Analysis of Telomerase Activity and Telomere Lengths in Human Umbilical Cord Cell Populations During *Ex Vivo* Amplification of Hematopoietic Stem Cells

A THESIS

Submitted to the faculty

of the

WORCESTER POLYTECHNIC INSTITUTE

In partial fulfillment of the requirements for the

Degree of Master of Science

in

Biotechnology

By

Manish Rajkumarji Chomal

January 8, 2003

APPROVED:

David S. Adams, Ph.D. Major Advisor WPI	Jill Rulfs, Ph.D. Committee Member WPI	Dan Gibson, Ph.D. Committee Member WPI

ABSTRACT

Human umbilical cord blood (CB) hematopoietic stem cells (HSCs) have well established applications for cellular therapy. Current protocols for isolating HSCs from bone marrow or cord select for $CD34^+$ cells, however some $CD34^-$ populations have recently been shown to also contain strong HSC activity. Thus the positive selection of HSCs based on cell surface markers remains controversial. However, it is clear from the literature that differentiated hematopoietic cells (lineage positive, Lin^+), representing the vast majority (>90%) of most blood populations, contain no long-term reconstitution potential. Thus Viacell Inc. (Worcester, MA) expands and enriches its populations of cells containing HSCs by removing only those Lin^+ cells known not to contain HSCs. This is accomplished on two separation columns (post-sep-1, and post-sep-2) (separated by 7 days of cell growth) that contain a variety of antibodies to known differentiation surface markers. Although this process strongly enriches functional HCSs, these primitive cell populations remain biochemically uncharacterized.

Because HSC populations containing long chromosomal telomeres and high telomerase activity (which helps maintain telomeres) have been shown to display the strongest long-term reconstitution potential, the purpose of this thesis was to investigate these two parameters in selected samples of Viacell's *ex vivo* amplification procedure. Two specific hypotheses were tested: 1. the removal of Lin^+ cells will appear to increase the telomerase activity and telomere lengths in the remaining cell population, and 2. these two parameters will decrease upon hematopoietic cell differentiation and proliferation. Telomerase activity was assayed using a telomeric repeat amplication protocol (TRAP), and normalized relative to a cancer cell line positive control. Relative to fresh cord

blood, telomerase activity was found to increase significantly in post-sep-1 (from 8.5 ± 1.5% to 76.2 ± 4.9%, p = 0.0001, n = 5) and post-sep-2 (8.5 ± 1.5% to 111.3 ± 4.9%, p = 0.0001, n = 5) fractions following the removal of Lin^+ cells. This increase was found to be highly reproducible, showing very low intra-cord and inter-cord variability. Telomere lengths were assayed using a telomere length assay (TLA). Relative to fresh cord blood, telomere lengths increased significantly in post-sep-1 (from 10 to 12 kb, n = 2) and post-sep-2 (from 10 to 14 kb, p = 0.001, n = 2) fractions. These apparent increases likely result from the direct removal of cells low in telomerase activity with short telomeres since the Lin^+ cells from the post-sep-1 column were found to contain relatively low telomerase activity (32.1 ± 15%, p = 0.001, n = 2) and short telomeres (7.5 kb, p = 0.001), which supports our first hypothesis. Finally, we show that telomerase activity and telomere lengths decreased in Day-14 cells (expanded and differentiated 14 days) relative to post-sep-2 (from 111.8 ± 19.6% to 54 ± 21.2%, p = 0.001, n = 3 for the TRAP, and from 14 kb to 9 kb, p = 0.0001, n = 2 for the TLA). Those two parameters also decreased in pre-sep-3 cells (terminally differentiated by treatment with All Trans Retinoic Acid for 14 days) relative to post-sep-2 (from 111.3 ± 4.9% to 14.8 ± 1.7%, p = 0.0001, n = 6 for the TRAP, and from 14 kb to 7.5 kb, p = 0.001 for the TLA), supporting our second hypothesis. Telomerase activity was found to not directly correlate with $CD34^+CD38^-$ content, supporting recent observations that a significant portion of HSCs reside outside this population.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ACKOWLEDGEMENTS

I would like to express my gratitude and thank Dr. David Adams, my major advisor and great mentor, for encouraging and guiding me throughout my Master's program, and providing me with his insight and wisdom that was necessary to complete this project. He is one of the best professors I have ever known and a very good human being. I would like to thank the members of my committee: Dr. Jill Rulfs and Dr. Daniel Gibson, for their advice and guidance throughout this project. ViaCell, Inc. (Worcester, MA) kindly provided all of the umbilical cord blood samples and other materials used in this project. A special thanks to Dr. Elizabeth Ryder and Sal Beatini for helping me with the statistical analysis of the data. I couldn't accomplish this without the blessings of my Late Guruji Rajyogi Paramhanse Shree Parasramji Babaji and my Late grandparents. I would also like to thank my family for their never-ending support. Last but not the least, I am thankful to Suchi for encouraging me throughout this project, when sometimes my experiments didn't work, and especially, for being with me in the lab at times even past midnight!! Thank you all.

BACKGROUND

Hematopoietic Stem Cells

Hematopoietic stem cells (HSCs) (fig. 1) are multipotent cells with the ability to reconstitute all hematopoietic lineages, including the formation of additional stem cells (Quesenberry et al., 1998). Hematopoiesis is a complex biological process in which

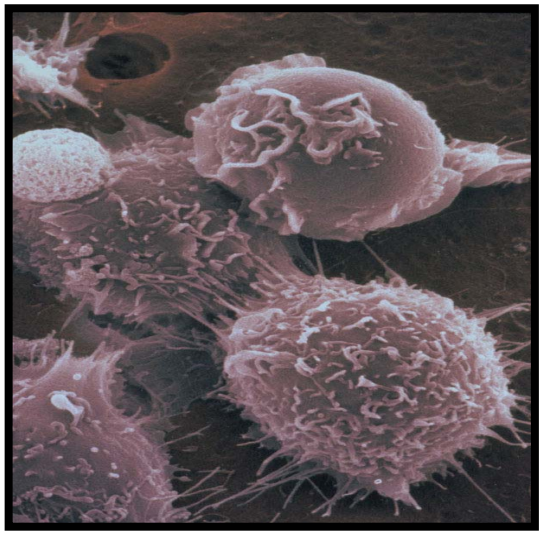


Figure 1. Scanning Electron Micrograph of HSCs in Bone Marrow
(McLaren, 2001)

HSCs differentiate by two basic pathways to give rise to lymphoid, myeloid and erythroid cell lineages (fig. 2). Because of this potential, HSCs are used for cell therapy following chemotherapy or radiation therapy.

How are HSCs Identified?

What constitutes hematopoietic stem cells has been the subject of intense debate in the literature. The cell surface marker sialomucin-like adhesion molecule CD34 is

frequently used as a convenient marker for HSCs (Civin et al., 1985; Andrews et al., 1989). The CD34 antigen is expressed on 1%-3% of all mononuclear bone marrow (BM) cells, and 1% of all nucleated cells in cord blood from full term deliveries (Civin and Gore, 1993; Kinniburgh and Russell, 1993). However, the $CD34^+$ fraction lacking the B-lymphoid marker CD38 in umbilical cord blood accounts for 4% of the $CD34^+$ fraction compared to only 1% in bone marrow, indicating that umbilical cord blood may be relatively enriched in stem cells (Cardoso et al., 1993). CD34 is found on both HSCs and early committed progenitors (Krause et al., 1996). Using the most definitive assays to date for HSC function, $CD34^+$ cells have been shown to possess colony forming potential in

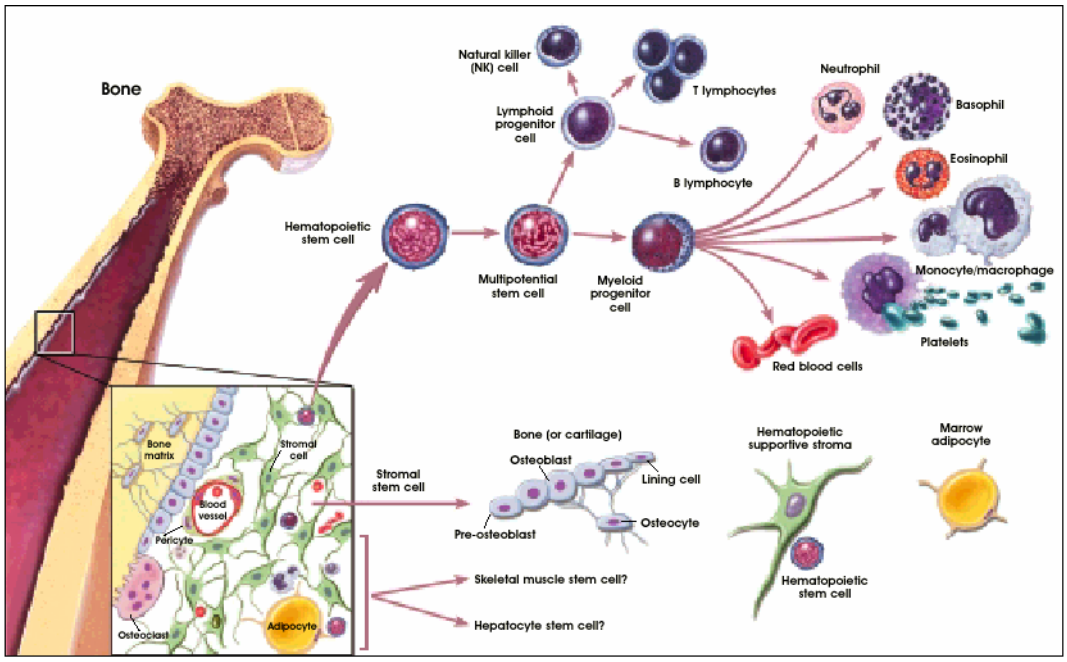


Figure 2: Differentiation of Hematopoietic Stem Sells from Bone Marrow (NIH, 2001)

in vitro cultures (Sutherland et al., 1989) and to allow the differentiation of blood cell lineages in immunocompromised mice (Bhatia et al., 1997). Moreover, CD34 plays an important role in the formation of progenitor cells in both fetal and adult hematopoiesis

(Quensberry et al., 1998).

In addition to the CD34 marker, Ziegler et al (1999) showed that the vascular endothelial growth factor receptor 2 (VEGFR2, also known as kinase domain receptor, KDR) is a positive functional HSC marker that distinguishes HSCs from progenitors. HCSs were located in the $CD34^{+}KDR^{+}$ cell fraction, whereas lineage-committed hematopoietic progenitor cells (HPCs) are restricted to the $CD34^{+}KDR^{-}$ subset.

Several additional cell surface markers allow the distinction of HSCs from their more specialized offspring. Terstappen et al (1991) showed that lineage commitment of the $CD34^{+}$ cells into the erythroid lineage could be assessed by the coexpression of high levels of the CD71 antigen, the myeloid lineage by coexpression of the CD33 antigen, and the B-lymphoid lineage by coexpression of the CD10 and CD38 antigens. The $CD34^{+}CD38^{+}$ cell populations are known to be heterogeneous in morphology and contain myeloblasts, erythroblasts, and lymphoblasts. In contrast, the $CD34^{+}CD38^{-}$ cell population lacks differentiation markers and are homogeneous primitive blast cells by morphology, and has the ability to form primitive colonies (Terstappen et al., 1991). HLA-DR is absent or is expressed at low levels on adult HSCs, but is present on fetal and neonatal hematopoietic stem/ progenitor cells (Lansdorp et al., 1993). Based on the results of long-term culture-initiating cells (LTC-IC) and various other assays, human HSCs are also frequently defined as $Lin^{-}CD34^{+}DR^{-}$ (Srour et al., 1993; Galy et al., 1998; Ogawa et al., 1996; Cashman et al., 1997; Hogan et al., 1997). Table 1 lists some of the known phenotypes of adult hematopoietic stem and progenitor cells.

However, CD34 is not a perfect marker for HSCs. Recent studies in murine and human models have indicated that $CD34^{-}$ HSCs possess engraftment potential and distinct HSC characteristics (Dao and Nolta, 2000; Ogawa, 2002). These studies challenge the dogma that HSC are exclusively found in the $CD34^{+}$ subset, and question

whether primitive HSCs are $CD34^+$ or $CD34^-$ (Engelhardt et al., 2002). Recent data reveal the presence of Lin^-CD34^- subpopulations with long-term reconstitution potential, and

Table 1. Phenotypes of Adult Human Hematopoietic Stem and Progenitor Cells
(Quesenberry et al., 1998).

Stem Cells	Progenitor Cells
CD34+	CD34+
KDR+	KDR-
AC133+	AC133+
Lin-	CD33+, CD54+, CD7+, CD19+, CD24+(3%-30%) CD9+, CD18+, CD29+, CD31+, CD38+, CD44+
$CD45\text{-}RA^{lo}$ (>70%)	CD45+
Thy-1+	Thy-1+ (5%-25%)
HLA-DR-	HLA-DR+
c-kit+	kit+ (70%-80%)
Flk-2+	Flk-2+ (20%-50%)
$MDR1^{hi}$	$MDR1^{lo}$
$Rhodamine^{dull}$	$Rhodamine^{bright}$

suggest the absence of long-term reconstitution potential in the $CD34^+$ fraction. However, this data contrasts with a variety of human transplant experiments (Goodell et al., 1996; 1999; Dao and Nolta, 2000; Andrews et al., 2000; Ogawa, 2002). Moreover, cord blood (CB) Lin^-CD34^- cells have been shown to generate $CD34^+$ HSCs when cultured in the presence of a murine BM stromal cell line which suggests that Lin^-CD34^- cells possess extensive potential for generation of $CD34^+$ HSCs *in vitro*. These derived $CD34^+$ cells produce colony forming units (CFU) and LTC-IC with multilineage differentiation potential, all of which are characteristics of HSCs (Summers et al., 2001). In addition, the demonstration of $CD34^-$ HSCs in NOD/SCID mice (Galy et al., 1998; Sato et al., 1999), and the development of $CD34^+$ cells from cultures of initially $CD34^-$ HSCs (Sato et al., 1999; Ando et al., 2000; Tajima et al., 2000; 2001; Ogawa, 2002) suggest that HSC activity exists within the murine and human Lin^-CD34^- population,

raising doubts on the frequent practice of using the positive selection of $CD34^+$ cells for cell therapy.

ViaCell defines their HSCs obtained from umbilical cord blood (UCB) as $CD34^+CD38^-Lin^-$. However, their negative selection process (which removes >90% of Lin^+ cells) enriches a combination of $CD34^-$, $CD34^+$, and primitive cells. Thus, ViaCell believes that their process of isolating HSCs by removing only those cells (Lin^+) known not to be HSCs is superior to the standard art of CD34 positive selection.

Umbilical Cord Blood HSCs

In 1974, Knudtzon showed that relatively mature hematopietic progenitor cells (HPCs) are present in human UCB. Eight years later, Nakahata and Ogawa (1982) documented the presence of primitive HPCs in UCB. Later, Broxmeyer et al (1989) provided experimental evidence that UCB is a rich source of hematopietic stem/ progenitor cells (HSPCs). Because of these studies and others, UCB has gained tremendous acceptance over the last decade as a potential source of transplantable stem cells. Gluckman et al (1989) reported the first human HSC transplant in which UCB was used instead of BM as the source of HSCs.

Studies have shown that significant functional differences exist between HSCs and HSPCs from UCB versus adult BM. UCB contains a higher proportion of primitive HSCs than adult BM and peripheral blood (PB) (Lansdorp et al., 1993; Hirao et al., 1994; Traycoff et al., 1995). HSCs derived from UCB possess higher proliferation and expansion potential than BM and PB HSCs (Vaziri et al., 1994; Cairo et al., 1997; Traycoff et al., 1995; Hao et al., 1995). Unlike BM and PB, UCB cells appear to be in a relatively naïve immunological state (Johnson et al., 1998), which is thought to result in

reduced incidences of graft versus host disease (GVHD) in allogeneic transplants (Rocha et al., 2000). UCB provides other advantages as a source of HSCs in its ability to be banked and stored following birth (Rubeinstein et al., 1993), the lower potential risk of transmissible infectious diseases, the absence of donor attrition, the absence of donor collection risks, and the ability to develop high definition donor pools to better target ethnic and racial minorities who are vastly under represented in current BM registries (Wagner et al., 1998).

Ex- vivo Expansion of Hematopoietic Stem Cells

Although cord blood is an attractive alternative to BM or PB as a source of transplantable hematopoietic tissue, a major limitation is the relatively low cell number available, which is believed to contribute to delayed engraftment in CB transplantations as compared to BM or PB transplantations. The time to engraftment has been reported to be inversely related to the cell dose given, suggesting that the injection of more HSCs might lead to accelerated engraftment (Kurtzberg et al., 1996; Rubeinstein et al., 1998). Further, because of the low overall HSC cell number in CB there may be enough HSCs from one cord to reconstitute one child, but the ability to fully reconstitute an adult requires *ex-vivo* expansion (Piacibello et al., 1998).

The *in vitro* expansion of HSCs depends on both intrinsic (biological properties of the cell subpopulation) and extrinsic (cytokines and culture conditions) properties including such variables as non-HSC cell types, cytokines that form the microenvironment, the type of culture medium, the medium change schedule, temperature, the presence or absence of serum, etc. (Mayani and Lansorp, 1998). The most common current expansion technology involves CD34 enrichment (Kohler et al.,

1999). This type of enrichment displays optimum results when a combination of three growth factors: stem cell factor (SCF), FLT-3L and IL-3 is included (Kohler et al., 1999). However, in other experiments, the best results were obtained using combinations of FL, thrombopoietin (Tpo), KL and IL-6 (Piacibello et al., 1998; Gilmore et al., 2000).

ViaCell: Company Information and Research Interests

ViaCell, Inc. is a new cellular medicine company merged from two previous companies: Viacord, Inc. in Boston, Massachusetts, and t. Breeders, Inc. in Worcester, Massachusetts. Viacord specializes in storing cord blood stem cells from the umbilical cords of newborns for future use within that family. T. Breeders is a biotechnology company with proprietary technology for expanding UCB derived HSCs. The goal of the new combined biotechnology company is to use its high quality cord blood banking service and patented stem cell expansion technology to develop a premier cellular pharmaceutical company to provide high quality cellular medicines for the treatment of human diseases, such as cancer, certain genetic disorders, organ transplant tolerance, and autoimmune diseases (Craig, 2000; ViaCell, Inc. Annual Report, 2001). ViaCell has an Investigational New Drug (IND) application approved by the FDA and is initiating a Phase I clinical trial for testing its proprietary selective amplification technology.

