



**Role of oxidative stress and telomerase on
haematopoietic stem and progenitor cell ageing**

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Thesis submitted in fulfillment of the requirements
of the regulations for the degree of Doctor of Philosophy

Newcastle University

Faculty of Medical Sciences

Institute of Genetic Medicine

April 2014

ACKNOWLEDGMENTS

I would like to sincerely thank my supervisor Prof. Ioakim Spyridopoulos (Kim), who was always guiding, encouraging, and supporting me throughout the years. Kim, I can't thank you enough. Thanks to my co-supervisors Prof. Maya Sieber-Blum and Dr. Jola Weaver. Thanks to my examiners Prof. Susan Lindsay and Prof. Helen Arthur for their valuable comments during my past assessments. A special thanks to Dr. Colin Miles for guiding me in the laboratory with the mice work and for his tremendous support and advice that I really appreciate it. Many thanks to Dr. Gabriele Saretzki, who kindly provided me with the mice samples; she was always nice and very helpful during discussions and when analysing my data. I would also like to thank the people who taught me in the laboratory when I first arrived: Anuradha Doddaballapur and Monika Loehner; I appreciate their patience and amazing skills. I am really grateful for my best friend in Newcastle, Bothinah Altaf, who was a true sister and was very patient with me during the years. Many thanks to my friend Bashayer for her support and prayers. Thanks to my friends, Aisha and Maryam, who I met here and they left but never stopped asking and caring. Thanks to my friends in the institute, Mahsa, Marina, Vipul, Charlie, Mohammad, Lisa, and Harsh for being always funny, supportive and encouraging. Thanks to my group and to the institute of genetic medicine for providing us with an excellent research environment. Last but not least, I would like to express my deepest thanks and gratitude to my loving family and friends back home who always cared, loved, supported, and pushed me to accomplish good things I had never imagined doing before. Thank you dear father, mother, my brother Saud and his wife Fatimah, my brother Abdulrahman, and my sisters Sarah, Wadha, and Fatimah. Many thanks to my friends since high school Maha, Shaikha, Nawal, and Fatimah. I truly love you all.

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ABSTRACT

Ageing is associated with the functional decline of both haematopoietic stem and endothelial progenitor cells (HSPCs and EPCs), leading to an imbalance between cellular damage and repair. Telomeres are the end caps of chromosomes that maintain chromosomal integrity and shorten with age. Telomerase is the enzyme responsible for telomere replication. The absence of telomerase leads to premature ageing. Oxidative stress as well as metabolic stress and short telomeres are key contributors to the manifestation of different age-related diseases. However, the exact effect of these factors on HSPCs and EPCs is not clear.

The effects of metabolic stress were studied by the addition of different glucose concentrations to low passage (early) cells in culture. Metabolic stress impaired the growth of EPCs and CD34+ HSPCs. There was no change in telomerase enzyme activity under metabolic stress in CD34+ HSPCs. However, metabolic stress upregulated the metabolic co-activator PGC-1 α in EPCs but not in CD34+ HSPCs.

The effects of oxidative stress were investigated by incubating peripheral blood EPCs, and cord blood CD34+ expanded HSPCs under 40% O₂ in culture. While early EPCs show resistance to oxidative stress, CD34+ HSPCs showed impaired growth and differentiation potential. This impairment was associated with increased telomerase activity, no changes to TERT or TERC expression, and maintenance of telomere length. Oxidative stress limited CD34+ HSPC myeloid differentiation. In particular, CD15+ granulocytes were more sensitive to oxidative stress than CD14+ monocytes. Furthermore, CD15+ granulocytes reduced the expression of TERC during myeloid differentiation. In contrast to CD34+ HSPCs under growth conditions, there was no increase in telomerase activity during myeloid differentiation under oxidative stress.

To investigate the effects of ageing in vivo with telomerase dysfunction, HSPCs from bone marrow of aged telomerase deficient TERT^{-/-} and TERC^{-/-} first generation mice were studied. Ageing resulted in the accumulation of Lineage⁻Sca-1⁺CKit⁺ stem cells and CFU-GM colonies in wild type mice. TERT^{-/-} mice without telomere shortening showed a normal phenotype at young age (1.5-7.5 months) and augmented ageing of bone marrow with increased age (22 months). On the other hand, TERC^{-/-} mice with short telomeres led to a premature ageing bone marrow phenotype, even at young ages (8-12 months). Interestingly, both TERT^{-/-} and TERC^{-/-} showed more erythroid progenitor colonies. Furthermore, short- (7 months) and long-term (16 months) dietary restrictions ameliorated the ageing bone marrow phenotype.

Together, the data presented demonstrates the damaging effect of oxidative and metabolic stress on humans in early EPCs and CD34+ HSPCs. In a mouse model, normal ageing disrupted HSPCs. Telomerase deficiency augmented normal ageing, whilst short telomeres appear to be a major determinant of ageing. These ageing phenotypes in mice can be ameliorated by dietary restriction.

ABBREVIATIONS

ABBREVIATION	FULL NOMENCALTURE
•OH	Hydroxyl radical
4-OHT	4-Hydroxytestosterone
4E-BP	Eukaryotic translation initiation factor 4E-binding protein
AAV	Adeno-associated virus
acLDL	Acetylated low-density lipoprotein
ADP	Adenosine diphosphate
AGM	Aorta-gonad-mesonephros
Agtr1a	Angiotensin II receptor type 1a
AKT	Protein kinase B
ALT	Alternative lengthening of telomeres
AML	Acute myeloid leukemia
AMP	Adenosine monophosphate
AMPK	Adenosine monophosphate AMP-activated protein kinase
AngII	Angiotensin II
ATP	Adenosine triphosphate
BFU-E	Burst-forming unit-Erythroid
BM	Bone marrow
C-Kit	Mast/stem cell growth factor receptor (SCFR) or CD117
CACs	Circulating angiogenic cells
CD34	Cluster of differentiation 34
Cdkn1a and 2a	Cyclin-dependent kinase inhibitor 1a and 2a
CEPs	Circulating endothelial precursors
CFC	Colony-forming cell
CFU-Bas	Colony-forming unit-Basophils
CFU-ECs	Endothelial colony-forming units
CFU-Eo	Colony-forming unit-Eosinophil
CFU-GEMM	Colony forming unit-Granulocytes, erythroid, megakaryocytes, and monocytes
CFU-GM	Colony forming unit-Granulocytes monocytes
CFU-Hill	Colony forming unit-Hill
CFU-Meg	Colony-forming unit-Megakaryocytes
CLP	Common lymphoid progenitor
CMF	Common myeloid progenitor
CR	Caloric restriction
DDR	DNA damage response
DNA	Deoxyribonucleic acid

DR	Dietary restriction
ECFC	Endothelial colony-forming cell
eNOS	Endothelial nitric oxide synthase
EPC	Endothelial progenitor cells
EPCR	Endothelial protein C receptor or CD201
ES	Embryonic stem
FACS	Fluorescence-activated cell sorting
Flk-1 and 2	Fetal liver kinase-1 and 2
Flow-FISH	Flow cytometry-fluorescence in situ hybridization
Flt-1	Fms-related tyrosine kinase 1
Flt-3L	Fms-related tyrosine kinase-3 ligand
FOXO	Forkhead box
G6Pase	Glucose-6-Phosphatase
GFP	Green fluorescent protein
GPx-1	Glutathione peroxidase-1
Grdl	Gridlock
GVHD	Graft-versus-host disease
H₂O₂	Hydrogen peroxide
HGF	Hepatocyte growth factor
HIF	Hypoxia induced factor
HIF-1α	Hypoxia induced factor-1 α
HLA	Human leukocyte antigen
HSCs	Haematopoietic stem cells
HSPCs	Haematopoietic stem and progenitor cells
HUVECs	Human endothelial umbilical vein endothelial cells
ICM	Inner cell mass
IGF-1	Insulin-like growth factor type one
IIS	Insulin and insulin-like growth factor 1 (IGF1) signaling pathway
iPS	Induced pluripotent stem cells
KDR or VEGFR2	Kinase insert domain receptor or vascular endothelial growth factor receptor 2 or CD309
KSL cells	Lineage ⁻ C-Kit ⁺ Sca-1 ⁺
LT-HSCs	Long-term reconstituting haematopoietic stem cells
LTL	Leukocyte telomere length
Ly-bi	Lymphoid-biased
MPP	Multipotent progenitors
mRNA	Messenger ribonucleic acid
mtDNA	Mitochondrial DNA
mTOR	Mammalian target of rapamycin
mTORC1 and 2	Mammalian target of rapamycin complex 1 and 2

My-bi	Myeloid-biased
MΦ	Macrophage
NAC	N-acetyl cysteine
NAD	Aldehyde dehydrogenase
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NO	Nitric oxide
NOD/SCID	Non-obese diabetic/severe combined immunodeficient
NSC	Neural stem cell
O₂^{•-}	Superoxide anion
ox-LDL	Oxidized low-density lipoprotein
PBMNCs	Peripheral blood mononuclear cells
PDGF	Platelet-derived growth factor
PECAM-1	Platelet endothelial cell adhesion molecule-1 or CD31
PGC1-α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PI3K	phosphatidylinositide 3-kinases
PIGF	Phosphatidylinositol-glycan biosynthesis class F protein

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Chapter 1 Introduction

1.1 Ageing

Ageing is commonly characterised as generalised progressive impairment of function leading to an increased vulnerability to environmental challenges and the risk of death and disease. Ageing affects most living organism and is also associated with a decrease in fertility. Ageing corresponds with molecular damage in lipids, proteins, and nucleic acids with the failure of maintenance and repair. These are the basis of all age-related diseases which gerontology research is aimed to understand in order to increase the lifespan and improve the quality of life during old age (Rattan and Kassem, 2006). Although there is a clear heritable aspect in human longevity (Cournil and Kirkwood, 2001), the ageing phenotype significantly differs, even between monozygotic human twins (Herskind *et al.*, 1996).

Among the numerous ageing theories is the mitochondrial free radical theory of ageing (or oxidative stress theory) put forward by Harman (1956). This theory states that ageing organismal deterioration occurs as a consequence of free radical-mediated damage, which gradually compromises the function of cells, tissues, and, eventually, the organism. Oxidative stress is known to induce reactive oxygen species (ROS), mainly by-products of mitochondria, including superoxide anion ($O_2^{\bullet-}$), hydroxyl radical ($\bullet OH$), and hydrogen peroxide (H_2O_2). The accumulation of ROS causes oxidative stress during which redox balance between oxidants and antioxidants is disrupted, leading to oxidative cellular damage, senescence, and apoptosis and, eventually, to ageing. Numerous data are in support of mitochondrial free radical theory. For example, metabolic syndrome patients showed a significant increase in oxidative stress (Hansel *et al.*, 2004). However, the mitochondrial free radical theory is under an intense re-evaluation due to recent multiple data that challenged the theory (Hekimi *et al.*, 2011). Specifically, the unexpected observations challenging the theory include the following: 1) ROS may prolong the lifespan in yeast and worms (Van Raamsdonk and Hekimi, 2009; Mesquita *et al.*, 2010); 2) genetic manipulations which impair mitochondrial but do not elevate ROS accelerate ageing (Hiona *et al.*, 2010); 3) genetic manipulation in mice which increases

mitochondrial ROS, and oxidative damage does not accelerate ageing (Van Remmen *et al.*, 2003); and 4) increased antioxidant defences in mice do not extend lifespan (Perez *et al.*, 2009). These controversies lead to the emergence of new theories, mainly based on the notion that, despite their deleterious role, ROS are essential in signalling molecules. In this sense, ROS's primary effect will be the activation of a compensatory homeostatic response, which as age advances, ROS accumulates and eventually aggravates, leading to age-associated damage (Hekimi *et al.*, 2011).

1.1.1 Age-related diseases

There are 10.8 million people aged 65 or above in the UK who are expected to pass the 20 million mark by 2031, according to the Office for National Statistics in the UK (2013). In the last three generations, a significant increase of human life expectancy was achieved by a reduction in birth-related deaths and as a result of improvements in health interventions. The risks of age-associated conditions, such as infections, frailty, cancers, anaemia, autoimmune diseases, cardiovascular diseases, and diabetes, may be high with this rapid rise of aged populations (Rattan and Kassem, 2006). Among the common age-related diseases is the metabolic syndrome, which is associated with different cardiovascular risk factors, such as hypertension, obesity, atherogenic dyslipidemia, insulin resistance, and glucose intolerance. These risk factors ultimately lead to abnormal lipid and carbohydrate metabolism and increase the chances of atherosclerosis, diabetes, stroke, and cardiovascular disease (C. K. Roberts and Sindhu, 2009).

Diabetes encompasses a group of metabolic diseases characterised by high blood glucose that is the result of either insufficient insulin production (Type 1) or cells resistant to the production of insulin (Type 2). Diabetes can also occur only during pregnancy (gestational diabetes), which might proceed to type 2. Nowadays, the advanced medical therapies along with the discovery of insulin have improved the life expectancy and quality of life for patients with type 1 and 2 diabetes. However, the majority of these patients will develop chronic diabetic complications over the years that will lead to mortality and morbidity. There are several diabetic complications, including macrovascular events that lead to accelerated atherosclerosis and microvascular events that affect the nervous system (neuropathy), kidneys (nephropathy), and eyes

(retinopathy) (Kojima *et al.*, 2014). Hyperglycemia is the underlying cause of these complications that occurs possibly via inducing dysregulated biochemical pathways that cause injury and death in the affected organs. In diabetic nephropathy, hyperglycemia stimulate mesangial cell proliferation and hypertrophy with the accumulation of an excessive extracellular matrix and the thickening of the glomerular basement membrane. It further leads to the loss of podocytes, tubular hypertrophy, and the thickening of the tubular basement membrane (Kanwar *et al.*, 2011). Whereas in diabetic retinopathy, hyperglycemia lead to the secretion of vasoregulatory factors from Muller cells and the loss of pericytes and endothelial cells, causing acellular capillary formation without perfusion (Hammes *et al.*, 2011). Furthermore, hyperglycemia in diabetic peripheral neuropathy disrupts endothelial cells of the vasa nervorum in the peripheral nerves, neurons, and Schwann cells (Vincent *et al.*, 2011). The bone marrow was not considered a target organ for chronic diabetic complications until recently. It has been observed that endothelial progenitor cells (EPCs) and haematopoietic stem and progenitor cells (HSPCs) decreased in diabetic humans and rodents (Fadini *et al.*, 2005; Fadini *et al.*, 2006b). This implies their potential involvement in the manifestation of diabetes.

1.1.2 Cellular mechanisms of ageing

There are several complex mechanisms underlying ageing and hallmarks, including the loss of proteostasis, epigenetic alterations, genomic instability, and, of particular interest to this thesis, mitochondrial dysfunction, deregulated nutrient sensing, altered intercellular communication, cellular senescence, telomere attrition, and stem cell exhaustion (Lopez-Otin *et al.*, 2013).

Mitochondrial dysfunction can contribute to ageing as direct evidence in mice showed that proofreading deficiency by mutated mitochondrial DNA polymerase γ (POLG) can cause a number of age-related changes, such as a reduced lifespan, anaemia, hair loss, osteoporosis, the induction of apoptosis markers in different tissues, and reduced fertility (Trifunovic *et al.*, 2004; Kujoth *et al.*, 2005). It has been shown that the impaired mitochondrial function in these mice was, unexpectedly, not accompanied with increased ROS production (Hiona *et al.*, 2010). Furthermore, it has been reported that reduced mitochondrial biogenesis can occur as a consequence of telomere attrition in telomerase

deficient mice heart, liver, and HSCs with subsequent p53-mediated suppression of the transcriptional coactivators Peroxisome Proliferator-Activated Receptor Gamma (PPAR γ)-Coactivator-1 α (PGC-1 α) and - β (Sahin *et al.*, 2011). During physiological ageing, this mitochondrial decline occurs in wild type mice and can be partially reversed through the activation of telomerase (Bernardes de Jesus *et al.*, 2012). PGC-1 α is a master regulator of a complex metabolic response involved in mitochondrial biogenesis such as mitochondrial DNA (mtDNA) replication and transcription and mitochondrial respiration (Wu *et al.*, 1999). Mitochondrial biogenesis can be modulated by SIRT1 through a process involving PGC-1 α (Rodgers *et al.*, 2005). In fact, it has been shown recently that PGC-1 α is reduced in wild type mice muscle with ageing and that in ageing mice, the muscle-specific knockout of PGC-1 α impaired glucose tolerance and led to insulin resistance and inflammatory markers in liver and white adipose tissue (Sczelecki *et al.*, 2014).

Deregulated nutrient sensing during ageing can involve four main pathways; insulin- and IGF-1 signalling (IIS), mTOR (mammalian target of rapamycin), sirtuins, and AMPK (adenosine monophosphate AMP-activated protein kinase). In mammals, the somatotrophic axis consists of the growth hormone (GH), produced by the anterior pituitary gland, and its secondary mediator IGF-1 (insulin-like growth factor-1), produced in response to GH by many cell types, though mostly hepatocytes. The IGF-1 intracellular signalling pathway is the same as the one triggered by insulin, which informs cells of a glucose presence. This is the reason this pathway was called the “insulin and IGF-1” IIS pathway (Barzilai *et al.*, 2012). Mutations or genetic polymorphisms that reduce the IGF-1 receptor, GH, insulin receptor, or downstream effectors (e.g., FOXO (forkhead box), mTOR, and AKT (protein kinase B)) have been linked to longevity in model organisms and humans (Barzilai *et al.*, 2012). GH and IGF-1 levels decline during normal ageing and, paradoxically, in mouse models with premature ageing (Schumacher *et al.*, 2008). These contradictory observations can be accommodated in a model where IIS downmodulation can reflect a defensive response to systemic damage that aims to minimise cell metabolism and growth (Garinis *et al.*, 2008). However, extremely lower low levels of IIS signalling are incompatible with life, as seen by PI3K or AKT kinases null mice are embryonically lethal (Renner and Carnero, 2009). Furthermore,

supplementation with IGF-1 in progeroid mice can ameliorate premature ageing (Marino *et al.*, 2010).

The mTOR is part of two multiprotein complexes, mTORC1 and mTORC2. mTOR is a kinase that regulates signals for cell growth control in response to nutrients and essentially all aspects of anabolic metabolism. This pathway is widely studied due to its important role in cancer development and apoptosis. It has been shown that mTOR signalling activation increases proliferative capacity of cancer cells and reduces apoptosis (Morgensztern and McLeod, 2005). Furthermore, it has been reported that mTOR can act as a link between energy and nutrients signalling pathways (Tokunaga *et al.*, 2004). Several studies suggested that the mTOR pathway inhibition extends the lifespans of yeast, flies, worms, and mice (Hansen *et al.*, 2008; Selman *et al.*, 2009; Bjedov *et al.*, 2010). Although the inhibition of TOR activity retards multiple aspects of ageing, it also has undesirable side effects, such as insulin resistance, testicular degeneration, cataracts, and impaired wound healing (Wilkinson *et al.*, 2012).

In contrast to IIS and mTOR, the nutrient sensors sirtuins and AMPK signal nutrient catabolism and scarcity rather than nutrient anabolism and abundance. Thus, their upregulation favours healthy ageing.

Sirtuins are members of NAD (aldehyde dehydrogenase)-dependent protein deacetylases and ADP ribosyltransferases which have been studied extensively due to their anti-ageing potential. An extra copy of SIR2 has been shown to extend the lifespan in yeast, worms, and flies (Kaeberlein *et al.*, 1999; Tissenbaum and Guarente, 2001; Rogina and Helfand, 2004). SIRT1 can deacetylate and activate PGC-1 α (Rodgers *et al.*, 2005). Furthermore, overexpression of SIRT3 has been shown to improve the regenerative capacity of aged HSCs in mice (Brown *et al.*, 2013).

AMPK is regarded as the “fuel gauge” of the cell which is activated by the increase of cellular ratio of AMP+ADP to ATP. The activation of AMPK has multiple effects on metabolism, shuts off mTORC1 (Akers *et al.*, 2012), and may mediate lifespan extension in worms and mice (Onken and Driscoll, 2010; Anisimov *et al.*, 2011). Moreover, it has been reported that SIRT1 and AMPK can engage in a positive feedback loop and hence act in a unified response, connecting both sensors of low-energy (Price *et al.*, 2012).

The **altered intercellular communication** involves more inflammation during ageing or ‘inflammageing,’ which is defined as a pro-inflammatory phenotype accompanying ageing in mammals. This inflammageing interconnects with dysfunctional immune system cells or immunosenescence, leading to the failure of clearing out infectious agents and infected cells. Furthermore, inflammation is involved in the pathogenesis of several age-related diseases including obesity, type 2 diabetes, and atherosclerosis (Franceschi *et al.*, 2000). The presence of pro-inflammatory phenotype is evident by (1) higher expression of genes linked to immune responses and inflammation in the tissues of old humans and mice (Csiszar *et al.*, 2003; de Magalhaes *et al.*, 2009), (2) increased levels of cytokines (Fagiolo *et al.*, 1993), and (3) activation of the master regulator of inflammatory responses NF- κ B (Helenius *et al.*, 1996).

Cellular senescence can be defined as a signal transduction program that leads to irreversible growth arrest associated with morphological changes in cells and structural changes in nuclear chromatin (Shay and Roninson, 2004). Cellular senescence was originally described by Leonard Hayflick, who first observed that human fibroblasts stop dividing after approximately 50 population doublings known as the “Hayflick limit” (Hayflick and Moorhead, 1961). When dividing, cells are marked by decline in the proliferative potential (replicative senescence) triggered by critically shortened end caps of chromosomes termed telomeres (Harley *et al.*, 1990). Cell division in a mammalian organism requires the duplication of its chromosomal DNA. DNA polymerases lack the capacity to completely replicate the terminal ends of linear DNA molecules called telomeres—a function performed by a specialized DNA polymerase known as telomerase. However, as most somatic cells do not express telomerase, this leads to the progressive loss of telomere-protective sequences from chromosomal ends, to the reaching of the Hayflick limit, and, eventually, to becoming senescent (Olovnikov, 1996). However, there are other ageing-associated stimuli that trigger cellular senescence like non-telomeric DNA damage and derepression of the INK4/ARF locus, also called CDKN2a and CDKN2b (encodes for p16^{INK4a} and P19^{ARF}). The most important mechanisms, which implement senescence in response to an oncogenic insult, include p16^{INK4a}/Rb and p19^{ARF}/p53 pathways (Serrano *et al.*, 1997). Strikingly, the levels of p16^{INK4a} and, to a lesser extent, P19^{ARF} correlate to the age of all tissues analysed in both

humans and mice (Krishnamurthy *et al.*, 2004; Ressler *et al.*, 2006). INK4a/ARF locus was identified with the highest number of age-associated pathologies like Alzheimer's disease, glaucoma, cardiovascular diseases, and diabetes (Jeck *et al.*, 2012). Nevertheless, an additional copy of the INK4/ARF locus in transgenic Super-Ink/Arf mice can protect against the age-associated alterations in glucose homeostasis with enhanced sensitivity and signalling and lower glucose levels (Gonzalez-Navarro *et al.*, 2013). These studies suggest that the activation of INK4a/ARF can be a beneficial compensation response to avoiding the spread of damaged cells and their effects on ageing and disease.

Telomere shortening is observed in the normal ageing of mice and humans (Flores and Blasco, 2010). In humans, telomerase deficiency is associated with the premature development of diseases that involve the loss of the regenerative capacity of different tissues such as aplastic anaemia (Vulliamy *et al.*, 2002), pulmonary fibrosis (Armanios *et al.*, 2007), and dyskeratosis congenita (Vulliamy *et al.*, 2001). Mice that are genetically modified with shortened or lengthened telomeres exhibit shorter or longer lifespans, respectively (Blasco *et al.*, 1997; Herrera *et al.*, 1999; Rudolph *et al.*, 1999; Tomas-Loba *et al.*, 2008). A recent study had shown that ageing could be reverted by telomerase activation in aged mice (Jaskelioff *et al.*, 2011). Furthermore, the systemic viral transduction of telomerase can delay normal physiological ageing in adult wild type mice without increasing the incidence cancer (Bernardes de Jesus *et al.*, 2012). A recent meta-analysis in humans was in favour of a strong relation between risk of mortality and short telomeres, particularly at younger ages (Boonekamp *et al.*, 2013).

Among the most obvious characteristics of ageing is the regenerative decline of tissues, which is implied by **stem cell exhaustion**. The decline of haematopoiesis with age leads to less production of adaptive immune cells (immunosenescence) and therefore to increased incidence of myeloid malignancies and anaemia (Franceschi *et al.*, 2000). Aged mice have shown an overall decrease in cell cycle activity of haematopoietic stem cells (HSCs) with fewer cell divisions in old HSC compared to young ones and with the accumulation of DNA damage (Rossi *et al.*, 2007). It has been shown that the overexpression of inhibitory proteins of the cell cycle, such as p16^{INK4a}, also correlates to DNA damage. Old INK4a^{-/-} HSCs showed increased cell cycle activity and enhanced

engraftment compared with old wild type HSCs (Janzen *et al.*, 2006). Stem cell decline is also caused by telomere shortening with ageing in multiple tissues (Flores *et al.*, 2005; Sharpless and DePinho, 2007). Furthermore, the quiescence of stem cells has been shown to be important for their long-term functionality. In *Drosophila* intestinal stem cells, the excessive proliferation of these cells has led to premature exhaustion and premature ageing (Rera *et al.*, 2011). Similar findings were observed in p21^{-/-} mice, which led to HSCs (Cheng *et al.*, 2000) and neural stem cells' (Kippin *et al.*, 2005) premature exhaustion. In particular, p21^{-/-} mice maintained HSCs quiescence and increased their numbers, however, in serial transplantation, their self-renewal ability was impaired (Cheng *et al.*, 2000). It has recently been shown that LT-HSCs (long-term reconstituting haematopoietic stem cells), upon irradiation, activate the cell cycle inhibitor p21 independently of p53. This upregulation of p21 inhibited p53 induction and prevented apoptosis (Insinga *et al.*, 2013). Thus, these studies indicate that the long-term maintenance of the organism requires a balance in the proliferation of stem and progenitor cells as excessive proliferation can lead to the exhaustion of stem cell niches. Recent work has supported the importance of extrinsic pathways in the decline of stem cell function. Interestingly, the extrinsic stem cell environment rather than telomere dysfunction impaired lymphopoiesis during the transplantation of mice with telomere shortened bone marrow (BM) cells (G3 TERC^{-/-}) compared to controls (Song *et al.*, 2010). When muscle-derived stem cells from young mice were transplanted to progeroid mice, it extended their lifespan and improved degenerative alterations. The therapeutic effects of stem cells have been suggested to be caused by secreted factors (Lavasani *et al.*, 2012). Furthermore, muscle regeneration by muscle stem cell activation after injury in old mice can be restored in parabiosis experiments by exposure to systemic factors from young mice. This effect was mediated at least in part by restoration of the normal signalling of the Delta-Notch pathway (Conboy *et al.*, 2005). It has been shown that ageing induces Wnt signalling in muscle stem cells and increases fibrosis in parabiosis studies (Brack *et al.*, 2007).

1.1.3 Anti-ageing interventions

Ageing effects on the immune system have implications for clinical medicine. One example is the influenza virus vaccination which has 30-40% efficiency in protecting the elderly from influenza (McElhaney and Dutz, 2008). Another clinical implication was also seen after transplantation, as bone marrow transplantation from older individuals showed a lower capacity of reconstitution of the immune system in recipients (Kollman *et al.*, 2001). Such examples highlighted the importance of research translation into clinical developments. Anti-ageing or rejuvenating therapies do not necessarily restore immune function to young levels as long as modest enhancement in the immune function can potentially be clinically beneficial. Ideally, these interventions should be cost effective, readily available, and easy to administer to a large number of individuals. Some of these interventions are discussed below.

1.1.3.1 Dietary restriction

Dietary or caloric restriction (DR or CR) is the most powerful intervention that increases the lifespans of several organisms and decreases the incidence of age-related phenotypes that are delayed by DR including cancer, diabetes, cardiovascular disease, neurodegenerative diseases, and kidney disease (Everitt *et al.*, 2006). DR or caloric restriction (CR) refers to food intake limitation by 20-40% of ad libitum consumption without malnutrition (Bronson and Lipman, 1991; Fontana *et al.*, 2010). There are other dietary modifications used in ageing research, including changes of food content in the diet such as the alterations in protein, some amino acids, or fat intake (Zimmerman *et al.*, 2003; Miller *et al.*, 2005).

In 1935, McCay initially proposed that DR may be able to prolong lifespan. He has noted that feeding of the laboratory rats with a reduced calorie diet while maintaining their micronutrient levels had extended their lifespans compared to controls (McCay *et al.*, 1989). Subsequently, numerous reports pertaining to yeast (S. J. Lin *et al.*, 2000), invertebrate animals such as worms (*C. elegans*) (Klass, 1977), flies (Cooper *et al.*, 2004), mammals like rodents (Weindruch *et al.*, 1988; Swindell, 2012), and dogs (Kealy *et al.*, 2002) have shown the beneficial effect of DR on lifespan extension and health improvement. However, contradictory influences of DR on lifespan were also reported,

depending on mice genotype (Liao *et al.*, 2010) and initial basal metabolic rate (Brzek *et al.*, 2012). In fact, two studies from long-term DR in non-human primates have reported health benefits, such as the reduction of the incidence of cancer and diabetes in dietary-restricted rhesus monkeys compared to controls, but were discordant on the effect of DR on life extension (Colman *et al.*, 2009; Mattison *et al.*, 2012). The conflicting results might arise from the different study designs as different diet composition of dietary restrictions and controls as well as different strains were used. In humans, a randomized clinical study on non-obese sedentary subjects has shown that a 12-month 20% reduction in caloric intake improves insulin sensitivity, decreases fat mass, reduces circulating inflammatory markers, decreases plasma triiodothyronine levels, increases adiponectin concentration, and reverses some of the ageing deterioration in cardiac diastolic function (Weiss *et al.*, 2008; Fontana *et al.*, 2010; Stein *et al.*, 2012).

The physiological benefits of DR are implicated by lowering body mass and fat content, improving insulin sensitivity, and lowering blood glucose in rats (Zheng *et al.*, 2009). Although the exact mechanism of dietary restriction remains to be defined, theories suggested the protection against cellular damage by reducing cellular metabolism (Bordone and Guarente, 2005). It has been shown on many animal models that DR enhances mitochondrial biogenesis and function (Martin-Montalvo and de Cabo, 2013). It has been reported that DR can reduce oxidative damage in rodents' muscles and protect against sarcopenia, even with a key antioxidant enzyme deletion ($Sod1^{-/-}$) (Y. C. Jang *et al.*, 2012). Furthermore, some reports confirmed significant mitochondrial ROS reduction and decreased oxidative stress in the liver and muscle of rodents (Zainal *et al.*, 2000; C. Wang *et al.*, 2010). The mitochondrial and metabolic effects of DR were suggested to be due to the induced expression of transcriptional co-activator PGC-1 α (Civitarese *et al.*, 2007). On the other hand, other groups suggested the induction of oxidative stress resistance with DR by the increased production of ROS, which induced catalase activity (Schulz *et al.*, 2007). The authors proposed that DR promotes the production of ROS that leads to the ignition of stress defence mechanisms and to the long-term reduction of oxidative damage. This leads to another hypothesis, known as "mitochondrial hormesis," which proposes that DR acts as a low-intensity stressor that

induces mitochondrial biogenesis and subsequent cellular defence and adaptation to metabolic alterations to reduce oxidative stress (Ristow and Zarse, 2010).

1.1.3.2 Dietary restriction pathways

There are several nutrient sensing pathways implicated in the beneficial effect of DR on the ageing process. Since the first paper by Kenyon *et al.* (1993) in which the reduction of the insulin/IGF-1/FOXO pathway was shown to prolong the lifespan of worms, accumulating data have shown that this pathway is evolutionary conserved (Partridge and Gems, 2002). It has been shown that DR reduced IGF-1 blood levels in mice (Huffman *et al.*, 2008). In human skeletal muscle, DR has been also shown to inhibit the insulin/IGF-1 pathway in microarray studies (Mercken *et al.*, 2013). Another important nutrient sensor pathway is mTOR. In mice, it has been shown that the deletion of S6K (a main mTORC1 substrate) increased lifespan and resistance to age-related pathologies such as loss of insulin sensitivity (Selman *et al.*, 2009). The lifespan extension effect of DR in *Drosophila* was mediated by the translational repressor 4E-BP, a downstream target of mTOR, which enhanced mitochondrial activity (Zid *et al.*, 2009). The third pathway involves sirtuins, which can mediate the effects of DR as evidenced by several studies. First, it has been shown that DR induces the expression of sirtuins SIRT1 (Cohen *et al.*, 2004), SIRT3 (Lombard *et al.*, 2007), and SIRT5 (Nakagawa *et al.*, 2009) in mice and SIRT1 in humans (Civitarese *et al.*, 2007). Furthermore, the loss of function mutations in sirtuins can decrease DR output. For example, SIRT1 knockout mice do not show a typical life extension (Mercken *et al.*, 2014) or physical activity (D. Chen *et al.*, 2005) seen by DR. Also, the overexpression of SIRT1 in transgenic mice can mitigate diseases, such as diabetes, much like DR (Herranz *et al.*, 2010). Conversely, the compromise of sirtuin activity can contribute to diabetes and metabolic syndrome in mice (Chalkiadaki and Guarente, 2012) and humans (Biaison-Lauber *et al.*, 2013). The DR increases the insulin-stimulated glucose uptake in mouse skeletal muscle by an AMPK α 2-dependent induction of SIRT1 activity (P. Wang *et al.*, 2012). It has recently been shown that AMPK^{-/-} mice skeletal muscles impaired DR-mediated glucose tolerance compared to wild type mice (Silvestre *et al.*, 2014). In this study, the lack of AMPK under DR abolishes the SIRT1 downregulation seen in ad libitum mice. This suggests that the deficiency of AMPK impairs the beneficial effects of DR on glucose tolerance,

possibly through SIRT1 gene downregulation (Silvestre *et al.*, 2014). An interesting and recent study suggested that the balance between macronutrients rather than caloric intake determines cardiometabolic health and longevity in mice (Solon-Biet *et al.*, 2014). This study showed that a high carbohydrate and low protein diet optimizes longevity and health (lower blood pressure, improved glucose tolerance, and lower triglycerides) compared to 24 ad libitum diets. The beneficial effects were associated with hepatic mTOR activation and mitochondrial function (Solon-Biet *et al.*, 2014).

Resveratrol (antioxidant in red wine) has been shown to prolong in mice fed with a high fat diet (Baur *et al.*, 2006). This life extension effect mimics DR effects with reduced IGF-1 levels, increased AMPK, and PGC-1 α activity. Furthermore, it has been reported that resveratrol delays the onset of senescence in human endothelial progenitor cells and induces telomerase activity dose-dependently (X. B. Wang *et al.*, 2011). The oral administration of red wine and resveratrol in normal rats has been shown to preserve vascular function by delaying ageing (improved aerobic capacity, decreased p53 and p16 in the aorta, and increased telomere length and telomerase activity) although no effect on lifespan was observed (da Luz *et al.*, 2012).

1.1.3.3 Other anti-ageing interventions

Beyond caloric restriction and resveratrol, metabolic strategies have been proposed to delay ageing acting on sirtuins, mTOR signalling, the IIS pathway, and AMPK. Some drugs, such as metformin, may create a cellular milieu that facilitates longevity because of their specific mechanism of action through AMPK (Suwa *et al.*, 2006). Also, angiotensin II inhibitors may potentially be useful for knocking out an At-II type I receptor (Agtr1a) in mice's prolonged lifespan and increased number of mitochondria, SIRT3 expression, and nicotinamide phosphoribosyltransferase (Benigni *et al.*, 2009). Furthermore, it has been clearly established that using HMG-CoA reductase inhibitors, statins, for lipid-lowering therapy reduces cardiovascular events in patients with or without coronary artery diseases (Baigent *et al.*, 2010). Statins may exert anti-ageing pleiotropic effects by targeting the accumulation of farnesylated proteins that causes several human progeria syndromes (Fong *et al.*, 2006; Varela *et al.*, 2008). Moreover, a natural extract isolated from *Astragalus membranaceus*, called TA-65, induced telomerase activity in vitro, increased mRNA expression of TERT, and improved mouse health-span

indicators such as glucose tolerance (Bernardes de Jesus *et al.*, 2011). Of note, AGS-499 has recently been demonstrated to protect human mesenchymal stem cells from oxidative stress by telomerase activation (Tichon *et al.*, 2013). This novel compound has also been reported to delay the progression of amyotrophic lateral sclerosis disease in mice (Eitan *et al.*, 2012). However, no current human longitudinal study investigates the potential effects of telomerase-activating drugs on lifespan.

1.2 Endothelial progenitor cells

The vascular system plays a primary role in the repair and regeneration of various tissues in the human body. In vascular tissues, a complex network of capillaries and blood vessels is formed to provide sufficient nutrients and oxygen and to remove waste products. This network is formed and maintained through three main mechanisms: vasculogenesis, angiogenesis, and arteriogenesis. Vasculogenesis is the primary formation of blood vessels and the vascular system by the endothelial cell precursors during foetal development, whereas angiogenesis is the formation of new blood vessels sprouting from pre-existing ones—for instance, due to ischemia, as shown in Figure 1.1. Arteriogenesis occurs when arterioles' lumen widens as a result of the growth or remodelling of the adjacent arteries (Risau, 1997).

Furthermore, the endothelium plays a critical role in the homeostasis of the vascular system. It forms a layer lining for all blood vessels in the body. It functions as an interface between vessels and blood-borne elements and tissues. The endothelium regulates coagulation, thrombosis, platelet adhesion, leukocyte recruitment, vascular tone, and release growth factors and cytokines (J. Z. Chen *et al.*, 2004). Endothelial dysfunction is associated with endothelial cell loss (Libby, 2002) and a decrease in nitric oxide (NO) availability, which is one of the most important factors in maintaining vascular homeostasis (Wassmann and Nickenig, 2003; Laufs *et al.*, 2004). Additionally, endothelial dysfunction has been linked to coronary artery disease and cardiovascular risk factors (Verma *et al.*, 2004). It also leads to atherosclerosis, which, besides endothelial cell damage, causes the invasion of inflammatory cells and the proliferation of vascular smooth muscle cells (Libby and Theroux, 2005).

Therefore, maintaining the integrity of the endothelial layer is crucial for healthy vessels and the prevention of vascular diseases. Several studies, *in vivo* and *in vitro*, confirmed

the beneficial properties of endothelial progenitor cells (EPCs) in vascular growth and repair (Asahara *et al.*, 1997; Kocher *et al.*, 2001; Griese *et al.*, 2003)

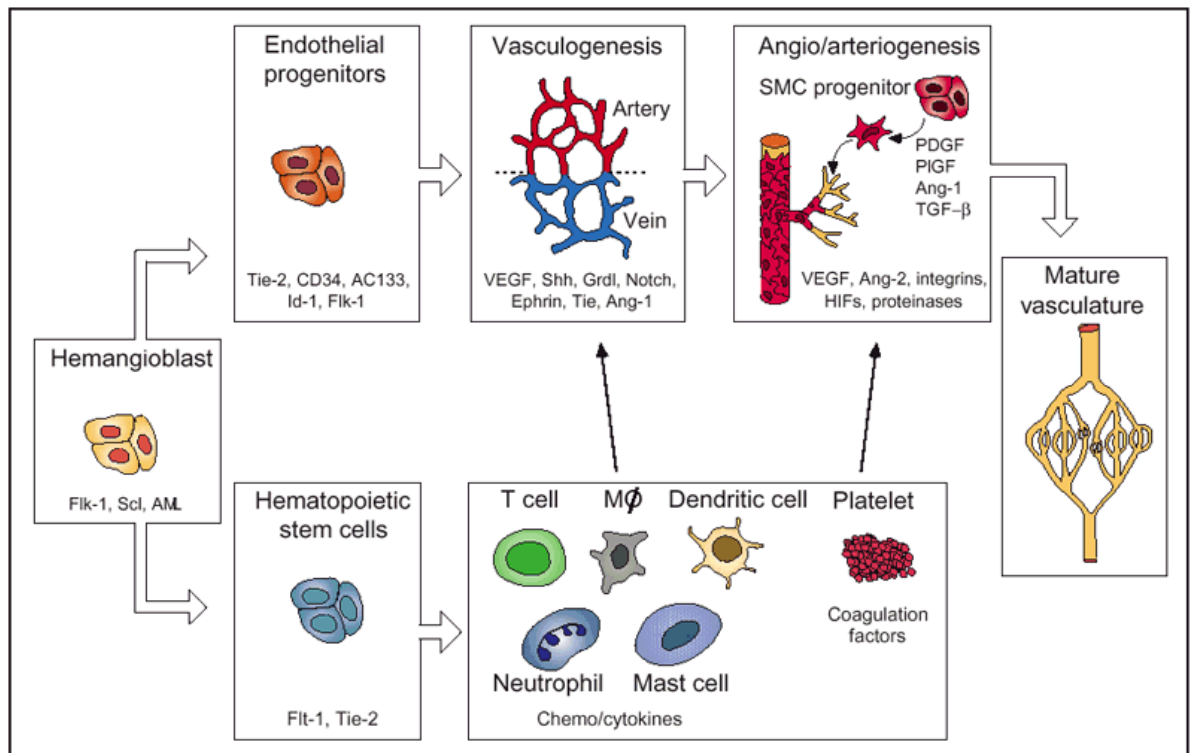


Figure 1.1: Vasculogenesis and angiogenesis.

Endothelial progenitor cells differentiate into venous and arterial endothelial cells that assemble in a primitive capillary plexus. Cells aid in vasculogenesis and angiogenesis by producing different cytokines, which recruit other cells to form the new vessels. As differentiating from their progenitors, vessels then sprout and become stabilised by smooth muscle cells (SMC) to form a mature vasculature. Haematopoietic progenitors and their descendants contribute to angiogenesis directly and indirectly by differentiating from leukocytes and platelets and releasing angiogenic factors. Some factors produced by different cells as indicated in the figure. Flk-1 (Fetal liver kinase-1), AML (Acute myeloid leukemia), Scl (Stem cell leukemia), Tie-1 and -2 (Tyrosine kinase-1 and -2), VEGF (Vascular endothelial growth factor), Shh (Sonic hedgehog), Grdl (Gridlock), MΦ (Macrophage), HIF (Hypoxia induced factor), PDGF (Platelet-derived growth factor), PIGF (Phosphatidylinositol-glycan biosynthesis class F protein), and TGF- β (Transforming growth factor- β). Source: Carmeliet (2003).

1.2.1 Origins of endothelial progenitor cells

Vascular and haematopoietic systems develop in parallel and interdependently during embryogenesis. Multi-lineage haematopoietic progenitors are derived from endothelium within embryo proper from the inner cell mass of the blastocyst (Zovein *et al.*, 2008) and yolk sac (Goldie *et al.*, 2008). After that, haemtopoiesis migrate in the fetal liver (Palis *et al.*, 2001) and intra-embryonic sites: the aorta-gonad-mesonephros (AGM) region and in head and major blood vessels (Z. Li *et al.*, 2012). There is a close physical association between endothelial and haematopoietic stem cells (Morrison and Spradling, 2008). Many cell surface markers are shared between vascular endothelial and hematopoietic cells (Jackson *et al.*, 2001). Therefore, several studies identified EPC populations that exhibit haematopoietic characteristics. There are specialised areas in embryonic tissue composed of endothelial cells termed haemogenic endothelium. These are able to generate HSCs and differentiated haematopoietic cells (Medvinsky and Dzierzak, 1996). A haemogenic endothelium was shown to give rise to haematopoietic cells (Lancrin *et al.*, 2009). Furthermore, studies have shown other anatomical regions where haemogenic potential of endothelial cells is located, including umbilical arteries (Yokomizo and Dzierzak, 2010), head vasculature (Z. Li *et al.*, 2012), yolk sac developing capillaries (W. Li *et al.*, 2005b), placenta (Gekas *et al.*, 2005), and endocardium (Nakano *et al.*, 2013). Moreover, accumulating evidence is in favour of a new concept interconnecting endothelial and haematopoietic cells (Chao and Hirschi, 2010). However, the presence of haemogenic cells in adult hematopoietic tissues is not directly proven. A current view supports a model that suggests new HSCs, during adult life, are not generated through hamogenic endothelium but rather through self-renewal from pre-existing HSCs (Hirschi, 2012). Nevertheless, the labelling of hemogenic endothelium before definitive haemtopoiesis and following their progeny during adult life showed that embryonic HSCs remain functional in adult life but do not contribute to all the haematopoietic cells generated postnatally (Zovein *et al.*, 2008). Therefore, the existence of functional endothelium in adult haematopoietic tissues cannot be excluded.

1.2.2 Uses of endothelial progenitor cells

Premature ageing of the vascular system is characterised by decreased repair and neovascularization. Endothelial progenitor cells became a popular research material due to their pro-angiogenic and regenerative potential; in particular, EPCs were investigated in diabetes (Tepper *et al.*, 2002; Loomans *et al.*, 2004; Fadini *et al.*, 2006a; Fadini *et al.*, 2007; Ling *et al.*, 2012), cancer (Lyden *et al.*, 2001; Mancuso *et al.*, 2001; Mancuso *et al.*, 2006; Shaked *et al.*, 2006; Nolan *et al.*, 2007), and cardiovascular disorders (Eizawa *et al.*, 2004; N. Werner *et al.*, 2005; Kunz *et al.*, 2006; Hughes *et al.*, 2007). One of the most important advantages of endothelial progenitor cells is that they do not elicit an immune response when allogenic grafts are transplanted in vivo and in vitro (Ladhoff *et al.*, 2010). In this study, EPCs were protected against allospecific cytotoxic T lymphocyte activity and alloantibody/complement lysis. Also, allogenic aortic transplantation showed no signs of rejection (Ladhoff *et al.*, 2010). The injection of EPCs in ischemic animal models was shown to significantly enhance neovascularization, leading to the recovery of injured tissue and better blood flow (Murohara *et al.*, 2000; Kawamoto *et al.*, 2001; Kawamoto *et al.*, 2003; Urbich *et al.*, 2003).

Clinical studies have shown a negative correlation between the number of circulating EPCs and the risk of cardiovascular disease (e.g., age, hypertension, diabetes, smoking) and morbidity (Vasa *et al.*, 2001a; Vasa *et al.*, 2001b; N. Werner *et al.*, 2005). Furthermore, human clinical trials using EPCs transplantation in peripheral artery disease and myocardial infarction were safe, but associated with absent or very modest clinical benefit (Assmus *et al.*, 2002; Tateishi-Yuyama *et al.*, 2002; Leistner *et al.*, 2011). A recent meta-analysis of acute myocardial patients has suggested that the time of cell delivery as well as the type, quantity, and mobility of delivered cells are important determinants of the response to the therapy. The most promising studies are those with high purified CD34⁺ doses on the intra-coronary infusion (Poole and Quyyumi, 2013). In fact, a phase III randomized study was designed on a large set of refractory angina and chronic myocardial ischemia patients, involving intramyocardial administration of a 1×10^5 autologous CD34⁺ cell/kg (Povsic *et al.*, 2013).

1.2.3 Characterisation of endothelial progenitor cells

Asahara and colleagues first characterised endothelial progenitor cells (EPCs) in 1997. They isolated a proliferative heterogeneous population of cells in animal models with endothelial cells markers (CD34+, kinase insert domain receptor KDR + or VEGFR2) contributing to new blood vessel formation in vitro (Asahara *et al.*, 1997). Since then, extensive studies were performed to identify those cells due to their therapeutic potential, mainly in cardiovascular disease (Rafii and Lyden, 2003). Two major mechanisms are thought to be involved in vascular repair by EPCs. The first is carried out by the physical incorporation of EPCs into the growing angiogenic network, whereas the second mechanism is via secreting pro-angiogenic cytokines (e.g., VEGF-A, stromal cell-derived factor-1 (SDF-1), insulin-like growth factor-1 (IGF-1), and hepatocyte growth factor (HGF)) (Rehman *et al.*, 2003; Urbich *et al.*, 2005).

Different approaches and nomenclature were used to classify endothelial progenitor cells. These can be divided into two main approaches: cell culture techniques and cell biomarkers. The first cell culture technique, originally described by Asahara *et al.* (1997), involves the isolation of mononuclear cells from the peripheral blood and the growth them on fibronectin-coated plates. Adherent cells are harvested and identified as acetylated low-density lipoprotein (acLDL) positive cells. These cultures are short-term producing colonies after 4-9 days and termed early EPCs (Hur *et al.*, 2004). Hill *et al.* (2003) used a different isolation technique in which non-adherent cells were cultured instead of adherent cells after 48 hours of preplating. Those cells were referred to as colony forming unit-Hill (CFU-Hill) and are characterised by a cluster of round cells surrounded by spindle shape cells. The third cell culture technique is used to identify an endothelial colony forming cell (ECFC). It differs from the first one in that cells are cultured from the cobblestone appearance in culture on collagen-coated plates and are seeded for extended period of time (Ingram *et al.*, 2004). Other cell culture techniques might involve using commercial kits for isolation purposes, such as endothelial cell colony-forming units (CFU-ECs) (Yoder *et al.*, 2007).

