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# Alternative splicing and extra-telomeric role of telomerase reverse transcriptase isoforms in human embryonic stem cells

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# Abstract

Human telomerase, a ribonucleoprotein complex consisting of a catalytic subunit (hTERT) and a RNA template component (TERC), prevents telomere attrition in human embryonic stem cells (hESCs) providing these cells with prolonged replicative capacity. Interestingly, hTERT has several alternatively spliced isoforms, most of which are catalytically inactive. One alternative splicing event involving a complete loss of exons 7 and 8 results in a catalytically inactive isoform called β-hTERT. The β-hTERT is highly expressed in certain cancer types and hESCs. Full-length hTERT (FL-hTERT) is known to translocate from nuclei to mitochondria when cells are exposed to either chronic (oxygen tension) or acute (hydrogen peroxide) oxidative stress. In this study, I examined the alternative splicing of endogenous hTERT transcript in hESC, intra-cellular localization of FL-hTERT and β-hTERT in hESCs, and the effect of overexpressing recombinant hTERT on hESC mitochondria. Human ESCs (HES2 line) were cultured under 2% oxygen and 3xFLAG tagged FL-hTERT and β-hTERT were induced by doxycycline prior to 100μM $H_2O_2$ treatment. My results demonstrate that overexpression of transgenic FL-hTERT resulted in a significant increase in endogenous β-hTERT transcript level. Additionally, doxycycline and $H_2O_2$ altered the splicing pattern of hTERT in favor of β-hTERT. I demonstrate that not only the full-length- but also β-hTERT can bind TERC, and that β-hTERT overexpression inhibits telomerase activity *in vitro*. However, I failed to observe the mitochondrial localization of recombinant FL-hTERT or β-hTERT. Lastly, my results demonstrate the pro-apoptotic role of β-hTERT and the pro-survival effect of FL-hTERT under $H_2O_2$-induced oxidative stress.



**Key Words**: Telomerase catalytic subunit, alternative splicing, oxidative stress, doxycycline

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# List of Abbreviations

| | |
|---|---|
| **ALT** | Alternative lengthening of telomere |
| **APC** | Adenomatosis polyposis coli |
| **BIO** | 6-bromoindirubin-3-oxime |
| **BRG1** | Brahma-related gene 1 |
| **BSA** | Bovine serum albumin |
| **Abl** | Abelson murine leukemia viral oncogene homolog 1 |
| **CHIP** | C terminus of HSC70-Interacting Protein |
| **CRM1** | Chromosomal maintenance 1 |
| **Ct** | Cycle threshold |
| **DHS** | DNAse I hypersensitive site |
| **DMEM** | Dulbecco's modified eagle medium |
| **DMSO** | Dimethylsulfoxide |
| **DNA** | Deoxyribonucleic acid |
| **DSB** | Double-stranded break |
| **ESC** | Embryonic stem cell |
| **ESE** | Exonic splicing enhancer |
| **ESS** | Exonic splicing silencer |
| **ETC** | Electron transport chain |
| **FBS** | Fetal bovine serum |
| **FL** | Full length |
| **GSK3β** | Glycogen synthase kinase 3 beta |
| **hESC** | Human embryonic stem cell |

| | |
|---|---|
| **HIF1A** | Hypoxia-inducible factor 1 alpha |
| **HIF2A** | Hypoxia-inducible factor 2 alpha |
| **HK2** | Hexokinase 2 |
| **hnRNP** | Heterogeneous ribonucleoprotein |
| **hPSC** | Human pluripotent stem cell |
| **HSP90** | Heat shock protein 90 |
| **iPSC** | Induced pluripotent stem cell |
| **ISE** | Intronic splicing enhancer |
| **ISS** | Intronic splicing siliencer |
| **LRP** | Lipoprotein receptor-related protein |
| **MEF** | Mouse embryonic fibroblast |
| **MFN1/2** | Mitofusin 1/2 |
| **MKRN1** | Makorin ring finger protein 1 |
| **MMP** | Mitochondria membrane potential |
| **mtDNA** | Mitochondrial DNA |
| **mTHF** | L-methyl-tetrahydrofolate |
| **mTOR** | Mechanistic target of rapamycin |
| **NLS** | Nuclear localization signal |
| **NMD** | Nonsense mediated decay |
| **NP-40** | Nonidet-P40 |
| **NS** | Nucleostemin |
| **ORF** | Open reading frame |
| **OXPHOS** | Oxidative phosphorylation |
| **PBS** | Phosphate buffered saline |

| | |
|---|---|
| **PCR** | Polymerase chain reaction |
| **PDH** | Pyruvate dehydrogenase |
| **PFK** | Phophofructokinase |
| **PI3K** | Phosphatidylinositol-4,5-bisphosphate 3-kinase |
| **PKC** | Protein kinase C |
| **Plk1** | Polo-like kinase 1 |
| **PP2A** | Protein phosphatase 2A |
| **PSC** | Pluripotent stem cell |
| **PTB** | Polypyrimidine tract binding protein |
| **PTDC** | Pyrrolidinethiocarbamate |
| **RdRP** | RNA-dependant RNA Polymerase |
| **RISC** | RNA-induced silencing complex |
| **RMRP** | RNA component of the mitochondrial RNA-processing endoribonuclease |
| **RNA** | Ribonucleic acid |
| **RNP** | Ribonucleoprotein |
| **ROS** | Reactive oxygen species |
| **RT** | Reverse transcriptase |
| **S1P** | Sphingosine-1-phosphate |
| **siRNA** | Small-interfering RNAs |
| **snRNP** | Small nucleolar ribonucleoprotein |
| **SSEA** | Stage-specific embryonic antigen |
| **TBE** | Tris/Borate/EDTA |
| **TCA** | Tricarboxylic acid |
| **TCF/LEF** | T-cell factor/lymphoid enhancer factor |

| | |
|---|---|
| **TDH** | Threonine dehydrogenase |
| **TEN** | Telomerase essential N-terminal |
| **TERC** | Telomerase RNA component |
| **TERT** | Telomerase reverse transcriptase |
| **THF** | Tetrahydrofolate |
| **TNF** | Tumor necrosis factor |
| **UCP2** | Uncoupling protein 2 |

# Chapter 1: Introduction and Literature Review

## *1.1 Human Embryonic Stem Cells*

In 1998, Jamie Thomson isolated human embryonic stem cells (hESCs) from the inner cell mass of a day 5-6 blastocyst [1]. Two defining characteristics of hESCs that define their therapeutic potential are their indefinite self-renewal capability and their ability to differentiate into cell types that comprise all three germ layers (endoderm, mesoderm, ectoderm) of a developing embryo including the germ cell lineage. Morphologically, hESCs grow in tight, spherical colonies when cultured on plastic dishes. Their cell size is small with very large nuclei compared to a small cytoplasmic space. hESCs are positive for various pluripotency markers such as alkaline phosphatase activity, pluripotency transcription factors (such as OCT4, NANOG, SOX2), glycolipids (stage specific embryonic antigens SSEA-3 and SSEA-4), and keratan sulfate antigens (Tra-1-60 and Tra-1-81) [2]. For their pluripotent nature and its stark resemblance to other immortal cell types, hESCs stand in the centre of research for human development, aging, cancer, and regenerative medicine. To address the ethical and technical difficulties associated with the derivation of hESCs, it was demonstrated that pluripotency could be induced directly from differentiated somatic cells of mice [3] and humans [4,5]. Despite many studies confirming the differentiation competency of these so called induced-pluripotent stem cells (iPSCs), iPSCs vary significantly in their self-renewal capacity due, in part, to their heterogeneity in telomerase activity levels and/or telomere lengths [6,7]. Human ESCs, like other PSCs, ubiquitously express high levels of telomerase activity [8]. In support of this, hESCs have a unusually long S phase of the cell cycle where telomere elongation by telomerase takes place [9] and have distinctive cell properties such as heavy reliance on glycolytic metabolism [10-13], and elevated DNA-repair/defenses [14-16].

### *1.1.1 Signaling pathways in hESCs*

Typical PSC media for feeder-free growth conditions includes factors such as insulin-like growth factor (IGF) a strong stimulant for the canonical phosphatidylinositol-3 kinase (PI3K) signaling pathway [17,18]. PI3K/AKT signaling is crucial for maintenance of hESC pluripotency [19, 20]. PI3K/AKT is a signaling pathway best known for its regulatory role in cell cycle regulation. Its function is especially important in actively proliferating cell types such as cancer cells and hESCs. A microarray experiment by Armstrong *et al.* revealed a highly active PI3K/AKT signal-transduction in undifferentiated ESCs [18]. Inhibition of PI3K/AKT in hESCs resulted in a significant loss of core pluripotency marker (OCT4, NANOG, SOX2) expression, confirming the requirement of PI3K signaling in the maintenance of hESC pluripotency. When activated, AKT1 initiates a signaling cascade by activating mTOR and by inhibiting GSK3[19]. Inhibition of PI3K/AKT signaling by small molecules such as LY294002 or by removal of PI3K activators leads to an eventual loss of pluripotency in hPSCs [19, 20, 23]. The mechanisms of how PI3K controls the pluripotent state in hPSCs are unclear. It is speculated that PI3K/AKT regulates protein synthesis through mTOR, thereby regulating the protein synthesis of core factors of the pluripotency network [21]. Inhibition of mTOR by rapamycin results in similar effects to that of AKT inhibition, triggering spontaneous hPSC differentiation [19, 24]. GSK3 inhibition by AKT signaling may also contribute to PSC maintenance through Wnt/β-catenin-mediated expression of c-myc, a key regulator of PSC maintenance [25, 26]. This mechanism could also apply in hPSCs, but has not been evaluated fully.

The role of canonical Wnt/β-catenin pathway in human ESCs is a controversial one. At first, the activation of Wnt/β-catenin pathway via either Wnt3A (ligand of Wnt-receptor) or GSK3 inhibitor 6-bromoindirubin-3′-oxime (BIO) was shown to have a significant role in

the self-renewal of hESCs (H1 and H9 cell line) under feeder-free conditions [27-29]. In presence of BIO, Wnt-signaling sustained the expression of Oct-3/4, Rex1, and Nanog. The effect was reversed when BIO was withdrawn from the culture, resulting in spontaneous multi-differentiation of hESCs. However, the consequences of blocking GSK3 are not limited to Wnt/β-catenin pathway, and can affect many other pathways through mTOR stabilization/cross-talk between signaling pathways. For instance, inhibition of GSK3 via BIO results in increased phosphorylated Smad2/3 in hESCs, a downstream Nodal signaling pathway characteristic of the undifferentiated state of hESCs [26]. In support of this view, contradicting results emerged afterwards, demonstrating that Wnt/β-catenin activation by Wnt3A/BIO does not support the self-renewal of hPSCs in long term cultures [30-32]. Moreover, their functional reporter assay showed that β-catenin-mediated transcription activity was nearly absent in undifferentiated hESCs (H1 and H9 cell lines). Further studies with a chimeric (estrogen-receptor tagged) β-catenin model showed that 4-hydroxytamoxifen-induced activation of Wnt/β-catenin signaling resulted in the differentiation of hESCs into primitive streak and mesoderm progenitors [33, 34]. Inhibition of Wnt/β-catenin signaling by blocking the secretion of Wnt [31] or by stabilizing β-catenin destruction complex [28] augments the self-renewal and long term propagation of human ESCs. It was additionally shown that Wnt-signaling promotes cell proliferation of hESC with little effect on apoptosis. This agrees well with the known gene targets upregulated by Wnt-signaling pathway such as cyclin D1 [32, 33], and c-Myc [34]. In short, while Wnt-pathway's pro-proliferative effect may be important in self-renewal of hESC, its importance in the maintenance of pluripotent state is unclear.

*1.1.2 The role of oxygen tension in embryonic stem cells.*

The natural niche of early embryonic cells is enriched with essential growth factors and the proper microenvironment for the maintenance of self-renewal and pluripotency, and this often includes low oxygen tensions [36, 37]. Early embryonic cells grow under low oxygen availability starting from fertilization and continuing through implantation and fetal development. During implantation, a hypoxic environment is created due to the absence of oxygen provided from maternal circulation [37]. The uterine surface typically has oxygen concentrations of 2% in early pregnancy. The oxygen tension rises to approximately 8% once the maternal vasculature is established [38]. Oxygen acts as the final electron carrier in the ETC of OXPHOS-dependent energy metabolism in mammalian cells [39]. During early embryo development, cells adapt to the low oxygen environment by switching between OXPHOS-dependent and glycolysis-dependent energy production [40].

hESCs derived from the early embryo grow relatively well under ambient oxygen tension (~21%) without apparent negative consequences. However, closer examination revealed the effect of oxygen tension on altered hESC morphology [42-44], pluripotency and differentiation marker expression [44, 45], cloning efficiency [43], chromosomal stability[44] and differentiation capacity [42, 43]. In the majority of the studies, low oxygen tension (2–5%) improved human ES cell pluripotency and maintenance [44– 47]. In addition, low oxygen condition (5%) was shown to improve the efficiency of iPS generation from human dermal fibroblast cells [48], further supporting the notion that stemness of hESC is augmented by hypoxia. Notably, conflicting results do exist to suggest that oxygen tension has minimal to no effect on the stemness of hESCs [43, 48].

In murine ESCs, hypoxia-inducible-factor1 alpha (HIF1A) plays a central role in regulating the cellular response to oxygen availability, mediating the expression of many oxygen-related genes [50]. While HIF1A controls a broader spectrum of oxygen-dependent genes, HIF2A is a direct upstream regulator of POU5F1 (OCT4) in mouse ES cells [51]. In hESCs, HIF1A is only transiently expressed for approximately 48 hours following hypoxic exposure [52], suggesting a long-term hypoxic regulation by a factor other than HIF1A. Indeed, HIF2A was described to be responsible for the regulation of energy metabolism and self-renewal in hESCs grown under 5% oxygen tensions [53].

*1.1.3 Energy Metabolism of Pluripotent Stem Cells*

Prior to vasculature formation, early-stage embryonic cells grow in a hypoxic environment (1-5%) [38, 53]. PSCs such as hESCs and mouse epiblast-derived stem cells (mEpiSC) exhibit low-level cellular respiration and down-regulation of electron transport chain (ETC) activity, while enzymes involved in glycolytic pathways are up-regulated [11]. This reliance on glycolytic metabolism is partly explained by the hypoxic micro-environmental condition of pre-implantation embryos [55]. Consequently, ATP synthesis in PSCs is primarily produced via glycolysis and pyruvate is converted into lactate instead of being fed into the Krebs cycle [13, 55]. hESCs' reliance on glycolysis is achieved by up-regulation of Glut1/3 [57], hexokinase2 (HK2) [55], phosphofructokinase (PFK) [58], and inactivation of mitochondrial ETC enzymes such as pyruvate dehydrogenase (PDH) [55]. Moreover, hESCs up-regulate UCP2 uncoupler protein, which in turn suppresses oxidative phosphorylation (OXPHOS) by actively shunting out C4 metabolites from mitochondria matrices [59]. In agreement with the suppressed mitochondrial glucose oxidation, mitochondria of hESC are structurally and functionally immature with underdeveloped cristae [55]. Conversely, differentiation of hESC is accompanied by rapid maturation of

mitochondria and increased metabolism via OXPHOS [60]. Glycolytic energy metabolism of hESCs serves to protect the cells from DNA damage by minimizing reactive oxygen species (ROS) generated through OXPHOS [55]. Although oxygen consumption is decoupled from ATP generation in ESCs, some level of oxygen consumption appears to be important, possibly to maintain functional tricarboxylic acid (TCA) cycle for lipid and protein synthesis by NADH oxidation to $NAD^+$. Interestingly, PSCs do not experience a metabolic shift from glycolysis to OXPHOS in presence of sufficient oxygen, which closely resembles Warburg metabolism in cancer cells [55]. In the context of cancer cells, the aerobic glycolysis provides an anabolic advantage to support rapid cell growth. It is speculated that glycolysis endows a similar advantage in PSCs, whose key defining characteristic is high proliferative capacity. For instance, mouse ESCs show increased activity in pentose phosphate pathway and use it to generate carbon intermediates, which allows rapid nucleic acid synthesis [61]. Moreover, 3-phosphoglycerate can be converted into 3-phosphoserine and eventually into serine/glycine to be used for nucleotide biosynthesis and mono-carbon metabolism. One of the defining hallmarks of the Warburg phenomenon is rapid glutamine consumption that is used as an alternative carbon source for TCA cycle. Glutamine is the most abundant free amino acid in human plasma [62]. Glutamine is first converted into glutamate and subsequently to a key carbon component of TCA cycle alpha-ketoglutarate. The carbon intermediates generated through TCA minimally contribute to OXPHOS-dependent ATP synthesis. Instead, they are used for anabolic processes such as lipid (from citric acid pathway) and amino acid synthesis (alpha-ketoglutarate, oxaloacetate). In addition to methionine, threonine is another amino acid critical for mESC metabolism and proliferation. Threonine contributes to the pool of acetyl-CoA and glycine through L-threonine dehydrogenase (TDH) in mitochondria. Glycine then contributes directly to nucleotide biosynthesis or indirectly to S-adenosyl-methionine

(SAM) production by modulating tetrahydrofolate's (THF) conversion to L-methyl-tetrahydrofolate (mTHF). Threonine deprivation in mESCs leads to the reduction in the trimethylation of histone 3 lysine 4, increasing the propensity to differentiate [63].

The importance of mitochondria is also seen in reprogramming of somatic cells to induced pluripotent stem cells (iPSCs). During conversion of somatic cells to a pluripotent state, depleting mitofusin proteins (MFN1/2) promotes the reprogramming efficiency by increased mitochondria fission [64]. Similarly, the reprogramming efficiency of somatic cells into iPSCs is enhanced three- to four-fold when glycolysis is promoted, and is reduced when glycolysis is inhibited [48]. Several pieces of evidence suggest that mitochondrial maturation and subsequent rise in intra-cellular level of reactive oxygen species (ROS) are required signaling elements in regulating both stem cell function and differentiation potential [54, 63–65]. Conversely, use of potent antioxidant vitamin C improves somatic cell reprograming efficiency by reducing intra-cellular ROS and by modifying the epigenetic landscape [68]. The significance of mitochondrial function and nuclear-mitochondrial cross-talk in stem cell biology remains yet poorly understood. The functional significance of mitochondria-dependent metabolites in the self-renewal, pluripotency, and induction of PSCs calls for a closer investigation.

### *1.1.4 Telomerase and Telomere in Human Embryonic Stem Cells*

Telomeres are linear DNA structures at the end of chromosomes. They are non-coding DNA buffer regions that shorten with each DNA replication event due to the "end-replication problem" of eukaryotic DNA polymerase. For actively proliferating cells such as hESCs, maintaining the telomere homeostasis is critical for the function and longevity of the cells. A few mechanisms exist to maintain telomere lengths. In mice, both the telomerase and

alternative lengthening of telomere (ALT) pathway are utilized to maintain telomere lengths. In human cells, ALT is utilized to a lesser extent and telomerase activity is the primary means for maintaining telomere homeostasis. A ribonucleoprotein called telomerase is highly upregulated in hESCs for this reason, and the stable telomerase activity and long telomere lengths are characteristic of hESCs. Telomere lengthening via telomerase is activated as early as the blastocyst stage of embryo development in mice and high telomerase expression becomes absent in most adult somatic cells with exception of the germ line [69] and activated lymphocytes [65-69]. Of note, several stem cell types, their proliferative capacity notwithstanding, exhibit low to moderate telomerase activity and experience telomeric shortening. To date, most telomerase/telomere studies have been conducted with mouse embryonic stem cells (mESCs). In mESCs, the loss of telomerase activity results in telomere shortening, genomic instability, and reduced growth rates. Moreover, PSCs with long telomere lengths exhibit higher chimera success rate in tetraploid complementation assays than those with short telomeres [72]. These results suggests that telomere maintenance by telomerase is crucial for the proper functioning of pluripotent stem cells.

## *1.2 Human Telomerase Catalytic Subunit hTERT*

### *1.2.1 Canonical Function of hTERT*

The telomerase ribonucleoprotein (RNP) complex was first discovered in *Tetrahymena* by Elizabeth Blackburn and Carol W. Greider [73]. The human homologue of telomerase RNP was soon characterized [74], and was found to consist of catalytic protein subunit hTERT (127kDa) and RNA component called hTERC [75]. The active form of the telomerase complex requires dimerization of these components, and lack of either of these

components or the failure to dimerize result in loss of telomerase activity [76]. hTERC is a non-coding RNA that acts as a template for telomere sequence elongation. Using hTERC, hTERT adds multiple hexameric DNA repeats (TTAGGG) to the telomere ends. This canonical function of telomerase is strictly regulated at multiple levels. Transcriptionally, the pre-messenger RNA of hTERT gets spliced to generate various alternative transcripts (23 different alternatively spliced transcripts to date) with disrupted catalytic domain, ultimately resulting in a decreased pool of mRNA for catalytically active hTERT [75, 76]. Several studies have observed that the splicing pattern changes in a cell type-, and developmental stage-dependent manner, suggesting that alternative splicing is likely a tightly controlled regulatory mechanism for telomerase activity [77– 80]. However, the means or the effect of alternative splicing of hTERT on telomerase activity remains largely unknown (*see* section 1.2.2). Telomerase activity is also regulated post-translationally through phosphorylation and ubiquitination (*see* section 1.2.4). hTERT has a relatively short half-life of approximately 24 hours due to its fast-turnover rate through ubiquitination [83]. Interestingly, the RNA component hTERC is rarely the limiting factor for telomerase activity [73, 82]. Unlike hTERT, hTERC is ubiquitously expressed in most cell types, which appears to be independent of TERT levels [75]. However, in a pathological setting such as in the premature aging syndrome dyskeratosis congenita where TERC could be mutated or lacking, telomerase activity is inhibited [83, 84].

### *1.2.2 Alternative splicing of hTERT*

Ninety five percent of protein coding genes are affected by alternative splicing in multicellular eukaryotes, generating >100,000 proteins from 20,000 protein-coding

sequences [87]. The canonical mode of RNA splicing entails joining of exonic sequences via the removal of the noncoding intron sequences. Splicing events occur on the nascent pre-mRNA, simultaneously with transcription [86, 87]. The event is orchestrated by a large and dynamic ribonucleoprotein called the spliceosome. A spliceosome is composed of snRNP core components (U1, U2, U4, U5, and U6) and other accessory proteins (~300 proteins) [90]. An exon may be constitutively included in the final transcript (constitutive splicing) or be conditionally included (alternative splicing), leading to alternative transcripts. Various local *cis*-regulatory sequences such as exonic splicing enhancers (ESEs), exonic splicing silencers (ESSs), intronic splicing enhancers (ISEs), and intronic splicing silencers (ISSs) regulate alternative splicing with the help of *trans*-acting elements such as snRNPs (activators), hnRNPs (repressors), and polypyrimidine tract binding protein (PTB)[89-91]. A clear understanding of splicing mechanisms is hampered by the vast number of splicing factors (>500), and by involvement of other cellular processes that affect spliceosome assembly. The selection of *cis*-elements is influenced by the molecular kinetics of transcription, 5′-end capping, and 3′-end polyadenylation [92, 93]. The complexity of alternative splicing is compounded by the competition of various transcripts for the splicing machinery [95].

The human TERT (hTERT) gene contains 15 introns and 16 exons and its transcript is alternatively spliced to produce various insertion and deletion isoforms [96]. Multiple alternative splicing events can occur on a single transcript, greatly expanding the pool of possible hTERT transcript variants. To date, 22 hTERT transcript variants have been identified [97]. However, translational competency of the identified alternatively spliced transcripts remains largely unknown. Amongst these, only the full-length hTERT (***hereafter***

***referred to as FL-hTERT***) constitutively spliced transcript (transcript with sixteen uninterrupted exons) results in a TERT protein capable of reverse transcriptase activity [97, 98].

An hTERT isoform called the β-deletion hTERT (***hereafter referred to as β-hTERT***) results from a 182-nucleotide out-of-frame deletion and is amongst the best characterized. The deletion skips exons 7 and 8, and joins exon 6 to exon 9 (*see* Fig. 1.1).The frame shift introduces a premature termination codon (PTC) and results in truncation of the hTERT protein [99, 100]. Splicing of β-hTERT is enhanced by serine/arginine-rich splicing factor 11 (SRSF11), and repressed by hnRNPH2 and hnRNPL [102]. Another study conducted by Wong *et al.* identified three highly conserved (amongst primates) direct repeat sequences in introns 6 and 8 [103]. The repeats in intron 6 (called *block 6* and *direct repeat 6* by the author) promoted β-deletion while the repeat in intron 8 (called *direct repeat 8*) repressed it. Importantly, block 6 is necessary for β-deletion splicing. Despite the PTC, it was reported that β-deletion transcripts escape non-sense mediated decay (NMD) and are translated into proteins [102]. There is currently no report on β-hTERT's propensity to degrade through protein degradation pathway. The β-hTERT contains an incomplete reverse-transcriptase domain and therefore was not considered a direct contributor to telomeric lengthening. However, Listerman *et al.* reported that β-hTERT acts as dominant-negative regulator of telomerase activity by sequestering hTERC [102]. Interestingly, β-hTERT transcript persists during human fetal development even when FL-hTERT splicing diminishes in various tissues in a development-stage-specific manner during fetal gestation [104]. Accumulating evidence indicates that either an increase or decrease in FL-hTERT transcript levels (and subsequent telomerase activity) may come at the expense of β-hTERT transcript abundance, further increasing the likelihood of regulatory functions by β-hTERT [100, 104-106].

The α-deletion hTERT (hereby noted as α-hTERT) results from a partial in-frame deletion (36bp) of exon 6, leading to the partial loss of RT-motif A. This catalytically inactive form of hTERT is a dominant negative inhibitor of telomerase activity when overexpressed in telomerase positive immortal cells [98, 107]. Considering the relatively low transcript abundance of the α-hTERT [108, 110], the level of telomerase activity inhibition by α-hTERT may be insufficient for it to have significant impact on telomere maintenance of indefinitely dividing cells. Following the discovery of the α-hTERT, Hisatomi *et al.* discovered another catalytically inactive variant (γ-hTERT) with two missing RT-motifs [110]. Like the α-hTERT, the γ-hTERT was suggested as a negative regulator of telomerase activity. However, γ-hTERT transcript levels, relative to those of α-hTERT and β-hTERT, are extremely low or nearly undetectable in all tested cell types [111-112]. Deletions at α and β sites can occur simultaneously on a single transcript generating truncated and catalytically inactive α-β-hTERT [112]. The function, splicing mechanisms, and protein stability of this isoform yet remain to be elucidated.

Telomerase activity requires dimerization of two catalytically active forms of hTERT [76]. Therefore, all translated hTERT splice variants that contain an oligomerization domain can potentially suppress telomerase and/or extra-telomeric functions of FL-hTERT by forming heterodimeric complex with it (Figure 1.1)

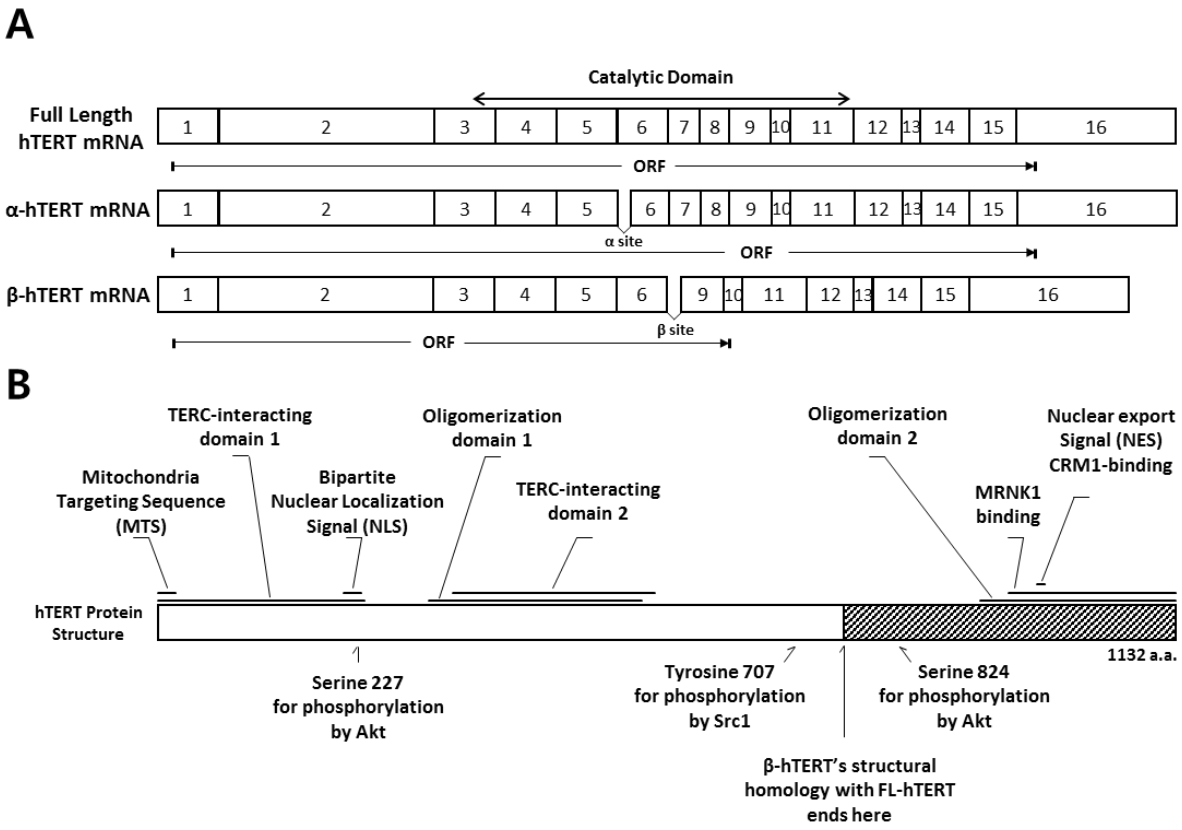



**Figure 1.1: mRNA and protein structure of the major hTERT isoforms.** A) Transcript structures of FL-hTERT, α-hTERT, and β-hTERT post-splicing. The deletion site and the open reading frame (ORF) of each isoform are indicated. B) Protein structure of hTERT. Regulatory domains/residues on the protein are annotated.

### *1.2.3 Transcriptional Regulation of hTERT*

An intricate regulation of hTERT expression is orchestrated by several transcription factors and DNA/histone modifying complexes at the promoter region. HSP90 (whose involvement is important for telomerase DNA binding and association with hTERC [113], [123, 124] was found to interact with the hTERT promoter in immortalized and cancer cells, regulating hTERT transcription [116]. Inhibition of HSP90 causes hTERT transcription and telomerase activity to decrease, which occurs concurrently with the diminished interaction of HSP90 with the hTERT promoter [116]. It is postulated that HSP90 may associate with GC-box-binding transcription factors such as Sp1 [116]. GC-boxes are important regulatory regions of the hTERT promoter that lacks a TATA-box [126, 127]. The hTERT promoter contains a minimum of five GC-boxes, and mutations in these regions severely impair the transcriptional activity at the site [116]. Notably, Sp1's interaction with the hTERT promoter is not disrupted by inhibition of HSP90, but there has been a report on their cooperative coordination of other GC-box-containing gene expression [119], suggesting an auxiliary function of HSP90 in Sp1-driven hTERT transcription. The significance of Sp1 in hTERT regulation is a controversial one. As demonstrated by Cheng *et al.*, both Sp1 and its related transcription factor Sp3 are required at the hTERT promoter for its transcription [120]. This agrees with previous reports that Sp1-family proteins generally activate hTERT transcription. Conversely, the repressive role of Sp1/3 has also been reported in normal human somatic cells [121]. Notably, the epigenetic state of the hTERT promoter appears to have little effect on Sp1 binding. Conversely, binding of Sp1/3 does not affect H3K9 (histone 3 lysine 9) acetylation or trimethylation status. It is postulated that hTERT transcriptional activation/repression by Sp1/3 may be dependent on other cell-type/-state specific factors, and that the recruitment of the complex to the promoter is relatively insensitive to the

epigenetic state of the target site [121]. Nonetheless, the heterochromatin-like state of the hTERT gene must become more open landscape for transcription factors' access to the GC-boxes and the eventual promoter activity in telomerase positive cells [120].

E-box transcription factors, Myc family proteins, and USF1/2 are believed to be the key de-repressors of the hTERT promoter via the recruitment of histone-modifying complexes [120]. The E-box transcription factors were found to be clustered around the hTERT promoter, possibly to promote an open conformation of the site. However, such occupation by E-box transcription factors at the hTERT promoter is seen even in telomerase-negative cells. This implies an involvement of additional factors to initiate the remodeling of heterochromatins [120]. In the same study, it was demonstrated that the rate of hTERT transcription was directly proportional to the establishment of a nucleosome-free region (also known as a DNAse I hypersensitive site (DHS; euchromatic) in the hTERT promoter of telomerase positive cells [120]. Chromatin remodeling events are not unique for GC-boxes as they can also take place at estrogen response elements (EREs) in the hTERT promoter of estrogen-receptor-α-positive cells [122]. In addition, retinoic acid hormone receptor family proteins can repress hTERT transcription by reducing H3K9 acetylation (change to heterochromatic state) and by promoting methylation at the promoter region (repressive marker) [132, 133]. Such hormonal control of hTERT transcription has significant impact on the up-regulation of hTERT by oncogenic transcription factors such as c-Myc and HIF1A. [134, 135], both of which are direct positive-regulators of hTERT transcription [136, 137]. While the transcriptional control by various transcription factors and chromatin remodeling proteins is an important step in hTERT regulation, mounting evidence indicates that post-translational modification mechanisms play critical roles in determining the end-function of the hTERT protein (discussed in the following section).

### *1.2.4 Post-translational activation/repression of hTERT*

hTERT is primarily regulated at the transcriptional level [113, 114], but regulation by post-translational mechanisms, such as phosphorylation and ubiquitination, has been reported and thought to be important [113, 115]. New discoveries regarding the post-translational regulation of hTERT are continuously emerging.

Two known mechanisms of post-translational regulation of hTERT are by phosphorylation and ubiquitination (Figure 1.2). The best characterized degradation process of hTERT occurs through ubiquitination by Makorin Ring Finger Protein 1 (MKRN1, interacts with hTERT C-terminal residues 946–1132)[129], C terminus of HSC70-Interacting Protein (CHIP)[130], and HDM2 (E3 ligases)[131]. Ubiquitinated hTERT is subsequently targeted for destruction by proteasomes in the cytoplasm. The exact mechanisms of ubiquitination remain to be elucidated. The fact that the ubiquitination and degradation processes of hTERT take place in the cytoplasmic space makes hTERT's sub-cellular localization a critical factor in hTERT regulation. In line with this, one dominant negative form of hTERT was proposed to promote the degradation of FL-hTERT by exporting itself out of nuclei with the FL-hTERT (as heterodimeric complex), where hTERT becomes available for ubiquitination and for the subsequent proteolysis [132] in MCF-7 cells, ultimately reducing telomerase activity [133]. Sphingosine-1-phosphate (S1P) is another bioactive lipid mediator of hTERT degradation [134]. Allosteric binding of S1P to hTERT mimics a phosphorylated residue at aspartate 684, which in turn prevents the interaction of MKRN1 with hTERT (therefore stabilizes hTERT against proteolysis). It was also shown by the same group that a similar phosphomimetic substitution by S1P occurs at serine 921, protecting hTERT from degradation while keeping them in the nuclear region.

Activation of hTERT is primarily regulated by a series of phosphorylation events caused by various kinases and adaptor proteins. Phosphorylation of hTERT by protein kinase C (PKC) isoenzymes alpha, beta, delta, epsilon, and zeta phosphorylate hTERT, thereby enhancing telomerase activity [144, 145]. The mechanism of this remains unclear. hTERT contains a consensus bipartite nuclear localization signal (NLS) consisting of two clusters of basic amino acids ($^{222}$*RRR*$^{224}$ and $^{236}$*KRPRR*$^{240}$)[137]. Additionally, putative Akt-phosphorylation sites are located at $^{220}$*GARRRGGSAS*$^{229}$ and $^{817}$*AVRIRGKSYV*$^{826}$, and the phosphorylation of serine 227 (but not serine 824) is required for nuclear translocation of hTERT [138]. Chung et al. [137] predicted that the observed post-translational modification by Akt may result in a conformational change of hTERT, generating new interaction sites for binding partners like p65 of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) family proteins [139]. Similarly, Polo-like kinase 1 (Plk1) is believed to augment telomerase activity by promoting the nuclear retention of hTERT (thereby preventing the proteosomal degradation of hTERT in the cytoplasm) [140]. In support of this, overexpression of Plk1 resulted in increased hTERT protein levels without changing the steady-state transcript level of hTERT [140]. However, Plk1 does not phosphorylate hTERT, nor does it affect its interaction with hTERT [140]. Conversely, extra-nuclear localization of hTERT is regulated by the phosphorylation of Src tyrosine kinase at tyrosine 707 [141]. Src-mediated tyrosine 707 phosphorylation enables binding of Ran GTPase to hTERT, and the subsequent nuclear export via the chromosomal maintenance 1 (CRM1) export receptor [142]. 14-3-3 protein can bind hTERT and prevent its cytoplasmic localization by inhibiting CRM1-hTERT interaction [143]. Contrarily, dephosphorylation of hTERT by the phosphatase PP2A blocks the interaction between 14-3-3 and hTERT, thereby disrupting the nuclear localization and telomerase activity [144]. In the context of mitochondrial

localization, Src (mitochondrial)-mediated phosphorylation at Y707 was also shown to be responsible for the depletion of mitochondrial hTERT following $H_2O_2$-treatment [141]. In the same study, $H_2O_2$ treatment led to the reduced activity of mitochondria-resident Akt, suggesting Akt may regulate hTERT function not only in the nuclei, but also in the mitochondria [141]. Another tyrosine kinase Abelson murine leukemia viral oncogene homolog 1 (c-Abl) is a negative regulator of telomerase activity. c-Abl directly interacts with hTERT (at $^{308}$*PSTSRPPRP*$^{316}$) and induces tyrosine phosphorylation of hTERT in MCF-7 cells following ionizing radiation, reducing telomerase activity. However, the exact location of phosphorylated amino acid residue and the mechanism remain to be investigated [133].

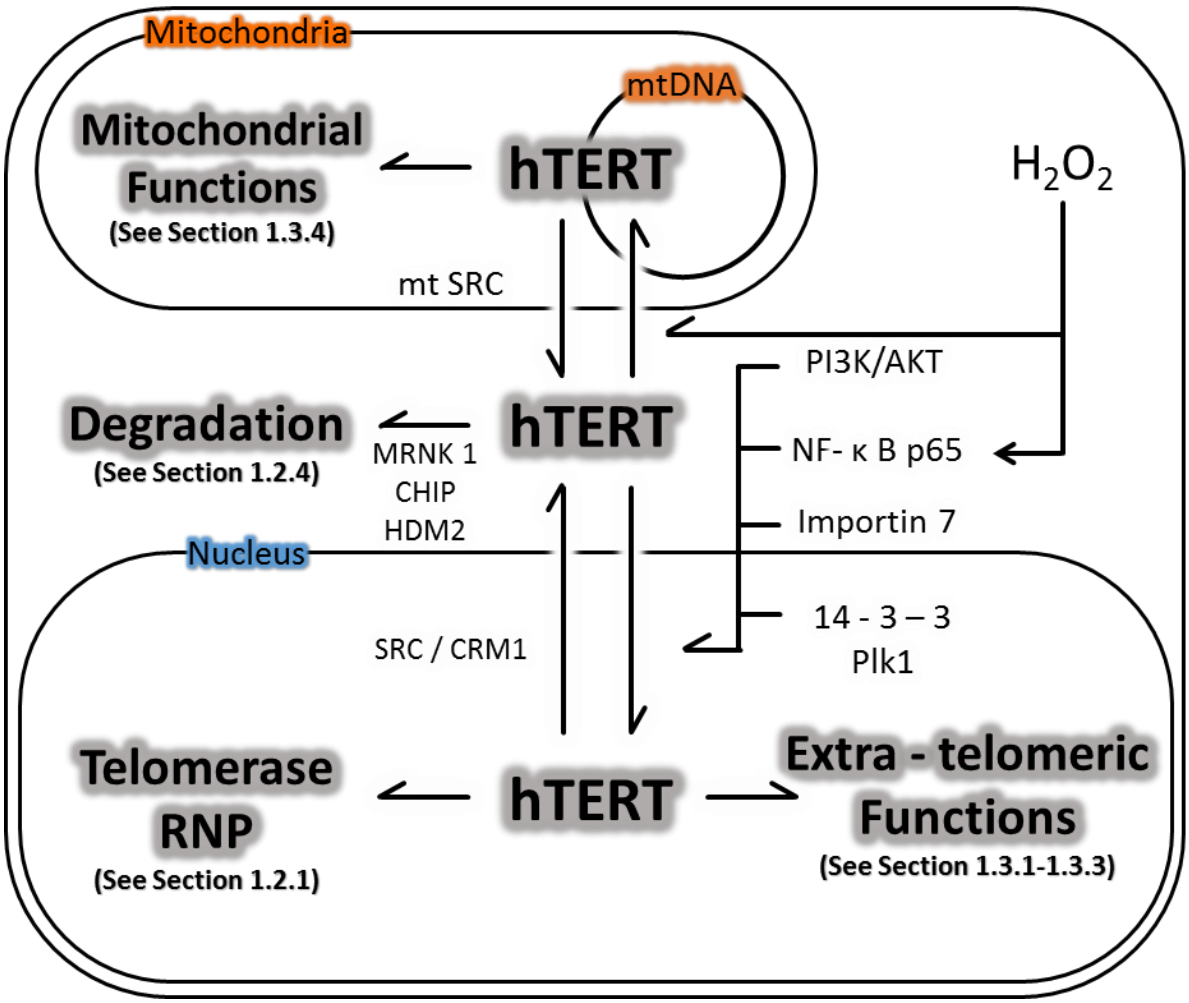



**Figure 1.2: Post-translational modification and function of hTERT.** Summary of signaling molecules/pathways that regulate the intra-cellular movement/stability of hTERT. hTERT is recruited to nuclear space for telomerase activity (section 1.2.1) and extra-telomeric functions (section 1.3.1-1.3.3). Conversely, hTERT is exported out of nucleus for ubiquitin-mediated degradation (section 1.2.4) or is targeted to mitochondria for mitochondrial functions (section 1.3.4). Factors/signaling-pathways that regulate hTERT translocation are indicated beside the arrows.