Selective Clonogenic Amplification ^{TM}

ViaCell, Inc. has developed a patented *ex-vivo* system called Selective Clonogenic Amplification ^{TM} (SCA), to select and amplify a population of cells containing HSCs. SCA enables simultaneous selection and amplification of stem cells from BM, mobilized PB, or CB through the use of highly specific markers on

differentiated cells (t. Breeders, 2000). This method utilizes the "negative selection separation" of HPSCs. This separation method, as shown in figure 3, uses a cocktail of

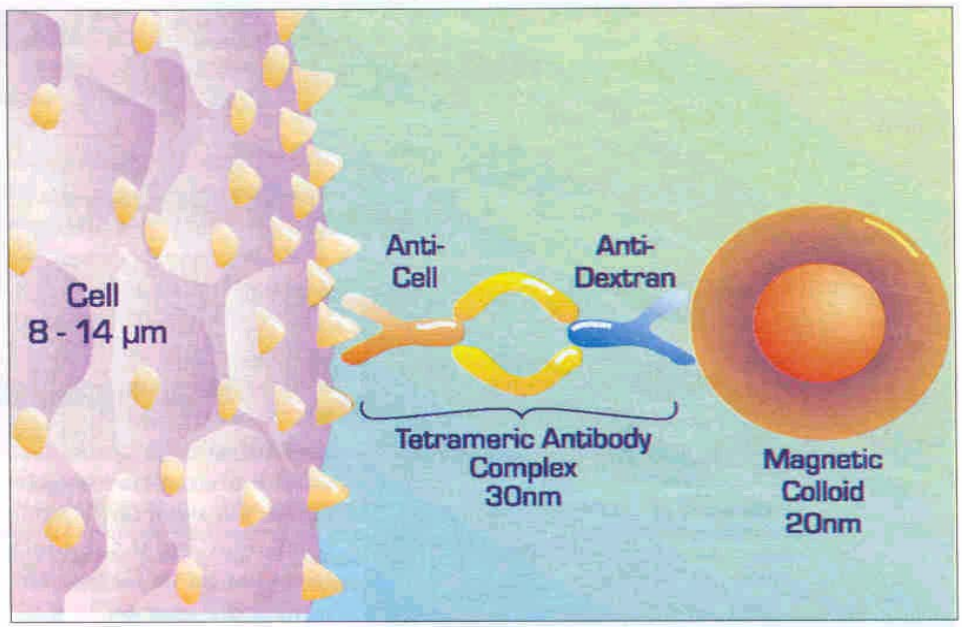


Figure 3. 'Negative Selection Separation' to Remove Unwanted Differentiated Cells
(Zimmerman, 1998)

antibodies that bind to mature and differentiated cell surface markers from the mononuclear cell population, tagging them for removal using magnetic beads. This separation results in a subpopulation of cord blood mononuclear cells that ViaCell terms $CD34^{+}CD38^{-}Lin^{-}$ cells, but which also contain $CD34^{-}$ cells, both of which are believed to contain stem cells (Hao et al., 1995). Lin^{-} cells are negative for the differentiated markers CD2, CD3, CD14, CD16, CD19, CD24, CD56, CD66b, and Gly A, which are expressed on the surface of mature red blood cells, monocytes, natural killer cells, and T cells. After amplification of UCB-derived HSCs, ViaCell believes that these new cells now have the capability of reconstituting a 200-kg person(s), instead of a 20-kg child before

amplification; Phase I clinical trials are underway to prove this. ViaCell's 14-day expansion process includes the following steps as shown in figure 4:

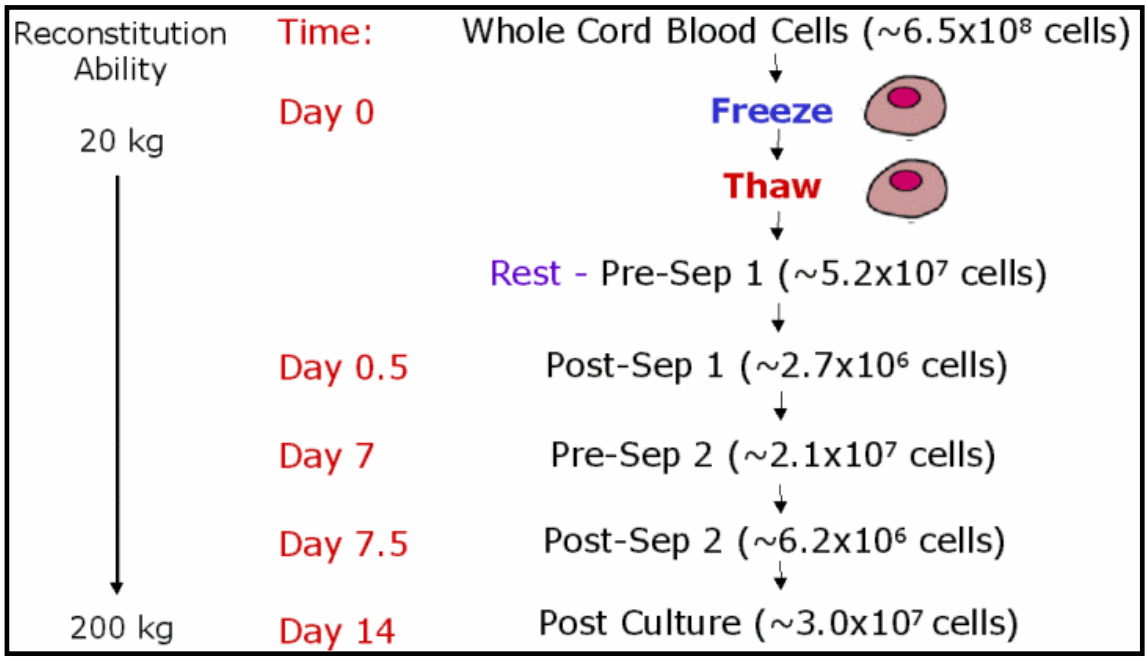


Figure 4. ViaCell's UCB Cell Selection and Amplification Process Time Course

Telomerase Activity and Telomere Length in Hematopoietic Stem Cells

Telomeres, the nucleoprotein complex at the ends of eukaryotic chromosomes, are characterized by the presence of a large number of highly conserved tandem repeats of G-rich DNA (Blackburn, 1991). In humans and other vertebrates, the telomeric sequence contains TTAGGG repeats (Moyzis et al., 1988). Studies in various species have shown that telomeres mediate important chromosome/nuclear matrix interactions (Mathog et al., 1984), protect encoding DNA from enzymatic breakdown (Sandell and Zakian, 1993), may exert effects on regional subtelomeric gene transcription (Levis et al., 1985; Gottschling et al., 1990), interact critically with cell-cycle regulatory mechanisms (Sandell and Zakian, 1993; Weinert and Hartwell, 1988; Schiestl et al., 1989), and

prevent chromosome dicentric fusion and other chromosomal aberrations (de Lenge and DePinho, 1999; Smith and Blackburn, 1999). The length of telomeres is remarkably variable because of the variability in the number of TTAGGG repeats (de Lenge et al., 1990). Telomere lengths show wide inter-individual variation, and they also vary among cells in the same tissue and among chromosomes in the same cell (Blackburn, 2000).

Table 2: Average Telomere Lengths in Various Human Cell Types

Size	Source	Telomere Length (Kb)	Reference
Long	Fetal Liver ($CD34^+$)	11	Engelhardt et al., 1997
	Cord Blood ($CD34^+$)	10.4	Engelhardt et al., 1997
Medium	B Lymphocytes (Naive & Memory)	8- 9	Weng et al., 1997
	Bone Marrow	7.6	Engelhardt et al., 1997
	Peripheral Blood	7.4	Engelhardt et al., 1997
Short	Fibroblasts	6- 6.8	Allsopp et al., 1992

Table 2 shows average telomere lengths in various human cell types. Due to the inability of DNA polymerase to replicate the ends of eukaryotic chromosomes completely (Watson, 1972; Olovnikov, 1973), each cell division results in a loss of 50 bp to 200 bp of telomere repeats in normal human somatic cells (Smith and deLange, 2000; Harley et al., 1990; de Lange et al., 1990; Allsopp et al., 1992; Vaziri et al., 1994; Chang and Harley, 1995). If a minimal telomere length is essential for chromosomal integrity and replication, short telomere lengths could limit the replicative lifespan of cells. Hence,

telomere shortening is considered a molecular or "mitotic clock" that counts the number of cell divisions and determines the onset of cellular senescence (Harley, 1991; Shay et al., 1996).

In contrast to differentiated cells, cells with essentially unlimited replicative potential such as reproductive cells, immortal cell lines and cancer tissues have stable telomeres (de Lange et al., 1990; Hastie and Dunlop, 1990; Allsopp et al., 1992). The maintenance of telomere lengths in these cells is highly correlated with the presence of telomerase, a ribonucleoprotein enzyme which synthesizes telomeric repeats *de novo* (Greider an Blackburn, 1985; Morin, 1989; Harley, 1991; Counter et al., 1994; Kim et al., 1994). Studies have shown that telomerase activation is necessary for stabilizing telomere lengths, thereby maintaining the replicative capacity of self-renewing cells such as germ line cells, tumor cells, and possibly stem cells of various tissue types (Harley et al., 1990; 1991; Allsopp et al., 1992).

Sensitive assays for telomerase (Kim et al., 1994) have revealed low but detectable activity in human hematopoietic cells (Counter et al., 1995) and in highly enriched human hematopoietic progenitors (Shay, 1995; Lansdorp et al., 1996). This basal level of telomerase activity was found to increase in very early hematopoietic progenitors when they were "stimulated" with growth factors (Engelhardt et al., 1997). Interestingly, the average telomere lengths of primitive HSCs decrease upon proliferation despite thier having detectable telomerase activity (Vaziri et al., 1994). Studies carried out in peripheral blood cells as a function of age (Hastie and Dunlop, 1990; Vaziri et al., 1994) and in cultures of hematopoietic cells (Vaziri et al., 1994) suggest that although these cells contain detectable telomerase, this telomerase activity is not sufficient to prevent the telomere shortening induced by cell division.

The level of telomerase activity is different in human HSCs and their differentiated progeny (Counter et al., 1995; Hiyama et al., 1995; Chiu et al., 1996; Zhang et al., 1996) as depicted in figure 5. Primitive HSCs are likely to be quiescent most of the time, and thus these cells exhibit a low level of telomerase activity and no proliferation-induced shortening. However, upon stimulation with cytokines, which cause the cells to proliferate, telomerase activity appears to be upregulated in the HSC's immediate progeny, which may help slow down the rate of telomere erosion induced by cell division. The more mature cells then become quiescent again and downregulate telomerase activity upon differentiation (Lansdorp et al., 1996; Engelhardt et al., 1997; Yui et al., 1998). Supporting the above observations, Vaziri and Benchimol (1998), also found a low basal level of telomerase activity in the most primitive quiescent HSCs (with a $CD34^{+}CD71^{Low}CD45^{+}RA^{low}$ phenotype), and in the more mature $CD34^{+}$ cells, and an upregulation of telomerase activity in the cycling $CD34^{+}CD71^{+}$ progenitors. Thus telomerase activity appears to correlate best with $CD34^{+}$ cell cycling than simply the presence of CD34. Whether low, but detectable telomerase activity in the primitive HSCs is related to their functional "self-renewal potential" is currently under investigation.

What is the physiological basis of this upregulation and downregulation of telomerase activity? Telomerase activity is increased when the cells are in the G_1, S, and G_2+M phases of the cell cycle, but is repressed when such cells enter G_0 due to growth factor deprivation or differentiation (Zhang et al., 1996; Holt et al., 1996a; Engelhardt et al., 1997). These observations lead to the important concept that one major factor influencing the level of telomerase activity observed in a population of telomerase-

competent cells is the fraction of cells undergoing proliferation. If most of the cells are in G_0, telomerase activity will be very low, while if most are dividing the telomerase

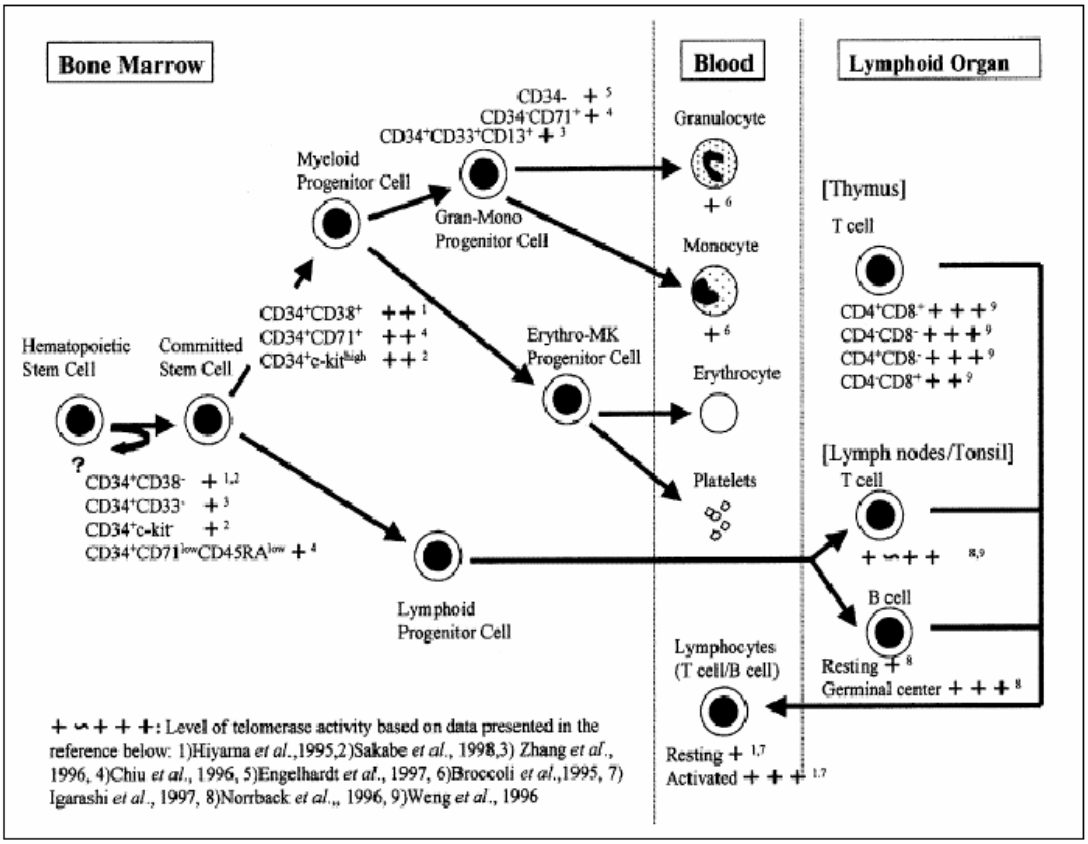


Figure 5. Telomerase Activity in Normal Hematopoietic Cell Populations.
(Ohyashiki et al., 2002)

activity will be much higher (Shay et al., 1996). Telomerase-positive immortal cells express telomerase activity at each stage of the cell cycle. As cells exit the cell cycle and enter a state of nondivision (G_0) via quiescence, senescence, or differentiation, telomerase activity is dramatically decreased until it becomes virtually undetectable. However, the process is reversible. Quiescent cells are able to reenter the cell cycle and reexpress telomerase activity (Holt et al., 1996b).

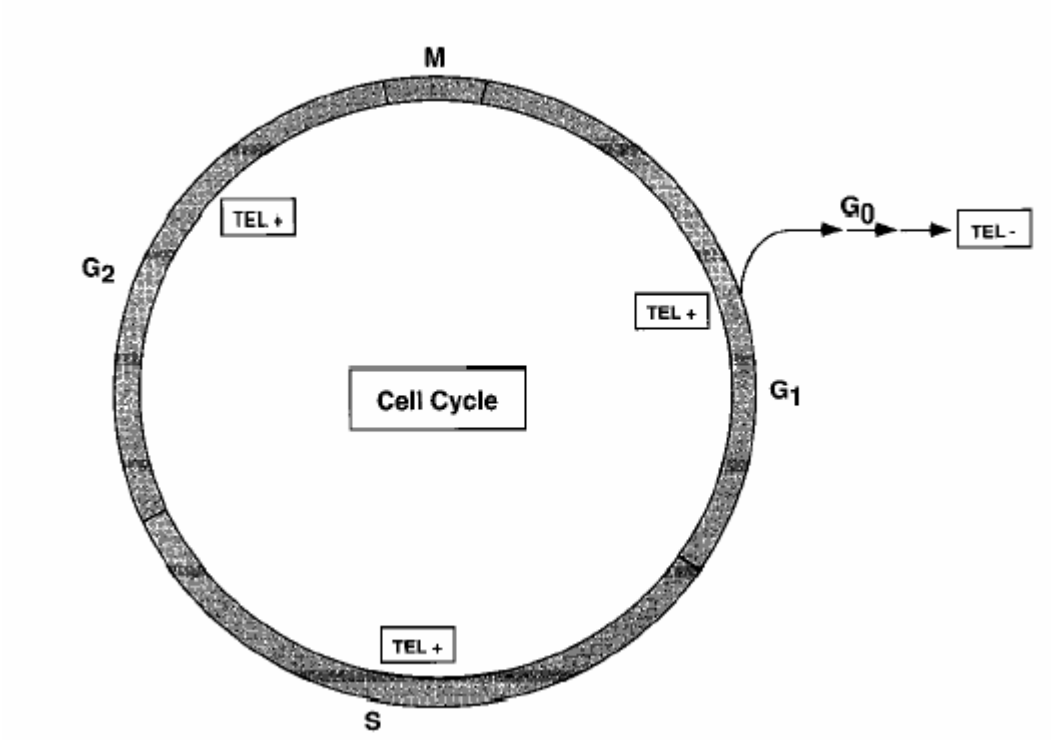


Figure 6. Model for the Repression of Telomerase Activity in Cells that Exit the Cell Cycle. (Holt et al., 1996b)

Recent studies have suggested that telomerase activity in human BM and PB could be almost exclusively assigned to the hematopoietic progenitor cell fraction expressing the CD34 antigen (Hohaus et al., 1997). $CD34^+$ cells lacking co-expression of myeloid marker CD33 demonstrated higher levels of telomerase than myeloid committed $CD34^+CD33^+$ cells (Hohaus et al., 1997). Hohaus et al (1997) suggested that at least a portion of the hematopoietic stem/ progenitor cell fraction expresses telomerase, and downregulates its expression during differentiation. Further, *ex-vivo* expansion of human $CD34^+$ cells derived from BM, fetal liver (FL), PB and CB in the presence of a cytokine combination (KL, IL-3, IL-6, erythropoietin, granulocyte colony-stimulating factor and stem cell factor) showed upregulation of telomerase activity which peaked after one week of expansion, and sharply declined during the second week, decreasing to baseline levels

after 3-4 weeks (Engelhardt et al., 1997). Engelhardt et al also showed that $CD34^+$ cells from CB display long telomeres relative to BM and PB. During four weeks of *ex-vivo* expansion of $CD34^+$ cells, telomere length progressively shortened, with the smallest telomere lengths detected in highly differentiated cells after 3-4 weeks of expansion (Engelhardt et al., 1997). These results suggest that cell populations high in telomerase activity initially show increased telomere lengths, then shortening upon prolonged expansion.

