CTC
Centro de Terapia Celular

HEMOCENTRO DE RIBEIRÃO PRETO

RELATÓRIO CIENTÍFICO

PROCESSO Nº 98/14247-6

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FAPESP

2006

Principal Investigators

Name	Institution	Position/Responsibility
Marco Antonio Zago	FMRP/USP	Coordinator Center of Cell-Based Therapy.
Dimas Tadeu Covas	FMRP/USP	Coordinator of Technology Transfer.
Marisa Ramos Barbieri	FUNDHERP	Coordinator of Education and Dissemination.
Marco Antonio Zago	FMRP/USP	**Subproject Coordinator:** ▪ Functional genomics of B-cell malignancies: the gene expression profiles of chronic lymphocytic leukemias and mantle cell lymphomas ▪ The impact of gene polymorphisms on the response to treatment with cell therapy. ▪ The impact of gene polymorphisms on the susceptibility to hematological diseases ▪ The functional genomics of cells used for cell therapy: the gene expression profiles of human mesenchymal stem cells obtained from different sites ▪ The functional genomics of cells used for cell therapy: the comparison of the gene expression profiles of human CD34+ cells obtained from bone marrow, umbilical cord and peripheral blood ▪ The early gene expression changes in the hematopoiesis: the erythroid and granulocytic-monocytic pathways
Dimas Tadeu Covas	FMRP/USP	**Subproject Coordinator:** ▪ Generation, characterization and in vitro manipulations of mesenchymal stem cells aiming at their use for cell therapy ▪ The impact of gene polymorphisms on the response to HIV and HTLV infection. ▪ The functional genomics of cells used for cell therapy: the gene expression profiles of human mesenchymal stem cells obtained from different sites ▪ The functional genomics of cells used for cell therapy: the comparison of the gene expression profiles of human CD34+ cells obtained from bone marrow, the umbilical cord and peripheral blood ▪ Cloning and expression of recombinant human coagulation factor VIII in mammalian cells using retrovirus as a vector. ▪ Brazil Cord Blood Bank. ▪ Development of an animal model for the study of mesenchymal stem cell differentiation in vivo. ▪ Assessment and treatment of iron overload in β thalassemia homozygous patients.
Eduardo Magalhães Rego	FMRP/USP	**Subproject Coordinator:** ▪ Animal model of dyskeratosis congenita ▪ Analysis of the molecular basis of leukemogenesis in the transgenic model of acute promyelocytic leukemia

		▪ Analysis of leukemic cells adhesion and tethering upon Histone Deacetylases inhibitors and G-CSF treatment in acute promyelocytic leukemia ▪ Analysis of FLT-3 mutations in acute myelogenous leukemia by single strand polymorphism ▪ Analysis of the effect of vitamin E isomers in acute promyelocytic leukemia ▪ Study of the effect of Histone Deacetylase Inhibitors on gene transcription in acute promyelocytic leukemia cells. ▪ Analysis of PRAME antigen expression in normal lymphoid cells ▪ Study of the pathogenesis of disseminated intravascular coagulation in the transgenic model of acute promyelocytic leukemia.
Roberto Passetto Falcão	FMRP/USP	**Subproject Coordinator:** ▪ The expression of adhesion molecules in the leukemic phase of non-Hodgkin's lymphomas ▪ Analysis of blasts adhesion and tethering upon histone decaetylase inhibitors and G-CSF treatment in acute promyelocytic leukemia ▪ Analysis of the expression of glucocorticoid receptors in non-Hodgkin's lymphomas
Júlio César Voltarelli	FMRP/USP	**Subproject Coordinator:** ▪ Treatment of immunological diseases by high dose chemotherapy and autologous bone marrow transplantation. ▪ Treatment of late onset type II diabetes mellitus by bone marrow transplantation ▪ Development of animal models for testing cell therapy for lung disorders.
Lewis Joel Greene	FMRP/USP	**Subproject Coordinator:** ▪ Evaluation of gene expression during differentiation and maturation of cord blood CD34-derived dendritic cells using proteomic analysis. ▪ Proteome modification during the early stages of melanoma malignization. ▪ Proteomic analysis of human metastatic cells treated with antitumoral drugs..
Marisa Ramos Barbieri	FUNDHERP	**Subproject Coordinator:** ▪ The cells, the genome and you.

Name	Institution	Position/Responsibility
Aparecida Maria Fontes	FUNDHERP	**Subproject Coordinator:** ▪ Cancer vaccine for chronic myeloid leukemia. ▪ Cloning and expression of recombinant human coagulation factor VIII in mammalian cells ▪ Cloning and expression of recombinant human coagulation factor IX in mammalian cells ▪ Isolation and characterization of murine mesenchymal cells ▪ Gene modification of stem cells
Wilson Araújo da Silva Jr	FMRP/USP	**Subproject Coordinator:** ▪ Initiative to validate of the human transciptome ▪ The comparison of gene expression in human acute leukemias, with especial emphasis in interluekines, adhesion molecules and angiogenesis. ▪ Analysis of the sequences generated by the Human Genome of Cancer project. ▪ Clinical Genomics Project – Bioinformatics Laboratory. ▪ Genome Data Mining. ▪ Gene expression of MicroRNAs in hematopoietic stem cells

Senior Investigators

Name	Institution	Subproject
Roger Chammas	FM/USP	▪ The use of pulsed autologus dendritic cells for the treatment of melanoma ▪ Gene expression profile during melanoma progression ▪ Analysis of cell membrane molecules changes during melanoma progression ▪ Gangliosides in hematological malignancies and normal lymphoid cells
Vanderson Rocha	EUROCORD	▪ Facilitating cord blood cells for engraftment: importance of specific lymphocyte subpopulations. ▪ Expansion of cord blood mononuclear cells in coculture with autologous human umbilical vein endothelial cells (HUVEC). ▪ The impact of gene polymorphisms on the response to treatment with cell therapy. ▪ The impact of gene polymorphisms on the susceptibility to hematological diseases
José César Rosa	FMRP/USP	▪ Proteome modification during the differentiation of dendritic cells from CD34+ cells of human umbilical cord, during the early stages of melanoma malignization, and of human metastatic cells treated with antitumoral drugs
Evamberto Garcia de Góes	FUNDHERP - FAPESP	▪ Use of Telecobalt therapy for the prevention of graft versus host disease asociated with transfusion: dosimetry and quality control of irradiated blood ▪ Effects of diagnostic X-ray dose on peripheral blood mononuclear cells

Junior Investigators

Name	Institution	Subproject
Greice A Molfetta	FMRP/USP - FAPESP	▪ Changes of gene expression in the early differentiation of CD34+ along the erythroid and the granulocytic-monocytic pathways
Rita de Cássia Viu Carrara	FUNDHERP - FAPESP	▪ Identification of genes differentially expressed in CD34+ Bcr/Abl+ cells of patients with chronic myeloid leukemia
Clarice Izumi	FMRP/USP	▪ Proteome modification during the differentiation of dendritic cells from CD34+ cells of human umbilical cord, during the early stages of melanoma malignization, and of human metastatic cells treated with antitumoral drugs
Paulo Peitl Júnior	FUNDHERP - FAPESP	▪ Changes of gene expression in human cells treated *in vitro* with antitumoral drugs

Post Doctoral Fellows

Name	Institution	Adviser
Paulo Peitl Jr	FMRP/USP-FAPESP	Marco Antonio Zago
Rodrigo Proto-Siqueira	FMRP/USP-FAPESP	Marco Antonio Zago
Rita de Cássia Viu Carrara	FMRP/USP-FAPESP	Dimas Tadeu Covas

Ph D Students

Name	Institution	Adviser
Ana Paula Costa Nunes da Cunha Cozac	FUNDHERP	Dimas Tadeu Covas
André Marinato	FMRP/USP	Roberto Passetto Falcão
Barbara Amélia Santana	FMRP/USP-FAPESP	Eduardo Magalhães Rego
Carolina Boschi Cabral	FMRP/USP – FAPESP	Lewis Joel Greene
Ciane Martins de Oliveira	FMRP/USP - CAPES	Wilson Araújo da Silva Júnior
Daniel Guariz Pinheiro	FMRP/USP - FAPESP	Wilson Araújo da Silva Júnior
Daniel Mazza	FMRP/USP-FAPESP	Roberto Passetto Falcão
Eliana da Silva Nascimento	FMRP/USP - CNPq	Wilson Araújo da Silva Júnior
Elisa Maria de Sousa Russo Carbolante	CNPq	Dimas Tadeu Covas
Flora Cristina Lobo Penteado	CAPES	Dimas Tadeu Covas
Gislaine da Silva Pimentel Pereira	FMRP/USP - FAPESP	Wilson Araújo da Silva Júnior
Gustavo Antonio de Souza	FMRP/USP – FAPESP	Lewis Joel Greene
Hamilton Luis G. Teixeira	FMRP/USP-CAPES	Eduardo Magalhães Rego
Israel Tojal da Silva	FMRP/USP - FAPESP	Wilson Araújo da Silva Júnior
Kelson Roberto Kodama	FMRP/USP - CAPES	Wilson Araújo da Silva Júnior
Lorena Lobo e Figueiredo	FMRP/USP-FAPESP	Eduardo Magalhães Rego
Lyris Martins Franco de Godoy	FMRP/USP – FAPESP	Lewis Joel Greene
Maria Carolinia Tostes Pintão	FMRP/USP	Eduardo Magalhães Rego
Maria Fernanda de Castro Amarante	FAPESP	Dimas Tadeu Covas
Patricia dos Santos Pereira Lima	FMRP/USP - CAPES	Wilson Araújo da Silva Júnior
Rodrigo Abreu e Lima	FMRP/USP-FAPESP	Eduardo Magalhães Rego
Rodrigo Alexandre Panepucci	FMRP/USP – FAPESP	Marco Antonio Zago
Rodrigo Haddad	FUNDHERP	Dimas Tadeu Covas
Rodrigo Proto Siqueira	FMRP/USP-FAPESP	Marco Antônio Zago
Sandra Rodrigues Pereira	FMRP/USP - FAPESP	Lewis Joel Greene
Simone Kashima Haddad	FUNDHERP	Dimas Tadeu Covas
Virginia Proença Picanço	FUNDHERP	Dimas Tadeu Covas

Master Students

Name	Institution	Adviser
Evandra Strazza Rodrigues	FUNDHERP	Dimas Tadeu Covas
Elaine Cristina P. Vitorelli	FUNDHERP	Aparecida Maria Fontes
Andrielle de C. Fernandes	FUNDHERP	Dimas Tadeu Covas
Fabíola Singaretti	FUNDHERP	Aparecida Maria Fontes

FUNDHERP Fundação Hemocentro de Ribeirão Preto
FAPESP Fundação de Amparo à Pesquisa do Estado de São Paulo
HCRP/USP Hospital das Clínicas de Ribeirão Preto / Universidade de São Paulo
FMRP/USP Faculdade de Medicina de Ribeirão Preto / Universidade de São Paulo
CAPES Coordenação de Aperfeiçoamento de Pessoal de Nível Superior.
CEPID Centro de Pesquisa Inovação e Difusão.
CNPq Conselho Nacional de Desenvolvimento Cientifico e Tecnológico.

OVERVIEW

B) List of Publications

B. RESULTS OBTAINED IN BASIC RESEARC

B.1 – Articles Published in International Journals with Selective Editorial Politics

B 2.1 Souza, C.; Vigorito, A.; Ruiz, M. A.; Nucci, M.; Funcke, V.; Tabak, D.; Voltarelli, J. C.; Pasquini, R. . Validation of the EBMT risk score in chronic myeloid leukemia in Brazil and allogeneic transplant outcome. Haematologica, v. 90, p. 232-237, 2005.

B 2.2 Hamerschlak, N.; Maluf, E.; Ricardo Pasquini; Eluf-Neto, J.; FR, J. M.; Cavalcanti, A. B.; Okano, I. R.; Falcão, R. P.; Pita, M. T.; Loggetto, S. R. . Incidence of aplastic anemia and agranulocytosis in Latin America: the LATIN study. São Paulo Medical Journal, São Paulo, v. 123, p. 101-104, 2005.

B 2.3 Silva Jr, WA; Marrero, AR; Leite, FPN; Carvalho, BA; Peres, LM; Kommers, TC; Cruz, IM; Salzano, FM; Ruiz-Linhares, A; Bortolini, MC . Heterogeneity of the Genome Ancestry of Individuals Classified as White in the State of Rio Grande do Sul, Brazil. American Journal of Human Biology, v. 17, n. 1, p. 1-17, 2005.

B 2.4 Estalote AC; Proto-Siqueira R; Silva-Jr WA; Zago MA; Palatnik M . The mutation G298A-->Ala100Thr on the coding sequence of the Duffy antigen/chemokine receptor gene in non-caucasian Brazilians. Genetics and molecular research, v. 4, p. 166-173, 2005.

B 2.5 Wunsch Filho V; Zago MA . Modern cancer epidemiological research: genetic polymorphisms and environment. Revista de Saúde Pública, v. 39, p. 490-497, 2005.

B 2.6 Rizzatti EG; Falcao RP; Panepucci RA; Proto-Siqueira R; Anselmo-Lima WT; Okamoto OK; Zago MA. Gene expression profiling of mantle cell lymphoma cells reveals aberrant expression of genes from the PI3K-AKT, WNT and TGFbeta signalling pathways. British Journal of Haematology, v. 130, p. 516-526, 2005.

B 2.7 Carneiro AA; Fernandes JP; Araujo DB; Elias JJr; Martinelli AL; Covas DT; Zago MA; Angulo IL; Baffa O . Liver iron concentration evaluated by two magnetic methods: Magnetic resonance imaging and magnetic susceptometry. Magnetic Resonance in Medicine : Official Journal of the Society of Magnetic Resonance in Medicine / Society of Magnetic Resonance in Medicine, v. 54, p. 122-128, 2005.

B 2.8 Zago MA; Covas DT; Santos SEB; Guerreiro JF; Kashima S . Variation in the FcgammaR3B gene among distinct Brazilian populations. Tissue Antigens, v. 62, p. 178-182, 2005.

B 2.9 Matos DM, Rizzatti EG, Fernandes M, et al. Gamma/delta and alfa/beta T-cell acute lymphoblastic leukemia: comparison of the clinical and immunophenotypic features. Haematologica/The Hematological Journal, Itália, v. 90, p. 264-266, 2005.

B 2.10 Lima RSA, Baruffi MR, Lima ASG, et al. The co-expression of PML/RARalfa and AML1/ETO fusion genes is associated with ATRA resistance. British Journal of Haematology, Inglaterra, v. 128, p. 407-409, 2005.

B 2.11 Souza C, Vigorito A, Ruiz MA, et al. Validation of the EBMT risk score in chronic myeloid leukemia in Brazil and allogeneic transplant outcome. Haematologica, v. 90, p. 234-239, 2005.

B 2.12 Faça VM, Pereira SR, Laure HJ, et al. Determination and reoxidation of the disulfide bridges of a squash-type trypsin inhibitor from Sechium edule (SETI-II). The Protein Journal, v. 23, p. 309-315, 2004.

B 2.13 Mcnamara JC, Rosa JC, Greene LJ, et al. Free amino acid pools as effectors of osmotic adjustment in different tissues of the freshwater shrimp macrobrachium ollfersii (crustacea, Decapoda) during long-term salinity acclimation. Mar. Fresch Behav. Physiological, v. 37, n. 3, p. 193-208, 2004.

B 2.14 Martinelli AL, Franco RF, Tavela MH, et al. Liver iron deposits in hepatitis B patients: Association with severity of liver disease but not with hemochromatosis gene mutations. Journal of Gastroenterology and Hepatology, v. 19, p. 1036-1041, 2004.

B 2.15 Calado RT, Pintao MC, Rocha V, et al. Lack of mutations in the human telomerase RNA component (hTERC) gene in Fanconi' anemia. Haematologica, Itália, v. 89, p. 1014-1015, 2004.

B 2.16 Santos FLS, Dore AI, Lima ASG, et al. Características hematológicas e perfil de expressão de antígenos mielóides de pacientes com leucemia promielocítica aguda. análise de fatores prognósticos para o desenvolvimento da síndrome do ácido retinóico. Revista da Associação Médica Brasileira, São Paulo, v. 50, p. 286-292, 2004.

B 2.17 Ximenes VF, Costa M, Brunetti IL, et al. p-Iodophenol-enhancedluminol chemiluminescent assay applied to discrimination between acute lymphoblastic amd minimally differentiated acute myeloid (FAB-M0) or acute megakaryoblastic (FAB-M7) leukemias. The hematology Journal, Inglaterra, v. 5, p. 496-499, 2004.

B 2.18 Voltarelli JC. Transplante de células tronco hematopoéticas no diabete melito do tipo I. Revista Brasileira de Hematologia e Hemoterapia, São José do Rio Preto-SP, . 26, p. 43-45, 2004.

B 2.19 Voltarelli JC. Perspectivas da terapia celular na esclerose lateral amiotrófica. Revista Brasileira de Hematologia e Hemoterapia, São José do Rio Preto-SP, v. 26, p. 155-156, 2004.

B 2.20 Voltarelli JC, Oliveira MCB, Stracieri ABP, et al. Haematopoietic stem cell transplantation for refractory Takayasu's arteritis. Rheumatology, v. 43, p. 1308-1309, 2004.

B 2.21 Scridelli CA, Queiroz RGP, Kashima S, et al. T cell receptor gamma (TCRG) gene rearrangements in Brazilian children with acute lymphoblastic leukemia: analysis and implications for the study of minimal residual disease. Leukemia Research, p. 267-273, 2004.

B 2.22 Panepucci RA, Siufi JLC, Silva-Jr WA, et al. Comparison of Gene Expression of Umbilical Cord Vein and Bone Marrow Derived Mesenchymal Stem Cells. Stem Cell, v. 22, n. 7, p. 1263-1278, 2004.

B 2.23 Nunes FMF, Valente V, Sousa JF, et al. The use of Open reading frame ESTs (ORESTES) as an additional strategy for analysis of the honey bee transcriptome. Bmc Genomics, v. 5, p. 84, 2004.