Several biomarkers were used to identify EPCs by fluorescence-activated cell sorting (FACS). Peichev *et al.* used the expression of CD34, CD133, and CD309, which is the vascular endothelial growth factor receptor 2 (VEGFR2 or KDR in humans), to

identify EPCs and referred to them as circulating endothelial precursors (CEPs) (Peichev *et al.*, 2000). Other studies have used CD31 (Platelet Endothelial Cell Adhesion Molecule 1, PECAM-1), CD105 (endoglycan), CD146 (MUC-18), CD144, UEA-1 (Ulex Europaeus Agglutinin-1), vWF (Von Willbrand Factor), CD144 (VE-cadherin), and CD202b (Tie-2) (Timmermans *et al.*, 2007). However, until this point, investigators have failed to identify unique markers for EPCs.

The different methods of isolation and culture have resulted in different characterisations of EPCs and observations of functions. Some groups compared EPCs as early and late EPCs according to their time in the culture. Hur *et al.* showed that these two types, early EPCs and late EPCs, differ in their lifespan, appearance in culture, expression of genes, and *in vitro* capillary tube formation. Early EPCs were spindle-shaped and lasted for 4 weeks in the culture, whereas late EPCs appeared as cobblestones and grew up to 12 weeks in the culture. Unlike early EPCs, late EPCs expressed VE-cadherin, Flt-1, KDR, and CD45 and formed a better capillary tube. However, both early EPCs and late EPCs showed comparable potential for induction *in vivo* vasculogenesis (Hur *et al.*, 2004; Yoon *et al.*, 2005). Yoder and colleagues compared CFU-ECs and ECFCs and discovered interesting differences. Mainly, ECFCs have the ability to form blood vessels *in vivo* and have robust proliferative potential. CFU-ECs express haematopoietic antigens and phagocyte bacteria, whereas ECFCs do not. A clonal analysis performed by Yoder *et al.* showed the distinct origin of these two types of cells, in which CFU-ECs are related to hematopoietic origin while ECFCs are not. Therefore, according to Yoder *et al.* (2007), CFU-ECs progeny are hematopoietic related cells with macrophage antigens and function (phagocytosis) as well as endothelial antigens that have some proliferative potential and cannot form vessel *in vivo*.

Although these studies suggested that ECFCs are the best candidate for regenerative vascular therapy, they have not been used clinically yet. A potential limitation of these cells is the paucity of these cells in the peripheral blood. It has been shown that ECFCs were grown from a subset (14%) of PBMNCs (peripheral blood mononuclear cells) from acute coronary syndrome (Campioni *et al.*, 2013). Therefore, an improved methodology for ECFC cultures is needed to achieve adequate cells for therapeutic applications.

1.2.4 Vascular repair during ageing

Dysfunctional endothelium is considered a common ground for cardiovascular disease and type 2 diabetes among other diseases (Loomans *et al.*, 2004; Fadini *et al.*, 2005). The vascular homeostatic response is the balance between cellular damage and repair, and it involves the mobilization of EPCs from their bone-marrow niche. This integral part of mobilisation is associated with rapid and significant increase in circulating EPCs in acute ischemic events such as vascular injury secondary to burns or surgery (Gill *et al.*, 2001), acute coronary syndrome (George *et al.*, 2004), and myocardial infraction (Shintani *et al.*, 2001).

The link between ageing and EPCs is supported by several studies. Although there was no significant difference in the number of circulating EPCs (CD34+/KDR+ or CD133+/KDR+) between young (25 years old) and old (61 years old) subjects, early EPCs showed significant impairment in cells survival, migration, and proliferation (Heiss *et al.*, 2005). Furthermore, young but not old donor mice bone marrow derived EPCs transplantation prevented a decline in the angiogenic platelet-derived growth factor (PDGF)-B induction pathway and cardiac angiogenesis in the ageing murine model (Edelberg *et al.*, 2002). In addition, bone marrow-derived EPCs (CD31+CD45-) showed exhausted atheroprotective property with old ApoE^{-/-} mice compared to young ones (Rauscher *et al.*, 2003).

Additional factors affecting EPC ageing are described in Table 1. The first factor linked to EPC ageing is **oxidative stress**. Several studies proved the effect of oxidative stress during ageing on EPCs. Early EPCs were found to be more resistant to oxidative stress as they express high levels of antioxidants compared to adult microvascular endothelial cells and HUVECs (human umbilical vein endothelial cells) (He *et al.*, 2004). Furthermore, in aged subjects, early EPCs were found to be more sensitive to oxidative stress as they showed less antioxidant enzyme glutathione peroxidase-1 (GPx-1) (He *et al.*, 2009). Early EPCs were also shown to have an upregulation of proatherogenic factor Angiotensin II (AngII), which enhances ROS production and cellular senescence of early EPCs (M. Wang *et al.*, 2003; Imanishi *et al.*, 2005). Another important factor during ageing connected with EPCs is **inflammation**. Low grade inflammation was shown to be associated with increased proinflammatory cytokine tumour necrosis factor-alpha (TNF-

α) in aged rats' coronary arteries compared to young ones (Csiszar *et al.*, 2003). Long-term treatment of EPCs in culture, isolated from human umbilical cord blood (UCB), with TNF- α , increased premature senescence associated with an induction of the cell cycle inhibitor p16^{INK4a} (Y. Zhang *et al.*, 2009). **Pro-angiogenic factors** including the vascular endothelial factor (VEGF) and the stromal derived factor-1 (SDF-1) have been shown to impair EPC (Flk-1⁺/CD11b⁻) trafficking to ischemic sites and to depress the hypoxia-inducible factor alpha (HIF-1 α) (E. I. Chang *et al.*, 2007). Estrogen might also act as a pro-angiogenic factor by which estrogen treatment of post-ovariectomy young female mice increased levels of EPCs (Sca-1⁺/Flk⁺) compared to the untreated group (Strehlow *et al.*, 2003). Another factor affecting EPC ageing is the reduction in telomere length in early EPCs associated with age in healthy sedentary men (Kushner *et al.*, 2009). Additionally, a 57% reduction of telomerase activity was seen in older men compared to young men (Kushner *et al.*, 2011). Moreover, the overexpression of human TERT in circulating angiogenic cells (CACs) conserved telomerase activity, enhanced EPC function, and delayed senescence after the ischemic hind limb (Murasawa *et al.*, 2002). The last factor related to EPC ageing is reduced **nitric oxide bioavailability** where decline of EPC levels and function may be related to the reduced nitric oxide (NO) generation of ageing endothelium. The importance of nitric oxide production and nitric oxide synthase (eNOS) expression for EPC mobilisation was proved where increased NO bioavailability by physical exercise induced EPCs (Sca-1⁺/Flk⁺), which was not seen with eNOS null mice or with an NOS inhibitor (Laufs *et al.*, 2004). In addition, oxidized low density lipoprotein (ox-LDL) accumulation with age may also contribute to a reduction of EPCs during ageing. Early EPCs were shown to be impaired in survival and function with an ox-LDL inhibitory effect on EPC eNOS activity and expression (Ma *et al.*, 2006). Therefore, ageing implicated both environmental and intracellular changes in EPCs homing, generation, and mobilisation from bone marrow leading to a senescent phenotype.

Factor	Remarks	Reference
Oxidative stress	<ul style="list-style-type: none"> • Early EPCs express high levels of antioxidant enzymes leading to more oxidative stress resistance compared to adult microvascular endothelial cells and HUVECs • Reduced antioxidant enzyme GPx-1 and more oxidative stress sensitivity in early EPCs from aged subjected • Upregulation of proatherogenic AngII during ageing which enhances ROS production and cellular senescence in early EPCs 	<ul style="list-style-type: none"> • (He <i>et al.</i>, 2004) • (He <i>et al.</i>, 2009) • (M. Wang <i>et al.</i>, 2003; Imanishi <i>et al.</i>, 2005)
Inflammation	<ul style="list-style-type: none"> • Low grade inflammation during ageing with increased TNF-α • Chronic treatment of EPCs with TNF-α induce premature senescence associated with increased cell cycle inhibitor p16^{INK4a} 	<ul style="list-style-type: none"> • (Csiszar <i>et al.</i>, 2003) • (Y. Zhang <i>et al.</i>, 2009)
Pro-angiogenic factors	<ul style="list-style-type: none"> • Decreased VEGF and SDF-1 in aged tissues impair EPC (Flk-1⁺/CD11b⁺) trafficking to ischemic sites and depress HIF-1α • Lower levels of EPCs (Sca-1⁺/Flk⁺) after ovariectomy of young female mice which is prevented by estrogen treatment 	<ul style="list-style-type: none"> • (E. I. Chang <i>et al.</i>, 2007) • (Strehlow <i>et al.</i>, 2003)
Molecular dysfunction	<ul style="list-style-type: none"> • Reduction in telomere length with age in early EPCs in healthy sedentary men • 57% decrease in early EPCs telomerase activity in older men compared to younger men • Overexpression of human TERT in CACs conserve telomerase activity, enhance EPC function, and delay senescence after ischemic hind limb in mice 	<ul style="list-style-type: none"> • (Kushner <i>et al.</i>, 2009) • (Kushner <i>et al.</i>, 2011) • (Murasawa <i>et al.</i>, 2002)
Decreased nitric oxide bioavailability	<ul style="list-style-type: none"> • Physical exercise, which increases NO bioavailability, increases EPCs (Sca-1+/Flk+) not found in eNOS null mice or mice treated with an NOS inhibitor • ox-LDL accumulation with age impairs early EPCs survival and function 	<ul style="list-style-type: none"> • (Laušs <i>et al.</i>, 2004) • (Ma <i>et al.</i>, 2006)

Table 1: Factors affecting EPC ageing.

VEGF: vascular endothelial growth factor; SDF-1: stromal derived factor-1; HIF-1 α : hypoxia-inducible factor-1 α ; Flk-1: fetal liver kinase; HUVECs: human umbilical vein endothelial cells; GPx-1: glutathione peroxidase-1; AngII: angiotensin II; NO: nitric oxide; ox-LDL: oxidized low-density lipoprotein.

1.3 Haematopoietic stem and progenitor cells

Stem cells are defined as cells that are capable of self-renewal and differentiation. Self-renewal is the process of producing one or two daughter cells identical to the parent cell in the genetic material and epigenetic modification. Those daughter cells are also identical in function with multipotency (full range of differentiation ability) and also self-renewal potential. Differentiation is the process in which cells become more specialised mature functional cells. Stem cells are least prone to differentiation without cell division. The differentiation involves epigenetic change via cell division and the loss of self-renewal or some part of multipotency (Kondo, 2010). Furthermore, stem cells can be classified according to their potency (differentiation potential) into totipotent, pluripotent, multipotent, and unipotent as shown in Table 2.

Cells potency	Definition	Example
Totipotent	Differentiate into all embryonic layers (endoderm, mesoderm, ectoderm) and extraembryonic tissue	Fertilized egg (zygote)
Pluripotent	Differentiate into all embryonic tissues	ES, ICM, iPS cell
Multipotent	Differentiate into all lineages of a tissue/organ	NSC, HSC
Oligopotent	Differentiate into some but not all lineages of a tissue/organ	CLP, CMP
Unitipotent	Differentiate into one type of lineage in a tissue/organ	Marcophage progenitor

Table 2: Stem cells potency

ES: embryonic stem; ICM: inner cell mass; iPS: induced pluripotent stem cell; NSC: neural stem cell; HSC: haematopoietic stem cell; CLP: common lymphoid progenitor; CMP: common myeloid progenitor (Seita and Weissman, 2010)

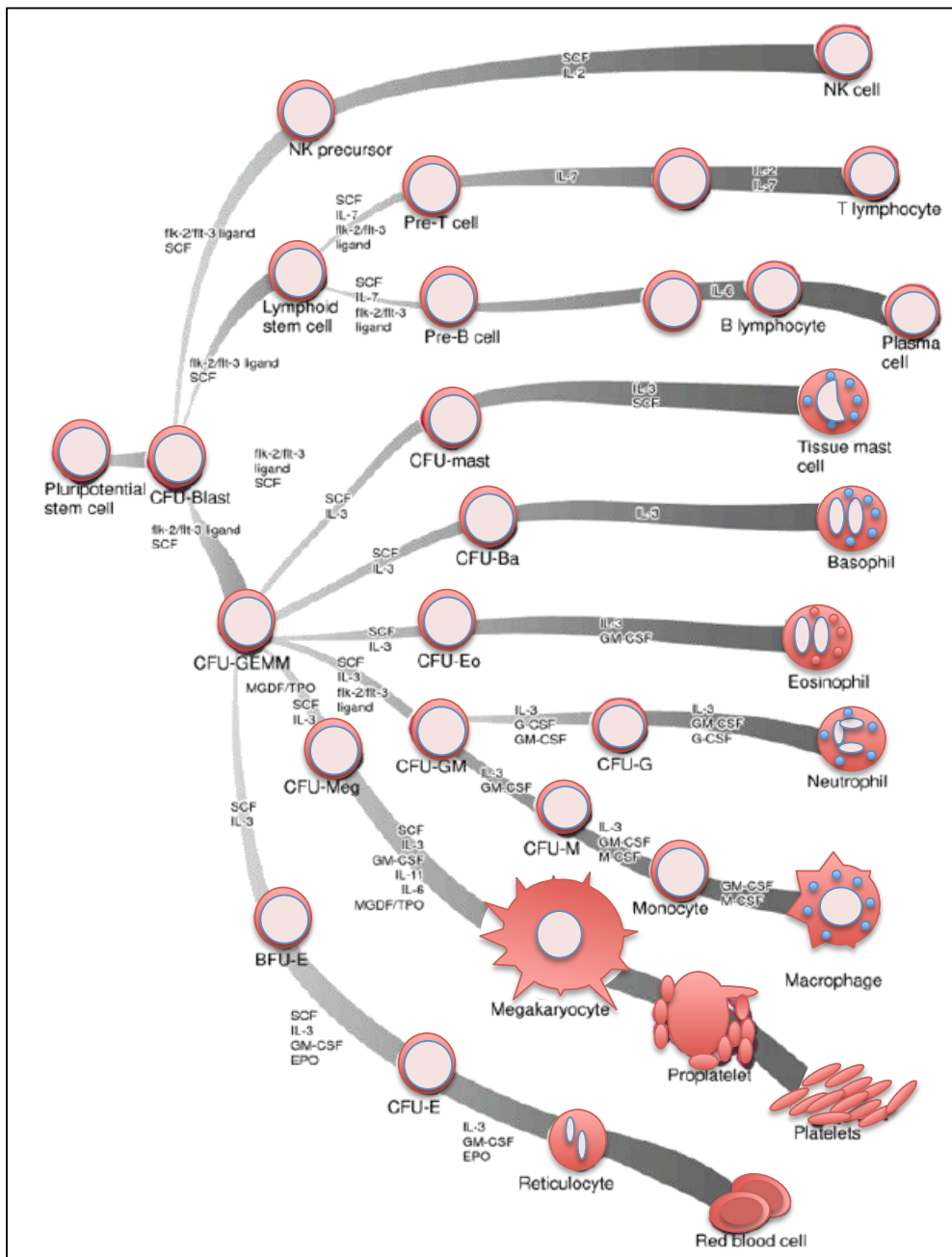


Figure 1.2: Haematopoiesis.

The process of haematopoiesis involves the differentiation of haematopoietic stem cells (CFU-Blast) into either myeloid or lymphoid progenitors. Myeloid precursors differentiate into granulocytes, erythroids, monocytes, and megakaryocytes (CFU-GEMM) mature blood cells which produce granulocytes and monocytes (CFU-GM), erythrocytes from burst-forming units (BFU-E), megakaryocytes (CFU-Meg), mast cells (CFU-mast), basophils (CFU-Bas), and eosinophils (CFU-Eo). Modified from Kufe et al. (2003).

Transplantation assays provide the gold standard functional definition of HSC, which includes self-renewal and differentiation. In mice, for example, HSCs isolated from the bone marrow can rescue the recipient after sublethal irradiation and showed long-term repopulating ability in serial transplantation (Krause *et al.*, 2001). This study has also shown that bone marrow HSCs can differentiate into epithelial cells in the liver, lung, GI tract, and skin of the recipients.

HSCs can be isolated primarily from bone marrow and peripheral blood, but these are limited due to donor compatibility. Umbilical cord blood is an alternative source of HSCs that provides a rapidly available and more youthful population than in peripheral blood and bone marrow. However, limited volumes of UCB lead to lower engraftment and more susceptibility to infections as well as other complications (Ballen *et al.*, 2012). Therefore, to identify ideal HSC generation and expansion, a better understanding of molecular and cellular network involved is crucial. HSCs are mainly slow cycling (Wilson *et al.*, 2008) and reside in the bone marrow for a lifetime but do not originate there. During embryonic development, HSCs are produced and expanded before residing in the bone marrow before birth (Christensen *et al.*, 2004).

1.3.1 Origins of haematopoietic stem cells

The generation of adult HSCs occurs during embryonic development. The initial site for haematopoietic production is the yolk sac. During mouse development on embryonic day (E), 7.25 extra-embryonic tissue, containing both visceral endoderm and mesoderm, produces large nucleated erythrocytes termed erythroblasts (Palis *et al.*, 2001). Surrounding these blood cells, a layer of endothelial cells is formed, and these specialized structures are called blood islands. The haematopoietic and endothelial cells are thought to be generated from a common mesodermal precursor referred as the haemangioblast (Ferkowicz and Yoder, 2005). In addition, it was shown that by E7.5-E8, erythroid-myeloid progenitors start to appear in the yolk sac and in the allantois and chorion (that fuse later, forming the placenta and umbilical cord blood). It was also shown by Medvinsky and Dzierzak (1996) that AGM (aorta-gonads-mesonephros) can develop HSC *de novo* in mice. In mammalian development, two overlapping waves emerge haematopoietic cells known as primitive (short-term and limited haematopoietic cell types)

and definitive (long-term and produce all haematopoietic cell types) haematopoiesis. Definitive haematopoiesis occurs in the extra-embryonic yolk sac later in the development and characterised by lifelong production of all haematopoietic lineages, including HSCs (Medvinsky *et al.*, 2011).

The perspective of the yolk sac as the origin of primitive haematopoiesis was broadly accepted. However, mechanisms pertaining to how sites for definitive haematopoiesis were colonized have been intensively debated in the last century. During development, it was suggested that HSC production migrates sequentially from the yolk sac to multiple niches, including the fetal thymus, spleen, liver, and bone marrow. Supporting this theory, Moore and Owen (1965) showed that haematopoietic stem cells do not appear *de novo* in the same way with the yolk sac but rather originated and migrated from the yolk sac to colonize other organs' rudiments. Furthermore, the principle of migration from the yolk sac was also supported by Hossaint (1981), who demonstrated grafting mouse liver rudiments during early development (10 days before gestation) in chick/quail do not produce haematopoietic cells. While grafting the liver, when it was taken after the circulatory system had been established at a later stage, resulted in haematopoiesis. However, this study shows the migration of haemangioblast in the colonization of fetal liver but does not directly illustrate that these haematopoietic stem cell progenitors originated from the yolk sac and possibly have migrated from alternative haematopoietic niche. Alternative niches of the haemangioblast from the embryo proper from quails (Dieterlen-Lievre, 1975), the aorta-gonads-mesonephros (AGM) region in mouse (Godin *et al.*, 1995) and human (Tavian *et al.*, 1999).

In summary, HSC origin studies illustrated that the site of haematopoietic development and haematopoiesis is not fixed and involves sequential niches in the two developmental waves, beginning with short-term presence at the blood islands of the yolk sac (primitive) to their long-term residence in the bone marrow (definitive) (Figure 1.3).

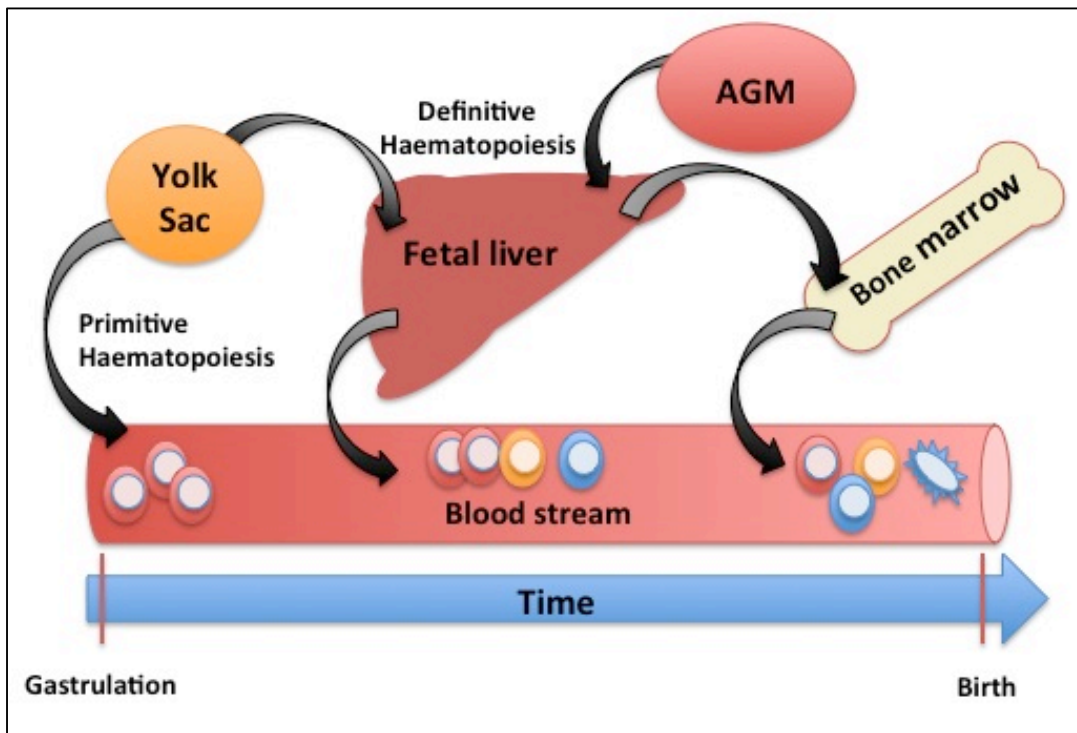


Figure 1.3: Haematopoietic niche model during development.

Haematopoiesis occurs in two developmental waves: the primitive wave, which involves the yolk sac, and the definitive wave in the fetal liver, where haematopoietic progenitor cells migrate from the yolk sac and AGM (aorta-gonads-mesonephros). Then, the bone marrow becomes the major niche and the primary haematopoietic progenitors source for a lifetime. Reproduced from McGrath et al. (2005).

1.3.2 Uses of haematopoietic stem cells

The first experiment of the stem cell concept was proved by McCulloch and Till by performing bone marrow cell transplantation into irradiated mice (Till and McCulloch, 1961). These bone marrow cells produced myeloid multilineage colonies in the spleens of the transplanted animals, which was proportional to the number of injected cells. Successful human stem cell transplantation was reported since 1957 and then was performed on nuclear workers and leukemia patients (Thomas *et al.*, 1957; Mathe *et al.*, 1959). This provided formal evidence that intravenous injection of bone marrow allows the long-term repopulation of new blood cells. For over 50 years now, such transplantations have been performed to treat patients with malignancies, bone marrow failure, and immunodeficiencies. After conditioning regimens, such as total body irradiation and chemotherapy to suppress the immune system, HSCs are infused intravenously to myeloablated patients. Long-term reconstitution of the haematopoietic system is achieved gradually with donor cells if infection and immunological reaction are under control (Kondo, 2010). There are two types of stem cell transplantation, autologous and allogeneic, based on the source of haematopoietic cells. Autologous stem cell transplantation is performed to rescue patients after doses of radiation and/or chemotherapy with their own mobilised peripheral blood. The allogeneic hematopoietic stem cell transplantation differs from the autologous in that the haematopoietic cells obtained from an appropriate HLA-matched donor can rescue patients and also induce graft-versus-host disease (GVHD) (Ferrara *et al.*, 1999). For this reason, these patients require immunosuppressive therapy. Furthermore, hematopoietic stem cell transplantation can also be used to induce tolerance to solid organ grafts. At Stanford University, it has been shown that with kidney transplantation in patients with an HLA-matched sibling, followed by total irradiation and then infusion of T cells and CD34+ stem cells from the same donor, 8 out of 12 patients successfully discontinued all immunosuppressive drugs (followed up from 12-36 months) (Scandling *et al.*, 2011). However, a recent follow up study of HLA-mismatched renal transplantation without maintenance of immunosuppression reported 4 out of 10 patients without rejection, 2 patients who developed rejection, and 1 patient who developed recurrent disease; the

remaining 3 patients could not stop the immunosuppression due to rejection (Kawai *et al.*, 2013).

In addition to bone marrow, cord blood and mobilised peripheral blood are sources of HSCs used in transplantation. However, HSC numbers remain limited, which is a major obstacle for basic and clinical research. Despite the extensive research and current better understanding of intrinsic and extrinsic HSC regulation, it remains very difficult to reproduce HSCs *in vitro* without inducing differentiation (Hofmeister *et al.*, 2007). A potential promising source of HSCs includes induced pluripotent stem cells (iPS), which are created by the transcription factor-mediated reprogramming of somatic cells (K. Takahashi and Yamanaka, 2006). Interestingly, a recent study reported a strategy where by authors induced a megakaryocyte progenitor cell line from a human embryonic stem cell and iPS was able to show thrombogenic activity in mouse models of thrombocytopenia (S. Nakamura *et al.*, 2014). These findings suggest the potential of megakaryocyte progenitor cell lines for clinical application.

1.3.3 Characterisation of haematopoietic stem cells

Since 1988, Weissman and collaborators pioneered in the identification of HSCs through a set of protein markers on the surface of mouse blood cells using multi-colour fluorescence-activated cell sorting (FACS) (Spangrude *et al.*, 1988). The identified population was capable of haematopoietic system reconstitution when transplanted into lethally irradiated mice. This mouse HSC-enriched population accounted for about 0.05% of the total bone marrow cells and expressed Thy-1^{low}Lineage⁻Sca-1⁺ markers (Spangrude *et al.*, 1988). The most thoroughly characterised HSC population relies on the expression of membrane glycoprotein Sca-1 and the tyrosine kinase receptor C-Kit (CD117) along with the lack of lineage markers (Ter119, Gr-1, Mac-1, B220, CD4, and CD8) (Okada *et al.*, 1992). Furthermore, C-Kit⁺Sca-1⁺lineage⁻ (KSL) cells contain a variety of progenitor cells capable of haematopoietic reconstitution. Further strategies to enrich KSL cells with HSCs involved other markers: CD34 (CD^{neg/low}KSL) (Osawa *et al.*, 1996), Flk2 (CD34⁻Flk2⁻KSL) (Christensen and Weissman, 2001), and the efflux of supravital dye Hoechst 33342 (Goodell *et al.*, 1996). Other methods to identify HSCs have been described, including Endoglin (C. Z. Chen *et al.*, 2002), SLAM (Signalling

lymphocyte activation molecule) family markers CD150⁺CD48⁻ (Slamf2 and Slamf2) (Kiel *et al.*, 2005; I. Kim *et al.*, 2006), Tie-2 (Arai *et al.*, 2004), CD133 (Yin *et al.*, 1997), and a CD201 endothelial protein C receptor (EPCR) (Balazs *et al.*, 2006). Recently, it has been shown that CD49f⁺ cells, specifically Lin⁻CD34⁺CD38⁻CD45RA⁻Thy1⁺Rho^{lo}CD49f⁺, were highly efficient in long-term multilineage engraftment in NOD-SCID (nonobese diabetic severe combined immunodeficient)-IL-2 γ c^{-/-} mice (Notta *et al.*, 2011).

Similar to technologies used for the isolation of mouse HSCs, human HSCs were also isolated on the basis of functional assays and cell-surface marker phenotype. In human HSCs, the long-term reconstitution property was evaluated with xenotransplantation models (Baum *et al.*, 1992). CD34 is considered the first cell surface marker to enrich human HSCs. It is a ligand for L-selectin expressed by 0.5-5% of blood cells in human fetal liver, cord blood, and adult BM (Krause *et al.*, 1996). In-vitro assays revealed that these populations are very heterogeneous and mostly have multipotency and oligopotency (Civin *et al.*, 1984; DiGiusto *et al.*, 1994; Krause *et al.*, 1996). Human CD34⁺ has been used clinically in numerous autologous and allogeneic transplantations and proved potential engraftment (Vogel *et al.*, 2000). In contrast, CD34⁻ is reported to engraft NOD/SCID mice by intra-BM injection, which gives rise to CD34⁺ HSCs in vivo and in vitro. Such a finding suggests the presence of more primitive cells within the CD34⁻ population (Y. Nakamura *et al.*, 1999). Studies have identified two further markers within the Lin⁻CD34⁻ cells from human CB HSC, which was able to repopulate SCID mice, CD93 (Anjos-Afonso *et al.*, 2013), and CD133 (M. Takahashi *et al.*, 2013). However, engraftment is only achieved by direct intra-BM injection, suggesting homing difficulties in comparison to CD34⁺ (Yahata *et al.*, 2003; M. Takahashi *et al.*, 2013).

1.3.4 HSCs and ageing

The dysfunction of HSCs, which give rise to both myeloid and lymphoid lineages, might underlie the ageing of the immune system. The innate and adaptive immune systems coordinate the immune response by myeloid leukocytes and lymphoid cells (B and T lymphocytes), respectively. As age progresses, the production of naïve T cells declines

and the clonal expansion of memory and effector T cells increases, which leads to less immunity and more autoimmunity in the elderly (Dorshkind *et al.*, 2009). B cells decrease in number with age and produce antibodies with less diversity and affinity (Han *et al.*, 2003). In comparison, myeloid lineage expands with age, thus providing a pro-inflammatory environment (Franceschi *et al.*, 2000). Other cells, such as dendritic antigen-presenting cells and natural killers, also diminish and become functionally impaired during ageing (Mocchegiani and Malavolta, 2004). These phenotypes of inflammation and immunosenescence are contributed in several pathologies during ageing, such as cancer and autoimmune diseases. The ageing of HSCs is discussed in this section in several levels, including population, cellular, and molecular.

At the **population level**, in mouse HSC ageing, the HSC number was increased and myeloid-skewed with lymphoid deficiency, despite differences from strain to strain (Sudo *et al.*, 2000; M. Kim *et al.*, 2003; Dykstra *et al.*, 2011). Similarly, immunophenotypically defined haematopoietic stem and progenitor cells (CD34+CD38- or Lin-CD34+CD38-CD90+CD45RA-) increase in elderly individuals' bone marrow (>65 years) (Kuranda *et al.*, 2011; Pang *et al.*, 2011). However, the increase in the number of HSCs does not compensate for the loss of their function during ageing, thus leading to an overall decline in the regenerative capacity (Sudo *et al.*, 2000). Aged HSCs show reduced self-renewal activity in serial transplantation assays (Rossi *et al.*, 2005). To explain HSC ageing, two models were proposed: first, a clonal alteration model and, second, a population shift model (Muller-Sieburg and Sieburg, 2008). The clonal alteration model implies that HSCs change uniformly as a homogenous population by which individual aged stem cells differentiate into a myeloid, rather than a lymphoid, lineage. However, recent evidence supported the population shift, as evidence suggested that HSC are heterogeneous and ageing HSCs are derived from a different composition of distinct classes of HSCs (Cho *et al.*, 2008; Beerman *et al.*, 2010; Dykstra *et al.*, 2011). There are three classes of HSCs in mice; balanced, myeloid-biased (my-bi) and lymphoid-biased (ly-bi) stem cells. Both young and old marrows show all three subsets of HSCs; however, during ageing the composition shift over to the my-bi HSC become dominant in old marrow. On the other hand, human HSC ageing in the bone marrow is largely unknown, due to insufficient information for HSCs markers (Cavazzana-Calvo *et al.*, 2011).

At the **cellular level**, ageing is associated with metabolic byproducts, genomic damage, and telomere shortening, thus leading to the functional decline of stem cells (Nijnik *et al.*, 2007; Sahin and Depinho, 2010; Yahata *et al.*, 2011). The proliferative response of HSCs to early-acting cytokines flt3 ligand (FL), c-kit (KL), and thrombopoietin (TPO) decreases with ageing (Henckaerts *et al.*, 2002). However, aged HSCs are more actively cycling, in which more frequent cells were in the S/G2/M phases of cell cycle compared to the HSCs of young mice (Morrison *et al.*, 1996). Old HSCs that were transplanted into myeloablated recipient mice show less reconstitution (Morrison *et al.*, 1996), more mobilization to the peripheral blood (Xing *et al.*, 2006), and less homing and engrafting in the bone marrow of recipients (Liang *et al.*, 2005).

At the **molecular level**, microarray analysis revealed a general ageing signature of HSCs in comparison to young individuals. The expression of genes linked to protein aggregation, inflammatory response, and oxidative stress increases with age, while genes involved in transcriptional regulation and genomic integrity (chromatin remodeling and DNA repair) decrease with age in both mice and humans (Rossi *et al.*, 2005; Pang *et al.*, 2011).

Mitochondria and ROS can also affect HSC ageing. It has been shown that the lack of mitochondrial DNA polymerase catalytic subunit gamma (PLOG) expression in transgenic mice leads to increased mtDNA mutations that developed anaemia and lymphopenia (Trifunovic *et al.*, 2004). Furthermore, the reduced self-renewal potential of HSCs in serial transplantations can be rescued with N-acetyl cysteine (NAC)-mediated ROS inhibition (Ito *et al.*, 2006). Interestingly, HSCs with high levels of interacellular ROS showed an increase in the p38MAPK activity and mTOR, both of which have been shown to induce stem cell exhaustion after serial transplantation (Y. Y. Jang and Sharkis, 2007). Moreover, mice lacking the FOXO protein family of transcription factors (an integral part of the IIS pathway) showed higher ROS levels and less quiescence than wild-type mice (Miyamoto *et al.*, 2007). However, it has also been shown that the ROS levels in physiologically aged HSCs and HSCs from Plog^{-/-} mice are not elevated. Thus, one may question whether or not ROS and mtDNA can directly affect HSC ageing (Norrdahl *et al.*, 2011).

A new insight into the molecular regulation of HSC ageing linked intercellular activity of RhoGTPase Cdc42 to the declined HSC functionality during ageing (Florian *et al.*, 2012). Authors have found that Cdc42 activity in HSCs increases with ageing and the genetically enforced activation of Cdc42 leads to a premature ageing phenotype. Cdc42 distribution in the cytoplasm has been shown to be polarised in young HSCs, but diffused in aged HSCs. Even more interestingly, in aged HSCs the pharmacological inhibition of Cdc42 restores polarity and rejuvenates them to a youthful state. Recently, the same group has shown that Wnt5a activates Cdc42 and induces an ageing phenotype in young LT-HSCs (Lin-Sca-1+C-Kit+CD34-Flk-) (Florian *et al.*, 2013).

1.4 Telomere length and telomerase

1.4.1 Telomeres

Telomeres are specialised nucleoproteins at the ends of mammalian chromosomes and are composed of long hexa nucleotide repeats (TTAGGG)_n and that are associated with shelterin multiprotein complex (Figure 1.4). There are six proteins in the human shelterin complex: telomere repeat factor 1 (TRF1) and 2 (TRF2), TRF1-interacting nuclear protein 2 (TIN2), TPP1, protection of telomeres 1 (POT1), and repressor/activator protein 1 (RAP1) (de Lange, 2002). Telomere length varies among species, ranging from 2-15 kilobase pairs (kb) in humans (Martens *et al.*, 1998) and 40-80kb in laboratory mice (Blasco *et al.*, 1997). The main function of telomeres and their associated proteins is the maintenance of chromosomal integrity and stability by (1) the protection of chromosomal ends from enzymatic degradation, (2) the prevention of end-to-end chromosome fusion, and (3) protection from DNA damage response by the prevention of chromosomal DNA to be recognised as a double-stranded breaks (de Lange, 2002). Telomeres are regarded as a biological clock, as they shorten with each cell division as a result of the end replication problem and oxidative stress. The end replication problem occurs when the DNA-polymerase cannot fully replicate chromosomal ends (more details are described in the next section). When reaching a critical length, cells either enter senescence or apoptosis. In most somatic cells, unlike stem cells, telomeres shorten with each cell division, thus leading to a finite limit known as the “Hayflick limit” (Allsopp *et al.*, 1992;

von Zglinicki *et al.*, 1995; Blasco *et al.*, 1997). However, cellular senescence can also occur through telomere-independent signaling, such as altered mitogenic signaling, cyclin-dependent kinase inhibitors signaling, and non-telomeric DNA damage response, as well as other signaling pathways (Rodier and Campisi, 2011).

Telomere length has been postulated as a biomarker of human ageing since it is an indicator of cellular senescence and oxidative stress (von Zglinicki and Martin-Ruiz, 2005). Remarkable epidemiologic researchers measured telomere length, mostly the average leukocyte telomere length (LTL), to investigate the links to ageing and disease. LTL shortening is associated with age, several age-related diseases, environmental risk factors linked to vascular ageing (e.g., high BMI, insulin resistance, sedentary lifestyle, and smoking), and atherosclerosis (Samani *et al.*, 2001). In fact, a human longitudinal study recently indicated that smoking and metabolic syndrome variables (waist-hip ratio, blood glucose, cholesterol level) accelerate telomere attrition (Huzen *et al.*, 2014). Therefore, these cells may serve as biomarkers of chronic immune activation, a cumulative burden of inflammation, or oxidative stress. Furthermore, hereditary diseases caused by telomerase mutations implicated in the telomere length include dyskeratosis congenita (Vulliamy *et al.*, 2001), idiopathic pulmonary fibrosis (Armanios *et al.*, 2007), and bone marrow failure syndromes (Vulliamy *et al.*, 2002). As haematopoietic stem cells are on the top of the hierarchy of leukocytes, telomere length in HSCs and LTL are mirrored at any age. The maximum length of telomeres is observed at birth and then it progressively shortens with age (Flores *et al.*, 2008). Furthermore, the haematopoietic system undergoes the highest telomere attrition early in life, probably due to its high proliferative potential to maintain blood homeostasis (Allsopp *et al.*, 1992; Rufer *et al.*, 1999).

Telomere length maintenance can be either by telomerase (described in the next section) or via an alternative lengthening of telomeres (ALT) mechanism. About 10% of tumours stabilise telomere length with ALT, which involves DNA homologous recombination (Bryan *et al.*, 1997). Furthermore, it was shown that during the early embryo cleavage stage, telomeres are also lengthened by an ALT mechanism (Liu *et al.*, 2004). ALT was recently found to exist in normal mouse somatic cells by using a telomere-tagged transgenic mouse strain, but not in the germline (Neumann *et al.*, 2013).

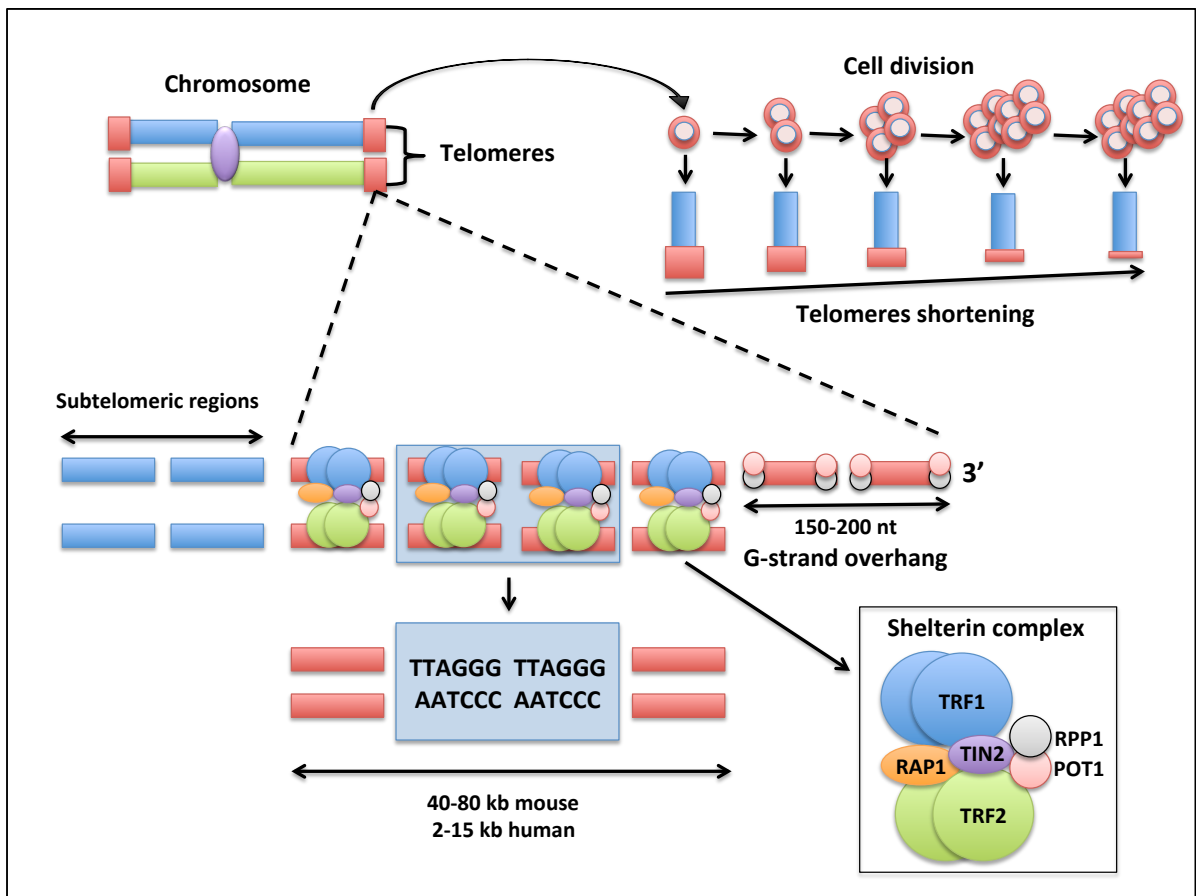


Figure 1.4: The structure of telomeres.

Telomeres are end caps of chromosomes bound by a complex of proteins regarded as telomerase or shelterin, which is composed of TRF1, TRF2, RAP1, POT1, TPP1, and TIN2. POT1/TPP1 heterodimer is also bound to the G-strand overhang. Telomere length shortens with each division, until it reaches a critically short length. Telomere erosion leads to cell arrest and replicative senescence. Modified from Hoffman et al. (2011).

1.4.2 Telomerase

Since telomerase was first described by Nobel Prize winners Elizabeth Blackburn, Carol Greider, and Jack Szostak (Greider and Blackburn, 1985), it has attracted many scientists from multidisciplinary fields since it represents the possible key to the fountain of youth. Telomerase plays a pivotal role in stem cell rejuvenation by telomere maintenance and is, therefore, a crucial factor in ageing. Ageing is associated with tissue atrophy, reduced telomerase activity, telomere shortening, and stem cell senescence (Flores and Blasco, 2010). Telomerase structure, expression, function, diseases, and mutations will be discussed in this section.

1.4.2.1 Telomerase structure

Unlike somatic cells, stem cells maintain telomere length by a unique reverse transcriptase called telomerase. Telomerase replicates telomeres (the ends of telomeres) (Greider and Blackburn, 1989). Telomerase is composed of the catalytic subunit reverse transcriptase (TERT), the RNA template (TERC) for telomere synthesis, and associated proteins (such as dyskerin and heat-shock protein hsp90) (Lingner *et al.*, 1997; T. M. Nakamura *et al.*, 1997). For the catalytic activity (canonical function) of telomere maintenance and elongation, both TERT and TERC are required (Lingner *et al.*, 1997). The structure of TERT and TERC are similar in mice and humans. Mammalian TERT mRNA is composed of 16 exons and 15 introns that extend over 35kb (Y. S. Cong *et al.*, 1999). Mouse gene TERT is located on chromosome 13, whereas human TERT is located on chromosome 5 (Greenberg *et al.*, 1998). Mouse and human TERT genes have a similar promoter region which differ in regulation (Pericuesta *et al.*, 2006). In contrast, the non-coding RNA component of telomerase (TERC) is similar between mice and humans and both are located on chromosome 3. Mature mammalian TERC is composed of 451 nucleotides, while its template part is complementary to the telomeric sequence which is composed of 11 nucleotides (5'-CUAACCCUAAC-3') (Feng *et al.*, 1995; Egan and Collins, 2012). Furthermore, there is a high functional similarity between mouse and human telomerase. When telomerase-activity-deficient mouse cells (TERC knockout) were transfected with human telomerase genes, telomerase was fully restored indicating

the structural conservation and functional interchangeability between mice and humans (Martin-Rivera *et al.*, 1998).

1.4.2.2 Telomerase expression

There is a tissue-specific expression of telomerase activity in mammals. Mice show telomerase activation across different cells and tissues including intestine, muscle, liver, testes, and lymphocytes throughout life. During human embryonic development, telomerase is active; after 20 weeks of gestation, it gradually decreases in the embryo. However, adult stem cells, germ cells, endothelial cells, and lymphocytes still show detectable telomerase activity (Ulaner *et al.*, 1998). Furthermore, epigenetic regulation was shown to modify telomerase activity during stem cells via acetylation/deacetylation, as well as methylation/demethylation of histone H3 in the TERC promoter and histone H3 and H4 in the TERT promoter region (Saretzki *et al.*, 2008; Yang *et al.*, 2008).

The functions of telomerase can be divided into canonical and non-canonical. With each cell division, chromosomes must be replicated to share DNA between two daughter cells. The end caps of chromosomes (telomeres) get shorter, due to the “end replication problem” (Figure 1.5). During mammalian cell division, the chromosomal DNA is duplicated with DNA polymerases. DNA polymerases can only synthesise DNA in a 5'-3' direction. As DNA polymerases need a template to start the DNA synthesis, they use an 8-12 base segment of RNA as a starting primer. While the leading strand can be continuously synthesised, the lagging strand is not fully synthesised, leading to short, RNA-primed segments termed Okazaki fragments. After DNA extension, the gaps filled in by DNA polymerase priming from upstream DNA 3' ends and the RNA segments are removed. An 8-12 base pairs gap is created after the removal of the outer 5'-RNA primer, leading to a daughter strand that is shorter than the parental strand. This inability to achieve full DNA replication causes a loss of the terminal sequence during each replication cycle (Watson, 1972; Levy *et al.*, 1992). This short fragment of telomere (≈ 20 bp) is lost with each division. Once a critical level length of telomere is reached, cells face cell cycle arrest and replicative senescence. Telomerase recognises the strand (3'-G-rich) and elongates it with the 5' TTAGGG telomeric repeats that are complementary to RNA template on the TERC component (Blackburn, 2001). This

telomeric shortening is even significantly accelerated with oxidative stress ($\approx 50\text{-}100\text{bp}$) (von Zglinicki *et al.*, 1995).

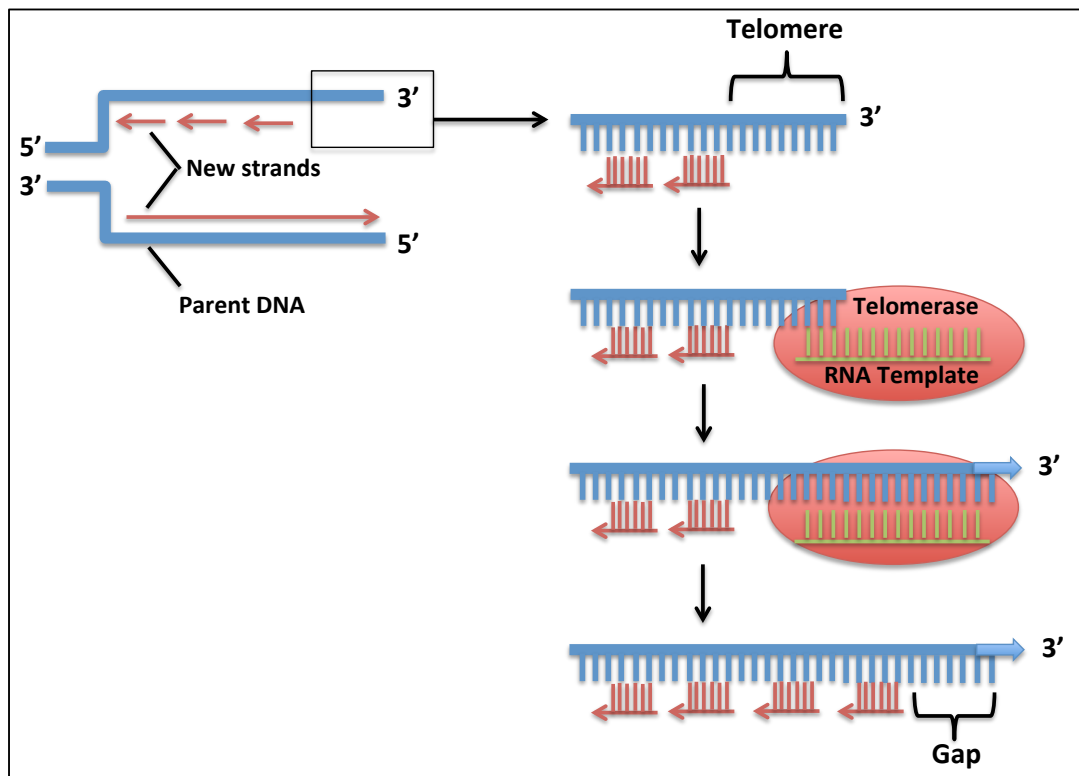


Figure 1.5: Telomerase function.

