

**Telomerase Activity in Human Umbilical Cord Cell Populations  
Containing Hematopoietic Stem Cells**

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## ABSTRACT

Hematopoietic cell populations exhibiting detectable telomerase activity and elongated telomere lengths display strong engraftment survivability in humans during transplants. We investigated telomerase activity and telomere length in umbilical cord blood hematopoietic cell populations obtained from ViaCell Inc. at various intervals of a two-week *ex vivo* stem cell amplification process. Telomerase activity is increased with time in ViaCell's amplification process, perhaps in response to the removal of differentiated cells and expansion of primitive hematopoietic stem cell populations in tissue culture media containing a mixture of growth factors. Two of ViaCell's cell culture fractions were analyzed for telomere length using a TLA. Our results showed relatively long telomere lengths for day-0 and day-14 cord populations, and that despite an upregulation of telomerase activity in Day-14 samples, a loss of about 2 kb of telomeric DNA occurs. Our data are consistent with a model in which the increase in telomerase activity in day-14 *ex vivo* amplified cord blood hematopoietic cells relative to fresh cord is sufficient to reduce, but not prevent, telomere shortening caused by cell proliferation. Lastly, we investigated various culture conditions that could potentially upregulate telomerase activity in the Day-14 amplified cells. However none of the treatments tested altered telomerase activity. Our detection of increased telomerase activity and relatively long telomere lengths in ViaCell's Day-14 *ex vivo* amplified cord blood stem cell fraction provides support for ViaCell's *Selective Clonogenic Amplification*<sup>TM</sup> indicating a high engraftment potential for these cells.

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*For  
Appa and Bhagu  
With  
Love and gratitude*

# BACKGROUND

## Stem Cells

Stem cells have the ability to divide for indefinite periods in culture and to give rise to specialized cells. They are characterized by their capacity for extensive proliferation and differentiation (de Wynter et al., 1998). They are best described in the context of normal human development. The fertilized egg is said to be totipotent (see fig. 1, upper diagram) because it has the potential to generate all the cells and tissues that

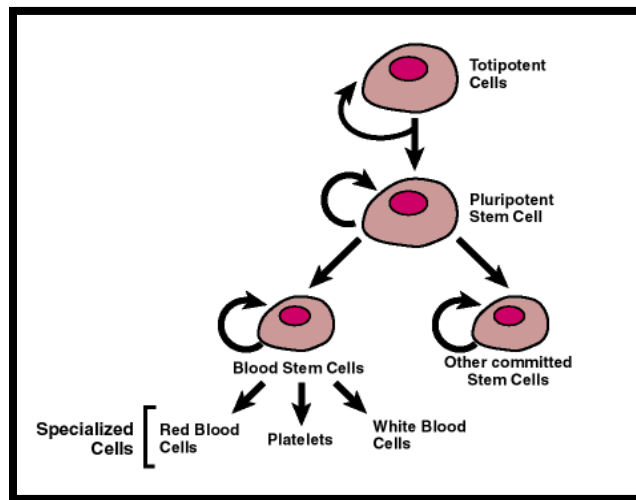


Figure 1. Levels of stem cell differentiation. (NIH 2000).

make up an embryo, including those that support the egg's development *in utero* (like the extra-embryonic tissues, placenta, and umbilical cord).

The term pluripotent (see fig. 1, middle diagram) is used to describe stem cells that can give rise to cells derived from all three embryonic germ layers: mesoderm, endoderm, and ectoderm. These three germ layers are the embryonic source of all cells of the body. The embryonic stem (ES) cell is pluripotent (see fig. 2). The ES cell is defined by its origin from one of the earliest stages of the development of the embryo, the



blastocyst. Specifically, ES cells are derived from the inner cell mass of the blastocyst at



**Figure 2. A microscopic view of ES cells** (Newsweek, July 9, 2001).

a stage before it would implant in the uterine wall. The defining properties of an ES cell include the following: they are capable of long-term self-renewal (exhibit and maintain a stable diploid, normal karyotype); are clonogenic (a single cell can give rise to genetically identical cells); can be induced to continue proliferating or to differentiate (lack the G1 checkpoint in the cell

cycle and spend most of their time in the S phase of the cell cycle, during which they synthesize DNA), show stable developmental potential to form derivatives of all three embryonic germ layers even after prolonged culture (Smith, 2001; Thomson & Marshall, 1998).

The pluripotent stem cells undergo further specialization into stem cells that are committed to give rise to cells that have a particular function. These more specialized stem cells are called multipotent (see fig. 1, lower diagram). Examples of multipotent cells include hematopoietic stem cells (HSC) that give rise to the various types of blood cells.

Unipotent stem cells, a term that is usually applied to a stem cell in adult organisms, means that these cells are usually capable of differentiating along only one lineage. Adult stem cells in many differentiated tissues give rise to just one cell type under normal conditions, in a process that allows a steady state self-renewal for the tissue

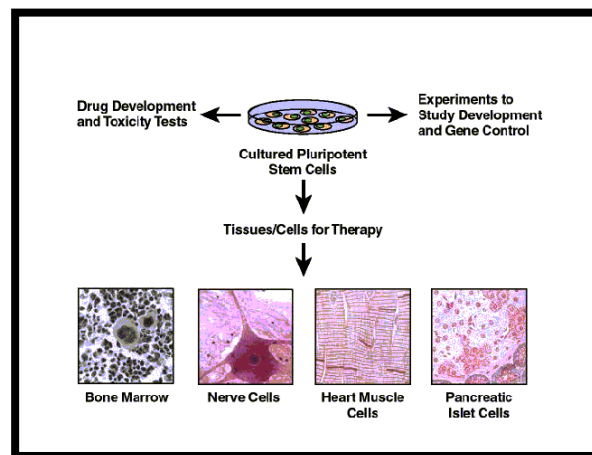
(NIH, 2001). Recent data indicate that adult and multipotent stem cells may be capable of more versatile differentiation than previously thought. For example, HSCs may have the capacity to differentiate into neuronal cells (McGovern et al., 2001).

### **Potential Uses of Human Stem Cells**

A new era in stem cell biology began in 1998 with the derivation of embryonic stem (ES) cells from the inner cell mass of human blastocysts by Thomson et al (1998) and from fetal tissue by John Gearhart (Shamblott et al., 1998). These breakthroughs showed that ES cells provided a possible source of cells for cell based therapies for many human diseases. The ES cells isolated from human blastocysts showed normal karyotypes and expressed high levels of telomerase activity. These cells were grown with mouse feeder fibroblasts, and after undifferentiated proliferation *in vitro* for 4-5 months, these cells still maintained the developmental potential to produce gut epithelium, cartilage, bone, muscle, neural epithelium, embryonic ganglia, and stratified squamous epithelium. When injected into SCID mice, these cells formed teratomas, which are tumors containing a mix of differentiated human cell types (Thomson et al., 1998). In another study, human primordial germ cells cultured *in vitro* retained their karyotype while producing large, ES-like cell colonies capable of repeated passages (Shamblott et al., 1998). Reports showed that specific media hold promise for a directed differentiation of ES cells (Pittenger et al., 1999). In another key publication, eight growth factors were tested on a human ES cell line expressing receptors for each growth factor. Molecular markers identified all three germ layers, eleven tissues, and specific gene expression in germ layers and tissues (Schuldiner et al., 2001). These studies show

that directed differentiation of stem cells into germ layers or specific tissues will become possible in the near future.

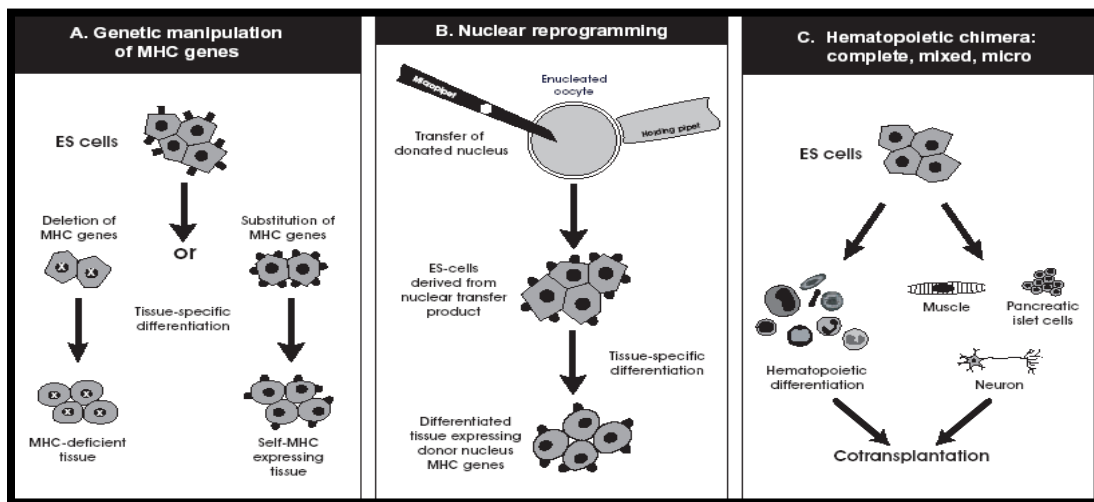
Many uses have been proposed for human embryonic stem cells. The most often discussed is their potential use in transplant therapy, to restore tissue that has been damaged by disease or injury. Diseases that might be treated by therapeutic transplants with ES-derived cells include Parkinson's disease, diabetes, traumatic spinal cord injury, Purkinje cell degeneration, Duchenne's muscular dystrophy, heart failure, and osteogenesis imperfecta. However, treatments for any of these diseases require that human ES cells be directed to differentiate into specific cell types prior to transplantation



**Figure 3. Promise of stem cell research.** (NIH, 2000)

(See fig. 3). At this stage, any therapies based on the use of human ES cells are still hypothetical and highly experimental (Odorico et al., 2001, Pedersen, 1999). One of the current advantages of using ES cells compared to adult stem cell is that ES cells have an unlimited ability to proliferate *in vitro*, and are more likely to be able to generate a broad range of cell types through directed differentiation. The potential disadvantages of the use of human ES cells for transplant therapy include greater ethical considerations, and

the propensity of undifferentiated ES cells to induce the formation of tumors (teratomas), which are typically benign (NIH, 2001). Because it is the undifferentiated ES cells rather than their differentiated progeny that have been shown to induce teratomas, tumor formation might be avoided by devising methods for removing any undifferentiated ES cells prior to transplant (NIH, 2001). The potential immunological rejection of human ES-derived cells might be avoided by using nuclear transfer technology to generate ES cells that are genetically identical to the person who receives the transplant. It has been suggested that this can be accomplished by using somatic cell nuclear transfer technology or “therapeutic cloning” (Odorico et al., 2001) (see fig. 4).



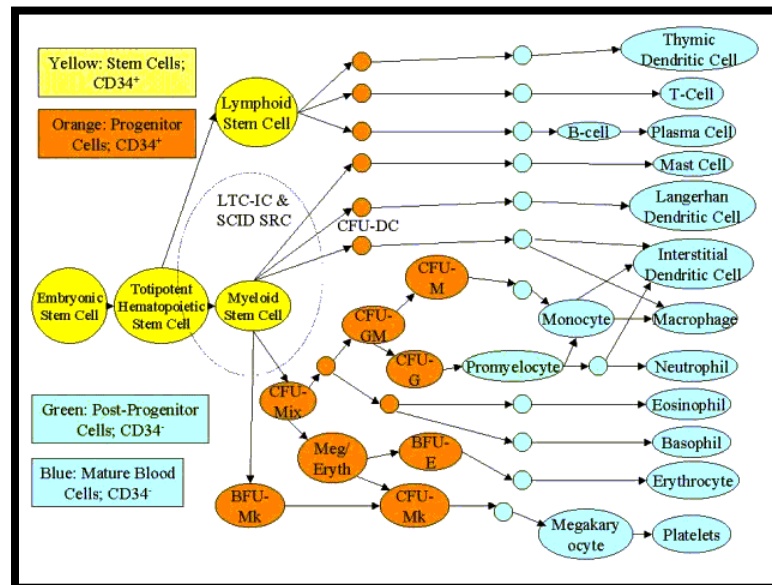
**Figure 4. Genetic manipulation of human ES cells. (NIH, 2001).**

Another potential use of ES cells that does not involve transplantation, is their use in studying the early events in human development, for example to identify genetic, molecular, and cellular events that lead to congenital birth defects, placental abnormalities that lead to spontaneous abortions, and to identify methods for preventing them (Rathjen et al., 1998). Human ES cells can also be used to test candidate

therapeutic drugs because the ES- derived cells may be more likely to mimic the *in vivo* response of the cells/tissues to drugs being tested, and so offer safer and potentially cheaper models for drug screening. Human ES cells could also be employed to screen potential toxins. However, to meet these objectives ES cells must be directed to differentiate into specific cell types (NIH, 2001).

## Hematopoiesis

Hematopoiesis (see fig. 5) is the formation of red and white blood cells from hematopoietic stem cells (HSC). In human ontogeny, hematopoiesis begins in the yolk



**Figure 5. Hematopoiesis.** Shows formation of red and white blood cells from hematopoietic stem cell (Tissue Therapeutics, 2000).

sac and migrates to the fetal liver and then to the spleen. As the gestation continues, the bone marrow becomes the major hematopoietic organ, and by the time of birth hematopoiesis has ceased within the liver and spleen. Early in hematopoiesis, the

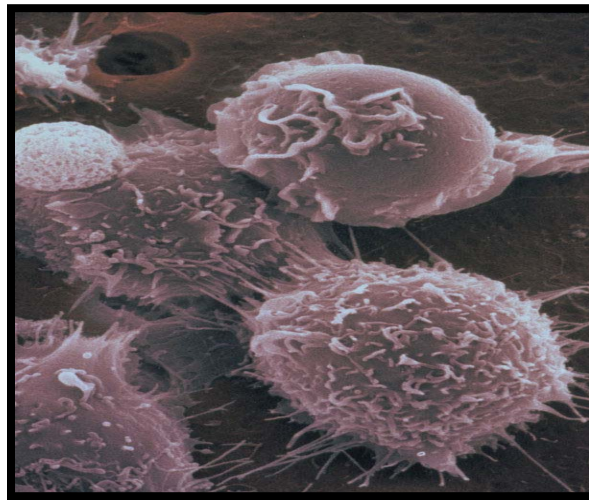
multipotent HSC differentiates along one of two pathways, giving rise to either a lymphoid stem cell (upper pathway in the figure) or a myeloid stem cell (lower pathway in the figure). The lymphoid stem cell generates T and B progenitor lymphocytes. The myeloid stem cell generates progenitor cells for erythrocytes, neutrophils, eosinophils, basophils, monocytes, mast cells, and platelets (Kuby, 1992). The earliest evidence proving that the various cell lineages in bone marrow originate from HSCs came from the classic experiment of Till and McCulloch (1961).

The complex orchestration of hematopoiesis through which the elaborate array of blood cells is produced requires three physiologic components, each of which is essential: 1) the stem cell pool itself, 2) hematopoietic cytokines, which regulate hematopoiesis through both endocrine and paracrine mechanisms, and 3) the hematopoietic inductive microenvironment, which is made up of the bone marrow stroma and vasculature. The unique microenvironment influences the growth and differentiation of hematopoietic stem cells by providing a hematopoietic inducing microenvironment consisting of a cellular matrix and either membrane bound or diffusible growth factors (Sullivan, 2000).

### **Hematopoietic Stem Cells**

Hematopoietic stem cells (HSCs) (figure 6) are usually found in the bone marrow, umbilical cord blood, and can be found in the peripheral blood if they are stimulated in the bone marrow by factors such as granulocyte colony-stimulating factor (G-CSF) (t. Breeders, 2000). An HSC can renew itself, can differentiate to a variety of specialized cells, or can undergo apoptosis (NIH, 2001). HSCs are few in number, occurring with a frequency of one stem cell per  $10^4$  bone marrow cells. Studies have revealed that there

appear to be two kinds of HSCs: 1) long-term stem cells that are capable of self-renewal and can regenerate all the different types of cells, and 2) short term progenitor or precursor cells which are relatively immature and are precursors to a fully differentiated cell of the same tissue type. It is the long-term replicating HSCs that are most important for developing HSC-based cell therapies (NIH, 2001).