B 2.24 Marques-Jr W, Davis MB, Abou-Sleiman PM, et al. Hereditary motor and autonomic neuronopathy 1 maps to chromosome 20q13.2-13.3. Brazilian Journal of Medical and Biological Research, v. 37, n. 11, p. 1757-1762, 2004.

B 2.25 Neder L, Marie SK, Carlotti-Jr CG, et al. Galectin-3 as an immunohistochemical toll to distinguish pilocytic astrocytomas from diffuse astrocytomas, and glioblastomas from anaplastic oligodendrogliomas. Brain Pathology, v. 14, p. 399-405, 2004.

B 2.26 Albuquerque LMM, Garcia AB, Mengel JO, et al. The higher expression of alpha and beta isoforms of the human glucocorticoid receptor in leukemic B-progenitors compared to normal CD10(+) BM cells does not correlate with methylprednisolone-induced apoptosis. Leukemia, v. 18, n. 4, p. 890-892, 2004.

B.2 – Books and Chapters

Título do Livro: Hematologia – Fundamentos e Prática – Edição Revista e Atualizada

Editora Atheneu

Editores: Marco Antonio Zago
Roberto Passetto falcão
Ricardo Pasquini

Dimas Tadeu Covas
B 2.1 Suporte Transfusional do Paciente com Neoplasia Hematopoética (cap. 40)
B 2.2 Retrovírus (cap. 60)
B 2.3 Antígenos Eritrocitários, Leucocitários e Plaquetários (cap. 83)
B 2.4 Doenças Infecciosas transmissíveis por Transfusões Sangüíneas (cap. 84)

Eduardo Magalhães Rego
B 2.5 Hematopoese. Regulação e Microambiente (cap. 2)
B 2.6 Classificação das Neoplasias Hematológicas. Marcadores. Imunofenotipagem (cap. 38)
B 2.7 Classificação das Leucemias Agudas: Citologia, Citoquímica e Imunofenotipagem (cap. 42)
B 2.8 Leucemia Linfóide Crônica (cap. 51)

Júlio César Voltarelli
B 2.9 Ontogênese e diferenciação do tecido linfóide. Dinâmica dos linfócitos. Imunidade Celular e Humoral (cap. 6)

Marco Antonio Zago
B 2.10 Eritropoese e Eritropoetina. Produção e Destruição de Hemácias (cap.3)
B 2.11 Granulócitos: Produção, dinâmica e função (cap. 4)
B 2.12 Monócitos e macrófagos. Sistema de Fagócitos Mononucleares (cap. 5)
B 2.13 O paciente com anemia (cap. 11)
B 2.14 O paciente com Esplenomegalia (cap. 12)
B 2.15 Deficiências de vitamina B12 e de Folatos: Anemias Megaloblásticas (cap. 21)
B 2.16 Deficiência de Glicose-6-Fosfato Desidrogenase (cap. 27)
B 2.17 Estrutura, Síntese e Genética das Hemoglobinas (cap. 28)
B 2.18 Defeitos hereditários das Hemoglobinas (cap. 29)
B 2.19 Talassemias (cap. 31)
B 2.20 Bases Moleculares e Citogenéticas. Oncogenes e antioncogenes (cap. 37)
B 2.3 Antígenos eritrocitários, Leucocitários e Plaquetários (cap. 83)

Roberto Passetto Falcão

B 2.21 Heterogeneidade das células do sangue. Órgãos Hematopoéticos e Linfopoéticos (cap. 1)
B 2.9 Ontogênese e diferenciação do tecido linfóide. Dinâmica dos Linfócitos. Imunidade Celular e Humoral (cap. 6)
B 2.22 O Paciente com Linfonodomegalia (cap. 13)
B 2.23 Matebolismo do Ferro (cap. 22)
B 2.24 Anemia Ferropriva (cap. 23)
B 2.6 Classificação das Neoplasias Hematológicas. Marcadores. Imunofenotipagem (cap. 38)
B 2.7 Classificação das Leucemias Agudas: Citologia, Citoquímica e Imunofenotipagem (cap. 42)
B 2.8 Leucemia Linfóide Crônica (cap. 51)
B 2.25 Tricoleucemia (Leucemia de Células Pilosas) (cap. 53)

OVERVIEW

C) Technological Achievements

The technology transfer program develops within two defined frameworks:

1. A significant part of the Center is physically located in and functionally related to the Regional Blood Center (Hemocentro). This independently funded entity is responsible directly or indirectly for the collection and supply of blood derived components, donor selection and personnel training in an area that covers 237 municipalities with ca. 3.5 million inhabitants.
2. The researchers' expertise is related to transfusion medicine, hematology, cancer, immunology, bone marrow transplantation, genome sequencing and gene expression

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D) Educational Activities

There are many approaches by which the researchers of the center transfer knowledge directly to the population. Very often they participate in interviews, TV programs or talks to the general population concerning topics related to the research. However, as a regular and planned activity we have focused our education program on teachers and students from the middle and high school, especially from the poor areas at the periphery of the city, as well as from smaller towns around Ribeirão Preto.

Results of Basic Research

The Research Program

The first version of the Research Project for the center, discussed with the evaluating committees and approved by FAPESP, is based on the broad concept of using cells for therapy, under different conditions and from several sources, including transfusion of blood components, hematopoietic stem cell transplantation from bone marrow, umbilical cord, and peripheral blood, for instance. The program approved considers also the actual or potential targets of the treatment, particularly individuals affected with neoplastic blood diseases. This is consistent with the origin of the investigators and the laboratories. Accordingly, the first version of the research proposal stated:

> *The research project focuses on basic cellular mechanisms (cell differentiation, cell recognition, cell-to-cell interaction, cell mediators, inflammation, coagulation, and apoptosis) and processing (isolation, expansion, selection, purging) which are relevant for cell-based therapy and its relation with gene structure and protein expression, on four levels: a. Characteristics and manipulation of the cells used for therapy, b. Characteristics of the recipients, affected with neoplastic blood diseases, c. Relationship of the host with parasites transmitted by cell therapy, d. Transgenic animals as experimental models.*

Although the main purpose of the center focus on basic cellular mechanisms and processing which are relevant for cell-based therapy and its relation with gene structure and protein expression, we are progressively increasing the clinical and applied component of the center research. Thus, mesenchymal stem cells are now starting to be tested for their possible therapeutic uses, and the experimental approach with dendritic cells is also starting to move to the clinical application. Moreover, innovative clinical trials with hematopoietic stem cell transplantation are underway or starting, for autoimmune diseases and *diabetes mellitus*.

Briefly, the research carried out in the center can be enumerated under the following titles:

Mesenchymal and Hematopoietic Stem Cells
Therapeutic Applications
Dendritic Cells
Other Basic and Applied Research on Neoplasias and Immune Diseases
Other Research not Directly Linked to Cell Therapy

Mesenchymal and Hematopoietic Stem Cells

We are dealing with two general types of stem cells: hematopoietic precursors, represented by CD34+ found in bone marrow, adult peripheral blood and umbilical cord blood, and mesenchymal stem cells.

Mesenchymal stem cells from the bone marrow

Mesenchymal stem cells (MSC) have been obtained from different sources. MSC are multipotent precursors present in adult bone marrow, that differentiate into osteoblasts, adipocytes and myoblasts, and play important roles in hematopoiesis. We determined the gene expression profile of MSC obtained from human bone marrow, comparing it with the gene expression of CD34+ hematopoietic stem cells[1]. We examined the gene expression of these cells by SAGE (serial analysis of gene expression), and found that collagen I, SPARC (osteonectin), transforming growth factor beta-induced, cofilin, galectin 1, laminin-receptor 1, cyclophilin A and matrix metallo-proteinase 2 are among the most abundantly expressed genes. Comparison with a library of CD34+ cells revealed that MSC had a larger number of expressed genes in the categories of cell adhesion molecule, extracellular and development. The two types of cells share abundant transcripts of many genes. IL-11, IL-15, IL-27 and IL-10R, IL-13R and IL-17R were the most expressed genes among the cytokines and their receptors in MSC, and various interactions can be predicted with the CD34+ cells. This study identified the important contribution of extracellular protein products, adhesion molecules, cell motility, TGF-beta signaling, growth factor receptors, DNA repair, protein folding and ubiquination as part of the MSC transcriptome.

MSC from the umbilical cord vein

In addition to bone marrow, we have obtained MSC from other sites in the adult or the fetus. There is controversy if MSC can be obtained from the umbilical cord (UC). Instead of using cord blood, we obtained MSC starting from cells detached from the UC vein, in a similar manner as for initiating HUVEC cultures[2]. The cells had morphological features, immuno-phenotypic markers and differentiation ability similar to BM-MSC, and we used SAGE to compare the gene expression profile of BM-MSC and of the MSC derived from umbilical cord vein (UC-MSC)[3]. The two libraries share almost all of the first thousand most expressed transcripts, some of which were validated by RT-PCR, including the genes VIM, LGALS1, SPARC, COL1A1, COL1A2, TPT1, TAGLN, TAGLN2, ANXA2 and MMP2. Nevertheless, a set of genes related to anti-microbial activity, to osteoblast differentiation and adherence to the matrix, and to osteogenesis was expressed at higher levels in BM-MSC, whereas higher expression in UC-MSC was observed for genes that participate in pathways related to matrix remodeling via metalloproteinases and angiogenesis.

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[1] Silva-Jr WA, Covas DT, Panepucci RA, Proto-Siqueira R, Siufi JLC, Zanette DL, Santos ARD, Zago MA – The profile of gene expression of human marrow mesenchymal stem cells. **Stem Cells** 21: 661-669, 2003

[2] Covas DT, Siufi JL, Silva AR, Orellana MD. Isolation and culture of umbilical vein mesenchymal stem cells. **Brazilian Journal of Medical and Biological Research** 36:1179-83, 2003

[3] Panepucci RA, Siufi JLC, Silva-Jr WA, Proto-Siqueira R, Neder L, Orellana M, Rocha V, Covas DT, Zago MA Comparison of gene expression of umbilical cord vein and bone marrow derived mesenchymal stem cells. **Stem Cells,** *in press*

The similarities observed between cultured MSC derived from the UC vein and from BM identifies UC-MSC as a new potential source of cells for use in cell-based therapies or tissue engineering and we are now starting to explore this. Umbilical cord is a more accessible source than bone marrow, and its availability tends to increase. In spite of their similarities, the genes differentially expressed between the two types of MSC may reflect functional differences related to their sites of origin: BM-MSC would be more committed to osteogenesis whereas UCV-MSC would be more committed to angiogenesis.

MSC from other sources

We have successfully cultured mesenchymal stem cells obtained from adult vascular wall, both from the saphenous venous and from the artery, and from the adipose tissue removed during the lipoaspiration surgery. MSCs cultured from the saphenous vein were compared with MSC obtained from bone marrow and umbilical cord vein, in relation to immunophenotypic characteristics, differentiation potential and expression of a set of selected genes.

Identification of MSC

In all studies, mesenchymal stem cells have been extensively characterized by their morphological and immunophenotypic properties and differentiation potential. In culture they originate a cell population that assumes a spindle-shaped morphology in confluent wave-like layers and can be replated several (20 or more) times. The cells harvested are negative for hematopoietic lineage markers (CD34, CD45, and CD133), monocytic (CD14), and for endothelial markers such as KDR, cadherin-5, CD31 and CD133. As observed with other mesenchymal stem cells, the majority of cells were positive for CD13, CD29, CD44, CD54, CD90 and HLA class I, and negative for HLA class II. In appropriate conditions they differentiate *in vitro* into osteoblasts, or adypocytes or chondroblasts/ condocytes, identified by the morphological features, histochemical or immunohistochemical staining.

Age-related functional changes of $CD34^+$ cells, T, B and NK lymphocytes

P-gp is expressed by stem cells and thought to represent a physiological mechanism of protection against toxic substances and metabolites. We demonstrated that the P-glycoprotein-mediated rhodamine 123 efflux in bone marrow stem cell from normal subjects correlates with age, with stem cells from older individuals expressing significantly higher Rh123 efflux[4]. These findings reinforce the idea of the existence of functional differences in the biology of normal stem cells. We also demonstrated a decline of P-glycoprotein function in T CD4+ and CD8+ lymphocytes with age that may contribute to the decrease in T cell cytolytic activity with aging. A similar decrease of function was not observed in $CD19^+$ B or $CD16^+CD56^+$ natural killer cells.

[4] Calado RT, Machado CG, Carneiro JJ, Garcia AB, Falcao RP. Age-related changes of P-glycoprotein-mediated rhodamine 123 efflux in normal human bone marrow hematopoietic stem cells. **Leukemia**. 2003 Apr;17(4):816-8.

Research projects in development

Early changes of gene expression during CD34+ cell differentiation

CD34+ cells obtained from the bone marrow or the peripheral blood plated at low densities in semi-solid methylcellulose with cytokines and growth factors give origin to individual colonies, each derived from a single CD34+ progenitor cell. The colonies may be formed either by differentiating erythroid cells (BFU-E), myeloid cells (GM-CFU) or mixed (GEMM-CFU). In order to identify the early changes in gene expression that accompany the commitment to either the myeloid or the erythroid differentiation pathway, we isolate CD34+ cells from normal human marrow by magnetic activated cell sorting and submit the cells to a 12-40 hour "conditioning" period in liquid culture either with myeloid (SCF, Flt-3 ligand, IL-3, IL-6 and intermediate concentration of GM-CSF) or erythroid (low concentration of GM-CSF, IL-3 and erythropoietin) factors. The cells are then cultured as usual in semi-solid methylcellulose with the usual cocktail of growth factor. The period of "conditioning" changes significantly the proportion of erythroid, myeloid or mixed colonies obtained after 14 days of culture, demonstrating the effectiveness of the procedure to force differentiation along one of the pathways. Cells collected after the conditioning period are processed for RNA extraction, and the profiles of gene expression of the erytrhoid or myeloid-conditioned cells are being analyzed by cDNA microarray and SAGE.

Comparison of gene expression profiles of hematopoietic precursors from the bone marrow or from the umbilical blood

There are three possible sources of hematopoietic stem cells for clinical use in transplants: donor bone marrow, mononuclear cells from the peripheral blood and umbilical cord blood. Hematopoietic precursors represent a low proportion of these cells (less than 0.1%), and are enriched in the fraction of CD34+ cells. Clinical behavior, complications and efficiency of transplants vary depending on the source of the hematopoietic precursors, and the variation may be at least in part caused by the precursor cells. In order to identify functional differences between precursor cells from diverse sources, we are comparing the gene expression profiles of CD34+ cells form the normal bone marrow and the umbilical cord blood. The CD34+ cells are separated by magnetic activated cell sorting (purity verified by flow cytometry >95%), and the RNA extracted for evaluation of gene expression by SAGE. The results of a set of genes found differentially expressed will be validated by real-time PCR, and selected pathways or functional group of genes will be further explored.

Therapeutic Applications

Cell markers of prognosis in cell therapy

Our findings reveal the clinical relevance of gene polymorphisms to the outcome of bone marrow transplantation (BMT) and suggest that therapeutic strategies should be individualized on the basis of genetic and clinical factors in order to decrease toxicities and graft-versus-host disease

Inflammatory response and defense genes polymorphism

The important contribution of polymorphisms of genes related to defense and the immunologic and inflammatory responses was demonstrated in 107 pairs of donors and patients with acute or chronic leukemia[5]. Genotyping was performed for gene polymorphisms of cytokines (tumor necrosis factor-α[TNF-α] and TNF-β, interleukin-1 receptor antagonist [IL-1Ra], IL-6, and IL-10), adhesion molecules (CD31 and CD54), Fcγ-receptors (Fcγ RIIa, IIIa, IIIb), mannose-binding lectin (MBL), and myeloperoxidase (MPO). In multivariate analysis, first overall infections were increased in patients with the Fcγ RIIa R-131 genotype, and severe bacterial infections were increased when the MPO donor genotype was AG or AA. Viral and invasive fungal infections were not influenced by any genetic factor studied. Interestingly, we also found that (1) time to neutrophil recovery was shorter when donors were Fcγ RIIIb HNA-1a/HNA-1b; (2) donor IL-1Ra (absence of IL-1RN*2) increased the risk for acute graft-versus-host disease (GVHD) (II-IV); and (3) recipient IL-10 (GG) and IL-1Ra genotypes increased the risk for chronic GVHD. Finally, 180-day transplantation-related mortality rates were increased when donors were Fcγ RIIIb HNA-1a/HNA-1a or HNA-1b/HNA-1b and donor MPO genotype was AA.

Drug metabolism genes polymorphism

More recently[6], we demonstrated in the same 107 patients the role of polymorphisms of genes that may interfere with drug metabolism used in the preparative regimen and GvHD prevention of HLA-identical sibling BMT. The following candidate genes were investigated: P450 cytochrome family (CYP2B6*2, *3, *4, *5, *6), glutathione-S-transferases (GSTM1, GSTT1, GSTT1), thiopurine-S-methyl transferase (TPMT), multiresistance drug (MRD1), methylene-tetrahydrofolate reductase (MTHFR C677T) and vitamin-D receptor (VDR). In multivariate analysis, increased incidence of hemorrhagic cystitis was associated with a gene involved in CY metabolism. Venous occlusive disease was associated with donor to recipient CYP2B6*6 (GG). No clinical or genetic factors were associated with interstitial pneumonitis. We found increased GVHD associated with specific alleles of recipient MTHFR, VDR, IL10, donor GSTP1 and IL1-Ra, lower bone marrow cell dose, patient age and female donor. Thus we demonstrated that polymorphisms of genes that interfere with the toxicity and

[5] Rocha V, Franco RF, Porcher R, Bittencourt H, Silva WA Jr, Latouche A, Devergie A, Esperou H, Ribaud P, Socie G, Zago MA, Gluckman E – Host defense and inflammatory gene polymorphisms are associated with outcomes after HLA-identical sibling bone marrow transplantation. **Blood** 100:3908-3918, 2002

[6] Rocha V, Porcher R, Filion A, Bittencourt H, Silva Jr WA, Vilela G, Zanette D, Ferry C, Larghero J, Devergie A, Esperou H, Ribaud P, Socie G, Gluckman E and Zago MA Association of drug metabolism gene polymorphisms with toxicities, graft-versus-host disease and survival after HLA-identical sibling bone marrow transplantation. **Journal of Clinical Oncology**, *submitted*

efficacy of drugs used in the preparative regimen and GvHD prophylaxis are important genetic factors for outcomes after HLA identical BMT.

