University of Alberta

OVERCOMING REPLICATIVE SENESCENCE IN HUMAN CELLS AND TISSUES USING TELOMERASE GENE THERAPY

by

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ABSTRACT

Despite expansion of the donor pool to include older donors, aging remains to impact on the functional success of allografts. Late graft dysfunction is believed to occur via replicative senescence instigated by both chronological aging and the impacts of various stressors accompanying transplantation. Replicative senescence is reached when telomeres approach the Hayflick limit, at which cells cease to further proliferate, leading to reduced ability to thrive and repair injury, especially in aging allografts, which ultimately result in organ failure.

Telomere shortening can be overcome by telomerase gene therapy which has been shown to extend cellular life span beyond the Hayflick limit in normal human cells. Telomerase (hTERT) expression is virtually absent in normal diploid cells. Various gene transfection protocols were examined in our laboratory for the delivery of exogenous hTERT DNA into human endothelial cells (HUVECs) used as an *in vitro* model of cell senescence. *In vitro* endothelial senescence was demonstrated by changes in morphology, increased senescence-associated-βgal expression, impaired wound healing and shortened telomeres. To establish proof of principle, HUVECs of increasing *in vitro* age were transfected with the hTERT gene using Effectene lipids and yielding HUVECs with ectopic telomerase activity. This transient activity led to an increase in telomere length between 240-540 bp in hTERT-expressing HUVECs. These hTERT-modified cells showed increased proliferative index, however, the enhanced growth potential did not lead to a transformed phenotype as the HUVECs retained both normal contact-inhibited growth and cell cycling profile.

hTERT gene delivery was further conducted in human islets *in vitro* and mouse kidneys *in vivo* resulting in positive hTERT expression. In the latter, hTERT expression was induced with doxycycline thereby providing a switch for gene regulation. These 2 studies provided the framework by which further *in vivo* experimentation could be conducted and telomerase induction can be fine-tuned to maximize its potential therapeutic effects in clinical transplantation.

In summary, we have established telomerase transfection in human cells and tissues, both *in vitro* and *in vivo*, as well as demonstrated the advantages of hTERT expression to cell survival. Ultimately, we hope hTERT gene modification will yield more robust grafts to enhance the long-term survival of tissue and organ transplants.

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DEDICATION

This thesis is dedicated to my mother who believes in the power of knowledge like I do. She is one of the most important people in my life and has given me the chance to pursue the life I want. My mother, Molly, has always encouraged me to walk the path of my heart's desire to find my dreams and make them come true.

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LISTS OF ABBREVIATIONS

53BP1	p53 Binding Protein 1
ALT	Alternative Lengthening of Telomeres
APB	ALT-associated PML bodies
ATM	Ataxia Telangiectasia Mutated (protein mediating apoptosis)
AU	Arbitrary Unit
BM	bone marrow
bp	base pair
BSA	Bovine Serum Albumin
CAN	Chronic Allograft Nephropathy
$CaCl_2$	Calcium Chloride
$CaPO_4$	Calcium Phosphate
CDKs	Cyclin Dependent Kinases
CHK1	DNA damage CHeckpoint Kinase 1
CHK2	DNA damage CHeckpoint Kinase 2
CLL	Chronic lymphocytic leukemia
CTL	Cytotoxic Lymphocytes
d	day
DC	Dyskeratosis congenita
D-loop	Displacement loop
DMEM	Dulbecco's Modified Eagle's Medium
DSBs	Double Stranded Breaks

DNA-PK	DNA-dependent Protein Kinase
ECs	Endothelial Cells
ECM	Endothelial Cell Matrix
ECTR	Extrachromosomal Telomeric Repeats
E2F	Elongation Factor 2
ES Cells	Embryonic Stem Cells
FBS	Fetal Bovine Serum
FISH	Fluorescence In Situ Hybridization
Flow-FISH	FISH analyzed by flow cytometry
FACS	Fluorescent Activating Cell Sorting or flow cytometry
FBS	Fetal Bovine Serum
G	generation
G0	the gap 0 cell cycle phase
G1	the gap 1 cell cycle phase
G2	the gap 2 cell cycle phase
GADPH	glyceraldehyde-3-phosphate dehydrogenase
G-rich	Guanosine-rich
h	hours
H2AX	histone H2A variant
H/ACA	box H/box ACA of NoLEs
hn RNP	heterogeneous nuclear ribonucleoprotein
Hsp	Heat shock protein
hTERT	human catalytic subunit of telomerase reverse transcriptase

hTR	human telomerase RNA subunit
HUVECs	Human Umbilical Vein Endothelial Cells
ICAM-1	Intercellular Adhesion Molecule-1
kbp	kilobase pair
kDa	kiloDalton
KO	Knock out
M	molar
M0	Mortality stage 0
M1	Mortality stage 1
M2	Mortality stage 2
M199	Medium 199
MCS	Multiple Cloning Site
MDC1	Mediator of Damage Checkpoint 1
MEF	Mouse Embryonic Fibroblasts
MHC I/II	Major Histocompatibility Complex I/II
min	minutes
ml	milliliter
mM	millimolar
mTERT	mouse telomerase reverse transcriptase
mTR	mouse telomerase RNA
MTT	3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide
MW	Molecular Weight
NoLD	Nucleolar Localization Domain

NoLEs	Nucleolar Localization Elements
NSB1	Nijmegen breakage syndrome 1 DNA damage response protein
nt	nucleotide
P	Passage number
PARP	Poly (ADP-Ribose) Polymerase
PBS	Phosphate Buffer Saline
pCMV	Cytomegalovirus promoter
PCR	Polymerase Chain Reaction
PD	Population Doubling
PECAM-1	Platelet Endothelial Cell Adhesion Molecule-1
PI	Propidium Iodide
PIN	Protein Interacting with NIMA (Never in Mitosis gene A)
PINX1	PIN2/TRF1 Interacting Factor
PIP1	POT1 Interacting Protein
PML	Promyelocytic Leukemia
PNA	Peptide Nucleic Acid
POT1	Protection Of Telomeres 1
PTOP	POT1 and TIN2 Organizing Protein
PTLD	Post-Transplant Lymphoproliferative Disorder
p16	protein of 16 kilodaltons; a cyclin-dependent kinase inhibitor
p21	protein of 21 kilodaltons; a cyclin-dependent kinase inhibitor
p53	protein of 53 kilodaltons; a tumor suppressor protein
pRb	Retinoblastoma protein

RNA	RiboNucleic Acid
RNP	RiboNucleoProtein
RPMI	Roswell Park Memorial Institute medium
RT-PCR	Reverse transcription-Polymerase Chain Reaction
RT	Reverse Transcriptase (motifs in telomerase)
rtTA	reverse tetracycline-controlled transactivator
S phase	the DNA synthesis phase of the cell cycle
SA-βgal	Senescence-Associated Beta-galactosidase
SIPS	Stress-Induced Premature Senescence
SSBs	Single Stranded Breaks
STASIS	STress or Aberrant Signalling Induced Senescence
STELA	Single Telomere Length Analysis
Tet-OFF	turn off by tetracycline or its analogue doxycycline
Tet-ON	turn on by tetracycline or its analogue doxycycline
TGF-β 1	Transforming Growth Factor beta 1
TIN2	TRF-Interacting Nuclear Factor
T-loop	Telomere loop
TPE	Telomere Position Effect
TRAIL	tumor necrosis factor (TNF)-related apoptosis-inducing ligand
TRAP	Telomere Repeat Amplification Protocol
TRF	Terminal Restriction Fragment
TRF	Telomere Repeat binding Factor
T-SCE	Telomere Sister Chromatid Exchange

TTAGGG	Telomere sequence motif
VEGF	Vascular Endothelial Growth Factor
wk	weeks
yr	years

CHAPTER 1

GENERAL INTRODUCTION

1-A) CELL INJURY AND SENESCENCE IN CHRONIC REJECTION

Chronic graft failure compounds the profound problem of organ shortage and together they are the two major obstacles hindering long-term success of clinical transplantation (1). The Canadian transplant waiting lists have been steadily increasing over the last five years, driving the need to expand the donor pool (2). Xenotransplantation has the potential to generate a foreign pool of organs (1). Relaxing donor acceptance criteria has led to more organ donations coming from older donors (> 70 years) and living unrelated donors (3). Unfortunately, these adaptations have not been able to overcome the existing clinical burdens.

Currently, there is no therapy to prevent or treat chronic rejection (allograft nephropathy) (4). This disease remains a huge obstacle or challenge in solid organ transplantation, particularly renal transplantation. It is a principal cause of late graft loss (5). Potential causes include transplantation-related injuries, viral infection, and donor characteristics; all categorized as immune and non-immune related factors (5-8). These multifactorial causes have made treatment difficult and refractory. As a result, long-term graft survival is severely compromised in spite of progressive improvements in immunosuppressive therapy (1, 9).

It has been observed that late graft function deteriorates with donor age. A 20% decrease in five-year graft survival rates in kidney and heart transplants from older

donors has been reported (3). Effecting a cure by treating the individual cause of rejection seems more difficult than to treat the main target site of injury. The quiescent endothelium, which is an immediate interface between graft and host, is the major target for graft rejection (10-12). Many studies have pointed out the strong link between endothelial activation and chronic rejection (6, 10-12). Activation of endothelial cells promotes release of cytokines and adhesion molecules as well as up-regulating constitutive MHC class II expression, all of which attract cellular infiltration (6, 13-15). Both humoral- and cell-mediated immunity attacks the endothelium and further activates more endothelial cells creating a positive feedback loop (16-18). This recurrent attack finally exhausts the cell's reparative ability and results in organ failure. Vascular lesions, glomerulosclerosis and tubular atrophy are the salient histopathologies of Chronic Allograft Nephropathy (CAN) (5, 19). These lesions, resembling aging, result from multiple cell type involvement. The predominant and limiting lesion appears to be obliterative endarteritis.

The leading role of endothelial cells in rejection is irrefutable, making them the focus of many research studies (10-12, 17). These cells are widely studied because of the simplicity of endothelial cell isolation and culture compared to other cell types of the kidney. The complexity of the kidney architecture poses a major limitation for clinical gene applications. Efficient gene transfer into renal allograft is limited by current gene transfer technology (20-23). In prior studies, transgene expression was localized mainly in the endothelial cells with expression occasionally reported in glomerular and tubular cells using the most efficient adenoviral vector (20, 24, 25). Even though endothelial

cells may not be the only affected cell type mediating kidney graft dysfunction, it has been shown to be the most prominent one.

Accelerating endothelial cell injury, in turn, accelerates the progression of chronic renal graft failure (6, 8, 11). Cells have an inherent ability to repair themselves as dictated by their proliferative capacity, which correlates with telomere length. However, this telomere reserve is depleted as cells divide, leading to replicative senescence, which is strongly believed to be the cause of chronic organ dysfunction (26-29). Expression of senescence-associated markers has been noted in chronically rejected rat kidney grafts (30-32). One important trigger of replicative senescence is telomere length. Telomeres are the physical structures located at the distal extremities of eukaryotic chromosomes and possess thousands of hexameric repeat units of the $(TTAGGG)_n$ sequence. *In-vivo* telomere attrition in aging human kidneys has been documented (33). Telomere shortening also occurs in aging human islets (34). The most compelling evidence that correlated telomere shortening to organismal survival came from Dolly, the sheep who died prematurely at 6 years of age and had short telomeres (35). A causal relationship between telomeres and replicative senescence has been well established. The focus of this Introduction Chapter is to review the increasing body of experimental data on telomeres and its causative role in cellular senescence. Successful cloning of the human telomerase gene in 1997 by different laboratories opened up a new era of potential gene therapy for the treatment of age-related diseases and cancer. Furthermore, the dual roles of telomerase in the attenuation of senescence and development of cancers are discussed. Finally, the validity of utilizing telomerase knock-out mice to model the impact of telomere shortening on aging *in vivo* is also discussed in the remainder of this chapter.

1-B) CELL SENESCENCE

A cell is the basic building block of life, yet its replicative capacity is limited and genetically preset at the Hayflick limit by telomeres located at the physical extremities of eukaryotic chromosomes. The Hayflick limit is a natural upper limit (60-80 doublings) of normal cell division, after which the cell ceases to divide. This irreversible growth arrest is known as cell or replicative senescence, an *in vitro* phenomenon first described in 1961 by Leonard Hayflick and Paul Moorhead (36-39). Cells at the Hayflick limit have critically short and unstable telomeres. Telomeres, specifically the length of a telomere, may act as a "replicometer" or a "mitotic clock" to keep track of a cell's replicative history and define the limit of proliferation (40). When telomeres reach a critical length threshold (that is too short for chromosome end protection), the cell exits the cell cycle expressing a unique senescent phenotype and ultimately awaits cell death (41). While quiescence is a reversible process in terminally differentiated cells, cell senescence is irreversible despite mitogenic stimulation. Nonetheless, senescent cells remained viable for up to three years *in-vitro* and resistant to apoptosis (42). Accumulation of senescent cells could alter tissue homeostasis and impact on both aging and cancer (43, 44). Senescent cells show progressive morphological changes, polyploidy and differential gene expression (45, 46). Senescent cells are also characterized by increased cell volume and a distinct flat morphology in culture. Changes in gene expression include elevated senescence-associated β-galactosidase (SA-βgal) activity, as well as increased expression of p16, p21 and hypophoshorylated retinoblastoma protein (pRb) (30). In addition, aging cells secrete factors *in vivo* that are

potentially destructive to surrounding tissues, thereby contributing to the various age-related conditions and cancers (47).

By restricting the replicative life span of a cell via a telomere length setpoint, tissues only undergo a finite number of mitoses. Lacking an infinite capacity of self-renewal, body system functions gradually decline with age. As an evolutionary selection measure, a telomere length setpoint is inherent to prevent uncontrollable cell proliferation and lethal mutations in human cells. Cell division inherits risks by involving a cascade of signaling events and, if not well coordinated; resulting genomic mutations would induce a premature onset of disease. As a result, the human species have evolved defense mechanisms to limit cell divisions in proliferating somatic tissues and enable appropriate cellular DNA maintenance and repair during the functional life span of a cell.

Telomeres are stable native termini that are functionally and structurally distinct from randomly broken ends. Dysfunctional telomeres can be recognized as damaged DNA resembling broken chromosome ends if not capped. Uncapped telomeres can activate cell cycle arrest or programmed cell death (apoptosis) depending on the extent of telomere dysfunction and p53 expression (48-50). p53 can induce several different cell fates, which include a G1 checkpoint arrest, apoptosis or replicative senescence. Activation of whichever endpoint is determined by a multiple of cellular and environmental factors (51). In any event, p53 activated by dysfunctional telomeres can induce any one of those destined endpoints. Senescent cells with shortened dysfunctional telomeres display molecular markers that are found also with DNA double-stranded breaks (DSBs). DNA damage response proteins, such as γ-H2AX, CHK1/CHK2, 53 BP1, MDC1 and NSB1, are recruited to damaged telomeres to enforce checkpoint arrest,

and to halt cell cycle progression (52). Telomeres are therefore required as unique physical structures that function to protect and mask the vulnerable "ends" from inappropriate DNA damage signaling.

I-C) TELOMERES

1. Structure

Given their evolutionary importance, telomeric DNA sequences are conserved from the most primitive ciliated protozoan, Tetrahymena thermophila, to the most complex vertebrate, the Homo sapiens (53). Telomeres are double-stranded DNA characterized by short tandem G-rich repeats, which vary in number between organisms and even between different telomeres of the same cell. The first identified telomeres in Tetrahymena with a sequence motif of $d(TTGGG)_n$, are on average 20-30 bp in length, which differs significantly from the size of mouse telomeres that can be as long as 150 kbp (54, 55). Human telomere length is intermediate and is made up of hexanucleotide repeats containing the sequence motif of $d(TTAGGG)_n$. The short tandem repeats comprised a telomere length between 5-15 kbp in human cells (56-58). Although mice possess longer telomeres, they only have a maximum life span of 3 yr. In culture, mice cells only divide for 10 or fewer population doublings (PD) (59, 60). Given that rodents possess longer telomeres than humans, yet live a shorter life span, a correlation between cell aging and critically short telomeres is weakly established (61-63). Such findings also suggest additional telomere-independent mechanisms might be involved in limiting the replicative life span of certain cell types (60), particularly those of rodents, or perhaps the

critical cell size is variable from mammal to mammal; or perhaps the long size in the rodents, is an artifact of their inbreeding.

As early as the 1930's, telomeres (in Greek, *telo* means "end" and *mero* means "part") were recognized to be specialized end structures, postulated to cap and protect linear chromosomes from illegitimate recombination, end-to-end fusion and nucleolytic degradation (64). The structure of human telomeres has a bipartite arrangement in which proximal repeats are organized into nucleosomal arrays and distal repeats are arranged in a special non-nucleosomal conformation. Chromosomes are anchored to the nuclear matrix by telomeres spatially distributed throughout the G0/G1 and S phases of the cell cycle (65). This spatial arrangement is re-organized in the G2 phase where all telomeres are aligned on a central telomeric disk to permit proper chromosomal segregation (66). Distortion of this 3-dimensional (3-D) organization is evident in cancer cells resulting in telomere aggregates and a partially altered telomeric disk that can affect chromosomal positioning and equal division. A non-random 3-D dynamic arrangement of telomeres is crucial to gene regulation and genomic stability.

2. End-Replication Problem

Linear eukaryotic chromosomes pose a special problem for DNA maintenance. The inherent mechanism of DNA replication causes telomere loss in cycling cells. Normal DNA replication is unidirectional and requires RNA primers to initate DNA synthesis. Unfortunately, DNA polymerase proceeds only from the 5' to 3' direction of the replication fork (Figure 1-1A) resulting in incomplete copying of the parental DNA. Degradation of RNA primers leaves non-replicated gaps at the extreme 5' end of newly synthesized DNA producing shorter daughter strands with a 3' G-rich overhang (Figure

1-1B) (67). With each round of DNA replication, a variable loss of approximately 50-200 bp per generation of telomeric DNA has been reported (68). This loss of genetic information is known as the end-replication problem (Figure 1-1C) first recognized independently by Olovnikov and Watson (69, 70). This incomplete replication is a consequence of the inability of conventional DNA polymerase to fully copy a linear strand of DNA during semi-conservative DNA synthesis prior to mitosis.

Human telomeric DNA is synthesized throughout S phase of the cell cycle (71). Telomere repeat sequences do not encode genetic information, but rather provide a buffer of expendable DNA to protect against the loss of gene-encoding sequences. Genes positioned within or near the telomeric regions are transcriptionally repressed. This is a phenomenon called telomere position effect (TPE) or telomere silencing (72, 73). Gene silencing is derepressed as telomeres progressively shorten leading to the activation of senescence-related genes found on chromosomes 1, 4 and 7 (74, 75). Although human TPE has been demonstrated in HeLa cells, its existence remains inconclusive until further proof of affected genes is found (76-79). The heterochromatin structure has been implicated in human TPE regulation rather than telomere length *per se* (80). Whether TPE has any actual physiological impact on human senescence has yet to be elucidated (76).

3. T-Loop and D-Loop

Mammalian telomeres do not end as linear DNA molecules. Griffith and de Lange showed that telomeres form large terminal loops at chromosome ends known as a telomere loop (T-loop), inside of which the 3' single-stranded overhang (resulting from incomplete DNA replication) is buried (Figure 1-1E) (81). The 3' G-rich single-stranded

overhang (2-8 G residues) mediates strand invasion of the duplex telomeric DNA forming a displacement loop (D-loop) to seal off the linear termini. The long stretch of double-stranded telomeric DNA folds back on itself forming a lariat structure. The D-loop junction is formed by the single-stranded DNA terminus that is tucked back inside the double-stranded DNA molecule via base pairing with the internal CCCTAA tract consisting of 100-200 nucleotides (nt). This large number of nucleotides in the single stranded DNA overhang does not result from DNA replication alone since primer removal after DNA synthesis only creates an overhang of 12-20 nt. Other mechanisms in C-strand processing and G-strand extension possibly exist to create the long G-overhangs critical to T-loop formation regulation (82-87). These so-called G-tails were found in almost all chromosome ends varying in length between 12-16 nt in ciliates to 50-210 nt in mouse and human telomeres (88). *In vitro*, long G-tails of eukaryotic cells can form G-G base pairing, triple helices and G-quartets that as yet have unknown biological significance (89). Possibly, post-replicative telomere loss occurs at both chromosome ends due to 5' strand degradation in order to produce long 3' G-overhangs at all telomere ends (Figure 1-1D). This revised model of telomere shortening is strongly supported by the need to bury all single-stranded termini inside the T-loop as a means to protect against nuclease digestion and inappropriate recombination (86). T-loop helps to distinguish chromosome ends from double-stranded DNA breaks and prevent telomere shortening via a capping function. Otherwise, exposed telomeres become dysfunctional, analogous to double-stranded breaks, and trigger cell cycle arrest with accumulation of DNA damage foci at the termini (49, 52, 90, 91).

4. Telomere-Binding Proteins

Telomere organization as nucleoprotein complexes is both dynamic and complex. Telomere repeat binding factors (TRF), TRF1 and TRF2, are major telomere-binding proteins that play pivotal roles in telomere length regulation and chromosome end protection respectively. These two homodimeric proteins strictly bind duplex $(TTAGGG)_n$ tandem repeats. TRF1 binds along the T-loop and TRF2 binds at the D-loop junction forming a lariat structure that is dynamic in telomere maintenance. Each TRF forms separate multiprotein complexes with other regulatory proteins in modulating telomere length. Overexpression of the TRF proteins leads to an increased rate of telomere shortening (92-95). These complexes negatively regulate telomere length by preventing accessibility to telomerase, a reverse transcriptase enzyme that synthesizes telomere DNA *de novo*. The binding of TRF1 to PINX1 inhibits telomerase activity and thus, forbids telomere elongation (96). Similar inhibitory effects were shown through interactions of TRF1 with a 3-subunit complex, DNA-PK/Ku70/Ku80, that is involved in DSB repair and telomere capping (97-99). Furthermore, binding of TRF1 to telomere is controlled by tankyrase 1 which has poly(ADP-ribose) polymerase (PARP) activity. Ribosylation of TRF1 by tankyrase prohibits binding to telomeric DNA. Overexpressed tankyrase protein targets TRF1 dissociation from telomeres and its subsequent proteosome-mediated degradation, thereby allowing elongation (100). On the other hand, telomere's association with POT1 leads to length suppression. POT1 binds to single-stranded overhangs and protects them from degradation but, in turn, prohibits telomere extension. Interaction between POT1 and TRF1 is mediated by PTOP (also called PIP1 or TINT1). The simultaneous interaction between PTOP and TIN2 helps recruit TIN2 to

the TRF1 multiprotein complex. TIN2 functions as both a PARP modulator within this complex and a negative regulator of telomere length (101). The pairing of telomere tracks mediated by TIN2 activity obstructs telomerase access to telomeres.

In addition, crosstalk between TRF1 and TRF2 is mediated by TIN2 through its dual interactions with both TRFs. Interaction between TRF1 and TRF2 is reinforced by POT1, also a TRF2-binding protein. TRF2 is important to telomere end protection. It interacts with nuclease, helicase, polymerase, recombinase and molecular chaperons. Because TRF2 mediates T-loop formation, its level of expression is critical to telomere maintenance. Loss of TRF2 results in uncapped telomeres which consequently triggers DNA damage checkpoints mediated by tumor suppressor proteins, p53 and retinoblastoma (Rb). PARP1, a DNA-damaged sensing protein, is recruited to dysfunctional telomeres and catalyzes ribosylation of TRF2, which then becomes dissociated from telomeres to allow repair (102). Telomere uncapping can be induced by expression of dominant negative TRF2 mutants leading to the activation of p53-dependent apoptosis. Overexpression of TRF2 increased the rate of telomere shortening by 50-80%, possibly due to increased TRF2-associated nuclease activity at telomeric ends (95). On the other hand, inhibition of TRF2 reduced 3' overhang signals and eventually caused cell death due to the unraveling of the terminal DNA configuration maintained by TRF2 (93, 94, 103). Assembly of these protein factors onto telomeres is part of a maturation process of becoming functional telomeres. Complexes assembled by the different human telomere-associated proteins, along with additional as yet unidentified ones, form the telomere interactome, a molecular machinery involved in

mammalian telomere regulation (104). However, the exact underlying mechanism of telomere length regulation remains uncertain.

5. Telomeres and Senescence *In Vitro*

An increasing body of evidence has pointed towards a telomere length trigger of cellular senescence. The shortening of telomeres *in vivo* as a function of age and *in vitro* with cellular proliferation is well documented in different human cell types since the 1990s. The serious implication of not meeting the "ends" were noticed independently by Watson and Olovnikov, both of whom recognized the end-replication problem as a cause of incomplete inheritance of genetic information (69, 70). A.M. Olovnikov further suggested the gradual loss of chromosome ends lead to cell cycle exit (69, 105). Recently with the advent of molecular DNA technology, this concept has been re-visited and extended by Calvin Harley, the Chief Scientist of Geron™ Corporation (106). Harley's telomere hypothesis of aging states that chromosomes lose terminal sequences with each cell division due to incomplete DNA replication, to a point where chromosome ends are no longer protected. Such a phenomenon represents a molecular equivalent of aging displayed by hallmarks of cell cycle exit and senescence. Harley and co-workers were pioneers in demonstrating telomere shortening with age in normal dividing cells and believed this process may be responsible for aging *in vivo* (106). With the cloning of human telomerase, the causal role of telomere shortening in human cell senescence was established. The initial study by Bodnar et al. (107) has therefore fuelled many subsequent studies on telomere-driven cell senescence occurring in different cell types, including human fibroblasts, keratinocytes, leukocytes, epithelial cells, endothelial cells and hematopoeitic stem cells (108-110).

Cells cultured *in vitro* demonstrated telomere shortening at rates between 50-200 bp/PD at individual telomeres resulting in a net loss of 2 kbp of tandem repeats at senescence (72, 106). A higher rate of telomere loss happened in cells cultured from donors with Hutchinson Gilford progeria, a premature aging disease. In comparison with normal age-matched controls, progeria cells inherited a narrower Hayflick limit. Telomere attrition occurred at much slower rates *in vivo*, for example at rates between 15-40 bp/yr in blood and skin cells (36, 106, 111, 112). Telomere shortening occurs only in actively dividing cells leading to reduced replicative potential. The lower rates of telomere attrition found in human tissues are related to the slow cell turnover in most human body systems. For instance, no significant telomere shortening was found in quiescent cerebral and myocardial tissues (113-114). In contrast, human liver, kidney and spleen experienced annual telomere loss at rates between 29-60 bp (114). Since only 0.1% of glial cells in rodent brain tissue is actively dividing *in vivo*, telomere reduction seems insignificant as to pose any major physiological influence on tissue function (115).

Increased cell turnover can be accelerated by stress leading to increased rate of telomere shortening. Telomere length in the vascular intima was shorter than those in the smooth muscle layer of blood vessels as a result of direct contact with greater hemodynamic stress (116). Likewise, senescent endothelial cells were also observed in stressed regions of atherosclerotic plaques (117). Therefore, telomere shortening depends largely, if not entirely, on cell divisions both *in vitro* and *in vivo*, but little on chronological age *per se*.

Allsopp et al. found weak correlations between telomere length and donor age and between donor age and proliferative potential (118). Similarly in a larger study

conducted by Cristofalo and co-workers, no correlation was reported between donor age and proliferative potential in skin fibroblasts isolated from 42 human donors age 12 wk to 92 yr (119). A similar lack of correlation was presented in an adjunct study comparing growth of cells cultured at different ages from the same donor. However, results documented by Sierra et al. were in contradiction (120). These authors found a stronger negative correlation between donor age and proliferative potential in 14 healthy fibroblast strains (aged 28-90 yr). Interestingly, these authors did not find a significant correlation between telomere length and donor age, as well as telomere length and replicative capacity. Overall, the link between replicative potential and donor age seems weak and inconsistent. Larger sample sized studies may be necessary to resolve this inconsistency in the experimental data.

To the contrary, the link between telomeres and replicative senescence was convincingly established by Allsopp and co-workers (36). Telomere length was reported to be a better predictor of proliferative potential than donor age. They found a striking correlation ($r = 0.76$) between initial telomere length and replicative capacity from 31 human donors (aged 0-93 yr). Initial length of telomeres at the time of culture is the factor limiting replicative life span. However, this is not true for rodent cells which can only be cultured for several population doublings, yet rodent cells possess longer telomeres (59, 60). Thus, rodent cells do not appear to be influenced by telomere-based senescence which suggests other potential mechanisms may exist. Although telomere-initiated senescence occurs in cultured human cells, senescence is thus far strictly an *in vitro* phenomenon that has yet to have proven biological significance *in vivo*.

6. Telomere and Senescence *In Vivo*

Evidence linking cell senescence to human aging *in vivo* was first provided by Dimri et al. (30). Senescent cells were detected in aging skin *in vivo* using the SA-βgal marker. The number of senescent cells observed strongly correlated with donor age. A high number of senescent cells were also found in aging kidney biopsies from patients diagnosed with CAN (121). However, use of the SA-βgal as a marker of aging has been criticized based on findings that culture conditions and cell density could affect staining results. Hence, the exclusive use of SA-βgal as a senescence marker is still debatable and not widely accepted.

Given that telomere shortening is the putative mechanism underlying cell senescence, then shortened telomeres found in aging human tissues could be attributed to senescence occurring *in vivo*. Shortened telomeres were reported in the aging liver, kidney and spleen (33, 114). A convincing correlation between organismal survival and short telomeres has been recently been shown by Dolly, the sheep cloned from a mammary cell derived from a 6 yr old adult ewe. At 1 yr of age, Dolly's telomere length was 20% shorter than age-matched controls (35). Instead, her telomere size was more comparable to that of a cultured 6 yr old mammary cell. Dolly developed arthritis in the hind leg, knee and hip at 5 years of age but these aging pathologies normally should not occur at her age. She died prematurely at 6 years of age from a particular type of lung disease that is commonly seen in older sheep. These findings suggest that Dolly's cells were older than her birth age which means she could have been 6 yr old at the time of birth. Dolly would then be 11 yr old at her death and would have attained the normal life span (12-15 yr) for a sheep. Dolly's age suggests that her telomeres remained at donor

length during the cloning procedure and there onward continued to shorten leading to premature death. Despite this circumstantial evidence, the belief that replicative senescence occurs *in vivo* still needs to be substantiated with further proof of actual physiological relevance and contribution to human aging.

7. Mechanisms on Telomere-Based Senescence

In spite of active research in the field of telomeres and telomerase for just over two decades, many speculations and uncertainties still remain involving the underlying mechanism of telomere-initiated cell senescence. Karlseder and co-workers proposed that change in the protected status of shortened telomeres rather than a complete loss of telomeres is responsible for replicative senescence (95). For example, manipulation of TRF2 would alter the protective status granted to telomeres. It was shown that TRF2 expression attenuated senescence by lowering the telomere length threshold from 7 kbp to 4 kbp. It is believed that not just critical telomere length *per se*, but rather, a threshold level of telomere-associated proteins forming a functional telomere cap governs cell senescence. Telomeres are recognized as dysfunctional when chromosome ends are uncapped. It is the shortening of the 3' G-overhang that leads to telomere uncapping and irreversible growth arrest (122). Approximately 60-85% of 3' G-overhangs were lost at senescence and the termini became a "free" open tail that signals cell cycle arrest. Loss of the G'-overhang also affects telomere elongation when the single-stranded DNA needed to prime telomere replication is destroyed. Von Zglinicki laboratory believes that telomeres do not act as replicometers but instead, are sentinels for genomic damage. Damage preferentially accumulates at "gene-less" telomeres and shortening was accelerated to stop cell cycling in order to prevent mutational risks from accumulating

with additional cell divisions. Loss of telomeres also occurred due to stochastic events such as oxidative stress (123). Generation of free radicals can cause single-stranded breaks (SSBs) in telomere regions and repair is inefficient compared to other DNA sequences (124). In one study half of the telomeric lesions remained unrepaired after 19 d whereas, breaks induced in the minisatellites (other non-transcribed repetitive sequences) and bulk genomic DNA were repaired within 24 h. As a result, significant steady-state levels of SSBs accumulated (84). Presence of lesions can stall DNA polymerase processivity and terminate DNA replication prematurely leading to a loss of telomere sequences (125). Aging also exacerbates the inefficiency of DNA repair at telomeres (83). Evidently, telomere length is not a constant function of cell division. Telomere loss is associated with both cell proliferation and oxidative stress, neither of which is mutually exclusive.

Recently, there is evidence to suggest that the onset of cell senescence is triggered by one or few of the shortest telomeres that constitute telomere dysfunction rather than average telomere length (126, 127). In these studies short telomeres were detected on chromosomes 17p, 19p, 20q and 22q. Based on the single telomere length analysis (STELA) assay developed by Baird and co-workers, telomeres on chromosome 17p were the shortest telomeres found in senescent cells (128, 129). The work of Martens et al. does not support the hypothesis of shortest telomeres acting as a trigger of cell senescence (130). Ultrashort telomeres (<0.5 kb) have been shown to exist, albeit at very low frequency. Furthermore some studies have suggested that the different telomere length among chromosomes just happens to be related to individual chromosome size (120, 131). A donor-specific telomere length threshold might also be involved in

signalling senescence in cells. Blackburn proposed that senescence is determined by the combined effects of telomerase inactivity and telomere shortening (132). Her laboratory provided further evidence that telomere capping is a critical factor in promoting cell growth, not just absolute telomere length *per se* (133). There appears to be various schools of thoughts concerning the mechanistic role of telomeres in replicative aging. Regardless of whichever pathway leads to general telomere dysfunction, this endpoint unmistakenly drives cellular senescence.

8. Senescence and Immortalization

A two-phase cell cycle checkpoint model has been proposed to explain senescence and immortalization, with the former being a default mechanism against the development of cancer (Figure 1-2) (134, 135). Human cells possess a finite capacity to proliferate. As the cells divide, telomeres continue to get shorter at successive cell cycles. At maximum proliferation (Hayflick limit), telomeres become critically short and signal cell cycle arrest leading to the cessation of growth. This stage is termed mortality stage 1 (M1) or cell senescence mediated by tumor suppressor proteins, p53 and retinoblastoma (Rb). Pre-senescent cells transformed *in vitro* with viral oncogenes (ie. SV40 large T antigen, adenovirus E1A, human papilloma virus E6 or E7) can overcome senescence by disrupting the M1 checkpoint guarded by p53 and pRb. These cells gain an additional 20-100 PDs, but not immortality. Transformed cells with extended replicative life span approach a second checkpoint termed crisis or mortality stage 2 (M2) punctuated by another surge of cell death (136-139). At this stage, genetic mutations have already accumulated, with continuing telomere attrition during cell divisions resulting in widespread chromosomal abnormalities that ultimately promote

carcinogenesis. Overcoming replicative senescence is one critical rate-limiting step towards tumorigenesis. Even though increased apoptosis seen at the M2 checkpoint serves to barricade further proliferation, M2 survivors still emerge from crisis at a frequency between 10^{-7} to 10^{-5} and become immortalized (140-142). While a few or possibly even one gene mutation could lead to spontaneous immortalization in rodent cells, a higher frequency of mutations is needed in humans (140). Likewise, cells cultured from chicken, bovine and horse are not spontaneously immortalized. Human senescence is a dominant trait (143, 144). Human cells are required to bypass both the M1 and M2 stages to achieve immortality, whereas rodent cells are restricted only by senescence (145). In order to bypass crisis, telomeres must be stabilized via one of two mechanisms, either involving telomerase reactivation or the alternative lengthening of telomeres (ALT) pathway. Through these means, cells maintain chromosomal integrity, but most importantly, they obtain unlimited replicative life span.

I-D) TELOMERASE

1. Function

Telomeres are a direct target for telomerase whose function requires two key components, a catalytic subunit (hTERT; human telomerase reverse transcriptase; 1132 aa) and an intrinsic RNA moiety (hTR; human telomerase RNA; 451 nt) (146, 147). Human telomerase is a large ribonucleoprotein (RNP) complex that functions as a terminal transferase with a conserved reverse transcriptase (RT) domain. Although many accessory proteins have been implicated in the RNP complex, the integral RNA template with sequence complementary to $(TTAGGG)_n$ and hTERT protein subunit are the two

basic components necessary for telomerase function. While the hTR subunit is ubiquitously expressed throughout human development, the catalytic hTERT protein component is differentially expressed in adult tissues (61, 148). Telomerase expression is essentially downregulated or absent in virtually all normal diploid cells whereas germ line cells and cancer cells show stable expression (148). Constitutive telomerase activity enables cancer and reproductive cells to proliferate indefinitely throughout life due to telomere maintenance. Despite the presence of telomerase activity in renewable somatic tissues (i.e. blood, gastrointestinal tract, epidermis, blood), telomeres continue to shorten, albeit at slow rates. Stem or progenitor cells, skin cells, crypt cells and activated lymphocytes, all lose telomeric repeats progressively with cell divisions. Presumably, telomerase activation in these cells is below the threshold required for telomere maintenance (149-153). Nevertheless, telomerase activity in proliferating tissues slows the rate of telomere attrition during clonal expansion to maintain the telomere reserve from rapid depletion. Telomerase activity is therefore only sufficient to uphold proliferative requirements during a normal life time. Progressive telomere shortening persists as a defensive front against human malignancy. This stringent regulation of telomerase throughout human development reflects an evolutionary pressure to preserve the human species and concurrently prevent deleterious somatic mutations.

Telomerase of Tetrahymena was first characterized by Greider and Blackburn in 1985 (154). In 1989, a similar enzymatic activity was discovered in HeLa cells derived from uterine cervical carcinoma by Morin (155). In the same year, the gene encoding the RNA template of Tetrahymena telomerase was cloned followed by the protein subunit in 1995 (156, 157). The gene sequences of the human RNA and TERT homologues were

also reported around this period (158). Greater than 90% of human tumors express telomerase as a means to sustain tumor mass and the virtual lack thereof in normal somatic cells renders cell loss to aging unavoidable (159). Telomerase activation is a double-edged sword and this interesting discovery has led to explosive studies in the fields of cell aging and carcinogenesis.

2. Telomerase and Immortality

The catalytic hTERT subunit is the rate-limiting component necessary for the *in vitro* reconstitution of telomerase activity in negative cells (160). Telomerase activation provides a means to overcome telomere attrition in normal somatic cells whereas germline and cancer cells have acquired constitutive telomerase activity rendering them immortal. Various human cells (epithelial cells, endothelial cells, fibroblasts, T-lymphocytes) transfected with the hTERT gene demonstrated ectopic telomerase activity with concomitant increase in telomere length and enhanced replicative life span well beyond the Hayflick limit (107, 161-164). In human keratinocytes however, reconstituted telomerase activity alone was insufficient to achieve this transient immortality unless further cell cycle checkpoints were disrupted (165, 166). Similar observations were documented for mammary epithelial cells, but later found that the premature growth arrest in these cells could be rescued by hTERT transfection alone (167, 168). The initial conflicting evidence was due to culture artifacts. Depending on the anatomical location, individual tissues might harbor different mechanisms governing cellular immortalization.

The immortality issue raises an important concern, which is whether telomerase expression leads to potential oncogenesis. At present, no solid conclusion can be drawn,

however a large number of studies have supported telomerase role in extending cellular life span, and not tumorigenesis (169-175). Activation of oncogenes and/or deletion of tumor suppressor genes, along with telomerase expression are required to induce tumorigenesis (173, 174). Although a few studies have misconstrued immortalization, resulting from the extension of cellular life to tumorigenicity, these two processes are not the same (176, 177). For example, it has been demonstrated that the immortalized cell line, 3T3 Swiss, cannot grow in soft agar culture nor form tumors in nude mice (178, 179). The ability to grow in agar or form tumors are two widely-used assays to support malignant transformation (178, 180, 181). To date no mutations have been reported in cell cycle checkpoints in telomerase-transfected normal cells. As well, none of these transfected cells have been able to grow in soft agar culture, nor develop tumor *in-vivo*. However, some studies have shown immortalized cells can be transformed by hTERT expression alone and form tumor (175-177). This is understandable, in that immortalized cells have bypassed the senescence (M1) checkpoint and may have acquired some mutations, potentially in tumor suppressor genes, to promote tumorigenesis along with telomerase activation (182-186). Therefore, immortalized cells may have a higher susceptibility to oncogenesis, but this is not unlike the higher risk of post-transplant lymphoproliferatve disorder (PTLD) associated with immune suppression. Transient telomerase expression alone should not confer immortality in normal cells *per se*, but merely the extension of replicative life span in the absence of malignant potential.

3. Telomere Elongation by Telomerase

Key function of telomerase is to synthesize new telomeric DNA *de novo* to counteract telomere shortening during cell divisions. The hTERT subunit catalyzes the

polymerization of multiple copies of noncoding hexanucleotide $(TTAGGG)_n$ repeats onto the 3' ends of chromosomes creating a buffer zone against the loss of functional genes. Versatility of telomerase is illustrated by its characteristic DNA polymerase and reverse transcriptase domains. Telomerase has a 5' to 3' polymerase activity and proofreading ability to allow editing and removal of mismatched and non-telomeric sequences at the 3' terminus during telomere replication. It differs from conventional reverse transcriptase in that telomerase transcribes only a short specific template region of the RNA whereas the conventional enzymes transcribe long stretches of variable RNA molecules. The addition of repeats is reiterated approximately 50 times at each chromosome terminus and the rate of synthesis appears to be determined by the hTR template (187, 188). Enzyme activity is highly processive, attributed to the constant engagement of the RNP particle to the DNA substrate recognized by the RNA template sequence motif of 3'-^{56}CAAUCCCAAUC^{46}-5'. As telomerase proceeds unidirectionally from the 5' to 3' end, the DNA substrate is elongated through reverse transcription of the RNA template (Figure 1-1F). The complementary strand is later filled in by conventional DNA polymerase. Following a next round of repeat addition, the DNA molecule is bound at the 5' end to the anchor site within the enzyme complex as the 3' end translocates back to the starting nucleotide in the template region. Substrate anchorage is essential during translocation to ensure DNA attachment to the RNP particle in order to avoid premature termination of telomere replication.