**Figure 6. Electron micrograph of a hematopoietic stem cell** (McLaren, 2001).

### **Identification (Phenotype) of Hematopoietic Stem Cells**

Both HSCs and lineage committed hematopoietic progenitor cells (HPC) express the CD34 antigen (see Table 1). CD34<sup>+</sup> cells constitute 1%-5% of cells in the adult bone marrow, and 5%-10% of fetal bone marrow Cells (Krause et al., 1996) and 1% of all nucleated cells in cord blood from full term deliveries (Civin and Gore, 1993). About 1 in every 100,000 cells in the marrow is a long term HSC (NIH, 2001). CD34 plays an important role in the formation of progenitor cells in both fetal and adult hematopoiesis. The CD34 antigen is an integral membrane glycoprotein of 90-120 kD and has been

suggested to function as a regulator of hematopoietic cell adhesion to stromal cells of the hematopoietic microenvironment (Healy et al, 1995).

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

**Table 1. Phenotypes of adult human hematopoietic stem and progenitor cells** (Stem cell biology and gene therapy, edited by Quesenberry PJ, Stein GS, Forget BG, and Sherman MW, 1998 Wiley-Liss, Inc.

Primitive HSCs lack the differentiation antigens that are present on lineage-committed progenitors and are thus CD38-, CD45<sup>lo</sup>, CD71<sup>lo</sup> (Craig et al., 1993). HLA-DR is absent or is expressed at low levels on adult stem cells but is present on fetal and neonatal hematopoietic stem cells (Lansdorp et al., 1993). Thy-1 antigen is present on all human fetal and neonatal hematopoietic cells but is only expressed on a proportion of lineage-committed progenitors in the adult (Craig et al., 1993). The MDR1 gene is strongly expressed in HSCs and confers on them the ability to exclude the mitochondrial binding dye rhodamine 123 (Chaudhary and Roninson, 1991). The receptors kit and flk-2, which have intrinsic tyrosine kinase activity, are expressed on both stem cells and progenitors, but some kit+ cells with stem cell function can be flk-2- (Zeigler et al., 1994). AC133 is a recently described antigen whose function is unknown. The AC133 antibody selects a subset of CD34+ cells which contain both short and long term repopulating cells and therefore offers an alternative to the CD34 antigen for cell selection (Yin et al., 1997).



Mayani and Lansdorp (1998) have reported that most primitive hematopoietic stem/progenitor cells (HSPC) present in UCB are small mononuclear cells with the following immunophenotype: CD34<sup>+</sup>CD38<sup>-</sup>CD45RA<sup>lo</sup>CD71<sup>lo</sup>Thy-1<sup>+</sup>c-kit<sup>+</sup>Rh<sup>low</sup>.

### **Sources of HSC**

The classic source of HSCs is bone marrow. HSCs can be obtained from the bone marrow, usually by puncturing the hipbone after anesthetizing the stem cell donor. About 1 in every 100,000 cells in the marrow is a long term HSC (NIH, 2001). A small number of stem and progenitor cells circulate in the peripheral blood. HSCs can also be obtained from the peripheral blood by mobilizing the hematopoietic stem/progenitor cells from the marrow by using a wide variety of cytokines and cytotoxics, alone or in combination. Administration of antibodies to the adhesion factor VLA-4 results in rapid mobilization (within 30 minutes) of progenitor cells (Papayannopoulou and Nakamoto, 1993). G-CSF treatment increases the numbers of CD34<sup>+</sup> cells 4-62 fold in peripheral blood (Pettengell and Testa, 1995).

In the late 1980s and early 1990s, physicians recognized that blood from the human umbilical cord (UCB) and placenta was a rich source of HSCs. The presence of relatively mature hematopoietic progenitor cells (HPC) in human UCB was demonstrated by Knudtzon in 1974 (Knudtzon, 1974). Later Ogawa and colleagues documented the presence of primitive HPC in UCB (Nakahata and Ogawa, 1982). Studies by Broxmeyer et al (1989) showed that the frequency of hematopoietic stem/progenitor cells in umbilical cord blood equals or exceeds that of marrow, and greatly surpasses that of adult blood (Broxmeyer et al., 1989). Since the first successful umbilical cord blood (UCB)

transplants in children with Fanconi anemia (Gluckman et al., 1989), the collection and therapeutic use of these cells has grown quickly (NIH, 2001). Volume for volume, human UCB is as rich a source of hematopoietic progenitor cells as bone marrow (Broxmeyer and Carow, 1993). The proliferative potential of long-term culture-initiating cells (LTC-IC) from UCB exceeds that of the adult bone marrow (Mayani and Lansdorp, 1995). This compensates in part for the lower number of cells that can be obtained from a single donor compared with a conventional bone marrow harvest.

Sustained hematopoietic engraftment after myoablation has been obtained with as few as  $2 \times 10^4$  LTC-IC from UCB (Wagner et al., 1996). Reports indicate that the UCB provides sufficient transplantable HSCs for children with human leukocyte antigen (HLA)-identical or single HLA antigen-disparate sibling donors, but whether this will prove adequate for two or three HLA-antigen-disparate sibling donors and adults remains to be determined. Recently, a successful engraftment was reported in three adult patients of >50 kg transplanted with UCB (Kurtzberg et al., 1996). In reported UCB transplants, the incidence of graft-versus-host-disease is low. An important source of HSCs in research, but not in clinical use, is the developing blood-producing tissues of fetal animals. Fetal hematopoietic progenitors have a greater growth potential than those in UCB, adult bone marrow, or leukapheresis product (Lansdorp et al., 1993).

## **Therapeutic Applications of HSCs**

### *Stem Cell Replacement Therapy*

Among the first clinical uses of HSCs were the treatment of cancers of the blood such as leukemia and lymphoma. Since HSCs are the source of all differentiated blood

cells, their destruction during cancer therapy is a major source of cancer treatment morbidity and mortality. A key element of cancer treatment therefore is the replacement of HSCs following radiation or chemotherapy, a procedure known as stem cell replacement therapy. The HSCs used for replacement can be obtained from the patient's own bone marrow or peripheral blood (autologous) prior to chemotherapy, from the peripheral circulation of an HLA-matched donor (allogeneic), or from UCB taken at birth (allogeneic) (t. Breeders, 2000).

One of the problems with autologous HSC transplants in cancer therapy is that cancer cells are sometimes inadvertently collected and reinfused back into the patient along with the stem cells. Studies have shown that this can be prevented by purifying the cells and preserving only the CD34+, Thy-1+ cells (Negrin et al., 2000). Since most solid tumors do not express CD34, the selection of CD34+ cells has been used to reduce tumor cell contamination of hematopoietic products used for autologous transplantation for patients with these tumors. CD34+ cell selection can reduce tumor cell contamination by a factor of  $10^{-10^4}$  using immunomagnetic beads or biotin- avidin columns (Farley et al., 1997).

One of the most exciting new uses of HSC transplantation is the graft-versus-tumor treatment of cancer. A study by Joshi et al. (2000) shows that human UCB HSCs show antitumor activity in the test tube against leukemia cells and breast cancer cells. In recent years, researchers have contemplated hematopoietic stem cell therapy for autoimmune diseases. Reports suggest that HSC replacement therapy may fundamentally alter the patient's immune system. Lupus patients, who underwent this therapy, remained

free from active lupus and improved continuously after transplantation, without the need for immunosuppressive medications (Traynor et al., 2000).

### *UCB Transplantation*

The first allogeneic UCB transplant (UCBT) was performed successfully in 1989, to treat a child with Fanconi's anemia; the UCB donor was his HLA-identical sister (Gluckman et al., 1989). Several years post-transplant, this patient is doing well, with full donor hematopoietic and lymphoid reconstitution. This first success opened the way to an entire new field in the domain of allogeneic HSC transplant, as it showed that a single UCB unit contained sufficient numbers of HSCs to reconstitute a child's lympho-hematopoietic compartment. It also showed that a UCB unit could be collected at birth without any harm to the newborn infant, and that a UCB HSC graft could be cryopreserved and transplanted to a myeloablated host after thawing without losing its repopulating ability (Gluckman, 2000).

Since then a number of advantages of using UCB stem cells for transplantation have become apparent. Simultaneously, UCB banks have been established for related or unrelated UCBTs, with >30,000 units currently available, and >1,500 UCBTs having been performed in children (and increasing numbers in adults) with malignant and nonmalignant diseases (Rubinstein et al, 1998). The methods of UCB collection and cryopreservation are easy and safe; as soon as the baby is delivered, the cord is clamped and cut; cells are collected either by catheterization of umbilical veins, by aspiration of cord and placental vessels, by gravity or by flushing through catheters inserted into the umbilical artery and vein. The mean volume obtained by these various methods is 100-

150 ml, with a mean total number of nucleated cells of  $15 \times 10^8$  (Gluckman et al, 1993). UCB cells have many advantages as grafts for stem cell transplantation because of the immaturity of newborn cells. Hematopoietic progenitors from UCB are enriched for in vivo long-term repopulating stem cells. Compared to adult stem cells, UCB HSCs produce larger hematopoietic colonies in vitro, are able to expand in long-term culture in vitro, engraft SCID-human mice in the absence of additional human growth factors, and have longer telomeres (Noort and Falkenburg, 2000). These properties should theoretically compensate for the relatively low number of cells contained in a single UCB donation, and through rapid expansion reconstitute myeloablated adult patients. The second advantage of UCB grafts relates to the immaturity of the immune system at birth. This property should decrease the alloreactive potential of the lymphocytes within a cord blood graft and consequently should reduce the incidence and severity of GVHD after HLA-matched or HLA-mismatched transplants which are limitations of allogeneic bone marrow transplants. Clinical analyses have shown that most UCBTs have been performed with donors having one, two or three HLA antigen mismatches, compared to unrelated BMTs where complete HLA identity for class I and class II antigens is required (Gluckman, 2000).

The other practical advantages of using UCB as an alternative source of stem cells are the relative ease of procurement, absence of risk to donors, reduced risk of transmitting infection, and prompt availability of cryopreserved samples to transplant centers. The other advantages of UCBT are the large donor pool, faster allocation process and decreased risk of viral transmission. The most important factor in predicting a positive outcome for transplant is that the number of nucleated cells infused be  $>3 \times$

$10^7/\text{kg}$  (Gluckman et al., 1997). For the purposes of optimizing the chances of finding a suitable UCB donor for the recipient, Netcord and Eurocord registries, which are a cooperative network of large experienced UCB banks, were founded (Gluckman, 2000). The major concern with UCBT has been engraftment, as all studies show delayed neutrophil and platelet recovery, whereas long-term engraftment was similar after UCBT and BMT. However, a cord blood nucleated cell dose  $>0.37 \times 10^8/\text{kg}$  increased the speed and probability of engraftment (Wagner et al., 1996).

### *Stem Cell Based Gene Therapy*

The multipotent HSCs form an ideal candidate for gene therapy because they are a self-renewing population of cells and thus may reduce or eliminate the need for repeated administrations of the gene therapy. Several investigators have reported on the successful introduction of particular genes into primitive hematopoietic cells from BM, and similar approaches are being used with UCB cells (Mayani and Lansdorp, 1998). HSCs have been a delivery cell of choice for several reasons: 1) although small in number, they are readily isolated from the body from the circulating blood, bone marrow, or UCB, 2) they give rise to many different types of blood cells, and once the engineered stem cells differentiate, the therapeutic transgene will reside in all the different types of blood cells, 3) HSCs 'home' in to a number of different spots in the body- bone marrow, liver, spleen and lymph nodes. These may be strategic locations for localized gene delivery of therapeutic agents. The only type of human stem cell used in gene therapy trials so far is the HSC (NIH, 2001). Generally however, gene therapies using HSCs have encountered a phenomenon known as 'gene silencing' where over time, the

therapeutic transgene gets turned off due to cellular mechanisms that alter the structure of the chromosome where the transgene is inserted (Challita and Kohn, 1994). Stem cell gene therapy could also allow the development of novel methods for immune modulation in autoimmune diseases. The goal is to modify the aberrant, inflammatory immune response that is characteristic of autoimmune diseases. Studies in a lupus mouse model have shown that genetic modification of HSCs with a 'decoy' receptor for the inflammatory cytokine interferon gamma, arrested disease progression (Lawson et al., 2000). Long-term in vivo gene transfer studies in mice have shown that recombinant murine retroviruses are able to infect murine HSCs with high efficiency. Because of the success in murine studies, it was believed that gene therapy would soon be applicable to treat a wide variety of congenital or acquired human diseases associated with the hematopoietic system. Human congenital diseases which are manifested predominantly in one or more of the blood lineages are, in principle, target diseases for stem cell gene therapy, since all blood cells are derived from a common ancestor, the HSC. There are, however, some limitations. First, the precise genetic defect causing the disease must be known. Second the defect should not be dominant. In general, those diseases that can be treated by allogeneic bone marrow transplantation are candidates for stem cell gene therapy. The aberrant gene in the HSC can be replaced by a correct copy in a process known as homologous recombination, or correct copies of the gene can be inserted into the host genome using viral delivery (Havenga et al., 1997). Recently genetically manipulated CD34+ UCB cells have been used in the treatment of patients with SCID (Kohn et al., 1995).

## **Ex-vivo HSC Amplification**

The use of UCB as a source of marrow repopulating cells for the treatment of pediatric malignancies is well established. However, the major potential limitation to the widespread use of UCB as a source of HSCs for marrow replacement and gene therapy is that the ability to engraft an adult might require ex vivo manipulations (Piacibello et al., 1997). The proliferation potential of hematopoietic stem/progenitor cells as well as their expansion potential appear to be biologic features that depend upon intrinsic factors. These are related to whether the cell is already committed to a particular lineage of differentiation and, if so, the specific hematopoietic lineage to which it specifically belongs and its stage of maturation. However, the ability of a cell to exhibit such potentials depends on extrinsic factors that include different cell types and cytokines that form part of the microenvironment in which the cell develops (Mayani et al., 1992). *In vitro* proliferation and expansion of hematopoietic stem/progenitor cells (HSPC) also depend on variables such as type of medium, medium change schedule, temperature, presence or absence of serum, number of cells plated per culture, etc. Several groups have assessed the in vivo expansion and proliferation of UCB progenitors using either total CD34+ cells, or CD34+ cell subsets. In general, it is clear that primitive subpopulations of CD34+ cells possess greater expansion potential than their more mature counterparts (Mayani and Lansdorp, 1998).

The ability of HSPCs to express expansion and proliferation potential in vitro depends predominantly on the cytokines present in culture. In terms of HSPC expansion, the best results have been obtained when cytokines are used in combinations that include early acting factors, such as SCF, flt-3 ligand (FL), and Tpo. The greatest expansion of



UCB-derived CD34<sup>+</sup> cells reported to date (146,000-fold expansion in CD34<sup>+</sup> cell numbers, and  $2 \times 10^6$  fold expansion in CFC numbers) was achieved using both FL and Tpo (Piacibello et al., 1997). Reports have shown that in a simpler medium, with two cytokines, flt-3 (FL) and thrombopoietin/c-mpl ligand (TPO/ML), significant expansion of HSC populations was observed, including LTC-IC that could be maintained long-term (up to six months) (Gilmore et al., 2000). Addition of late acting factors, such as Epo, usually contribute to the production of large numbers of mature cells, however they do not seem to have an effect on HSPC expansion (Mayani et al., 1993). In contrast to cytokines, hematopoietic inhibitors, such as transforming growth factor- $\beta$ , tumor necrosis factor- $\alpha$ , and macrophage inflammatory protein-1 $\alpha$  have been shown to significantly reduce both expansion and proliferation of CD34<sup>+</sup> cell populations from UCB (Mayani et al., 1995). Some investigators have used an antitransforming growth factor- $\beta$  monoclonal antibody, together with stimulatory cytokines, to achieve a significant expansion of primitive progenitor cells (Cardoso et al., 1993). Investigators have shown that UCB-derived HSPC possess higher expansion and proliferation potentials than their BM counterparts (Hows et al., 1992).

