Developmentally Interesting Cytokines Upregulated During Human Hematopoietic Stem Cell Amplification In Vitro

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ABSTRACT

Amplification of hematopoietic stem cells (HSCs) from human cord blood has applications for a variety of cell therapy protocols. The purpose of this thesis (performed in collaboration with ViaCell, Inc.) was to analyze differential gene expression (especially related to cytokines) during the process of human HSC amplification in vitro. When applied to markers previously shown to be specific for HSC's and/or progenitor cells, the analysis validates ViaCell's cellular product. Total cellular RNA was isolated from cord blood samples at various stages of amplification and used to synthesize cDNAs as probes for hybridization arrays. mRNA candidates increased in cell populations enriched for stem cells were first identified using hybridization arrays, then confirmed by RT-PCR. Restriction mapping confirmed RT-PCR amplicons. The results identified several developmentally interesting cytokines (CD117, Jagged-2, Manic Fringe, and Notch) upregulated in stem cell enriched fractions. Analysis of one candidate previously shown to be a marker for HSCs and progenitors, CD117, was extended using Western blots to show a CD117-related protein upregulation. The observed upregulations did not contain many inflammatory cytokines, which could hinder survival of HSC grafts. The future hope for the non-CD117 candidates is as potential growth modifiers for stem cell samples isolated by clonogenic amplification.

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BACKGROUND

What Are Stem Cells?

In 1909, Alexander Maximow was the first person to suggest that there was a hematopoietic stem cell (HSC) with the morphological appearance of a "lymphocyte" capable of migrating through the blood to microecological niches that would allow them to proliferate and differentiate along lineage specific pathways (Fliedner, 1998). However these HSC's were not actually identified until 1984 from human bone marrow. Curt Civin, professor of Oncology and Pediatrics at John Hopkins University, developed in 1984 (Civin and Loken, 1987) the CD34 monoclonal antibody that makes it possible to identify, isolate, and collect hematopoietic stem cells (Rienzi, 1999). Prior to Civin's discovery in 1984, doctors harvested patient's bone marrow before chemotherapy and then returned it to the patient's body. Unfortunately, this method easily spread the cancer to the rest of the body since the transplanted bone marrow still contained cancerous cells. However, using the CD34 antibody to bind stem cells, followed by FACS to remove these "stained" cells, a patient's noncancerous stem cells can be harvested from the marrow and introduced back into the patient, in turn renewing the body's blood and immune system (Rienzi, 1999).

Stem cells are characterized by their capacity for extensive proliferation and differentiation (de Wynter et al., 1998), as they have the ability to divide for indefinite periods in culture and can give rise to specialized cells (NIH, 2000).

> As stem cells proliferate, they give rise to lineage-committed cells with more proliferative potential, which in turn mature to functional end cells (de Wynter et al., 1998).

Figure 1 shows the most important scientific breakthrough of the 20th century, the discovery of the stem cell.

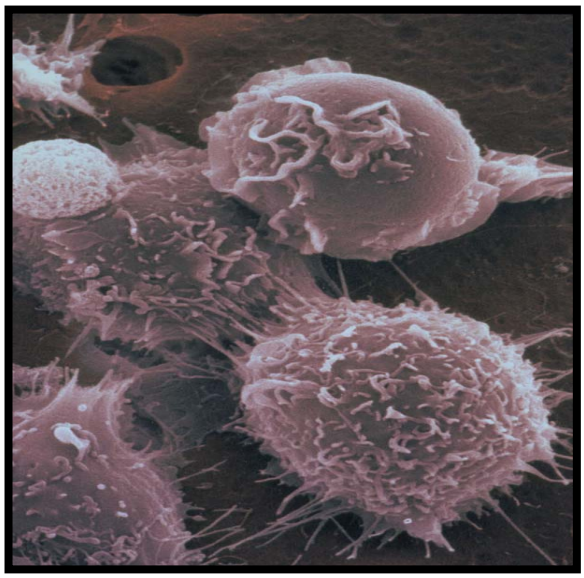

Figure 1. A microscopic look at a hematopoietic stem cell (McLaren, 2001).

Levels of Stem Cell Potency

During normal human development, when a sperm fertilizes an egg, it creates a new cell that has the potential to form an entire human being, as seen in Figure 2. This fertilized egg is totipotent, which means that it has the capacity to specialize into extraembryonic membranes and tissues, the embryo, and all postembryonic tissues and organs (NIH, 2000).

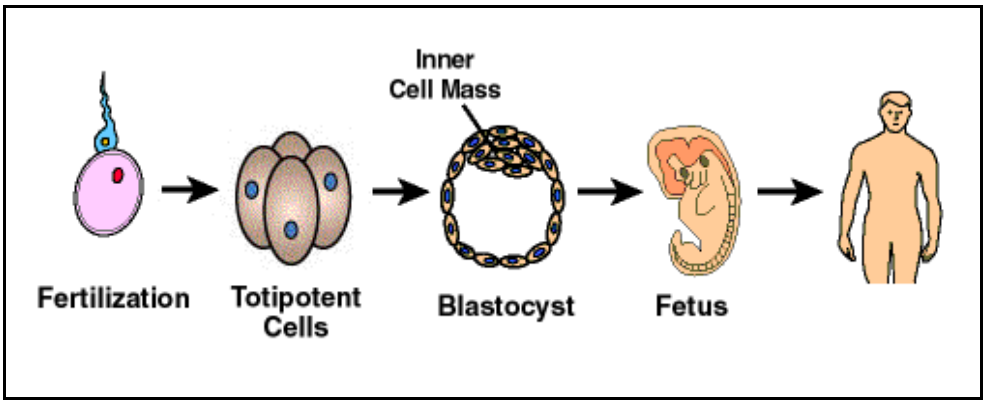

Figure 2. Summary of stem cell development into a human being.

(NIH, 2000) This diagram displays the development of totipotent cells into an adult human being. The fertilized egg is itself totipotent and will eventually give rise to specific cells and tissues.

About four days after fertilization, these totipotent cells begin to form a hollow sphere of cells called a blastocyst (NIH, 2000). This blastocyst has an outer layer of cells and an inner cell mass (middle diagram, figure 2). These inner cell mass cells are pluripotent, which means they are capable of giving rise to most tissues of an organism (NIH, 2000) but they do not have the ability to develop into a fetus. Pluripotent cells undergo further specialization into multipotent stem cells that are committed to give rise to cells with a particular function (NIH, 2000). The more specialized multipotent stem cells can be found in adult tissue (NIH, 2000). These stem cells (including HSCs as an example) are needed to replenish older cells that are continuously dying off, such as skin and blood cells. Figure 3 shows the levels of differentiation competence of various stem cells.

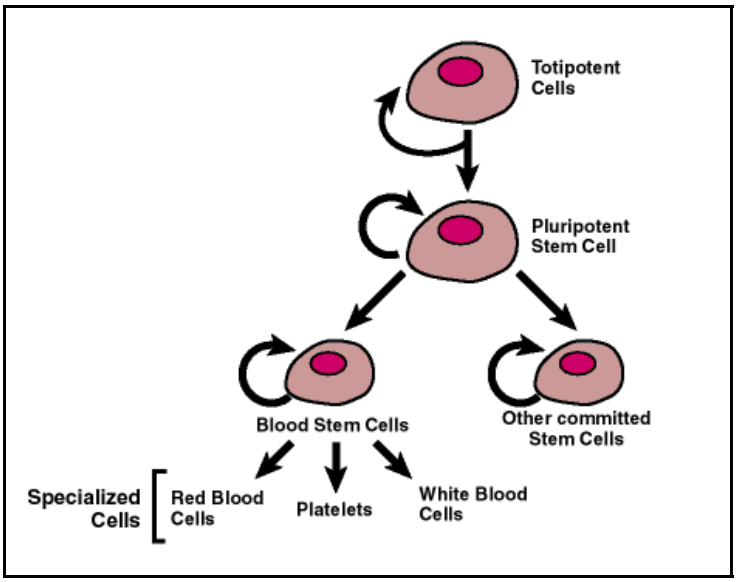

Figure 3. Levels of stem cell differentiation.

(NIH, 2000) This diagram displays the various competence levels of totipotent cells, pluripotent cells, and multipotent stem cells.

Stem Cells and Hematopoiesis

One example of a multipotent stem cell is the hematopoietic stem cell (HSC). HSCs are the source for red blood cells, white blood cells, and platelets (t. Breeders, 2000). HSCs are usually found in bone marrow, umbilical cord blood, and in the peripheral blood if they are stimulated to be released from the bone marrow by factors such as granulocyte colony-stimulating factor (G-CSF) (t. Breeders, 2000). However, the HSC can differentiate into myeloid stem cells and lymphoid stem cells (Brown, 1996), which define two basic pathways (Figure 4).

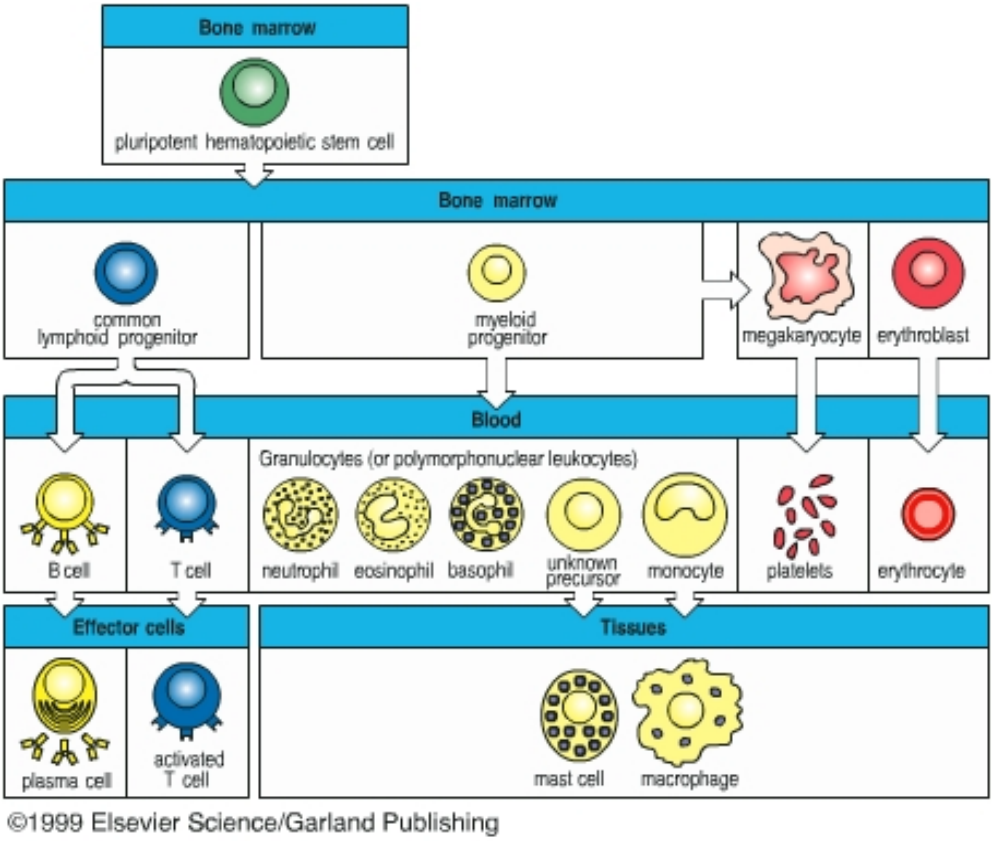

Figure 4. Hematopoiesis proliferation and differentiation pathways.

This diagram shows the differentiation of a hematopoietic stem cell by two major pathways, lymphoid and myeloid (Capra et al., 1999).

Myeloid stem cells further differentiate into leukocytes, erythrocytes, and megakaryocytes. The erythroid line gives rise to red blood cells (Sullivan, 2000). The megakaryocytic line provides blood platelets, which are the primary defense against hemorrhage, and maintaining and repairing the endothelium (Sullivan, 2000). The phagocytic line divides into two subdivisions, the monocytic pathway (which gives rise to blood monocytes) and the granulocytic pathway (which gives rise to neutrophils, eosinophils, and basophils) (Sullivan, 2000). Lymphoid progenitor cells give rise to T- and B-lymphocytes (Capra et al., 1999). Many cytokines are produced by the T-lymphocyte cell lineage (Babakhanian, 1995).

The T- and B- lymphocytes are distinguished by their sites of differentiation- T cells in the thymus and B cells in the bone marrow – and by their antigen receptors as seen in Figure 5. B-lymphocytes differentiate on activation into antibody-secreting plasma cells, and T-lymphocytes differentiate into cells that can kill infected cells or activate other cells of the immune system (Capra et al., 1999).

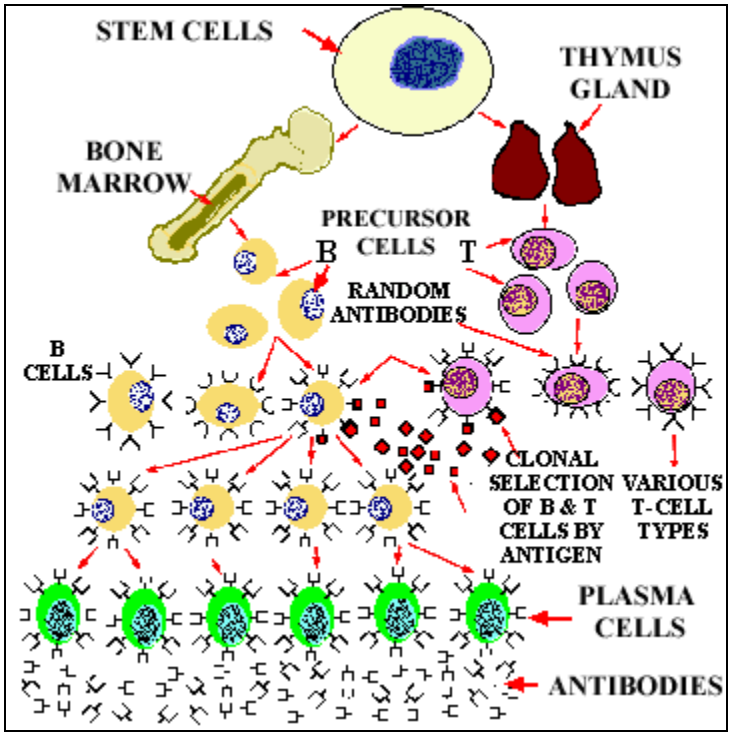

Figure 5. Lymphocytes and Immunity.

Lymphocytes are made from bone-marrow stem cells, and then processed -- B cells in the bone marrow and T cells in the thymus (Hurlburt, 1999).

How are Stem Cells Identified?

The CD34 antigen is a defining cell surface marker of hematopoietic stem/progenitor cells (HSPC). It is expressed in virtually all human hematopoietic progenitors and their precursors, including most (if not all) mammalian stem cells (Opie et al., 1998). It has been suggested that this cell surface molecule functions as a regulator of hematopoietic cell adhesion to stromal cells of the hematopoietic environment (Healy

et al., 1995). The frequency of CD34+ cells in adult bone marrow has been estimated to be 1%-3% of all nucleated cells (Civin and Loken, 1987), and 1% in umbilical cord blood (UCB) (Kinniburgh and Russell, 1993).

The vast majority of UCB CD34+ cells coexpress FLT3, the receptor for the early-acting cytokine FLT3-ligand (FL). However, HSPC lack the expression of both HLA-DR and CD38 antigens. This differs from adult bone marrow, where the majority of CD34+ cells express both HLA-DR and CD38 antigens. The frequency of CD34+HLA-DR- and CD34+CD38- cells in UCB are higher than in adult bone marrow, suggesting that UCB possesses a higher proportion of immature HPC than human bone marrow. Other antigens also found on the UCB 34+ cells are lymphoid- and myeloid-associated antigens, CD13, CD58, and CD44. Figure 6 displays the steps of t-cell development and the key antigens involved in the process (Piascik, 2001).

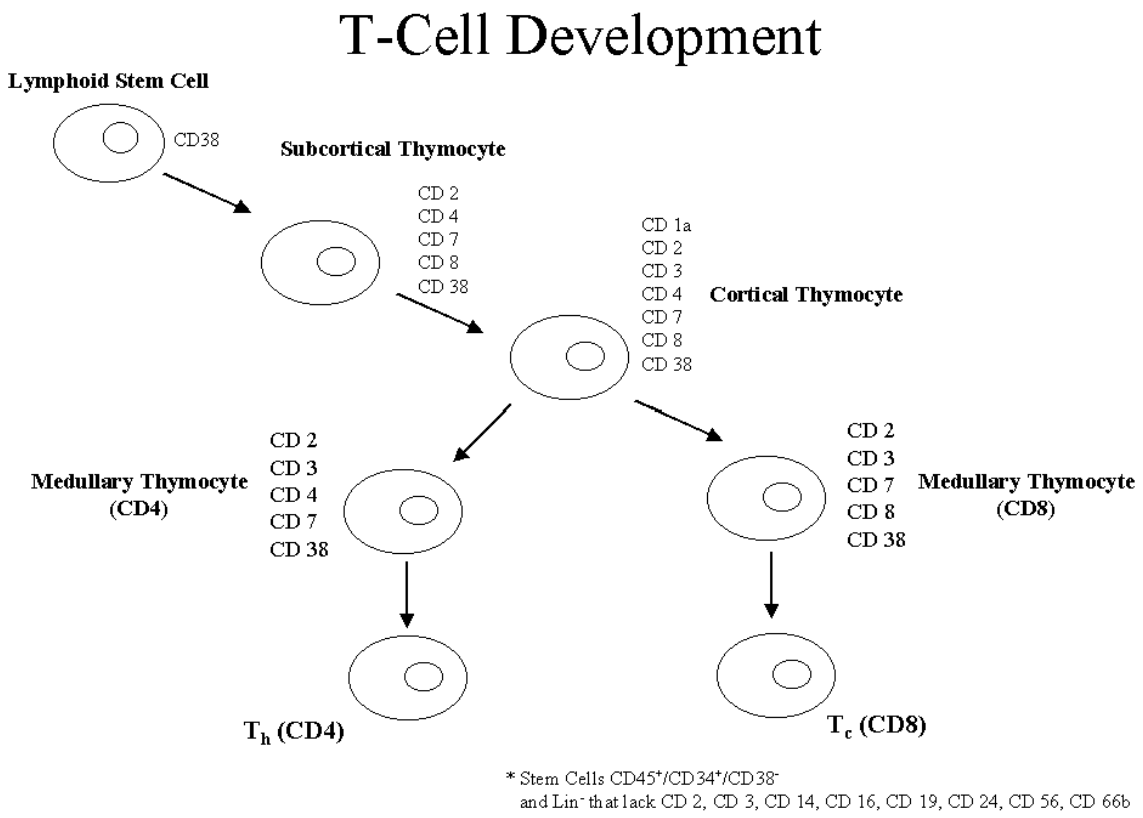

Figure 6. T-cell development and the key antigens involved.

From Where Can Hematopoietic Stem Cells Be Isolated?

Hematopoietic stem cells can directly be obtained from the bone marrow of a patient using a general anesthetic. Indirectly, stem cells can be increased in the marrow (and spill over into the peripheral blood) through various treatments, including the use of growth factors (CancerBACUP, 1999).

Since 1989, umbilical cord blood has proven to be a rich source of hematopoietic stem/progenitor cells (Broxmeyer et al., 1989) as an alternative source to bone marrow (Kim et al., 1999). It was found that the frequency of progenitors in UCB equals or exceeds that of marrow, and greatly surpasses that of adult blood (Broxmeyer et al., 1989). Their work also included examining the prospect of storing the UCB for future

research or medical use. Their results suggested that the UCB from a single donor could serve as a good source of autologous or major histocompatibility complex-matched allogenic transplantable hematopoietic repopulating cells (Broxmeyer et al., 1989).

HSCs can be isolated from umbilical cord blood after the birth of a newborn baby. The blood is collected, treated, cultured, and counted for use in the laboratory or for cryopreservation (Rubinstein et al., 1995). *Knudtzon's* experiments (1974) were the first to demonstrate the presence of UCB stem cells. He found that when human UCB and peripheral blood cells are cultured, increased concentrations of colony-forming cells are seen in the UCB cultures (Knudtzon, 1974). The presence of colony-forming cells in culture are a good indication that stem cells are present in the original cell population that can divide and proliferate in culture. He also demonstrated the presence of relatively immature hematopoietic progenitor cells in human cord blood (Knudtzon, 1974). However, it was not until 1989 that UCSCs were used in clinical settings (Mayani and Lansdorp, 1998). That same year, Gluckman et al. (1989) reported the first hematopoietic cell transplant in which umbilical cord blood was used instead of bone marrow. These scientists were also instrumental in work pertaining to cryopreservaton of UCB cells for later use in transplantation from both genetically related and unrelated patients (Gluckman et al., 1993). Survival following cryopreservation of UCB cells was found after they assessed the hematopoietic reconstitution in a boy with Fanconi's anemia who received cryopreserved UCB from his sister (Gluckman et al., 1989). Mayani and Lansdorp (1998) cultured individual UCB samples supplemented with different cytokine combinations and found that cytokines play a permissive role in hematopoietic cell commitment.

