Published in final edited form as:

Mol Diagn Ther. 2009 ; 13(3): 181–193. doi:10.2165/01250444-200913030-00003.

Global Transcriptional Analysis for Biomarker Discovery and Validation in Cancer and Hematological Malignancy Biologic Therapies

David F. Stroncek, Ping Jin, Ena Wang, Jiagiang Ren, Marianna Sabatino, and Francesco M. Marincola

Department of Transfusion Medicine, Clinical Center, National Institutes of Health, Bethesda, Maryland, USA

Abstract

Potency testing is an important part of the evaluation of cellular therapy products. Potency assays are quantitative measures of a product-specific biologic activity that is linked to a relevant biologic property and, ideally, a product's *in vivo* mechanism of action. Both *in vivo* and *in vitro* assays can be used for potency testing. Since there is often a limited period of time between the completion of production and the release from the laboratory for administration to the patient, *in vitro* assays such as flow cytometry, ELISA, and cytotoxicity are typically used. Better potency assays are needed to assess the complex and multiple functions of cellular therapy products, some of which are not well understood. Gene expression profiling using microarray technology has been widely and effectively used to assess changes of cells in response to stimuli and to classify cancers. Preliminary studies have shown that the expression of non-coding microRNA (miRNA), which plays an important role in cellular development, differentiation, metabolism, and signal transduction, can distinguish between different types of stem cells and leukocytes. Both gene and miRNA expression profiling have the potential to be important tools for testing the potency of cellular therapies. Potency testing, the complexities associated with potency testing of cellular therapies, and the potential role of gene and miRNA expression microarrays in potency testing of cellular therapies are discussed.

1. Background: Cellular Therapies

Cellular and gene therapies are making a major contribution to the emerging field of biologic therapy for cancer and hematologic malignancies. The possibilities for the clinical application of new cellular therapy products are expanding rapidly, as is their clinical promise. The diversity and effectiveness of cellular therapies that are now available has encouraged the development of new clinical applications and improved the quality of life of patients. These therapies include adoptive immune therapy utilizing enriched or *in vitro* manipulated autologous or allogeneic immune cells,[1,2] hematopoietic stem cell (HSC) transplantation, [3,4] mesenchymal stem cells, and genetic modification of immune cells.[5] As this field matures, the ability to produce large quantities of biologic products with predictable quality and quantifiable potency is becoming critical.

The complexity of cellular cancer therapies is increasing as new knowledge about the function of specific cell types and their biologic status becomes available. For example, the initial

Correspondence: Dr David F. Stroncek, Department of Transfusion Medicine, Clinical Center, National Institutes of Health, Building 10, Room 1C711, 10 Center Drive-MS-C-1184, Bethesda, MD 20892-1184, USA. dstroncek@cc.nih.gov.

The authors have no conflicts of interest directly related to the content of this review.

adoptive immune therapy protocols to treat cancer once involved only the administration of autologous tumor-infiltrating leukocytes (TILs)[6] or leukocyte activated killer cells.[7] Now adoptive immune therapy protocols are combination therapies that include high-dose chemotherapy, the administration of *in vitro* activated and primed TILs, tumor vaccines, dendritic cells (DCs), autologous HSCs,[8] and genetically engineered cells. Immunosuppressive chemotherapy depletes the patient's naturally occurring repertoire of lymphocytes, including T regulatory (T_{reg}) cells. The lack of T_{reg} cells and increased levels of cytokines, including interleukin (IL)-7, that are associated with leukopenia allow for the rapid and marked *in vivo* expansion of TILs administered with hematopoietic progenitor cells.[8] Some adoptive immune therapy protocols use peptide vaccines both with and without DCs. Gene therapy is also becoming an important part of adoptive immune cancer therapy. Both gene-modified T cells and DCs are being used in cancer immunotherapy.[9]

The production of cellular therapies is also becoming more complex. Most clinical cellular therapy products require cell mobilization, collection, subset isolation, *in vitro* or *in vivo* stimulation, and culture and expansion of cells over a period of several days. The production of some cellular therapies involves serial isolation steps and multiple stimulation and/or culturing steps. Cellular therapy product manufacture is further complicated by donor or patient genetic and physiologic heterogeneity. The final product is often markedly different from the starting material. It is important to produce cellular therapy products that provide the desired clinical effect without resulting in adverse effects. An adequate dose of cells must be provided to each patient, each product must meet release specifications, and lot-to-lot variation should be minimized. In order to produce consistently high-quality products, quality assurance has become a critical part of cellular therapy laboratories.

Cell therapies must be safe, pure, stable, and potent. To ensure that cell therapies have these properties, they are tested in many ways both during and at the end of their production. The analysis of cell therapies has become an important part of the delivery of effective clinical cellular therapies. Most cell therapies are currently being assessed using automated cell counting and analysis, morphology, enzyme-linked immunosorbent assay (ELISA), flow cytometry, colony-forming culture assays, cytokine release assays, cytotoxicity assays, and sterility assays. Although molecular assays are in common use in the clinical diagnostic and research laboratory, these techniques have not yet achieved widespread usage in the cellular therapy laboratory. The molecular methods that are now widely used to analyze cells and cellular systems in the research laboratories are seldom used to assess clinical cellular therapies. Global gene transcriptional analysis has become a cornerstone in the analysis of cancer and host response to cancer. These assays also have the potential to become a powerful tool for the analysis of cellular therapies. They could be used to identify biomarkers that correlate with the safety, purity, and potency of the product; confirm that therapies prepared using different methods are equivalent; or assess changes in the cellular therapy manufacturing process.

Although global transcriptional and microRNA (miRNA) analysis has tremendous potential for assessing cellular therapies, biomarkers of the viability, stability, purity, and potency of each cellular therapy must first be identified. This paper reviews the need to identify cellular therapy biomarkers, and the emerging and future applications of molecular assays to assess cellular therapies.

2. Potency Testing

One of the most important and difficult analyses required for cellular therapies is potency testing. One of the most important aspects of assessing the quality of cellular therapy products is to ensure that all products meet established minimal levels or ranges of potency, and that

potency levels are consistent across manufacturing lots. Potency testing involves quantitative measurement of the biologic activity of a product.[10-12]

Potency assessments are meant to measure the critical biologic activity of a cellular therapy product within a complex mixture by quantifying the activity of the product in a biologic system. Measurement of the potency of a product is not the same as measuring clinical efficacy but, rather, is a means to control product consistency. Generally, potency testing is performed at the time of product lot release and across all production lots.

2.1 Complexities Associated with Potency Testing of Cellular Therapies

Potency testing of cellular therapies is particularly challenging for several reasons. Specifically, such testing is often limited by the:

- small quantity of final product available to test
- short time to perform potency testing at the time of lot release
- limited stability of most cellular therapy products
- insufficient availability of reference standards
- high variability among lots

Since most cellular therapies produced in academic health centers are patient specific, there is usually a limited quantity of suitable source material and, therefore, a limited amount of final (i.e. ready to administer) biologic material to use for lot release and potency testing. The starting materials for most cellular therapies are cells collected from human subjects. The subjects may be the person being treated (autologous products), or a living donor (allogeneic products). For both situations, the quantity of starting material that can be collected is limited and consequently the amount of material produced is limited. As a result, an entire production lot of a cellular therapy is usually administered to a single patient, and the use of large quantities of the product for lot release testing may adversely affect the dose and clinical effectiveness of the product. This limitation on the quantity of material available prevents the use of some assays and/or limits the number of analytes that can be tested. This is generally the case with DC and HSC therapies.