Given the enormous diversity that exists in the primary RNA sequence, telomerase activity is conserved among the species unless the RNA secondary structure is altered (189, 190). The entire RNA structure, consisting of stem loop and pseudoknot, is

required for proper function. Even the slightest base mutations in the template region of the RNA will cause nucleotide misincorporation, at rates approaching 50%, and premature abortion of telomere synthesis. Particularly important is the conserved H/ACA [(box H and box ACA of nucleolar localization elements (NoLEs)] domain at the 3' end of hTR. This domain is used for hTR targeting to the nucleoli where it accumulates and integrates into the hTERT protein complex.

Human telomerase assembles specifically during S-phase and disassembles thereafter due to hTERT disintegration, hence activity is restricted to only this phase of the cell cycle. The two key components of telomerase are separately compartmentalized for the sake of regulation (191). The hTR-containing Cajal bodies reside in the subnuclear compartment while the inactive form of hTERT resides in the cytoplasm. When activated by phosphorylation, hTERT is translocated from the cytoplasm into the nucleus (192). Efficient retention and accumulation of hTERT in the nucleus entails binding of the 14-3-3 protein to the hTERT C-terminus where the reverse transcriptase domain is situated. In addition, this C-terminus is utterly essential for biological function in human cells as reported by Ouellette et al. (193). Moreover, the importance of this terminus to telomerase biological function *in vivo* was further emphasized by Middleman et al. (194). A functional C-terminal domain is critical to telomere maintenance and stabilization as well as to achieving immortalization by regulating hTERT protein levels. Undoubtedly, this domain plays a key role in the post-transcriptional processing of hTERT and replication of telomeres *in vivo*.

As well, the extreme N-terminus of the catalytic subunit is pivotal to hTERT trafficking in the subnuclear compartment. The discrete nucleolar localization domain

(NoLD) at the N-terminus directs hTERT translocation into the nucleoli independent of hTR binding (195). hTR trafficking into the nucleoli likewise occurred as directed by a H/ACA localization domain. This domain binds to H/ACA proteins, such as Dyskerin, to facilitate nuclear hTR accumulation and stabilization. Binding of hTR with hTERT promotes stability of the template RNA sequence. Mutations in Dyskerin causes X-linked Dyskeratosis congenita (DC), a disease affecting multiple proliferating tissues (skin, nail, hair, bone marrow) in the body (196, 197). Dyskerin involves in ribosome biogenesis and telomerase complex stabilization. The autosomal dominant form of DC is caused by mutations in the core RNA component of telomerase, thus it is highlighted to be a disease of defective telomere maintenance. DC is a rare inherited disorder affecting the bone marrow, lungs, liver and bones thereby, underscoring the significance of telomerase function in tissues of high renewal (196-198). Graying of hair and a high incidence of cancer are evident in inflicted individuals (199). As a form of premature aging syndrome, affected individuals have shorter telomeres than normal and die early from bone marrow failure (200, 201).

Both the hTR and hTERT subunits are partially enriched in the nucleolus (202, 203). Cajal body-associated hTR accumulates at the nucleolar periphery and hTERT is spatially distributed throughout the nucleoli. To date, active telomerase assembly in the nucleolus remains speculative as this compartment might serve other roles as well (147, 202). hTERT nucleolar localization might also assist in sequestering telomerase from chromosome ends when activity is not required in the nucleoplasm (147, 204, 205). Assembly of the active holoenzyme is mediated by molecular chaperones, Hsp90 and p23 (206, 207). The core enzyme comprises both hTERT and hTR dimers (208).

Cooperation of the two hTR molecules is required for function of the multimeric complex. It was further suggested that the ratio of hTR:hTERT expression in cells affect enzymatic activity *in vivo* as it relates to the degree of dimerization in the multimeric holoenzyme (209). Association between telomeres and telomerase is possibly established by the hnRNP A1 protein which acts as a linker through its binding with hTR. The colocalization of Cajal body-associated hTR and hTERT foci observed at telomeres suggests active telomerase assembly in S phase cells. hTERT localized predominantly in the nucleolus of primary cells in G1 and early S phases (210). In late S/G2 phase, hTERT localization is no longer restricted to this subnuclear compartment. The telomerase complex is released into the nucleoplasm during S phase for telomere synthesis at chromosome ends. hTERT was found at telomeres of HeLa cells in a recent study by Cristofari and Lingner (211). The recruitment of the RNA complexes to only subsets of telomeres indicates that not all telomeres are elongated in a given cycle of replication. As reported by some, telomerase preferentially elongates the shortest telomeres due to reason of accessibility (211, 212). In spite of potential recruitment by both long and short telomeres, the abundant negative regulatory proteins associated with long telomeres block telomerase access to the termini. To the contrary, short telomeres frequently approach an "open" conformation granting easier access to the core holoenzyme. The switch between a telomerase-extendible versus a –nonextendible state emphasizes to the importance of coordination between telomeres and telomerase in telomere length maintenance and regulation.

4. Telomerase in Diagnosis and Prognosis

The positive effects of telomerase on senescence are offset by its significant role in human malignancies. In 1994, readily detectable telomerase activity was first reported in a variety of human cells and tumors using the newly available PCR-based TRAP assay. Telomerase activity was found in 98% of immortal cell lines and 90% of actual tumor tissues representing 12 distinct tumor types, while no activity was found in mortal cell cultures and normal somatic tissues (213). This high prevalence of telomerase in human cancers and absence in nearly all normal somatic cells render it an effective diagnostic tumor marker. Reactivation of telomerase in cancer cells is a pre-requisite step for tumor development. Telomeres are stabilized at a shorter, yet still functional length. Adverse effects resulting from progressive telomere shortening or telomere uncapping are thus avoided. Stabilization of the malignant phenotype renders cells with unrestricted proliferative and metastatic potentials. Early detection of telomerase in pre-malignant lesions helps prescribe preventative measures against progression to advanced cancers (152). Increased telomerase activity was observed in intraepithelial neoplasia from the cervix, prostate, bladder, colon and kidney during transit to full-blown cancers. Specifically, the high numbers of human cancers showing telomerase positivity are the neuroblastomas (94%), hepatocelluar carcinomas (93%) as well as cancers of lung (80%), colon (93%), stomach (85%), bladder (90%) and breasts (93%) (214-220). In some cancers however, activity is less definitive due to low telomerase expression that was found with the sarcomas, astrocytoma, retinoblastoma and glioblastoma multiforme. In some benign tumors, telomerase activity was undetectable (221). Detection of telomerase may be interfered by enzyme inhibition and sample contamination (222, 223).

Sample degradation and unspecific enzyme inhibition in cancerous tissues may create false negative results. In contrast, contamination with cells normally expressing telomerase, such as activated lymphocytes in inflammation, could falsely identify these normal tissues as cancerous. In spite of a few drawbacks, telomerase assessment provides ease, speed, high sensitivity and high specificity of detection rendering it a promising diagnostic marker for routine clinical settings.

Presence of telomerase activity provides a strong indication of prognosis. As illustrated in a study of breast cancer patients at 12 yr follow-up, a 98% survival rate was found in a group of 201 patients showing very low telomerase activity compared to 0% survival in those yielding very high protein expression (224, 225). Poor prognosis was also reported in patients with leukemia, meningioma and gastric cancers and in whom telomerase levels were high (226). Telomerase activity in a high number of human tumors therefore suggests this enzyme to be the key mechanism that allows cancer cells to divide without limit.

5. Telomerase and Anti-cancer Therapy

Telomere length in cancer cells is often shorter than in normal somatic cells as a result of increased cell divisions en route to malignant transformation. Tumor cells often end up with extremely short telomeres that require protection by telomerase reactivation if transformed cells were to continue propagating in the absence of genomic instability. Telomerase confers resistance to apoptosis and promotes cell growth as functions exclusive to telomere maintenance (122). The Shay laboratory was the first to show a strong correlation between cancer and telomerase (159). A great number of studies have explored telomerase inhibition in combating cancer. Therefore, strategies to undermine

the protective effects of telomerase in cancer cells are under intensive investigation in an attempt to find countermeasures for tumor invasion.

Given the antagonistic roles of telomerase, a delicate balance between senescence and tumorigenesis exists but are not mutually exclusive. A tip of the balance in either direction can have dire consequence. Telomerase reactivation in normal cells that escape from the M1 and M2 checkpoints are highly susceptible to tumorigenesis. Gorbunova et al. showed that senescence could be re-initiated in immortalized cells as part of an anti-cancer strategy (227). Approximately 20% of hTERT-immortalized fibroblasts displayed senescence. hTERT was highly overexpressed in this cell fraction compared with the rest of the immortal cell population. Instead, increased levels of p21, hypophosphorylated Rb, as well as intense SA-βgal staining were found in these cells. Excessive build-up of hTERT proteins in the immortal cells may cause a shift in the equilibrium towards senescence. Although recent evidence presented the possibility of re-initiating senescence as a form of anti-cancer strategy, little is known of the underlying mechanism.

Various forms of anti-telomerase strategies have been examined including immunotherapy, inhibition therapy and promoter-driven gene therapy. The rationale behind the latter is to utilize the hTERT promoter for the delivery of harmful genes to malignant cells knowing that this promoter is highly active in cancer cells but poorly responsive in most normal cells. Apoptotic genes such as caspases 6 and 8, TRAIL and Bax are being examined under the hTERT promoter (228-230). Currently, telomerase immunotherapy is an active form of treatment undergoing clinical trials. Patients are immunized against tumor antigens and, in this case, it is a short peptide from the reverse

transcriptase domain. Specific immune responses are triggered to eradicate the tumor cells that express telomerase antigens. Geron™ Corporation has initiated a Phase 1 clinical trial for metastatic prostate cancers in 24 patients (231, 232). Specific CTL responses were activated in the treated patients leading to tumor reduction and a lack of general toxicity. Telomerase immunotherapy clinical trial for advanced breast cancer is also being undertaken. A different strategy utilizing telomerase inhibitors, in particular the GRN163 molecule, developed by Geron™ has just entered Phase 1-2 clinical trials (233, 234). Various protocols of telomerase inhibition are presently under active investigation in many different cancer cell types. For instance, antisense technology makes use of short modified nucleic acids (oligonucleotide) to target complementary mRNAs of hTR and hTERT for destruction. GRN163 is a hTR antagonist that is being used in patients with chronic lymphocytic leukemia (CLL). *In vitro* data showed telomere shortening and apoptosis in GRN163-treated myeloma cells. Just over a decade ago, Feng et al. first reported successful tumor inhibition using antisense hTR oligonucleotides in HeLa cells (146, 235). These cells proliferated for another 23 to 26 more doublings prior to cell death. Apoptosis in HeLa cells was attenuated until telomeres became critically short after some rounds of replication and caused genomic instability. Although slow acting, telomerase inhibitors work synergistically with chemotherapy by enhancing the drug effects. However, telomerase inhibition in tumors with extremely long telomeres (9-11 kbp) may not be as effective because these cells would have to undergo endless cell divisions for telomeres to reach critical length. Therefore, the effects of telomerase inhibition may take longer to occur in tumors with long telomeres. Interestingly, results from Yatabe et al's study were quite different (236).

Rapid apoptosis was induced in cervical cancer cells after 3 to 6 days of antisense hTR treatment. No telomere erosion occurred in these cells. Resultant apoptosis was not caused by uniform telomere shortening as documented in Feng et al's study, but instead related to the disruption of telomerase capping at telomeric ends. Uncapping of as few as one telomere per cell is hypothesized to induce spontaneous apoptosis independent of telomere shortening. Use of antisense hTERT oligonucleotides in colon and breast cancer cell lines induced rapid apoptosis and no telomere shortening (237, 238). Evidently, apoptosis in telomerase inhibited cancer cells is mediated by both telomere shortening and telomere uncapping. The efficacy of telomerase inhibition is dependent on which of these telomere-based mechanisms is activated in cancer cells.

Dominant negative hTERT and hTR mutants also showed inhibition of telomerase by competing with the wild-type counterparts in assembly of the RNP complex. These dominant negative mutants are usually expressed at higher levels, therefore they are frequently assembled with the enzyme. As a result, the RNP complex fails to synthesize the correct telomeric sequences leading to progressive telomere shortening and eventual cell death. Telomestatin, a natural product found in Streptomyces anulatus, also inhibits telomerase activity by encouraging formation of intermolecular structures at telomeres to block telomerase access (239). Telomestatin causes apoptosis in acute leukemia cells. Further assessment of the long-term effects of this product is needed before initiating clinical trial. Much remains to be learned about the telomerase signaling pathways that influence growth and apoptosis in tumor cells. Two major concerns revolve around anti-telomerase therapy; one involves telomerase positive normal cells and another, telomerase negative tumors. While almost all normal human cells lack telomerase,

several cell types including germ cells, stem cells, activated lymphocytes, gastrointestinal tract, endometrium, skin cells and hair follicles, express telomerase. Although these self-renewing cells express telomerase, endogenous activity is below that needed to maintain telomere length, thus significant harmful effects resulting from short-term anti-telomerase treatment is not expected. Realistically, any potential adverse effect appears minor in life threatening scenarios in cancer patients. Anti-telomerase therapy is a promising approach for treating the majority, if not all, of human malignancies.

6. Alternate Lengthening of Telomeres (ALT)

Telomerase is not the exclusive mode of telomere maintenance as exemplified by a minority of human tumors whose telomeres are preserved via a recombination-based alternative lengthening of telomeres (ALT) mechanism. This fraction constitutes up to 10% of the malignancies refractory to telomerase inhibition (240). Therefore, telomerase-based anti-cancer strategies would produce minimal effects in the treatments of astrocytomas, osteosarcomas and soft tissue sarcomas, all of which displayed ALT-mediated telomere maintenance (241). ALT cells are characterized by a heterogeneity in telomere sizes (>20 kbp to <2 kbp) plus an infinite capacity to divide despite undetectable telomerase activity. Another hallmark of ALT cells is the presence of ALT-associated promyelocytic leukemia (PML) bodies (APBs). Packaged within these subnuclear APB structures are extrachromosomal telomeric repeat (ECTR) DNA in linear, t-loop and circular conformations, as well as telomere-associated proteins including TRF1 and TRF2 (242). These telomere-related components are found only in PML bodies specific to ALT cells and not in PML bodies of other cell types. PML proteins in conjunction with proteins involved in replication and recombination are also

found within APBs. Apparently, APBs housed the machinery used for telomere extension via homologous recombination involving telomeric sister chromatid exchange (T-SCE). APBs appear instantly upon ALT activation and disappear when ALT is repressed (243, 244). While telomerase activity is present in self-renewable tissues, existence of ALT has not been reported in normal human cells. Operating ALT has only been documented in tumors (7-10%) and some immortal cell lines *in vitro* (35%) (245). ALT positive tumors are relatively benign due to their ineptness to form robust metastases in mouse models. A fibroblast cell line positive for ALT did not form tumors in immunodeficient mice lacking telomerase activity (246). These findings suggest that ALT tumors do not exhibit the same degree of tumorigenicity and metastatic capability offered by telomerase. However, ALT tumors are no less lethal *in vivo* (122). Spontaneously arising ALT-positive tumors are infrequent which implicates the irreplaceable function of telomerase in tumor progression (122). As well, ALT cells may require additional mutations to reach a physiological state equivalent to that achieved by telomerase positive tumors. As proven, telomere maintenance via an ALT mechanism is not equivalent to that maintained by telomerase (247). Although telomerase prevails in the vast majority of human cancers, experimental evidence remains inconclusive as to which mechanism predominates. In telomerase-ALT cell hybrids, telomerase can either be repressed or activated. Telomerase reactivation in ALT cells can likewise demonstrate an ALT-positive or –negative phenotype in the presence of telomerase. It is not surprising to find the co-existence of telomerase and ALT activities in the same cell (248). However, it is perplexing that Cerone et al. did not find features of ALT or telomerase in an ALT-derived immortal cell line (249). These recent observations may

suggest existence of another alternate mechanism of telomere maintenance, which may be a variation of ALT. Currently, the ALT mechanism is still not completely understood given that research has been in progress for approximately a decade.

7. Telomeres and Mice

Telomere dynamics in humans and mice are very different. Size of telomeres between these two species can vary by as much as 10 fold (15 kbp in humans versus 150 kbp in mice) yet the life time of rodents does not exceed a few years. Even though endowed with longer telomeres, mice have not gained any survival advantage. Explanted mouse embryonic fibroblasts (MEFs) in culture failed to expand beyond 30 PDs at most, in contrast to human adult fibroblasts that divided up to 100 PDs (39). Mus musculus has very long telomeres (up to 150 kbp) and likewise their cells underwent rapid growth arrest after only a few population doublings and shortened telomeres were evident (250). Although Mus spretus possess an average telomere length similar to humans, their cells also divided to the same extent *in vtiro* as those of Mus musculus (251). These observations raise the important question as to whether telomeres behave like a "mitotic clock" in mouse senescence. Unless senescence is triggered by the shortest telomeres and not average critical telomere length, mice do not appear to use a telomere-based mechanism to track and delimit replicative life span. The shortest mouse telomeres are approximately 10 kbp, a length within the human range, but whether this length is considered critically short amidst the longer ones involved in initiating cellular senescence remains questionable (252). Studies have shown that the senescent-like state observed in mice is not telomere-dependent (168, 253). Instead, this senescence-like state can be triggered by inadequate culture conditions, such as oxidative stress. This *in*

vitro phenomenon, called STASIS or SIPS, was also induced in human cells by culture shock or stress (227, 254, 255). STASIS is not true senescence in that telomeres are not the rate-limiting factor. Shay and Wright proposed that STASIS is responsible for the stochastic loss of proliferative capacity, particularly when mouse cells do not divide frequently to cause any significant shortening in telomere length (256). Despite much longer telomeres that decay at rates comparable to human's, mice do not possess increased replicative life span (257, 258). This lack of reconciliation between telomere length and rodent life span reinforces the belief that mouse senescence may not be telomere-driven and therefore, challenges the telomere hypothesis of aging.

8. Telomerase and Mice

Not only do mouse telomeres remain long, telomerase is also widely expressed in primary adult mouse tissues. High levels of mTERT expression were found in the thymus, intestine, skin, uterus and testes (259-261). Organs with low proliferative indices such as the adult brain, heart, skeletal muscle and kidney displayed weak to undetectable levels of mTERT mRNA. Similar to humans, mTR component remains constant throughout life. mTERT expression is differentially regulated with high telomerase activity present only during proliferation and absent during differentiation. Telomerase activity is widespread and under less stringent control in mouse cells providing the ease of spontaneous immortalization. Due to the ubiquitous expression of telomerase in normal mouse tissues, cancer formation is merely an upregulation of existing activity concomitant with the loss of mortality control. Appreciable telomere shortening is difficult to detect in the presence of extremely long telomeres and promiscuous telomerase expression to allow proper analysis of mouse senescence. In this

case, telomerase knock-out (KO) mice were developed as model systems to assess the impact of telomere shortening on organismal survival and, in particular, to corroborate results documented in human cells.

9. Telomerase-Transgenic Mice

Long telomeres (40-60 kbp) in laboratory mice interfere with the assessment of telomere loss as a function of age *in vivo*. Successive generations of telomerase KO mice caused telomeres to shorten to a length approaching that of humans and the resulting phenotypic abnormalities became obvious in proliferative tissues lacking telomerase expression. The $mTR^{-/-}$ KO mouse, deficient in the template RNA subunit of telomerase, is a transgenic prototype created by Blasco et al. in 1995 and since has been a widely tested model (262). In early generation (G) mice, from G1 to G3, no deleterious phenotypic changes were detected other than some skin lesions, hair graying and hair loss (alopecia). No other organ dysfunctions were reported. Retention of long telomeres in these early-G mice explains the underlining normal phenotypes. Just the fact that telomerase KO mice were born alive suggests that telomerase activity is not too critical to the survival of younger generations of animals whose telomeres remained at sufficient length to sustain organ homeostasis. However, long-term viability of highly proliferative organ systems is adversely affected in late-G mice. Telomerase KO mice, starting from G4 onward, exhibited impairments in numerous capacities including hematopoesis, spermatogenesis, liver function, neural tube closure and wound healing (263, 264). Pathologies were also noted in the heart and kidney leading to reduced survival with age. mTR-deficient mice also exhibited similar phenotypic abnormalities (265). The most profound defect is sterility in G6 mice cutting off future generations. Infertility was

noticed as early as G4 of pure bred mTR-deficient C57BL/6 mice as opposed to mice with a mixed background (62). Telomeres were much shorter in these animals than those found on a C57BL6/129Sv mixed background. Reports of splenic and intestinal atrophies, defective hematopoeisis and reduced lymphocytic proliferation were described (266). Size of littermates declined and depletion of germ cells were obvious resulting in testicular atrophy in the G6 progeny. Testicular atrophy is also a phenotype that spontaneously arises in normal aging mice, thus implicating the crucial role of telomeres and telomerase in mouse senescence. These abnormalities were associated with increased apoptosis and decreased proliferation in KO mouse tissues (267). Increased fusion events and aneuploidy attributed to shortened telomeres became evident in the G3 $mTR^{-/-}$ littermates. A steady decline in telomere length, with continuous organ renewal *in vivo* and cell proliferation *in vitro,* inevitably develops genetic instability that leads to senescence or tumorigenesis. Espejel and Blasco reported senescence in $mTR^{-/-}$ MEFs due to telomere dysfunction (60). The Blasco laboratory further showed that telomerase rescued $mTR^{-/-}$ mice from accumulating chromosomal instability and premature aging (62). Examination of Y chromosomes from the G4 $mTR^{-/-}$ progeny illustrated an additional reduction of approximately 3 kbp in telomere length compared to paternal G3 $mTR^{-/-}$ mice. In contrast, G4 $mTR^{+/-}$ progeny expressing telomerase had increased telomere length and only 0.2% of all chromosome ends lacked detectable TTAGGG repeats. This percentage was elevated by 30 fold in the $mTR^{-/-}$ progeny. These findings are proof that the role of telomere/telomerase complex in aging remains conserved between species that are biologically different. Conclusively, the appearance of numerous defects in different organ systems due to telomere shortening in $mTR^{-/-}$ mice

strongly supports the importance of telomerase function in telomere maintenance and organismal survival. The essential role of telomerase in human survival is demonstrated in dyskeratosis, a disorder affecting proliferating tissues as discussed above.

Telomerase deficiency also leads to another extreme, which is tumorigenicity reported in tissues with high rates of renewal in late-G $mTR^{-/-}$ mice (263, 268). Apparently, mouse tumorigenesis does not require telomerase activation, and so challenges the efficacy of anti-telomerase therapy in the treatment of human malignancies. Shortened telomeres appear to have dual effects; either triggering senescence and apoptosis or promoting tumor formation depending on the context of genomic instability (269, 270). Accumulating deleterious mutations in mTR-deficient mice create a high susceptibility to tumorigenicity. High incidences of tumors were found in late-G $mTR^{-/-}$ mice and was amplified in a p53 null genetic background (271, 272). These $p53^{-/-}$/ $mTR^{-/-}$ double KO mice reached G8 as opposed to G6 in mice with $mTR^{-/-}$ alone. This increase in progeny generation from the double KO line is associated with the attenuation of negative consequences resulting from telomere dysfunction. p53-mediated pathways that signal apoptosis and senescence (271, 273, 274). P53 was mutated in more than half of human malignancies and its loss underlines a strong selection pressure against p53 in tumorigenesis (275). Tumor development reached 100% frequency in p53 KO mice (276, 277). Mouse senescence is mediated by p53 alone at the M1 checkpoint unlike human cells, in which the M2 checkpoints prevents p53/Rb-deficient cells from continuous divisions. The genetic context is therefore important to the outcomes produced by telomere dysfunction.

Mice are inherently prone to cancer via a telomere-independent mechanism (278, 279). Overexpression of telomerase in mTERT-transgenic mice resulted in higher spontaneous tumor incidence at old age (280). Increased incidence of mammary carcinoma in aging $mTERT^{+/+}$ mice was observed even though they possess an abundance of telomere reserve. Such findings suggest a novel function of telomerase in tumor promotion (281). Incidence of cancer and reduced viability were more pronounced in a $p53^{+/-}$ hemizygous context illustrating the synergistic effects of TERT activity and loss of p53 function in tumor formation (280). Although telomerase activity was detected in wild-type ($mTERT^{+/+}$) and hemizygous ($mTERT^{+/-}$) mice, telomere shortening occurred only in mice with the haploinsufficiency. Increased aneuploidy, but no end-to-end fusions, was reported in the hemizygous animals since remaining telomerase activity was adequate to maintain the residual amounts of telomeres at all chromosome ends. Similar to humans, effects of telomerase expression on telomere length is dosage-dependent (282, 283). hTERT dosage is critical to telomere maintenance that is defective in dyskeratosis and aplastic anemia. Telomerase function is highly complex and manifestation of phenotypes largely depends on the overall genetic background. Delay in phenotypic expression caused by telomere shortening in mouse models is mainly attributed to inherently long telomeres compared to humans. Nonetheless, mouse models are continually being utilized to provide valuable insights in human senescence and carcinogenesis.

1-E) SUMMARY

In the last two decades, organ transplantation has been the adopted treatment modality for end-stage disease. The universal application of this treatment protocol relies heavily on the availability of donor resources, that are in shortage. Given that demand for organ transplantation continues to rise, organs from older donors are being utilized. In addition, lengthening of the waiting lists is in part, attributed to the general dysfunction of the transplanted organ as constitutive cells of an aging organ have a much reduced ability to thrive following transplantation. Despite advances in immunosuppression therapy, long-term graft survival has yet to be achieved. Chronic graft rejection is currently an untreatable disease, which continues to impact on the long-term success of clinical organ transplantation. Chronic Allograft Nephropathy is the leading cause of late graft dysfunction and is refractory to current treatment protocols. Findings of aging pathology in failed allografts have suggested an underlying mechanism relating to cell senescence that affects the use of older donor organs. The limited longevity of aging allografts associates with a state of irreversible growth arrest triggered in tissue cells. Shortening of telomeres at successive cell division, will eventually exhaust a cell's replicative capacity and trigger cell senescence. Telomeres, located at the ends of eukaryotic chromosomes, functions to protect the chromosomal termini from genetic mutations and instability. Telomeres function as a biological clock that tracks the inherent replicative life span of a cell. Virtually all somatic cells undergo telomere attrition as the cells divide until they reach the Hayflick limit, at which point proliferation ceases and the cells become senescent or otherwise immortal with telomerase activation. Telomerase, present mainly in germ line and cancer cells, counteracts telomere

shortening by the *de-novo* synthesis of telomeric DNA. Given that telomeres in mice are lengthy and their highly proliferative organs harbor telomerase activity, the shortest telomere is likely to be the single most important determinant for cell entry into senescence rather than average telomere length. Therefore, the importance of developing a telomerase KO mouse model cannot be overstressed. Telomeres were shortened in these KO animals resulting in multiple organ dysfunction and sterility in late-G mice (*vide supra*). The antagonistic roles of telomerase in senescence and tumor formation require strict developmental control of gene expression throughout the human life time. Given that telomerase deficiency or overexpression causes human disease, therapeutic significance may be acquired in a very narrow window of activity and fine-tuning of telomerase is required to effect a cure. In summary, human telomerase gene therapy provides hopeful prospects in treating age-related diseases and chronic pathologies, such as cancers and transplant rejection, in the near future.

I-F) OBJECTIVES AND GENERAL OUTLINE OF THESIS

Our **Overall AIM** is to improve graft survival using human telomerase gene therapy to attenuate cell senescence. In particular, we believe this gene therapy strategy will improve the survival of renal transplant and other allografts. Numerous studies have demonstrated that decreasing telomere length in human cells causes replicative senescence. Telomere shortening can be overcome via the ectopic expression of telomerase, either transient or stable in nature, which forms part of the proof provided by this thesis. The primary objective of this thesis is to provide "proof of principle" on the role of telomerase and established the necessary tools by which the hTERT gene can be delivered, expressed and effect telomere maintenance in human cells and tissues.

Overall **HYPOTHESIS**: The longevity of cell and tissue transplants can be extended via *ex vivo* or *in vivo* telomerase gene therapy.

SPECIFIC AIM # 1: To examine and optimize various transfection parameters to achieve high efficiency of transgene expression in human endothelial cells.

Overview/Experimental Rationale: Suffice it to say, telomerase offers therapeutic potential in reviving aging human cells, however, there is currently no efficacious gene vehicle available for a safe delivery *in vivo*. The greatest obstacle preventing the advance of gene medicine from an experimental technology into a viable therapeutic option is the lack of an efficient and safe gene delivery strategy. Although the majority of gene transfer protocols use recombinant viral vectors for their high transfection efficiency and stable gene expression, namely adenovirus and retrovirus respectively, the grave concern has always been about biosafety in humans (284). The biological hazards associated with viral vectors urge a nonviral-mediated alternative.

Different transfection parameters (i.e. temperature, media, glucose contents, transfection time, DNA/lipid concentrations, lipid reagents) will be assessed and optimized in human umbilical vein endothelial cells (HUVECs) using a nonviral-mediated strategy. Reasons for using endothelial cells in our *in vitro* studies include the ease of isolation and ECs' critical role in mediating vascularized graft rejection. Knowing that the transfection strategy has no adverse effects on endothelial cells *in vitro* would likely be non-toxic to the endothelium *in vivo*. To be able to evaluate the effects of telomerase expression on cell growth and survival, this enzyme first needs to be adequately expressed in HUVECs, and therefore it is essential to find an effective transfection method to deliver hTERT into HUVECs. In addition, the importance of these initial studies is to obtain, and then to translate the optimized *in vitro* transfection parameters to whole organ transfection, with the hope of achieving efficient delivery of protective genes into organ transplants to create more robust grafts.

SPECIFIC AIM # 2: To examine telomerase activity and telomere maintenance at pre- and post-hTERT transfection in HUVECs. As well, to assess the growth advantage and potential malignant changes in phenotype of hTERT-transfected HUVECs.

Overview/Experimental Rationale: We have introduced the different mechanisms (i.e. telomere length, telomere capping function, oxidative stress) proposed for initiating replicative senescence in normal human cells. Despite a lack of consensus on the underlying mechanism, we believe that telomere length plays a more critical role in signalling cell senescence, based on some convincing evidence provided by the studies of Bodnar et al. (107) and Yang et al. (161). Given that short telomeres prevent T-loop

formation, the length of telomeres then has a direct effect on the capping function. Therefore, we believe telomere length to be the primary cause of human replicative senescence.

To establish "proof of concept", telomere length in normal HUVECs will be determined prior to telomerase (hTERT) transfection. HUVECs will be transfected with hTERT to determine whether transient telomerase activity is able to maintain telomere length post-treatment. Furthermore, the proliferative ability and normal function of HUVECs, at a late passage number indicative of *in vitro* aging, will be assessed in untransfected and hTERT-transfected cells. Because telomerase expression is also a hallmark of cancer, potential malignant transformation will be evaluated. It is technically difficult to assess the direct effects of telomere shortening on replicative senescence using a mouse model due to the presence of long telomeres in this species. Because telomere length is much longer in rodents than in humans, the loss of capping function may have a more significant and direct impact on rodent senescence than telomere length.

SPECIFIC AIM # 3: To examine islet transfection using the EGFP reporter and hTERT genes.

Overview/Experimental Rationale: We have the privilege of using human islets kindly provided by Dr. Jonathan Lakey (University of Alberta, Edmonton, AB, Canada), who is the Director of both the Comprehensive Tissue Centre and the Clinical Islet Laboratory (Edmonton, AB, Canada). Islets are a simplified form of human tissue that can be cultured without the need for highly controlled matrix and differentiation and growth factors required with more complex organs. Human islets provide us a simple tissue model for which we can further assess the effects of telomerase expression in culture.

Prior to conducting hTERT transfection studies, telomere length and telomerase activity will first be evaluated to substantiate the degree of telomere shortening *in vivo*, if any, in human islets that are not expected to express telomerase to establish "proof of principle".

Gene transfection in islets will be assessed using DOTAP and FuGene 6 with the EGFP reporter. These 2 lipid reagents are selected because more DNA can be prepared with a small of volume of lipids to avoid potential toxicity, if any, associated with lipofection. After optimization of the different transfection parameters, the hTERT gene will be delivered and expression assessed. Pending on the successful transfection and expression of hTERT, we look forward to conducting transplantation studies in diabetic nude mice to assess the enhanced effects of hTERT on islet survival *in vivo* in future studies.

SPECIFIC AIM # 4: To examine telomerase expression *in vivo* in transfected kidney grafts derived from C57BL/6 rtTA-transgenic mice. To compare survival outcomes from BALB/c recipients receiving renal transplants that stably express either the hTERT gene or TRE control.

Overview/Experimental Rationale: This study focuses on kidney transfection with the hTERT gene cloned into an inducible retroviral dual expression system, followed by renal transplantation. Donor kidneys will be obtained from C57BL/6 mice constitutively expressing the reverse tetracycline-controlled transactivator (rtTA) in mouse tissues. This rtTA regulatory protein is needed for induced activation of the hTERT gene in a response vector that is directly delivered into the kidneys via infusion through the abdominal aorta into the renal artery. Administration of Doxycycline (Dox) induces hTERT activity, and lack of the drug results in no transgene expression. A shift from the

use of lipofection to the retroviral-mediated Tet-ON system will be adopted in this study in a novel attempt to achieve stable expression of telomerase *in vivo* for assessment of the long-term effects of telomerase on the survival of grafts experiencing chronic stress. Pending on the development of chronic rejection in the selected animal model (C57BL/6 and BALB/c combination), the *in vivo* effects of telomerase on long-term graft function of renal allotransplants will be evaluated. Ultimately, we hope to improve the long-term survival of allografts using the Tet-ON system with hTERT to enable the precise regulation of *in vivo* telomerase expression within a therapeutic range. Specifically, survival rates in genetically modified pre-transplant renal and islet allografts as well as other solid organs will hopefully increase, thereby shortening the transplant waiting lists.

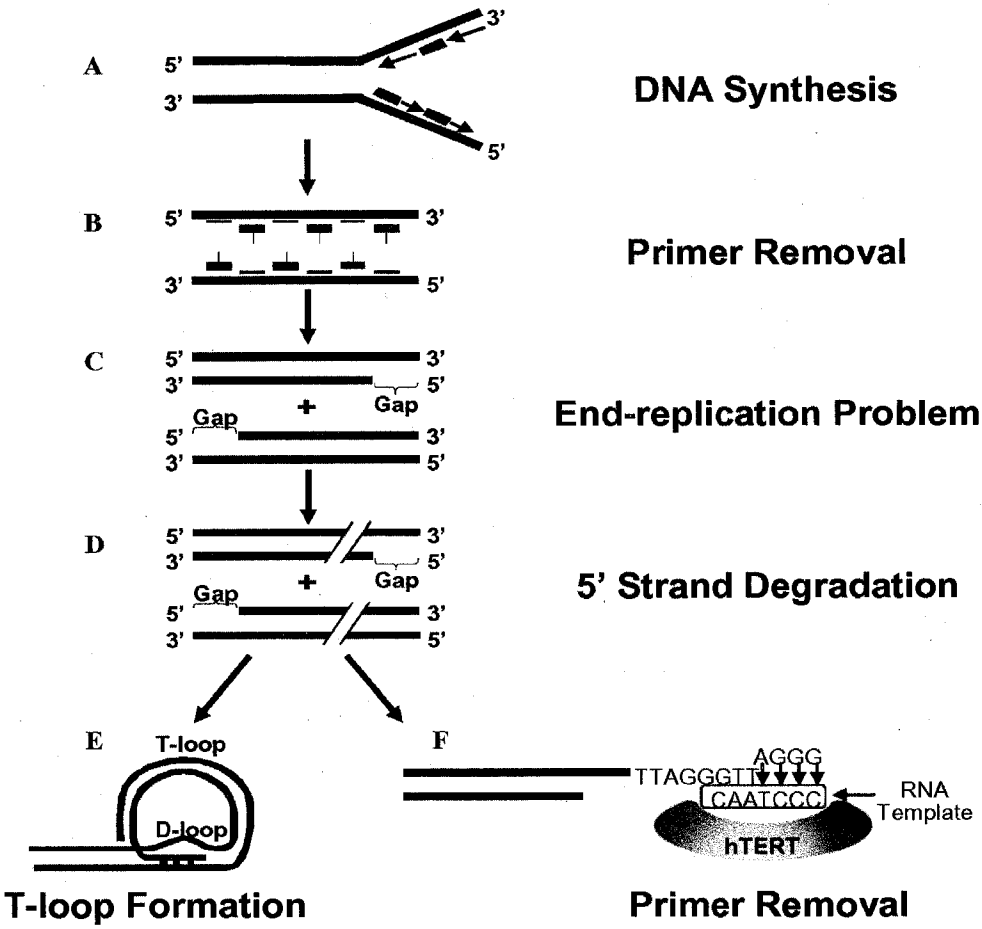

Figure 1-1: DNA replication at telomeres. **(A)** DNA fork opens up for DNA synthesis initated by RNA primers. **(B)** After DNA synthesis is completed, primers are degraded and gaps filled by DNA polymerase **(C)** for the 5' ends resulting giving rise to the End-replication Problem. **(D)** Further nucleolytic processing occurred at the 5' termini. **(E)** The 5' termini are tucked inside the duplex DNA or **(F)** telomeres are extended in the presence of telomerase during replication.

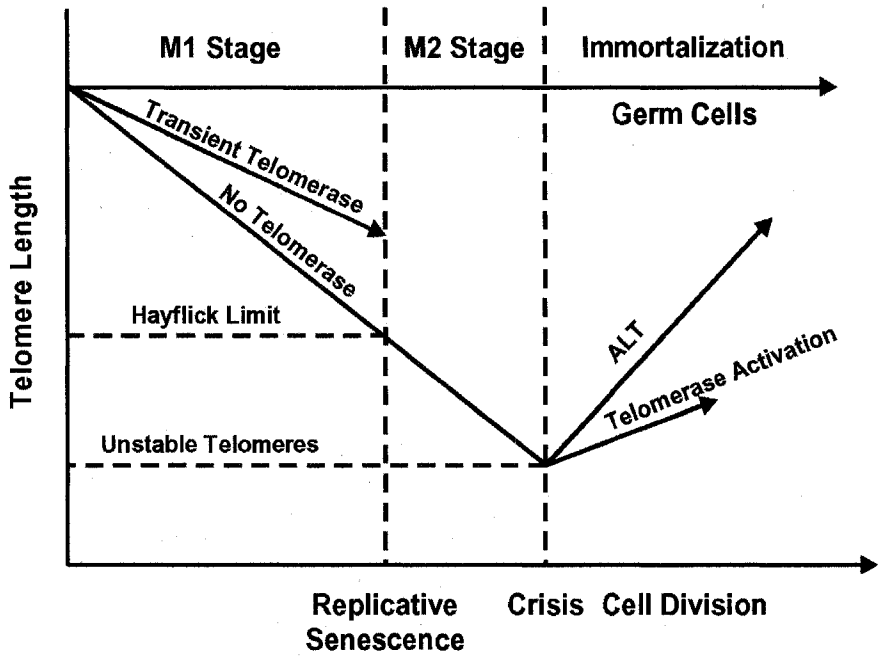

Figure 1-2: Senescence and Immortalization. Germ line cells express telomerase therefore telomere length is stable. Normal somatic cells lacking telomerase activity undergo telomere shortening and eventually reach the Hayflick limit and senesce. A rare fraction of cells achieves transient immortalization via oncogenic transformation. These transformed cells continue to divide until they reach crisis at which their telomeres are very unstable and cells undergo apoptosis. However, emergence of survivors is evident at extremely low frequencies and these clones are immortalized via the activation of telomerase or ALT as a means to stabilize telomere length. Normal cells of self-renewal tissues (ie. skin, lymphocytes) possess low transient telomerase activity to slow the rate of telomere attrition.

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CHAPTER II

GENE THERAPY: A LIPOFECTION APPROACH FOR GENE TRANSFER INTO PRIMARY ENDOTHELIAL CELLS**

II-A) INTRODUCTION

The basis of gene therapy is the introduction of a normal gene into somatic cells, which express the desired gene product that may function either in: 1) correcting a cellular dysfunction, 2) imparting a new function, or 3) inhibiting an unfavorable cellular process (1, 2). The rationale is to provide remedy at the most fundamental level; that is, to effect a cure by intervening in the disease process via somatic gene manipulation (3).

Gene therapy was originally developed for inherited disorders (4). Since the initial reported success from the first human clinical trial in 1990 for adenosine deaminase (ADA) deficiency, gene therapy has become the focus of numerous treatment protocols (2, 3, 5, 6). Application of gene therapy has been extended to treatment of chronic diseases including cystic fibrosis, AIDS, cancer, diabetes, and vascular diseases (1, 7-9). Several hundred human gene therapy clinical trials have been approved worldwide with over 100 protocols implemented in the United States alone (1, 2, 10). Unfortunately, gene therapy has never reached beyond the stage of human clinical trial (3). One of several major obstacles is the development of a safe, efficient, and efficacious vector system for in vivo gene application (10). This impediment remains at the forefront in the field of gene therapy (11).

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Approximately 75% of human clinical trials have adopted viral-mediated gene transfer technologies that are much more efficient than nonviral-mediated vector systems (1). The majority of the protocols have employed retrovirus as a method of gene delivery due to its stable long-term expression and high infection rates in proliferating cells (12-14). Slowly replicating or nondividing endothelial cells (ECs) of the vascular endothelium become problematic for this particular viral vector (1, 15). Efficiency of gene transfer has been as low as 0.5% to 1% (16, 17). Nonetheless, successful in vivo gene transfer to specific sites using retroviral and liposomal vectors has been documented albeit at extremely low efficiencies compared to adenovirus (18-20). Although liposome-mediated transfection is second to adenovirus in efficiency, nonviral vectors are biologically safer for in vivo application (15, 21).

