known as the telomere position effect (Gottschling et al., 1990; Kulkarni et al., 2010). This silencing of telomere-adjacent genes involves increased DNA CpG methylation (Pedram et al., 2006) and changes in chromatin conformation (Tham and Zakian, 2002). Interestingly, transcriptional repression by the telomere position effect becomes reduced as telomeres shorten (Tham and Zakian, 2002). Therefore, it is possible that during NPSC culture, hTERT becomes derepressed as telomeres shorten, leading to the telomerase reactivation observed. This idea is supported by a recent study in which TERT knockout ESCs with short, dysfunctional telomeres were observed to express significantly higher levels of Nanog and other pluripotency factors. This transcriptional dysregulation was reversed when telomeres were re-elongated via the re-introduction of TERT (Pucci et al., 2013). TERT knockout ESCs with short telomeres also displayed reduced levels of the repressive histone mark H3K27me3 at the Nanog promoter and these levels were restored upon telomere elongation by TERT. In addition, genome-wide CpG methylation was significantly reduced in TERT knockout ESCs. The results of this study demonstrated that critically short telomeres affect chromatin organisation, CpG methylation and gene expression at loci distal to telomeres (Pucci et al., 2013). This raises the possibility that as a result of telomere

shortening in our NPSCs, genome-wide chromatin changes and CpG demethylation occurs which result in hTERT reactivation.

Chapter 5. The impact of telomerase in human embryonic stem cells and their neural derivatives

5.1 Introduction

A number of studies have suggested that TERT has non-telomeric functions independent of its catalytic activity, in particular the promotion of proliferation and modulation of transcription of certain genes (Park et al., 2009; Sarin et al., 2005). However, other studies have contradicted these data (Strong et al., 2011; Vidal-Cardenas and Greider, 2010). Therefore, the question still exists as to what, if any, functions telomerase may have in addition to telomere elongation. Research is ongoing into the potential roles of telomerase in cellular self-renewal, proliferation and differentiation. As shown in Chapter 3, hESCs have high telomerase expression and long telomeres. During neural differentiation, there is a dynamic pattern of telomerase expression and telomere length and following extended culture, late NPSCs display reactivation of telomerase. Based on these observations, hESCs and their derived NPSCs provide a good model to further study the function and impact of telomerase in normal stem cells.

Telomerase expression, limited by levels of hTERT, can be readily detected in the early embryo and is particularly high in the inner cell mass (ICM) of the preimplantation blastocyst. During human development however, hTERT expression becomes closely restricted and in the adult organism, hTERT is only expressed in germ cells and some adult stem cell compartments. Corresponding to the early embryo, hESCs derived from the ICM show high hTERT mRNA levels and therefore high telomerase activity.

It was found previously that endogenous hTERT expression and telomerase activity are dramatically downregulated upon differentiation of hESCs (Gerrard et al., 2005; Hay et al.,

2008). It is unknown, however, if hTERT downregulation is a requirement for or merely a consequence of differentiation. Since telomerase restriction in somatic cells is an important tumour suppressor mechanism, it could be speculated that telomerase downregulation is necessary for hESC differentiation Therefore, I hypothesised that sustained hTERT expression might to some extent inhibit differentiation.

To address the role of telomerase in hESC self-renewal and differentiation, both H1 and H7 hESC lines were previously stably transfected with a plasmid containing the full hTERT coding cDNA under the control of the mouse PGK promoter. The construct also contained a puromycin resistance cassette under the control of the SV40 promoter. Puromycin resistant colonies were clonally expanded and hTERT transgene integration was confirmed by Southern blotting. Several clones of empty vector transfected cells were also obtained for controls. I aimed to investigate with these cells whether the ectopic expression of hTERT in hESCs has any effect on their differentiation to neural progenitors and terminal differentiated neurons and glia.

5.2 Results

5.2.1 Overexpression of hTERT in hESCs

The various hTERT-overexpressing (hTERT-ov) hESC clones overexpressed hTERT at different levels; hTERT mRNA expression of a representative H7 hTERT-ov clone compared to one control clone was shown by RT-PCR (Figure 5.1A). I used primers located at the coding cDNA to detect both transgenic and endogenous hTERT mRNA expression and primers located at the hTERT 3'UTR to detect the specific expression of endogenous hTERT. Whilst total hTERT mRNA was higher in the hTERT-ov hESCs, the endogenous level was similar to, if not lower than, the control hESCs. Expression of the transgenic SV40 polyA signal was used to confirm transgene expression, since it is a non-human sequence and therefore not endogenous to the cells. Although SV40 polyA expression was lower in the hTERT-ov clone than empty vector controls (perhaps due to the nature of the constructs in which SV40 polyA is closer to the promoter), its expression was clearly visible. TRAP assay of H7 parental, control and hTERT-ov hESC clones (Figure 5.1B) shows that telomerase activity was highest in the hTERT-ov clone (although the control clone also had higher telomerase activity than the parental cells, probably due to clonal variation). Consistent with this, the average telomere length of the hTERT clone, shown by TRF Southern blotting, was increased compared to that of the control (Figure 5.1C). In addition, the TRF signal in the hTERT-ov hESCs was less spread out than that of the control indicating reduced telomere length heterogeneity. Moreover, it appeared that the lower molecular weight telomeres were lost from the hTERT-ov hESC sample compared to the control; the shortest telomere length in the control clone is around 9 kb while in the hTERT-ov clone, it is about 12 kb. This suggests that the increased telomerase in the hTERT-ov clone has acted at the shorter

telomeres to elongate them, which supports the theory that telomerase preferentially extends shorter telomeres (Marcand et al., 1999; Ouellette et al., 2000).

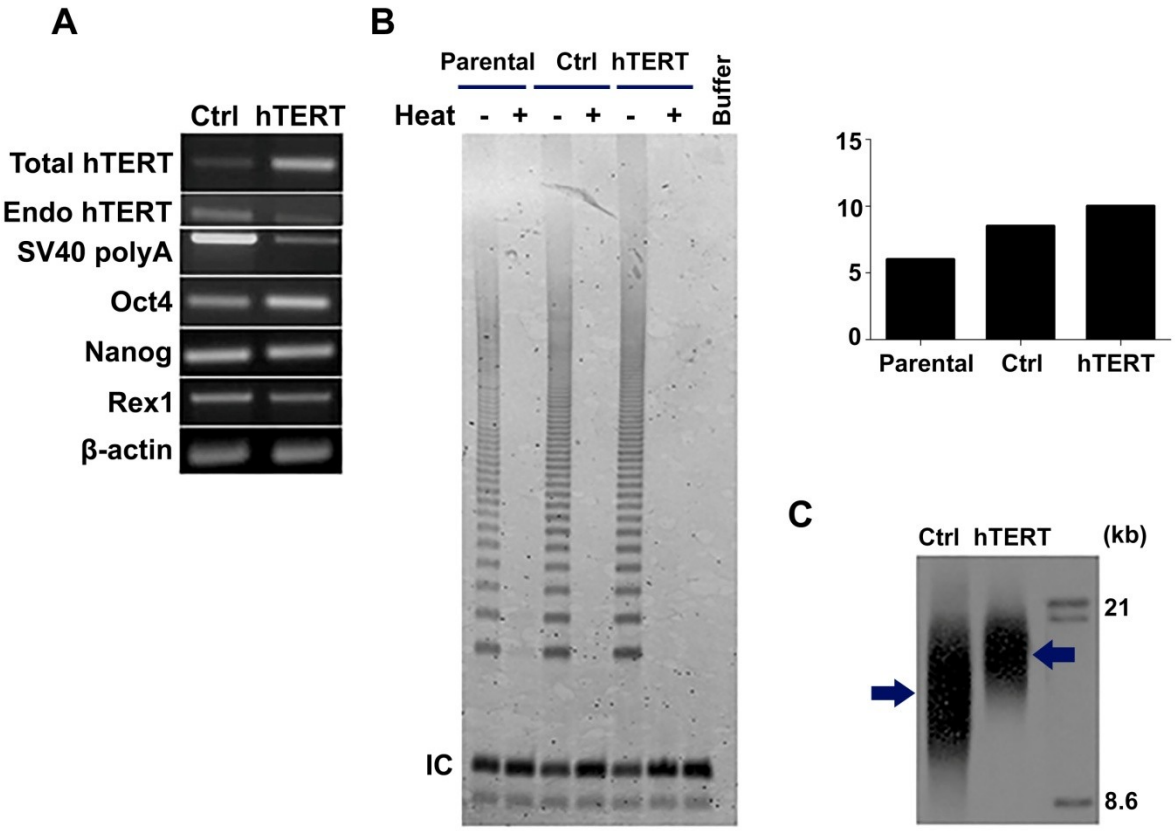


Figure 5.1: Effect of hTERT overexpression on pluripotent gene expression and telomere length in hESCs

(A) Comparison of mRNA expression levels of total hTERT, endogenous (endo) hTERT, transgenic SV40 poly A and pluripotency-related genes (Oct4, Nanog and Rex1) between H7 hTERT-overexpressing hESCs and control (ctrl) hESCs, by RT-PCR. β-actin is used as a loading control for total cDNA. **(B)** Telomerase activity in H7 parental, ctrl and hTERT hESCs, by TRAP assay. Heat inactivated sample reactions are included to demonstrate telomerase-dependency of ladder. An internal control sequence (IC) shows the relative PCR efficiency of each reaction. Quantification by gel densitometry is displayed to the right. **(C)** Telomere length in H7 ctrl and hTERT hESCs, by TRF Southern blot, with the average telomere length of each clone indicated with arrows. A molecular weight ladder is present in the right hand lane.

