ANALYSIS OF TELOMERE LENGTH OF SUBPOPULATIONS OF PRIMARY HUMAN HEMATOPOIETIC CELLS.

by

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Abstract

Telomeres are structures at the ends of eukaryotic chromosomes; they protect the ends from degradation and end-to-end fusions. Mammalian telomeres consist of a tandem array of G-rich repeats, with a length of two to 12 kb in human somatic cells. Somatic cell telomere length shortens with each cell division, leading to senescence or apoptosis. Critically short telomeres can lead to genomic instability, thus in cells that divide continually, such as germ cells, telomere length is maintained by telomerase. In a rare autosomal dominant form of dyskeratosis congenita, patients carry a mutation in the RNA template of telomerase, resulting in half maximal telomerase activity. Patients with this genotype die of aplastic anemia, indicating that maintenance of telomere length is likely critical, and may be particularly important in hematopoietic stem cells. Telomere length can be measured by a quantitative fluorescence *in situ* hybridisation and flow cytometry based method (Flow-FISH). In the present study, this technique was used to measure telomere length in lymphocytes and in "candidate" stem cell populations isolated from eight cadaveric marrow samples from normal adults (aged 14 to 48 years). Telomere length analysis of B and T cells – two populations that undergo activation-induced telomerase up-regulation – revealed that $CD20^+$ B cells in the bone marrow had longer telomeres than $CD3^+$ T cells ($p<0.002$). The $CD34^+CD38^-$ populations from each of the eight donors also had longer telomeres than the T cell subsets. The telomerase activity in T lymphocyte populations thus appears insufficient to prevent telomere shortening during differentiation from the pluripotent stem cell. To compare telomere length in different subsets of primitive hematopoietic cells, we analysed FACS-purified $CD34^+CD38^-$ and $CD34^+CD38^+$ populations. The $CD34^+CD38^-$ cells had significantly longer telomeres than the $CD34^+CD38^+$ cells ($p<0.02$, $n=8$). The Side Population (SP) cells identified by FACS as Hoechst 33342^{lo} cells did not have significantly longer telomeres than the

$CD34^+CD38^-$ population. In two donor samples, additional populations were sorted, and the data is consistent with the proposed hierarchical pattern of these cell populations, with $CD34^+CD38^-$ thought to have the greatest proliferative potential and the longest telomeres. This data supports the hypothesis that hematopoietic cells with the greatest proliferative potential will have the longest telomere length. It is still unclear if a hematopoietic cell population exists within the SP with "germ-line" or "fetal" length telomeres. While we cannot exclude that a small subset of more primitive cells capable of maintaining long telomere length exists within the sorted SP cells that were analysed, we conclude that telomere length declines in the majority of the primitive hematopoietic cells examined here.

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List of Abbreviations

AA	Aplastic anemia
AD	Autosomal Dominant
AR	Autosomal Recessive
BM	Bone Marrow
BSA	Bovine Serum Albumin
CRU	Competitive Repopulating Unit
DKC	Dyskeratosis congenita
DMEM	Dulbecco's Modified Eagle's Media
DN	Dominant Negative
FACS	Fluorescence Activated Cell Sorter
FBS	Fetal Bovine Serum
FITC	Fluorescein isothiocyanate
Flow FISH	Flow Cytometry-based Fluorescence *in situ* Hybridisation
HSC	Hematopoietic Stem Cell
HSCT	Hematopoietic Stem Cell Transplantation
LTC-IC	Long Term Culture-Initiating Cell
PBS	Phosphate Buffered Saline
PE	Phycoerythrin
PNA	Peptide Nucleic Acid
Q-FISH	Quantitative Fluorescence *in situ* Hybridisation
SF	Serum Free
SP	Side Population
TERC	Telomerase RNA Component
TERT	Telomerase

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Chapter 1. Introduction

The replicative potential of hematopoietic cells is of critical importance for the maintenance of hematopoiesis during the lifetime of an organism. Stem cells are typically defined as pluripotent cells with self-renewal potential. Although the term self-renewal suggests unlimited replicative potential, it is as yet unclear whether the replicative potential of normal hematopoietic stem cells (HSC) is finite. Telomere length is a parameter that determines the proliferative potential of many cells *in vivo* and *in vitro*. Abnormal regulation of telomere length in hematopoietic stem cells, or the cells they generate, is associated with certain diseases, including aplastic anemia and dyskeratosis congenita [1, 2]. Characterising telomere length in HSC populations and more differentiated hematopoietic cells is therefore important in understanding normal development and disease states. Telomere length of different types of hematopoietic cells was investigated in this thesis project.

1.1. Telomeres and Telomerase

Telomeres are structures at the ends of eukaryotic chromosomes. They function to protect chromosome ends from degradation and to prevent end-to-end fusions, which would lead to genomic instability. Mammalian telomeres consist of a tandem array of G-rich repeats (T_2AG_3) and associated proteins [3]. In human somatic cells the telomere repeats are two to 15 kb [4]. In most somatic cells, telomeres shorten progressively with each cell division. This process can lead to instability of the genome and apoptosis [5]. The progressive shortening of telomeres with each cell division is presumably due to the inability of the conventional DNA polymerase complex to fully replicate the ends of a linear DNA molecule [6, 7] (Figure 1.1).

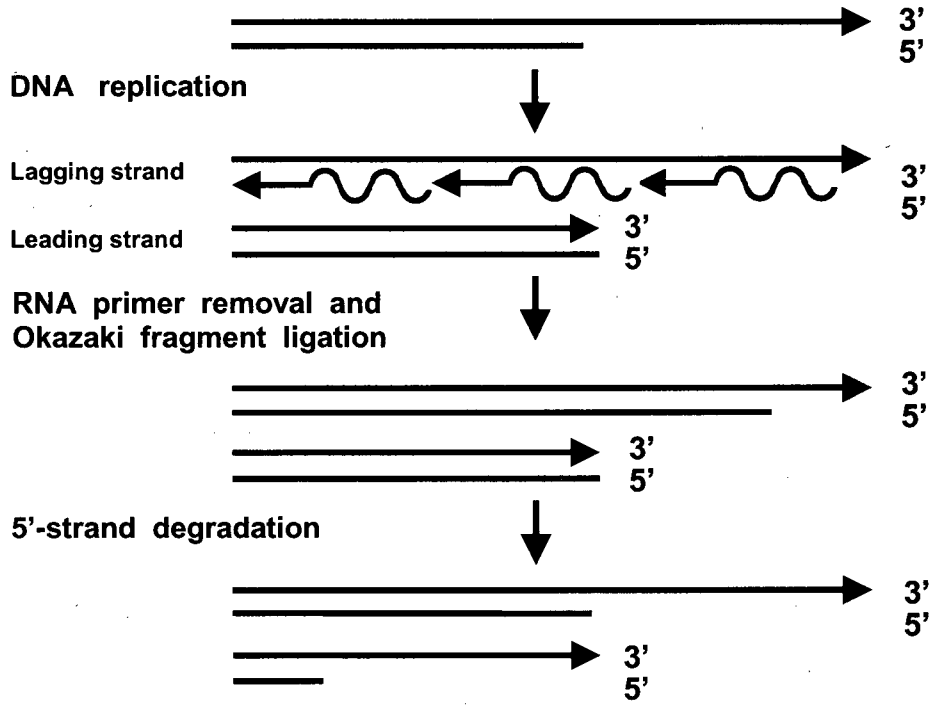

Figure 1.1 The End Replication Problem. The ends of a linear DNA duplex cannot be fully replicated by the conventional DNA polymerase complex. Lagging strand DNA synthesis produces a series of Okazaki fragments covalently attached to RNA primers at their 5' ends. Subsequent degradation of the primers, gap repair, and ligation replaces all but the last primer with DNA resulting in an unfillable gap. DNA is lost from both ends of the chromosome subsequent to replication due to degradation of 5' strands, leaving a long 3' overhang at both ends. Adapted from [8].

Cells of the germ line, such as spermatogonial stem cells, are able to maintain relatively long telomeres, 15 to 20 kb in humans, throughout the lifetime of the organism [9]. These long telomeric ends are maintained by the reverse transcriptase telomerase. Normal human cells possess a finite life span *in vitro*, known as the Hayflick limit; this is due to the loss of replicative ability in a continuously increasing fraction of cells at every population doubling [10]. The progressive shortening of chromosome ends is thought to represent a "mitotic clock", contributing to cellular senescence, and mortality of normal somatic cells [11]. In the hematopoietic system, there is an overall age-related decline in telomere length in nucleated blood cells [12].

1.1.1. Telomere Structure: The T-Loop

The telomere is thought to take on the structure of a T-loop, which is a loop of several kilobases of telomeric DNA postulated to sequester the natural chromosome ends. It is proposed that formation of the T-loop is by invasion of the 3' overhang into the double helix of the telomeric repeat array [13]. There is extensive variability in loop size between different species, ranging from 0.3 kb in trypanosomes to 30 kb in in-bred mice [14, 15]. The crucial feature of the T-loop is the sequestering of the terminal telomeric sequence into the double stranded telomeric DNA. While the exact base structure of the loop is unknown, it is postulated that it is formed by a short segment of single stranded DNA, possibly representing the D-loop of T_2AG_3 repeats that are displaced by the invading 3' overhang. A short segment of the C-strand (the 5' end) may also invade, resulting in the formation of a Holliday junction. Thus, the T-loop resembles a DNA recombination intermediate as it occurs during re-initiation of a stalled replication fork or during homologous recombination.

Pot1p (protection of telomeres protein) is a candidate for the factor that binds the telomeric end. Pot1p was recently identified in *Schizosaccharomyces pombe* and humans based on orthology to

a similar protein from the ciliate *Oxytricha nova* [16]. Pot1p is postulated to play a role in prevention of degradation of the chromosome ends and loss of Pot1p in *S. pombe* leads to immediate chromosome instability [16]. This is in contrast to loss of telomerase function, which leads to the gradual shortening of telomeres over many generations; no immediate effect on chromosome stability or cell viability is observed [17, 18]. In *S. pombe*, Pot1p appears to be more important than telomerase for the short-term maintenance of telomere structure and function. It is postulated that Pot1p is involved in the recruitment of telomerase to the telomere, and/or the stabilisation of the T-loop structure by capping the 3' overhang of telomeric DNA [16].

1.1.2. Telomere Associated Proteins: Roles of TRF1 and TRF2

The telomere structure is stabilised by two main telomere-associated proteins, TRF1 and TRF2, which bind to tandem arrays of duplex T_2AG_3 repeats [19, 20]. TRF1 functions as a dimer, and is involved in regulation of telomere length in human cells [21]. TRF1 is thought to behave as a negative regulator of telomere maintenance by inhibiting the activity of telomerase at the ends of individual telomeres [22]. TRF2 coats the entire length of all human telomeres during all stages of the cell cycle; it is a small ubiquitously expressed protein, thought to be present at 100 copies per chromosome end. It is assumed that the specific sequence, T_2AG_3, of the telomere is requisite for TRF2 binding. The expression of a mutant telomerase that adds aberrant repeats lacking TRF2 binding sites results in telomere dysfunction similar to that seen with TRF2 inhibition [23, 24]. The inhibition of TRF2 by expression of a dominant negative (DN) form, $TRF2^{\Delta B\Delta M}$, results in loss of a functional telomere; G-strand overhangs are lost from the telomeric ends and consequently, chromosomal ends covalently fuse [21]. In primary lymphocytes, TRF2 inhibition via expression of the

DN form leads to apoptosis. However, in primary fibroblasts, similar inhibition of TRF2 leads to senescence, not apoptosis (reviewed in [20]).

1.1.3. The Telomerase Complex

The telomerase enzyme is approximately 1000 kDa, and consists of two main components: hTERC, an RNA molecule encoded on human chromosome 3q26.3 that acts as a template, and hTERT, encoded on human chromosome 5p15.33, which has reverse transcriptase activity. Telomeric DNA at the linear 3' end of a chromosome is extended by the catalytic activity of telomerase (Figure 1.2). Telomerase action results in the *de novo* synthesis of 6 bp tandem T_2AG_3 repeats at the 3' ends of chromosomes. The telomeric extension is completed by normal lagging strand synthesis.

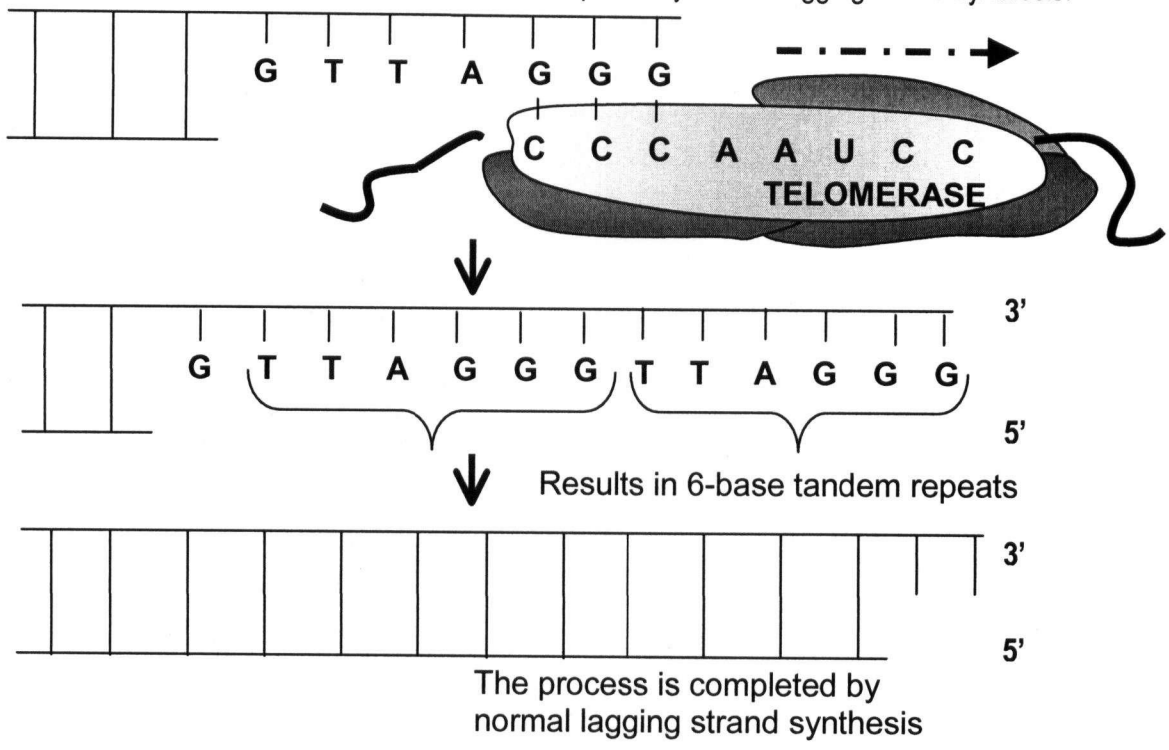

Figure 1.2 Elongation of the Telomere by Telomerase. Telomerase extends the 3' end of the chromosome leaving behind 6 bp tandem repeats of the sequence T_2AG_3. Normal lagging strand synthesis completes the extension of the telomere, leaving behind a 3' overhang.