What is Cellular Medicine?

Stem cells can be used to reconstitute healthy cells and tissues needed for medicinal purposes through a concept called cellular medicine. The aim of this type of therapy is to replace, repair or enhance the important biological functions of tissues and cells that have been damaged by cancer, trauma, etc (Gage, 1998). By isolating the multipotent stem cells of different organs, transplantation can be performed to a target organ to induce healthy function again (Gage, 1998). Several cell sources are outlined in Table 1.

Cell Source	Advantages	Disadvantages	Solution
Autologous (same patient)	Immunologically privileged; No ethical issues	Limited supply; Time constraints for donor and host	Cryopreserve; Amplify in vitro
Allogeneic (same species)	Greater supply; Fewer time constraints on donor	Cellular immunity; Ethical issues: fetal tissues	Immunosuppress; Encapsulate
Xenograft (different species)	Greater supply; No time constraints on donor	Cellular and humoral immunity; Possible transfer of new virus across species	Immunosuppress; Encapsulate; Genetically mask immunity
Cell line (immortalized or tumorigenic)	Infinite supply; No time constraints for donor or host; Safety test and standardization simplified	Cellular and humoral immunity; Tumorigenicity and neoplasia	Immunosuppress; Encapsulate; Genetically mask immunity

Table 1. Cell source considerations (Gage, 1998). The table outlines the advantages and disadvantages of individual cell sources from which multipotent stem cells can be isolated.

This stem cell method of transplantation is needed because many diseases result from the disruption of cellular function or destruction of tissues of the body (NIH, 2000). Currently, organ and tissue transplantation is performed to replace damaged parts of the body (for example, the liver, heart, etc.), but the need is much greater than the supply.

> Hematopoietic stem cell transplantation is effective as a treatment for selected high-risk patients with hematological malignancies or solid tumors treated with high-dose chemotherapy. Over the past decade,

several advances have been made in the collection and manipulation of hematopoietic progenitor cells for transplantation (Gage, 1998).

Figure 7 demonstrates the promising stem cell technology that will eventually affect all areas of medicine.

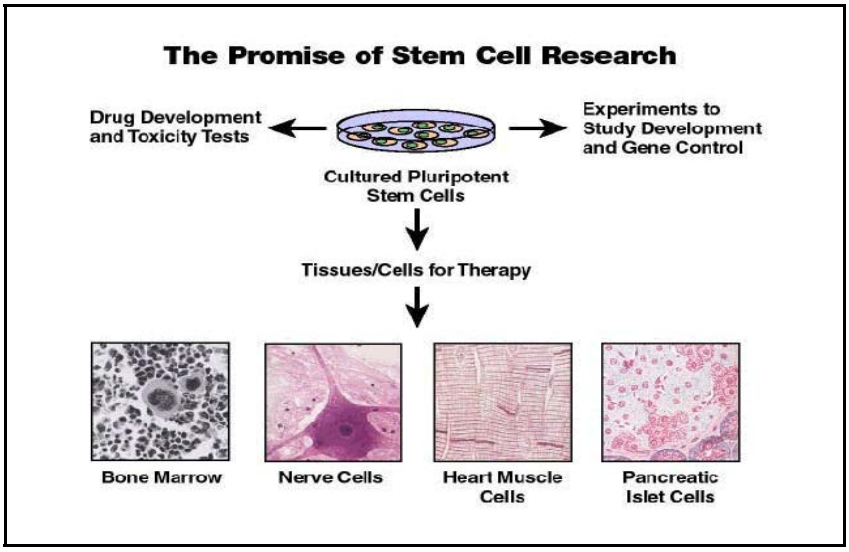

Figure 7. The promise of stem cell research.

(NIH, 2000) This figure displays the possible uses of stem cells as cellular medicine.

HSCs are destroyed during cancer therapy. This destruction represents a major source of cancer treatment morbidity and mortality (t. Breeders, 2000). Therefore, the replacement of HSCs is crucial after an individual has undergone radiation or chemotherapy. In stem cell replacement therapy, HSCs are isolated from the patient's own bone marrow or peripheral blood prior to chemotherapy, from a histocompatible individual, or from blood taken from an umbilical cord at birth (t. Breeders, 2000). These HSCs are amplified then injected back into the patient.

The principal limitations of stem cell replacement therapy using HSCs isolated from the bone marrow are histocompatibility differences, and cell numbers. It is

important for the number of HSCs transplanted to be sufficient to provide a good source of blood cells for the patient, and to minimize the time to repopulate the patient's depleted bone marrow.

The first allogeneic UCB transplant was performed in 1988 on a patient with Fanconi's anemia (Gluckman et al., 1989). Today the patient is doing well, with full cell reconstitution. In this historical case, the UCB was collected at birth, cryopreserved, and transplanted after thawing, without losing its repopulating ability (Gluckman et al., 1989). In 1997, Broxmeyer and Cooper determined that UCB cells cryopreserved for up to 10 years have a high efficiency recovery of immature and mature progenitors (Broxmeyer and Cooper, 1997). To date, hundreds of UCB transplants have been performed, and the data was reported to the Eurocord and Netcord Registry. Data has been obtained from 527 UCB transplants at 121 transplant centers in 29 countries (Gluckman, 2000). The results from these clinical analyses were evaluated from related donors in 138 cases, and unrelated in 399 cases, which ultimately showed that there is a definite promise in UCB transplantation. It is important to obtain $3x10^7$-nucleated cells per kilogram for a successful transplantation (Gluckman, 2000).

The success and advantages of using UCSCs for transplantation include the ease of procurement, no risk to donors, larger donor pool, less risk of transmitting infection or viral transmission, prompt availability of cryopreserved samples, and low incidence of complications due to graft-vs-host disease (GVHD) (Gluckman, 2000). These advantages make stem UCSC replacement therapy a promising procedure in the future of medicine.

Thus far, multipotent stem cells have not been found for all adult tissues present in the body and are very rare. Tissues that have identified stem cells display only minute quantities within the tissue, which makes it difficult to isolate and purify the stem cells. Recently, a population of neural stem cells (NSCs) was found to be present in the adult mammalian central nervous system (Rietze, 2001). Rietze and colleagues isolated the NSC, but its precise identity, location, and potential still remain unclear (Rietze, 2001). This limits usage of these cells in therapeutic cases. Since stem cells have a unique reconstituting ability, they are also an important target for genetic-based therapies for the treatment of hematopoietic disorders (Rosu-Myles et al., 2000).

Cytokines and Their Effect on Inflammation

The term cytokine is used as a generic name for proteins released by cells that affect the behavior of other cells. These proteins serve as a means of communication between cells to modify the functional activities of individual cells and tissues (Capra et al., 1999). Cytokines attach to receptors on the outside of cells causing the target cell to produce a certain reaction, depending on the cell and the cytokine (Babakhanian, 1995). Table 2 displays a list of all known cytokines found to date and their functions.

Family	Cytokine	Actions
Hematopoietins	Epo	Stimulates erythroid progenitors
	IL-2	T-cell proliferation
	IL-3	Synergistic action in early hematopoiesis
	IL-4	B-cell activation, IgE switch, supresses TH1 cells
	IL-5	Eosinophil growth, differentiation
	IL-6	T- and B-cell growth and differentiation, acute phase protein production, fever
	IL-7	Growth of pre-B cells and pre-T cells
	IL-9	Mast cell enhancing activity
	IL-11	Synergistic action with IL-3 and IL-4 in hematopoiesis
	IL-13	B-cell growth and differentiation, inhibits macrophage inflammatory cytokine production and TH1 cells
	G-CSF	Stimulates neutrophil development and differentiation
	IL-15	IL-2-like, stimulates growth of intestinal epithelium, T cells, and NK cells
	GM-CSF	Stimulates growth and differentiation of myelomononocytic lineage
	OSM	Stimulates Kaposis's sarcoma cells, inhibits melanoma growth
	LIF	Maintains embryonic stem cells, like IL-6, IL-11, OSM
	SCF	Mast/stem cell growth factor
Interferons	IFN-gamma	Macrophage activation, increased expression of MHC molecules and antigen processing components, Ig class switching
	IFN-alpha	Anti-viral, increased MHC class I expression
	IFN-beta	Anti-viral, increased MHC class I expression
Immunoglobulin superfamily	B7.1	Co-stimulation of T-cell responses
	B7.2	Co-stimulation of T-cell responses
TNF family	TNF-alpha	Local inflammation, endothelial activation
	TNF-beta	Killing, endothelial activation
	LT-beta	Lymph node development
	CD40 ligand	B-cell activation, class switching
	Fas ligand	Apoptosis, Ca2+-independent cytotoxicity
	CD27 ligand	Stimulates T cell proliferation
	CD30 ligand	Stimulates T and B cell proliferation
	4-1BBL	Co-stimulates T and B cells
Unassigned	TGF-beta	Inhibits cell growth, anti-inflammatory
	IL-1alpha	Fever, T-cell activation, macrophage activation
	IL-1beta	Fever, T-cell activation, macrophage activation
	IL-1 RA	Binds to but doesn't trigger IL-1 receptor, acts as a natural antagonist of IL-1 functions
	IL-10	Potent suppressant of macrophage functions
	IL-12	Activates NK cells, induces CD4 T-cell differentiation to TH1-like cells
	MIF	Inhibits macrophage migration, stimulates macrophage
	IL-16	Chemoattractant for CD4 T cells, monocytes and eosinophils, anti-apoptotic for IL-2-stimulated T cells
	IL-17	Induce cytokine production by epithelia, endothelia, and fibroblasts
	IL-18	Induces IFN-gamma production by T cells and NK cells, favors TH1 induction

Table 2. Cytokines. A list of currently known cytokines and their functions (Capra et al., 1999).

By interacting through a series of cascades, many cytokines induce other cytokines to be secreted (Giasuddin et al., 1997). Whether a cytokine's effect is local or more distant is likely to reflect the amounts released, the degree to which this release is focused on the target cell, and the stability of the cytokine *in vivo* (Capra et al., 1999). Cytokines have powerful effects on hematopoietic stem cells. For example, cytokines such as Interleukin 4 (IL-4) and IL-5 are responsible for the differentiation of individual HSCs into other cell types, such as lymphocytes, platelets, neutrophils and macrophages (Brown, 1996). Other factors that control the migration, proliferation and differentiation of stem cells are also induced by cytokines (Rosu-Myles et al., 2000). The ability of hematopoietic progenitor cells to expand in vitro depends on the kind of cytokines present (Mayani and Lansdorp, 1998). Several cytokines have been linked to the proliferation of umbilical cord blood-derived cells, but the best stem cell amplification results have been obtained when cytokines are used in combinations that include early-acting factors, such as stem cell factor (SCF), FLT-3 ligand (FL), and thrombopoietin (TPO) (Mayani and Lansdorp, 1998).

What is Expansion Technology?

The successful proliferation and expansion of a HSC depends on whether the cell is already committed to a specific lineage of differentiation, and if so, the specific hematopoietic lineage to which it belongs and its stage of maturation (Mayani and Lansdorp, 1998). In order for the cell to exhibit the potential it needs for expansion, it needs specific factors that would normally make up the microenvironment in which the cell develops. HSC proliferation and expansion in vitro depends on variables that include

non-HSC cell types, cytokines that form the microenvironment, the type of culture medium, medium change schedule, temperature, presence or absence or serum, etc.

Expansion technologies include using CD34 enrichment, which leads to a significant increase in the expansion of $CD34^+$ cells compared with unmanipulated human UCB cells (Kohler et al., 1999). This type of enrichment displays optimum results when a combination of SCF, FLT-3L (at 300 ng/ml) and IL-3 (at 50 ng/ml) is included (Kohler, 1999). *Hunnestad et al.* (1999) found that adding Thrombopoietin (TPO), an established powerful stimulant of megakaryocyte differentiation and platelet production both in vivo and in vitro, to combinations of other factors causes a significant increase in $CD34^+$ cells cultured for 14 days (Hunnestad et al., 1999).

Gilmore and colleagues at the Western Pennsylvania Cancer Institute, found that stimulating the UCB cells, ex vivo, with a combination of two growth factors, flt-3 ligand (FL) and thrombopoietin/c-mpl ligand (TPO/ML) caused the UCB-HSC cells to expand (Gilmore et al., 2000). Brugger et al. cultured cells for 12-14 days in a combination of SCF, IL-1, IL-3, IL-6, and EPO, which produced a population of cells with the ability to restore neutrophils and platelets rapidly in patients treated with high-dose chemotherapy (Brugger, 2000).

The optimal choice of factors in the expansion of UCB-HSCs has not yet been determined, but the findings of these and other researchers are proving to be important to the final goal of obtaining the amount of cells needed for successful transplantations. The work that already has been done has lead hematologists to explore ex vivo expansion for clinical use.

ViaCell's Research Interests

ViaCell, Inc. is a new cellular medicine company merged from two companies: Viacord, Inc. in Boston, Massachusetts, and t. Breeders, Inc. in Worcester, Massachusetts. Viacord specializes in storing cord blood stem cells from the umbilical cords of newborns for future use within that family. t. Breeders is a biotechnology company with proprietary technology for expanding UCSCs. The merger of these two companies made ViaCell's mission:

> To provide pharmaceutical grade cellular medicines for the treatment of human diseases, such as cancer, certain genetic disorders, organ transplant tolerance, and autoimmune diseases. "The research and development arm of t. Breeders combined with the commercialized service of Viacord will catalyze the development and delivery of new cellular medicines to the marketplace," said Morey Kraus, Chief Technical Officer of ViaCell (Craig, 2000).

ViaCell has an Investigational New Drug (IND) application approved by the FDA and is initiating a Phase I/II clinical trial for testing of its proprietary selective amplification technology.

ViaCell's Selective Clonogenic Amplification™

ViaCell's Expansion Technology involves amplifying the number of stem cells present in a given sample to end up with a higher yield. t. Breeders (now ViaCell) has developed a process called *Selective Clonogenic Amplification™*, which 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 creating culture conditions that foster the outgrowth of stem cells. In the strict sense, t. Breeders' *Selective Clonogenic Amplification* 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).

This amplification process uses a sample of bone marrow, peripheral blood, or umbilical cord blood from either the patient (autologous) or a donor (allogeneic) (t. Breeders, 2000). The features of this system include:

- Removal of differentiated cells and their by-products during cell culture;
- Production of highly defined target cell populations;
- Active purging of co-isolated cancer cells; and,
- Efficient, cost-effective production (t. Breeders, 2000).

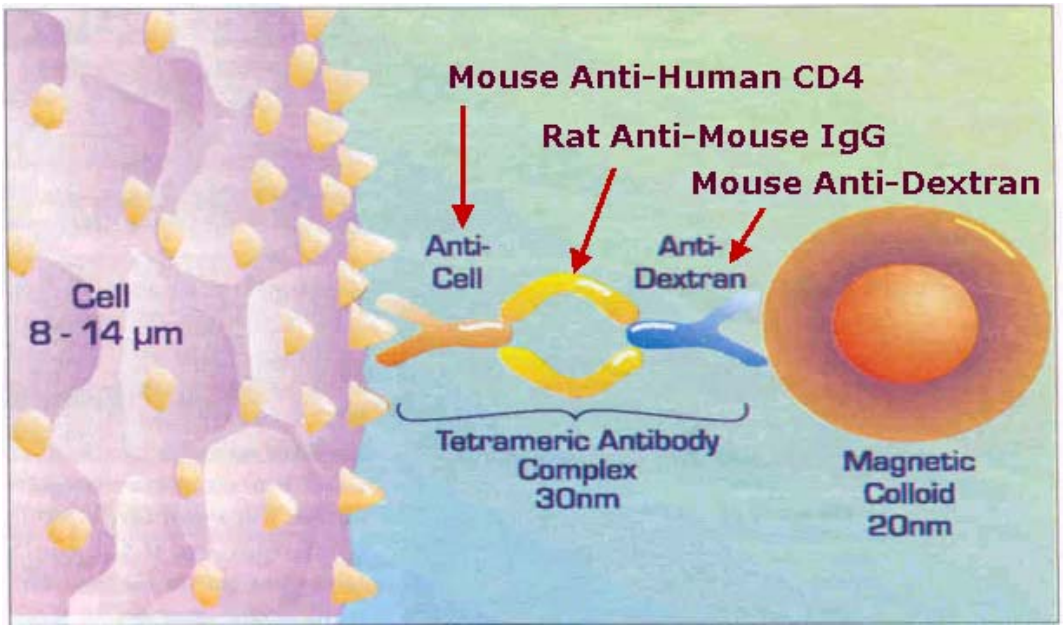

Figure 8. Negative Selection Process.

(Zimmerman, 1998) Shown is the removal of unwanted $CD4^+$ T-cells.

This technique (shown in Figure 8) is especially needed for stem cell replacement therapy (SCRT). It uses a cocktail of antibodies that binds to mature and differentiated surface cell markers from the mononuclear cell population (Piascik, 2001). A subpopulation of cord blood mononuclear cells termed $CD34^+/CD38^-/Lin^-$ cells that is believed to contain stem cells results from this separation process. These Lin^- cells are defined as those that lack CD2, CD3, CD14, CD16, CD19, CD24, CD56, CD66b, and glycophorin A. These markers are expressed on the surface of mature red blood cells,

monocytes, natural killer cells, and T-cells. The hope of ViaCell, Inc. is that these $CD34^{+}/CD38^{-}/Lin^{-}$ cells are in fact primitive hematopoietic stem cells completely depleted of mature T-cells, which can cause GVHD. The FDA has approved the initiation of clinical trials for engraftment into human cancer patients. ViaCell's 14-day expansion process is shown in Figure 9. After amplification of the UCSC's from one cord, ViaCell believes that these cells now have the capability of reconstituting a 200-kg person(s), instead of a 20-kg child before amplification; this needs to be proven clinically.

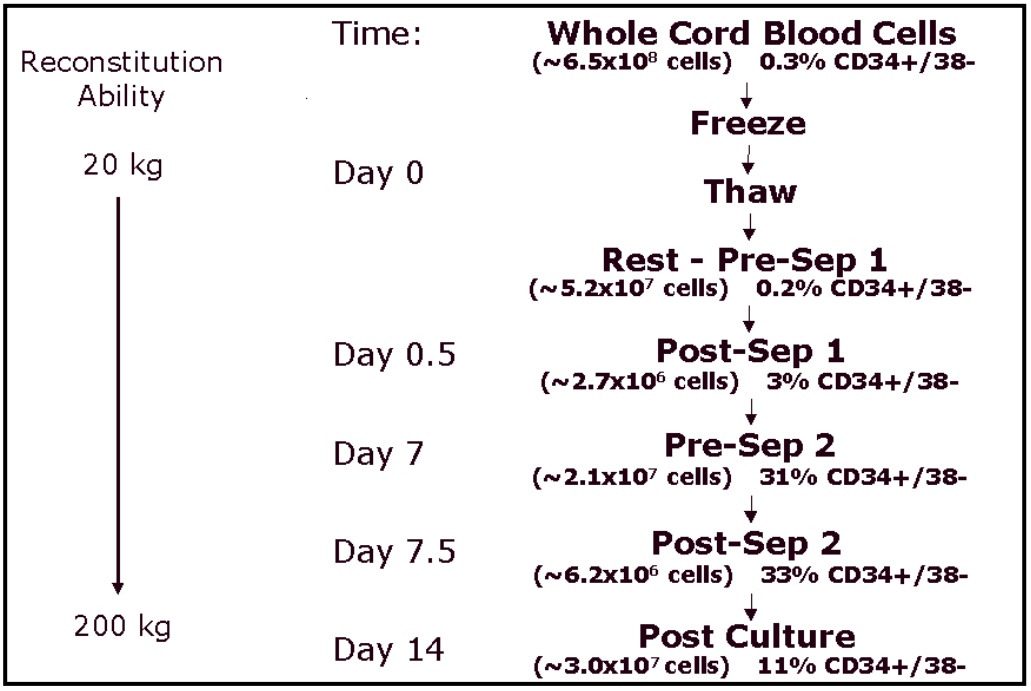

Figure 9. UCB cell selection and amplification process time course.