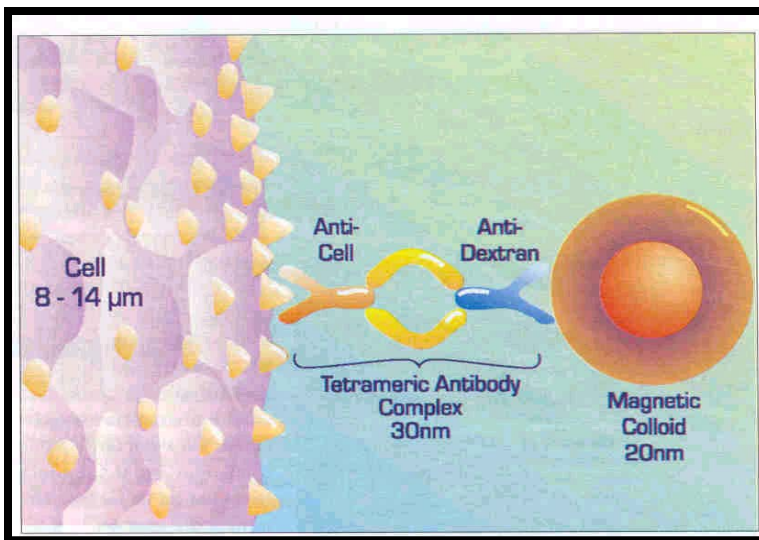
Currently, there are several unresolved issues about the ex vivo expansion and transplantation of HSPC. The first question surrounds the problem of defining the cells responsible for short and/or long term hematopoietic recovery after transplantation. The most controversial and important issue regarding the clinical use of ex vivo manipulated cells is whether on eventual exhaustion of stem cells might result from prolonged growth factor stimulation ex vivo (Brugger et al., 2000).

Two kinds of enrichment methods are currently used for the purification of CD34+ stem cells from UCB. Purification of HSCs is performed either with a combination of monoclonal antibodies to remove unwanted differentiated cells (negative selections) using the Stem Sep method (discussed in the Viacell section), or with a positive cell selection based on their surface CD34 antigens using the Mini Macs system (Pafumi et al., 2001).

### **ViaCell, Inc.**

ViaCell, Inc. is a new cellular medicine company merged from two companies: Viacord, Inc. (Boston, MA) and t. Breeders, Inc. (Worcester, MA). The goal of the new combined biotechnology company is to use its high quality cord blood banking service and patented stem cell expansion technique to develop a premier cellular pharmaceutical company providing the highest quality products and services for the treatment of diseases using stem cells (ViaCell, Inc. Annual Report 2000). In June 2001, ViaCell filed an investigational New Drug (IND) application for approval from the FDA for a phase I/II clinical trial for its proprietary selective amplification technology, which involves expansion of rare hematopoietic stem cells and other rare primary cell types. The Phase 1 study involving one patient every 3 months is currently underway. The targeted population for therapy currently includes myoablative therapy patients, patients with genetic diseases, patients with hematological malignancies, and those with neurological disorders (Craig, 2000). ViaCell will still continue to offer cord blood banking to their clients under the name of Viacord, and will continue research and development of expanded stem cell products under the name of t. Breeders (Stringer, 2000).

ViaCell's patented method of expansion called *Selective Clonogenic Amplification*<sup>™</sup> enables simultaneous selection and amplification of stem cells from bone marrow, mobilized peripheral blood, or cord blood through the use of highly specific markers on stem cells and amplifying these cells under culture conditions that foster the outgrowth of stem cells. In the strict sense, *Selective Clonogenic Amplification*<sup>™</sup> is a process for "breeding" cells, i.e., selecting preferred events of biological fission to produce target populations from among a variety of irrelevant derivative populations (t. Breeders, 2000). The salient features of the *Selective Clonogenic Amplification*<sup>™</sup> process include: removal of differentiated cells and their by-products during cell culture production of highly defined target populations, active purging of co-isolated cancer cells



**Figure 7. 'Negative Selection Separation' Process.**  
(Zimmerman, 1998)

(which do not carry the CD 34+ antigen), and efficient, cost-effective production (t.Breeders, 2000).

The *Selective Clonogenic Amplification*<sup>™</sup> utilizes the 'negative selection separation' of

hematopoietic stem/progenitor

cells. This separation

technique, as shown in fig 7, works by immunomagnetically labeling and removing the unwanted cells in the column. Cells are labeled by the use of unmodified colloidal magnetic dextran iron (orange in the figure) and non-covalent bispecific antibody cross-

linking reagents called tetrameric antibody complexes. The tetrameric antibody complex is comprised of two murine IgG monoclonal antibodies (orange and blue in the figure), held in a tetrameric array by two rat anti-mouse IgG monoclonal antibody molecules (yellow in the figure). One murine antibody molecule recognizes the differentiated cell surface antigen (orange) and the other (blue) recognizes the dextran on the magnetic particle. The cell suspension is passed through a column and the unwanted differentiated magnetically labeled cells bind to the column, while the unlabeled cells (containing CD 34+ cells) pass through (StemCell Technologies, 2001). A cocktail of monoclonal antibodies against differentiation surface markers is used to weed out the differentiated cells from the UCB cell population. The above separation process results in the separation of a subpopulation of hematopoietic stem/progenitor cells characterized as CD34+/CD38-/Lin- cells. Cells that lack 13-14 different mature blood-lineage markers including: CD2, CD3, CD14, CD16, CD19, CD24, CD56, CD66b, and glycoporphin A (collectively referred to as Lin- cells). These markers are expressed on the surface of mature red blood cells, monocytes, natural killer cells, and T-cells. ViaCell's *Selective Clonogenic Amplification*<sup>TM</sup> is a 14-day long amplification process and includes the following steps, as shown in table 2.

<b>Time- Point</b>	<b>Fraction</b>	<b>Total Cells</b>	<b>%CD34+/CD38-</b>
Day-0	Whole Cord Blood	~6.5x10 <sup>8</sup>	0.3%
	Freeze		
	Thaw		
	Pre-Sep-1	~5.2x10 <sup>7</sup>	0.2%
Day-0.5	Post-Sep-1	~2.7x10 <sup>6</sup>	3%
Day-7	Pre-Sep-2	~2.1x10 <sup>7</sup>	31%
Day-7.5	Post-Sep-2	~6.2x10 <sup>6</sup>	33%
Day-14	Post Culture	~3.0x10 <sup>7</sup>	11%

**Table 2. ViaCell's UCB amplification time course.**

## **Telomerase**

### *Structure and Function of Telomeres*

Telomeres are nucleoprotein structures located at the ends of eukaryotic chromosomes that contain protein-bound, simple repeat units of a nucleotide sequence (Rhyu, 1995). Telomeres protect chromosomes from shortening and unraveling during each replication cycle. It has been suggested that telomeres protect chromosome ends, because damaged chromosomes lacking telomeres undergo fusion, re-arrangement and translocation (Blackburn, 1991). Telomeres play an essential role in the stable maintenance of the eukaryotic chromosome within a cell by specifically binding to structural proteins. These proteins cap the ends of linear chromosomes, preventing nucleolytic degradation, end-to-end fusion, irregular recombination and other specific events that are normally lethal to a cell. Additionally telomeres are involved in nuclear architecture, and interact with other proteins to repress the expression of adjacent genes (Blackburn, 1991).

Telomeres have been studied in a variety of eukaryotic organisms. For example, *Tetrahymena* contains up to 40,000 telomere repeats per DNA macromolecule, each containing the repeat sequence GGGGTT (Blackburn and Gall, 1978). Telomeres of many insects and Lepidopteran species contain the pentanucleotide repeat sequence TTAGG (Sasaki and Fujiwara, 2000). In the diploid human cell, there are 46 chromosomes, each containing two telomeres, and each telomere contains the nucleotide repeat sequence TTAGGG, which may be repeated up to 15 Kb per telomere (Moyzis et al., 1998). The telomere repeats in most species tend to be G-C rich, with a strand bias so that the G-rich strand is oriented with its 3' end towards the end of the DNA (Kurenova

and Mason, 1997). In humans the 3' -terminal G-rich strand is about 200 nucleotides longer than the C-rich strand, leaving a 3' overhang (Wright et al., 1997).

The functional telomere is organized into a special chromatin structure, the 'telosome' (Wright et al., 1992), which contains telomeric DNA complexed with sequence-specific telomere binding proteins such as TRF1, TRF2 and more loosely with proteins such as tankyrase (Broccoli et al., 1997). The single stranded, G-rich 3' extension is not only hidden by association with numerous telomere binding proteins, it is folded back and entangled in internal double stranded telomeric DNA and thus forms the telomeric t-loop (Griffith et al., 1999). The 200 bp G-rich, 3' terminal, single stranded extensions are required for binding of TRF2, and failure to do so results in genome instability by chromosomal end-to-end fusions or, depending on the cell type, in apoptotic cell death (van Steensel et al., 1998).

### *End Replication Problem*

In somatic cells, telomere length is progressively shortened with each cell division both in vivo and in vitro (Harley et al., 1990; Lindsey et al., 1991), due to the inability of the DNA polymerase complex to replicate the very 5' end of the lagging strand (Watson, 1972; Olovnikov, 1973). DNA replication in the S-phase of the cell cycle starts by extending small RNA primers by DNA polymerases, which are unable to start *de novo* synthesis. After generation of the new DNA strand, the RNA primers are removed and all internal gaps are filled with DNA. The primers can be replaced everywhere except at the extreme 5' end which makes this new strand slightly shorter than the parental strand. This phenomenon is the molecular basis of the 'end replication problem', which was

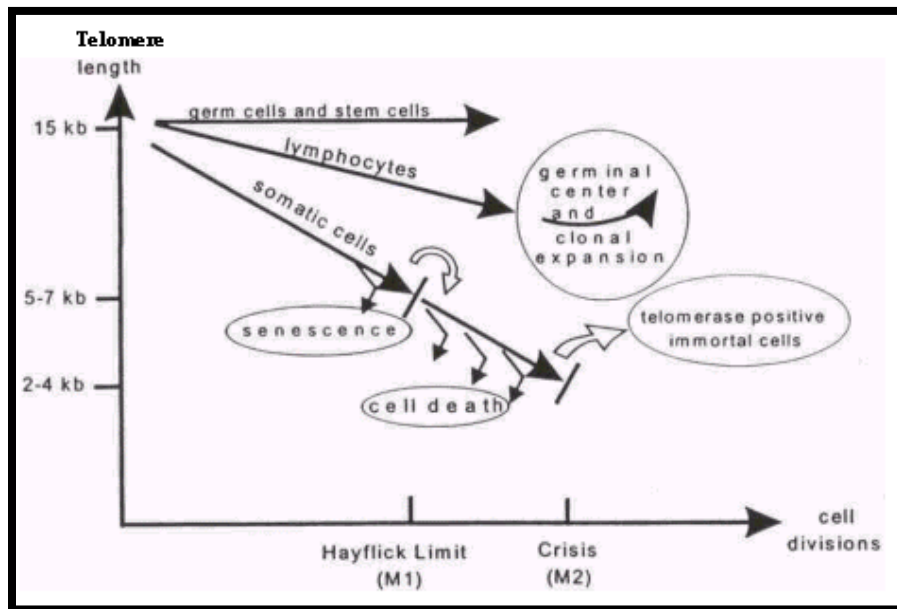
described long before the structure of the chromosomal ends was known (Olovnikov, 1973). Although the chromosomal loss is potentially very small, this loss will occur every cell division, and must eventually compromise cell or chromosomal viability following the removal of essential DNA sequences, either functional genes or telomeric sequences required for an essential end protective function (Kipling, 2001). Human telomeres are programmed to undergo gradual shortening by about 100 bp per cell division, and when several kilobases of the telomeric DNA are lost, cells stop dividing and senesce (De Lange, 1998).

### *Mitotic Clock*

Due to the ‘end replication problem’, successive shortening of the telomeres with each cell division results in a ‘mitotic clock’, and it was shown *in vitro* that this clock limits the replicative capacity of cell proliferation (Klapper et al., 2001). Telomere shortening provides an explanation for a phenomenon observed long ago: the ‘Hayflick limit’ (also called M1 or mortality stage one (see fig. 8), which postulates that the replicative potential of somatic cells *in vitro* is strictly limited by the number of consecutive cell divisions, but not in a time dependent manner. Consequently, proliferation stops after a defined number of cell divisions, independently of the time a cell needs to carry out the divisions (Harley, 1991). Once a cell reaches the Hayflick limit, which is defined by a short, critical telomere length, the cell irreversibly exits the cell cycle and enters a stage called senescence (Klapper et al., 2001). The senescent cells are metabolically active but cannot proliferate and can be considered as replicative or telomeric aged (Harley, 1991). Some rare events can abolish the M1 barrier of the

proliferation; the best-studied alterations are the expression of viral oncogenes that inactivate p53 and retinoblastoma (Rb) (Shay et al., 1991; 1993). But infrequent accumulation of these genetic aberrations leaves only a few cells that proliferate beyond the Hayflick limit (Harley, 1991), resulting in further telomere shortening.

A second checkpoint is reached at a critical telomere length called crisis (mortality stage two or M2).



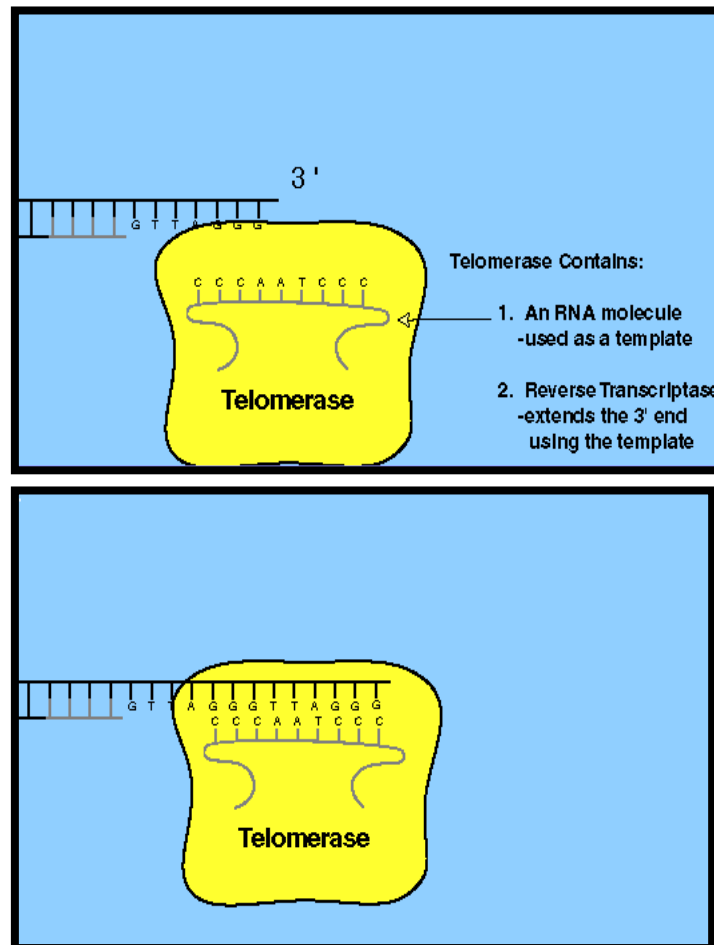
**Figure 8. Mitotic Clock** (Klapper et al., 2001) Shows that telomeres in somatic cells shorten with each cell division and enter senescence, while in telomerase positive germ line and stem cells, the telomere lengths are kept constant.

At this stage, almost all cells die due to extensive chromosomal aberrations, caused by short and dysfunctional telomeres; however, very rarely some immortal cells arise. To overcome crisis (M2) and become immortal, the cell activates telomerase activity (Harley, 1991; Klapper et al., 2001).



## Telomerase- Discovery and Function

The molecular basis of telomere replication came to light in 1985 with the discovery by Greider and Blackburn of the enzyme ‘telomerase’ in the protozoa *Tetrahymena thermophila* (Greider and Blackburn, 1985). Telomerase is a specialized



**Figure 9. Telomerase Function.** (Donald.F.Slish at SUNY, Plattsburgh). Shows de novo synthesis of telomeres by telomerase. Using its RNA as a template, telomerase synthesizes new telomeric hexamer repeats on the chromosome end.