The accumulation of mature hTERC requires the presence of an H/ACA RNA motif, which is composed of primary and secondary structural elements in an overall hairpin-Hinge-hairpin-ACA configuration [25, 26]. The H/ACA motif is phylogenetically conserved among vertebrate telomerase RNA [27]. In human cells, telomerase is partially localised to the nucleolus [25]. This localisation may be regulated by transformation and cell cycle status (unpublished data referred to in [28]). The mammalian hTERC molecule is folded into a secondary structure, and interacts with a number of proteins such as dyskerin, Tep1, a Staufen homolog, ribosomal L22 protein, and foldasome pathway proteins (reviewed in [29]). While hTERT and hTERC are the basal requirements for an active telomerase enzyme, the *in vivo* telomerase complex is likely much larger, involving many additional components.

1.1.4. Telomerase Regulation during Normal Human Development

Telomerase is active in human germ line and developing embryonic tissues, but not in the majority of adult somatic tissues [30, 31]. Telomerase activity has been detected in fetal, newborn and adult testes, and fetal ovaries, but not in mature spermatocytes or oocytes (reviewed in [32]). These data are consistent with the quiescent nature of mature germ cells and the observation that quiescent cells are telomerase negative, irrespective of their potential ability to express telomerase [32]. Telomerase levels are high at the blastocyst stage, a level that is maintained in most tissues until the end of the first trimester. Telomerase activity persists in a subset of fetal somatic cells as gestation proceeds (liver, intestine, lung, skin, muscle, adrenal glands, and kidney) but is absent from brain and bone extracts. Telomerase activity declines in adrenal gland, muscle, lung, skin and liver as fetal development progresses [31]. While the majority of human adult somatic tissues are telomerase negative, telomerase remains active in a selected set of adult somatic cells. Telomerase activity has

been detected in hematopoietic cells, basal keratinocytes, and epithelial cells in the endometrium, oesophagus, prostate, and pancreas [33]. The activity of telomerase, and its potential effect on telomere length has been studied in the hematopoietic system, and will be discussed in detail to follow.

1.1.5. Telomere Shortening and Senescence

Unprotected chromosome ends, resulting from a telomere length insufficient to adopt the T-loop structure, may trigger a senescence-inducing signal within the cell. The role for telomere shortening in senescence has been supported by a number of studies. First, normal human cells proliferating in culture display progressive telomere shortening [34-36]. Second, cells collected from older organisms, which are presumed to have undergone an increased number of cell divisions *in vivo*, display a shorter life span in culture and shorter telomeric DNA [37, 38]. Third, ectopic expression of hTERT restores telomere length in fibroblasts and several other cell types, and allows extended population doublings in culture [36, 39-41]. Finally, nuclei of senescent cells used for nuclear transfer cloning of animals results in escape of the nucleus from a senescent state [42]. The telomere length of senescent nuclei is critically short upon nuclear transfer; however, telomere length is extended in the cells of the cloned animals during development and the resulting cell is released from its senescent state [42]. Inhibition of TRF2 in G_1 cells results in the induction of apoptosis prior to the onset of DNA replication [20]. This indicates that telomere replication and chromosome segregation are not required to generate the apoptotic signal [20]. In some cell types senescence is induced by TRF2 inhibition or telomere shortening; this response largely resembles that of extensive DNA damage (reviewed in [20]).

1.1.6. Telomere Shortening and Oxidative Damage

Telomere shortening has been shown to be stress-dependent. Normal fibroblasts cultivated under conditions of oxidative stress, such as mild hyperoxia, enter replicative senescence prematurely, and have an increased rate of telomere length shortening [43]. The mechanism of this enhanced telomere shortening is thought to involve damage to the telomeric DNA by free oxygen radicals [44]. It has been reported that the G-rich repeats of telomeric DNA preferentially accumulate DNA lesions, specifically single strand DNA breaks, and loss of bases from the DNA backbone. The accumulation of these lesions is higher in telomeric DNA than in both repetitive non-transcribed regions of the genome (such as minisatellite sequences) and the rest of the coding and non-coding genome [45]. The model of telomere shortening and signalling suggests that the T-loop is able to prevent the DNA repair machinery from accessing the damaged telomeric DNA. This leads to the accumulation of single strand DNA breaks and sites lacking DNA bases at the telomere. These lesions may lead to telomere shortening by transient stalling of the replication machinery, or if the lesions are severe, the T-loop may be destabilised, leading to opening of the capping structure and exposure of the G-rich 3' overhang. This is thought to signal a DNA damage response, such as p53-mediated cellular senescence and apoptosis [44].

1.2. The Hematopoietic System

Hematopoiesis is the formation of newly differentiated blood cells. These cells originate during embryogenesis from primitive mesodermal cells, which become specified to a hematopoietic fate early during development. The hematopoietic system develops in two phases. Initially, embryonic hematopoiesis is established in the yolk sac. Following that, adult or definitive hematopoiesis is established within the embryo proper [46]. Establishment of a circulatory system coincides with the fetal liver being designated as the primary hematopoietic organ. Subsequently, the bone marrow and lymphoid tissues are colonised, becoming the primary sites of hematopoiesis in the adult [47-49].

HSC are defined as cells that can give rise to all of the lineages of the blood for the lifespan of the organism (Figure 1.3). These include the three classes of cellular elements of the blood: erythrocytes, leukocytes, and platelets. HSC self-renew and replenish the blood with these cellular elements. A multi-step process is thought to accomplish the sequential restriction of lineage options. This model is supported by the identification in mice of a common lymphoid progenitor, which then develops into B, T and NK cells, and a common myeloid progenitor, from which all of the other cells of the blood develop (including erythrocytes, platelets, monocytes, neutrophils, eosinophils, and basophils). During normal hematopoiesis, the number of new mature blood cells produced by the hematopoietic system equals the number of blood cells that die during a given period of time [50]. The number of cell divisions undergone by human HSC during steady state hematopoiesis and regeneration of the hematopoietic system following bone marrow injury (such as total body irradiation) is not known, but is expected to be 20 to 100 cell divisions [51].

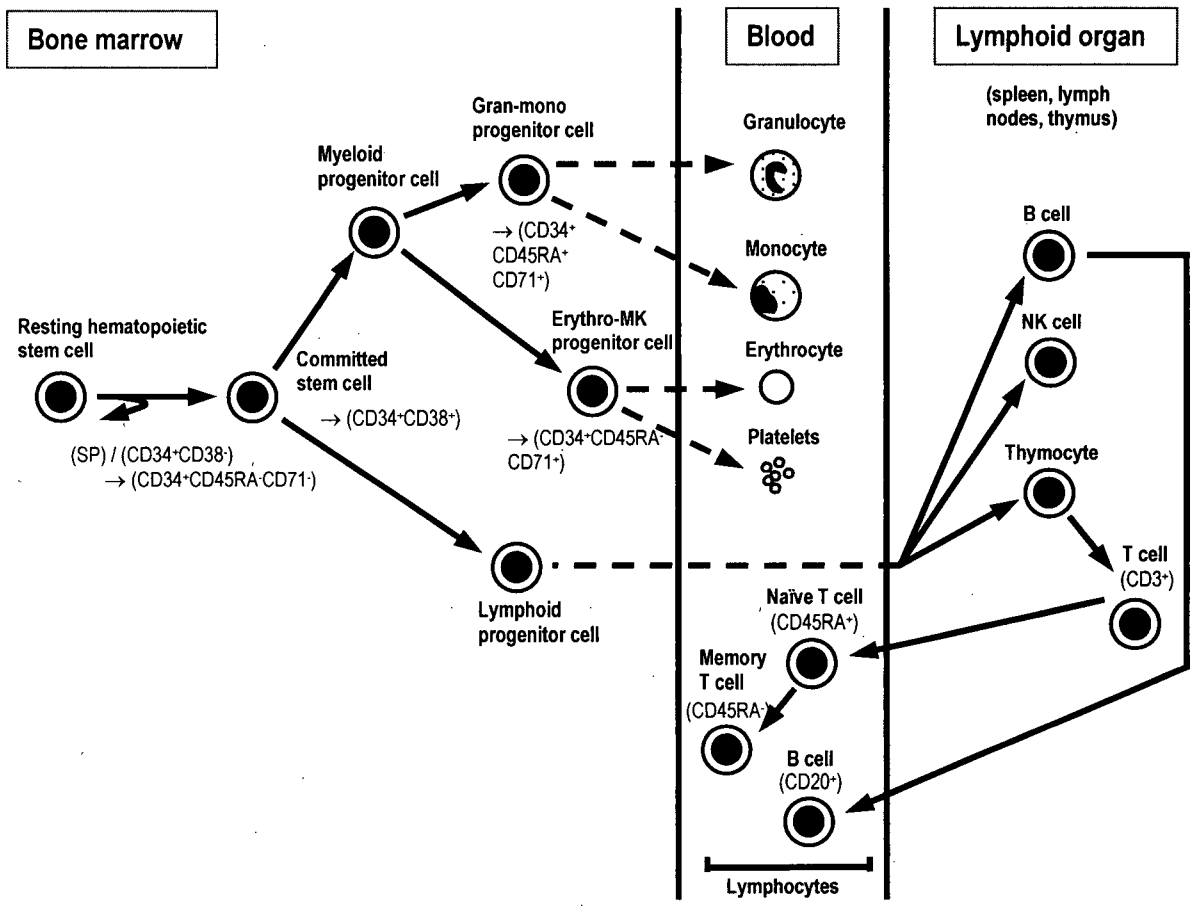

Figure 1.3 Normal hematopoiesis: a hematopoietic stem cell gives rise to all lineages of the blood. Cellular markers in grey brackets are used throughout this work to classify the indicated populations phenotypically based on current data. Adapted from [52].

1.2.1. Hematopoietic Stem Cells

1.2.1.1. Characterisation of Hematopoietic Stem Cells

Hematopoietic progenitor cells are defined using various *in vitro* and *in vivo* assays. These have allowed pluripotent, and various lineage-restricted types of myeloid progenitor populations to be distinguished. The mouse has been used to study hematopoietic stem cell activity *in vivo* using competitive repopulating units (CRU). This assay has provided evidence of the ability of some individual mouse hematopoietic cells to reconstitute the hematopoietic system of the recipient (reviewed in [50]).

Quantitative studies of transplantable human HSC cannot be readily performed in human recipients; however, the use of immune deficient mice (such as NOD/SCID mice) for xenogeneic transplantation has indicated that human hematopoietic cells can home to the bone marrow of these mice and function to repopulate the hematopoietic system, producing both lymphoid and myeloid progeny. This has been used to define human CRU using limiting dilution assays of cells able to generate both myeloid and lymphoid cells in NOD/SCID mice (reviewed in [50]). The *in vitro* assay, LTC-IC (long-term culture-initiating cells), is also used for the detection of primitive hematopoietic cells and is based on their ability to produce clonogenic progenitors of mature granulocytes and macrophages for at least five weeks when co-cultured with marrow stromal cells (reviewed in [50]). These assays allow for the biological identification and characterisation of cells with stem cell properties and their characterisation in terms of cell surface marker expression.

1.2.1.2. Cell Surface Markers of Hematopoietic Cells

A widely used phenotypic marker of human hematopoietic stem cells is the CD34 antigen [53]. CD34 is a sialomucin-like adhesion molecule selectively expressed on human hematopoietic progenitor cells [54]. Human cell populations highly enriched for the CD34 antigen are capable of repopulating the hematopoietic system of both sub-lethally irradiated immune deficient mice [55, 56] and human stem cell transplant recipients [57-59]. $CD34^+$ cells can be further classified into a population of stricter definition by including additional markers, such as CD38, CD45RA and CD71. For example, cells that are $CD34^+CD38^-$ contain a population with greater proliferative potential than the population delineated by the CD34 antigen alone. Human CD38 is a type II trans-membrane glycoprotein, which possesses an enzymatic activity, is able to mobilise calcium, to transduce signals and to adhere to hyaluronan and other ligands [60]. In humans, the CD38 antigen is expressed on >80% of medullary thymocytes, activated T cells, >90% of bone marrow B cell progenitors, plasma cells, Natural Killer cells, myeloid cells, circulating monocytes, erythrocytes, and platelets [61]. The passage from primitive or uncommitted progenitor cells ($CD34^+CD38^-$) to a committed progenitor population ($CD34^+CD38^+$) is delineated by expression of the CD38 antigen [62].