PROJECT PURPOSE

The purpose of this thesis was to use molecular analyses to analyze gene expression patterns in ViaCell's cellular products. When the RNA analyzed was previously known to be a marker for HSCs (i.e. CD117) the analysis provides a new way to validate ViaCell's product. For other RNAs shown to be elevated in cell populations enriched for HSCs, the analysis provides the identification of new potential HSC markers, or growth factors that can stimulate HSC growth during amplification. Umbilical cord blood samples from all stages of the amplification process were obtained from ViaCell and used to isolate total cellular RNA. mRNA candidates increased in cell populations enriched for stem cells were first identified using hybridization arrays then confirmed by RT-PCR. Restriction mapping confirmed RT-PCR amplicons. Finally, one specific HSC marker, CD117, was further investigated using Western blots.

The identification of new markers for HSCs besides $CD34^+$ is important to further validate cell products to be perfused into a patient. A molecular analysis also offers the potential to identify new HSC markers or growth factors that would not necessarily be detectable in whole UCB.

Introduction to Atlas Arrays

Atlas Arrays (Clontech) are a type of microarray, specially designed to perform hybridization experiments to enable gene profiling. The history of microarrays began 25 years ago with the Southern blot, a technique which introduced the principle of anchoring nucleic acids to a solid support for analysis by hybridization, and the use of the blots to screen filter lifts of bacterial clones (Qiagen, 2000). Nylon-filter-based arrays rely on the

same principles, but use synthetic cDNAs instead of bacterial colonies (Qiagen, 2000). The filter array is densely packed with sample dots of known sequences of cDNA, making it possible to study the potential expression of thousands of known genes in one single hybridization (www.clontech.com).

Microarrays offer a number of advantages over other approaches to gene expression analysis, since this approach allows small hybridization volumes, high array densities, and the use of fluorescence labeling and detection schemes (Schena et al., 1996). In addition, a study performed by Schena et al. (1996) concluded that microarrays can also be used to analyze the expression of unknown genes when attached clones are chosen at random from any library of interest. Therefore, microarrays may allow rapid mechanistic examination of hormones, drugs and other small molecules. For example, functional analysis of growth factors, cytokines and receptors should also be possible (Schena et al., 1996). cDNA microarrays have already been used to profile complex diseases such as rheumatoid arthritis, melanoma and Ewing's sarcoma (Lueking et al., 1999).

Many commercially available types of microarrays are on the market. Genemed Biotechnologies, Inc. sells the Star Profiler™ Array, which allows accurate, reproducible gene expression profiles. Clontech sells the Atlas™ cDNA Expression Array which contains hundreds of carefully selected, well-characterized cDNAs. Because we were specifically interested in analyzing potential changes in cytokine expression, and Clontech offered a special filter containing 268 carefully chosen cytokine synthetic cDNAs, we chose Clontech as our commercial source.

MATERIALS AND METHODS

Cord Blood Samples

Human umbilical cord blood samples were donated to ViaCell Inc. from UMass Memorial Hospital. When RNA was to be isolated, cells were usually pooled from 3 different donors to provide enough material (>10^7 $CD45^+$ cells) to obtain a good yield of total cellular RNA. When cell lysates were to be made for immunoblot analysis, aliquots from single cords containing 10^5 $CD45^+$ cells were used. 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 concentrated by centrifugation and left in an aliquot of original culture media. The cells were then transported to WPI on ice.

Isolation of Total Cellular RNA

Total cellular RNA was isolated from 1-3 x 10^7 $CD45^+$ cord blood cells using the Clontech Atlas Pure Total RNA Labeling System (#K1038-1). Cells were centrifuged at 12,000 rpm (Sanyo Micro Centaur) for 15 seconds at 4°C, and the supernatant was discarded. A volume of 1 ml of denaturing solution was added to the cell pellet, and the solution was separated into two 1.5-2 ml eppendorf tubes (0.5 ml per tube). Cells were resuspended by pipetting up and down, and vortexing. The lysate solution was incubated on ice for 5-10 minutes. After vortexing the solution again, the lysate was microcentrifuged at 12,000 rpm (Sanyo Micro Centaur) for 5 minutes at 4°C to remove cellular debris. The entire supernatant was then transferred to new 2 ml centrifuge tubes (0.5 ml per tube), and 1 ml of TE-saturated phenol (i.e. 2 volumes) was added to each

tube. The tubes were capped securely, vortexed vigorously, and incubated on ice for 5 minutes. Next, 0.3 ml of chloroform (i.e. 0.6 original volumes) was added per tube, and the sample was shook and vortexed vigorously for 1-2 minutes. It was then incubated on ice for another 5 minutes. The organic/aqueous mixture was microcentrifuged at 12,000 rpm for 10 minutes at 4°C, and the upper aqueous phase containing the RNA was transferred to new tubes, making sure not to pipet any material from the white interphase or lower organic phase. A second round of the phenol: chloroform extraction was performed on the aqueous phase using 0.8 ml of phenol and 0.3 ml of chloroform per tube. The twice extracted upper aqueous phase was then transferred to new tubes, and 1 ml of isopropanol was added per tube. The sample was mixed, incubated on ice for 10 minutes, and then microcentrifuged at 12,000 rpm for 15 minutes at 4°C to pellet the RNA. Supernatants were immediately removed without disturbing the RNA pellets. A volume of 0.5 ml of 80% ethanol was then added per tube; the tubes were inverted several times, then microcentrifuged at 12,000 rpm for 5 minutes at 4°C to again pellet the RNA. Immediately the supernatant was carefully discarded, and the RNA pellet was air-dried (usually for 10 min). The pellets from both tubes were resuspended in a total of 50 µl of RNase-free dH_2O. In order to assess RNA yield, 5 µl of sample was added to 1 ml of dH_2O, and the absorbance was taken at 260 nm. Calculations were based on the assumption that 1 OD_{260} in a 1 cm light path = 40 µg/ml RNA. The remaining sample was aliquotted and stored at -70°C.

Microscale RNA Isolation

This protocol was used when the cell sample received was below 10^7 cells. The procedure was as described above except for the following. All of the reagent volumes were used at ¼ of the above stated volumes. Only one round of the phenol: chloroform extraction was performed. Also, when separating the upper aqueous layer from the white interphase and lower organic phase, 1 µl of glycogen was added to the sample as a "carrier" for the RNA in the sample. This glycogen provides us with a visible pellet after performing the alcohol precipitation, making it harder to accidentally remove the hard to see RNA pellet. The glycogen does not absorb UV light and therefore does not hinder RNA quantitation from the spectrophotometer.

DNase Treatment of Total Cellular RNA

The following reagents were combined in a 1.5 ml microcentrifuge tube for each sample to make a 100 µl reaction: 50 µl Total RNA (1 mg/ml), 10 µl 10X DNase I Buffer, 5 µl DNase I (1 unit/µl), and 35 µl deionized H_2O. This reaction(s) was incubated at 37°C in an air incubator for 30 minutes. A volume of 10 µl of 10X Termination Mix was added and the solution was mixed well by pipetting. In order to make a final volume of 550 µl, 440 µl of deionized H_2O was added to the tube. A volume of 500 µl of saturated phenol and 300 µl of chloroform was added, and the tube was vortexed thoroughly. In order to separate the phases, the sample was microcentrifuged at 13,000 rpm (Sanyo Micro Centaur) at 4°C for 10 minutes. The top aqueous layer was carefully transferred to a fresh 1.5 ml microcentrifuge tube while trying not to pipet any material from the interface or lower phase of the sample. A volume of 550 µl of chloroform was added. Tubes were vortexed thoroughly, then

microcentrifuged at 13,000 rpm at 4°C for 10 minutes to separate the phases. The top aqueous layer was transferred to a new 2 ml microcentrifuge tube. A volume of 50 µl of 2M NaOAc and 1.5 ml of 100% ethanol was added, vortexed, and samples were incubated on ice for 10 minutes. The sample was then spun in a microcentrifuge at 13,000 rpm at 4°C for 15 minutes to pellet the RNA. The supernatant was carefully removed without disturbing the pellet and 500 µl of 100% ethanol was added. The sample was microcentrifuged at 13,000 rpm at 4°C for 5 minutes and the supernatant was then carefully removed. The RNA pellet was then air dried for approximately 10 minutes and the precipitate was dissolved in 50 µl of RNase-free dH_2O. RNA yield was determined as previously described. The remaining sample was stored at -70°C.

Assessing the Yield and Purity of Total RNA

In order to assess the yield and purity of the total RNA samples, a denaturing agarose gel was prepared and run. First, a mini-gel box and combs were thoroughly washed with deionized water. A 250 ml beaker containing a magnetic stir-bar was obtained, and 1 g of agarose was weighed and mixed with 82.5 ml of water. The solution was heated in a microwave oven for 2 minutes and placed on a magnetic stir-plate to stir slowly for 2 minutes to cool. While the solution was stirring, 10 ml of 10X MOPS buffer and 7.5 ml of formaldehyde were added. The stirring was continued for 1 minute, then the solution was poured into the gel tray. The gel was left to solidify at room temperature for 1 hour, then the gel comb was removed and the gel was submerged in the gel box with 1X MOPS buffer.

The RNA loading solution was prepared immediately before running the gel by mixing the following (for 6-10 samples): 45 µl of formaldehyde, 45 µl deionized formamide, 10 µl 10X MOPS buffer, 3.5 µl EtBr (10 mg/ml), 1.5 µl 0.1M EDTA (pH 7.5), and 8 µl 10X bromophenol blue dye (in 50% glycerol). A volume of 15 µl of RNA loading solution was added to 2 µg (usually 2 µl) of total RNA and mixed well. The solution was heated at 70°C for 15 minutes and cooled on ice for 1 minute. The sample solution was then loaded onto the gel, and electrophoresed at 50-60V until the blue dye reached the midpoint of the gel tray. RNA was visualized on a UV transilluminator.

cDNA Probe Synthesis

Probe synthesis from total cellular RNA was performed using Clontech's Atlas cDNA Expression Array Protocol (#PT3140-1). First, a Master Mix was prepared for all labeling reactions. The following reagents were combined in a 0.5-ml microcentrifuge tube at room temperature for <u>each</u> sample of RNA: 2 µl of 5X Reaction Buffer, 1 µl of 10X dNTP Mix, 3.5 µl of [α-^{32}P]dATP (3,000 Ci/mmol), and 0.5 µl of DTT. Then, a PCR thermocycler was preheated to 70˚C. For each reaction, 2 µl RNA (usually containing 2 µg) and 1 µl CDS Primer Mix (a special combination of primers designed specifically for the Atlas filter) were combined in a labeled 0.5-ml PCR tube. Next, the tubes were mixed well by pipetting, and spun briefly in a microcentrifuge. Then, these tubes were incubated in the PCR thermocycler at 70˚C for 2 minutes. After 2 minutes, the temperature of the thermocycler was reduced to 50˚C, and the tubes were incubated for 2 more minutes. During this incubation, 1 µl MMLV Reverse Transcriptase was added per reaction to the Master Mix, it was mixed by pipetting, and incubated at room

temperature for 2 minutes. After the incubation of two minutes was completed, 8 µl of Master Mix was added to each ~3 µl reaction sample, ensuring that the RNA samples in the 50˚C thermocycler were not removed for longer than was necessary to add the Master Mix. The contents of the tubes were mixed by pipetting and then immediately returned to the thermocycler. The tubes were then incubated in the PCR thermocycler at 50˚C for 25 minutes. After incubation, 1 µl of 10X Termination Mix was added to each tube. The tubes were stored on ice until column chromatography.

To purify the labeled cDNA from unincorporated ^{32}P-labeled nucleotides and small cDNA fragments, the following procedure was performed. Before using the Buffer NT3, 15 ml of 95% ethanol was added to the bottle. First, the probe synthesis reactions were diluted by adding 190 µl of Buffer NT2. Second, a NucleoSpin Extraction Spin Column was placed into a 2-ml Collection Tube, and the sample was pipetted into the column. The tube was then microcentrifuged at 14,000 rpm for 1 minute. The Collection Tube and the flowthrough containing the waste were then discarded into the appropriate container for radioactive waste. Then, the NucleoSpin column was inserted into a fresh 2-ml Collection Tube, and 400 µl of Buffer NT3 wash was added to the column. The sample was microcentrifuged at 14,000 rpm for 1 minute, and the tube and flowthrough were discarded again. The addition of Buffer NT3 wash and the centrifugation steps were repeated twice more. The NucleoSpin column was then transferred into a clean 1.5-ml microcentrifuge tube, 100 µl of elution Buffer NE was added, and the column was allowed to soak for 2 minutes. The tube was microcentrifuged at 14,000 rpm for 1 minute to elute the purified cDNA probe. The approximate radioactivity of the liquid eluted into the microcentrifuge tube versus that remaining in the column were assessed

using a Ludlum hand-held Geiger counter to monitor efficient elution from the column. The counts should be higher in the eluate tube than in the column, otherwise the elution was repeated.

Probe Hybridization

Hybridization of cDNA probe to the Atlas Array filter was performed using Clontech's Atlas cDNA Expression Array Protocol (#PT3140-1). First, 5 ml of ExpressHyb was prewarmed at 68˚C. Next, 50 µl (0.5 mg) of sheared salmon testes DNA was heated at 97˚C for 5 minutes to denature the DNA, then quickly chilled on ice to prevent snap-back. Then, the denatured salmon testes DNA and the 5 ml prewarmed ExpressHyb aliquot were mixed, and kept at 68˚C. A Tupperware container was filled with deionized water and the Atlas Array (catalog #7744-1, stored at -20˚C) was placed in the Tupperware to wet it. All the water was then poured off and 5 ml of the 68˚C testes DNA/ExpressHyb solution was added to the membrane. This step was performed quickly to ensure that the membrane would not dry. Then, the membrane was prehybridized for 30 minutes with continuous agitation at 68˚C in an air incubator. Next, the probe was prepared for hybridization by adding 5 µl C_ot-1 DNA (1 mg/mL) to the entire 100 µl pool of labeled probe. The probe was then incubated in a boiling water bath for 2 minutes to denature it, and incubated on ice for 2 minutes. This mixture was added to the prehybridization solution and membrane in the Tupperware, ensuring that the probe was not directly added to the membrane, and that the two solutions were thoroughly mixed together. The membrane was then hybridized overnight with continuous agitation at 68˚C. If necessary, 2-3 ml of extra prewarmed ExpressHyb was

added to the solution to ensure that the entire membrane was in contact with hybridization solution at all times.

The next day, Wash Solution 1 and Wash Solution 2 were prewarmed at 68˚C. The hybridization solution was discarded in an appropriate radioactive waste container, and was replaced with 200 ml of prewarmed Wash Solution 1. The membrane was washed for 30 minutes with continuous agitation at 68˚C. This process was repeated three more times. Then, one 30-minute wash was performed using 200 ml of prewarmed Wash Solution 2 with continuous agitation at 68˚C. One final 5-minute wash was performed in 200 ml of 25˚C 2X SSC with agitation at room temperature. Using forceps, the membrane was removed from the container, and the excess Wash Solution was shaken off, never allowing the membrane to dry. The membrane was immediately wrapped in a plastic bag and sealed with a bag sealer. Finally, the membrane was exposed to BioMax x-ray film at -70˚C with an intensifying screen for varying lengths of time (3- and 5-day exposures). When the exposure was complete, the membrane was kept wrapped in plastic, and stored at -20˚C until stripped for re-use. In our hands, membranes could only be used a total of 3 times.

Probe Removal

To strip the cDNA probes from the membrane, 500 ml of 0.5% SDS solution was heated to boiling in a 2-L beaker. Then the membrane was removed from the plastic bag, and placed in the boiling solution for 5-10 minutes. The SDS solution was then removed from the heat and allowed to cool for 10 minutes. The Atlas Array filter was then removed from the solution and immediately re-wrapped in a new plastic bag and sealed.

To ensure the stripping procedure was effective, the membrane was checked with the hand-held Geiger counter, and by exposure to x-ray film for 3 days. Once the efficacy of the stripping was verified, the membrane was stored at ˉ20˚C until needed.

RT-PCR

Reverse Transcription

RT-PCR was performed to verify the upregulation of candidates identified from the arrays. Buffers, Taq polymerase, RT, and RNase Inhibitor were purchased from Ambion. Primers for these candidates were ordered from IDT based on sequences provided by Clontech. Each reaction was performed in a 0.5-µl-eppendorf tube. First, 2 µg of total cellular RNA (usually 2 µl) was added to 5µM (2 µl of a 50 µM stock) of the antisense primer and brought up to 12 µl with nuclease-free dH_2O. The tube(s) was then mixed, spun briefly, and heated for 3 minutes at 85˚C. The tube(s) was then removed to ice, spun briefly, and the following components were added (for each 12 µl sample to make a 20 µl reaction volume): 2 µl of 10X RT Buffer, 4 µl of dNTP mix, 1 µl of RNase Inhibitor, and 1 µl (100 units) of Reverse Transcriptase (a negative control that lacks Reverse Transcriptase should also be included when RT-PCR is performed). The tube(s) was then mixed gently, spun briefly, and incubated at 44˚C for one hour. Once the hour was over, an additional incubation period of 10 minutes at 92˚C inactivated the Reverse Transcriptase. These reactions were either stored at -20˚C or analyzed by agarose electrophoresis.

PCR

The following components were mixed in a separate tube for each PCR reaction, 5 µl of the RT reaction described above, 5 µl of 10X PCR buffer, 2.5 µl of dNTP mix, and nuclease-free dH_2O to make 50 µl. Then the following were added: 2.5 µl of 50 µM sense primer and 0.4 µl (2 U) of Thermostable DNA Polymerase. The templates were denatured by heating the samples at 94˚C for 2 minutes. The samples were incubated for 30 cycles of 94˚C for 30 seconds, 55˚C annealing for 30 seconds, and 72˚C elongation for 40 seconds. After these 30 cycles, a final polishing extension for the samples was at 72˚C for 5 minutes. The tube(s) were held in the thermocycler for 4˚C until the next day, stored at -20˚C, or analyzed by agarose gel analysis of RT-PCR products.

Analysis of RT-PCR Products

To analyze the RT-PCR products, a 1X dye buffer at $1/10^{th}$ the volume of RT-PCR product (approximately 5 µl) was added. A 10 µl aliquot of the 50 µl reactions were loaded into the wells of a 2.5% agarose gel containing 0.5 µg/ml of ethidium bromide and 1X TAE. The marker λ/Pst I (10 µl of 0.2 µg/µl stock) was used to determine the size of the bands produced. The remainder of the reactions were stored at -20˚C.

Western Blotting

Preparation of Whole Cell Lysates

The microcentrifuge tube(s) containing the cell samples (10^7 $CD45^+$ cells) were microcentrifuged for 15 seconds at 12,000 rpm (Sanyo Micro Centaur) at 4°C to pellet the cells. The supernatant (medium) was discarded, and this centrifugation was repeated to thoroughly remove all liquid. A volume of 200 µl of CHAPS lysis buffer (10

mM Tris-HCl, pH 7.5, 1 mM MgCl2, 1 mM EGTA, 0.1 mM Benzamidine, 5 mM β-mercaptoethanol, 0.5% CHAPS, 10% Glycerol, supplied from Intergen #S7700) was added per pellet. The cells were resuspended by gently pipetting up and down. The tube(s) were then incubated on ice for 30 minutes and microfuged at 12, 000 rpm for 20 minutes at 4°C to pellet cell debris. Cleared lysates were aliquotted and stored at ⁻80°C.

Western Gels

Western Blots were performed to analyze protein upregulations. First, a lower resolving gel (10% Acrylamide, 0.38 M Resolving Gel Buffer, 0.1% SDS, 0.1% Ammonium Persulfate, 0.05% TEMED, and dH_2O) was made and polymerized for 20 minutes. Next, an upper stacking gel (5% Acrylamide, 0.125 M Stacking Gel Buffer, 0.1% SDS, 0.1% Ammonium Persulfate, 0.1% TEMED, and dH_2O) was added and polymerized for 20 minutes or overnight.

Whole cell lysates (15 µl) were added to 5 µL 4X protein sample buffer (dH_2O, 0.5 M Stacking Gel Buffer, 8% SDS powder, 20% glycerol, 40% β-mercaptoethanol, 0.4% bromphenol blue powder). The samples were boiled for 2 minutes, loaded onto the gel, and electrophoresed for about 3 hours at 150 V.