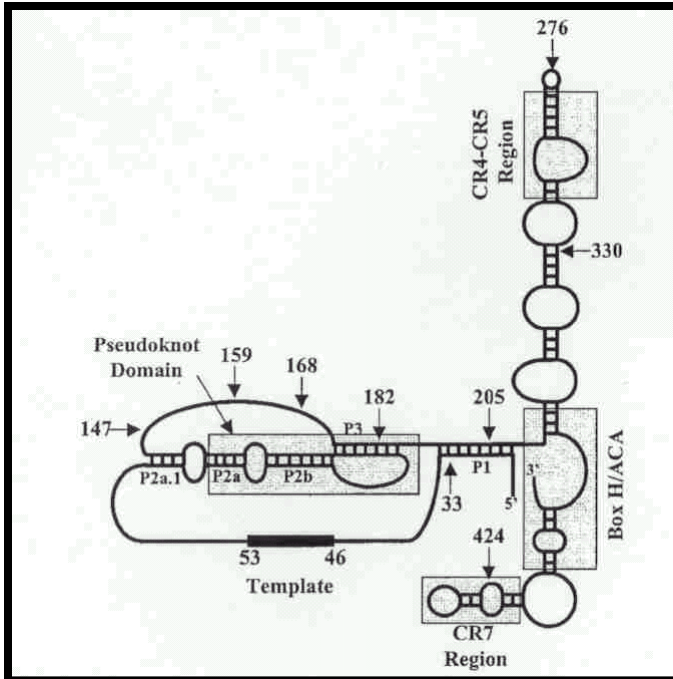
reverse transcriptase that synthesizes new telomeric repeats on the chromosome end. It thus compensates the telomeric loss due to the ‘end replication problem’ and provides the basis for unlimited proliferative capacity (Collins, 2000)(See fig.11). Telomerase is a ribonucleoprotein, that is composed of two core components, the catalytic subunit

hTERT and the RNA component hTR. Using its RNA component as the template, it synthesizes and directs telomeric repeats onto the 3' end of existing telomeres. In this respect, telomerase is acting as a reverse transcriptase, insofar as it is synthesizing DNA based upon an RNA template (Greider and Blackburn, 1989; Morin, 1989). In vitro synthesized hTERT and hTR can assemble to form catalytically active telomerase holoenzyme, thus demonstrating that these two components can form a minimal core enzyme (Weinrich et al., 1997).

### *Telomerase Structure*

The telomerase complex represents a specialized terminal reverse transcriptase with an estimated molecular mass of ~1000 kDa (Dhaene et al., 2000). The telomerase RNA component was first cloned in *Tetrahymena thermophila*. Later, the homologous genes were identified in ciliates such as *Oxytrichia* and *Euplotes*, in yeast *S. cerevisiae* (TLC1), and in mammals such as mouse (mTR) and human (hTR, currently referred to as hTERC for human telomerase RNA component) (Feng et al., 1995). hTERC is a single copy gene present on chromosome 3 (3q26.3). In humans, the length of the mature hTERC gene transcript is 451 nucleotides and lacks polyadenylation. In all organisms analyzed to date, a 'template' region complementary to the sequence of the telomere repeats is embedded in the integrated telomerase RNA sequence. For humans, the hTERC template consists of 11 nucleotides: 5'CUAACCCUAAC 3'. Mammalian telomerase RNAs resemble small nucleolar RNAs (snoRNAs)- an RNA family required for pseudouridine modification and precursor processing of rRNA – because of the presence of an H/ACA box in their 3' domain (Mitchell et al., 1999). The primary

structure of the RNA component has evolved rapidly between species, but there seems to be a secondary structure core that is highly conserved even between distant groups (Blackburn, 2000). Four conserved structural elements are universally present in the



**Figure 10. Secondary structure of hTERC** (Chen et al., 2000). Shows the RNA template sequence (nucleotides 46-53) and its 5' and 3' ends.

predicted secondary structure of RNA: these are the pseudoknot domain, the CR4-CR5 domain, the H/ACA box, and the CR7 domain (see fig 10).

Telomerase reverse transcriptase is a special class of reverse transcriptases that functions as the rate limiting step in telomerase activity. It has been

identified in yeast (*Sc-Est2p*), the ciliate *E. aediculatus* (*Ea-p123*),

*Tetrahymena thermophila* (*Tt-*

*TERT/p133*), and in mammals such as mouse (*mTERT*) and human (*hTRT/hEst2/hTCS1/TP2*, currently referred to as *hTERT*). *hTERT* contains a telomerase specific amino acid motif (T motif) and seven conserved reverse transcriptase motifs (RT motifs), making it phylogenetically related to RTs (Dhaene et al., 2000; Nakamura et al., 1997). Substitution of conserved amino acid residues in the RT domain of *hTERT* completely abolishes telomerase activity. The 40-kB single copy *hTERT* gene, located on chromosome 5 (5p15.33), codes for a 127-kDa protein of 1132 amino

acids contained in 6 exons (Meyerson et al., 1997). The human telomerase reverse transcriptase subunit (hTERT) has been cloned by Nakamura et al., (1997).

Another telomerase-associated protein includes the mammalian p80 homologue identified in rat, mouse and human (TP1/TLP1, currently referred to as hTEP1 for human telomerase-associated protein1), but similar to hTERC, the expression of this protein does not correlate with telomerase activity in cells and tissues. It has been suggested that hTEP1 may play a role in some aspect of ribonucleoprotein structure, function or assembly (Harrington et al., 1997).

### *Telomerase vs. Cancer*

In the mid-1990s, the hypothesis emerged that the upregulation or re-expression of telomerase is a critical event responsible for continuous tumor cell growth. In contrast to normal cells, in which a gradual mitosis-related erosion of telomeres eventually limits replicative life span, tumor cells have telomerase activity and show no loss of chromosomal ends. It was thus suggested that telomere stabilization might be required for cells to escape replicative senescence and to proliferate indefinitely (Dheane et al., 2000). But a key debate emerged on whether telomerase upregulation by itself induce a malignant phenotype, i.e. does telomerase act as an oncogene. And if so, then how does this relate to the debatable levels of tolerance in HSCs.

One point is clear; telomerase activity has been demonstrated in the vast majority of tumor biopsies (85%) (Kim et al., 1994). Moreover, cell lines immortalized either spontaneously or after transformation by oncogenic viruses (such as simian virus 40 or human papillomavirus types 16 or 18) are usually telomerase-positive (Belair et al.,

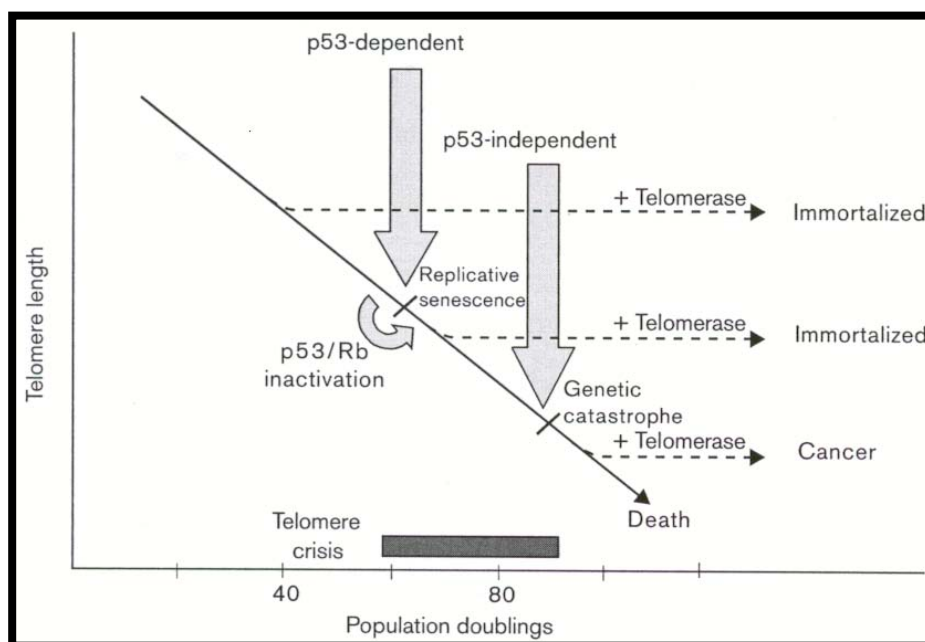
1997). Such observations lead to the current hypothesis that telomerase is activated during immortalization *in vitro* and tumorigenesis *in vivo* (De lange, 1994). However, telomerase activity is not always detectable in immortal cell lines (Bryan et al., 1995).

Most results have shown that normal somatic cells are telomerase negative, whereas germ cells and stem cells in renewable tissues are telomerase positive (Belair et al., 1997). It has been suggested that normal cells contain an inhibitor of telomerase, possibly on chromosome 3, whose deletion or inactivation is required for immortalization and tumorigenic transformation (Seachrist, 1995). Telomerase activity has also been demonstrated in highly proliferative non-cancerous tissues such as the basal layer of the epidermis, endometrial tissue during the proliferative phase of the menstrual cycle, and oral mucosa (Belair et al., 1997). These latter studies are not consistent with a model in which activation of telomerase occurs during tumorigenic transformation. Instead, they suggest that telomerase activity may more directly be associated with cell proliferation.

Belair et al. (1997) demonstrated using both normal and tumorous human uroepithelial tissues that telomerase activity is a marker for cell proliferation, not malignant transformation. They showed that normal cells do have the capability to express telomerase activity given their proliferative conditions *in vitro*. Uncultured normal human uroepithelial cells (HUCs) were telomerase negative. However, the same cells, when established as proliferating cultures *in vitro*, showed telomerase activity but at lower levels than in tumorous cells. Here they attribute the relatively high telomerase activity in tumor biopsies, in part to their high proliferative ability. These results support a model in which the detection of telomerase in tumor biopsies, but not in uncultured normal cells, reflects differences in proliferation between tumor and normal cells *in vivo*

(Belair et al., 1997). hTERT transfection experiments have convincingly shown that hTERT is rate limiting for telomere elongation (Nakayama et al., 1998). Most somatic human cells do not express the reverse transcriptase subunit of telomerase but contain all other components of the enzyme so that expression of the missing hTERT component leads to reconstitution of enzyme activity (Weinrich et al., 1997). Transfection of pre-senescent cultures of telomerase-negative retinal pigment epithelial cells, human vascular endothelial cells and young/midlife and old fibroblasts (Bodnar et al., 1998; Vaziri & Benchimol, 1998; Yang et al., 1999) as well as pre-crisis cells, with hTERT gene resulted in an increase in telomerase activity, elongation of telomeres and indefinite replicative growth, thus establishing a causal relationship between telomere shortening and *in vitro* cellular senescence. While sufficient for immortalization, this ectopic expression of telomerase did not result in changes typically associated with malignant transformation, such as increased growth rate, loss of contact inhibition, acquisition of serum-independent growth, disturbances in the pRB and p53-mediated cell cycle checkpoints, and cytogenetic abnormalities, indicating that telomerase expression per se is not oncogenic (Jiang et al., 1999).

Most recently, studies conducted with mice doubly null for mTR and p53 (mTR<sup>-/-</sup>p53<sup>-/-</sup>mice) or INK4a/ARF (mTR<sup>-/-</sup>INK4a<sup>-/-</sup>mice) showed that telomerase may play a paradoxical role, either promoting or inhibiting tumor formation depending on the genetic context of the would be cancer cell (Chin et al., 1999; Greenberg et al., 1999). Progressive telomere shortening occurs with the division of primary human cells and can



**Figure 11. Telomere Hypothesis** (Artandi and DePinho, 2000) Shows telomere shortening in primary human cells leads to replicative senescence, a checkpoint that is dependant on p53 and Rb. Inhibition of p53/Rb allows continued cell division and entry into telomere crisis, a period of chromosomal instability and cell death. In the absence of p53, cell growth goes unchecked and telomere function continues to deteriorate until genetic catastrophe- a point at which secondary genetic changes occur that result either in cell death or transformation. Genetic catastrophe is probably an important step in the development of most human cancers.

trigger at least two cellular responses depending on genetic context: senescence or crisis.

As telomeres shorten during the earliest steps of carcinogenesis, nascent cancer cells encounter the proliferative barrier of replicative senescence. Cells that escape this checkpoint via tumor suppressor loss enter telomere crisis. Analysis of cancers arising in telomerase-deficient mouse, have led to the theory that the massive chromosomal instability of telomere crisis is an important step in development of cancer (Artandi and DePinho, 2000). According to the “Telomere Hypothesis” (fig. 11), telomere shortening prevents tumorigenesis and telomere crisis promotes tumorigenesis. The

telomere hypothesis was formulated to explain the important role of telomeres in senescence, the observation that telomerase is reactivated in 80-90% of human cancers, and the observation that telomeres in tumor lines are often shorter than in primary somatic cells. The model states that in a developing cancer cell both senescence and crisis represent barriers to continued tumor growth (Artandi and DePinho, 2000).

It has recently been shown that transcription of the hTERT gene is regulated directly by the immortalizing oncoprotein Myc, whose upregulation is an obligate feature of all cancers (Greenberg et al, 1999). Inhibition of telomerase or experimental interference with telomere function arrests and often kills cells even if they are transformed (van Steensel et al., 1998). Thus telomerase activity appears to make an important contribution to the viability of transformed cells, but its action does not fit the usual roles ascribed to oncogenes and tumor suppressors (de Lange & DePinho, 1999).

#### *Telomerase and Aging (Telomerase- the immortality enzyme?)*

Is telomerase really all that is needed for cellular immortalization? Will enforced somatic expression of telomerase lead to a cancer-prone condition? Definitive answers to these questions have yet to emerge. However, the first major advance was provided with the finding that ectopic expression of hTERT in primary human cells could confer endless growth in culture (De Lange & DePinho, 1999). The cloning of the cDNA encoding the catalytic subunit of telomerase (hTERT) (Meyerson et al., 1997), made it possible to test the telomere hypothesis. Two telomerase-negative somatic human cell types, retinal pigment epithelial cells and foreskin fibroblasts, were transfected with hTERT. The telomerase-expressing clones had elongated telomeres, divided vigorously,



and showed reduced staining for Senescence-associated  $\beta$ -galactosidase (SA- $\beta$ -Gal), a biomarker for senescence. These cells also showed a normal karyotype and exceeded their normal life span by at least 20 doublings, thus establishing a causal relationship between telomere shortening and in vitro cellular senescence (Bodnar et al., 1998). These reports also indicate that, a very low level of telomerase activity is insufficient to prevent telomere shortening. This is consistent with the observation that hematopoietic stem cells have low but detectable telomerase activity; yet continue to exhibit shortening of their telomeres throughout life. Thus it appears that a threshold level of telomerase activity is required for actual life-span extension (Bodnar et al., 1998). Similar findings were observed in a similar study in which Vaziri & Benchimol (1998) expressed hTERT in normal fibroblasts, which lack telomerase activity. Similar results were also reported with endothelial cells (Yang et al., 1999). Other cell types like keratinocytes and mammary epithelial cells may need, in addition to hTERT expression, additional genetic changes to extend their life span beyond crisis. These cells arrest prematurely as a result of accumulation of p16<sup>INK4A</sup>, a critical inhibitor of the RB pathway and key mortality gene (Kiyono et al., 1998). These cells are immortal but do not show any changes associated with the transformed phenotype. The ability of telomerase to prevent the senescence of primary human cells without causing any overt change to a more cancerous phenotype has created great excitement in the gerontological community as a potential route to therapeutic intervention in human aging (Kipling, 2001).

Telomere based barriers to unlimited cell division can be imposed in several ways (Holt et al., 1996). One is via the triggering of replicative senescence, as is seen in normal fibroblasts (Bodnar et al., 1998). The second is the triggering of apoptosis, as has

been described following telomerase repression and subsequent telomere erosion on several human cancer cell lines (Hahn et al., 1999). The third is the ultimate loss of telomere protective function and the triggering of non-specific “genome crisis” (Halvorsen et al., 1999). All three outcomes can be prevented by telomerase (Kipling, 2001).

All pathological syndromes associated with accelerated aging show alterations in telomere biology. Telomere defects in Werner syndrome, Bloom syndrome, Hutchinson-Gilford progeria, Down syndrome, Dyskeratosis congenital, and Ataxia telangiectasia have been reported (Klapper et al., 2001). Forced expression of hTERT in primary fibroblasts isolated from Werner syndrome patients confers detectable telomerase activity and leads to extension of cellular life span. These studies indicate a potential route to therapeutic intervention in a human ageing syndrome (Kipling, 2001). Cellular senescence is believed to contribute to multiple conditions in the elderly, and could in principle be remedied by cell life span expansion *in situ* (Bodnar et al., 1998). Expansion of normal cells *in vitro*, followed by reimplantation might be a future form of cell based therapy for several aging related diseases that are based on loss of irreplaceable cells. Attempts to use telomerase-immortalized cells for *in vitro* tissue engineering of adrenal, vascular, skin, pancreatic or muscle tissue are already underway (Yang et al., 1999).