In addition, the time to test the product is limited, since cellular therapy products must be tested at the time production is complete but prior to release of the product for clinical use. This is particularly problematic for cellular therapies, since the potency of many living cells is affected by prolonged storage at physiologic temperatures. Although some cellular therapies can be cryopreserved, the freeze-thaw process results in cell loss, and most cell therapies are administered within hours upon production completion. Handling also affects the potency of some products.

Finally, cellular therapy products typically show a large degree of lot-to-lot variability. Product variability is due, in part, to inherent variability in the starting cells or tissues.

2.2 Factors Affecting the Potency of Cellular Therapies

Despite the difficulties associated with potency testing of cellular therapies, potency is particularly important for these products, since the complexities associated with their production can result in considerable differences in potency among different lots of the same product. These differences arise from variations in the starting cellular material (including genetic differences among individuals donating the starting cells), and are related to the multiple steps required, and the multiple biologic products used, to produce most cellular therapies. Potency is also affected by the limited stability of the final product. Further

complexities stem from the fact that clinical effectiveness may be dependent on multiple cellular functions and complex mechanisms of action.

Advanced cellular therapies may incorporate multiple components. For example, cellular products used for cancer vaccines may require more than one peptide to educate immune cells *in vitro*, followed by cytokine stimulation. A manipulated lymphocyte component prepared for an HSC transplant donor may involve isolating and recombining multiple cells of different types. The multiple cell types present in many cellular therapies have the potential to interfere with one another or to act synergistically.

Many cellular therapies are subject to extensive manipulation, including manufacturing processes such as cytokine, growth factor, or antigen stimulation; culture; expansion; and treatment with vectors or toxins. For these products, slight variations in the starting cellular material, reagents, processing methods, or culture conditions may result in significant variation in the final product, which leads to heterogeneous clinical outcomes of the same therapies.

Donor genetic factors are likely to contribute to differences in potency of the final cellular therapy product. Genetic polymorphisms in cytokines, growth factors, and their receptors affect the cellular immune response.[13-16] It is likely that these polymorphisms affect the response of cells to cytokine and growth factor stimulation *in vitro* and the behavior of cells during culture. Epigenetic changes may also be important. The same type of cells obtained from different donors at different time points and under different physiologic conditions could vary significantly because of genetic heterogeneities, epigenetic differences, or transcription regulation diversities.

Finally, the *in vivo* function of most cellular therapies is dependent on multiple factors in the host environment. HSCs must traffic to specific sites, expand, and differentiate into several mature cell types. Immune therapies must migrate from the site of administration, interact with tumor or other immune cells, and respond to stimuli and/or stimulate other cells.

2.3 Measuring Potency of Cellular Therapies

Potency can be tested in a number of ways, including *in vivo* and *in vitro* systems (table I). Testing potency using *in vivo* animal models is generally preferred over *in vitro* test systems, since animal models assays have the ability to directly measure the functional activity of a product. However, existing animal models may not be relevant, and new animal models may be difficult to develop.[10] In addition, the results of *in vivo* tests are often variable and difficult to reproduce. Furthermore, these assays usually take a considerable amount of time to complete, which makes it difficult to use these assays for routine lot release testing. Many *in vivo* assays are best suited for use in product development, as an in-process control, or to evaluate the potential effect of changes in the manufacturing process or materials.[11]

In vitro assays involve the measurement of biochemical or physiologic responses at the cellular level.[10] The *in vitro* measurement of cell surface markers, activation markers, secretion of factors, or protein expression does not directly measure the function of a cellular product; however, such measurements have been used as surrogates for potency. When an *in vitro* assay is used as a surrogate for potency, a correlation should be demonstrated between the assay results and the intended biologic activity. Typical *in vitro* assays used as surrogates for potency testing include ELISA, enzyme-linked immunosorbent spot (ELISPOT) assays, flow cytometry, proteomic analysis, and cytotoxicity assays.

When the mechanism of action of a cellular therapy can be attributed to the expression of specific cell surface antigens, the measurement of antigens by flow cytometry can be used as an *in vitro* potency assay. In fact, the measurement of biomarkers by flow cytometry is often

used as a surrogate measure of cell potency. Flow cytometry is useful because of the large number of reagents and assays that are available, as well as the relatively quick turnaround time. It can be used to measure the expression of cell surface markers, viability, and the production of cytokines. Extensive analysis of cell surface markers using flow cytometry has been used to assess cellular therapies, but the maximum number of markers that can be analyzed is limited by the availability of specific antibodies, instrumental detection limits, and final product quantity. In addition, the markers that may be most useful may not be known

In vitro cell function assays have also been used to measure cell potency. Cytotoxicity assays are sometimes used to reflect the function of adoptive immune therapies. Cytokine release by stimulated cells can also be used to measure cell function. However, cell function assays have many limitations. Although they may be able to detect differences in relevant biologic activity, these assays are typically highly specialized for each cell type, are labor intensive, and require highly skilled staff. Different types of cells and cell subsets require completely different types of cell function technologies. Many cell types require the measurement of multiple functions to adequately assess potency. Furthermore, the function(s) that best predict cell potency may not be known. In fact, for many cellular therapies, all aspects that contribute to *in vivo* activity are not completely understood. In addition to these limitations, many cell processing laboratories working with cellular therapies in phase I and II clinical trials prepare several different types of cellular therapies. It is possible, but may not always be feasible, for a centralized cell processing laboratory to perform several different types of cell function assays.

2.4 Gene Expression Microarrays for the Identification of Cellular Therapy Biomarkers

Measurements of the expression of genes related to a specific cellular activity or function could be used as an *in vitro* biomarker of potency. Quantitative real-time PCR assays are useful tools for analyzing the expression of individual genes in order to assess the activity of various types of cells. The measurement of changes in interferon (IFN)- γ transcription by quantitative real-time PCR has been used as a marker for T-cell activation following stimulation with a recall antigen.[17-20] Quantitative real-time PCR has recently been used to measure the production of messenger RNA (mRNA) encoding IFN- γ , IL-2, IL-4, and IL-10 by stimulated T cells. [18] Quantitative real-time PCR arrays are also available to assess angiogenesis, apoptosis, cell cycle, insulin signaling pathways, cytokines and receptors, nitric oxide signaling pathways, and JAK/STAT signaling pathways.

Although using quantitative real-time PCR to measure the expression of single genes or groups of genes is helpful in assessing cell function, the complete assessment of the function of cellular therapies requires the measurement of a broad range of gene transcripts, especially when the mechanisms responsible for effective therapy are not thoroughly understood. The analysis of cells using gene expression microarrays allows the simultaneous assessment of the expression of thousands of genes. One practical advantage of gene expression microarray assays over other analytic assays is that very few cells are needed. Enough RNA can be isolated from 1×10^4 to 1×10^6 cells for analysis with a 17,500 gene complementary DNA (cDNA) expression microarray.[21]

Microarrays with 15,000–40,000 genes or oligonucleotide probes have been used clinically to characterize lymphomas,[22] prostate cancer,[23] ovarian cancer,[24] small cell lung cancer,[25] melanoma,[26] and many other cancers. We have used cDNA gene expression microarrays with 17,500 genes to investigate the immunologic changes associated with high-dose IL-2 therapy for renal cell carcinoma,[27] and imiquimod (a TLR-7 ligand) therapy for basal cell carcinoma.[28] We have also used cDNA microarrays to assess the effects of IL-10 on natural killer (NK) cells,[29-31] and several different types of IFN on lipopolysaccharide (LPS)-stimulated mononuclear cells, the *in vitro* response of mononuclear cells to IL-2,[32] and the molecular basis of cutaneous wound healing.[33]

Whereas gene expression microarrays have been widely used to assess changes in cells in response to stimuli or to classify different types of cancers, they have only been used to a limited extent to identify and validate markers of the potency of cellular therapies. However, since gene expression microarrays simultaneously measure the expression of thousands of genes, they capture a snapshot of all possible gene expression signatures that are associated with cellular function and, therefore, could be a very important tool for assessing the potency of cellular therapies. The comprehensive nature of gene expression microarray analysis makes them ideal for measuring biomarkers of both expected and unexpected cell functions. This is particularly important for the analysis of cells with complex and multiple critical functions, such as DCs, embryonic stem cells, and HSCs.