Antibody Binding

Next the gels were electrically transblotted onto a Nitrocellulose membrane (BA-45, 0.45 µm), placed in blocking solution (1X PBS pH 7.5, 1% Casein, 0.2% Tween-20, and dH_2O) for 1 hour, and then 20 µl of primary antibody (mouse anti-human CD117, BD PharMingen catalog # 555713) in 10 ml of blocking solution was added and left to

incubate at 25°C on a rocker overnight. Subsequent washes were as follows. A volume of 50 mL of 1X PBS-Tween was added to the membrane and incubated at 25°C for 5 minutes. This wash was repeated 3 times. The wash solution was removed and 10 µl of secondary antibody (goat anti-mouse IgG conjugated to HRP) in 10 ml of blocking solution was added and incubated at 25°C for 2 hours. A subsequent wash was performed as stated above. Chemiluminescence (5 ml Luminol/Enhancer Solution plus 5 ml of Stable Peroxide Solution, Pierce # 34080) was used to detect the HRP on the membrane. Membranes were exposed to X-ray film for 30 minutes (with an intensifying screen) and developed as usual using GBX developer.

RESULTS

The goal of this project was to determine which cytokine mRNAs were upregulated or downregulated during ViaCell's stem cell amplification treatment. A hybridization array assay was chosen as a preliminary screen because of its speed in screening hundreds of candidates at one time. Clontech was chosen as a commercial source for the array filter because they offer an array (cat# 7744-1) with 268 carefully chosen cytokine and cytokine receptor cDNAs.

In a hybridization array assay, a population of cDNAs is synthesized from total cellular RNA or poly(A)$^+$ mRNA by reverse transcriptase. During the synthesis reaction, the cDNAs can be radiolabeled or biotinylated. This cDNA population is then hybridized to a filter containing attached cDNAs representing a variety of known mRNAs. The filter-attached cDNAs are often short synthetic oligos unique to each mRNA. With the Clontech arrays the cDNA dots are pre-spotted in duplicates. Following hybridization, the filter is washed, then exposed for autoradiography or chemiluminescence. The intensity of each dot signal on the film reflects the abundance of the corresponding mRNA in the original RNA sample.

For this project we chose to synthesize cDNAs from total cellular RNA instead of poly(A)$^+$ RNA because the former is more likely to be isolated intact from a tissue (blood) rich with nucleases. We also chose to radiolabel the cDNAs with ^{32}P because it gives more reliable signals in our hands.

FACS Analysis of Day-0 and Day-14 Cells

Umbilical cord blood cell samples obtained from ViaCell for RNA analysis represent day-0 and day-14 samples. When day-0 is referred to (see Table 3), it represents a set of un-amplified, un-selected $CD45^+$ cells containing little or no red blood cells. Day-14 cell samples represent cells that have undergone two column selections and amplification in the presence of added growth factors. Samples obtained for Western analysis came from all points of the amplification process.

Fraction	**% $CD34^+/CD38^-$**
Pre-Freeze (Day-0)	0.26
Pre-Sep 1	0.17
Post-Sep 1 (Day-0.5)	3.20
Pre-Sep 2	31.25
Post-Sep 2	33.20
Cell Product (Day-14)	10.53
Thawed Cell Product (Day-14.5)	11.54

Table 3. Percent of $CD34^+/CD38^-$ cells at each stage of the amplification process.

"Pre-freeze" (or day-0) samples are fresh mononuclear cells and contain about 0.26% of what ViaCell defines as hematopoietic stem cells. The cell population before the first separation step of the amplification process is termed "pre-sep 1" and contain 0.17% HSCs. The cell population after the first round of separation is termed "post-sep 1" and contains 3.20% HSCs. The cells then undergo a week of growth in culture and are

termed “pre-sep 2” right before the second separation (containing about 31.25% HSCs). After the second round of separation, the cells are termed “post-sep 2” and contain 33.20% HSCs. After undergoing a second week of growth in culture they are termed “cell product” or (day-14) and contain 10.53% HSCs. Cells are frozen for storage and thawed (then termed “thawed cell product) and contain 11.54% HSCs.

RNA Isolation and Integrity

The first phase of this project was to isolate total cellular RNA from human cord blood samples. Pooled cord samples (usually 3) were obtained from ViaCell, and RNA was isolated by a modified phenol extraction procedure (see Methods). Table 4 summarizes the cord blood samples obtained from ViaCell, the number of cells, and the corresponding RNA yield after the isolation procedure.

Table 4. Cord Cell Numbers and Corresponding RNA Yields.

Date	Sample	$CD45^+$ Cell #'s	Microscale technique?	RNA yield (μg)	Conc. (μg/μl)
12/1/00	Day 0	2x10^6	no	6.8	0.136
12/6/00	Day 0	8.51x10^7	no	58.40	1.170
12/12/00b	Day 14	*1.3x10^8	no	322.40	6.450
12/15/00a	Day 0	*7.30x10^8	no	83.60	1.670
12/15/00a	Day 14	*2.17x10^8	no	148.40	2.970
12/15/00b	Day 14	*2.17x10^8	no	114.40	2.290
2/14/01a	Day 14	*7.79x10^7	no	123.60	2.470
2/14/01b	Day 14	*7.79x10^7	no	149.20	2.980
9/6/01a	Post Thaw	*2.6x10^7	no	2.00	0.040
9/6/01b	Post Thaw	*2.6x10^7	no	1.60	0.032
9/7/01a	Pre-Sep 1	*5.6x10^7	no	6.80	0.136
9/7/01b	Pre-Sep 1	*5.6x10^7	no	10.40	0.208
9/10/01a	Post Thaw	*approx.3.0x10^8	no	46.00	0.920
9/10/01b	Post Thaw	*approx.3.0x10^8	no	46.40	0.928
9/13/01a	Day 0	*approx.4.0x10^8	no	49.60	0.992
9/13/01b	Day 0	*approx.4.0x10^8	no	36.80	0.736
9/13/01c	Day 0	*approx.4.0x10^8	no	21.60	0.432

Table 4, continued.

Date	Sample	$CD45^+$ Cell #'s	Microscale technique?	RNA yield (μg)	Conc. (μg/μl)
9/13/01	Day 14	2x10^6	yes	6.40	0.128
9/14/01a	Pre-Sep 1	*approx.1.0x10^8	no	8.40	0.168
9/14/01b	Pre-Sep 1	*approx.1.0x10^8	no	14.00	0.280
9/14/01a	Pre-Sep 2	*approx.1.0x10^7	no	43.20	0.864
9/14/01b	Pre-Sep 2	*approx.1.0x10^7	no	38.00	0.760
9/17/01a	Day 0	*approx.1.0x10^8	yes	14.80	0.296
9/17/01b	Day 0	*approx.1.0x10^8	yes	90.40	1.808
9/17/01c	Day 0	*approx.1.0x10^8	yes	38.80	0.776
9/17/01d	Day 0	*approx.1.0x10^8	yes	110.40	2.208
9/17/01e	Day 0	*approx.1.0x10^8	yes	82.40	1.648
9/17/01f	Day 0	*approx.1.0x10^8	yes	132.80	2.656
9/17/01g	Day 0	*approx.1.0x10^8	yes	72.80	1.456
9/17/01h	Day 0	*approx.1.0x10^8	yes	74.00	1.480
9/18/01a	Day 0	4ml off 1 cord	yes	102.40	2.048
9/18/01b	Day 0	4ml off 1 cord	yes	36.40	0.728
9/20/01a	Day 0	4ml off 1 cord	yes	9.60	0.192
9/20/01b	Day 0	4ml off 1 cord	yes	3.60	0.072
9/25/01a	Day 0	~10^6	yes	9.60	0.192
9/25/01b	Day 0	~10^6	yes	33.60	0.672
9/27/01a	Post Thaw	4.5x10^6	yes	1.60	0.032
9/27/01b	Post Thaw	1.2x10^6	yes	0.40	0.008
9/27/01c	Post Thaw	4.68x10^6	yes	0.80	0.016
9/27/01d	Post Thaw	6.75x10^6	yes	1.20	0.024
9/28/01a	Pre-Sep 1	5.10x10^6	yes	17.20	0.344
9/28/01b	Pre-Sep 1	1.02x10^6	yes	8.00	0.160
9/28/01c	Pre-Sep 1	5.45x10^6	yes	10.00	0.200
9/28/01d	Pre-Sep 1	8.95x10^6	yes	6.00	0.120
10/01/01a	Day 0	6.9x10^6	yes	10.80	0.216
10/01/01b	Day 0	3.2x10^6	yes	28.00	0.560
10/01/01c	Day 0	3.04x10^6	yes	38.80	0.776
10/01/01d	Day 0	2.56x10^6	yes	163.60	3.272
10/04/01a	Post Thaw	3.40x10^6	yes	4.40	0.088
10/04/01b	Post Thaw	2.62x10^6	yes	4.40	0.088
10/04/01c	Post Thaw	3.64x10^6	yes	6.40	0.128
10/05/01a	Pre-Sep 1	3.99x10^7	yes	4.00	0.080
10/05/01b	Pre-Sep 1	1.24x10^8	yes	1.20	0.024
10/05/01c	Pre-Sep 1	5.93x10^7	yes	6.40	0.128
10/05/01d	Pre-Sep 1	~10^8	yes	16.00	0.320
10/12/01a	Pre-Sep 2	2x10^6	yes	4.40	0.088
10/12/01b	Pre-Sep 2	1.34x10^6	yes	1.20	0.024
10/12/01c	Pre-Sep 2	1.01x10^6	yes	2.00	0.040
10/12/01a	Post-Sep 2	2x10^6	yes	2.40	0.048
10/12/01b	Post-Sep 2	1x10^6	yes	0.00	0.000
10/16/01a	Day 14	9.20x10^5	yes	0.80	0.016
10/16/01b	Day 14	1.03x10^6	yes	1.60	0.032

Table 4, continued.

Date	Sample	$CD45^+$ Cell #'s	Microscale technique?	RNA yield (μg)	Conc. (μg/μl)
10/17/01a	Day 14	*9.10x10^6	yes	16.40	0.328
10/17/01b	Day 14	*9.10x10^6	yes	8.00	0.160
10/17/01c	Day 14	2.56x10^6	yes	2.80	0.056
10/19/01a	Day 14	1.87x10^7	yes	91.20	1.824
10/19/01b	Day 14	8.23x10^6	yes	27.60	0.552
10/25/01a	Day 14-thawed	2.70x10^6	yes	8.40	0.168
10/25/01b	Day 14-thawed	2.80x10^7	yes	145.20	2.904
10/26/01a	Day 14-thawed	5.98x10^6	yes	56.80	1.136
10/26/01b	Day 14-thawed	1.50x10^7	yes	20.80	0.416
3/11/02	Day-0	1.0x10^7	yes	11.6	0.232
3/25/02	Day-14	1.0x10^7	yes	18.4	0.368

As can be seen in the Table, the final yield of RNA did not correlate with the total $CD45^+$ cells received, therefore the status of the cords could have been a factor affecting yield.

In order to determine which RNA samples were intact following the RNA isolation protocol, denaturing formaldehyde agarose gel electrophoresis was performed. Discrete 28S and 18S ribosomal RNA bands were used as evidence of intact RNA. Figure 10 displays an example of an RNA integrity gel. The 28S and 18S ribosomal RNA bands can clearly be seen. Samples in lanes 2 and 3 were not taken for further analysis.

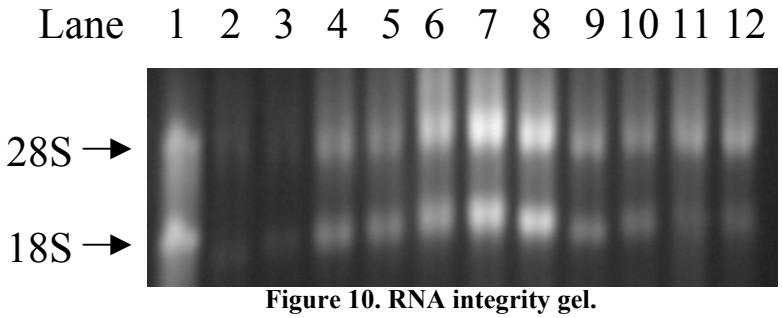

Figure 10. RNA integrity gel.

Extraction procedures used for isolating total cellular RNA sometimes co-isolate trace amounts of DNA. This contaminating DNA is capable of being radiolabeled during probe preparation, so DNase treatment was performed on two cord RNA samples. The reason all samples were not DNase treated was because the RNA yield fell to 0 μg after the treatment. Therefore, it was concluded that the Clontech DNase treatment protocol was not an effective way to rid DNA so it was discontinued. Using the information obtained from the RNA gels, it was possible to determine which RNA samples were most intact and likely to give the best hybridization results.

Hybridization Arrays for Day-0 Cells

Day-0 cord cells represent un-amplified, un-selected CD45+ (non RBC) cord cells. The Atlas Array (Clontech, cat# 7744-1) chosen for this experiment contained 268 known cytokine and cytokine receptor cDNAs. Figure 11 shows the result of hybridization 1-29-01 of day-0 cDNA synthesized from cord RNA isolated on December 15, 2000, tube a. All dots are in duplicate on the membrane and equal signals were observed for all duplicate dots. The membrane on the right hand side of the figure is overlaid with a grid provided by Clontech to identify the dots.

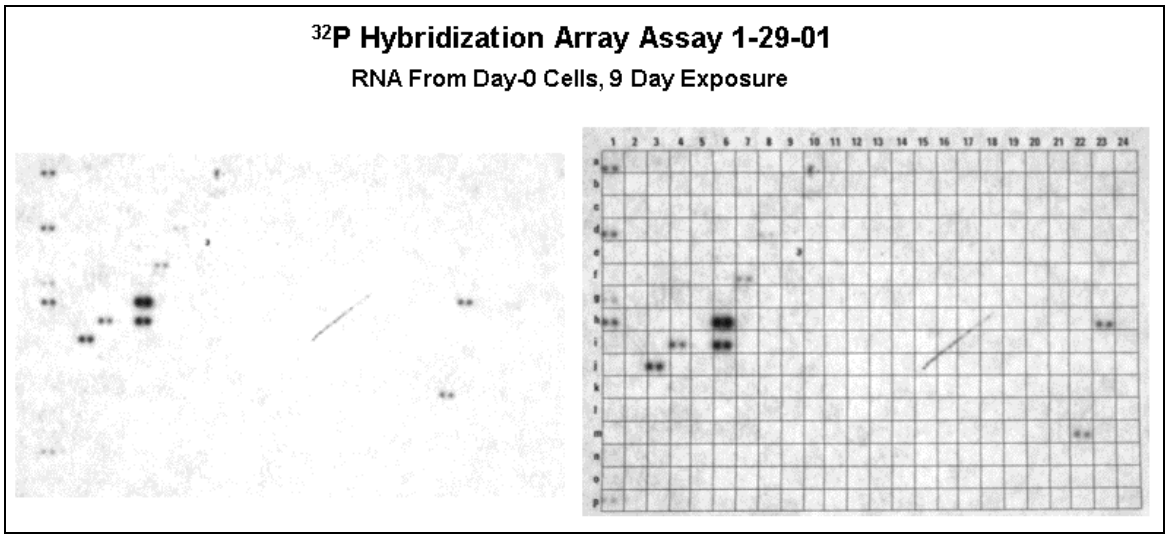

Figure 11. Trial 1, Hybridization Array for the First Sample of Day-0 Cells.

Results from the 9-day exposure on XAR-5 film of the Day-0 cord sample. From RNA isolated on December 15, 2000, tube a. The membrane on the right contains a grid in order to identify the cytokine mRNAs that are upregulated.

All of the dots seen in column 1 (rows A-I) are "housekeeper" controls. These are present to ensure that the hybridization was complete to the membrane, as well as aiding in the alignment of the grid to properly identify the dots. In Figure 11 housekeeper mRNAs are seen at positions 1A, 1D, and 1H. The other housekeepers that should have appeared did not in this hybridization, most likely due to the standard XAR-5 type of x-ray film used (low sensitivity), or due to a low specific activity of the probe. Also, the 9-day exposure is longer than usually done according to the Clontech Manual. Therefore, in our subsequent hybridizations, BioMax x-ray film was used, as well as a higher specific activity probe, which gave us much better results. Column 1, rows J-L are negative controls, and no signal was observed for these. One interesting finding from this figure is that no inflammatory cytokine mRNAs appear to be expressed in the day-0 RNA. This lack of inflammatory cytokines could be highly significant if the cord

samples are to be reperfused into a patient. Table 5 (at the end of the hybridization array results) summarizes the cytokine mRNAs seen on this membrane.

To assay intra-sample variability, a second cDNA probe was synthesized from the same RNA examined in Figure 11. The probe was hybridized to the same membrane again. This data (Figure 12) shows us the same dots as in Figure 11, although many more are seen, and they are darker. Obviously, the probe used in this figure was of much higher specific activity than the previous figure. Again, the duplicate dots are identical in each case indicating a reproducible signal. The housekeepers that were the darkest on the previous filter were also the darkest here. All housekeepers are visible on this array (column 1, rows A-I). Therefore, we have concluded that although probe specific activity varies from one reaction to another from the same RNA sample, which alters the total number of dots observed, the strongest dots remained nearly identical in the duplicate determination. Regarding cytokine mRNAs (and their receptors), strong new signals were observed for IL-8 precursor (3J), IL-2-R (4I), Leukocyte interferon-inducible peptide (6F), calgranulin-A (6H), calgranulin-B (6I), small inducible cytokine A5 (7F), and thymosin-β (23H). Weak or no signals were observed for TNF-α (14I), and TNF-R (14J) (14K).

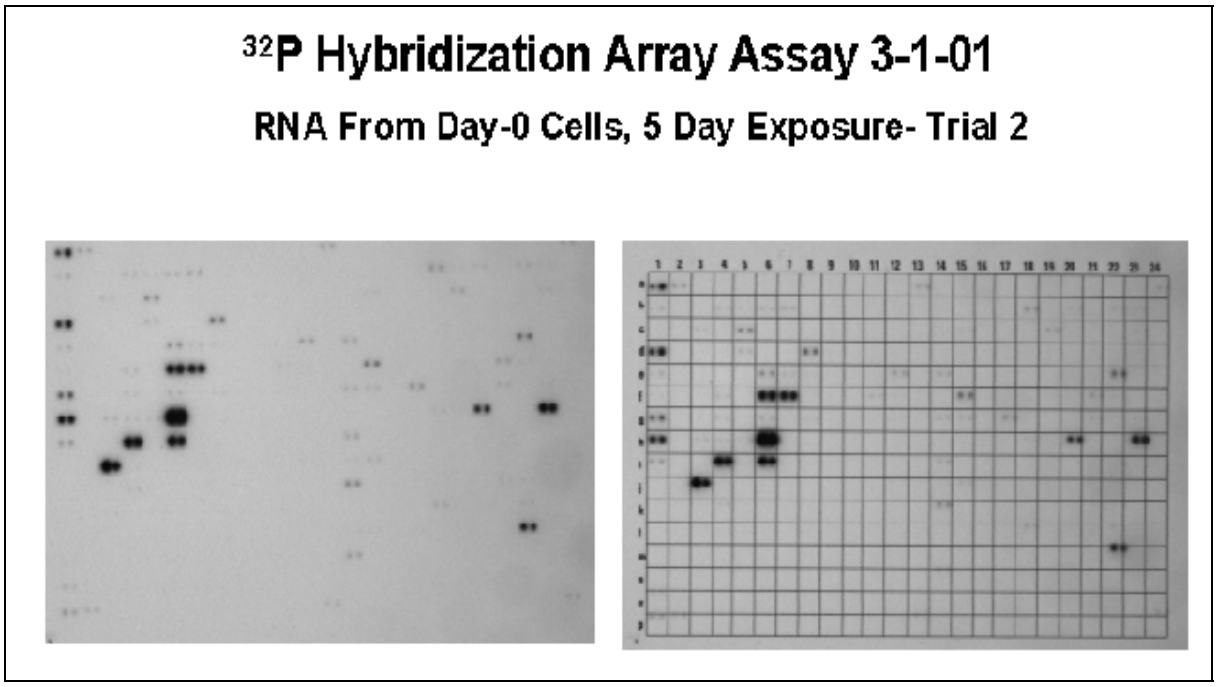

Figure 12. Trial 2, Hybridization Array for the First Sample of Day-0 Cells.

Results from the 5-day exposure on BioMax film of the Day-0 cord sample. From RNA isolated on December 15, 2000, tube a. This second trial focuses on day-0 intra-sample variability.