### *Telomerase and Stem Cells*

In most somatic cells, telomerase activity is lacking. However, primitive hematopoietic cells have shown to exhibit low but detectable telomerase activity (Hiyama

et al., 1995; Broccoli et al.; 1995; Chiu et al., 1996). But despite having detectable telomerase activity, telomere shortening is observed in blood leukocytes with age, and in vivo hematopoietic progenitor cultures (Vaziri et al., 1994). In their study, telomerase activity in human BM and PB was assigned to the hematopoietic progenitor cell fraction expressing the CD34 antigen. CD34+ cells lacking co-expression of CD33 demonstrated higher levels of telomerase than myeloid committed CD34+/CD33+ cells. The presence of growth factors inducing differentiation resulted in a decrease of telomerase activity. In addition, telomerase activity increased in PB during cytokine-induced mobilization of hematopoietic progenitor cells. Based on these results, it has been suggested that at least a portion of the hematopoietic stem/progenitor cell fraction expresses telomerase, and downregulates its expression during differentiation (Hohaus et al., 1997)

Overall, the observed levels of telomerase activity in stem cells appear to be related to the mitotic or cycling state of the cell population. Reports indicate that telomerase is generally present in rapidly expanding cells, upregulated at cell cycle entry as cells progress through S-phase, and repressed in quiescent G<sub>0</sub> cells (Holt et al., 1996; Engelhardt et al., 1997). Telomerase activity in CD34+/CD38+ cells (non-quiescent), from bone marrow (BM), Peripheral blood (PB), cord blood (CB) and fetal liver (FL), exceeded levels in CD34+/CD38-, CD34- (quiescent), and mononuclear cells (Engelhardt et al., 1997). Telomerase activity was reduced in noncycling FL and CB CD34+ cells compared to more actively cycling PB CD34+ and BM CD34+ cells (Engelhardt et al., 1997). Recent studies have established the role of hematopoietic cytokines in ex-vivo expansion systems (Moore & Hoskins, 1994). Stem cell self-renewal, as measured by increases in the numbers of long-term culture initiating cells, can be achieved in

particular with KL and Flk-L cytokine combinations. Cytokine synergistic growth promoting interactions have been reported on CD34+ cells from different sources such as CB, PB and BM (Petzer et al., 1996). In the absence of growth factors, CD34+ cells undergo apoptosis. Single cytokines preserve cells in expansion cultures and block apoptotic death, but do not induce noncycling progenitors into cycle, whereas cytokine combinations result in the progression of cells into DNA synthesis and induction of cell cycle proteins (Moore & Hoskins, 1994). In vitro culture of CD34+ cells derived from BM, PB, CB, FL in the presence of a cytokine combination (KL, IL-3, IL-6, erythropoitin, granulocyte colony-stimulating factor) showed upregulation of telomerase activity which peaked after 1 week of culture, and decreased to baseline levels or below detection after 3-4 weeks. In contrast, stimulation of CD34+ cells with single cytokines resulted in no (or minor) telomerase upregulation (Engelhardt et al., 1997).

It has been shown that telomerase activity is low in CB derived CD34+CD38- and CD34+c-kit- cells compared to CD38+ or c-kit (high or low) cells, suggesting that CD34+CD38- or c-kit- cells are likely to be more quiescent. These results suggest that the CD34+CD38- and CD34+c-kit- cell populations are primitive stem/progenitor cells, and that the telomerase activity of these cells correlates with their proliferative capacity as well as their stage of differentiation (Sakabe et al., 1998). Telomerase activity has been attributed more to actively dividing mature subsets (CD34+71+45+) than to more primitive progenitors with a CD34+71low45low phenotype or to CD34- cells (Chiu et al., 1996). Telomerase was found in actively cycling CD34+/CD38+ cells exceeding the levels found in CD34- cells and in quiescent CD34+/CD38- cells. Non-expanding CD34+ cells showed a low or undetectable telomerase activity. Secondary CD34+ cells,

however, showed a reduced ability to upregulate telomerase activity and to proliferate after 1 week of expansion compared with primary CD34+ cells, which suggests that CD34+ cells lose telomerase activity and may undergo replicative aging on cell proliferation. The secondary CD34+ cells refer to primary CD34+ cells that were harvested from a delta culture and selected for CD34+ for a second time using immunomagnetic beads (Engelhardt et al., 1997). Elevated telomerase activity is found in BM progenitor stem cells and activated lymphocytes *in vitro* as well as *in vivo*, indicating that cells with high growth requirements can readily upregulate telomerase (Norrback & Roos, 1997). The reason for elevated telomerase activity in lymphocytes may be that the repeated expansion of individual clones during antigen exposure throughout their life span requires telomerase to slow down the rate of telomere erosion that normally occurs in normal somatic cells without telomerase activity (Holt et al., 1997).

Cell expansion analyses have shown that telomerase is highly expressed in populations where the greatest proliferation and cell expansion takes place. But, telomerase decreases with the reduction of cell renewal and expansion potential (Engelhardt et al., 1997). A “cell cycle” model has been suggested, which postulates that telomerase is repressed in quiescent stem cells (CD34+CD38-), is activated on cell proliferation, expansion, cell cycle entry, and progression into progenitor compartment (CD34+/CD38+), and is repressed again on terminal cell differentiation (CD34-).

From these reports it can be concluded that telomerase is upregulated in response to multi-cytokine-induced proliferation and cell cycle activation in primitive

hematopoietic cells, and that induction of a differentiation program downregulates telomerase activity.

## PROJECT PURPOSE

Hematopoietic cell populations showing elevated CD34+/CD38- cells (HSCs), detectable telomerase activity and elongated telomere lengths display increased graft survivability in humans during transplants. The goal of this project was to investigate telomerase activity and telomere length in umbilical cord blood cell populations enriched for HSCs during ViaCell's amplification process. The first aim of this study was to assay telomerase activity in each of ViaCell's amplification fractions comprising cord cell samples obtained at various stages of a two-week *ex vivo* stem cell amplification process. The second aim was to determine the average telomere length of these fractions. The third aim was to investigate various culture conditions that could potentially upregulate telomerase activity and thus elongate the telomere length of the final cell fraction slated for perfusion into the patient to improve engraftability.

## MATERIALS AND METHODS

### **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,  $10^5$  CD45+ cells were provided at various time points during ViaCell's stem cell amplification process. Cord cell samples from three different donors were tested using this assay. For the telomerase length assay (TLA),  $10^7$  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**

#### *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<sub>2</sub>, 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  $10^5$  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  $\mu$ l 1X CHAPS lysis buffer by pipetting up and down. For  $10^6$  cells, 200  $\mu$ l 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  $\times$ g for 20 min at 4°C to pellet cell debris. The supernatant was aliquoted and stored at -80°C. One of the aliquots of each of the samples was heat inactivated by incubating at 85°C for 10 min, to serve as a negative control in the assay. 5  $\mu$ l of supernatant of each sample was transferred into a fresh eppendorf tube to determine the protein concentration.

#### *Determination of Protein Concentration*

Protein concentration was 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  $\mu$ g/ml, 2.50  $\mu$ g/ml, 5  $\mu$ g/ml, 10  $\mu$ g/ml, 20  $\mu$ g/ml, and 40  $\mu$ g/ml. In the first tube, 500  $\mu$ l distilled water was added. In the second tube, 5  $\mu$ l of cell extract was diluted with 495  $\mu$ l of distilled water. In the remaining tubes 500  $\mu$ l 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, then the OD was read at 595 nm relative to the tube containing only distilled water.

#### *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 [ $\gamma$ - $^{32}$ P] ATP (ICN Pharmaceuticals) 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  $\mu$ l reaction: 10  $\mu$ l of TS primer, 2.5  $\mu$ l of [ $\gamma$ - $^{32}$ P] ATP (3,000 Ci/mmol), 2  $\mu$ l of 10X kinase buffer, 0.5  $\mu$ l T4 polynucleotide kinase (10 units/ $\mu$ l) (Ambion, #2310) and 5  $\mu$ l of PCR grade water. These reagents were then mixed and spun briefly in a microcentrifuge. The reagent mix was then 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. 2  $\mu$ l of kinased TS primer was used per TRAP assay reaction.

#### *Telomerase Reaction and PCR*

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

lysis buffer (primer-dimer/PCR contamination control), 2  $\mu$ l of heat inactivated extract (negative control), 2  $\mu$ l of cancer cell positive control, or a volume of cord cell extract containing 1  $\mu$ g of protein (usually 0.5-2  $\mu$ l). 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 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 acrylamide gel solution was prepared by mixing 10 ml of 30% polyacrimide / bisacrylamide, 1.5 ml of 10X TBE, 3 ml of 5% ammonium persulfate (to make 0.1%), dH<sub>2</sub>O to make 30 ml, and 30  $\mu$ l 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  $\mu$ l of 10X loading dye-containing bromophenol blue and xylene cyanol (0.05%) and 10% glycerol was added to each PCR reaction tube. The tubes were then vortexed and spun. 5  $\mu$ l 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 dismantled. 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 quantitated 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 telomerase reaction products (i.e. all bands  $\geq 50$  – mer) 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**

#### *Cord Blood Samples*

Human umbilical cord blood samples containing  $10^7$  CD45+ cells were obtained from 2-3 pooled donors at two time points (Day-0 and Day-14) during ViaCell's stem

cell amplification process. For the purposes of the Telomere Length Assay (TLA),  $10^7$  cells were required to obtain a good yield of genomic DNA. Before being transported to WPI, the cultured cells were left in an aliquot of the original culture media. The cells were then transported to WPI on ice.

#### *Isolation of Genomic DNA*

Genomic DNA isolation 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  $1 \times 10^7$  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 in an eppendorf tube by 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 4 ml of diluted lysis buffer solution was added to the pelleted cells split into 4 eppendorf tubes and the tubes were vortexed gently. The cell solution was mixed with 200  $\mu$ l of proteinase K (50  $\mu$ l per each of the 4 eppendorf tubes) and vortexed twice for 10 sec. This treatment helps ensure cell lysis and inactivation of nucleases. Then 10 Magnetic Glass Particles (MGP) tablets (approx. 3 tablets per eppendorf tube) were added to the lysate to immobilize the DNA by binding to it. 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 on a rotating mixer. Next, the MGP beads were separated by placing the eppendorf tubes in a magnetic particle separator (Roche # 1641794) for 2 min, and the supernatant was discarded. In a separate tube, washing buffer solution containing RNase was prepared by mixing 10  $\mu$ l 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°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°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°C.

#### *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 17µl. Handling of all solutions and pipeting was done on ice. The following reagents were mixed in a 0.5 eppendorf tube to make a 20 µl reaction: 2 µl of 10X digestion buffer, 1 µl of Hinf 1 (40 U/µl), 1 µl of Rsa 1 (40 U/µl). Depending on the assay, 1 µg genomic DNA (high molecular weight control DNA (high molecular weight telomeres, 100 ng/µl), low molecular weight control DNA (low molecular weight telomeres, 100 ng/µl) or cord sample) in 16 µl volume was added. The above reaction mixture was then incubated for 2 hours at 37°C. Before loading onto the gel, 5 µl of 5X loading buffer was added to each 20 µl reaction mix 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 highly pure nucleic acid grade agarose (International Biotechnologies Inc.) was added to 100 ml 1X TAE buffer in an Erlenmeyer flask. The solution was microwaved 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 DIG molecular weight marker reaction mix was prepared just before loading the samples onto the gel, the following reagents were mixed in a 0.5 ml eppendorf tube: 4 µl of DIG molecular weight marker, 12 µl of nuclease free water, 4 µl of 5X loading buffer. This 20 µl marker sample was microfuged briefly and incubated at

65°C for 10 min. 25 µl of each cord sample was loaded per lane and 10 µl of the DIG labeled molecular weight marker was 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 approx.  $\frac{3}{4}$  the length of the gel.

### *Southern Blotting*

Southern transfer of the digested genomic DNA was done by high salt capillary transfer to nitrocellulose membrane using a 20X SSC (Sodium Saline Citrate) 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 depurinate the DNA. The gel was rinsed 2 times with distilled water, then was denatured 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 2 times with distilled water, and neutralization by submerging it 2 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 (3 M NaCl, 0.3 M Sodium Citrate, pH 7.0) 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 overlaid with a piece of dry 3MM paper, which in turn was overlaid with several layers of dry paper towels to make a stack about 10 cm thick. The paper towels were placed in such way 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. 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°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°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°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°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 hybridization solution, and mixed. After pre-hybridization of membrane, the pre-hybridization 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°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.

### *DIG Antibody Binding*

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. The membrane was then incubated in freshly prepared Blocking solution for 30 min on a

gyrotory at 25°C. The antibody solution was prepared as follows: The vial containing the Anti-DIG –AP antibody (0.75 U/μl, F<sub>ab</sub> 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. 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°C. Excess substrate solution was squeezed out from the plastic sheets, and the membrane was exposed to X-ray film for 1 hour at 25°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 goal of this project was to investigate telomerase activity in umbilical cord blood cell populations during ViaCell's hematopoietic stem cell (HSC) amplification process. A TRAP assay was used for this purpose. Detection of this activity in the day-14 fraction could serve as a new means for validating ViaCell's product. Second, a TLA assay was used to investigate telomere length in these cell populations. Third, because cell populations elevated in telomerase have previously been shown to contain elevated engraftment potential, different HSC culture conditions that could potentially upregulate telomerase activity were also investigated.

### **TRAP Assay**

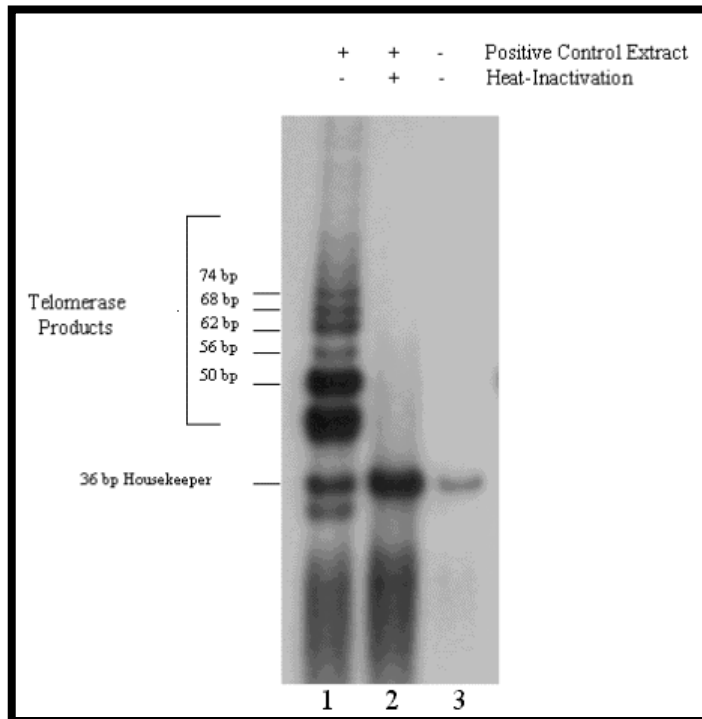
A TRAP (Telomerase Repeat Amplification Protocol) assay was used to measure telomerase activity. The TRAPeze telomerase detection kit (Intergen, # S7700) was chosen because this kit features several improvements over the original method described by Kim et al., (1994), such as inclusion of a modified reverse primer sequence which eliminates the need for a wax barrier PCR hot start, reduces amplification artifacts, and permits better quantitation of telomerase activity. Each reaction mixture also contains an additional primer (TK) and a template (TSK1) for amplification of a 36 bp internal PCR control. Incorporation of this control makes it possible to identify false-negative samples that contain Taq polymerase inhibitors. The TRAP assay is a highly sensitive in vitro assay system for detecting telomerase activity in as little as 0.5 µg of total cell lysate. The technique is based on the ability of telomerase to recognize and elongate in vitro an 18-mer artificial oligonucleotide substrate TS, 5'-AATCCGTCGAGCAGAGTT-3'. In the

first step of the reaction, telomerase adds a number of telomeric repeats (TTAGGG) onto the 3' end of a substrate oligonucleotide (TS). In the second step, the resulting hexamer-extension products are amplified via PCR using as primers the original TS oligonucleotide and a reverse primer, a 14-mer oligonucleotide, RP. For a telomerase-extended product to be amplified by TS and RP primers, it must have at least 3 telomeric repeats. Therefore, the shortest band on the “telomere ladder” is a 50-mer (18 nucleotides of TS, 14 of RP and 18 of the 3 telomeric repeats). A ladder spanning a range from 50, 56, 62, 68, 74 etc. is expected in telomerase positive samples.