CD45 is a transmembrane protein tyrosine phosphatase expressed by all nucleated human hematopoietic cells [63]. CD45RA is one of the isoforms of CD45, formed by alternative splicing of exons that encode the extracellular domain of CD45. Alternative splicing leads to isoforms that associate differently with the T-cell receptor to facilitate antigen recognition [64]. CD45RA is expressed on naïve T lymphocytes, a proportion of $CD4^+$ and $CD8^+$ T cells, Natural Killer cells, and B cells [65, 66]. CD71 is the transferrin receptor [67] and is ubiquitously expressed on actively growing human cells, and its expression is particularly elevated in erythroid precursors and their terminally differentiating progeny where iron is required at a high level for hemoglobin production [68].

A novel method has been described for identifying mouse HSC capable of contributing to all peripheral blood lineages in long-term bone marrow reconstitution of lethally irradiated recipients. This method is based solely on the dual wavelength flow cytometric analysis of Hoechst 33342-stained cells [69]. Hoechst 33342 is a DNA dye that is actively pumped out by a specific pump expressed on the surface of a small population of cells [70]. Flow cytometric analysis of Hoechst 33342-stained cells reveals a population of Hoechst^{low} cellular events resembling a horse's tail to the side of the bulk population, hence termed Side Population, or SP. The SP is formed by the active removal of Hoechst 33342 from the small population of cells expressing a pump specific for this expulsion; this population includes candidate hematopoietic stem cells.

In mice, a small fraction of SP cells are Sca-1^{+} lin$^{neg/low}$ [69]. Sca-1 is a marker for a population containing primitive progenitor cells, and lin$^{neg/low}$ indicates the cell population does not contain cells with particular markers of mature blood cells (lineage negative). The Sca-1^{+}lin$^{neg/low}$ population has been independently established as containing the HSC activity in adult mouse bone marrow [71-73]. SP cells have been isolated from miniature swine, rhesus and human whole bone marrow, and human umbilical cord blood [74].

The populations phenotypically defined as CD34^{+}CD38^{-} and lin^{-}CD34^{+}SP are "candidate" stem cell populations since, while having stem cell properties and likely containing a primitive progenitor, they are not functionally homogeneous. Both the CD34^{+}CD38^{-} population and the lin^{-}CD34^{+}SP cells contain committed progenitor cells and other cell types, and do not phenotypically describe all of the HSC in the bone marrow [75, 76].

1.2.2. Replicative Potential of Hematopoietic Stem Cells

HSC play a central role in the production of all hematopoietic lineages over the lifespan of an organism. HSC are generally defined as clonogenic cells having the capacity for both self-renewal and multilineage differentiation [77]. In adult bone marrow most HSC are quiescent [78-80] and it is believed that rare divisions are sufficient to supply the homeostatic needs for new blood cell output. Evidence of stem cell self-renewal is observed in the recovery of mouse bone marrow after repeated ablation with cytotoxic agents [81], the reconstitution of the hematopoietic system of lethally irradiated mice, retroviral marking studies [82], and the repopulation of NOD/SCID mice with human HSC [83]. It is estimated that 10^{12} blood cells are produced daily in adult life, totalling $4x10^{15}$ blood cells during an entire lifespan [51]. In order to fulfil this number requirement, a single stem cell would have to divide at least 52 times and even 100 HSC would have to be able to divide 47 times. It is therefore likely that HSC have a mechanism for maintaining telomere length, a mechanism that may not be complete, resulting in an increased but finite replicative potential.

Several observations suggest that the most primitive HSC have a limited replicative potential. *In vitro* expansion potential appears to decline in a developmentally regulated fashion [84, 85]. Similarly, *in vivo* expansion potential also appears to decline [86]. Serial transplantation of either whole bone marrow, or highly purified HSC candidates results in an apparent loss of multilineage reconstituting capacity over time [87]. These observations suggest that stem cells have only a limited replicative potential, and are unable to undergo absolute self-renewal. Telomere length and telomerase may play a role in determining the replicative potential of HSC. However, other work suggests that the replicative potential of HSC is maintained during serial transplantation, and that the apparent exhaustion of HSC is in fact due to stem cell dilution [88]. Extensive *in vitro* expansion of some totipotent HSC does not result in a reduction of the long-term *in vivo* repopulating ability of these

cells [89]. It has been suggested that it is intrinsic and extrinsic variables, such as the *in vivo* cytokine environment, that may regulate and limit the ability of HSC to regenerate to normal levels after transplantation [86].

Stem cells can divide asymmetrically, resulting in the uneven distribution of proliferative potential and cell cycle properties to daughter cells [90]. HSC dividing *in vitro* undergo an alteration in cellular phenotype through differentiation into mature cell types and alterations in functional properties [91-93]. However, it is unclear if the observed changes involve cellular senescence. Phenotypically identical "candidate" stem cells derived from fetal versus adult tissues show remarkably different functional properties in terms of asymmetric cell division potential. These cells do not have the same capacity to produce identical as well as lineage restricted daughter cells [51]. Another indication of age-related changes in the stem cell compartments is CD34 expression. It has been shown that CD34 expression is subject to modulation on HSCs. In the mouse, CD34 expression changes during development. The HSC of perinatal and early juvenile mice are predominantly $CD34^+$, shifting to predominantly $CD34^-$ as the mice age beyond 7 to 10 weeks [94]. Furthermore, CD34 expression on adult mouse HSCs reflects the activation state of the stem cells, and is reversible [95].

In normal, healthy, elderly humans there are no signs of anemia or lymphopenia [5, 96, 97], indicating that the HSC pool continues to function normally throughout old age. However, increased skewing of X-chromosome inactivation in blood cells with age may suggest a depletion of the stem cell pool over time. An alternate explanation for this observed increase in skewing is that one of the two Xs offers a slight selective difference due to allelic variation (reviewed in [98]).

1.2.3. Telomerase Regulation in the Immune System

The immune system is maintained by the continual production of clonally expanded cells. Lymphocytes (both B and T cells) are the progeny of a bone marrow-derived common lymphoid progenitor. Lymphoid progenitors that stay in the bone marrow give rise to B cells. The stimulation of naïve B cells by exposure to antigen results in their concomitant expansion through multiple cell cycles. This leads to the production of germinal centre cells and eventually to memory B cells. Germinal centre cells, namely centroblasts and centrocytes, have high telomerase activity relative to resting naïve, activated naïve, and mature memory B cells, which have low or no telomerase activity [99-101]. Telomerase activity is strictly regulated during the germinal centre reaction, and is capable of causing elongation of telomeres in the B cell compartment [102] 103].

Thymocytes differentiate from a bone marrow-derived common lymphoid progenitor within the thymus. Mature thymocytes differentiate into naïve T cells upon emigration to the periphery. In the periphery, antigen contact leads to T cell activation and clonal expansion. Naïve T cells have a basal level of telomerase activity, which is up regulated during antigen-induced T cell activation [104, 105]. Telomerase activity is maintained at a high level throughout this antigen-induced T cell activation. Cytokine-induced activation of naïve T cells leads to the up-regulation of telomerase activity during clonal expansion [106-108]. While telomerase activity is high in activated T cells, it is transient and insufficient to maintain a stable telomere length. Thus, while an initial increase in telomere length has been observed in activated T cells, it is followed by a general decline in telomere length [106, 109, 110].

The hematopoietic system is telomerase competent. It has the ability to control telomerase activity according to the specific requirements of the immune system response. Cells of the immune system that are not actively dividing have undetectable or low telomerase activity. Telomerase activity

is coupled to cell cycle progression in lymphocytes [99, 107], and thus activity increases with cellular proliferation.

Telomerase activation during embryogenesis and early fetal development presumably compensates for the loss associated with rapid cell proliferation, and establishes cells with sufficient telomere length for future cell divisions during adult life. Tissues with particularly high proliferative demands, such as the hematopoietic system, see a more dramatic loss in telomere length in the first few years of life than that seen throughout adult life [12]. Telomerase up-regulation occurs in cells with high proliferative potential; this increase in activity is transient, and corresponds with increased cellular proliferation. Furthermore, activation of telomerase does not necessarily lead to a stable elongation of the telomere; it may simply function to maintain telomeres above a critical length.

1.2.4. Telomeres and Telomerase in Human Hematopoietic Stem Cell Candidates

Subpopulations of adult bone marrow that are enriched in HSC have shorter telomeres than those derived from both fetal liver and cord blood cells [111]. This suggests that HSC may be unable to fully maintain telomere length since these cells would seem the most likely candidate cell population to use telomerase-dependent telomere maintenance. $CD34^+CD38^-$ cells from fetal liver exhibit relatively high telomerase activity, and exhibit telomere length decline upon proliferation *in vitro*. $CD34^+CD38^-$ cells from adult bone marrow have relatively low levels of telomerase, and telomere length declines upon proliferation *in vitro* [111]. Telomerase activity has also been detected in non-malignant bone marrow, cord blood, and peripheral blood leukocytes, including granulocyte, T lymphocyte, and monocyte / B cell enriched fractions [104, 112]. Telomerase levels in these cell types ranged from less than 1% to approximately 40% of that seen in a telomerase-positive control HeLa cancer cell line. The level of telomerase activity in $CD34^+CD38^-$ is at least an order of magnitude less

than that of an equal number of cancer cells *in vitro* [111]. Telomere shortening is observed with proliferation in $CD34^+CD38^-$ cells, implying that the level of telomerase activity is insufficient to prevent overall telomere shortening. A proportion of mitotically hyperactive descendants of $CD34^+CD38^-$ cells may use telomerase to maintain or extend telomere length [28]. Thus, in a few cell types that are activated and required to undergo further divisions, such as lineage-restricted myeloid progenitors, B and T cells of the immune system, telomerase is up regulated.

Multiple groups have demonstrated that excessive telomere shortening occurs in multiple lineages of the blood following bone marrow transplantation (BMT) [113-115]. This suggests that increased telomere loss occurs in the HSC compartment. The accelerated proliferation demanded of HSC in recipients following BMT is thought to be the dominant cause of the accelerated telomere loss [115]. In the first year post transplant the loss in telomere length is 400 to 600 bp in neutrophils [115] and one to two kbp in monocytes and lymphocytes [114]. The magnitude of this loss is thought to represent a less drastic loss in telomere length than that experienced in hematopoietic lineages within the first year of life. However, both phenomena are thought to be due to increased proliferative demands on HSC leading to telomere attrition that is not compensated for by the low level telomerase activity.

1.2.5. Aplastic Anemia

Aplastic anemia (AA) is characterised by a decrease in cell numbers of erythrocytes, granulocytes, and platelets. Severe AA is diagnosed based on a hypocellular bone marrow for the patients age and two of the three following criteria: a platelet count of less than 20,000/mm^3, a corrected reticulocyte count of less than 1%, and a granulocyte or absolute neutrophil count of less than 500/mm^3. AA leads to fatigue, dyspnea and cardiac symptoms. Thrombocytopenia leads to

bruising and mucosal bleeding. Neutropenia leads to sharply increased susceptibility to infection [116]. The onset of AA has been associated with exposure to certain drugs and chemicals, such as chloramphenicol and benzene [116]. However, the majority of acquired AA cases are thought to result from T-cell-mediated, organ-specific destruction of bone marrow hematopoietic cells [117].

AA is generally associated with decreased numbers of immature hematopoietic cells, as measured by CD34 expression and *in vitro* assays that detect hematopoietic progenitor cell activity (discussed in [118-122]). This suggests that the pathological process of AA may also affect the stem cell compartment. A significant decrease in telomere length is observed in granulocytes from patients with AA [1]. The inability to maintain a stable or adequate telomere length may contribute to the exhaustion of the stem cell compartment. This could be due to an increased loss of telomeric DNA caused by increased proliferative demands on the remaining functional HSC. This supports the hypothesis that HSC have a limited proliferative potential, possibly influenced by telomere length.

1.2.6. Dyskeratosis Congenita

DKC is a progressive bone marrow failure syndrome characterised by abnormal skin pigmentation, leukoplakia, and nail dystrophy [2]. The cytogenetic characteristics of DKC include an increased frequency of chromosomal breaks, hyperdiploidy, and premature centromere disjunction [123]. The most common form of DKC is X-linked, followed by the autosomal dominant (AD) and putative autosomal recessive (AR) forms. The gene mutated in X-linked DKC is *dyskerin*, the human ortholog of yeast *CBF5* [124]. AD DKC is caused by a mutation mapping to 3q21-q28. In a large pedigree study, the mutation was found to be an 821 bp deletion removing the 3' 74 bp of the *hTERC* gene in all affected members [2]. The proposed interaction of dyskerin with the H/ACA motif in hTERC [25] suggests that DKC is caused by a telomerase deficiency [28]. In fact, an approximately 5-fold

reduction in hTERC levels was observed in affected males relative to maternal carriers in two pedigrees with different dyskerin alleles [125]. The similarity of phenotypes resulting from mutations in dyskerin and hTERC provides evidence that DKC is caused by a telomerase deficiency. It has been suggested that X-linked DKC will lead to a maximal telomerase activity of 20% whereas AD DKC will lead to maximal telomerase activity of 50% [28]. This could explain the increased severity and earlier age of onset seen in X-linked DKC relative to AD DKC (reviewed in [126]).

DKC is a multi-system disease, affecting highly proliferative tissues, such as the bone marrow, gut, and skin [2]. These tissues require a constant renewal of cells, suggesting that dyskeratosis may be due to a defect in stem cell turnover or proliferative capacity [2]. This stem cell defect could in turn be due to impaired telomere length maintenance, caused by a sub-maximal level of telomerase activity, especially affecting highly proliferative tissues [28]. Thus, the regulation of telomerase activity and telomere length in the hematopoietic stem cell compartment is likely of critical importance for long-term maintenance of hematopoiesis.

1.3. Telomere Length Measurement

In order to understand the regulation, function and importance of telomere length, it is critical to use appropriate methods for telomere length measurement. Three methods have been described for the measurement of telomere length: Terminal Restriction Fragment (TRF) analysis by Southern blot, Quantitative Fluorescence *in situ* Hybridisation (Q-FISH) by digital fluorescence microscopy [127], and Quantitative Fluorescence *in situ* Hybridisation by flow cytometry (Flow FISH) [128]. These three methods will be discussed, focusing on the advantages of Flow FISH as a method for telomere length measurement.