DNA polymerase lacks the ability to replicate the lagging strand ($3'\text{-}5'$) forming Ozaki fragments on the lagging strand, thus leading to an “end replication problem”. Therefore, telomeres shorten with each cell replication. Telomerase elongates telomeres by binding to a $3'$ overhang and extending it with TTAGGG repeats. DNA polymerase then synthesises a new lagging $5'$ end strand of telomeres. Modified from Sadava (2007).

1.4.2.3 Functions of telomerase

The **canonical function** of telomerase involves the protection of telomeres against erosion, chromosomal end fusion, and the instability of chromosomes. Therefore, telomerase enzymatic activity is required for the indefinite potential of immortal cell proliferation (e.g., embryonic stem cells, germ cells, and cancer cells) (Greenberg *et al.*, 1998; Martin-Rivera *et al.*, 1998). When telomerase is absent (i.e., somatic cells), telomeres shorten with each cell division. Eventually, telomere length reaches a critical point that activates a p53-dependent DNA damage response (DDR) in the telomeric region that may lead to cell cycle arrest and apoptosis or senescence (Saretzki *et al.*, 1999). DDR is activated by the sensor kinases, such as ATM/ATR or DNA-PK or the phosphorylation of γ H2A.X, leading to the activation of Ck1 and Ck2 (cell cycle checkpoint proteins) and a p53/p21 pathway, causing cell-cycle arrest (d'Adda di Fagagna *et al.*, 2003).

Many studies suggest several **non-canonical functions** of telomerase. Mounting experimental data suggests that TERT is involved in telomere-independent effects on cell survival, proliferation, stem cell biology, cellular transformation, mitochondrial function, DNA damage response, regulation of gene expression, and chromatin remodeling (Y. Cong and Shay, 2008). The overexpression of TERT was reported to activate epidermal stem cell proliferation and mobilisation in transgenic mice with enhanced hair growth, increased keratinocyte proliferation, and augmented skin hyperplasia independent of its reverse transcriptase activity (Flores *et al.*, 2005). Furthermore, TERT can act as an integrator and transcriptional co-activator of signaling pathways, as in Wnt/ β -catenin and Myc (Choi *et al.*, 2008). TERT also acts as a co-factor that promotes Wnt target genes through interaction with the Wnt- β -catenin transcriptional complex (Park *et al.*, 2009). Another study showed a regulatory feedback loop between Wnt and TERT by which it has been suggested that β -catenin, which is an effector in Wnt signaling, might be a direct regulator of mouse TERT expression (Hoffmeyer *et al.*, 2012). In addition, human TERT interacts with the RNA component of mitochondrial RNA-processing endoribonuclease (RMRP), which has a function in processing ribosomal RNA in the nucleus. This gene is mutated in pleiotropic syndrome cartilage-hair hypoplasia and an hTERT-RMRP complex generates other small-interfering RNAs that regulate gene expression at the

post-transcriptional level (Maida *et al.*, 2009). Nitta *et al.* (2011) studied a TERT^{-/-} and ATM^{-/-} knockout mouse model and demonstrated that TERT is required for HSC function and tissue renewal in ageing. These double-knockout models (TERT^{-/-}ATM^{-/-}) showed higher senescence markers (γ H2AX and p16), oxidative stress, and apoptosis, but such findings were not present in the single-knockout model. Moreover, it has been shown that mitochondrial TERT plays a protective function by reducing reactive oxygen production, protecting mtDNA against damage, increasing the membrane potential, and inhibiting apoptosis induction (Ahmed *et al.*, 2008; Haendeler *et al.*, 2009). In fact, it has been demonstrated recently that mitochondrial telomerase localisation specifically decreases cellular oxidative stress and mitochondrial ROS generation after induction of exogenous stress generated by irradiation or H₂O₂ in cancer cells and thereby prevents nuclear DNA damage (Singhapol *et al.*, 2013).

Telomerase function is regulated by genetic, epigenetic, and environmental factors. Several polymorphisms in the TERT promoter region and telomerase-associated protein genes have been shown to regulate telomerase expression and activity (Hsu *et al.*, 2006). The transcriptional activators that target the TERT promoter include c-Myc, Sp1, Est-1, nuclear factor kappa B (NF κ B), nuclear factor of activated T-cells, cAMP response element protein, hypoxia-inducible factor, and signal transducer and activator of transcription 3 (STAT3). The transcriptional repressors include myeloid zinc finger 2, p53, Wilms tumor 1, AP-1, AP-2, AP-4, CCCTC binding factor, menin, and Smad3 (Kyo *et al.*, 2000). Furthermore, the TERT promoter contains CpG islands and, thus, is susceptible to epigenetic modification by DNA methylation (Kyo *et al.*, 2008). Histone acetylation and deacetylation also became increasingly important in TERT transcriptional regulation. Histone deacetylase inhibitors activate the TERT promoter by SPI (Doetzlhofer *et al.*, 1999), with recruitment mimicking TERT mRNA expression by T-cell antigen receptor stimulation through maintaining H3/H4 acetylation (Hou *et al.*, 2002). TERT expression is also regulated by an mRNA splicing mechanism that contains at least six splice sites and α exerts a dominant negative effect on normal transcripts (Colgin *et al.*, 2000). In addition, microRNAs are involved in the control of TERT gene expression and translation, whereas high levels of telomerase activity in NK/T-cell lymphomas is associated with diminished miRNA-150 and transfection with exogenous

miRNA-150-reduced telomerase activity via reducing Akt kinase expression (Watanabe *et al.*, 2011). Telomerase activity is also regulated at the post-translational level by phosphorylation and protein folding. TERT protein has multiple serine residues that are putative sites for phosphorylation and Akt kinase activation (Kang *et al.*, 1999). TERT phosphorylation and activation also occur via protein kinase C (PKC α) (H. Li *et al.*, 1998). Moreover, lifestyle factors can implicate telomerase function. Exercise induces aortic telomerase activity and telomere-stabilizing proteins in mouse models. Exercise decreased apoptosis regulators and vascular cell cycle inhibitors were absent in TERT^{-/-} mice. Telomerase activity and telomere proteins were upregulated and pro-apoptotic proteins were downregulated in young and middle-aged athletes compared to untrained subjects (C. Werner *et al.*, 2009).

1.4.2.4 Telomerase dysfunction in disease

Several diseases are linked to mutations or changes in telomerase gene expression. Telomerase was found to be active in over 90% of human cancers and is responsible for their unlimited proliferative potential. Therefore, numerous studies in vitro and in vivo investigated telomerase as a major pharmacological target for cancer therapy (Ruden and Puri, 2013). However, the other 10% of tumours stabilise telomere length without telomerase activity via the ALT mechanism (Bryan *et al.*, 1997). Other than cancer, a number of diseases are related to dysfunctional telomerase genes, including aplastic anaemia, pulmonary fibrosis, and hepatic cirrhosis, which are all associated with short telomeres (Young, 2012). Another example, dyskeratosis congenita, shows some features of premature ageing (anaemia, premature graying, thrombocytopenia, predisposition to cancer) with autosomal-dominant deletion in the human TERC gene. A common feature of these patients is their extraordinary short telomeres (Vulliamy *et al.*, 2001). Furthermore, telomerase is a vital factor in ageing, due to its role in rejuvenating stem cells through telomere maintenance. In ageing tissues, stem cell ageing diminishes the regeneration potential. It was shown that telomerase is activated in adult stem cells with longer telomeres than the surrounding tissues (Flores *et al.*, 2008).

1.4.2.5 Telomerase dysfunction in mouse models

Telomerase mutation studies in animal models have advanced the research field. $TERT^{-/-}$ telomerase-deficient mice show direct evidence of a loss of differentiation and proliferation capacity of somatic and germ cell lines with telomere shortening (Allsopp *et al.*, 2003). In comparison, the overexpression of TERT in embryonic stem cells enhanced proliferation, enriched apoptosis and oxidative stress resistance, and improved differentiation (Armstrong *et al.*, 2005; Yang *et al.*, 2008).

Mouse TERT and TERC knockout models that lack telomerase activity only show defects in the late generations with increased age-related disease incidence and premature tissue degeneration affecting high cellular turnover tissues, such as intestine and bone marrow (Blasco, 2003). Late generations of $TERT^{-/-}$ mice lacking functional telomerase show reduced testes mass and fertility, as well as increased intestinal cell apoptosis (Allsopp *et al.*, 2003; Meznikova *et al.*, 2009).

$TERT^{-/-}$ G1 and G2 did not show any phenotypic changes in the testes, ovary, brain, gastrointestinal tract, liver, pancreas, bone marrow; other tissues were all normal in comparison to wild types (Yuan *et al.*, 1999). However, telomere length-shortening occurred in TERT-deficient mice. TERT-heterozygous mice were able to elongate TL to a level equivalent to the wild type, unlike the deficiency seen by $TERC^{+/-}$ (Chiang *et al.*, 2004). Moreover, when TERT and TERC mutations were introduced by backcrossing with mice with an already-shortened telomere background (CAST/EiJ), progressive telomere shortening and reduced survival of $TERT^{+/-}$ mice with progressive breeding (9 generations) have been shown (Strong *et al.*, 2011). Interestingly, these mice showed a less severe phenotype than the TERT-null mice (skewed myeloid/erythroid in the bone marrow, extramedullary hematopoiesis in the liver and spleen, intestinal villous atrophy and crypt depletion, crypt hyperplasia and microadenomas, and typhocolitis in the large intestine).

Similarly, $TERC^{-/-}$ that was compared to wild types demonstrated an impaired regeneration of organs, shorter lifespans, skewed differentiation of stem cells, kyphosis, and decreased body weights (Lee *et al.*, 1998; Rudolph *et al.*, 1999). Studies by Lee *et al.* (1998) on the first-generation G1 $TERC^{-/-}$ revealed no adverse effect on the overall clinical condition (weight, motor behaviour/activity, lifespan,) through 20 months of life.

G1 $TERC^{-/-}$ showed a normal morphology and capacity of haematopoietic progenitor cells to grow and differentiate in clonogenic assays in vitro, however, in G3 and G6, colonies were significantly reduced. Furthermore, the authors noticed infertility with no offspring produced after G6 intercrosses. The male reproductive systems demonstrated smaller testes, impaired spermatogenesis, and increased apoptosis in the testes (G6). Similarly, the G6 $TERC^{-/-}$ female reproductive system exhibited smaller ovaries and a decreased number of oocytes. Interestingly, Rudolph *et al.* (1999) studied $TERC^{-/-}$ mice extensively under ageing and stress. G3 and G6, when compared to $TERC^{+/+}$, showed increased hair graying and alopecia (hair loss) at a younger age (less than 6 months) and during ageing (older than 15 months). In those ageing late generations (G6) of $TERC^{-/-}$ mice, a spectrum of consequences were shown, including ulcerative skin lesions that were negatively correlated with telomere length, decreased body weight, and decreased wound healing. As a measure of stress response, blood cell ablation was performed with 5-fluorouracil (5-FU) to measure an induced stress response. Unlike young mice (1-3 months), old mice showed a decline in blood cells during ageing, with an increased loss of G6 in comparison to G3 and $TERC^{+/+}$. Furthermore, there was an increased frequency of chromosomal fusion during ageing in G3 and an increased incidence of cancer (i.e.: teratocarcinoma, lymphomas, squamous cell carcinomas), especially in late generations (G4-G6). In addition, in conditions with stress, as in serial transplantation of G1 HSCs from both $TERC^{-/-}$ and $TERT^{-/-}$, repopulating activity was reduced and a loss of telomere length was noted (Allsopp *et al.*, 2003). Furthermore, the lack of telomerase activity and telomere dysfunction in late generations of $TERT^{-/-}$ and $TERC^{-/-}$ mice is associated with impaired mitochondrial function and biogenesis. In particular, $TERT^{-/-}$ mice showed increased ROS production in heart, liver, and haematopoietic stem cells, cardiomyopathy, and decreased gluconeogenesis. These defects were shown to be through PGC-1 α -dependent activation of p53 (Sahin *et al.*, 2011). Interestingly, a reversible TERT knockout was engineered by using an allele encoding a 4-OHT-inducible telomerase reverse transcriptase-oestrogen receptor (TERT-ER). In late-generation (f4) $TERT^{-/-}$, telomerase was reactivated by a 4-OHT (4-Hydroxytestosterone) treatment that reduced DNA damage signaling and restored tissue function by re-establishing the stem cell population; for example, in interstitial crypts and the brain (Jaskelioff *et al.*, 2011).

Another promising genetic manipulation study showed that telomerase gene therapy might substantially delay or reduce age-related decline of mouse health span. An adeno-associated virus (AAV) was introduced; carrying TERT to mature and old mice showed better neuromuscular coordination, reduced biomarkers of ageing, improved insulin sensitivity and increased lifespans without an increased incidence of cancer (Bernardes de Jesus *et al.*, 2012).

1.4.3 Telomerase and haematopoietic stem and progenitor cells regulation

Haematopoiesis relies on the self-renewal and differentiation of HSCs. Studies in both humans and mice show clear evidence that short telomeres cause quantitative and qualitative defects in HSCs that manifest in the form of stem cell exhaustion (Allsopp *et al.*, 2003; Hao *et al.*, 2005; Rossi *et al.*, 2007). Serial transplantation of extensive self-renewal induced by stimulating NA10hd-transduced TERT^{-/-} HSCs showed deficient reconstituting ability, reduced telomere length, and accumulating DNA damage (Sekulovic *et al.*, 2011). Similarly, the presence of short telomeres in G3 TERC^{-/-} long-term reconstituting haematopoietic stem cells (LT-HSCs) decreases their ability to repopulate irradiated mice. Regardless of the HSC pool expansion with age in these mice, this decline in the regenerative function is exacerbated in old G3 TERC^{-/-} HSCs (Rossi *et al.*, 2007). Furthermore, in this study, the majority of LT-HSCs from old wild-type mice stained for the DNA-damage response marker H2AX indicated that DNA damage accumulates with age. The cellular mechanisms of telomere-mediated stem cell failure are not completely known, but the deletion of cyclin-dependent kinase inhibitor 1a (Cdkn1a) that encodes p21 (transcriptional target of p53) rescues the HSC self-renewal defect in mice (Choudhury *et al.*, 2007). The aplastic anaemia phenotype can be reversed with an allogeneic stem cell transplantation in humans, which indicates that telomere-related stem cell disruption occurs cell autonomously. Other tissues are also involved with telomere dysfunction, such as the intestinal epithelium, which manifests as villous atrophy due to the loss of crypt stem cells (Rudolph *et al.*, 1999; Hao *et al.*, 2005; Choudhury *et al.*, 2007).

1.5 Aims

As oxidative and metabolic stress contributes to ageing and age-related diseases, my thesis is focused on investigating their roles on both endothelial and haematopoietic stem progenitor cells (EPCs and HSPCs). To assess the impact of oxidative stress, cells were incubated under hyperoxia (40% O₂) culture conditions and compared to normoxia (20% O₂) and hypoxia (3% O₂). On the other hand, metabolic stress was induced in culture by the addition of different glucose concentrations, as can be found in diabetic patients (10mM, 25mM, 30mM), as well as supraphysiological levels (50mM, 100mM, and 150mM). In the first results chapter, EPCs were isolated from healthy donors' peripheral blood and incubated under metabolic stress. In particular, I have studied the role of hyperglycemia on the metabolic regulator PGC-1 α . I also have examined the role of the anti-diabetic drug metformin on PGC-1 α and downstream targets in EPCs in vitro. In the second results chapter, HSPCs were isolated from umbilical cord blood samples. I examined the role of telomerase in HSPCs during CD34⁺ cell expansion under oxidative and metabolic stress. I have further tested the effects of oxidative stress on telomerase in myeloid cells after differentiation from CD34⁺ HSPCs. To evaluate the role of telomerase in depth, I studied HSPCs' ageing in bone marrow isolated from telomerase-deficient mice (TERT^{-/-} and TERC^{-/-}) in the last two results chapters. These two mouse models allowed me to analyse the role of telomerase without telomere shortening (TERT^{-/-}) and with telomere shortening (TERC^{-/-}) in vivo. Ageing in BM, the HSPCs of these models were assessed by evaluating the stem cell expression and clonogenic potential. Furthermore, in the last results chapter, I was interested in examining the effects of anti-ageing interventions, specifically dietary restriction and telomerase activation by the telomerase activator TA-65. A brief outline of aims for each results chapter are shown in Figure 1.6 and detailed aims are separately described in each chapter.

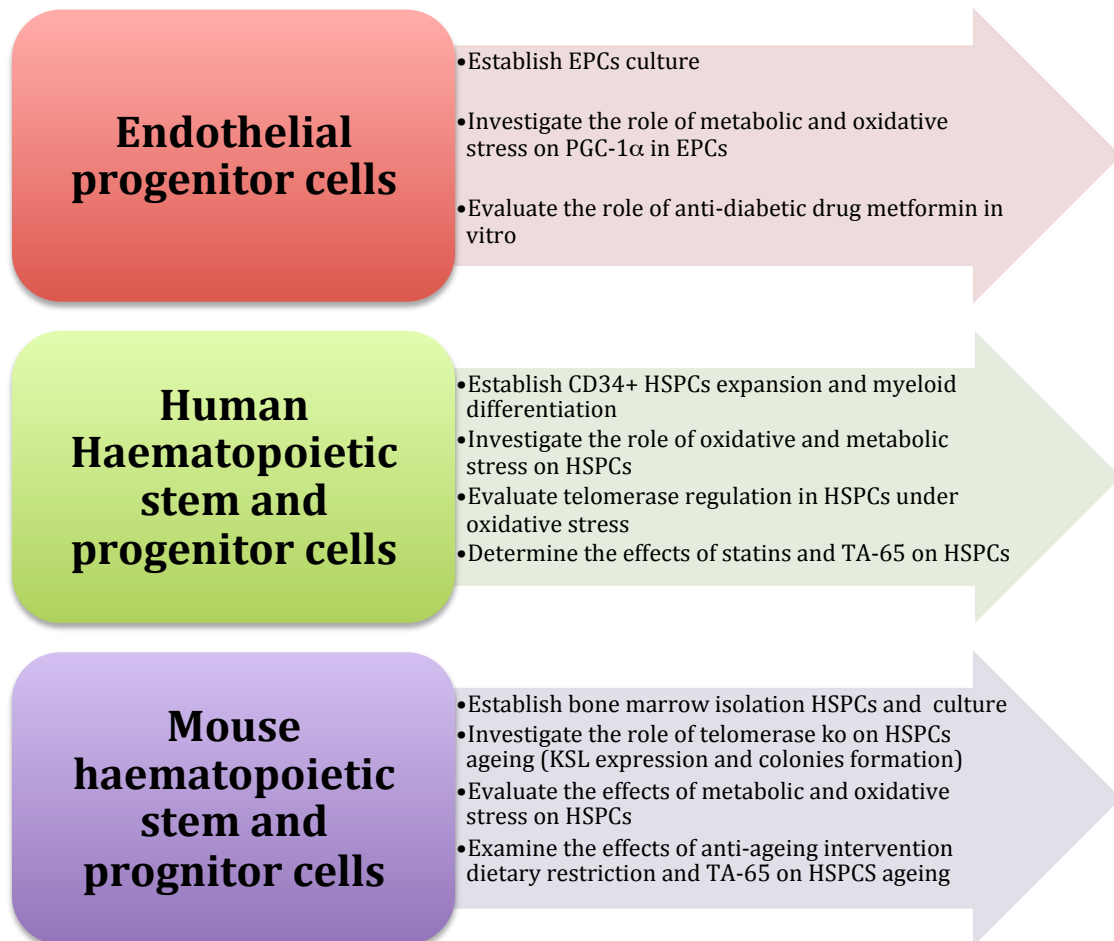


Figure 1.6: Aims of thesis.

Chapter 2 Materials and methods

2.1 Animals

TERT and TERC mouse lines were purchased from Jackson Laboratory. The mice lines included the TERT^{-/-} strain: B6.129S-Tert, tm1Yjc/J (Chiang *et al.*, 2004) and TERC^{-/-} strain: B6.Cg-Terc, tm1Rdp/J (Blasco *et al.*, 1997), both on a C57BL/6 background. Mice were inbred to obtain a wild-type, first-generation (G1) knockout and heterozygous mice for the TERT and TERC genotypes. The mice used for the TA-65 treatment had a genotype C57BL/Icrfa(t) (Rowlatt *et al.*, 1976). Animals were housed in the same room and provided with sawdust, paper bedding, and had ad libitum access to water. Mice were housed at 20 +/-2°C under a 12h light/12h dark photoperiod. Ethical approval was granted by the LERC Newcastle University, UK. The work, which was licensed by the UK Home Office (PPL 60/3864), complied with the guiding principles for the care and use of laboratory animals.

As shown in Figure 2.1, according to Chiang *et al.* (2004) TERT-deficient mice were constructed in two main steps. First the constructing of the targeting vector that was performed with a 6kb-mTERT genomic DNA fragment was derived from strain 129, which consisted of exon 1 and exon 2 of the mTERT gene. The DNA fragment was inserted into the pBluescript-KS vector at the EcoRI site (KS-tert). After cutting a BamHI-XbaI fragment from KS-tert, the fragment was subcloned into pBluescript-KS vector (KS-BX). A unique enzyme restriction site (XhoI) in KS-BX was created by PCR proximally to an ATG, which corresponds to the translation initiation code of the mTERT mRNA. Neomycin resistance and green fluorescent protein (GFP) genes were inserted in the XhoI site of the vector. This resulted in a BamHI-XbaI DNA fragment with neomycin and GFP-resistant genes, which was used to replace the BamHI-XbaI fragment of KS-tert. Outside the genomic DNA fragment, at a NotI site the thymidine kinase gene was inserted. The final construct was used as an mTERT gene-targeting construct. The second step was to target the gene in embryonic stem cells and mTERT knockout mice production. The mTERT-targeting construct was linearised by using SmaI and electroporated into embryonic stem cells. G418-resistant colonies were selected and isolated. Embryonic stem cell colonies with homologous recombination events were

identified with a BmHI-XbaI fragment from KS-tert as a probe in the Southern blot analysis. Mutant mice were generated with the selected embryonic stem cell clones. To confirm the disruption of the mTERT gene, southern blotting, RT-PCR, and telomerase activity were used.

The $TERC^{-/-}$ mice construction (Figure 2.1) was performed as described by Blasco *et al.* (1997). For the $TERC$ genomic deletion, two new unique *SacI* restriction sites were introduced by site-directed mutagenesis into a 4kb *XhoI* genomic fragment and a 3.3kb *XbaI* genomic fragment. These sites allowed the correct homologous recombination events to be recognised by the digestion of genomic DNA with *SacI*. *SacI*-digested DNA, by using a 1.2kb probe, generated a 6.3kb wild-type band or a 4.7kb band from the correct targeted allele. The site-directed mutagenesis was performed by using the Amersham Sculptor In-vitro Mutagenesis System and the sites were confirmed by sequencing. The mutagenised fragments were cloned into the targeting vector pPNT to generate pPNT-mTR Δ . The plasmid contained the neomycin resistance gene for the replacement of the mTR gene and the HSV TK gene for the counterselection in the presence of ganciclovir of randomly integrated constructs. The plasmid pPNT-mTR Δ was linearised with *NotI* to generate embryonic stem cells with targeted integration, and the DNA was electroporated into WW6 embryonic stem cells. Using G418 and ganciclovir, cells were selected and positive clones were picked after 9 to 10 days. Four clones (Tel-1, Tel-2, Tel-3, and Tel-4) correctly targeted the mTR locus out of the 449 neomycin-/ganciclovir-resistant clones that were initially screened. The four clones were injected into C57BL/6J blastocysts and implanted into pseudopregnant mice. The chimeric progeny were identified by their mosaic coat colour. Mice from the first microinjections (Tel-1) were mated to C57BL/6J to test for germline transmission of the mTR gene replacement, and heterozygous progeny mTR $^{+/-}$ were identified. Further generations of the telomerase-null mice were obtained by mating mTR $^{-/-}$ with each other.

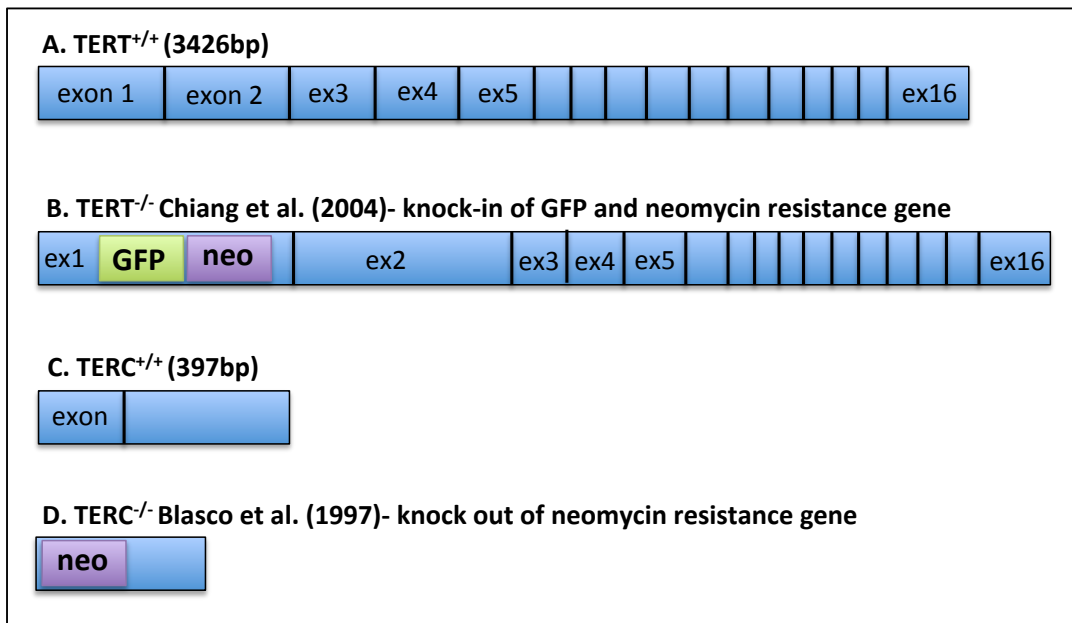


Figure 2.1: Expression of TERT and TERC knockout constructs

Comparison of wild-type TERT mRNA (**A**) and TERT knockout constructs in mice (**B**). Wild-type TERT^{+/+} contains 16 exons. The knockout described by Chiang et al. (2004) contains a GFP and a neomycin-resistant gene (neo) inserted into exon 1. The TERC noncoding gene consists of one exon, as shown in wild-type TERC. The exon was replaced with neomycin, as described by Blasco et al. (1997).

2.1.1 Bone marrow isolation

Tibiae and femura from both legs of mice were collected and carefully cleaned from adherent soft tissue with sterile tissue paper. The tips of each bone were cut open by using sterile scissors and forceps. The bone marrow was collected by inserting the needle into one end of the bone and flushing it out from the other open end. Each bone was flushed with 3ml PBS; after that, the cells were washed twice with 15ml PBS and counted with a haemocytometer.

2.1.2 Cell culture

Cell culture was performed as previously described (Samper *et al.*, 2002) by using MyeloCult culture media [H5100 05150, Stem cell Technologies, Canada]. Isolated bone marrow cells were plated in a 6-well plate at 16×10^6 per 2 ml MyeloCult for 3 days. Two concentrations of glucose [43835 Hameln Pharmaceuticals, Germany] (10mM or 20mM) were added daily to bone marrow cells compared to controls. TA-65, a gift from T.A.

Sciences, was dissolved in DMSO in three concentrations (1 μ M, 5 μ M, and 10 μ M) and added only once initially with media and compared to controls. Expansion was performed by using a cocktail of cytokines for a week (100ng/ml SCF, 100ng/ml TPO, and 100ng/ml FLT-3L).

2.1.3 Treatments

The diet that was used was standard rodent pelleted chow (CRM (P); Special Diets Services, Witham, UK) for the DR experiment and powdered food for the TA-65 treatment.

1. **DR:** During the dietary restriction experiments, the control group had ad libitum access to food (AL), whereas the dietary-restricted (DR) group was limited by 40% as described previously (Cameron *et al.*, 2011). Long-term DR started at 6 months of age and lasted for 16 months, (i.e., mice were 22 months old when used). Short-term DR started at 4 months of age and lasted for 7 months, so that the mice were 11 months old when used. Only males were used for the DR experiments.
2. **TA-65 treatment:** TA65® is a telomerase activator purified from Astragalus membranaceous and licensed to TA-Science Inc., USA. Twenty-four-month-old female wild-type mice with the genotype C57BL/ICrfa(t), as well as 9-month-old TERC^{+/-} and TERC^{-/-}, were treated with TA-65 for 3-4 months. TA65® was solubilised in DMSO and further diluted into water to form a suspension. This substance was mixed with powdered food (5 g/mouse per day) and administered orally at a daily dose of 25 mg/kg per mouse, as described earlier (Bernardes de Jesus *et al.*, 2011). The control mice received the same food with only DMSO in water (0.33%). All work complied with the guiding principles for the care and use of laboratory animals in the UK.

2.2 Human endothelial progenitor cells

2.2.1 Peripheral blood isolation

Mononuclear cells were isolated from peripheral blood by density gradient centrifugation. The methodology is based on using a high-density polymer solution in

the centrifugation of different cell components (shown in Figure 2.3). Briefly, a leucocyte cone from the Blood Donor Center, Newcastle, was diluted 1:2 with 1X Phosphate Buffered Saline (PBS) [GIBCO, Invitrogen 1218564, USA]. The sample was then layered over 15ml of Bicolll separating solution [density 1.077 g/ml, L6113 Biochrom AG, Germany]. Using a density gradient centrifugation at 800g for 20 minutes without a brake [Centrifuge 5810, Eppendorf, Germany], the white layer (buffy coat) of the mononuclear cells was collected along with the serum above it. Two washes were performed with PBS and a full brake centrifugation at 800g for 10 minutes. Cells were counted by using a haemocytometer under a light microscope in the four squares, as shown in Figure 2.2.

2.2.2 Cell culture

The cell culture was performed as described by Urbich and colleagues (Urbich *et al.*, 2003). After cell isolation from peripheral blood, cells (15×10^6 /well) were resuspended in EBM-2™ [CC3156 Lonza, Switzerland] media with SingleQuots®EGM-2™ (FBS 10 ml, Hydrocortisone 0.2 ml, hFGF-B 2ml, VEGF 0.5 ml, R3-IGF- 1,0.5 ml, Ascorbic acid 0.5 ml, hEGF 0.5 ml, GA-1000 0.5 ml, and Heparin 0.5 ml) and 10% fetal bovine serum (FBS) [12003C Sigma, USA]. After coating 6 wells of the polystyrene plates [734-1599 Iwaki, Japan] with 0.001% human fibronectin-coat [F0895 Sigma Aldrich, USA] for at least 30 minutes at room temperature, the cells were then seeded. The cells were treated daily with the following glucose [43835 Hameln Pharmaceuticals, Germany] concentrations: 10mM, 20mM, 30mM, 50mM, 100mM, 150mM, 200mM, and 250mM. Conditions with metformin [D150959 Sigma, USA] dissolved in water to concentrations (0.1mM, 0.5mM, 1mM, and 10mM) were added when the cells were first plated. Cells were incubated in humidified 37°C, 95% air conditioned, 5% CO₂, and 20% oxygen were placed in a CO₂ ThermoForma steri-cycle incubator for 7 days or 3 days depending on the experiment. For oxidative stress testing, cells were incubated in the same incubator conditions (humidified 37°C, 95% air conditioned, 5% CO₂), except for the oxygen levels in which 3% oxygen and 40% oxygen levels were used. Cells were harvested from wells using a foam tool [9010-320 Iwaki, Japan] after washing with PBS and then centrifuged

at 800g for 5 minutes. For the rest of the cells, the media were changed every 2 days and cells were harvested at days 3, 5, and 7.

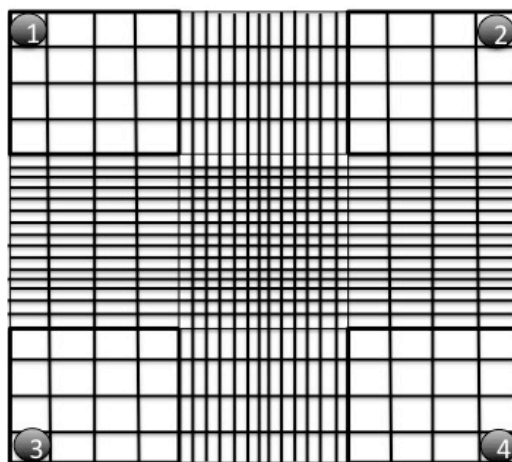


Figure 2.2: The haemocytometer.

Haemocytometer used for manual cell counting under light microscope for cells isolated and grown in cell culture. The squares labeled with numbers 1, 2, 3, and 4 were counted. Any outlying cells were not counted.

2.3 Human haematopoietic stem and progenitor cells

2.3.1 Human umbilical cord blood isolation

Umbilical cord blood samples were collected only from Caesarean section births, due to logistical considerations and homogeneity of treatment reasons. Patients consented and ethical approval was obtained from the Local Research Ethics Committee prior to any umbilical cord blood collection. Umbilical cord blood samples were collected in Baxter collection bags provided with CPD-A (citrate phosphate dextrose adenine) anticoagulant [Baxter, USA]. All samples were processed within 24 hours of collection. Whole umbilical cord blood was diluted 1:1 with 1X PBS and layered very slowly on 15ml of Bicolll separating solution. Layered samples were centrifuged for 20 minutes without deceleration at 800g, as shown in Figure 2.3. The mononuclear cells layer was carefully aspirated in a new tube and filled with PBS and centrifuged at 800g for 10 minutes; this step was performed twice. Another washing step was performed at 350g for 15 minutes. Cells were then were resuspended in 50ml MACS buffer, which is

1X PBS, 2% FBS, and 2mM EDTA [AM9260 Life technologies, USA], and counted in a haemocytometer.

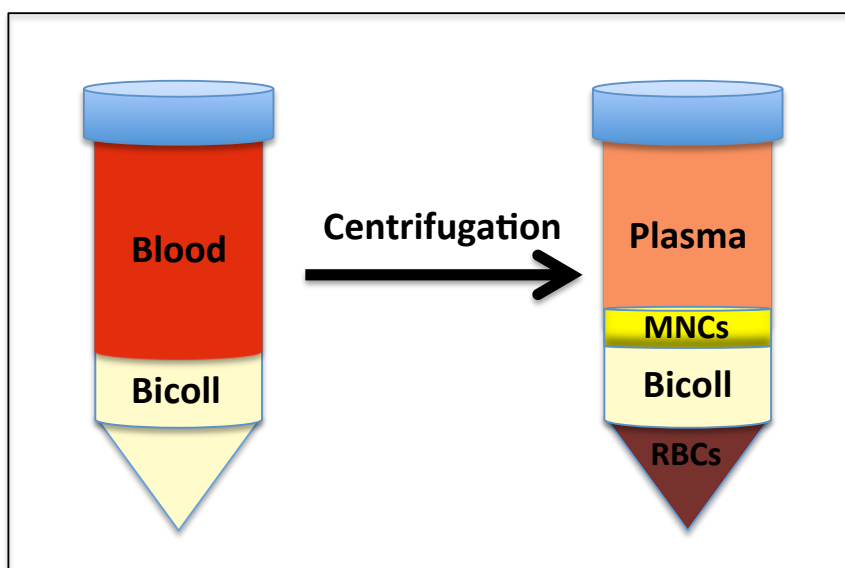


Figure 2.3: Density gradient centrifugation.

Blood is diluted with PBS and layered carefully over Bicoll. After centrifugation with Bicoll, the top layer is plasma. Then, cells separate into the yellow MNCs (mononuclear cells) layer or the buffy Bicoll layer, followed by granulocytes and RBCs (red blood cells or erythrocytes). Mononuclear cells are carefully harvested for further experiments.

2.3.2 Immunomagnetic sorting of human CD34+ cells

MACS® technology is now the gold standard in magnetic cell separation according to the cell surface antigens. As shown in Figure 2.4, cells of interest are labeled specifically with super-paramagnetic MACS microbeads. Cells are then are passed through separation columns that are placed in a strong magnet. Labeled cells with microbeads are retained in the column while unlabeled cells pass through. After removing the column from the magnet, the retained cells are eluted to give the positive fraction of cells.

MACS super-paramagnetic microbeads are only 50nm in diameter, which is comparable to the size of a virus and about one million times smaller in volume than eukaryotic cells.

Microbeads are composed of iron oxide and polysaccharide, which make them, together with their small size, biodegradable. Microbeads do not influence function and viability, nor do they activate cells. When cells are cultured, microbeads are decomposed, thus allowing cells to retain their physiological functions. Therefore, there is no need for bead detachment and positively selected cells can be used directly after separation for experiments (Biotec, 1999).

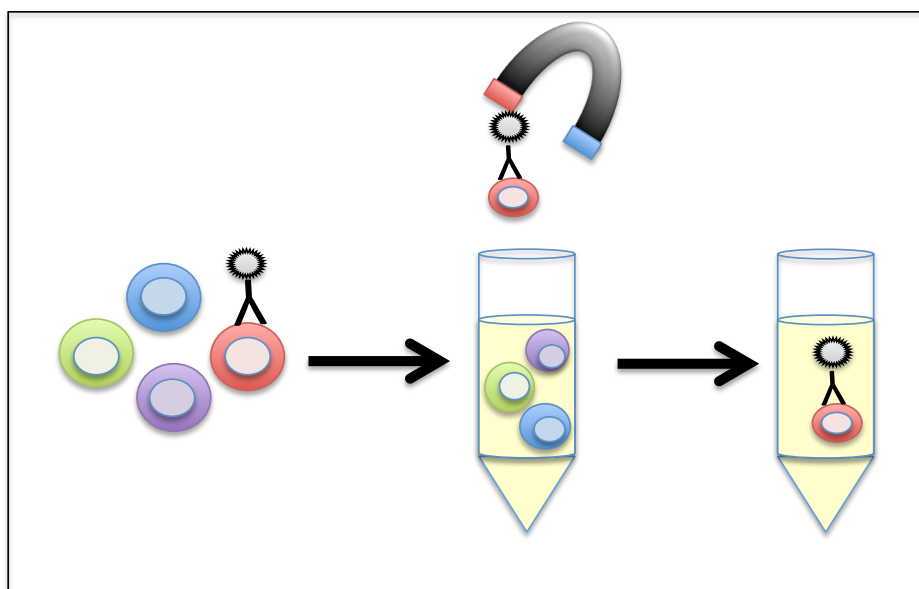


Figure 2.4: Principle of immunomagnetic beads labeling.

Microbeads bind specifically to a cell's antigen (such as CD34) and are retained in a magnetic field. Unlabeled cells pass through with buffer washes then labeled cells are removed from magnetic field and flushed out. Pure positively selected cells then are ready for use in experiments.

The isolation of CD34⁺ cells was performed by using CD34⁺ microbead kit for humans, as described by the manufacturer [Miltenyi biotec, 130046702, Germany]. After the isolation of umbilical cord blood, mononuclear cells in the previous methods section (i.e., 2.2.1) cells were resuspended in a final volume of 300 μ l per 10⁸ total cell. 100 μ l of FcR blocking reagent per 10⁸ cells was incubated at room temperature for 5 minutes. CD34⁺ labeling was performed by adding 100 μ l per 10⁸ cells and incubated at 4°C for 30 minutes. Cells were then were washed with MACs buffer at 350g for 15 minutes.

Columns were primed with MACS buffer and cells were applied to the columns in a magnetic field, letting the CD34⁻ unlabeled cells pass through and trapping the CD34⁺ labeled cells. Washing was performed 3 times with 3ml-MACS buffer to ensure that nonspecific binding was washed away. The column was removed from the magnetic field and flushed with 5ml-MACS buffer. Another column was used and a CD34⁻ fraction passed again in the column to ensure that no CD34⁺ cells were missed. The second column was flushed with 2.5ml and CD34⁺ cells were collected. CD34⁺ cells were used for flow cytometry and cell culture.

Similar to the isolation of CD34⁺ microbeads, CD14 and CD15 microbeads were also used to separate monocytes and granulocytes, respectively, from mononuclear cells isolated from peripheral blood [CD14: 130050201, CD15: 130046601, Miltenyi Biotec, Germany].

2.3.3 Cell culture

CD34⁺ cells then were counted by using haemocytometer and expanded for a week in 20% FBS 200mM IMDM media [21980032 Invitrogen, USA] supplemented with 100ng/ml stem cell factor (SCF) [3007 Peprotech, USA], 100ng/ml thrombopoietin (TPO) [30018 Peprotech, USA], 100ng/ml Fms-related tyrosine kinase-3 ligand (FLT-3L) [30019 Peprotech, USA], and 50µg/ml gentamycin [15710049 Invitrogen, USA]. Furthermore, cells were differentiated into granulocytes by using a supplementary mixture: 20ng/ml SCF, 50ng/ml FLT-3L, and 100ng/ml G-CSF [30023 Peprotech, USA], as described by Schuller et. al. (2007). Table 3 shows the different cytokines mixtures that were used. The schematic set up of expansion and differentiation of CD34⁺ cells that were isolated from umbilical cord blood are shown in Figure 2.5. Oxidative stress was tested by using different oxygen levels in an incubator (3%, 20%, and 40%). Different concentrations (10mM, 20mM, and 30mM) of glucose were added daily to CD34⁺ HSPCs for a week. TA-65 [T.A. Sciences, USA] was dissolved in DMSO to working solution of 1µM, 5µM and 10µM. Those TA-65 concentrations were added initially to CD34⁺ HSPCs and once during media change and compared to controls. Atorvastatin was kindly donated by Pfizer and prepared in DMSO [472301 Sigma, USA] to a working dilution of 0.3µM dissolved in DMSO, as found by our group to be the

optimal for cell growth. Atorvastatin (0.3 μ M) was added during myeloid differentiation of HSPCs for one week, initially with media and then once with media change.

Stem cells expansion cytokines	100ng/ml SCF
	100ng/ml TPO
	100ng/ml FLT-3L
Granulocytes differentiation cytokines	20ng/ml SCF
	50ng/ml FLT-3L
	100ng/ml G-CSF

Table 3: Cytokines mixtures for expansion and differentiation of CD34+ HSPCs isolated from cord blood.

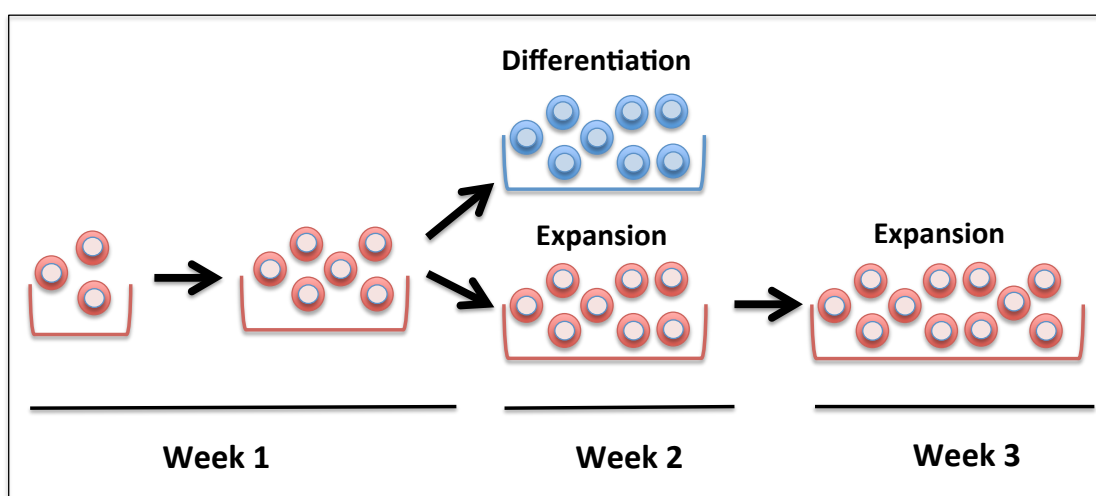


Figure 2.5: CD34+ cells from umbilical cord blood expansion and differentiation to granulocytes set up in culture.

Cells were expanded for 1 week under cytokines, then either further expanded for another two weeks or differentiated for one week. A different cytokines mixture was used for the differentiation. Media were changed twice a week.

2.4 Samples cryopreservation

Human PBMNCs, cord blood cells, and mouse bone marrow cells were cryopreserved in -80°C before or at different days of the cultures. Cryopreserved cells were usually used before performing different experiments. The medium that was used included RPMI 1640 [R0883 Sigma, USA] with 10% FBS and 10% DMSO. After centrifugation 800g for 10 minutes, the supernatant media were discarded. The freezing media were added as 100 μl per 1 million cell and mixed well, stored in a cryovial, and placed under -80°C in Mr frosty container [5100-0001 Thermo Scientific, USA] until experiments were conducted.

2.5 RT-qPCR

A real-time quantitative polymerase chain reaction (qPCR) is used to accurately measure the active or inactive state of any gene of interest within the genome. It is a further development of PCR technology which is widely applied in the field of molecular diagnostics. The essential components required for RT-qPCR include a DNA or RNA template, DNA polymerase, primers, nucleotides, and the conversion of RNA into cDNA with reverse transcriptase. The real-time quantitative PCR reaction copies the specific sequence into billions of amplicons with a few steps. These steps include denaturation to separate strands, annealing to allow primer binding, extension to make new DNA and, lastly, exponential amplification. In RT-qPCR, SYBR green dye was used for the detection of double-stranded DNA with high affinity. However, SYBR green also binds to single-stranded DNA, albeit with a lower affinity. When SYBR green is excited at 488nm wavelength (blue light), it emits at 522nm (green light) (Biosystems, 2011).

2.5.1 Total Ribo-nucleic Acid (RNA) extraction

RNA total extraction was performed using guanidinium thiocyanate phenol chloroform extraction, which was originally developed by Chomczynski and Sacchi (1987). Nowadays, this chemical is TRIzol, which is a monophasic solution containing phenol, guanidine isothiocyanate, and other components that facilitate the RNA isolation of a variety of species. TRIzol maintains RNA integrity by inhibiting RNase activity and dissolving and disrupting cells.

The RNA was isolated from the cell culture by TRIzol [15596026 Life Technologies, USA], according to the manufacturer's protocol. After taking off the media off of the well, the plate was washed once with PBS. One ml of TRIzol was added and incubated for 5 minutes at room temperature. After incubating with 250µl of chloroform [9722 life technologies, USA] for 5 minutes, the samples were centrifuged at 10,000 rpm for 5 minutes. Three layers formed; the top clear, aqueous layer contained RNA, the middle contained white precipitated DNA, and the organic phase was found in the bottom pink layer. The top RNA layer was taken carefully, mixed with 550 µl isopropanol [190764 Sigma, USA], and incubated for 5 minutes. The pellets should have been barely visible after 30 minutes of centrifugation at maximal speed. Before adding 1 ml of 75% ethanol [7023 Sigma, USA] in DEPC-[57585 Sigma, USA] treated water, the isopropanol was poured off. Again, samples were centrifuged at 9500rpm for 5 minutes and the supernatant was discarded. The pellets were left to air dry and then 20 µl of DEPC-treated water was added. The total RNA concentration was measured by using the Nanodrop spectrophotometer ND-1000.

The sensors of the Nanodrop were cleaned with 2µl of distilled water in each channel and then wiped off and blanked with another fresh 2µl of distilled water. Then sensors were cleaned again before inserting 2µl of the extracted RNA sample. The RNA results were recorded and expressed as ng per µl and the upper limit 3000µg/µl, which should not be reached to avoid inaccurate measurements. The RNA quality was measured by the ratio of absorbance at a wavelength of 260 (260; RNA) and at a wavelength of 280 (280; protein). Generally, a very pure RNA sample would result in a 260/280 ratio of 2.0 or closer.

2.5.2 Reverse transcription

The first strand of cDNA was synthesised using 25ng/ µl total RNA, 0.5 µg random primers [C1101 Promega, USA] and 0.5mM deoxy-nucleotide triphosphate (dNTP) mix [10297018 Invitrogen, USA]. The mixture was incubated for 5 minutes at 65°C and then placed on ice for 1 minute. After that, 5mM dithiothreitol (DTT), 5X First Strand Buffer, 40 units RNaseOut, and 200 units Superscript III Reverse Transcriptase [18080044 Invitrogen, USA] were added to the mix and mixed well, followed by an incubation in

Hybaid PxE Thermal Cycler [Thermo Scientific, USA] with the following program: 55°C for 60 minutes and 70°C for 15 minutes. After these incubations, 2 units of RNase H [EN0201 Fermentas, USA] were added to the mix and returned to the thermal cycler for the following incubation at 37°C for 20 minutes and 65°C for 10 minutes.

2.5.3 Real-time quantitative PCR

The StepOnePlus Real-Time PCR system [Applied Biosystems, USA] was used for the quantification of the gene transcripts. Triple samples were used in PCR assays in 96-well plates [4346907 Applied biosystems, USA]. Each reaction mixture (20 µl) contained cDNA (27.5ng RNA), 0.13µM of gene specific primers, and 1X Fast SYBR Green Master Mix [4385612 Applied Biosystems, USA]. The comparative Ct method was used to quantify the target gene expression. Forty cycles were performed for each sample. After preheating the PCR plate for 10 minutes at 95°C, each cycle involved 95°C for 15 seconds, annealing for 60 seconds at optimum primer temperature, and heating at 72°C for the specific elongation time. Data were collected during the extension step and analysis was carried out using StepOne Software v2.0 [Applied Biosystems, USA]. The specificities of primers used in experiments are listed in Table 4 on the next page.