HSC transplantat for severe auto-immune diseases

Few effective therapeutic alternatives are available for patients with autoimmune disorders refractory to conventional therapy. Therefore, the possibility of using Hematopoietic Stem Cell (HSC) transplantation after intensive chemo- and radiotherapy for these diseases would be of great benefit. Autologous HSC transplantation was performed for 3 cases of refractory lupus erythematosus with impairment of renal function. Two of the 3 patients achieved clinical remission of the disease and do not require further immunosuppression at days +360 and +700 post transplant. In addition, 7 patients with refractory multiple sclerosis were transplanted with autologous HSC. In 5/7 patients the progression of neurological impairment was halted by the transplant and the patients became independent of the use of immunosuppressors. Two patients died of transplant-related disorders. Finally, the same approach was applied to one patient with Takayasu´s arteritis[7] and one with pemphigus. However, the follow-up is still too short to be evaluated. In general, our results suggest that HSC transplantation is feasible and may be a therapeutic option for refractory severe autoimmune disorders[8].

Tumor associated antigens

There is considerable interest in tumor-associated antigens (TAA) because they may be the basis of therapeutic approaches to malignant disease and may also be used to measure minimal residual disease. The most extensively studied families are MAGE, GAGE, BAGE, LAGE, NY-ESO1 and PRAME, which are expressed exclusively in cancer, testis and trophoblasts. Thus far, we have demonstrated the expression of mRNA of these antigens in: a) 15/58 (26%) cases of chronic lymphoproliferative disorders[9]; b) 24/28 (86%) cases of osteosarcoma; c) 22/33 (67%) cases of squamous cell carcinoma of head and neck.

Monoclonal anti-PRAME antibody

PRAME (Preferentially Expressed Antigen in Melanoma) gene, located on chromosome 22 (22q11.22), is the most frequently expressed TAA gene in the diseases that we have studied; for instance, it was expressed in 68% of head and neck cancers, and was the only antigen in 25% of cases. Nevertheless, the expression of PRAME protein in normal and neoplastic tissues is unknown. We produced a monoclonal antibody against PRAME protein, using a 562-nt fragment of the PRAME cDNA (in collaboration with V. Filonenko, from Kiev, Ukraine). The 196-amino acid peptide was expressed in BL21(DE3) cells as His-tagged protein. The monoclonal antibody (MoAb) against PRAME was analyzed by flow cytometry in samples of peripheral blood (PB, n=15), bone marrow (BM, n=3) from healthy donors; in tonsils (n=3) from patients submitted to tonsilectomy

[7] Voltarelli JC, Oliveira MCB, Stracieri ABPL, Godoi DF, Moraes DA, Coutinho MA. Autologous hematopoietic stem cell transplantation for Takayasu's arteritis: Report of the first case of the literature. **Biology of Blood and Marrow Transplantation** 10(1), Suppl: 62, 2004.

[8] Voltarelli JC, Ouyang J. Hematopoietic stem cell transplantation for autoimmune diseases in developing countries: current status and future prospectives. **Bone Marrow Transplantation** 32 Suppl 1:S69-71, 2003.

[9] Proto-Siqueira R, Falcao RP, de Souza CA, Ismael SJ, Zago MA. The expression of PRAME in chronic lymphoproliferative disorders. **Leukemia Research**. 2003 May;27(5):393-6.

for non-malignant diseases and in PB samples from 26 patients with chronic lymphocytic leukemia and 7 with mantle cell lymphoma. PRAME positive cells represented less than 15% of cells from normal PB, BM and tonsil cells, whereas it represented more than 20% in CLL and MCL. Furthermore, PRAME expression in normal lymphocytes was dimmer than in CLL and MCL cells. The lymphoid cells from normal BM and tonsils that expressed PRAME were $CD19^+$ $CD10^+$ $CD27^+$ CD38 TdT^- $cIgM^-$ $CD5^-$ suggesting that PRAME is expressed early during lymphoid ontogenesis and that its expression in CLL and MCL cells is aberrant[10]. These findings indicate that this antigen may be further explored as a target for diagnostic, to detect minimal residual disease detection and for therapeutic approaches.

Mechanisms of interferon-alpha effect on chronic myelogenous leukemia

The mechanisms of the antileukemic effect of interferon-alpha in chronic myelogenous leukemia are still unclear. To investigate the immunological effects of interferon-alpha in CML patients, markers of cellular activation and apoptosis, natural killer (NK) cell cytotoxicity and production of intracellular cytokines (IFN-gamma, IL-2 and IL-4) were determined by flow cytometry in the peripheral blood mononuclear cells (PBMC) of 26 CML-CP patients before and 3, 6 and 9 months after IFN-alpha treatment. The results were correlated with the hematological response, and indicate that IFN-alpha use in CML reduces the number of $CD34^+$ cells, activates T cells, enhances stem cell apoptotic markers and increases the production of intracellular IFN-gamma and IL-2 by lymphocytes. Taken together, these results indicate that the therapeutic effect of IFN-alpha in CML is mediated at least in part by immunological mechanisms[11].

Donor lymphocytes for chronic leukemia relapsing after transplant

The success of treatment of CML with bone marrow transplantation depends at least in part of a graft-versus-leukemia effect. This reaction can also be induced by donor lymphocyte infusion (DLI), which produces a remission in most patients with CML who relapse after transplantation. The mechanisms of this effect are poorly understood. We observed an increase of the percent of activation markers on T and NK cells (CD3/DR, CD3/CD25 and CD56/DR), of lymphocytes producing IL-2 and IFN-gamma, of NK activity, and of *in vivo* lymphocyte proliferation in the patients who achieved remission after DLI. The percent of apoptotic markers (Fas, FasL and Bcl-2) on lymphocytes and CD34-positive cells did not change. These preliminary results suggest that the therapeutic effect of DLI in the chronic phase of CML is mediated by classic cytotoxic and proliferative events involving T and NK cells but not by the *Fas* pathway of apoptosis.

[10] Proto-Siqueira R, Figueredo-Pontes L, Rego EM, Rizzatti EG, Nascimento FM, Lima RSA, Falcão RP, Filonenko V, Zago MA – PRAME protein is aberrantly expressed in chronic lymphocytic leukemia and mantle cell lymphoma. Submitted abstract to the **46th Annual Meeting of the American Society of Hematology,** 2004.

[11] de Castro FA, Palma PV, Morais FR, Simoes BP, Carvalho PV, Ismael SJ, Lima CP, Voltarelli JC. Immunological effects of interferon-alpha on chronic myelogenous leukemia. **Leukemia Lymphoma**. 2003 Dec;44(12):2061-7.

Research projects in development

Is HSC transplant an approach to cure severe diabetes mellitus?

In January 2004 we started an innovative transplant approach for patients with severe newly diagnosed type-I *diabetes mellitus*. The aim of this approach is to "salvage" the remaining beta-pancreatic cells from the immunological attack. An immunoablative therapy started no later than 6 weeks after the diagnosis is followed by autologous hematopoietic stem cell tranplantation. Thus far, 4 patients have been submitted to this therapy; although the follow-up is still too short, the three last patients are no longer taking insulin injections.

Gene modification of mesenchymal cells

Examination of the clinical therapeutic efficacy of using gene modified mesenchymal stem cells (MSC) has recently been the focus of much investigation. We developed a strategy for delivering a gene marker into human mesenchymal stem cells (hMSCs) using a retrovirus system. First a retrovirus-producer cell line with 98% of GFP expression was obtained by electroporation followed by the treatment with G418. We next transduced and The gene transfer rate from the retrovirus supernatant into MSCs was in the range of 10% but after treatment with G418 the level of GFP expression increased to 75% of the mesenchymal cell population. The immunophenotypic characteristics do not change after the gene modification and the bilineage mesenchymal differentiation potential of the MSC/GFP+ cells is maintained, as they differentiated into adipocytes and osteocytes.

Neurological differentiation of MSC

In collaboration with Suely K. Marie from the Department of Neurology at the Faculty of Medicine of USP in S. Paulo we are evaluating the potential of generating neurological cells starting from mesenchymal stem cells. Semi-confluent cultures of MSC from the human bone marrow after the 3rd in vitro passage are induced to differentiate with bFGF for two weeks followed by FGF8b in the third week. Cells develop striking morphological changes with prolonged extensions that at the electronic microscopy have significant amount of filaments and tubules. Immunohistochemical staining is moderately/strongly positive for nestin and N-cadherin, weekly positive for N-CAM and negative for GFAP. The experiments are being repeated for the third time, with the objective of carrying out immunological staining in electronic microscopy preparations and the evaluation of electric potential of individual cells.

Mesenchymal stem cells in chronic myeloid leukemia: are they normal?

Are the bone marrow mesenchymal stem cells also affected by the molecular deffect in chronic myeloid leukemia? To investigate this point we analyzed the MSCs from two previously untreated patients. Although the RT-PCR demonstrated the presence of BCR/ABL mRNA in both cases, FISH showed a very low percentage (0.54%) of cells with the rearrangement. To investigate the presence of chimerism of the MSC after bone marrow transplantation, we analyzed the presence of Y chromosome by nested PCR and FISH in three CML patients who underwent sex-mismatched allogeneic bone marrow transplants. Our results thus far showed that 2/3 of CML patients had MSC positive for Y chromosome by nested PCR, however the analysis by FISH showed a very low percentage of MSCs with Y chromosome, 0.14 and 0.34%. The presence of very low level of BCR/ABL+ MSC in both cases is probably caused by hematopoetic

cells contaminations. We conclude that the MSC of patients with CML do not carry the mutation characteristic of the disease. The cells therefore originate from an early precursor and the autologous MSC may be used for eventual therapeutic purposes.

Dendritic Cells

Culture of dendritic cells (DC)

As described previously, we established a method for the induction of dendritic differentiation from peripheral blood mononuclear cells based on *in vitro* culture in the presence of GM-CSF + IL-4 for 5 days, followed by TNF-α + PGE-1 or LPS treatment. In the last year, we compared the dendritic differentiation of mononuclear cells from patients with melanoma and normal subjects using the above method. The pattern of expression of CD1a, CD83 and CD86 upon stimulation was similar between the two groups, indicating that autologous cells from melanoma patients may be used as a source for *ex-vivo* dendritic differentiation.

Protein changes during DC maturation

We used the proteomic approach based on two-dimensional electrophoresis and mass spectrometry to identify the protein changes that occur during differentiation of dendritic cells[12]. Sixty-three differentially expressed proteins (± 2-fold) were unambiguously identified with sequence coverage greater than 20%. They corresponded to only 36 different proteins, because 11 were present as 38 electrophoretic forms. Some proteins such as tropomyosin 4 and heat shock protein 71 presented differentially expressed electrophoretic forms, suggesting that many of the changes in protein expression that accompany differentiation and maturation of DCs occur post-translationally. The largest differences in expression were observed for actin (21-fold in Mo), Rho GDP-dissociation inhibitor 2 (20-fold in Mo), vimentin (8-fold in immature DCs), lymphocyte-specific protein 1 (12-fold in mature DCs) and thioredoxin (14-fold in mature DCs). Several proteins are directly related to functional and morphological characteristics of DCs, such as cytoskeletal proteins (cytoskeleton rearrangement) and chaperones (antigen processing and presentation). Only a few proteins identified here were the same as those reported in proteomic studies of DCs, which used different stimuli to produce the cells (GM-CSF/IL-4 and TNF-α). These data suggest that the DC protein profile depends on the stimuli used for differentiation and especially for maturation.

Research projects in development

Pulsed autologous dendritic cells for the

We have shown that patients bearing advanced stage melanomas had a smaller proportion of dendritic cell (DC) precursors, as compared to healthy individuals. However, *ex vivo* differentiation and maturation of DC from these patients were normal. In keeping with this, a melanoma vaccination protocol in a group of

[12] Pereira SR, Faca VM, Gomes GG, Chammas R, Fontes AM, Covas DT, Greene LJ – The changes in the proteomic profile during differentiation and maturation of human monocyte-derived dendritic cells stimulated with GM-CSF/IL-4 and LPS. **Proteomics**, in press

treatment of human melanoma

patients, conducted at *FMUSP* and *Hospital do Câncer* had unsatisfactory results, likely associated with the decreased immune functions of the patients. Different lines of evidence indicate that patients with advanced stage tumors are indeed immunossuppressed, prompting us to design a protocol based on the use of these cells as an adjuvant to the vaccination protocol. In this protocol, stage III or IV melanoma patients will be enrolled. After tumor excision, melanoma cells will be cultured. Three weeks after surgery, a first delayed hipersensitivity test will be done, using lysed autologous melanoma cells. A punch biopsy of the intradermal reaction on day 2 will be analyzed. Peripheral blood will be drawn for lymphocyte and DC precursors isolation. DC will be differentiated *in vitro*, pulsed with irradiated autologous cells (apoptotic cells), maturated *in vitro* and then used as a cellular vaccine (intradermal injection). A total of 6 doses of this cellular vaccine will be administered. Immune function will be followed during the vaccination protocol. The tests for immune function will include: (1) analysis of the intradermorreaction triggered by lysed melanoma cells by routine histopathology and immunhistochemistry for qualification of the cellular infiltrate before and after at least 4 doses of the cellular vaccine; (2) production of specific anti-melanoma antibodies; (3) production of IFN-γ by lymphocytes cocultured with DC pulsed with irradiated melanoma cells; (4) integrity of IFN-α signaling cascade in melanoma cells. These parameters will be compared to the clinical follow-up of the immunized patients.This protocol will also serve for in vitro testing of the role of tumor associated gangliosides in the immunization against melanoma. Depending on these results a second trial will be then suggested.

Other Basic and Applied Research on Neoplasias and Immune Diseases

Chronic neoplasias of the lymphoid system

The chronic neoplastic diseases of the human lymphoid system represent one of the largest and more heterogeneous groups of human malignant disorders. The affected cells belong to one of the the developemntal stages of the B or T lymphoid lineages, and a large variety of molecular mechanisms are involved in the genesis and progression of the diseases. We have thus far focused in two diseases of this group: chronic lymphocytic leukemia and mantle cell lymphoma.

TGF-β sinalling in mantle cell lymphoma

Mantle cell lymphoma (MCL) is a subtype of B-cell lymphoma associated with the t(11;14)(q13;32) and consequent ectopic overexpression of cyclin D1. Ontogenetically, MCL is considered the malignant counterpart of pre-germinal-center naive B-cells. In addition to the overexpression of cyclin D1, other molecular mechanisms are needed to develop the disease. We compared the gene expression profile of magnetic sorted MCL and normal naive B-cells using oligonucleotide microarrays representing 10,000 genes[13]. Real-Time RT-PCR confirmed the data obtained for 10 selected genes (6 overexpressed and 4 underexpressed in lymphoma cells). Our microarray results revealed an aberrant expression of several genes from the TGF-β signaling pathway in MCL: ACVR1, ACVR2, ACVR2B, BMP4, TGIF, Smad2 and Smad6. Except for TGIF and Smad6, all other genes induce the TGF-β signaling pathway. The activin receptors ACVR1, ACVR2 and ACVR2B are receptors of the TGF-β superfamily, which consists of TGF-β, activins, bone morphogenic proteins (BMPs) and others. Upon ligand binding, activin receptors induce anti-proliferative and pro-apoptotic responses, acting as tumor suppressors in early tumorigenesis. In advanced cancer, however, there is a loss of growth-inhibitory responsiveness downstream the core TGF-β signaling pathway, and it may be used as a tumor-progression factor by inducing immune supression, angiogenesis, epithelial-mesenchymal transdifferentiation and increased potential for metastasis. Our results suggest an activation of the TGF-β signaling pathway in MCL, and point to potential new therapeutic targets for this yet incurable lymphoma.

MDR1 in aplastic anemia

Our data[14] indicated that P-gp activity was decreased in aplastic anemia not only during the development of the disease, but also after remission, suggesting that it may contribute to drug-induced injury to hemopoietic cells in some cases of aplastic anemia, by increasing the proportion of susceptible cells.

RNA telomerase gene

We also studied the relevance of genetic abnormalities in a group of disorders characterized by the failure of hematopoiesis. We demonstrated that acquired aplastic anemia, an important target for hematopoietic transplantation, is not associated with mutations of the RNA telomerase gene (hTR), as had been suggested by others[15]. We sequenced the relevant segment of the hTR gene in 42 unselected patients with aplastic anemia and none of the three mutations

[13] Rizzatti EG, Panepucci RA, Proto-Siqueira R, Anselmo-Lima WT, Okamoto OK, Falcao RP, Zago MA – Gene expression profiling of mantle cell lymphoma in the leukemic phase reveals aberrant expression of genes from the TGF-β signaling pathway. Abstract submittted to the 46th Anunual Meeting of the **American Society fo Hematology**, 2004.

[14] Calado RT, Garcia AB, Gallo DA, Falcao RP. Reduced function of the multidrug resistance P-glycoprotein in CD34+ cells of patients with aplastic anaemia. **British Journal of Haematology**. 2002 Jul;118(1):320-6.

[15] Calado RT, Pintao MC, Silva WA Jr, Falcao RP, Zago MA. Aplastic anaemia and telomerase RNA mutations. **Lancet**. 2002 Nov 16;360(9345):1608.

previously described were observed in this population. Although intriguing, our findings were later confirmed in a larger population. Finally, in 45 patients with Fanconi anemia we found only one example of the mutation, which should more appropriately be interpreted as a clinically silent polymorphism[16].