1.3.1. Terminal Restriction Fragment Length Analysis

The classical method for telomere length measurement is based on Southern blot analysis. Genomic DNA, extracted from the cells of interest (usually requiring ~10^6 cells), is digested with a frequently cutting restriction enzyme. The digested DNA is then separated by gel electrophoresis, transferred to a nylon membrane, and hybridised with a labelled $(C_3TA_2)_n$ oligonucleotide probe. A smear of hybridising DNA fragments results, representing the heterogeneity of the TRF length of all the cells analysed. Comparison of the sample to DNA length markers allows for the calculation of mean telomere length. The restriction enzyme used in TRF analysis results in the inclusion of a varying amount of interstitial telomeric sequence in the TRFs. This introduces one source of error in telomere length measurement using this technique.

1.3.2. Quantitative Fluorescence *in situ* Hybridisation (Q-FISH)

Q-FISH is a technique based on the use of $(C_3TA_2)_3$ oligonucleotide peptide nucleic acid (PNA) probes that are hybridised to the denatured DNA of chromosome metaphase spread preparations. PNA probes are synthetic mimics of single stranded DNA with bases attached to an uncharged peptide backbone [129, 130]. PNA molecules form duplex structures with both DNA and RNA of the complementary sequence. PNA/DNA duplexes have increased stability relative to DNA/DNA or RNA/DNA, and can be formed in relatively harsh hybridisation conditions that do not support DNA/DNA interactions. Thus, PNA probes can be used in a quantitative and reproducible way to measure telomere fluorescence. Images of the metaphase chromosome spreads are captured using digital fluorescence microscopy. Q-FISH allows for the accurate calculation of telomere repeat length at the ends of each individual chromosome arm. While detailed information can be obtained using the Q-FISH procedure, it is a complex protocol, requiring significant technical expertise.

1.3.3. Flow Cytometry-based Fluorescence *in situ* Hybridisation (Flow FISH)

Flow FISH is a modified Q-FISH procedure, where $(C_3TA_2)_3$ oligonucleotide PNA probes are hybridised to the denatured interphase DNA of fixed cells. The average fluorescence is then measured by flow cytometry. Flow FISH offers particular advantages over both TRF and Q-FISH. Advantages over TRF analysis include the ability to analyse multiple cell types within one sample, and process many samples with relatively low cell counts (10^5) at the same time. This aspect is of particular importance in efforts to measure telomere length in "candidate" stem cell populations, which are inherently limited in obtainable cell numbers. An advantage of Flow FISH over Q-FISH is its relative speed as compared to the time and technical skill required for cytogenetic analysis. However, Flow

FISH does not allow for the quantification of telomere length of individual chromosome arms; this data can only be obtained using Q-FISH of metaphase spreads [4, 131]. The usefulness and power of Flow FISH has been demonstrated in studies measuring telomere length in human myeloid cells and granulocytes [132], and human lymphocytes [12]. Recently, efforts have been made to limit the amount of variation in telomere length measurement during the Flow FISH procedure by optimising the various steps involved in cell processing [133]. This rigorously tested method was employed for the telomere length measurements of cell populations analysed in this thesis.

Flow FISH, a reproducible and accurate technique for the measurement of telomere length, is ideal for measuring small differences in overall telomere length of cell populations. Furthermore, the ability to process low cell numbers by Flow FISH allows for telomere length analysis of cell populations of limited availability. Thus, Flow FISH offers a promising method for the measurement of rare HSC populations, which was not feasible by other measurement techniques. Flow FISH provides a useful technique to tests the hypothesis that telomere length is an indicator of proliferative potential, and that HSC with the greatest proliferative potential will have the longest telomeres.

1.4. Objectives and Rationale

The data from dyskeratosis congenita patients [2], aplastic anemia patients [1], and the age-related decline in telomere length [12], all discussed in detail above, indicate that the overall telomere length or the maintenance of the telomere is of critical importance in the hematopoietic stem cell compartment. A functional telomere appears to be required for normal sustained hematopoiesis. It is as yet unclear as to whether a hypothesised "fetal" length telomere or the basal telomerase level of "candidate" stem cells [111] is responsible for the high proliferative potential observed in these populations. In an attempt to address the question that there is a population with "fetal" length (~15 kb) telomeres, telomere length was measured in human bone marrow subpopulations by Flow FISH.

It is currently unclear how much telomeric DNA is lost during the differentiation of HSC into mature blood cells. Several questions relating to this issue are addressed in this thesis. It was of interest to determine if the activation-induced up-regulation of telomerase in T and B lymphocyte populations is sufficient to maintain their telomere length relative to less mature bone marrow subpopulations from the same human donor. As lymphocytes typically undergo extensive clonal expansion and have high telomerase activity during this time, it was of interest to determine the length at which telomeres are maintained in mature $CD20^+$ B and $CD3^+$ T cell populations. Does this clonal expansion require the telomere length to be maintained at a length similar to that of the stem cell compartment? Furthermore, are the telomeres of bone marrow-derived $CD20^+$ B and $CD3^+$ T cells of similar length to each other, or are there consistent differences between these two populations, as is seen in data generated from peripheral blood $CD20^+$ and $CD45RA^{+/-}$ lymphocytes (G. Baerlocher *personal communication*).

Another important question being addressed is whether telomere length is an indicator of proliferative potential. It is postulated that adult HSC with the longest telomeres will have the greatest proliferative potential. In order to determine if telomere length correlates with increasing enrichment of cells with high proliferative potential, telomere length was measured in SP and $CD34^{+}CD38^{-}$ cells. These measurements were compared to telomere length from populations with a decreased proliferative potential. The populations with decreased proliferative potential as compared to the "candidate" stem cell populations have an increase in the cellular heterogeneity and an increased content of mature hematopoietic cells. The data presented in this thesis suggests that cell populations with increased proliferative potential have the longest telomere length.

Chapter 2. Materials and Methods

2.1. Human Cadaver Bone Marrow Purification, Staining and Sorting

2.1.1. Primary Tissue

Previously frozen heparinized human bone marrow samples were obtained with informed consent from the vertebral bodies of organ donors (Puget Sound Blood Centre, Northwest Tissue Bank, Seattle, WA). The frozen human cadaver bone marrow was thawed quickly in a 37°C water bath in the presence of 0.1 mg/mL DNase. The cells were slowly diluted into 50% fetal bovine serum (FBS), and centrifuged at 1200 rpm for 5 minutes. The pellet was re-suspended in one volume DNase, brought to a cell concentration of $3.5x10^7$ cells/mL in Hanks Balanced Salt Solution + 2% FBS, then layered onto Ficoll-Hypaque (Amersham Biosciences, Uppsala, Sweden). Density separation was performed by centrifugation at 2300 rpm for 20 minutes. Cells from the interphase layer were recovered and further processed by lineage depletion, antibody staining, or Hoechst 33342 staining. Viable cells were counted on a hemocytometer after samples were stained with trypan blue to identify dead cells.

2.1.2. Lineage Depletion

Two donor samples (Cad 14 and Cad 17) of density separated human cadaver bone marrow (BM) cells were lineage depleted using the StemSep™ Hematopoietic Progenitor Enrichment (StemCell Technologies, Inc, Vancouver, BC). Briefly, the magnetic column was primed with 1X

Phosphate Buffered Saline (PBS), then washed three times with Hanks Balanced Salt Solution + 2% FBS. The cells to be depleted were incubated with the antibody cocktail (anti-CD2, -CD3, -CD14, -CD16, -CD19, -CD24, -CD56, -CD66b, -glycophorin A) at room temperature for 15 minutes. The magnetic colloid mix was then added to the cell-antibody reaction, and incubated at room temperature for 15 minutes. The sample was loaded onto the primed column using a peristaltic pump, and the lineage-depleted cells were collected. Dr. Shelly Heimfeld (Fred Hutchinson Cancer Centre, Seattle, Washington) kindly provided the third donor sample (FHCad1) which was processed by CD34-positive selection using magnetic beads.

2.1.3. Hoechst 33342 and Monoclonal Antibody Staining

Human bone marrow (BM) SP cells were stained and sorted as described by Goodell *et al.* [69] with minor modifications. Briefly, human BM cells were thawed as above, stored in Serum Free (SF) media (StemCell Technologies Inc) at 4°C for 16 hours, then re-suspended at 10^6 cells per mL in pre-warmed SF media. Hoechst 33342 (Sigma) was added to the cell suspension to a concentration of 5 μg/mL and incubated at 37°C for 90 minutes. A control aliquot of $5x10^5$ cells was prepared using 5 μg/mL Hoechst 33342 (Sigma-Aldrich) and 50 μM verapamil (Sigma-Aldrich). The inclusion of verapamil during the incubation procedure blocks the formation of the SP profile.

Following Hoechst staining, the cells were spun down at 4°C and re-suspended in cold Hanks + 0.1% Bovine Serum Albumin (BSA) at $1x10^7$ cells/mL. When required, the cells were stained at 4°C for 30 minutes with monoclonal antibodies to CD34-FITC (8G12) (Terry Fox Lab, Vancouver, BC) and CD38-PE (Becton-Dickinson, San Jose, CA). Antibodies were used at a 1:100 dilution. To sort for lymphocyte populations, Ficoll treated cadaver BM was co-stained with anti-CD3-PE and anti-CD20-

FITC (Becton-Dickinson, San Jose, CA). Two cadaver BM samples were co-stained with anti-CD34-FITC (8G12), anti-CD45RA-PE (8D2) and anti-CD71 (OKT9) (Terry Fox Lab, Vancouver, BC). Cells were washed once with Hanks + 0.1% BSA, and once with Hanks + 0.1% BSA + 2 μg/mL propidium iodide (PI) for dead cell discrimination during FACS sorting.

2.1.4. Fluorescence-Activated Cell Sorter (FACS) Analysis and Sorting

Cells were analysed and sorted on a FACS Vantage (B-D, San Jose, CA). An ultraviolet laser at 350nm was used to excite Hoechst stained cells, and fluorescence was measured with Hoechst blue and red filters as described [69]. Cells with low PI staining were gated as the viable cell population. FITC or PE conjugated antibodies were excited at 488 nm and detected in a standard way. SP cells were collected using a non-linear sorting gate based on the population set by the verapamil control.

2.1.5. Flow Cytometry based Fluorescence *In Situ* Hybridisation

The average telomere repeat length of individual cells was determined by flow cytometry-based fluorescence *in situ* hybridisation (Flow FISH), as described previously [128, 133]. Sorted cells were frozen at –135°C in 100 to 200 μL of SF media including 15% DMSO until processed by Flow FISH. .2x10^5 fixed Cow thymocytes (control cells) were added to ~1x10^5 sample cells in 5% Dextrose containing 10mM HEPES and 0.2% BSA in Nunc tubes. The cells were spun at 4°C, 1200 rpm for 5 minutes. The sorted cells were then further manipulated in 96-well plates on the Hydra96 (Robins Scientific, Sunnyvale, CA). The pellet was aspirated to 10 μL, mixed, then 170 μL of hybridisation mix {20 mM Tris pH 7.1, 75% Formamide, 1% BSA, 20 mM NaCl, 0.3 μg/mL PNA-FITC (for stained sample)} was added. The cells were incubated for 10 minutes at room temperature in the dark, then

denatured at 87°C for exactly 15 minutes. The cells were then hybridised in the dark at room temperature for at least 90 minutes. Following hybridisation, the cells were washed once with 900 μL Wash I (75% Formamide, 10 mM Tris pH 7.1, 0.1% BSA, 0.1% Tween 20), then three times with 1 mL Wash I. Between each wash the cells were spun at 1500 g, 5 minutes, 16°C, and aspirated to a volume of 100 μL following each spin. The cells were washed a final time with Wash II (0.1% BSA, 0.1% Tween 20, 5% Dextrose, 10 mM HEPES), spun at 900 g, 5 minutes, 16°C, and aspirated to 20 μL. The cells were counter-stained with the DNA dye LDS 751 at 0.1μg/mL (Sigma). The cells were incubated at 4°C overnight prior to acquisition on the FACSCalibre (Becton-Dickinson, San Jose, CA).

Chapter 3. Results

The telomere length of mature hematopoietic cells and HSC "candidates" was measured by Flow FISH. The sample cells were obtained by flow-cytometric sorting of cadaver bone marrow samples from eight human donors (Table 3.1).

Donor ID	Sex/Age	Sorted Cells	Number of Cells Sorted	Number of Sample Tubes
Cad 18	F/17	$CD34^+CD38^-$	$1.0x10^5$	2
		$CD34^+CD38^+$	$1.6x10^6$	4
		$CD3^+$	$3.3x10^5$	2
		$CD20^+$	$3.4x10^5$	2
Cad 19	F/48	$CD34^+CD38^-$	$1.7x10^4$	2
		$CD34^+CD38^+$	$1.9x10^5$	2
		$CD34^+CD45RA^-CD71^-$	$8.0x10^4$	2
		$CD34^+CD45RA^+CD71^+$	$5.9x10^5$	3
		$CD3^+$	$2.4x10^5$	2
		$CD20^+$	$2.4x10^5$	2
Cad 20	F/16	$CD34^+CD38^-$	$1.6x10^5$	2
		$CD34^+CD38^+$	$9.1x10^5$	4
		$CD34^+CD45RA^-CD71^-$	$4.8x10^5$	3
		$CD34^+CD45RA^+CD71^+$	$5.0x10^5$	3
		$CD3^+$	$5.0x10^5$	3
		$CD20^+$	$8.5x10^5$	4
Cad 21	M/14	$CD34^+CD38^-$	$1.2x10^5$	2
		$CD34^+CD38^+$	$1.2x10^6$	4
		$CD3^+$	$1.2x10^6$	4
		$CD20^+$	$1.7x10^6$	4
Filter 70	M/28	$CD34^+CD38^-$	$2.4x10^5$	2
		$CD34^+CD38^+$	$6.2x10^5$	4
		$CD3^+$	$5.4x10^5$	3
		$CD20^+$	$5.6x10^5$	3
Cad 14	M/34	SP	$3.2x10^5$	2
		$CD34^+CD38^-$	$1.8x10^5$	2
		$CD34^+CD38^+$	$2.1x10^6$	4
		$CD3^+$	$9.0x10^5$	4
		$CD20^+$	$1.5x10^6$	4
Cad 17	M/17	SP	$6.8x10^4$	2
		$CD34^+CD38^-$	$1.1x10^5$	2
		$CD34^+CD38^+$	$6.1x10^5$	4
		$CD3^+$	$3.9x10^5$	3
		$CD20^+$	$6.7x10^5$	4
FH Cad 1	M/29	SP	$1.5x10^5$	2
		$CD34^+CD38^-$	$7.1x10^4$	2
		$CD34^+CD38^+$	$1.1x10^6$	4
		$CD3^+$	$1.0x10^6$	4
		$CD20^+$	$7.1x10^5$	4

Table 3.1 Summary of Cadaver marrow donor identification, sex, age, and hematopoietic cells sorted.