Primer	Sequence 5'-3'		Annealing temperature (°C)
	Forward	Reverse	
TBP	GCCACGCCAGCTTCGGAGAG	TCAGTGCCGTGGTTCGTGGC	60
PGC-1α	TGTTTTTGACGACGAAGCAG	AATGAATAGGATTGCGTGCC	60
AMPKα1	GACAGCCGAGAAGCAGAAAC	AGGATGCCTGAAAAGCTTGA	60
GLUT4	AGAGCGTGTCG TTCACGCGG	CCCACAACGGGTCTGGTGGC	60
G6Pase	GGTGGTGCGAGCAGCCAGAA	TGCAGGAGGACGAGGGAGGC	60
hTERT	TTCTGCACTGGCTGATGAG	CCGGTAAGAAAAAGAGCCTGTTC	60
hTERC	GCCTTCCACCGTTCATTCTA	CCTGAAAGGCCTGAACCTC	60
mGAPDH	AACTTTGGCATTGTGGAAGG	ACACATTGGGGGTAGGAACA	60
mβ-actin	TGCCGCATCCTCTTCCTC	CCACAGGATTCCATACCCAAG	60
mTERT	GGATTGCCACTGGCTCCG	TGCCTGACCTCCTCTTGAC	60
mTERC	TCATTAGCTGTGGGTTCTGGT	TGGAGCTCCTGCGCTGACGTT	60

Table 4: Different specificities of studied primers.

2.6 Quantitative-telomeric repeat amplification protocol (Q-TRAP)

Quantitative-telomeric repeat amplification protocol (Q-TRAP) is an assay used for telomerase activity detection using real-time PCR. This technique is developed from the original TRAP technique and involves two steps process of telomerase product formation and amplification. In the first, if telomerase is present within sample lysate it will bind to substrate and dNTPs (deoxynucleotide triphosphate) within the reaction solution to make telomeric repeats. In the second step, specific forward and reverse primers are used to amplify these products in real-time PCR by using SYBR green. The advantages of this method are increased throughput, reduced carry-over contamination, and single-step detection and quantification. Conversely, the drawback of this technique is the non-specificity of SYBR green, which can interfere with correct data interpretation.

However, this issue can be resolved by primer concentration and annealing temperature optimisation (Herbert *et al.*, 2006).

2.6.1 Q-TRAP

Telomerase activity was tested by using the quantitative telomeric repeat amplification protocol (Q-TRAP), as reported previously. A telomerase substrate (TS) (5'-AATCCGTCGAGCAGAGTT-3') and a reverse primer ACX (5'-GCGCGGCTTACCCTTACCCTTACCCTAACC -3') were used in the PCR mastermix. Furthermore, the mismatches in the ACX primer reduce primer-dimer artifacts. The mastermix contained 1xSYBR Green [Applied Biosystems Mastermix 4309155, USA], double-distilled H₂O, and 1mM EGTA with 0.1µg of each primer. Cell extracts (50,000 cells) were added to the mastermix and distributed in triplicates (25µl each) in MicroAmp Fast-tubes [4358293 Applied Biosystems, USA]. SYBR green bound to the double-stranded DNA as amplicons were produced and PCR program first started with 25°C for 30 minutes to allow the telomerase in the protein extracts to elongate the TS primer by adding TTAGGG repeats. This was followed by 95°C for 10 minutes, then cycled x 40 with 95°C for 15 seconds and 60°C for 1 minute. A meltcurve was produced at the end with 95°C for 15 seconds, 60°C for 1 minute, and 95°C for 15 seconds. Data was collected and analysed by StepOne software [Applied Biosystems, USA]. Telomerase activity was calculated in samples based on threshold cycle values (C_T).

2.7 Fluorescence activated cell sorting (FACS)

Flow cytometry is a quantitative single cell analysis technique that has been developed since the 1970s. It has become fundamental in clinical and research applications. The wide application of flow cytometry is not only due to phenotypic cellular characteristics, but also attributed to the intracellular measurements of DNA and RNA, and functional characteristic such as cell death. FACS analysis in the flow cytometer stands for fluorescence-activated cell sorting and was invented by BD. It is a technology used to analyse single particles based on size and internal complexity or granularity. The flow cytometer is divided into three main systems: fluidics, optics, and electronics, as shown in Figure 2.6. The fluidics system transports samples one at a time by hydrodynamic focusing of the sheath fluid to laser beams. The laminar flow separates

the sheath fluid from the sample and ensures that they do not come into contact and, once cells are analysed, they flow to waste along with the sheath fluid. The optics system consists of lasers and lenses that illuminate samples to specific optical filters and then recorded to appropriate detectors. The electronics system is the converter of light signals into electronic signals that can be processed by the computer. When light strikes a cell, it is either scattered or absorbed. Fluorescence can be re-emitted from the cell if the absorbed light is at the appropriate wavelength. Fluorescence occurs when the cell has a naturally fluorescent particle or is conjugated with fluorochrome antibodies that bind cellular or intracellular antigens. Forward-scattered light (FSC) and side-scattered light (SSC) are the main parameters collected when light strikes cells, depending on the cells' size and internal complexity, respectively. The FSC is collected at an angle of approximately $<10^\circ$, while the SSC is collected at 90° , as shown in Figure 2.7.

In addition to considerable information gathered in regard to the physical properties of cells from the side and forward scatter, the flow cytometers are frequently used to measure fluorescent probes or fluorochromes. There are numerous antibodies, mostly monoclonal, that are used for staining available in the market directed to specific antigens with different types of fluorochromes. Dyes of choice, such as R-phycoerythrin (PE), fluorescein (FITC) or peridinin chlorophyll protein (PerCP), might target intracellular content such as DNA (Hoechst 33342 or propidium iodide (PI)). When using two or more fluorochromes in one sample, a spectral overlap can interfere with results; therefore, compensation is essential to eliminate it. Fluorochromes absorb light, resulting in an unstable state of excited electrons. The energy absorbed generates vibration and heat, which leads to the emission of a longer wavelength of photons (i.e., termed the emission wavelength) for the molecule. The photomultiplier tube (PMT) converts photons into a proportional voltage by using a photocathode, which results in the generation of pulse. This electrical pulse or signal is then converted by analog to a digital converter to a numerical signal. Recorded fluorescence quantity and intensity are displayed in the computer system by single (such as histograms) or multiple parameters plots (such as a dot plot) (Scheffold and Kern, 2000; Stewart, 2000; Bakke, 2001; Givan, 2001).

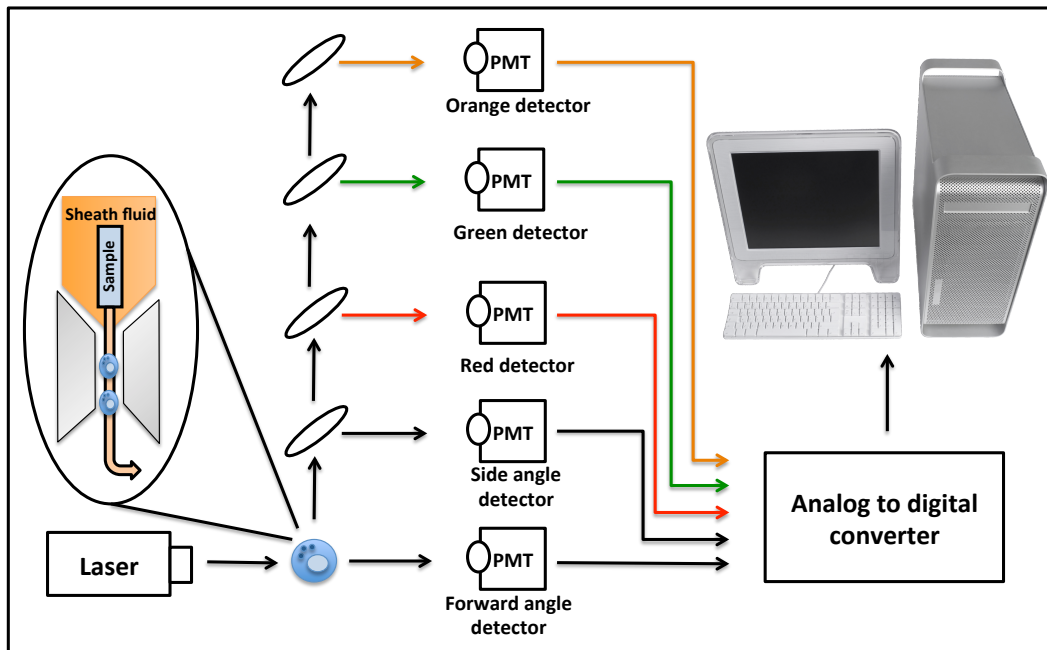


Figure 2.6: Principle of flow cytometry.

Cells in sheath fluid are analysed by laser beams according to their physical properties and fluorescence intensity via different specific lenses and detectors. A photomultiplier tube (PMT) transmits signals to an analog-to-digital-converter which translates information into the computer.

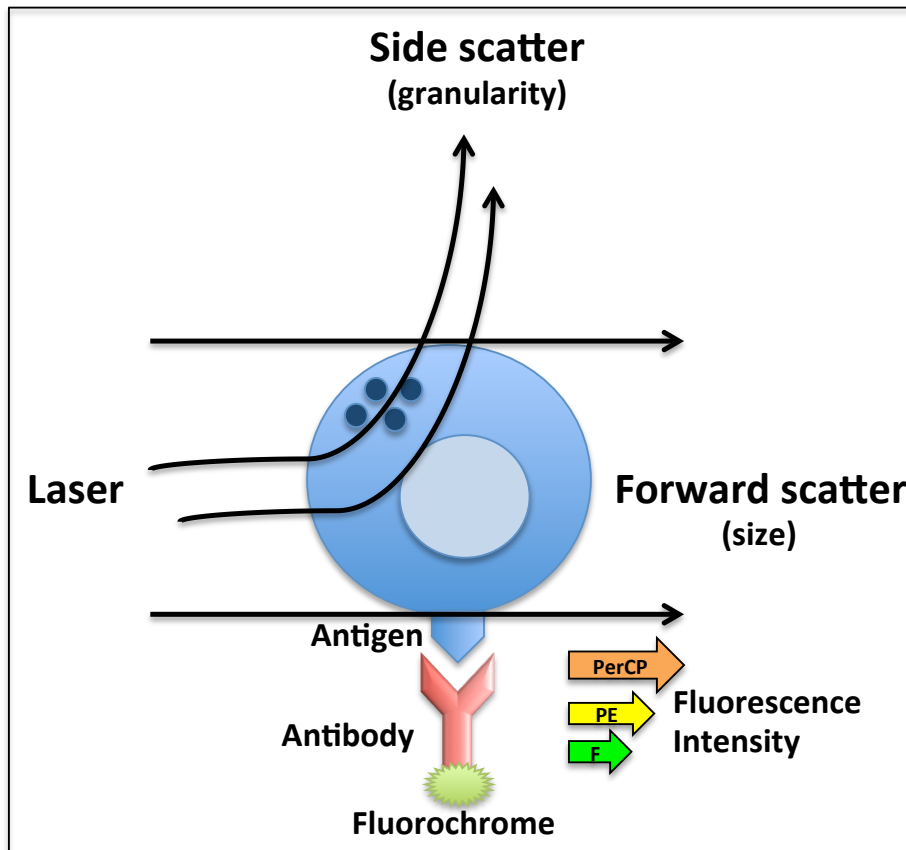


Figure 2.7: Flow cytometry analysis of a single cell.

When laser beams hit the cell, relative directions of forward-scatter light and side-scatter light and fluorescence of different fluorochromes become evident. PE: R-Phycoerythrin and F: Fluorescein (FITC). Reproduced from (Dimmick, 2009).

CD34⁺ purity check was performed by the addition of 5 μ l of CD34 antibody to 1 x 10⁶ cells after the centrifugation of freshly isolated cells (shown in Figure 2.8). Furthermore, mononuclear cells isolated from cord blood samples were characterised for the presence of a CD14 monocytes marker and a CD15 granulocyte marker (Figure 2.9). Antibodies were incubated in the dark 4°C for 30 minutes (antibodies listed in Table 5). The cells then were washed using BD FACS lyse/wash assistant and analysed in FACSCanto II 424 [BD, USA].

Target and conjugate	Manufacturer	Catalogue number
CD45 anti-human PerCP-eFluor 710	eBioscience	46045942
CD45 anti-human FITC	BD Pharmingen	555482
CD34 anti-human PE	BD Pharmingen	555822
CD15 anti-human FITC	Miltenyi Biotec	13008101
CD14 anti-human Pacific blue	Invitrogen	MHCD1428

Table 5: Antibodies used in human umbilical cord blood immunophenotyping.

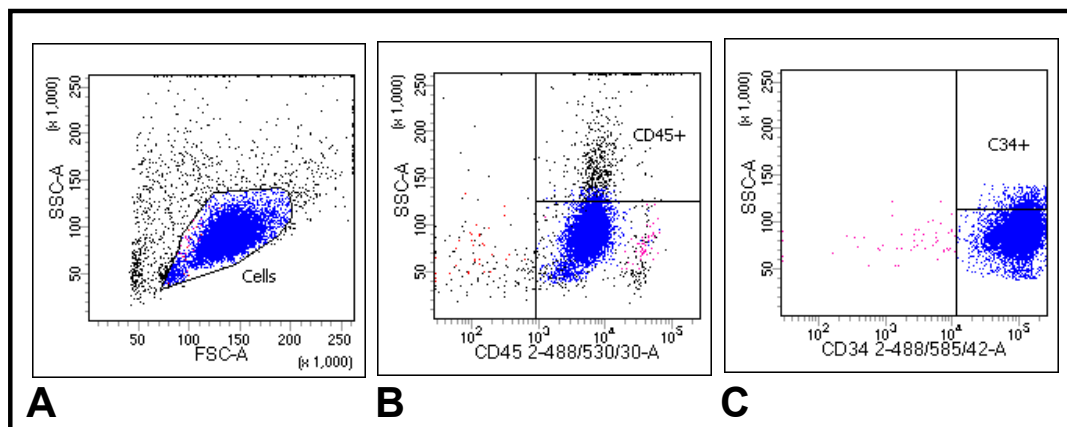


Figure 2.8: Purity check of CD34+ cells isolated from human umbilical cord blood samples.

After CD34+ cells were separated by using immunomagnetic beads, CD34+ cells were analysed using FACS for CD34 antigen expression (purity check). **A:** Side scatter (SSC) vs. forward scatter (FSC). **B:** SSC vs. CD45 expression to exclude non-cellular content. **C:** SSC vs. CD34 to check CD34+ cells percentage. Over 90% purity was obtained with the positive selection. CD34- fraction was measured as a negative control and did not contain CD34+ cells.

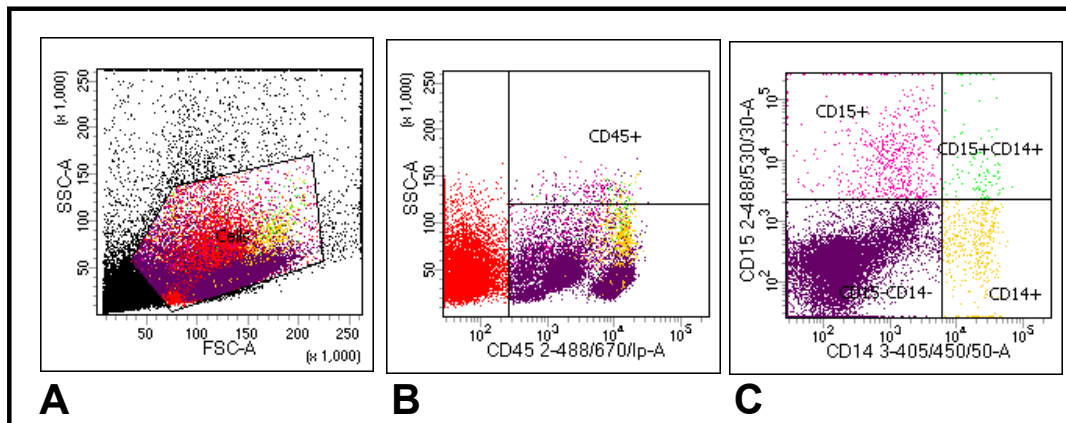


Figure 2.9: Immunophenotyping of CD15+ and CD14+ cells isolated from human umbilical cord blood samples.

CD34+ cells isolated from human umbilical cord blood were characterised during 3 weeks of expansion and 1 week of granulocytes differentiation. **A:** Side scatter (SSC) vs. forward scatter (FSC). **B:** SSC vs. CD45 expression to exclude non-cellular content. **C:** CD15 vs. CD14 to check percentages of CD15+ granulocytes and CD14+ monocytes. Unstained cells were used as a negative control.

To identify haematopoietic stem and progenitor cells in mouse bone marrow, a lineage kit was used to exclude specific lineage cells including CD3e/CD3 ϵ chain (T cells marker), CD11b/Mac-1 α chain (leukocytes marker), CD45R/B220 (B cells marker), Ly-6G and Ly-6C/Gr-1 (granulocytes and neutrophils), TER-119/Ly-76 (erythroid cells). Gating strategy is shown in Figure 2.10 and antibodies specifications are listed in Table 6.

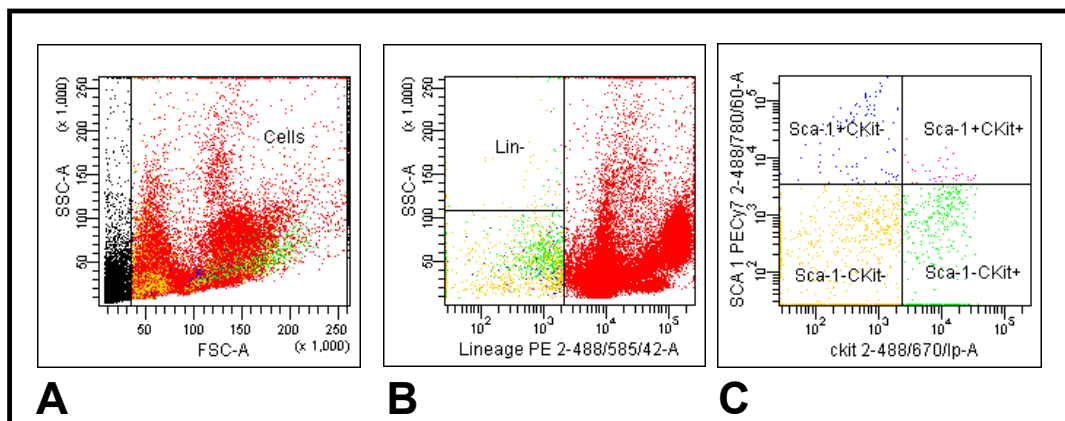


Figure 2.10: Haematopoietic stem and progenitor cells (HSPCs) gating using lineage kit.

Side scatter (SSC) vs. forward scatter (FSC) were first evaluated to exclude debris (A). The following gate was SSC vs. lineage negative to select the (Lin-) cells (B). According to lineage negative cells in B, LSK (lineage-Sca-1+CKit+) stem cells were defined as Sca-1+CKit+ cells (C). Unstained cells were used as a negative control.

Target and conjugate	Manufacturer	Catalogue number
Lineage kit:	BD Pharmingen	559971
CD3e anti-mouse Biotin		
CD11b anti-mouse Biotin		
CD45R anti-mouse Biotin		
Gr-1 anti-mouse Biotin		
TER-119 anti-mouse Biotin		
Streptavidin PE	BD Pharmingen	554061
CKit anti-mouse PerCP-eFluor 710	eBioscience	46117180
Sca-1 anti-mouse PE-Cy7	BD Pharmingen	558162
Mouse BD Fc Block	BD Pharmingen	553142

Table 6: Antibodies used in mouse bone marrow stem cells immunophenotyping

2.7.1 Cell sorting

Sorting using flow cytometry is the process of physical separation of cells or particles of interest from a heterogenous population. Electrostatic sorting is the most common technology used in analytical flow cytometers, as in FACSAria II [BD, USA]. It is based on the same principle used in ink-jet printers involving the electrostatic deflection of charged droplets. Generally, hydrodynamic focusing of ejected samples in sheath fluid passes through a light source from one or more lasers. Scattered light and fluorescence signals are then produced, detected, and measured. Cells are subsequently removed by vacuum to a waste reservoir (Davies, 2012). Cells isolated from mTert-GFP mice bone marrow were sorted into GFP high, intermediate, and low.

2.8 Flow-fluorescent in situ hybridization (Flow-FISH)

The Flow-FISH method is a further development of quantitative fluorescence in situ hybridization (Q-FISH). It combines FISH with flow cytometry, which allows simultaneous measurement of fluorescence and light properties of cells. Unlike Q-FISH, Flow-FISH can also be performed on non-metaphase therefore non-dividing cells. However, Flow-FISH protocol requires accurate measurement of weak fluorescence signals with proper sample preparation, instrument calibration, and internal controls to ensure reproducibility (Rufer *et al.*, 1998; Baerlocher and Lansdorp, 2004).

Telomere length measurement was performed as described previously (Spyridopoulos *et al.*, 2004; Spyridopoulos *et al.*, 2008). Briefly, each sample contained 100,000 bovine thymocytes as an internal standard and 200,000 haematopoietic stem and progenitor cells from umbilical cord blood or mouse bone marrow resuspended in a hybridization mixture with a telomere-specific N-terminal FITC-conjugated $(C_3TA_2)_3$ PNA probe. After washing and counterstaining with propidium iodide, cells were analysed by flow cytometry on a FACSCanto with FACSDiva version 6.1. software [BD, USA]. Relative telomere length was determined by comparing mean fluorescence intensity of the sample with that of the internal control. The internal control, bovine thymocytes, was used to express the telomere length in kilobases. Mean telomere length of samples was calculated as molecular equivalents of soluble fluorchrom (MESF) in samples divided by MESF in thymocytes multiplied by mean telomere length of thymocytes determined

directly by Southern Blotting prior to the study (13.37kb). An example of murine bone marrow cells Flow-FISH plots is shown in Figure 2.11.

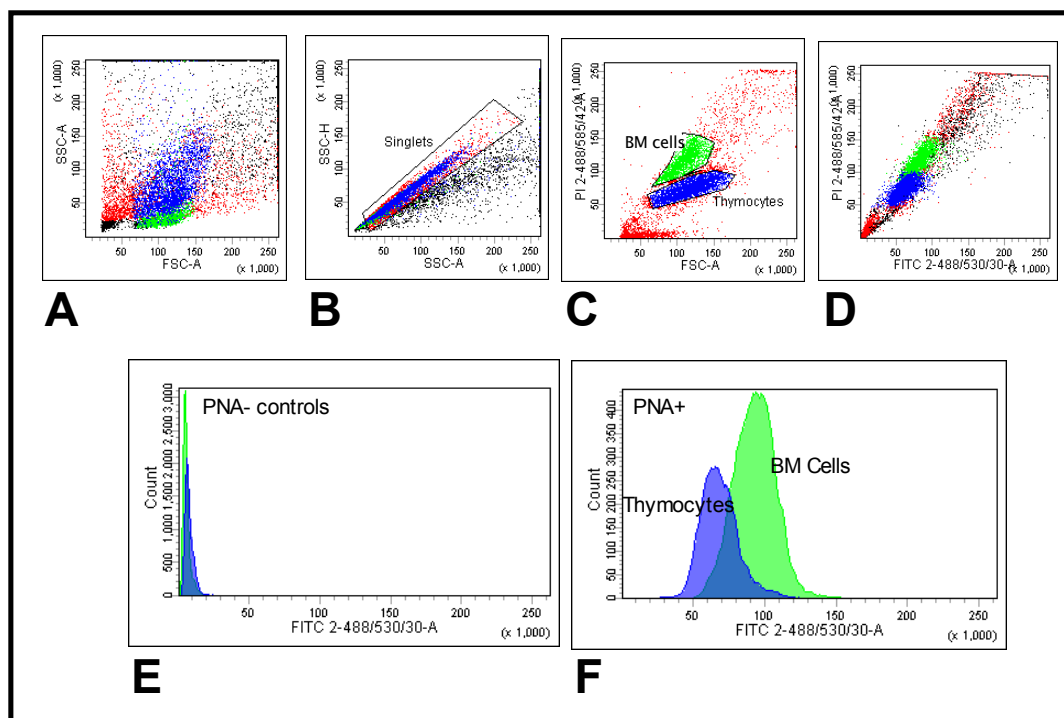


Figure 2.11: An example of Flow-FISH analysis of mouse bone marrow cells.

Flow-FISH is based on DNA denaturation and hybridization with a PNA (peptide nucleic acid) probe that is labelled with a fluorescein fluorophore (FITC). Excess probe is washed away, and a reading is made through FACS analysis.

A: Side scatter (SSC) vs. forward scatter (FSC). **B:** Singlet cells were identified according to height and side scatter (SSC). **C:** According to PI staining, negative unstained controls, and single staining tubes, signals from bovine thymocytes and bone marrow (BM) cells were gated separately. **D:** The PNA signal corresponding to molecular equivalents of soluble fluorochrome (MESF) was detected, and different populations were measured in non-stained PNA histograms (**E**) and stained cells PNA+ (**F**).

2.9 Clonogenic Assay

Haematopoietic colony-forming cell (CFC) assay has been used extensively since 1961 in clinical and research applications. This functional assay assesses the haematopoietic stem and progenitor cells' ability to divide and differentiate in response to cytokines stimulation in a semi-solid medium. The results of this assay provide an excellent measurement of the original cell population since each haematopoietic colony is derived

from a single progenitor cell that gives rise to haematopoietic cells. The applications of CFC assays included testing cell manipulations such as CD34+ enrichment, T-cell depletion, cryopreservation, and gene therapy (Gordon, 1993).

A semi-solid methylcellulose-based medium, methocult [GF M3434, Stemcell Technologies, Canada], was thawed slowly from -20°C inside a fridge at 4°C overnight, and 8×10^5 of haematopoietic stem and progenitor cells isolated from mouse bone marrow were mixed with 4 ml of Methocult. The mixture of cells and methocult (1.1ml) was divided with 16-g blunt-end needle into triplicate 35mm cell culture dishes accompanied by an additional well with a sterile water well in 100mm petri to keep the appropriate humidity (shown in Figure 2.12). Cultures were then placed in a 37°C incubator maintained with 5% CO₂ and >95% humidity. Samples were counted blindly, and a gridded scoring dish was used to enumerate colony types and numbers according to criteria described by the manufacturer [Stemcell Technologies, Canada], shown in Figure 2.13.

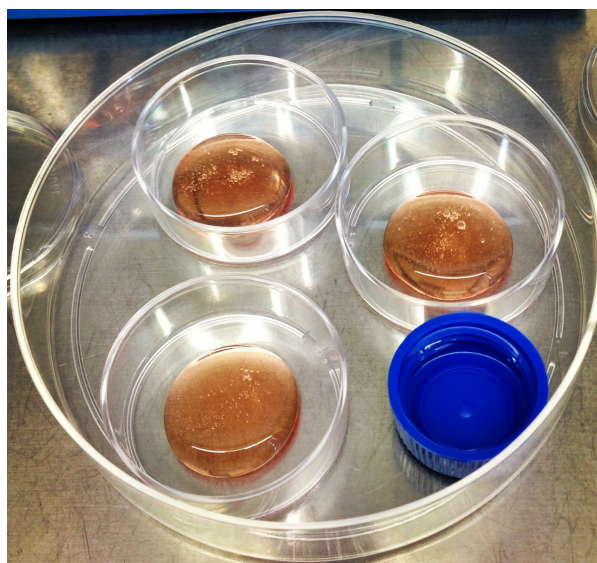


Figure 2.12: Preparation of clonogenic assay plates

Haematopoietic colonies were set up with bone marrow cells mixed with Methocult in triplicate 35mm culture dishes placed in a 100mm petri dish. The blue well contained sterile water to ensure humidity for the 14-day assay.

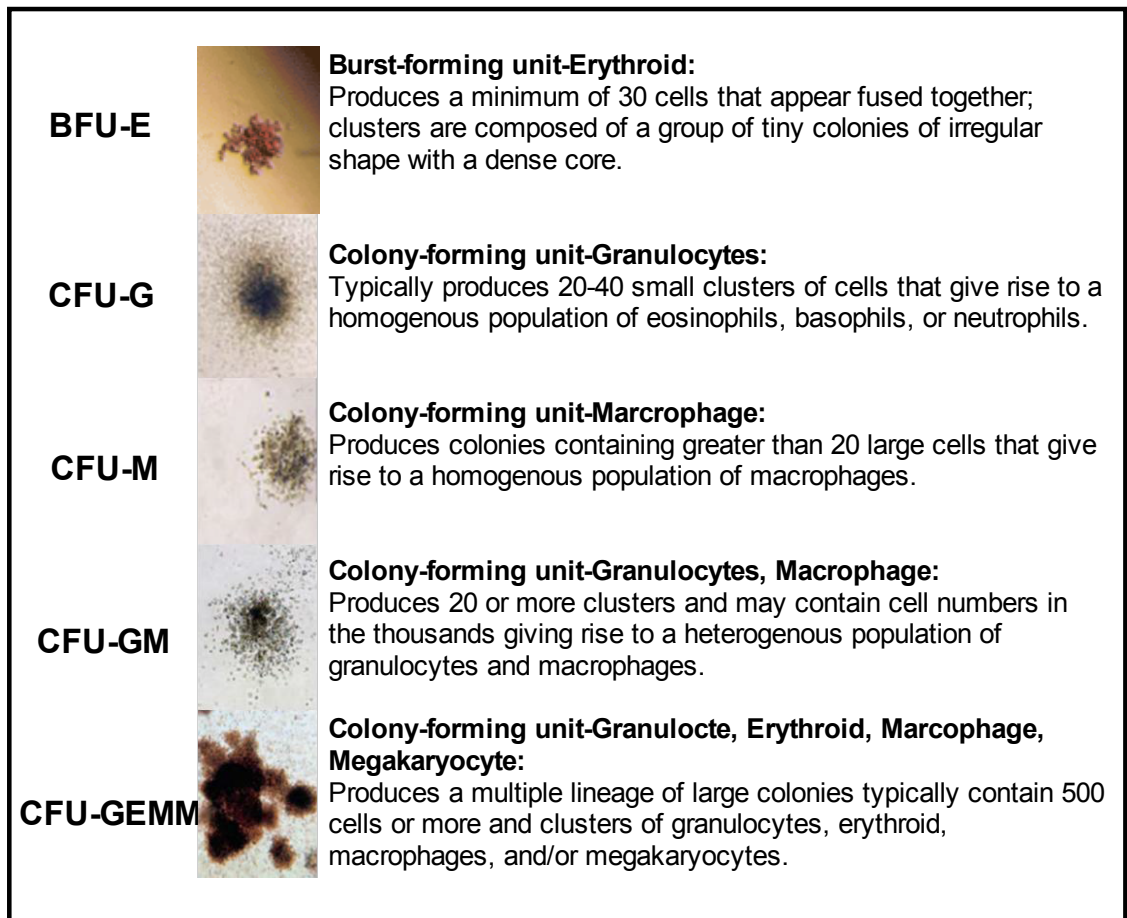


Figure 2.13: Colony-forming units morphological identification.

Different colonies produced from the haematopoietic clonogenic assay can be identified according to their morphology into one of the colony-forming unit types: BFU-E, CFU-G, CFU-M, CFU-GM, or CFU-GEMM. Adapted from MethoCult User Instructions (Stemcell Technologies).

2.10 Statistical analysis

All results were analysed for statistical significance with the threshold of probability (p-value) defined at 0.05 (5%) for significant results. This measures how confidently one can reject the null hypothesis, which states that there are no relationships between measured variables. The null hypothesis can be true if the observed data have at least a 95% probability occurring purely by chance (Lieber, 1990). The Mann-Whitney nonparametric test was used in the calculation of statistical significance between two variables. For more than two variables, Kruskal-Wallis with Dunn's post hoc test was used. All tests were performed using GraphPad Prism version 6.0d.

Chapter 3 Metabolic stress induces PGC-1 α in endothelial progenitor cells

3.1 Introduction

Vascular endothelial dysfunction is a major manifestation of diabetes that leads to numerous cardiovascular complications including limb ischemia, which could potentially result in limb amputation (S. J. Hamilton *et al.*, 2007).

EPCs play a critical role in angiogenesis and vascular repair. Early EPCs were reported to fail to form tubelike structures and to have limited proliferative potential (Hur *et al.*, 2004). However, their ability to augment new blood vessels appears to be by secreting chemokines, angiogenic growth factors, and cytokines without direct incorporation in the vascular network (Yoon *et al.*, 2005). Studies have reported that hyperglycemia decreases the number and function of circulating blood-derived early EPCs both in vitro (Seeger *et al.*, 2005; Y. H. Chen *et al.*, 2007) and in diabetic patients in vivo (Fadini *et al.*, 2005).

PGC-1 α (Peroxisome Proliferator-Activated Receptor Gamma (PPAR γ)-Coactivator-1 α) is a key metabolic coactivator that expresses various functions in different tissues. It has been shown that PGC-1 α upregulation mediates hyperglycemia in the diabetic liver (Herzig *et al.*, 2001). Furthermore, PGC-1 α was found to promote vasculogenesis in the heart and muscles (Arany *et al.*, 2008). However, little is known about the link between hyperglycemia and PGC-1 α in early EPCs.

In this chapter, EPCs isolated from human peripheral blood were incubated with different diabetic (10mM, 15mM, 20mM, and 25mM) and supraphysiological (50mM, 100mM, and 150mM) glucose concentrations to assess the role of hyperglycemia as a metabolic stress in culture. Additionally, cultured EPCs were also studied under different metformin concentrations (0.1 mM, 0.5 mM, 1 mM, and 1mM) to investigate their effects. PGC-1 α and downstream targets GLUT4 (Glucose Transporter type 4) and G6Pase (Glucose-6-Phosphatase) expressions were measured using real-time qPCR.

Therefore, three main aims were investigated in this chapter:

- a) To establish EPCs in culture
- b) To assess the role of metabolic stress on PGC-1 α and its downstream targets in EPCs
- c) To investigate the effects of metformin on EPCs

3.2 Results

3.2.1 Impact of oxidative stress and metabolic stress on EPC cell count

EPCs were isolated from peripheral blood of healthy volunteers. Cells were cultured for one week using specific endothelial media with growth factors. As seen in Figure 3.1A, EPCs showed the distinctive colonies with a cluster of cells and spindle-shape cells radiating off them. EPCs did not proliferate, and the adherent cell number was reduced during culture (Figure 3.2). However, due to the limitation of techniques used (cell count and microscopic morphology), I cannot exclude the contamination of other cells in culture.

As diabetes has been shown to lead to reduction in EPC numbers (Fadini *et al.*, 2005) and function (Vasa *et al.*, 2001b), I have investigated the effects of metabolic stress on EPCs. For that, cells were cultured under different glucose concentrations (15mM, 20mM, 25mM). Of note, the EBM-2 medium used for cell culture has already contained 5mM glucose. In particular, critical glucose levels of >13.8 mmol/l and >33.3 mmol/l are implicated in severe lethal complications of diabetes in diabetic ketoacidosis and the hyperosmolar hyperglycemic state, respectively (Kearney and Dang, 2007). Whereas the physiological level of glucose in a healthy person ranges from 4.9–5.9 mmol/L according to the national institute for clinical excellence (NICE) (NHS, 2008).

Oxidative stress (40% oxygen level) has been shown to reduce the growth of endothelial colony-forming cells (ECFCs), proposed to be “true” EPCs, via disruption of VEGF-NO signalling in human preterm cord blood samples compared to normoxic conditions (Fujinaga *et al.*, 2009). Therefore, I tested the effect of oxidative stress on EPCs by incubating them under different oxygen levels (3%, 20%, and 40%). Although the sample number was small (n=2), the cell number was reduced under metabolic stress. Vice versa, cell counts increased with decreasing glucose concentrations (Figure 3.3).

Cells were noticed to be smaller under the light microscope in high glucose concentrations and failed to form the distinctive colonies (Figure 3.1B). In comparison to metabolic stress, oxidative stress did not affect the EPC count in a short-term culture (Figure 3.3). Furthermore, more cell count was noticed under atmospheric oxygen conditions (20%) compared to 3% and 40%.

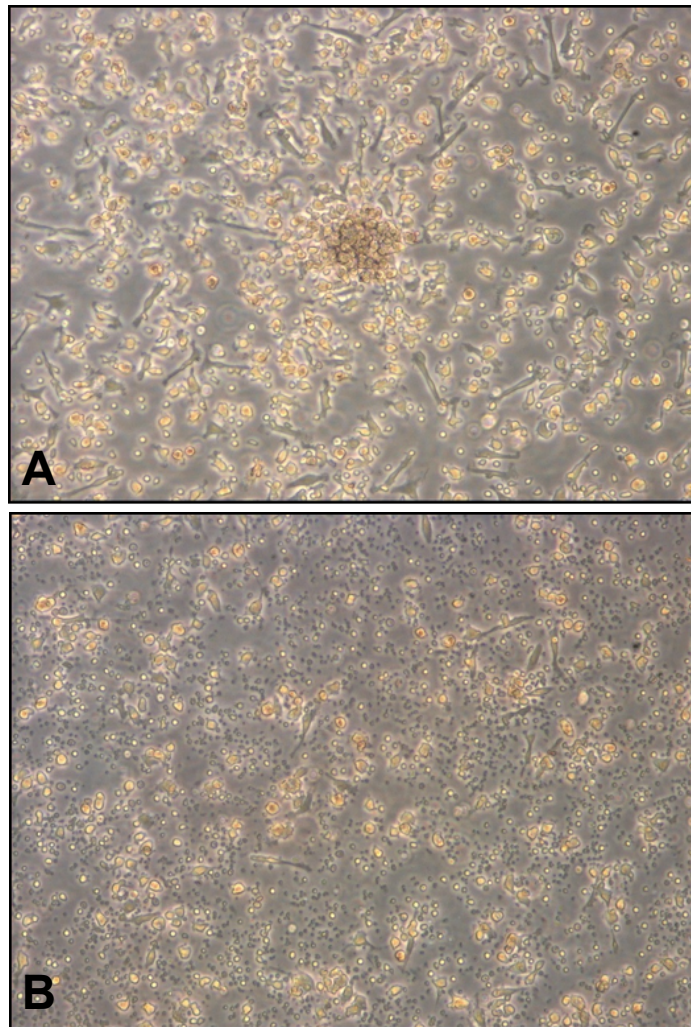


Figure 3.1: Morphology of EPCs in culture.

EPCs were isolated by density-gradient centrifugation from peripheral blood of healthy volunteers. Cells were cultured in EBM-2 media supplemented with endothelial growth factors (hFGF-B, VEGF, R3-IGF-1, and hEGF). **A:** A cluster of cells was observed under the light microscope by day 4. This distinctive pattern of EPCs was seen as a cluster of cells surrounded by spindle-shaped cells. **B:** Cells were cultured with 100mM glucose concentration. On day 5, EPCs appeared smaller in size and failed to form distinctive EPCs colonies (10x magnification).

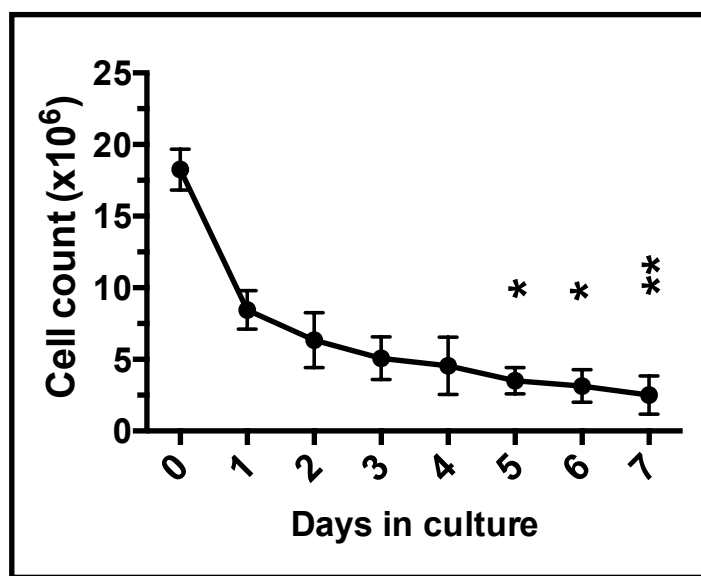


Figure 3.2: EPCs count in culture.

Endothelial progenitor cells were isolated by density-gradient centrifugation from peripheral blood of healthy volunteers. Cells were cultured for one week in EBM-2 media supplemented with endothelial growth factors (n=4). Media were changed every two days, and adherent cells were counted. Statistical significance was calculated by the Kruskal-Wallis multiple comparisons test compared to Day 0 (*P<0.05, **P<0.01). Error bars stand for mean \pm SEM.

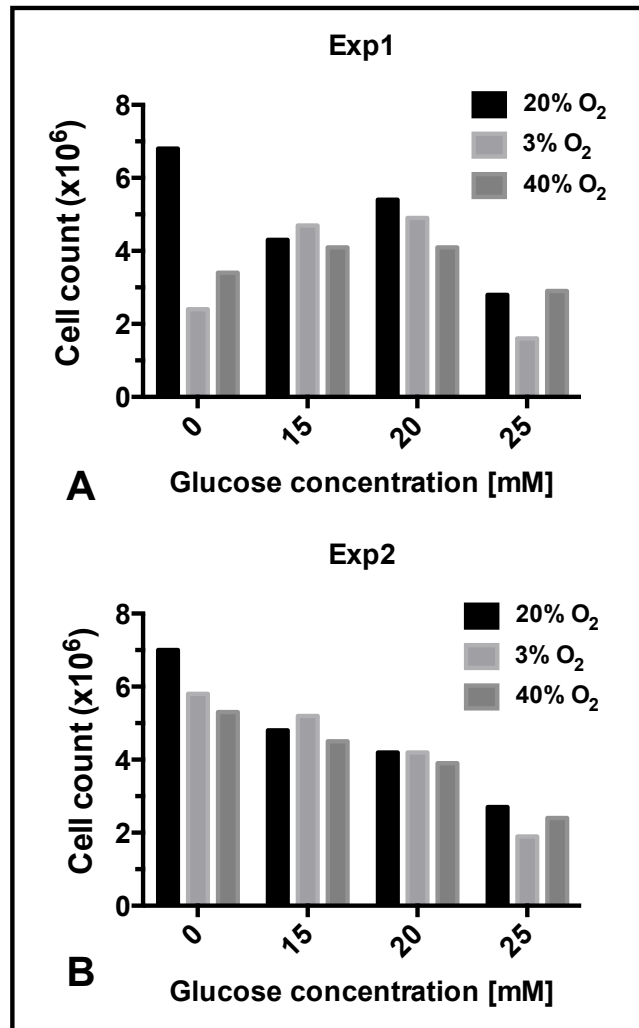


Figure 3.3: EPC count under different oxygen levels and glucose concentrations.

EPCs were isolated by gradient centrifugation from healthy volunteers' peripheral blood. Cells were cultured under different glucose concentrations (15mM, 20mM, and 25mM) and incubated under 3%, 20%, and 40% oxygen levels for 3 days. Two experiments were performed experiment A and experiment B. Adherent cells were counted using a haemocytometer. The calculation of statistical significance was not possible due to small n numbers. Error bars stand for mean \pm SEM.

3.2.2 Effects of metabolic stress and metformin on PGC-1 α signalling pathways in EPCs

The onset of diabetes and insulin resistance has been correlated with human genetic variants in PGC-1 α (Hara *et al.*, 2002). PGC-1 α has been also shown to induce angiogenesis in skeletal muscle (Arany *et al.*, 2008). Thus, I was interested to examine the effect of metabolic stress (mimicking hyperglycemia in diabetes) on PGC-1 α expression and downstream targets (GLUT4 and G6Pase). AMPK α 1 was evaluated, as it has been reported that AMPK acts as an upregulator of PGC-1 α in skeletal muscles (Jager *et al.*, 2007). I used diabetic and supraphysiological glucose doses (10mM, 25mM, 50mM, 100mM, and 150mM) to assess the effects on PGC-1 α and associated genes. Contrary to expectation, as seen in Figure 3.4, glucose induced PGC-1 α and targets (GLUT4 and G6Pase) in EPCs dose-dependently. Supraphysiological glucose levels lead up to a 30-, 46-, and 26-fold increase in PGC-1 α , G6Pase, and GLUT, respectively. However, AMPK α 1 was 1.8-fold induced under 150mM glucose concentration compared to control. Whereas under glucose levels that are relevant to diabetic patients (10-25mM), the induction of mRNA expression was two-, three- and fivefold in PGC-1 α , G6Pase, and GLUT4, respectively, compared to control. Furthermore, under diabetic glucose levels, AMPK α 1 was not changed.

Metformin is an insulin-sensitizing bioguanide (dimethylbiguanide), a commonly used drug to treat type 2 diabetes as well as for prevention of some vascular complications (Gong *et al.*, 2012). To evaluate the effects of this anti-diabetic drug on PGC-1 α in EPCs, different doses of metformin (0.1mM, 0.5mM, 1mM, and 10mM) were added to EPCs in culture. There was no change of the EPC number with metformin (Figure 3.5). High metformin concentrations significantly induced PGC-1 α and downstream targets GLUT4 and G6Pase up to 28-, 26-, and 84-fold in EPCs, respectively. However, AMPK α 1 was not changed with different doses of metformin (Figure 3.6).

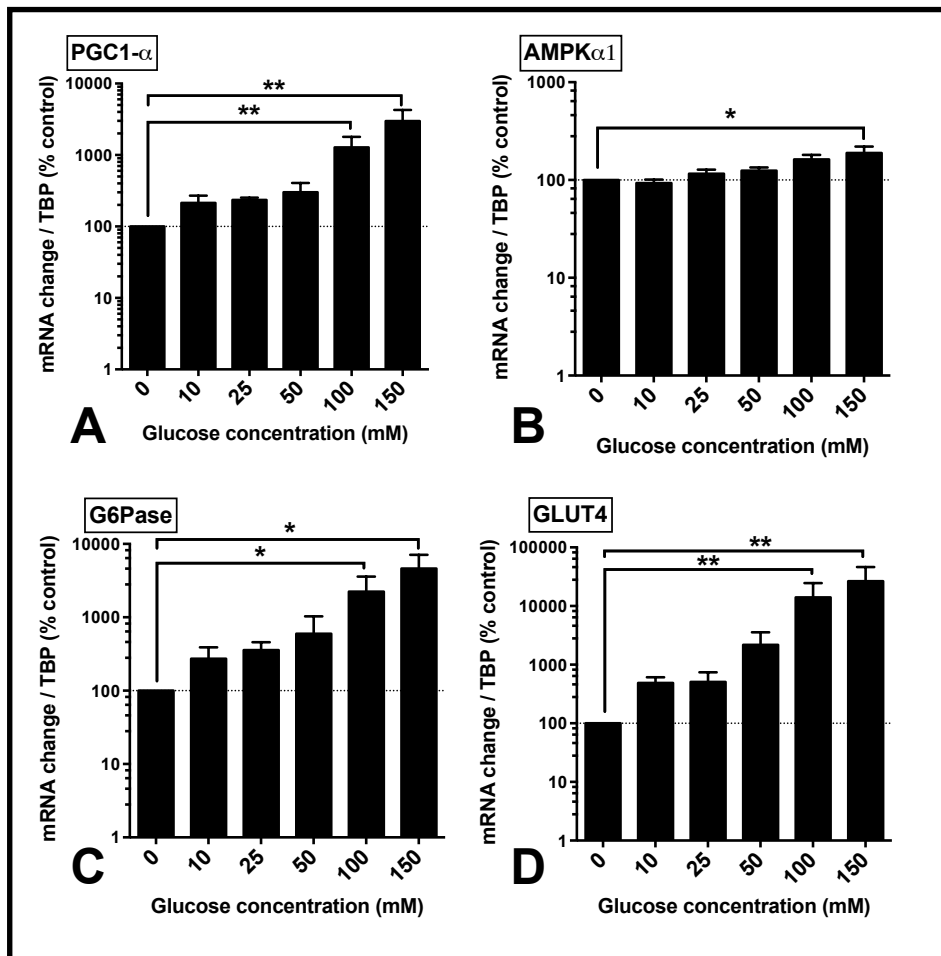


Figure 3.4: Effects of metabolic stress on the expression of PGC1- α and downstream targets in EPCs.

EPCs were isolated by gradient centrifugation from peripheral blood of healthy volunteers. Cells were incubated for three days with different glucose concentrations (10mM, 25mM, 50mM, 100mM, 150mM) (n=4). The percentage of mRNA expression compared to control EPCs without glucose. Genes examined were PGC-1 α (A), AMPK α 1 (B), GLUT4 (C), and G6Pase (D). Gene expression was referenced to the housekeeping gene TBP (TATA-box binding protein). Real Time PCR [StepOnePlus, Applied Biosystems] was used to measure the gene expression by the $\Delta\Delta C_T$ method. Statistical significance was calculated by Kruskal-Wallis and Dunn's multiple comparison tests (*P<0.05 and **P<0.01). Error bars stand for mean \pm SEM.