5.2.2 The effect of hTERT overexpression on hESC cell cycle

Overexpression of telomerase has been shown to increase proliferation rates of both somatic and stem cells (Cui et al., 2002; Flores et al., 2005). However, I did not observe a significant increase in cell proliferation of the hTERT-ov hESCs. Both control and hTERT-ov hESCs were propagated at similar rates. To further assess the effect of hTERT overexpression on cell cycle in hESCs, hTERT-ov and control H1 hESCs were stained with PI and analysed by flow cytometry, producing a cell cycle profile of the cells. However, the percentage of cells in the G1-, S- or G2-phases of the cell cycle varied very little between parental, control and hTERT-ov H1 hESCs (Figure 5.2). This indicates that hTERT overexpression did not cause cell cycle changes. This discrepancy with published data in the literature could be due to the fact that hESCs already express high levels of telomerase in the absence of the hTERT transgene and are different from somatic cells and somatic stem cells, which express no or low levels of telomerase. Therefore, slightly increasing hTERT expression does not have a distinct effect on hESC proliferation.

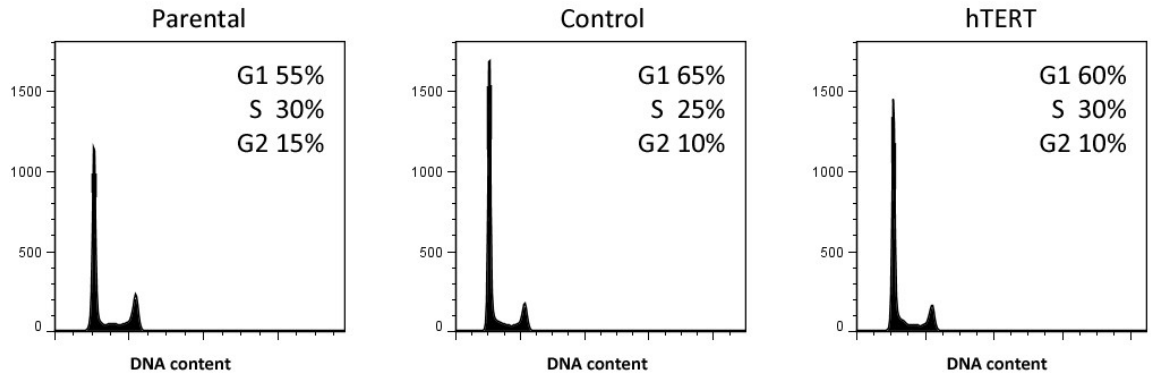


Figure 5.2: Cell cycle analysis of parental, control and hTERT-overexpressing H1 hESCs.

There was little difference between parental, control and hTERT-overexpressing H1 hESCs in the proportion of cells in each phase of the cell cycle, as shown by propidium iodide staining and flow cytometry analysis of subconfluent hESCs.

5.2.3 The effect of ectopic hTERT expression upon hESC differentiation to neural progenitor/stem cells

To study the effect of hTERT on hESC properties, I examined the expression of pluripotent genes in these hTERT-overexpressing cells cultured in hESC culture conditions. hTERT-ov hESCs appeared to have higher Oct4 expression than control cells but Nanog and Rex1 expression did not exhibit clear differences between them (Figure 5.1A). Moreover, the hTERT-hESCs appeared as typical hESC colonies, exhibiting similar morphology to the controls, suggesting that overexpression of hTERT does not have a detrimental effect on hESC self-renewal.

To test the possibility that ectopic hTERT expression might inhibit differentiation, hTERT-ov and control hESCs were differentiated down the neural lineage using the established monolayer differentiation protocol in our laboratory (Figure 3.1). hESCs can be efficiently differentiated into NPSCs by this protocol, in which the hESC self-renewal culture medium (MEF-conditioned media supplemented with bFGF) is replaced with chemically defined neural-supportive media supplemented with noggin, an antagonist of BMP signalling pathways. BMP signalling is well documented to inhibit neural differentiation and neural development, therefore supplementing differentiation medium with noggin promotes hESC differentiation towards the neural lineage.

The neural differentiation was carried out in hTERT-ov clones and their corresponding controls derived from H1 parental hESCs. hTERT mRNA expression was examined in these cells by RT-PCR, which showed higher hTERT expression in the hTERT-ov hESCs than in the control and parental cells (Figure 5.3A). As indicated previously, both hTERT-ov and control hESC lines displayed typical hESC colonies, with cells exhibiting normal hESC morphology, as shown in Figure 5.3B, left panels. There was no morphological difference

between the hTERT-ov and control cells at the start of the differentiation. However, by day 5 of the neural differentiation, neural progenitor-like cells started to emerge in the controls, although the cell numbers were very low, while hTERT-ov clones were still present predominantly with hESC-like morphology (Figure 5.3B, right panels).

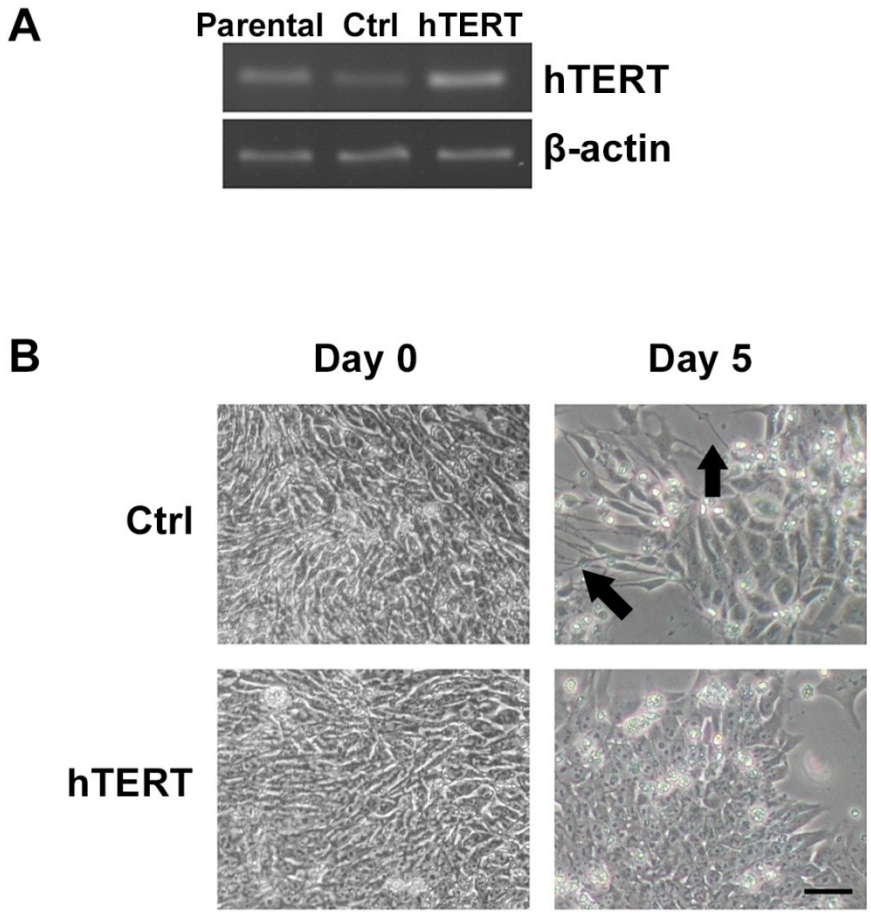


Figure 5.3: hTERT overexpression delays neural differentiation in H1 hESCs

(A) hTERT mRNA expression was higher in H1 hTERT hESCs than in parental or control (ctrl) cells, as shown by RT-PCR, with β-actin mRNA used as a loading control. **(B)** H1 ctrl and hTERT hESCs displayed colony morphology prior to neural differentiation (Day 0). At Day 5 of neural differentiation, ctrl cells began to display neural progenitors (indicated by arrows) while hTERT cells did not. Scale bar represents 20 μm for all images.