There are some limitations concerning the use of gene expression microarrays for potency testing. Gene expression microarray analysis involves multiple steps, including RNA isolation, amplification, fluorescent labeling, hybridization, and data analysis. It is impossible at the current technological stage to complete the whole procedure within a few hours, and so these global expression microarrays cannot yet be used for lot-release testing. However, if global microarrays can identify specific sets of genes whose expression is associated with potency, tailored chips or quantitative real-time PCR kits that only assess specific ‘potency genes’ could be developed and used for lot release testing.

2.5 MicroRNA Expression Analysis for the Identification of Cellular Therapy Biomarkers

Another potentially important indicator of hematopoietic and immune cell potency is miRNA expression. miRNAs are an abundant class of endogenous, non-protein-coding, small RNAs of 19–23 nucleotides that are derived from pre-miRNA of 60–120 nucleotides. Mature miRNAs negatively regulate gene expression at the post-transcriptional level. They reduce the levels of target transcripts as well as the amount of protein encoded. More than 800 human miRNAs have been identified so far.[34] In general, miRNAs are phylogenetically conserved and, therefore, have conserved and defined post-transcription inhibition function. Some miRNAs are expressed throughout an organism, but most are developmentally expressed or are tissue specific. miRNAs play an important role in many cellular development and metabolic processes, including developmental timing, signal transduction, tissue differentiation, and cell maintenance.

Assessment of miRNA expression seems ideally suited for distinguishing primitive from committed hematopoietic, embryonic, and other stem cells, as well as different types of lymphocytes and mononuclear phagocytes. Microarrays containing probes that recognize up to 827 miR have been used to assess cardiac myocytes,[35] HSCs,[36,37] and some peripheral blood leukocytes.[38,39] Although miRNA profiles of mononuclear phagocytes and DCs have not been studied extensively using microarrays, if miRNA profiles differ between immature and mature DCs, they may be useful in assessing the potency of DCs produced *in vitro*.

The use of miRNA expression microarrays for biomarker identification is limited by the fact that a relatively large quantity of RNA is required. The analysis of miRNAs requires at least ten times greater quantities of cells than gene expression profiling, since miRNAs contribute only approximately 1% of the total mRNA of a cell. miRNA amplification methods have not yet been fully validated and, therefore, are not considered reliable. However, targeted miRNA analysis requires a relatively small number of cells (1×10^6).

2.6 Potential Applications of Gene and miRNA Expression for the Assessment of Cellular Therapies

2.6.1 Predicting the Confluence of Human Embryonic Kidney 293 Cells—Gene expression microarrays have been demonstrated to be useful for some cell therapy applications.

They can be used to predict the quality of cells used to manufacture biologic products. Human embryonic kidney (HEK) 293 cells are often used to manufacture products such as adenoviral gene therapy vectors and vaccines.[40] These cells can be grown in bioreactors, tissue culture flasks, and roller bottles. However, when HEK 293 cells grow to form a confluent monolayer, their phenotype changes, as does the quality of the vector or vaccine produced by these cells. Cell confluence can be readily assessed by visual inspection of cells grown in flasks and roller bottles, but for cells grown in bioreactors, the assessment of confluence by visual inspection is not always possible. Gene expression profiling has been used to identify genes whose expression predicts cell confluence.[40] HEK 293 cells that have been grown to 90% confluence have a unique gene expression signature compared with those grown to 40% confluence. A set of 37 of these signature genes is able to predict the quality and confluence of HEK 293 cells. Although this use of gene expression profiling does not represent a potency assay, it demonstrates the potential of the use of gene expression profile assays.

2.6.2 Molecular Biomarkers of Hematopoietic Stem Cell Potency—There are many different sources of HSCs. HSCs can be obtained from bone marrow, umbilical cord blood, and mobilized peripheral blood stem cells (PBSCs). HSC products are widely used for clinical transplantation and regenerative medicine applications, and better potency assays for these therapies are needed. Potency assays for HSC products used for transplantation should measure the ability of the product to reconstitute bone marrow hematopoietic cells and PBSCs in the transplant recipient. The potency assay should reflect the period of time that neutrophil, platelet, and red blood cell (RBC) counts return to and remain above specified levels independent of transfusion therapy. In other words, if the potency assay indicates that a product meets the minimum criteria, the therapy should result in at least the minimum acceptable neutrophil, platelet, and RBC counts in the recipient for a minimum specified duration of time.

Liquid culture of long-term culture-initiating cells and the repopulation of marrow in non-obese diabetic (NOD)/severe combined immunodeficiency (SCID) mice assays are considered to be the best measure of the quantity and quality of HSCs. However, these assays require several weeks to complete, highly specialized reagents, and highly trained staff. As a result, these assays have seldom, if ever, been used as potency assays.

The measurement of myeloid, erythroid, and mixed colony formation in methylcellulose culture systems has been the standard method for assessing bone marrow and PBSC concentrates, but they have been used mainly as in-process controls. The measurement of colony formation in methyl cellulose is an effective biologic assay that directly measures a relevant function of HSCs; however, these assays take approximately 14 days to complete and consequently cannot be used as a potency assay.

The measurement of CD34+ cells by flow cytometry has been used as a potency assay for HSCs collected from the blood as PBSC concentrates. PBSCs are collected from the peripheral blood by apheresis; however, to collect sufficient quantities of HSCs for a successful transplant, ‘mobilizing’ agents are given to the HSC donor to increase the concentrate of stem cells in the blood. For many years, granulocyte colony-stimulating factor (G-CSF) has been the standard agent for mobilizing stem cells from the marrow to the blood. The measurement of CD34+ cells has been the standard potency assay for G-CSF-mobilized PBSC concentrates. A dose of $3\text{--}5 \times 10^6$ CD34+ cells per kg of recipient weight is required for a successful allogeneic transplant, and $1\text{--}2 \times 10^6$ CD34+ cell per kg for a successful autologous transplant. However, CD34 is expressed by many different types of hematopoietic cells. In fact, several different subpopulations of CD34+ hematopoietic progenitor cells have been described. HSCs obtained from other sources, such as bone marrow or umbilical cord blood, are likely to contain different subpopulations of CD34+ cells than G-CSF-mobilized PBSC concentrates, and CD34+ cell counts may not necessary reflect the potency of other HSC sources.