3.1. Telomere Length Measurement of Lymphocytes

In order to have a mature hematopoietic cell population as a telomere length reference for comparison to "candidate" stem cell populations, lymphocyte populations were analysed from eight human cadaver bone marrow donors. The CD3/T-cell receptor antigen complex is composed of several invariable CD3 polypeptides (gamma, delta, epsilon, and zeta) closely associated with the T-cell receptor. CD3 is expressed in 70-80% of normal peripheral blood T cells and on 10-20% of thymocytes [134]. An antibody to CD3 epsilon was used to define a T cell population ($CD3^+$). The CD20 antigen is a human B lymphocyte surface molecule that is widely expressed during B cell ontogeny, from early pre-B cell developmental stages until final differentiation into plasma cells [135]. It was thus used to define a bulk B cell population ($CD20^+$). The data generated from bone marrow (BM) B and T cell populations was compared to data previously generated from age-matched peripheral blood (PB) donors in $CD20^+$ B cells, $CD45RA^+$ naïve T cells and $CD45RA^-$ memory T cells. Naïve and memory T cell populations are both $CD3^+$, but their relative proportions within the CD3 subset change during normal human ageing, such that there is an increase in the number of memory T cells, and a decrease in the number of naïve T cells [136].

3.1.1. Telomere Length of Bone Marrow B and T cell Populations

$CD20^+$ B cell populations, and $CD3^+$ T cell populations were sorted from eight human cadaver marrow donors and telomere lengths were measured by Flow FISH (Table 3.1). The telomere length of the $CD20^+$ B cell populations was consistently longer than that of the $CD3^+$ T cell populations in seven of eight human donors (Figure 3.1). In donor M/29, the $CD3^+$ T cell population had longer

telomere length than the CD20+ B cell population. The increased telomere length observed in the CD20+ B cell compartment was statistically significant (Student's paired t-test; p>0.002) (Figure 3.2).

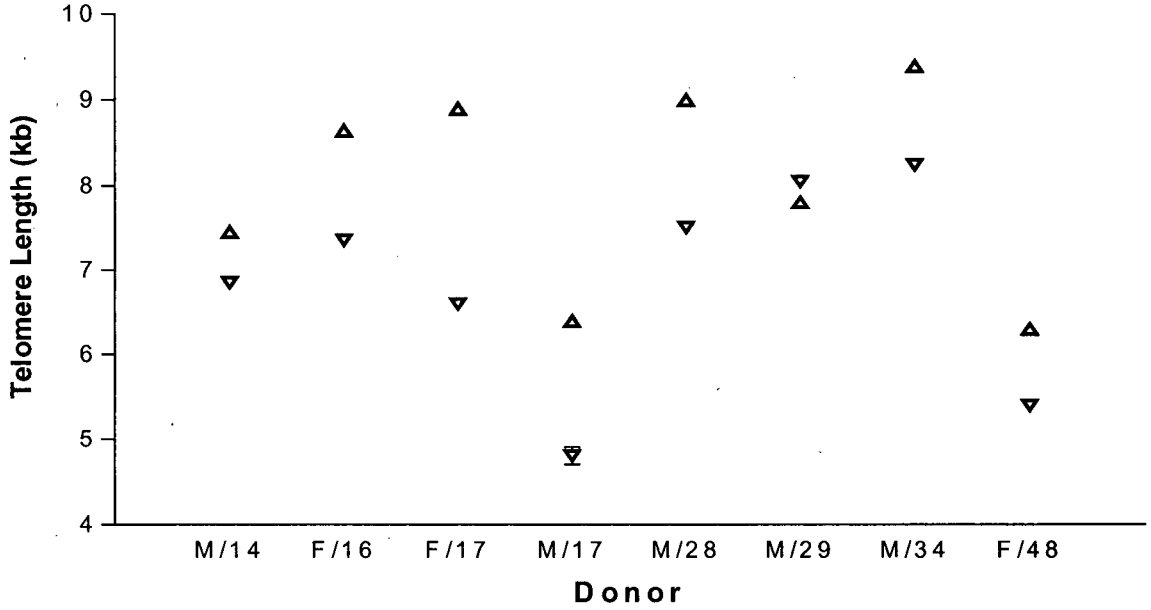

Figure 3.1 Telomere length of CD3+ T cells (▽) and CD20+ B cells (△) sorted from eight cadaver marrow donors. The CD20+ B cells have longer telomeres than the CD3+ T cells in seven of eight donor populations; M/29 had longer telomeres in the T cell compartment

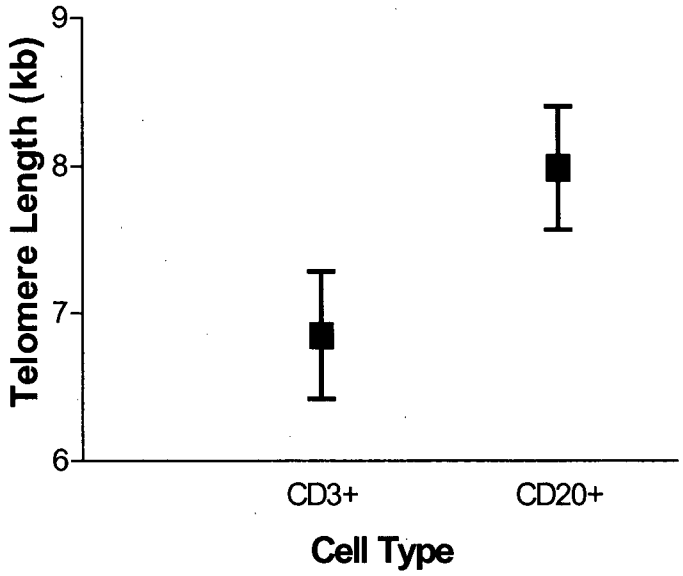

Figure 3.2 Mean telomere length of the CD3+ T cell and CD20+ B cell populations (n=8). The mean telomere length of the CD3+ T cell populations is 6.9 ± 0.4 kb. The mean telomere length of the CD20+ B cell populations is 8.0 ± 0.4 kb.

3.1.2. The Telomere Length of Bone Marrow B and T Cells Corresponds with the Telomere Length of Peripheral Blood Lymphocytes

The telomere lengths of $CD3^+$ T cells sorted from the bone marrow and naïve ($CD45RA^+$) and memory ($CD45RA^-$) T cells from the peripheral blood, as well as BM versus PB $CD20^+$ B cell populations were analysed to determine if there were differences in telomere length between lymphocytes isolated from the bone marrow versus those isolated from the peripheral blood. Telomere length data points generated from these BM lymphocyte populations ($CD3^+$ T cells and $CD20^+$ B cells) were compared to the 95% confidence interval, representing the normal range of telomere length for the given cell type from age-matched PB donors, generated by Gabriela Baerlocher in the Lansdorp lab. The confidence interval was based on the biphasic curve drawn to represent the age-related decline in telomere length – 90% of the donors at a particular age fall within the error bars.

The telomere length of bone marrow $CD3^+$ T cells was within the 95% confidence interval of both naïve ($CD45RA^+$) and memory ($CD45RA^-$) T cells from the peripheral blood (Figure 3.3). Of the eight donor samples, three had $CD3^+$ T cells that were within the normal range of telomere length of the PB naïve ($CD45RA^+$) T cells. The $CD3^+$ T cell populations from four of the eight donors had telomere lengths at the lower end of the normal range, and one of the eight donors had telomere length below the 95% confidence interval (Figure 3.3A). Seven of the eight donors had telomere length within the normal range (90% of normal PB donors) of PB memory T cells ($CD45RA^-$). One of the eight donors (M/29) had telomere length slightly above the normal range for PB memory T cell populations (Figure 3.3B).

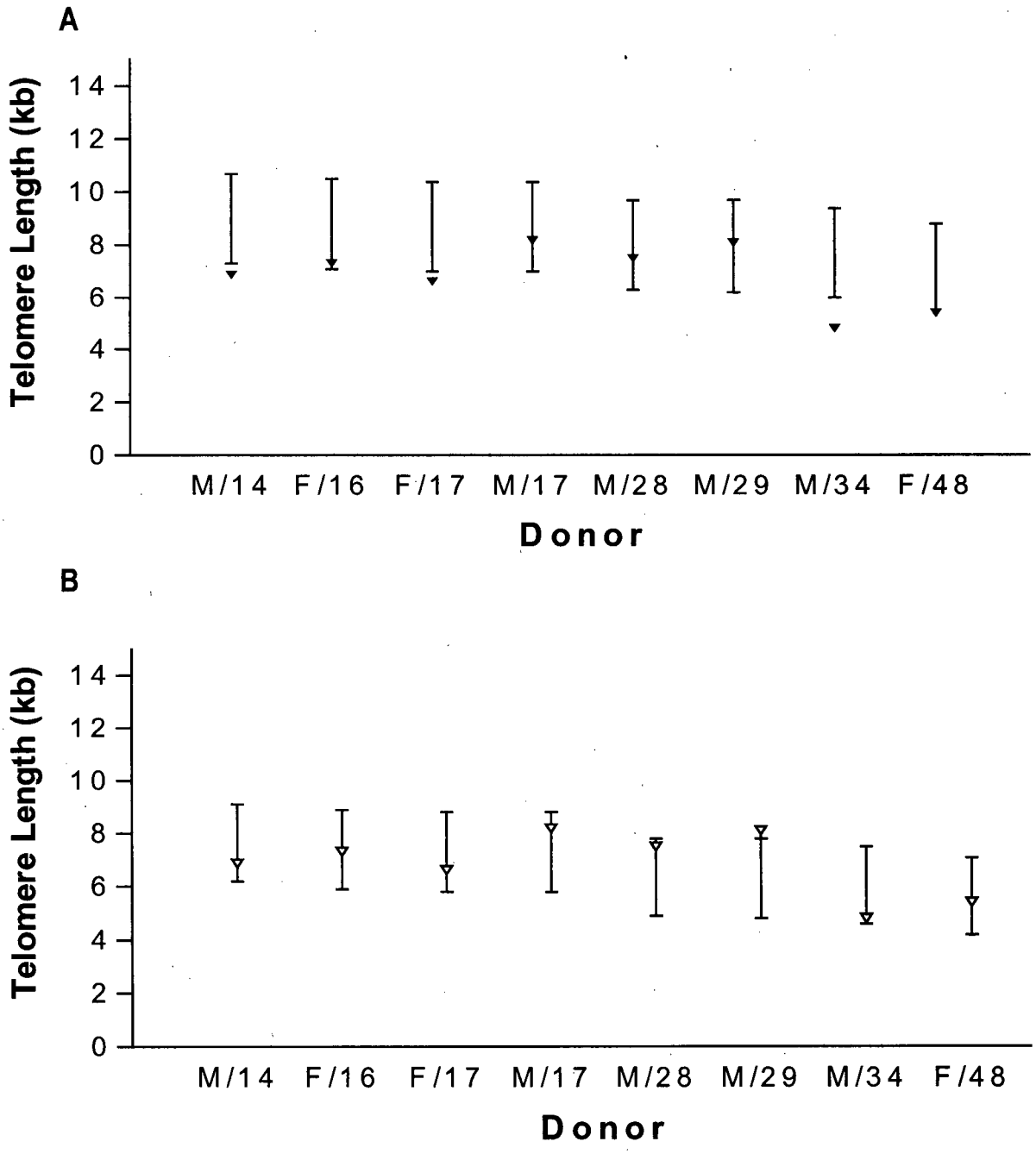

Figure 3.3 Telomere length of CD3+ T cell populations from eight cadaver marrow donors, shown with the 95% confidence interval (representing the normal range within which 95% of PB donors fall) of age-matched donors from the PB (n=400 over entire age range). **A** The BM-derived CD3+ T cell populations (▼) had telomere lengths that, for all but one donor (M/34), were within the 95% confidence interval derived from telomere length analysis of PB CD45RA+ naïve T cells. **B** All of eight cadaver marrow donors had CD3+ T cell populations (▽) with telomere length within the 95% confidence interval derived from telomere lengths of PB CD45RA- memory T cells.

The 95% confidence interval calculated from the plot of telomere length versus donor age of 400 PB donors was used to determine if the BM sorted CD20+ B cells match the data generated from the PB CD20+ cells. The CD20+ B cell populations from all eight cadaver marrow donors had telomere length within the normal range (95% of human PB donors) of PB CD20+ populations (Figure 3.4).