By day 9 of the differentiation, neural-like cells also started to appear in hTERT-ov cell culture. RNA was collected from those cells and tested for gene expression. Oct4 and Nanog were still expressed in both control and hTERT-ov cells. Sox2, which is important for both pluripotency and neural differentiation, was upregulated in both control and hTERT-ov cells, but with higher expression in the differentiated control cells compared with hTERT-ov cells (Figure 5.4). This is consistent with the previous finding that Sox2 was upregulated during neural differentiation of hESCs (Gerrard et al., 2005), indicating that cells of both hESC clones may have had initiated neural differentiation. These data suggest that hTERT probably has no significant effect on neural differentiation, although it might slightly delay the differentiation process.

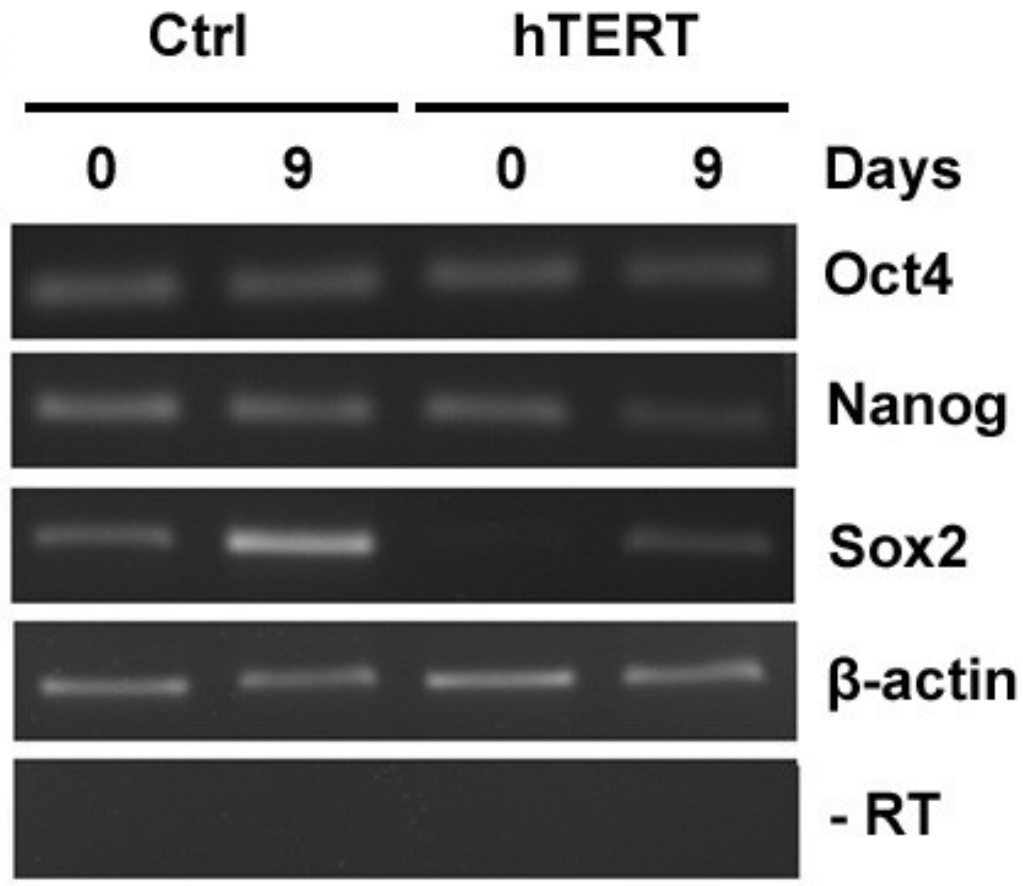


Figure 5.4: Gene expression of H1 control and hTERT hESCs during neural differentiation

RT-PCR shows mRNA expression of pluripotency genes (Oct4 and Nanog) and the neural lineage-associated gene Sox2 in control (ctrl) and hTERT-hESCs before and 9 days after the start of neural differentiation. β-actin mRNA was used as a loading control, a RNA sample prepared without reverse transcriptase (-RT) was used to demonstrate that PCR products were not due to amplification of genomic DNA.

In order to further validate this initial finding, I carried out neural differentiation experiments in the H7 hTERT-ov and control clones and continued the differentiation and culture for a few months. As expected, both control and hTERT-ov hESCs were able to efficiently differentiate to the neural lineage, with initial formation of neural rosettes followed by the appearance of bipolar cells, although hTERT-ov hESCs exhibited neural rosette formation at a slightly later timepoint following differentiation compared with ctrl hESCs. However, once neural progenitor cells were formed, they were able to further develop a triangular morphology (Figures 5.5 and 5.6), consistent with previous studies of wild-type hESC differentiation using this protocol (Wu et al., 2010).

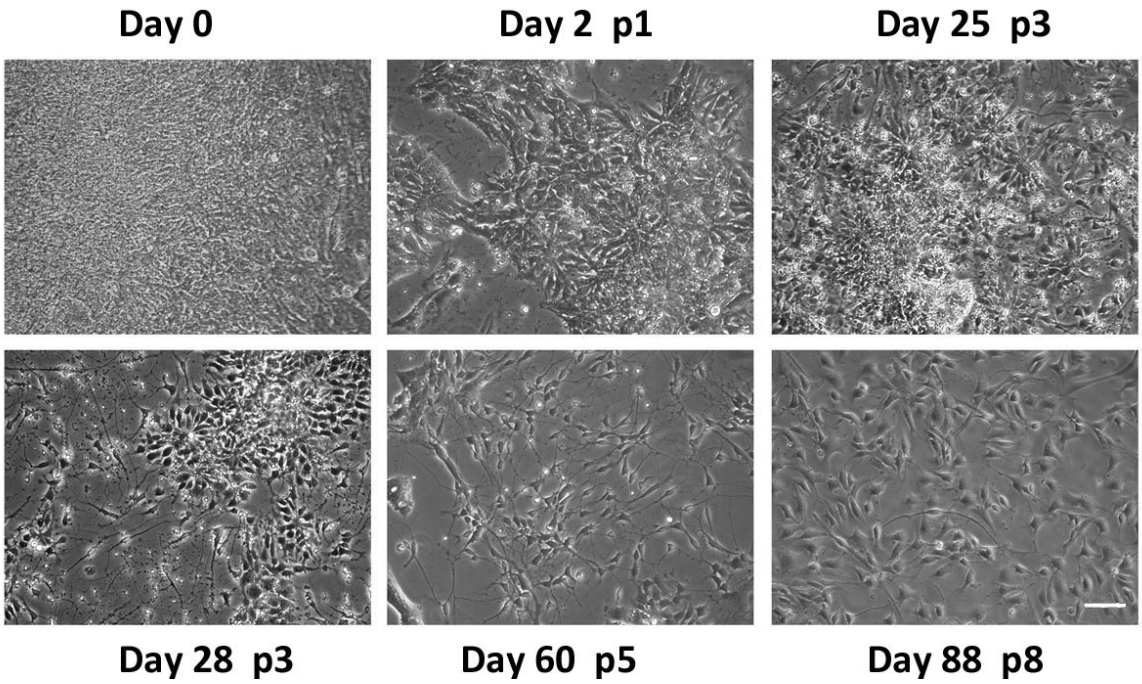


Figure 5.5: Morphology of H7 control hESCs during neural differentiation

Scale bar represents 30 μm for all images.