Maintenance of telomere length is of immense significance in cell therapy experiments since studies have shown that long telomere lengths result in improved graft survivability (Lansdorp et al., 1997; Notaro et al., 1997; Vaziri and Benchimol, 1998). Moreover, increased telomerase activity in hematopoietic cells is associated with self-renewal potential (Morrision et al., 1996). Because of these correlations, ViaCell has an interest in analyzing telomerase activity and telomere lengths throughout their entire selection/ amplification protocol.

PROJECT PURPOSE

The purpose of this project was to test two hypotheses that 1. telomerase activity and telomere lengths increase upon removal of differentiated cells, and 2. decrease upon differentiation of hematopoietic cells. To test these hypotheses, telomerase activity and telomere lengths were assayed in each of ViaCell's amplification fractions, which comprised human umbilical cord blood cell samples obtained at various intervals of a two-week *ex vivo* stem cell amplification process. Telomerase activity was quantified by a TRAP assay, which was found to produce low inter-cord and intra-cord variability. We also analyzed the quantitation data by analysis of variance (ANOVA).

In the work presented here, I show that telomerase activity is apparently increased on a per cell average during ViaCell's 14-days of *ex-vivo* expansion of UCB-derived HSCs and primitive cells. Telomerase activity is further apparently increased after removal of differentiated cells, which are known to be low in telomerase activity. Telomere lengths also increased in the cell populations enriched for primitive cells including HSCs after removal of differentiated cells. Differentiated Lin^+ cells showed a 300% decrease in telomerase activity compared to Post-Sep-2 and showed very short telomere lengths. This work also shows that telomerase activity and telomere lengths decrease upon cell amplification and differentiation. Finally, we show that telomerase activity and telomere lengths do not correlate with $CD34^+CD38^-$ content.

MATERIALS AND METHODS

Acquisition of Cord Blood Samples

Human umbilical cord blood samples were provided by ViaCell Inc. (Worcester, MA). The cord blood samples were donated to ViaCell from UMass Memorial Hospital. For the TRAP assay, at least 10^5 $CD45^+$ cells were provided at various time points during ViaCell's stem cell amplification process. Using this assay, cord cell samples from five different donors were tested. For the telomerase length assay (TLA), at least 6 X 10^6 $CD45^+$ cells from three pooled donors were required for genomic DNA isolation. Cells were cultured in Stem Span Medium (Stem Cell, Vancouver B.C., Cat #09650) supplemented with chemically defined lipid (0.2% final concentration) (Gibco, Cat #11905-031) and gentamycin (0.1% final concentration) (Mediatech, Cat #30-005-CR). Before being transported to WPI, the cultured cells were left in an aliquot of original culture media or PBS on ice.

TRAP (Telomerase Repeat Amplification Protocol) Assay

This assay (Kim et al., 1994; Wright et al., 1995) was performed as described in the specification manual (Intergen) with a few exceptions.

Cell Extract/Lysate Preparation

Cord blood whole cell extract was prepared using 1X CHAPS lysis buffer (10 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 1 mM EGTA, 0.1 mM Benzamidine, 5 mM β-mercaptoethanol, 0.5% CHAPS, 10% Glycerol) supplied with the TRAPeze telomerase detection kit (Intergen, #S7700). Cord blood cell samples containing at least 10^5 CD45+

cells were microfuged for 15 sec at room temperature to pellet the cells. The supernatant was discarded. This centrifugation was performed twice to thoroughly remove all the media or PBS that the cells were suspended in. Cell pellets from 10^5 cells were resuspended in 20 μl 1X CHAPS lysis buffer by pipetting up and down. For 10^6 cells, 200 μl of 1X CHAPS lysis buffer was used. The suspension was incubated on ice for 30 min. The lysate was then spun in a microcentrifuge at 10,000 xg for 20 min at 4°C to pellet cell debris. The supernatant was aliquoted and stored at –80°C. 5 μl of supernatant of each sample was transferred into a fresh eppendorf tube to determine the protein concentration.

Determination of Protein Concentration

Protein concentrations were determined for whole cell lysates using a Coomassie assay (Pierce) and a BSA standard curve. BSA standard dilutions were prepared at the following concentrations: 1.25 μg/ml, 2.50 μg/ml, 5 μg/ml, 10 μg/ml, 20 μg/ml, and 40 μg/ml. In the first tube, 500 μl distilled water was added. In the second tube, 5 μl of cell extract was diluted with 495 μl of distilled water. In the remaining tubes 500 μl of each of the BSA standard dilutions were added. To equalize the temperature, all the tubes were incubated at 37°C for 1 min. 0.5 ml of Coomassie protein assay reagent (Pierce) was added to each tube. Samples were mixed, and then the OD was read at 595 nm relative to the tube containing only distilled water as "blank".

TS Primer Kination

End labeling of the TS primer was performed according to Intergen's TRAPeze Telomerase detection protocol (#S7700). The TS primer (5'-

AATCCGTCGAGCAGAGTT-3') was 5' end labeled with [γ-^{32}P]-ATP (ICN Pharmaceuticals, 3000 Ci / mmol) using T4 polynucleotide kinase (Ambion). All the reagents were thawed and kept on ice. The following reagents were combined in a 0.5 ml eppendorf tube to make a 20 µl reaction: 10 µl of TS primer, 2.5 µl of [γ-^{32}P]-ATP (3,000 Ci/mmol), 2 µl of 10X kinase buffer, 0.5 µl T4 polynucleotide kinase (10 units/µl) (Ambion, #2310) and 5 µl of PCR grade water. These reagents were then mixed and spun briefly in a microcentrifuge. The reagent mix was incubated for 20 min at 37°C, then for 5 min at 85°C to inactivate the kinase. The kinased samples were stored at –20°C in a lead pig. 2 µl of kinase-labelled TS primer were used per TRAP assay reaction.

Telomerase Reaction and PCR

A 'Master Mix' was prepared for the PCR amplification according to Intergen's TRAPeze Telomerase detection protocol (#S7700). The master mix was prepared by combining the following reagents in a 1.5 ml eppendorf tube. All reagents were thawed and kept on ice. The amount of reagents used for <u>each</u> assay was as follows: 5 µl of 10X TRAP reaction buffer (200 mM Tris-HCl, pH 8.3, 15 mM $MgCl_2$, 630 mM KCl, 0.5% Tween 20, 10 mM EGTA), 1 µl of 50X dNTP mix (2.5 mM each dATP, dTTP, dGTP, dCTP), 2 µl ^{32}P-labeled TS primer, 1 µl TRAP primer mix (RP primer, K1 primer, TSK1 template), 0.4 µl of Taq polymerase (5 units/µl, Amersham Pharmacia Biotech, #27-0799-01), and 38.6 µl of PCR grade water. The tubes were vortexed and spun briefly in a microcentrifuge. For each assay, 48 µl of the 'Master Mix' was aliquoted into a 0.5 ml eppendorf tube. One of the following sample cell extracts or controls was added to the master mix aliquoted in each tube: 2 µl of CHAPS lysis buffer (primer-dimer/PCR

contamination control), 2 μl of heat inactivated extract (negative control), 2 μl of cancer cell line positive control, or a volume of cord cell extract containing 1 μg of protein (usually 0.5-2 μl). The tubes were then mixed and spun briefly in a microcentrifuge. The tubes were placed in a thermocycler and incubated at 30°C for 30 min to allow ladder extension of the TS primer. A 2-step PCR was then performed at 94°C/30 sec, and 59°C/30 sec for 27 cycles. Following PCR, the samples were stored at 4°C, or the PCR products were analyzed on a 10% non-denaturing polyacrylamide gel.

TRAP Gel Electrophoresis

The TRAP reaction products were analyzed on a 0.8 mm 10% non-denaturing polyacrylamide gel containing 0.5x TBE. First, the BRL V-16 glass plates were set up using 0.8 mm thick spacers and comb. A narrow toothed comb was used to analyze more samples. 30 ml of gel solution was prepared by mixing 10 ml of 30% polyacrimide / bisacrylamide, 1.5 ml of 10X TBE, 0.6 ml of 5% ammonium persulfate (to make 0.1%), dH_2O to make 30 ml, and 15 μl TEMED to make a 0.8 mm thick, 7 inches long, 10% gel. The gel was left to polymerize for 30 min, then the comb and lower spacer were removed. The gel was mounted into the electrophoresis unit, and the upper and lower reservoirs were filled with 0.5X TBE buffer. Before loading the samples, the gel was pre-electrophoresed at 287 V for 15 min. 5 μl of 10X loading dye-containing bromophenol blue and xylene cyanol (0.05% each) and 10% glycerol was added to each PCR reaction tube. The tubes were then vortexed and spun in a microfuge. 5 μl from each of the reaction tubes was loaded per lane. The remaining reaction mixes were stored at 4°C. The gel was then electrophoresed at 287 V for 1 hour and 30 min, until the xylene cyanol ran 70-75% of the gel length.

Gel Drying and Autoradiography

After electrophoresis, the radioactive electrode buffer was discarded in the isotope sink and the PAGE unit was dismounted. The gel was separated from the glass plates, and the lower right corner of the gel was marked for orientation. The gel was then carefully spread out on 2 layers of 3 MM filter paper and was covered with saran wrap. The gel covered with saran wrap was placed in the gel drier and dried for 1 hour at 80°C. The telomerase reaction products on the dry gel were then visualized by autoradiography using Kodak X-OMAT AR X-ray film.

TRAP Assay Quantitation

The telomerase products were quantified using a Dupont Benchtop Radioisotope Counter. Radioactive India ink was used to orient the gel with the X-Ray film. Then the portion of the gel corresponding to the P^{32}-labeled telomerase reaction products (i.e. all bands ≥ 50 bp) was carefully cut out from the gel, squished into an eppendorf tube, and placed in the counter. The radioactive signal was read as counts per minute (CPM).

Telomere Length Assay

This assay (Chang and Harley, 1995; Lansdorp et al., 1996) was performed essentially as described in the specification manual (Roche) with a few exceptions.

Acquisition of Cord Blood Samples

Human umbilical cord blood samples containing at least 6 X 10^6 CD45+ cells were obtained from 2-3 pooled donors at various time points during ViaCell's stem cell amplification process. For the Telomere Length Assay (TLA), at least 6 X 10^6 cells were

required to obtain a good yield of genomic DNA. The cultured cells were transported to WPI in an aliquot of original culture media or PBS on ice.

Isolation of Genomic DNA

A genomic DNA isolation procedure based on magnetic bead technology was performed at room temperature according to Roche's DNA isolation protocol (Roche, #2032805). This method utilizes the ability of nucleic acids to adsorb to silica (glass) in the presence of a chaotropic salt. The volume of reagents used for DNA extraction was taken from Roche's chart for 1 X 10^7 cells. All the reagents used were supplied in the DNA isolation kit for Blood/Bone Marrow/Tissue (Roche, #2032805). First the media containing the cells was split into 4 eppendorf tubes. Cord blood cells were pelleted by low speed centrifugation at 2000-3000 rpm for 2-3 min. The following reagents were pipetted into a fresh 15 ml plastic tube to prepare the lysis buffer solution: 2 ml of lysis buffer, 2 ml of distilled water. The contents of the tube were then mixed. The four ml of diluted lysis buffer solution were added to the pelleted cells split into four eppendorf tubes (1 ml per pellet) and the tubes were vortexed gently. The cell solution was mixed with 200 µl of proteinase K (50 µl per each of the 4 eppendorf tubes) and vortexed twice for 10 seconds. This treatment helps ensure cell lysis and inactivation of nucleases. Then 10 Magnetic Glass Particles (MGP) tablets (approx. 2-3 tablets per eppendorf tube) were added to the lysate to immobilize the DNA. The lysate with the beads was vortexed for 10 sec, causing the beads to break into a powdered form to bind DNA more efficiently. The lysate was incubated for 5 min at room temperature with slow continuous inverted mixing by hand. Next, the MGP beads were separated by placing the eppendorf tubes in a magnetic particle separator (Roche # 1641794) for 2 minutes, and

the supernatant was discarded. In a separate tube, washing buffer solution containing RNase was prepared by mixing 10 μl RNase solution with 5 ml of washing buffer. The separated MGP pellet was suspended in the RNase mixture (1.25 ml for each of the 4 eppendorf tubes) and incubated for 5 min at 37^{o}C. This treatment with RNase was done to remove minor contaminations of the DNA sample with RNA. The MGP pellet was again separated in a magnetic particle separator and the supernatant was removed. Next, the MGP pellet was washed by repeated steps of separation and resuspension. The MGP pellet was washed twice using washing buffer solution without RNase, as follows: the separated MGP was suspended by pipetting in 5 ml (1.25 ml for each of the 4 eppendorf tubes) of washing buffer, and separated by placing the tube in a magnetic particle separator for 2 min. The wash supernatants were completely removed and discarded. Finally the DNA was eluted from the MGP pellet in the following manner: the MGP containing the DNA was resuspended in 1 ml (0.25 ml per eppendorf tube) of elution buffer, and incubated for 5 min at 70^{o}C on a heating block with intermittent vortexing. This was followed by microcentrifugation for 4 min at 13000 rpm. The supernatant containing the DNA was then aliquoted and stored at –20^{o}C.

Ethanol Precipitation of Isolated DNA

In some instances, the DNA isolated using the above procedure was not pure enough to be cut by the restriction enzymes used in the TLA. Hence, I ethanol precipated the DNA. To 400 μl of the DNA solution obtained from the above procedure, 40 μl of 3.0 M NaOAc, pH 7.0 (one tenth the volume of the DNA sample) was added. 1 μl of glycogen (10 mg/ ml, 10 μg) was added to act as carrier. This solution was mixed well before adding 1.1 ml of ethanol (2.5 times the volume of the DNA sample). This mixture

was incubated overnight at –20° C. It was then microfuged for 15 minutes at room temperature. The supernatant was carefully decanted leaving the pellet undisturbed. The pellet was then washed by adding 0.5 ml of 100 % ethanol and inverted mixing. It was then immediately microfuged for 15 minutes. The supernatant was very carefully removed without dislodging the loose pellet. The DNA pellet was then air dried and dissolved in 1X TE buffer (to give about 1 μg/ μl concentration of DNA, usually 30 μl), and heated for five minutes at 50° C to aid dissolution.

Digestion of Genomic DNA

The digestion of genomic DNA isolated from cord blood cells was performed according to Roche's TeloTAGGG Telomere Length Assay protocol (#2209136). Per sample, 1 μg of extracted genomic DNA was diluted with nuclease free water (supplied in the TeloTAGGG Kit) to a final volume of 16 μl. Handling of all solutions and pipeting was done on ice. The following reagents were added to the 16 μl to make a 20 μl reaction: 2 μl of 10X digestion buffer, 1 μl of Hinf 1 (40 U/μl), and 1 μl of Rsa 1 (40 U/μl). As controls, 1 μg of high molecular weight control DNA (high molecular weight telomeres, 100 ng/μl), and low molecular weight control DNA (low molecular weight telomeres, 100 ng/μl) or cord sample) in 16 μl volume were also tested. The reaction mixture was then incubated for 6 hours at 37° C. Again 1 μl of Hinf 1 (40 U/μl) and 1 μl of Rsa 1 (40 U/μl) were added to each of the reaction mixtures and left overnight at 37° C. This was an exception to Roche's protocol. Before loading onto the gel, 5 μl of 5X loading buffer was added to each 20 μl reaction to make a final volume of 25 μl.

Genomic DNA Electrophoresis

Digested genomic DNA was separated by agarose gel electrophoresis. A 0.8% horizontal agarose gel was prepared as follows: 0.8 g of highly pure nucleic acid grade agarose (International Biotechnologies Inc.) was added to 100 ml of 1X TAE buffer in an Erlenmeyer flask. The solution was heated in a microwave oven for 2-3 min until the agarose was fully dissolved. The hot agarose solution was then poured into an 8 cm x 10 cm electrophoresis tray, and left to solidify at room temperature for 45 min. Once the gel solidified, the gel comb was removed and the electrophoresis unit was filled with 1X TAE running buffer. The digoxygenin DIG molecular weight marker mix was prepared just before loading the samples onto the gel by mixing in a 0.5 ml eppendorf tube: 4 µl of DIG molecular weight marker, 12 µl of nuclease free water, and 4 µl of 5X loading buffer. This 20 µl marker sample was microfuged briefly and incubated at 65^{o}C for 10 min. 25 µl of each cord sample were loaded per lane and 10 µl of the DIG labeled molecular weight marker were loaded on each side of the gel. The gel was electrophoresed at 22 V for 5 hours until the Bromophenol blue tracking dye had traveled approximately ¾ the length of the gel.

Southern Blotting

Southern transfer of the digested genomic DNA was performed by high salt capillary transfer to nitrocellulose membrane using a 20X SSC (Sodium Saline Citrate, 3 M NaCl, 0.3 M Sodium Citrate, pH 7.0) transfer buffer. After electrophoresis, a small piece from the lower right corner of the gel was cut for orientation purposes. All the gel-washing steps were performed with gentle agitation on a gyrotory shaker at 25°C in a tupperware dish. The gel was first submerged in for 5-10 min in HCl solution (0.25 M

HCl) until the BPB went yellow. This step was done to fragment the DNA, to facilitate the transfer. The gel was rinsed 2 times with distilled water, then was denatured to single strands by submerging 2 times for 15 min in Denaturation solution (0.5 M NaOH, 1.5 M NaCl). This was followed by rinsing the gel two times with distilled water, and neutralization by submerging it two times for 15 min in Neutralization solution (0.5 M Tris-HCl pH 7.5, 3 M NaCl). All washes were decanted to waste.