To date, many studies have shown that transfection efficiency using lipid-based gene transfer technologies is remarkably low in primary cells (8, 22-24). We are interested inoptimizing these nonviral vectors for potential ex vivo transfection of allografts prior to transplantation. We assessed the in vitro transfection of primary human umbilical vein endothelial cells (HUVECs) utilizing three different commercial lipid-based reagents, Effectene (Qiagen™, Mississauga, ON, Canada), Fugene 6 (Roche™, Laval, QC, Canada), and DOTAP (Roche™), at experimental temperatures of 37°C, 24°C, and 6°C. Using the enhanced green fluorescent protein (EGFP) as a reporter, the performance of each lipofection agent was evaluated based on percent transfection.

II-B) MATERIALS AND METHODS

1. Human Umbilical Vein Endothelial Cell Isolation and Culture

Primary endothelial cells (ECs) were isolated from human umbilical cord vein (HUVECs) as previously described (25). Briefly, the cord was digested with Type V collagenase (0.2 mg/ml, Sigma™, Oakville, ON) for 10 min at 37°C and then flushed thoroughly with phosphate-buffered saline (PBS, pH 7.4) to remove ECs from the cords (25). ECs were collected in a 50-ml conical tube and pelleted at 1000 X g for 10 min. Isolated ECs were cultured in 3 ml of complete M199 medium (Invitrogen™, Carlsbad, CA, USA) supplemented with 20% fetal bovine serum, L-glutamine (1.4 mM), and penicillin/streptomycin (200 U/ml, 200 mg/ml) at 37°C and 5% CO_2 in 95% air. Endothelial cell growth supplement (5 mg/ml ECGS, Becton Dickensen™, San Jose, CA, USA) was also provided to the cell culture. Upon confluence in the uncoated 25-cm^2 flask (VWR™, Mississauga, ON, Canada) the cells were washed with 1 X Hanks solution (Invitrogen™, Carlsbad, CA, USA), removed with 0.5 X trypsin/EDTA (Invitrogen™, Carlsbad, CA, USA), and spun down at 1000 X g for 10 min. After removal of the supernatant the cell pellet was resuspended in complete M199 growth medium and transferred to a 75-cm^2 (VWR™, Mississauga, ON, Canada) gelatin-coated flask. Subsequent confluent flasks were subcultured at a 1:3 split ratio into 75-cm^2 gelatinized flasks.

2. Primary Endothelial Cell Transfection Studies

The temperature dependence with lipid transfection was assessed by experiments carried out at temperatures of 37°C, 24°C, and 6°C. Confluent flasks of HUVECs of low passages ($p \leq 6$) were plated onto six-well Trans-well plates for transfection studies. The

plated cell density varied, ranging from 1.2 to 3 X 10^5 cells/well and cultured for 1 to 3 days depending on their growth rate. When they reached approximately 80% to 90% confluence the plated HUVEC monolayer was transfected at experimental temperatures of 37°C, 24°C, and 6°C with one of the three commercial lipid-based reagents: Fugene 6, DOTAP, and Effectene. Optimization of the provided commercial protocols to achieve high levels of reporter gene expression for these lipid transfectants was done at 37°C. These optimized transfection parameters (DNA/lipid rations, DNA/lipid doses, duration in transfection mixture) were maintained for transfections at 24°C and 6°C. In addition to a negative control, a 37°C temperature control was also carried out in transfection experiments done at temperatures other than 37°C.

Transfection mixtures containing reporter DNA and lipid reagent were prepared according to commercial protocols. The HUVEC monolayer was washed twice with serum-free medium, Opti-MEM I (Invitrogen™, Carlsbad, CA, USA), then overlayed with 1 ml Opti-MEM I. Transfection mixtures were added dropwise while swirling the monolayer of cells. At the end of the incubation period the transfection mixture was removed, the monolayer washed twice with M199 growth medium (no antibiotic), and then cultured in the same medium in an incubator at 37°C and 5% CO_2/balance humidified air for 3 days.

3. Reporter Gene and Tranfection Efficiency

Efficiency of transfection was determined using the enhanced green fluorescent protein (EGFP) as a reporter driven by a pCMV promoter and cloned in a pEGFP-NI vector (Clontech™, Mountain View, CA, USA). Transfected ECs expressed EGFP and fluoresced green under darkfield fluorescence microscopy, whereas untransfected control

cells had no fluorescence. Endothelial cells were detached from the culture dish with 0.5 X trypsin-EDTA, washed, and resuspended in PBS. An aliquot of cell suspension was spread on a glass slide for assessment of transfection efficiency based on expression of the green fluorescent protein. Transfection efficiency was evaluated by counting the number of fluorescing ECs under darkfield from the total number of cells present in the same field viewed under light microscopy. A minimum of 100 cells were evaluated, in duplicate, from each experimental group.

A negative control vector was prepared in our laboratory using the same plasmid backbone as the pEGFP-NI vector by removing the EGFP gene (771 bp fragment) with the restriction enzymes (Invitrogen™, Carlsbad, CA, USA), EcoRI and NotI. Incompatible termini of the remaining vector fragment were filled by an end-filling reaction using the Klenow fragment of DNA polymerase I and ligated with T4 DNA ligase. Cloned DNA plasmids were then purified using an endotoxin-free maxi-prep kit (Qiagen™, Mississauga, ON, USA). Transfection with this empty vector was done under the same experimental conditions as the test samples. Results of percent transfection were expressed as the mean±SEM. Statistical significance between the three experimental groups at different temperatures was evaluated by ANOVA followed by the Scheffé *F* test, and $p<0.05$ was considered significant.

II-C) RESULTS

1. Optimization of Lipofection

To avoid in vitro toxicity and achieve optimal transfection efficiency with lipofection, it is necessary to optimize the transfection protocol for each lipid-based

reagent. Parameters evaluated included the DNA and lipid dose, DNA/lipid ratio, and duration of incubation in the transfection mixture. The amounts of plasmid DNA and lipid reagent used and found to be optimal for each protocol were as follows: 0.5 μg DNA/6 μl of Fugene 6, 2.5 μg DNA/15 μl DOTAP, and 0.2 μg DNA/10 μl of Effectene. Recommended incubation time in the transfection mixture also varied among the lipid reagents: 5 h for both Fugene 6 and DOTAP and 3 h for Effectene (Figure 2-1). For these incubation periods minimal toxicity was observed. Fugene 6 appeared the least toxic by the low degree of cell detachment observed after incubation in the DNA/lipid mixture.

2. Temperature-Dependent Effect on Lipid Transfection

Rate of lipid-based transfection was temperature dependent (ANOVA; $p<0.0001$) (Figures 2-2, 2-3, 2-4), and the combined mean efficiency from all three lipid reagents (Effectene, Fugene 6, DOTAP) decreased significantly (37°C vs. 24°C, $p<0.0001$; 37°C vs. 6°C, $p<0.0001$; 24°C vs. 6°C, $p<0.0115$) as the temperature decreased. Highest mean efficiency was observed at 37°C with 29±1.7% (n=25, mean±SEM) cells expressing EGFP, whereas the lowest percentage of EGFP-expressing ECs occurred at 6°C with a mean expression level of 6±0.6% (n=6). At 24°C, the mean efficiency was 18 ± 2.1% (n=17). Efficiency of transfection using lipofection appears to be adversely affected by the decreasing temperatures.

3. Comparison of Performance Among Individual Lipid Reagents

The performance of individual lipotransfectant was compared based on percent transfection (Figure 2-5). Effectene reagent is a unique nonliposomal formulation that is used with a DNA-condensing enhancer and optimized buffer to achieve high transfection

efficiency in eukaryotic cells. Transfection of HUVECs with Effectene resulted in 34±1.3% (n=13) mean efficiency at 37°C. Lowering the temperature to 24°C resulted in a decrease in mean efficiency to 24±3.1% (n=7). Further decline in transfection efficiency was noted at 6°C at which gene expression was 6±0.5% (n=2). Untransfected control HUVECs had no fluorescence.

Fugene 6 is another nonliposomal reagent comprised of a blend of lipids and other compounds combined in 80% ethanol. Comparable transfection efficiency to Effectene was achieved with Fugene 6 at 37°C, which was 30±2.5% (n=7). Similarly, as the temperature declined mean transfection efficiency also decreased, but the magnitude of decrease was greater for Fugene 6 than Effectene at 24°C. Mean transfection efficiency was 14±2.9% (n=6) and at 6°C it was 8±1.5% (n=2).

Among the three commercial lipid-based reagents studied lowest transfection efficiency was obtained with DOTAP transfection. DOTAP is a cationic liposomal formulation and this difference may account for the lower transfection results. At 37°C, mean transfection efficiency was 18±2.3% (n=7), which was only half as efficient as both Effectene and Fugene 6. However, at 24°C, the magnitude of decline in percent transfection was small, mean efficiency was 15±3.2% (n=5), and even lower at 6°C, with resultant mean efficiency of 6±0.0% (n=2). Among the three lipid formulations, it appears that Effectene performs relatively better at the different temperatures assessed (Figure 2-5).

II-D) DISCUSSION

Gene therapy has arisen from a well-supported theory in the early 1970s to having immense practical implications in clinical medicine today (26). Although it is still a developing clinical strategy, the versatility of gene therapy has opened up new doors to different treatment approaches for both genetic and acquired diseases. Two forms of gene therapy, *ex vivo* and *in vivo*, have been utilized in human clinical trials with thrice the number of successful protocols adopting an ex vivo approach (5). *Ex vivo* gene manipulation involves the removal, modification *in vitro*, and reintroduction of the modified autologous cells back into the host to deliver a specific transgene product (4). Using this approach a specific cell type can be targeted for gene transfer and the level of transgene expression can be evaluated prior to reimplantation (15). Unfortunately, *ex vivo* gene manipulation is a multistep process involving long preparation time. Another disadvantage is the requirement to use autologous cells for gene modification to eliminate the risk of alloimmunity following transplantation (15, 18). These drawbacks can be overcome by *in vivo* gene therapy where the exogenous gene can be directly transferred to any specific site (18) using various delivery strategies. Direct gene transfer *in vivo*, though it is a method of relative ease and has widespread applicability, is limited by the low efficiency of gene delivery (2, 16, 27).

Located at the interface between the circulation and the surrounding tissues the vascular endothelium is a tremendously appealing site for gene therapeutics. Although the quiescent endothelium is an excellent target site for gene therapy ECs are difficult to transfect because of their slow proliferative rate both *in vivo* and *in vitro* (23, 28, 29). Low efficiency of gene transfer has been demonstrated in primary ECs using replication-

deficient recombinant viral vectors, which usually give high infection rates (16). However, unlike adenovirus, retrovirus is effective in transducing only replicating cells (6). To increase in vitro transduction DEAE-dextran was added to the retroviral-infected cultures and efficiency reached 50-90% (16). Seeding experiments showed high coverage of retroviral-transduced ECs on denuded vessel walls after being sparsely implanted following *ex vivo* manipulation (13, 28, 30-32). Retroviral transduction of vascular cells *in vivo* is highly inefficient compared with *in vitro* manipulation rendering this vector less competent than adenovirus for *in vivo* gene transfer into quiescent cell types (5, 16).

Adenovirus is the predominant vector used almost entirely for *in vivo* human gene transfer trials (5). It transfects dividing and nondividing cells with high efficiency both *in vivo* and *in vitro*. *In vitro* transduction with adenovirus is 10-100 fold greater than nonviral vectors, ranging from 50% to 100% efficiency in previous studies (8, 21, 22, 29, 33-35). Adenvector is also a far more superior vector *in vivo*. Successful gene transfer to the intact endothelium has been documented (19, 21, 29). Transfection of porcine coronary arteries *in vivo* with recombinant adenovirus reached 100 times greater efficiency than with Lipofectin (33). Although transgene expression is transient, it may last from weeks to months (9, 21, 29). Use of this viral vector, reported in these studies, resulted in no significant inflammation, except for a study completed by French et al. (33). Periadventitial cellular infiltrate in adenoviral-infected arteries was found that might be responsible for the decline in transgene expression. Immunogenicity and systemic toxicity associated with high titral administration are safety issues limiting the widespread use of adenovirus in human gene therapy (2, 11, 36). As with all other

biological vectors, biosafety is a major limitation for human clinical trial (37). Insertional mutagenesis and reversion to replication-competent virus are huge limiting factors when using retrovirus for *ex vivo* gene manipulation (26). Finding a safer and more efficacious gene delivery protocol has now become the focus and ultimate goal of gene therapy studies before this gene approach can advance beyond human trial.

A safer alternative to viral gene transfer is lipofection. Lipofection is neither immunogenic nor pathogenic (11). However, lipofection generally gives much lower efficiency, especially in primary ECs (22, 23, 38, 39). Transfection in primary cell types is more problematic because they contain abundant nucleases that degrade foreign DNA upon endocytosis (5). There have been developments in pH-sensitive liposomes and improved formulations of cationic liposomes and other lipid transfectants that are better capable of evading DNA degradation. These modifications have improved transfection efficiency astronomically, from less than 1% up to 70% (24, 34, 39-44). Improvement in efficiency has been documented in studies with primary ECs of porcine and bovine origins that were transfected at 15.2% with Lipofectamine and 60-70% with Fugene 6 (21, 34). The degree of procedural optimization, cell type, and lipid reagent selected are all crucial factors affecting the outcome of transfection. Undoubtedly, Fugene 6 appears to be relatively efficient in delivering gene(s) into refractory cell types as shown by several earlier studies (34, 45, 46). New lipid formulas may facilitate the escape of the DNA/lipid complexes from enzymatic degradation by rupturing the endosomes to make their immediate release prior to endo-lysosomal fusion (47). Alternatively, DNA uptake can be mediated by fusion of DNA/lipid complexes to the cell membrane, thereby avoiding the endo-lysosomal pathway by this direct transfer to the cytosol (47). Gene

therapy is being revitalized for treatment of human disease through these improvements in gene transfer technologies.

Unfortunately, primary cells of human origin have always been more difficult to transfect with nonviral-mediated vectors (23, 24, 38, 46). Teifel et al. reported an *in vitro* efficiency of 0.68% for electroporation and 0.45% for DOTAP (24). In contrast, transfection with DOTAP was 18% in our study (Figure 2-5). Transfection of an endothelial cell line, EA.hy 926, was also extremely low with a reported efficiency of less than 0.5% for EA.hy 926 with Lipofectamine (22, 24). Although Sipehia et al. achieved an efficiency of 22.6% with ApoE-lipo-fection, which is targeted to specifically HUVECs, this result was lower than what we achieved with lipofection alone using Effectene and Fugene 6 (23). We were also able to transfect HUVECs at low temperatures, albeit at low efficiency, yet the levels of gene expression achieved were higher than those obtained in some previous studies (23, 24, 38, 44). The decline in transfection efficiency as a result of lowering the temperature may be associated with a reduction in cellular metabolism affecting gene expression as opposed to transfection. In addition, the DNA/lipid complexes may be more stable at colder temperature, thereby sequestering the DNA and preventing its release and translocation to the nucleus for transcription (48). At lower temperatures the ECs appeared retracted and shriveled up, consistent with a low metabolic state. This observation was more pronounced at 6°C than at room temperature. Shorter incubation times were used in our study than what were used in previous studies (23, 24, 34, 49). Ideally, the minimal exposure time to lipotransfectant needed to achieve optimal *in vivo* transfection is preferred to minimize the risks of systemic toxicity and tissue pathology. In summary, lipofection is an

efficient method of gene transfer to primary HUVECs as demonstrated by our study (Figures 2-2, 2-3, 2-4). We also showed that efficiency of transfection using lipid reagents is largely temperature dependent (Figure 2-5). We presume that the observed transfection results from this study could be extended to relevant ECs of other origins (i.e., aortic, microvascular) given the appropriate lipid reagent and sufficient optimization of the transfection protocol. Nonetheless, actual transfection experiments would have to be preformed in other EC types to be certain about the level of efficiency. We chose HUVECs rather than other EC types due to the ease of attaining these ECs and that they could be propagated to a sufficient mass in reasonable time for experimental studies. Other studies have demonstrated transfection in other EC types, including brain microvessel ECs and pulmonary aortic ECs (34, 50). Kovala et al. achieved efficiencies of approximately 50% as reported by GFP expression in aortic ECs using Fugene 6 with adequate optimization, which is crucial for a lipofection strategy (34). The type of lipid reagent, amounts of DNA and lipid used, as well as their proportion in the complexation and the duration of transfection are some important parameters to consider for optimization. Also using Fugene 6, other primary cell types, such as chondrocytes of bovine and human origins, were transfected with efficiencies between 21% and 41%, which further supports the efficacy of the lipid reagents chosen for our study and the importance of optimization (45).

In vivo studies attempted with lipofection in the pulmonary vasculature and arterial segments resulted in successful transfection predominantly in ECs (18, 21, 44, 51). These improved lipid formulations appeared safe to use *in vivo*. Biochemical abnormalities and organ histopathologies, however, were not reported in the animal

models exposed to lipid-designed vectors, even at liposomal concentrations of 1000 fold higher than those used with previous formulations (6, 27, 52, 53). DOTAP was one of the lipid reagents found to be nontoxic for in vivo administration at concentrations optimal for effective gene transfer, and resultant gene expression has been shown in the lung, liver, heart, spleen, and kidney (49, 54). Lipid-based vectors appear to be a promising gene delivery vehicle for *in vivo* gene therapy. Furthermore, these agents hold promise for our ultimate goal of altering the endothelium of allografts by *ex vivo* perfusion. Specifically, these noninfective agents are able to transfect at preservation temperature, albeit with low efficacy. Future studies of whole-organ *ex vivo* perfusion are planned.

In conclusion, we showed that transfection efficiency can be improved with proper optimization of the lipofection protocol. This study showed the temperature dependence of lipid-based transfection in primary ECs. We showed that primary ECs can be transfected at higher rates than what has been previously documented using the commercial lipid-based reagents assessed in this study. Furthermore, we conclude that efficiency of lipofection is adversely affected by the decreasing temperatures, and among the three lipid reagents Effectene appears to perform relatively better at the experimental temperatures evaluated in this study.

In future studies we are proposing to use lipofection to deliver the telomerase gene into endothelial cells. Our aim is to assess for the expression of telomerase in primary ECs and whether these telomerase-transfected ECs have delayed replicative senescence and to what degree. Ultimately, we want to move onto translational studies in

an allogeneic mouse model to assess the effects of telomerase-transfected ECs on the longevity of allografts prior to proposing human trials.

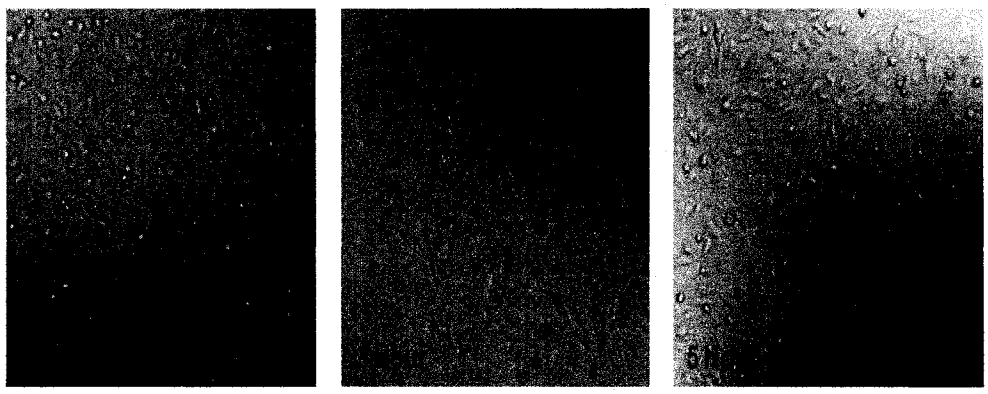

Figure 2-1: Monolayer of HUVECs transfected with the EGFP reporter vector using DOTAP, and viewed under a confocal microscope. HUVECs were incubated in this DNA/lipid mixture for 3 h and 5 h during the transfection period.

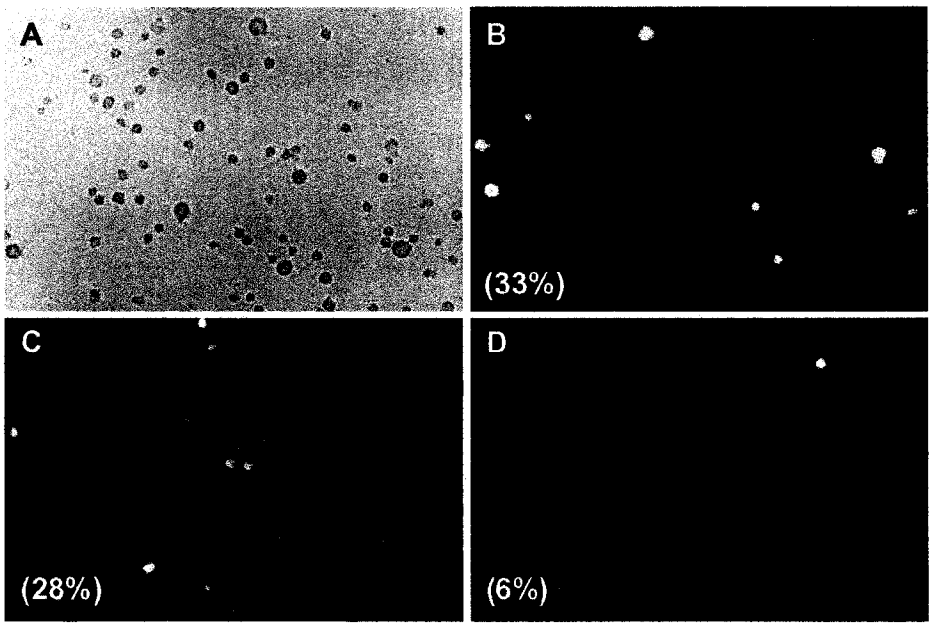

Figure 2-2: Transfection of HUVECs with Effectene at **(B)** 37°C, **(C)** 24°C, and **(D)** 6°C. **(B-D)** EGFP expression in trypsinized HUVECs was evaluated using fluorescent microscopy. Percent expression was **(B)** 33%, **(C)** 28%, and **(D)** 6%. **(A)** Field was viewed under light microscopy.

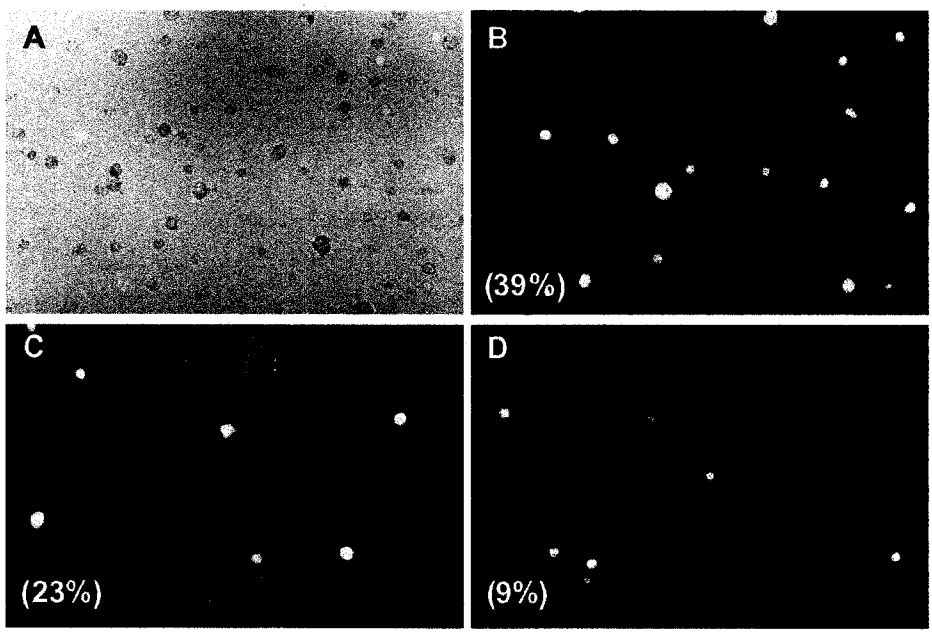

Figure 2-3: HUVECs were transfected with FuGene 6 at **(B)** 37°C, **(C)** 24°C, and **(D)** 6°C. Cells were trypsinized 3 d post-transfection and efficiency was determined by the levels of EGFP expression. Percent transfection was **(B)** 39%, **(C)** 23%, and **(D)** 9% under fluorescent microscopy. **(A)** Same field as **(B)** viewed under light microscope.

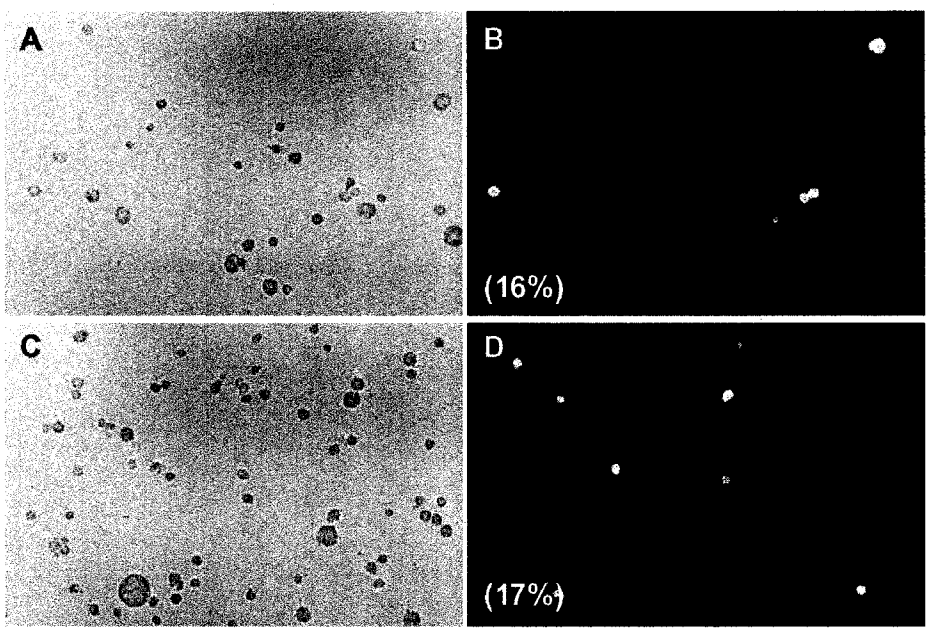

Figure 2-4: Transfection of HUVECs with DOTAP at **(A, B)** 37°C and **(C, D)** 24°C. HUVECs were first trypsinized, washed, and resuspended in PBS before spreading on glass slide for microscopic assessment. **(B)** and **(D)** were taken from the same field of view under fluorescent microscopy as **(A)** and **(C)**, respectively. EGFP expression was **(B)** 16% and **(D)** 17%. **(A, C)** Viewed under light microscope.

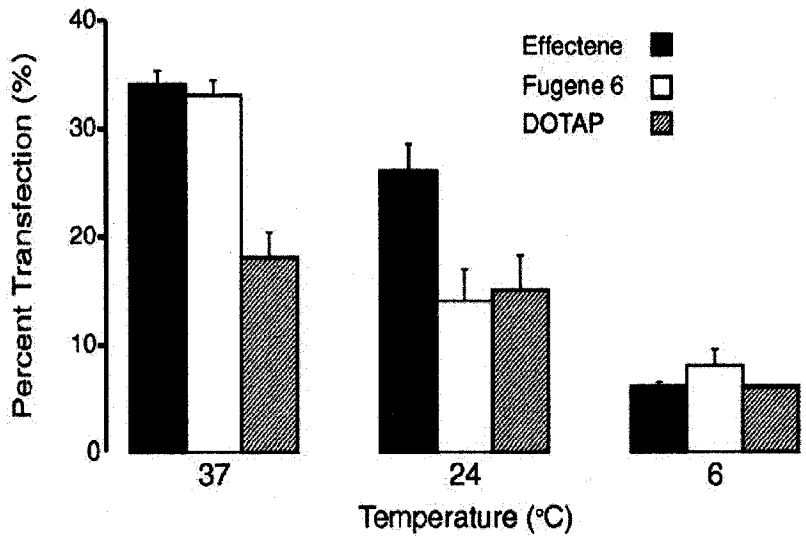

Figure 2-5: Transfection efficiency at different temperatures. Percent transfection was expressed as the mean±SEM. Resulting efficiencies were 34± 1%, (n=3), 33±1% (n=5), and 18±2% (n=7) for Effectene, FuGene 6 and DOTAP, respectively, at 37°C. Mean efficiencies were 26±2% (n=6) for Effectene, 14±3% (n=6) for FuGene 6 and 15±3% (n=5) for DOTAP at 24°C. At 6°C, percent transfection was 6±1% (n=2) for Effectene, 8±2% (n=2) for FuGene 6 and 6±0% (n=2) for DOTAP.

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CHAPTER III

ASSESSMENT OF DIFFERENT TRANSFECTION PARAMETERS IN EFFICIENCY OPTIMIZATION**

III-A) INTRODUCTION

The primary focus of many research projects nowadays is to overcome the primary obstacle of gene therapy, namely the improvement in gene transfer efficiency into human cells (1-3). High efficiency can be achieved in cell lines as opposed to primary cells. As a follow-up to our first transfection study in primary human endothelial cells reported in chapter I and published in *Cell Transplant* (4), we wanted to further investigate some transfection parameters that may aid in achieving optimal efficiency. As shown in many studies, efficiency is affected by many factors including concentrations of lipids and DNA, purity of DNA, transfection media and cell type (2, 5, 6). Another critical parameter is the use of serum-free media for transfection to avoid interference by serum proteins (7-9).

Based on our initial studies, Effectene lipid transfectant gave the highest rate of transfection which is the reason it was chosen as a lipotransfectant in this study. Here we examined the use of different cell culture media (DMEM, M199, Opti-MEM I) for transfection, as well as the potential effects of various glucose concentrations on transfection efficiency. Glucose being an important cellular metabolite has been shown to induce cell proliferation at high concentrations in some cell types (10-15), thereby

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possibly affecting transfection efficiency. Cellular proliferation is a critical factor for exogenous DNA uptake, which may explain the low efficiency observed in some primary cells (16, 17). Further aims of the work reported here also included assessing the effects of different reporter DNA concentrations on the rate of transfection and potential reagent toxicity that may be detrimental to cell transfectability and viability outcome.

III-B) MATERIALS AND METHODS

1. Endothelial cell culture

Both isolation and culture of endothelial cells from human umbilical vein (HUVECs) were described in detail in the previous chapter and have been published (4). HUVECs were cultured in normal M199 media supplemented with 20% fetal bovine serum, 1.4 mM L-glutamine and antibiotics (penicillin/streptomycin). Transfection experiments were carried out on cell cultures within the first 6 passages from cell isolation. These cells were plated onto 6-well Transwell plates (Falcon™) and incubated under standard cell culture conditions (37°C, 5% CO_2, humidified air) until they were ready for transfection at approximately 90% density (confluence).

2. Transfection studies

Prior to transfection, cells were first incubated in reduced serum concentration (7.5%) for 3-4 h. Transfection mixtures of DNA-Effectene were prepared according to the standard commercial protocol. Enhanced green fluorescent protein (EGFP) DNA (pEGFF-NI vector, Clontech™) used was at 0.2 μg per 10 μl Effectene. Negative controls included untransfected HUVECs and those transfected with an empty vector (pEGFP-NI vector without the EGFP gene). Cell monolayer was washed twice with

serum-free media and then overlaid with 1 ml of serum-free transfection media. The three serum-free transfection media used include DMEM (Invitrogen™), M199 (Invitrogen™) and Opti-MEM-I (Invitrogen™). The DNA-Effectene mixture was added dropwise with swirling after which the cells were incubated for 3 h under standard culture conditions. After removal of the transfection mixture the cells were washed with culture media and further incubated in normal culture media (M199 for HUVECs) for 2 days prior to detection.

Transfection studies examining the effects of glucose and DNA concentration on DNA uptake were also carried out. Standard glucose DMEM (4.5 g/L) and low glucose (1 g/L) DMEM media were used for transfection. Efficiency was also assessed in M199 of high (5.5 g/L) and normal (1g/L) glucose concentrations. For the latter, transfection experiments using double the amount of reporter DNA (0.4 μg) were also conducted. All experiments were performed in replicates of 5 or greater.

3. EGFP detection and analysis

Flow cytometry (FACS) and manual cell counting under darkfield fluorescent microscopy were used for detection after 2 days of culture. Duplicate counts of greater than 100 cells were recorded and 10, 000 events were counted using FACS analysis.

The Kruskal-Wallis, Mann-Whitney and student's t-test were used to establish statistical significance on the results, expressed as a mean ± standard error of the mean (SEM). A p value less than or equal to 0.06 was considered significant ($p \leq 0.06$). Nonparametric tests such as the Kruskal-Wallis and Mann-Whitney were used to assess statistical significance in transfection efficiency between ≥ 3 and 2 experimental groups respectively.

III-C) RESULTS

1. Efficiency in different transfection media

Cells fluorescing green indicated positive transfection and negative controls gave no fluorescence under darkfield. Using FACS analysis, less than 0.5% positivity was detected in negative control cells.

Opti-MEM I media was superior to standard DMEM media showing greater than 10% difference ($p=0.001$) in transfection efficiency whereas, no statistical significance was detected in comparison to M199 ($p=0.455$) media (Figure 3-1). Rates of transfection for Opti-MEM I, standard DMEM and M199, respectively, were 29±2.28% ($n=13$), 14±0.28% ($n=5$) and 24±1.54% ($n=5$). Use of M199 as a transfection media also produced a significantly higher efficiency compared to DMEM ($p=0.009$). Overall, the use of Opti-MEM I as a transfection media resulted in higher gene transfer *in vitro*. Therefore, we concluded, that in our hands, Opti-MEM I is a better performing transfection media.

2. Effects of glucose concentrations on transfection efficiency

Mean transfection efficiencies obtained in low and high glucose DMEM were significantly different ($p=0.001$). A mean of 17±0.38% ($n=7$) positive cells resulted from transfection in low glucose DMEM whereas, only 14±0.28% ($n=5$) were detected from using the high glucose media (Figure 3-2). No statistical significance was detected between M199 of normal and high glucose concentrations ($p=0.824$). Mean efficiencies of 27±1.35% ($n=9$) and 27±0.86% ($n=17$) were obtained for both normal and high glucose M199 respectively (Figure 3-2).

Furthermore, we examined whether priming cells in high glucose prior to transfection has any effects on efficiency. Note most preservation solutions have a high glucose concentration for both tonicity and energy requirements to prevent cell swelling. Rather than just exposing HUVECs to high glucose during transfection the cells were incubated in high glucose M199 media supplemented with 20% fetal bovine serum overnight and then transfected in serum-free, high glucose M199. The mean transfection rate from glucose primed HUVECs was 25±1.18% (n=6) which was not significantly different from non-glucose primed HUVECs with a mean transfection rate of 26±1.23% (n=10; p=0.590).

When comparing efficiencies between the high glucose media, M199 gave significantly ($p<0.001$) higher transfection results than DMEM, likewise for low glucose media. DMEM showed significantly ($p<0.001$) lower mean transfection efficiency compared to M199 at the same glucose concentration, which suggests that M199 is more optimal for transfection of HUVECs.

3. Effects of a higher DNA concentration with Effectene on transfection

Transfection in the presence of higher DNA concentration (0.4 μg) than 0.2 μg did not significantly ($p=0.273$) alter the mean efficiency of HUVEC transfection in normal glucose M199 media. Mean rates of DNA uptake were 24±1.54% (n=5) and 27±1.35% (n=9) respectively for 0.2 μg and 0.4 μg EGFP DNA (Figure 3-3).

4. Reagent toxicity

Significant reagent toxicity was observed when the DNA-lipid transfection mixture was not removed after the 3 h incubation period. The average cold storage time in preservation solution varies for each organ. For kidneys the average cold storage time

is approximately 19 h. As such, potential toxicity would occur with this prolonged contact of the transfecting agent, again suggesting the need for alternative storage such as cold pulsitile perfusion, where the perfusate could be altered. Extensive cell death resulted when the DNA-Effectene/DMEM was left for 2 days prior to detection. The overall mean efficiency observed from such treatment was 5±0.87% (n=4). The lengthened incubation of HUVECs with DNA-lipid mixture in low glucose DMEM transfection media resulted in a mean transfection rate of 7±0.80% (n=2) and 4±0.73% (n=2) in high glucose DMEM. Whereas, significantly greater efficiencies were obtained from both low and high glucose DMEM with removal of the transfection mixture after 3 h incubation (Figure 3-4). The mean transfection rates obtained were 20±3.79% (n=3; p=0.060) and 16±1.39% (n=6; p=0.001) for low and high glucose DMEM respectively.

III-D) DISCUSSION

Increasing donor organ supply is the most pressing issue in clinical transplantation today, not only because organ transplantation has become the accepted therapeutic modality for end-stage human diseases (18), but also because recipients are living longer and healthier and outlasting their allograft. Therefore urgency also arises from the need of a retransplantation due to the limited longevity of allografts that further perpetuate the problem of organ shortage. Although advances in immunosuppressive therapy have significantly improved short-term graft survival rates, long-term graft survival has yet to be achieved (19).

One area of focus to enhance graft survival is the *ex* vivo genetic manipulation of allografts made possible with the advanced understanding of molecular biology and

improvement in gene therapy technologies. Modification of organs by the introduction of human genes (i.e. immunomodulatory, anti-apoptotic and anti-oxidative stress genes) into grafts increases their protective mechanisms against chronic rejection, a major cause of late graft failure (20). These protective effects from exogenous gene expression in modified allografts will hopefully help improve long-term graft survival that has not yet been accomplished with current systemic immunosuppressive regimens.

Overcoming the low efficiency of transfection in primary human cells is an area of diligent research to further human gene therapy beyond clinical trials. In addition, biosafety is a crucial issue in the selection of an appropriate gene transfer vector (1, 3). Although many human gene therapy clinical trials have adopted some form of viral vector for gene delivery, the risk of administering a biological agent into the body remains a major concern, particularly after the report of the Gelsinger case in Philadephia in 1999 (21). As a result, non-viral vector systems are more appealing from a biosafety perspective. However, a critical drawback of non-viral vectors is the low efficiency of transfection compared to viral vectors (1-3, 5). Improving upon the rate of gene transfection by non-viral means will undoubtedly lead to a wider acceptance of gene therapy being part of routine clinical medicine in the future.

Efficiency of transfection may be affected by a number of factors (1-3, 5, 6). One of them is the type of media used for transfection. Opti-MEM I was used in our previous transfection study and gave optimal efficiency (4). Nonetheless, this particular media maintained its superior performance also in this study in comparison to other media utilized consisting of DMEM and M199. Although the mean rate of transfection achieved in Opti-MEM I (29±2.28%) was not significantly higher than M199

(24±1.54%), both of these media, performed significantly better compared to DMEM (14±0.28%).

One difference among the three media used for transfection was the glucose concentration. DMEM is high in glucose concentration at 4.5 g/L whereas M199 is low at 1 g/L and Opti-MEM I is intermediate at 2.5 g/L. Since Opti-MEM I yielded the highest transfection efficiency it appears that maintaining this glucose level may be optimal for gene transfer experiments. However, a much higher glucose concentration used in M199 for transfection did not appear detrimental to either HUVEC viability or transfectability. Rates of transfection acquired in low and high glucose M199 were similar. Surprisingly, the mean efficiency of transfection in low glucose DMEM (1 g/L) was higher than that obtained from high glucose DMEM. The glucose content in this media type did appear to make a difference in transfection rates.

Interestingly, the base media formulation for M199 contains twice as many ingredients, such as the amino acid and vitamin contents, as DMEM. Furthermore, Opti-MEM is also well supplemented with growth factors, trace elements and proteins although the detailed formulation is proprietary. We believed that the inhibitory effects of high glucose may be compensated by the vast amounts of growth supplements in the enriched base media. Therefore, we believed that the glucose concentration in transfection media is not the sole determinant of transfection efficiency. Rather, the base media formulation also seems to play an important role. Certainly, the above studies support that the additional glucose found in most preservation solutions is not likely to have an adverse effect on transfection.

Some studies however, have found that a high glucose content in the culture media can be inhibitory to endothelial cells. DNA damage, altered endothelial cell function and decline in cell proliferation have been reported (22-26). However, glucose-mediated damage, as reported by some, may not be evident in short term cultures (5 days and under) (24, 25, 27, 28). We did not see increased cell death when HUVECs were exposed to high glucose levels during transfection or when cultured in high glucose media (Figure 3-6A) for 2 days. Similar findings on cell viability were reported in another study by Porta et al. (29, 30). Although one study claimed that inhibition of endothelial cell replication by elevated glucose has been observed within 24 h as evident by a reduction in DNA synthesis by more than 25% (24), we did not observe any significant change. Likewise, Donnini et al. reported that the glucose-mediated alterations in cell behavior were mild (23). Even after a one day culture in high glucose M199 prior to transfection (glucose-priming) we did not observe a change in transfectability and viability of the HUVECs. In contrast, Lorenzi et al. have found only 30% of HUVEC isolates were unresponsive to high glucose levels (24, 25). Our study and others would suggest little if any pronounced effects from short term culture in high glucose.

As shown in Figures 3-5 and 3-6, HUVECs cultured in either high or low glucose containing DMEM had no significant effect on cell viability. Instead, the continuous incubation of cells in DNA-lipid transfection complexes till the day of detection (2^{nd} day) resulted in significant cell death in both high ($p=0.001$) and low ($p=0.060$) glucose DMEM leading to a significant reduction in transfection rates. Alternative strategies to cold storage preservation with transfectant will be needed, both on toxicity of prolonged

contact and low efficiency at low temperatures. Strategies to overcome this include transfection prior to organ retrieval or brief pulsatile perfusion at low temperatures greater than 6°C.