Although G-CSF has been the standard agent for mobilizing HSCs, a new agent, AMD3100 or plerixafor, is being used to mobilize PBSCs in autologous donors, and the results have been promising. This agent will soon be used to mobilize HSCs in allogeneic stem cell donors. The mechanisms by which AMD3100 and G-CSF alter HSC trafficking and mobilization are different, suggesting that HSCs with different intrinsic properties may be mobilized by these agents. AMD3100, as a CXC chemokine receptor 4 (CXCR4) antagonist, mobilizes HSCs within 6 hours by disrupting the engagement of stem cell surface CXCR4 with its ligand SDF-1 (CXCL12), which is expressed on marrow osteoblasts.[41-49] In contrast, G-CSF mobilizes stem cells indirectly by downregulating the expression of SDF-1 on marrow osteoblasts and releasing neutrophil and monocyte proteolytic enzymes, including neutrophil elastase, cathepsin G, and matrix metalloproteinase-9. These in turn degrade important HSC trafficking and adhesion molecules KIT, VCAM1, CXCR4, and SDF-1.[50] In animal studies, AMD3100 mobilizes a CD34+ cell population with a greater long-term marrow repopulating capacity than G-CSF,[51-53] possibly due to differences in mechanisms of mobilization.

We have shown that gene and miRNA expression is likely to be useful in assessing the potency of HSCs using G-CSF- and AMD3100-mobilized PBSCs as a model. We first applied gene and miRNA expression profiling analysis to mobilized peripheral blood HSCs and used T cells, B cells, monocytes, and NK cells as a reference. We hypothesized that miRNA and gene expression analysis would be useful for characterizing HSCs. In preliminary studies, we assessed PBSCs mobilized with two different types of mobilizing agents, G-CSF and AMD3100, and isolated with two different monoclonal antibodies, CD34 and CD133. When these two types of HSCs were compared with peripheral blood leukocytes, we found that the genes with the greatest fold upregulation in HSCs were *GATA2*, *MYCN*, *MYB*, *TRH*, *SOC2*, *FLT3*, and *KIT* (table II).[38] Using a miRNA expression array, we found 11 miRNAs whose expression was increased 14- and 2-fold more in mobilized peripheral blood HSCs than in peripheral blood leukocytes, respectively. These miRNAs included miR-126, -10a, -19a, -19b, -93, -20b, and -34a (table III).[38]

We next demonstrated that gene and miRNA expression can be used to distinguish different types of HSCs. To determine if miRNA and gene expression profiling would be beneficial in distinguishing different types of HSCs, we compared AMD3100- and G-CSF-mobilized PBSCs isolated with anti-CD34 in a rhesus monkey model.[54] We found that CD34+ cells mobilized with G-CSF and AMD3100 have different gene expression profiles. G-CSF-mobilized CD34+ cells were more likely to express neutrophil and mononuclear phagocyte markers, and AMD3100-mobilized CD34+ cells were more likely to express B-cell, T-cell, and NK cell markers. Not only did the combination of AMD3100 plus G-CSF mobilize more CD34+ cells, but the CD34+ cells mobilized by AMD3100 plus G-CSF had a gene expression signature that differed from those mobilized by either AMD3100 alone or G-CSF alone. G-CSF plus AMD3100-mobilized CD34+ cells were enriched for B-cell markers. Since molecular assays can distinguish HSCs from different sources and isolated with different antibodies, they should be useful in measuring HSC potency. These genes and miRNAs are potential biomarkers for HSC potency.

2.6.3 Molecular Biomarkers of Dendritic Cell Potency—DCs are potent professional antigen-presenting cells capable of capturing and processing antigens in order to present peptides to prime T cells.[55] They express both human leukocyte antigen (HLA) class I and class II molecules, and present peptides to CD4+ and CD8+ T cells. They also express co-stimulatory molecules, such as CD80, CD86, CD40, intercellular adhesion molecule-1 (ICAM1), and lymphocyte function-associated antigen-3 (LFA3). For immune therapy, DCs can be generated from PBMCs after granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4 stimulation *in vitro*, or they can be generated by co-culturing *in vitro* with irradiated tumor cells or virus infected cells, proteins, or peptides. Mature DCs are then

administered to patients to stimulate cytotoxic T cells *in vivo*. Immunotherapies with DCs are being used to treat melanoma, renal cell carcinoma, prostate cancer, and leukemia.[55]

Since few DCs are present in the blood, they must be produced from other types of cells. DCs for clinical therapies produced from CD34+ cells are known as plasmacytoid DCs and those produced from circulating mononuclear cells are known as myeloid-derived DCs. Either mature or immature DCs can be produced. Immature DCs express lower levels of HLA class II antigens and co-stimulatory molecules but higher levels of Fc and mannose receptors. The ability of immature DCs to phagocytose and process antigens is better than that of mature DCs, but mature DCs present antigens better than immature DCs. Although the function of mature and immature DCs differ, it is not possible with standard analytic assays to precisely distinguish the degree of maturation of DCs.

The potency of DCs can be tested by assessing the ability of DCs loaded with antigen to stimulate autologous T cells.[56] However, this is difficult because of the low percentage of T cells in most patients that are responsive to tumor antigens. One alternative to overcome the low number of autologous T cells is to generate and expand T cell clones that respond to specific antigens. Even so, only T cells with the same HLA restriction elements and antigen specificity could be used in a DC potency assay. For example, HLA-A*0201 T cell clones specific to a melanoma antigen such as MART1 would not be useful for testing DCs prepared from subjects with other HLA types, such as HLA-A*03, or other antigens, such as cytomegalovirus pp65. Consequently, separate clones must be developed for each antigen and HLA restriction being studied.

The potency of DCs can be assessed by using test peptides from recall antigens that are able to stimulate memory T-cell responses.[56] These antigens include HLA-restricted tetanus toxin, influenza virus, and Epstein-Barr virus antigens, since most people have been immunized against these antigens. However, assays using recall antigens do not directly test the ability of DCs to present tumor-associated antigens and efficacy to stimulate tumor-specific T cells. Therefore, these assays cannot be used as a lot-release test for DCs used for cancer therapy, although testing the ability of DCs to present recall antigens and stimulate T cells is useful as an in-process control.

The measurement of DC co-stimulatory activity has been used to assess the potency of DCs. Co-stimulation plays a critical role in the induction of antigen-specific immunity. One method to measure co-stimulation is the mixed lymphocyte culture reaction, which is based on the stimulation of responder cells with replication-competent allogeneic DC stimulator cells. However, it is not known to what degree alloreactivity and co-stimulation contribute to T-cell stimulation.

Alternatively, gene expression profiling is likely to be useful in assessing the potency of DCs used for clinical therapies. It has been used to characterize the differentiation of monocytes into macrophages and their polarization to macrophages with a type 1 or type 2 phenotype, [57] and has also been used to characterize the response of monocytes to LPS and cytokine stimulation.[30,31] Preliminary data in our laboratory has also found that gene expression profiling can distinguish monocytes from immature DCs,[58] and immature DCs from mature DCs. Genes with the most significant differences in expression in immature DCs compared with monocytes are shown in table IV. The ability of gene expression microarrays to assess cells globally may allow them to determine the potency of DCs by evaluating unstimulated cells or cells that have been stimulated with a recall antigen. However, genes whose expression reflects DC maturation as well as specific DC functions must be identified before gene expression profiling can be used a potency assay for DCs.