Nitrocellulose membrane (BA-45, 0.45 μm pore size) and two 3MM filter papers cut to the size of the gel were pre-soaked in 2X SSC buffer for 30 min before blotting the gel to the membrane. This was done to decrease the chance of bubble formation and to facilitate the transfer of the DNA. The digested DNA from the gel was blotted to the nitrocellulose membrane by capillary transfer at 25°C using 20X SSC as a transfer buffer. The southern blot transfer was performed as follows: a tupperware dish was used as the transfer unit, and a piece of dry 3MM filter paper served as a wick in the transfer unit. The tupperware dish was then filled with 20X SSC buffer and the ends of the wick were submerged in the buffer. Extra buffer was poured over the wick, and all the air bubbles were removed by smoothing out the wick using a gloved hand. One of the pre-moistened 3MM filter paper squares was then placed on top of the wick. The gel was placed on the 3MM sheet and all air bubbles were removed. The pre-moistened nitrocellulose membrane was then placed over the gel, and its corner corresponding to the gel was also cut, and all air bubbles were removed. Another pre-moistened 3MM filter paper was then layered over the membrane. Next, a sheet of saran wrap was placed over the whole unit and the center of the saran wrap corresponding to the size of the gel was cut out. The saran wrap was then overlayered with a piece of dry 3MM paper, which in turn was overlayered with several layers of dry paper towels to make a stack about 10 cm thick.

The paper towels were placed so that they did not directly touch the SSC buffer in the tupperware dish, as this would short-circuit the flow of buffer through the gel. The paper towels were covered with a glass plate, and a big book was placed on top of the plate as a weight. The blot was allowed to sit overnight for maximum sensitivity and reproducibility of transfer. After blotting, the membrane was washed in 2X SSC solution. The membrane was then placed between 2 sheets of dry 3MM filter paper cut to the size of the membrane, and baked at 120^{o}C in a glassware drying oven for 2 hours. If not used immediately for hybridization and chemiluminescence detection, the membrane was wrapped in a foil and stored at 4^{o}C.

DNA Hybridization

The hybridization and chemiluminescence detection steps were performed according to Roche's TeloTAGGG Telomere Length Assay protocol (Roche, #2209136). The hybridization and wash temperatures were precisely controlled for maximum sensitivity and reproducibility of results. The hybridization was performed as follows: the DIG hybridization solution was pre-warmed to 42^{o}C. For pre-hybridization, the membrane was submerged in 10 ml of pre-warmed DIG hybridization solution in a hybridization bag, and incubated for 30-60 min at 42^{o}C on a gyrotory shaker. Hybridization solution was prepared by adding 1 µl of telomere probe (DIG labeled telomere specific hybridization probe, Roche, #2209136) to 5 ml pre-warmed hyb-solution, and mixed. After pre-hyb incubation of membrane, the pre-hyb solution was discarded and the 5 ml Hybridization solution containing the telomere probe was immediately added. The membrane was incubated in a hybridization bag for 3 hours at 42^{o}C on a gyrotory shaker. After hybridization, the Hybridization solution was discarded, and the membrane was washed 2 times with 100 ml stringent wash buffer-I (2X SSC, 0.1

SDS) for 5 min at 25°C with gentle agitation. The membrane was then washed 2 times with pre-warmed stringent wash buffer-II (0.2X SSC, 0.1 SDS) at 50°C with gentle agitation. These washes were followed by rinsing the membrane in washing buffer-1X (supplied with the Roche kit # 2209136) for 1-5 min at 25°C on a gyrotory shaker.

DIG Antibody Binding

The membrane was then incubated in freshly prepared Blocking solution (by mixing 15 ml of 10X Roche Blocking solution with 135 ml maleic acid buffer) for 30 min on a gyrotory shaker at 25°C. The antibody solution was prepared as follows: The vial containing the Anti-DIG –AP antibody (0.75 U/µl, F_{ab} fragments of a polyclonal antibody from sheep, conjugated to alkaline phosphatase (AP), Roche, #2209136) was microfuged at 13,000 rpm for 5 min. This was done to remove particulates to reduce background by aggregated antibody. The antibody was then diluted 1:10,000 with fresh blocking solution by adding 5 µl antibody to 50 ml blocking solution. The membrane was incubated in this solution for 30 min at 25°C on a gyrotory shaker. This was followed by washing the membrane 2 times with 100 ml washing buffer-1X at 25°C on a gyrotory.

TLA Chemiluminescence Detection

The membrane was then incubated in 100 ml detection buffer-1X for 2-5 min at 25°C on a gyrotory. The membrane with the DNA side up was then placed on a dry 3MM filter paper, placed on top of a clear plastic sheet, so that the membrane did not dry completely. 3 ml of substrate solution (containing CDP-Star, a highly sensitive chemiluminescence substrate) was applied immediately. A second plastic sheet was immediately used to cover the membrane so that the substrate solution spread evenly. All

bubbles over the membrane were removed, and the membrane was incubated for 5 min at 25^{o}C. Excess substrate solution was squeezed out from the plastic sheets, and the membrane was exposed to Kodak XAR-5 X-ray film for 1 hour at 25^{o}C. Luminescence continued for 24 hours allowing multiple exposures. The signal intensity increased during the first few hours, so weak initial exposures were strengthened by waiting 1-2 hrs.

RESULTS

The purpose of this project was to test two hypotheses that telomerase activity and telomere lengths 1) appear to increase upon removal of differentiated Lin^+ cells, and 2) decrease upon differentiation and proliferation of hematopoietic cells. We investigated telomerase activity and telomere lengths in human cord blood hematopoietic cell populations obtained from Viacell Inc. at various intervals of a two-week *ex vivo* stem cell amplification process. We also quantified the data and estimated the inter-cord and intra-cord variability by analysis of variance (ANOVA).

Time Course of ViaCell's HSC Amplification Protocol

The main goal of this project was to analyze telomerase activity and telomere lengths throughout ViaCell's entire selection/ amplification protocol. Table 3 lists various time points of ViaCell's two-week *ex vivo* stem cell amplification process, with typical cell numbers and the typical $CD34^+CD38^-$ content of each time point. The time course corresponds to ViaCell's 14-day long amplification process. During this process, fresh whole cord blood mononuclear cells, which are un-amplified and termed 'Pre-Freeze' or Day-0, are first frozen and thawed. After thawing ('Post-Thaw' and 'Pre-Sep-1'), these cells undergo the first of two rounds of 'Negative Selection' separation to remove differentiated cells. The cell population is termed 'Pre-Sep-1' before passage over the column, and 'Post-Sep-1 after the first separation. After the first separation, the cells are grown for a week in medium supplemented with a combination of cytokines

known to stimulate HSC growth. These cells then undergo a second round of separation. The cell populations are called 'Pre-Sep-2' and 'Post-Sep-2, before and after the second

Table 3. Steps During ViaCell's HSC Amplification Protocol.

Time	Cell Sample Fraction	Typical Cell Number	Percentage of $CD34^+CD38^-$
Day 0 Fresh Cord	Pre-freeze	~6.5×10^8	0.26
Day 0	Post-Freeze Pre-Sep-1	~5.2×10^7	0.17
Day 0.5	Post-Sep-1	~2.7×10^6	3.20
Day 7	Pre-Sep-2	~2.1×10^7	31.25
Day 7.5	Post-Sep-2	~6.2×10^6	33.20
Day 14	Post Culture With proliferation-inducing cytokines	~3.0×10^7	10.53
Day 14 (Pre-Sep-3)	Post Culture With differentiation-inducing Cytokines	N/A	N/A

FACS data for cord-3 is shown representing typical $CD34^+CD38^-$ content for each time point.

separation step, respectively. These two stages correspond to 'Day-7' and 'Day-7.5' respectively. After the second separation, the cells are grown in culture for an additional week and are called 'Cell Product' or 'day 14'. These cells are usually frozen for storage. Upon thawing the cells are termed 'thawed day 14' (not shown on the time course). In one experiment, after the second separation, the cells were grown for a week in culture supplemented by a combination of cytokines including all-trans-retinoic acid (ATRA) known to induce differentiation of HSCs and primitive progenitor cells. This cell fraction is termed as 'Pre-Sep-3'. Note that the Post-Sep-2 sample contains the highest

percentage of CD34+/CD38- cells, representing approximately 128-fold enrichment of these cells over fresh cord.

An Overview of Telomerase Activity throughout ViaCell's Process

A TRAP (Telomerase Repeat Amplification Protocol) assay was used to measure telomerase activity throughout ViaCell's entire selection/ amplification protocol. For the time course experiments, at least 10^5 CD45+ (marker for hematopoietic cells) cells were provided at various time-points in ViaCell's amplification process. Because the TRAP assay is so sensitive, samples could be obtained from individual cords without pooling. Whole cell lysates were prepared from each sample. 1 μg of cord whole cell lysate protein load, previously determined to be the optimum protein load for the assay for cords-1 and 2 (Murthy, 2002) was used for the time course experiments. A single 'Master Mix' for the PCR amplification was used to assay all the samples from one cord, which proved to be critical for obtaining an even amplification of the internal 36 bp PCR control. The 36 bp internal PCR control was observed in all lanes, which indicates no cord sample contained an unusual amount of Taq Polymerase inhibitor.

Figure 7 shows duplicate determinations for cord-3. As expected, high telomerase activity was detected in the cancer cell extract positive control (lane M). Telomerase activity was undetected early in ViaCell's process, in 'pre-freeze' (Day-0), 'Post thaw' and 'Pre-Sep-1' time points (lanes 1-3) that theoretically represent quiescent cells. Telomerase activity was low but detectable in 'Post-Sep-1' (lane 4), was high in 'Pre- Sep-2' (lane 5), and peaked at 'Post-Sep-2' (lane 6). Telomerase activity at 'Post-Sep-2' (Day-7) was higher than that of the cancer cell extract positive control (lane M). At 'Day-14', however, a decrease in telomerase activity was observed. The activity increased following freeze/thaw of the 'Day-14' fraction. These results indicate that

telomerase activity is low or undetectable when the cells are quiescent ('pre-freeze' (Day 0), 'Post thaw' and 'Pre-Sep-1') and increase when the cells are stimulated by cytokines to proliferate ('Post-Sep-1', 'Pre- Sep-2', 'Post-Sep-2'). The highest telomerase activity

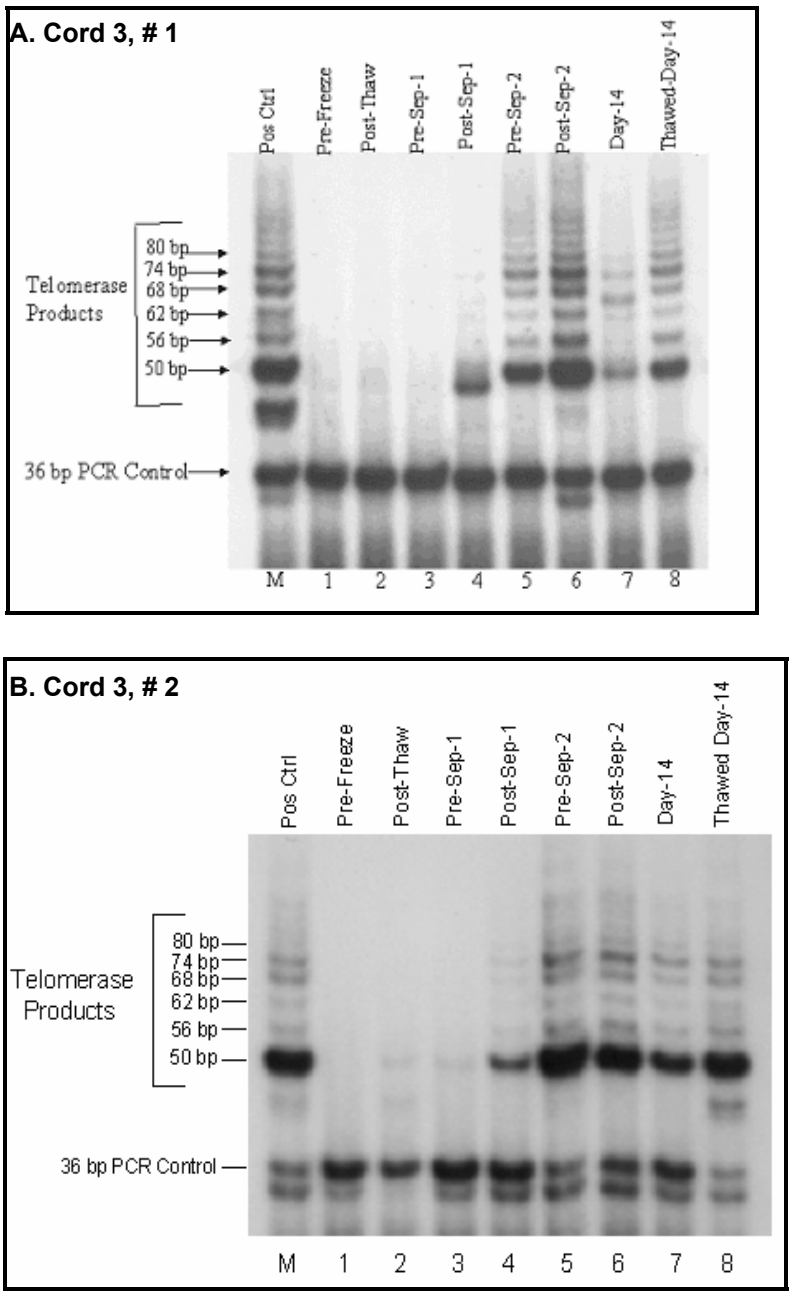


Figure 7. Telomerase Activity is Low in Quiescent Cells and Increases in Proliferating Cell Populations. TRAP assay on cord 3, #1 (top), and # 2 (bottom)

was seen in the 'Post-Sep-2' cell fraction, which superficially appears to correlate with cell population containing the highest percent $CD34^{+}CD38^{-}$ cells (which are known to be high in telomerase activity). A dip in telomerase activity at 'Day-14' is expected in rapidly proliferating cell population, which is also known to contain differentiated cells.

Surprisingly, there was resurgence in telomerase activity in 'thawed-day 14' cells which only differ from the 'day 14' cells by a single round of freeze/thaw. We speculate this may result from a higher survivability of cells during the freeze/thaw that contain telomerase. However, a more detailed investigation is required to prove this.

Figure 7 lower panel represents trial # 2 for Cord 3. This second trial showed the same trends in telomerase activity as trial 1. Figure 8 shows the quantitation data for cord 3 determined by counting the ^{32}P cut from the dried gel that corresponds directly with the telomerase ladder rungs from the x-ray films. The Post-Sep-2 sample (130%) showed a 120% increase in activity over fresh cord (10%) ($p = 0.0001$, Tukey-ANOVA test).

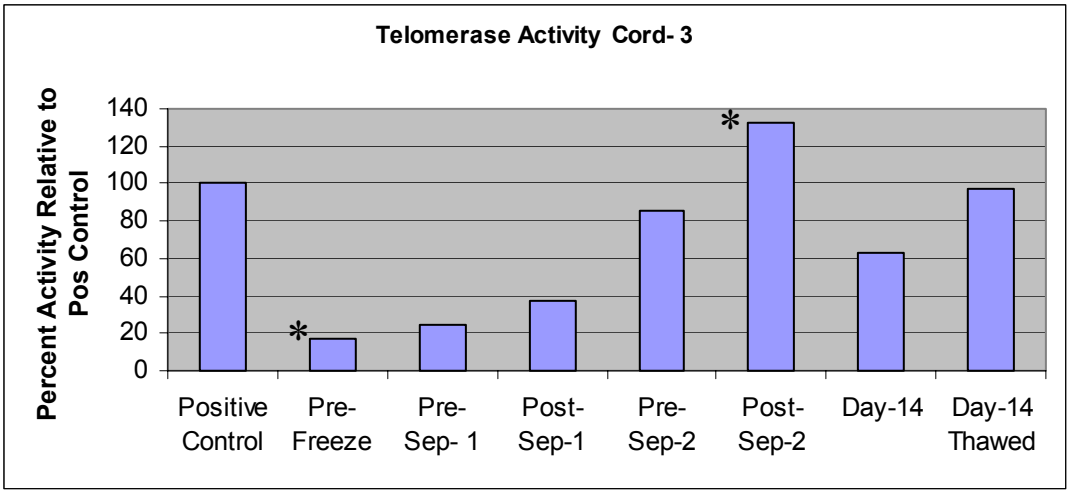


Figure 8. Quantitation of Telomerase Activity in Cord-3. The Y-axis shows values as percent activity relative to the cancer cell positive control. Histobars represent the mean telomerase activity of the two trials for cord 3. The Post-Sep-2 sample (130%) showed a 120% increase in activity over fresh cord (10%) ($p = 0.0001$, Tukey-ANOVA test) (*).

Telomerase Activity Apparently Increases in Post-Sep Cells Relative to Pre-Sep and Decreases upon Differentiation and Proliferation

To test the hypotheses that telomerase activity will appear to increase upon the removal of differentiated cells, and decrease upon differentiation of hematopoietic cells specific fractions from cord 4 were analyzed. After post-sep-2, we let the selected primitive cells mature and differentiate for a week (Pre-Sep-3) under the influence of a combination of differentiation-inducing cytokines (ATRA). Table 4 shows FACS data for cord-4. In this particular cord (unlike cord-3) note the strong increase in CD34+CD38$^-$ cells Post-Sep-1 relative to Pre-Sep-1, but only a slight percent increase in CD34+CD38$^-$ cells Pre-Sep-2 vs Post-Sep-2. Also note the substantial decrease in percent CD34$^+$CD38$^-$ cells for the Pre-Sep-3 sample.

Table 4. Percent CD34$^+$CD38$^-$ Content for Cord-4.