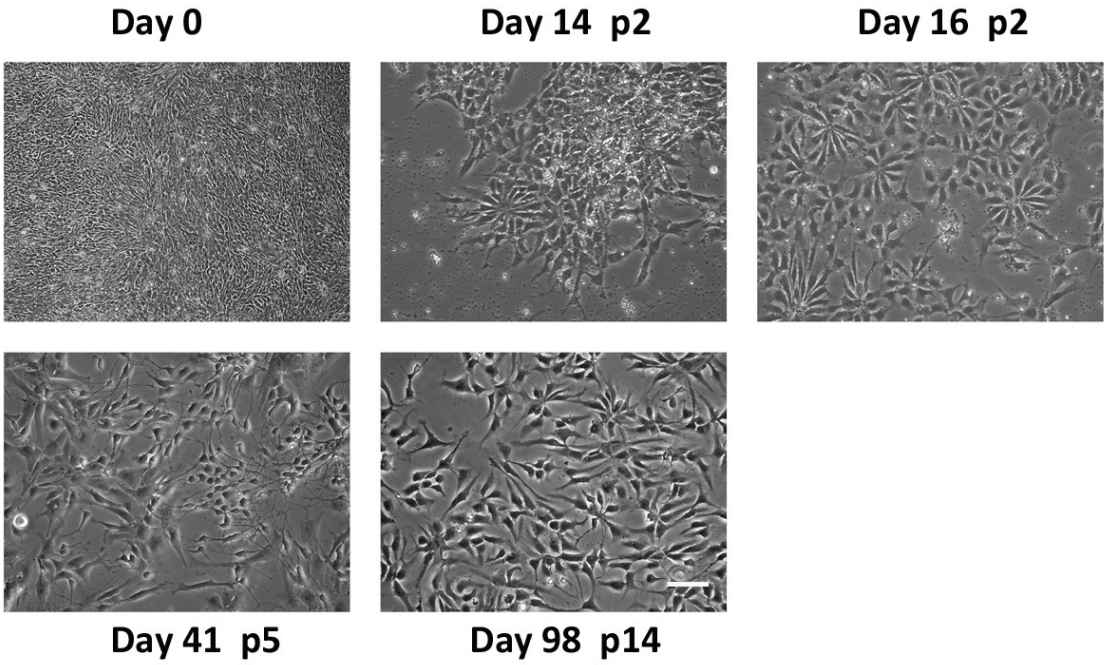


Figure 5.6: Morphology of H7 hTERT hESCs during neural differentiation

Scale bar represents 30 μm for all images.

The expression of pluripotency and neural-lineage specific genes was examined using RT-PCR in control and hTERT-ov H7 hESCs (Figures 5.7 and 5.8, respectively). The results showed that the gene expression profile of differentiating hTERT-ov hESCs was similar to that of the control, with the neural-specific transcription factors Sox1 and Mash1 switching on in both clones, after differentiation for two weeks. In addition, the pluripotency marker Rex1 was undetectable by the second passage. Oct4 and Nanog expression appeared to be maintained for longer during the differentiation of the control hESCs compared to the previous study, in which parental H7 hESCs showed downregulation of Oct4 and Nanog by day 30 of the differentiation (Gerrard et al., 2005), although this could be an artefact of the differentiation, sample preparation or due to something intrinsic within the hESC clone (for example, an effect of clonal selection). By passage 6 of the differentiation, Oct4 and Nanog were shown to be silenced. In contrast, despite initial gradual downregulation of Oct4 and Nanog in the hTERT-ov clone, these transcription factors appeared to be maintained for an extended time during the differentiation. Taken together, my results indicate that telomerase overexpression has no significant effect on the overall neural differentiation of hESCs to generate neural progenitor/stem cells although it appears to have slightly slowed down the neural initiation.

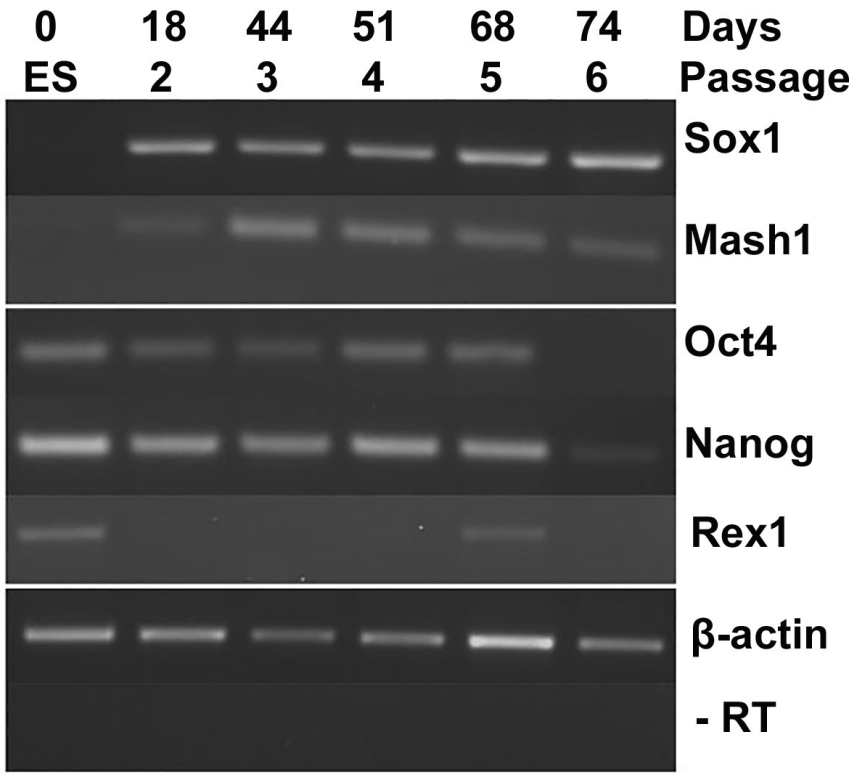


Figure 5.7: Gene expression profile of H7 control hESCs during neural differentiation

RT-PCR shows mRNA expression levels of neural lineage genes (Sox1, Mash1) and pluripotency genes (Oct4, Nanog, Rex1) in H7 control hESCs during differentiation. β-actin mRNA was used as a loading control, a RNA sample prepared without reverse transcriptase (-RT) was used to demonstrate that PCR products were not due to amplification of genomic DNA.

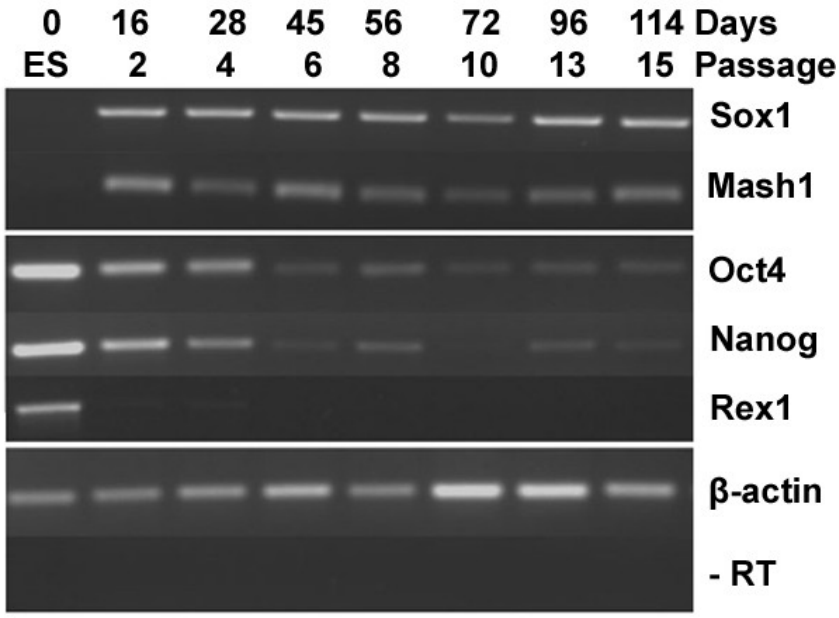


Figure 5.8: Gene expression profile of H7 hTERT hESCs during neural differentiation

RT-PCR shows mRNA expression levels of neural lineage genes (Sox1, Mash1) and pluripotency genes (Oct4, Nanog, Rex1) in H7 hTERT-overexpressing hESCs during differentiation. β-actin mRNA was used as a loading control, a RNA sample prepared without reverse transcriptase (-RT) was used to demonstrate that PCR products were not due to amplification of genomic DNA.

5.2.4 The effect of ectopic hTERT expression upon differentiation of neural progenitor/stem cells to generate postmitotic neural cells

5.2.4.1 Neural progenitor/stem cells derived from hTERT-hESCs

Despite the slightly delayed timing and irregular Oct4/Nanog expression, the hTERT-ov hESCs were able to differentiate efficiently down the neural lineage, producing a homogenous population of NPSCs, as did the control hESCs. These NPSCs strongly expressed the neural stem/progenitor cell markers Nestin and Sox2 (Figure 5.9).