Since many different day-0 samples were received from ViaCell, several independent observations of day-0 cells could be performed. Figure 13 shows the analysis of a second day-0 sample from RNA isolated on December 6, 2000. Fewer dots are seen here than in Figure 11. Only 3 housekeepers are visible. Unlike the previous day-0 sample, IL-1-R antagonist protein (4F) was the strongest dot on the filter. This indicates that some significant inter-sample variability can be expected between pooled cord samples.

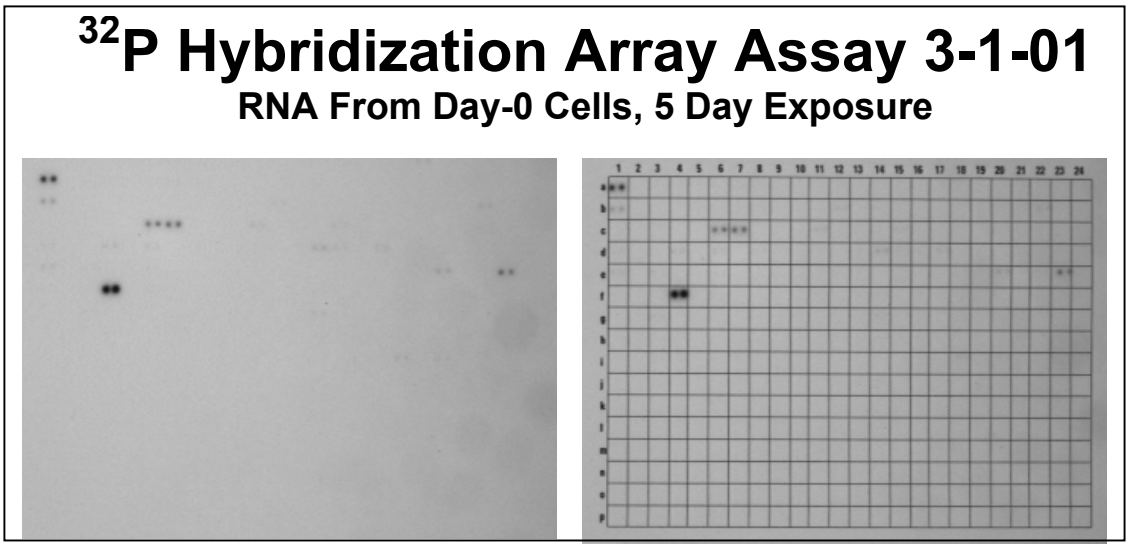

Figure 13. Trial 1, Hybridization Array for the Second Sample of Day-0 Cells.

Results from the 5-day exposure on BioMax film of the Day-0 cord sample. From RNA isolated on December 6, 2000. This trial focuses on day-0 inter-sample variability.

To assay intra-sample variability for this second day-0 sample, a second cDNA probe was synthesized from the same RNA assayed in Figure 13. This new cDNA was hybridized to the same membrane again after its first probe was removed. Unfortunately Figure 14 shows us few of the same dots as in Figure 13, although many more are seen, and they are much darker. Obviously, the probe used in Figure 14 is of much higher specific activity than the previous figure. Again, the duplicate dots are identical in each case, and almost all of the housekeepers are seen (although darker in this figure). We can conclude that the RNA assayed in the previous Figure 13 was good, but the cDNA from that batch was low specific activity.

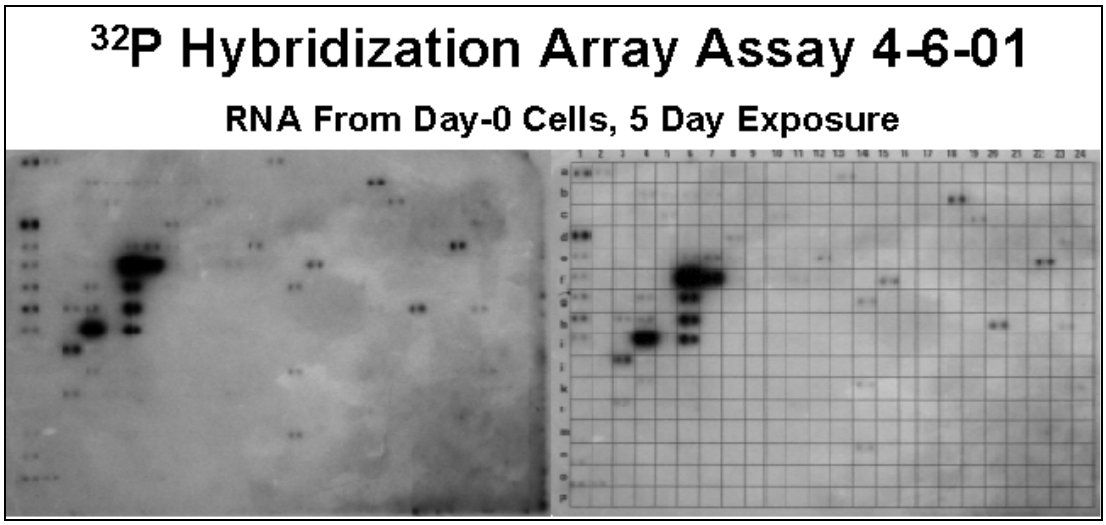

Figure 14. Trial 2, Hybridization Array for the Second Sample of Day-0 Cells.

Results from the 5-day exposure on BioMax film of the Day-0 cord sample. From RNA isolated on December 6, 2000.

In order to determine whether different membranes obtained from Clontech were identical to each other, we performed another cDNA synthesis on the same RNA isolated on December 6, 2000, and hybridized to a new different membrane. Figure 15 contains most of the same dots seen in Figure 14, although some differences were observed. The best way to perform this experiment is to prepare two cDNA reactions from the same RNA, pool them, and hybridize to two membranes. This will be done in future experiments.

We conclude from the array analysis of day-0 cells that although significant signal variability results from low and high specific activity probes, when comparing only high-specific activity data, the patterns are mostly reproducible.

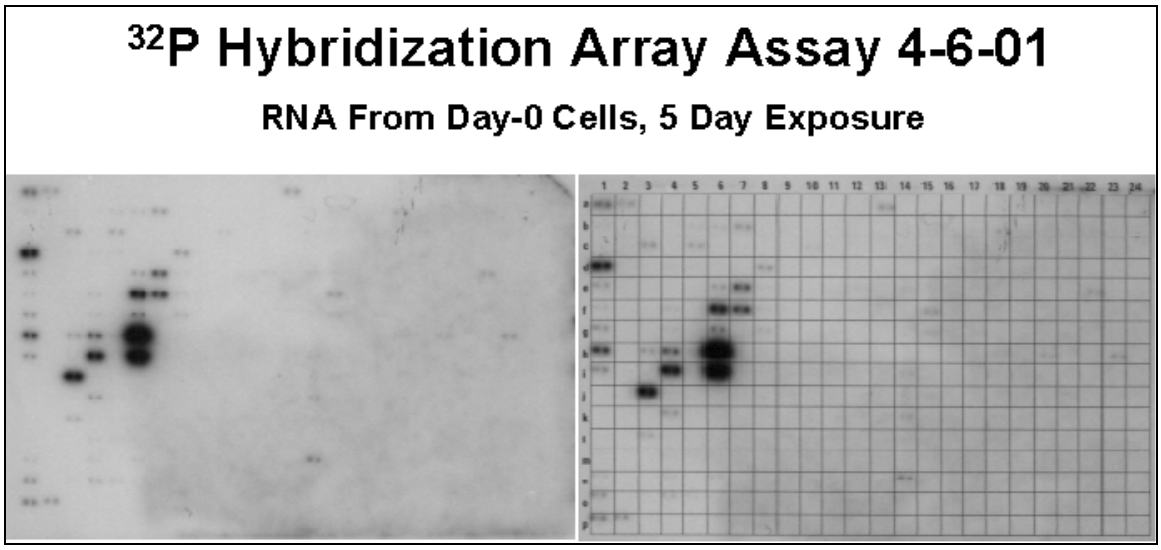

Figure 15. Trial 3, Hybridization Array for the Second Sample of Day-0 Cells.

Results from the 5-day exposure on BioMax film of the Day-0 cord sample. From RNA isolated on December 6, 2000. This experiment focuses on membrane-to-membrane production variability.

Hybridization Arrays for Day-14 Cells

As was done for the day-0 samples, day-14 samples were also analyzed on arrays. Day-14 samples represent cells that have undergone two column selections and cell amplification. Figure 16 shows the results for day-14 RNA isolated on 12/15/00. Many more dots are seen here, especially to the right hand side of the membrane, than was ever observed for day-0 samples, in spite of this particular probe being low specific activity (few housekeepers are visible). This area of dots signify some of the developmentally important cytokines, as well as growth factors. If these are subsequently proven to be upregulated in RT-PCR assays, it may imply that these "stem" cell enriched populations at Day-14 may have in part returned to an undifferentiated state. Upregulated candidates not seen in day-0 samples include frizzled homologs (20L), jagged homologs (21D and

21E), wingless-related MMTV protein (20H), and growth factors such as vascular endothelial growth factors (19D) and transforming growth factor (15F and 15G).

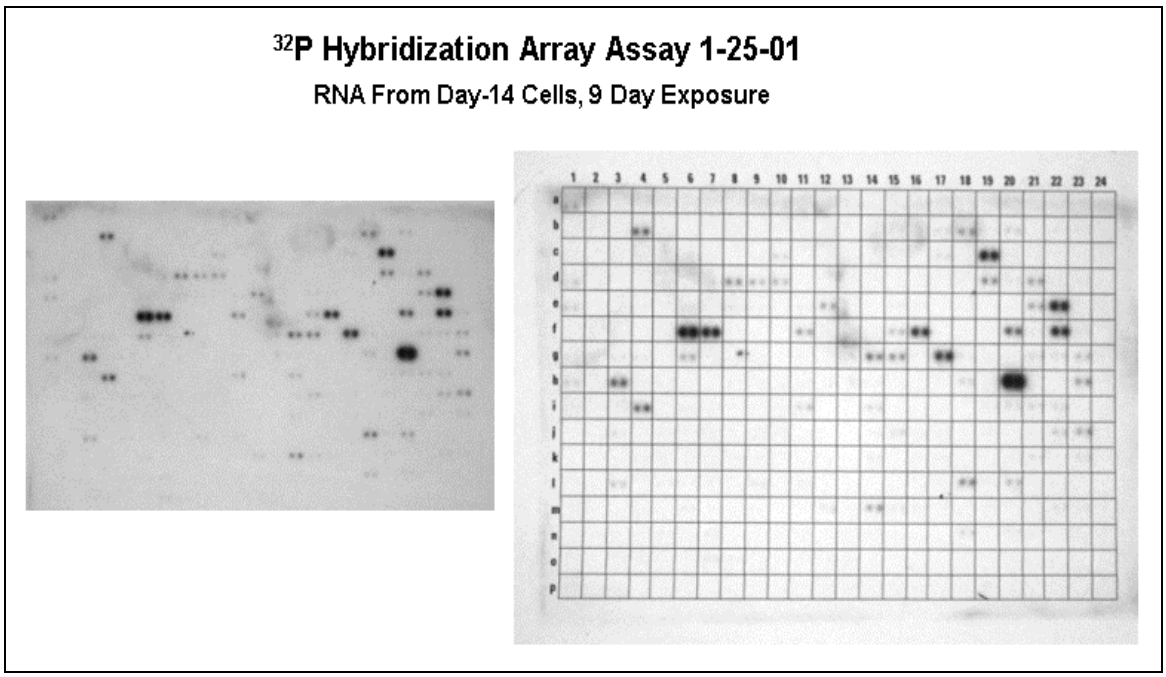

Figure 16. Trial 1, Hybridization Array for the First Sample of Day-14 Cells.

Results from the 9-day exposure on XAR-5 film of the Day-14 cord sample. From RNA isolated on December 15, 2000, tube a.

A complete list of the apparently upregulated cytokine mRNAs is seen in Table 5 at the end of the hybridization array results. Some of the strongest dots on the day-14 array include mRNAs in common with day-0 samples, including Leukocyte interferon inducible peptide (6F), and small inducible cytokine A5 (7F). As was the case for the day-0 samples, no signal was observed for inflammatory cytokine TNF-α (14J).

To assay day-14 intra-sample variability, a second cDNA batch from the same RNA analyzed in the previous figure (Figure 16) was hybridized to the same membrane again after its first probe was removed. Figure 17 shows the results of this experiment. This probe gave the highest number of cytokine dots observed on any array so far under conditions in which all but 3 housekeepers are visible. The resulting darker spots in some cases result from the use of stronger probe and film. For example, the dots seen at positions 6F and 7F are strongly seen in both figures, but the dots are darker in Figure 17. Figures 16 and 17 show basically the same dot patterns, so this again proves that expression patterns are somewhat reproducible when using high-specific activity probes.

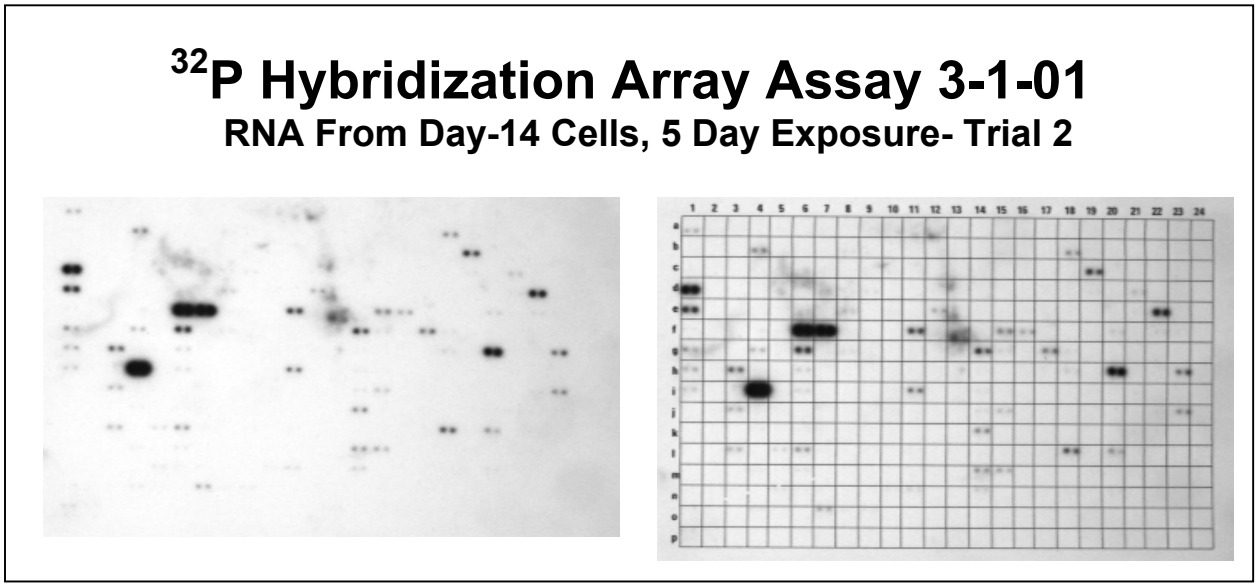

Figure 17. Trial 2, Hybridization Array for the First Sample of Day-14 Cells.

Results from the 5-day exposure on BioMax film of the Day-14 cord samples. From RNA isolated on December 15, 2000, tube a. This second trial focuses on intra-sample variability.

In the future, we plan to demonstrate this more rigorously by synthesizing one large batch of cDNAs from one day-14 sample, then splitting the cDNA sample into halves, and hybridizing to two membranes.

Since many day-14 samples were received from ViaCell, a thorough analysis of this cell population was performed. Figure 18 shows a second day-14 sample from RNA isolated on February 14, 2001. This RNA sample yielded our highest specific activity probe to date. All housekeepers are visible, and many are quite strong. As observed with the first day-14 sample, the membrane contains many more dots than for any day-0 sample.

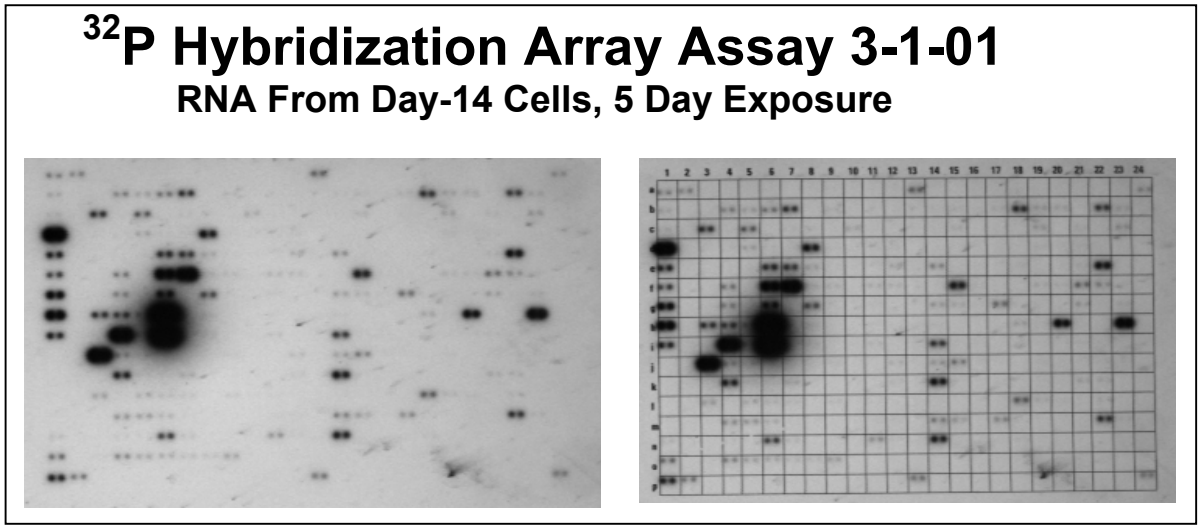

Figure 18. Trial 1, Hybridization Array for the Second Sample of Day-14 Cells.

Results from the 5-day exposure on BioMax film of the Day-14 cord sample. From RNA isolated on February 14, 2001 tube a. This trial enables us to assay inter-sample variability relative to the previous figures.

This second day-14 sample was reanalyzed for intra-sample variability. Unfortunately, this cDNA did not have quite the same high specific activity as trial-1, but the pattern of strongest dots is the same. In each day-14 case, however, more dots are seen in these membranes than in any day-0 membranes. In all cases, the duplicate dots have the same intensities.

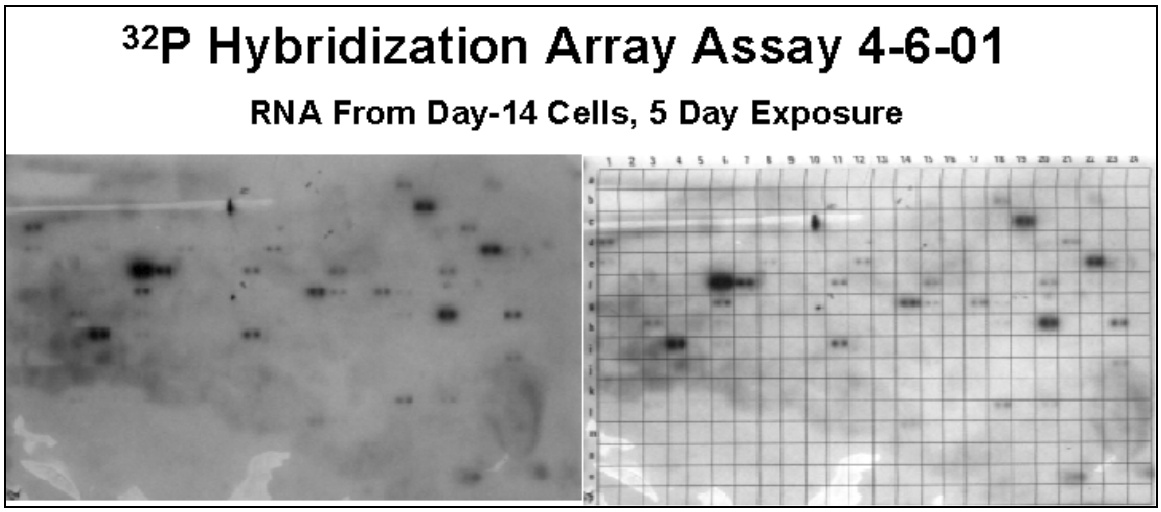

Figure 19. Trial 2, Hybridization Array for the Second Sample of Day-14 Cells.