Cell markers in promyelocytic leukemias

Also related to the identification of cell markers with prognostic significance, we analyzed the immunophenotypical profile of 71 patients with acute promyelocytic leukemia. Our aim was to determine whether the intensity of expression of myeloid antigens could predict the occurrence of the retinoid syndrome, a life-threatening complication observed rarely in APL patients receiving all *trans* retinoic acid (ATRA) treatment. Neither the fluorescence intensity nor the coefficient of variation of CD33, CD13 and CD117 fluorescence in the blasts from APL patients were significantly correlated to a higher risk for the development of retinoid syndrome.

A luminescent assay for the diagnosis of acute leukemia

Our group also developed a different approach for the diagnosis and classification of acute leukemias based on a chemiluminescent assay. The hydrolysis of 2-methyl-1-propenylbenzoate mediated by monocytic esterases generates acetone phosphorescence, which can be quantified with a luminometer, and applied to the assessment of monocytic lineage commitment[17]. The results obtained with this method were the same as those obtained with α-naphthyl acetate esterase (ANAE) in 97% of 23 monocytic or myelomonocytic leukemias and the method was capable of distinguishing between acute myelogenous leukemias, with and without monocytic, commitment from a pool of 66 AML cases.

Genome projects

Our group participated in the *Xyllela* Genome project funded by FAPESP, and in the Cancer Genome Project funded by FAPESP and Ludwig Institute. In the last project we coordinated a group of five laboratories, and we were responbile for a substantial proportion of the sequencing. The project generated over one million ESTs from normal and cancer cells, and the results were published[18, 19, 20].

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[16] Calado RT, Pintao MC, Rocha V, Falcao RP, Bittencourt MA, Silva-Jr WA, Gluckman E, Pasquini R, Zago MA – Lack of mutations in the telomerase RNA component (*hTERC*) gene in Fanconi anemia. **Haematologica**, *in press*

[17] da Fonseca LM, Brunetti IL, Campa A, Catalani LH, Calado RT, Falcao RP. Assessment of monocytic component in acute myelomonocytic and monocytic/monoblastic leukemias by a chemiluminescent assay. **The Hematology Journal.** 2003;4(1):26-30.

[18] Camargo AA, Samaia HP, Dias-Neto E, Simao DF, Migotto IA, Briones MR, Costa FF, Nagai MA, Verjovski-Almeida S, Zago MA, *et al*. The contribution of 700,000 ORF sequence tags to the definition of the human transcriptome. **Proceedings of the National Academy of Sciences USA** 98:12103-12108, 2001.

[19] Brentani H, Caballero OL, Camargo AA, Da Silva AM, Da Silva WA Jr, *et al*, Zago MA, Zalcberg H – The generation and utilization of a cancer-oriented representation of the human transcriptome by using expressed sequence tags. **Proceedings of the National Academy of Sciences USA.** 100:13418-13423, 2003

[20] Sogayar MC, Camargo AA, *et al*, Silva WA Jr, Silva ID, Silva NP, Soares-Costa A, Sonati MF, Strauss BE, Tajara EH, Valentini SR, Villanova FE, Ward LS, Zanette DL; Ludwig-FAPESP Transcript Finishing Initiative. A transcript finishing initiative for closing gaps in the human transcriptome. **Genome Research**. 2004 Jul;14(7):1413-23.

Research projects in development

What are the effects of antineoplastic drugs on cancer or normal cells?

The main mechanism of action and the effect of the drug on the metabolic pathway most directly affected are known for most antineoplastic agents. However, the general effect of an antineoplastic drug upon the cell is not generally known, particularly the differential effect on normal as compared with cancer cells. We are investigating this subject with two parallel approaches: by evaluating gene expression and by proteomics. The effects of the following drugs on a normal human melanocyte line, a radial growth phase melanoma line and a metastatic melanoma line (LB373-MEL) are being evaluated: cisplatin, vincristine, tamoxifen. After *in vitro* treatment of the culture for varied periods and with different drug concentrations, in order to obtain measurable effect on the cell viability, total RNA or/and protein are extracted. For the proteomics approach, proteins are separated by 2D electrophoresis, using IPG strips of pH 4-7 and SDS-PAGE in 12.5% polyacrylamide. Reproducibility tests on gels with an average of 603 spots demonstrated low variation for *p*I and MW, but intensity varied an average of 30% between replicates, thus leading to a minimum of 200% variation between samples to identify differential expression. Selected spots are cut off the gel, the proteins are digested locally by trypsin and the peptides are identified by ESI/MS/MS using peptide mass fingerprinting or peptide sequencing by collision induction dissociation (CID). Gene expression is carried out by analysis of RNA by RASH. The libraries obtained are automatically sequenced and selected over- or under-expressed genes are validated by semiquantitative RT PCR.

Other Research not Directly Linked to Cell Therapy

Population Genetics and Polymorphisms as Risk-factors for Diseases

Researchers of the CTC Center had a strong interest in population genetics and the association of gene polymorphisms with human diseases. These subjects are being phased down in the recent years, but still contribute to a significant percentage of the groups production.

Population genetics

Based on the sequencing of a 8.8-kb mitochondrial DNA segment of 40 individuals in a collaborative project coordinated by our Center, we obtained results that demonstrate that peopling of Americas was probably carried out by a single wave of Asian migrants who arrived around 20-thousand years ago, much earlier than generally accepted[21]. This work was the focus of considerable attention by the lay press (*Folha de São Paulo*, *O Estado de São Paulo*, both important daily newspapers) and was the object of a cover article by the monthly science review edited by FAPESP (*Pesquisa FAPESP*).

The origin of the Y-chromosome was analyzed in six different Brazilian populations, including two semi-isolated Afro-Brazilian communities ("quilombos"), and compared with the historical and social data of the formation of our population[22].

The genetic relationship between seven different Amerindian groups was analyzed on the basis of HLA class II diversity, and revealed a link of the Ache, whose relationship to other Amerindian populations is unclear, with the Guarani[23].

Polymorphism as risk factor

We demonstrated that *MDR-1* and methylenetetrahydrofolate reductase gene polymorphisms are risk factors for the development of acquired aplastic anemia and childhood acute lymphoblastic leukemia, respectively[24].

We found that fasting hyper-homocysteinemia is a risk factor for venous thrombosis in patients without known acquired thrombophilia. Although fasting total homocysteine levels are significantly higher in those homozygous for the MTHFR C677T mutation, this genotype does not increase the thrombotic risk in our study population[25].

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[21] Silva Jr WA, Bonatto SL, Holanda AJ, Ribeiro-Dos-Santos AK, Paixao BM, Goldman GH, Abe-Sandes K, Rodriguez-Delfin L, Barbosa M, Paco-Larson ML, Petzl-Erler ML, Valente V, Santos SE, Zago MA – Mitochondrial genome diversity of native Americans supports a single early entry of founder populations into America. **American Journal of Human Genetics** 71:187-192, 2002

[22] Abe-Sandes K, Silva WA Jr, Zago MA – Heterogeneity of the Y chromosome in Afro-Brazilian populations. **Human Biology** 76:77-86, 2004.

[23] Tsuneto LT, Probst CM, Hutz MH, Salzano FM, Rodriguez-Delfin LA, Zago MA, Hill K, Hurtado AM, Ribeiro-Dos-Santos AK, Petzl-Erler ML – HLA class II diversity in seven Amerindian populations. Clues about the origins of the Ache. **Tissue Antigens** 62:512-526, 2003

[24] Calado RT, Franco RF, Zago MA, Falcao RP. MDR1 gene C3435T polymorphism and the risk of acquired aplastic anaemia. **British Journal of Haematology**. 2002 Jun;117(3):769.

[25] Franco RF, Simoes BP, Tone LG, Gabellini SM, Zago MA, Falcao RP. The methylenetetrahydrofolate reductase C677T gene polymorphism decreases the risk of childhood acute lymphocytic leukaemia. **British Journal of Haematology**. 2001 Dec;115(3):616-8.

Thrombin activatable fibrinolysis inhibitor (TAFI) plays an important role in hemostasis, functioning as a potent fibrinolysis inhibitor. We identified 6 novel single nucleotide polymorphisms in the promoter region of the TAFI gene[26]. Polymorphisms in the TAFI promoter are linked to plasma antigen levels and with the risk of venous thrombophilia.

The frequency of the different alleles of thiopurine methyltransferase, a key enzyme in the metabolism of the anti-neoplastic drug 6-mercapto uridine, in Brazilians was described[27].

The similarity of prevalence for type-2 diabetes in Afro- and Euro-Brazilians may be related to the extensive gene flow that occurred between them[28].

Hemoglobin Diseases

Sickle cell anemia and thalassemia are the two most common single gene diseases in Brazil. The molecular basis, and clinical and epidemiological aspects of hereditary disorders of hemoglobin was an important research topic of three researchers of the group. A few contributions in this area are still produced.

Effect of hydroxyurea on adhesion molecules

Hydroxyurea treatment of sickle cell anemia patients leads to clinical improvement and reduces the incidence of vaso-occlusive episodes. We examined the effect of hydorxyurea on the expression on the adhesion molecule phosphatidylserine on the surface of erythrocytes and platelets of sickle cell anemia patients under treatment. We found that the treatment reduces the expression of phsphatidylserine on the surface of erythrocytes and platelets, thus contributing to the favorable effects of this therapy[29].

Heterogeneity of the thalassemia mutations

Thalassemia major, the symptomatic severe for of the disease, is more common in the South and Southeast of Brazil, where it was intorduced by massive Italian immigration[30]. As expected, the molecular basis is very similar to that observed in Italy, with presominance of the β39 null mutation. However, we observed a high prevalence of a milder symptomatic form of the disease, β-thalassemia intermedia, in the Northeast of the country, a region not reached by the Italian immigration. Molecular analysis revealed a completely different pattern of mutations, with predominance of the IVS-I-6 T→C (63%) and IVS-I-1 G→A (15%), probably revealing the contribution of ancient Portuguese colonists.

Animal Models of Human Diseases

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Dyskeratosis congenita

Dyskeratosis Congenita is a rare disease characterized by premature aging and increased tumor susceptibility. It is an X-linked recessive disease caused by point mutations in the DKC1 gene, which codes for dyskerin, a putative pseudouridine synthetase that mediates posttranscriptional modification of ribosomal RNA (rRNA). In addition, dyskerin can bind to the RNA component of telomerase. In a collaborative effort between the Memorial Sloan-Kettering Cancer Center, the Baylor College of Medicine and the Center for Cell-Based Therapy, we generated hypomorphic Dkc1 mutants. Cells from hemizygous male and heterozygous female mutants displayed a decreased level of Dkc1 expression[31]. About 60% of the Dkc1 mutants presented bone marrow failure and developed a variety of tumors, the most common being lung and mammary gland tumors. We analyzed the telomere status/telomerase activity and the ribosomal RNA pseudouridylation. Whereas the impairment in ribosome biogenesis was detected from the first generations of mutant mice, reductions of telomere length became evident only in later generations. These results represent the first demonstration that the deregulation of ribosomal function is important for oncogenesis, being important in the initiation of dyskeratosis congenita, whereas telomere shortening may modify and/or exacerbate the disease

Cell cycle and apoptosis in myeloid progenitors of PML-RARα trans genic mice

Acute promyelocytic leukemia (APL) is characterized by the expansion of malignant myeloid cells that morphologically resemble promyelocytes. In the vast majority of APL cases, the t(15;17) is present and causes the fusion between retinoic receptor a (RARα) gene locus located on chromosome 17 with the promyelocytic leukemia (PML) gene *locus* located on chromosome 15, yielding a fusion gene translated in an aberrant PML-RARα fusion protein. We study a transgenic mouse model of APL generated by Prof. Pier Paolo Pandolfi from the *Memorial Sloan Kettering Cancer Center*, in which the fusion gene PML-RARα is expressed under the control of a cathepsin G promoter[32]. Ten percent of these transgenic mice develop a form of acute promyelocytic leukemia that closely resemble human APL. The long pre-leukemic phase and low incidence of leukemia, suggest that other mutagenic events must occur before the development of overt leukemia. We are analyzing the apoptosis, cell cycle and the expression of cell cycle regulators, such as p16 and p15, in myeloid progenitors from the bone marrow at different time points (birth to 12 months and after the development of leukemia). In addition, we are performing comparative genomic hybridization leukemic cells to identify additional mutagenic

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events. We have detected aneuploidy in half of the leukemic mice tested so far, with non-significant increase of the percentage of cells in S phase. The precise chromosomal aberrations are being characterized.

Research projects in development

Analysis of the NPM-RARα oncogenic activity on a transgenic mouse model

In the first phase of the project, we have determined that transgenic mice harboring the NPM-RARα fusion gene expressed under the control of the cathepsin G promoter develop a myelomonocytic leukemia, characterized by the infiltration of blood, bone marrow and lymph nodes by blasts CD68+, MPO+, lysozyme+. Recently, it has been demonstrated by Pellici´s group that the wild type NPM protein interacts physically with the p53 protein. We are currently trying to determine whether the expression of the fusion protein NPM-RARα results in functional inactivation of p53.

The Profile of Gene Expression of Human Marrow Mesenchymal Stem Cells

Stem Cells 21: 661-669, 2003

Wilson A. Silva-Jr, Dimas T. Covas, Rodrigo A. Panepucci, Rodrigo Proto-Siqueira, Jorge L. C. Siufi, Dalila L. Zanette, Anemari R. D. Santos, Marco A. Zago

Center for Cell Therapy and Regional Blood Center, Department of Clinical Medicine, Faculty of Medicine, Ribeirão Preto, Brazil

Mesenchymal stem cells (MSC) are multipotent precursors present in adult bone marrow, that differentiate into osteoblasts, adipocytes and myoblasts, and play important roles in hematopoiesis. We examined gene expression of these cells by SAGE (serial analysis of gene expression), and found that collagen I, SPARC (osteonectin), transforming growth factor beta-induced, cofilin, galectin 1, laminin-receptor 1, cyclophilin A and matrix metalloproteinase 2 are among the most abundantly expressed genes. Comparison with a library of CD34+ cells revealed that MSC had a larger number of expressed genes in the categories of cell adhesion molecule, extracellular and development. The two types of cells share abundant transcripts of many genes, some of which are highly expressed in myeloid progenitors (thymosin-β4 and β10, fos and jun). IL-11, IL-15, IL-27 and IL-10R, IL-13R and IL-17R were the most expressed genes among the cytokines and their receptors in MSC, and various interactions can be predicted with the CD34+ cells. MSC express several transcripts for various growth factors and genes suggested to be enriched in stem cells. This study reports the profile of gene expression in MSC and identifies the important contribution of extracellular protein products, adhesion molecules, cell motility, TGF-beta signaling, growth factor receptors, DNA repair, protein folding and ubiquination as part of their transcriptome.

Table 3. Comparison of MSC and CD34+ marrow cell gene expression, as measured by the number of tags of the corresponding genes (ribossomal proteins excluded)

Exclusively or higly represented in MSC[1]
collagens type I and type VI, SPARC, matrix metaloproteinase 2 (gelatinase), transforming growth factor beta 1 induced, eukaryotic translation elongation factor 1 alpha 1, fibronectin 1, light peptide 9 of myosin, transgelin, calgranulin A, heat shock protein 47, latent transforming gorwth factor beta binding protein 2, gap junction protein alpha 1, biglycan, annexin A2, IGF binding proteins 4 and 6, hemeoxygenase 1, tropomyosin 2, connective growth factor, brain abundant membrane attached signal protein 1, galectin 1, nexin, integrins alpha 2 and alpha V, endocytic receptor, CD151 antigen
Exclusively or highly represented in CD34+ stem cells[2]
CD2 antigen, kinesin family member 5, MAP2K3, MHC class II DR alpha, eukaryotic translation elongation factor 3, aldolase C frutose-biphosphate, pulmonary surfactant associated protein C, glucose phosphate isomerase, myeloperoxydase, phosphatic acid phosphatase type 2A
Highly represented in both types of cells[3]
filamin alpha, early growth response, annexin 2 ligand (calpactin I), serotonin receptor 1 D, calcyclin, calgizzarin, cofilin 1, COX8, tropomyosin 1, 5'3' nucleotidase, benzodiazapine receptor, IGF binding protein 7, cysteine and glycine rich protein 1, vigilin, MAP2K2, pyruvate kinase, phsphoglycerate mutase 1, integrin-linked kinase, cyclophillin A and B, vimentin, thymosin beta 10, milk fat globule EGF 8 protein, zyxin, heavy polyptide 1 of ferritin, glyceraldehyde-3-phospate dehydrogenase, heat shock 70 kD, light peptide 6 of myosin, high motility group protein 1, Erb-b3, CD74 antigen, cell division cycle-2 like 5, 5'-nucleotidase, transmembrane gamma-carboxyglutamic acid protein 4, B-cell CLL/lymphoma 7A

[1] Tags for these transcripts were at least 50 times more abundant in the MSC as compared with CD34 cells
[2] Tags for these transcripts were at least 50 times more abundant in the CD34 as compared with MSC
[3] Tags correspond to at least 0.05% of total tags from one of the cells, and the relative abundance in one type of cell does not exceeds 50 times the other

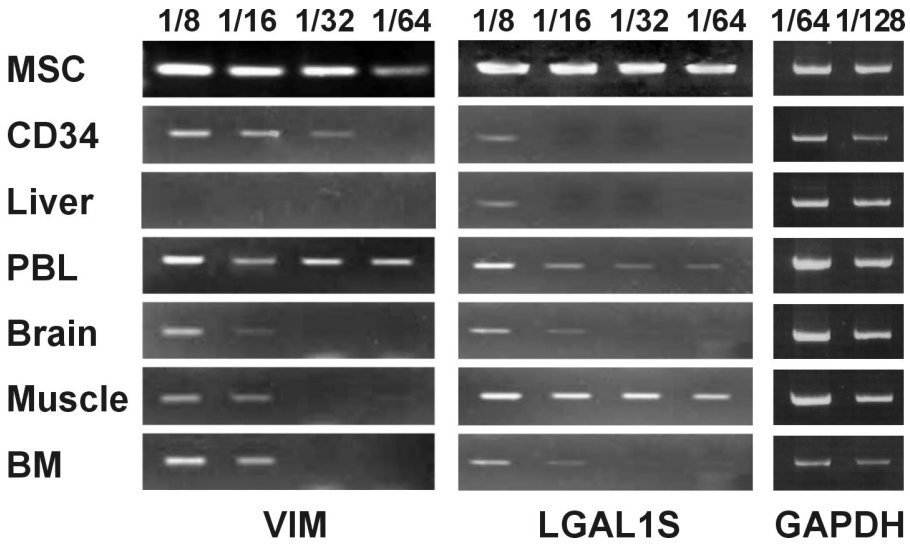

Figure 1. Semi-quantitative evaluation of mRNA abundance by RT-PCR. Total RNA was diluted 1/2 to 1/128 (only 1/8 to 1/64 dilutions are shown), reverse transcribed into cDNA and then a 30-cycle PCR with specific primers located in different exons was performed. At the left is shown the reaction for vimentin (VIM) and at the right the reaction for galectin 1 (LGAL1S). MSC: mesenchymal stem cells; CD34: $CD34^+$ hematopoietic progenitor cells; Liver: adult human liver; PBL: peripheral blood leukocytes; Brain: control human normal brain (temporal); Muscle: skeletal muscle; BM: normal human unfractioned bone marrow. A control with GAPDH primers was carried out and gave positive results up to 1/128 dilution for all samples (last two columns).