## *1.3 Non-canonical functions of Human Telomerase Catalytic Subunit hTERT*

### *1.3.1 Non-Canonical Functions of hTERT in Wnt/β-catenin pathway*

hTERT participates directly with Wnt/β-catenin to regulate downstream gene expression [145]. Wnt-signaling governs cell proliferation, and its involvement is critical for proper axis formation during embryo development. Three known modes of Wnt-signaling pathways are Wnt/calcium pathway, planar cell polarity pathway, and the canonical Wnt/β-catenin pathway [145]. The canonical signaling cascades starts when extra-cellular signaling Wnt family proteins bind to the N-terminal extra-cellular cysteine-rich domain of Fizzled (Fz) family receptor and lipoprotein receptor-related protein (LRP)-5/6 [145]. The binding of Wnt to Fz and LRP-5/6 leads to the dephosphorylation of a negative regulator of Wnt-signaling called Axin. This subsequently causes the disruption of protein degradation machinery (consisting of adenomatosis polyposis coli (APC), protein phosphatase 2A (PP2A), glycogen synthase kinase 3 β (GSK3β), which otherwise marks β-catenin for degradation by ubiquitination. The stabilized β-catenin eventually accumulates in nuclei to initiate the cellular response with the help of other transcription factors such as T-cell factor/lymphoid enhancing factor (TCF/LEF) and BRG1 [146]. It was demonstrated by Park *et al.* [147] that murine TERT (mTERT) modulates Wnt/β-catenin by acting as a cofactor of transcription complexes that consists of TCF/LEF and BRG1 (SMARCA4). Another group also confirmed TERT's association with BRG1 (Brahma-related gene 1) in human cells [148]. However, a contradicting result presented by Elizabeth Blackburn's group [149] showed that hTERT did not associate with BRG1. The discrepancy may arise from cell type-, and species specific differences. Interestingly, β-catenin was found to be a positive and direct regulator of hTERT gene expression through the interaction with Klf4 (in mouse ES cells) [150], and TCF4 (in human cancer cell) [151] at the transcription start site, suggesting a feed-

forward regulation between Wnt-signaling and hTERT expression. BRG1, on the other hand, suppresses hTERT expression by forming a ternary complex with HDAC2, leading to deacetylation of H3K9ac and H4ac at the transcription start site of hTERT in human cancer cells [152].

*1.3.2 Non-Canonical Functions of hTERT in NF-kB pathway*

Several groups have shown that TERT regulates NF-κB-dependent gene transcription in the absence of any telomere-mediated effects [156, 166-168]. The NF-κB signaling pathway is a master regulator of cell stress, inflammatory response, and developmental signaling [169- 171]. NF-κB protein family proteins include p50, p52, RelA (p65), RelB, c-Rel. The canonical NF-κB signaling involves dimerization of p65/p50 subunits (stabilized through the degradation of I-kappa-B-alpha (IκBα)) to regulate the expression of target genes. NF-κB is strongly activated in cancer cells, which led more researchers to investigate the potential regulatory cross-talk between hTERT and NF-κB. The studies have revealed that NF-κB positively regulates TERT by directly binding to its promoter [157]. In agreement, it was demonstrated that hTERT's nuclear translocation and telomerase activity/expression were enhanced by the binding of p65 in MM.1S cells [173, 174]. Additionally, Ghosh *et al.* recently discovered that hTERT directly regulates the expression of the NF-κB-dependent genes by binding to p65, and localizing to a subset of NF-κB sites, on promoters of IL-6, TNF-α and IL-8 [166, 167]. In the same study, results indicated that TERT is not merely a co-factor, but a requirement for optimal p65 binding to a few NF-κB target genes when NF-κB signaling was induced by TNF-α stimulation [153]. Ghosh *et al.* clarified this further by identifying telomere like sequences (TTGGG) within the promoter regions of IL6A, IL8A and TNF-α genes [153].

Not much is known about NF-κB signaling with respect to the pluripotency and self-renewal of PSC. However, the gene expression/proteome profiling of undifferentiated and differentiated ESCs show that components of the NF-κB signaling pathway are enriched in undifferentiated hESC, and downregulated during differentiation. Immunohistochemistry of RelA showed that it is only present in the nucleus of undifferentiated hESC, indicating active NF-κB signaling. To test the potential importance of NF-κB signaling in the maintenance of pluripotency, a specific NF-κB inhibitor sodium pyrrolidinethiocarbamate (PTDC) was added to the cells. PTDC prevents RelA (p65) entry into the nucleus. Inhibition of NF-κB signaling by PTDC caused massive cell differentiation within ESC colonies, as well as significant cell death, demonstrating that NF-κB, or at least the nuclear translocation of hTERT mediated by NF-κB molecule p65, is essential to maintain ESC pluripotency [18]. The downregulation of NF-κB signaling was also observed in spontaneously differentiating human ESCs. In another study with three independent human iPSC lines, the augmented level of NF-κB signaling was confirmed. Moreover, the knockdown of NF-κB resulted in decreased Nanog and Oct-3/4 expression levels, suggesting an important role of NF-κB in the maintenance of undifferentiated human PSCs [161]. In contrast, NF-κB signaling is suppressed in undifferentiated mouse ESCs and activated in differentiating mouse ESCs [176, 177]. Torres *et al.* reported that a pluripotency transcription factor Nanog represses NF-κB signaling to promote pluripotency. Taken together, the studies point to the importance of NF-κB signaling and its cross-talk with hTERT in modulation of pluripotency in human PSCs.

### *1.3.3 Non-Canonical function of hTERT as an RNA-dependant RNA Polymerase (RdRP)*

Several pieces of scientific evidence exist to show that TERT plays a direct role in the regulation of gene expression, including Wnt/β-catenin and NF-κB pathways just reviewed.

In more recent years, several reports show that TERT may regulate gene expression at the post-transcriptional level in an extra-telomeric fashion [157, 178, 179]. Maida *et al.* showed that hTERT can interact with non-TERC RNA species called RNA component of the mitochondrial RNA-processing endoribonuclease (RMRP) to form a ribonucleoprotein complex that has RNA-dependent RNA polymerase activity. The resulting reverse transcriptase TERT-RMRP complex generates double stranded RNA (dsRNA) using RMRP as a template RNA strand. The dsRNAs are subsequently processed by Dicer1 into 22-nucleotide small-interfering RNAs (siRNAs) that selectively cleave and silence themselves. RMRP is a 267 nucleotide-long non-coding RNA that is highly expressed in many human and murine tissues. Notably, RMRP expression is activated by Wnt/β-catenin and YAP proteins in human carcinoma cells [166], suggesting TERT-RMRP complex may be a part of much broader signaling pathway. The canonical role of RMRP is implicated in mitochondrial DNA replication where it initiates the replication process by cleaving another mitochondrial RNA (inhibits progression of mitochondrial DNA replication) bound at the priming site [167]. Mutations in the RMRP gene are associated with cartilage hair hypoplasia (CHH) [182, 183]. Homozygous deletion of RMRP in mouse results in embryonic lethality, indicating RMRP is essential for early mouse embryonic development [165]. Sharma *et al.* (a team led by a pioneering scientist to show mitochondrial effects of hTERT) suggested the mitochondrial hTERT binds different mitochondrial RNAs and carries out its tentative mitochondrial functions (*see* section 1.2.4) in TERC-independent manner [170]. The study raised possibilities that there may be more RNA binding partners other than RMRP to regulate gene expression in the mitochondria.

Outside of mitochondrial context, hTERT was shown to regulate heterochromatin assembly at centromeres and transposon elements on chromosomes [148]. hTERT associates

with BRG1 and nucleostemin (NS) to form a TBN (TERT, BRG1, and Nucleostemin) complex with RdRP activity. The complex produces dsRNAs homologous to centromeric alpha-satellite (alphoid) repeat elements. The dsRNA is then processed into siRNAs that target the centromeric alphoid regions to promote the recruitment of heterochromatin assembly complexes to the site. In a different study by the same group, it was shown that siRNA generated via RMRP-hTERT RdRP and RNA-induced silencing complex (RISC) negatively regulated PHYHIPL transcript level whose cellular functions are yet unclear [171]. Even though there has been no report to indicate the functional significance of hTERT as TERC-independent RdRP in hESCs, the mounting evidence of it in other cell types suggest hTERT may carry out important nuclear and mitochondrial roles in hESCs independently of TERC.

*1.3.4 Mitochondrial Functions of hTERT*

Santos *et al.* (2003) [172] have shown that ectopically expressed TERT exacerbates $H_2O_2$-mediated mitochondrial damage in human cells. They delineated this phenomenon by identifying the putative 20 amino acid-long mitochondrial localization signal at the very N-terminal end of hTERT. hTERT associates with mitochondrial membrane translocases TOM20/40 and TIM23 proteins, further suggesting the mitochondrial localization of hTERT [173]. Mitochondrial DNA has 22 sites with telomere-like sequences for hTERT binding, and hTERT's binding to two of these sites has been confirmed [173]. The bound regions of mitochondrial DNA each code for complex 1 NADH-Ubiquinone Oxidoreductase chain 1 and 2 subunits [173]. Ectopic expression hTERT up-regulates complex I activity [173]. Conversely, ectopic expression of catalytically inactive hTERT results in decreased complex I activity. Other known mitochondrial effects of overexpressed FL-hTERT include increased mitochondrial membrane potential, down-regulated UCP2 (uncoupler of mitochondria that is

responsible for repressing OXPHOS in PSCs), reduced mitochondrial superoxide, mitochondrial DNA (mtDNA) content, and mitochondria mass [174].

It was suggested that ectopically expressed hTERT lead to increased $H_2O_2$-mediated mtDNA damage through increased availability of metal ions [175]. However, other studies demonstrate that FL-hTERT protects mtDNA from $H_2O_2$-induced oxidative stress[142], [174]. In addition, FL-hTERT protects mtDNA from other genotoxins such as ethidium bromide and UV-light. In general, hTERT appears to augment mitochondrial function and maturation. A growing body of research suggests that TERT has important effects on mitochondrial function in a telomere-independent manner [187, 190-193]. However, the mechanism by which TERT protects mitochondrial function is not completely understood, and remains to be elucidated

### *1.4 Rationale*

Mitochondria in undifferentiated hESCs are localized to the peri-nuclear space, and remain low in copy number and are punctuate in morphology [60]. In contrast, hESC differentiation is accompanied by the formation of a reticular network of maturing mitochondria with a simultaneous increase in OXPHOS and ROS production [66]. Taken together, mitochondrial function and biogenesis is key to understanding the regulation of pluripotency in undifferentiated hESCs and in early differentiation processes [178].

hTERT can interact with a wide range of biomolecules such as DNA [187, 195], transcription factors [156, 166], a helicase [180], and various species of RNA [157, 185, 196-

198]. Thus, hTERT has various functions in the nucleus and mitochondria. Interestingly, a number of these hTERT functions require catalytically activity of hTERT [181] and is often inhibited by catalytically inactive forms [98, 108, 187]. Many past studies [175–177, 187, 188] and our lab's previous work by Radan *et al.* [184] demonstrated that high oxygen tension and oxidative stress on cells induce the extra-nuclear/mitochondrial localization of hTERT. Radan *et al.* additionally showed that pharmacological inhibition of telomerase activity, but not TERC, resulted in spontaneous differentiation of hESC. Similarly, disrupting β-splicing by morpholino oligonucleotides in hESC resulted in loss of pluripotency, indicating the importance of hTERT and its non-catalytic variants in stem cell function [184]. Yang et al. [185] had also shown the importance of hTERT in hESC maintenance through knockdown studies. β-hTERT, like its full-length counterpart, has an intact MTS [172], RNA-interacting domains [199, 200], and oligomerization sequence[188], and retains some phosphorylation sites for nuclear import [201, 152] and export [142] (Figure 1.1). Listerman and colleagues recently demonstrated that β-hTERT is capable of mitochondrial localization in HeLa cells [102]. However, the cause and the pattern of β-hTERT localization is currently unknown. This mitochondrial localization may allow β-hTERT to alter the function of FL-hTERT in any subcellular compartment by sequestering key functional components such as specific RNA components or by disrupting the formation of a functional homodimer of catalytically active hTERT subunits.

Following the relatively recent discovery of hTERT localization to mitochondria, several studies indicated that mitochondrial FL-hTERT may function through modulation of mitochondria biogenesis, retrograde response to oxidative stress, and changes in divalent metal ion homeostasis [186-188]. It was demonstrated that FL-hTERT interacts with mtDNA, and enhances mitochondrial function [173]. Notably, overexpression of a reverse-

transcriptase incompetent hTERT mutant abrogated this effect [173]. Considering β-hTERT is a reverse-transcriptase incompetent form, there may exist an interaction between FL-hTERT and β-hTERT in mitochondria to fine-tune mitochondria function, possibly through hetero-dimerization. Collectively, targeting of β-TERT to mitochondria and its potential to modulate mitochondrial function and growth may imply biological significance in the regulation of pluripotency, self-renewal and differentiation potential of PSCs.

***Hypothesis***: Extra-telomeric hTERT β-isoform localizes to mitochondria under $H_2O_2$-induced oxidative stress, modulating mitochondria function in hESCs.

***Objectives:***

1. To characterize FL-hTERT and β-hTERT expression and their role in modulating telomerase activity in hESCs.
2. To characterize the mitochondrial localization pattern of FL-hTERT and β-hTERT in hESCs under $H_2O_2$-induced oxidative stress conditions.
3. To determine the effect of overexpressed FL-hTERT and β-hTERT on mitochondrial biogenesis and function in hESCs.

# Chapter 2: Material and Methods

## *2.1 Cell Culture*

### *2.1.1 Mouse Embryonic Fibroblasts Feeder Cells*

Mouse embryonic fibroblasts (MEF) secrete several soluble growth factors to enhance the self-renewal and pluripotency of hESCs. For this reason, hESCs are often co-cultured with irradiated MEF-feeder cells. Four drug (neomycin, hygromycin, puromycin, and 6-thioguanine)-resistant MEF(4DR-MEF) cells were purchased for the future use in drug-selection of cloned transgenic hESC populations. 4DR-MEF was cultured in DMEM medium (ThermoFisher) containing 10% fetal bovine serum, 1X Non-Essential Amino Acid (NEAA; ThermoFisher) under 5% $CO_2$ at 37ºC. A bulk batch of MEFs was γ-irradiated to halt the cell proliferation and was cryo-preserved in small aliquots. γ-irradiated 4DR MEFs (approximately 60,000 cells and $10^6$ for each well of 6-well plate and 10cm dish, respectively) were thawed and allowed to settle onto the culture dishes or plates for one day prior to being used as feeder-layer for hESC culture.

### *2.1.2 Human Embryonic Stem Cell Line HES2*

The human ESC line HES2 was originally established in a fertility clinic in Singapore [190] and was purchased from ATCC for utilization in this study. For maintenance purposes, HES2 cells were cultured on γ-irradiated 4DR MEFs layer for a single passage (3-4 days to reach 70-80% confluency). hESC growth medium (83% KnockOut™ DMEM/F-12 (Gibco™), 15% KnockOut™ serum replacement (ThermoFisher), 1% GlutaMax™ (X100; Gibco™), 1% NEAA (X100; Gibco™), 0.1% DMSO (Sigma), and 8ng/ml bFGF

(ThermoFisher)) was refreshed daily. The stably growing HES2 cells were subsequently expanded onto a feeder-free basement membrane Geltrex$^{TM}$ (Life technologies) in 2% $O_2$ (37$^{o}$C, in 5% $CO_2$) for experimental use. Fresh Essential 8$^{TM}$ Medium (ThermoFisher) was supplied daily for feeder free hESC culture. Cells were treated with 100μM $H_2O_2$ for up to 4 hours.

*2.1.3 Establishment of monoclonal transgenic HES2 lines.*

The lack of a reliable hTERT-specific antibody has been a challenge in studying FL-hTERT, β-hTERT, and other TERT isoforms. To address this problem, my lab established clonally selected transgenic hESC cell lines capable of doxycycline-inducible overexpression of 3xFLAG-tagged FL-hTERT and 3xFLAG-tagged β-hTERT (hereby called HES2 'FL' and HES2 'B' for 3xFLAG-FL-hTERT and 3xFLAG-β-hTERT lines, respectively), enabling the use of commercially available anti-FLAG antibody to trace/isolate overexpressed FL-hTERT and β-hTERT transgenic proteins in hESCs (Fig. 2.1 A). Briefly, endogenous FL-hTERT and β-hTERT transcripts were reverse-transcribed and PCR-amplified into library DNA sequence using hTERT specific primers (appendix A1: Table 2.1). The library hTERT sequences were extended to include Gateway LR-Clonase (ThermoFisher) adaptor sequences attL1 (5'GTACAAAAAAGCAGGCT), attL2 (TGGGTCGAAAGAACATG3'), 5'Kozak sequence (GCCGCCACC), 3xFLAG sequence (GACTACAAGGACCACGACGGTGACTACAAGGACCACGACATCGACTACAAGGACGACGACGACAAGTGA), and β-GEO drug resistance gene (appendix A1: Table 2.1) by PCR. The clone sequence of FL- and β-hTERT was ligated into the open reading frame of Tet-inducible/transposable entry vector of PiggyBac (PB) Transposon System (donated by

Nagy Lab, Mt Sinai Hospital). The correct orientation and sequence of ligated inserts was validated by DNA sequencing (Robarts Genomic facility). The entry vector plasmid and two other PB plasmid constructs containing PiggyBac rtTA gene (transactivator of gene of interest (GOI) expression; genome-integrated), and PiggyBac transposase gene (facilitates integration of GOI and rtTA into the genome; short-lived without genomic integration) were introduced to HES2 genome via electroporation (170V, 1050 μF). Successfully transformed HES2 cells were selected by blasticidin (10μg/ml; 1 day) and G418 (1mg/ml; 7 days). The surviving cell clusters were single-celled and clonally picked to establish the final transgenic HES2 clones.

*2.1.4 Cell treatment scheme*

Preliminary experiments were carried out to determine the optimal treatment time and dose of doxycycline for the induction of hTERT transgene expression in HES2 clones (appendix A3: Fig 3.1). Non-transgenic HES2 (hereby referred to as HES2) and HES2 B and HES2 FL clones were treated with doxycycline-containing (1μg/ml) Essential 8 media for 2 days. The culture medium was renewed daily. Based on studies that demonstrated extra-nuclear localization of hTERT following oxidative stress treatments ($H_2O_2$ and/or high oxygen tension)[144, 174, 185], $H_2O_2$ (100μM)-containing growth medium was directly added to the culture 2 hours prior to cell harvest. Cells were kept under 2% oxygen tension for optimal cell growth of hESC (*see* section 1.1.2).

## *2.2 PCR protocols*

### *2.2.1 General Real-Time quantitative PCR protocol*

A phenol-chloroform extraction protocol was performed for RNA isolations as outlined in the product instructions (TRIzol reagent; ThermoFisher). Isolated RNA was reverse transcribed to cDNA using SuperScript III (ThermoFisher). cDNA concentrations were estimated with a NanoDrop (ThermoFisher) spectrophotometer and equal amount of template was ensured for each set of PCR reactions. RT-qPCR reactions consisted of SensiFAST$^{TM}$ (Bioline) PCR mix, gene-specific primer sets, and cDNA template. DNA polymerase was heat-activated at 95$^{o}$C for 2 minutes prior to PCR cycles. Temperature cycling conditions were 95 $^{o}$C for 10 seconds (denaturation), pre-determined temperature (primer specific; appendix A1: Table 2.1), 72$^{o}$C for 30 seconds (elongation). HPRT1 was used as reference gene in all RT-qPCR experiments. Relative transcript quantity was determined based on ΔCt(gene of interest)ΔCt(HPRT1) values.

### *2.2.2 Detection and quantitation of hTERT isoforms by Hydrolysis-probe assisted PCR*

TaqMan® Hydrolysis probe-assisted RT-qPCR was used to detect hTERT isoform transcripts. Exon-exon junctions unique for each of hTERT isoforms (α+β+hTERT, α-hTERT, β-hTERT, α-β-hTERT) were used as PCR priming sites (Fig. 2.1 B). α+β+hTERT are transcript species with α and β site intact, and includes FL-hTERT transcript. Importantly, the TaqMan primers are designed to detect any transcripts with the target junctions, meaning ***they do not distinguish between transcripts that are different outside of the PCR-amplified region*** (*see* Fig. 2.1 description). ***Additionally, the TaqMan primers do not distinguish recombinant 3xFLAG-hTERT transcript from endogenous hTERT transcript.*** FAM-

conjugated DNA probes specific for the hTERT gene were employed to further increase the specificity and sensitivity of the RT-qPCR (Fig. 2.1 B). 1X TaqMan Universal PCR Master Mix (Applied Biosystems), 200nM forward and reverse primers, and 250nM hTERT specific FAM-probe were included per PCR reaction (20 µl). For reference, HPRT1 transcript abundance was measured alongside by SensiFAST PCR as outlined above.

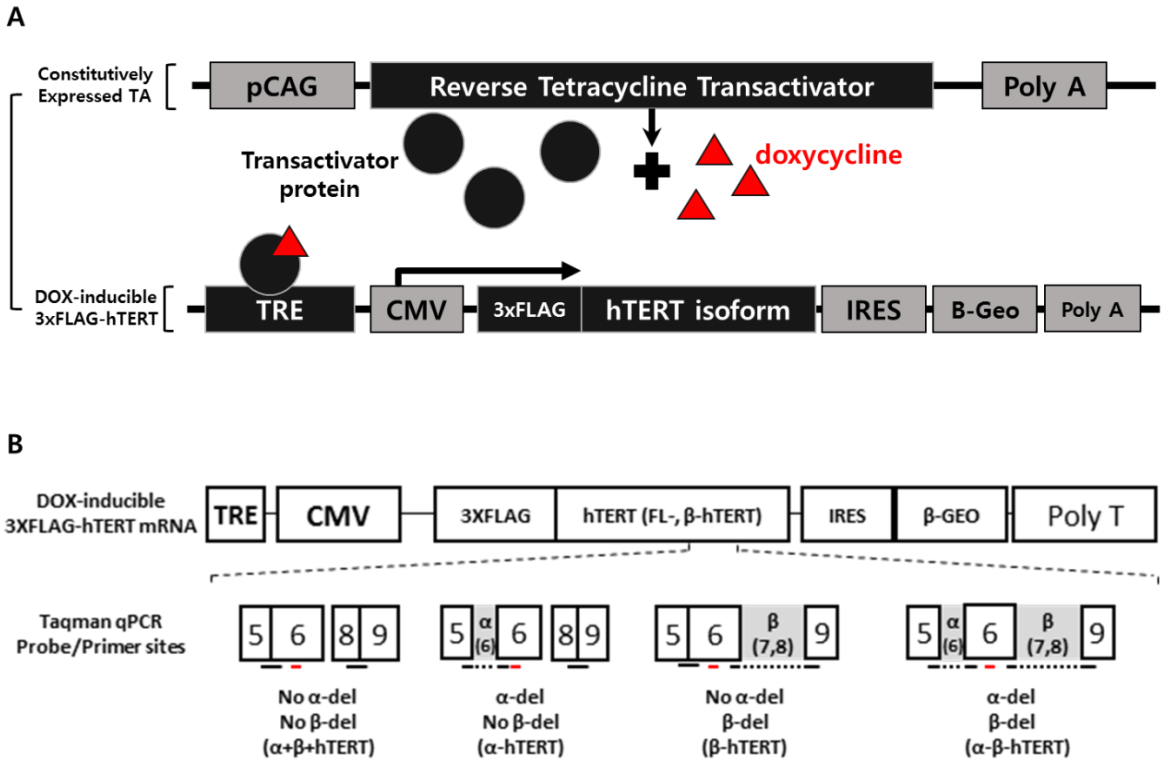



**Fig. 2.1 Transgene structure and TaqMan qPCR probe-/primer-binding sites.** Dox-inducible overexpression cassette for 3xFLAG-tagged FL-hTERT and 3xFLAG-tagged β-hTERT were cloned and integrated into the genome of HES2 using the PiggyBac Transposon System (vectors donated by Nagy Lab, Mt Sinai Hospital; work of Dr. Jonathan H. Teichroeb). Two transgenes were genomically integrated **A)** PiggyBac rtTA gene for transactivator expression (required for transcription of 3xFLAG-hTERT) and DOX-inducible 3xFLAG-tagged-hTERT expression sequence. **B)** Primers (black) and TaqMan™ probe (red) annealing sites for hTERT variants. ***The primers cannot detect the differences in transcripts with alternatively spliced sites outside of the targeted regions. (i.e. α+β+hTERT primers will non-discriminately detect all transcripts with intact α and β sites regardless of alternative splicing that may be present outside of exon5/exon6 junction-to-exon8/exon9 junction region). Moreover, the TaqMan primers do not differentiate between recombinant 3xFLAG-hTERT transcripts and endogenous hTERT transcripts.***

*2.2.3 RNA-immunoprecipitation and PCR-assisted detection of TERC*

1 x $10^7$ HES2 transgenic clonal cells were trypsinized and lysed in cold non-denaturing buffer (20mM Tris pH8.0, 137mM NaCl, 10% Glycerol, 1% IGEPAL (Sigma), 2mM EDTA, 1X Protease Inhibitor Cocktail Set I (Calbiochem). The lysate was kept on ice for 30 minutes with constant agitation to help complete lysis. Cell debris was isolated and removed by centrifugation at 800g for 10 minutes. The cleared lysate was co-incubated with Anti-FLAG M2 Magnetic beads (30µl packed volume; Sigma), which was precleared with salmon sperm DNA (10 µg/ml) to prevent non-specific binding of nucleotides to the beads. The incubation was done at 4$^o$C on a rotator overnight. Beads were washed 3 times with the lysis buffer and 2 times with TBE buffer (89mM Tris, 0.5mM EDTA, 89mM boric acid). TRIzol reagent (ThermoFisher) was added to the washed beads to extract bound RNA from 3xFLAG-hTERT proteins. TERC transcript abundance was quantified and compared in doxycycline-negative and doxycycline-positive sample groups.

*2.2.4 PCR amplification of mitochondrial DNA for mitochondria DNA copy number*

A set of PCR primers (appendix A1: Table 2.1) targeting a small segment of mitochondrial DNA ($tRNA^{Leu}$) was used to estimate the relative mitochondrial DNA copy number in the sample groups (appendix A1: Table 2.1). A Mammalian Genomic DNA Miniprep Kit (Sigma) was used to extract genomic DNA. Nucleotide content in each sample was measured using a NanoDrop (ThermoFisher) spectrophotometer. Extracted gDNA was preheated to 75$^o$C for 15 minutes to ease the supercoiling of circular mitochondrial DNA. The PCR reaction volume consisted of SensiFAST PCR master mix (5µl; Bioline), 10µM forward/reverse primers (0.2µl), 2µl template gDNA (6ng in mass), and 2.8µl RT-qPCR

grade water. PCR Cycling conditions were 95$^{o}$C for 2mins (DNA polymerase activation; 1cycle), 95$^{o}$C for 10 seconds (denaturation), 62$^{o}$C for 10 seconds (annealing), and 72 $^{o}$C for 20 seconds (synthesis). A single copy nuclear gene, beta-2-microglobulin (B2M) was used as a reference to normalize the data to the number of genomes per sample.

### *2.3 Telomerase Activity Assay*

To confirm the telomerase activity of 3xFLAG-tagged transgenic hTERT, a telomeric repeat amplification protocol (TRAP) assay was performed as previously described [191]. HES2 and 3xFLAG-hTERT-expressing lines were treated with doxycycline (1μg/ml) for 2 days. Cells were harvested by trypsinization and lysed in CHAPS lysis buffer (150mM KCl, 50mM HEPES pH7.4, 0.1% CHAPS). RNaseOUT$^{TM}$ (Recombinant RNase Inhibitor; ThermoFisher) was included in the lysis buffer to prevent the degradation of TERC. To avoid protein degradation from freezing, all downstream steps were performed immediately and the samples were kept on ice. Total protein content in each sample was quantified by DC$^{TM}$ Protein Assay (BioRad). In the initial step of the tandem polymerase reactions, any TERC-bound hTERT species in the lysate would elongate (by telomerase activity) the TS substrate primer (AATCCGTCGAGCAGAGTT) *in vitro* for 30 mins at 25$^{o}$C. This was followed by heat-activation of DNA polymerase (SensiFAST; Bioline) and simultaneous heat-inactivation of hTERT protein at 95$^{o}$C for 2 minutes. The *de novo*-synthesized telomeric sequences was amplified using a return primer ACX (GCGCGGCTTACCCTTACCCTTACCCTAACC). The temperature settings for this PCR (32 cycles; predetermined to ensure exponentially amplifying PCR cycle across all sample groups) were 95$^{o}$C for 10 seconds (denaturation), 58$^{o}$C for 10 seconds (annealing), 72$^{o}$C 30 seconds (elongation). An internal control primers

TSNT (AATCCGTCGAGCAGAGTTAAAAGGCCGAGAAGCGAT) and NT (ATCGCTTCTCGGCCTTTT) were included. TSNT is semi-competitively amplified by the annealing of TS primer and its dedicated return primer NT during PCR. The primary purpose of this is to rule out potential false-negative outcomes resulting from hindered DNA polymerase during PCR. Secondly, consistent amplification of TSNT amplicon (36bp) is indicative of equal PCR reaction efficiency. The finished PCR reaction mix was electrophoresed through a vertical native 12% polyacrylamide gel at 400V for 45 minutes in 1X TBE buffer (89mM Tris, 0.5mM EDTA, 89mM boric acid) to resolve the product bands. The PAGE gel was stained with X1 SYBR Green I (ThermoFisher) nucleic acid dye in TBE for 30 minutes at room temperature. Bands were visualized using a Gel Doc$^{TM}$ EZ (BioRad) and UV. The band intensity values of telomeric repeat products were first normalized to TSNT internal control band intensity.

## *2.4 Immunodetection of 3xFLAG-hTERT isoforms*

### *2.4.1 Western Blot Analysis*

HES2 B6 and F12 clones (n≥3/group) were grown under feeder-free condition under 2%$O_2$ and $H_2O_2$ treatments. 3xFLAG-hTERT overexpression was induced by direct addition of doxycycline (1µg/ml) to the culture medium 48-hours prior to cell harvest. Cells were lysed and fractionated using a mitochondria isolation kit per its instructions (Mitochondria Isolation Kit for Cultured Cells; ThermoFisher). Protease Inhibitor Cocktail Set I (Calbiochem) was added to all lysis/resuspension buffers to prevent protein degradation. The isolated mitochondria and nuclei of mitochondria-enriched, and nuclei-enriched samples were examined by Western Blot analysis with antibodies against voltage-dependent anion

channel (VDAC; mitochondria marker), Prohibitin (mitochondria marker), and histone H3 (nuclear marker) (*see* appendix A2: Table 2.2). VDAC and histone H3 were used for normalization. 3xFLAG- hTERT in nuclear and mitochondrial fractions was traced using an anti-FLAG antibody (Sigma) and quantified by densitometry analysis using Image Lab™ (BioRad). All membranes were developed using a VersaDoc Imaging System (BioRad)

*2.4.2 Immunocytochemistry*

For immunofluorescence microscopy, HES2 B6 and F12 clones were grown in feeder-free conditions on ChamberSlides™ (Nunc), and were treated with $H_2O_2$ as previously indicated (*see* section 2.1.4). Cells were fixed and permeabilized in 4% formaldehyde (15 minutes) and in 0.25% Triton™ X-100 for (10 minutes), respectively. Cells were washed with phosphate buffered saline three times in between steps. Cells were blocked with 10% bovine serum albumin solution for 45 minutes at 37 ºC. Cells were incubated with anti-TOM20 (1:500; rabbit polyclonal; ProteinTech) and anti-FLAG antibody (1:200; mouse monoclonal; Sigma) in 3% BSA solution overnight at 4ºC. Lastly, cells were counterstained with NucBlue (nuclear stain; ThermoFisher). The subcellular localization of 3xFLAG-hTERT was investigated by co-localization using a confocal microscope (Zeiss LSM 5 Duo Vario) and 63X oil-immersion objective lens. Image analysis software Zen (Zeiss) and Image J were utilized to analyze sample images.

*2.4.3 Cell count analysis for intra-nuclear localization of 3xFLAG-hTERT isoforms*

HES2 B6 and F12 clones were grown in feeder-free condition on ChamberSlides™ (Nunc) for 3 days until ~70% confluency was reached. Cells were treated with doxycycline-containing (1μg/ml) Essential 8 media for 2 days. The culture medium was renewed daily. $H_2O_2$ was directly added to the media (100 μM final concentration) 2 hours prior to harvest. Field-of-view images of HES2 B6 (n=11) and HES2 FL (n=12) were taken as outlined in section 2.4.2. All images contained either a whole colony or a part of a colony. Only the clear and round nuclei (DAPI stain) were counted. Partial nuclei at the edge of the images were excluded from analysis. The intra-nuclear localization of 3xFLAG-β-hTERT and 3xFLAG-FL-hTERT were categorized into an uniform distribution or a peripheral distribution (Fig. 2.2). Nuclei with uniform distribution of 3xFLAG-hTERT was defined as nuclei with two or more nucleoli (dark and hollow space indicated by arrows in Fig. 2.2 left) as delineated by 3xFLAG-hTERT signal (green). Nuclei with peripheral distribution of 3xFLAG-hTERT was defined as nuclei that has only one nucleolus two thirds of whose boundary is near the nuclear membrane (as delineated by DAPI stain; DAPI not shown in Fig. 2.2). Images were first assigned randomized numbers and alphabets by a third-party member. The blinded images were presented to three volunteers from a third party in random order. Peripheral distribution was defined as an intra-nuclear distribution that exhibits only one nucleolus whose boundary is in close proximity with the nuclear boundary. (Fig. 2.2). Uniform distribution was defined as an intra-nuclear distribution that exhibits more than one nucleolus. The percentage of nuclei with peripheral distribution (out of total nuclei count) determined by the volunteers was averaged for statistical analysis.

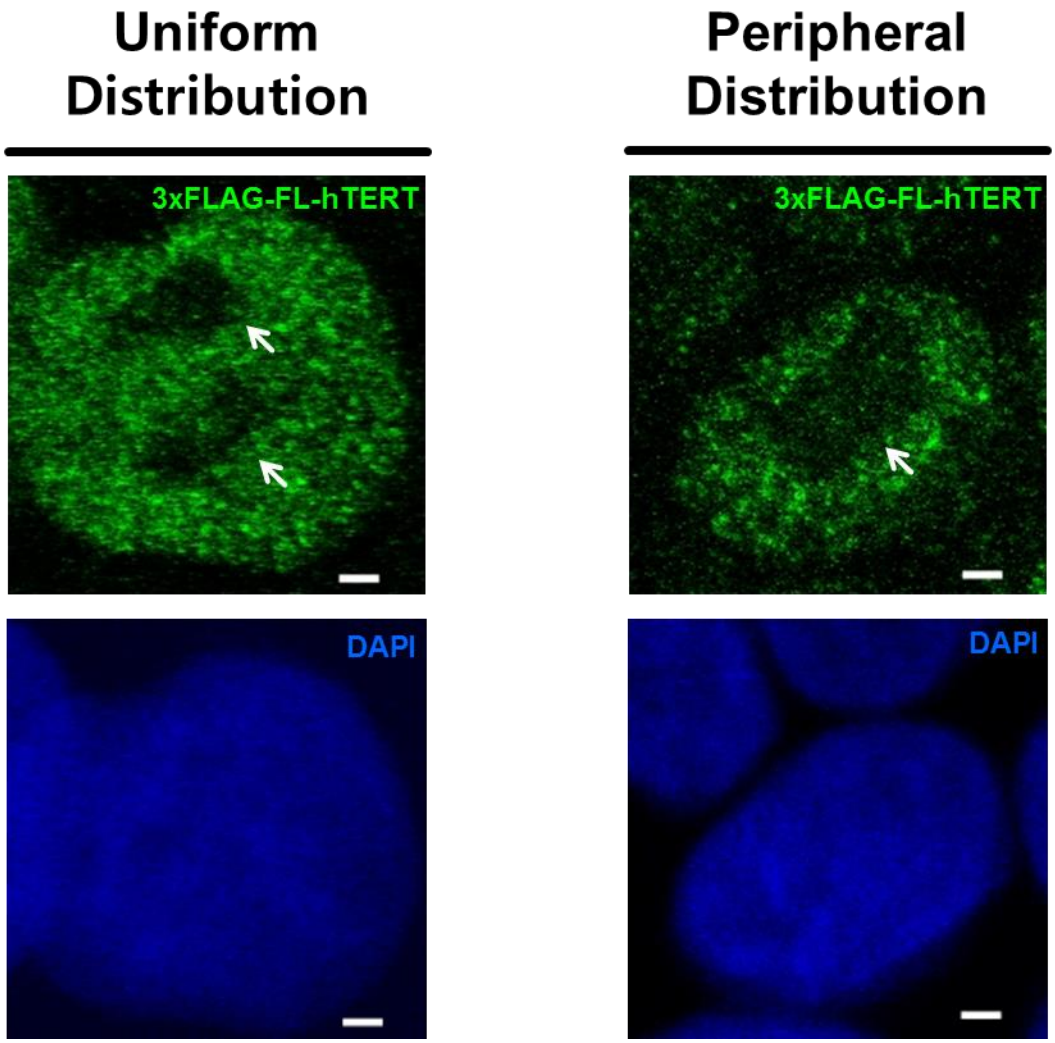



**Fig. 2.2: Representative confocal microscopy images of 'uniform' and 'peripheral' intra-nuclear distribution of 3xFLAG-FL-hTERT within *nuclei.*** The intranuclear distribution of 3xFLAG-FL-hTERT was visualized with anti-FLAG antibody and Alexa Fluor 488 secondary antibody (green). DAPI counter stain was used to mark nuclei. Nucleoli are indicated with white arrows. Scales bars (white) = 2 μm.

## *2.5 Flow Cytometry*

### *2.5.1 Mitochondria Membrane Potential*

The cyanine dye JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolocarbo-cyanine iodide; Sigma) [192, 193] is widely used for microscopic and cytometric estimation and measurement of mitochondrial membrane potential (Δψm). Aggregates of the dye (J-aggregate) form at the high concentrations reached in energized mitochondria of cells exposed to near-micromolar external concentrations of the dye. J-aggregates are spectrally distinguishable from dye monomers. JC-1 is advantageous over other types of cationic Δψm dyes in that it selectively accumulates in mitochondria. JC-1 is excited at 488nm with the highest efficiency. When excited at 488 nm, JC-1 monomers emit green fluorescence with a maximum at 530 nm (green), whereas J-aggregates emit orange-red fluorescence with a maximum at 595 nm (orange-red). A subset of HES2, B6, and FL12 clones were treated with 1µg/ml doxycycline for 2 days. All cells were treated with 100 µM $H_2O_2$ for 2 hours to induce oxidative/apoptotic stress. Thirty minutes prior to the harvest, cells were treated with JC-1 (10 µg/ml; dissolved in DMSO) for 30 minutes in the incubator. Cells were washed with PBS ($Ca^{2+}$ free) once and collected by trypsinizing/centrifugation at 300g for 5 minutes. Cell pellets were re-suspended in flow-cytometry staining buffer ($Ca^{2+}$ free PBS, 0.5% BSA). Spectral readings were taken immediately with BD Accuri$^{TM}$ C6 using a blue laser (488nm), bandpass filters FL2 (586±20nm; J-aggregate) and FL1 (530 ±15nm; JC-1 monomer). Twenty thousand events were recorded per group at all channels. 20µM Carbonyl cyanide 4-l (trifluoromethoxy) phenylhydrazone (FCCP; chemical dissipater of Δψm; Molecular Probes®)-treated samples were included as a positive control to represent a depolarized membrane. MMP variation was deduced by calculating FL2 (J-aggregate; high Δψm) to FL1 ratio (JC-1 monomer; low Δψm).