### **Telomerase Activity in Cord Cell Fractions**

#### *Test of Controls*

First a test of positive and negative controls was performed, as shown in fig 12. Cancer cell (HeLa) extract lane 1, a rich source of telomerase, was used as the positive control. The presence of the 36 bp internal control indicates no inhibition of the PCR reaction. Heat inactivation of the cancer sample (lane 2) is a negative control: telomerase is a heat sensitive enzyme. The sample was heat treated by incubating at 85°C for 10 min. Only the 36 bp internal PCR control is observed in this assay (lane 2). A Primer-Dimer/ PCR contamination control (lane 3), in which cell extract was substituted with CHAPS lysis buffer indicated no telomerase activity, only the 36 bp internal PCR control was observed as expected.

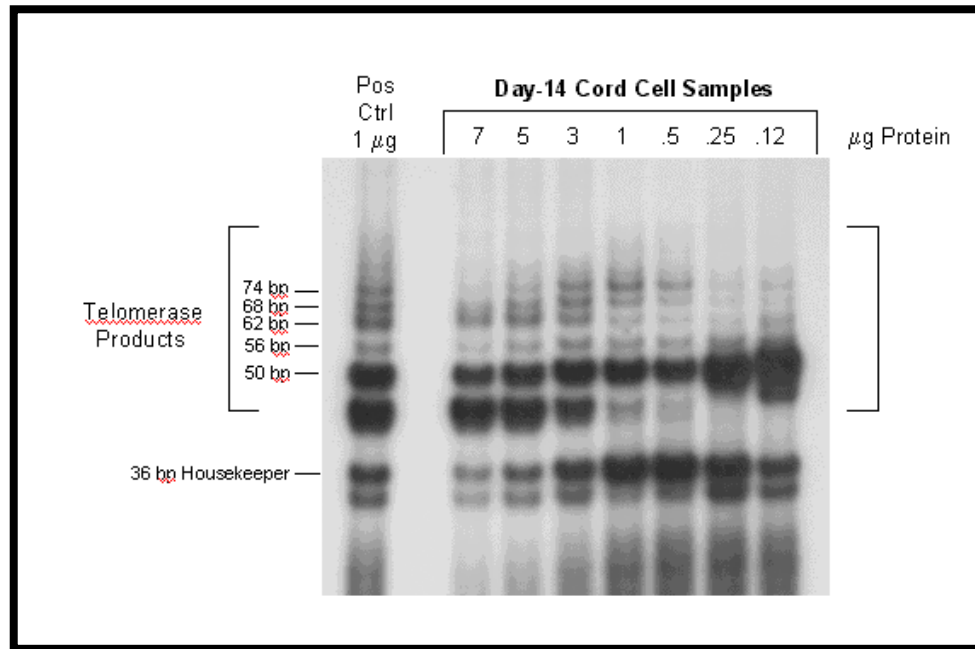


**Figure 12. Test of positive and negative controls of the TRAP Assay.** Lanes show cancer cell extract positive control (lane 1), heat inactivation negative control (lane 2), cell extract substituted with CHAPS lysis buffer-(negative control) (lane 3)

### *Optimizing the Protein Concentration in Cord Cell Samples*

In order to determine the optimum cord cell lysate protein concentration to use for the assay, it was performed with decreasing amounts of protein (fig 13). The optimum protein concentration was found to be 0.5 to 1  $\mu\text{g}$ , because at this concentration, the telomerase ladder of products for cord samples extends higher and darker than the other protein concentrations tested with no inhibition of the internal PCR standard. Note that the telomerase activity exhibited by the optimized 1  $\mu\text{g}$  cord cell samples appears to be equal to 1  $\mu\text{g}$  of cancer cell extract positive control. Also note that when the protein mass

is too high, amplification of the 36 bp internal PCR control is diminished, and when the protein mass is too low, the amplification of the telomerase ladder is diminished.



**Figure 13. TRAP Assay with descending protein mass.** Shows the effect of different amounts of protein on telomerase activity.

### *Time Course Experiments*

The first phase of this project was to investigate telomerase activity in Viacell's cord blood populations. These populations are variously enriched in CD34+ Hematopoietic Stem Cells, and some fractions are amplified by growth in a rich medium containing a mixture of cytokines known to stimulate HSC growth. Table 3, shows the percentage of CD34+/CD38- at various time points during the amplification process. For this project, I conducted time course experiments on three different sets of cord blood samples obtained from three different donors. For the time course experiments,  $10^5$  CD45+ cells were provided at various time-points in ViaCell's amplification process.

Because the assay is so sensitive,  $10^5$  cells provided enough material for multiple determinations, and cords did not need to be pooled.

<b>Cord-Sample</b>	<b>Pre-Freeze (Day-0)</b>	<b>Pre-Sep-1</b>	<b>Post-Sep-1 (Day-0.5)</b>	<b>Pre-Sep-2 (Day-7)</b>	<b>Post-Sep-2 (Day-7.5)</b>	<b>Cell Product (Day-14)</b>	<b>Thawed Cell Product (Thawed Day-14)</b>
Cord-1	0.13	0.08	2.04	22.82	23.09	5.62	5.29
Cord-2	0.06	0.19	3.54	16.79	19.91	7.25	7.75
Cord-3	0.26	0.17	3.2	31.25	33.2	10.53	11.54

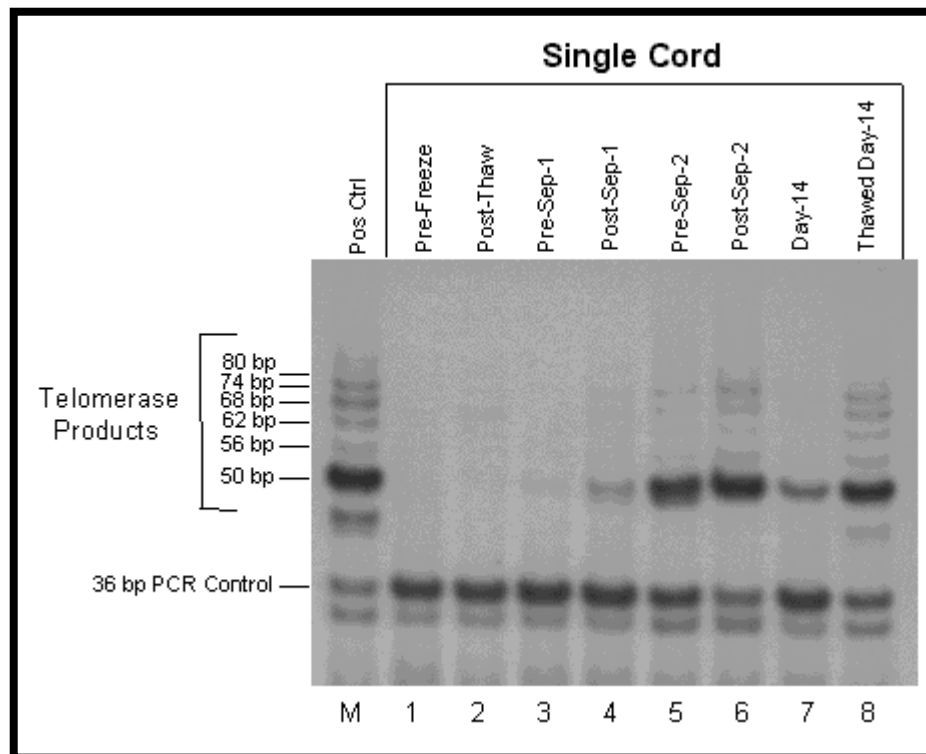
**Table 3. Percent of CD34+/CD38-cells at each time point during ViaCell’s amplification of three cord samples.**

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 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 in culture for a week. 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 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 then frozen for storage and thawed, which correspond to ‘thawed day 14’ on the time course. Note that the Post-Sep-2 sample contains the highest percentage of CD34+/CD38- cells in each cord tested, representing 127-331 fold enrichment of these cells over fresh cord.



### *Telomerase Activity in Cord-1 Time Course*

An ascending telomerase activity profile was observed during the time course experiment on cord-1 (sample# EXPO91001A) as shown in figure 14. As expected, the cancer cell extract positive control (lane 1) showed high telomerase activity. 1  $\mu$ g cord cell lysate protein load was used for the time course experiments in accordance with the optimization experiments. The same 'Master Mix' for the PCR amplification was used to assay all the samples, which proved to be critical for obtaining an even amplification of the PCR control.



**Figure 14. TRAP Assay on Cord-1, N=1**

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). 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 comparable to the cancer cell extract positive control (lane 1). At 'Day 14', however, a dip in telomerase activity was observed. 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. The 36 bp internal PCR control was observed in all the lanes, which indicates no sample contained an unusual amount of Taq Polymerase inhibitor.

To determine the reproducibility of the results obtained in cord 1 and to assay the intra-sample variability, a trial 2 of the time course experiment on cord 1 was conducted (figure 15). This second trial showed the same trends as trial 1. Because the  $^{32}\text{P}$  for trial-2 was fresh, it proved sufficient to quantitate the telomerase bands cut out of the gel (fig 16). The histoplot determined by counting  $^{32}\text{P}$  corresponds with the telomerase activity estimated by eye from the x-ray films. The Post-Sep-2 sample contained the highest activity at 5.7x the fresh cord level.

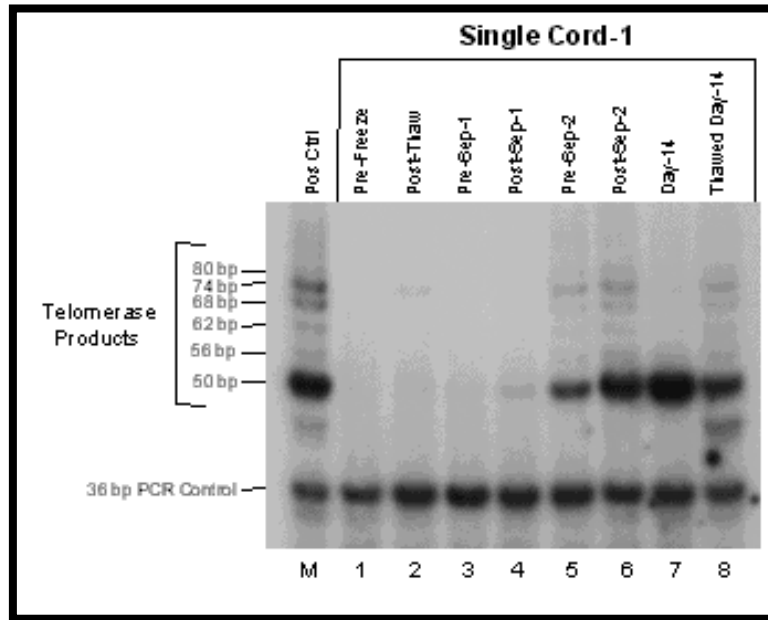


Figure 15. TRAP Assay on Cord-1, N=2

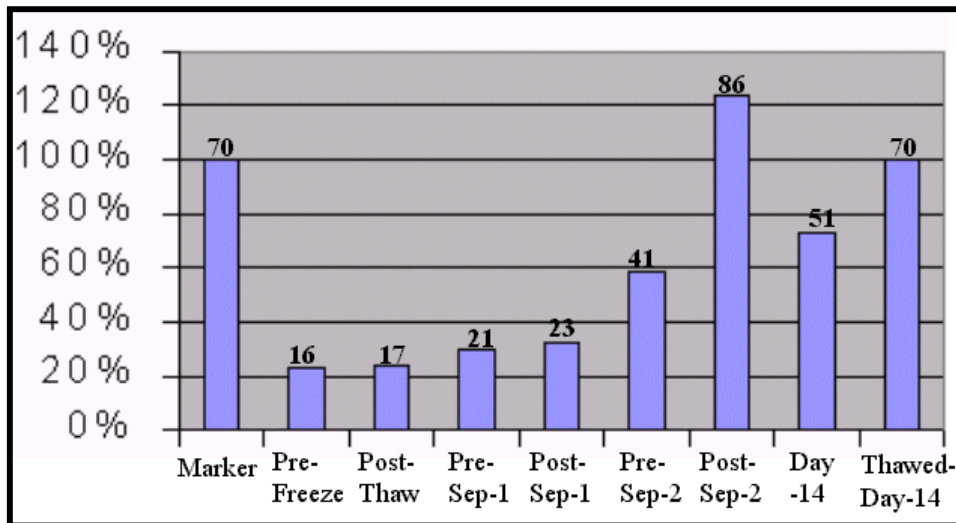
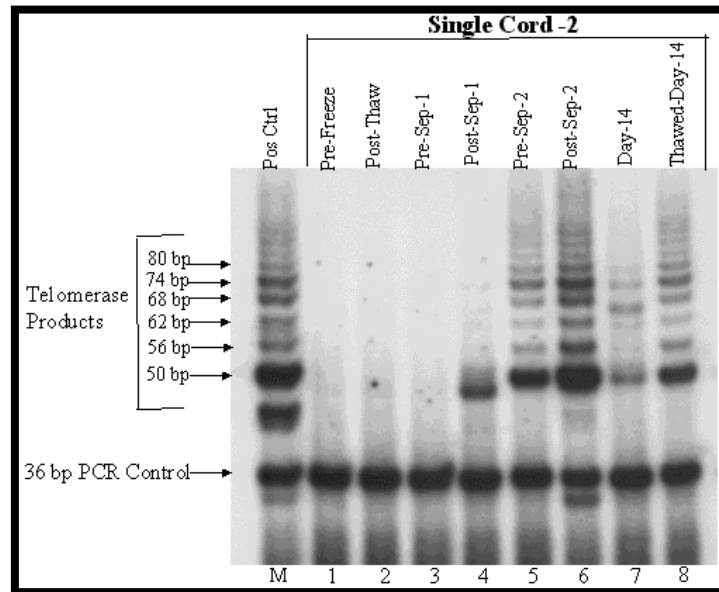


Figure 16. Quantitation of Telomerase Activity in Cord-1. The Y-axis shows values as percent relative to the cancer positive control. The counts per minute are shown on the histobars.

### Telomerase Activity in Cord-2 Time Course

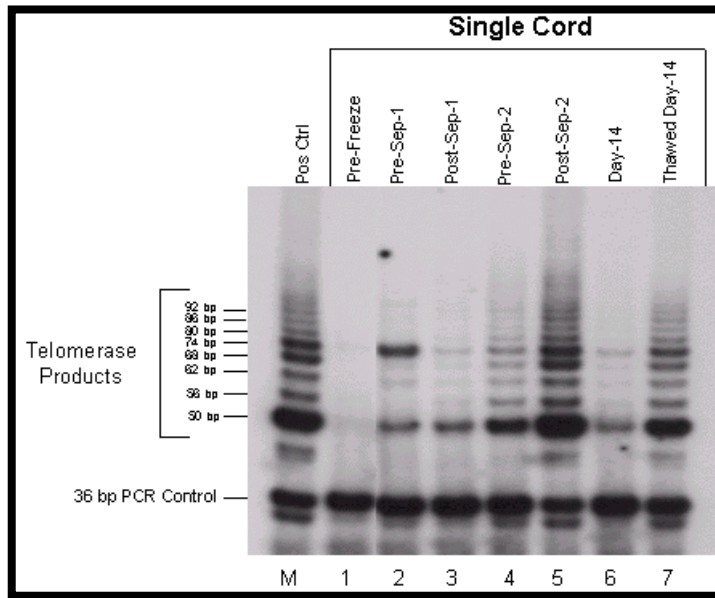
Telomerase activity in cord-2 (Sample # EXPO91001B) reflected the same pattern as in cord-1 under conditions in which the internal control was equally amplified

(figure 17). This particular analysis provided the most extended ladders of this entire thesis.