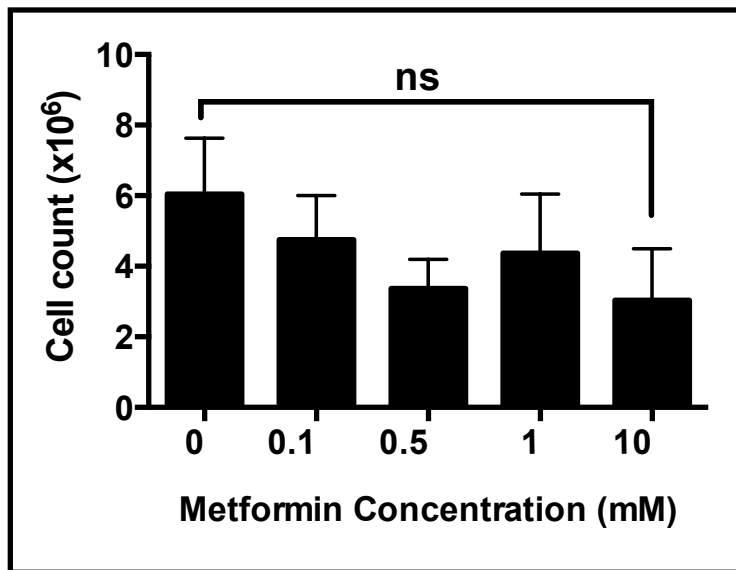


Figure 3.5: EPC count under metformin in culture.

EPCs were isolated by gradient centrifugation from peripheral blood of healthy volunteers. Cells were incubated with different doses of metformin (0.1mM, 0.5mM, 1mM, and 10mM) for three days in culture (n=4). Adherent cells were counted using a haemocytometer. Statistical significance was calculated by the Kruskal-Wallis test (ns= nonsignificant). Error bars stand for mean \pm SEM.

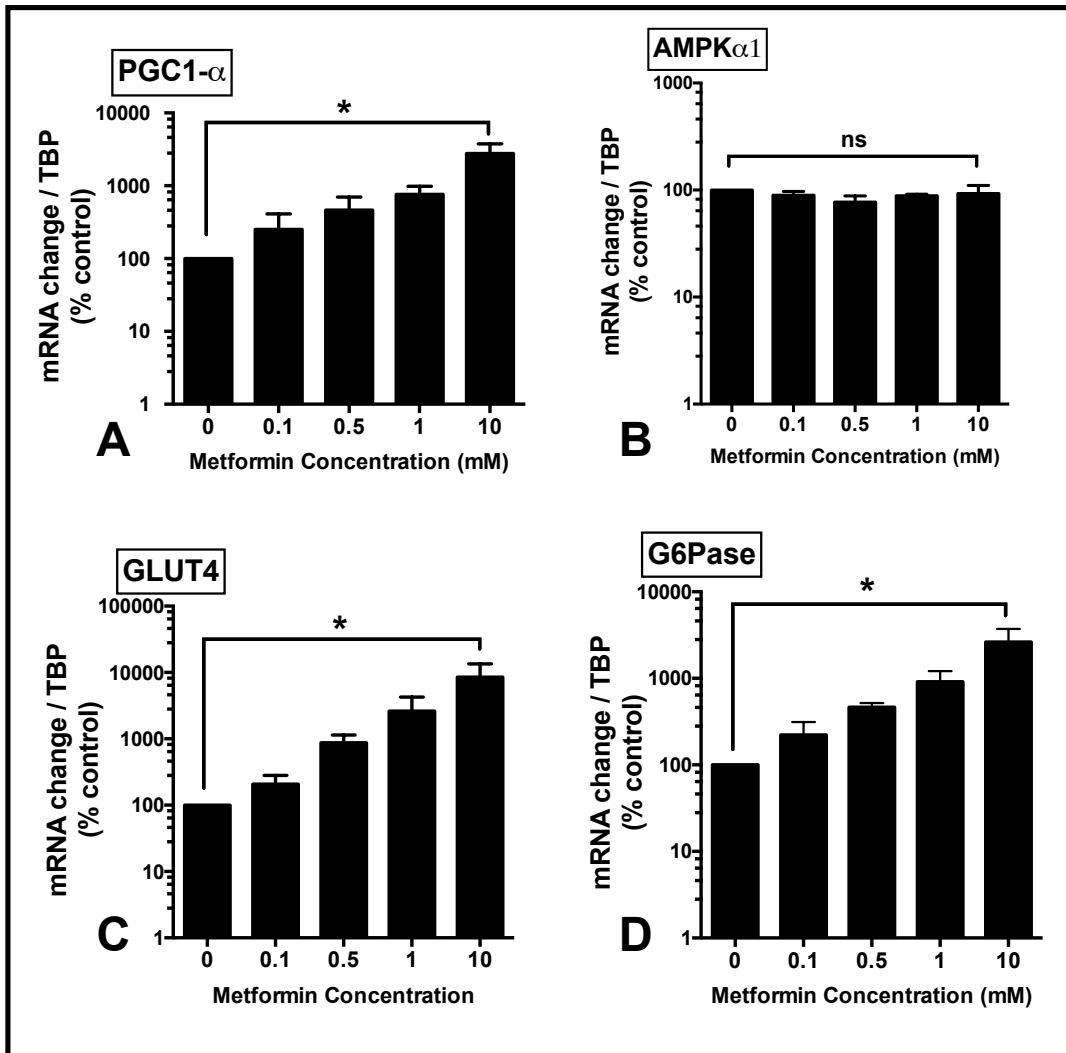


Figure 3.6: Effects of metformin on PGC1- α expression and downstream targets.

EPCs were isolated by gradient centrifugation from peripheral blood of healthy volunteers. Cells were cultured under different concentrations of metformin (0.1mM, 0.5mM, 1mM, 10mM) for three days (n=3). The percentage of mRNA expression compared to control EPCs without metformin. PGC-1 α (A), AMPK α 1 (B), GLUT4 (C), and G6Pase (D) were referenced to housekeeping gene, TBP. Real-time PCR [StepOnePlus, Applied Biosystems] was used to measure the gene expression by the $\Delta\Delta C_T$ method. Statistical significance was calculated by Kruskal-Wallis and Dunn's multiple comparison tests compared to controls (* $P < 0.05$ and ns= nonsignificant). Error bars stand for mean \pm SEM.

3.3 Discussion

PGC-1 α plays a fundamental role in energy regulation, such as in mitochondrial biogenesis, lipid oxidation, and glucose metabolism as well as angiogenesis (Arany *et al.*, 2008; Ventura-Clapier *et al.*, 2008). Since ageing has been associated with impaired repair of vascular lesions (Gennaro *et al.*, 2003), several studies have demonstrated the potential of endothelial progenitor cells to restore vascular repair (Asahara *et al.*, 1997; Kalka *et al.*, 2000; Sheng *et al.*, 2013). To promote angiogenesis and vascular repair, endothelial progenitor cells act directly via physical incorporation into the growing angiogenic network or indirectly by secreting angiogenic factors, known as the “paracrine effect” (Rehman *et al.*, 2003). In diabetes, significant impairment of angiogenesis occurs (Fadini *et al.*, 2006b). However, the underlying mechanisms of diabetes and the role of PGC-1 α in EPCs are poorly understood. Therefore, the goal of this study was to investigate the role of PGC-1 α in EPCs under oxidative and metabolic stress.

I could not detect any EPC proliferation in culture in agreement to previous reports (Hur *et al.*, 2004). Oxidative stress showed no obvious tendency to reduce EPCs. However, the number of samples tested in my experiments was low (n=2), and it was difficult to reach a definitive conclusion. In fact, EPCs have been shown previously to express high levels of antioxidant enzymes and thus were more resistant to oxidative stress compared with adult microvascular endothelial cells and HUVECs (Dernbach *et al.*, 2004; He *et al.*, 2004; Cai *et al.*, 2006). Therefore, such an enhanced antioxidant system in EPCs allows them to promote vascular regeneration in the setting of ischemic injury. However, during ageing, the system is disrupted. It was demonstrated that the activity of antioxidant enzyme glutathione peroxidase-1 (GPx-1) was significantly reduced in EPCs isolated from old subjects and more sensitive to stress-induced apoptosis compared to younger ones (He *et al.*, 2009).

In my experiments, metabolic stress or hyperglycemia reduced early EPCs in culture. Similarly, in diabetic patients EPC numbers and function were reduced (Fadini *et al.*, 2005). It has been shown that hyperglycemia (25mM) in mouse heart myocardial endothelial cells induced angiopoietin-2, which led to impairment of angiogenesis (J. X. Chen and Stinnett, 2008). Furthermore, studies have suggested that hyperglycemia reduces the number and function of circulating blood-derived EPCs, mostly early, both in

vitro (Krankel *et al.*, 2005; Seeger *et al.*, 2005; Y. H. Chen *et al.*, 2007) and in vivo (Tepper *et al.*, 2002; Loomans *et al.*, 2004; Fadini *et al.*, 2005). In identifying the underlying mechanism of hyperglycemia-induced damage to EPCs, it has been suggested that glucose results in an overproduction of ROS, leading to EPC senescence (Kuki *et al.*, 2006). Chen *et al.* argued that the reduction of EPCs under high glucose conditions is associated with nitric oxide modification, not oxidative stress (Y. H. Chen *et al.*, 2007). One group found that inhibition of p38 MAP kinase activation led to improved cell viability under glucose (Seeger *et al.*, 2005). Moreover, another study reported that glucose reduces EPCs via downregulation of SIRT1, a metabolic sensor, expression levels, and enzyme activity (Balestrieri *et al.*, 2008).

I reported that high glucose concentrations in EPCs induced PGC-1 α expression as well as GLUT4 and G6Pase. GLUT4 is a glucose transporter while G6Pase is an enzyme that hydrolyzes glucose-6-phosphate during gluconeogenesis and glycogenolysis. Both GLUT4 and G6Pase are downstream targets of PGC-1 α (Baar *et al.*, 2002; Matsumoto *et al.*, 2007). PGC-1 α has been shown to act differently depending on tissues. For example, it was shown that PGC-1 α activates mitochondrial biogenesis in muscle (J. Lin *et al.*, 2002), drives thermogenesis in response to cold in brown fat (Puigserver *et al.*, 1998), and increases in diabetic liver (Herzig *et al.*, 2001). Furthermore, induction of PGC-1 α has been shown by others to improve vasculogenesis in the heart and muscles (Arany *et al.*, 2008). Therefore, the result I found was rather unexpected, as PGC-1 α was associated with enhanced vasculogenesis in the heart and muscles. Accordingly, I expected to see a reduction of PGC-1 α expression because hyperglycemia has been shown to disrupt angiogenesis in diabetes. Unfortunately, due to this contradictory result and lack of clear data at the time of these experiments (my first year), I decided to move my studies towards a more primitive cell type, such as haematopoietic stem cells. However, a very recent publication from Zoltan Arany's group at Harvard University confirmed my findings in EPCs and endothelial cells and for the first time revealed that PGC-1 α induction in EPCs is associated with defective endothelial migration via notch signalling that blunts the Rac/Akt/eNOS pathway (Sawada *et al.*, 2014). The authors further confirmed that diabetic endothelial dysfunction could be rescued in both type 1 and 2 diabetic mouse models when lacking endothelial PGC-1 α . This deletion

accelerated wound healing and recovered blood flow after hind limb ischemia (Sawada *et al.*, 2014).

To investigate the effects of the anti-hyperglycemic drug metformin on PGC-1 α in EPCs, I tested the effect of adding different doses of metformin on the expression of PGC-1 α and downstream targets. The hypothesis was that metformin could induce PGC-1 α in endothelial progenitor cells. As expected, metformin induced mRNA expression of PGC-1 α and downstream targets, but not AMPK α 1. However, metformin doses used in this study exceeded the recommended dose and are considered supratherapeutic (0.1–10mM), by which some groups considered 20 μ M to be clinically relevant in vitro. However, it can be argued that these culture conditions are already under the overabundant nutrient environment, and thus higher metformin doses were needed to try to mimic the in-vivo effects (Cantrell *et al.*, 2010). Similar to my finding, metformin has been reported to induce expression of PGC-1 α by almost twofold in HUVECs compared to controls. They have also shown that metformin reduces intracellular ROS production by promoting mitochondrial biogenesis through activating the AMPK-PGC-1 α pathway in HUVECs (Kukidome *et al.*, 2006). Prior studies have noted the positive effect of metformin in the treatment of type 2 diabetes patients and its association with improved vascular function (Abbasi *et al.*, 2004). It has been shown that metformin does not influence insulin secretion but promotes glucose utilization by an AMP-activated protein kinase (AMPK)-mediated stimulation in white adipocytes (Gong *et al.*, 2012). Moreover, treatment with metformin has been shown to reduce incidence and improved survival for many common cancers such as liver, pancreatic, and breast cancer (P. Zhang *et al.*, 2013). Metformin showed contrasting reports regarding the pro-angiogenic (Phoenix *et al.*, 2009) and anti-angiogenic (Tan *et al.*, 2009) effect. In fact, a recent study in HUVECs suggested that metformin acts in a paradoxical way. Metformin inhibited angiogenesis in HUVECs partially dependent on AMPK in vitro and in vivo. However, the gene expression profiling revealed modulation of several angiogenesis-associated genes and proteins by metformin. This was associated with induction of VEGF and rapid induction of inflammatory response mediators COX2 (cyclooxygenase 2) and CXCR4 (CXC chemokine receptor 2), while antibody array analysis showed the opposite

regulation of angiogenesis-associated proteins in endothelial cells such as IL-8 (Dallaglio *et al.*, 2014).

Taken together, both metabolic stress and metformin treatment induced PGC-1 α in cultured EPCs. However, exact mechanisms underlying metformin use require further extensive research as published data are still controversial.

Chapter 4 Effects of metabolic and oxidative stress on HSPC expansion and differentiation

4.1 Introduction

Ageing is associated with an overall decline in the functional capacity of stem cells to maintain homeostasis under different physiological stimuli. Haematopoietic stem cells are the common ancestor cells forming different lineage cells of the blood and immune system. They are a rare cell type at the top of the hierarchy of stem and progenitor cells. HSPCs are ultimately responsible for the production of numerous new blood cells every day. HSPCs can be isolated from the blood or bone marrow and have the full potential for cell renewal, differentiation, or mobilization into circulating blood. Among the markers commonly used to identify the heterogeneous HSPC population is CD34. CD34 is a highly glycosylated transmembrane protein strongly expressed on HSPCs. It has been shown that CD34+ cells can reconstitute haematopoiesis in the bone marrow of a myeloablated recipient for a lifetime during transplantation assays (Krause *et al.*, 1996). Interestingly, also in transplantation assays, HSCs from aged donors have demonstrated a significantly reduced function compared with HSCs from young donors (Sudo *et al.*, 2000). Furthermore, current research indicates that cell intrinsic and extrinsic factors can contribute to the functional decline of stem cells during ageing. This functional reduction is associated with age-associated changes in gene-expression profiles of HSCs (Rossi *et al.*, 2005).

Umbilical cord blood is a valuable source of HSPCs that can be easily collected and usually discarded after the delivery process. In some studies, umbilical cord blood extended the availability of haematopoietic stem cell transplantation when there was no suitable donor (Ballen *et al.*, 2012). However, due to the usually limited volume of umbilical cord blood compared to bone marrow and peripheral blood, ex-vivo expansion has been considerably studied as an important way to amplify HSPCs.

In most somatic cells, telomeres shorten with age. After each cell division, telomere length shortens until they reach a critically short length. This telomere erosion leads to cell-cycle arrest, chromosomal instability, and replicative senescence (Harley *et al.*, 1990). However, telomeres are maintained in the germ line and almost all tumour cells

by a specialized ribonucleoprotein reverse transcriptase called ‘telomerase.’ Telomerase counteracts the attrition of telomeric repeats from incomplete end replication by the addition of telomeric repeats de novo onto the 3’ overhang (Feng *et al.*, 1995). In addition to the germ line, telomerase is present at detectable levels in haematopoietic stem cells, though in limited amounts (Counter *et al.*, 1995). Although HSPCs have extensive replicative potential, there is a consistent decline in telomere length in their progeny as humans age (Rufer *et al.*, 1999). Hence, telomerase levels in human HSPCs are not sufficient to maintain replication indefinitely (Vaziri *et al.*, 1994). In addition, telomerase activity decreases with age (Engelhardt *et al.*, 2000). In haematopoietic stem cells, the assumption that telomerase is required to prevent telomere loss with age or proliferation is not fully supported by fundamental data. Studies on the role of telomerase and telomeres in HSCs from healthy individuals are challenging because they are very rare cells lacking definitive markers. In contrast, blood cells derived from HSCs are easily accessible for analysis. Indeed, if one assumes that the number of cell divisions between granulocytes and HSPCs is relatively constant (Hills *et al.*, 2009), then the average telomere length in granulocytes can be an indicator for the telomere length in HSPCs (Shepherd *et al.*, 2004). In fact, data from our group has showed that human telomere length in peripheral blood correlates very well with their bone marrow-residing CD34+ progenitors ($r=0.95$) (Spyridopoulos *et al.*, 2008; Spyridopoulos *et al.*, 2009). Furthermore, in humans, individuals with heterozygous mutations in hTERT or hTERC genes can present with a wide spectrum of diseases including bone marrow failure, dyskeratosis congenital (DKC), and pulmonary fibrosis (Young, 2012).

Several reports studied ex vivo expansion of UCB HSPCs and effects on telomerase. It has been shown that despite telomerase upregulation as a response to cytokine-induced expansion, telomeres shorten in UCB CD34+ cells compared to unexpanded controls (Engelhardt *et al.*, 1997). Similar induction in telomerase during CD34+ cells’ ex vivo expansion was reported; however, telomere length was maintained for 16 weeks when a coculture system was used (Gammaitoni *et al.*, 2004). Another study also confirmed telomerase induction during expansion and has shown that telomere length in CD34+ might predict erythroid proliferative potential (Schuller *et al.*, 2007). Furthermore, a

recent study reported that telomerase induction during CD34⁺ expansion was associated with mRNA TERT expression (Ge *et al.*, 2013).

Oxidative stress has been implicated in a number of age-related chronic medical conditions, such as atherosclerosis and metabolic syndrome. Metabolic syndrome is defined as a group of risk factors including hypertension, obesity, atherogenic dyslipidemia, glucose intolerance, and insulin resistance, which increases cardiovascular disease risk and other diseases such as diabetes (Frisard and Ravussin, 2006). However, the effects of metabolic and oxidative stress on HSPC expansion and myeloid differentiation are largely unknown.

In this chapter, CD34⁺ cells isolated from human umbilical cord blood were studied in order to investigate the effects of ageing stress cues (oxidative and metabolic stress) on expanded CD34⁺ HSPCs and myeloid-differentiated cells in culture. Cells were isolated from human UCB, and CD34⁺ cells were obtained by immunomagnetic beads separation. The main aims of this chapter were first to establish a protocol to expand CD34⁺ HSPCs in culture, and second, to assess the effects of oxidative stress on cell growth, telomerase activity, and colony formation in CD34⁺ HSPCs. Another aim of this chapter was to examine the effects of metabolic stress on CD34⁺ HSPCs expansion and telomerase activity. Finally, I wanted to examine if there was any role for telomerase in HSPC expansion or differentiation and whether or not I can enhance CD34⁺ HSPCs expansion and telomerase activity using the telomerase activator TA-65.

4.2 Results

4.2.1 Growth characteristics of human CD34⁺ HSPCs during in-vitro expansion

To establish CD34⁺ expansion in culture, CD34⁺ cells isolated by immunomagnetic bead separation from umbilical cord blood were expanded using a cocktail of expansion cytokines to specifically induce HSPC growth as described by Schuller *et al.* (2007) (details are described in Chapter 2 section 2.3). As seen in Figure 4.1, HSPCs can be expanded over 37 times in a 2-week culture under atmospheric oxygen concentration (20% O₂). Cell growth drops by 20-fold in three-week culture under 20% oxygen.

Furthermore, to check how CD34⁺ HSPCs behave in culture conditions during ex-vivo expansion for three weeks, cells were characterized using flow cytometry for the stem cell marker CD34, the granulocyte marker CD15, and the monocyte marker CD14. As shown in Figure 4.2, when CD34⁺ HSPCs were cultured, they lost the stem cells marker CD34 and expressed more differentiation markers (CD15 and CD14).

To assess the role of telomerase during CD34⁺ HSPCs expansion, telomerase activity was measured by Q-TRAP. Telomerase activity increased to reach the maximum of 25-fold at two weeks and then dropped by the third week (Figure 4.3). This induction was matching the proliferation pattern seen in Figure 4.1.

Additionally, to assess the effects of expansion on telomere length and to find out whether or not telomerase induction is associated with changes in telomere length, I measured telomere length using flow-FISH in CD34⁺ HSPCs at different stages of expansion (day 0, 1 week, and 2 weeks). There was no detectable change in telomere length detected, as shown in Figure 4.4.

To further investigate whether there was a transcriptional regulation of TERT or TERC genes during CD34⁺ HSPCs expansion in culture, TERT and TERC expression were analysed by qRT-PCR. As seen in Figure 4.5, neither TERT nor TERC expression were influenced during CD34⁺ HSPCs expansion in a three-week culture.

Moreover, as PGC-1 α and downstream targets were induced during metabolic stress in EPCs (as shown in the previous chapter, Figure 3.4), I was interested to evaluate the expression of PGC-1 α during CD34⁺ HSPCs expansion. As shown in Figure 4.6, no significant difference in the expression of PGC-1 α occurred during CD34⁺ expansion.

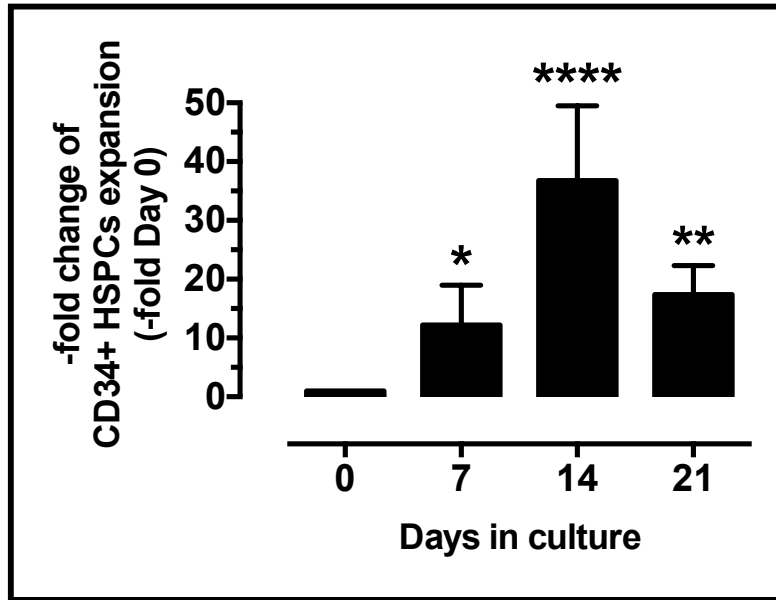


Figure 4.1: Expansion of CD34+ HSPCs in culture.

CD34+ cells were isolated by immunomagnetic labelling from human umbilical cord blood of Caesarean deliveries. Cells were expanded in IMDM media supplemented with a mixture of cytokines (SCF, TPO, and FLT-3L) for three weeks in culture (n=10). Cell counts were performed with a haemocytometer under light microscopy. Cells were grown under normoxic oxygen levels (20%), and media were changed twice a week. Statistical significance was calculated by the Kruskal-Wallis multiple comparisons test, comparing each time point with the baseline (Day 0) (*P<0.05, **P<0.01, and ****P<0.0001). Error bars stand for mean \pm SEM.

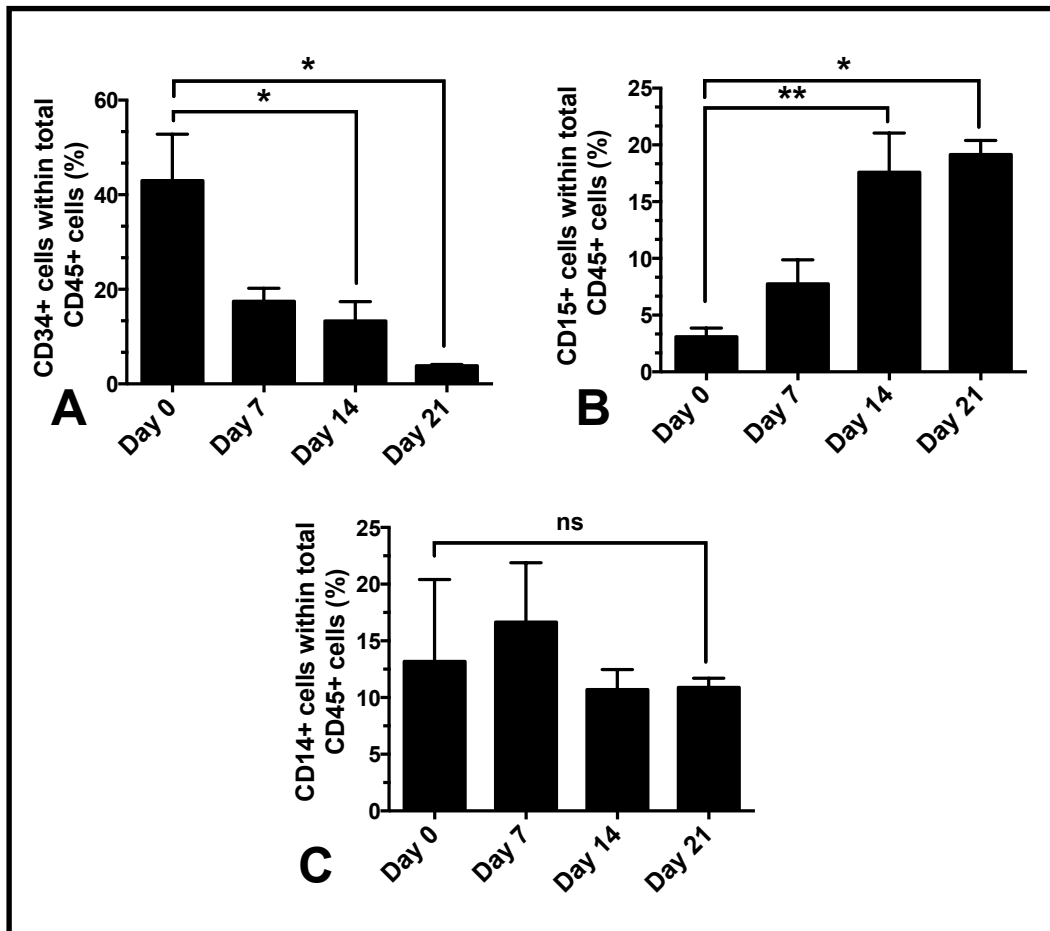


Figure 4.2: Expression of CD34, CD15, and CD14 during CD34+ HSPCs expansion.

CD34+ HSPCs were isolated using immunomagnetic labelling isolated from umbilical cord blood samples from Caesarean deliverers. Cells were expanded in IMDM media supplemented with a cocktail of cytokines for three weeks. Cultured CD34+ HSPCs were characterized using FACS analysis for stem cell marker CD34 (n=10) and differentiation markers CD15 (n=8) and CD14 (n=7). Cells were cultured under atmospheric (normoxic) oxygen levels (20% O₂), and media were changed twice a week. Statistical significance was calculated using Kruskal-Wallis and Dunn's multiple comparison tests (*P<0.05, **P<0.01, and ns= nonsignificant). Error bars stand for mean ± SEM.

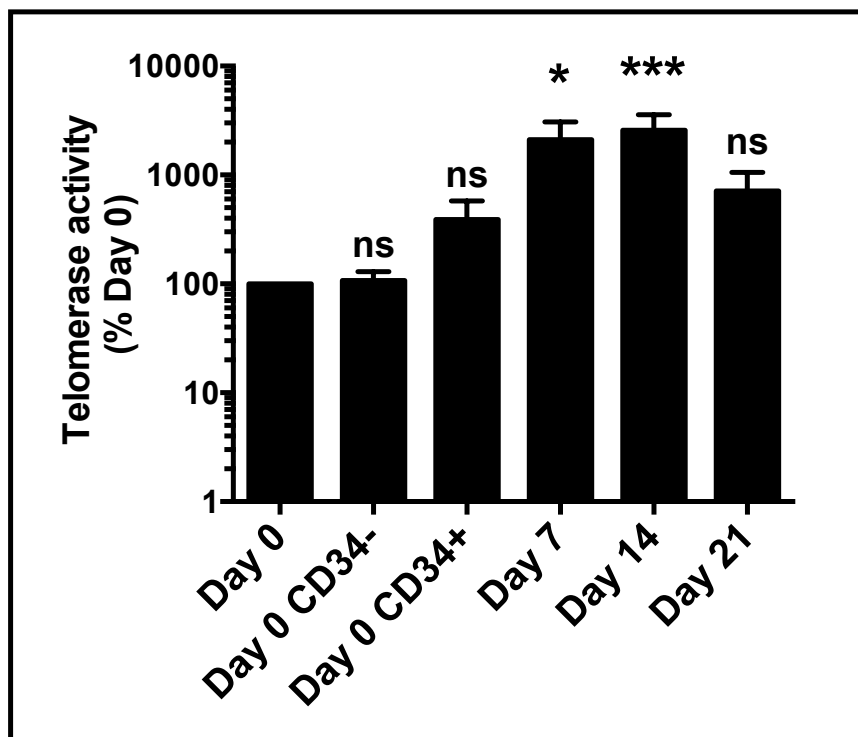


Figure 4.3: Telomerase activity during CD34+ HSPCs expansion.

CD34+ HSPCs were isolated by immunomagnetic labelling from umbilical cord blood of Caesarean deliveries. Cells were expanded using a cytokines mixture for three weeks in culture (n=9). Telomerase activity was measured by Q-TRAP at different time points and compared to the day 0 sample (control). Statistical significance was calculated using Kruskal-Wallis and Dunn's multiple comparison tests (*P<0.05, ***P<0.001). Error bars stand for mean \pm SEM.

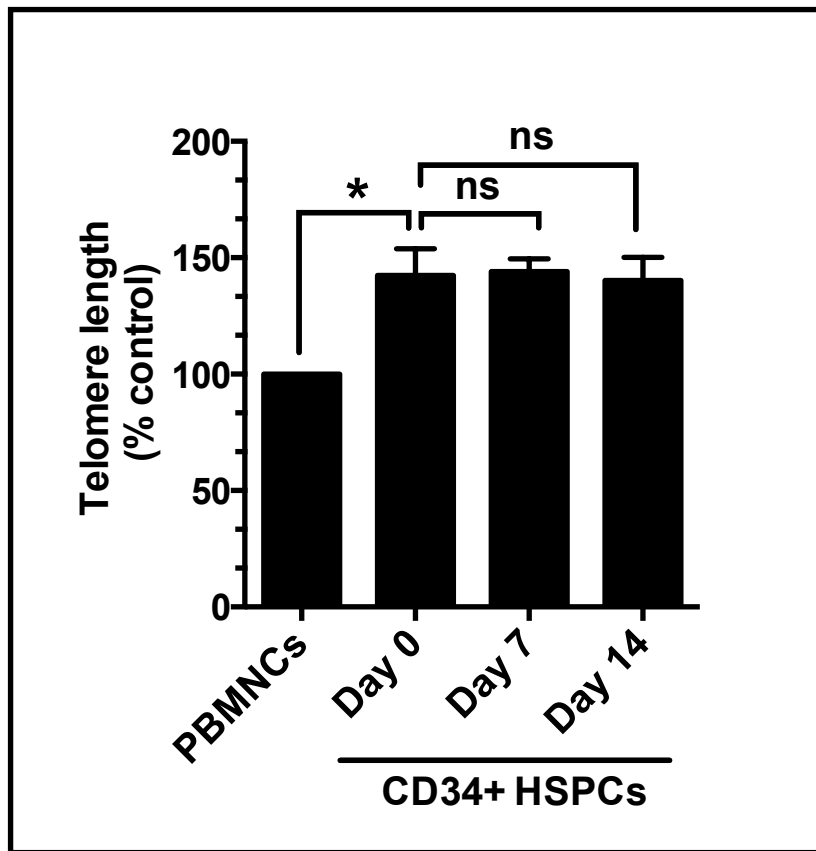


Figure 4.4: Telomere length during CD34+ HSPCs expansion.

CD34+ HSPCs were isolated by immunomagnetic labelling from umbilical cord blood of Caesarean deliveries. Cells were expanded using a cytokines mixture for three weeks in culture. Telomere length was measured by Flow-FISH for HSPCs at day 0 and after one and two weeks (n=6). Measurements were compared to human peripheral blood mononuclear cells (PBMNCs). Statistical significance was calculated using Kruskal-Wallis and Dunn's multiple comparison tests (ns= nonsignificant). Error bars stand for mean \pm SEM.

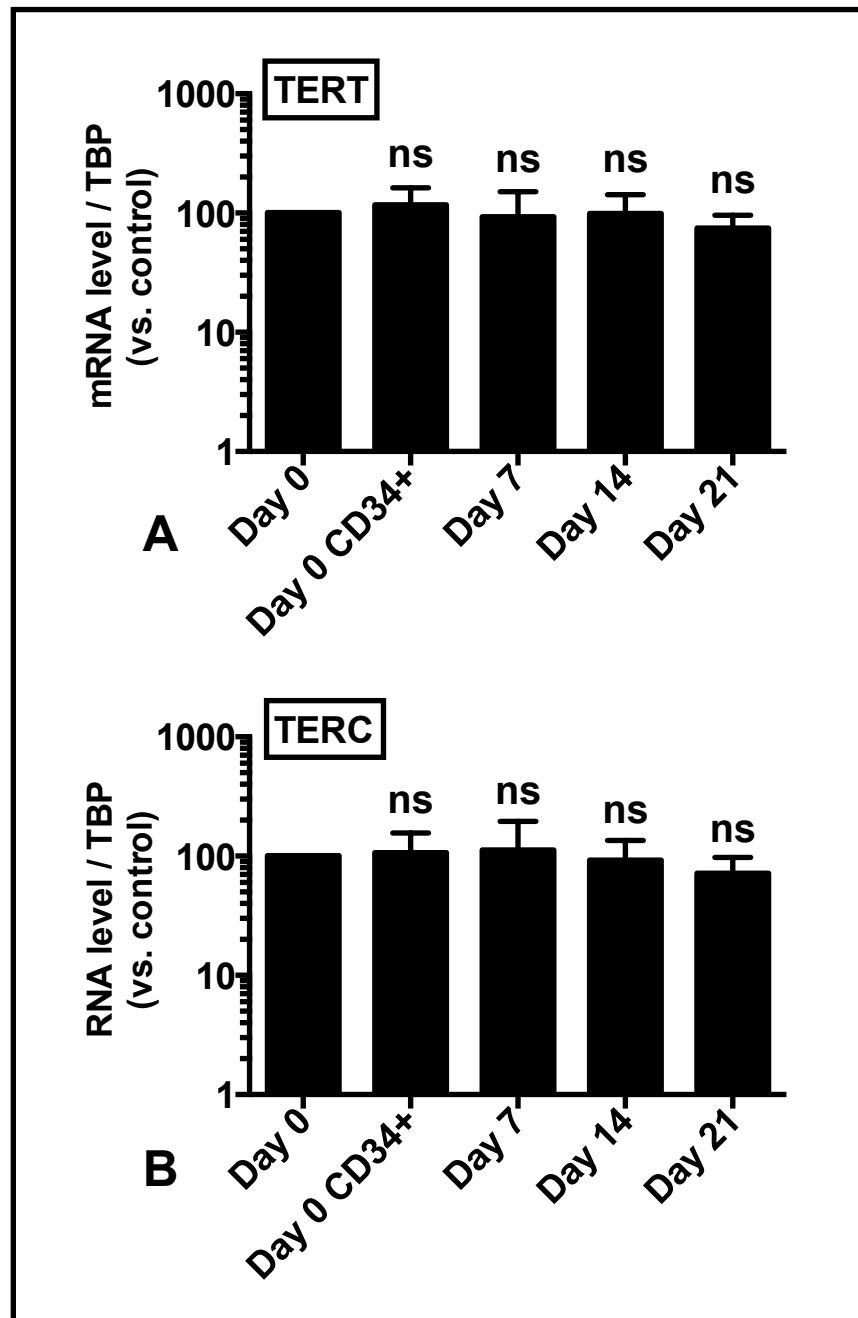


Figure 4.5: Effects of CD34+ HSPCs expansion on TERT and TERC expression.

CD34+ HSPCs were isolated by immunomagnetic labelling from umbilical cord blood samples from Caesarean deliverers. Cells were expanded with a cocktail of cytokines over three weeks. TERT mRNA (A) and TERC RNA (B) expression were measured using qRT-PCR, normalized to the housekeeping gene TBP and compared to day 0 samples (control) (n=3). Statistical significance was calculated using Kruskal-Wallis and Dunn's multiple comparison tests (ns= nonsignificant). Error bars stand for mean \pm SEM.

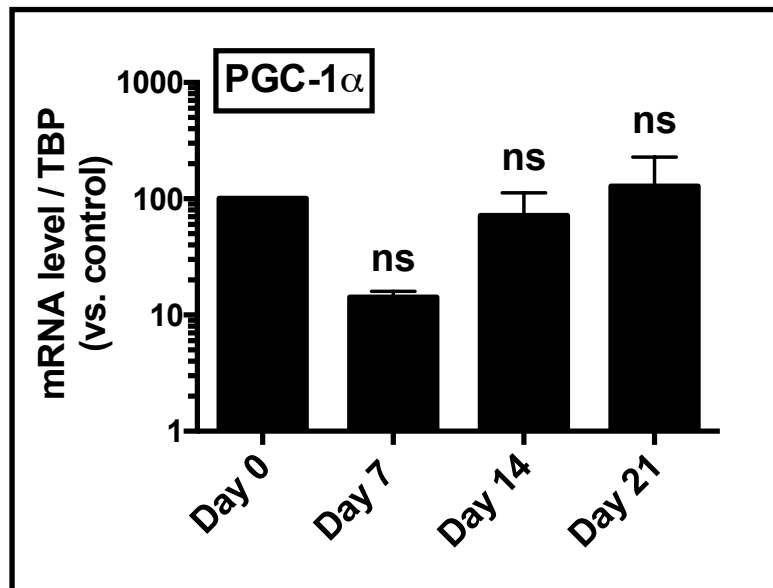


Figure 4.6: The transcription of PGC-1 α during CD34+ HSPCs expansion.

CD34+ HSPCs were isolated by immunomagnetic labelling from umbilical cord blood samples from Caesarean deliverers. Cells were expanded with a cocktail of cytokines for three weeks. PGC-1 α expression was measured using qRT-PCR, normalized to the housekeeping gene TBP, and compared to day 0 samples (control) (n=5). Statistical significance was calculated using Kruskal-Wallis and Dunn's multiple comparison tests (ns= nonsignificant). Error bars stand for mean \pm SEM.

4.2.2 Impact of oxidative and metabolic stress on HSPC expansion

Oxidative stress was induced by incubating cells under hyperoxic conditions (40% O₂), as published by Saretzki *et al.* (2004). HSPCs were also incubated under atmospheric (20% O₂) and hypoxic conditions (3% O₂) for comparison of the impact of oxidative stress. Hyperoxia (40% O₂) impaired HSPC growth in culture, as HSPC cell counts were significantly reduced compared to normoxia and hypoxia in two-week expansion.

Notably, more HSPCs were expanding under atmospheric oxygen conditions compared to hypoxic and hyperoxic ones (Figure 4.7).

Furthermore, I was interested in evaluating the effects of oxidative stress on telomerase activity. Surprisingly, 40% oxygen significantly induced telomerase activity during expansion of HSPCs over one week (Figure 4.8). This was contradictory to the association of telomerase activity with cell proliferation that I had seen during CD34+ HSPCs expansion (see Figure 4.1 and Figure 4.3).

To investigate the effect of oxidative stress on clonogenicity, cells were isolated from umbilical cord blood and cultured in a clonogenic assay using Methocult. This media was supplemented with specific growth factors (SCF, GM-CSF, G-CSF, IL-3, and EPO) and other supplements that optimize colony growth. Colonies were incubated under hyperoxia (40% O₂) and compared with hypoxic (3% O₂) and normal culture conditions (20% O₂). At the start of clonogenic assay experiments, cells isolated after CD34+ immunomagnetic beads failed to form colonies in culture. I therefore used MNCs isolated freshly from umbilical cord blood samples for these CFU assays, and colonies were formed successfully. However, there was no growth of colonies under oxidative stress. Whereas under hypoxic (3% O₂) culture condition, the monocytic (CFU-M) and more progenitor colonies (CFU-GEMM) were significantly induced (Figure 4.9).

The effect of metabolic stress on CD34+ HSPCs expansion was assessed *in vitro* by incubating cells with different concentrations of glucose (10mM, 20mM, and 30mM). This was performed to mimic diabetes in which high blood glucose causes cellular stress and damage. Similar to the effect seen with high glucose impairment of cell count in EPCs in the previous chapter, high glucose levels also reduced CD34+ HSPCs growth significantly by fourfold compared to controls at one week of culture (Figure 4.10).

Therefore, I investigated whether HSPC cell-count decrease under hyperglycemia was

associated with changes in telomerase activity. As seen in Figure 4.11, metabolic stress did not influence telomerase activity in expanded over one week CD34+ HSPCs.

I also had tested the telomerase activator TA-65 in culture to investigate its effects on HSPC expansion. CD34+ HSPCs were incubated under different concentrations (1 μ M, 5 μ M, and 10 μ M). As seen in Figure 4.12, there was no change in CD34+ HSPCs cell count under the treatment with TA-65. To further assess the effects of TA-65 on CD34+ HSPC expansion in culture, telomerase activity was evaluated. TA-65 showed a threefold induction of telomerase at 5 μ M compared to control (0 μ M TA-65); however, this induction was not statistically significant. This might be due to the high variability of samples. Interestingly, a concentration of 10 μ M TA-65 significantly lowered telomerase activity (Figure 4.13).

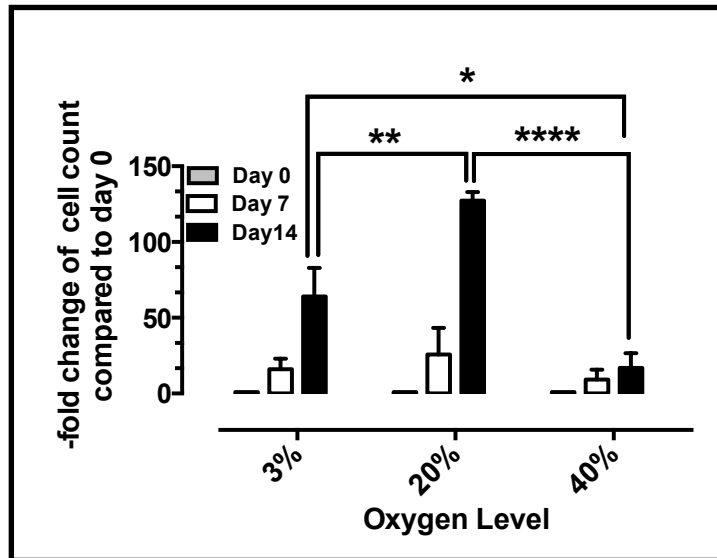


Figure 4.7: Impact of oxidative stress on CD34+ HSPC expansion.

CD34+ HSPCs were isolated from umbilical cord blood from Caesarean deliveries. Cells were expanded using a cocktail of cytokines for 14 days. Expansion of CD34+ HSPCs cells was performed under different oxygen levels (3%, 20%, and 40%) in independent samples (n=3). Cell count was performed with a haemocytometer under light microscopy. Statistical significance was calculated using Two-way ANOVA and Tukey's multiple test (*P<0.05, **P<0.01, and ****P<0.0001). Error bars stand for mean \pm SEM

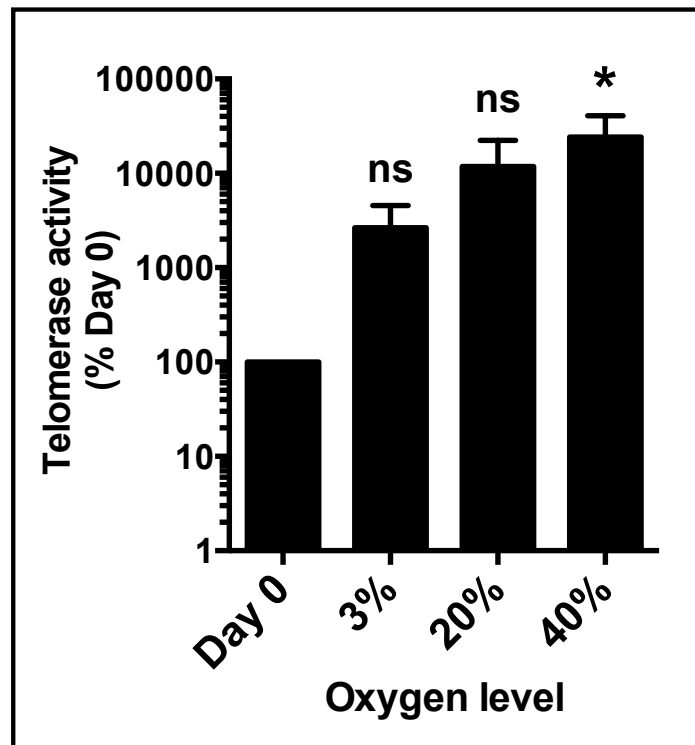


Figure 4.8: Effects of oxidative stress on telomerase activity on HSPC expansion.

CD34+ HSPCs were isolated from umbilical cord blood from Caesarean deliveries. Cells were expanded using a cocktail of cytokines under different oxygen levels in culture (3%, 20%, and 40%) for one week (n=3). Telomerase activity was measured using Q-TRAP and compared to the day 0 sample (control). Statistical significance was calculated using Kruskal-Wallis and Dunn's multiple comparison tests (*P<0.05). Error bars stand for mean ± SEM.

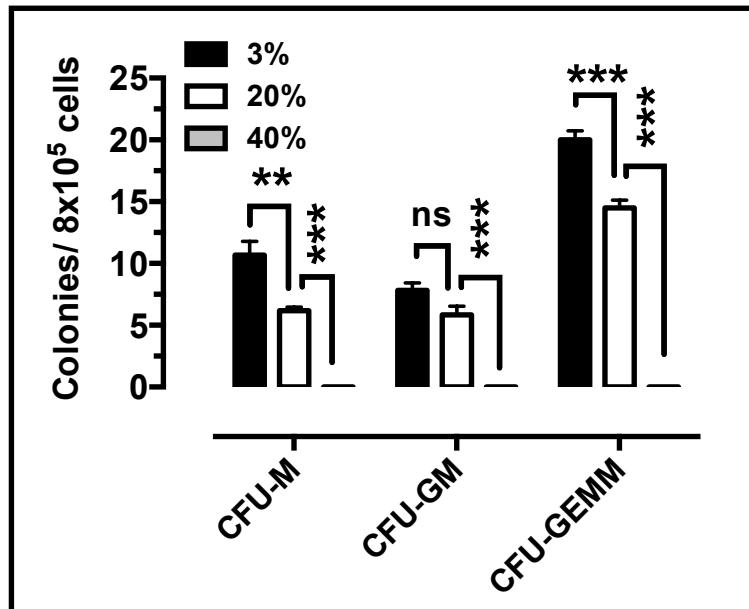


Figure 4.9: Impact of oxygen concentration on colony-forming potential of umbilical cord blood cells.

Fresh whole umbilical cord blood cells were isolated from Caesarean deliveries. Cells were grown in clonogenic assay under 3%, 20%, and 40% oxygen levels (n=3). Different colony types were scored under an inverted microscope after 14 days of culture. CFU = colony-forming units, -M= macrophage, -GM= granulocyte macrophage, -GEMM= granulocyte, erythroid, macrophage, and megakaryocyte. Statistical significance was calculated using Two-way ANOVA and Tukey tests (**P<0.01, ***P<0.001, ns=nonsignificant). Error bars stand for mean \pm SEM.

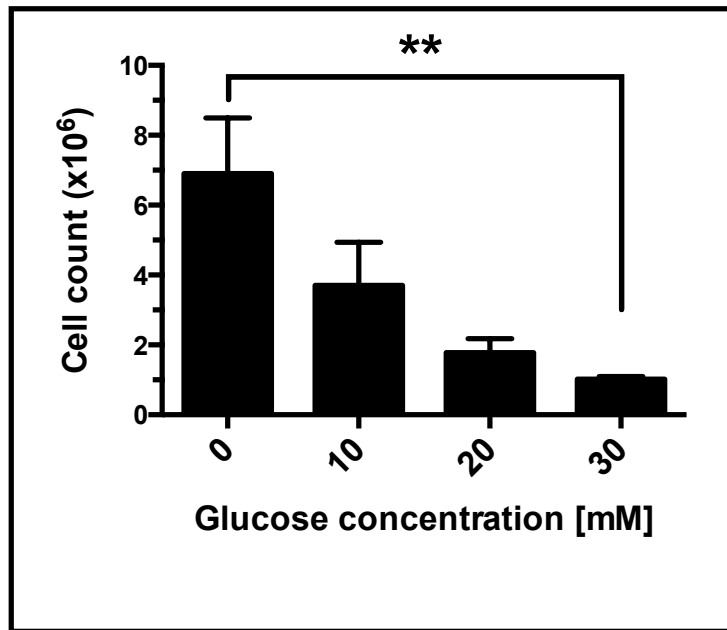


Figure 4.10: Effects of metabolic stress on HSPC growth in culture.