*2.5.2 Detection of apoptosis*

To assess the pro-survival effect of overexpressed 3xFLAG-hTERT in HES2, cells were stained with Annexin V-FITC (ThermoFisher)/7-amino-actinomycin (7-AAD; eBioscience), following 100 µM $H_2O_2$ treatment. Annexin V/7-AAD staining provides a way to discriminate between early apoptosis and late apoptosis. When a cell undergoes apoptotic cell death, phosphatidyl serine (PS; normally on the inner leaflet of plasma membrane) becomes exposed on the outer leaflet of the plasma membrane. Annexin V binds PS with high affinity and marks early-apoptotic cells. 7-AAD is a DNA intercalating dye that can enter dead cells with compromised membrane integrity. A subset of HES2, B6, and FL12 clones were treated with 1µg/ml doxycycline for 2 days. All cells were treated with 100 µM $H_2O_2$ for 2 hours to induce oxidative/apoptotic stress. Following cell harvest by trypsinization/centrifugation (as outlined above), cells were re-suspended in Annexin V-staining buffer (10mM HEPES, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) to $10^6$ cells per/ml. Annexin V-FITC (15 minutes, at room temperature) and 7-AAD (5 minutes, at room temperature) were added to a 100 µl aliquot of the resuspension. Spectral readings were taken immediately with BD Accuri$^{TM}$ C6 using a blue laser (488nm), bandpass filters FL3 (670 nm longpass; 7-AAD) and FL1 (530±15nm; FITC Annexin V-FITC). A total of 30000 events were recorded per group at both channels.

## *2.6 Statistical Analyses*

All statistical analyses were performed using Prism 6 (Graph Pad Inc.), and are presented in mean ±SEM. An unpaired t-test was used for comparisons between DOX-treated and non-DOX-treated samples. Paired t-tests were used for flow cytometer analysis of DOX-

and non-DOX- groups. An One-Way ANOVA with Tukey's post hoc test was used for analyses involving more than two hTERT isoforms or HES2 lines. Statistically significant data are indicated with asterisks (* for $p<0.05$, ** for $p<0.01$, *** for $p<0.001$). Non-significant results were indicated with 'NS'.

# Chapter 3: Results

## 3.1 Basic cell characterization of human embryonic stem cells

### *3.1.1 Characterization of pluripotency markers in human embryonic stem cells*

Human embryonic stem cells (HES2 cell line) grow as flat and compact round colonies indicative of the primed pluripotent stem cell state [194] (Fig. 3.1 A). Assessed by immunofluorescence microscopy, HES2 cells were positive for pluripotency markers SSEA-4 and OCT-4, but negative for SSEA-1 (Fig. 3.1 C). Transcripts of the α+β+TERT isoform (includes FL-hTERT transcript) and the most prominent hTERT splice variants (β-, α-, α-β-) were detected in HES2 cells that had been cultured under 21% oxygen tension (Fig. 3.1 B). Both β-hTERT and α+β+hTERT transcript levels were detected at about 40% of hTERT variant pool measured by TaqMan RT-qPCR (Fig. 2.1), and were significantly higher in abundance (~5-10 fold higher) than α-hTERT and α-β-hTERT transcript levels (Fig. 3.1 B).

### *3.1.2 Characterization of hTERT isoforms in human embryonic stem cells*

HES2 cells were grown under 2% oxygen conditions, with and without doxycycline (DOX) and 100 μM $H_2O_2$ treatment to assess the potential side-effect of each treatment. The percent (%) proportion of selected hTERT isoforms in each treatment group was investigated by TaqMan RT-qPCR (Fig. 2.1). When cells were grown under 2% oxygen, the transcript composition of α+β+hTERT and β-hTERT remained at approximately 40% and dominant over α-hTERT and α-β-hTERT (in comparison to when the cells grew in 21% oxygen; Fig. 3.2 A and E). However, the splicing pattern changed when $H_2O_2$ or doxycycline was singly added to the culture media (Fig. 3.2 B and C). The proportion of β-hTERT transcript level

were increased in relation to α+β+hTERT and other isoforms when doxycycline and $H_2O_2$ treatments were applied individually (Fig. 3.2 B and C). In case of doxycycline treatment, the percentage of α-β-hTERT transcript decreased slightly (statistical significance; Fig. 3.2 E). A similar decease in α-β-hTERT transcript levels were also observed when both $H_2O_2$ and doxycycline were applied in combination. Neither oxygen tension nor different combinations of doxycycline and $H_2O_2$ resulted in significant change in any hTERT isoform transcripts except in α-β-hTERT (Fig. 3.2 E). Interestingly, the difference between % α+β+hTERT and % β-hTERT was not observed when DOX and $H_2O_2$ were applied simultaneously (Fig. 3.2 D). In all circumstances, β-hTERT was the most abundant hTERT transcript species expressed in the HES2 hESC line.

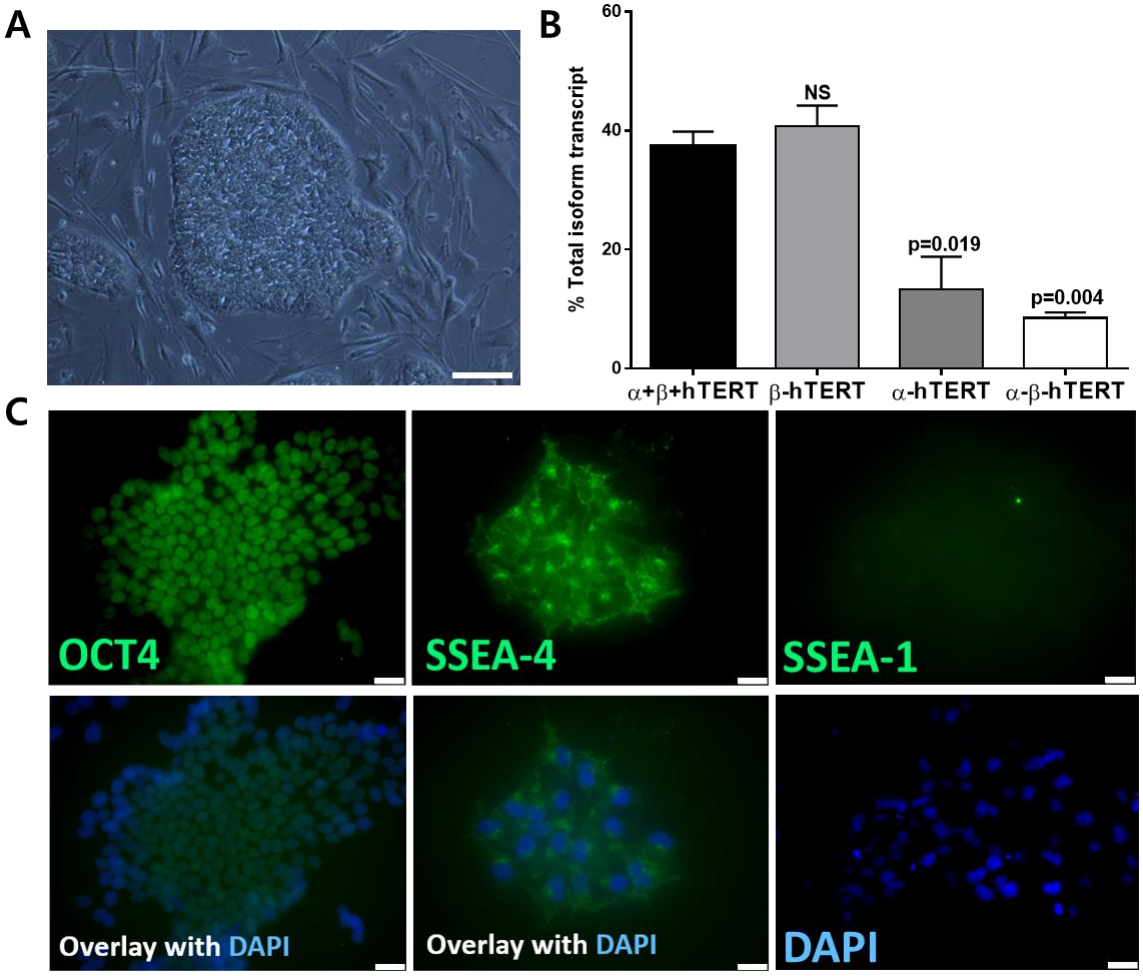



**Fig. 3.1: HES2 is a hESC cell line that expresses various markers of pluripotency and different proportions of endogenous telomerase reverse transcriptase (TERT) isoform transcripts.** **A)** HES2 (Non-transgenic) hESC colony grown on mitotically inactivated mouse embryonic fibroblast (MEF) feeder cells. Scale bar (white) = 200µm **B)** HES2 was cultured under ambient oxygen tension (~21% $O_2$) and was harvested for RT-qPCR analyses. Primers specific for four different hTERT isoforms (α+β+, β-, α-, α-β-) were used to examine TERT isoform composition (n=4, error bars = ±SEM). **C)** HES2 was cultured under 2% $O_2$ in MEF-free environment to sub-confluency. Cells were fixed and assayed by immunofluorescence microscopy for OCT4, SSEA-4 (positive pluripotency markers), and SSEA-1 (negative pluripotency marker). Cells were counter-stained with DAPI. Scale Bars (white) = 25µm. NS = non-significant.

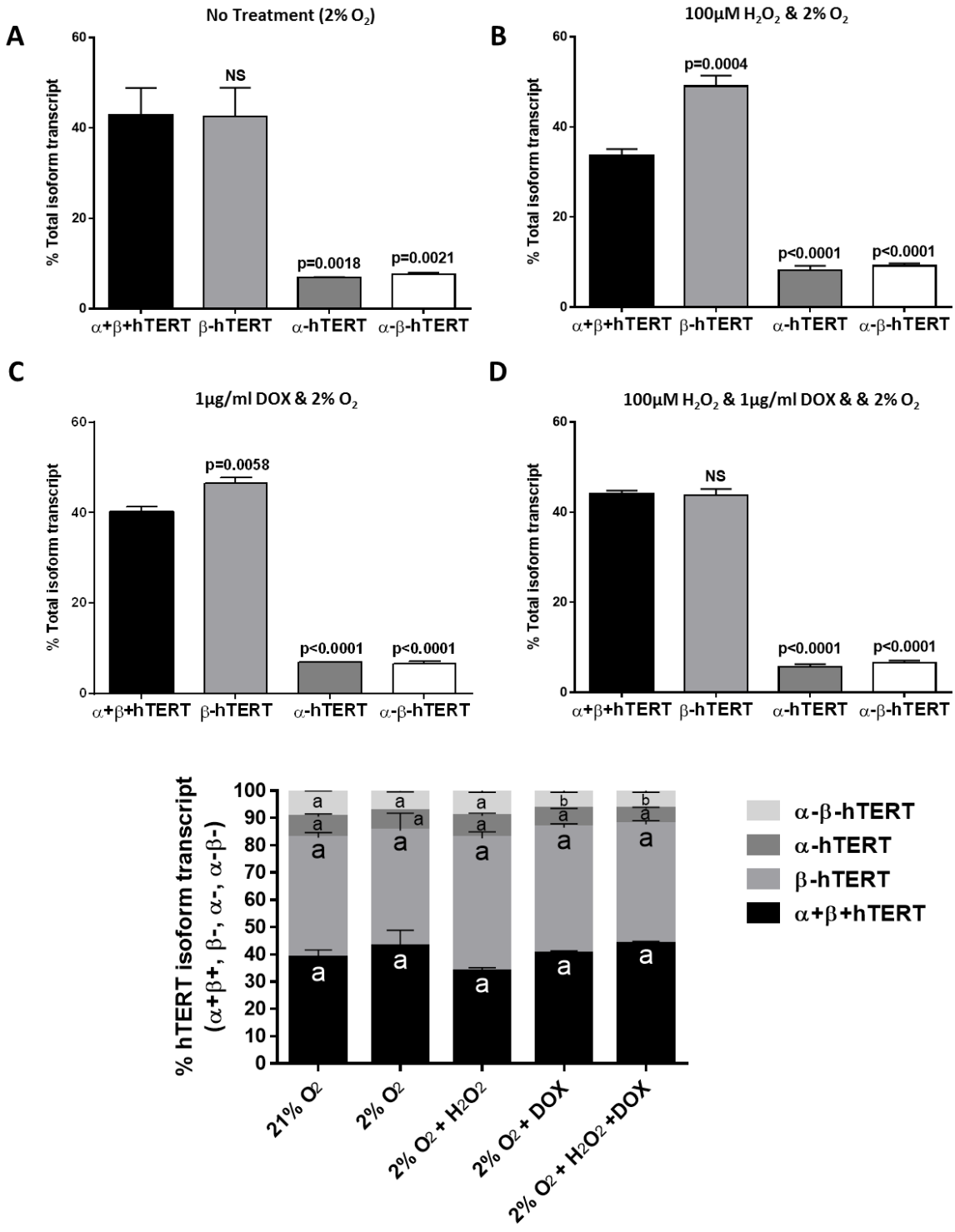



**Fig. 3.2: Endogenous β-hTERT transcript is ubiquitously present at high levels in human embryonic cells grown under low oxygen tension conditions, with and without $H_2O_2$ and/or doxycycline (DOX) treatments.** hESCs (HES2 cell line) were grown under low oxygen conditions (2% $O_2$) with **A)** no treatment, **B)** 100μM $H_2O_2$, **C)** 1μg/ml DOX, **D)** 100μM $H_2O_2$ and 1μg/ml DOX, **E)** Combined representation of %hTERT isoform change under conditions specified in A-D. Endogenous hTERT variant transcript levels were examined by RT-qPCR (n=3, error bars = ±SEM). NS = non-significant.

## 3.2 Characterization of 3xFLAG-hTERT transgenic clones

### *3.2.1 Characterization of 3xFLAG-hTERT transgene expression level in transgenic hESC clones*

DOX-induced 3xFLAG-hTERT isoform expression was examined in a broad panel of HES2 clones by Western blot analysis to select the number of lines for closer examination based on their transgene expression levels. Four HES2 clonal hESC lines overexpressing 3xFLAG-ß-hTERT (B2, B3, B6, B7) and four HES2 clones overexpressing 3xFLAG-FL-hTERT (FL7, FL9, FL10, FL12) were randomly chosen and analyzed for protein expression (appendix A3: Fig. 3.1). Based on the preliminary result of 3xFLAG-hTERT induction 12-hours post doxycycline, the selection was further narrowed down to the least expressive β-clone HES2 B3, the most expressive HES2 B6, a FL-clone with similar level of protein induction as B6 HES2 FL9, and the most expressive FL-clone HES2 FL12. HES2 B3 and HES2 B6 hESC lines exhibited the highest levels of 3xFLAG-β-hTERT transcripts. HES2 FL7 and HES2 FL12 hESC clonal cell lines were the least and the most expressive clones for 3xFLAG-FL-hTERT, respectively (appendix A3: Fig 3.1). 3xFLAG-hTERT expression in the selected HES2 hESC clones (B3, B6, FL9, FL12) were examined more closely over a doxycycline time course via RT-qPCR (n=1) and Western blot analysis (Fig. 3.3). Clones B3 and B6 had similar transgene expression at both the transcriptional and translation level. B3 and B6 clones, however, showed much lower transgenic steady-state protein expression levels than FL9 and FL12 hESC clones. Of all, FL12 exhibited the highest FLAG-tagged protein levels at all DOX treatment times. Clone FL9 had markedly lower protein levels than the FL12 clonal hESC line (Fig.3.3 A and C).

*3.2.2 Characterization of Pluripotency in HES2 B6 and HES2 FL12 hTERT cell lines*

HES2 B6 and FL12 were treated with DOX (2 days; 1 μg/ml) and with $H_2O_2$ (final 4 hours; 100μM) to assess the potential side effect of DOX and $H_2O_2$ treatment on pluripotency marker expression. Positive markers of pluripotency (OCT-4, SSEA-4) were present under all treatment conditions, while the negative marker of pluripotency (SSEA-1) remained absent (Fig. 3.4 and Fig. 3.5).

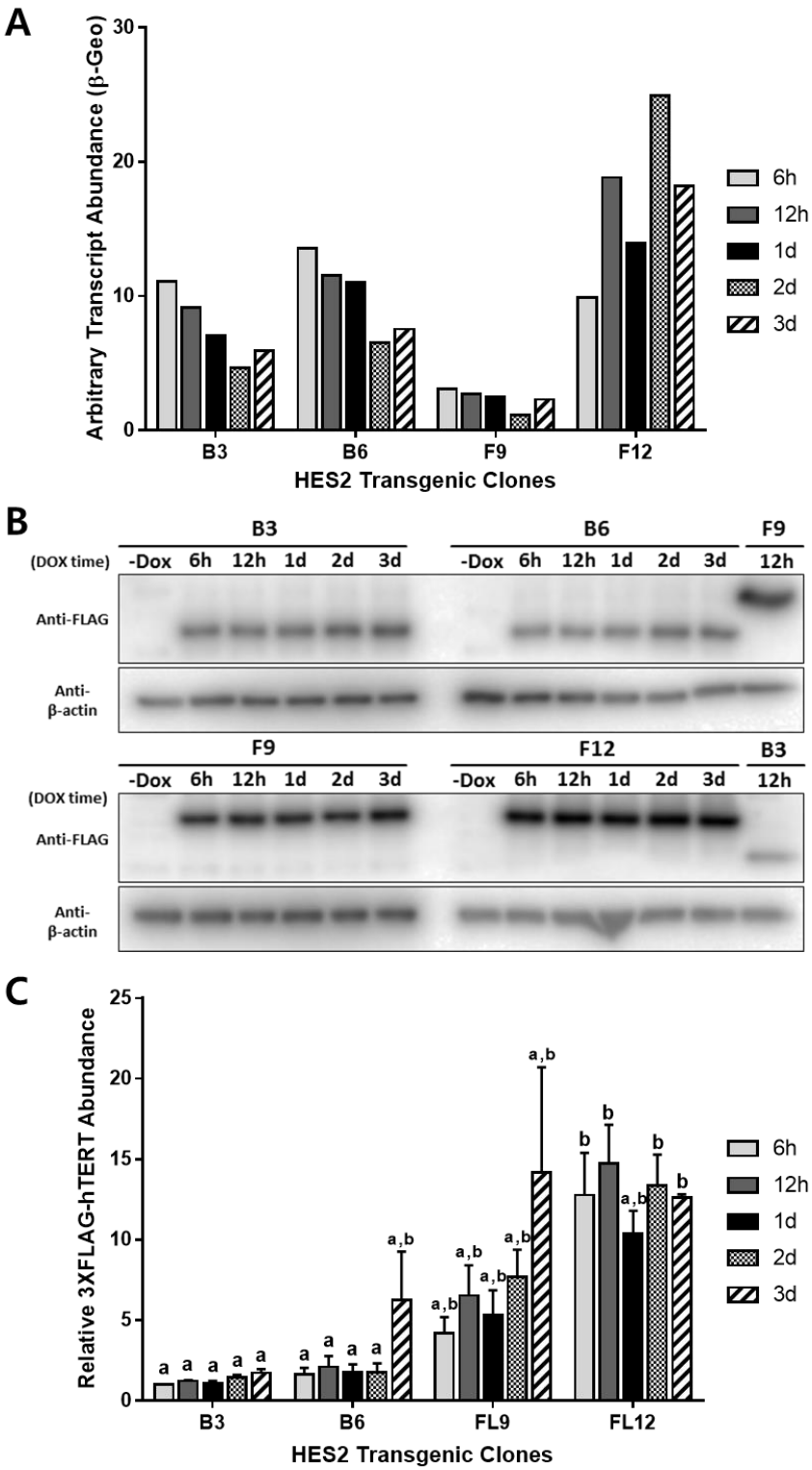



**Fig. 3.3: RT-qPCR and Western blot analysis of doxycycline-induced expression of 3xFLAG-hTERT isoforms in HES2 clones over 3-day time course.** HES2 clones (HES2 B3 and B6 for DOX-inducible 3xFLAG-β-hTERT, and HES2 FL9 and FL12 for DOX-inducible 3xFLAG-FL-hTERT) were cultured under 2% $O_2$ and were treated with varying times (h = hours, d = days) of doxycycline (DOX) prior to harvest. **A)** In a pilot study, the abundance of various 3xFLAG-hTERT isoform transcripts was examined by RT-qPCR (n=1) **B)** Western blot using anti-FLAG antibody. **C)** Densitometry analysis was performed for quantification of the proteins. Data was normalized to β-actin (n=3, error bars = ±SEM).

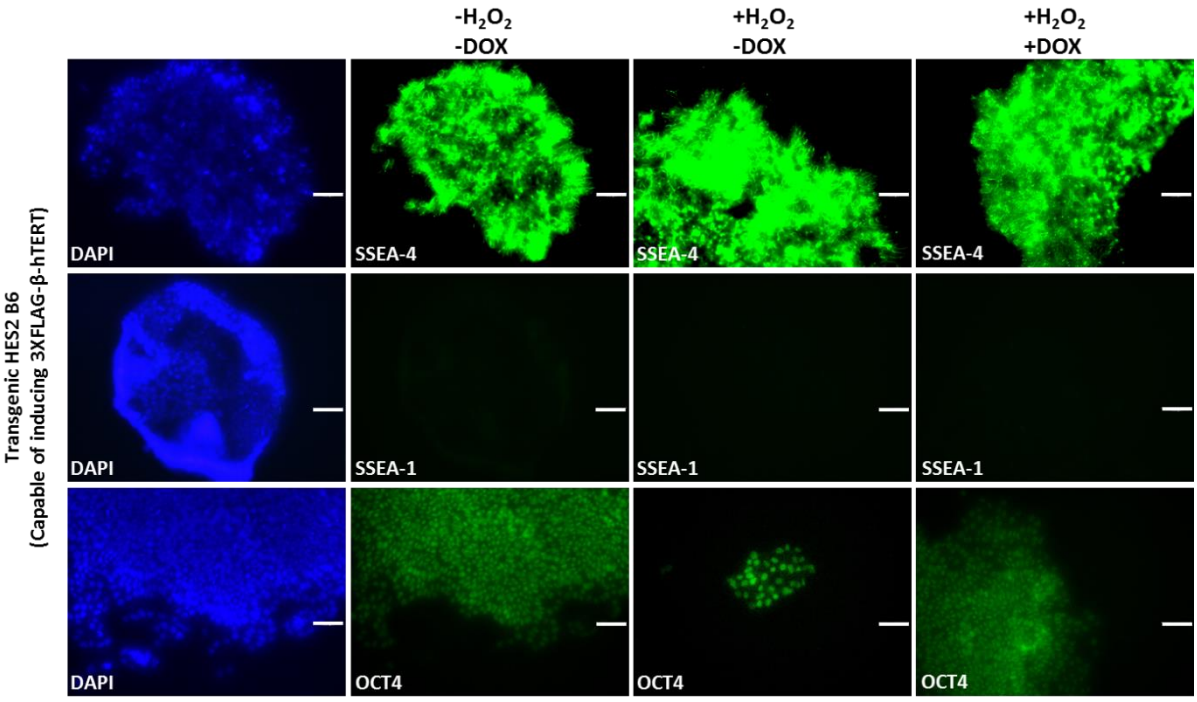



**Fig. 3.4: $H_2O_2$ and DOX treatments do not affect pluripotency marker expression in the 3xFLAG-β-hTERT transgenic HES2 B6 line.** HES2 B6 (transgenic hESC line with DOX-inducible 3xFLAG-β-hTERT gene) was cultured in feeder free conditions under 2% $O_2$. Cells were treated with or without DOX (2 days; 1 µg/ml) and $H_2O_2$ (4 hours; 100µM) prior to fixation. Cells were stained for positive markers of pluripotency (OCT4 & SSEA-4; green fluorescence) and a negative marker of pluripotency (SSEA-1). DAPI (blue fluorescence) was used as counterstain and representative DAPI images are only shown for the first treatment group (-$H_2O_2$, -DOX). Scale bars = 60µm.

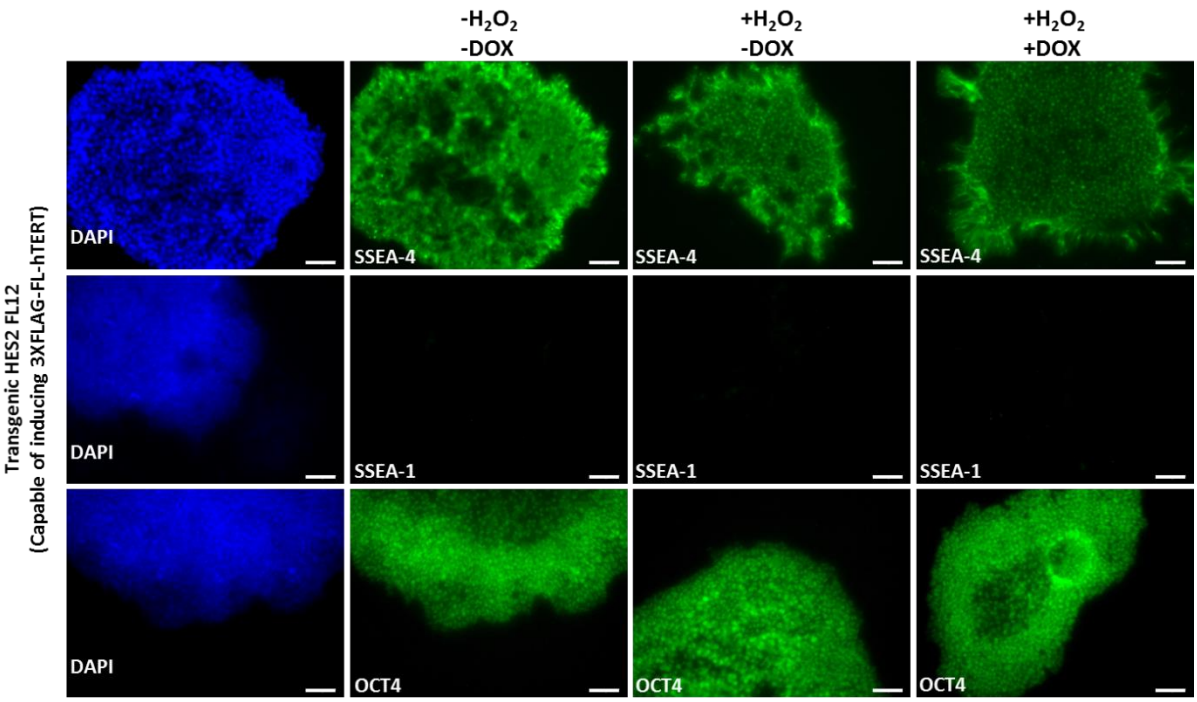



**Fig. 3.5: $H_2O_2$ and DOX treatments do not affect pluripotency of the 3xFLAG-FL-hTERT transgenic HES2 FL12 lines.** HES2 FL12 (transgenic hESC line with DOX-inducible 3xFLAG-FL-hTERT gene) were cultured in feeder free condition under 2% $O_2$. Cells were treated with or without DOX (2 days; 1 µg/ml) and $H_2O_2$ (4 hours; 100µM) prior to fixation. Cells were stained for positive markers of pluripotency (OCT4 & SSEA-4; green fluorescence) and a negative marker of pluripotency (SSEA-1). DAPI (blue florescence) was used as counterstain and representative DAPI images are only shown for the first treatment group (-$H_2O_2$,-DOX). Scale bars = 60µm.

## 3.3 Characterization of DOX-induced recombinant hTERT in HES2 B6 and HES2 FL12

### *3.3.1 Endogenous TERC-binding of 3xFLAG-β-hTERT and 3xFLAG-FL-hTERT subunits*

RNA-immunoprecipitation (RIP) using a FLAG antibody was performed to determine whether the 3xFLAG-hTERT transgenic proteins have the ability to bind to the telomerase RNA-component (TERC). TERC recovery from the RIP eluents of DOX-treated samples and non-DOX-treated samples were compared. Overexpression of 3xFLAG-hTERT by doxycycline prior to RIP resulted in approximately 60-fold increase in TERC recovery for 3xFLAG-FL-hTERT, and 10-fold increase for 3xFLAG-β-hTERT (Fig. 3.6 A, B, C). Doxycycline-treated HES2 (non-transgenic; negative control) did not result in any change in TERC recovery, and resulted in a very low TERC PCR-amplification (Fig. 3.6 D).

### *3.3.2 Endogenous RMRP-binding of 3xFLAG-β-hTERT and 3xFLAG-FL-hTERT subunits*

Another RIP using a FLAG antibody was performed to determine whether the 3xFLAG-hTERT transgenic proteins have the ability to bind to the RNA component of RNase MRP (RMRP). Overexpression of 3xFLAG-hTERT by doxycycline prior to RIP resulted in an approximately 4-fold increase in RMRP recovery for 3xFLAG-FL-hTERT, and a 2-fold increase for 3xFLAG-β-hTERT (Fig. 3.7 A, B, C). RMRP recovery from DOX-treated HES2 (non-transgenic; negative control) was not different from its control. Notably, the amount of PCR-amplified RMRP from HES2 control group (Fig. 3.7 D) was significant and was comparable to that from 3xFLAG-β-hTERT RNA immuoprecipitates (Fig. 3.7 B).

*3.3.3 Telomerase activity in HES2 B6 and HES2 FL12 hESCs*

The effect of 3xFLAG-β-hTERT and 3xFLAG-FL-hTERT overexpression on total telomerase activity of HES2 B6 and FL12 cell lines was investigated by the telomerase repeat amplification protocol (TRAP) assay (Fig. 3.8). Negative controls (heat-inactivated lysate and no-lysate control) did not generate any product bands (Fig.3.8 A). Doxycycline in non-transgenic HES2 had no effect on the endogenous telomerase activity (Fig. 3.8 B top). DOX-induction of recombinant β-hTERT in HES2 B6 resulted in ~30% decrease in the total telomerase activity, whereas DOX-induction of recombinant FL-hTERT in HES2 FL12 had no effect (Fig. 3.8 B bottom left and right, respectively).

*3.3.4 Re-visiting DOX-induction of 3xFLAG-hTERT transcription in HES2 clones*

Since overexpression of 3xFLAG-FL-hTERT did not result in an the expected increase in telomerase activity in HES2 FL12 (Fig. 3.8 B), we re-investigated the 3xFLAG-hTERT transgene expressions and endogenous hTERT isoform transcript abundance in the trangenic HES2 clones. The transcript level of different hTERT isoforms in HES2 FL12 and HES2 B6 was examined after DOX-induction by hydrolysis-probe-assisted RT-qPCR. The addition of DOX in HES2 FL12 resulted in ~500-fold increase in FL-hTERT transcript, but suprisingly a ~300-fold increase in β-hTERT transcript, and ~200-fold increase in α-β-hTERT transcript was also observed compared to non-induced controls (Fig.3.9 A). Interestingly, the HES2 B6 cell line expressed only the β-deletion-containing TERT isoforms upon doxycycline treatment. β-hTERT abundance rose sharply (~1000-fold in comparison with its non-induced control) after doxycycline treatment in HES2 B6. A ~150-fold increase in α-β-hTERT was also observed (Fig.3.9 B).

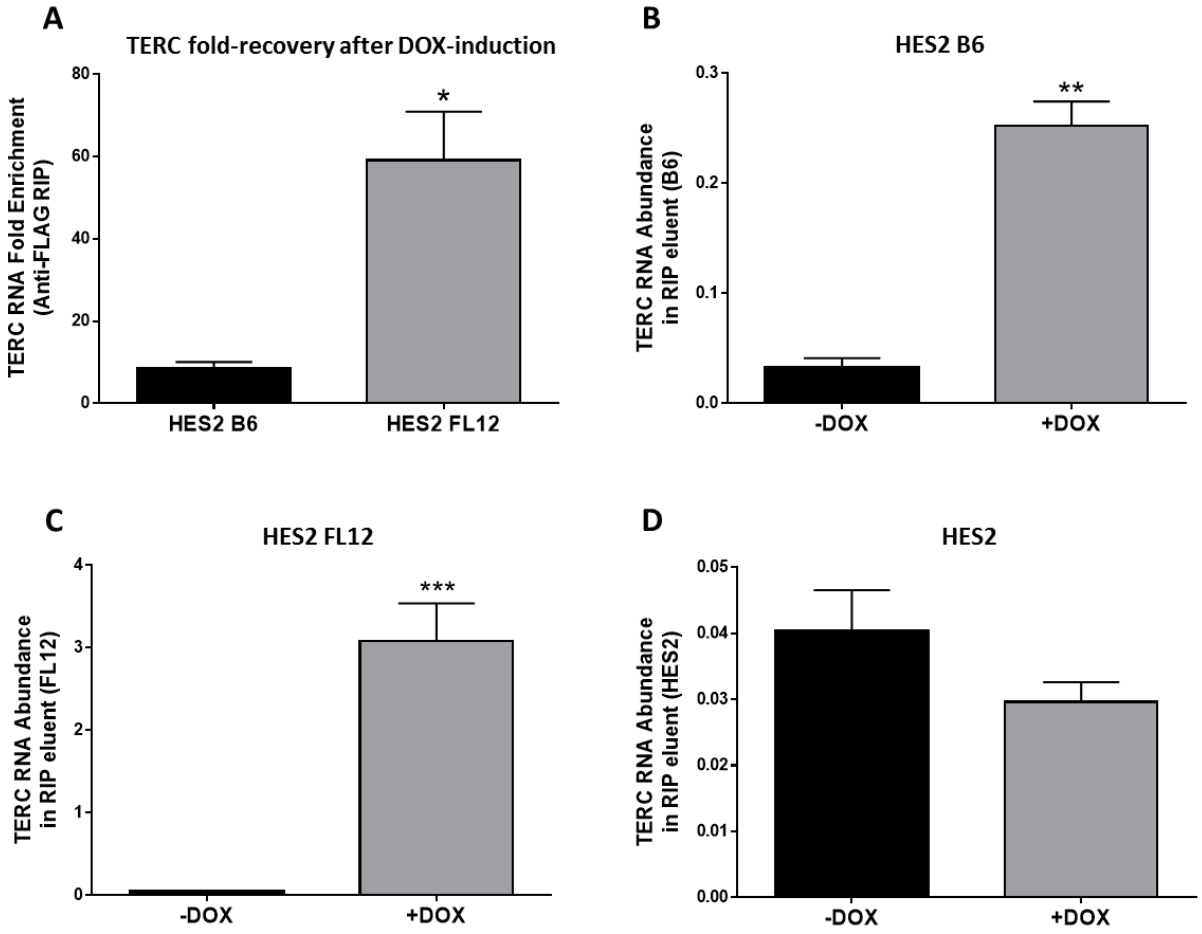



**Fig. 3.6: Doxycycline-induced transgenic telomerase proteins (3xFLAG-β-hTERT and 3xFLAG-FL-hTERT) bind to the telomerase RNA component (TERC).** Cells were grown under 2% $O_2$ and were treated with 1μg/ml doxycycline (DOX) for two days prior to harvest. DOX-induced 3xFLAG-hTERT isoforms were isolated from their respective clones by immunoprecipitation using anti-FLAG-magnetic beads. Eluted proteins were subsequently denatured to release any bound RNA for downstream RT-qPCR analysis. **A)** Comparison of results in HES2 transgenic lines, and individual TERC enrichment data from **B)** HES2 B6 **C)** HES2 FL12 **D)** Non-transgenic HES2 control (n=3, error bar=±SEM, **=P<0.01, ***= P<0.001).

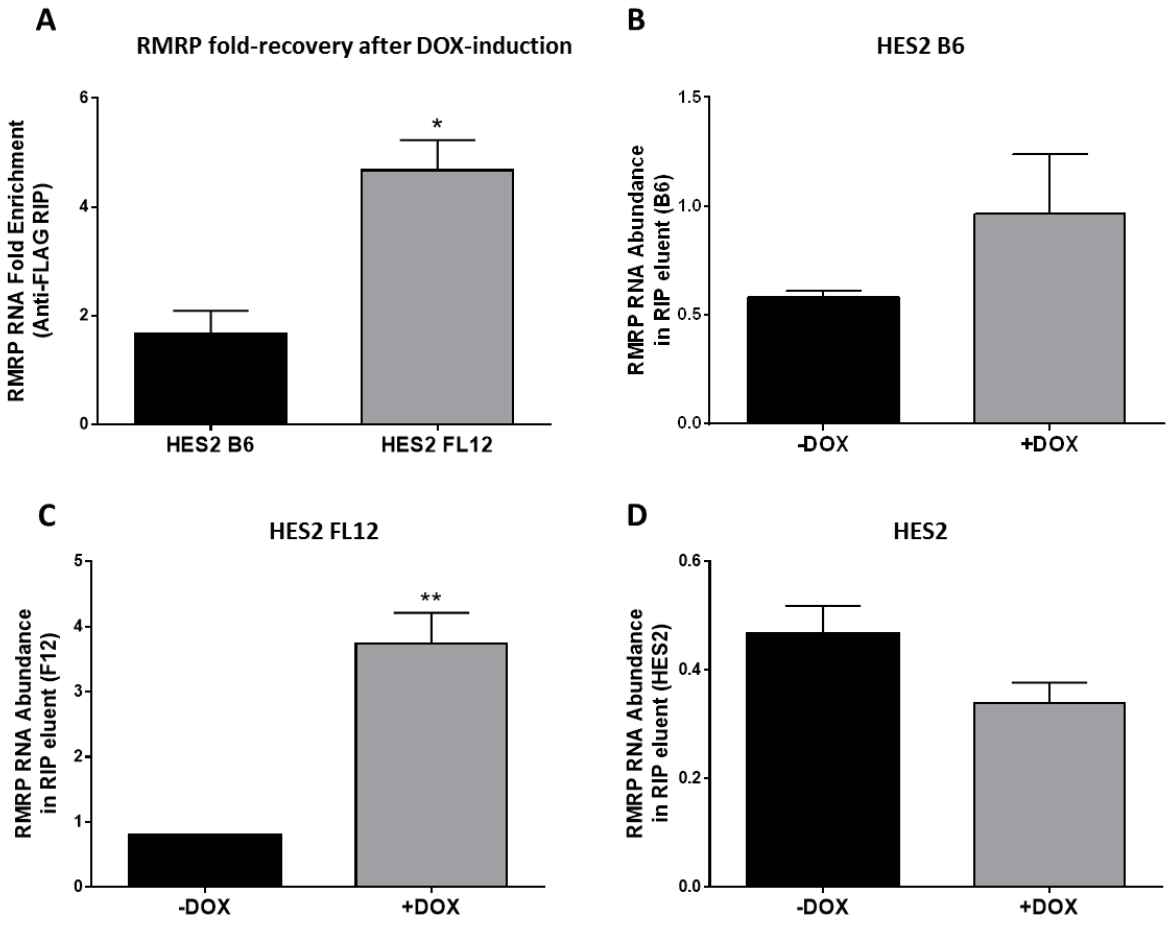



**Fig. 3.7: Enrichment of RNA component of mitochondrial RNA processing endoribonuclease (RMRP by doxycycline-induced transgenic telomerase proteins (3xFLAG-β-hTERT and 3xFLAG-FL-hTERT).** Cells were grown under 2% $O_2$ and were treated with 1μg/ml doxycycline (DOX) for two days prior to harvest. DOX-induced 3XFLAG-hTERT isoforms were isolated from their respective clones by immunoprecipitation using anti-FLAG-magnetic beads. Eluted proteins were subsequently denatured to release any bound RNA for downstream RT-qPCR analysis. **A)** Comparison of results in HES2 transgenic lines, and individual RMRP enrichment data from **B)** HES2 B6 **C)** HES2 FL12 **D)** Non-transgenic HES2 control (n=3, error bar = ±SEM).