Even though endothelial cells are more susceptible to glucose overload than other cell types (26, 27) cell toxicity was evident from the lipid transfectant rather than high glucose levels. Nevertheless, lower glucose levels in the culture media seemed less harmful subjectively to the cells in culture. Slightly better cell viability was observed in low glucose DMEM (Figures 3-5B, 3-5C, 3-6B) with the presence of lipid transfectant compared to its counterpart.

In conclusion, understanding the interplay of various transfection parameters is critical to attaining high transfection efficiency. We continue to strive to make non-viral mediated gene therapy a successful reality in the ex-vivo modification of human tissue.

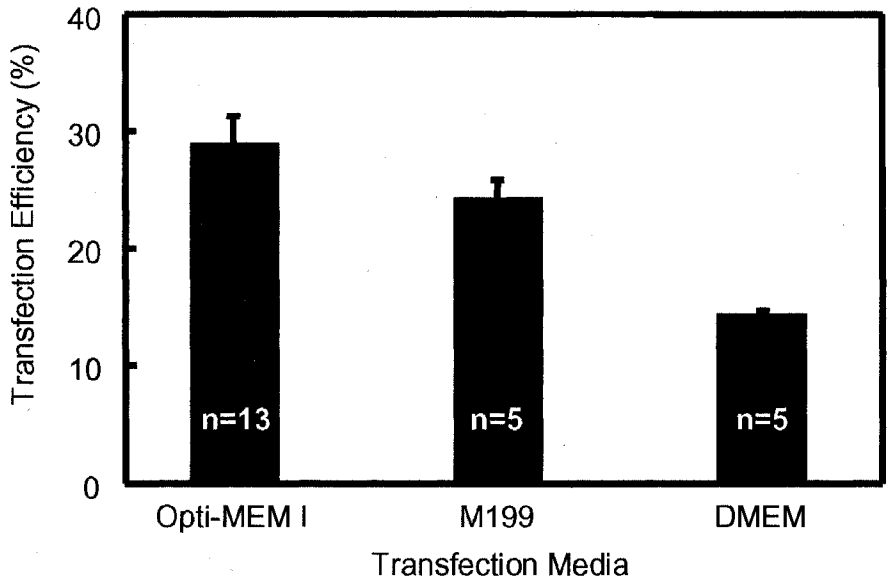

Figure 3-1: Efficiency of transfection of HUVECs in different types of media. Highest efficiency was achieved using Opti-MEM I media (29±2.28%) which was significantly better compared to DMEM (14±0.28%; p=0.001). Although no statistical significance (p=0.455) was observed between the performance of Opti-MEM I and M199 media (24±1.54%), the latter also produced significantly higher transfection rates than DMEM. Overall, Opti-MEM I media remains superior in terms of transfection outcome.

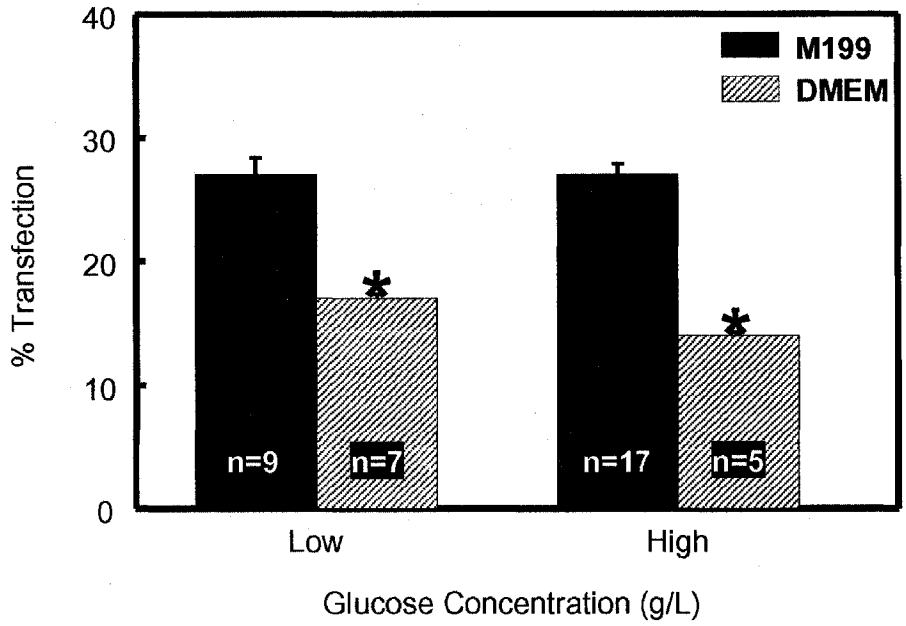

Figure 3-2: Effects of glucose concentrations on the percent transfection of HUVECs. Statistical significance was only observed for the mean rates of transfection between low and high glucose DMEM ($p=0.001$) media. M199 performed significantly better than DMEM in both low ($p=0.001$) and high ($p=0.001$) glucose concentrations resulting in rates of 27±1.35% and 27±0.86% as opposed to 17±0.38% and 14±0.28% respectively.

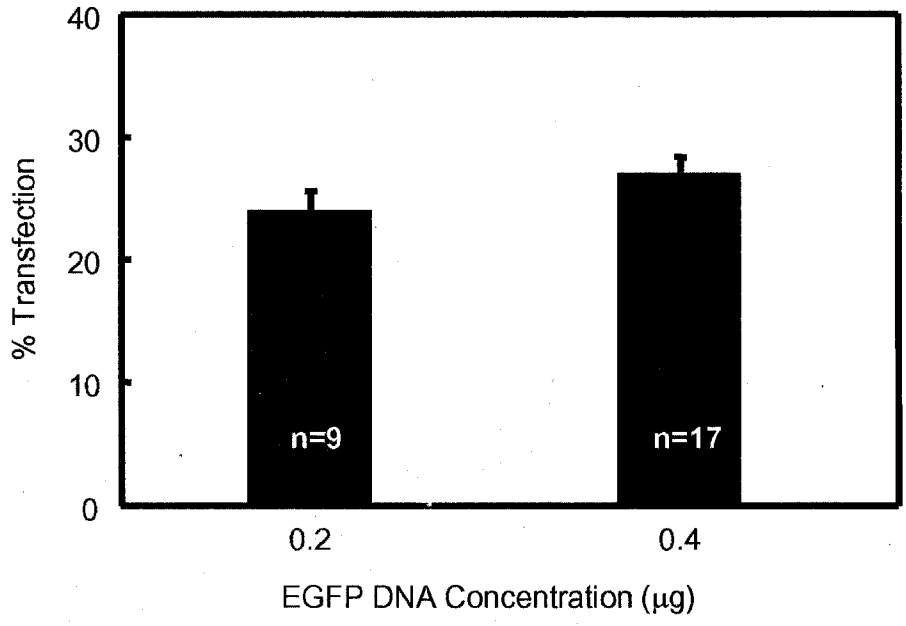

Figure 3-3: Effects of DNA concentrations on transfection efficiency from HUVECs. Transfection rates ranged from 24±1.54% to 27±1.35%. Doubling the DNA concentration had no significant effect on the percent transfection of HUVECs ($p=0.273$).

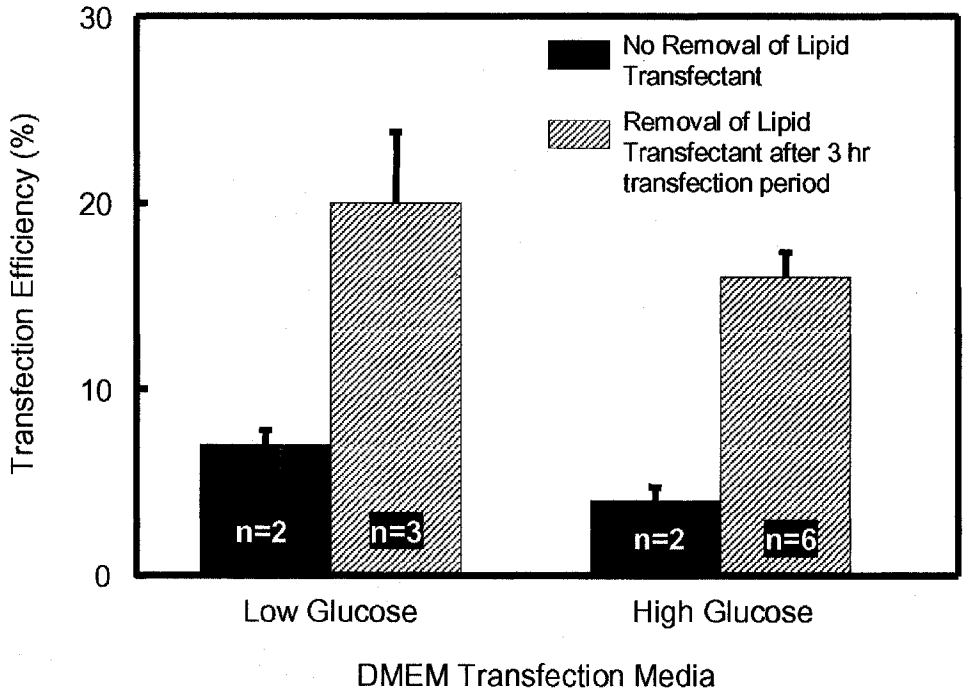

Figure 3-4: Reagent toxicity. Long-term (2 days) incubation of HUVECs with DNA-lipid transfection mixture resulted in significantly lower efficiencies both in low (p=0.060) and high glucose (p=0.001) DMEM media. Mean transfection rates were 7±0.8% and 4±0.73% respectively as opposed to 20±3.79% and 16±1.39% under relatively shorter incubation time.

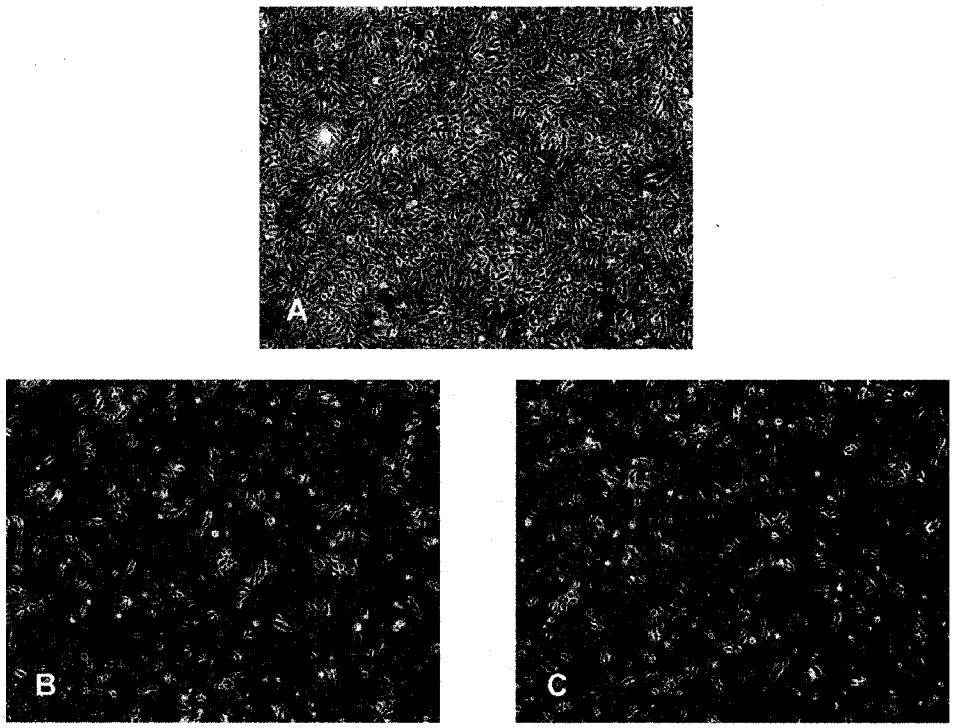

Figure 3-5: Reagent toxicity. Appearance of HUVECs in culture 2 days post transfection either with an empty vector **(A, B)** or the EGFP-Ni vector (Clontech^{TM}). **(A)** Removal of DNA-Effectene lipid mixture after 3 hr incubation and media replacement led to significantly ($p=0.060$) higher mean transfection rates and cell viability compared to **(B, C)** no removal of transfectant in low glucose DMEM until the day of detection (2^{nd} day).

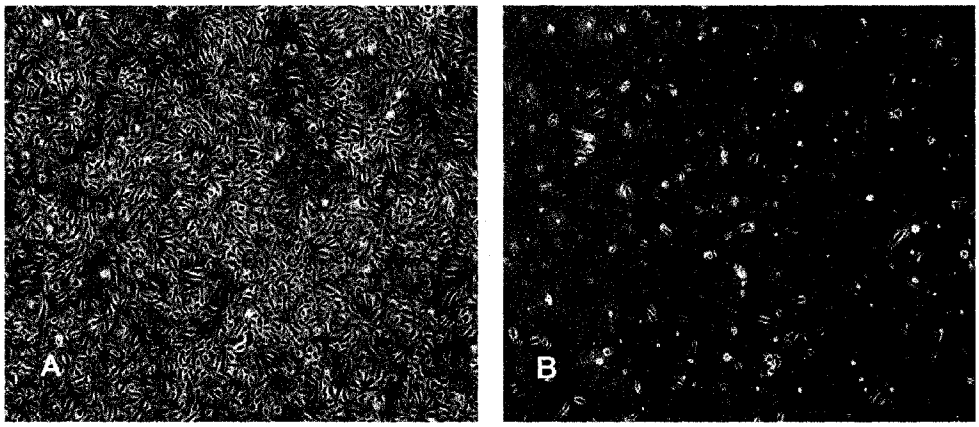

Figure 3-6: Reagent toxicity. HUVECs were transfected with a EGFP-Effectene mixture in the presence of high glucose DMEM. After 2 days of culture a significantly higher number of cell deaths occurred when the DNA-lipid transfectant **(B)** was not removed from initial transfection as opposed to **(A)** removal and media replacement following 3 hours of transfection ($p=0.001$).

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CHAPTER IV

RESCUE OF TELOMERE SHORTENING AND INCREASE GROWTH VIA TELOMERASE EXPRESSION IN VITRO

IV-A) INTRODUCTION

Replicative or cell senescence is an *in-vitro* aging phenomenon that is widely studied in various cell types (1-3). In particular, endothelial cells have been commonly used as a model of cell senescence, being examined within the context of cell morphology, chromosomal abnormalities, gene expression profiles, and metabolic processes (4-10). Endothelial cells form the interface between the blood circulation and underlying tissues thus these cells are crucial to maintaining physiological homeostasis (11, 12). Their involvement with the circulation includes blood clotting and blood pressure control, inflammation as well as angiogenesis. In addition, the endothelium regulates the passage of substances and cells trafficking between this endothelial interface. In recent years, evidence has suggested a potential role of telomeres in human senescence (13-19). Telomeres cap and protect chromosomal ends from recombinant aberrations and loss of essential genetic information. They are critical to genomic stability and cell viability. The length of telomeres represents a mitotic clock that keeps track of the cell's replicative history. Shortening of telomeres has been found in aging human kidneys as well as in the dermal and vascular tissues of older donors (15, 16, 19). However, it remains inconclusive as to the causal role of telomere attrition in organismal aging as a whole.

Aging pathologies have been found in chronically rejected organ transplants (20-22). Such findings are due in part to chronological age; however, transplantation-related stress factors also impact on the functional age of the organ. This leads to late graft dysfunction, which is a significant obstacle in clinical transplantation today (20-22). The shortening of telomeres triggers cell senescence potentially causing organ failure (17, 22). When cells reach the Hayflick limit, as dictated by telomere length, they undergo senescence and their tissue repair ability is impaired (13, 17). Therefore, overcoming cell senescence by means of telomere maintenance has significant clinical implications and this therapeutic potential should not be ignored. As constitutive cells of an organ reach their finite replicative life span due to the shortening of telomeres, organ function becomes compromised (23, 24). This functional deterioration is triggered by cell senescence when telomeres are critically short (17, 24, 25). Aside from chronological aging, stress-induced telomere shortening also contributes to graft senescence (26, 27). Attenuation of replicative senescence and extension of cellular life span can be achieved through the activation of telomerase, a reverse transcriptase that synthesizes telomeric DNA de-novo (28, 29). Telomerase expression is a hallmark of germ cells and malignant cells, whereas almost all normal diploid cells lack telomerase and therefore senesce (18). The diminished thriving ability of aging somatic cells, that don't normally possess telomerase activity, can be restored via ectopic expression of the human telomerase catalytic subunit (hTERT). This is the rate-limiting component of the ribonucleoprotein complex that also consists of a RNA template (hTR) constitutively expressed at various levels in different organs (30, 31). Many studies have shown that the forced expression of hTERT led to an increase in the replicative life span of human cells (28, 29, 32).

When telomere length is maintained or extended in normal somatic cells by telomerase activation, the growth capacity of cells can be renewed and cell aging can be prevented. The stable expression of telomerase in transfected cells allowed them to overcome senescence or the M1 checkpoint at the Hayflick limit, a point of growth arrest triggered by critically short telomeres (27-29). Even though telomerase has been found to reverse *in vitro* replicative senescence, its expression is also a hallmark of cancer (28, 29, 33-35). Greater than 90% of cancers express telomerase (34). Both *in vitro* and *in vivo* studies have shown that the inhibition of key components of telomerase resulted in tumor regression (36-38). The strong implication of telomerase in promoting tumor growth necessitates the examination for potential malignant transformation in telomerase-transfected normal cells. Results from many studies have been inconclusive in demonstrating tumorigenicity in cells having hTERT expression alone (39-42). It is important given the overall aim of this study to undoubtedly demonstrate that HUVECs expressing telomerase do not exhibit any tumor/malignant phenotypic changes. To accomplish this we propose using a soft agar assay and assessment of tumor suppressor proteins. Malignant cells acquire the capability to grow in an anchorage-independent manner and form colonies in soft agar (43, 44). Furthermore, cell cycle checkpoints are often found disrupted in transformed cells acquiring uncontrollable, contact-independent cell growth (45-47). These cells can easily form tumors in nude animals (43, 44, 48). The primary defense against tumorigenesis is elicited by checkpoint guardians such as the retinoblastoma (pRb) and p53 proteins. They are either present in a mutated form or absent in transformed cells leading to cancer (45-47).

Given the risk of cancer associated with stable long-term telomerase expression, a non-viral gene transfection strategy was used in this study (23, 31, 49). A non-viral gene strategy results in only transient gene expression and thereby eliminates potential biosafety problems arising from independent, long-term expression associated with viral vectors. In preceding chapters, which have been published, a mean efficiency of approximately 30% was achieved in human endothelial cells using lipofection (50, 51). The overall aim of this study was to utilize optimized lipofection to deliver the human telomerase gene into endothelial cells and examine resulting telomerase activity and telomere length. Functional phenotype of hTERT-transfected endothelial cells was also assessed as "proof of concept" that human telomerase gene therapy alone leads to the "fountain of youth" in the absence of tumorigenesis. As proof of concept, this current study has demonstrated and characterized replicative senescence in human endothelial cells. This *in-vitro* model was then used to further elucidate the relationship between *in-vitro* cell senescence and telomere length using a modified flow-FISH technique. This study provides support for conducting gene transfection studies, with telomerase, in an attempt to revitalize cells through telomere length manipulation as a means to prolong allograft (cell or organ) survival.

IV-B) MATERIALS AND METHODS

1. Cell Culture and Morphology

Isolation of primary endothelial cells from human umbilical veins was described in an earlier chapter and published (50). HUVECs were cultured on 0.1% gelatin-coated 100 mm Falcon™ tissue culture dishes (VWR™, Mississauga, ON, Canada) to promote

cell attachment. M199 medium supplemented with 20% FBS, 2.8 mM L-glutamine, penicillin/streptomycin (100 U/ml:100 mg/ml) and 50 μg/ml endothelial cell growth supplement (ECGS, Becton Dickinson, Bedford, MA, USA) was used to culture HUVECs under standard conditions (37°C, 5% CO_2 in humidified air). When standard culture medium is supplemented with nutrient additives, it is classified as "complete" medium. *(Note: Unless specified, otherwise all tissue culture media and reagents were purchased from Invitrogen™, Carlsbad, CA, USA)* Change of culture medium was performed every 2 to 3 days.

Morphological images of HUVECs at increasing passage numbers were captured with a Nikon™ digital camera connected to a phase contrast inverted microscope at 100X the original magnification.

2. Wound Healing

Early- and late-passage (P) HUVECs were cultured in gelatin-coated 24-well plate. At confluence, a wound was chemically induced in the center of the cell monolayer with 2 ul of 0.1 M NaOH that was immediately aspirated off to create a circular area devoid of cells (52). The monolayer was carefully washed 3X with reconstituted M199 media to remove residual NaOH and cultured in the same media being replenished every 2 d to 3 d. Healing of the wound was monitored under phase contrast microscopy.

3. Senescence-Associated β-galactosidase (SA-βgal) Staining

The use of SA-βgal activity as a marker of cell aging was first described by Dimri et al. (2). β-gal activity is optimal at pH 4 and it is normally expressed in cells at this pH. At the suboptimal pH 6 however, only residual activity due to the overexpression of the

β-gal enzyme in senescent cells would be detected. SA-βgal staining is therefore used to assess cell aging. Cells that are positive for βgal would be stained blue in color using the commercially available Senescent Cells Staining Kit (Sigma™, Oakville, ON, Canada). This assay was used to determine βgal expression in HUVEC cultures at various passages (P7, P14 and P21) as a quantitative marker of senescence. HUVECs were plated, in duplicates, at 5 X 10^4 cells per gelatin-coated 35 mm Falcon™ tissue culture dishes (VWR™). After 2 d of standard culture, the cells were briefly treated with fixation buffer essentially containing 2% formaldehyde and 0.2% glutaraldehyde in 1X PBS for 5 min. Cells were washed 3X with 1X PBS and incubated overnight in staining solution containing a mixture of Reagent B (potassium ferricyanide), Reagent C (potassium ferrocyanide) and X-gal provided in the Sigma™ staining kit. The percentage of blue-stained cells was recorded from a count of 100-200 cells.

4. Vector Cloning

The hTERT gene sequence (red) was kindly provided by Geron™ Corporation (Menlo Park, CA, USA) in a retroviral vector identified as pGRN145 (Figure 4-1B). To prepare the recombinant pNI-Tel plasmid, the procedure entails removing this hTERT gene from pGRN145 and re-inserting it into an empty pEGFP-NI vector (3929 bp fragment, Figure 4-1A) already having the EGFP gene (771 bp fragment) first removed with restriction enzymes EcoRI and NotI. Similarly, the hTERT gene was digested with the same 2 enzymes to remove it from pGRN145 giving a hTERT fragment of 3415 bp in size flanked by EcoRI and NotI. Since the empty vector backbone and hTERT gene were flanked by the same restriction enzymes sites, these 2 segments were directly ligated

together using T4 DNA ligase (Invitrogen™, Carlsbad, CA, USA) to create a new recombinant plasmid, pNI-Tel, of 7344 bp in size.

5. Telomere Repeat Amplification Protocol (TRAP) Assay

The commercial protocol provided with the TRAPeze XL Kit (Chemicon™, Temecula, CA) was used for the detection of telomerase activity examined at 3 h to 5 d post-hTERT transfection. The TRAP kit provides a substrate oligonucleotide to which telomeric repeats will be added in the presence of telomerase activity to the 3' end of this substrate. The following reaction entails PCR amplification of the extended substrates using fluorescein-labelled primers which will fluoresce if incorporated into the TRAP product. As a result, the emitted fluorescence is directly proportional to the amount of TRAP products generated. Total protein lysates were prepared from untransfected and hTERT-transfected HUVEC. Extracts from HeLa cells were run as positive controls. A 1.0 μg protein extract was analyzed from all samples for telomerase activity.

6. Southern Blot Detection of Telomere Length

The TeloTAGGG Telomere Length Assay Kit (Roche™, Laval, QC) in conjunction with a Southern blotting procedure were used to assess telomere length in normal HUVECs at P7, P14 and P21. Briefly, the procedure involved isolating genomic DNA using the DNAzol reagent with accompanying commercial protocol and 2 μg DNA was digested with HinfI and RsaI restriction enzymes according to Roche protocol provided in the TeloTAGGG assay kit. These 2 frequently used cutting restriction enzymes do not cut into the telomeric DNA and subtelomeric DNA due to the special sequence characteristics of the repeats, but they do digest non-telomeric DNA to low molecular weight (MW) fragments. As a result, the Southern blot measures the average

telomere length (that includes both telomeric and subtelomeric DNA) and is the midpoint of the DNA smear which consists of these 2 regions. The digested DNA was separated on a 0.8% agarose gel, from which the DNA was then transferred onto a Hybond-N membrane (Amersham™ Biosciences, Piscataway, NJ, USA). The membrane was probed with a digoxigenin-labeled telomere probe, which was detected with a digoxigenin-specific antibody covalently linked to alkaline phosphatase. This enzyme metabolized a substrate in the detection solution to emit chemiluminescence that was captured on Kodak film.

7. Co-transfection, Detection and Cell Sorting by Fluorescence Activated Cell Sorting (FACS)

HUVECs plated onto Falcon™ 6-well plate (VWR™) were transfected with a commercial Effectene lipid transfection kit (Qiagen™, Mississauga, ON, Canada). Transfection mixtures were prepared with 10 μl of Effectene reagent added to a buffered solution containing 0.4 μg DNA and 3.2 μl enhancer provided in the kit. The pEGFP-NI (Clontech™, Mountain View, CA, Canada) and pNI-Tel vectors were mixed in a 1:2 ratio that comprised the 0.4 μg in the transfection mixture, which was added to the monolayer HUVECs overlaid with 1 ml of OptiMem I medium. After a 3 h incubation period the transfection mixture was removed, the cells were washed 3X with complete M199 medium and re-incubated in the same medium for 2 d prior to detection. Co-transfection efficiency based on EGFP expression was determined by FACS detection and co-transfected HUVECs were concurrently sorted for hTERT-transfected HUVECs at P7, P14, P18 and P21. Telomere length assessment was performed in both normal HUVECs and hTERT-transfected HUVECs at P7, P14 and P21 after 2 d of culture post-

transfection. FACS-sorted hTERT-transfected HUVECs at P18 were used for the matrigel and soft agar assays.

8. Flow-FISH Technique for Telomere Length Assessment

Telomere length in HUVECs was assessed using a modified technique referred to as flow-FISH, a fluorescence *in*-situ hybridization method coupled to flow cytometric detection (40, 53, 54). The advantages of flow-FISH over Southern blot include (1) simplicity, (2) time requirement, (3) sample size, and (4) the use of a fluorescent labeled probe specific to the telomeric region only (53-55). HUVECs were fixed and permeabilized with Cytofix/Cytoperm Kit (BD Pharmingen™, CA, USA) according to a commercial protocol, after which the cells were resuspended in 0.5 ml of hybridization buffer [(70% formamide, 20 mM Tris at pH 7.2, 1% bovine serum albumin (BSA)] containing 0.3 μg/ml $(CCCTAA)_3$-FITC conjugated peptide nucleic acid (PNA) probe (Applied Biosystems™, Foster City, MA, USA) with sequence complementary to only telomeric DNA. This telomere probe solution was pre-heated at 50°C for 10 min before use. HUVECs were resuspended in 0.5 ml probe solution and heated at 87°C for 10 min, at which penetration of the probe and DNA denaturation occurred. The samples were left at room temperature, in the dark, for 3 h hybridization. The cell suspension was repeatedly washed twice with a wash buffer (70% formamide, 10 mM Tris at pH 7.2, 0.1% BSA, 0.1% Tween 20) and once with 1X phosphate buffer saline (PBS). The sample was resuspended in 1X PBS for detection with flow cytometry using a FACS Calibur Flow Cytometer (Becton Dickinson™, San Jose, CA, USA) with channel FL1 set for detection of fluorescein signal. Analysis of 10,000 gated events was performed using a CELL-Quest software (Becton Dickinson™).

Quantum 24PC fluorescent bead solution (Bangs Laboratories™, San Juan, IN, USA) containing 4 subpopulations of beads in which each has a fixed amount of fluorescence measured in molecular equivalence of soluble fluorochrome (MESF). This bead suspension was used for instrument calibration and creation of a standard curve from which the fluorescent intensity of each sample was equated to MESF values, which directly correlate to telomere length. Longer telomeres bind more 5'-CCCTAA-3' specific fluorescent probes corresponding to higher MESF values.

9. Tubular Network Formation on Matrigel

Matrigel-mediated assays are now frequently adopted to evaluate tumor invasion and angiogenesis (56-58). The matrigel matrix, supplied by BD Biosciences, is a soluble basement membrane preparation extracted from Engelbreth-Holm-Swarm mouse tumor enriched with extracellular matrix proteins. The primary component of matrigel is laminin, followed by collagen IV, heparin sulfate proteoglycans and entactin. Matrigel can evoke endothelial tube formation within 24 h providing a means for a rapid assessment of in vitro angiogenesis (58). Matrigel also provides a basement membrane for *in-vitro* cell growth and differentiation (59). Its resemblance to mammalian cellular basement membrane allows evaluation of normal physiological responses to stimuli. Matrigel is therefore an attractive medium to use in the evaluation of normal biological endothelial function following hTERT transfection.

To examine capillary tube-like structure formation, 150 µl of 10 mg/ml matrigel (BD Biosciences™, Bedford, MA) basement membrane matrix was thinly coated onto a Falcon™ 24-well plate (VWR™) and incubated at 37°C for 30 min to promote solidification. Both untransfected and hTERT-transfected HUVECs at P18 were plated

onto the matrigel-coated plate at a cell density of 1 X 10^5/1.88 cm^2 and examined the next day for tubule formation. The degree of tubular network formation was quantitated by counting the number of polygons forming the mesh-like structure in 6 consecutive fields of view at 40X magnification (60). HeLa cells were used as negative controls.

10. Anchorage-dependent Growth in Soft Agar

Anchorage-independent growth in soft agar is often used as a surrogate test for tumorigenicity. Therefore, a soft agar assay was adopted to examine any changes to normal contact-inhibited cell growth. Colony formation of hTERT-transfected HUVECs at P18 was evaluated in soft agar prepared with 200 µl of 1% agarose diluted in 200 µl of 2X M199 medium forming a bottom layer on a Falcon™ 24-well. The top layer of agar contained a suspension of 1 X 10^4 cells mixed with 200 µl of 0.5% agarose diluted in 100 µl of 3X M199 medium. After the agar solidified with embedded hTERT-transfected HUVECs, 500 µl of complete M199 was added to the well and the plate was placed into a 37°C incubator. Concurrently, untransfected HUVECs and HeLa cells were seeded separately in soft agar as controls and cultured respectively in complete DMEM and M199 media. Culture medium was changed every 2 to 3 d.

11. Cell Cycle Analysis with Propidium Iodide (PI)

HUVECs were plated onto gelatin-coated 6-well Falcon™ plates for transfection with Effectene. Transfection mixtures containing 10 µl Effectene, 3.2 µl enhancer and 0.4 µg (hTERT or EGFP) were prepared and added to the HUVEC monolayer. After 3 h of incubation under standard culture conditions, the cell monolayer was washed twice with 1X PBS and trypinized with 0.5 ml of trypsin/EDTA. The cell suspension was transferred to 60 mm gelatin-coated Falcon™ dishes and incubated with 4 ml of complete

M199 for 2 d prior PI staining. Cells were cultured in larger-sized dishes after transfection to prevent growth arrest from contact-inhibition. Cell cycle analysis was assessed by staining for DNA content with propidium idodide (PI) and detecting by flow cytometry. Cells were first fixed in 70% ethanol overnight in -20°C and washed twice with 1X PBS buffer. Cells were resuspended in 500 µl of 1X PBS containing 100 µg ribonuclease A (Sigma™) and left at room temperature for 20 min, after which 4 µl of 10 mg/ml PI solution was added to the cells for 1 h staining prior to FACS analysis. Stained samples were evaluated by FACS with instrument settings at 10,000 events. The percentage of HUVECs in different phases of the cell cycle was determined using a MODFIT software (Becton Dickinson™).

12. MTT Cell Proliferation Assay

HUVECs isolated from 3 different humans were cultured on a 6-well Falcon™ plate and transfected at P18 with either the hTERT or irrelevant EGFP vector. At 2 d post transfection, HUVECs were starved in M199 medium supplemented with 0.5% of FBS (61). After 48 h of serum starvation, the medium was replaced with complete M199 medium regularly supplemented with 20% FBS and re-incubated for 3 d prior to conducting the MTT [3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide] assay. The MTT method was first described by Mosmann in 1983 based on the ability of viable cells to metabolize the yellow-colored MTT (62). Mitochondrial dehydrogenase cleaves the tetrazolium rings of MTT to form insoluble formazan crystals that are impermeable to cell membrane and therefore accumulates within viable cells. Addition of a detergent solubilizes the crystals resulting in a purple-colored solution that was detected by spectrophotometer. An MTT Cell Proliferation Kit (Promega™, Madison,

WI, USA) that included a commercial protocol was used to determine the extent of cell viability and proliferation. Briefly, cells were incubated in 0.5 ml MTT solution for 4 h in a 37°C incubator and washed 3 times with 1X PBS before adding 1 ml solubilization solution and re-incubated for 1 h until spectrophotometric detection at 562 nm. A standard curve relating absorbance to viable cell counts was generated to calculate the actual increase in cell number with respective to sample absorbance at 562 nm. The number of viable cells was directly proportional to the amount of formazan product created.

13. Western Blot Detection of p53 and pRb

Total protein lysates were extracted from HUVECs transfected with the Effectene reagent at P18 with 0.4 μg hTERT DNA vector or an irrelevant vector lacking the hTERT gene (negative control). Total protein was extracted with RIPA (1X PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate) buffer, supplemented with 10 ul/ml PMSF, 30 μl/ml aprotinin and 10 μl/ml sodium orthovanadate, and a 3 h incubation on ice with periodic mixing. Protein extracts analyzed on a Western blot for p53 and pRb gene expression according to the protocol provided by Santa Cruz™ Biotechnology (Santa Cruz, CA, USA). Anti-p53 (DO-1), purchased from Santa Cruz™, is a mouse monoclonal antibody raised against amino acids 11-25 of p53 of human origin. Anti-pRb is also a mouse monoclonal antibody that is raised against the retinoblastoma gene product of 110 kDa. *(Note: Monoclonal antibody is highly specific for only one antigen. It is a single type of highly purified antibody that is directed against a specific epitope (antigen, antigenic determinant) and is produced by a single clone of cells.)* Both monoclonal antibodies were conjugated to

horse radish peroxidase (HRP-conjugated) for direct detection. HUVEC extracts containing 35 μg of total proteins, along with a Santa Cruz™ molecular weight marker, were ran on a 7.5% polyacrylamide gel and transferred onto a hybond-P membrane (Amersham™ Biosciences). Prior to antibody detection, protein loading was checked by staining the membrane in Coomassie blue R250 for 5 min and rinsed in several washes of 100% methanol until the bands became distinct. The membrane was further rinsed in methanol to remove remnants of the Coomassie blue (BioRad™ Laboratories, Hercules, CA, USA) stain and a final rinse in double distilled water before blocking. The membrane was blocked for 1 h with blocking reagent (1X TBS, 5% non-fat dried milk, 0.05% Tween-20) at room temperature and then probed with 1:100 dilution of primary p53 antibody or 1:100 dilution of pRb antibody for 1 h. The membrane was washed three times for 10 min each with wash buffer (1X TBS and 0.05% Tween-20) and a final wash in 1X TBS before detection. Chemiluminescence detection was accomplished with Luminol reagent (Santa Cruz™) containing the substrate for the HRP enzymatic reaction and the emitted chemiluminescence was captured on Kodak film.

Another mouse anti-human pRb monoclonal antibody purchased from BD Pharmingen™ (Mississauga, ON, Canada) was tested on Western blot. This pRb antibody recognizes an epitope located between amino acids 300-380 of human Rb with molecular weight of 110-116 kDa. The primary pRb antibody was used at a 1:100 dilution for 1 h incubation at room temperature and the blot washed three times. The blot was subsequently incubated with a secondary goat anti-mouse HRP-conjuated antibody (Santa Cruz™) at a 1:1000 dilution for 45 min and detected with Luminol reagent.

14. Statistical Analysis

Results were expressed as a mean and standard error of the mean (±SEM). Experimental data collected from assays examining HUVECs at the 3 different passages (P7, P14, P21) were analyzed using ANOVA followed by a post-hoc Tukey test if the overall $p<0.05$. The Tukey test is a post-test to compare all pairs of means to determine between which pair of means does the statistical difference lie. A value of $p<0.05$ denotes a significant difference between experimental groups. Student's t-test was used to statistically compare results obtained from hTERT-transfection with the untreated control group.

IV-C) RESULTS

1. Morphology

As HUVECs aged *in vitro* due to continuous subculturing from P2 to P20, they gradually lost their normal "cobblestone" appearance, starting at a low incidence at P7. These senescent HUVECs acquired a more flattened and elongated morphology (Figure 4-2). The large flattened morphology is contributed by a loss of cytokerin 7 in senescent cells, that causes destabilization of the cytoskeleton (63). Senescent HUVECs were further characterized by being multinucleated and displayed vacuolization as well as spindly cytoplasmic protrusions in culture. The cell monolayer was populated by cells heterogeneous in both size and shape. As passage number was approaching the Hayflick limit, HUVEC cultures could not easily reach confluence even with adequate growth provisions.

2. Absence of wound healing in senescent HUVECs

Wound healing of a disrupted cell monolayer was more rapid in early-passage (≤P11) as opposed to late-passage (≥P22) HUVECs. Recovery from the wound would normally require both cell proliferation and migration. The wounded area was completely healed in early-passage HUVEC cultures in all 5 replicate experiments while healing was absent on the monolayer of late-passage cells (Figure 4-3). Younger cell cultures seemed to thrive more due to a higher proliferative index, whereas a lack of proliferation and migration was apparent in senescent HUVEC cultures. Cells near the periphery of the wound seemed to retract or die off due to a loss of cellular contact and communication. HeLa cells also showed rapid wound recovery indicative of a high proliferation rate. HeLa cell clusters were evident on the cell monolayer due to rapid and contact-independent growth characteristics of cancer cells. To the contrary, growth appeared arrested as a result of contact-inhibition in the normal HUVECs at P11 after the wound was repaired.

3. Fraction of senescent cells increased with *in vitro* age

Normal HUVECs at increasing passage numbers yielded a larger fraction of SA-βgal stained cells indicative of cell aging *in vitro* (Figure 4-4). SA-βgal activity was assessed in 5 donor HUVEC strains, each at P7, P14 and P21 which showed the highest fraction of senescent cells. The fraction of senescent cells at P21 was 62±4%, which was significantly larger than cultures stained at both P14 (**$p<0.001$) and P7 (*$p<0.001$). The percentage of SA-βgal positive cells at P7 was 25±3% which was comparable to that of P14 showing 36±4% senescent HUVECs ($p>0.050$). Overall however, HUVEC cultures at all 3 different passages had significantly higher numbers of senescent cells

that the HeLa cell culture ($p<0.001$). The percentage of HeLa cells that demonstrated senescence was 7±2% (n=31) and this fraction did not change with continuous subculturing. The proportion of HeLa cells that stained positive for SA-βgal remained at 3% to 11% over all time points with continuous culturing. In conclusion, HeLa cells did not display *in vitro* aging indicative of immortality, whereas normal HUVECs demonstrated cell senescence with aging.

4. Telomerase activity in hTERT-transfected HUVECs

Both untransfected and heat-inactivated hTERT-transfected HUVECs yielded negligible levels of telomerase activity compared with hTERT-transfected cells (Figure 4-5). Heat denatured the holoenzyme required for activity. Telomerase activity was negligible at 3 h post transfection as illustrated by a low yield of mean fluorescence of 1913±81 AU (arbitrary unit of fluorescence), which was similar to those of the untransfected and heated controls. A mean fluorescence of 13,646±1715 AU was first detected at 1 d after hTERT transfection of HUVEC cultures. Activity of telomerase peaked on day 2, as represented by maximum fluorescence of 18,820±3413 AU, and gradually declined thereafter due to transient gene expression. The positive HeLa control cells yielded a mean fluorescence of 24,597±3203 AU resulting with the highest level of telomerase activity, yet comparable to the peak activity found in hTERT-expressing HUVECs at 2 d post-transfection ($p=0.285$).

5. Declining telomere length in normal aging cells

Telomere length was addressed in HUVECs when cultures reached P7, P14 and P21. Both the Southern blotting and flow-FISH techniques were utilized in the assessment of telomere length from 5 donor isolates (Figure 4-6A). Results acquired

from both methods were compared and used to establish a correlation between lengths measured in fluorescence units (flow-FISH) to base pair equivalence. Telomere length obtained in fluorescence unit was equated to actual base pair using this equation: $y = 0.044x + 3.8$ (Figure 4-7). The y-intercept of 3.8 kbp corresponds to subtelomeric DNA. This correlation would be important for small sample size where the Southern blot technique is not applicable.

A proportional decline in telomere length was found in normal HUVECs from cultures of increasing passages. A decrease from 6.0 kbp at P7 (n=5) to 3.4 kbp at P14 (n=5) and to 1.3 kbp at P21 (n=3) virtually at a point of senescence (Figure 4-6B). Significant telomere loss of 4.7 kbp was observed between P7 and P21, as HUVECs progressively aged *in vitro* (p=0.014). One donor HUVEC strain reached the Hayflick limit at P16 and another strain had not yet approach P21 at the time of assessment therefore, only 3 HUVEC isolates were evaluated at P21. Mean TRF value for HUVECs at senescence (P21) was 5.1 kbp (3.8 kbp + 1.3 kbp), a length similar to that reported by Yang et al. and Hastings et al. (28, 64). Evidently, telomere shortening occurred with aging in human endothelial cells.