CD34+ HSPCs were isolated from umbilical cord blood from Caesarean deliveries. Cells were expanded using a cocktail of cytokines for one week with different doses of glucose (10mM, 20mM, and 30mM) (n=3). Cell count was performed with a haemocytometer under light microscopy. Statistical significance was calculated using Kruskal-Wallis and Dunn's multiple comparison tests compared to an untreated sample (**P<0.01). Error bars stand for mean \pm SEM.

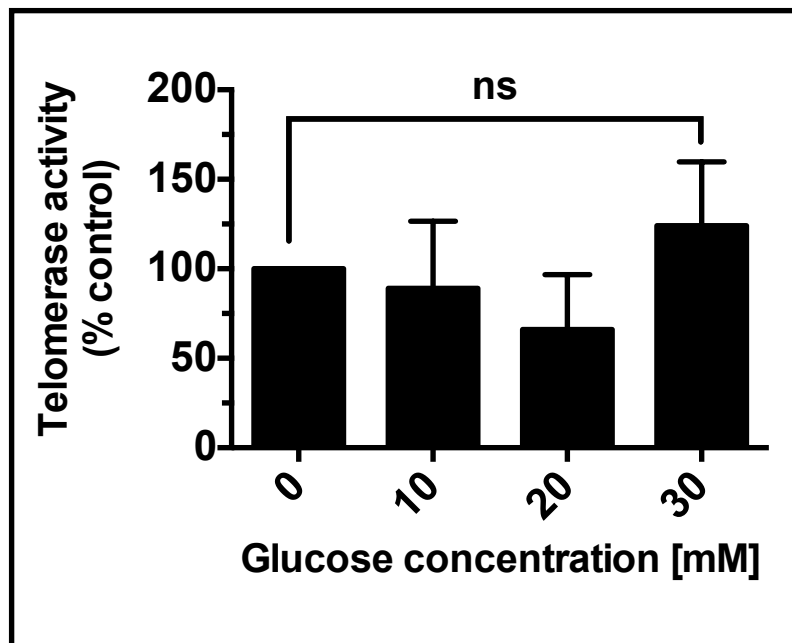


Figure 4.11: Impact of metabolic stress on telomerase activity in HSPCs.

CD34+ HSPCs were isolated from umbilical cord blood from Caesarean deliveries. Cells were expanded using a cocktail of cytokines. Different glucose concentrations were added in culture for one week (n=3). Telomerase activity was measured using Q-TRAP and compared to an untreated sample (control). Statistical significance was calculated using Kruskal-Wallis and Dunn's multiple comparison tests (ns= nonsignificant). Error bars stand for mean \pm SEM.

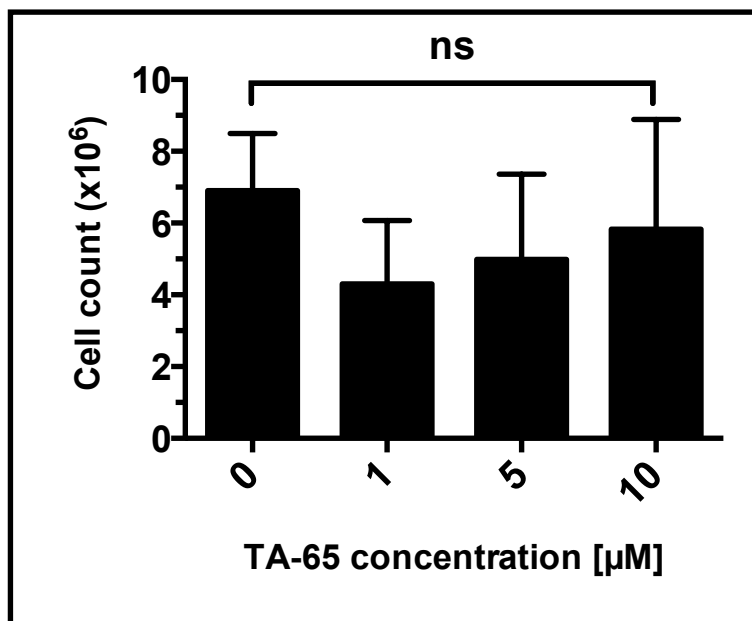


Figure 4.12: Effects of TA-65 on HSPC count in culture.

CD34+ HSPCs were isolated from umbilical cord blood from Caesarean deliveries. Cells were expanded using a cocktail of cytokines. Different doses of TA-65 (1 μM , 5 μM , and 10 μM) were added to HSPCs for one week in culture (n=3). Cell count was performed with a haemocytometer under light microscopy. Statistical significance was calculated by Kruskal-Wallis and Dunn's multiple comparison tests (ns= nonsignificant). Error bars stand for mean \pm SEM.

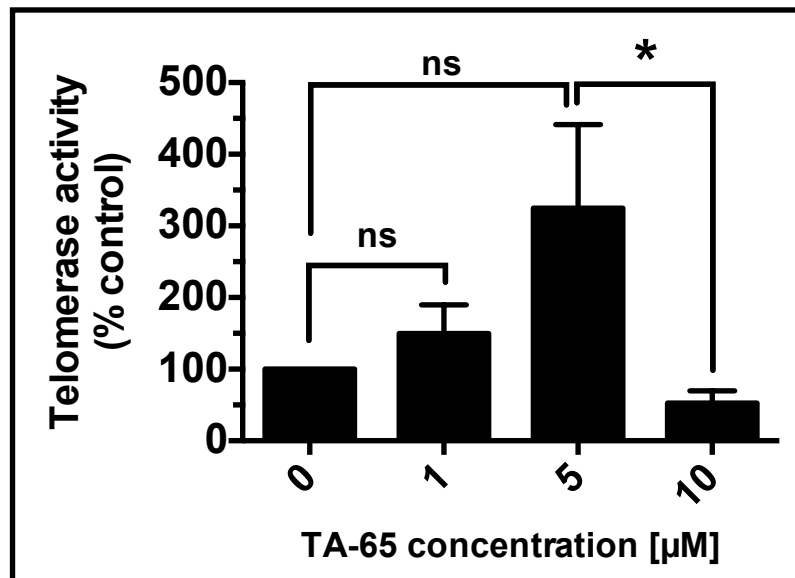


Figure 4.13: Effects of TA-65 on telomerase activity.

CD34+ HSPCs were isolated from umbilical cord blood from Caesarean deliveries and were expanded using a cocktail of cytokines. Different doses of TA-65 (1μM, 5μM, and 10μM) were added to expanded CD34+ HSPCs in culture for one week in culture (n=3). Telomerase activity was measured using Q-TRAP compared to the day 0 sample (control). Statistical significance was calculated by Kruskal-Wallis and Dunn's multiple comparison tests (*P<0.05). Error bars stand for mean ± SEM

4.2.3 Effect of oxidative stress on myeloid differentiation from CD34+ HSPCs

As I have shown in the previous section that oxidative stress impairs CD34+ HSPC expansion (Figure 4.7), I was interested to see what happens during myeloid differentiation of these cells. I chose myeloid cells, as they reflect the majority of leukocytes. In addition to leukocytes' role in innate immunity, telomere length in these cells, or LTL (leukocyte telomere length), has been widely used and suggested as a predictor marker for ageing and several diseases such as diabetes (J. Zhao *et al.*, 2014).

CD34+ HSPCs were isolated from UCB and differentiated into myeloid cells using specific cytokines (described in Chapter 2). As shown in Figure 4.14, more adherent cells, possibly macrophages, were seen under oxidative stress. The cell number was also significantly reduced by fourfold under high oxygen levels (40%) compared to low oxygen levels in culture (Figure 4.15). Furthermore, atorvastatin, a cholesterol-lowering drug, was tested according to its proven beneficial effects on endothelial progenitor cells as well as improvement of cardiovascular health (Baran *et al.*, 2012). However, as shown in Figure 4.15, there was no increase in myeloid cellularity by atorvastatin *in vitro*.

To confirm the differentiation of myeloid cells isolated from CD34+ umbilical cord blood, expression of the stem cell marker CD34, the granulocyte marker CD15, and the monocyte marker CD14 were measured by flow cytometry after one week of differentiation in culture. Furthermore, myeloid cells were incubated under different oxygen levels (3%, 20%, and 40%) to assess the role of oxidative stress on cellular composition. As seen in Figure 4.16, during one week of myeloid differentiation, the expression of granulocyte differentiation marker CD15 was increased from 7% to 41% under normoxic culture conditions (i.e., 20% O₂) compared to CD34+ HSPC expansion (See Figure 4.2, day 7 expression). Additionally, CD15+ granulocytes were significantly reduced by almost fourfold under oxidative stress (40% O₂) compared with hypoxic conditions. Whereas CD14+ monocytes were more resistant to oxidative stress with twofold increase compared with hypoxia (Figure 4.16). Therefore, granulocytes were very sensitive to oxidative stress while monocytes were more resistant during myeloid differentiation.

As CD34+ HSPCs showed induction of telomerase under oxidative stress in Figure 4.8, I was interested to test the effect of oxidative stress on telomerase activity during myeloid-

differentiated cells. In addition, atorvastatin was added to those cells to test whether it could induce telomerase activity *in vitro*. Unlike during CD34⁺ HSPC expansion (Figure 4.8), oxidative stress did not induce telomerase activity during myeloid differentiation. Telomerase activity was significantly upregulated during hypoxia and normoxia compared to day 0. However, when the comparison was performed with the right controls of the experiment (i.e., using hypoxic conditions as control), there was no difference across the groups. It is worth noting that the myeloid differentiation showed almost threefold higher telomerase activity compared to CD34⁺ HSPCs expansion (compare day 7 in Figure 4.3 with 20% in Figure 4.17). Moreover, atorvastatin induced more telomerase activity in myeloid cells only under normoxia (Figure 4.17).

To check if there were any transcriptional regulation coincidentally with telomerase activity in myeloid-differentiated cells under different oxygen levels, I also analysed the gene expression of TERT and TERC. The RNA expression of these genes was measured using real-time qPCR. In contrast to the unchanged TERT and TERC expression during CD34⁺ expansion in Figure 4.5, TERC expression was significantly reduced during myeloid differentiation under oxidative stress (Figure 4.18). Interestingly, this reduction was similar to CD15⁺ granulocyte reduction. Therefore, I investigated TERC gene expression for CD15⁺ and CD14⁺ following immunomagnetic separation of cells. Peripheral blood samples were taken from healthy volunteers, and mononuclear cells were collected and sorted into CD15⁺ and CD14⁺ with appropriate controls. As shown in Figure 4.19, TERC was significantly upregulated in CD15⁺ granulocytes. Therefore, the reduction in TERC gene expression during myeloid differentiation is most likely the consequence of decreasing granulocyte numbers during oxidative stress.

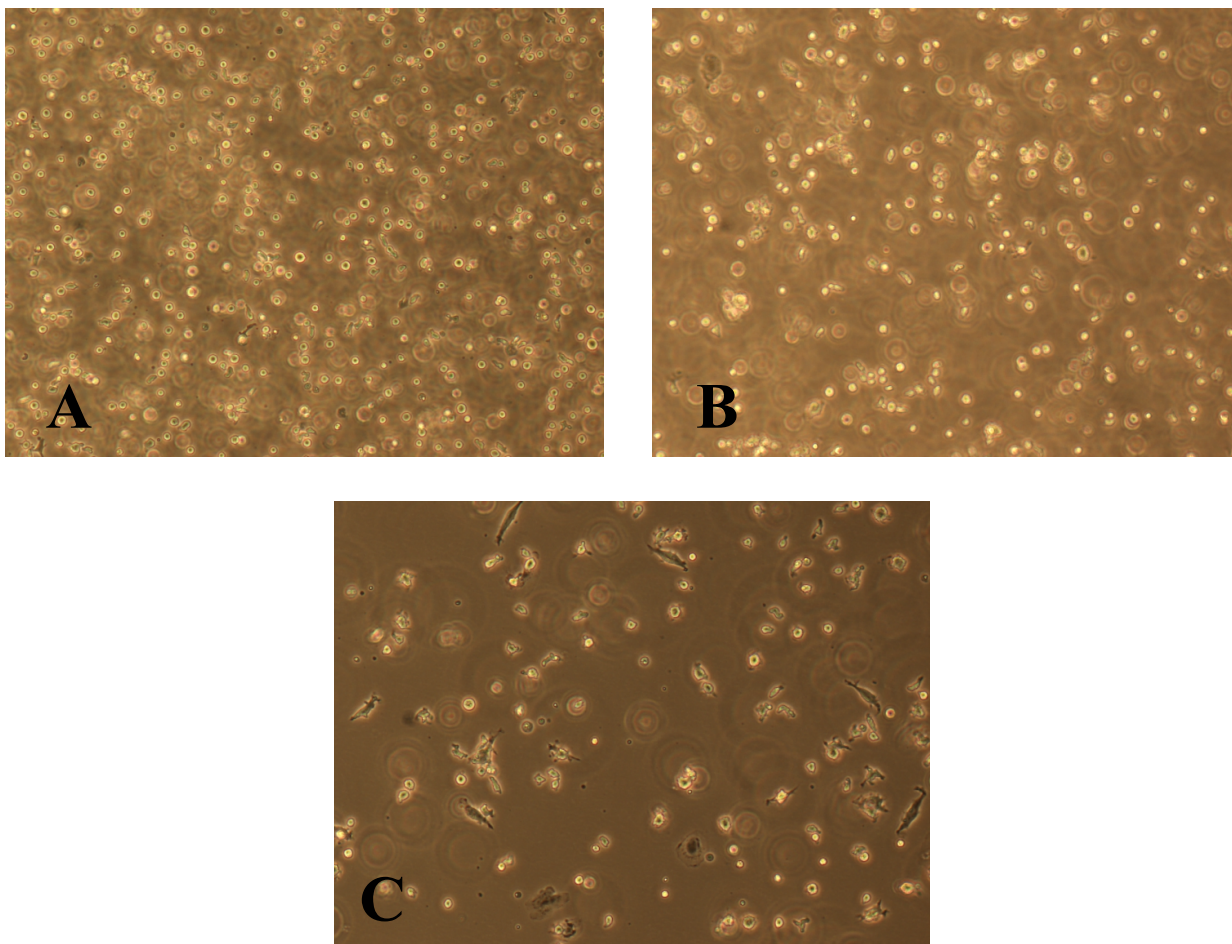


Figure 4.14: Morphology of cells under different oxygen levels.

CD34⁺ HSPCs were isolated from Caesarean deliveries' umbilical cord blood. Cells were differentiated in culture into myeloid cells using a cytokine mixture (SCF, FLT3-L, TPO, and G-CSF) for one week. **A**, **B**, and **C**: Myeloid differentiated cells under 3%, 20%, and 40% oxygen levels, respectively. Fewer but more adherent cells were seen during oxidative stress under light microscopy (10x magnification).

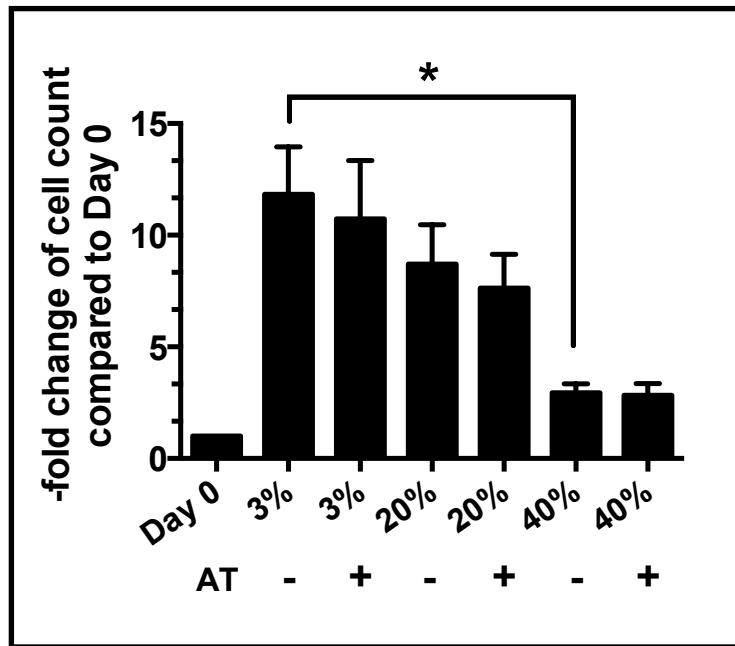


Figure 4.15: Effects of oxidative stress on myeloid differentiation.

CD34⁺ HSPCs were isolated from umbilical cord blood of Caesarean deliveries. Cells were differentiated in culture into myeloid cells using a cytokine mixture for one week (n=4). Myeloid cells were incubated under different oxygen levels (3%, 20%, and 40%). AT: 0.3 μ M atorvastatin was supplemented with media. Cell count was performed with a haemocytometer under light microscopy. Statistical significance was calculated by Kruskal-Wallis and Dunn's multiple comparison tests (*P<0.05). Error bars stand for mean \pm SEM.

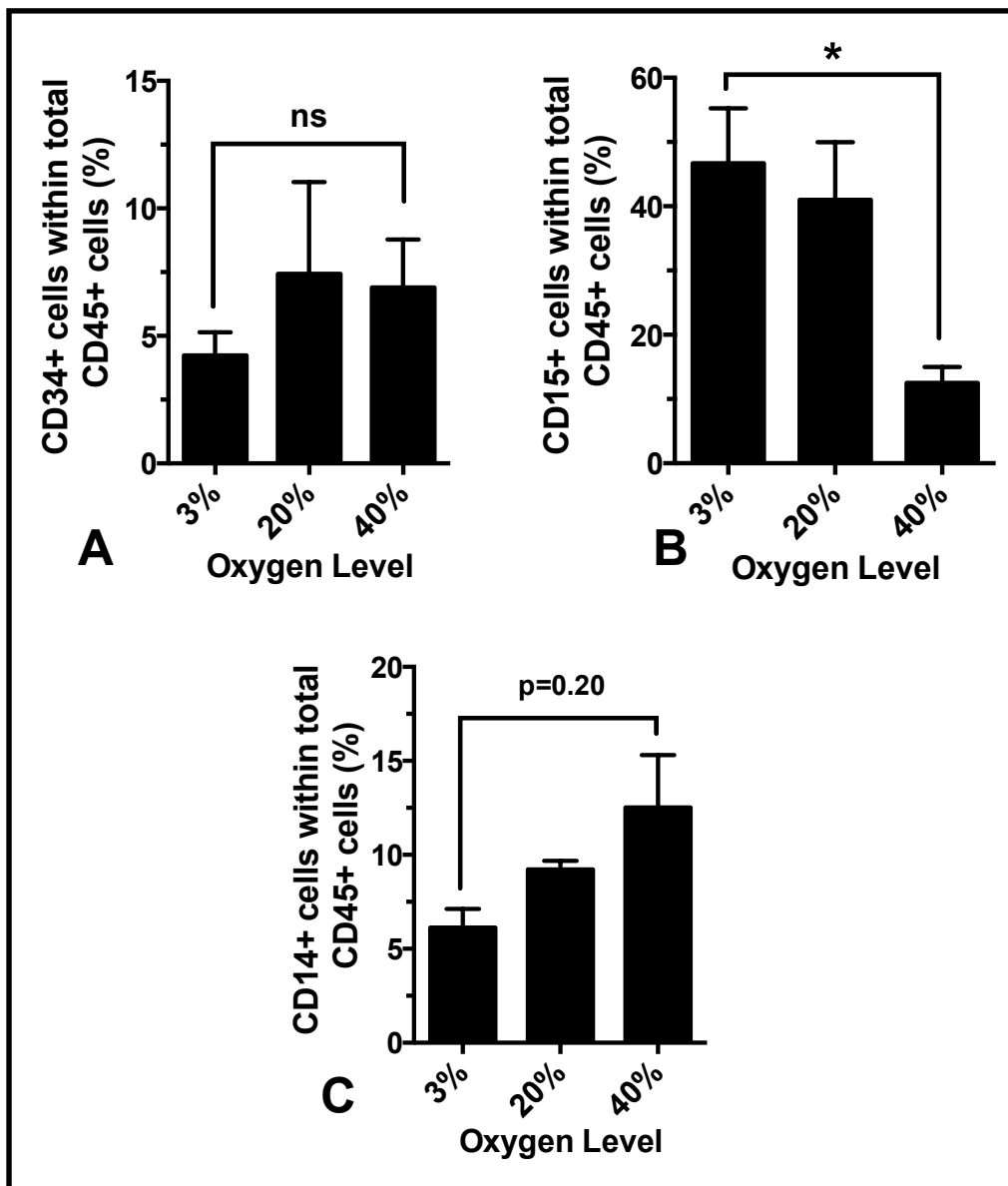


Figure 4.16: Impact of oxidative stress on the expression of CD34, CD15, and CD14 during myeloid differentiation.

CD34+ HSPCs were isolated from umbilical cord blood of Caesarean deliveries. Cells were differentiated in culture into myeloid cells using a cytokine mixture for one week. The expression of stem cell markers CD34 (n=6) (A), granulocyte marker CD15 (n=7) (B), and monocyte marker (n=4) (C) were measured using FACS analysis. Statistical significance was calculated by Kruskal-Wallis and Dunn's multiple comparison tests (*P<0.05 and ns= nonsignificant). Error bars stand for mean ± SEM.

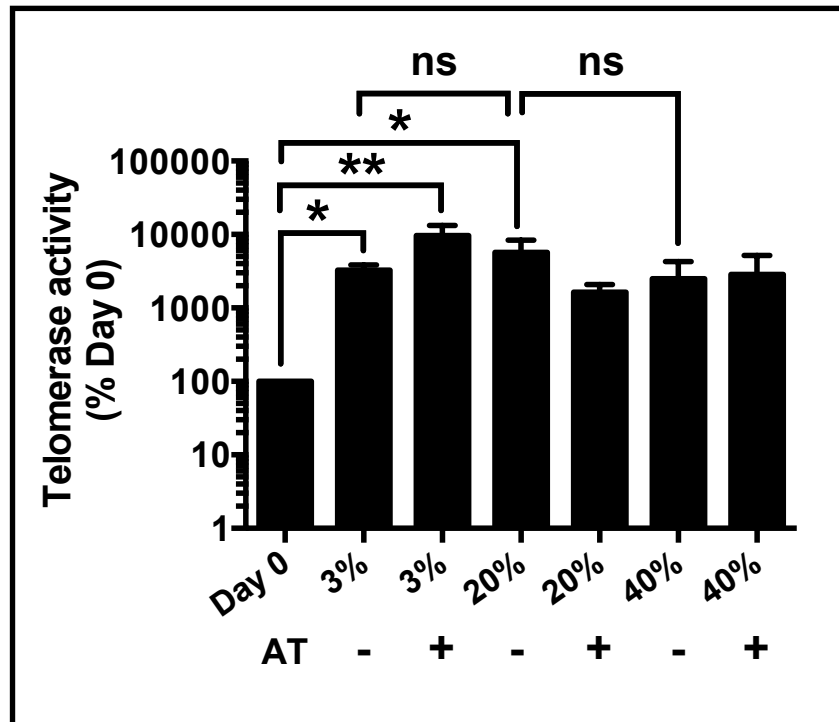


Figure 4.17: Effects of oxidative stress on telomerase activity in myeloid differentiation.

CD34⁺ HSPCs were isolated from umbilical cord blood and differentiated into myeloid cells using cytokines. Myeloid-differentiated cells were incubated under different oxygen levels (3%, 20%, and 40%) and supplemented with 0.3 μ M atorvastatin (AT) at each oxygen condition. Telomerase activity was measured using Q-TRAP normalized to day 0 samples (control) (n=4). Statistical significance was calculated using Kruskal-Wallis and Dunn's multiple comparison tests (*P<0.05, **P<0.01, and ns= nonsignificant). Error bars stand for mean \pm SEM.

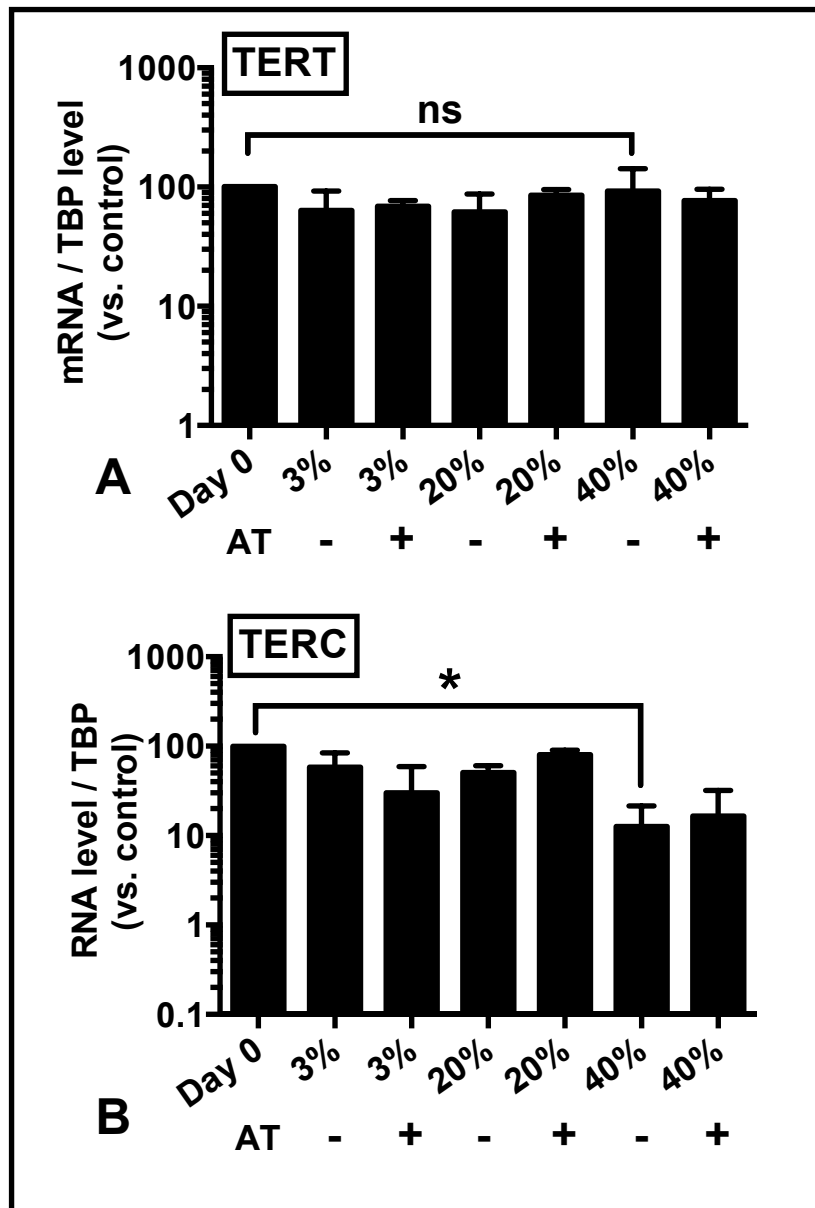


Figure 4.18: Impact of oxidative stress on TERT and TERC expression during myeloid differentiation.

CD34⁺ HSPCs were isolated from umbilical cord blood samples. Cells were differentiated into myeloid cells using cytokines. TERT mRNA (A) and TERC RNA (B) expression were measured using qRT-PCR after one week in culture. Expression was normalized to day 0 and referenced to housekeeping gene TBP (n=3). Statistical significance was calculated using Kruskal-Wallis and Dunn's multiple comparison tests (*P<0.05 and ns= nonsignificant). Error bars stand for mean ± SEM.

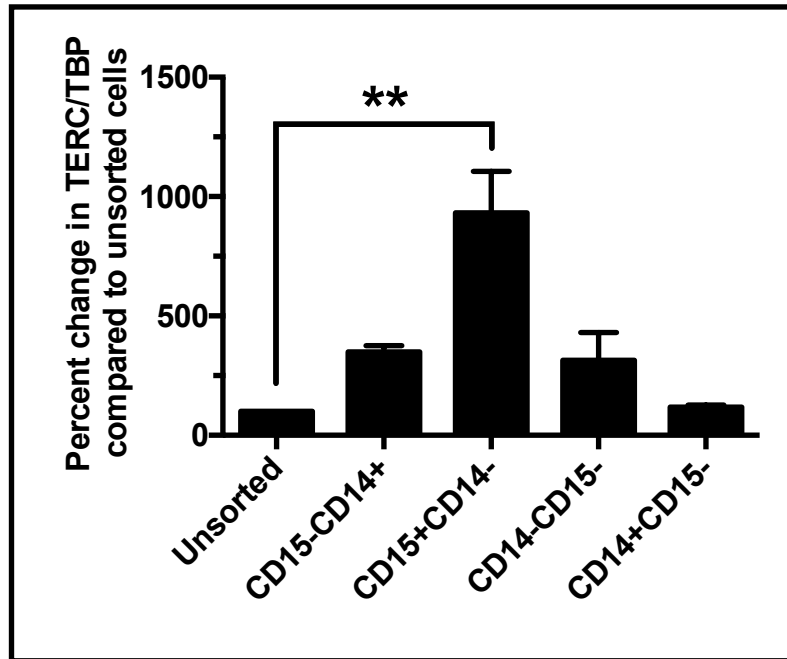


Figure 4.19: TERC expression in CD15+ granulocytes.

Immunomagnetic separation of CD14 and CD15 was performed from healthy volunteers' peripheral blood. TERC expression was measured using qRT-PCR, normalized to unsorted cells, and referenced to housekeeping gene TBP (n=3). Statistical significance was calculated using Kruskal-Wallis and Dunn's multiple comparison tests (**P<0.01). Error bars stand for mean \pm SEM.

4.3 Discussion

Ageing is associated with high levels of oxidatively damaged molecules (M. L. Hamilton *et al.*, 2001) and less-functional stem cells. Haematopoietic stem cells play a vital role in the homeostasis of the human body throughout life. Many studies have aimed to expand these cells *in vitro* to obtain sufficient cell numbers for therapeutic transplantation purposes. Furthermore, other studies focused on *ex-vivo* expansion and regulations of telomeres and telomerase (Gammaitoni *et al.*, 2004; Schuller *et al.*, 2007; Ge *et al.*, 2013). However, no current data examined the effects of oxidative stress and metabolic stress on telomerase in CD34⁺ UCB HSPCs during *ex-vivo* expansion and myeloid differentiation. Hence, I have used cytokines to expand CD34⁺ HSPCs isolated from umbilical cords under different oxygen levels (3%, 20%, and 40%), measured telomerase activity and telomere length, and analysed the transcription of TERT and TERC. Additionally, these measurements were also repeated under the same conditions but after myeloid differentiation of CD34⁺ HSPCs.

In my experiments, CD34⁺ HSPCs were expanded successfully in culture with cytokines, reaching 37-fold cell count after only two weeks. However, CD34⁺ expression was lost during culture and more differentiated cells appeared. Similarly, it has been shown that by nine days, CB CD34⁺CD38⁻ HSPCs differentiated, as they lost CD34 in culture and reduced the repopulating potential in SCID mice (Bhatia *et al.*, 1997). Telomerase activity was upregulated during CD34⁺ HSPCs expansion consistent with previous reports (Gammaitoni *et al.*, 2004). This induction was associated with the proliferation pattern, confirming the published relationship between telomerase activity and cell growth (S. Li *et al.*, 2005a).

4.3.1 Oxidative stress

In my experiments, oxidative stress induced by hyperoxia (40% O₂) did reduce CD34⁺ HSPC expansion and myeloid differentiation. Neither the telomerase activator TA-65, nor statins enhanced this impairment of cellularity. Interestingly, high oxygen levels completely inhibited the growth of colonies, whereas hypoxia induced their formation in clonogenic assays. My data suggest that hypoxia is a better *in-vitro* environment for maintaining colonies in comparison to normoxia and hyperoxia. This is in agreement

with favourable stem and progenitor growth under 1–6% oxygen levels in human bone marrow (Eliasson and Jonsson, 2010).

Oxidative stress is implicated in several diseases and can cause cellular damage. The reduction-oxidation (redox) modulation of signalling pathways is involved in the regulation of many metabolic processes. Although oxidative reactions are an essential part of several biological systems, they can be toxic depending on the balance between oxidative stimuli and defence response (Sies, 1997). The generation of ROS is dependent on the oxygen content surrounding the cells. Higher oxygen content generates more ROS and vice versa (Sauer *et al.*, 2001). Moreover, suitable ROS content acts as an intracellular second messenger, regulating adhesion, cell growth, transcription factor activation, and apoptosis (Kowaltowski *et al.*, 2009). Therefore, maintaining dynamic equilibrium under physiological circumstances by which the body produces and removes ROS continuously is crucial (Allen and Balin, 2003). It has been shown that the level of ROS in HSCs must be controlled to preserve the capacity for quiescence and cell renewal (Ito *et al.*, 2004). Although low levels of ROS are required for HSCs functioning, the underlying mechanisms that regulate and ameliorate oxidative stress in these cells remain unclear. However, it is widely known that ROS are mainly generated in the mitochondria of eukaryotic cells. Alterations in mitochondrial function increase ROS production leading to several oxidative stress-associated diseases and accelerated ageing (Kobayashi and Suda, 2012). Therefore, the number of mitochondria in HSCs is lower than in differentiated cells to reduce ROS generation (M. Kim *et al.*, 1998). Furthermore, the level of oxygen was found to influence muscle satellite cells (Csete *et al.*, 2001), neural precursor cells (Limoli *et al.*, 2004), and murine embryonic stem cells (Saretzki *et al.*, 2004).

The biological functions of HSCs probably change when exposed to a different environment with higher oxygen levels than the bone marrow, where HSCs reside. The bone marrow microenvironment is hypoxic with dissolved O₂ concentration varying from 0.1–4% according to radial position of the marrow (Chow *et al.*, 2001). Hypoxia has been shown to retain the long-term biological functions of HSCs, such as haematopoietic reconstruction, proliferation, self-renewal, and multi-differentiation (Naka *et al.*, 2008; Warren and Rossi, 2009). In culture, 5% hypoxia improved expansion of human cord

blood HSC (Roy *et al.*, 2012). Recently, 10% O₂ tension was found to be optimal for CD34⁺ CD90⁺ expansion of UCB stem/progenitor cells in co-culture with BM mesenchymal stem/stromal cells (MSC) (Andrade *et al.*, 2013). In fact, it has been shown that when BM lineage-negative cells hyperoxia (20% O₂), hypoxia (1% O₂) then hyperoxia (20% O₂) reoxygenation decreased stem cells, increased cell senescence, and caused cell-cycle arrest in culture. These experiments were performed in a mouse model of Fanconi's anaemia (Fanc^{c-/-}) (X. Zhang *et al.*, 2005).

In contrast, hyperoxia contributes to the pathogenesis of bronchopulmonary dysplasia (BPD) (R. J. Roberts *et al.*, 1983), abnormal vascular growth in the developing retina causing retinopathy (Gu *et al.*, 2003), and decreases growth of endothelial colony-forming cells (Fujinaga *et al.*, 2009). Higher oxygen partial pressure (21–40%) increased levels of intracellular peroxides (Lorenz *et al.*, 2001) and protein carbonyls (Starke-Reed and Oliver, 1989). Furthermore, hyperoxia decreased the replicative lifespan and increased the rate of telomere shortening four- to tenfold (von Zglinicki *et al.*, 1995). On the other hand, murine embryonic stem cells were found to be resistant to potential oxidative stress with increased levels of antioxidant defence and good DNA repair capacity (Saretzki *et al.*, 2004).

These studies confirm the importance of hypoxic conditions for HSPCs growth, which was supported by my data (induced colony formation under hypoxic conditions).

However, no current data have examined the role of hyperoxia on CD34⁺ HSPCs isolated from UCB. My results showed that hyperoxia could reduce cellularity and colony formation ability of HSPCs in culture, suggesting its toxic role.

4.3.2 Hyperglycemia and stem cell expansion

In this study, metabolic stress (30mM glucose) impaired CD34+ HSPC growth and myeloid differentiation in culture, suggesting a toxic effect in a dose relevant to the case seen in diabetes. Although the role of metabolic stress has not been directly examined in the context of haematopoiesis in the literature, several studies suggest that metabolic conditions can affect HSC homeostasis. An example is increased risk for acute myelogenous leukemia (AML) and acute lymphoblastic leukemia (ALL) in children exposed to high glucose levels in utero (Hjalgrim *et al.*, 2003; Hjalgrim *et al.*, 2004). Consequently, hyperglycemia is linked to higher mortality patients with AML (Ali *et al.*, 2007). Furthermore, ALL was found to be more frequent in children with diabetes (Feltbower *et al.*, 2004). In addition, cord blood from diabetic mothers displayed decreased neutrophil mobility (Mehta and Petrova, 2005) and platelet function (Strauss *et al.*, 2010). Telomerase is upregulated in mononuclear cells from UCB from mothers with type 1 diabetes (Cross *et al.*, 2010). Furthermore, mouse models of type 1 diabetes (induced by streptozotocin injections that destroy pancreatic islet cells inducing chronic hyperglycemia) has been shown to decrease BM Lin-Sca-1+C-Kit+ stem cells, lower engraftment and repopulation capacity, and downregulate growth factors IGF-1, insulin growth factor binding protein 5 (IGFBP-5), and proangiogenic cytokine VEGF compared to control mice (Orlandi *et al.*, 2010). It has been shown that the depletion of BM HSPCs (LSK cells) was associated with increased oxidative stress, DNA damage, and apoptosis in type 1 diabetic mice (Oikawa *et al.*, 2010). Likewise, in human bone marrow samples of type 2 diabetes, CD34 immunosorted BM progenitors have been shown to be reduced compared to controls. Furthermore, diabetes in those patients with upregulated apoptotic factor FOXO3a and downstream targets p21 and p27 and forced expression of mircoRNA-155 has been shown to reverse these transcriptional changes (Spinetti *et al.*, 2013). In a retrospective study of bone marrow transplant patient records, it has been reported that diabetes was correlated with poor mobilization of CD34+ HSPCs compared to non-diabetic patients. The authors also studied mouse models of diabetes type 1 (streptozotocin-induced) and type 2 (leptin receptor knockout db/db), which also showed impaired mobilization of HSPCs, increased LSK, increased adhesion, and decreased chemotaxis of HSPCs compared to controls. Interestingly, HSPC numbers and

mobilization were reverted to normal when transplanted into a non-diabetic host implying that the defects seen are due to a dysfunctional bone marrow niche (Ferraro *et al.*, 2011). On the contrary, a recent report confirmed that glucose exposure enhanced HSC formation in zebrafish via HIF-1 α in response to ROS sensing. Conversely, the dose used was only 1% in fish water (Harris *et al.*, 2013). Collectively, these data suggest that metabolic stress can affect HSC homeostasis.

4.3.3 Role of telomerase in expansion and differentiation

In this chapter, telomere length and telomerase activity were measured during ex vivo CD34⁺ HSPC expansion and differentiation. Telomerase, among other factors, is reported to play a critical role in the maintenance of telomere length that is important for both differentiation and self-renewal of haematopoietic stem cells (Gammaitoni *et al.*, 2004). It was also suggested that telomerase activity is directly linked to cell proliferation capacity (Sakabe *et al.*, 1998; S. Li *et al.*, 2005a). Unlike stem/progenitor cells, most adult somatic cells do not have telomerase activity and thus telomeres gradually shorten, limiting cell division (Harley *et al.*, 1990). Furthermore, the increasing interest in telomerase regulation is due to evidence that telomerase is activated in the majority of cancers, leading to unlimited growth of cancer cells (N. W. Kim *et al.*, 1994; M. W. Chang *et al.*, 2005).

From my findings, telomere length was maintained in culture whereas telomerase activity was upregulated in a similar way to the HSPC proliferation pattern seen with expansion and consistent with reported studies (Gammaitoni *et al.*, 2004; Schuller *et al.*, 2007; Ge *et al.*, 2013). Telomerase activity was upregulated with oxidative stress contradicting reported telomerase induction under hypoxia (5% O₂) in hTERT-transduced bone marrow endothelial cells (Napier *et al.*, 2010). However, telomerase activity was elevated in rats under physical stress (i.e., swim, fox urine exposure, water bottle removal, cage tilt, and weak tail pinch) (Beery *et al.*, 2012). Similarly, telomerase was also induced in lens epithelium of dogs after exposure to tertiary butyl-hydroperoxide (TBHP)-induced oxidative stress (Colitz *et al.*, 2004). These data suggest telomerase might play a protective role during oxidative stress.

Furthermore, more CD14⁺ monocytes were seen under oxidative stress in our experiments in comparison with CD15⁺ granulocytes, suggesting that monocytes are

resistant to oxidative stress in comparison to granulocytes. This is in agreement with a published report where fibroblasts damaged with oxidative stress from peroxide treatment showed a higher migration of CD14⁺ cells from PBMNCs (Geiger-Maor *et al.*, 2012).

Another report showed that CD14^{+low}CD16⁻ monocytes isolated from human PBMNCs have been showed to be more susceptible than CD14⁺CD16⁻ monocytes to undergo apoptosis, express higher proapoptotic genes and proteins such as cytochrome c and TNF α , and produce higher ROS, whereas CD14⁺CD16⁻ were more resistant to oxidative stress compared to CD14^{+low}CD16⁺ monocytes (C. Zhao *et al.*, 2010).

In addition, suppression of CD15⁺ granulocytes during oxidative stress was responsible for the downregulation in TERC gene expression during myeloid differentiation. Two main findings should be pointed out. First, it has been shown in hepatocellular carcinoma that accumulation of CD15⁺ cells correlate with the tumour progression and produced metalloproteinase-9 (MMP-9) that triggered tumour angiogenesis (Kuang *et al.*, 2011). Second, it has been reported that reducing the hTR mRNA by siRNA in oral squamous carcinoma Tca8113 cells successfully inhibited xenograft tumour growth in nude mice (Y. Li *et al.*, 2011). Therefore, these data suggest that the CD15⁺ and the associated TERC reduction might be a protective response to hyperoxia.

In conclusion, metabolic and oxidative stress influence HSPCs proliferation, clonogenic potential, and telomerase activity, suggesting a disrupted homeostasis in culture. To further assess the role of telomerase in HSPCs, TERT- and TERC-deficient mice were studied in the next chapter.

Chapter 5 Effects of telomerase on bone marrow HSPCs ageing

5.1 Introduction

Ageing is associated with a functional decline in tissues and stem cells. This dysfunction of stem cells has been shown to be due to intrinsic and extrinsic factors. The contribution of intrinsic factors to HSC ageing was seen in transplantation assays where HSCs from aged donors exhibited a significantly reduced function compared with young ones (Sudo *et al.*, 2000), while extrinsic factors were implicated in parabiosis experiments in which aged stem cells have been rejuvenated by exposure to a young systemic environment (Conboy *et al.*, 2005). However, there is no clear-cut distinction between these factors, and it is also possible that the cellular environment could lead to intrinsic stem cell alteration that may also persist even if the environment rejuvenated. Therefore, this interrelation can only be addressed by defining the underlying molecular processes such as DNA damage. During HSC ageing, an accumulation of DNA damage was observed (Rossi *et al.*, 2007) as well as telomere dysfunction (Choudhury *et al.*, 2007). Telomere shortening limits the proliferation capacity to a finite number of cell divisions (Allsopp *et al.*, 1992). Critically short telomeres induce DNA damage checkpoints, resulting in cell cycle arrest or apoptosis, and thus limiting cell viability (Rudolph *et al.*, 1999). To maintain telomeres, telomerase is required to enhance replicative capacity of regenerative cells such as stem and progenitor cells (Lee *et al.*, 1998). An important tool to understand telomere biology is the genetically engineered telomerase-deficient mouse model. Both telomerase knockout mice, $TERT^{-/-}$ and $TERC^{-/-}$, lack telomerase activity and are characterised by progressively shortening telomeres (Blasco *et al.*, 1997; Chiang *et al.*, 2004; Sahin *et al.*, 2011).

Although telomerase deficient models were studied extensively, mainly at later generations, to assess the role of telomere shortening (Blasco, 2003), no data are available with regard to normal bone marrow ageing in $TERT^{-/-}$ and $TERC^{-/-}$ first generation mice.

The aim of this chapter was to assess HSPC ageing. Haematopoietic stem and progenitor cells were isolated from $TERT^{-/-}$ and $TERC^{-/-}$ telomerase-deficient mice. Bone marrow

counts and HSPC content were assessed, and clonogenic assays were performed. HSPCs isolated from bone marrow were harvested and cultured in order to learn the effects of ageing on bone marrow HSPC cellularity, stem cell content, and colonies formation. The other aim of this chapter was to investigate if bone marrow ageing is mediated by telomerase or shortening of telomere length.

5.2 Results

5.2.1 Effects of ageing on bone marrow HSPCs

To confirm the impact of ageing on mouse HSPCs described previously (Dykstra *et al.*, 2011) in our system, freshly isolated bone marrow cells were counted and analysed for the expression of stem cell markers in young (1.5-7.5 months) and old (22 months) wild type mice on strain C57BL/6. As shown in Figure 5.1, there was no change in bone marrow cellularity during ageing. Furthermore, haematopoietic stem and progenitor cells (HSPCs) were assessed using flow cytometry characterisation of Lineage⁻Sca-1⁺CKit⁺ (LSK) in young and aged mice. LSK cells increased from 0.22±0.15% in young mice to 1.3±0.5% in old mice (p<0.01, Figure 5.2), similar to what is reported by Etrl and colleagues (2008).

Clonogenic assays are used clinically to assess the ability of various haematopoietic stem cells to divide and differentiate after ex-vivo manipulations (e.g., CD34⁺ cell enrichment, cryopreservation, gene therapy, and T-cell depletion) (Pereira *et al.*, 2007). Therefore, assessing colonies during ageing is a valuable tool that enables the comparison of HSC potential. During ageing, in vitro clonogenic assays revealed a significant increase in the CFU-GM progenitors of normal morphology (Figure 5.3) from 17.3 to 22.4 (p<0.01, Figure 5.4). Therefore, collectively, these data are consistent with disrupted haematopoiesis during ageing, manifesting as an increase in LSK cells and change in the clonogenic assay and possibly consistent with the myeloid skewing of haematopoiesis previously reported by others (Dykstra *et al.*, 2011).

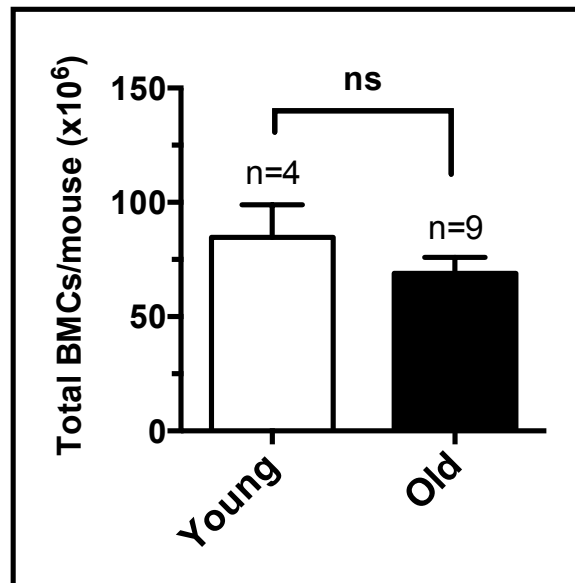


Figure 5.1: Bone marrow cellularity during ageing.

Bone marrow cell count from freshly isolated bones of wild type young (1.5-7.5 months) and old (22 months) C57BL/6 mice. Statistical significance calculated by Mann-Whitney test (ns= nonsignificant). Error bars stand for mean \pm SEM.

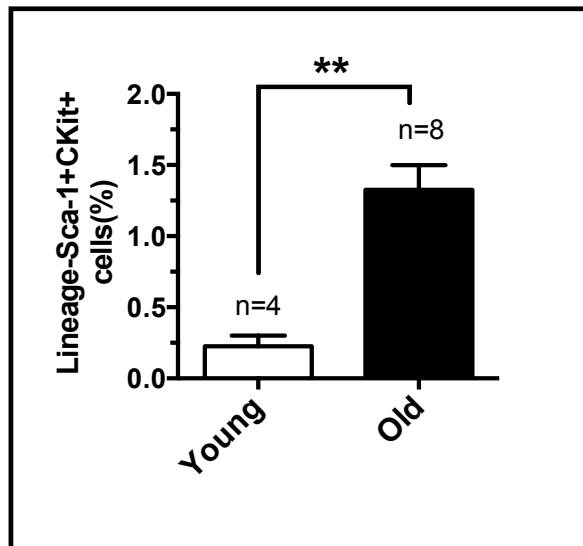


Figure 5.2: Stem cells content during ageing.

Lineage⁻Sca-1⁺CKit⁺ (LSK) stem cell content in freshly isolated bone marrow cells were measured by FACS analysis from young (1.5-7.5 months) and old (22 months) C57BL/6 mice. All p-values calculated by Mann-Whitney test (**P<0.01). Error bars stand for mean \pm SEM.