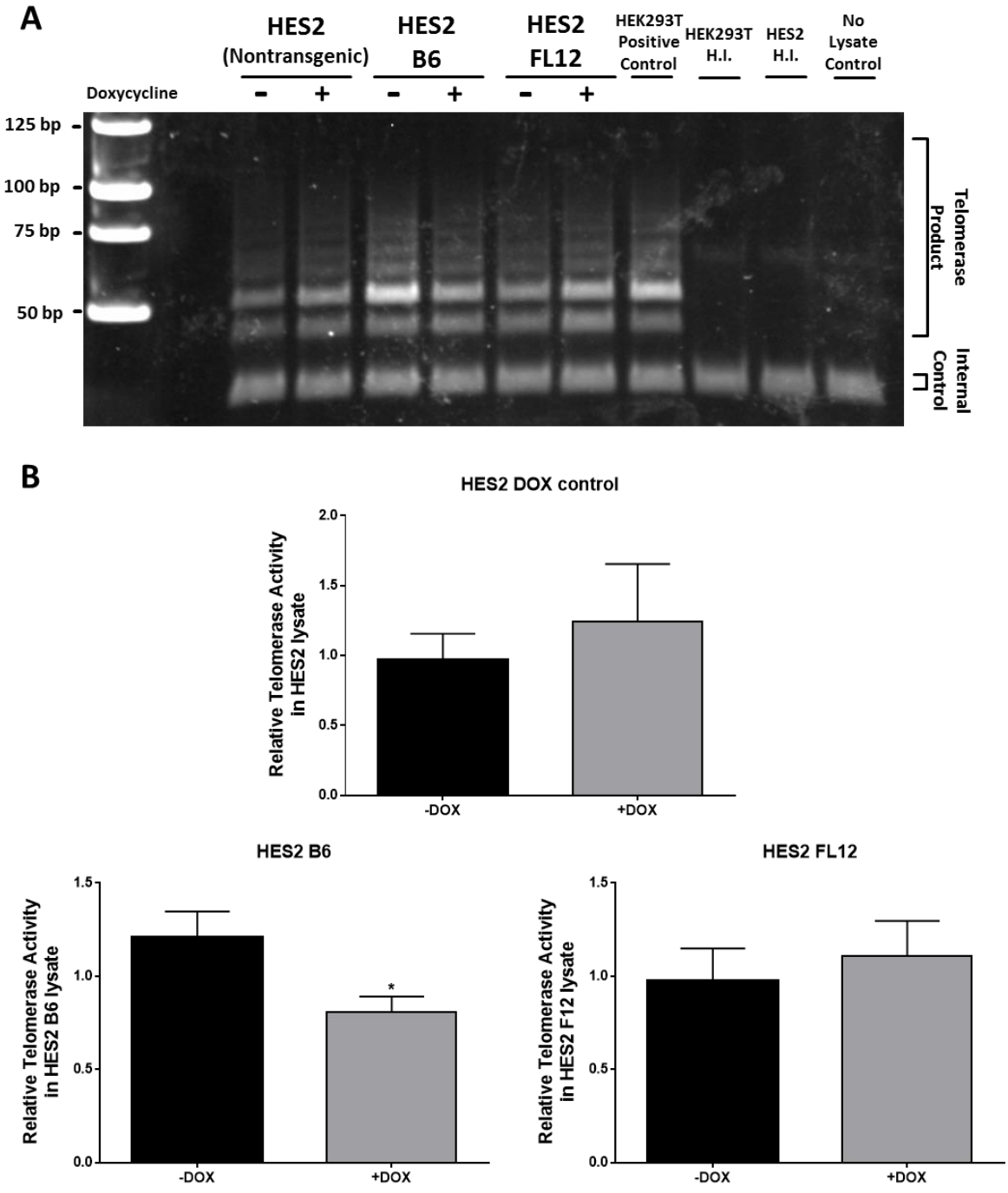



**Fig. 3.8: Altered telomerase activity levels in doxycycline (DOX)-induced transgenic telomerase proteins (3xFLAG-hTERT) in hESCs.** Cells were grown under 2% $O_2$ and were treated with 1µg/ml doxycycline for two days prior to harvest. **A)** Representative DNA gel of telomerase-repeat amplification assay by qPCR. Relative telomerase activity between samples is reflected in the intensity and the range of telomerase product bands. H.I. = Heat-Inactivated **B)** Band intensity of amplified telomerase product was measured by densitometry analysis and represented as relative telomerase activity. Data were normalized to internal control band intensities (n=4, error bar=±SEM).

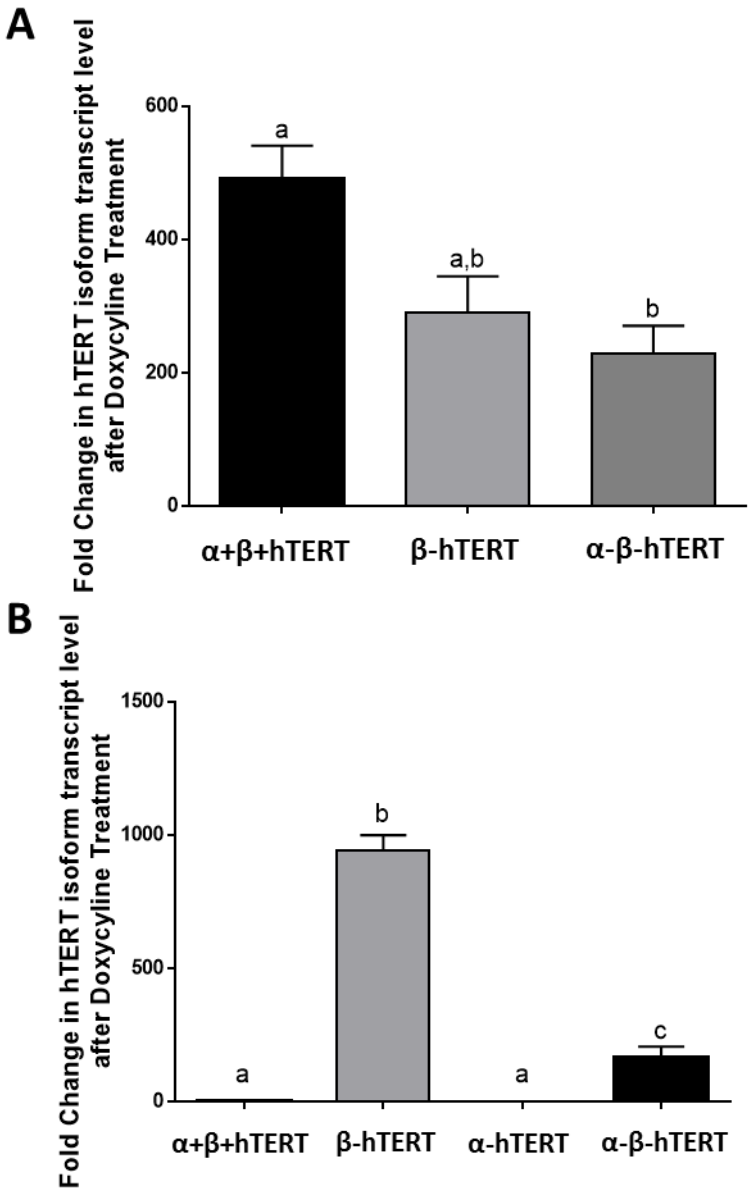



**Fig. 3.9: DOX-induction of 3xFLAG-hTERT isoforms results in a simultaneous increase in the amount of other hTERT transcript variants containing β-deletion. A)** HES2 FL12 and **B)** B6 were grown under 2% $O_2$ and were treated with 1μg/ml doxycycline for two days prior to harvest. hTERT variant transcript abundance in each sample was examined by RT-qPCR using primer sets specific for α+β+, α-, β-, α-β-. *The primers do not discriminate endogenous hTERT species from transgenic hTERT species.* n=3, error bars = ±SEM.

## 3.4 3xFLAG-hTERT localization under $H_2O_2$-induced oxidative stress

### *3.4.1 Sub-cellular Western Blot Analysis of 3xFLAG-FL-hTERT and 3xFLAG-β-hTERT expressing hESC lines*

$H_2O_2$ (100μM) was applied to promote mitochondrial localization of 3xFLAG-β-hTERT and 3xFLAG-FL-hTERT in the respective transgenic cell lines. Cells were fractionated into nuclear fraction and mitochondrial fraction for protein analysis. Western blot analysis of sub-cellular fractions demonstrated that the level of mitochondrial 3xFLAG-β-hTERT appears to increase with 1h $H_2O_2$ (100μM) treatment, but fluctuates with longer $H_2O_2$ exposure (Fig. 3.10 Column A). However, no statistical significance ($p>0.05$) was observed between different treatment groups. Similarly, 3xFLAG-FL-hTERT levels in the mitochondrial fraction initially increased at 30-minute post $H_2O_2$ (100μM) treatment, but decreased over longer exposure time (Fig. 3.11 Column A). Again, no statistical significance ($p>0.05$) was detected between different treatment groups. Similarly, neither the levels of 3xFLAG-β-hTERT nor 3xFLAG-FL-hTERT changed significantly in the nuclear fractions over the $H_2O_2$ treatment time course (Fig. 3.10 B and Fig. 3.11 B). Additionally, I failed to observe significant intra-cellular movement of 3xFLAG-hTERT when I performed the same fractionation experiment using prohibitin as a mitochondrial marker (appendix A4: Fig. 3.2).

### *3.4.2 Immunocytochemistry and Image analysis of 3xFLAG-FL-hTERT and 3xFLAG-β-hTERT localization in hESCs*

Due to the inconclusive results obtained by the cellular fractionation studies, I monitored 3xFLAG-β-hTERT and 3xFLAG-FL-hTERT cellular localization by immunofluorescence microscopy. I failed to see co-localized signal from MitoTracker (mitochondria) and M2-anti-FLAG/Alexa Fluor 488 (3xFLAG-hTERT) in either HES2

transgenic clones (Fig. 3.12). Much of 3xFLAG-β-hTERT and 3xFLAG-FL-hTERT remained in nuclei regardless of different durations of $H_2O_2$ exposure (Fig. 3.12). Such nuclear localization of 3xFLAG-FL-hTERT persisted even when HES2 FL12 cells were exposed to 21% $O_2$ for 3 days (appendix A5: Fig.3.3). Interestingly, there appeared to be distinct patterns of 3xFLAG-hTERT species' distribution within the nuclei. Most cells exhibited 'uniform' intra-nuclear distribution of 3xFLAG-hTERT (indicated by the red arrow in Fig. 3.12). Some nuclei exhibited 'peripheral' intra-nuclear distribution of 3xFLAG-hTERT (indicated by the white arrow in Fig. 3.12). The number of cells exhibiting the different intra-nuclear distributions of 3xFLAG-hTERT was counted from immunofluorescence images. However, the addition of doxycycline to HES2 B6 (Fig. 3.13 A) and HES2 FL12 (Fig. 3.13 B) resulted in no significant change in the intra-nuclear localization of 3xFLAG-hTERT.

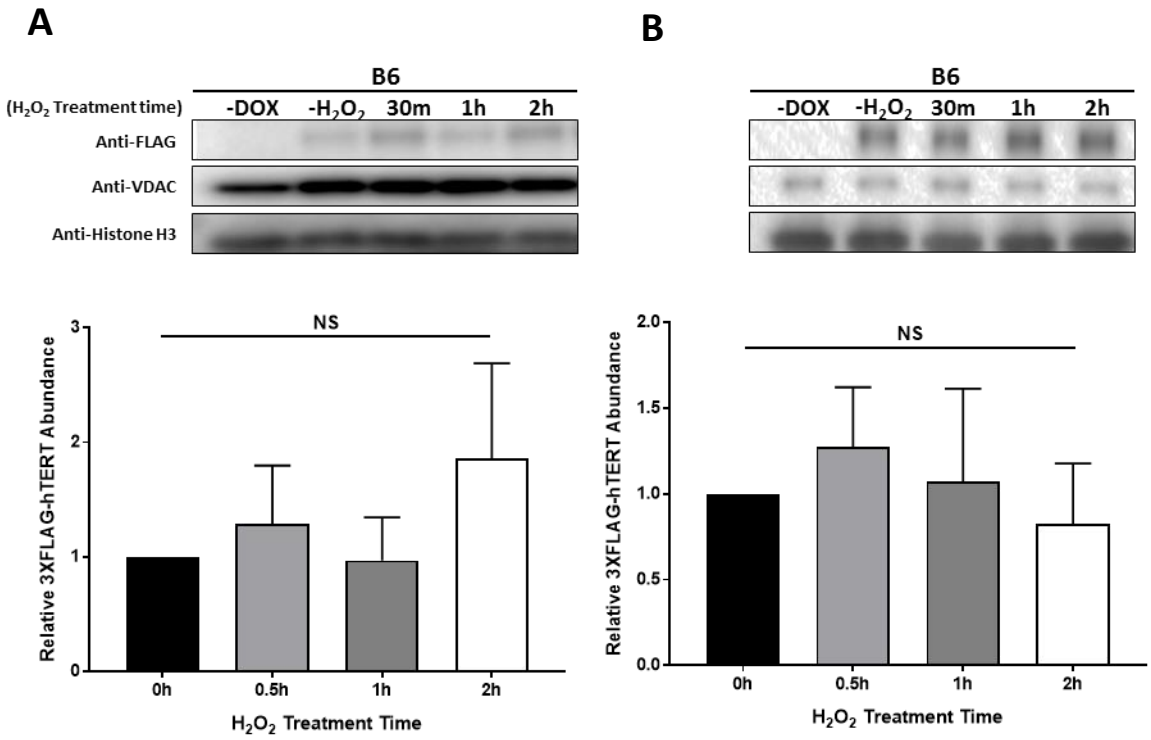



**Fig. 3.10: $H_2O_2$-induced Oxidative stress and its effect on the cellular localization of 3xFLAG-β-hTERT.** Cells were grown under 2% $O_2$ and were treated with 1μg/ml DOX for two days prior to harvest. 100μM $H_2O_2$ was applied at different times (30m, 1hr, 2hr) prior to harvest. Nuclei-enriched fraction was isolated via fractionation by differential centrifugation. The amount of 3xFLAG-hTERT species in respective sample groups were investigated by Western blot/densitometry analysis. Results were normalized to VDAC and Histone H3 for mitochondrial and nuclear fractions, respectively. Densitometry results of treatment groups are relative to $H_2O_2$ negative control. Western Blot and densitometry analysis plots of **A)** mitochondrial fraction and **B)** nuclear fraction. n=3, error bar=±SEM. NS = non-significant

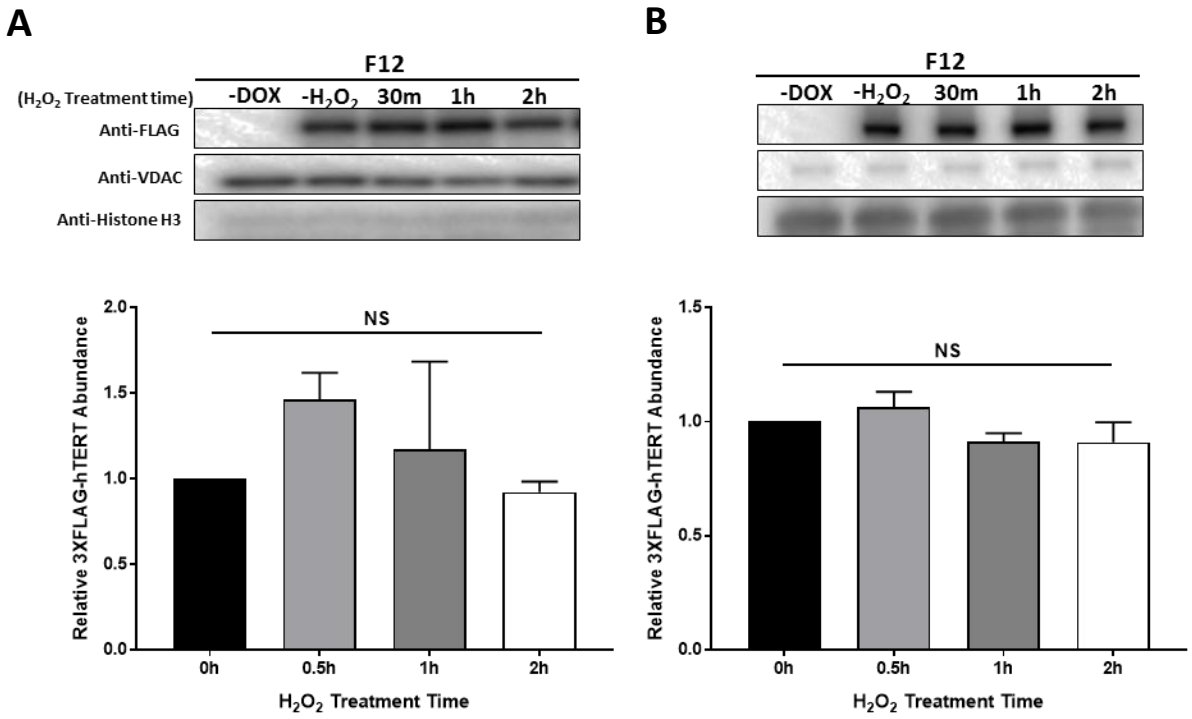



**Fig. 3.11: $H_2O_2$-induced Oxidative stress and its effect on the cellular localization of 3xFLAG-FL-hTERT.** Cells were grown under 2% $O_2$ and were treated with 1µg/ml DOX for two days prior to harvest. 100µM $H_2O_2$ was applied at different times (30m, 1hr, 2hr) prior to harvest. Nuclei-enriched fraction was isolated via fractionation by differential centrifugation. The amount of 3xFLAG-hTERT species in respective sample groups were investigated by Western blot/densitometry analysis. Results were normalized to VDAC and Histone H3 for mitochondrial and nuclear fractions, respectively. Densitometry results of treatment groups are relative to $H_2O_2$ negative control. Western Blot and densitometry analysis plots of **A)** mitochondrial fraction and **B)** nuclear fraction. n=3, error bar=±SEM. NS = non-significant

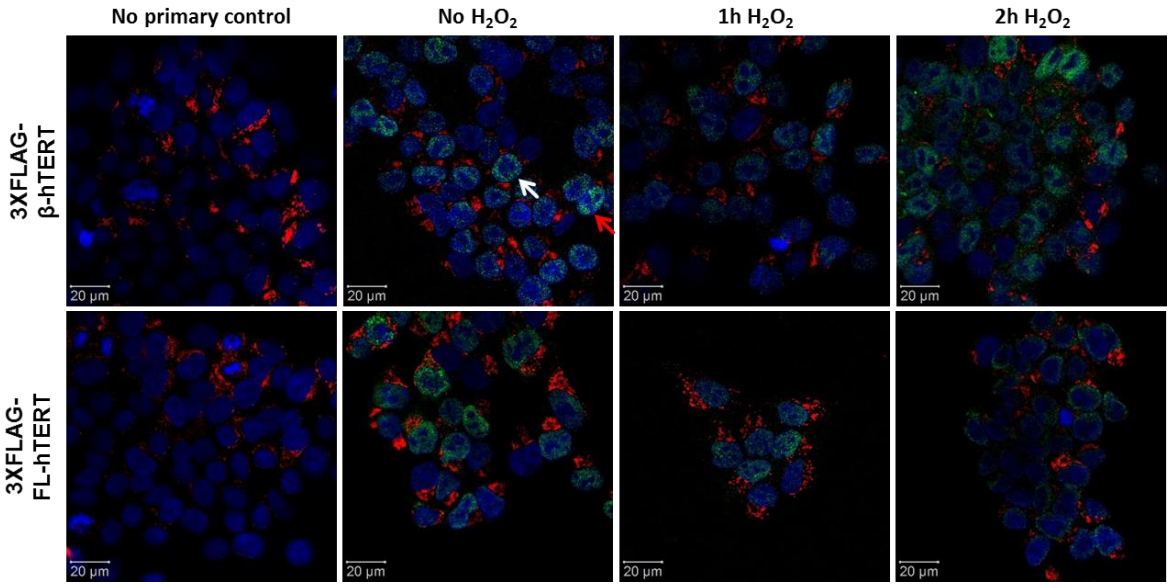



**Fig. 3.12: $H_2O_2$-induced effect on the intra-cellular localization of 3xFLAG-β-hTERT and 3xFLAG-FL-hTERT in hESCs.** Transgenic hESCs were grown under 2% $O_2$ and transgenes induced by treated with 1µg/ml DOX for two days prior to harvest. 100µM $H_2O_2$ was applied at different durations (1hr, 2hr) prior to fixation. Immunofluorescence-microscopy images of 3xFLAG-hTERT (green fluorescence), mitochondria (MitoTracker; red fluorescence), and nuclei (DAPI; blue fluorescence) in HES2 transgenic clones. Images were taken using a confocal microscope (63X oil-immersion objective lens, x10 eyepiece lens). 3xFLAG-hTERT were predominantly nuclear, and exhibited either peripheral localization (white arrow; upper row second column) or more uniform distribution within nuclei (red arrow). Scale bar = 20 µm.

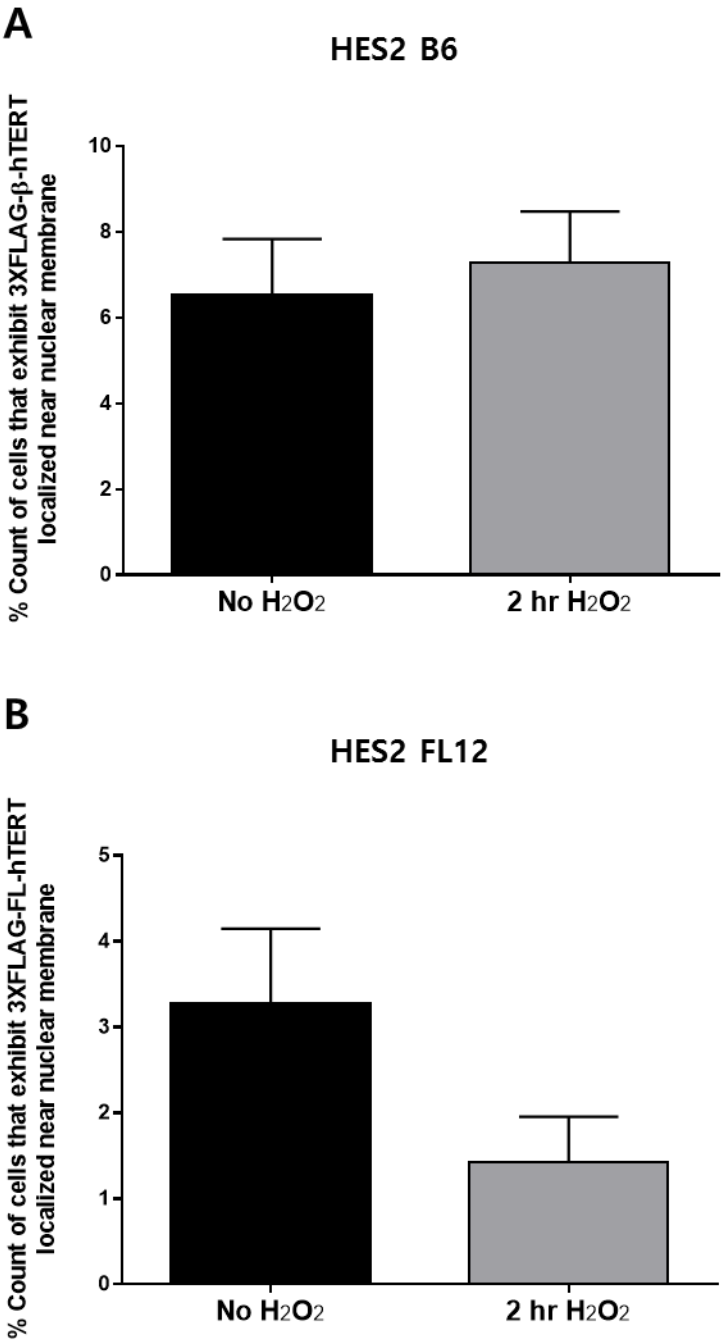



**Fig. 3.13: Microscopic analysis of intra-nuclear localization of 3xFLAG-β-hTERT and 3xFLAG-FL-hTERT under $H_2O_2$-induced oxidative stress.** Multiple confocal images of HES2 B6 and HES2 FL12 (see Fig. 3.12) were taken and blinded. Percentages of cells that exhibited peripheral 3xFLAG-hTERT localization was counted and averaged by three volunteers. Only the whole and fully visible cells (nuclei) were taken into account. **A)** 3xFLAG-β-hTERT (n=11) **B)** 3xFLAG-FL-hTERT (n=12). Error bars ±SEM

## 3.5 The effect of overexpressed 3xFLAG-hTERT on mitochondrial function

### *3.5.1 The effect of DOX-induced 3xFLAG-hTERT on mitochondria membrane potential*

It was previously shown that ectopic expression of FL-hTERT augments mitochondria membrane potential in human embryonic lung fibroblast cells [195]. Human embryonic stem cells (HES2 cell line) were stained with JC-1 dye, which accumulates within the mitochondria and varies its fluorescence excitation in an MMP-dependent manner. The results were examined and analyzed by flow cytometry (Fig. 3.14). Both 3xFLAG-β-hTERT and 3xFLAG-FL-hTERT overexpression in hESCs appeared to slightly decrease MMP activity ($2^{nd}$ and $3^{rd}$ bars) compared to the doxycycline control group in non-transgenic HES2 cells ($1^{st}$ bar) (Fig. 3.15). However, no significant change in MMP was observed for either transgenic clones.

### *3.5.2 The effect of DOX-induced 3xFLAG-hTERT on mitochondrial DNA copy number*

A previous report has shown that ectopically expressed hTERT results in decreased mitochondrial DNA copy number in human embryonic lung fibroblast cells under 21% oxygen tension [195]. The relative mitochondrial DNA copy number was compared in HES2 B6 and HES2 FL12 clones before and after DOX-induction of 3xFLAG-hTERT species. Approximately a 20% decrease (statistically significant) in mitochondrial DNA copy number was observed when HES2 cells were treated with doxycycline (Fig. 3.16 A). Interestingly, doxycycline treatment of HES2 B6 and HES2 FL12 cells resulted in neither reduction nor increase of mitochondrial DNA copy number (Fig. 3.16 B and C).

*3.5.3 The effect of DOX-induced 3xFLAG-hTERT on mitochondria biogenesis and function*

Mitochondria biogenesis is regulated by several nuclear genes. A panel of markers for mitochondria biogenesis was monitored by RT-qPCR after doxycycline treatment in HES2 clonal lines (Fig. 3. 17). No significant change in steady-state transcript abundance was observed for any of the examined genes. Notably, POLG (mitochondrial DNA polymerase subunit) did not change with doxycycline treatment in any of the HES2 lines (Fig. 3.17 first bar plot). *SOD2* (mitochondrial anti-oxidant defense enzyme) and *UCP2* (potent mitochondria uncoupler protein) expression levels were also estimated by RT-qPCR. In agreement with previous literatures, UCP2 transcript was highly abundant in comparison to the biomarkers of mitochondria biogenesis (Figs. 3.17 and 3.18). No significant change was observed with either of these genes following doxycycline treatment.

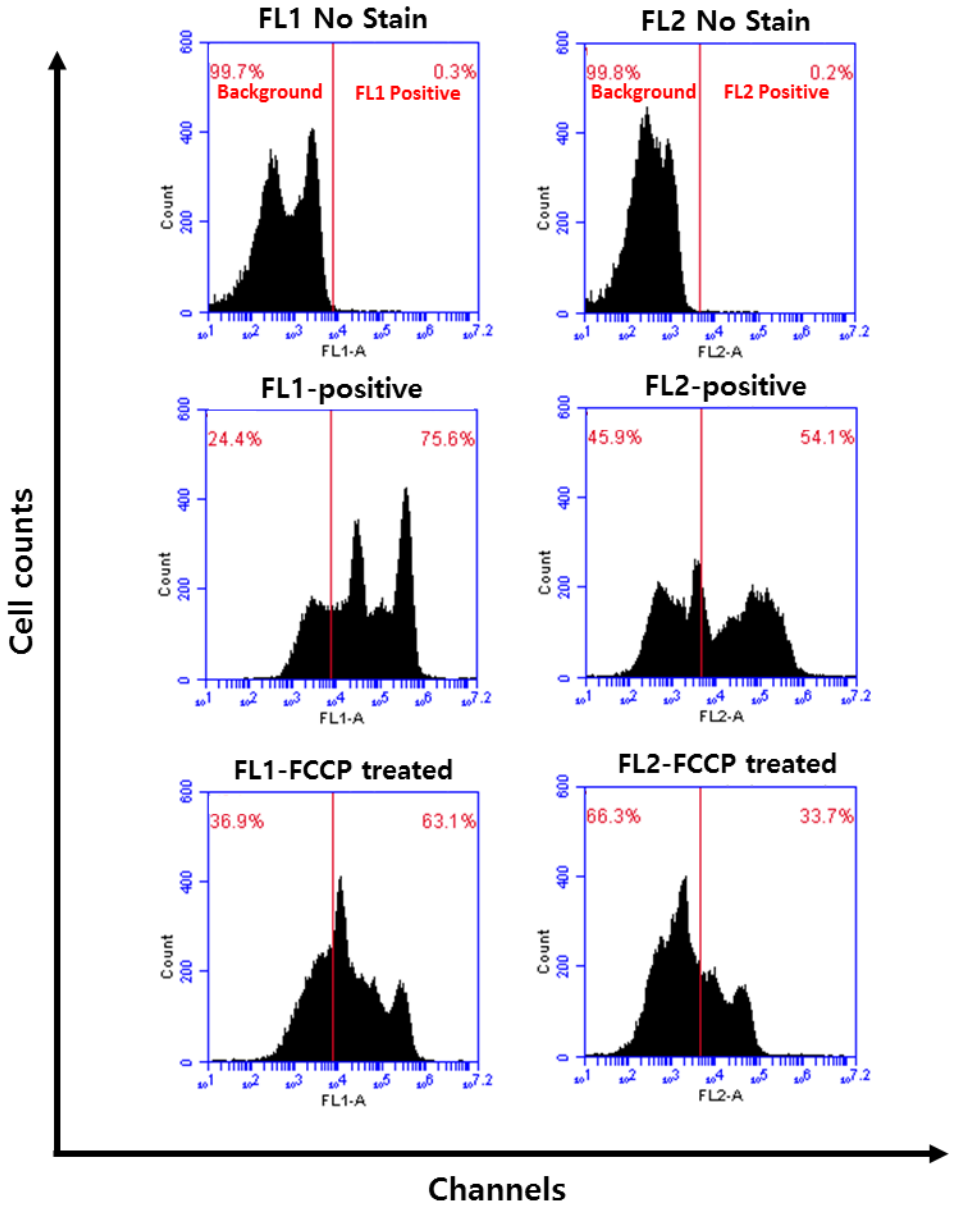



**Fig. 3.14: Mitochondrial membrane potential (MMP) gating strategy and controls for flow cytometry experiment with JC-1 dye measurements.** 3xFLAG-hTERT isoform expression was induced via 1μg/ml DOX (2 days) in HES2 B6 and HES2 FL12. Cells were grown under 2% $O_2$ and were stained with JC-1 dye for 30 minutes. Control groups were no JC-1 control, 20μM FCCP (chemical uncoupler of mitochondria electron transport chain). Mean fluorescence from FL1 and FL2-positive populations were measured for FL2:FL1 ratio calculation. Cell debris was excluded from analysis. Gated-events (15,000) were recorded and analyzed.

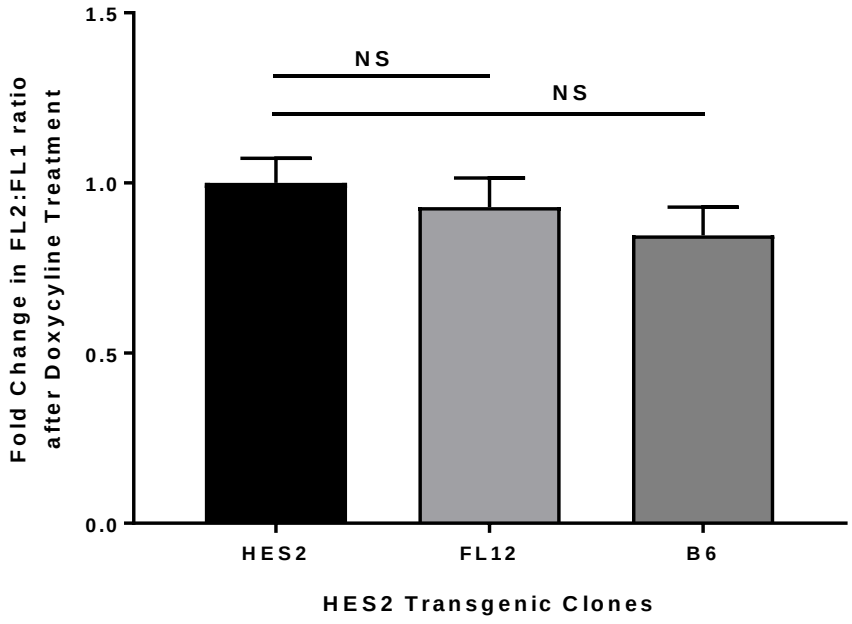



**Fig. 3.15: 3xFLAG-β-hTERT and 3xFLAG-Full-Length-hTERT do not affect mitochondrial membrane potential (MMP) in hESCs.** Gated mean fluorescence ratio of J-aggregate (FL2; orange-red; bandpass filter 586 ± 20nM) to monomer (FL1; green; bandpass filter 530 ± 15nM) was measured and calculated by flow cytometry. The fold change in FL2:FL1 ratio before and after the addition of doxycycline in HES2, HES2 FL12, HES2 B6 is shown as a bar plot. n=4, error bars = ±SEM, NS = non-significant.

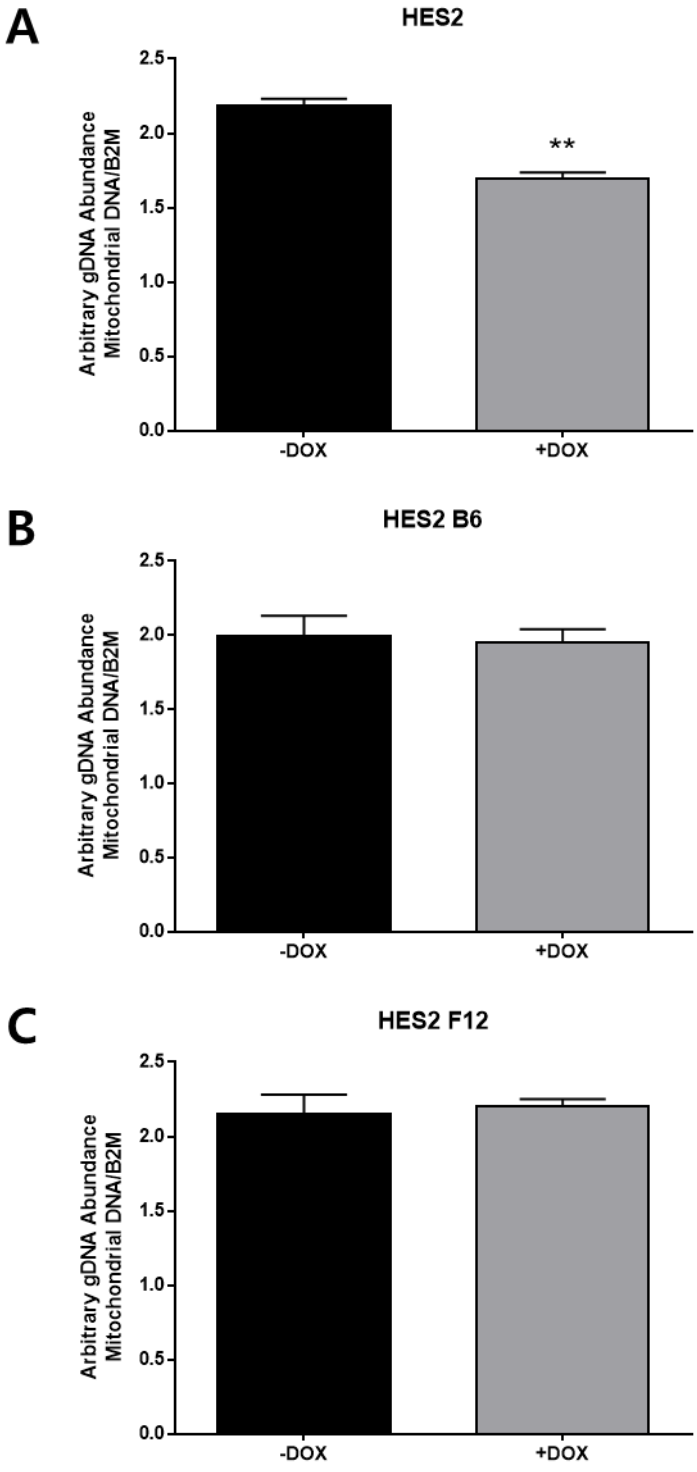



**Fig. 3.16: Doxycycline affects mitochondria copy number in hESCs.** 3xFLAG-hTERT isoform expression was induced via 1µg/ml DOX (2 days) in HES2 B6 and HES2 FL12 clonal cell lines. HES2 (nontransgenic) was also treated with doxycycline (as drug control). All cells were grown under 2% $O_2$ tension. The mitochondria DNA copy number was estimated by PCR-amplifying a small segment of mitochondria DNA. Results were normalized to B2M (a single copy nuclear gene). **A)** HES2 **B)** HES2 B6 **C)** HES2 FL12 (n=3, error bars = ±SEM).

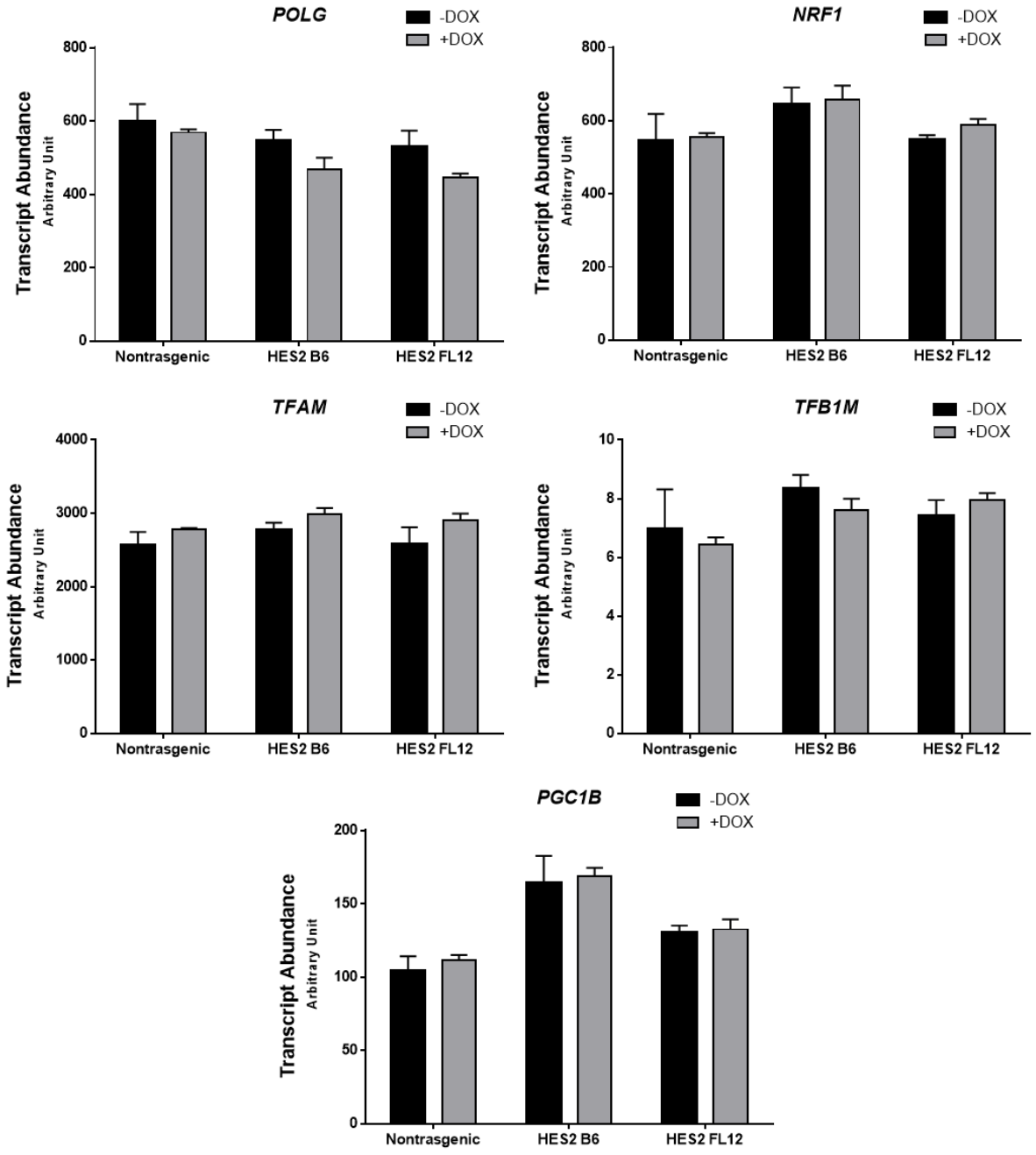



**Fig. 3.17: The effect of 3xFLAG-β-hTERT and 3xFLAG-Full-Length-hTERT overexpression on mitochondria biogenesis regulator gene expression.** The transcript levels of mitochondria biogenesis regulators were examined by RT-qPCR. HES2, HES2 B6, HES2 FL12 clonal hESC lines were treated with 1μg/ml DOX for 2 days prior to analysis. n=3, error bars = ±SEM

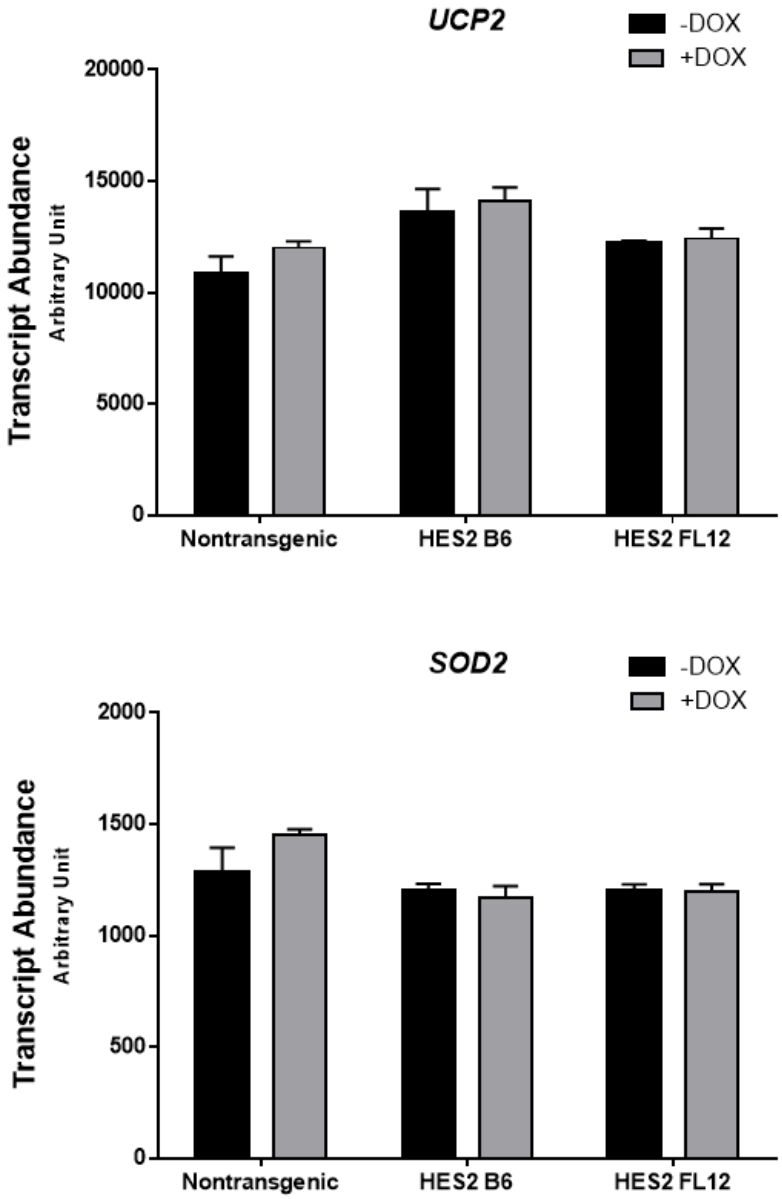



**Fig. 3.18: Dox-induced 3xFLAG-β-hTERT and 3xFLAG-Full-Length-hTERT overexpression does not affect UCP2 and SOD2 transcript abundance levels.** The transcript levels of *SOD2* (antioxidant enzyme) and *UCP2* (endogenous mitochondrial uncoupler) were examined via RT-qPCR. HES2, HES2 B6, HES2 FL12 clonal hESC lines were treated with 1μg/ml DOX for 2 days prior to analysis (n=3, error bars = ±SEM).