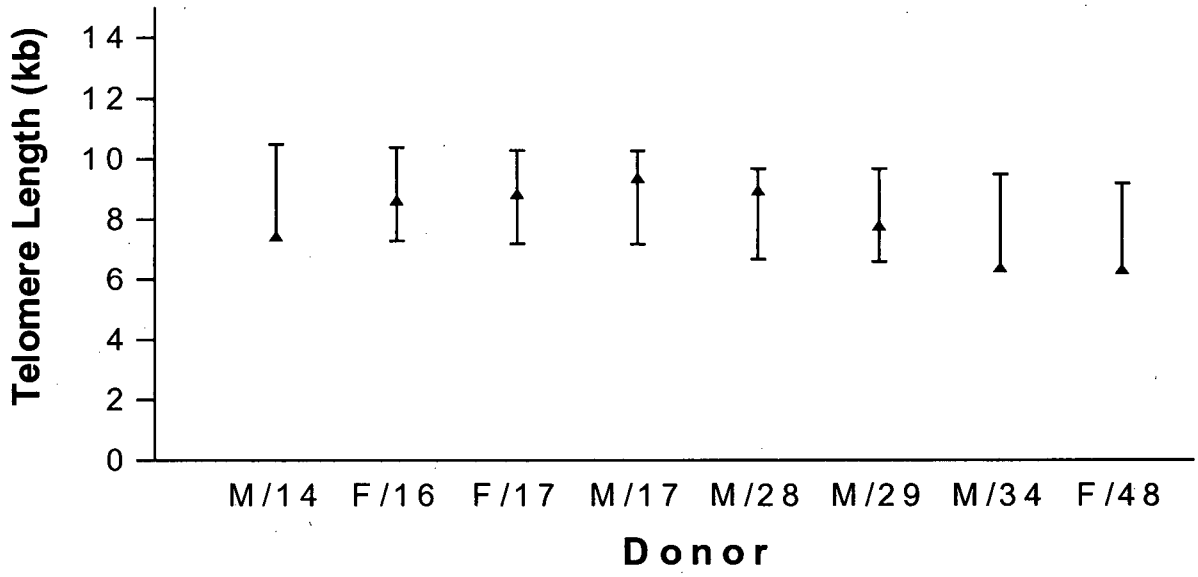

Figure 3.4 Telomere length of CD20+ B cell populations sorted from eight cadaver bone marrow donors (▲) shown with the 95% confidence intervals generated from age-matched PB donor CD20+ cells (n=400). The telomere lengths of all eight bone marrow donor CD20+ B cell populations were within the 95% confidence interval.

3.1.3. T and B cell Populations have Shorter Telomeres than a Candidate Stem Cell Population

T and B cell populations both undergo activation-induced telomerase up-regulation [100, 108] that is capable of either elongating (as is the case in B cells) or preventing excessive telomere shortening. It was of interest to determine if the telomerase activity present in these two cell populations is capable of elongating or maintaining the telomeres at a length similar to that of the stem cell compartment. To this end, the telomere lengths of $CD3^+$ T cells, $CD20^+$ B cells, and $CD34^+CD38^-$ cells (discussed in detail later) were compared. In the majority of donor cases, the $CD34^+CD38^-$ "candidate" stem cell population had longer telomeres than both the $CD20^+$ B cell population and the $CD3^+$ T cell population (Figure 3.5).

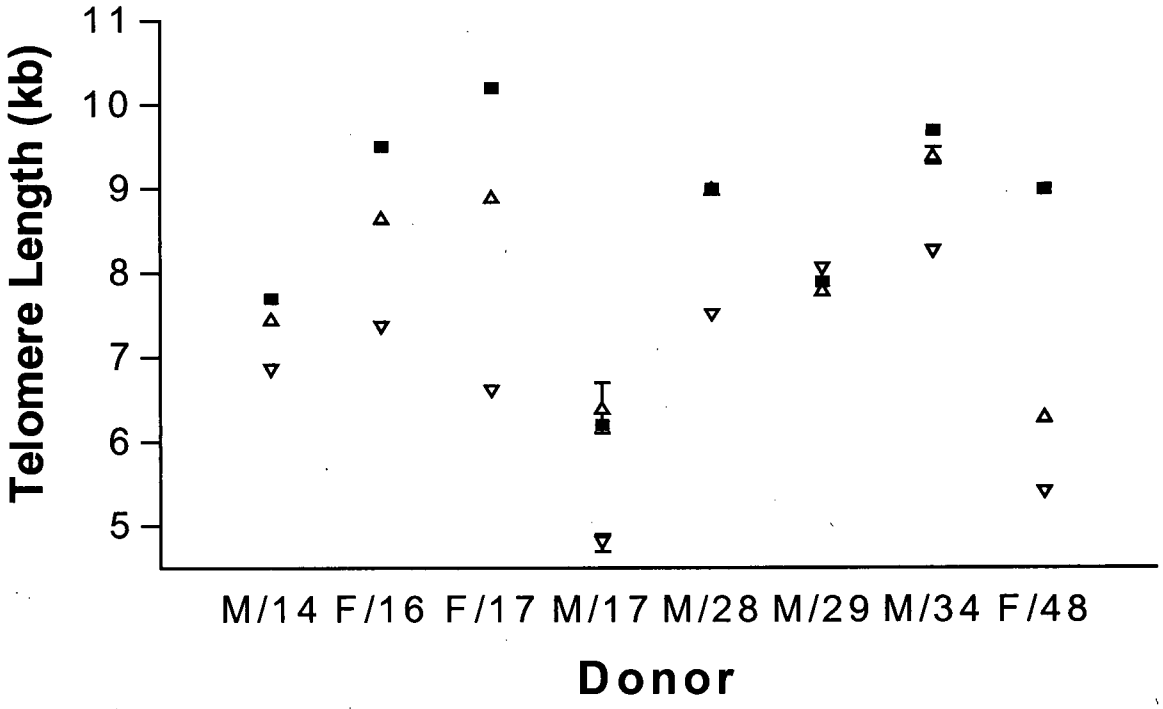

Figure 3.5 Telomere length data of eight cadaver marrow donors $CD34^+CD38^-$ (■), $CD20^+$ B cell (Δ) and $CD3^+$ T cell (∇) populations. In six of eight individuals, the $CD34^+CD38^-$ population had the longest telomeres. Donor M/29 had overlapping telomere length in all three populations. Donor M/34 had overlapping telomere length in the $CD34^+CD38^-$ and $CD20^+$ populations.

3.2. Cadaver Marrow Cell Number Limitations and Flow FISH

Stem cells are of limited number in adult bone marrow. Thus, the number of "candidate" stem cells one is able to collect from human donor bone marrow sources is also limited, leading to the potential for low cell numbers when isolating "candidate" stem cell populations. In order to determine the lower limit for analysis of a single cell population sorted from human donor BM, a cell number titration was conducted. $3x10^5$, $1x10^5$, $3x10^4$, and $1x10^4$ human cadaver BM cells with $2x10^5$ control cells (fixed cow thymocytes) were processed by Flow FISH.

It is possible to detect a population with sample input of $1x10^4$ cells (Figure 3.6). The major problem appears to be an increased variability in the loss of sample cells with decreasing cell numbers. Thus, not all replicates of $1x10^4$ or $3x10^4$ cells contain adequate cell numbers following the Flow FISH procedure to allow for a reliable telomere length calculation. In order to ensure adequate cell numbers for telomere length measurement, the optimal cell number per tube is $3x10^5$. This ensures that in every replicate there is an excess of events to acquire for telomere length measurement. In order to obtain this optimal number of cells per tube in two duplicates of one stained and one unstained sample (equalling four tubes), $1.2x10^6$ cells of interest would ideally be sorted. This was not always possible, particularly when sorting $CD34^+CD38^-$ and SP cells from cadaver marrow. The cell number limits of the Flow FISH procedure were thus thoroughly explored in its application to the measurement of telomere length of rare $CD34^+CD38^-$ and SP cell populations.

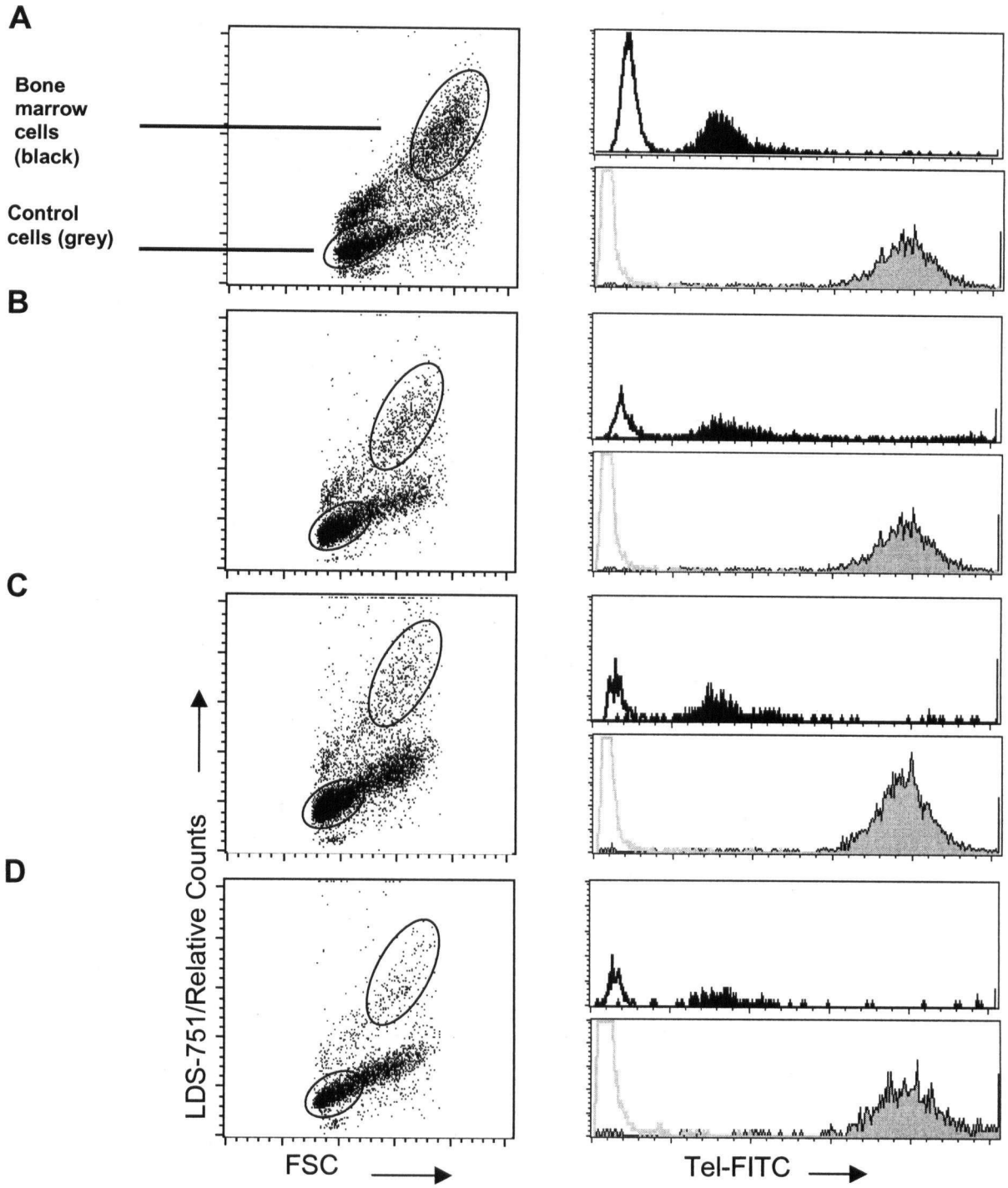

Figure 3.6 Flow FISH was used to analyse $2x10^5$ control cells with **A** $3x10^5$, **B** $1x10^5$, **C** $3x10^4$, and **D** $1x10^4$ human bone marrow cells. The sample tubes with $3x10^5$ or $1x10^5$ input cells had easily gated populations based on DNA dye content (LDS 751). A population with relatively homogeneous telomere length was identifiable by telomere fluorescence (Tel-FITC PNA probe). The sample tubes with $3x10^4$ cells had sparse populations based on LDS 751; however, a population was identifiable by telomere fluorescence. The black histogram represents the sample cells; the grey histogram is the telomere fluorescence of the control cells. The filled peak represents the cells stained with the telomere FITC-PNA probe, and the open peak represents the background, or unstained fluorescence.

3.3. Sources for Side Population Cells

In order to obtain the required number of cells (at least $1.2x10^5$ sorted cells) to ensure an accurate telomere length measurement by Flow FISH, various human cell sources and treatments of these cells were investigated for ability to provide increased proportions of SP. One potential source was fresh bone marrow filters from allogeneic bone marrow transplants. These are bone marrow aspirations, and thus are largely composed of peripheral blood. Lineage depletion of cells recovered from the bone marrow filters, while increasing the relative proportion of $CD34^+CD38^-$ cells in the sample, did not function to increase the proportion of SP; this was therefore not an ideal source, as low to no SP cells were obtained. Previously frozen human cadaver bone marrow stained with Hoechst 33342 directly after thawing also resulted in no detectable SP fraction. It is thought that the cells require time to recover from the freezing process and DMSO treatment. Cadaver marrow incubated in Serum Free media at 4^oC overnight, then stained with Hoechst 33342 showed an SP fraction of 0.05% of Ficoll treated BM cells (Figure 3.7A). Lineage depletion of cadaver marrow increased the relative proportion of SP from 0.05% to 0.8% (Figure 3.7B). CD34-enrichment of cadaver marrow resulted in an even more drastic increase in relative proportion of SP: from 0.05% to nearly 7% (Figure 3.7C).