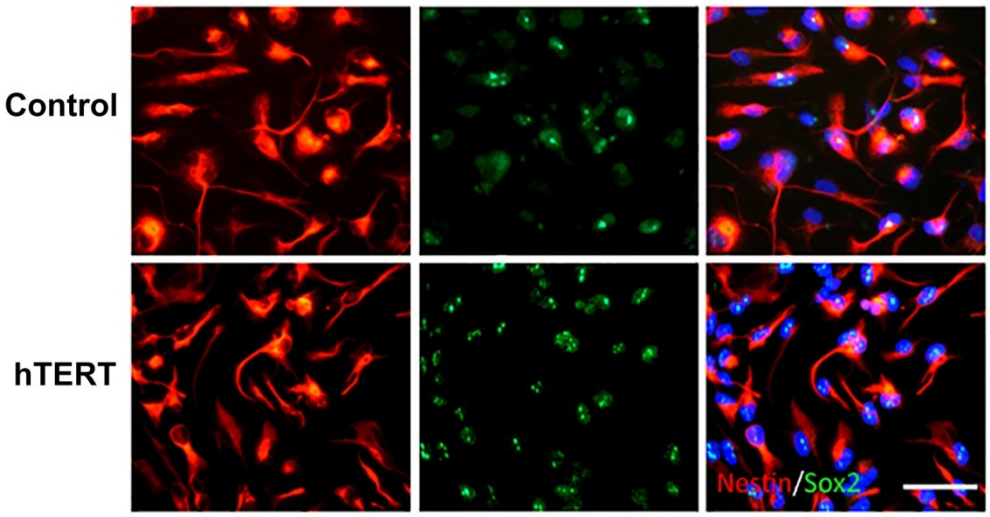


Figure 5.9: Expression of neural progenitor markers in NPSCs derived from hTERT- and control- hESCs

Neural progenitor/stem cells derived from H7 control (top panel) and hTERT (bottom panel) hESCs were positive for Nestin (red) and Sox2 (green) expression, as shown by immunocytochemistry; DAPI counterstain, blue; scale bar represents 20 μm for all images.

Although undifferentiated hTERT-ov hESCs did not show a clear increase in proliferation compared with parental or control hESCs (Figure 5.2), NPSCs derived from these hTERT-ov hESCs displayed faster cell proliferation than the NPSCs derived from control hESCs. The growth curves show that the hTERT-NPSCs have a shorter population doubling time than the controls, with the trendline producing a gradient of 0.53 versus 0.27 for the control cells (Figure 5.10). These results are consistent with the idea that hTERT expression increases proliferation (Choi et al., 2008; Park et al., 2009; Sarin et al., 2005).

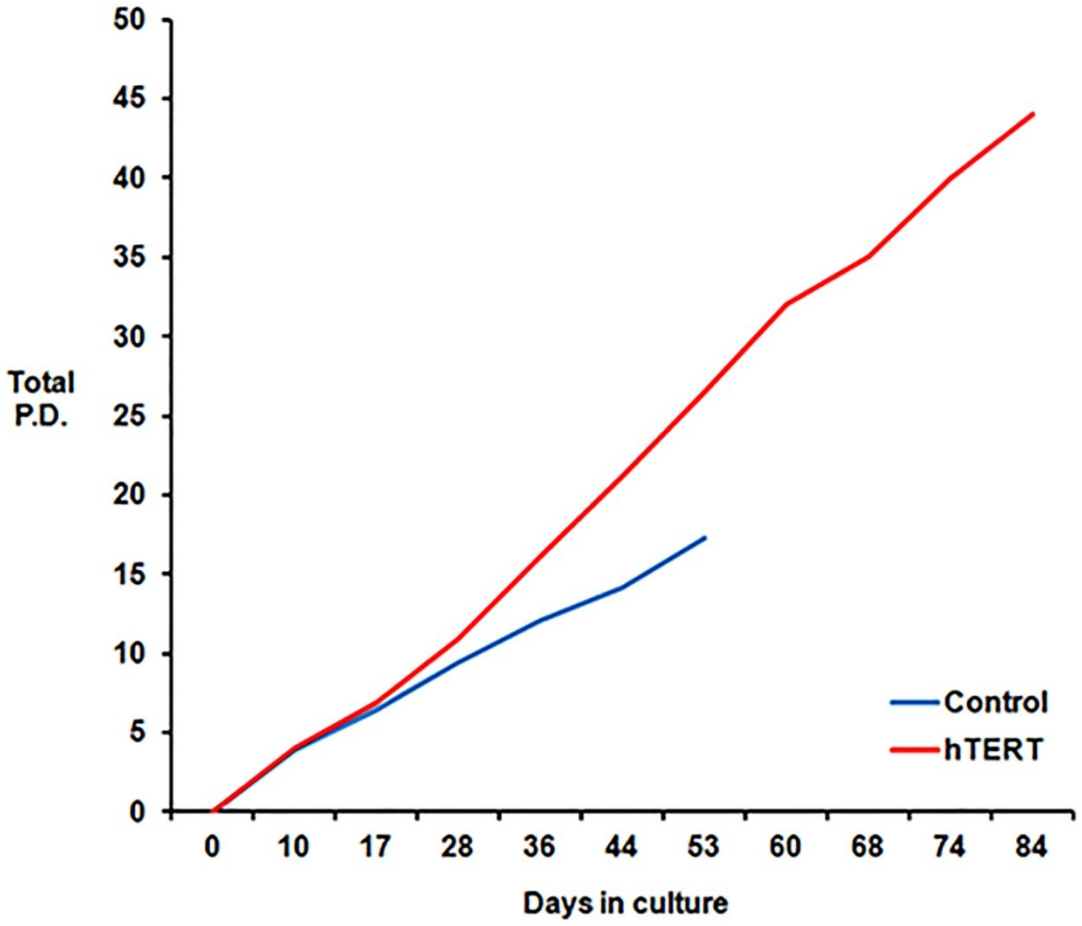


Figure 5.10: Growth curve of hTERT-NPSCs and control-NPSCs

NPSCs derived from H7 hTERT-overexpressing hESCs (red) proliferated more rapidly than NPSCs derived from H7 control hESCs (blue), shown by the total number of population doublings (P.D.) plotted against number of days in culture.

5.2.4.2 Differentiation of hTERT-hESC-derived neural progenitor/stem cells to neurons and glia

As discussed in Chapter 3.2.2, NPSCs are multipotent stem cells which are able to self-renew in culture when they are supplemented with growth factors bFGF and EGF. They can be induced to cease self-renewal and differentiate further down the neural lineage to become postmitotic neurons and glia simply by withdrawing the growth factors. Telomerase has been found to be expressed in certain adult stem cell compartments but is not detectable in somatic cells. Therefore, I asked whether constitutive expression of hTERT would affect the ability of NPSCs to further differentiate into postmitotic cell types. To this end, I assessed the NPSCs derived from hTERT-ov hESCs (hTERT-NPSCs) for their competence in differentiating into neurons and glia upon withdrawal of growth factors.

Upon withdrawal of growth factors, parental NPSCs readily differentiated, becoming postmitotic neuronal and glial cells. This process was visualised as loss of the typically triangular NPSC morphology and development of an axon-sprouting neuronal or enlarged/flattened dendritic glial morphology. These morphological changes were accompanied by striking upregulation of neuronal and glial markers, classically the cytoskeleton proteins β-III-tubulin and GFAP respectively (see Chapter 3.2.2 for further data and explanation). This upregulation was shown by immunocytochemical staining of parental NPSCs after growth factor withdrawal for one week (Figure 5.11A, left panel). In contrast, although hTERT-NPSCs developed a differentiated morphology upon growth factor withdrawal (Figure 5.11B), they failed to upregulate β-III-tubulin or GFAP (Figure 5.11A, right panel). The lack of β-III-tubulin/GFAP upregulation after one week without growth factors suggests that the differentiation of the hTERT-ov hESC-derived NPSCs was inhibited or delayed.

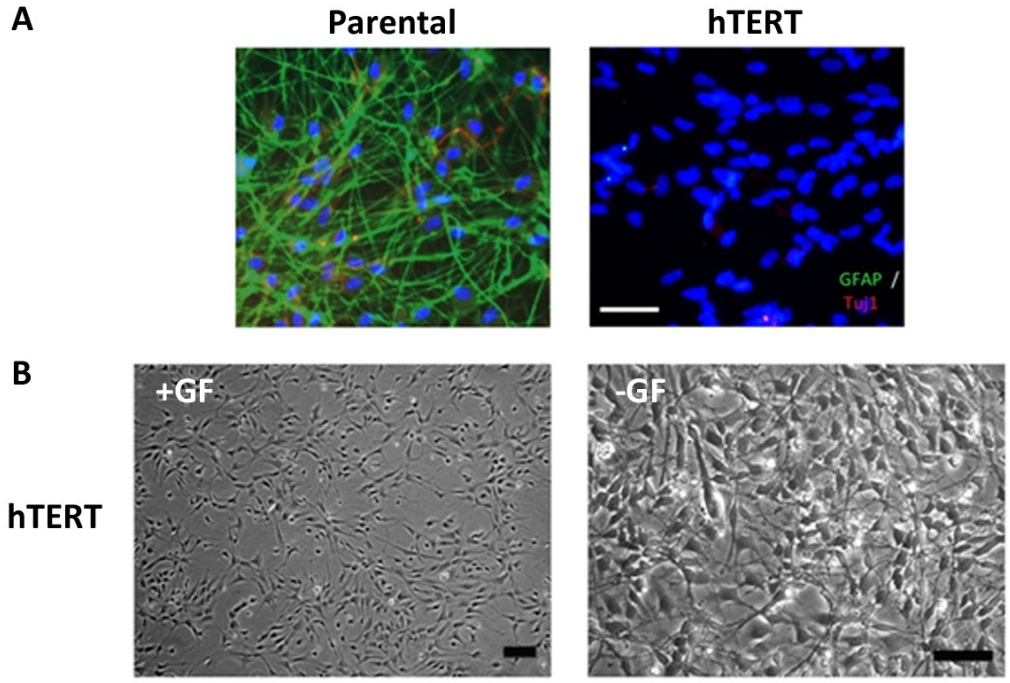


Figure 5.11: hESC-NPSC differentiation after withdrawal of bFGF

(A) Following growth factor withdrawal, NPSCs derived from parental hESCs (left panel) strongly expressed GFAP and β-III-tubulin while hTERT-overexpressing hESC-derived NPSCs (right panel) did not, as shown by immunocytochemical staining for GFAP (green) and β-III-tubulin (red), with DAPI counterstain (blue). Scale bar represents 20 μm for all images. **(B)** Brightfield images of hTERT-overexpressing NPSCs show a typical triangular morphology in the presence of growth factors (GF) and a neuronal morphology in the absence of GF. Scale bars represent 20 μm for all images.