### 3.6 The effect of doxycycline-induced 3xFLAG-hTERT on cell survival in HES2 lines under $H_2O_2$-induced oxidative stress

Cell viability, apoptosis, and cell death were measured by flow cytometry. Viable, apoptotic, and dead cell groups were defined as annexin V-FITC$^-$/7-AAD$^-$, annexin V-FITC$^+$/7-AAD$^-$, and 7-AAD$^+$ populations, respectively. (Fig. 3.19). Doxycycline treatment in HES2 resulted in no change in the assessed cell populations (Fig. 3.20 A, Fig. 3.21 A, and Fig. 3.22 A). The percent viability of HES2 B6 decreased by ~5% following DOX-induction (Fig. 3.20 B). At the same time, the percent apoptotic population of HES2 B6 increased by ~5% following DOX treatment, but the percent dead cell population was unaffected (Fig. 3.21 B and Fig. 3.22B). Conversely, the percent viability increased by ~14% with DOX-treatment in HES2 FL12 (Fig. 3.20 C). This was concurrent with a ~19% decrease in the apoptotic population, and a ~5% increase in the dead cell population in HES FL12 cells (Fig. 3.21 C and Fig. 3.22 C).

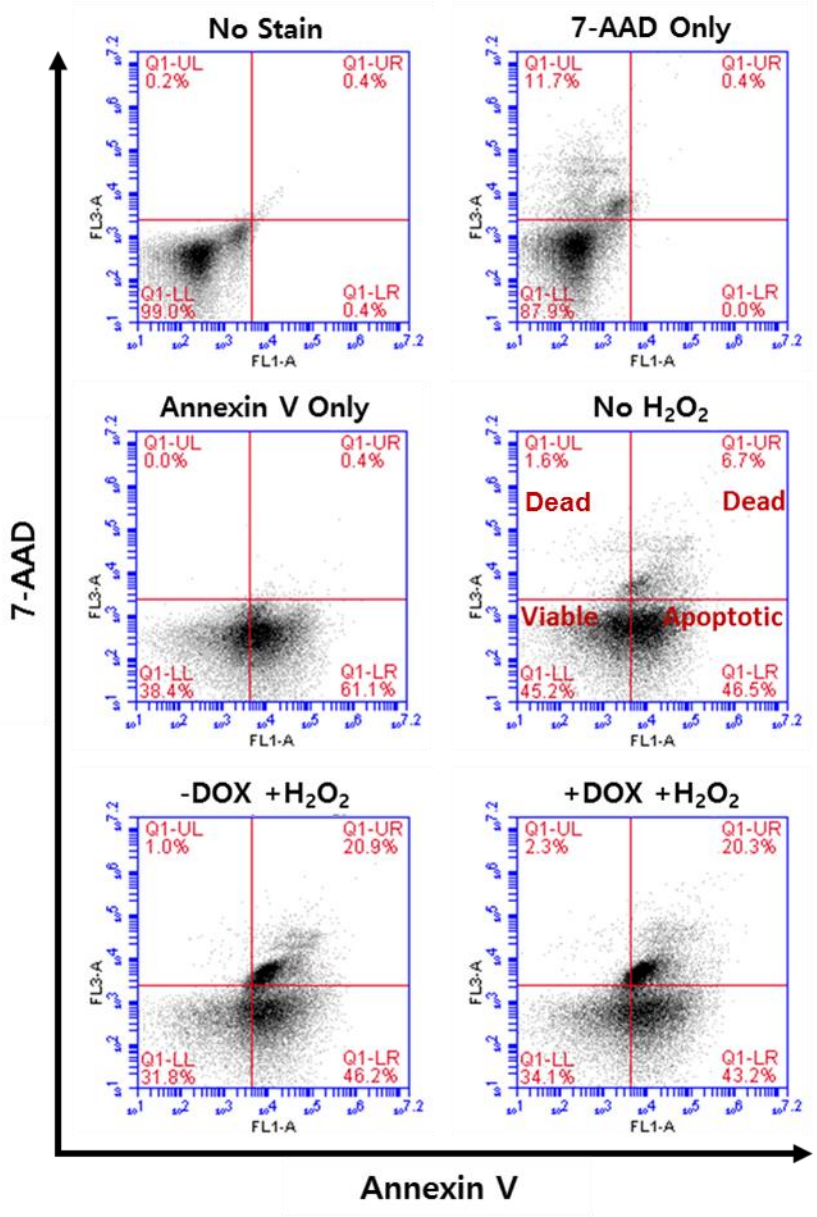



**Fig. 3.19: Representative plots showing stain controls and gating strategies for flowcytometry experiment with 7-AAD and Annexin V for measuring cell death upon $H_2O_2$ treatment.** 3xFLAG-hTERT isoform expression was induced via 1μg/ml DOX (2 days). Cells were grown under 2% $O_2$ and 100μM $H_2O_2$ was added the growth medium 2-hour prior harvest. Cells were stained with annexin V-FITC and 7-AAD to label apoptotic and dead cells, respectively. Measured cell population was first gated to exclude cell debris and background signals. Q1-UR: +Annexin V/+7-AAD (dead), Q1-UL: -Annexin V/+7-AAD(dead), Q1-LR: +Annexin V/-7-AAD (apoptotic), Q1-LL: -Annexin V/-7-AAD (viable).

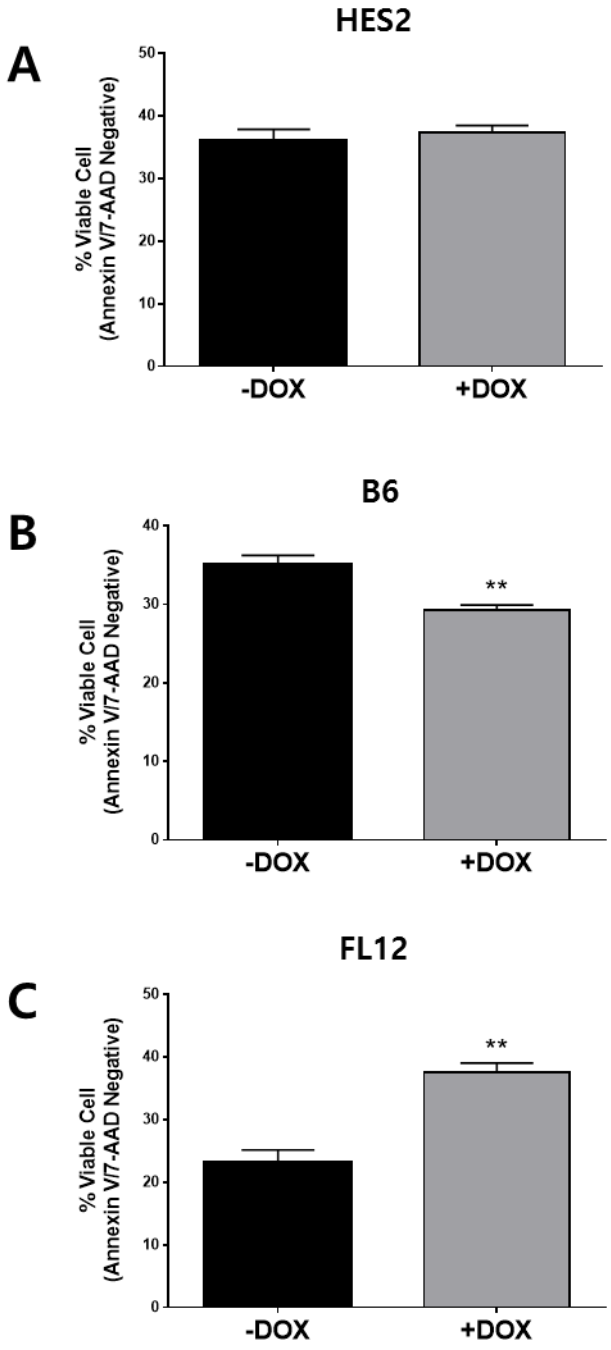



**Fig. 3.20: Effect of DOX-induced 3xFLAG-β-hTERT and 3xFLAG-Full-Length-hTERT on cell viability in HES2 under $H_2O_2$-induced cell stress.** Percent viable cell (Annexin V-FITC/7-AAD negative) plots in **A)** HES2, **B)** HES2 B6 **C)** HES2 FL12 (n=4, error bars = ±SEM).

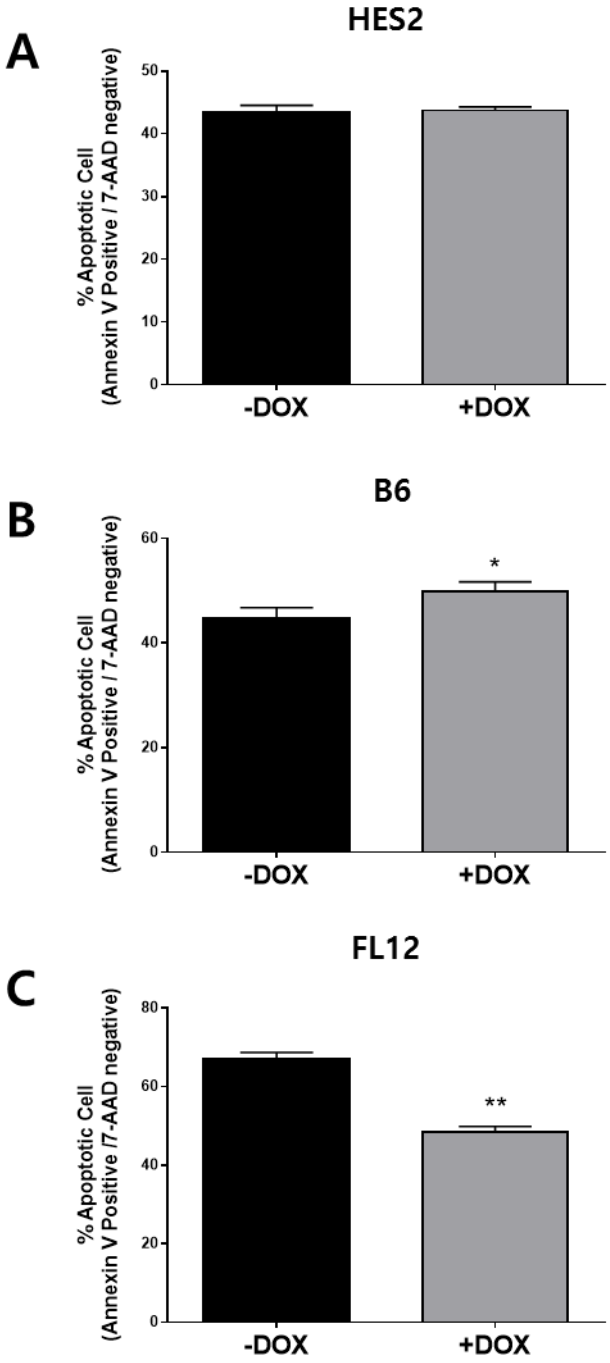



**Fig. 3.21: Effect of DOX-induced 3xFLAG-β-hTERT and 3xFLAG-Full-Length-hTERT on apoptosis in HES2 under $H_2O_2$-induced cell stress.** Percent apoptotic cell (Annexin V-FITC positive/7-AAD negative) plots in **A)** HES2, **B)** HES2 B6 **C)** HES2 FL12 (n=4, error bars = ±SEM).

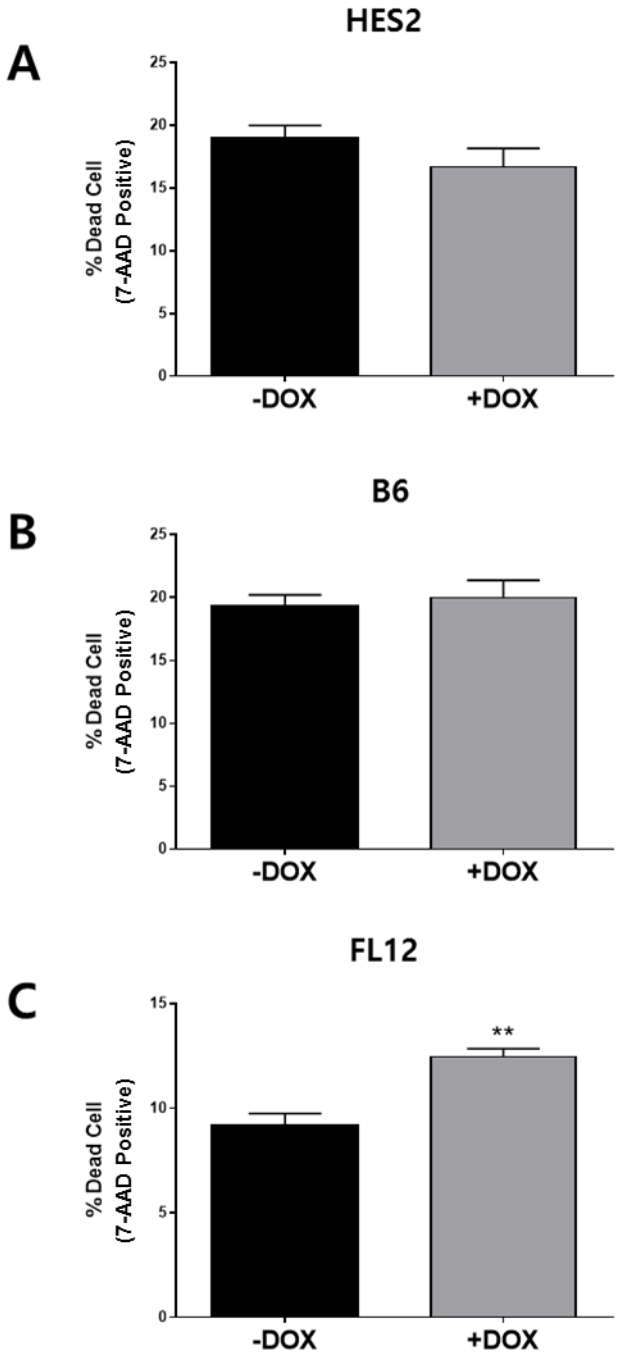



**Fig. 3.22: Effect of DOX-induced 3xFLAG-β-hTERT and 3xFLAG-Full-Length-hTERT on cell death in HES2 under $H_2O_2$-induced cell stress.** Percent dead cell (Annexin V-FITC/7-AAD positive) plots in **A)** HES2, **B)** HES2 B6 **C)** HES2 FL12 (n=4, error bars = ±SEM).

# Chapter 4: Discussion

The telomerase RNP complex plays a crucial role in maintaining telomere homeostasis. To enable rapid and indefinite cellular proliferation, cancer cell utilizes telomere-lengthening cellular machinery such as alternative lengthening of telomere (ALT) or telomerase. Several studies in human cancer and fibroblast cells have pointed to extra-nuclear translocation of telomerase catalytic subunit hTERT and its significance in extra-telomeric functions in modulating cellular behavior. Notably, alternative splicing of the TERT transcript to β-hTERT and its extra-nuclear localization have been previously reported in a subset of cancer cells. hESCs and cancer cells share similar cell physiology including high proliferative capacity, glycolysis-dependent energy metabolism, and high telomerase expression. In light of recent discoveries of hTERT's mitochondrial roles, I hypothesized that extra-nuclear translocation of FL-hTERT and β-hTERT will occur in hESC, and that they will collect at mitochondria under hydrogen peroxide-induced oxidative stress, thereby modulating mitochondrial functions. Owing to the lack of hTERT-specific antibody and the intricacy of hTERT alternative splicing, hTERT-overexpressing cell lines (doxycycline-inducible) were established by PiggyBac genomic integration of Flag-tagged (N-terminal) FL-hTERT and Flag-tagged β-hTERT mRNA sequences (*see* section 2.1.3). Various effects of doxycycline-induced hTERT expression were assessed in the clonal hESCs while paying extra-attention to the potential side effects of doxycycline and the Flag-fusion at the N-terminal of the TERT transgene.

## 4.1 Pluripotency and alternative splicing of endogenous hTERT in hESC

Prior to the overexpression studies, I began by verifying the pluripotent state of the hESC line HES2, cultured under 21% oxygen, by immunofluorescence microscopy. HES2

cells were marked immuno-positive for OCT4 and SSEA-4, and negative for SSEA-1 (Fig. 3.1 C) supporting their pluripotent state.

TaqMan RT-qPCR was used to detect endogenous hTERT transcripts isoforms containing neither α- nor β-deletion (α+β+hTERT), β-deletion (β-hTERT), α-deletion (α-hTERT), and both α- and β-deletion (α-β-hTERT). Importantly, each of the four primer sets (α+β+, β-, α-, α-) used for the PCR detects the presence of only the respective deletion, but does not distinguish between any other insertions/deletions that may be present on the same target transcript. For example, α+β+hTERT, as detected by TaqMan primers and probe, includes not only the full-length-(FL-)hTERT transcript but also α+β+ transcript species with other deletions/insertions present elsewhere. To increase the specificity of detection, one could employ long-range PCR to amplify the whole lengths of hTERT transcripts.

My PCR results revealed that HES2 was indeed expressing certain hTERT isoforms exhibiting different combinations of α- and β-deletion when cultured under 21% oxygen. The transcript abundance of β-hTERT was proportionately the highest among α+β+, β-, α-, α-β-hTERT transcripts, and was as high as that of α+β+hTERT (Fig. 3.1 B). In a previous study by Ulaner *et al.* [78, 102], it was demonstrated that the splicing pattern of hTERT was tissue-, and developmental-stage-specific. In human embryonic kidney tissue, β-splicing occurs at the expense of FL-hTERT transcript, and this was proposed to be a mechanism involved in downregulation of telomerase activity. Similarly, Yan and colleagues [111] demonstrated a negative correlation of telomerase activity with the constituent ratio of deletion hTERT variants, especially β- hTERT. In hESCs, telomere length homeostasis is strictly maintained as excessively long telomeres can cause telomere instability and replication stress [196]. Therefore, it is highly possible that a similar regulation of telomerase activity by alternative splicing may be reinforced in hESC to prevent excessive lengthening

of telomeres. Notably, β-hTERT transcript persisted in human fetal kidney development even in the absence of all other hTERT isoforms (including the FL-hTERT), raising the possibility of β-hTERT's involvement outside canonical telomerase function.

I then examined how the splicing of endogenous hTERT changed when cells were cultured with 2% oxygen and different combinations of doxycycline and $H_2O_2$ (Fig. 3.2). When cells were cultured under 2% oxygen, the transcript composition of α+β+hTERT and β-hTERT remained nearly equal in quantity and higher than that of α-hTERT and α-β-hTERT compared to when the cells were cultured in 21% oxygen (Fig. 3.2 A and E). In contrast, our lab's previous work in H9 hESC line showed that α+β+hTERT and α-hTERT transcripts dramatically decreased going from 21% $O_2$ to 2% $O_2$ culture condition [184]. Radan *et al.* proposed that the change in splicing pattern might be attributed to the function of hypoxia-inducible-factor (HIF), based on its ability to regulate alternative splicing in hypoxic conditions [209 - 213]. The apparent discrepancy in our results may arise from cell line-specific differences and/or from the differences in our methodology. Although all hESC lines share the expression of characteristic pluripotency markers, they exhibit several differences owing to the wide range of factors such as variable culture conditions and the inherent genetic variation of the embryos from which embryonic stem cells were derived [199]. Thus, it is possible that the two cell lines used in this study (HES2) and by Radan *et al.* (H9) respond differently to the changing oxygen tension. Secondly, the outcome of the result may have greatly depended on the oxygen tension the cells were exposed to during maintenance prior to the hypoxic/normoxia treatment. Lastly, the duration of hypoxia and normoxia treatment varied between the two studies (5 days in the study by Radan *et al.* and 3 days for the current thesis). It would be interesting to see whether the alternative splicing patterns converge when the experimental differences are removed.

The splicing pattern changed when $H_2O_2$ or doxycycline was singly added to the culture media (Fig. 3.2 B and C). After 2-hour treatment of 100 μM $H_2O_2$, the percent transcript composition of β-hTERT was significantly higher than that of α+β+hTERT (Fig. 3.2 B). It appeared that the increased β-splicing came at the expense of α+β+splicing. However, no statistically significant change was seen with any of the four hTERT isoforms between control group (2% oxygen) and $H_2O_2$-treated group. A small sample size (n=3) may be preventing the observation of a statistically true event. Nonetheless, it appears evident that $H_2O_2$ alters the splicing pattern of hTERT in favor of β-hTERT. Other studies have shown a similar effect of $H_2O_2$ on alternative splicing of different genes through oxidative stress [212, 213] and mitochondrial damage [202]. Gilbert *et al.*[201] demonstrated that $H_2O_2$ directly affects the protein abundance of spliceosome complex proteins (hnRNPs, PTBP1), which suggests $H_2O_2$ can potentially alter the transcript splicing of many genes and, perhaps, hTERT. $H_2O_2$ is a cell membrane-permeable molecule that is naturally generated as byproduct of aerobic respiration [203], and can act as inter- and intra-cellular signaling molecule [204]. In a pathological context, $H_2O_2$ concentration greater than 50μM is generally considered cytotoxic, but this is largely cell-type and culture media-dependent as different cells possess differential resistance to oxidative stress [205, 206]. In mESC, 24 hour 150μM $H_2O_2$ treatment results in apoptosis, whereas shorter treatment time (2 hours, same concentration) resulted in transient cell cycle arrest [207]. Research conducted by Kumar *et al.* [208] demonstrated that treating hESC with 200 μM $H_2O_2$ for 30 minutes resulted in a significant increase in DNA damage. The concentration of $H_2O_2$ used in my study, 100μM, albeit lower than 200μM, was applied for much longer duration (two hours) than 30 minutes, and may have caused significant amount of DNA damage in the cells, triggering cell stress- and apoptotic signaling cascades. $H_2O_2$, in fact, is a poorly reactive molecule and undergoes

Fenton-reaction with intra-cellular iron ($Fe^{2+}$) to become the deleterious hydroxyl radical [209]. Intriguingly, Santos et al. [172] showed that ectopically expressed FL-hTERT in normal human fibroblasts cells promotes $H_2O_2$-induced mitochondrial DNA damage by increasing bioavailability of divalent iron pool. Therefore, it is possible that the observed shift towards β-splicing may have been a rescue attempt to lessen the damage-amplifying effect of FL-hTERT.

Similarly, 2-day treatment of 1μg/ml doxycycline resulted in higher percent composition of β-hTERT transcript relative to that of α+β+hTERT (Fig. 3.2 C). This result indicates that doxycycline treatment has a side-effect on the splicing pattern of hTERT (and possibly other pre-mRNA transcripts) in hESCs. Whether doxycycline acts directly or indirectly on the splicing machinery of hTERT is unclear at this stage. Doxycycline is categorized as a tetracycline family antibiotic that is used to cure a broad spectrum of bacterial infections [209 – 211]. The antibacterial activity by tetracyclines is mediated by inhibiting the bacterial ribosome, effectively blocking bacterial protein synthesis [210]. Despite its frequent use in inducible mammalian transgene systems, significant evidence exists to show that tetracyclines can impair mitochondrial function in eukaryotic cells through the inhibition of mitochondrial ribosome, which may be explained by the stark similarity of bacterial ribosomes and eukaryotic mitochondrial ribosomes (in support of endosymbiotic bacterial origin of mitochondria) [212 - 215]. The effect of doxycycline extends to hESC and iPSC. Work by Chang *et al.* [216] showed that doxycycline improves viability and self-renewal of hESCs and iPSCs through activation of PI3K/AKT signaling, an important pathway for self-renewal of PSCs (*see* section 1.1.1). However, it is not known whether the activation of PI3K/AKT signaling is a result of mitochondrial inhibition by doxycycline. The PI3K/AKT signaling pathway controls numerous cellular processes such as

glucose metabolism, protein synthesis, and RNA processing. AKT can influence splicing regulation indirectly by phosphorylating serine/threonine-protein kinase 2 (SRPK2) and serine/threonine-protein kinase 1 (SRPK1; auto-phosphorylation), which in turn activates SR family proteins [217, 218]. Alternatively, AKT can directly phosphorylate SRSF1, SRSF7 and SRSF5 [219-221]. Considering β-deletion is also mediated by a SRSF family protein (SRSF11; *see* section 1.2.2), it is likely the case that doxycycline treatment acts on the splicing machinery of hTERT by first activating the PI3K/AKT signaling pathway and subsequently SRSF proteins. In addition, the fact that hTERT splicing pattern changed in favor of β-hTERT following the speculated activation of PI3K/AKT hints at a novel extra-telomeric role of β-hTERT in survival/self-renewal of hESCs and supports the currently existing evidence for hTERT regulation by alternative splicing. Additionally, DOX-treated samples showed reduced splicing of α-β-hTERT in comparison to non-doxycycline treated groups (Fig. 3.2 E $4^{th}$ and $5^{th}$ columns). This relative reduction of α-β-hTERT transcript population may account for the shift of splicing toward β-hTERT transcript following doxycycline treatment.

When doxycycline and hydrogen peroxide treatments were combined, strikingly, the proportion of β-hTERT transcript dropped down to the level of α+β+hTERT, while the α-β-hTERT transcript remained low as seen in doxycycline-treatment only group. It is tempting to look for an explanation for this phenomenon through mitochondria since they are the known off-targets of doxycycline and are the central hub of intra-cellular free iron sequestration for the downstream heme and iron sulfur syntheses [221, 222]. In this context, the side effect of 2-day long doxycycline treatment (impairment of mitochondria) might have had a serendipitous effect of curtailing the free bivalent iron pool by the time $H_2O_2$ was introduced to the cells, reducing the generation of highly deleterious hydroxyl radicals.

Assuming the previously seen increase in β-hTERT splicing played a protective role (when doxycycline and hydrogen peroxide were applied singly), one may speculate that the cytotoxic effect of $H_2O_2$ might have been alleviated by mitochondrial impairment and AKT activation by doxycycline, leading to the overall reduction of intra-cellular stress and the resultant decrease in β-splicing. Future experiments that examine changes in intra-cellular ROS and AKT activation after DOX- and $H_2O_2$ treatments will be necessary to better explain this multi-faceted result. α-β-hTERT, despite the reduced β-splicing, remained unchanged, which suggest an independent role for α-β-hTERT isoform may exist.

## 4.2 Transgenic HES2 line B6 and FL12 remain pluripotent following doxycycline and $H_2O_2$ treatment.

For delineating TERT isoform function and to enable reliable detection of hTERT transgenic proteins, multiple transgenic HES2 lines were clonally established for DOX-inducible overexpression of 3xFLAG-tagged β-hTERT and FL-hTERT transgenes. Four clonal cell lines established from HES2 β-hTERT (B2, B3, B6, B7) and HES2 FL-hTERT (FL7, FL9, FL10, FL12) were randomly chosen and analyzed for FLAG-tagged protein expression (appendix A3: Fig. 3.1). Based on preliminary results of 3xFLAG-hTERT transgene induction 12-hours post doxycycline, clonal cell line selection was further narrowed down to the least expressive β-clone HES2 B3, the most expressive HES2 B6, a FL-clone (FL9) with similar level of protein induction as B6 HES2, and the most expressive FL-clone HES2 FL12. The level of transgene induction in the selected clones was re-examined by RT-qPCR using transgene-specific primers (n=1; I focused more on the protein because the goal of my study relies on the protein stability/expression rather than the

transcript stability) and by Western blot (Fig. 3.3). In all clones, both 3xFLAG-β-hTERT and 3xFLAG-FL-hTERT expression peaked after 6 hour of doxycycline treatment and remained relatively unchanged over the 3 day-long doxycycline time course.

Interestingly, the steady-state protein level of 3xFLAG-β-hTERT was significantly lower (approximately by 7-fold) than that of 3xFLAG-FL-hTERT (Fig. 3.3 C) regardless of the duration of doxycycline. This can arise from multiple factors including transcriptional activity at the promoter site of the recombinant gene, the stability of 3xFLAG-β-hTERT transcript, and protein degradation.

The low protein expression of 3xFLAG-β-hTERT may result from diminished transcription of the transgene. 3xFLAG-β-hTERT and 3xFLAG-FL-hTERT expression cassettes utilize identical promoter (pCAG), transactivator (TA), activator (DOX), and transactivator responsive element (TRE) (Fig. 2.1). Therefore, the differences in transcriptional activity of 3xFLAG-β-hTERT and 3xFLAG-FL-hTERT would depend on the site of and the multiplicity of transgene integration.

In my preliminary characterization of transgenic transcript level by RT-qPCR (Fig. 3.3 A, n = 1), I observed that 3xFLAG-β-hTERT transcripts slowly decrease over 3 days of doxycycline treatment in both HES2 B3 and B6, despite daily renewal of doxycycline in the growth media. Previously, it was shown that the NMD activity on β-hTERT transcript can vary in different cell types [102]. Greater than 90% depletion of UPF1 (core component of NMD machinery) resulted in 10-fold increase in β-hTERT transcript in UM-UC-3 cell line (bladder cancer), an indication that β-hTERT transcript can be targeted by NMD. Conversely, β-hTERT transcript in BT-549 cells (breast cancer; normally expresses high level of β-hTERT) did not change significantly with the knockdown of UPF1, suggesting β-hTERT

transcript can escape NMD pathway depending on the cell type. Therefore, it would be interesting to perform a similar experiment to test the NMD sensitivity of β-hTERT transcript in hESC.

The steady-state protein abundance of 3xFLAG-β-hTERT (B6) was consistently ~5-fold less than that of 3xFLAG-FL-hTERT (FL12) at all doxycycline treatment times (Fig. 3.3 C), whereas 3xFLAG-β-hTERT is only ~2-fold less than 3xFLAG-FL-hTERT at transcript level (Fig. 3.3 A). Adding to this, the steady-state transcript of 3xFLAG-FL-hTERT in FL9 clone was ~3 times less than 3xFLAG-β-hTERT (B6), yet the protein abundance was ~3 times higher at all doxycycline durations (Figure. 3.3 A and C). Listerman *et al.* and our lab previously confirmed the translational competency of β-hTERT transcript [100, 186] and α-β-hTERT transcript [184] by polysome profiling. Collectively, these findings point to the possibility that 3xFLAG-β-hTERT protein has higher propensity to degrade than 3xFLAG-FL-hTERT. FL-hTERT is known to degrade by ubiquitination pathway [131– 133], but little is known about the protein degradation of β-hTERT. β-hTERT lacks a large portion of the C-terminal end (Fig. 1.1 B), which is necessary for MKRN1-mediated ubiquitination/ degradation of hTERT[129]. Therefore, if 3xFLAG-β-hTERT is indeed degrading via ubiquitination, CHIP [130] or Hdm2 [131] may be at the focus of such regulation (*see* section 1.2.4).

I decided to work with the most expressive clones HES2 B6 and HES2 FL12 for the robustness of potential downstream effects and to adequately compare functional differences between overexpression of the two TERT isoform transgenes in hESCs. For the treatment time of doxycycline, I settled with 2 days of transgene induction by doxycycline for both B6 and FL12 clones for maximum transgene expression (Fig. 3.3 A).

In the light of discoveries highlighting the importance of hTERT [185] and telomere maintenance [224-228], I performed immunofluorescence microscopy to assess the effect of TERT isoform transgene expression on the pluripotent status of the selected clones. Both HES2 B6 (Fig. 3.4) and HES2 FL12 (Fig. 3.5) stained positively for OCT4/SSEA-4, and negatively for SSEA-1, indicating their pluripotent state under normal culture conditions (2% oxygen, no $H_2O_2$ or doxycycline added). Following doxycycline (1μg/ml for 2 days) and/or $H_2O_2$ treatment (100μM for 4 hours), the cells stained in the same pattern and apparent intensity, suggesting, at least at the visible level, that pluripotency was not altered by the cell treatments (Fig. 3.4 and Fig. 3.5). This supports a previous study in mESC which showed that short-term treatment of $H_2O_2$ (150μM for 2 hours) did not affect the expression of pluripotency marker genes Oct4, Sox2, and Nanog [207]. However, no quantification was performed on the immunofluorescence intensities so it is possible that the pluripotency markers were changing over the course of cell treatments. Many cells were non-adherent and round in morphology (data not shown) by the end of 4-hour $H_2O_2$ treatment, which suggests the cells were responding to the acute oxidative stress by switching on the apoptotic pathway rather than by differentiating (loss of pluripotency). In support of this, Guo *et al.* demonstrated that mESCs, despite their increased resistance to senescence by oxidative stress, undergo apoptosis when treated with $H_2O_2$ at dose higher than 150 μM for 24 hours [207]. In the same study, a significant portion of mESCs treated with 150μM $H_2O_2$ for 3 hours lost their ability to adhere to the surface of culture dish, and failed to recover when resuspended in fresh culture medium. This closely resembles my observation with $H_2O_2$-treated hESC, and likely signifies a pre-apoptotic event that ultimately contributes to $H_2O_2$-induced cell cycle arrest and apoptosis.

### 4.3 3xFLAG-hTERT in HES2 B6 and HES2 FL12 bind TERC and RMRP.

To show the competency of the 3xFLAG-tagged-hTERT species in carrying out the known functions of non-tagged versions of hTERT, I tested their RNA-binding ability (Fig. 3.6 and 3.7) and telomerase activity levels (Fig. 3.8) of the FLAG-tagged hTERT isoforms in HES2 hESCs. In FLAG-RIP experiments for TERC-binding, the amount of TERC recovered was significantly higher when doxycycline was added for both HES2 B6 and HES2 FL12 (Fig. 3.7 B and C, respectively), but not for parental HES2 cells. The insignificant change observed with parental HES2 demonstrates that the significant differences observed in HES2 B6 and HES2 FL12 were not due to a side-effect of doxycycline and/or because of nonspecific binding of TERC to the FLAG-antibody-conjugated beads. Together, the experiment confirms that both 3xFlag-tagged β-hTERT and FL-hTERT transgenic proteins can bind TERC *in vitro*. In a side-by-side comparison (Fig. 3.7 A), the fold-recovery of TERC was significantly higher for 3xFLAG-FL-hTERT (~60-fold increase) than for 3xFLAG-β-hTERT (~10-fold increase). Interestingly, a similar RIP experiment by Listerman et al. [102] confirmed Flag-tagged β-hTERT bound to TERC. In stark resemblance with my results, Flag-tagged FL-hTERT co-immunoprecipitated only a 3-fold higher amount of TERC than Flag-tagged β-hTERT. It is tempting to conclude that FL-hTERT has greater affinity for TERC than β-hTERT. However, making any conclusions on the affinity would be inappropriate for a few reasons. First, my data for DOX-induced recombinant protein expression showed that the steady-state abundance of 3xFLAG-FL-hTERT was significantly higher than that of 3xFLAG-β-hTERT by approximately 5-fold (Fig. 3.3, *see* section 4.2). This disparity between the two cell lines can also lead to similar reduction in TERC-recovery by 3xFLAG-β-hTERT. Second, there is no way of knowing whether FLAG-hTERT proteins were bound to the immunoprecipitation beads to saturating capacity or not. In other words, if

the same volume (30µl packed volume) of anti-FLAG beads were binding 3xFLAG-FL-hTERT to saturation, but not 3xFLAG-β-hTERT (possibly because of significantly low steady-state protein abundance of 3xFLAG-β-hTERT; see the beginning part of this section), no proper comparison can be made between 3xFLAG-FL-hTERT and 3xFLAG-β-hTERT since the amount of immunoprecipitated 3xFLAG-hTERT species differs. Thirdly, the fact hTERT can form dimers adds another layer of complexity. As shown in Fig. 1.1, β-hTERT lacks one of two oligomerization domains. If 3xFLAG-β-hTERT is less likely to self-dimerize than 3xFLAG-FL-hTERT *in-vitro*, it is possible that the same amount of saturated anti-FLAG-beads were pulling down different amount of 3xFLAG-β-hTERT and 3xFLAG-FL-hTERT. Further investigation is required to clarify this.

RMRP is another RNA partner of hTERT. The domain for RMRP-binding on the protein is currently unknown [181]. I performed the identical RIP experiment as above to estimate the recombinant hTERTs' ability to bind RMRP. As expected, no difference was seen amongst HES2 control group samples (Fig. 3.7 D). Similarly, RMRP recovery in HES B6 hESCs treated with doxycycline was not statistically different from its non-DOX-treated counterpart. The inherently low expression level of 3xFLAG-β-hTERT of HES2 B6 clone may have contributed to the low RMRP yield in DOX-treated group (Fig. 3.3, *see* section 4.1). However, considering the identical doxycycline treatment (thus, presumably the same level of 3xFLAG-β-hTERT protein expression) used for TERC-RIP resulted in significant TERC recovery (Fig. 3.6 B), the low protein expression by HES2 B6 may not be the limiting factor. This points to the possibility that β-hTERT has impaired RMRP binding. Importantly, HES2 control group samples (Fig. 3.7D) and HES2 B6 samples (Fig. 3.7 B) precipitated comparable amount of RMRP, which suggests a substantial amount of RMRP might have been bound to the RIP beads non-specifically. The hypothesized non-specific precipitation

could have led to false positive reading of RMRP in non-DOX-treated samples, masking the truly immunoprecipitated (by 3xFLAG-β-hTERT) RMRP in DOX-treated HES2 B6. In support of a previous study by Maida *et al.* [164], I observed ~5-fold increase in RMRP recovery upon the addition of doxycycline to HES2 FL12, indicating the interaction of 3xFLAG-FL-hTERT with RMRP (Fig. 3.7 C). In a side by side comparison of 3xFLAG-β-hTERT and 3xFLAG-FL-hTERT (Fig. 3.7 A), 3xFLAG-FL-hTERT co-precipitated with approximately 2-fold more RMRP than 3xFLAG-β-hTERT. However, as discussed above, more optimization and redesigning of the experiment is necessary to compare binding affinities of RNA components between TERT isoforms.

## 4.4 The effect of 3xFLAG-β-hTERT and 3xFLAG-FL-hTERT on the telomerase activity in HES2 B6 and HES2 FL12.

The effect of overexpressing 3xFLAG-β-hTERT and 3xFLAG-FL-hTERT on telomerase activity in hESCs was investigated (Fig. 3.8). hESCs express endogenous telomerase reverse transcriptase (TERT) and exhibit high telomerase activity [184]. β-hTERT can act as a dominant negative inhibitor of telomerase activity by sequestering TERC [102]. It was proposed by the same study that this mode of inhibition is effective only when TERC is the limiting factor for telomerase activity. Potential side-effects of doxycycline on endogenous telomerase activity in non-transgenic HES2 was first tested. I demonstrated that doxycycline had no effect on endogenous telomerase activity levels since no significant difference in the activity was observed with doxycycline treatment compared to non-treated controls hESCs (Fig. 3.8 B first bar plot).