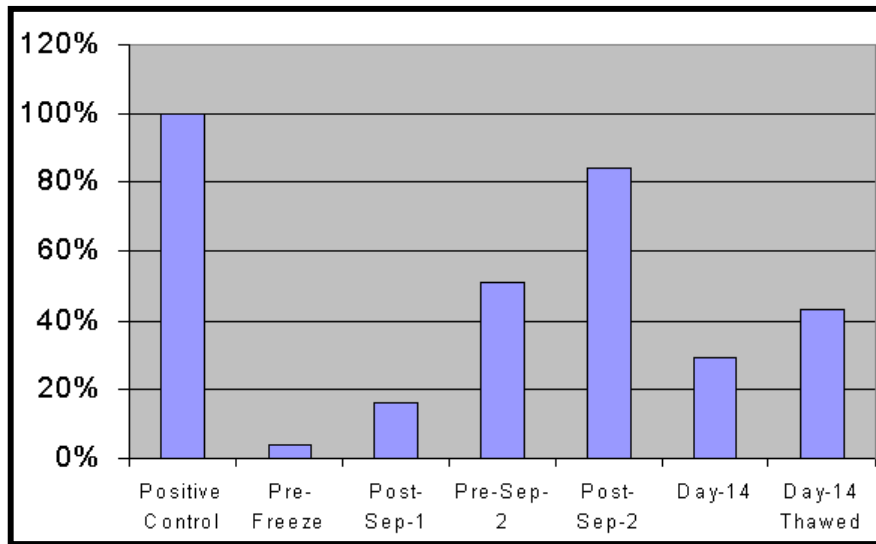


**Figure 17. TRAP Assay on Cord-2, N=1**

Similar results were obtained in Trial 2 for Cord-2 (figure 18), except for the appearance of an unusual band in the Pre-Sep-1 sample (lane 2). Because trial-2 for cord-2 used fresh  $^{32}\text{P}$ , quantitation was performed (fig 19), except on the unusual Pre-Sep-1 sample. The trends reflect what was seen earlier in cord-1. The Post-Sep-2 sample showed a 12x or an 80% increase over fresh cord.



**Figure 18. TRAP Assay on Cord-2, N=2**

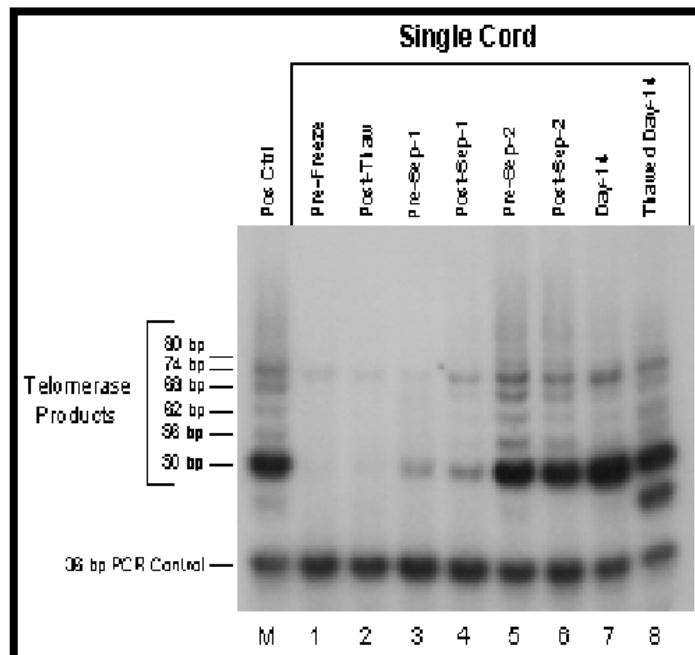


**Figure 19. Quantitation of Telomerase activity in Cord-2.** The Y-axis shows values as percent relative to cancer positive control.

### *Telomerase Activity in Cord-3 Time Course*

Telomerase activity in cord-3 (figure 20) (sample # EXPO91001C) exhibited an identical trend as observed in cord-1 and cord-2 samples. The assay continued to show low intra-sample variability (fig 21), and the quantitation for cord-3 (fig 22) indicated an 8.0x or a 120% increase in telomerase activity for Post-Sep-2 relative to fresh sample.

The above TRAP results show that there is little intra-sample variability in the assay. Although differences were observed between cords regarding the fold-increase in activity, the main trend of telomerase activity observed in the three time courses was identical. Table 4, shows percent CD34+ content and telomerase quantitation of each of the cords tested. Although for each cord tested the highest telomerase activity occurred for fraction containing the highest percent CD34+ cells, a direct correlation was not always observed.



**Figure 20. TRAP Assay on cord-3, N=1**

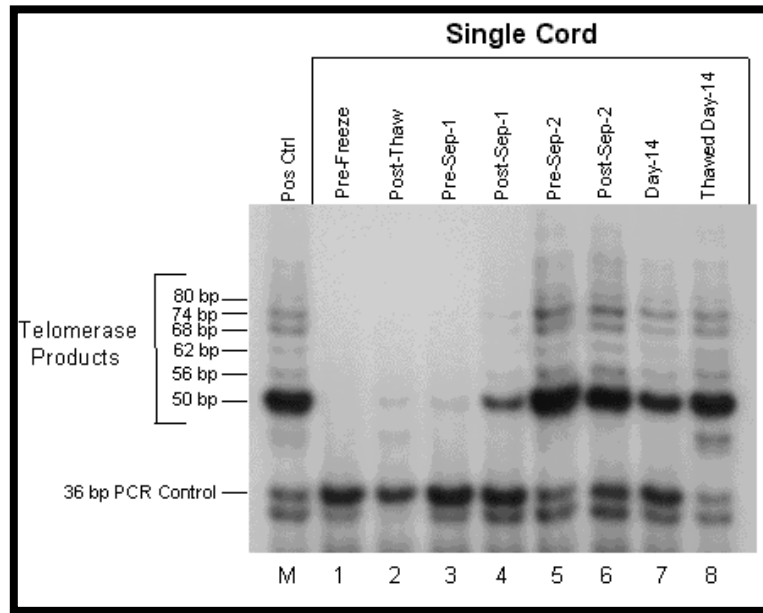


Figure 21. TRAP Assay on cord-3, N=2

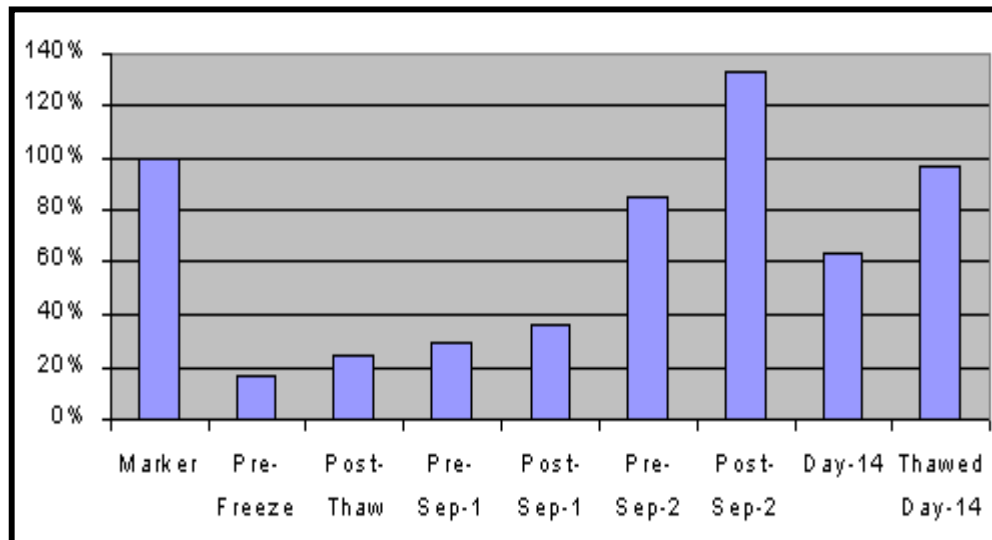


Figure 22. Quantitation of Telomerase activity in Cord-3. The Y-axis shows values as percent relative to cancer positive control.

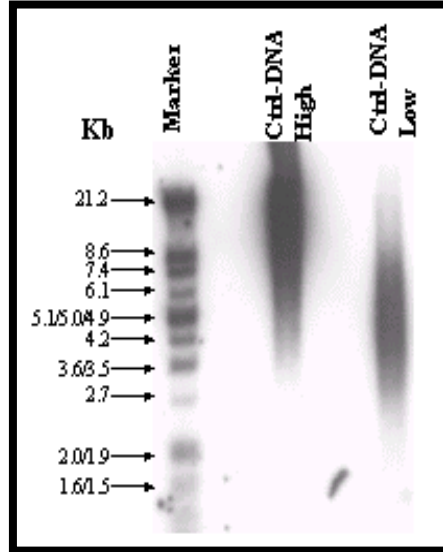
Fraction	CORD-1			CORD-2			CORD-3		
	%CD34+	Percent Telomerase Quantitation	Ratio (%CD34/%Quant)	%CD34+	Percent Telomerase Quantitation	Ratio	%CD34+	Percent Telomerase Quantitation	Ratio
<b>Pre-Freeze</b>	0.13	23	0.005	0.06	4	0.015	0.26	17	0.015
<b>Pre-Sep-1</b>	0.08	30	0.002	0.19			0.17	30	0.005
<b>Post-Sep-1</b>	2.04	33	0.061	3.54	16	0.221	3.2	37	0.086
<b>Pre-Sep-2</b>	22.82	59	0.386	16.79	51	0.329	31.25	85	0.367
<b>Post-Sep-2</b>	23.09	123	0.187	19.91	84	0.237	33.2	133	0.249
<b>Day-14</b>	5.62	73	0.076	7.25	29	0.250	10.53	63	0.167
<b>ThawedDay-14</b>	5.29	100	0.052	7.75	43	0.180	11.54	97	0.118

**Table 4. Shows %CD34+ content and Telomerase Quantitation for each cord.**

### **Telomere Length Assay**

In the second phase of this project, the telomere lengths of two of Viacell's cord populations were investigated via a telomere length assay (TLA). Various methods have been described to detect telomeres and to measure telomere length (Harley, 1995; Lansdorp et al., 1996). The TeloTAGGG Telomere Length Assay (Roche, # 2209136) was chosen as the commercial source. This method utilizes Southern blot analysis of terminal restriction fragments (TRF) obtained by digestion of genomic DNA using frequently cutting restriction enzymes such as Rsa I and Hinf I. The specificity of the enzymes is such that the telomeric DNA (TTAGGG)<sub>n</sub> is not cut. After digestion, the DNA fragments are separated by gel electrophoresis, blotted and the TRFs are visualized by hybridization with DIG-labeled telomere-specific probe. Finally, after exposure of the blot to an X-ray film, an estimate of the mean TRF length was obtained by visually comparing the mean size of the smear to the DIG-labeled molecular weight marker. Telomere length of human cell samples may range over one order of magnitude. Even within a population of cell lines and on a single cell level, considerable heterogeneity of





**Figure 23. Test of Controls for Telomere length Assay.** Shows the DIG-labeled molecular weight marker and two positive control DNAs. The mean TRF length of control-DNA-High and control-DNA-Low has been estimated at 10.2 kbp and 3.9 kbp respectively.

telomere length is observed. Therefore, analyzing a population of cells provides the average telomere length of the telomeres in the sample, indicated by a smear whose average size is compared to the molecular weight marker. TRFs comprise not only the variable terminal telomeres but also a short sub-telomeric region. In addition to a molecular weight marker, two positive control DNAs (Control-DNA-low and Control-DNA-high) obtained from immortal cell lines and supplied with the TeloTAGGG kit were used to compare the mean TRF length of each sample. The mean TRF length of these positive control cell lines has been estimated at 3.9 kb and 10.2 kb respectively. After several false starts with this tricky assay, the controls (fig. 23) produced their expected profiles.

### *Telomere Length in Hematopoietic Cord Cell Populations*

Day-0 and Day-14 samples, representing 2 time-points before and after amplification, were chosen for analysis by the TLA assay. Because  $10^7$  CD45+ cells were required to provide sufficient DNA for analysis, pooled cords (3) were used, and only two time points were analyzed. Day-0 ('Pre-Freeze') samples represent fresh umbilical cord blood CD45+ cells that contain about 0.26% CD34+/CD38- cells. Day-14 cells have undergone two weeks of amplification and two rounds of separation (day 0.5 and day 7.5). These Day-14 samples contained about 10.53% CD34+/CD38- cells (40.5 fold enrichment) as analyzed by FACS (table 4).

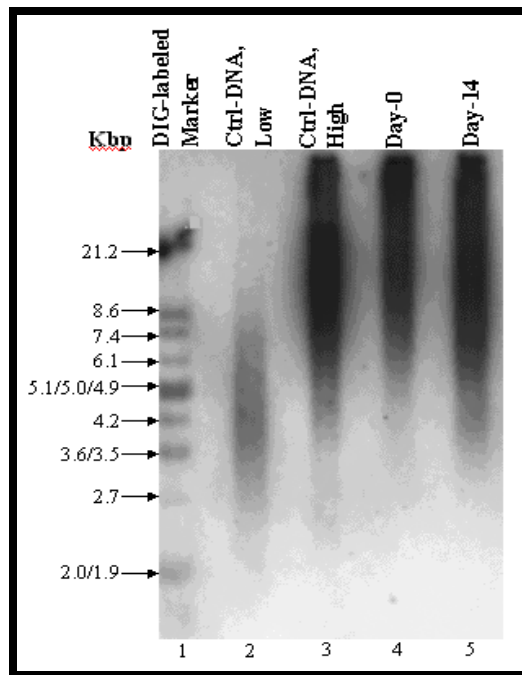
<b>Time Point</b>	<b>% CD34+/CD38- Cells</b>
'Pre-Freeze' (Day-0)	0.26
'Cell Product' (Day-14)	10.53

**Table 5. FACS Analysis of Day-0 and Day-14 Cells used for the TLA**

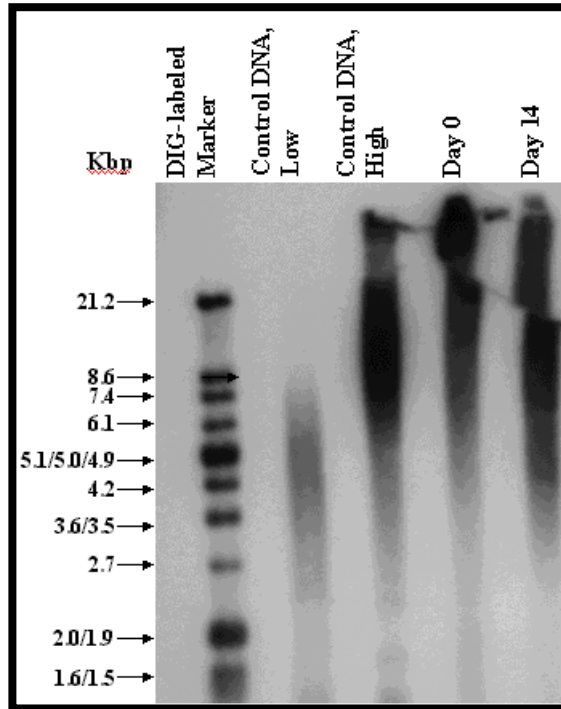
The TLA analysis of the two samples is shown in fig 24. Each of the two cord samples showed TRF smears corresponding more to the high control (lane 3) than the low (lane 2), in agreement with previous studies showing long telomeres in hematopoietic populations. Based on the mean TRF length of the control DNAs (3.9 kb and 10.2 kb) the mean TRF length of the Day-0 sample was approximately 11 kb, and the mean TRF length of Day-14 cells was 9 kb. Thus the telomere length of the Day-0 cells was longer than those of the Day-14 cells by about 2 kb, which is consistent with a population of cells strongly induced towards proliferation for a period of two weeks.

Trial 2 of the TLA analysis of the two samples showed that the results obtained were reproducible (fig. 25).

Thus, despite our detection of telomerase activity at Day-14 compared to none detected at Day-0, an average telomere loss of about 2 kb occurs after the two-week amplification. So, the activity increase is not sufficient for fully maintaining telomere length in the cells pushed towards proliferation. Therefore, the above results show that the presence of telomerase activity does not necessarily correspond to longer telomeres.