Cord-Sample (Cord-4)	Pre-Sep-1	Post-Sep-1 (Day-0.5)	Pre-Sep-2 (Day-7)	Post-Sep-2 (Day-7.5)	Pre-Sep-3 (Day-14)
CD34$^+$CD38$^-$ (%)	0.81	85.09	75.42	79.78	22.30

Figure 9 shows the TRAP data for cord-4, and figure 10 its quantitation. The TRAP analysis revealed that both post-column separation cell populations (Post-Sep-1 and Post-Sep-2; lane 2 and lane 4 respectively) showed apparent increases in telomerase activity compared to their corresponding pre-column separation cell populations (Pre-Sep-1 and Pre-Sep-2 respectively; lane 1 and lane 3). For this cord, the increase was especially prevalent for the Post-Sep-1 sample that showed the greatest increase in CD34+CD38$^-$ cells relative to before the column. Thus the removal of differentiated cells can have a substantial effect on the apparent telomerase activity of the remaining cells.

The Pre-Sep-3 cell fraction showed low to undetectable telomerase activity. This indicates that telomerase activity apparently decreases upon differentiation and proliferation of ViaCell's hematopoietic cells. Post-Sep-1 and Post-Sep-2 showed about

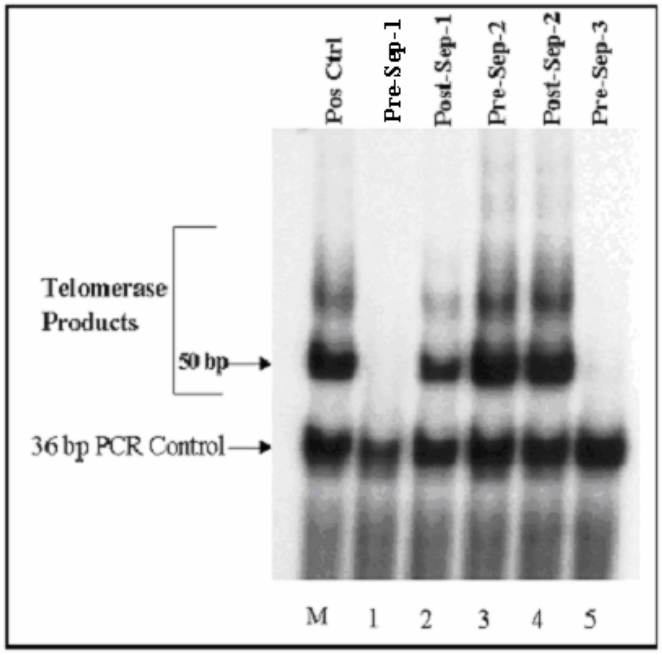


Figure 9. Telomerase Activity Apparently Increases in Post-Sep Relative to Pre-Sep Cells and Decreases Upon Cell Differentiation and Proliferation. TRAP assay on Cord 4, # 1.

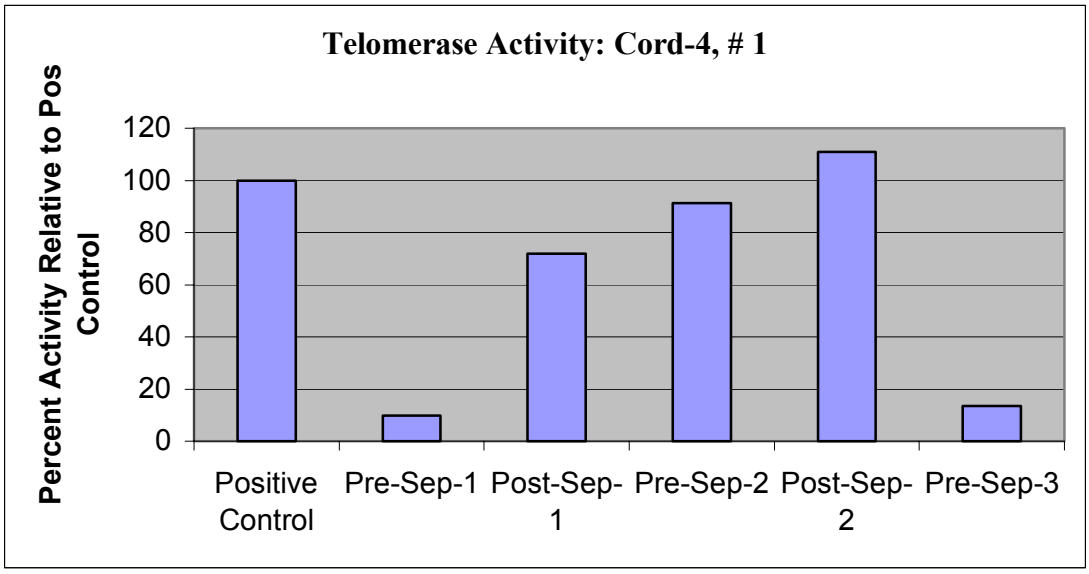


Figure 10. Quantitation of Telomerase Activity in Cord-4, # 1. The Y-axis shows values as percent activity relative to cancer cell positive control.

707% and 22% increase in telomerase activity over Pre-Sep-1 and Pre-Sep-2 cell populations respectively (p = 0.0001, Tukey-ANOVA test). Pre-Sep-3 showed about 702 % decrease in telomerase activty compared to Post-Sep-2 (p = 0.0001, Tukey-ANOVA test).

The TRAP Assay is Highly Reproducible with Low Intra-Cord Variability

In order to determine the intra-cord variability in the TRAP assays, we analyzed the telomerase activity in cord-4 five more times (Figure 11). All five TRAP assays

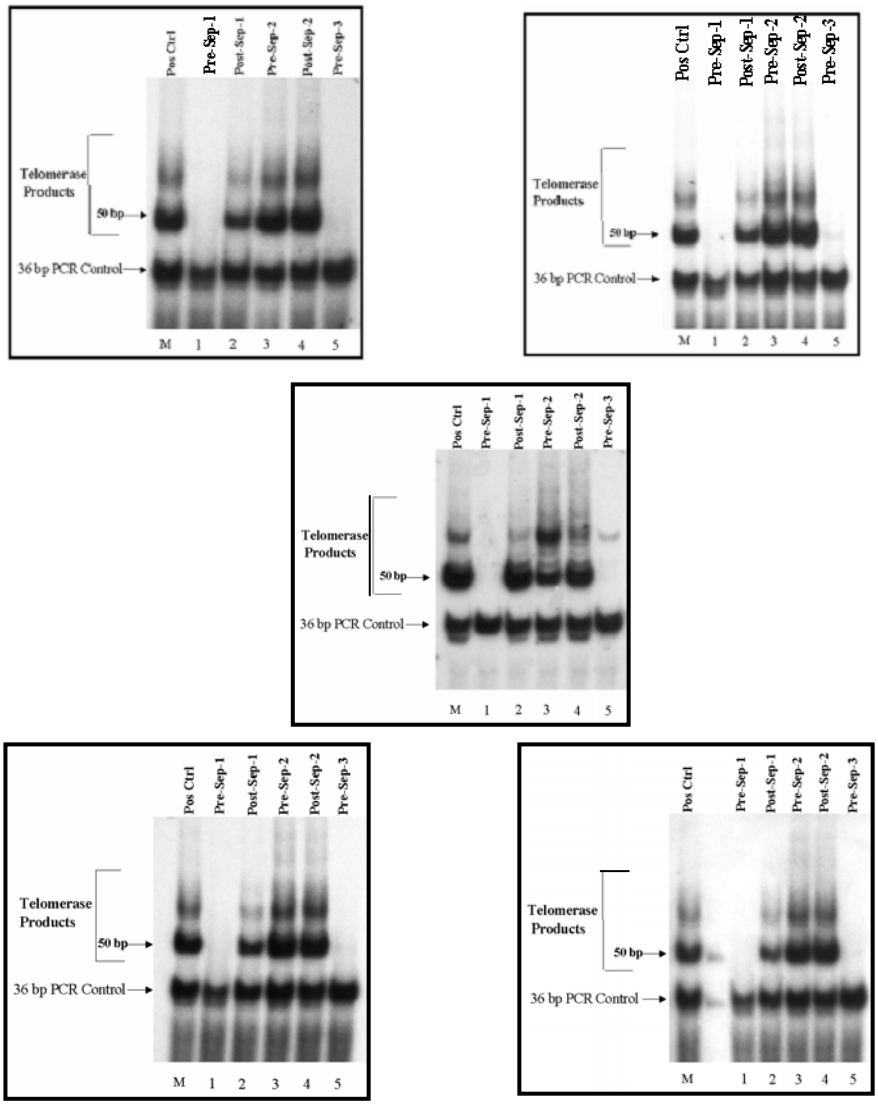


Figure 11. Several TRAP Assays on Cord 4. # 2 (top left), # 3 (top right), # 4 (center), # 5 (bottom left), # 6 (bottom right).

showed the same trend in telomerase activity as in the previous figure. All six determinations for cord-4 are quantitated in figure 12. The low standard deviations indicate that the TRAP assay is highly reproducible with low intra-cord variability. Moreover, the quantitation data is in agreement with the trends in telomerase activity for cord-3 as seen in figures 10 and 11, although the sep-1 difference was smaller, and the sep-2 difference was larger.

Sample	# 1	# 2	# 3	# 4	# 5	# 6	Mean	Std Dev
Positive control	100.0	100.0	100.0	100.0	100.0	100.0	100.0	
Pre-Sep-1	9.9	8.9	9.3	9.6	7.0	6.1	8.5	1.5
Post- Sep 1	79.0	75.6	78.7	79.5	66.7	77.6	76.2	4.9
Pre- Sep 2	91.4	87.8	84.0	88.0	82.5	89.8	87.2	3.4
Post- Sep 2	109.9	108.9	114.7	116.9	103.5	114.3	111.3	4.9
Pre- Sep 3	13.6	12.2	16.0	16.9	15.8	14.3	14.8	1.7

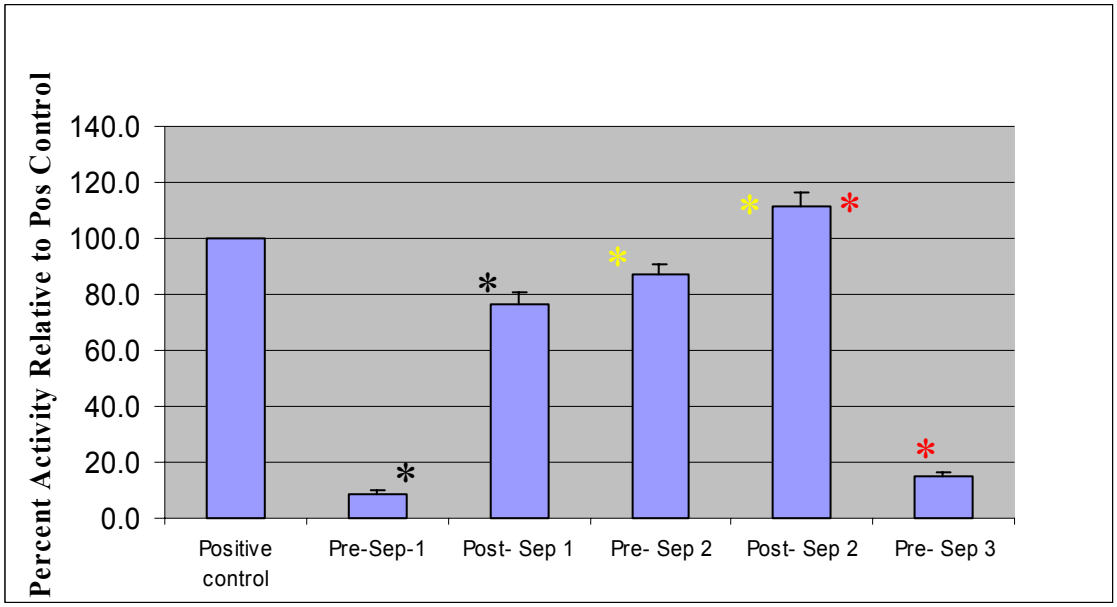


Figure 12. The TRAP Assay is Highly Reproducible with Low Intra-Cord Variability. Telomerase activity was measured relative to a cancer cell extract positive control set at 100%. Histobars represent the mean telomerase activity for six TRAP assays on cord 4. Error bars represent the standard deviations from the mean. Statistical analysis was performed on logarithm-transformed data using the Tukey-ANOVA test (logarithms are more normally distributed than ratios). Post-Sep samples were found to be significantly different than Pre-Sep samples (**P < 0.0001). Telomerase activity sharply declined in Pre-Sep-3 compared to Post-Sep-2 (*P < 0.0001).

Inter-Cord Variability

Serum cytokine levels are known to vary among cord donors, which could affect hematopoiesis. Inter-cord variability was investigated for four cords (the first two cords were analyzed by Vidya Murthy and the next two by myself) as shown in figure 13. Unlike intra-cord variability, higher inter-cord variability in telomerase activity was observed with standard deviations from the mean activity ranging from 9% to 33%. Although the stand deviations are larger than for single cords, the data still support both the hypotheses.

	Vidya's Data		Manish's Data			
Sample	N1	N2	N3	N4	Mean	Std Dev
Marker	100	100	100	100	100	
Pre-Freeze	22	5	18	ND	15.0	8.9
Post-Thaw	22	ND	24	ND	23.0	N/A
Pre-Sep-1	28	ND	30	10	22.7	11.0
Post-Sep-1	35	18	38	78	42.3	25.4
Pre-Sep-2	60	50	82	85	69.3	17.0
Post-Sep-2	122	85	130	110	111.8	19.6
Day-14	70	30	62	ND	54.0	21.2
Thawed Day-14	100	42	98	ND	80.0	32.9

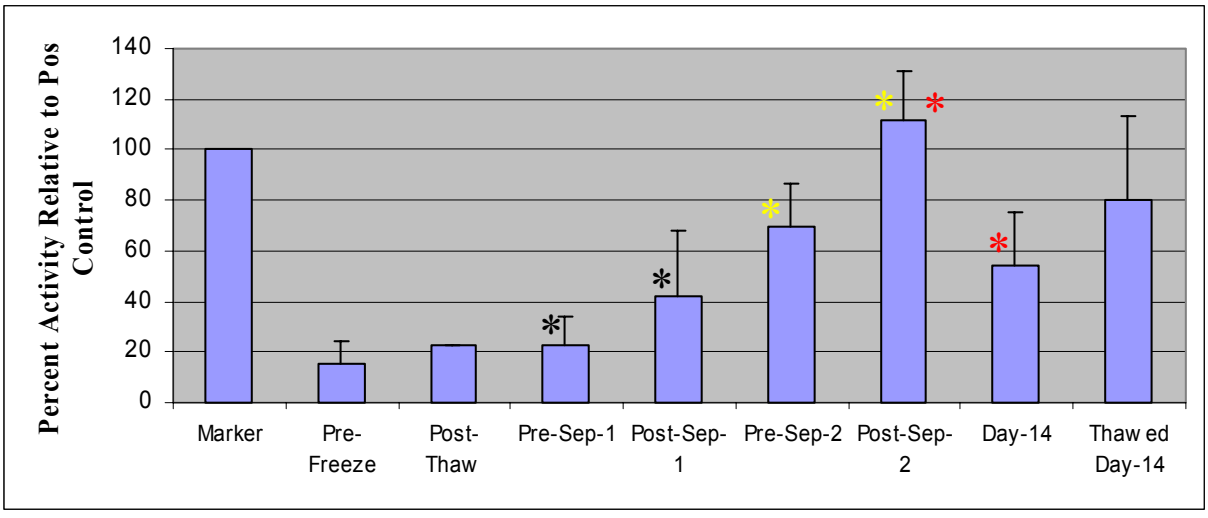


Figure 13. Inter-Cord Variability. Telomerase activity was measured relative to a cancer cell extract positive control set at 100%. Histobars represent the mean telomerase activity for four different cords, Cords 1-4. Error bars represent the standard deviations from the mean. Statistical analysis was performed on logarithm-transformed data. The Post-Thaw sample could not be statistically analyzed because only two of these cell fractions were available. Post-Sep-1 and Post-Sep-2 showed an 87% and 60% increase in activity over Pre-Sep-1 and Pre-Sep-2 cells respectively (** $p = 0.001$). Day-14 cells showed a 52% decrease in telomerase activity relative to Post-Sep-2 (* $p = 0.001$).

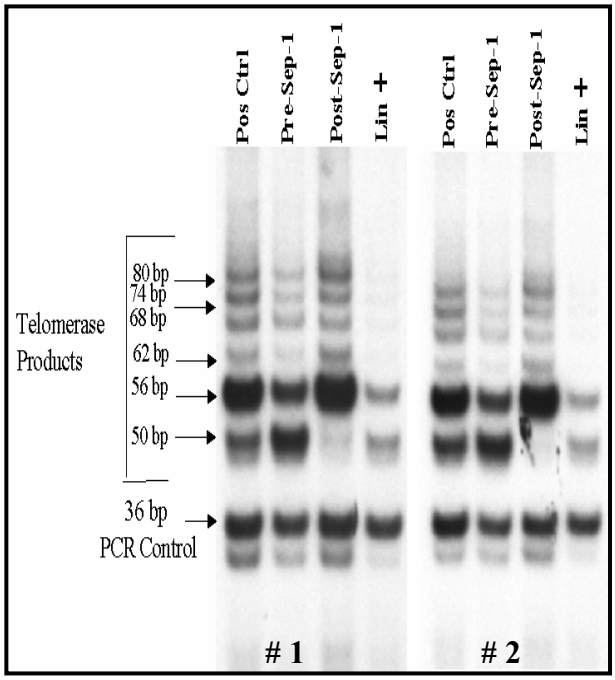
Removal of Telomerase-Low Differentiated (Lin^+) Cells Apparently Increases Telomerase Activity in Post-Sep Cells

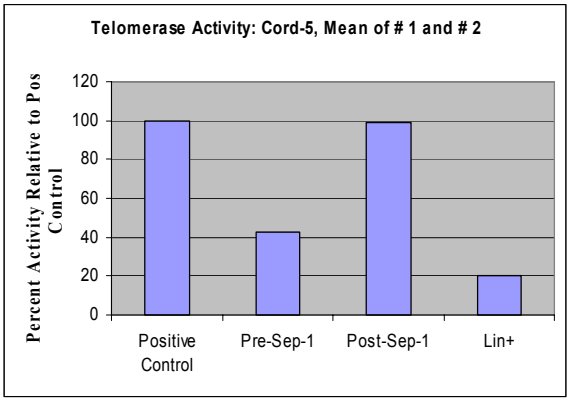
From our TRAP data, we hypothesized that removal of telomerase-low differentiated (Lin^+) cells is responsible for the apparent increase in telomerase activity in Post-Sep cells. To test this hypothesis, we analyzed telomerase activity in Lin^+ cells during a sep-1 separation. Table 5 shows the percent $CD34^+CD38^-$ cells in Pre- and Post-Sep-1 fractions for cords 5 - 7. The FACS data for Lin^+ cells was unavailable for these cords.