Comparison of Gene Expression of Umbilical Cord Vein and Bone Marrow Derived Mesenchymal Stem Cells

Stem Cells, *in press*

Rodrigo A. Panepucci, Jorge L. C. Siufi, Wilson A. Silva-Jr, Rodrigo Proto-Siqueira, Luciano Neder, Maristela Orellana, Vanderson Rocha, Dimas T. Covas, Marco A. Zago

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Mesenchymal stem cells (MSC) give origin to the marrow stromal environment that supports hematopoiesis. These cells present a wide range of differentiation potentials and a complex relationship with hematopoietic stem cells (HSC) and endothelial cells. In addition to bone marrow (BM), MSC can be obtained from other sites in the adult or the fetus. We isolate MSC from the umbilical cord vein (UC) that are morphologically and immunophenotpically similar to MSC obtained from the BM. In culture, these cells are capable of differenttiating in vitro into adipocytes, osteoblasts and condrocytes. The gene expression profiles of BM-MSC and of UC-MSC were compared by serial analysis of gene expression (SAGE), validated by RT-PCR of selected genes. The two lineages shared almost all of the first thousand most expressed transcripts, including vimentin, galectin 1, osteonectin, collagens, transgelins, annexin A2 and MMP2. Nevertheless, a set of genes related to anti-microbial activity and to osteogenesis were more expressed in BM-MSC, whereas higher expression in UC-MSC was observed for genes that participate in pathways related to matrix remodeling via metalloproteinases and angiogenesis. Finally, cultured endothelial cells, CD34+ HSC, MSC, blood leukocytes and bulk BM clustered together, separated from other 7 normal non-hematopoietic tissues, on the basis of shared expressed genes. MSC isolated from UC vein are functionally similar to BM-MSC, but differentially expressed genes may reflect differences related to their sites of origin: BM-MSC would be more committed to osteogenesis whereas UCV-MSC would be more committed to angiogenesis.

Flow cytometry: In cultures started from cells detached from the umbilical vein by collagenase treatment, we have regularly obtained a cell population that assumes a spindle-shaped morphology in confluent wave-like layers and can be replated several (20 or more) times. The cells harvested are negative for hematopoietic lineage markers (CD34, CD45, and CD133), monocytic (CD14), and for endothelial markers such as KDR, cadherin-5, CD31 and CD133. As observed with other mesenchymal stem cells, the majority of cells were positive for CD13, CD29, CD44, CD54, CD90 and HLA class I, and negative for HLA class II.

Table 3. Differentially expressed transcripts in BM and UC MSC (only a ummary of the table is shown)**.** Transcripts correspond to tags with at least 10-fold higher levels in one type of MSC in comparison with the other (*P* value <0.001). Out of 83 tags selected by these criteria, the 58 that mapped to a known gene or EST are shown. The best UniGene cluster for the tag is indicated in the CGAP column. Transcripts in bold were selected for validation by RT-PCR.

Higher expression in Bone Marrow derived MSC
calgranulin A, calgranulin B, alpha 1 defensin (myeloid-related sequence), class I major histocompatibility complex, laminin A/C, lysozyme, neutrophil-specific defensin alpha 3, alpha 1 type I collagen, periostin (osteoblast specific factor), biglycan
Higher expression in Umbelical Vein derived MSC
chemokine (C-X-C motif) ligand 6, matrix metalloproteinase 1 (interstitial collagenase), chemokine (C-X-C motif) ligand 1, interleukin 8, galectin 1

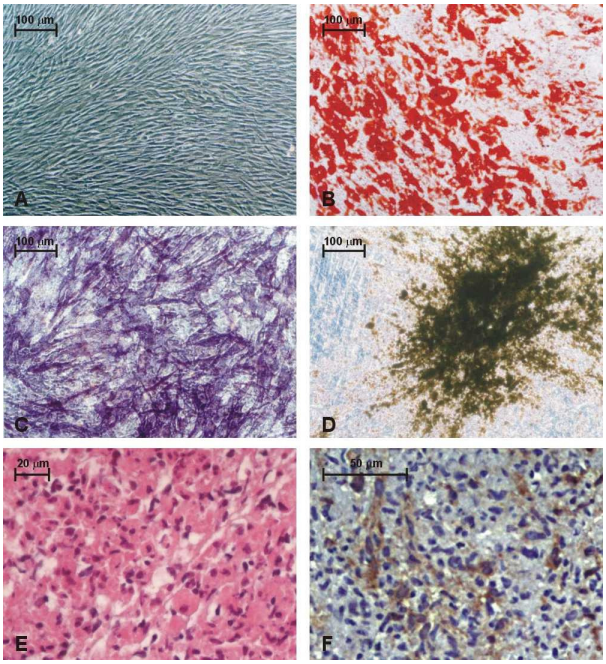

Figure 1. (A) A culture of MSC obtained from the umbilical vein. (B) Sudan III staining of adipocytes derived from the MSC. (C,D) Osteogenic differentiation: positive staining for alkaline phosphatase (C) and Ca^{++} (von Kossa reation) (D). (E,F) Chondrocyte differentiation of MSC: HE stained sections of the firm mass of cells with characteristic features of condrocytes or condroblasts (E); abundant collagen bundles in the extracellular matrix stained with anti-collagen II (F),

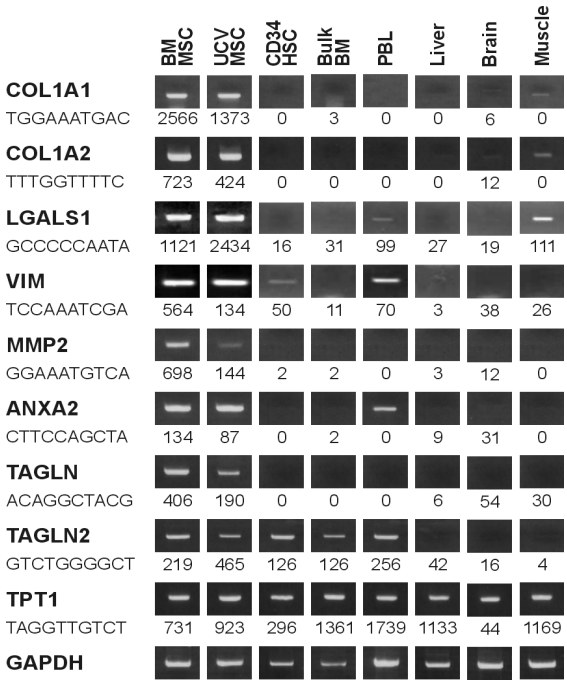

Figure 2. Comparison of gene expression by RT-PCR for nine genes in the MSC obtained from two different sources and in six additional tissues. Underneath each band, the normalized number of tags obtained by us (BM MSC and UCV MSC) or from the literature is indicated. The expression of GAPDH was used as reference for evalauting *the quality of mRNA.*

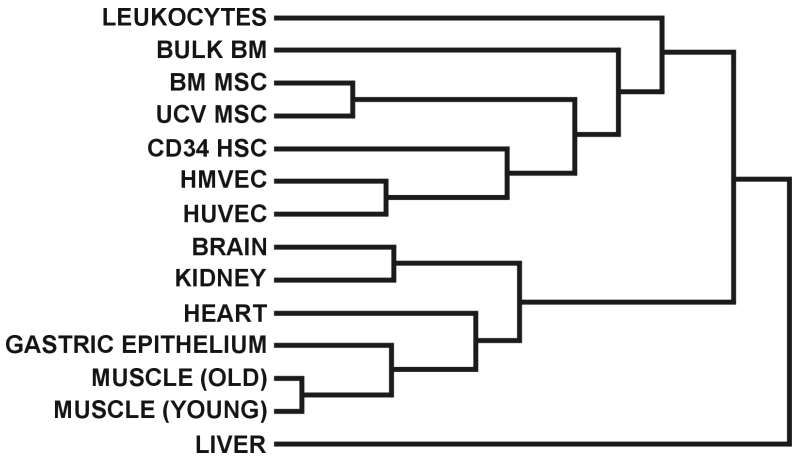

Figure 4. Dendrogram generated by hierarchical clustering (uncentered Pearson correlation, average linkage). Clustering was carried out with the first 500 most frequent tags of each of 14 libraries obtained from normal human tissues.

CLINICAL OBSERVATIONS, INTERVENTIONS, AND THERAPEUTIC TRIALS

Host defense and inflammatory gene polymorphisms are associated with outcomes after HLA-identical sibling bone marrow transplantation

Blood 100:3908-3918, 2002

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From the Hematology Department and Bone Marrow Transplant Unit and the Biostatistics Department (INSERM U444), Hôpital Saint Louis, Paris, France; and Center for Cell Therapy, Faculty of Medicine of Ribeirão Preto, Brazil.

We made the hypothesis that donor and recipient gene polymorphisms that drive the host response to microorganisms could be associated with infections after bone marrow transplantation (BMT). HLA-identical BMT was performed for patients with acute (n = 39) or chronic leukemia (n = 68). Genotyping was performed in 107 D/R DNA pairs for gene polymorphisms of cytokines (tumor necrosis factor-α [TNF-α] and TNF-β, interleukin-1 receptor antagonist [IL-1Ra], IL-6, and IL-10), adhesion molecules (CD31 and CD54), Fcreceptors (FcγRIIa, IIIa, IIIb), mannose-binding lectin (MBL), and myeloperoxidase (MPO). First infection (overall) and first episodes of bacterial, viral, or invasive fungal infection were studied retrospectively for 180 days after BMT. Univariate and multivariate analyses, using death as a competing event, were performed to study risk factors. In multivariate analysis, first overall infections were increased in patients with the FcγRIIa R-131 genotype (hazard ratio [HR] = 1.92; *P* = .04), and severe bacterial infections were increased when the MPO donor genotype was AG or AA (HR = 2.16; *P* = .03). Viral and invasive fungal infections were not influenced by any genetic factor studied. Interestingly, we also found that (1) time to neutrophil recovery was shorter when donors were FcRIIIb HNA-1a/HNA-1b (HR = 1.77; *P* = .002); (2) donor IL-1Ra (absence of IL-1RN*2) increased the risk for acute graft-versus-host disease (GVHD) (II-IV) (HR = 2.17; *P* = .017); and (3) recipient IL-10 (GG) and IL-1Ra genotypes increased the risk for chronic GVHD (*P* = .03 and *P* = .03, respectively). Finally, 180-day transplantation-related mortality rates were increased when donors were FcRIIIb HNA-1a/HNA-1a or HNA-1b/HNA-1b (HR = 2.57; *P* = .05) and donor MPO genotype was AA (HR = 5.14; *P* = .004). In conclusion, donor and recipient gene polymorphisms are informative genetic risk factors for selecting donor/recipient pairs and could help in the understanding of mechanisms involved in host defenses of BM transplant recipients.

Table 5. Causes of non leukemic deaths at day 180 according to donor FcγIIIb and MPO genotypes

Causes	Donor Fcγ IIIb			Donor MPO		
	HNA-1a/1a (n= 13)	HNA 1a/1b (n=42)	HNA-1b/1b (n=50)	GG (n=64)	AG (n=37)	AA (n=6)
GvHD	2	1	4	4	3	0
Infections	1	2	6	7	1	1
Graft failure/infections	1	-	1	1	-	1
Interstitial pneumonitis	-	2	1	2	-	1
Others	1	1	1	2	-	1

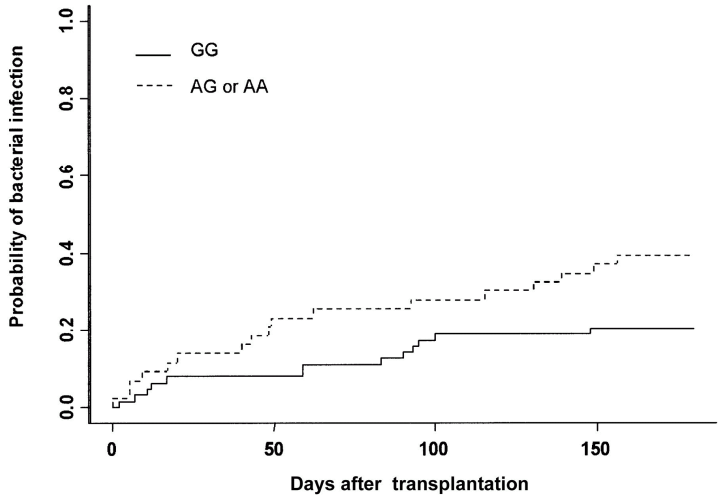

Figure 1. Severe bacterial infection. Cumulative incidence of severe bacterial infection after HLA-identical BMT according to bone marrow donor MPO genotype.

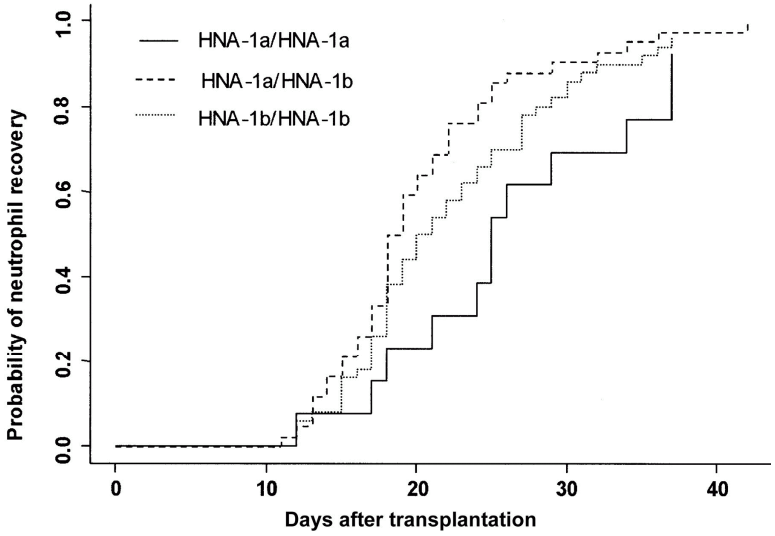

Figure 2. Neutrophil recovery. Cumulative incidence of neutrophil recovery after HLA-identical BMT according to bone marrow donor FcγIIIb genotype

Association of Drug Metabolism Gene Polymorphisms with Toxicities, Graft-versus-Host Disease and Survival After HLA-Identical Sibling Bone Marrow Transplantation

Journal of Clinical Oncology, *submitted*

V. Rocha, R. Porcher, A. Filion, H. Bittencourt, W. Silva Jr, G. Vilela, D. L. Zanette, C. Ferry, J. Larghero, A. Devergie, H. Esperou, P. Ribaud, G. Socie, E. Gluckman and M. A. Zago

From Hematology Department and Bone Marrow Transplant Unit and Biostatistics Department (INSERM Erm 321), University of Paris 7, Hôpital Saint Louis, Paris, France and Center for Cell Therapy, Faculty of Medicine of Ribeirão Preto, University of Sao Paulo, Brazil.

Individual differences in drug efficacy or toxicity can be influenced by genetic factors. We investigated whether polymorphisms of pharmacogenes that interfere with metabolism of drugs used in conditioning regimen and graft-versus-host disease (GvHD) prophylaxis could be associated with outcomes after HLA-identical bone marrow transplantation (BMT). Candidate pharmacogenes and their polymorphisms were studied in 107 recipients (R) with leukemia and donors (D) of BMT: [P450cytochrome family (CYP2B6), glutathion-S-transferase (GST), thiopurine-S-methyl transferase (TPMT), multiresistance-drug (MRD1), methylenetetrahydrofolate-reductase (MTHF) and vitamin-D receptor (VDR)]. The endpoints studied were mucositis, hemorrhagic cystitis (HC), toxicity and veno-occlusive disease of the liver (VOD), GvHD, transplantation-related mortality and survival. Multivariate analysis, using death as competing event, were performed adjusting for clinical factors. In a multivariate analysis, polymorphism of gene that interfere with cyclophosphamide metabolism was associated with oral mucositis [R-AG or GG genotype of CYP2B6*4 (785G), p= 0.0067], HC [R-CT or TT genotype of CYP2B6*2A (C64T), p=0.03] and VOD [D-GG to R-GG CYP2B6*6 (G516T), p=0.03]. Recipient-MTHRF (C677T) (genotypes CT or TT), that interfere with methotrexate, and D-Interleukine-1Ra (absence of IL-1RN*2) were associated with acute GvHD (grade II-IV) (p< 0.0001). Acute GvHD (grade III-IV) was associated with R-MTHFR (genotypes CT or TT) (p=0.05), and R-VDR TaqI (genotype TT) (p=0.04). Finally, in a multivariate analysis for survival R-VDR TaqI (Tt or TT) increased the risk of death (Hazard Ratio=4.61 for each T, p=0.035). We conclude that genetic factors that interfere with drug metabolisms used in BMT are associated with toxicities, GvHD and survival after HLA-identical BMT for patients with leukemia.