6. Telomere length extension after hTERT transfection in HUVECs

To counteract telomere shortening with age, HUVECs were transfected with the hTERT catalytic subunit at P7, P14 and P21 cell cultures using Effectene lipofection. The mean efficiency of co-transfection, based on EGFP reporter expression, ranged from 11% to 14%, with the highest percent gene transfection in P7 HUVEC cultures (Figure 4-8). Despite aging *in vitro*, gene transfer efficiency did not seem to be affected in older cell cultures that were transfected to a similar extent. No significant decrease in mean

efficiency of transfection was observed from early to late-passage cells (p=0.620), indicating that Effectene lipofection is equally effective for gene delivery into both young and old cells. The appearance of hTERT-transfected HUVECs was similar to those transfected with a control vector.

The transient induction of telomerase activity in Effectene-transfected HUVECs led to telomere extension at all 3 passages examined (Figure 4-9). Although telomeres were elongated, the number of base pairs added to telomeric ends were insignificant between the hTERT-transfected versus control cells at P7 (p=0.754), P14 (p=0.641) as well as P21 (p=0.825). Telomeres of HUVECs, assessed after 2 d of culture post-hTERT transfection, increased by a mean length of 540 bp at P7, 320 bp at P14 and 240 bp at P21. In early-passage HUVECs expressing hTERT, their telomeres did not appear to be significantly elongated compared to the telomeres of late-passage HUVECs (p=0.162). HUVECs were reported to have an average population doubling time of approximately 2 d, which means elongation occurred between a rate of 240 bp/PD to 540 bp/PD (65) Given that HUVECs lose telomeric repeats at a constant rate of 90 bp/PD, the addition of 240 bp to 540 bp of telomeric DNA in hTERT-transfected HUVECs represented a gain of 3 to 6 extra population doublings for hTERT-transfected HUVECs at P21, P14 and P7 respectively (65). In spite of elongation by just a few hundred nucleotides, telomeres were, nonetheless, preserved by the induction of transient telomerase activity in normal HUVECs.

7. Normal functional phenotype retained in matrigel

A distinct normal phenotype of endothelial cells is that they form blood vessels *in vivo* and show tubule formation *in vitro* in matrigel basement membrane. HUVECs

formed interconnected tube-like extensions in matrigel after a 24 h culture. Both untreated and hTERT-treated HUVECs demonstrated vessel sprouting, whereas HeLa cells showed no such capacity (Figure 4-10A). Clonal expansion of HeLa cells in matrigel was instead observed. As expected, normal P7 cultures were capable of forming more tube-like extensions than HUVEC cultures at P18 in which a larger fraction of SA-βgal stained cells would be present. Interestingly, hTERT-transfected endothelial cells showed a significantly enhanced ability in tubule formation compared to normal untransfected HUVECs at P7 ($p=0.015$) and P18 ($p=0.026$). At P7, the numbers of polygon shapes counted on the mesh-like structure were 22±4 and 38±4 for the negative controls and hTERT-transfected HUVECs respectively (Figure 4-10B). Fewer polygons were counted for the control and transfected cells in senescing cultures (P18). While HUVECs expressing hTERT yielded 26±4, untransfected HUVECs had a significantly lower number of polygons (15±3) formed in matrigel. Since telomerase has been shown to have growth promoting capabilities other than telomere maintenance, the enhanced tubule formation of hTERT-expressing HUVECs may be attributed to the potential effects telomerase has on growth-promoting genes (66).

8. Rapid cell recovery from serum starvation

HUVECs transfected with hTERT showed remarkable cell recovery from a 48 h period serum starvation than cells transfected with an irrelevant EGFP vector. The MTT assay revealed a higher mean absorbance measured in hTERT-treated cultures than cultures expressing EGFP at 3 d post serum replacement. In order to equate absorbance values to actual cell number, a standard curve was generated with different cell densities and their respective absorbance at 562 nm (Figure 4-11A). Cell proliferation in hTERT-

expressing HUVECs was significantly higher than that quantitated in the irrelevant control cultures (p=0.043). Cell number in hTERT-transfected cultures increased approximately five times compared with the EGFP control cultures (7051 cells versus 1474 cells) following serum replacement and cultivation for 3 d (Figure 4-11B).

A study conducted by Endemann et al. has raised concerns regarding potential toxicity associated with the use of pEGFP vectors (67). These authors found the pEGFP-C2 (Clontech™) vector construct to be hazardous to cell viability due to a C-terminal peptide sequence within the multiple cloning sites (MCS). To eliminate concerns regarding any potential toxicity associated with the pEGFP-NI vector, this subsequent experiment evaluated cell proliferation after serum starvation between EGFP-transfected HUVECs and untransfected cells. Comparable proliferative indices were found, with cell numbers of 23,825 and 17,788 in EGFP-treated and untreated HUVECs respectively (p=0.322, Figure 4-11C). Apparently the green fluorescent protein, isolated from bioluminescent jelly fish and normally absent in mammalian cells, did not produce any toxicity in human endothelial cells.

9. Normal contact-inhibited cell growth in hTERT-transfected HUVEC culture

Although expressing telomerase activity, transfected HUVECs did not display any abnormalities in growth pattern, such that these cells retained contact-inhibited cell growth. No colony formation was observed in HUVECs expressing hTERT suggesting that cell growth remained anchorage-dependent (Figure 4-12). Similarly, untransfected HUVECs also did not form colonies in soft agar. All HUVEC cultures were followed for as long as 6 wks and still no colonies were established during this period. Unlike normal somatic cells, HeLa cancer cells started showing colony formation at 1 wk post seeding

in soft agar. HeLa cell colonies grew in number and size over the 6 wks culture period indicative of anchorage-independent cell growth, a hallmark of transformed cells.

10. Normal cell cycling in hTERT-transfected HUVECs

No significant changes were observed in the cell cycle profile of HUVECs transfected with hTERT compared with the untransfected or those transfected with the irrelevant EGFP vector (Figure 4-13). The percentage of HUVECs in S-phase was similar in all 3 groups ($p=0.371$). In addition, hTERT transfection did not cause any significant alterations to the percentage of cells cycling in the G0-G1 phase either ($p=0.616$). A decreasing trend was found in the proportion of cells in S-phase, albeit insignificant, upon subculturing (P7 to P21) as a reflection of *in vitro* aging in normal diploid cells, leading to a corresponding increase in the fraction of G0-G1 cells. The percentages of hTERT-transfected, EGFP-transfected and untreated HUVECs in S-phase ranged from 10-17%, 11-19% and 12-18% respectively from cultures examined at P7 to P21. In contrast, a significantly larger mitotic fraction (30±1%) of immortalized HeLa cells was observed in S-phase compared to normal cycling HUVECs ($p=0.010$). Nonetheless, hTERT treatment did not lead to abnormal cell cycle pattern and hTERT-transfected HUVECs apparently retained a normal growth phenotype.

11. Tumor suppressors p53 and pRb expressions in hTERT-transfected HUVECs

Protein lysates examined from 3 HUVEC isolates (strains) transfected with hTERT (T1-T3) showed higher p53 expression than the negative controls not transfected with the hTERT gene. Although similar amounts of proteins were loaded (Figure 4-14A) hTERT-transfected HUVECs, particularly donor strains 1 and 3 (T1 and T3) had more

p53 proteins whereas strain 2 (T2) yielded comparable amounts to its corresponding control (N2, Figure 4-14B).

The retinoblastoma gene product could not be detected using 2 mouse monoclonal pRb antibodies purchased from Santa Cruz™ and Pharmingen™. However, pRb was detected in Jurkat cell lysates (Pharmingen™), serving as a positive control, using the Pharmingen™ mouse monoclonal pRb antibody. pRb was detected in 5 μg, 7 μg and 10 μg of loaded Jurkat protein extracts (Figure 4-14C). With a loading of 35 μg of HUVEC protein lysates, antibodies from both suppliers failed to detect pRb in all HUVEC samples examined. This under detection of pRb could be explained by culture confluence which prevented the contact-inhibited growth of HUVECs from entering S phase due to an absence of Rb phosphorylation and, as a result, pRb stays sequestered. Especially in cultures approaching senescence, elevated levels of kinase inhibitors in senescing cells keeps pRb in a dephosphorylated state so its activity remains dormant, and therefore cannot be easily detected with a procedure (Western blot) that lacks high sensitivity compared to PCR methods.

IV-D) DISCUSSION

It is the shortest telomeres that attract telomerase (68). This preferential recruitment of telomerase activity by cis-acting telomere binding proteins to the shortest telomeres assists in maintaining their length while longer telomeres get shortened. Therefore cell senescence, found to be triggered by the shortest rather than the average length of telomeres, can be bypassed with hTERT transfection (69). Although telomeres in hTERT-transfected human fibroblasts were shorter than would normally be tolerated,

they remained replicative (68). This observation, in part, is due to a capping function of telomerase that is independent of its catalytic role in telomere elongation (32, 68, 70). Telomere capping protects chromosomal ends from recombination, fusion and degradation. As demonstrated in our study, transfection of HUVECs with the hTERT catalytic subunit of telomerase resulted in telomere maintenance. Both Bodnar et al. (29) and Yang et al. (28) illustrated extension of replicative life span in normal somatic cells stably expressed hTERT through the preservation of telomere length (28, 29). These authors utilized retroviral transduction and evaluated the effects of long-term telomerase expression on telomere length, while our study reported the immediate effects of transient telomerase activity on telomeres shortly after gene transfection. They reported the size of telomere restriction fragments (TRF) as a representation of telomere length, whereas we reported the length of just the (TTAGGG) repeats using the flow-FISH technique. Unlike Bodnar and Yang who focussed very little on HUVECs, in contrast, our study used HUVECs as an *in vitro* cell model.

Both short and long telomeres could be elongated by ectopic telomerase activity. Long telomeres are equally dynamic and eligible for extension. The only determinant preventing elongation of long telomeres is limiting telomerase activity. Telomeres switching between a telomerase-extendible versus a -non-extendible state is a function of telomere length (71). Telomerase being recruited to the shortest telomeres under limiting expression is inherently a survival signal (68, 72). If short telomeres are not recognized and maintained, their progressive shortening would have resulted in DNA damage checkpoint activation and senescence despite telomerase acting on longer telomeres. Logically, it would have to be the shortest telomeres that are elongated, since the longer

counterparts have sufficient buffer DNA reserve to undergo shortening. The signal for telomerase recruitment is potentially mediated by the extent of telomere-binding proteins associated with telomeres that provides feedback on telomere length. Under copious telomerase activity, long and short telomeres are equally capable of transiting between the 2 states encompassing telomerase-extensibility versus –non-extensibility. Cristofari et al. recently proposed a third state in which transition between a telomerase-extendible and an extending state depends on telomerase concentration (73). Being in this third state results in telomere elongation. We found that neither telomerase activity nor *in-vitro* age of HUVECs dictated the degree of telomere length extension. However, there seems to be a trend, albeit insignificant ($p=0.162$), towards greater telomere extension in early-passage HUVECs (P7) expressing hTERT. Longer telomeres in early-passage cells probably facilitate an easier fastening of telomerase onto telomeres and subsequent activity. Even a very small average telomere elongation by telomerase resulted in a 50% increase in the cellular lifespan as shown in human BJ fibroblasts (72). In fact, Ouellette et al. demonstrated that hTERT transfection had resulted in extension of cellular life span even though telomeres were not elongated (32, 74). This observation suggests that hTERT activity alone, in the absence of telomere extension, is favorable to cell survival.

HTERT-transfected HUVECs in our study only showed transient telomerase activity with plasmid DNA transfection. Telomerase activity peaked on day 2 post-transfection and gradually fell over a 5 d period. Nonetheless, telomerase activity of day 2 was comparable to that observed in HeLa cells known to constitutively express high levels of telomerase to maintain immortality ($p=0.285$) (70). The rapid decline in enzymatic activity is due to the nonviral vectors not being integrated into the human

genome. During the period of telomerase activation, telomeres were elongated to a small extent. On the contrary, retroviral vectors yielded stable telomerase activity that was observed for >100 PDs in human fibroblasts (29). Aside from the inherent biological risks associated with retroviral vectors, the caveats of long-term stable telomerase expression are increased cell proliferation and survival (anti-apoptotic) as observed in cancer cells rendering plasmid vectors more appealing (66, 75, 76). Stem cells and immune cells also possess telomerase activity, but they too, underwent telomere shortening, albeit at a slower rate (23, 27, 77). Endogenous telomerase activity in early-passaged HUVECs (up to P3) observed by Hsiao et al. was soon lost with continuous subculturing *in*-vitro (78). However in our study, HUVECs of higher passage numbers were used and endogenous telomerase activity was negligible.

As opposed to many studies that have evaluated senescence in terms of protein expression, aneuploidy, apoptosis and oxidative state in aging cells (2, 4, 8, 9), we have examined the biological responses to injury from young and old HUVECs. The ability to form capillary-like tubes on matrigel was also assessed in this study. We observed a reduced angiogenic ability in forming vessel-like tubules from aging HUVECs, but increased tubule formation was found in HUVECs expressing hTERT. Furthermore, the responsiveness of older HUVEC cultures was dampened and the wounded monolayer could not be healed compared to younger cultures (Figure 4-3), due to defective angiogenesis in senescent ECs. Angiogenesis, function as part of the wound healing process, involves a complex cascade of events beginning with growth factor production and secretion, followed by vessel sprouting, basement membrane dissolution, and finally vascular tube formation and differentiation. Both EC-to-EC and EC-to-ECM interactions

are crucial to the activation of intracellular/intercellular signaling pathways regulating cell proliferation, survival, differentiation and apoptosis (79). In angiogenesis, EC activation also promotes ECM degradation in combination with sprouting and migration of ECM from existing capillaries. The binding of ECM receptors with integrins on activated ECs regulates the formation of capillary-like tubes (80). Integrins serve as a tether, as well as having an information transfer function. This integrin-ligand binding triggers reorganization of the cytoskeleton at specific sites on the cell membrane to either facilitate cell movement or tissue stability. Adhesion molecules (i.e. PECAM-1, VE-cadherin), in association with the actin cytoskeleton and vimentin filament network, redistribute themselves to the border of monolayer cells to mediate cell-to-cell contact and establish direct intercellular communication (81, 82). Gap junctions, which are intercellular membrane channels are also formed, via the noncovalent interactions between the structural protein connexin 43 (cx43), of adjacent cells (83). These channels permit the intercellular exchange of small molecules, ions and second messengers. In senescent HUVECs however, these cell-to-cell and cell-to-matrix interactions are disrupted. Redistribution of intercellular adhesion molecules on cell surface was observed (84). For example, β-catenin proteins accumulated at regions of cell-to-cell contacts, which may decrease motility of senescent HUVECs. Xie et al. detected decreased levels of cx43 mRNAs and proteins as HUVECs aged *in vitro* (85). Furthermore, these authors reported amplified levels of IL-1α mRNAs in senescent HUVECs (86). IL-1α, an inhibitor of endothelial cell proliferation, suppressed gap junction activity that is required for angiogenesis (87). In addition, this inflammatory cytokine promotes tissue degradation by stimulating the production of matrix

metalloproteinase (MMPs; i.e. collagenase and stromelysin), as well as inhibiting tissue repair (88). IL-1α also alters the cytoskeleton by spatially distributing the stress fibers throughout the cytoplasm, which differs from an arrangement of fibers with polarity found in migrating cells (89). The depolymerization and repolymerization of the actin cytoskeleton are affected in senescent cells leading to defective lamellipodium formation, and thereby reducing cell movement. Given the altered expression profile of surface adhesion molecules and modification of the cytoskeleton, changes in cell morphology at senescence were inevitable. In addition to the irreversible growth arrest, a senescent phenotype includes alterations in endothelial gene expression and activation of tumor suppressors guarding cell cycle progression.

Telomerase activity in normal somatic cells is repressed through post-transcriptional modification of the hTERT mRNA (23, 31, 77). The alternate splicing mechanism creates nonfunctional hTERT transcripts as a means of dominant repression in normal cells. In gene transfection however, plasmids containing intronless cDNA do not activate the intrinsic splicing machinery in normal cells and therefore, exogenous telomerase activity is induced (90). The half-life of this active protein in human cells was shorter than that observed in cultured mouse cells having a half-life of 36 h and 72 h (31). The tight regulation of telomerase activity in human cells is associated with telomere-implicated replicative senescence and to guard against tumorigenesis (23, 91). Even though hTERT-transfected endothelial cells recovered much quicker than untransfected cells from serum starvation ($p=0.043$), this may only be due to a transient increase in proliferative rate rather than a malignant phenotype as illustrated by the absence of colony formation in soft agar. It was expected that colonies would not established from

hTERT-expressing HUVECs given that Effectene-mediated lipofection resulted in only transient expression. Even stable, long-term expression of hTERT in HUVECs in other studies did not lead to tumorigenic transformation (92). Given that rare cells immortalize at a frequency as low as 10^{-7}, the probability of finding immortal clones from examining 3 hTERT-transfected donor strains would be astronomically low (93). The transience of vector expression in HUVECs and the multiple mutations required to achieve tumorigenicity preclude the ability of telomerase alone to form tumors *in vitro* and similarly *in vivo* as well.

The concomitant loss of function of Rb and p53 is a critical step to tumorigenesis (46, 94, 95). To substantiate findings in other studies that endothelial cells cannot be easily transformed with telomerase alone, the levels of pRb and p53 were examined on Western blot with cell extracts prepared from untransfected and hTERT-transfected HUVECs. Expression of these tumor suppressors indicates intact cell cycle checkpoints that would normally be disrupted in transformed cells (94, 95). We found that endogenous p53 expression seemed intact, but increased p53 levels were observed in hTERT-transfected HUVECS compared to their respective controls. With plasmid transfection, many copies of the gene vector can be taken up by the cells resulting in hTERT overexpression. Between 2000 and 10,000 copies of plasmids are delivered per cell using lipofection (96-98). Because hTERT is normally not expressed in most human diploid cells, natural mechanisms must exist to suppress hTERT transcription and p53 has been shown by others to mediate that suppression (99, 100). Thus, p53 overexpression observed in hTERT-transfected HUVECs may be an attempt to downregulate hTERT promoter activity. However, hTERT was under the control of a

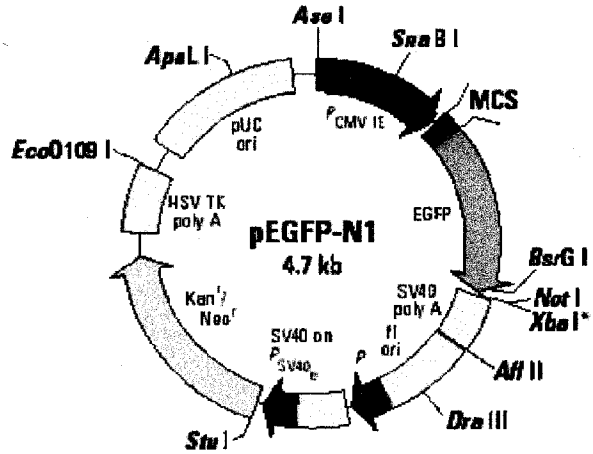
pCMV promoter in the pNI-Tel vector, hence action of hTERT on telomeres in transfected HUVECs was not affected as hTERT transcription could not be repressed. The observations here imply that hTERT expression must reach a certain critical threshold for detectable activity due to the existence of natural tumor suppressor mechanism against immortalization (101, 102).

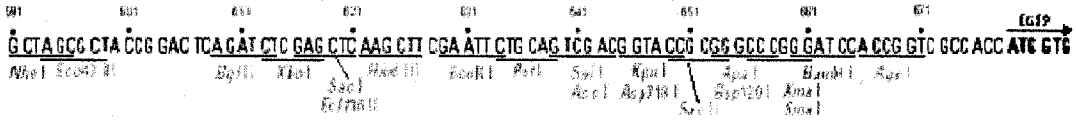
With regards to the other tumor suppressor protein, pRb's expression, or lack thereof, can be explained by confluent cell cultures. Studies by both Jiang et al. and Morales et al. showed extremely low to undetectable pRb expression from protein lysates extracted from confluent cultures (47, 103). Furthermore, phosphorylated pRb status in hTERT-expressing BJ fibroblasts was null from confluent cultures too. pRb detection on Western blot remains difficult even in subconfluent cultures. All HUVEC cultures in our study, transfected and controlled, reached high cell density following a 2 d culture period post-treatment, which explains the undetectable pRb expression on Western blots. The level of pRb proteins was most likely downregulated at confluence as previously reported by Jiang et al. (103). pRb negatively regulates entry into S phase by sequestering transcription factors, such as E2F, necessary for the G1/S phase transition (104, 105). Hyperphosphorylation of Rb, by cyclin-dependent kinases (CDKs), is required to abolish this sequestration in order to activate E2F and allow cell cycle progression (106). Elevated expression of CDK inhibitor p16 in senescent cells causes G1 cell cycle arrest by specific inhibition of the CDKs (i.e. CDK 2, CDK 4, CDK6), and thus prevents Rb hyperphosphorylation that is required for S phase entry in mammalian cells. Wagner et al. (6) reported a significant accumulation of hypophosphorylated Rb in senescent HUVECs suggesting cell cycle arrest in the G1 phase due to an abundance of CDK

inhibitors. Furthermore, Wagner et al. also observed increased levels of other CDK inhibitors, p21 and p27, with increasing passage numbers (6). The induction of p21 is mediated by p53 and p21 causes Rb hypophosphorylation (107). Therefore, engagement of the p53 pathway in cell senescence also, in turn, engages the Rb pathway. It is reasonable to presume that levels of pRb from the treated and control groups were unaltered based on previous studies on hTERT-transduced cells (47, 87, 103).

In conclusion, the findings from our study showed that normal endothelial cells underwent cell senescence with subculturing and resulted in telomere shortening with age. Aging HUVECs were slow to respond to stress compared to younger cells. Senescent cultures also demonstrated slower rates of proliferation in the absence of telomerase expression. Telomerase transfection imparted positive effects on cell survival without altering cell cycle progression and causing malignant transformation. With human telomerase gene therapy, it is of the utmost importance to balance the scale between cell senescence and tumorigenesis in order to achieve desirable therapeutic significance. Further examination of the role of telomerase in bypassing senescence and extending replicative life span will be assessed in future studies with human tissues and animal model. The ultimate goal is to be able to genetically modify allografts, either *in situ* or *ex vivo*, prior to transplantation to create more robust grafts with the hopes that long-term allograft survival could improved significantly.

A.





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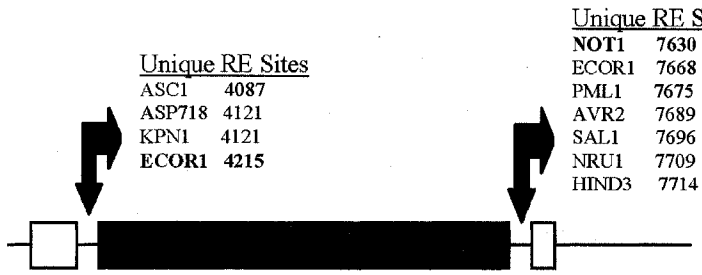

Figure 4-1: (A) Restriction Map and Multiple Cloning Site of pEGFP-NI (Clontech™, CA), with unique restriction sites labeled in orange bold color (bottom). **(B)** The hTERT gene sequence (3.4 kbp) is flanked by multiple restriction enzyme sites. The hTERT gene (red) was directly inserted into the pEGFP-NI empty vector, already with the EGFP gene first removed, utilizing the EcoRI and Not1 (black bold) restriction enzymes followed by a ligation reaction thereby creating a new recombinant vector, pNI-Tel.

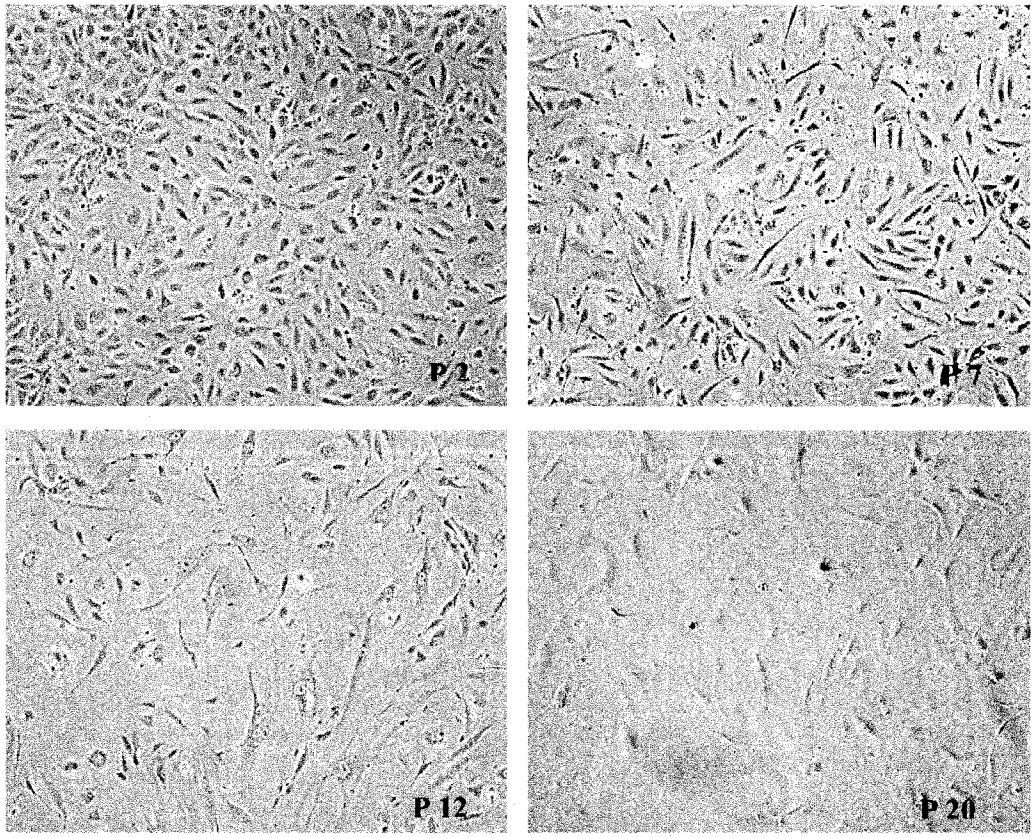

Figure 4-2: *In vitro* cell senescence of HUVECs with continuous subculturing. As the passage number increased HUVECs started to lose the normal "cobblestone" morphology and high cell density. Late-passage HUVECs were larger in size, multinucleated and more spindly in appearance. (original maginification x 100)

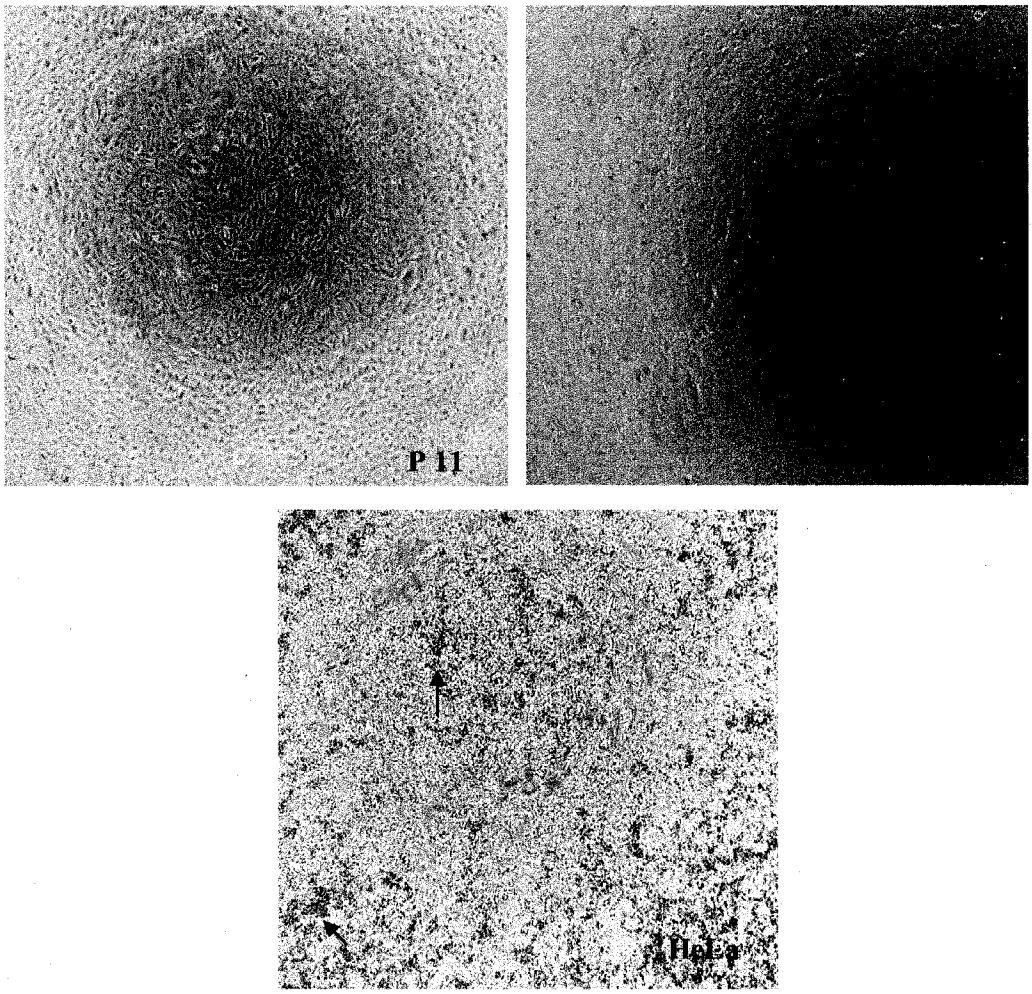

Figure 4-3: The HUVEC monolayer was wounded at P11 (early) and P22 (late). Cell recovery was observed in P11 HUVECs wheres P22 endothelial cultures did not show any recovery from the wound. HeLa cells also showed a completely healed wound on the cell monolayer. HeLa cells also displayed clustering (black arrows) on the monolayer. A representative well for each group is shown. (original magnification x 40)

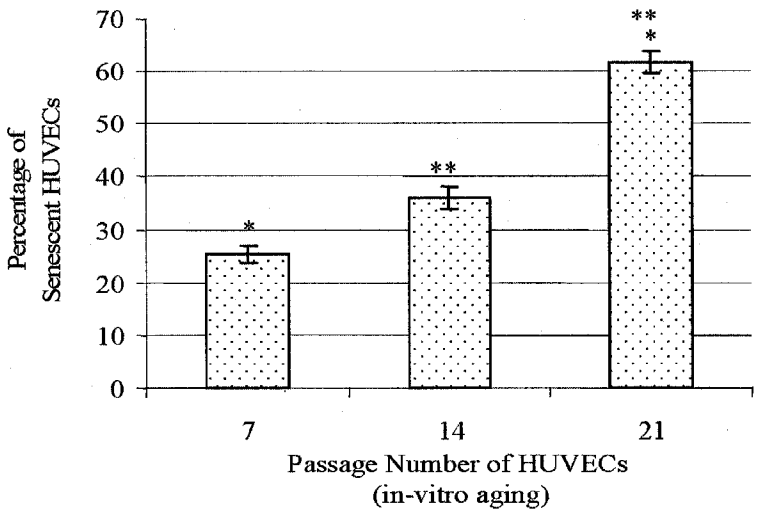

Figure 4-4: Increased fraction of senescent HUVECs was observed with continuous subculturing. The number of senescent cells between cultures of P7 and P21 (*$p<0.001$), and between P14 and P21 (**$p<0.001$) were significantly different as determined by SA-βgal staining.

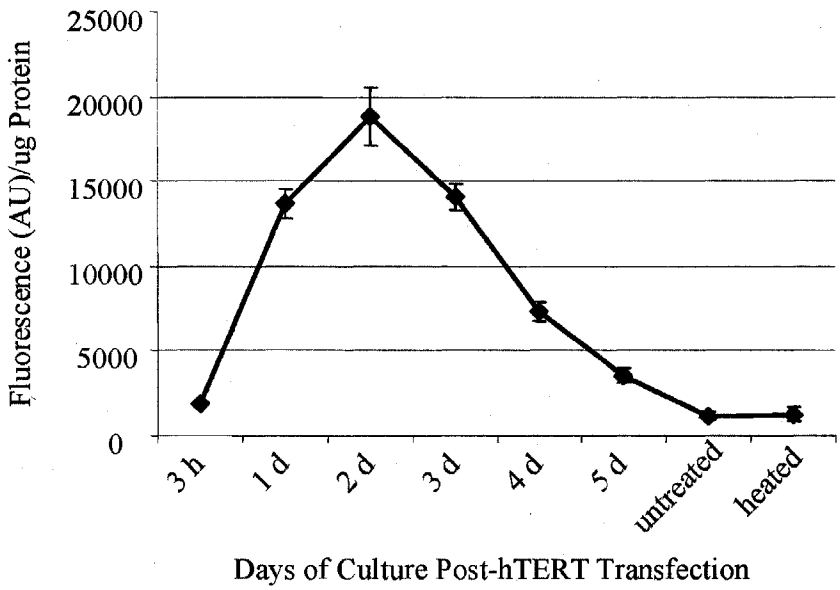

Figure 4-5: Telomerase activity in 1 μg of total protein lysate extracted from transfected HUVECs (n=4) was examined using the TRAP assay and resultant fluorescent intensity (arbitrary unit) is proportional to enzyme activity. Telomerase activity peaked on day 2 and declined thereafter. Untransfected HUVECs showed negligible activity and heated controls were also negative for telomerase activity as the enzyme was denatured by heat.

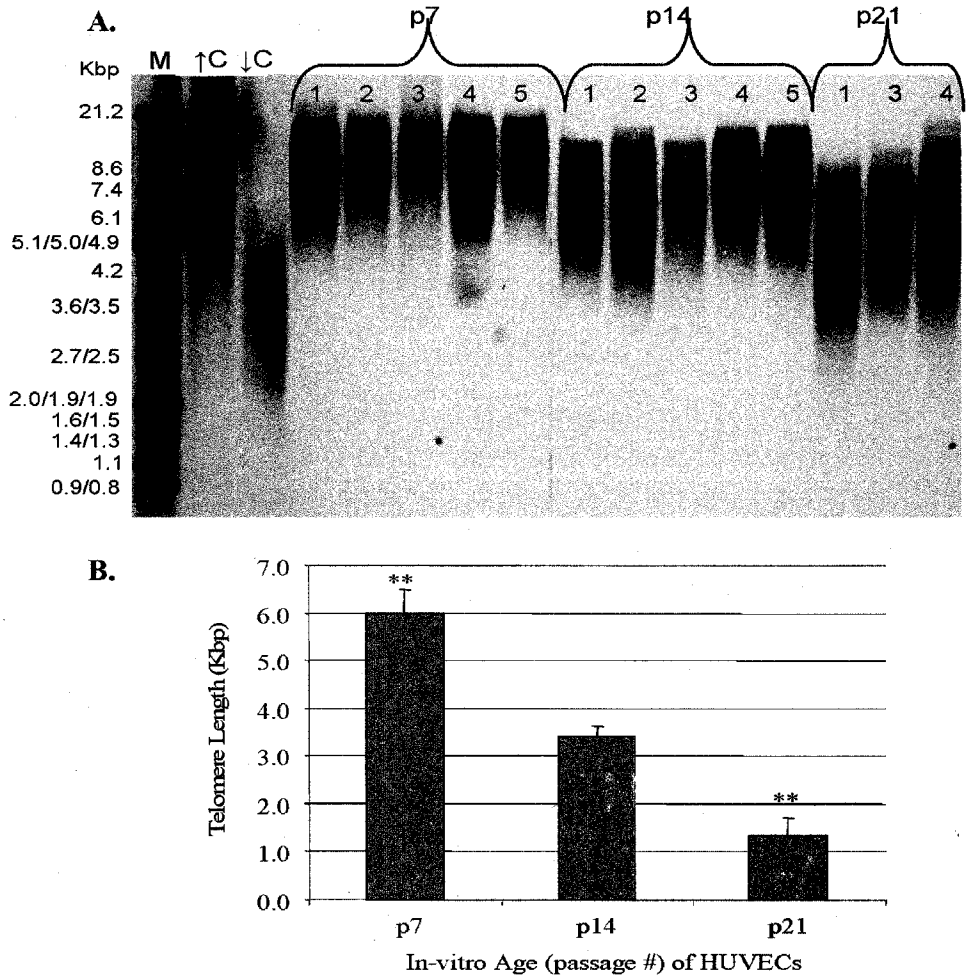

Figure 4-6: Both the **(A)** Southern blot and **(B)** flow-FISH technique used for telomere length assessment in HUVEC cultures of P7, P14 and P21. **(A)** The donor HUVEC isolates used were identified numerically at the top of the blot for each passage number. The midpoint of each DNA smear, indicative of average telomere length, declined with aging. (M = DNA MW marker, ↑C = high DNA MW control, ↓C = low MW DNA control) **(B)** Telomere length decreased from 6 kbp at P7 to 3.4 kbp at P14 and 1.3 kbp at P21. Significant telomere loss was observed between P7 and P21 (**p=0.014).

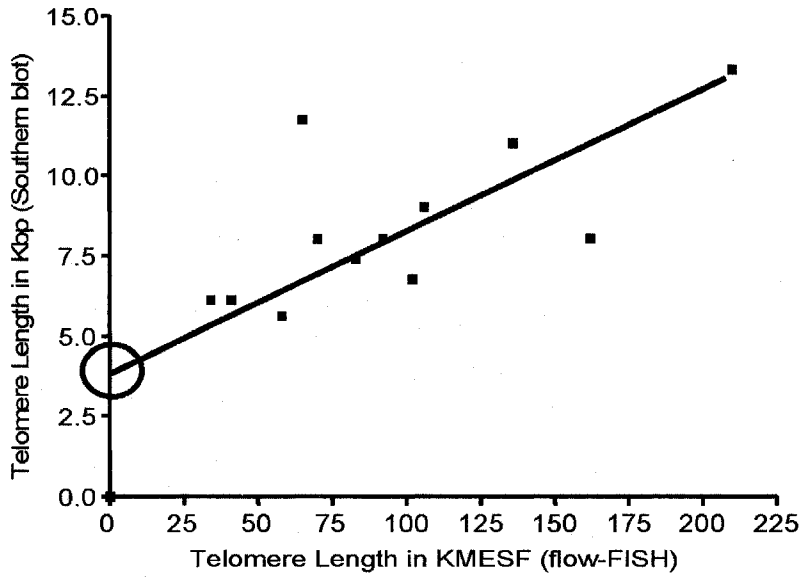

Figure 4-7: This correlation curve (r^2=0.5718) was generated from experimental data on telomere length collected from 2 assays, the Southern blot and flow-FISH methods. The y-intercept of 3.8 kbp corresponded to subtelomeric DNA and telomeric DNA in arbitrary fluorescence unit measured by the flow-FISH method was converted to bp length using the equation y=0.044x.

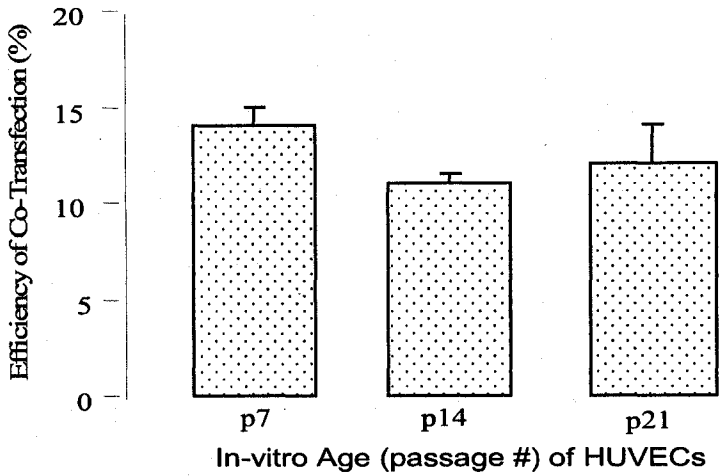

Figure 4-8: Efficiency of EGFP:hTERT (1:2 ratio) co-transfection of HUVECs at P7, P14 and P21. Efficiency ranged from 11-14%. No significant difference in transfection efficiency was observed at the 3 different passage numbers (p=0.620).