Furthermore, growth factor withdrawal in wild-type NPSCs results in rapid cell cycle exit and termination of DNA synthesis, demonstrated by a complete lack of BrdU incorporation (Chapter 3, Figure 3.6). However, after one week of culture in the absence of growth factors, hTERT-ov NPSCs were still proliferating and with a majority of them becoming labelled after incubation with BrdU (Figure 5.12A). RT-PCR confirmed that hTERT was still highly expressed in the NPSCs at passage 17, albeit at a lower level than in the hESCs from which they were derived (Figure 5.12B). In contrast, hTERT is downregulated in NPSCs derived from wild-type hESCs by passage 12 (Figure 5.12C). This shows that after neural differentiation of hTERT-hESCs, the transgene was still actively expressed. These results suggest that sustained hTERT expression possibly inhibits the differentiation of NPSCs into postmitotic neurons and glia.

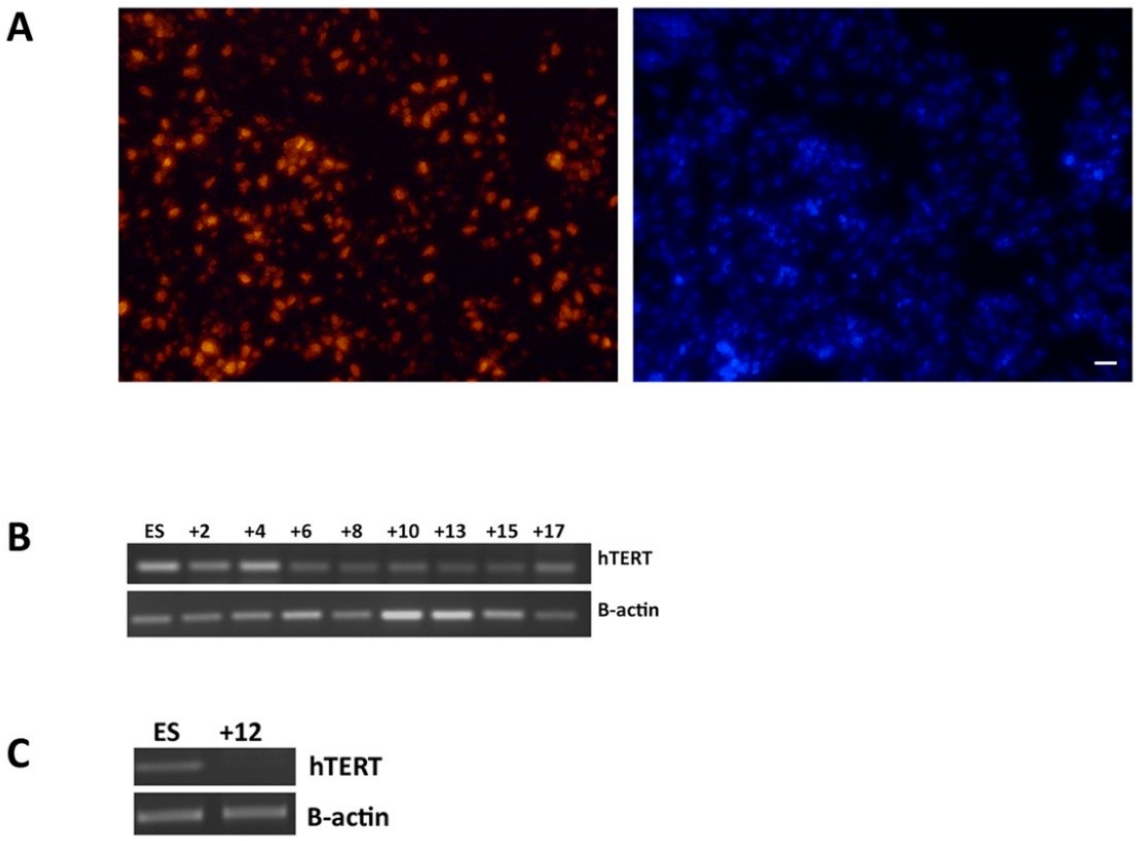


Figure 5.12: hTERT-NPSC proliferation after withdrawal of bFGF

(A) hTERT-overexpressing NPSCs were still proliferating one week after growth factor withdrawal, as shown by BrdU labelling and immunocytochemistry. BrdU (red) and DAPI (blue). Scale bar represents 20 μm for both images. **(B)** hTERT mRNA was still expressed in p17 NPSCs derived from hTERT-overexpressing hESCs. **(C)** hTERT mRNA was undetectable in p12 NPSCs derived from parental hESCs. RT-PCR for mRNA expression with β-actin mRNA used as a loading control.

5.3 Discussion

For the purpose of testing the effect of ectopic hTERT expression upon hESC self-renewal and differentiation, hESCs were transfected with a constitutive hTERT expression vector and stable transfectants were picked and expanded. Comparing hTERT and control hESC clones showed that the hTERT-ov hESCs did indeed have higher hTERT expression and telomerase activity. Furthermore, their average telomere length was increased. However, it appeared that overexpression of hTERT does not affect hESC proliferation and self-renewal. Also, it has no significant effect on the cell morphology or the expression of pluripotent marker genes, Oct4, Nanog and Rex1. Therefore, we consider hTERT overexpression to have no detrimental effect on hESC properties.

Experiments to determine if ectopic hTERT affects the differentiation potential of hESCs demonstrated that ectopic hTERT slightly delayed neural initiation, resulting in delayed differentiation to neural stem/progenitor cells, which was shown by the delay in the morphological changes of the cells. However, hTERT-ov hESCs are able to differentiate to neural progenitor stem cells efficiently, exhibiting classical neural progenitor stem cell morphology with clear upregulation of the neural progenitor markers, Sox1 and Mash1 and high expression of Sox2 and Nestin. Hence, our results demonstrate that higher levels of telomerase do not appear to affect the ability of hESCs in differentiating to neural progenitors. These results are consistent with our previous finding (Chapter 3 & 4) and a published report (Koch et al., 2009) that telomerase can be expressed at higher levels in neural progenitor/stem cells.

In contrast, the effect of constitutive hTERT expression on NPSCs was more evident. NPSCs overexpressing hTERT proliferated faster and their differentiation potential was

compromised. In the absence of growth factors, they failed to upregulate neuronal or glial markers and they continued to proliferate. This is consistent with other reports of sustained hTERT expression inhibiting the differentiation of adult stem cells (Cerezo et al., 2003; Richardson et al., 2007).

Since the expression levels of transgenic hTERT are not substantially higher than the endogenous hTERT level in our hTERT-ov hESCs, it is not possible to draw any firm conclusions about the role of telomerase in hESC differentiation. This is made further difficult by the fact that the hESCs studied were all individual clones and therefore subject to clonal variation, which is particular prevalent in hESC lines. A better approach to answering the question of the role of telomerase in differentiation would be to derive hESCs carrying an inducible hTERT expression vector containing hTERT cDNA under a strong promoter, for higher hTERT expression. In this way, ectopic hTERT expression could be easily switched on in hESCs and then their differentiation compared to the same cells but with hTERT expression not induced.

Chapter 6. General discussion

The ribonucleoprotein complex telomerase is required to maintain telomeres, the specialised structures that cap the chromosome ends, throughout successive rounds of DNA replication; as such it is fundamental to continued cell division. Telomerase and telomere biology is of central importance to the fields of both cancer and ageing and much research has been carried out in cancer cells. However, our understanding of telomerase and telomerase regulation is still incomplete and knowledge is particularly lacking with respect to telomerase regulation in normal cells.