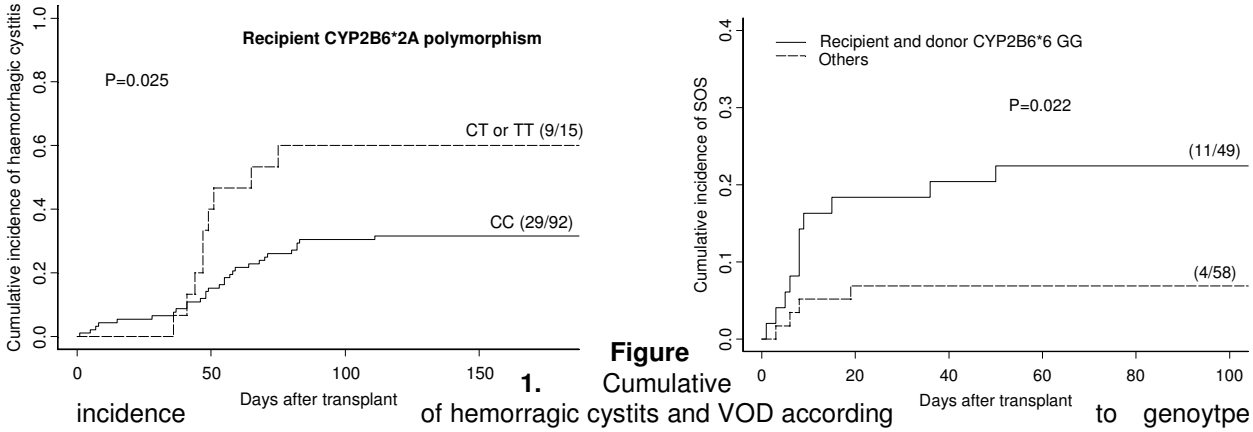

Figure 1. Cumulative incidence of hemorragic cystits and VOD according to genoytpe of CYP2B6*2A (left) and CYP2B6*6 (right), respectively.

Table 5. Multivariable analysis for outcomes after HLA identical BMT for patients with leukemia.

Outcomes	**Variables increasing the risk of outcomes**	**Odds Ratio (OR)°° or Hazard ratio (HR) (95%CI) , p value**
Mucositis (moderate to very severe)	CYP2B6*4 (AG or GG)	°°3.03 (1.37; 6.73), 0.0067
Hemorrhagic Cystitis (I-IV)	CYP2B6*2A (CT or TT) Chronic leukemia	2.22; (1.07;4.63),0.033. 4.69 (1.85;11.9), 0.0012
VOD (mild to severe)	Donor to Recipient CYP2B6*6 GG	3.49; (1.12;10.88); 0.031
Acute GvHD (II-IV) at day 100	Donor IL1Ra (absence of IL-1RN*2) to recipient MTHFR C677T (CT or TT)	3.47 (1.94;6.19); 0.0002
Acute GvHD (III-IV) at day 100	Lower bone marrow cell dose, (per 10^8/kg) Recipient MTHFR C677T (TT or CT genotype) Recipient VDR *taq*I(presence of T)	1.40 (0.99;1.98); 0.055 2.96 (0.97;9.07); 0.057 2.61 (1.23;5.56); 0.013
Chronic GvHD at 2 years	Recipient age (per 10 y) ***Recipient IL-10 (AA)*** Female donor Donor GSTP1 (GG or GA)	1.51 (1.24;1.85);<0.0001 2.70 (1.06;6.92); 0.038 1.8 (1.04;3.13); 0.035 2.25 (1.32;3.84); 0.0030
Transplant related mortality at day 180	Recipient age (per 10 y) Advanced stage of disease Donor MPO (AA) Donor FcγRIIIb HNA-1a/HNA-1a or HNA-1b/HNA-1b Recipient VDR*taq*I (for each T)	1.84 (1.28;2.63); 0.0010 7.24 (1.91;27.51); 0.0036 3.60 (1.18;10.96); 0.024 3.53 (1.34;9.31); 0.011 3.13 (1.38;7.10); 0.0063
Survival at 5 years	ABO major incompatibility Recipient age (per 10 y) Conditioning other than BuCY Recipient VDR*taq*I (Tt or TT)	2.01 (1.03;3.91); 0.039 1.40 (1.13;1.73); 0.0023 2.24 (1.25;4.01); 0.0069 4.61 (1.11;19.14); 0.035

Abbreviations: CMV= cytomegalovirus, MPO= myeloperoxidase, ys= years, kg= kilo, 95%CI=95% confidence interval, p=p value, BuCY= busulfan+ Cyclophosphamide, FcγRIIIb: Fcγ receptor IIIb (see ref 11)

The Changes in the Proteomic Profile during Differentiation and Maturation of Human Monocyte-Derived Dendritic Cells Stimulated with GM-CSF/IL-4 and LPS

Proteomics, *in press*

Sandra R Pereira, Vitor M Faça, Glauce G Gomes, Roger Chammas, Aparecida M Fontes, Dimas T Covas and Lewis J Greene

Departamento de Bioquímica, Universidade Federal de São Paulo; Centro de Química de Proteínas, Departamento de Biologia Celular e Molecular e Bioagentes Patogênicos, Centro Regional de Hemoterapia, Centro de Terapia Celular - CEPID, Departamento de Clínica Médica, Faculdade de Medicina de Ribeirão Preto, USP, Ribeirão Preto; Laboratório de Oncologia Experimental, Faculdade de Medicina, USP, São Paulo, Brazil.

Dendritic cells (DCs) are highly specialized antigen-presenting cells that play an essential role in the immune response. We used the proteomic approach based on two-dimensional electrophoresis and mass spectrometry to identify the protein changes that occur during differentiation of DCs from monocytes (Mo) stimulated with GM-CSF/IL-4 and during the maturation of immature DCs stimulated with LPS. Sixty-three differentially expressed proteins (± 2-fold) were unambiguously identified with sequence coverage greater than 20%. They corresponded to only 36 different proteins, because 11 were present as 38 electrophoretic forms. Some proteins such as tropomyosin 4 and heat shock protein 71 presented differentially expressed electrophoretic forms, suggesting that many of the changes in protein expression that accompany differentiation and maturation of DCs occur in post-translationally modified proteins. The largest differences in expression were observed for actin (21-fold in Mo), Rho GDP-dissociation inhibitor 2 (20-fold in Mo), vimentin (8-fold in immature DCs), lymphocyte-specific protein 1 (12-fold in mature DCs) and thioredoxin (14-fold in mature DCs). Several proteins are directly related to functional and morphological characteristics of DCs, such as cytoskeletal proteins (cytoskeleton rearrangement) and chaperones (antigen processing and presentation), but other proteins have not been assigned specific functions in DCs. Only a few proteins identified here were the same as those reported in proteomic studies of DCs, which used different stimuli to produce the cells (GM-CSF/IL-4 and TNF-α). These data suggest that the DC protein profile depends on the stimuli used for differentiation and especially for maturation.

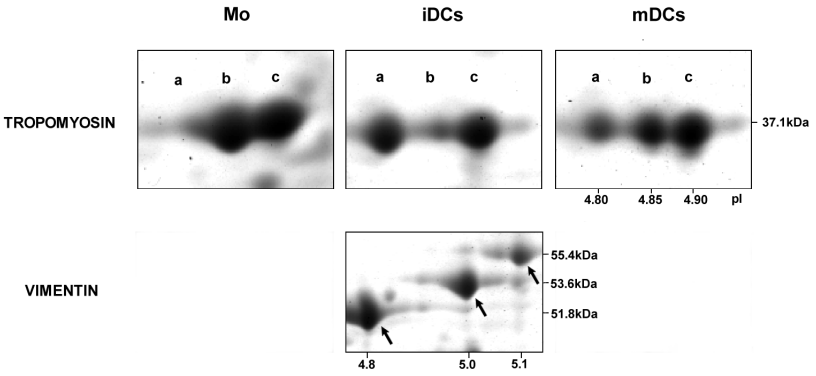

Figure 4. **Multiple electrophoretic forms of tropomyosin and vimentin.** The two forms of tropomyosin 4 (forms *a* and *b*) were identified as two electrophoretic forms with similar migration (mass) but different pI values and were differentially expressed in Mo, iDCs and mDCs (see relative volume of spots 49 and 50 in Table 1, column 3). Spot c was identified as tropomyosin 3, and was expressed to the same extent by the three cell types. The protein vimentin was identified in 10 different molecular forms. In this figure we present 3 forms of vimentin (spots 57, 58 and 59) which showed variations in both pI and mass.

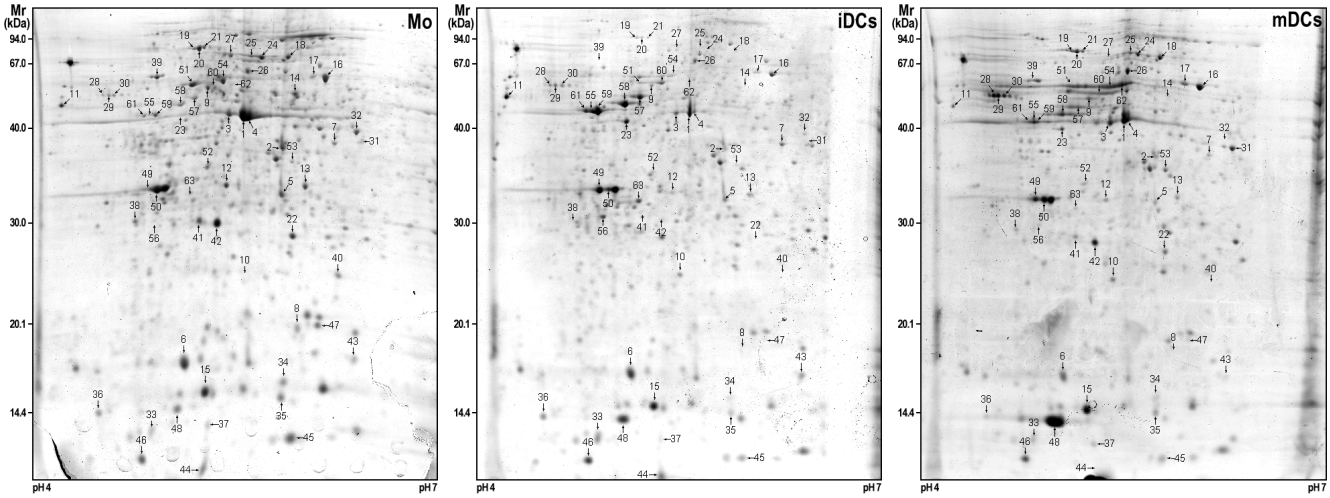

Figure 3. **Two-dimensional electrophoretic profile of monocytes, immature DCs and mature DCs.** 2DE Gels of total extracts of monocytes (Mo), immature DCs (iDCs) and mature DCs (mDCs). The number of spots detected was 336 spots in the monocyte profile, 385 in the immature DC profile and 280 in the mature DC profile. The 103 differentially expressed proteins were submitted to *in situ* trypsin digestion. The 63 spots that were identifyed by peptide mass fingerprint in a MALDI-TOF mass spectrometer are numbered in the gel and listed in Table 1.

Table 1 (part)**.** Differentially expressed proteins during differentiation and maturation of DCs

Spot Number [a]	Protein Name [b]	Relat. Volume (Mo:iDCs:mDCs) [c]			Mass/ pI 2DE [d]	Mass/ pI DataBank [e]	Masses Matched [f] # / (%)	Protein Covered (%) MS-FIT / Automatic	Acess Number Swiss-Prot	Biological Process [g]
1	2	3			4	5	6	7	8	9
1	[1] Actin, cytoplasmic 1	**3**	1	1	53,2/5,5	41,7/5,3	15 (57)	43 / 44	P02570	f
2	[1] Actin, cytoplasmic 1	**7**	1	2	45,1/5,7	41,7/5,3	9 (50)	30 / 35	P02570	f
3	[1] Actin, cytoplasmic 1	2	1	**3**	52,8/5,4	41,7/5,3	10 (62)	35 / 38	P02570	f
4	[1] Actin, cytoplasmic 1	**21**	1	6	53,2/5,5	41,7/5,3	13 (56)	46 / 44	P02570	f
5	[1] Actin, cytoplasmic 1	**2**	1	1	36,2/5,7	41,7/5,3	7 (70)	17 / 29	P02570	f
6	[1] Actin, cytoplasmic 1*	**3**	2	1	16,4/5,0	42,0/5,2	7 (33)	18 / 16	P02570	f
7	[2] Alpha enolase	**2**	**2**	1	45,8/6,1	49,5/5,8	5 (26)	14 / 34	Q05524	b
8	[3] ARP2/3 16 kDa	**1**	-	-	20,0/5,8	16,3/5,5	7 (63)	45 / 45	O15511	a,f
9	[4] ATP synthase beta chain	**1**	-	**1**	60,0/5,2	56,6/5,3	20 (50)	39 / 48	P06576	a,b
10	[5] ATP synthase D chain	-	1	**2**	24,7/5,4	18,5/5,2	11 (78)	73 / 73	O75947	a
11	[6] Calreticulin	2	**4**	1	56,2/4,1	48,1/4,3	9 (75)	32 / 31	P27797	b,c
12	[7] Chloride intracellular channel (RNCC)	**3**	1	1	37,2/5,3	26,9/5,1	12 (70)	65 / 49	O00299	a
13	[8] F-actin capping protein beta subunit	**3**	1	1	37,2/5,9	31,3/5,4	16 (64)	49 / 42	P47756	a
14	[9] Fibrinogen gamma chain [Precursor]	**5**	1	-	58,6/5,9	51,5/5,4	10 (40)	21 / 31	P02679	a,h,i,m,n
15	[10] Galectin-1	1	1	2	14,0/5,2	14,7/5,3	9(34)	72 / 59	P09382	c,k,l
16	[11] Glucose-regulated protein 58 kDa (Grp58)	**3**	1	**3**	62,5/6,1	56,7/6,0	23 (67)	42 / 40	P30101	a,b,c
17	[11] Glucose-regulated protein 58 kDa (Grp58)	2	1	**4**	63,1/6,0	56,8/6,0	19 (73)	36 / 34	P30101	a,b,c
18	[12] Glucose-regulated protein 75 kDa (Grp75)	**1**	-	**1**	70,9/5,8	73,7/5,9	15 (71)	28 / 28	P38646	-
19	[13] Glucose-regulated protein 78 kDa (Grp78)	2	1	**3**	73,2/5,1	72,3/5,1	25 (71)	40 / 39	P11021	-
20	[13] Glucose-regulated protein 78 kDa (Grp78)	**5**	1	3	73,2/5,1	72,3/5,1	20 (80)	35 / 36	P11021	-
21	[13] Glucose-regulated protein 78 kDa (Grp78)	**2**	1	1	73,3/5,2	72,3/5,1	25 (71)	40 / 40	P11021	-
22	[14] Glutathione S-transferase P	**4**	1	1	29,3/5,8	23,3/5,4	7 (24)	55 / 45	P09211	b,d
23	[15] Heat shock cognate 71 kDa protein	-	**3**	1	49,5/5,0	70,9/5,4	9 (42)	19 / 22	P11142	b
24	[15] Heat shock cognate 71 kDa protein	**2**	-	1	71,4/5,6	70,9/5,4	24 (75)	39 / 47	P11142	b
25	[15] Heat shock cognate 71 kDa protein	3	1	**5**	69,2/5,5	70,9/5,4	10 (50)	17 / 24	P11142	b
26	[16] Heat shock protein 60 kDa	2	1	**3**	65,2/5,5	61,0/5,7	24 (92)	47 / 48	P10809	a,b
27	[17] Lamin B1	**1**	-	-	71,0/5,4	66,4/5,1	24 (82)	39 / 37	P20700	-
28	[18] Lymphocyte-specific protein 1	1	3	**12**	60,0/4,4	37,1/4,7	5 (41)	22 / 28	P33241	c,e,f,g
29	[18] Lymphocyte-specific protein 1	1	1	**6**	60,0/4,5	37,1/4,7	7 (46)	27 / 33	P33241	c,e,f,g
30	[18] Lymphocyte-specific protein 1	1	2	**4**	60,0/4,5	37,1/4,7	7 (43)	27 / 33	P33241	c,e,f,g
31	[19] Macrophage capping protein	2	1	**3**	46,3/6,3	38,5/5,9	8 (50)	25 / 24	P40121	b,e,g
32	[19] Macrophage capping protein	**9**	1	-	48,2/6,3	38,6/5,9	9 (37)	25 / 26	P40121	b,e,g
33	[20] MHC I	1	**5**	1	12,1/4,8	-	-	- / 35	P30480	-
34	[21] Migration inhibitory factor-related (MRP14)	**1**	-	-	15,6/5,7	13,2/5,7	9 (64)	56 / 57	P06702	c,e,g
35	[21] Migration inhibitory factor-related (MRP14)	**6**	1	2	13,7/5,8	13,2/5,7	11 (40)	82 / 75	P06702	c,e,g

Technological Results

Technology Transfer Program

Projects related to blood transfusion

Quality control system for blood irradiation using a teletherapy unit

We developed an automated system for blood bag storage during irradiation, using a teletherapy unit. The device has two thermal compartments made of acrylic and foam for the storage of blood bags, and an automatic acquisition system, coupled with an amplifier and a thermal sensitive probe, to check blood temperature during irradiation. The thermal device kept the blood temperature below 6° C for more than 2 h. Our system allowed the simultaneous irradiation of two different blood components while maintaining a constant temperature. Dosimetric measurements showed a homogenous dose distribution in a phantom constructed to simulate our device. The results of this project were published in *Vox Sanguinis* 2004; 86:105, and was the object of an article by the monthly science review edited by FAPESP (*Pesquisa FAPESP,* in August 2004).

Dissemination of quality control programs and certification

The Regional Blood Center (Hemocentro) developed and installed a quality control program (ISO 9002) certified in 10/29/1999. The institute responsible for the certification process had had no previous experience in certifying a blood center, so that the process had to be developed by our Center. In December of 2003 the Hemocentro's certification was upgraded to ISO 9001/2000. The certification itself is now being extended nine additional units of the Hemocentro. In September 2003 the Hemocentro was evaluated and preliminarily certified by the AABB (American Association of Blood Banks), thus becoming the first Blood Bank in Brazil and one of the five in America that has double certification (ISO and AABB). In July 2004 the Hemocentro led a national workshop to disseminate its experience related to certification.