Results from the 5-day exposure on BioMax film of the Day-14 cord sample.
From RNA isolated on February 14, 2001, tube a. This second trial analyzes intra-sample variability for the second day-14 sample.

Finally, once more in order to determine whether different membranes obtained from Clontech were identical to each other, we performed another cDNA synthesis on the same RNA isolated on February 14, 2001, and hybridized to a new membrane. Unfortunately, Figure 20 shows that a very poor cDNA reaction occurred. Very few dots are seen, including a lack of housekeepers. Since the same RNA yielded better results in previous hybridizations, we can conclude that the cDNA synthesis for trial-3 was most likely very weak.

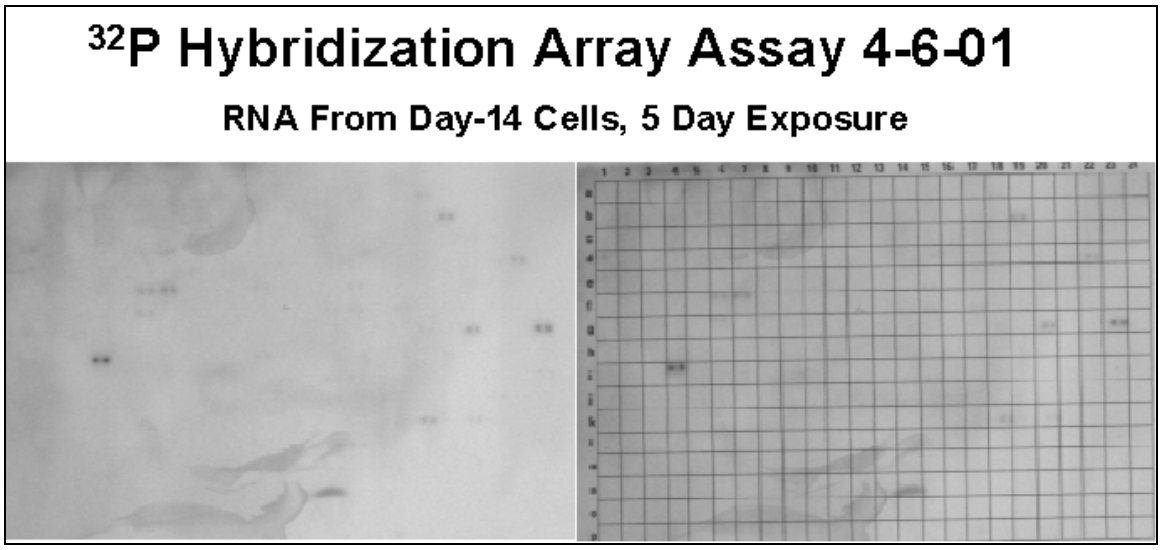

Figure 20. Trial 3, Hybridization Array for the Second Sample of Day-14 Cells.

Results from the 5-day exposure on BioMax film of the Day-14 cord sample. From RNA isolated on February 14, 2001, tube a. The lack of dots in this case was most likely due to a poor cDNA synthesis and weak hybridization signals.

One additional hybridization was performed with an RNA sample isolated on December 15, 2000, tube b. When the film was exposed, it contained no dots whatsoever. When the RNA integrity was re-analyzed on a denaturing agarose gel, the sample displayed a weak 18S band, so perhaps this RNA sample was somewhat degraded.

Array Summary

Table 5 (page 59) summarizes all the hybridization array figures. The symbol +++ signifies a strong signal (dark dots on the membrane), ++ signifies a moderate signal (medium dots), + signifies a low signal (light dots), and blank signifies no observable signal. Because signal patterns are so strongly influenced by probe specific activity, our

best comparison can be made only between those arrays showing most of the housekeepers, i.e. array-5 versus array-8.

Some mRNAs were commonly represented in day-0 and day-14 samples. These include: interleukin-8 precursor, interleukin-10 precursor, interleukin-2 receptor alpha subunit precursor, interleukin-6 receptor beta subunit precursor, leukocyte interferon inducible peptide, B-cell growth factor-1 precursor, migration inhibitory factor-related protein 8, calgranulin-B, small inducible cytokine subfamily B5, small inducible cytokine A5, insulin receptor precursor, secreted apoptosis related protein, tumor necrosis factor alpha precursor, transforming growth factor beta, transforming growth factor beta-2 precursor, smoothened homolog, jagged homolog-2, neurogenic locus Notch protein homolog-1 precursor, platelet-activating factor receptor, thymosin beta-10, and orientation marks. Each of these cytokines were upregulated at day-0 as well as day-14.

Some cytokine mRNAs were upregulated greatly at day-14: interleukin-1 beta precursor, interleukin-6 precursor, interleukin-13 precursor, interleukin-1 receptor type II precursor, interleukin-2 receptor gamma subunit, interleukin-7 receptor alpha subunit precursor, interferon alpha/beta receptor beta subunit, interferon gamma antagonist, interferon regulatory factor-1, hepatocyte growth factor-like protein, macrophage-specific colony-stimulating factor, macrophage inflammatory protein-1 alpha precursor, macrophage inflammatory protein-1 beta precursor, macrophage inflammatory protein-2 alpha, C5a anaphylatoxin receptor, CDW40 antigen, mast/stem cell growth factor receptor precursor (CD117 antigen), granulocyte colony stimulating factor receptor precursor, macrophage colony-stimulating factor-1 receptor precursor, glia maturation factor beta, neurotrophin-3 precursor, Trk-T3, insulin-like growth factor-binding protein-

2, neurotrophin-4, colon carcinoma kinase-4 precursor + transmembrane receptor PTK7, secreted apoptosis related protein, tumor necrosis factor alpha precursor, tumor necrosis factor receptor superfamily member-1A, tumor necrosis factor receptor superfamily member-1B, WSL protein + TRAMP + Apo-3 + death domain receptor-3, platelet-derived growth factor-A subunit precursor, inhibin beta-A subunit precursor, cysteine-rich fibroblasts growth factor receptor-1, heparin-binding EGF-like growth factor, ErbB3 proto-oncogene, endothelin-2, SL cytokine precursor, vascular endothelial growth factor precursor, vascular endothelial growth factor receptor-3, endothelial-monocyte activating polypeptide-2, wingless-related MMTV, frizzled homolog, jagged homolog-1, neurogenic locus Notch protein (not the same as the previously discussed neurogenic locus Notch protein homolog-1), Manic Fringe homolog precursor, BIGH3, corticotropin-releasing factor receptor 1 precursor, basigin precursor, hepatoma-derived growth factor, G-protein-coupled receptor HM74, Oncostatin M, and epithelial discoidin domain receptor-1 precursor.

Table 5. Summary of Array Data.

	Array	1	2	3	4	5	6	7	8	9	10
	Cord Day	Day 0	Day 0	Day 0	Day 0	Day 0	Day 14	Day 14	Day 14	Day 14	Day 14
	Hybridization Date	1/29/01	3/1/01	3/1/01	4/6/01	4/6/01	1/25/01	3/1/01	3/1/01	4/6/01	4/6/01
	RNA Isolation Date	12/15a	12/15a	12/6/00	12/6/00	12/6/00	12/15a	12/15a	2/14a	2/14a	2/14a
	Trial Number	Trial 1	Trial 2	Trial 1	Trial 2	Trial 3	Trial 1	Trial 2	Trial 1	Trial 2	Trial 3
Location	**Gene/Protein Name**										
1A	Housekeeper Control	++	++	+++	++	+	+	+	+		
1B	Housekeeper Control		+	+					+		
1D	Housekeeper Control	++	++		+++	+++	+	+++	+++	++	
1E	Housekeeper Control		+		+	+	+	+++	+++	+	
1F	Housekeeper Control				+	+			+++		
1G	Housekeeper Control	+	++		+	+		+	+++		
1H	Housekeeper Control	++	++		++	++	+	+	+++		
1I	Housekeeper Control		+		+	+		+	+++		
1O	Calibration Marker		+		+	+			++		
1P	Calibration Marker	+	+		+	+			+++		
2A	Orientation Mark		+		+	+			+		
2P	Orientation Mark		+		+	+			++		
3C	Interleukin 1 beta precursor; catabolin					+			+++		
3H	Interleukin 6 precursor; B-cell stimulatory factor 2; interferon beta 2, hybridoma growth factor		+		+	+	++	++	+++	+	
3J	Interleukin 8 precursor; monocyte-dervied neutrophil chemotactic factor; T-cell chemotactic factorl neutrophil-activation protein 1; lymphocyte-derived neutrophil-activation factor; protein 3-10c; granulocyte chemotactic protein 1; emoctakin		+++		++	+++		+	+++		
3L	Interleukin 10 precursor; cytokine synthesis inhibitory factor				+	+		+	+		
4B	Interleukin 13 precursor; NC30		+		+		++	++	++		
4F	Interleukin 1 receptor antagonist protein precursor		+	+++					++		
4G	Interleukin 1 receptor type II precursor; IL1R-alpha; p80; CDW121A antigen		+		+			++	++		
4H	Interleukin 1 receptor type II precursor; IL1R-beta		+		+	++			+++		
4I	Interleukin 2 receptor alpha subunit precursor; TAC antigen; CD25 antigen		+++		+++	+++	++	+++	+++	+++	++
4J	Interleukin 2 receptor beta subunit precursor; p70-75; CD122 antigen								+		
4K	Interleukin 2 receptor gamma subunit; cytokine receptor common gamma chain precursor		+		+	+			+++		
4M	Interleukin 4 receptor alpha subunit precursor; CD124 antigen								++		
4O	Interleukin 6 receptor alpha subunit precursor; CD126 antigen					+			++		
5B	Interleukin 6 receptor beta subunit precursor; membrane glycoprotein 130; oncostatin M receptor; CDW130 antigen		+		+				+		

Table 5, continued.

5C	Interleukin 7 receptor alpha subunit precursor; CDW127 antigen		+		+				+++		
5D	High-affinity interleukin 8 receptor alpha; IL8 receptor type 1; CDW128 antigen		+						+		
5H	Interferon alpha/beta receptor beta subunit precursor; type I interferon receptor								++		
5L	Interferon gamma precursor; immune interferon							+	+		
5M	Interferon gamma antagonist								++		
5O	Interferon gamma receptor; CDW119 antigen								+		
6B	Interferon gamma receptor beta subunit precursor; IFN-gamma accessory factor 1; IFN-gamma transducer 1		+		+	+			++		
6C	Cytokine humig; interferon gamma-induced monokine			++							
6E	Interferon regulatory factor 1		+		+	+			+++		
6F	Leukocyte Interferon Inducible Peptide		+++		+++	+++	+++	+++	+++	+++	+
6G	B cell growth factor 1 precursor; 12-kDa BCGF		+		+++	+	+	+++	+++	++	
6H	Migration inhibitory factor-related protein 8; calgranulin A; leukocyte L1 complex light chain; S100 calcium-binding protein A8; cystic fibrosis antigen		+++		+++	+++		+	+++	+	
6I	Calgranulin B; migration inhibitory factor-related protein 14; leukocyte L1 complex heavy chain; S100 calcium-binding protein A9	+++	+++		+++	+++		+	+++	+	
6L	Hepatocyte growth factor-like protein; macrophage-stimulating protein							++	+		
6M	Macrophage-specific colony-stimulating factor								+		
6N	Macrophage inflammatory protein 1 alpha precursor; tonsillar lymphocyte LD78 alpha protein; GOS19-1 protein; PAT 464.2; SIS-beta; small inducible cytokine A3								++		
6O	Macrophage inflammatory protein 1 beta precursor; T-cell activation protein 2; PAT 744; H400; SIS-gamma; lymphocyte activation gene 1 protein; HC21; small inducible cytokine A4; G 26 T-lymphocyte secreted protein								+		
7B	Macrophage inflammatory protein 2 alpha; growth-regulated protein beta		+		+	+			+++		
7C	Macrophage migration inhibitory factor; glycosylation-inhibiting factor			++							
7E	Small inducible cytokine subfamily B5; epithelial-derived neutrophil-activating peptide 78		+		+	++			+++		
7F	Small inducible cytokine A5, regulated on activation normal T-cell expressed and secreted protein precursor	+	+++		+++	++	+++	+++	+++	+++	+
7K	C5a anaphylatoxin receptor; CD88 antigen								+		
7N	CDW40 antigen; CD40L receptor precursor; nerve growth factor receptor-related B-lymphocyte activation molecule								+		

Table 5, continued.

7O	c-kit proto-oncogene; mast/stem cell growth factor receptor precursor; CD117 antigen							+	+		
8D	Granulocyte colony stimulating factor receptor precursor; CD114 antigen	+	++		+	+	+		+++		
8E	Granulocyte-macrophage colony-stimulating factor receptor alpha; CSW116 antigen				+			+	+	+	
8G	Macrophage colony-stimulating factor I receptor precursor; c-fms proto-oncogene; CD115 antigen								+++		
8O	Glia maturation factor beta								+		
9D	Neurotrophin 3 precursor; nerve growth factor 2						+				
9O	Trk-T3; P68 TRK-T3 oncoprotein								+		
10C	Ephrin type A receptor 1 precursor				+				+		
10D	Tyrosine-protein kinase receptor tyro3 precursor; rse; sky; dtk						+				
10F	Eph-related receptor tyrosine kinase ligand 1 precursor; LERK-1; immediate early response protein B61; tumor necrosis factor-alpha-induced protein 4		+								
11F	Colon carcinoma kinase 4 precursor + transmembrane receptor PTK7						+	+++	+	++	
11I	Neurotrophin 4; NT5; NT6-alpha; NT6-beta; NT6-gamma							++	+	++	
11N	Insulin-like growth factor-binding protein 2							+	+		
12E	Insulin Receptor precursor		+		+		+	+	+	++	
12F	Insulin-like growth factor I receptor								+		
12J	CD30 ligand; CD 153 antigen								+		
13A	Orientation Mark		+		+	+			++		
13P	Orientation Mark		+						++		
14E	Lymphotoxin-beta; tumor necrosis factor C		+						++		
14G	Secreted apoptosis related protein		+		+		+	+++	+	+++	
14I	Tumor necrosis factor alpha precursor; cachectin		+			+			+++		
14J	Tumor necrosis factor receptor superfamily member 1A; tumor necrosis factor receptor 1; tumor necrosis factor-binding protein 1; CD120A antigen							+	+		
14K	Tumor necrosis factor receptor superfamily member 1B; tumor necrosis factor receptor 2 precursor; tumor necrosis factor-binding protein 2; CD120B antigen		+		+	+		++	+++		
14M	WSL protein + TRAMP + Apo-3 + death domain receptor 3						+	++	++	+	
14N	Platelet-derived growth factor A subunit precursor		+		+	+		+	+++		
15F	Transforming growth factor beta		++		+	+	+	++	+++	+	
15G	Transforming growth factor beta2 precursor; glioblastoma-derived T-cell suppressor factor; bsc-1 cell growth inhibitor; polyergin; cetermin		+				+	+	+	+	
15J	Bone morphogenetic protein 2A		+					+	++		
15M	Bone morphogenetic protein 4; BMP2B							+	+		
16F	Inhibin beta A subunit precursor; activin AB alpha polypeptide						+++	+			
17G	Cysteine-rich fibroblasts growth factor Receptor 1; Golgi membrane sialoglycoprotein MG160; GLG1		+				+++	++	++	++	

Table 5, continued.

17M	Heparin-binding EGF-like growth factor; diphtheria toxin receptor								++		
18B	Teratocarcinoma-derived growth factor 1; epidermal growth factor-like CRIPTO protein 1; CR3; CRIPTO-1 growth factor; teratocarcinoma derived growth factor		+		++	+	+	++	+++	+	
18G	WebB2 receptor protein-tyrosine kinase; neu proto-oncogene; c-erbB2; HER2 receptor							+			
18H	ErbB3 proto-oncogene; HER3							+	+		
18L	Endothelin 2		+				+	++	+++		+
19B	Ribonuclease/angiogenin inhibitor; placental ribonuclease inhibitor								+		
19C	SL cytokine precursor; FMS-related tyrosine kinase 3 ligand		+		+		+++	+++	+	+++	+
19F	Vascular endothelial growth factor precursor; vascular permeability factor								+		
19L	Vascular endothelial growth factor receptor 3 precursor; FMS-related tyrosine kinase 4								+		
19D	Vascular endothelial growth factor B precursor and VEGF-related factor 186						+				
20B	Endothelial-monocyte activating polypeptide 2						+		+		
20F	Smoothened homolog			+			++	+	+	+	
20H	Wingless-related MMTV Integration Site 5a Protein		++		++	+	+++	+++	+++	+++	+
20L	Frizzled homolog						+	+		+	
21D	Jagged homolog 1						+	+		+	
21E	Jagged homolog 2				++		+				
21F	Neurogenic locus Notch protein		+						++		
21G	Neurogenic locus Notch protein homolog 1 precursor; translocation-associated Notch protein		+						+		
21K	Manic Fringe homolog precursor								+		
22B	BIGH3		+						+++		
22C	Calcitonin receptor			+							
22E	Corticotropin-releasing factor receptor 1 precursor, corticotropin-releasing hormone receptor 1		++			+	+++	+++	+++	+++	+
22F	Basigin precursor; leukocyte activation anitgen M6; collagenase stimulatory factor; ECM metalloproteinase inducer; 5F7; CD147 Antigen						+++		+		
22K	Hepatoma-derived growth factor								+		
22L	G-protein-coupled receptor HM74								+		
22M	CXC chemokine receptor type 4; stromal cell-derived factor 1 receptor; fusin; leukocyte-derived seven transmembrane domain receptor; LCR1	++	++						+++		
23B	Oncostatin M								+		
23C	Platelet-activating factor receptor		+						+		
23F	Renin-binding protein			++							
23G	Thrombin Receptor; F2R; PAR1						+				
23H	Thymosin beta 10; PTMB10	++	+++		+	+	+	++	+++	++	++
23J	Epithelial discoidin domain receptor 1 precursor; cell adhesion kinase; TRKE; RTK6; protein tyrosine kinase 3A; neuroepithelial tyrosine kinase						+	+		+	
24A	Orientation Mark		+						+		
24P	Orientation Mark		+						++		

List of the all spots seen on all array membranes. Key: +++ indicates strong signals (dark dots on membrane); ++ indicates moderate signals (medium dots); + indicates low signals (light dots).

RT-PCR

Four main candidates were chosen from the hybridization array results for further analysis by RT-PCR. All four candidates were seen as potentially upregulated in day-14 cell on the arrays and were chosen because of their known roles in stem cell function. Figure 21 shows the RT-PCR results using primers for CD117.

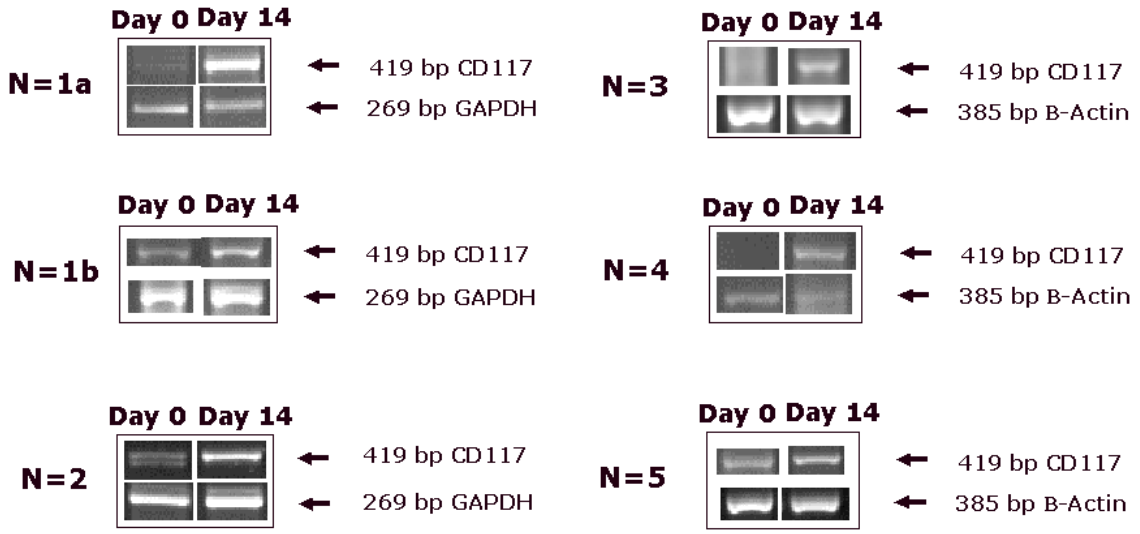

Figure 21. RT-PCR Results for Candidate CD117.