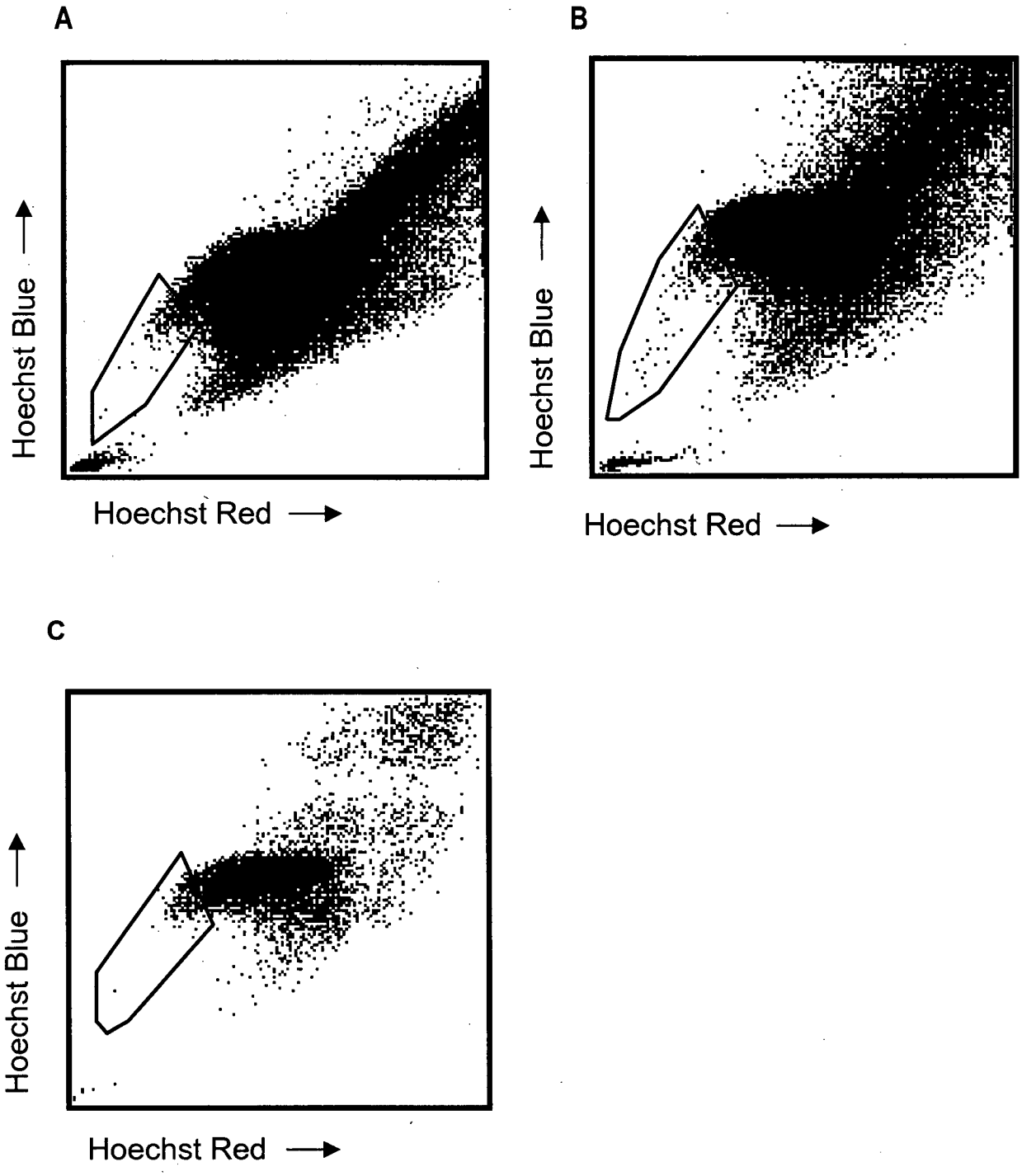

Figure 3.7 SP profile of **A** Density separated frozen cadaver bone marrow, incubated overnight at 4°C prior to staining with Hoechst 33342, and sorting. The SP fraction was ~0.05%. **B** Lineage depleted density separated cadaver marrow, incubated at 4°C overnight. The SP fraction was ~0.8%. **C** CD34-enriched human cadaver bone marrow incubated overnight at 4°C. The SP fraction was ~7%. In each case, the sort gate was based on verapamil inhibition of the SP profile.

3.4. Telomere Length in "Candidate" Hematopoietic Stem Cell Populations

Flow FISH was used to measure telomere length in sorted cell populations from eight previously frozen normal human cadaver BM donors. Of the eight donors, five were analysed for telomere length in the $CD34^+CD38^-$ and $CD34^+CD38^+$ populations. Two of the eight donors were analysed for telomere length in the $CD34^+CD71^-CD45RA^-$ and $CD34^+CD71^+CD45RA^+$ compartments, and three donors were analysed for telomere length in the SP (Table 3.1). In order to obtain sufficient numbers of cells for telomere length analysis, the frozen marrows were centrifuged through Ficoll to remove multinucleated cells and dead cells. The three donors analysed for SP were then further processed by lineage depletion or CD34-enrichment, resulting in a $CD34^+$ enriched bone marrow cell population. This increased the relative proportion of SP cells within the marrow samples from ~0.05% in whole bone marrow up to 7% in CD34 enriched marrow.

3.4.1. Telomere Length Measurements in Hematopoietic Cells Defined by the CD34 Antigen

The telomere length difference between the $CD34^+CD38^-$ and $CD34^+CD38^+$ compartments in the eight cadaver marrow donors is illustrated in Figure 3.8. Seven of the eight donors had longer telomere repeat length in the $CD34^+CD38^-$ compartment as compared to the $CD34^+CD38^+$ compartment; the maximum telomere length difference was 2.7 kb (donor F/17).

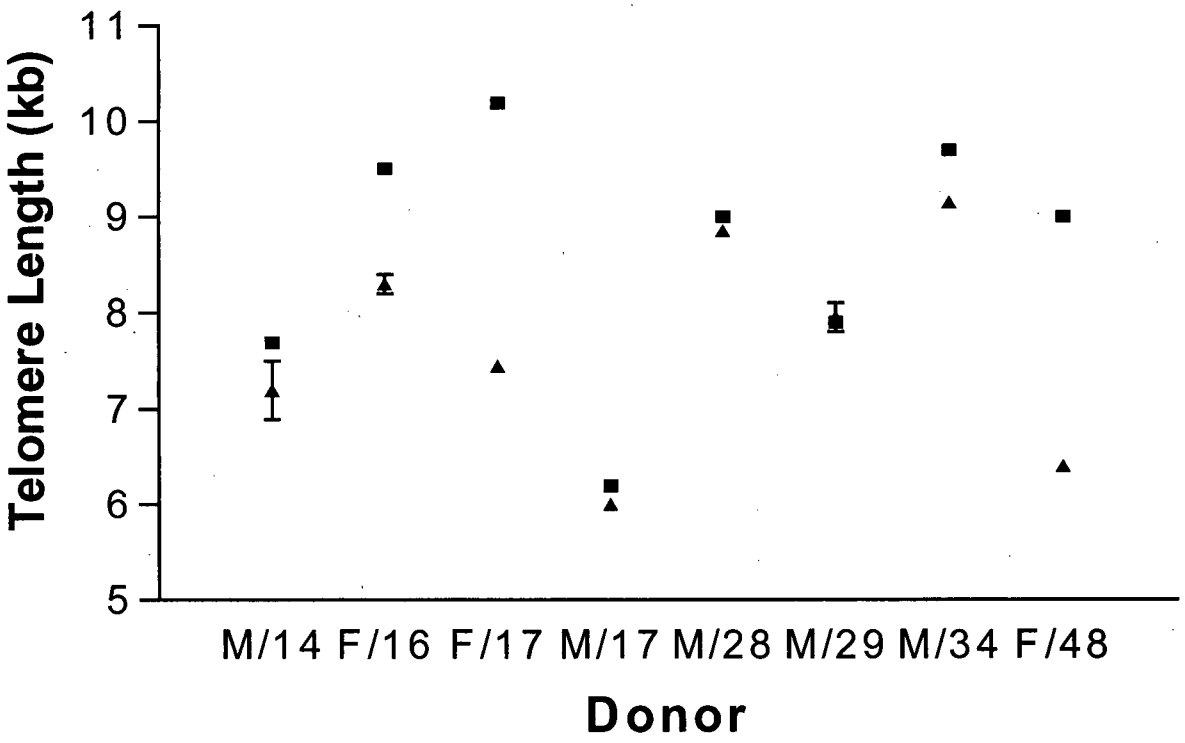

Figure 3.8 Telomere length in kb of $CD34^+CD38^-$ (■) and $CD34^+CD38^+$ (▲) cell populations sorted from eight cadaver marrow donors. Seven of eight donors had longer mean telomere length in the $CD34^+CD38^-$ populations versus the $CD34^+CD38^+$ populations. In three of the donors (M/17, M/28, M/29) the telomere lengths are very close, or overlap in absolute value.

The mean telomere length in the $CD34^+CD38^-$ compartment was 8.6 ± 0.5 kb (n=8) and the mean telomere length in the $CD34^+CD38^+$ compartment was 7.6 ± 0.4 kb (n=8). Overall, the difference in mean telomere length of the $CD34^+CD38^-$ cells compared to that of the $CD34^+CD38^+$ cells from each of the eight donor samples was statistically significant (Student's paired t-test; $p<0.02$).

In two cadaver marrow donors, additional populations were sorted: $CD34^+CD45RA^-CD71^-$, and $CD34^+CD45RA^+CD71^+$. In both donor samples, the $CD34^+CD38^-$ populations had the longest telomeres; however, in only one sample the telomere length was different in the three additional populations analysed, descending in the following order: $CD34^+CD38^-$ > $CD34^+CD45RA^-CD71^-$ > $CD34^+CD45RA^+CD71^+$ > $CD34^+CD38^+$ populations (Figure 3.9).

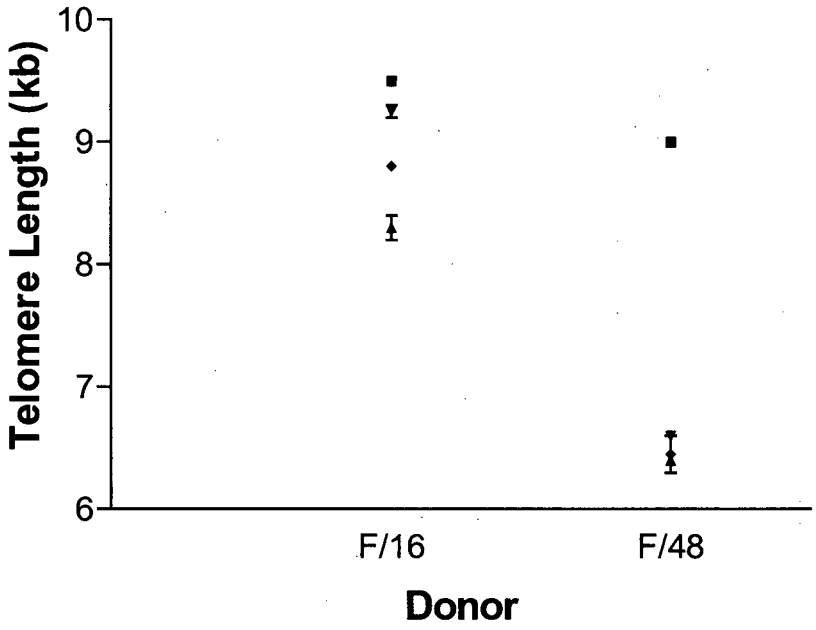

Figure 3.9 Telomere length of $CD34^+CD38^-$ (■), $CD34^+CD45RA^-CD71^-$ (▼), $CD34^+CD45RA^+CD71^+$ (◆) and $CD34^+CD38^+$ (▲) populations sorted from two cadaver marrow donors. In donor F/16 the $CD34^+CD38^-$ population had the longest telomeres, followed by the $CD34^+CD45RA^-CD71^-$ population, the $CD34^+CD45RA^+CD71^+$ population, and the $CD34^+CD38^+$ population.

3.4.2. Telomere Length in the Side Population

There is some evidence that suggests the SP contain hematopoietic cells with greater proliferative potential than contained within the $CD34^+CD38^-$ population, or that perhaps the SP contains a greater proportion of cells with greater proliferative potential. If there is a correlation between long telomere length and high proliferative potential, and if the SP does in fact contain cells with greater proliferative potential than that contained within the $CD34^+CD38^-$ fraction, it would be expected that the SP would contain a population with longer telomeres.

The range of telomere length difference observed between the SP and $CD34^+CD38^-$ compartments was limited to 0 to 0.2 kb (Figure 3.10). The SP populations from M/17 and M/34 had longer telomeres than the $CD34^+CD38^-$, which in turn were longer than the $CD34^+CD38^+$ populations. Donor M/29 had over-lapping telomere length in all three populations, but the SP and $CD34^+CD38^-$ populations were slightly longer than the $CD34^+CD38^+$ population.

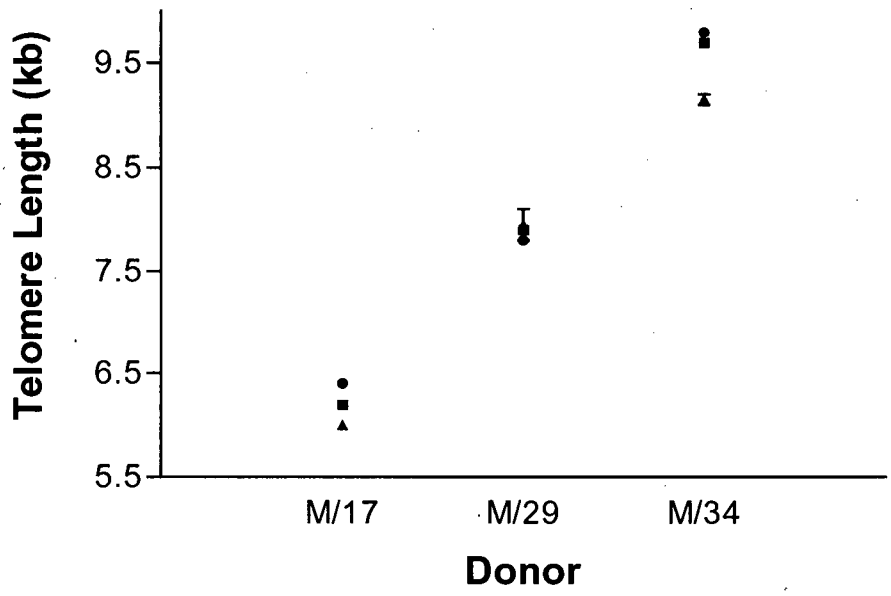

Figure 3.10 Telomere length of SP (●), $CD34^+CD38^-$, (■) and $CD34^+CD38^+$ (▲) cell populations from three human cadaver marrow samples. In two donors (M/17 and M/34) the SP populations had longer telomeres than the $CD34^+CD38^-$ populations. In one donor (M/29), the three cell populations had overlapping telomere length.