6.1 Telomerase is dynamically expressed during neural differentiation of hESCs and culture of their neural progeny

This thesis focussed on telomerase expression and telomere dynamics in hESCs and their derived NPSCs. Similar to during embryonic development, hTERT expression and telomerase activity are dramatically downregulated during the differentiation of hESCs (Gerrard et al., 2005; Golebiewska et al., 2009; Hay et al., 2008) and work in our laboratory has previously shown that hESCs downregulate hTERT when differentiated down the neural lineage (Gerrard et al., 2005). hESC-derived NPSCs had been expanded and continuously cultured in order to study their neural stem cell properties. Since it was thought that these cells did not express telomerase, we had expected them to enter senescence at some point in their extended culture, as a result of telomere erosion. However, we observed that two NPSC populations (one derived from H1 and one from H7 hESC lines) were still proliferating vigorously after more than one year of continuous culture.

To explore this, I assessed the telomerase expression and telomere length of the long-term cultured NPSCs at different stages of culture. This showed that although telomerase expression was downregulated after hESC neural differentiation, the derived NPSCs maintained a very low level of telomerase, which is consistent with the low telomerase expression found in some stem cell compartment in *vivo* but different from the complete absence of telomerase in cultured fibroblasts and other somatic cells *in vivo*. This divergence between NPSCs and fibroblasts may reflect a difference between progenitor/stem cells and somatic cells.

Due to low telomerase expression, extensive telomere shortening occurred with successive cell divisions in early passage NPSCs. After continuous culture, telomerase was shown to become reactivated in the late passage NPSCs. Furthermore, telomerase reactivation started to appear around the period of time that telomeres had shortened to the critical lengths. Reactivated telomerase was functional and capable of gradually elongating the telomeres, accounting for the sustained proliferation of the NPSCs. Late passage telomerase-high NPSCs exhibited a similar morphology and marker expression as the early cells and maintained the ability to further differentiate into postmitotic neurons and glia. Moreover, they were growth-anchorage dependent, karyotypically normal, subject to contact inhibition and a normal DNA damage response, demonstrating that they were not transformed. Therefore, the data presented in this thesis represent the first report of normal, untransformed cells showing telomerase reactivation and telomere homeostasis.

6.2 CpG methylation in the hTERT promoter may play a role in regulating hTERT expression in NPSCs

In order to study the mechanisms underlying hTERT regulation, and specifically hTERT reactivation, in our cells, I examined transcriptional and epigenetic factors known to affect hTERT expression. Gene expression analysis of a panel of candidate transcriptional regulators of hTERT suggested that the ETS family member ETV1 is a likely transcriptional activator of hTERT in our cell system, since the mRNA levels of both were closely correlated in hESCs and NPSCs of different stages in culture. It will be interesting to examine ETV1 binding to the hTERT promoter in hESCs and telomerase-high NPSCs and to test the effect of ETV1 knockdown upon hTERT expression in these cells.

Analysis of DNA methylation at the core hTERT promoter revealed abundant CpG methylation at a small ~200 bp region that is 400 bp upstream of the transcriptional start site (-400 bp to -665 bp) in early telomerase-low NPSCs. This methylation was largely absent in hESCs and telomerase-high NPSCs thus CpG methylation is negatively correlated with hTERT expression in hESCs and their derived NPSCs. Furthermore, ChIP analysis demonstrated that the known hTERT transcriptional activator, Sp1, was bound to the proximal hTERT promoter region in hESCs and NPSCs and that the level of Sp1 binding was correlated with hTERT expression and blocked by CpG methylation. Together these data suggest that Sp1 may function to activate hTERT transcription in hESCs and particularly in NPSCs and that its binding to the hTERT promoter is blocked in early passage NPSCs by CpG methylation, resulting in downregulation of hTERT expression.

In order to confirm this it will be necessary: 1) to compare the expression of reporter genes that are under the control of the wild-type hTERT promoter and an hTERT promoter in which the Sp1 binding motif in the BS1 region is mutated, in late NPSCs; and 2) to induce

demethylation of the hTERT BS1 promoter region (e.g. by treatment with 5'-azacytidine) and assess the effect upon Sp1 binding and hTERT expression. However, the latter approach may also affect methylation in other genes that affect hTERT expression. In addition, it will also be important to explore the hypothesis that telomere shortening in NPSCs may result in the de-repression of hTERT transcription, by mechanisms involving either the telomere-position effect or genome-wide epigenetic changes (as recently demonstrated by Pucci et al.). A starting point for this could be to use NPSCs derived from hTERT-overexpressing hESCs. As discussed in Chapter 5, these hTERT-NPSCs constitutively express hTERT. If these cells were thereby able to maintain their telomeres during continuous culture, it might be expected that endogenous telomerase would not be reactivated as it is in wild-type hESC-derived NPSCs. If this were the case, it would be useful to conduct a comprehensive study of the telomeric and genome-wide epigenetic changes that occur in NPSCs during their extended culture.

6.3 Challenges in the field of telomerase and telomere biology

Telomerase expression in the adult organism is restricted to germ cells and certain somatic stem cells. However, due to the difficulty of mimicking the stem cell niche in a culture dish and the low expression level of telomerase in these cells, it is currently unclear if telomerase is expressed in all adult stem cell compartments. Furthermore, the lack of a robust technique for detecting telomerase expression *in situ* also makes these studies extremely difficult. Further research using modern techniques is necessary to determine if telomerase expression can be detected in more somatic stem cell types than have currently been reported, in particular in the central nervous system. Moreover, it will be important to explore the mechanisms and factors which repress telomerase in the majority of somatic cells and

particularly the mechanisms which result in telomerase repression during embryonic development. The data in this thesis demonstrate that CpG methylation of the hTERT promoter plays an important role in repressing hTERT during hESC differentiation therefore this should be studied during the process of embryonic development. More generally, it would be interesting to discover if CpG methylation at the hTERT promoter occurs widely *in vivo*. Also, more research is likely to be carried out regarding which factors activate hTERT transcription in different adult stem cell types. Finally, although some adult stem cells are currently known to express telomerase, the levels in those cells are too low to prevent telomere shortening. This raises the question of what role telomerase plays in adult stem cells *in vivo*.

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Appendix

Table A1: Antibodies used in this thesis

Antibody	Supplier	Cat. No.	Dilution
β-Actin	Sigma	A5441	1:5000
β-III-tubulin	Sigma	T8660	1:1000
BrdU	DSHB	G3G4	1:1000
c-myc	DSHB	9E 10	1:500
GFAP	DAKO	Z0334	1:500
H3Ac	Millipore	06-599	1:250
Nestin	Millipore	MAB 5326	1:200
p21	BD Biosciences	C24420	1:250
p53	Cell Signaling Technology	2527	1:1000
Sox2	Abcam	ab86818	1:500
Sp1	Millipore	07-645	1:500
TRF1	Millipore	04-638	1:1000
TRF2	Millipore	05-521	1:250
Normal rabbit IgG	Santa Cruz	sc-2027	1:250
Goat Anti-Mouse IgM Alexa Fluor 555	Invitrogen	A21426	1:400
Goat Anti-Mouse IgG Alexa Fluor 568	Invitrogen	A11004	1:400
Goat Anti-Rabbit IgG Alexa Fluor 488	Invitrogen	A-11008	1:400
Goat anti-mouse IgG-HRP	Santa Cruz	sc-2005	1:2000
Goat anti-mouse IgM-HRP	Santa Cruz	sc-2064	1:2000
Goat anti-rabbit IgG-HRP	Santa Cruz	sc-2030	1:2000

Table A2: RT-PCR primer sequences used in this thesis

Gene	Forward	Reverse
β-actin	TCACCACCACGGCCGAGCG	TCTCCTTCTGCATCCTGTCG
Endo hTERT	CATTCCTGCTCAAGCTGACTCGAC	CCTCAGACTCCCAGCGGTGCG
Nanog	AGCCTCTACTCTTCCTACCACC	TCCAAAGCAGCCTCCAAGTC
Oct4	GACAACAATGAAAATCTTCAGGAGA	TTCTGGCGCCGGTTACAGAACCA
Pax6	AACAGACACAGCCCTCACAAACA	CGGGAACTTGAACTGGAACTGAC
Rex1	TGAAAGCCCACATCCTAACG	CAAGCTATCCTCCTGCTTTGG
Sox1	CAATGCGGGGAGGAGAAGTC	CTCTGGACCAAACTGTGGCG
Sox2	CCCCCGGCGGCAATAGCA	TCGGCGCCGGGGAGATACAT
Total hTERT	GCCTGAGCTGTACTTTGTCAA	CGCAAACAGCTTGTTCTCCATGTC