2.6.4 Mesenchymal Stem Cells—Another potential cell therapy application for gene expression profiling is the analysis of mesenchymal stem cells (MSCs). MSCs or bone marrow stromal cells are adherent cells obtained through the culture of bone marrow aspirates. The cells have the ability to differentiate into bone, cartilage, and adipocytes. MSCs also have immune modulatory effects and are used in phase I and II clinical trials to treat autoimmune disease and graft-versus-host disease.[59] When injected systemically or into tissue, MSCs also induce or support the growth of other types of stem cells and mature cells, and are being used in cardiac and neurologic regenerative applications. Despite the widespread use of these cells, good biomarkers of MSC quality and potency are lacking. MSCs are typically characterized by flow cytometry; however, the markers used are not specific for MSCs. Furthermore, there are no other analytic markers to assess the potency of any of the multiple functions of these cells. Molecular analysis may allow the identification of more specific MSC biomarkers.

There are several applications for the use of molecular biomarkers for the evaluation of MSC products. The commonly used methods to produce MSCs for laboratory research and in preliminary clinical trials differ from those used for many other clinical applications. Fetal bovine serum (FBS) is typically used as a media supplement for MSC culture and expansion; however, whenever possible, it is best to avoid the use of animal-derived reagents in the production of cellular therapies. When products are moved from the research laboratory to clinical cell processing laboratory, the manufacturing process is often revised to eliminate FBS in order to avoid allergic reactions to foreign proteins or possible exposure to pathogens. Some labs have grown MSCs with media supplemented with platelet lysate rather than FBS and have used media with any serum or platelet lysate supplement.

MSCs are also typically grown in flasks, but the production of the quantities of MSCs required for clinical studies would require the use of a large number of flasks. The culture of MSCs in flasks presents a significant risk of bacterial contamination, and it would be better to produce clinical MSCs in a closed system, such as bags or bioreactors. The comparison of MSCs produced using different conditions currently requires the use of animal models that access the ability of MSCs to support hematopoiesis and bone formation. These assays are laborious and time consuming. MSCs grown without FBS have not yet been shown to be equivalent to those grown with FBS, nor have those grown in bags or bioreactors been shown to be equivalent to those grown in flasks. Gene expression profiling may be useful in comparing the potency of MSCs grown in different media and different containers.

2.7 Global Transcriptional Analysis for Process Validation

The processes used to manufacture cellular therapies must often be changed. These changes may result from the need to substitute a new reagent for one that is no longer available, a change of instrumentation or equipment used in the manufacturing process, or a need to accommodate a change in clinical practice. We have found that gene expression profiling is useful in assessing changes in the manufacturing process.

The production of clinical cellular therapies often involves multiple centers. Cells such as peripheral blood mononuclear cells (PBMCs) are collected from a donor or patient at one center and then shipped to a specialized cellular therapy laboratory, where they are processed and cells such as DCs are produced. When processing is complete, the product is shipped to the site where it is administered to a specific patient. The centralized cell processing laboratory may be hundreds or thousands of miles from the collection center, and it may take up to 48 hours after the collection is complete for the starting cellular material to reach the cell processing laboratory. We used gene expression profiling to show that although significant changes occur in PBMCs that have been stored at 4°C for 48 hours, there was no difference in the gene expression signature of immature DCs by the culture of monocytes in GM-CSF and

IL-4 prepared from fresh and PBMCs stored for 48 hours. We found that among 17,500 genes on a cDNA microarray, 711 differed between elutriated monocytes prepared from fresh and PBMCs stored for 48 hours, but only three genes differed between immature DCs prepared from fresh and PMBCs stored for 48 hours.[58]

For many clinical applications, immature DCs are treated with cytokines or inflammatory signals to produce mature DCs. Immature DCs are often treated *ex vivo* with LPS and IFN- γ to produce mature DCs for clinical therapy. We used gene expression profiling to determine if the DC maturation cocktail LPS plus IFN- γ could be improved by the addition of two other DC maturation agents, IL-1 β and tumor necrosis factor (TNF) α . Monocytes were isolated from the PBMC concentrates for healthy subjects by elutriation and were incubated for 3 days with GM-CSF and IL-4 to produce immature DCs. Immature DCs from each subject were divided in thirds and were incubated for 24 hours with LPS plus IFN- γ , LPS plus IFN- γ and IL-1 β , or LPS plus IFN- γ , IL-1 β and TNF- α to produce mature DCs. There were no differences in the expression of co-stimulatory molecules (CD80, CD83, and CD86), HLA-DR and CCR7, and production of IL-12p70 and IL-10 among the mature DCs produced with the three cocktails. Global gene expression analysis found that the expression of 9576 genes differed between the immature and mature DCs, but the expression of only 13 differed among the three different groups of mature DCs. Global gene expression profiling demonstrated that there was no benefit of adding IL-1 β and TNF- α to LPS and IFN- γ to order to produce mature DCs.

3. Conclusions

As more new and complex cellular therapies are being developed and used to treat an increasing variety of diseases and patients, the characterization of the final clinical product is becoming a more critical part of the production of cellular therapies. Existing assays, such as function, flow cytometry, and ELISA are important but limited by the number of factors analyzed. Gene and miRNA expression microarrays have the potential to become important in potency testing. They are well suited to the assessment of the potency of cellular therapies in phase I and II clinical trials. As data is collected during clinical trials, the results of analysis of the expression of gene and miRNA biomarkers should be compared with the results of traditional function assays and clinical outcomes. After molecular biomarkers whose expression is associated with critical biologic function and clinical outcome are identified, assays that rapidly measure the expression of these biomarkers can be developed. Molecular biomarker analysis is also useful for validating new cell therapy production methods.

Acknowledgments

The authors thank the Department of Transfusion Medicine, Clinical Center, National Institutes of Health for their support of this work.

References

- [1]. Powell DJ Jr, Dudley ME, Hogan KA, et al. Adoptive transfer of vaccine-induced peripheral blood mononuclear cells to patients with metastatic melanoma following lymphodepletion. *J Immunol* Nov 1;2006 177(9):6527–39. [PubMed: 17056585]
- [2]. Leen AM, Myers GD, Sili U, et al. Monoculture-derived T lymphocytes specific for multiple viruses expand and produce clinically relevant effects in immunocompromised individuals. *Nat Med* Oct; 2006 12(10):1160–6. [PubMed: 16998485]
- [3]. Kinnaird T, Stabile E, Burnett MS, et al. Local delivery of marrow-derived stromal cells augments collateral perfusion through paracrine mechanisms. *Circulation* Mar 30;2004 109(12):1543–9. [PubMed: 15023891]