The mean telomere length in SP cells from Cad 14 (M/34), Cad 17 (M/17), and FH Cad1 (M/29) was 8.0 ± 0.9 kb (Figure 3.11). There was no statistical difference in telomere length between SP and $CD34^+CD38^-$ cells sorted from the same cadaver marrow, nor any difference upon mean telomere length comparison (Figures 3.10 and 3.11). Three donor samples were used to calculate the mean telomere length for the SP fraction. The individual telomere lengths of these donors are illustrated in Table 3.2.

Donor	Telomere Length of SP (Kbp)
Cad14 (M/34)	6.4
Cad17 (M/17)	9.8
FH Cad1 (M/29)	7.8

Table 3.2 Telomere length of SP fraction from three donors. The telomere length data from these three donors indicates the great variation in telomere length between individuals.

The large error bar observed on the mean telomere length for the SP fraction (Figure 3.11) was a result of the low donor number (n=3) and the great individual variation in telomere length between these three donors.

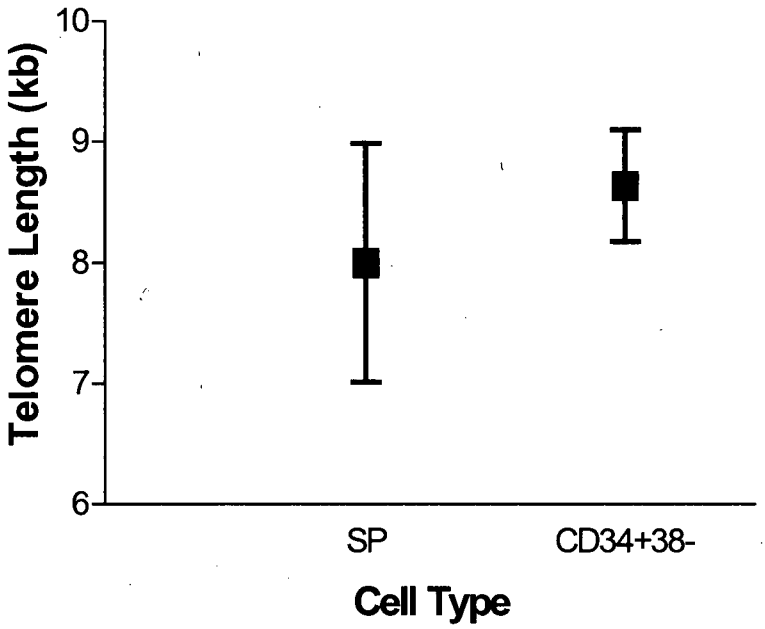

Figure 3.11 Mean telomere length of SP and $CD34^+CD38^-$ sorted bone marrow cells. The mean telomere length of the SP subset was 8.0 ± 1.0 kb. The mean telomere length of the $CD34^+CD38^-$ subset was 8.6 ± 0.5 kb. There was no statistical difference between the mean SP telomere length and the mean $CD34^+CD38^-$ telomere length from these three donors.

Chapter 4. Discussion

It is currently unknown how much telomeric DNA is lost during the differentiation of HSC into mature blood cells. Thus, it was of interest to measure the telomere length in bone marrow sub-populations from individual donors. This was undertaken in order to determine if there are tangible differences in telomere length between different "candidate" stem cell populations, and more mature hematopoietic cells, such as B and T lymphocytes. Flow FISH, a reliable and accurate technique that can accommodate lower sample cell numbers than other available telomere length measurement techniques, was used to measure telomere length in sorted bone marrow cells. This made it feasible to measure the telomere length in "candidate" stem cell populations.

In each of eight donor samples, the $CD34^+CD38^-$ population had longer telomeres than the $CD34^+CD38^+$ population. Analysis of two donor samples showed that the $CD34^+CD45RA^-CD71^-$ population had longer telomeres than the $CD34^+CD45RA^+CD71^+$ population. In these two donors, the $CD34^+CD38^-$ population had the longest telomere length of all the sorted populations. Interestingly, in three donor samples analysed, it was found that the SP populations did not have significantly longer telomeres than the $CD34^+CD38^-$ populations; however, the telomere length ranges of the SP and $CD34^+CD38^-$ populations overlap.

The $CD34^+CD38^-$ populations from the majority of donors had longer telomeres than both the $CD20^+$ B and the $CD3^+$ T lymphocyte populations sorted from the same individual. The $CD20^+$ B cell populations had longer telomeres than the $CD3^+$ T cell populations from the same individual donors, and this pattern of telomere length is consistent with data generated in the peripheral blood (G. Baerlocher *personal communication*). Furthermore, the mean telomere length of BM-derived $CD20^+$ B

cells and BM-derived $CD3^+$ T cells correlated with the age-matched mean telomere length measured for these respective populations in the peripheral blood.

The finding that the telomere length in $CD34^+CD38^-$ populations was longer than the telomere length in $CD34^+CD38^+$ populations agrees with previous telomere length data generated by terminal restriction fragment length analysis [111]. The telomere length analysis of additional subpopulations of hematopoietic cells shown here further supports the hypothesis that the cells with high proliferative potential will have long telomeres. The $CD34^+CD38^-$ population contains cells with high proliferative potential, followed by the $CD34^+CD45RA^-CD71^-$, $CD34^+CD45RA^+CD71^+$, and $CD34^+CD38^+$ populations [137]. The telomere length decreased following the same order in these populations. Interestingly, the $CD34^+CD38^-$ population is a subset of the $CD34^+CD45RA^-CD71^-$ population, and thus may be enriched for a population with longer telomere length, likely a population with stem cell activity. It has been suggested that the SP is more highly enriched for primitive progenitors than the $CD34^+CD38^-$ population [74]. Following with the previously described hypothesis of higher proliferative potential, it could be proposed that a fraction of cells within part or all of the SP, perhaps the most primitive progenitors, have significantly long, or even "fetal" length telomeres. The data presented indicates that the SP had telomere length similar to the $CD34^+CD38^-$ population, but was unable to identify a population within the SP that had longer telomeres than the $CD34^+CD38^-$ cells. This data does not, however, exclude the possibility that an elusive HSC exists with "fetal" length telomeres. If such a population represented a significant minority – for example, greater than five per cent – within a particular sorted subpopulation, we would have expected an abnormal distribution of the frequency histogram. The median, or midpoint of the telomere length frequency distribution, would be shifted from the mean telomeric value to the right, or skewed toward a longer telomere repeat length.

Two bone marrow subpopulations analysed in this study, $CD34^+CD38^-$ and SP, are less heterogeneous populations than the cell population defined by the CD34 antigen alone. This provides an opportunity to more precisely determine the telomere length of bone marrow populations with some stem cell activity. However, these populations are still heterogeneous, containing cells that do not read out as HSC in well-established assays for stem cell function [50]. These cell populations may not be of sufficient purity to enable the identification of a population with "fetal" length telomeres, if one exists. The heterogeneity of the populations may shift the frequency distribution of telomere length toward that of the more mature cells, with shorter telomere lengths. It may therefore be prudent to analyse populations of increased purity using additional phenotypic markers. However, the inherent limitations to the number of obtainable stem cells from the bone marrow may be a complicating factor.

Telomerase levels are thought to be limiting in HSC. This is based on the established donor age-related decline in telomere length of normal human granulocytes and lymphocytes [12], telomere length decline in the first year following stem cell transplantation [113-115], short telomeres observed in patients with aplastic anemia [1], and the evidence for a critical role of telomerase-dependent telomere length maintenance in HSC from dyskeratosis congenita patients [2]. Telomere length in HSC and their progeny is predicted to be an indicator of proliferative potential. The observed decline in telomere length described above supports this hypothesis, as no age-related telomere length decline would be expected in mature hematopoietic cells if their precursors expressed telomerase at levels sufficient to maintain telomere length.

Telomerase may be variably regulated depending on cellular conditions. Data from two studies suggests that telomerase is up regulated in response to the *ex vivo* expansion of hematopoietic progenitor cells [92, 138]. The data presented here suggests that the basal telomerase activity observed in the $CD34^+CD38^-$ compartment [139] is insufficient to prevent overall telomere shortening

and thus contributes to the observed telomere loss with age. This is also supported by data from dyskeratosis congenita patients, where half-maximal telomerase activity is insufficient to maintain hematopoiesis through adult life, and leads to bone marrow failure [2]. Furthermore, patients with aplastic anemia have significantly shorter telomeres in granulocytes than age-matched controls [1] suggesting that short telomere length resulting from increased proliferative demands in the HSC compartment may be contributory to bone marrow failure. Telomerase activity may be required to maintain only the shortest telomeres within HSC, allowing for extensive proliferation in the absence of the maintenance of "fetal" length telomeres. Data generated from mice null for the telomerase RNA template gene (*mTERC-/-*) suggests that the critical parameter for telomere function and cell survival is in fact the shortest telomere, and not overall telomere length [140]. This phenomenon could be at play in HSC, leading to an overall decline in telomere length and maintenance of short telomeres above a critical level.

The telomere length of hematopoietic stem cells is determined by multiple factors. The telomere length of cells in an upstream progenitor population will affect the initial telomere length of hematopoietic cells. Presumably this is not of consequence for the stem cell at the very top of the hematopoiesis hierarchy; however, it would be of consequence for cells that have some stem cell properties, but are in fact daughter cells of a more primitive precursor. The number of cell divisions a cell undergoes, and the extent of telomere loss with each round of mitosis will affect telomere length. The degree of attenuation of telomere loss by telomerase will also have a significant effect on the overall maintenance, or loss of telomere length in the stem cell compartment.

The great variation in telomere length in individual donors of cells with the same surface phenotype prevents direct donor-to-donor comparisons of telomere length data from a particular cell population. This phenomenon is obvious in the three donors from which SP cells were sorted (Figure 3.11). In this example, the overall pattern in telomere length is similar between the donors (with the SP

and $CD34^+CD38^-$ populations having similar telomere length, both longer than the $CD34^+CD38^+$ populations), but the actual telomere length values for the particular cell populations can vary by several kilobases. This increases the need to determine the telomere length in subpopulations from the same donor in order to establish a reference point for telomere length comparisons. The additional populations can therefore act as internal controls, increasing the value of the information obtainable from telomere length measurements.

Previous studies have shown that both B and T cells undergo activation-induced telomerase up-regulation [99-101, 104, 105]. Other studies have indicated that telomerase activity in B cells maintains and possibly even elongates telomere length, whereas telomerase activity in T cells is unable to prevent overall telomere shortening [102, 106, 109, 110]. In our analysis, the mean telomere lengths of the $CD20^+$ B cells and the $CD3^+$ T cells analysed were shorter than the $CD34^+CD38^-$ populations. This does not mean that all B and T cell subsets have shorter telomere length than cells of the stem cell compartment, only that the mean telomere length of the cells within the phenotypic category is shorter. Given the ability of B cells to undergo telomere length elongation [102, 106, 109, 110] it is possible that different subsets of B cells have widely different telomere length, one subset of which may have longer telomere length than cells within the $CD34^+CD38^-$ compartment.

Maintenance of telomeres in the HSC compartment is likely important for the production of hematopoietic cells even in telomerase competent lineages, such as lymphocytes. In populations that undergo massive clonal expansion, such as the B and T cell populations, an additional boost in telomere length may be required in order to avoid critical shortening of telomeres. However, if the HSC has short telomeres initially, the subsequent telomerase activity in telomerase competent cells may be insufficient to extend telomere length beyond a critical threshold length. In daughter cells that do not have active telomerase, the telomere length decline resulting from cell divisions during expansion and

differentiation will result in critically short telomeres. Thus, critically short telomeres in the stem cell compartment will result in a lack of viable progeny that have presumably undergone apoptosis via a DNA-damage response to non-functional or uncapped telomeric ends. Maintenance of telomeres in the stem cell compartment has an impact on the telomere length of its progeny cells, and thus an overall effect on the proliferative potential of these cells and the normal regeneration of the hematopoietic system.

The Flow FISH technique allows for the analysis of single cells, and the potential to identify single events with greater than mean telomere length. However, this data is less informative if lacking the presence of a population of cells with a telomere length that is relatively homogeneous and distinguishable from the defined population. It will be interesting to use additional phenotypic markers to refine the stem cell populations analysed and determine if there is indeed a phenotypically identifiable population of HSC with "germ-line" or "fetal" length telomeres. However, extensive fractionation of the stem cell compartment leads to rapid decreases in obtainable cell numbers. This could push the limits of detection for telomere length measurement by Flow FISH, which has already allowed for the measurement of samples with relatively low cell numbers.

4.1. Conclusions

Telomere length is an important parameter for the determination of cellular proliferative potential. It is of critical importance in the hematopoietic system. Abnormal maintenance of telomeres in the HSC compartment can lead to bone marrow failure, as seen in patients with dyskeratosis congenita [141]. Increased demands on stem cell proliferation, as seen in patients with aplastic anemia can lead to increased telomere length shortening in mature hematopoietic cells [1]. Telomere length measurements of hematopoietic stem cell candidates will increase our knowledge regarding the role of the telomere in maintenance of normal hematopoiesis. This will allow a further understanding of alterations in telomere biology of the hematopoietic system in disease states. Telomere length measurement also has the potential to act as a selection parameter in order to identify HSC with the greatest proliferative potential. In situations where HSC with great proliferative potential are required, such as hematopoietic stem cell transplantation, or *ex vivo* expansion of stem cells for gene therapy, HSC could be chosen based on their telomere length.

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