Table A3: qRT-PCR primer sequences used in this thesis

Gene	Forward	Reverse
β-actin	TGTCTGGCGGCACCACCATG	AGGATGGAGCCGCCGATCCA
Bmi1	TGGACTGACAAATGCTGGAGA	GAAGATTGGTGGTTACCGCTG
c-myc	TCGGAAGGACTATCCTGCTG	GTGTGTTCGCCTCTTGACATT
DKC	AATCCTCTTCTCGACTGGGC	CCTTCAAGGCTGAACTCAAGA
E2F1	TGTTTCCAGTGCAGAACCAA	GCATCTGAGGTTTCCTCCAA
ETS2	GACTTTGTGGGTGACATTCTCTG	CGGAGGTGAGGTGTGAATTTT
ETV1	AGTTTGTACCAGACTATCAGGCT	CTCATTCCCACTTGTGGCTTC
GAPDH	TCTGCTCCTCCTGTTCGACA	AAAAGCAGCCCTGGTGACC
GFAP	CACCACGATGTTCCTCTTGA	GTGCAGACCTTCTCCAACCT
hTERT	TCAACCGCGGCTTCAAG	TCCAGAAACAGGCTGTGACACT
hTR	TCTAACCCTAACTGAGAAGGGCGTAG	GTTTGCTCTAGAATGAACGGTGGA
Jun	TGCCTCCAAGTGCCGAAAAA	TGACTTTCTGTTTAAGCTGTGCC
MAD1	TACGTGTTGTTGCCATCCAG	AACTTGCATTCAGGGGTAAAAA
MEN1	CAGCACCAAAGGCTCAGCTT	CCTTGCTTCTAACCCATCATATCC
Nanog	TGATTTGTGGGCCTGAAGAAAA	GAGGCATCTCAGCAGAAGACA
Oct4	TCGAGAACCGAGTGAGAGGC	CACACTCGGACCACATCCTTC
Pax6	TCCGTTGGAACTGATGGAGT	GTTGGTATCCGGGGACTTC
PCNA	TGTCCTTACTCAAGTTCTCAGAAGA	GAGGCCACATAACTGCACTG
SIP1	GTCAGTTCATCTGAGCGGG	AGAGTTCCCATCTCAAGGCA
Sox1	AACACTTGAAGCCCAGATGGA	GCAGGCTGAATTCGGTTCTC
Sp1	GCCGCTCCCAACTTACAGAA	CCCATCAACGGTCTGGAACT
AP2-A	CAGCAAATGTCACGTTACTCACC	GCCGGTTCAAATACTCAGAGAC
AP2-B	CTGTTGCTGCACGATCAGACA	CAGGGACTGAGCAGAAGACCT
TRF1	AATTTGTTGAACCCTGCCAC	ACAGGGTTGAGGTCAGCCTA
TRF2	CCGTTCTCAACCAACCCCTC	GCTGCCTGAACTTGAAACAGT
WT1	CTATACAGCCGACAAGGCTACC	ACCCACAGGGATGAGGTTAAA

Table A4: Other primer sequences used in this thesis

Primer	Forward	Reverse	Application
hTR	CATGTGAGGCTTGCTGGGTC		RT
β-actin	TCCTCGGGTCTACAAGCCATGCTT		RT
XpYp	GAGTGAAAGAACGAAGCTTCC	CCCTCTGAAAGTGGACCTAT	ChIP
hTERT BS1	TCACGTCCGGCATTCGTGGT	TCGAATCGGCCTAGGCTGTG	ChIP
hTERT BS3	AGCCCCTCCCCTTCCTTTCC	AGCGCACGGCTCGGCAGC	ChIP
XpYp	TTTTTATTGTGAGGTATTGATGTAAATATT	AAAAATATAACTACTCCCTTTACCAC	Bisulphite
hTERT BS1	GGGTTTGTGTTAAGGAGTTTAAGT	CATAATATAAAAACCCTAAAAACAAAT	Bisulphite
hTERT BS2	TTGTTTTTAGGGTTTTTATATTATGGT	CAAAACTAAAAAATAAAAAAACAAAAC	Bisulphite
hTERT BS3	TAGTTTTGTTTTTTTATTTTTTAGTTT	CCAACCCTAAAACCCCAAAC	Bisulphite
hTERT BS1 MSP	TCGCGGGGAAGTGTTGTAGGGA	ACGCGAAACGTCGAACTCCGA	MSP
GAPDH uMSP	AAAAAGTGGGGAGAAAGTAGGG	CCCACCAAACTCAACCAATC	MSP

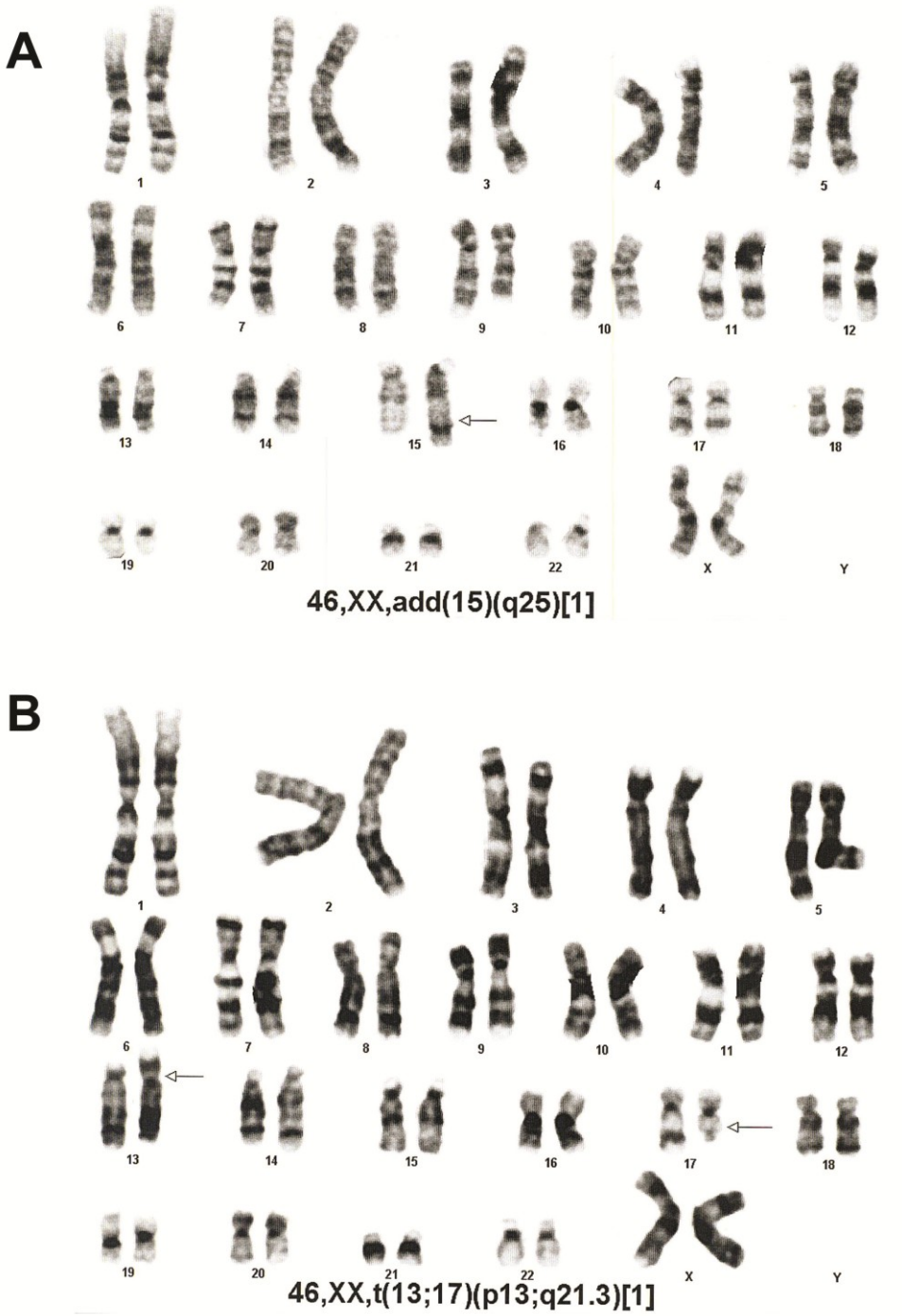


Figure A1: Abnormal karyotypes observed in late passage NPSCs

Late passage (pxx) NPSCs displayed 2/20 abnormal metaphase spreads. One spread had a 46,XX,add(15)(q25) karyotype **(A)** and one spread had a 46,XX,t(13;17)(p13;q21.3) karyotype **(B)**. Arrows indicate chromosomal anomalies.