Table 5. Percent $CD34^+CD38^-$ Content of the Pre- and Post-Sep-1 Fractions of Cords 5-7.

Cord-Sample	Pre-Sep-1	Post-Sep-1	Fold Enrichment
Cord-5	1.34 %	26.09 %	19.5
Cord-6	1.72 %	36.57 %	21.3
Cord-7	0.57 %	13.88 %	24.3

Figure 14 shows the TRAP data for the Sep-1 samples of cords 5- 7.





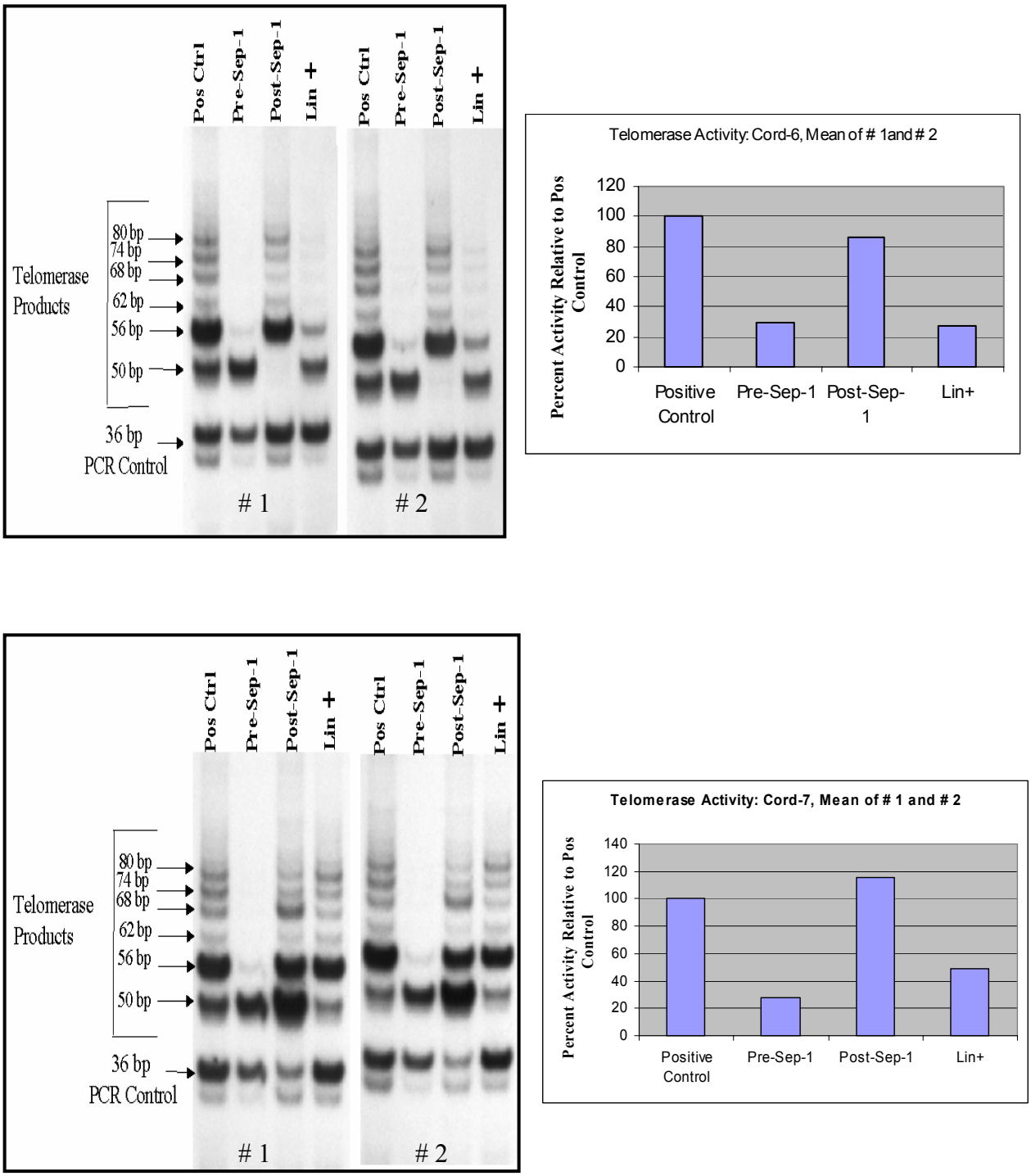


Figure 14. Lin+ Cells are Low in Telomerase Activity. TRAP Assay on Cords 5- 7, # 1 and # 2 (left). Lane 1 shows the cancer cell extract positive control, lane 2 shows Pre-Sep-1, lane 3 shows Post-Sep-1, and Lane 4 shows Lin+. Quantitation data (right) represents the mean of # 1 and # 2. As stated earlier, telomerase activity was measured relative to the positive control.

The statistical summary of the sep-1 fractions for cords 5- 7 is shown in figure 15. The Post-Sep-1cell fraction showed 2.98-fold increase in telomerase activity over Pre-Sep-1 (* $P < 0.0001$). Supporting our hypothesis Lin^+ cells were observed to be 3.12-fold less in telomerase activity compared to Post-Sep-1 (* $P < 0.0001$). Lin^+ cells were removed from the column by pushing about 50 ml 1% BSA/ Saline through the column

Sample	Mean Activity Cord-5	Mean Activity Cord-6	Mean Activity Cord-7	Mean	Std Dev
Pos Control	100	100	100	100	
Pre-Sep-1	28	42.7	30	33.6	8.0
Post-Sep-1	115.7	98.9	85.9	100.2	14.9
Lin+	49	20.3	27	32.1	15.1

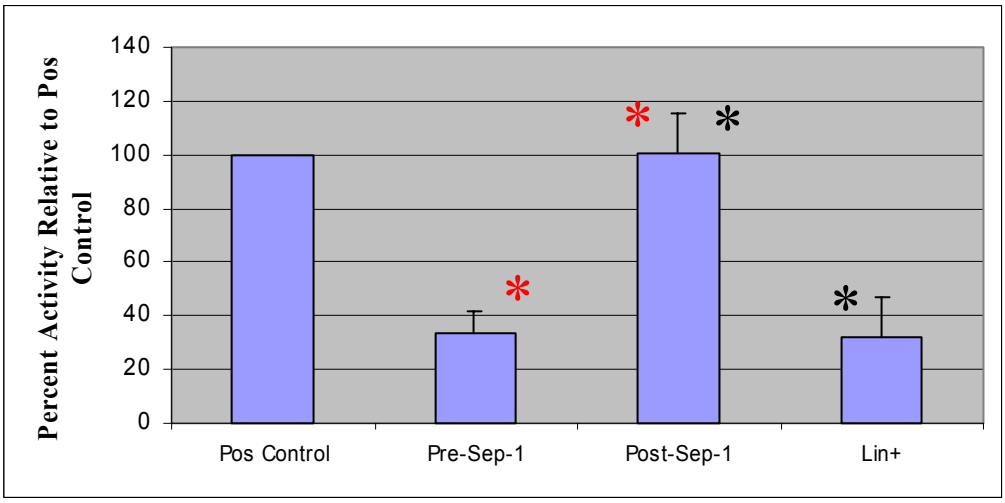


Figure 15. Statistical Summary of Cords - 5, 6, and 7. Telomerase activity was measured relative to the cancer cell extract positive control set at 100%. Histobars represent the mean telomerase activity of # 1 and # 2 for each of the three cords (i.e., two TRAP assays per cord). Error bars represent the standard deviations from the mean. Statistical analysis was performed on logarithm-transformed data using the Tukey-ANOVA test (logarithms are more normally distributed than ratios). ** $p = 0.0001$

at a very high flow rate. Cells were then collected by centrifugation. It is interesting to note that Lin^+ cells showed some telomerase activity. This could be attributed to the presence of some cycling mature progenitors present in the Lin^+ cell population.

Telomere Length Assay

The second goal of this thesis was to test the hypothesis that the telomere lengths will appear to increase upon the removal of differentiated cells, and decrease upon differentiation and proliferation of hematopoietic cells. We investigated telomere lengths throughout ViaCell's entire selection/ amplification protocol. Since there is no reliable method of directly measuring the complex assortment of telomere lengths in human cells, we and others have assayed mean telomeric restriction fragment (TRF) lengths to detect changes in the length of the terminal TTAGGG repeats (Harley et al., 1990; Hastie et al., 1990; Lindsey et al., 1991). The mean TRF lengths in human cord blood hematopoietic cells were measured using a telomere length assay (TLA) (Harley et al., 1990).

The length of telomeres is remarkably variable because of the variability in the number of TTAGGG repeats (de Lenge et al., 1990). Telomere lengths show wide inter-individual variation, and they also vary among cells in the same tissue and among chromosomes in the same cell (Blackburn et al., 2000). Therefore, analyzing a population of cells provides the average telomere length of all the telomeres in the sample, indicated by a smear whose average size is compared to a molecular weight marker. TRFs comprise not only the variable terminal telomeres but also a brief sub-telomeric region. In addition to a molecular weight marker, two positive control DNAs were analyzed: Control-DNA-low (mean TRF = 3.7 Kb, obtained from a late-passage fibroblast cell line) and Control-DNA-high (mean TRF = 9.5 Kb, obtained from a cancer cell line) supplied with the TeloTAGGG kit.

Telomere Lengths Decrease upon Expansion and Proliferation of Hematopoietic Cells

To determine the telomere lengths of ViaCell's hematopoietic cells before and after their selection/ amplification protocol, we analyzed the Day-0 and Day-14 cell populations by TLA (figure 16). The Day-14 cell fraction (selected and amplified, ave. 10.53% $CD34^{+}CD38^{-}$) (see table 6) was observed to have slightly shorter telomere lengths (avg. 9 Kb) compared to the Day-0 cell fraction (fresh cord blood cells) (avg. 10 Kb) (0.26% $CD34^{+}CD38^{-}$). However, as expected of human cord-blood hematopoietic cell populations, the telomere lengths of both ViaCell's fractions were comparable to the high DNA control. Combining the TRAP and TLA data, despite the presence of significant telomerase activity in the Day-14 sample, I observed some proliferation-associated telomeric DNA loss. Therefore, the telomerase activity in that population may not be sufficient to completely prevent telomere shortening.

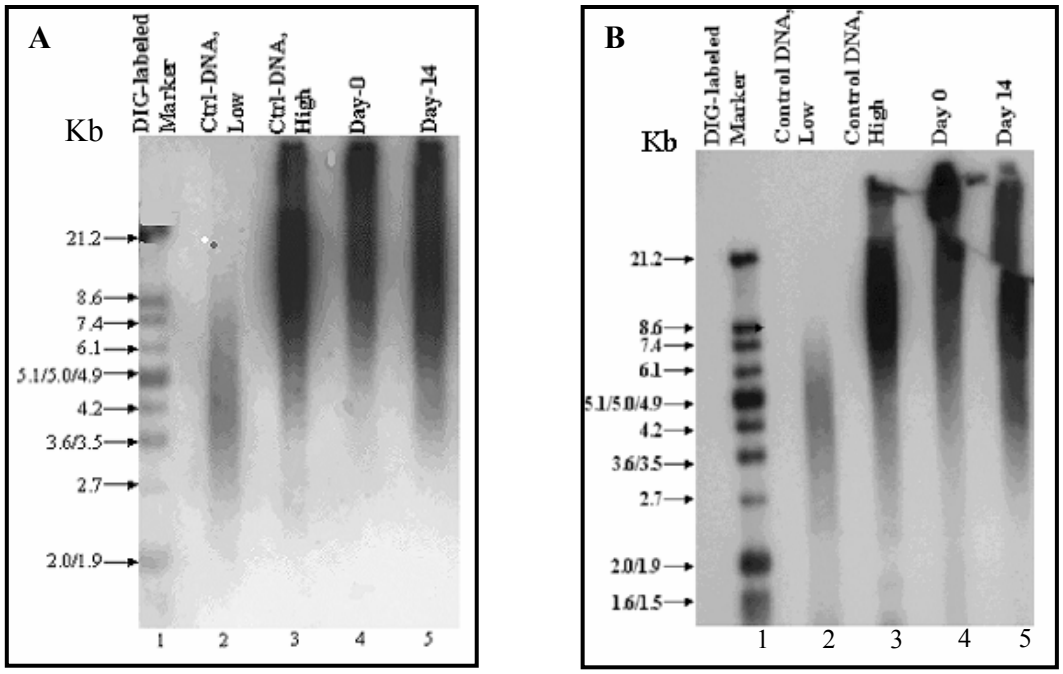


Figure 16. Telomere Lengths Decrease Upon Cell Proliferation and Differentiation. A. # 1, and B. # 2 of the TLA assay on Day-0 and Day-14 cell populations. Lane 1 shows a molecular weight marker, lane 2 shows control DNA- low, lane 3 shows control DNA-high, lane 4 shows Day 0 telomeric DNA and lane 5 shows Day 14 telomeric DNA.

Telomere Lengths Appear to Increase in Post-Sep Cell Populations

To determine the effect of the removal of differentiated cells (Lin^+) on telomere lengths, we analyzed telomere lengths of the Post-Sep cell populations. In general, as expected in cell populations enriched with primitive cells and HSCs, we observed long telomeres in Post-Sep-1 and Post-Sep-2 cell fractions, averaging 12 Kb and 14 Kb respectively, and 41.58% and 43.57% $CD34^+CD38^-$ cells (Figure 17). In another experiment, we again found average telomere lengths of Post-Sep-2 to be 14 Kb (data not shown) and Post-Sep-1 to be 12 Kb (figure 18) compared to 10 Kb for Pre-Sep-1. This data supports the hypothesis that the removal of differentiated cells will appear to increase telomere lengths in the remaining population.

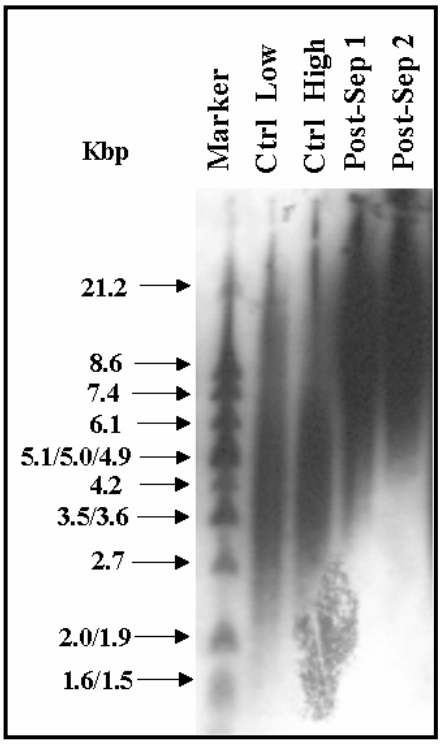


Figure 17. Telomere Lengths Appear to Increase in Post-Sep Cell Populations. TLA assay on Post-Sep-1 and Post-Sep-2 cell populations. Lane 1 shows a molecular weight marker, lane 2 shows control DNA- low, lane 3 shows control DNA-high, lane 4 shows Post-Sep-1 telomeric DNA and lane 5 shows Post-Sep-2 telomeric DNA.

Lin$^+$ Cells Have Shorter Telomeres Than Post-Sep-1 Populations

To determine if the differentiated Lin$^+$ cells removed by column-separation really have shorter telomeres than a corresponding Post-Sep population, we analyzed average telomere lengths in these cell populations (Figure 18). Post-Sep-1 cells (63.16% CD34$^+$CD38$^-$ cells) (avg. 12 Kb) exhibited longer telomeres compared to Pre-Sep-1 cells (1.29% CD34$^+$CD38$^-$ cells) (avg. 10 Kb). As expected of differentiated cell populations, we observed relatively small telomere lengths in Lin$^+$ cells (7.6 Kb). Thus, this data indicates that the apparent increase in telomere lengths of Post-Sep samples likely results from the removal of mature and differentiated cells with shorter telomere lengths.

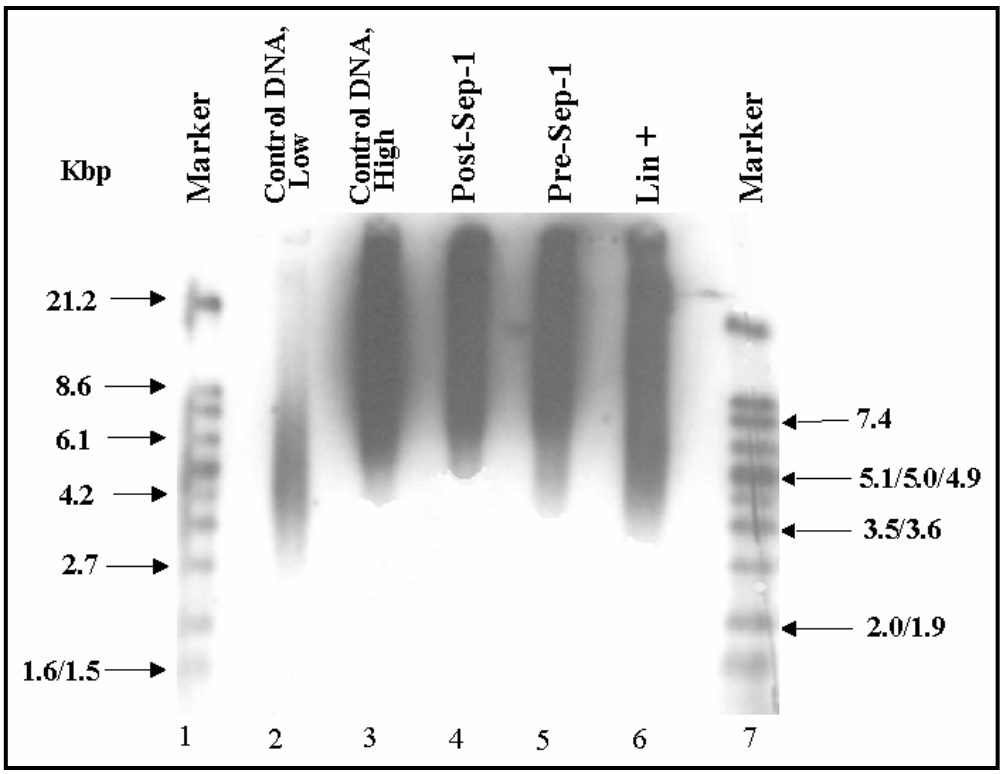


Figure 18. Lin$^+$ Hematopoietic Cells Have Shorter Telomeres. TLA assay on Post-Sep-1, Pre-Sep-1, and Lin$^+$ cell populations. Lanes 1 and 7 show a molecular weight marker, lane 2 shows control DNA- low, lane 3 shows control DNA-high, lane 4 shows Post-Sep-1 telomeric DNA, lane 5 shows Pre-Sep-1 telomeric DNA, lane 6 shows Lin+ telomeric DNA from the sep-1 column.