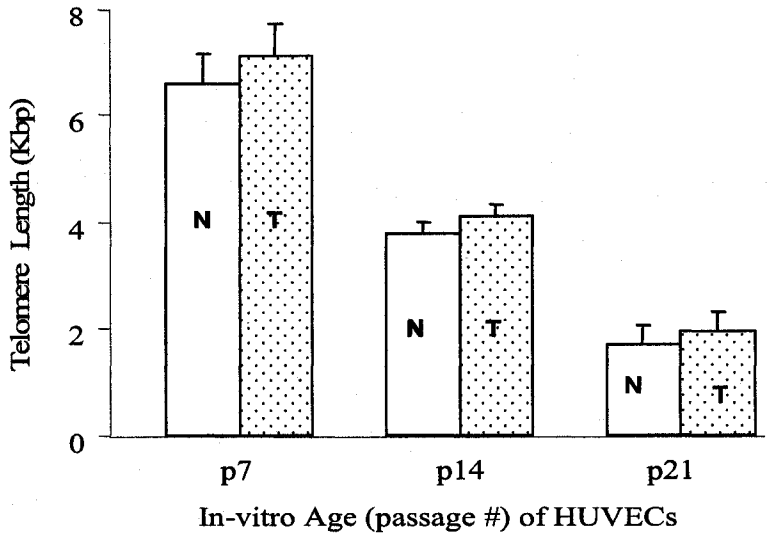

Figure 4-9: Telomere length assessment 2 d post-hTERT transfection of HUVECs at P7, P14 and P21. Although not a significant extension, the length of telomeres showed an increase by an additional 540 bp at P7 (p=0.754), 320 bp at P14 (p=0.641) and 240 bp at P21 (p=0.825) compared with their respective negative controls. (N=untreated negative controls, T=hTERT-transfected)

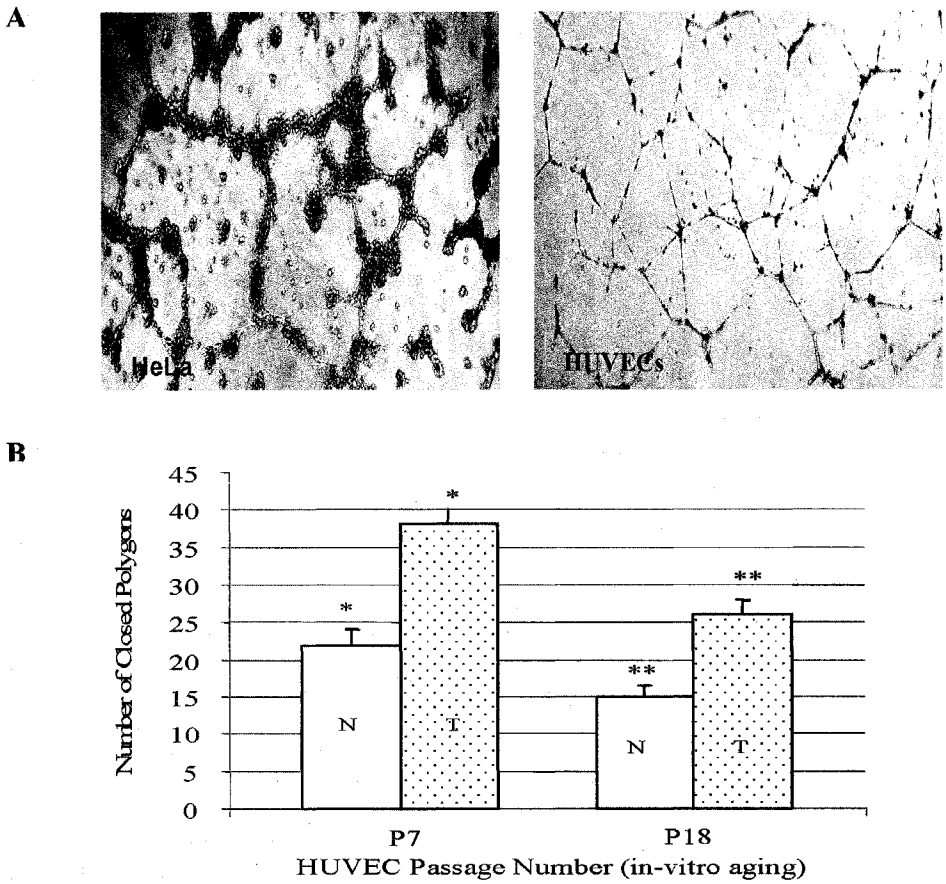

Figure 4-10: Formation of tube-like structures on matrigel by HUVECs. **(A)** Endothelial cells normally formed tubules on matrigel whereas, HeLa cancer cells do not. HeLa cells formed clusters. (original magnification x 40) **(B)** Tubule-like structures significantly increased in cultures of hTERT-transfected HUVECs, at P7 (*p=0.015) and P18 (**p=0.026) compared to untreated controls as shown by a greater number of closed polygons formed on matrigel. Senescing HUVECs, untreated (N) and hTERT-treated (T), showed a reduction in the numbers of tubules formed at P18 compared to P7.

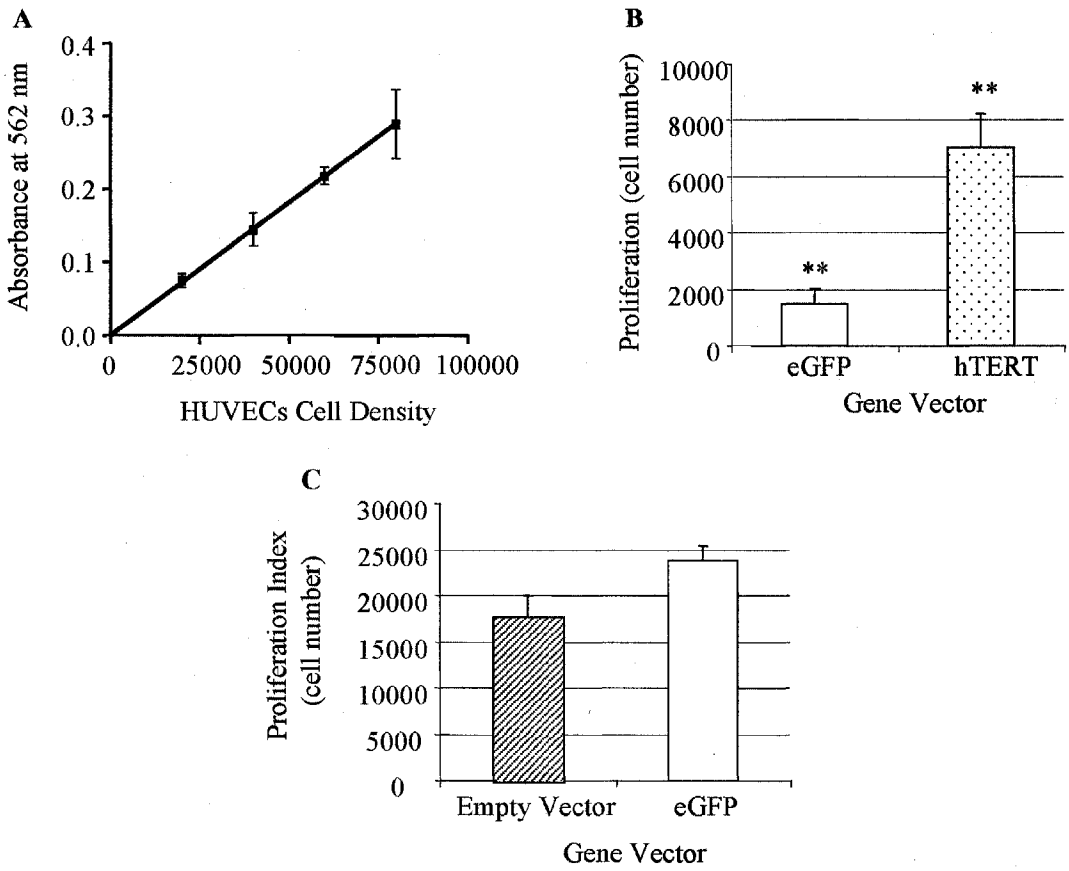

Figure 4-11: Proliferative index of hTERT-treated, EGFP-treated and untreated HUVECs at P18 after serum starvation. **(A)** A standard curve was generated from a MTT assay to correlate absorbance with cell number. **(B)** hTERT-treated HUVECs showed rapid cell recovery after serum replacement compared to EGFP-treated cells (**p=0.043). **(C)** Comparable proliferative indices were observed between EGFP-treated and untreated HUVECs in subsequent experiments suggesting an absence of toxicity from the EGFP vector (p=0.322).

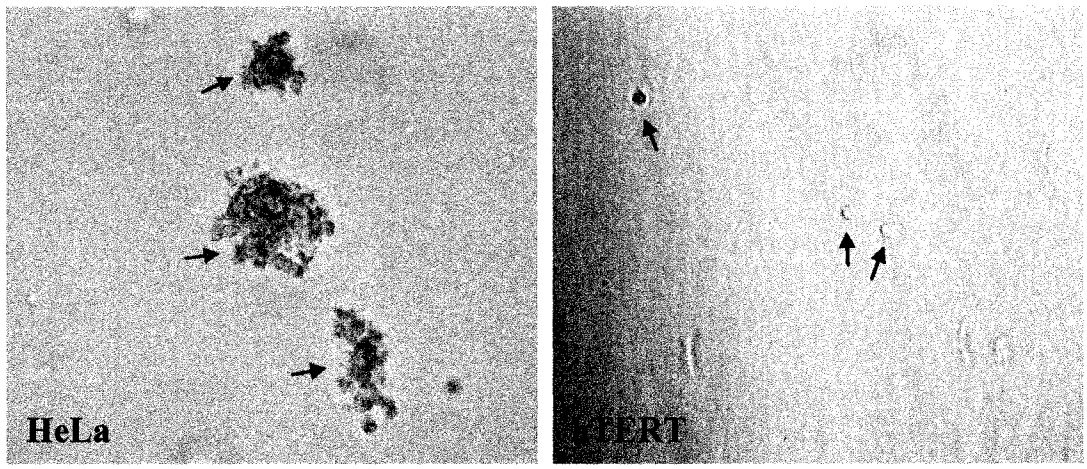

Figure 4-12: Anchorage-dependent growth in soft agar. Growth of HUVECs was examined in soft agar following hTERT-transfection. Colony formation of HeLa cells (red arrow) was observed at 2 weeks and no colonies were found with hTERT-transfected (blue arrow) cells seeded in soft agar at a density of 1 X 10^4 cells. (original magnification x 200)

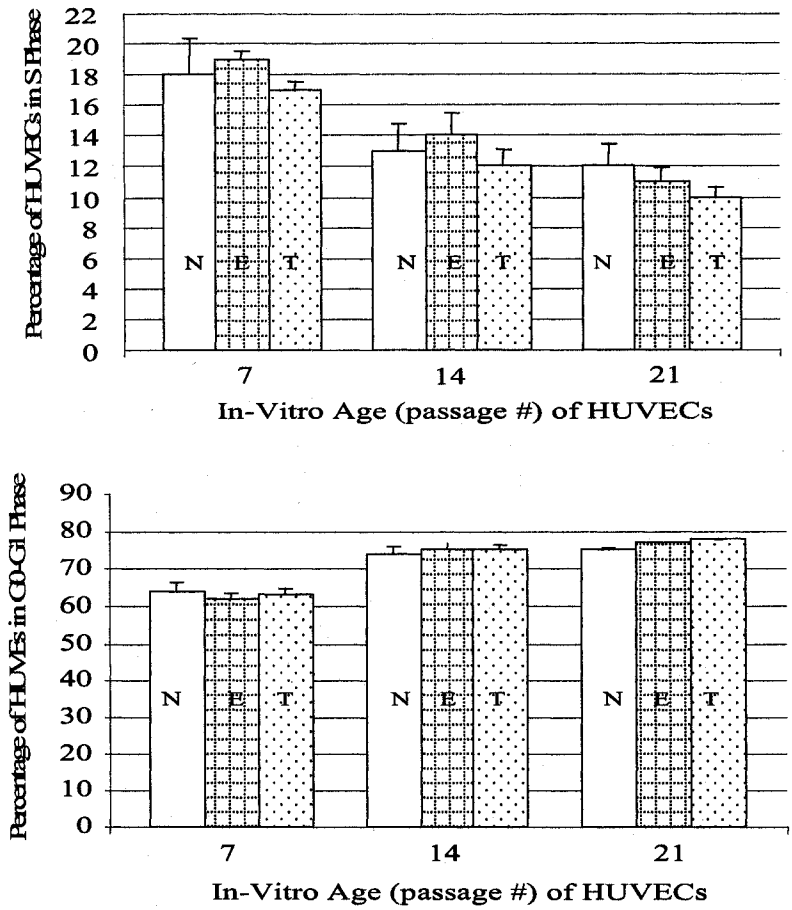

Figure 4-13: Cell cycle profile of HUVECs after hTERT transfection. Although expressing hTERT, HUVECs retained a normal cell cycle profile compared with the untransfected HUVECs and HUVECs transfected with the irrelevant EGFP vector. Although not significant, a decreasing trend was found in the S phase fraction of the cell cycle as HUVECs of all 3 treatment groups underwent aging *in vitro* (p=0.371). G0-G1 fraction did not show any significant changes between the untransfected (N), EGFP-treated (E) and hTERT-treated (T) cells (p=0.616).

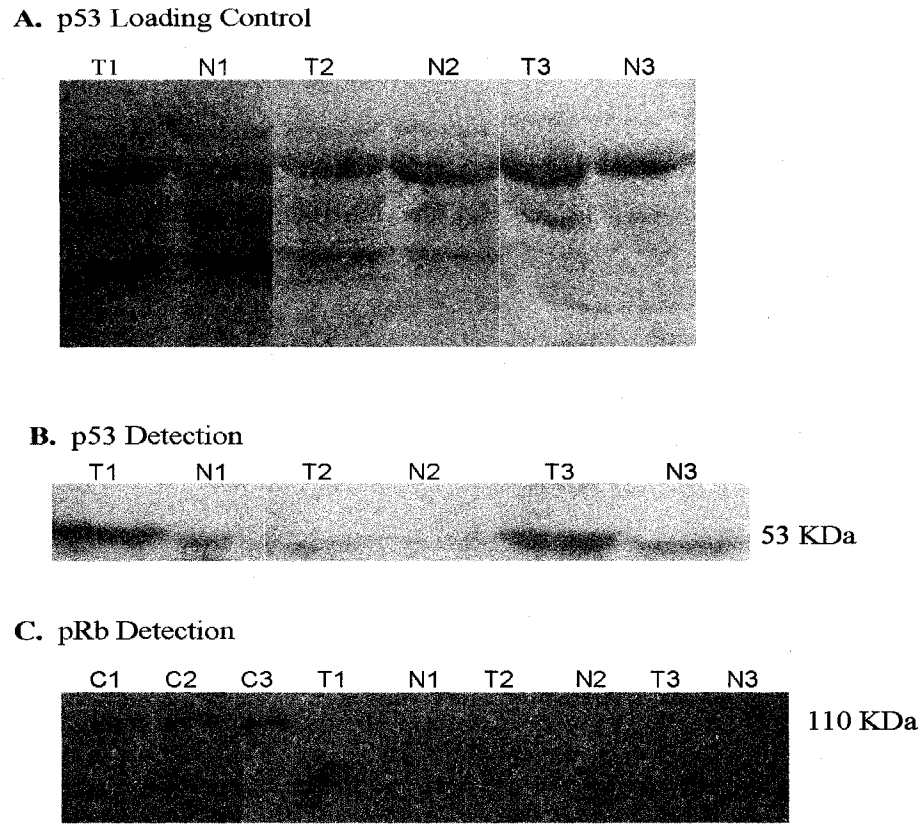

Figure 4-14: Tumor suppressor proteins p53 and Rb. **(A)** 35 μg total proteins were loaded for gel electrophoresis. **(B)** P53 expression was retained in HUVECs transfected with hTERT or with a control vector. hTERT-transfected HUVECs appeared to express higher p53 although loading was similar for both transfected and controlled HUVECs. **(C)** Rb protein was not detected for either treatment groups due to low phosphorylated Rb levels at steady state in normal human cells. Positive controls loaded at 5 μg (C1), 7 μg (C2) and 10 μg (C3) were detected whereas all samples were not detected. (T=hTERT-transfected, C=Control)

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CHAPTER V

HTERT AND EGFP GENE TRANSFECTION IN HUMAN ISLETS USING A NONVIRAL-MEDIATED GENE STRATEGY

V-A) INTRODUCTION

Recent clinical success in pancreatic islet cell transplantation, as a treatment for insulin-dependent (type I) diabetes mellitus in humans, has been attributed to "The Edmonton protocol". On this protocol all patients receiving islet tissue transplants have demonstrated insulin independence (1). Although a major accomplishment, the wide application of islet transplantation is still limited by the scarcity of donor tissues and the variable success rates in islet isolation and recovery (2). Given the simplicity and minimally invasive nature, as well as the reduction in both the costs and use of immunosuppression, clinical islet transplantation is a promising alternative to whole organ transplant as a potential cure for diabetes (1, 3). The drive to establish islet transplantation as a routine treatment for type I diabetes, therefore fuels the need to find new sources of insulin-producing cells, as millions of people are affected by diabetes worldwide (4, 5). Diabetes mellitus is characterized by a juvenile onset of severe insulin deficiency, due to the autoimmune destruction of the pancreatic islets of Langerhans.

To date, the major focus of diabetes research is to find or produce an alternative source of islet tissues. Either from the *ex vivo* culture and/or the expansion of existing tissues or induce the differentiation of functional endocrine cells from stem cells to grow new islets. Islet tissue is a collection of various differentiated cell types that do not

divide, and as a result, pose extreme difficulties for islet regeneration. *In-vitro* expansion of the 3D islet cell mass in fibrin gels along with a cocktail of growth factors has yielded limited success, as these normal cells have limited proliferative potential (6). At birth, normal somatic cells inherit a defined number of cell divisions, that is known as the Hayflick limit, dictated by the length of telomeres. In the absence of telomerase activity in these normal cells, telomeres are inevitably shortened with cell division leading to replicative senescence, an irreversible state of growth arrest and possible apoptosis (7, 8). Difficulties in cultivating these cells *in-vitro* is possibly associated with critically short telomeres triggering premature replicative senescence, and crisis with any stressor. As the regenerative capability and functioning cell mass diminishes, graft function is ultimately compromised (8-10). The genetic engineering of human islets to express telomerase therefore may trigger islet cell division, and prolong tissue survival (11-15).

Telomerase gene therapy has been used to overcome replicative cell senescence in various human cell types and tissues. One example of this is the bioengineering of human blood vessels in bioreactors (16). To create autologous vascular grafts, smooth muscle cells (SMCs) and endothelial cells (ECs) isolated from human blood vessels of a 67 and 74 yr old donor were genetically modified to express hTERT. These 2 cell types successfully regenerate *in vitro* new and robust blood vessels in bioreactors. In contrast, both untransfected SMCs and ECs showed low cell density and failed *in vitro* to re-create a vessel, possibly due to cells reaching their Hayflick limit. Replicative senescence has been suggested as a possible cause for the unsuccessful expansion of human islets in tissue culture (6, 11). To overcome senescence, successful delivery of the human telomerase (hTERT) gene is required. We have shown in the previous chapters and

published that human endothelial cells transfected with telomerase were more robust and proliferated with retained contact inhibition (17). Very little, however, is known of the potential role played by telomerase and telomeres in islet growth and viability. As proof of concept, in this study we examined telomerase expression, and telomere length in native human islets prior to conducting telomerase transfection. This is the first study to use a fluorescent *in-situ* hybridization technique accompanied by flow-cytometry (flow-FISH) to address telomere length in isolated human islets. As another part of this study, we investigated the efficiency of 2 different lipofection strategies, DOTAP and FuGene 6, to effect islet transfection. The initial obstacle in gene therapy is to find an efficient transfection strategy to deliver a therapeutic gene. Despite poor efficiency, non-viral based transfection offers exemplary biosafety and clinical feasibility (18-21). Although recombinant viral vectors are commonly employed due to higher transfection rates, mean efficiencies above 90% and 30% have been achieved in intact canine islets and primary human endothelial cells respectively using lipid formulations in our laboratory (22-26). This paper reports on the further refinement of lipofection in intact human islets as opposed to transfection of partially dispersed or dissociated islets conducted in a great number of other islet transfection studies. Once the transfection parameters were optimized for human islets, as evaluated by EGFP reporter expression, telomerase expression was subsequently assessed in transfected islets. The primary aim of this study was to find an efficient gene delivery strategy for hTERT transfection and assessment of gene expression in human islets. Firstly however, telomerase activity and telomere length were assessed *a priori*, otherwise it defeats the purpose of hTERT gene therapy without any knowledge of telomere shortening, or possible endogenous enzyme activity.

The working hypothesis is that the rescue of cell senescence with telomerase gene transfection will provide a potential means to generate unlimited islet cells *in vitro* to meet the increasing demands for islet transplantation to treat type 1 diabetes mellitus.

V-B) MATERIALS AND METHODS

1. Islet Isolation and Culture

Human islet isolation was performed at the Human Islet Isolation Laboratory (University of Alberta, Edmonton, AB, Canada) from which fresh and cryopreserved islets were obtained from human donors with research consent (1). Cryopreserved human islets were quickly thawed in a 37°C water bath and spun down at 1500 rpm (250 g) for 1 min. After removal of the DMSO-containing supernatant, 1 ml of 0.75 M sucrose solution was used to resuspend the islets and left to equilibrate on ice for 30 min. Islets were then washed successively with 1 ml (twice), 2 ml and lastly, 4 ml of complete M199 media (Invitrogen™, Calrsbad, CA), supplemented with 10% fetal bovine serum (FBS, Invitrogen™, Calrsbad, CA) and standard antibiotics, at 5-min intervals. The revived islets were centrifuged at 1500 rpm (250 g) for 1 min and cultured overnight in complete CMRL media (Invitrogen™, Calrsbad, CA), reconstituted with 10% FBS, 2.8 mM L-glutamine and penicillin/streptomycin, under standard conditions (37°C, 5% CO_2 in balanced air). Islets were isolated and used from a total of 21 donors for this study.

2. Islet Dissociation for Flow-FISH

Thawed intact islets were dissociated through a series of chemical digestions with trypsin/EDTA, followed by mechanical break-up using needle injection with decreasing bore-size (16 to 22 guage (G)). After addition of 2 ml of 1X trypsin/EDTA

(Invitrogen™, Calrsbad, CA) and 1 ml of 0.2 mg/ml EDTA/PBS (Ca^{2+} and Mg^{2+} free), the islet suspension was vigorously shaken for 5 minutes in a 37°C water bath. Following this partial enzymatic digestion, the islet suspension was aspirated through decreasing gauge-size needles (16G, 18G, 21G, 22G), with 10 passages through each gauge. The whole series of enzymatic and mechanical dissociations were repeated 3 times. The dissociated islets were washed with complete CMRL media, yielding a near single cell suspension as assessed by light microscopy with Trypan Blue staining.

3. Flow-FISH Technique for Telomere Length Assessment

After dissociation and washing, islet cells were fixed and permeabilized with Cytofix/Cytoperm Kit (BD Pharmingen™, San Diego, CA, USA) according to commercial protocol. Islet were then resuspended in 0.5 ml of hybridization buffer (70% formamide, 20 mM Tris at pH 7.2, 1% BSA) containing 0.3 μg/ml $(CCCTAA)_3$-FITC conjugated peptide nucleic acid (PNA) probe (Applied Biosystems™, Foster City, MA) with sequence complementary to only telomeric DNA (27-29). This telomere probe solution was pre-heated at 50°C for 10 min before use. Islets were resuspended in 0.5 ml probe solution and heated at 87°C for 10 min after which it was left at room temperature, in the dark, for a 3 h hybridization. The cell suspension was repeatedly washed, twice with a buffered wash (70% formamide, 10 mM Tris at pH 7.2, 0.1% BSA, 0.1% Tween 20) and once with 1X PBS. The sample was resuspended in 1X PBS for detection with flow-cytometry using a FACS Calibur flow cytometer (Becton Dickinson™, San Jose, CA) with channel FL1 set for detection of fluorescein signal. Analysis of 10,000 gated events was performed using the CELL-Quest software (Becton Dickinson™).

Quantum 24PC fluorescent bead solution (Bangs Laboratories™, San Juan, IN) containing 4 subpopulations of beads in which each has a standard fixed amount of fluorescence measured in molecular equivalence of soluble fluorochrome (MESF) was used for instrument calibration. In addition, a standard curve was created from which the fluorescent intensity of each sample was equated to MESF values that directly related to telomere length. Longer telomeres bind more 5'-CCCTAA-3' specific fluorescent probes corresponding to higher MESF values.

4. Western Blot

Total protein extracted from both cryopreserved and fresh human islets were analyzed on Western blot for telomerase gene expression according to the protocol provided by Santa Cruz™ Biotechnology (Santa Cruz, CA, USA). Total protein was extracted with RIPA (1X PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate) buffer, supplemented with 10 ul/ml PMSF, 30 µl/ml aprotinin and 10 µl/ml sodium orthovanadate, during a 3 h incubation on ice with periodic mixing. A fixed amount of proteins (60 µg) were run on a 7.5% polyacrylamide gel and transferred onto a hybond-P membrane. The membrane was blocked for 1 h with blocking reagent (1X TBS, 5% non-fat dried milk, 0.05% Tween-20) at room temperature and then probed with mouse monoclonal anti-hTERT primary antibody (NCL-hTERT, Vector™, ON) at a 1:500 dilution for 1 h. The membrane was washed three times for 5 min each with wash buffer (1X TBS and 0.05% Tween-20), after which the membrane was incubated for 45 min at room temperature with a horse radish peroxidase (HRP)-conjugated goat anti-mouse secondary antibody (Santa Cruz™ Biotechnology, Santa Cruz, CA) at a dilution of 1:2000. *(Note: Monoclonal antibody is*

highly specific for only one antigen. It is a single type of highly purified antibody that is directed against a specific epitope (antigen, antigenic determinant) and is produced by a single clone of cells.) The membrane was washed three times with wash buffer at 5 min each and once with 1X TBS before detection. Chemiluminescence detection was accomplished with Luminol reagent (Santa Cruz™ Biotechnology, Santa Cruz, CA) containing the substrate for the HRP enzymatic reaction and the emitted chemiluminescence was captured on Kodak film.

5. Telomere Repeat Amplification Protocol (TRAP) Assay

The commercial TRAPeze XL Kit (Chemicon™, CA) was used for the detection of telomerase activity. The Chemicon™ TRAP kit provides a substrate oligonucleotide to which telomeric repeats are added in the presence of telomerase activity to the 3' end of this substrate. The following reaction entails PCR amplification of the extended substrates using fluorescein-labeled primers which will fluoresce if incorporated into the TRAP product. As a result, the emitted fluorescence is directly proportional to the amount of TRAP products generated. A 1.0 μg total protein sample extracted from islets was analyzed for each human islet donor. Extracts from HeLa cells were ran as positive controls.

6. Islet Transfection

EGFP reporter gene was used to determine transfection efficiency using commercial protocols provided with the DOTAP (Roche™, Laval, QC, Canada) and FuGENE 6 (Roche™) transfection reagent kits. Parameters evaluated include DNA dosage (3 μg and 6 μg), ratio of DNA:lipid (1:4 and 1:6) and percent FBS (5% and 10%) used in the transfection protocol. A mixture of EGFP DNA:DOTAP (1:6) was prepared

in 1 M HEPES (Invitrogen™, Calrsbad, CA). This mixture containing 6 μg DNA was incubated at room temperature for 15 min prior to drop-wise addition to cultures containing 850 – 1000 islet equivalents suspended in 2 ml of complete CMRL media, either supplemented with 5% or 10% serum. Islet equivalents of each islet sample were determined by staining and counting an aliquot of islet suspension with Diphenylthiocarbazone (dithizone, DTZ), a zinc chelating agent that stains the islets of Langerhans red due to an abundance of zinc in constitutive β cells. Islets suspended in CMRL medium are Dithizone-stained intact islets, which were viewed and counted under a dissecting microscope to calculate the islet equivalent using a mathematical formula. Likewise, an EGFP DNA:FuGene 6 (1:6) mixture prepared in Optimem I (Invitrogen™, Calrsbad, CA) was incubated for 15 min and added drop-wise to the islet suspension. The transfection mixture was removed from the islet culture after 20±4 hr incubation and replaced with fresh CMRL media for further incubation. Levels of EGFP expression were assessed in 100 to 200 islets for each transfected human samples, on the second day post-transfection, by standard fluorescent microscopy. Transfection efficiency was determined by the percentage of intact whole islets exhibiting green fluorescence. Untreated islets were used as negative controls.

7. Static Incubation

Islet viability was evaluated via insulin production and secretion initiated by glucose-stimulation. The ratio of insulin secreted in high and low glucose solutions is known as the stimulation index, an indicator of islet function. Following a 2 day culture period, EGFP-transfected and control islets were briefly washed twice with no-glucose-RPMI-1640 medium (Invitrogen™, Calrsbad, CA, USA) to remove the CMRL culture

medium. Afterwards, the islets were incubated consecutively in RPMI-1640 medium supplemented with 2.8 mM (low) d-glucose then in the same medium supplemented with 25 mM (high) d-glucose, for 1 h each respectively at 37°C. Supernatants were collected and insulin contents assessed by the University of Alberta hospital laboratory (Edmonton, AB, Canada) using a radioimmunoassay kit.

8. Confocal Microscopy

An aliquot of EGFP-transfected islets were examined by confocal microscopy (Carl Zeiss™, Canada) fluorescent labeling. A drop of 5-10 ul islet suspension was placed onto a slide and before coverslipping, grease was applied along the edges of the slide to prevent dehydration and crushing of the islets by the coverslip. This technique was used to scan whole intact islets to yield optical cross-sections to further assess the efficiency of transfection, based on the EGFP reporter protein expression in an islet.

9. Islet Transfection with hTERT

Frozen-thawed human islets, a total of 200 single islets averaging between 50-150 μm in diameter, from 3 individual pancreas donors were handpicked and placed into 35 mm suspension culture dishes. *(Note: Islets were picked based on morphology because dithizone staining might affect transfection results and gene expression profile.)* DOTAP was utilized to deliver the hTERT (recombinant pNI-Tel plasmid from Chapter 3) gene into islets. Transfection mixtures containing 6 μg of hTERT and 36 μl of DOTAP (DNA:lipid ratio of 1:6) were added to islets cultured in complete CMRL with 10% FBS. CMRL medium was changed every day until islets were collected at 2 d post transfection and cell pellets stored in -80°C prior to RNA isolation. Untransfected islets were used as negative controls.

10. RNA Isolation

RNA extraction started with 1 ml Trizol (Invitrogen™, Calrsbad, CA) and mixed well before setting samples on ice for 3 h with mixing between intervals. For phase extraction, 300 µl of chloroform was added and mixed by inversion and brief vortexing thrice. The suspension was spun down at 12,000 rpm using an Eppendorf bench top microcentrifuge set at 4°C for 30 min. The aqueous phase was removed to a clean eppendorf tube and 1 ml of isopropanol was added to the supernatant which was left at -20°C overnight. Following nucleic acid precipitation, the suspension was centrifuged again for 30 min at 12,000 rpm at 4°C. The pellet was washed twice with 1 ml of 70% ethanol with each washing spun down at 12,000 rpm for 15 min. The pellet was briefly air dried and resuspended it in 75 µl diethylpyrocarbonate (DEPC; Invitrogen™, Calrsbad, CA) sterile water.

10. Real-time RT-PCR

An iScript One-Step RT-PCR Kit with SYBR Green (BioRad Laboratories™, Hercules, CA) was used to set up the real-time RT-PCR reactions. Each reaction tube was set up as follows: 10 µl 2X SYBR Green RT-PCR reaction mix, 0.34 µM each of forward and reverse primers, 0.1 µg RNA, 1 ul iScript reverse transcriptase and nuclease-free water to make up a final volume of 20 µl. The primer sequences for the hTERT gene were 5'-GCC TGA GCT GTA CTT TGT CAA-3' (TERT-2164S; forward) and 5'-CGC AAA CAG CTT GTT CTC CAT GTC-3' (TERT-2620A; reverse). Glyceraldehyde-3-phosphate dehydrogenase (GH) was used as a housekeeping gene (normalizer) with sequences of 5'-TTC ATT GAC CTC AAC TAC ATG-3' (forward) and 5'-GTG GCA GTG ATG GCA TGG AC-3' (reverse). Reverse transcription was carried out at 50°C for

10 min, followed by a general denaturation step at 95°C for 5 min. Each PCR reaction began with denaturation at 95°C for 10 sec followed by annealing at 60°C for 45 sec for a total of 35 cycles. The level of gene expression is represented by a Ct (cycle threshold) value. A lower Ct reflects a higher number of copies of the nucleic acid target at the start of the reaction hence, the sooner a significant increase in fluorescent signal is observed, and vise versa. Cycle threshold (Ct) is the relative cycle number at which fluorescence is first detected indicating that the gene of interest is being specifically amplified using specially designed primers. Ct of the GH housekeeping gene in the same sample was also determined. HeLa, a human cervical cancer cell line known to express endogenous telomerase, was used as a positive control. A water blank was included with every RT-PCR run. A second real-time RT-PCR run was repeated on the same samples.

11. Statistical Analysis

Both transfection efficiency and stimulation indices were expressed as the mean ± standard error of the mean (mean±SEM), where n is the number of individual transfections conducted. Non-parametric (Mann-Whitney and Kruskal-Wallis) tests were adopted to establish statistical significance ($p<0.05$) in transfection efficiency and stimulation index. Furthermore, correlation between telomere length and age was established through Pearson's coefficient r. Student's t-test was applied to evaluate hTERT expression between DOTAP-transfected and native human islets.

V-C) RESULTS

1. Telomerase gene expression and activity in human islets

Telomerase expression was not detected in either cryopreserved or fresh human islets as revealed on Western blot (Figure 5-1). However, it was highly expressed in cell lines propagated from human cervical carcinoma (HeLa) and bladder carcinoma (MGH-U3) since telomerase expression is a hallmark of cancer cells (30).

Absence of telomerase expression on Western blot was confirmed by the lack of enzyme activity shown by the TRAP assay. Telomerase activity was examined in islet cultures at 1d, 2d, 4d, and 7d post-thaw to determine if longer revival time is required to allow telomerase reactivation in cryopreserved islets. No activity was detected at the different time intervals. To rule out the presence of tissue inhibitor, HeLa protein extract was also included in a subset of islet protein samples in which telomerase activity was detected as a result. This observation demonstrated the absence of tissue inhibitor effect on telomerase activity. Hence, telomerase expression is apparently absent or downregulated in human islets.

2. Telomere length assessment in frozen-thawed human islets

Length of telomeres was evaluated in dissociated islets using flow-FISH and a telomere-specific fluorescent probe. Telomeres in intact islets were found to decrease with age (Pearson $r = -0.328$) as illustrated by a decreasing rate of fluorescence, proportional to telomere length, of 206±188 MESF/yr (Figure 5-2). This mean value of decrease reflects minimal islet cell division *in vivo* leading to low rate of telomere loss.

3. Optimization of lipofection

Successful transfection of intact canine islets has been achieved in a previous study using a DNA:lipid ratio of 1:4 and 1:6 with 3 μg and 6 μg reporter DNA (21). Therefore, we continued to pursue islet transfection, in particular, using human islets in this study. Poor efficiency of transfection was observed with 3 μg DNA as opposed to 6 μg DNA. Using the latter DNA amount, transfection rates were significantly higher for cryopreserved islets than fresh samples at a 1:4 DNA:lipid ratio ($p=0.005$) but not at 1:6 ratio ($p=0.419$). Mean efficiencies were 30±5% (n=7, bar 3*, Figure 5-3) and 27±8% (n=4, bar 3**, Figure 5-3) at 5% and 10% FBS respectively. At these respective serum concentrations, transfection rates were significantly lowered to 4±3% (n=2, bar 4*, Figure 5-3) and 6±1% (n=2, bar 4**, Figure 5-3) for fresh islets. Transfection efficiency did not differ greatly with the use of either FBS concentrations ($0.232<p<1.000$). However, a DNA:lipid ratio of 1:6 gave higher rates of transfection, for both cryopreserved (bar 1, Figure 5-3) and fresh (bar 2, Figure 5-3) islets in both 5% and 10% FBS than a 1:4 ratio, ranging from 36±4% to 45±4% in a total of n=49 observations ($p=0.003$). To summarize, optimal transfection efficiency was achieved at a 1:6 DNA:lipid ratio with 6 μg DNA and in either 5% or 10% serum.

4. Comparison between DOTAP and FuGene 6 at a 1:6 DNA:lipid ratio

DOTAP demonstrated to be a significantly better lipofection reagent for the transfection of frozen-thawed human islets (Figure 5-4). Mean efficiencies of transfection achieved were 50±5% (n=12) and 42±4% (n=13) at 5% and 10% FBS respectively as opposed to the corresponding rates of 28±2% (n=3) and 26±3% (n=7) using FuGene 6 (*$p=0.025$ at 5%, **$p=0.014$ at 10%). Overall, the increases in

transfection efficiency of DOTAP over FuGene 6 were 16% and 22% at both FBS concentrations. In addition, DOTAP continued to perform well with fresh human islets (Figure 5-5). At 5% FBS, mean efficiency was 45±12% (n=4) whereas FuGene 6 demonstrated a mean rate of 31±7% (n=5, p=0.413). However, at 10% FBS, transfection rates were similar for both lipid reagents (36% for DOTAP vs 37% for FuGene 6, p=1.000). DOTAP-transfected islets also fluoresced more intensely consistent with increased number of EGFP-expressing loci (Figure 5-6).

5. Insulin responsiveness of DOTAP- and FuGene 6-transfected islets

EGFP-expressing islets were equally responsive to glucose as unmodified islets demonstrative of the lack of toxicity from transfection. Both DOTAP- and FuGene 6-transfected islets in 5% FBS had stimulation indices of 2.21±0.30 (n=10) and 1.98±0.57 (n=3) respectively compared to 1.89±0.17 (n=13) for untreated controls (p=0.461, Figure 5-7). Transfection done in the presence of 10% serum also demonstrated insulin responsiveness to glucose. Stimulation indices of 1.72±0.29 (n=9) and 1.43±0.19 (n=3) were obtained for DOTAP- and FuGene 6-treated islets respectively compared to a mean control value of 1.49±0.24 (n=10, p=0.767). Hence, genetically modified islets remained functional, suggesting that these lipid formulations are not toxic.

6. Functional comparison between cryopreserved and fresh islets following DOTAP transfection

Fresh human islets seemed more responsive to glucose than cryopreserved islets following transfection (p=0.031, Figure 5-8). DOTAP-transfected fresh islets showed stimulation indices of 2.38±0.34 (n=8) in 5% FBS and 2.01±0.46 (n=5) in 10% FBS whereas, their respective controls showed 1.85±0.22 (n=8) and 2.06±0.46 (n=4). The

EGFP-transfected islets and controls showed comparable insulin secretion (p=0.117). However, insulin secretion was reduced in cryopreserved islets displaying stimulation indices of 1.53±0.39 (n=2) and 1.35±0.15 (n=4) post-transfection in 5% and 10% serum respectively compared to control values of 1.89±0.30 (n=5) and 1.11±0.12 (n=6). Moreover, no significant difference in islet function, in terms of insulin production, was observed between the test and control samples (p=0.574). The DOTAP method does not appear to jeopardize islet function in insulin production while achieving adequate rates of gene transfection. Overall, fresh human islets were stimulated to produce and secrete more insulin than cryopreserved islets. This observation may be related to potential damaging effects of cryopreservation on human tissues thereby affecting any function when revived. To date, cryopreservation is a necessary means to preserve the scarce availability of donor supplies in the face of organ shortages, especially when islets cannot be adequately maintained in culture at the current time.

7. EGFP fluorescence and hTERT expression in DOTAP-transfected islets

Strong fluorescence was observed in transfected islet cells. Optical cross-sections of representative islets showed EGFP fluorescence appearing mainly near the surface of an islet. The scattered EGFP fluorescence was observed in various cell populations existing at the islet periphery (Figure 5-9).

HTERT-transfected islets yielded lower Ct values than untransfected islets, indicative of higher amounts of initial hTERT transcripts after transfection. When a sample contains a higher number of copies of the gene of interest, the products, from PCR amplication of this gene, can be detected sooner. As a result, the amplified fluorescent labeled products start to appear at a lower cycle threshold (Ct) than in

samples with initial low levels of the gene expressed. As exemplify by similar Ct values obtained for hTERT and GH ($p=0.595$), HeLa cells expressed hTERT ($Ct=25.5$) mRNA at levels comparable to that of the GH ($Ct=24.3$) housekeeping gene (Figure 5-10). In hTERT-transfected islets, however, the initial levels of hTERT ($Ct=14.6$) mRNA were significantly higher than the constitutive levels of GH ($Ct=26.5$) expressed ($p=0.014$). This finding was reversed in untransfected islets, which expressed significantly lower amounts of the hTERT ($Ct=28.0$) transcripts than those of GH ($Ct=23.0$, $p=0.044$). Following transfection, hTERT-expressing islets had significantly more hTERT mRNA than in HeLa cells ($p=0.034$) and untransfected controls ($p=0.017$).

V-D) DISCUSSION

Very few transfection studies have been conducted with human islets particularly in combination with a nonviral gene transfection approach. This study and that of Mahato et al. (18) examined nonviral transfection in intact human islets. Mahato et al. observed only minimal GFP expression in islets transfected with Lipofectamine and Superfect (18). In contrast, we achieved higher rates of transfection using DOTAP and FuGene 6 lipid reagents. Differences between the protocols included the amount of DNA used, DNA:lipid ratio, number of islet equivalents per transfection, as well as the incubation time with liposomal carrier. Mahato et al. incubated fresh islets in smaller volumes of transfection mixture in 1.5 ml eppendorf tubes, in contrast to this study, in which islets were transfected in 35 mm suspension culture dishes. Islets in the former culture condition were more likely to experience hypoxia, possibly resulting in lower transfection rates and greater cell death. Furthermore, we used an approximate 10-hour

longer incubation time in the transfection medium containing serum in this study. Serum might be beneficial for longer incubation periods, as it was found to increase transfection efficiency in a study reported by Nchinda et al. (31). Both DOTAP and FuGene 6 lipid transfections did not generate toxicity and are highly efficient in the presence of serum, suggesting suitability for *in-vivo* application. Islet function, as demonstrated by the stimulation index, was not affected by transfection in either 5% or 10% serum (Figure 5-7). Both the untransfected and EGFP-transfected islets had similar stimulation indices. In comparison, fresh islets were better insulin secretor than frozen-thawed islets as reported in this study and a prior study by Kneteman et al. (32). Piemonti et al. also noted lower stimulation indices for cryopreserved islets (33). The cryopreservation procedure is likely damaging to the islets, thereby affecting insulin production. Despite this observation, frozen-thawed islets were more susceptible to transfection. The membrane stabilization with DMSO may allow for higher fusion rates between lipid carriers and the frozen-thawed islets. Freshly isolated human islets were more difficult to transfect, particularly at a DNA:lipid ratio of 1:4 (Figure 5-3). Possibly, more lipid carriers may be required to coat the negatively charged DNA to prevent repulsion at the cell membrane, which too contains negative charges. The combined effects of; reduced cell entry and abundant nucleases in primary cells, severely hamper transfection rates. The degree to which lipofection, successfully delivers functional genetic material into frozen-thawed human islets, makes it a more attractive transfection strategy. Overall, DOTAP demonstrated higher efficiencies of transfection in fresh (Figure 5-5) and cryopreserved (Figure 5-4) islets among the 2 lipid transfectants used in our study. Furthermore, an absence of toxicity from DOTAP transfection is also critical to its

potential use in future gene therapy clinical trials involving genetically modified human islet transplants.