As seen in the figure CD117, stem cell growth factor receptor, was upregulated in day-14 vs. day-0 cells in five independent RT-PCR experiments and one repeat experiments. The first trial (N=1a and N=1b) shows intra-sample results.

Notch-2, a receptor involved in the Notch signaling pathway, was shown to be downregulated (Figure 22) in trials 1, 2 and 3 and upregulated in trial 4 and 5. The downregulation contracts with the upregulation seen in the hybridization arrays. Again, trial one was repeated to test intra-sample results.

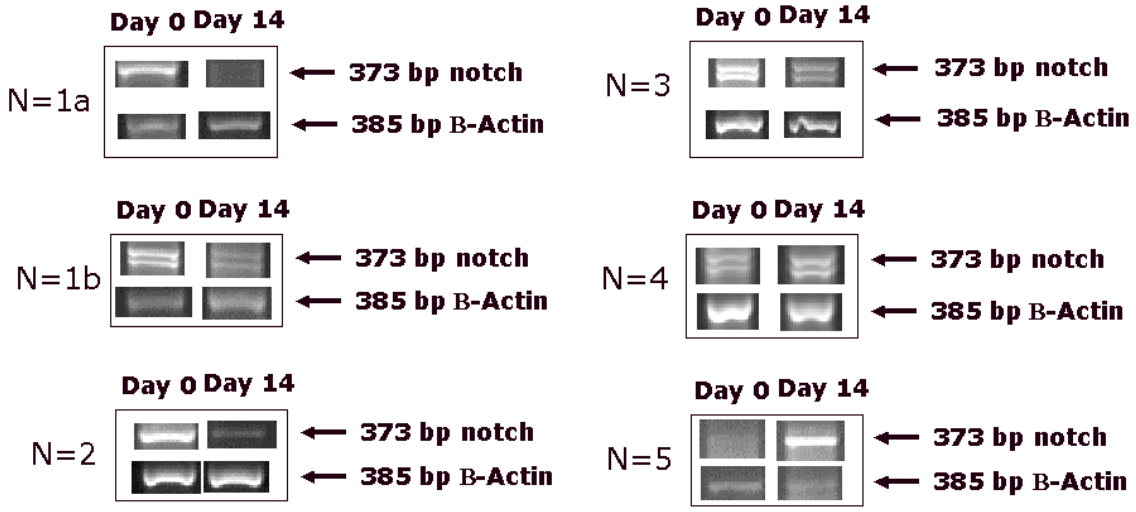

Figure 22. RT-PCR Results for Candidate Notch-2.

Jagged-2, a ligand involved in the Notch signaling pathway was seen to be upregulated in day-14 vs. day-0 samples in all four RT-PCR trials shown in Figure 23. This correlates nicely with the results obtained from the hybridization arrays. A trial to test RT-PCR intra-sample variability for Jagged-2 was not performed due to a lack of RNA samples.

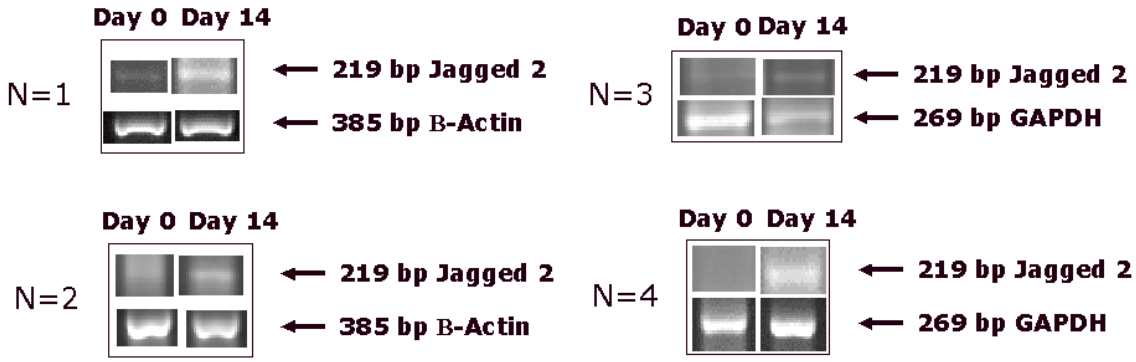

Figure 23. RT-PCR Results for Candidate Jagged-2.

Lastly, the result from the RT-PCR analysis of Manic Fringe, which is a modifier in the Notch signaling pathway, is shown (in Figure 24) to be upregulated in day-14 vs.

day-0 in four independent trials, and about equal in expression for one trial. This correlates with the results seen in the hybridization arrays.

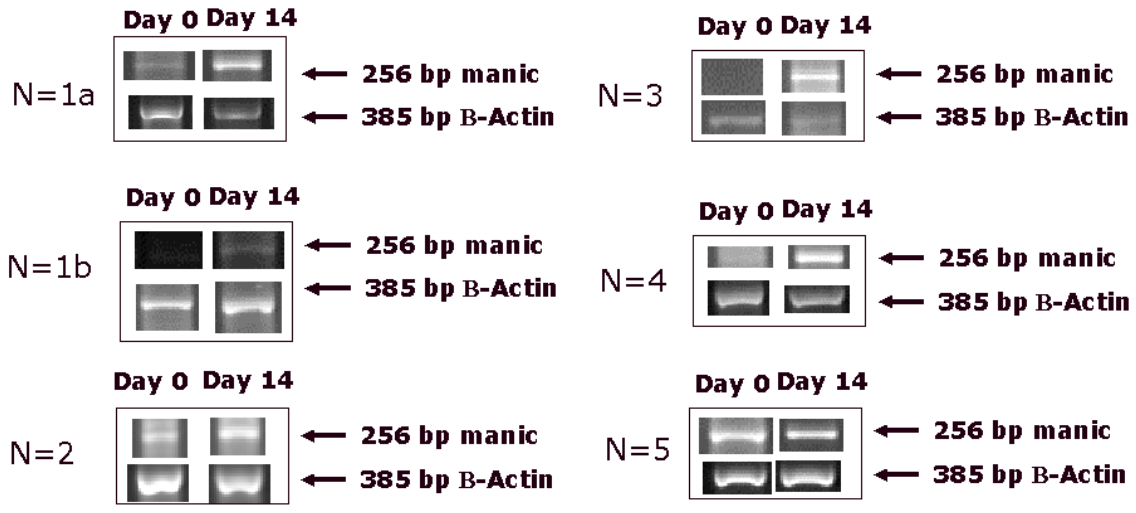

Figure 24. RT-PCR Results for Candidate Manic Fringe.

Restriction Mapping

Restriction mapping was performed on the amplicon for Notch-2 to prove the identity of the PCR band amplified (Figure 25).

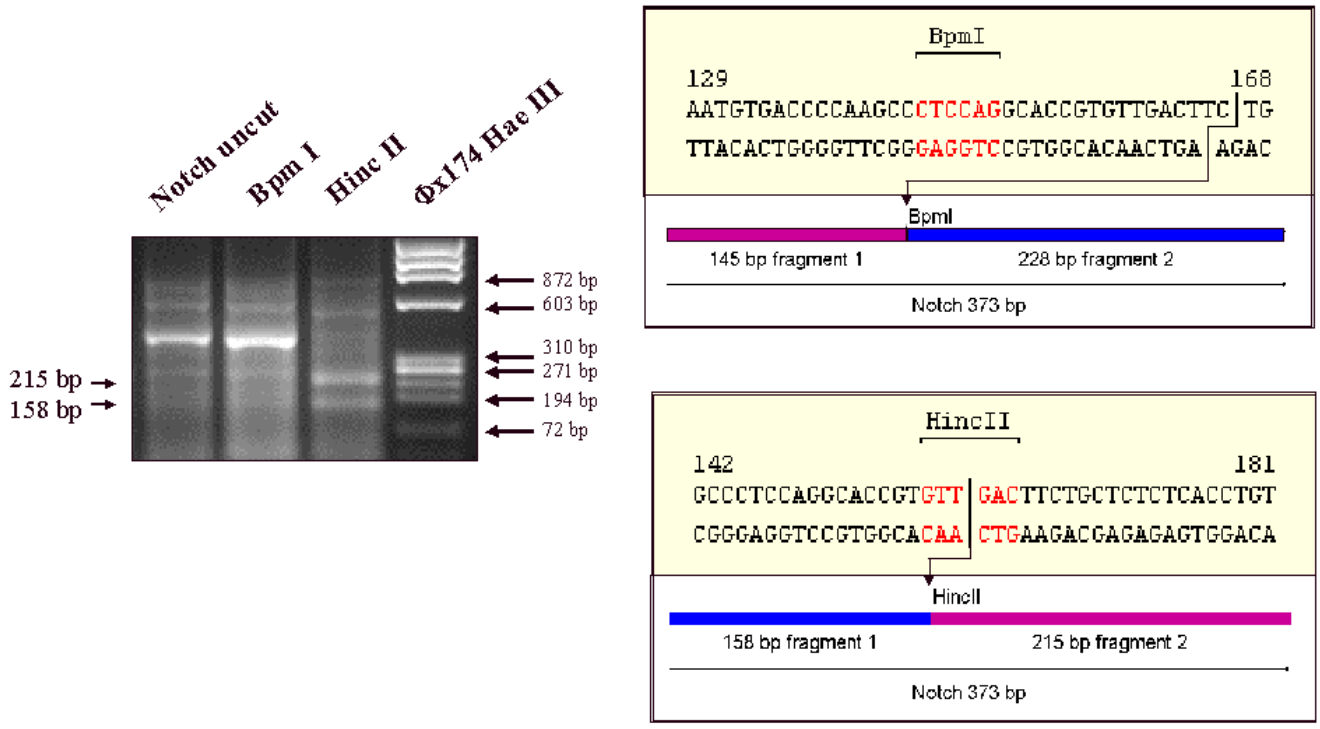

Figure 25. Restriction Mapping of Candidate Notch-2.

In the sequence figure, only a portion of the amplified fragment is shown.

Enzyme HincII produced fragment of the expected size (lane 3), but BpmI unexpectedly did not cut. The success in obtaining fragments of the correct size for Notch-2 cut with HincII could be due to the fact that HincII tends to cut DNA easily under many different salt conditions. The other three RT-PCR amplicons also remained uncut by their enzymes. To try to solve this problem, the PCR products were ethanol precipitated and dissolved in buffers that correspond to the preferred buffer for a given restriction enzyme, but again the bands remained uncut (data not shown). In the future the amplicons will be sequenced for positive identification.

Western Blots

Western Blots were used to determine whether CD117 protein increased at day-14 like its mRNA. The antibody for CD117 was commercially available and purchased from BD PharMingen (catalog number 555714). Figure 26 displays a Western blot performed on cell lysates from a full time course of amplification.

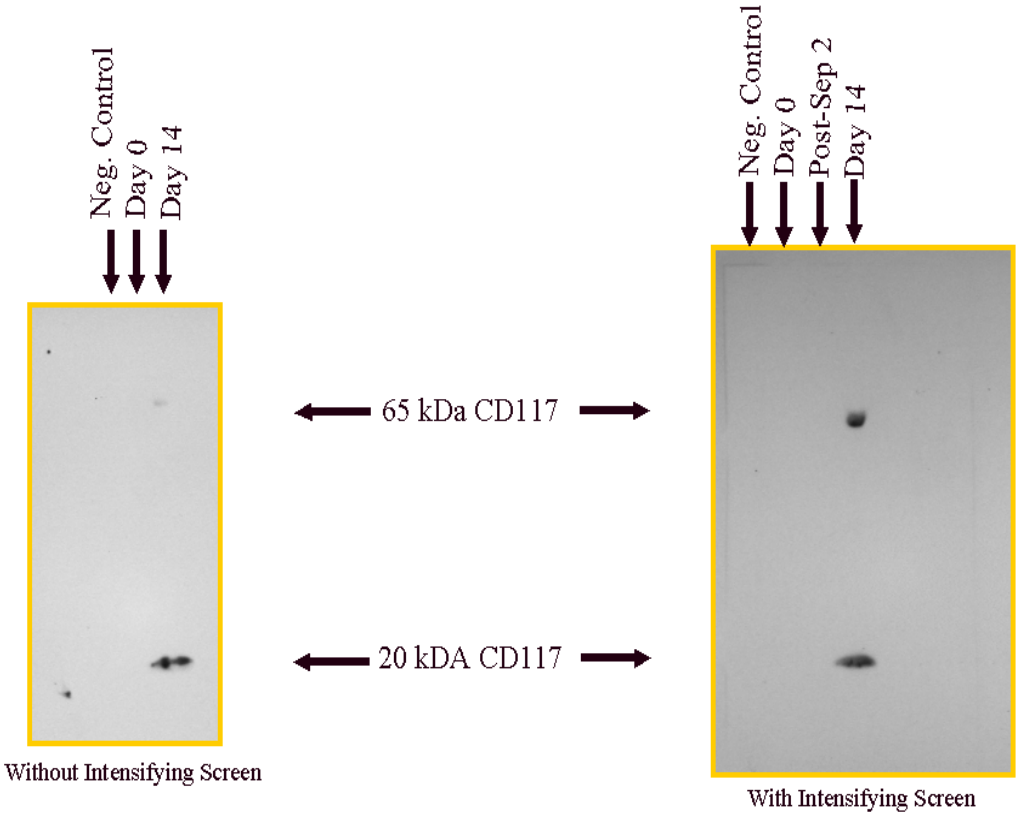

Figure 26. CD117 Western blot.

A biotinylated marker was not available when performing this time course. The sizes on the gel were predicted by comparison to a kaleidoscope marker. From those results, an approximate size for the upper band seen in the day-14 sample is 65 kDa and 20 kDa for the lower band. This does not correlate with the published molecular weight of CD117,

which is 145 kDa (Elmore et al., 2001), but the bands were repeatedly seen so perhaps they represent a CD117-related protein or a denatured fragment.

If this band represents CD117, the data are contradictory to what would be expected. The highest number of HSCs in ViaCell's cellular product is at post-sep-2, not at day-14 so the post-sep-2 sample should have shown the strongest CD117 band. This would suggest that the upregulation of CD117 is not due to the higher number of HSCs in the population, but from the growth factors being given to the cells in culture. Further investigation of CD117 with fresh cell lysates and with a molecular weight marker need to be done.

DISCUSSION

The purpose of this project was to analyze the differential expression of cytokine mRNAs in unamplified UCB cells (containing ~0.3% $CD34^+/CD38^-$ cells on average) versus cells amplified by ViaCell's *Selective Clonogenic Amplification* patented Technology (containing ~11% $CD34^+/CD38^-$ cells on average). This goal was accomplished by isolating total cellular RNA from pooled UCB cells obtained from ViaCell, synthesizing ^{32}P-labelled cDNA, then hybridizing the cDNA to commercially available nylon membranes containing 268 carefully chosen cytokine and cytokine receptor synthetic cDNAs. The resulting membranes were exposed to film and the expression patterns were identified.

RNA Isolations

Regarding the RNA isolations, our data indicate that the Clontech procedure for isolating total cellular RNA from blood works well for HCB samples when $>10^7$ $CD45^+$ cells were used. Yields varying from 0.4 μg to 322 μg were routinely obtained, and denaturing agarose electrophoretic analyses displayed sharp, discrete 28S and 18S rRNAs indicating intact RNA. Yields were usually sufficient for multiple analyses (both arrays and RT-PCR). However, yields were highly variable, perhaps due to cord differences or technique. A microscale procedure, using glycogen as a carrier applied to 10^6 $CD45^+$ cells, usually provided 0 μg-160 μg RNA, however this yield was usually not sufficient for repeated analyses.

Hybridization Arrays

The hybridization array approach used in this project had the advantage of being able to simultaneously screen 268 carefully chosen cytokine mRNAs, but this approach also had some disadvantages. One disadvantage is that using Clontech's recommended RNA isolation protocol, so many cells were required ($>10^7$) that cords were pooled (usually 3) to obtain enough material. When we used less than this, no RNA pellet was obtained. This meant we were unable to assay individual cords, which might have shown interesting cytokine differences between individual donors. Secondly, the Clontech procedure did not produce RNA clean enough to survive 37°C incubations with DNase. In the future, different RNA isolation procedures could be tried to lower the required starting material to one cord (after all, only 1 μg total RNA is needed per cDNA reaction), and to produce cord RNA samples clean enough (free of RNases) to allow 37°C DNase treatments. Lastly, the hybridization arrays can only be used a maximum of three times. Therefore new arrays must be purchased after the maximum of three uses.

Regarding the arrays, in our hands the variable having the greatest impact on dot profiles was the specific activity of the probes. Within a given RNA sample, the number of dots displayed varied from experiment to experiment. Nevertheless, when high specific activity probes were used, all housekeepers were usually present, and the main expression profiles were mostly reproducible for a given sample. Our most accurate comparisons were achieved under conditions in which the housekeepers were equally visible.

The variability and reproducibility of these hybridization array methods were addressed in several ways. Inter-sample variability was tested by performing the same

array protocol on different day-0 samples or day-14 samples. Although some differences were observed, the main expression patterns were mostly reproducible. Stripping the original membrane and reusing it with a new batch of cDNA prepared from the same RNA tested intra-sample variability, the variability seen using one specific sample. These assays showed the greatest consistency, however in our hands, a given membrane could only be used a total of 3 times. In the future we also need to test inter-filter variation more accurately by using one large cDNA reaction split in half. Because differences in the specific activity of individual ^{32}P-cDNA samples varied so much, (which greatly affected the total number of dots displayed) future attempts will be made to assay probe specific activity before proceeding with the experiment, or to analyze only those arrays hybridized to high specific activity probes.

Several candidate mRNAs appeared to be expressed equally between the day-0 and day-14 samples. These candidates included: several interleukins (IL-8, IL-10), interleukin receptors (IL-2R, IL-6R), growth factors (B-cell growth factor, leukocyte IFN inducible protein, small inducible cytokines A5 and B5, TGF-β), inhibitory factors (migration inhibitory factor), developmental homologs (smoothened homolog), and others (platelet-activating-R, thymosin-β, insulin-R, and calgranulin-B).

For the day-14 samples, however, many more types of mRNAs were repeatedly observed, including: several interleukins (IL-1β, IL-6, IL-13), interleukin receptors (IL-1R, IL-2R, IL-7R), growth factors (hepatocyte growth factor-like protein, platelet-derived growth factor, MCSF, small inducible cytokines A3 and A4, inhibin-β, glial maturation factor, NGF-2, Trk-T3 oncoprotein, PTK-7 colon carcinoma kinase, neurotrophin-4, insulin-like growth factor-binding protein, oncostatin-M, EGF-like

growth factor, hepatoma-derived growth factor, ErbB3 proto-oncogene, endothelin-2, SL cytokine, vascular endothelial growth factor, endothelial monocyte activating polypeptide), growth factor receptors (FGF-R, CD88, CDW40, CD117 stem cell growth factor-R, CD114 GCSF-R, CD115 MCSF-R, epithelial discoidin-R, G-protein-coupled-R, CRF-R, vascular endothelial-R), developmentally interesting proteins (Manic Fringe homolog, wingless, frizzled, jagged-1, Notch protein), interferon-related proteins (IFN-α-R, IFN-β-R, IFN-γ antagonist, IFN regulatory factor), and other proteins (BIGH3, humig, basigin).

This is exciting information since the observed upregulations did not contain many inflammatory cytokines, which could hinder survival of these therapeutic cells as grafts in the future. Only small upregulations of macrophage inflammatory protein-1 α and β, TNF-α, and TNF-R were observed.

Further Analysis of Selected Candidates by RT-PCR

Several developmentally interesting mRNA candidates were upregulated in the day-14 samples. Jagged-1 was detected in three day-14 experiments, but in none of the five day-0 experiments. Jagged-1 represents a growth factor of human stem cells, and is expressed by uncommitted human hematopoietic cells and cells that comprise the putative blood stem cell microenvironment (Karanu et al., 2000). Another developmentally interesting cytokine expressed in day-14 cells was the Frizzled Homolog. It was detected in three of the day-14 experiments, but in none of the five day-0 experiments. Frizzled is a protein, which can stimulate the survival and proliferation of hematopoietic progenitors (Austin et al., 1997). However these two interesting

candidates were not pursued further because initial attempts at RT-PCR failed to produce amplicons of the corrected size, so perhaps in the future different primer sets could be tested.

Four other candidates identified using the hybridization arrays were chosen for further analysis by RT-PCR. These candidates include CD117 (c-kit), Jagged-2, Manic Fringe, and Notch.