As expected, overexpression of 3xFLAG-β-hTERT led to significantly reduced levels of telomerase activity (Fig. 3.8 B second bar plot). This inhibition could result from several reasons. As previously suggested, the available pool of free TERC transcripts may be in limiting supply in hESCs and reduced by binding to catalytically inactive 3xFLAG-β-hTERT, decreasing the number of active telomerase RNP complexes [102]. Although there is no confirmation on the formation of heterodimeric β-hTERT/FL-hTERT to date, such dimerization seems plausible given β-hTERT retains oligomerization domain for interaction with another hTERT protein [188]. Hence, one can speculate that the heterodimerization of 3xFLAG-β-hTERT with the endogenous FL-hTERT may have caused an inactivation of the complex, which could additionally account for the observed inhibition of telomerase activity.

Based on previous studies that showed TERC is in excess for telomerase complex formation in human immortalized cells [82, 226], I had anticipated that overexpressing 3xFLAG-FL-hTERT would lead to increased total telomerase activity. Surprisingly, the overexpression of 3xFLAG-FL-hTERT did not significantly increase telomerase activity levels (Fig. 3.8 B third bar plot). To seek an explanation for this unexpected outcome, I stepped back and examined what was happening to the total hTERT (endogenous and transgenic) population at the transcript level (Fig. 3.9) by TaqMan qPCR (Fig. 2.1 B). Upon adding doxycycline to HES2 FL12, not only did the transcript abundance of α+β+hTERT increased by ~500 fold, those containing β- transcript species (β- and α-β-hTERT) also increased greatly by ~300 fold (Fig. 3.9 A). The transgenic DNA sequence for 3xFLAG-FL-hTERT directly codes for the processed TERT mRNA species and does not include introns which are required for the alternatively splicing of β-hTERT [102]. This suggests that the observed increase in β-hTERT- and α-β-hTERT transcripts is due to the increased β-splicing of ***endogenous*** hTERT pre-mRNA. Moreover, doxycycline is unlikely a part of this as I had

seen no such tremendous increase in β-splicing in DOX-treated non-transgenic HES2 (Fig. 3.2 C and E). This is very interesting result which demonstrates the cell's ability to respond to ectopic expression of 3xFLAG-FL-hTERT. In other words, a feedback mechanism may exist in hESCs to sense the aberrant increase in α+β+hTERT transcript abundance and/or telomerase activity levels (canonical and/or non-canonical) of FL-hTERT and to downregulate it by switching to β-favoring splicing. In support of this, similar hTERT regulation by alternative splicing had been reported previously [79, 80, 110] (*see* section 1.2.2). Thus, the significant increase in non-catalytic hTERT transcripts (β-hTERT and α-β-hTERT) due to DOX-induced 3xFLAG-hTERT overexpression may be attributed to my failure to observe an increase in telomerase activity within this transgenic cell line. Notably, DOX-treatment in HES2 B6 only induced β-hTERT (~1000-fold induction) and α-β-hTERT (~200-fold induction). Since α-deletion is mediated by an alternative splice acceptor within exon 6 (therefore present in the 3xFLAG-β-hTERT transgene), The increase in α-β-hTERT transcript level may result from an additional α-deletion on the DOX-induced 3xFLAG- β-hTERT transcript.

The N-terminal end of hTERT is essential for telomerase activity as it contains an anchor site (TEN domain) to stabilize telomeric DNA-to-hTERT interaction [226], and TEN domain mutations are known to cause defective telomere maintenance *in vivo* [227]. Therefore, one may speculate that the N-terminal 3xFLAG could potentially disrupt this particular function of the TEN domain, leading to the failure of 3xFLAG-FL-hTERT to remain bound to the telomere-like DNA substrate in telomerase activity assay. However, several previous reports demonstrated unobstructed telomerase activity by N-terminal 3xFLAG-tagged hTERT, suggesting that N-terminal 3xFLAG-tagging does not affect the telomerase activity *in-vitro* [71, 231, 232].

### 4.5 The intra-cellular localization of 3xFLAG-β-hTERT and 3xFLAG-FL-hTERT in hESCs treated with $H_2O_2$-induced oxidative stress.

Several studies previously reported mitochondrial localization of FL-hTERT [187, 189, 203, 208, 214] and β-hTERT [149] under oxidative stress. We previously shown that hTERT was more extra-nuclear localized in hESCs cultured under 21% oxygen conditions compared to the prominently nuclear TERT observed in cells cultured in 2% oxygen [184]. Western blot of sub-cellular fractions and immunofluorescence microscopy were used in this study to determine the intra-cellular localization of 3xFLAG-β-hTERT and 3xFLAG-FL-hTERT when cells were treated with the characterized dose and duration of $H_2O_2$.

The amount of 3xFLAG-β-hTERT in mitochondrial and nuclear fractions did not change significantly over the time course of hydrogen peroxide treatment (Fig. 3.10 A and B). Similarly, no change in 3xFLAG-FL-hTERT was observed with $H_2O_2$ treatment in either fractions (Fig. 3.11 A and B). An independent set of the identical experiment was conducted with prohibitin as mitochondrial marker protein (for normalization of mitochondrial protein; appendix A4: Fig. 3.2). It showed similar results to when VDAC was used as the mitochondrial fractionation and loading control. Importantly, the fractionation quality (as determined by the presence of mitochondrial/nuclear markers in fractions) was rather inconsistent between experimental replicates (Fig. 3.10 and Fig. 3.11; data not shown), leading to highly variable results (Fig. 3.10, Fig. 3.11; appendix A4: Fig. 3.2; *see* error bars). hESCs exhibit high nuclear-to-cytoplasm ratio [230], and mitochondria of hESC are located in close proximity with the nuclei [230, 231]. Such intrinsic cell characteristics make clean separation of the two organelles very difficult [232– 234], a task I could not fully overcome.

As an alternative method to fractionation, immunofluorescence imaging was performed on fixed HES2 B6 and HES2 B12 cells after $H_2O_2$ treatment (Fig. 3.12). There was no apparent co-localization of either 3xFLAG-β-hTERT or 3xFLAG-FL-hTERT with mitochondria after $H_2O_2$ treatments. 3xFLAG-β-hTERT and 3xFLAG-FL-hTERT were predominantly nuclear with only minute amount of 3xFLAG-hTERT localized to the cytoplasmic space. Our lab previously observed extra-nuclear localization of endogenous hTERT in H9 line under chronic exposure to 21% $O_2$ for 5 days [184]. However, I failed to observed this in HES2 FL12 that had been exposed to 21% $O_2$ for 3 days (appendix A5: Fig. 3.3). there were some nuclei with 3xFLAG-hTERT located very close to the nuclear boundary (Fig 3.12 indicated by white arrow). I believed this intra-nuclear localization pattern might be the influence of $H_2O_2$-induced oxidative stress, and performed a blinded cell-count analysis on immunofluorescence microscopy images (Fig. 2.2 and Fig. 3.13). Unfortunately, the analysis revealed no significant effect of hydrogen peroxide on 3xFLAG-β-hTERT and 3xFLAG-FL-hTERT localization within hESCs.

The observed localization pattern of 3xFLAG-FL-hTERT is particularly puzzling as it directly contradicts previous studies that demonstrated extra-nulcear-[184] and mitochondrial [185, 186, 197, 240] localization of FL-hTERT under $H_2O_2$-induced oxidative stress. These studies employed wide range of dose and duration of oxygen tension/hydrogen peroxide [186, 187, 197], or no oxidative stress at all [175]. The dose of hydrogen peroxide used in these studies vary from 200μM to 500μM, and the duration of $H_2O_2$ treatment ranged from 3 hours to 5 days. The different cell types used in the studies (NHF, HeLa [175], fetal human lung fibroblast cell MRC5 [195], breast cancer cell MCF7 [183]) add extra layer of complexity to this, making it nearly impossible to predict the right dose and time window of $H_2O_2$ to use in hESC for inducing mitochondrial localization of hTERT. In my preliminary

immunofluorescence experiments with earlier (15 minutes, 30 minutes, 1 hour) and longer duration (4 hours, at which point the cells were apoptotic; *see* last paragraph of section 4.1) of 100 μM $H_2O_2$ treatment (data not shown), 3xFLAG-β-hTERT and 3xFLAG-FL-hTERT remained mostly nuclear. Based on this, it is likely that I am targeting right (inducing cell stress) dose and sufficiently wide time window of $H_2O_2$ treatment. Considering the hTERT investigated in previous studies were either endogenously expressed protein [195] or tagged with relatively small tag such as myc (1.2 kDa) [186, 240], the large size of 3xFLAG tag (2.8 kDa) used in my study may be physically hindering the trans-membrane translocation of 3xFLAG-β-hTERT and 3xFLAG-FL-hTERT. However, EGFP (32.7 kDa)-tagged hTERT used by Santos et al.[175] was able to cross both nuclear and mitochondrial membrane, precluding this possibility. Importantly, the mitochondrial targeting sequence (MTS) of hTERT lies at the N-terminal end. Thus, the location (N-terminal vs C-terminal) and the amino acid (a.a.) constituents of the tagging can greatly influence the proper function of MTS. Mitochondrial targeting sequences, as outlined in a review by Walter Neupert [237], are generally 20-60 amino acid long, rich in positively charged a.a., and have the tendency to fold into amphiphilic α-helices. 3xFLAG is particularly enriched in negatively charged aspartate residues [238]. The negative charge on N-terminally tagged 3xFLAG may negate the positive charge on MTS, ultimately disabling MTS and abolishing mitochondrial localization. However, this does not fully explain my failure to observe not only the mitochondrial translocation but also the extra-nuclear localization of 3xFLAG-hTERT.

Phosphorylation by Src at tyrosine 707 is required for nuclear export of hTERT by CRM1/Ran interaction near the C-terminal of hTERT [142] (Fig 1.2 B). β-hTERT does not have this C-terminal region due to PTC-mediated truncation, and it is possible that 3xFLAG-β-hTERT was trapped inside nuclei because of the impaired CRM1/Ran-mediated nuclear

export. Under the assumption that the two ends of the protein come into close proximity in the native state, the N-terminal 3xFLAG-tag may block CRM1-hTERT interaction through steric hindrance, leading to nuclear trapping of 3xFLAG-FL-hTERT. For clarification, it would be necessary to investigate whether Y707 phosphorylation of FLAG-tagged TERT isoforms occurs upon $H_2O_2$ treatment in hESCs.

## 4.6 Investigation of the effect of 3xFLAG-β-hTERT and 3xFLAG-FL-hTERT on mitochondria function in hESCs.

Ahmed *et al.* [195] demonstrated that ectopic expression of FL-hTERT in human fetal lung fibroblast cells results in increased mitochondria membrane potential and decreased mitochondria DNA content. Of note, the study did not consider whether mitochondrial localization of FL-hTERT was required for such changes. In my study, the potential mitochondrial changes in hESC after overexpressing 3xFLAG-β-hTERT and 3xFLAG-FL-hTERT were assessed.

Mitochondria membrane potential (MMP) was estimated by FL2 (fluorescence signal from J-aggregate, indicator of high membrane potential) to FL1 (fluorescence signal from JC-1 monomer, indicator of low membrane potential) ratio using flow cytometry (see section 2.5.1; Fig. 3.14 and 3.15). To account for the possibility of doxycycline side effect, DOX-treated non-transgenic HES2 was include in the experiment. The fold change in mitochondrial membrane potential (MMP) with DOX-treated HES2 F12 and HES2 B6 did not differ from DOX-treated non-transgenic HES2 control (Fig. 3.15). My failure to observe increase in MMP in DOX-treated HES2 FL12 may owe to the fact that DOX-induction in this cell line increases not only the 3xFLAG-FL-hTERT expression but also other hTERT

isoforms containing β-deletion (*see* section 4.2 of discussion). Certain mitochondrial roles of hTERT, such as DNA damage-amplifying [235] and mitochondrial ROS-reducing effects [173] requires the catalytically active form of hTERT. To date, there is no concrete evidence indicating that MMP-enhancing role of FL-hTERT strictly requires its presence in mitochondria, a large body of MMP-determining factors are nuclearly transcribed [65, 243]. control In this context, sufficiently expressed β-hTERT, a catalytically inactive form, could mask, and even inhibit the MMP-enhancing role of FL-hTERT from nuclei (recall 3xFLAG-β-hTERT remained in nuclei; *see* section 4.5). The fold change in MMP of DOX-treated HES2 B6 decreased slightly in comparison to that of HES2 control, but was not statistically significant ($p = 0.0635$, paired t-test). Considering the low level of DOX-induction of 3xFLAG-β-hTERT in HES2 B6 (see section 4.2), I believed that a significant change in MMP may be observed if 3xFLAG-β-hTERT can be overexpressed more robustly.

The effect of overexpressed 3xFLAG-β-hTERT and 3xFLAG-FL-hTERT on mitochondrial DNA (mtDNA) copy number was examined by qPCR. I confirm a side-effect of doxycycline by showing that mtDNA content decreases by ~20% in non-transgenic HES2 after doxycycline treatment. (Fig. 3.16 A). As previously mentioned, several past studies have shown that tetracycline family drugs can impair eukaryotic mitochondrial function by inhibiting mitochondrial ribosome [212 - 215], which suggests doxycycline may be hindering the mtDNA synthesis process. Interestingly, the reduction in mtDNA content was not detected when 3xFLAG-β-hTERT and 3xFLAG-FL-hTERT were overexpressed with doxycycline (Fig. 3.16 B and C). It was proposed that mtDNA synthesis involves priming of a nascent RNA from light strand promoter (LSP) [240] by a RNase formed between mitochondrial RNA processing endoribonuclease (RNase MRP) and RMRP (RNA component of the mitochondrial RNA-processing endoribonuclease) [241]. Studies by Maida

et al. [178, 185] and my earlier results (Fig. 3.7) demonstrated the interaction of RMRP with FL-hTERT and β-hTERT. In the context of successful mitochondrial localization of RMRP-bound 3xFLAG-β-hTERT and 3xFLAG-FL-hTERT, the available pool of RMRP in mitochondria may increase, promoting mtDNA synthesis. However, my previous results indicate no mitochondrial localization of 3xFLAG-β-hTERT and 3xFLAG-FL-hTERT in hESCs. Therefore, an intricate interplay between hTERT and RNase MRP (also known to be both nuclear and mitochondrial [242]) may exist to regulate mitochondria DNA synthesis from the nucleus.

To further investigate the regulation of mitochondria by nuclear 3xFLAG-β-hTERT and 3xFLAG-FL-hTERT, the expression of genes involved in mitochondria biogenesis (Fig. 3.17) and function (Fig. 3.18) was measured by RT-qPCR. PGC1 family proteins (PGC1A, PGC1B, PRC) are well known regulators of mitochondria biogenesis [243]. PGC1B, together with NRF1 (nuclear respiratory factor 1), stimulates the expression of target genes including regulators of mitochondria DNA replication (TFAM and POLG) [244] and mitochondrial transcription (TFAM and TFB1M) [240]. In support of the results from my mtDNA copy number experiment, the overexpression of 3xFLAG-β-hTERT and 3xFLAG-FL-hTERT did not affect the transcript abundance of any of the mitochondria biogenesis regulators (Fig. 3.17). Moreover, doxycycline treatment did not change the transcript abundance in HES2 (non-transgenic hESCs) (Fig. 3.17). This result indicates that DOX-induced reduction in mtDNA copy number observed earlier (Fig. 3.16) is not mediated by changes in nuclear transcribed genes, but more likely through direct intervention of doxycycline with the mitochondria. In terms of function, mitochondria of hPSC contain high levels of Mn-SOD2 (antioxidant enzyme)[66] and UCP2 (uncoupler protein)[245]. The steady-state transcript level of these genes also did not change with doxycycline treatment in any of the HES2 cell

lines (Fig. 3.18). Notably, UCP2 transcript level in all HES2 cells lines was much higher than other genes measured simultaneously, which supports the notion that HES2 is pluripotent cell with metabolic reliance on glycolysis (through down-regulation of OXPHOS through removal of carbon intermediates in the mitochondria matrix) [246].

## 4.7 The effect of 3xFLAG-β-hTERT and 3xFLAG-FL-hTERT on cell survival of HES2 lines under $H_2O_2$-induced oxidative stress.

$H_2O_2$ exposure at a high concentration can lead to generation of highly deleterious hydroxyl radicals and damage various cell components including lipids, proteins, and DNA. I conducted flow-cytometry experiments to measure cell survival/death in the various HES2 hESC lines under $H_2O_2$-induced oxidative stress.

First, the potential side effect of doxycycline on cell viability (Annexin V-FITC$^-$/7-AAD$^-$), apoptosis (Annexin V-FITC$^+$/7-AAD$^-$), and cell death (7-AAD$^+$) was assessed by flow cytometry in nontransgenic HES2 cells (Fig. 3.20-22 A). Our results show that doxycycline has no effect on cell survival under $H_2O_2$-induced cell stress (Fig. 3.20-22 A).

When 3xFLAG-β-hTERT overexpression was induced via doxycycline, % cell viability decreased (Fig. 3.20 B) while the apoptotic population increased in HES2 B6 (Fig. 3.21 B). However, the dead cell population remained unchanged (Fig. 3.22 B). On the other hand, when 3xFLAG-FL-hTERT was overexpressed via DOX, the viable population dramatically increased (Fig. 3.20 C) while the apoptotic population decreased (Fig. 3.21 C). In cancer research, there have been several reports demonstrating anti-apoptotic role of hTERT against chemotherapeutic drugs [251-254]. In research conducted by Cao *et al.* [248], a short-term knock down of endogenous hTERT (measurements were taken 24 hours post

antisense RNA-mediated knock down) in breast cancer cells resulted in increased apoptosis without change in telomere length. The hTERT-depletion-mediated apoptosis was overcome by overexpression of catalytically active *and* inactive hTERT, suggesting anti-apoptotic/extra-telomeric role of hTERT and its non-catalytic versions. In my study, however, the cell survival of $H_2O_2$-treated hESC is enhanced by 3xFLAG-FL-hTERT, not by 3xFLAG-β-hTERT. It is highly possible that 3xFLAG-β-hTERT and hTERT mutant used by Cao *et al.* behave differently due to their structural differences. Moreover, the types of cells and stress used in the studies are completely different, which can also contribute to the disparate outcome. The pro-survival role of hTERT is mediated through modulation of Bcl2/Bax and the resultant mitochondria-dependent apoptosis pathway[251, 253]. Importantly, the inhibition of Bax-dependent apoptosis signaling was simultaneous with mitochondrial localization of hTERT. Therefore, my failure to observe mitochondrial localization of 3xFLAG-hTERT suggests that the augmented cell viability following doxycycline treatment in HES2 FL12 is possibly independent of Bax pathway. There has been several reports of the protective role of hTERT on mitochondrial [188, 225] and nuclear[183] DNA. All three studies proposed that DNA-protection by hTERT is mediated through reduction of mitochondrial generated ROS. hESCs have minimally active OXPHOS activity, and have lower mitochondrial ROS than its differentiated progeny [66]. Therefore, it is likely that the cells were exposed to an exogenous source of ROS ($H_2O_2$) that is far more damaging than the endogenous ROS generated by mitochondria of hESC. It is speculated that the anti- and pro-survival effect exhibited by 3xFLAG-β-hTERT and 3xFLAG-FL-hTERT, respectively, would result from fundamental changes in anti-oxidant/DNA-repair mechanisms.

In addition, we show that DOX-induction in FL12 slightly increased dead cell population by ~2.5% (Fig. 3.22 C). At first glance, this may be puzzling because we demonstrated in the same experiment that DOX treatment in FL12 led to increase viability and decreased apoptosis. However, knowing that DOX-treated FL12 accompanies a significant increase in β-hTERT and α-β-hTERT transcript (Fig. 3.9 A), one can speculate that the molecular interactions between DOX-induced 3xFLAG-FL-hTERT and endogenous β-hTERT isoforms may lead to increased cell death.

Considering 3xFLAG-β-hTERT and 3xFLAG-FL-hTERT are predominantly nuclear under the treatment conditions (Fig. 3.12), I believe that the effect of 3xFLAG-β-hTERT and 3xFLAG-FL-hTERT on cell survivability under oxidative stress is likely mediated through its nuclear regulation, not through mitochondria. NF-kB is a key regulator of pluripotent state of hESC (*see* 1.3.2) and executes diverse cell functions including survival and apoptosis. Importantly, the nuclear translocation of p65 (one of five transcription factors in NF-kB signaling) is facilitated by its direct molecular interaction with hTERT and *vice versa* [255, 256]. In the light of recent discovery demonstrating $H_2O_2$-activated p65 and its nuclear translocation [253], it is highly likely that NF-kB plays a major role in the nuclear localization/retention of 3xFLAG-β-hTERT and 3xFLAG-FL-hTERT following $H_2O_2$ treatment, and their transcriptional activity in the nucleus to modulate cell survival under oxidative stress. For a future experiment, the molecular interaction between p65 and 3xFLAG-β-hTERT/ 3xFLAG-FL-hTERT should be tested using anti-FLAG-immunoprecipitation.

## 4.8 Concluding remarks and proposed future studies

hTERT remains a difficult protein to study due to its vastly diverse functions in different organelles, and for the poorly characterized mechanisms of its alternative splicing and isoforms. In this thesis, I show that hTERT transcript is alternatively spliced into isoforms carrying both α-deletion and β-deletion (α-β-), an α-deletion (α-), a β-deletion (β-), and neither deletion (α+β+) in hESC. The alternative splicing of hTERT pre-mRNA was affected significantly by both doxycycline and $H_2O_2$ treatments. It would be worthwhile to examine the physiological significance of the altered splicing in a future study. Using 3xFLAG-tagged recombinant hTERT proteins, I showed that 3xFLAG-β-hTERT overexpression inhibited the endogenous telomerase activity. The overexpression of 3xFLAG-FL-hTERT, on the other hand, did not change the telomerase activity, which I tried to explain through an aberrant increase in endogenous β-containing hTERT transcripts following DOX-induction in FL12 cells. To delineate the differences in isoform-specific functions, a better-controlled method to overexpress hTERT isoforms should be devised. Importantly, I failed to observe any physical/functional association between the recombinant hTERT isoforms and mitochondria in hESCs. To validate this, future experiments should focus on investigating the phosphorylation status of hTERT and kinases involved in hTERT cross-membrane translocation. Additionally, differently tagged- (smaller tag size and C-terminal location), or non-tagged hTERT isoforms should be considered. Lastly, I demonstrated the pro-survival role of FL-hTERT and the pro-apoptotic role of β-hTERT in hESCs under $H_2O_2$-induced stress. Based on my results, previous literature, and the known/observed side effects of doxycycline, I conclude that more extensive assessment on intra-cellular ROS and ROS-induced damage is required for an appropriate interpretation of the results.

Despite the great amount of evidence pointing to the extra-telomeric roles of the general hTERT population, surprisingly little effort has been made to tease out the functional significance of different hTERT isoforms and the regulation of hTERT splicing. For better understanding of hTERT, more attention to developing better techniques and tools for the detection of all hTERT isoforms is required so that TERT isoform-specific functions can be elucidated.

# Chapter 5: Reference List


[1] J. A. Thomson, "Embryonic Stem Cell Lines Derived from Human Blastocysts," *Science*, vol. 282, no. 5391. pp. 1145–1147, 1998.

[2] W. M. Schopperle and W. C. DeWolf, "The TRA-1-60 and TRA-1-81 human pluripotent stem cell markers are expressed on podocalyxin in embryonal carcinoma.," *Stem Cells*, vol. 25, no. 3, pp. 723–730, 2007.

[3] K. Takahashi and S. Yamanaka, "Induction of Pluripotent Stem Cells from Mouse Embryonic and Adult Fibroblast Cultures by Defined Factors," *Cell*, vol. 126, no. 4, pp. 663–676, 2006.

[4] J. Yu, M. A. Vodyanik, K. Smuga-Otto, J. Antosiewicz-Bourget, J. L. Frane, S. Tian, J. Nie, G. A. Jonsdottir, V. Ruotti, R. Stewart, I. I. Slukvin, and J. A. Thomson, "Induced pluripotent stem cell lines derived from human somatic cells.," *Sci. (New York, NY)*, vol. 318, no. 5858, pp. 1917–1920, 2007.

[5] K. Takahashi, K. Tanabe, M. Ohnuki, M. Narita, T. Ichisaka, K. Tomoda, and S. Yamanaka, "Induction of Pluripotent Stem Cells from Adult Human Fibroblasts by Defined Factors," *Cell*, vol. 107, no. 5, pp. 861–872, 2007.

[6] R. Allsopp, "Telomere length and iPSC re-programming: survival of the longest," *Cell Res.*, vol. 22, no. 4, pp. 614–615, 2012.

[7] F. Wang, Y. Yin, X. Ye, K. Liu, H. Zhu, L. Wang, M. Chiourea, M. Okuka, G. Ji, J. Dan, B. Zuo, M. Li, Q. Zhang, N. Liu, L. Chen, X. Pan, S. Gagos, D. L. Keefe, and L. Liu, "Molecular insights into the heterogeneity of telomere reprogramming in induced pluripotent stem cells.," *Cell Res.*, vol. 22, no. 4, pp. 757–68, 2012.

[8] S. Zeng, L. Liu, Y. Sun, P. Xie, L. Hu, D. Yuan, D. Chen, Q. Ouyang, G. Lin, and G. Lu, "Telomerase-mediated telomere elongation from human blastocysts to embryonic stem cells.," *J. Cell Sci.*, vol. 127, no. Pt 4, pp. 752–62, 2014.

[9] J. White and S. Dalton, "Cell cycle control of embryonic stem cells," *Stem Cell Rev*, vol. 1, no. 2, pp. 131–138, 2005.

[10] J. Ramalho-Santos, S. Varum, S. Amaral, P. C. Mota, A. P. Sousa, and A. Amaral, "Mitochondrial functionality in reproduction: From gonads and gametes to embryos and embryonic stem cells," *Human Reproduction Update*, vol. 15, no. 5. pp. 553–572, 2009.

[11] A. Prigione, B. Fauler, R. Lurz, H. Lehrach, and J. Adjaye, "The senescence-related mitochondrial/oxidative stress pathway is repressed in human induced pluripotent stem cells," *Stem Cells*, vol. 28, no. 4, pp. 721–733, 2010.

[12] B. R. Adams, S. E. Golding, R. R. Rao, and K. Valerie, "Dynamic dependence on ATR and ATM for double-Strand break repair in human embryonic stem cells and neural descendants," *PLoS One*, vol. 5, no. 4, 2010.

[13] B. R. Adams, A. J. Hawkins, L. F. Povirk, and K. Valerie, “ATM-independent, high-fidelity nonhomologous end joining predominates in human embryonic stem cells.,” *Aging (Albany. NY).*, vol. 2, no. 9, pp. 582–596, 2010.

[14] C. Xu, M. S. Inokuma, J. Denham, K. Golds, P. Kundu, J. D. Gold, and M. K. Carpenter, “Feeder-free growth of undifferentiated human embryonic stem cells.,” *Nat. Biotechnol.*, vol. 19, no. 10, pp. 971–974, 2001.

[15] M. Amit, M. K. Carpenter, M. S. Inokuma, C. P. Chiu, C. P. Harris, M. a Waknitz, J. Itskovitz-Eldor, and J. a Thomson, “Clonally derived human embryonic stem cell lines maintain pluripotency and proliferative potential for prolonged periods of culture,” *Dev Biol*, vol. 227, no. 2, pp. 271–278, 2000.

[16] A. B. McLean, K. a D’Amour, K. L. Jones, M. Krishnamoorthy, M. J. Kulik, D. M. Reynolds, A. M. Sheppard, H. Liu, Y. Xu, E. E. Baetge, and S. Dalton, “Activin a efficiently specifies definitive endoderm from human embryonic stem cells only when phosphatidylinositol 3-kinase signaling is suppressed.,” *Stem Cells*, vol. 25, no. 1, pp. 29–38, 2007.

[17] S. C. Bendall, M. H. Stewart, P. Menendez, D. George, K. Vijayaragavan, T. Werbowetski-Ogilvie, V. Ramos-Mejia, A. Rouleau, J. Yang, M. Bossé, G. Lajoie, and M. Bhatia, “IGF and FGF cooperatively establish the regulatory stem cell niche of pluripotent human cells in vitro.,” *Nature*, vol. 448, no. 7157, pp. 1015–21, 2007.

[18] L. Armstrong, O. Hughes, S. Yung, L. Hyslop, R. Stewart, I. Wappler, H. Peters, T. Walter, P. Stojkovic, J. Evans, M. Stojkovic, and M. Lako, “The role of PI3K/AKT, MAPK/ERK and NFκβ signalling in the maintenance of human embryonic stem cell pluripotency and viability highlighted by transcriptional profiling and functional analysis,” *Hum. Mol. Genet.*, vol. 15, no. 11, pp. 1894–1913, 2006.

[19] S. Ohtsuka and S. Dalton, “Molecular and biological properties of pluripotent embryonic stem cells,” *Gene Ther.*, vol. 15, no. 2, pp. 74–81, 2008.

[20] A. M. Singh, D. Reynolds, T. Cliff, S. Ohtsuka, A. L. Mattheyses, Y. Sun, L. Menendez, M. Kulik, and S. Dalton, “Signaling network crosstalk in human pluripotent cells: A Smad2/3-regulated switch that controls the balance between self-renewal and differentiation,” *Cell Stem Cell*, vol. 10, no. 3, pp. 312–326, 2012.

[21] J. Zhou, P. Su, L. Wang, J. Chen, M. Zimmermann, O. Genbacev, O. Afonja, M. C. Horne, T. Tanaka, E. Duan, S. J. Fisher, J. Liao, J. Chen, and F. Wang, “mTOR supports long-term self-renewal and suppresses mesoderm and endoderm activities of human embryonic stem cells.,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 106, no. 19, pp. 7840–7845, 2009.

[22] A. M. Singh, M. Bechard, K. Smith, and S. Dalton, “Reconciling the different roles of Gsk3?? in ‘na??ve’ and ‘primed’ pluripotent stem cells,” *Cell Cycle*, vol. 11, no. 16, pp. 2991–2996, 2012.

[23] P. Cartwright, C. McLean, A. Sheppard, D. Rivett, K. Jones, and S. Dalton, “LIF/STAT3 controls ES cell self-renewal and pluripotency by a Myc-dependent mechanism.,” *Development*, vol. 132, no. 5, pp. 885–896, 2005.

[24] U. Ullmann, C. Gilles, M. De Rycke, H. Van de Velde, K. Sermon, and I. Liebaers, "GSK-3-specific inhibitor-supplemented hESC medium prevents the epithelial-mesenchymal transition process and the up-regulation of matrix metalloproteinases in hESCs cultured in feeder-free conditions," *Mol. Hum. Reprod.*, vol. 14, no. 3, pp. 169–179, 2008.

[25] L. Cai, Z. Ye, B. Y. Zhou, P. Mali, C. Zhou, and L. Cheng, "Promoting human embryonic stem cell renewal or differentiation by modulating Wnt signal and culture conditions," *Cell Res.*, vol. 17, no. 1, pp. 62–72, 2007.

[26] D. Besser, "Expression of nodal, lefty-A, and lefty-B in undifferentiated human embryonic stem cells requires activation of Smad2/3," *J. Biol. Chem.*, vol. 279, no. 43, pp. 45076–45084, 2004.

[27] G. Dravid, Z. Ye, H. Hammond, G. Chen, A. Pyle, P. Donovan, X. Yu, and L. Cheng, "Defining the role of Wnt/β-catenin signaling in the survival, proliferation, and self-renewal of human embryonic stem cells," *Stem Cells*, vol. 23, no. 10, pp. 1489–1501, 2005.

[28] K. C. Davidson, a. M. Adams, J. M. Goodson, C. E. McDonald, J. C. Potter, J. D. Berndt, T. L. Biechele, R. J. Taylor, and R. T. Moon, "Wnt/b-catenin signaling promotes differentiation, not self-renewal, of human embryonic stem cells and is repressed by Oct4," *Proc. Natl. Acad. Sci.*, vol. 109, no. 12, pp. 4485–4490, 2012.

[29] H. Kim, J. Wu, S. Ye, C.-I. Tai, X. Zhou, H. Yan, P. Li, M. Pera, and Q.-L. Ying, "Modulation of β-catenin function maintains mouse epiblast stem cell and human embryonic stem cell self-renewal.," *Nat. Commun.*, vol. 4, no. 61, p. 2403, 2013.

[30] T. Sumi, N. Tsuneyoshi, N. Nakatsuji, and H. Suemori, "Defining early lineage specification of human embryonic stem cells by the orchestrated balance of canonical Wnt/beta-catenin, Activin/Nodal and BMP signaling.," *Development*, vol. 135, no. 17, pp. 2969–2979, 2008.

[31] D. ten Berge, D. Kurek, T. Blauwkamp, W. Koole, A. Maas, E. Eroglu, R. K. Siu, and R. Nusse, "Embryonic stem cells require Wnt proteins to prevent differentiation to epiblast stem cells.," *Nat. Cell Biol.*, vol. 13, no. 9, pp. 1070–5, 2011.

[32] M. Shtutman, J. Zhurinsky, I. Simcha, C. Albanese, M. D'Amico, R. Pestell, and A. Ben-Ze'ev, "The cyclin D1 gene is a target of the beta-catenin/LEF-1 pathway.," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 96, no. 10, pp. 5522–7, 1999.

[33] O. Tetsu and F. McCormick, "Beta-catenin regulates expression of cyclin D1 in colon carcinoma cells.," *Nature*, vol. 398, no. 6726, pp. 422–426, 1999.

[34] T. C. He, A. B. Sparks, C. Rago, H. Hermeking, L. Zawel, L. T. da Costa, P. J. Morin, B. Vogelstein, K. W. Kinzler, J. Groden, B. Rubinfeld, I. Albert, B. Souza, B. Rubinfeld, P. Polakis, B. Rubinfeld, M. Molenaar, J. Behrens, V. Korinek, P. J. Morin, B. Vogelstein, K. W. Kinzler, V. E. Velculescu, L. Zhang, B. Vogelstein, K. W. Kinzler, L. Zhang, V. E. Velculescu, K. J. Smith, M. van de Wetering, M. Oosterwegel, D. Dooijes, H. Clevers, K. Giese, A. Amsterdam, R. Grosschedl, L. Zawel, K. B. Marcu, S. A. Bossone, A. J. Patel, G. J. Kato, C. V. Dang, B. Amati, K. Alevizopoulos, J. Vlach, K. Sikora, M. D. Erisman, J. K. Scott, R. A. Watt, S. M.

Astrin, G. G. Finley, H. Imaseki, D. R. Smith, T. Myint, H. S. Goh, R. Dalla-Favera, S. Collins, M. Groudine, R. Dalla-Favera, F. Wong-Staal, R. C. Gallo, M. D. Erisman, M. C. Goyette, C. Rodriguez-Alfageme, E. J. Stanbridge, S. M. Astrin, A. Kamb, C. J. Sherr, W. R. Sellers, W. G. Kaelin, J. Jen, M. Ohhara, M. Esumi, Y. Kurosu, K. Alevizopoulos, J. Vlach, S. Hennecke, B. Amati, V. Korinek, and T.-C. He, "Identification of c-MYC as a target of the APC pathway.," *Science*, vol. 281, no. 5382, pp. 1509–12, 1998.

[35] R. Stewart, M. Stojkovic, and M. Lako, "Mechanisms of self-renewal in human embryonic stem cells.," *Eur. J. Cancer*, vol. 42, no. 9, pp. 1257–72, 2006.

[36] M. C. Simon and B. Keith, "The role of oxygen availability in embryonic development and stem cell function.," *Nat. Rev. Mol. Cell Biol.*, vol. 9, no. 4, pp. 285–96, 2008.

[37] F. Rodesch, P. Simon, C. Donner, and E. Jauniaux, "Oxygen measurements in endometrial and trophoblastic tissues during early pregnancy.," *Obstet. Gynecol.*, vol. 80, no. 2, pp. 283–285, 1992.

[38] O. Genbacev, "Regulation of Human Placental Development by Oxygen Tension," *Science (80-. ).*, vol. 277, no. 5332, pp. 1669–1672, 1997.

[39] J. G. Lees, J. Rathjen, J. R. Sheedy, D. K. Gardner, and A. J. Harvey, "Distinct profiles of human embryonic stem cell metabolism and mitochondria identified by oxygen," *Reproduction*, vol. 150, no. 4, pp. 367–382, 2015.

[40] P. Je??ek, L. Plecit??-Hlavat??, K. Smolkov??, and R. Rossignol, "Distinctions and similarities of cell bioenergetics and the role of mitochondria in hypoxia, cancer, and embryonic development," *International Journal of Biochemistry and Cell Biology*, vol. 42, no. 5. pp. 604–622, 2010.

[41] T. Ezashi, P. Das, and R. M. Roberts, "Low O2 tensions and the prevention of differentiation of hES cells.," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 102, no. 13, pp. 4783–4788, 2005.

[42] C. E. Forristal, K. L. Wright, N. A. Hanley, R. O. C. Oreffo, and F. D. Houghton, "Hypoxia inducible factors regulate pluripotency and proliferation in human embryonic stem cells cultured at reduced oxygen tensions.," *Reproduction*, vol. 139, no. 1, pp. 85–97, 2010.

[43] T. E. Ludwig, M. E. Levenstein, J. M. Jones, W. T. Berggren, E. R. Mitchen, J. L. Frane, L. J. Crandall, C. A. Daigh, K. R. Conard, M. S. Piekarczyk, R. A. Llanas, and J. A. Thomson, "Derivation of human embryonic stem cells in defined conditions.," *Nat. Biotechnol.*, vol. 24, no. 2, pp. 185–187, 2006.

[44] N. R. Forsyth, A. Musio, P. Vezzoni, a H. R. W. Simpson, B. S. Noble, and J. McWhir, "Physiologic oxygen enhances human embryonic stem cell clonal recovery and reduces chromosomal abnormalities.," *Cloning Stem Cells*, vol. 8, no. Icm, pp. 16–23, 2006.

[45] H. F. Chen, H. C. Kuo, W. Chen, F. C. Wu, Y. S. Yang, and H. N. Ho, "A reduced oxygen tension (5%) is not beneficial for maintaining human embryonic stem cells in

the undifferentiated state with short splitting intervals," *Hum. Reprod.*, vol. 24, no. 1, pp. 71–80, 2009.

[46] C. E. Forristal, K. L. Wright, N. A. Hanley, R. O. C. Oreffo, and F. D. Houghton, "Hypoxia inducible factors regulate pluripotency and proliferation in human embryonic stem cells cultured at reduced oxygen tensions," *Reproduction*, vol. 139, no. 1, pp. 85–97, 2010.

[47] T. E. Ludwig, M. E. Levenstein, J. M. Jones, W. T. Berggren, E. R. Mitchen, J. L. Frane, L. J. Crandall, C. a Daigh, K. R. Conard, M. S. Piekarczyk, R. a Llanas, and J. a Thomson, "Derivation of human embryonic stem cells in defined conditions.," *Nat. Biotechnol.*, vol. 24, no. 2, pp. 185–187, 2006.

[48] Y. Yoshida, K. Takahashi, K. Okita, T. Ichisaka, and S. Yamanaka, "Hypoxia Enhances the Generation of Induced Pluripotent Stem Cells," *Cell Stem Cell*, vol. 5, no. 3. pp. 237–241, 2009.

[49] N. R. Forsyth, A. Kay, K. Hampson, A. Downing, R. Talbot, and J. McWhir, "Transcriptome alterations due to physiological normoxic (2% O2) culture of human embryonic stem cells.," *Regen. Med.*, vol. 3, no. 6, pp. 817–833, 2008.

[50] C.-J. Hu, S. Iyer, A. Sataur, K. L. Covello, L. A. Chodosh, and M. C. Simon, "Differential regulation of the transcriptional activities of hypoxia-inducible factor 1 alpha (HIF-1alpha) and HIF-2alpha in stem cells.," *Mol. Cell. Biol.*, vol. 26, no. 9, pp. 3514–26, 2006.

[51] K. L. Covello, J. Kehler, H. Yu, J. D. Gordan, A. M. Arsham, C. J. Hu, P. A. Labosky, M. C. Simon, and B. Keith, "HIF-2{alpha} regulates Oct-4: effects of hypoxia on stem cell function, embryonic development, and tumor growth," *Genes Dev*, vol. 20, no. 5, pp. 557–570, 2006.

[52] C. M. Cameron, F. Harding, W.-S. Hu, and D. S. Kaufman, "Activation of Hypoxic Response in Human Embryonic Stem Cell–Derived Embryoid Bodies," *Exp. Biol. Med.*, vol. 233, no. 8, pp. 1044–1057, 2008.

[53] C. E. Forristal, D. R. Christensen, F. E. Chinnery, R. Petruzzelli, K. L. Parry, T. Sanchez-Elsner, and F. D. Houghton, "Environmental Oxygen Tension Regulates the Energy Metabolism and Self-Renewal of Human Embryonic Stem Cells," *PLoS One*, vol. 8, no. 5, 2013.

[54] E. Jauniaux, A. Watson, O. Ozturk, D. Quick, and G. Burton, "In-vivo measurement of intrauterine gases and acid-base values early in human pregnancy," *Hum. Reprod.*, vol. 14, no. 11, pp. 2901–2904, 1999.