**Figure 24. TLA on Cord Samples, N=1.** Lane 1 shows DIG labeled 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.

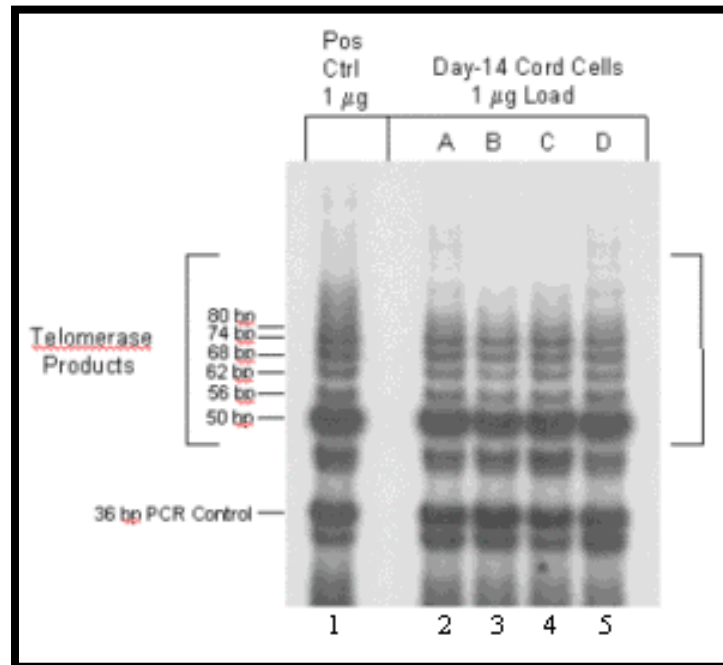


**Figure 25. TLA on Cord Samples, N=2.** Lane 1 shows the DIG labeled molecular weight marker, lane 2 shows control DNA-low, lane 3 shows control DNA-high, lane 4 shows Day 0 telomeric DNA, and lane 5 shows Day 14 telomeric DNA.

*Initial Investigation of Culture Conditions that Could Potentially Alter Telomerase Activity*

Based on previous studies showing that cell populations with elevated telomere lengths and detectable telomerase activity show higher engraftment survival, ViaCell may eventually be interested in exploring various culture conditions that can increase telomerase activity further in their product and therefore compensate for the 2 kb telomere loss observed during the amplification. Two preliminary experiments were performed here. In the first set of treatments (fig. 26), Day-14 cord blood cells were treated as follows A) ½ cord treated with Annexin (to rid apoptotic cells), B) whole cord treated with Annexin, C) ¼ cord treated with 30% BSA (Bovine Serum Albumin) and D)

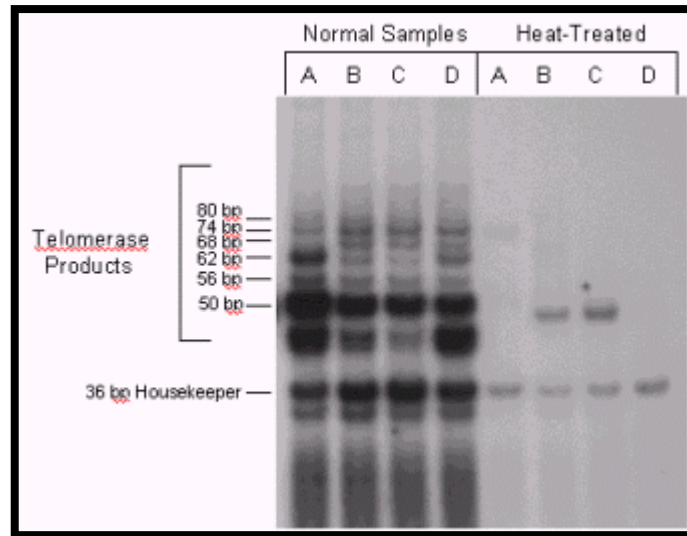
$\frac{1}{4}$  cord treated with 20% HSA gradients (to remove non-viable cells), then were analyzed for telomerase activity. All four culture treatments (lanes A-D) showed equal telomerase activity, which was comparable to the cancer cell extract positive control.



**Figure 26.** Treatment of Day-14 Cord Cells with Annexin, BSA and HSA. Lane 1 shows cancer cell extract positive control. A= $\frac{1}{2}$  cord treated with Annexin, B= whole cord treated with Annexin, C=  $\frac{1}{4}$  cord treated with 30% BSA, D=  $\frac{1}{4}$  cord treated with 20% HSA.

In the second set of treatments (fig. 27), day-14 cord cells were first put through a density centrifugation with 20% HSA (to remove non-viable cells). Samples A and C were  $\frac{1}{2}$  cord cells treated with HSA coated plastics, and samples B and D were  $\frac{1}{2}$  cord cells treated with uncoated plastics. These samples were then analyzed for telomerase activity. As shown in fig 27, both the uncoated and coated plastic pre-treatments showed equal telomerase activity.

Thus no cord treatment was identified in this preliminary analysis that altered the telomerase activity of the day-14 cell population.



**Figure 27.** Day-14 cord cells have undergone density centrifugation with 20% HSA. Coating refers to a brief pre-treatment of the plastics with 5% HSA.

A= with HSA coat of plastics, B= without coat, C= with HSA coat of plastics, D= without coat. These samples were also heat inactivated to serve as negative controls. However, samples B and C were not completely heat inactivated and therefore show faint telomerase activity.

## DISCUSSION

Hematopoietic cell populations showing elevated telomerase activity (Morrison et al., 1996) and elongated telomere lengths (Kobari et al., 2000; Lansdorp et al., 1997; Vaziri et al., 1994; Notaro et al., 1997; Wynn et al., 1998;) display strong engraftment survivability and higher replicative potential in humans during bone marrow transplants. Thus the telomerase activity and telomere length are important parameters to analyze in hematopoietic cell fractions slated for transplant into patients. In this study, we assessed telomerase activity and telomere length in selectively amplified umbilical cord blood (UCB) cell populations prepared using ViaCell's patented stem cell amplification process. The first aim of this project was to assay telomerase activity throughout ViaCell's entire *ex vivo* cell amplification process. The second aim was to analyze telomere length in two of their samples. The third aim was to investigate various culture conditions that could potentially upregulate telomerase activity in ViaCell's final day-14 cell fraction slated for perfusion into a patient.

### *Telomerase Activity Assay*

We have demonstrated using a PCR-based TRAP assay that telomerase activity is undetectable early on in Day-0 ('Pre-Freeze'), 'Post-Thaw' and 'Pre-Sep-1' (Day-0.5) samples), increases following removal of differentiated cells in Post-Sep-1 samples, and increase by as much as 120-fold after 1 week of ex-vivo expansion (as observed in 'Post-Sep-2' (Day-7) samples). Because telomerase activity was relatively high in all fractions induced for proliferation (Pre-Sep-2, Post-Sep-2, Day-14, Thawed Day-14) compared to fresh cord, Post-Thaw or Pre-sep-1, our results agree with others (Engelhardt et al., 1997;

Holt et al., 1996; Zhu et al., 1996) indicating that telomerase is present in rapidly expanding cells. It has also been previously shown that the expression of human telomerase reverse transcriptase (hTERT) was low in freshly isolated cord blood cells, and was significantly increased when these cells were cultured *in vitro* along with optimal cytokines (Ma and Zou, 2001). Reports have previously shown that telomerase is upregulated at cell cycle entry as cells progress through S-phase, and repressed in quiescent G<sub>0</sub> cells Sakabe et al., (1998). Our low activity in the unamplified, unselected, cell population may simply reflect the low abundance of HSCs in this population, or the quiescent primitive nature of these stem/progenitor cells. Telomerase activity increased (in all 3 cords tested) in Post-Sep-1 samples compared to Pre-Sep-1. This increase may simply reflect the removal of Lin<sup>+</sup> cells known to be low in telomerase activity.

After 2 weeks of *ex vivo* expansion, telomerase activity showed a slight decline in all 'Day-14' samples relative to Day-7 (although it was still above fresh cord, Post-Thaw, and Pre-sep-1). This decline may be due to the differentiation of a subset of hematopoietic stem/progenitor cells in the Post-Sep-2 samples into more mature telomerase-low blood cells during the weeklong growth (indicated by the decrease from 33.2% to 10.53% of CD34<sup>+</sup>/CD38<sup>-</sup> cells in the Day-14 sample). These results agree with previous studies showing that the induction of a differentiation program decreases telomerase activity in the hematopoietic sample (Engelhardt et al., 1997).

Surprisingly the telomerase activity was again upregulated after the 'Day-14' samples were frozen for storage and thawed. The reason for this is unknown. However, this observation could be explained in part by an increase in the survival of telomerase-rich cells following freezing, stimulation of an unknown telomerase activator, or



denaturation of a telomerase inhibitor. The increase is not likely due to an increase in percent CD34+ cells in this fraction because the CD34 count goes up by only 1% by FACS analysis.

### *Telomere Length Assay*

In concordance with previous studies (Vaziri et al., 1994; Chiu et al., 1996), we report telomere shortening of hematopoietic cells on proliferation despite the presence of detectable levels of telomerase activity. Southern blot analysis of telomere length in the total nucleated cell population obtained at 2 different time points (Day-0 and Day-14), demonstrated relatively long telomeres in these hematopoietic fractions compared to the DNA from the differentiated cell controls, and a 2 kb loss of telomeric DNA in these cells after 14 days of amplification. These results are consistent with a model in which the upregulation of telomerase activity in the Day-14 sample (compared to fresh cord) is insufficient to maintain telomere lengths following cell amplification. These results agree with previous studies by Engelhardt et al., 1997; Chiu et al., 1996, showing telomere shortening in amplified hematopoietic populations. Direct analysis of telomeres in HSCs by *in situ* hybridization during serial transplantation of murine HSCs also revealed a reduction in telomere size (Allsopp et al., 2001). Telomerase activity is upregulated in primitive hematopoietic cells following their entry into cell cycle, which is sufficient to reduce (not to completely prevent) telomere loss when bulk cell turnover, cell expansion, and massive cell proliferation takes place (Engelhardt et al., 1997).

It has been suggested that most primitive hematopoietic cells lose telomeric DNA at a rate that is roughly comparable to other somatic cells (50-100 bp per doubling)

(Allsopp et al., 1992; Vaziri et al., 1993). From our data it can be inferred that the hematopoietic cell population has undergone around 20 doublings, assuming a constant rate of loss of 100 bp telomeric DNA per cycle. A large body of evidence on telomere length in somatic cells *in vitro* and *in vivo* indicates that telomere length serves as a biomarker of the replicative history of cells (Harley et al 1990; Vaziri et al., 1994). It has been suggested that the replicative senescence within a hematopoietic lineage may be causally linked to functional differences such as a decrease in the production of CD34+ cells, and a decreased proliferation rate of CD34+ cells and those cells responding to a mixture of hematopoietic cytokines (Lansdorp et al., 1993).

The proliferative lifespan of stem cells to sustain hematopoiesis throughout life is not known. Lansdorp et al (1997) propose that HSCs like other somatic cells may have only a limited replicative potential (<100 divisions). This hypothesis is supported by the consideration that, in theory, 55 divisions can yield  $4 \times 10^{16}$  cells, which is about the same as the estimated number of blood cells produced over a lifetime. Lansdorp et al., (1996) have shown that the proliferative potential of most, if not all, HSCs is limited, decreases with age, and correlates directly with telomere length.

Telomerase studies have widespread implications for hematopoietic transplantations, as well as gene therapy. Reports have shown that the proliferative potential of HSCs is decreased after hematopoietic reconstitution of myelo-ablated patients. The mean TRF length was shown to be consistently shorter in the bone marrow transplant (BMT) recipient than in the respective donor. One interpretation of this finding is that the fewer the HSCs transferred to a recipient, the more cell divisions are needed for reconstitution of hematopoiesis. Consequently, a greater consumption of telomeres

takes place. The donor stem cells must presumably undergo a larger number of telomere shortening rounds in the engrafting recipient than have naturally occurred in the donor (Notaro et al., 1997). Similar conclusions were obtained for autologous peripheral blood stem cell transplantation (Lee et al., 1999). Direct analysis of telomeres in HSCs by *in situ* hybridization during serial transplantation of murine HSCs also revealed a reduction in telomere size (Allsopp et al., 2001).

The fate of telomeres may also be crucial for the outcome of gene therapy protocols in which one or few stem cells are expected to repopulate the bone marrow (Notaro et al., 1997). Another factor affecting gene therapy is that hematopoietic engraftment imposes replicative stress on stem cells, resulting in aging effect, which would carry the risk of an increased frequency of clonal hematopoietic disorders during later life. This is particularly important in young recipients with a lifetime of hematopoietic demand before them (Wynn et al., 1998). In this regard, cord blood cells would be a better source for allogeneic transplantation. Studies have shown significant functional differences between UCB and adult bone marrow (BM) and peripheral blood (PB) cells. The UCB cells have longer telomeres compared with PB and BM cells. This suggests that CB has higher replicative potential than adult PB or BM cells, which combined with their greater expansion potential, would support the use of such cells for allogeneic transplantation (Mayani and Lansdorp, 1998).

In conclusion, our TLA and TRAP data support the prevailing hypothesis that telomerase activity in hematopoietic cells reduces rather than completely eliminates telomere loss on proliferation, and may thus help extend the proliferative life span of hematopoietic cells. Reports have shown that the developmental characteristic most

consistently associated with telomerase expression is self-renewal potential (Morrison et al., 1996). Therefore, the relatively high telomerase activity and telomere lengths in the Day-14 samples is encouraging because it provides a new way to validate ViaCell's clonogenic amplification protocol for the cell populations that will be perfused into an immunosuppressed patient and indicates a high self-renewal potential for these cells.

#### *Treatments to Elevate Telomerase Activity*

Different culture conditions and treatments that could potentially elevate telomerase activity were investigated. Day-14 cord blood fractions were treated with annexin, 30% BSA (Bovine Serum Albumin), 20% HSA, or first put through a density centrifugation with 20% HSA and treated with HSA coated plastics. These treatments were performed to remove apoptotic cells or non-viable cells from the population, which are known to be low in telomerase activity. Unfortunately, none of the treatments altered telomerase activity, so perhaps these unwanted cells only constituted a small percent of the population. Viability studies should be performed to ascertain the abundance of these unwanted cells in the population. Therefore in our analysis, no cord blood was identified that could potentially elevate telomerase activity.

#### **Future Investigations**

In future, telomere length should be assessed in all the different time points of ViaCell's amplification process, especially Post-Sep-1 (unamplified, but selected) and Post-Sep-2 (amplified and selected). Alternative methods of telomere length measurement that are less labor intensive than a TLA could be tested, such as a "telomere

amount and length assay” (TALA) (Gan et al., 2001). TALA is based on solution hybridization and does not require blotting, pre-hybridization and washing. Compared to the TLA, one lab claims TALA shows a 4-fold greater sensitivity, >2 fold-higher reproducibility and 4-fold less time requirement (Gan et al., 2001). However at this moment this assay is not commercially available. Other methods such as flow cytometry-based fluorescent *in situ* hybridization (FISH) can also be used for measuring telomere length *in situ*, in single cells. A TelBam8 probe that is unique for the subtelomeric region of the long arm of chromosome 7 can also be used to measure the telomere length of one end of a single chromosome pair. This method reduces the variation size of the telomeric length that is seen in blots hybridized to a (TTAGGG)<sub>n</sub> telomeric repeat probe (Notaro et al., 1997).

The telomerase experiments could 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. Such antibodies have recently become commercially available following the cloning of the hTERT gene by Nakamura et al. (1997). *In vitro* studies with telomerase inhibitors can be conducted to further understand the specific role of telomerase in telomere maintenance in hematopoietic stem cells. Recent studies indicate the existence of an alternative “lengthening of telomeres” (ALT) system in which telomere maintenance occurs in the absence of telomerase activity (Bryan et al., 1997). Previous studies have shown the presence of ALT in yeast, and in a subset of tumors and tumor-derived cell lines (Bryan et al., 1997). This could be investigated by treatment of CB derived cell populations with telomerase inhibitors such as TRF1 and TRF2, differentiation inducing agent “all-trans retinoic acid” (ATRA), and

other putative telomerase inhibitors such as alterperyleneol (a fungus metabolite) (Togashi et al., 1998) or a bis-dimethylaminoethyl derivative of quindoline (an alkaloid from the west African shrub *Cryptolepis sanguinolenta*), which stabilizes the folded G-quadruplex structures and thus inhibit telomerase activity (Caprio et al., 2000). Such studies could shed new light on the means whereby ALT is repressed in normal hematopoietic cells and HSCs.

Finally, since the level of telomerase activity is insufficient to fully maintain telomere lengths in hematopoietic stem cells, perhaps its activity could be increased by transfecting these cells with plasmids encoding the hTERT gene. The expression of the hTERT gene parallels telomerase activity, while the RNA component is ubiquitously expressed in all cells, therefore, such gene therapy could be successful with only the hTERT gene transfection. Perhaps such treated cells would show further elevated telomerase activity which would fully maintain telomere length in spite of the proliferation, and would show increased survivability of the graft in the host.

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