CD117

CD117 is a stem cell growth factor receptor. Using RT-PCR CD117 was shown to be upregulated in day-14 cells relative to day-0 in five independent trials. The upregulation of CD117 is exciting because it validates ViaCell's clonogenic amplification protocol for this stem cell-enriched fraction.

Stem Cell Growth Factor receptor (c-kit, CD117) is expressed on almost all hematopoietic progenitor cells, and the receptor/ligand interaction is crucial for the development of hematopoietic stem cells. CD117 is a tyrosine kinase cell surface receptor for stem cell factor (Carson et al., 1994) known to support the cycling of CD34+ cells in the absence of other cytokines (Birkmann et al., 1997).

Since many contradictory reports have been published on the expression of CD117 in conjunction with HSCs, a study was done by Ratajczak et al. (1999) to reevaluate the role of CD117. They validated CD117's status as a stem cell marker by finding that human HSCs do not reside within a CD34+KIT- cell population. Cells isolated from human bone marrow were sorted by FACS and evaluated for their ability to engraft in an immunodeficient SCID mouse model. Only CD34+KIT+ cells, but not

CD34+KIT- or CD34-KIT- cells, possess the ability to establish a human-murine hematopoietic chimerism in the mice. They argue that CD117 is a very useful marker for identifying human HSCs, present in CD34+KIT+ cell populations.

Many studies have been performed to determine the role that CD117 and its ligand have on hematopoietic progenitor cells. A study done by Simmons et al. (1994) examined the expression of c-kit protein on primitive $CD34^{+}$ hematopoietic cells isolated from human bone marrow. They looked for coexpression of c-kit and early lymphoid markers in the CD34+ populations by multiparameter flow cytometry, and found coexpression of c-kit only on a minority of cells with markers of B or T lineages. However, the vast majority of early lymphoid cells lacked c-kit expression.

Notch Signaling Pathway

The Notch protein is a surface receptor that transmits signals received from outside the cell to the cell's interior. Its main function is to repress the adoption of differentiation by cells that carry the Notch protein (Society for Developmental Biology, 2000) as seen in Figure 27.

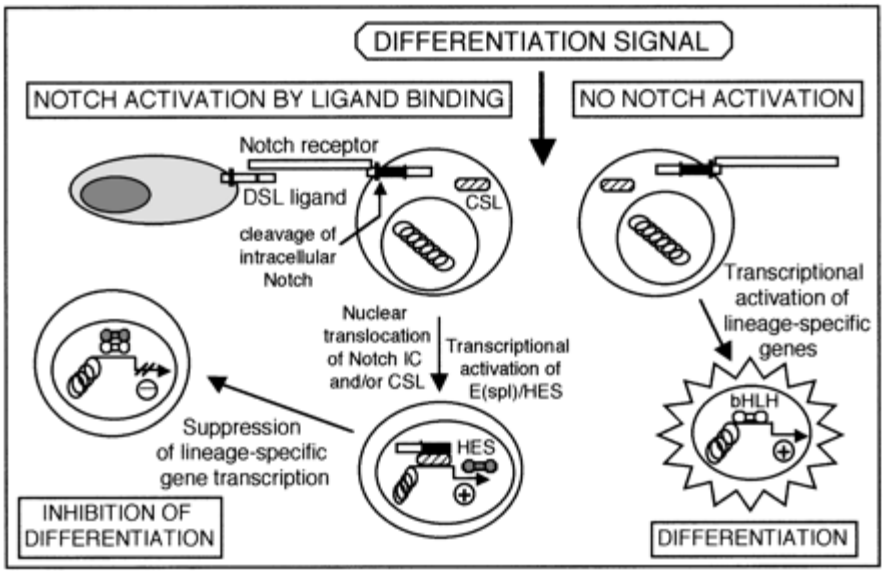

Figure 27. The role of Notch in differentiation.

When DSL proteins (the ligands for Notch) bind, an inhibition of differentiation is observed. Differentiation is observed when the receptor/ligand interaction does not occur (Milner and Bigas, 1999).

Members of the Notch family have a critical role in hematopoiesis by regulating cell fate and self-renewal of progenitors (Milner and Bigas, 1999). Table 6 summarizes the components involved in the Notch-signaling pathway in flies, worms, and mammals.

	C. elegans	*Drosophila*	Mammals
NOTCH receptors	Lin-12 Glp-1	Notch	Notch-1, Notch-2, Notch-3, Notch-4
Extracellular ligands (DSL proteins)	Lag-2 Apx-1	Delta Serrate	Delta-1, Delta-like-1, Delta-like-3 Jagged-1 (Serrate-1), Jagged-2 (Serrate-2)
Intracellular effectors	Lag-1	Suppressor of Hairless Deltex	CBF-1/RBP-J_K Deltex NF_KB
Target genes		Enhancer of split bHLH Groucho	HES (Hairy/Enhancer of split) bHLH TLE
Processing molecules	SUP-17	Kuzbanian	Kuzbanian
Modifiers		Fringe Numb Dishevelled	Lunatic Fringe, Manic Fringe, Radical Fringe Numb,Numb-like Dishevelled 1,2,3

Table 6. Components of the Notch Signaling Pathway.

(Milner and Bigas, 1999)

Notch signaling can occur either among a group of equivalent cells (homotypic interactions) or between nonequivalent cells (heterotypic interactions) (Milner and Bigas, 1999). In homotypic interactions, a group of equipotent cells exposed to a specific differentiation signal will adopt a specific cell fate, whereas adjacent cells (that express more Notch) are inhibited from differentiating (Milner and Bigas, 1999). The uncommitted cells may undergo alternative fates, which again is regulated by Notch. Heterotypic interactions are regulated by the binding of Notch with its ligands DSL proteins, making Notch activation dependent on those cells that are in direct contact with ligand-expressing cells and by other molecules such as Fringe and Wingless (Milner and Bigas, 1999). The overall regulation of Notch is viewed as inhibiting differentiation of cells along a specific pathway, leaving undifferentiated cells capable of expanding or adopting a default pathway. In the context of the normal hematopoietic

microenvironment, regulation of Notch activity through extracellular ligand binding will permit only those cells maintaining the highest relative levels of *Notch* expression to remain multipotent (Milner et al., 1996). Other factors activate or inactivate the Notch pathway. When Granulocyte Colony Stimulating Factor (G-CSF) is introduced, it activates Notch through its effects on the Notch-1 receptor (Bigas et al., 1998). In the same way, when Granulocyte Macrophage Stimulating Factor (GM-CSF) is introduced, it activates Notch through its effects on the Notch-2 receptor (Bigas et al., 1998). Better understanding of the conditions of activation and inactivation of the Notch signaling pathway may help in further studies of stem cell expansion techniques.

Notch-2

Although Notch-2 mRNA appeared to be increased in day-14 populations relative to day-0 by array analyses, in agreement with its role in preventing differentiation, it actually was down regulated according to three RT-PCR trials. Notch 2 is one of the four known Notch receptors. Notch receptors are present on the surface of the cell as heterodimers, generated by cleavage of full-length Notch protein and reassociaton by disulfide bonds of extracellular and intracellular cleavage products (Milner and Bigas, 1999). It has been shown that the Notch-2 receptor (as well as Notch 3 and 4) is expressed by human hematopoietic progenitors (Milner et al., 1994). A detailed investigation of the Notch receptors by Milner and colleagues showed:

> the ability of two mammalian Notch homologs, mouse Notch1 and Notch2, to inhibit the granulocytic differentiation of 32D myeloid progenitor cells. 32D cells undergo granulocytic differentiation when stimulated with either granulocyte colony-stimulating factor (G-CSF) or granulocyte-macrophage colony-stimulating factor (GM-CSF). Expression of the activated intracellular domain of Notch1 inhibits the differentiation

> induced by G-CSF but not by GM-CSF; conversely, the corresponding domain of Notch2 inhibits differentiation in response to GM-CSF but not to G-CSF. The region immediately C-terminal to the cdc10 domain of Notch confers cytokine specificity on the cdc10 domain. The cytokine response patterns of Notch1 and Notch2 are transferred with this region, which we have termed the Notch cytokine response (NCR) region. The NCR region is also associated with differences in posttranslational modification and subcellular localization of the different Notch molecules. These findings suggest that the multiple forms of Notch found in mammals have structural differences that allow their function to be modulated by specific differentiation signals (Bigas et al., 1998).

The effects of Notch1 and Notch2 may vary among individual cells. The signaling of Notch receptors with its ligands could prove to be a mechanism in which progenitor cells "communicate" with each other to signal differentiation of cells while also inhibiting differentiation of other cell types in order to keep a pool of mulitpotent progenitors. The apparent disconnect between our array and RT-PCR data for Notch-2 warrants further investigation.

Manic Fringe

Manic Fringe homolog was upregulated in three independent RT-PCR trials. Manic Fringe homolog, one of three mammalian homologs of Drosophila Fringe (Manic, Lunatic, and Radical), modulates the activation of the Notch signal transduction pathway. Expression studies in mouse embryos support a conserved role for mammalian Fringe family members in participation in the Notch signaling pathway leading to boundary determination during segmentation (http://stemcell.princeton.edu/table1.html). Manic Fringe, specifically, was found to be highly expressed in uncommitted precursor cells as well as in committed neutrophil and macrophage precursors by Singh and colleagues

(2000). Thus our RT-PCR and array data for Manic Fringe further validates the ViaCell purification protocol.

The Fringe genes encode molecules that modulate interactions between Notch and ligands (Milner and Bigas, 1999). The Fringe protein modulates the ability of Notch ligands to activate Notch signaling (Moloney et al., 2000). The molecular mechanism in which this happens has not been determined. Recent work by Moloney et al. (Ibid.) proposes that Fringe modulates Notch signaling by direct modification of *O*-linked fucose residues within EGF repeats of the Notch extracellular domain. These results are inconsistent with the recent proposal that Fringe functions by forming a stable complex with Notch (Ju, 2000). Even though the mechanism in which Fringe influences Notch is unknown, it is certain that it has a role in affecting the differentiation of cells through the Notch-signaling pathway.

Manic Fringe is a modifier of the Notch-signaling pathway. Its effect on differentiation is seen in Figure 28. Manic Fringe must be expressed on the same cell as Notch in order to have an effect on the Notch-signaling pathway. Manic Fringe potentiates signaling from Delta while inhibiting signaling from Jagged (Haltiwanger, 2001).

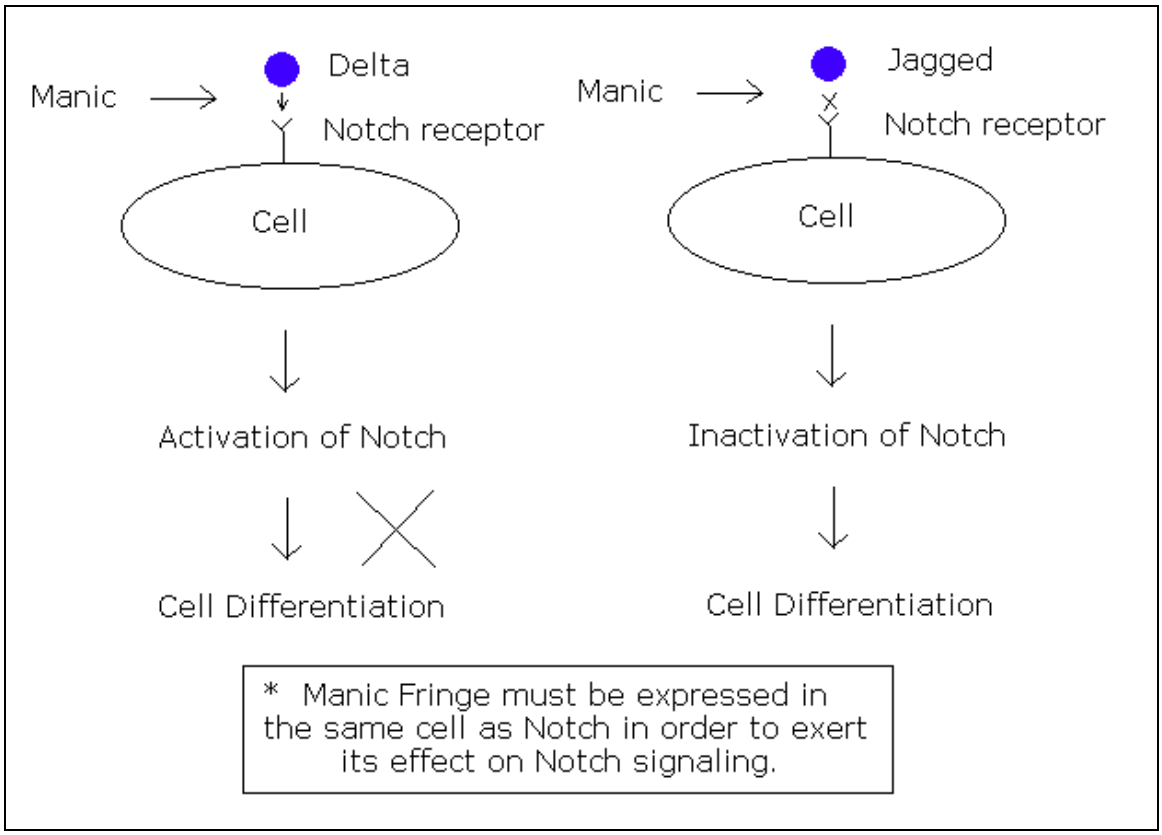

Figure 28. The Effect of Manic Fringe on the Notch-Signaling Pathway.

Jagged-2

Jagged-2 was seen to be upregulated in day-14 cells in four independent RT-PCR experiments. A higher expression of Jagged-2 could mean that the amplified stem cells are in fact uncommitted HSCs because it is known that Jagged-2 is expressed by uncommitted HSCs that comprise the putative blood stem cell microenvironment (Karanu et al., 2000). This upregulation could be used as a marker for HSCs in future cell lines to be used for human grafting.

Jagged-2 is an extracellular ligand involved in the Notch-signaling pathway. Coexpression of Jagged-2 and Notch-1 in the developing thymus suggests that Jagged-2 is a ligand for the Notch-1 receptor (Milner and Bigas 1999). Work by Luo et al. (1997)

found that a culture of fibroblasts expressing human Jagged-2 in murine C2C12 myoblasts inhibited myogenic differentiation accompanied by increased Notch-1. The exposure of C2C12 cells to Jagged-2 led to increased amounts of Notch mRNA. The binding of Jagged-2 to Notch-1 caused a positive feedback control of genes seen when active forms of Notch-1 induced increased levels of the same set of mRNAs. This feedback control may function in vivo to coordinate the differentiation of progenitor cells adopting identical cell fates (Luo et al., 1997). Work done by Singh et al. (2000) found that activation of endogenous Notch by Jagged-2 decreased proliferation and reduced differentiation of normal human $CD34^+$ cells.

Studies presently being done to examine the role of the Notch-signaling pathway on differentiation suggest that Notch ligands, such as Jagged-2, may be useful for in vitro expansion of hematopoietic stem/progenitor cells. However, the complex interactions involving various Notch molecules, ligands, and cytokines may present a considerable challenge (Milner and Bigas, 1999). A brief summary of the effects of the expression of Notch receptors (such as Notch-2) and ligands (such as Jagged-2) on differentiation of cells is seen in Figure 29.

If its true that Manic Fringe blocks the interaction of Jagged-2 with Notch receptor (Haltiwanger, 2001) it is not immediately clear how to explain our observed increases in both Manic Fringe and Jagged-2. Perhaps the subset of cells showing the increase in Manic Fringe act to facilitate Notch signaling via Delta proteins (blocking cell differentiation), while a different subset of cells express Jagged-2 to also stimulate Notch signaling.

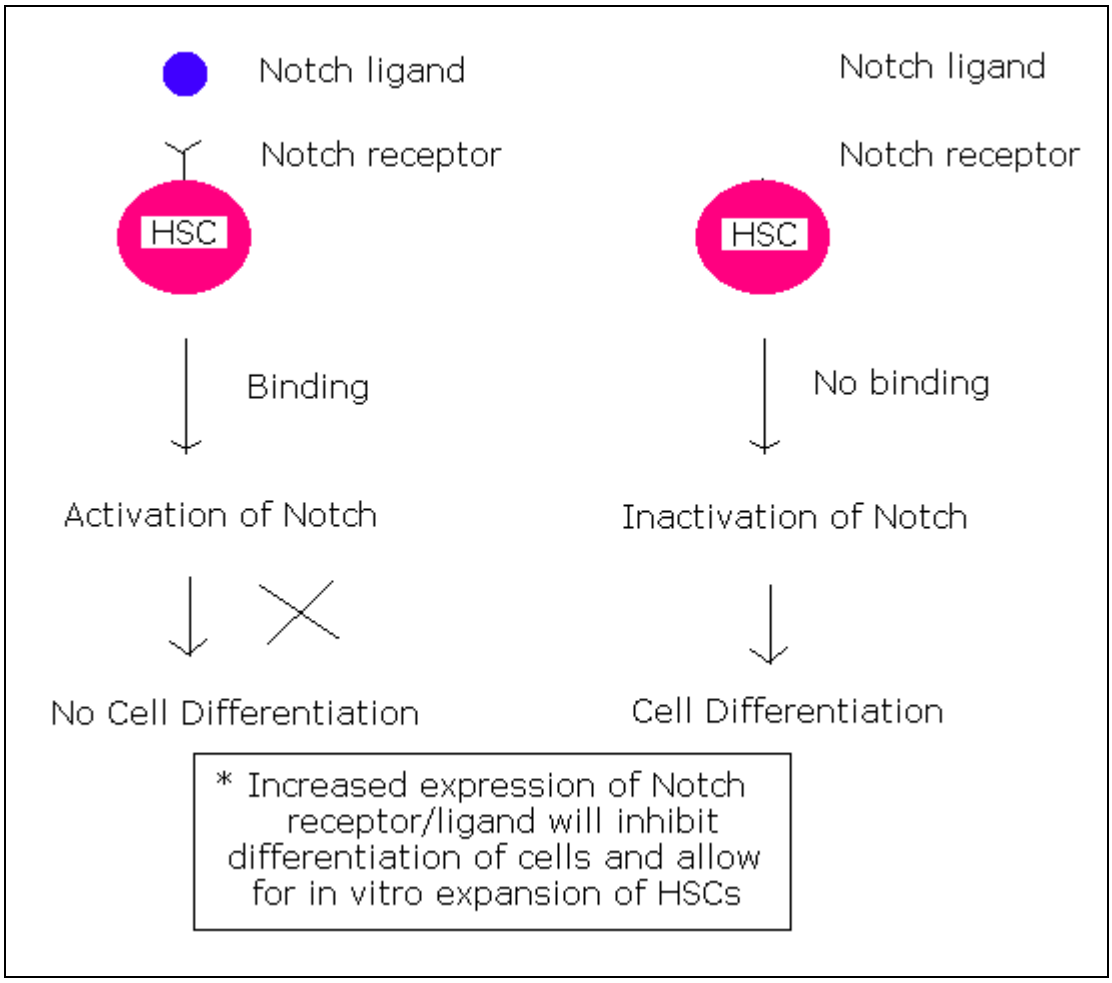

Figure 29. The Effects of the Expression of Notch Receptors and Ligands on Differentiation.

Future Experiments

In the future, the array work could be expanded to include post-sep-2 samples which are especially enriched (˜33% of the cell population) for CD34+/CD38-/Lin- stem cells. Only high specific activity probes should be used. For the RT-PCR experiments, those candidates showing a disconnect with the arrays could be further investigated by designing different primers for that mRNA to ensure the correct mRNA is being analyzed. The Western blots need to be improved by running markers on all gels, and by

extending the analysis to candidates other than CD117 if appropriate antibodies are available.

Regarding new potential treatments of the cultured stem cells, more work could be done to try to activate the Notch-signaling pathway (i.e. by adding Jagged-2 to the medium). This activation could be very beneficial since it inhibits the differentiation of cells and could minimize production of unwanted mature cells in grafts. This activation allows for in vitro expansion of a culture of HSCs, since the cells are inhibited from further differentiation. To test for specific Notch pathway activation a microarray, similar to the one used for this project, could be custom designed that contains all of the components of the Notch signaling pathway. In order to better design an optimal array for ViaCell, preliminary experiments could be done by testing the expression of genes on an array other than one specific for human cytokines and their receptors. The developmentally interesting non-cytokine related candidates could be added to the custom designed array. The results of this array would give a better understanding of the mechanisms affecting differentiation of the cell samples obtained from ViaCell.

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