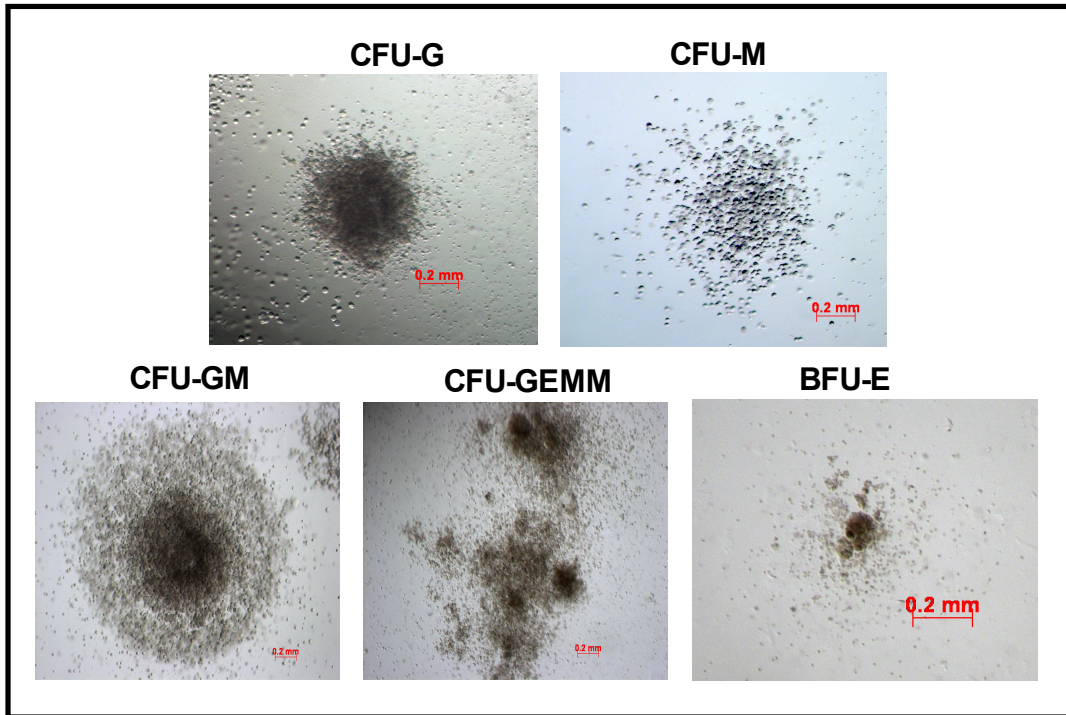


Figure 5.3: Morphology of different colonies in clonogenic assay.

Freshly isolated HSPCs were grown in clonogenic assays for 2 weeks. Using inverted microscopy, colony types were scored according to morphological appearance to CFU-G, CFU-M, CFU-GM, CFU-GEMM, and BFU-E. CFU=colony-forming unit, -G=granulocyte, -M=monocyte, -GM=granulocyte/monocyte, -GEMM=granulocyte, erythroid, megakaryocyte, monocyte, and red bar=0.2mm.

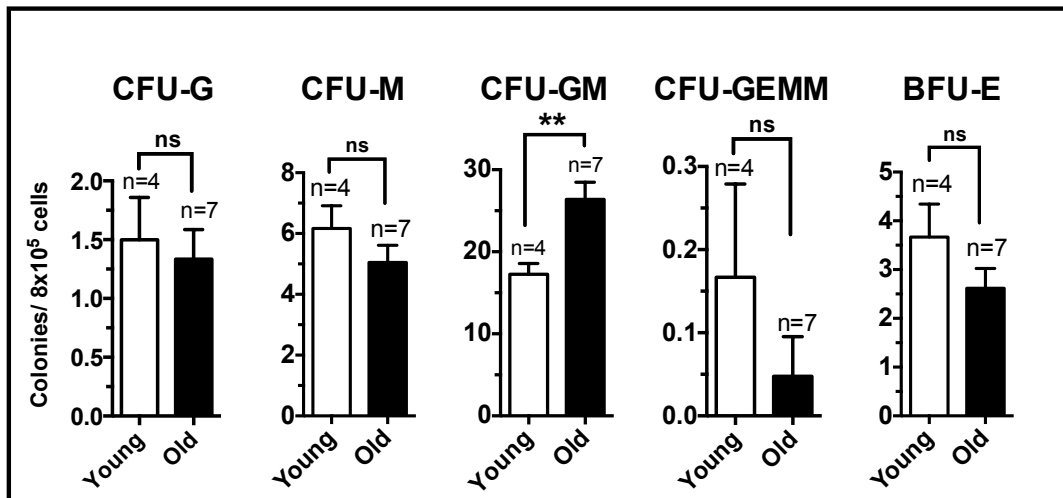


Figure 5.4: Clonogenic assay during ageing.

HSPCs were isolated from young (1.5-7.5 months) and old (22 months) C57BL/6 mice bone marrows. Clonogenic assays of bone marrow cells were scored after two weeks of culture. All p-values calculated by Mann-Whitney test (**P<0.01, ns=nonsignificant). Error bars stand for mean \pm SEM.

5.2.2 Impact of lack of TERT on HSPCs ageing

The impact of critically short telomeres on bone marrow functionality has been described previously using telomerase-negative late generation $TERC^{-/-}$ mice (Ju *et al.*, 2007). However, telomere-independent roles for the telomerase protein TERT have been described recently (Saretzki, 2009). Therefore, we were interested in whether the lack of the catalytic subunit TERT has a direct influence on the HSPC ageing.

As expected, splenocytes from $TERT^{-/-}$ mice did not possess any telomerase activity when compared to wild type cells (data not shown from our group). Flow-FISH analysis of bone marrow from young and old wild type mice as well as $TERT^{-/-}$ mice showed no significant change in telomere length (Figure 5.5). To identify a potential effect of telomerase deficiency on the phenotype of the bone marrow found in wild type aged mice, we compared young (1.5-7.5 month old) and old (22 month old) $TERT^{-/-}$ mice to their age-matched wild type littermates. Young and old $TERT^{-/-}$ bone marrow showed no evidence of accelerated ageing compared with wild type controls (Figure 5.6). Neither HSPC (LSK cells) frequency nor CFU-activity differed significantly between young $TERT^{-/-}$ and wild type groups (Figure 5.7 and Figure 5.8). Similarly in aged mice, TERT

deficiency did not augment the age-related increase in HSPCs found in wild type mice but did result in a significant increase in granulocyte-, monocyte-, and erythroid progenitor colonies (Figure 5.7 and Figure 5.8) consistent with aged $TERT^{-/-}$ bone marrow exhibiting a greater change in the clonogenic assay than wild type. Thus, $TERT$ deficiency augments the normal ageing process of HSPCs, implying that the lack of telomerase per se is not responsible for changes seen in HSPCs during normal ageing of wild type mice.

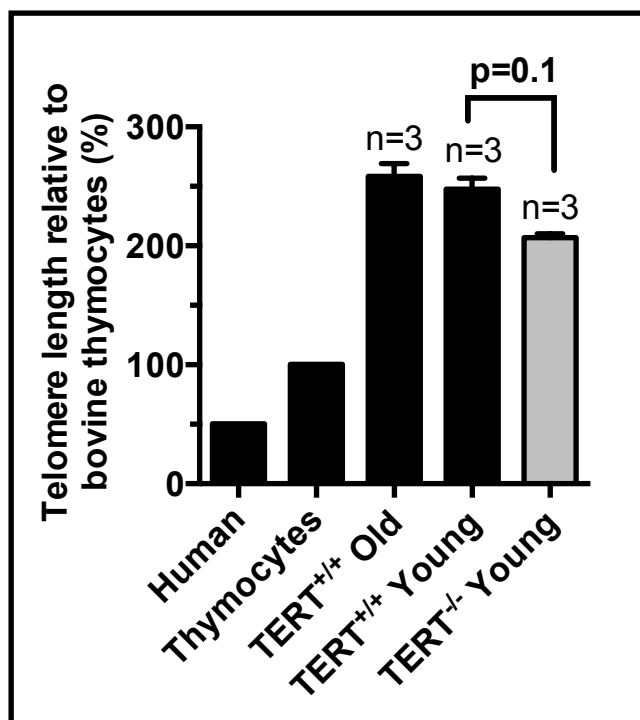


Figure 5.5: Telomere length in $TERT^{-/-}$ bone marrow HSPCs.

HSPCs were freshly isolated from mice bone marrow. Samples were cryopreserved until experiment. Telomere length was measured by flow-FISH of $TERT$ wild type and knockout C57BL/6 mice. Human=human PBMCs and Thymocytes=bovine thymocytes were used as controls. Statistical significance calculated by Mann-Whitney test. Error bars stand for mean \pm SEM.

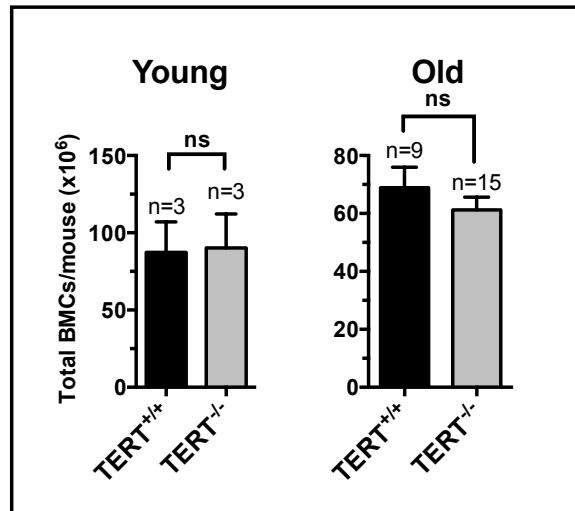


Figure 5.6: Bone marrow cellularity of TERT^{-/-} during ageing.

Total cell count of freshly isolated total bone marrow from young (1.5-7.5 months) and old (22 months) C57BL/6 mice. Statistical significance calculated by Mann-Whitney test (ns=non-significant). Error bars stand for mean \pm SEM.

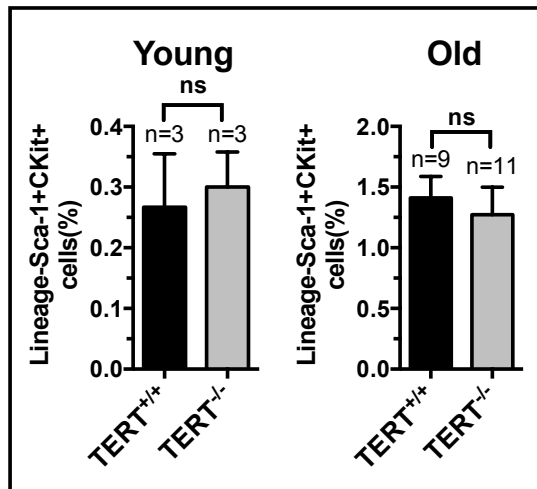


Figure 5.7: LSK stem cell content in TERT^{-/-} during ageing.

Lineage-Sca-1⁺CKit⁺ (LSK) stem cell measured by FACS analysis from freshly isolated bone marrow HSPCs from young (1.5-7.5 months) and old (22 months) C57BL/6 mice. Statistical significance calculated by Mann-Whitney test (ns=non-significant). Error bars stand for mean \pm SEM.

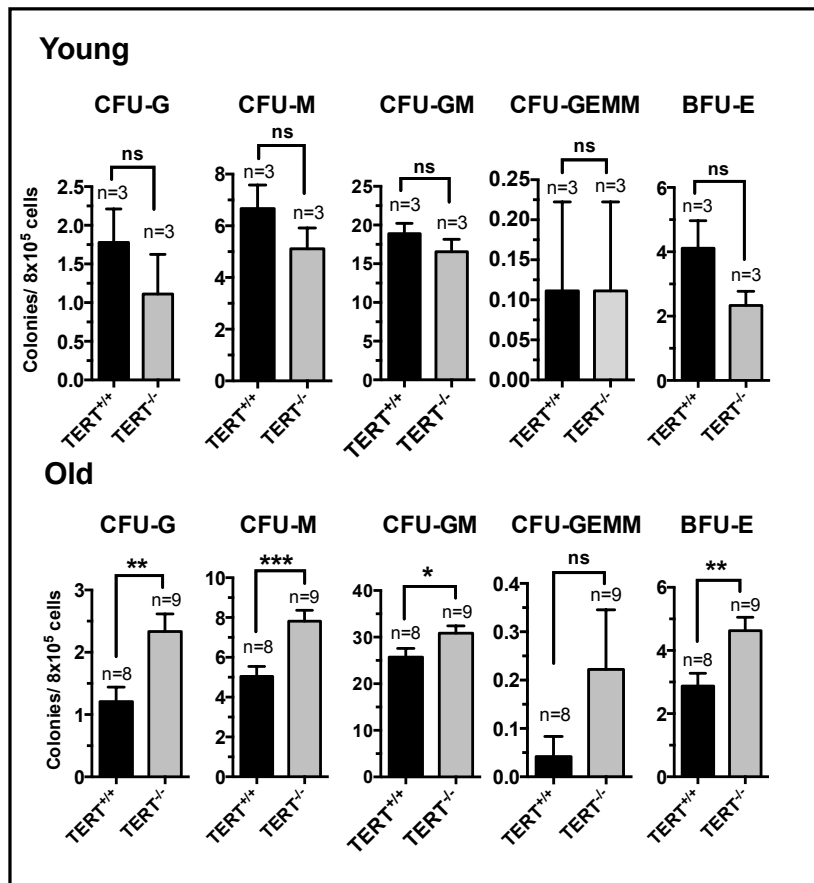


Figure 5.8: Clonogenic assay of TERT^{-/-} mice during ageing.

HSPCs isolated from young (1.5-7.5 months) and old (22 months) C57BL/6 mice were cultured in clonogenic assay for 14 days. Colonies were scored according to morphology under inverted microscopy. All p-values calculated by Mann-Whitney test (ns=non-significant, *P<0.05, **P<0.01, ***P<0.001). Error bars stand for mean ± SEM.

5.2.3 Effects of lack of TERC^{-/-} on bone marrow ageing

The absence of TERC abolishes telomerase activity and leads to shortened telomere: bone marrow haematopoietic stem cells (HSCs) from TERC^{-/-} mice display impaired replicative capacity, manifested as a reduction in serial bone marrow transplantation potential, and accompanied by rapid telomere shortening, compared with wild type mice (Allsopp *et al.*, 2003). In order to investigate the effect of shortened telomeres on HSPC ageing, we analysed LSK cells and CFU-potential in bone marrow from 8-12 month (young) and 15 month (old) old TERC^{-/-} mice. Flow-FISH analysis revealed a significant reduction in bone marrow HSPC telomere length in TERC^{-/-} mice compared with wild type controls $P < 0.05$ (Figure 5.9), unlike in TERT^{-/-} mice (compare Figure 5.5 and Figure 5.9). This telomere shortening is not reported elsewhere in the first generation of TERC^{-/-} mice because they normally are backcrossed with heterozygous TERC^{+/-} mice with intact telomeres. However, this was not performed with our mouse model, leading to shorter telomeres in the first generation of mice already.

Young TERC^{-/-} mice showed a significant decrease in bone marrow cellularity compared to old TERC^{-/-} mice (Figure 5.10). We also observed a significant induction of LSK stem cells (Figure 5.11) comparable to that seen during ageing (Figure 5.2) and significantly higher CFU-G, CFU-M, CFU-GM, and BFU-E formation in young and old TERC^{-/-} mice compared to wild types in vitro (Figure 5.12). Therefore, it seems that short telomeres, rather than telomerase deficiency itself, results in an ageing bone marrow profile. However, older TERC^{-/-} mice show a slightly different phenotype with no clear increase in LSK stem cell frequency whilst retaining a myeloid-skewed colony formation potential.

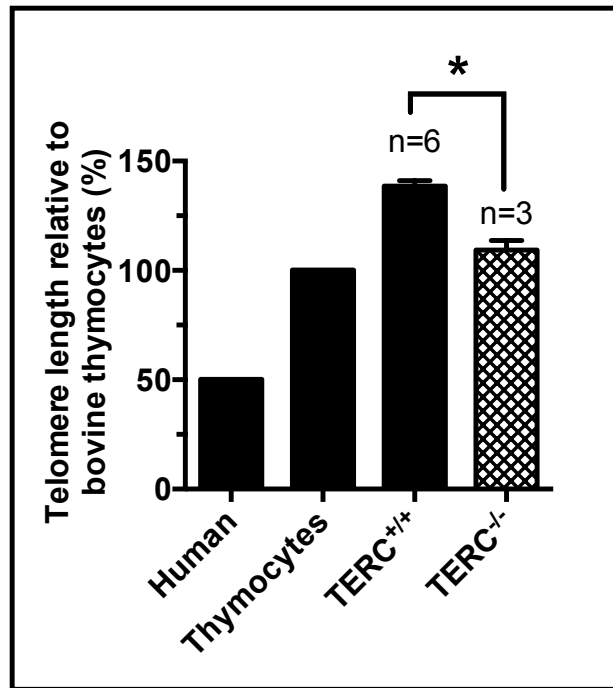


Figure 5.9: Telomere length in TERC^{-/-} bone marrow HSPCs.

HSPCs were freshly isolated from mice bone marrow. Samples were cryopreserved until the experiment. Telomere length was measured by flow-FISH of TERC wild type and knockout C57BL/6 mice. Human=human PBMNCs and Thymocytes=bovine thymocytes were used as controls. Statistical significance calculated by Mann-Whitney test (*P<0.05). Error bars stand for mean \pm SEM.

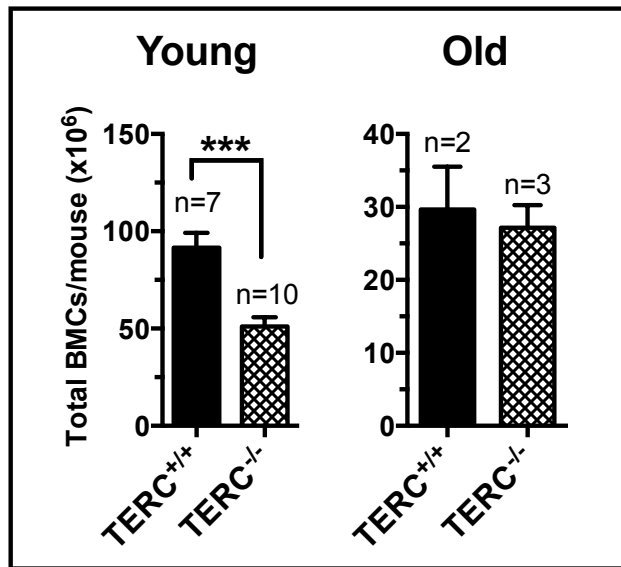


Figure 5.10: Bone marrow cellularity of TERC^{-/-} mice.

Total bone marrow cell count from freshly isolated bones from young (8-12 months) and old (15 months) C57BL/6 mice. Statistical significance calculated by Mann-Whitney test (***)P<0.001 and ns=nonsignificant). Error bars stand for mean ± SEM except n=2 which represents the range.

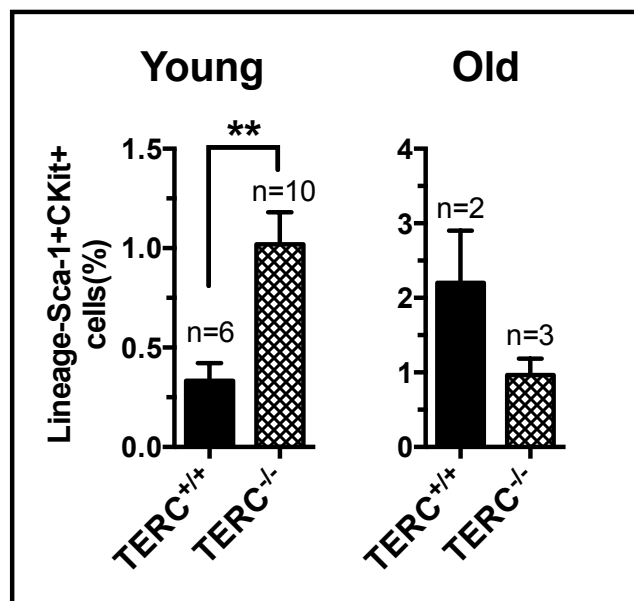


Figure 5.11: LSK stem cell content in TERC^{-/-} mice.

Lineage-Sca-1⁺CKit⁺ (LSK) stem cell measured by FACS analysis from freshly isolated bone marrow HSPCs from young (8-12 months) and old (15 months) C57BL/6 mice. Statistical significance calculated by Mann-Whitney test (ns=nonsignificant). Error bars stand for mean ± SEM except n=2 which represents the range.

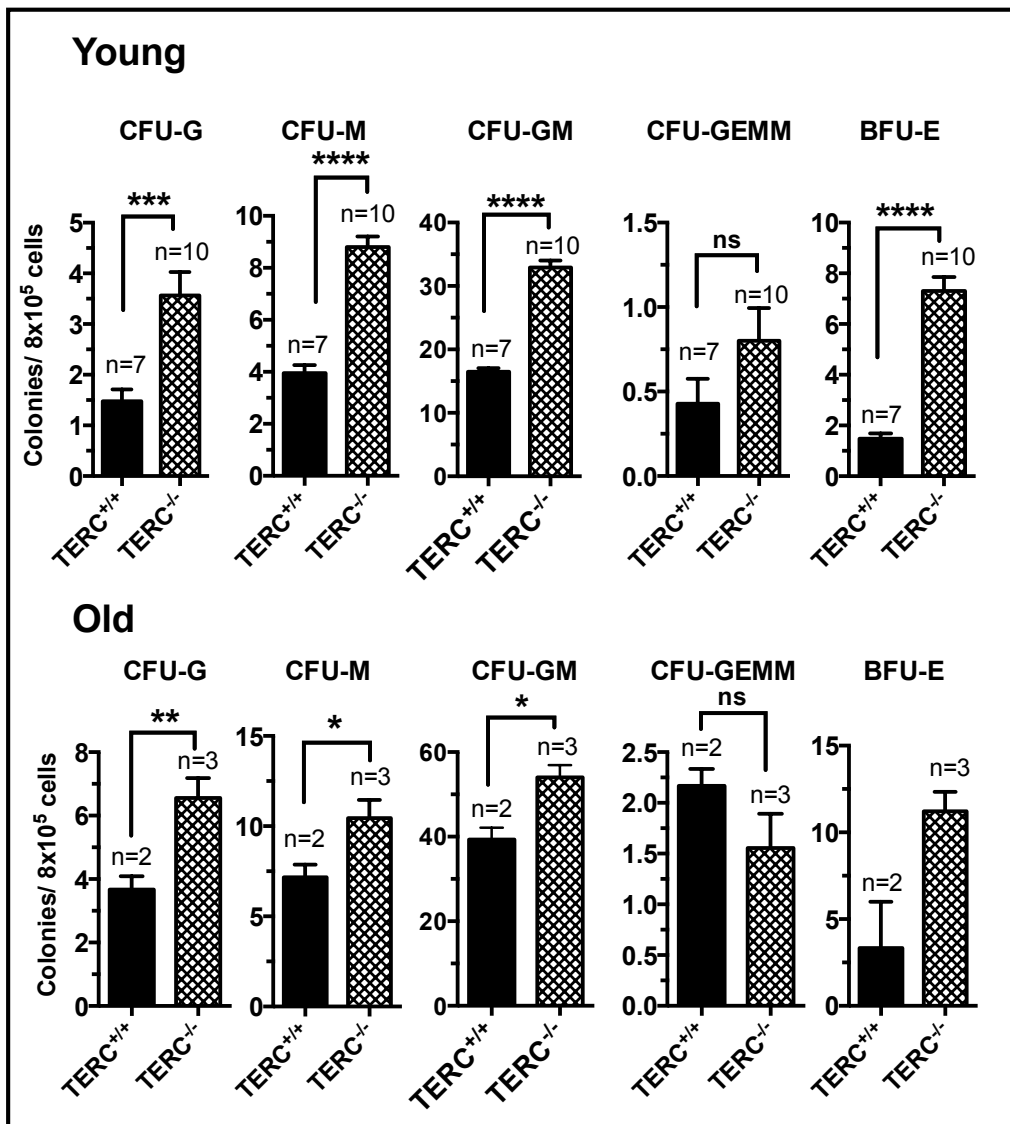


Figure 5.12: Clonogenic assay of TERC^{-/-} mice.

HSPCs were isolated from young (8-12 months) and old (15 months) C57BL/6 mice and cultured in a clonogenic assay for 14 days. Colonies were scored according to morphology under inverted microscopy. All p-values calculated by Mann-Whitney test (ns=nonsignificant, *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001). Error bars stand for mean \pm SEM except n=2 which represents the range.

5.3 Discussion

Ageing is a complex process that involves dysfunction in haematopoietic stem cells due to cell intrinsic and extrinsic alterations, which ultimately causes compromise in different mature blood lineages. This dysfunction leads to several haematological diseases preferentially found in older age including anemia, myelodysplastic syndrome, and myeloid leukemia (Franceschi *et al.*, 2000). Tremendous research is directed towards deciphering ageing mechanisms to combat age-related diseases and to extend healthy lifespans. Mutations in telomerase genes limit telomere maintenance and lead to shorter lifespans in mice and humans (Herrera *et al.*, 1999; Rudolph *et al.*, 1999; Vulliamy *et al.*, 2004). However, little is known about the link between bone marrow ageing and telomerase. In this study we analysed the ageing haematopoietic system *in vivo*, without bone marrow transplantation, employing fluorescence antibody cell sorting (FACS) and colony-forming assays to measure haematopoietic stem and progenitor cells (HSPCs) from wild type and telomerase deficient mice up to 22 months of age.

In our models, ageing increased Lineage⁻Sca-1⁺CKit⁺ HSPCs in the bone marrow of TERT^{+/+} wild types. This induction is accompanied by more myeloid progenitors (CFU-GM), which might be consistent with myeloid skewing with ageing as reported by others in humans and mice (Waterstrat and Van Zant, 2009; Dykstra *et al.*, 2011; Pang *et al.*, 2011). In humans, CFU-GM grown from bone marrow also increased with age (Marley *et al.*, 1999). However, the influence of ageing on HSCs numbers from murine models has been reported to be strain-specific (J. Chen *et al.*, 2000). Furthermore, the number of CD34⁺CD8⁻ progenitors increased with age in humans, with no change in the repopulating activity with colonies in humans (Kuranda *et al.*, 2011), whereas another study showed no difference between young and elderly HSC in particular myeloid progenitors in mononucleated cells (Chatta *et al.*, 1993). Conversely, elderly adults displayed less mixed granulocyte/macrophage colonies compared to young (Marley *et al.*, 1999).

Telomeres are specialized end caps of chromosomes that protect and maintain chromosomal integrity and prevent DNA damage responses (Blackburn, 2001; de Lange, 2002). Telomeres shorten as age progresses in humans, limiting the proliferative capacity of a cell to a certain number of cell divisions (Allsopp *et al.*, 1992). Critically short

telomeres lose the capping function and induce DNA damage checkpoints leading to apoptosis or cell cycle arrest. Although mice have very long telomeres, they also shorten in stem and somatic cell compartments and accumulate telomere-associated DNA damage when sensitive methods are applied (Flores *et al.*, 2008; Hewitt *et al.*, 2012). The organ systems most sensitive to telomere shortening are those with high cell turnover rates, supporting the finding that telomere dysfunction impairs stem cell maintenance (Lee *et al.*, 1998; Rudolph *et al.*, 1999; Kirwan and Dokal, 2008; Sahin *et al.*, 2011). Furthermore, various groups have shown recently that telomere length also influences mitochondrial function and thus contributes to metabolic changes (Passos *et al.*, 2010; Sahin *et al.*, 2011).

Telomerase is a DNA reverse transcriptase consisting of an RNA template (TERC) and catalytic subunit (TERT) that replicates telomeres. Although telomerase is activated in some adult stem cells, its expression in stem cells is not sufficient to prevent telomere shortening during ageing (Flores and Blasco, 2010). The TERC^{-/-} mouse model at later generations has been reported to have less haematopoietic stem cell repopulating potential and a dysfunctional microenvironment (Allsopp *et al.*, 2003; Ju *et al.*, 2007). In agreement with these studies, our data confirm that telomere shortening in TERC^{-/-} telomerase deficient mice lead to an ageing bone marrow phenotype, reflected by a fivefold increase in LSK cells as well as CFU-GM colonies. In comparison, telomerase-deficient TERT^{-/-} mice without telomere shortening showed no evidence of premature hematopoietic stem cell ageing at the level of LSK cell frequency (Rossi *et al.*, 2007). However, lack of telomerase did lead to a significant increase in granulocyte, monocyte, and erythroid colonies, augmenting the normal ageing process and providing the first example of a specific erythroid progenitors increase associated with ageing—notably, that it can be ameliorated by DR. Of note, erythroid progenitor (BFU-E) increase was found only in telomerase-deficient mice, suggesting a telomerase dependent disruption of normal bone marrow homeostasis. A possible explanation of the erythroid colonies induction seen in our TERT^{-/-} mouse models is anaemia, which has been reported as an independent predictor of mortality and morbidity in ageing (Guralnik *et al.*, 2005). The induction seen in erythroid progenitor colonies in aged mice might be a good example of how the bone marrow responds to anaemic shortage of erythrocytes as a compensation

mechanism. Whilst erythropoiesis has yet to be studied extensively in ageing mice, a recent study showed aged, anaemic mice to have a significant increase in splenic CD71^{hi}Ter119^{hi} progenitors with a similar trend in bone marrow (Guo *et al.*, 2013). These findings are of particular importance, given the clinical burden of age-related anaemia. The only other case to our knowledge of a marked erythroid induction of progenitor populations has been demonstrated in a disease model of myeloproliferative neoplasm (Mullally *et al.*, 2010). Others have described a certain function of the tumour suppressor gene p15Ink4b in the commitment of blood progenitors to the erythroid lineage (Humenuik *et al.*, 2013). Interestingly, p15INK4b and telomerase/hTERT have been found in a complex recently where hTERT inhibits translation of p15 mRNA (Iannilli *et al.*, 2013). One could speculate that without TERT p15 expression stays high and promotes erythroid differentiation of myeloid progenitors. In conclusion, telomerase deficiency without telomere attrition leads to HSPCs ageing in the bone marrow, which is exacerbated with short telomeres.

Chapter 6 The impact of anti-ageing interventions on HSPCs

6.1 Introduction

Ageing is a progressive process of functional decline of various tissues that cannot be reversed. However, current research identified some interventions that can ameliorate such as dietary restriction and TA-65 supplementation. Dietary restriction (DR) is a well-known anti-ageing intervention that increases lifespan as well as health in different organisms and decreases the incidence of cancer during ageing (Weindruch *et al.*, 1988; Bronson and Lipman, 1991; Swindell, 2012). DR also delays some age-related diseases such as diabetes, atherosclerosis, kidney disease, and neurodegenerative diseases (Mattison *et al.*, 2012). However, some studies reported contradicting influences of DR on lifespan depending on the genotype of mice (Liao *et al.*, 2010). The exact mechanism of dietary restriction is still not clear; nevertheless, theories are pointing towards protection against cellular damage by reducing cellular metabolism (Bordone and Guarente, 2005). Although the beneficial effect of DR on hematopoietic stem-cell ageing has been demonstrated previously (Ertl *et al.*, 2008), little is known about its mechanism, particularly with respect to telomeres. Since telomere shortening is associated with ageing and accelerated in human diseases with telomerase mutations such as dyskeratosis congenita and aplastic anaemia (Garcia *et al.*, 2007), telomerase activation might be a potential anti-ageing target. In fact, experimental activation of telomerase using TA-65 in mice has been shown to rescue telomere length in mouse embryonic fibroblasts and enhance their health span (Bernardes de Jesus *et al.*, 2011).

However, data are lacking with regard to the effects of DR and TA-65 on HSPCs ageing in telomerase-deficient mouse models.

In this chapter, haematopoietic stem and progenitor cells (HSPCs) isolated from the bone marrow of different telomerase knockout mice were investigated. Anti-ageing dietary restriction and the herbal extract telomerase activator TA-65 were also tested in HSPCs in vivo and in vitro, respectively. Parameters of bone marrow ageing studied included bone marrow cell counts, HSPCs content, and clonogenic assays. HSPCs isolated from bone marrow were harvested and cultured to find out if the telomerase activator TA-65 can ameliorate ageing of bone marrow stem cells in vivo and in vitro. The second aim of

this chapter was to investigate if dietary restriction can ameliorate ageing in a telomerase-dependent manner.

6.2 Results

6.2.1 Effects of TA-65 on $TERC^{-/-}$ and $TERT^{+/+}$ bone marrow ageing

TA-65 is a herbal telomerase activator that is commercially available and is reported to ameliorate the amount of short telomeres, and decrease the percentage of critically short telomeres and DNA damage in mouse fibroblasts that harbour critically short telomeres (Fauce *et al.*, 2008; Bernardes de Jesus *et al.*, 2011; Harley *et al.*, 2011). Therefore, we tested in vivo the treatment of different telomerase knockout mice to see if TA-65 shows any ageing amelioration in the bone marrow. In vitro treatment of 12-month-old $TERC^{+/-}$ and $TERC^{-/-}$ mice HSPC cells with TA-65 did not affect bone marrow cellularity (Figure 6.1), stem cells (Figure 6.2), or colonies in culture (Figure 6.3). On the contrary, TA-65 treatment of 28-month-old $TERT^{+/+}$ mice showed a tendency to induce bone marrow cellularity by 1.7-fold (Figure 6.4), whereas no change was seen in HSPC content (Figure 6.5) or colonies (Figure 6.6). In addition, TA-65 showed the tendency to increase TERT transcription by 2.5-fold, similar to what has been reported by de Jesus and colleagues in other cell types (2011). We also found a trend of telomerase activity induction (fivefold) with TA-65 in 28-month-old $TERT^{+/+}$ mice bone marrow compared to controls (Figure 6.7). In contrast, old $TERT^{+/+}$ mice treatment showed significantly reduced transcription of TERC and no change in the proliferation marker c-myc, as shown in Figure 6.8. Furthermore, when colonies were incubated under different oxygen levels to assess the effect of oxidative stress, there was a significant induction of CFU-GM colonies with TA-65 under 3% oxygen level, while there was a trend of increased CFU-GM colonies with TA-65 under oxidative stress (Figure 6.9). To examine the effect of oxidative stress on colony formation, data were combined from TA-65 treated and non-treated mice and oxygen levels were compared. As shown in Figure 6.10, oxidative stress significantly reduced CFU-G, CFU-M, and CFU-GM colonies compared to the 3% oxygen level. Moreover, in an attempt to track cells lacking TERT in clonogenic assay, TERT-GFP tagged bone marrow cells were isolated and sorted using FACS (FACSAria II [BD,

USA] from mice described by Breault *et al.* (2008). Unfortunately, sorted cells were unable to produce colonies in culture, and the reason might have been the loss of cellular properties as unsorted cells grew colonies as normal.

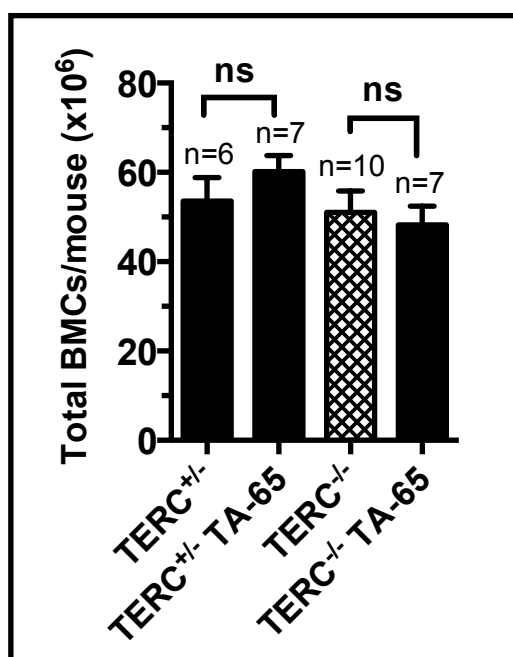


Figure 6.1: Effects of TA-65 treatment in TERC^{+/-} and TERC^{-/-} mice bone marrow cellularity.

8-12-month-old C57BL/6 mice were treated with either TA-65 or control for 3-4 months. Bone marrow cells were isolated and quantified. Statistical significance calculated by Mann-Whitney test. Error bars stand for mean \pm SEM.

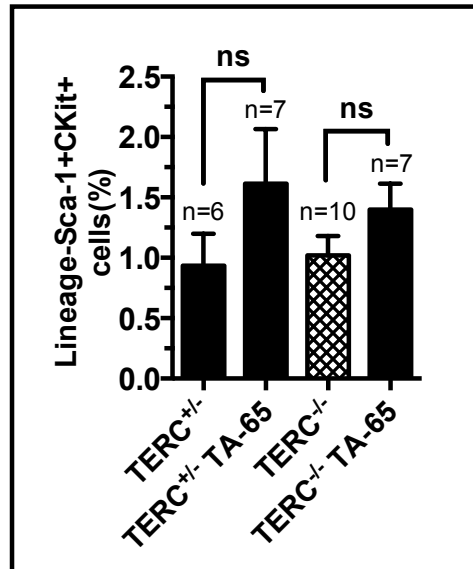


Figure 6.2: Effects of TA-65 treatment on LSK stem cell content of TERC^{+/-} and TERC^{-/-} mice.

8-12-month-old C57BL/6 mice were treated with either TA-65 or control for 3-4 months. Bone marrow cells were isolated and lineage⁺Sca-1⁺CKit⁺ (LSK) stem cell measured by FACS analysis. Statistical significance calculated by Mann-Whitney test. Error bars stand for mean \pm SEM.

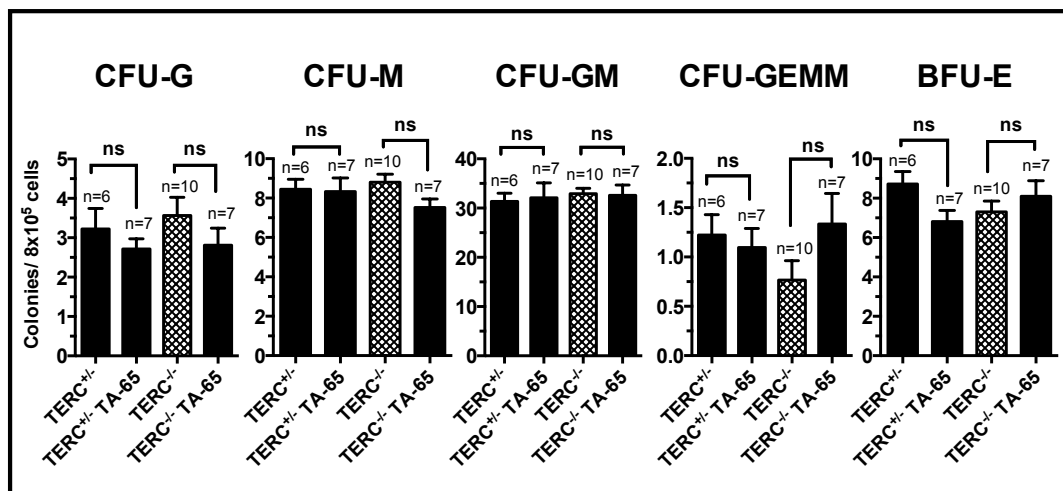


Figure 6.3: Effects of TA-65 treatment on clonogenic assay of TERC^{+/-} and TERC^{-/-} mice.

8-12-month-old C57BL/6 mice were treated with either TA-65 or control for 3-4 months. Bone marrow cells were isolated and cultured in clonogenic assay for 14 days. Colonies were scored according to morphology under inverted microscop. All p-values calculated by Mann-Whitney test. Error bars stand for mean \pm SEM.

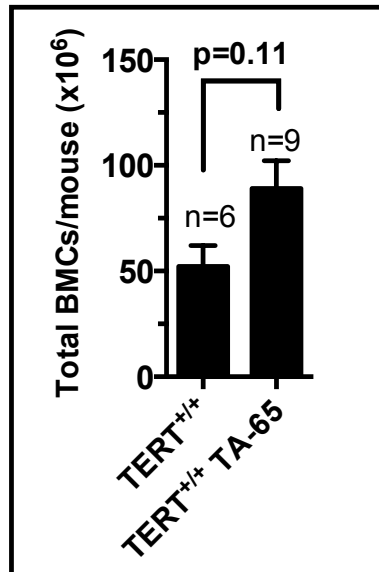


Figure 6.4: Effects of TA-65 treatment in TERT^{+/+} mice bone marrow cellularity.

28-month-old TERT^{+/+} C57BL/1crfa(t) mice were treated with either TA-65 or control for 3-4 months. Bone marrow cells were isolated and quantified. Statistical significance calculated by Mann-Whitney test (ns=nonsignificant). Error bars stand for mean ± SEM.

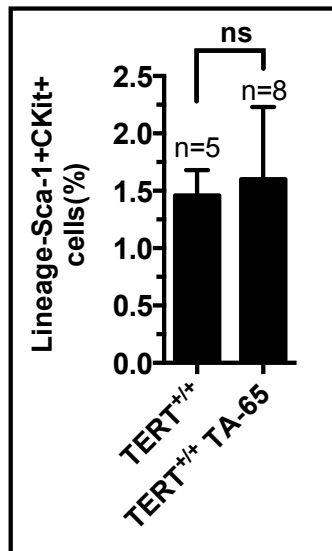


Figure 6.5: Effects of TA-65 treatment on LSK content of TERT^{+/+} mice.

28-month-old TERT^{+/+} C57BL/1crfa(t) mice were treated with either TA-65 or control for 3-4 months. Bone marrow cells were isolated and lineage⁻Sca-1⁺CKit⁺ (LSK) stem cells were measured by FACS analysis. Statistical significance calculated by Mann-Whitney test (ns=nonsignificant). Error bars stand for mean ± SEM.

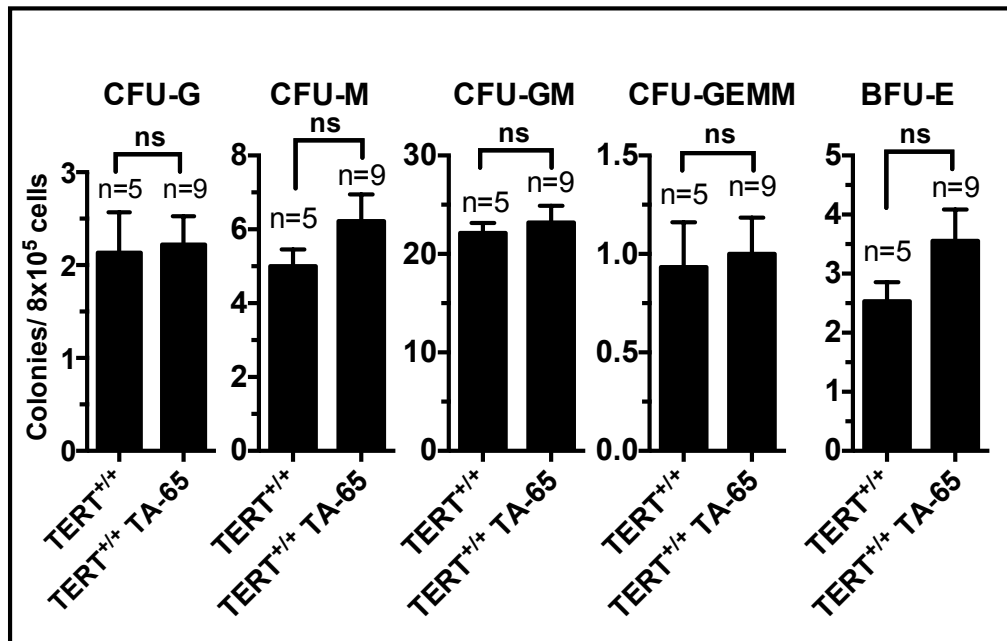


Figure 6.6: Effects of TA-65 treatment on clonogenic assay of TERT^{+/+} mice.

28-month-old TERT^{+/+} C57BL/1crfa(t) mice were treated with either TA-65 or control for 3-4 months. Bone marrow cells were isolated and cultured in clonogenic assay for 14 days. Colonies were scored according to morphology under inverted microscope. All p-values calculated by Mann-Whitney test (ns= nonsignificant). Error bars stand for mean \pm SEM.

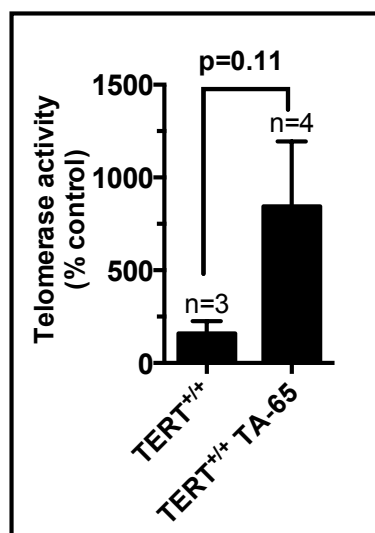


Figure 6.7: Effects of TA-65 treatment on telomerase activity in TERT^{+/+} mice.

28-month-old TERT^{+/+} C57BL/1crfa(t) mice were treated with either TA-65 or control for 3-4 months. Bone marrow cells were isolated and telomerase activity was measured by Q-TRAP. Statistical significance calculated by Mann-Whitney test (ns= nonsignificant). Error bars stand for mean \pm SEM.

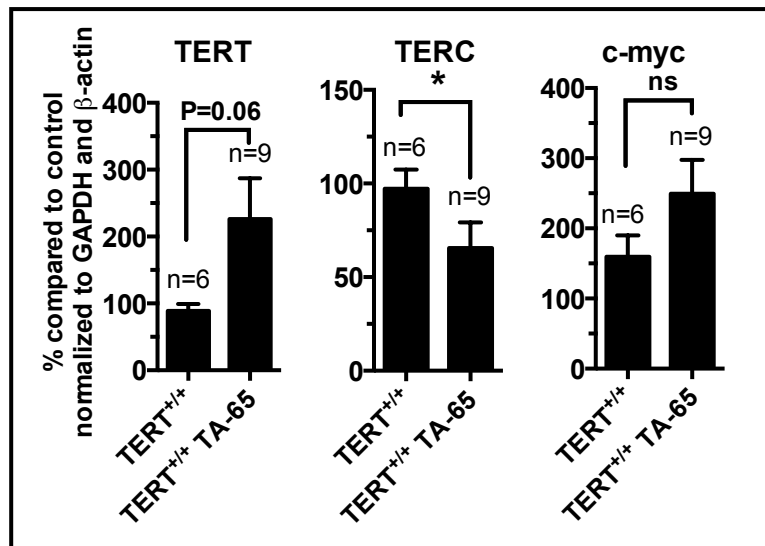


Figure 6.8: Effects TA-65 treatment on TERT, TERC, and c-myc expression in TERT^{+/+} mice.

28-month-old TERT^{+/+} C57BL/1crfa(t) mice were treated with either TA-65 or control for 3-4 months. Bone marrow cells were isolated and real-time quantitative PCR was used to analyse the expression of: TERT, TERC and c-myc. Real Time PCR [StepOnePlus, Applied Biosystems] was used to measure the gene expression by the $\Delta\Delta C_T$ method. Expression was normalized to housekeeping genes GAPDH and β -actin and referenced to one TERT^{+/+} control. All p-values calculated by Mann-Whitney test (ns= nonsignificant, *P<0.05). Error bars stand for mean \pm SEM.

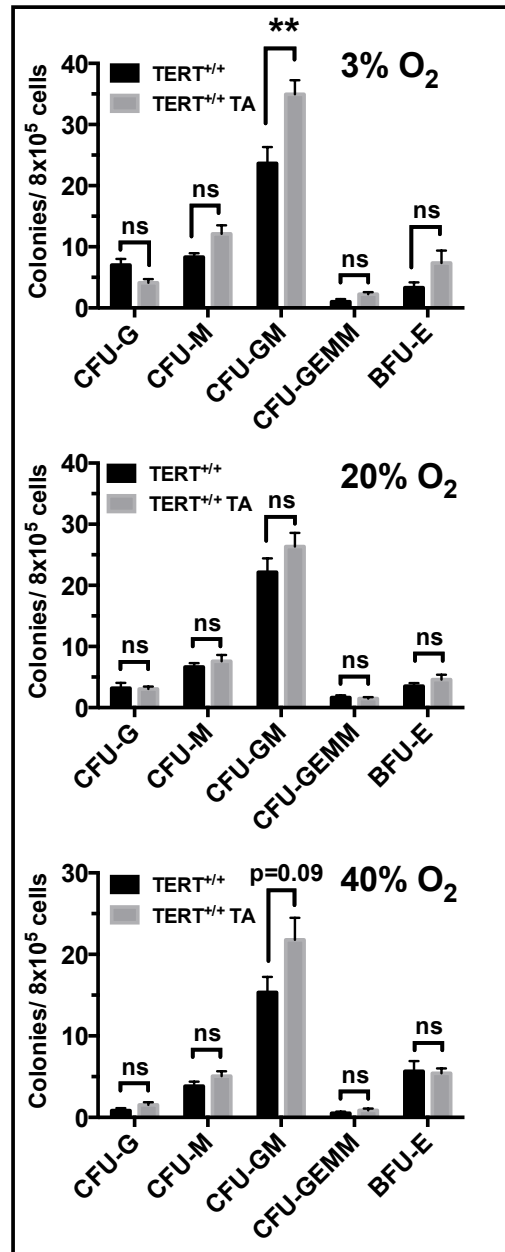


Figure 6.9: Effects of oxidative stress on clonogenic assay in TA-65 treated TERT^{+/+} mice.