DOTAP-transfected islets showed intense gene expression based on the EGFP reporter protein. Positive transfection was noted mainly at the islet periphery (Figure 5-9). β-cells are as likely to be transfected as other constitutive cell types in human islets since β-cells are also located on the islet surface. Human islets are markedly different from rodent islets (34, 35). The different subpopulations of α-, β- and δ-cells are dispersed throughout the human islet reflecting a random and heterogeneous cell arrangement. Unlike humans however, mouse and rat islets have a defined architecture organized by a β-cell core surrounded by α and δ cells residing at the periphery. As a result, transfection of β-cells in rodent islets has shown to be difficult, even with adenovirus, which is known to be an efficient vector in both dividing and non-dividing cells. Narushima et al. found no GFP reporter gene expression in the inner core of mouse islets (36). In his study, GFP expression was mostly localized on the surface of an islet. Penetration of the transfection reagent or biological agent may be affected by the close clustering of different cell types within the assembled islet. Or perhaps, the loss of blood supply in cultured islets causes cell necrosis in the islet core, and therefore the cells were lost and no transfection resulted.

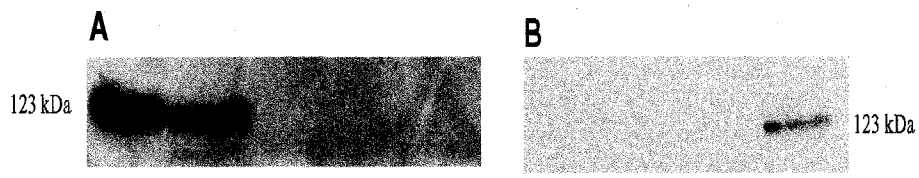
We demonstrated successful hTERT transfection in human islets (frozen-thawed tissues). Significantly higher levels of hTERT transcripts were found in hTERT-transfected islets than untreated controls (Figure 5-10). The mRNA levels in hTERT-transfected islets were even higher than those observed in the HeLa positive control. β-cells should be as readily accessible as other cell types to the hTERT:DOTAP

transfection complexes given that this cell type is also located at the periphery and intermingles with other cell types. Since freshly isolated human islets were better insulin secretor than cryopreserved tissues (Figure 5-8), it is worthwhile to examine telomerase induction in fresh islets, particularly if the goal is to prolong islets in tissue culture to avoid possible damaging effects from freezing the tissue. Therefore, the effects of telomerase on growth and survival of fresh islets in culture will be assessed in future studies.

The importance of hTERT transfection is to endow dividing cells with unlimited proliferative potential for tissue generation. This technology has clinical relevance in that cell expansion *in vitro* facilitates creation of skin grafts, microvessels and β-cell lines to treat patients suffering from burns, cardiac problems and diabetes, respectively. However, cell expansion is limited by replicative senescence (6, 11). Telomere attrition was found to be a cause of growth arrest in β-cells (6, 11, 15). Growth arrest was initiated after only 10 to 15 cell divisions in the β-cell cultures. As a means to overcome this limitation, Halvorsen et al. transduced β-cell-encriched cultures with hTERT. Unfortunately, they did not observe extended proliferative potential in the hTERT-transduced β-cells. This observation is in marked contrast with other studies that have shown increased proliferative capacity in hTERT-transfected cells (37, 38, 39). However, there are studies in which hTERT transfection alone did not result in increased replicative life span (40, 41). Likewise, β-cells could belong to this category and their rapid senescence may be similar to that reported in epithelial cells, that often displayed a senescent-like phenotype termed mortality stage 0 (M0). Induction of telomerase activity and loss of p16, a cyclin-dependent kinase inhibitor, were required to achieve

immortalization. As reported by Narushima et al., immortalization of β-cells required the combined transduction of SV40 large T antigen and hTERT genes (15). The continuous propagation of a β-cell line is facilitated by the stable transfection of primary β-cells with the SV 40 large T antigen and hTERT (14, 15). Clearly, humans have evolved safeguard mechanisms against telomerase expression, such that even when telomerase alone is expressed in some normal somatic cells and germline cells, humans do not easily develop tumors.

In future studies, the effects of hTERT expression in human islets may be further investigated by examining telomerase activity and telomere length in hTERT-transfected islets and untransfected controls, prior to conducting studies that assess the *in vivo* function of hTERT-modified islet grafts implanted into diabetic nude mice. Telomerase gene therapy may have therapeutic potential in better preserving islet tissues *ex vivo* before transplantation, and prolonging graft function *in vivo* following engraftment.



Tissue / Cell Sample	Characteristics
HeLa	Human cervical cancer
MGH-U3	Human bladder cancer
Rat	Frozen rat islets
Donor 1	Frozen human islets
M-ECs	Mouse Endothelial Cells

Tissue / Cell Sample	Characteristics
Donor 2	Frozen human islets
Donor 3	Frozen human islets
Donor 4	Fresh human islets
HeLa	Human cervical cancer cells

Figure 5-1: Telomerase expression in human islets. Telomerase detection in adult islets on Western blots **(A)** and **(B)**. Telomerase was not detected in both fresh (donor 4) and frozen-thawed (donors 1-3) human islets using a mouse monoclonal anti-hTERT antibody. Telomerase was present in cancer cells, HeLa (human cervical carcinoma) and MGH-U3 (human bladder carcinoma), used as positive controls. However, the monoclonal anti-hTERT antibody showed no cross-reactivity with rodent tissues and cells. Therefore, telomerase expression could not be detected in rat islets and mouse ECs using this monoclonal antibody.

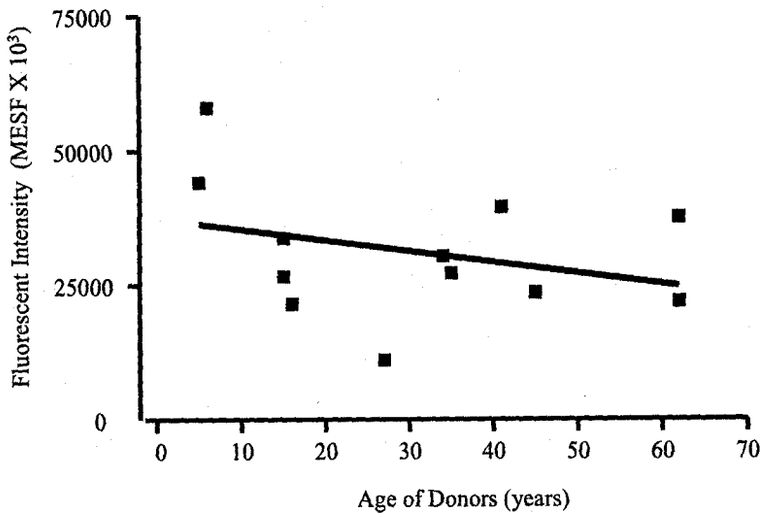


Figure 5-2: Telomere shortening in human islets. Telomere length was measured in MESF units where higher MESF values are indicative of longer telomeres. *In-vivo* telomere shortening was observed in intact islets isolated from donors of increasing age ($r = -0.328$).

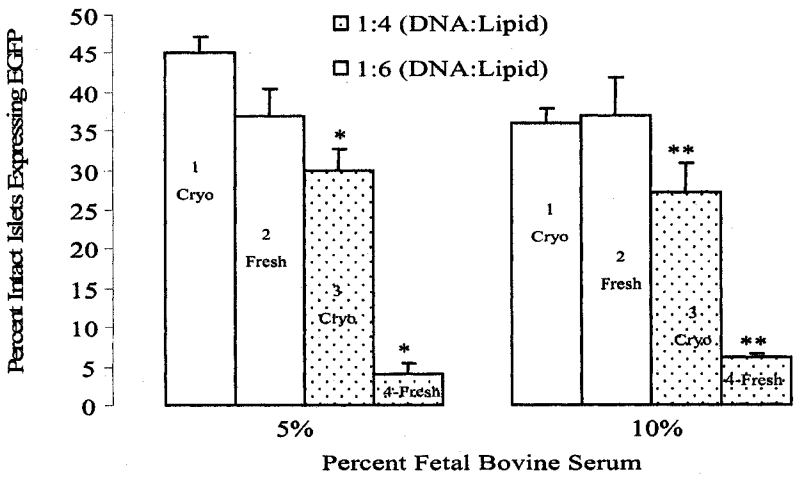


Figure 5-3: Optimization of transfection efficiency in cryopreserved and fresh human islets. Optimal efficiency was achieved with a DNA to lipid ratio of 1:6 with 6 μg DNA. Transfection efficiency was not affected by the different serum concentrations ($0.232<p<1.000$). However, at a 1:4 DNA to lipid ratio, significantly higher rates of transfection was obtained for cryopreserved islets as opposed to fresh ($p=0.005$, * and **) whereas no significant difference in efficiency was observed at a 1:6 ratio ($p = 0.419$) for the two groups. (1=cryo, 1:6; 2=fresh, 1:6; 3=cryo, 1:4, 4=fresh, 1:4)

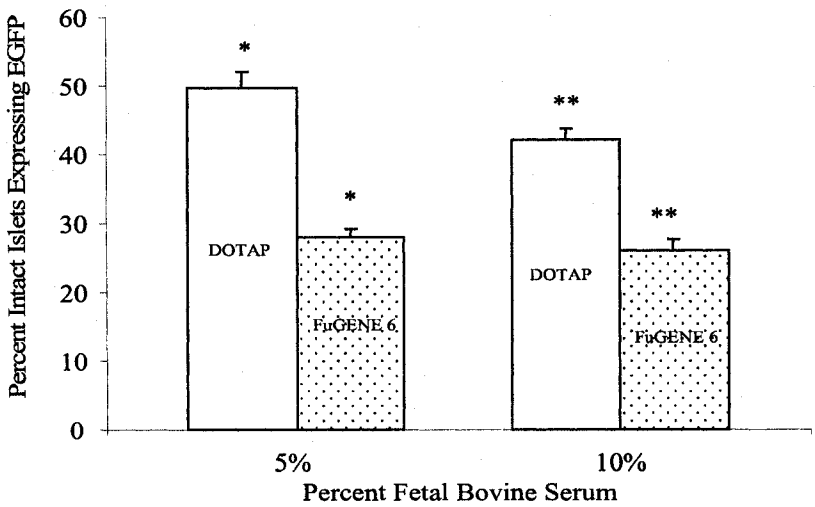


Figure 5-4: Transfection efficiency in cryopreserved human islets. Significantly higher rates of transfection were achieved in cryopreserved islets using DOTAP than FuGENE 6 in the presence of 5% (*p=0.025) and 10% (**p=0.014) serum. Mean efficiencies of transfection in the former serum concentration were 50±5% (n=12) for DOTAP and 28±2% (n=3) for FuGENE 6. In 10% serum, transfection rates of 42±4% (n=13) and 26± 3% (n=7) were obtained for DOTAP and FuGENE 6 respectively.

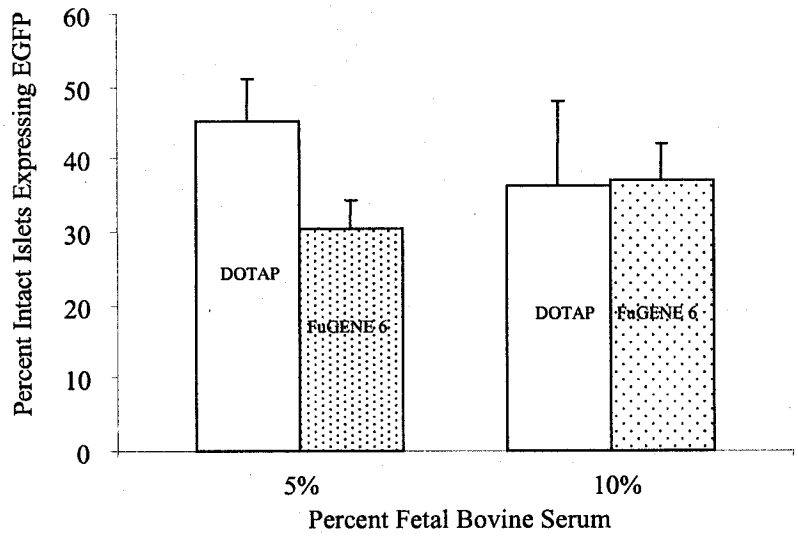


Figure 5-5: Transfection efficiency in fresh human islets. Mean efficiencies achieved with DOTAP and FuGENE 6 were comparably similar in both 5% ($p=0.413$) and 10% FBS ($p=1.000$). Transfection of fresh islets with DOTAP yielded 45±12% (n=4) and 36±23% (n=3) for 5% and 10% FBS, respectively, whereas FuGENE 6 yielded rates of 31±7% (n=5) and 37±11% (n=5).

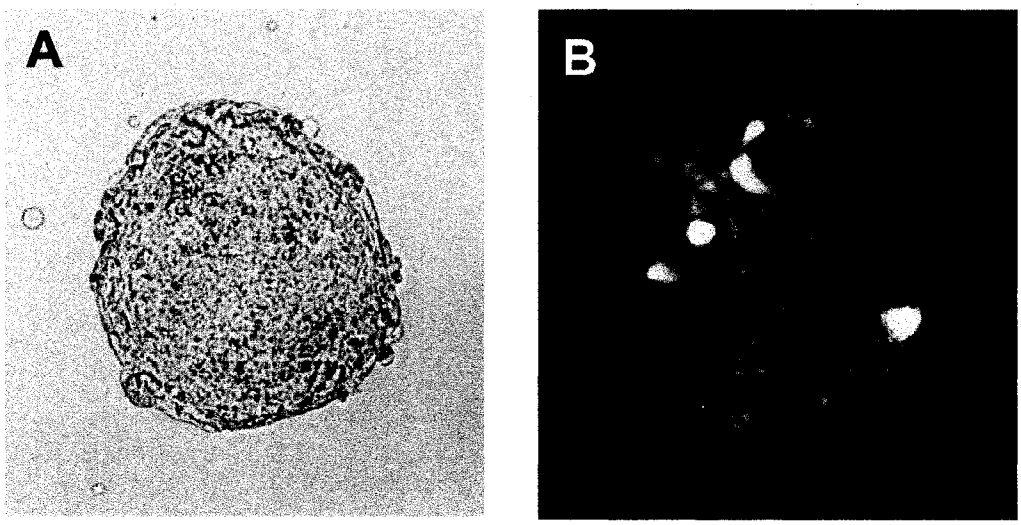


Figure 5-6: Human islet expressing the enhanced green fluorescent protein (EGFP) reporter following DOTAP transfection. Intact islet viewed under both **(A)** light and **(B)** fluorescent microscopy. Loci expressing EGFP were highly fluorescent on the surface of an islet.

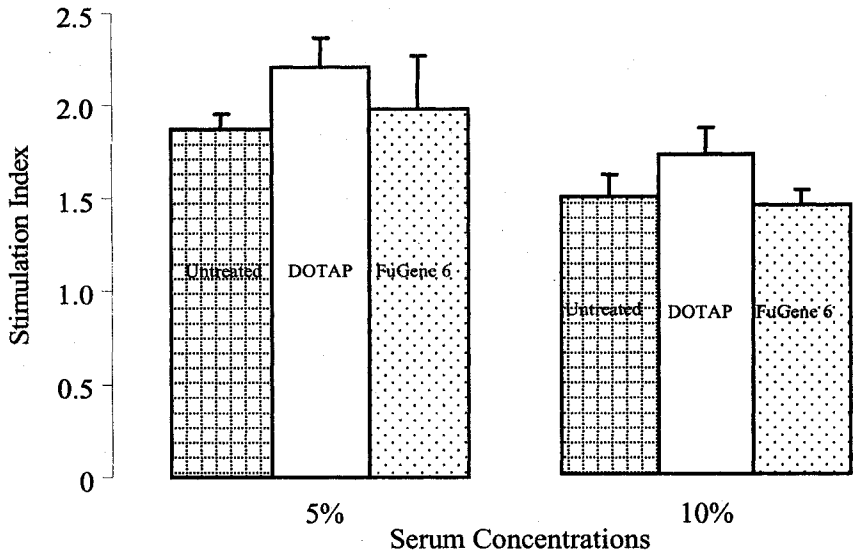


Figure 5-7: Comparative stimulation indices (SI) between treated and untreated islets. Transfection of human islets did not result in toxicity from the use of either DOTAP or FuGENE 6 lipid reagent. GFP-transfected islets had similar stimulation indices as the untreated controls ($p=0.461$ at 5% and $p=0.767$ at 10% serum concentrations). Transfection in 5% serum resulted in indices of 1.89 ± 0.17 ($n=13$) for untreated, 2.21 ± 0.30 ($n=10$) for DOTAP-treated and 1.98 ± 0.57 ($n=3$) for FuGENE 6-treated islets. In 10% serum, stimulation indices for islets transfected with DOTAP and FuGENE 6 were 1.72 ± 0.29 ($n=9$) and 1.43 ± 0.19 ($n=3$), respectively, similar to the untreated control value of 1.49 ± 0.24 ($n=10$).

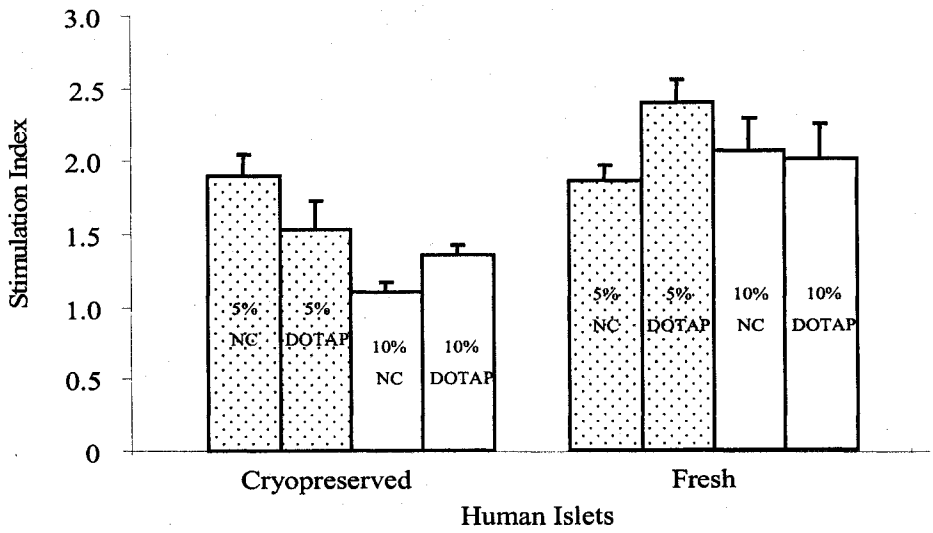


Figure 5-8: Comparison of stimulation indices (SI) between fresh and cryopreserved islets. Fresh human islets were better responsive to glucose, and therefore produced higher levels of insulin. Significantly higher stimulation indices were obtained for fresh than cryopreserved islets ($p=0.031$). However, at both 5% and 10% serum concentrations, DOTAP-transfected islets and untreated (NC) islets displayed similar stimulation indices ($p=0.574$).

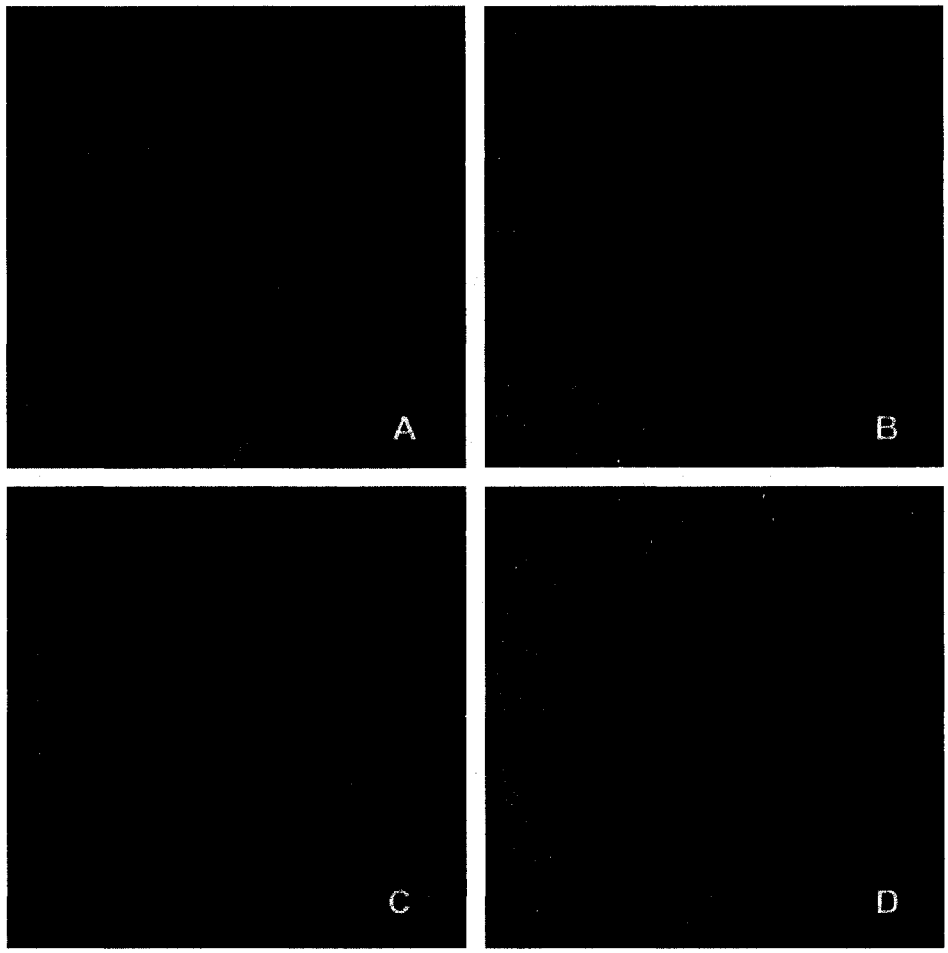


Figure 5-9: Optical cross-sections were taken from islets transfected with EGFP:DOTAP transfectants. In contrast to the **(A)** untransfected negative control, **(B-D)** EGFP-expressing islets showed strong fluorescence at the periphery of an islet. These islets are representative examples of positive transfection.

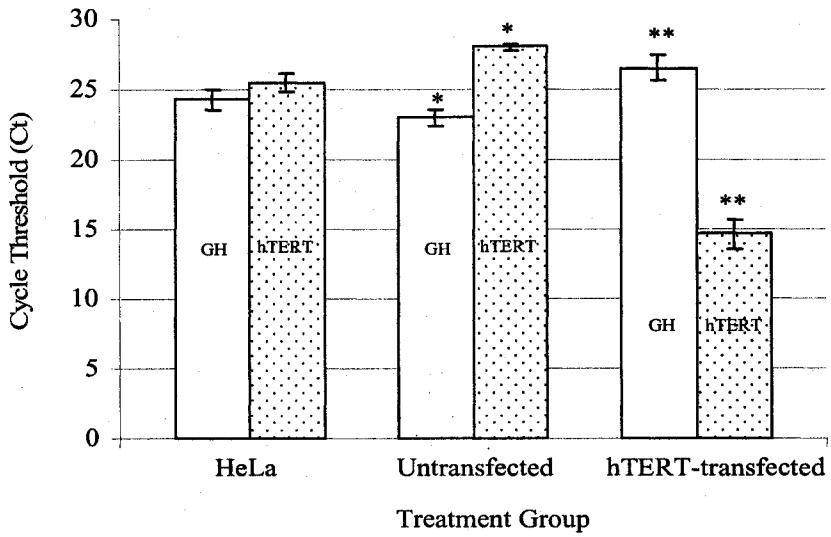


Figure 5-10: hTERT mRNA levels in human islets (n=3) measured by real-time RT-PCR. Ct values are used to indicate the relative amounts of initial hTERT mRNA in the untreated and hTERT-treated islets, as well as in the HeLa positive controls. A lower Ct reflects a higher number of copies of the nucleic acid target at the start of the reaction hence, the sooner a significant increase in fluorescent signal is observed, and vise versa. HeLa cells expressed hTERT (Ct=25.5) mRNA at levels comparable to that of GH (Ct=24.3, p=0.595). In hTERT-transfected islets however, hTERT (Ct=14.6) mRNA expression was significantly higher than that of GH (Ct=26.5, p=0.014). Significantly lower amounts of the hTERT (Ct=28.0) transcripts were found in untransfected islets compared with those of GH (Ct=23.0, p=0.044). Following transfection, hTERT-expressing islets had significantly more hTERT mRNA than in HeLa cells (p=0.034) and untransfected controls (p=0.017).

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CHAPTER VI

IN VIVO hTERT GENE EXPRESSION INDUCED BY DOXYCYCLINE IN Tet-ON TRANSGENIC MICE

VI-A) INTRODUCTION

In vitro gene transfer into many different cell types has been easily accomplished utilizing both viral and nonviral gene vectors (1-6). Unfortunately, these successful applications do not mirror the *in vivo* situations with tissue and organ manipulation (7, 8). Demonstration of high-efficiency gene transfer into vascular cells has been reported with the use of adenovirus, adeno-associated virus and vaccinia virus (9-13). However, they showed inefficient rates of infection *in vivo* in the quiescent endothelium and induced cell death (9, 13, 14). Retrovirus has been shown to efficiently infect endothelial cells and smooth muscle cells of arterial walls (15, 16). Retrovirus-mediated transductions have often resulted in long-term transgene expression with the absence of toxicity; these advantages offset the observed suboptimal transduction rate (17-20). Furthermore, if telomerase expression can be maintained indefinitely with retroviral transduction, the stable positive cell clones will always be present to replace senescent cells at times of renal endothelial cell injury. This therapy could also be extended to other organs.

A drawback in nonviral transfection strategy (Effectene lipofection) is transient gene expression that limits the long-term culture of telomerase-expressing cells (7, 8). Therefore telomere extension is very limited, due to rapid clearance of the hTERT vector as opposed to stable transfection (7, 21). It is also difficult to establish the long-term

effects of telomerase expression on replicative senescence using transient gene transfection. To overcome these technical limitations, this study explored the use of a retroviral vector combined with the Tet-ON system. This tetracycline (Tc) inducible gene expression system is a two-vector (RevTet and pTRE vectors) gene strategy that permits switching on/off gene expression as desired with tetracycline (Tc) and/or Doxycycline (Dox) (22-24). The simultaneous transfection of two plasmids, one of which encodes for the transactivator (tTA in Tet-OFF; rtTA in Tet-ON) that binds to the Tet response element (TRE) in the silent promoter ($PCMV_{min}$) of the second plasmid (response vector) to activate transcription (*in cis*) of the target gene (Figure 6-1). This is a valuable dual transfection system providing controlled expression of the target gene through activity of an inducible transactivator. The Tet systems are widely used to generate high-level gene expression, both *in vitro* and *in vivo* (25-27). The Tet system also provides very high specificity in transgene activation and transcription without affecting host genes (23, 24). Range of dosage of Tc and Dox required for high-level gene expression does not produce any adverse effects on cell proliferation *in vitro* and animal growth *in vivo*, even with continuous treatment (22-24). Dosage variation allows one to fine tune gene expression rendering them attractive vectors for physiological studies of gene effects, as opposed to constitutive gene expression that could be life-threatening (22, 24). Even though there are a few drawbacks with the RevTet/pTRE system, its advantages far outweigh any shortcomings (22, 23). Tet-ON/Tet-OFF systems (Clontech™, Mountain View, CA, USA) are commercially available in either retroviral (RevTet) or adenoviral (Adeno-Tet) formats. Other formats have also been used in private laboratories (26-31).

The use of the RevTet/pTRE system allows gene expression to be induced when desired due to chromosomal integration, in contrast to both lipofection and adenoviral-mediated strategies (25, 30). Severe systemic toxicity has been a major concern with the application of adenoviral vectors that require high viral titres for efficient gene transduction. Studies have also shown that adenovirus combined with the Tet-ON system is not as efficient a system at transfection (29, 32). Efficiency of greater than 40% in primary cells that are not easily transfected, has been observed with the use of the RevTet/pTRE system (24). These are important observations since retrovirus has been known to be an inefficient vector, partially due to promoter silencing, in slowly dividing primary human cells (9, 13, 14, 17).

Tet-ON transgenic mice are available through Jackson Laboratories that constitutively express the reverse tetracycline-controlled transactivator (rtTA) protein required to induce expression of the target gene (33, 34). This new transgenic animal technology was used in this study to minimize technical manipulation *ex vivo*. Since these Tet-ON transgenic mice, on a C57BL/6, are already expressing the transactivator gene that is induced with Doxycycline (Dox) administration, only the response vector containing the hTERT gene needs to be delivered into the animal for telomerase expression. Together, the rtTA, bound to Dox, activate the minimal CMV promoter in the response element to activate hTERT transcription (Figure 6-1). Systemic injection of the transgenic mice with the pRevTRE/target gene vector leads to prolonged telomerase expression. The transfected organ will be exposed to the environment only at the time of vector infusion and organ harvest. This transgenic animal model enables us to conduct experiments closely resembling the normal clinical situations, where organ procurement

and transplantation are carried out in a narrow window of time to minimize injuries. External variables will be greatly minimized due to the reduced handling of the grafts *ex-vivo*. Given that the C57BL/6 and BALB/c strain combination only yielded a 20% spontaneous graft acceptance, rejection is likely to occur. Therefore, this rodent transplant model can be used to assess the beneficial effects, if any, of telomerase on graft function measured by animal survival.

VI-B) MATERIALS AND METHODS

1. Vector Cloning

The human telomerase gene (hTERT) insert (details in Chapter IV, Figure 4-1B) from the pGRN145 retroviral vector was re-cloned into a pRevTRE vector (Clontech™, Mountain View, CA, USA) by Technologies Inc.(Gaithersburg, MD, USA). The recombinant pRevTRE-hTERT vector was created by excising the hTERT gene insert from the pGRN145 vector with Eco RI digestion, after which the ends were blunted by a klenow fill-in reaction. The pRevTRE vector was digested with Hind III (at MCS site) and the ends were also filled by a klenow reaction. The "opened" vector and insert were ligated and transformed into E. coli DH10 B bacteria to mass produce the recombinant pRevTRE-hTERT vector (Figure 6-2B).

The pRevTRE-EGFP recombinant vector was cloned, by GenScript Corporation™ (Piscataway, NJ, USA), by ligating the EGFP gene insert excised from the pEGFP-NI vector (Clontech™) using BamH1 (vector position 661) and Hpa1 (vector position 1521). The EGFP insert was directly cloned into the pRevTRE vector after first being digested with the same 2 enzymes at the MCS (Figure 6-2A).

2. Optimization of Transient Transfection of AmphoPack™-293 Packaging Cells

AmphoPack-293 packaging cell line (Clontech™) was used to produce recombinant retroviral particles. AmphoPack-293 is a human embryonic kidney, HEK 293-derived cell line for rapid transient production of high tittered amphotropic retrovirus that can infect a broad range of mammalian cell types. The packaging cell line contains the gag, pol and env genes required to package the recombinant provirus into infectious particles. These cells are cultured in complete DMEM medium (reconstituted with 10% FBS, 2 mM L-glutamax and 1% penicillin/streptomycin). Plating out cells at 4 to 6 h prior to transfection was sufficient time to specifically allow for AmphoPack-293 cell attachment to culture dish.

DOTAP and calcium phosphate were the two methods evaluated for the efficiency of gene transfer into AmphoPack-293 cells using an EGFP reporter plasmid. Optimization parameters for DOTAP (Roche™, Laval, QC) included; the amount of DNA (1 ug - 7 ug) and percent FBS (2% - 5%) in the DMEM medium used to prepare the lipid transfection mixture. The DNA:lipid ratio was kept at 1:6. Procedures for preparing transfection mixture were detailed in Roche™ DOTAP reagent kit. Briefly, DNA was diluted to 0.1 ug/ul in HBS buffer in a sterile microcentrifuge tube. In a separate sterile tube DOTAP was mixed with HBS buffer at a 1:3 ratio. The nucleic acid solution was transferred to the reaction tube containing DOTAP and gently mixed. The transfection mixture was incubated at room temperature for 10 min before gently adding to cells plated in 6-well plates at a density of 1 X 10^6/9.4 cm^2. *(Note: AmphoPack-293 cells become detached from culture vessel very easily, so transfectant needed to be added to cells very gently.)* The cells with transfection mixture in a final volume of 1 ml

complete DMEM medium, were incubated under standard culture conditions (37°C and 5% CO_2 in a humidified incubator) for 24 h prior to detection using flow cytometry.

The CalPhos™ Mammalian Transfection Kit (Clontech™) was used with different optimization parameters including; percent FBS (2% - 10%), DNA amount (1 ug - 4 ug) and cell density ($3.5 \times 10^5 - 1 \times 10^6$) (35, 36). At the beginning of preparing transfection mixtures all reagents were at room temperature as they should be. The final volume of tube # 1 was 100 µl containing sterile water, DNA and 12.4 µl of 2 M $CaCl_2$ added in this order. To a second sterile microcentrifuge tube, 100 µl 2X HBS buffer solution was added. Tube # 2 was continually vortexed at intermediate speed while adding in the content of tube # 1 dropwise. The reaction tube was incubated between 1 min to 5 min at room temperature prior to adding dropwise to the plated AmphoPack-293 cells. The plates were then gently swirled before placing in a standard culture incubator for 24 h until detection by flow cytometry. Cell densities below 1×10^6 cells were plated on 12-well plates and a density of 1×10^6 cells/3.83 cm^2 were plated on 6-well plates. The final volume of the transfection mixture in complete DMEM medium was 1 ml.

EGFP fluorescence was detected using a FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA) with channel FL1 set for detection of fluorescein signal. Analysis of 10,000 gated events was performed using CELL-Quest software (Becton Dickinson™).

3. Virus Production and Infection

AmphoPack-293 packaging cells were plated on 12-well plates at a cell density of 3.5×10^5/3.83 cm^2 at 4 to 6 h prior to transfection using the CalPhos™ Mammalian Transfection Kit. The content of tube # 1, containing sterile water, 2 µg DNA

(pRevTRE, pRevTRE-EGFP, pRevTRE-hTERT) and 12.4 μl of 2 M $CaCl_2$, was added dropwise to tube 2 containing 2X HBS with vortexing. After a brief incubation at room temperature, the transfection mixture was added to cells overlaid with complete DMEM medium at 1 ml final volume. The cells were incubated under standard culture conditions for 48 h prior to collection of the virus-containing medium for infection. Viral stock solution was prepared by filtering the virus-containing medium through a 0.45 um polysulfonic (low protein binding) Whatman filter. *(Note: Nitrocellulose binds viral proteins destroying virus and should not be used.)* To avoid titer reduction the viral stock solution was used immediately at the time of collection.

4. Determination of Viral Titer

Protocol was modified from that described in the Retroviral Gene Transfer and Expression User Manual provided by Clontech™. NIH 3T3 cells (ATCC™, Manassas, VA, USA), cultured in complete DMEM medium, were plated onto 24-well plates at a cell density of 2 X 10^4/1.88 cm^2, 1 d prior to transduction. Six fold serial dilutions were prepared from the filtered viral stock solution in 6 sterile microcentrifuge tubes filled with 270 μl of medium containing 8 μg/ml polybrene. *(Note: Polybrene is a polycation used to reduce charge repulsion between the virus and cellular membrane.)* From the viral stock solution, 30 μl of virus-containing medium was added to tube # 1 and gently mixed. Then 30 μl of viral stock dilution from tube # 1 was added to tube # 2 and continued with the serial dilutions by transferring 30 μl of each successive dilution to the next prepared tube. DMEM medium in NIH 3T3 cells was replaced with 400 μl fresh DMEM containing 8 μg/ml polybrene. NIH 3T3 cells were infected by adding 200 μl of the diluted virus medium from each tube to separate wells. At 24 h the virus-containing

medium was removed and cells washed twice prior to adding fresh DMEM medium to the wells. Antibiotic selection started 24 h after infection. NIH 3T3 cells infected with virus containing the pRevTet-ON vector was selected in the presence of 1.2 mg/ml of Geneticin (Invitrogen™, Carlsbad, CA, USA) for a week. Cells infected with either the control vectors (pRevTRE and pRevTRE-EGFP) or pRevTRE-hTERT vector were selected in the presence of 0.4 mg/ml Hygromycin (Sigma™, Oakville, ON, USA). Uninfected NIH 3T3 cells were used as control for the antibiotic selection. The viral titer corresponds to the number of colonies present at the highest dilution that contains colonies, multiplied by the dilution factor.

5. Mice

Female rtTA-transgenic mice [Tg(rtTAhCMV)4Uh/J; *Stock No.* 003273] on a C57BL/6 background at 8-12 wks were purchased from the Jackson Laboratory™ (Bar Harbor, Maine, USA) at $229 US dollar per animal. Reported sites of constitutive rtTA expression from a human cytomegalovirus (hCMV) minimal promoter include the muscle, kidney, stomach, thymus, heart, and pancreas (jaxmice.jax.org/models/tet). These transgenic mice were maintained in a regular housing facility operated by Animal Services at the University of Alberta (Edmonton, AB, Canada). Normal Balb/c mice at 8-12 wks were purchased from Animal Services and also kept in a regular facility. All the animals had unrestricted access to food and water supplies.

6. Renal Microsurgery

a) Donor Procedure:

Renal microsurgeries were performed on the rtTA-transgenic mice by Dr. Lin Fu Zhu from the Microsurgery Laboratory at the University of Alberta. Prior to surgery, the

animals were anesthetized by inhalation of 1.5% Isoflurane and ophthalmic ointment was applied to the eyes. Each mouse was injected intravenously with 0.5 ml of warm lactated Ringer's solution to maintain blood pressure during the surgery. The abdomen was shaved, prepped with Betadine (10% povidone-iodine) and draped in a sterile fashion. The midline abdomen was incised open through the linea alba and the aorta below the renal arteries exposed. Aorta was isolated between 2 vascular clamps; one above the renal arteries and another above the bifurcation of the iliac arteries. The aorta was cannulated with a 24G angiocath. The right renal artery was occluded and the left kidney directly infused, via the cannulated aorta into the renal artery, with a solution containing the exogenous gene. Isolated perfusion to the left kidney was retained by leaving the distal end of the aorta clamped to prevent back flow of the gene solution. The gene solution contained the retrovirus carrying either pRevTRE-, pRevTRE-EGFP or pRevTRE-hTERT gene insert. After 20 min incubation *in situ*, the distal clamps were removed to re-establish blood flow. Doxycycline (Dox), at 2 mg/ml in 5% sucrose solution, was administered to the animals through drinking water for 3 d to 5 d to permit gene expression *in vivo* and allow sufficient time for the animal to recover from the surgery (37, 38). Following recovery the animals were again anesthetized, prepped and draped and the kidney harvested through the midline incision. The animals were sacrificed by cervical dislocation immediately after kidney harvest. At this stage, either gene expression was assessed or the kidney was transplanted into BALB/c recipients.

b) Recipient Procedure

BALB/c mice were used as transplant recipients of the C57BL/6 rtTA-transgenic kidney allografts tranduced with retrovirus carrying a control or hTERT gene. As for the

transplantation procedure, BALB/c mice were given 0.8 ml to 1.2 ml of warm lactated Ringer's solution intravenously for blood pressure maintenance. The abdomen was shaved, prepped with Betadine (10% povidine-iodine) and draped in a sterile fashion. The midline abdomen was incised open through the linea alba and the aorta below the renal arteries exposed. Aorta was isolated between 2 vascular clamps; one below the renal arteries and another above the bifurcation of the iliac arteries. After mobilization of the abdominal aorta and inferior vena cava, the kidney was transplanted by an end-to-end anastomosis between the aortic segment of the kidney graft and recipient aorta using 10-0 nylon sutures. The inferior vena cava patch of the graft was anastomosed end to side to the inferior vena cava with 10-0 nylon sutures. Body temperature of the recipient was retained with a heating blanket, overhead heating lamp and warm fluid infusion into the abdominal cavity.

c) Post-operative Care

After surgery, BALB/c transplant recipients were maintained in a warmed nursery incubator for the first 48 h in a small animal intensive unit in the Microsurgery Laboratory. Buprenorphine of 0.05 mg/kg was administered twice daily for the first 2 post-operative days. Mice also received 100 mg/kg Ancef intraperitoneally once as a prophylactic antibiotic pre-operatively. Animals had unrestricted access to food and water after transplantation. Fresh Dox (2 mg/ml in 5% sucrose) antibiotic solution was administered to the drinking water every 3-4 d to induce gene expression (39). Between 14 d and 17 d post-transplant, recipient BALB/c mice were sacrificed by anesthetic overdose. The criteria for euthanasia at an earlier date included; lethargy, dehydration

and moribund state. Predicted mortality and morbidity are approximately 5% due to surgical complications and was met in this study.