- [4]. Nanjundappa A, Raza JA, Dieter RS, et al. Cell transplantation for treatment of left-ventricular dysfunction due to ischemic heart failure: from bench to bedside. *Expert Rev Cardiovasc Ther* Jan; 2007 5(1):125–31. [PubMed: 17187464]
- [5]. Ariga T. Gene therapy for primary immunodeficiency diseases: recent progress and misgivings. *Curr Pharm Des* 2006;12(5):549–56. [PubMed: 16472146]
- [6]. Rosenberg SA, Spiess P, Lafreniere R. A new approach to the adoptive immunotherapy of cancer with tumor-infiltrating lymphocytes. *Science* Sep 19;1986 233(4770):1318–21. [PubMed: 3489291]
- [7]. Rosenberg SA, Lotze MT, Muul LM, et al. A progress report on the treatment of 157 patients with advanced cancer using lymphokine-activated killer cells and interleukin-2 or high-dose interleukin-2 alone. *N Engl J Med* Apr 9;1987 316(15):889–97. [PubMed: 3493432]
- [8]. Wrzesinski C, Paulos CM, Gattinoni L, et al. Hematopoietic stem cells promote the expansion and function of adoptively transferred antitumor CD8 T cells. *J Clin Invest* Feb;2007 117(2):492–501. [PubMed: 17273561]
- [9]. Yang S, Rosenberg SA, Morgan RA. Clinical-scale lentiviral vector transduction of PBL for TCR gene therapy and potential for expression in less-differentiated cells. *J Immunother* Nov;2008 31(9):830–9. [PubMed: 18833004]
- [10]. European Medicines Agency Committee for Medicinal Products for Human Use (CHMP). Guideline on potency testing of cell based immunotherapy medicinal products for the treatment of cancer. [Accessed 2009 May 19]. [online]. Available from URL: <http://www.emea.europa.eu/pdfs/human/bwp/27147506enfin.pdf>
- [11]. US FDA Cellular, Tissue, and Gene Therapies Advisory Committee. Potency measurements for cell and gene therapy products. Meeting #41; Gaithersburg (MD). 2006 Feb 9;
- [12]. Specifications: test procedures and acceptance criteria for biotechnological/biological products. ICH tripartite harmonized guideline no. Q6B; International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use; Geneva. ICH Secretariat; [Accessed 2009 May 19]. Mar 101999 [online]. Available from URL: <http://www.ich.org/cache/compo/363-272-1.html#Q6B>
- [13]. Howell WM, Rose-Zerilli MJ. Interleukin-10 polymorphisms, cancer susceptibility and prognosis. *Fam Cancer* 2006;5(2):143–9. [PubMed: 16736283]
- [14]. Howell WM, Turner SJ, Bateman AC, et al. IL-10 promoter polymorphisms influence tumour development in cutaneous malignant melanoma. *Genes Immun* Feb;2001 2(1):25–31. [PubMed: 11294564]
- [15]. McCarron SL, Edwards S, Evans PR, et al. Influence of cytokine gene polymorphisms on the development of prostate cancer. *Cancer Res* Jun 15;2002 62(12):3369–72. [PubMed: 12067976]
- [16]. Hollegaard MV, Bidwell JL. Cytokine gene polymorphism in human disease: on-line databases, supplement 3. *Genes Immun* Jun;2006 7(4):269–76. [PubMed: 16642032]
- [17]. Provenzano M, Mocellin S, Bettinotti M, et al. Identification of immune dominant cytomegalovirus epitopes using quantitative real-time polymerase chain reactions to measure interferon-gamma production by peptide-stimulated peripheral blood mononuclear cells. *J Immunother* Jul;2002 25(4):342–51. [PubMed: 12142557]
- [18]. Provenzano M, Mocellin S, Bonginelli P, et al. Ex vivo screening for immunodominant viral epitopes by quantitative real time polymerase chain reaction (qRT-PCR) [abstract]. *J Transl Med* Dec 15;2003 1(1):12. [PubMed: 14675481]
- [19]. Kammula US, Marincola FM, Rosenberg SA. Real-time quantitative polymerase chain reaction assessment of immune reactivity in melanoma patients after tumor peptide vaccination. *J Natl Cancer Inst* Aug 16;2000 92(16):1336–44. [PubMed: 10944556]
- [20]. Panelli MC, Wang E, Monsurro V, et al. The role of quantitative PCR for the immune monitoring of cancer patients. *Expert Opin Biol Ther* Jun;2002 2(5):557–64. [PubMed: 12079490]
- [21]. Wang E, Miller LD, Ohnmacht GA, et al. High-fidelity mRNA amplification for gene profiling. *Nat Biotechnol* Apr;2000 18(4):457–9. [PubMed: 10748532]
- [22]. Dave SS, Fu K, Wright GW, et al. Molecular diagnosis of Burkitt's lymphoma. *N Engl J Med* Jun 8;2006 354(23):2431–42. [PubMed: 16760443]

- [23]. Halvorsen OJ, Oyan AM, Bo TH, et al. Gene expression profiles in prostate cancer: association with patient subgroups and tumour differentiation. *Int J Oncol* Feb;2005 26(2):329–36. [PubMed: 15645116]
- [24]. Wang E, Ngalame Y, Panelli MC, et al. Peritoneal and subperitoneal stroma may facilitate regional spread of ovarian cancer. *Clin Cancer Res* Jan 1;2005 11(1):113–22. [PubMed: 15671535]
- [25]. Taniwaki M, Daigo Y, Ishikawa N, et al. Gene expression profiles of small-cell lung cancers: molecular signatures of lung cancer. *Int J Oncol* Sep;2006 29(3):567–75. [PubMed: 16865272]
- [26]. Basil CF, Zhao Y, Zavaglia K, et al. Common cancer biomarkers. *Cancer Res* Mar 15;2006 66(6):2953–61. [PubMed: 16540643]
- [27]. Panelli MC, White R, Foster M, et al. Forecasting the cytokine storm following systemic interleukin (IL)-2 administration [abstract]. *J Transl Med* Jun 2;2004 2(1):17. [PubMed: 15175100]
- [28]. Panelli MC, Stashower ME, Slade HB, et al. Sequential gene profiling of basal cell carcinomas treated with imiquimod in a placebo-controlled study defines the requirements for tissue rejection [abstract]. *Genome Biol* Jan 12;2007 8(1):R8. [PubMed: 17222352]
- [29]. Mocellin S, Panelli M, Wang E, et al. IL-10 stimulatory effects on human NK cells explored by gene profile analysis. *Genes Immun* Dec;2004 5(8):621–30. [PubMed: 15573087]
- [30]. Nagorsen D, Deola S, Smith K, et al. Polarized monocyte response to cytokine stimulation [abstract]. *Genome Biol* 2005;6(2):R15. [PubMed: 15693944]
- [31]. Stroncek DF, Basil C, Nagorsen D, et al. Delayed polarization of mononuclear phagocyte transcriptional program by type I interferon isoforms [abstract]. *J Transl Med* Jun 13;2005 3:24. [PubMed: 15953390]
- [32]. Jin P, Wang E, Provenzano M, et al. Molecular signatures induced by interleukin-2 on peripheral blood mononuclear cells and T cell subsets [abstract]. *J Transl Med* 2006;4:26. [PubMed: 16805915]
- [33]. Deonaraine K, Panelli MC, Stashower ME, et al. Gene expression profiling of cutaneous wound healing [abstract]. *J Transl Med* 2007;5:11. [PubMed: 17313672]
- [34]. Wellcome Trust Sanger Institute miRBase. [online]
<http://microrna.sanger.ac.uk/sequences/index.shtml>
- [35]. Lagos-Quintana M, Rauhut R, Yalcin A, et al. Identification of tissue-specific microRNAs from mouse. *Curr Biol* Apr 30;2002 12(9):735–9. [PubMed: 12007417]
- [36]. Georgantas RW III, Hildreth R, Morisot S, et al. CD34+ hematopoietic stem-progenitor cell microRNA expression and function: a circuit diagram of differentiation control. *Proc Natl Acad Sci USA* Feb 9;2007 104(8):2750–5. [PubMed: 17293455]
- [37]. Felli N, Fontana L, Pelosi E, et al. MicroRNAs 221 and 222 inhibit normal erythropoiesis and erythroleukemic cell growth via kit receptor down-modulation. *Proc Natl Acad Sci U S A* Dec 13;2005 102(50):18081–6. [PubMed: 16330772]
- [38]. Jin P, Wang E, Ren J, et al. Differentiation of two types of mobilized peripheral blood stem cells by microRNA and cDNA expression analysis [abstract]. *J Transl Med* Jul 22;2008 6(1):39. [PubMed: 18647411]
- [39]. Chen CZ, Li L, Lodish HF, et al. MicroRNAs modulate hematopoietic lineage differentiation. *Science* Jan 2;2004 303(5654):83–6. [PubMed: 14657504]
- [40]. Han J, Farnsworth RL, Tiwari JL, et al. Quality prediction of cell substrate using gene expression profiling. *Genomics* Apr;2006 87(4):552–9. [PubMed: 16413166]
- [41]. Nagasawa T, Hirota S, Tachibana K, et al. Defects of B-cell lymphopoiesis and bone-marrow myelopoiesis in mice lacking the CXC chemokine PBSF/SDF-1. *Nature* Aug 15;1996 382(6592):635–8. [PubMed: 8757135]
- [42]. Deichmann M, Kronenwett R, Haas R. Expression of the human immunodeficiency virus type-1 coreceptors CXCR-4 (fusin, LESTR) and CKR-5 in CD34+ hematopoietic progenitor cells. *Blood* May 15;1997 89(10):3522–8. [PubMed: 9160656]
- [43]. Mohle R, Bautz F, Rafii S, et al. The chemokine receptor CXCR-4 is expressed on CD34+ hematopoietic progenitors and leukemic cells and mediates transendothelial migration induced by stromal cell-derived factor-1. *Blood* Jun 15;1998 91(12):4523–30. [PubMed: 9616148]
- [44]. Aiuti A, Webb IJ, Bleul C, et al. The chemokine SDF-1 is a chemoattractant for human CD34+ hematopoietic progenitor cells and provides a new mechanism to explain the mobilization of CD34+ progenitors to peripheral blood. *J Exp Med* Jan 6;1997 185(1):111–20. [PubMed: 8996247]