[55] S. Varum, A. S. Rodrigues, M. B. Moura, O. Momcilovic, C. A. Easley IV, J. Ramalho-Santos, B. van Houten, and G. Schatten, "Energy metabolism in human pluripotent stem cells and their differentiated counterparts," *PLoS One*, vol. 6, no. 6, 2011.

[56] J. Zhang, I. Khvorostov, J. S. Hong, Y. Oktay, L. Vergnes, E. Nuebel, P. N. Wahjudi, K. Setoguchi, G. Wang, A. Do, H.-J. Jung, J. M. McCaffery, I. J. Kurland, K. Reue, W.-N. P. Lee, C. M. Koehler, and M. A. Teitell, "UCP2 regulates energy metabolism

and differentiation potential of human pluripotent stem cells," *The EMBO Journal*, vol. 30, no. 24. pp. 4860–4873, 2011.

[57] M. Pantaleon and P. L. Kaye, "Glucose transporters in preimplantation development.," *Rev. Reprod.*, vol. 3, pp. 77–81, 1998.

[58] K. Ito and T. Suda, "Metabolic requirements for the maintenance of self-renewing stem cells.," *Nat. Rev. Mol. Cell Biol.*, vol. 15, no. 4, pp. 243–56, 2014.

[59] L. Zhang, G. Marsboom, D. Glick, Y. Zhang, P. T. Toth, N. Jones, A. B. Malik, and J. Rehman, "Bioenergetic shifts during transitions between stem cell states (2013 Grover Conference series)," *Pulm Circ*, vol. 4, no. 3, pp. 387–394, 2014.

[60] J. C. St John, J. Ramalho-Santos, H. L. Gray, P. Petrosko, V. Y. Rawe, C. S. Navara, C. R. Simerly, and G. P. Schatten, "The expression of mitochondrial DNA transcription factors during early cardiomyocyte in vitro differentiation from human embryonic stem cells.," *Cloning Stem Cells*, vol. 7, no. 3, pp. 141–153, 2005.

[61] R. L. Krisher and R. S. Prather, "A role for the Warburg effect in preimplantation embryo development: Metabolic modification to support rapid cell proliferation," *Mol. Reprod. Dev.*, vol. 79, no. 5, pp. 311–320, 2012.

[62] P. Gao, I. Tchernyshyov, T.-C. Chang, Y.-S. Lee, K. Kita, T. Ochi, K. I. Zeller, A. M. De Marzo, J. E. Van Eyk, J. T. Mendell, and C. V Dang, "c-Myc suppression of miR-23a/b enhances mitochondrial glutaminase expression and glutamine metabolism.," *Nature*, vol. 458, no. 7239, pp. 762–5, 2009.

[63] N. Shyh-Chang, J. W. Locasale, C. A. Lyssiotis, Y. Zheng, R. Y. Teo, S. Ratanasirintrawoot, J. Zhang, T. Onder, J. J. Unternaehrer, H. Zhu, J. M. Asara, and G. Q. Daley, "Influence of threonine metabolism on S-adenosylmethionine and histone methylation.," *Science*, vol. 339, no. 6116, pp. 222–6, 2013.

[64] M. J. Son, Y. Kwon, M.-Y. Son, B. Seol, H.-S. Choi, S.-W. Ryu, C. Choi, and Y. S. Cho, "Mitofusins deficiency elicits mitochondrial metabolic reprogramming to pluripotency," *Cell Death Differ.*, pp. 1–13, 2015.

[65] C.-T. Chen, Y.-R. V Shih, T. K. Kuo, O. K. Lee, and Y.-H. Wei, "Coordinated changes of mitochondrial biogenesis and antioxidant enzymes during osteogenic differentiation of human mesenchymal stem cells.," *Stem Cells*, vol. 26, no. 4, pp. 960–8, 2008.

[66] Y. M. Cho, S. Kwon, Y. K. Pak, H. W. Seol, Y. M. Choi, D. J. Park, K. S. Park, and H. K. Lee, "Dynamic changes in mitochondrial biogenesis and antioxidant enzymes during the spontaneous differentiation of human embryonic stem cells," *Biochem. Biophys. Res. Commun.*, vol. 348, no. 4, pp. 1472–1478, 2006.

[67] A. Prigione and J. Adjaye, "Modulation of mitochondrial biogenesis and bioenergetic metabolism upon in vitro and in vivo differentiation of human ES and iPS cells," *International Journal of Developmental Biology*, vol. 54, no. 11–12. pp. 1729–1741, 2011.

[68] M. A. Esteban, T. Wang, B. Qin, J. Yang, D. Qin, J. Cai, W. Li, Z. Weng, J. Chen, S. Ni, K. Chen, Y. Li, X. Liu, J. Xu, S. Zhang, F. Li, W. He, K. Labuda, Y. Song, A.

Peterbauer, S. Wolbank, H. Redl, M. Zhong, D. Cai, L. Zeng, and D. Pei, “Vitamin C Enhances the Generation of Mouse and Human Induced Pluripotent Stem Cells,” *Cell Stem Cell*, vol. 6, no. 1, pp. 71–79, 2010.

[69] W. E. Wright, M. A. Piatyszek, W. E. Rainey, W. Byrd, and J. W. Shay, “Telomerase activity in human germline and embryonic tissues and cells,” *Dev. Genet.*, vol. 18, no. 2, pp. 173–179, 1996.

[70] K. Hiyama, Y. Hirai, S. Kyoizumi, M. Akiyama, E. Hiyama, M. A. Piatyszek, J. W. Shay, S. Ishioka, and M. Yamakido, “Activation of telomerase in human lymphocytes and hematopoietic progenitor cells.,” *J. Immunol.*, vol. 155, no. 8, pp. 3711–3715, 1995.

[71] N. P. Weng, L. Granger, and R. J. Hodes, “Telomere lengthening and telomerase activation during human B cell differentiation,” *Proc. Natl. Acad. Sci.*, vol. 94, no. 20, pp. 10827–32, 1997.

[72] J. Jiang, W. Lv, X. Ye, L. Wang, M. Zhang, H. Yang, M. Okuka, C. Zhou, X. Zhang, L. Liu, and J. Li, “Zscan4 promotes genomic stability during reprogramming and dramatically improves the quality of iPS cells as demonstrated by tetraploid complementation.,” *Cell Res.*, vol. 23, no. 1, pp. 92–106, 2013.

[73] C. W. Greider and E. H. Blackburn, “Identification of a specific telomere terminal transferase activity in tetrahymena extracts,” *Cell*, vol. 43, no. 2 PART 1, pp. 405–413, 1985.

[74] T. M. Nakamura, G. B. Morin, K. B. Chapman, S. L. Weinrich, W. H. Andrews, J. Lingner, C. B. Harley, and T. R. Cech, “Telomerase catalytic subunit homologs from fission yeast and human.,” *Science*, vol. 277, no. 5328, pp. 955–9, 1997.

[75] J. Feng, W. D. Funk, S. S. Wang, S. L. Weinrich, A. A. Avilion, C. P. Chiu, R. R. Adams, E. Chang, R. C. Allsopp, J. Yu, and et al., “The RNA component of human telomerase.,” *Science*, vol. 269, no. 5228, pp. 1236–41, 1995.

[76] A. Sauerwald, S. Sandin, G. Cristofari, S. H. W. Scheres, J. Lingner, and D. Rhodes, “Structure of active dimeric human telomerase.,” *Nat. Struct. Mol. Biol.*, vol. 20, no. 4, pp. 454–60, 2013.

[77] R. Hrdlickova, J. Nehyba, and H. R. Bose Jr., “Alternatively Spliced Telomerase Reverse Transcriptase Variants Lacking Telomerase Activity Stimulate Cell Proliferation,” *Mol. Cell. Biol.*, vol. 32, no. 21, pp. 4283–4296, 2012.

[78] S. Amor, S. Remy, G. Dambrine, Y. Le Vern, D. Rasschaert, and S. Laurent, “Alternative splicing and nonsense-mediated decay regulate telomerase reverse transcriptase (TERT) expression during virus-induced lymphomagenesis in vivo.,” *BMC Cancer*, vol. 10, p. 571, 2010.

[79] G. A. Ulaner and L. C. Giudice, “Developmental regulation of telomerase activity in human fetal tissues during gestation,” *Mol Hum Reprod*, vol. 3, no. 9, pp. 769–773, 1997.

[80] G. A. Ulaner, J. F. Hu, T. H. Vu, L. C. Giudice, and A. R. Hoffman, “Telomerase activity in human development is regulated by human telomerase reverse transcriptase

(hTERT) transcription and by alternate splicing of hTERT transcripts," *Cancer Res.*, vol. 58, no. 18, pp. 4168–4172, 1998.

[81] G. A. Ulaner, J. F. Hu, T. H. Vu, H. Oruganti, L. C. Giudice, and A. R. Hoffman, "Regulation of telomerase by alternate splicing of human telomerase reverse transcriptase (hTERT) in normal and neoplastic ovary, endometrium and myometrium," *Int. J. Cancer*, vol. 85, no. 3, pp. 330–335, 2000.

[82] G. A. Ulaner, J. F. Hu, T. H. Vu, L. C. Giudice, and A. R. Hoffman, "Tissue-specific alternate splicing of human telomerase reverse transcriptase (hTERT) influences telomere lengths during human development," *Int. J. Cancer*, vol. 91, no. 5, pp. 644–649, 2001.

[83] S. E. Holt, D. L. Aisner, J. W. Shay, and W. E. Wright, "Lack of cell cycle regulation of telomerase activity in human cells.," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 94, no. 20, pp. 10687–92, 1997.

[84] A. A. Avilion, M. A. Piatyszek, J. Gupta, J. W. Shay, S. Bacchetti, and C. W. Greider, "Human telomerase RNA and telomerase activity in immortal cell lines and tumor tissues.," *Cancer Res.*, vol. 56, no. 3, pp. 645–650, 1996.

[85] T. Vulliamy, A. Marrone, F. Goldman, A. Dearlove, M. Bessler, P. J. Mason, and I. Dokal, "The RNA component of telomerase is mutated in autosomal dominant dyskeratosis congenita.," *Nature*, vol. 413, no. 6854, pp. 432–5, 2001.

[86] D. Ruggero, S. Grisendi, F. Piazza, E. Rego, F. Mari, P. H. Rao, C. Cordon-Cardo, and P. P. Pandolfi, "Dyskeratosis congenita and cancer in mice deficient in ribosomal RNA modification.," *Science*, vol. 299, no. 5604, pp. 259–62, 2003.

[87] T. W. Nilsen and B. R. Graveley, "Expansion of the eukaryotic proteome by alternative splicing.," *Nature*, vol. 463, no. 7280, pp. 457–63, 2010.

[88] U. Schmidt, E. Basyuk, M. C. Robert, M. Yoshida, J. P. Villemin, D. Auboeuf, S. Aitken, and E. Bertrand, "Real-time imaging of cotranscriptional splicing reveals a kinetic model that reduces noise: Implications for alternative splicing regulation," *J. Cell Biol.*, vol. 193, no. 5, pp. 819–829, 2011.

[89] A. C. Goldstrohm, A. L. Greenleaf, and M. A. Garcia-Blanco, "Co-transcriptional splicing of pre-messenger RNAs: Considerations for the mechanism of alternative splicing," *Gene*, vol. 277, no. 1–2. pp. 31–47, 2001.

[90] M. S. Jurica and M. J. Moore, "Pre-mRNA splicing: Awash in a sea of proteins," *Molecular Cell*, vol. 12, no. 1. pp. 5–14, 2003.

[91] A. J. Matlin, F. Clark, and C. W. J. Smith, "Understanding alternative splicing: towards a cellular code.," *Nat. Rev. Mol. Cell Biol.*, vol. 6, no. 5, pp. 386–98, 2005.

[92] X. Roca, A. R. Krainer, and I. C. Eperon, "Pick one, but be quick: 5′ splice sites and the problems of too many choices," *Genes and Development*, vol. 27, no. 2. pp. 129–144, 2013.

[93] B. R. Graveley, "Coordinated control of splicing and translation.," *Nat. Struct. Mol. Biol.*, vol. 12, no. 12, pp. 1022–3, 2005.

[94] M. J. Moore, “From birth to death: the complex lives of eukaryotic mRNAs.,” *Science*, vol. 309, no. 5740, pp. 1514–8, 2005.

[95] M. S. Wong, W. E. Wright, and J. W. Shay, “Alternative splicing regulation of telomerase: a new paradigm?,” *Trends Genet.*, vol. 30, no. 10, pp. 430–438, 2014.

[96] A. Kilian, D. D. Bowtell, H. E. Abud, G. R. Hime, D. J. Venter, P. K. Keese, E. L. Duncan, R. R. Reddel, and R. A. Jefferson, “Isolation of a candidate human telomerase catalytic subunit gene, which reveals complex splicing patterns in different cell types.,” *Hum. Mol. Genet.*, vol. 6, no. 12, pp. 2011–2019, 1997.

[97] R. Hrdlickova, J. Nehyba, and H. R. Bose, “Alternatively Spliced Telomerase Reverse Transcriptase Variants Lacking Telomerase Activity Stimulate Cell Proliferation,” *Molecular and Cellular Biology*, vol. 32, no. 21. pp. 4283–4296, 2012.

[98] S. Saebøe-Larssen, E. Fossberg, and G. Gaudernack, “Characterization of novel alternative splicing sites in human telomerase reverse transcriptase (hTERT): analysis of expression and mutual correlation in mRNA isoforms from normal and tumour tissues.,” *BMC Mol. Biol.*, vol. 7, p. 26, 2006.

[99] X. Yi, D. M. White, D. L. Aisner, J. A. Baur, W. E. Wright, and J. W. Shay, “An Alternate Splicing Variant of the Human Telomerase Catalytic Subunit Inhibits Telomerase Activity1,” *Neoplasia*, vol. 2, no. 5, pp. 433–440, 2000.

[100] a Kilian, D. D. Bowtell, H. E. Abud, G. R. Hime, D. J. Venter, P. K. Keese, E. L. Duncan, R. R. Reddel, and R. a Jefferson, “Isolation of a candidate human telomerase catalytic subunit gene, which reveals complex splicing patterns in different cell types.,” *Hum. Mol. Genet.*, vol. 6, no. 12, pp. 2011–2019, 1997.

[101] M. Wick, D. Zubov, and G. Hagen, “Genomic organization and promoter characterization of the gene encoding the human telomerase reverse transcriptase (hTERT),” *Gene*, vol. 232, pp. 97–106, 1999.

[102] I. Listerman, J. Sun, F. S. Gazzaniga, J. L. Lukas, and E. H. Blackburn, “The major reverse transcriptase-incompetent splice variant of the human telomerase protein inhibits telomerase activity but protects from apoptosis,” *Cancer Res.*, vol. 73, no. 9, pp. 2817–2828, 2013.

[103] M. S. Wong, L. Chen, C. Foster, R. Kainthla, J. W. Shay, and W. E. Wright, “Regulation of Telomerase Alternative Splicing: A Target for Chemotherapy,” *Cell Rep.*, vol. 3, no. 4, pp. 1028–1035, 2013.

[104] G. A. Ulaner, J. F. Hu, T. H. Vu, L. C. Giudice, and A. R. Hoffman, “Tissue-specific alternate splicing of human telomerase reverse transcriptase (hTERT) influences telomere lengths during human development,” *Int. J. Cancer*, vol. 91, no. 5, pp. 644–649, 2001.

[105] S. Amor, S. Remy, G. Dambrine, Y. Le Vern, D. Rasschaert, and S. Laurent, “Alternative splicing and nonsense-mediated decay regulate telomerase reverse transcriptase (TERT) expression during virus-induced lymphomagenesis in vivo.,” *BMC Cancer*, vol. 10, p. 571, 2010.

[106] C. J. Anderson, S. F. Hoare, M. Ashcroft, A. E. Bilsland, and W. N. Keith, “Hypoxic regulation of telomerase gene expression by transcriptional and post-transcriptional mechanisms,” *Oncogene*, vol. 25, no. 1, pp. 61–69, 2006.

[107] L. M. Colgin, C. Wilkinson, A. Englezou, A. Kilian, M. O. Robinson, and R. R. Reddel, “The hTERTalpha splice variant is a dominant negative inhibitor of telomerase activity.,” *Neoplasia*, vol. 2, no. 5, pp. 426–32, 2000.

[108] E. Mavrogiannou, A. Strati, A. Stathopoulou, E. G. Tsaroucha, L. Kaklamanis, and E. S. Lianidou, “Real-time RT-PCR quantification of human telomerase reverse transcriptase splice variants in tumor cell lines and non-small cell lung cancer,” *Clin. Chem.*, vol. 53, no. 1, pp. 53–61, 2007.

[109] S. Y. Rha, H. C. Jeung, K. H. Park, J. J. Kim, and H. C. Chung, “Changes of telomerase activity by alternative splicing of full-length and beta variants of hTERT in breast cancer patients.,” *Oncol. Res.*, vol. 18, no. 5–6, pp. 213–220, 2009.

[110] H. Hisatomi and K. Ohyashiki, “Expression profile of a gamma-deletion variant of the human telomerase reverse transcriptase gene,” *Neoplasia*, vol. 5, no. 3, pp. 193–197, 2003.

[111] Y. Liu, B. Wu, H. Zhong, X. Tian, and W. Fang, “Quantification of Alternative Splicing Variants of Human Telomerase Reverse Transcriptase and Correlations with Telomerase Activity in Lung Cancer,” *PLoS ONE*, vol. 7, no. 6. p. e38868, 2012.

[112] X. Yi, D. M. White, D. L. Aisner, J. A. Baur, W. E. Wright, and J. W. Shay, “An Alternate Splicing Variant of the Human Telomerase Catalytic Subunit Inhibits Telomerase Activity1,” *Neoplasia*, vol. 2, no. 5, pp. 433–440, 2000.

[113] O. a Toogun, D. C. Dezwaan, and B. C. Freeman, “The hsp90 molecular chaperone modulates multiple telomerase activities.,” *Mol. Cell. Biol.*, vol. 28, no. 1, pp. 457–467, 2008.

[114] S. E. Holt, D. L. Aisner, J. Baur, V. M. Tesmer, M. Dy, M. Ouellette, J. B. Trager, G. B. Morin, D. O. Toft, J. W. Shay, W. E. Wright, and M. A. White, “Functional requirement of p23 and Hsp90 in telomerase complexes,” *Genes Dev.*, vol. 13, no. 7, pp. 817–826, 1999.

[115] B. R. Keppler, A. T. Grady, and M. B. Jarstfer, “The biochemical role of the heat shock protein 90 chaperone complex in establishing human telomerase activity,” *J Biol Chem*, vol. 281, no. 29, pp. 19840–19848, 2006.

[116] R. H. Kim, R. Kim, W. Chen, S. Hu, K. H. Shin, N. H. Park, and M. K. Kang, “Association of hsp90 to the hTERT promoter is necessary for hTERT expression in human oral cancer cells,” *Carcinogenesis*, vol. 29, no. 12, pp. 2425–2431, 2008.

[117] Y. S. Cong and S. Bacchetti, “Histone deacetylation is involved in the transcriptional repression of hTERT in normal human cells,” *J. Biol. Chem.*, vol. 275, no. 46, pp. 35665–35668, 2000.

[118] S. Kyo, M. Takakura, T. Taira, T. Kanaya, H. Itoh, M. Yutsudo, H. Ariga, and M. Inoue, “Sp1 cooperates with c-Myc to activate transcription of the human telomerase

reverse transcriptase gene (hTERT)," *Nucleic Acids Res.*, vol. 28, no. 3, pp. 669–677, 2000.

[119] S. A. Wang, J. Y. Chuang, S. H. Yeh, Y. T. Wang, Y. W. Liu, W. C. Chang, and J. J. Hung, "Heat Shock Protein 90 Is Important for Sp1 Stability during Mitosis," *J. Mol. Biol.*, vol. 387, no. 5, pp. 1106–1119, 2009.

[120] D. Cheng, Y. Zhao, S. Wang, W. Jia, J. Kang, and J. Zhu, "Human telomerase reverse transcriptase (hTERT) transcription requires Sp1/Sp3 binding to the promoter and a permissive chromatin environment," *J. Biol. Chem.*, vol. 290, no. 50, pp. 30193–30203, 2015.

[121] J. Won, J. Yim, and T. K. Kim, "Sp1 and Sp3 recruit histone deacetylase to repress transcription of human telomerase reverse transcriptase (hTERT) promoter in normal human somatic cells," *J. Biol. Chem.*, vol. 277, no. 41, pp. 38230–38238, 2002.

[122] W. K. Aldous, A. J. Marean, M. J. DeHart, L. A. Matej, and K. H. Moore, "Effects of tamoxifen on telomerase activity in breast carcinoma cell lines," *Cancer*, vol. 85, no. 7, pp. 1523–1529, 1999.

[123] S. M. O. Phipps, W. K. Love, T. White, L. G. Andrews, and T. O. Tollefsbol, "Retinoid-induced histone deacetylation inhibits telomerase activity in estrogen receptor-negative breast cancer cells," *Anticancer Res.*, vol. 29, no. 12, pp. 4959–4964, 2009.

[124] a Azouz, Y.-L. Wu, J. Hillion, I. Tarkanyi, a Karniguian, J. Aradi, M. Lanotte, G.-Q. Chen, M. Chehna, and E. Ségal-Bendirdjian, "Epigenetic plasticity of hTERT gene promoter determines retinoid capacity to repress telomerase in maturation-resistant acute promyelocytic leukemia cells.," *Leukemia*, vol. 24, no. 3, pp. 613–22, 2010.

[125] M. J. Choi, K. H. Cho, S. Lee, Y. J. Bae, K. J. Jeong, S. Y. Rha, E. J. Choi, J. H. Park, J. M. Kim, J.-S. Lee, G. B. Mills, and H. Y. Lee, "hTERT mediates norepinephrine-induced Slug expression and ovarian cancer aggressiveness.," *Oncogene*, vol. 34, no. 26, pp. 3402–12, 2015.

[126] S. H. Kim, K. H. Cho, Y. N. Kim, B. Y. Jeong, C. G. Park, G. M. Hur, and H. Y. Lee, "Resveratrol attenuates norepinephrine-induced ovarian cancer invasiveness through downregulating hTERT expression," *Arch. Pharm. Res.*, vol. 39, no. 2, pp. 240–248, 2016.

[127] H. Nishi, T. Nakada, S. Kyo, M. Inoue, J. W. Shay, and K. Isaka, "Hypoxia-inducible factor 1 mediates upregulation of telomerase (hTERT).," *Mol. Cell. Biol.*, vol. 24, no. 13, pp. 6076–6083, 2004.

[128] J. Wang, L. Y. Xie, S. Allan, D. Beach, and G. J. Hannon, "Myc activates telomerase," *Genes Dev.*, vol. 12, no. 12, pp. 1769–1774, 1998.

[129] J. H. Kim, S. M. Park, M. R. Kang, S. Y. Oh, T. H. Lee, M. T. Muller, and I. K. Chung, "Ubiquitin ligase MKRN1 modulates telomere length homeostasis through a proteolysis of hTERT," *Genes Dev.*, vol. 19, no. 7, pp. 776–781, 2005.

[130] J. H. Lee, P. Khadka, S. H. Baek, and I. K. Chung, "CHIP promotes human telomerase reverse transcriptase degradation and negatively regulates telomerase activity.," *J. Biol. Chem.*, vol. 285, no. 53, pp. 42033–45, 2010.

[131] W. Oh, E.-W. Lee, D. Lee, M.-R. Yang, A. Ko, C.-H. Yoon, H.-W. Lee, Y.-S. Bae, C. Y. Choi, and J. Song, "Hdm2 negatively regulates telomerase activity by functioning as an E3 ligase of hTERT.," *Oncogene*, vol. 29, no. 28, pp. 4101–12, 2010.

[132] B. N. Nguyen, L. W. Elmore, and S. E. Holt, "Mechanism of dominant-negative telomerase function," *Cell Cycle*, vol. 8, no. 19, pp. 3227–3233, 2009.

[133] S. Kharbanda, V. Kumar, S. Dhar, P. Pandey, C. Chen, P. Majumder, Z. M. Yuan, Y. Whang, W. Strauss, T. K. Pandita, D. Weaver, and D. Kufe, "Regulation of the hTERT telomerase catalytic subunit by the c-Abl tyrosine kinase," *Curr. Biol.*, vol. 10, no. 10, pp. 568–575, 2000.

[134] S. Panneer Selvam, R. M. De Palma, J. J. Oaks, N. Oleinik, Y. K. Peterson, R. V. Stahelin, E. Skordalakes, S. Ponnusamy, E. Garrett-Mayer, C. D. Smith, and B. Ogretmen, "Binding of the sphingolipid S1P to hTERT stabilizes telomerase at the nuclear periphery by allosterically mimicking protein phosphorylation," *Sci. Signal.*, vol. 8, no. 381, p. ra58-ra58, 2015.

[135] H. Li, L. Zhao, Z. Yang, J. W. Funder, and J. P. Liu, "Telomerase is controlled by protein kinase Calpha in human breast cancer cells," *J Biol Chem*, vol. 273, no. 50, pp. 33436–33442, 1998.

[136] J. T. Chang, Y.-C. Lu, Y.-J. Chen, C.-P. Tseng, Y.-L. Chen, C.-W. Fang, and a-J. Cheng, "hTERT phosphorylation by PKC is essential for telomerase holoprotein integrity and enzyme activity in head neck cancer cells.," *Br. J. Cancer*, vol. 94, no. 6, pp. 870–8, 2006.

[137] J. Chung, P. Khadka, and I. K. Chung, "Nuclear import of hTERT requires a bipartite nuclear localization signal and Akt-mediated phosphorylation," *J. Cell Sci.*, vol. 125, no. Pt 11, pp. 2684–2697, 2012.

[138] S. S. Kang, T. Kwon, D. Y. Kwon, and S. Il Do, "Akt protein kinase enhances human telomerase activity through phosphorylation of telomerase reverse transcriptase subunit," *J. Biol. Chem.*, vol. 274, no. 19, pp. 13085–13090, 1999.

[139] K. Liu, R. J. Hodes, and N. -p. Weng, "Cutting Edge: Telomerase Activation in Human T Lymphocytes Does Not Require Increase in Telomerase Reverse Transcriptase (hTERT) Protein But Is Associated with hTERT Phosphorylation and Nuclear Translocation," *J. Immunol.*, vol. 166, no. 8, pp. 4826–4830, 2001.

[140] Y. Huang, L. Sun, N. Liu, Q. Wei, L. Jiang, X. Tong, and X. Ye, "Polo-like Kinase 1 (Plk1) up-regulates telomerase activity by affecting human telomerase reverse transcriptase (hTERT) stability," *J. Biol. Chem.*, vol. 290, no. 30, pp. 18865–18873, 2015.

[141] N. Büchner, T. C. Zschauer, M. Lukosz, J. Altschmied, and J. Haendeler, "Downregulation of mitochondrial telomerase reverse transcriptase induced by H2O2 is Src kinase dependent," *Exp. Gerontol.*, vol. 45, no. 7–8, pp. 558–562, 2010.

[142] J. Haendeler, J. Hoffmann, R. P. Brandes, A. M. Zeiher, and S. Dimmeler, “Hydrogen peroxide triggers nuclear export of telomerase reverse transcriptase via Src kinase family-dependent phosphorylation of tyrosine 707.,” *Mol. Cell. Biol.*, vol. 23, no. 13, pp. 4598–4610, 2003.

[143] H. Seimiya, H. Sawada, Y. Muramatsu, M. Shimizu, K. Ohko, K. Yamane, and T. Tsuruo, “Involvement of 14-3-3 proteins in nuclear localization of telomerase.,” *EMBO J.*, vol. 19, no. 11, pp. 2652–61, 2000.

[144] P. Xi, L. Zhou, M. Wang, J. P. Liu, and Y. S. Cong, “Serine/threonine-protein phosphatase 2A physically interacts with human telomerase reverse transcriptase hTERT and regulates its subcellular distribution,” *J. Cell. Biochem.*, vol. 114, no. 2, pp. 409–417, 2013.

[145] T. P. Rao and M. Kühl, “An updated overview on wnt signaling pathways: A prelude for more,” *Circulation Research*, vol. 106, no. 12. pp. 1798–1806, 2010.

[146] B. T. MacDonald, K. Tamai, and X. He, “Wnt/??-Catenin Signaling: Components, Mechanisms, and Diseases,” *Developmental Cell*, vol. 17, no. 1. pp. 9–26, 2009.

[147] J.-I. Park, A. S. Venteicher, J. Y. Hong, J. Choi, S. Jun, M. Shkreli, W. Chang, Z. Meng, P. Cheung, H. Ji, M. McLaughlin, T. D. Veenstra, R. Nusse, P. D. McCrea, and S. E. Artandi, “Telomerase modulates Wnt signalling by association with target gene chromatin.,” *Nature*, vol. 460, no. 7251, pp. 66–72, 2009.

[148] Y. Maida, M. Yasukawa, N. Okamoto, S. Ohka, K. Kinoshita, Y. Totoki, T. K. Ito, T. Minamino, H. Nakamura, S. Yamaguchi, T. Shibata, and K. Masutomi, “Involvement of TERT in heterochromatin maintenance.,” *Mol. Cell. Biol.*, 2014.

[149] I. Listerman, F. S. Gazzaniga, and E. H. Blackburn, “An investigation of the effects of the core protein telomerase reverse transcriptase on Wnt signaling in breast cancer cells,” *Mol. Cell. Biol.*, vol. 34, no. 2, pp. 280–289, 2014.

[150] K. Hoffmeyer, A. Raggioli, S. Rudloff, R. Anton, A. Hierholzer, I. Del Valle, K. Hein, R. Vogt, and R. Kemler, “Wnt/ -Catenin Signaling Regulates Telomerase in Stem Cells and Cancer Cells,” *Science*, vol. 336, no. 6088. pp. 1549–1554, 2012.

[151] Y. Zhang, L. Toh, P. Lau, and X. Wang, “Human telomerase reverse transcriptase (hTERT) is a novel target of the Wnt/β-catenin pathway in human cancer.,” *J. Biol. Chem.*, vol. 287, no. 39, pp. 32494–511, 2012.

[152] S. Wu, Y. Ge, L. Huang, H. Liu, Y. Xue, and Y. Zhao, “BRG1, the ATPase subunit of SWI/SNF chromatin remodeling complex, interacts with HDAC2 to modulate telomerase expression in human cancer cells,” *Cell Cycle*, vol. 13, no. 18, pp. 2869–2878, 2014.

[153] A. Ghosh, G. Saginc, S. C. Leow, E. Khattar, E. M. Shin, T. D. Yan, M. Wong, Z. Zhang, G. Li, W.-K. Sung, J. Zhou, W. J. Chng, S. Li, E. Liu, and V. Tergaonkar, “Telomerase directly regulates NF-κB-dependent transcription.,” *Nat. Cell Biol.*, vol. 14, no. 12, pp. 1270–81, 2012.

[154] J. Choi, L. K. Southworth, K. Y. Sarin, A. S. Venteicher, W. Ma, W. Chang, P. Cheung, S. Jun, M. K. Artandi, N. Shah, S. K. Kim, and S. E. Artandi, “TERT

promotes epithelial proliferation through transcriptional control of a Myc- and Wnt-related developmental program," *PLoS Genet.*, vol. 4, no. 1, pp. 0124–0138, 2008.

[155] J. Zhou, B. Mao, Q. Zhou, D. Ding, M. Wang, P. Guo, Y. Gao, J. W. Shay, Z. Yuan, and Y. S. Cong, "Endoplasmic reticulum stress activates telomerase," *Aging Cell*, vol. 13, no. 1, pp. 197–200, 2014.

[156] A. Oeckinghaus, M. S. Hayden, and S. Ghosh, "Crosstalk in NF-κB signaling pathways.," *Nat. Immunol.*, vol. 12, no. 8, pp. 695–708, 2011.

[157] L. Yin, A. K. Hubbard, and C. Giardina, "NF-??B regulates transcription of the mouse telomerase catalytic subunit," *J. Biol. Chem.*, vol. 275, no. 47, pp. 36671–36675, 2000.

[158] M. Akiyama, T. Hideshima, T. Hayashi, Y. T. Tai, C. S. Mitsiades, N. Mitsiades, D. Chauhan, P. Richardson, N. C. Munshi, and K. C. Anderson, "Cytokines modulate telomerase activity in a human multiple myeloma cell line," *Cancer Res*, vol. 62, no. 13, pp. 3876–3882, 2002.

[159] Q. P. Zuo, S. K. Liu, Z. J. Li, B. Li, Y. L. Zhou, R. Guo, and L. H. Huang, "NF-kappaB p65 modulates the telomerase reverse transcriptase in the HepG(2) hepatoma cell line," *Eur J Pharmacol*, vol. 672, no. 1–3, pp. 113–120, 2011.

[160] D. Ding, P. Xi, J. Zhou, M. Wang, and Y. S. Cong, "Human telomerase reverse transcriptase regulates MMP expression independently of telomerase activity via NF-??B-dependent transcription," *FASEB J.*, vol. 27, no. 11, pp. 4375–4383, 2013.

[161] O. Takase, M. Yoshikawa, M. Idei, J. Hirahashi, T. Fujita, T. Takato, T. Isagawa, G. Nagae, H. Suemori, H. Aburatani, and K. Hishikawa, "The Role of NF-kappaB Signaling in the Maintenance of Pluripotency of Human Induced Pluripotent Stem Cells," *PLoS One*, vol. 8, no. 2, p. e56399, 2013.

[162] Y.-E. Kim, H.-B. Kang, J.-A. Park, K.-H. Nam, H.-J. Kwon, and Y. Lee, "Upregulation of NF-kappaB upon differentiation of mouse embryonic stem cells.," *BMB Rep.*, vol. 41, no. 10, pp. 705–9, 2008.

[163] J. Torres and F. M. Watt, "Nanog maintains pluripotency of mouse embryonic stem cells by inhibiting NFkappaB and cooperating with Stat3.," *Nat. Cell Biol.*, vol. 10, no. 2, pp. 194–201, 2008.

[164] Y. Maida, M. Yasukawa, M. Furuuchi, T. Lassmann, R. Possemato, N. Okamoto, V. Kasim, Y. Hayashizaki, W. C. Hahn, and K. Masutomi, "An RNA-dependent RNA polymerase formed by TERT and the RMRP RNA.," *Nature*, vol. 461, no. 7261, pp. 1–7, 2009.

[165] J. Rosenbluh, D. Nijhawan, Z. Chen, K. K. Wong, K. Masutomi, and W. C. Hahn, "Rmrp is a non-coding rna essential for early murine development," *PLoS One*, vol. 6, no. 10, 2011.

[166] J. Park and S. Jeong, "Wnt activated β-catenin and YAP proteins enhance the expression of non-coding RNA component of RNase MRP in colon cancer cells.," *Oncotarget*, vol. 6, no. 33, pp. 34658–68, 2015.

[167] D. D. Chang and D. A. Clayton, “A novel endoribonuclease cleaves at a priming site of mouse mitochondrial DNA replication.,” *EMBO J.*, vol. 6, no. 2, pp. 409–17, 1987.

[168] M. Ridanpää, H. Van Eenennaam, K. Pelin, R. Chadwick, C. Johnson, B. Yuan, W. VanVenrooij, G. Pruijn, R. Salmela, S. Rockas, O. Mäkitie, I. Kaitila, and A. De la Chapelle, “Mutations in the RNA component of RNase MRP cause a pleiotropic human disease, cartilage-hair hypoplasia,” *Cell*, vol. 104, no. 2, pp. 195–203, 2001.

[169] D. D. Chang and D. A. Clayton, “A mammalian mitochondrial RNA processing activity contains nucleus-encoded RNA.,” *Science*, vol. 235, no. 4793, pp. 1178–1184, 1987.

[170] N. K. Sharma, A. Reyes, P. Green, M. J. Caron, M. G. Bonini, D. M. Gordon, I. J. Holt, and J. H. Santos, “Human telomerase acts as a hTR-independent reverse transcriptase in mitochondria,” *Nucleic Acids Research*, vol. 40, no. 2. pp. 712–725, 2012.

[171] Y. Maida, S. Kyo, T. Lassmann, Y. Hayashizaki, and K. Masutomi, “Off-target effect of endogenous siRNA derived from RMRP in human cells,” *Int. J. Mol. Sci.*, vol. 14, pp. 9305–9318, 2013.

[172] J. H. Santos, J. N. Meyer, M. Skorvaga, L. A. Annab, and B. Van Houten, “Mitochondrial hTERT exacerbates free-radical-mediated mtDNA damage,” *Aging Cell*, vol. 3, no. 6, pp. 399–411, 2004.

[173] J. Haendeler, S. Dröse, N. Büchner, S. Jakob, J. Altschmied, C. Goy, I. Spyridopoulos, A. M. Zeiher, U. Brandt, and S. Dimmeler, “Mitochondrial telomerase reverse transcriptase binds to and protects mitochondrial DNA and function from damage,” *Arterioscler. Thromb. Vasc. Biol.*, vol. 29, pp. 929–935, 2009.

[174] S. Ahmed, J. F. Passos, M. J. Birket, T. Beckmann, S. Brings, H. Peters, M. A. Birch-Machin, T. von Zglinicki, and G. Saretzki, “Telomerase does not counteract telomere shortening but protects mitochondrial function under oxidative stress.,” *J. Cell Sci.*, vol. 121, no. Pt 7, pp. 1046–1053, 2008.

[175] J. H. Santos, J. N. Meyer, M. Skorvaga, L. A. Annab, and B. Van Houten, “Mitochondrial hTERT exacerbates free-radical-mediated mtDNA damage,” *Aging Cell*, vol. 3, no. 6, pp. 399–411, 2004.

[176] O. A. Kovalenko, M. J. Caron, P. Ulema, C. Medrano, A. P. Thomas, M. Kimura, M. G. Bonini, U. Herbig, and J. H. Santos, “A mutant telomerase defective in nuclear-cytoplasmic shuttling fails to immortalize cells and is associated with mitochondrial dysfunction,” *Aging Cell*, vol. 9, no. 2, pp. 203–219, 2010.

[177] I. R. Indran, M. P. Hande, and S. Pervaiz, “Tumor cell redox state and mitochondria at the center of the non-canonical activity of telomerase reverse transcriptase,” *Molecular Aspects of Medicine*, vol. 31, no. 1. pp. 21–28, 2010.

[178] S. Mandal, A. G. Lindgren, A. S. Srivastava, A. T. Clark, and U. Banerjee, “Mitochondrial function controls proliferation and early differentiation potential of embryonic stem cells.,” *Stem Cells*, vol. 29, no. 3, pp. 486–95, 2011.

[179] C. W. Greider and E. H. Blackburn, “Identification of a specific telomere terminal transferase activity in Tetrahymena extracts.,” *Cell*, vol. 43, no. 2 Pt 1, pp. 405–413, 1985.

[180] T. Lassmann, Y. Maida, Y. Tomaru, M. Yasukawa, Y. Ando, M. Kojima, V. Kasim, C. Simon, C. Daub, P. Carninci, Y. Hayashizaki, and K. Masutomi, “Telomerase Reverse Transcriptase Regulates microRNAs,” *Int. J. Mol. Sci.*, vol. 16, pp. 1192–1208, 2015.

[181] Y. Maida, M. Yasukawa, M. Furuuchi, T. Lassmann, R. Possemato, N. Okamoto, V. Kasim, Y. Hayashizaki, W. C. Hahn, and K. Masutomi, “An RNA-dependent RNA polymerase formed by TERT and the RMRP RNA.,” *Nature*, vol. 461, no. 7261, pp. 230–235, 2009.

[182] N. Büchner, T. C. Zschauer, M. Lukosz, J. Altschmied, and J. Haendeler, “Downregulation of mitochondrial telomerase reverse transcriptase induced by H2O2 is Src kinase dependent,” *Exp. Gerontol.*, vol. 45, no. 7–8, pp. 558–562, 2010.

[183] C. Singhapol, D. Pal, R. Czapiewski, M. Porika, G. Nelson, and G. C. Saretzki, “Mitochondrial Telomerase Protects Cancer Cells from Nuclear DNA Damage and Apoptosis,” *PLoS One*, vol. 8, 2013.

[184] L. Radan, C. S. Hughes, J. H. Teichroeb, F. M. Vieira Zamora, M. Jewer, L.-M. Postovit, and D. H. Betts, “Microenvironmental Regulation of Telomerase Isoforms in Human Embryonic Stem Cells.,” *Stem Cells Dev.*, 2014.

[185] C. Yang, S. Przyborski, M. J. Cooke, X. Zhang, R. Stewart, G. Anyfantis, S. P. Atkinson, G. Saretzki, L. Armstrong, and M. Lako, “A key role for telomerase reverse transcriptase unit in modulating human embryonic stem cell proliferation, cell cycle dynamics, and in vitro differentiation.,” *Stem Cells*, vol. 26, no. 4, pp. 850–63, 2008.

[186] K. Collins and L. Gandhi, “The reverse transcriptase component of the Tetrahymena telomerase ribonucleoprotein complex.,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 95, no. 15, pp. 8485–8490, 1998.

[187] S. Rouda and E. Skordalakes, “Structure of the RNA-Binding Domain of Telomerase: Implications for RNA Recognition and Binding,” *Structure*, vol. 15, no. 11, pp. 1403–1412, 2007.