A non invasive method for measurement of iron in human liver

Iron overload is a common problem in patients with chronic anemia who are submitted to regular blood transfusion. Chelation therapy is effective in eliminating the effects of iron toxicity, thus increasing life expectancy, and needs regular and accurate determination of iron overload. Due to the large amounts of iron usually stored in liver, this organ is the main target for the direct measurement iron stores by liver biopsy, which causes significant discomfort and risk to the patient. In collaboration with the Medical Physics Department of the Faculty of Science we developed a non-invasive method for iron measurement in the human liver based on magnetic susceptometry. We performed measurements in a group of 34 normal individual and 20 patients with iron overload showing the ability of the instrument to distinguish normal and pathological conditions. The instrument is now used routinely for the follow up of patients. The results of this project were published in part in *Review of Scientific Instruments* 74(6):3098, 2003 and *Physiological Measurements* 23:683-93, 2002.

Development of a GMP self-diagnosis software

The Regional Blood Center developed in partnership with GMP, a private company, a software intended to perform the auto-diagnosis of Good Manufacturing Practices in blood banks and blood processing units. The software was installed and implemented in 121 units of the Blood Center network and two GMP evaluations have been performed thus far in each unit. The preliminary results were presented at a Seminar conducted by FAPESP in August 2001. This program was supported by a Public Policy grant from FAPESP.

Stem cell therapy

In 1999 researcher of the CTC and from other centers established a collaboration program to setup a network of public umbilical cord banks in the country, called BrasilCord, to make the cells available for non-related hematopoietic stem cell transplantation for patients who do not have a compatible related donor. They prepared the technical and the political documents and started negotiations with the Federal Government to implant the system. Lacking funds, the government postponed the decision to launch the program.

Umbilical Cord Blood Bank (UCBB) and Brasil-Cord Program

The Center set up a UCBB that is now operating in a pilot phase in an adapted laboratory situated in the Blood Center and established a collaboration with an Obstetrics Hospital for regular collection of cord blood. Thus far, the material collected is used for research and for training of the personnel before starting the operational phase of the BrasilCord Program.

Cloning and expression of proteins of biotechnological interest

Production of HIV-1 recombinant proteins

Our objective is to obtain proteins that may be used in diagnostic tests (ELISA and Western Blot) of HIV-1 infection. The HIV-1 p24 capsid protein is an important early marker of HIV infection. We recently isolated a 666-bp fragment corresponding to the p24 HIV. This DNA product was isolated by nested PCR from DNA isolated from peripheral blood mononuclear lymphocytes of an asymptomatic HIV-1 seropositive human subject from University Hospital of Ribeirão Preto. The fragment was further sub-cloned in an expression vector and transfected into the HEK293 cell line. By RT-PCR we have detected the expression of mRNA p24 in the cell population after geneticin selection, and the presence at the protein was detected by immunologically

Production of HTLV –1 recombinant proteins

The gp21 gene of HTLV-1 was cloned into vector system (Gateway™ Technology, Invitrogen Life Technologies) for expression in *E.coli* (Rosetta(DE3)pLYsS strain, Novagen). Soluble and insoluble fractions of the induced culture were covered and analyzed by SDS-PAGE showing an intense band with an approximate molecular weight of 45,000 (expected size for this recombinant protein which was tagged with GST in the N-terminal portion). This recombinant fusion protein (GST-rgp21) was identified by immunological detection using anti-gp21 monoclonal antibody. Also it was tested against HTLV-1 positive serum samples and negative serum. We conducted purification using GST columns showing lower degree of recovery. Other *env* regions were isolated and cloned into TOPO system as: gp21-HTLV-2, gp46 – HTLV1 and HTLV-2, the whole *env* gene for HTLV-1. Further steps of recombination into expression vector systems will be conducted.

Production of human clotting factor VIII

With additional financial support from FINEP we started a pilot project aiming at cloning and expressing human clotting factors VIII and IX. These factors are absent or altered in patients with hemophilia A and B, respectively, who depend on infusion of exogenous factors for the normalization of the coagulation process. We generated two B-domain deleted recombinant factor VIII molecules named rFVIIIΔB_{SQ}*BMN* and

rFVIIIΔB$_{SQG}$*MFG*. Using two different strategies, which consist of sorting by flow cytometry or treatment with two therapeutic drugs we generated two Hek-293 cell lines. Stable expression of recombinant factor VIII was demonstrated by RT-PCR and the analysis of factor VIII biological activity showed levels of 246% e 543%, respectively, in Hek-293/FVIIIΔB$_{SQ}$*BMN* and Hek-293/FVIIIΔB$_{SQG}$*MFG* cell lines These preliminary results demonstrate that we developed a viable strategy to generated FVIII in retrovirus cell system that may be a good candidate for further development aiming at producing a the FVIII replacement therapy for hemophilia.

Production of human clotting factor IX

Hemophilia B, also known as Christmas disease, arises from mutations in the factor IX gene. The cornerstone of therapy has been replacement of the deficient factor, historically with pooled-plasma derivatives, with risks of transmission of blood-borne infectious diseases. Thus, recombinant factors have become the most attractive treatment approach for hemophilia. Herein, by screening a human kidney cDNA library we isolated a full-length cDNA encoding human factor IX. The factor IX recombinant molecule named rFIX-LXIN was transfected into HepG2 and a cell line that constitutively express human factor IX was established using a retrovirus system. The stable expression of rFIX was demonstrated by RT-PCR and the biological activity of rFIX molecules present in the supernatant was 274%. The identity of the rFIX present in the serum-free medium was confirmed by SDS-PAGE and immunoblotting, and its purification was obtained by reversed-phase HPLC. The strategy developed seems viable and highly valuable for the production of recombinant human coagulation factors into mammalian cell system.

Other activities in technology transfer

Setup of a small business incubator

In November 2002, the Small Business Incubator linked to the CEPID (INBIOS – *Incubadora de Biotecnologia e Saúde*) was officially launched. The Incubator is located in a space of 126 m^2 (figure): it has the capacity to support initially up to 5 small business initiatives, three of which are being installed now. Two of the small companies are dedicated to the production of diagnostic reagents developed by researchers of the Center for blood banks (immuno-hematology) and for the screening of infectious diseases (Chagas´s disease, HIV and HTLV infections). The third small company is dedicated to software development and bioinformatics.

Collaboration with local company

During this period, the Transfer Coordinator promoted meetings with a local pharmaceutical company Indústria Farmacêutica JP (JP Pharmaceutical Industry), which is a partner in the development of plastic products for cell collection and culture. Some projects have been outlined and efforts are currently underway to make this cooperation operational

Education Results

Educational Program

The Science House

The **Science House** was first set up near the Blood Center and then transferred temporarily to the Grounds of the Blood Center. The USP High Administration has already authorized the use of one of the houses owned by the university for its definitive installation on the USP Campus. The place is currently being renovated, and should be ready for occupation within one month. The Science House is the physical center from where the educational activities of the CTC Center are coordinated. The House establishes contact with the researchers, acts as an intermediary between teachers and students in the schools, and keeps records about the various activities. It is a place for meetings, for storing educational material, for planning activities, and for producing the educational material.

The Cells, the Genome and You

That is the title of our main education program aimed at the high and secondary school. It includes four components: an extension course for teachers, a talent-hunt project, the *Journal da Ciência* (Science Newsletter), and the Science Education Portal.

Extension course

The Cell, the Genome and You, Teacher is an extension course recognized by the University of S. Paulo and counts credits for continuous education. We have already complete two versions of the course. During this period, the activities continued with the Saturday classes and with 6 research groups under the guidance of Center researchers during the week. The 22 teachers who started in 2001 were officially supposed to attend until December 2002, but 18 of them continued to attend until May 2003. At the present we are starting the 3rd version of the course.

Talent-hunt project

This project is closely associated with the extension course for teachers. We stimulate the teachers to bring the students to the center and to participate in activities with the researchers. Many of the young researchers participate regularly in seminars, bring students to visit the laboratories and help them to plan experiments that can be performed in at home or at school. The end product is quite variable; it can be a poster, a plastic model, a monograph, a protocol, a comic strip or a theatrical performance. In the years 2001-2003 the program involved 83 teachers from Ribeirão Preto and 33 other towns, and 494 students, of which 30 helped the teachers as monitors in classes, laboratories, workshops, and science exhibitions.

Junior research fellowship

FAPESP started this year, for the first time, to give Junior Fellowships for high school students who wish to engage in a scientific training program. On a competitive basis, four of the fellowships offered were assigned to students of our program. Each one is under the supervision of a young researcher of the center.

The education portal

It has been functioning and actualized regularly during the four years since the center's organization. It has been recently redesigned, and can be accessed at *http://ctc.fmrp.usp.br/casadaciencia/*

Science Newletter

Since the center's organization, 9 numbers of the ***Jornal da Ciência*** have been published, with 3,500-4,000 copies each edition. It is organized by the team of the

Education Coordinator, with the help of teachers, students and journalists, and is used in the classroom by teachers in different areas. Starting in number 8, there is a new section *Trail* concerning the experimental work of the students and of their advisers in the Talent-hunt program.

Summer courses

We have thus far organized 5 two-week summer courses entitled ***Traveling through the Genome*** or ***Genome, Proteome and the Cellular Universe***. This course is aimed at university undergraduate students of the biological and medical science areas (biology, medicine, dentistry, pharmacy, etc). A total of 188 students from most of the Brazilian States attended these courses, and some of them returned later as candidates for longer periods of research work at our center.

Dissemination Activities

The Center's researchers are often the source of information or opinion on topics related to their expertise. The following are a few examples of this activity.

Contribution to the Parliamentary debate

A new federal law on biosafety is now under discussion in the Federal Senate in Brasilia. The Center's coordinator presented a talk to the Senators and participated in a public debate in the Social Issues Subcommittee on the topic of the use of stem cells

Public debate

On 12/14/2001 there was a public debate on the use of human stem cells at the Auditorium of *A Folha de São Paulo* (one of the most prestigious Brazilian daily newspapers). Invited speakers were MA Zago, M Zatz, D Brunoni and R Romano. Moderator was R Bonalume Neto. News appeared in the national edition of the following day.

Articles in the monthly science review edited by FAPESP

MA Zago – *Injeções de vida: clonagem e terapia celular* [Injections of life: cloning and cell therapy] Pesquisa FAPESP 67, August 2001.

MA Zago – *Opinião: Um debate fora de foco* (sobre células embrionárias) [Opinion: A biased debate (on the embryonic cells)] Pesquisa FAPESP 67, August 2001.

MA Zago – *Genoma humano e a era pós-genômica no Brasil* [Human genome and the post-genomic era in Brazil] Pesquisa FAPESP 54, June 2000.

Press and Television

Information for the general public is an important item in the CTC program. In numerous occasions the CTC has been the focus or the source of news and information in the press and television. Topics such as blood transfusions, diseases transmitted by transfusion, cancer, cancer therapy, organ transplant, transgenic products, genetics of the Brazilian population, the genome project, for instance, are often the subject of interviews and reports.

Changes in Plan

The focus of the research and the technology transfer projects is the use of cells for therapy, from allogeneic and autologous stem cell transplantation to novel techniques of cell collection, culture and modification of cell's genetic material to manipulate cell populations or the cells themselves to increase their therapeutic potential. Within this wide and rapidly expanding field we are progressively focusing on applications for the treatment of malignant diseases, especially hematopoietic malignancies and melanoma, autoimmune diseases and some infections. As a consequence of the interaction developed in the period of 2001-2004, we have now selected two priority subjects for research:

- **The use of stem cells for the treatment of neoplastic and inflammatory diseases**
- **Search for new therapeutic targets in hematological malignancies**

STEM CELLS FOR THE TREATMENT OF NEOPLASTIC AND INFLAMMATORY DISEASES

Adult mammalian bone marrow contains two discrete populations of adult stem cells. The first and the most fully characterized are the hematopoietic stem cells responsible for the continuous production of blood cells throughout the life. The second marrow resident population of stem cells termed bone marrow stromal cells or mesenchymal stem cells (MSC) are much less characterized.

MSC are multipotent with the capacity to give rise to each of the differentiated cell lineages found in the marrow stroma, bone and cartilage; in culture, these cells have extensive differentiation potential to osteocytes, chondrocytes, adipocytes, neurons and myocytes. These properties, coupled with the apparent ease of *ex vivo* culture, have engendered considerable interest in potential therapeutic applications of these cells in a wide range of settings. However, many aspects of MSC biology, including the cellular identity and anatomical location of their precursors, and their *in vivo* functions remain obscure.

This project aims at understanding various aspects of MSC biology that may be relevant for their use in medicine, including basic aspects and therapeutic applications in pre-clinical and clinical studies, and the pathways that control hematopoietic stem cell differentiation.

Basic Research

Mesenchymal stem cells

In previous studies, we characterized the MSC derived from bone marrow and umbilical cord blood in terms of gene expression. We demonstrated their similarities revealed by the gene expression profile, although there are differences in the expression of various genes, especially those related to bone formation and angiogenesis. Expanding these studies, we have isolated MSC from various other human tissues, including the subendothelium of the umbilical vein, of the saphena vein and of the umbilical artery, and from various fetal tissues such as the liver, the gonads, the skin,

the amniotic fluid, the muscle fascia, and the carotids. All the MSCs isolated from these tissues exhibit the capacity to give rise to osteocytes, chondrocytes and adipocytes in culture, and express a set of genes specific for MSC. In all these experiments we used cells derived from the multicolonies culture of MSC after 3-9 passages. These cultures were composed by true stem cells and by cells with a certain degree of differentiation, compromised with osteoblastic, adypogenic and chondrogenic lineages. The next step is to separate the most primitive MSC from the remaining differentiated cellular population. To do this we intend to isolate MSC using specific markers such as STRO1, CD146, CD106, CD73 and CD63, by flow sorting or by purification with magnetic beads labeled with the specific antibodies. The phenotype and the biological properties of the more primitive MSC will be studied by analyses of surface expression of mesenchymal markers, by assay of colony efficiency formation, by *in vitro* and *in vivo* differentiation into osteocytes, chondrocytes and adipocytes, and by determining the gene expression profile and the pattern of expressed protein by proteomics approaches. We are developing animal models suitable for MSC transplantation to perform the *in vivo* studies,

Animal models

We plan to study the behavior of MSC *in vivo* using animal models of various diseases, including post bone marrow transplantation graft versus host disease (GVHD), acute liver injury induced by carbon tetrachloride, chronic cardiac insufficiency induced by adriamycin, and in acute radiation disease.

Mesenchymal stem cells and liver regeneration

There have been a number of reports showing the potential of adult rodent bone marrow cells to differentiate into a variety of cell types, including hepatocytes. Although bone marrow cells repopulate the damaged liver of mice with fumar*y*lacetoacetate hydrolase deficiency, this correction is caused by fusion of donor bone marrow cells with recipient hepatocytes, and there is little or no contribution of bone marrow mononuclear cells to form hepatocytes to replace injured livers. Recently, however, Seo *et al* (Bioch Bioph Res Comm 2005) have demonstrated that human adipose tissue derived stromal cells differentiate into functional hepatocytes *in vitro* and *in vivo* in a model of liver injury by carbon tetrachloride.

We intend to test the capacity of MSC to form hepatocyte *in vitro* and *in vivo* in liver injury models. The MSC obtained from de umbilical cord blood will be transfected with GFP using a retroviral system. The *in vitro* hepatocyte differentiation will be stimulated incubating the MSC with Hank´s balanced salt solution without serum, containing 0,1% dimethyl sulfoxide, 10ng/mL rhHGF and rhOSM. The gene expression profile will be evaluated by SAGE or by microarray before and after stimulation,.

NOD/SCID mice will be submitted to acute liver injury by carbon tetrachlorid (CCL4) treatment and transplanted as described by Seo *et al*. The *in vivo* hepatocyte differentiation will be tested by immunocytochemistry and by immunohistochemistry by using antibodies to human albumin. The functional properties of the hepatocytes will be tested by RT-PCR using primers for orosomucoid 2, alpha-fetoprotein and albumin, and by their ability to uptake acetylated low-density-lipoprotein (LDL). Models of chronic liver disease such as fumarylacetoacetate hydrolase knockout mouse and hepatitis B transgenic mouse should be tested in the second part of this project.

Mesenchymal stem cells and experimental GVHD

Hematopoietic stem cells transplantation (HSCT) is the first-choice treatment for many malignancies (leukemias, lymphomas, myeloma) and non-neoplastic diseases (aplastic anemia, primary immunodeficiencies, genetic diseases). Its main limitation is the necessity of HLA compatibility between donor and receptor which restricts the availability of donors. Cases with a partial compatibility are submitted to a variable risk from 10 to 80% to develop a potentially lethal complication called GVHD (Graft Versus Host Disease).

Acute GVHD begins with the recognition of MHC molecules in the surface of receptors cells by donor T limphocytes. These alloreactives T cells infiltrate the receptor's organs such as liver, intestine and skin where they proliferate and start an inflammatory response, causing tissue injuries. The most effective way to block the development of GVHD is submitting the HSC preparation to T-cell depletion. However, T-cell depletion increases the transplant rejection ratio, opportunistic infections, tumor relapse and secondary limphoproliferative diseases.

Mesenchymal stem cells have a remarkable immunomodulatory capacity *in vitro* and *in vivo.* They inhibit T-cell and dendritic cell proliferation, a process that involves cell-cell interactions and secretion of inhibitory cytokines.