- [45]. Kim CH, Broxmeyer HE. In vitro behavior of hematopoietic progenitor cells under the influence of chemoattractants: stromal cell-derived factor-1, steel factor, and the bone marrow environment. *Blood* Jan 1;1998 91(1):100–10. [PubMed: 9414273]
- [46]. Liles WC, Broxmeyer HE, Rodger E, et al. Mobilization of hematopoietic progenitor cells in healthy volunteers by AMD3100, a CXCR4 antagonist. *Blood* Oct 15;2003 102(8):2728–30. [PubMed: 12855591]
- [47]. Devine SM, Flomenberg N, Vesole DH, et al. Rapid mobilization of CD34+ cells following administration of the CXCR4 antagonist AMD3100 to patients with multiple myeloma and non-Hodgkin's lymphoma. *J Clin Oncol* Mar 15;2004 22(6):1095–1102. [PubMed: 15020611]
- [48]. Broxmeyer HE, Orschell CM, Clapp DW, et al. Rapid mobilization of murine and human hematopoietic stem and progenitor cells with AMD3100, a CXCR4 antagonist. *J Exp Med* Apr 18;2005 201(8):1307–18. [PubMed: 15837815]
- [49]. Flomenberg N, Devine SM, Dipersio JF, et al. The use of AMD3100 plus G-CSF for autologous hematopoietic progenitor cell mobilization is superior to G-CSF alone. *Blood* Sep 1;2005 106(5):1867–74. [PubMed: 15890685]
- [50]. Nervi B, Link DC, Dipersio JF. Cytokines and hematopoietic stem cell mobilization. *J Cell Biochem* Oct 15;2006 99(3):690–705. [PubMed: 16888804]
- [51]. Larochelle A, Krouse A, Metzger M, et al. AMD3100 mobilizes hematopoietic stem cells with long-term repopulating capacity in nonhuman primates. *Blood* May 1;2006 107(9):3772–8. [PubMed: 16439684]
- [52]. Hess DA, Bonde J, Craft TP, et al. Human progenitor cells rapidly mobilized by AMD3100 repopulate NOD/SCID mice with increased frequency in comparison to cells from the same donor mobilized by granulocyte colony stimulating factor. *Biol Blood Marrow Transplant* Apr;2007 13(4):398–411. [PubMed: 17382247]
- [53]. Fruehauf S, Seeger T, Maier P, et al. The CXCR4 antagonist AMD3100 releases a subset of G-CSF-primed peripheral blood progenitor cells with specific gene expression characteristics. *Exp Hematol* Aug;2006 34(8):1052–9. [PubMed: 16863911]
- [54]. Donahue RE, Jin P, Bonifacino AC, et al. AMD3100 and granulocyte colony-stimulating factor (G-CSF) mobilize different CD34+ cell populations base on global gene and micro RNA expression [abstract]. *Blood* Nov 16;2008 112(11):496.
- [55]. Schmitt A, Hus I, Schmitt M. Dendritic cell vaccines for leukemia patients. *Expert Rev Anticancer Ther* Mar;2007 7(3):275–83. [PubMed: 17338648]
- [56]. Hinz T, Buchholz CJ, van der ST, et al. Manufacturing and quality control of cell-based tumor vaccines: a scientific and a regulatory perspective. *J Immunother* Sep;2006 29(5):472–6. [PubMed: 16971803]
- [57]. Martinez FO, Gordon S, Locati M, et al. Transcriptional profiling of the human monocyte-to-macrophage differentiation and polarization: new molecules and patterns of gene expression. *J Immunol* Nov 15;2006 177(10):7303–11. [PubMed: 17082649]
- [58]. Shin JW, Jin P, Fan Y, et al. Evaluation of gene expression profiles of immature dendritic cells prepared from peripheral blood mononuclear cells. *Transfusion* Apr;2008 48(4):647–57. [PubMed: 18282241]
- [59]. Le BK, Frassoni F, Ball L, et al. Mesenchymal stem cells for treatment of steroid-resistant, severe, acute graft-versus-host disease: a phase II study. *Lancet* May 10;2008 371(9624):1579–86. [PubMed: 18468541]

Table I

Assays used for potency testing

<i>In vivo</i> assays
Animal models
<i>In vitro</i> assays
Cell-based assay systems
ELISA
Flow cytometry
ELISPOT
Quantitative real-time PCR

Table II

Genes differentially expressed between hematopoietic stem cells (HSCs) and peripheral blood leukocytes (PBLs)^a [Reproduced from Jin et al.[38] with permission]