[188] K. Arai, K. Arai, K. Masutomi, K. Masutomi, S. Khurts, S. Khurts, S. Kaneko, S. Kaneko, K. Kobayashi, K. Kobayashi, S. Murakami, and S. Murakami, “Two independent regions of human telomerase reverse transcriptase are important for its oligomerization and telomerase activity.,” *J. Biol. Chem.*, vol. 277, no. 10, pp. 8538–44, 2002.

[189] C. Frohnert, S. Hutten, S. Wälde, A. Nath, and R. H. Kehlenbach, “Importin 7 and Nup358 promote nuclear import of the protein component of human telomerase,” *PLoS One*, vol. 9, no. 2, 2014.

[190] B. E. Reubinoff, M. F. Pera, C. Y. Fong, a Trounson, and a Bongso, “Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro.,” *Nat. Biotechnol.*, vol. 18, no. 4, pp. 399–404, 2000.

[191] P. P. Banerjee and S. Jagadeesh, “Non-radioactive assay methods for the assessment of telomerase activity and telomere length,” *Methods Mol. Biol.*, vol. 1288, pp. 305–316, 2015.

[192] M. Reen, T. W. Smith, and L. B. Chens, “J-Aggregate Formation of a Carbocyanine as a Quantitative Fluorescent Indicator of Membrane Potential+,” *Biochemistry*, vol. 30, pp. 4480–4486, 1991.

[193] S. T. Smiley, M. Reers, C. Mottola-Hartshorn, M. Lin, A. Chen, T. W. Smith, G. D. Steele, and L. B. Chen, “Intracellular heterogeneity in mitochondrial membrane potentials revealed by a J-aggregate-forming lipophilic cation JC-1.,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 88, no. 9, pp. 3671–3675, 1991.

[194] L. Kent, “Culture and maintenance of human embryonic stem cells.,” *J. Vis. Exp.*, no. 34, pp. 2–5, 2009.

[195] S. Ahmed, J. F. Passos, M. J. Birket, T. Beckmann, S. Brings, H. Peters, M. A. Birch-Machin, T. von Zglinicki, and G. Saretzki, “Telomerase does not counteract telomere shortening but protects mitochondrial function under oxidative stress,” *J. Cell Sci.*, vol. 121, no. 7, pp. 1046–1053, 2008.

[196] T. Rivera, C. Haggblom, S. Cosconati, and J. Karlseder, “A balance between elongation and trimming regulates telomere stability in stem cells,” *Nat. Struct. Mol. Biol.*, vol. 24, no. 1, pp. 30–39, 2016.

[197] X. Hang, P. Li, Z. Li, W. Qu, Y. Yu, H. Li, Z. Shen, H. Zheng, Y. Gao, Y. Wu, M. Deng, Z. Sun, and C. Zhang, “Transcription and splicing regulation in human umbilical vein endothelial cells under hypoxic stress conditions by exon array,” *BMC Genomics*, vol. 10, p. 126, 2009.

[198] J. a Sena, L. Wang, L. E. Heasley, and C.-J. Hu, “Hypoxia Regulates Alternative Splicing of HIF and non-HIF Target Genes.,” *Mol. Cancer Res.*, pp. 1233–1244, 2014.

[199] I. S. C. Initiative, O. Adewumi, B. Aflatoonian, L. Ahrlund-Richter, M. Amit, P. W. Andrews, G. Beighton, P. A. Bello, N. Benvenisty, L. S. Berry, S. Bevan, B. Blum, J. Brooking, K. G. Chen, A. B. Choo, G. A. Churchill, M. Corbel, I. Damjanov, J. S. Draper, P. Dvorak, K. Emanuelsson, R. A. Fleck, A. Ford, K. Gertow, M. Gertsenstein, P. J. Gokhale, R. S. Hamilton, A. Hampl, L. E. Healy, O. Hovatta, J. Hyllner, M. P. Imreh, J. Itskovitz-Eldor, J. Jackson, J. L. Johnson, M. Jones, K. Kee, B. L. King, B. B. Knowles, M. Lako, F. Lebrin, B. S. Mallon, D. Manning, Y. Mayshar, R. D. McKay, A. E. Michalska, M. Mikkola, M. Mileikovsky, S. L. Minger, H. D. Moore, C. L. Mummery, A. Nagy, N. Nakatsuji, C. M. O’Brien, S. K. Oh, C. Olsson, T. Otonkoski, K. Y. Park, R. Passier, H. Patel, M. Patel, R. Pedersen, M. F. Pera, M. S. Piekarczyk, R. A. Pera, B. E. Reubinoff, A. J. Robins, J. Rossant, P. Rugg-Gunn, T. C. Schulz, H. Semb, E. S. Sherrer, H. Siemen, G. N. Stacey, M. Stojkovic, H. Suemori, J. Szatkiewicz, T. Turetsky, T. Tuuri, S. van den Brink, K. Vintersten, S. Vuoristo, D. Ward, T. A. Weaver, L. A. Young, and W. Zhang, “Characterization of human embryonic stem cell lines by the International Stem Cell Initiative,” *Nat. Biotechnol.*, vol. 25, no. 7, pp. 803–816, 2007.

[200] D. Sumi, C. Takeda, D. Yasuoka, and S. Himeno, “Hydrogen peroxide triggers a novel alternative splicing of arsenic (+3 oxidation state) methyltransferase gene,” *Biochem. Biophys. Res. Commun.*, vol. 480, no. 1, pp. 18–22, 2016.

[201] G. J. Cote, W. Zhu, A. Thomas, E. Martin, F. Murad, and I. G. Sharina, “Hydrogen Peroxide alters splicing of soluble Guanylyl cyclase and selectively modulates expression of splicing regulators in human cancer cells,” *PLoS One*, vol. 7, no. 7, 2012.

[202] A. Maracchioni, A. Totaro, D. F. Angelini, A. Di Penta, G. Bernardi, M. T. Carrì, and T. Achsel, “Mitochondrial damage modulates alternative splicing in neuronal cells: Implications for neurodegeneration,” *J. Neurochem.*, vol. 100, no. 1, pp. 142–153, 2007.

[203] B. Halliwell, M. V Clement, J. Ramalingam, and L. H. Long, “Hydrogen peroxide. Ubiquitous in cell culture and in vivo?,” *IUBMB Life*, vol. 50, no. 4–5, pp. 251–257, 2000.

[204] E. Veal and A. Day, “Hydrogen Peroxide as a Signaling Molecule,” *Antioxid. Redox Signal.*, vol. 15, no. 1, pp. 147–151, 2011.

[205] M. B. Hampton and S. Orrenius, “Dual regulation of caspase activity by hydrogen peroxide: Implications for apoptosis,” *FEBS Lett.*, vol. 414, no. 3, pp. 552–556, 1997.

[206] M. V. Clément, A. Ponton, and S. Pervaiz, “Apoptosis induced by hydrogen peroxide is mediated by decreased superoxide anion concentration and reduction of intracellular milieu,” *FEBS Lett.*, vol. 440, no. 1–2, pp. 13–18, 1998.

[207] Y.-L. Guo, S. Chakraborty, S. S. Rajan, R. Wang, and F. Huang, “Effects of oxidative stress on mouse embryonic stem cell proliferation, apoptosis, senescence, and self-renewal.,” *Stem Cells Dev.*, vol. 19, no. 9, pp. 1321–31, 2010.

[208] K. J. Vinoth, J. Manikandan, S. Sethu, L. Balakrishnan, A. Heng, K. Lu, A. Poonepalli, M. P. Hande, and T. Cao, “Differential resistance of human embryonic stem cells and somatic cell types to hydrogen peroxide-induced genotoxicity may be dependent on innate basal intracellular ROS levels,” *Folia Histochem. Cytobiol.*, vol. 53, no. 2, pp. 169–174, 2015.

[209] E. González-Suárez, E. Samper, A. Ramírez, J. M. Flores, J. Martín-Caballero, J. L. Jorcano, and M. A. Blasco, “Increased epidermal tumors and increased skin wound healing in transgenic mice overexpressing the catalytic subunit of telomerase, mTERT, in basal keratinocytes,” *EMBO J.*, vol. 20, no. 11, pp. 2619–2630, 2001.

[210] I. Chopra and M. Roberts, “Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance.,” *Microbiol. Mol. Biol. Rev.*, vol. 65, no. 2, p. 232–60 ; second page, table of contents, 2001.

[211] M. Gossen and H. Bujard, “Tight control of gene expression in mammalian cells by tetracycline-responsive promoters.,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 89, no. 12, pp. 5547–51, 1992.

[212] M. C. Roberts, “Tetracycline therapy: update.,” *Clin. Infect. Dis.*, vol. 36, no. 4, pp. 462–467, 2003.

[213] R. S. Fife, G. W. Sledge, B. J. Roth, and C. Proctor, “Effects of doxycycline on human prostate cancer cells in vitro.,” *Cancer Lett.*, vol. 127, no. 1–2, pp. 37–41, 1998.

[214] B. H. Cohen and R. P. Saneto, “Mitochondrial translational inhibitors in the pharmacopeia,” *Biochimica et Biophysica Acta - Gene Regulatory Mechanisms*, vol. 1819, no. 9–10. pp. 1067–1074, 2012.

[215] E. E. McKee, M. Ferguson, A. T. Bentley, and T. A. Marks, “Inhibition of mammalian mitochondrial protein synthesis by oxazolidinones,” *Antimicrob. Agents Chemother.*, vol. 50, no. 6, pp. 2042–2049, 2006.

[216] M.-Y. Chang, Y.-H. Rhee, S.-H. Yi, S.-J. Lee, R.-K. Kim, H. Kim, C.-H. Park, and S.-H. Lee, “Doxycycline Enhances Survival and Self-Renewal of Human Pluripotent Stem Cells,” *Stem Cell Reports*, vol. 3, no. 2, pp. 353–364, 2014.

[217] S. W. Jang, X. Liu, H. Fu, H. Rees, M. Yepes, A. Levey, and K. Ye, “Interaction of Akt-phosphorylated SRPK2 with 14-3-3 mediates cell cycle and cell death in neurons,” *J. Biol. Chem.*, vol. 284, no. 36, pp. 24512–24525, 2009.

[218] Z. Zhou, J. Qiu, W. Liu, Y. Zhou, R. M. Plocinik, H. Li, Q. Hu, G. Ghosh, J. A. Adams, M. G. Rosenfeld, and X. D. Fu, “The Akt-SRPK-SR Axis Constitutes a Major Pathway in Transducing EGF Signaling to Regulate Alternative Splicing in the Nucleus,” *Mol. Cell*, vol. 47, no. 3, pp. 422–433, 2012.

[219] M. Blaustein, F. Pelisch, T. Tanos, M. J. Muñoz, D. Wengier, L. Quadrana, J. R. Sanford, J. P. Muschietti, A. R. Kornblihtt, J. F. Cáceres, O. a Coso, and A. Srebrow, “Concerted regulation of nuclear and cytoplasmic activities of SR proteins by AKT.,” *Nat. Struct. Mol. Biol.*, vol. 12, no. 12, pp. 1037–1044, 2005.

[220] J. C. Shultz, R. W. Goehe, D. S. Wijesinghe, C. Murudkar, A. J. Hawkins, J. W. Shay, J. D. Minna, and C. E. Chalfant, “Alternative splicing of caspase 9 is modulated by the phosphoinositide 3-kinase/Akt pathway via phosphorylation of SRp30a,” *Cancer Res.*, vol. 70, no. 22, pp. 9185–9196, 2010.

[221] R. Lill, “Function and biogenesis of iron-sulphur proteins.,” *Nature*, vol. 460, no. 7257, pp. 831–8, 2009.

[222] R. S. Ajioka, J. D. Phillips, and J. P. Kushner, “Biosynthesis of heme in mammals,” *Biochimica et Biophysica Acta - Molecular Cell Research*, vol. 1763, no. 7. pp. 723–736, 2006.

[223] H. Vaziri, K. B. Chapman, a Guigova, J. Teichroeb, M. D. Lacher, H. Sternberg, I. Singec, L. Briggs, J. Wheeler, J. Sampathkumar, R. Gonzalez, D. Larocca, J. Murai, E. Snyder, W. H. Andrews, W. D. Funk, and M. D. West, “Spontaneous reversal of the developmental aging of normal human cells following transcriptional reprogramming.,” *Regen. Med.*, vol. 5, no. 3, pp. 345–363, 2010.

[224] F. Pucci, L. Gardano, and L. Harrington, “Short telomeres in ESCs lead to unstable differentiation,” *Cell Stem Cell*, vol. 12, no. 4, pp. 479–486, 2013.

[225] L. Xi and T. R. Cech, “Inventory of telomerase components in human cells reveals multiple subpopulations of hTR and hTERT,” *Nucleic Acids Res.*, vol. 42, no. 13, pp. 8565–8577, 2014.

[226] N. F. Lue, “A physical and functional constituent of telomerase anchor site,” *J. Biol. Chem.*, vol. 280, no. 28, pp. 26586–26591, 2005.

[227] J. C. Schmidt, A. B. Dalby, and T. R. Cech, “Identification of human TERT elements necessary for telomerase recruitment to telomeres,” *Elife*, vol. 3, no. October2014, pp. 1–20, 2014.

[228] A. J. Zaug, S. M. Crary, M. Jesse Fioravanti, K. Campbell, and T. R. Cech, “Many disease-associated variants of hTERT retain high telomerase enzymatic activity,” *Nucleic Acids Res.*, vol. 41, no. 19, pp. 8969–8978, 2013.

[229] T. W. Chu, D. E. MacNeil, and C. Autexier, “Multiple mechanisms contribute to the cell growth defects imparted by human telomerase insertion in fingers domain mutations associated with premature aging diseases,” *J. Biol. Chem.*, vol. 291, no. 16, pp. 8374–8386, 2016.

[230] I. Ginis, Y. Luo, T. Miura, S. Thies, R. Brandenberger, S. Gerecht-Nir, M. Amit, A. Hoke, M. K. Carpenter, J. Itskovitz-Eldor, and M. S. Rao, “Differences between human and mouse embryonic stem cells,” *Dev. Biol.*, vol. 269, no. 2, pp. 360–380, 2004.

[231] J. C. St John, A. Amaral, E. Bowles, J. F. Oliveira, R. Lloyd, M. Freitas, H. L. Gray, C. S. Navara, G. Oliveira, G. P. Schatten, E. Spikings, and J. Ramalho-Santos, “The analysis of mitochondria and mitochondrial DNA in human embryonic stem cells.,” *Methods Mol. Biol.*, vol. 331, pp. 347–374, 2006.

[232] F. Shekari, H. Baharvand, and G. H. Salekdeh, “Organellar proteomics of embryonic stem cells,” *Adv. Protein Chem. Struct. Biol.*, vol. 95, no. September 2015, 2014.

[233] M. Dreger, “Subcellular proteomics,” *Mass Spectrom. Rev.*, vol. 22, no. 1, pp. 27–56, 2003.

[234] W. Yan, R. Aebersold, and E. W. Raines, “Evolution of organelle-associated protein profiling,” *Journal of Proteomics*, vol. 72, no. 1. pp. 4–11, 2009.

[235] J. H. Santos, J. N. Meyer, and B. Van Houten, “Mitochondrial localization of telomerase as a determinant for hydrogen peroxide-induced mitochondrial DNA damage and apoptosis,” *Hum. Mol. Genet.*, vol. 15, no. 11, pp. 1757–1768, 2006.

[236] J. Haendeler, S. Dröse, N. Büchner, S. Jakob, J. Altschmied, C. Goy, I. Spyridopoulos, A. M. Zeiher, U. Brandt, and S. Dimmeler, “Mitochondrial telomerase reverse transcriptase binds to and protects mitochondrial DNA and function from damage,” *Arterioscler. Thromb. Vasc. Biol.*, vol. 29, no. 6, pp. 929–935, 2009.

[237] W. Neupert and J. M. Herrmann, “Translocation of Proteins into Mitochondria,” *Annu. Rev. Biochem.*, vol. 76, no. 1, pp. 723–49, 2007.

[238] T. P. Hopp, K. S. Prickett, V. L. Price, R. T. Libby, C. J. March, D. Pat Cerretti, D. L. Urdal, and P. J. Conlon, “A Short Polypeptide Marker Sequence Useful for Recombinant Protein Identification and Purification,” *Nat. Biotechnol.*, vol. 6, no. 10, pp. 1204–1210, 1988.

[239] S. Leigh-Brown, J. A. Enriquez, and D. T. Odom, “Nuclear transcription factors in mammalian mitochondria.,” *Genome Biol.*, vol. 11, p. 215, 2010.

[240] M. Falkenberg, N.-G. Larsson, and C. M. Gustafsson, “DNA replication and transcription in mammalian mitochondria.,” *Annu. Rev. Biochem.*, vol. 76, pp. 679–99, 2007.

[241] G. S. Shadel and D. A. Clayton, “Mitochondrial DNA maintenance in vertebrates,” *Annu. Rev. Biochem.*, vol. 66, pp. 409–435, 1997.

[242] K. Li, C. S. Smagula, W. J. Parsons, J. A. Richardson, M. Gonzalez, H. K. Hagler, and R. S. Williams, “Subcellular partitioning of MRP RNA assessed by ultrastructural and biochemical analysis,” *J. Cell Biol.*, vol. 124, no. 6, pp. 871–882, 1994.

[243] D. Shao, Y. Liu, X. Liu, L. Zhu, Y. Cui, A. Cui, A. Qiao, X. Kong, Y. Liu, Q. Chen, N. Gupta, F. Fang, and Y. Chang, “PGC-1??-Regulated mitochondrial biogenesis and function in myotubes is mediated by NRF-1 and ERR??,” *Mitochondrion*, vol. 10, no. 5, pp. 516–527, 2010.

[244] R. C. Scarpulla, “Nuclear control of respiratory gene expression in mammalian cells,” *J. Cell. Biochem.*, vol. 97, no. 4, pp. 673–683, 2006.

[245] J. Zhang, I. Khvorostov, J. S. Hong, Y. Oktay, L. Vergnes, E. Nuebel, P. N. Wahjudi, K. Setoguchi, G. Wang, A. Do, H.-J. Jung, J. M. McCaffery, I. J. Kurland, K. Reue, W.-N. P. Lee, C. M. Koehler, and M. A. Teitell, “UCP2 regulates energy metabolism and differentiation potential of human pluripotent stem cells,” *EMBO J.*, vol. 30, no. 24, pp. 4860–4873, 2011.

[246] A. Vozza, G. Parisi, F. De Leonardis, F. M. Lasorsa, A. Castegna, D. Amorese, R. Marmo, V. M. Calcagnile, L. Palmieri, D. Ricquier, E. Paradies, P. Scarcia, F. Palmieri, F. Bouillaud, and G. Fiermonte, “UCP2 transports C4 metabolites out of mitochondria, regulating glucose and glutamine oxidation.,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 111, no. 3, pp. 960–5, 2014.

[247] C. Massard, Y. Zermati, a-L. Pauleau, N. Larochette, D. Métivier, L. Sabatier, G. Kroemer, and J.-C. Soria, “hTERT: a novel endogenous inhibitor of the mitochondrial cell death pathway.,” *Oncogene*, vol. 25, no. 33, pp. 4505–4514, 2006.

[248] Y. Cao, H. Li, S. Deb, and J.-P. Liu, “TERT regulates cell survival independent of telomerase enzymatic activity.,” *Oncogene*, vol. 21, no. 20, pp. 3130–3138, 2002.

[249] D. Del Bufalo, a Rizzo, D. Trisciuoglio, G. Cardinali, M. R. Torrisi, U. Zangemeister-Wittke, G. Zupi, and a Biroccio, “Involvement of hTERT in apoptosis induced by interference with Bcl-2 expression and function.,” *Cell Death Differ.*, vol. 12, no. 11, pp. 1429–1438, 2005.

[250] J. Haendeler, J. Hoffmann, R. P. Brandes, A. M. Zeiher, and S. Dimmeler, “Hydrogen Peroxide Triggers Nuclear Export of Telomerase Reverse Transcriptase via Src Kinase Family-Dependent Phosphorylation of Tyrosine 707,” *Mol. Cell. Biol.*, vol. 23, no. 13, pp. 4598–4610, 2003.

[251] A. Ghosh, G. Saginc, S. C. Leow, E. Khattar, E. M. Shin, T. D. Yan, M. Wong, Z. Zhang, G. Li, W.-K. Sung, J. Zhou, W. J. Chng, S. Li, E. Liu, and V. Tergaonkar,

"Telomerase directly regulates NF-κB-dependent transcription.," *Nat. Cell Biol.*, vol. 14, pp. 1270–81, 2012.

[252] Q. P. Zuo, S. K. Liu, Z. J. Li, B. Li, Y. L. Zhou, R. Guo, and L. H. Huang, "NF-kappaB p65 modulates the telomerase reverse transcriptase in the HepG(2) hepatoma cell line," *Eur J Pharmacol*, vol. 672, no. 1–3, pp. 113–120, 2011.

[253] W. Wang, J. P. Zheng, S. X. Zhu, W. J. Guan, M. Chen, and N. S. Zhong, "Carbocisteine attenuates hydrogen peroxide-induced inflammatory injury in A549 cells via NF-??B and ERK1/2 MAPK pathways," *Int. Immunopharmacol.*, vol. 24, no. 2, pp. 306–313, 2015.

[254] E. Mavrogiannou, A. Strati, A. Stathopoulou, E. G. Tsaroucha, L. Kaklamanis, and E. S. Lianidou, "Real-time RT-PCR quantification of human telomerase reverse transcriptase splice variants in tumor cell lines and non-small cell lung cancer," *Clin. Chem.*, vol. 53, no. 1, pp. 53–61, 2007.

[255] R.-K. Bai, J. Chang, K.-T. Yeh, M. A. Lou, J.-F. Lu, D.-J. Tan, H. Liu, and L.-J. C. Wong, "Mitochondrial DNA content varies with pathological characteristics of breast cancer.," *J. Oncol.*, vol. 2011, p. 496189, 2011.

# Appendix: Supplemental Figures and Tables

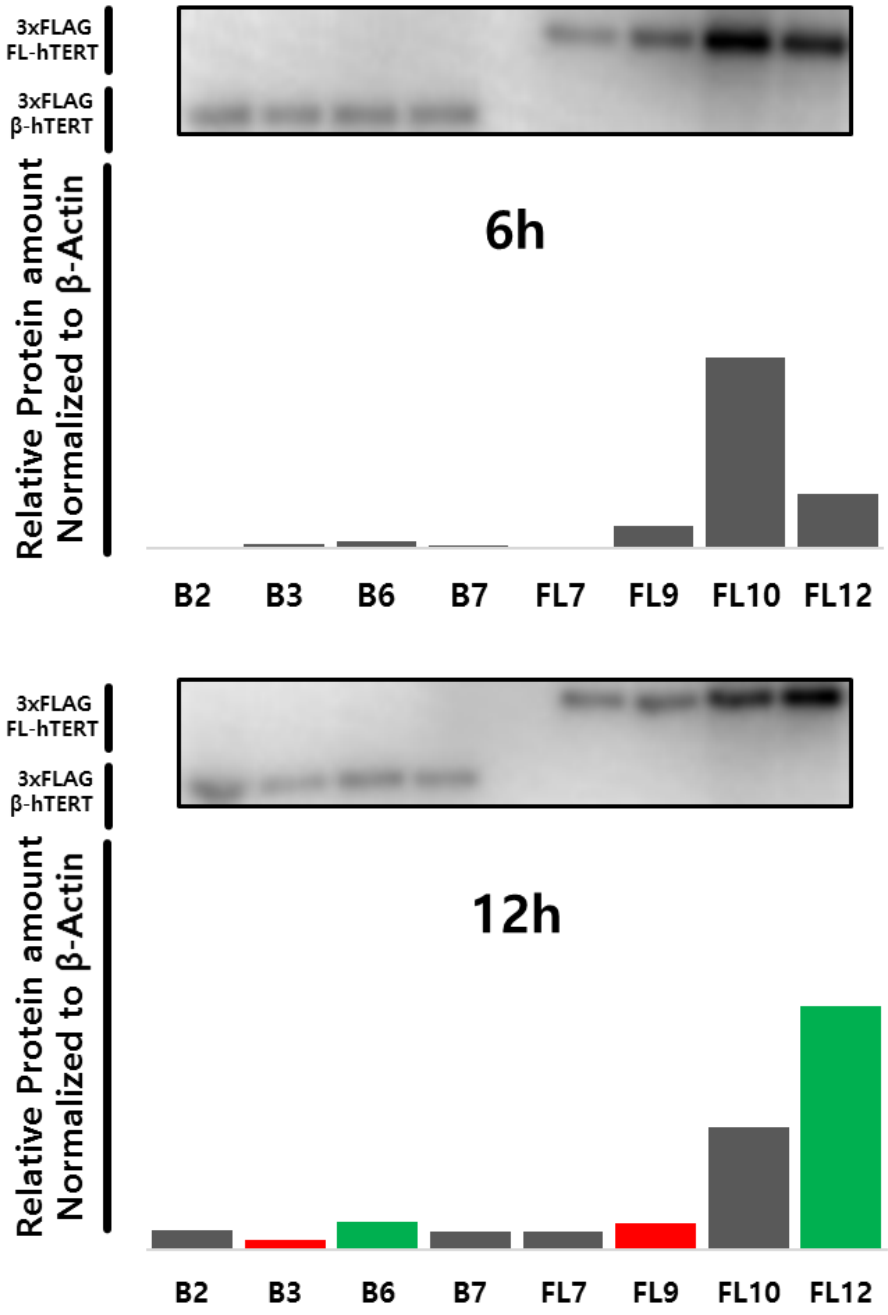
**A1: Table 2.1** List of PCR primers

| Primer Target | Sequence (Forward/Reverse) | Reference |
|---|---|---|
| Taqman α+β+ | TCAAGGTGGATGTGACGGG/ GGACTTGCCCCTGATGCG | [254] |
| Taqman β- | TCAAGGTGGATGTGACGGG/ GGCACTGGACGTAGGACGTG | [254] |
| Taqman α- | CCTGAGCTGTACTTTGTCAAGGA/ GGACTTGCCCCTGATGCG | [254] |
| Taqman α-β- | CCTGAGCTGTACTTTGTCAAGGA/ GGCACTGGACGTAGGACGTG | [254] |
| Taqman Probe | [6FAM] CGTGTTCTGGGGTTTGATGATGCTGGCGA[6FAM] | [254] |
| β-GEO | CCTGCTGATGAAGCAGAACA/ TTGGCTTCATCCACCACATA | |
| TERC | CGCCTTCCACCGTTCATTCT/ GCTGACAGAGCCCAACTCTT | |
| RMRP | AAAGTCCGCCAAGAAGCGTA/ CTGCCTGCGTAACTAGAGGG | |
| β-actin | TCAAGATCATTGCTCCTCCTGAG/ ACATCTGCTGGAAGGTGGACA | |
| HPRT1 | CCCTGGCGTCGTGATTAGTG/ TCGAGCAAGACGTTCAGTCC | |
| TS | AATCCGTCGAGCAGAGTT | [191] |
| ACX | GCGCGGCTTACCCTTACCCTTACCCTAACC | [191] |
| TSNT | AATCCGTCGAGCAGAGTTAAAAGGCCGAGAAGCGAT | [191] |

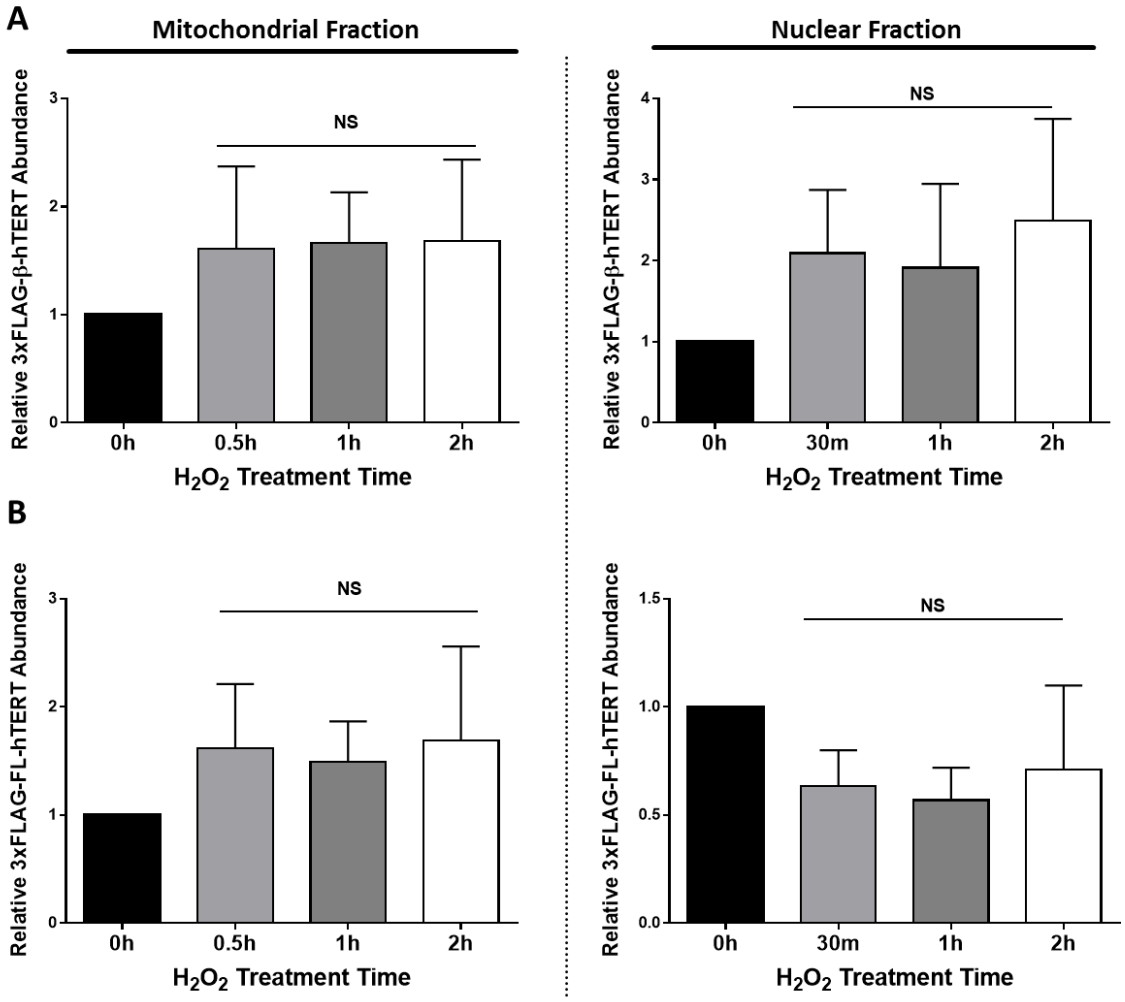
| | | |
|---|---|---|
| NT | ATCGCTTCTCGGCCTTTT | [191] |
| Mitochondrial tRNA | CACCCAAGAACAGGGTTTGT/ TGGCCATGGGTATGTTGTTA | [255] |
| B2M | CCAGCAGAGAATGGAAAGTCAA/ TCTCTCTCCATTCTTCAGTAAGTC | [255] |
| POLG | CATTGGACATCCAGATGCTG/ CCTGATATGAGCTCGGTCAA | |
| PGC1B | CCACATCCTACCCAACATCAAG/ CACAAGGCCGTTGACTTTTAGA | |
| NRF1 | GGAGTGATGTCCGCACAGAA/ CGCTGTTAAGCGCCATAGTG | |
| TFAM | TATCAAGATGCTTATAGGGC/ ACTCCTCAGCACCATATTCT | |
| TF1BM | TCTGCAATGTTCGACACATC/ ACCTATATAAGAAGCTCCAC | |
| SOD2 | GGGATTGATGTGTGGGAGCA/ TGACTAAGCAACATCAAGAAATGC | |
| UCP2 | ACAAGACCATTGCCCGAGAG/ AGGAGGGCATGAACCCTTTG | |

**A2: Table 2.2** List of antibodies and dyes

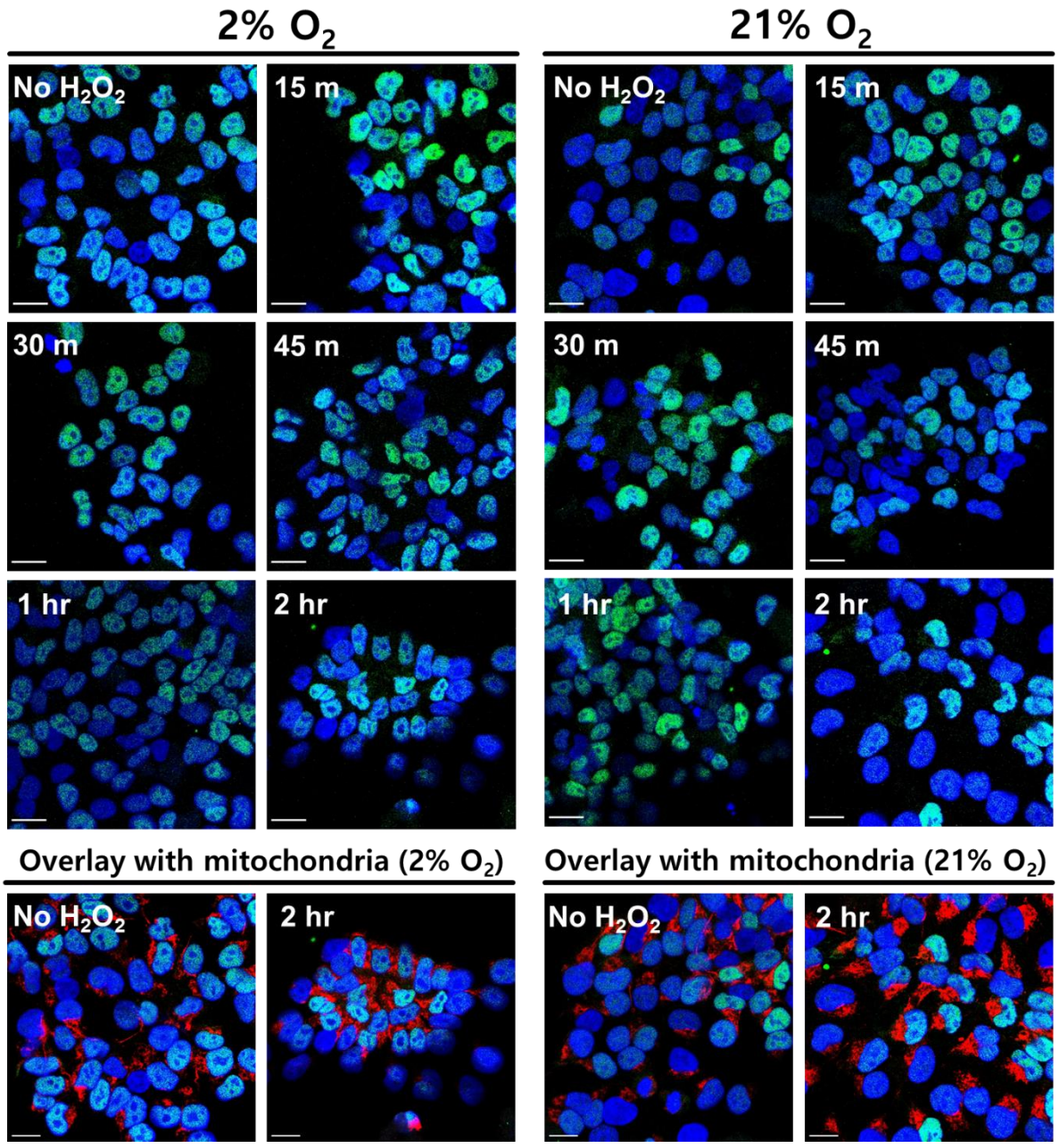
| Target | Company | Host | Catalogue # |
|---|---|---|---|
| 3xFLAG | Sigma | Mouse Monoclonal | F1084 |
| β-ACTIN | Cell signaling | Rabbit Monoclonal | 5125S |
| Histone H3 | Cell signaling | Rabbit Monoclonal | 4499 |
| Prohibitin | Abcam | Mouse Monoclonal | ab55618 |
| VDAC | New England Biolabs | Rabbit Monoclonal | 4661S |
| TOM20 | Protein Technologies | Rabbit Polyclonal | 11802-1-AP |
| Alexa Fluor 488 | Abcam | Goat | ab150113 |
| Alexa Fluor 594 | Abcam | Goat | ab150080 |
| MitoTracker CMX ROS | ThermoFisher | - | M7512 |
| DAPI | ThermoFisher | - | R37606 |
| JC-1 | ThermoFisher | | T3168 |
| Calcein AM | eBioscience | - | 65-0853-39 |
| 7-AAD | eBioscience | - | 00-6993 |
| Annexin V-FITC | ThermoFisher | - | A13199 |




**A3: Fig. 3.1 The amount of 3xFLAG-hTERT in the respective HES2 clones after 6-hours and 12- hours post doxycycline induction.** 3xFLAG-hTERT isoform expression was induced via 1µg/ml doxycycline (6 and 12 hours) in HES2 transgenic clones grown under normoxia (21% $O_2$). Cells were harvested and processed for Western blot. Membranes were probed for 3xFLAG-hTERT isoforms using M2 anti-FLAG mouse monoclonal antibodies (Sigma). The protein level of 3xFLAG-hTERT isoforms in each clone at each time point of doxycycline treatment was compared by Western blot & densitometry analysis. Clonal names are in forms of B# for 3xFLAG-β-hTERT-expressing lines, and FL# for 3xFLAG-FL-hTERT-expressing lines.




**A4: Fig. 3.2 $H_2O_2$-induced oxidative stress and its effect on the cellular localization of 3xFLAG-hTERT.** Cells were grown under 2% $O_2$ and were treated with 1μg/ml DOX for two days prior to harvest. 100μM H2O2 was applied at different times (30m, 1hr, 2hr) prior to harvest. Mitochondria- and Nuclei-enriched fractions were isolated by differential centrifugation. The amount of **A)** 3xFLAG-β-hTERT and **B)** 3xFLAG-FL-hTERT in mitochondrial (left side)/nuclear (right side) fractions was investigated by Western blot (data not shown)/densitometry analysis. Results were normalized to prohibitin and histone H3 for mitochondrial and nuclear fractions, respectively. Densitometry results of treatment groups are relative to $H_2O_2$ negative control n=3, error bar=±SEM. NS = non-significant.




**A5: Fig. 3.3: The effect oxygen tension on the intra-cellular localization of 3xFLAG-FL-hTERT in hESCs.** Transgenic hESCs were grown under 21% $O_2$ and 2% $O_2$. HES2 FL12 were treated with 1µg/ml DOX for two days prior to harvest. 100µM $H_2O_2$ was applied at different time points (15min, 30min, 45min, 1hr, 2hr prior to fixation). Immunofluorescence of 3xFLAG-FL-hTERT (green fluorescence), nuclei (DAPI; blue fluorescence), and mitochondria (TOM20; red) was visualized with a confocal microscope (Zeiss LSM 5 Duo Vario). Images were taken using 63X oil-immersion objective lens and x10 eyepiece lens. Scale bars = 20 µm.

# Curriculum Vitae Joohwan Kim

## Education

| | |
|---|---|
| 2006 - 2012 | B.Sc. (Honors) in Biochemistry at University of Guelph, Ontario, Canada (2 years spent as Republic of Korea Army in the duration of undergraduate studies) |
| 2013 | Lab internship at an embryology laboratory Gyeongsang National University, Korea |
| Jan 2014 - | M.Sc. student at University of Western Ontario, Department of Physiology and Pharmacology. |

## Relevant Laboratory Skills

- Mammalian Cell/Micro-organism culturing
- Embryo culturing/manipulation
- Basic lab techniques for molecular analysis (e.g. PCR, Immunoprecipitation, etc.)

## Publication

J. H. Teichroeb, J. Kim, and D. H. Betts (2016), "The Role of Telomeres and Telomerase Reverse Transcriptase Isoforms in Pluripotency Induction and Maintenance," *RNA Biol.*, vol. 6286, no. January, pp. 00–00,.

**Research Experiences**

May 2012 – Aug 2012 | Research Project as undergraduate student, department of molecular and cellular biology, University of Guelph (supervised by Dr. Jnanankur Bag)

**Poly(A) binding proteins and the qualitative investigation of their interactions with translation regulators in HeLa.**

Relevant techniques:

- Maintenance of cell culture (HeLa)
- Western Blot

Jul 2013 – Dec 2014 | Research Project at Theriogenology Lab, College of Veterinary Medicine, Gyeongsang National University, South Korea (supervised by Dr. Gyu-Jin Rho)

**The effect of melatonin on proliferation/pluripotency of porcine mesenchymal stem cells (MSC):**

Relevant techniques:

- Maintenance and directed differentiation of MSCs

Jan 2014 – Current | M.Sc. Student, Physiology and Pharmacology, University of Western Ontario, (supervised by Dr. Dean H. Betts)

**FL- and β-variant of human telomerase reverse transcriptase in the regulation of mitochondria of human embryonic stem cell.**

Relevant techniques:

- Maintenance of human embryonic stem cell
- Immunofluorescence (Confocal microscopy)
- Various types of PCR
- RNA-immunoprecipitation
- Cloning