Subsequent analyses were attempted using New England Biolab's restriction enzymes for genomic DNA digestion (including their digestion buffer instead of Roche's) (the latter was depleted quickly); however, the DNA was not effectively digested using these enzymes (data not shown). The TLA data is summarized in table 6.

Table 6. Summary Table of Telomere Lengths and $CD34^+CD38^-$ Content at Various Time Points in ViaCell's Amplification Process.

Time- point in ViaCell's Amplification	Percent $CD34^+CD38^-$	Avg. Telomere Length (Kb)
Day 0	0.26	10
Pre-Sep-1	1.29	10
Post-Sep-1	63.16	12
Post- Sep-2	43.57	14
Day 14	10.53	9
Lin +	N/A	7.5

Average telomere lengths of two TLA trials on each time point are shown.

Telomerase Activity and Telomere Lengths Do Not Always Correlate with $CD34^+CD38^-$ Content

To determine whether telomerase activity and telomere lengths correlate with the percent $CD34^+CD38^-$ cells in a population we compared a summary of cords 1- 7 TRAP and TLA data with FACS analysis data (Table 7). Upon initial observation, they appear to correlate. For example, for cords 1- 7, the fraction containing the highest telomerase activity (Post-Sep-2) also contains the highest percent $CD34^+CD38^-$ cells. And the fraction containing the lowest telomerase activity (Day-0 Pre-Freeze) also contains the lowest percent $CD34^+CD38^-$ cells. Upon closer observation, however, we observed that

telomerase activity and average telomere lengths did not always correlate with $CD34^+CD38^-$ content. We analyzed individual cord data (cords 1- 4) specifically for Post-Sep-2 cell fractions that are relatively enriched with $CD34^+CD38^-$ cells (Table 8). Although cord-4 contained the highest percentage of

Table 7. Summary Table of Cords 1- 7: Average of FACS Data with TRAP and TLA Results.

Time	Cell Sample Fraction	Percentage of $CD34^+CD38^-$ Cells	Relative Telomerase Activity (% relative to a positive control)	Average Telomere Length (Kb)
Day 0	Pre-freeze	0.2	13.8	10
Day 0	Pre-Sep 1	0.7	29	10
Day 0.5	Post-Sep 1	24.3	42.3	12
Day 7	Pre-Sep 2	36.6	69.3	N/A
Day 7.5	Post-Sep 2	39.0	111.8	14
Day 14	Post Culture With proliferation-inducing cytokines	7.8	54	9
Day 14 (Pre-Sep-3)	Post Culture With differentiation-inducing cytokines	N/A	14.8	7.5

Mean $CD34^+CD38^-$ content, telomerase activity and TLAs for cords 1-7 are shown. Average telomere lengths of all the cell fractions is represented, except Pre-Sep-2. N/A = not available.

Table 8. Telomerase Activity Does Not Correlate with $CD34^+CD38^-$ Content.

Cord #	Post-Sep-2		
	Percentage of $CD34^+CD38^-$ Cells	Relative Telomerase Activity (% relative to a positive control)	Ratio Activity/ % CD34+CD38-
Cord- 1	23.09	123	5.3
Cord- 2	19.91	84	4.2
Cord- 3	33.2	133	4.0
Cord- 4	79.78	110	1.4

FACS data and telomerase activity for cords 1-4 are shown for the Post-Sep-2 fractions.

$CD34^+CD38^-$ cells (79.78%) it exhibited lower telomerase activity than cord-1 and cord-3. Thus, our results indicate that telomerase activity does not always directly correlate with $CD34^+CD38^-$ content, implying that this activity also resides in other kinds of cells.

In summary, our data indicate that the removal of telomerase-low telomere-short differentiated (Lin^+) cells appears to increase the telomerase activity and telomere lengths in the remaining cell population. Moreover, our data also suggests that telomerase activity and telomere lengths decrease upon hematopoietic cell differentiation and proliferation. Lastly, we show that telomerase activity and average telomere length do not directly correlate with $CD34^+CD38^-$ content.

DISCUSSION

Viacell Inc. (Worcester, MA) expands and enriches its populations of cells containing HSCs by removing only those Lin^+ cells known not to contain HSCs. This is accomplished on two separation columns (post-sep-1, and post-sep-2) (separated by 7 days of cell growth) that contain a variety of antibodies to known differentiation surface markers. Although this process strongly enriches functional HCSs, these primitive cell populations remain biochemically uncharacterized. Because telomerase activity and telomere lengths are known to correlate with the long term proliferation and reconstitution potential of hematopoietic cells, it is of utmost importance to Viacell to analyze these parameters in their hematopoietic cell fractions slated for transplant into patients.

We quantified the telomerase activity in all the cord blood cell fractions using a radioisotope-count based method. Several attempts have been made by other labs at quantifying telomerase activity, ranging from subjective ratings of assay signal intensities, to computerized densitometric ladder analyses that control for background noise and assay inhibitors. Quantitative results are probably most meaningfully expressed on a per cell basis, but such extremely sensitive assays are often difficult or impossible to do. Therefore our results were presented on a per protein basis. Even on a per cell basis however, the results would not be informative unless one also knows the surface marker or functional phenotype of the cell being analyzed. For example, tumor metastases often have higher levels of telomerase activity than those found in the original tumor. The primary tumor is often much more heterogeneous, including normal epithelial and stromal cells, as well as varying amounts of extracellular matrix and

connective tissue, all of which may act to decrease the proportion of cancer cells in the sample (Kim et al., 1994). The cloning of the genes for the RNA component (Feng et al, 1995) and, more recently, the catalytic protein subunit (Nakamura et al., 1997) of human telomerase opens up the prospects of developing new *in situ* assays for the detection and detailed localization of these critical enzyme components. These new tools will provide information on the important issues of the tissue localization and regulation of this enzyme.

The first objective of the present study was to analyze telomerase activity and telomere lengths throughout ViaCell's entire amplification protocol. We show that telomerase activity differs throughout their procedure. Low telomerase activity was detected early in ViaCell's process, that is, in 'Pre-Freeze' (Day-0), 'Post-Thaw', and 'Pre-Sep-1' fractions. This data is as expected. Day-0 cells theoretically represent quiescent cells, so our data support the findings of Lansdorp et al (1996) and Engelhardt et al (1997) that quiescent cells are low in telomerase activity. Telomerase activity was found to be significantly upregulated on cell proliferation and expansion stimulated with a proliferation-inducing cytokine combination in Pre-Sep-2, and decreased upon further proliferation and differentiation in Day-14 cells. These results are in line with others (Engelhardt et al., 1997; Holt et al., 1996a) who showed that telomerase is upregulated in rapidly expanding cells, and downregulated upon differentiation. Moreover, Day 14 cells were observed to have shorter telomere lengths (avg. 9 Kb) compared to Day-0 cells (avg. 10 Kb). These results agree with previous studies by Engelhardt et al (1997) and Chiu et al (1996) that telomere lengths shorten upon cell proliferation and differentiation. Despite the presence of significant telomerase activity, we observed a proliferation-associated telomeric DNA loss of about 1 Kb in Day-14 cell populations during the two weeks of ex vivo expansion compared to Day-0. Thus, our data suggest that telomerase

activity in hematopoietic cells reduces, but does not prevent, telomere shortening on proliferation.

The next goal was to specifically test our first hypothesis that telomerase activity and telomere lengths (markers of the cellular proliferation and long-term reconstitution potential) appear to increase upon removal of differentiated Lin^+ cells. We observed a significant increase in telomerase activity in post-column separation cell populations (Post-Sep-1 and Post-Sep-2) compared to their pre-column equivalents. Moreover, consistent with high telomerase activity in Post-Sep cells, we observed long telomeres in Post-Sep-1 (avg. 12 Kb) and Post-Sep-2 (avg. 14 Kb) compared to Pre-Sep-1 (avg. 10 Kb). We believe that this could be because the Post-Sep cell populations were enriched with HSCs and primitive progenitor cells known to be high in telomerase activity, after removal of differentiated Lin^+ cells that are known to be low in telomerase activity. To test this, we analyzed Lin^+ cells from the sep-1 process. We observed low telomerase activity in Lin^+ cells off the column and short telomere lengths (avg. 7.5 Kb) in these differentiated cell populations. These results support those of (Engelhardt et al., 1997) who showed that differentiated hematopoietic cells contain low to undetectable telomerase activity and short telomere lengths. These results support our hypothesis that removal of telomerase-low telomere-short Lin^+ cells appears to increase telomerase activity and telomere lengths in the remaining cells. Although it is possible that our observed telomerase increase could be due to the removal of Lin^+ cells that had been secreting a repressing lymphokine that reduced telomerase activity in the pre-sep-1 cells, we believe this is unlikely since very little time elapsed following removal of the lin^+ cells (perhaps 30 minutes), thus the cells would have very little time in which to upregulate telomerase activity. Our proposed mechanism remains the simplest explanation.

We then tested our second hypothesis that telomerase activity and telomere lengths decrease upon hematopoietic cell differentiation and proliferation. We observed that telomerase activity decreased in Day-14 cells (expanded and differentiated 14 days) relative to Post-Sep-2 (selected 7 days). Day 14 cells exhibited shorter telomere lengths (avg. 9 Kb) compared to Post-Sep-2 cells (avg. 14 Kb). This is in line with findings of Counter et al (1995), Hiyama et al (1995), and Zhang et al (1996) who showed that upon cell differentiation, telomerase activity is repressed and telomere lengths are shortened. Moreover, significantly low telomerase activity was detected in Pre-Sep-3 cells (upon ATRA treatment) indicating that telomerase activity is repressed in terminally differentiated cells. Thus our data supports Holt et al (1996a) who showed low telomerase activity in terminally differentiated cells. These results support our second hypothesis that telomerase activity and telomere lengths decrease upon hematopoietic cell differentiation and proliferation.

Our data supports that of Chiu et al (1996) who showed that the presence of telomerase activity in hematopoietic subpopulations is insufficient to overcome the net telomere loss in the total population. We speculate that a threshold level of telomerase activity may be required to maintain telomere lengths in a population of cells challenged mitotically. Currently available technology does not allow us to determine whether our observed telomerase expression in ViaCell's Day-14 cells functioned to slow the rate of telomere loss.

The possibility that telomerase activity detected in ViaCell's cell extracts does not correlate with its functional activity in the intact cells cannot be ruled out. For instance, telomere-binding proteins (Zhong et al., 1992; McKay and Cooke, 1992; Cardenas et al., 1993) or chromatin structures may play a role in regulating the accessibility of telomeres to the telomerase enzyme. Moreover, telomerase activity might be expressed in only a

small subpopulation of cells (for example, $CD34^+KDR^+$ or $CD34^+CD71^+$, etc.), and stabilization of telomere length in this subpopulation could be masked when the cultures are analyzed as a whole. If there exists a subpopulation of cells that maintains stable telomeres, and does not differentiate, it should theoretically have an unlimited replicative capacity. Unfortunately since ViaCell does not positively select for $CD34^+$ cells in any of their procedures, we were unable to analyze telomerase activity and telomere lengths in this specific fraction. Given that there are currently no *in vitro* growth conditions in which primitive hematopoietic cells can selectively self-renew, the presence of such a population would not be easily identified (Chiu et al., 1996). Eventually, only the use of single cell assays for telomerase activity, and more sensitive techniques for telomere length measurements, could help to resolve this issue.

Recent studies have suggested that HSC activity exists within the murine and human Lin^-CD34^- population (Sato et al., 1999; Ando et al., 2000; Tajima et al., 2000; 2001; Ogawa, 2002). This redirects us to consider which cell population to select for therapeutic applications. If HSCs exist within the $CD34^-$ population, the enrichment of $CD34^+$ cells, the current practice in stem cell transplantation and to date one of the most efficient and effective HSC separation methods, may be of concern, since this may result in the loss of at least a portion of HSCs. Hence, ViaCell believes that their process of isolating HSCs by removing only those cells (Lin^+) known not to be HSCs is superior to the standard art of CD34 positive selection since it leaves intact a mixed population of cells that include HSCs, even if they are biochemically uncharacterized. Although this process strongly enriches functional HCSs, these primitive cell populations remain biochemically uncharacterized. Therefore, HSCs will need further extensive investigation to conclusively answer this question: what constitutes a HSC?

Based on the positive selection of CD34 cells as current standard art, our next aim was to determine if telomerase activity and telomere lengths in ViaCell's hematopoietic cell fractions correlate with the $CD34^+CD38^-$ content. Our data shows that telomerase activity and telomere lengths do not directly correlate with the $CD34^+CD38^-$ content. So indeed telomerase activity in ViaCell's samples may reside, in part, elsewhere. Although telomere lengths did not correlate with $CD34^+CD38^-$ content, they did show some correlation with telomerase activity. Thus, our data supports that of others (Shay, 1995; Holt et al., 1996a; Engelhardt et al., 1997) that telomerase is responsible for elongation and stabilization of telomere lengths in hematopoietic cells. We speculate that there exits a subpopulation of hematopoietic cells other than $CD34^+CD38^-$ that accounts for some of the telomerase activity. Such candidates might include $CD34^-$ cells, early progenitors $CD34^+CD38^+$, $CD34^+CD71^+$, $CD34^+KDR^+$, and other hematopoietic stem/ progenitor cell fractions. Future experiments could include sorting the fresh cord blood, and the cell fractions stimulated to proliferate and differentiate, and specifically determining which hematopoietic cell subpopulations are low or high in telomerase. The telomerase experiments could also be expanded by conducting Northern blots or RT-PCR for telomerase RNA, or alternatively by performing western blots using antibodies against the reverse transcriptase subunit. Moreover, alternative methods of telomere length measurement, for example, "telomere amount and length assay" (TALA, Gan et al., 2001) that are less labor intensive than the TLA could be tested. Although TALA is not commercially available yet, is claimed to show a 4-fold greater sensitivity, >2 fold-higher reproducibility and 4-fold less time requirement (Gan et al., 2001).

In summary, our data indicates that ViaCell's removal of telomerase-low differentiated (Lin^+) cells with short telomeres appears to increase the telomerase activity

and telomere lengths in the remaining cell population. Also telomerase activity and telomere lengths decrease upon hematopoietic cell differentiation and proliferation. Lastly, we show that telomerase activity and average telomere length do not directly correlate with the $CD34^{+}CD38^{-}$ content.

BIBLIOGRAPHY

Allsopp RC, Vaziri H, Patterson C, Goldstein S, Young-lai EV, Futcher AB, Greider CW, Harley CB (1992) Telomere length predicts replicative capacity of human fibroblasts. *Proc. Natl. Acad. Sci.USA* **89**: 10114-10118.

Ando K, Nakamura Y, Tsuji T, Kato S, Hotta T (2000) Extensive generation of human cord blood $CD34^+$ stem cells from Lin-$CD34^-$ cells in a long-term in vitro system. *Exp. Hematol.* **28**: 690-699.

Andrews RE, Singer JW, Bernstein ID (1989) Precursors of colony-forming cells in humans can be distinguished from colony-forming cells by expression of CD33 and CD34 antigen and light scatter. *J. Exp. Med.* **169**: 1721-1731.

Andrews RG, Peterson LJ, Morris J, Potter J, Heyward S, Keim H, Gough M (2000) Differential engraftment of genetically modified $CD34^+$ and $CD34^-$ hematopoietic subsets in lethally irradiated baboons. *Exp. Hematol.* **28**: 508-518.

Bhatia M, Wang JC, Kapp U, Dick JE (1997) Purification of primitive human hematopoietic cells capable of repopulating immune-deficient mice. *Proc. Natl. Acad. Sci.* **94**: 5320-5325.

Blackburn EH and Gall JG (1978) A tandemly repeated sequence at the termini of the extrachromosomal ribosomal RNA genes in Tetrahymena. *J. Mol. Biol.* **120**: 33-53.

Blackburn EH (1991) Structure and function of telomeres. *Nature* **350**: 569-573.

Blackburn EH (2000) The end of the (DNA) line. *Nat. Struct. Biol.* **7**: 847-850.

Broxmeyer HE, Douglas GW, Hangoc G, Cooper S, Bard J, English D, Arny M, Thomas L (1989) Human umbilical cord blood as a potential source of transplantable hematopoietic stem/progenitor cells. *Proc. Natl. Acad. Sci. USA.* **86**: 3828-3832.

Cairo MS, Wagner JE (1997) Placental and/ or umbilical cord blood: an alternative source of hematopoietic stem cells for transplantation. *Blood* **90**: 4665-4678.

Cardenas ME, Bianchi A, de Lange T (1993) A xenopus egg factor with DNA-binding properties characteristic of terminus-specific telomeric proteins. *Genes Dev.* **7**: 883-894.

Cardoso AA, Li ML, Batard P, Hatzfeld A, Brown EL, Levesque JP, Sookdeo H, Panterne B, Sansilvestri P, Clark SC (1993) Release from quiescence of CD34+ CD38- human umbilical cord blood cells reveals their potentiality to engraft adults. *Proc. Natl. Acad. Sci. U S A.* **90**: 8707-8711

Cashman JD, Lapito T, Wang JCY, Lansdorp P, Dick JE, Eaves C (1997) Kinetic evidence of the regeneration of multilineage hematopoiesis from primitive cells in normal human bone marrow transplanted into immunodeficient mice. *Blood* **89**: 4307-4316.

Chang E, Harley CB (1995) Telomeres and aging. In *Telomeres. Cold spring Harbour Laboratory, New York.*

Chiu CP, Dragowska W, Kim NW, Vaziri H, Yui J, Thomas T E, Harley CB, Lansdorp PM (1996) Differential expression of telomerase activity in hematopoietic progenitors from adult bone marrow. *Stem Cells* **14**: 239-248.

Civin CI, Strauss LC, Shaper JH (1985) Antigenic analysis of hematopoiesis III. *J. Immunol.* **133:** 157-164.