7. Immunohistochemistry for hTERT

a) Histology

All histological work was performed in the laboratory of HistoBest Inc., (Edmonton, AB, Canada). The samples were fixed in 4% neutral buffered formalin (Protocol™, Fisher Scientific Company, Ottawa, ON, Canada) for 24±1 h, dehydrated through a series of graded non-denatured ethanols, cleared and tissue-banked as previously described (40, 41). After re-grossing, the tissue samples were infiltrated under vacuum in paraffin for 6 hours (Peel-A-Way®, Micro-Cut Paraffin, Gold Standard Series, 56-58°C, Polysciences Inc., Warrington, PA, USA).

Sectioning was performed at 4-5 microns using R35 disposable microtome blades on a Jung 2235, rotary microtome (Leica Microsystems GmbH, Nussloch, Germany). Semi-serial sections were affixed onto premium aminosilane coated slides (TissuePath Superfrost® Plus Gold microscope slides, Fisher Scientific Company). The format used was of custom macroarrays with internal controls (liver, testes) included for every slide. The archived tissue biopsies for internal controls were provided by HistoBest Inc. After drying overnight at 37°C the slides were baked at 58°C for 5 minutes and used for IHC within 72 hours.

b) Immunohistochemical staining for hTERT on paraffin sections

Sections were deparaffinized via xylene, graded ethanols and rehydrated. The final protocol for immunostaining was determined after an extensive series of experiments using positive control animal/tissue and it was optimized for kidney.

Endogenous peroxidase was inactivated with 1% H2O2 in Tris buffered saline, 20mM Tween20, (DAKO™, Canada Inc.), for 15 minutes. Antigen retrieval was performed by microwaving in 10 mM citrate buffer, pH 6.0.

The hTERT primary antibody was a polyclonal Rabbit Ab (ab27573, recombinant fragment, encoding amino acids 174-341 of human telomerase (Abcam™ plc., Cambridge, UK), 4 μg/mL, for 60 mins, RT, diluted with Antibody diluent (DAKO™, Canada Inc.). Isotype controls at matching concentrations were performed throughout: Rabbit serum (ab7487, Abcam plc.) and Rabbit normal Immunoglobulin fraction (X0903, DAKO Canada Inc.). Detection was performed with Envision+, Rabbit (polyclonal IgG), HRP and DAB^+ (DAKO™, Canada Inc.). Color development was monitored under the microscope, (6 minutes, ±30 seconds). Nuclear counterstaining was done with 0.5% chloroform-purified Methyl green, in acetate buffer, pH 4.0 (HistoBest Inc.). After a brief washing with deionized water, dehydration was executed via butanol. Slides were cleared with xylene and mounted with Cytoseal 60 (Stephens Scientific™, Riverdale, NJ, USA).

c) Microscopy and scoring

The slides were examined, scored and photographed on a Nikon Eclipse 90i microscope, equipped with a DS-5M-L1 digital camera (Nikon™ Canada Inc., Mississauga, ON, Canada). At least five fields of cortex and three of medulla were examined at 200X magnification. Ten additional individual glomeruli were randomly selected for the glomerular readings. Blind scoring was performed by a scientist and a medical staff at the Histobest Inc. and hTERT expression was reported based on the average of 2 scores.

Positive staining was scored as follows:

0 = no staining

1 = faint tan colour

2 = faint, but distinct brown stippling evident

3 = moderate brown stippling

4 = strong brown stippling

5 = strong, uniform chestnut brown colour

VI-C) RESULTS

1. Transfection efficiency achieved in AmphoPack 293 cells using DOTAP and Calcium Phosphate methods

The highest mean efficiency achieved with the $CaPO_4$ method in the transfection of the AmphoPack-293 cell line after optimization of various parameters (%FBS, μg DNA, cell density) was 75±4% (n=8). The parameters used were 3.5 X 10^5 cells plated on 12-well plates and transfected in 10% FBS with 2 μg EGFP DNA. The plate was incubated under standard culture condition for 24 h prior to FACS analysis. On the other hand, transfection with DOTAP only yielded a mean efficiency of 8±1.5% (n=3) at 2% FBS and 2.5μg EGFP reporter plasmids. From all the different parameters assessed, the average efficiency of 8% was the best achieved for DOTAP in the AmphoPack-293 retroviral packaging cell line.

2. *In vivo* perfusion of rtTA-transgenic mouse kidneys with pRevTRE-EGFP

In vivo transduction in mouse kidneys was rapidly assessed by using an EGFP reporter gene. Dox was used to successfully induce EGFP expression at a concentration

of 2 mg/ml immediately administered following gene infusion. At concentrations below 2 mg/ml, only partial induction between 6% and 53% was reported (37). EGFP expression was observed mostly in the kidney tubules when organs were harvested at 3 d post-transduction (Figure 6-3). Transgene expression was also observed in the renal vasculature. Some studies have reported significant induction occurring as soon as 4 h post Dox administration in some organs, such as the kidney, pancreas and thymus, and was complete after 24 h unless Dox is continually replenished (37, 42). Other organs may take 4 d to 7 d for full induction of gene expression by Dox. Stability and sensitivity of the EGFP protein, which is not normally expressed in vertebrates, permitted rapid detection of green fluorescence on cryosections under confocal microscopy, well beyond the peak induction period (43). EGFP-expressing cells were also found in the liver. This finding was expected since the re-establishment of blood flow in the animal flushed the gene solution out of the kidney with resultant circulation to the liver delivering residual vectors to the hepatocytes.

3. Induced hTERT expression in kidney tubules after Dox administration

Normally the kidney has little, if any, telomerase expression *in vivo* (44). Following retroviral transduction and post-Dox induction, hTERT transgene expression was observed exclusively in the renal tubules and no protein was detected in the glomeruli (Figure 6-4). At each time interval (3 d, 7 d, 12 d), one animal was used for each treatment (pRevTRE versus pRevTRE-hTERT), except for the group at 12 d in which 2 animals were treated with the pRevTRE vector. With a continuous supply of fresh Dox, induction was observed up to 12 d on which the animals were sacrificed suggesting stable hTERT expression was achieved with retroviral vectors. Higher

background staining was observed on tissue sections prepared from kidneys harvested on day 7, which is most likely an isolated case and may be related to tissue processing of this batch (Figure 6-4). Increased endogenous peroxidase activity or other tissue variables could contribute to higher background staining, as well the use of a polyclonal secondary IgG antibody may cross react with tissue proteins. The anti-hTERT rabbit polyclonal antibody appeared to cross react with mTERT, leading to intense staining in the testis known to be positive for TERT and weak in the mouse liver. Moreover, this primary anti-hTERT antibody from Abcam™ has not been tested for cross reactivity in mouse by the supplier. However, since the nucleotide sequence homology between mTERT and hTERT is 74% and protein similarity is 69% it would be reasonable to find cross reactivity, particularly when using a polyclonal primary antibody (44).

3. Genetically modified kidney allografts transplanted into BALB/c mice

Gross morphological changes were observed in the C57BL/6 kidney grafts genetically modified with either the pRevTRE control (Figure 6-5) or pRevTRE-hTERT vector (Figure 6-6). Macroscopic findings of tissue discoloration and swelling, suggestive of acute rejection, were found in all grafts (n=3 for each vector treatment group) harvested at approximately 2 wk post-transplantation. The pRevTRE-treated kidney (Figure 6-5C) seemed more severely rejected compared to the pRevTRE-hTERT-infused graft (Figure 6-6C) based on the extent of tissue discoloration and swelling. In contrast, the BALB/c recipient's native kidney appeared normal in color and morphology thus remained healthy-looking. In a second part of the experiment, in which the native kidney was removed from the BALB/c recipient between 10-12 d post-engraftment and renal function relied solely on the allograft, animal survival did not surpass 5 d in both

treatment groups (pRevTRE versus pRevTRE-hTERT). Similar survival rates were found when the experiment was repeated with another set of animals. These observations suggest severe allograft rejection. Genetic modification with hTERT did not appear to improve the survival outcomes of both grafts and recipients in this preliminary study as would be expected with untreated acute rejection. The hypothesized role for hTERT rejuvenation being with long-term chronic rejection which would require protracted immunosuppression.

VI-D) DISCUSSION

Allograft rejection occurs when the recipient's immune system recognizes donor tissue as foreign and attacks the graft. The primary exposure to graft alloantigens evokes acute rejection which occurs at 1-2 weeks following transplantation. With the use of immunosuppression, graft loss due to acute rejection is less than 10% (45). In the absence of immunosuppression, vascularized solid organ grafts are severely damaged by the infiltration of host leukocytes that mediates rapid rejection and causes graft cell necrosis and vessel thrombosis. The bulk of the immune response is directed against donor MHCs in acute rejection, whereas the specific target antigen(s) is still undefined in chronic rejection, but MHC incompatibility also influences the latter process (46). Even though the precise relationship between acute and chronic rejection remains uncertain, the incidence of acute rejection increases the risk of chronic rejection that develops over months or years. Any previous tissue injuries (i.e. brain-death injury, ischemia/reperfusion, prolonged cold storage, hypertension/hyperlipidemia, CMV infection, nephrotoxic drugs) also add to the risk of chronic rejection (46, 47). Chronic

rejection results from an insidious, low grade immune response stimulated against cryptic organ-specific antigens exposed during transplant injury (46) resulting in chronic inflammation, of which immunosuppression is ineffective. Chronic rejection, in particular Chronic Allograft Nephropathy (CAN), is characterized by excessive tissue fibrosis and sclerosis in the graft parenchyma and vasculature, thereby destroying the normal tissue architecture. Unfortunately, our animal transplant model failed to develop chronic rejection as the grafts were rapidly rejected without using immunosuppression. The reported success rate of spontaneously developing lesions of chronic rejection using this mouse strain combination, C57BL/6 versus BALB/c, was only 20% (48). All 3 harvested kidney grafts in our study showed signs of acute rejection, portrayed by tissue discoloration and swelling. The endpoint of acute graft dysfunction was death of the transplant recipients who had their native kidneys removed. Function of the rejecting C57BL/6 graft alone could not support the survival of BALB/c recipients beyond 5 d. Both the TRE- and hTERT-modified renal allografts had no effects on the progress of acute rejection, which is expected. Telomerase interventions will be for dividing cells subjected to chronic stress and, as a result, experience significant telomere loss leading to the loss of normal replicative capacity. When normal cells approach replicative crisis due to critical telomere shortening, they become senescent and their ability to mount tissue repair is impaired, which gradually results in graft loss.

Experimental evidence suggests that renal cell senescence plays a critical role in the progressive deterioration of kidney graft function (49-51). Markers of senescence (i.e. SA-βgal, telomere shortening) are found in the tubular epithelium contributing to the lesions of tubular atrophy and interstitial fibrosis (49, 50, 52). A senescent phenotype is

not restricted to only replicative exhaustion, as senescent cells also express a pronounced inflammatory phenotype that contributes to tissue aging and age-related pathologies (53). Expression of pro-inflammatory cytokines and cell adhesion molecules were high in senescent cells (54). For instance, adhesion molecule ICAM-1 is predominantly upregulated in senescent human fibroblasts and endothelial cells (55). This cell adhesion molecule aids in the recruitment of lymphocytes to inflamed or injured tissue (56). Elevated expression of p53 in senescent cells directly activates ICAM-1 expression (57), which provides costimulatory signal for T cell activation (58, 59). When graft cells exhaust their reparative capability in the face of persistent immune-mediated injury, they eventually become replicatively senescent. In turn, these senescent graft cells, which are still metabolically active, produce inflammatory mediators that help perpetuate the chronic process of inflammation in a feedback loop, leading to chronic rejection (60). Kidney allografts, diagnosed with CAN, showed increased expression of cell adhesion molecules along with tissue infiltration by macrophages and T cells consistent with an immune-mediated inflammation (46). This low grade immune reaction is, in part, contributed by the presence of senescent graft cells adopting an inflammatory phenotype. We hypothesize that telomerase gene therapy is effective in subduing this chronic picture by endowing graft cells with unlimited reparative ability to prevent telomere-driven cell senescence.

Even though the concept of gene therapy is intriguing and holds a great promise in effecting a cure at the most fundamental level, gene medicine still remains in the experimental phase. Progress in gene therapy is practically stalled by limitations in gene vehicles being the biggest obstacle. Preferably among the available viral vectors,

adenoviral vectors have been utilized in many *in vivo* studies due to high transgene expression in both mitotic and quiescent cells (61-63). Despite this fact, adenoviral vectors, as well as other forms of viral and nonviral vectors, have varying degrees of success in genetically modifying solid organs. Efficiency of gene transfer however tends to be low and sporadic, thus, absolute transfection has yet to be achieved in whole organs. In our study, both the EGFP and hTERT genes were mainly expressed in the renal tubules following retroviral transduction, and no expression found in the glomeruli. As low as 1% transfection efficiency was documented in the glomeruli (64). As reported by many authors, transgene expression was found almost exclusively in either the glomerular or tubular compartment (65-69). Such an observation may be related to the anatomical function of the individual structure within the kidney. In spite of an easily accessible route via the renal circulation, the glomerulus is generally difficult to transfect. Perhaps it is due, in part, to the rapid and constant filtration of fluid through this structure that prevents productive contacts between the gene vectors and the glomerular surface to effect significant *in vivo* transduction. Gene vehicles that do not require active cell division to facilitate exogenous DNA uptake tend to produce transfection outcomes in the glomerular compartment. For instance, these methods include adenovirus, lipofection and electroporation. The steady-state proliferative index in the glomerulus is less than 0.1% whereas 0.44% of tubular cells exhibit proliferation in human kidneys (70). Given a higher proliferative index in kidney tubules, this compartment was relatively easier to transduce than others by retrovirus, as observed in our study and that of Bosch et al. who reported an efficiency of 0.5-1% in tubular cells (69). We have achieved transduction of the renal tubules, as well as the blood vessels. Since tubular cells reabsorb the filtrated

viral vectors that pass the filtration barrier will be collected in the tubules, which partially explain the EGFP and hTERT expression found exclusively in renal tubules and not the glomeruli. We also observed EGFP expression in the liver and this was expected (71, 72). Retrovirus is not commonly adopted for gene transfer into kidney *in vivo* because the overall proliferative index of all solid organs, is extremely low. To our knowledge, our study is the second one only conducted after Bosch et al. which uses a retroviral vector.

Despite hTERT expression in renal tubules, the majority of protein was localized in the cytoplasm even though some speckles were seen in the nucleus. Nuclear translocation upon phosphorylation of the catalytic subunit is required for assembly of the functional holoenzyme complex that acts on telomeres in the nucleus during DNA replication in S phase (73, 74). However, we observed negligible hTERT expression in the nuclear compartment of transduced tubular cells suggesting that the inactive translational products of hTERT accumulated, but not translocated. The absence of signalling for nuclear translocation is perhaps due to the undividing status of virtually all differentiated tissue cells. To the contrary, cells normally expressing telomerase, such as HeLa cells and MEFs, displayed TERT protein in the nucleus throughout all stages of the cell cycle (44). Martin-Rivera et al. found a 3-fold increase in hTERT proteins in both HeLa and MEFs as more cells entered S phase. Different from normal somatic cells, telomerase expression did not appear to be regulated by nuclear transport in these cells, as activity is constantly expressed (74, 75).

To continue testing our hypothesis, we propose to use immunosuppression to prevent acute rejection in the animal model used in this study in an attempt to develop

chronic rejection. Another strategy is to use a different strain combination, a C57BL/6 versus C3H/HeN model (48). This transplant model has shown to develop features of chronic rejection at 100 d after the graft has been spontaneously accepted in the absence of immunosuppression. Future studies will also include transplantation of normal and mTERT-transgenic kidneys into normal allogeneic mice and into recipients having senescence-accelerated disease (76, 77, 78). If telomerase imparts survival advantage, mTERT-transgenic kidney grafts should be able to sustain renal function longer than control kidneys in both allogeneic and premature aging recipients. These experiments will permit the direct evaluation of telomerase in graft survival.

Although chronic rejection did not develop in our mouse model to allow testing of the telomere hypothesis, this study, nevertheless, provided preliminary data on the *in vivo* gene transfection of kidneys. Furthermore, telomerase gene induction by Dox was also demonstrated to establish the proof of principle. If transduction efficiency remains low using retroviral vectors, which is anticipated, in slowly dividing cells, a closed-circuit perfusion system (79, 80) may be the other feasible alternative in future studies. The ultimate goal is to deliver telomerase into kidneys to build up the telomere reserve, at times of chronic stress, by turning on telomerase expression with Dox whenever this enzyme is needed. This Dox switch is a safeguard against potential tumorigenesis from constitutive telomerase overexpression (81). We believe that this strategy of using telomerase to prolong graft survival may help meet the high demands of clinical organ transplantation.

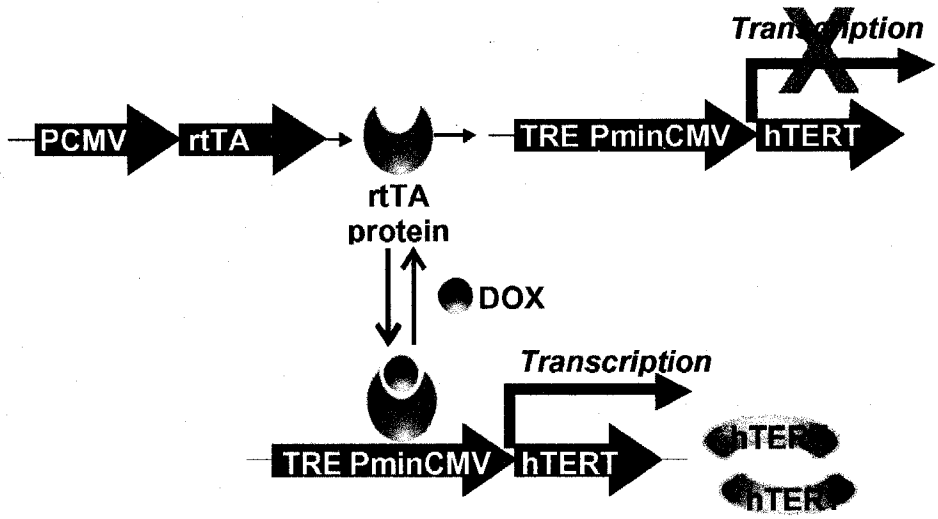

Figure 6-1: Tet-ON dual vector system. The expression of the rtTA transactivator from the first expression cassette is bound by Dox. Together, this complex binds to the minimal CMV promoter of the response vector to activate transcription of the hTERT gene (bottom). The absence of Dox results in no transcription of hTERT (top).

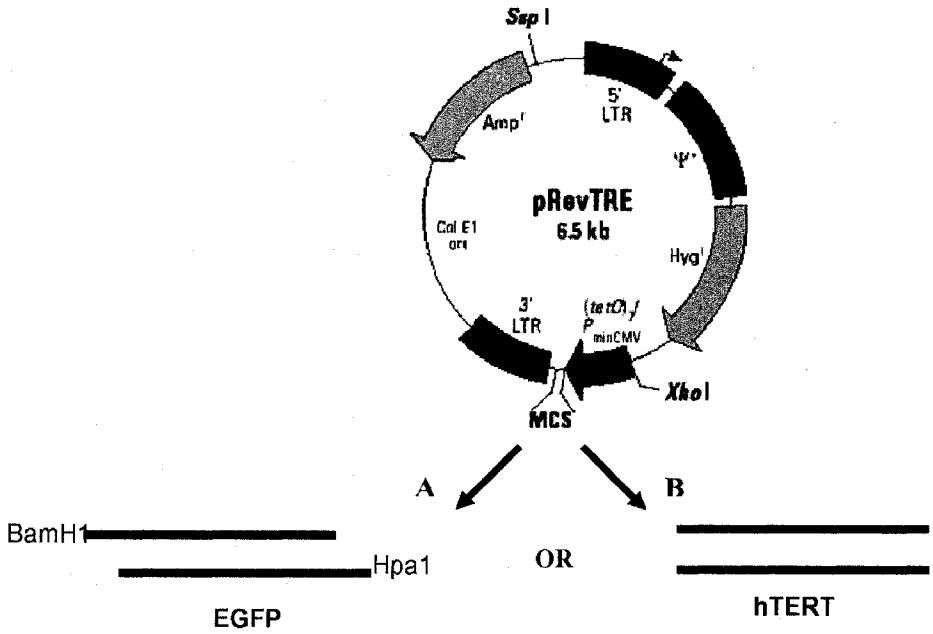

Figure 6-2: Restriction map and multiple cloning site (MCS) of pRevTRE vector (Clontech™). The EGFP and hTERT genes were cloned into the MCS site of the pRevTRE vector, using restriction enzyme digestion, to produce the **(A)** pRevTRE-EGFP and **(B)** pRevTRE-hTERT recombinant vectors.

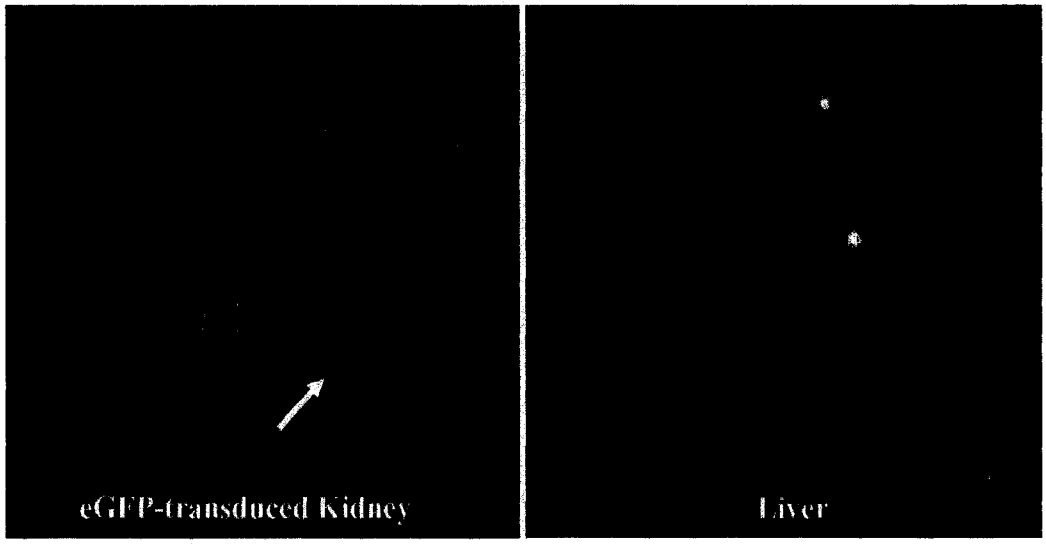

Figure 6-3: *In vivo* EGFP reporter gene expression was observed in the kidney of C57BL/6 rtTA-transgenic mice infused with the pRevTRE-EGFP vector via the abdominal aorta directly into renal artery. EGFP expression was found in both the renal tubules (yellow arrow) and vasculature (blue arrow) at 3 d post-transduction. The liver also expressed EGFP due to circulation of the EGFP vectors to the liver when blood flow was re-established after infusion. Propidium iodide was used as a counterstain (red nuclei). (original magnification x 400)

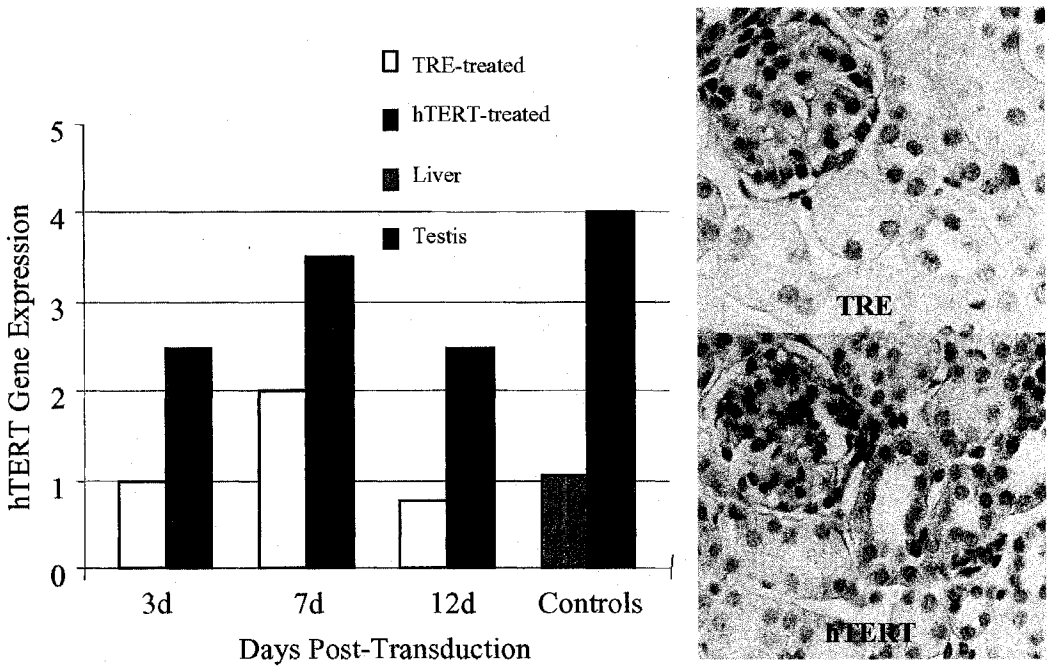

Figure 6-4: Expression of hTERT in the renal tubules after Dox induction. pRevTRE-hTERT infused kidneys of rtTA-transgenic C57BL/6 mice showed presence of the hTERT protein in tubular cells. Negligible hTERT expression was found in the glomeruli. hTERT expression was detected until the animals were sacrificed at 12 d. Strong hTERT expression was observed in the testis and weak in the liver. Each bar represents a single mouse treated either with pRevTRE or pRevTRE-hTERT vector, except for the pRevTRE group at 12 d in which 2 animals were treated. An average score was obtained from 2 blind readings of the staining on kidney sections. (picture original magnification x 400)

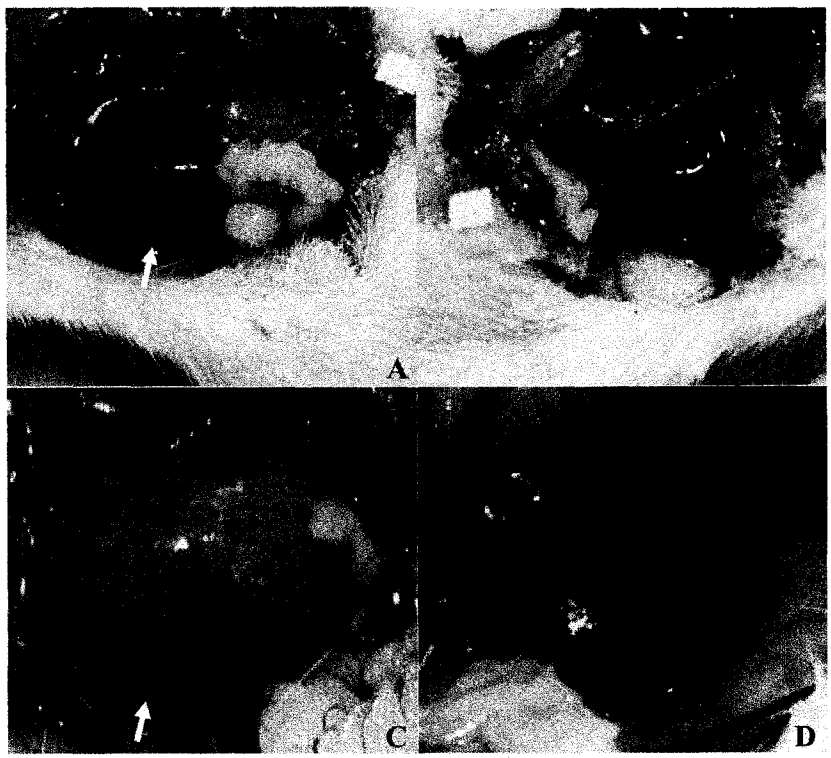

Figure 6-5: C57BL/6 kidney allografts transduced with pRevTRE control vectors. **(A, C)** Control pRevTRE-infused kidneys harvested from BALB/c transplant recipients. Tissue discoloration and swelling occurred in the genetically modified renal allografts (yellow arrow) due to rejection. **(B, D)** These were native kidneys harvested from the same transplanted BALB/c recipients. They appeared normal in color and morphology macroscopically. Pictures were taken with a Nikon digital camera from 2 sets of experiments.

Figure 6-6: C57BL/6 kidney allografts transduced with pRevTRE-hTERT vectors. **(A, C)** hTERT-expressing kidney allografts harvested from BALB/c transplant recipients. Abnormal tissue discoloration and minor swelling were observed in the pRevTRE-hTERT transduced kidneys suggestive of allograft rejection (yellow arrow). **(B, D)** The native kidneys harvested from the same BALB/c mice appeared normal in color and morphology. Pictures were taken with a Nikon digital camera from 2 sets of experiments.

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CHAPTER VII

GENERAL DISCUSSION AND CONCLUSIONS

VII-A) GENERAL DISCUSSION

Given that the endothelium is the immediate barrier between the circulation and tissues, as well as being a medium for the transport of cells and blood components, it is often believed to be the primary target of insults and transplant rejection. Endothelial damage, initiated in the pre-transplant and early post-transplant periods, evokes a non-specific inflammatory response to injury, that if not properly controlled, leads to neointimal hyperplasia, and ultimately obliterative endarderitis. Cold organ storage is employed to slow the rate of cellular metabolism at low temperature, and thereby delay the onset of cell/tissue injury (1, 2). Unfortunately, transfection efficiency at a low temperature would be significantly lower, as observed in our endothelial cell model *in vitro*. A slow metabolic rate and reduced fluidity of cell membrane at a cold temperature may contribute to low DNA uptake. Additionally, increased stability of the DNA:lipid transfection complexes at a low temperature may also slow the release of DNA inside the cell. Given the low transfection rates we found at 6°C, hTERT transgene delivery *ex vivo* into solid organ transplants will be limited during cold storage, unless gene delivery into organs can be maximized by raising the temperature for transfection. The alternative approach would be to improve efficacy of nonviral-mediated gene transfection through extended periods of *ex vivo* perfusion of kidneys physiologically maintained during transport in a bioartificial system that permits temperature control and pulsatile perfusion (3, 4).

Because hTERT-transfected HUVECs transiently expressed telomerase, such activity only yielded a small bp extension in HUVEC telomeres. This few hundred bp addition to telomeres suggests that telomerase may or may not be a therapeutically important gene remedy for prolonging allograft survival. The low efficiency of gene transfer and unstable expression of telomerase, not normally activated in human somatic cells, have urged researchers to examine different approaches to telomere maintenance. The rolling circle mechanism is a telomerase-independent mechanism proposed by Kool et al. (5, 6). The use of nanocircular DNA, containing the 5'-CCAATCCCAATCCCA-3' sequence motif, represents the telomerase RNA template to the telomeric 3' end that acts as a primer for DNA polymerase-catalyzed elongation, by rolling of the nanocircle to produce new telomeric repeats. These nanocircles appear similar to the extrachromosomal telomeric DNA circles store in APBs found in ALT-activated cells. The use of DNA nanocircles in proposed mechanisms mirroring the ALT mechanism is still a far fetched approach because, to date, the ALT pathway does not exist in normal human cells (7). Its presence in human tumors is less than 10% (8) suggesting that telomerase is the prevalent mechanism of telomere maintenance in humans. Despite this, Telomolecular™ Corporation (Sacramento, CA, USA) has designed DNA nanocircles that have been shown to elongate telomeres *in vitro*. They are looking further into delivering vTERT, a synthetic enzyme that synthesizes and repairs telomeres, with biodegradable nanoparticles into human cells.

In an *in vitro* HUVEC model, we have demonstrated the shortening of telomeres in senescent cells lacking telomerase activity. Wound healing was impaired in senescing HUVECs due to low rates of cell proliferation and migration. A differentiated phenotype

of endothelial cells to form tubules on a basement membrane was also compromised, reflecting defective angiogenesis due to cell aging. However, when young (P7) and senescing (P18) HUVECs were transfected with telomerase, vessel sprouting on matrigel was enhanced. These *in vitro* findings underscore the therapeutic relevance of telomerase gene therapy in graft revascularization. Impaired neovascularization would be detrimental to the survival of solid organ and tissue transplants.

Graft survival beyond 5 years is well below 50% as seen in cases of Chronic Allograft Nephropathy (CAN), the second biggest threat to long-term survival of kidney transplants besides patient death (9). Senescent tubular cells were found in 67% of renal patient biopsies that showed signs of severe CAN (10). Telomeres were shorter in senescent tubular cells than their non-senescent counterparts. Chronological age of donor organs correlated strongly with the number of senescent cells detected in CAN biopsies. Therefore, it is clinically relevant that we have achieved telomerase transfection in the tubules of mouse kidneys infused with retroviral vectors. The induced telomerase expression in tubular cells provides a means to overcome telomere shortening, as demonstrated in HUVECs. The main drawback of retroviral vectors is the requirement for cell division, which poses a huge hurdle for delivering telomerase into solid organ transplants. The shortcoming of this recombinant virus commonly used in human gene therapy clinical trials is accepted in our experimental design when stable transfection is desired. Unfortunately, long-term transgene expression imparted by retroviral vectors may be lost due to DNA methylation of the promoter (11-13). Promoter silencing happens in plasmid vectors as well, leading to a much shorter expression period than retroviral promoters (14). This transient telomerase expression led to only a short

extension of telomere length, which may have limited long-term clinical application. Even though retrovirus permits a more stable transgene expression, gene silencing *in vitro* (11, 12) and *in vivo* (13) has occurred as a result of methylation of the enhancer sequences in retroviral long terminal repeats (LTRs) or of the coding regions. Lentivirus can be another choice of vector to achieve stable transgene expression in both dividing and non-dividing cells. However, lentiviral vectors still face the same problem of low *in vivo* transduction efficiency in kidneys (15).

To date, the persistent clinical problem of chronic rejection remains a big challenge for transplantation medicine. Despite improvement in immunosuppression therapy, long-term allograft survival has yet to be achieved. Late graft failure caused by chronic rejection continues to be the reason for lengthening of the transplant waiting lists. Overlapping pathological lesions found in chronically rejected and aging kidneys suggest that renal cell senescence contributes to late graft dysfunction. There are 2 ways to approach senescence. Either by turning on genes that allow the bypass of senescence, such as telomerase, or by shutting off genes that are associated with senescence. Inhibiting expression of senescence-associated genes may help attenuate the aging process and prolong allograft survival. Elevated expression of p16, p53, p21 and TGF-β1 were detected in aging kidneys (16-18). If gene therapy can be used to activate certain genes, RNA interference (RNAi) can be used to inactivate them. Small interference RNA (siRNA) technology can be used to elicit degradation of specific mRNAs of the senescence genes to attenuate renal senescence in allografts.

Gene therapy and siRNA are restricted by the number of genes they can affect in cells. Cell therapy is one way to overcome this limitation by replacing the cells

themselves. Cell therapy is a technology that uses healthy functional cells (autologous or allogeneic) modified *ex-vivo* or unmodified, to replace diseased or dysfunctional cells. Stem cells are unspecialized cells that possess unlimited self-renewal capacity and are capable of differentiating into any specialized cell type. Embryonic stem (ES) cells are totipotent and can differentiate into any organ cells. In turn, pluripotent adult or tissue-specific stem cells, derived from ES cells, give rise to lineage-restricted cells comprised of an organ. Currently, it remains unknown whether an adult kidney harbors any pluripotent stem cells among its multiple cell types (19). No single progenitor cell capable of regenerating the whole kidney (i.e. giving rise to all epithelial cell types of a nephron) has yet been found (20). Apparently bone marrow (BM)-derived progenitor cells serve as the renal stem cell compartment, from both mesenchymal stem cells (MSCs) and hematopoeitic stems cells (HSCs). Both of these regenerate damaged renal tissues after ischemic and chemical injury (21-23). Most, if not all, HSCs have a limited proliferative potential that decreases with age due to telomere shortening during cell division (24). The low and transient telomerase activity was unable to prevent telomere attrition in these cells (25). In particular, not all human stem cell populations demonstrated telomerase expression. In the MSC population, telomerase activity above background level was undetected, resulting in telomere shortening during the *in vitro* expansion of these cells leading to replicative senescence (26-28). Therefore, telomerase may indeed play an important role in stem cell therapy as well. Use of stem cells in cell-based therapeutics, with or without hTERT gene modification, provides an attractive alternative to gene therapy. The discovery of progenitor cells is anticipated to have a significant impact in the field of clinical transplantation.

VII-B) CONCLUSIONS

Overall **HYPOTHESIS:** The longevity of cell and tissue transplants can be extended via *ex vivo* or *in vivo* telomerase gene therapy. Our **Overall AIM** is to improve the long-term survival of renal allografts, as well as other solid organ transplants, using human telomerase gene therapy to attenuate cell senescence.

SPECIFIC AIM # 1: To examine and optimize various transfection parameters to achieve high efficiency of transgene expression in human endothelial cells.

Lipofection is an efficient gene transfection method for human endothelial cells. A mean efficiency above 30% was achieved at 37°C. Although transfection efficiency was compromised at lower temperature, successful transfection at 6°C was still being achieved with lipofection, albeit at a mean efficiency below 10%. Transfection in the presence of high glucose in the medium had no adverse effects on cell viability and transfection rates. However, an increased exposure time to the lipid transfectant caused significant toxicity.

SPECIFIC AIM # 2: To examine telomerase activity and telomere maintenance at pre- and post-hTERT transfection in HUVECs. As well, to assess the growth advantage and potential malignant changes in phenotype of hTERT-transfected HUVECs.

Despite transient telomerase activity, telomerase maintained telomere length in human endothelial cells that senesced with age in culture. Normal senescent cells were defective in wound repair. Expression of the enzyme enhanced vessel sprouting on the matrigel basement membrane. These observations have therapeutic implications in wound healing and angiogenesis. hTERT-expressing HUVECs also recovered faster

from serum-deprived stress and resulted in a higher proliferative index. Although hTERT-transfected HUVECs showed increased proliferative potential, they retained a normal functional phenotype. Growth of hTERT-expressing HUVECs remained contact-inhibited and cell cycle intact.

SPECIFIC AIM # 3: To examine islet transfection using the EGFP reporter and hTERT genes.

Native human islets do not express telomerase and the rate of telomere attrition *in vivo* is slow. Lipofection is an effective strategy for islet transfection, and it does not cause apparent toxicity. Islet function measured by insulin secretion remained intact. Transfection of human islets with hTERT resulted in significantly higher hTERT mRNA levels than in untransfected controls. Majority of the transfected cells within an islet was at the periphery.

SPECIFIC AIM # 4: To examine telomerase expression *in vivo* in transfected kidney grafts derived from C57BL/6 rtTA-transgenic mice. To compare survival outcomes from BALB/c recipients receiving renal transplants that stably express either the hTERT gene or TRE control.

In vivo hTERT expression was successfully induced with Dox in kidneys infused with retroviral vectors. Stable expression of hTERT was localized in the renal tubules. Kidney allografts showed signs of acute rejection when assessed at approximately 2 weeks post-transplantation. A major limitation of the mouse strain combination (C57BL/6 donors to BALB/c recipients) used in our transplant model is acute rejection. Immunosuppression is needed to stop the acute rejection and, in time, hopefully the mice develop chronic rejection, specifically features of CAN. Since we did not use

immunosuppression, chronic rejection did not develop in the C57BL/6 allografts, which prevented us from evaluating the therapeutic significance of telomerase gene therapy using this model. Currently, a good animal model harboring pathological lesions of CAN is lacking and, as a result, research is needed to develop or expand on the current model system.

In **FUTURE STUDIES**, senescence and telomere shortening in kidney transplants will be conducted with kidneys obtained from female C57BL/6, telomerase knock-out (KO) mice engrafted into syngeneic female C57BL/6 recipients. The degrees of *in vivo* cell senescence and telomere shortening are not expected to be accelerated in syngeneic recipients; in fact, these findings will be comparable to those exhibit by the donors. However, when KO kidneys are transplanted into an allogeneic strain, both senescence and telomere shortening are expected to increase under transplantation-related stress. Transplantation of KO kidneys from female C57BL/6 mice into syngeneic male recipients may develop features of chronic rejection, in particular CAN. If this is the case, the relationship between senescence/chronic stress and the clinical course of the disease can be established.

To use human tissues in future *in vivo* experiments, we plan to transplant human islets into diabetic nude mice. This animal model allows us to assess whether chronic disease, such as diabetes, exacerbates telomere shortening in islets and causes cell senescence. Some studies showed that glucose responsiveness of human islets transplanted into diabetic nude mice declined with time (29, 30) due to loss of β-cells (31). Whether this loss of function can be attributed to cell senescence remains to be determined with senescence markers and telomere length assessment. In turn, can

hTERT-expressing islets maintain telomere length and prolong islet function *in vivo*? Protective effects of telomerase from senescence can also be assessed in hTERT-transfected β-cells by quantifying the β-cell mass and insulin production as an indicator of islet function. We expect to find improved survival of hTERT-modified islet grafts with normal *in vivo* function. Human islets provide us a simple tissue model for gene manipulation and for the evaluation of transplantation-related telomere shortening and cell senescence. Given that our laboratory has achieved optimal transfection of human islets, we are hopeful that telomerase gene therapy has therapeutic potential in telomere-driven cell senescence providing that this mechanism contributes to human islet graft dysfunction.

After the establishment of the "proof of principle" *in vitro* and demonstration of successful hTERT gene delivery *in vivo*, the studies in this thesis provide a framework for future studies in utilizing telomerase gene therapy as a strategy to prolong cell and tissue functions. The therapeutic importance of stem cell research should not be overlooked; knowledge in areas of stem cell mobilization, proliferation, and differentiation is particularly essential to clinical transplantation. The combination of stem cell therapy with hTERT gene modification could, in the future, help alleviate the shortage of organ donation by creation of a more robust supply of human tissues. This thesis on telomerase gene therapy is an important stepping stone towards the ultimate goal of improving the long-term survival of solid organ transplants and may potentially increase the effectiveness of stem cell therapy.

VII-C) REFERENCES

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