Phase I studies with MSC infusion in patients submitted to bone marrow transplantation have been reported. The main goals of MSC infusion were to promote de HSC graft and GVHD prevention. Preliminary results suggest that MSC can contribute to GVHD reduction. We plan to study the effect of MSC infusion in animal models of GVHD. We will use C57BL/6 (H-2kb) mice as bone marrow donors, and BALB/c (H-2kd) as receptors. The bone marrow transplantation between these animals results in acute GVHD. MSC from C57BL/6 mice will be isolated, expanded and labeled with GFP using a retroviral vector. Appropriate amounts of MSC-GFP will be administered to the recipient animals at determined time intervals and we will observe the effects upon the development of GVHD. We will observe a numbers of variables including clinical parameters, pattern of tissue injury determined by GVHD, the profile of cytokine secretion and the tissue grafting of GFP-labeled MSC.

Human mesenchymal stem cell transplantation in an experimental model of heart failure

We will use an experimental model in rats to evaluate the potential impact of human mesenchymal bone marrow stem cells (hMSC) transplantation in chronic heart failure. The chronic heart failure will be induced in rats by using doxorubicin (15 mg/kg i.p. over 2 weeks). Four weeks after drug infusion, the left ventricular ejection fraction and volumes will be assed by 2D-echocardiography. The animals will be assigned to 3 groups: 1. controls (n=20); 2. One procedure of peripheral infusion i.v. of hMSC (tail vein, $4x10^6$ cells, n=20); 3. Repeated procedures of hMSC peripheral infusion (tail vein, $4x10^6$ cells, n=20): 1 infusion/week over 4 consecutive weeks. Four weeks after treatment, the animals will be submitted to a final 2D-echocardiographic left ventricular function evaluation, subsequent euthanasia and tissue collection for histological analysis.

Hematopoietic progenitors

The hemangioblast – the cell that originated endothelial and hematopoietic cells in the embryo – is a transient cell type that develops early and disappears quickly during embryonic development. In human postnatal life, $CD133^+$, $CD34^+$, or $CD34^+KDR^+$ cell subsets in bone marrow, peripheral blood and cord blood possess the functional activity of hemangioblasts and are capable to differentiate into both hematopoietic and endothelial cells. We plan to separate pure populations of bone marrow, peripheral blood, and umbilical cord blood $CD133^+$, $CD34^+$, $CD34^+KDR^+$, $CD133^+KDR^+$, $CD34^-$ $CD133^+$, $CD34^+CD133^+$ cells to study their capacity to form hematopoietic colonies on long term culture (LTC-IC), and endothelial colonies in matrigel plaques. Next, we will examine the gene expression profile of $CD133^+$ and $CD34^+$ cells in order to characterize them. We also intend to transplant these cells in animal models of hematopoietic and endothelial injury to evaluate their clinical potentials.

We are also examining the genes involved in the early differentiation of $CD34^+$ cells either along the erythrocytic or the granulocytic-monocytic lines. $CD34^+$ cells isolated from the normal bone marrow by immunomagnetic techniques are incubated briefly (up to 40 hours) in medium that promotes differentiation along one of the hematopoietic lines, and the gene expression is analyzed by SAGE and microarrays. If the primed $CD34^+$ cells are culture for 2-3 weeks in semisolid medium they will form preferentially colonies of the red or the white types. The effect of treatment with demethylating agents and histone deacetylase inhibitors upon the properties and gene expression of CD34+ precursors is also being evaluated.

Embryonic Stem Cells

Embryonic stem cells (ES) are cells derived from the early embryo that can be propagated indefinitely in the undifferentiated state while remaining pluripotent. ES can be isolated from the inner cell mass of blastocysts, from the embryonic ectoderm and from primordial germ cells. When transfected back into early embryo, they contribute to all the tisues of the embryo except to the placenta; they are not, thus, able to generate a complex organism. Most of the knowledge of ES biology are based on research with mouse embryonal carcinoma stem cells. The culture and frozen storage of human embryonic stem cells remains difficult, slow and labor-intensive. The ability to direct ES into specific differentiation pathways and to obtain pure populations of differentiated phenotypes remains limited and is the object of great interest.

We plan to study several biologic characteristics of ES lineages donated by Dr. D A Melton (Howard Hughes Medical Institute, Harward University). The objectives will be:

1. Evaluate the ES gene expression profiles by quantitative and qualitative methods.
2. Compare the ES gene expression profile with the gene expression profile of adult stem cells including hematopoietic stem cells (CD34+, CD133+) and mesenchymal stem cells from bone marrow and umbilical cord blood.
3. Understand the genetic and molecular mechanisms involved in the initial phase of ES differentiation.

In the second phase of the project, we plan to isolate, characterize and propagate ES lineages derived from the inner cell mass of blastocysts from frozen embryos obtained from the Fertilization Clinic of the University Hospital.

Clinical Research

Autologous hematopoietic stem cell transplantation for therapy of autoimmune and inflammatory diseases

The clinical trials focusing in the use of high-dose immunosuppression associated to autologous hematopoietic stem cell transplantation (HSCT) for therapy of autoimmune and inflamatory diseases will continue and evolve to new developments. Treatment of multiple sclerosis with HSCT in phase I/II trials was performed in 30 patients and the course of the disease stabilized or improved in most patients. Thus, we plan to engage in a phase III international trial comparing HSCT with the best available pharmacological treatment for MS (mitoxantrone). Current phase I/II trial of HSCT for early-onset type I diabetes mellitus which shows very promising results will be completed and a new one for early-onset amyotrophic lateral sclerosis will be open. More experience should be gained in rheumatic diseases (systemic lupus, systemic sclerosis and others) before engaging in phase III trials.In addition, experimental models of autoimmune/inflammatory diseases (inflammatory bowel disease, idiopathic pulmonary fibrosis and others) will be developed and treated with various forms of HSCT. A further development of these studies will be the use of stem cells from different sources (hematopoietic, mesenchymal and embryonic) for tissue regeneration in cardiac, neurologic and degenerative diseases.

The use of mesenchymal stem cells as suppressor cells in haploidentical stem cell transplantation for advanced neoplastic diseases.

Stem cell transplantation is the only curative approach for several hematological and non hematological diseases. Unfortunately a large proportion of patients will not have a matched sibling for transplant. Thus, grafts from other sources, not only from unrelated donors but also cord blood has been used. The relatively small number of hematopoietic stem cells in a cord blood unit limits its use in adult patients, whereas the chance to find a suitable donor in the national and international donor registries is limited by the frequency of HLA phenotype and the time required to identify the right donor. Most of the patient will progress from their disease or die while waiting for a donor. Since virtually all patients will have a full-haplotype mismatched donor in the family this could be an interesting option in patients with advanced neoplastic diseases. The use of mismatched family donors has been evaluated for more than 20 years. Unsatisfactory early results, especially because of graft versus host diseases and graft failure, could be reverted after the use of high doses of stem cell infusion introduced since 1990. The demonstration that megadose CD34 positive cells exert suppressor activity against activated T cells opened the way for the use of these cells in mismatched transplants.

More recently, mesenchymal stem cells have been shown to have similar imunossupressive effects in animal tumor models and in T cell proliferation and dendritic function *in vitro*. As mesenchymal stem cells also have been used for better engraftment, we supposed that the use of mesenchymal stem cells in a full-haplotype mismatched stem cell transplant could be able to improve engraftment while preventing GVHD. The use of full-haplotype mismatched mesenchymal stem cell infusion was already used in a case of severe GVHD with resolution of the disease.

The project involves the use of mesenchymal stem cells together with bone marrow infusion on day zero in a full-haplotype mismatched stem cell transplantation and a additional infusion on day +6. Further mesenchymal stem cell infusions will be used if severe GVHD appears. For this objective we will select patients with renal carcinoma and patients with relapsed acute myeloid leukemia who do not have an HLA-matched related donor.

Search for new therapeutic targets in hematological malignancies

As result of the joined effort of different laboratories, originally focused on distinct aspects of molecular and cellular biology, it was possible to identify disease models to which the present project might now contribute by identifying new therapeutic targets. Two groups of diseases were selected for further analysis: lymphoproliferative disorders and acute myelogenous leukemia.

Lymphoproliferative disorders

Mantle cell lymphoma (MCL) is a distinct subtype of non-Hodgkin lymphoma that is associated with the translocation t(11;14)(q13;32) and ectopic overexpression of cyclin D1. The disease is predominantly disseminated at diagnosis and a frank leukemic phase occurs in one third of patients. The pre-germinal-center naive B-cells, which populate the mantle zone of the secondary lymphoid follicles, are thought to be the cells that give origin to MCL. However, overexpression of cyclin D1 alone is not sufficient to cause lymphoma, and a better understanding of the additional molecular lesions may provide insights toward pathogenesis and new therapeutic approaches. In this context, large-scale gene expression studies may be useful in the investigation of such molecular alterations. To address this issue, we intend to compare the gene expression profiles of mantle cell lymphoma cells and normal naive B-cells using oligonucleotide microarrays. Lymphoma cells and naive B-cells (IgD+CD38±CD27-) are isolated by magnetic activated cell sorting, from the peripheral blood of patients with mantle cell lymphoma in the leukemic phase and from tonsils of normal controls. The analyses are performed in replicates using the Amersham CodeLink Human UniSet I Bioarrays with 10,000 genes. Our preliminary results identified 106 genes differentially expressed with a fold change of at least three times, 63 induced and 43 repressed in mantle cell lymphoma in comparison to naive B-cells. Ten genes were selected (6 induced and 4 repressed in lymphoma cells) for quantification by real-time RT-PCR for confirmation of microarray results. We have already stored samples from 21 patients with mantle cell lymphoma in the leukemic phase, as well as from 14 patients with other chronic lymphoproliferative diseases and 7 normal individuals for real-time RT-PCR analysis. Amongst these candidate genes, there are key regulators of the PI3K/AKT1, WNT and TGFβ signaling pathways.

Chronic Lymphocytic Leukemia (CLL) is characterized by the proliferation of mature B-lymphocytes. Several recurrent genetic abnormalities have been described in CLL, however none of them is detected in all cases and a few are associated with the clinical outcome. Recently, abnormalities in the TGFβ, WNT and PI3K/AKT1 pathways were reported. In addition, based on the microarray analysis mentioned above that also

suggest that these pathways are involved in the pathogenesis of MCL, we opted to quantify the expression of genes of the WNT and PI3K/AKT1 pathways by Real Time PCR as well as to perform SAGE analysis of CLL cells. We currently have samples from 60 CLL patients and the methods have been established in our lab.
We will also analyze the *SPARC* gene, which may play a role in lymphoproliferative disorders (candidate genes approach). The SPARC protein was recently associated with the TGF-beta pathway and is involved in cell adhesion, metastasis and matrix metalloproteins production (such as MMP2) in many types of neoplasias. We aim to inactivate the *SPARC* gene in a MCL cell line using a *SPARC* antisense construct pCML/SP-AS. The ability of mutants to adhere and migrate will be evaluated *in vitro* using Matrigel. If the inactivation of *SPARC* changes the MCL-cell properties, we intend to analyze downstream targets of SPARC using microarray/real time RT-PCR approach. Finally, we also intend to test the effect of halofuginone, a TGF-beta inhibitor, on MCL-cell lines (with and without *SPARC* inactivation).

A potential target already identified in this project is the tumor-associated antigen *PRAME*. We have so far demonstrated that *PRAME* is expressed at higher levels by Mantle Cell Lymphoma and Chronic Lymphocytic Leukemia cells compared with normal peripheral blood lymphocytes (as we have also demonstrated that it is often expressed in squamous cell carcinomas and osteosarcomas). We will analyze PRAME expression in other lymphoproliferative disorders, namely the NK lymphomas and related disorders, in which the development of new diagnostic markers would be helpful. Moreover, in order to determine if anti-PRAME antibodies might be of potential clinical use, we plan now: a) to demonstrate that it is expressed on the cell membrane, by means of confocal microscopy of normal, MCL and CLL lymphoid cell using the anti-PRAME MoAb and an anti-CD20 commercial Ab; b) to determine its spectrum of expression by flow cytometry in lymphoid organs (spleen, tonsils and lymphnodes), and by immuno-histochemistry on other tissues (liver, kidney, gut, esophagus, heart, brain, testicle, skin, eye, aorta, lung). Additionally, we plan to evaluate *in vitro* the eventual anti-leukemic effect of the anti PRAME antibody. In order to do that, we will: a) incubate tumour cells (cell lines Granta-19 and K562) with increasing concentrations of anti-PRAME (0; 0.02; 0.2; 2 and 20 µg/mL) and determine the percentage of apoptotic cells after 24, 48 and 72 h of culture using annexin V and propidium iodide staining; b) quantify the phagocytosis of tumour cells opsonized with the MAb; c) evaluate the ADCC in the presence and absence of antibody, and d) determine complement mediated lysis of tumour cells incubated with the anti PRAME in presence of human serum (inactivated and not inactivated).

Acute Myelogenous Leukemia

Acute myelogenous leukemia (AML) is characterized by the abnormal proliferation of progenitors that present a block of differentiation. Acute promyelocytic leukemia (APL) is a distinct subtype of AML associated with the translocation between chromosomes 15 and 17, involving the *PML* and *RARα* loci. The fusion protein encoded by the chimeric *PML-RARα* gene retains the main functional domains of the parental proteins and, therefore can: *i*) bind to retinoic acid (RA) responsive elements as either homodimers or multimeric complexes containing retinoid X receptors (RXR); *ii*) bind to the ligand (RA) with an affinity comparable to that of wild-type RARα and *iii*) physically

interact with native PML delocalizing it from discrete subnuclear structures called nuclear bodies (NB). In absence of ligand, PML-RARα and RARα affect transcription by forming heterodimers with RXR, which recruit nuclear corepressors (N-CoR) and histone deacetylase (HDAC) complexes, thus altering chromatin structure. Upon addition of physiological concentrations of RA, complexes containing native RARα are released and the receptor associates with coactivators. In contrast, the PML-RARα containing repressive complexes are stable, due the presence of the coiled-coil region of PML, which cause the formation of PML-RARα oligimers. Therefore, PML-RARα oncoprotein acts as a constitutive transcriptional repressor, through an epigenetic mechanism.

In collaboration with Prof. Pier Paolo Pandolfi from the Memorial Sloan Kettering Cancer Center from New York, we have been analyzing a transgenic model of APL, in which the *PML/RARα* cDNA is under the control of the *human Cathepsin G* (*hCG*) promoter. The PML/RARα transgenic mice (TM) develop a form of leukemia that closely resembles human APL. However, the disease is developed only by mice older than one year, suggesting that other mutagenic events have to occur prior to diagnosis. We aim to characterize cell cycle, apoptosis and *cyclin dependent kinase inhibitors* (*CDKis*) gene expression at different TM age groups in order to determine which abnormalities precede the development of leukemia. We will study the distribution of myeloid progenitors CD117 positive in the different phases of the cell cycle using flow cytometry. Proliferation *in vivo* of this cell subset will be assessed by measuring BuDU incorporation after i.p. injection. Apoptosis will be induced by gamma irradiation and the percentage of apoptotic myeloid progenitors will be studied by flow cytometry using Annexin V and PI staining. Expression of the *p21* and *p16 CDKis* will be evaluated semi-quantitatively by RT-PCR.

The transgenic model is a useful tool for the development of new therapeutic strategies. We are currently interested on the potential anti-leukemic activity of a derivative of vitamin E, α-tocopherol, which was demonstrated to inhibit cell growth of APL cell lines. Its effects were associated with the inhibition of the NFκB pathway. We will determine the *in vivo* effects of α-tocopherol, using the hCG-PML/RARα TM. Leukemic mice will be treated with α-tocopherol alone or in association with the all-trans retinoic acid or arsenic trioxide, which are the currently available therapy for APL. The clearence of leukemia cells, survival and toxicity will be evaluated. Moreover, we plan to analyze the changes in the gene expression profile of leukemic cells treated with α-tocopherol using a macroarray technique already set up in our laboratory.

A second class of drugs under investigation are HDAC inhibitors, which are synergic with RA in the induction of differentiation and may revert RA-resistance in APL patients harboring mutations in the ligand binding domain of PML/RARα. Among the HDACis are trichostatin A (TSA), sodium phenylbutirate (NaB) and valproic acid. We are currently characterizing genes which have their expression affected by RA associated or not with TSA. We have so far analyzed by macroarray the expression of genes associated with cell cycle and apoptosis control in the APL cell line NB4. Based on these results, five genes were selected: *GADD153, CDC37, NEDD5, Cyclin D2, RAD23* and their expression will be quantified by real-time PCR in primary APL cells tretaed in vitro with RA with or without TSA.

Between 5-30% of APL patients treated with retinoic acid develop the retinoid syndrome (RS), characterized by respiratory distress, fever, gain of weight, renal

function impairment and pleural effusion. RS pathogenesis has been associated with changes in the expression of adhesion molecules, cytokine imbalance and release of cellular enzymes, among others. We aim to study the role of adhesion molecules (AMs) in RS pathogenesis. For that, we will evaluate CD11a, CD11b, CD18, CD29, CD54, CD62L and CD162 expression in leukemic cells from patients with APL and in NB4 cells (APL cell line) treated with retinoic acid, as well as quantify the adhesion of treated cells to Matrigel. Moreover, the effect on AMs expression of new therapeutic agents for APL such as histone deacetylase inhibitors (HDACis) or filgrastim will be evaluated.

Recently, Pandolfi's group have demonstrated that mouse embryonic fibroblasts in which the *Pml* gene was inactivated by homologous recombination (*Pml -/-*) were resistant to TGFβ induced apoptosis and growth arrest. On the other hand, Raza et al. demonstrated up-regulation of TGFβ and prolongation of S phase in bone marrow biopsy samples of 23 APL patients. These data suggest that PML/RARα may induce resistance to apoptosis by repressing native PML and interfering with the TGFβ pathway. We aim to study the role of TGFβ pathway deregulation on APL pathogenesis. We will construct a retrovirus harboring the PML/RARα cDNA under the control of tetracycline responsive element (Tet-on system). Murine hematopoietic progenitors (Sca1+ Lineage-) will be co-transfected with the tet-PML/RARα and Tta retroviruses, in order to generate a conditional model. TGFβ induced apoptosis, growth arrest as well as the activation of downstream targets of TGFβ will be analyzed in presence and absence of PML/RARα expression (controlled by the addition of doxocycline to the media). In addition, the effect of halofunginone, a small molecule inhibitory TGFβ will be assessed.