28-month-old TERT^{+/+} C57BL/1crfa(t) mice were treated with either TA-65 (n=5) or control (n=3) for 3-4 months. Bone marrow cells were isolated and clonogenic assay of bone marrow cells after two weeks and TERT^{+/+} TA: TA-65 treated. Clonogenic assay was performed under different oxygen levels (3%, 20%, and 40%). All p-values calculated by two-way ANOVA (**P<0.01); other comparisons were not significant. Error bars stand for mean ± SEM.

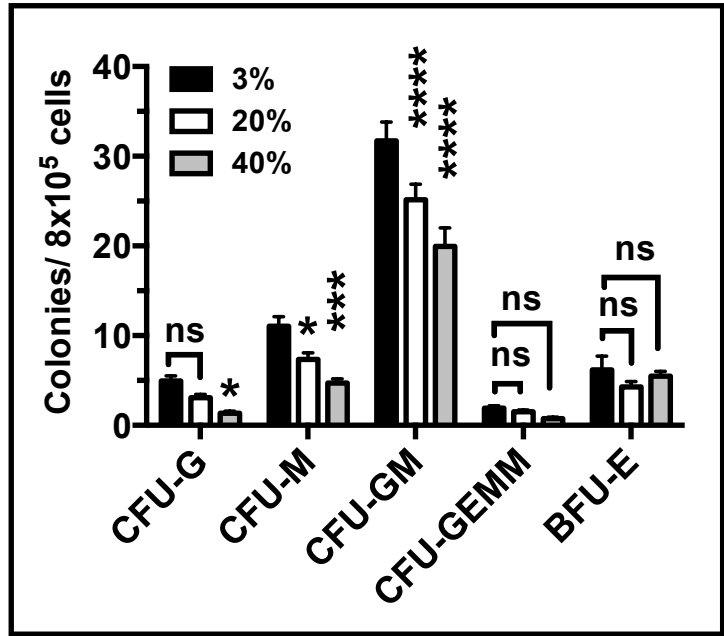


Figure 6.10: Effects of oxidative stress on colony formation in culture.

Clonogenic assay of bone marrow cells after two weeks' culture of 28-month-old C57BL/1crfa(t) mice TA-65 treated and controls were combined to assess oxygen impact on colonies (n=7). Colonies were incubated in different oxygen levels 3%, 20%, and 40%. Statistical significance displayed in comparison to 3% oxygen. All p-values calculated by two-way ANOVA (*P<0.05, ***P<0.001, ****P<0.0001). Error bars stand for mean ± SEM.

6.2.2 Impact of short-term expansion of HSPCs, metabolic stress, and TA-65 in culture

Previous reports confirmed the role of telomerase in the maintenance of haematopoietic stem cells through serial transplantation *in vivo* (Gluckman *et al.*, 1989; Allsopp *et al.*, 2003; Ballen *et al.*, 2012). Nevertheless, to assess the effect of telomerase in HSPCs *in vitro*, cell count was quantified under expansion, metabolic stress, and TA-65 addition. These experiments were performed to compare mouse HSPCs to CD34+ HSPCs isolated from human UCB (Chapter 4). HSPC expansion was induced using a different cytokine cocktail over 1 week of culture, including 100ng/ml stem cell factor (SCF), 100ng/ml thrombopoietin (TPO), and 100ng/ml FLT-3 ligand (FLT-3L). As shown in Figure 6.11, telomerase does not affect the expansion level of HSPCs *in vitro* in both old (22 months) and young (1.5-8 months) mice. Unlike EPCs and cord blood HSPCs, HSPCs from $TERT^{-/-}$ and $TERC^{-/-}$ showed resistance to metabolic stress, in which different doses of glucose were administered *in vitro* (Figure 6.12A). Furthermore, treatment of mice HSPCs with TA-65 did not enhance their growth in culture (Figure 6.12B).

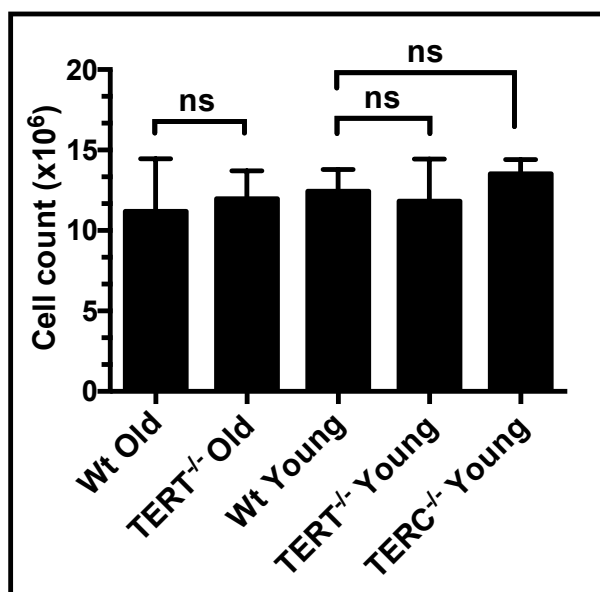


Figure 6.11: Effects of short-term HSPCs expansion in culture.

Bone marrow cells were expanded in culture using cytokines. Cells were seeded on the first day and recounted after 7 days of culture. Wild types were $TERT^{+/+}$, young: 8-12 months, old: 22 months C57BL/6 mice (n=3). Statistical significance was calculated using Kruskal-Wallis test (ns= nonsignificant).

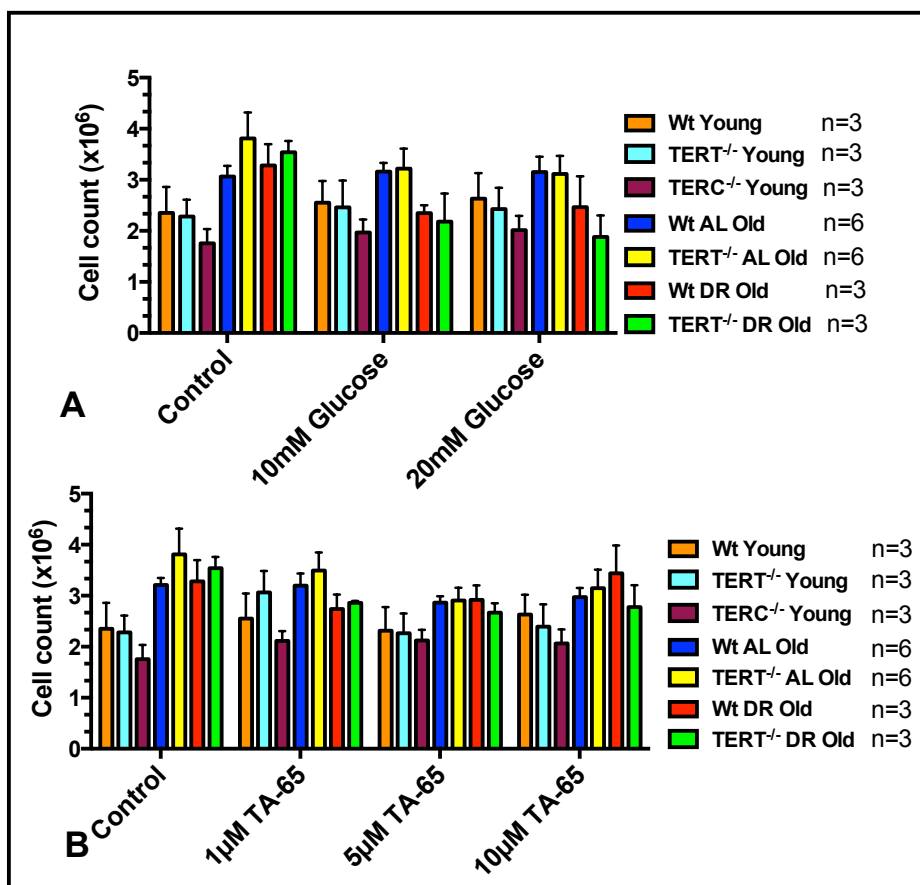


Figure 6.12: Effects of metabolic stress and TA-65 treatment on HSPCs in short-term culture.

Bone marrow cells from different knockout mice were grown in culture for 3 days under different concentrations of glucose to assess the effect of metabolic stress and TA-65 on haematopoietic stem and progenitor cells (HSPCs) growth. Two-way ANOVA revealed no significant differences between groups and treatments. Wt young: TERT^{+/+} wild type. Ages were 1.5-7.5 month old for young wild types TERT^{+/+} and TERT^{-/-}, 8-12 month old for young TERC^{-/-}; all old mice were 22 months old. All mice were on C57BL/6 strain.

6.2.3 Impact of short- and long-term dietary restriction on bone marrow HSPCs

A previous study provided evidence that ageing causes an increase in short-term haematopoietic stem cells (ST-HSCs) in wild type mice that can be partly reversed by dietary restriction (DR) (Ertl *et al.*, 2008). To identify whether the previously described effect of DR on ageing of haematopoietic stem cells is mediated by telomerase, we compared TERT^{+/+} or TERT^{+/-} with TERT^{-/-} mice under conditions of short-term (7 month) and long-term (16 months) DR.

Despite variability in bone marrow cellularity, both short-term (Figure 6.13) and long-term (Figure 6.16) DR reduced the percentage of LSK cells within the bone marrow of aged mice in both TERT^{+/+} or TERT^{+/-} and TERT^{-/-} mice. This was almost the same level found in young mice (Figure 6.14 and Figure 6.18). Of particular note, TERT^{-/-} mice showed a similar result under DR (Figure 6.14 and Figure 6.18), suggesting a telomerase-independent effect of DR on the accumulation of LSK HSPCs with age. Furthermore, DR reduced the change in the clonogenic assay during aging, as indicated by statistically significant reductions in CFU-M and CFU-GM following both short- and long-term DR in wild type and mutant mice (Figure 6.15 and Figure 6.18). In 22-month-old mice subjected to long-term DR, the accumulation of BFU-E and CFU-G specifically associated with TERT deficiency was also reversed in TERT^{-/-} mice to young and wild type levels (Figure 6.18). Together, these data show that dietary restriction is able to ameliorate the phenotypes of ageing in haematopoietic stem cells, mostly independent of TERT and telomerase.

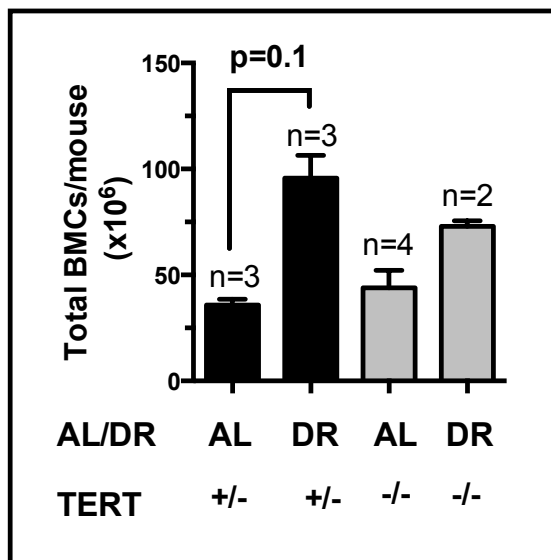


Figure 6.13: Effects of short-term DR on bone marrow cellularity in $TERT^{+/-}$ and $TERT^{-/-}$ mice.

Bone marrow counts were isolated freshly from bones of 11-month-old mice under dietary restriction (DR) for 7 months and ad libitum (AL) control C57BL/6 mice. Statistical significance was calculated by Mann-Whitney test. Error bars stand for mean \pm SEM.

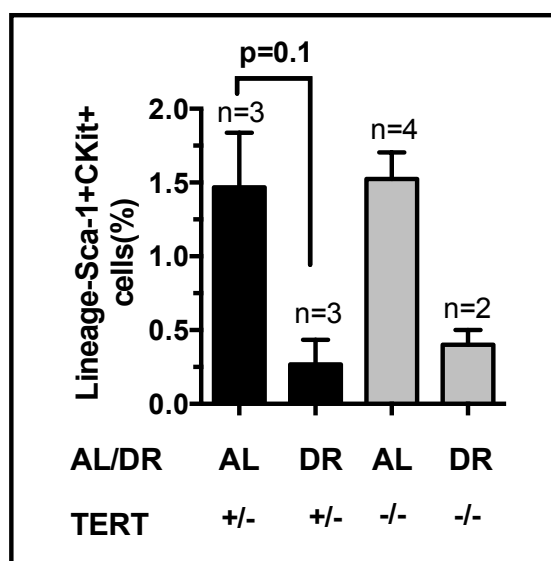


Figure 6.14: Effects of short-term DR on LSK stem cell content in $TERT^{+/-}$ and $TERT^{-/-}$ mice.

Lineage⁻Sca-1⁺CKit⁺ (LSK) stem cells were measured using FACS analysis from freshly isolated bone marrows of 11-month-old mice under dietary restriction (DR) for 7 months and compared ad libitum (AL) control C57BL/6 mice. All p-values calculated by Mann-Whitney test. Error bars stand for mean \pm SEM.

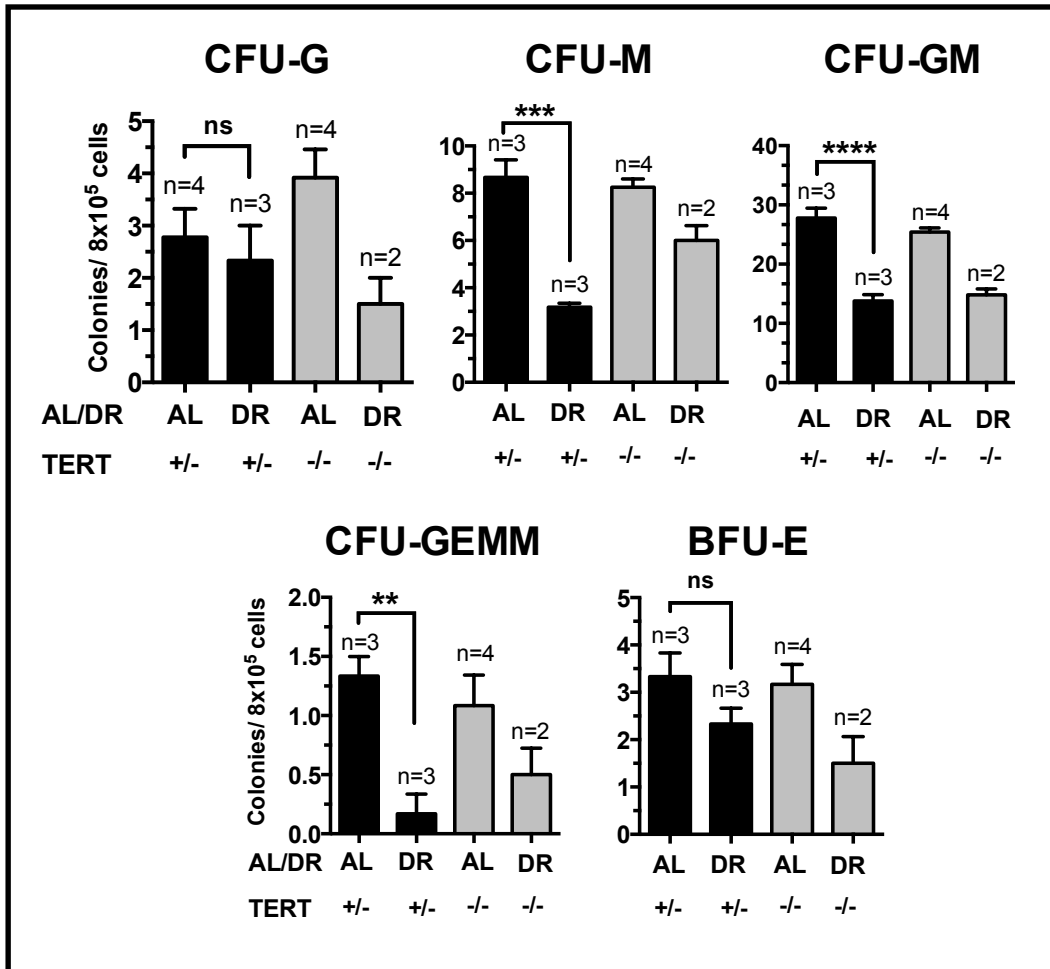


Figure 6.15: Effects of short-term DR on clonogenic assay.

Clonogenic assay of bone marrow cells after two weeks' culture from 11 month old bone marrow under dietary restriction (DR) for 7 months and ad libitum (AL) control C57BL/6 mice. All p-values calculated by Mann-Whitney test (**P<0.01, ***P<0.001, ****P<0.0001). Error bars stand for mean \pm SEM.

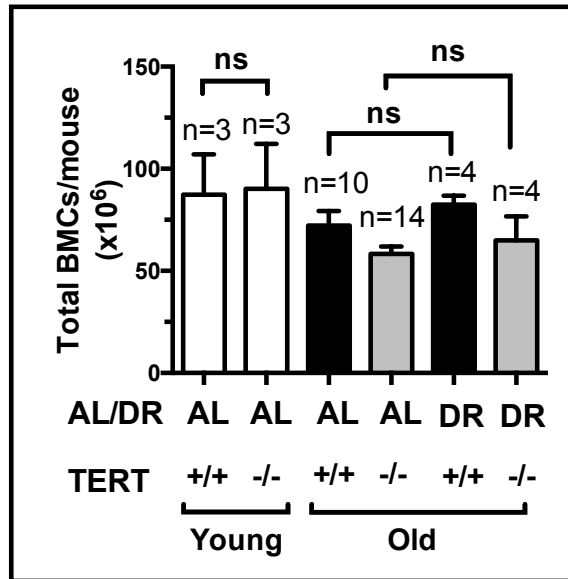


Figure 6.16: Effects of long-term DR on bone marrow cellularity.

Bone marrow cell count of freshly isolated bones from young (1.5-7.5 month old) and old (22 month old) under dietary restriction (DR) for 16 months and ad libitum (AL) control C57BL/6 mice. Statistical significance calculated by Mann-Whitney test. Error bars stand for mean \pm SEM.

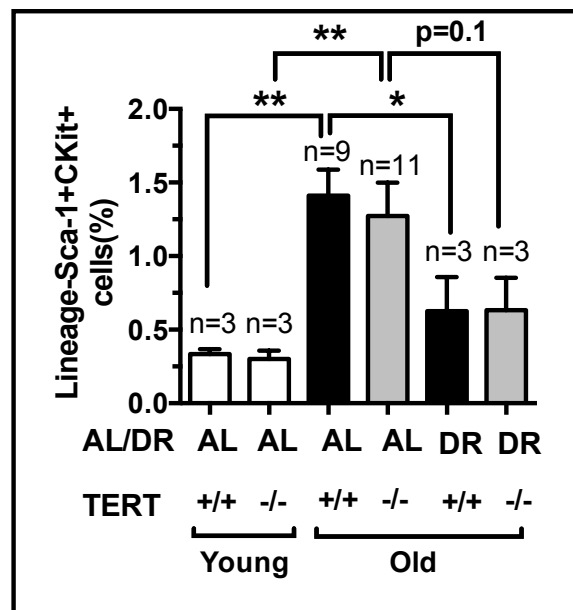


Figure 6.17: Effects of long-term DR on LSK stem cell content.

Lineage-Sca-1⁺CKit⁺ (LSK) stem cells were measured using FACS analysis from freshly isolated bone marrows from young (1.5-7.5 month old) and old (22 month old) under dietary restriction (DR) for 16 months and ad libitum (AL) control C57BL/6 mice. All p-values calculated by Mann-Whitney test (*P<0.05 and **P<0.01). Error bars stand for mean \pm SEM.

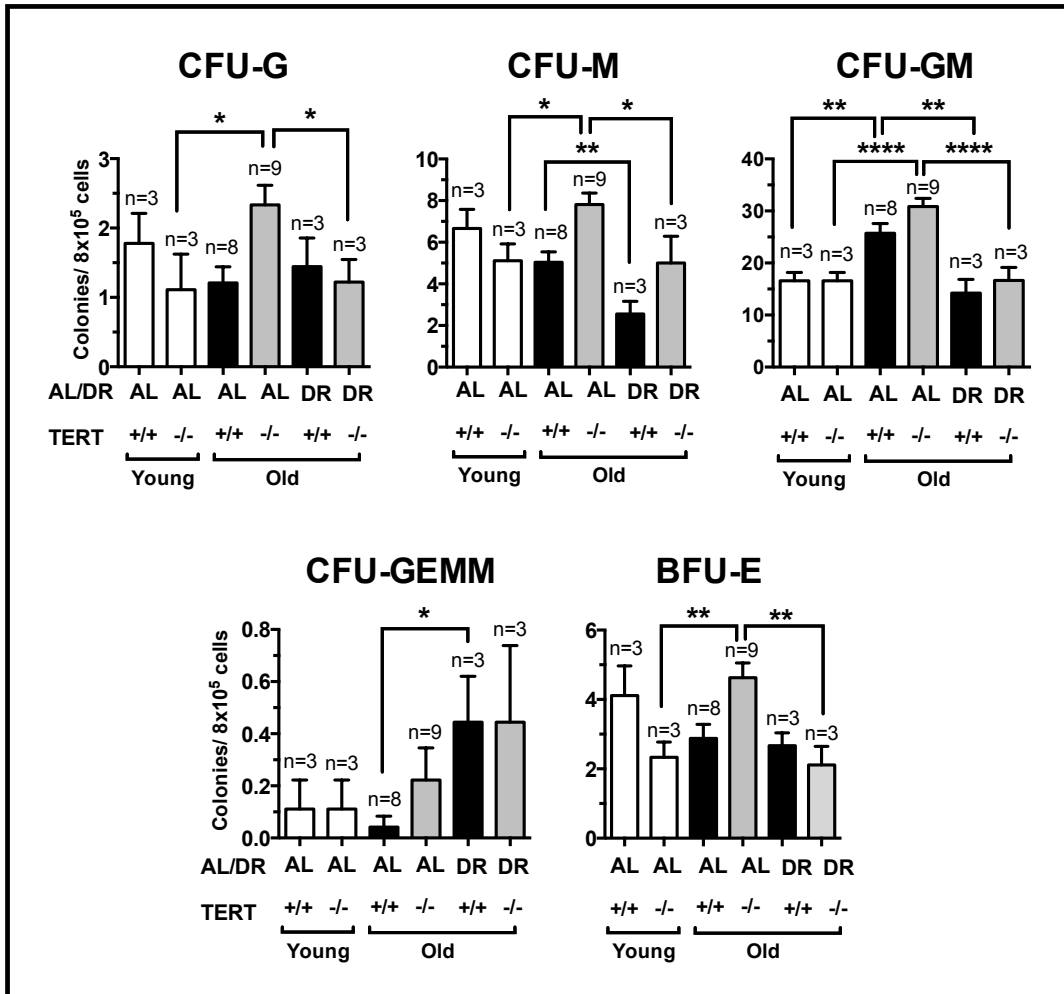


Figure 6.18: Effects of long-term DR on clonogenic assay.

Clonogenic assay of bone marrow cells after two weeks' culture from young (1.5-7.5 month old) and old (22 month old) under dietary restriction (DR) for 16 months and ad libitum (AL) control C57BL/6 mice. All p-values calculated by Mann-Whitney test (** $P < 0.01$, **** $P < 0.0001$). Error bars stand for mean \pm SEM.

6.3 Discussion

The anti-ageing effect of telomerase on haematopoietic stem cells is mediated by both its canonical function through elongating telomeres (Tomas-Loba *et al.*, 2008; Bernardes de Jesus *et al.*, 2011) as well by telomere-independent mechanisms (Nitta *et al.*, 2011). The latter study showed that TERT can mitigate ageing of HSPCs by antagonising the effects of oxidative stress on DNA damage. Vera *et al.* reported that mice overexpressing the catalytic subunit TERT (TgTERT) show less telomere shortening and less telomere damage, resembling the effect of caloric restriction (Vera *et al.*, 2013). The authors also found that caloric restriction of TgTERT mice leads to a significant lifespan extension compared to wild types. Ertl and coauthors have found that the effect of DR on haematopoietic stem-cell ageing is a general phenomenon in mice from different genetic backgrounds, although its effects on function per HSC are genotype-dependent (Ertl *et al.*, 2008). These observations are confirmed by my study, where I provide evidence that telomerase does not seem to be required for the beneficial effect of ameliorating ageing in HSPCs under dietary restriction. In fact, even short-term caloric restriction reversed the “ageing” phenotype of bone marrow stem cells. To our surprise, this beneficial effect occurred independently of the presence of telomerase (TERT ko mice).

The telomerase activator TA-65, purified from the root of *Astragalus membranaceus*, has been shown to ameliorate the amount of short telomeres and decrease the percentage of critically short telomeres as well as DNA damage in mouse embryonic fibroblasts that harbour critically short telomeres (Bernardes de Jesus *et al.*, 2011). In humans, it has also been shown that the small molecule telomerase activator (TAT2) isolated from *Astragalus membranaceus* induced telomerase activity in peripheral blood mononuclear cells and T lymphocytes and increased their antiviral functions (Fauce *et al.*, 2008). Furthermore, human supplementation with TA-65 as well as other dietary supplements have been shown to reduce the percentage of cells with short telomeres (Harley *et al.*, 2011).

Although there were no effects of TA-65 on the bone marrow in young $TERC^{-/-}$ and $TERC^{+/-}$ mice, I found a tendency of boosting bone marrow cellularity and telomerase activity with TA-65 in aged $TERT^{-/-}$ mice. Furthermore, this effect of TA-65 was accompanied by a tendency of increased TERT transcription, matching what has been

reported previously (Bernardes de Jesus *et al.*, 2011) and also a downregulation of TERC expression using real-time PCR. However, TA-65 did not affect LSK cells or haematopoietic stem cell function, as quantified by clonogenic assays. Additionally, TA-65 does not protect colonies in culture from oxidative stress. Taken together, TA-65 negative results might be due to the short period of TA-65 treatment and given that even the TERT ko mouse has a very mild phenotype regarding the bone marrow. Moreover, another possibility is that the dose and/or the uptake of TA-65 in food might not be sufficient to induce telomerase. Therefore, further testing of doses as well as route of uptake are required.

Recently, one week of treatment of young heterozygous TERT mice with GRN510, a small molecule on the basis of cycloastragenol (GRN665 or TAT2 also isolated from the plant *Astragalus membranaceus*), has been shown to activate telomerase in bone marrow progenitor cells compared to controls. Perhaps this activation is either due to the much younger age of the mice or to the small molecule activator being more potent than the plant extract TA-65 (Le Saux *et al.*, 2013).

To sum up, the effects of ageing discussed in the previous chapter (Chapter 5), including the lineage change in the clonogenic assay associated with lack of telomerase, can be ameliorated by dietary restriction but not TA-65 supplementation.

Chapter 7 General discussion

Ageing implicates the progressive functional decline of different cells and tissues, affecting all species. Recently, it has been shown that there are several complex mechanisms underlying ageing summarized into 9 interconnecting hallmarks. Lopez-Otin and colleagues have proposed a hierarchical relationship between those hallmarks. In brief, ageing hallmarks can be divided into three: primary (causes of damage, all negative), antagonistic (responses to damage, depend on intensity), and integrative (culprits of the phenotype, directly affect tissue homeostasis). Primary hallmarks include genomic instability, telomere attrition, epigenetic alteration, and loss of proteostasis. Antagonistic hallmarks include deregulated nutrient signalling, mitochondrial dysfunction, and cellular senescence. Integrative hallmarks include stem cell exhaustion and altered cellular communication (Lopez-Otin *et al.*, 2013). Of particular interest for this thesis are telomeres and telomerase and their involvement in ageing and disease. Telomeres are specialized protective nucleoprotein complexes at the end of chromosomes that shorten with each cell division. Critically short telomeres lose the capping function at the chromosomal ends as these dysfunctional telomeres induce DNA damage checkpoints leading to apoptosis or cell cycle arrest. Organ systems with the higher rates of cell turnover are more sensitive to telomere shortening (Lee *et al.*, 1998). Therefore, telomere length has been suggested to be a biomarker of ageing as it can be an indicator of cellular senescence and oxidative stress (von Zglinicki and Martin-Ruiz, 2005). Telomerase is a unique reverse transcriptase that replicates de novo telomeric DNA to extend telomere length. Telomerase mutations are implicated in several human diseases including; dyskeratosis congenita (Vulliamy *et al.*, 2001), idiopathic pulmonary fibrosis (Armanios *et al.*, 2007), and bone marrow failure syndromes (Vulliamy *et al.*, 2002). The focus of this thesis was to investigate underlying cellular mechanisms of ageing by oxidative and metabolic stress in early EPCs and HSCs, and a particular focus of my thesis was the relation to telomerase.

7.1 PGC-1 α and metabolic stress

Ageing is associated with a defect in the repair of vascular lesions (Gennaro *et al.*, 2003). Many researchers have shown the therapeutic potential of EPCs in restoring vascular repair (Asahara *et al.*, 1997; Kalka *et al.*, 2000; Sheng *et al.*, 2013). Although PGC-1 α plays a critical role in energy regulation, such as in mitochondrial metabolism, lipid oxidation, glucose metabolism, and angiogenesis (Arany *et al.*, 2008; Ventura-Clapier *et al.*, 2008), little is known about its role in EPCs and underlying mechanisms of diabetes. I showed in my thesis that metabolic stress and metformin in early EPCs induced PGC-1 α and downstream targets similar to that reported in HUVECs (Kukidome *et al.*, 2006). It has been reported for the first time recently that the expression of PGC-1 α is induced in diabetic mice and humans and blocks endothelial migration in vitro and vasculogenesis in vivo via induction of Notch signalling (Sawada *et al.*, 2014). In their elegant study, the authors showed that transgenic overexpression of PGC-1 α in the endothelium resembles multiple diabetic phenotypes, including blunted wound healing, aberrant re-endothelialization after carotid injury, and decreased blood flow recovery after hind limb ischemia. Conversely, endothelial specific PGC-1 α knockout mice rescued wound healing and recovery after hind limb ischemia in type 1 and type 2 diabetes (Sawada *et al.*, 2014).

The role of metabolic stress has not been directly examined in the context of haematopoiesis; however, several studies suggest that metabolic conditions can affect HSC homeostasis. The risk for acute myelogenous leukemia (AML) and acute lymphoblastic leukemia (ALL) increased in children exposed to high glucose levels in utero (Hjalgrim *et al.*, 2003; Hjalgrim *et al.*, 2004). Furthermore, hyperglycemia is linked to higher mortality in patients with AML (Ali *et al.*, 2007). In addition, ALL was found to be more frequent in children with diabetes (Feltbower *et al.*, 2004). Cord blood from diabetic mothers displayed decreased neutrophil mobility (Mehta and Petrova, 2005) and platelet function (Strauss *et al.*, 2010). A recent report confirmed that glucose exposure enhanced HSC formation in zebrafish (Harris *et al.*, 2013). Telomerase is upregulated in mononuclear cells from UCB from mothers with type 1 diabetes (Cross *et al.*, 2010). In contrast, in my experiments no difference in telomerase activity was seen

in CD34+ HSPCs with hyperglycemia in vitro suggesting that metabolic stress acts in a telomerase-independent mechanism in these cells.

7.2 Oxidative stress

Oxidative stress contributes to ageing and several diseases and leads to cellular damage. In order to avoid toxicity of oxidative reactions, there must be a balance between oxidative stimuli and defence response (Sies, 1997). ROS generation depends on oxygen sensing around the cells; high oxygen leads to more ROS and vice versa (Sauer *et al.*, 2001). However, suitable ROS content is important as an intracellular second messenger that regulates cell growth, adhesion, transcription factor activation, and apoptosis (Kowaltowski *et al.*, 2009). Therefore, the equilibrium in ROS production and removal must be maintained continuously (Allen and Balin, 2003; X. Zhang *et al.*, 2005). Furthermore, hyperoxia increases the rate of telomere shortening from four- to tenfold and decreases the replicative lifespan of fibroblasts (von Zglinicki *et al.*, 1995). Interestingly, murine embryonic stem cells have increased levels of antioxidant defence and good DNA repair capacity, making them resistant to potential oxidative stress (Saretzki *et al.*, 2004). Similarly, in this study, when early EPCs were challenged under oxidative stress, there was no difference in their proliferation, suggesting that these cells are particularly resistant to oxidative stress. This is consistent with reported high levels of antioxidant enzymes in EPCs and more resistance to oxidative stress compared with adult microvascular endothelial cells and HUVECs (Dernbach *et al.*, 2004; He *et al.*, 2004; Cai *et al.*, 2006). In addition, when CD34+ HSPCs isolated from UCB were expanded and differentiated to myeloid cells under hyperoxia, their proliferation was impaired. Interestingly, oxidative stress reduced CD15+ granulocytes while CD14+ monocytes were more resistant. In fact, it has been shown that CD14⁺CD16⁻ monocytes were more resistant to oxidative stress compared with CD14^{+/low}CD16⁺ monocytes (Geiger-Maor *et al.*, 2012). Furthermore, when fibroblasts were damaged by oxidative stress using peroxide treatment, higher migration of CD14+ cells was seen (C. Zhao *et al.*, 2010). Additionally, I also showed that the downregulation of TERC expression during myeloid differentiation was due to the suppression CD15+ granulocytes. CD15+ granulocytes have also been shown to trigger tumour angiogenesis by producing proangiogenic MMP-9 (Kuang *et al.*, 2011). Therefore, I propose that this suppression of

CD15+ differentiation and the associated decrease in TERC under hyperoxia might be a protective cellular mechanism. Furthermore, hyperoxia reduced the clonogenic capacity of HSPCs from both human UCB and mouse models in my experiments. Unexpectedly, telomerase activity was induced during hyperoxia in CD34+ HSPCs expansion. On the contrary, it has been reported that telomerase is induced under hypoxic 5% oxygen in hTERT-transduced bone marrow endothelial cells (Napier *et al.*, 2010). However, the difference might be explained by the different cells they used compared to my expanded CD34+ HSPCs. Data from our group has also shown that telomerase activity is suppressed in T lymphocytes under hyperoxia (Bennaceur *et al.*, manuscript submitted). However, under physical stress, in vivo telomerase activity is elevated in PBMNCs of rats (Beery *et al.*, 2012).

7.3 Telomerase in HSCs ageing

Telomerase expression is readily detected in most haematopoietic stem cells (Counter *et al.*, 1995), but this activity is not sufficient to prevent overall loss of telomeric DNA with age or proliferation (Rufer *et al.*, 1999). Primarily, telomerase is required to directly act on chromosome ends in haematopoietic stem cells; however, indirect effects of telomerase via cells that support cell proliferation have also been shown (Ju *et al.*, 2007). An accumulation of DNA damage was shown during ageing in HSCs from mice and humans (Rossi *et al.*, 2007; Rube *et al.*, 2011). Genetically engineered mouse models demonstrated that telomere dysfunction and the accumulation of DNA damage can contribute to the age-related decline of stem cell function and maintenance (Choudhury *et al.*, 2007; Rossi *et al.*, 2007). Telomerase knockout mice ($TERT^{-/-}$ and $TERC^{-/-}$) have been investigated to understand the role of telomeres and telomerase biology (Blasco, 2003). Despite the fact that these models were studied extensively, normal ageing in those models is poorly understood. Therefore, I investigated the role of telomerase on HSPC content and their clonogenic potential.

Ageing in wild type mice bone marrow HSPCs was associated with increased LSK cells and CFU-GM colonies. This data might be in line with studies reporting myeloid skewing with ageing and accumulation of stem cells both in mice and humans (Sudo *et al.*, 2000; Rossi *et al.*, 2007; Waterstrat and Van Zant, 2009; Dykstra *et al.*, 2011; Pang *et al.*, 2011). I then compared this phenotype with old $TERT^{-/-}$ (lack of telomerase activity,

but intact telomeres), which showed an augmented ageing phenotype of the bone marrow particularly in the composition of colonies (CFU-G, -M, -GM and BFU-E) but not LSK cells in aged mice. In contrast, young $TERC^{-/-}$ mice that lack telomerase and have shortened telomeres showed even more HSCs ageing with increased LSK cells and induction of the same colonies. These effects suggest a gradient effect of telomerase and telomeres on HSCs ageing in the bone marrow. As shown in Figure 7.1, lack of telomerase per se without telomeres shortening ($TERT^{-/-}$ mice) augments ageing, but lack of telomerase with telomeres shortening ($TERC^{-/-}$ mice) accelerate ageing of the BM HSCs.

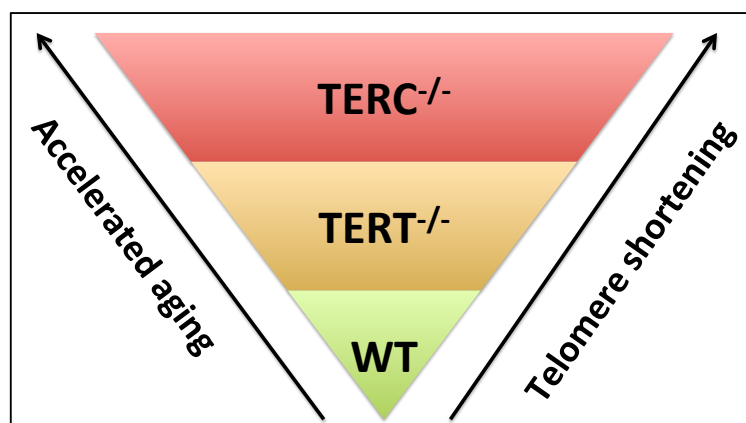


Figure 7.1: The effect of telomerase deficient mice on bone marrow ageing.

From results of chapter 6, it is speculated that the genetic lack of telomerase TERT and TERC show a gradient effect with TERC on the top affecting bone marrow HSPCs ageing.

Of note, the erythroid BFU-E colonies were only skewed with telomerase knockouts ($TERT^{-/-}$ and $TERC^{-/-}$), indicating a telomerase-dependent imbalance in the bone marrow homeostasis. Anaemia might be a good explanation of erythroid skewness where the bone marrow responds to anaemic shortage of cells in a compensatory mechanism. A recent study confirmed a significant induction in splenic $CD71^{hi}Ter119^{hi}$ progenitors with a similar tendency in bone marrow (Guo *et al.*, 2013). Given the clinical burden of age-related anaemia, these findings might suggest new approaches to resolve it.

7.4 Dietary restriction

Dietary restriction is a well-known anti-ageing intervention that extends lifespans of many organisms including worms (Klass, 1977), flies (Cooper *et al.*, 2004), mice

(Bronson and Lipman, 1991), dogs (Kealy et al., 2002), and monkeys (Colman et al., 2009). DR has several health benefits including reduction of the incidence of cancer, diabetes, brain atrophy, and cardiovascular disease (Colman et al., 2009). However, recently, it has become apparent that the effects of DR in extending lifespans are not universal (Mattison *et al.*, 2012). Indeed, DR can either extend or decrease longevity depending on the genetic background (Liao *et al.*, 2010). Although the exact mechanism of dietary restriction is not well understood, theories suggest the protection against cellular damage by reducing cellular metabolism (Bordone and Guarente, 2005). In particular, little is known about the DR mechanism with respect to telomeres and telomerase. Thus, I was interested to find out if the effect of DR in HSPC ageing is influenced by the lack of telomerase. I have shown that long-term DR (16 months) can ameliorate HSCs ageing in the bone marrow. In my study, even short-term (7 months) DR ameliorated bone marrow ageing. In fact, DR almost reversed the ageing BM HSPCs phenotype (more LSK stem cells and more colonies) seen with ageing to a similar BM HSPCs seen in young mice (less LSK stem cells and colonies). This is in agreement with previous studies (Ertl *et al.*, 2008). Remarkably, Ertl and colleagues have demonstrated that DR can also ameliorate HSC function in repopulation assays *in vivo*. Nevertheless, this functional benefit of DR *in vivo* was found to be mouse strain-specific (Ertl *et al.*, 2008). In my experiments, I investigated the role of lack of telomerase (TERT^{-/-} mice) on DR, but there was no association of telomerase with DR in our models. Therefore, the DR beneficial effect in ageing HSPCs is TERT-independent and, to my knowledge, this finding is quite novel. Interestingly, a recent report confirmed that TERT overexpression in mice “synergizes” with the beneficial effects of CR, including improved glucose tolerance, protection from osteoporosis, enhanced neuromuscular coordination, decreased rate of telomere shortening with ageing, decreased DNA damage, and increased longevity (Vera *et al.*, 2013).

In addition, the same group has shown that supplementation of mice with TA-65, purified from the root of *Astragalus membranaceus*, improves the health span and is capable of telomerase-dependent elongation of short telomeres (Bernardes de Jesus *et al.*, 2011), suggesting that TA-65 has an anti-ageing potential. Therefore, I investigated the role of dietary supplementation of the telomerase activator TA-65 in aged telomerase deficient

(TERT^{-/-}) and heterozygous mice (TERT^{+/-}). I reported here that TA-65 shows a tendency to increase bone marrow cellularity, induce telomerase activity, and TERT expression in bone marrow HSPCs. However, no effect was seen on bone marrow HSCs ageing. In comparison, data from our group has shown that TA-65 can induce T lymphocytes proliferation in vitro (data not shown).

7.5 Limitations and perspectives

There are several limitations in my study, which can only be addressed by further investigation. The first limitation is in the characterisation of cells. This limitation involves EPCs from PBMNCs, and HSPCs from both human and mice. As I have shown, EPCs do not proliferate in culture and ECFCs are thought to be the real angiogenic endothelial progenitor cells; data are limited in those cell types. It will be interesting to find out how ECFC can behave under metabolic and oxidative stress and how telomerase is regulated under those conditions. Furthermore, HSPCs can be better characterised with reported markers CD93 and CD133 (Anjos-Afonso *et al.*, 2013; M. Takahashi *et al.*, 2013). The second limitation in my mice study: treatment of mice with TA-65 might be limited in this study due to three main reasons. First, the strain of mice used for the TA-65 treatment is different than the one used in the ageing HSPCs (chapter 5). Although the right control wild type mice were used, which were fed with DMSO as TA-65 was dissolved in DMSO for the treated group, the strain might show a different ageing phenotype in their BM HSPCs (Ertl *et al.*, 2008). Second, the dietary supplementation might not be sufficient to induce HSPCs in the bone marrow. Data from our group showed induction of T lymphocytes with TA-65. Unlike lymphocytes, HSPCs are a rare cell type that reside in the bone marrow niche. Therefore, it might be possible that the absorbed amount of TA-65 could not reach the bone marrow cells. Third, the duration of TA-65 treatment was only 4 months in aged mice. It will be interesting if the route of TA-65 administration and time course can be tested and compared in mice in further experiments (e.g., injections vs dietary supplementation).

7.6 Summary and conclusion

In summary, my studies have examined the effects of metabolic and oxidative stress on EPCs and HSPCs with the aim of understanding the underlying influences of ageing and telomerase. To start with, metabolic and oxidative stresses were induced in culture with glucose addition and incubation under different oxygen levels, respectively. Metabolic stress disrupted the cellularity of both EPCs and HSPCs. Unlike HSPCs, EPCs showed an increased expression of the metabolic co-activator PGC-1 α under diabetic conditions. In contrast, oxidative stress limited the cellularity of both EPCs and HSPCs. Indeed, when HSPCs were differentiated into myeloid cells, the expression of TERC was blunted. This reduction was essentially due to suppression of CD15⁺ granulocytes under oxidative stress. Furthermore, oxidative stress stimulated more growth in CD14⁺ monocytes suggesting their resistance. Surprisingly, telomerase activity was increased under oxidative stress in HSPCs. This induction was opposite to the proliferation pattern and was neither associated with telomere shortening, nor a difference in TERT or TERC expression. To investigate the role of telomerase and telomeres on HSPCs, mice lacking telomerase activity with (TERC^{-/-}) and without (TERT^{-/-}) telomere shortening were evaluated. Ageing in wild type mice was associated with a specific bone marrow HSPCs phenotype, with increased LSK stem cells and more CFU-GM accumulation. Telomerase deficiency in aged TERT^{-/-} mice augmented this bone marrow ageing phenotype whereas telomerase deficiency plus telomere shortening exaggerated the effect even in young TERC^{-/-} mice, suggesting that shorter telomeres cause ageing. I also investigated the role of DR in TERT^{-/-} mice and showed that DR can ameliorate the ageing of bone marrow HSPCs in a telomerase-independent fashion. However, lack of telomerase was correlated with induced erythroid colonies, implying that telomerase disruption might impact haematopoiesis. In addition, in vivo treatment with the telomerase activator TA-65 did not change bone marrow ageing, at least in my mouse models. A schematic summary of my results is shown in Figure 7.2.

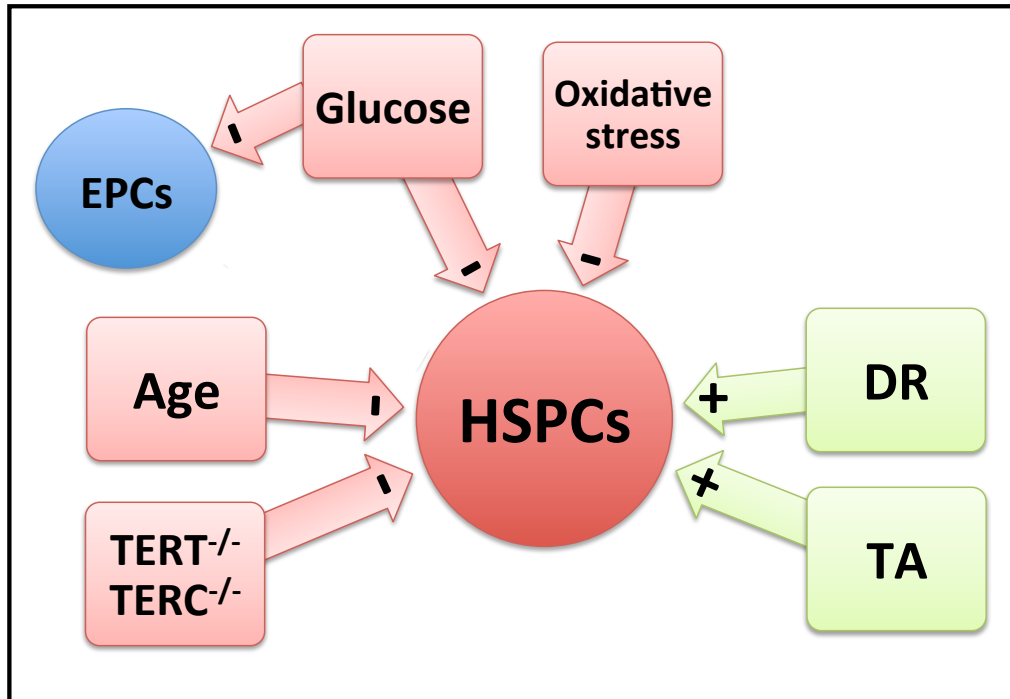


Figure 7.2: Summary of thesis findings.

Metabolic stress and oxidative stress limited HSPCs proliferation as well as age and lack of telomerase ($TERT^{-/-}$ and $TERC^{-/-}$ mice). Metabolic stress also blunted EPCs cell count. Both short-term and long-term dietary restriction can ameliorate HSPCs ageing. Telomerase activator (TA) might also have the potential to improve bone marrow HSPCs ageing.

In conclusion, my data suggest a pivotal role of metabolic and oxidative stress on early EPC and HSPC homeostasis. Although most of my experiments were limited to in vitro effects, our mice models showed interesting and novel findings with basic ageing effects. Experiments in mice were a measure of bone marrow in situ ageing, unlike bone marrow transplantation studies where homing is a complication. However, future experiments should reveal more in-depth pathways associated with those ageing phenotypes that will open gates to new medical interventions.

Appendix

Presentation and publication

- **Presentation:** Role of telomere biology and oxidative stress in the regenerative capacity of haematopoietic stem cells. For Newcastle University vice-chancellor Prof. Chris Brink. **Venue:** Hanger Lab, IGM, Newcastle University (03/02/2012).
- **Poster:** Role of telomere biology and oxidative stress in the regenerative capacity of haematopoietic stem cells. Saudi International conference 2012. **Venue:** Brunel University, London (26/10/2012).
- **Poster:** Role of telomere biology and oxidative stress in the regenerative capacity of haematopoietic stem cells. North East Postgraduate Conference (NEPG) 2012. **Venue:** Hancock museum, Newcastle University (26/10/2012).
- **Talk:** The role of telomerase and telomeres in haematopoietic stem and progenitor cells during ageing and dietary restriction. For Prof. Thomas von Zglinicki group. **Venue:** IAH Edwardson meeting room, Newcastle University (03/12/12).
- **Poster:** Role of telomere biology and oxidative stress in the regenerative capacity of haematopoietic stem cells. IAH/IGM research day. **Venue:** IGM, Newcastle University (25/01/2013).
- **Poster:** Role of telomere biology and oxidative stress in the regenerative capacity of haematopoietic stem cells. NESCI research day. **Venue:** Newcastle University (24/06/13).
- **Poster:** Role of telomere biology and oxidative stress in the regenerative capacity of haematopoietic stem cells. 12th International conference on oxidative stress, redox states & antioxidants. **Venue:** Paris, France (03/07/2013).
- **Manuscript:** Nouf Al-Ajmi, Gabriele Saretzki, Colin Miles and Ioakim Spyridopoulos. Dietary restriction ameliorates telomerase-dependent and -independent haematopoietic ageing in situ. Submitted to the Journal of Experimental Gerontology (26/02/2014).

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