Expression increased in HSCs			Expression increased in PBLs		
Gene symbol	Description	Fold change	Gene symbol	Description	fold change
<i>GATA2</i>	GATA-binding protein 2	237.82	<i>CARD14</i>	Caspase recruitment domain protein 14	25.59
<i>MYCN</i>	v-myc myelocytomatosis viral related oncogene, neuroblastoma derived (avian)	97.75	<i>LTBR</i>	Lymphotoxin-β receptor	18.83
<i>CRHBP</i>	corticotropin-releasing hormone binding protein	65.52	<i>GPR65</i>	G protein-coupled receptor 65 (TDAG8)	18.33
<i>FHL1</i>	Four and a half LIM domains 1	58.2	<i>FCGR3A</i>	Fc-γ-receptor IIIa (CD16a)	18.15
<i>ERG</i>	v-ets erythroblastosis virus E26 oncogene homolog (avian)	43.57	<i>ITGAM</i>	Integrin, α-M (complement component 3 receptor 3 subunit)	16.96
<i>NPR3</i>	Natriuretic peptide receptor C/guanylate cyclase C (atrioatriuretic peptide receptor C)	42.04	<i>SGSH</i>	N-sulfolucosamine sulfohydrolase (sulfamidase)	16.64
<i>SCHIP1</i>	Schwannomin interacting protein 1	41.77	<i>ITGB7</i>	Integrin, β-7	16.39
<i>MSRB3</i>	Methionine sulfoxide reductase B3	35.29	<i>GNLY</i>	Granulysin	15.21
<i>MYB</i>	v-myb myeloblastosis viral oncogene homolog (avian)	32.99	<i>ALOX5AP</i>	Arachidonate 5-lipoxygenase-activating protein	15.11
<i>DEPDC6</i>	DEP domain containing 6	28.85	<i>CD48</i>	CD48 antigen (BLAST-1)	14.81
<i>EPDR1</i>	Ependymin related protein 1 (zebrafish)	27.88	<i>IL10RA</i>	IL-10 receptor, α	13.71
<i>TRH</i>	Thyrotropin-releasing hormone	26.63	<i>CX3CR1</i>	Chemokine (C-X3-C motif) receptor 1	13.64
<i>NGFRAP1</i>	Nerve growth factor receptor (TNFRSF16) associated protein 1	25.86	<i>COTL1</i>	Coactosin-like 1 (Dictyostelium)	13.4
<i>SERPING1</i>	Serpin peptidase inhibitor, clade G (C1 inhibitor), member 1, (angioedema, hereditary)	25.83	<i>ADAM19</i>	ADAM metalloproteinase domain 19 (meltrin β)	13.24
<i>MAP7</i>	Microtubule-associated protein 7	25.11	<i>IL2RB</i>	IL-2 receptor β chain	13
<i>SOC52</i>	Suppressor of cytokine signaling 2	24.44	<i>ITK</i>	IL-2-inducible T cell kinase	11.91
<i>TSC22D1</i>	TSC22 domain family, member 1	24.12	<i>KLRC4</i>	Killer cell lectin-like receptor subfamily C, member 4	11.36
<i>HIF0</i>	H1 histone family, member 0	22.71	<i>CCL4</i>	Chemokine (C-C motif) ligand 4 (MIP-1 β)	11.22
<i>RBPMS</i>	RNA binding protein with multiple splicing	22.67	<i>CTSH</i>	Cathepsin H	11.18

Expression increased in HSCs			Expression increased in PBLs		
Gene symbol	Description	Fold change	Gene symbol	Description	fold change
<i>CTDSP1</i>	CTD (carboxy-terminal domain, RNA polymerase II, polypeptide A) small phosphatase-like	22.64	<i>EBI2</i>	Epstein-Barr virus induced gene 2 (lymphocyte-specific G protein-coupled receptor)	10.69
<i>CDC47</i>	Cell division cycle associated 7	22.49	<i>GIMAP4</i>	GTPase, IMAP family member 4	10.28
<i>CYTL1</i>	Cytokine-like 1	20.9	<i>POU2F2</i>	POU- domain, class 2, transcription factor 2	10.22
<i>FLT3</i>	Fms-related tyrosine kinase 3	20.45	<i>CXCR4</i>	CXC chemokine receptor 4	10.11
<i>PRKAR2B</i>	Protein kinase, cAMP-dependent, regulatory, type II, β	20.09	<i>CYP4A11</i>	Cytochrome P450, family 4, subfamily A, polypeptide 11	10.1
<i>TRIM58</i>	Tripartite motif-containing 58	19.18	<i>LGALS3</i>	Lectin, galactoside-binding, soluble, 3 (galectin 3)	9.61
<i>FSCN1</i>	Fascin homolog 1, actin-bundling protein (<i>Strongylocentrotus purpuratus</i>)	18.91	<i>SNX27</i>	Sorting nexin family member 27	9.44
<i>Clorf150</i>	Chromosome 1 open reading frame 150	18.83	<i>TRIM26</i>	Tripartite motif-containing 26	9.23
<i>TRH</i>	Thyrotropin-releasing hormone	17.56	<i>SNX27</i>	Sorting nexin family member 27	9.05
<i>KIT</i>	V-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog	17.27	<i>CSPG2</i>	Chondroitin sulfate proteoglycan 2 (versican)	8.89
<i>WASF1</i>	Wiskott-Aldrich syndrome protein family, member 1	16.75	<i>IL32</i>	IL-32	8.76

^aThe groups were compared using t-tests ($p < 0.001$).

Table III

MicroRNAs (miRNAs) whose expression differed among 13 hematopoietic stem cell (HSC) and 28 peripheral blood leukocyte (PBL) samples^a [Reproduced from Jin et al.,[38] with permission]

miRNAs with increased expression in HSCs	Fold increase	miRNAs with increased expression in PBLs	Fold increase
hsa-miR-126	14.43	hsa-miR-142-3p	15.66
hsa-miR-10a	13.46	hsa-miR-218	11.07
hsa-miR-19a	3.89	hsa-miR-21	8.46
hsa-miR-19b	3.11	hsa-miR-379	7.63
hsa-miR-595	2.98	hsa-miR-381	4.61
hsa-miR-146a	2.72	hsa-miR-29b	3.83
hsa-miR-93	2.56	hsa-miR-26b	3.54
hsa-miR-221	2.38	hsa-miR-30c	3.12
hsa-miR-20b	2.35	hsa-miR-142-5p	2.51
hsa-miR-130a	2.25	hsa-miR-29a	2.49
hsa-miR-34a	2.12	hsa-let-7g	2.49
hsa-miR-363	1.92	hsa-let-7i	2.42
hsa-miR-17-5p	1.89	hsa-miR-191	2.3
		hsa-miR-30b	2.03
		hsa-let-7b	1.88
		hsa-miR-26a	1.85
		hsa-miR-16	1.78
		hsa-let-7c	1.75
		hsa-miR-30a-5p	1.73
		hsa-miR-373	1.62
		hsa-miR-594	1.6
		hsa-miR-610	1.34

^aThe expression of miRs between the two groups were compared using t-tests ($p < 0.005$).

Table IV

Monocyte genes whose expression were increased and decreased the most in immature dendritic cells (DCs) [p < 0.005]

Genes upregulated in immature DCs			Genes downregulated in immature DCs		
symbol	name	fold increase	symbol	name	fold decrease
<i>CYP27A1</i>	Cytochrome P450, family 27, subunit A, polypeptide 1	70.6	<i>FOSB</i>	FBJ murine osteosarcoma viral oncogene homolog B	76.9
<i>FCER2</i>	Fc of IgE, low-affinity II, receptor (CD23)	58.2	<i>FPR1</i>	Formyl peptide receptor 1	71.4
<i>MRC1</i>	Mannose receptor, C type 1	58.4	<i>SELL</i>	Selectin-L (CD62L)	40.0
<i>CCL18</i>	Chemokine (C-C motif) ligand 18	36.2	<i>S100A8</i>	S100 calcium binding protein A8	37.8
<i>PLA2G7</i>	Phospholipase A2, group VII	28.1	<i>THBD</i>	Thrombomodulin	11.9
<i>VEGFB</i>	Vascular endothelial growth factor B	21.0	<i>FCGR3A</i>	Fc γ -receptor IIIa (CD16)	9.4
			<i>FCGR1A</i>	high-affinity immunoglobulin Fc γ -receptor 1A (CD64)	9.4