Civin CI and Gore SD (1993) Antigenic analysis of hematopoiesis: a review. *J. Hematother.* **2**: 137-144.

Counter CM, Hirte HW, Bacchetti S (1994) Telomerase activity in human ovarian carcinom. *Proc. Natl. Acad. Sci.* **91**: 2900-2904.

Counter CM, Gupta J, Harley CB, Leber B, Bacchetti S (1995) Telomerase activity in normal leukocytes and in hematologic malignancies. *Blood* **85**: 2315-20.

Craig J (2000) ViaCord, Inc. and t. Breeders, Inc. Merge to from ViaCell, Inc., ViaCell-Press Release

Dao MA, Nolta JA (2000) CD34: to select or not to select? That is the question. *Leukemia* **14**: 773-776 (review).

de Lange T, Myers RM, Cox DR, Varmus HE (1990) Telomeres shorten with age. *Mol. Cell. Biol.* **10**: 518-527.

de Lange T & DePinho RA (1999) Unlimited mileage from telomerase? *Science* **283**: 947-949.

Engelhardt M, Kumar R, Albanell J, Pettengell R, Han W, Moore MA (1997) Telomerase regulation, cell cycle, and telomere stability in primitive hematopoietic cells. *Blood* **90**: 182-193.

Engelhardt M, Lubbert M, Guo Y (2002) $CD34^+$ or $CD34^-$: which is the more primitive? *Leukemia* **16**: 1603-1608.

Feng J, Funk WD, Wang SS, Weinrich SL, Avilion AA, Chiu CP, Adams RR, Chang E, Allsopp RC, Yu J (1995) The RNA component of human telomerase. *Science* **269**: 1236-1241.

Gan YB, Engelke KJ, Brown CA, Au JLS (2001) Telomere amount and length assay. *Pharmaceutical Research* **18**: 1655-1659.

Gilmore GL, DePasquale DK, Lister J, Shadduck RK (2000) Ex vivo expansion of human umbilical cord blood and peripheral blood CD34+ hematopoietic stem cells. *Exp. Hematol.* **28**: 1297-1305.

Gluckman E, Broxmeyer H, Auerbach AD, Friedman HS, Douglas GW, Devergie A, Esperou H, Thierry D, Socie G, Lehn P, Cooper S, English D, Kurtzberg J, Bard J, Boyse EA (1989) Hematopoietic reconstitution in a patient with Fanconi's anemia by means of umbilical cord blood from an HLA-identical sibling. *N. Engl. J. Med.* **321**: 1174-1178.

Goodell MA, Brose K, Conner AS, Mulligan R (1996) Isolation and function properties of murine hematopoietic stem cells that are replicating *in vivo*. *J. Exp. Med.* **183**: 1797-1806.

Goodell MA (1999) $CD34^+$ or $CD34^-$: does it really matter? *Blood* **15**: 2545-2547.

Gottschling DE, Aparicio OM, Billington BL (1990) Position effect at *S. cerevisiae* telomeres: reversible repression of PolII transcription. *Cell* **63**: 751–762.

Greider CW and Blackburn EH (1985) Identification of a specific telomere terminal transferase activity in Tetrahymena extracts. *Cell* **43**: 405-413.

Greider CW and Blackburn EH (1989) A Telomeric sequence in the RNA of Tetrahymena telomerase required for telomere repeats synthesis. *Nature* **337**: 331-337.

Greider CW & Blackburn EH (1996) Telomeres, telomerase and cancer. *Sci. Am.* **274**: 92-97.

Hao Q-L, Shah AJ, Thiemann FT (1995) A functional comparison of $CD34^+CD38^-$ cells in cord blood and bone marrow. *Blood* **86**: 3745–3753.

Harley CB, Futcher AB and Greider CW (1990) Telomeres shorten during aging of human fibroblasts. *Nature* **345**: 458-460.

Harley CB (1991) Telomere loss: mitotic clock or genetic time bomb? *Mutat. Res.* **256**: 271-282.

Hastie ND, Dunlop MG (1990) Telomere reduction in human colorectal carcinoma and with aging. *Nature* **346**: 866-868.

Healy L, May G, Gale K, Grosveld F, Greaves M, Enver T (1995) The stem cell antigen CD34 functions as a regulator of hematopoietic cell adhesion. *Proc. Natl. Acad. Sci. USA* **92:** 12240-12244.

Hirao A, Kawano Y, Takaue Y (1994) Engraftment potential of peripheral and cord blood stem cells evaluated by a long-term culture system. *Exp. Hematol.* **22**: 521–526.

Hiyama K, Hirai Y, Kyoizumi S, Akiyama M, Hiyama E, Piatzsek MA, Shay JW, Ishoka S, Yamakido M (1995) Activation of telomerase in human lymphocytes and hematopoietic progenitor cells. *J. Immunol.* **155**: 3711-3715.

Hogan CJ, Shpall EJ, Dick JE, Keller G (1997) Engraftment and development of human $CD34^+$-enriched cells from umbilical cord blood in NOD/Lt-Sz-scid mice. *Blood* **90**: 85-96.

Hohaus S, Voso MT, Barbera OL, Cavallo S, Bellacosa A, Rutella S, Rumi C, Genuardi M, Neri G, Leone G (1997) Telomerase activity in human hematopoietic progenitor cells. *Haematologica* **82**: 262-268.

Holt SE, Shay JW, Wright WE (1996a) Refining the telomere-telomerase hypothesis of ageing and cancer. *Nat. Biotech.* **14**: 836-839.

Holt SE, Wright WE, Shay JW (1996b) Regulation of telomerase activity in immortal cell lines. *Mol. Cell Biol.* **16**: 2932-2939.

Johnson PWM, Simnett SJ, Temkin V (1998) Bone marrow and peripheral blood stem cell transplantation for malignancy. *Health Technology Assessment.* Vol 2: No. 8.

Kim NW, Piatyszek MA, Prowse KR, Harley CB, West MD, Ho PL, Coviello GM, Wright WE, Weinrich SL, Shay JW (1994) Specific association of human telomerase activity with immortal cells and cancer. *Science* **266**: 2011-2015.

Kinniburgh D, Russell NH (1993) Comparitive study of CD34 positive cells and subpopulations in human umbilical cord blood and bone marrow. *Bone Marrow Transplant* **12**: 489-494.

Knudtzon S (1974) In vitro growth of granulocyte colonies from circulating cells in human cord blood. *Blood* **43**: 357-361.

Kohler T, Plettig R, Ordemann R, Bornhauser M (1999) Defining optimum conditions for the *ex vivo* exapansion of human umbilical cord blood cells. *Stem Cells* **17**: 19-24.

Koller MR, Bender JG, Miller WM (1992) Reduced oxygen tension increases hematopoiesis in long-term culture of human stem and progenitor cells from cord blood and bone marrow. *Exp. Hematol*. **20**: 264-270.

Krause DS, Fackler MJ, Civin CI, May WS (1996) CD34: structure, biology and clinical utility. *Blood* **87:** 1-3.

Kurtzberg J, Laughlin M, Graham ML, Smith C, Olson JF, Halperin EC, Ciocci G, Carrier C, Stevens CE, Rubinstein P (1996) Placental blood as a source of hematopoietic stem cells for transplantation into unrelated recipients. *N. Eng. J. Med.* **335**: 157-166.

Lansdorp PM, Dragowska W, Mayani H (1993) Ontogeny related changes in proliferative potential of human hematopoietic cells. *J. Exp. Med.* **178**: 787-791.

Lansdorp PM, Verwoerd NP, Van de Rijke FM, Dragowska V, Little MT, Dirks RW, Raap AK, and Tanke HJ (1996) Heterogeneity in telomere length of human chromosomes. *Hum. Mol. Genet.* **5**: 685-691.

Lansdorp PM, Poon S, Chavez E, Dragowska V, Zijlmans M, Bryan T, Reddel R, Egholm M, Bacchetti S, Martens U (1997) Telomeres in the hematopoietic system. *Ciba Found Symp.* **211**: 219-222.

Levis R, Hazelrigg T, Rubin GM (1985) Effects of genomic position on the expression of transduced copies of the white gene of Drosophila. *Science* **229**: 558–561.

Mathog D, Hochstrasser M, Gruenbaum Y (1984) Characteristic folding pattern of polytene chromosomes in Drosophila salivary gland nuclei. *Nature* **308**: 414–421.

Mayani H and Lansdorp PM (1998) Biology of human umbilical cord blood-derived hematopoietic stem/progenitor cells. *Stem Cells* **16**: 152-165.

McKay S, Cooke H (1992) A protein which specifically binds to single stranded TTAGGG repeats *in vitro*. *Nucleic Acids Res.* **20**: 1387-1391.

Moore MA and Hoskins J (1994) Ex vivo expansion of cord blood-derived stem cells and progenitors. *Blood Cells* **20**: 468-481.

Morin GB (1989) The human telomere terminal transferase enzyme is a ribonucleoprotein that synthesizes TTAGGG repeats. *Cell* **59**: 521-529.

Morrison SJ, Prowse KR, Ho P, Weissman IL (1996) Telomerase activity in hematopoietic cells is associated with self-renewal potential. *Immunity* **3**: 207-216.

Moyzis RK, Buckingham JM, Dani M, Deaven LL, Jones MD, Meyne J, Ratliff RL, Wu JR, Cram LS (1988) A highly conserved repetitive DNA sequence, (TTAGGG)n, present at the telomeres of human chromosomes. *Proc. Natl. Acad. Sci. USA* **85**: 6622-6626.

Nakahata T and Ogawa M (1982) Hematopoietic colony-forming cells in umbilical cord blood with extensive capability to generate mono- and multipotential hematopoietic progenitors. *J. Clin. Invest.* **70**: 1324-1328.

Nakamura TM, Morin GB, Chapman KB, Weinrich SL, Andrews WH, Lingner J, Harley CB, Cech TR (1997) Telomerase catalytic subunit homologs from fission yeast and human. *Science* 277: 955-959.

NIH (2001) Stem Cells: Scientific progress and future research directions. http://www.nih.gov/news/stem cell.

Notaro R, Cimmino A, Tabarini D, Rotoli B, and Luzzatto L (1997) In vivo telomere dynamics of human hematopoietic stem cells. *Proc. Natl. Acad. USA* **94**: 13782-13785.

Ogawa M, Kawashima I, Zanjani E, Flake A, Zeng H (1996) $CD34^+$ human marrow cells that express low levels of Kit protein are enriched for long-term marrow-engrafting cells. *Blood* **87**: 4136-4142.

Ogawa M (2002) Changing phenotypes of hematopoietic stem cells. *Exp. Hematol*. **30**: 3-6.

Ohyashiki JH, Sashida G, Tauchi T, Ohyashiki K (2002) Telomeres and telomerase in hematologic neoplasia. *Oncogene* **21**: 680-687.

Olovnikov AM (1973) A theory of marginotomy. The incomplete copying of template margin in enzymic synthesis of polynucleotide and biological significance of the phenomenon. *J. Theor. Biol.* **41:** 181-190.

Osawa M, Hanada K, Hamada H, Nakachi H (1996) Long-term lymphohematopoietic reconstitution by a single CD34-low/negative hematopoietic stem cell. *Science* **273**: 242-245.

Piacibello W, Sanavio F, Garetto L, Severino A, Dane A, Gammaitoni L, Aglietta M (1998) Differential growth factor requirement of primitive cord blood hematopoietic stem cell for self-revewal and amplification *vs* proliferation and differentiation. *Leukemia* **12**: 718- 727.

Quesenberry P, Stein G, Forget B, and Weissman S (1998) Stem Cell Biology and Gene Therapy. New York: John Wiley & Sons, Inc.

Rocha V, Wagner JE, Zhang MJ (2000) Cord blood transplantations in unrelated and related donors. *N. Engl. J. Med.* **342**: 1846-1854.

Rubinstein P, Rosenfield RE, Adamson JW (1993) Stored placental blood for unrelated bone marrow reconstitution. *Blood* **81**: 1679–1690.

Rubinstein P, Carrier C, Scaradavou A, Kurtzberg J, Adamson J, Migliaccio AR, Berkowitz RL, Cabbad M, Dobrila NL, Taylor PE, Rosenfield RE, Stevens CE (1998) Outcomes among 562 recipients of placental blood transplants from unrelated donors. *N Engl. J. Med.* **339**: 1565-1577.

Sandell LL, Zakian VA (1993) Loss of a yeast telomere: arrest, recovery, and chromosome loss. *Cell* **75**: 729–739.

Sato T, Laver JH, Ogawa M (1999) Reversible expression of CD34 by murine hematopoietic stem cells. *Blood* **94**: 2548-2554.

Schiestl RH, Reynolds P, Prakesh S (1989) Cloning and sequence analysis of the *Saccharomyces cerevisiae* RAD9 gene and further evidence that its product is required for cell cycle arrest induced by DNA damage. *Mol. Cell Biol.* **9**:1882–1896.

Shay JW, Pereira-Smith OM, Wright WE (1991) A role for both RB and p53 in the regulation of human cellular senescence. *Exp. Cell Res.* **196**: 33-39.

Shay JW, Wright WE, Brasiskyte D, Van der Haegen BA (1993) E6 of human papillomavirus type 16 can overcome the M1 stage of immortalization in human mammary epithelial cells but not in human fibroblasts. *Oncogene* **8**: 1407-1413.

Shay JW (1995) Aging and cancer: are telomeres and telomerase the connection? *Mol. Med. Today* **1**: 378-384.

Shay JW, Werbin H, Wright WE (1996) Telomeres and telomerase in human leukemias. *Leukemia* **10**: 1255-1261. Review

Shay J W (1999) Toward identifying a cellular determinant of telomerase repression. *J. Natl. Cancer Inst.* **91**: 4-6.

Smith AG (2001) Origins and properties of mouse embryonic stem cells. *Annu. Rev. Cell. Dev. Biol.* **53**: 275-287.

Smith CD, Blackburn EH (1999) Uncapping and deregulation of telomeres lead to detrimental cellular consequences in yeast. *J. Cell Biol.* **145**: 203–214.

Smith S and De Lange T (2000) Tankyrase promotes telomere elongation in human cells. *Curr. Biol.* **10**: 1299-1302.

Srour E, Briddell R, Hoffman R (1993) Long-term generation an expression of human primitive hematopoietic progenitor cells *in vitro*. *Blood* **81**: 661-669.

Summers YJ, Chang J, Testa NG (2001) Cord blood G0 $CD34^+$ cells have a thousand-fold higher capacity for generating progenitors in vitro than G1 $CD34^+$ cells. *Stem Cells* **19**: 505-513.

Sutherland HJ, Eaves AC, Eaves CJ, Lansdorp PM (1989) Characterization and partial purification of human marrow cells capable of initiating long-term hematopoiesis *in vitro*. *Blood* **74**: 1563-1570.

t. Breeders, Inc. (2000) Amplifying opportunities in cellular therapy. Corporate Overview.

Tajima F, Sato T, Laver JH, Ogawa M (2000) CD34 expression by murine hematopoietic stem cells mobilized by granulocyte colony-stimulating factor. *Blood* **96**: 1989-1993.

Tajima F, Deguchi T, Laver JH, Zeng H, Ogawa M (2001) Reciprocal expression of CD38 and CD34 by adult murine hematopoietic stem cells. *Blood* **97**: 2618-2624.

Terstappen LW, Huang S, Safford M, Lansdorp PM, Loken MR (1991) Sequential generations of hematopoietic colonies derived from single nonlineage-committed CD34+CD38- progenitor cells. *Blood* **77**: 1218-1227.

Traycoff CM, Kosak ST, Grigsby S (1995) Evaluation of *ex vivo* expansion potential of cord blood and bone marrow hematopoietic progenitor cells using cell tracking and limiting dilution analysis. *Blood* **85**: 2059–2068.

Vaziri H, Dragowska W, Allsopp RC, Thomas TE, Harley CB, Lansdorp P M (1994) Evidence for a mitotic clock in human hematopoietic stem cells: Loss of telomeric DNA with age. *Proc. Natl. Acad. Sci. USA*. **91**: 9857-9860.

Vaziri H, Benchimol S (1998) Reconstitution of telomerase activity in normal human cells leads to elongation of telomeres and extended replicative life span. *Curr Biol* **8**: 279-282.

ViaCell, Inc. Annual Report. 2000.

ViaCell, Inc. Annual Report. 2001.

Wagner JE, Rosenthal J, Sweetman R, Shu XO, Davies SM, Ramsay NK, McGlave PB, Sender L, Cairo MS (1998) Successful transplantation of HLA matched and HLA mismatched umbilical cord blood from unrelated donors: Analyses of engraftment and acute graft versus host disease. *Blood* **88**: 795-802.

Watson JD (1972) Origin of concatemeric T7 DNA. *Nature New Biol.* **239**: 197-201.

Weinert TA, Hartwell LH (1988) The RAD9 gene controls the cell cycle response to DNA damage in *Saccharomyces cerevisiae*. *Science* **241**: 317–322.

Weng N, Granger L, Hodes RJ (1997) Telomere lengthening and telomerase activation during human B cell differentiation. *Proc. Natl. Acad. Sci.* **94**: 10827-10832

Wright WE, Shay JW, Piatyszek MA (1995) Modifications of a telomeric repeat amplification protocol (TRAP) result in increased reliability, linearity and sensitivity. *Nucleic Acids Res.* **23**: 3794-3795.

Yui J, Chiu CP, Lansdorp PM (1998) Telomerase activity in candidate stem cells from fetal liver and adult bone marrow. *Blood* **91**: 3255-3262.

Zhang W, Piatyszek MA, Kobayashi T, Estey E, Andreeff M, Deisseroth AB, Wright WE, Shay JW (1996) Telomerase activity in human acute myelogenous leukemia: inhibition of telomerase activity by differentiation-inducing agents. *Clin. Cancer Res.* **2**: 799-803.

Zhong Z, Shiue L, Kaplan S (1992) A mammalian factor that binds telomeric $TTAGGG_n$ repeats. *Mol. Cell. Biol.* **12**: 4834-4843.

Ziegler BL, Valtieri M, Porada GA, De Maria R, Miller R, Casella I, Bock T, Zanjani ED, Peschle C (1999) KDR Receptor: A key marker defining hematopoietic stem cells. *Science* **285**: 1553-1558.

Zimmermann J (1998) Stem Cell Isolation Comes of Age. *Genetic Engineering News*, April 1 issue, pg. 17.